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Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

AcGI1, a novel genomic island carrying antibiotic resistance integron In687 in multidrug resistant *Achromobacter xylosoxidans* in a teaching hospital in Thailand

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One sentence summary: The first identification of a novel genomic island, AcGI1, containing multiple antimicrobial resistance genes in *A. xylosoxidans*.

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ABSTRACT

This study investigated the genetic basis of multidrug resistance in two strains of *Achromobacter xylosoxidans* isolated from patients attending a hospital in Thailand in 2012. These isolates were highly resistant to cephalosporins, aminoglycosides, fluoroquinolones, co-trimoxazole and carbapenems. Whole genome sequencing revealed that the two isolates were not clonally related and identified a carbapenem resistance gene-habouring integron (In687), residing in a novel genomic island, AcGI1. This In687 shares 100% identical nucleotide sequence with ones found in *Acinetobacter baumannii* Aci 16, isolated from the same hospital in 2007. We report the first analysis of multidrug-resistant *A. xylosoxidans* isolated in Thailand, and the first example of this island in *A. xylosoxidans*. Our data support the idea that resistance has spread in Thailand via horizontal gene transfer between species and suggest the possibility of *A. xylosoxidans* may serve as a reservoir of antibiotic resistance, especially in hospital setting.

Keywords: antibiotic resistance; *Achromobacter xylosoxidans*; class 1 integron; In687; horizontal gene transfer; AcGI1

INTRODUCTION

Members of the genus *Achromobacter* are Gram-negative bacteria of growing importance as opportunistic pathogens, particularly in relation to patients with Cystic Fibrosis (CF) (Hansen *et al.* 2013) and immunocompromised patients (Bellissimo *et al.* 2013). Amongst *Achromobacter* isolated from chronic lung infections of CF patients, *Achromobacter xylosoxidans* is the most abundant species (40–60%) (Spilker, Vandamme and LiPuma 2012; Barrado *et al.* 2013; Hansen *et al.* 2013; Coward *et al.* 2015). In part

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the clinical problems associated with these bacteria are due to the fact that a number of isolates display high levels of antibiotic resistance. All *A. xylosoxidans* isolates show high levels of innate resistances to cephalosporin and aminoglycoside antibiotics and some of the genes associated with this innate resistance have been described (Doi *et al.* 2008; Bador et al. 2011, 2013). Additionally, there is evidence that a number of resistance genes have been obtained by horizontal transfer (Yamamoto *et al.* 2012).

A number of different metallo-β-lactamases, carried in class 1 integrons, have been described in *A. xylosoxidans*. These include VIM-type (Sofianou *et al.* 2005; Di Pilato, Pollini and Rossolini 2014) and IMP-type β-lactamases (Yamamoto *et al.* 2012). Also, extended-spectrum β-lactamases, such as Vietnamese extended-spectrum β-lactamase (VEB)-type (Neuwirth *et al.* 2006), are found in *A. xylosoxidans*. There are currently more than 50 types of IMP β-lactamases, based on amino acid sequence variations(Zhao and Hu 2011; Pournaras *et al.* 2013; Shakibaie, Azizi and Shahcheraghi 2017). In addition to β-lactam resistance, genes associated with the resistance to fosfomycin, aminoglycosides and sulfamethoxazole/trimethoprim have been identified previously from the complete genome sequence data of *A. xylosoxidans* (Jakobsen *et al.* 2013).

In this study, we investigated two multidrug resistant isolates of *A. xylosoxidans* from Thailand, using whole genome sequencing to reveal the presence and localization of genes responsible for carbapenem resistance, within a novel genomic island called *Achromobacter* genomic island 1 (AcGI 1).

