J Antimicrob Chemother 2013; **68**: 2498–2505
doi:10.1093/jac/dkt250 Advance Access publication 21 June 2013

Journal of Antimicrobial Chemotherapy

# Characterization of a major facilitator superfamily (MFS) tripartite efflux pump EmrCABsm from *Stenotrophomonas maltophilia*

**Yi-Wei Huang[1], Rouh-Mei Hu[2–4], Fang-Yeh Chu[5,6], Hui-Rung Lin[5] and Tsuey-Ching Yang[1]***

[1]*Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei 112, Taiwan;* [2]*Department of Biotechnology, Asia University, Taichung 413, Taiwan;* [3]*Department of Biomedical Informatics, Asia University, Taichung 413, Taiwan;* [4]*School of Chinese Medicine, China Medical University, Taichung 404, Taiwan;* [5]*Department of Clinical Pathology, Far Eastern Memorial Hospital, New Taipei City 220, Taiwan;* [6]*Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu 300, Taiwan*

*Corresponding author. 155 Section 2, Lie-Nong Street, Taipei 112, Taiwan, ROC. Tel: +886-2-28267289; Fax: +886-2-28264092; E-mail: tcyang@ym.edu.tw

*Received 19 April 2013; accepted 23 May 2013*

**Objectives:** To characterize the *emrRCABsm* operon of *Stenotrophomonas maltophilia*.

**Methods:** The presence of the *emrRCABsm* operon was verified by RT–PCR. The regulatory role of EmrRsm was investigated by Δ*emrRsm* mutant construction and promoter transcriptional fusion assay. A susceptibility test was employed to assess the substrate spectrum of the EmrCABsm efflux pump. The requirement for each component of the EmrCABsm pump was assessed by individual mutant construction and susceptibility testing. The expression of the *emrRCABsm* operon was evaluated by an induction assay, using different compounds as inducers.

**Results:** *emrRsm*, *emrCsm*, *emrAsm* and *emrBsm* formed a four-member operon that was negatively regulated by the MarR-type transcriptional regulator EmrRsm. The *emrRCABsm* operon was intrinsically poorly expressed and the EmrCAB pump favoured extrusion of the uncoupling agents carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and tetrachlorosalicylanilide (TCS), and the hydrophobic antibiotics nalidixic acid and erythromycin. However, the *emrRCABsm* operon could not be derepressed by CCCP, nalidixic acid, TCS, 2-chlorophenylhydrazine hydrochloride or salicylate, which are known to be possible inducers for MarR-type regulons. Each component of the EmrCABsm pump was apparently essential for pump function.

**Conclusions:** The EmrRsm-regulated EmrCABsm efflux pump is involved in the extrusion of hydrophobic compounds.

**Keywords:** bacteria, antibiotic resistance, efflux pump

## Introduction

*Stenotrophomonas maltophilia*, a non-fermentative Gram-negative bacillus, is considered to be an opportunistic agent. The treatment of *S. maltophilia* is greatly hindered by its innate antibiotic multiresistance, including resistance to β-lactams, aminoglycosides and quinolones.[1] Several mechanisms have been proposed to be involved in the resistance of *S. maltophilia*, including altered drug targets, decreased membrane permeability, antibiotic inactivating enzymes and active efflux pumps.[2] Among them, efflux pump systems are capable of providing resistance to a variety of compounds with unrelated structures, including antibiotics, toxins, detergents, disinfectants, organic solvents, metabolite waste products, dyes and fatty acids, and cause many failures in the treatment of *S. maltophilia* infection.[3]

To date, five efflux systems have been described: resistance nodulation cell division (RND) family, major facilitator superfamily (MFS), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE) and ATP-binding cassette (ABC).[4] Of these systems, the MFS transporter is the largest group and is present in all organisms.[5] In general, MFS transporters function as a single-component pump that transports small solutes across the membrane using electrochemical gradients.[6,7] In addition, some MFS transporters can associate with a membrane fusion protein and an outer membrane protein to form a tripartite complex spanning the inner and outer membranes of Gram-negative bacteria.[8–10] Several efflux systems of *S. maltophilia* have been characterized, including five RND-type efflux systems, SmeABC,[11] SmeDEF,[12] SmeVWX,[13] SmeIJK and SmeYZ,[14] and an ABC, SmrA.[15] However, MFS-type efflux pumps in *S. maltophilia* have not yet been reported.

© *The Author 2013. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com*



Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

The MarR-type transcriptional regulator was first reported in the *marRAB* operon of *E. coli*.[16] Many MarR-type transcriptional regulators have since been identified and have been shown to modulate the expression of genes involved in multiple antibiotic resistance.[17–19] The molecular mechanisms of MarR family transcriptional regulators have been elucidated. In general, MarR-type regulators work as repressors, inhibiting the expression of the genes they regulate. They exist as dimers and bind palindromic sequences within the regulated gene's promoters, resulting in transcriptional repression. As soon as the MarR-type regulator binds with certain inducers (ligands), the ligand-mediated attenuation of DNA binding causes the derepression of transcription.[20]

A typical representative of MFS-type efflux pump in *Escherichia coli*, *emrAB*, was first described in 1992.[8] Later, the expression of *E. coli emrAB* was proved to be regulated by a MarR-type regulator, *emrR*.[21] EmrAB is considered to form a tripartite efflux pump with the outer membrane protein TolC.[8] Overexpression of the *emrAB* of *E. coli* confers resistance to uncouplers of oxidative phosphorylation, such as 3-chlorophenylhydrazone (CCCP) and tetrachlorosalicylanilide (TCS), and unrelated hydrophobic compounds such as thiolactomycin and nalidixic acid.[8,22] In this study, the orthologue of *E. coli emrAB* in *S. maltophilia* – *emrRCABsm* – was identified by a homology search-based strategy, and its characteristics were further assessed.

