Burkholderia multivorans Exhibits Antibiotic Collateral Sensitivity

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Burkholderia multivorans is a member of the *Burkholderia cepacia* complex whose members are inherently resistant to many antibiotics and can cause chronic lung infections in patients with cystic fibrosis. A possible treatment for chronic infections arises from the existence of collateral sensitivity (CS)—acquired resistance to a treatment antibiotic results in a decreased resistance to a nontreatment antibiotic. Determining CS patterns for bacteria involved in chronic infections may lead to sustainable treatment regimens that reduce development of multidrug-resistant bacterial strains. CS has been found to occur in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Here, we report that *B. multivorans* exhibits antibiotic CS, as well as cross-resistance (CR), describe CS and CR networks for six antibiotics (ceftazidime, chloramphenicol, levofloxacin, meropenem, minocycline, and trimethoprim-sulfamethoxazole), and identify candidate genes involved in CS. Characterization of CS and CR patterns allows antibiotics to be separated into two clusters based on the treatment drug to which the evolved strain developed primary resistance, suggesting an antibiotic therapy strategy of switching between members of these two clusters.

Keywords: collateral sensitivity, antibiotic resistance, *Burkholderia*

Introduction

CHRONIC BACTERIAL INFECTIONS are associated with medical conditions such as nonhealing wounds, indwelling medical devices, and genetic diseases such as cystic fibrosis. While some of those infections may be addressed by treating the underlying condition, for cystic fibrosis patients clinicians rely on periodic use of antibiotics, which alleviate symptoms despite evidence that antibiotics do not necessarily eradicate or reduce *in vivo* bacterial populations.^{1,2} Periodic antibiotic use drives evolution of multidrug-resistant bacteria, which complicates treatment by requiring antibiotics with greater toxicity or eliminating options of some antibiotic classes.

Exposure to an antibiotic selects for mutants resistant to that antibiotic, but the mutation may have pleiotropic effects, including changed susceptibility to nontreatment antibiotics. Resistance to an antibiotic due to selective pressure by that antibiotic is direct resistance.³ An increased resistance to nontreatment antibiotics is termed cross-resistance (CR) and a decreased resistance to nontreatment antibiotics is termed collateral sensitivity (CS).^{3–17} This phenomenon has been reported to occur in *Escherichia coli*,^{3,5–11} *Pseudomonas aeruginosa*,^{12–14} and *Staphylococcus aureus*.^{15–17} The two Gram-negative pathogens had different and sometimes opposing collateral interactions,¹² so collateral changes may be

species specific. Even within species, there can be different collateral reactions depending on the particular mutations that arise during experimental evolution.^{11,18}

Because cross-resistance (CR) and collateral sensitivity (CS) are assumed to result from the mutation that conferred resistance to the evolved strain, they are a fitness gain/cost of resistance acquisition, which is detected in environments with the collaterally involved antibiotics. The fitness cost theory of CS^{7,12} has at least three types of genetic changes that can result in CS: a mutated gene product exerting direct pleiotropic effects resulting in both the selected-for resistance and collateral changes; a mutation in a regulatory gene (either cis- or trans-acting); or coincidental acquisition of multiple mutations. Whole-genome sequencing, to identify molecular mechanism of the phenomenon, and repeated testing, to decrease the likelihood of coincidental acquisition of uncorrelated mutations, are required to characterize the observed CS interactions.

Burkholderia multivorans is a member of the *Burkholderia cepacia* complex (Bcc), a group of closely related species that cause chronic and debilitating lung infections in patients with cystic fibrosis¹⁹ and are inherently resistant to many antibiotics. Mechanisms of antibiotic resistance reported in *Burkholderia* spp. include decreased intracellular accumulation, enzymatic modification of drugs, and alteration of target.²⁰ The limited antibiotic options for treating

Bcc infections have led to alternate antibacterial strategies using combination treatments.²¹ While Bcc prevalence is low in cystic fibrosis, the risks associated with Bcc infections are high, including the potential for “cepacia syndrome.”^{19,22} While transient infections can occur,²³ Bcc infections are often chronic once established. Patients usually take antibiotics prophylactically and receive additional antibiotics in response to a pulmonary exacerbation. Because dominant bacterial taxa/species inhabiting cystic fibrosis lungs are relatively stable,^{1,2,24} antibiotic therapy creates an environment that selects for resistant mutants. These species are ideal for susceptibility research due to persistence of infections, inherent resistance, and potential for fatality.

