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The Novel Macrolide-Lincosamide-Streptogramin B Resistance Gene *erm*(44) Is Associated with a Prophage in *Staphylococcus xylosus*

Juliette R. K. Wipf, Sybille Schwendener, Vincent Perreten

Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

A novel erythromycin ribosome methylase gene, *erm*(44), that confers resistance to macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics was identified by whole-genome sequencing of the chromosome of *Staphylococcus xylosus* isolated from bovine mastitis milk. The *erm*(44) gene is preceded by a regulatory sequence that encodes two leader peptides responsible for the inducible expression of the methylase gene, as demonstrated by cloning in *Staphylococcus aureus*. The *erm*(44) gene is located on a 53-kb putative prophage designated ΦJW4341-pro. The 56 predicted open reading frames of ΦJW4341-pro are structurally organized into the five functional modules found in members of the family *Siphoviridae*. ΦJW4341-pro is site-specifically integrated into the *S. xylosus* chromosome, where it is flanked by two perfect 19-bp direct repeats, and exhibits the ability to circularize. The presence of *erm*(44) in three additional *S. xylosus* strains suggests that this putative prophage has the potential to disseminate MLS_B resistance.

Staphylococcus xylosus is a ubiquitous bacterium and a commensal of human and animal skin (1). It is also used in the fermentation and production of cheeses and raw meat products (2, 3). Although rarely associated with human infections (4–6), *S. xylosus* plays a major role in the pathogenesis of subclinical bovine mastitis, in which it is in frequent contact with intramammary antibiotics used for mastitis treatment, such as macrolides and lincosamides (7–10). Simultaneous resistance to both of these drugs is due mainly to the acquisition of erythromycin ribosome methylase (*erm*) genes (11). Erm methylases are enzymes that add one or two methyl groups to adenine A2058 of the 23S rRNA, preventing the binding of macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics. To date, 35 Erm methylases have been identified in bacterial species (12, 13), 10 of which [Erm(A), Erm(B), Erm(C), Erm(F), Erm(G), Erm(Q), Erm(T), Erm(Y), Erm(33), and Erm(43)] have been detected on plasmids, transposons, or integrated elements in different *Staphylococcus* species (12, 14, 15).

Staphylococci can also acquire new genes by temperate-bacteriophage-mediated gene transfer (16, 17). The vast majority of staphylococcal bacteriophages belong to *Siphoviridae*, a family of the double-stranded DNA virus order *Caudovirales* (18). Members of the family *Siphoviridae* display a distinct genomic structure that corresponds to five functional modules, which are lysogeny, DNA replication, DNA packaging and capsid morphogenesis, tail morphogenesis, and host cell lysis (18, 19). In addition, they possess accessory genes, generally situated downstream of the lysis module. Those genes can be expressed by the bacteria following lysogenic conversion, providing novel phenotypes to the host, like virulence in staphylococci (e.g., Panton-Valentine leukocidin, superantigens, toxic shock syndrome toxins) (18, 20).

S. xylosus strain JW4341, isolated from bovine mastitis milk in Switzerland, was found to exhibit resistance to erythromycin, together with inducible resistance to clindamycin, suggesting the presence of an MLS_B Erm methylase (10). However, the mechanism could not be attributed to a known methylase. In this study, we identified and characterized the novel MLS_B resistance gene *erm*(44) carried by ΦJW4341-pro, a 52,814-bp putative prophage in *S. xylosus*.

MATERIALS AND METHODS

Bacterial strains, species identification, and growth conditions. *S. xylosus* strains were isolated from bovine milk samples in Switzerland as described previously (10). The strains were grown aerobically on Trypticase soy agar plates containing 5% sheep blood (Becton, Dickinson & Company, Franklin Lakes, NJ) at 37°C. Identification at the species level was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Microflex LT; Bruker Daltonics GmbH, Bremen, Germany).

