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Antibiotic Resistance in Plant-Pathogenic Bacteria

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Abstract

Antibiotics have been used for the management of relatively few bacterial plant diseases and are largely restricted to high-value fruit crops because of the expense involved. Antibiotic resistance in plant-pathogenic bacteria has become a problem in pathosystems where these antibiotics have been used for many years. Where the genetic basis for resistance has been examined, antibiotic resistance in plant pathogens has most often evolved through the acquisition of a resistance determinant via horizontal gene transfer. For example, the *strAB* streptomycin-resistance genes occur in *Erwinia amylovora*, *Pseudomonas syringae*, and *Xanthomonas campestris*, and these genes have presumably been acquired from nonpathogenic epiphytic bacteria colocated on plant hosts under antibiotic selection. We currently lack knowledge of the effect of the microbiome of commensal organisms on the potential of plant pathogens to evolve antibiotic resistance. Such knowledge is critical to the development of robust resistance management strategies to ensure the safe and effective continued use of antibiotics in the management of critically important diseases.

INTRODUCTION

The classical definition of antibiotics by Waksman, the discoverer of streptomycin in 1944, is "a compound produced by a microbe with killing or growth-inhibiting activity against other microbes" (125). Following the discovery and deployment of penicillin, streptomycin, and the sulfonamides in clinical medicine, antibiotics were quickly viewed as silver bullets that would eradicate all infectious diseases (57, 126). Indeed, antibiotic therapy has played a significant role in curing diseases and saving lives and continues to be critically important today in clinical medicine and animal and plant agriculture. However, the enthusiasm concerning antibiotic use has been eroded by the widespread development of antibiotic resistance; this has become most critical from a human health perspective in clinical bacterial pathogens.

Antibiotic resistance most commonly evolves in bacteria either through mutation of a target-site protein, through the acquisition of an antibiotic-resistance gene (ARG) that confers resistance through efflux or inactivation of the antibiotic, or through synthesis of a new target protein that is insensitive to the antibiotic (21). An extensive body of knowledge has been gained from studies of antibiotic resistance in human pathogens and in animal agriculture. The ability of bacterial pathogens to acquire ARGs and to assemble them into blocks of transferable DNA encoding multiple ARGs has resulted in significant issues that affect successful treatment interventions targeting some specific human infections. The current global antibiotic resistance crisis in bacterial populations has been fueled by basic processes in microbial ecology and population dynamics, engendering a rapid evolutionary response to the global deployment of antibiotics by humans in the millions of kilograms per year. What was not anticipated when antibiotics were discovered and introduced into clinical medicine is that ARGs preexisted in bacterial populations (6, 54, 83). Furthermore, the extent to which ARGs could be transferred between bacteria, and even between phylogenetically distinct bacteria, was not understood 70 years ago but is becoming more apparent through a number of elegant studies identifying the microbial antibiotic resistome. The collection of all known ARGs in the full-microbial pan-genome is defined as the antibiotic resistome (132). What is most important conceptually about the antibiotic resistome is the potential accessibility of individual ARGs to all bacteria.

In this review, we focus on our current knowledge of the evolution of antibiotic resistance in plant-pathogenic bacteria. To frame this topic, we must first detail our understanding of the evolution of antibiotic resistance, namely that antibiotic selection impacts ecosystems and not just individual bacterial pathogens and that this ecosystem selection has affected the collective evolution of antibiotic resistance in bacterial communities, which ultimately impacts individual bacterial pathogens. We also elaborate on the concept of the antibiotic resistome, discussing the impact of the resistome on the evolution of antibiotic resistance in animal agriculture systems and identifying gaps in our knowledge of the resistome in plant agricultural systems.

ANTIBIOTIC RESISTANCE MECHANISMS

Depending on the modes of action, structures, and biochemical properties of different antibiotics, bacteria encode different resistance mechanisms. Those antibiotic resistance mechanisms can be classified into the following major strategies: modifications of the antimicrobial molecule, prevention of the antibiotic from reaching its cellular target (by reducing uptake or active export of the antimicrobial compound), synthesis of an antibiotic-insensitive alternate target protein, protection of the target, and alteration of the target protein via mutation (71, 73). The frequency of occurrence of a particular resistance mechanism is dependent upon the antibiotic; for example, 28 different classes of efflux proteins have been shown to be involved in tetracycline resistance in gram-negative and gram-positive bacteria (37), but this mode of action is not utilized for

streptomycin resistance. Because streptomycin and oxytetracycline are the most widely used antibiotics in plant agriculture, we briefly describe the important resistance mechanisms known for these two antibiotics.

Antibiotic Resistance Against Streptomycin

Streptomycin is an aminoglycoside antibiotic produced by *Streptomyces griseus* and was one of the first antibiotics discovered (in 1944) (92). Streptomycin is a broad-spectrum antibiotic with activity against both gram-negative and gram-positive bacteria. The streptomycin antibiotic functions as an inhibitor of protein synthesis and binds within the ribosome to four nucleotides of the 16S RNA and the ribosomal protein S12 (11). As a human therapeutic drug, streptomycin has most often been utilized in the chemotherapy of tuberculosis and can also be administered in the treatment of other diseases, including tularemia and plague. Streptomycin was initially evaluated for the control of bacterial diseases of plants in the early 1950s (55), and by the late 1960s, it was deployed for the management of fire blight in apple and pear orchards (69). Streptomycin resistance is distributed on a global scale and has been characterized in clinical, animal, and plant pathogens as well as a wide range of environmental bacteria. Here, we describe the most commonly encountered mechanisms of resistance to streptomycin.

