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ABSTRACT

THE MECHANISMS OF STREPTOMYCIN RESISTANCE IN *Erwinia amylovora*

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The mechanism of streptomycin resistance in *Erwinia amylovora,* the fire blight pathogen causing the most destructive bacterial disease of apple and pear, has been studied on a molecular genetic and biochemical basis. Streptomycin-resistant mutants of *E. amylovora* were isolated from an apple orchard in Michigan in 1990. In colony hybridization, a portion of the resistance gene (SMP3) from strain Psp36 of *Pseudomonas syringae* pv. *papulans* hybridized with all streptomycin-resistant strains of *E. amylovora*, but not with streptomycin-sensitive strains. A 34-kb plasmid (pEa34) was present in all streptomycin-resistant field strains but not in streptomycin-sensitive strains. Streptomycin resistance and pEa34 were cotransferred by conjugation into four streptomycin-sensitive recipients.

A class II Tn*3*-type transposable element, designated Tn*5393* and located on plasmid pEa34 from streptomycin-resistant strain CA11 of *E. amylovora*, was identified by its ability to move from pEa34 into recipient plasmid replicons pGEM3Zf(+) and pUCD800. Nucleotide sequence analysis reveals that Tn*5393* consists of 6,705 bp with 81-bp terminal inverted repeats and generates 5-bp duplications of the

target DNA following insertion. Two open-reading frames, separated by a 194-bp putative recombination site (res), encode a putative transposase (*tnpA*) and resolvase (*tnpR*) of 961 and 181 amino acids, respectively. Two streptomycin resistance gene, *strA* and *strB*, were identified on the basis of their DNA sequence homology to the resistance genes in plasmid RSF1010. Between *tnpR* and *strA* is a 1.2-kb insertion sequence designated IS*1133*. The 3.2-kb *tnpA*-res-*tnpR* was detected in *P. syringae* pv. *papulans* Psp36 and in many other gram-negative bacteria harboring *strA-strB* isolated from Michigan apple orchards. Except for some strains of *Erwinia herbicola*, these other gram-negative bacteria lack insertion sequence IS*1133*. The prevalence of *strA-strB* could be accounted for by transposition of Tn*5393* to conjugative plasmids that are then disseminated widely among gram-negative bacteria.

The plasmid-borne *strA* and *strB* genes from *Erwinia amylovora* strain CA11 were characterized by genetic and biochemical analyses. In deletion experiments, deletions in *strB* resulted in a reduction in the minimum inhibitory concentration (MIC) from 500 to 100 μg streptomycin ml^{-1} and in *strA* from 500 to 25 μg streptomycin ml^{-1} or less. When *strA* and *strB* were cloned separately on a *lacI*^{Q}/Ptac-based expression vector in *Escherichia coli*, the protein encoded by *strA*, but not the one by *strB*, was overexpressed. Sequence analysis of the overlapping genes indicated that the distal *strB* gene lacked a Shine-Dalgarno sequence and

that the initiation codon was in the double-stranded region of the stable stem-loop structure. Conversely, the Shine-Dalgarno sequence and the initiation codon in *strA* were exposed in the single-stranded loop of a stable stem-loop structure. The *strB* gene was overexpressed and resistance restored to a MIC of 100 μg streptomycin ml^{-1} by introducing a Shine-Dalgarno sequence and by altering the mRNA secondary structure. ^{13}C-NMR analysis of the respective phosphorylated streptomycin products indicated that *strA-strB* encoded aminoglycoside-3"-phosphotransferase [APH(3")-Ib] and aminoglycoside-6-phosphotransferase [APH(6)-Id], respectively. These data suggest that the high level of resistance to streptomycin exhibited by bacteria with *strA-strB* genes is due to the coexistence in the cells of APH(3")-Ib and APH(6)-Id enzymes and that the differential expression of these enzymes is regulated by the mRNA secondary structures.

DEDICATION

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GENERAL INTRODUCTION AND LITERATURE REVIEW

Fire blight is the most devastating bacterial disease of pomaceous fruit trees (106). It is very destructive to pear (*Pyrus communis* L.) and less so to apple (*Malus sylvestris* Mill.), quince (*Cydonia oblonga* Mill.), and several other members of the family *Rosaceae*. Fire blight may result in tremendous economic losses on pear and apple crops when it occur epidemically.

The first reference to fire blight is considered to be a description of a problem on apple trees taken from a letter by W. Denning in 1793. Thereafter, the possible cause of fire blight was debated until 1878 when T. J. Burrill first proposed that bacteria caused the disease. However, the isolation of the bacterium in pure culture and reinoculating it into healthy plants to prove definitely the real causal agent was not completed until 1884 by J. C. Arthur. Fire blight was apparently indigenous to North America. The disease probably occurred on native American plants, such as crab apple, hawthorn, and mountainash. From these native hosts the bacterium probably spread to the susceptible cultivated pears and apples planted by the early American settlers.

The occurrence of fire blight was restricted to North America for a century since 1800s. Outside North America, fire blight was first reported in New Zealand in 1919. In Europe the disease was report in England in 1957. The disease has since spread through much of Europe and the Mediterranean area causing serious losses to pome fruits and rosaceous ornamentals. It has not been reported in Asia.

The causal organism of fire blight is the bacterium *Erwinia amylovora* (Burrill 1882) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers, and Smith 1920. *E. amylovora*, a member of the family *Enterobacteriaceae*, is a Gram-negative, facultatively anaerobic, peritrichously flagellal, rod-shaped (av. 1.1-1.6 x 0.6-0.9 μm) bacterium. The identification of *E. amylovora* is usually based on characteristic growth of colonies on one or more differential media (21, 47, 70), immunological tests, the production of bacterial ooze on immature pear fruit following inoculation, and the hypersensitive reaction of *Nicotiana tabacum* L. to infiltrated bacteria (43, 52, 53, 54). Recently, a specific DNA probe derived from the universal plasmid pEA29 in *E. amylovora* has been developed for routine, cost-effective identification of this bacterium (32).

The methods for the control of fire blight are difficult, expensive, and not very effective. Streptomycin is the most effective bactericide compared to the alternatives, copper-based bactericides, oxytetracycline, and flumequine.

Therefore, the emergence of streptomycin-resistant pathogen of fire blight has become a severe problem to the pear and apple industry in North America.

Streptomycin is an aminoglycoside-aminocyclitol antibiotic produced by *Streptomyces* spp. (23, 45). It was first isolated in 1944 by Schatz et al. (86) and introduced as an antituberculosis drug in 1949. However, it frequently produced serious toxic effects, notably, severe vestibular dysfunction and, occasionally, partial or complete deafness. Perhaps its most serious disadvantage lies in the rapidity with which resistant strains emerge. In plant pathology, streptomycin was mainly introduced to control fire blight. Extensive experimentation on the efficacy of streptomycin for the control of fire blight was conducted in North America in the early 1950s. After streptomycin was registered in the United States in the late 1950s, it was used extensively for the control of fire blight on pears in the West and on apples in the East. Besides fire blight, streptomycin has been used to control bacterial spot of tomato and pepper and blister spot of apple. Outside the United States, streptomycin has also been used in New Zealand and Canada.

As early as 1958, M. H. Dye had demonstrated the development of streptomycin resistance in a plant bacterial pathogen in the laboratory (31). In the field, the occurrence, frequency, and distribution of streptomycin resistance in the causal organism of bacterial spot in

tomato and pepper, *Xanthomonas campestris* pv. *vesicatoria*, were first reported in the early 1960s (98, 104). Resistance to streptomycin has also been reported in several pathovars of *Pseudomonas syringae* (13, 26, 49, 85, 102, 109) and in strains of *X. campestris*. pv. *dieffenbachiae* (55).

Streptomycin-resistant *E. amylovora* was first detected in pear orchards of California in 1971 (70) and then in pear orchards of Washington and Oregon (20, 63). Except for a report of resistant strains on apple in Missouri (91), detection of streptomycin-resistant *E. amylovora* has until recently been limited to the western United States. In the mid-1970s, attempts to detect resistant strains in apple and pear orchards in western New York State and in apple orchards in Michigan failed (7, 103). A second attempt to detect resistant *E. amylovora* in 1993 in New York apple growing areas was also unsuccessful (14). In Michigan, the first detection of streptomycin-resistant strains of *E. amylovora* was in 1990 in an apple orchard in Van Buren County. The characterization of the streptomycin-resistant *E. amylovora* detected in Michigan in 1990 is the subject of this thesis.

Bacteria have versatile ways to cope with antibiotics and other toxic elements (5, 11, 22). The mechanisms of resistance include inactivation of the antibiotics by enzymes, alteration of the antibiotic target, and reduction of drug accumulation (12). Some groups of antibiotics are inactivated by modifying enzymes (22, 93) or destructive

enzymes (1, 3). Inactivation of antibiotics by enzymes is the most important and commonest mechanism of resistance since the genes encoding the enzymes are usually carried on self-transmissible or mobilizable plasmids and transposons. Antibiotics effectively inhibit the cellular components and enzymes related to bacterial cell wall synthesis, protein synthesis, and DNA replication (12). It has been found that modification with enzymes and mutations of the target sites may reduce the binding affinity of the target sites to the antibiotics (58, 75, 95, 107). Overproduction of the target protein (41) or production of an insensitive form of the target protein by genes on a plasmid and transposon may also result in resistance (4, 84). Decreased permeability of the plasma membrane to toxic substances or decreased binding of toxic compounds are initially proposed to explain the mechanism of reduced accumulation by resistant cells (48, 57). Recently, active efflux have been proposed to explain the mechanism of reduced drug accumulation (2, 60, 68). In addition to bacteria, active efflux systems, which are responsible for resistance to a variety of structurally unrelated antibiotics and toxic compounds, have been reported in fungi (28), protozoan parasites (56, 66), and mammalian cancer cells (29). In bacteria, active efflux systems may contribute to mechanism of resistance to antibiotic, heavy metals, and other toxic substances (60). Efflux proteins usually contain membrane-spanning units and ATP-binding domains that allow them to serve as energy-

dependent transporters, and their genes may occur on plasmids, transposons, or chromosomes. Recent studies have shown that active efflux systems are involved in multidrug resistance in bacteria (19, 77) and in mammalian cancer cells (29); therefore, mechanisms associated with active efflux have become of considerable scientific interest.

The first suggestion that the ribosome was the site of action of streptomycin came in 1961 (97). Since that time the ribosome has been positively identified as the target site for all the aminoglycoside-aminocyclitol drugs that have been tested. The binding of streptomycin to the ribosome is reversible (6, 16); however, the uptake of streptomycin is irreversible (15, 78). The binding of streptomycin to ribosome induces misreading and inhibition of protein synthesis. It has been proposed (15, 25) that the irreversible uptake and misreading (mistranslating) and inhibition of protein synthesis is associated with the bactericidal, not bacteristatic, action of streptomycin; however, the detailed mechanism of action is still unclear.

Bacteria use the same strategies to cope with streptomycin as with other antibiotics and toxic elements. The mechanisms of resistance used for streptomycin include alteration of the ribosomal target site, inactivation by modifying enzymes, and reduced accumulation of streptomycin in the cells (5, 23). Biochemical and genetics studies have revealed that streptomycin primarily interacts with 16S ribosomal RNA (rRNA). Cross-linking experiments have shown

that streptomycin can be linked to a fragment of *Escherichia coli* 16S rRNA spanning residues 892 to 917 and 1394-1415 (39), and chemical footprinting studies indicated that bases 909, 911, 912, and particularly bases 913 to 915, which have been designated as the 915 region, are protected by streptomycin (72). Streptomycin resistance can result from changes at the bases equivalent to *E. coli* 523 (designated 530 loop) and 912 to 915 in the 16S rRNA or changes of amino acid in ribosomal protein (r-protein) S4, S5 and S12. It has been observed that mutations at position 912, 914, or 915 (*E. coli* numbering) in chloroplast 16S rRNA confer resistance to streptomycin (42, 74), and mutations at position 912, 913, and 915 have been shown to lower the response of ribosomes to streptomycin when introduced into *E. coli* 16S rRNA (30, 73). Although the 530 loop and r-proteins S4, S5, and S12 are located on a distinct site from the streptomycin binding site (59, 69), they are also involved in streptomycin binding. A mutation at position 523 in the 530 loop of 16S rRNA has been proved to cause resistance to streptomycin (33, 69). Protein S12, when altered, can produce a phenotype of streptomycin resistance (37, 62, 81, 101) in bacteria and chloroplasts, or streptomycin dependence (9). Mutations in protein S12 confer streptomycin resistance by impairing the binding of the drug to the ribosome, whereas, mutations in protein S4 or S5 (*ram* mutations) have the opposite effect since they enhance streptomycin binding (10, 59).

Aminoglycoside-modifying enzymes include acetyltransferases (AAC), nucleotidyltransferases (ANT) (adenylyltransferases, AAD), and phosphotransferases (APH) (23, 93). Among the enzymes two nucleotidyltransferases, ANT(3") and ANT(6), and two phosphotransferases, APH(3") and APH(6), are associated with resistance to streptomycin (93). ANT(3") confer by resistance to streptomycin and spectinomycin (24, 46). The enzyme modifies the 3"-hydroxyl position of streptomycin and the 9-hydroxyl position of spectinomycin. The *ant(3")* gene has been cloned in association with several transposons (46, 87) and is ubiquitous among gram-negative bacteria. ANT(6), APH(3") and APH(6) are characterized by resistance to streptomycin only. ANT(6) is found in gram-positive organisms (82) and reacts at the 6-hydroxyl position of streptomycin. APH(3") and APH(6) modify streptomycin at the 3"- and 6-hydroxyl groups, respectively. Both phosphotransferases have been found in streptomycin producing strains of *Streptomyces griseus* (45, 65), but APH(6) is the major enzyme and its gene is clustered with the genes encoding enzymes involved in streptomycin biosynthesis (65). APH(3") has been found in many clinically important gram-negative bacteria (38, 50, 80) and in phytopathogenic bacteria (36, 108). Because bacteria with reduced drug accumulation exhibit low-level resistance, whereas bacteria with altered ribosome and inactivating enzymes exhibit high-level resistance, the mechanism of reduced accumulation has not been studied in

detail even though it is potentially clinical importance. Whether or not this mechanism results from the development of an active efflux system remains to be investigated.

Horizontal and vertical transfer of resistance to drugs and toxic metal ions in bacteria has been shown to be mostly conveyed by plasmids and transposons which carry resistance genes (34, 51, 64, 92). Different species of bacteria harbor characteristic types of plasmids, some of which can mediate their own transfer by conjugation. However, except some groups of broad-host-range plasmids most plasmids can only exist and replicate themselves in a limited number of bacterial species (105). Transposons have the capacity to transpose from one DNA molecule to another. This has undoubtedly contributed to the rapid dissemination of antibiotic resistance by providing an efficient mechanism for incorporating resistance determinants into new plasmid vectors which can transfer to and stably replicate in diverse hosts. Recent studies on the horizontal transfer of tetracycline resistance in the gram-positive clinical pathogen, *Streptococcus*, have shown that several nonplasmid conjugative elements, designated conjugative transposons, mediate the transfer of resistance genes (17, 35, 90). Conjugative transposons transpose from donor (usually chromosome-borne) to recipient by a circular DNA intermediate rather than by a conjugative plasmid. Conjugative transposons may be more commonly involved in the

dissemination of drug resistance than plasmids in some species of the clinically important streptococci.

Transposons have been divided into two groups based on the organization of their genes (51). Class I transposons, for example Tn*5*, carry genes for antibiotic resistance bounded by two directed or inverted copies of an insertion sequence (IS) element in the form of a compound transposon. Class II transposons contain short inverted terminal repeats, a *tnpA* encoding transposase of about 1,000 amino acids, a *tnpR* encoding resolvase of about 185 amino acids, and drug or heavy metal ion resistance gene(s). Some class II transposons, especially those belonging to the Tn*21* subfamily, also contain a gene encoding integrase which mediates a site-specific integration mechanism responsible for the acquisition of other resistance genes. Therefore many class II transposons carry several genes for multidrug resistance which has resulted in the failure of many antibiotics in chemotherapy (67, 100). The presence of a transposon is usually detected by the movement of the marker gene(s) to a recipient replicon, a small multi-copy plasmid or a conjugative plasmid with an selection marker (27, 35, 44, 99). The movement of transposon (transposition) is accomplished by transposase and site-specific recombinase (resolvase) that are encoded by the transposon (40, 92). Usually, a direct repeat in the target DNA is generated following the transposition.

It has been suggested that the aminoglycoside resistance genes in clinically important bacterial pathogens were derived from organisms that produce the aminoglycosides (8). The presence of these enzymes in aminoglycoside-producing strains could provide a mechanism of self-protection against the antibiotic produced. Therefore, the actinomycetes could have provided the initial gene pool from which some of the present-day aminoglycoside resistance genes were derived. A second theory is that aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism (83). Recent studies reveal that overexpression of a cellular gene may result in multiple antibiotic resistance in some bacteria (18, 94) as well as multiple resistance to anti-cancer drugs in mammalian cancer cells (30). The gene *aac(6')-Ic* was detected in both susceptible and resistant strains of *Serratia*, but the AAC(6') enzyme was overexpressed in resistant strains (94). Another example is the *mar* (multiple antibiotic resistance) locus in *E. coli*. Mar mutants overexpress Mar proteins that mediate the cross-resistance to several unrelated antibiotics and their genes have been found in other members of the family *Enterobacteriaceae*, including *Salmonella*, *Shigella*, and *Klebsiella* spp. (19). Although these genes are limited to a few genera, it is possible that further genetic selection will result in the mobilization of these genes via association with plasmids or transposons and will speed the

dissemination of the resistant determinants through species barriers.

Although streptomycin resistance in phytopathogenic bacteria have been noticed since 1958 (31), the mechanism of resistance was not studied until recent years. Schroth et al. assumed that resistance to streptomycin in the strains of *E. amylovora* was caused by a chromosomal mutation because no plasmid could be detected (89). Later, resistance in *P. syringae* pv. *papulans* and *X. campestris* pv. *vesicatoria* were found to be carried on plasmids (13, 71). A specific probe, SMP3, cloned from *P. syringae* pv. *papulans* strain Psp36 was developed for detecting the occurrence, frequency, and distribution of the resistance gene in the pathogen and many epiphytic bacteria in New York apple orchards (79). The probe also hybridized to plasmids in distinct sizes in the epiphytic bacteria suggesting that different plasmids had carried the same resistant determinant (79). Probe SMP3 was also used for a study on streptomycin-resistant *E. amylovora* and epiphytic bacteria isolated from Michigan apple orchards (96). This study indicated that 97% of 152 strains of epiphytic gram-negative bacteria contained DNA homologous to the DNA associated with resistance in *P. syringae* pv. *papulans* but none of 28 gram-positive bacteria contained DNA that hybridized with SMP3. Restriction fragment length polymorphism of the resistance gene was present among the resistant epiphytic bacteria and one resistant strain of *E. amylovora* (79, 96). The resistance determinant in the

streptomycin- and copper-resistant *P. syringae* pv. *syringae* has also been studied (102). A restriction mapping indicated that organization of resistance genes in *P. syringae* pv. *syringae* strain A2 was homologous to streptomycin resistance genes *strA-strB* in plasmid RSF1010 (88, 102).

The appearance of antibiotic-resistant human pathogenic microbes has become a worldwide crisis in this decade. Rapid development of resistance, especially multidrug resistance, has stun the pharmaceutical industry in creating new antibiotics and caused the failure of many antibiotics in treatment (61, 76). The deciphering of drug resistance mechanisms will not only allow a better understanding of incipient clinical crises but may also suggest strategies for reversing resistance and preventing the appearance of new resistant microbes (107). Similarly, the reasons for studying streptomycin resistance mechanisms in *E. amylovora* are to develop a better understanding of the problem and methods for combating resistant strains.

In Michigan, streptomycin-resistant strains of *E. amylovora* were first isolated from an apple orchard in southwest in the summer of 1990. More resistant strains of *E. amylovora* and many epiphytic bacteria were collected from the orchards in the same area and Grand Rapids area in 1990 and 1991. The objective of this research is to determined the mechanism of resistance in the streptomycin-resistant *E. amylovora* strain CA11 on a molecular genetic and biochemical

basis. Part I describes the molecular genetic analysis of the resistance determinant. In Part II the entrapment of a transposon (Tn*5393*) carried with the streptomycin resistance in *E. amylovora* strain CA11, nucleotide sequence analysis of the transposon, and investigation of the distribution of the transposon in other gram-negative bacteria are described. Part III describes the identification of two modifying enzymes, APH(3")-Ib and APH(6)-Id, encoded by the resistance genes *strA-strB* located in Tn*5393*. The role of the two modifying enzymes on the level of resistance and the regulatory expression of the genes are investigated. The methods and procedures for identifying mechanisms of streptomycin resistance in bacteria are illustrated as a flowchart on Appendix C.

REFERENCES

1.Abrahams, E. P., and E. Chain. 1940. An enzyme from bacteria able to destroy penicillin. Nature **146:**837.

2. Allard, J. D., and K. P. Bertrand. 1993. Sequence of a class E tetracycline resistance gene from *Escherichia coli* and comparison of related tetracycline efflux proteins. J. Bacteriol. **175:**4554-4560.

3. Amyes, S. G. B. 1990. The success of plasmid-encoded resistance genes in clinical bacteria: an examination of plasmid-mediated ampicillin and trimethoprim resistance genes and their resistance mechanisms. J. Med. Microbiol. **28:**73-83.

4. Amyes, S. G. B., and K. J. Towner. 1990. Trimethoprim resistance; epidemiology and molecular aspects. J. Med. Microbiol. **31:**1-19.

5. Amyes, S. G. B., and C. G. Gemmell. 1992. Antibiotic resistance in bacteria. J. Med. Microbiol. **36:**4-29.

6. Andry, K., and R. C. Bockrath. 1974. Dihydrostreptomycin accumulation in *E. coli*. Nature **251:**534-536.

7. Beer, S. V., and J. L. Norelli. 1976. Streptomycin-resistant *Erwinia amylovora* not found in western New York pear and apple orchards. Plant Dis. Rep. **60:**624-626.

8. Benveniste, R., and J. Davies. 1973. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. USA **70:**2276-2280.

9. Birge, E. A., and C. G. Kurland. 1969. Altered ribosomal protein in streptomycin-dependent *Escherichia coli*. Science **166:**1282-1284.

10. Bock, A., A. Petzet, and W. Piepersberg. 1979. Ribosomal ambiguity (*ram*) mutations facilitate dihydrostreptomycin binding to ribosomes. FEBS Lett. **104:**317-321.

11. Bryan, L. E. (ed.). 1984. Antimicrobial drug resistance. Academic Press, Inc., Orlando, Fla.

12. Bryan, L. E. 1988. General mechanisms of resistance to antibiotics. J. Antimicrob. Chemother. (Suppl. A) **22**:1-15.

13. Burr, T. J., J. L. Norelli, B. Katz, W. F. Wilcox, and S. A. Hoying. 1988. Streptomycin resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. Phytopathology **78**:410-413.

14. Burr, T. J., J. L. Norelli, C. L. Reid, L. K. Capron, L. S. Nelson, H. S. Aldwinckle, and W. F. Wilcox. 1993. Streptomycin-resistant bacteria associated with fire blight infections. Plant Dis. **77**:63-66.

15. Busse, H.-J., C. Wostmann, and E. P. Bakker. 1992. The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of *Escherichia coli* and subsequent caging of the antibiotic inside the cells due to degradation of these proteins. J. Gen. Microb. **138**:551-561.

16. Chang, F. N., and J. G. Flaks. 1972. Binding of dihydrostreptomycin to *Escherichia coli* ribosomes: characteristics and equilibrium of the reaction. Antimicrob. Agents Chemother. **2**:294-307.

17. Clewell, D. B. 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Ann. Rev. Microbiol. **40**:635-659.

18. Cohen, S. P., H. Hachler, and S. B. Levy. 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175**:1484-1492.

19. Cohen, S. P., W. Yan, and S. B. Levy. 1993. A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria. J. Infect. Dis. **168**:484-488.

