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A novel *erm*(44) gene variant from a human *Staphylococcus saprophyticus* confers resistance to macrolides, lincosamides but not streptogramins

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ABSTRACT

A novel *erm*(44) gene variant, *erm*(44)$_{v,}$ has been identified by whole genome sequencing in a *Staphylococcus saprophyticus* isolated from the skin of a healthy person. It has the particularity to confer resistance to macrolides and lincosamides, but not to streptogramins B when expressed in *S. aureus*. The *erm*(44)$_{v}$ gene resides on a 19,400-bp genomic island which contains phage-associated proteins and is integrated into the chromosome of *S. saprophyticus*.

Staphylococcus saprophyticus is a bacterium which is widespread in the environment and in animals, and may also occur on the skin of humans. It is known as a major cause of urinary tract infection and cystitis in humans (1). Although macrolides and lincosamides are not used for the treatment of urinary tract infections, they are amongst the antibiotics of choice for the treatment of other infection diseases, such as pulmonary infection, and their use may contribute to the selection of resistance in bacteria of the normal human flora, including staphylococci (2). Resistance to macrolide, lincosamide and streptogramin (MLS_B) antibiotics in staphylococci have been associated with erythromycin ribosome methylase (*erm*) genes which methylate the 23S rRNA at position A2058 preventing binding of the MLS_B antibiotics (Fig.1) (3). The *erm*(44) gene, originally found in *Staphylococcus xylosus* from bovine mastitis milk (4), has also been recently identified in a *S. saprophyticus* isolate from river water (5), and now in *S. saprophyticus* from human skin.

Three of ten healthy human volunteers who did not receive MLS_B antibiotics and who were participating to a large project aiming at determining the effects of antibiotic administration on the emergence and persistence of antibiotic-resistant bacteria in humans (ANTIRESDEV project (www.ucl.ac.uk/antiresdev); UK ethics approval number EC 10/H0806/12) were found to harbor *Staphylococcus saprophyticus* on the skin. The strains were isolated on sheep blood agar plates and identified using MALDI-TOF (microflex LT, Bruker Daltonic GmbH, Bremen, Germany). Minimal inhibitory concentrations of MLS_B antibiotics erythromycin, clindamycin, virginiamycin S1 and pristinamycin 1A were determined by microdilution method in Mueller-Hinton broth and one strain (N041) showed resistance to erythromycin and clindamycin according to the EUCAST interpretation criteria (6). As this strain did not contain any known *erm* genes as determined using a microarray (7), whole genome sequencing was performed at the UZH/ETH Functional Genomics Center (Zurich, Switzerland) by life technologies Ion Torrent™ semiconductor sequencing using a 400-bp library on a 314v2 chip. Comparison of all contigs with currently annotated *erm* genes using

BLAST™ identified a *erm* gene which showed the closest relatedness to *erm*(44) from *S. xylosus* JW4341 with 84% amino acid (aa) and 86% DNA identity, and to *erm*(44) from *S. saprophyticus* A ER Ab-7 with 84% aa sequence identity and 83.1% DNA identity (Fig. 1). The newly detected *erm* gene encodes a 243-aa protein containing an rRNA adenine dimethylase signature (PS01131) as found in other *erm* methylases (8). It was not preceded by any intact leader peptides, neither by a complete IFVI motif nor by inverted repeat sequences, which are essential for induction and translational attenuation of *erm* genes (3, 9-11), likely explaining constitutive expression of this *erm* gene as determined by MIC (Table 1). Putative -10 (TTTTAAAAT) and -35 (TTGCCT) promoter sequences were found 27 bp and 48 bp upstream of the start codon, respectively.

The functionality of the *erm* gene of strain N041 was assessed after cloning into the shuttle vector pBUS-HC(12) generating plasmid pBSC0714, where the gene was expressed with its own promoter. Presence of pBCS0714 in *S. aureus* RN4220 led to an increase of the MIC of erythromycin to 16µg/ml and of clindamycin to ≥256µg/ml, while the MICs for the streptogramins pristinamycin Ia and virginiamycin S1 did not increase compared to *S. aureus* RN4220 recipient strain alone and a RN4220 strain harboring pBUS-HC or pBUS-P_{cap}. To verify this uncommon phenotype, the *erm* gene was placed under the control of a strong *cap* promoter in plasmid pBSC0814 confirming both the erythromycin and clindamycin phenotype and the absence of increased MIC to streptogramins B pristinamycin and virginiamycin in RN4220 (Table 1), in contrast to the closely related *erm*(44) from *S. xylosus* JW4341 and from *S. saprophyticus* A ER Ab-7 (4, 5). Due to the sequence identity being above the 80% threshold for a new *erm* determinant and to an altered phenotype compared to the original *erm*(44) from *S. xylosus* when expressed in *S. aureus*, the *erm* gene identified in *S. saprophyticus* N041 was assigned the name *erm*$(44)_v$ according to the nomenclature of the MLS_B resistance genes (http://faculty.washington.edu/marilynr/) (13). However, it cannot be

excluded that *erm*(44)$_v$ might confer resistance to streptogramins B in *S. saprophyticus* due to the presence of a specific inducer which is absent in *S. aureus* RN4220.

