

Novel Antibiotic Resistance Determinants from Agricultural Soil Exposed to Antibiotics Widely Used in Human Medicine and Animal Farming

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ABSTRACT Antibiotic resistance has emerged globally as one of the biggest threats to human and animal health. Although the excessive use of antibiotics is recognized as accelerating the selection for resistance, there is a growing body of evidence suggesting that natural environments are “hot spots” for the development of both ancient and contemporary resistance mechanisms. Given that pharmaceuticals can be entrained onto agricultural land through anthropogenic activities, this could be a potential driver for the emergence and dissemination of resistance in soil bacteria. Using functional metagenomics, we interrogated the “resistome” of bacterial communities found in a collection of Canadian agricultural soil, some of which had been receiving antibiotics widely used in human medicine (macrolides) or food animal production (sulfamethazine, chlortetracycline, and tylosin) for up to 16 years. Of the 34 new antibiotic resistance genes (ARGs) recovered, the majority were predicted to encode (multi)drug efflux systems, while a few share little to no homology with established resistance determinants. We characterized several novel gene products, including putative enzymes that can confer high-level resistance against aminoglycosides, sulfonamides, and broad range of beta-lactams, with respect to their resistance mechanisms and clinical significance. By coupling high-resolution proteomics analysis with functional metagenomics, we discovered an unusual peptide, PPP^{AZI} 4, encoded within an alternative open reading frame not predicted by bioinformatics tools. Expression of the proline-rich PPP^{AZI} 4 can promote resistance against different macrolides but not other ribosome-targeting antibiotics, implicating a new macrolide-specific resistance mechanism that could be fundamentally linked to the evolutionary design of this peptide.

IMPORTANCE Antibiotic resistance is a clinical phenomenon with an evolutionary link to the microbial pangenome. Genes and protogenes encoding specialized and potential resistance mechanisms are abundant in natural environments, but understanding of their identity and genomic context remains limited. Our discovery of several previously unknown antibiotic resistance genes from uncultured soil microorganisms indicates that soil is a significant reservoir of resistance determinants, which, once acquired and “repurposed” by pathogenic bacteria, can have serious impacts on therapeutic outcomes. This study provides valuable insights into the diversity and identity of resistance within the soil microbiome. The finding of a novel peptide-mediated resistance mechanism involving an unpredicted gene product also highlights the usefulness of integrating proteomics analysis into metagenomics-driven gene discovery.

KEYWORDS agriculture, antibiotic resistance, biosolids, functional metagenomics, manure, soil microbiology

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Antimicrobial resistance is a serious threat to human and animal health on a global scale, with bacteria now resistant to the “last-resort” antibiotics, including carbapenems and polymyxins (1–4). It is estimated that without any international-level efforts to revert our current situation, antibiotic resistance would kill an additional 10 million people and cost the global economy 100 trillion U.S. dollars by 2050 (5). To safeguard the future of medicines required for treating otherwise fatal infections, there is an urgent need to advance our understanding of the many mechanisms that are exploited by bacteria to promote resistance.

The ancestral origin of clinical resistance is largely unknown and remains an subject that is elusive to both the medical and research communities. In the perspective of evolutionary genetics, bacteria are capable of adapting to stressful conditions through chromosomal mutations and/or acquisition of genetic materials that can provide them with survival advantages. As such, exposure to antibiotics is a key evolutionary driver for the development of various resistance mechanisms, including, but not limited to, enzymatic drug modification, alteration of drug targets, reduced cell membrane permeability, and transporters-mediated drug efflux (6). These resistance mechanisms are typically encoded by the so-called antibiotic/antimicrobial resistance genes (ARGs) and can be disseminated among microorganisms through horizontal gene transfer. The use of antibiotics will accelerate the selection for ARGs-carrying bacterial pathogens in clinical settings (7). Likewise, anthropogenic inputs of antibiotics into the environment through wastewater effluent, agricultural use of manure and biosolids, and aquaculture will increase the diversity and size of the environmental ARG reservoir, contributing to the spread of antibiotic resistance (8–10).

Antibiotic resistance is ancient and prevalent in the natural environment, and soil is a significant reservoir of ARGs (11, 12). Soil is a very complex and dynamic environmental matrix, typically containing billions of microorganisms and thousands of bacterial species in each kilogram (13). With such microbial diversity and richness, it is not surprising that soil harbors a large diversity of ARGs (14). In fact, recent studies analyzing soil samples collected from sites geographically or temporally distant from human activities recovered not only genetic material orthologous to known ARGs found in contemporary pathogenic bacteria but also novel resistance mechanisms that have potential clinical implications (15–17).

In the present study, we sought to explore the ARG content of agricultural soil from an experimental farm located in London, Canada, by using functional metagenomics. Soil samples collected from antibiotic-amended and control field plots (18, 19) were mined for ARGs that can promote resistance against clinically important antimicrobial agents. We report here the identification of 34 new ARGs and the functional characterization of some of these resistance determinants. We uncovered several previously unknown resistance genes, notably, one that encodes a novel peptide-associated macrolide resistance mechanism.

RESULTS

To explore the content of antibiotic resistance genes (ARGs) borne by soil microorganisms, three metagenomic fosmid libraries were constructed by cloning soil microbial DNA into *Escherichia coli* (Table 1). These libraries encompassed over 36 Gb of metagenomic DNA, equivalent to the combined size of about 7,200 *E. coli* genomes, assuming an average genome size of 5 Mb. Phenotypic selection for resistance against 12 antimicrobial agents belonging to six different antibiotic classes recovered ca. 250 fosmid clones from these metagenomic libraries. After restriction pattern analyses were performed to reduce clonality among candidates selected by the same antibiotic, retransformation confirmed at least 44 individual fosmids that are capable of conferring antibiotic resistance. High-throughput fosmid sequencing also revealed selection redundancy of using different and (un)related antibiotics, implying that some of the recovered fosmids can promote multidrug resistance. Of the 31 nonredundant fosmids that can confer resistance, 28 had their metagenomic DNA insert sequence resolved to completion (ranging from 24.2 to 43.9 kb in size), whereas the remaining three were

TABLE 1 Metagenomic fosmid libraries constructed using DNA extracted from antibiotic-exposed and control field plot soils

Library name	No. of annual applications (expt period)	Antibiotic composition (application concn, ^a mg kg ⁻¹ soil)	No. of clones	Avg insert size (kb)	Cloned bases (Mb)
UTC	0 (2010–2014)	None	111,000	28	3,100
MACRO	5 (2010–2014)	Erythromycin (10), azithromycin (10), clarithromycin (10)	593,000	30	17,900
SCT	16 (1999–2014)	Sulfamethazine (1, 10), chlortetracycline (1, 10), tylosin (1, 10)	488,000	31	15,100
Total			1,192,000		36,100

^aFor the SCT treatment, the annual application concentration for each drug was increased from 1 to 10 mg kg⁻¹ soil since 2005.

partially assembled into separated contigs (8.8 to 26.2 kb) (see Data Set S1 in the supplemental material).

Based on the retransformant’s antibiotic susceptibility profiles (Fig. 1A; see Table S1 in the supplemental material), the fosmid-conferred resistance against aminoglycosides, sulfonamides, and most of the observed β-lactams was not associated with co- or cross-resistance against other classes of antibiotics (Fig. 1B). On the contrary, all tetracycline-resistant fosmid clones displayed reduced susceptibility toward at least one additional class of antibiotic, including macrolides (Fig. 1B). Among the nine clones

