ANTIMICROBIAL RESISTANCE

Molecular analysis of the macrolide-lincosamide resistance gene region of a novel plasmid from *Staphylococcus hyicus*

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Resistance to macrolides and lincosamides in *Staphylococcus hyicus* has been shown to be encoded by a 4.0-kb plasmid designated pSES21. It differed distinctly in its restriction map from all other staphylococcal macrolide resistance plasmids reported so far. Southern blot hybridisation with gene probes specific for staphylococcal *erm* genes demonstrated that the macrolide resistance gene belonged to hybridisation class C. Analysis of the *ermC* gene revealed that the deduced amino-acid sequence of the pSES21-encoded ErmC methylase exhibited c. 93% identity with the ErmC methylase encoded by plasmid pE194. The *ermC* gene of pSES21 was expressed constitutively and sequence analysis of the regulatory region showed multiple base-pair insertions and substitutions in the translational attenuator. As a consequence of these mutations, the reading frame of the small regulatory peptide was destroyed and a novel pair of inverted repeated sequences was generated. Previous studies identified sequence deletions and sequence duplications in the *ermC* regulatory region as the basis for constitutive *ermC* gene expression. The multiple point mutations shown in the pSES21-encoded *ermC* translational attenuator represent a novel kind of structural alteration in this regulatory region and may explain constitutive *ermC* gene expression by pairing of the newly generated inverted repeated segments in the presence of a functionally deleted reading frame for the small regulatory peptide.

Introduction

Staphylococcal resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics is commonly mediated by the genes *ermA*, *ermB* or *ermC* [1, 2]. While the transposon-encoded genes *ermA* and *ermB* have rarely been detected in staphylococci, the plasmid-encoded gene *ermC* is the predominant MLS resistance gene in staphylococcal isolates from both man and animals [3]. The *ermC* gene has been found on small plasmids of 2.3–4.0 kb [1, 4–16]. Two different types of plasmids have been described so far, between which homology has been limited to the *ermC* region. The 3.7-kb plasmid pE194 from *Staphylococcus aureus* [7] represents one type of *ermC* plasmid, the 2.35-kb plasmid pNE131 from *S. epidermidis* [8] the other type. Expression of the *ermC*

gene has been found to be either inducible by 14- and 15-membered macrolides or constitutive [17, 18]. The genetic basis for the differences of both types of *ermC* gene expression is in the *ermC* regulatory region, which is located immediately upstream of the *ermC* structural gene. The regulatory region of inducible *ermC* genes is referred to as the translational attenuator and comprises two pairs of inverted repeated sequences as well as a small open reading frame for a regulatory peptide [7, 8, 19–21]. Changes from the inducible to the constitutive type of *ermC* gene expression involve structural alterations in the translational attenuator. So far, two types of structural changes have been reported: (i) sequence deletions of varying extent [4, 8, 10, 22] and (ii) sequence duplications at different locations in the translational attenuator [9, 12, 23].

This study identified a novel *ermC*-encoding MLS-resistance plasmid, designated pSES21, from *S. hyicus* and describes its *ermC* structural gene and the regulatory region.

Materials and methods

Bacterial strains and determination of macrolide-lincosamide resistance

The strain of *S. hyicus* was isolated from the skin swab of a tylosin-fed pig and identified by the ID32 STAPH system (bioMérieux, La Balme les Grottes, France). Resistance to macrolide-lincosamide antibiotics was determined by the agar diffusion test [24] with disks (Becton-Dickinson, Heidelberg, Germany) containing erythromycin 15 µg, lincomycin 10 µg, or clindamycin 10 µg or tablets (Hiss Diagnostics, Freiburg, Germany) containing azithromycin 30 µg, spiramycin 200 µg or tylosin 150 µg. *S. aureus* RN4220 served as the recipient for plasmid pSES21 in protoplast transformation experiments [25]. During the further course of the study, the original *S. hyicus* strain, as well as *S. aureus* RN4220:pSES21 were grown on sheep blood agar (Oxoid) supplemented with erythromycin 30 mg/L. *S. aureus* RN4220 carrying plasmid pSES5, which encodes the inducible *ermC* gene [11], served as control for the determination of the type of *ermC* gene expression.

