THE EVOLUTION OF COPPER AND STREPTOMYCIN RESISTANCE IN *PSEUDOMONAS SYRINGAE*

By

GEORGE W. SUNDIN

Bachelor of Science
The Pennsylvania State University
State College, Pennsylvania
1986

Master of Science
Michigan State University
East Lansing, Michigan
1988

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1994

THE EVOLUTION OF COPPER AND STREPTOMYCIN RESISTANCE IN *PSEUDOMONAS SYRINGAE*

Thesis Approved:

Carol Bender
Thesis Advisor

Ulrich Melcher

John V. Morr

Bruce A. Stevens

Jan Bell

Thomas C. Collins
Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Carol Bender for her guidance, patience, support, excellent editorial skills, and willingness to allow me to pursue many of the projects detailed within this dissertation. I also thank my committee members Dr. Ulrich Melcher, Dr. Robert Miller, Dr. Bruce Stermer, and Dr. Sharon von Broembsen for their good ideas concerning my research.

I would like to give special thanks to my wife, Janette, for her encouragement, suggestions, and caring love during this process.

I thank Dave Monks and Carol Rodgers for valuable technical assistance with the research reported in Chapters IV and VIII. Lastly, I thank the Oklahoma State University Foundation for partial financial support through a fellowship endowed by Mr. and Mrs. Eugene Miller.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	3
	Bactericide resistance in phytopathogenic bacteria	3
	Characterization of plasmids in phytopathogenic pseudomonads	10
	Evolution of antibiotic resistance in clinical bacteria	13
	Objectives	14
III.	ECOLOGICAL AND GENETIC ANALYSIS OF COPPER AND STREPTOMYCIN RESISTANCE IN *PSEUDOMONAS SYRINGAE* PV. SYRINGAE	15
	Summary	15
	Materials and Methods	19
	Bacterial strains, plasmids, and media	19
	Source and identification of bacteria	19
	Phenotypic characterization of copper and streptomycin resistance in *Pseudomonas syringae* pv. syringae	21
	Characterization of plasmids	21
	Molecular genetic techniques	22
	Cloning and characterization of the streptomycin-resistance genes from pPSR1	23
	Hybridization experiments with DNA probes containing copper-resistance determinants from phytopathogenic bacteria	24
	Results	25
	Source and identification of bacteria	25
	Phenotypic characterization of copper and streptomycin resistance	25
	Characterization of plasmids	26
	Cloning and characterization of the streptomycin-resistance genes from *Pseudomonas syringae* pv. syringae	28
	Hybridization experiments with copper-resistance gene probes	32
	Discussion	32
IV.	THE EVOLUTION OF COPPER AND STREPTOMYCIN RESISTANCE IN *PSEUDOMONAS SYRINGAE*	36
	Summary	36
	Materials and Methods	41
	Bacterial strains, plasmids, and media	41
	Source of *P. syringae* isolates	41
	Characterization of plasmids	44
	Molecular genetic techniques	44
	Phenotypic characterization of the *P. syringae* isolates	45

Chapter Page

Genetic analysis of a subset of *P. syringae* isolates using AP-PCR 46
Data analysis 47
Results 47
Colonization of *P. calleryana* trees by *P. syringae* . 47
Characterization of plasmids 49
Phenotypic characterization of a subset of *P. syringae* isolates 59
AP-PCR analysis of *P. syringae* pv. syringae strains 59
Discussion 69

V. RELATIVE FITNESS *IN VITRO* AND *IN PLANTA* OF *PSEUDOMONAS SYRINGAE* CONTAINING COPPER AND STREPTOMYCIN RESISTANCE PLASMIDS 77

Summary 77
Materials and Methods 80
Bacterial strains, plasmids, antibiotics, and media 80
Population dynamics of FF5.1 and FF5.1(pPSR1) on ornamental pear leaves 82
Plasmid stability experiments 83
Competition experiments 84
Results 85
Population dynamics of FF5.1 and FF5.1(pPSR1) on ornamental pear leaves 85
Plasmid stability 88
In vitro competition experiments 88
In planta competition experiments 89
Discussion 94

VI. DETECTION OF IS*6100* WITHIN THE STREPTOMYCIN RESISTANCE TRANSPOSON TN*5393* IN *XANTHOMONAS CAMPESTRIS* PV. VESICATORIA 98

Summary 98
Introduction 99
Results and Discussion 102
Detection of IS*6100* within Tn*5393* in *X. campestris* pv. vesicatoria 102
Codon usage analysis of the putative transposase protein of IS*6100* 104
Conclusions 109

VII. GENETIC ANALYSIS OF SEQUENCES DIRECTING EXPRESSION OF THE *strA/strB* STREPTOMYCIN RESISTANCE GENES IN *PSEUDOMONAS SYRINGAE* AND *XANTHOMONAS CAMPESTRIS* 110

Summary 110
Materials and Methods 113
Bacterial strains, plasmids, and media 113
Cloning and sequencing of regions from Tn*5393* from *P. syringae* 113
Construction and analysis of *strA-uidA* fusions . . . 116
Analysis of the transcriptional start site located within *res* in *P. syringae* pv. syringae 117
Results 117
Cloning and sequencing of regions from Tn*5393* from *P. syringae* 117

Chapter Page

Quantification of *strA* promoter activity in *P. syringae* and *X. campestris* 118
Analysis of the transcriptional start site located within *res* in *P. syringae* pv. syringae 120
Discussion 123

VIII. DETECTION OF SEQUENCES FROM THE STREPTOMYCIN-RESISTANCE TRANSPOSON TN*5393* IN DIVERSE PHYLLOPLANE AND SOIL BACTERIA IN MANAGED AGRICULTURAL ECOSYSTEMS 128
Summary 128
Materials and Methods 131
Bacterial strains, plasmids, and media 131
Isolation of bacteria from leaf and soil samples . . 132
Characterization of bacterial isolates 134
Molecular genetic techniques 134
Hybridization experiments 135
Results 135
Isolation of bacteria from leaf and soil samples . . 135
Phenotypic and genetic characterization of bacterial isolates 137
Resistance to tetracycline in the streptomycin-resistant isolates 138
Discussion 141

IX. CONCLUSIONS 143

BIBLIOGRAPHY 147

LIST OF TABLES

Table		Page
1.	Characterization of copper- and streptomycin-resistance determinants in phytopathogenic bacteria	5
2.	Distribution of the *strA/strB* streptomycin resistance and the *sulII* sulfonamide resistance determinants among bacterial residents and pathogens of humans, animals, and plants	8
3.	Bacterial strains and plasmids and their relevant characteristics (Chapter 3)	20
4.	Number of *P. syringae* pv. syringae strains with resistance to copper and/or streptomycin	26
5.	Bacterial strains and plasmids and their relevant characteristics (Chapter 4)	42
6.	Colonization of *Pyrus calleryana* cv. Aristocrat leaves by *Pseudomonas syringae* at three nurseries in Oklahoma and recovery of *P. syringae* isolates with resistance or sensitivity to copper and streptomycin	48
7.	Number of *Pseudomonas syringae* strains belonging to 18 distinct plasmid profile groups.	57
8.	Characterization of 14 profiles groups of plasmids encoding copper and/or streptomycin resistance and three profile groups of cryptic plasmids from *Pseudomonas syringae* isolates recovered from three *Pyrus calleryana* cv. Aristocrat nurseries in Oklahoma	60
9.	Relevant characteristics of 100 *Pseudomonas syringae* pv. syringae strains whose chromosomal genotypes were examined using the arbitrarily-primed polymerase chain reaction technique. The strains were isolated from two *Pyrus calleryana* cv. Aristocrat nurseries in Oklahoma. The order of the strains corresponds to the order in the accompanying dendrogram (Fig. 5)	67
10.	Bacterial strains and plasmids and their relevant characteristics (Chapter 5)	81
11.	Bacterial strains and plasmids and their relevant properties (Chapter 6)	101
12.	Codon usage of the putative transposase gene of IS*6100*, and 37 and 16 coding sequences of *Xanthomonas campestris* and *Mycobacterium* spp., respectively	105

Table Page

13. Correspond analysis of codon usage tables from the putative transposase gene of IS*6100* with codon usage tables from five bacterial genera 106

14. Bacterial strains and plasmids and their relevant characteristics (Chapter 7) 114

15. Mean bacterial populations (phylloplane and soil samples) from *Pyrus calleryana* cv. Aristocrat nurseries and tomato fields in Oklahoma recovered on medium with and without added streptomycin 136

16. Number of streptomycin-resistant phylloplane and soil isolates from *Pyrus calleryana* cv. Aristocrat nurseries and tomato fields which contain Tn*5393* 139

17. Resistance to tetracycline among the streptomycin-resistant isolates recovered from the phylloplane and soil of *Pyrus calleryana* cv. Aristocrat nurseries and tomato fields 140

LIST OF FIGURES

Figure — Page

1. Plasmids profiles of *Pseudomonas syringae* pv. syringae strains isolated from woody hosts. Lanes: 1, FF3 (pPSR5); 2, A2 (pPSR1); 3, AA2 (pPSR6); 4, E2 (pPSR2); 5, H12 (pPSR3). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA . . 27

2. Agarose gel electrophoresis (A) and hybridization (B) of plasmid DNA from Sm^r phytopathogenic bacteria with the 1.5-kb *Sac*I - *Eco*RV fragment containing *strA* and *strB* from plasmid RSF1010. Lanes 1 - 5 contain the following strains of *P. syringae* pv. syringae: 1, FF3 (pPSR5); 2, A2 (pPSR1); 3, E2 (pPSR2); 4, H12 (pPSR3); 5, G1 (pPSR3); 6, *P. syringae* pv. papulans Psp36; 7, *Xanthomonas campestris* pv. vesicatoria BV5-4a; 8, *E. coli* (RSF1010). LPC is linearized plasmid and chromosomal DNA. 29

3. Conservation of restriction sites over a 1.7 kb region encoding *strA* and *strB* in RSF1010 and streptomycin resistance in pSM1. E, *Eco*RI (8676)*; H, *Hinc*II (8682); S, *Ssp*I (75); N, *Nru*I (169,441); CII, *Sac*II (194); CI, *Sac*I (201); AI, *Ava*I (258); R, *Rsa*I (324, 520, 1594); O, *Nco*I (605); AII, *Ava*II (638, 845); D, *Dde*I (1209); B, *Bst*YI (1429); T, *Not*I (1664); EV, *Eco*RV (1702). * Position numbers are from the published RSF1010 sequence (Scholz et al, 1989). Arrows indicate the direction of transcription. 31

4. The percentage of *Pyrus calleryana* cv. Aristocrat leaves colonized by *Pseudomonas syringae*, with each phenotype of resistance or sensitivity to copper and streptomycin, recovered from three nurseries in Oklahoma. S = sensitive to copper and streptomycin (Cu^s Sm^s), Cu = copper resistant (Cu^r) Sm^s, Sm = Cu^s Sm^r, CuSm = Cu^r Sm^r, + = strains with different phenotypes were recovered from a single leaf. Fifteen foliar applications of copper (2.4 g/l) and streptomycin (0.2 g/l) bactericides per season in Nursery I; 20 foliar applications of copper (2.4 g/l) bactericides per season in Nursery II; no bactericides were applied in Nursery III. Two hundred leaves were sampled over two growing seasons at Nurseries I and II; 75 leaves were sampled during one growing season at Nursery III. 50

Figure Page

5. Plasmid profiles (A) and (B) from isolates of *Pseudomonas syringae* with resistance to copper and/or streptomycin which were recovered from three *Pyrus calleryana* cv. Aristocrat nurseries in Oklahoma. Lanes (A) 1 to 9 and (B) 1 to 5 contain the following isolates (and plasmids and plasmid profile designation): (A) 1, 7B44 (pPSR7 - 1); 2, 2H12 (pPSR8 - 2); 3, 3C1 (pPSR9 - 3); 4, 8B48 (pPSR10 - 4); 5, 7G14 (pPSR7, pPSC4 - 1A); 6, 7A36 (pPSR11, pPSC4 - 5); 7, 7C12 (pPSR7, pPSR15 - 6); 8, 7B12 (pPSR1 - 7); 9, 8C32 (pPSR14 - 8). (B) 1, 2E49 (pPSR2 - 9); 2, 7B22 (pPSR12 - 10); 3, 7F14 (pPSR3 - 11); 4, 9A26 (pPSR14, pPSC6 - 8A); 5, 9A22 (pPSR13 - 12). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA . . 53

6. Characterization of individual plasmids from plasmid profile groups identified in *Pseudomonas syringae* isolates from three *Pyrus calleryana* cv. Aristocrat nurseries in Oklahoma. Cu = copper, sm = streptomycin, r = resistant. Group 1 plasmids contained sequences which hybridized to a probe containing the *oriV* and *par* loci from pOSU22 (Mukhopadhyay et al 1990). Group 2 comprised plasmids of 190 - 220 kilobase pairs 55

7. Genomic fingerprinting of *Pseudomonas syringae* pv. syringae strains using the arbitrarily-primed polymerase chain reaction technique and the REP primers (Panel A) of the IS*50* primer (Panel B). Panels A and B (lanes 1 - 12 each) contain the following strains (and PCR group): (Panel A) 1, 8F21 (B); 2, 8F35 (B); 3, 8F43 (B); 4, 8G5 (B); 5, 8G10 (B); 6, 8G17 (A); 7, 8C43 (A); 8, 8D2 (B); 9, 8D6 (B); 10, 8D17 (B); 11, 8C5 (B); 12, 8D34 (B). (Panel B) 1, 9B36 (B); 2, 9D8 (B); 3, 8F21 (B); 4, 7F29 (A); 5, 9A3 (A); 6, 9C39 (B); 7, 8C32 (B); 8, 8C43 (A); 9, 8D6 (B); 10, 8D17 (B); 11, 8D29 (A); 12, 8E17 (B). Size standards (in base pairs) are indicated at left . . 62

8. Dendrogram of *Pseudomonas syringae* pv. syringae strains derived from PCR fingerprints generated from the IS*50* and REP primers. The dendrogram was generated from the combined IS*50* and REP data sets 65

9. Populations of *Pseudomonas syringae* pv. syringae FF5.1 [panel **A**] and FF5.1(pPSR1) [panel **B**] on nontreated *Pyrus calleryana* leaves (•); leaves treated weekly with $Cu(OH)_2$ at 2.4 g/l and streptomycin sulfate at 0.2 g/l (■); leaves treated weekly with $Cu(OH)_2$ at 2.4 g/l (□); and leaves treated weekly with streptomycin sulfate at 0.2 g/l (▲). The bactericide spray program was initiated two days following inoculation. The vertical bars represent the standard error of the mean $\log_{10}$ bacterial population sizes 86

10. Competition between plasmid-containing and plasmid-free strains of *Pseudomonas syringae* pv. syringae in a glucose-limited minimal medium. The plasmid-free strain FF5 (•) was competed with (**A**) FF5(pPSR4), □; (**B**) FF5(pPSR5), ▲; and (**C**) FF5(pPSR4::Tn5393), ■ 90

Figure Page

11. Competition between plasmid-containing and plasmid-free strains of *Pseudomonas syringae* pv. syringae grown epiphytically on the surfaces of bean leaves. The plasmid-free strain FF5 (•) was competed against (**A**) FF5(pPSR4), □; (**B**) FF5(pPSR5), ▲; and (**C**) FF5(pPSR4::Tn*5393*), ■. 92

12. Structure of the streptomycin-resistance (Sm^r) transposon Tn*5393* (41). In *Erwinia amylovora*, IS*1133* is inserted downstream of the transposase (*tnpR*) gene and upstream of the *strA* Sm^r gene. Putative promoter sequences are identified by arrows 100

13. (A) Characterization of the region within Tn*5393* upstream of *strA* in *Xanthomonas campestris* pv. vesicatoria. An 880-bp sequence, identical to that of IS*6100* is inserted within the *tnpR* gene. The 14-bp inverted repeats are indicated by arrows. The putative transposase gene of IS*6100* is indicated by the line with the direction of transcription indicated by the arrow. (B) Putative physical map of Tn*5393* within *X. campestris* pv. vesicatoria. The *strA*/*strB* genes were mapped previously (215). The region sequenced included all of *res*, IS*6100*, *tnpR*, and 420 bp of *tnpA* upstream of *res* 103

14. Genetic maps of Tn*5393* from *Erwinia amylovora* (41), *Pseudomonas syringae* pv. syringae, and *Xanthomonas campestris* pv. vesicatoria (217). Genes which have been characterized are boxed; open boxes refer to genes which have been partially sequenced. IR = inverted repeat, tnpA = transposase, tnpR = resolvase, res = recombination site, strA and strB are aminoglycoside phosphotransferase genes, E = *Eco*RV, S = *Sst*I. Putative promoter sequences detected within Tn*5393* in *E. amylovora* and the direction of transcription are identified by arrows 119

15. Analysis of β-glucuronidase (GUS) activity determined fluorimetrically from lysates of (A) *P. syringae* pv. syringae FF5 and (B) *X. campestris* pv. vesicatoria XV16 containing *strA-uidA* fusion clones. GUS activity is reported as specific activity (units per milligram protein) ± the standard deviation of the mean of two replicates from two independent experiments. tnpA = transposase, res = recombination site, tnpR = resolvase, strA = aminoglycoside phosphotransferase genes of Tn*5393*. E = *Eco*RV, H = *Hin*dIII, N = *Nru*I, P = *Pst*I, R = *Rsa*I, S = *Ssp*I, T = *Sst*I 121

16. Determination of the transcriptional start site of *strA* by primer extension. The ^{32}P-labeled cDNA product was electrophoresed in parallel with G/A/T/C sequence reactions generated using the same primer. 122

Figure Page

17. Identification of a putative promoter sequence within the intergenic region between the resolvase (*tnpR*) gene and the coding sequence of the streptomycin-resistance gene *strA* on Tn*5393*. The termination codon of *tnpR* (position 1) is shown in underlined bold characters. A putative σ^{70} promoter is shown in overlined bold characters. The initiation codon of *strA* (TTG - position 70) is also the first three nucleotides of the putative -35 sequence . . 126

18. Nucleotide sequence of the recombination site (*res*) and partial sequence of the *tnpR* gene of Tn*5393* from *Pseudomonas syringae* pv. syringae A2. The transcriptional start site is shown by the asterisk. A putative σ^{70} promoter is shown in overlined bold characters. The translational initiation codon of *tnpR* (position 196) is shown in underlined bold characters. The sequence of the oligonucleotide primer utilized in the primer extension reaction is indicated, and is the complement of nucleotides 205 - 221. 127

19. Location of *Pyrus calleryana* cv. Aristocrat nurseries (A - E) and tomato fields (a - c) sampled in the present study 133

CHAPTER I

INTRODUCTION

The phytopathogen Pseudomonas syringae causes many important diseases on a wide variety of host plants. The original strain was isolated from diseased lilac (Syringa vulgaris) by van Hall in 1902 (173). Over the following decades, pathogenic bacteria with similar characteristics to P. syringae, but differing in traits such as host range, symptoms produced, and nutrient utilization patterns, were isolated from several plant species. Although these bacteria were originally assigned as new species, the overall similarity of the group resulted in all individual species being lumped into P. syringae (71). However, phytobacteriologists needed to effectively distinguish these different P. syringae "species", and the term "pathovar" or pathological variant was proposed as a subspecies designation (74). Genetic relationships between pathovars are becoming better understood, and in some cases, individual pathovars may be distantly-related enough to warrant separate species designations (67).

P. syringae pv. syringae is a wide-host-range phytopathogen (25), an important epiphyte or phylloplane resident (98), and an active ice nucleus at temperatures slightly below freezing (138). P. syringae pv. syringae is the causal agent of several important diseases on woody hosts including bacterial canker of stone fruits

(103,184), blossom blast of pear (148,233), blister bark of apple (94), and tip dieback of ornamental trees (32). The life cycle of P. syringae pv. syringae on tree hosts involves overwintering in dormant buds (29,72,77,185,219); epiphytic colonization of leaf and floral tissue as they unfold from the bud (98,128); a leaf spot, canker, and epiphytic phase during the summer (124,186,219,239); followed by colonization of dormant buds in the fall (31,56,57,219). Bactericides are heavily utilized in most P. syringae pv. syringae disease management programs on woody hosts, since they are crops of high economic value. Copper and streptomycin are both recommended for use in the reduction of populations of P. syringae pv. syringae on various woody hosts (20,58,165,166,193,238). Spray programs are usually initiated early in spring while the trees are still dormant, and continue throughout the growing season. Recently, the development of resistance to bactericides has been observed in populations of P. syringae pv. syringae and other phytopathogenic bacteria. A disease problem on ornamental trees in nurseries in Oklahoma, and the evolution of resistance to copper and streptomycin within populations of the pathogen, P. syringae pv. syringae, are the focus of the research reported in this dissertation.

CHAPTER II

LITERATURE REVIEW

Bactericide resistance in phytopathogenic bacteria

Plant diseases of economic importance caused by phytopathogenic bacteria occur on virtually every important agricultural crop in all agricultural regions of the world. While effective management of bacterial diseases includes cultural practices and the use of available resistant plant varieties, chemical control agents have been heavily utilized on crops of high economic value. Copper, streptomycin, and tetracycline are the most commonly utilized bactericides. Copper, a heavy metal atom, is utilized as a foliar spray in the formulations $Cu(OH)_2$ or $CuSO_4$, and is toxic to bacterial cells when present in the Cu^{2+} free ion state (248). Copper compounds, which are also applied to some crops as fungicides, are the most inexspensive of the bactericides, and the most heavily utilized. The antibiotics streptomycin and tetracycline are usually registered for use either on crops such as apple on which copper is phytotoxic, or on nonfood crops such as ornamental trees. An important sideline of the use of streptomycin and tetracycline on plants is that these antibiotics are also used in human medicine, and administered to livestock as growth promotants.

The evolution of resistance to bactericides in populations of phytopathogenic

bacteria has become an important constraint to effective disease management. While copper resistance has only been extensively characterized in the last decade, strains of copper-resistant (Cu^r) phytopathogenic bacteria isolated from 1960 - 1980 were detected in one culture collection (149). Resistance to copper or streptomycin has been reported in three genera of phytopathogenic bacteria (Table 1). Recently, there have been two reports of resistance to both copper and streptomycin within individual strains of two phtopathogens, Pseudomonas syringae pv. syringae and Xanthomonas campestris pv. vesicatoria (182,215). Tetracycline has not been utilized as much as streptomycin and, to date, tetracyline-resistance has not been observed in phytopathogenic bacteria (143).

Researchers studying bactericide resistance have been interested in the genetic determinants and mechanisms of resistance in individual phytopathogens, the horizontal dissemination of bactericide-resistance determinants on conjugative plasmids, and the effect of bactericide-resistant strains on crop production. Copper-resistance determinants have been cloned from P. syringae pv. tomato (13) and X. campestris pvs. juglandis (129) and vesicatoria (84). The Cu^r determinant from P. syringae pv. tomato is organized as an operon (cop operon) (156) which consists of four genes (copA, copB, copC, copD). Transcription of the cop operon occurs from a promoter sequence located within an 87-bp region upstream from the translational start site of copA (157). Transcription is induced by subinhibitory levels of Cu^{2+} ions, and is under the control of copR and copS, regulatory genes which have homology to two component bacterial regulatory systems which respond to

Table 1. Characterization of copper- and streptomycin-resistance determinants in phytopathogenic bacteria.

Genus/Species	Genomic Location[a]	Relevant characteristics[b]	Reference
Copper			
Pseudomonas syringae			
pv. syringae	P, tra+	cherry; MI, USA; plasmids (multiple sizes)	218
	P, tra+	ornamental trees; OK, USA; plasmids (multiple sizes)	215
pv. tomato	P, tra-	tomato; CA, USA; pPT23D *cop* operon	13
P. syringae	C	CA, USA	5
	P, tra-	Impatiens; CA, USA; pPSI1; *cop* operon	48
Pseudomonas spp.	P	tomato; CA, USA; *cop* operon	50
Xanthomonas campestris			
pv. juglandis	C	almond; CA, USA; *cop* operon	129
pv. vesicatoria	P, tra+	pepper; FL, USA; pXvCu-type plasmids	210
	P, tra+	pepper; OK, USA; pXvCu-type plasmids	14
	NT	pepper; Mexico	1
	NT	pepper; NC, USA	182
	P	tomato; CA, USA; *cop* operon	50
Streptomycin			
Erwinia amylovora	C	apple; CA, USA	199
	C	apple; Pacific Northwest, USA	143
	P, tra+	apple; MI, USA; *strA*/*strB*; Tn*5393*::IS*1133*	40,41
E. carotovora	NT	Japan	81
P. lachrymans	NT	cucumber; Japan	245

Table 1 (continued)

Genus/Species	Genomic Location[a]	Relevant characteristics[b]	Reference
P. syringae			
pv. papulans	P, tra+	apple; NY, USA; pCPP501 strA/strB; Tn5393	30
		apple; MI, USA; plasmids (multiple sizes) strA/strB; Tn5393	41, 170
pv. syringae	P, tra+	ornamental trees; OK, USA; plasmids (multiple sizes); strA/strB; Tn5393	215,217
P. syringae	NT	cherry laurel; Canada	64
	NT	apricot; New Zealand	246
X. campestris			
pv. dieffenbachiae	NT	Dieffenbachia	112
pv. vesicatoria	P, tra-	pepper; FL, OH, USA; strA/strB; Tn5393::IS6100	163a,215
	NT	pepper; NC, USA	182

[a] C = chromosome, P = plasmid, tra+ = self-transmissible via conjugation, tra- = nonconjugative, NT = not tested.

[b] CA = California, FL = Florida, MI = Michigan, NC = North Carolina, NY = New York, OH = Ohio, OK = Oklahoma; strA/strB are homologs of the aminoglycoside phosphotransferase genes from RSF1010 (Scholz et al 1989).

environmental stimuli (163). Regulation of expression of the cop operon also appears to be controlled by at least one chromosomal gene, which shares homology to the cop operon (136). The resistance mechanism of the cop operon appears to be through the sequestration of toxic Cu^{2+} ions in the periplasm of the cell. The CopA protein is the

main Cu-binding protein, and binds 11 Cu^{2+} ions per molecule (34). The sequence of the chromosomal Cu^r determinant from X. campestris pv. juglandis is organized similarly to, and shares nucleotide sequence and amino acid sequence homology to the cop operon (129). The Cu^r determinant from X. campestris pv. vesicatoria has limited homology to copA from P. syringae pv. tomato although the determinants appear to have genus-specific promoter elements (229).

Although streptomycin resistance has been reported in many phytopathogenic bacteria, genetic analyses of Sm^r determinants have been conducted only recently. The Sm^r determinants from P. syringae pv. papulans and X. campestris pv. vesicatoria were the first to be cloned (163a,170). A probe constructed from the Sm^r determinant from X. campestris pv. vesicatoria hybridized to the Sm^r plasmid pCPP501 from P. syringae pv. papulans, but did not hybridize to total DNA of Sm^r E. amylovora strains from California (163a). Sundin and Bender (215) demonstrated that the Sm^r determinants from P. syringae pvs. papulans and syringae, and X. campestris pv. vesicatoria were highly similar to the tandem strA/strB aminoglycoside phosphotransferase genes which are present on small nonconjugative plasmids such as RSF1010. Concurrently, Chiou and Jones (41) determined the nucleotide sequence of the strA/strB homologs from E. amylovora. The strA/strB homologs shared 99.8% nucleotide sequence identity with the strA/strB genes from RSF1010, and were harbored on a 6.7-kb transposable element, Tn5393 (41). Tn5393 has since been detected in the strains of P. syringae pv. syringae and X. campestris pv. vesicatoria which contained strA/strB (217), and in a variety of gram-negative bacteria inhabiting

apple orchards in regions in which streptomycin is utilized (170,209). The strA/strB genes are widespread among bacterial residents and pathogens of humans, animals, and plants (Table 2). The worldwide distribution of strA/strB among such diverse bacteria from varied hosts suggests a common gene pool may be available to ranges of bacteria which are wider than previously thought.

Table 2. Distribution of the strA/strB streptomycin-resistance and the sulII sulfonamide-resistance determinants among bacterial residents and pathogens of humans, animals, and plants.

			Resistance genes[a]			
Genus/Species	Host[b]	Loc.[c]	strA	strB	sulII	Reference
Actinobacillus pleuropneumoniae	A	USA	X	X	X	85
	A	CAN	X	X	X	85
	A	CAN	X	X	X	237
Bordetella bronchiseptica	A	UK	X	X	X	126
Eikenella corrodens	H	---	X	§		119
Enterobacter cloacae	H	JPN	X	X	X	7
Erwinia amylovora	P	USA	X	X		41
Erwinia herbicola	P	USA	X	X		41
Escherichia coli	H	---	X	X	X	92
	A	UK	X	X	X	114
	H	MEX	X	X	X	114
	H	JPN	X	X	X	114
	H	GER	X	X	X	227
	H	JPN	X	X	X	114
	H	MEX	X	X	X	114
	H	USR	X	X	X	114
	H	UK	X	X	X	114
	A	UK	X	X	X	114

Table 2 (continued)

Genus/Species	Host[b]	Loc.[c]	Resistance genes[a] strA	strB	sulII	Reference
Escherichia coli	H	SWE	X	X	X	179
	H	UK	X	X	X	114
	A	UK	X	X	X	114
	H	---	X	X		192
	H	---	X	X	X	199a
	H	SWE	X	X	X	179
	H	FRA	X	X	X	159
Haemophilus ducreyi	H	KEN			X	2
Klebsiella pneumoniae	H	JPN	X	X	X	7
Neisseria sicca	H	SPA	X	X	X	188
Pasteurella haemolytica	A	---	X	†		35
Proteus morganii	H	SAF	X	X	X	10
Proteus mirabilis	H	GER	X	X	X	10
Providencia sp.	H	USA	X	X	X	10
Pseudomonas aeruginosa	H	CAN	X	X	X	10
Pseudomonas syringae						
pv. papulans	P	USA	X	X		170
pv. syringae	P	USA	X	X		215
	P	USA	X	X		215
	P	USA	X	X		215
	P	USA	X	X		215
	P	USA	X	X		215
Salmonella dublin	H	DEN	X	X	X	10
Salmonella senftenberg	H	DEN	X	X	X	10
Salmonella typhimurium	H	UK	X	X	X	10
	H	UK	X	X	X	207
	H	NZ	‡		X	80
	H	JPN	X	X	X	7
Shigella flexneri	H	USA	X	X	X	39
	H	JPN	X	X	X	7
Xanthomonas campestris						
pv. vesicatoria	P	ARG	X	X		163a

Table 2 (continued)

[a] strA and strB are streptomycin phosphotransferase genes; sulII encodes a type II sulfonamide resistant dihydropteroate synthase (Scholz et al 1989). X = indicated gene is present, a blank space = indicated gene is absent.

[b] Host of isolation; A = animal, H = human, P = plant.

[c] Location where bacterium was isolated; ARG = Argentina, CAN = Canada, DEN = Denmark, FRA = France, GER = Germany, JPN = Japan, KEN = Kenya, MEX = Mexico, NZ = New Zealand, SAF = South Africa, SPA = Spain, SWE = Sweden, UK = United Kingdom, USA = United States, --- indicates country of origin not reported.

[d] tra+ = self-transmissible via conjugation, plasmids not designated tra+ are nonconjugative; kb = kilobase pairs; plasmids followed by another plasmid in parentheses are synonymous.

§ 323 bp of strB downstream of strA, nucleotide identity ends after that.

† 97 bp of strB downstream of strA, nucleotide identity ends after that.

‡ substitution of type III trimethoprim-resistant dihydrofolate reductase 30 bp within strA.

Characterization of plasmids in phytopathogenic pseudomonads

The detection of important bactericide-resistance determinants on plasmids in many phytopathogenic bacteria indicates the importance of plasmids in the vertical and horizontal dissemination of these determinants within populations. Plasmids are autonomously replicating, extrachromosomal DNA elements which tend to encode traits that are beneficial to the host bacterial cell during some stages of its life cycle. Plasmid-encoded traits such as antibiotic and metal resistance, the ability to catabolize unusual substances, bacteriocin and toxin production, and plant invasion, all benefit

cells under certain conditions (76). All plasmids encode at least one oriV sequence, a sequence at which plasmid replication is initiated (117). Although plasmids observed in natural populations are usually diverse, similarities of oriV sequences between different plasmids have been utilized to categorize related plasmids and establish lineages (53). Plasmids are disseminated vertically within cell lineages during cell division. Some large, low copy number plasmids encode partition (par) determinants which ensure plasmid stability through processes which are not fully understood (8,9,235). Self-transmissible plasmids encode genes whose products are involved in horizontal transfer between cells (234). Several recent observations indicate plasmid transfer via conjugation can occur between distantly-related bacteria (154).

Plasmids have been isolated from all genera of phytopathogenic bacteria (52). In P. syringae, indigenous plasmids have been isolated from several pathovars (12,17,30,44,60-62,66,88,167,168,176,177,194,215,218,221,230). Obukowicz and Shaw labeled cryptic P. syringae plasmids with Tn3, but determined that functions associated with tabtoxin production and pathogenicity were not encoded on the plasmids they studied (172). However, some phenotypic traits including coronatine production (17), indole acetic acid production (44), avirulence (113), and copper and streptomycin resistance (12,30,215,218) have been associated with plasmids in P. syringae. Although several plasmid-encoded genes have been characterized, little is known about the relation of plasmids among pathovars, and the evolution of plasmids within populations. Curiale and Mills (61) and Quant and Mills (177) determined that individual plasmids from P. syringae pvs. glycinea and phaseolicola share extensive

homology. Piwowarski and Shaw (176) and von Bodman and Shaw (230) have shown that individual plasmids from different P. syringae pathovars (angulata, coronofaciens, and tabaci) shared a high degree of homology. von bodman and Shaw suggested the plasmids may encode regions important for coexistence with a plant host (230). Mukhopadhyay et al isolated the oriV and par sequences from pOSU900, a cryptic plasmid isolated from a P. syringae pv. syringae strain from Oregon (167). A probe constructed from these sequences hybridized to indigenous plasmids from several pathovars of P. syringae (168) indicating that individual plasmids may be transferred among P. syringae pathovars.

The observation of closely-related plasmids within and among P. syringae pathovars suggests these plasmids have coevolved with their host bacterial cells, are stable throughout the life cycle of their host, and may encode traits conferring advantages in fitness during specific periods of the P. syringae life cycle. The effect of plasmids on host fitness has been examined in Escherichia coli, mostly with laboratory constructs, but some natural plasmids have been studied (33,87,158,171,242). Regarding P. syringae, there are only two studies regarding the effect of indigenous plasmids on fitness in planta. Both of these plasmids encoded a compound which enhanced the virulence of the pathogen, the phytotoxin coronatine in P. syringae pv. tomato (16), and the phytohormone indole acetic acid in P. syringae pv. savastonoi (204). The effect of indigenous plasmids on epiphytic fitness of P. syringae is essentially unknown.

Evolution of antibiotic resistance in clinical bacteria

The evolution of antibiotic resistance in clinical bacterial pathogens occurs through: (*i*) the spread of a resistant strain; (*ii*) the spread of an antibiotic-resistance plasmid among bacterial species or genera; or (*iii*) the spread of a specific antibiotic-resistance determinant among different plasmids or chromosomes (202). The dissemination of resistance determinants among plasmids has occurred mostly through the association of these determinants with transposable elements. Transposable elements are discrete DNA sequences which are capable of mobilizing between two sequences by a process called transposition (220). Transposons typically encode genes involved in the transposition process, inverted-repeat sequences which are specifically recognized by transposase enzymes, and one to several genes encoding beneficial traits such as antibiotic or metal resistance (111).

The evolution of bactericide resistance in phytopathogenic bacteria is much less understood. In P. syringae pv. tomato, each of 11 strains isolated in California contained a 35-kb plasmid indistinguishable from the Cu^r plasmid pPT23D (45). Also, genetic analysis of chromosomal DNA of these strains revealed they were closely related, and distinguishable from other Cu^s strains (51). Cooksey interpreted these results to indicate the Cu^r P. syringae pv. tomato strains represented a single or closely-related clones which were recently disseminated (47). The 103-kb conjugative Sm^r plasmid pCPP501 was detected in all Sm^r P. syringae pv. papulans strains isolated in New York apple orchards (30). Burr et al noted the relatively rapid

evolution and dissemination of streptomycin resistance within the P. syringae pv. papulans population and suggested conjugation in planta was responsible for this process (30). Although conjugative bactericide-resistance plasmids have been isolated from several other phytopathogens, little is known about the extent of plasmid transfer within natural populations.

Objectives

The objectives of the research reported in this dissertation were: (*i*) to determine the extent of a tip dieback and canker disease problem caused by P. syringae pv. syringae on woody ornamental trees in commercial nurseries in Oklahoma; (*ii*) to characterize isolates of P. syringae pv. syringae which were resistant to copper and/or streptomycin; (*iii*) to clone and genetically analyze the plasmid encoded streptomycin-resistance determinant; (*iv*) to assess the genetic diversity within plasmid and chromosomal genomes of P. syringae pv. syringae isolates from natural populations; (*v*) to examine the molecular relatedness of individual plasmids recovered; (*vi*) to examine the expression of the strA/strB homologs from Tn5393 in P. syringae and X. campestris; (*vii*) to examine the effect of individual resistance plasmids on ecological fitness of P. syringae in vitro and in planta; and (*viii*) to determine the distribution of Tn5393 among diverse phylloplane and soil bacteria in regions exposed to streptomycin and in regions where streptomycin has not been applied as a bactericide.

CHAPTER III

ECOLOGICAL AND GENETIC ANALYSIS OF COPPER AND STREPTOMYCIN RESISTANCE IN PSEUDOMONAS SYRINGAE PV. SYRINGAE

SUMMARY

Strains of Pseudomonas syringae pv. syringae resistant to copper, streptomycin, or both compounds were recovered from symptomless and diseased tissue of four woody hosts in three nurseries in Oklahoma. In strains resistant to copper and streptomycin (Cu^r Sm^r), resistance to both compounds was co-transferred with a single plasmid which was either 68, 190, or 220 kilobase pairs (kb). All Cu^s Sm^r strains contained a 68-kb conjugative plasmid. Cu^r Sm^s strains contained one plasmid which varied in size from 60 - 73 kb. All conjugative plasmids which transferred streptomycin resistance contained sequences homologous to the strA and strB Sm^r genes from the broad-host-range plasmid RSF1010. The Sm^r determinant was subsequently cloned from a 68-kb Cu^r Sm^r plasmid designated pPSR1. A restriction map detailing the organization of the homologous Sm^r genes from pPSR1, RSF1010, and cloned Sm^r genes from P. syringae pv. papulans and Xanthomonas campestris pv. vesicatoria revealed a conservation of all sites studied. The Cu^r genes cloned from P.

syringae pv. tomato PT23 and X. campestris pv. vesicatoria XV10 did not hybridize to the Cu^r plasmids identified in the present study indicating that copper resistance in these P. syringae pv. syringae strains may be conferred by a distinct genetic determinant.