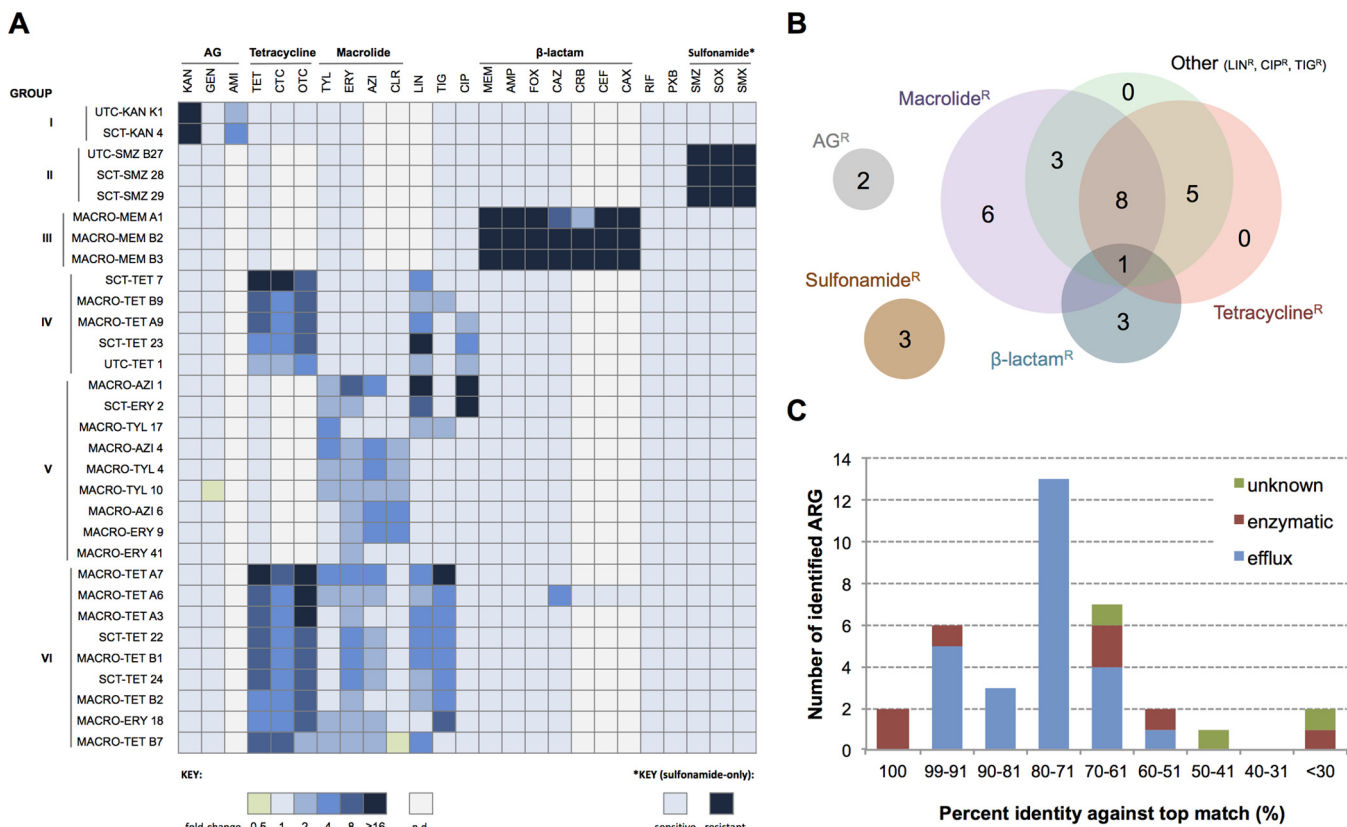


FIG 1 Antibiotic susceptibility profiles and resistance phenotypes of selected soil metagenomic fosmid clones and distributions of their associated antibiotic resistance genes (ARGs). (A) The MICs of antibiotics (except sulfonamides) for 31 nonredundant metagenomic fosmid clones were determined using the broth microdilution method and are presented as fold change relative to those for a control strain. Light gray indicates that the MIC not determined (n.d.). For detecting sulfonamide resistance, serial dilutions of culture were spotted onto agar plates supplemented with the appropriate sulfonamide antibiotic. (B) Resistance phenotypes of the soil metagenomic clones. Each number represents the number of unique clones that displayed resistance against antibiotics belonging to the labeled drug classes. (C) Distribution of putative ARGs based on their predicted resistance mechanism and sequence homology against best matches found by blastX.

that exhibited a classic multidrug resistance phenotype (group VI), resistance against a third-generation cephalosporin, ceftazidime (CAZ), was detected only in the MACRO-TET A6 clone (Fig. 1A). Macrolide-specific resistance was observed in six fosmid clones, with MACRO-TYL 10 also demonstrating mildly enhanced sensitivity toward an aminoglycoside (Fig. 1A).

Using random transposon mutagenesis, 36 putative ARGs were identified from the resistance-associated fosmids (Table 2). Functional annotations suggested that the majority of the identified ARGs encode efflux-mediated mechanisms, while two known and five presumed enzymatically based resistance determinants were also uncovered (Fig. 1C). Additional open reading frame (ORFs) that showed no apparent linkage with any prominent resistance mechanisms were affiliated with resistance against macrolides (Table 2). To ascertain their ARG identity and to gain insight into the underlying resistance principles encoded by some of these soil-derived resistance determinants, a subcloning strategy was employed with the corresponding characterization findings presented in the following sections.

Efflux-mediated resistance. Individual versus combinational subcloning of two putative ATP-binding cassette (ABC) transporter-encoding ORFs identified from fosmid MACRO-TET A7 (i.e., *orf-45*^{TET A7} and *orf-46*^{TET A7}) revealed a corequisite of these two ARGs for conferring multidrug resistance (Table 3). The restored antibiotic sensitivity of transposon mutants that had either one of the two transporter-encoding ARGs inactivated supports the notion that these two ARGs constitute a single functional efflux system. Susceptibility testing against the anticancer drugs daunorubicin (DAU) and doxorubicin (DOX) further demonstrated the capability of this ABC multidrug efflux pump to protect the bacterial host against the anthracycline class of antibiotics (Fig. 2A). It was discovered that homologues of this two-component efflux system of MACRO-TET A7 were present in seven other multidrug-resistant fosmid clones (Table 2), all of which displayed similar reductions in susceptibility toward both DAU and DOX (Fig. 2B).

Enzymatic resistance mechanisms: drug or target modifications. ARGs identical to the well-characterized resistance determinants *aph-3'*, which encodes an aminoglycoside *O*-phosphotransferase (20), and *sul2*, which encodes a type II sulfonamide-resistant variant of dihydropteroate synthase (DHPS) (also known as FolP) (21), were uncovered (Table 2). Both ARGs were identified within genomic neighborhoods where elements implicated in lateral gene transfer (e.g., insertion sequence IS903, transposase, integrase, and conjugative transfer protein) and homologous recombination (e.g., DNA helicases and restriction endonuclease) were in close proximity (see Data Set S1 and Table S2 in the supplemental material).

Expression of *orf-7*^{KAN 4} derived from the fosmid clone SCT-KAN 4 was confirmed to promote resistance against three clinically significant aminoglycosides not only in *E. coli* but also in the opportunistic pathogen *Pseudomonas aeruginosa* (Table 4). Given its sequence similarity (~60% shared identity) to an aminoglycoside 6'-*N*-acetyltransferase (Table 2), these results suggest that *orf-7*^{KAN 4} encodes a potentially new member of this aminoglycoside-modifying enzyme family, which confers antibiotic resistance through drug inactivation.

The broad-spectrum β -lactam resistance determinants recovered from the meropenem-selected fosmid clones (Fig. 1A, group III) were both inhibited by EDTA but not by the classic serine-type β -lactamase inhibitor clavulanic acid (Table 5), in agreement with their predicted functional entities as metallo- β -lactamases (Table 2). The extensive β -lactam resistance phenotype was also recapitulated in *E. coli* and *P. aeruginosa* strains carrying the subcloned *orf-27*^{MEM A1}, which was found proximal to additional β -lactamase-related ARG-like genetic elements in MACRO-MEM A1 (Data Set S1 and Table S2).

A novel sulfonamide resistance gene, here designated *folP*^{SMZ B27}, was discovered from the fosmid clone UTC-SMZ B27. *FolP*^{SMZ B27} shares 35% sequence identity with the *E. coli* DHPS (22), but, reminiscent of the case for the two *sul2*-carrying fosmid clones,

TABLE 2 Putative ARGs identified from resistant metagenomic fosmid clones

Group and fosmid	ORF no.	Description of best blastX match (bacterial species or strain)	Predicted mechanism	Sequence identity (%)	Sequence coverage (%)	Accession no.
I						
UTC-KAN K1	B5	APH(3') family aminoglycoside O-phosphotransferase (<i>Bacteria</i> , multispecies)	Enzymatic	100	99	WP_000338412.1
SCT-KAN 4	7	Aminoglycoside N(6')-acetyltransferase (<i>Chloroflexi</i> bacterium OLB14)	Enzymatic	57	99	KXX13532.1
II						
UTC-SMZ B27	20	Dihydropteroate synthase (" <i>Candidatus</i> Peribacteria" bacterium GW2011_GWB1_54_5)	Enzymatic	62	95	KKW39307.1
SCT-SMZ 28	17	Sulfonamide-resistant dihydropteroate synthase Sul2 (<i>Bacteria</i> , multispecies)	Enzymatic	100	99	WP_001043260.1
SCT-SMZ 29	A6	Sulfonamide-resistant dihydropteroate synthase Sul2 (<i>Bacteria</i> , multispecies)	Enzymatic	100	99	WP_001043260.1
III						
MACRO-MEM A1	27	Subclass B3 metallo-beta-lactamase (<i>Duganella zoogloeooides</i>)	Enzymatic	69	89	WP_019923984.1
MACRO-MEM B2	21	Beta-lactamase class B (bacterium enrichment culture clone cep-06)	Enzymatic	98	99	AIQ86556.1
MACRO-MEM B3	5	Beta-lactamase class B (bacterium enrichment culture clone cep-06)	Enzymatic	98	99	AIQ86556.1
IV						
SCT-TET 7	6	Bcr/CflA family drug resistance efflux transporter (<i>Rhizobium</i> sp. strain YS-1r)	Efflux	95	97	WP_037146735.1
MACRO-TET B9	32	Transmembrane secretion effector (uncultured bacterium)	Efflux	70	97	AIA12822.1
MACRO-TET A9	2	Transmembrane secretion effector (uncultured bacterium)	Efflux	79	99	AIA15249.1
SCT-TET 23	25	Drug resistance transporter, EmrB/QacA subfamily (" <i>Candidatus</i> Saccharibacteria" bacterium GW2011_GWC2_48_9)	Efflux	52	97	KKW00845.1
UTC-TET 1	29	Drug resistance transporter EmrB/QacA subfamily (<i>Ktedonobacter racemifer</i>)	Efflux	64	96	WP_007922578.1
V						
MACRO-AZI 1	B14	MFS antibiotic efflux pump (uncultured bacterium)	Efflux	71	94	AIA18803.1
SCT-ERY 2	14	MFS antibiotic efflux pump (uncultured bacterium)	Efflux	76	99	AIA18803.1
MACRO-TYL 17	12	MATE family efflux transporter (<i>Thermobaculum terrenum</i>)	Efflux	61	97	WP_012876167.1
MACRO-AZI 4	28	None found	— ^c	—	—	—
	<i>ppp</i> ^{AZI 4a}	None found	Unknown	—	—	—
MACRO-TYL 4	18	30S ribosomal protein S21 (" <i>Candidatus</i> Uhrbacteria" bacterium GW2011_GWA2_53_10)	Unknown	49	89	KKW32987.1
MACRO-TYL 10	16	GTPase HflX (<i>Simkania negevensis</i>)	Unknown	64	96	WP_013943053.1
MACRO-AZI 6	2	RND transporter (<i>Acidovorax</i> sp. strain Root275)	Efflux	91	99	WP_057227541.1
	3	RND transporter (<i>Acidovorax</i> sp. strain Root275)	Efflux	98	98	WP_057227539.1
MACRO-ERY 41	17	Major facilitator superfamily (uncultured bacterium)	Efflux	66	93	AIA17006.1
VI						
MACRO-TET A7	45	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	83	99	AIA14488.1
	46	ABC-2-type transporter (uncultured bacterium)	Efflux	86	98	AIA14415.1
MACRO-TET A6	11	Penicillin-binding protein (<i>Streptomyces avermitilis</i>)	Enzymatic	29	79	WP_037649119.1
	12	ABC-2-type transporter (uncultured bacterium)	Efflux	96	99	AIA10928.1
	13 ^b	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	96	95	AIA10927.1
MACRO-TET A3	7	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	82	96	AIA14162.1
	8 ^b	ABC-2-type transporter (uncultured bacterium)	Efflux	77	98	AIA14382.1
SCT-TET 22	4	ABC-2-type transporter (uncultured bacterium)	Efflux	76	99	AIA14382.1
	5	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	78	95	AIA15370.1
MACRO-TET B1	12 ^b	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	80	99	AIA14577.1
	13	ABC-2-type transporter (uncultured bacterium)	Efflux	80	99	AIA15441.1
SCT-TET 24	23	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	80	99	AIA14577.1
	24	ABC-2-type transporter (uncultured bacterium)	Efflux	80	99	AIA15441.1
MACRO-TET B2	32	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	76	99	AIA14488.1
	33	ABC-2-type transporter (uncultured bacterium)	Efflux	73	98	AIA14382.1

