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The diversity of inducible and constitutively expressed \textit{erm}(C) genes and association to different replicon types in staphylococci plasmids

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The aim of this study was to analyze the diversity of the macrolide resistance gene, \textit{erm}(C) in relation to structural alterations affecting the gene expression. In addition, the association of \textit{erm}(C) to mobile genetic elements (MGEs) in staphylococci mainly from Danish pigs was investigated. In total, 78 erythromycin-resistant isolates were screened for \textit{erm}(C) by PCR. The \textit{erm}(C) genes incl. the upstream regulatory region were sequenced and the expression types were characterized phenotypically (agar diffusion test) and genotypically (sequence analysis). Phylogenetic analysis of \textit{erm}(C) was compared with structural alterations affecting the gene expression. Plasmids carrying \textit{erm}(C) from seven selected isolates were fully or partially sequenced. Thirty-seven isolates were shown to be \textit{erm}(C) positive and \textit{erm}(C) from pigs were all constitutively expressed, mainly caused by different sized deletions (118, 111, 107, 70, 66, 16 and 3 bp) in the regulatory region. Duplication (63 bp) and substitutions were also found to cause a constitutive phenotype. Only one horse isolate had an inducible expression type. Phylogenetic analysis showed that structural alterations have happened in different \textit{erm}(C) allele groups and not only in one group. Furthermore \textit{erm}(C) was found mainly on plasmids (~2.4–8 kb) and gene sequence types correlated with plasmid replication (\textit{rep}) gene types. One \textit{erm}(C) type was linked to an IS257 element able to circularize. In conclusion, structural alterations giving rise to constitutive expression of \textit{erm}(C) have happened several times in the evolution of \textit{erm}(C). Interestingly, the diversity of \textit{erm}(C) appears to be linked to the plasmid type or MGE carrying the gene.

Introduction

Staphylococci are part of the natural skin flora and count for some of the most important veterinary pathogens, e.g., \textit{Staphylococcus aureus} and \textit{Staphylococcus hyicus}.

A very high prevalence of macrolide-lincosamide-streptogramin B (MLS\textsubscript{B}) resistance isolates has been found among staphylococcal isolates from animals, and the erythromycin ribosome methylase (\textit{erm}) gene \textit{erm}(C) is the most predominant MLS\textsubscript{B} resistance gene in staphylococcal isolates from both humans and animals. The \textit{erm}(C) gene has mostly been found on small multi-copy plasmids (2.3–2.5 kb), but also larger and more diverse plasmids (3.7–4 kb) with mobilization (\textit{mob}) and/or plasmid recombination (\textit{pre}) genes have been reported.

The expression of \textit{erm}(C) can be either inducible or constitutive. Macrolide antibiotics are characterized by a lactone ring containing 12–16 members, but only 14- and 15-membered macrolides like erythromycin can induce \textit{erm}(C) expression by translational attenuation.

Inducer, IR1 pairs with IR2 and IR3 pairs with IR4 in a two loop structure which renders the \textit{erm}(C) start codon non-accessible to the ribosome, and only a leader peptide located just upstream of \textit{erm}(C) is translated. Upon induction, inducers bind to the ribosome translating the leader, which leads to alteration in the mRNA secondary structure so that IR2 pairs with IR3, the \textit{erm}(C) start codon becomes accessible, and \textit{erm}(C) is translated. Structural alterations within the upstream region of \textit{erm}(C) can result in constitutive expression of \textit{erm}(C) which also confers resistance to 16-membered macrolides (e.g., tylosin) and lincosamides, streptogramin B and ketolides.

Clinical reports and in vitro studies have shown that the gene expression can change from inducible to constitutive under selective pressure of non inducers. Although the use of non-inducing growth promoters such as tylosin was discontinued in Denmark after 1998, tylosin is still the predominant macrolide used for therapeutic treatment of infections in pigs. Alterations causing constitutive expression of \textit{erm}(C) are believed to be a result of high concentrations of non-inducible macrolides like e.g., tylosin in the environment.