20. Coyier, D. L., and R. P. Covey. 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. **59**:849-852.

21. Crosse, J. E., and R. N. Goodman. 1973. A selective medium for and a definitive colony characteristic of *Erwinia amylovora*. Phytopathol. **63**:1425-1426.

22. Cundliffe, E. 1989. How antibiotic-producing organism avoid suicide. Annu. Rev. Microbiol. **43**:207-233.

23. Davies, J. 1986. Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes. P. 790-809 in: Antibiotics in Laboratory Medicine. V. Lorian, ed. Williams and Wilkins, Baltimore, MD. 1259 pp.

24. Davies, J., and D. I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32:**469-518.

25. Davis, B. D. 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol. Rev. **51:**341-350.

26. DeBoer, S. H. 1980. Leaf spot of cherry laurel caused by *Pseudomonas syringae*. Can. J. Plant Pathol. **2:**235-238.

27. de la Cruz, F., and J. Grinsted. 1982. Genetic and molecular characterization of Tn*21*, a multiple resistance transposon from R100.1. J. Bacteriol. **151:**222-228.

28. de Waard, M. A., and J. G. M. Van Nistelrooy. 1980. An energy-dependent efflux mechanism for fenarimol in a wild type and fenarimol resistant mutants of *Aspergillus nidulans*. Pestic. Biochem. Physiol. **13:**255-266.

29. Deuchars, K., and V. Ling. 1989. P-glycoprotein and multidrug resistance in cancer chemotherapy. Semin. Oncol. **16:**156-165.

30. Douglass, J., and L. M. Steyn. 1993. A ribosomal gene mutation in streptomycin-resistant *Mycobacterium tuberculosis* isolates. J. Infect. Dis. **167:**1505-1506.

31. Dye, M. H. 1958. Development of streptomycin-resistant variants of *Pseudomonas syringae* van Hall in culture and in peach seedlings. N. Z. J. Agric. Res **1:**44-50.

32. Falkenstein, H., P. Bellemann, S. Walter, W. Zeller, and K. Geider,. 1988. Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. **54:**2798-2802.

33. Finken, M., P. Kirschner, A. Meier, A. Wrede, and E. C. Bottger. 1993. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol. Microbiol. 9:1239-1246.

34. Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47:**361-409.

35. Franke, A. E., and D. B. Clewell. 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145:**494-502.

36. Fukusawa, K., H. Sakurai, S. Shimizu, H. Naganawa, S. Kondo, H. Kawabe, and S. Mitsuhashi. 1980. 3"-phosphoryldihydrostreptomycin produced by the inactivating enzyme of *Erwinia carotovora*. J. Antibiot. **33**:122-123.

37. Galili, S., H. Fromm, D. Aviv, M. Edelman, and E. Galun. 1989. Ribosomal protein S12 as a site for streptomycin resistance in *Nicotiana* chloroplasts. Mol. Gen. Genet. **218**:289-292.

38. Ganelin V. L., L. G. Vinogradova, V. N. Stepanshina, R. M., Petyushenko, A. I., Chernyshev, S. E. Esipov, Y. O. Sazykin, and S. M. Navashin. 1980. Streptomycin-3"-phosphotransferases from streptomycin-resistant cells of *Escherichia coli* strains. Biochimia **45**:2198-2205.

39. Gravel, M., P. Melancon, and L. Brakier-Gingras. 1987. Cross-linking of streptomycin to the 16S ribosomal RNA of *Escherichia coli*. Biochemistry **26**:6227-6232.

40. Grindley, N. D. F., and R. R. Reed. 1985. Transpositional recombination in prokaryotes. Ann. Rev. Biochem. **54**:838-896.

41. Hamilton-Miller, J. M. T. 1984. Resistance to antibacterial agents acting on antifolate metabolism. P. 173-190. In: Bryan, L. E. (ed.) Antimicrobial drug resistance. Orlando, Academic Press.

42. Harris, E. H., B. D. Burkhart, N. W. Gillham, and J. E. Boynton. 1989. Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of *Chlamydomonas reinhardii*: correlation of genetic and physical maps of the chloroplast genome. Genetics 123:281-292.

43. He, S. Y., H.-C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* $Harpin_{pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell **73**:1255-1266.

44. Heffron, F., C. Rubens, and S. Falkow. 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular mature and specificity of insertion. Proc. Nat. Acad. Sci. USA 72:3623-3627.

45. Heinzel, P., O. Werbitzky, J. Distler, and W. Piepersberg. 1988. A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phosphotransferase. Arch. Microbiol. **150**:184-192.

46. Hollingshead, S., and D. Vapnek. 1985. Nucleotide sequence analysis of a gene encoding a

streptomycin/spectinomycin adenyltransferase. Plasmid 13:17-30.

47. Ishimaru, C., and E. J. Klos. 1984. A new medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology **74:**1342-1345.

48. Izaki, K., and K. Arima. 1963. Disappearance of oxytetracycline accumulation in the cells of multiple drug resistant *Escherichia coli*. Nature (London) **200:**384-385.

49. Jones, A. L., J. L. Norelli, and G. R. Ehret. 1991. Detection of streptomycin-resistant *Pseudomonas syringae* pv. *papulans* in Michigan apple orchards. Plant Dis. **75:**529-531.

50. Kawabe, H., F. Kobayashi, M. Yamaguchi, R. Utahara, and S. Mitsuhashi. 1971. 3"-phosphoryldihydrostreptomycin produced by the inactivating enzyme of *Pseudomonas aeruginosa*. J. Antibiot. **24:**651-652.

51. Kleckner, N. 1981. Transposable elements in prokaryotes. Annu. Rev. Genet. 15:341-404.

52. Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature **199:**299-300.

53. Klement, Z. 1982. Hypersensitivity. In Phytopathogenic Prokaryotes, Volume 2, Mount, M. S. and Lacy, G. H. eds. New York: Academic Press, PP. 149-177.

54. Klement, Z., G. L. Farkas, and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology **54:**474-477.

55. Knauss, J. F. 1972. Resistance of *Xanthomonas dieffenbachiae* isolates to streptomycin. Plant Dis. Rep. **56:**394-397.

56. Krogstad, D. J., I. Y. Gluzman, D. E. Kyle, A. M. J. Oduola, S. K. Martin, W. K. Milhous, and P. H. Schlesinger. 1987. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science 238:1283-1285.

57. Kushner, D. J., and S. R. Khan. 1968. Proflavine uptake and release in sensitive and resistant *Escherichia coli*. J. Bacteriol. 96:1103-1114.

58. Leclerc, D., P. Melancon, and L. Brakier-Gingras. 1991. Mutations in the 915 region of *Escherichia coli* 16S ribosomal RNA reduce the binding of streptomycin to the ribosome. Nucl. Acids Res. **19:**3973-3977.

59. Leclerc, D., P. Melancon, and L. Brakier-Gingras. 1991. The interaction between streptomycin and ribosomal RNA. Biochimie **73**:1431-1438.

60. Levy, S. B. 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36**:695-703.

61. Levy, S. B. 1993. Confronting multidrug resistance: a role for each of us. JAMA **269**:1840-1842.

62. Liu, X.-Q., N. W. Gillham, and J. E. Boynton. 1989. Chloroplast ribosomal protein gene *rps12* of *Chlamydomonas reinhardtii*: wild-type sequence, mutation to streptomycin resistance and dependence, and function in *Escherichia coli*. **264**:16100-16108.

63. Loper, J. E., and M. D. Henkels, R. G. Roberts, G. G. Grove, M. J. Willett, and T. J. Smith. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. Plant Dis. **75**:287-290.

64. Lupski, J. R. 1987. Molecular mechanisms of transposition of drug-resistance genes and other movable genetic elements. Rev. Infect. Dis. **9**:357-368.

65. Mansouri, K., K. Pissowotzki, J. Distler, G. Mayer, P. Heinzel, C. Braun, A. Ebert, and W. Piepersberg. 1989. Genetics of streptomycin production, p. 61-67. In C. L. Hershberger, S. W. Queener, and G. Hegeman (ed.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, DC.

66. Martin, S. K., A. M. J. Oduola, and W. K. Milhous. 1987. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science **235**:899-901.

67. Martinez, E., and F. de la Cruz. 1990. Genetic elements involved in Tn*21* site-specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. EMBO J. **9**:1275-1281.

68. McMurry, L., R. E. Petrucci, Jr., and S. B. Levy. 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:3974-3977.

69. Melancon, P., C. Lemieux, and L. Brakier-Gingras. 1988. A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin. Nucl. Acids Res. **16**:9631-9639.

70. Miller, T. D., and M. N. Schroth. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology **62:**1175-1182.

71. Minsavage, G. V., B. I. Canteros, and R. E. Stall. 1991. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **80:**719-723.

72. Moazed, D., and H. F. Noller. 1986. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature **327:**389-394.

73. Montandon, P.-E., R. Wagner, and E. Stutz. 1986. *E. coli* ribosomes with a C912 to U base change in the 16S rRNA are streptomycin resistant. EMBO J. **5:**3705-3708.

74. Montandon, P.-E., P. Nicolas, P. Schurmann, and E. Stutz. 1985. Streptomycin-resistance of *Euglena gracilis* chloroplasts: identification of a point mutation in the 16S rRNA gene in an invariant position. Nucl. Acids Res. **13:**4299-4310.

75. Nakamura, S., M. Nakamura, T. Kojima, and H. Yoshida. 1989. *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob. Agents Chemother. **33:**254-255.

76. Neu, H. C. 1992. The crisis in antibiotic resistance. Science **257:**1064-1073.

77. Neyfakh, A. A., V. E. Bidnenko, and L. B. Chen. 1991. Efflux-mediated multidrug resistance in bacteria: similarities and dissimilarities with mammalian system. Proc. Natl. Acad. Sci. USA **88:**4781-4785.

78. Nichols, W. W., and S. N. Young. 1985. Respiration-dependent uptake of dihydrostreptomycin by *Escherichia coli*: its irreversible nature and lack of evidence for a uniport mechanism. Biochem. J. **228:**505-512.

79. Norelli, J. L., T. J. Burr, A. M. Lo Cicero, M. T. Gilbert, and B. H. Katz. 1991. Homologous streptomycin resistance gene present among diverse gram-negative bacteria in New York State apple orchards. Appl. Environ. Microbiol. **57:**486-491.

80. Ozanne, B., R. Benveniste, D. Tipper, and J. Davies. 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. **100:**1144-1146.

81. Ozaki, M., S. Mizushima, and M. Nomura. 1969. Identification and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli*. Nature **222:**333-339.

82. Ounissi, H., E. Derlot, C. Carlier, and P. Courvalin. 1990. Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci. Antimicrob. Agents Chemother. **34:**2164-2168.

83. Piepersberg, W., J. Distler, P. Heinzel, and J.-A. Perez-Gonzalez. 1988. Antibiotic resistance by modification: many resistance genes could be derived from cellular control genes in actinomycetes - a hypothesis. Actinomycetologica **2:**83-98.

84. Radstrom, P. and G. Swedberg. 1988. RSF1010 and a conjugative plasmid contain *sulII*, one of two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. Antimicrob. Agents Chemother. **32:**1684-1692.

85. Russell, P. E. 1975. Variation in the virulence of some streptomycin resistant mutants of *Pseudomonas phaseolicola*. J. Appl. Bacteriol. **39:**175-180.

86. Schatz, A., E. Bugie, and S. A. Waksman. 1944. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc. Soc. Exptl. Biol. Med. **55:**66-69.

87. Schmidt, F. R. J., E. J. Nucken, and R. B. Henschke. 1988. Nucleotide sequence analysis of 2"-aminoglycoside nucleotidyltransferase ANT(2") from Tn*4000*: its relationship with AAD(3") and impact on Tn*21* evolution. Mol. Microbiol. **2:**709-717.

88. Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger. 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271-288.

89. Schroth, M. N., S. V. Thomson, and W. J. Moller. 1979. Streptomycin resistance in *Erwinia amylovora*. Phytopathology **69:**565-568.

90. Scott, J. R. 1992. Sex and the single circle: conjugative transposition. J. Bacteriol. **174:**6005-6010.

91. Shaffer, W. H., and R. N. Goodman. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. (Abstr.) Phytopathology **75:**1281.

92. Shapiro, J. A. (ed.). 1983. Mobile Genetic Elements. Academic Press, New York.

93. Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes

and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Reviews **57:**138-163.

94. Shaw, K. J., P. N. Rather, F. J. Sabatelli, P. Mann, H. Munayyer, R. Mierzwa, G. L. Petrikkos, R. S. Hare, G. H. Miller, P. Bennett, and P. Downey. 1992. Characterization of the chromosomal *aac(6')-Ic* gene from *Serratia marcescens*. Antimicrob. Agents Chemother. **36:**1447-1455.

95. Skeggs, P. A., J. Thompson, and E. Cundliffe. 1985. Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimariensis*. Mol. Gen. Genet. **200:**415-421.

96. Sobiczewski, P., C.-S. Chiou, and A. L. Jones. 1991. Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Dis. **75:**1110-1113.

97. Spotts, C. R., and R. Y. Stanier. 1961. Mechanism of streptomycin action on bacteria; a unitary hypothesis. Nature **192:**633-637.

98. Stall, R. E., and P. L. Thayer. 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Dis. Rep. 46:389-392.

99. Stanisich, V. A., P. M. Bennett, and M. H. Richmond. 1977. Characterization of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in *Pseudomonas aeruginosa*. J. Bacteriol. **129:**1227-1233.

100. Stokes, H. W., and R. M. Hall. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669-1683.

101. Stuy, J. H., and R. B. Walter. 1992. Cloning, characterization, and DNA base sequence of the high-level streptomycin resistance gene *strA1* of *Haemophilus influenzae* Rd. J. Bacteriol. **174:**5604-5608.

102. Sundin, G. W., and C. L. Bender. 1993. Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. **59:**1018-1024.

103. Sutton, T. B., and A. L. Jones. 1975. Monitoring *Erwinia amylovora* populations on apple in relation to disease incidence. Phytopathology **65:**1009-1012.

104. Thayer, P. L., and Stall, R. E. 1962. A survey of *Xanthomonas vesicatoria* resistance to streptomycin. Proc. Fla. State Hortic. Soc. **75:**163-165.

105. Thomas, C. M. (ed.). 1989. Promiscuous plasmids of gram-negative bacteria. London, San Diego, Academic Press, 276 pp.

106. van der Zwet, T., and H. L. Keil. 1979. Fire blight: A bacterial disease of Rosaceous Plants. U. S. Dep. Agric. Agric. Handb. 510. 200pp.

107. Walsh, C. T. 1993. Vancomycin resistance: decoding the molecular logic. Science **261:**308-309.

108. Yano, H., H. Fujii, H. Mukoo, M. Shimura, T. Watanabe, and Y. Sekizawa. 1978. On the enzymatic inactivation of dihydrostreptomycin by *Pseudomonas lachrymans*, cucumber angular leaf spot bacterium: isolation and structural resolution of the inactivated product. Ann. Phytopath. Soc. (Japan) **44:**413-419.

109. Young, J. M. 1977. Resistance to streptomycin *Pseudomonas syringae* from apricot. N. Z. J. Agric. Res. **20:**249-251.

PART I

THE ANALYSIS OF PLASMID-MEDIATED STREPTOMYCIN RESISTANCE IN *Erwinia amylovora*

The Analysis of Plasmid-Mediated Streptomycin Resistance in *Erwinia amylovora*

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ABSTRACT

Chiou, C.-S., and Jones, A. L. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology 81:710-714.

Streptomycin-resistant mutants of *Erwinia amylovora* were isolated from an apple orchard in Michigan and from crabapple trees adjacent to the same orchard in 1990. Isolates that grew on King's medium B amended with 100 μg/ml of streptomycin sulfate were considered to be resistant strains, whereas isolates that failed to grow on this medium were considered to be sensitive strains. Growth of the resistant strains was not inhibited in a filter-paper disk assay (0.06–5 μg of streptomycin sulfate), but growth of sensitive strains was inhibited at concentrations as low as 0.06 μg of streptomycin sulfate. Only sensitive strains were detected in an additional 19 apple orchards sampled for resistant strains. In colony blot hybridizations, an internal portion of the streptomycin-resistance gene (probe SMP3) from strain Psp36 of *Pseudomonas syringae* pv. *papulans* hybridized with all streptomycin-resistant strains of *E. amylovora*, but not with streptomycin-sensitive strains. Probe SMP3 hybridized to a 2.7-kb restriction fragment from *Ava*I-digested total genomic and plasmid DNA of two resistant strains of *E. amylovora* and to a 1.5-kb fragment in DNA from strain Psp36 of *P. s. papulans*. The probe did not hybridize with digested DNA from sensitive strains. A 33-kb plasmid was present in all streptomycin-resistant field strains but not in streptomycin-sensitive strains. Streptomycin resistance was transferred by matings to four streptomycin-sensitive recipient strains of *E. amylovora* from each of two streptomycin-resistant donor strains. Transconjugants also contained the 33-kb plasmid. DNA from resistant strain Ea88-90 from Washington did not hybridize with the probe, indicating that this strain contains a resistance system unrelated to that in streptomycin-resistant strains from Michigan.

Fire blight, caused by *Erwinia amylovora*, is a devastating disease of apples and pears in North America, New Zealand, much of Europe, and the Mediterranean region (1). Extensive experimentation on the efficacy of streptomycin for the control of fire blight was conducted in North America in the early 1950s. After streptomycin was registered in the United States in the late 1950s, it was used extensively for the control of fire blight on pears in the West and on apples in the East.

Streptomycin-resistant *E. amylovora* was first detected in pear orchards of California in 1971 (14) and soon thereafter in pear orchards of Washington and Oregon (4); in 1988, resistant strains were ubiquitous in pear orchards of Washington (11). In the mid-1970s, attempts to detect resistant strains in apple and pear orchards in western New York state and in apple orchards in Michigan failed (2,22). Except for a report of resistant strains on apple in Missouri (19), detection of streptomycin-resistant *E. amylovora* has been limited to the western United States. However, streptomycin resistance in *Pseudomonas syringae* pv. *papulans* has been a problem in apple orchards of the cultivar Mutsu in the eastern United States (3,8,17).

In California, resistance to streptomycin in *E. amylovora* is caused by a chromosomal mutation (18), but streptomycin resistance in *P. s. papulans* and *Xanthomonas campestris* pv. *vesicatoria* is plasmid-borne (3,15,17). No hybridization was obtained when a streptomycin-resistance probe from *X. c. vesicatoria* was used to probe DNA from a streptomycin-resistant strain of *E. amylovora* from California (15). But the resistance probe did hybridize with DNA from a streptomycin-resistant strain of *P. s. papulans* from New York.

This study reports the detection of streptomycin-resistant *E. amylovora* on apple in Michigan. It shows that resistance is plasmid-borne, and that a DNA probe specific for streptomycin-resistance in *P. s. papulans* hybridizes with DNA from streptomycin-resistant strains of *E. amylovora* but not with DNA from streptomycin-sensitive strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. Streptomycin-resistant strain Ea88-90 of *E. amylovora* was supplied by R. G. Roberts, Tree Fruit Research Laboratory, Wenatchee, WA. All other strains of the bacterium were isolated from apple trees in Michigan during the course of this study. Strains Psp36 and Psp32 of *P. s. papulans* were supplied by T. J. Burr, New York State Agricultural Experiment Station, Geneva. Plasmid pCPP505, containing an internal portion of the streptomycin resistance gene from strain Psp36 of *P. s. papulans* (17), was supplied by J. L. Norelli, New York State Agricultural Experiment Station, Geneva.

Detection of streptomycin-resistant *Erwinia amylovora*. In June 1990, samples of fire blight were received from an apple grower in southwest Michigan (orchard SW) who suspected a problem with streptomycin-resistant *E. amylovora*. Bacteria were isolated from the samples by placing small bits of tissue from infected spurs and shoots on King's medium B (KB) containing 100 μg/ml of streptomycin and 50 μg/ml of cycloheximide (KBsc). The pathogenicity of eight strains isolated from orchard SW was established by inoculating immature Jonathan apple fruit in the laboratory. Upon reisolation, five strains from orchard SW and two strains from an orchard located at the Botany and Plant Pathology Farm, Michigan State University, East Lansing, were tested for resistance to streptomycin by evenly spreading 10^7 colony-forming units per milliliter onto nutrient-yeast-dextrose agar (4). About 5 h later, six 12.7-mm-diameter filter-paper disks (Schleicher & Schuell Inc., Keene, NH) were placed equidistant on the surface of the plates. Aliquots (50 μl) of solution from a streptomycin sulfate dilution series were applied to the disks.

Each treatment was replicated four times. Plates were incubated at 22 C, and inhibition zones were measured after 4 days.

Orchard survey. Spurs and terminals with active fire blight lesions were collected in June and early July 1990 from 20 apple orchards throughout the western Michigan fruit belt, including orchard SW. Infected twigs were also collected from seedling crabapple trees in a 2-ha block adjacent to orchard SW. After pulling back the bark, two small pieces of tissue were removed from the water-soaked tissue distal to the necrosis. One piece of tissue was placed on KB with 50 μg/ml of cycloheximide (KBc), and the second was placed on KBsc. Isolations were made from up to 10 samples per location (25 samples, however, were taken from orchard SW). Nonfluorescent white, semimucoid colonies typical of *E. amylovora* that formed on KBc or KBsc in 72 h at 20 C were streaked onto the selective high-sucrose medium of Crosse and Goodman (5). Bacteria characteristic of *E. amylovora* on the high-sucrose medium were saved for study.

Plasmid characterizations. Sensitive and resistant strains of *E. amylovora* from orchard SW and from crabapple trees adjacent to orchard SW were screened for plasmids using a modification of the method of Kado and Liu (9) as described by Burr et al (3). Plasmid DNA was electrophoresed on 0.5% agarose gel, stained, and photographed as described by Sundin et al (21).

Hybridizations. Plasmid pCPP505 was received, maintained, and amplified in *Escherichia coli* strain DH1. Probe SMP3 was prepared as described by Norelli et al (17). Plasmid DNA, extracted by the alkaline lysis method described by Maniatis et al (12) from cultures grown for 16 h at 37 C in Luria-Bertani (LB) medium containing 50 μg/ml of ampicillin and 50 μg/ml of streptomycin, was digested with restriction enzymes *Bam*HI and *Ava*I. A 500-bp restriction fragment (SMP3) was excised from a low melting temperature agarose gel following electrophoresis in Tris-borate EDTA buffer. The fragment was radiolabeled with ^{32}P by the randomized oligonucleotide labeling procedure (Random Primed DNA Labeling Kit, U.S. Biochemical, Cleveland, OH).

Colony hybridizations with DNA probe SMP3 were performed with streptomycin-sensitive and streptomycin-resistant *E. amylovora*. Up to five sensitive strains from each orchard and all resistant strains were transferred to Colony/Plaque Screen hybridization transfer membranes (New England Nuclear Research Products, Boston, MA) that had been placed on the surface of KB agar plates and incubated for 48 h at 20 C. Colonies of *E. coli* with pCPP505 and strain Psp36 of *P. s. papulans* (streptomycin-resistant) and strain Psp32 of *P. s. papulans* (streptomycin-sensitive) were included on each membrane as positive and negative controls, respectively. The bacteria were lysed and the DNA denatured and fixed to the membranes according to the manufacturer's instructions. Hybridizations were performed overnight and the membranes washed according to the manufacturer's recommended procedures. Autoradiographs of membranes were carried out with XAR X-ray film at −70 C.

Restriction enzyme digests and Southern blots. Plasmid DNA was isolated by alkaline lysis extraction followed by cesium chloride centrifugation (12). Total genomic DNA was prepared by a miniprep procedure (23). The purified plasmid and total genomic DNAs were digested with *Ava*I, and following gel electrophoresis and Southern transfer to GeneScreen hybridization transfer membrane, hybridizations were carried out as described for colony hybridizations.

