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The New Macrolide-Lincosamide-Streptogramin B Resistance Gene *erm*(45) Is Located within a Genomic Island in *Staphylococcus fleurettii*

Juliette R. K. Wipf,[a,b] Sybille Schwendener,[a] Jesper Boye Nielsen,[c] Henrik Westh,[c,d] Vincent Perreten[a]

Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland[a]; Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland[b]; Department of Clinical Microbiology, Hvidovre University Hospital, Hvidovre, Denmark[c]; Institute of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark[d]

Genome alignment of a macrolide, lincosamide, and streptogramin B (MLS_B)-resistant *Staphylococcus fleurettii* strain with an MLS_B-susceptible *S. fleurettii* strain revealed a novel 11,513-bp genomic island carrying the new erythromycin resistance methylase gene *erm*(45). This gene was shown to confer inducible MLS_B resistance when cloned into *Staphylococcus aureus*. The *erm*(45)-containing island was integrated into the housekeeping gene *guaA* in *S. fleurettii* and was able to form a circular intermediate but was not transmissible to *S. aureus*.

Staphylococcus fleurettii is a commensal bacterium of various animal species and an occasional cause of bovine mastitis (1–3). It naturally contains the methicillin resistance gene *mecA* within its chromosome and is therefore suspected to have been the source of the *mecA* gene found in staphylococcal cassette chromosome *mec* (SCC*mec*) of methicillin-resistant staphylococci, including methicillin-resistant *Staphylococcus aureus* (MRSA) (4).

Due to this intrinsic resistance to β-lactams, other antibiotic classes such as macrolides or lincosamides are being used for the treatment of mastitis caused by *S. fleurettii* (5, 6).

S. fleurettii strain JW205, recently isolated from bovine milk in Switzerland, exhibited resistance to erythromycin and inducible resistance to clindamycin (3). This suggested the presence of a macrolide, lincosamide, and streptogramin B (MLS_B) resistance methylase (Erm) (7). However, none of the *erm* genes commonly occurring in staphylococci were detected by microarray analysis (8, 9). We therefore examined *S. fleurettii* strain JW205 for a novel MLS_B resistance mechanism by genome comparison with MLS_B-susceptible *S. fleurettii* strain JW404 (Table 1).

Detection and characterization of *erm*(45). Genomes of strain JW205 and JW404 were sequenced using Ion Torrent (Life Technologies, Grand Island, NY) at the UZH/ETH Functional Genomics Center (Zurich, Switzerland) and Illumina MiSeq (Illumina, San Diego, CA) at the Department of Clinical Microbiology at Hvidovre Hospital (Hvidovre, Denmark), respectively. Contigs of the sequenced strains were aligned using the progressive Mauve algorithm (10). This revealed an 11,513-bp integrated genomic island in JW205, which was absent in strain JW404. The island contained 18 open reading frames (ORFs) (Fig. 1), which were identified by the Prokaryotic Dynamic Programming Genefinding Algorithm (Prodigal) (11) and compared to protein sequences and conserved domains in the BLASTp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Swiss Institute of Bioinformatics PROSITE database (http://prosite.expasy.org/). The rightmost ORF of the island encoded a 245-amino-acid (aa) protein, which contained the rRNA adenine dimethylase PROSITE signature PS01131, which is present in nearly all Erm 23S rRNA methylases (12). Of all 36 currently described Erm determinants, this methylase exhibited the highest similarity to Erm(B), with 64% aa and 67% nucleotide (nt) identity (Fig. 2). The novel gene was assigned the name *erm*(45) according to the established MLS_B resistance gene nomenclature (http://faculty.washington.edu/marilynr/), which defines novel Erm determinants by amino acid sequence identities of ≤79% compared to those of their closest Erm protein (13). Like *erm*(B), the *erm*(45) gene was preceded by a leader region encoding a single 27-aa leader peptide (Lp) (14). The Lp of *erm*(B) and the Lp of *erm*(45) shared 81% aa and 81% nt identity and each harbored the MRNVD motif, which is crucial for inducible expression of *erm* methylases (14). The leader region of *erm*(45) also contained 2 different pairs of inverted repeats (IRs), identical to those found in *erm*(B), which have been shown to form stem-loops involved in translational attenuation (14).

