An acquired efflux system is responsible for copper resistance in *Xanthomonas* strain IG-8 isolated from China

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Abstract

The genus *Xanthomonas* contains plant pathogens exhibiting innate resistance to a range of antimicrobial agents. In other genera, multidrug resistance is mediated by a synergy between a low-permeability outer membrane and expression of a number of multidrug efflux systems. This report describes the isolation of a novel gene cluster *xmeRSA* from *Xanthomonas* strain IG-8 that mediates copper chloride resistance. Subsequent analysis of these genes showed that they were responsible for the high level of multiple resistance in this strain and were homologues of the *sme* system of *Stenotrophomonas maltophilia*. Knock-out mutants of this gene cluster indicate that these genes are required for the copper resistance phenotype of strain IG-8. Expression analysis using *lacZ* fusions indicates that the genes are regulated by copper and other antimicrobials. Bioinformatic analysis suggests that these genes were acquired by horizontal gene transfer.

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Introduction

Multidrug resistance (MDR) presents a serious problem in the treatment of bacterial infections (Srikumar *et al.*, 1997; van Veen & Konings, 1998; Lee *et al.*, 2000; Poole, 2000; Sardessai & Bhosle, 2002; Schweizer, 2003). The MDR phenomenon is often associated with the overexpression of the transporters that recognize and efficiently expel from the cell a broad range of structurally unrelated compounds. Analysis of the available genome sequences of various bacteria revealed that known and putative drug efflux transporters constitute from 6% to 18% of all transporters (Nikaido, 1998; Poole, 2000). Thus, evolution has tailored bacteria with considerable capabilities to survive in a deleterious environment (Schweizer, 2003; Piddock, 2006).

Several types of nonspecific efflux pumps, mediating multiple resistance phenotypes, have recently been described in the literature (Nikaido, 1998; Poole, 2000; Schweizer, 2003; Piddock, 2006). One more recent MDR system to be identified and characterized in *Stenotrophomonas maltophilia* is SmeRSABC (Zhang *et al.*, 2000, 2001a; Li *et al.*, 2002). The SmeRSABC multidrug system is unique among the multidrug efflux systems in Gram-negative bacteria as it is regulated by a two-component regulatory system encoded by the *smeRS* genes (Li *et al.*, 2000; Zhang *et al.*, 2001a, b). A member of the RND family, this efflux system encodes resistance to several antimicrobials, including amino glycosides, β-lactams and fluoroquinones (Alonso & Martinez, 1997, 2000; Li & Poole, 1999; Li *et al.*, 2000).

The bacterial strain *Xanthomonas* IG-8 was originally isolated as a glyphosate-resistant organism from soil samples from a glyphosate production facility in the HeiBei province, China. The soil from this site was heavily contaminated with glyphosate and heavy metals. Here, the isolation and molecular analysis of an MDR system mediating copper resistance from this strain in *Escherichia coli* are reported. In addition, bioinformatic evidence that this phenotype is due to a possible horizontal gene transfer (HGT) event is presented.

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Materials and methods

Bacterial strains, growth conditions and plasmids

All the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C and *Xanthomonas* strains at 30 °C in Luria–Bertani (LB) broth.

16S rRNA gene and BIOLOG analysis of strain IG-8

The following PCR primer set was developed to clone the 16S rRNA gene: forward primer [20-mer (5′-AGAGTTT GATCKTGGCTCAG-3′)] and reverse primer [20-mer (5′-KAAGGAGGTGKTCCAGCC-3′)]. PCR was run using standard methods described previously (Sambrook *et al*., 1989). DNA sequencing of primer amplicons was carried out by Complement Genomics, Sunderland, UK. The sequence for the first part of the 16S rRNA gene, *c*. 500 bp in length, was determined using four consecutive sequencing reactions on either DNA strand (accession no. AY360452). Further characterization of the IG-8 strain was carried out using BIOLOG^{TM}.

DNA preparation and amplification

Genomic DNA from pure cultures was obtained using the Wizard® Genomic DNA purification kit as per the manufacturer's instructions. Additional DNA techniques were performed by published protocols (Birnboim & Doly, 1979; Sambrook *et al*., 1989).

Chromosomal library construction

A cosmid library of IG-8 was constructed as described previously (Sun *et al*., 2005), 10 000 clones were obtained, and these were pooled into two groups of 5000 and stored in 15% glycerol stocks at −70 °C.