## Materials and methods

### *Bacterial strains, plasmids and primers*

The bacterial strains, plasmids and primers used in this study, as well as their properties, are listed in Table 1. Unless noted otherwise, all cells were grown in Luria Bertani broth.

### *Construction of the emrRsm and emrRCABsm deletion mutants*

The deletion mutants were constructed by the allelic replacement strategy described previously.[23] Four PCR amplicons, labelled 1 to 4 in Figure 1, were obtained by PCR using primers EmrRN-F/EmrRN-R, EmrRC-F/EmrRC-R, EmrCC-F/EmrCC-R and Emr23N-F/Emr23N-R, respectively (Figure 1 and Table 1). These PCR amplicons contained the following segments: the 518 bp upstream of the *emrRsm* gene and partial N-terminus of the *emrRsm* gene for amplicon 1, the partial C-terminus of the *emrRsm* gene and partial N-terminus of the *emrCsm* gene for amplicon 2, the partial C-terminus of the *emrCsm* gene and partial N-terminus of the *emrAsm* gene for amplicon 3 and the partial C-terminus of the *emrBsm* gene for amplicon 4. Amplicons 1 and 2 were subsequently cloned into pEX18Tc, yielding plasmid pΔEmrR, which contained an internally deleted *emrRsm* gene. pΔEmrC, pΔEmrAB and pΔEmrRCAB were similarly construction from the assembly of amplicons 2/3, 3/4 and 1/4, respectively. Plasmids pΔEmrR, pΔEmrC, pΔEmrAB and pΔEmrRCAB were mobilized from the *E. coli* strain S17-1 to the wild-type KJ and KJEmr23, respectively, to delete the target gene(s) by homologous double crossover recombination. The correctness of mutants was verified by PCR analysis.

### *Construction of emrRCABsm-xylE single-copy fusion strain KJEmr23*

Strain KJEmr23 was constructed by incorporating a *xylE* gene downstream of the *emrBsm* gene (Figure 1) through homologous double crossover recombination, which occurred between the wild-type KJ chromosome and plasmid pEmr23 in *E. coli* S17-1. Plasmid pEmr23 was constructed as follows: a 794 bp DNA fragment containing the C-terminus of the *emrBsm* gene (labelled as amplicon 4 in Figure 1) and a 714 bp DNA fragment containing the downstream region of the *emrBsm* gene (labelled as amplicon 5 in Figure 1) were obtained by PCR using the primer sets of Emr23N-F/Emr23N-R and Emr23C-F/Emr23C-R (Table 1), respectively. The PCR amplicons were subsequently cloned into pEX18Tc, yielding plasmid pEmr23P. A *xylE* cassette was retrieved from pTxylE[24] and inserted into the KpnI site of pEmr23P, generating plasmid pEmr23. The orientation of *xylE* was confirmed by sequencing and was the same as that of the *emrBsm* gene. The conjugation between *S. maltophilia* KJ and *E. coli* S17-1(pEmr23) was performed as previously described.[23] The *xylE* gene of pEmr23 was inserted into the intergenic region downstream of the *emrBsm* gene, without disrupting any gene. This construction yielded an *emrRCABsm-xylE* operon in the KJEmr23 chromosome and the expression of the *xylE* gene represents the expression of the *emrRCABsm* operon. Accordingly, strain KJEmr23 was used to study *emrRCABsm* operon expression in single-copy fusion in this study.

### *Construction of the transcription fusion plasmids*

A 688 bp PCR amplicon, primed by EmrRN-F and EmrRN-R (labelled as amplicon 1 in Figure 1) and containing the predicted promoter region and the partial N-terminal portion of the *emrRsm* gene, was cloned into the pRK415, yielding plasmid p688EmrR. A *xylE* cassette retrieved from pTxylE[24] was inserted downstream of the PCR amplicon, forming a transcription fusion construct, pEmrR$_{xylE}$. To assess whether the region upstream of the *emrCsm* gene has the promoter activity, the 481 bp PCR amplicon (labelled as amplicon 2 in Figure 1) containing the 227 bp upstream of the *emrCsm* gene was used to construct pEmrC$_{xylE}$.

### *Catechol 2,3-dioxygenase (C23O) activity assay*

The activity of C23O, encoded by the *xylE* gene, was measured as described previously.[25] The hydrolysis rate of catechol was calculated by using 44 000 $M^{-1}$ $cm^{-1}$ as the extinction coefficient. One unit of enzyme activity (Uc) was defined as the amount of enzyme that converts 1 nmol of catechol/min. The specific activity was expressed as Uc/$A_{450}$.

### *Susceptibility testing*

MICs were determined as recommended by the CLSI.[26] After incubation of Mueller–Hinton agar plates at 37°C for 18 h, the MIC was determined by observing the lowest concentration of the antimicrobial at which bacterial growth was inhibited.

### *RT–PCR*

Total RNA was extracted from mid-logarithmic phase *S. maltophilia* culture using the PureLink™ Total RNA Purification System (Invitrogen, Carlsbad, CA, USA). Extracted RNA was further purified by treating with 1 U of RNase-free DNaseI to remove residual DNA (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the purified RNA was analysed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized using the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre Biotechnologies, Taiwan) with random primers. The resultant cDNA was subjected to PCR using the primers listed in Table 1. A PCR control of the same samples without reverse transcription was included to assure that there was no DNA contamination. Amplicons were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

### *Quantitative real-time PCR (qRT–PCR)*

cDNA was prepared as in the aforementioned protocol. qRT–PCR was then performed in the ABI Prism 7000 Sequence Detection System (Applied