Staphylococcus aureus strains RN4220 (21) and 80CR5 (22) were grown in brain heart infusion (BHI) medium, and *Escherichia coli* DH5α was grown in Luria broth.

Antimicrobial susceptibility testing. D-zone testing was used to screen for constitutive macrolide resistance and inducible clindamycin resistance (23). MICs of erythromycin, clindamycin (Sigma-Aldrich, St. Louis, MO), and pristinamycin IA (Molcan Corporation, Richmond Hill, Ontario, Canada) for *S. xylosus* and *S. aureus* strains were determined by broth microdilution with Mueller-Hinton broth (23). MICs for inducible resistance to clindamycin and pristinamycin IA were measured in the presence of 2 μg/ml erythromycin (23).

Antibiotic resistance genes were detected with a custom-made microarray (AMR+ve-4 array tubes; Alere GmbH, Jena, Germany) (24).

DNA extraction. Crude genomic DNA of staphylococci for PCR and microarray analyses was obtained by lysostaphin lysis (10). The genomic DNA used for next-generation sequencing and for long-range PCR experiments was isolated by acid guanidinium thiocyanate phenol-chloroform extraction (25).

Plasmid DNA of the *S. xylosus* strains was extracted by the protocol of Anderson and McKay (26). Plasmid DNA of the cloning vectors and derived constructs was obtained with PeqGold Plasmid miniprep kit 1

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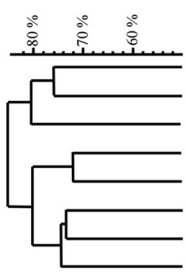

Protein	aa%	nt%	Protein acc. no.	Species
Erm(43)	73	78	CCF55073	*S. lentus*
Erm(44)	**100**	**100**	**CDL65151**	***S. xylosus***
Erm(B)	47	62	AAC44378	*S. lentus, S. aureus, S. pseudintermedius, S. hyicus*
Erm(A)	60	67	CAA26964	*S. aureus, S. epidermidis*
Erm(33)	57	70	CAC86410	*S. sciuri*
Erm(T)	56	67	CAY48681	*S. aureus*
Erm(Y)	54	70	BAB20748	*S. aureus*
Erm(C)	54	69	CAA24591	*S. aureus, S. hyicus, S. chromogenes, S. haemolyticus, S. simulans, S. epidermidis, S. hominis, S. saprophyticus, S. equorum, S. arlettae, S. cohnii*

FIG 1 Relatedness of erythromycin resistance methylases (Erm proteins) of different *Staphylococcus* species. Amino acid and nucleotide sequence identity percentages were obtained by sequence alignment with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences chosen for comparison are from the species for which the Erm protein was initially described. Clustering of Erm amino acid sequences was performed by BioNumerics 7.1 (Applied Maths). The comparison settings were standard algorithm for pairwise alignment, an open gap penalty of 100%, a unit gap penalty of 0%, and the unweighted-pair group method using average linkages. Methylase genes that were detected in *Staphylococcus* only by PCR and/or hybridization and for which sequences are not available [e.g., *erm*(F), *erm*(G), *erm*(Q)] were not included (http://faculty.washington.edu/marilynr/).

(Peqlab, Erlangen, Germany) by following the manufacturer's instructions.

PCR, sequencing, and annotation. *Taq* DNA polymerase (Solis BioDyne, Tartu, Estonia) was used for standard PCRs. Promega GoTaq Long PCR master mix (Promega Corporation, Madison, WI) was used to obtain amplicons of up to 20 kb. The reaction mixtures were cycled 35 times for amplification by using the primers and conditions indicated below.