Bacterial conjugation. Recipient strains were rifampicin-resistant variants of streptomycin-sensitive parental strains EL01, BC06, GR05, and MA05. Donor (HO62-1 and CA11) and recipient strains were grown for 16–24 h at about 22 C on a rotary shaker in 5 ml of LB medium amended with 50 μg/ml of streptomycin (donor strains) or 150 μg/ml of rifampicin (recipient strains). The cultures, each with about 10^{10} cells per milliliter, were mixed in a 1:1 ratio, and 10-μl aliquots were plated on KB medium and incubated 24 h at 22 C. Cell mixtures were suspended in 10 ml sterile distilled water, vortexed, serially diluted, and plated on LB medium amended with 50 μg/ml of streptomycin or 150 μg/ml of rifampicin to determine donor and recipient populations, respectively. Cell mixtures were also plated on LB medium amended with both 50 μg/ml of streptomycin and 150 μg/ml of rifampicin to determine the population of transconjugants. Colonies with good growth on LB medium amended with both antibiotics were considered putative transconjugants. The frequency of spontaneous resistant mutants was determined by plating donor and recipient strains on LB medium amended with rifampicin and streptomycin, respectively.

RESULTS

Detection of resistance. Colonies of *E. amylovora* were recovered on KBsc medium from eight samples of fire blight from orchard SW. When immature fruit were inoculated with these bacteria, all of the strains caused typical symptoms of fire blight, including the production of bacterial ooze.

Level of resistance. Pathogenic strains initially recovered on KBsc medium were highly resistant to streptomycin. Each strain grew to the margin of filter-paper disks containing the highest level (5 μg per disk) of streptomycin sulfate tested. Two strains from the orchard in East Lansing were sensitive to streptomycin, as indicated by the development of clear zones around each disk. Mean size of zones of inhibition for the two sensitive strains were 13.6, 14.2, 15.8, 19.4, 22.2, and 25.0 mm for disks that received 0.06, 0.31, 0.62, 1.25, 2.50, and 5.0 μg of streptomycin sulfate, respectively.

Distribution of resistant strains. *E. amylovora* was recovered on KBc from 137 samples and on KBsc from 20 samples of fire blight collected from 20 apple orchards in western Michigan. All streptomycin-resistant strains (20 out of 25 samples) were recovered from orchard SW. No streptomycin-resistant strains were detected in samples from the remaining 19 orchards. Five streptomycin-resistant strains of the bacterium were also recovered from 15 samples of fire blight collected from crabapple trees adjacent to orchard SW.

Colony hybridization studies. The probe hybridized with plasmid pCPP505 and with DNA from streptomycin-resistant strain Psp36, but not with DNA from streptomycin-sensitive strain Psp32 of *P. s. papulans*. Total DNA from all 25 streptomycin-resistant colonies of *E. amylovora* from orchard SW and the adjacent crabapple trees (but no DNA from 78 colonies of streptomycin-sensitive *E. amylovora*, including five strains from orchard SW) hybridized with the probe in colony blot hybridizations. Probe SMP3 did not hybridize with DNA from streptomycin-resistant *E. amylovora* strain Ea88-90 isolated from pear in Washington.

Plasmid characterizations. Streptomycin-resistant strains from orchard SW and the neighboring crabapple trees contained a plasmid of approximately 33 kb that was not present in streptomycin-sensitive strains isolated from these same locations (Fig. 1). In addition, all strains from these orchards and all other strains of *E. amylovora* examined for plasmid content, including strain Ea88-90 from pear in Washington state, contained a plasmid of approximately 30 kb. Confirmation that this plasmid was the ubiquitous plasmid common to *E. amylovora* (10) was obtained by comparing fragment sizes from *Sal*I, *Pst*I, and *Kpn*I restriction digests of the isolated plasmid with fragment sizes reported in Table 1 of Falkenstein et al (7). Single digests with each of these restriction enzymes of the plasmid isolated from strain Ea88-90 yielded 4, 9, and 5 restriction fragments, respectively. Streptomycin-sensitive strain BC06 contained a large plasmid in addition to the 30-kb plasmid.

Southern analysis. A single 2.7-kb fragment in *Ava*I digests of both plasmid and total genomic DNAs of two streptomycin-resistant strains of *E. amylovora* hybridized with probe SMP3 (Fig. 2, lanes 5, 6, 9, and 10). None of the *Ava*I fragments of DNAs from two streptomycin-sensitive strains of *E. amylovora* hybridized with the probe (Fig. 2, lanes 7, 8, 11, and 12). Hybridization with probe SMP3 also occurred with a 1.5-kb fragment in *Ava*I digests of plasmid and of total genomic DNAs from streptomycin-resistant *P. s. papulans* strain Psp36 (Fig. 2, lanes 1 and 2) and with a 3.7-kb fragment in digested DNAs from

E. coli with plasmid pCPP505 (Fig. 2, lanes 3 and 4).

Bacterial conjugation. Transfer of the plasmid carrying the gene for resistance to streptomycin in donor strains HO62-1 and CA11 was achieved in matings with four recipient strains (Table 1). The frequency of transfer of resistance varied from 2.7×10^{-5} to 4.5×10^{-2}. Spontaneous mutations to streptomycin resistance were less than 2.0×10^{-10}, and spontaneous mutations to rifampicin resistance was about 5.0×10^{-9} per donor cell.

Transfer of streptomycin resistance in *E. amylovora* was associated with transfer of the 33-kb plasmid. All transconjugants resulting from the mating of donor strains CA11 and recipient strains BC06 rif^r and EL01 rif^r contained a 33-kb plasmid not present in the recipient strains (Fig. 3). DNA of putative transconjugants, randomly chosen from antibiotic selection plates, always contained an *Ava*I restriction fragment identical to one in restriction digests of DNA from donor strains but absent in restriction digests of DNA from recipient strains (Fig. 4A). Plasmid DNA from transconjugant BC06 rif^r × HO62-1 contained a 23-kb restriction fragment associated with donor HO62-1 and CA11 but not with recipient BC06 rif^r (Fig. 4A, lanes 4–6). A 2.7-kb *Ava*I restriction fragment in streptomycin-resistant donor and transconjugant strains, but not in streptomycin-sensitive recipient strains, hybridized with probe SMP3 (Fig. 4B, lanes 2–4, 6, and 7).

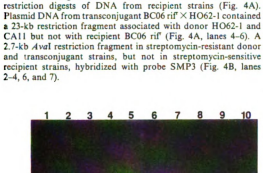

Fig. 1. Plasmids in field strains of *Erwinia amylovora* isolated from apple orchard SW in southwest Michigan and from adjacent crabapple trees. Streptomycin-resistant strains CA13, CA11, HT16, HT01-1, and HT06 are in lanes 1, 3, 6, 7, and 8, respectively, and streptomycin-sensitive strains CA10, CA06, HT03, and HT01 are in lanes 2, 4, 9, and 10, respectively. Lane 5 contains *Erwinia stewartii* strain SW2.

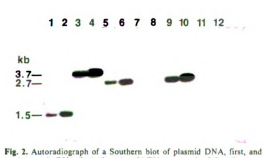

Fig. 2. Autoradiograph of a Southern blot of plasmid DNA, first, and total genomic DNA (including plasmid DNA), second, of *Pseudomonas syringae* pv. *papulans* strain Psp36 (lanes 1 and 2), and DH1 with plasmid pCPP505 (lanes 3 and 4) and streptomycin-resistant (CA11, lanes 5 and 6; HO62-1, lanes 9 and 10) and streptomycin-susceptible (BC06, lanes 7 and 8; EL01, lanes 11 and 12) strains of *Erwinia amylovora* digested with *Ava*I and hybridized to probe SMP3. The sizes (in kb) of hybridizing fragments calculated from *EcoR*I-*Hind*III-digested bacteria phage lambda DNA are given at the far left.

DISCUSSION

The detection of a putative streptomycin-resistance gene in *E. amylovora* that grew on media amended with streptomycin is strong evidence that streptomycin-resistant strains of the bacterium are present in Michigan. In addition, our conjugation and hybridization studies provide evidence that resistance in *E. amylovora* from Michigan is plasmid-borne. In our study, probe SMP3 did not hybridize with DNA from streptomycin-resistant strain Ea88-90 of *E. amylovora* from Washington. The failure of probe SMP3 to hybridize with a streptomycin-resistant strain from the western United States is evidence that the resistance system in the two regions is unrelated.

DNA sequences from streptomycin-resistant *E. amylovora* from Michigan hybridized with DNA sequences from the internal portion of a streptomycin-resistant gene cloned from strain Psp36 of *P. s. papulans*. In previous studies on streptomycin resistance in field strains of *E. amylovora* from California, resistance was found to be chromosomal (18). In addition, a 4.9-kb DNA subclone from streptomycin-resistant *X. c. vesicatoria* did not hybridize with DNA from streptomycin-resistant strain UCBPP 929 of *E. amylovora* from California, but it did hybridize with DNA from strain Psp36 of *P. s. papulans* from New York (15). This is evidence that the DNA sequences in the two pathogens are closely related. Base sequencing and other forms of genetic analyses are needed to establish whether the genes in the two pathogens are identical. Because the DNA sequences associated with resistance in *P. s. papulans* and *X. c. vesicatoria* are related (15), the DNA associated with resistance in *E. amylovora* must also be closely related to the DNA associated with streptomycin-resistance in *X. c. vesicatoria*.

The detection of streptomycin-resistant *E. amylovora* in orchard SW and in crabapple trees next to orchard SW, but not in 19 other orchards, is evidence that resistant strains of the bacterium are currently localized in Michigan's large apple-growing region. The high frequency of transfer of streptomycin resistance from donor strains HO62-1 and CA11 is evidence that transfer of resistance to other strains of the bacterium could result in a rapid increase in streptomycin-resistant *E. amylovora* in Michigan orchards. The spread and buildup of streptomycin-resistant *E.*

TABLE 1. Frequency of conjugational transfer of resistance from streptomycin-resistant to streptomycin-sensitive *Erwinia amylovora*

Recipient strains	Donor strains		Recipient means
	HO62-1	CA11	
EL01	2.8×10^{-5}	2.7×10^{-5}	2.8×10^{-5}
BC06	6.2×10^{-4}	3.8×10^{-4}	5.0×10^{-4}
GR05	3.2×10^{-2}	1.0×10^{-2}	2.1×10^{-2}
MA05	4.5×10^{-2}	2.5×10^{-2}	3.5×10^{-2}

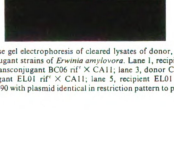

Fig. 3. Agarose gel electrophoresis of cleared lysates of donor, recipient, and transconjugant strains of *Erwinia amylovora*. Lane 1, recipient BC06 rif^r; lane 2, transconjugant BC06 rif^r × CA11; lane 3, donor CA11; lane 4, transconjugant EL01 rif^r × CA11; lane 5, recipient EL01 rif^r; lane 6, strain Ea88-90 with plasmid identical in restriction pattern to pEA29 (7).

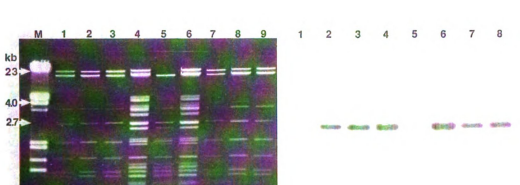

Fig. 4. Ethidium bromide-stained gel (A) and autoradiograph of a Southern blot (B) of plasmid DNAs of donor, recipient, and transconjugant strains of *Erwinia amylovora* digested with *Ava*I and hybridized to probe SMP3. Lane 1, recipient EL01 rif^r; lane 2, transconjugant EL01 rif^r × CA11; lane 3, donor CA11; lane 4, transconjugant **BC06** rif^r × CA11: lane 5, recipient BC06 rif^r; lane 6, transconjugant BC06 rif^r × HO62-1; lane 7 donor HO62-1; lane 8, transconjugant EL01 rif^r × HO62-1; and lane 9, recipient EL01 rif^r. The sizes (in kb) of hybridizing fragments calculated from *Eco*RI-*Hin*dIII-digested bacteria phage lambda DNA are given at the far left.

amylovora presents a serious economic threat to apple and pear growers because of the lack of alternative bactericides for the control of fire blight.

To limit further spread of resistant strains and reduce the likelihood of selecting resistant strains elsewhere in Michigan, it is important that apple and pear growers limit applications of streptomycin to a period from bloom to about the first cover stage of bud development (a maximum of four applications per season). The frequency of spray applications could be reduced even more by using a forecast system to aid in the timing of applications of streptomycin for the control of fire blight (20). Although the occurrence of streptomycin-resistant *E. amylovora* in California and Washington could not be correlated with the use of streptomycin (11,18), it was suggested that selection pressure was not sufficient in the East, due to the limited use of streptomycin, to cause resistant strains to build up to detectable levels (16). Orchard SW has had a history of fire blight problems for over 20 yr, and the management program for fire blight in this orchard has been one of the most intensive in Michigan (A. L. Jones, *personal observation*). Grower records indicate that in 1988, 1989, and 1990 streptomycin was applied in 6, 7, and 8 applications (5.4 kg/ha of actual streptomycin over 3 yr), respectively. The continued and frequent use of streptomycin in orchard SW could have resulted in the buildup of streptomycin-resistant *E. amylovora* to detectable levels. Although Terramycin-streptomycin combinations have been shown to delay resistance in laboratory trials (6), this strategy cannot be utilized until Terramycin is registered on apples.

Because we detected streptomycin-resistant *E. amylovora* in crabapple trees, there is evidence that the resistant strains have started to move out of orchard SW. Now these infected crabapple trees are a potential source of resistant strains for reinfecting orchard SW and for infecting other orchards currently free of resistant strains. Removal of these crabapple trees would reduce the potential for further movement of resistant strains in southwest Michigan. It is also likely that the resistant strains in the two orchards have a common source. Strain HO62-1 from orchard SW and strain CA11 from crabapples carried the resistance gene on the same size plasmid. Also, no restriction-length polymorphism was observed among *Ava*I DNA fragments from the two strains, but *Ava*I digests of DNA from some sensitive strains produced different restriction patterns.

Unlike *P. s. papulans*, with its high diversity in plasmid content (3,8), the plasmid content of *E. amylovora* is predictable and not diverse. All strains have a plasmid of approximately 30 kb, and some strains have an additional plasmid of approximately 65 kb (13). The failure to detect a plasmid of approximately 33 kb in earlier studies on plasmids in *E. amylovora* suggests that it may be a recent introduction into the bacterium. It is possible that a transposable element carrying the resistance gene has been inserted into the approximately 30-kb plasmid, or that *E. amylovora* has acquired a new plasmid. Characterization of the plasmid of approximately 33 kb would help to determine if one of these possibilities is correct.

The streptomycin-resistance gene identified in *E. amylovora* is widely geographically distributed in *P. s. papulans* and in *X. c. vesicatoria* (3,8,15). It is also widely distributed among a diverse group of gram-negative bacteria isolated from apple orchards in New York (17) and in Michigan, including orchard SW (P. Sobiczewski, C.-S. Chiou, and A. L. Jones, *unpublished data*). The presence of a homologous streptomycin-resistance gene in both saprophytic and pathogenic bacteria that inhabit apple suggests that acquisition of streptomycin resistance by *E. amylovora* was by conjugation. We are currently investigating the possibility that the gene can be transferred from saprophytic gram-negative bacteria to *E. amylovora*.

LITERATURE CITED

1. Beer, S. V. 1990. Fire blight. Pages 61-63 in: Compendium of Apple and Pear Diseases. A. L. Jones and H. S. Aldwinckle, eds. American Phytopathological Society, St. Paul, MN.
2. Beer, S. V., and Norelli, J. L. 1976. Streptomycin-resistant *Erwinia amylovora* not found in western New York pear and apple orchards. Plant Dis. Rep. 60:624-626.
3. Burr, T. J., Norelli, J. L., Katz, B., Wilcox, W. F., and Hoying, S. A. 1988. Streptomycin resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. Phytopathology 78:410-413.
4. Coyier, D. L., and Covey, R. P. 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. 59:849-852.
5. Crouse, J. E., and Goodman, R. N. 1973. A selective medium for and a definitive colony characteristic of *Erwinia amylovora*. Phytopathology 63:1425-1426.
6. English, A. R., and Van Halsema, G. 1954. A note on the delay

in emergence of resistant *Xanthomonas* and *Erwinia* strains by the use of streptomycin plus terramycin combinations. Plant Dis. Rep. 38:429-431.
7. Falkenstein, H., Bellemann, P., Walter, S., Zeller, W., and Geider, K. 1988. Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. 54:2798-2802.
8. Jones, A. L., Norelli, J. L., and Ehret, G. R. 1991. Detection of streptomycin-resistant *Pseudomonas syringae* pv. *papulans* in Michigan apple orchards. Plant Dis. 75:529-531.
9. Kado, C. I., and Liu, S. T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
10. Laurent, J., Barny, M.-A., Kotaujansky, A., Dufriche, P., and Vanneste, J. L. 1989. Characterization of a ubiquitous plasmid in *Erwinia amylovora*. Mol. Plant-Microbe Interact. 2:160-164.
11. Loper, J. E., Henkels, M. D., Roberts, R. G., Grove, G. G., Willett, M. J., and Smith, T. J. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. Plant Dis. 75:287-290.
12. Maniatis, R., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
13. Merckaert, C., Thiry, M. E., Thiry, G. J., and Ledous, L. 1982. Characterization of the plasmids present in *Erwinia amylovora*. Arch. Int. Physiol. Biochem. 90:B54-B55.
14. Miller, T. D., and Schroth, M. N. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62:1175-1182.
15. Minsavage, G. V., Canteros, B. I., and Stall, R. E. 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 80:719-723.
16. Moller, W. J., Schroth, M. N., and Thomson, S. V. 1981. The scenario of fire blight and streptomycin resistance. Plant Dis. 65:563-568.
17. Norelli, J. L., Burr, T. J., Lo Cicero, A. M, Gilbert, M. T., and Katz, B. H. 1991. Homologous streptomycin resistance gene present among diverse gram-negative bacteria in New York apple orchards. Appl. Environ. Microbiol. 57:486-491.
18. Schroth, M. N., Thomson, S. V., and Moller, W. J. 1979. Streptomycin resistance in *Erwinia amylovora*. Phytopathology 69:565-568.
19. Shaffer, W. H., and Goodman, R. N. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. (Abstr.) Phytopathology 75:1281.
20. Steiner, P. W. 1990. Predicting apple blossom infections by *Erwinia amylovora* using the Maryblyt model. Acta Hortic. 273:139-148.
21. Sundin, G. W., Jones, A. L., and Fulbright, D. W. 1989. Copper resistance in *Pseudomonas syringae* pv. *syringae* from cherry orchards and its associated transfer in vitro and in planta with a plasmid. Phytopathology 79:861-865.
22. Sutton, T. B., and Jones, A. L. 1975. Monitoring *Erwinia amylovora* populations on apple in relation to disease incidence. Phytopathology 65:1009-1012.
23. Wilson, K. 1988. Preparation of genomic DNA from bacteria. Pages 2.4.1-2.4.5 in: Current Protocols in Molecular Biology. Vol. 1. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. John Wiley & Sons, New York.

PART II

NUCLEOTIDE SEQUENCE ANALYSIS OF A TRANSPOSON (Tn*5393*) CARRYING STREPTOMYCIN RESISTANCE GENES IN *Erwinia amylovora* AND OTHER GRAM-NEGATIVE BACTERIA

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Nucleotide Sequence Analysis of a Transposon (Tn*5393*) Carrying Streptomycin Resistance Genes in *Erwinia amylovora* and Other Gram-Negative Bacteria

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A class II Tn*3*-type transposable element, designated Tn*5393* and located on plasmid pEa34 from streptomycin-resistant strain CA11 of *Erwinia amylovora*, was identified by its ability to move from pEa34 to different sites in plasmids pGEM3Zf(+) and pUCD800. Nucleotide sequence analysis reveals that Tn*5393* consists of 6,705 bp with 81-bp terminal inverted repeats and generates 5-bp duplications of the target DNA following insertion. Tn*5393* contains open reading frames that encode a putative transposase (*tnpA*) and resolvase (*tnpR*) of 961 and 181 amino acids, respectively. The two open reading frames are separated by a putative recombination site (res) consisting of 194 bp. Two streptomycin resistance genes, *strA* and *strB*, were identified on the basis of their DNA sequence homology to streptomycin resistance genes in plasmid RSF1010. *StrA* is separated from *tnpR* by a 1.2-kb insertion element designated IS1133. The *tnpA*-res-*tnpR* region of Tn*5393* was detected in *Pseudomonas syringae* pv. papulans Psp36 and in many other gram-negative bacteria harboring *strA* and *strB*. Except for some strains of *Erwinia herbicola*, these other gram-negative bacteria lacked insertion sequence IS1133. The prevalence of *strA* and *strB* could be accounted for by transposition of Tn*5393* to conjugative plasmids that are then disseminated widely among gram-negative bacteria.

The bacterium *Erwinia amylovora* (Burrill 1882) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers, and Smith 1920 causes fire blight, a disease of apple and pear trees and other rosaceous plants. Streptomycin has been used commercially in the United States, Canada, and New Zealand since the late 1950s to control fire blight and certain other bacterial diseases of plants. Streptomycin-resistant *E. amylovora* has been detected in fruit-growing areas of the western and midwestern United States (3, 4, 33, 34), but little is known about the molecular basis for the resistance in this bacterium. The development of resistant strains of *E. amylovora* is particularly significant because of the lack of alternative methods of control for this devastating disease.

Resistance to streptomycin in *Pseudomonas syringae* pv. papulans, *Xanthomonas compestris* pv. vesicatoria, and *E. amylovora* is often plasmid borne (2, 3, 14, 24). Sequences of plasmid DNAs from these streptomycin-resistant bacteria and from numerous epiphytic gram-negative bacteria isolated from apple orchards cross-hybridized (3, 24, 27, 36). Although the DNA sequences associated with streptomycin resistance in these bacteria were related, the size of the plasmids harboring these sequences varied markedly.

The widespread distribution among plant-pathogenic and plant-associated gram-negative bacteria of related plasmid-borne genes for resistance raises the basic question of how a common determinant for streptomycin resistance has developed on different plasmids. Spread of antibiotic resistance by transposable elements in the bacterial population of human and animal pathogens is well documented (25), but to date no active transposable element has been demonstrated in the movement of antibiotic resistance among plant-pathogenic bacteria.

We report that the genes for streptomycin resistance in *E. amylovora* are located on a transposable element designated Tn*5393* and that resistance in *P. syringae* pv. papulans Psp36 and in many epiphytic gram-negative bacteria from apple orchards is associated with Tn*5393*. In addition, we report that the nucleotide sequence of the genes conferring resistance to streptomycin in *E. amylovora* and epiphytic gram-negative bacteria from apple orchards is homologous to the streptomycin resistance genes on plasmid RSF1010 (10, 32).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) agar or in LB broth at 37°C. All other bacteria were grown on King's medium B (16) agar or in broth at 22°C. Unless otherwise indicated, the media were supplemented with appropriate antibiotics at concentrations in micrograms per milliliter as follows: streptomycin, 50; kanamycin, 50; and ampicillin, 100.

Hybridization studies. Colony and Southern hybridizations were performed by using Colony/Plaque Screen and GeneScreenPlus (New England Nuclear, Boston, Mass.) as described by the manufacturer. Probes were radiolabeled with [α-^{32}P]dCTP by the randomized oligonucleotide labeling procedure (Random Primed DNA Labeling Kit, United States Biochemical, Cleveland, Ohio). Hybridizations were performed overnight, and the membrane was washed according to the manufacturer's recommended procedures. Autoradiography was performed at −70°C with XAR X-ray film.

