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Brief Report

Whole genome analysis of spontaneous antimicrobial resistance in *Liberibacter crescens* suggests long-term efficacy for antimicrobial treatment of citrus greening disease

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Abstract

Currently, oxytetracycline and streptomycin are being applied to citrus groves in Florida for the control of citrus greening disease caused by the unculturable bacterium 'Candidatus Liberibacter asiaticus'. Here, the closest cultured relative, *L. crescens*, was used to estimate the frequency of spontaneous antimicrobial resistance of, as yet, uncultured 'Ca. Liberibacter asiaticus'. Results yielded thirteen streptomycin and zero oxytetracycline mutants after exposing 13 billion cells to the antimicrobials. These low rates, alongside the restrictive habitats of the vector and pathogen, suggest resistance may develop very slowly, if at all. Thus, the treatments will likely remain useful long enough before pathogen-resistant or -tolerant genotypes are deployed.

Keywords: 'Candidatus Liberibacter asiaticus', Streptomycin, Oxytetracycline, AMR, HLB

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Introduction

Only three antimicrobials are registered by the Environmental Protection Agency (EPA) for use in plant agriculture: oxytetracycline (OTC), streptomycin, and kasugamycin. Prior to 2016, streptomycin comprised 90% of the antimicrobials used, largely to control plant diseases on temperate fruit trees such as apples, pears, and peaches (Stockwell and Duffy 2012). Nowadays, the use of antimicrobials in plant agriculture has increased dramatically with streptomycin and OTC registered by the EPA and Food and Drug Administration (FDA) for use on citrus canker disease (FDA 2013). Recently, the EPA's emergency exemption for use of streptomycin sprays for citrus greening disease was vacated (9th Cir 2023). Meanwhile, under FIFRA Section 24(c), OTC trunk injections were approved for citrus in Florida in 2022 (Archer et al. 2023). Currently, field tests have shown OTC trunk injections are more effective than sprays on 'Candidatus Liberibacter asiaticus' (CLas)-infected trees because of the systemic nature of CLas.(Hu and Wang 2016; Hu et al. 2018; Hu et al. 2018; Li et al. 2019; Killiny et al. 2020; Archer et al. 2022; Vincent et al. 2022). In addition, these antibiotics are stable in citrus tissues for months after injection (Hu and Wang 2016; Al-Rimawi et al. 2019).

Low or moderate levels of streptomycin resistance (up to 200 µg/ml) are often conferred by lateral transfer of the *strA-strB* gene pair which inactivates streptomycin by phosphorylation catalyzed by an aminoglycoside phosphotransferase (Chiou and Jones 1995). High levels of resistance (up to 2000 µg/ml) are conferred by single base mutations in *rpsL* gene which codes for ribosomal protein S12, a constituent of the 30S ribosomal subunit (Chiou and Jones 1995; Finken et al. 1993). Less commonly observed mechanisms include 16S rRNA gene modifications and the lack of N-7 methylation in 16S rRNA at position 527 caused by a mutation in *rsmG* (Nishimura et al. 2007; Okamoto et al. 2007; Demirci et al. 2014). Since lateral gene transfer is unlikely in CLas given its restrictive habitat, and the lack of *strA-strB* in the CLas and *L. crescens* genomes, we focused on *rpsL* and 16S rRNA mutations as potential streptomycin resistance factors in CLas.

Among plant pathogens, streptomycin resistance has been studied mostly in *Erwinia amylovora*, the causative agent of fire blight disease on apple, pear, and other plants. Resistance in this pathogen has been reported in Michigan, New York, and California (Chiou and Jones 1995; Förster et al. 2015; McGhee et al. 2011; McGhee and Sundin 2012; Tancos et al. 2016; Tancos and Cox 2016). Reports of spread across southwestern Michigan between 2003 and

2009 indicate 98.7% of all streptomycin resistance was conferred by *strA-strB* genes; the remaining 1.3% was conferred by a single base mutation in *rpsL* (McGhee et al. 2011). In contrast, other reports highlight 102 strains of streptomycin-resistant *E. amylovora* possessed mutations in *rpsL* (Chiou and Jones 1995), while a separate report showed 32 of 34 resistant strains possessed the *strA-strB* gene pair and the remaining 2 *rpsL* mutations (Tancos et al. 2016). Consequently, *rpsL* mutations and presence of *strA-strB* were examined here.