Primers erm(44)-F (5′-TACAAAATACATGTCCAATATAGC) and erm(44)-R (5′-GAGATTAAAGATTTGTGCTGC) were used to amplify a 420-bp fragment of *erm*(44) at an annealing temperature of 55°C and an extension time of 50 s. The flanking region and attachment site of *erm*(44)-containing inserts were amplified and sequenced with primers IC11-F9 (5′-AGGTAGAGGTTCAACAATCC) and IC11-R1 (5′-CACTG CTCTTATCTCCTTGC) to detect *attL* (annealing temperature, 50°C; extension time, 30 s) and with primers ClpP-R (5′-CGTGAGTAAATGTC ATAGG) and erm(44)-F to detect *attR* (annealing temperature, 50°C; extension time 20 min). Circular forms were detected with primers IC11-F10 (5′-AATAGG[A/T]GGGTTGTTTTGTC) and IC11-R1 (annealing temperature, 50°C; extension time, 40 s). Primers IC11-F9 and ClpP-R (annealing temperature, 50°C; extension time, 2 min) were used to detect *attB* in *erm*(44)-negative strains.

Sequence analysis of the PCR products was performed with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Whole-genome sequencing of *S. xylosus* JW4341 was performed at the UZH/ETH Functional Genomics Center (Zurich, Switzerland) by Life Technologies Ion Torrent semiconductor sequencing with a 400-bp library on a 314v2 chip.

Genomic sequence analysis to detect open reading frames (ORFs) was performed with the Prokaryotic Dynamic Programming Genefinding Algorithm (Prodigal) (27). Deduced amino acid sequences of predicted ORFs were 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/).

Cloning of *erm*(44). A 1,134-bp fragment containing *erm*(44), along with a 292-bp upstream region and a 110-bp downstream region, was amplified from *S. xylosus* JW4341 DNA by PCR with *Pfu* DNA polymerase (Promega Corporation) and primers erm(44)sal1-F (5′-**CATGTCGAC**T ATCCATATTCTAATGTTTTAGATTAG) and erm(44)sac1-R (5′-**CAT GAGCTC**AATCACAAGTGTATTTTAACAC) (annealing temperature, 53°C; extension time, 2 min). The fragment was cloned into the SalI and SacI sites of *E. coli-S. aureus* shuttle vector pBUS1 (28), generating plasmid pBJW5. The 732-bp *erm*(44) gene was amplified with primers erm(44)cap-F (5′-**CATACATA**TGAATAACAAAAATCCTAAAAACTC) and erm(44)cap-R (5′-**CATAGTCGACTAGTC**AATTAAACAATTTAT AACTATG) (annealing temperature, 50°C; extension time, 1 min) and cloned into the NdeI and SalI sites of pBUS1-P_{cap} to generate plasmid pBJW13. In this vector, *erm*(44) is under the control of the *S. aureus* type 1 capsule polysaccharide biosynthesis gene cluster (*cap*) promoter (29). The primers were supplemented with linkers (boldface letters) containing a restriction site (underlined) to facilitate vector cloning. Plasmids pBJW5 and pBJW13 were obtained in *E. coli* DH5α and subsequently electroporated into *S. aureus* RN4220 with 10 μg/ml tetracycline in the selection plates (30).

Transfer experiments. Conjugation was carried out by filter mating with *S. xylosus* JW4341 as the donor strain and *S. aureus* 80CR5 as the recipient strain (31). Selection occurred on BHI agar containing 100 μg/ml rifampin; 25 μg/ml fusidic acid; and 2, 4, or 8 μg/ml erythromycin. Electrotransformation of *S. aureus* RN4220 with the plasmid DNA of JW4341 was performed as previously described with 2, 4, and 8 μg/ml erythromycin in the selective plates (30).

Nucleotide sequence accession numbers. The sequences of *erm*(44)-containing prophage ΦJW4341-pro and its insertion region in *S. xylosus* JW4341 and *erm*(44) in *S. xylosus* JW3659 have been deposited in the EMBL database under accession numbers HG796218 and LK392593, respectively.