Cloning, isolation, and restriction mapping of pEa34. Plasmid pEa34, which carries the genes for streptomycin resistance, was introduced into *E. coli* JM109 by conjugation from *E. amylovora* CA11 as previously described (3). Transconjugants were selected on LB agar supplemented with streptomycin and incubated at 37°C. The presence of plas-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Relevant characteristic(s)[a]	Source or reference(s)
Bacterial strains		
Erwinia amylovora		
EL01, BC06	Sm^s	3
CA11	Sm^r, containing pEa34	3
Escherichia coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* λ^- Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔM15]	44
JMEa34-1	Sm^r, JM109 containing pEa34	This study
JMUCD8001	Km^r, JM109 containing pUCD800	This study
JMGEM3	Amp^r, JM109 containing pGEM3Zf(+)	This study
Pseudomonas syringae pv. papulans Psp36	Sm^r, containing pCPP501, isolated in New York	2, 27
Other gram-negative bacteria (147 strains)[b]	Sm^r, various genera	36
Plasmids		
pCPP505	A 2.1-kb fragment with *strA* and *strB* from pCPP501 cloned on pBR322	27
pEa34	Containing Tn*5393* with *strA* and *strB*	This study
pGEMTN-1	Tn*5393* inserted on nucleotide 347 of pGEM3Zf(+)	This study
pGEMTN-7	Tn*5393* inserted on nucleotide 331 of pGEM3Zf(+)	This study
pGEM3Zf(+)	3.2 kb with Amp^r gene	Promega Corp.
pSTRBS	A 2.5-kb *Bam*HI-*Sal*I fragment with *strA* and *strB* from pCPP505 cloned on pGEM3Zf(+)	This study
pUCD800	14.5 kb with Km^r gene	8

[a] Sm^r, streptomycin resistance; Sm^s, streptomycin sensitivity; Km^r, kanamycin resistance; Amp^r, ampicillin resistance. All Sm^r strains contained DNA that hybridized with probe SMP3.

[b] One hundred forty of these strains contained DNA that hybridized with probe SAC32; five strains also contained DNA that hybridized with a 25.6-kb *Sma*I fragment from pEa34.

mid pEa34 in the transconjugants was confirmed by the method of Kado and Liu (15).

Plasmid pEa34 was isolated and purified from transconjugant *E. coli* JMEa34-1 by alkaline lysis extraction followed by centrifugation on CsCl gradients (21). The plasmid was digested with restriction enzymes *Apa*I, *Bam*HI, *Not*I, *Pst*I, *Sac*I, *Sma*I, and *Xba*I, and the sizes of restriction fragments were estimated by coelectrophoresis with 1-kb DNA ladder markers (GIBCO-BRL, Grand Island, N.Y.). Restriction fragments were cloned into vector pGEM3Zf(+) to investigate the location of the streptomycin resistance genes.

Detection of a transposon from pEa34. To test whether the determinant for streptomycin resistance on pEa34 was transposable, high-copy-number plasmids pGEM3Zf(+) and pUCD800 were used as recipient replicons to entrap the element. Plasmid pEa34 was introduced into *E. coli* JMGEM3 and JMUCD800 by conjugation from *E. amylovora* CA11 or *E. coli* JMEa34-1. Twenty-four transconjugant colonies were chosen randomly from each combination and grown in 5 ml of LB medium with streptomycin and either ampicillin or kanamycin for 16 h at 37°C and then subjected to plasmid analysis (15). Plasmid DNA from each transconjugant clone was isolated by alkaline lysis (21) and then transformed into competent *E. coli* JM109 cells (26). Transformants were selected by plating onto LB medium supplemented with ampicillin or kanamycin, followed by replica plating of the resultant colonies onto LB medium supplemented with the appropriate antibiotic plus streptomycin. Colonies resistant to streptomycin and either ampicillin or kanamycin and containing a plasmid only about 6.7 kb larger than the recipient replicons were selected for restriction enzyme analysis. Restriction mapping with *Sac*I, *Ava*I, *Pst*I, and *Hin*dIII and hybridization with radiolabeled pEa34 DNA were used to determine which portion of pEa34 had inserted into the plasmids and the insertion sites.

Cloning and sequencing of DNA. Plasmids pSTRBS, pGEMTN-1, and pGEMTN-7 were used as the source DNA for nucleotide sequencing. Plasmid pSTRBS was constructed by cloning a 2.5-kb *Bam*HI-*Sal*I DNA fragment of pCPP505 (27) in vector pGEM3Zf(+). Plasmids pGEMTN-1 and pGEMTN-7 resulted from the capture of transposon Tn*5393* in opposite directions on nucleotides 347 and 331, respectively, of pGEM3Zf(+). Deletions of the plasmids were performed with ExoIII (Erase-a-Base kit; Promega Corp., Madison, Wis.). A nested series of deletion subclones of 200 to 250 bp each was selected, and their sequences were determined by the dideoxy chain-termination method using denatured plasmid templates (11, 31). Nucleotide dGTP was replaced by dITP in some sequencing reactions to reduce band compression of the nucleotide sequence (Sequenase version 2.0, United States Biochemical). All sequences were confirmed by sequencing both strands from at least two overlapping clones. The nucleotide sequences and the deduced amino acid sequences were analyzed with software from the University of Wisconsin Genetics Computer Group (UWGCG software, version 7.0; 5).

Detection of Tn*5393* and pEa34 in other gram-negative bacteria. A 3.2-kb *Sac*I fragment from Tn*5393*, designated SAC32 (Fig. 1B), was used as a probe in colony and Southern hybridizations to detect transposon Tn*5393*. Colony hybridization was used to screen 147 strains of streptomycin-resistant epiphytic gram-negative bacteria, which contained DNA that previously had hybridized with probe SMP3 (36). Southern hybridization was conducted to analyze *Sac*I-digested plasmid DNAs from *P. syringae* pv. papulans Psp36, *E. amylovora* EL01 and CA11, eight strains

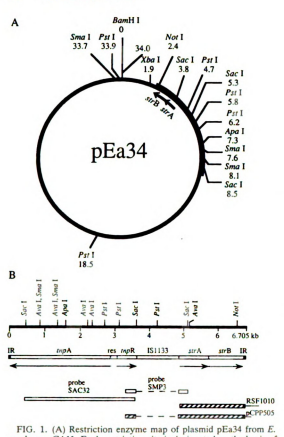

FIG. 1. (A) Restriction enzyme map of plasmid pEa34 from *E. amylovora* CA11. Each restriction site is designated on the basis of its distance (in kilobases) from the unique *Bam*HI site. The location of streptomycin resistance genes *strA* and *strB* is indicated with a double arrow, and the location of transposon Tn*5393* is indicated with a thick line. (B) The proposed genetic map for transposon Tn*5393*. The proposed elements in Tn*5393* are IR, transposase gene (*tnpA*), region res, resolvase gene (*tnpR*), insertion element (IS1133), *strA* and *strB* genes, and IR, respectively. The directions of transcription of the genes are indicated with arrows. The locations of probe SMP3 from *P. syringae* pv. papulans Psp36 and of probe SAC32 are shown by the open boxes immediately below the genetic map. The locations of DNA sequences from RSF1010 and pCPP505 that are identical to the sequence in Tn*5393* are shown by the slashed boxes, and the IR sequences are shown by the filled boxes.

of the epiphytic bacteria which hybridized to SAC32 in colony hybridization, and two strains that did not hybridize.

To detect plasmid pEa34, a 25.6-kb *Sma*I fragment from pEa34 was used as a probe in colony hybridizations with *E. amylovora* EL01 and the 147 strains mentioned above. Plasmid pEa34 was used as the probe in Southern analysis of *Ava*I-digested plasmid DNAs from six selected strains of *Erwinia herbicola*.

Nucleotide sequence accession number. The GenBank accession number for the 6,705-bp transposon sequence is M96392.

RESULTS

Restriction map of pEa34. Plasmid pEa34 was transferred from *E. amylovora* CA11 into *E. coli* JM109 by conjugation; plasmid DNA was isolated, and its restriction map was determined. The unique *Bam*HI site was used as a reference point for the restriction map of plasmid pEa34 (Fig. 1A). The plasmid also contained unique *Apa*I, *Not*I, and *Xba*I sites; three *Sac*I sites; three *Sma*I sites; and five *Pst*I sites. On the basis of the size of the restriction fragments, the plasmid was determined to be 34 kb in size. The smallest restriction fragment to confer resistance to streptomycin when ligated into plasmid pGEM3Zf(+) was located between the *Xba*I and *Pst*I (4.7) sites and was 2.8 kb in size.

Detection of a transposable element from pEa34. When plasmid DNAs from 24 transconjugant colonies with pGEM3Zf(+) as the recipient replicon were examined, cells from 12 colonies contained a 9.9-kb plasmid that was not detected in the donor or recipient strains (data not shown). Cells of *E. coli* JM109 transformed with the 9.9-kb plasmid were resistant to both streptomycin and ampicillin. The high-copy-number plasmid was about 6.7 kb larger than the recipient replicon. Restriction mapping and Southern analysis of 9.9-kb plasmids from several transformants revealed that a 6.7-kb fragment from pEa34 had inserted into different sites in pGEM3Zf(+). Similar results were obtained when plasmid DNAs from 24 transconjugant colonies with pUCD800 as the recipient replicon were examined. The 6.7-kb fragment from pEa34 was considered to be a transposable element and was designated transposon Tn*5393*.

Sequence and coding regions of transposon Tn*5393*. The nucleotide sequence of Tn*5393* (Fig. 2) was analyzed to produce a restriction enzyme map and proposed genetic map for the transposon (Fig. 1B). A search of the GenBank data base indicated that Tn*5393* contained four functional open reading frames (ORFs) located between the 81-bp inverted repeats. The size and orientation of ORFs C and D suggested that ORF C encodes a transposase (*tnpA*) and ORF D encodes a resolvase (*tnpR*). ORF C encodes a polypeptide of 961 amino acids, is transcribed in a direction opposite to that of the other ORFs, and is terminated by a stop codon within the inverted repeat (IR). When the data base was searched for a putative polypeptide encoded by ORF C, identity of 29, 40, 31, 38, and 32% and similarity of 51, 61, 54, 57, and 56% with transposases of Tn*3*, Tn*21*, Tn*917*, Tn*2501*, and Tn*4430*, respectively, were found (1, 12, 20, 41, 42). ORF D encodes a polypeptide of 181 amino acids. It exhibits 34, 34, 36, and 37% identity and 56, 52, 60, and 56% similarity with resolvases of Tn*3*, Tn*21*, Tn*917*, and Tn*2501*, respectively (6, 12, 23, 35). However, ORF D revealed higher homology to integrases of *E. coli* and bacteriophage Mu and recombinases of *Shigella boydii* and bacteriophage P1 with 48, 47, 47, and 44% identity and 65, 63, 63, and 62% similarity, respectively (13, 28, 29, 40). A stretch of 194 bp, with two putative promoter sequences (nucleotides 2945 to 2940 and 3057 to 3062) for the transcription of *tnpA* and *tnpR*, separates ORFs C and D.

ORFs A and B encoded polypeptides of 267 and 278 amino acids, respectively. Computer-aided sequence comparisons indicated that ORFs A and B share DNA sequence identity with ORFs H and I of plasmid RSF1010. The nucleotide sequence of ORF A differed by 3 bp (nucleotides 5163, 5426, and 5429) from the sequence of ORF H, and the sequence of ORF B was identical to the sequence of ORF I in RSF1010. These ORFs were designated *strA* and *strB* by Scholz et al. (32). A 1.2-kb sequence (nucleotides 3660 to 4891) with 27-bp

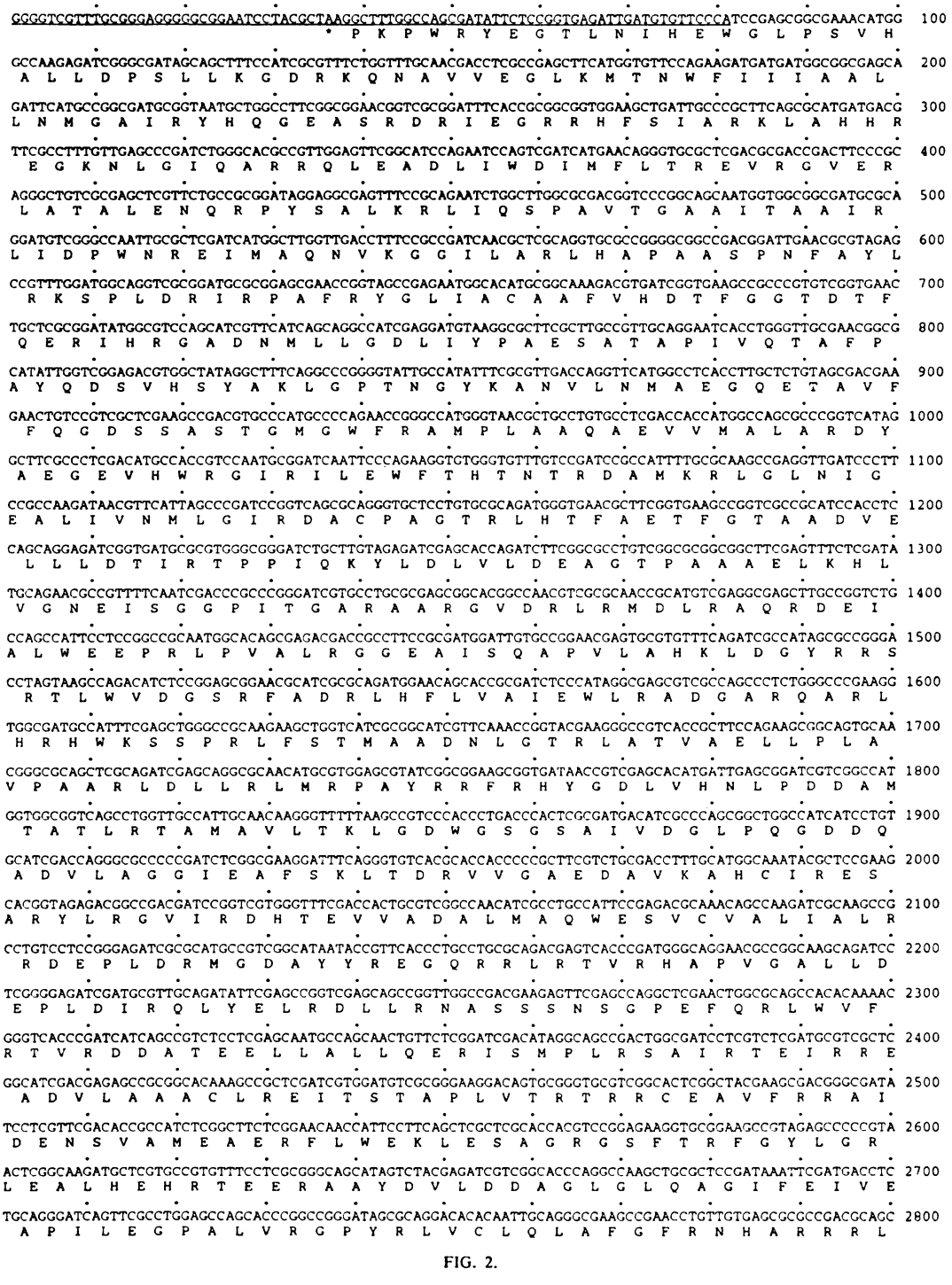
GGGGTCGTTTGCGGGAGGGGGCGGAATCCTACGCTAAGGCTTTGGCCAGCGATATTCTCCGGTGAGATTGATGTGTTCCCATCCGAGCGGCGAAACATGG 100
* P K P W R Y E G T L N I H E W G L P S V H
GCCAAGAGATCGGGCGATAGCAGCTTTCCATCGCGTTTCTGGTTTGCAACGACCTCGCCGAGCTTCATGGTGTTCCAGAAGATGATGATGGCGGCGAGCA 200
A L L D P S L L K G D R K Q N A V V E G L K M T N W F I I I A A L
GATTCATGCCGGCGATGCGGTAATGCTGGCCTTCGGCGGAACGGTCGCGGATTTCACCGCGGCGGTGGAAGCTGATTGCCCGCTTCAGCGCATGATGACG 300
L N M G A I R Y H Q G E A S R D R I E G R R H F S I A R K L A H H R
TTCGCCTTTGTTGAGCCCGATCTGGGCACGCCGTTGGAGTTCGGCATCCAGAATCCAGTCGATCATGAACAGGGTGCGCTCGACGCGACCGACTTCCCGC 400
E G K N L G I Q A R R Q L E A D L I W D I M F L T R E V R G V E R
AGGGCTGTCGCGAGCTCGTTCTGCCGCGGATAGGAGGCGAGTTTCCGCAGAATCTGGCTTGGCGCGACGGTCCCGGCAGCAATGGTGGCGGCGATGCGCA 500
L A T A L E N Q R P Y S A L K R L I Q S P A V T G A A I T A A I R
GGATGTCGGGCCAATTGCGCTCGATCATGGCTTGGTTGACCTTTCCGCCGATCAACGCTCGCAGGTGCGCCGGGGCGGCCGACGGATTGAACGCGTAGAG 600
L I D P W N R E I M A Q N V K G G I L A R L H A P A A S P N F A Y L
CCGTTTGGATGGCAGGTCGCGGATGCGCGGAGCGAACCGGTAGCCGAGAATGGCACATGCGGCAAAGACGTGATCGGTGAAGCCGCCCGTGTCGGTGAAC 700
R K S P L D R I R P A F R Y G L I A C A A F V H D T F G G T D T F
TGCTCGCGGATATGGCGTCCAGCATCGTTCATCAGCAGGCCATCGAGGATGTAAGGCGCTTCGCTTGCCGTTGCAGGAATCACCTGGGTTGCGAACGGCG 800
Q E R I H R G A D N M L L G D L I Y P A E S A T A P I V Q T A F P
CATATTGGTCGGAGACGTGGCTATAGGCTTTCAGGCCCGGGGTATTGCCATATTTCGCGTTGACCAGGTTCATGGCCTCACCTTGCTCTGTAGCGACGAA 900
A Y Q D S V H S Y A K L G P T N G Y K A N V L N M A E G Q E T A V F
GAACTGTCCGTCGCTCGAAGCCGACGTGCCCATGCCCCAGAACCGGGCCATGGGTAACGCTGCCTGTGCCTCGACCACCATGGCCAGCGCCCGGTCATAG 1000
F Q G D S S A S T G M G W F R A M P L A A Q A E V V M A L A R D Y
GCTTCGCCCTCGACATGCCACCGTCCAATGCGGATCAATTCCCAGAAGGTGTGGGTGTTTGTCCGATCCGCCATTTTGCGCAAGCCGAGGTTGATCCCTT 1100
A E G E V H W R G I R I L E W F T H T N T R D A M K R L G L N I G
CCGCCAAGATAACGTTCATTAGCCCGATCCGGTCAGCGCAGGGTGCTCCTGTGCGCAGATGGGTGAACGCTTCGGTGAAGCCGGTCGCCGCATCCACCTC 1200
E A L I V N M L G I R D A C P A G T R L H T F A E T F G T A A D V E
CAGCAGGAGATCGGTGATGCGCGTGGGCGGGATCTGCTTGTAGAGATCGAGCACCAGATCTTCGGCGCCTGTCGGCGCGGCGGCTTCGAGTTTCTCGATA 1300
L L L D T I R T P P I Q K Y L D L V L D E A G T P A A A E L K H L
TGCAGAACGCCGTTTTCAATCGACCCGCCCGGGATCGTGCCTGCGCGAGCGGCACGGCCAACGTCGCGCAACCGCATGTCGAGGCGAGCTTGCCGGTCTG 1400
V G N E I S G G P I T G A R A A R G V D R L R M D L R A Q R D E I
CCAGCCATTCCTCCGGCCGCAATGGCACAGCGAGACGACCGCCTTCCGCGATGGATTGTGCCGGAACGAGTGCGTGTTTCAGATCGCCATAGCGCCGGGA 1500
A L W E E P R L P V A L R G G E A I S Q A P V L A H K L D G Y R R S
CCTAGTAAGCCAGACATCTCCGGAGCGGAACGCATCGCGCAGATGGAACAGCACCGCGATCTCCCATAGGCGAGCGTCGCCAGCCCTCTGGGCCCGAAGG 1600
R T L W V D G S R F A D R L H F L V A I E W L R A D G A R Q A R L
TGGCGATGCCATTTCGAGCTGGGCCGCAAGAAGCTGGTCATCGCGGCATCGTTCAAACCGGTACGAAGGGCCGTCACCGCTTCCAGAAGCGGCAGTGCAA 1700
H R H W K S S P R L F S T M A A D N L G T R L A T V A E L L P L A
CGGGCGCAGCTCGCAGATCGAGCAGGCGCAACATGCGTGGAGCGTATCGGCGGAAGCGGTGATAACCGTCGAGCACATGATTGAGCGGATCGTCGGCCAT 1800
V P A A R L D L L R L M R P A Y R R F R H Y G D L V H N L P D D A M
GGTGGCGGTCAGCCTGGTTGCCATTGCAACAAGGGTTTTTAAGCCGTCCCACCCTGACCCACTCGCGATGACATCGCCCAGCGGCTGGCCATCATCCTGT 1900
T A T L R T A M A V L T K L G D W G S G S A I V D G L P Q G D D Q
GCATCGACCAGGGCGCCCCCGATCTCGGCGAAGGATTTCAGGGTGTCACGCACCACCCCCGCTTCGTCTGCGACCTTTGCATGGCAAATACGCTCCGAAG 2000
A D V L A G G I E A F S K L T D R V V G A E D A V K A H C I R E S
CACGGTAGAGACGGCCGACGATCCGGTCGTGGGTTTCGACCACTGCGTCGGCCAACATCGCCTGCCATTCCGAGACGCAAACAGCCAAGATCGCAAGCCG 2100
A R Y L R G V I R D H T E V V A D A L M A Q W E S V C V A L I A L R
CCTGTCCTCCGGGAGATCGCGCATGCCGTCGGCATAATACCGTTCACCCTGCCTGCGCAGACGAGTCACCCGATGGGCAGGAACGCCGGCAAGCAGATCC 2200
R D E P L D R M G D A Y Y R E G Q R R L R T V R H A P V G A L L D
TCGGGGAGATCGATGCGTTGCAGATATTCGAGCCGGTCGAGCAGCCGGTTGGCCGACGAAGAGTTCGAGCCAGGCTCGAACTGGCGCAGCCACACAAAAC 2300
E P L D I R Q L Y E L R D L L R N A S S S N S G P E F Q R L W V F
GGGTCACCCGATCATCAGCCGTCTCCTCGAGCAATGCCAGCAACTGTTCTCGGATCGACATAGGCAGCCGACTGGCGATCCTCGTCTCGATGCGTCGCTC 2400
R T V R D D A T E E L L A L L Q E R I S M P L R S A I R T E I R R E
GGCATCGACGAGAGCCGCGGCACAAAGCCGCTCGATCGTGGATGTCGCGGGAAGGACAGTGCGGGTGCGTCGGCACTCGGCTACGAAGCGACGGGCGATA 2500
A D V L A A A C L R E I T S T A P L V T R T R R C E A V F R R A I
TCCTCGTTCGACACCGCCATCTCGGCTTCTCGGAACAACCATTCCTTCAGCTCGCTCGCACCACGTCCGGAGAAGGTGCGGAAGCCGTAGAGCCCCCGTA 2600
D E N S V A M E A E R F L W E K L E S A G R G S F T R F G Y L G R
ACTCGGCAAGATGCTCGTGCCGTGTTTCCTCGCGGGCAGCATAGTCTACGAGATCGTCGGCACCCAGGCCAAGCTGCGCTCCGATAAATTCGATGACCTC 2700
L E A L H E H R T E E R A A Y D V L D D A G L G L Q A G I F E I V E
TGCAGGGATCAGTTCGCCTGGAGCCAGCACCCGGCCGGGATAGCGCAGGACACACAATTGCAGGGCGAAGCCGAACCTGTTGTGAGCGCGCCCGACGCAGC 2800
A P I L E G P A L V R G P Y R L V C L Q L A F G F R N H A R R R L

FIG. 2.

