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Comparative resistomic analyses of *Lysobacter* species with high intrinsic multidrug resistance

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ABSTRACT

Objectives: Ubiquitous Gram-negative *Lysobacter* species are known to confer intrinsic antibiotic resistance and are being considered as new sources for novel anti-methicillin-resistant *Staphylococcus aureus* (MRSA) antibiotics. This study aimed to determine the intrinsic antibiotic resistance profiles of *Lysobacter enzymogenes* strain C3 (LeC3) and *Lysobacter antibioticus* strain ATCC29479 (LaATCC29479), and to in silico identify their intrinsic resistomes and compare with *Xanthomonas campestris*, a close relative and plant pathogen.

Methods: The intrinsic resistant profiles of LeC3 and LaATCC29479 were determined by minimum inhibitory concentration (MIC) and disk diffusion assays. Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD) was used to predict resistomes. Selected resistance genes were mutated and their roles in resistance to antibiotics were determined by spot dilution assays.

Results: MIC and disk diffusion assays revealed that both LeC3 and LaATCC29479 exhibited high levels of multidrug resistance to 12 common antibiotics. Comparative resistomic analyses using the RGI revealed possible antibiotic resistance genes (ARGs) related to the antibiotic resistance profiles in LeC3 and LaATCC29479, and the core resistome of *Lysobacter* spp. Functional studies confirmed that three ARGs (*bla*, *aac* and *sph*) conferred antibiotic resistance in LeC3, and also in *X. campestris* when expressed in trans.

Conclusion: The findings show that LeC3 and LaATCC29479 exhibited multidrug resistance at very high levels and the resistomes of *Lysobacter* strains were more abundant than those of *X. campestris*, which might provide novel targets for studies in the intrinsic antibiotic resistance of *Lysobacter* and other environmental bacterial species.

1. Introduction

Antibiotics are among the most important tools in medicine. The global emergence of multidrug-resistant (MDR) bacteria is a growing threat in the ability to effectively treat bacterial infections with antibiotics [1,2]. Antibiotics are also used in diverse settings in agriculture food production, including animals, fruit trees, aquaculture, and may persist in soil and aquatic environments [3]. In both clinical and agricultural settings, antibiotic use exerts a great pressure that promotes the evolution and spread of antibiotic resistance genes (ARGs). However, human antibiotic usage is not the only source of selective pressure for ARGs in nature. It has been suggested that antibiotics have been produced in natural environments for more than 500 million years [4]. Most antibiotics in nature are produced by microbes, exerting additional selective pressure for antibiotic-resistant mutations in surrounding microorganisms [5]. Moreover, bacterial ARGs are probably intrinsically carried by antibiotic-producing microbes, and are usually in the same cluster with antibiotic biosynthetic pathway genes in order to protect from antimicrobial activities [6,7]. Intrinsic antibiotic resistance in environmental bacteria provides resources for understanding antibiotic resistance mechanisms. However, the reservoirs of resistance determinants in non-clinical and non-agricultural environments are poorly understood.

The *Lysobacter* genus, belonging to the Xanthomonadaceae family in *Gamma-proteobacteria*, was first described in 1978 and currently includes >30 species [8,9]. Widely found in diverse ecosystems – including soil, rhizosphere, and freshwater habitats – the non-pathogenic *Lysobacter* species possess two unique

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characteristics: it is rich in antibiotic natural products and antifungal compounds, and has intrinsic resistance to multiple antibiotics [10,11]. Strains from several *Lysobacter* species are also considered as promising biocontrol agents against plant pathogens [12,13] and as new sources for novel anti-MRSA (methicillin-resistant *Staphylococcus aureus*) and other antibiotics [10,14]. The genomes of several *Lysobacter* species are currently available [9,15], thus making it possible to identify genes or gene clusters for the biosynthesis and regulation of natural products. However, little is known about the degree of intrinsic resistance of *Lysobacter* species against multiple antibiotics and the molecular mechanisms underlying this unique characteristic.

This study aimed to determine the intrinsic antibiotic resistance profiles of two *Lysobacter* strains: *Lysobacter enzymogenes* (*L. enzymogenes*) strain C3 (LeC3) and *Lysobacter antibioticus* (*L. antibioticus*) strain ATCC29479 (LaATCC29479), and to in silico identify their intrinsic resistomes. Comparative resistomic analyses of the *Lysobacter* species and *Xanthomonas campestris* (*X. campestris*) revealed that the resistomes of *Lysobacter* strains were more abundant than that of *X. campestris*, which is a plant pathogen.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB, Lennox) broth was used for routine bacterial growth at 28 °C. In addition, 10% tryptic soy medium (10% TSB, 1.5 g/L tryptone, 0.5 g/L soytone, and 0.5 g/L NaCl) was used for culture of Lysobacter strains at 28 °C and to determine antibiotic sensitivity. Antibiotics were used at the following concentrations unless otherwise noted: 100 μg/mL kanamycin (Km) and 100 μg/mL gentamicin (Gm). Primers used in this study for mutant construction and cloning were listed in Table S1 (Supplementary information).