(Continued on next page)

TABLE 2 (Continued)

Group and fosmid	ORF no.	Description of best blastX match (bacterial species or strain)	Predicted mechanism	Sequence identity (%)	Sequence coverage (%)	Accession no.
MACRO-ERY 18	17	ABC-2-type transporter (uncultured bacterium)	Efflux	77	99	AIA13096.1
	16	Daunorubicin resistance ABC transporter, ATP-binding protein (uncultured bacterium)	Efflux	79	96	AIA13097.1
MACRO-TET B7	12	MATE efflux family protein (uncultured bacterium)	Efflux	79	99	AIA12080.1

^aAn alternative, oppositely oriented ORF present within ORF28 of MACRO-AZI 4.

^bORF not directly specified by transposon mutagenesis but found within the same transposon-disrupted operon.

^c—, not available.

folP^{SMZ B27}-expressing *E. coli* cells were recalcitrant to the bacteriostatic effect of sulfonamide antibiotics (Fig. 3), which is mediated through the inhibition of dihydropyrimidine synthase (DHPS)-dependent folate biosynthesis. Based on its ability to confer growth competence to a thymidine-auxotrophic *folP*-knockout *E. coli* strain (23) under a thymidine-deficient condition (Fig. 4B and C), it was verified that *folP*^{SMZ B27} encodes a functional DHPS. The ability to withstand the antagonistic effect of sulfonamides specific to DHPSs (Fig. 4D and E) also implies that *FolP*^{SMZ B27} represents an insensitive variant of these enzymes.

By selecting for MACRO-TET A6-derived transposon mutants with restored CAZ sensitivity, a new cephalosporin resistance determinant, ORF11 (here designated *pbp*^{TET A6}), was identified adjacent to an ABC multidrug transporter-encoding operon (i.e., ORF12 and -13) (Table 2). *PBP*^{TET A6} consists of putative N-terminal transglycosylase and C-terminal transpeptidase domains, resembling the bifunctional class A penicillin-binding proteins (PBPs) (24). Subcloning of *pbp*^{TET A6} confirmed the ability of this ARG-encoding product to render *E. coli* resistant to selected third- and fourth-generation cephalosporins (Table 6). Introduction of *pbp*^{TET A6} into *P. aeruginosa* further demonstrated its versatility and potential in promoting resistance against the same spectrum of antibiotics in a clinically significant pathogen (Table 6).

Unknown macrolide resistance mechanisms. The MACRO-TYL 10-derived ORF-16^{TYL 10} has 64% sequence identity to the GTPase HflX of a *Chlamydia*-related bacterium, *Simkania negevensis*, and promoted mild resistance against macrolides when subcloned into different *E. coli* hosts (Table 7). Intriguingly, expression of ORF-16^{TYL 10} in strain AG100A, a macrolide-sensitive derivative of AG100 that is devoid of the significant multidrug resistance-nodulation-division (RND) efflux system component AcrAB (25), did not result in any appreciable level of macrolide resistance. Consistent with this, the MACRO-TYL 10-associated reduction of macrolide susceptibilities was also compromised by the RND pump inhibitor phenyl-arginine- β -naphthylamide (PA β N). Yet, *orf-16*^{TYL 10} expression did not provide any resistance against other ribosome-targeting antibiotics, including tetracycline, lincomycin, and chloramphenicol (Fig. 1A and Table 7), despite some of them being excellent substrates of the AcrAB efflux pump (26). Collectively, these observations rule out a general upregulation of AcrAB's efflux

TABLE 3 Corequirement of two ABC transporter-encoding genes identified in fosmid MACRO-TET A7 for multidrug resistance

<i>E. coli</i> strain	Vector	Genotype ^a		MIC (μ g/ml) ^b							
		<i>orf-45</i> ^{TET A7}	<i>orf-46</i> ^{TET A7}	TET	CTC	OTC	TYL	ERY	AZI	LIN	TIG
DH10 β	pCF430	—	—	ND	ND	ND	2,048	512	32	128	4
	pCL027	+	—	ND	ND	ND	2,048	512	32	128	4
	pCL028	—	+	ND	ND	ND	2,048	512	32	128	4
	pCL029	+	+	ND	ND	ND	8,192	2,048	128	256	16
EPI300T1 ^R	pCC1FOS	—	—	4	64	4	2,048	256	16	256	1
	MACRO-TET A7	+	+	64	512	64	8,192	1,024	64	1,024	16
	MACRO-TET A7/ Δ <i>orf-45</i> ::Tn	—	+	4	64	4	2,048	256	16	256	1
	MACRO-TET A7/ Δ <i>orf-46</i> ::Tn	+	—	4	64	4	2,048	256	16	256	1

^a+, present; —, absent or transposon disrupted.

^bND, not determined. MIC values higher than that for the corresponding empty-vector control are in bold. AZI, azithromycin; CTC, chlortetracycline; ERY, erythromycin; LIN, lincomycin; OTC, oxytetracycline; TET, tetracycline; TIG, tigecycline; TYL, tylosin.

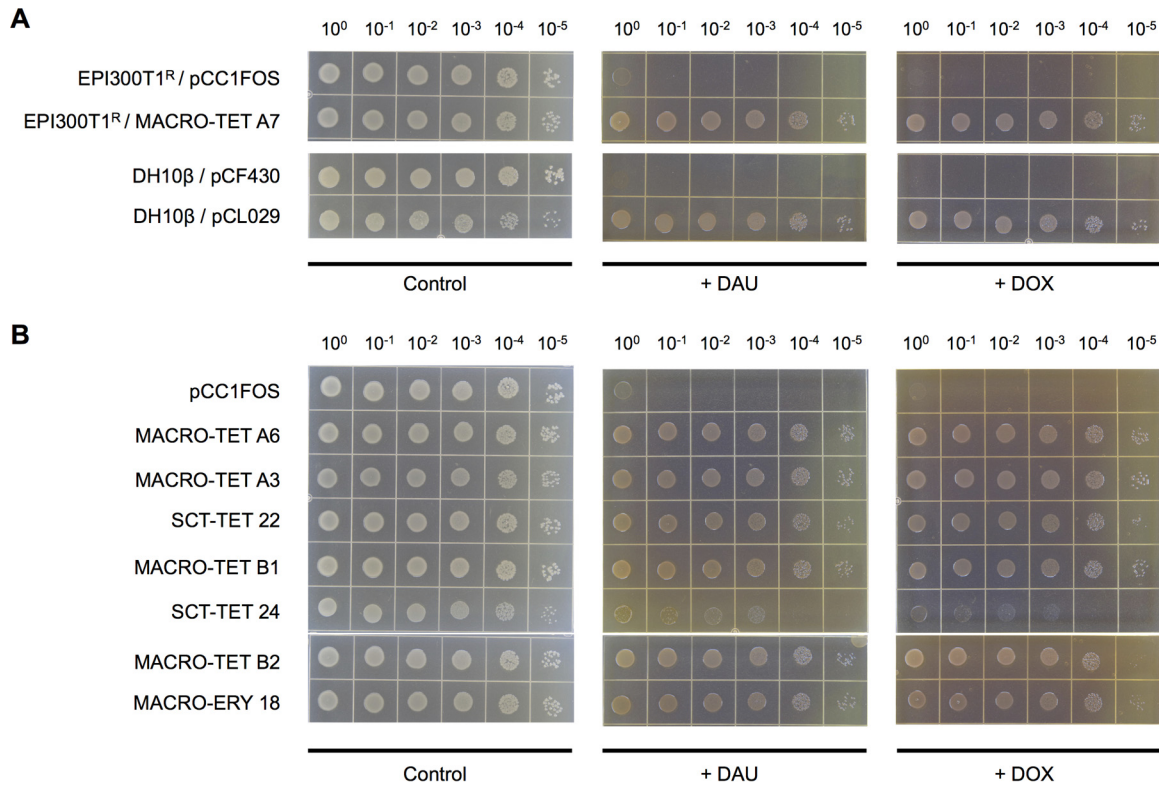


FIG 2 Resistance against the anticancer drugs daunorubicin (DAU) and doxorubicin (DOX) mediated by soil metagenome-derived multidrug ABC transporter genes in *E. coli*. Five microliters of serially diluted overnight *E. coli* cultures with the starting OD_{600} normalized to 0.5 was spotted onto LB agar plates supplemented with 100 $\mu\text{g/ml}$ of DAU (+ DAU) or DOX (+ DOX). LB agar plates with no anticancer drug (Control) were also included. (A) Strains carrying fosmid MACRO-TET A7, pCF430 derivatives containing the ABC transporter-encoding *orf-45^{TET A7}* and *orf-46^{TET A7}* (pCL029), or the appropriate empty-vector controls. (B) EPI300T1^R strains with fosmid carrying close homologues of the multidrug ABC transporter genes identified in MACRO-TET A7.

activity in the *orf-16^{TYL 10}*-expressing cell and indicate that ORF-16^{TYL 10} constitutes a new macrolide-specific resistance mechanism which is somehow dependent on the macrolide extrusion mediated by AcrAB.