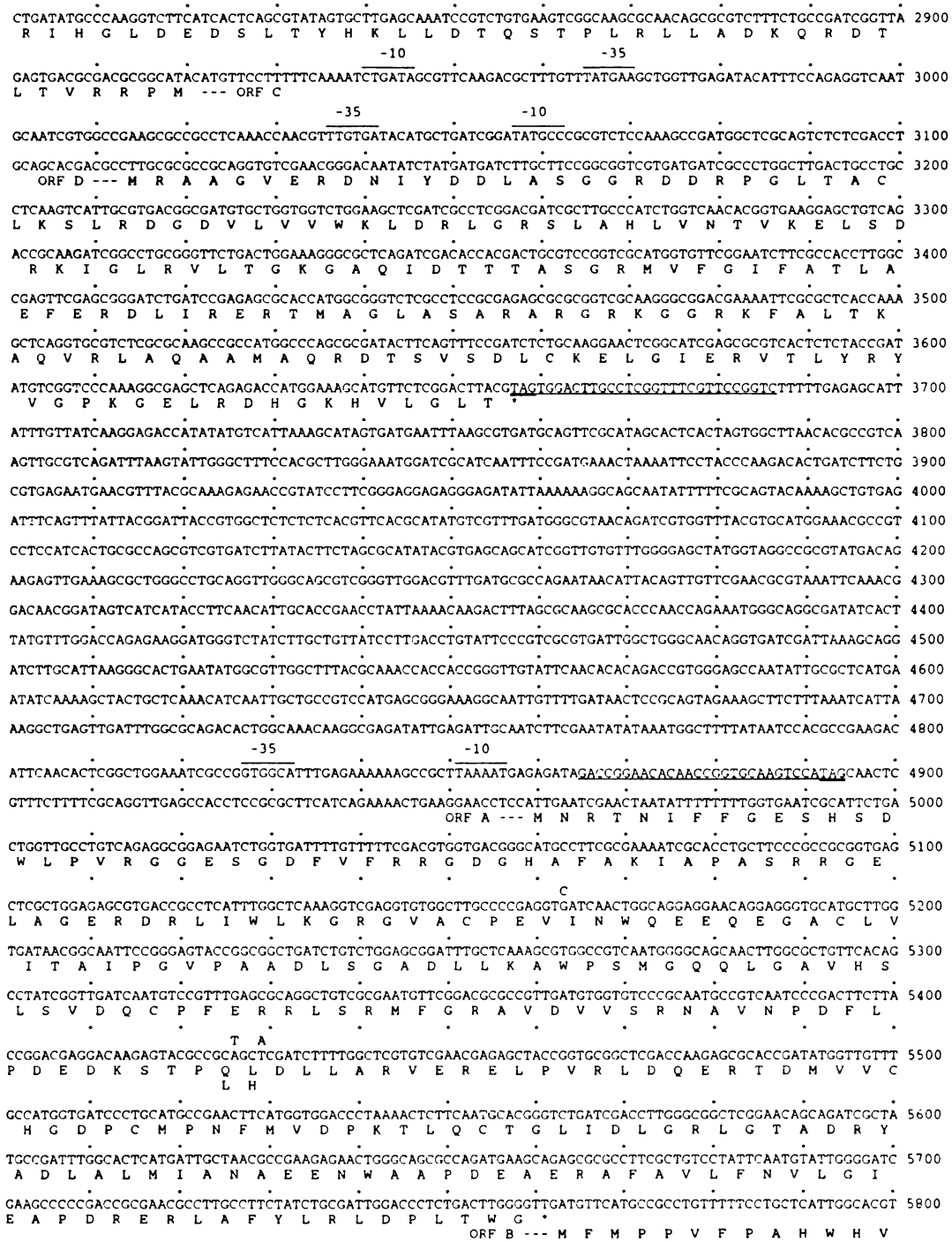

FIG. 2—*Continued.*

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TTCGCAACCTGTTCTCATTGCGGACACCTTTTCCAGCCTCGTTTGGAAAGTTTCATTGCCAGACGGGACTCCTGCAATCGTCAAGGGATTGAAACCTATA 5900
 S  Q  P  V  L  I  A  D  T  F  S  S  L  V  W  K  V  S  L  P  D  G  T  P  A  I  V  K  G  L  K  P  I

GAAGACATTGCTGATGAACTGCGCGGGGCCGACTATCTGGTATGGCGCAATGGGAGGGGAGCAGTCCGGTTGCTCGGTCGTGAGAACAATCTGATGTTGC 6000
E  D  I  A  D  E  L  R  G  A  D  Y  L  V  W  R  N  G  R  G  A  V  R  L  L  G  R  E  N  N  L  M  L  L

TCGAATATGCCGGGGAGCGAATGCTCTCTCACATCGTTGCCGAGCACGGCGACTACCAGGCGACCGAAATTGCAGCGGAACTAATGGCGAAGCTGTATGC 6100
  E  Y  A  G  E  R  M  L  S  H  I  V  A  E  H  G  D  Y  Q  A  T  E  I  A  A  E  L  M  A  K  L  Y  A

CGCATCTGAGGAACCCCTGCCTTCTGCCCTTCTCCCGATCCGGGATCGCTTTGCAGCTTTGTTTCAGCGGGCGCGCGATGATCAAAACGCAGGTTGTCAA 6200
 A  S  E  E  P  L  P  S  A  L  L  P  I  R  D  R  F  A  A  L  F  Q  R  A  R  D  D  Q  N  A  G  C  Q

ACTGACTACGTCCACGCGGCGATTATAGCCGATCAAATGATGAGCAATGCCTCGGAACTGCGTGGGCTACATGGCGATCTGCATCATGAAAACATCATGT 6300
T  D  Y  V  H  A  A  I  I  A  D  Q  M  M  S  N  A  S  E  L  R  G  L  H  G  D  L  H  H  E  N  I  M  F

TCTCCAGTCGCGGCTGGCTGGTGATAGATCCCGTCGGTCTGGTCGGTGAAGTGGGCTTTGGCGCCGCCAATATGTTCTACGATCCGGCTGACAGAGACGA 6400
  S  S  R  G  W  L  V  I  D  P  V  G  L  V  G  E  V  G  F  G  A  A  N  M  F  Y  D  P  A  D  R  D  D

CCTTTGTCTCGATCCTAGACGCATTGCACAGATGGCGGACGCATTCTCTCGTGCGCTGGACGTCGATCCGCGTCGCCTGCTCGACCAGGCGTACGCTTAT 6500
 L  C  L  D  P  R  R  I  A  Q  M  A  D  A  F  S  R  A  L  D  V  D  P  R  R  L  L  D  Q  A  Y  A  Y

GGGTGCCTTTCCGCAGCTTGGAACGCGGATGGAGAAGAGGAGCAACGCGATCTAGCTATCGCGGCCGCGATCAAGCAGGTGCGACAGACGTCATACTAGA 6600
G  C  L  S  A  A  W  N  A  D  G  E  E  E  Q  R  D  L  A  I  A  A  A  I  K  Q  V  R  Q  T  S  Y  *

TATCAAGCGACTTCTCCTATCCCCTGGGAACACATCAATCTTACCGGAGAATATCGTTGGCCAAAGCCTTAGCGTAGGATTTCGCCCTCTCCCGCAAACG 6700
ACCCC                                                                                                    6705
```

FIG. 2. Complete nucleotide sequence of Tn*5393*, numbered from the IR on the *tnpA* end, is shown in 5' to 3' orientation. The positions of ORFs corresponding to four predicted polypeptides (A, B, C, and D) are indicated with their encoded amino acid sequence. ORF C is transcribed from the strand opposite to ORFs A, B, and D. The nucleotide sequence of insertion sequence IS1133 is from nucleotides 3660 to 4891. The 3-bp direct repeats that border IS1133 (TAG) are double underlined, and the IRs of Tn*5393* and IS1133 are underlined. The −10 and −35 consensus sequences of three putative promoters are indicated above the sequence by a line and the number. Asterisks indicate stop codons. The nucleotide and amino acid sequences of ORF A that differ from those of ORF I in RSF1010 are indicated above the nucleotide and beneath the amino acid, respectively. Sequence 4928 to 6705 of Tn*5393* is homologous to sequence 31 to 1808 of RSF1010 (32).

IRs and 3-bp duplicates separated *tnpR* and *strA* and was designated insertion element IS1133. Its nucleotide sequence shared 45% identity with insertion sequence IS3 (39). IS1133 provided a promoter (nucleotides 4851 to 4856) for transcription of the resistance genes. When nucleotides were deleted from pGEMTN-7, streptomycin resistance was lost when deletions were made downstream, but not when deletions were made upstream, of the promoter sequence (data not shown).

The 81-bp IRs of Tn*5393* were about twice the length of IRs typically found in Tn*3*-type transposons. The sequences of the two IRs (IR-5393o and IR-5393t) differed by 4 bp (Fig. 3A), and both IRs were flanked by 5-bp direct repeats. The stop codon for ORF C was located in nucleotide positions 34 to 36 of IR-5393t. A stop codon was also found in the IRs of transposons Tn*3*, Tn*21*, and Tn*2501*, but not in the IRs of transposon Tn*917* or Tn*4430* (Fig. 3B). The sequence of IR-5393t differed by 1 bp, and the sequence of IR-5393o differed by 4 bp, from nucleotides 1728 to 1808 in plasmid RSF1010 (32; Fig. 3A).

Identity of streptomycin resistance in *P. syringae* pv. papulans and *E. amylovora*. The nucleotide sequence of the 2.5-kb *Bam*HI-*Sal*I fragment from pCPP505, a DNA clone from *P. syringae* pv. papulans Psp36 that confers resistance to streptomycin in *E. coli* (27), was compared with the sequence of Tn*5393*. The first 241 nucleotides in the DNA fragment from pCPP505 were identical to nucleotides 3419 to 3659 in *tnpR* of Tn*5393*, and nucleotides 242 to 2052 were identical to the sequence for the *strA* and *strB* genes of Tn*5393*. The nucleotide sequence of IS1133 and a direct repeat (TGA) in Tn*5393* were absent in the fragment from pCPP505. Fragment SMP3 from *P. syringae* pv. papulans (27), which has been used as a probe for the detection of streptomycin resistance genes in many gram-negative bacteria (27, 36), consists of sequences from the *tnpR* and *strA* genes of Tn*5393* (Fig. 1B).

Tn*5393* in other gram-negative bacteria. In colony hybridizations, probe SAC32 hybridized with DNA from 140 of 147 epiphytic gram-negative bacteria from the study of Sobiczewski et al. (36). In Southern hybridizations, the probe hybridized with a 3.2-kb *Sac*I restriction fragment from *P. syringae* pv. papulans Psp36, *E. amylovora* CA11, and eight strains of epiphytic bacteria, which reacted positively to SAC32 in colony hybridizations. However, the fragment was

A.

```
                10         20         30         40         50
IR-5393o  GGGGTCGTTT GCGGGAGAGG GCGAAATCCT ACGCTAAGGC TTTGGCCAAC
IR-5393t  GGGGTCGTTT GCGGGAGGGG GCGGAATCCT ACGCTAAGGC TTTGGCCAGC
RSF1010   GGGGTCGTTT GCGGGAAGGG GCGGAATCCT ACGCTAAGGC TTTGGCCAGC

                60         70         80
IR-5393o  GATATTCTCC GGTAAGATTG ATGTGTTCCC A
IR-5393t  GATATTCTCC GGTGAGATTG ATGTGTTCCC A
RSF1010   GATATTCTCC GGTGAGATTG ATGTGTTCCC A
```

B.