RESULTS AND DISCUSSION

Detection and characterization of *erm*(44). To detect the unknown macrolide resistance gene of *S. xylosus* JW4341, whole-genome sequencing was performed. *De novo* assembly generated 53 contigs obtained from a total of 160.9 Mbp in 582,303 reads, with a mean read length of 276 bp. The nucleotide sequences of the 53 contigs were compared to *erm*(A) (GenBank accession no. X03216) with BLASTn megablast for the alignment of several discontinuous sequences. An ORF of 732 bp was identified that displayed 67% nucleotide and 60% amino acid identity to Erm(A). Alignment of this putative methylase with all known Erm proteins revealed the highest identity to Erm(43) of *Staphylococcus lentus* (12), with 73% amino acid and 78% nucleotide identity. This new methylase gene was named *erm*(44) according to the nomenclature for MLS_B resistance genes (http://faculty.washington.edu/marilynr/) (32). The relatedness of Erm(44) with other described Erm proteins of *Staphylococcus* is presented in Fig. 1.

The new *erm*(44) gene encodes a 243-amino-acid (aa) protein. This protein contains an rRNA adenine dimethylase signature with the PROSITE pattern PS01131, which can be found in almost all described Erm methylases (12). Erm(44) is preceded by two

TABLE 1 MIC of erythromycin, clindamycin, and pristinamycin Ia 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
S. aureus								
80CR5	Recipient strain for conjugation, plasmid free	22		≤0.25	≤0.25	NA	4	NA
RN4220	Recipient strain for electrotransformation, plasmid free	21		0.25	≤0.25	NA	4	NA
RN4220/pBUS1	RN4220 with cloning vector pBUS1	28, this study	*tet*(L)	0.25	≤0.25	NA	4	NA
RN4220/pBUS1-P_{cap}[c]	RN4220 with pBUS1 containing *cap* promoter	S. Schwendener	*tet*(L)	0.25	≤0.25	NA	4	NA
RN4220/pBJW5	RN4220 with *erm*(44) and its regulatory region cloned into pBUS1	This study	*tet*(L), *erm*(44)	8	≤0.25	8	4	8
RN4220/pBJW13	RN4220 with *erm*(44) cloned into pBUS1-P_{cap}	This study	*tet*(L), *erm*(44)	>256	>256	>256	16	32
S. xylosus								
JW4341	Subclinical bovine mastitis milk	10, this study	*erm*(44), *mph*(C)	16	0.5	8	8	128
JW1049	Subclinical bovine mastitis milk	10, this study	*erm*(44)	16	1	8	16	256
JW3659	Subclinical bovine mastitis milk	This study	*erm*(44)	128	0.5	16	32	256
JW4305	Subclinical bovine mastitis milk	This study	*erm*(44)	32	0.5	8	16	256

[a] Antibiotic resistance genes and functions: *tet*(L), tetracycline efflux gene; *mph*(C), macrolide phosphotransferase gene; *erm*(44), 23S rRNA methylase gene.
[b] Abbreviations: 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); NA, not applicable.
[c] Vector pBUS1-P_{cap} is a pBUS1 derivate that harbors the *cap* promoter of the *S. aureus* type 1 capsular polysaccharide biosynthesis gene cluster.

small ORFs encoding leader peptide 1 (Lp1) of 14 aa and Lp2 of 17 aa, similar to the regulatory region of *erm*(A) in Tn*554* (33). Lp2 contains the IFVI motif, which is known to play a key role in the induction mechanism of *erm* genes (34). Putative −10 (TATTAT) and −35 (TTCAAT) promoter sequences were found 222 and 240 bp upstream of the *erm*(44) start codon, respectively. Two pairs of inverted repeats (IRs) (IR1, 5′-AAGTTCATTAT; IR2, 5′-ATAAT GAACTT; IR3, 5′-TTCGTTATGAT; IR4, 5′-ATATAATGAA) were found within the putative regulatory sequence of *erm*(44). In *erm*(C), a similar structure of two IR pairs within the leader region is known to regulate translational attenuation (35). The regulator sequences of *erm*(44), *erm*(A) (accession no. X03216), *erm*(C) (accession no. M19652), and *erm*(43) (accession no. HE650138) were aligned by using a fragment that includes the putative ribosomal binding site of Lp1 and the first 2 nucleotides (nt) after the start codon, which is 208 nt for *erm*(44). Overall, the putative translational attenuator region of *erm*(44) showed the closest identity (83%) with that of *erm*(43) in a 207-bp overlap (208 nt/207 nt).