Enzymatic inactivation of streptomycin. The majority of known streptomycin resistance determinants encode enzymes that confer resistance through inactivation of the streptomycin molecule through either phosphorylation or adenylylation (100). Streptomycin is an antibiotic that is produced naturally in soil by *Streptomyces griseus*, and the phosphotransferase enzymes Aph(6)-Ia and Aph(6)-Ib were cloned from *S. griseus* and *Streptomyces glaucescens* (hydroxystreptomycin producer), respectively (**Table 1**) (100). These enzymes presumably evolved as self-protection mechanisms for the antibiotic-producing streptomycetes; their escape to other organisms via horizontal gene transfer represents one method in which horizontal gene transfer (HGT) facilitated the evolution of antibiotic resistance (5). On a global scale, the two most widely distributed streptomycin-resistance determinants are the *strAB* gene pair (also reported as *strA-strB*) and the *aadA* (and variant alleles) gene (**Table 1**). *strAB* is associated with the transposon Tn*5393* and with small, nonconjugative broad-host-range plasmids such as pBP1 and RSF1010 (114). *strAB* most commonly occurs as a gene pair and is sometimes linked to the sulfonamide-resistance gene *sul2*; the *sul2-strA-strB* gene organization is present on plasmid RSF1010 (96), but *sul2* is not found within Tn*5393* (13). These genes are detected in almost any culture-independent sequencing experiment assessing the presence of antibiotic resistance in environmental and agricultural habitats. *strAB* has also been detected in bacteria recovered from permafrost environments (85), signifying that this gene combination evolved long before the introduction of antibiotic use through human activity. The *aadA* gene encodes resistance to both streptomycin and spectinomycin and is associated with integrons, which are mobile genetic elements that have increased in frequency because of an ability to acquire and add additional resistance genes as cassettes (26, 38). *aadA* is located on a conserved region of the integron, thus facilitating its rapid increase in frequency through coselection with other antibiotic resistance determinants. Three other streptomycin-resistance determinants, *aph*(*6*)*-1c*, *ant*(*3″*), and *ant*(*6*), are more limited in distribution at the current time (**Table 1**).

Spontaneous resistance to streptomycin. Mutational resistance to streptomycin also occurs in bacteria in some cases and can be important clinically or in agricultural situations. Mutations in the *rrs* or *rpsL* genes that lead to an alteration of the streptomycin binding site in the ribosome are most commonly associated with spontaneous streptomycin resistance (78). Likely the most important example of mutational streptomycin resistance occurs in the tuberculosis pathogen *Mycobacterium*

Table 1 Streptomycin resistance genes, the enzyme they encode, and representative bacterial genera, transposons, and plasmids known to harbor each gene. Genus names in bold contain plant pathogens or plant-associated bacteria

Gene name	Enzyme, function	Representative bacterial genera, transposons, and plasmids harboring these genes[a]
strA [*aph*(3″) *strB*, *aph(6)-1d*]	Phosphotransferase (enzymes typically occur as a gene pair)	*Actinobacillus*, *Aeromonas*, *Alcaligenes*, *Bordetella*, *Brevibacterium*, *Brevundimonas*, *Citrobacter*, *Corynebacterium*, *Dietzia*, *Eikenella*, *Enterobacter*, ***Erwinia***, *Escherichia*, *Haemophilus*, *Klebsiella*, *Moraxella*, *Ochrobactrum*, *Neisseria*, ***Pantoea***, *Pasteurella*, *Proteus*, *Providencia*, ***Pseudomonas***, *Salmonella*, *Shigella*, ***Xanthomonas*** Tn*5393* pBP1, R300B, RSF1010
aph(6)-1a	Phosphotransferase	*Streptomyces*
aph(6)-1b	Phosphotransferase	*Streptomyces*
aph(6)-1c	Phosphotransferase	*Citrobacter*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, *Salmonella* Tn*5*
ant(3″)	Nucleotidyltransferase	*Aeromonas*, *Citrobacter*, *Enterobacter*, *Leclaria*, *Proteus*, *Providencia*, *Rhodococcus* Tn*1826*
ant(6)	Nucleotidyltransferase	*Lactococcus*, *Staphylococcus*
aadA and variant alleles	Nucleotidyltransferase	*Acinetobacter*, *Campylobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Salmonella*, ***Xanthomonas*** Tn7, Tn*21*, Tn*2670*, Tn*1401* R1, R100, R483

[a]Distribution data of specific genes among bacterial genera, transposons, and plasmids were taken from the following sources: 42, 62, 82, 88, 106, 114, 123, and 131.

tuberculosis (35). Mutational resistance to streptomycin also occurs in *E. amylovora* populations in the western United States and also occurs in low frequencies in populations in Michigan (14, 69). This high-level resistance enables strains to grow in the presence of as much as 4,096 ppm streptomycin (14).

Antibiotic Resistance Against Oxytetracycline (Tetracycline)

Tetracycline antibiotics are broad-spectrum agents and polyketide in nature and exhibit antimicrobial activity against gram-negative and gram-positive bacteria, spirochetes, and obligate intracellular bacteria as well as protozoan parasites (15, 36). Tetracyclines bind to the ribosome and inhibit translation by preventing the binding of aminoacylated tRNA to the A site (15). Both the bacteriostatic and bactericidal effect have been reported for tetracyclines (36). The tetracyclines were first isolated from *Streptomyces aureofaciens* in the 1940s (25), whereas oxytetracycline was discovered in 1950 (28). Many more tetracycline derivatives were produced either by actinomycetes (tetracycline and demethylchlortetracycline) or synthesis (methacycline, rolitetracyclinee, lymecycline, doxycycline, and minocyclineeravacycline) (16, 20, 51, 72). Tetracycline resistance has been shown to be prevalent in clinical environments, which has rendered several tetracycline derivatives currently unusable. However, tetracycline resistance in agriculture seems not to be such an alarming issue. Mainly based on clinical studies, tetracycline resistance has resulted from mutations or horizontal gene transfer events affecting transportation and mechanism of action. Here, we summarize some major mechanisms underlying tetracycline resistance.