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

**Table 1.** Bacterial strains, plasmids and primers used in this study

| Strain or plasmid | Genotype or properties | Reference |
|---|---|---|
| ***S. maltophilia*** | | |
| KJ | Wild-type, a clinical isolate from Taiwan | 24 |
| KJEmr23 | *S. maltophilia* KJ with a chromosomal *emrRCABsm-xylE* transcriptional fusion construct | this study |
| KJΔEmrR | *S. maltophilia* KJ *emrRsm* deletion mutant; Δ*emrRsm* | this study |
| KJΔREmr23 | *S. maltophilia* KJEmr23 *emrRsm* deletion mutant | this study |
| KJΔTolC | *S. maltophilia* KJ *tolCsm* deletion mutant; Δ*tolCsm* | 31 |
| KJΔTolCΔEmrR | *S. maltophilia* KJ *tolCsm* and *emrRsm* deletion mutant; Δ*tolCsm,* Δ*emrRsm* | this study |
| KJΔTolCΔEmrRCAB | *S. maltophilia* KJ *tolCsm* and *emrRCABsm* operon deletion mutant; Δ*tolCsm,* Δ*emrRCABsm* | this study |
| KJΔTolCΔEmrRΔEmrC | *S. maltophilia* KJ *tolCsm*, *emrRsm* and *emrCsm* deletion mutant; Δ*tolCsm,* Δ*emrRsm,* Δ*emrRsm* | this study |
| KJΔTolCΔEmrRΔEmrAB | *S. maltophilia* KJ *tolCsm*, *emrRsm* and *emrABsm* deletion mutant; Δ*tolCsm,* Δ*emrRsm,* Δ*emrAsm,* Δ*emrBsm* | this study |
| ***Escherichia coli*** | | |
| DH5α | F-φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* ($r_k^-$ $m_k^+$) *phoA supE44*$\lambda^-$ *thi-1 gyrA96 relA1* | invitrogen |
| S17-1 | λ pir+ mating strain | 40 |
| **Plasmids** | | |
| pEX18Tc | *sacB oriT*, $Tc^r$ | 41 |
| pRK415 | mobilizable broad-host-range plasmid cloning vector, RK2 origin; $Tc^r$ | 42 |
| pTxylE | plasmid containing the *xylE* gene, $Amp^r$ | 24 |
| pΔEmrR | pEX18Tc with an internally deleted *emrRsm* gene; $Tc^r$ | this study |
| pΔEmrC | pEX18Tc with an internally deleted *emrCsm* gene; $Tc^r$ | this study |
| pΔEmrAB | pEX18Tc with an internally deleted *emrABsm* gene; $Tc^r$ | this study |
| pΔEmrRCAB | pEX18Tc with an internally deleted *emrRCABsm* operon; $Tc^r$ | this study |
| pΔTolC | pEX18Tc with an internally deleted *tolCsm* gene; $Tc^r$ | 31 |
| pEmr23 | pEX18Tc with a *xylE* gene inserted into the intergenic region downstream *emrBsm* gene; $Tc^r$ | this study |
| $pEmrR_{xylE}$ | pRK415 with a 517 bp DNA fragment upstream from the *emrRsm* start codon and a $P_{emrRsm}$::*xylE* transcriptional fusion | this study |
| $pEmrC_{xylE}$ | pRK415 with a 227 bp DNA fragment upstream from the *emrCsm* start codon and a $P_{emrCsm}$::*xylE* transcriptional fusion | this study |
| **Primers** | | |
| Emr23N-F | 5′-TGGCCTACGCGTCGTTCTTCAGCGTGG-3′ | this study |
| Emr23N-R | 5′-AGGAAGCTTCAGTGTCCACCACCGGC-3′ | this study |
| Emr23C-F | 5′-TGCGGGTACCAATTCCGCCACC-3′ | this study |
| Emr23C-R | 5′-CGGAGCTCTGACCAACTACCTG-3′ | this study |
| EmrRN-F | 5′-CAGCATGCAGGGATCGGCGGGCA-3′ | this study |
| EmrRN-R | 5′-CCGTGCTCGAGGCCATGTGCGAGAG-3′ | this study |
| EmrRC-F | 5′-CAGGTACCCGCCTGCTGGACAAG-3′ | this study |
| EmrRC-R | 5′-CAGCGAATTCCACCAGTCCTGC-3′ | this study |
| EmrCC-F | 5′-CCTGCAGTCGCAGCAGGTGCAGTC-3′ | this study |
| EmrCC-R | 5′-GAAGTCTTCGCGCACGCGCTTCAG-3′ | this study |
| CQ-F | 5′-AGCAGCAGGCGGTGGATAC-3′ | this study |
| CQ-R | 5′-AGGAATCGGAATGGGAGGCG-3′ | this study |
| AQ-F | 5′-GCCGCATCCAGAGCCTTG-3′ | this study |
| AQ-R | 5′-GTGTCGAACACCGTGCCC-3′ | this study |
| BQ-F | 5′-AACGTCTCGCTGCCGACC-3′ | this study |
| BQ-R | 5′-ATGCCCATGCTCTGCGCC-3′ | this study |
| rDNA-F | 5′-GACCTTGCGCGATTGAATG-3′ | 23 |
| rDNA-R | 5′-CGGATCGTCGCCTTGGT-3′ | 23 |
| RC-F | 5′-ACGCTGCTGCTGTCCCTC-3′ | this study |
| RC-R | 5′-GTGCAGGCTGTCCACGTC-3′ | this study |
| CA-F | 5′-AGCAGGTGCAGTCCTCGGTG-3′ | this study |
| CA-R | 5′-CAGTGCGGCAAGCAGCAG-3′ | this study |
| AB-F | 5′-GCGACCAGAAGGGCGAAG-3′ | this study |
| AB-R | 5′-GCTGGAGCTTGTGCGGAC-3′ | this study |