2.2. Antibiotic susceptibility assays

To determine minimum inhibitory concentrations (MIC), *Lysobacter* strains were cultured overnight in 10% TSA (15 g/L agar in 10% TSB), harvested by centrifugation and washed twice using phosphate-buffered saline (PBS). After the final wash, cells were resuspended in fresh 10% TSB medium and amended with serial two-fold dilution of antibiotics. The initial concentration of bacterial suspension was adjusted to OD_{600} = 0.01. After incubation at 28 °C with shaking at 250 rpm for 22 h, bacterial growth (OD_{600}) was measured. MIC was defined as the lowest concentration of a certain antibiotic at which bacterial growth (OD_{600}) was <5% of that of no-antibiotic control. In addition, disk diffusion assays were also used to determine the inhibition zone of antibiotics against *Lysobacter* strains. Briefly, 50 μL of bacterial suspension (OD_{600} = 0.01) was spread-cultured on 10% TSA plates and different amounts (μg) of a certain antibiotic dissolved in corresponding solutions were added onto the paper disks. The plates were then incubated at 28 °C for 2–3 days and the distance from the edge of the inhibition zone to that of the paper disk was measured. Statistical analysis of the data was performed by one-way ANOVA followed by Fisher's LSD test (P = 0.05).

2.3. In silico identification of resistomes

Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD) [16] was used to predict resistomes from protein or nucleotide data, based on homology and single nucleotide polymorphism (SNP) models in curated CARD. Several previous studies have shown that sequences sharing >40% identity are very likely to share functional similarity [17]; however, it has been revealed that a bit score of 40 is significant in searches of reference database with <7000 entries [18]. Therefore, sequence identity of 40% and a bit score of 40 were used as thresholds to filter all antibiotic resistance ontologies (AROs). The genome sequences used in this study were obtained from the National Center for

Table 1
Bacterial strains and plasmids used in this study.

Strains, Plasmids	Description	Reference or source
Strains		
Escherichia coli DH10B	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–	Invitrogen (Carlsbad, CA, USA)
Lysobacter enzymogenes C3 (LeC3)	Wild-type, isolated from Kentucky bluegrass foliage, Nebraska, Km^R	[12]
Lysobacter antibioticus ATCC29479 (LaATCC29479)	Wild-type, isolated from soil in Central Experimental Farm, Ottawa, Ontario, Canada	[8]
Xanthomonas campestris pv. campestris B24-79	Wild-type	From A Poplawsky
Δaac	*aac* (*GLE_1155*) in-frame deletion mutant in LeC3, Km^R	This study
Δsph	*sph* (*GLE_5415*) in-frame deletion mutant in LeC3, Km^R	This study
Δant	*ant* (*GLE_2537*) in-frame deletion mutant in LeC3, Km^R	This study
Δbla	*bla* (*GLE_4415*) Tn5 transposon-insertional mutant in LeC3, Km^R	This study
Plasmids		
pEX18GM	Suicide vector with a sacB gene, Gm^R	[19]
pBBR1MCS-5	Broad-host-range vector with a Plac promoter, Gm^R	[21]
pAac	1670-bp DNA fragment containing promoter and coding sequence of *pdxH* and *aac* of LeC3 (*GLE_1154-1155*) in pBBR1MCS-5	This study
pSph	1419-bp DNA fragment containing promoter and coding sequence of *sph* of LeC3 (*GLE_5415*) in pBBR1MCS-5	This study
pAnt	1384-bp DNA fragment containing promoter and coding sequence of *ant* of LeC3 (*GLE_2537*) in pBBR1MCS-5	This study
pBla	1472-bp DNA fragment containing promoter and coding sequence of *bla* and one hypothetical gene of LeC3 (*GLE_4415-4416*) in pBBR1MCS-5	This study

LeC3: *Lysobacter enzymogenes* strain C3, gene bank accession # CP013140.

Biotechnology Information (NCBI): *Lysobacter capsici* strains 55 (Lc55) and KNU-14 (LcKNU-14); *L. antibioticus* strains ATCC29479 (LaATCC29479) and 76 (La76); *L. enzymogenes* strains C3 (LeC3) and M497-1 (LeM497-1); *Lysobacter gummosus* strain 3.2.11 (Lg3.2.11); and *X. campestris pv. campestris* strain ATCC 33913 (XccATCC33913).

2.4. Construction of mutants and mutant complementation in L. enzymogenes

Mutants of *L. enzymogenes* strain C3 (LeC3) were generated by either transposon mutagenesis or a homologous recombination approach, as previously described [19]. The EZ-Tn5^{TM} <KAN-2> Tnp Transposome^{TM} kit (Epicentre, Madison, WI, USA) was used for random mutagenesis following the manufacturer's instructions. A mutant library of 3135 clones was screened and 12 mutants sensitive to ampicillin were recovered. Inverse PCR was performed, as previously described, to determine the transposon insertion site [20]. Transposon insertion sites were then confirmed by PCR using primers listed in Table S1. One mutant harboured mutation in the gene encoding a beta-lactamase was included in this study and the rest of the mutants will be reported later (Table 1). In-frame deletion mutants of *L. enzymogenes* strain C3 (LeC3) were generated by homologous recombination. Briefly, two flanking regions of target genes were amplified by PCR using primers listed in Table S1 and cloned into the suicide-vector pEX18Gm. Deletion constructs were then transformed into LeC3 competent cell by electroporation. Positive colonies with a single-crossover event were selected from LB plates containing Km (100 μg/mL) and Gm (150 μg/mL). After cultivation on LB plates amended with 10% (w/v) sucrose, the sucrose-resistant and gentamicin-sensitive double-crossover candidates were selected and confirmed using PCR with corresponding primers (Table S1).