Expression of *erm*(44). The functionality of Erm(44) was assessed by cloning *erm*(44) with its own regulatory sequence (pBJW5) and by cloning *erm*(44) solely downstream of the *cap* promoter (pBJW13) (Table 1). When *erm*(44) was expressed from plasmid pBJW5 in *S. aureus* RN4220, the erythromycin MIC increased 32-fold, while the MICs of clindamycin and pristinamycin Ia remained unchanged. Only after induction with erythromycin, resistance to clindamycin increased at least 32-fold and resistance to pristinamycin Ia doubled. The RN4220 transformants carrying pBJW13 expressed *erm*(44) constitutively and exhibited higher MICs, with a >1,024-fold increase in resistance to erythromycin and clindamycin and a 4-fold increase in resistance to pristinamycin Ia. RN4220 harboring the empty vectors remained susceptible to these antibiotics.

Even if situated above the CLSI resistance breakpoint for staphylococci, the erythromycin MIC of 16 μg/ml conferred by Erm(44) in *S. xylosus* JW4341 is low for a 23S rRNA methylase resistance mechanism. In the presence of Erm methylases like Erm(B) and Erm(C), which are the most frequent among coagulase-negative staphylococci (CoNS) from bovine mastitis, the erythromycin MICs were higher than 128 μg/ml (10, 36). The lower MIC generated by *erm*(44) is likely due to weak expression from its own regulatory sequence, since a higher MIC of >256 μg/ml was measured when *erm*(44) was under the control of the *cap* promoter in pBJW13. Furthermore, in *S. xylosus* strain JW3659, which contained a different *erm*(44) regulatory and promoter region, the MIC of erythromycin was higher at 128 μg/ml (see below).

Characterization of ΦJW4341-pro harboring *erm*(44). Alignment of the 737,222-bp contig containing *erm*(44) with the draft genomes of *S. xylosus* DMB3-Bh1 isolated from mouse feces (GenBank accession no. of contig 3, AURW01000003.1) and *S. xylosus* NJ from human nasal cavities (GenBank accession no. of contig 6, ANMR01000006.1) revealed that *erm*(44) is present on a 52,814-bp insert in JW4341, which is absent from the *erm*(44)-negative *S. xylosus* NJ and DMB3-Bh1 genomes.

This insert was integrated at a specific 19-bp (TTATAAGTCC CCTCGAAGG) core integration site (*attB*) situated between two housekeeping genes, one coding for a putative DNA-binding helix-turn-helix protein (WhiA) and the other coding for a putative caseinolytic protease (ClpP) (Fig. 2). In *S. xylosus* strains JW4341 and NJ, an additional *orf* (*orfA*) is present between the *whiA* gene and the *attB* site. Both *erm*(44)-negative strains NJ and DMB3-Bh1 contain only the empty *attB* site. In *S. xylosus* JW4341, two perfect copies of the *att* site (*attL* and *attR*) are present at each site of the insert.

Sequence analysis of this insert showed a G+C content of 32%, which is similar to that of *S. xylosus* and in the range of staphylococcal bacteriophages (19). Additionally, 29 of the 56 ORFs detected (see Table S1 in the supplemental material) in this insert could be assessed with regard to the genetic organization of the