Prevention against reaching the tetracycline target. Efflux is one of the major determinants for tetracycline resistance, functioning to expel tetracycline from the cell. Twenty-eight different

classes of efflux proteins have been shown to be involved in tetracycline resistance in gram-negative and gram-positive bacteria (37). Among them, *tetA* is the most widespread determinant encoding tetracycline-resistance efflux in gram-negative bacteria, and this gene has been identified in more than 1,000 bacterial species.

Tetracyclines are hydrophilic molecules that often use water-filled diffusion channels (porins) to cross the outer membrane (79). Some bacteria have mechanisms utilizing the outer membrane and its accessories (lipopolysaccharides) to decrease the uptake and penetration of tetracyclines. Mutation of the OmpF porin protein reduces the uptake of tetracycline by *E. coli* cells (120). In addition, tetracycline is also reported to enter cells as an uncharged form by diffusion through the outer membrane lipid barrier (74).

Protection of the cellular target of tetracycline. Ribosomal protection is another major determinant for tetracycline resistance in both gram-positive and gram-negative species. Twelve distinct classes of ribosome protection proteins (RPPs) have been reported to confer resistance to tetracycline. RPPs share high homology among themselves and might have been derived from OtrA, which confers tetracycline resistance in *Streptomyces rimosus*, a native tetracycline producer (23). RPPs are similar to elongation factors and also to GTPases. RPPs bind and hydrolyze GTP in a ribosome-dependent manner (8, 9). RPPs confer tetracycline resistance by dislocating tetracycline from the ribosome, thus liberating the ribosome from the inhibitory effects of tetracycline, such that aa-tRNA can bind to the A site and protein synthesis can continue (19). The ability of RPPs to dislodge tetracycline is strictly dependent on the presence of GTP (9, 122). The most common RPPs are TetO and TetM (18, 19).

Modifications of the tetracycline molecule. It has been reported that *Bacteroides* encodes a flavin-dependent monooxygenase (104, 135). The monooxygenase hydroxylates tetracyclines in the presence of NADPH and O_2. The hydroxylated tetracycline has reduced affinity for the ribosome and also undergoes a nonenzymatic decomposition (73). Two tetracycline-modifying monooxygenase genes, *tetX* and *tet37*, have been reported (104, 135).

Changes to target sites of tetracycline. Tetracyclines bind the decoding center of the small subunit to cause translation arrest. Tetracyclines bind at one primary binding site and multiple secondary sites on the 30S subunit (7, 86). The primary binding pocket might consist of G693, A892, U1052, C1054, G1300, and G1338 of 16S rRNA (68, 76). In the primary binding site, the hydrophilic surface of tetracycline interacts with the irregular minor groove of helix 34 and the loop of helix 31 of the 16S rRNA. Mutations of interaction sequences of 16S rRNA (G1058C, A926T, G927T, A928C, and ΔG942) have abolished the interaction of tetracycline with the rRNA, thus conferring resistance to the antibiotic (73).

Besides the aforementioned mechanisms, changes within intrinsic regulatory networks reduce the uptake and intracellular accumulation of tetracycline, thus affecting bacterial resistance to tetracycline. Owing to their indirect contribution, those mechanisms are not discussed here, but the readers are referred to several excellent reviews on this topic (e.g., 15, 36).

ORIGIN AND ECOLOGY OF ANTIBIOTIC RESISTANCE DETERMINANTS

Antibiotic resistance currently observed in bacterial pathogens has evolved from three major resources: the escape through horizontal gene transfer of natural resistance genes encoded by the antibiotic-producing microbes, the presence and ultimate movement of resistance genes extant

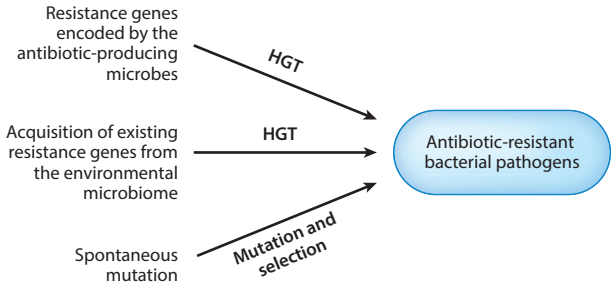

Figure 1

Schematic illustration of the origin of antibiotic-resistant bacterial pathogens. Abbreviation: HGT, horizontal gene transfer.

within the microbiome to pathogenic organisms under antibiotic selection, and mutations encoding target-site alterations (**Figure 1**). Today, most of the medical and agricultural antibiotics in use are either derived from or produced by soil actinomycete bacteria. These organisms may be the original source of many ARGs; natural ARGs are assumed to have evolved billions of years ago and coevolved with antibiotic production in bacteria that originally functioned in a self-protection mechanism (5, 6, 46). For example, *S. rimosus* is known to carry multiple tetracycline-resistant determinants, including *otrA*, *otrB*, and *otrC* (67, 77). The original escape of these natural ARGs is hypothesized to provide the origins of known antibiotic-modifying enzymes that exist today.

ARGs have been detected in present day pristine environments and in ancient samples, including metagenome samples from 30,000-year old permafrost (22), a separate sample of 5,000-year old permafrost (84), and the gut microbiome of an approximately 900-year old mummy (91). ARGs have been identified in the microbiome associated with different environments, including soil, Antarctic ice, phyllosphere, insects, animals, and humans (1, 10, 24, 27, 33, 127). ARGs mainly cluster by ecology; for example, the suite of ARGs present in soils and wastewater treatment plants is significantly distinct from that of human pathogens (34, 70). The human and mammalian gut microbiomes contain the widest diversity of clinically relevant ARGs, and human microbiomes also harbor a high relative abundance of ARGs, whereas abundance varies greatly in the marine and soil microbiomes (29).