From the macrolide-resistant fosmid MACRO-AZI 4, transposon mutagenesis identified the *in silico*-predicted *orf-28^{AZI 4}*, *orf-29^{AZI 4}*, and *orf-30^{AZI 4}* as potential resistance determinants (Fig. 5A). A blastX homology search against the NCBI nonredundant (nr) protein database using these three theoretical ORFs as queries resulted in no significant similarity found for *orf-28^{AZI 4}* and two hypothetical proteins for *orf-29^{AZI 4}* and *orf-30^{AZI 4}*. Through constructing a series of pCF430-based subclones (pCL043 to pCL047) (Fig. 5A), it was deduced that the macrolide resistance, which is independent of the RND pump-mediated efflux, was attributed solely to the region encompassing *orf-28^{AZI 4}* (Table 8). Compared to the control strain (Fig. 5B), the pCL047 transformant was clearly less susceptible to the growth-inhibitory effect of azithromycin (Fig. 5C). Furthermore,

TABLE 4 New aminoglycoside resistance determinant, *orf-7^{KAN 4}*, found in fosmid SCT-KAN 4 can confer antibiotic resistance to both *E. coli* and *P. aeruginosa*

Species and strain	Vector	MIC ($\mu\text{g/ml}$) ^a		
		GEN	AMI	KAN
<i>E. coli</i> DH10 β	pCF430	2	4	4
	pCL030 (pCF430:: <i>orf-7^{KAN 4}</i>)	4	16	64
<i>P. aeruginosa</i> PAO1	pUCP19	2	4	256
	pCL031 (pUCP19:: <i>orf-7^{KAN 4}</i>)	16	256	2,048

^aMIC values higher than that for the corresponding empty-vector control are in bold. AMI, amikacin; GEN, gentamicin; KAN, kanamycin.

TABLE 5 Soil metagenome-derived metallo- β -lactamases conferring broad-spectrum β -lactam resistance

Species and strain	Vector	MIC ($\mu\text{g/ml}$) ^a								
		AMP	CRB	FOX	CAZ	CEF	CAX	MEM	MEM + CLV ^b	MEM + EDTA ^c
<i>E. coli</i>										
EPI300T1 ^R	pCC1FOS	8	16	16	2	0.5	0.125	0.03	0.03	0.03
	MACRO-MEM A1	256	32	256	16	128	64	32	32	0.03
	MACRO-MEM B2	> 1,024	> 2,048	256	64	16	8	16	16	0.03
DH10 β	pCF430	2	8	16	2	0.5	0.125	0.015	ND	ND
	pCL032 (pCF430:: <i>orf-27</i> ^{MEM A1})	64	16	256	16	32	16	8	ND	ND
<i>P. aeruginosa</i> PAO1										
pRK415	pRK415	256	32	1,024	4	64	8	0.25	ND	ND
	pCL033 (pRK415:: <i>orf-27</i> ^{MEM A1})	2,048	32	2,048	32	512	256	256	ND	ND

^aMIC values higher than that for the corresponding empty-vector control are in bold. AMP, ampicillin; CAX, cefotaxime; CAZ, ceftazidime; CEF, ceftiofur; CLV, clavulanic acid; CRB, carbenicillin; FOX, foxitoxin; MEM, meropenem. ND, not determined.

^bCLV was included at 4 $\mu\text{g/ml}$.

^cEDTA was included at 5 mM.

the growth deficiency associated with MACRO-AZI 4 (Fig. 5D) and only certain sub-clones (Fig. 5E) failed to correlate with the generally observed macrolide-resistant phenotype (Fig. 5A and Table 8).

To verify the expression of any hypothetical protein that could be related to resistance, total proteomic analyses were performed to search for fosmid-specific proteins in the MACRO-AZI 4 fosmid clone and its resistant derivatives (pCL043 and pCL047). Unexpectedly, no peptide fragment corresponding to the theoretical translational product of *orf-28*^{AZI 4} was detected (see Data Set S2 in the supplemental material). Instead, a 61-residue-long polyproline peptide, here designated PPP^{AZI 4}, encoded by an alternative reading frame present within *orf-28*^{AZI 4} (Fig. 5A) was consistently expressed in all three resistant clones. Closer examination of this unusual proline-rich peptide revealed that the bulk of PPP^{AZI 4} is composed of 15 direct-repeats of an (im)perfect "PPX" motif (Fig. 5F) and that it shares no homology with proteins in the NCBI nr database. While the macrolide-resistant phenotype and PPP^{AZI 4} expression were fully recapitulated in cells carrying pCL048, deletion of the first 38 bp of the PPP^{AZI 4} gene and its upstream sequence from pCL047 (yielding pCL049) resulted in no detect-

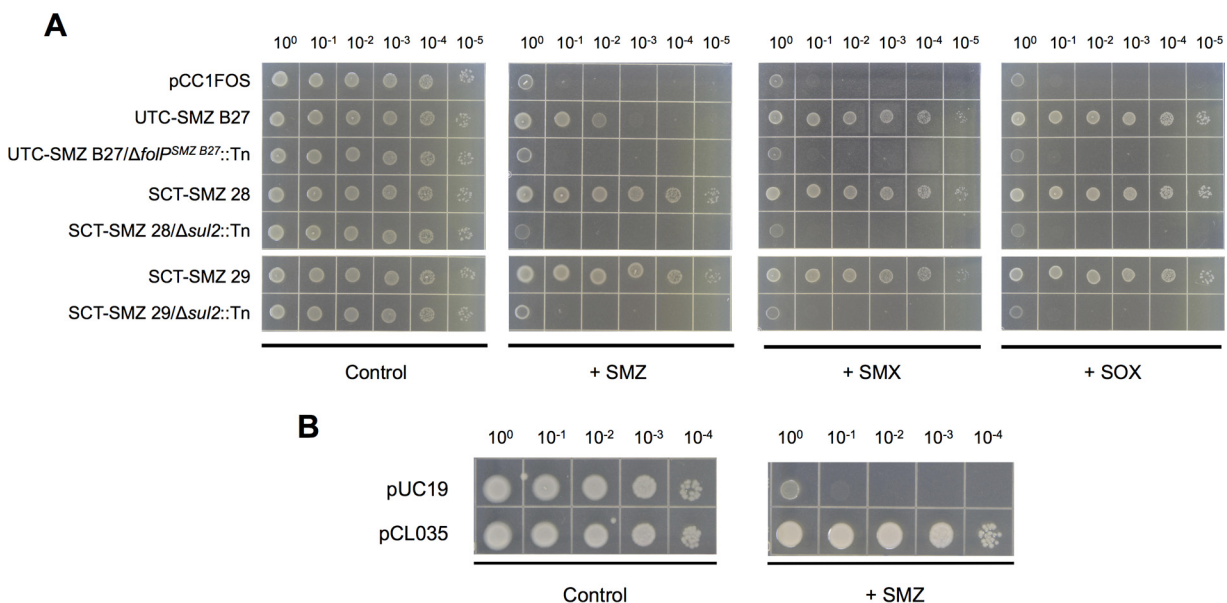


FIG 3 Resistance against sulfonamide antibiotics mediated by soil metagenome-derived ARGs *folP*^{SMZ B27} and *sul2*. Five microliters of serially diluted overnight *E. coli* EPI300T1^R (A) or DH10 β (B) cultures with starting OD₆₀₀ normalized to 0.5 were spotted onto Iso-Sensitest agar plates supplemented with 2,000 $\mu\text{g/ml}$ of sulfamethazine (+ SMZ), 1,000 $\mu\text{g/ml}$ of sulfamethoxazole (+ SMX), or 1,000 $\mu\text{g/ml}$ of sulfisoxazole (+ SOX). Iso-Sensitest agar plates with no sulfonamide added (Control) were also included. (A) pCC1FOS-derived metagenomic fosmid clones and their respective transposon insertion mutants. (B) *E. coli* strains carrying pUC19 or its *folP*^{SMZ B27}-expressing derivative pCL035.

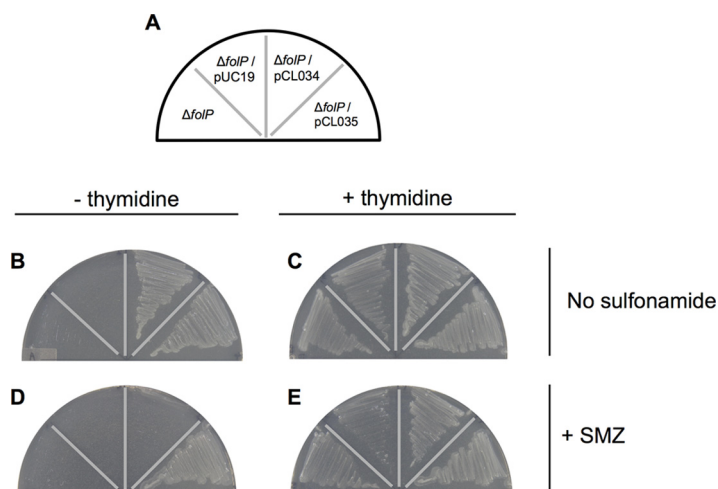


FIG 4 Growth complementation and sulfonamide tolerance conferred by *folP*^{SMZ B27} to a thymidine-auxotrophic *folP*-knockout *E. coli* strain. Single colonies of the C600 $\Delta folP::Km^r$ strain ($\Delta folP$) and its derivatives transformed with pUC19, the prototypic *folP*^{E. coli}-expressing pCL034, and the *folP*^{SMZ B27}-expressing pCL035 were streaked according to the plate map shown (A) onto Iso-Sensitest agar without (B) or with (C) the supplement of 200 μ g/ml of thymidine. For assessing sulfonamide susceptibility, 2,000 μ g/ml of sulfamethazine (+ SMZ) was included in separated thymidine-deficient (D) and thymidine-amended (E) plates.

able PPP^{AZI 4} expression and, most importantly, a complete loss of resistance (Fig. 5A and Table 8). To examine the selectivity of PPP^{AZI 4}-associated resistance, susceptibility against two additional and structurally distinct macrolides, telithromycin (TLT) (a 14-member ring ketolide) and josamycin (JOS) (a 16-member ring), was also examined. Again, reduced susceptibility against TLT and JOS was observed with only the PPP^{AZI 4}-expressing cells and not their nonexpressing counterparts, consistent with the case for other macrolide antibiotics. Taking the results together, although the mechanism underlying this peptide-associated macrolide resistance is currently unclear (see Discussion for proposed mechanisms), PPP^{AZI 4} expression is evidently linked to general resistance against macrolides, implicating the PPP^{AZI 4} gene as a novel ARG.

