Inducible or constitutive expression of resistance in clinical isolates of streptococci and enterococci cross-resistant to erythromycin and lincomycin

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Thirty-five of 40 clinical isolates of enterococci and streptococci cross-resistant to erythromycin and lincomycin and harbouring erm genes were inducibly resistant to these drugs, suggesting that ribosomal methylation is predominantly inducibly expressed in these bacterial genera. Regulatory regions located upstream of the erm genes of four inducible and three constitutive strains were amplified and sequenced. Expression of constitutive resistance in two strains of Streptococcus pneumoniae and Enterococcus faecalis could be accounted for by a large deletion or a DNA duplication within the regulatory regions, respectively.

Introduction

Ribosomal modification is a major mechanism of resistance to macrolide antibiotics in streptococci and enterococci.1,2 Resistance is due to the synthesis of a methylase which N6-dimethylates a specific adenine residue in 23S ribosomal RNA. Methylases are encoded by erm genes and confer cross-resistance to macrolides, lincosamides and streptogramins B, the so-called MLSB phenotype.2

In streptococci, MLSB resistance is encoded by genes belonging to the ermB group of genes. The ermB group includes closely related genes such as ermAM, ermB and ermBC genes which have been reported in a variety of Gram-positive and Gram-negative organisms.1,3 The inducible expression of MLSB resistance is putatively controlled at a post-transcriptional level by a regulatory region located upstream from the structural erm gene.2 Constitutive expression of the MLSB resistance mediated by plasmid pAMβ1 from E. nterococcus faecalis has been related to a deletion of most of the regulatory region of the erm gene.6 In a recent study, we have shown that a similar deletion was responsible for constitutive expression of the erm gene from Streptococcus agalactiae HM 1081.7 In this report, we have determined the inducibility of 40 streptococci and enterococci that carry MLSB resistance and determined the molecular structure of the regulatory regions of three erm genes expressed constitutively.

Materials and methods

Bacterial strains

Forty clinical isolates of streptococci and enterococci which had no zone of inhibition around discs containing erythromycin (30 µg), spiramycin (100 µg) and lincomycin (15 µg) (all discs from SanofiDiagnostics Pasteur, Marnes-la Coquette, France) were studied. The strains were epidemiologically unrelated. They included 11 E. faecalis, two E. nterococcus faecium, 14 Streptococcus pneumoniae, six oral streptococci and seven S. agalactiae (including S. agalactiae HM 1081, as a control) from various hospitals located in France, Spain, the UK and the USA.

Antibiotic susceptibility testing

MICs of erythromycin, rokitamycin and lincomycin were determined by a standard agar-dilution technique using Mueller–Hinton medium (Sanofi Diagnostic Pasteur) supplemented with 5% horse blood.8 Erythromycin (Hoechst-Marion-Roussel, Romainville, France), lincomycin (Upjohn Laboratories, Val de Reuil, France) and rokitamycin (Pierre Fabre Laboratories, Boulogne, France) were provided by their respective manufacturers.

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Induction assays

To test inducibility, MICs of erythromycin, lincomycin and rokitamycin against the strains cultured in the absence or in the presence of erythromycin (0.06 mg/L) were determined.

Analysis of the regulatory regions of erm A M -related genes

The sequences of the erm attenuators were amplified from bacterial DNA by PCR using primers SR3 (5‘-CCTTGGACGCAAACATTAAAAGTGTTGT-3‘) and SR5 (5‘-GTTTGAGTACCTTTTCATTCGTTA-3‘) designed to amplify a fragment including the regulatory region and the 24 first nucleotides of the ermB group genes. PCR experiments were carried out as follows: 35 cycles of amplification including 30 s of denaturation at 94°C, 1 min of annealing at 50°C and 1 min of elongation at 72°C. Nucleotide sequencing was performed with an ABI PRISM 310 sequencer.

Results and discussion

Expression of MLSB resistance

All 40 streptococci and enterococci were cross-resistant to erythromycin (MICs 16–8000 mg/L) and lincomycin (MICs 128–1000 mg/L). MICs of these antibiotics against streptococci and enterococci were already high in the absence of induction with erythromycin and did not increase or increased only by one dilution after induction, probably because of rapid self-induction by the tested antibiotic. By contrast, MICs of rokitamycin were lower and had a greater range (1–2000 mg/L). After induction, the rokitamycin MICs increased for 32 out of the 40 strains studied by more than two dilutions, probably because of a weaker inducing capacity of this antibiotic. These 32 strains were thus considered inducibly resistant to erythromycin. This study confirms that for streptococci and enterococci, inducible MLSB resistance cannot be distinguished from constitutive resistance on the sole basis of elevated MICs of erythromycin and lincomycin. High-level cross-resistance to erythromycin and lincomycin in our clinical isolates of streptococci and enterococci was mostly due to inducible expression of MLSB resistance. This is in contrast to staphylococci where the MLSB phenotype is related to constitutive synthesis of the ribosomal methylase.1,2 The high prevalence of the MLSB-inducible phenotype in streptococci explains why macrolides that are very weak inducers, such as the ketolides, are active against most of these Gram-positive organisms despite MLSB cross-resistance.3,4

