Molecular characterization of ketolide-resistant \textit{erm}(A)-carrying \textit{Staphylococcus aureus} isolates selected \textit{in vitro} by telithromycin, ABT-773, quinupristin and clindamycin

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The aim of this study was to investigate whether a \textit{Staphylococcus aureus} strain that carried an inducibly expressed \textit{erm}(A) gene might exhibit resistance to the non-inducers telithromycin, ABT-773, clindamycin, quinupristin, dalfopristin or the combination quinupristin–dalfopristin after incubation in the presence of inhibitory concentrations of any of these compounds. Whenever resistant mutants were obtained, these were investigated for the molecular basis of the altered resistance phenotype. Resistant mutants were not selected with dalfopristin or quinupristin–dalfopristin, but were obtained with the other four agents. Irrespective of which drug was used for selection, all mutants were cross-resistant to clindamycin, quinupristin, telithromycin and ABT-773, and exhibited structural alterations in the \textit{erm}(A) translational attenuator. The structural alterations observed included deletions of 14, 83, 131, 147 or 157 bp, three different tandem duplications of 23, 25 or 26 bp, two different types of point mutation, as well as the insertion of IS\textsubscript{256}. All these alterations either completely prevented the formation of mRNA secondary structures in the \textit{erm}(A) regulatory region or favoured the formation of those mRNA secondary structures that allowed translation of the \textit{erm}(A) transcripts. Deletions, which were observed in almost two-thirds of the mutants, might be explained by illegitimate recombination between different parts of the \textit{erm}(A) regulatory region.

Introduction

Ribosome methylases of the five classes A, B, C, F and Y are known to mediate resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS\textsubscript{B}) in staphylococci.\textsuperscript{1–3} While methylases of the classes B, F and Y have been detected only rarely,\textsuperscript{1,3} those of classes A and C are predominant among staphylococci of human and animal sources.\textsuperscript{4–7} Expression of the respective genes, \textit{erm}(A) and \textit{erm}(C), is either inducible by only 14- and 15-membered macrolides or constitutive.\textsuperscript{2–10} Inducible and constitutive resistance mediated by the same \textit{erm} gene is associated with different resistance phenotypes.\textsuperscript{2,7–10} Strains carrying inducibly expressed \textit{erm}(A) or \textit{erm}(C) genes show initially only resistance to the inducers (M\textsubscript{14–15} phenotype), while those harbouring constitutively expressed \textit{erm}(A) or \textit{erm}(C) genes show resistance to all macrolides, lincosamides and streptogramin B antibiotics (M\textsubscript{14–16LS\textsubscript{B}} phenotype).\textsuperscript{2,8} Sixteen-membered ring macrolides, lincosamides, streptogramins and also the new ketolides do not exhibit inducing properties for these two genes.\textsuperscript{7,8,11,12} Previous studies revealed that inducible expression of \textit{erm}(A) or \textit{erm}(C) will quickly and irreversibly turn into constitutive expression when the corresponding strains are cultivated in the presence of non-inducers, such as the 16-membered macrolide tylosin or the lincosamide clindamycin.\textsuperscript{7,13,14}

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Little is known about whether other antimicrobial agents such as the new ketolides, streptogramin A, streptogramin B, or the combination of streptogramin A and B compounds are also able to cause a conversion of the type of \textit{erm} gene expression.

The aim of the present study was to cultivate a \textit{Staphylococcus aureus} strain that carried an inducibly expressed \textit{erm}(A) gene in the presence of the lincosamide clindamycin, the streptogramin A dalfopristin, the streptogramin B quinupristin, the combination quinupristin–dalfopristin, or the ketolides telithromycin and ABT-773, and to analyse the resulting resistant mutants—if available—for the genetic basis of this altered resistance phenotype.