DISCUSSION

The present study set out to uncover ARGs from soil exposed to antibiotics heavily used in human medicine or food animal production. The idea of mining this antibiotic-amended agricultural soil for novel ARGs is justified not only by the clinical significance of these antimicrobial agents (27) and their high potential for soil exposure through manure or biosolid fertilization but also by the fact that the soil microbial communities

TABLE 6 Cephalosporin resistance in *E. coli* and *P. aeruginosa* conferred by a probable penicillin-binding protein gene homologue, *pbp*^{TET A6}, recovered from a multidrug-resistant fosmid clone

Species and strain	Vector	Genotype ^a		MIC (μ g/ml) ^b										
		<i>pbp</i> ^{TET A6}	<i>orf-12</i> ^{TET A6}	CAZ	CAX	FEP	TET	CTC	OTC	TYL	ERY	AZI	LIN	TIG
<i>E. coli</i>														
EPI300T1 ^R	pCC1FOS	–	–	2	0.125	1	4	64	4	2,048	256	16	256	1
	MACRO-TET A6	+	+	8	0.125	1	32	256	64	4,096	512	32	512	4
	MACRO-TET A6/ $\Delta orf-12::Tn$	+	–	8	ND	ND	4	64	4	2,048	256	16	256	1
	MACRO-TET A6/ Δpbp ^{TET A6::Tn}	–	+	2	ND	ND	32	256	64	4,096	512	32	512	4
DH10 β	pUC19	–	–	2	0.125	1	ND	ND	ND	ND	ND	ND	ND	ND
	pCL050	+	–	32	0.5	4	ND	ND	ND	ND	ND	ND	ND	ND
<i>P. aeruginosa</i> PAO1														
PAO1	pUCP19	–	–	4	16	1	ND	ND	ND	ND	ND	ND	ND	ND
	pCL051	+	–	128	32	4	ND	ND	ND	ND	ND	ND	ND	ND

^a+, present; –, absent or transposon disrupted.

^bND, not determined. MIC values higher than that for the corresponding empty-vector control are in bold. AZI, azithromycin; CAZ, ceftazidime; CAX, cefotaxime; CTC, chlortetracycline; ERY, erythromycin; FEP, cefepime; LIN, lincomycin; OTC, oxytetracycline; TET, tetracycline; TIG, tigecycline; TYL, tylosin.

TABLE 7 Reduced macrolide susceptibility conferred by *orf-16*^{TYL 10} is dependent on the efflux activity of RND multidrug pump AcrAB in *E. coli*

<i>E. coli</i> strain	Vector ^a	Genotype ^b		MIC ($\mu\text{g/ml}$) ^c						
		<i>orf-16</i> ^{TYL 10}	<i>acrAB</i>	TYL	ERY	AZI	CLN	GEN	LIN	CAM
EPI300T1 ^R	pCC1FOS	–	+	2,048	256	16	256	8	256	ND
	MACRO-TYL 10	+	+	4,096	512	32	512	4	256	ND
	pCC1FOS (+ PA β N)	–	+	16	4	1	2	8	ND	ND
	MACRO-TYL 10 (+ PA β N)	+	+	16	4	1	2	4	ND	ND
DH10 β	pUC19	–	+	2,048	256	16	256	2	256	4
	pCL036	+	+	4,096	1,024	32	512	2	256	4
	pCL037	+	+	4,096	1,024	32	512	2	256	4
AG100	pUC19	–	+	2,048	128	16	128	4	ND	ND
	pCL036	+	+	4,096	256	32	256	4	ND	ND
AG100A	pUC19	–	–	64	8	4	8	4	ND	ND
	pCL036	+	–	64	8	4	8	4	ND	ND

^a+ PA β N, the RND pump inhibitor phenyl-arginine- β -naphthylamide was included at 25 $\mu\text{g/ml}$. pCL036 and pCL037 are identical except that the *orf-16*^{TYL 10}-carrying insert fragment was cloned in opposite orientations.

^b–, absent; +, present.

^cMIC values higher and lower than that of the corresponding empty-vector control are in bold and in italic, respectively. AZI, azithromycin; CAM, chloramphenicol; CLN, clarithromycin; ERY, erythromycin; GEN, gentamicin; LIN, lincomycin; TYL, tylosin. ND, not determined.

displayed adaptability and responsiveness toward these specific drug exposures in recent studies (18, 19, 28). Using functional metagenomics, a number of ARGs were discovered, most of which encode (multi)drug transporters of all five superfamilies. This finding is consistent with those of others (29, 30), supporting drug efflux as one of the most prominent strategies employed by environmental bacteria to resist antibiotics that are either produced by other soil microorganism or introduced anthropogenically. The identification of new antibiotic-inactivating enzymes that can promote resistance against aminoglycosides and β -lactams in both *E. coli* and *P. aeruginosa* reinforces the notion that soil is a significant reservoir of as-yet-unknown ARGs that could seriously exacerbate clinical resistance if recruited by human pathogens. Furthermore, the proteomics-driven discovery of a potentially new peptide-mediated resistance mechanism against macrolides underscores the value of deploying high-resolution analytical proteomic technology in functional metagenomics studies.

We demonstrated substrate polyspecificity in a homologous group of ABC efflux systems that can confer resistance against a spectrum of structurally distinct antibiotics and anticancer drugs. These probable two-component transporters all display moderate sequence homology (30 to 50% shared identity) against the DrrAB multidrug efflux system of the DAU-/DOX-producing *Streptomyces peucetius* (31) and are predicted to originate from bacteria of diverse phyla, including *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (see Table S3 in the supplemental material). While the identification of novel DrrAB homologues in different bacterial taxa is not unexpected, the ligand promiscuity of these functionally conserved, but sequence-wise diverse, ABC transporters (DrrAB included) raises insightful questions of what drove the development of polyspecific substrate recognition and whether these transporters have shared evolutionary history with similar multidrug efflux system found in now-pathogenic organisms.

The discovery of *foIP*^{SMZ B27} from the metagenome of soil with no history of exposure to the synthetic sulfonamide antibiotics is intriguing. *FoIP*^{SMZ B27} is distantly related (\sim 30% shared sequence identity) to three highly prevalent sulfonamide-resistant DHPs alternatives, Sul1, Sul2, and Sul3 (32), and shows the greatest homology with deduced DHPs of several uncultured bacteria belonging to the candidate phyla radiation (CPR) group (Fig. 6). CPR organisms are estimated to