A plasmid-based method was used to complement LeC3 mutants and to express LeC3 genes in *X. campestris*. Briefly, DNA fragments containing the full length of target genes and their corresponding promoter regions were amplified by PCR using different primer pairs, as listed in Table S1. Purified PCR products were cloned into pBBR1MCS-5 [21]. After confirmation by DNA sequencing using the primer pair M13For-40/M13Rev-48 (Table S1), the final constructs were electroporated into the corresponding LeC3 mutants or *X. campestris*. The transformants were again verified by PCR.

2.5. Spot dilution assay

Spot dilution assay was performed as previously described [22]. Overnight bacterial cells were harvested by centrifugation and washed twice using PBS. Bacterial cells were resuspended and adjusted to an initial concentration of OD_{600} = 1 in PBS. Ten-fold serial dilution of bacterial suspensions was made in PBS. For each dilution, 5 μL was spotted on the plates with or without antibiotics and incubated at 28 °C for 3 days, and bacterial growth was visually observed. Growth on plates without antibiotics was used as control. If growth of the one strain was observed at least one dilution lower/higher than the other on plates with antibiotics, this indicated that the strain was more sensitive/resistant to antibiotics and vice versa. The experiments were performed in triplicate and repeated at least three times.

3. Results

3.1. Both Lysobacter strains LeC3 and LaATCC29479 were resistant to multiple antibiotics but differed in the degree of resistance to certain antibiotics

Screening for resistance to 12 common laboratory antibiotics showed that both LeC3 and LaATCC29479 exhibited high levels of resistance to multiple antibiotics (Table 2). The MICs of ampicillin and carbenicillin were 4096 μg/mL and 1024 μg/mL for LeC3, respectively, as compared with 64 μg/mL for LaATCC29479. Both LeC3 and LaATCC29479 exhibited high levels of resistance to spectinomycin, nitrofurantoin, nalidixic acid, and trimethoprim, but the MICs were one-fold to eight-fold higher for LeC3 than those for LaATCC29479 (Table 2). LeC3 was also highly resistant to rifampicin (MIC at 1024 μg/mL), whereas LaATCC29479 was not. Resistance to kanamycin, gentamicin, tetracycline and polymyxin B for LeC3 was not as high as other antibiotics, but still much higher as compared with LaATCC29479 (Table 2). Interestingly, the MIC of chloramphenicol for LaATCC29479 was two-fold higher than that for LeC3 (Table 2).

Table 2
Antibiotic resistance profiles of *Lysobacter* strains.

Antibiotics	MIC (μg/mL)[a]	
	LeC3	LaATCC29479
Ampicillin	4096	64
Carbenicillin	1024	64
Spectinomycin	8192	1024
Kanamycin	128	8
Gentamicin	16	1
Tetracycline	16	2
Rifampicin	>1024	1
Nalidixic acid	128	64
Nitrofurantoin	>256	256
Trimethoprim	512	128
Chloramphenicol	8	16
Polymyxin B	4	1

LeC3: *Lysobacter enzymogenes* strain C3; LaATCC29479: *Lysobacter antibioticus* strain ATCC29479. The *Xanthomonas campestris* strain used in the study did not resist to any of the above antibiotics tested (MIC < 1).

[a] Minimum inhibitory concentration (MIC) was defined as the lowest concentration of a certain antibiotic at which growth (OD_{600}) of the bacterium was <5% of that of the control without antibiotic. For rifampicin and nitrofurantoin, the concentration shown in the table was the highest concentration that could be made due to the low antibiotic solubility in 10% TSB.

The results of disk diffusion assay for LeC3 and LaATCC29479 were consistent with the MIC results (Fig. 1). Smaller inhibition zones were observed for LeC3 than LaATCC29479 for ampicillin, carbenicillin, spectinomycin, kanamycin, gentamicin, tetracycline, rifampicin, nalidixic acid, and trimethoprim (Fig. 1); LaATCC29479 showed similar and more resistance for polymyxin B and chloramphenicol, respectively (Fig. 1 KL). For nitrofurantoin, due to its solubility in DMSO, both strains exhibited almost no inhibition zone in the amount of antibiotics tested (Fig. 1 I). These results indicated that both LeC3 and LaATCC29479 are resistant to multiple antibiotics at high concentrations. Based on genome sequences, both LeC3 and LaATCC29479 contain no plasmid [9], suggesting that these strains do not harbour plasmid-borne antibiotic resistance genes.

3.2. Resistomes of Lysobacter strains are more abundant as compared with X. campestris

Both the *Lysobacter* and *Xanthomonas* genus belong to the Xanthomonadaceae family. Phylogenetic analysis showed that seven *Lysobacter* strains are clustered together and distinct from *X. campestris pv. campestris* strain XccATCC33913 (Fig. S1). RGI detected hundreds of ARGs in both *Lysobacter* and *Xanthomonas* genomes (Table 3). On average, there are 433 ARGs detected in each *Lysobacter* genome, 24% more than those detected in the XccATCC33913 genome (Table 3). In terms of potential resistance mechanisms, the number of ARGs in an antibiotic target