The literature contains a number of reports of structural alterations detected in the upstream regulatory region of...
erm(C). Until now, three different types have been identified (Fig. 1): Sequence deletions of varying length, duplications of parts of the erm(C) gene as well as multiple point mutations. However, no one has studied the evolution of the erm(C) gene in comparison with different structural alterations causing a constitutive gene expression, and how the gene diversity is related to associated mobile genetic elements (MGEs). Thus, the aim of this study was to analyze the diversity of erm(C) in relation to structural alterations affecting the gene expression, and to characterize associated MGEs in staphylococci mainly from Danish pigs. Specifically, erythromycin resistant isolates were screened for the erm(C) gene, and a phenotypic test revealed the erm(C) expression types. Sequence analysis identified the genetic background for observed phenotypes. Finally, phylogenetic analysis of the erm(C) gene were compared with structural alterations causing a constitutive phenotype and with replicon types (rep gene) found on erm(C) carrying plasmids.

Results

Screening S. aureus and S. hyicus isolates for erm(C). Out of 78 erythromycin-resistant staphylococcal isolates, 16 S. aureus and 21 S. hyicus isolates were shown to be positive for erm(C) (all 37 isolates are listed in Table 1). The highest prevalence of erm(C) was found among S. aureus isolates from pigs, with 60.9% compared with 40.4% among S. hyicus isolates. Expression types for erm(C). Agar diffusion tests showed that only one S. aureus horse isolate (7504026-1) had an inducible clindamycin resistance phenotype (Fig. S1), and sequence analysis showed the upstream regulatory region of erm(C) to be identical or highly similar to corresponding regions from isolates known to have an inducible phenotype: S. aureus pT48 (GenBank, M19652), pE5 (GenBank, M17990), pWG738 (GenBank, DQ088624), pE194 (GenBank, V01278), S. hominis pSE55 (GenBank, Y09001) and S. lentus pSTE2 (GenBank, AJ888003). For these sequences, IR1:IR2 (ΔG = -44.43 kJ/mol, -10.62 kcal/mol) and IR3:IR4 (ΔG = -52.72 kJ/mol, -12.60 kcal/mol) were predicted to pair in the absence of an inducer with a total change in Gibbs free energy of ΔG_total = -97.15 kJ/mol (-23.22 kcal/mol) (Fig. 1A). In the presence of an inducer, IR3 and IR4 were predicted to pair, ΔG = -66.11 kJ/mol (-15.80 kcal/mol) (Fig. 1A).

Thirty-six S. hyicus and S. aureus isolates (35 from pig and 1 from horse isolates) had a constitutively expressed clindamycin resistance phenotype (Table 1; Fig. S1). Sequence analysis of the upstream regulatory region showed that 31 isolates contained different size deletions (118 bp, 111 bp, 107 bp, 70 bp, 66 bp, 16 bp and 3 bp), predicted to result in alternative secondary structures of the erm(C) mRNA, leaving IR4 and SD2 accessible for translation of erm(C) (Fig. 1B). Observed deletions of size, 118 bp, 111 bp and 107 bp included IR1-IR3. Deletions of size 70 bp, 58 bp and 66 bp only included IR1, and pairing of IR2 with IR3 were predicted to be more stable (ΔG = -66.11 kJ/mol, -15.80 kcal/mol) than the pairing of IR3 with IR4 (ΔG = -57.24 kJ/mol, -12.60 kcal/mol) (Fig. 1A and B). Finally, deletions of size 6 bp and 3 bp included all or part of IR3. No pairing of IR3 containing a 3 bp deletion with IR4 was predicted, since the calculated change in Gibbs free energy (ΔG = -15.90 kJ/mol, -3.80 kcal/mol) was much higher than the threshold for pairing (< -41.84 kJ/mol, -10 kcal/mol) (Fig. 1B).