Plasmid analysis and Southern blot hybridisation

The single plasmid found in the original *S. hyicus* isolate was transformed into *S. aureus* RN4220 by polyethylene glycol-mediated protoplast transformation [25, 26]; *S. aureus* RN4220:pSES21 transformants were subsequently selected on DM3 regeneration plates [26] supplemented with erythromycin 30 mg/L. The plasmid, designated pSES21, was isolated by affinity chromatography [27] (Qiagen Midi System, Hilden, Germany) and subjected to restriction endonuclease digests (Boehringer Mannheim, Germany). Uncleaved plasmid pSES21 as well as its restriction fragments were separated in agarose 1–1.5% w/v gels with 1 × TAE buffer as running buffer. The sizes of the fragments were calculated from logarithmic plots by comparison with the 1-kb ladder (Gibco-BRL, Eggenstein, Germany). To determine the class of *erm* gene, hybridisation experiments were conducted with the following gene probes: the 472-bp *Hae*III/*Hinc*II internal fragment of the *ermC* gene of plasmid pE194 served as *ermC* gene probe [7]; the 1.46-kb *Cla*I fragment of plasmid pSES20 as *ermB* gene probe [28]; and the 1.21-kb *Cla*I-*Eco*RV fragment of transposon Tn554 as *ermA* probe [29]. Plasmid DNA was transferred from agarose gels to nitrocellulose membranes by the capillary blot procedure [27]. The probes were labelled with the non-radioactive enhanced chemiluminescence system (ECL, Amersham-Buchler, Braunschweig, Germany). High stringency hybridisation conditions were as previously described [27] and the signal was detected with the detection reagents supplied with the ECL kit.

Cloning and sequence analysis

Plasmid pSES21 was linearised by digestion with the restriction endonucleases *Eco*RI, *Xba*I, *Bam*HI, *Hpa*II, *Hind*III and *Hae*III. The entire linearised plasmids were cloned into pBluescript II SK+ (Stratagene, Heidelberg, Germany) and the recombinant plasmids were transformed into *Escherichia coli* JM107 [30, 31]. To localise the position of the pSES21-encoded *erm* gene by its functional inactivation, the transformants were grown overnight at 37°C in nutrient agar supplemented with erythromycin 150 mg/L.

Plasmid pBluescript II SK+, which carried the *Hind*III-linearised pSES21, was chosen for sequence analysis. Initial sequence analyses were performed with the commercially available M13 universal and T3 primers (Stratagene). For additional sequence analyses, four 14–18-mer primers derived from the sequence data obtained were used. Sequence analyses were performed on both strands by the dideoxy chain termination method [32] with the Sequenase 2.0 kit and [α^{32} S]dATP or by automated non-radioactive sequencing with the AutoRead™ sequencing kit (Pharmacia) and the ALF DNA analysis system (Pharmacia). The calculation of mRNA stabilities followed the recommendations of Tinoco *et al.* [33]. Dice coefficients of similarity [34] were calculated to compare the deduced amino-acid sequences of the *ErmC* methylases encoded by the plasmids pE194 [7], pIM13 [22], pT48 [5], pNE131 [8], pSES4a [11], pSES5 [11], pSES6 [9, 11] and pSES21.

Nucleotide sequence accession number

The nucleotide sequence of the pSES21-encoded *ermC* gene and its regulatory region has been submitted to the EMBL database and assigned accession number Y09003.

Results

Analysis of the pSES21-encoded ermC gene

The 4.0-kb plasmid pSES21 from *S. hyicus* was shown by protoplast transformation to mediate macrolide-lincosamide resistance. Hybridisation with specific gene probes for staphylococcal *erm* genes demonstrated that the pSES21-encoded *erm* gene belonged to the hybridisation class C. Although pSES21 resembled the MLS resistance plasmids pE194 and pSES6 in size, restriction mapping did not reveal homology of pSES21 to either of the two types of *ermC* plasmids, so far described in staphylococci (Fig. 1). Particularly, the characteristic arrangement of *Sac*I, *Hae*III, *Bcl*I and *Cla*I sites within the *ermC* gene region was missing in pSES21.

Fig. 1. Comparison of the restriction maps of the *ermC*-encoding plasmids pE194 from *S. aureus*, pSES6 from *S. equorum*, pNE131 from *S. epidermidis* and pSES21 from *S. hyicus*. Restriction endonuclease cleavage sites are abbreviated as follows: Ac, *Acc*I; B, *Bcl*I; Ba, *Bam*HI; C, *Cla*I; Cf, *Cfo*I; E, *Eco*RI; H, *Hpa*I; Ha, *Hae*III; Hi, *Hind*III; Hp, *Hpa*II; M, *Mbo*I; Ps, *Pst*I; S, *Sac*I; T, *Taq*I; X, *Xba*I. A distance scale in kb is shown below each map; the arrows indicate the extent and the direction of transcription of the *ermC* gene.