It is well accepted that one important source for ARGs found in human-pathogenic bacteria is the environment (133), with soil bacteria serving as the most important reservoir (31). In fact, many ARGs in pathogenic bacteria originated from nonpathogenic bacterial sources in the environmental microbiome and were acquired by HGT (40, 87). The initial HGT of ARGs likely happened long before the production and use of antibiotics by humans. For example, it has been reported that plasmid-borne Oxa-type β-lactamase resistance was able to transfer between bacterial species millions of years ago (3). More recently, the massive deployment of antibiotics by humans has resulted in increases in the abundance of ARGs and likely in the breadth of organisms to which these ARGs have been disseminated. In a study of soil samples collected between 1940 and 2008, the frequency of specific ARGs conferring resistance to β-lactams, tetracyclines, erythromycins, and glycopeptides significantly increased, with one tetracycline ARG approximately 15 times more abundant in 2008 compared to the 1970s (54). As detailed below, the use of antibiotics in plant agriculture also impacted plant environments and, in some cases, has resulted in the evolution of antibiotic resistance through spontaneous mutation or via the acquisition of ARGs by plant-pathogenic bacteria.

ANTIBIOTIC USE IN PLANT AGRICULTURE

Effective management of bacterial plant diseases is difficult and is exacerbated by factors such as the large size of bacterial pathogen populations on susceptible plant hosts and the few available bactericides. In the absence of durable and robust host disease resistance, antibiotics have represented the best option for bacterial disease control in many pathosystems because these materials provide the most efficacious means of reducing bacterial population size and limiting disease outbreaks. Although many new types of antibiotics were rapidly tested and then deployed in animal agriculture starting in the 1950s, antibiotic use for plant disease control was tempered by several factors, including lack of efficacy at lower doses, phytotoxicity problems at higher doses, and expense compared to other existing methods of disease control. Thus, although penicillin, streptomycin, aureomycin, chloramphenicol, and oxytetracycline were tested for plant disease control in the late 1940s (2, 55), only streptomycin and oxytetracycline were ultimately deployed in plant agriculture and only in specific disease pathosystems.

Streptomycin is the main antibiotic currently in use for plant disease control around the world, targeting pathogens such as *Erwinia amylovora*, which causes fire blight of apple and pear; *Pseudomonas syringae*, which causes flower and fruit infection of apple and pear trees; and *Xanthomonas campestris*, which causes bacterial spot of tomato and pepper (66). Oxytetracycline has been used as the primary antibiotic in specific disease control situations, including the control of *Xanthomonas arboricola* pv. *pruni*, causal agent of bacterial spot of peach and nectarine (66). In addition, oxytetracycline has been used as a secondary antibiotic for fire blight management in the United States, most prominently in situations in which streptomycin resistance has become a problem (65, 69). More recently, in 2016, a Section 18 emergency exemption was granted by the US Environmental Protection Agency for the use of streptomycin and oxytetracycline on citrus trees in Florida for management of citrus Huanglongbing (HLB) disease (44, 45, 129).

Regarding other antibiotics, gentamicin has been used in Mexico for fire blight control and in Chile, Mexico, and Central American countries for vegetable disease control, while oxolinic acid (OA) has been used only in Israel for fire blight management (101, 124). Lastly, kasugamycin is used in Japan and other Asian countries to control the fungal disease rice blast and bacterial seedling diseases of rice (49) and has recently been registered for use in the United States and Canada for managing fire blight (64). Concerns regarding the use of antibiotics in plant disease control and potential impacts on human health have led to the banning of antibiotic use by the European Union. However, streptomycin is still utilized for fire blight management in Austria, Germany, and Switzerland under strict control parameters.

EVOLUTION OF ANTIBIOTIC RESISTANCE IN PLANT-PATHOGENIC BACTERIA

As discussed above, bacteria typically evolve resistance to antibiotics either through spontaneous mutation generating an altered target site or through acquisition of a resistance gene that may confer resistance through modification of the antibiotic, efflux of the antibiotic, or synthesis of a substitute nonsusceptible target. What we have learned over the years in working with the evolution and dissemination of antibiotic resistance in plant-pathogenic bacteria is that plant pathogens have also been able to access ARGs from the environmental gene pool and reorganize particular resistance determinants within their genome.

Streptomycin Resistance

The lack of effective bactericide alternatives in several plant disease systems has resulted in a decades-long dependence or overdependence on streptomycin. As streptomycin has been used

Table 2 Reports of antibiotic resistance in plant-pathogenic bacteria

Antibiotic	Organism	Location	Genetic mechanism	Reference
Kasugamycin	*Acidovorax avenae* ssp. *avenae*	Japan	*aac(2′)-IIa*	138
	Burkholderia glumae	Japan	*aac(2′)-IIa*	138
Oxolinic acid	*Erwinia amylovora*	Israel	Probable chromosomal mutation	53
	Burkholderia glumae	Israel	Probable chromosomal mutation	53
		Japan	Probable chromosomal mutation	41
Streptomcyin	*E. amylovora*	California, USA	Chromosomal mutation	97
		California, USA	*rpsL* mutation	14
		Michigan, USA	*rpsL* mutation	14
		Oregon, USA	*rpsL* mutation	14
		Washington, USA	*rpsL* mutation	14
		New Zealand	*rpsL* mutation	14
		California, USA	*strAB* on plasmid RSF1010	80
		California, USA	Tn*5393*a	32
		Michigan, USA	Tn*5393* on pEa34	13
		Michigan, USA	Tn*5393* on pEa29	63
		New York, USA	Tn*5393* on pEa29	119
	Pseudomonas syringae			
	P. syringae	Oregon, USA	*strAB*[a]	93
	P. syringae pv. *actinidiae*	Japan	Tn*5393*a	39
		Japan	*rpsL* mutation	
	P. syringae pv. *papulans*	New York, USA	*strAB*[b]	75
		Michigan, USA	*strAB*[b]	52
	P. syringae pv. *syringae*	Oklahoma, USA	Tn*5393*a	111
	X. axonopodis pv. vesicatoria	Argentina	Tn*5393*b	113
	X. citri subsp. *citri*	Korea	*strB*[c]	48
	X. oryzae pv. *oryzae*	China	*aadA1*	134