However, four S. hyicus isolates (9805143-1, 9811071-1, 7630009-4, 7430116-4) contained an upstream erm(C) region with a complete leader sequence and four IR sequences, these were shown to have a constitutive phenotype. An agar disk diffusion test of an E. faecalis recipient (JH2-2) transformed with the plasmid carrying erm(C) from one of these four S. hyicus isolates (9811071-1), ruled out the possibility that another gene could have caused the observed constitutive phenotype. The sequence analysis showed that, for these four isolates, the upstream erm(C) region contained four IR sequences with a few substitutions (Fig. 1C) that have not been characterized previously, and these substitutions could explain the observed phenotype. The pairing of IR2:IR3 was predicted (ΔG = -83.68 kJ/mol, -20.00 kcal/mol) instead of IR1:IR2 and IR3:IR4, thus leaving the IR4 and SD2 accessible for translation of erm(C) (Fig. 1C). Finally, one isolate that was lost (S. aureus 65–5) could not be tested phenotypically, but sequence analysis showed it to contain a 63 bp duplication including an additional SD2 /IR4' in the upstream regulatory region (Fig. 1D). This erm(C) gene was predicted to be constitutively expressed by the pairing of IR1:IR2 (ΔG = -49.04 kJ/mol, -11.72 kcal/mol) and IR3:IR4' (ΔG = -52.71 kJ/mol, -12.60 kcal/mol) with a total change in Gibbs free energy of ΔG_total = -101.75 kJ/mol (-24.32 kcal/mol) and leaving IR4 and the erm(C) start site accessible for translation (Fig. 1C).
Table 1. Staphylococci strains carrying \textit{erm}(C) used in this study

| Strain       | Species | Source /year | Other resistance phenotypes | \textit{erm}(C) plasmid or DNA ring$^1$ | \textit{erm}(C) phylogenetic group$^2$ | Expression type of \textit{erm}(C)$^3$ | GenBank accession number |
|--------------|---------|--------------|-----------------------------|----------------------------------------|---------------------------------------|--------------------------------------|-------------------------|
| 9710013-1    | \textit{S. hyicus} | pig/1997     | STR                         | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968519                |
| 9730769-3    | \textit{S. hyicus} | pig/1997     | PEN, SPT, STR, SUL, TMP     | 1.7 kb                                  | 2                                     | Constitutive                       | JF968541                |
| 9805143-1    | \textit{S. hyicus} | pig/1998     | PEN, STR, SUL, TET, TMP     | 3.7–4 kb                                | 1b                                    | Constitutive                       | JF968536                |
| 9805598-1*   | \textit{S. hyicus} | pig/1998     | -                           | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968536                |
| 9810320-1    | \textit{S. hyicus} | pig/1998     | PEN, STR, TET, TMP          | 1.7 kb                                  | 2                                     | Constitutive                       | JF968538                |
| 9811071-1    | \textit{S. hyicus} | pig/1998     | PEN, SUL, TET, TMP          | 3.7–4 kb                                | 1b                                    | Constitutive                       | JF968540                |
| 9831219-1*   | \textit{S. hyicus} | pig/1998     | -                           | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968524                |
| 9905227-1*   | \textit{S. hyicus} | pig/1999     | PEN, STR                    | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968528                |
| 9911527-1    | \textit{S. hyicus} | pig/1999     | -                           | 2.4 kb                                  | 4b                                    | Constitutive                       | JF968522                |
| 9911757-1*   | \textit{S. hyicus} | pig/1999     | PEN                         | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968529                |
| 7213504-1    | \textit{S. hyicus} | pig/2000     | CIP, PEN, STR, TET, TMP     | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968521                |
| 7231274-2    | \textit{S. hyicus} | pig/2000     | -                           | 2.4 kb                                  | 4c                                    | Constitutive                       | JF968517                |
| 7313178-1    | \textit{S. hyicus} | pig/2001     | TET                         | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968543                |
| 7313624-1    | \textit{S. hyicus} | pig/2001     | PEN, TET                    | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968520                |
| 7330561-6    | \textit{S. hyicus} | pig/2001     | PEN, SPT, STR, SUL, TET, TIA| 2.4 kb                                  | 4a                                    | Constitutive                       | JF968514                |
| 7410443-1    | \textit{S. hyicus} | pig/2002     | PEN, SPT, STR               | 2.4 kb                                  | 4c                                    | Constitutive                       | JF968515                |
| 7411659-1    | \textit{S. hyicus} | pig/2002     | CIP, PEN, STR               | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968516                |
| 7430116-4    | \textit{S. hyicus} | pig/2002     | PEN                         | 3.7–4 kb                                | 1b                                    | Constitutive                       | JF968534                |
| 7510871-1    | \textit{S. hyicus} | pig/2003     | PEN, STR                    | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968531                |
| 7514773-1    | \textit{S. hyicus} | pig/2003     | PEN, TET                    | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968518                |
| 7630009-4    | \textit{S. hyicus} | pig/2004     | PEN, STR                    | 3.7–4 kb                                | 1b                                    | Constitutive                       | JF968535                |
| 7311242-1    | \textit{S. aureus} CC398 | pig/2001 | SPT, STR, TET               | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968512                |
| 7312429-1    | \textit{S. aureus} | pig/2001     | SPT                         | 2.4 kb                                  | 4c                                    | Constitutive                       | JF968510                |
| 7411141-1    | \textit{S. aureus} CC9 | pig/2002 | PEN, TET                    | 4 kb                                    | 3                                     | Constitutive                       | JF968526                |
| 7413727-1    | \textit{S. aureus} CC398 | pig/2002 | PEN, TET                    | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968513                |
| 7414035-2    | \textit{S. aureus} CC398 | pig/2002 | PEN, STR, TET               | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968509                |
| 7511314-2    | \textit{S. aureus} CC398 | pig/2003 | SPT, TET, TEMP              | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968508                |
| 7512166-1    | \textit{S. aureus} CC398 | pig/2003 | PEN, STR, TET, TEMP         | 7–8 kb                                  | 1a                                    | Constitutive                       | JF968537                |
| 7512986-1    | \textit{S. aureus} CC398 | pig/2003 | SPT, TET                    | 4 kb                                    | 3                                     | Constitutive                       | JF968527                |
| 7504026-1    | \textit{S. aureus} CC30 | horse/2003 | PEN, STR                    | 2.4 kb                                  | 4                                     | Inducible                          | JF968532                |
| 7612628-4    | \textit{S. aureus} CC398 | pig/2004 | SPT, TET                    | 7–8 kb                                  | 1a                                    | Constitutive                       | JF968539                |
| 200610584-1  | \textit{S. aureus} CC9 | pig/2006 | PEN, TET, TEMP              | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968530                |
| 200640995-1  | \textit{S. aureus} | horse/2006   | PEN                         | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968511                |
| 65-5         | \textit{S. aureus} | pig/2007     | ND                          | ND                                      | 4a                                    | Constitutive                       | JF968533                |
| 66-1         | \textit{S. aureus} CC9 | pig/2007 | -                           | 2.4 kb                                  | 4a                                    | Constitutive                       | JF968507                |
| 71-1         | \textit{S. aureus} CC398 | pig/2007 | PEN, TET, TEMP              | 4 kb                                    | 3                                     | Constitutive                       | JF968525                |
| 9b           | \textit{S. aureus} CC398 | pig/2007 | CEF, PEN, SPT, STR, TET, TEM| 2.4 kb                                  | 4a                                    | Constitutive                       | JF968542                |