Determining CS patterns for bacteria involved in chronic infections may lead to sustainable treatment regimens that reduce development of multidrug-resistant bacterial strains. Elucidation of CS networks may allow treatment cycling regimens to become part of antimicrobial stewardship programs for chronic infections. Here, we report the first study on collateral resistance and susceptibility in a Bcc species.

Materials and Methods

Strains, culture conditions, and antibiotics

B. multivorans strain AS149,²⁴ used to evolve all others in this study, is a clinical isolate from a patient with cystic fibrosis. Bacteria were grown on LB broth, Miller (Fisher) with 1.5% agar (Fisher) for routine culturing and during experimental evolution. Mueller–Hinton broth 2 (Sigma-Aldrich) with 1.7% agar was used for antimicrobial susceptibility testing. All cultures were incubated at 37°C in ambient air.

Antibiotics involved in this study, as listed in Table 1, were chosen due to their inclusion in the CLSI²⁵ list of standard antibiotics tested against *B. cepacia* and for having varied targets. BBL Sensi-Disc antimicrobial susceptibility test disks (BD) were used for all except minocycline, which were Oxoid antimicrobial susceptibility disks (Thermo Scientific). The minimum inhibitory concentration (MIC) was determined using ETEST gradient strips (bioMérieux).

Antibiotics and abbreviations used, antimicrobial susceptibility testing, and the number of strains evolved for each treatment drug (TD) are given in Table 1. Clusters were determined *post hoc* based on result grouping, and were named (β LA and non- β LA) based on antibiotic similarity.

Experimental evolution

Strains were evolved for resistance to a TD using a previously described, plate-based method.²⁶ In brief, we swabbed AS149 onto LB agar to create a lawn, then added an antibiotic-impregnated disk in the center. After 16–24 hours incubation at 37°C, the growth closest to the zone of inhibition (ZOI) was collected and used to inoculate the next plate until resistance was achieved. Strain evolution was stopped when antimicrobial susceptibility testing through disk diffusion on Mueller–Hinton agar plates exhibited a ZOI that was considered “resistant” by the CLSI breakpoints, or there was growth up to the disk for the antibiotics that do not have disk diffusion breakpoints; that is, LVX and CHL. At least 11 strains were independently evolved to be resistant to each drug as shown in Fig. 1; we refer to all strains evolved to be resistant to a particular drug as the “resistance group” for that drug.⁸ To detect and exclude mutations that occur with only laboratory growth conditions as the selective pressure, we grew the parental strain on LB plates for 20 days as a negative control.

Antimicrobial Susceptibility Testing and Interpretation

Disk diffusion testing was performed on strains selected for resistance to a TD to determine susceptibility to five non-TDs, as well as the TD to ensure the strain was resistant and not a persister or cheater.²⁷ The antibiogram of the evolved strain was compared with the antibiogram of the parental strain to determine CS and cross-resistance (CR). Any change in ZOI of $\geq 20\%$ for a non-TD reflects collateral changes in susceptibility, with an increase in ZOI indicating CS and a decrease in ZOI indicating CR.

To better quantitate CS interactions, an ETEST[®] was used to determine the MIC on all strains having CS as indicated by $\geq 20\%$ increase in ZOI with disk diffusion testing.

TABLE 1. ANTIBIOTICS USED FOR EXPERIMENTAL EVOLUTION

Antibiotic	Abbreviation	Disk content, μ g	Mechanism of action	Group
Chloramphenicol	CHL	30	Terminates polypeptide synthesis by binding to 50 seconds ribosomal subunit	n- β LA
Levofloxacin	LVX	5	Inhibits relaxation of supercoiled DNA and promotes breakage of DS DNA by inhibiting DNA gyrase	n- β LA
Minocycline	MIN	30	Inhibits bacterial protein synthesis by binding with 30 seconds ribosomal subunit	n- β LA
Trimethoprim-sulfamethoxazole	SXT	1.25/23.75	Inhibits folic acid synthesis	n- β LA
Meropenem	MEM	10	Inhibits cell wall synthesis Targets penicillin-binding proteins	β LA
Ceftazidime	CAZ	30	Inhibits cell wall synthesis Inhibits peptidoglycan cross-linkage	β LA

Abbreviations, antimicrobial susceptibility testing disk content and mechanism of action are given. Groupings were determined *post hoc* based on clustering seen in number of collateral sensitivity changes and the drugs involved.