Cloning and expression of *erm*(45). To test functionality and inducibility of *erm*(45) a 1,098-bp region of strain JW205 including *erm*(45), its leader peptide and promoter (represented by green arrows in Fig. 1) was amplified by PCR (*Pfu* DNA Polymerase; Promega Corporation, Madison, WI) using primers erm(45)-Sal1-F2 (5′-cacagggtcgacATAAGTTGTTAGTAAATAGTATTCAAC) and erm(45)-XbaI-R (5′-cacaggtctagaCACCTATTTCAATACTAGG) (annealing temperatures, 50°C for cycles 1 to 3 and 56°C for cycles 4 to 24; elongation time, 2 min 30 s). The primers also contained polylinkers (lowercase) with restriction site sequences (underlined) to facilitate cloning. A 772-bp region containing *erm*(45) alone without its leader sequence was also amplified by PCR using primers erm(45)-NdeI-F2 (5′-cacacacggcatatgAATCAAAATATTAAGTTTACTC) and erm(45)-XbaI-R under

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Address correspondence to Vincent Perreten, vincent.perreten@vetsuisse.unibe.ch.

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TABLE 1 MICs of erythromycin, clindamycin, and pristinamycin IA for different *S. aureus* and *S. fleurettii* strains as determined by broth microdilution

Strain	Origin and characteristic(s)	Reference(s)	Antibiotic resistance gene(s)[a]	MIC (μg/ml) for:[b]				
				ERY	CLI	iCLI	PIA	iPIA
S. aureus								
80CR5	Recipient strain for conjugation, plasmid free, Rif^r Fus^r	26		≤0.5	≤0.5	NA[c]	4	NA
RN4220	Recipient strain for electrotransformation, plasmid free	27		≤0.5	≤0.5	NA	4	NA
RN4220/pBUS1-HC[d]	RN4220 with *S. aureus-E. coli* shuttle vector pBUS1-HC	28	*tet*(L)	≤0.5	≤0.5	NA	4	NA
RN4220/pBUS1-P$_{cap}$-HC[d]	RN4220 with *S. aureus-E. coli* shuttle vector pBUS1-P$_{cap}$-HC	28	*tet*(L)	≤0.5	≤0.5	NA	4	NA
RN4220/pBJW15	RN4220 with *erm*(45) and its regulatory region cloned into pBUS1-HC	This study	*tet*(L), *erm*(45)	32	≤0.5	256	4	8
RN4220/pBJW16	RN4220 with *erm*(45) cloned into pBUS1-P$_{cap}$-HC	This study	*tet*(L), *erm*(45)	>256	>256	>256	64	64
S. fleurettii								
JW205	Bovine milk	3, this study	*erm*(45), *mecA*, *tet*(K)	16	1	64	4	8
JW404	Bovine mastitis milk	3, this study	*mecA*	≤0.5	≤0.5	NA	2	NA

[a] Antibiotic resistance genes and functions: *tet*(L) and *tet*(K), tetracycline efflux genes; *mecA*, penicillin binding protein 2A gene; *erm*(45), 23S rRNA methylase gene.
[b] ERY, erythromycin; CLI, clindamycin; PIA, pristinamycin IA; iCLI and iPIA, 2 μg/ml erythromycin added to the broth for the detection of inducible resistance to clindamycin (iCLI) and pristinamycin IA (iPIA).
[c] NA, not applicable.
[d] pBUS1-HC is a promoterless cloning vector and pBUS1-P$_{cap}$-HC is an expression vector that harbors the strong *cap* 1A promoter of the *S. aureus* type 1 capsular polysaccharide biosynthesis gene cluster.

the same conditions. The former amplicon was cloned into the SalI and XbaI restriction sites of pBUS1-HC, generating plasmid pBJW15, where *erm*(45) was under the control of its own promoter (Table 1). The latter amplicon was placed into the NdeI and XbaI restriction sites of pBUS1-P$_{cap}$-HC, generating plasmid pBJW16, where *erm*(45) was under the control of the strong *S. aureus* type I capsule gene 1A promoter provided by the vector (Table 1). Plasmids pBJW15 and pBJW16 were first transformed by heat shock into *Escherichia coli* DH5α and were subsequently electroporated into *S. aureus* RN4220 (15) (Table 1). All transfor-