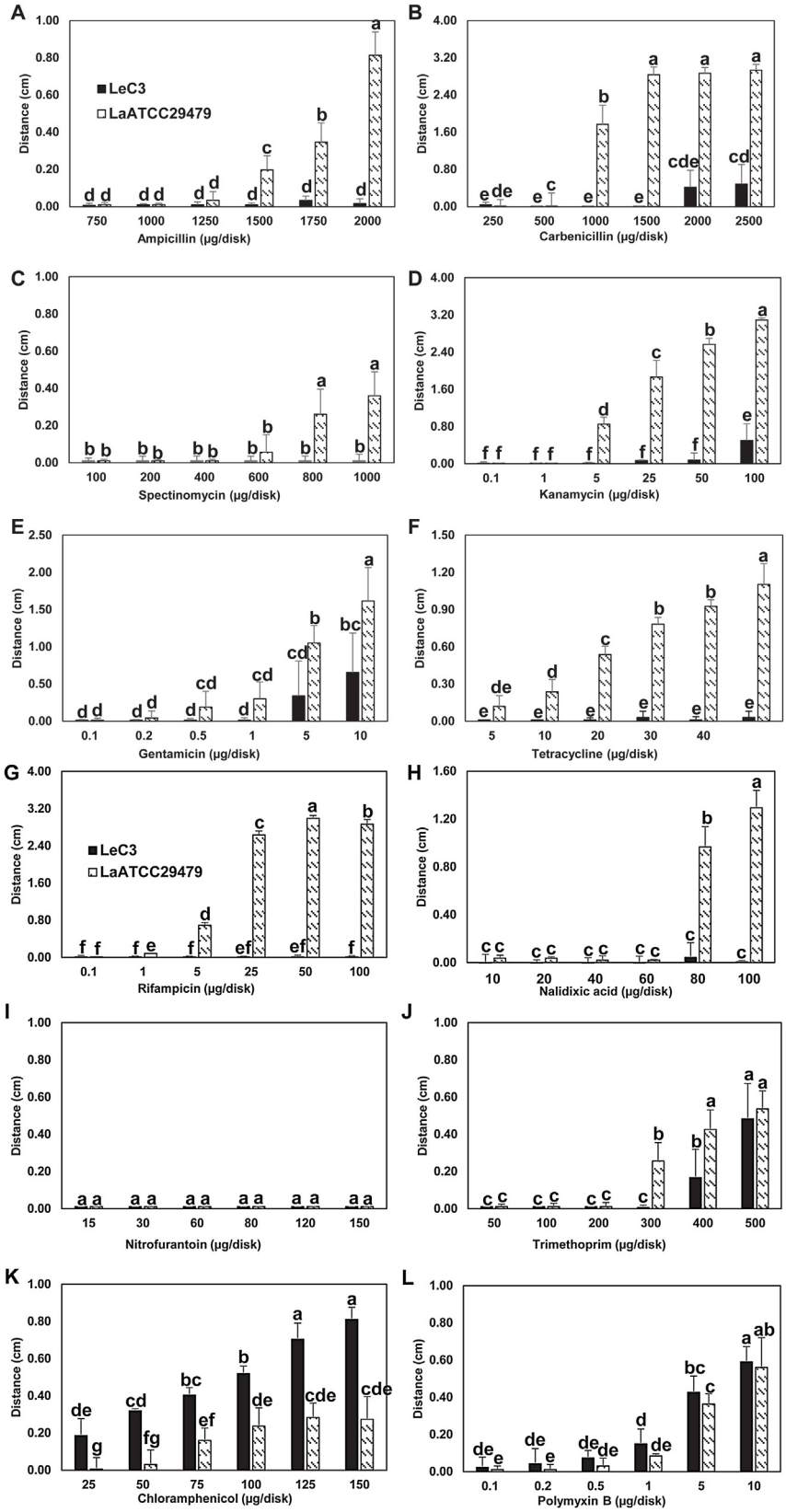

Fig. 1. Inhibition of *Lysobacter* strains by antibiotics using disk diffusion assay.
Bacterial suspension was cultured on 10% TSA plates and different amounts (μg) of antibiotic in corresponding solution were added onto the paper disks. The plates were then incubated at 28 °C for 2–3 days and the diameter of the inhibition zone was measured. The distance (cm) shown in the corresponding bar charts (A–L) was the distance from the edge of inhibition zone minus the diameter of the disk. Error bars indicate standard deviation of three replicates. Bars marked with the same letter were not significantly different ($P < 0.05$).
LeC3: *Lysobacter enzymogenes* strain C3; LaATCC29479: *Lysobacter antibioticus* strain ATCC29479.

Table 3
Summary of predicted resistomes by Resistance Gene Identifier (RGI) in Comprehensive Antibiotic Resistance Database (CARD).

Strain	Lc55	LcKNU-14	LaATCC29479	La76	LeC3	LeM497-1	Lg3.2.11	XccATCC33913
Drug classes	35	35	37	37	36	36	35	35
Antibiotic resistant genes	448	443	403	409	429	455	443	348
Antibiotic inactivation	70	67	57	60	65	73	66	40
Antibiotic target protection	50	49	48	48	44	52	49	33
Antibiotic target replacement	5	5	7	7	7	5	5	6
Antibiotic target alteration	112		107	113	121	129	104	100
Antibiotic efflux	222	221	197	194	202	209	229	178

Lc55: *Lysobacter capsica* strain 55, gene bank accession # CP011130; LcKNU-14: *Lysobacter capsica* strain KNU-14, gene bank accession # CP023465; LaATCC29479: *Lysobacter antibioticus* strain ATCC29479, gene bank accession # CP013141; La76: *Lysobacter antibioticus* strain 76, gene bank accession # CP011129; LeC3: *Lysobacter enzymogenes* strain C3, gene bank accession # CP013140; LeM497-1: *Lysobacter enzymogenes* strain M497-1, gene bank accession # AP014940; Lg3.2.11: *Lysobacter gummosus* strain 3.2.11, gene bank accession # CP011131; XccATCC33913: *Xanthomonas campestris pv. campestris* strain ATCC33913, gene bank accession # AE008922.

replacement is similar in all eight analysed genomes. In contrast, there are 64%, 47%, 14%, and 18% more ARGs found in *Lysobacter* genomes than those predicted in XccATCC33913 genome in enzymatic antibiotic inactivation, antibiotic target protection from antibiotic binding, antibiotic target alteration, and antibiotic efflux, respectively (Table 3). These results suggest that resistomes of *Lysobacter* strains are more abundant as compared with *X. campestris*, which is not resistant to any of the tested antibiotics.

