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Novel tetracycline resistance gene *tet*(65) located on a multi-resistance *Corynebacterium* plasmid

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Background: *Corynebacterium* (*C.*) sp. 22KM0430 related to *C. oculi* and isolated from a dog exhibited resistance to tetracycline, and its WGS analysis revealed a putative resistance gene on a 35 562-bp plasmid also harbouring the MLS_B resistance gene *erm*(X).

Objectives: To characterize the novel tetracycline resistance gene *tet*(65) and demonstrate its functionality by expression in *C. glutamicum* and *Escherichia coli* and plasmid curing of the host strain.

Methods: *tet*(65) was cloned with and without its repressor *tetR*(65) and expressed in *C. glutamicum* DSM20300 and *E. coli* DH5α. Plasmid was cured by non-selective passages. Minimal inhibitory concentrations (MICs) of tetracyclines were determined according to CLSI guidelines. Association of *tet*(65) with efflux was shown by the addition of reserpine to MIC assays. Phylogenetic position and transmembrane structure of Tet(65) were analysed using MEGA11 and DeepTMHMM.

Results: Tet(65) shows 73% amino acid identity with the closest related Tet(Z), contains 12 transmembrane domains and is structurally related to the Major Facilitator Superfamily. The tetracycline MICs decreased in the plasmid-cured strain and increased when *tet*(65) was expressed in *C. glutamicum* and in *E. coli*. The MICs of tetracycline decreased in the presence of reserpine indicating that *tet*(65) functions as an efflux pump. A GenBank search also identified *tet*(65) in *C. diphtheriae* and *Brevibacterium* (*B.*) *casei* and *B. luteolum*.

Conclusions: A novel tetracycline efflux gene *tet*(65) was identified in a *C. oculi* related species and was also present in the human pathogen *C. diphtheriae* and in *Brevibacterium* species indicating broader potential for dissemination.

Introduction

Several species of corynebacteria are major pathogens in mammals including *Corynebacterium diphtheriae*, causing diphtheria in humans and *C. pseudotuberculosis* causing pseudotuberculosis in small ruminants.[1] Recently, *C. oculi* was described as a novel species associated with human ocular infections.[2] We isolated two strains of a closely related species from an ocular specimen of a dog with keratitis and a dog urine sample.[3] The isolate from urine harboured the 35 562-bp plasmid p22KM0430 (GenBank accession no. CP123906) containing the 23S rRNA methylase gene *erm*(X) conferring resistance to macrolides, lincosamides and streptogramin B (MLS_B) and a putative tetracycline efflux pump annotated by PGAP as encoding a putative Tet(A)/Tet(B)/Tet(C) family tetracycline efflux Major Facilitator Superfamily (MFS) transporter.[3] Both erythromycin and tetracycline are used for treatment of ocular infection in veterinary medicine (https://www.msdvetmanual.com/pharmacology/systemic-pharmacotherapeutics-of-the-eye/antimicrobial-use-in-animals, 06.11.2023). Tetracycline is also used for topical ocular treatment in humans.[4]

Tetracycline resistance is widespread among both Gram-positive and Gram-negative bacteria.[5] Tetracycline resistance genes can be divided into three groups according to mechanism, including energy-dependent efflux proteins, ribosomal protection proteins and tetracycline inactivating enzymes.[5] So far 36 efflux genes, 13 ribosomal protection genes and 13 enzymatic genes have been described (http://faculty.washington.edu/marilynr/, accessed 21 July 2023). In the genus *Corynebacterium*, five different *tet* genes have been found, two for ribosomal protection (*tet*(M), *tet*(W)) and three for efflux (*tet*(Z), *tet*(33) and *tet*(39)). Tetracycline efflux proteins can be subdivided into six groups with the three found in corynebacteria

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Table 1. MICs of tetracyclines for bacterial strains and transformants