FIG. 1. Number of evolved strains exhibiting collateral sensitivity and cross-resistance. The black bars indicate the total number of strains evolved to each treatment drug, shown on the X axis (CAZ=ceftazidime; MEM=meropenem; CHL=chloramphenicol; LVX=levofloxacin; MIN=minocycline; and SXT=trimethoprim-sulfamethoxazole). The dark gray bars indicate the number of evolved strains exhibiting cross-resistance (CR). The light gray bars indicate the number of evolved strains exhibiting collateral sensitivity (CS). White bars indicate number of evolved strains exhibiting both CS and CR.

The 20% value was chosen after determining which of 15% and 20% changes correlated better with MIC results. A decrease in MIC, indicating increased sensitivity, was considered a positive CS interaction.

Statistical analysis

GraphPad Prism was used to run observed versus expected binomial one-tailed tests for cross-resistance clustering. Numbers of cross-resistant interactions were used as opposed to strains since strains could contain more than one interaction. Null hypothesis is that any of the five non-TDs had an equal chance to be the drug in the observed interaction, so expected values are 20% beta-lactam antibiotic (β LA) (1 of 5) and 80% nonbeta-lactam antibiotic (non- β LA) (4 of 5) when the TD was a β LA; 40% β LA (2 of 5) and 60% non- β LA (3 of 5) when the TD was a non- β LA.

Genomic analysis

Thirteen independently evolved *B. multivorans* isolates demonstrating CS, three parental AS149 biological replicates, and eight negative control biological replicates (2 $\times$ five exposures, 10 exposures, 15 exposures, and 20 exposures) were subjected to whole-genome sequencing. A single colony from each strain and two morphologically distinct colonies from control plates were sent to Omega Bioservices, where WGS was performed using 151 bp paired end reads with an Illumina HiSeq 2500 platform.

Raw files were visualized in FastQC-0.11.8²⁸ for quality. Adaptor reads and contigs >151 bp were trimmed, and bases with quality reads falling below a phred score of 20 were trimmed in Trimmomatic-0.35.²⁹ Genomes were globally aligned to the reference genome *B. multivorans* ATCC BAA-247 (NCBI: Accession: PRJNA264318) using Bowtie2-2.3.4.3.³⁰ Duplicate reads were marked and removed using Picard-2.8.26 and INDELS realigned using GATK3 Indel-Aligner. Variants were called in GATK3 HaplotypeCaller

with a standard confidence call of 30 in Discovery mode.^{31,32} Variants below coverage <10 $\times$ were filtered for the final variant calls using VCFtools.³³

Python scripts and SAMtools were used to remove all called variants in parental and control strains from individual CS strains to validate that all variants called in each strain were induced in the evolution experiment.³⁴ Final called variants (SNP/INDEL) were annotated using snpEFF-4.3T, and functional characterization was achieved through NCBI, Burkholderia Database (Burkholderia.com), STRING database, and KEGG database.³⁵

Results

Evolved strains and collateral changes in susceptibility

A sputum isolate of *B. multivorans* obtained from a cystic fibrosis patient (36) was exposed to each of six treatment antibiotics $\sim$ 11 independent times for each drug to generate a total of 73 evolved strains. Of these, antimicrobial susceptibility testing documented that 16% exhibited CS, 79% exhibited cross-resistance (CR), and 5% exhibited both CS and CR. When strains within a resistance group were analyzed, the percentage of strains with CS ranged from 0% (SXT) to 36% (MEM); the percentage of strains with CR ranged from 27% (MEM) to 100% (CHL and LVX). With analysis of different parameters of CS and resistance (ex. number of strains exhibiting CS or CR), the evolved strains grouped into two clusters (Table 1) with the β LA making up one cluster and the non- β LA in the second.