In recent years, the severity of a tip dieback and canker disease of woody plants has increased in nurseries in eastern Oklahoma. The symptoms of this disease are similar to those observed on nursery trees in the Pacific Northwest which are infected with Pseudomonas syringae van Hall (32). Although bactericides containing copper and streptomycin have been used extensively in nurseries in Oklahoma for control of this disease, a reduction in disease incidence has not been observed.

The occurrence of copper-resistant (Cu^r) strains of the phytopathogens Pseudomonas syringae (5,12,218) and Xanthomonas campestris pv. vesicatoria (1,14,50,149) has been established. These bacteria were isolated from plant hosts on which copper bactericides had been utilized for disease control. Streptomycin was first utilized as a bactericide in agriculture in the late 1950s, and streptomycin resistance has since been reported in the phytopathogens Erwinia amylovora (40,54,143,199), E. carotovora (81), P. lachrymans (245), P. syringae (30,64,104,246), and X. campestris pv. vesicatoria (163a,211).

Genetic studies of bactericide resistance in phytopathogenic bacteria have increased in recent years (47). Copper-resistance determinants have been localized to plasmid DNA in all phytopathogenic bacteria studied. In P. syringae pv. tomato, the

copper-resistance genes reside on a 35-kb plasmid in strains isolated in California (45). In X. campestris pv. vesicatoria, the Cu^r determinant resides on large plasmids in strains isolated in Florida and Oklahoma (14,210). Cu^r determinants have been cloned from P. syringae pv. tomato PT23 and X. campestris pv. vesicatoria XV10 (13,84).

Sm^r determinants have been cloned from plasmids which reside in P. syringae pv. papulans (170) and X. campestris pv. vesicatoria (163a). The 4.9-kb insert from the X. campestris pv. vesicatoria clone hybridized with plasmids indigenous to Sm^r P. syringae pv. papulans and other Sm^r X. campestris pv. vesicatoria strains (163a). Additional hybridization work using a 500-bp fragment with specificity for the Sm^r determinant in P. syringae pv. papulans indicated that Sm^r gene homologs are found in Erwinia amylovora (41), P. syringae pv. papulans (104), and X. campestris pv. vesicatoria (163a). Although this Sm^r determinant appears widespread in distribution, the Sm^r genes from P. syringae pv. papulans and X. campestris pv. vesicatoria did not hybridize with DNA from all strains of Sm^r phytopathogenic bacteria tested, indicating that another resistance determinant may be present in some strains (41,163a).

Although streptomycin resistance has been intensively studied in clinical bacteria, only four types of streptomycin-inactivating enzymes are known, those being aminoglycoside phosphotransferase {APH(6) and APH(3'')} and aminoglycoside nucleotidyltransferase {ANT(6) and ANT(3'')} (63). The conservation of these four mechanisms in both gram-negative and gram-positive bacteria suggests that gene

transfer has played an important role in the dissemination of Sm^r genes. For example, the nonconjugative IncQ plasmid RSF1010 (92), which encodes two tandem Sm^r genes strA and strB (198), has been recovered from clinical isolates of Escherichia coli, Proteus spp., Providencia sp., Pseudomonas aeruginosa, Salmonella spp., and Shigella flexneri (10). The strA gene product is reported to be an aminoglycoside phosphotransferase {(APH(3'')} (107). Two reports have indicated that Sm^r phytopathogenic bacteria also inactivate streptomycin by the APH(3'') mechanism (81,245).

Although copper or streptomycin resistance has been identified in several phytopathogenic bacteria, we are aware of only one study reporting strains with resistance to both compounds (182). The involvement of plasmids in the resistance phenotypes of these strains was not studied, nor was the relatedness of the resistance genes contained in these strains compared to previously cloned resistance determinants.

The long term use of copper and streptomycin in nurseries in Oklahoma and the lack of disease control led us to believe that strains of P. syringae pv. syringae with resistance to both compounds were present in these nurseries. In this study, we surveyed three nurseries in Oklahoma to determine if Cu^r and Sm^r strains of P. syringae pv. syringae were present and if the resistance genes were encoded on self-transmissible plasmids. The relatedness of the Cu^r and Sm^r determinants contained in strains of P. syringae pv. syringae was compared to previously characterized resistance genes from other phytopathogenic bacteria. In this study, we detected

sequences homologous to strA and strB from RSF1010 on conjugative plasmids which reside in strains of the phytopathogenic bacteria P. syringae pv. syringae, P. syringae pv. papulans, and X. campestris pv. vesicatoria. A preliminary report of this work has been published previously (214).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids and their relevant characteristics are listed in Table 3. The medium used for isolation of P. syringae from plant samples was medium B of King et al (109) (KB) amended with 100 μg/ml cycloheximide (KBc). Resistance to copper and streptomycin was determined using mannitol-glutamate medium (108) containing 0.25 g/L yeast extract (MGY) and amended with 250 μg/ml cupric sulfate (MGYcu) or 25 μg/ml streptomycin sulfate (MGYsm). Cupric sulfate and streptomycin sulfate were added to autoclaved, cooled medium from filter-sterilized stock solutions. The spontaneous mutants FF5.1 and FF5.2 were selected on MGY medium using 100 and 50 μg/ml of rifampicin (MGYrif) and chloramphenicol (MGYcm), respectively. E. coli was cultured on LB medium (162) and the antibiotics ampicillin and streptomycin were added to LB at concentrations of 40 and 25 μg/ml, respectively.

Source and identification of bacteria. Bacteria were isolated from cottonwood (Populus spp.), dogwood (Cornus florida L.), ornamental pear (Pyrus calleryana Dcne.) and willow (Salix spp.) in July 1988 and July 1990 from three nurseries in the eastern Oklahoma counties of Cherokee and Muskogee. Copper and streptomycin bactericides had been applied to trees in these nurseries for at least three consecutive

Table 3. Bacterial strains and plasmids and their relevant characteristics.

Strain	Host[a]	Relevant characteristics	Reference
Pseudomonas syringae			
pv. syringae			
A2	P	Cu^r Sm^r, pPSR1 (68-kb)	This study
AA2	P	Cu^r Sm^s, pPSR6 (70-kb)	This study
E2	C	Cu^r Sm^r, pPSR2 (190-kb)	This study
FF3	P	Cu^s Sm^r, pPSR5 (68-kb)	This study
FF5	P	Cu^s, Sm^s	This study
FF5.1		Rif^r	This study
FF5.2		Cm^r	This study
G1	W	Cu^r Sm^r, pPSR3 (220-kb)	This study
H12	W	Cu^r Sm^r, pPSR3 (220-kb)	This study
pv. papulans			
Psp36		Sm^r, pCPP501	170
Xanthomonas campestris			
pv. vesicatoria			
BV5-4a		Sm^r, 68-kb plasmid	163a

Plasmids	Relevant characteristics	Reference
RSF1010	*strA*, *strB* Sm^r determinant	198
pSM1	3.7-kb insert containing Sm^r determinant from pPSR1 cloned into pBluescript SK+	This study
pCPP505	2.5-kb insert containing Sm^r determinant from pCPP501 cloned into pBR322	170
pBV10	4.9-kb insert containing Sm^r determinant from BV5-4a cloned into pLAFR3	163a
pCOP2	4.5-kb insert containing Cu^r determinant from PT23 cloned into pRK415	13
pCuR2	6.4-kb insert containing Cu^r determinant from XV10 cloned into pRK415	84

[a] P, ornamental pear; C, cottonwood; W, willow.

years. Epiphytic bacteria were isolated by washing leaf surfaces in 0.01 M potassium phosphate buffer, pH 7.0 (K-Buffer) and plating dilutions on KBc medium. Bacteria were also recovered from cankers, dormant buds, and stem lesions using previously described methods (195). Individual plant samples were selected randomly from different trees. Isolates which were fluorescent on KBc and oxidase-negative (195) were further characterized for gelatin liquefaction, aesculin hydrolysis, tyrosinase activity, and utilization of tartrate (GATTa determinative tests; 125). Isolates which liquefied gelatin, hydrolyzed aesculin, lacked tyrosinase activity, and did not utilize tartrate were identified as P. syringae pv. syringae.

Phenotypic characterization of copper and streptomycin resistance in P. syringae pv. syringae. Resistance to copper and streptomycin was tested by inoculating strains to MGYcu and MGYsm. Prior to testing, strains were grown for 48 h on KBc. Strains which exhibited growth on MGYcu or MGYsm equivalent to that on MGY after 72 h growth at 24 C were considered resistant. Minimal inhibitory concentrations (MICs) of copper and streptomycin were determined for resistant strains by inoculating them to MGY agar amended with 375, 500 or 750 μg/ml copper sulfate or 50, 75, or 100 μg/ml streptomycin.

Characterization of plasmids. Plasmids from P. syringae pv. syringae were isolated using the procedure of Crosa and Falkow (55) with slight modifications (12). Plasmids were separated on 0.7% agarose gels, and their sizes were estimated using previously characterized plasmid size standards. The size of pPSR3 was calculated from the sum of fragments generated by five restriction enzymes. The plasmids were

initially grouped according to size and phenotype of the host strain. Representative strains from each plasmid group were chosen for conjugation experiments. To determine if plasmids were conjugative, matings were conducted using P. syringae pv. syringae strains AA2 and FF3 as donors and FF5.1 as the recipient. The matings were conducted by mixing together 25 μl of donor and recipient cells (10^8 cfu/ml), inoculating the 50 μl suspension to KB agar, and incubating the cells for 12 h at 24 C. The cells were then suspended in K-buffer, and appropriate dilutions were plated on MGY amended with 100 μg/ml rifampicin (MGYrif) to enumerate recipients, and MGYrif cu or MGYrif sm to enumerate transconjugants. Matings involving P. syringae pv. syringae strains A2, E2, and G1 with FF5.1 were conducted in the same manner and transconjugants were initially selected on MGYrif cu and MGYrif sm. Twenty five colonies from each medium were then transferred to the other medium to determine whether both resistance markers were transferred.

Molecular genetic techniques. Agarose gel electrophoresis, DNA restriction digests, and Southern transfers to nylon membranes (Amersham Corp., Arlington Hts., IL) were done using standard procedures (147). Specific DNA fragments for cloning or labeling were separated on agarose gels and isolated by electroelution (147). DNA fragments used as probes were labeled with digoxigenin (Genius kit; Boehringer Mannheim, Indianapolis, IN) as described by the manufacturer. Prehybridizations were conducted for a minimum of 1 h at 68 or 37 C for high or moderate stringency conditions, respectively. Hybridizations at high stringency were conducted at 68 C followed by two post-hybridization washes of 5 min at 24 C in 2X

SSC, 0.1% sodium dodecyl sulfate (SDS) (1X SSC is 0.15 M NaCl, 0.015 M Na-citrate; pH 7.0) and two washes of 15 min at 68 C in 0.1X SSC, 0.1% SDS. Hybridizations at moderate stringency were conducted at 37 C followed by two post-hybridization washes of 5 min at 24 C in 2X SSC, 0.1% SDS and two washes of 15 min at 37 C in 0.1X SSC, 0.1% SDS.

Cloning and characterization of the streptomycin-resistance genes from pPSR1. The 1.5-kb SacI - EcoRV fragment, which contains the Sm^r genes strA and strB from the broad-host-range plasmid RSF1010 (198), was used to identify homologous sequences on plasmids from Cu^s Sm^r and Cu^r Sm^r strains of P. syringae pv. syringae. The SacI site is located 138 bp downstream of the translational initiation site for strA, and the EcoRV site is located 3 bp downstream from the stop codon for strB. Plasmids from Sm^r strains of the phytopathogens P. syringae pv. papulans Psp36 and X. campestris pv. vesicatoria BV5-4a were also included in the analysis. pPSR1, a 68-kb Cu^r Sm^r plasmid from P. syringae pv. syringae A2, hybridized with the SacI - EcoRV fragment and was chosen for further study. BamHI, EcoRI, HindIII, and PstI, which do not cut strA or strB internally (198), were used to identify restriction fragments in pPSR1 containing the strA and strB homologs.

A 3.7-kb PstI fragment in pPSR1 hybridized to the SacI - EcoRV fragment and was chosen for cloning experiments because it was the smallest hybridizing fragment and would presumably contain less extraneous DNA. This fragment was ligated into the PstI site of pBluescript SK (Stratagene, La Jolla, CA), transformed into E. coli

DH5α, and plated onto LB amended with streptomycin. pSM1, a recombinant plasmid resulting from this experiment, contained the 3.7-kb PstI fragment from pPSR1 and conferred streptomycin resistance to E. coli DH5α.

The coding sequence of strA and strB in RSF1010 encompasses positions 63 - 863 and 866 - 1699, respectively, using the numbering convention from the published sequence (198). Restriction sites internal to strA and strB in RSF1010 and additional restriction sites outside of the coding sequence were obtained from the published sequence (198). To determine whether the restriction sites in strA and strB and adjacent sequences were conserved in the Sm^r genes from the phytopathogenic bacteria, pSM1, pBV10, and pCPP505 were digested with the following enzyme combinations: SspI - EcoRV, NruI, NruI - EcoRV, SacII - EcoRV, AvaII, SacI - AvaII, SacI - EcoRV, AvaI - EcoRV, RsaI, NcoI - EcoRV, AvaII - EcoRV, DdeI - EcoRV, DdeI - SacI, BstYI - SacI, NotI - SacI, EcoRI - EcoRV, and HincII - EcoRV. The digestion products were electrophoresed in agarose gels adjacent to RSF1010 digested with the same enzyme combinations, blotted, and probed with the 1.5 kb SacI - EcoRV fragment from RSF1010.

Hybridization experiments with DNA probes containing copper-resistance determinants from phytopathogenic bacteria. DNA-DNA hybridization experiments were conducted to evaluate the relationship of the Cu^r determinants in strains of P. syringae pv. syringae with Cu^r genes from other phytopathogens. DNA fragments used as probes were: a) the 4.5-kb PstI fragment in pCOP2 containing the Cu^r determinant from P. syringae pv. tomato PT23 (13); and b) a 4.6-kb EcoRI -

HindIII fragment in pCuR2 containing the Cu^r determinant from X. campestris pv. vesicatoria (84). Southern blot hybridizations were first conducted under the high stringency conditions described above, and following negative results, under the conditions described for low stringency.

RESULTS

Source and identification of bacteria. In 1988, 11 isolates, each from a single colony, were recovered from two stems of willow showing tip dieback symptoms. In 1990, six, four, and eight isolates were recovered from stems of cottonwood, dogwood, and willow, respectively, which also exhibited tip dieback symptoms. Ten isolates were recovered in 1990 from the surface of two leaves of cottonwood, three isolates were recovered from two symptomless petioles of ornamental pear, and an additional 66 isolates were recovered from the interior of 13 symptomless buds of ornamental pear. All of these isolates were oxidase-negative, liquefied gelatin, hydrolyzed aesculin, lacked tyrosinase activity, and did not utilize tartrate as a sole carbon source. These biochemical traits are characteristic of P. syringae pv. syringae (125).

Phenotypic characterization of copper and streptomycin resistance. Growth of bacteria on MGYcu (250 μg/ml) or MGYsm (25 μg/ml) was sufficient to identify strains with resistance to copper or streptomycin because Cu^s Sm^s strains of P. syringae pv. syringae did not grow on these media. A summary of the resistance phenotypes from the 108 P. syringae pv. syringae strains is presented in Table 4. The MIC of cupric sulfate varied among Cu^r strains from 375 to 500 μg/ml. The

MIC of streptomycin was 75 μg/ml for all Sm^r strains studied.

Table 4. Number of Pseudomonas syringae pv. syringae strains with resistance to copper (Cu) and/or streptomycin (Sm).

Host[a]	Resistance Phenotype (# strains)			
	Cu^sSm^s	Cu^rSm^s	Cu^sSm^r	Cu^rSm^r
Cottonwood	0	0	0	16
Dogwood	4	0	0	0
Ornamental pear	7	46	11	5
Willow	8	0	0	11

[a] Host plants originated from three nurseries in eastern Oklahoma.

Characterization of plasmids. A single plasmid of 68 kb (pPSR1, Fig. 1, lane 2) was detected in all Cu^r Sm^r strains of P. syringae pv. syringae isolated from ornamental pear. A single plasmid of 190 kb (pPSR2, Fig. 1, lane 4) was detected in all Cu^r Sm^r strains of P. syringae pv. syringae isolated from cottonwood. A 220-kb plasmid (pPSR3, Fig. 1, lane 5) was detected in all 11 Cu^r Sm^r strains isolated from willow. Ten of these strains also contained a smaller plasmid from 60 - 90 kb. All Cu^r Sm^s and Cu^s Sm^r strains isolated from ornamental pear contained one plasmid which varied in size from 60 - 73 kb (Fig. 1, lanes 1 and 3). No plasmids were detected in Cu^s Sm^s strains of P. syringae pv. syringae isolated from dogwood, ornamental pear, or willow.

Plasmids representing all sizes and all resistance phenotypes were tested for conjugative ability in plate matings with the Cu^s Sm^s Rif^r recipient, FF5.1. The spontaneous mutation frequency to streptomycin and copper resistance in FF5.1 was

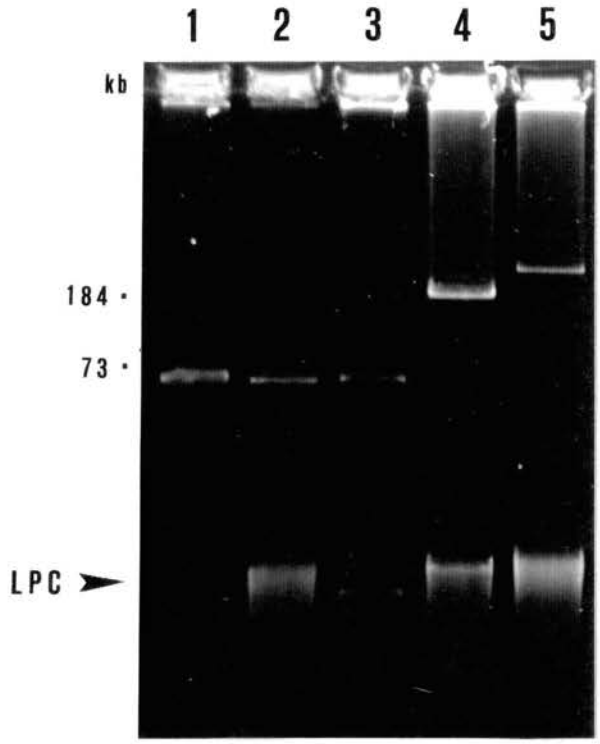


Fig. 1. Plasmids profiles of Pseudomonas syringae pv. syringae strains isolated from woody hosts. Lanes: 1, FF3 (pPSR5); 2, A2 (pPSR1); 3, AA2 (pPSR6); 4, E2 (pPSR2); 5, H12 (pPSR3). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA.

MIC of streptomycin was 75 μg/ml for all Sm^r strains studied.

Table 4. Number of Pseudomonas syringae pv. syringae strains with resistance to copper (Cu) and/or streptomycin (Sm).

Host[a]	Resistance Phenotype (# strains)			
	Cu^sSm^s	Cu^rSm^s	Cu^sSm^r	Cu^rSm^r
Cottonwood	0	0	0	16
Dogwood	4	0	0	0
Ornamental pear	7	46	11	5
Willow	8	0	0	11

[a] Host plants originated from three nurseries in eastern Oklahoma.

Characterization of plasmids. A single plasmid of 68 kb (pPSR1, Fig. 1, lane 2) was detected in all Cu^r Sm^r strains of P. syringae pv. syringae isolated from ornamental pear. A single plasmid of 190 kb (pPSR2, Fig. 1, lane 4) was detected in all Cu^r Sm^r strains of P. syringae pv. syringae isolated from cottonwood. A 220-kb plasmid (pPSR3, Fig. 1, lane 5) was detected in all 11 Cu^r Sm^r strains isolated from willow. Ten of these strains also contained a smaller plasmid from 60 - 90 kb. All Cu^r Sm^s and Cu^s Sm^r strains isolated from ornamental pear contained one plasmid which varied in size from 60 - 73 kb (Fig. 1, lanes 1 and 3). No plasmids were detected in Cu^s Sm^s strains of P. syringae pv. syringae isolated from dogwood, ornamental pear, or willow.

Plasmids representing all sizes and all resistance phenotypes were tested for conjugative ability in plate matings with the Cu^s Sm^s Rif^r recipient, FF5.1. The spontaneous mutation frequency to streptomycin and copper resistance in FF5.1 was

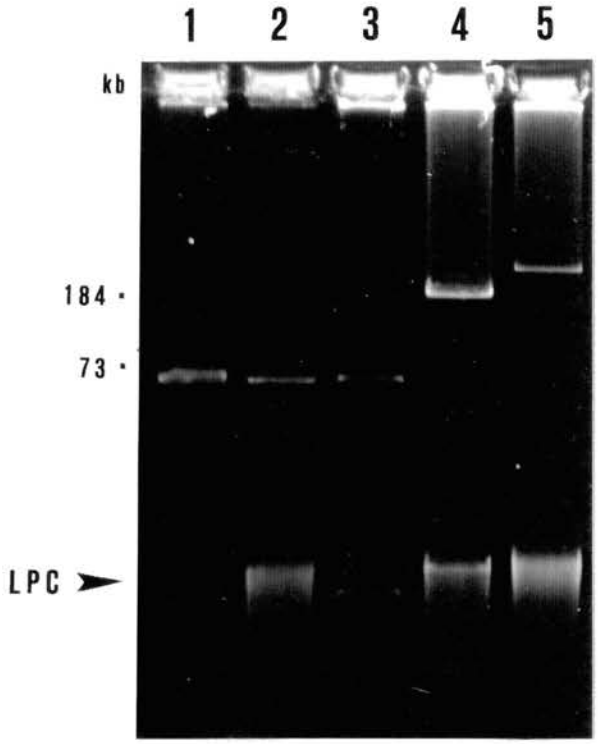


Fig. 1. Plasmids profiles of Pseudomonas syringae pv. syringae strains isolated from woody hosts. Lanes: 1, FF3 (pPSR5); 2, A2 (pPSR1); 3, AA2 (pPSR6); 4, E2 (pPSR2); 5, H12 (pPSR3). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA.

2.0×10^{-9} and less than 1.8×10^{-10}, respectively. Strains containing pPSR1, pPSR2 and pPSR3 transferred Cu^r and Sm^r to FF5.1 at frequencies of 9.3×10^{-8}, 1.4×10^{-2}, and 1.8×10^{-6} per recipient cell, respectively. Following selection on MGYrif cu or MGYrif sm, all Cu^r or Sm^r colonies grew after transfer to the other medium (MGYrif sm or MGYrif cu, respectively). Putative transconjugants of FF5.1 contained the appropriate plasmid when examined by agarose gel electrophoresis (data not shown). Since pPSR1 was transferred to FF5.1 at a low frequency, a Cu^r Sm^r Rif^r transconjugant of FF5.1 containing pPSR1 was mated with FF5.2 (Cm^r Rif^s Cu^s Sm^s). Transfer of pPSR1 to FF5.2 and acquisition of Cu^r Sm^r was detected at a frequency of 9.6×10^{-6} which is higher than the spontaneous mutation frequency to Rif^r in FF5.2 and Cm^r in FF5.1. pPSR5, a 68-kb plasmid from the Cu^s Sm^r strain FF3, transferred Sm^r to FF5.1 at a frequency of 5.8×10^{-6} per recipient cell. Transfer of Cu^r was not detected when the Cu^r Sm^s strain AA2 and five additional Cu^r Sm^s strains were used as donors.

Cloning and characterization of the streptomycin-resistance genes from P. syringae pv. syringae. The SacI - EcoRV fragment from RSF1010 hybridized to self-transmissible Cu^r Sm^r plasmids of 68, 190, and 220 kb in four P. syringae pv. syringae strains (Fig. 2, lanes 2 - 5), and to a 68-kb self-transmissible Sm^r plasmid in one strain (Fig. 2, lane 1). This fragment also hybridized to the smaller plasmid present in eight of ten Cu^r Sm^r strains isolated from willow. Although these strains presumably contained copies of the Sm^r determinant on plasmids which varied in size, only pPSR3 (220 kb) was self-transmissible. Additionally, the MIC of streptomycin

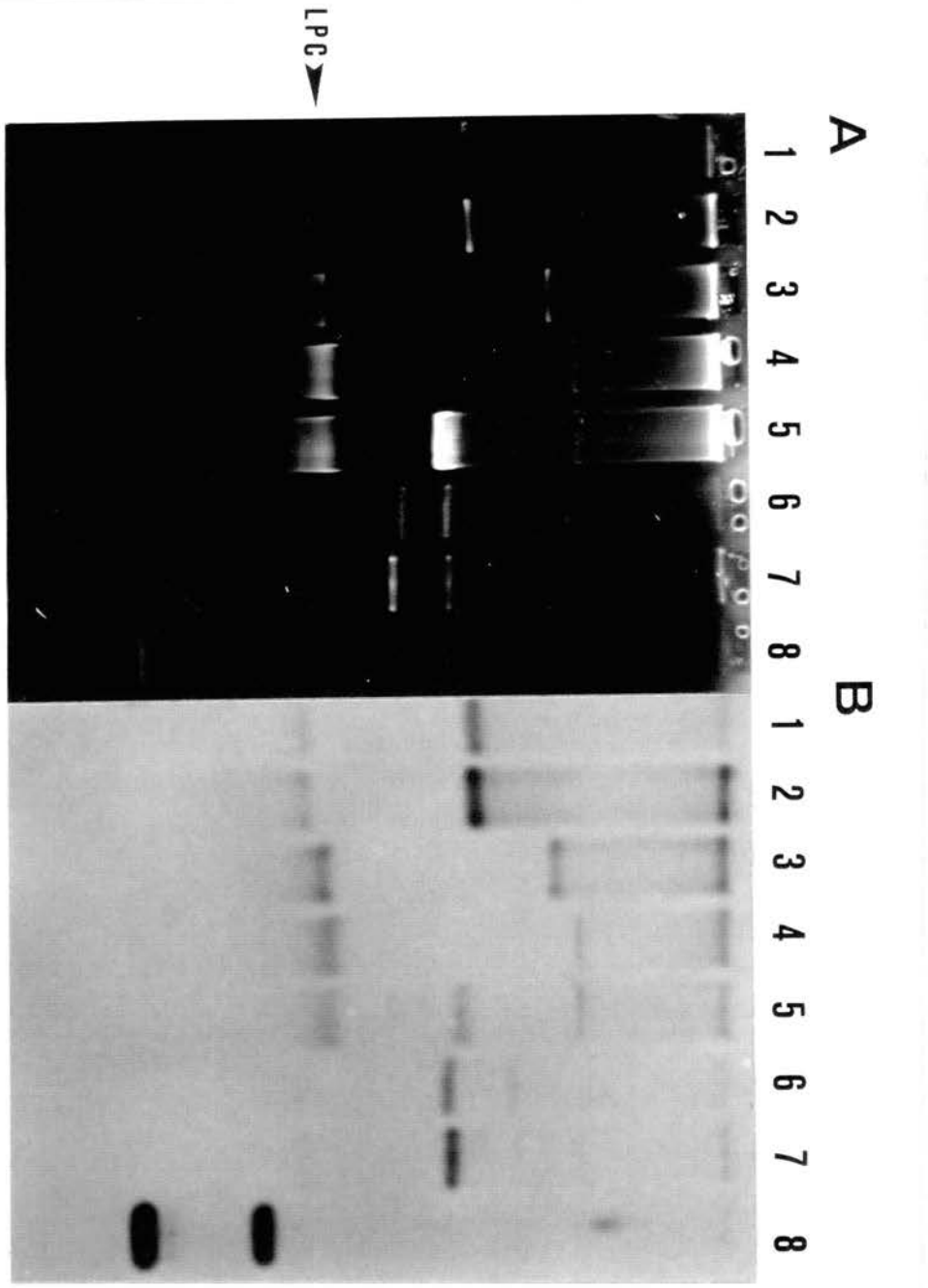


Fig. 2. Agarose gel electrophoresis (A) and hybridization (B) of plasmid DNA from Sm^r phytopathogenic bacteria with the 1.5-kb SacI - EcoRV fragment containing strA and strB from plasmid RSF1010. Lanes 1 - 5 contain the following strains of P. syringae pv. syringae: 1, FF3 (pPSR5); 2, A2 (pPSR1); 3, E2 (pPSR2); 4, H12 (pPSR3); 5, G1 (pPSR3); 6, P. syringae pv. papulans Psp36; 7, Xanthomonas campestris pv. vesicatoria BV5-4a; 8, E. coli (RSF1010). LPC is linearized plasmid and chromosomal DNA.

was identical for P. syringae pv. syringae strains isolated from willow regardless of whether they contained pPSR3 alone or pPSR3 plus a smaller plasmid. The probe did not hybridize to plasmid DNA contained in Cu^r Sm^s strains of P. syringae pv. syringae (data not shown). However, it did hybridize to plasmids present in the Sm^r strains P. syringae pv. papulans Psp36 (Fig. 2, lane 6) and X. campestris pv. vesicatoria BV5-4a (Fig. 2, lane 7).

The SacI - EcoRV fragment from RSF1010 hybridized to restriction fragments from pPSR1 which were 16.0 kb (BamHI), 10.0 kb (HindIII), 9.6 kb (EcoRI), and 3.7 kb (PstI). The 3.7 kb PstI fragment was selected for cloning and was ligated into pBluescript SK creating the plasmid pSM1. Transformants of E. coli DH5α containing pSM1 expressed streptomycin resistance. Restriction mapping and hybridization analyses indicated that the relative positions of selected restriction sites internal to strA and strB in RSF1010 were conserved in the P. syringae pv. syringae clone pSM1 (Fig. 3). The EcoRV site (1702) in RSF1010 was also present in pSM1. The restriction sites shown in Fig. 3 for pSM1 were also present in the same relative positions in pBV10 and pCPP505 (data not shown). The EcoRI and HincII sites which are located 71 and 65 bp upstream from the translational start site in strA (Fig. 3; RSF1010 map) were not present in pSM1, pBV10, or pCPP505. The restriction mapping data, combined with the hybridization data at high stringency, demonstrated that the Sm^r determinant in P. syringae pv. syringae, P. syringae pv. papulans, and X. campestris pv. vesicatoria was similar if not identical to strA and strB from RSF1010.

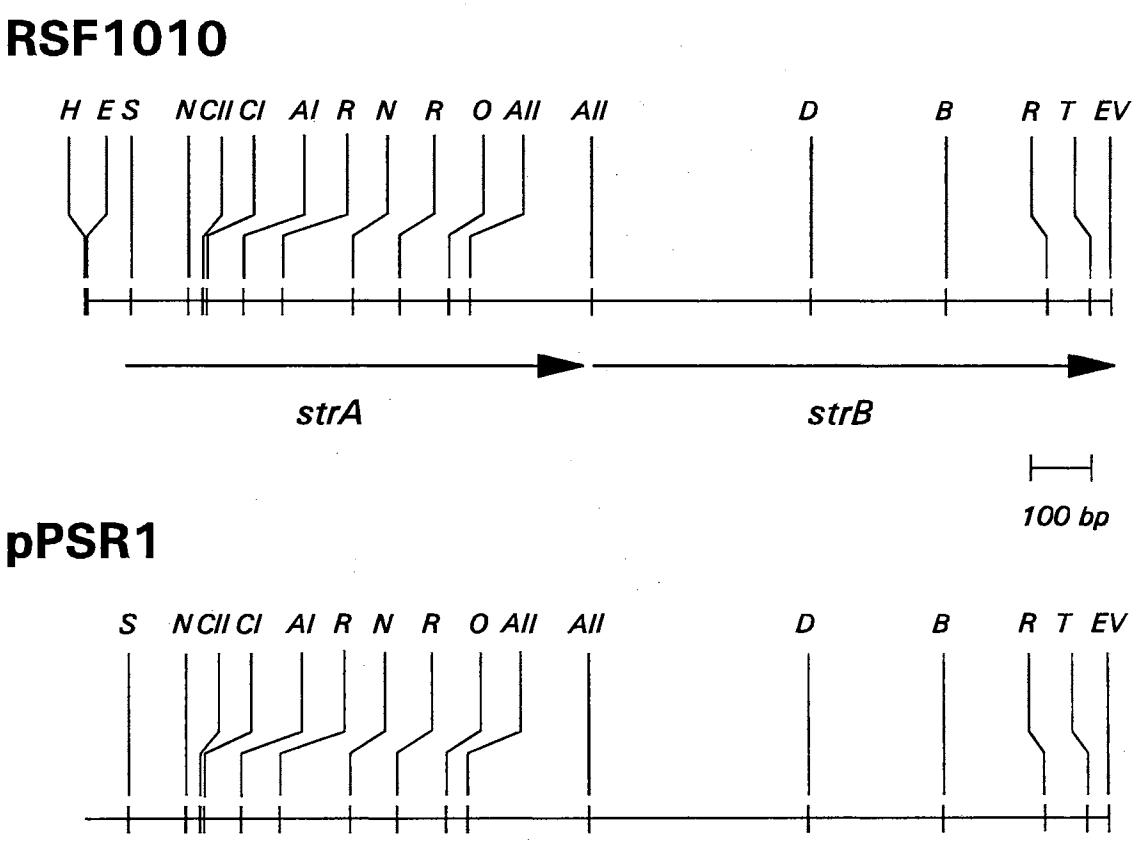


Fig. 3. Conservation of restriction sites over a 1.7 kb region encoding strA and strB in RSF1010 and streptomycin resistance in pSM1. E, EcoRI (8676)*; H, HincII (8682); S, SspI (75); N, NruI (169,441); CII, SacII (194); CI, SacI (201); AI, AvaI (258); R, RsaI (324, 520, 1594); O, NcoI (605); AII, AvaII (638, 845); D, DdeI (1209); B, BstYI (1429); T, NotI (1664); EV, EcoRV (1702). * Position numbers are from the published RSF1010 sequence (198). Arrows indicate the direction of transcription.

Hybridization experiments with copper-resistance gene probes. Restriction fragments containing the Cu^r determinants from P. syringae pv. tomato PT23 and X. campestris pv. vesicatoria XV10 were used as probes in Southern blot hybridization experiments with plasmids from 18 Cu^r Sm^r, Cu^r Sm^s, or Cu^s Sm^r strains of P. syringae pv. syringae. The restriction fragments containing the Cu^r determinants did not hybridize to any of the P. syringae pv. syringae plasmids under conditions of high or moderate stringency (data not shown).

DISCUSSION

To our knowledge, this is the first report demonstrating the linkage of Cu^r and Sm^r determinants on conjugative plasmids in a phytopathogenic bacterium. Presumably, in response to the selection pressure of copper and streptomycin bactericidal sprays, the P. syringae pv. syringae population has developed resistance to one or both of these compounds. Surprisingly, the majority of strains isolated from ornamental pear were resistant to copper or streptomycin alone. This could indicate a more recent evolution of the plasmids which encode both Cu^r and Sm^r determinants.

We identified plasmids of three distinct sizes which transferred copper and streptomycin resistance to Cu^s Sm^s recipients in laboratory matings. In our survey, a 68-kb plasmid designated pPSR1 was detected in each of five Cu^r Sm^r strains isolated from ornamental pear; a 190-kb plasmid, pPSR2, was detected in each of 16 Cu^r Sm^r strains isolated from cottonwood; and a 220-kb plasmid, pPSR3, was detected in 11 Cu^r Sm^r strains isolated from willow. Plasmids detected in Cu^r Sm^s or Cu^s Sm^r

strains from ornamental pear ranged in molecular weight from 60 - 73 kb. Resistance plasmids of different sizes have been isolated previously from local populations of P. syringae (104). The diversity in plasmid size may indicate the ability of resistance determinants to transfer among indigenous plasmids in a bacterial population.

In the present study, the Sm^r determinants from P. syringae pv. papulans, P. syringae pv. syringae, and X. campestris pv. vesicatoria were shown to be homologous to the strA/strB genes of the broad-host-range enterobacterial plasmid RSF1010 (198). Recently, Chiou and Jones (41) sequenced the Sm^r determinant in E. amylovora and P. syringae pv. papulans and demonstrated that they are identical to the strA/strB genes in RSF1010. Sequence analysis has also indicated that a portion of the strA homolog in pSM1 is identical to strA in RSF1010 (217). Because RSF1010 encodes an APH(3''), it is possible that Sm^r strains of E. carotovora and P. lachrymans from Japan, which produce an APH(3'') (81,245), also contain strA and strB homologs. Among Sm^r bacteria isolated from humans and animals, sequences homologous to strA and strB from RSF1010 have been identified on related IncQ plasmids in Bordetella bronchiseptica (126), Branhamella spp. (175), E. coli (192) and Neisseria spp. (188). strA and strB homologs also occur on nonconjugative replicons of unknown incompatibility in Actinobacillus pleuropneumoniae (85,237), E. coli (199a,229), and S. flexneri (39) and on a conjugative plasmid from E. coli (178). Clearly, this Sm^r determinant has been disseminated to plasmids inhabiting widely varied bacteria worldwide. Recently, Lacroix and Walker (119) identified a homolog of strA associated with sequences of the transposon Tn3 in chromosomal DNA of

Eikenella corrodens. Sequence analysis of the region flanking the strA/strB genes in both E. amylovora and P. syringae pv. papulans has also suggested that the Sm^r genes in these bacteria are contained on a transposable element (41). Although we have not demonstrated transposition of the Sm^r genes encoded by P. syringae pv. syringae, the acquisition of this determinant by transposition could explain the presence of these genes on plasmids of variable size.

The discovery of strA and strB homologs in bacteria pathogenic to humans and in phytopathogenic bacteria is a powerful example of the ability of bacterial populations to respond to selection pressure. Dissemination of this Sm^r determinant may be impacted by selection pressure, plasmid transfer, transfer of the determinant among plasmids, and gene expression. In environments where streptomycin is used as a bactericide, Sm^r is selected in populations of phytopathogenic bacteria and in nontarget bacteria inhabiting plant surfaces and soil. For example, a DNA probe specific for the Sm^r determinant from P. syringae pv. papulans was used to study the distribution of Sm^r homologs in target and nontarget bacteria. Homologs were detected in miscellaneous Gram-negative, Sm^r bacteria isolated from leaves and soil of apple orchards in New York (170), and from apple leaves in Michigan (209). Streptomycin had been previously applied in some but not all of the orchards sampled. Exposure of plant surfaces and soil to streptomycin may selectively enrich organisms carrying this determinant, eventually resulting in its transfer to phytopathogenic bacteria.