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

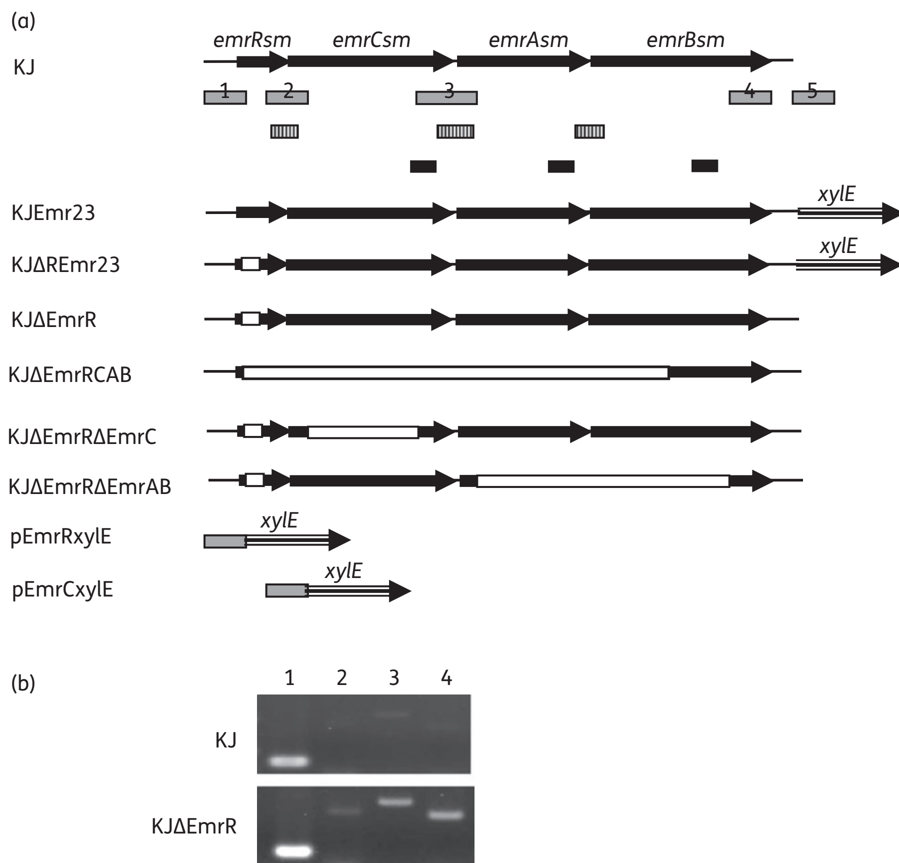


**Figure 1.** (a) Schematic organization of the *emrRCABsm* operon from *S. maltophilia* KJ and the structure of constructed mutants. The *emrRCABsm* operon contains genes for a MarR-type transcriptional regulator (*emrRsm*), an outer membrane protein (*emrCsm*), a membrane fusion protein (*emrAsm*) and an MFS transporter (*emrBsm*). The solid grey bars (labelled 1–5) indicate the PCR products that were used for the plasmid construction. The bars with vertical lines indicate the products of RT–PCR. The solid black bars indicate the products of qRT–PCR. Solid arrows represent open reading frames and the direction of transcription. The empty bars represent the deleted region for each mutant construct. (b) RT–PCR assay. Agarose gels of RT–PCR products of strains KJ and KJΔEmrR. Lane 1: 16S RNA transcript; Lane 2: transcript of the intergenic region of the *emrRsm* and *emrCsm* genes; Lane 3: transcript of the intergenic region of the *emrCsm* and *emrAsm* genes; Lane 4: transcript of the intergenic region of the *emrAsm* and *emrBsm* genes.

Biosystems) using the Smart Quant Green Master Mix (Protech Technology Enterprise) according to the manufacturers' protocols. Amplification of 16S rDNA was used as the endogenous reference for relative quantification. Individual target genes were amplified with the primers listed in Table 1. The $\Delta\Delta Ct$ method[27] was used to quantify the relative amounts of mRNA from each gene of interest. Each experiment was performed three times.

### *Nucleotide sequence accession numbers*

The nucleotide sequences of *S. maltophilia* KJ have been deposited in GenBank under accession no. KC017848 for partial *emrRsm* and *emrCsm* genes (PCR amplicons 1 and 2 in this study), KC017846 for partial *emrBsm* gene (PCR amplicon 4 in this study) and KC017847 for partial LysR-type transcriptional regulator (PCR amplicon 5 in this study).

## Results

### *Smlt1527–Smlt1530 genes cluster in S. maltophilia K279a*

A BLAST analysis of the *S. maltophilia* K279a genome using the EmrA and EmrB proteins of *E. coli* as queries revealed Smlt1529 and Smlt1530 to have significant homology. Smlt1529 and Smlt1530 displayed 43% and 49% protein identity with the EmrA and EmrB proteins of *E. coli*, respectively. *smlt1529*, *smlt1530* and two predicted upstream genes (*smlt1527* and *smlt1528*) likely constituted a four-member operon, because of a 4 bp nucleotide overlapping for *smlt1527* and *smlt1528* predicted genes as well as short intergenic regions for *smlt1528–smlt1529* and *smlt1529–smlt1530* predicted genes. The protein encoded by *smlt1527* was a MarR-type transcriptional regulator, which was 30% identical to the EmrR of *E. coli*.[20] The *smlt1528* determinant encoded a 495 amino acid outer membrane protein. By analysing the 528 amino acid Smlt1530 protein, the conserved motif A and B of the MFS-type transporters[28] can be identified from residues 87–97 and 171–176. The proteins encoded by *smlt1528, smlt1529* and *smlt1530* may therefore consist of a tripartite MFS-type efflux pump.

The *smlt1527–smlt1530* cluster displayed significant similarity to the reported *emrRAB* and *emrYK* systems of *E. coli*,[8,9,21] the *vceAB* system of *Vibrio cholerae*[10] and the *farRAB* system of *Neisseria gonorrhoeae*[29,30] (Table 2).