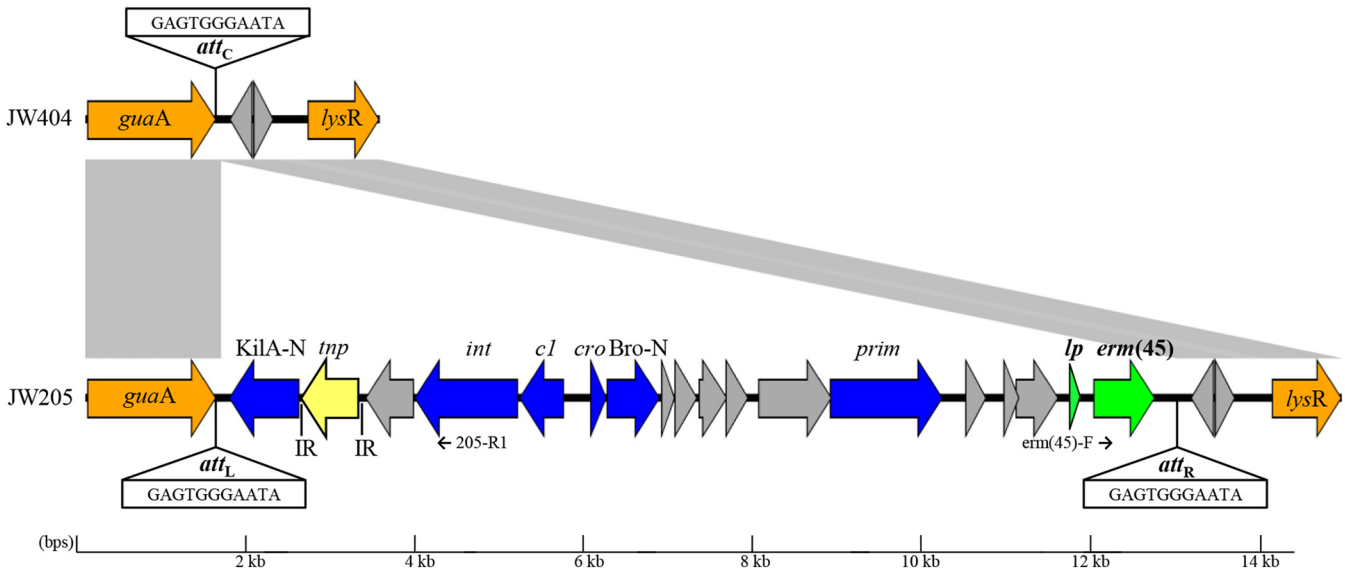

FIG 1 Schematic gene map showing the *erm*(45)-containing island and flanking region of *S. fleurettii* JW205 (ENA accession number LN680996), as well as the chromosomal glutamine amidotransferase gene (*guaA*) region of *erm*(45)-negative strain JW404. Gray areas represent regions showing high similarity at nucleotide level (>98%). Arrows represent position and orientation of open reading frames (ORFs). The *erm*(45) and its leader peptide (*lp*) gene are shown in green. The 11-bp (GAGTGGGAATA) chromosomal hot spot situated within the 3′ end of *guaA* is abbreviated as att$_C$. This attachment site is illustrated as att$_L$ and att$_R$ at each extremity of the island. The IS*431* transposase gene (*tnp*) illustrated in yellow is flanked by 16-bp inverted repeats (IR) (GGTTCTGTTGCAAAGT). Other putative genes and functions: KilA-N, KilA-N domain-carrying putative DNA-binding protein; *c1*/*cro*, repressors; Bro-N, Bro-N domain-carrying putative DNA-binding protein; *lysR*, transcriptional regulator; *int*, integrase; *prim*, primase. Color code: *S. fleurettii* core genome, orange; *erm*(45)-carrying island genes related to SaPI and bacteriophage genomes, blue; antibiotic resistance, green; no known function, significant similarity to proteins in the database used, or any known protein signatures, gray. Primers used to detect circular forms of the *erm*(45)-carrying island are indicated by small black arrows. The figure was generated using the program Easyfig (25).

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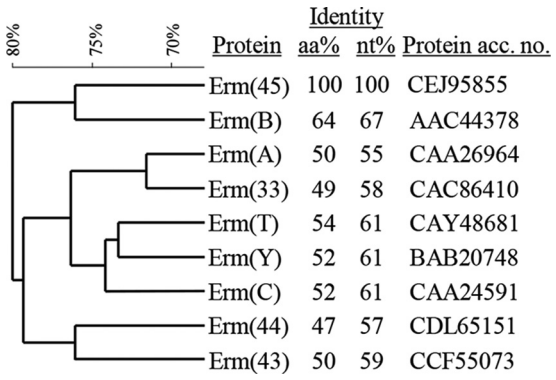

FIG 2 Relatedness of the novel erythromycin resistance methylase Erm(45) with other methylases commonly detected in *Staphylococcus* species (9, 12). Amino acid (aa) and nucleotide (nt) identity percentages were obtained by Clustal Omega sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). Erm aa sequence clustering was performed by BioNumerics 7.1 (Applied Maths). Comparison settings used the standard algorithm for pairwise alignment, open gap penalty 100%, unit gap penalty 0%, and the unweighted-pair group method using average linkages.