Natural populations of P. syringae pv. syringae in Oklahoma appear to be in a

state of flux regarding copper and streptomycin resistance. Plasmids encoding Cu^r or Sm^r phenotypes are still detected in addition to Cu^s Sm^s strains which do not contain plasmids. However, the continued practice of repeated applications of copper and streptomycin in Oklahoma nurseries, may eventually result in a population which is uniformly resistant to both bactericidal compounds.

The choice of bactericides is currently limited in agriculture. Accompanying this problem for agricultural producers is the prospect of resistance to bactericides in pathogen populations following intensive use of currently available bactericidal agents. The use of medically-important antibiotics in agriculture was discouraged by the Bacteriology committee of the American Phytopathological Society in 1978, because the selection of resistance genes in plant-inhabiting bacteria could possibly impact human health (4). Our results indicate that antibiotic resistance genes which have been selected in bacterial pathogens of humans have also been selected in distinct populations of phytopathogenic bacteria. Although alternative bactericides are needed in agriculture, the use of other registered antibiotics such as tetracycline may select for additional gene transfer events and result in the further development of antibiotic-resistance determinants in phytopathogenic bacteria.

CHAPTER IV

THE EVOLUTION OF COPPER AND STREPTOMYCIN RESISTANCE IN PSEUDOMONAS SYRINGAE

SUMMARY

The evolution of resistance to copper and streptomycin was studied in populations of Pseudomonas syringae isolated from three ornamental pear nurseries in eastern Oklahoma. The bactericide spray regime differed at each nursery with copper and streptomycin, copper only, and no bactericides applied at nurseries I, II, and III, respectively. Resistance to copper (Cu^r) and streptomycin (Sm^r) was determined for 1,938 isolates of P. syringae; isolates from nursery I and II were generally Cu^r Sm^s; whereas most isolates from nursery III were Cu^s Sm^s. The plasmid profile of 366 isolates was determined, and six, one, seven, and four plasmid profiles were identified from Cu^r, Sm^r, Cu^r Sm^r, and Cu^s Sm^s isolates, respectively. All Sm^r plasmids contained sequences homologous to the strA and strB Sm^r genes from the broad-host-range plasmid RSF1010 that were associated with the Sm^r transposon Tn5393. Plasmids were placed into two groups based on hybridization to the oriV and par sequences from pOSU900, a cryptic plasmid in P. syringae pv. syringae. Restriction fragment length polymorphism analysis indicated relatedness among many

plasmids in group 1; however, plasmids in group 2 plasmids were more diverse. One hundred randomly chosen P. syringae isolates from nursery I and III were analyzed for phenotypic diversity and genetic diversity using arbitrarily-primed polymerase chain reaction (AP-PCR). Analysis of chromosomal genotypes by AP-PCR revealed a high degree of genetic diversity among the isolates and indicated that the isolates could be clustered into two distinct groups. Plasmid profiles were specific to isolates from a particular AP-PCR group. Within each AP-PCR group, identical plasmid profiles were present in isolates with different chromosomal genotypes implying that plasmid transfer has played an important role in the dissemination of Cu^r and Sm^r within these populations.

The effective management of many important plant diseases caused by phytopathogenic bacteria has been compromised during the past three decades due to the evolution of resistance to the commonly used bactericides copper and streptomycin. Plasmid-encoded resistance to copper and streptomycin has become increasingly widespread in Erwinia amylovora, Pseudomonas syringae, and Xanthomonas campestris (47).

Several copper resistance (Cu^r) determinants from P. syringae and X. campestris have been cloned and characterized (13,84,129,227). The plasmid-encoded Cu^r determinant in P. syringae pv. tomato (cop operon) has been most extensively studied (49) and has been detected in other Cu^r bacteria (47,129,136,203). However, Cu^r strains have been isolated which do not show homology to the cop operon (215) or

differ from the cop operon in the mechanism of resistance (5).

Streptomycin-resistance (Sm^r) determinants have been cloned from a number of phytopathogenic bacteria, and recent results indicated that the Sm^r determinants from P. syringae pv. papulans, P. syringae pv. syringae, and X. campestris pv. vesicatoria were homologous (215). These bacteria were isolated from diverse geographic origins, indicating that the determinant had been widely disseminated. Further characterization of the Sm^r determinant demonstrated homology to the strA/strB genes of the broad-host-range plasmid RSF1010 (215), and showed that it was encoded by Tn5393, a member of the Tn3 family (41). Tn5393 was originally characterized from E. amylovora strains isolated from orchards in Michigan and was also detected in bacteria inhabiting the phylloplane and soil of apple orchards in regions where streptomycin was applied as a bactericide (170,209).

In clinical bacteria, the evolution of antibiotic resistant populations occurs through the spread of a resistant strain, the spread of a plasmid among bacterial species or genera, or the spread of a specific resistance determinant among different plasmids and chromosomes (202). The evolution of copper and streptomycin resistance in phytopathogenic bacteria probably occurs through these same mechanisms, although their contribution to the evolution of resistance in natural populations is mostly unknown. Conjugal transfer of indigenous plasmids encoding copper and/or streptomycin resistance has been demonstrated between in vitro for E. amylovora, P. syringae, and X. campestris (12,30,40,210,215,218). However, the dissemination of resistance plasmids within natural populations of phytopathogenic bacteria is not well

understood. In P. syringae pv. tomato, a single Cu^r plasmid, pPT23D, was detected in all Cu^r strains examined, and the genomic digests of these strains could be distinguished from Cu^s strains (45,51). Since the genetic diversity within P. syringae pv. tomato is relatively low (67), the lack of many competing genotypes, coupled with selection pressure from copper sprays, may have enhanced the selection of a single Cu^r P. syringae pv. tomato genotype in California. However, the genetic diversity within P. syringae pv. syringae is higher relative to P. syringae pv. tomato (67,131). The outcome following exposure of a heterogenous population of P. syringae pv. syringae to selection from copper and streptomycin bactericides is unknown. In previous studies, resistance plasmids varying in size were recovered from P. syringae pv. syringae inhabiting different tree hosts, implying the presence of different plasmid genotypes within these populations (215,218). However, information about the chromosomal genotypes within indigenous populations of P. syringae pv. syringae is also necessary in order to understand the evolution of copper and streptomycin resistance in this bacterium.

Arbitrarily-primed polymerase chain reaction (AP-PCR) is a sensitive method which can be utilized to fingerprint genomes (231,236). Welsh et al used AP-PCR analysis with three oligonucleotides to differentiate a collection of Eurasian and North American isolates of Borrelia burgdorferi into three groups (232). The AP-PCR data were correlated with previous analyses where multilocus enzyme electrophoresis, DNA-DNA hybridization, and restriction fragment length polymorphisms were used to differentiate strains (232). AP-PCR analysis has also been utilized to distinguish

strains of P. syringae pv. glycinea which produce the phytotoxin coronatine (225). The use of primer sets targeted at specific sites within the genome, such as the repetitive extrachromosomal palindromic (REP) regions of gram-negative bacteria (228), increase the utility of this technique in differentiating closely-related bacterial strains. The relative positions of REP sequences in the genome of gram-negative bacteria appear to be conserved in closely related strains and distinct in unrelated species and genera (144). The REP primers have been used to fingerprint genomes of a number of gram-negative soil genera (65,228), and data generated from one study was correlated with previous multilocus enzyme electrophoresis data (65). Also, Judd et al have recently used the REP primers and another primer set to differentiate closely-related Bradyrhizobium japonicum serocluster 123 strains which could not be effectively differentiated with other methods (105).

In Oklahoma, copper and streptomycin bactericides have been intensively utilized in the nursery industry for at least 10 and 5 years, respectively. We have previously isolated Cu^r, Sm^r, and Cu^r Sm^r strains of P. syringae pv. syringae from three woody plant hosts from nurseries in Oklahoma (215). Our long-term goal is to understand the evolution and persistence of plasmid-encoded genetic traits in populations of P. syringae. Our objective in the present study was to examine the evolution of copper and streptomycin resistance in epiphytic P. syringae which colonized Pyrus calleryana trees exposed to different Cu and Sm spray regimes. To achieve this, populations of epiphytic P. syringae were surveyed at three nurseries, and the frequencies of resistance phenotypes were determined. We examined plasmid diversity within a

subset of isolates from each population and utilized AP-PCR to investigate the genetic diversity within populations from two nurseries. The distribution of individual plasmids among different chromosomal genotypes of P. syringae was also determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, including a representative from each plasmid profile group, and plasmids utilized in the present study are listed in Table 5. The characteristics of 100 P. syringae pv. syringae strains which were utilized in the AP-PCR analysis are listed in Table 9. The medium used for isolation of P. syringae from plant samples was medium B of King et al (109) (KB) amended with 100 μg/ml cycloheximide (KBc) to inhibit fungal growth. Resistance to copper and streptomycin was determined with mannitol-glutamate medium (108) amended with 250 μg of cupric sulfate per ml (MGcu) or 25 μg of streptomycin sulfate per ml (MGsm). E. coli was cultured on LB medium (162). The antibiotics ampicillin, chloramphenicol, streptomycin, and tetracycline were added to LB and MG medium at concentrations of 40, 50, 25, and 12.5 μg/ml, respectively.

Source of P. syringae isolates. Sampling took place at three ornamental pear (Pyrus calleryana cv. Aristocrat) nurseries located in the eastern Oklahoma counties of Cherokee and Muskogee. The distance between nurseries was 45 km (I and II), 47 km (II and III), and 2 km (I and III). The trees were subject to the following bactericide spray regimes during each growing season (March to August): nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/l) and streptomycin

Table 5. Bacterial strains and plasmids and their relevant characteristics.

Strain	Chromosomal phenotype	Plasmid profile	Relevant characteristics[a]	Reference
Pseudomonas syringae				
pv. syringae				
7B44		1	Cu^r Sm^s; pPSR7	This study
7G14		1A	Cu^r Sm^s; pPSR7, pPSC4	This study
2H12		2	Cu^r Sm^s; pPSR8	This study
3C1		3	Cu^r Sm^s; pPSR9	This study
8B48		4	Cu^r Sm^s; pPSR10	This study
7A36		5	Cu^r Sm^s; pPSR11, pPSC4	This study
7C12		6	Cu^r Sm^r; pPSR7, pPSR15	This study
7B12		7	Cu^r Sm^r; pPSR1	This study
8C32		8	Cu^r Sm^r; pPSR14	This study
9A26		8A	Cu^r Sm^r; pPSR14, pPSC6	This study
2E49		9	Cu^r Sm^r; pPSR2	This study
7B22		10	Cu^r Sm^r; pPSR12	This study
7F14		11	Cu^r Sm^r; pPSR3	This study
9A22		12	Cu^s Sm^r; pPSR13	This study
7G43		C1	Cu^s Sm^s; pPSC1	This study
7E42		C2	Cu^s Sm^s; pPSC2	This study
8B24		C3	Cu^s Sm^s; pPSC3	This study
7C27		C4	Cu^s Sm^s; pPSC4, pPSC5	This study
9C11.2	Cm^r			This study
B48				66
B61				66
B64				66
B76				66
B78				66
PSC1B				66
B86-6			Sm^r plasmid	170
pv. coronafaciens				
PC27			pJP27.00	230
pv. morsprunorum				
3714			INA-	J.M. Young
pv. tabaci				
PT81			pJP50.00	230

Table 5 (continued)

Plasmids	Relevant characteristics	Reference
RSF1010	strA, strB Sm^r determinant	198
pPSR1	Cu^r Sm^r; contains Tn5393	215
pOSU22	oriV, par loci from pOSU900	167

[a] Cu = copper, Sm = streptomycin, Cm = chloramphenicol, r = resistant, s = sensitive, INA- = ice nucleation negative.

sulfate (0.2 g/l); nursery II, 20 applications of cupric hydroxide (2.4 g/l); and nursery III, no applications of copper or streptomycin bactericides. Following an initial application while the trees were dormant, the bactericides were applied once during the bloom period and then weekly throughout the growing season. Epiphytic bacteria were isolated from symptomless leaf surfaces on 13 Apr, 11 May, 3 Jun, 2 Jul and 30 Jul 1991 and 27 Apr, 26 May, and 24 Jun 1992. Single leaves at approximately the same canopy position were selected randomly from each of 25 trees, placed individually into plastic bags, and transported to the laboratory on ice. Leaves were washed individually in 20 ml of 0.01 M potassium phosphate buffer (pH 7.0) supplemented with 0.1% peptone (PK buffer) for 1 h at 250 rpm on a rotary shaker. Fifty-microliter aliquots from appropriate serial dilutions were plated on KBc medium and incubated for 48 h at 28 C. Oxidase negative colonies (195) which were fluorescent on KBc and which exhibited the appropriate colony morphology were considered to be P. syringae (215). Up to 10 isolated P. syringae colonies per leaf were randomly chosen and examined for bactericide resistance by replica plating to

MGcu and MGsm as previously described (215).

Characterization of plasmids. Plasmids were isolated from one or two randomly-selected P. syringae isolates per colonized leaf. The plasmid isolation method utilized was that of Crosa and Falkow (55) with slight modifications (12). Phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) extractions were done before restriction enzyme digests. Plasmids were separated on 0.7% agarose gels, and their sizes estimated by comparison to known plasmid standards and by the additive sizes of restriction fragments generated by three different enzymes. Plasmids from different isolates were placed into profile groups on the basis of size and Cu^r and Sm^r phenotype. The conjugative ability of resistance plasmids belonging to each profile group was assessed by conducting matings on KB medium using a method described previously (215). A chloramphenicol-resistant (Cm^r) spontaneous mutant of the plasmid-free Cu^s Sm^s strain 9C11 was designated 9C11.2 and used as the recipient.

Molecular genetic techniques. Restriction enzyme digests, agarose gel electrophoresis, Southern transfers, and isolation of DNA fragments from agarose gels using electroelution were done using standard procedures (147). When plasmid profile groups included two plasmids, the resistance plasmids of interest were conjugated into the plasmid-free strain P. syringae 9C11.2. Hybridization analyses were conducted to determine the location of specific genetic determinants on plasmids from each profile group, and whether restriction fragment length polymorphisms (RFLPs) were associated with the location of the determinants. DNA fragments used

as probes were (*i*) the 1.5-kb SstI - EcoRV fragment containing the Sm^r genes strA and strB from the broad-host-range plasmid RSF1010 (198); (*ii*) the 3.2-kb SstI fragment which contains portions of the tnpA and tnpR genes and the res site from the Sm^r transposon Tn5393 (41); and (*iii*) the 1.2-kb HindIII and 1.4-kb EcoRI fragments from pOSU22 containing the oriV and par loci from pOSU900 (167). DNA fragments were labeled with digoxigenin-11-dUTP following the instructions of the manufacturer (Genius Labeling and Detection Kit; Boehringer Mannheim; Indianapolis, Ind.). Hybridizations were conducted at 68 C followed by high stringency washes as previously described (215).

Phenotypic characterization of the P. syringae isolates. Phenotypic characterization of the 100 P. syringae isolates used in AP-PCR analyses was further investigated by assessing for the presence of oxidase (195), hydrolysis of aesculin, liquefaction of gelatin, utilization of sodium tartrate, and tyrosinase activity (103). The ability of the strains to elicit a hypersensitive response in a nonhost plant, tobacco (Nicotiana tabacum cv. Xanthi), was evaluated by infiltrating cell suspensions (approximately 10^7 cells/ml in PK), into the intercellular spaces of the leaves. Plants were incubated for 24 h on a greenhouse bench and then rated for the presence of collapsed tissue characteristic of the hypersensitive reaction (195). Ice nucleation activity was assessed by incubating a 1 ml suspension (approximately 10^7 cfu/ml in PK) at -5 C in a controlled temperature bath. Suspensions which froze within 15 min were scored as ice nucleation active (INA+); PK buffer alone or a suspension of the INA- strain P. syringae pv. morsprunorum, were used as negative controls.

Genetic analysis of a subset of P. syringae isolates using AP-PCR. One hundred P. syringae isolates were randomly selected from the pool of isolates recovered from nurseries I and III in 1992. Genomic fingerprinting of these bacteria was done using AP-PCR (231,236) with the following oligonucleotide primer sets: (1) the 18 bp REP primers (228) 5'-ICGICTTATCIGGCCTAC-3' and 5'-IIIICGICGICATCIGGC-3' (Genosys Biotechnologies, Inc.; The Woodlands, TX), which were used in combination; (2) and a 20 bp oligonucleotide 5'-GGTTCCGTTCAGGACGCTAC-3'(Oklahoma State University Recombinant DNA/Protein Resource Facility), complementary to the IS50 portion of Tn5 (181), which was used singly. PCR reactions were conducted in a 25-μl volume containing the REP primers (50 pmol each) or the IS50 primer (100 pmol), 4 μg bovine serum albumin (Boehringer Mannheim), 1.25 mM deoxynucleoside triphosphates (Boehringer Mannheim), 10% dimethylsulfoxide (Fluka Chemical Corp.; Ronkonkoma, NY), and 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus; Norwalk, Conn.) in a salt buffer (115). To this reaction mixture, approximately 10^6 cells (previously grown on KB medium for 12 h) were introduced with a sterile toothpick, and the mixture was covered with a drop of mineral oil. Amplifications were also performed on purified DNA of plasmids from each plasmid profile group. The plasmid DNA was purified either on CsCl-ethidium bromide gradients or by electroelution from 0.7% agarose gels. The amplifications were performed with a DNA thermal cycler (Perkin Elmer Cetus). The cycles used were as follows: 1 cycle at 95 C for 6 min; 5 cycles at 94 C for 1 min, 40 C for 1 min, 65 C for 8 min; 25

cycles at 94 C for 1 min, 50 C for 1 min, 65 C for 8 min; 1 cycle at 65 C for 16 min; and a final soak at 4 C. The fidelity of the technique was checked by performing all the amplifications at least twice, and performing amplifications on five replicate colonies of six strains. After the reaction, 8 μl of the PCR products were electrophoresed for 1.5 h at 60 volts on 1.5% agarose gels, and the gels were stained with ethidium bromide and photographed using Polariod type 55 film.

Data analysis. After the number of PCR products generated from the two primer sets was determined, the data were converted to a two dimensional binary matrix where 1 = the presence of a PCR product and 0 = the absence of a PCR product. Analysis was done using the biostastical analysis program NTSYS-pc (Applied Biostatistics, Inc.; Setauket, NY). A similarity matrix was computed using Dice's coefficient, $2n_{xy} / (n_x + n_y)$, where n_{xy} equals the number of common bands between isolates x and y, and n_x and n_y equals the total number of bands in isolates x and y, respectively. Cluster analysis was done using the unweighted pairgroup method arithmetic average (UPGMA) (208).

RESULTS

Colonization of P. calleryana trees by P. syringae. Epiphytic P. syringae was detected at all sampling times in all locations (Table 6). Populations were generally higher during samples taken in early spring which is consistent with observations reported for epiphytic colonization of tree hosts by P. syringae (98). Populations generally decreased in the summer months when the ambient temperatures were high and rainfall was low. Fig. 4 shows the total percentage of leaves colonized by each

Table 6. Colonization of Pyrus calleryana cv. Aristocrat leaves by Pseudomonas syringae at three nurseries in Oklahoma and recovery of P. syringae isolates with resistance or sensitivity to copper and streptomycin.

Nursery[b]	Sampling Time[c]	Colonized Leaves[d]	Mean Population[e]	No. P. syringae isolates[a] Cu^s Sm^s	Cu^r Sm^s	Cu^s Sm^r	Cu^r Sm^r
I	1	25	5.73 (0.79)	63(25)	99(40)	0	88(35)
	2	21	4.21 (0.81)	17(10)	110(67)	0	38(23)
	3	22	4.30 (0.59)	42(23)	71(39)	0	71(39)
	4	18	4.81 (0.74)	2(4)	27(54)	0	21(42)
	5	10	4.31 (0.53)	0	6(16)	0	32(84)
	6	19	4.47 (1.03)	11(7)	103(69)	0	36(24)
	7	18	4.11 (0.74)	7(5)	71(54)	0	53(40)
	8	12	4.19 (0.47)	0	17(33)	3(6)	31(61)
TOTAL		145	4.59 (0.93)	142(14)	504(49)	3(0.3)	370(36)

Nursery[b]	Sampling Time[c]	Colonized Leaves[d]	Mean Population[e]	No. P. syringae isolates[a] Cu^s Sm^s	Cu^r Sm^s	Cu^s Sm^r	Cu^r Sm^r
II	1	1	5.66 (0.00)	0	0	0	10(100)
	2	21	5.23 (0.84)	6(3)	156(84)	0	24(13)
	3	14	3.83 (0.97)	0	65(86)	0	11(14)
	4	9	3.28 (0.66)	2(6)	25(74)	0	7(21)
	5	2	2.43 (0.12)	3(100)	0	0	0
	6	13	4.05 (1.07)	11(12)	81(86)	0	2(2)
	7	16	3.95 (0.85)	13(10)	114(90)	0	0
	8	8	3.88 (0.61)	0	52(100)	0	0
TOTAL		84	4.17 (1.10)	35(6)	493(85)	0	54(9)

Nursery[b]	Sampling Time[c]	Colonized Leaves[d]	Mean Population[e]	No. P. syringae isolates[a] Cu^s Sm^s	Cu^r Sm^s	Cu^s Sm^r	Cu^r Sm^r
III	6	16	4.51 (0.91)	54(41)	0	0	78(59)
	7	15	4.79 (0.51)	96(66)	1(1)	0	48(33)
	8	13	3.76 (0.47)	40(67)	0	0	20(33)
TOTAL		44	4.38 (0.79)	190(56)	1(0.3)	0	146(43)

[a] Number of P. syringae isolates recovered at each sampling. Cu = copper; Sm = streptomycin; r = resistant; s = sensitive. Percentage of total isolates recovered at the sampling time is indicated in parentheses.

[b] Bactericide spray regimes were as follows: nursery I, 15 application of a mixture containing cupric hydroxide (2.4 g/l) and streptomycin sulfate (0.2 g/l); nursery II, 20 applications of cupric hydroxide (2.4 g/l); and nursery III, no applications of copper or streptomycin bactericides.

Table 6 (continued)

[c] Sampling times were on the following dates: (1), 13 Apr; (2), 11 May; (3), 3 Jun; (4), 2 Jul.; (5), 30 Jul 1991; (6), 27 Apr; (7), 26 May; (8), 24 Jun 1992.

[d] Number of leaves colonized by P. syringae from a total of 25 leaves sampled.

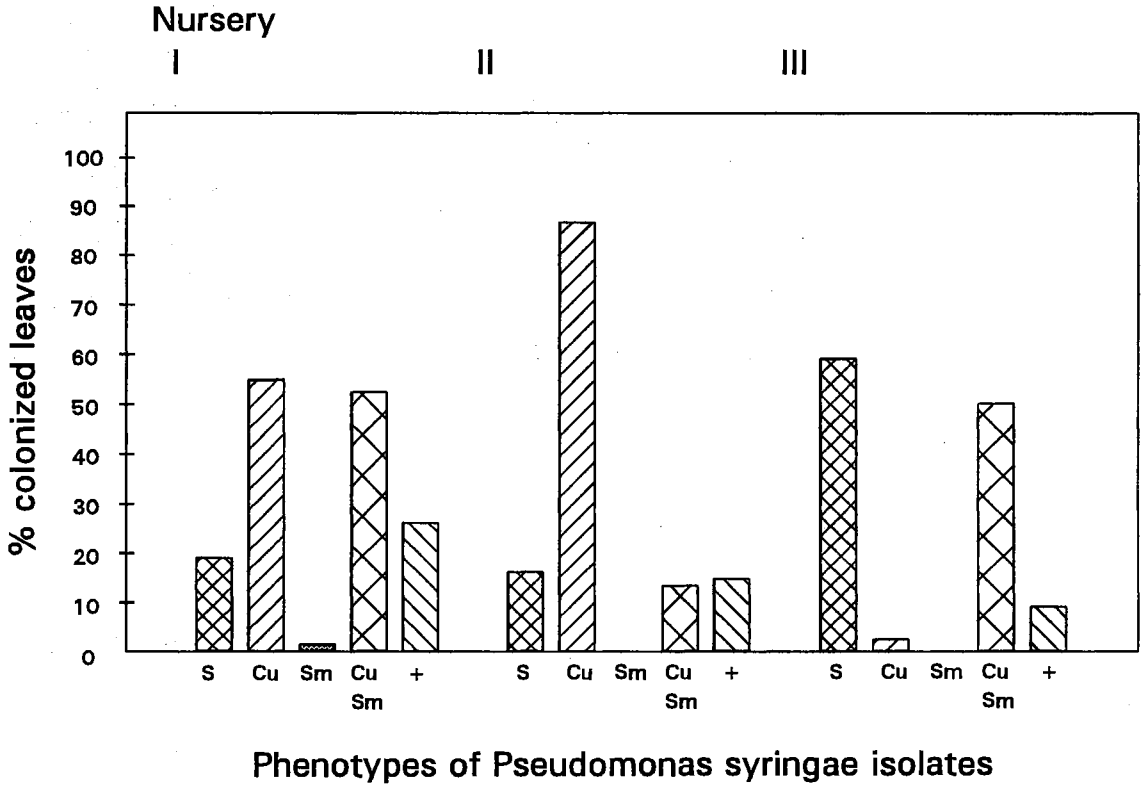
[e] Log_{10} mean populations (and standard deviation) of P. syringae per gram leaf tissue.

---

resistance phenotype at each location. Although both copper and streptomycin were applied at nursery I, Cu^s Sm^s strains were detected on 19.1% of the colonized leaves at this site. Cu^r Sm^s and Cu^r Sm^r P. syringae were detected on an approximatley equal number of leaves at nursery I. At nursery II, a large percentage (86.6%) of colonized leaves harbored Cu^r Sm^s P. syringae. Although streptomycin was not sprayed at nursery II, Cu^r Sm^r P. syringae was detected on 13.4% of colonized leaves. Neither copper nor streptomycin was sprayed at nursery III; however, an approximately equal numbers of colonized leaves harbored Cu^s Sm^s or Cu^r Sm^r P. syringae. Cu^s Sm^r P. syringae was rarely detected at any nursery. 26.2, 14.6, and 9.1% of the colonized leaves from nurseries I, II, and III, respectively, contained heterogenous populations with regard to resistance phenotypes with two or three resistance phenotypes detected on a single leaf (Fig. 4; +). The total number of P. syringae isolates with each resistance phenotype recovered at each sampling time is shown in Table 6. The phenotype Cu^s Sm^r was extremely rare in the nurseries surveyed; only 3 of 1,938 (0.15%) P. syringae isolates during the entire survey had this phenotype.

Characterization of plasmids. Plasmid isolations were performed on 366 P.

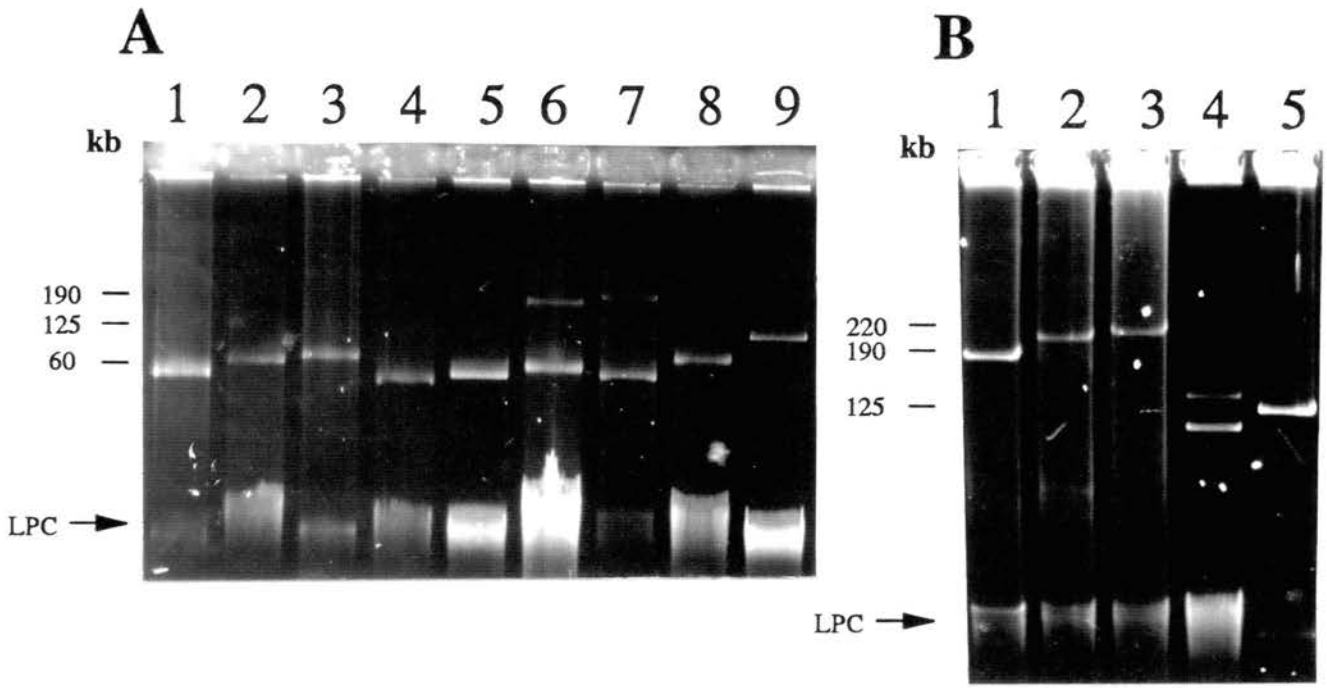
Fig. 4. Percentage of Pyrus calleryana cv. Aristocrat leaves colonized by Pseudomonas syringae. Values for nurseries I and II represent a total of 200 leaves sampled in 1991 and 1992; the percentages reported for nursery III represent 75 leaves sampled in 1992. S = (Cu^s Sm^s), Cu = (Cu^r) Sm^s, Sm = Cu^s Sm^r, CuSm = Cu^r Sm^r, + = strains with different phenotypes were recovered from a single leaf. Bactericide spray regimes were as follows: nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/l) and streptomycin sulfate (0.2 g/l); nursery II, 20 applications of cupric hydroxide (2.4 g/l); nursery III, no applications of copper or streptomycin bactericdes.


Nursery
I
II
III
% colonized leaves
100
90
80
70
60
50
40
30
20
10
0
S
Cu
Sm
Cu Sm
+
Phenotypes of Pseudomonas syringae isolates

syringae isolates (18.9% of the total number). 314 isolates were resistant to copper or streptomycin and contained at least one plasmid. Plasmids varied in size and were assigned to one of 14 profile groups according to size and resistance phenotype (Fig. 5). Ten plasmid profile groups consisted of a single plasmid; these were found in Cu^r Sm^s (Fig. 5A, lanes 1 - 4), Cu^r Sm^r (Fig. 5A, lanes 8 - 9; 2B, lanes 1 - 3), and Cu^s Sm^r (Fig. 5B; lane 5) strains. The other four plasmid profile groups contained two plasmids each and were recovered from Cu^r Sm^s (Fig. 5A, lanes 5 and 6) and Cu^r Sm^r (Fig. 5A, lane 7; Fig. 5B, lane 4) isolates. Cryptic plasmids in Cu^s Sm^s strains were examined (data not shown) and assigned to four plasmid profile groups (C1, C2, C3, and C4). Cryptic plasmids were detected in 11 of 14, 5 of 7, and 14 of 31 isolates from nurseries I, II, and III, respectively. Plasmid profile group C4 included the 190-kb cryptic plasmid pPSC4 which was also contained in two Cu^r Sm^s plasmid profiles (Fig. 5A; lanes 5 and 6). This plasmid was not detected alone in a Cu^s Sm^s P. syringae isolate.

Southern hybridization and conjugal transfer of selected plasmids into strain 9C11.2 were used to determine which plasmid(s) in each profile group harbored a particular resistance determinant and if they contained a common replicon. The Cu^r determinant was located on conjugative plasmids of 53 - 68 kb in all Cu^r Sm^s P. syringae strains (Fig. 6, 1 - 5, 1A), and on conjugative plasmids ranging from 68 to 220 kb in Cu^r Sm^r strains (Fig. 6, 6 - 11). The strA/strB genes were located by hybridization to the plasmid contained in profile group 12, and on the plasmid containing the Cu^r determinant in plasmid profiles 7 - 11 and 8A (Fig. 6). In plasmid

Fig. 5. Plasmid profiles from Pseudomonas syringae isolates with resistance to copper and/or streptomycin. Plasmid designations and profile group numbers follow the strain in parentheses. Lanes (A) 1 to 9 and (B) 1 to 5 contain the following isolates: (A) 1, 7B44 (pPSR7 - 1); 2, 2H12 (pPSR8 - 2); 3, 3C1 (pPSR9 - 3); 4, 8B48 (pPSR10 - 4); 5, 7G14 (pPSR7, pPSC4 - 1A); 6, 7A36 (pPSR11, pPSC4 - 5); 7, 7C12 (pPSR7, pPSR15 - 6); 8, 7B12 (pPSR1 - 7); 9, 8C32 (pPSR14 - 8). (B) 1, 2E49 (pPSR2 - 9); 2, 7B22 (pPSR12 - 10); 3, 7F14 (pPSR3 - 11); 4, 9A26 (pPSR14, pPSC6 - 8A); 5, 9A22 (pPSR13 - 12). Plasmid size standards are listed at left. LPC is linearized plasmid and chromosomal DNA.


A
1 2 3 4 5 6 7 8 9
kb
190
125
60
LPC
B
1 2 3 4 5
kb
220
190
125
LPC

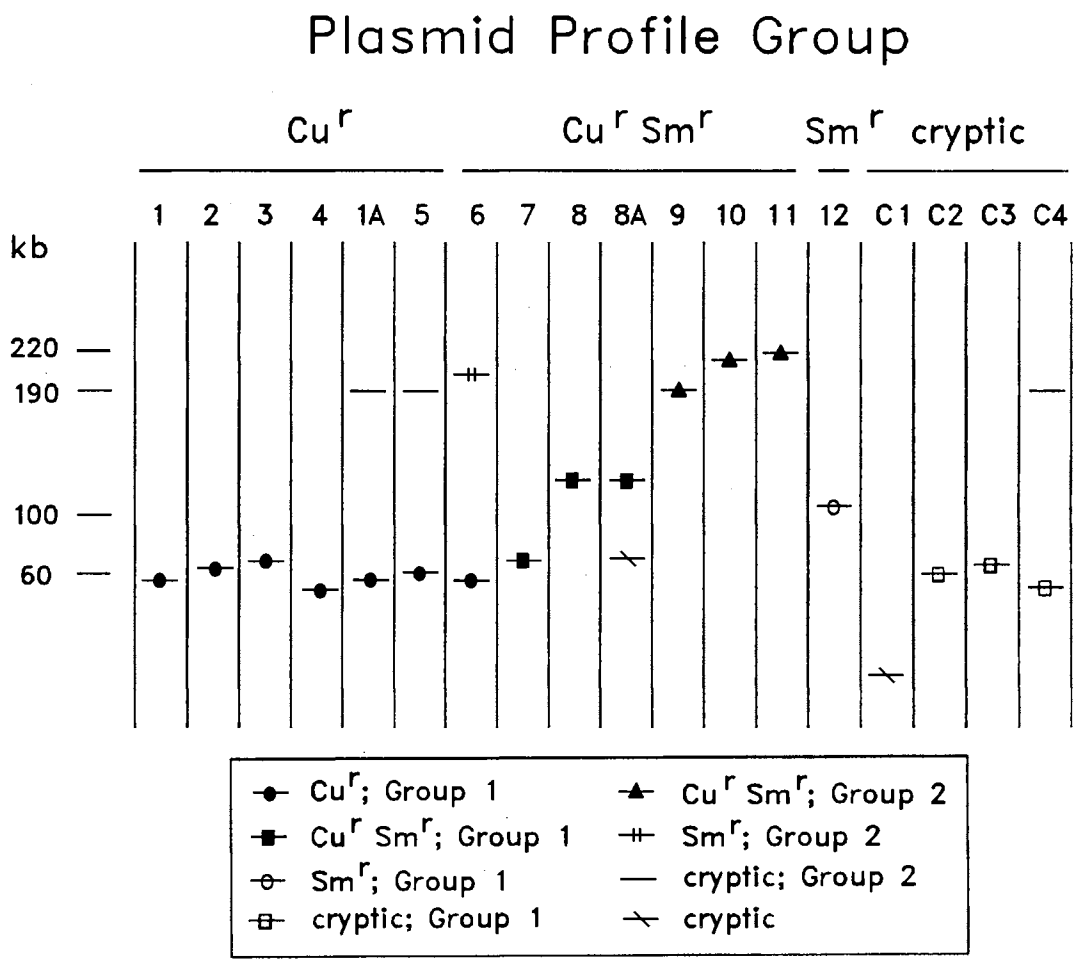


Fig. 6. Characterization of 18 plasmid profile groups identified in Pseudomonas syringae strains isolated from Pyrus calleryana cv. Aristocrat. Cu = copper, Sm = streptomycin, r = resistant. Plasmid profiles assigned to group 1 contained sequences which hybridized to a probe containing the oriV and par loci from pOSU22 (Mukhopadhyay et al 1990). Group 2 comprised plasmids of 190 - 220 kilobase pairs which did not hybridize to the pOSU22 probe.

profile group 6, the Cu^r and Sm^r determinants were not linked and were localized to the 58 and 196 kb plasmids, respectively (Fig. 6).

The resistance plasmids could be separated into two main groups by size and by hybridization to the pOSU22 replicon. Plasmids assigned to group 1 ranged in size from 53 - 125 kb and contained sequences which hybridized to a probe constructed from pOSU22, a clone containing the oriV and par loci from pOSU900, a cryptic plasmid isolated from P. syringae pv. syringae (167). Group 1 comprised all of the Cu^r plasmids (Fig. 6, 1 - 6, 1A), three Cu^r Sm^r (Fig. 6, 7 - 8, 8A), one Sm^r (Fig. 6, 12), and three cryptic (Fig. 6, C1, C2, C4) plasmids. The pOSU22 probe also hybridized to pJP27.00, pJP50.000, and the Sm^r plasmid, from P. syringae pv. coronafaciens PC27, P. syringae pv. tabaci PT81, and P. syringae pv. syringae B86-6, respectively (data not shown). Group 2 plasmids did not hybridize to pOSU22, ranged from 190 - 220 kb, and comprised the large Cu^r Sm^r plasmids from profiles 9 - 11, the Sm^r plasmid from profile 6, and the large cryptic plasmid pPSC4 from profiles 1A, 5, and C4. The cryptic plasmids in profile groups 8A and C1 did not hybridize to pOSU22 but were smaller than the plasmids in group 2 and thus were not assigned to either of the two plasmid groups. Restriction endonuclease analysis indicated that the Cu^r Sm^r plasmids from profiles 7, 9, and 11 were essentially identical to pPSR1, pPSR2, and pPSR3, respectively, which were previously isolated from P. syringae pv. syringae (215).