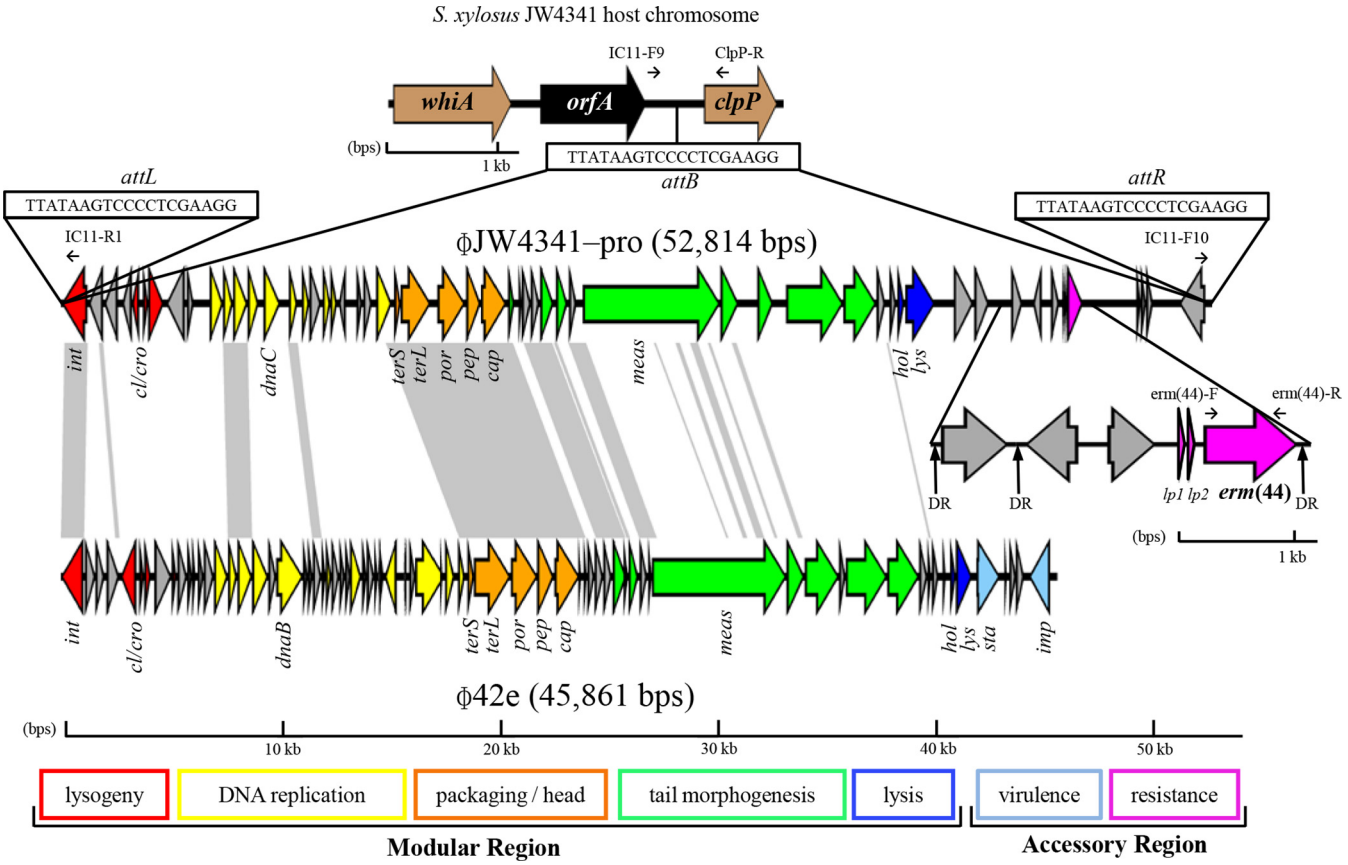

FIG 2 Schematic gene map showing *erm*(44)-containing *S. xylosus* prophage ΦJW4341-pro and its integration region (EMBL accession no. HG796218) and comparison with *S. aureus* Φ42e (accession no. AY954955), a related representative of the *Siphoviridae* family of phages. Gray areas represent high (>66%) similarity at the nucleotide level. Arrows represent the positions and orientation of ORFs. New MLS_B resistance gene *erm*(44) and its two leader peptide genes (*lp1* and *lp2*) are shown in pink. The 19-bp (TTATAAGTCCCCTCGAAGG) core integration site of *erm*(44)-containing bacteriophages is designated *attB* in the genome of *S. xylosus* and *attL* and *attR* at both sites of ΦJW4341-pro. The 29-bp DR (AGGCAGTCGTTAATTCGGCTGTCTTTTTT) flanking *erm*(44) in the prophage accessory region is indicated by upward-pointing black arrows. Selected putative gene products and functions: *whiA*, DNA-binding helix-turn-helix protein; *orfA*, hypothetical protein; *clpP*, caseinolytic protease; *int*, integrase; *cI*/*cro*, transcriptional regulators; *dnaB*/*dnaC*, DNA replication proteins; *terS* and *terL*, small- and large-subunit terminases, respectively; *por*, portal protein; *pep*, prohead peptidase; *cap*, major capsid protein; *meas*, tail tape measure protein; *hol*, holin; *lys*, lysin; *sta*, staphylokinase; *imp*, IMP dehydrogenase. Prophage modules are color coded as follows: lysogeny, red; DNA replication, yellow; DNA packaging and capsid morphogenesis, orange; tail morphogenesis, green; host cell lysis, blue; virulence, sky blue; resistance, pink; unattributed, gray. Primers used to detect *erm*(44) and ΦJW4341-pro insertion sites are indicated by small black horizontal arrows. This image was generated with the program Easyfig (47).

five functional modules of the family *Siphoviridae* (Fig. 1). This genetic structure suggested that this insert is the genome of a temperate siphoviral bacteriophage (18, 19). This putative *S. xylosus* prophage was designated ΦJW4341-pro. By using the *S. aureus* phage classification proposed by Kwan et al., which is based on genome size, comparative nucleotide and amino acid sequence analysis, and gene map organization with special regard