Localisation of the *ermC* gene in pSES21 was performed by the marker inactivation strategy. *E. coli* JM107 transformants of recombinant pBluescript II SK+ which carried the *Eco*RI-, *Xba*I-, *Bam*HI-, *Hpa*II- or *Hae*III-linearised pSES21 plasmid as an insert grew on nutrient agar containing erythromycin 150 mg/L. However, those with the *Hind*III-linearised pSES21 as insert showed no growth after 24 h at 37°C on that medium. This suggested that the *Hind*III site was located either within the *ermC* structural gene or within its essential regulatory region. Sequence analysis of this *Hind*III clone showed that the *Hind*III site was located within an open reading frame for a protein of 244 amino acids (Fig. 2). Among the 36 base-pair substitutions seen within the *ermC* reading frame, 18 were involved in changes of the amino-acid sequence. Another five were responsible for the generation of new recognition sites for the enzymes *Hind*III (position 269, A–C), *Taq*I (position 233, A–G) and *Cfo*I (position 605, A–G), but also for the destruction of the recognition sites for *Hae*III (position 314, C–G) and *Bcl*I (position 408, G–A). Comparison of this reading frame with the *ermC* gene of pE194 showed 95.1% homology in the nucleotide sequences and 93.4% identity in the deduced amino-acid sequences. The structural relationship of the pSES21-encoded *ErmC* methylase to other adenine methylases of this hybridisation class is shown in Fig.

3a. The cladogram (Fig. 3b) confirmed that the pSES21-encoded adenine methylase differed the most from the *ErmC* methylases known so far.

Analysis of the ermC translational attenuator and transcriptional terminator

Plasmid pSES21 conferred combined resistance to 14-, 15- and 16-membered macrolides and also to lincosamides without previous induction by erythromycin. This resistance pattern confirmed that the pSES21-encoded *ermC* gene was expressed constitutively. A comparison of the pSES21-encoded *ermC* translational attenuator with the corresponding region of pE194 revealed the presence of 14 base-pair insertions and another 21 base-pair substitutions within the 188-bp region preceding the *ermC* structural gene (Fig. 2). Conversion of C to T at position 17 deleted the *Sac*I cleavage site while the insertion of an additional G at position 19 and the substitution of T by C at position 20 generated a new *Cfo*I cleavage site. Five base-pair insertions were located in the reading frame of the small regulatory peptide with one of them deleting the start codon (ATG–ATTG). No other start codon was found in this region that could functionally replace this deleted start codon. The inverted repeated segments were also affected by base-pair substitutions (Fig. 2). As inducible *ermC* gene expression requires the

a	pT48	: -----Q	- 50
	pIM13	: -----Q	- 50
	pSES4a	: -----	- 50
	pSES5	: -----K-----D-----Q	- 50
	pE194	: MNEKNIKHSQNFITSKHNIDKIMTNIRLNEHDNIFEIGSGKGHFTLELVK	- 50
	pNE131	: -----Q	- 50
	pSES21	: --K--D--R-V--S-----Q	- 50
	pT48	: -----Q-----F-	- 100
	pIM13	: -----Q-----F-	- 100
	pSES4a	: -----F-	- 100
	pSES5	: -----K-----F-	- 100
	pE194	: RCNFVTAIEIDHKLCKTTENKLVLDHDFQVLNKDILEFKFKPNQSYKIYG	- 100
	pNE131	: -----F-	- 100
	pSES21	: -----N-----F-	- 100
	pT48	: -----D-----	- 150
	pIM13	: -----D-----F-	- 150
	pSES4a	: -----D-----F-	- 150
	pSES5	: -----D-----F-	- 150
	pE194	: NIPYNISTDIIRKIVFDSIANEIIYLIVEYGFARLLNTRSLALLLMAEV	- 150
	pNE131	: -----D-----F-	- 150
	pSES21	: S-----V-E--D-S-----	- 150
	pT48	: -----N-----	- 200
	pIM13	: -----N-----	- 200
	pSES4a	: -----N-----	- 200
	pSES5	: -----N-----	- 200
	pE194	: DISILSMVPREYFHPKPKVNSSLIRLSRKKSRIHKDKQKYNFYVMKWVN	- 200
	pNE131	: -----N-----	- 200
	pSES21	: -----N-----Y-----	- 200
	pT48	: -----	- 244
	pIM13	: -----	- 244
	pSES4a	: -----	- 244
	pSES5	: -----	- 244
	pE194	: KEYKKIFTKNQFNNSLKHAGIDDLNNISFEQFLSLFNSYKLFNK	- 244
	pNE131	: -----	- 244
	pSES21	: -----K-----	- 244