The distribution of the plasmid profiles among isolates from the three nurseries indicates the predominance of specific plasmids at each nursery (Table 7). At nursery

Table 7. Number of Pseudomonas syringae strains belonging to 18 distinct plasmid profile groups.

	Plasmid Profile Group[a]																		
	Cu^r Sm^s						Cu^r Sm^r							Cu^s Sm^r	Cu^s Sm^s				
Nursery[b]	1	1A	2	3	4	5	6	7	8	8A	9	10	11	12	C1	C2	C3	C4	0[c]	Total
I	34	25	4	4	0	25	3	6	56	15	4	3	0	3	0	1	8	2	3	196
II	47	0	30	4	3	0	0	5	3	0	0	1	0	0	0	0	5	0	2	100
III	0	0	0	0	0	1	0	10	2	0	0	18	4	0	8	6	0	0	17	66

[a] Cu = copper, Sm = streptomycin, r = resistant, s = sensitive.
[b] Bactericide spray regimes were as follows: nursery I, 15 applications of a mixture containing cupric hydroxide (2.4 g/l) and streptomycin sulfate (0.2 g/l); nursery II, 20 applications of cupric hydroxide (2.4 g/l); and nursery III, no applications of copper or streptomycin bactericides.
[c] Plasmid profile group 0 refers to Cu^s Sm^s P. syringae isolates which contained no detectable plasmids.

I, the Cu^r plasmid profiles detected at highest frequency were 1, 1A, and 5. Since the Cu^r determinant in profiles 1 and 1A was borne by pPSR7, this plasmid encoded Cu^r in 64.1% of the Cu^r Sm^s P. syringae strains isolated in nursery I. Furthermore, 42.4% of these strains contained pPSC4 in addition to pPSR7. 75.9% of the Cu^r Sm^r P. syringae isolates at nursery I contained pPSR14 (plasmid profile groups 8 and 8A) (Table 7). P. syringae strains from nursery I contained 15 different plasmid profiles and showed the most variability with regards to plasmid diversity with respect to the other two nurseries sampled. At nursery II, the predominant Cu^r plasmid profiles were 1 and 2. Profiles 1A and 5 were not detected, suggesting that the 190-kb cryptic plasmid pPSC4 may not be present in P. syringae isolates inhabiting nursery II. Nine Cu^r Sm^r isolates were examined for plasmids at nursery II, and five contained pPSR1 (plasmid profile group 7) which was only a minor component of the Cu^r Sm^r population at nursery I. At nursery III, only one Cu^r Sm^s isolate was detected and it contained plasmids of profile group 5. The major Cu^r Sm^r plasmid profiles at nursery III were 7 and 10, which were infrequently isolated at nursery I (Table 7). The cryptic plasmids also differed at each nursery; profile C3 predominated at nurseries I and II, and profiles C1 and C2 were more frequently isolated at nursery III (Table 7).

Resistance plasmids from the 14 plasmid profile groups were next examined for genetic variability and conjugative ability. Southern hybridizations to digested plasmid DNA were preformed to determine if RFLPs were present in sequences flanking known genetic markers. The pOSU22 probe hybridized to 1 or 2 EcoRI

fragments per plasmid and to SstI fragments of six sizes among the plasmids in group 1 (Table 8). The RFLP analysis with the pOSU22 probe indicated the plasmids in group 1 could be further subdivided into five subgroups (1a - 1e) based on the RFLP banding pattern generated from the EcoRI digests (Table 8). Probes containing the strA/strB and tnpA/tnpR loci hybridized to the same sized EcoRI fragment on pPSR3 and pPSR12 (Table 8). These probes hybridized to different sized EcoRI fragments on pPSR1, pPSR2, pPSR13, pPSR14, and pPSR15 (Table 8).

With the exception of pPSR15, each plasmid could be conjugated into the recipient strain 9C11.2 at frequencies ranging from 3.42×10^{-7} to 5.73×10^{-3} exconjugants per donor cell (Table 8). Conjugation frequencies among plasmids within group 1 or 2 were similar, with the notable exception of pPSR2 (Table 8).

Phenotypic characterization of a subset of P. syringae isolates. Analysis of phenotypic traits of 100 randomly-selected P. syringae isolates from nurseries I and III at sampling dates 6, 7, and 8 indicated that 98/100 strains were homogeneous with respect to 7 tests. These strains were oxidase negative, hydrolyzed aesculin, liquefied gelatin, did not utilize Na-tartrate, had no tyrosinase activity, were ice nucleation active at -5 C, and elicited a hypersensitive response in tobacco. Based on these characteristics, the strains were identified as P. syringae pv. syringae. Only two strains, 8H1 and 8H2, differed with respect to having no ice nucleation activity at -5 C.

AP-PCR analysis of P. syringae pv. syringae strains. Genome analysis was performed on the 100 P. syringae pv. syringae strains identified as described above,

Table 8. Characterization of 14 profiles groups of plasmids encoding copper and/or streptomycin resistance and three profile groups of cryptic plasmids from Pseudomonas syringae isolates recovered from three Pyrus calleryana cv. Aristocrat nurseries in Oklahoma.

Profile frequency[e]	Plasmid[b]	Group[c]	Phenotype[d]	Fragments used as probes in Southern hybridizations[a]: oriV EcoRI	oriV SstI	strA/strB EcoRI	tnpA/tnpR EcoRI	Conjugation
1	pPSR7	1a	CU	3.6;2.1	14.1	----	----	5.85×10^{-7}
1A	pPSR7	1a	CU	3.6;2.1	14.1	----	----	4.47×10^{-7}
2	pPSR8	1b	CU	5.2;2.3	21.9	----	----	4.96×10^{-7}
3	pPSR9	1b	CU	5.2;2.3	21.9	----	----	6.11×10^{-7}
4	pPSR10	1a	CU	3.6;2.1	14.1	----	----	3.57×10^{-7}
5	pPSR11	1a	CU	3.6;2.1	14.1	----	----	3.78×10^{-7}
6	pPSR7	1a	CU	3.6;2.1	14.1	----	----	6.94×10^{-7}
	pPSR15	2	SM	---	----	10.2	10.2	0
7	pPSR1	1b	CU,SM	5.2;2.3	9.6	10.1	10.1	3.95×10^{-7}
8	pPSR14	1c	CU,SM	7.2	19.7	6.4	6.4	1.48×10^{-6}
8A	pPSR14	1c	CU,SM	7.2	19.7	6.4	6.4	2.12×10^{-6}
9	pPSR2	2	CU,SM	---	----	15.7	15.7	5.73×10^{-3}
10	pPSR12	2	CU,SM	---	----	17.4	17.4	3.77×10^{-7}
11	pPSR3	2	CU,SM	---	----	17.4	17.4	3.42×10^{-7}
12	pPSR13	1d	SM	4.5	14.1	11.3	11.3	6.22×10^{-6}
C2	pPSC2	1e	CRYP	4.8;2.2	18.5	----	----	ND
C3	pPSC3	1b	CRYP	5.2;2.3	19.7	----	----	ND
C4	pPSC4	1a	CRYP	3.6;2.1	17.5	----	----	ND

[a] Indicates the size of restriction fragments [in kilobase pairs (kb)] hybridizing to each probe. (---) indicates no hybridization observed. Probes were: (oriV and par) from pOSU900; (strA/strB) from RSF1010; (tnpA/tnpR) from Tn5393.

[b] Plasmid = resistance plasmid (profiles 1 - 12, 1A, 8A) or cryptic plasmid from Group 1 (profiles C2, C3, C4). Profiles 1A, 5, and 8A also contain a cryptic plasmid which was not included in the table.

[c] Plasmid group designations: (1) 53 - 125 kb, contain DNA hybridizing to the oriV and par sequences from pOSU22 (Mukhopadhyay et al 1990); (2) 190 - 220 kb, do not hybridize to pOSU22. Plasmid group 1 is further subdivided into subgroups a - e based on the size of EcoRI fragments hybridizing to the oriV probe.

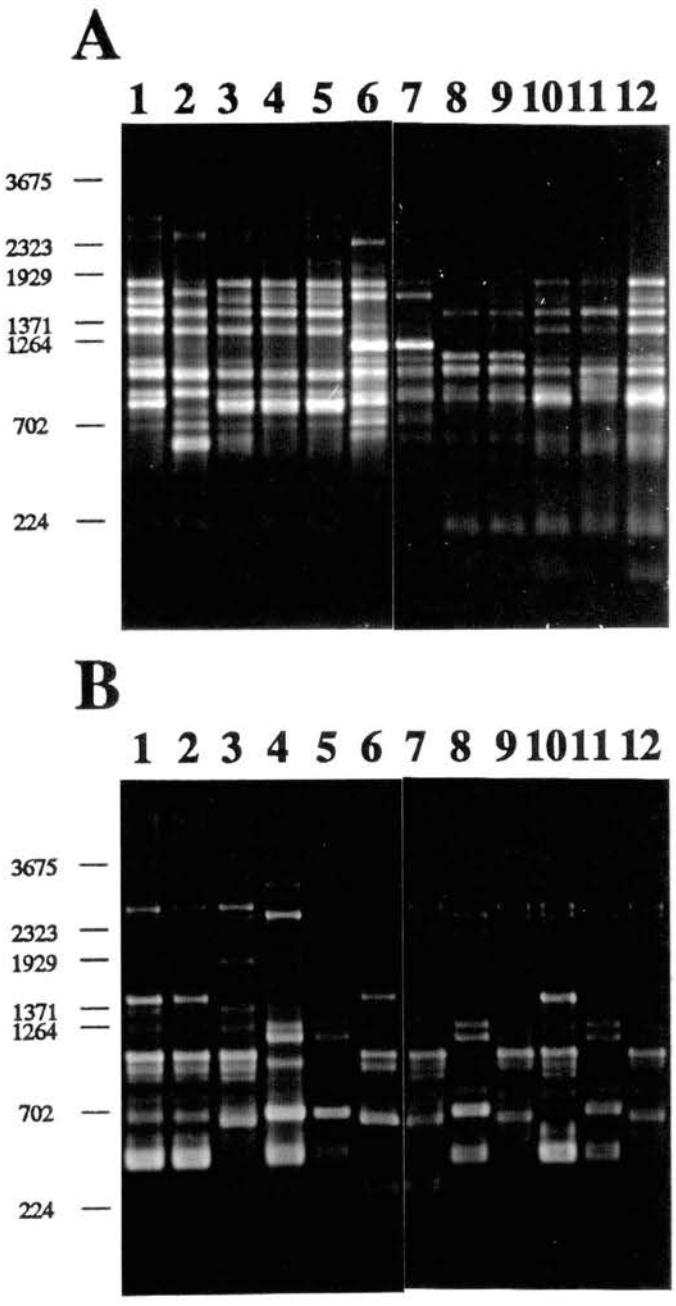
[d] Cu = copper resistant, Sm = streptomycin resistant, CRYP = cryptic.

[e] Indicates the frequency of exconjugants per donor strain recovered in agar plate matings. P. syringae pv. syringae 9C11.2 was used as the recipient strain in each experiment. The strains utilized as donors for each plasmid profile are listed in Table 1. ND = not determined.

and on six P. syringae pv. syringae strains which were previously examined for phenotypic and genetic diversity (66,67). Total genomic DNA released from intact cells of the 106 P. syringae pv. syringae strains were used as a template for PCR amplification with the IS50 primer and the REP primers. A total of 29 and 36 DNA products, ranging in size from approximately 0.3 to 4.0 kb, were observed among the 106 strains using the IS50 and the REP primers, respectively. No bands were observed when gel-purified plasmids from each plasmid profile group were used as a template for PCR, indicating differences in plasmid content among the strains were not reflected in the PCR analysis. Patterns of PCR products amplified from specific strains were compared with others run on the same gel. An example of the results obtained with the IS50 and the REP primer set is shown in Fig. 7.

Cluster analysis of the data matrices generated with the IS50 or the REP primers indicated the 106 P. syringae pv. syringae strains could be differentiated into two distinct groups (A and B) which were separated at 39.7% and 47.0% genetic similarity, respectively (data not shown). A comparison of the dendrograms generated with the IS50 and REP primers indicated that they were not completely identical. Four strains (B48, B61, B64, and PSC1B) were placed into group A in the IS50-PCR dendrogram and group B in the REP-PCR dendrogram. One strain (B76) was placed into group B in the IS50-PCR dendrogram and group A in the REP-PCR dendrogram. The IS50 and REP dendrograms also showed minor differences in the grouping of closely-related strains between the IS50 and REP dendrograms. The differences generated with the two primer sets suggested that each primer combination

Fig. 7. Genomic fingerprinting of Pseudomonas syringae pv. syringae strains using the arbitrarily-primed polymerase chain reaction technique with the REP primers (Panel A) and an IS50 primer (Panel B). Panels A and B (lanes 1 - 12 each) contain the following strains with the PCR group indicated in parentheses: Panel A, Lane 1, 8F21 (B); 2, 8F35 (B); 3, 8F43 (B); 4, 8G5 (B); 5, 8G10 (B); 6, 8G17 (A); 7, 8C43 (A); 8, 8D2 (B); 9, 8D6 (B); 10, 8D17 (B); 11, 8C5 (B); 12, 8D34 (B). Panel B, Lane 1, 9B36 (B); 2, 9D8 (B); 3, 8F21 (B); 4, 7F29 (A); 5, 9A3 (A); 6, 9C39 (B); 7, 8C32 (B); 8, 8C43 (A); 9, 8D6 (B); 10, 8D17 (B); 11, 8D29 (A); 12, 8E17 (B). Size standards (in base pairs) are indicated at left.


A
1 2 3 4 5 6 7 8 9 10 11 12
3675
2323
1929
1371
1264
702
224
B
1 2 3 4 5 6 7 8 9 10 11 12
3675
2323
1929
1371
1264
702
224

detected differences in the genome of strains which were not evidenced when the other primer combination was used. A combined matrix was then generated using the IS50 and REP PCR data. When this matrix was analyzed, 84 distinct patterns were observed among the 106 P. syringae pv. syringae strains. Of these, 70 patterns were unique, 14 patterns were observed with more than one individual, and the maximum number of individuals with identical IS50 and REP PCR patterns was six. The dendrogram generated from the combined matrices also differentiated the strains into two groups at 45.8% similarity (Fig. 8).

Information regarding the plasmid content and origin of the 100 P. syringae pv. syringae strains identified in this study is listed in Table 9. Fourteen clusters containing multiple strains with identical IS50 and REP PCR patterns, and differences in plasmid profiles were observed among strains within five of the clusters (Table 9; e.g. cluster A1, A3). When the plasmid data are included in the analysis, a total of 87 unique P. syringae pv. syringae genotypes were observed among the 100 strains isolated from Pyrus calleryana in the present study. The two PCR groups (A and B) contained strains with distinct plasmid profiles; group A contained Cu^r Sm^s strains with plasmid profiles 1, 1A, and 5 dispersed among several clusters, and one Cu^r Sm^r strain (7C12) with plasmid profile 6 (Fig. 8; Table 9). Group B contained Cu^r Sm^r strains with plasmid profiles 7 - 11 and 8A dispersed among several genotypes, and two Cu^s Sm^r strains of identical genotype with plasmid profile 12 (Table 9). Also, 21 Cu^s Sm^s strains, 11 of which did not contain a plasmid, were found in several clusters throughout this group.

Fig. 8. Dendrogram of Pseudomonas syringae pv. syringae strains derived from PCR fingerprints using the IS50 and REP primers. The dendrogram was generated from the combined IS50 and REP primer data sets.

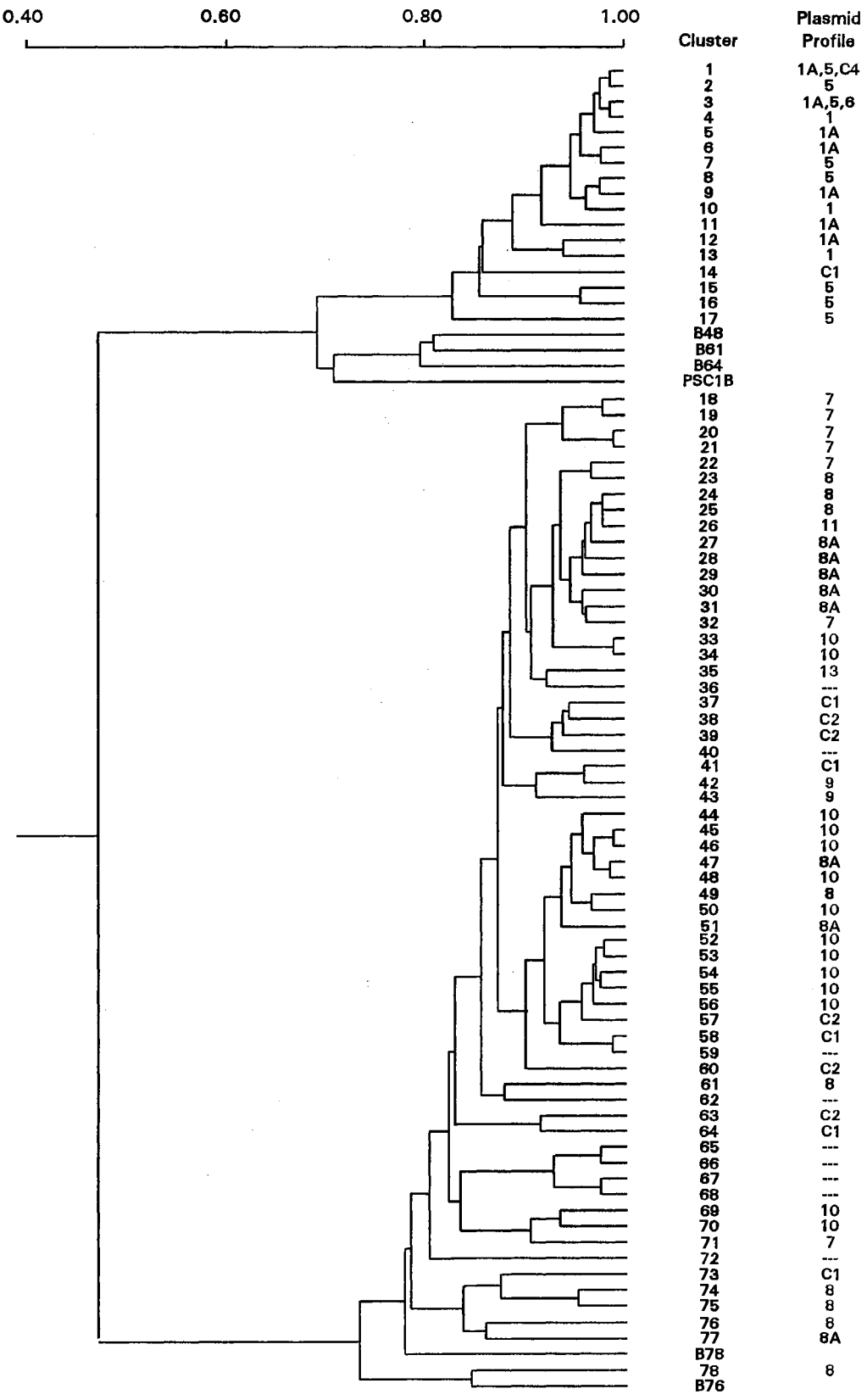

Relative Genetic Similarity
0.40
0.60
0.80
1.00
Cluster
Plasmid Profile
1 1A,5,C4
2 5
3 1A,5,6
4 1
5 1A
6 1A
7 5
8 5
9 1A
10 1
11 1A
12 1A
13 1
14 C1
15 5
16 5
17 5
B48
B61
B64
PSC1B
18 7
19 7
20 7
21 7
22 7
23 8
24 8
25 8
26 11
27 8A
28 8A
29 8A
30 8A
31 8A
32 7
33 10
34 10
35 13
36 ---
37 C1
38 C2
39 C2
40 ---
41 C1
42 9
43 9
44 10
45 10
46 10
47 8A
48 10
49 8
50 10
51 8A
52 10
53 10
54 10
55 10
56 10
57 C2
58 C1
59 ---
60 C2
61 8
62 ---
63 C2
64 C1
65 ---
66 ---
67 ---
68 ---
69 10
70 10
71 7
72 ---
73 C1
74 8
75 8
76 8
77 8A
B78
78 8
B76

Table 9. Relevant characteristics of 100 Pseudomonas syringae pv. syringae strains whose chromosomal genotypes were examined using the arbitrarily-primed polymerase chain reaction technique. The strains were isolated from two Pyrus calleryana cv. Aristocrat nurseries in Oklahoma. The order of the strains corresponds to the order in the accompanying dendrogram (Fig. 8).

Strain	Cluster[a]	Plasmid Resistance/ Profile[b]		Nursery	Strain	Cluster	Plasmid Resistance/ Profile		Nursery
PCR Group A					8G24	32	CS	7	III
7A3	1	C	1A	I	9C39	33	CS	10	III
7A12	1	C	5	I	9C40	34	CS	10	III
7A15	1	C	5	I	9A15	35	S	13	I
7C19	1	C	5	I	9A21	35	S	13	I
7C27	1	---	C4	I	9C49	36	---	--	III
7C35	1	C	5	I	7F31	37	---	C1	III
7D21	2	C	5	I	7H6	38	---	C2	III
7A7	3	C	5	I	7H9	38	---	--	III
7A36	3	C	5	I	9C5	39	---	C2	III
7C12	3	CS	6	I	9C11	40	---	--	III
8C23	3	C	1A	I	8F21	41	---	C1	III
8B31	4	C	1	I	8H1	42	CS	9	III
7C6	5	C	1A	I	8H2	43	CS	9	III
7C7	5	C	1A	I	7B22	44	CS	10	III
7G13	6	C	1A	I	7H12	45	CS	10	III
7G38	7	C	5	I	8H31	46	CS	10	III
7A29	8	C	5	I	9B36	47	CS	8A	I
8F2	8	C	5	I	9D8	48	CS	10	III
7G14	9	C	1A	I	8F43	49	CS	8	III
8E33	9	C	1A	I	8G31	50	CS	10	III
8A6	10	C	1	I	9A24	51	CS	8A	I
8B37	10	C	1	I	7E41	52	CS	10	III
7F41	11	C	1A	I	9D14	53	CS	10	III
8H38	12	C	1A	I	8G36	54	CS	10	III
8H48	12	C	1A	I	9C12	55	CS	10	III
9A3	13	C	1	I	9D5	55	CS	10	III
7F29	14	---	C1	III	9A31	56	CS	10	I
8C43	15	C	5	I	8G48	57	---	C2	III
8D29	15	---	C4	I	8C9	58	---	C1	III
8D39	15	C	1A	I	9C41	59	---	--	III
8G17	15	C	5	III	8H32	60	---	C2	III
8F19	16	C	5	I	7B34	61	CS	8	I
7D28	17	C	5	I	9C19	62	---	--	III
PCR Group B					7E44	63	---	C2	III
7B12	18	CS	7	III	8F35	64	---	C1	III
7D5	19	CS	7	III	8D2	65	---	--	III
8G5	20	CS	7	III	8D6	65	---	--	III
8G10	21	CS	7	III	8E15	66	---	--	III
7B13	22	CS	7	III	8E17	67	---	--	III

Table 9 (continued)

Strain	Cluster[a]	Plasmid Resistance/ Profile[b]		Nursery	Strain	Cluster	Plasmid Resistance/ Profile		Nursery
8I2	23	CS	8	I	8E23	68	---	--	III
7E50	24	CS	8	I	8D17	69	CS	10	I
9B3	25	CS	8	I	8D34	70	CS	10	I
9B5	25	CS	8	I	8D36	71	CS	7	I
9B10	26	CS	11	I	7D12	72	---	--	III
7H24	27	CS	8A	I	8C5	73	---	C1	III
9A26	28	CS	8A	I	8C15	74	CS	8	I
7F45	29	CS	8A	III	8C16	75	CS	8	I
7F49	30	CS	8A	I	8C42	76	CS	8	I
7G22	31	CS	8A	I	9A30	77	CS	8A	I
7G23	31	CS	8A	I	8C32	78	CS	8	I

[a] Clusters were deduced from the dendrogram generated from the combined matrix of IS*50* and REP-PCR analysis of 100 *P. syringae* pv. syringae strains.

[b] Resistance phenotype and plasmid profile of the *P. syringae* pv. syringae strain; C = copper resistant, S = streptomycin resistant, --- = sensitive to copper and streptomycin, -- = contains no plasmids.

DISCUSSION

Population sizes of P. syringae on individual Pyrus calleryana leaves were similar at all three nurseries indicating that the intensive bactericide spray program at nurseries I and II was not effective in reducing epiphytic P. syringae populations. Isolates of P. syringae with resistance to copper and streptomycin were present in populations recovered from all three nurseries. At nurseries I and II, the prevalence of resistant strains indicates that copper and streptomycin resistance are important traits in these populations and are probably responsible for the maintenance of P. syringae populations on these trees. At nursery I, the majority of the P. syringae isolates were Cu^r Sm^s, despite the fact that both copper and streptomycin bactericides were applied weekly in this nursery during the growing season. Because copper has been used as a bactericide in Oklahoma on nursery trees for a longer period of time than streptomycin, it is possible that copper resistance evolved in P. syringae prior to the evolution of streptomycin resistance. Subsequent usage of streptomycin probably selected for increased resistance to this antibiotic and a population containing a larger percentage of Sm^r strains. It is unclear why the Cu^s Sm^r phenotype was so rare in the P. syringae populations studied. In the nurseries receiving bactericide sprays, the ratio of resistant to sensitive P. syringae isolates was large; therefore, there may be fewer Cu^s Sm^s isolates in these populations which could acquire streptomycin resistance and exhibit the Cu^s Sm^r phenotype. At nursery II, 9.3% of the P. syringae isolates were Cu^r Sm^r even though streptomycin was not applied at this nursery, and 43.6% of the P. syringae isolates at nursery III were Cu^r Sm^s or Cu^r Sm^r, even

though neither copper nor streptomycin were applied at this nursery. These results suggest that P. syringae strains with resistance to copper or streptomycin colonize the phylloplane in the absence of selection from bactericides, thus corroborating previous observations (5,46,216,218).

The plasmid content of Cu^r Sm^s, Cu^s Sm^r, and Cu^r Sm^r P. syringae was diverse, and 12 resistance plasmids were grouped into 14 plasmid profile groups on the basis of size and resistance phenotype. The skewed frequency of individual plasmid profile groups detected among P. syringae isolates from nurseries I, II, and III suggests different plasmids were selected at each nursery. It is unlikely that P. syringae isolates would be restricted to only one of the three sites because the relative proximity of each nursery would favor dissemination between nurseries from plant surfaces by rain and wind. P. syringae cells are disseminated from plant surfaces by rain and wind. The dominance of different plasmids within a nursery could result from physical or biological factors including the influence of variations in physical or biological parameters of the phylloplane environment which select other genetic determinants on the plasmids that enhance fitness or from the influx of P. syringae cells from other hosts.

In this study, streptomycin resistance in P. syringae was conferred by the strA/strB homologs present on the Sm^r transposon Tn5393. This transposon was located on variable EcoRI fragments among group 1 and group 2 plasmids, an observation which suggests Tn5393 was mobile in the P. syringae populations studied and transposed to several different plasmids. Other investigators have reported the

detection of copper and streptomycin determinants on plasmids which varied in size (50,104,170,215,218), thus indicating that mobilization of resistance determinants among plasmids does occur.

The probe derived from the oriV and par sequences in the present study was originally constructed from pOSU900, a cryptic plasmid in P. syringae pv. syringae J900, which was originally isolated in Oregon (167). In the present study, this probe hybridized to a Sm^r plasmid in P. syringae pv. syringae B86-6, and to the cryptic plasmids pJP50.000 and pJP27.00 from P. syringae pv. coronafaciens and P. syringae pv. tabaci, respectively. pJP27.00 and closely-related variants were harbored by a group of strains of the tobacco pathogens P. syringae pvs. angulata and tabaci which were isolated worldwide and as long ago as 1941 (230). pJP50.000 and variants were detected in strains of the oat pathogens P. syringae pvs. coronafaciens and striafaciens which were isolated from North America and Europe as early as 1940 (230). The probe containing the oriV and par determinants from pOSU900 also hybridized to plasmids containing the coronatine biosynthetic pathway in P. syringae pvs. atropurpurea, glycinea, morsprunorum, and tomato (11). Therefore, the origin of replication and stability determinants from plasmid pOSU900 may be widely distributed in P. syringae and may encode additional determinants important for association of P. syringae with plant hosts.

Genomic fingerprinting of the 100 P. syringae pv. syringae strains characterized in the present study indicated a high level of diversity, and many unique AP-PCR patterns were detected. Cluster analysis of the combined data set differentiated the

strains into two distinct groups; however, strains within the groups were closely related, with 88.6 and 94.4% of the strains in PCR groups A and B distinguished at >80% similarity, respectively. The separation into two distinct groups, and the occurrence of strains from each group at nursery I, may indicate that these strains belong to distinct subpopulations of P. syringae which are both capable of colonizing Pyrus calleryana. This situation could be analagous to the coexistence of P. syringae pv. syringae with other P. syringae pathovars on the phylloplane of the same host. For example, P. syringae pv. syringae can coexist with P. syringae pvs. morsprunorum and papulans on cherry and apple, respectively (28,125).

An examination of resistance phenotypes and plasmid profiles of individuals within PCR groups A and B yielded interesting results. Group A consisted primarily of Cu^r Sm^s strains from nursery I which contained plasmid profiles 1, 1A, or 5. The Cu^r Sm^r strain 7C12 and the Cu^s Sm^s strains 7C27 and 8D29 were also clustered within group A. These strains contained plasmids in profile groups 6 and C4, respectively, which are closely related to profile group 1A (see Table 9). Thus, the plasmids distributed among strains assigned to PCR group A were closely related. When the plasmid content of individual strains is included in the analysis, strains with identical IS50 and REP PCR patterns could be further differentiated. For instance, the first cluster in PCR group A contains five strains (Fig. 8), three of which (e.g. 7A3, 7A12, 7C27) contain different plasmid profiles (Table 9). Since these plasmid profiles are closely related (Table 8), these results suggest that plasmid rearrangements have occurred in the same genetic background.

Results of AP-PCR analysis of the six P. syringae pv. syringae strains isolated from diverse hosts such as peach, tomato, and wheat, indicated the strains were neither closely related to each other, nor to the strains isolated in the present study. This finding corroborates results of other studies indicating some strains of P. syringae pv. syringae exhibit host specificity (38,190,191) and strains from one host may be unrelated to strains isolated from other hosts (67,131). In a previous analysis, Denny et al (67) using multi-locus enzyme electrophoresis and RFLP analyses, could differentiate the strains isolated from dicots (B48, B76, B78) from those isolated from monocots (B61, B64, PSC1B). However, the AP-PCR analysis done in the present study did not differentiate the dicot and monocot strains.

Studies of Ti plasmid-chromosome associations in Agrobacterium tumefaciens indicate specific plasmid and chromosomal genotypes are highly correlated, implying plasmid transfer between strains with different chromosomal backgrounds is rare in nature (24,174). However, other reports state Ti plasmids have been detected in different chromosomal backgrounds of A. tumefaciens suggesting Ti plasmids can be transferred in some situations (161,169). In Rhizobium leguminosarum, identical Sym plasmids were detected in different chromosomal backgrounds, and strains with the same chromosomal genotype contained different plasmids, suggesting that plasmid transfer occurs in natural populations of this species (122,123,197,247). In P. syringae pv. syringae, the location of plasmids such as pPSR11 (plasmid profile group 5) or pPSR14 (plasmid profile group 8 and 8A) in strains containing different chromosomal genotypes within PCR group A and B, respectively, suggests plasmid

transfer has occurred in these populations. However, the lack of dissemination of these plasmids between strains in PCR groups A and B also implies limitations exist barring the establishment of particular plasmids in certain chromosomal backgrounds. Young and Wexler hypothesized that the species R. leguminosarum is effectively compartmentalized, with plasmid transfer occurring within compartments but not between them (247). The species P. syringae may be compartmentalized as well; for example, Cu^r plasmids from P. syringae pv. syringae could not be transferred to P. syringae pv. morsprunorum strains in vitro and were not detected in natural populations of P. syringae pv. morsprunorum (218). Our observation that some plasmids were apparently confined to strains of a particular chromosomal genotype is interesting since group 1 plasmids which contain the pOSU900 replicon were detected among strains in both PCR groups. Factors contributing to the apparent compartmentalization of these plasmids are unclear since transfer of plasmids such as pPSR11 between group A strains and the group B strain 9C11.2 could be demonstrated in vitro in the present study.

The failure to identify Cu^r Sm^r strains with plasmid profiles other than 6 in PCR group A implies pPSR7, the most common Cu^r plasmid we detected, has not acquired the Sm^r transposon Tn5393. It is currently unknown why pPSR7 has not been a recipient of Tn5393. Strains 7C27 and 8D29, which contain pPSR7, have acquired Tn5393; however, the transposon was inserted into the cryptic plasmid pPSC5. The question of whether pPSR7 is able to acquire a copy of Tn5393 will be the subject of further study.

The AP-PCR, plasmid profile, and resistance phenotype analyses illustrate the large number of subpopulations of P. syringae in the phyllosphere in each nursery. There is evidence in the literature that the frequencies of subpopulations of P. syringae are dynamic, with temporal population shifts occurring in response to alterations of physical and/or biological environmental parameters (78,99,100,132,160). In the present study, selective pressure from bactericide usage has apparently resulted in the colonization of many P. syringae genotypes by individual resistance plasmids, a phenomenon which may contribute to the ultimate persistence of resistance plasmids in these populations. The evolution of resistance to the bactericides copper and streptomycin within P. syringae pv. syringae appears to have involved a relatively large number of plasmid and chromosomal genotypes, both of which could be differentiated into two distinct groups. This may reflect the response of a population which was initially heterogenous, a characteristic which would tend to inhibit the rapid dissemination of a single plasmid or clone. The isolation of different plasmid profile groups and the genome analysis of strains from nurseries I and III indicated that the P. syringae populations at each nursery were distinct. The populations at each nursery could have been influenced by a number of factors, including the initial microbial community inhabiting the trees before bactericides were applied, immigration and colonization of P. syringae genotypes from other plant hosts, environmental factors, and the bactericide spray regimes. AP-PCR analysis of chromosomal genotypes of the populations from nurseries I and III suggested that plasmid transfer has probably occurred, although it is unknown

whether the frequency of plasmid transfer was affected by bactericide selection pressure. Previous observations have indicated that bactericide resistance plasmids are stably maintained in P. syringae and do not impact epiphytic fitness (216). Thus, effective management of P. syringae pv. syringae is compromised by the ability of populations to evolve resistance to bactericides. Management alternatives, such as host resistance or biological control, may likewise be confounded by the inherent heterogeneity of the pathogen populations.

CHAPTER V

RELATIVE FITNESS OF IN VITRO AND IN PLANTA OF PSEUDOMONAS SYRINGAE STRAINS CONTAINING COPPER AND STREPTOMYCIN RESISTANCE PLASMIDS

SUMMARY

The effect of resistance plasmids encoding copper resistance (Cu^r), streptomycin resistance (Sm^r), and both Cu^r and Sm^r on competitive fitness of Pseudomonas syringae pv. syringae was studied in vitro and in planta. The Cu^r Sm^r plasmid pPSR1 provided a selective advantage to its bacterial host (P. syringae pv. syringae FF5.1) on Pyrus calleryana leaves which were treated weekly with copper and/or streptomycin bactericides. However, populations of the plasmid-free Cu^s Sm^s FF5.1 were reduced 10 to 1000-fold over a 12-week period on trees treated with bactericides. The resistance plasmids pPSR4 (Cu^r), pPSR5 (Sm^r), and pPSR4::Tn5393 (Cu^r Sm^r) were highly stable for over 200 generations of growth in glucose-limited batch culture. Results of competition experiments in vitro indicated that P. syringae pv. syringae FF5 containing pPSR4, pPSR5, or pPSR4::Tn5393 was reduced to less than 5% of the total culture in competition with wild-type FF5. In growth chamber studies, the resistance plasmids studied did not impact epiphytic

fitness of P. syringae pv. syringae. Our data suggest that resistance plasmids will persist in populations of P. syringae pv. syringae following their initial selection regardless of the bactericidal spray regime.

The effect of plasmids on the relative fitness of bacterial cells is of interest from evolutionary, medical, and industrial viewpoints. Results of studies using small, nonconjugative plasmids or various cloning vector constructs have indicated that plasmid carriage generally reduces bacterial fitness in the absence of selection pressure (23,75,97,250). Thus, cells in which the plasmid has been eliminated generally grow faster and dominate in a mixed culture containing plasmid-bearing cells. In some studies, this negative effect or burden was attributed to expression of specific plasmid-encoded genes (19,118). However, in other studies where natural antibiotic-resistance plasmids such as RP1 were investigated, the wild-type plasmid or deletion derivatives persisted in culture (33,73,87,171,242).

Although these studies and others have greatly enhanced our knowledge of plasmid biology, the effect of plasmids on reproductive fitness of bacterial hosts in their native ecological niche has not been fully elucidated. In studies where the fitness of plasmid-containing strains was investigated in vivo, the transfer and persistence of resistance plasmids generally occurred in the absence of selection. E. coli strains carrying antibiotic-resistance plasmids successfully transferred the plasmids and sometimes persisted in animal or human hosts without antibiotic selection (151,152,222). Other studies have indicated that antibiotic selection may not

be required for maintenance of antibiotic-resistant populations after resistance is selected in vivo (36,134). These observations suggest that resistance plasmids contain genes which enhance survival in specific ecological niches (18).

In phytopathogenic bacteria, plasmids encode a variety of important traits which may confer selective advantages in planta (52). The Ti plasmid of Agrobacterium tumefaciens and the Sym plasmid of Rhizobium spp. encode genes which are essential for the successful colonization of plant hosts (142). In Pseudomonas syringae, plasmid-encoded traits include biosynthesis of indoleacetic acid (IAA) and the phytotoxin coronatine (15,44). The 73-kb plasmid pIAA from P. syringae subsp. savastanoi increased fitness of this pathogen in oleander leaves (204), and the coronatine plasmid, pPT23A, significantly increased the growth of P. syringae pv. tomato in tomato leaves (15,16). Therefore, the maintenance of pIAA and pPT23A significantly benefits phytopathogenic P. syringae strains during their interaction with plant hosts.