$^1$ Determined as the approximate size of the PCR product generated with primers pointing out of the \textit{erm}(C) gene (primers 2020 and 2021 or 2022).

$^2$ Determined by a phylogenetic analysis (Fig. 2). $^3$ Determined by agar diffusion test, except for 65-1 that was predicted to be constitutively expressed. $^{*}$ PFGE pattern of 9805598-1 was shown to be identical with 9831219-1 and the pattern of 9905227-1 was shown to be identical with 9911757-1 (Fig. S5). $^{5}$ Type that could be deduced from \textit{spa} types (Table S1). Isolates predicted to be of CC398 represented different related \textit{spa} types such as t034, t2876, t571. CEF, Ceftiofur; CIP, Ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; FLO, florfenicol; PEN, penicillin; SPE, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TIA, tiamulin; TMP, trimethoprim; ND, not determined.
Phylogenetic analysis of \( \text{erm}(C) \) compared with expression types. Comparing the \( \text{erm}(C) \) gene and the corresponding amino acid sequences from all 37 staphylococcal isolates revealed 7 unique sequence types both on DNA and amino acid level. A phylogenetic analysis of the 37 \( \text{erm}(C) \) gene sequences and 19 \( \text{erm}(C) \) GenBank sequences divided them into four \( \text{erm}(C) \) groups (Fig. 2). A comparison of the corresponding amino acid sequences also resulted in the same grouping of four Erm(C) groups (data not shown). As illustrated in Figure 2, all 56 \( \text{erm}(C) \) gene sequences were highly similar (95.1–100% DNA identity). The \( \text{erm}(C) \) sequences within groups 2, 3 and 4 were highly related (98.8–99% DNA identity) whereas \( \text{erm}(C) \) of group 1 were more distinct (95.1–95.9% DNA identity) compared with the other groups.