MATERIALS AND METHODS

Bacterial strains

Multidrug-resistant *A. xylosoxidans* strains R4 and R8 were isolated from an emergency unit between 2010 and 2012, at Ramathibodi Hospital in Bangkok, Thailand. Strain R4 was isolated from a urine sample and strain R8 from a peripheral blood culture. The collection of these clinical isolates was approved by the ethical review committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Thailand (MURA 2009/1396/S8; ID 03-52-93). The *A. xylosoxidans* reference strain, NCIMB11015 (Braker, Fesefeldt and Witzel 1998), was purchased from NCIMB, UK, and used as a non-clinical isolate control. The relationship of the isolates to previously reported strains of *A. xylosoxidans* was determined using multilocus sequence typing (MLST) assignment (Jolley, Bray and Maiden 2018) (http://pubmlst.org/achromobacter/).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by disk diffusion assays, using guidelines of the European Society Committee on Antimicrobial Susceptibility Testing (The European Committee on Antimicrobial Susceptibility Testing 2020). All isolates were grown on Iso-Sensitest agar (Oxoid, UK). All antimicrobial disks were purchased from Thermo Scientific Inc., Waltham, MA, USA. The minimum inhibitory concentrations (MIC) were determined using the Sensititre (Trek diagnostic systems Ltd, UK). The antibiotics used in this study were cefuroxime (CXM), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), meropenem (MEM), imipenem (IPM), ertapenem (ETP), ciprofloxacin (CIP), levofloxacin (LVX), gentamicin (GEN), amikacin (AMK), co-trimoxazole (SXT) and Piperacillin/tazobactam (TZP). Metallo-β-lactamase detection assay was performed using disk diffusion with 10 μg MEM + 750 μg EDTA disk (Thermo Scientific Inc.). β-lactamase activity was assessed using a nitrocefin broth method (O'Callaghan *et al.* 1972). Briefly, nitrocefin was added into mid-log culture to reach a final concentration of 51.6 μg/mL. Optical density (OD) at 486 nm and 390 nm was measured over 30 min, when incubated at 37°C. Activity was expressed as the ratio of OD at 486 nm to OD at 390 nm (OD486/OD390 ratio). Assays were conducted in triplicate. The significance of differences in the OD486/OD390 ratio between three isolates was determined by one-way ANOVA with Tukey's test. For the comparison of OD486/OD390 ratio of each isolate between 0 and 30 min, the significance of differences in the OD486/OD390 ratio was evaluated using paired t-test. Statistical significance was defined as a *P*-value of < 0.05.

Whole genome sequencing

Achromobacter xylosoxidans strains R4, R8 and NCIMB11015 were grown in LB broth at 37°C overnight. Genomic DNA from all *A. xylosoxidans* strains was extracted using DNeasy Blood and Tissue kit (QIAGEN, UK). Whole genome sequencing was performed using the Pacific Bioscience® RS II platform by the Centre for Genomic Research, University of Liverpool. Genome assemblies of the isolates were obtained using the HGAP software (Chin *et al.* 2013) and annotated using Prokka 1.13 (Seemann 2014). Comparative genomic analysis between sequenced genomes and a reference genome NH44784-1996 (Jakobsen *et al.* 2013) was performed to identify the genomic difference between multidrug resistance isolates and the reference isolate. Briefly, three newly sequenced genomes of *A. xylosoxidans* were compared against one another using BLAST algorithm, implemented in BLAST+ (Camacho *et al.* 2009), to generate comparison files. In order to identify genes that are associated with antibiotic resistance, coding sequences called by Prokka were searched against an antibiotic resistance database using Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD) (McArthur *et al.* 2013; Jia *et al.* 2017). The prediction of genomic islands was performed using a combination of the observation of GC content of the genome sequence and *in silico* prediction in IslandViewer 4 (Bertelli *et al.* 2017). Then the comparison was visually investigated using Artemis Comparison Tool (Carver *et al.* 2005) and comparison figures were generated using EasyFig version 2.2.2 (Sullivan, Petty and Beatson 2011).

Genome submission and accession

The nucleotide sequences mentioned in this report are available in the GenBank nucleotide database under accession number LN890476 (strain R4), LN890477 (strain R8), LN890335 (strain NCIMB11015), KJ406505 (IMP-14-containing In687 in strain R4) and KJ406506 (IMP-14-carrying In687 in strain R8).