mants were grown on LB agar plates containing 10 μg/ml tetracycline. MIC values of erythromycin, clindamycin (Sigma-Aldrich, St. Louis, MO), and pristinamycin IA (Molcan Corporation, Richmond Hill, ON, Canada) of *S. fleurettii* and *S. aureus* strains were determined by broth microdilution using Mueller-Hinton broth according to CLSI guidelines (16). MICs for inducible resistance were measured in the presence of 2 μg/ml erythromycin (16). When *erm*(45) was expressed from its own promoter in plasmid pBJW15 in *S. aureus* RN4220, the MIC of erythromycin increased at least 64-fold, while the MICs of clindamycin (>512-fold) and pristinamycin IA (2-fold) increased only after induction with erythromycin (Table 1). The RN4220 transformant carrying pBJW16 and expressing *erm*(45) constitutively exhibited a >512-fold increase of the MICs of erythromycin and clindamycin and a 16-fold increase of the MIC for pristinamycin IA (Table 1).

Characterization of the *erm*(45)-containing genomic island. The 11,513-bp island containing *erm*(45) was flanked by 11-bp direct repeats (DRs) (GAGTGGGAATA) situated within the 3′ end of the glutamine amidotransferase (GMP) synthetase housekeeping gene *guaA* (Fig. 1), a known integration hot spot for genetic islands, transposons, and bacteriophages in different bacterial species (17, 18). For instance, identical DRs and *guaA* integration sites were associated with the pathogenicity islands of staphylococci (SaPIs) SaPIbov1 and SaPIbov2, identified in *S. aureus* from bovine mastitis milk (19). SaPIs are characterized by their specific set of bacteriophage-related genes and functions, the ability to exploit the life cycle of bacteriophages in favor of their own, and sizes ranging from 3 to 28 kb with most of them being 14 to 17 kb (19). Although the size of the element (11.5 kb) suggested a potential relation to SaPIs, the *erm*(45)-carrying island did not show significant DNA similarity to SaPI genomes in the GenBank and lacked the conserved genes *rep*, *xis*, *pif*, and *terS*, which are involved in the replication and excision of SaPIs, and in their interferences with phages (19). Nevertheless, six of the putative ORFs on the *erm*(45)-carrying island contained conserved domains related to SaPI proteins. In each element, these ORFs are structurally organized in a similar fashion at the 5′ end (Fig. 1). In the *erm*(45)-carrying island, they consisted of one putative integrase (Pfam signature cd00397), a putative *cro* repressor and *c1* antirepressor (PROSITE signature PS50943), one putative primase (PS51206), and two ORFs containing the conserved DNA-binding domains KilA-N (PS51301) and Bro-N (SMART accession number 01040; no defined PS), domains which have been found in proteins of bacterial DNA viruses (20–23). The integrase, harboring the conserved similarity regions of tyrosine recombinases (24), was most likely responsible for integration into the *S. fleurettii* chromosome and the formation of a circular intermediate of the *erm*(45)-containing island. This intermediate form was detected by PCR (GoTaq Green master mix PCR; Promega, Madison, WI) using primers 205-R1 (5′-GTAACCCTATGGCTCTATCATC) and erm(45)-F (5′-CATAATTTATGAGGTTGGAACTGG), which read outwards from the island (annealing temperature, 55°C; elongation time, 5 min). Due to the structure and the ability to form a circular intermediate, the element carrying *erm*(45) was classified as a genomic island. Although a circular conformation was observed, transmission of erythromycin resistance was not observed either by electrotransformation into *S. aureus* RN4220 or by conjugation with *S. aureus* 80CR5 using conditions previously described (9). An IS*431* flanked by 16-bp IRs (GGTTCTGTTGCAAAGT) was situated within the SaPI-like region between the KilA-N domain-containing ORF and the integrase (Fig. 1). The IRs flanking this transposase were the only repeats found that indicate integration of additional DNA into the island.

In conclusion, the detection of the new methylase gene *erm*(45) in *S. fleurettii* underlines the role of this bacterium as a reservoir of antibiotic resistance genes. The island containing *erm*(45) appears to have evolved from SaPIs and bacteriophages, emphasizing the potential of phage and phage-related structures to act as vehicles for antibiotic resistance.

Nucleotide sequence accession number. The nucleotide sequence of the *erm*(45)-carrying island of *S. fleurettii* JW205 and its flanking region was deposited into the ENA database under accession number LN680996.

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