The phylogenetic tree showed structural alterations in the \( \text{erm}(C) \) upstream regulatory region to be located in all four \( \text{erm}(C) \) groups (Fig. 2). Interestingly, inducible \( \text{erm}(C) \) genes were only present within \( \text{erm}(C) \) group 3 and 4 (e.g., V01278; pE194 and M19652;pT48/ M117990; pE5) (Fig. 2). Thus complete regulatory sequences of inducible \( \text{erm}(C) \) genes have not been reported for sequences belonging to \( \text{erm}(C) \) group 1 and 2. Overall, deletions appear to be the most common form of alteration causing a constitutive \( \text{erm}(C) \) expression and specific type/sizes of deletions appear to be specific for the different \( \text{erm}(C) \) groups. Only deletions of 16 bp were present in more than one of the \( \text{erm}(C) \) groups; the highly related \( \text{erm}(C) \) group 3 and 4 (Fig. 2). Altogether, this showed that structural alterations...
giving rise to constitutive expression have happened in different allele groups of erm(C) as opposed to in one type only.

The erm(C) gene sequences are linked to the plasmid carrying the gene. In order to test whether the sequenced erm(C) genes were located on small plasmids, which is most often the case, 5, 9 PCR reactions using primers pointing in opposite directions out of erm(C) (Fig. S2), were performed. If erm(C) was located on a plasmid or a circular DNA molecule, PCR products would be generated also when the primers were pointing in opposite directions. In this case, different size PCR products were amplified for 36 isolates (except S. aureus 65-5 that was lost during the study), and the product sizes were shown to correlate with the four erm(C) groups (Fig. 2; Table 1). Full or partial sequencing of these PCR products, representing each of the four groups, showed erm(C) of group 1, 3 and 4 to be located on rep containing plasmids of approximately 2.4–8 kb, whereas erm(C) of group 2 was associated with a IS257-like transposase containing element of 1.7 kb (Fig. S3).

In order to study the relationship between erm(C) and associated plasmids, a phylogenetic tree based on sequenced rep genes from this study and rep genes from erm(C) carrying plasmids available from GenBank was constructed. The rep genes were divided into three main groups of repU, repE and repL corresponding to erm(C) phylogenetic group 1, 3 and 4 respectively (Fig. 2; Fig. S4). Thus, the erm(C) gene sequences appear to be linked to the plasmid (or the mobile element) carrying them.

Discussion

The screening of erythromycin resistant staphylococci showed approximately half of the pig isolates to contain the erm(C) gene (60.9% of S. aureus and 40.4% of S. hyicus) which is in overall agreement with former prevalence studies.2, 3, 21

Phenotypic testing revealed all pig isolates containing erm(C) to be constitutively expressed, and only one horse isolate was shown to contain an inducible erm(C) gene. In environments with high concentrations of non-inducing macrolides (e.g., tylosin), staphylococci with constitutively expressed erm(C) genes are believed to have a selective advantage both to sensitive staphylococci and to staphylococci containing a regulated erm(C) gene.5, 16 Macrolides are one of the most commonly used antimicrobial agents for therapeutic treatment of infections in pigs in Denmark, and tylosin is the predominant macrolide used. Also large amounts of non-inducing lincosamides are used in pigs.20 In 2008, a total of 14,181 kg macrolides and lincosamides were sold for therapeutic use in horses compared with 1.418 kg sold for the use in pigs.20 Although, this would have to be investigated further in a study designed to compare expression types between different animal reservoirs.

In this study, we identified structural alterations causing constitutive expression of erm(C) that have not previously been reported. These included deletions of 3 bp, 66 bp, 118 bp, a duplication of 63 bp, and new substitutions within the IR sequences. The latter alteration was verified to be responsible for the observed phenotype by transforming the S. hyicus plasmid carrying erm(C) (9811071-1) into an Enterococcus recipient strain. This strongly suggests that the erm(C) located on the S. hyicus plasmid was responsible for the observed phenotype, even if erm(C) induction might work slightly different in different genera.