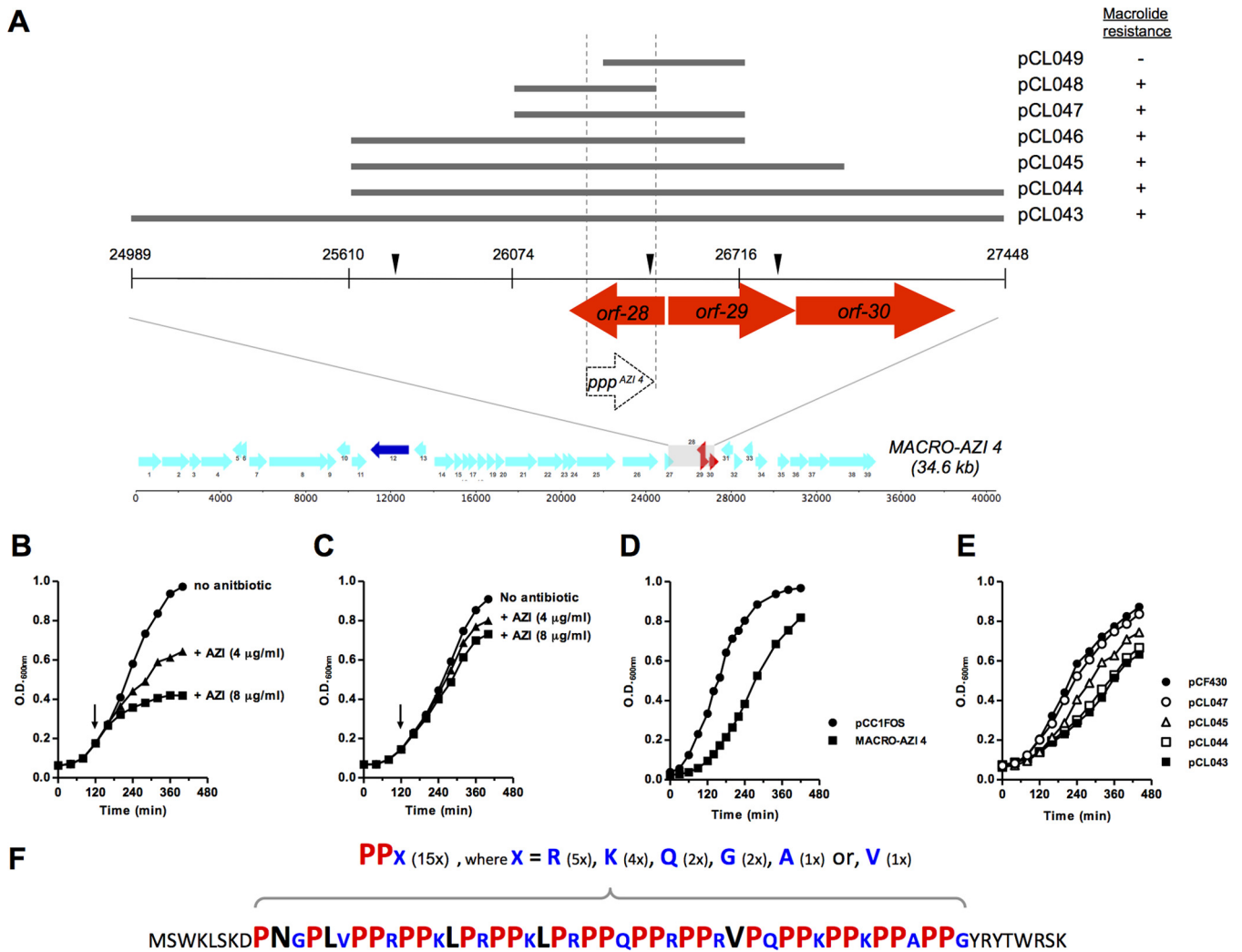


FIG 5 Characterization of soil metagenome-derived fosmid MACRO-AZI 4 and identification of a novel macrolide resistance determinant, the $PPP^{AZI 4}$ gene. (A) Schematic presentation of the subcloned fragments derived from fosmid MACRO-AZI 4. The approximate locations of individual transposon insertions found on fosmid MACRO-AZI 4 that restored macrolide sensitivity are indicated by inverted black triangles. Colored arrows depict the *in silico*-predicted ORFs, while the white arrow represents an alternative ORF, the $PPP^{AZI 4}$ gene. Macrolide susceptibilities of subclones are stated as sensitive (-) or resistant (+) based on result in Table 8. (B and C) Azithromycin susceptibilities of the pCF430 (B)- and pCL047 (C)-carrying DH10β strains. Cell growth in LB medium at 37°C with mild agitation was monitored by measuring the OD₆₀₀ every 20 to 30 min using a spectrophotometer. Arrows indicated when azithromycin was introduced. (D and E) Growth impairment associated with subcloned MACRO-AZI 4 insert fragments. EPI300T1^R (D) and DH10β (E) strains carrying the indicated vector were grown in LB medium, without any antibiotic supplements, exactly as described above. (F) Amino acid sequence of the polyproline peptide encoded by the $PPP^{AZI 4}$ gene. PPX motifs are in bold and colored in red (proline residues), blue (variable residues [X]), and black (nonconserved residues). Numbers in parentheses specify the number of occurrences of the indicated residue or motif.

constitute >15% of the bacterial domain and have relatively small genomes that lack numerous biosynthetic pathways (33). It is therefore tempting to speculate that some bacterial members of this largely uncharacterized phylum might possess alternative folate-biosynthetic mechanisms (e.g., novel DHPS enzyme variants) that are naturally insensitive to the antagonistic effect of sulfonamide. If so, such kinds of underappreciated metabolic pathways might offer biological clues to the ancestry of specific resistance determinants (e.g., FolP^{SMZ B27} and PBPT^{TET A6}), which can act incidentally against artificial antimicrobial agents.

In a genomic context, some of the ARGs are syntenic to elements that indicate lateral gene transfer and acquisition (Table S2). Apart from the most striking case seen with *sul2*, three unrelated ARGs (*pbp*^{TET A6}, a metallo-β-lactamases gene, and a transporter gene) were found in close proximity to genes encoding homologues of two bacterial nonhomologous end-joining (NHEJ) complex components, the Ku and ligase

TABLE 8 Macrolide susceptibility of the metagenomic fosmid clone MACRO-AZI 4 and its derivatives carrying different subcloned fragments

Strain	Vector ^a	Genotype ^b			MIC ($\mu\text{g/ml}$) ^c					
		<i>orf-28</i> ^{AZI 4}	<i>orf-29</i> ^{AZI 4}	<i>orf-30</i> ^{AZI 4}	TYL	ERY	AZI	CLN	TLT	JOS
EPI300T1 ^R	pCC1FOS	–	–	–	2048	256	16	256	ND	ND
	MACRO-AZI 4	+	+	+	8,192	512	64	512	ND	ND
	pCC1FOS (+ PA β N)	–	–	–	16	4	1	2	ND	ND
	MACRO-AZI 4 (+ PA β N)	+	+	+	64	8	2	4	ND	ND
DH10 β	pCF430	–	–	–	2,048	512	32	512	128	512
	pCL043	+	+	+	8,192	2,048	128	1,024	512	1,024
	pCL044	+	+	+	8,192	2,048	128	1,024	512	1,024
	pCL045	+	+	–	8,192	2,048	128	1,024	512	1,024
	pCL046	+	–	–	8,192	2,048	128	1,024	512	1,024
	pCL047	+	–	–	8,192	2,048	128	1,024	512	1,024
	pCL048	–*	–	–	8,192	2,048	128	1,024	512	1,024
	pCL049	–	–	–	2,048	512	32	512	128	512

^a+ PA β N, the RND pump inhibitor phenyl-arginine- β -naphthylamide was included at 25 $\mu\text{g/ml}$.

^b–, absent; +, present; *, a partial sequence of *orf-28*^{AZI 4} corresponding to the PPP^{AZI 4} gene is present.

^cND, not determined. MIC values higher than that for the corresponding empty-vector control are in bold. AZI, azithromycin; CLN, clarithromycin; ERY, erythromycin; JOS, josamycin; TLT, telithromycin; TYL, tylosin.

D (LigD) proteins (Table S2). NHEJ carries out DNA double-strand break repair and was discovered first in eukaryotes and subsequently in bacteria (34). The association of ARGs with a putative NHEJ apparatus hints at possible horizontal gene transfer events. Although Ku and LigD are not universal among the bacterial domain, ARG acquisition by similar recombination-independent mechanism has also been reported in *E. coli* (35). Collectively, these observations suggest that the end-joining DNA repair mechanism might play a role in the spread of environmental ARGs.

While the molecular principles underlying the new macrolide resistance determinants reported have yet to be fully elucidated, the evidence here is consistent with the ribosome being central to these resistance mechanisms. ORF-16^{TYL 10} has substantial

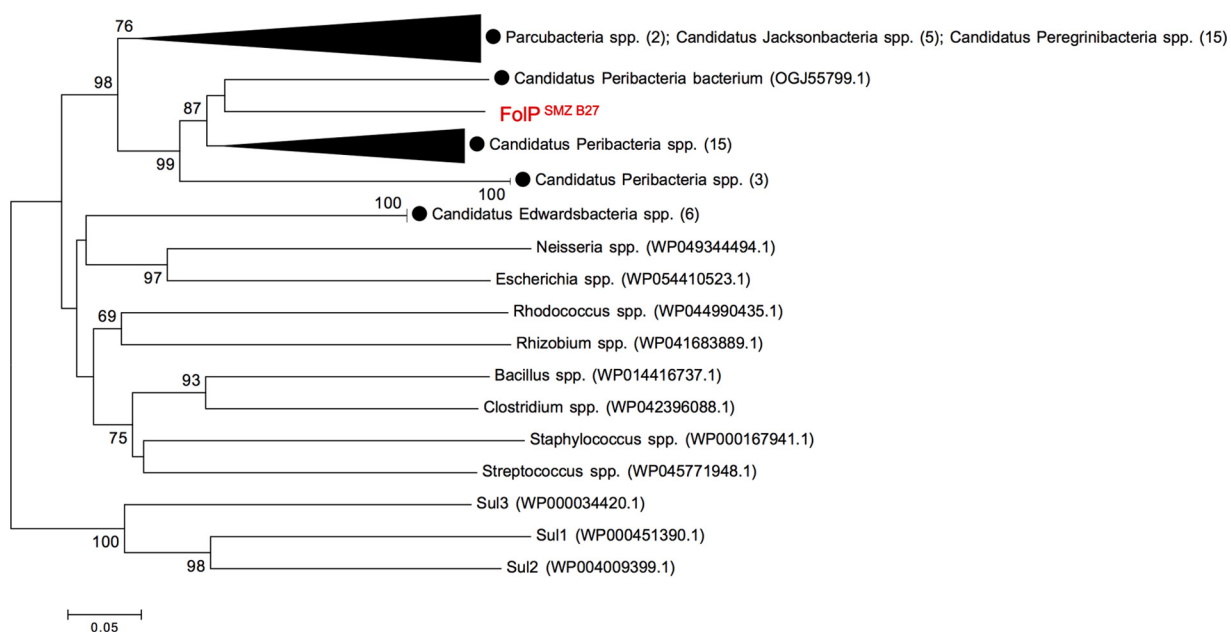


FIG 6 Neighbor-joining tree of aligned amino acid sequences of FoIP^{SMZ B27} and bacterial dihydropteroate synthases (DHPSs). The alignment was performed with prototypic DHPSs and sulfonamide-resistant DHPS variants (Sul1, Sul2, and Sul3) using the ClustalW program within MEGA 7. Genera and species are followed by their GenBank accession numbers in parentheses. DHPSs from the candidate phyla radiation (CPR) group bacteria are specified with black circles. Some of the branches were grouped to improve the visibility of the tree (the number of sequences grouped is indicated in parentheses). Bootstrap values were calculated as a percentage of 1,000 replicates, and those above 60% are shown at the branching points. The sequence of FoIP^{SMZ B27} obtained in this study is depicted in red. Scale bar = 0.05 change/site.

homology to the ribosome-associated GTPase HflX (36). HflX can interact with the peptidyl transferase center (PTC) and nascent peptide exit tunnel (NPET) of the ribosome to induce dissociation of 30S and 50S subunits in a nucleotide-dependent manner, thereby rescuing stalled ribosomes during cellular stress (37). Interestingly, both the NPET-binding macrolides (e.g., erythromycin) and the PTC-binding antibiotics (e.g., chloramphenicol) can inhibit the ribosome-induced GTPase activity of HflX (38). Hence, we hypothesize that ORF-16^{TYL 10} functions as an alternative ribosome-splitting factor to alleviate the macrolide-mediated translational arrest. We postulate that ORF-16^{TYL 10} might be able to better “sense” the drug-induced translational stalling event at the PET (and presumably not at the PTC) and/or to withstand the inhibitory effect of macrolides on its ribosome-induced GTPase activity. The dissociation of macrolide-bound ribosome promoted by ORF-16^{TYL 10} is believed to reset the stalled ribosome to allow productive translation. Concerning the apparent AcrAB dependency of the ORF-16^{TYL 10}-conferred resistance, observations highly similar to ours, in which the macrolide resistance caused by ribosomal mutation was “masked” by drug efflux pump deficiency, have been reported for several bacteria, including *E. coli* (39). This so-called “drug target mutation masking” effect in response to efflux pump inhibition is fundamentally linked to growth bistability and is favored by factors including high-affinity drug binding to its target and high target abundance within bacterial cells (39), both of which are applicable in the case of macrolide antibiotics and their ribosomal target. As the exact mechanism remains to be unraveled, our data lean toward a conceivable theory of ORF-16^{TYL 10} acting on the ribosome target, instead of selectively modulating the efflux pump activity, to confer macrolide-specific resistance in an efflux-dependent manner.