\textbf{Material and methods}

\textit{Bacterial strain, MIC determination and in vitro selection experiments}

The \textit{S. aureus} strain SA1 was obtained from a blood culture and carried a single chromosomal copy of an inducibly expressed \textit{erm}(A) gene indistinguishable from that of Tn554.\cite{6,15,16} The MICs of erythromycin, clindamycin, dalfopristin, quinupristin, quinupristin–dalfopristin, telithromycin and ABT-773 for \textit{S. aureus} SA1 were determined by the broth microdilution method following NCCLS guidelines.\cite{17} Plate selection experiments were carried out as described earlier.\cite{18} In brief, in \textit{vitro} selection was performed by spreading $10^9$ to $10^{10}$ cfu/mL of \textit{S. aureus} SA1 on blood agar plates containing clindamycin 1 mg/L, dalfopristin 2 mg/L, quinupristin 1 mg/L, quinupristin–dalfopristin 0.5 mg/L, telithromycin 0.5 mg/L or ABT-773 0.25 mg/L. These concentrations corresponded approximately to 4 $\times$ MIC for the original \textit{S. aureus} strain SA1. After overnight incubation at 37°C, the frequency of appearance of mutants with elevated MICs of telithromycin, ABT-773, quinupristin or clindamycin was calculated as the ratio of mutants arising divided by the number of cfu originally inoculated.\cite{18} Plate selection experiments were repeated twice on independent occasions. Of the mutants observed, c. 10–20\% were subsequently used for PCR and sequence analysis. All selected mutants were typed using pulsed field gel electrophoresis (PFGE) following \textit{SmaI} digestion of DNA in order to confirm clonal relatedness to the original strain SA1, as described previously.\cite{19}

\textbf{PCR and sequence analysis}

Preparation of whole cell DNA for PCR experiments followed a previously described protocol.\cite{6,10} To detect structural alterations in the \textit{erm}(A) regulatory region we used a PCR assay, which enabled the amplification of the entire regulatory region including c. 200 bp upstream of the regulatory region and c. 150 bp of the 5’ end of the \textit{erm}(A) gene.\cite{10} An inducibly expressed \textit{erm}(A) gene that carries a complete attenuator—as known from \textit{erm}(A) of Tn554—yielded a PCR product of 593 bp.\cite{10} PCR amplifications were carried out using a GeneAmp PCR System 2400 (Perkin Elmer, Weiterstadt, Germany). All reagents (GeneAmp dNTPs, High Fidelity \textit{Taq} DNA polymerase and 10 $\times$ PCR buffer) were purchased from Perkin Elmer or Boehringer Mannheim (Mannheim, Germany). PCR-amplified DNA was sequenced by the dye terminator method on both strands. The reaction was carried out using 50 ng DNA, 0.1 $\mu$mol of primers, and Ready Reaction Dye Terminator Cycle Sequencing Kit (Perkin Elmer) according to the manufacturer’s instructions. The products were resolved and automatically analysed using a 310 DNA sequencer (Perkin Elmer). The primers used for amplification\cite{10} were also used for sequence analysis. For a specific subset of amplicons, another two primers derived from the IS256 sequence (GenBank accession no. M18086) were used to complete the sequence analysis. The sequence of the IS256 element identified in the \textit{erm}(A) translational attenuator was submitted to the EMBL database and assigned accession no. AJ416751. Stabilities of predicted mRNA secondary structures were calculated as described.\cite{20}

\textbf{Results}

\textit{Selection of constitutively expressed \textit{erm}(A) mutants}

The original \textit{S. aureus} SA1 showed MICs of clindamycin, dalfopristin, quinupristin, quinupristin–dalfopristin, telithromycin and ABT-773 of 0.25, 0.5, 0.25, 0.125, 0.125 and 0.06 mg/L, respectively. In \textit{vitro} selection with either dalfopristin or quinupristin–dalfopristin failed to yield resistant mutants in repeated experiments. However, the remaining four non-inducers proved able to select for resistant mutants from telithromycin selection, 26 from ABT-773 selection. In contrast to the original \textit{S. aureus} SA1, all these mutants exhibited common high MICs of clindamycin, quinupristin, telithromycin and ABT-773 of $>1024$, $>64$, $>512$ and $>256$ mg/L, respectively. The observation that all mutants derived from the macrolide-resistant \textit{S. aureus} SA1 exhibited cross-resistance to clindamycin, quinupristin and the two ketolides suggested that inducible expression of the \textit{erm}(A) gene might have been changed into constitutive expression during the \textit{in vitro} selection process. Since a switch from inducible to constitutive \textit{erm}(A) gene expression is usually accompanied by structural alterations in the \textit{erm}(A) translational attenuator,\cite{10,13,14} further molecular studies concentrated on the analysis of the \textit{erm}(A) regulatory region.
In vitro selection of ketolide resistance

Molecular analysis of constitutively expressed erm(A) mutants

PCR amplification of the \textit{erm}(A) regulatory region of 94 of the 146 mutants investigated (64.4\%) revealed amplicons that were slightly larger or smaller than the 593 bp amplicon produced by amplification of a complete \textit{erm}(A) translational attenuator. Another 49 mutants revealed the presence of a distinctly larger amplicon of c. 1.9 kb while the amplicons of the remaining three mutants were indistinguishable in their sizes as compared with the amplicon obtained from the original \textit{S. aureus} SA1. Sequence analysis revealed four different types of structural alteration in the \textit{erm}(A) translational attenuator: deletions, tandem duplications, point mutations and disruption of the translational attenuator by the integration of the insertion sequence IS\text{256}.