### *Involvement of emrRsm in the expression of emrCABsm*

The homologues of *smlt1527*, *smlt1528*, *smlt1529* and *smlt1530* in *S. maltophilia* KJ are denoted as *emrRsm*, *emrCsm, emrAsm*

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

and *emrBsm* (*emr* homologues in *S. maltophilia*), respectively (Figure 1a). EmrR is a negative regulator of the EmrAB pump in the *E. coli* system.[20] Whether EmrRsm could regulate the EmrCABsm pump in a similar manner in *S. maltophilia* was therefore investigated. A Δ*emrRsm* allele was introduced into KJ, resulting in a mutant KJΔEmrR. The *emrCsm*, *emrAsm* and *emrBsm* transcripts of KJ and KJΔEmrR were determined using qRT–PCR with primer pairs within *emrCsm* (primers CQ-F/CQ-R), *emrAsm* (primers AQ-F/AQ-R) and *emrBsm* (primers BQ-F/BQ-R) (Figure 1a and Table 1). Compared with KJ, ∼8.3±1.2-fold, 29±3.3-fold and 13±2.5-fold increases in the transcripts of *emrCsm*, *emrAsm* and *emrBsm*, respectively, were observed in KJΔEmrR, indicating that *emrRsm* acts as a repressor of the expression of the *emrCsm, emrAsm and emrBsm* genes.

## Transcriptional analysis of the emrRCABsm gene cluster

To verify the presence of the *emrRCABsm* operon, the transcript of the *emrRCABsm* gene cluster was analysed by RT–PCR. The transcripts encompassing two flanking genes, *emrRCsm*, *emrCAsm*, and *emrABsm* from the wild-type KJ and mutant KJΔEmrR were obtained by amplification using primers RC-F/RC-R, CA-F/CA-R and AB-F/AB-R, respectively (Figure 1a and Table 1). Products of the expected size, which could only be produced if *emrRsm*, *emrCsm*, *emrAsm* and *emrBsm* were cotranscribed, were clearly obtained from KJΔEmrR (Figure 1b); however, few transcripts were obtained from the wild-type KJ. These results indicate that the *emrRCABsm* genes were cotranscribed and the *emrRCABsm* operon was intrinsically feebly expressed.

For the studies of *emrRCABsm* operon expression, a chromosomal *emrRCABsm-xylE* transcriptional fusion strain, KJEmr23, was constructed (see the Materials and methods section). Strain KJEmr23 is genetically identical to strain KJ, except that KJEmr23 is equipped with an *emrRCABsm-xylE* transcriptional fusion instead of an *emrRCABsm* operon. Mutant KJΔREmr23 (an *emrRsm* isogenic mutant of KJEmr23) was also prepared. The transcripts of *emrCsm*, *emrAsm* and *emrBsm* produced in KJEmr23 and KJΔREmr23 were determined using qRT–PCR. The transcripts of *emrCsm*, *emrAsm* and *emrBsm* in KJΔREmr23 were, respectively, 7.6±2.1-fold, 32±4.1-fold and 11.4±2.8-fold higher than those in KJEmr23, indicating that the insertion of a *xylE* gene into the intergenic region downstream of the *emrBsm* gene did not affect the operon expression. In agreement with the results from qRT–PCR, Table 3 also shows that the C23O activity significantly increased in KJΔREmr23 versus KJEmr23, further confirming that EmrRsm acts as a repressor of the *emrRCABsm* operon.

To identify the possible promoter(s) for the *emrRCABsm* cluster, the 518 bp and 227 bp fragments upstream of the *emrRsm* and *emrCsm* genes were cloned in the *xylE*-fusion constructs, yielding the transcriptional fusion constructs $pEmrR_{xylE}$ and $pEmrC_{xylE}$. These constructs were transformed into strains KJ and KJΔEmrR. The C23O activities in cell lysates of KJ($pEmrR_{xylE}$), KJΔEmrR($pEmrR_{xylE}$), KJ($pEmrC_{xylE}$) and KJΔEmrR($pEmrC_{xylE}$) were determined. Table 3 demonstrates that the C23O activity of KJΔEmrR($pEmrR_{xylE}$) increased >7-fold compared with that of KJ($pEmrR_{xylE}$); however, no significant C23O activity was detected in KJ($pEmrC_{xylE}$) and KJΔEmrR($pEmrC_{xylE}$). This indicates the absence of a promoter immediately upstream of *emrCsm*. Accordingly, the *emrRCABsm* operon was driven by the promoter upstream of the *emrRsm* gene and this promoter was negatively regulated by EmrRsm.

## Substrate spectrum of the EmrCABsm pump

Bearing in mind that the *emrRCABsm* operon is intrinsically poorly expressed and that *emrRsm* plays a negative regulatory role in the expression of the *emrRCABsm* operon, we compared the susceptibility toward different compounds between strains KJ and KJΔEmrR to elucidate the substrate spectrum of the EmrCABsm pump.

KJ and KJΔEmrR exhibited the same MICs of antimicrobials examined, except for nalidixic acid and CCCP (Table 4). Compared with the wild-type KJ, mutant KJΔEmrR had a 2-fold increase in the MICs of nalidixic acid and CCCP in multiple reproducible experiments. In our recent study, *tolCsm* was shown to be involved in the intrinsic resistance of *S. maltophilia* KJ to a wide range of antibiotics, including nalidixic acid and CCCP.[31] Therefore, we considered that TolCsm-associated efflux pumps may mask the contribution of the EmrCABsm efflux pump. To test this possibility, a Δ*tolCsm* allele was introduced into KJΔEmrR, yielding mutant KJΔTolC-ΔEmrR. KJΔTolC (a *tolCsm* isogenic mutant reported in our previous study) was also included for comparison (Table 4). Compared with KJΔTolC, KJΔTolCΔEmrR notably exhibited increased resistance to nalidixic acid (8-fold MIC), CCCP (4-fold MIC), TCS (4-fold MIC) and erythromycin (2-fold MIC), further emphasizing the role of the EmrCABsm pump in the extrusion of hydrophobic compounds (Table 4).