Host strain	Plasmid	*tet* genes present	MIC (in mg/L) of				
			TET	DOX	MIN	TGC	TET with 40 mg/L RES
Corynebacterium sp. 22KM0430	none	none	1	0.5	0.25	≤0.25	≤1
Corynebacterium sp. 22KM0430	p22KM0430[3]	*tet*(65) with its regulator *tetR*(65)	64	8	0.5	≤0.25	32
Corynebacterium sp. 22KM0430	ptetA65	*tet*(65)	16	1	0.25	≤0.25	8
C. glutamicum DSM20300	none	none	1	1	0.5	≤0.25	≤1
C. glutamicum DSM20300	pPBEx2[11]	none	1	0.5	0.5	≤0.25	≤1
C. glutamicum DSM20300	p22KM0430[3]	*tet*(65) with its regulator *tetR*(65)	8	1	0.25	≤0.25	2
C. glutamicum DSM20300	ptetAR65	*tet*(65) with its regulator *tetR*(65)	16	2	0.5	≤0.25	8
C. glutamicum DSM20300	ptetA65	*tet*(65)	64	4	0.5	≤0.25	16
E. coli DH5α	none	none	2	2	2	≤0.25	2
E. coli DH5α	ptetAR65	*tet*(65) with its regulator *tetR*(65)	16	2	1	≤0.25	8
E. coli DH5α	ptetA65	*tet*(65)	16	2	1	≤0.25	8

For each host strain, MICs are shown for the plasmid free version and the generated transformants. The effect of the efflux pump inhibitor reserpine is shown in the last column.
TET, tetracycline; DOX, doxycycline; MIN, minocycline; TGC, tigecycline; RES, resperpine.

belonging to group one.[6] The Tet proteins of group one contain 12 transmembrane domains and are regulated by an associated repressor encoded by *tetR*, which is in inverse orientation to the *tetA* gene with an overlapping promotor operator region.[7] These genes are frequently found on transposons or plasmids.[6]

In this study we characterize the novel, plasmid borne tetracycline efflux gene officially designated *tet*(65) (https://faculty.washington.edu/marilynr/) following the nomenclature for new tetracycline resistance determinants.[8]

Material and methods

Bioinformatic analysis

Transmembrane domains of Tet(65) were predicted using by DeepTMHMM v.1.0.24 (https://dtu.biolib.com/DeepTMHMM). Motif A and motif C, typical of the MFS,[9] were annotated manually using Geneious Prime® v.2019.2.3.

Comparative analysis of Tet(65) with tetracycline efflux reference sequences obtained from GenBank and construction of the phylogenetic tree were performed using the Maximum Likelihood method and JTT matrix-based model using MEGA11.[10] Promoters for *tet*(65) and its regulator *tetR*(65) were annotated using BPROM (http://www.softberry.com).

BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect *tet*(65) in other sequences present in GenBank. The *tet*(65) encoding regions were extracted and compared to p22KM0430 using clinker v.0.0.27.

Cloning and expression of tet*(65)*

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid p22KM0430 (GenBank accession no. CP123906) was extracted from *Corynebacterium* sp. 22KM0430 using Peqlab Plasmid Miniprep Kit applying the protocol for low copy plasmids. Vector pPBEx2[11] was used as PCR template backbone for cloning experiments. Curing of plasmid p22KM0430 from strain 22KM0430 was performed by passaging the culture on sheep blood agar at 37°C under 5% CO_2 every 48 h until the plasmid was no longer detected by PCR for *erm*(X) as previously described.[3] Plasmid-cured *Corynebacterium* sp. 22KM0430, *C. glutamicum* DSM20300 and *E. coli* DH5α were used as recipients. Competent cells of *C. glutamicum* for electroporation were prepared following the optimized protocol with NCM medium.[12] Competent cells of the plasmid-cured strain 22KM0430 were prepared as for *C. glutamicum* except that the brain heart infusion broth was supplemented with 1% Tween 80.