Isolation of the genes involved in copper resistance

LB agar containing a concentration of 2.5 mM copper chloride was used to screen the library for copper-resistant clones. A number of clones were isolated expressing resistance to 2.5 mM copper and 100 μg mL ampicillin in *E. coli*. One clone termed pITCCOS38 was selected with an insert size of 38 kb. Subcloning experiments were then carried out to isolate a minimal copper-resistant clone. A series of partial BamHI digests were ligated with pCOSI vector DNA restricted with BamHI. One subclone, pITCCOS22, had an insert size of 22 kb and conferred the copper-resistant phenotype to *E. coli*. The subcloning experiment was repeated using this plasmid, yielding a subclone pITCCU4, containing an insert size of 9.2 kb that still conferred copper resistance on *E. coli* cells. A minimal 6 kb subclone made in vector pUC119 (pITCCU5) was found to confer copper resistance and was used for further studies.

Susceptibility to antimicrobial agents

Susceptibility of sub clones to the following antimicrobials was examined: ampicillin (100 μg mL^{-1}), nalidixic acid (20 μg mL^{-1}), chloramphenicol (20 μg mL^{-1}), penicillin (20 μg mL^{-1}), kanamycin (20 μg mL^{-1}), rifampicin (50 μg mL^{-1}), tetracycline (20 μg mL^{-1}), zinc (2.5 mM), copper (2.5 mM), nickel (2.5 mM), arsenic (7.5 mM), tellurite (500 μM), cobalt (2.5 mM) and cadmium (500 μM) by streaking the selected colonies on LB agar with the added antimicrobial agent. In order to estimate the minimum inhibitory concentration (MIC) for antibiotics and heavy metals, a concentration series of all metals and antibiotics

Table 1. Bacterial strains and plasmids and their relevant characteristics

Strain	Relevant characteristics	Reference
Escherichia coli JM109	*EndA1*, *recA1*, *gyrA96*, *thi*, *lacI*^{q}ZΔM15, *rel*A1	Sambrook *et al*. (1989)
Xanthomonas IG-8	Cu^{R} isolate	This study
pK18mobtet	Tet^{R}	Schäfer *et al*. (1994)
pK18mobsac	Tet^{R}, 10% (w/v) sucrose	Schäfer *et al*. (1994)
pMP220	*E. coli*, Tet^{R} promoterless lacZ cloning vector	Spaink *et al*. (1987)
pUC119	Plasmid vector, Amp^{R}	Sambrook *et al*. (1989)
PCOSI	Cosmid cloning vector, Amp^{R} Nal^{R}	Stratagene Inc.
pITCCU4	9.2 kb fragment of *Xanthomonas* IG-8 cloned into pCosI, Cu^{R}	This study
pITCCU5	6 kb fragment containing *smeRSA* system cloned into pUC119, Cu^{R}	This study
pITCCU41	*E. coli*, pMP220, (partial *xmeRSA* fragment), Amp^{R} Cu^{R}	This study
pITCCU43	*E. coli*, pMP220, (partial *xmeRSA* fragment), Amp^{R} Cu^{R}	This study
pITCCU44	*E. coli*, pMP220, (partial *xmeRSA* fragment), Amp^{R}	This study
pITCCU45	*E. coli*, pMP220, (*xmeA* fragment), Amp^{R}	This study
IG-8 ▲ *xmeR*	*Xanthomonas* IG-8, ▲ *xmeR* unmarked deletion mutant created using pK18mobsac	This study
IG-8 ▲ *xmeS*	*Xanthomonas* IG-8, ▲ *xmeS* unmarked deletion mutant created using pK18mobsac	This study
IG-8 ▲ *xmeA*	*Xanthomonas* IG-8, ▲ *xmeA* unmarked deletion mutant created using pK18mobsac	This study

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was used as previously described (Ryan *et al.*, 2005). Two approaches were used to assess solvent and detergent tolerance. The first involved overlaying various solvents on 25 mL LB agar plates inoculated with *E. coli* containing sub clones and the second was using the efficiency of plating (EOP) method as previously described (Li *et al.*, 1998).