3.3. Diversity of resistomes for four Lysobacter species and X. campestris

Non-repetitive AROs inside each of the four *Lysobacter* species and *X. campestris* were then compared from resistomes predicted by RGI. The number of AROs in each *Lysobacter* species ranged from 234 in *L. antibioticus* to 295 in *L. enzymogenes*, which is 24–57% more than those in *X. campestris* (188) (Fig. 2A and B). Clustering of the predicted AROs of all five species showed that 89 non-repetitive AROs were shared within the four *Lysobacter* species and *Xanthomonas* (Fig. 2A); whereas 114 AROs were shared in the four *Lysobacter* species, representing the core resistome of the *Lysobacter* genus (Fig. 2B). The AROs in the core *Lysobacter* resistome, but not in XccATCC33913 genome, include 10, 2, 15 AROs functioning in antibiotic efflux, antibiotic inactivation, and antibiotic target alteration/protection/replacement, respectively (Table 4). In addition, some unique conserved AROs predicted in the *Lysobacter* resistomes are listed in Table S2.

3.4. The probable genetic bases of differential resistant phenotypes in LeC3 and LaATCC29479

Comparison of predicted resistomes of LeC3 and LaATCC29479 showed that similar numbers of ARGs functioning in protection/replacement of antibiotic target and antibiotic efflux were detected in both genomes. In contrast, there were about 14% and 13% more ARGs functioning in antibiotic inactivation and antibiotic target alteration, respectively, in LeC3 than those in LaATCC29479 (Table 3). These results suggest that enzymatic inactivation of antibiotic and alteration of antibiotic targets are probably the main mechanisms leading to differential resistant profiles in LeC3 and LaATCC29479.

Comparative resistomic analyses of LeC3 and LaATCC29479 genomes showed that there were 61 non-repetitive AROs (coming from a total of 70 predicted ARGs) in LeC3 that were not found in either LaATCC29479 or XccATCC33913 (Table S3), which would probably explain the difference in resistance to various antibiotics between LeC3 and LaATCC29479. Among the 70 ARGs, 36, 22, and 12 function in antibiotic efflux, antibiotic inactivation and antibiotic target alteration/protection, respectively, and these ARGs confer resistance to 24 kinds of antimicrobial agents. Thirty

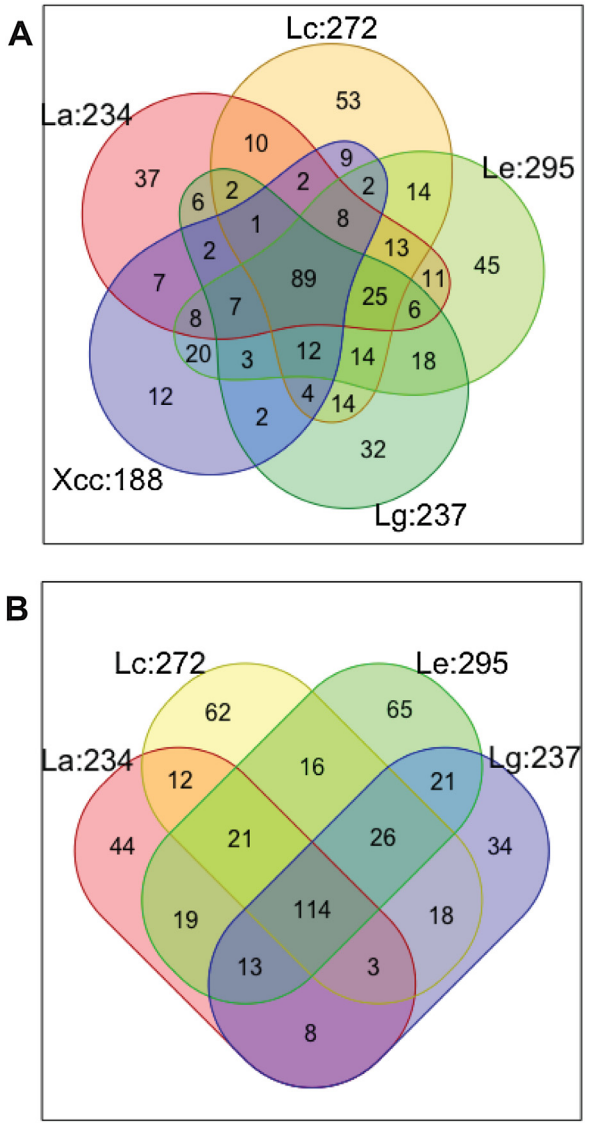

Fig. 2. Comparative resistomic analyses of the four *Lysobacter* species and *Xanthomonas campestris. pv. campestris* (Xcc).
(A) Venn diagram showed the number of unique and common antibiotic resistance ontologies (AROs) shared by the *Lysobacter* species and Xcc.
(B) Venn diagram showed the number of unique and common antibiotic resistance ontologies (AROs) shared by the *Lysobacter* species.
The total number of non-repetitive AROs within each resistome was listed after the species name. Overlapping regions showed the number of AROs existed only within the specific resistomes. Numbers in non-overlapping portions showed the number of AROs unique to each species.
La: *Lysobacter antibioticus*; Lc: *Lysobacter capsici*; Le: *Lysobacter enzymogenes*; Lg: *Lysobacter gummosus*; Xcc: *Xanthomonas campestris pv. campestris*.