```
                10         20         30         40
IR-5393t  GGGGTCGTTT GCGGGAGGGG GCGGAATCCT ACGCTAAGGC TTTGGCCA····
IR-3t     GGGGTCTGAC GGTCAGTGGA ACGGAAACTC ACGCTAAAG             (22)
IR-21t    GGGGTCGTCT CAGAAAACGG AAAATAAAGC ACGCTAAG              (22)
IR-917t   GGGGTCCCGA GCGCTTAGTG GGAATTTGTA TCGACTAAG            (19)
IR-2501t  GGGGTCCGCC TGGAAAACGG AAATTATCCC ACGCTAAGAC TGTTTTTT  (22)
IR-4430t  GGGGTACCGC CAGCATTTCG GAAAAAAACC ACGCTAAG             (19)
```

FIG. 3. Comparison of IR sequences. (A) The 81-bp IRs of Tn*5393* (IR-5393t and IR-5393o, where t is the *tnpA* end and o is the other end) and the sequence of plasmid RSF1010. Boldface type shows positions where residues were different. (B) IRs in representative transposons from the Tn*3* family (Tn*5393*, Tn*3*, Tn*21*, Tn*917*, Tn*2501*, and Tn*4430*) are all listed from the *tnpA* end. The stop codons for the transposases in Tn*5393*, Tn*3*, Tn*21*, and Tn*2501* are underlined. The number in parentheses is the number of identical base pairs between each IR and IR-5393t. Sequences are from Scholz et al. (32) for RSF1010, Heffron et al. (12) for Tn*3*, Zheng et al. (45) for Tn*21*, An and Clewell (1) for Tn*917*, Michiels and Cornelis (22) for Tn*2501*, and Mahillon and Lereclus (20) for Tn*4430*.

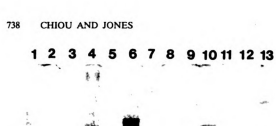

FIG. 4. Autoradiograph of a Southern blot of plasmid DNAs from streptomycin-resistant (except lane 3) gram-negative bacteria digested with *Sac*I and hybridized to probe SAC32. Lane 1, *P. syringae* pv. papulans Psp36; lane 2, *E. amylovora* CA11; lane 3, *E. amylovora* EL01; lane 4, *Acinetobacter* sp. strain 1; lane 5, *E. herbicola* 6a; lane 6, *E. herbicola* 34; lane 7, a yellow *Pseudomonas* strain, 40; lane 8, *P. syringae* pv. syringae 45; lane 9, *Pseudomonas fluorescens* biovar III, 57; lane 10, *Pseudomonas aeruginosa* 60; lane 11, *P. putida* 61; lane 12, *Aeromonas* sp. strain 145; lane 13, *E. herbicola* 144. The size of the 3.2-kb hybridizing fragment is indicated at the far left.

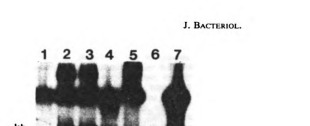

FIG. 5. Hybridization of a 25.6-kb *Sma*I fragment from plasmid pEa34 with *Ava*I-digested total plasmid DNAs from streptomycin-resistant strains of *E. herbicola*. Lanes 1 to 6 contain DNAs from strains 52, 50a, 144, 35a, 18, and 6a, respectively. Lane 7 contains plasmid pEa34 from *E. amylovora* CA11. Strain 6a was a negative control. The three *Ava*I fragments indicated (by size in kilobases) at the far left are internal fragments of Tn*5393*. A 127-bp fragment is not seen.

not detected in streptomycin-sensitive *E. amylovora* EL01 and streptomycin-resistant *Pseudomonas putida* 61 or *Aeromonas* sp. strain 145 (Fig. 4).

Plasmid pEa34 in other gram-negative bacteria. In colony hybridizations, a 25.6-kb *Sma*I fragment from pEa34 hybridized with DNAs from five strains of *E. herbicola* and from streptomycin-sensitive *E. amylovora* EL01 but not with DNAs from 142 additional epiphytic gram-negative bacterial strains. When plasmid DNA was separated by agarose gel electrophoresis, the five strains of *E. herbicola* each contained a 34-kb plasmid, whereas *E. amylovora* EL01 contained a 28-kb plasmid. Among the five strains of *E. herbicola*, only three strains showed *Ava*I restriction patterns identical to that of plasmid pEa34 in Southern blots when hybridized with radiolabeled pEa34 DNA (Fig. 5), indicating that the transposon inserted on different sites of the prototype plasmid. In conjugation studies, a 34-kb plasmid was transferred from *E. herbicola* 144 to *E. coli* JM109, JM-GEM3, and JMUCD800 and to *E. amylovora* BC06 in high frequency (data not shown).

DISCUSSION

We suggested in a previous paper that the plasmid pEa34 may have arisen from the insertion of a transposable element into an existing plasmid or that *E. amylovora* may have acquired a new plasmid (3). Except for streptomycin-resistant *E. amylovora* from Michigan, a 34-kb plasmid resembling pEa34 has not been reported among strains of *E. amylovora* examined for cryptic plasmids (7, 15, 37). Plasmid pEa34 likely originated from the insertion of Tn*5393* into a 28-kb plasmid; we detected a 28-kb plasmid with DNA homologous to pEa34 in streptomycin-sensitive strain EL01 of *E. amylovora*. Hybridization studies indicated that the 28-kb plasmid and plasmid pEa34 were not related to the 29-kb universal plasmid found in pathogenic strains of *E. amylovora* (7, 18). Whether sensitive strains of *E. herbicola* harbor a 28-kb plasmid homologous to the plasmid found in strain EL01 needs to be determined.

We classify Tn*5393* as a class II, Tn*3*-like transposon because it makes a 5-bp target duplication, and its *tnpA* and *tnpR* are transcribed in opposite directions (17). A large number of antibiotic resistance transposons isolated from gram-negative bacteria associated with humans and animals are Tn*3*-like elements carried often by conjugative plasmids. In this study, we extend the host range of Tn*3*-like transposons to gram-negative bacteria associated with plants. The detection of streptomycin resistance in many gram-negative bacteria associated with plants may be explained by the transposition of Tn*5393* to another plasmid followed by conjugational transfer of the plasmid within a bacterial species and later between ecologically associated species.

Tn*3* transposons have been divided into five subgroups (9). The near-perfect 81-bp IRs at the ends of Tn*5393* were much longer than the 38- to 45-bp IRs common to other transposons in this family (9), and amino acid sequence identity of transposases and resolvases between Tn*5393* and other Tn*3*-like transposons was low (29 to 40%). Therefore, our results suggest that Tn*5393* should belong to a new subgroup in the Tn*3* family.

The nucleotide sequences associated with streptomycin resistance in *E. amylovora* CA11 and *P. syringae* pv. papulans Psp36 were identical to the *strA* and *strB* genes for streptomycin resistance in the enterobacterial plasmid RSF1010 (32). Streptomycin resistance in *P. syringae* pv. syringae is also related on the basis of sequence analysis to the streptomycin genes in RSF1010 (38). Our sequence data confirmed that probe SMP3 is part of the streptomycin resistance gene from *P. syringae* pv. papulans (Fig. 1B). Previously, DNA homologous with probe SMP3 was detected in many streptomycin-resistant gram-negative bacteria from plants (27, 36), and DNA similar to or homologous with the *strA* and *strB* genes of RSF1010 was detected in many streptomycin-resistant gram-negative bacteria of animal, including human, origin (19, 30, 43). The finding that bacteria from plants have the streptomycin resistance genes

found in bacteria from human and veterinary clinics extends the importance of this resistance determinant. Furthermore, RSF1010 contains an 81-bp sequence downstream of *strA* and *strB* that is homologous to the IRs of Tn*5393*, suggesting that the streptomycin resistance genes on RSF1010 and Tn*5393* are derived from a common ancestor. The transposition of Tn*5393* to various mobilizable plasmids could have played an important role in the widespread distribution of the streptomycin resistance genes among gram-negative bacteria.

Restriction fragment length polymorphism was observed in *Ava*I digests of plasmid DNAs from various epiphytic gram-negative bacteria isolated from apple orchards in New York and Michigan (27, 36). In most orchard bacteria, probe SMP3 hybridized with a single *Ava*I fragment of either 2.7 or 1.5 kb (27, 36). We noted that the difference of about 1.2 kb corresponded to the size of insertion sequence IS1133 and subsequently confirmed by restriction analysis that IS1133 was present in the 2.7-kb fragments but not in the 1.5-kb fragments (data not presented). The detection of two forms of transposon Tn*5393* indicates that the resistance in *E. amylovora* and some strains of *E. herbicola* probably evolved independently, or at least that its evolution has diverged, from that in *P. syringae* pv. papulans and most other fluorescent pseudomonads. For bacteria in which probe SMP3 hybridized with a single *Ava*I fragment smaller than 1.5 kb, DNAs from such bacteria often contained the *strA* and *strB* genes but not the whole transposon (data not shown). Evolution of streptomycin resistance in these bacteria probably resembles that for plasmid RSF1010.

We detected insertion sequence IS1133 in transposon Tn*5393* from streptomycin-resistant *E. amylovora* CA11. Although transposon Tn*5393* was common in streptomycin-resistant bacteria isolated from apple orchards, it did not generally harbor IS1133. However, a few strains of streptomycin-resistant *E. herbicola* harbored not only Tn*5393* and IS1133 but also a plasmid that hybridized with pEa34 from *E. amylovora* CA11. The presence of Tn*5393* and IS1133 inserted in a plasmid common to two species of bacteria indicates a possible epidemiological association between *E. amylovora* and *E. herbicola*.

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REFERENCES

1. **An, F. Y., and D. B. Clewell.** 1991. Tn917 transposase sequence correction reveals a single open reading frame corresponding to the *tnpA* determinant of Tn3-family elements. Plasmid **25**:121–124.
2. **Burr, T. J., J. L. Norelli, B. Katz, W. F. Wilcox, and S. A. Hoying.** 1988. Streptomycin resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. Phytopathology **78**:410–413.
3. **Chiou, C.-S., and A. L. Jones.** 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology **81**:710–714.
4. **Coyier, D. L., and R. P. Covey.** 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. **59**:849–852.
5. **Devereux, I., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence programs for the VAX. Nucleic Acids Res. **12**:387–395.
6. **Diver, W. P., J. Grinsted, D. C. Fritzinger, N. L. Brown, J. Altenbuchner, P. Rogowsky, and R. Schmitt.** 1983. DNA sequences of and complementation by the *tnp*R genes of Tn21, Tn501, and Tn1721. Mol. Gen. Genet. **191**:189–193.
7. **Falkenstein, H., P. Bellemann, S. Walter, W. Zeller, and K. Geider.** 1988. Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. **54**:2798–2802.
8. **Gay, P., D. LeCoq, M. Steinmetz, T. Berkelman, and C. I. Kado.** 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J. Bacteriol. **164**: 918–921.
9. **Grinsted, J., F. de la Cruz, and R. Schmitt.** 1990. The Tn21 subgroup of bacterial transposable elements. Plasmid **24**:163–189.
10. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
11. **Hattori, M., and Y. Sakai.** 1986. Dideoxy sequencing method using denaturated plasmid templates. Anal. Biochem. **152**:232–238.
12. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn3: three genes and three sites involved in transposition of Tn3. Cell **18**:1153–1163.
13. **Hiestand-Nauer, R., and S. Iida.** 1983. Sequence of the site-specific recombinase gene *cin* and of its substrates serving in the inversion of the C segment of bacteriophage P1. EMBO J. **2**:1733–1740.
14. **Jones, A. L., J. L. Norelli, and G. R. Ehret.** 1991. Detection of streptomycin-resistant *Pseudomonas syringae* pv. *papulans* in Michigan apple orchards. Plant Dis. **75**:529–531.
15. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
16. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44**:301–307.
17. **Kleckner, N.** 1981. Transposable elements in prokaryotes. Annu. Rev. Genet. **15**:341–404.
18. **Laurent, J., M.-A. Barny, A. Kotoujansky, P. Dufriche, and J. L. Vanneste.** 1989. Characterization of a ubiquitous plasmid in *Erwinia amylovora*. Mol. Plant-Microbe Interact. **2**:160–164.
19. **Lax, A. J., and C. A. Walker.** 1986. Plasmids related to RSF1010 from *Bordetella bronchiseptica*. Plasmid **15**:210–216.
20. **Mahillon, J., and D. Lereclus.** 1988. Structural and functional analysis of Tn4430: identification of an integrase-like protein involved in the co-integrate-resolution process. EMBO J. **7**:1515–1526.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Michiels, T., and G. Cornelis.** 1984. Detection and characterization of Tn*2501*, a transposon included within the lactose transposon Tn*951*. J. Bacteriol. **158**:866–871.
23. **Michiels, T., G. Cornelis, K. Ellis, and J. Grinsted.** 1987. Tn*2501*, a component of the lactose transposon Tn*951*, is an example of a new category of class II transposable elements. J. Bacteriol. **169**:624–631.
24. **Minsavage, G. V., B. I. Canteros, and R. E. Stall.** 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **80**:719–723.
25. **Mitsuhashi, S.** 1982. Drug resistance in bacteria: genetics, biochemistry and molecular biology. Japan Scientific Societies Press, Tokyo.
26. **Nishimura, A., M. Morita, Y. Nishimura, and Y. Sugino.** 1990. A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. Nucleic Acids Res. **18**:6169.
27. **Norelli, J. L., T. J. Burr, A. M. Lo Cicero, M. T. Gilbert, and B. H. Katz.** 1991. Homologous streptomycin resistance gene present among diverse gram-negative bacteria in New York State apple orchards. Appl. Environ. Microbiol. **57**:486–491.
28. **Plasterk, R. H., A. Brinkman, and P. van de Putte.** 1983. DNA inversions in the chromosome of *Escherichia coli* and in bacteriophage Mu: relationship to other site-specific recombination

systems. Proc. Natl. Acad. Sci. USA 80:5355–5358.
29. **Plasterk, R. H., and P. van de Putte.** 1985. The invertible P-DNA segment in the chromosome of *Escherichia coli*. EMBO J. 4:237–242.
30. **Rotger, R., F. Rubio, and C. Nombela.** 1986. A multi-resistance plasmid isolated from commensal *Neisseria* species is closely related to the enterobacterial plasmid RSF1010. J. Gen. Microbiol. 132:2491–2496.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
32. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 75:271–288.
33. **Schroth, M. N., S. V. Thomson, and W. J. Moller.** 1979. Streptomycin resistance in *Erwinia amylovora*. Phytopathology 69:565–568.
34. **Shaffer, W. H., and R. N. Goodman.** 1985. Appearance of streptomycin resistance in *Erwinia amylovora* in Missouri apple orchards. Phytopathology 75:1281. (Abstr.)
35. **Shaw, J. H., and D. B. Clewell.** 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. 164: 782–796.
36. **Sobiczewski, P., C.-S. Chiou, and A. L. Jones.** 1991. Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Dis. 75:1110–1113.
37. **Steinberger, E. M., G.-Y. Cheng, and S. V. Beer.** 1990. Characterization of a 56-kb plasmid of *Erwinia amylovora* Ea322: its noninvolvement in pathogenicity. Plasmid 24:12–24.
38. **Sundin, G. W., and C. L. Bender.** 1991. Characterization of copper- and streptomycin-resistant strains of *Pseudomonas syringae*. Phytopathology 81:1145. (Abstr.)
39. **Timmerman, K. P., and C.-P. D. Tu.** 1985. Complete sequence of IS3. Nucleic Acids Res. 13:2127–2139.
40. **Tominaga, A., S. Ikemizu, and M. Enomoto.** 1991. Site-specific recombinase genes in three *Shigella* subgroups and nucleotide sequences of a *pinB* gene and an invertible B segment from *Shigella boydii*. J. Bacteriol. 173:4079–4087.
41. **Turner, A. K., and J. Grinsted.** 1987. The nucleotide sequence of the *tnpA* gene of Tn2501. Nucleic Acids Res. 15:10049.
42. **Ward, E., and J. Grinsted.** 1987. The nucleotide sequence of the *tnpA* gene of Tn21. Nucleic Acids Res. 15:1799–1806.
43. **Willson, P. J., H. G. Deneer, A. Potter, and W. Albritton.** 1989. Characterization of a streptomycin-sulfonamide resistance plasmid from *Actinobacillus pleuropneumoniae*. Antimicrob. Agents Chemother. 33:235–238.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.
45. **Zheng, Z. X., M. Chandler, R. Hipskind, M. Clerget, and L. Caro.** 1981. Dissection of the r-determinant of the plasmid R100.1: sequence at the extremities of Tn21. Nucleic Acids Res. 9:6265–6278.

PART III

IDENTIFICATION OF TWO AMINOGLYCOSIDE PHOSPHOTRANSFERASES, APH(3")-Ib AND APH(6)-Id, ENCODED BY THE *aph(3")-Ib (strA)* AND *aph(6)-Id (strB)* GENES FROM STREPTOMYCIN-RESISTANT *Erwinia amylovora*

ABSTRACT

The plasmid-borne *strA* and *strB* genes from *Erwinia amylovora* strain CA11 were characterized by genetic and biochemical analyses. In deletion experiments, deletions in *strB* resulted in a reduction in the minimum inhibitory concentration (MIC) from 500 to 100 μg streptomycin ml^{-1} and in *strA* from 500 to 25 μg streptomycin ml^{-1} or less. When *strA* and *strB* were cloned separately on a *lacI*^{Q}/*Ptac*-based expression vector in *Escherichia coli*, the protein encoded by *strA*, but not the one encoded by *strB*, was overexpressed. Sequence analysis of the overlapping genes indicated that the distal *strB* gene lacked a Shine-Dalgarno sequence and that the initiation codon was in the double-stranded region of the stable stem-loop structure. Conversely, the Shine-Dalgarno sequence and the initiation codon in *strA* were exposed in the single-stranded loop of a stable stem-loop structure. The *strB* gene was overexpressed and resistance restored to a MIC of 100 μg streptomycin ml^{-1} by introducing a Shine-Dalgarno sequence and by altering the mRNA secondary structure. ^{13}C-NMR analysis of the respective phosphorylated streptomycin products indicated that *strA-strB* encoded aminoglycoside-3"-phosphotransferase [APH(3")-Ib] and aminoglycoside-6-phosphotransferase [APH(6)-Id],

respectively. These data suggest that the high level of resistance to streptomycin exhibited by bacteria with *strA-strB* genes is due to the coexistence in the cells of APH(3")-Ib and APH(6)-Id enzymes and that the differential expression of these enzymes is regulated by the mRNA secondary structure.

INTRODUCTION

The DNA associated with streptomycin resistance in strain CA11 of *Erwinia amylovora* is carried on transposon Tn*5393* which is inserted on a 34-kb conjugative plasmid pEa34 (3, 4). The region of pEa34 affecting resistance consists of two open reading frames that have sequence identity with the *strA* and *strB* genes of plasmid RSF1010 (4, 10, 19). Hybridization studies indicate that these genes are also present in many gram-negative streptomycin-resistant bacteria isolated in Michigan and New York from the apple orchard environment (2, 16, 21).

Streptomycin is a member of the aminoglycoside-aminocyclitol class of antibiotics. Aminoglycosides can be inactivated by phosphorylation, adenylylation, or acetylation. The enzymes reported to modify streptomycin include aminoglycoside-3"-phosphotransferase, APH(3")-I; aminoglycoside-6-phosphotransferase, APH(6)-I; aminoglycoside-3"-nucleotidyltransferase, ANT(3")-I; and aminoglycoside-6-nucleotidyltransferase, ANT(6)-I (5, 20). Based on sequence comparison analysis, Shaw et al. (20) proposed that the *strA-strB* genes in RSF1010 encode APH(3")-Ib and APH(6)-Id, respectively. The identity of the enzymes encoded by *strA-strB* remains to be confirmed by biochemical

analysis.

In this research, we analyzed the *strA-strB* genes from *E. amylovora* plasmid pEa34 by deletion analysis and investigated the role of the stem-loop structure around the ribosome-binding site on the translational regulation of *strB*. Finally, the enzymes encoded by *strA* and *strB* were identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in this study are described in Table 1. *E. coli* JM109 (25) was routinely used as the host for plasmids and gene expression. Strains of *E. coli* were grown at 37°C on Luria-Bertani (LB) agar or in LB broth (13). Plasmid pMMB66HE (7) was kindly supplied by M. Bagdasarian, Michigan State University, East Lansing. The primers were synthesized with an automatic 380B DNA Synthesizer (Applied Biosystems, Foster City, CA) at the Macromolecular Facility Laboratory, Michigan State University.

DNA manipulations and construction of expression vector pTWNHE. Plasmid preparations, DNA restriction digests, agarose gel electrophoresis, isolation of DNA fragments, ligations, and fill-in reactions were done using standard methods (13). A 4,018-bp multiple copy expression vector pTWNHE was constructed by ligation of a 1,550-bp *Sca*I-*Pvu*II fragment from pGEM3Zf(+)(25) and a 2,468-bp *Eco47*III-*Sca*I fragment from pMMB66HE (7). The hybrid plasmid consisted of the replication origin of pGEM3Zf(+), a β-lactamase gene (*bla*), *lacI*^{Q}, *lac* operator site, *tac* promoter, a polylinker, and two transcriptional terminators of the *E. coli rrnB*

TABLE 1. Bacterial strains, plasmids and primers

Designation	Relevant characteristic(s)	Source or reference(s)
Bacterial strains		
Escherichia coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* λ^- Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q} ZΔM15]	Promega Corp. 25
SMAB39	JM109 (pTWNHE::*strA-B39*)	This study
SMBC15	JM109 (pTWNHE::*strB-C15*)	This study
SMBE1	JM109 (pTWNHE::*strB-AJ24*)	This study
SMB1	JM109 (pTWNHE::*strB-AJ25*)	This study
SMB10	JM109 (pTWNHE::*strB-AJ26*)	This study
SMB10N	JM109 (pTWNHE::*strB-AJ26N*)	This study
SMBA9	JM109 (pTWNHE::*strB-AJ27*)	This study
SMBD3	JM109 (pTWNHE::*strB-AJ28*)	This study
SMBC5	JM109 (pTWNHE::*strB-AJ34*)	This study
Plasmids[a]		
pEa34	Sm^r, *strA-strB*, Tn*5393*, conjugative	4
pTWNHE	Ap^r, a multiple copy expression vector derived from pMMB66HE and pGEM3Zf(+)	This study
pGEM3Zf(+)	Ap^r, multiple copy plasmid vector	Promega Corp.
pMMB66HE	Ap^r, a broad-host-range expression vector derived from RSF1010	7
pSTRXP	A 2.8-kb *Xba*I-*Pst*I fragment with *strA-strB*	

Table 1 (cont'd)

	from pEa34 cloned on pGEM3Zf(+)	This study
pC2, pF16	Deletion derivatives of pSTRXP containing both intact *strA* and *strB*	This study
pE21	Derivative of pSTRXP *strA* disrupted by removing a 272-bp *Nru*I fragment	This study
pC8, pC15	Deletion derivatives of pSTRXP with intact *strB*, disrupted *strA*	This study
pB5, pB39	Deletion derivatives of pSTRXP with intact *strA*, disrupted *strB*	This study
pA17	Deletion derivative of pSTRXP with disrupted *strA* and disrupted *strB*	This study
Primers[b]		
AJ23	d(GGAGAATTCGCTTGATATCTAGTA)	This study
AJ24	d(CTCTAAGGAAGGGTTGATGTTCATG)	This study
AJ25	d(TAAGGAGTTAACGTTATGTTCATGCCGCCTG)	This study
AJ26	d(TAAGGAGGTTAACGTTATGTTCATGCCGCCTG)	This study
AJ27	d(TAAGGAGTTAACGTTATGCCGCCTGTTTTTC)	This study
AJ28	d(TAAGGAGGTTAACGTTATGCCGCCTGTTTTTC)	This study
AJ30	d(ATGTGTGGAATTGTGAGCGG)	This study
AJ34	d(TAAGGAGGTAGGGTTGATGTTCATGCC)	This study

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[a] Sm^r = streptomycin resistance; Ap^r = ampicillin resistance.

[b] Nucleotides in each sequence that could pair with the 3' end of *E. coli* 16S rRNA (AUUCCUCCACU... 5') are shown in larger size print. Nucleotides not present in the original *strB* template are underlined.

operon from pMMB66HE.

Enzyme assays. APH enzyme activity was detected using a phosphocellulose paper binding assay (5). All bacteria were grown in LB broth at 37°C in a shaking incubator until the optical density at 600 nm was 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added (final concentration 0.5 mM) to the culture and shaking was continued for 4 h. Crude enzyme extracts were prepared by sonication (Vibra CellTM, Sonics & Materials Inc., Danbury, CT). Incubation of streptomycin and [γ-P^{32}]ATP with the sonicated enzyme extract, washing of the phosphocellulose paper, and radioactivity counting were performed as described (5). The nomenclature proposed by Shaw et al (20) for the aminoglycoside resistance genes and the enzymes they encode is used in this paper.