DNA sequencing and Bioinformatic analysis

Clone pITCCU4-mediating resistance to copper was sequenced by MWG-biotech using primer walking in both directions. DNA sequences were screened for ORFs using CHROMAS and OMEGA software™. Screening of the GenBank database was performed using BLAST analysis (Altschul *et al.*, 1990). Expasy Tools (http://us.expasy.org/tools/) was used to predict protein structure and function. Further study of the *Xanthomonas* IG-8 copper resistance gene system was carried out using codon usage analysis. Correspondence analysis of the relative synonymous codon usage (RSCU) values for each gene in the copper-resistant fragment was carried out using the general codon usage analysis (GCUA) program (http://bioinf.nuim.ie/gcua/download.html) (Gouy & Gautier, 1982; McInerney, 1998a, b).

Construction of *lacZ* transcriptional fusions

The restriction and ligation procedure was carried out as detailed in Sambrook *et al.* (1989). The construction of the broad host range *lacZ* reporter vector pMP220 was described by Spaink *et al.* (1987). A 6-kb fragment from pITCCU5 (encoding copper resistance) was cloned into pMP220 to generate pITCCU41. The presence and orientation of the fragment was confirmed by restriction analysis. To obtain pITCCU43, a 3526 bp region from pITCCU41 was excised by digestion with PvuI and EcoRI then relegated into pMP220. To obtain pITCCU44, an AvaI-EcoRI fragment measuring 2648 bp isolated from pITCCU41 was religated to the pMP220. Finally, to obtain pITCCU45 a SacI-EcoRI fragment measuring 1192 bp (which contains the *xmeA* gene only) was isolated from pITCCU41 and relegated with pMP220. Fusions were individually introduced into *E. coli* by transformation and IG-8 by tri-parental conjugation using pRK2013 as a helper plasmid (Spaink *et al.*, 1987). The β-galactosidase assay was carried out as previously described (Spaink *et al.*, 1987).

Construction of deletion mutants in *Xanthomonas* IG-8

In-frame deletion mutants were constructed as described previously using the suicide vector pK18mobsac (Schäfer *et al.*, 1994; Niebisch & Bott, 2001). To construct in-frame *xmeR* and *xmeS* deletion mutants, primers were designed to amplify *c.* 250 bp on either side of the gene to be deleted. These primers were selected to amplify the fragment in-frame. For deletion mutants of *xmeR* and *xmeS*, fragments of 1902 and 1800 bp were amplified, respectively, from pITCCU4 using primers for ▲*xmeS* (xmeSfwd- 3′ CTGCCATCGGCGCGGCCGAA 5′, xmeSrev- 3′ CACGC CATCGCGCTGCGGCA 5′) and ▲*xmeR* (xmeRfwd- 3′ GTGGAAAGGAGGGCGGCGCG 5′, *xme*Rrev- 3′ TTGTA GAAGTTCAGATCGTT 5′). To isolate the 2021 bp fragment containing the *xmeA* gene, a digestion of pITCCU4 by SacI/BstI was used. These constructs were named pK18mobsac-*xmeR*, pK18mobsac-*xmeS* and pK18mobsac-*xmeA*. These suicide vectors were reintroduced into *Xanthomonas* IG-8. Mutants were verified by PCR. The flanking regions in the genome were amplified for IG-8 (▲*xmeS*) producing an amplicon of 410 bp in the deleted mutant, IG-8 (▲*xmeR*) producing an amplicon of 530 bp in the deleted mutant and IG-8 (▲*xmeA*) producing an amplicon of 450 bp in the deleted mutant.

Results

Isolation and characterization of *Xanthomonas* sp. strain IG-8

The IG-8 strain was isolated from a soil sample containing *c.* 50% vegetation/rhizosphere and 50% soil. The soil had a matrix consisting of 8% clay, 50% silt and 42% sand. The soil from this site at HeiBei province (China) was heavily contaminated with glyphosate and heavy metals (copper – 454 p.p.m., zinc – 2257 p.p.m., lead – 9644 p.p.m., cadmium – 16 p.p.m.).

The IG-8 strain was found to have elevated levels of resistance to copper chloride (4 mM) in addition to other heavy metals and glyphosate. The strain was closely related to *Xanthomonas campestris* based on BIOLOG™ analysis (99% confidence level) and this was confirmed by 16S rRNA gene sequencing and phylogenic analysis. Partial 16S rRNA gene sequence (first 502 bp) (accession no. AY360452) analysis using nucleotide BLAST gave the closest hit as *Xanthomonas* (98%).