The phylogenetic analysis illustrated that different types (sizes) of structural alterations causing a constitutive erm(C) expression may appear to be specific for the erm(C) groups, with the exception of 16 bp deletions that have occurred both in erm(C) groups 3 and 4. However, a larger test population would be required to draw any broader conclusions from the observed trend. A former study showed the recombination system of a host cell to play a role in the development of different types of structural alterations associated with constitutive erm(C) gene expression, and a model for the development of deletions in the erm(C) regulatory region by homologous recombination suggested the sequence region beginning just before IR3 until SD2 to be involved in a 16 bp deletion.14 The sequence in this region is identical in erm(C) group 2, 3 and 4 but differs from erm(C) group 1 which explains why 16 bp deletions have been observed in erm(C) group 3 and 4, but not in group 1. Replication slippage or illegitimate recombination may result in tandem duplications observed in the erm(C) translational attenuator.15 Altogether, our results showed that different types of alterations causing constitutive expression of erm(C) have happened in different erm(C) attenuators, and that the size and/or type of alterations that have happened appear to depend mainly on the sequence surrounding the alteration. However, it has also been suggested that the frequency and type of alteration may depend on the selecting antimicrobial agent.22
Interestingly, this study show that the erm(C) gene sequences are linked to the replicon type of the plasmid (or IS element) carrying the gene. We found erm(C) to be located mainly (25/36 isolates) on small repL containing plasmids of approx. 2.4 kb, but we also identified erm(C) on larger repF plasmids of approx. 3.8 kb (3/36 isolates) and on repU plasmids of approx. 4–8 kb (6/36 isolates), also containing a prel mob gene. In addition, erm(C) was also found (for 2/36 isolates) to be associated with an IS257 transposase (also known as IS431), an active staphylococcal insertion sequence previously found to be associated with other resistance genes in staphylococcus species, but this is the first report of erm(C) associated with IS257. Regarding the gene regulations, it is worth noting that no complete regulatory sequence of an inducible erm(C) has been reported for erm(C) group 1 and 2 linked to repU plasmids and IS257 transposase elements, respectively. Thus it is not known whether possible structural alterations conferring constitutive expression for these two erm(C) types may have occurred before or after the genes were associated with their respective MGE. In fact, it remains to be proven whether these two erm(C) types have actually developed from an inducible gene type at all.

As illustrated by the phylogenetic tree (Fig. 2), erm(C) encoding plasmids have been detected in different staphylococcal species from various animal sources and also from humans. These plasmids can be spread by mobilization between members of different staphylococcal species, but also between staphylococci and bacillus. Most of the small erm(C) carrying plasmids, however, do not encode any mobilization genes. In correlation with this, the phylogenetic erm(C) tree illustrates that within erm(C) group 4, three subgroups of identical erm(C) sequences with identical size deletions in the upstream region have been detected both in S. aureus and S. hyicus (16 bp and 111 bp deletion) and S. aureus, Bacillus subtilis and Neisseria meningitides (107 bp deletion). Thus, horizontal transfer of erm(C) on small plasmids without genes encoding mobilization has not only occurred between different species of staphylococci but also between bacteria from different genera. Such a transfer may have occurred by transduction or transformation, or alternatively mobilization proteins encoded by other plasmids may facilitate mobilization of small erm(C) plasmids carrying an oriT. The dissemination is believed to be controlled by the plasmids stability in the new host or alternatively by the occurrence of chromosome integration, which appears to be the case for erm(C) observed in Neisseria meningitides. In this study we also identified erm(C) on a mobilizable plasmid (repU) containing a prel mob gene in S. hyicus. This plasmid was related to previously reported plasmids from Gram positive bacteria; with a prel mob gene identical to pUB10, pC16, pG01, pSK41 and a recombin-ation site, RSA (oriT) identical to a corresponding site in S. saprophyticus plasmid pSES22 (AM159501). Interestingly, an almost identical region (1534 bp) from this S. hyicus plasmid, including erm(C) and starting just before the RSA (data not shown) has also been detected on a large (20 kb) plasmid from Lactobacillus reuteri isolated from a pig in the 1970s. This strongly suggests that exchange of erm(C) between Lactobacillus and Staphylococcus has occurred. Finally, the finding of erm(C) associated with an IS257 element on a circular DNA element without any rep gene suggests a large mobilization potential for this erm(C) gene, since it appears to have the ability to integrate both in chromosomal, transposon and plasmids sites containing IS257 elements.