Determination of minimum inhibitory concentration (MIC). MICs were determined by the agar dilution method (1). Four single colonies from each clone were transferred to LB broth and incubated overnight at 37°C with shaking. A 3 μl aliquot of each of the bacterial suspensions (10^7 cfu/ml; 100X dilution of the overnight culture) was spotted onto two sets of LB agar plates containing 0, 10, 25, 50, 100, 200, or 500 μg streptomycin ml^{-1}. One set of plates was amended with 0.1 mM IPTG. Bacterial strains without confluent growth in the spots after 16 h incubation at 37°C were considered sensitive to the respective level of streptomycin.

Deletion analysis in the *strA* and *strB* region. A 2.8-kb *Xba*I-*Pst*I fragment from pEa34, with the *strA-strB* genes and a promoter derived from IS1133, was cloned into the *Xba*I-*Pst*I site of pGEM3Zf(+), downstream from a *lac* promoter, to produce plasmid pSTRXP. Deletions in the *strA* and *strB* region were performed with the Erase-a-Base System (Promega Corp., Madison, WI). The deletion derivatives were transformed into competent *E. coli* JM109 cells (15). The extent of each deletion was verified by DNA sequencing. MICs and in vitro APH enzyme activity for each clone were determined as described above. In the complementation assay, deletion derivative pB39 was transformed into *E. coli* JM109 harboring pC15 or pE21. Transformants were selected on LB agar amended with 100 μg streptomycin ml^{-1}. Four transformed clones from the two complementation assays were selected and their MICs determined as described above.

Modification of *strB* and cloning into pTWNHE. A series of primers were used to modify and amplify *strB* by the polymerase chain reaction (PCR; Table 1). The forwarded primers AJ24 to AJ28, and primer AJ34 contained a potential Shine-Dalgarno sequence with 6 to 9 nucleotides complementary to the 3'-flanking sequence of the 16S rRNA. The reverse primer AJ23 contained an *Eco*RI site to facilitate cloning of the amplified DNA. The PCR was run through 30 cycles at 90°C for 1 min, 50°C for 1 min, and 72° C for 1 min. The reaction mixture (100 μl) consisted of 200 pmol each of two primers (AJ23 and one of AJ24, AJ25, AJ26,

AJ27, AJ28, and AJ34), 0.01 ng of plasmid pC15 as template, 10 μl of 10X Taq reaction buffer (200 mM Tris.HCl, pH 8.4, 500 mM KCl), 2.5 mM $MgCl_2$, 200 μM of each of four dNTPs, and 2.5 units Taq DNA polymerase. The reactions produced 0.84-kb *strB* variants *strB-AJ24*, *strB-AJ25*, *strB-AJ26*, *strB-AJ27*, *strB-AJ28*, and *strB-AJ34*. They were separated from the reaction mixtures with a DNA purification system kit (Magic PCR Preps, Promega Corp., Madison, WI), digested with *Eco*RI, and then cloned into the *Hind*III-*Eco*RI site of pTWNHE, 46-bp downstream from the *tac* promoter. Modifications in each *strB* variant were examined by DNA sequencing with primer AJ30, which complemented the sequence 28-bp upstream from the *Hind*III site of pTWNHE. Variant *strB-AJ26N* was made by filling-in the *Not*I site of *strB-AJ26*. It encoded a polypeptide with 14 additional amino acids than the authentic *strB* protein due to a frameshift at codon 268. Variants *strA-B39* and *strB-C15* were *Hind*III-*Eco*RI fragments from pB39 and pC15 with wild-type *strA* and *strB* coding sequences, respectively. They were cloned into the *Hind*III-*Eco*RI site of pTWNHE for expression analysis.

Expression and analysis of *strA* and *strB* gene products. Each clone was grown in LB broth at 37°C with shaking until the optical density at 600 nm was 0.6 and then IPTG was added (final concentration 0.5 mM). No IPTG was added to the controls. The bacteria were incubated for an additional 4 h and harvested by centrifugation. The cell pellets were resuspended in 1/5 volumes of sample buffer (62.5 mM

Tris.HCl pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue), heated for 10 min at 90°C, and the proteins separated by electrophoresis on 13.5% SDS-polyacrylamide gels (12).

N-terminal amino acid sequence determination. The protein encoded by *strA* from strain SMAB39 and those encoded by the *strB* variants from strains SMB10 and SMBD3 were separated on a 1.5-mm thick 13.5% SDS-polyacrylamide gel. Proteins were then electroblotted onto a polyvinylidene difluoride membrane (14). The proteins were excised and their N-terminal amino acid sequences determined using the automatic Edman degradation method and a 477A Protein Sequencer (Applied Biosystems, Foster City, CA) at the Macromolecular Facility Laboratory, Michigan State University.

Preparation and ^{13}C-NMR analysis of the phosphorylated streptomycin. *E. coli* SMAB39 and SMB10 were grown in 500 ml of LB broth with IPTG until the optical density at 600 nm was 1.0. Crude enzyme extracts were prepared by ultrasonication (5). To prepare phosphorylated streptomycin, 50 ml of each crude enzyme extract was added to an end volume of 150 ml of a mixture of 10 mM streptomycin sulfate, 16.6 mM ATP, 60 mM Tris.HCl, 4 mM $MgSO_4$, 10 mM β-mercaptoethanol, and 0.25 mM phenyl-methyl-sulfonyl-fluoride (PMSF), pH 8. After the reaction mixture was incubated for 48 h at 30°C, protein was removed by ultra-filtration (Diaflo YM3, Amicon Corp., Beverly, MD) and the streptomycin phosphate purified over a 2.5 x 30 cm

Amberlite CG50 column eluted with a 2N KCl gradient. Fractions containing streptomycin phosphate were detected using the maltol assay (18) and then pooled. The excess KCl salt was precipitated with 3.5 volumes of acetone at pH 2 (24). After removal of the precipitated KCl salt, the filtrate was titrated with 1N KOH until a streptomycin phosphate precipitate formed. The precipitate was collected, dissolved in 10 ml H_2O, adjusted with 1N HCl to pH 6, and lyophilized. ^{13}C-NMR studies of the respective streptomycin phosphate (The Max T. Rogers NMR Facility, VXR 500 spectrometer; Michigan State University) were conducted in D_2O with dioxane as the external standard.

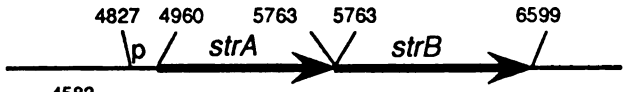
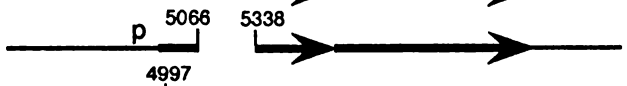
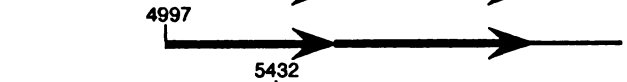
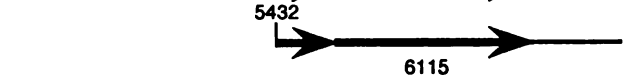
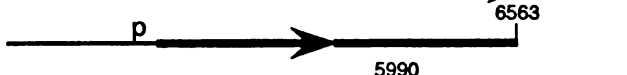
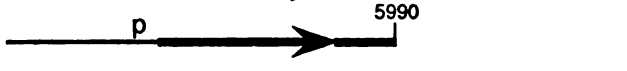
Secondary structures. The stem-loop structures and free energy for *strA* and *strB* were determined using the computer program Hairpin Loop Search, DNASIS Version 3.0 (Hitachi Software Engineering Co. LTD).

RESULTS

Deletion and complementation analysis of *strA* and *strB*. Plasmids pSTRXP and pC2 contained *strA-strB* and a promoter from IS1133, while plasmid pF16 contained *strA-strB* but no IS1133 promoter (Fig. 1A). Cells of *E. coli* JM109 transformed with pSTRXP and pC2 were as resistant to streptomycin under both inducing and noninducing conditions as cells transformed with *E. amylovora* plasmid pEa34. Cells with pF16 were less resistant (MIC = 100 μg streptomycin ml^{-1}) under noninducing than under inducing (MIC >500 μg streptomycin ml^{-1}) conditions. Under inducing conditions, APH enzyme activity was high in extracts from cells with pSTRXP, pC2, and pF16, but low in extracts from cells with the low-copy-number plasmid pEa34. Cells transformed with pC8 and pC15, plasmids with deletions from the 5' end of the *strA* region, exhibited a dramatic loss of resistance and APH enzyme activity. Cells transformed with pE21, which had a 272-bp *Nru*I fragment deleted from *strA*, and cells transformed with pA17, a plasmid containing deletions in both *strA* and *strB*, were as susceptible to streptomycin as cells transformed with pGEM3Zf(+). Phosphorylation activity in extracts from these cells was low. Cells with pB5 and pB39, plasmids with *strA* and the IS1133 promoter but

Fig. 1. A. Resistance to streptomycin and aminoglycoside phosphotransferase (APH) activity in extracts from cells of *Escherichia coli* JM109 transformed with various deletions in the *strA* and *strB* genes from *Erwinia amylovora* strain CA11. Deletion derivatives were cloned in vector pGEM3Zf(+) with the genes oriented downstream from a *lac* promoter. Bacteria were grown in the presence and absence of isopropyl-β-D-thiogalactopyranoside (IPTG). Bold lines with arrows represent the coding regions of *strA* and *strB* and the transcriptional direction for each gene. Numbers above the lines are nucleotide sequence numbers for Tn*5393* (4) and "P" indicates a promoter from IS1133. **B.** Complementation of deletions in *strA* and *strB*.

A. Deletion study

Plasmid		MIC (μg streptomycin ml^{-1}) 0.5 mM IPTG		APH (relative activity)
		+	-	+
pSTRXP		> 500	> 500	52.3 ± 3.0
pC2		> 500	> 500	70.0 ± 1.0
pF16		> 500	100	70.1 ± 2.1
pE21		< 10	< 10	2.4 ± 0.4
pC8		25	< 10	4.0 ± 0.3
pC15		25	< 10	3.8 ± 1.3
pA17		< 10	< 10	1.1 ± 0.2
pB5		100	100	80.5 ± 0.5
pB39		100	100	80.0 ± 3.2
pEa34		> 500	> 500	4.4 ± 0.1
pGEM3Zf(+)		< 10	< 10	1.0 ± 0.1

B. Complementation study

pB39 + pC15	500	500	ND
pB39 + pE21	500	500	ND

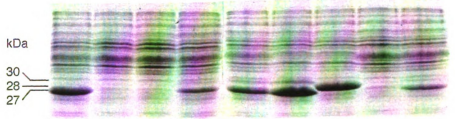
nucleotide deletions in *strB*, were 5-fold less resistant than cells containing pSTRXP or pC2 under both inducing and noninducing conditions. But, extracts from these cells exhibited a high level of APH enzyme activity. When cells were transformed with two plasmids, one with functional *strB* (pE21 or pC15) and the other with functional *strA* (pB39), resistance was restored to MIC = 500 μg streptomycin ml^{-1} (Fig. 1B).

Effects of Shine-Dalgarno sequence and mRNA secondary structure on the expression of *strB*. When induced with IPTG, strain SMAB39(pTWNHE::*strA-B39*) produced the expected 27-kDa encoded polypeptide in abundance (13% of the total cellular protein), but strain SMBC15(pTWNHE::*strB-C15*) did not produce the expected 28-kDa encoded polypeptide in abundance (Fig. 2, lanes *strA-B39* and *strB-C15*). Analysis of the primary sequences for *strA* and *strB* revealed that *strA* contained a Shine-Dalgarno consensus sequence of AAGGA in the ribosome-binding site while *strB* lacked a Shine-Dalgarno consensus sequence (Fig. 3). Introducing TAAGGA into *strB* 7-bp upstream from the initiation codon did not increase the amount of the 28-kDa protein produced by strain SMBE1 nor affect its resistance to streptomycin (Fig. 2, lane *strB-AJ24*). When the computer-predicted mRNA secondary structures for *strA* and *strB* were assessed, stable stem-loop structures surrounded the ribosome-binding site (Fig. 3, *strA-B39* and *strB-C15*). The Shine-Dalgarno sequence and the initiation codon for *strA* were located in the single-

Fig. 2. A. SDS-PAGE analysis of proteins from strains of *Escherichia coli* transformed with expression vector pTWNHE with *strA*, *strB*, or modified *strB* variants. Proteins were separated on a 13.5% SDS-polyacrylamide gels and stained with Coomassie blue. Bands labeled 27, 28, and 30 kDa are protein products from *strA*, *strB*, and *strB-AJ26N*, respectively. **B.** The percentage of the expressed proteins from *strA* and *strB* variants to the total cellular protein for strains grown in LB medium with (+) or without (-) IPTG. Data were obtained by scanning four individual lanes for each strain on SDS gels using AMBIS Core Software Version 4.0 (AMBIS, Inc. San Diego, California). **C.** Resistance of each strain to streptomycin on LB agar with (+) and without (-) IPTG.

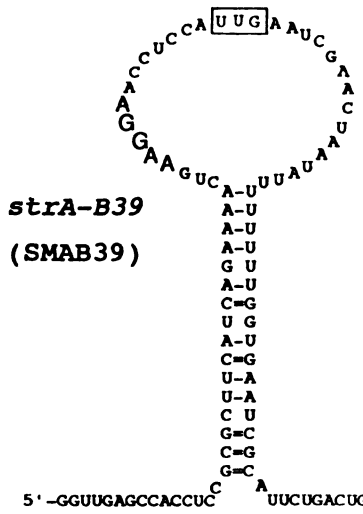
A. *E. coli* strains and gene variants

strA-B39	*strB-C15*	*strB-AJ24*	*strB-AJ34*	*strB-AJ25*	*strB-AJ26*	*strB-AJ26N*	*strB-AJ27*	*strB-AJ28*
SMAB39	SMBC15	SMBE1	SMBC5	SMB1	SMB10	SMB10N	SMBA9	SMBD3

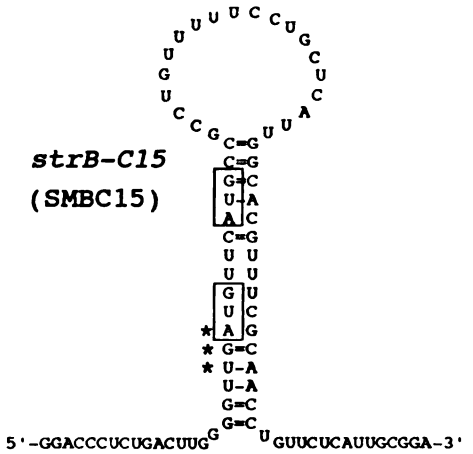


B. Expression by *E. coli* strains (% of total cellular protein)	+	13	< 1	< 1	4	12	16	16	< 1	4
	-	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
C. MIC (μg streptomycin ml^{-1})	+	200	< 10	< 10	100	100	100	< 10	25	100
	-	100	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10

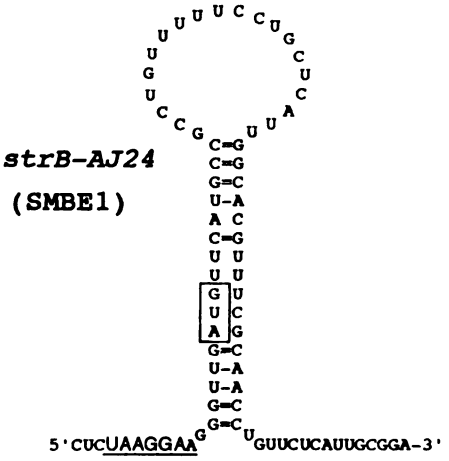
Fig. 3. Predicted mRNA secondary structures surrounding the ribosome-binding site for *strA*, *strB*, and six *strB* variants. The stem-loop structures and free energy (ΔG; in kcal mol^{-1}) were determined with the computer program Hairpin Loop Search, DNASIS version 3.00 (Hitachi Software Engineering Co. LTD). Nucleotides in ribosome-binding sites that can pair with the 3' end of *E. coli* 16S rRNA (AUUCCUCCACU...5') are indicated as larger characters and those that cannot pair with *strB* sequence are underlined. Initiation codons are enclosed by a box and the stop codon from *strA* is marked with asterisks. The secondary structures for *strB-AJ24* and *strB-AJ34* were unaltered, the structures for *strB-AJ25*, *strB-AJ26*, *strB-AJ27*, and *strB-AJ28* were altered by replacing nucleotides between the Shine-Dalgarno sequence and the initiation codon. Six nucleotides (the first two codons of *strB*) on *strB-AJ27* and *strB-AJ28* were omitted. The strain of *Escherichia coli* transformed with each gene is shown in parenthesis under the gene designation.



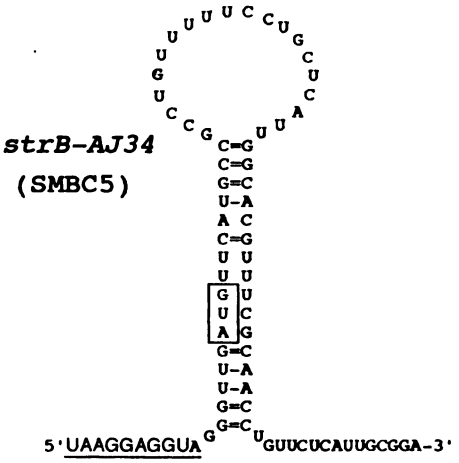
ΔG= -10.66



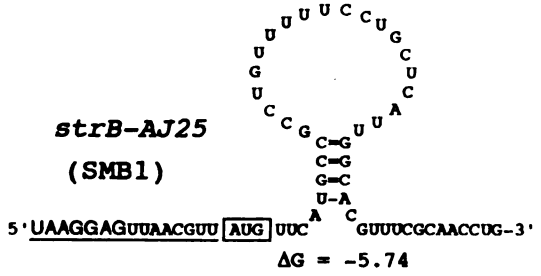
ΔG = -15.94



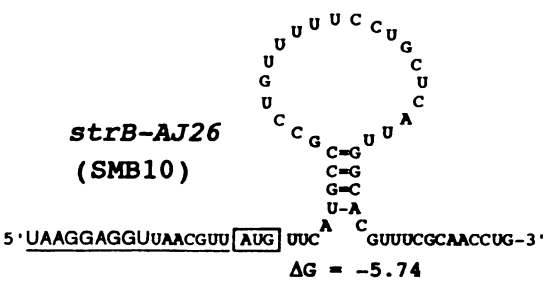
ΔG = -15.94



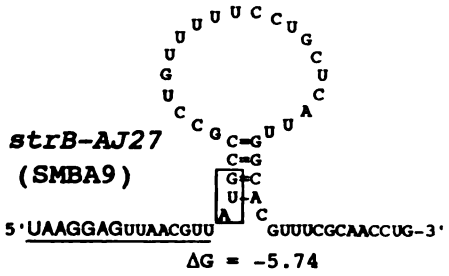
ΔG = -15.94



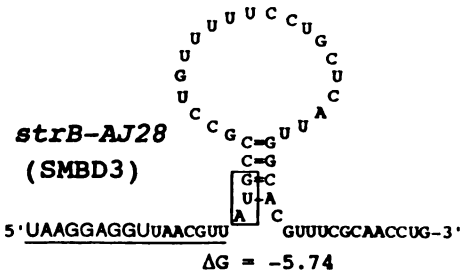
ΔG = -5.74



ΔG = -5.74



ΔG = -5.74



ΔG = -5.74

stranded region but the initiation codon for *strB* was located in the double-stranded region of the stem-loop structure.

To assess the effect of the strength of the Shine-Dalgarno sequence on the expression of *strB*, the sequences TAAGGAG (primers AJ25 and AJ27) and TAAGGAGGT (primers AJ26, AJ28, and AJ34) were introduced into the sequence 7- and 8-bp upstream from the initiation codon. To study the effect of the stem-loop structure on expression of *strB*, the structure was altered by nucleotide replacement. Each of the stem-loop structures of *strB-AJ25*, *strB-AJ26*, *strB-AJ27*, and *strB-AJ28* had a free energy less negative (ΔG = -5.74 kcal mol^{-1}, Fig. 3) than that for *strB-AJ34* (ΔG = -15.94 kcal mol^{-1}). The initiation codons for *strB-AJ25* and *strB-AJ26* were free from the stem-loop, those for *strB-AJ27* and *strB-AJ28* were located, due to the removal of two codons ATGTTC, in the double-stranded region of the stem-loop structure.

When induced with IPTG, strains SMB1, SMB10, SMBD3, and SMBC5 were resistant to 100 μg streptomycin ml^{-1} and produced the 28-kDa polypeptide at levels of 12, 16, 4, and 4% of total cellular protein, respectively (Fig. 2, lanes *strB-AJ25*, *strB-AJ26*, *strB-AJ27*, and *strB-AJ28*). Strains SMB10, SMBD3, and SMBC5 with Shine-Dalgarno sequence TAAGGAGGT produced more *strB* protein than strains SMB1 and SMBA9 with Shine-Dalgarno sequence TAAGGAG. Strain SMB1 (initiation codon free from the stem) produced more gene product than strains SMBC5 and SMBD3 (initiation

codon located in the stem) even though strains SMBC5 and SMBD3 contained a longer Shine-Dalgarno sequence. Overproduction of *strB* protein in strains SMB1, SMB10, SMBD3, and SMBC5 was accompanied with an increase in the level of resistance, but MICs did not increase above 100 μg streptomycin ml^{-1} even though the encoded polypeptide increased from 4 to 16% of the total protein. Strain SMB10N produced a 30-kDa protein (with a modification in the C-terminal of *strB* protein) consisting of 16% of the total protein, but was sensitive to streptomycin (Fig. 2. lane *strB-AJ26N*).

Confirmation of *strA* and *strB* proteins. The first 12 N-terminal amino acids of the protein from *E. coli* SMAB39 were consistent with the amino acid sequence predicted from the nucleotide sequence for *strA* (4, 19). The first 11 N-terminal amino acids of the proteins from *E. coli* SMB10 and SMBD3 were M-F-M-P-P-V-F-P-A-H-W-H and M-P-P-V-F-P-A-H-W-H-V-S. These sequences were identical to the amino acid sequences predicted from the nucleotide sequences for *strB-AJ26* and *strB-AJ28*, respectively.

Identification of the phosphorylated streptomycin products. The ^{13}C-NMR spectrum for the 21 carbons on the streptomycin phosphate produced by incubating streptomycin with *strA* encoded protein was consistent with the spectrum for streptomycin-3"-phosphate (8, 11). The difference in chemical shift for carbon position C3" of streptomycin and of phosphorylated streptomycin was 2.8 ppm and the signals

at carbons C2", C3", and C4" were split by coupling with the phosphorous group (coupling constants were 2.6 Hz at C2", 5.3 Hz at C3", and 3.1 Hz at C4"). Therefore, the enzyme encoded by *strA* was APH(3")-Ib. The ^{13}C-NMR spectrum for the streptomycin phosphate produced when streptomycin was reacted with *strB* encoded protein showed split chemical shifts at carbons C6 and C1 (coupling constants were 5.3 Hz at C6 and 3.2 Hz at C1). The difference of 3.5 ppm in the chemical shifts at carbon C6 between streptomycin and the phosphorylated streptomycin was similar to that reported for carbon C6 in streptomycin-6-phosphate (23). This established that the enzyme encoded by *strB* was APH(6)-Id.

DISCUSSION

Our biochemical analysis of the enzymes produced by *strA* and *strB* support predictions made from protein sequence data (20) that the *strA-strB* genes encode two aminoglycoside phosphotransferases, APH(3")-Ib and APH(6)-Id, respectively. We also confirmed in the deletion and complementation studies that both genes were required for a high level of resistance to streptomycin. Interaction between APH(3")-Ib and APH(6)-Id appear essential for high resistance since bacteria with only *strA* or *strB* were 2.5 to 5 fold more sensitive to streptomycin than bacteria with both genes.

When *strA* and *strB* were cloned separately, only *strA* was expressed. Examination of the primary sequence data revealed that *strA-strB* were overlapping genes and that the genes were translationally coupled. When *strB* was separated from *strA* by cloning, cloned *strB* was devoid of an reinitiation region and translation was inhibited until a suitable Shine-Dalgarno sequence was inserted 5' of the initiation codon.

It is also possible that the differential expression of *strA-strB* is regulated by mRNA secondary structure surrounding the ribosome binding site. The predicted stem-loop structure for *strA* mRNA revealed that the Shine-

Dalgarno sequence and the initiation codon, two elements within the ribosome-binding site that allow translation initiation (9), were located in the single-stranded region of a stable stem-loop structure. However, no Shine-Dalgarno sequence was present in the predicted stem-loop structure for *strB* and the initiation codon was located in the double-stranded region of a stable stem-loop structure. We found that a weak Shine-Dalgarno sequence in the single-stranded region upstream from an initiation codon in the double-stranded region was not sufficient for APH(6)-Id production. When the Shine-Dalgarno sequence and the initiation codon were both located in the single-stranded region, APH(6)-Id production was maximized and bacteria with this DNA were resistant to streptomycin.

The gene encoding APH(6)-Ia, but not the one encoding APH(3")-Ia, is part of the gene cluster involved in streptomycin biosynthesis in the streptomycin-producing *Streptomyces griseus* (6, 11). It was suggested that APH(6)-Ia may protect *S. griseus* from the toxic action of streptomycin (17, 22). However, the biological function of APH(3")-Ia in *S. griseus* is unclear. Our study suggests that the coexistence of APH(3")-Ia and APH(6)-Ia in *S. griseus* may enable it to tolerate high levels of streptomycin just as the coexistence of APH(3")-Ib and APH(6)-Id in bacteria enable them to tolerate high levels of streptomycin.

ACKNOWLEDGMENTS

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REFERENCES

1. Barry, A. L. 1976. The antimicrobic susceptibility test: principles and practices. The Lea & Febiger, Philadelphia.

2. Burr, T. J., J. L. Norelli, C. L. Reid, L. K. Capron, L. S. Nelson, H. S. Aldwinckle, and W. F. Wilcox. 1993. Streptomycin-resistant bacteria associated with fire blight infections. Plant Dis. **77:**63-66.

3. Chiou, C.-S., and A. L. Jones. 1991. The analysis of plasmid-mediated streptomycin resistance in *Erwinia amylovora*. Phytopathology **81:**710-714.

4. Chiou, C.-S., and A. L. Jones. 1993. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J. Bacteriol. **175:**732-740.

5. Davies, J. E. 1986. Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes. p. 790-809. In V. Lorian (ed.). Antibiotics in Laboratory Medicine. The Williams & Wilkins Co., Baltimore.

6. Distler, J., A. Ebert, K. Mansouri, K. Pissowotzki, M. Stockmann, and W. Piepersberg. 1987. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucl. Acids Res. **15:**8041-8056.

7. Furste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tac*P expression vector. Gene **48:**119-131.

8. Ganelin V. L., L. G. Vinogradova, V. N. Stepanshina, R. M., Petyushenko, A. I., Chernyshev, S. E. Esipov, Y. O. Sazykin, and S. M. Navashin. 1980. Streptomycin-3"-phosphotransferases from streptomycin-resistant cells of *Escherichia coli* strains. Biochimia **45:**2198-2205.

9. Gold, L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Ann. Rev. Biochem. **57:**199-233.

10. Guarry, P., J. van Embden, and S. Falkow. 1974. Molecular nature of two nonconjugative plasmids carrying

drug resistance genes. J. Bacteriol. **117:**619-630.

11. Heinzel, P., O. Werbitzky, J. Distler, and W. Piepersberg. 1988. A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phosphotransferase. Arch. Microbiol. **150:**184-192.

12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.

13. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

14. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035-10038.

15. Nishimura, A., M. Morita, Y. Nishimura, and Y. Sugino. 1990. A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. Nucleic Acids Res. **18:**6169.

16. Norelli, J. L., T. J. Burr, A. M. Lo Cicero, M. T. Gilbert, and B. H. Katz. 1991. Homologous streptomycin resistance gene present among diverse gram-negative bacteria in New York State apple orchards. Appl. Environ. Microbiol. **57:**486-491.

17. Piwowarski, J. M., and P. D. Shaw. 1979. Streptomycin resistance in a streptomycin-producing microorganism. Antimicrob. Agents Chemother. **16:**176-182.

18. Schenk, J. R., and M. A. Spielman. 1945. The formation of maltol by the degradation of streptomycin. J. Am. Chem. Soc. **67:**2276-2277.

19. Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger. 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271-288.

20. Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Reviews **57:**138-163.

21. Sobiczewski, P., C.-S. Chiou, and A. L. Jones. 1991. Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Dis. **75:**1110-1113.

22. Sugiyama, M., H. Mochizuki, O. Nimi, and R. Nomi. 1981.

Mechanism of protection of protein synthesis against streptomycin inhibition in a producing strain. J. Antibiot. **34:**1183-1188.

23. Tohyama, H., T. Shigyo, and Y. Okami. 1984. Cloning of streptomycin resistance gene from a streptomycin producing streptomycete. J. Antibiot. **37:**1736-1737.

24. vander Brook, M. J., A. N. Wick, W. H. DeVries, R. Harris, and G. F. Cartland. 1946. Extraction and purification of streptomycin, with a note on streptothricin. J. Biol. Chem. **165:**463-468.

25. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

APPENDIX A

PROPOSED RESEARCH ON RIBOSOME-MEDIATED STREPTOMYCIN RESISTANCE IN *Erwinia amylovora*

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INTRODUCTION

Fire blight, caused by *Erwinia amylovora*, is the most important and destructive bacterial disease of apples and pears in North America, New Zealand, much of Europe, and the Mediterranean region (29). The methods for control of fire blight are difficult, expensive, and not always effective. Streptomycin is the most reliable and effective bactericide for the control of fire blight. However, intensive use of streptomycin has been accompanied by the development of resistant strains of *E. amylovora* (18, 2, 13, 26). Streptomycin-resistant *E. amylovora* were first detected in Michigan in an apple orchard in Van Buren county in 1990 (Part I, this thesis). By 1993, resistant strains had been detected in eight more apple orchards in Van Buren county, two orchards in Kent county, and three orchards in Newaygo county (14).