An exciting discovery in this study is the macrolide resistance associated with a novel polyproline peptide, PPP^{AZI 4}, which is encoded by an alternative ORF not recalled by the gene prediction algorithm. Together with its exceptionally high proline content, the repeated appearance of diproline motifs in PPP^{AZI 4} suggests that this small peptide functions in the context of macrolide-induced translational arrest. Sequences containing consecutive proline residues (especially with three or more) can trigger significant stalling of the bacterial ribosome, which is effectively rescued by the translation elongation factor EF-P (40–43). The likelihood of a nascent peptide becoming arrested at its diproline motif is determined by the identity of residues flanking the proline pair (41, 44–46). In contrast to strong stalling sequences (e.g., PPP), PPR and RPP motifs (where R = Arg) confer weak stalling activity, as their translation has little reliance on the activity of EF-P (41, 45). Furthermore, *in vivo* PPK (where K = Lys) is translationally arrested only when preceded by an acidic residue (44). These findings illustrate the context-specific nature of EF-P-dependent stalling of diproline motifs. Strikingly, PPP^{AZI 4} consists of 15 (im)perfect PPX motifs in direct tandem arrangement, with Arg (R) and Lys (K) being most frequently found between diproline sequences and no PPP motif present (Fig. 5F). Our preliminary data have shown that PPP^{AZI 4} can be detected in both an *efp* deletion mutant (47) and its parental strain carrying plasmid pCL047 and that the macrolide resistance associated with PPP^{AZI 4} expression is EF-P independent (see Data Set S2 and Table S4 in the supplemental material). Taken together, these results suggest that the sequence of PPP^{AZI 4} is evolutionarily optimized to ensure its proper synthesis, probably by minimizing the appearance of any strong EF-P-alleviated ribosomal stalling signals, for a reason(s) that could be related to macrolide resistance.

How can the effective translation of PPP^{AZI 4} be linked to macrolide resistance? We believe our finding is reminiscent of the previously reported peptide-mediated resistance, in which the synthesis of a pentapeptide-encoding ORF renders both Gram-positive (48) and Gram-negative (49) bacteria resistant against erythromycin. Lovmar et al. (50) proposed a *cis*-acting model implicating a specific interaction between the nascent pentapeptide and the tunnel-bound macrolide, which can lead to erythromycin removal from its ribosomal binding site upon peptidyl-tRNA release at the end of translation cycle. However, PPP^{AZI 4} is significantly longer than the described pentapeptides. Also, unlike the macrolide selectivity of different resistant pentapeptides,

PPP^{AZI} 4 can promote resistance against broad range of macrolides, suggesting an alternative mechanism of action. Notably, PPP^{AZI} 4 shares the first four residues with one of the ketolide-resistant pentapeptides (MSWKL) (51). We speculate that this N terminus sequence facilitates the initial threading of the PPP^{AZI} 4 nascent peptide through the macrolide-obstructed NPET of the ribosome, analogous to how appending the N-terminal sequence of the H-NS protein to the OsmC protein, whose syntheses are erythromycin resistant and erythromycin sensitive, respectively, allowed the latter to evade the macrolide-induced translational attenuation (52). Although the mere presence of a consensus pentapeptide sequence at the N termini of longer peptides did not confer erythromycin resistance (48), PPP^{AZI} 4 comprises a unique multi-PPX segment that has Arg (R) and Lys (K) as the dominant spacer residues and Leu (L) as the major proline substitute in the imperfect PPX motifs. These amino acids are thought to give peptides a high propensity to adopt a distinctive secondary structure called the type II polyproline (PPII) helix (53). In structural terms, the PPII helix is a left-handed helix that is relatively open reading frame and flexible and has no internal hydrogen bonding (54). It is plausible that formation of the PPII helical structure, after the initial threading through of the PPP^{AZI} 4 nascent peptide, can eject the antibiotic, resulting in ribosomal “cleaning” to restore ribosomal translation. This hypothetical model is somewhat analogous to the previously proposed “bottle brush” theory (51), with the exception that PPP^{AZI} 4 can confer *cis*-acting resistance against general, instead of specific, macrolide members through the complete synthesis of its polyproline segment. The lack of antibiotic resistance in subclone that expressed a truncated version of the PPP^{AZI} 4 gene encoding only the first 25 amino acids (data not shown) is in support of the theory that the multi-PPX sequence is key to the peptide’s ability to confer macrolide resistance. Certainly, given that PPII helices are implicated in diverse molecular functions, including cell signaling, transcription, cell motility, and bacterial pathogenesis (54), we cannot exclude the possibility that PPP^{AZI} 4 may possess other biological functions that can render bacteria less susceptible to macrolide antibiotics.

MATERIALS AND METHODS

Additional method details are provided in Text S1 in the supplemental material.

Soil treatments and sampling. Soil samples were collected in fall 2014 from two series of antibiotic-amended (and control) field plots established at the Agriculture and Agri-Food Canada (AAFC) experimental farm in London, Canada (18, 19). The drug application history of the three groups of field plots used in the present study, namely, UTC (untreated control), MACRO (macrolides), and SCT (agricultural antibiotics), is presented in Table 1.

Soil DNA extraction and metagenomic clone library construction. High-molecular-weight metagenomic DNA was extracted from 100 g of sieved soil using an indirect extraction method as described by to Liles et al. (55). Pulse-field gel electrophoresis (PFGE) was used to size fractionate the DNA onto a 1% agarose gel. Nucleic acid with the chosen size of 25 to 70 kb was electroeluted from the excised gel block and concentrated by ethanol precipitation. Three individual metagenomic libraries were constructed using a pCC1FOS fosmid library production kit (Epicentre) with the DNA derived from the aforementioned field plots (UTC, MACRO, and SCT) and the *E. coli* host strain EPI300-T1^R. Fosmid clones from each library were aseptically scraped from the plates, pooled into subpools of ca. 6,000 clones ml⁻¹, and stored at -80°C in LB broth supplemented with 12.5 μg ml⁻¹ chloramphenicol and 15% (vol/vol) glycerol. To estimate the average insert size of each library, fosmid DNA was extracted from 30 randomly selected clones, followed by NotI digestion and PFGE analysis (56).

Selection for antibiotic-resistant metagenomic fosmid clones. Each library was replicated by inoculating scrapes of frozen subpool stocks into 50 ml of LB broth supplemented with 12.5 μg ml⁻¹ chloramphenicol for 2 h at 37°C. Ten- and 100-fold-diluted library cultures were plated separately onto LB agar or Iso-Sensitest agar plates supplemented with one of 12 antibiotics. Colonies formed after the initial 16 to 18 h of incubation at 37°C, together with those, if any, that appeared after further incubation at room temperature for 24 h were subjected to clonality assessment. To preclude any spontaneous mutation of the *E. coli* host strain that might be (co)selected for during the selection process, fosmid DNA with a unique NotI restriction profile was reelectrotransformed into EPI300-T1^R cells to confirm the fosmid-associated antibiotic resistance phenotypes.

Fosmid sequencing and identification of putative ARGs. To resolve the sequence of cloned metagenomic DNA, resistance-associated fosmid targets were sequenced using a MiSeq (multiplex) sequencing system (Illumina). To identify putative antibiotic resistance genes (ARGs) harbored on each resistance selected fosmid, transposon mutagenesis was performed using either the EZ-Tn5 <KAN-2> or <TET-1> insertion kit (Epicentre). Transposon-bearing fosmid DNA isolated from mutants with restored susceptibility toward the selection antibiotic was sequenced to locate the transposon insertion site using a transposon-specific primer set.