Cross-resistance patterns

Cross-resistance (CR) to at least one of the five non-TDs was present in the majority of strains in all resistance groups except for that of meropenem (Fig. 1). The combined percentages of strains exhibiting CR differed for β LA and non- β LA TDs; 96% of 51 evolved strains had cross-resistance

		TREATMENT DRUG					
		n-βLA				βLA	
		CHL	LVX	MIN	SXT	CAZ	MEM
NON-TREATMENT DRUG	Number of strains exhibiting CR	12	11	12	14	6	3
	n-βLA	CHL	11	11	12		3
		LVX	10		12	2	2
		MIN	2	11			
		SXT	12	11	8	1	1
	βLA	CAZ		3	1		2
		MEM			1	5	

FIG. 2. Heat maps of cross-resistance interactions. The number of strains that demonstrated cross-resistance (CR) is indicated in each box. The treatment drug used to evolve the strain is listed across the top, and the nontreatment drug to which the strain exhibits CR is on the left. The heat map reflects percentage of evolved strains exhibiting CR to each nontreatment drug, with greater intensity indicating a higher percentage of reactions observed, and the absolute number of CR strains given in each box. Clustering is seen within the non-βLA group, with highest percentages of reactions seen within group members, as indicated by the box formed by dashed lines.

when a non-βLA was the TD, and 40% of 22 strains had cross-resistance when a βLA was the TD.

As the TD impacted the percentage of evolved strains exhibiting cross-resistance (CR), so too did the TD affect to which non-TD CR was observed. When the TD was a non-βLA, CR was observed to other non-βLA than to βLA

(Fig. 2). For example, when the TD was LVX (non-βLA), 100% of evolved strains demonstrated cross-resistance to all three of the other non-βLA drugs (CHL, MIN, and SXT), but only 27% demonstrated cross-resistance to a βLA. For the two βLAs used in this study, a similar pattern was observed for CAZ, but not MEM. When CAZ was the TD, five of six evolved strains demonstrated cross-resistance to MEM (βLA), but only two to LVX, one to SXT, and none to CHL or MIN (non-βLA). The one exception to this pattern is when MEM was the TD, for which there was no distinct CR pattern of clustering observed. We performed an observed versus expected, one-tailed binomial statistical test (see Materials and Methods section); clustering was statistically significant for all TDs except MEM with p -values of ≤ 0.0001 for CHL, LVX, MIN, and SXT and $p = 0.0104$ for CAZ.

Collateral susceptibility patterns

Although within-cluster cross-resistance was common, all CS interactions were between clusters (Fig. 3). The five βLA strains exhibiting CS did so to non-βLA. One interesting pattern is seen with MEM, which had CS interactions as either the TD or non-TD. In the MEM resistance group, four of 11 evolved strains had a decrease in MIC to at least one non-TD, and one of those four strains exhibited CS to three drugs (LVX, SXT, and MIN). All of the seven non-βLA strains exhibiting CS did so to MEM (Fig. 1); CS was observed when a strain was evolved to be resistant to the non-βLAs LVX (3 of 11 strains), CHL (2 of 12 strains), or MIN (2 of 13 strains). Of the 15 strains evolved to be resistant to SXT, none demonstrated CS.

Quantitative reduction in MIC for CS interactions

We considered any decrease in MIC for a non-TD as measured by ETEST to be positive for collateral susceptibility. Since the MIC is a quantitative measurement, the amount of decrease in MIC reflects the degree of increased

FIG. 3. Network of collateral sensitivity changes. Each sphere represents an antibiotic (CAZ=ceftazidime; MEM=meropenem; CHL=chloramphenicol; LVX=levofloxacin; MIN=minocycline; and SXT=trimethoprim-sulfamethoxazole). Gray spheres are members of the β-lactamase antibiotic group (βLA), and black spheres are members of the non-β-lactamase antibiotic group (non-βLA). Lines originate at a treatment drug and terminate at a nontreatment drug, which had a collateral change in sensitivity. The line weight is proportional to the relative number of reactions. (A) Shows the cross-resistant network depicted with lines that end in a ball. (B) Shows the collateral susceptibility network depicted with lines that end in an arrowhead.

sensitivity the evolved strain had to the parental strain. For all strains demonstrating CS, we observed a range of decreases in MIC of 1.5–3.4-fold.