[a]Presence of the *strAB* genes was determined by hybridization, but structural genes of Tn*5393* were not screened for.
[b]The probe SMP3 was utilized to detect streptomycin resistance; this probe contains portions of the *strA* and *tnpR* genes from Tn*5393*a.
[c]Presence of the *strB* gene was determined by PCR but *strA* or structural genes of Tn*5393* were not screened for.

the longest, over the largest geographic area, and for treatment of the largest variety of crops, streptomycin resistance is relatively widespread among plant-pathogenic bacteria. Although the first streptomycin-resistant (Sm^R) plant-pathogenic bacteria detected were strains of *E. amylovora* harboring a chromosomal resistance mutation, the majority of Sm^R plant pathogens encode the transmissible Sm^R transposon Tn*5393* (66) (**Table 2**). Tn*5393* is a Tn*3*-type transposon originally isolated from *E. amylovora* that harbors *strAB*, a tandem resistance gene pair that confers streptomycin resistance through covalent modification of the streptomycin molecule (13). The Tn*5393* transposon is composed of genes required for the transposition process (*tnpA* and *tnpR*), a central site that contains outwardly directed promoters for expression of both *tnpA* and *tnpR* as well as the *strAB* Sm^R genes. Expression of the *strAB* genes from Tn*5393* in *E. amylovora* is driven by a promoter present in the 3′ end of the insertion sequence IS*1133* that is inserted directly upstream of the *strA* gene (113). Two closely related variants of Tn*5393* have also been found in plant pathogens: Tn*5393*a, an element that does not contain IS*1133*, has been detected in *P. syringae*

and in a group of *E. amylovora* strains from California exhibiting a moderate level of resistance, and Tn*5393*b, an element that does not contain IS*1133* but instead contains an insertion of IS*6100* within the *tnpR* gene, has been characterized in *X. campestris* (32, 113).

There are two other reports of additional genetic mechanisms of streptomycin resistance in plant pathogens; these include the occurrence of the small, nonconjugative but mobilizable broad-host-range plasmid RSF1010 in some strains of *E. amylovora* isolated in California (80) (**Table 2**). This observation carries further significance because RSF1010 has been distributed globally among a number of bacterial genera and also occurs in some human-pathogenic bacteria (114). A recent report detailing an analysis of streptomycin-resistant *X. oryzae* subsp. *oryzae* from China indicated that four strains harbored the *aadA1* gene associated with class 1 integron sequences (134) (**Table 2**). This observation is significant because of the importance of integrons in both the transfer of antibiotic resistance in human and animal pathogens and the accumulation of antibiotic resistance genes within one multiresistance element.

To date, streptomycin resistance mediated by Tn*5393* or the closely related variants, has been reported in *E. amylovora*, *P. syringae*, and *X. campestris* isolated from North and South America and Asia (32, 39, 63, 108, 109, 111, 113, 115, 119). The location of essentially the same genetic element in different genera of plant pathogens isolated from distinct crop hosts and from different continents is confirmatory evidence of the role of horizontal gene transfer (HGT) in the dissemination of antibiotic resistance in these pathosystems. The source of Tn*5393* to the plant pathogens was likely not from the antibiotic preparations themselves as a study of 18 available agricultural streptomycin formulations revealed no contamination with the *strA* Sm^R gene (89). Instead, the acquisition of Tn*5393* by bacterial plant pathogens was likely from commensal co-occurring epiphytic bacteria via HGT. For example, Tn*5393* was thought to have been acquired by *E. amylovora* on the plasmid pEa34 from *Pantoea agglomerans*, a common orchard epiphyte (13). The transfer event most likely occurred on the apple flower stigma, a surface where *E. amylovora* grows to high population densities and where *Pantoea agglomerans* can also grow. *Pseudomonas syringae* and *X. campestris* pv. *vesicatoria* both have epiphytic phases where the pathogens grow on leaf surfaces, providing opportunities for HGT with other epiphytes.

It should be noted that high-level streptomycin resistance, conferred by a spontaneous mutation within the *rpsL* gene that encodes the ribosomal target protein for streptomycin, does occur in some populations of *E. amylovora*, particularly within populations from the western United States as well as in a small number of strains isolated in Michigan and New Zealand (14, 69, 97). The minimal inhibitory concentration (MIC) of streptomycin in these highly resistant spontaneous mutants is greater than 4,096 μg/mL (14). In contrast, Sm^R strains of *E. amylovora* harboring Tn*5393* exhibit MICs of streptomycin ranging from 512 to 1,024 μg/mL (14). Streptomycin solutions used for fire blight management are typically applied at 100 μg/mL; thus, it is unclear whether the increased level of resistance exhibited by the spontaneous mutants provides a survival advantage in streptomycin-treated orchards.

Tetracycline Resistance

Tetracycline resistance has been reported in a few plant-pathogenic bacteria, including *P. syringae* (47, 105) and *Agrobacterium tumefaciens* (59) (**Table 2**). Other studies have reported on sensitivity; for example, in one study, 138 strains of *E. amylovora* from the Pacific Northwest, USA, were all determined to be sensitive to oxytetracycline (58). Although there are few reports of resistance, multiple tetracycline resistance genes homologous to *tetA* and *tetM* are present within the genomes of many different plant-pathogenic bacteria (N. Wang, unpublished results). Efflux pump proteins that belong to the same protein family as TetA have been identified in *Ralstonia solanacearum*;

Erwinia piriflorinigrans; multiple *Xanthomonas* species, including *Xanthomonas citri*, *Xanthomonas phaseoli*, *Xanthomonas perforans*, and *X. campestris*; multiple *Pseudomonas* species, including *P. syringae*, *Pseudomonas aeruginosa*, and nonpathogenic *Pseudomonas putida* and *Pseudomonas fluorescens*. However, even though putative tetracycline-resistance proteins have been annotated in the NCBI database for plant-pathogenic bacteria such as *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, and *Ralstonia*, their function in tetracycline resistance remains to be characterized.