To further confirm the role of the EmrCABsm pump in substrate efflux, the EmrCABsm pump of KJΔTolCΔEmrR was inactivated by

**Table 2.** Protein identities and similarities of the EmrRCABsm cluster and its homologues

| Bacterium | Protein [identity/similarity (%)] | | | |
|---|---|---|---|---|
| *S. maltophilia* K279a | Smlt1527 (100/100) | Smlt1528 (100/100) | Smlt1529 (100/100) | Smlt1530 (100/100) |
| *E. coli* | EmrR (30/48) | | EmrA (43/63) | EmrB (49/68) |
| *E. coli* | | | EmrK (44/62) | EmrY (45/65) |
| *V. cholerae* | | | VceA (40/58) | VceB (43/62) |
| *N. gonorrhoeae* | | | FarA (49/65) | FarB (46/66) |

**Table 3.** Transcriptional analysis for the *emrRCABsm* cluster

| | C23O activity ($Uc^a/A_{450}$) |
|---|---|
| KJEmr23 | 16±1.7 |
| KJΔREmr23 | 254±30 |
| KJ($pEmrR_{xylE}$) | 69±8 |
| KJΔEmrR($pEmrR_{xylE}$) | 489±52 |
| KJ($pEmrC_{xylE}$) | 8±1.1 |
| KJΔEmrR($pEmrC_{xylE}$) | 7±0.9 |

Results are expressed as the mean±SD of three independent determinations.
[a]One unit of C23O activity is defined as the hydrolysis of 1 nmol of catechol/min.

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

**Table 4.** MICs of compounds for *S. maltophilia* KJ and its derived mutants

| | MIC (mg/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | KJ | KJΔEmrR | KJΔTolC | KJΔTolCΔEmrR | KJΔTolCΔEmr RCAB | KJΔTolCΔEmrRΔEmrC | KJΔTolCΔEmrRΔdEmrAB |
| Chloramphenicol | 8 | 8 | 4 | 8 | 8 | 8 | 8 |
| **Quinolones** | | | | | | | |
| nalidixic acid | 8 | 16 | 2 | 16 | 2 | 2 | 2 |
| norfloxacin | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| **Tetracyclines** | | | | | | | |
| tetracycline | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| doxycycline | 1 | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| **Aminoglycosides** | | | | | | | |
| kanamycin | 256 | 256 | 16 | 16 | 16 | 16 | 16 |
| gentamicin | 1024 | 1024 | 8 | 8 | 8 | 8 | 8 |
| amikacin | 1024 | 1024 | 16 | 16 | 16 | 16 | 16 |
| **Macrolides** | | | | | | | |
| erythromycin | 64 | 64 | 32 | 64 | 32 | 32 | 32 |
| **Others** | | | | | | | |
| fusaric acid | 512 | 512 | 256 | 256 | 256 | 256 | 256 |
| menadione | 64 | 64 | 32 | 32 | 32 | 32 | 32 |
| paraquat | 1024 | 1024 | 512 | 512 | 512 | 512 | 512 |
| CCCP | 16 | 32 | 8 | 32 | 8 | 8 | 8 |
| plumbagin | 32 | 32 | 8 | 8 | 8 | 8 | 8 |
| CHH | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| TCS | 8 | 8 | 2 | 8 | 2 | 2 | 2 |
| Crystal violet | 8 | 8 | 4 | 4 | 4 | 4 | 4 |
| proflavine | 512 | 512 | 512 | 512 | 512 | 512 | 512 |
| acriflavine | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 |
| palmitic acid | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 |
| oleic acid | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 |
| capric acid | 512 | 512 | 512 | 512 | 512 | 512 | 512 |
| Triton X-100 | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 | >4096 |

deletion. The resultant strain, KJΔTolCΔEmrRCBA, showed restored susceptibility to nalidixic acid, CCCP, TCS and erythromycin to the level of strain KJΔTolC (Table 4), indicating that overexpression of the EmrCABsm pump in a Δ*tolCsm* background made a contribution to resistance to nalidixic acid, CCCP, TCS and erythromycin.

### *Evaluation of the requirement for EmrCsm and EmrABsm for pump function*

To assess whether EmrCsm and EmrABsm are required for the function of the EmrCABsm pump, the Δ*emrCsm* and Δ*emrABsm* alleles were introduced into the chromosome of KJΔTolCΔEmrR, yielding mutants KJΔTolCΔEmrRΔEmrC and KJΔTolCΔEmrRΔEmrAB, respectively. The expression of *emrAsm* and *emrBsm* of strain KJΔTolCΔEmrRΔEmrC was evaluated by qRT–PCR, indicating that inactivation of *emrCsm* makes no polar effect on the expression of the downstream *emrAsm* and *emrBsm* genes. Table 4 shows that inactivation of either *emrCsm* or *emrABsm* of KJΔTolCΔEmrR restored drug susceptibility to the same level as that of KJΔTolCΔEmrRCAB, signifying that EmrCsm and EmrAB are essential for the function of the EmrCABsm pump.