Isolation of copper resistance genes from *Xanthomonas* IG-8 and expression in *E. coli*

A chromosomal library of IG-8 was prepared using the pCOSI cosmid cloning vector. Approximately 10^3 CFU mL^{-1} of the *Xanthomonas* IG-8 library were plated on 2.5 mM copper chloride plates to select copper-resistant isolates. The cosmid pITCCOS38 was isolated from *E. coli* and restricted with BamHI, revealing an insert of *c.* 38 kb; partial restriction with BamHI and ligation produced the subclone pITCCU4 with an approximate size of 9.2 kb, containing ORFs 1–8, which confers copper resistance at a level of

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4 mM in *E. coli*. A 6-kb fragment from pITCCU4 was subcloned into pUC119 and this maintains the copper resistance phenotype in *E. coli* (construct pITCCU5).

Phenotypic analysis of the parent *Xanthomonas* IG-8 strain and *E. coli* (pITCCU5) revealed that elevated levels of resistance to several antimicrobial agents including kanamycin (20 μg mL^{-1}), chloramphenicol (20 μg mL^{-1}), octanol (100%), xylene (100%) and copper 4.5 mM were also evident in the *E. coli* strain containing pITCCU5 (Table 2).

Table 2. Phenotypic examination of *Xanthomonas* IG-8, and *Escherichia coli* containing the pITCCU5 clone and the pUC119 vector

	Bacterial strain and plasmid		
Antimicrobial agent	*Xanthomonas* IG-8	*E. coli*+ pITCCU5	*E. coli*+ pUC119
Antibiotics (μg mL^{-1})			
Nalidixic acid	15	15	5
Tetracycline	10	10	5
Chloramphenicol	20	20	5
Kanamycin	20	20	5
Rifampicin	1001	5	5
Spectinomycin	20	5	5
Gentamycin	20	5	5
Organic solvents (%)			
Diphenyl ether	100	10	10
Hexane	100	100	10
p-Xylene	100	100	10
Octanol	100	100	10
Toluene	50	10	10
Benzene	25	10	10
Detergents (%)			
Triton X–100	50	50	5
Tween 20	25	25	5
Tween 80	25	25	5
Heavy metal			
Zinc	500 μM	500 μM	500 μM
Copper	6 mM	4.5 mM	1 mM
Cadmium	2.5 mM	1 mM	500 μM
Cobalt	500 μM	500 μM	500 μM
Nickel	2.5 mM	1 mM	500 μM
Arsenic	5 mM	2 mM	2 mM

A multidrug efflux system from *Xanthomonas* sp. IG-8 confers copper resistance in *E. coli*

The DNA sequence of pITCCU5 revealed five ORFs (ORF4–ORF8) (accession no. AY359472). The nucleotide sequence was analysed using BLASTN, revealing homology to genes of two families of multidrug-resistant proteins found in *S. maltophilia* (Fig. 1).

The ORF4 spanned nucleotides 304–994 and has similarity to an outer membrane protein in *X. campestris* and *Pseudomonas syringae*. The ORF5 had high similarity to transferase proteins in *X. campestris*. The ORFs 6 and 7 spanned nucleotides 2769–4907 and are homologous to *smeSR* of *S. maltophilia*. These genes were termed *xmeS* and *xmeR*, respectively. The ORF8 (termed *xmeA*) spanned the region of sequence 2559–5919 with similarity to the inner-membrane fusion protein SmeA, found in the SmeRSABC system of *S. maltophilia*. Orientations of these ORFs were from the 5′ to the 3′ direction in the case of ORFs 4, 5, 6 and 7 while ORF8 read in the 3′–5′ direction.

The *xmeRSA* genes play a major role in the multi-resistant phenotype of *Xanthomonas* IG-8

Deletion of *xmeA* in IG-8 leads to the reduction of copper/arsenic/cadmium tolerance to background levels (250 μM, 1 mM and 1 mM, respectively), indicating that this gene plays a major role in the copper-resistant phenotype of this strain. To investigate the role of *xmeSR* in relation to the copper resistance phenotype of IG-8, deletions of the *xmeR* and *xmeS* genes were also engineered into the chromosome of *Xanthomonas* IG-8 and their impact on copper susceptibility was examined. The deletion of *xmeR* in IG-8 lowered copper resistance to a level to 500 μM, whereas the *xmeS* deletion mutant showed a fourfold reduction in copper resistance (to a level of 1 mM).