Resistance to Oxolinic Acid and Kasugamycin

There are a few reports documenting resistance to other antibiotics used in plant disease management. OA was introduced in 1997 for fire blight management in Israel as a replacement for streptomycin, and OA resistance in *E. amylovora* was first detected in 1999 (60) and expanded in range by 2001 (61) (**Table 2**). However, populations of OA-resistant *E. amylovora* fluctuated, with OA-resistant strains becoming undetectable in orchards where they previously occurred. Laboratory analyses of OA-resistant strains suggested that these strains were reduced in fitness compared to OA-sensitive strains (53). Analysis of OA-resistant strains of *Burkholderia glumae* also showed that the strains were reduced in fitness, as these strains could not survive in rice paddy fields (41).

Kasugamycin was discovered in Japan and has been used since the 1960s in Asia for the control of rice blast caused by the fungus *Magnaporthe grisea* and for the control of bacterial grain and seedling rots of rice. This antibiotic has also been used to control diseases of sugar beet, kiwi, and Japanese apricot in at least 30 countries (103). More recently, kasugamycin has been utilized for management of the blossom blight phase of fire blight disease in Canada and the United States. Resistance to kasugamycin was reported for two bacterial rice pathogens in Japan, *Acidovorax avenae* subsp. *avenae* and *Burkholderia glumae* (43, 117) (**Table 2**). Kasugamycin resistance in *A. avenae* subsp. *avenae* and *B. glumae* was conferred by a novel *aac*(2′)-*IIa* acetyltransferase gene located within an IncP genomic island and likely acquired by HGT (138). A promoter mutation that resulted in a fourfold increase in expression of the *aac*(2′)-*IIa* gene was found to confer an increased level of kasugamycin resistance in strain 83 of *A. avenae* subsp. *avenae* (139). Kasugamycin resistance has not been reported in *E. amylovora*; one study assessing the potential for spontaneous resistance revealed that a two-step mutational process was required and that spontaneous kasugamycin-resistant mutants were substantially reduced in fitness (64).

ECOLOGICAL IMPLICATIONS OF ANTIBIOTIC RESISTANCE GENE ACQUISITION BY PLANT-PATHOGENIC BACTERIA

As mentioned above, there are at least two examples [*strAB*, *aac*(2′)-*IIa*] in which the evolution of antibiotic resistance in plant-pathogenic bacteria involved the acquisition of a resistance gene(s) via HGT. The most likely immediate source of the resistance determinants acquired by the plant pathogens is the co-occurring nonpathogenic microflora. A series of papers highlighted the occurrence of Sm^R nontarget bacteria in orchards treated with streptomycin and that a large subset of these bacteria harbored *strAB* or, where studied, Tn*5393* (13, 75, 102, 116). These studies demonstrate that plant disease control agents such as streptomycin also affect the native phyllosphere and soil microflora and further indicated that ARGs can be selected in epiphytic bacteria in antibiotic-sprayed plant habitats and could provide a route of acquisition by plant pathogens. Furthermore, an additional study has shown that several tetracycline-resistance genes, including *tetA*, *tetB*, *tetC*, and *tetG*, were present in tetracycline-resistant epiphytic bacteria in two apple orchards with no or limited exposure to oxytetracycline (95). However, to date, tetracycline resistance has not been observed in the target plant pathogens *E. amylovora* from apple and

X. arboricola pv. *pruni* from peach. In one other study examining effects of spraying oxytetracycline and gentamicin onto field-grown coriander plants, the authors found no effects of the antibiotic treatment on the abundance of bacteria resistant to the two antibiotics or on the occurrence of ARGs in the antibiotic-sprayed or control plots (90).

Analyses of the genomic location of Tn*5393* in *E. amylovora* and Tn*5393*b in *P. syringae* suggest that these transposons are located in regions that minimize potential negative effects on ecological fitness. For example, the earliest Sm^R strains of *E. amylovora* recovered in Michigan contained pEa34 encoding Tn*5393* as well as the nonconjugative virulence plasmid pEa29 (13, 63). Approximately 10 years after these strains were isolated, a new survey revealed some Sm^R *E. amylovora* strains containing Tn*5393* copies on both pEa34 and pEa29; however, the majority of strains only contained Tn*5393* on pEa29 and had lost pEa34 (63). Two distinct insertion sites were detected on pEa29, both of which were intergenic (63). Because pEa29 is a virulence plasmid, it was hypothesized that the location of Tn*5393* on this plasmid had stabilized the Sm^R determinant within *E. amylovora* (63). Likewise, in *P. syringae*, insertions of Tn*5393*b were detected within several pPT23A-family plasmids, a group of plasmids that is known to be native to the *P. syringae* species (98, 110, 115). Further work has shown that carriage of Tn*5393*b-containing plasmids did not have a negative impact on fitness of *P. syringae* in vitro or when the organism was grown as an epiphyte on plant leaf surfaces (112).

THE ANTIBIOTIC RESISTOME

Thus, results from studies of plant-pathogenic bacteria mirror those in clinical bacteria, as the movement of ARGs from commensal bacteria into pathogenic bacteria is generally accepted as a typical pathway to antibiotic resistance development in clinical bacteria (56). Knowledge of the importance of commensal bacteria to the overall evolution of antibiotic resistance in pathogens eventually grew over time to a point at which information attained through community microbiome analyses fostered the development of the concept of the antibiotic resistome.