Plasmids ptetAR65 and ptetA65 (Figure S1, available as Supplementary data at *JAC* Online) were constructed by Gibson assembly[13] using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs). Primers were designed with Geneious Prime® v.2019.2.3 (Figure S1). A 4934-bp segment of pPBEx2 containing *oriE.c.*, *oriC.g* and *aph(3')-Ia* (Kan^R) was amplified using primers vec_875_F and vec_5808_R and Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) under the following conditions: 30 s 98°C (10 s 98°C, 10 s 49°C, 2.5 min 72°C) ×35; 7 min 72°C. For construction of ptetAR65 the region encoding the *tet*(65) gene and its repressor *tetR*(65) was amplified from p22KM0430 with primers insert3_1_F and insert3_2064_R with the same polymerase and the following conditions: 30 s 98°C (10 s 98°C, 10 s 70°C, 1 min 72°C) ×35; 7 min 72°C. For construction of ptetA65, only the *tet*(65) gene and its promoter region were amplified replacing primer insert3_1_F with insert4_1_F. PCR products were subsequently treated with DpnI to remove template DNA and then purified with the High Pure PCR Product Purification kit (Roche). Vector and inserts were then combined at a molar ratio of 1:1.8 using (100 ng) in a total reaction volume of 10 µL. The reaction mix was incubated for 1 h at 50°C and then 1.5–2 µL were used for electrotransformation of *C. glutamicum* DSM20300 and *Corynebacterium* sp. 22KM0430 following the protocol of Ruan *et al.*[12] *E. coli* DH5α was also transformed by electroporation using the same settings (1 mm cuvette, 1.8 kV, 5 ms).

Transformants were selected on brain heart infusion agar plates containing 10 mg/L tetracycline and plates containing

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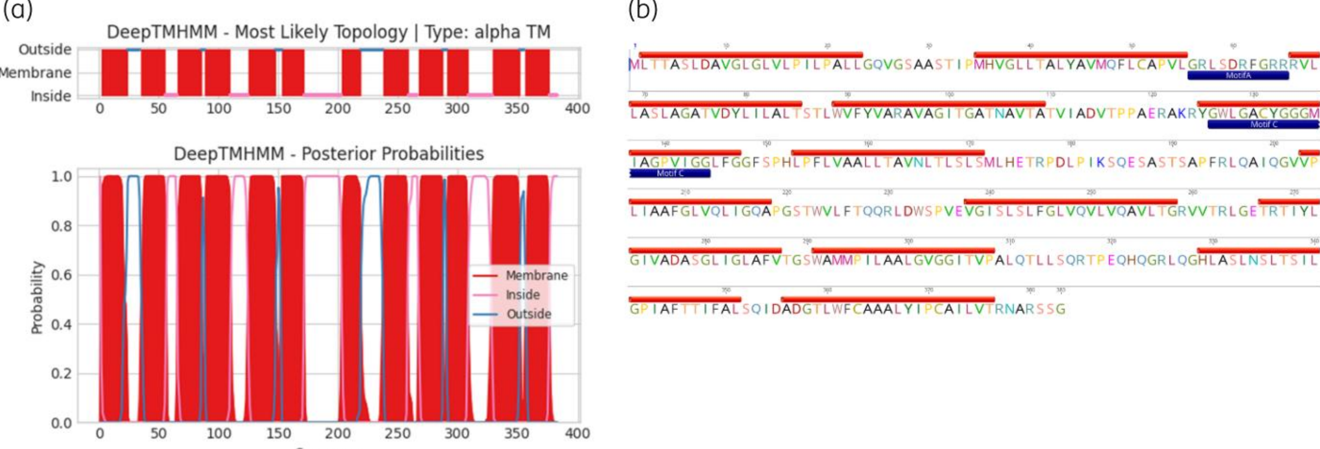

Figure 1. Location of transmembrane domains of Tet(65) as predicted by DeepTMHMM (https://dtu.biolib.com/DeepTMHMM) in the probability graph (a) and on the sequence (b). The model predicts the location of the sequence as transmembrane (red) outside (blue) or inside (pink) (upper graph). This is based on the probabilities calculated by a deep learning model (lower graph). The location of the motif A 'G (X)3 D R/K X G R R' after the second transmembrane helix and antiporter motif C 'G (X)8 G (X)3 G P (X)2 G G' in the fifth transmembrane helix are shown on the sequence in (b). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

25 mg/L kanamycin. *Corynebacterium* transformants were incubated at 30°C for 48 h as recommended,[12] except for *Corynebacterium* sp. 22KM0430 that was incubated for 5 d due to slower growth. *E. coli* transformants were incubated at 37°C for 24 h. Presence of the correct insert in the selected transformants was verified by PCR using primers col_F 5′-GCT ACT AAG CGT GAT CTG AA-3′ and col_R 5'-TCT TTA TAG TCC TGT CGG GT-3′ at a concentration of 1 µM with GoTaq® DNA Polymerase (Promega) under the following conditions 3 min 94°C (30 s 94°C, 30 s 50°C, 1 min 72°C) ×35 7 min 72°C (Figure S1).