Patterns of collateral susceptibility reactions within antibiotic groups

If CS is to influence treatment regimes, strains that demonstrated CS need to do so in a predictable pattern, which was observed in resistance groups. Except for MEM, a consistency was seen for all TDs; 100% of strains that exhibited CS had increased susceptibility to only one non-TD. When the TD was a non- β LA, 100% of all CS-exhibiting strains had increased susceptibility to MEM, a β LA. When the TD was a β LA, 80% (MEM) to 100% (CAZ) of the CS-exhibiting strains had increased susceptibility to MIN, a non- β LA.

Genetic determinants of CS

Whole-genome sequencing and mutational analysis were conducted on 13 of the evolved strains, as well as the parental strain. Thirty-six genes accumulated nonsynonymous mutations in the evolved strains. The average mutation load was ~ 36.3 mutations/strain with range 13–61. Most mutations were found to be synonymous (average 10.9; range 3–19) or in the intergenic region (average 17.1; range 6–33). There was an average of 7.6 nonsynonymous coding mutations/strain (range 4–15).

To identify candidate genes most likely involved in direct resistance and CS, we focused on the mutations in two strains with similar phenotype: TD of CAZ and CS to MIN. Based on known gene function, we identified two candidate genes that have the identical mutation in both strains. The first gene is *penA*, with a conservative in-frame deletion [p.Thr180_Glu181del]. The second gene is *mpl*, with a missense variation [p.Ala147Val] seen in the Mur_Ligase_C (CDD:332156) region.

The 11 remaining strains did not have mutation profiles to correlate with collateral susceptibility profile. As a result, candidate genes were identified based on gene product functional analysis (Table 2). For example, mutations in transcriptional regulators of efflux pumps: a disruptive insertion in the *bpeR* gene at the TetR_N region (CDD:332510) was found in a LVX-MEM (TD-NTD) strain, and a SNP [p.Val143Met] occurring in the *bpeT* gene in the PBP2_CrgA_like_3 region (CDD:176161) was found in a MIN-MEM (TD-NTD) strain.

Because of the role membrane proteins play in antibiotic transport, it is worth noting that 12 of the 13 strains obtained at least one mutation in a membrane protein: 61% strains obtained a nonsynonymous mutation in one membrane protein, 31% strains obtained nonsynonymous mutations in two membrane proteins, and 8% of strains obtained nonsynonymous mutations in three membrane proteins. Membrane proteins affected were involved in cell structure formation (AsmA, RfaF), lipoprotein synthesis (Int), peptidoglycan synthesis and remodeling (PenA, Mpl), and putative transmembrane and lipoproteins (NP80_3676, NP80_4832) (see Table 3).

Discussion

We have documented that *B. multivorans* exhibits both cross-resistance and CS in laboratory-evolved strains. This

study focused on CS, which occurs when a TD-adapted strain has a concomitant increase in sensitivity to a non-TD. We chose the six antibiotics used (ceftazidime, chloramphenicol, levofloxacin, meropenem, minocycline, and trimethoprim-sulfamethoxazole) because they varied by target and mechanism of action, and because they have defined antimicrobial susceptibility testing interpretations for resistant/intermediate/sensitivity using MICs for *Burkholderia*.²⁵

Our initial characterization of cross-resistance (CR) patterns allowed antibiotics to be separated into two clusters (Fig. 2). If a single mutation is responsible for CR, we hypothesize that either there is likely a common target or underlying specific resistance mechanism between the TD and the CR-exhibiting non-TD, or resistance is due to a generalized resistance mechanism such as increased efflux or decreased permeability. The first hypothesis is supported by some of the data involving the β LA meropenem and ceftazidime, which have a common target of cell wall synthesis inhibition and had a high number of CR responses. However, that CR interactions were often seen within non- β LA cluster members that have different mechanisms of action or cellular target is not consistent with the first hypothesis. The second hypothesis is currently investigated.