Analysis of the regulatory regions of erm genes

The ermB group genes are preceded by an attenuator composed of an ORF encoding a putative control peptide (36 amino acids) and of a set of numerous inverted repeats which could fold into several successive stem–loops.1,2 Using oligonucleotides SR3 and SR5, we obtained an amplification product from the 40 strains studied. DNA fragments of approximately 380 bp were amplified for 38 strains whereas 170 bp fragments were amplified from the constitutive strains of S. pneumoniae H M 36 and S. agalactiae H M 1081. The sequence of the PCR product from three of the constitutive strains, E. faecalis U M H 1 and S. pneumoniae H M 36 and H M 35, and from four of the inducible strains, S. agalactiae H M 1 and H M 12, and oral streptococci H M 6 and H M 18, was determined and compared with that from gene banks. A ligament of the sequence of the constitutive erm gene of S. pneumoniae H M 36 with those of the inducible genes from plasmid pA M 77 and transposons Tn917 and Tn1545 revealed a large deletion within the regulatory region similar to that observed upstream from the erm genes of constitutively resistant strains of E. faecalis harbouring plasmid pA M 41 and of S. agalactiae H M 1081 (Figures 1 and 2).5,6 This deletion could result in constitutive expression of MLSB resistance since it removed the inverted repeats and the sequence of the leader peptide, thus making the ribosome binding site and the start codon for the methylase accessible to the ribosomes. However, occurrence of a deletion was not the only explanation for constitutive expression of MLSB resistance, as shown by the analysis of nucleotide sequences of the two remaining constitutive strains, E. faecalis U M H 1 and S. pneumoniae H M 35. E. faecalis U M H 1 displayed a duplication of a GTGTAATGT sequence which generated two successive stop codons, resulting in a truncated leader peptide of 12 or 20 amino acids (Figures 1 and 2). The contribution of this marked alteration in constitutive expression of MLSB resistance remains unknown. The sequence of the regulatory region of the last constitutive strain, S. pneumoniae H M 35, was similar to that of inducible strains (Figure 1). Therefore, modifications of other still unidentified structures could be responsible for constitutive expression of MLSB resistance.

The N-terminal region of the leader peptide in our strains contained a duplication of a TAA A A motif which was absent from the inducible erm genes of pA M 77 and Tn917, but present in the gene of the pneumococcal transposon Tn1545 (Figure 1). The duplication generated a stop codon in the leader peptide sequence and could thus result in a shorter peptide (Figure 2). This change was observed in both inducible and constitutive strains and therefore did not appear to alter the expression of resistance. Various point mutations were observed in the inducibly resistant strains which do not seem to alter the secondary structure of the messenger of the erm genes (Figure 1 and data not shown). Duplication of a GT sequence in the consensus region of several strains (Figure 1) brought the -10 sequence closer to the consensus promoter of E. faecalis. However, we could not find any relationship between this duplication and the mode of expression of MLSB resistance.
Resistance to erythromycin

Figure 1. Sequence alignment of the regulatory regions of \textit{ermB} genes. Only differences relative to the sequence of pAM77 are indicated.\textsuperscript{3} Deletions are indicated by dashes. The sequences of pAM77, Tn917 and pAM \textit{\beta}1 are from \textit{Streptococcus sanguis}, \textit{Enterococcus faecalis} and \textit{Streptococcus pneumoniae}, respectively.\textsuperscript{3-5} HM1 and HM12: \textit{S. agalactiae} HM1 and HM12; UMH1: \textit{E. faecalis} UMH1; HM35 and HM36: \textit{S. pneumoniae} HM35 and HM36; HM6 and HM18: oral streptococci HM6 and HM18. \textit{erm} genes from pAM \textit{\beta}1, \textit{E. faecalis} UMH1 and from \textit{S. pneumoniae} HM35 and HM36 are expressed constitutively. Putative promoters (–35, –10), ribosome-binding sites (SD\textsubscript{1} and SD\textsubscript{2}), and sequences for the control peptide and the methylase are underlined. Stop codons are underlined and italicized.

Figure 2. Schematic representation of alterations of the regulatory sequences of \textit{erm} genes in constitutively resistant strains of \textit{Streptococcus pneumoniae} HM35 and HM36, and \textit{Enterococcus faecalis} UMH1 and in inducibly resistant strains of \textit{Streptococcus agalactiae} HM1 and HM6 and oral streptococci HM12 and HM18. SD1 and SD2: ribosome-binding sites for the control peptide and the \textit{erm} gene, respectively. Deletions are indicated by dashes.
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References


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