TABLE 9 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> strains		
EPI300T1 ^R	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU</i> <i>galk</i> λ ⁻ <i>rpsL</i> (Str ^r) <i>nupG</i> <i>trfA</i> <i>tonA</i>	Epicentre
DH10β	Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>fhuA</i> <i>galk16</i> <i>galE15</i> e14 ⁻ <i>rpsL</i> (Str ^r) <i>nupG</i> <i>rph</i> <i>spoT1</i>	New England BioLabs
C600 Δ <i>folP</i> ::Kmr ^r	C600 derivative with insertion of a 1,250-bp kanamycin resistance gene and deletion of 330 bp within the <i>folP</i> coding region; Kan ^r	23
AG100	K-12 <i>argE3</i> <i>thi-1</i> <i>rpsL</i> <i>xyl</i> <i>mtl</i> Δ(<i>gal-uvrB</i>) <i>supE44</i>	25
AG100A	Δ <i>acrAB</i> ::Tn903 derivative of AG100; Kan ^r	25
BW25113	Wild-type parental strain used for the Keio collection	47
MW1014	Δ <i>efp</i> derivative of Keio collection strain with kanamycin resistance cassette removed	47
<i>P. aeruginosa</i> strain		
K767	PAO1 prototroph (wild type)	58
Plasmids		
pCC1FOS	Host fosmid vector for metagenomic library; Cam ^r	Epicentre
pUC19	<i>E. coli</i> cloning vector; Amp ^r	59
pCF430	Broad-host-range cloning vector with <i>araC</i> -P _{BAD} cassette; Tet ^r	60
pUCP19	Broad-host-range <i>P. aeruginosa</i> cloning vector; Amp ^r	61
pRK415	<i>P. aeruginosa</i> - <i>E. coli</i> shuttle cloning vector; Tet ^r	62
pCL027	pCF430 derivative carrying <i>orf-45</i> ^{TET A7} of fosmid MACRO-TET A7	This study
pCL028	pCF430 derivative carrying <i>orf-46</i> ^{TET A7} of fosmid MACRO-TET A7	This study
pCL029	pCF430 derivative carrying <i>orf-45</i> ^{TET A7} and <i>orf-46</i> ^{TET A7} of fosmid MACRO-TET A7	This study
pCL030	pCF430 derivative carrying <i>orf-7</i> ^{KAN 4} of fosmid SCT-KAN 4	This study
pCL031	pUCP19 derivative carrying <i>orf-7</i> ^{KAN 4} of fosmid SCT-KAN 4	This study
pCL032	pCF430 derivative carrying <i>orf-27</i> ^{MEM A1} of fosmid MACRO-MEM A1	This study
pCL033	pRK415 derivative carrying <i>orf-27</i> ^{MEM A1} of fosmid MACRO-MEM A1	This study
pCL034	pUC19 derivative carrying wild-type <i>folP</i> of <i>E. coli</i>	G. Swedberg, unpublished
pCL035	pUC19 derivative carrying <i>folP</i> ^{SMZ B27} of fosmid UTC-SMZ B27	This study
pCL036	pUC19 derivative carrying <i>orf-16</i> ^{TYL 10} of fosmid MACRO-TYL 10	This study
pCL037	pUC19 derivative carrying <i>orf-16</i> ^{TYL 10} of fosmid MACRO-TYL 10 in opposite-orientation	This study
pCL043	pCF430 derivative carrying 2,460-bp fragment encompassing <i>orf-28</i> ^{AZI 4} , <i>orf-29</i> ^{AZI 4} , and <i>orf-30</i> ^{AZI 4} of fosmid MACRO-AZI 4	This study
pCL044	pCL043 derivative carrying 1,839-bp fragment encompassing <i>orf-28</i> ^{AZI 4} , <i>orf-29</i> ^{AZI 4} , and <i>orf-30</i> ^{AZI 4} of fosmid MACRO-AZI 4	This study
pCL045	pCL044 derivative carrying 1,385-bp fragment encompassing <i>orf-28</i> ^{AZI 4} and <i>orf-29</i> ^{AZI 4} of fosmid MACRO-AZI 4	This study
pCL046	pCL045 derivative carrying 1,107-bp fragment encompassing <i>orf-28</i> ^{AZI 4} of fosmid MACRO-AZI 4	This study
pCL047	pCL046 derivative carrying 643-bp fragment encompassing <i>orf-28</i> ^{AZI 4} of fosmid MACRO-AZI 4	This study
pCL048	pCL047 derivative carrying 391-bp fragment encompassing PPP ^{AZI 4} gene of fosmid MACRO-AZI 4	This study
pCL049	pCL047 derivative missing a 263-bp, KpnI-restricted fragment	This study
pCL050	pUC19 derivative carrying <i>pbp</i> ^{TET A6} of fosmid MACRO-TET A6	This study
pCL051	pUCP19 derivative carrying <i>pbp</i> ^{TET A6} of fosmid MACRO-TET A6	This study

^aCam^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant.

Subcloning of putative ARGs. The bacterial strains and plasmids used in this study are summarized in Table 9. For *E. coli*, selected ORFs (including the predicted promoters) were cloned into pUC19 or pCF430 using PCR-amplified and enzyme-restricted fragments derived from fosmid DNA and introduced into the DH10β strain, unless otherwise specified. The PCR conditions and primer pairs used are provided in Table 10. To construct pCL049, pCL047 was digested with KpnI to remove a 263-bp fragment encompassing the 5' end of the PPP^{AZI 4} gene, followed by purification and religation of the remaining DNA fragment. For *Pseudomonas aeruginosa*, DNA insert fragments were extracted from the pUC19- or pCF430-based constructs by restriction digestion and recloned into the *P. aeruginosa*-compatible plasmid pUCP19 or pRK415, followed by electrotransforming into the PAO1 strain.

Antibiotic susceptibility assays. The susceptibility of *E. coli* and *P. aeruginosa* to antibiotics was assessed using the 2-fold serial microtiter broth dilution method (57) and the MICs recorded after 18 h of incubation at 37°C. To evaluate sulfonamide and anthracycline susceptibility, serial dilutions of overnight cultures were spotted onto Iso-Sensitest agar (for sulfonamides) or LB agar (for anthracyclines) plates supplemented with the appropriate antibiotic, followed by 24 h of incubation at 37°C.

Proteomics analysis. Briefly, *E. coli* cultures with final optical densities at 600 nm (OD₆₀₀) of ca. 2 were pelleted, resuspended in 50 mM ammonium bicarbonate, and sonicated on ice using a Q125 sonicator (Qsonica). After centrifugation, 100 μg of protein was reduced, alkylated, and digested with

TABLE 10 PCR primers and extension times used in this study

Plasmid	Primer	Oligonucleotide sequence (5'→3') ^a	Extension time (s)
pCL027	TET A7- <i>abc1</i> -XbaI-F TET A7- <i>abc1</i> -KpnI-R	GAGCGTCTAGATTTGACCGATCTCCGTGG GGATCAGGTACCGAAGTCGAGATAGCTGATGC	40
pCL028	TET A7- <i>abc2</i> -XbaI-F TET A7- <i>abc2</i> -KpnI-R	GAGACTCTAGACGATACGGCTTACCAGTCTGC GATCAGGTACCGTTACCAAGCAGTAGCAGCAG	40
pCL029	TET A7- <i>abc1</i> -XbaI-F TET A7- <i>abc2</i> -KpnI-R		75
pCL030	KAN 4- <i>aac6'</i> -XbaI-F KAN 4- <i>aac6'</i> -KpnI-R	GATGTCTAGAGTTGCACAACGTCCTCGCAC GCATGGTACCGAGCAGGTTAAGCAGTGG	30
pCL032	MEM A1- <i>mbI</i> -XbaI-F MEM A1- <i>mbI</i> -KpnI-R	AGCGATCTAGAGTGGGTGCGACAG GCAAGGTACCGAACTGGGACATGGATG	30
pCL035	SMZ B27- <i>folP</i> -XbaI-F SMZ B27- <i>folP</i> -EcoRI-R	CAGTTCTAGATACCAGTATCCCAACCGCGTC GGACCTGAATTCGCACCTTGACAGTCTTG	30
pCL036	TYL 10- <i>hflX</i> -XbaI-F TYL-10- <i>hflX</i> -Sall-R	GCACCTCTAGACGTTGCTTTTGTGCCAAG GACTGTCGACGAACCTCATCAGCCGCTC	45
pCL037	TYL 10- <i>hflX</i> -Sall-F TYL 10- <i>hflX</i> -XbaI-R	CGACTGTCGACGTTGCTTTTGTGCCAAG CGACATCTAGAGAACCTCATCAGCCGCTC	45
pCL043	AZI 4-2460-XbaI-F AZI 4-2460-PstI-R	GACGTCTAGAGCAAATCGCCTATCCAACGC GCATCTGCAGTCTCGCACACCACTCACTATC	75
pCL044	AZI 4-1839-XbaI-F AZI 4-2460-PstI-R	CGAACTCTAGACTAAGGTCCAGGAGGAAAC	50
pCL045	AZI 4-1839-XbaI-F AZI 4-1385-PstI-R	GCATCTGCAGGCGCTTTATAGATCCCAATCG	40
pCL046	AZI 4-1839-XbaI-F AZI 4-1107-PstI-R	GCATCTGCAGCGGTTTTCTGGCTAACACG	30
pCL047	AZI 4-643-XbaI-F AZI 4-1107-PstI-R	CGAGGTCTAGAGCATTATTGGGTACTTGAC	30
pCL048	AZI 4-643-XbaI-F AZI 4-391-PstI-R	CGAGGTCTAGAGCATTATTGGGTACTTGAC GCATCTGCAGTCATTTAGATCTCCAAGTA	15
pCL050	TET A6- <i>pbp</i> -XbaI-F TET A6- <i>pbp</i> -EcoRI-R	GGACGTCTAGAATCGCATCCTTAACAGGGTG GGACGGAATTCACCTGTCTGAAGGCATTTG	75

^aRestriction endonuclease cleavage sites are underlined. Repeated primer sequences are not shown.

trypsin in the presence of RapiGest surfactant (Waters Corp) The tryptic peptides were resolved using an Easy-nLC 1000 nano system with the Acclaim C₁₈ PepMap column (75 μm by 15 cm) coupled to a Q-Exactive Orbitrap (Thermo-Fisher Scientific). The tandem mass spectrometry (MS/MS) scans were searched against the target/reverse proteome of *E. coli* MG1655 (Proteome ID UP0000625, accessed April 2016) with an additional 39 annotated gene products derived from fosmid MACRO-AZI 4. A second search was completed using additional amino acid sequences deduced from all six possible reading frames (including PPP^{AZI 4}) in the subcloned fragment of pCL043.

Accession number(s). All sequences reported in this paper have been deposited in GenBank (accession no. KY705323 to KY705356) and the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00989-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.7 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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C.H.-F.L. and E.T. conceived and designed the study. C.H.-F.L., K.V.E., and J.R. performed experiments. C.H.-F.L., K.V.E., S.G., J.R., and E.T. analyzed data. C.H.-F.L., J.R., and E.T. wrote the paper.

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