Intriguingly, the observed CS pattern creates the same two clusters as is seen for cross-resistance. However, while CR was most often seen within clusters, CS was always between clusters (Fig. 3). This pattern is not surprising for the β LA MEM and CAZ, which have a similar mechanism of action: inhibition of peptidoglycan cross-linking to disrupt cell wall synthesis. The between-cluster CS pattern was less expected when a non- β LA was the TD because they have a wider range mechanism of action. Interestingly, strains with CS to one beta-lactam drug did not always exhibit increased sensitivity to the other β -lactam drug. The lack of shared CS patterns may be due to the differences in the drugs' resistance to beta-lactamases, chemical structure, or other parameter of their activity. More firm conclusions cannot be drawn from the available data.

One strategy to elucidate the genetic mechanisms involved in CS as well as to allow prediction of CS is through mutation analysis. Toward this end, we analyzed genomic sequences of all 13 strains generated in this study. For the two strains having a similar phenotype (direct resistance to ceftazidime, collateral susceptibility to minocycline), both strains had mutations in the *penA* and *mpl* genes. PenA is a beta-lactamase,³⁶ and *penA* mutations have been implicated for ceftazidime resistance in *Burkholderia pseudomallei*.³⁷ Mpl is a murein peptide ligase involved in cell wall recycling to synthesize peptidoglycan.³⁸ Disruptions within the peptidoglycan synthesis pathway could result in a compromised cell wall, which could increase resistance to ceftazidime and increase diffusion of hydrophobic antibiotics such as minocycline.³⁹ Consistent with this hypothesis is that strains of *P. aeruginosa* that were experimentally adapted to penicillins or cephalosporins also had mutations in penicillin-binding protein and *mpl* genes.¹² In addition, mutations inactivating LdcA reduce meropenem resistance in *B. thailandensis*⁴⁰; LdcA is a precursor to Mpl in the peptidoglycan recycling pathway.

TABLE 2. GENES THAT ACCUMULATED NONSYNONYMOUS MUTATIONS GROUPED BY FUNCTIONAL ANALYSIS

Function	Gene name	Locus tag	Number of mutations	Number of strains	Avg Mut/Strain
Cell division	ftsK	NP80_1063	1	1	1.00
	spoOJ	NP80_2201	8	8	1.00
DNA repair/replication	dut	NP80_2659	1	1	1.00
	recN/recF	NP80_3671	1	1	1.00
	uvrD	NP80_3677	1	1	1.00
	dprA	NP80_52	2	2	1.00
	topB	NP80_54	2	1	2.00
Enzyme	copper-translocating P-type ATPase	NP80_250	1	1	1.00
	kumamolisin	NP80_5103	1	1	1.00
	methyltransferase	NP80_530	1	1	1.00
Membrane	asmA	NP80_1631	4	4	1.00
	Int	NP80_2833	2	1	2.00
	rfaF	NP80_2834	4	2	2.00
	putative lipoprotein; transmembrane protein	NP80_3676	14	9	1.56
	DUF1275	NP80_4832	2	2	1.00
	penA	NP80_4859	2	2	1.00
Metabolism	mpl	NP80_646	2	2	1.00
	prs	NP80_2919	1	1	1.00
	araC	NP80_4081	1	1	1.00
	guaB	NP80_4504	15	8	1.88
	rhaT	NP80_5143	3	2	1.50
	pyrD	NP80_5414	2	2	1.00
	mopB	NP80_584	2	2	1.00
	phage lysozyme	NP80_5617	2	2	1.00
Phage	phage tail sheath	NP80_90	3	2	1.50
	phage late control	NP80_96	5	3	1.67
Ribosome	rph	NP80_1086	2	2	1.00
	rimO	NP80_1213	14	7	2.00
	Rne/Rng	NP80_1846	1	1	1.00
Secretion system	epaP	NP80_4783	1	1	1.00
	virB	NP80_4784	1	1	1.00
	fimV	NP80_3943	3	1	3.00
Transcription factor	bpeR	NP80_2751	1	1	1.00
	bpeT	NP80_2792	1	1	1.00

Each of nine categories shows the representative genes and their NCBI locus tag, the number of nonsynonymous mutations that accumulated over the 13 strains in that gene and the number of strains (of 13) that acquired a mutation. The average number of mutations per gene per strain is given.