MIC testing

Minimal inhibitory concentrations (MICs) were determined according to CLSI standards VET06Ed1E[14] for corynebacteria and VET01SEd6[15] for *E. coli*. Reserpine, an efflux pump inhibitor,[16] was added to the Müller–Hinton broth (supplemented with 5% horse blood for corynebacteria) at a concentration of 40 mg/L before tetracycline MIC testing to demonstrate that Tet(65) acts as an efflux pump.

Results and discussion

Location and characterization of tet*(65)*

Tet(65) consists of a 383aa protein with 12 transmembrane domains indicating that it belongs to the tetracycline efflux proteins of group one (Figure 1a).[6] It has both motif A and motif C[9] placing Tet(65) within the MFS (Figure 1b).

Tet(65) shows the closest aa identity (73%) with Tet(Z) (GenBank accession no. AAD25063.1) and clusters together with Tet(Z) and Tet(33) in the phylogenetic analysis, both determinants being also present among *Corynebacterium* (Figure 2).

The *tet*(65) and *tetR*(65) genes were located on the 35 562-bp plasmid p22KM0430 in inverse orientation as is common for the *tetA*/*tetR* combination.[18] The putative promoter region of *tet*(65) overlaps with the promoter of *tetR*(65) with a −10 box (CTTTATCGT) 55 bp upstream and a −35 box (TTGAAC) 75 bp upstream of the coding sequence. The −35 box of *tetR*(65) (GTGACG) is located 53 bp upstream while the −10 box (CGATAAAGT) is located 30 bp upstream overlapping with the −10 box of *tet*(65). This is similar to what is described for other *tetAR* systems.[18]

The *tet*(65) and *tetR*(65) genes are stably linked with two coding sequences (NAD(P)H-binding protein, DUF1772 domain-containing protein) that are also found together with *tet*(65) in *C. diphtheriae* plasmids pNG3 (GenBank accession no. MZ348427) and FRC0402_p2 (GenBank accession no. OV884290) as well as in the chromosome of *Brevibacterium casei* FDAARGOS_1100 (GenBank accession no. CP068173) and *Brevibacterium luteolum* NEB1784 (GenBank accession no. CP035810) (Figure 3). Nevertheless, the origin of *tet*(65) remains unknown. In *Corynebacterium*, *tet*(65) was situated next to IS*1628*, a member of the IS*6*/IS*26* family found in *Actinobacteria*,[19] which may have contributed to the integration of the gene into the plasmids (Figure 3). Two copies of IS*1628* are flanking the *tet*(65) containing region in the two *C. diphtheriae* plasmids FRC0402_p2 and pNG3 while only one copy is present upstream of *tet*(65) in p22KM0430 (Figure 3). In *B. luteolum* NEB1784 and *B. casei* FDAARGOS_1100, the *tet*(65) containing region was almost identical to that of the *Corynebacterium* plasmid, but lacks IS*1628* including its inverted repeats. Additionally, neither *B. luteolum* nor *B. casei* seem to be the precursor of *tet*(65), since the gene was not present among the 14 additional *B. luteolum* and 30 *B. casei* genomes available in GenBank (accessed 13 February 2024). *B. luteolum* NEB1784 was isolated from a cell culture contamination and the origin of *B. casei* FDAARGOS_1100 is unclear, which makes it impossible to clearly determine the origin of the gene in these species.