Oxytetracycline carries a single hydroxyl group present on the B ring of the naphthacene core that is missing in tetracycline (Nguyen et al. 2014). Tetracycline class antibiotics prevent protein synthesis by the binding of aminoacyl-tRNAs to the A site of the 30S subunit of the ribosome (Schnappinger and Hillen 1996). As tetracycline and oxytetracycline share similar structure and mode of action, their activity is blocked by the same resistance mechanisms. The four mechanisms of tetracycline resistance include: ribosomal protection, efflux, degradation, and 16S rRNA mutations (Nguyen et al. 2014).

The objective of this work was to determine which of these resistance mechanisms, if any, are developed spontaneously in large populations of *L. crescens* cells by comparing the genomes of resistant mutants to wild-type sensitive strains. *L. crescens* was chosen because it is the closest cultured bacterial relative of phloem-limited uncultured CLas and very similar physiologically and genetically (Fagen et al., 2014; Leonard et al., 2012). These results, along with our knowledge of the etiology of the disease, anticipate a slow pace for the appearance of antimicrobial resistance in *C*Las.

Materials and Methods

L. crescens BT-1 was cultured in liquid BM7 medium at 28°C with shaking (125 rpm) as described previously (Fagen, Leonard, McCullough, et al., 2014) to an OD_{600} of 0.3-0.4. Fifty mL of this culture was plated on 3 sets of 12 BM7 agar plates; each set was supplemented with either 2.5 mg/mL streptomycin, 0.25 mg/mL oxytetracycline, or 2.5 mg/mL streptomycin and 0.25 mg/mL oxytetracycline. These concentrations were chosen as they were the minimum concentrations required for inhibition of *L. crescens*. Serial dilutions of the same culture were done from 10^{-4} to 10^{-9} in liquid BM7 medium and 50 mL of each dilution were plated on BM7 agar in triplicates with and without the appropriate antimicrobial to determine the rate of spontaneous mutation. Plates were incubated at 28°C for about 25 days. Colonies resistant to the antimicrobials were selected and grown in liquid BM7 medium supplemented with the appropriate antibiotic and concentration for about seven days. The antimicrobial-resistant bacterial cultures were stored in 25% glycerol at 80°C.

For whole genome sequencing, the wild-type strain (M1) and the mutant strains (A2, A3, K1, K2, K3, K4, and K5) were cultured from the frozen glycerol stocks on BM7 plates supplemented with 2.5 mg/mL streptomycin. After incubating for about fourteen days at 28°C, DNA from these subcultures was extracted using the E.Z.N.A.® Bacterial DNA Kit (D3350) and sent out for PacBio RS II whole genome shotgun sequencing, as described previously (Leonard et al., 2012), using the PacBio platform with the P6-C4 chemistry (Pacific Biosciences, Menlo, CA, USA). A single SMRT cell was used for each genome and the reads obtained were assembled to generate a single contig using the HGAP_Assembly.3 protocol on the PacBio SMRT Analysis server.

Once *rpsL* was shown to be the only gene with mutations in seven mutants through whole genome sequencing, the remaining five streptomycin resistant mutants (A5, A6, A7, A8, and A9) were subjected to polymerase chain reaction. The *L. crescens rpsL* gene was amplified using the primer set (F246: 5'GTGTGGTGGTCGTGTAAAAGA3') and (R363:5'ACGCTTTGCCCCATACTTGG3') targeting a region spanning the 43rd and 88th codons. The amplification conditions were set as follows: initial denaturation at 95°C for 2min, 30 cycles of 95°C for 20s, 55°C for 30s, and 72°C for 30s, and final elongation step of 72°C for 30s. The amplicons were submitted for Sanger sequencing through Azenta Life Sciences.