RESULTS

Bacterial strain typing and antibiotic resistance

Analysis using the pubMLST database typing (accessed 18th May 2020) revealed that the Thai *A. xylosoxidans* strains R4 and R8 were ST-183 and ST-185, respectively, and they were not clonally related (Table 1a and Fig. 1). The initial antibiotic susceptibility screen using the disk diffusion method revealed that strains R4 and R8 were less susceptible to multiple antibiotics, compared to the reference strain NCIMB11015, especially the carbapenems (IPM, MEM and ETP) and co-trimoxazole (Table 1a). However,

Table 1a. Characteristics and antimicrobial susceptibility profiles of multidrug-resistant *A. xylosoxidans* strains. Screening for antimicrobial resistance using disk diffusion.

Strain	Source	MLST type	Zone of inhibition (mm)										
			CXM	CTX	CRO	CAZ	IPM	MEM	MEM + EDTA	ETP	CIP	LVX	GEN	AMK	TGC	SXT
R4	Urine	183	5 (R)	5 (R)	5 (R)	5 (R)	17 (R)	5 (R)	23 (S)	12 (R)	16 (R)	20 (R)	5 (R)	13 (R)	25 (S)	5 (R)
R8	Blood	185	5 (R)	5 (R)	22 (R)	21 (R)	16 (R)	5 (R)	25 (S)	13 (R)	12 (R)	12 (R)	16 (R)	14 (R)	22 (S)	14 (R)
NCIMB11015	Soil	166	5 (R)	12 (R)	16 (R)	34 (S)	36 (S)	32 (S)	32 (S)	32 (S)	22 (S)	22 (S)	14 (S)	16 (S)	32 (S)	26 (S)

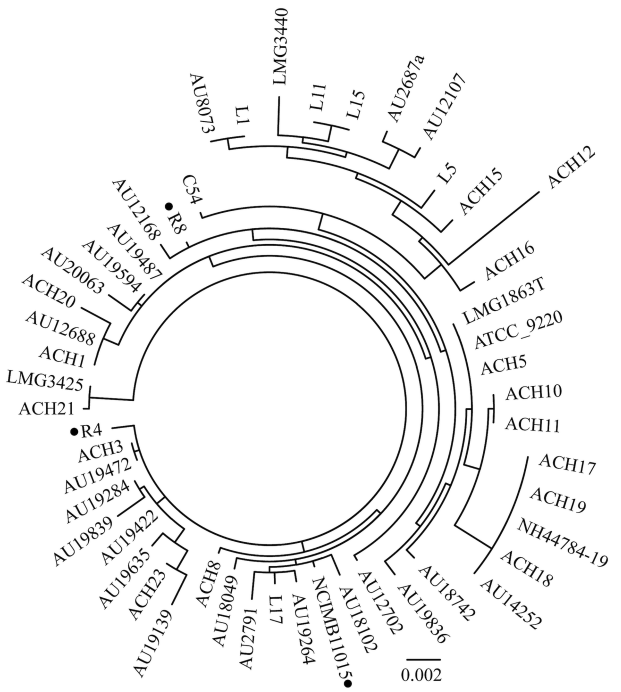

Figure 1. Maximum-likelihood phylogenetic relationship with 1000 bootstraps based on concatenated nucleotide sequences of seven housekeeping genes (eno, gltB, lepA, nrdA, nuoL, nusA and rpoB) of *A. xylosoxidans* strain R4, R8, NCIMB11015 and 48 other *A. xylosoxidans* strains (data obtained from pubMLST (http://pubmlst.org/achromobacter)). Strains R4, R8 and NCIMB11015 are labeled with a black dot.

strains R4 and R8 were more susceptible to MEM with the addition of EDTA (Table 1a). This suggests that a metallo-β-lactamase plays a role in the resistance exhibited by these strains.

The multidrug-resistant strains R4 and R8, were further analysed in order to determine MIC values (Table 1b). The two strains were resistant to aminoglycosides (GEN and AMK) and cephalosporins (CXM, CTX, CAZ and CRO), but varied in their susceptibility to fluoroquinolones, with strain R8 being less susceptible than strain R4. Moreover, both strains were resistant to SXT, a combination of sulfamethoxazole and trimethoprim, but susceptible to TGC. Unlike strain NCIMB11015, both strains R4 and R8 exhibited resistance to IPM, MEM and ETP (Table 1b).