The *erm*(44)$_v$ gene was located on a putative 19,400-bp genomic island (GenBank acc. no. LN623525), which is absent in the MLS$_B$-susceptible strain *S. saprophyticus* KACC16562 (GenBank acc. no. AHKB01, Fig. 2). In contrast to *erm*(44) from *S. xylosus* JW4341 which is situated on a pro-phage ΦJW4341-pro (4), the genomic composition of the island described here shows a rather heterogeneous composition of ORFs remotely resembling that of a temperate siphoviral bacteriophage SaPImw2 with the common presence of one terminase, two primases, two transcriptional regulators and an integrase belonging to the tyrosine type of bacterial phage integrases (*Int-Ssapro1*, NCBI conserved domain number: cd01189, Fig. 2)(14). The genomic island contains an additional integrase of the same type (*Int-Ssapro2*, NCBI conserved domain number: cd01189) at its distal end which potentially played a role in the integration and recombination of the genomic island into the *S. saprophyticus* genome. However, no conjugal transfer of macrolide resistance into *S. aureus* 80Cr5 (Rif^R)(15) and *S. saprophyticus* 7108R (a rifampicin-resistant mutant of 7108) (16) was observed by filter mating (17) using different donor-recipient rations (10^6:10^8, 10^8:10^8, 10^8:10^6 cells/ml) and 10µg/ml erythromycin and 100 µg/ml rifampicin in the BHI agar selective plates. No circular form could be observed by PCR using GoTaq© polymerase (Promega) and plasmid DNA (NucleoBond® PC 100, Macherey-Nagel) as template and using primer1 (5'-CCCGTTGTTACGGGGTTTCT) and primer2 (5'-GCGATAAAGAGCATTTTGATTTTCC) (annealing temperature: 55°C, extension time 2 min) reading outwards of the genomic island (Fig. 2).

Analysis of *Staphylococcus* whole genome sequences using the MaGe Microscope Platform (https://www.genoscope.cns.fr/agc/microscope/home/) revealed that the genetic island containing *erm*(44)$_v$ inserted into a chromosomal hotspot, as most strains annotated in MaGe

show large sequence variation at this specific locus. The genomic island integrated at a specific 19-bp integration site (*attC*: CCCTCCCAGGACACTAAAA) situated between a metal-dependent phosphodiesterase and two tandem-transposases (*InsO_Ssapro* and *InsE_Ssapro*, NCBI conserved protein family number: COG2801, COG2963, Fig. 2). The attachment site *attC* was duplicated in the N041 strain with one perfect copy downstream (*attR*) and one imperfect copy upstream of the genomic island (*attL*) (Fig. 2).

This study describes an *erm*(44) gene variant, *erm*(44)$_v$, in a human isolate of *S. saprophyticus*, which does not confer decreased susceptibility to streptogramin B in *S. aureus*, in contrast to the *erm*(44) from *S. xylosus* from milk and from *S. saprophyticus* from river water. However, besides this uncommon phenotype, the *erm*(44)$_v$ was found, like *erm*(44) from *S. xylosus* (4), on an element containing genes associated with phages, indicating that phage associated elements may play a role in the spread of MLS_B resistance.

Nucleotide sequence accession numbers. The *erm*(44)$_v$-containing element and its insertion region in *S. saprophyticus* N041 has been deposited in the DDBJ/ENA/GenBank database under accession number LN623525.