The collection of all known ARGs in the full-microbial pan-genome is defined as the antibiotic resistome (132). What is most important conceptually about the antibiotic resistome is the potential accessibility of individual ARGs to all bacteria. The concept of bacterial species existing within genetic exchange communities (GECs) can be informative when considering the access that individual bacterial species have to the resistome, and thus the potential for acquisition of ARGs by distinct species inhabiting unlinked environmental niches. A GEC is defined by Jain et al. (50) as "a collection of organisms that can share genes by HGT, but need not be in physical proximity." The concept of a GEC is not limited by time, and thus includes all examples of HGT-mediated genome evolution, even though partners in the transfer process are typically unknown. Regarding HGT, ARGs, and recent bacterial evolution, the close sequence similarity of specific ARGs among disparate host genotypes illustrates the large ecological breadth of particular GECs and the powerful selection effect of antibiotic deployment by humans (81).

However, as more resistome data are acquired, a disconnect has arisen regarding the actual potential for HGT within microbiomes, in particular within soil microbiomes. Genomic analyses of Jain et al. (50) indicate that HGT preferentially occurs among organisms that share similar factors, including genome size and percent G+C composition. Also, HGT of resistance genes in soil does not appear to be very frequent, as soil ARG content is strongly correlated with bacterial species composition (30). Thus, although the resistome is theoretically accessible to all bacteria, individual ARGs cluster by ecology, and environmental (communities inhabiting soil or water) and human-associated microbial communities harbor distinct resistance genes (34).

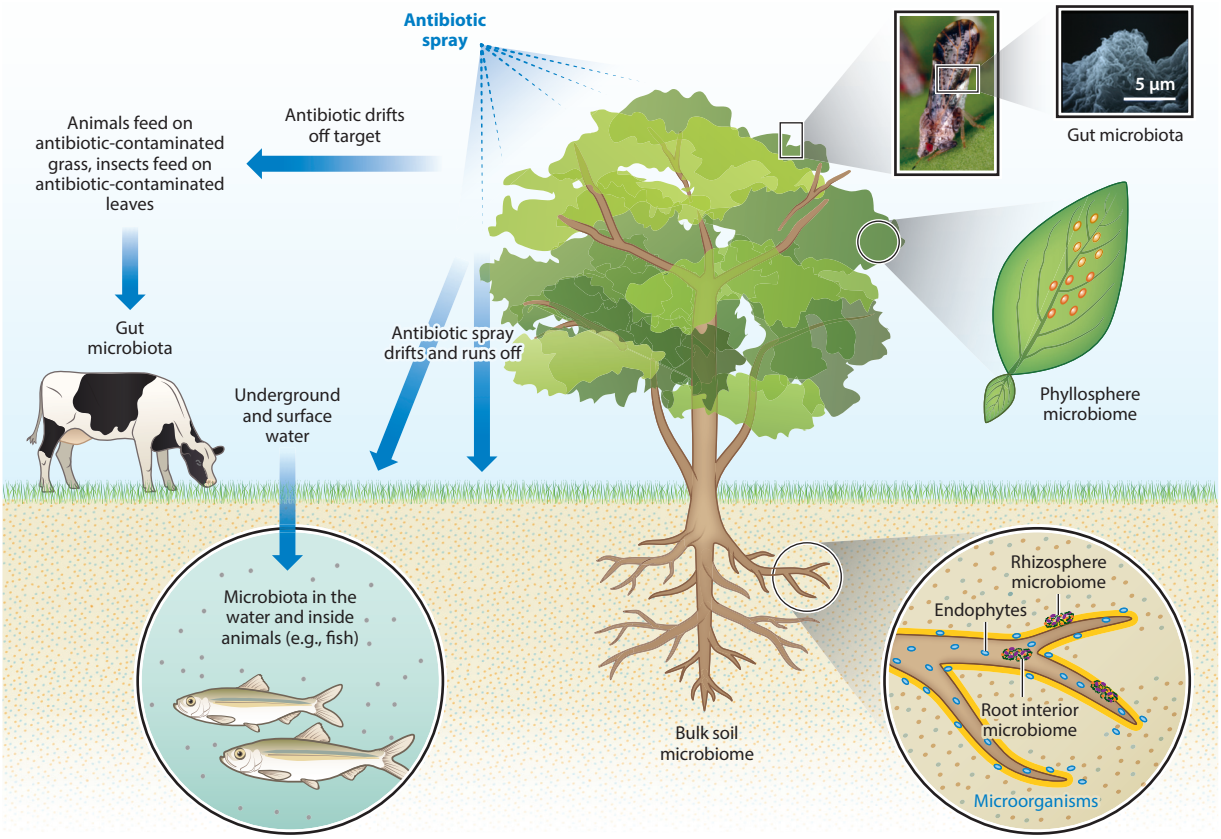

Figure 2

Schematic illustration of the effect of antibiotic application on the microbiome in plant orchard agricultural systems. The most direct effects of antibiotic application to orchard trees are to the phyllosphere microbiome present on sprayed leaves. Effects on soil and rhizosphere microbiomes and surface and underground water are through antibiotic deposition to soil and transfer through soil via runoff and spray drift. Photo insets at the top right illustrate a feeding insect that could be exposed to sprayed antibiotics; if the insect ingests the antibiotic, this could also affect its gut microbiota. Off-target spray drift of antibiotics outside of orchards are be predicted to be of minor consequence; however, if such spray drift occurs, and antibiotics land on plants such as grass, these could be consumed by animals, affecting gut microbiota. Figure adapted from Reference 130.