The evolution of resistance to the bactericides copper and streptomycin in phytopathogenic bacteria is a developing problem in agriculture. Although genetic studies have indicated that most resistance determinants in phytopathogenic bacteria are plasmid-encoded (47), little is known about the stability of these plasmids, their effect on fitness of their bacterial hosts, and their persistence in their bacterial host in the absence of bactericidal sprays. We have been studying the evolution of plasmid-encoded resistance to copper (Cu^r) and streptomycin (Sm^r) in P. syringae pv. syringae (215). We are especially interested in the ecological impact of resistance in P.

syringae pv. syringae, namely, whether resistance plasmids can persist in this bacterium and how they impact competitive fitness. Our objectives in the present study were to: (*i*) assess the colonization ability and persistence of a P. syringae pv. syringae strain containing a Cu^r Sm^r plasmid relative to a plasmid-free strain in planta; (*ii*) examine the stability of representative resistance plasmids in vitro; and (*iii*) examine the effect of plasmid carriage on the competitive fitness of cells in vitro and in planta.

MATERIALS AND METHODS

Bacterial strains, plasmids, antibiotics, and media

The bacterial strains and plasmids used in the present study are listed in Table 10. P. syringae pv. syringae FF5 was originally isolated from a dormant bud of Pyrus calleryana cv. Aristocrat, and spontaneous mutants with rifampicin resistance (FF5.1), and chloramphenicol resistance (FF5.2) were previously selected (215). pPSR1 was introduced into FF5.1 by conjugation as described previously (215), and pPSR4, pPSR5, and pPSR4::Tn5393 were introduced into FF5 by electroporation (84). After plasmid introduction into FF5 or FF5.1, a single colony was transferred to medium containing the appropriate resistance markers and then stored in 15% glycerol at -80°C. Subsequent experiments were initiated with cells obtained from these stocks. The medium used in the plasmid competition and plasmid stability experiments was a modified version of a minimal medium developed by Hoitink and Sinden (HS medium; 101) in which the concentration of glucose was reduced from 10.0 g/l to 0.25 g/l (HSg). Cell densities of stationary-phase cultures of FF5 and all derivatives

Table 10. Bacterial strains and plasmids and their relevant characteristics[a].

Strain or Plasmid	Chromosomal phenotype	Relevant characteristics	Reference
Pseudomonas syringae pv. syringae			
FF5		Cu^s Sm^s; no detectable plasmids	215
FF5.1	Rif^r		215
FF5.2	Cm^r		215
pPSR1		Cu^r Sm^r; 68 kb	215
pPSR4		Cu^r Sm^s; 60 kb	This study
pPSR5		Cu^s Sm^r; 68 kb	215
pPSR4::Tn*5393*[b]		Cu^r Sm^r; 65.5 kb	215

[a] Rif = rifampicin, Cm = chloramphenicol, Cu = copper, Sm = streptomycin, r = resistant, s = susceptible, kb = kilobase pairs.
[b] Tn*5393* is a 5.4-kb streptomycin-resistance transposon containing the *strA*/*strB* Sm^r genes (41). Streptomycin resistance in pPSR1 and pPSR5 is conferred by an indigenous copy of Tn*5393* which is encoded by these plasmids. In order to compare the fitness of strains containing plasmids with a single resistance phenotype (Cu^r encoded by pPSR4) with those containing plasmids with two resistance phenotypes (Cu^r Sm^r), a copy of Tn*5393* was inserted into pPSR4 in the laboratory (217).

of this strain were approximately 1 x 10^8 cfu/ml in HSg medium. For enumeration, cells were plated on MG medium (108) and MG amended with cupric sulfate (MGcu), streptomycin (MGsm) or cupric sulfate and streptomycin (MGcu sm). Chloramphenicol (cm) or rifampicin (rif) were added to these media when appropriate. For enumeration of cells from plant surfaces, cycloheximide was added

to MG medium at 100 μg/ml to inhibit fungal growth. Cells were grown on medium B of King et al. (109) before inoculation to plants. Cupric sulfate was added to media at 250 μg/ml, and chloramphenicol, rifampicin, and streptomycin were added to media at 50, 100, and 25 μg/ml, respectively.

Population dynamics of FF5.1 and FF5.1(pPSR1) on ornamental pear leaves

A field experiment was conducted to evaluate epiphytic colonization and persistence of FF5.1 and FF5.1(pPSR1) in the presence and absence of copper and streptomycin bactericides. One-yr-old Pyrus calleryana cv. Aristocrat trees were grown in 37.8 l containers spaced 0.5 m apart in plots consisting of 18 trees. In the field experiment, a randomized complete block design with four blocks was used. Prior to inoculation, cells were grown on KB agar medium for 36 h, suspended into 0.01 M potassium phosphate buffer, pH 7.0 (K-buffer), and adjusted turbidimetrically to a cell density of approximately 10^7 cfu/ml. FF5.1 or FF5.1(pPSR1) cells were individually inoculated onto expanding P. calleryana leaves using a mist sprayer until the leaves were uniformly wet. The bactericide spray regimes for individual blocks were as follows: (1) cupric hydroxide at 2.4 g/l and streptomycin sulfate at 0.2 g/l; (2) cupric hydroxide at 2.4 g/l; (3) streptomycin sulfate at 0.2 g/l; and (4) no bactericide application (nothing sprayed as a control). Bactericide applications were initiated two days following inoculation and continued weekly thereafter for the duration of the experiment. Samples were taken at 1 h and 1, 2, 4, 8, and 12 wk after inoculation and consisted of five leaves which were individually removed from each inoculated tree, placed into plastic bags, and transported to the laboratory on ice.

Each leaf was washed separately in 20 ml K-buffer on a rotary shaker for 1.5 h at 250 rpm, following which serial dilutions were made and plated onto MG, MGrif, and MGcu sm rif. P. syringae population sizes were expressed as $\log_{10}$ colony-forming-units (CFU) per gram leaf tissue, and the limit of detection was approximately 40 CFU/g.

Plasmid stability experiments

Following the field study, we decided to evaluate the stability of a Cu^r Sm^r plasmid which could be directly compared with the Cu^r plasmid pPSR4. Therefore, for this purpose we constructed pPSR4::Tn5393 in which streptomycin resistance is mediated by the strA/strB resistance genes contained in the 5.4-kb Sm^r transposon Tn5393 (41). Details concerning the construction of pPSR4::Tn5393 will be reported elsewhere (217). Plasmid stability experiments were designed to assess the stability of the plasmids in vitro in FF5 cells subjected to carbon limitation and grown without selection for bactericide resistance. Each plasmid/strain combination experiment was replicated twice. Bacterial inoculum grown on HS medium amended with cupric sulfate or streptomycin was suspended in K-buffer, and approximately 10^6 cells were inoculated into 20 ml of HSg. Cells were incubated at 25°C on a rotary shaker at 260 rpm. After each 24-h incubation period, 0.2 ml of culture was inoculated into 19.8 ml of fresh medium. The 100-fold daily increase in cell numbers corresponded to 6.64 doublings. In the present study, stability experiments were conducted for 31 days (approximately 206 doublings or generations). At intervals equivalent to 20 generations, serial dilutions were plated onto MG, MGcu, MGsm, and MGcu sm as

appropriate. Also, at each sampling interval, 50 colonies were replica plated from MG to MGcu, MGsm, or MGcu sm, and plasmid DNA was isolated from five colonies as previously described (55) to assess plasmid stability.

Competition experiments

In vitro competition experiments were set up between the Cm^r derivative FF5.2 and FF5 containing pPSR4, pPSR5, or pPSR4::Tn5393. FF5.2 was utilized because the Cm^r marker enabled us to monitor plasmid transfer by plating cells on MGcu cm, MGsm cm, or MGcu sm cm and to examine the plasmid content of any cells arising on these media. Plasmid loss was also monitored by screening for the Cu^r and Sm^r markers in Cm^s FF5 cells. A second series of competition experiments involved the substitution of wild-type FF5 as the plasmid- free strain. In these experiments, populations of the wild-type FF5 and plasmid-bearing cells were monitored by initially plating to a nonselective medium with subsequent screening on the appropriate selective medium.

Prior to the competition experiments, all strains were grown to stationary phase in 5 ml of HSg broth. 0.1 ml of plasmid-containing and plasmid-free strains were mixed and inoculated into 20 ml HSg broth. The experiments were conducted using the batch culture methods described for the plasmid stability experiments. The experiments consisted of two repetitions and were repeated twice. Total cells and cells carrying the appropriate plasmid markers were first enumerated 24 h after inoculation (6.64 generations), and subsequently every 48 h (13.32 generations) until the experiment was terminated at 15 days.

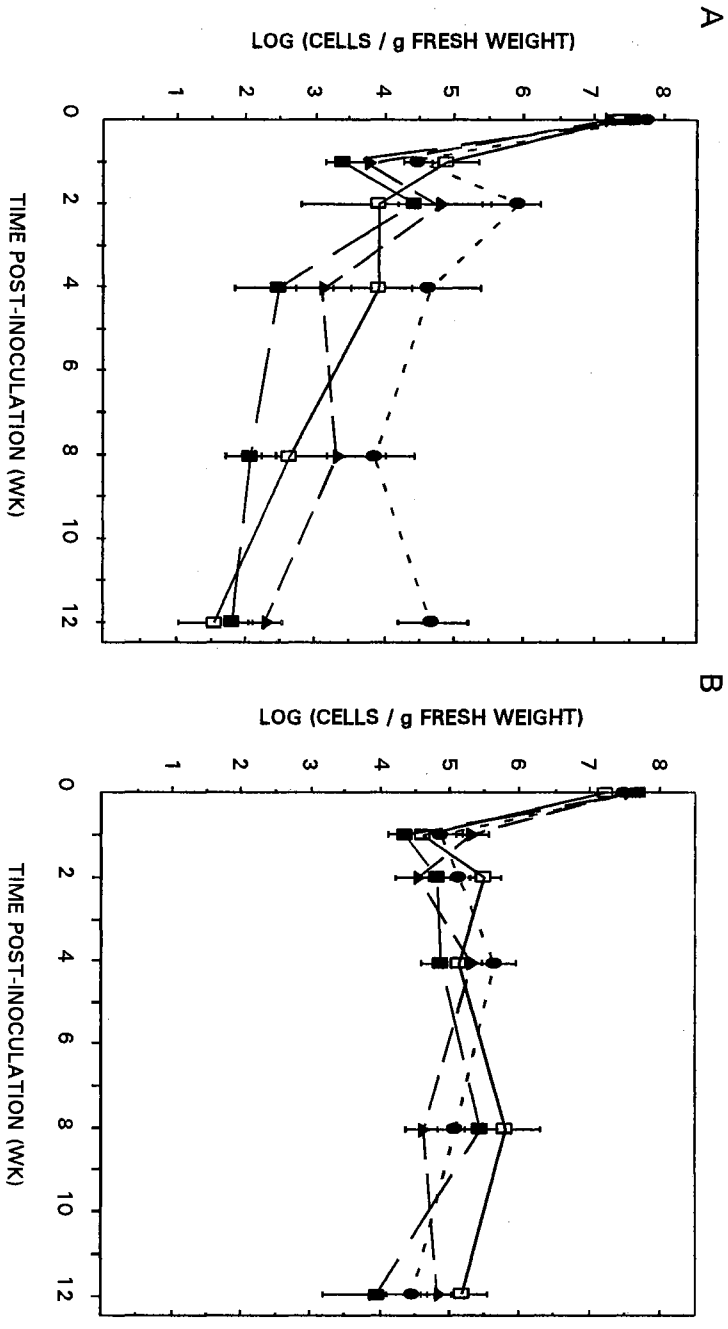
Competition experiments between strains were also conducted using bean plants (Phaseolus vulgaris cv. Blue Lake). Prior to inoculation, bean plants were grown on a greenhouse bench until the first trifoliate leaf was expanded, and care was taken to minimize the wetting of leaf surfaces during watering. Bacterial inoculum was grown for 36 h on KB medium, cells were then suspended in K-buffer, and cell densities were adjusted turbidimetrically to approximately 10^6 cfu/ml. Inoculum of competing strains was mixed in a 1:1 ratio and sprayed on the upper and lower surfaces of bean leaves using an air brush (Model 200; Badger, Franklin Park, Ill.) at 8 psi until leaf surfaces were evenly coated with the cell suspension. Sixteen to twenty plants were inoculated per experiment. The plants were incubated in a growth chamber at 24°C under high light intensity for 12 h per day with intermittent misting. Cell counts of leaf populations were determined by removing eight randomly-selected leaves from the group of inoculated plants at each sampling time. Leaves were washed individually in 20 ml K-buffer for 1.5 h on a rotary shaker at 250 rpm, and serial dilutions of the leaf washings were plated on MG and MGcu, MGsm, or MGcu sm to enumerate bacterial colonies.

RESULTS

Population dynamics of FF5.1 and FF5.1(pPSR1) on ornamental pear leaves

Both FF5.1 and FF5.1(pPSR1) colonized pear leaves on inoculated trees in all blocks (Fig. 9). An initial reduction in population size of the inoculated strains was observed in all blocks during the first week of sampling. Subsequently, in the unsprayed control blocks, slight increases in the population of the inoculated strains

Fig. 9. Populations of Pseudomonas syringae pv. syringae FF5.1 [panel **A**] and FF5.1(pPSR1) [panel **B**] on nontreated Pyrus calleryana leaves (●); leaves treated weekly with $Cu(OH)_2$ at 2.4 g/l and streptomycin sulfate at 0.2 g/l (■); leaves treated weekly with $Cu(OH)_2$ at 2.4 g/l (□); and leaves treated weekly with streptomycin sulfate at 0.2 g/l (▲). The bactericide spray program was initiated two days following inoculation. The vertical bars represent the standard error of the mean $\log_{10}$ bacterial population sizes.


A
LOG (CELLS / g FRESH WEIGHT)
1
2
3
4
5
6
7
8
TIME POST-INOCULATION (WK)
0
2
4
6
8
10
12
B
LOG (CELLS / g FRESH WEIGHT)
1
2
3
4
5
6
7
8
TIME POST-INOCULATION (WK)
0
2
4
6
8
10
12

were observed, and the populations then stabilized at levels typically observed for P. syringae pv. syringae on Pyrus calleryana in Oklahoma (Fig. 9A, 9B, ●; Chapter IV). However, by week 4, populations of the plasmid-free strain FF5.1 were reduced from 10 to 300-fold on bactericide-sprayed trees (Fig. 9A, ■, □, ▲) compared to populations of FF5.1 on unsprayed trees (Fig. 9A, ●). This trend continued through the conclusion of the experiment (week 12), when the population of FF5.1 on bactericide-sprayed trees was reduced by 100 to 1000-fold relative to the population of the same strain on unsprayed trees (Fig. 9A). Conversely, populations of FF5.1(pPSR1) on bactericide-sprayed trees (Fig. 9B, ■, □, ▲) were similar to or exceeded those of FF5.1(pPSR1) on unsprayed trees (Fig. 9B, ●). Furthermore, populations of FF5.1(pPSR1) [Fig. 9B, ●] were similar to or exceeded populations of FF5.1 on unsprayed trees (Fig. 9A, ●) during the 12-wk duration of the experiment.

Plasmid stability

pPSR4, pPSR5, and pPSR4::Tn5393 were highly stable in P. syringae pv. syringae FF5 for over 200 generations in vitro. Bacterial counts on MGcu, MGsm, and MGcu sm were similar throughout the experiment to counts on nonselective MG medium for FF5 containing pPSR4, pPSR5, and pPSR4::Tn5393, respectively. We did not detect any plasmid-free segregants when individual colonies were transferred from MG to MGcu, MGsm, or MGcu sm. Plasmids isolated from individual colonies at the sampling intervals did not differ in size from the wild-type plasmids.

In vitro competition experiments

Initially, competition experiments involved the spontaneous Cm^r mutant FF5.2 in

combination with FF5(pPSR4), FF5(pPSR5), or FF5(pPSR4::Tn5393) to determine if the plasmids were stable and whether there was evidence of plasmid transfer to FF5.2 under the conditions of the experiment. Each plasmid was stable in FF5; Cm^s plasmid-free cells were not detected at any time during these competition experiments. Selection for Cm^r exconjugants indicated that they did not exceed 0.0004% during experiments involving pPSR4, and exconjugants were not detected in experiments involving pPSR5 or pPSR4::Tn5393.

In vitro competition experiments were then conducted utilizing the wild-type FF5 as the plasmid-free strain (Fig. 10). Interestingly, the proportion of FF5 plasmid-free cells (Fig. 10A, B, C; ●) exceeded 95% of the total population after 100 generations regardless of the competing strain. The populations of the plasmid-containing cells (Fig. 10A, □; B, ▲; C, ■) remained consistently lower than the FF5 population throughout the course of all experiments. However, the plasmid-containing strains were never eliminated and were present at approximately 2% of the total population at the termination of the study. Each experiment was repeated with similar results.

In planta competition experiments

Experiments designed to evaluate epiphytic fitness were also performed with FF5 in competition with FF5 (pPSR4), FF5(pPSR5), or FF5(pPSR4::Tn5393). Bean, which is commonly used as an epiphytic host for P. syringae pv. syringae, was utilized in all experiments. Throughout the 11-day duration of these experiments, populations of plasmid-containing (□, ▲, ■) and plasmid-free strains (●) were very similar (Fig. 11A, B, C). There was no evidence of inhibition between competing

Fig. 10. Competition between plasmid-containing and plasmid-free strains of Pseudomonas syringae pv. syringae in a glucose-limited minimal medium. The plasmid-free strain FF5 (●) was competed with (**A**) FF5(pPSR4), □; (**B**) FF5(pPSR5), ▲; and (**C**) FF5(pPSR4::Tn5393), ■.

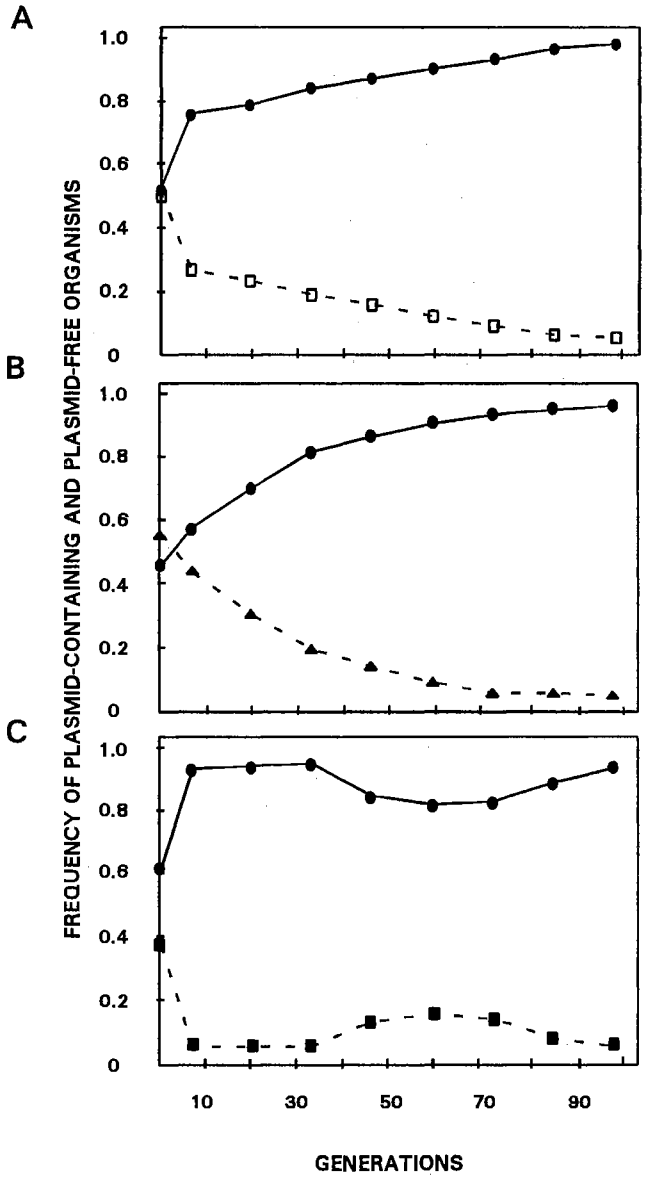
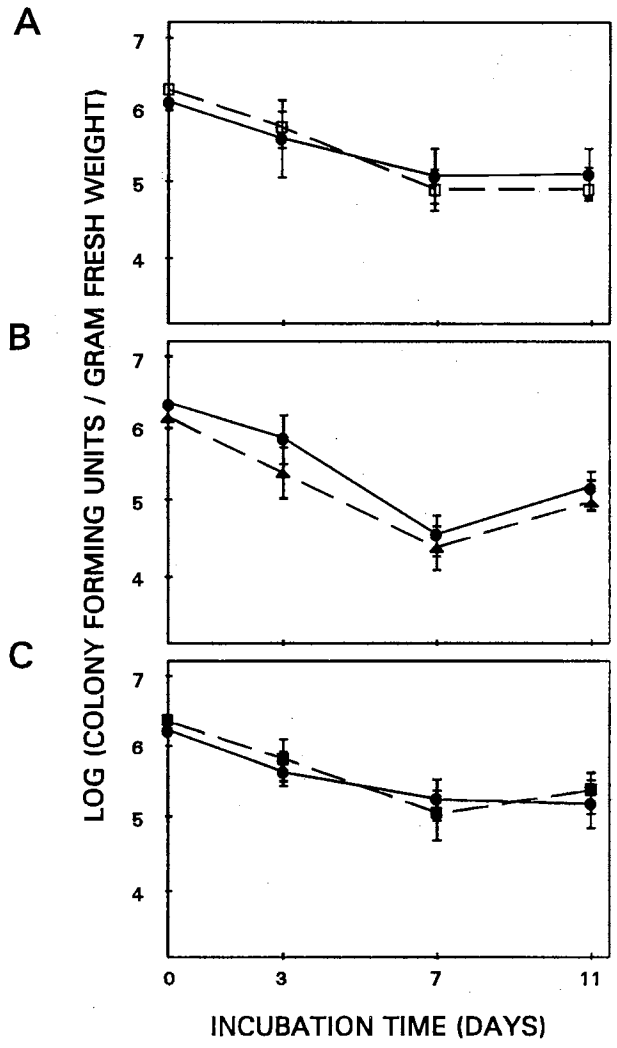

A
B
C
1.0
0.8
0.6
0.4
0.2
0
FREQUENCY OF PLASMID-CONTAINING AND PLASMID-FREE ORGANISMS
10
30
50
70
90
GENERATIONS

Fig. 11. Competition between plasmid-containing and plasmid-free strains of Pseudomonas syringae pv. syringae grown epiphytically on the surfaces of bean leaves. The plasmid-free strain FF5 (●) was competed against (**A**) FF5(pPSR4), □; (**B**) FF5(pPSR5), ▲; and (**C**) FF5(pPSR4::Tn5393), ■.


A
B
C
7
6
5
4
LOG (COLONY FORMING UNITS / GRAM FRESH WEIGHT)
0
3
7
11
INCUBATION TIME (DAYS)

strains because populations generally increased or decreased uniformly (Fig. 11A, B, C). The experiments were repeated with similar results.

DISCUSSION

To our knowledge, this is the first report addressing the effect of indigenous bactericide-resistance plasmids on competitive fitness of a phytopathogenic bacterium. The presence of the Cu^r Sm^r plasmid pPSR1 provided a selective advantage to FF5.1 on Pyrus calleryana trees sprayed with copper and streptomycin bactericides. The colonization of FF5.1(pPSR1) remained high on sprayed trees, but populations of FF5.1 declined about 10 to 1000-fold on trees treated with bactericides (Fig. 9A). Furthermore, we found that pPSR1 did not affect the ability of FF5.1 to colonize P. calleryana trees in the absence of bactericidal sprays (Fig. 9B), thus corroborating results obtained with other P. syringae strains containing different Cu^r plasmids (46,218).

The resistance plasmids pPSR4, pPSR5, and pPSR4::Tn5393 were highly stable in P. syringae pv. syringae FF5 in the absence of selection pressure. After introduction into strain FF5, each plasmid persisted for over 200 generations in batch culture grown under conditions of carbon limitation. An important factor in the stability of large, low-copy-number plasmids is the presence of genes encoding stability or partition functions (8). We recently cloned the vegetative origin (oriV) and a stability determinant from pPSR1 (217). A clone containing these functions hybridized to pPSR4, pPSR5, pPSR4::Tn5393, and pOSU22, a clone containing the replication region from pOSU900, an indigenous plasmid in P. syringae pv. syringae

J900 (167,217). However, the relatedness of pPSR4 and pPSR5 in regions flanking the origin of replication has not been firmly established. Interestingly, pOSU22 and other derivatives containing the pOSU900 replication region were highly stable in P. syringae in the absence of selection pressure (167). The conservation of the pOSU900 replication region in P. syringae strains isolated in Oklahoma strongly suggests that these plasmids have evolved replication functions which favor their stability in the absence of selection pressure.

Competition experiments in a minimal medium containing glucose in limiting concentrations indicated that pPSR4, pPSR5, and pPSR4::Tn5393 had a negative impact on competitive fitness of P. syringae pv. syringae. However, FF5 containing pPSR4, pPSR5, or pPSR4::Tn5393 persisted and was never completely eliminated from the cultures during any experiment.

An important phase in the life cycle of P. syringae is its growth as an epiphyte on host and nonhost plant surfaces (99). Although physical forces facilitate leaf colonization, bacterial traits such as motility and adherence to leaf surfaces are important as well (93,183). In natural environments, epiphytic P. syringae cells compete for survival with other P. syringae genotypes and various other components of the microbial flora. Competition for limiting nutrients and occupation of colonization sites on leaf surfaces are thought to play an important role in the growth and survival of closely-related epiphytic bacteria (21). However, other factors such as exclusion of competing strains and dissemination in the natural environment are also important in the success of a particular P. syringae genotype. In biological

control studies, organisms which function as competitors can inhibit target P. syringae strains through competitive exclusion, but cannot reduce established epiphytic strains (137,139,140). Our studies indicate that indigenous resistance plasmids in P. syringae pv. syringae do not compromise their host by reducing epiphytic fitness. Therefore, successful colonization of plant surfaces by plasmid-containing P. syringae is likely to be followed by persistence of these strains in the phylloplane. Furthermore, we have recovered P. syringae strains containing Cu^r Sm^r plasmids from P. calleryana buds, suggesting that these resistance plasmids do not prevent P. syringae from overwintering in the pear host (217).

The evolution of resistance to copper and streptomycin in P. syringae pv. syringae has apparently involved the dissemination of distinct resistance plasmids among many genotypes (215,Chapter III,IV). Colonization of plants by a specific genotype of P. syringae is strongly influenced by the indigenous P. syringae genotypes resident in the area (100). The heterogeneity of genotypes colonizing plants in a particular area may reflect strains which possess selective advantages during different environmental conditions or strains which are enhanced in dissemination or colonization of new hosts. A percentage of these P. syringae strains contain indigenous plasmids (52,176,230) which may acquire resistance genes after cells are exposed to copper and streptomycin sprays. Furthermore, these resistance plasmids may be conjugally transferred into other P. syringae genotypes, and may ultimately affect the stability of these plasmid genotypes in areas where bactericide use is discontinued.

Results obtained in the present study indicate that resistance plasmids and the strains which harbor them may remain associated with tree hosts even if bactericide selection is withdrawn. The location of unique plasmids in diverse genetic backgrounds and the diversity of resistance plasmids present in these P. syringae pv. syringae populations suggest that long-term maintenance of these plasmids in the population is possible. Also, the lack of negative effects on competitive fitness indicates that these resistance plasmids may harbor other genetic determinants which are beneficial to P. syringae in its ecological niche.

CHAPTER VI

DETECTION OF IS6100 WITHIN THE STREPTOMYCIN-RESISTANCE TRANSPOSON Tn5393 IN XANTHOMONAS CAMPESTRIS

SUMMARY

Resistance to streptomycin in the phytopathogen Xanthomonas campestris pv. vesicatoria is encoded by the strA/strB aminoglycoside phosphotransferase genes which are located on Tn5393. The nucleotide sequence of a region within Tn5393 upstream of the strA/strB genes was determined. An 880 bp sequence, identical to that of the insertion sequence IS6100, was detected 33 bp within the resolvase (tnpR) gene of Tn5393. IS6100, a member of the IS6 family, has previously been found on Tn610 in Mycobacterium fortuitum, and on nylon-degrading plasmids in Flavobacterium sp. and Pseudomonas sp. Codon usage within the putative transposase gene of IS6100 was compared to codon frequency tables generated for X. campestris and Mycobacterium spp., and to existing codon frequency tables for Bacillus subtilis, Pseudomonas spp. and Streptomyces spp. Comparative analysis using the computer program Correspond indicated that codon usage within IS6100 was most similar to that of X. campestris. The nucleotide sequence identity of

IS6100 in diverse organisms, combined with the similarity of codon usage of the putative transposase gene with that of gram-negative bacteria, suggests this IS element has been recently disseminated from gram negative to gram positive bacteria.

INTRODUCTION

The aminoglycoside antibiotic streptomycin has been utilized to control bacterial plant diseases in many regions of the world. Streptomycin-resistant strains of the phytopathogen X. campestris pv. vesicatoria have been isolated in four continents (163a). Streptomycin resistance in some of these strains is conferred by plasmid-encoded genes (163a) which were shown to be homologs of the strA/strB aminoglycoside phosphotransferase genes that are found on small, nonconjugative plasmids such as RSF1010 (198,215). In three genera of phytopathogenic bacteria, the strA/strB genes are encoded by Tn5393, a member of the Tn3 family (41,Chapter VII). In Tn3-type transposons, the transposase (tnpA) and resolvase (tnpR) genes are transcribed divergently from promoters located on opposite DNA strands within a central recombination site (res) (200). In Erwinia amylovora, Tn5393 is 6.7 kb and contains an insertion sequence (IS) element, IS1133, upstream of strA (Fig. 12) (41). Deletion analysis suggested the expression of Sm^r is controlled by a promoter sequence located near the end of IS1133 (41). Regions within Tn5393 upstream of strA have not been previously mapped in P. syringae or X. campestris pv. vesicatoria. Nucleotide sequence analysis of this region from X. campestris pv.

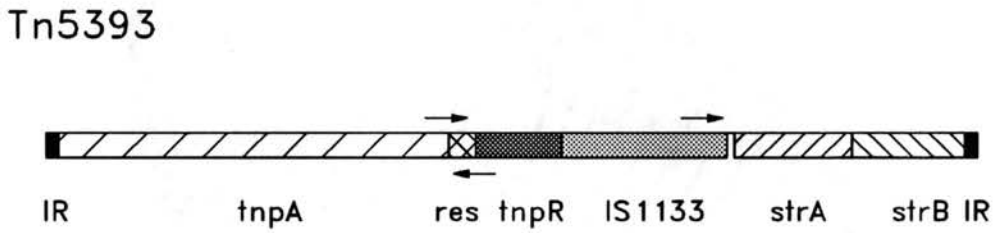


Fig. 12. Structure of the streptomycin-resistance (Sm^r) transposon Tn5393 (Chiou and Jones, 1993). In Erwinia amylovora, IS1133 is inserted downstream of the transposase (tnpR) gene and upstream of the strA Sm^r gene. Putative promoter sequences are identified by arrows.

vesicatoria indicated that the IS element IS6100 was inserted within the tnpR gene of Tn5393.

Table 11. Bacterial strains and plasmids and their relevant characteristics.

Strains, plasmids	Relevant characteristics	Reference
Bacterial strains[a]		
Escherichia coli		
DH5α		147
Xanthomonas campestris pv. vesicatoria		
BV5-4a	Sm^R; strA/strB on 68-kb plasmid	163a
79-2	Sm^R; strA/strB on 68-kb plasmid	163a
Plasmids[b]		
pBluescript SK+	Ap^R	Stratagene
pGWS3	3.7-kb EcoRV of 68-kb Sm^R plasmid plasmid from BV5-4a in pBluescript SK+	This study
pGWS4	2.2-kb HindIII of pGWS3 in pBluescript SK+	This study

[a] LB medium (Maniatis et al 1982) was used for cultivation of E. coli DH5α. Strains of X. campestris pv. vesicatoria were maintained on nutrient agar (Difco; Detroit, MI). Antibiotics were added to media at the following concentrations (μg/ml): Sm, 25; and Ap, 40.

[b] Indigenous plasmids from X. campestris pv. vesicatoria BV5-4a were purified on CsCl gradients, digested with EcoRV, electrophoresed on an agarose gel, blotted, and hybridized with a 1.5-kb SstI - EcoRV fragment from RSF1010 containing an internal fragment of strA/strB (198). A 3.7-kb EcoRV fragment which hybridized to the probe, was isolated by electroelution, ligated into the EcoRV site of pBluescript SK+ creating the recombinant plasmid pGWS3, and introduced into E. coli DH5α by transformation. Dideoxy sequencing of both strands of DNA was done using Sequenase (U.S. Biochemicals; Cleveland, OH) following the manufacturer's instructions.

RESULTS AND DISCUSSION

(a) Detection of IS6100 within Tn5393 in X. campestris pv. vesicatoria

Nucleotide sequence analysis of approximately 2.0 kb of Tn5393 upstream of the strA gene from X. campestris pv. vesicatoria BV5-4a indicated that the sequence was identical to that in P. syringae (41) except for the presence of an 888 bp sequence 33 bp within tnpR (Fig. 13A). A homology search of the EMBL database indicated that 880 bp of this sequence was identical to that of IS6100 which was also previously found on Tn610 from Mycobacterium fortuitum (153) and on the nylon-degrading plasmid pOAD2 in Flavobacterium sp. (106). Southern hybridization analyses indicated that sequences homologous to IS6100 were present on pNAD2, a nylon-degrading plasmid in Pseudomonas sp. (106). IS6100 is a member of the IS6 family of insertion sequences which are widespread within both gram-negative and gram-positive bacteria (82). An 8-bp duplication was detected at the insertion site in X. campestris pv. vesicatoria BV5-4a, which is characteristic of insertion of IS elements in the IS6 group (82). X. campestris pv. vesicatoria 79-2, a strain isolated eight years prior to BV5-4a from the same location in Argentina (163a), also contained IS6100 within Tn5393 on a 68-kb plasmid (data not shown). The insertion of IS6100 upstream of strA (Fig. 13B) was shown to increase the level of expression in X. campestris pv. vesicatoria when a fragment containing IS6100 was cloned in front of a promoterless β-glucuronidase gene (Chapter VII).

Alignments of amino acid sequences of the putative transposases in the IS6 family

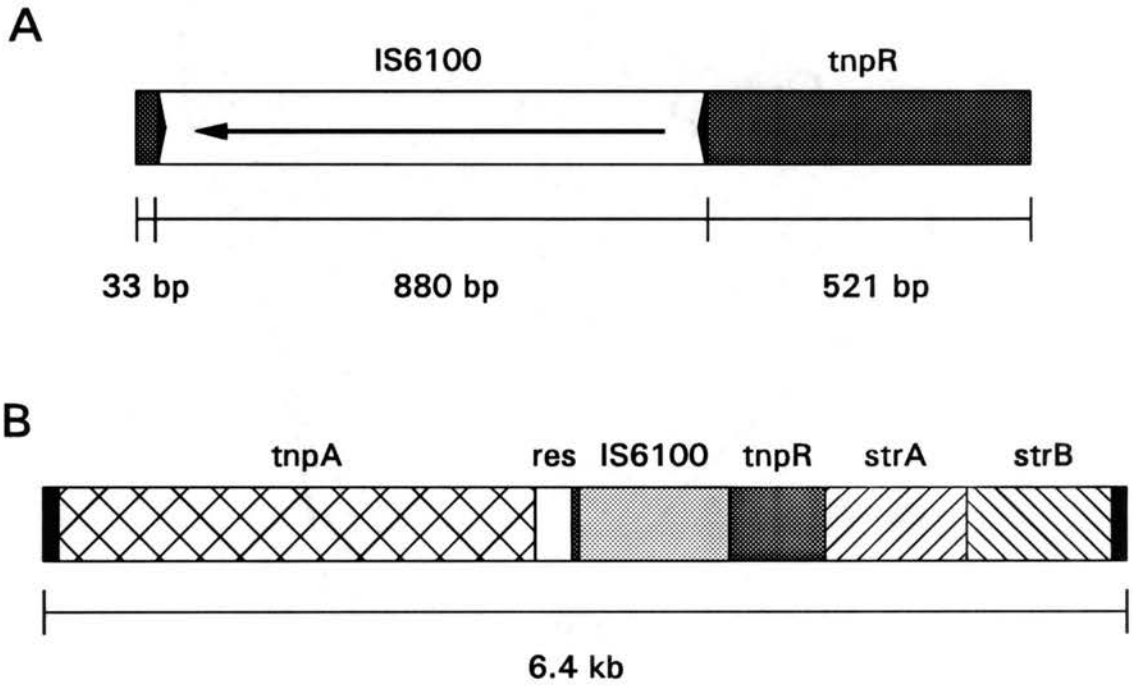


Fig. 13. (A) Characterization of the region within Tn5393 upstream of strA in Xanthomonas campestris pv. vesicatoria. An 880-bp sequence, identical to that of IS6100 is inserted within the tnpR gene. The 14-bp inverted repeats are indicated by arrows. The putative transposase gene of IS6100 is indicated by the line with the direction of transcription indicated by the arrow. (B) Putative physical map of Tn5393 within X. campestris pv. vesicatoria. The strA/strB genes were mapped previously (Sundin and Bender, 1993). The region sequenced included all of res, IS6100, tnpR, and 420 bp of tnpA upstream of res.

have been reported previously (106,153,180,189). Most of these elements flank antibiotic-resistance determinants and form compound transposons. Furthermore, IS15, IS26, and IS140 provide promoter sequences which activate adjacent genes (3,27,128a). Transcriptional activation by these IS elements is similar to other examples of IS-activated expression (82), with the IS providing a -35 region near its terminus and generating a hybrid promoter with an indigenous -10 region. In these cases, as also observed with other examples of IS-activated expression (82), the IS provides a -35 region in its outward end, creating a hybrid promoter upon insertion with an indigenous -10 region. However, the location of IS6100 within tnpR did not create a hybrid promoter; instead, promoter sequences increasing the expression of streptomycin resistance in X. campestris pv. vesicatoria were located within IS6100 (Chapter VII).