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TABLE 1 MIC of erythromycin, clindamycin, pristinamycin Ia and virginiamycin S1 for different *Staphylococcus* strains, as determined by broth microdilution

strain	characteristic(s) or origin	reference or source	antibiotic resistance gene(s)[a]	MIC (µg/ml)[b]						
				ERY	CLI	iCLI	PIA	iPIA	VS1	iVS1
S. saprophyticus										
N041	human nose skin sample	this study	$erm(44)_v$	128	>256	>256	32	32	32	32
S. aureus										
RN4220	Recipient strain for electrotransformation, plasmid free	(18)		<0.5	<0.25	NA	4	NA	8	NA
RN4220/pBUS-HC	RN4220 with cloning vector pBUS-HC	(12), this study	*tet*(L)	<0.5	<0.25	NA	4	NA	8	NA
RN4220/pBUS-P_{cap}[c]	RN4220 with pBUS-HC containing *cap* promoter	(12), this study	*tet*(L)	<0.5	<0.25	NA	2	NA	8	NA
RN4220/pBJW13	RN4220 with *erm*(44) from *S. xylosus* JW4341 cloned into pBUS-P_{cap}	(4), this study	*tet*(L), *erm*(44)	>256	>256	>256	8	8	16	32
RN4220/pLI50-*erm*(44)	RN4220 with *erm*(44) from *S. saprophyticus* A ER Ab-7 cloned into pLI50	(5), this study	bla_{TEM-1}, cat_{pC194}, *erm*(44)	>256	<0.25	256	4	64	32	128
RN4220/pBCS0714	RN4220 with $erm(44)_v$ from *S. saprophyticus* N041 and its regulatory region cloned into pBUS-HC	this study	*tet*(L), $erm(44)_v$	16	>256	>256	2	1	8	4
RN4220/pBCS0814	RN4220 with $erm(44)_v$ from *S. saprophyticus* N041 cloned into pBUS-P_{cap}	this study	*tet*(L), $erm(44)_v$	16	>256	>256	2	1	8	4

[a] Antibiotic resistance genes and functions: bla_{TEM-1}, β-lactamase gene; cat_{pC194}, chloramphenicol acetyltransferase; *tet*(L), tetracycline efflux gene; *erm*(44) and $erm(44)_v$, 23S rRNA methylase genes.
[b] Abbreviations: ERY, erythromycin; CLI, clindamycin; PIA, pristinamycin IA; VS1, virginiamycin S1; iCLI, iPIA and iVS1, 2 µg/ml erythromycin added to the broth for the detection of inducible resistance to clindamycin (iCLI), pristinamycin IA (iPIA) and virginiamycin S1 (iVS1); NA, not applicable.
[c] Vector pBUS-P_{cap} is a pBUS-HC derivate that harbors the *cap* promoter of the *S. aureus* type 1 capsular polysaccharide biosynthesis gene cluster.

FIGURE LEGEND

Figure 1

Relationship tree of erythromycin resistance methylases (Erm) detected in different *Staphylococcus* species. Amino acid (aa) and nucleotide (nt) identity were obtained by sequence alignment and clustering with BioNumerics 7.6 (Applied Maths). Comparison settings were standard algorithm for pairwise alignment, open gap penalty 100%, unit gap penalty 0% and UPGMA. Methylase genes that were detected in *Staphylococcus* only by PCR and/or hybridization and whose sequences are not available (e.g. *erm*(F), *erm*(G), *erm*(Q)) were not included (http://faculty.washington.edu/marilynr/).

Figure 2

Insertion site of genomic island in *S. saprophyticus* N041 (GenBank accession no. LN623525) and core genome of *S. saprophyticus* KACC16562 (GenBank accession no. NZ_AHKB00000000.1). Grey areas represent high similarity at nucleotide level (>98%). Arrows represent position and orientation of open reading frames (ORFs). New ML resistance gene *erm*(44)$_v$ is shown in pink. The 19-bp putative insertion site *attC* and the duplicated sites *attL* and *attR* in the N041 genome are shown. Two transposases of the core genome (*InsO_Ssapro* and *InsE_Ssapro*, short *InsO* and *InsE*) are indicated in yellow, the metal-dependent phosphodiesterase in red and the two flanking integrases of the genomic island (*Int-Ssapro1* and *Int-Ssapro2*) in orange. Additional genes are colored according to their sequence and function: transcription regulators are dark blue; replication genes (including the primase gene) are light blue; the terminase gene in green; genes encoding hypothetical proteins are grey. Primers for circular form test are indicated with a black arrow.

	Protein acc. no	aa %	nt %
Erm(C)	CAA24591	59.6	63.8
Erm(T)	AAA98096	62.6	62.8
Erm(Y)	BAB20748	59.7	62.0
Erm(A)	CAA26964	65.1	63.7
Erm(33)	CAC86410	63.1	63.6
Erm(44) *S. xylosus* JW4341 (milk)	CDL65151	87.1	85.1
Erm(44) *S. saprophyticus* A ER Ab-7 (river)	AJK31388	84.2	83.1
Erm(44)$_v$ *S. saprophyticus* N041 (human)	**CUU67654**	**100**	**100**
Erm(43)	CCF55073	80.6	76.6
Erm(B)	AAA27452	56	59
Erm(45)	CEJ95855	53.1	59.7