Mutations in additional candidate genes (Tables 2 and 3) were not found in strains sharing an antibiogram phenotype. However, mutations in some genes were found in multiple evolved strains having different antibiograms. Functional analysis revealed that those genes could be grouped into nine categories (Table 2). When focusing on those gene-encoding products involved in the membrane, four genes contain the identical mutation (*asmA*, *rfaF*, *penA*, and *mpl*) (Table 3). These results suggest that there is not one protein, or one combination of proteins, responsible for collateral susceptibility.

What complicates predicting CS patterns from mutation analysis is the recognition that the mutations responsible for a given pattern of resistance, cross-resistance, and collateral susceptibility can differ based on prior evolution and adaptation.^{11,17,41} That is, previous environmental exposure can lead to mutations that affect how selection for subsequent mutations impacts CS patterns. Therefore, correlating mutation patterns with predictable CS patterns will require analysis of a more extensive strain collection than is done here.

Of the six antibiotics examined in this study and in strains evolved from AS149, meropenem, which targets penicillin-binding proteins 2 and 3,⁴² exhibited unique collateral characteristics. Evolved strains in the MEM resistance group have the lowest percentage of strains exhibiting cross-resistance (3 of 11 total evolved strains) but the highest rate of CS. MEM-evolved strains had increased sensitivity to multiple drugs (CHL, LVX, MIN, and SXT), while every other resistance group had strains with CS to just one non-TD. If CS was observed in strains of the non-MEM resistance groups, MEM was most often the drug with increased sensitivity. We note that meropenem targets cell wall synthesis, which suggests an explanation for the observed characteristics as alterations of the cell wall may result in a change in the intracellular accumulation of other drugs.

Although neither the genetic nor biochemical mechanism involved in the observed interactions has been elucidated, such information is not required for these observations to impact treatment regimens. For example, if *B. multivorans* associated with a chronic infection became resistant to a β LA, selection of a non- β LA to treat the infection would

TABLE 3. MEMBRANE GENES CONTAINING NONSYNONYMOUS MUTATIONS

Locus tag	Gene name	Type of mutation	Protein mutation	Frequency	Warning
NP80_1631	asmA	SNP	p.Thr235Ala	1	
NP80_2834	rfaF	Deletion	p.Asp533_Leu537delinsVal	1	
		Deletion	p.Ala529_Ala531del	1	
NP80_2833	Int	Frameshift	p.Lys533fs	1	3' Realign
		SNP	p.Lys533Arg	1	
NP80_4859	penA	Deletion	p.Thr180_Glu181del	1	
NP80_646	Mpl	SNP	p.Ala147Val	1	
NP80_3676	putative transmembrane/lipoprotein	Deletion	p.Gly262_Ser264del	0.875	
		SNP	p.Ala346Thr	0.25	
		SNP	p.Ser327Gly	0.25	
NP80_4832	DUF1275/transmembrane	SNP	p.Pro249Leu	0.5	
		SNP	p.Ser203Ala	0.5	

Frequency refers to the number of strains that had that specific mutation out of the total number of strains with mutations in that gene. For example, all strains having a nonsynonymous mutation in *asmA* (which is four strains [Table 2]) carried the SNP at amino acid position 235, resulting in a frequency of 1.0.

still conform to the appropriate standard of care and may help avoid development of further resistance. Additional studies will increase the predictive value of collateral susceptibility interactions.¹¹ One particular focus being pursued is identifying reciprocal CS patterns using two antibiotics, when use of one as the TD leads to CS in the non-TD, and this pattern then continues when the initial non-TD is subsequently used as the TD. Such flipping patterns would allow long-term treatment of chronic infections to be limited to just two antibiotics with decreased concern of additional acquired antibiotic resistance.

Acknowledgment

This work was supported by funds provided by The University of North Carolina at Charlotte and a National Institutes of Health grant 1R15HL126122–01 to T.R.S.

Disclosure Statement

No competing financial interests exist for any author.

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