to the bacteriophage head region, ΦJW4341-pro could be grouped into class II clade B of the family *Siphoviridae* (19). Comparative analysis of the nucleotide sequences of ΦJW4341-pro and *S. aureus* phage Φ42e (accession no. AY954955), a prototype of the class II, clade B group, revealed that both phages show the highest DNA sequence identity within the integrase gene (73%, nt 2482 to 3519 of the sequence with accession no. HG796218), within the DNA replication region (68%, nt 9772 to 10898), within the packaging and head region (73%, nt 17638 to 22817), and within the tail morphogenesis region (63%, nt 23511 to 26037), indicating that ΦJW4341-pro belongs to the same group (Fig. 2). ΦJW4341-pro has the peculiarity of containing the *erm*(44) gene within the accessory region, which is situated toward the *attR* site downstream of the putative lysis module. To date, more than 100 *Staphylococcus* phage and prophage DNA sequences have been deposited in GenBank. However, to our knowledge, none of them has been reported to contain an antibiotic resistance gene within the accessory region (37). Otherwise, some of the most relevant virulence factors are located in this area of the phage genomes (18). In staphylococci, bacteriophage-mediated transfer of antibiotic resistance occurred mostly by transducing plasmids or resistance islands (38–40). Nonetheless, other bacterial species have been found to harbor phages that contained a resistance gene after the insertion of a transposon. Transposon Tn*6215*, which contains *erm*(B), was found to be integrated into transducing phage ΦC2 of *C. difficile* (41), and a *mef*(A)-carrying Tn*1207*.1-like transposon was found in phage Φm46.1 in *S. pyogenes* (42). In contrast, no genes associated with transposable elements were detected in the ΦJW4341-pro accessory region. However, the *erm*(44)-flanking region contains three 29-bp direct repeats (DRs) (AGGCAGTCGTTAATTC GGCTGTCTTTTTT), two situated upstream and one situated

downstream of *erm*(44) (Fig. 2). DRs longer than 100 bp were suggested to be involved in the spread of antibiotic resistance genes independently of a recombinase (43). Whether these DRs play a role in the integration of *erm*(44) into ΦJW4341-pro is not known.