In summary, we have shown that erm(C) genes in Danish staphylococci mainly from pigs are constitutively expressed, which is mainly caused by deletions in the regulatory region. Alterations giving rise to constitutive expression have happened in different allele groups of erm(C) and not only in one type, and the type and/or size appear to be linked to the sequence surrounding the alteration. Furthermore, erm(C) was found to be located mainly on small plasmids, and the gene sequence was shown to be linked to the plasmid or the element carrying the gene. Altogether this suggests the different erm(C) carrying plasmids found in staphylococci species until now have evolved independently.

**Materials and Methods**

**Strains.** The 78 erythromycin resistant isolates from Danish pigs, horses and lamb used in this study were identified as S. aureus or S. hyicus as described previously. Different clones were represented among the studied isolates as shown by spa typing or PFGE typing (Table S1; Fig. S5). All of the 52 S. hyicus and 20 of the 26 S. aureus were different diagnostic submissions to the National Food Institute, DTU (1997–2006), which originated from farms all across Denmark. Six S. aureus isolates were obtained from different healthy pigs from at least two different farms (2007). As part of the standard procedure at DTU, all isolates were tested for susceptibility to cefotiofur, chloramphenicol, erythromycin, florfenicol, penicillin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim by using the broth microdilution Sensititer method (Trek Diagnostic Systems Ltd, UK) as described previously and following CLSI guidelines (Table 1). Due to a change in the standard procedure, strains isolated from 2000 and later were also tested for susceptibility to tiamulin and ciprofloxacin (Table 1). Two isolates (9b and 71-1) were positive for a mecA screening PCR, showing them to be methicillin-resistant S. aureus (MRSA) (http://www.crl-ar.eu/data/images/meca-PCR_protocol%2006.02.08.pdf).

**PCR and sequencing.** All the 78 erythromycin resistant staphylococcal isolates were screened for erm(C) by PCR as described previously using DNA Taq polymerase (Ampliqon, Denmark) and the primers 28 and 29 (Table S2). The Bacillus subtilis strain B.3HU104 containing erm(C) on the plasmid pE194 was used as a positive control for all PCR reactions. The erm(C) gene including an upstream regulatory region (766-947 bp) of the 37 staphylococcal isolates that were positive for erm(C) (Table 1), was sequenced by Macrogen, Korea (www.macrogen.com/eng/sequencing/sequence_main.jsp) as outlined in Figure S2.

PCR reactions using the primers 2020 and 2021 or 2022 pointing in opposite directions out of erm(C) (Fig. S2) were performed for 36 isolates (all isolates that were positive for erm(C), except for S. aureus 65-5). PhusionTM High-Fidelity DNA Polymerase (Finnzymes, Finland) was used with conditions...
recommended by the manufacturer. Such PCR products were completely or partially sequenced for seven isolates which were selected to represent different ermA(C) phylogenetic groups as determined by the phylogenetic analysis: For three S. hyicus (7313178-1, 9730769-3, 9811071-1) and two S. aureus (9b, 7313242-9) isolates, the PCR products were completely sequenced, and for two S. aureus (7512984-1, 7612628-4) the PCR products were partially sequenced. The following combinations of sequencing primers were used: For isolates 7512984-1 and 9730769-3, primers 2019, 2022 and ned234 were used, for isolates 7313178-1 and 9b, primers 2020, 2022, ned234 and gr4ned_b, for isolate 9811071-1, primers 2020, 2021, op1SH, ned1, gr1upSH_b and gr1nedSH_SA_b and finally for isolate 7612628-4, primers 2020, 2021, gr1nedSH_SA_b and op1SA were used. All sequences were assembled, annotated and visualized in Vector NTI (Invitrogen). All primers used in this study are listed in Table S2.