THE EFFECT OF ANTIBIOTIC APPLICATION ON THE MICROBIOME IN PLANT AGRICULTURAL SYSTEMS

All of the antibiotic applied to trees in orchard systems using conventional air blast spraying systems does not reach the desired target; thus, the effects of antibiotic usage are potentially more complex than simply studying effects on the target pathogen and commensals colocated in the target plant habitat. Antibiotics reaching the target sites in the tree canopy impact the phyllosphere microbiome and flower microbiomes if applied during the bloom phase (**Figure 2**). Insects feeding within the tree canopy could also ingest the antibiotic, which could impact the insect gut microbiota. A portion of the antibiotic spray applied to trees will not reach the target because of spray drift or could be lost by runoff during spraying or runoff owing to rain events (**Figure 2**). It has been estimated that as much as 44%–71% of spray solutions applied by air blast sprayers is lost into the

environment (107). Whether it hits the target or not, once the antibiotic solution has been released into the environment the material is negatively affected by environmental parameters, including rainfall, sunlight (visible and ultraviolet radiation), and temperature, and other specific aspects of the plant leaf environment that may affect adsorption (**Figure 2**). For example, oxytetracycline residues are lost relatively rapidly from peach leaf surfaces because of weather parameters (17). Any antibiotic lost from the tree target by spray drift may land on other plant surfaces, such as the leaves of grasses or weeds, and thus impact the microbes inhabiting the phyllosphere of those plants. There is also the possibility of drift offsite to nontarget plants, and insect or animal may feed on the nontarget plants and potentially consume the antibiotic, which could impact the gut microflora of these animals. We are aware of one study in which the percentage of streptomycin-resistant *E. coli* isolates from feces of sheep feeding in a pasture that was sprayed with streptomycin was shown to increase (from 14.7% to 39.9% compared to 15.8% to 22.3% in a control group) (94). However, this study did not simulate actual conditions in commercial orchards as the streptomycin solution was sprayed directly onto the pasture grass and sheep were grazed in the pasture for 12 h immediately following application. Neither of these situations occur in commercial orchards.

Two studies have been published examining the effect of antibiotic application in apple orchards on phyllosphere bacteria. In one study using both culture-based and culture-independent approaches, Yashiro & McManus (136) examined phyllosphere bacteria from apple orchards that either had received streptomycin applications in spring for fire blight management for up to 10 previous years or had not been sprayed. The percentage of culturable isolates resistant to streptomycin was actually larger from the nonsprayed orchards (136). An examination of community structure using 16S rRNA clone libraries indicated that streptomycin treatment did not have long-term effects on the diversity or phylogenetic composition of the phyllosphere bacterial community in the examined apple orchards (136). A separate cultural study evaluated the effect of weekly applications of streptomycin (for 0, 3, 5, and 10 weeks) beginning at 80% bloom on specific components of the phyllosphere community (118). Testing of orchard epiphytes for streptomycin resistance indicated that 76.2%, 94.5%, 95.5%, and 98.5% of the bacterial isolates were resistant to streptomycin on trees receiving 0, 3, 5, and 10 applications within one season, respectively (118).

Further microbiome studies have also been conducted examining the effect of antibiotic usage on soil microbiomes in apple orchards. For example, Shade et al. (99) determined that streptomycin application to apple trees did not result in any observable difference in soil bacterial communities (soil collected beneath trees 8–9 days after streptomycin application). The authors concluded that application of the antibiotic had minimal impact on nontarget bacterial communities (99). A second microbiome study of apple orchard soil collected 14 days after streptomycin application also failed to detect any influence of the antibiotic on the soil bacterial community (128).

Lack of Knowledge of the Antibiotic Resistome Associated with Crop Plants

The microbiome studies detailed above have provided information that show limited impacts of antibiotics on the selection of antibiotic resistance at a period of time after application. However, there are no published studies to date assessing the resistome of crop plants and in particular the resistome of crop plants that have been treated with antibiotics. Interestingly, the application of struvite ($MgNH_4PO_4 \cdot 6H_2O$), which has been used as a plant fertilizer, alters the antibiotic resistome in the soil, rhizosphere, and phyllosphere (12). This might have resulted from the fact that struvite usually contains ARGs, antibiotic-resistant bacteria, and antibiotic residues (137). The need for knowledge of the antibiotic resistome in plant agricultural systems and especially in plant agricultural systems in which antibiotics are applied is critically important because we

need to understand whether the use of antibiotics in plant agriculture has the potential to select ARGs that could impact human health. This issue regarding potential impacts to human health is highly significant, with current implications for the use of antibiotics in animal agriculture (4, 121). Identification of particular ARGs, and the organisms harboring these genes, is important for risk assessments of pathogen acquisition of resistance based on close phylogenetic relationships with coinhabiting antibiotic-resistant commensals. If ARGs of importance in clinical medicine are identified in the resistome of plants sprayed with antibiotics, it is critical to determine whether their frequency and/or bacterial host range changes based on antibiotic exposure.

SUMMARY

Antibiotic resistance in plant-pathogenic bacteria is a problem in most plant pathosystems where these antibiotics have been used for many years. HGT has played a role in the dissemination of the streptomycin-resistance transposon Tn*5393* among plant pathogens from three genera in North America, South America, and Asia. Resistance management strategies for antibiotic use in plant agriculture are difficult mainly because of the lack of available bactericides having different modes of action that could be used in rotation. Another possible alternative, copper, has limited use for fire blight management on apple and pear because of the potential for phytotoxicity, mainly fruit russeting. The best strategies for resistance management have been to limit the frequency of use of antibiotics. For example, in regions of the eastern and midwestern United States, where streptomycin resistance has not evolved in *E. amylovora*, it is thought that limiting the number of applications during bloom and in the summer months has been a major factor in resistance management. The effect of the microbiome of commensal organisms on the potential of plant pathogens to evolve antibiotic resistance is still largely unknown. We are currently making the first assessments of the composition of the antibiotic resistome present in apple orchards and citrus groves where antibiotics have been applied (G.W. Sundin & N. Wang, unpublished information). These studies will inform us of the selection potential that antibiotic application to plants has on ARGs present in the plant and soil microbiome and of the potential for HGT of these resistance genes into pathogenic bacteria.

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