Whereas strain R4 was susceptible to TZP, strain R8 was resistant. The activity of β-lactamase was then assayed by using nitrocefin hydrolysis. Prior to incubation, there was no statistically significant difference between the OD486/OC390 ratios of the three strains (ANOVA, $F_{2,6} = 0.098$, $P = 0.908$). The assay revealed that the change of OD486/OD390 ratio for strain R4 (*T*-test, Mean $\pm$ SD; 0.633 $\pm$ 0.096 vs 1.097 $\pm$ 0.066; $P < 0.05$) and strain R8 (*T*-test, Mean $\pm$ SD; 0.668 $\pm$ 0.047 vs 1.098 $\pm$ 0.010; $P < 0.05$) after 30 min incubation was significant, whereas the change of the ratio for strain NCIMB11015 was not significant (Mean $\pm$ SD; 0.642 $\pm$ 0.133 vs 0.698 $\pm$ 0.076; $P = 0.262$). There was a significant difference in the ratio among the three strains after incubation for 30 min (ANOVA, F2,6 = 38.16, $P < 0.05$). Post-hoc comparison using Tukey's test revealed that the OD486/OD390 ratio was significantly different between strain R4 and strain NCIMB11015, and between strain R8 and strain NCIMB11015, whereas it was not significantly different between strain R4 and strain R8. Taken together, these results suggest the activity of a metallo-β-lactamase in strains R4 and strain R8.

Table 1b. Characteristics and antimicrobial susceptibility profiles of multidrug-resistant *A. xylosoxidans* strains. Determination of levels of resistance using minimum inhibitory concentration (MIC).

Strain	MIC (mg/L)											
	TZP	CXM	CTX	CRO	CAZ	IPM	MEM	ETP	CIP	LVX	GEN	AMK	TGC	SXT
R4	≤8	>16	>32	>32	>32	>8	>8	>4	1	1	>8	>32	≤0.25	>4
R8	≥32	>16	>32	>32	>32	>8	>8	>4	>2	8	>8	>32	1	>4

The identification of genes associated with Carbapenem resistance in strain R4 and R8

Whole genome sequencing provided complete chromosome assemblies for all three strains. Gene annotation using Prokka, together with RGI, identified 87 resistance genes, 92 resistance genes and 86 resistance genes in all for strain R4, R8 and NCIMB11015, respectively (Table S1, Supporting Information). Antibiotic resistance genes present in all three genomes included genes encoding a known species-specific β-lactamase, $bla_{\text{OXA-114}}$ (Turton *et al.* 2011) and an RND-type efflux pump, *axyA*, *axyB*, *axyM*, which has been implicated in resistance to aminoglycosides, fluoroquinolones and cephalosporins (Bador et al. 2011, 2013). Interestingly, the $bla_{\text{IMP-14a}}$ gene was identified in the two carbapenem resistant strains (R4 and R8), whereas it was not present in the carbapenem sensitive strain (NCIMB11015).

Carbapenem resistance mediated by a novel genomic island, *Achromobacter* genomic island 1 (AcGI1)

Focusing on genes associated previously with carbapenem resistance, WGS identified $bla_{\text{IMP-14a}}$ located on a class 1 integron In687 (*intI1*- $bla_{\text{IMP-14a}}$-*aac(6')-qacEdelta-sul1*). This integron was identified in several clinical isolates from Thailand, including two isolates of *Acinetobacter baumannii* (strain Aci16 (Kansakar *et al.* 2011) and strain RA-41 520 608 (Genbank GQ302618)), which were also from Ramathibodi Hospital in Thailand in 2007 and 2009, respectively, *Klebsiella pneumoniae* (Matsumura *et al.* 2017) and *Escherichia coli* ST 131 strain 732 (Stoesser *et al.* 2016).