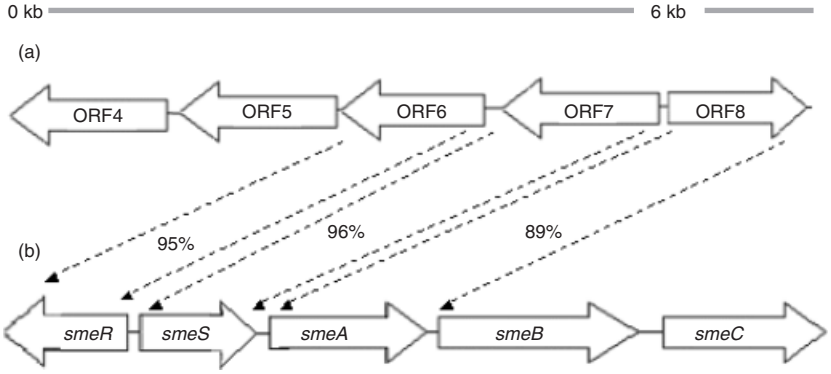

Fig. 1. Comparison of a fragment from *Xanthomonas* IG-8 (accession no. AY359472) (a) and the *SmeABC* operon organization of *Stenotrotromonas maltophilia* (accession no. X95923) (b). Percentage identities relate to translated amino acid sequences.

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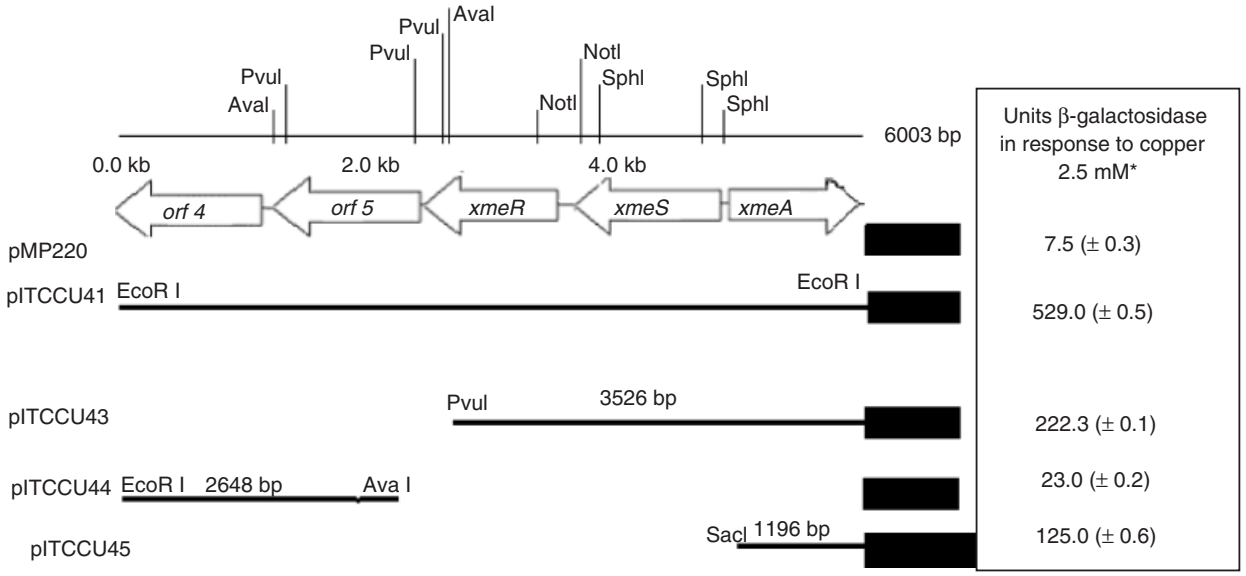

Fig. 2. Functional analysis of the *xmeRSA*-like region in *Escherichia coli* JM109 strains. The *lacZ* fusions are described in the text. *β-galactosidase activity in the absence of copper was 10 Miller units in *E. coli*.

Expression of *xme* genes is induced in the presence of copper

To investigate the region containing the *xmeRSA* genes of pITCCU5 for promoter activity, the region was inserted into a *lacZ* promoter-less transcription fusion vector, pMP220 (Spaink *et al.*, 1987). The fragment containing the *xmeRSA* genes in clone pITCCU5 was inserted into pMP220 as detailed in methods. The resulting *lacZ* fusions were introduced into *E. coli* JM109. The β-galactosidase activity was assessed following exposure to copper (2.5 mM).