All three *tet*(65)-containing plasmids from *Corynebacterium* also encode the MLS_B resistance gene *erm*(X), which was not present in the chromosome of the *tet*(65)-containing *Brevibacterium* (Figure 3). *C. diphtheriae* plasmid FRC0402_p2 was described by Hennart *et al.* in a tetracycline-resistant, non-toxigenic isolate

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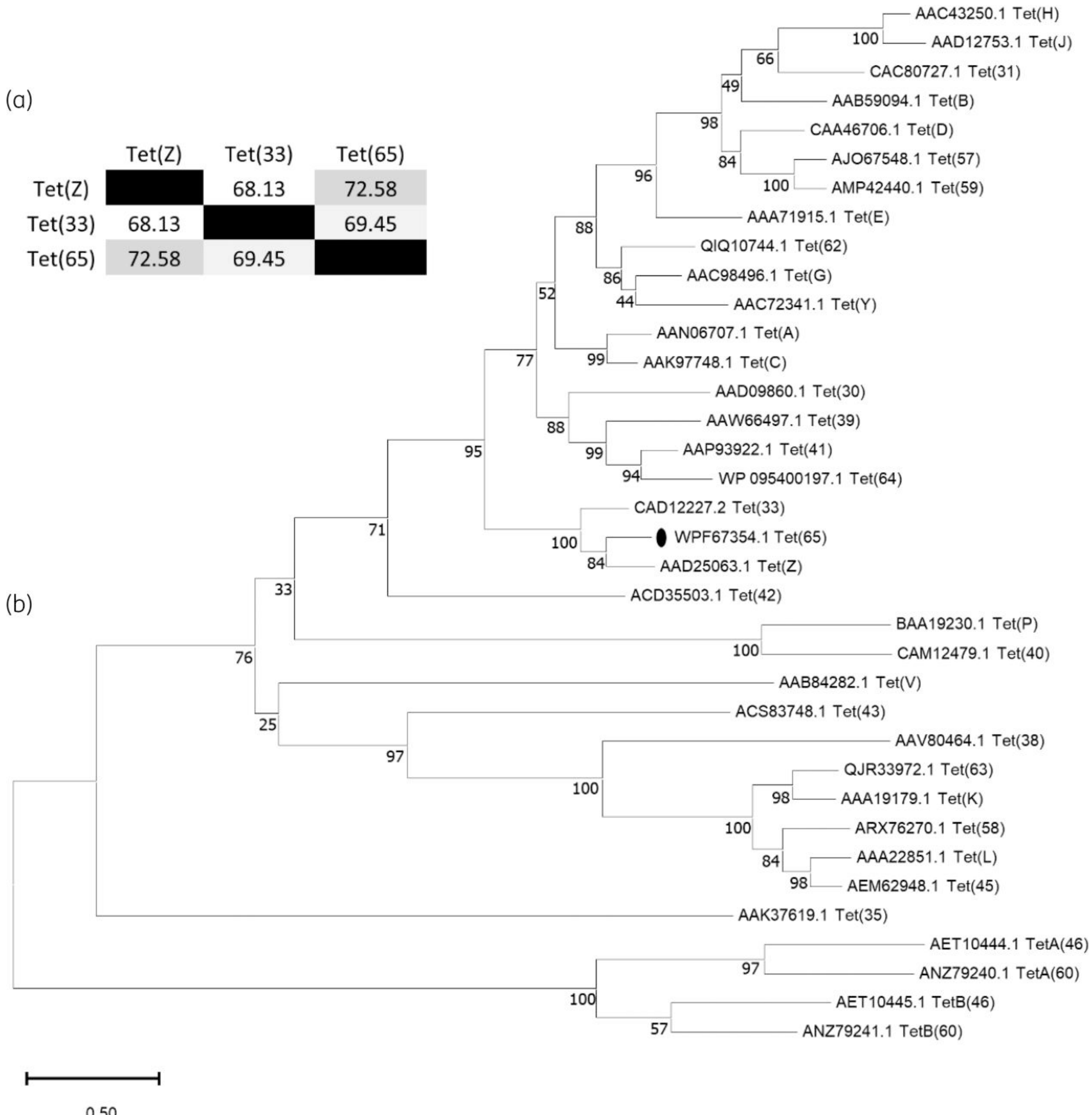

Figure 2. Comparison of Tet(65) with other Tet proteins. (a) Identity matrix. The identity was calculated with Geneious Prime® v.2023.2.1 of Tet(65) with the two most closely related Tet proteins. Numbers indicate the percentage amino acid identity. (b) Evolutionary analysis by maximum likelihood method. The evolutionary history was inferred by the maximum likelihood method and JTT matrix-based model implemented in MEGA11.[17] The percentage of trees in which the associated taxa clustered together is shown below each branch. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 36 amino acid sequences. There was a total of 610 positions in the final dataset.