Having identified the In687 integron in the chromosomes of *A. xylosoxidans* strains R4 and R8, we proceeded to analyse the neighbourhood of the integron to better understand the genomic context. As a consequence of combining visualised genome comparison with NCIMB11015 and IslandViewer 4, we determined that the integron was part of a novel genomic island, which was 121 613 bp long, and this island was named *Achromobacter* genomic island 1 (AcG1; Fig. 2). AcGI1 islands in the two strains share more than 99.0% nucleic acid identity. Similar to other genomic islands, AcGI1 was inserted after a $tRNA^{Gly}$ gene, however, direct repeats were not clearly identified. AcGI1 had a GC content of 61.5%, lower than the whole genome value of 67.5%.

This island contains 16 antibiotic resistance-associated genes responsible for resistance to β-lactam ($bla_{\text{IMP-14a}}$), aminoglycosides (*aac(6')*, aminoglycoside O-phosphotransferase APH(3')-Ib, APH(6)-I family aminoglycoside O-phosphotransferase), tetracycline (tetracycline repressor protein, tetracycline efflux MFS transporter, tetracycline resistance transcriptional regulator and tetracycline resistance protein) and quaternary compound (*qacEdelta*) antimicrobial agents. Also present were 44 mobile genetic element-associated genes and 38 hypothetical genes, suggesting that AcGI can be horizontally transferred between organisms.

We further investigated the structure of the element with sequence alignment against a non-redundant database. The best alignment result, with a high nucleotide identity (>90%), was to the genome of *Klebsiella pneumoniae* F128 (GenBank accession no. CP026149.1; 87% coverage of AcGI1, not including In687), indicating that *A. xylosoxidans* supporting the notion that AcGI1 can spread between species via horizontal gene transfer.

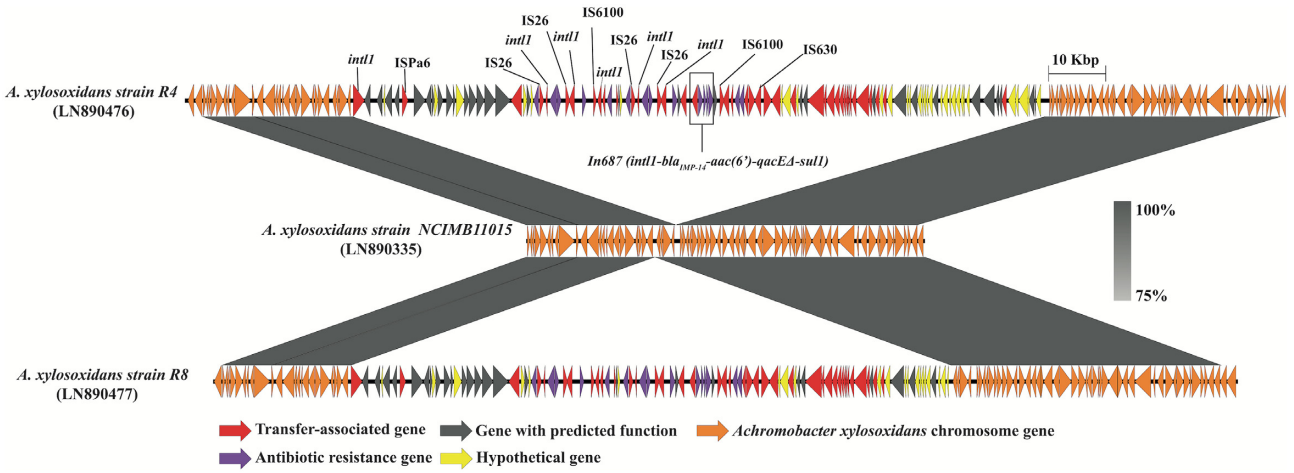

Figure 2. Schematic presentation of the *A. xylosoxidans* chromosome showing the insertion of Achromobacter genomic island (AcGI1) into the chromosome of *A. xylosoxidans* strain R4 (top) and strain R8 (bottom), compared with strain NCIMB11015 (middle) using EasyFig. The colored arrows indicate the direction of the transcription of predicted open reading frames. Grey regions indicate 80–100% sequence similarity.