The clones pITCCU41 and pITCCU43 in the *E. coli* background expressed resistance to copper (4 mM) and the other antimicrobials tested. These clones also showed copper-inducible *lacZ* expression in the presence of copper concentrations as low as 100 μM (Fig. 2). In the absence of copper, expression levels were 10 Miller units or less. This pattern of copper induction was also seen when these clones were introduced into the wild-type *Xanthomonas* IG-8 background (data not shown). The plasmids pITCCU44 and pITCCU45 showed no copper resistance phenotype in the *E. coli* JM109 background. However, pITCCU45 did show a slight increase in the level of *lacZ* expression in the presence of copper (Fig. 2).

Bioinformatic evidence that the *Xme* genes were recruited by IG-8 following HGT

The *X. campestris* genome (accession no. AE012131) was screened using *X. campestris* GENOMA NBLAST for nucleotide sequences similar to the 9.2-kb fragment from IG-8 (accession no. AY359472). The ORFs ORF1 to ORF5 showed similarity (85–95%) to the genes encoding the xanthan general secretion pathway. This was confirmed using *X. campestris* GENOMA PBLAST for protein products. However, *xmeRSA* showed low similarity to any area of the complete genome of *X. campestris* pv. *campestris* ATCC 33913 (accession no. AE012131) or *X. campestris* pv. *campestris* 8004 (accession no. NC_007086).

Codon usage analysis using GCUA was carried out on all eight ORFs identified from the 9.2 kb sequence isolated from *Xanthomonas* IG-8 as well a selection of 'house keeping' genes and highly expressed gene (mostly kinases) from *Xanthomonas campestris* (accession no. AE012131) to establish any adaptation patterns between the genes (Fig. 3). The IG-8 ORF1 to ORF5 (which are up-stream of the *xmeRSA* genes) had codon usage patterns similar to the genes from *X. campestris* pv. *campestris* ATCC 33913 genome (accession no. AE012131). This indicated that these ORFs used similar codons as did genes from the *X. campestris* genome. In contrast, XmeRSA have a significantly different codon usage pattern from ORFs 1 to 5 and the *X. campestris* genes tested. Although these genes have different functions they show a codon usage similar to each other. Taken together, these data suggest that *xmeRSA* may have been recruited to IG-8 following a gene transfer event from another microorganism.

A more detailed comparison of the 9.2 kb sequence from IG-8 using *X. campestris* GENOMA BLAST revealed a possible insertion point for these *xmeRSA* genes in the *Xanthomonas* IG-8 genome (Fig. 4). The region 500 bp downstream of *xmeRSA* contains a number of tandem and two inverted

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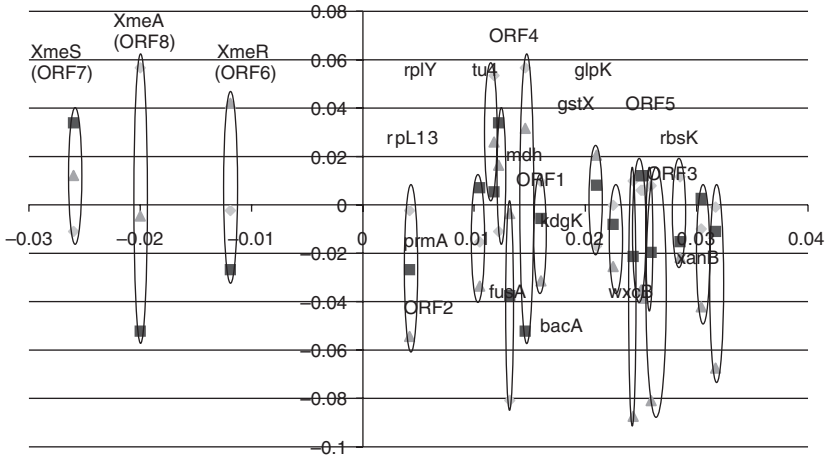

Fig. 3. Analysis of codon usage variation; Correspondence Analysis graph calculated using GCUA of genes from the *Xanthomonas* IG-8 copper resistance cluster compared with genes from *Xanthomonas campestris* ATCC 33913 (accession no. AE012131). *Note*: Correspondence analysis of the relative synonymous codon usage values for each gene in the copper resistant fragment was carried out using the GCUA program. The values are calculated by dividing the observed number of times that particular codon is used by the number of times it should be observed if codon usage for the amino acid was random.