Table 4
List of AROs in the core *Lysobacter* resistome, but not in XccATCC33913.

ARO	Drug class	Resistance mechanism	AMR gene family
macA	macrolides	antibiotic efflux	ATP-binding cassette antibiotic efflux pump (ABC)
tet(35)	tetracyclines		ABC
tet(D)	tetracyclines		major facilitator superfamily antibiotic efflux pump (MFS)
mgrA	tetracyclines peptide antibiotics fluoroquinolones β-lactams acridine dye		MFS and ABC
hmrM	acridine dye fluoroquinolones		multidrug and toxic compound extrusion transporter (MATE)
baeS	aminoglycosides aminocoumarin antibiotic		resistance-nodulation-cell division antibiotic efflux pump (RND)
mdtF	fluoroquinolones β-lactams macrolides		RND
MexL	triclosan tetracyclines macrolides		RND
emrE	aminoglycosides		small multidrug resistance antibiotic efflux pump (SMR)
AAC(6')-Iad	aminoglycosides	antibiotic inactivation	AAC(6')
ANT(4')-Ib	aminoglycosides		ANT(4')
fabI mutations	isoniazid triclosan	antibiotic target alteration	antibiotic resistant fabI
gidB mutation	aminoglycosides		antibiotic resistant gidB
vanC	glycopeptides		glycopeptide resistance gene cluster and van ligase
vanHA	glycopeptides		glycopeptide resistance gene cluster and vanH
pgpB	peptide antibiotics		lipid A phosphatase
basS	peptide antibiotics		pmr phosphoethanolamine transferase
vanRG	glycopeptides		antibiotic target alteration: vanR and glycopeptide resistance gene cluster
vanSL	glycopeptides		vanR and glycopeptide resistance gene cluster
soxR mutation	rifamycins β-lactams fluoroquinolones phenicols tetracyclines triclosan	antibiotic target alteration and efflux	RND, MFS and ABC
lsaA	streptogramins lincosamides pleuromutilins	antibiotic target protection	ABC-F ATP-binding cassette ribosomal protection protein
lsaB	streptogramins lincosamides pleuromutilins		ABC-F ATP-binding cassette ribosomal protection protein
vgaE	streptogramins pleuromutilins		ABC-F ATP-binding cassette ribosomal protection protein
otr(A)	tetracyclines		tetracycline-resistant ribosomal protection protein
tetT	tetracyclines		tetracycline-resistant ribosomal protection protein
dfrA19	diaminopyrimidines	antibiotic target replacement	trimethoprim resistant dihydrofolate reductase dfr

and 18 of the 70 ARGs in LeC3 are predicted to function in resistance of beta-lactam and tetracycline antibiotics, respectively (Table S3). Three ARGs were identified to function in resistance to aminoglycoside antibiotic, including: (i) *GLE_2598*, which encodes an AcrB multidrug efflux protein; (ii) *GLE_2597*, which encodes the MFP subunit of one efflux transporter from resistance-nodulation-cell division (RND) family; and (iii) *GLE_0095*, which encodes one acetyltransferase functioning in aminoglycoside enzymatic inactivation by adding acetyl groups. Moreover, two transporters (*GLE_0154* and *GLE_5347*) from a major facilitator superfamily might be involved in resistance to rifampicin.

3.5. One highly conserved antibiotic resistance gene predicted by Resistance Gene Identifier conferred ampicillin resistance

To examine the reliability of ARGs predicted by RGI, the mutant of one highly conserved ARG (bit score 241.9, identity 49.63%) (i.e. *GLE_4415* (*bla*)) in LeC3 resistome from the CTX-M-109 beta-lactamase family was identified using transposon mutagenesis. Spot dilution assay showed that the *bla* mutant exhibited high ampicillin sensitivity and that resistance to ampicillin was restored to the wild-type level by the cloned *bla* gene (Fig. 3A). Spot dilution assay further showed that introduction of the cloned *bla* gene from LeC3 conferred increased resistance to ampicillin in *X. campestris* (Fig. 3B). These results indicate that the highly conserved *bla* gene is functional and confers high level of resistance to ampicillin in LeC3 and in *X. campestris* when expressed in trans.