DISCUSSION

Horizontal gene transfer is recognised as an important mechanism contributing to the evolution of bacteria and the development of antibiotic resistance (Polz, Alm and Hanage 2013; Maddamsetti and Lenski 2018; Peterson and Kaur 2018; Sun *et al.* 2019). Genomic islands are genomic regions that often differ in %GC from the other parts of the chromosome, are locates near tRNA genes, and contain gene cassettes. Genomic islands can be located into several classes based on groups of genes carried, for example, pathogenicity island, resistance island and metabolism island, emphasizing the importance of these islands in bacterial evolution and adaptation.

Antibiotic resistance in bacterial pathogens has become a major public health concern, and the detection of resistance genes is urgently needed for the prevention of transmission of resistant organisms. Interestingly, antibiotic resistance can be genetically transferred between bacteria via horizontal gene transfer. Using Pacific Bioscience® single molecule real time sequencing, our comparative genomic analysis between two non-clonal carbapenem resistant clinical isolates of *A. xylosoxidans* (R4 and R8) and one carbapenem sensitive *A. xylosoxidans* (NCIMB11015) illustrates that a drug-resistance determining region was localized within a novel resistance island, named AcGI1. The high level of nucleotide sequence identity between the island in the genomes of strains R4 and R8 suggests that AcGI1 may have been transmitted within the hospital.

Multidrug-resistant bacteria have been identified in Thailand from various sources, including healthy adults, food contamination and hospital-associated infections. A previous study showed that the prevalence of carbapenem-resistant Enterobacteriaceae in Thailand is approximately 40–50%, which is the highest in the Asia–Pacific region. However, carbapenem-resistant *A. xylosoxidans* in Thailand have not been reported. Previous studies have shown that carbapenem resistance in *A. xylosoxidans* can be horizontally acquired, demonstrated by the identification of integron-associated resistance genes, such as VIM-type β-lactamase (Shin *et al.* 2005; Hishinuma *et al.* 2019). In this study, the carbapenem resistance of strains R4 and R8 is the result of a $bla_{IMP-14a}$-carrying In687 located in AcGI1. In687 was firstly described as a part of a plasmid pEC732-IMP14 (Genbank accession no. CP015139), in *E. coli*. The pEC732-IMP14-carrying *E. coli* ST-131 was recovered from urine culture in Thailand (Stoesser *et al.* 2016). Therefore, this study is the first report describing the integration of In687 in the chromosome rather than on a plasmid.

An IMP-type β-lactamase gene was first described in *Pseudomonas aeruginosa* in Japan, and there are currently more than 50 allelic variants (Shakibaie, Azizi and Shahcheraghi 2017). In Thailand, the first bla_{IMP-14} was identified in a clinical isolate of *P. aeruginosa* from the teaching hospital in 2004 (Genbank accession no. AY553332) (Khuntayaporn *et al.* 2013). Subsequently, $bla_{IMP-14a}$, with a single nucleotide difference, was identified in *Acinetobacter baumannii* in 2007 (Genbank accession no. HM036079) and 2009 (Genbank accession no. GQ302618), also in the same teaching hospital in Bangkok. Therefore, the presence of bla_{IMP-14} and $bla_{IMP-14a}$ in hospital-associated pathogens, including *A. xylosoxidans*, in Bangkok between 2004 and 2012, indicates that the circulation and distribution of this β-lactamase-containing mobile genetic element has been occurring for at least eight years in Bangkok.

CONCLUSIONS

Whole genome sequencing enables us to obtain a more comprehensive view of genetic of antibiotic resistance in bacteria. To our knowledge, this study is the first to identify a novel 122 kb genomic island in *A. xylosoxidans*, named AcGI1, containing $bla_{IMP-14a}$-habouring In687. Interestingly, AcGI1 was identified in two carbapenem-resistance isolates, which are not clonally related. This finding provides evidence that AcGI1 can be transferred horizontally and contribute to the spread of carbapenem resistance hospital-associated infections.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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