(b) Codon usage analysis of the putative transposase protein of IS6100

Martin et al. (153) suggested that IS6100 originated from Mycobacterium and cited similarity in the codon usage of the putative transposase gene in IS6100 and the M. bovis 65 kDa antigen protein. To more fully address the origin of IS6100, we used the program Correspond (90) from the University of Wisconsin Genetics Computer Group (UWGCG) to compare codon usage in the putative transposase of IS6100 with codon usage tables from Bacillus subtilus (201), Pseudomonas spp. (D.K. Willis, personal communication) and Streptomyces spp. (243). Additionally, we generated codon usage tables of 37 coding sequences from X. campestris and 16 coding sequences from Mycobacterium spp. from the GenBank database (Table 12)

TABLE 12. Codon usage of the putative transposase gene of IS6100, and 37 and 16 coding sequences of Xanthomonas campestris and Mycobacterium spp., respectively[a].

		IS6100		Xanthomonas		Mycobacterium	
AmAcid	Codon	No.[b]	/1000[c]	No.	/1000	No.	/1000
Gly	GGG	1.00	3.92	140.00	8.61	103.00	17.62
Gly	GGA	4.00	15.69	58.00	3.57	52.00	8.90
Gly	GGT	3.00	11.76	215.00	13.22	125.00	21.39
Gly	GGC	10.00	39.22	1159.00	71.28	362.00	61.93
Glu	GAG	8.00	31.37	354.00	21.77	267.00	45.68
Glu	GAA	7.00	27.45	313.00	19.25	106.00	18.14
Asp	GAT	6.00	23.53	331.00	20.36	94.00	16.08
Asp	GAC	6.00	23.53	458.00	28.17	272.00	46.54
Val	GTG	8.00	31.37	792.00	48.71	247.00	42.26
Val	GTA	0.00	0.00	43.00	2.64	22.00	3.76
Val	GTT	0.00	0.00	94.00	5.78	52.00	8.90
Val	GTC	5.00	19.61	250.00	15.38	214.00	36.61
Ala	GCG	6.00	23.53	635.00	39.06	236.00	40.38
Ala	GCA	5.00	19.61	237.00	14.58	51.00	8.73
Ala	GCT	2.00	7.84	119.00	7.32	64.00	10.95
Ala	GCC	12.00	47.06	1013.00	62.30	334.00	57.14
Lys	AAG	17.00	66.67	321.00	19.74	212.00	36.27
Lys	AAA	3.00	11.76	78.00	4.80	31.00	5.30
Asn	AAT	2.00	7.84	149.00	9.16	17.00	2.91
Asn	AAC	2.00	7.84	291.00	17.90	163.00	27.89
Met	ATG	6.00	23.53	85.00	16.03	92.00	15.74
Ile	ATA	1.00	3.92	7.00	1.32	7.00	1.20
Ile	ATT	2.00	7.84	31.00	5.84	35.00	5.99
Ile	ATC	10.00	39.22	165.00	31.11	190.00	32.51
Thr	ACG	9.00	35.29	328.00	20.17	75.00	12.83
Thr	ACA	0.00	0.00	67.00	4.12	18.00	3.08
Thr	ACT	0.00	0.00	72.00	4.43	26.00	4.45
Thr	ACC	5.00	19.61	652.00	40.10	239.00	40.89
Trp	TGG	10.00	39.22	205.00	12.61	72.00	12.32
End	TGA	1.00	3.92	29.00	1.78	11.00	1.88
Cys	TGT	1.00	3.92	10.00	0.62	9.00	1.54
Cys	TGC	2.00	7.84	132.00	8.12	36.00	6.16
End	TAG	0.00	0.00	0.00	0.00	2.00	0.34
End	TAA	0.00	0.00	8.00	0.49	2.00	0.34
Tyr	TAT	7.00	27.45	104.00	6.40	19.00	3.25
Tyr	TAC	5.00	19.61	255.00	15.68	96.00	16.42
Phe	TTT	2.00	7.84	75.00	4.61	16.00	2.74
Phe	TTC	8.00	31.37	297.00	18.27	134.00	22.93

Table 12 (continued)

AmAcid	Codon	IS6100 No.[b]	IS6100 /1000[c]	Xanthomonas No.	Xanthomonas /1000	Mycobacterium No.	Mycobacterium /1000
Ser	AGT	0.00	0.00	116.00	7.13	16.00	2.74
Ser	AGC	4.00	15.69	503.00	30.94	94.00	16.08
Ser	TCG	4.00	15.69	245.00	15.07	113.00	19.33
Ser	TCA	0.00	0.00	44.00	2.71	22.00	3.76
Ser	TCT	0.00	0.00	36.00	2.21	8.00	1.37
Ser	TCC	0.00	0.00	161.00	9.90	61.00	10.44
Arg	AGG	1.00	3.92	38.00	2.34	13.00	2.22
Arg	AGA	1.00	3.92	15.00	0.92	3.00	0.51
Arg	CGG	8.00	31.37	177.00	10.89	115.00	19.67
Arg	CGA	2.00	7.84	41.00	2.52	34.00	5.82
Arg	CGT	1.00	3.92	159.00	9.78	39.00	6.67
Arg	CGC	14.00	54.90	639.00	39.30	144.00	24.64
Gln	CAG	5.00	19.61	735.00	45.21	144.00	24.64
Gln	CAA	0.00	0.00	132.00	8.12	31.00	5.30
His	CAT	3.00	11.76	143.00	8.80	30.00	5.13
His	CAC	4.00	15.69	191.00	11.75	71.00	12.15
Leu	TTG	1.00	3.92	316.00	19.44	84.00	14.37
Leu	TTA	0.00	0.00	22.00	1.35	10.00	1.71
Leu	CTG	14.00	54.90	1099.00	67.59	272.00	46.54
Leu	CTA	0.00	0.00	30.00	1.85	23.00	3.93
Leu	CTT	2.00	7.84	58.00	3.57	27.00	4.62
Leu	CTC	4.00	15.69	229.00	14.08	85.00	14.54
Pro	CCG	6.00	23.53	548.00	33.70	155.00	26.52
Pro	CCA	0.00	0.00	86.00	5.29	29.00	4.96
Pro	CCT	1.00	3.92	75.00	4.61	23.00	3.93
Pro	CCC	4.00	15.69	196.00	12.05	96.00	16.42

[a]Total number of codons: IS6100, 255; Xanthomonas, 15,606; Mycobacterium, 5,845.

[b]The frequency each codon occurred in the examined sequences.

[c]The frequency a specific codon would occur per 1000 codons.

using the UWGCG program CodonFrequency (69). The formula used in analysis by Correspond is:

$$D^2 = [\text{Frequency}_{(\text{codon table 1})} - \text{Frequency}_{(\text{codon table 2})}]^2$$

The D^2 statistic decreases as patterns of codon usage become more similar. Analysis using Correspond indicated a greater similarity between the codon usage of the putative transposase of IS6100 and that of the two gram-negative genera (Xanthomonas and Pseudomonas) than the three gram-positive genera (Bacillus, Mycobacterium and Streptomyces) (Table 13).

TABLE 13. Correspond analysis of codon usage tables from the putative transposase gene of IS6100 with codon usage tables from five bacterial genera[a]

Organism	D^2
Xanthomonas campestris	0.952
Pseudomonas spp.	1.128
Mycobacterium spp.	1.577
Streptomyces spp.	2.796
Bacillus subtilis	3.984

[a] The values reported are the D^2 statistic which was calculated using the UWGCG program Correspond (Grantham et al., 1981).

The presence of identical copies of IS6100 among such taxonomically distinct genera as Flavobacterium, Mycobacterium, and Xanthomonas suggests this element has been disseminated both widely and recently among gram-negative and gram-

positive bacteria. Although it was once considered unlikely that conjugal transfer of plasmids could occur between gram-negative and gram-positive bacteria, many studies have demonstrated conjugation between these groups in vitro (89,127,154,155,1960 and in vivo (70). Evidence of recent natural transfer events comes from the close nucleotide sequence identity of the antibiotic-resistance genes aphA-3 and tetO in gram-positive cocci and Campylobacter spp. (224,249) and ermBC in gram-positive cocci and E. coli (27). In these cases, it was suggested that the natural transfer occurred from a gram-positive bacterium to a gram-negative bacterium. This was considered likely because antibiotic-resistance genes from gram-positive bacteria are usually expressed in gram-negative bacteria whereas the reverse is usually not true (116). In M. fortuitum, the composite transposon Tn610 consists of two copies of IS6100 flanking genes similar to the integrase and sulfonamide-resistance (sulI) gene of Tn1696, a transposon isolated from P. aeruginosa (241). Furthermore, sulI contains part of the conserved sequence from integrons, elements which are widespread in gram-negative bacteria (213). Thus, the available data regarding IS6100 suggest that this element was recently transferred from a gram-negative bacterium to Mycobacterium.

It is interesting to consider the dissemination of IS6100 among gram-negative bacteria and between gram-negative bacteria and M. fortuitum. The conjugal transfer of a shuttle plasmid, and the transfer of RSF1010 between E. coli and M. smegmatis (89,127) indicated that M. smegmatis can receive and express DNA from a gram-negative source. Recently, Mariani et al. characterized a family of IS elements

present in M. tuberculosis and soil-inhabiting plant pathogenic bacteria such as Agrobacterium tumefaciens and Rhizobium meliloti and suggested that DNA transfer among these diverse organisms may have involved the transfer of broad-host-range plasmids from an organism such as P. cepacia (150). The transfer of IS6100 to diverse organisms probably occurred in soil. M. fortuitum is a common soil organism, and the nylon-degrading Flavobacterium sp. was also isolated from soil (110). Furthermore, gram-negative bacteria containing Tn5393 are indigenous to soil in some regions (170). Our data suggest the transposition of IS6100 into Tn5393 probably occurred within a gram-negative organism similar to X. campestris which acquired Tn5393 and harbored IS6100 in another genomic location.

(c) Conclusions

(1) IS6100, a member of the IS6 family which is widely distributed among gram-negative and gram-positive bacteria, was detected upstream of the Sm^r genes strA/strB within the tnpR gene of Tn5393 in X. campestris pv. vesicatoria.

(2) Analysis of the codon usage of the putative transposase gene of IS6100 indicated that the codon usage was more similar to that of X. campestris and Pseudomonas spp. than three gram-positive genera. The detection of identical copies of IS6100 in gram-negative bacteria and M. fortuitum suggests this IS element was recently disseminated from gram-negative to gram-positive bacteria.

CHAPTER VII

GENETIC ANALYSES OF SEQUENCES DIRECTING EXPRESSION OF THE strA/strB STREPTOMYCIN-RESISTANCE GENES IN PSEUDOMONAS SYRINGAE AND XANTHOMONAS CAMPESTRIS

SUMMARY

Nucleotide sequence analysis of regions upstream of the strA/strB streptomycin-resistance genes on Tn5393 indicated the resolvase/repressor gene (tnpR) and the recombination site (res) shared 100% sequence identity with the same genes from Tn5393 in Erwinia amylovora. DNA fragments upstream of strA in Pseudomonas syringae and Xanthomonas campestris were assayed for promoter activity using a β-glucuronidase expression vector. A strong promoter sequence was located within the recombination site (res) of Tn5393 in P. syringae; expression from this promoter was reduced ≈5 fold if the tnpR resolvase/repressor gene was also expressed. The transcriptional start site within res was determined using primer extension; a putative promoter sequence was identified seven bp upstream of the start site. An additional putative promoter sequence, located within strA, was also involved in the expression of streptomycin resistance in P. syringae. In X. campestris, the insertion sequence IS6100, located within tnpR upstream of strA, increased expression 46% relative to the promoter sequence within res. The additional putative promoter sequence in P.

syringae was not active in X. campestris.

The aminoglycoside antibiotic streptomycin has been utilized in agriculture since the late 1950s to control populations of phytopathogenic bacteria. The effectiveness of streptomycin, however, has been reduced due to the appearance of resistance in strains of several important phytopathogens (47). Plasmid-encoded streptomycin-resistance (Sm^r) determinants have been cloned and characterized (41,163a,170,215). Hybridization analyses indicated that the Sm^r determinant present in E. amylovora, P. syringae pv. papulans, P. syringae pv. syringae, and X. campestris pv. vesicatoria strains isolated in Michigan, New York, Oklahoma, and Argentina, respectively, were similar (215). Subsequently, this Sm^r determinant was identified as a homolog of the tandem strA/strB aminoglycoside phosphotransferase genes which are also present on small nonconjugative plasmids such as RSF1010 (198,215). In E. amylovora, the strA/strB homologs were contained on Tn5393, a 6.7-kb transposon belonging to the Tn3 family (41). Nucleotide sequence analysis demonstrated the strA/strB genes from Tn5393 shared 99.8% sequence identity with those on RSF1010 (41). Tn5393 also appears to be widely disseminated among gram-negative bacteria inhabiting plant surfaces and soil in agricultural systems in regions where streptomycin is utilized as a bactericide (170,209).

Although Sm^r E. amylovora, P. syringae, and X. campestris each contain plasmid-encoded strA/strB homologs, the minimal inhibitory concentration (MIC) of

streptomycin is distinctly different among these bacteria. Reported MICs of streptomycin are 500 μg/ml in E. amylovora (41), 75 μg/ml in P. syringae pv. syringae (215), and less than 400 μg/ml in X. campestris pv. vesicatoria (163a). The location of promoter sequences directing expression of the strA/strB genes within Tn5393 is unknown. Previous sequence analysis has shown that the transcription signals for strA/strB in RSF1010 (198) were not present in DNA upstream of the strA/strB homologs in Tn5393 (41). In E. amylovora and E. herbicola, an insertion sequence element, IS1133, was detected upstream of the strA/strB genes and deletion analysis suggested a promoter sequence present within IS1133 was responsible for expression of these genes (41). IS1133, however, was only detected in E. amylovora and E. herbicola, and was not present in a collection of diverse gram-negative bacteria known to contain Tn5393 (41). Recently, IS6100 was detected upstream of strA/strB within Tn5393 in X. campestris pv. vesicatoria (Chapter VI), although the role of IS6100 in the expression of streptomcyin resistance in this organism has not been established.

Tn5393 is similar to elements of the Tn3 subgroup of the Tn3 family and Tn2501 (91), in that the transposase (tnpA) and resolvase (tnpR) genes are transcribed divergently from two promoters located on opposite DNA strands in a 135-bp central intergenic region termed the recombination site, or res (200). In Tn3, the resolvase protein (TnpR) is a negative regulator of transcription of both tnpA and tnpR, acting as a repressor through binding to res (42,86). The ampicillin-resistance gene (bla) of Tn3 is located downstream of tnpR; transcription of bla is initiated 34 bp upstream

from the translational initiation codon from a promoter sequence located within the tnpR - bla intergenic region (37,240). In Tn5393 in P. syringae, however, it is unknown whether transcription of strA/strB is initiated within res or within the 65 bp intergenic region between tnpR and strA. The relatively low MIC of streptomycin in P. syringae (75 μg/ml) indicated the possibility that strA and strB could be transcribed along with tnpR from the tnpR promoter, which is normally repressed.

Our objective in this study was to analyze the expression of strA/strB in P. syringae pv. syringae and X. campestris pv. vesicatoria. In this study, we characterized two putative promoter sequences involved in the expression of the strA/strB genes in P. syringae pv. syringae. We also determined the role of IS6100 in the expression of the strA/strB genes in X. campestris pv. vesicatoria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids and their relevant characteristics are listed in Table 14. Plasmids were propagated in E. coli DH5α or HB101 prior to transfer into P. syringae pv. syringae FF5. E. coli strains were cultured in LB medium (162) at 37 C; P. syringae, and X. campestris were grown in medium B of King et al (109), and nutrient agar (NA) (Difco; Detroit, MI), respectively at 28 C. Following triparental matings, P. syringae recipients were selected on mannitol-glutamate medium (108). The antibiotics ampicillin, spectinomycin, and streptomycin were added to media at concentrations of 40, 25, and 25 μg/ml respectively.

Cloning and sequencing of regions of Tn5393 from P. syringae. A cosmid

TABLE 14. Bacterial strains and plasmids and their relevant characteristics.

Strains, plasmids	Relevant characteristics	Reference
Bacterial strains[a]		
Escherichia coli		
DH5α		147
HB101		147
Pseudomonas syringae pv. syringae		
FF5	wild-type, no detectable plasmids	215
A2	Cu^r Sm^r; pPSR1	215
Xanthomonas campestris pv. vesicatoria		
BV5-4a	Sm^r; strA/strB on 68-kb plasmid	163a
XV16	wild-type, no detectable plasmids	84
Plasmids		
pBluescript SK+	Ap^r	Stratagene
pLAFR3	IncP1, Tc^r, Mob^+	212
pRG960sd	IncP, Sm/Sp^r, Mob^+, promoterless uidA and Shine and Dalgarno sequence	226
pRK2013	Mob^+, Km^r, Tra RK2	79
pPSR1	Cu^r Sm^r, Tn5393	215
pPSR1.7	pLAFR3 containing a 22-kb Sau3A pPSR1 fragment ligated into the BamHI site	This study
pPSR5	Sm^r, Tn5393	215
pSM1	3.7-kb PstI of pPSR1 in pBluescript SK+	215
pSM7	2.8-kb EcoRV of pPSR1.7 in pBluescriptSK+	This study
pGWS1	4.5-kb EcoRI of pPSR1.7 in pBluescriptSK+	This study
pGWS2	3.7-kb EcoRV of 68-kb Sm^r plasmid	

Table 14 (continued)

Strains, plasmids	Relevant characteristics	Reference
	from BV5-4a in pBluescript SK+; T7 orientation	This study
pGWS3	3.7-kb EcoRV of 68-kb Sm^r plasmid from BV5-4a in pBluescript SK+; T3 orientation	This study
pGWS4	2.2-kb HindIII of pGWS3 in pBluescript SK+	This study
pGWS6	0.4-kb SmaI - EcoRV of pGWS1 in pRG960sd	This study
pGWS8	1.45-kb RsaI of pSM7 in pRG960sd	This study
pGWS9	0.9-kb SmaI - RsaI of pSM1 in pRG960sd	This study
pGWS15	1.2-kb EcoRV - SspI of pGWS1 in pRG960sd	This study
pGWS20	0.9-kb NruI of pGWS3 in pRG960sd	This study
pGWS21	0.6-kb EcoRV - SspI of pGWS4 in pRG960sd	This study
pGWS32	2.0-kb EcoRV - SstI of pGWS2 in pRG960sd	This study

library was constructed from the 68-kb Cu^r Sm^r plasmid pPSR1 isolated from P. syringae pv. syringae A2. pPSR1 was partially digested with Sau3A generating fragments approximately 20-kb in size; these fragments were ligated into pLAFR3, which was prepared as described previously (212). Cosmids were packaged using an in vitro packaging kit (Boehringer Mannheim; Indianapolis, Ind.), and introduced into E. coli HB101 by transfection according to the instructions of the manufacturer. Cosmid clones were mobilized into the wild-type strain P. syringae pv. syringae FF5 via triparental matings using the helper plasmid pRK2013 as previously described

(79); selection of recipients was aided by the intrinsic ampicillin resistance of FF5. pPSR1.7, a cosmid clone which expressed streptomycin resistance in FF5, was utilized to construct a restriction map of Tn5393 from pPSR1. Subclones from pPSR1.7 were constructed in pBluescript SK+, and nucleotide sequence analysis of both DNA strands was done using Sequenase 2.0 (U.S. Biochemical; Cleveland, OH) following the instructions of the manufacturer.

Construction and analysis of strA-uidA fusions. Clones consisting of various regions of DNA upstream from strA/strB in P. syringae and X. campestris were constructed by ligating fragments into the promoter probe vector pRG960sd. In some cases, fragments were first cloned into pBluescript SK+, and adjacent blunt end restriction sites from the polylinker were utilized for cloning into pRG960sd. Clones were mobilized into P. syringae pv. syringae FF5 and X. campestris pv. vesicatoria XV16 via triparental mating and electroporation (84), respectively. Prior to processing, cells were grown overnight in KB and nutrient broth, respectively, containing spectinomycin.

β-glucuronidase (GUS) activity in bacteria grown in liquid medium was determined fluorometrically by monitoring the ability of bacterial lysates to catalyze the hydrolysis of 4-methylumbelliferyl glucuronide (102). Late-logarithmic phase cultures were harvested, suspended in GUS extraction buffer (102), and lysed by sonication. GUS activity, reported as specific activity (units per milligram protein) was determined essentially as previously described (244). Methylumbelliferone was estimated using a Fluoroskan spectrofluorometer (ICN). Protein in the bacterial

lysates was determined by the procedure of Bradford (26). Lysates of P. syringae pv. syringae FF5 and X. campestris pv. vesicatoria XV16 containing pRG960sd were utilized as negative controls in each experiment.

Analysis of the transcriptional start site located within res in P. syringae pv. syringae. Total cellular RNA from 250 ml overnight cultures of P. syringae pv. syringae FF5 containing pPSR5 was isolated using a published procedure (147). Primer extension reactions were conducted in a reaction mixture containing 100 mM Tris-HCl (pH 8.3), 140 mM KCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM each of dATP, dGTP, and dTTP (Boehringer Mannheim), 10 μCi [α-^{32}P]-dCTP, 32 U RNasin (ProMega Corp.; Madison, Wisc.), and 9.6 U AMV reverse transcriptase (U.S. Biochemical). Prior to addition to the mixture, 20 μg RNA and 20 ng of the synthetic oligonucleotide primer 5'- TGTCCCGTTCGACACCTG -3' (Oklahoma State University Molecular Biology Core Facility) were mixed, boiled for 1 min, and chilled quickly on ice. The reaction mixture was incubated at 42 C for 30 min, 0.3 μm of dNTP's was added, and the mixture was incubated an additional 30 min at 30 C. After incubation, the reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The primer extension reaction mixture was loaded on an 8% polyacrylamide-urea sequencing gel adjacent to sequencing reactions of pGWS1 which were generated with the same oligonucleotide primer.

RESULTS

A cosmid library of the Cu^r Sm^r plasmid pPSR1 from P. syringae pv. syringae A2

was constructed in the vector pLAFR3. Three cosmid clones which expressed streptomycin resistance in FF5 were identified, and the clone pPSR1.7 was chosen for analysis. Genetic maps of Tn5393 from E. amylovora (41), P. syringae pv. syringae, and X. campestris pv. vesicatoria (Chapter VI) were compared (Fig. 14). Nucleotide sequence analysis of 1,864 nucleotides was done in P. syringae from the Sau3A site [nucleotide number 1,921 from the published sequence of Chiou and Jones (41)] through nucleotide 5,020 which is within the strA gene. This sequence was identical to that in E. amylovora with the exception that the 1,235 bp insertion sequence element IS1133, and the sequence TAG, duplicated upon insertion of IS1133, were not present in P. syringae. The nucleotide sequence of the 1,864 bp region from P. syringae pv. syringae was also identical to that from X. campestris pv. vesicatoria except that the 880-bp IS element IS6100 and the 8-bp duplication ACAATATC within the tnpR gene were not present in P. syringae (217). The SstI site at position 414 within tnpA (41) was conserved within Tn5393 in P. syringae pv. syringae and X. campestris pv. vesicatoria (Fig. 14) (217).

Quantification of strA promoter activity in P. syringae and X. campestris. Analysis of expression of strA:uidA fusions in P. syringae indicated a strong promoter sequence was located within the 0.4-kb PstI fragment which encompassed res. Expression of GUS activity in FF5 containing this clone was $\approx$136X that of FF5 containing pRG960sd alone (Fig. 15A). This promoter is also apparently

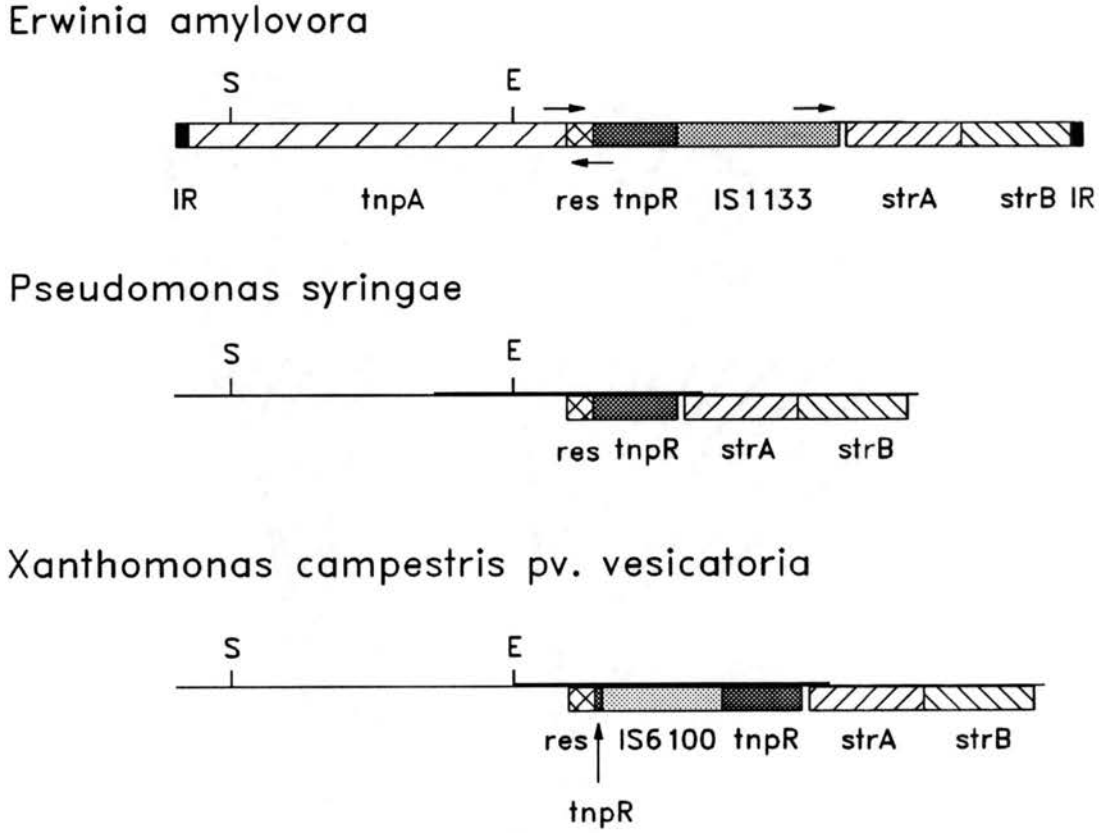


Fig. 14. Genetic maps of Tn5393 from Erwinia amylovora (41), Pseudomonas syringae pv. syringae, and Xanthomonas campestris pv. vesicatoria (Chapter VI). Genes which have been characterized are boxed; open boxes refer to genes which have been partially sequenced. IR = inverted repeat, tnpA = transposase, tnpR = resolvase, res = recombination site, strA and strB are aminoglycoside phosphotransferase genes, E = EcoRV, S = SstI. Putative promoter sequences detected within Tn5393 in E. amylovora and the direction of transcription are identified by arrows.

responsible for the expression of tnpR. The effect of TnpR as a repressor was observed in P. syringae containing pGWS8, a clone which included an intact tnpR sequence, as the expression level was reduced ≈5-fold (Fig. 15A). The expression of streptomycin-resistance from Tn5393 in P. syringae pv. syringae appeared to be enhanced by additional sequences located near the translational initiation site of strA. GUS activity in FF5 containing pGWS9, a clone which contains sequences downstream of res through 108 bp within strA, was ≈20X that of the control, while GUS activity in FF5 containing pGWS15, a clone containing res sequence through 12 bp within strA, was only ≈5X that of the control (Fig. 15A).

In X. campestris, GUS activity within the 0.4-kb PstI fragment was only 2.5X that of the control (Fig. 15B; pGWS6), while GUS activity expressed from pGWS32, a clone which contained intact res and IS6100 sequences, was ≈3.7X that of the control (Fig. 15B). However, only background levels of expression were observed from pGWS20 and pGWS21, clones which contained the right 14 and 146 bp, respectively, of IS6100 (Fig. 15B).

Analysis of the transcriptional start site located within res in P. syringae pv. syringae. An 18-bp synthetic oligonucleotide corresponding to the opposite strand 10 bp downstream of res was utilized to synthesize a cDNA from total cellular RNA of P. syringae pv. syringae FF5 containing pPSR5, a 68-kb Sm^r plasmid which harbors Tn5393. A single primer extension product was observed which indicated transcription was initiated within res, 106 bp upstream from the AUG translational start codon of tnpR (Fig. 16).

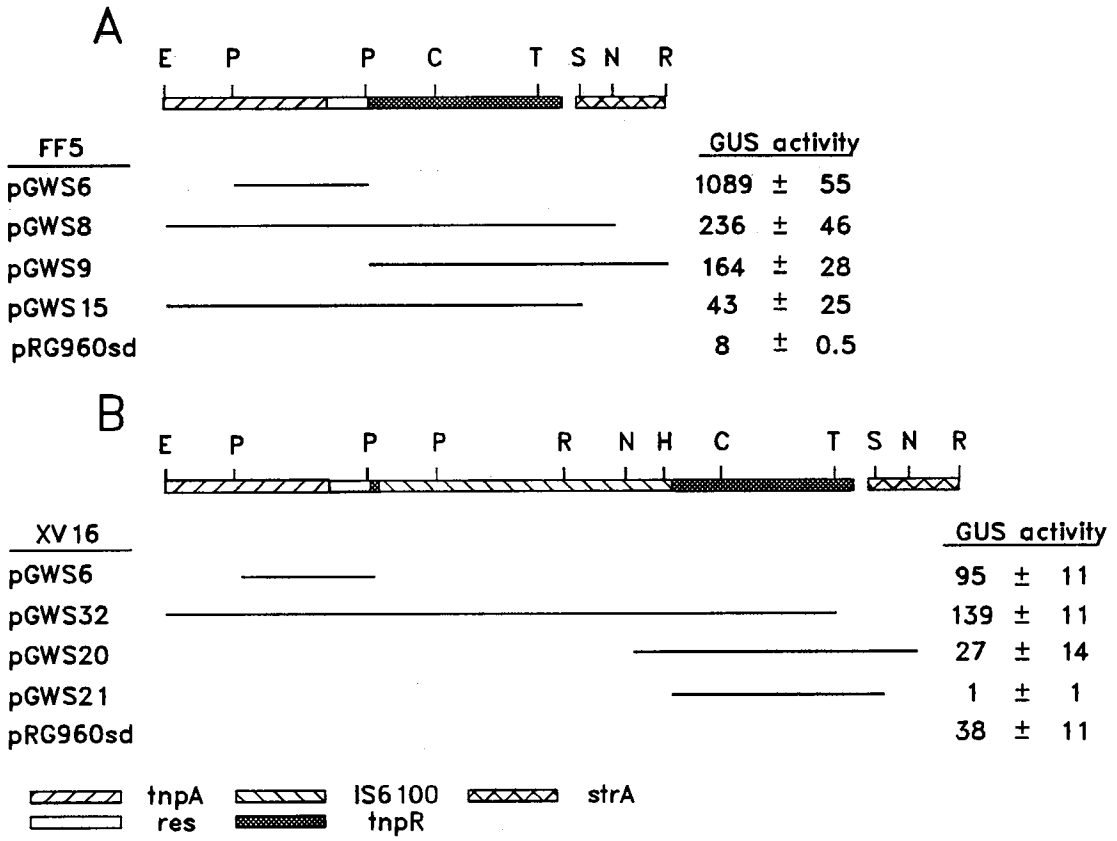


Fig. 15. Analysis of β-glucuronidase (GUS) activity determined fluorimetrically from lysates of (A) P. syringae pv. syringae FF5 and (B) X. campestris pv. vesicatoria XV16 containing strA-uidA fusion clones. GUS activity is reported as specific activity (units per milligram protein) ± the standard deviation of the mean of two replicates from two independent experiments. tnpA = transposase, res = recombination site, tnpR = resolvase, strA = aminoglycoside phosphotransferase genes of Tn5393. E = EcoRV, H = HindIII, N = NruI, P = PstI, R = RsaI, S = SspI, T = SstI.

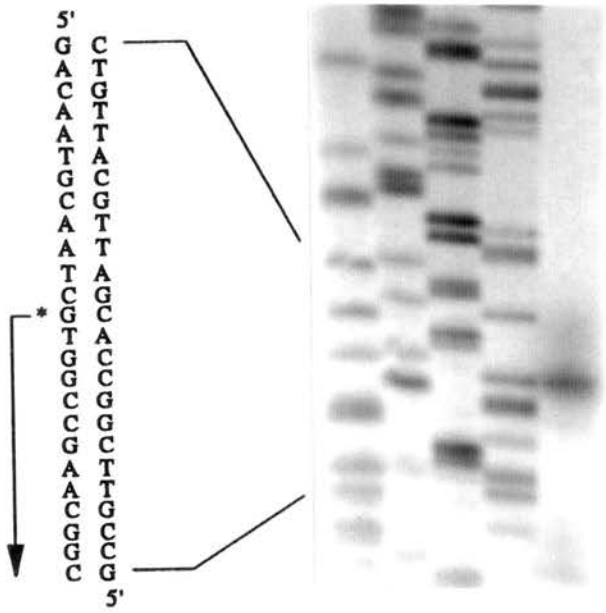


Fig. 16. Determination of the transcriptional start site of strA by primer extension. The ^{32}P-labeled cDNA product was electrophoresed in parallel with G/A/T/C sequence reactions generated using the same primer.

DISCUSSION

The Sm^r transposon Tn5393 and variants has been detected in three genera of phytopathogenic bacteria, and many genera of gram-negative bacteria isolated within North and South America (41,170,209,Chapter VI). Our data extend the range of Tn5393 to strains of P. syringae pv. syringae isolated from Pyrus calleryana in Oklahoma. The origin of Tn5393 is unknown, although the presence of a sequence downstream of strB within RSF1010 (198) which shares 77/81 bp sequence identity with IR-5393o (41) suggests this transposon was present on other plasmids besides those recently characterized in environmental bacterial isolates.

Analysis of strA:uidA fusions in P. syringae pv. syringae FF5 indicated a strong promoter sequence was located within a 400-bp PstI fragment encompassing res; GUS activity was markedly reduced when fusion clones included an intact copy of tnpR. The function of TnpR as a repressor, and its negative effect on the expression of downstream genes has been shown previously for Tn3 (42,86,96). The clones pGWS8 and pGWS15 only differed by an additional 94-bp sequence within pGWS8 which was downstream from the SspI site within strA. GUS activity in FF5 containing pGWS8, however, was $\approx$5.5X greater than in FF5 containing pGWS15. When combined, GUS activity of pGWS9 and pGWS15 was approximately equal to that of pGWS8. The results suggested the 94-bp sequence downstream of the SspI site within strA contained additional sequences involved in expression of streptomycin resistance in P. syringae. Putative -35 (TTGAAT) and -10 (TTTGGT) sequences, which would be disrupted by an SspI digest, were detected within strA (Fig. 17).

These sequences are present within pGWS8 and pGWS9, but not pGWS15.

Analysis of strA:uidA fusions in X. campestris pv. vesicatoria XV16 indicated expression from the 0.4-kb PstI fragment in pGWS6 was highly reduced compared to P. syringae pv. syringae. Also, GUS activity was not observed from pGWS20, a clone which contained the putative promoter sequence which was present in pGWS8 and pGWS9. These results suggest that some promoter sequences which are active in P. syringae do not function in X. campestris. Expression from a clone containing an intact copy of IS6100 was 46% higher than that from pGWS6 alone, indicating that the presence of IS6100 increased the expression of streptomycin resistance in X. campestris pv. vesicatoria. The overall low measurements of GUS activity in X. campestris compared to P. syringae may have been affected by the presence in cell lysates of the yellow nondiffusible pigment xanthamonadin produced by Xanthomonas spp. Activation of transcription of downstream genes by other members of the IS6 family occurs from the creation of a hybrid promoter comprised of a -35 sequence present in the right inverted repeat of the IS element and a -10 sequence downstream of the insertion site (82). However, GUS activity was not observed from two clones, pGWS20 and pGWS21, which contained the right inverted repeat and upstream sequences within IS6100. Thus, another sequence within IS6100 functioned as a promoter in X. campestris pv. vesicatoria. The nature of this sequence is currently unknown since the putative promoter sequences from P. syringae, which are similar to consensus E. coli and Pseudomonas promoter sequences (68,95), were nonfunctional in X. campestris.

Primer extension analysis of the transcriptional start site within res in P. syringae pv. syringae yielded a single cDNA product. Transcription was initiated at a guanine nucleotide located 108 bp upstream from the translational initiation codon of tnpR. Putative -35 (TTGTGG) and -10 (GTCAAT) sequences, which were similar to that of known Pseudomonas σ^{70} promoters (68), were identified within the res sequence, seven bp upstream of the transciptional initiation site (Fig. 18). This sequence differed from a putative E. coli σ^{70} promoter sequence previously identified within res of Tn5393 (41).

The expression of resistance genes from transposons is an important component of their selective value in dissemination into new genera. Tn5393 confers streptomycin resistance to P. syringae pv. syringae to a level of 75 μg/ml, which is high enough to provide a selective advantage to cells on plant surfaces sprayed with streptomycin (216). In X. campestris pv. vesicatoria, in which the promoter sequence located within res only functions at a low level, the problem of expression of streptomycin resistance has apparently been circumvented through the insertion of IS6100 within the transposon which enhances expression of strA/strB. A promoter sequence present in the right end of IS1133 upstream of strA in E. amylovora apparently increased the expression of streptomycin resistance in this organism (41). Thus, Tn5393 is a versatile transposon which is capable of acquiring IS elements to increase the expression of the Sm^r determinant in different bacterial genera. The wide dissemination of Tn5393 provides further evidence of the emerging importance of phytopathogenic bacteria as potential reservoirs of antibiotic resistance determinants in

the environment.

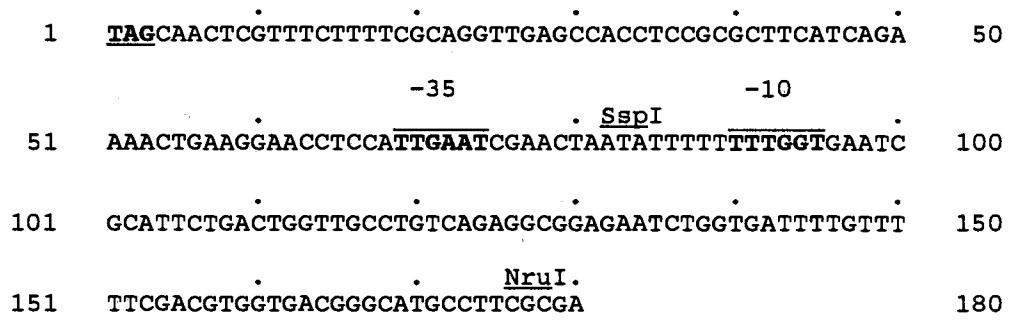


Fig. 17. Identification of a putative promoter sequence within the intergenic region between the resolvase (tnpR) gene and the coding sequence of the streptomycin-resistance gene strA on Tn5393. The termination codon of tnpR (position 1) is shown in underlined bold characters. A putative σ^{70} promoter is shown in overlined bold characters. The initiation codon of strA (TTG - position 70) is also the first three nucleotides of the putative -35 sequence.