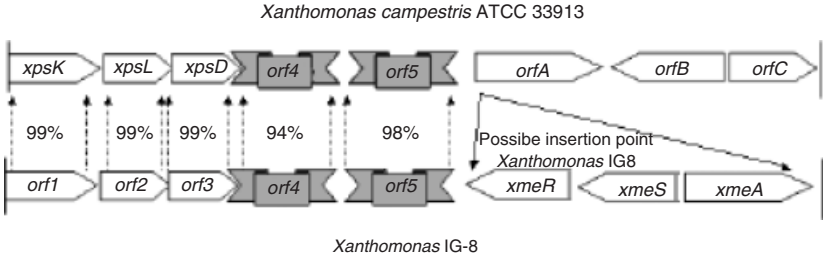

Fig. 4. Bioinformatic analysis of the probable IG-8 *xmeRSA* operon insertion site of *Xanthomonas campestris* (AE012131). *Note*: Dotted line show percentage homology between ORFs of *Xanthomonas* IG-8 and *Xanthomonas campestris* pv. *campestris (Xcc)* ATCC 33913 (http://cancer.lbi.ic.unicamp.br/xanthomonas/). Solid black lines show the possible insertion point of the gene cluster *xmeRSA*. Orf1 is 99% similar to XpsK of *Xcc*; Orf2 is 99% similar to XpsL of *Xcc*; Orf3 is 99% similar to XpsD precursor of *Xcc*; Orf4 is 94% similar to a hypothetical outer membrane protein of *Xcc*; Orf5 is 98% similar to a putative transcriptional regulator *Xcc*; OrfA is 98% similar to a putative transporter *Xanthomonas oryzae* pv. *oryzae*; OrfB is 98% similar to a putative siderophore receptor from *Xanthomonas oryzae* pv. *oryzae* and OrfC is an unknown protein found in *Xcc*. This information is adapted from the *Xanthomonas campestris* database of genome sequence *Xcc*. ATCC 33913

repeat sequences 25 bp long. This is suggestive of integration/recombination following HGT.

Discussion

Multidrug efflux systems play an important role in intrinsic as well as mutationally acquired MDR in Gram-negative bacteria (Schweizer, 2003). Recently, *S. maltophilia* was shown to possess efflux systems including SmeABC and SmeDEF (Alonso & Martinez, 1997; Alonso & Martinez, 2000). A partial homologue of the *smeABC* multidrug efflux systems of *S. maltophilia* was cloned using copper (2.5 mM) as a selection marker from *Xanthomonas* IG-8. The genes (*xmeRSA*), present in clone pITCCU5, encoded resistance to several antimicrobials but in addition encoded resistance to heavy metals including copper (4 mM), cadmium (1 mM) and nickel (3 mM), which has not been reported previously.

Targeted deletion of *xmeA* eliminates copper resistance in *Xanthomonas* IG-8; this confirms that this gene plays a major role in the copper-resistant phenotype of this strain. The 'knockout' mutants of *xmeR* and *xmeS* in IG-8 suggest that the presence of one or both of the putative two-component regulatory system is required for full resistance to copper. Transcriptional fusion analysis in *E. coli* revealed that the promoters in the *xmeRSA* region were induced in the presence of a number of antimicrobial agents tested.

The *xmeRSA* genes encode for resistance to these antimicrobials without an apparent designated efflux gene or outer membrane protein. CzcA has also demonstrated this feature in efflux of zinc alone (Goldberg *et al*., 1999). Homologues of *smeBC* of *S. maltophilia* were not identified up- or downstream of *xmeRSA*; however, they are possibly located elsewhere on the chromosome.

Heavy metal-polluted environments have been shown to provide a strong selective pressure for heavy metal resistance determinant transfer within soil systems (Hausner & Wuertz, 1999; Ryan *et al*., 2005). The possibility of the transfer, integration and subsequent expression of the *xmeRSA* genes from a donor strain to *Xanthomonas* IG-8 is highly plausible following bioinformatic analysis of the *X. campestris* genome (AE012131), the identification of a possible insertion point (Fig. 3) and codon usage analysis.

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The precise role of the *xmeA* gene and its relationship with *xmeRS* activity is yet to be determined. Elucidating the structures of the proteins and defining the interactions between them is the subject of future studies.

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