Bacteria use three strategies to cope with streptomycin: alteration of the ribosomal target site, inactivation of streptomycin by modifying enzymes, and reduced accumulation of streptomycin in the cells. The enzyme- and ribosome-mediated mechanisms are considered to be the most important

of the three mechanisms because they are associated with high levels of resistance. Among the streptomycin-resistant strains of *E. amylovora* isolated from Michigan apple orchards some exhibit an aminoglycoside phosphotransferase (APH) activity in enzyme assays, while other strains do not exhibit the enzyme activity. The molecular genetics and biochemistry of resistance in strains with the APH enzyme was the subject of this thesis. The resistant strains lacking APH activity exhibit cross resistance to myomycin, an antibiotic with a mode of action similar to streptomycin. Strains resistant to both streptomycin and myomycin often have mutations in ribosomal genes (3). Although enzyme-mediated resistance is more common than ribosome-mediated resistance in *E. amylovora* from Michigan, ribosome-mediated resistance is common in Washington, Oregon (13), and California (25). I propose to determine whether resistance in these strains of *E. amylovora* is due to mutations in ribosomal components, whether all field strains have the same or different mutations, and whether strains with different mutation sites have different levels of resistance and protein synthesis. I also propose to determine the nucleotide sequence for the 16S rRNA and r-protein S12 genes of *E. amylovora*.

OBJECTIVES

1. To test whether there are different resistance levels

present in the myomycin- and streptomycin-resistant strains of *E. amylovora*.

2. To determine the molecular basis and biochemical mechanism for streptomycin resistance in these strains.

3. To determine whether resistant field strains with altered ribosomes have lower levels of protein synthesis in vitro and lower growth rates than streptomycin-sensitive strains.

JUSTIFICATION

Developing an understanding of the mechanism of streptomycin resistance in *E. amylovora* may suggest methods for combating resistant strains. In this research project, the resistance mechanism will be investigated by comparing the nucleotide sequences of 16S rRNA and r-protein S12 genes from sensitive and the myomycin- and streptomycin-resistant *E. amylovora* and by determining the sensitivity of ribosomes from these strains to streptomycin in an in vitro protein synthesis assay. Although mutations in many ribosomal components and sites may result in insensitivity to streptomycin, mutations in the field strains may be limited to one or a few sites. If there is only one mutation site, the information suggests that the most stable and fittest mutation is unique. Thus, we can design specific primers for the detection of the point mutation by PCR-based methods. If mutation occurs in several sites on the ribosome that can be reflective to distinct resistance levels in the bacterial cells. The alteration on ribosome

may impair the protein synthesis and competition of the niche of the cell. This information is useful for the prediction for fitness of the resistant strains in nature.

Although mutations for streptomycin resistance may occur in several sites in r-protein S4, S5 and S12, and 16S rRNA, mutations in r-protein S12 and 16S rRNA are the most common. Resistance due to mutations on r-protein S12 and 16S rRNA has been reported not only in bacteria (5, 27) but also in chloroplast of plants and green blue algae (6, 7, 12, 20). Most mutations in the 16S rRNA gene are in the domains 530-loop and 915 region (*Escherichia coli* numbering) (5, 11, 16, 19).

The different levels of streptomycin resistance may result from mutations at different positions of r-protein S12 or 16S rRNA. Mutations at different positions of S12 and 16S rRNA genes having different levels of resistance have been reported (22, 24). If the field strains of *E. amylovora* exhibit different levels of resistance to streptomycin, they may have different mutation sites in r-protein S12 or 16S rRNA.

Ribosomes with mutated r-protein S12 gene often exhibit impaired protein synthesis in vitro. The efficiency of a natural messenger RNA (MS2 RNA)-directed protein synthesis is about 50% of the wild type (11). The impaired ribosome may slow the growth of the bacterium and be harmful to its fitness.

In a preliminary study, the streptomycin-resistant strains of *E. amylovora* which did not exhibit APH activity were found to be resistant to the antibiotic myomycin, while strains with APH activity were sensitive to myomycin. Except for plasmid pEA29, the myomycin- and streptomycin-resistant strains tested contain no other plasmid. Resistance in these strains may be chromosomal. It may result from mutations in the genes of ribosomal components S12 and 16S rRNA.

DESCRIPTION OF RESEARCH PLAN

To test if there are various resistance levels present in myomycin- and streptomycin-resistant strains of *E. amylovora* isolated from Michigan and other States, the minimum inhibitory concentration (MIC) will be determined in King's medium B (KB) (10) amended with 0.1, 0.5, 1, 2, 4, 6, 8, and 10 mg streptomycin ml^{-1}. If different resistance levels are present, two or three strains from each level will be selected for further analysis. Otherwise, 30 strains will be selected from the pool of resistant *E. amylovora*.

To clone the S12 gene a 0.6-kb fragment containing the complete S12 gene will be amplified from the selected resistant strains and a sensitive strain EL01 and a APH-producing resistant strain CA11 of *E. amylovora* using the polymerase chain reaction (PCR). Forward primer GGCCTGGTGATGATGGCGGG in the 5' noncoding region of S12 gene and the reverse primer CGTGGCATGGAAATACTCCG which

complementary to the neighboring S7 gene, will be synthesized for the PCR amplification. The two primers were designed based on the consensus sequences in the two cloned S12-S7 genes from *E. coli* and *Salmonella typhimurium* (9, 23), which with *E. amylovora* belong to the family *Enterobacteriaceae*. The genomic DNA from the *E. amylovora* strains will be prepared (28) and the PCR reactions will be conducted as the described in the Part III of this thesis.

To clone the 16S rRNA gene a 0.6-kb DNA fragment will be amplified by PCR and used as probe. Forward primer GCACAATGGGCGCAAGCCTG and reverse primer GGTAAGGTTCTTCGCGTTGC will be used in the PCR amplification. These primers were designed based on the consensus sequences of 16S rRNA genes from *E. coli*, *Yersinia enterocolitica*, and *Erwinia carotovora* (1, GenBank Accession number M59149 and M59292). Genomic DNA from *E. amylovora* EL01 will be prepared and a cosmid genomic library will be constructed in vector pHC79 (8). Colony hybridization with the probe will be conducted to identify and isolate recombinant clones containing the 16S rRNA gene. The complete nucleotide sequence of 16S rRNA gene from *E. amylovora* EL01 will be determined and used as the standard for sequence comparison. Since the 0.6-kb fragment contains the 530-loop and 915 region, the two primers can be used not only for DNA amplification from the resistant strains and strains CA11 but also for direct sequencing of the amplified fragments. The sequences of r-protein S12 and 16S rRNA from strain EL01 and the resistant

strains will be compared using the FASTA program of the Genetics Computer Group Sequence Analysis Software Package, Version 7 (4). If the mutation site in the resistant strains is unique, a specific primer will be synthesized based on the principle of the amplification refractory mutation system (ARMS; 21) for detection of the point mutation by PCR.

The efficiency of protein synthesis and the error frequency between normal and altered ribosomes will be assayed. Ribosomes will be isolated by sucrose-gradient centrifugation (17) from sensitive strain EL01 and resistant strains of *E. amylovora*. Protein synthesis efficiency will be tested under the direction of a natural messenger, MS2 RNA in the absence or the presence of streptomycin (17). To establish error frequency, I will measure the ratio of isoleucine incorporated per phenylalanine under the direction of poly(U) in the presence of streptomycin (17).

To test whether *E. amylovora* with altered ribosomes is incompetent to the cell with wild-type ribosome, competition between a mutant and a wild-type *E. amylovora* in rich medium and minimum medium will be conducted. The growth curve of strain EL01 and mutants will be determined in KB medium and in minimum medium M9 (15). To test the competition between the wild type and ribosome-altered mutants, EL01 and one of the tested mutants will be co-cultivated in the KB medium and the M9 medium and the growth curve of both strains will be determined. The colony forming units (cfu) of the

streptomycin-resistant mutant and the total cfu will be determined by plating in KB medium amended with and without 50 μg streptomycin ml^{-1}, respectively. A low cfu ratio of mutant to wild-type indicates the mutant is more incompetent.

REFERENCES

1. Carbon, P., C. Ehresmann, B. Ehresmann, and J.-P. Ebel. 1979. The complete nucleotide sequence of the ribosomal 16S RNA from *Escherichia coli*: experimental details and cistron heterogeneities. Eur. J. Biochem. **100:**399-410.

2. Coyier, D. L., and R. P. Covey. 1975. Tolerance of *Erwinia amylovora* to streptomycin sulfate in Oregon and Washington. Plant Dis. Rep. **59:**849-852.

3. Davies, J., M. Cannon, and M. B. Mauer. 1988. Myomycin: mode of action and mechanism of resistance. J. Antibiot. **41:**366-372.

4. Devereux, I., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence programs for the VAX. Nucleic Acids Res. **12:**387-395.

5. Finken, M., P. Kirschner, A. Meier, A. Wrede, and E. C. Bottger. 1993. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol. Microbiol. 9:1239-1246.

6. Galili, S., H. Fromm, D. Aviv, M. Edelman, and E. Galun. 1989. Ribosomal protein S12 as a site for streptomycin resistance in *Nicotiana* chloroplasts. Mol. Gen. Genet. **218:**289-292.

7. Harris, E. H., B. D. Burkhart, N. W. Gillham, and J. E. Boynton. 1989. Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of *Chlamydomonas reinhardii*: correlation of genetic and physical maps of the chloroplast genome. Genetics 123:281-292.

8. Hohn, B., and J. Collins. 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291-298.

9. Hughes, D., and R. H. Buckingham. 1991. The nucleotide sequence of *rpsL* and its flanking region in *Salmonella typhimurium*. Gene **104:**123-124.

10. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301-307.

11. Leclerc, D., P. Melancon, and L. Brakier-Gingras. 1991. Mutations in the 915 region of *Escherichia coli* 16S ribosomal RNA reduce the binding of streptomycin to the ribosome. Nucl. Acids Res. **19:**3973-3977.

12. Liu, X.-Q., N. W. Gillham, and J. E. Boynton. 1989. Chloroplast ribosomal protein gene *rps12* of *Chlamydomonas reinhardtii*: wild-type sequence, mutation to streptomycin resistance and dependence, and function in *Escherichia coli*. **264:**16100-16108.

13. Loper, J. E., M. D. Henkels, R. G. Roberts, G. G. Grove, M. J. Willett, and T. J. Smith. 1991. Evaluation of streptomycin, oxytetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. Plant Dis. **75:**287-290.

14. McManus, P., and A. L. Jones. 1993. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan apple orchards and evaluation of oxytetracycline for control. (Submitted)

15. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

16. Melancon, P., C. Lemieux, and L. Brakier-Gingras. 1988. A mutation in the 530 loop of *Escherichia coli* 16S ribosomal RNA causes resistance to streptomycin. Nucl. Acids Res. **16:**9631-9639.

17. Melancon, P., D. Leclerc, L. Brakier-Gingras. 1990. A deletion mutation at the 5' end of *Escherichia coli* 16S ribosomal RNA. Biochim. Biophys. Acta **1050:**98-103.

18. Miller, T. D., and M. N. Schroth. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology. **62:**1175-1182.

19. Montandon, P.-E., R. Wagner, and E. Stutz. 1986. *E. coli* ribosomes with a C912 to U base change in the 16S rRNA are streptomycin resistant. EMBO J. **5:**3705-3708.

20. Montandon, P.-E., P. Nicolas, P. Schurmann, and E. Stutz. 1985. Streptomycin-resistance of *Euglena gracilis* chloroplasts: identification of a point mutation in the 16S rRNA gene in an invariant position. Nucl. Acids Res. **13:**4299-4310.

21. Newton C. R., A. Graham, L. E. Heptinstall, et al. 1989. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res. **17:**2503-16.

22. O'Connor, M., E. A De Stasio, and A. E. Dahlberg. 1991. Interaction between 16S ribosomal RNA and ribosomal protein S12: Differential effects of paromomycin and streptomycin. Biochimie **73:**1493-1500.

23. Post, L. E., and M. Nomura. 1980. DNA sequences from the *str* operon of *Escherichia coli*. J. Biol. Chem. **255:**4660-4666.

24, Powers, T., and H. F. Noller. 1991. A functional pseudoknot in 16S ribosomal RNA. EMBO J. **10:**2203-2214.

25. Schroth, M. N., S. V. Thomson, and W. J. Moller. 1979. Streptomycin resistance in *Erwinia amylovora*. Phytopathology. **69:**565-568.

26. Shaffer, W. H., and R. N. Goodman. 1985. Appearance of streptomycin-resistant *Erwinia amylovora* in Missouri apple orchards. (Abstr.) Phytopathology. **75:**1281.

27. Stuy, J. H., and R. B. Walter. 1992. Cloning, characterization, and DNA base sequence of the high-level streptomycin resistance gene *strA1* of *Haemophilus influenzae* Rd. J. Bacteriol. **174:**5604-5608.

28. Wilson, K. 1988. Preparation of genomic DNA from bacteria. Pages 2.4.1-2.4.5. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, (ed.), Current protocols in molecular biology. Vol. 1. John Wiley & Sons, New York.

29. van der Zwet, T., and H. L. Keil. 1979. Fire blight: A bacterial disease of Rosaceous Plants. U. S. Dep. Agric. Agric. Handb. 510. 200pp.

APPENDIX B

CONJUGATIONAL TRANSFER OF pEa34

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Conjugative plasmids are important vehicles for horizontal transfer of new genetic material into plant pathogenic bacteria. Besides the ubiquitous plasmid pEA29 (1), only a 56-kb plasmid has been described in some strains of *E. amylovora* (2). Plasmid pEa34, the plasmid characterized in this thesis, has not been described previously and appears to be of recent origin in E. amylovora. Therefore, the opportunity for the acquisition of antibiotic resistance via plasmids must be rare in E. amylovora. This may be the reason that plasmid-borne resistance in *E. amylovora* was not detected until 1990, nearly four decades after streptomycin was registered for the control of fire blight.

To be a conjugative plasmid, pEa34 is quite small. Normally, the whole *tra* gene cluster (encoding proteins for conjugation process) spans about 30 kb. pEa34 must have the whole *tra* cluster arranged in DNA of less than 28 kb. pEa34 may also be a broad-host-range plasmid. In addition to *E. amylovora*, pEa34 also exists in *E. herbicola* and can move by conjugation from the donors into *E. coli* and *P. syringae* pv. *papulans* (Table 1).

The detection of pEa34 in five strains of *E. herbicola* and the conjugative transfer of the plasmid from *E. herbicola*

TABLE 1. The transfer frequency of pEa34 by conjugation among strains of *Erwinia amylovora*, *Escherichia coli*, *Pseudomonas syringae* pv. *papulans* and *Erwinia herbicola*.

Donor Strain	Recipient Strain	Conjugation Frequency	Genetic Marker and[a] Screening Conditions
E. amylovora	*E. coli*		
CA11	JM109	1.1×10^{-4}	Str^r, 37°C
	DH5α	1.4×10^{-4}	Str^r, 37°C
	JM109(pUCD800)	3.7×10^{-5}	Str^r + Kan^r, 37°C
	JM109(pGEM3Zf(+))	5.1×10^{-5}	Str^r + Amp^r, 37°C
E. amylovora	*P. syringae* pv. *papulans*		
CA11	Psp32	7.9×10^{-8}	Str^r + Rif^r, fluorescent on King's B medium
E. coli	*E. amylovora*		
JM109(pEa34)	BC06	5.0×10^{-5}	Str^r + Rif^r
E. coli	*E. coli*		
JM109(pEa34)	JM109(pUCD800)	3.2×10^{-3}	Str^r + Kan^r
E. coli	*P. syringae* pv. *papulans*		
JM109(pEa34)	Psp32	5.3×10^{-9}	Str^r + Rif^r, fluorescent on King's B medium
E. herbicola	*E. coli*		
144	JM109(pGEM3Zf(+))	1.7×10^{-2}	Str^r + Amp^r
E. herbicola	*E. amylovora*		
144	BC06	1.2×10^{-7}	Str^r + Rif^r, not 37°C

Table 1 (Cont'd)

[a] Str^r: streptomycin-resistant; Kan^r: kanamycin-resistant; Amp^r: ampicillin-resistant; Rif^r: Rifampicin-resistant; 37°C: screening the transconjugants at 37°C.

into *E. amylovora* suggests the possible movement of pEa34 between the two bacterial species. Dissemination of resistance on conjugative plasmid can be horizontal and vertical, but the dissemination of resistance on chromosome can only be vertical. Therefore, the occurrence of streptomycin resistance on pEa34 in *E. amylovora* is likely to hasten the spread of resistance into additional Michigan apple orchards via conjugational transfer of pEa34 into sensitive strains of the pathogen.

REFERENCES

Falkenstein, H., P. Bellemann, S. Walter, W. Zeller, and K. Geider,. 1988. Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. **54:**2798-2802.

Steinberger, E. M., G.-Y. Cheng, and S. V. Beer. 1990. Characterization of a 56-kb plasmid of *Erwinia amylovora* Ea322: its noninvolvement in pathogenicity. Plasmid **24:**12-24.

APPENDIX C

IDENTIFICATION OF THE MECHANISMS OF STREPTOMYCIN RESISTANCE

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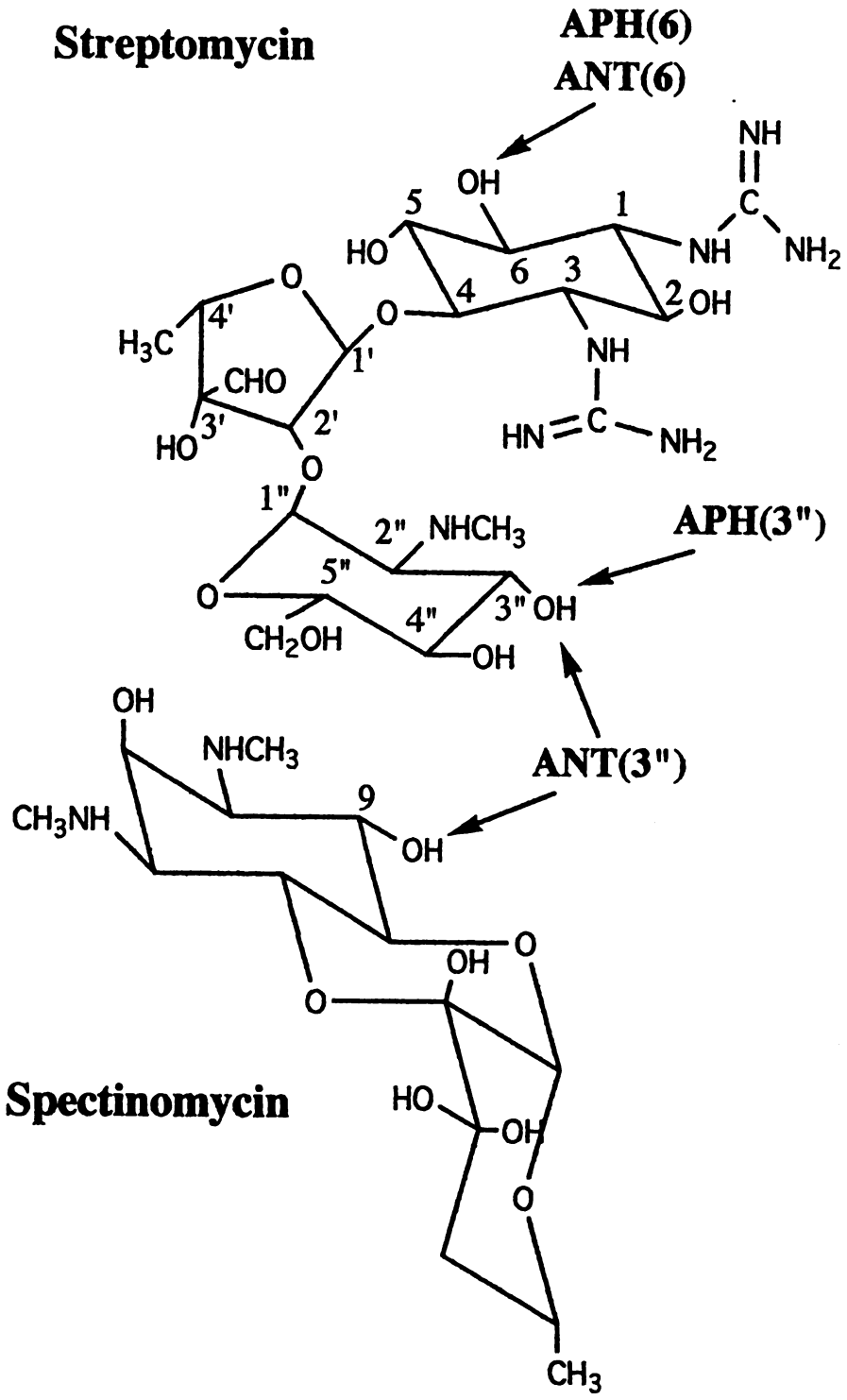
Among the mechanisms for streptomycin resistance, the resistance mechanisms based on modifying enzymes or on altered ribosomes are more important than the mechanism based on reduced accumulation because the former exhibit high level of resistance. Resistant strains isolated from orchards and that can grow on media plates with 20 μg streptomycin ml^{-1} or more are considered that resistance in the strains is enzyme-mediated or ribosome-mediated.

Streptomycin is inactivated via enzymatic modification by aminoglycoside phosphotransferases, APH(3") and APH(6), and aminoglycoside adenylyltransferases, ANT(3") and ANT(6). The modification is accomplished by transfer of a phosphate group (APH enzymes) or adenyl group (ANT enzymes) from ATP to the 3"- or 6-hydroxyl group (see Fig. 1).

Myomycin is an broad-spectrum antibiotic produced by a member of the *Nocardia* genus (3). The mode of action of myomycin in vivo and in vitro closely resembles that of streptomycin. Spontaneous myomycin-resistant mutants of *E. coli* are essentially indistinguishable from streptomycin-resistant mutants at the ribosomal RNA and ribosomal protein. However, myomycin is not a substrate for the known

Fig. 1. Molecular structures of streptomycin and spectinomycin and the reaction sites of APH(3"), APH(6), ANT(3"), and ANT(6).


Streptomycin
APH(6)
ANT(6)
APH(3")
ANT(3")
Spectinomycin

streptomycin-modifying enzymes and could be useful in the characterization of natural streptomycin-resistant isolates and in counterselecting against the presence of streptomycin-modifying enzymes.

Recent studies on streptomycin resistance have enabled the resistance mechanisms to be characterized in molecular level. Streptomycin resistant isolates can be divided into enzyme- and ribosome-mediated mechanisms by myomycin (Fig. 2). The modifying enzymes, APH and ANT, can be identified by phosphocellulose biding assay using [γ-^{32}P]-ATP and ^{14}C-ATP, respectively, as cosubstrates (1). APH(3") and APH(6) can be distinguished by analyzing the respective phosphorylated streptomycin using ^{13}C-NMR analysis and chemical reactions (Part III, 6). ANT(3") and ANT(6) can be distinguished by the resistance to spectinomycin (2, 4) or analyzing their adenylylated streptomycin with complicated chemical methods (8). However, the easiest way for identifying the enzyme-mediated mechanism is to probe the existence of the specific genes which have been cloned (7). Since altered ribosome is insensitive to streptomycin in protein synthesis and decoding of translation (5), to confirm the ribosome-mediated mechanism the sensitivity of ribosome to streptomycin in protein synthesis needs to be determined. Although alteration of several ribosomal subunits can reduce the affinity of ribosome to streptomycin, only mutations on 16S rRNA and ribosomal protein S12 are common. To characterize this mechanism

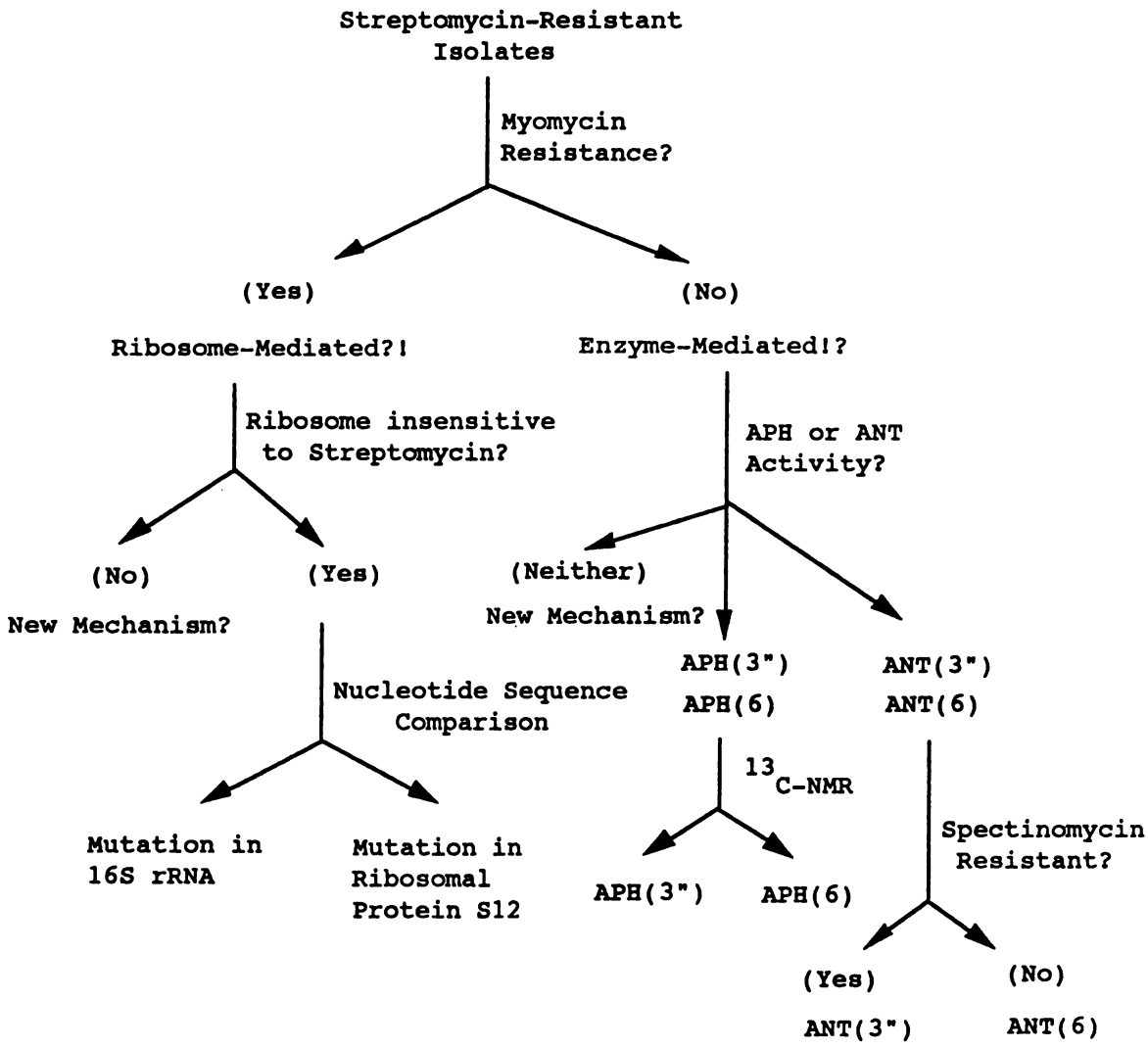


Fig. 2. Flowchart for identification of streptomycin resistance mechanisms

comparisons of the nucleotide sequences of the ribosome component genes from sensitive and resistant strain and test of the insensitivity of ribosome to streptomycin are required.

Studies with myomycin on the bacteria isolated from Michigan and New York State apple orchards have shown that myomycin can counterselect the resistance with modifying enzymes. *E. amylovora* CA11, *P. syringae* pv. *papulans* Psp36, *P. aeruginosa* 60, and *E. herbicola* 144, which exhibit an aminoglycoside phosphotransferase activity and contain DNA that hybridize to probe SMP3, are sensitive to myomycin (Table 1). The other resistant strains which contain no DNA homologous to the SMP3 are myomycin-resistant. The myomycin-resistant strains may have an altered ribosome because they are also highly resistant to streptomycin.

TABLE 1. Characterization of mechanisms of streptomycin resistance by the ability of resistance to streptomycin and myomycin, hybridization to SMP3, and in vitro streptomycin phosphotransferase (APH) and streptomycin adenylyltransferase (ANT) assay.

Designation	Streptomycin-Resistant	Myomycin-Resistant	Hybridization to SMP3[a]	Enzyme Activity[b]	
				APH	ANT
Erwinia amylovora					
EL01, BC06	No	No	No	No	No
CA11, HT62-1	Yes	No	Yes	Yes	No
R11, S5, Ea88	Yes	Yes	No	No	No
E. herbicola					
6a	Yes	No	Yes	Yes	No
180	Yes	Yes	No	No	No
Pseudomonas syringae pv. *papulans*					
Psp32	No	No	No	No	No
Psp36	Yes	No	Yes	Yes	No
MC37, MC38	Yes	Yes	No	No	No
Pseudomonas aeruginosa					
60	Yes	No	Yes	Yes	No

[a] A 0.5-kb *tnpR-strA* region from *P. syringae* pv. *papulans* Psp36.

[b] APH: aminoglycoside phosphotransferase; ANT: aminoglycoside nucleotidyltransferase.

REFERENCES

1. Davies, J. 1986. Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes, p. 790-809 In: V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore, MD.

2. Davies, J., and D. I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32:**469-518.

3. Davies, J., M. Cannon, and M. B. Mauer. 1988. Myomycin: mode of action and mechanism of resistance. J. Antibiot. **41:**366-372.

4. Hollingshead, S., and D. Vapnek. 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid 13:17-30.

5. Melancon, P., D. Leclerc, L. Brakier-Gingras. 1990. A deletion mutation at the 5' end of Escherichia coli 16S ribosomal RNA. Biochim. Biophys. Acta **1050:**98-103.

6. Ozanne, B., R. Benveniste, D. Tipper, and J. Davies. 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. **100:**1144-1146.

7. Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Reviews **57:**138-163.

8. Suzuki, I., N. Takahashi, S. Shirato, H. Kawabe, and S. Mitsuhashi. 1975. Adenylylation of streptomycin by *Staphylococcus aureus*: a new streptomycin adenylyltransferase, p. 463-473. In S. Mitsuhashi and H. Hashimoto (ed.), Microbial drug resistance. University Park Press, Tokyo.