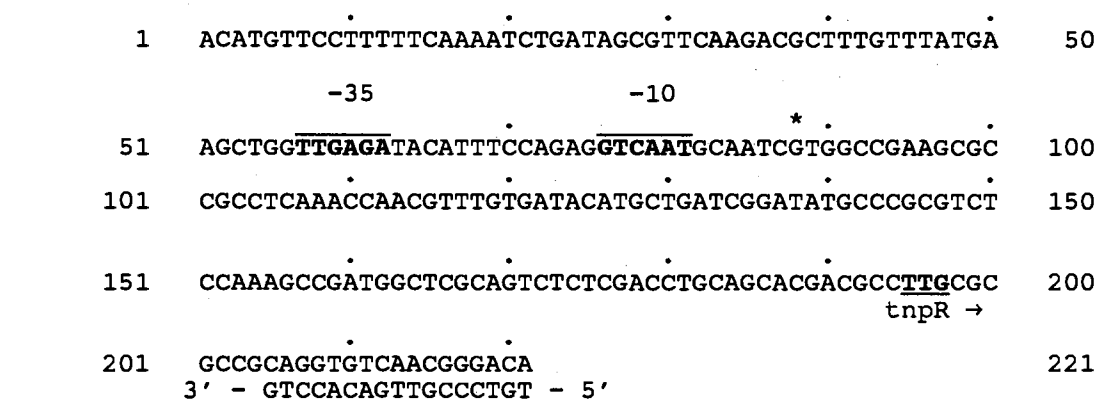


Fig. 18. Nucleotide sequence of the recombination site (res) and partial sequence of the tnpR gene of Tn5393 from Pseudomonas syringae pv. syringae A2. The transcriptional start site is shown by the asterisk. A putative σ^{70} promoter is shown in overlined bold characters. The translational initiation codon of tnpR (position 196) is shown in underlined bold characters. The sequence of the oligonucleotide primer utilized in the primer extension reaction is indicated, and is the complement of nucleotides 205 - 221.

CHAPTER VIII

DETECTION OF SEQUENCES FROM THE STREPTOMYCIN-RESISTANCE TRANSPOSON TN5393 IN DIVERSE PHYLLOPLANE AND SOIL BACTERIA IN MANAGED AGRICULTURAL ECOSYSTEMS

SUMMARY

Hybridization analyses were conducted to determine if the transposon Tn5393, which encodes streptomycin-resistance in three genera of phytopathogenic bacteria, was also present in diverse phylloplane and soil bacteria from ornamental pear nurseries and tomato fields. Streptomycin was utilized as a bactericide prior to sampling in two of the nurseries, but was never applied to the tomato fields. Tn5393 sequences were present in 20% and 11% of the streptomycin-resistant gram-negative phylloplane and soil isolates, respectively, from the ornamental pear nurseries. The highest concentration of Tn5393-containing bacteria was from a nursery in which streptomycin was heavily utilized. Tn5393 sequences were present in 22% and 10% of the streptomycin-resistant gram-negative phylloplane and soil isolates, respectively, from the tomato fields. The data imply that Tn5393 is widespread in environmental bacteria, and may be selected and ultimately transferred to populations of phytopathogenic bacteria inhabiting agricultural crops on which streptomycin is

utilized.

The appearance of phytopathogenic bacteria with resistance to the bactericide streptomycin on agricultural crops is of concern due to the possibility of disease control failures in crop production, and because of the possible impact of streptomycin-resistant (Sm^r) bacteria on human health. Streptomycin resistance in phytopathogenic bacteria is conferred by at least two determinants. Homologs of the strA/strB determinant from plasmids such as RSF1010 (198) have been detected in Erwinia amylovora (41), and in P. syringae pv. papulans, P. syringae pv. syringae, and X. campestris pv. vesicatoria (215). The Sm^r determinant is associated with a transposon, Tn5393, in each of these organisms (41,Chapter VII). A possible second Sm^r determinant has not been characterized and only presumed to exist because some Sm^r strains of P. syringae pv. papulans and X. campestris pv. vesicatoria have been isolated which did not hybridize to DNA probes containing portions of the strA/strB homologs (104,163a). Additionally, a chromosomal mutation conferring streptomycin resistance has been described in some strains of E. amylovora (199).

The importance of gene transfer in the evolution of antibiotic resistance (Ab^r) in clinical bacteria is illustrated by the wide dissemination of individual Ab^r determinants among unrelated bacterial genera. Among pathogenic and nonpathogenic organisms inhabiting the same environment, the frequency of Ab^r was higher among nonpathogens (134). The Ab^r nonpathogenic strains comprise a natural reservoir of resistance plasmids and genes which can ultimately be disseminated to pathogenic

strains (134). The introduction of specific Ab^r determinants to pathogenic genera is often accomplished via acquisition of the determinant from resident, nonpathogenic bacteria. Another problem confounding the use of antibiotics in medicine is the selection of multiple antibiotic resistance following the continued administration of a single antibiotic (43,135). Although observed in many instances, the evolutionary basis for this phenomenon is not well understood.

The aminoglycoside antibiotic streptomycin is registered for use in agriculture for management of diseases caused by phytopathogenic bacteria. However, bactericides applied to plant surfaces impact nontarget, indigenous bacteria as well as the phytopathogens. The effect of streptomycin bactericides on surface populations of phytopathogenic bacteria is well known; however, there is only limited evidence concerning the effect of streptomycin on the entire phylloplane and soil microbial community (6,145,164). Recently, Norelli et al demonstrated that Sm^r nontarget bacteria were present in leaf and soil samples from apple orchards in which streptomycin was previously applied, and from some orchards where streptomycin was previously applied (170). A total of 39% of the gram-negative isolates contained DNA which hybridized to a probe containing a portion of the strA gene from the apple pathogen, P. syringae pv. papulans (170), indicating the Sm^r determinant from a phytopathogenic bacterium was also present among nontarget gram-negative bacteria which co-inhabit apple orchards. Also, sequences from Tn5393 were detected in gram-negative bacteria isolated from leaf surfaces in apple orchards in Michigan (209). Norelli et al proposed that the strA/strB Sm^r determinant was initially selected

in nontarget bacteria and subsequently disseminated into the phytopathogen population (170).

In Oklahoma, streptomycin bactericides are regularly applied to tree crops in many nurseries. In previous studies from our laboratory, we identified Sm^r strains of the tree pathogen P. syringae pv. syringae (215), and determined that Sm^r strains harbored a plasmid-encoded copy of Tn5393 (Chapter VII). The objectives of this study were to determine if sequences from Tn5393 were present in nontarget bacteria inhabiting ornamental pear nurseries. We were also interested in the distribution of Tn5393 in regions which had not been exposed to streptomycin, and therefore included bacterial populations inhabiting tomato fields. In hybridization analyses, we utilized probes which were specific for the strA/strB genes, or the transposase (tnpA) and resolvase (tnpR) genes from Tn5393. Also, we tested the hypothesis that persistent antibiotic usage selects multiple antibiotic resistance by examining the isolates recovered in the study for resistance to tetracycline.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The medium used for isolation of bacteria from plant and soil samples was nutrient agar (Difco; Detroit, MI) amended with 100 μg of cycloheximide per ml (NAc) to inhibit fungal growth. NAc medium was further amended with 25 μg of streptomycin sulfate per ml (NAcs) or 12.5 μg of tetracycline per ml (NAct). P. syringae was cultured at 28 C on medium B of King et al (109), and E. coli was cultured at 37 C on LB medium (162). The antibiotics ampicillin and tetracycline were added to LB medium at concentrations of 40 and 12.5

μg/ml, respectively.

Isolation of bacteria from leaf and soil samples. Ornamental pear (Pyrus calleryana cv. Aristocrat) nurseries A and B were sampled on 24 June and 15 July 1992 and on 6 May 1993. Ornamental pear nurseries C, D, and E were sampled on 6 May 1993. Tomato field I was sampled on 12 August and 26 August 1992 and on 16 July 1993. Tomato fields II and III were sampled on 16 July 1993. All sites were located in Oklahoma; the nurseries were located in Cherokee, Haskell, and Muskogee counties, and the tomato fields were located in Oklahoma, Payne, and Tulsa counties (Fig. 19). According to information obtained from grower records, streptomycin bactericides were applied 15 and 2 times for the two previous years, respectively, in nurseries C and D. Streptomycin was not applied in nurseries A, B, and E and tomato field I, II, and III. Leaf samples consisted of approximately 200 leaves (2 leaves per plant) chosen at random. Soil samples consisted of 20 2-cm diameter cores to a depth of six cm taken at random locations under the dripline of the trees or tomato plants. The leaf and soil samples were each bulked, transported to the laboratory on ice, and processed within 12 h. The leaves were cut into approximately 2 cm^2 segments with 1 - 2 of the segments per leaf chosen at random for analysis. Five replicate 10-g portions from each leaf sample were separately placed into 500 ml flasks containing 100 ml of 0.01 M potassium phosphate buffer (pH 7.0) amended with 0.1% peptone (PK buffer), and washed for 1 h on a rotary shaker at 250 rpm. The bulked soil samples were mixed in the laboratory, and five replicate 10-g portions from each soil sample were washed in 50 ml PK buffer for 1 h on a rotary shaker at

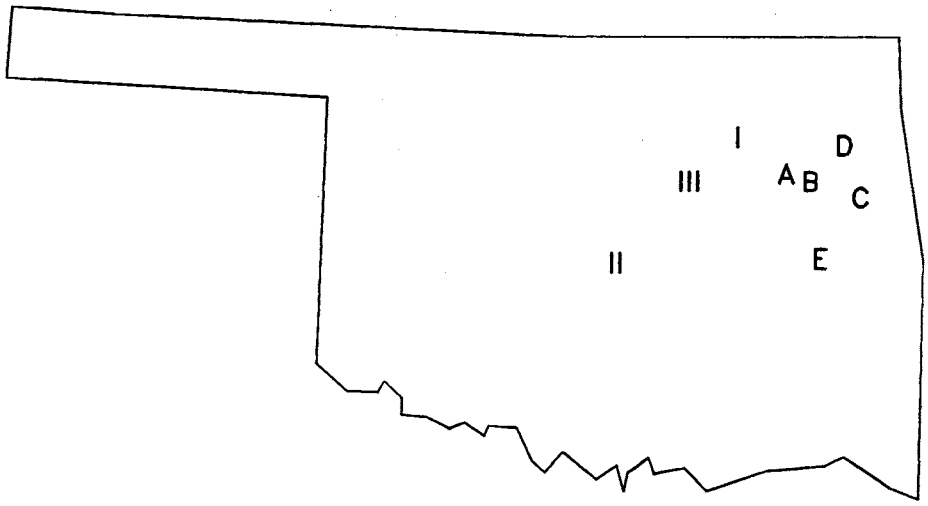


Fig. 19. Location of Pyrus calleryana cv. Aristocrat nurseries (A - E) and tomato fields (I - III) in Oklahoma which were sampled in the present study.

250 rpm. Aliquots from appropriate serial dilutions of the leaf and soil samples were plated on NAc and NAcs, and the samples were incubated at 28 C for 72 h.

Characterization of bacterial isolates. Following incubation, colonies appearing on NAc and NAcs were enumerated, and randomly-selected colonies were purified for phenotypic and genetic analysis. Isolates were characterized as to Gram reaction and fluorescence on KB medium; fluorescent isolates were also characterized as to oxidase reaction (195). Resistance to tetracycline was determined by patching each isolate to NAct medium. Isolates exhibiting confluent growth on NAct were considered resistant to tetracycline. Following phenotypic characterization, the isolates were maintained at 4 C on NAcs plates.

Molecular genetic techniques. Plasmids from specified isolates were isolated by the procedure of Crosa and Falkow (55). Agarose gel electorphoresis and Southern transfers to nylon membranes (Amersham Corp.; Arlington Heights, Ill.) were done by standard procedures (147). Specific DNA fragments for cloning or labeling were separated on 1% agarose gels and isolated by electroelution (147). DNA fragments used as probes were labeled with digoxigenin-11-dUTP (Genius kit; Boehringer Mannheim, Indianapolis, Ind.) as described by the manufacturer. For dot blots, cells grown for 12 h on NAcs medium were suspended in PK buffer to an approximate density of 10^8 cells/ml. Nylon membranes were placed on the surface of a NA plate, and 0.5 μl of the bacterial suspensions were spotted on the membranes in a 50 spots per plate grid pattern. Appropriate positive and negative control cultures were included in random locations on each membrane. The inoculated membranes were

incubated for 16 h at 28 C and processed by incubating the membrane on filter paper prewet with 10% sodium dodecyl sulfate for 5 min, followed by incubation on filter paper prewet with 0.5 M NaOH, 1 M NaCl for 15 min. Membranes were then air dried, and the DNA was fixed to the membranes with a 5 min exposure to UV light. Prehybridizations (minimum 6 h at 68 C), hybridizations at 68 C, and high stringency posthybridization washes were conducted as previously described (215).

Hybridization experiments. DNA-DNA hybridization experiments were conducted to determine if DNA homologous with internal regions of the Sm^r transposon Tn5393 were present in miscellaneous epiphytic and soil bacteria from agricultural habitats. DNA fragments used as probes were (a) the 1.5-kb SstI - EcoRV fragment in pSM1 containing an internal region of the strA/strB homologs from P. syringae pv. syringae A2 (215); and (b) the 3.2-kb SstI fragment from pPSR1 containing an internal region of the transposase (tnpA) and resolvase (tnpR) genes and the recombination site (res) of Tn5393 which was cloned from pPSR1 from P. syringae pv. syringae A2 (Chapter VII).

RESULTS

Isolation of bacteria from leaf and soil samples. Bacterial populations from leaf and soil samples from Pyrus calleryana cv. Aristocrat nurseries A - E and tomato fields I - III were enumerated on NAc and NAcs. Phylloplane populations recovered on NAcs were approximately equivalent on NAc from Nursery C, where streptomycin bactericides were the most heavily applied (Table 15). Populations were approximately 14 - 79 fold lower on NAcs at Nursery A, and 35 - 6,925 fold lower

on NAcs in the other nurseries. Populations recovered from tomato leaves were consistently 10 - 1,000 fold higher than those on P. calleryana leaves (Table 15). Although streptomycin had not been applied in any of the fields, leaf populations recovered on NAcs averaged approximately $\log_{10}$ 4.00 cfu/g in each field (Table 15). Soil populations recovered from both the P. calleryana nurseries and tomato fields were similar, with Sm^r bacterial populations approximately 10 - 100 fold lower than the total populations (Table 15).

Table 15. Mean bacterial populations (phylloplane and soil samples) from Pyrus calleryana cv. Aristocrat nurseries and tomato fields in Oklahoma recovered on medium with and without added streptomycin.

		Bacterial populations recovered[a]			
		Phylloplane		Soil	
Site[b]	Sample[c]	NAc	NAcs	NAc	NAcs
A	1	5.80±0.19	4.27±0.20	6.85±0.14	5.86±0.05
	2	5.47±0.68	4.31±0.31	6.80±0.11	5.83±0.08
	5	5.22±0.51	3.32±0.72	6.61±0.24	5.14±0.07
B	1	4.29±0.20	2.14±0.54	6.74±0.06	5.57±0.07
	2	3.90±0.35	2.35±0.29	6.72±0.03	5.50±0.12
	5	4.90±0.47	1.92±0.43	6.49±0.07	5.01±0.06
C	5	5.85±0.17	5.10±0.32	7.09±0.14	5.59±0.11
D	5	4.73±0.25	1.70±0.00	6.56±0.02	5.21±0.09
E	5	5.66±0.63	1.82±0.24	6.70±0.10	5.17±0.06
I	3	7.20±0.13	3.68±0.10	7.30±0.08	5.39±0.12
	4	7.31±0.15	4.18±0.13	7.27±0.10	5.80±0.15

Table 15 (continued)

Site[b]	Sample[c]	Bacterial populations recovered[a]			
		Phylloplane		Soil	
		NAc	NAcs	NAc	NAcs
I	6	6.99±0.16	4.05±0.75	6.77±0.03	5.23±0.09
II	6	7.37±0.53	4.37±0.17	7.26±0.08	5.69±0.04
III	6	6.68±0.11	3.39±0.14	7.10±0.36	5.28±0.05

[a] The bacterial populations are expressed as $\log_{10}$ cfu/g fresh weight. The media used for bacterial isolation from phylloplane and soil samples were nutrient agar amended with 100 μg/ml cycloheximide (NAc) and NAc amended with 25 μg/ml streptomycin (NAcs).

[b] Sites sampled were ornamental pear nurseries A - E and tomato fields I - III.

[c] Sampling dates were: (1) 24 June; (2) 15 July; (3) 12 August; (4) 26 August 1992; (5) 6 May; (6) 16 July 1993.

Phenotypic and genetic characterization of bacterial isolates. Bacterial isolates were randomly selected from NAcs plates and, after phenotypic testing, placed into the following groups: oxidase-negative fluorescent pseudomonads with colony morphology typical of P. syringae, oxidase-positive fluorescent pseudomonads, nonfluorescent gram-negative, and gram-positive.

DNA probes containing the strA/strB or the tnpA/tnpR genes from Tn5393 were then utilized in dot blot hybridization experiments to assay each isolate for the presence of hybridizing sequences. Each isolate which hybridized to the strA/strB probe also hybridized to the tnpA/tnpR probe. The strA/strB and tnpA/tnpR determinants were present in 77% of the Sm^r gram-negative phylloplane isolates from

Nursery C, in which streptomycin was heavily utilized, and in 10% of the Sm^r gram-negative phylloplane isolates from nursery A (Table 2). Although streptomycin was not applied in any of the tomato fields, 8 - 28% of the gram-negative Sm^r leaf isolates contained sequences which hybridized to strA/strB and tnpA/tnpR. The percentage of soil isolates which hybridized to strA/strB and tnpA/tnpR was similar to that observed for leaf isolates from all nurseries, and tomato field I (Table 16). In a combined analysis of data from nurseries and tomato fields, 22 and 11% of Sm^r gram-negative isolates from leaves and soil, respectively, hybridized to strA/strB and tnpA/tnpR. None of the 77 Sm^r gram-positive isolates in the study contained sequences which hybridized to strA/strB or tnpA/tnpR (Table 16).

Resistance to tetracycline in the streptomycin-resistant isolates. The observation that persistent usage of one antibiotic in clinical medicine selects multiple antibiotic resistance was evaluated by testing each Sm^r isolate recovered for resistance to the antibiotic tetracycline. Tetracycline was also chosen because this antibiotic is registered for use as a bactericide on some agricultural crops. Only a small number of phylloplane isolates from P. calleryana nurseries were also resistant to tetracyline, and the percentage of Tc^r isolates recovered was not significantly higher in Nursery C, in which streptomycin was heavily applied (Table 17). The percentage of Tc^r isolates recovered was higher among soil isolates, but was also not significantly higher among the isolates from Nursery C (Table 17). The percentage of Tc^r isolates from tomato leaves and soil was, on average, higher than that from the P. calleryana nurseries (Table 17).

Table 16. Number of streptomycin-resistant phylloplane and soil isolates from Pyrus calleryana cv. Aristocrat nurseries and tomato fields which contain Tn5393.

Bacteria isolated from:	Site/history[b]	No. of strains that hybridized with strA/strB / no. strains tested[a]				
		P. syringae	Fluorescent pseudomonads	Nonfluorescent gram negative bacteria	All gram negative bacteria	Gram positive bacteria
Phylloplane	A Cu	NT	NT	4/41 (10)	4/41 (10)	0/4 (0)
	B Cu	NT	0/1 (0)	0/13 (0)	0/14 (0)	0/5 (0)
	C Cu, Sm	8/8 (100)	NT	2/5 (40)	10/13 (77)	0/1 (0)
	D none	NT	NT	NT	NT	NT
	E Sm	NT	NT	0/3 (0)	0/3 (0)	NT
	Total	8/8 (100)	0/1 (0)	6/62 (10)	14/71 (20)	0/10 (0)
	I Cu	7/10 (70)	2/5 (40)	4/31 (13)	13/46 (28)	NT
	II Cu	NT	NT	3/14 (21)	3/14 (21)	0/1 (0)
	III none	NT	1/4 (25)	0/8 (0)	1/12 (8)	0/1 (0)
	Total	7/10 (70)	3/9 (33)	7/53 (13)	17/72 (24)	0/2 (0)
				Grand Total	31/143 (22)	0/12 (0)
Soil	A Cu	NT	2/3 (67)	5/40 (13)	6/43 (14)	0/26 (0)
	B Cu	NT	0/2 (0)	1/51 (2)	1/53 (2)	0/20 (0)
	C Cu, Sm	NT	2/2 (100)	6/11 (55)	8/13 (62)	0/2 (0)
	D none	NT	0/6 (0)	0/7 (0)	0/13 (0)	0/2 (0)
	E Sm	NT	NT	0/12 (0)	0/12 (0)	NT
	Total	NT	4/13 (31)	12/121 (10)	15/134 (11)	0/50 (0)
	I Cu	NT	NT	3/12 (25)	3/12 (25)	0/2 (0)
	II Cu	NT	NT	0/11 (0)	0/11 (0)	0/4 (0)
	III none	NT	NT	0/6 (0)	0/6 (0)	0/9 (0)
	Total	NT	NT	3/29 (10)	3/29 (10)	0/15 (0)
				Grand Total	18/163 (11)	0/65 (0)

[a] Numbers in parentheses indicate the percentage of positive strains; NT = not tested.

[b] Sites A - E are Pyrus calleryana cv. Aristocrat nurseries; sites I - III are tomato fields. History = the spray history for each site; Cu = copper sprayed at least once during each season, Sm = streptomycin was sprayed 15 and 2 times during each of the last two years at nurseries C and E, respectively; none = no bactericides sprayed at the site.

Table 17. Resistance to tetracycline among the streptomycin-resistant isolates recovered from the phylloplane and soil of Pyrus calleryana cv. Aristocrat nurseries and tomato fields.

Site[b]	No. of Sm^r Tc^r isolates / total Sm^r isolates[a]							
	Phylloplane				Soil			
	Gram neg.		Gram pos.		Gram neg.		Gram pos.	
A	1/41	(2)	0/4	(0)	13/43	(30)	0/26	(0)
B	3/14	(21)	1/5	(20)	29/53	(55)	6/20	(30)
C	3/13	(23)	1/1	(100)	6/13	(46)	0/2	(0)
D	0/0		0/0		10/13	(77)	0/2	(0)
E	0/3	(0)	0/0		11/12	(92)	0/0	
Grand Total	7/71	(10)	2/10	(20)	69/134	(51)	6/50	(12)
I	19/31	(61)	0/0	(0)	8/12	(67)	1/2	(50)
II	3/14	(21)	0/1	(0)	8/11	(73)	0/4	(0)
III	4/8	(50)	0/1	(0)	2/6	(33)	0/9	(0)
Grand Total	26/53	(49)	0/2	(0)	18/32	(56)	1/21	(5)

[a] Sm = streptomycin, tc = tetracycline, r = resistant; numbers in parentheses indicate the precentage of positive strains.

[b] Sites A - E are Pyrus calleryana cv. Aristocrat nurseries; sites a - c are tomato fields.

DISCUSSION

Previous studies have shown that the Sm^r transposon Tn5393 which is contained in phytopathogenic bacteria, is also present in diverse gram-negative bacteria in agricultural environments (41,170,209). Our data extend this observation to agricultural environments in another geographic region and demonstrate that Tn5393 sequences are also present in bacteria in enviroments which have presumably never been exposed to streptomycin through human usage. The presence of Tn5393 sequences in such diverse bacteria indicates this transposon is part of a gene pool which is accessible to a wide range of organisms.

The transfer of resistance plasmids among phytopathogenic and saprophytic plant-associated bacteria has been demonstrated in planta (120,121,146,218). Our data and that of others suggest transfer of sequences containing Tn5393 is a common occurrence. The movement of plasmid-borne resistance determinants between heterologous hosts is impacted by the transmissibility of the plasmids, the capacity for their establishment either through plasmid maintenance or transposition to a new genomic location, and the expression and selective value of the resistance determinant. The broad-host-range of Tn5393 is probably enhanced by the incorporation of insertion sequence elements such as IS1133 (41) and IS6100 (Chapter VI) which activate or increase expression of strA/strB.

The selection of multiple antibiotic resistance in human bacterial pathogens following continued use of a single antibiotic is a puzzling consequence of the overall widespread usage of antibiotics in medicine. A high proportion of resistance to

several antibiotics has been observed in the fecal flora of healthy individuals who were not ingesting antibiotics (22,133). This suggests the reservoir of antibiotic resistance in human enteric bacteria is widespread and large. Our data suggest the use of a single antibiotic does not select multiple antibiotic resistance among plant-associated bacteria. However, the presence of Tn5393 sequences in bacteria from areas in which streptomycin has not been used as a bactericide implies this determinant can be selected from the indigenous microbial communities in agricultural habitats and may be transferred to populations of plant pathogens.

The presence of Ab^r genes in environmental bacteria is of increasing concern due to the possible impact to human health. The distribution of Ab^r genes already present in human pathogens among environmental isolates is gaining increased attention. The nptII kanamycin-resistance gene from Tn5 and the sat streptothricin-resistance genes from a Tn7-like transposon were detected in environmental bacteria from habitats including river water, sewage, and soil (130,205,206). The strA/strB determinant on Tn5393 is also present on plasmids from a number of bacterial genera which cause diseases in humans (39,114). Thus, diverse ecological niches such as plant surfaces, soil, and water may serve as additional reservoirs of Ab^r bacteria, and the Ab^r genes they carry.

CHAPTER IX

CONCLUSIONS

The emergence of resistance to the bactericides copper and streptomycin in the phytopathogen Pseudomonas syringae pv. syringae served as an excellent model system for the study of the evolution of bactericide resistance in a phytopathogenic bacterium. The phenomenon of resistance was studied from both ecological and genetic standpoints. A populations approach was utilized to understand the problem. Hirano and Upper have championed the viewpoint that ecological studies of phylloplane dynamics of Pseudomonas syringae involve studying populations of bacteria and populations of habitats and that variablility is an inherent characteristic of the system (98-100). P. syringae pv. syringae is an organism in which the genetic diversity is high (67) indicating natural populations of this organism are heterogenous. The response of heterogenous populations of P. syringae pv. syringae to selection pressure from bactericide use was the basis of the work reported in this dissertation. From an ecological standpoint, the role of plasmids and transposons in the dissemination of resistance genes within populations was examined. The plasmid population was diverse with as many as 15 plasmid profiles detected wihtin a single nursery (Chapters III and IV). The host P. syringae pv. syringae populations

examined were also diverse (Chapter IV). In the final analysis, it is evident that the selection of a single resistant P. syringae pv. syringae strain or a single resistance plasmid was prohibited due to the inherent heterogeneity within these populations. Thus, heterogeneity was maintained within populations exposed to bactericide selection pressure due to the persistence of diverse plasmid and strain genotypes.

The selection of copper and streptomycin-resistance determinants on plasmids in P. syringae pv. syringae mirrors observations on the selection of antibiotic resistance determinants in clinical bacteria. Many of the resistance plasmids characterized in this dissertation contained the pOSU900 replicon (167,Chapter IV) which suggests that these plasmids comprise part of a plasmid lineage which is indigenous to P. syringae. In clinical bacteria, plasmid-encoded antibiotic resistance genes also tend to be located on plasmids indigenous to the bacterial species or on broad-host-range plasmids which are specialized in colonizing a diversity of bacterial species (134). The plasmid location of copper and streptomycin-resistance determinants in P. syringae pv. syringae enabled their dissemination within natural populations, presumably by conjugation (Chapter IV). However, a longstanding argument among plant pathologists regards the stability and persistence of bactericide resistance genes in phytopathogenic bacteria in the absence of bactericide use. We determined that several indigenous resistance plasmids were stable in vitro and did not impact competitive fitness of P. syringae pv. syringae in planta (Chapter V). Our observations that plasmid carriage had a negative effect on fitness of P. syringae pv. syringae in vitro were similar to results observed in many studies of plasmid biology

utilizing Escherichia coli as a model bacterium (e.g. 23,75,97,250). However, the effect of individual plasmids on host fitness has not been fully addressed in native ecological niches. Our observations suggest the presence of a plasmid does not impact fitness in the epiphytic niche of P. syringae pv. syringae, and the widespread distribution of the pOSU900 replicon-containing plasmids in P. syringae (167,168,Chapter IV) suggests these plasmids may contain other determinants which are beneficial to this bacterium during its life cycle.

Although resistance to streptomycin can be selected in many gram-negative bacteria as a spontaneous mutation, the evolution of streptomycin resistance in natural bacterial populations following the use of this antibiotic in medicine and agriculture has generally involved the acquisition of specific resistance determinants encoding streptomycin-inactivating enzymes (63). In most phytopathogenic bacteria, streptomycin resistance is encoded by the tandem strA/strB aminoglycoside phosphotransferase genes which are contained on Tn5393 (41,215,Chapters III - V). Tn5393 has been disseminated widely both geographically and in terms of the diversity of bacterial hosts the transposon now inhabits. Expression of the strA/strB genes on Tn5393 is essential for this transposon to provide a selective advantage to a host bacterium. The insertion sequence elements IS1133 and IS6100 are involved in expression of the strA/strB genes in Erwinia amylovora and Xanthomonas campestris pv. vesicatoria, respectively (41,Chapters VI and VII). In P. syringae pv. syringae, two indigenous promoters located within Tn5393 were identified (Chapter VII). Tn5393 is also widely disseminated among saprophytic bacteria from plant surfaces

and soil (170,209,Chapter VIII) indicating the versatility of this element in dissemination capability and expression in diverse organisms. The broad distribution of the strA/strB genes among bacterial commensals and pathogens of humans, animals, and plants (Chapters II and III) is a testament to the survival ability of bacteria in hostile enviroments created by human usage of bactericidal agents. It also indicates that organisms from such diverse hosts can interact and share a common gene pool. Finally, the location of resistance determinants on indigenous plasmids implies that bacteria which harbor these plasmids will not only survive bactericide exposure but also fluorish.

BIBLIOGRAPHY

1. **Adaskaveg, J.E., and R.B. Hine.** 1985. Copper tolerance and zinc sensitivity of Mexican strains of Xanthomonas campestris pv. vesicatoria, causal agent of bacterial spot of pepper. Plant Dis. **69**:993-996.

2. **Albritton, W., J.L. Brunton, L. Slaney, and I. MacLean.** 1982. Plasmid-mediated sulfonamide resistance in Haemophilus ducreyi. Antimicrob. Agents Chemother. **21**:159-165.

3. **Allmansberger, R., B. Brau, and W. Piepersberg.** 1985. Genes for gentamicin-(3)-N-acetyl-transferases III and IV. II. Nucleotide sequences of three AAC(3)-III genes and evolutionary aspects. Mol. Gen. Genet. **198**:514-520.

4. **American Phytopathological Society Bacteriology Committee.** 1978. Antibiotic use for control of bacterial diseases of plants. Phytopathol. News 12:54-55.

5. **Andersen, G.L., O. Menkissoglou, and S.E. Lindow.** 1991. Occurrence and properties of copper-tolerant strains of Pseudomonas syringae isolated from fruit trees in California. Phytopathology **81**:648-656.

6. **Andrews, J.H.** 1981. Effects of pesticides on non-target micro-organisms on leaves. p. 283-304 In J.P. Blakeman (ed.), Microbial ecology of the phylloplane. Academic Press, London.

7. **Araki, Y., M. Inoue, and H. Hashimoto.** 1987. Characterization and mobilization of nonconjugative plasmids encoding resistance to streptomycin and sulfanilamide. Microbiol. Immunol. **31**:1107-1111.

8. **Austin, S.J.** 1988. Plasmid partition. Plasmid **20**:1-9.

9. **Austin, S., and K. Nordstrom.** 1990. Partition-mediated incompatibility of bacterial plasmids. Cell **60**:351-354.

10. **Barth, P.T., and N.J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618-630.

11. **Bender, C.L.** Unpublished data.

12. **Bender, C.L., and D.A. Cooksey.** 1986. Indigenous plasmids in Pseudomonas syringae pv. tomato: conjugative transfer and role in copper resistance. J. Bacteriol. **165**:534-541.

13. **Bender, C.L., and D.A. Cooksey.** 1987. Molecular cloning of copper resistance genes from Pseudomonas syringae pv. tomato. J. Bacteriol. **169**:470-474.

14. **Bender, C.L., D.K. Malvick, K.E. Conway, S. George, and P. Pratt.** 1990. Characterization of pXV10A, a copper resistance plasmid in Xanthomonas campestris pv. vesicatoria. Appl. Environ. Microbiol. **56**:170-175.

15. **Bender, C.L., D.K. Malvick, and R.E. Mitchell.** 1989. Plasmid-mediated production of the phytotoxin coronatine in Pseudomonas syringae pv. tomato. J. Bacteriol. **171**:807-812.

16. **Bender, C.L., H.E. Stone, J.J. Sims, and D.A. Cooksey.** 1987. Reduced pathogen fitness of Pseudomonas syringae pv. tomato Tn5 mutants defective in coronatine production. Physiol. Mol. Plant Pathol. **30**:273-283.

17. **Bender, C.L., S.A. Young, and R.E. Mitchell.** 1991. Conservation of plasmid DNA sequences in coronatine-producing pathovars of Pseudomonas syringae. Appl. Environ. Microbiol. **57**:993-999.

18. **Bennett, P.M., and A.H. Linton.** 1986. Do plasmids influence the survival of bacteria? J. Antimicrob. Chemother. **18**:(Suppl. C)123-126.

19. **Bentley, W.E., N. Mirjalili, D.C. Andersen, R.H. Davis, and D.S. Kompala.** 1990. Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria. Biotechnol. Bioeng. **35**:668-681.

20. **Bethell, R.S., J.M. Ogawa, W.H. English, R.R. Hansen, B.T. Manji, and F.J. Schick.** 1977. Copper-streptomycin sprays control pear blossom blast. Calif. Agric. **31**:7-9.

21. **Blakeman, J.P.** 1991. Foliar bacterial pathogens: epiphytic growth and interactions on leaves. J. Appl. Bacteriol. Symposium Suppl. **70**:49S-59S.

22. **Bonten, M., E. Stobberingh, J. Philips, and A. Houben.** 1990. High prevalence of antibiotic-resistant Escherichia coli in faecal samples of students in the south-east of The Netherlands. J. Antimicrob. Chemother. **21**:585-592.

23. **Bouma, J.E., and R.E. Lenski.** 1988. Evolution of a bacteria/plasmid association. Nature(London) **335**:351-352.

24. **Bouzar, H., D. Ouadah, Z. Krimi, J.B. Jones, M. Trovato, A. Petit, and Y. Dessaux.** 1993. Correlative association between resident plasmids and the host chromosome in a diverse Agrobacterium soil population. Appl. Environ. Microbiol. **59**:1310-1317.

25. **Bradbury, J.F.** 1986. Guide to plant pathogenic bacteria. CAB International North America, Tucson, AZ.

26. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

27. **Brisson-Noel, A., M. Arthur, and P. Courvalin.** 1988. Evidence for natural gene transfer from gram-positive cocci to Escherichia coli. J. Bacteriol. **170**:1739-1745.

28. **Burr, T.J., and B. Katz.** 1982. Evaluation of a selective medium for detecting Pseudomonas syringae pv. papulans and P. syringae pv. syringae in apple orchards. Phytopathology **72**:564-567.

29. **Burr, T.J., and B.H. Katz.** 1984. Overwintering and distribution pattern of Pseudomonas syringae pv. papulans and pv. syringae in apple buds. Plant Dis. **68**:383-385.

30. **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.

31. **Cameron, H.R.** 1962. Mode of infection of sweet cherry by Pseudomonas syringae. Phytopathology **52**:917-921.

32. **Canfield, M.L., S. Baca, and L.W. Moore.** 1986. Isolation of Pseudomonas syringae from 40 cultivars of diseased woody plants with tip dieback in Pacific Northwest nurseries. Plant Dis. **70**:647-650.

33. **Caulcott, C.A., A. Dunn, H.A. Robertson, N.S. Cooper, M.E. Brown, and P.M. Rhodes.** 1987. Investigation of the effect of growth environment on the stability of low-copy-number plasmids in Escherichia coli. J. Gen. Microbiol. **133**:1881-1889.

34. **Cha, J.-S., and D.A. Cooksey.** Copper resistance in Pseudomonas syringae mediated by periplasmic and outer membrane proteins. Proc. Natl. Acad. Sci. USA **88**:8915-8919.

35. **Chang, Y.F., D.P. Ma, H.Q. Bai, R. Young, D.K. Struck, S.J. Shin, and D.H. Lein.** 1992. Characterization of plasmids with antimicrobial resistant genes in Pasteurella haemolytica A1. DNA Seq. **3**:89-97.

36. **Chaslus-Dancla, E., G. Gerbaud, M. Lagorce, J.-P. Lafont, and P. Courvalin.** 1987. Persistence of an antibiotic resistance plasmid in intestinal Escherichia coli of chickens in the absence of selective pressure. Antimicrob. Agents Chemother. **31**:784-788.

37. **Chen, S.-T., and R.C. Clowes.** 1987. Variations between the nucleotide sequence of Tn1, Tn2, and Tn3 and expression of β-lactamase in Pseudomonas aeruginosa and Escherichia coli. J. Bacteriol. **169**:913-916.

38. **Cheng, G.Y., D.E. Legard, J.E. Hunter, and T.J. Burr.** 1989. Use of a modified bean pod assay to detect strains of Pseudomonas syringae pv. syringae that cause bacterial brown spot of snap bean. Plant Dis. **73**:419-423.

39. **Chinault, A.C., V.A. Blakesley, E. Roessler, D.G. Willis, C.A. Smith, R.G. Cook, and R.G. Fenwick.** 1986. Characterization of transferable plasmids from Shigella flexneri 2a that confer resistance to trimethoprim, streptomycin, and sulfonamides. Plasmid **15**:119-131.

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

41. **Chiou, C.-S., and A.L. Jones.** 1993. Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in Erwinia amylovora and other gram-negative bacteria. J. Bacteriol. **175**:732-740.

42. **Chou, J., P.G. Lemaux, M.J. Casadaban, and S.N. Cohen.** 1979. Transposition protein of Tn3: identification and characterisation of an essential repressor-controlled gene product. Nature(London) **282**:801-806.