3.6. Antibiotic resistance genes with relatively low conservation conferred antibiotic resistance at lower concentration

To further examine the reliability of ARGs predicted by RGI, three less conserved ARGs were in-frame deleted by homologous recombination. These three aminoglycoside-inactivating ARGs are *GLE_1155* (*aac*) from AAC(6′) family encoding one aminoglycoside 6′-*N*-acetyltransferase; *GLE_5415* (*sph*) from APH(6) family encoding one streptomycin 3″-kinase; and *GLE_2537* (*ant*) from ANT(4′) family encoding one aminoglycoside 4′-O-nucleotidyltransferase. Spot dilution assay showed that the *sph* and *aac* mutants exhibited streptomycin and gentamicin susceptibilities, respectively (Fig. 4A). However, no decrease in aminoglycoside (i.e. streptomycin, gentamycin, and kanamycin) resistance was observed in the *ant* mutant. Spot dilution assay also showed that the cloned *aac* and *sph* from LeC3 conferred resistance to kanamycin and streptomycin in *X. campestris*, respectively (Fig. 4B). These results indicate that the *aac* and *sph* confer aminoglycoside resistance in both LeC3 and *X. campestris*.

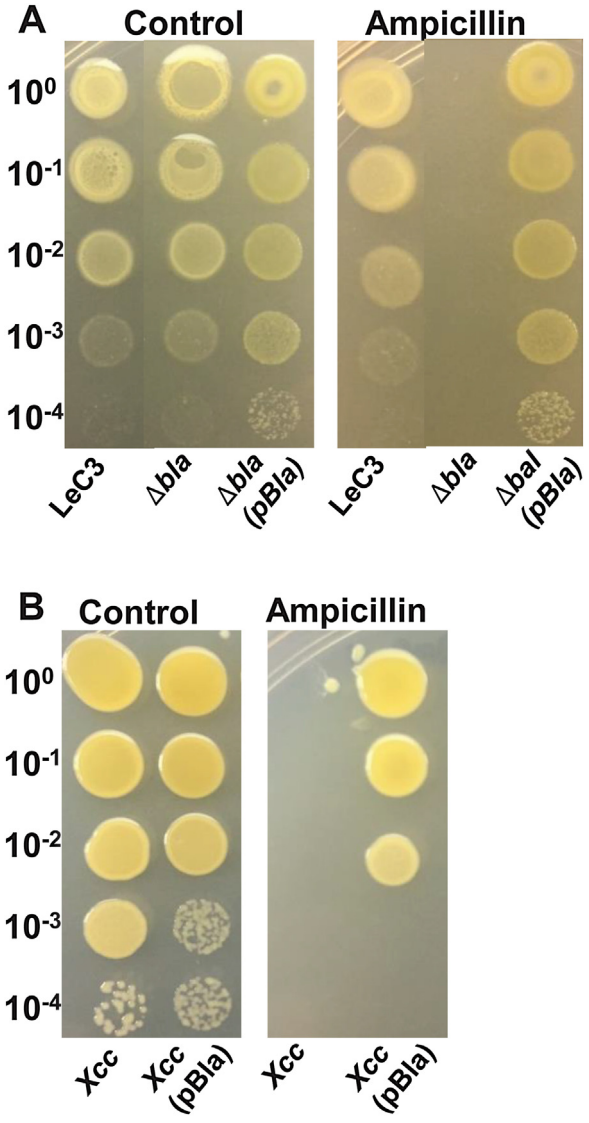

Fig. 3. The highly conserved *bla* (*GLE_4415*) gene rendered resistance to ampicillin.
(A) Deletion of the *bla* gene in LeC3 led to sensitivity to ampicillin.
(B) Expression of the *bla* gene from LeC3 in *Xanthomonas campestris pv. campestris* (*Xcc*) led to resistance to ampicillin.
In spot dilution assay, serial 10-fold dilutions were made from $OD_{600} = 1$ in $0.5 \times$ PBS. 5 μL of each dilution was added to 10% TSA (A) or LB (B) plates containing no antibiotics (control) or 1024 μg/mL ampicillin.
Pictures were taken 3 days post inoculation.

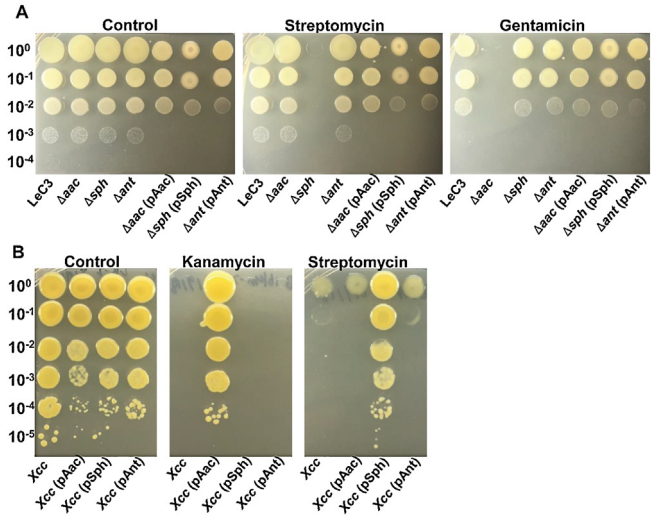

Fig. 4. The *aac* (*GLE_1155*) and *sph* (*GLE_5415*) conferred antibiotic resistance.
(A) Deletion of the *sph* and *aac* genes in LeC3 led to resistance to streptomycin and gentamicin, respectively.
(B) Expression of the *aac* and *sph* genes from LeC3 in *Xanthomonas campestris pv. campestris* (*Xcc*) led to resistance to kanamycin and streptomycin, respectively.
In spot dilution assay, serial 10-fold dilutions were made from $OD_{600} = 1$ in $0.5 \times$ PBS.
5 μL of each dilution was added to (A) LB plates containing no antibiotics (control), 16 μg/mL streptomycin, and 8 μg/mL gentamicin; (B) LB plates containing no antibiotics (control), 16 μg/mL kanamycin, and 8 μg/mL streptomycin.
Pictures were taken 2 days post inoculation.