from a from a patient living in La Réunion Island.[20] Plasmid pNG3 was recently described by Nguyen Thi Nguyen *et al.* as an erythromycin resistance conferring plasmid in *C. diphtheriae* isolates from Vietnam.[21] Erythromycin treatment would then lead to co-selection of tetracycline resistance. Tetracycline resistance is prevalent in *C. diphtheriae* with 18% of resistant strains reported in the study of Hennart *et al.*[20]

Cloning and expression of tet*(65)*

Corynebacterium sp. 22KM0430, which naturally harbours p22KM0430 encoding Tet(65) showed MICs of 64 mg/L for tetracycline, which decreased to 1 mg/L for the strain cured of p22KM0430 (Table 1). Expression of *tet*(65) with and without *tetR*(65) from constructs ptetA65 and ptetAR65, respectively, placed in *C. glutamicum* DSM20300 and *E. coli* DH5α led to

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tet(65)
tetR(65)

Brevibacterium casei chromosome
CP068173_extraction:1-37836

Brevibacterium luteolum chromosome
CP035810_extraction_(reversed):1-23430

Corynebacterium sp. p22KM0430
CP123906_extraction_(reversed):1-35494

Corynebacterium diphtheriae plasmid pNG3
MZ348427.1:1-18651

Corynebacterium diphtheriae plasmid FRC0402_p2
OV884290.1:1-37952

2.5kb
0 Identity (%) 100

- cupin domain containing protein
- NAD(P)H binding protein
- DUF1772 domain containing protein
- *tetR*(65)
- *tet*(65)
- hypothetical protein
- hypothetical protein
- MFS trasporter
- IS*3* family transposase
- replication initiation protein
- ParA family protein
- IS*1628* family transposase
- *erm*(X)
- LysR family transcription regulator
- classA betalactamase related serine hydrolase
- penicillin binding transpeptidase containing protein
- recombinase family protein
- mobF
- hypothetical protein
- hypothetical protein
- hypothetical protein
- TIR of IS*1628*: GGTGCTGTTGCAAA/C

Figure 3. Alignment of p22KM430 with the four sequences extracted from GenBank showing a Blastn match with *tet*(65): *C. diphtheriae* plasmids pNG3 and FRC0402_p2 and two *Brevibacterium* chromosomes. *tet*(65) is shown in pink. All three plasmids also encode *erm*(X) shown in green. Annotations are provided for all proteins found in more than one sequence. Terminal inverted repeats (TIR) are shown for IS*1628*.[19] The alignment was constructed with clinker v.0.0.27. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

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increased MIC of tetracycline (Table 1). The MIC increased from 1 to 16 mg/L when *tet*(65) was expressed with *tetR*(65) in *C. glutamicum* and to 64 mg/L when it was expressed without the repressor. Expression of *tet*(65) in *E. coli* DH5α also led to an increase of the tetracycline MIC from 2 to 16 mg/L, independent of the presence of *tetR*(65). Functional expression of *tet* efflux genes of Gram-positive bacteria in *E. coli* is not unusual and has been described before.[22] MICs for tigecycline and minocycline were not influenced by Tet(65) in both *C. glutamicum* and *E. coli* and neither was there a clear effect for doxycycline (Table 1).

Experimental confirmation of the efflux mechanism was obtained by efflux inhibition with reserpine, which led to a 2- to 4-fold MIC decrease in the corynebacteria and a 2-fold decrease in *E. coli* (Table 1). A similar phenomenon was already observed for Tet(63) originally identified in *Staphylococcus aureus*.[22]

Conclusions

The *tet*(65) gene encodes a novel tetracycline MFS efflux pump. It was identified on plasmids in a *C. oculi* related species and in *C. diphtheriae*, as well as within the chromosome of *Brevibacterium* indicating the potential of *tet*(65) to be acquired by different bacteria possibly through IS-driven transposition. Its plasmid co-location with *erm*(X) in *Corynebacterium* enhances the risk of co-selection.

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Transparency declarations

None to declare.

Data availability

The sequence of *tet*(65) was submitted to GenBank under accession no. OR494599. Plasmid p22KM0430 is available under GenBank accession no. CP123906.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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