Prophages can form free circular DNA in the host cell, and circular excision is the first step of the phage lytic pathway (42, 44). ΦJW4341-pro was detected in circular form with the sole *att* as a joining region in its host *S. xylosus* JW4341, indicating potential lytic activity. Excision and site-specific integration of ΦJW4341-pro are likely to be catalyzed by the ΦJW4341-pro integrase (*orf1*), which was found to harbor conserved similarity regions of tyrosine recombinases, including a potential active-site tyrosine residue (45). Tyrosine recombinases are predominant integrase types in *S. aureus* phages, while both tyrosine and serine recombinases have been found equally in CoNS phages (37, 46).

Distribution of *erm*(44) in *S. xylosus* and ΦJW4341-pro relatives. After the discovery of *erm*(44) in *S. xylosus* JW4341, 410 additional *S. xylosus* strains isolated from bovine milk were screened for the presence of constitutive macrolide resistance and inducible clindamycin resistance by D-zone testing. Three unrelated D-zone test-positive strains, JW1049, JW3659, and JW4305, were identified, all originating from cows with subclinical mastitis. The strains showed a phenotype similar to that of JW4341 and exhibited erythromycin resistance and inducible clindamycin and pristinamycin IA resistance, as confirmed by MIC measurements (Table 1). JW1049, JW3659, and JW4305 all carried *erm*(44), and no known *erm* or other resistance genes were detected. Of note, macrolide resistance in *S. xylosus* from bovine mastitis has previously always been associated with drug efflux mediated by *msr* (10, 36).

S. xylosus JW3659 exhibited high resistance to erythromycin, with an MIC 8-fold higher than that for JW4341. Erm(44) of JW3659 (accession no. LK392593) differed from that of JW4341 by 14 amino acid changes (94% amino acid identity), and their leader peptides differed by three amino acid changes in Lp1 and one amino acid change in Lp2. Additionally, the putative −10 and −35 promoter sequences of *erm*(44) were different in *S. xylosus* JW3659 (TATAAT and TCCACC, respectively), which may explain the MIC differences, which are likely due to possible different expression levels.

In the three additional *erm*(44)-positive *S. xylosus* strains, JW1049, JW3659, and JW4305, an *erm*(44)-containing fragment was integrated into the chromosome at the same core integration site and duplication of the *att* sequence was observed, suggesting a structure related to that of ΦJW4341-pro. In addition, circular forms with the sole *att* sequence as a joining region were detected in all of these *erm*(44)-positive strains, indicating that the putative prophage is capable of excision from the genome.

Transfer experiments. Although a circular form of ΦJW4341-pro was detected, no transfer of *S. xylosus* JW4341 macrolide resistance was observed by either electrotransformation or conjugation with *S. aureus*. The absence of conjugative and transposable elements from ΦJW4341-pro explains the lack of transfer of *erm*(44) by conjugation. On the other hand, the inability to transform *S. aureus* with *erm*(44)-containing DNA may be due to either low transformation efficiency or a deficient integration mechanism in *S. aureus*. Further experiments are necessary to determine the lytic and transducing properties of ΦJW4341-pro. Nevertheless, detection of similar prophages carrying *erm*(44) at the same integration site in four unrelated *S. xylosus* strains from bovine mastitis milk strongly suggests that this prophage has the capacity of transduction *in vivo*.

Conclusion. Detection of a new prophage containing the novel *erm*(44) resistance gene once again showed the ability of staphylococci to acquire and spread antibiotic resistance genes by multifaceted mechanisms. This study contains the first description of an MLS_B resistance gene within a prophage in staphylococci, highlighting the fact that phages may act as vehicles and disseminate antibiotic resistance in this clinically important group of bacteria. The presence of such an antibiotic resistance-carrying prophage in *S. xylosus* from bovine mastitis milk is a further demonstration of the role of animal bacteria as a reservoir of novel genetic elements carrying antibiotic resistance genes.

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