Results

Twelve streptomycin resistant *L. crescens* colonies were isolated after incubation and the frequency of spontaneous resistance was one in 500 million cells or 2×10^{-9}, substantially lower than the expected 1×10^{-8} typically found in resistant bacteria from a single base mutation (Martinez & Baquero, 2000). No oxytetracycline resistant colonies were recovered despite exposing over 13 billion cells to oxytetracycline, yielding a frequency of spontaneous resistance lower than one in 1.3×10^{-10} cells.

The full genome was sequenced for seven of the twelve streptomycin-resistant spontaneous mutants. Each of the seven mutant genomes was assembled into a single contig and analyzed for the presence of single nucleotide polymorphisms in the *rpsL*, 16S rRNA, and *rsmG* genes from *L. crescens*. Only *rpsL* mutations were observed in the streptomycin resistant mutants. Each mutant possesses one of these three single base mutations in *L. crescens rpsL*: A128G, G129T, or A263G which result in codon mutations K43R, K43N, and K88R respectively. Amino acid change K43R was present in genomes A1, K3, and K4 and K88R in genomes A3, K1, K2, and K5; these represent the two most common forms of streptomycin resistance in *Escherichia coli* (Pelchovich et al., 2014). Any *rpsL* gene mutations in the remaining five strains not subjected to WGS were identified by PCR and Sanger sequencing of the *rpsL* gene. These resulted in *rpsL* codon changes K43R in A5 and A6 mutants or K88R in A7, A8, and A9 mutants (Table 1).

Table 1
Locations of mutations in ribosomal protein S12 (*rpsL*), a constituent of the 30S ribosomal subunit, among the twelve *L. crescens* isolates spontaneously resistant to streptomycin. Mutation identified by whole genome sequencing[1] or PCR amplification and Sanger sequencing of *rpsL*[2].

Strain	*rpsL* nucleotide mutation	*rpsL* amino acid mutation
M1 (wildtype)	none	none
A2	A128G[1]	K43R
K2	A263G[1]	K88R
A3	G129T[1]	K43N
A5	A128G[2]	K43R
A6	A128G[2]	K43R
A7	A263G[2]	K88R
A8	A263G[2]	K88R
A9	A263G[2]	K88R
K1	A263G[1]	K88R
K3	A263G[1]	K88R
K4	A263G[1]	K88R
K5	A263G[1]	K88R

Discussion

This work has important implications for the application of antimicrobials in citrus groves. OTC and tetracycline have the same mode of action for which four mechanisms of resistance genes have been observed: 1) ribosomal protection (conferred by *tetM*, *tetO*, *tetQ*, *tetS*, *tetT*, *tetW*, *tetB*(P), *tet32*, *tet36*, *tet44*, *otrA*, and *tet*); 2) efflux (conferred by *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetH*, *tetJ*, *tetK*, *tetL*, *tetA(P)*, *tetV*, *tetY*, *tetZ*, *tet30*, *tet31*, *tet33*, *tet35*, *tet(38)*, *tet39*, *tet40*, *tet41*, *tet42*, *tet45*, *tetAB(46)*, *tcr3*, *otrC*, and *otrB*); 3) degradation (conferred by *tetX* and *tet37*); and 4) 16S rRNA mutations (G1058C, A926T, G927Y, A928C, ΔG942) (Nguyen et al., 2014). However, no cells were recovered for the OTC amended plates concluding that the frequency of spontaneous resistance through these mechanisms is absent in *L. crescens*.

In field studies, the minimum OTC concentration to suppress *C*Las in field citrus was 0.86 µg/g and the minimum inhibitory concentration against *L. crescens* in artificial media was 0.01 µg/mL (Li et al., 2019). Within the recommended range, at 0.72g of OTC per tree injections, the levels of OTC in the leaves were found to be 7 µg/g FWT (Vincent et al., 2022). Here, *L. crescens* was inoculated in BM7 media supplied with 0.25 µg/mL of OTC for which no cells were recovered. All taken together, the frequency of spontaneous resistance to OTC applications by *C*Las is expected to be insignificant.