4. Discussion

Although it has been reported that *L. enzymogenes* exhibits intrinsic antibiotic resistance to ampicillin, kanamycin, gentamycin, and chloramphenicol [10,11], the current study was the first to determine the antibiotic resistance profiles of the two most well studied *Lysobacter* strains – LeC3 and LaATCC29479 – and to demonstrate that *Lysobacter* strains exhibited MDR at very high levels, suggesting that the genetically tractable LeC3 could be used as a novel model system for unravelling the molecular mechanisms of intrinsic MDR in environmental microorganisms.

The RGI software on CARD integrates AROs, bioinformatic models and molecular reference data to predict ARGs at the genome level [16]. It analyses genomic sequences under three paradigms: Perfect, Strick and Loose. The Perfect algorithm detects perfect matches to CARD reference data; the Strict algorithm detects previously unknown variants of known ARGs; whereas the Loose algorithm discovers new emergent threats and more distant homologs of known ARGs (https://card.mcmaster.ca/analyze/rgi). The resistomes predicted by RGI in *Lysobacter* and *Xanthomonas* are mostly Loose, rarely Strict and with no Perfect hits, indicating that these non-clinical environmental bacteria could be new resources with which to provide novel insights into antibiotic resistance mechanisms. In this study, four ARGs predicted by RGI (i.e., *bla*, *aac*, *sph*, and *ant*) were either mutated in LeC3 or heterologously expressed in *X. campestris*, and three of them (*bla*, *aac* and *sph*) were confirmed to confer resistance to corresponding antibiotics. These results validated the ability of RGIs in predicting ARGs from genome sequences of *Lysobacter* species and suggested that *X. campestris* could be a good surrogate for in trans expressing resistance genes from *Lysobacter* species.

In addition, comparative resistomic analyses between *X. campestris*, which contains no known intrinsic antibiotic resistance, and *Lysobacter* species identified core and unique ARGs in *Lysobacter* species as well as in LeC3, which probably play important roles in their intrinsic resistance to antibiotics. In the core *Lysobacter* resistome, five AROs are: (i) *CTX-M* conferring resistance to β-lactams; (ii) mutation in *soxR* conferring resistance to β-lactams, fluoroquinolone, phenicol, rifamycin, tetracycline, and triclosan, functioning in target alteration and antibiotic efflux; (iii) *golS* conferring resistance to β-lactams and phenicol as RND antibiotic efflux pump; (iv) *tet* functioning in antibiotic target protection against tetracycline; and (v) *rgt1438* functioning in antibiotic inactivation against rifamycin, although four of them (i.e. *CTX-M*, *golS*, *tet* and *rgt1438*) were also found in XccATCC33913 resistome with either alternative from the same class or relatively lower sequence identity and bit score. However, *soxR* mutation conferring MDR was not found in XccATCC33913. SoxR is a conserved redox-sensitive transcriptional activator belonging to

the MerR family in Enterobacteriaceae [23]. The MerR family members share N-terminal winged helix-turn-helix (wHTH) DNA binding regions and mostly respond to stress signals (e.g. antibiotics, oxidative stress, and heavy metals) [24]. In *Escherichia coli* (*E. coli*), about 100 genes were found to be regulated by SoxR and its partner SoxS [25]. It has been reported that constitutive *soxR* mutations contributed to multiple antibiotic resistance in clinical *E. coli* isolates and were sufficient to confer multiple antibiotic resistance in a fresh genetic background [26]. In enteric bacteria, SoxR with reduced [2Fe-2S] cluster may bind to DNA, but is inactive for transcription initiation. In non-enteric bacteria, which lack SoxS, SoxR can also directly regulate some key genes involved in antibiotic resistance, quorum sensing, and detoxification of redox-active compounds [27–29].

In addition, 70 unique ARGs in LeC3 that are absent in both LaATCC29479 and XccATCC33913 resistomes probably play important roles in its strong intrinsic resistance (Table S3). Out of the four ARGs tested in this study, one highly conserved *bla* (i.e. *GLE_4415*) is among those listed in the 70 ARGs, indicating that a combination of resistant profiles and resistome information could provide some lead in ARG studies. The intrinsic resistome of *Paenibacillus* sp. LC231, a cave bacterial isolate in an underground ecosystem away from ground surface for more than 4 million years, was systematically investigated from a combination of antibiotic susceptibility assay and resistome prediction [30]. Moreover, comparison of transcriptomic and/or proteomic data with/without antibiotic treatment might also be beneficial for mining the intrinsic antibiotic resistance in *Lysobacter* species.

5. Conclusions

Fully understanding MDR in non-pathogenic *Lysobacter* could provide insight into antibiotic resistance reservoirs from natural environments, offer predictive capacity for the emergence of antibiotic resistance in agriculture and clinical environments, and deliver new targets for discovering novel therapeutics. Overall, this study examined the antibiotic resistance profiles in the two most well studied *Lysobacter* strains – LeC3 and LaATCC29479 – and revealed that LeC3 and LaATCC29479 exhibited MDR at very high levels. Comparative resistomic analyses based on RGI prediction revealed that the resistomes of *Lysobacter* strains were more abundant than that of *X. campestris*, which provided novel targets for studies in the intrinsic antibiotic resistance of *Lysobacter* and other environmental bacterial species.

Competing interests

None.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.05.008.

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