43. **Christie, P.J., and G.M. Dunny.** 1984. Antibiotic selection pressure resulting in multiple antibiotic resistance and localization of resistance determinants to conjugative plasmids in streptococci. J. Infect. Dis. **149**:74-82.

44. **Comai, L., and T. Kosuge.** 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in Pseudomonas savastonoi. J. Bacteriol. **143**:950-957.

45. **Cooksey, D.A.** 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of Pseudomonas syringae pv. tomato. Appl. Environ. Microbiol. **53**:454-456.

46. **Cooksey, D.A.** 1988. Reduction of infection by Pseudomonas syringae pv. tomato using a nonpathogenic, copper-resistant strain combined with a copper bactericide. Phytopathology **78**:601-603.

47. **Cooksey, D.A.** 1990a. Genetics of bactericide resistance in plant pathogenic bacteria. Annu. Rev. Phytopathol. **28**:201-219.

48. **Cooksey, D.A.** 1990b. Plasmid-determined copper resistance in Pseudomonas syringae from impatiens. Appl. Environ. Microbiol. **56**:13-16.

49. **Cooksey, D.A.** 1993. Copper uptake and resistance in bacteria. Mol. Microbiol. **7**:1-5.

50. **Cooksey, D.A., H.R. Azad, J.-S. Cha, and C.-K. Lim.** 1990. Copper resistance gene homologs in pathogenic and saprophytic species from tomato. Appl. Environ. Microbiol. **56**:431-435.

51. **Cooksey, D.A., and J.H. Graham.** 1989. Genomic fingerprinting of two pathovars of phytopathogenic bacteria by rare-cutting restriction enzymes and field inversion gel electrophoresis. Phytopathology **79**:745-750.

52. **Coplin, D.L.** 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Annu. Rev. Phytopathol. **27**:187-212.

53. **Couturier, M., F. Bex, P.L. Berquist, and W.K. Maas.** 1988. Identification and classification of bacterial plasmids. Microbiol. Rev. **52**:375-395.

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

55. **Crosa, J.H., and S. Falkow.** 1981. Plasmids, p. 266-282. In P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

56. **Crosse, J.E.** 1955. Bacterial canker of stone fruits. I. Field observations on the avenue of autumnal infection of cherry. J. Hort. Sci. **30**:131-142.

57. **Crosse, J.E.** 1956. Bacterial canker of stone fruits. II. Leaf scar infection of cherry. J. Hort. Sci. **31**:212-224.

58. **Crosse, J.E.** 1957. Bacterial canker of stone fruits. III. Inoculum concentration and time of inoculation in relation to leaf-scar infection of cherry. Ann. Appl. Biol. **45**:19-35.

59. **Crosse, J.E.** 1958. Streptomycin in the control of bacterial canker of cherry. Ann. Appl. Biol. **45**:226-228.

60. **Curiale, M.S. and D. Mills.** 1982. Integration and partial excision of a cryptic plasmid in Pseudomonas syringae pv. phaseolicola. J. Bacteriol. **152**:797-802.

61. **Curiale, M.S. and D. Mills.** 1983. Molecular relatedness among cryptic plasmids in Pseudomonas syringae pv. glycinea. Phytopathology **73**:1440-1444.

62. **Currier, T.J., and M.K. Morgan.** 1983. Plasmids of Pseudomonas syringae: no evidence of a role in toxin production or pathogenicity. Can. J. Microbiol. **29**:84-89.

63. **Davies, J.E., and D. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Ann. Rev. Microbiol. **32**:469-518.

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

65. **deBruijn, F.J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria. Appl. Environ. Microbiol. **58**:2180-2187.

66. **Denny, T.P.** 1988. Phenotypic diversity in Pseudomonas syringae pv. tomato. J. Gen. Microbiol. **134**:1939-1948.

67. **Denny, T.P., M.N. Gilmour, and R.K. Selander.** 1988. Genetic diversity and relationships of two pathovars of Pseudomonas syringae. J. Gen. Microbiol. **134**:1949-1960.

68. **Deretic, V., W.M. Konyecsni, C.D. Mohr, D.W. Martin, and N.S. Hibler.** 1989. Common denominators of promoter control in Pseudomonas and other bacteria. Bio/Technology **7**:1249-1254.

69. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. **12**:387-395.

70. **Doucet-Populaire, F., P. Trieu-Cuot, A. Andremont, and P. Courvalin.** 1992. Conjugal transfer of plasmid DNA from Enterococcus faecalis to Escherichia coli in digestive tracts of gnotobiotic mice. Antimicrob. Agents Chemother. **36**:502-504.

71. **Doudoroff, M., and N.J. Palleroni.** 1974. Genus I. Pseudomonas Migula. pp. 217-243 In Buchanan, R.E., N.E. Gibbons, eds., Bergey's Manual of Determinative Bacteriology 8th ed. Williams and Wilkins, Baltimore, MD.

72. **Dowler, W.M., and D.H. Petersen.** 1967. Transmission of Pseudomonas syringae in peach trees by bud propagation. Plant Dis. Rep. **51**:666-668.

73. **Duetz, W.A., and J.G. van Andel.** 1991. Stability of TOL plasmid pWW0 in Pseudomonas putida mt-2 under non-selective conditions in continuous culture. J. Gen. Microbiol. **137**:1369-1374.

74. **Dye, D.W., J.F. Bradbury, M. Goto, A.C. Hayward, R.A. Lelliott, and M.N. Schroth.** 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Pathol. **59**:153-168.

75. **Dykhuizen, D.E. and D.L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47**:150-168.

76. **Eberhard, W.G.** 1990. Evolution in bacterial plasmids and levels of selection. Quart. Rev. Biol. **65**:3-22.

77. **Endert, E., and D.R. Ritchie.** 1984. Overwintering and survival of Pseudomonas syringae pv. syringae and symptom development in peach trees. Plant Dis. **68**:468-470.

78. **Ercolani, G.L.** 1983. Variability among isolates of Pseudomonas syringae pv. savastonoi from the phylloplane of the olive. J. Gen. Microbiol. **129**:901-916.

79. **Figurski, D.H., and D.R. Helinski.** 1979. Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **79**:1648-1652.

80. **Fling, M.E., J. Kopf, and C. Richards.** 1988. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. Plasmid **19**:30-38.

81. **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.

82. **Galas, D.J., and M. Chandler.** 1989. Bacterial insertion sequences. pp. 109-162 In Berg, D.E., and Howe, M.M. (Eds.), Mobile DNA. American Society for Microbiology, Washington, D.C.

83. **Gardan, L., S. Cottin, C. Bollet, and G. Hunault.** 1991. Phenotypic heterogeneity of Pseudomonas syringae van Hall. Res. Microbiol. **142**:995-1003.

84. **Garde, S., and C.L. Bender.** 1991. DNA probes for detection of copper resistance genes in Xanthomonas campestris pv. vesicatoria. Appl. Environ. Microbiol. **57**:2435-2439.

85. **Gilbride, K.A., S. Rosendal, and J.L. Brunton.** 1989. Plasmid mediated antimicrobial resistance in Ontario isolates of Actinobacillus (Haemophilus) pleuropneumoniae. Can. J. Vet. Res. 53:38-42.

86. **Gill, R., F. Heffron, and S. Falkow.** 1979. Identification of the transposase and transposon-specific repressor encoded by Tn3. Nature(London) **282**:797-801.

87. **Godwin, D., and J.H. Slater.** 1979. The influence of the growth environment on the stability of a drug resistance plasmid in Escherichia coli K12. J. Gen. Microbiol. **111**:201-210.

88. **Gonzalez, C.F., S.K. Layher, A.K. Vidaver, and R.H. Olsen.** 1984. Transfer, mapping, and cloning of Pseudomonas syringae pv. syringae plasmid pCG131 and assessment of its role in virulence. Phytopathology **74**:1245-1250.

89. **Gormley, E.P., and J. Davies.** 1991. Transfer of plasmid RSF1010 by conjugation from Escherichia coli to Streptomyces lividans and Mycobacterium smegmatis. J. Bacteriol. **173**:6705-6708.

90. **Grantham, R., C. Gautier, M. Gouy, M. Jacobzone, and R. Mercier.** 1981. Codon catalog usage is a genome strategy modulated for gene expressivity. Nucl. Acids Res. **9**:r43-r74.

91. **Grinsted, J., F. de la Cruz, and R. Schmitt.** 1990. The Tn21 subgroup of bacterial transposable elements. Plasmid **24**:163-189.

92. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. 117:619-630.

93. **Haefele, D.M., and S.E. Lindow.** 1987. Flagellar motility confers epiphytic fitness advantages upon Pseudomonas syringae. Appl. Environ. Microbiol. **53**:2528-2533.

94. **Hattingh, M.J., I.M.M. Roos, and E.L. Mansvelt.** 1989. Infection and systemic invasion of deciduous fruit trees by Pseudomonas syringae in South Africa. Plant Dis. **73**:784-789.

95. **Hawley, D.K., and W.R. McClure.** 1983. Compilation and analysis of Escherichia coli promoter DNA sequences. Nucl. Acids Res. **11**:2237-2255.

96. **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.

97. **Helling, R.B., T. Kinney, and J. Adams.** 1981. The maintenance of plasmid-containing organisms in populations of Escherichia coli. J. Gen. Microbiol. **123**:129-141.

98. **Hirano, S.S., and C.D. Upper.** 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. **21**:243-269.

99. **Hirano, S.S., and C.D. Upper.** 1990. Population biology and epidemiology of Pseudomonas syringae. Annu. Rev. Phytopathol. **28**:155-177.

100. **Hirano, S.S., and C.D. Upper.** 1993. Dynamics, spread, and persistence of a single genotype of Pseudomonas syringae relative to those of its conspecifics on populations of snap bean leaflets. Appl. Environ. Microbiol. **59**:1082-1091.

101. **Hoitink, H.A.J., and S.L. Sinden.** 1970. Partial purification and properties of chlorosis-inducing toxins of Pseudomonas phaseolicola and Pseudomonas glycinea. Phytopathology **60**:1236-1237.

102. **Jefferson, R.A.** 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5**:387-405.

103. **Jones, A.L.** 1971. Bacterial canker of sweet cherry in Michigan. Plant Dis. Rep. **55**:961-965.

104. **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.

105. **Judd, A.K., M. Schneider, M.J. Sadowsky, and F.J. deBruijn.** 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related Bradyrhizobium japonicum serocluster 123 strains. Appl. Environ. Microbiol. **59**:1702-1708.

106. **Kato, K., K. Ohtsuki, H. Mitsuda, T. Yomo, S. Negoro, and I. Urabe.** 1994. Insertion sequence IS6100 on plasmid pOAD2, which degrades nylon oligomers. J. Bacteriol. **176**:1197-1200.

107. **Kawabe, H., T. Tanaka, and S. Misuhashi.** 1978. Streptomycin resistance and spectinomycin resistance mediated by plasmids. Antimicrob. Agents Chemother. **13**:1031-1035.

108. **Keane, P.J., A. Kerr, and P.B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of Agrobacterium isolates. Aust. J. Biol. Sci. **23**:585-595.

109. **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.

110. **Kinoshita, S., S. Kageyama, K. Iba, Y. Yamada, and H. Okada.** 1975. Utilization of a cyclic dimer and linear oligomers of ϵ-aminocaproic acid by Achromobacter guttatus K172. Agric. Biol. Chem. **39**:1219-1223.

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

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

113. **Kobayashi, D.K., S.J. Tamaki, and N.T. Keen.** 1990. Molecular characterization of avirulence gene D from Pseudomonas syringae pv. tomato. Mol. Plant Microbe-Interact. **3**:94-102.

114. **Korfmann, G., W. Ludtke, U. van Treeck, and B. Wiedemann.** 1983. Dissemination of streptomycin and sulfonamide resistance by plasmid pBP1 in Escherichia coli. Eur. J. Clin. Microbiol. **2**:463-468.

115. **Kogan, S.C., M. Doherty, and J. Gitschier.** 1987. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N. Engl. J. Med. **317**:985-990.

116. **Kreft, J., K. Bernhard, and W. Goebel.** 1978. Recombinant plasmids capable of replication in B. subtilis and E. coli. Mol. Gen. Genet. **162**:59-67.

117. **Kues, U., and U. Stahl.** 1989. Replication of plasmids in gram-negative bacteria. Microbiol. Rev. **53**:491-516.

118. **Kyslik, P., M. Dobisova, H. Maresova., and L. Sobotkova.** 1993. Plasmid burden in chemostat culture of Escherichia coli: its effect on the selection for overproducers of host enzymes. Biotechnol. Bioeng. **41**:325-329.

119. **Lacroix, J.-M., and C.B. Walker.** 1992. Identification of a streptomycin resistance gene and a partial Tn3 transposon coding for a b-lactamase in a periodontal strain of Eikenella corrodens. Antimicrob. Agents Chemother. **36**:740-743.

120. **Lacy, G.H.** 1978. Genetic studies with plasmid RP1 in Erwinia chrysanthemi strains pathogenic on maize. Phytopathology **68**:1323-1330.

121. **Lacy, G.H., and J.V. Leary.** 1975. Transfer of antibiotic resistance plasmid RP1 into Pseudomonas glycinea and Pseudomonas phaseolicola in vitro and in planta. J. Gen. Microbiol. **88**:49-57.

122. **Laguerre, G., S.I. Mazurier, and N. Amarger.** 1992. Plasmid profiles and restrictions fragment length polymorphism of Rhizobium leguminosarum bv. viciae in field populations. FEMS Microbiol. Ecol. **101**:17-26.

123. **Laguerre, G., E. Geniaux, S.I. Mazurier, R.R. Casartelli, and N. Amarger.** 1993. Conformity and diversity among field isolates of Rhizobium leguminosarum bv. viciae, bv. trifolii, and bv. phaseoli revealed by DNA hybridization using chromosome and plasmid probes. Can. J. Microbiol. **39**:412-419.

124. **Latorre, B.A., J. Gonzalez, J.E. Cox, amd F. Vial.** 1985. Isolation of Pseudomonas syringae pv. syringae from cankers and effect of free moisture on its epiphytic populations on sweet cherry trees. Plant Dis. **69**:409-412.

125. **Latorre, B.A., and A.L. Jones.** 1979. Pseudomonas morsprunorum, the cause of bacterial canker of sour cherry in Michigan, and its epiphytic association with P. syringae. Phytopathology **69**:335-339.

126. **Lax, A.J., and C.A. Walker.** 1986. Plasmids related to RSF1010 from Bordetella bronchiseptica. Plasmid 15:210-216.

127. **Lazraq, R., S. Clavel-Seres, H.L. David, and D. Roulland-Dussoix.** 1990. Conjugative transfer of a shuttle plasmid for Escherichia coli to Mycobacterium smegmatis. FEMS Microbiol. Lett. **69**:135-138.

128. **Leben, C.** 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. **3**:209-230.

129. **Lee, Y.-A., M. Hendson, N.J. Panopolous, and M.N. Schroth.** 1994. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from Xanthomonas campestris pv. juglandis: homology with small blue copper proteins and multicopper oxidase. J. Bacteriol. **176**:173-188.

130. **Leff, L.G., J.R. Dana, J.V. McArthur, and L.J. Shimkets.** 1993. Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl. Environ. Microbiol. **59**:417-421.

131. **Legard, D.E., C.F. Aquadro, and J.E. Hunter.** 1993. DNA sequence variation and phylogenetic relationships among strains of Pseudomonas syringae pv. syringae inferred from restriction site maps and restriction fragment length polymorphism. Appl. Environ. Microbiol. **59**:4180-4188.

132. **Legard, D.E., and H.F. Schwartz.** 1987. Sources and management of Pseudomonas syringae pv. phaseolicola and Pseudomonas syringae pv. syringae epiphytes on dry beans in Colorado. Phytopathology **77**:1503-1509.

133. **Lester, S.C., M. del Pilar Pla, F. Wang, I.P. Schael, H. Jiang, and T.F. O'Brien.** 1990. The carriage of Escherichia coli resistant to antimicrobial agents by healthy children in Boston, in Caracas, Venezuela, and in Qin Pu, China. N. Engl. J. Med. **323**:285-289.

134. **Levy, S.B.** 1986. Ecology of antibiotic resistance determinants. Banbury Report 24:17-29. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

135. **Levy, S.B., G.B. Fitzgerald, and A.B. Macone.** 1976. Changes in intestinal flora of farm personnel after introduction of tetracycline-supplemented feed on a farm. New Engl. J. Med. **295**:583-587.

136. **Lim, C.-K., and D.A. Cooksey.** 1993. Characterization of chromosomal homologs of the plasmid-borne copper resistance operon of Pseudomonas syringae. J. Bacteriol. **175**:4492-4498.

137. **Lindemann, J., and T.V. Suslow.** 1987. Competition between ice nucleation-active wild type and ice nucleation-deficient deletion mutant strains of Pseudomonas syringae and P. fluorescens Biovar I and biological control of frost injury on strawberry blossoms. Phytopathology **77**:882-886.

138. **Lindow, S.E.** 1983. The role of bacterial ice nucleation in frost injury to plants. Annu. Rev. Phytopathol. **21**:363-384.

139. **Lindow, S.E.** 1987. Competitive exclusion of epiphytic bacteria by Ice$^-$ Pseudomonas syringae mutants. Appl. Environ. Microbiol. **53**:2520-2527.

140. **Lindow, S.E., D.C. Arny, and C.D. Upper.** 1983. Biological control of frost injury: an isolate of Erwinia herbicola antagonistic to ice nucleation active bacteria. Phytopathology **73**:1097-1102.

141. **Lindow S.E., G.R. Knudsen, R.J. Seidler, M.V. Walter, V.W. Lambou, P.S. Amy, D. Schedding, V. Prince, and S. Hern.** 1988. Aerial dispersal and epiphytic survival of Pseudomonas syringae during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Microbiol. **54**:1557-1563.

142. **Long, S.R., and B.J. Staskawicz.** 1993. Prokaryotic plant parasites. Cell **73**:921-935.

143. **Loper, J.E., M.D. Henkels, R.G. Roberts, G.G. Grove, M.J. Willet, 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.

144. **Lupski, J.R., and G.M. Weinstock.** 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol. **174**:4525-4529.

145. **Manceau, C. J.P. Paulin, and L. Gardan.** 1984. Effect of streptomycin sprays on leaf microflora of pear trees. Acta. Hortic. **151**:87-91.

146. **Manceau, C., L. Gardan, and M. Devaux.** 1986. Dynamics of RP4 transfer between Xanthomonas campestris pv. corylina and Erwinia herbicola in hazelnut tissues, in planta. Can. J. Microbiol. **32**:835-841.

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

148. **Mansvelt, E.L., and M.J. Hattingh.** 1986. Pear blossom blast in South Africa caused by Pseudomonas syringae pv. syringae. Plant Pathol. **35**:337-343.

149. **Marco, G.M., and R.E. Stall.** 1983. Control of bacterial spot of pepper initiated by strains of Xanthomonas campestris pv. vesicatoria that differ in sensitivity to copper. Plant Dis. **67**:779-781.

150. **Mariani, F., E. Piccolella, V. Colizzi, R. Rappuoli, and R. Gross.** 1993. Characterization of an IS-like element from Mycobacterium tuberculosis. J. Gen. Microbiol. **139**:1767-1772.

151. **Marshall, B., D. Petrowski, and S.B. Levy.** 1990. Inter- and intraspecies spread of Escherichia coli in a farm environment in the absence of antibiotic usage. Proc. Natl. Acad. Sci. USA **87**:6609-6613.

152. **Marshall, B., S. Schluederberg, C. Tachibana, and S.B. Levy.** 1981. Survival and transfer of poorly mobilizable (pBR322) and of transferable plasmids from the same carrier E. coli. Gene **14**:145-154.

153. **Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel.** 1990. Transposition of an antibiotic resistance element in mycobacteria. Nature(London) **345**:739-743.

154. **Mazodier, P., and J. Davies.** 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25**:147-171.

155. **Mazodier, P., R. Petter, and C. Thompson.** 1989. Intergeneric conjugation between Escherichia coli and Streptomyces species. J. Bacteriol. **171**:3583-3585.

156. **Mellano, M., and D.A. Cooksey.** 1988a. Induction of the copper resistance operon from Pseudomonas syringae. J. Bacteriol. **170**:4399-4401.

157. **Mellano, M., and D.A. Cooksey.** 1988b. Nucleotide sequence and organization of copper resistance genes from Pseudomonas syringae pv. tomato. J. Bacteriol. **170**:2879-2883.

158. **Melling, J., D.C. Elwood, and A. Robinson.** 1977. Survival of R-factor carrying Escherichia coli in mixed cultures in the chemostat. FEMS Microbiol. Lett. **2**:87-89.

159. **Menard, R., C. Molinas, M. Arthur, J. Duval, P. Courvalin, and R. LeClercq.** 1993. Overproduction of 3'-aminoglycoside phosphotransferase type I confers resistance to tobramycin in Escherichia coli. Antimicrob. Agents Chemother. **37**:78-83.

160. **Mew, T.W., and B.W. Kennedy.** 1982. Seasonal variation in populations of pathogenic pseudomonads on soybean leaves. Phytopathology **72**:013-105.

161. **Michel, M.F., C.M. Brasileiro, C. Depierreux, L. Otten, F. Delmotte, and L. Jouanin.** 1990. Identification of different Agrobacterium strains isolated from the same forest nursery. Appl. Environ. Microbiol. **56**:3537-3545.

162. **Miller, J.H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

163. **Mills, S.D., C.A. Jasalavich, and D.A. Cooksey.** 1993. A two component regulatory system required for copper inducible expression of the copper resistance operon of Pseudomonas syringae. J. Bacteriol. **175**:1656-1664.

163a. **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.

164. **Mishra, R.R., and R.P. Tewari.** 1979. Studies on phyllosphere microflora-effect of antibiotics and fungicides on leaf surface fungi and bacteria. Acta. Bot. Ind. **7**:57-63.

165. **Montgomery, H.B.S., and M.H. Moore.** 1945. The control of bacterial canker and leaf spot in sweet cherry. J. Pomol. **21**:155-163.

166. **Moore, L.W.** 1988. Pseudomonas syringae: disease and ice nucleation activity. Ornam. Northwest Newsletter **12**:3-16.

167. **Mukhopadhyay, P., Mukhopadhyay, M., and Mills, D.** 1990. Construction of a stable shuttle vector for high-frequency transformation in Pseudomonas syringae pv. syringae. J. Bacteriol. **172**:477-480.

168. **Murillo, J., and N.T. Keen.** 1994. Two native plasmids of Pseudomonas syringae pv. tomato strain PT23 share a large amount of repeated DNA, including replication sequences. Mol. Microbiol. (in press).

169. **Nesme, X., C. Ponsonnet, C. Picard, and P. Normand.** 1992. Chromosomal and pTi genotypes of Agrobacterium strains isolated from Populus tumors in two nurseries. FEMS Microbiol. Ecol. **101**:189-196.

170. **Norelli, J.L., T.J. Burr, A.M. LoCicero, 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.

171. **Numan, Z., W.A Venables, and J.W.T. Wimpenny.** 1991. Competition between strains of Escherichia coli with and without plasmid RP4 during chemostat growth. Can. J. Microbiol. **37**:509-512.

172. **Obukowicz, M., and P.D. Shaw.** 1985. Construction of Tn3-containing plasmids from plant pathogenic pseudomonads and examination of their biological properties. Appl. Environ. Microbiol. **49**:468-473.

173. **Palleroni, N.J.** 1984. Genus I. Pseudomonas Migula. pp. 141-199 In: Krieg, N.R., and J.G. Holt ed., Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore, MD.

174. **Paulus, F., M. Ride, and L. Otten.** 1989. Distribution of two Agrobacterium tumefaciens insertion elements in natural isolates: evidence for stable association between Ti plasmids and their bacterial hosts. Mol. Gen. Genet. **219**:145-152.

175. **Pintado, C., C. Salvador, R. Rotger, and C. Nombela.** 1985. Multiresistance plasmid from commensal Neisseria strains. Antimicrob. Agents Chemother. 27:120-124.

176. **Piwowarski, J.M., and P.D. Shaw.** 1982. Characterization of plasmids from plant pathogenic pseudomonads. Plasmid **7**:85-94.

177. **Quant, R.L., and D. Mills.** 1984. An integrative plasmid and multiple-sized plasmids of Pseudomonas syringae pv. phaseolicola have extensive homology. Mol. Gen. Genet. **193**:459-466.

178. **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.

179. **Radstrom, P., G. Swedberg, and O. Skold. 1991.** Genetic analyses of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. Antimicrob. Agents Chemother. **35**:1840-1848.

180. **Rezsohazy, R., B. Hallet, J. Delcour, and J. Mahillon.** 1993. The IS4 family of insertion sequences: evidence for a conserved transposase motif. Mol. Microbiol. **9**:1283-1295.

181. **Rich, J.J., and D.K. Willis.** 1990. A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn5 insertion by the polymerase chain reaction. Nucl. Acids Res. **18**:6673-6676.

182. **Ritchie, D.F., and V. Dittapongpitch.** 1991. Copper- and streptomycin-resistant strains and host differentiated races of Xanthomonas campestris pv. vesicatoria in North Carolina. Plant Dis. **75**:733-736.

183. **Romantschuk, M.** 1992. Attachment of plant pathogenic bacteria to plant surfaces. Annu. Rev. Phytopathol. **30**:225-243.

184. **Roos, I.M.M., and M.J. Hattingh.** 1986a. Bacterial canker of sweet cherry in South Africa. Phytophylactica **18**:1-4.

185. **Roos, I.M.M., and M.J. Hattingh.** 1986b. Pathogenic Pseudomonas spp. in stone fruit buds. Phytophylactica **18**:7-9.

186. **Roos, I.M.M., and M.J. Hattingh.** 1986c. Resident populations of Pseudomonas syringae on stone fruit tree leaves in South Africa. Phytophylactica **18**:55-58.

187. **Roos, I.M.M., and M.J. Hattingh.** 1987. Pathogenicity and numerical analysis of phenotypic features of Pseudomonas syringae strains isolated from deciduous fruit trees. Phytopathology **77**:900-908.

188. **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.

189. **Rouch, D.A., and R.A. Skurray.** 1989. IS257 from Staphylococcus aureus: member of an insertion sequence superfamily prevalent among gram-positive and gram-negative bacteria. Gene **76**:195-205.

190. **Rudolph, K.** 1979. Bacterial brown spot disease of bush bean (Phaseolus vulgaris L.) in Germany, incited by Pseudomonas syringae van Hall s.s. pathovar phaseoli. Z. Pflanzenkr. Pflanzenschutz **86**:75-85.

191. **Saad, S.M., and D.J. Hagedorn.** 1972. Relationship of isolate source to virulence of Pseudomonas syringae on Phaseolus vulgaris. Phytopathology **62**:678-680.

192. **Saano, A.K., and V.V. Zinchenko.** 1987. A new IncQ plasmid R89S: properties and genetic organization. Plasmid **17**:191-201.

193. **Sands, D.C., and J.L. McIntyre.** 1977. Possible methods to control pear blast, caused by Pseudomonas syringae. Plant Dis. Rep. **61**:311-312.

194. **Sato, M., B.J. Staskawicz, and N.J. Panopolous.** 1982. Indigenous plasmids of Pseudomonas syringae pv. mori, the causal agent of bacterial blight of mulberry. Ann. Phytopath. Soc. Japan **48**:27-33.

195. **Schaad, N.W.** (ed.). 1988. Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. American Phytopathological Society, St. Paul, Minn.

196. **Schafer, A., J. Kalinowski, R. Simon, A. Seep-Feldhaus, and A. Puhler.** 1990. High-frequency conjugal transfer from gram-negative Escherichia coli to various gram-positive coryneform bacteria. J. Bacteriol. **172**:1663-1666.

197. **Schofield, P.R., A.H. Gibson, W.F. Dudman, and J.M. Watson.** 1987. Evidence for genetic exchange and recombination of Rhizobium symbiotic plasmids in a soil population. Appl. Environ. Microbiol. **53**:2942-2947.

198. **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.

199. **Schroth, M.N., S.V. Thomson, and W.J. Moller.** 1978. Streptomycin resistance in Erwinia amylovora. Phytopathology **69**:565-568.

199a. **Shareck, F., M. Zollinger, and A. Sasarman.** 1983. Structure and molecular properties of the R.SmSu plasmid pSAS1206. Plasmid **9**:215-217.

200. **Sherratt, D.** 1989. Tn3 and other related transposable elements. pp. 163-184 In Berg, D.E., and Howe, M.M. (Eds.), Mobile DNA. American Society for Microbiology, Washington, D.C.

201. **Shields, D.C., and P.M. Sharp.** 1987. Synonymous codon usage in Bacillus subtilis reflects both translational selection and mutational biases. Nucl. Acids Res. **15**:8023-8039.

202. **Shlaes, D.M., and C.A. Currie-McCumber.** 1989. The molecular epidemiology of antimicrobial resistance, p. 387-410. In L.E. Bryan (ed.), Handbook of Experimental Pharmacology Vol. 91: Microbial Resistance to Drugs. Springer-Verlag, Berlin.

203. **Silver, S., B.T.O. Lee, N.L. Brown, and D.A. Cooksey.** 1993. Bacterial plasmid resistances to copper, cadmium, and zinc, p. 38-53. In A.J. Welch and S.K. Chapman (ed.), The Chemistry of the Copper and Zinc Triads. The Royal Society of Chemistry, London.

204. **Silverstone, S.E., D.G. Gilchrist, R.M. Bostock, and T. Kosuge.** 1993. The 73-kb pIAA plasmid increases competitive fitness of Pseudomonas syringae subspecies savastanoi in oleander. Can. J. Microbiol. **39**:659-664.

205. **Smalla, K., R. Prager, M. Isemann, R. Pukall, E. Tietze, J.D. van Elsas, and H. Tschape.** 1993a. Distribution of streptothricin acetyltransferase encoding determinants among environmental bacteria. Mol. Ecol. **2**:27-33.

206. **Smalla, K., L.S. van Overbeek, R. Pukall, and J.D. van Elsas.** 1993b. Prevalence of nptII and Tn5 in kanamycin-resistant bacteria from different environments. FEMS Microbiol. Ecol. **13**:47-58.

207. **Smith, H.R., G.O. Humphreys, and E.S. Anderson.** 1974. Genetic and molecular characterisation of some non-transferring plasmids. Mol. Gen. Genet. **129**:229-242.

208. **Sneath, P.H.A., and R.R. Sokal.** 1973. Numerical taxonomy. Freeman, San Francisco.

209. **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.

210. **Stall, R.E., D.C. Loschke, and J.B. Jones.** 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in Xanthomonas campestris pv. vesicatoria. Phytopathology **76**:240-243.

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

212. **Staskawicz, B.J., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of Pseudomonas syringae pv. glycinea. J. Bacteriol. **169**:5789-5794.

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

214. **Sundin, G.W., and Bender, C.L.** 1991. Abstr. Annu. Meet. Am. Phytopathol. Soc. 1991, vol 81, p. 1145.

215. **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.

216. **Sundin, G.W., and C.L. Bender.** 1994. Relative fitness in vitro and in planta of Pseudomonas syringae strains containing copper and streptomycin resistance plasmids. Can. J. Microbiol. **40**:(in press).

217. **Sundin, G.W., and C.L. Bender.** Unpublished data.

218. **Sundin, G.W., A.L. Jones, and D.W. Fulbright.** 1989. Copper resistance in Pseudomonas syringae pv. syringae from cherry orchards and its associated transfer in vitro and in planta with a plasmid. Phytopathology **76**:861-865.

219. **Sundin, G.W., A.L. Jones, and B.D. Olson.** 1988. Overwintering and population dynamics of Pseudomonas syringae pv. syringae and P. s. pv. morsprunorum on sweet and sour cherry trees. Can. J. Plant Pathol. **10**:281-288.

220. **Syvanen, M.** 1984. The evolutionary implications of mobile genetic elements. Annu. Rev. Genet. **18**:271-294.

221. **Szabo, L.J., and D. Mills.** 1984. Characterization of eight excision plasmids of Pseudomonas syringae pv. phaseolicola. Mol. Gen. Genet. **195**:90-95.

222. **Timoney, J., and A. Linton.** 1982. Experimental ecological studies on H2 plasmids in the intestine and faeces of the calf. J. Appl. Bacteriol. **52**:417-424.

223. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from Escherichia coli to gram-positive bacteria. FEMS Microbiol. Lett. **48**:289-294.

224. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. In vivo transfer of genetic information between gram-positive and gram-negative bacteria. EMBO J. **4**:3583-3587.

225. **Ullrich, M., S. Bereswill, B. Volksch, W. Fritsche, and K. Geider.** 1993. Molecular characterization of field isolates of Pseudomonas syringae pv. glycinea differing in coronatine production. J. Gen. Microbiol. **139**:1927-1937.

226. **van den Eede, G., R. Deblaere, K. Goethals, M. van Montagu, and M. Holsters.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant-Microbe Interact. **5**:228-234.

227. **van Treeck, U., F. Schmidt, and B. Wiedemann.** 1981. Molecular nature of a streptomycin and sulfonamide resistance plasmid (pBP1) prevalent in clinical Escherichia coli strains and integration of an ampicillin resistance transposon (TnA). Antimicrob. Agents Chemother. **19**:371-380.

228. **Versalovic, J., T. Koeuth, and J.R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acids Res. **19**:6823-6831.

229. **Voloudakis, A.E., C.L. Bender, and D.A. Cooksey.** 1993. Similarity between copper resistance genes from Xanthomonas campestris and Pseudomonas syringae. Appl. Environ. Microbiol. **59**:1627-1634.

230. **von Bodman, S.B., and P.D. Shaw.** 1987. Conservation of plasmids among plant-pathogenic Pseudomonas syringae isolates of diverse origins. Plasmid **17**:240-247.

231. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. **18**:7213-7218.

232. **Welsh, J., C. Pretzman, D. Postic, I.S. Girons, G. Baranton, and M. McClelland.** 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves Borrelia burgdorferi into three distinct phyletic groups. Int. J. Syst. Bacteriol. **42**:370-377.

233. **Whitesides, S.K., and R.A. Spotts.** 1991. Induction of pear blossom blast caused by Pseudomonas syringae pv. syringae. Plant Pathol. **40**:118-127.

234. **Willetts, N. and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48**:24-41.

235. **Williams, D.R., and C.M. Thomas.** 1992. Active partitioning of bacterial plasmids. J. Gen. Microbiol. **138**:1-16.

236. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. **18**:6531-6535.

237. **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.

238. **Wimalajeewa, D.L.S., R. Cahill, G. Hepworth, H.G. Schneider, and J.W. Washbourne.** 1991. Chemical control of bacterial canker (Pseudomonas syringae pv. syringae) of apriocot and cherry in Victoria. Aust. J. Expt. Agric. **31**:705-708.

239. **Wimalajeewa, D.L.S., and J.D. Flett.** 1985. A study of populations of Pseudomonas syringae pv. syringae on stone fruit in Victoria. Plant Pathol. **34**:248-254.

240. **Wishart, W.L., C. Machida, H. Ohtsubo, and E. Ohtsubo.** 1983. Escherichia coli RNA polymerase binding sites and transcription initiation sites in the transposon Tn3. Gene **24**:99-113.

241. **Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tabguay, P.H. Roy, G.C. Gamboa, G.F. Barry, E. Aubert, J. Davies, and S.A. Kagan.** 1989. On the evolution of Tn21-like multiresistance transposons: sequence analysis of the gene (aacC1) for gentamicin acetyltransferase-3-I(AAC(3)-I), another member of the Tn21-based expression cassette. Mol. Gen. Genet. **217**:202-208.

242. **Wouters, J.T.M., and J.G. van Andel.** 1979. R-plasmid persistence in Escherichia coli grown in chemostat cultures. Ant. van Leeuwenhoek **45**:317-318.

243. **Wright, F., and M.J. Bibb.** 1992. Codon usage in the G + C-rich Streptomyces genome. Gene **113**:55-65.

244. **Xiao, Y., Y. Lu, S. Heu, and S.W. Hutcheson.** 1992. Organization and environmental regulation of the Pseudomonas syringae pv. syringae 61 hrp cluster. J. Bacteriol. **174**:1734-1741.

245. **Yano, H., H. Fujii, H. Mukoo, M. Shimura, T. Watanabe, and Y. Sekizawa.** 1978. On the enzymic 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.

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

247. **Young, J.P.W., and M. Wexler.** 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of Rhizobium leguminosarum. J. Gen. Microbiol. **134**:2731-2739.

248. **Zevenhuizen, L.P.T.M., J. Dolfing, E.J. Eshuis, and I.J. Scholten-Koerselman.** 1979. Inhibitory effects of copper on bacteria related to the free ion concentration. Microb. Ecol. **5**:139-146.

249. **Zilhao, R., B. Papadopoulou, and P. Courvalin.** 1988. Occurrence of the Campylobacter resistance gene tetO in Enterococcus and Streptococcus spp. Antimicrob. Agents Chemother. **32**:1793-1796.

250. **Zund, P., and G. Lebek.** 1980. Generation time-prolonging R plasmids: correlation between increases in generation time of Escherichia coli caused by R plasmids and their molecular size. Plasmid **3**:65-69.

VITA 2

George W. Sundin

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EVOLUTION OF COPPER AND STREPTOMYCIN RESISTANCE IN *PSEUDOMONAS SYRINGAE*

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Philadelphia, Pennsylvania, November 8, 1964, the son of Carl and Motoko Sundin.

Education: Graduated from Bishop Eustace Preparatory School, Pennsauken, New Jersey in May 1982; received Bachelor of Science degree in Biology from the Pennsylvania State University, State College, Pennsylvania in May 1986; received Master of Science degree in Plant Pathology from Michigan State University, East Lansing, Michigan, in December 1988. Completed the requirements for the Doctor of Philosophy degree with a major in Plant Pathology at Oklahoma State University in May 1994.

Experience: Undergraduate research assistant, Department of Biology, The Pennsylvania State University 1985-86; Graduate research assistant, Department of Botany and Plant Pathology, Michigan State University 1986-88; Associate Scientist, Crop Genetics International, Hanover, Maryland 1989-90; Graduate research assistant, Department of Plant Pathology, Oklahoma State University, 1990-94.

Professional Society Memberships: American Phytopathological Society, American Society for Microbiology, American Association for the Advancement of Science, Oklahoma Academy of Science, Gamma Sigma Delta, Phi Kappa Phi.

VITA

George W. Sundin

Candidate for the Degree of

Doctor of Philosophy

(continued)

Biographical:

Awards: Oklahoma State University Foundation Scholarship, 1990; Oklahoma State University Foundation Miller Distinguished Graduate Student Fellowship, 1991-94; Oklahoma State University Graduate Research Excellence Award, 1994.