**Sequence analysis.** GenBank was searched for full length ermA(C) genes based on the definition that the ermA(C) gene shares \( \geq 80\% \) similarity on the amino acid level.\(^4\) Nineteen gene sequences were selected based on the following criteria; the upstream regulatory sequence should be sequenced and the sequences should be published in a paper containing information about the phenotypic ermA(C) expression type. A neighbor-joining (NJ) tree based on a multiple alignment of the 37 ermA(C) gene sequences (735 bp) obtained in this study and 19 ermA(C) genes from GenBank was constructed in Clustal X\(^3\) and visualized by MEGA 4.0.2.\(^3\) The tree was rooted with the S. aureus ermA(B) gene (GenBank, AB300568) as outgroup. Another NJ tree based on 5 replication (rep) genes that were identified on the full or partial sequenced ermA(C) plasmids from this study and 14 rep genes located on full or partial ermA(C) containing plasmids deposit in GenBank, was constructed as described above.

Sequence analysis of the upstream region of ermA(C) was performed for the 37 isolates listed in Table 1 by alignment in ClustalX\(^3\) and manual checking and editing of alignments in Bioedit version 7.0.0.\(^4\) Changes in Gibbs free energy (\( \Delta G \)) for predicted pairing of IR sequences in the ermA(C) mRNA were calculated with the program RNAfold (http://bibiserv.techfak.uni-bielefeld.de/rnafold/submission.html) using sequence regions containing the specific IRs as queries.\(^4\)\(^-\)\(^4\)\(^3\) Pairing of IR sequences were predicted for \( \Delta G < -10 \text{ kcal/mol} (\sim -41.84 \text{ kJ/mol}) \). All pairwise sequence comparisons were performed with the EMBOSS program water (local alignments) and/or needle (global alignments) (http://www.ebi.ac.uk/emboss/align).\(^4\)\(^4\)

**Transforming a ermA(C) plasmids into a recipient strain.** Plasmid purification from one S. hyicus isolate (9811071-1) was performed using QIAfilter Plasmid Midi Kit (Qiagen). Electrococent Enterococcus faecalis, JH2-2RF cells were transformed with purified S. hyicus plasmid (9811071-1) or with an Enterococcus erythromycin resistant plasmid, PAT18 (positive control), as described previously.\(^4\)\(^5\) Transformants were selected on brain heart infusion (BHI) agar plates (Becton, Dickinson and Company, USA), supplemented with 12.5 mg/L of rifampicin and 12.5 mg/L of fusidic acid and either 4 mg/L or 8 mg/L erythromycin. Altogether, 10 transformants (five from each the BHI plates with 4 or 8 mg/L erythromycin, respectively) were selected and confirmed to carry a plasmid borne ermA(C) by two PCR sets; one ermA(C) PCR screen using primers 28 and 29, and a long PCR using the primers 2020 and 2021 pointing out of the ermA(C) gene. For one transformant (JH2-2-9811071-1 B1-RFE), the PCR product from the long PCR (primer 2020–2021) was partially sequenced with primer 2021, and the upstream region of ermA(C) was confirmed to be identical with the corresponding region from donor S. hyicus (9811071-1) ermA(C) plasmid.

**Agar diffusion test of ermA(C) expression types.** For 36 staphylococcal isolates (all isolates listed in Table 1, except 65-5) and the 10 E. faecalis transformants (inclusive JH2-2-9811071-1 B1-RFE), expression types were tested by an agar disk diffusion test, as described previously and in accordance with the standard CLSI disk diffusion method.\(^4\)\(^6\)\(^-\)\(^7\) For each of the tested strains, an erythromycin disc (15 \( \mu \)g) was placed in the middle surrounded by two clindamycin discs (2 \( \mu \)g) (lincosamide antibiotic) within a distance of 1–2 cm on a müller-hinton plate streaked with a standard 0.5 McFarland inoculum suspension. Plates were incubated for 16 to 18 h at 37°C. Flattening of the clindamycin zone adjacent to the erythromycin disc (a “D-zone”) was considered proof for an inducible expression type.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental material may be found here: www.landesbioscience.com/journals/mge/article/20109

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