The frequency of spontaneous resistance was very low for streptomycin in *L. crescens*. Single nucleotide changes in the *rpsL* gene were found in all streptomycin resistant mutants; this is not surprising given the relatively small *L. crescens* genome does not contain the classical streptomycin resistance genes, including *strA/B*. *rpsL* genes of *L. crescens* and *C*Las have 84% sequence homology, and since *rpsL* mutations confer streptomycin resistance in a diverse group of bacteria, it's very likely it does so in *C*Las as well. Yet, spontaneous resistance via *rpsL* mutations in *C*Las is expected to be negligible.

Certain mutations within the 16S rRNA gene can lead to a modest level of resistance to oxytetracycline and streptomycin; however, in *L. crescens* all three of its copies would need to be mutated to observe any robust resistance. Several of the appropriate mutations within a single copy of 16S rRNA are additive towards increased tetracycline resistance (Nguyen et al., 2014); but, this is not expected to provide protection at the level of selective pressure expected in the field. One can argue that once one of these alleles is mutated, resistance can be conferred to the other two by additional mutation or by homologous recombination; however, the slow growth rate of *Liberibacter* and multiple 16S rRNA gene copies likely mean a very slow appearance of resistance by this mechanism. Accordingly, it is not surprising that none of the *L. crescens* mutants isolated here contained 16S rRNA mutations predictive of resistance.

Antibiotic resistance in *C*Las in citrus groves is unlikely. First, *C*Las has a predicted slow growth rate – comparable to *L. crescens* (Cruz-Munoz et al., 2018; Fagen, Leonard, Coyle, et al., 2014) – meaning it will take a very long time for spontaneous resistance to dominate in the population compared to a typical bacterium. Second, *C*Las small genome size of 1.2 Mb shows a lack of genes commonly used by other bacteria for resistance, such as ribosome protection, tetracycline efflux and degradation, or the *strA/B* gene pair. And third, it is unlikely that *C*Las will acquire antimicrobial resistance genes through lateral transfer because of its very restrictive habitats; the citrus phloem, where only *C*Las has been found in infected trees (Tyler et al., 2009), and the Asian citrus psyllid gut, where only four bacteria – with reduced genomes and lack of antimicrobial resistance (AMR) genes – reside (Fagen et al., 2012; Petrone et al., 2022). This leaves spontaneous antimicrobial resistance as the only means by which *Liberibacter* could obtain resistance. However, *C*Las lacks nine out of ten factors that contribute to increased frequency of AMR in bacteria reviewed in Martinez & Baquero, (2000).

Initial levels of *C*Las antimicrobial resistance in Florida citrus groves can be estimated from the frequency of streptomycin resistance in *L. crescens*. The estimated number of *C*Las cells in an average infected tree ranges between 55 and 4,576 genomes per gram of tissue (Stover & McCollum, 2011) . Assuming 1000 genomes per gram

of living plant tissue and 10 kg of living tissue per tree, there are 10 million *C*Las cells per tree. This is 50-fold below *L. crescens* cells required (1 in 500 million) to isolate one streptomycin-resistant colony in the lab. Thus, if spontaneous resistance occurs at the same frequency in *C*Las, any set of 50 heavily infected trees would harbor one spontaneously resistant *C*Las mutant before antimicrobial selective pressure begins. The *C*Las-resistant cell number is expected to increase with selective pressure; however, the pace a cell can spread in a set of 50 trees through psyllid feeding is likely very slow, especially in groves with psyllid control. The frequency of spontaneous resistance to oxytetracycline is 1 in 13 million cells, meaning it would take more than 1300 infected trees to observe one resistant *C*Las cell. Nevertheless, with sufficient antibiotic exposure, significant resistance in the field will likely occur but we expect economically important levels of resistance will occur much faster with streptomycin compared to oxytetracycline. As antibiotic resistance in an uncultured pathogen is difficult to measure directly, the easiest means to assess resistance may be qPCR measurements of the pathogen as signs of decreased antibiotic efficacy.

Acknowledgments

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