Six different types of deletion of 14–157 bp were detected in the \textit{erm}(A) regulatory region (Figure, Table). The 14 bp deletion comprised part of the open reading frame (ORF) of the 19 aa peptide and part of the inverted repeat (IR) 3. Since the truncated IR3 is not able to form a stable mRNA secondary structure with IR4, IR4 will pair with IR5 and thereby render IR6 permanently accessible to ribosomes. The 83 bp deletion included the entire reading frame of the 19 aa peptide including its ribosome binding site SD2 as well as the IR2 and IR3 sequences. The 147 bp deletion, the IR4–IR6 sequences were present, but the entire upstream part, including the reading frames for the 15 aa and the 19 aa peptides as well as the IR1–IR3 sequences, was lost. In these two cases, stable mRNA secondary structures could be formed again by pairing IR4:IR5 with IR6 being accessible to ribosomes. The 121 and 131 bp deletions were closely related and comprised the IR4 and IR5 sequences in addition to the reading frame of the 19 aa peptide and the IR2 and IR3 sequences. Finally, the 157 bp deletion was characterized by a largely truncated translational attenuator in which only the 5’ end of the ORF of the 15 aa peptide including SD1 was present. These three types of deletion did not allow the formation of any mRNA secondary structures in the \textit{erm}(A) regulatory region. None of the six types of deletion affected the \textit{erm}(A)-associated ribosome binding site SD3 and the \textit{erm}(A) structural gene. The 14 bp deletion proved to be the most predominant deletion among mutants obtained in the clindamycin and telithromycin selection experiments, and was the only structural alteration seen in the ABT-773 selection experiments (Table). The reason for the preferential occurrence of this deletion is not known. All other deletions were observed in at least two independent mutants obtained in selection experiments with either clindamycin, quinupristin or telithromycin.

Three types of tandem duplication were observed in mutants obtained from selection in the presence of either clindamycin, quinupristin or telithromycin (Table). The 25 bp duplication seen in 20 mutants from clindamycin selection experiments comprised the \textit{erm}(A)-associated ribosome binding site SD3, the 5’ end of \textit{erm}(A) as well as the IR6 sequence. A similar tandem duplication of 26 bp was identified in a single mutant from quinupristin selection. In contrast to the 25 bp duplication, the SD3 sequence was only in part duplicated in the 26 bp duplication. In the 25 bp and 26 bp

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Structural alteration & clindamycin & quinupristin & telithromycin & ABT-773 \\
\hline
Deletion & 14 bp & 9\textsuperscript{a} & – & 25 & 26 \\
& 83 bp & 2 & – & – & – \\
& 121 bp & 2 & – & – & – \\
& 131 bp & 2 & – & – & – \\
& 147 bp & – & 4 & – & – \\
& 157 bp & – & – & 2 & – \\
Duplication & 23 bp & – & 1 & – & – \\
& 25 bp & 20 & – & – & – \\
& 26 bp & – & 1 & – & – \\
IS256 insertion & – & 23 & 4 & 22 & – \\
Point mutation in IR3 & – & 1 & – & – \\
Point mutations in IR3–IR5 & – & – & 1 & – \\
\hline
\end{tabular}
\caption{Number of constitutive \textit{erm}(A) mutants displaying different types of structural alteration in the \textit{erm}(A) translational attenuators after plate selection experiments with the non-inducers clindamycin, quinupristin, telithromycin or ABT-773.}
\end{table}

\textsuperscript{a}Number of mutants showing a specific type of structural alteration.
Figure. Schematic presentation of the regulatory regions of the inducibly expressed \textit{erm}(A) gene of Tn554 and the constitutively expressed \textit{erm}(A) genes detected in this study. SD1, SD2 and SD3 represent the Shine–Dalgarno sequences of the ORFs of the 15 aa peptide, the 19 aa peptide and the \textit{erm}(A) gene, respectively. The arrows indicate the inverted repeated sequences IR1–IR6. The truncated \textit{erm}(A) gene present in isolates that exhibit the 25 and 26 bp tandem duplications is displayed as a black box. The 8 bp direct repeat sequence at the integration site of IS256 is shown in capital letters.
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In this study, the presence of an 8 bp direct repeat of the downstream of the ORF of the 19 aa peptide and upstream in all 49 cases. The integration site was located immediately element IS translational attenuator into which a copy of the insertion

These inverted repeats are involved is unlikely.

amplicon of the amplicons that were indistinguishable in size from the IR5 and IR6. In this case, the truncated parts of IR5 and IR6—ΔIR5 and ΔIR6—present in the duplication prevented the formation of a stable mRNA between IR5 and IR6.