### *Induction survey of the emrRCABsm operon by multiple compounds*

Since the *emrRCABsm* operon is negatively regulated by a MarR-type regulator, the inducibility of the *emrRCABsm* operon by different compounds known to alter the activity of the MarR-type regulator[21,32,33] was investigated. The expressed C23O activity of KJEmr23 was determined in the absence or presence of different compounds. Each compound was tested at a subinhibitory concentration. The induction periods were set at 6 h and 24 h, respectively, to represent the induction potential of log phase and stationary phase bacterial culture. The compounds tested included tetracycline, kanamycin, chloramphenicol, nalidixic acid, erythromycin, salicylate, CCCP, 2-chlorophenylhydrazine (CHH) and TCS. The C23O activity of KJEmr23 remained constant upon the addition of the



Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

compounds tested, either for log phase or for stationary phase bacterial cultures.

## Discussion

MFS-type efflux pumps have been extensively described; however, this is the first time the system has been documented for *S. maltophilia*. The EmrCABsm pump is regulated by a MarR-type transcriptional regulator, EmrRsm. The homologues of the *S. maltophilia* EmrCABsm pump have also been revealed in other microorganisms, including EmrAB of *E. coli*,[8] EmrKY of *E. coli*,[9] VceAB of *Vibro cholerae*[10] and FarAB of *Neisseria gonorrhoeae*.[29] Most of these pumps favour extrusion of substances of high hydrophobicity, and they appear to form a tripartite pump, although the outer membrane components for some pumps have not yet been identified.[9,10] The outer membrane of Gram-negative bacteria is the major selective permeability barrier and displays low permeability to the entrance of hydrophobic substances. However, hydrophobic substances can cross the Gram-negative bacterial inner membrane with little impediment. Therefore, to directly extrude the noxious hydrophobic substances from the cytoplasm to the extracellular environment should be a more efficient mode of protecting bacteria from the threat of noxious hydrophobic compounds. A tripartite efflux pump, forming a channel spanning the entire membrane, is a device that meets this requirement. The majority of MFS transporters reported so far function as single-component transporters, such as CraA of *Acinetobacter baumannii*,[6] LmrS of *Staphylococcus aureus*[7] and EmrD-3 of *Vibrio cholerae* O395,[34] which catalyse the efflux of drugs from the cytoplasm into the periplasmic space. The MFS-type tripartite efflux pumps favouring the extrusion of hydrophobic substances are an exquisite device for increasing bacterial fitness.

The *emrKY* operon of *E. coli* displays growth phase-dependent induction in the presence of tetracycline, chloramphenicol or salicylate.[9] EmrK and EmrY display 44% and 45% identity, respectively, to EmrAsm and EmrBsm; however, no similar phenotype has been observed in the expression of the *emrCABsm* operon. The *emrRCABsm* operon is not induced in stationary phase cells upon challenge with tetracycline, kanamycin, chloramphenicol, nalidixic acid, erythromycin, salicylate, CCCP, CHH and TCS. Accordingly, the native physiological functions of the EmrCABsm pump may be less involved in growth phase-dependent drug tolerance.

A generally accepted mechanism for the function of a MarR-type regulator is: a basal-level expressed MarR-type regulator interacts with the promoter of MarR-regulated gene(s) and results in the repression of MarR-regulated gene(s) in the absence of inducers. In the presence of inducer, the inducer may bind to the MarR-type regulator, attenuate the affinity of the MarR-type regulator and promoter, and induce the expression of MarR-regulated gene(s).[20,35] The MarR-responsive inducers are generally anionic lipophilic and phenolic compounds.[32,36] For instance, the *emrRAB* operon of *E. coli* can be induced by CCCP, TCS, nalidixic acid, salicylate and 2,4-dinitrophenol.[21] Salicylate, plumbagin, 2,4-dinitrophenol and menadione can induce the expression of the *marRAB* operon of *E. coli*.[33] Interestingly, the expression of the *emrRCABsm* operon does not increase in the presence of tetracycline, kanamycin, chloramphenicol, nalidixic acid, erythromycin, salicylate, CCCP, TCS and CHH, although nalidixic acid, erythromycin, CCCP and TCS are the known substrates of the EmrCABsm pump.

Bacterial efflux pumps export not only antibiotics but also antimicrobial agents from the host cells or the natural environment.[37] *S. maltophilia* is ubiquitous in nature, including soil, water and plants, and can opportunistically cause human infection. Plant roots, microorganism secretions, plant exudates and host-derived compounds may contain a large number of antimicrobial agents that threaten bacterial survival. It has been proposed that bacterial multidrug resistance pumps play an ecological role in the soil biosphere, including in bacterial and plant interactions.[38] In our recent report, a fusaric acid extrusion efflux pump, FusABC, plays such a role in intermicrobial competition.[39] The EmrCABsm pump, like the EmrAB pump of *E. coli*,[8] the VceAB pump of *V. cholerae*[10] and the FarAB pump of *N. gonorrhoeae*,[29] favours the extrusion of hydrophobic substrates; nevertheless, the known hydrophobic antimicrobials, such as nalidixic acid, erythromycin, CCCP and TCS, cannot induce the expression of *emrCABsm* operon. This finding has led to the suggestion that the EmrCABsm pump may have a physiological role in evading naturally produced molecules from the environment or host-derived compounds, rather than in antimicrobial extrusion, allowing *S. maltophilia* to survive in its ecological niche.

## Funding

This work was supported by the National Science Council (NSC 101-2320-B-010-053-MY3).

## Transparency declarations

None to declare.

## References

**1** Brooke JS. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 2012; **25**: 2–41.

**2** Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. *Cell* 2007; **128**: 1037–50.

**3** Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. *Drugs* 2009; **69**: 1555–623.

**4** Piddock LJV. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* 2006; **19**: 382–402.

**5** Saidijam M, Benedetti G, Ren Q *et al*. Microbial drug efflux proteins of the major facilitator superfamily. *Curr Drug Targets* 2006; **7**: 793–811.

**6** Roca I, Marti S, Espinal P *et al*. CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2009; **53**: 4013–4.

**7** Floyd JL, Smith KP, Kumar SH *et al*. LmrS is a multidrug efflux pump of the major facilitator superfamily from *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2010; **54**: 5406–12.

**8** Lomovskaya O, Lewis K. *Emr*, an *Escherichia coli* locus for multidrug resistance. *Proc Natl Acad Sci USA* 1992; **89**: 8938–42.

**9** Tanabe H, Yamasaki K, Furue M *et al*. Growth phase-dependent transcription of *emrKY*, a homolog of multidrug efflux *emrAB* genes of *Escherichia coli* is induced by tetracycline. *J Gen Appl Microbiol* 1997; **43**: 257–63.

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024

**10** Colmer JA, Fralick JA, Hamood AN. Isolation and characterization of a putative multidrug resistance pump from *Vibrio cholera*. *Mol Microbiol* 1998; **27**: 63–72.

**11** Li XZ, Zhang L, Poole K. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2002; **46**: 333–43.

**12** Alonso A, Martínez JL. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2000; **44**: 3079–86.

**13** Chen CH, Huang CC, Chung TC *et al*. Contribution of resistance-nodulation-division efflux pump operon *smeU1-V-W-U2-X* to multidrug resistance of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2011; **55**: 5826–33.

**14** Gould VC, Okazaki A, Avison MB. Coordinate hyperproduction of SmeZ and SmeJK efflux pumps extends drug resistance in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2013; **57**: 655–7.

**15** Al-Hamad A, Upton M, Burnie J. Molecular cloning and characterization of SmrA, a novel ABC multidrug efflux pump from *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 2009; **64**: 731–4.

**16** Cohen SP, Hächler H, Levy SB. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *J Bacteriol* 1993; **175**: 1484–92.

**17** Oethinger M, Podglajen I, Kern WV *et al*. Overexpression of the *marA* or *soxS* regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* 1998; **42**: 2089–94.

**18** Kaatz GW, McAleese F, Seo SM. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. *Antimicrob Agents Chemother* 2005; **49**: 1857–64.

**19** Ruiz C, Levy SB. Many chromosomal genes modulate MarA-mediated multidrug resistance in *Escherichia coli*. *Antimicrob Agents Chemother* 2010; **54**: 2125–34.

**20** Perera IC, Grove A. Molecular mechanisms of ligand-mediated attenuation of DNA binding by MarR family transcriptional regulators. *J Mol Cell Biol 2010*; **2**: 243–54.

**21** Lomovskaya O, Lewis K, Matin A. EmrR is a negative regulator of *Escherichia coli* multidrug resistance pump EmrAB. *J Bacteriol* 1995; **177**: 2328–34.

**22** Furukawa H, Tsay JT, Jackowski S *et al*. Thiolactomycin resistance in *Escherichia coli* is associated with the multidrug resistance efflux pump encoded by *emrAB*. *J Bacteriol* 1993; **175**: 3723–9.

**23** Yang TC, Huang YW, Hu RM *et al*. AmpD$_I$ is involved in expression of the chromosomal L1 and L2 β-lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother 2009*; **53**: 2902–7.

**24** Hu RM, Huang KJ, Wu LT *et al*. Induction of L1 and L2 β-lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2008; **52**: 1198–200.

**25** Huang YW, Lin CW, Hu RM *et al*. AmpN-AmpG operon is essential for expression of L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 2010; **54**: 2583–9.

**26** Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20*. CLSI, Wayne, PA, 2010.

**27** Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta Delta C(T)) method. *Methods* 2001; **25**: 402–8.

**28** Rouch DA, Cram DS, DiBerardino D *et al*. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus:* common ancestry with tetracycline and sugar transport proteins. *Mol Microbiol* 1990; **4**: 2051–62.

**29** Lee EH, Shafer WM. The *farAB*-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. *Mol Microbiol* 1999; **33**: 839–45.

**30** Lee EH, Rouquette-Loughlin C, Folster JP *et al*. FarR regulates the FarAB-encoded efflux pump of *Neisseria gonorrhoeae* via an MtrR regulatory mechanism. *J Bacteriol* 2003; **185**: 7145–52.

**31** Huang YW, Hu RM, Yang TC. Role of the *pcm-tolCsm* operon in multidrug resistance of *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 2013; **68**: 1987–93.

**32** Seoane AS, Levy SB. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*. *J Bacteriol* 1995; **177**: 3414–9.

**33** Alekshun MN, Levy SB. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals *in vitro*. *J Bacteriol* 1999; **181**: 4669–72.

**34** Smith KP, Kumar S, Varela MF. Identification, cloning, and functional characterization of EmrD-3, a putative multidrug efflux pump of the major facilitator superfamily from *Vibrio cholera* O359. *Arch Microbiol* 2009; **191**: 903–11.

**35** Wilkinson SP, Grove A. Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins. *Curr Issues Mol Biol* 2006; **8**: 51–62.

**36** Sulavik MC, Gambino LF, Miller PF. The MarR repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*: prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. *Mol Med* 1995; **1**: 436–46.

**37** Ryan RP, Monchy S, Cardinale M *et al*. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol* 2009; **7**: 514–25.

**38** Martinez JL, Sánchez MB, Martínez-Solano L *et al*. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* 2009; **33**: 430–49.

**39** Hu RM, Liao ST, Huang CC *et al*. An inducible fusaric acid tripartite efflux pump contributes to the fusaric acid resistance in *Stenotrophomonas maltophilia*. *PLoS One* 2012; **7**: e51053.

**40** Simon R, Priefer U, Puhler AA. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* 1983; **1**: 784–90.

**41** Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ *et al*. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 1998; **212**: 77–86.

**42** Keen NT, Tamaki S, Kobayashi D *et al*. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 1988; **70**: 191–97.

Downloaded from https://academic.oup.com/jac/article/68/11/2498/834640 by guest on 24 April 2024