Two types of point mutation were observed in the three amplicons that were indistinguishable in size from the amplicon of the S. aureus SA1 (Table, Figure). The first type detected in single mutants from clindamycin and telithromycin selection experiments is characterized by a single bp exchange (C—to G) in IR3 at position 5363 in the Tn554 sequence. This bp exchange reduced the stability of the mRNA secondary position structure between IR3:4 from \( \Delta G = -12.0 \) kcal/mol to \( \Delta G = -7.8 \) kcal/mol, and thus favoured the formation of a more stable secondary structure between IR4:IR5, which in turn allowed ribosomes permanent access to SD3 and the start of erm(A).

The second type of point mutation seen in a single mutant from telithromycin selection also showed the aforementioned bp exchange in IR3, in addition to the exchange of 5329A→C in IR4 and another three alterations in IR5. These included the exchange of 5311T→AC and the insertion of a single ‘C’ between 5305A and 5304T, and also between 5303A and 5302A. These alterations in IR3–IR5 drastically decreased the stability of mRNA secondary structures, such as IR3:IR4 (from \( \Delta G = -54.3 \) kJ/mol to \( \Delta G = -23.4 \) kJ/mol), IR4:IR5 (from \( \Delta G = -86.9 \) kJ/mol to \( \Delta G = -18.4 \) kJ/mol) or IR5:IR6 (from \( \Delta G = -77.7 \) kJ/mol to \( \Delta G = -35.1 \) kJ/mol). Based on the low \( \Delta G \) values, the formation of stable mRNA secondary structures in which these inverted repeats are involved is unlikely.

The last type of structural alteration in the erm(A) translational attenuator was seen in 49 mutants (33.6%), all of which exhibited the c. 1.9 kb amplicon. Sequence analysis showed that this amplicon consisted of a complete erm(A) translational attenuator into which a copy of the insertion element IS256 had integrated (Figure). The orientation of this IS256 element and its site of integration were the same in all 49 cases. The integration site was located immediately downstream of the ORF of the 19 aa peptide and upstream of IR4. Analysis of the regions adjacent to the integration site revealed the presence of an 8 bp direct repeat of the sequence TCAAAATT (Figure).

Discussion

Early studies showed that inducible expression of erm(A) genes could be converted into constitutive expression by cultivation of staphyloocci in the presence of certain non-inducers, such as tylosin or lincosamines. The translational attenuator that is essential for inducible erm(A) expression was altered either by deletions, tandem duplications or point mutations at crucial positions in all these cases. Since the mid-1980s it has therefore been an accepted fact that certain non-inducers are able to catalyse the development of structural alterations that change the type of erm(A) gene expression, thereby also enlarging the spectrum of MLSB antibiotics to which strains that carry constitutively expressed erm(A) genes are resistant. During recent years, a new class of antimicrobial agents, the ketolides, has been developed to overcome MLSB resistance mediated by ribosome methylation. Ketolides such as telithromycin or ABT-773 can act at methylated ribosomes and their activity has been demonstrated in various genera of Gram-positive bacteria. The observation that ketolides failed to induce erm gene expression led us to two major questions: (i) are ketolides and other therapeutic agents such as dalfopristin, quinupristin and quinupristin–dalfopristin, respectively, also able to select for constitutive erm(A) gene expression? And, if so, (ii) are the mutations observed similar/identical to either those mutations seen after in vitro selection in the presence of clindamycin or the mutations previously detected in naturally occurring constitutively expressed erm(A) genes?

To answer the first question, in vitro selection experiments were conducted. Since erm(A)—even when expressed constitutively—did not confer resistance to dalfopristin or quinupristin–dalfopristin, constitutively expressed mutants were not obtained in selection experiments with these agents. However, any of the four remaining substances tested was able to select for constitutively expressed mutants although differences with regard to the mutation ratio and the types of mutations were observed (Table).

Point mutations were the most rarely encountered type of mutation and occurred in only three of the 146 mutants. The 5363C→G exchange in IR3 was seen in all three mutants and proved to be identical to the lin-71 mutation previously described by Murphy to cause constitutive erm(A) gene expression by destabilization of mRNA secondary structures in which IR3 is involved. The additional bp exchanges and insertions observed in IR4 and IR5 of one constitutive mutant during the course of this study were believed to enhance this effect by abolishing the formation of stable mRNA secondary structures involving IR3, IR4 and IR5.

As known from in vitro studies and analysis of naturally occurring constitutive erm(A) mutants, tandem duplications in the translational attenuator occurred rarely, possibly as a consequence of illegitimate recombination or replication slippage. The 26 and 25 bp duplications identi-
fied in this study (Figure) were closely related or identical to two 25 bp duplications seen in *S. aureus* strains from the SENTRY study. The third type of tandem duplication in which a segment carrying part of IR5 and IR6 was inserted into the original IR5 (Figure) has not been detected before.

The insertion of IS256 into the *erm(A)* translational attenuator is a novel observation. This type of structural alteration is limited to strains that harbour this insertion sequence. IS256 is commonly associated with the gentamicin–tobramycin–kanamycin resistance transposon Tn4001, however, independent copies of IS256 have also been seen in staphylococci. No specific integration sites have been described for IS256 or Tn4001, but analysis of the so far known integration sites might suggest a certain preference for AT-rich regions. The integration site in the *erm(A)* translational attenuator also consisted mainly of A and T residues. Integration of IS256 separated the promoter region from the *erm(A)* gene region by >1.3 kb. There are two possible ways in which transcription of *erm(A)* might be achieved. Since there is no transcriptional terminator detectable in the terminal part of IS256, transcription of a common mRNA of the transposase gene of IS256 and the downstream adjacent *erm(A)* region might be possible. In that case, the formation of mRNA secondary structures in the part upstream of *erm(A)* was believed to occur between IR4:IR5, thus rendering IR6 permanently accessible to ribosomes and thereby explaining constitutive *erm(A)* expression. However, the generation of a novel promoter by the integrated IS256 element must be considered as well. The terminal 6 bp of IS256, TTGACT, were in good accordance with the *Bacillus subtilis* −35 region (TTGACA). A further 15 bp downstream [8 bp arising from the target site duplication and 7 bp of the original *erm(A)* translational attenuator sequence], the sequence TATAAT, which exactly corresponded to the consensus sequence of the −10 region of *B. subtilis*, was detected. In this case, transcription might start between IR4 and IR5, and the 5′ end of that transcript might pair with part of the IR5 sequence so that IR6 remains accessible to ribosomes. This observation might also provide an explanation for the integration of IS256 in the same orientation and at the same position in 49 independent mutants selected by three different agents.

All six types of deletion either abolished any formation of mRNA secondary structures (121, 131 and 157 bp deletions) or favoured the formation of those mRNA secondary structures that did not involve IR6 (14, 83 and 147 bp deletions) and so allowed translation of the *erm(A)* transcripts independently of the presence or absence of inducers. Two of the deletions seen in the present study—the 83 and 121 bp deletions—were identical to deletions previously detected in *S. aureus* isolates from the SENTRY study. Moreover, all deletions detected in the present study were found in at least two independent mutants. The occurrence of the same type of deletion in the translational attenuators of *erm(C)* genes of independent staphylococcal isolates has been described before. A mechanism involving the recA-dependent recombination system of the host cell has been experimentally confirmed to account for the development of deletions at discrete positions in the translational attenuator. Since the translational attenuators of *erm(C)* and *erm(A)* are closely related, illegitimate recombination between parts of the attenuator that display a certain degree of sequence identity might also explain the deletions seen in the present study. The regions up- and downstream of the 14, 83, 121, 131, 147 and 157 bp deletions showed stretches of 10–17 bp with 60–80% sequence identity. The sizes of the potential recombination sites and their percentages of sequence identity are in good accordance with those identified in the *erm(C)* regulatory region.

In summary, the results of the present study showed that the four non-inducers, clindamycin, quinupristin, telithromycin and ABT-773, were able to select for constitutive *erm(A)* mutants, all of which showed structural alterations in the *erm(A)* translational attenuator. Irrespective of the type of structural alteration, common high MICs of telithromycin, ABT-773, quinupristin and clindamycin were recorded. In contrast to deletions, tandem duplications and point mutations, constitutive *erm(A)* expression by insertion of IS256 in the translational attenuator is a novel finding which enlarges understanding of the processes that can change the type of *erm(A)* expression. The observation that constitutive *erm(A)* mutants that also showed resistance to ketolides could be selected overnight supports the recommendation that ketolides should not be used for infections with staphylococci that display inducible resistance to MLSB antibiotics.

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References


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