Fig. 3. (a) Comparison of the amino-acid sequences of ErmC methylases from staphylococci and bacilli. Dashes (-) indicate the presence of the same amino acid as reported for the ErmC methylase of pE194. The methylases encoded by plasmids pSES6 and pE194 were indistinguishable by their amino-acid sequences (Fig. 3b, see over).

Fig. 3. (b) (contd) Cladogram showing the structural relationships of these ErmC methylases.

formation of different mRNA secondary structures by these inverted repeats, base-pair substitutions in the inverted repeats changed the stability of the stem-loop structures. Thus, the free energy of pairing of segments 1:2 was decreased from $\Delta G = -13.4$ kcal (c. -56.1 kJ)/mol in pE194 to $\Delta G = -5.0$ kcal (c. -20.9 kJ)/mol in pSES21, that of pairing of segments 2:3 from $\Delta G = -12.4$ kcal (c. -51.9 kJ)/mol in pE194 to $\Delta G = -9.4$ kcal (c. -39.4 kJ)/mol in pSES21, and finally that of pairing of segments 3:4 from $\Delta G = -12.2$ kcal (c. -51.1 kJ)/mol in pE194 to $\Delta G = -10.8$ kcal (c. -45.2 kJ)/mol in pSES21. The base-pair substitutions also generated a new pair of inverted repeated segments, designated 5:6, which encompassed parts of segments 2 and 3. The free energy of pairing of segments 5:6 was calculated to be $\Delta G = -11.0$ kcal (c. -46.1 kJ)/mol. The calculation of the free energies of the different mRNA secondary structures confirmed that there will be a greater tendency for segments 5:6 to form the energetically most stable secondary structure, than for segments 1:2 or 2:3 to form a stem-loop structure. If segments 5:6 pair, there is no partner left for segment 4 to form a secondary structure. Moreover, the unpaired state of segment 4 will render the ribosome-binding site for the *ermC* structural gene which is located within segment 4 accessible to ribosomes and so allow constitutive ErmC synthesis to occur.

The transcriptional terminator, composed of another two inverted repeated sequences 7 and 8, starts in the translational stop codon of the *ermC* structural gene. It also showed several base-pair exchanges which destabilised the secondary structure obtained from pairing of segments 7:8 from $\Delta G = -28.4$ kcal (c. -118.9 kJ)/mol in pE194 to $\Delta G = -21.4$ kcal

(c. -89.6 kJ)/mol in pSES21. Moreover, conversion of A to G at positions 932 and 936 (Fig. 3) destroyed the recognition sequence for the restriction enzyme *ClaI*.

Discussion

Antibiotics of the MLS group can be subdivided into two groups: (i) the inducers of *ermC* gene expression which include 14- and 15-membered macrolides and (ii) the non-inducers which comprise 16-membered macrolides, lincosamides and streptogramin B antibiotics [17, 18]. Staphylococci that carry inducible *ermC* genes are resistant only to inducers. This led to the assumption that inducibly *ermC* resistant staphylococci can effectively be treated with non-inducing members of the MLS group of antibiotics. However, such inducibly *ermC* resistant strains were found to be able to mutate to constitutive resistance in the presence of non-inducers [35]. First observations of this change of the type of gene expression [36] were made some time before the first *ermC* gene was sequenced and the translation attenuation model [19, 37] for inducible *ermC* gene expression was proposed. Several years later, in-vitro studies of constitutive mutants selected from inducibly *ermC* resistant strains on media containing tylosin, clindamycin, spiramycin or pristinamycin revealed structural alterations in the *ermC* translational attenuator [20, 23, 35, 38].

Two types of alterations were observed: sequence deletions and sequence duplications. The 59-bp deletion detected by Horinouchi and Weisblum [20] comprised the entire reading frame for the 19 amino-acid peptide including the inverted repeated segment 1. Constitutive mutants selected from clinical specimens also exhibited sequence deletions of varying extent within the translational attenuator [4, 8, 10]. A deletion of 107 bp which removed the reading frame for the small peptide and also the inverted segments 1, 2 and 3, was found to occur most frequently [10]. Besides these sequence deletions, a direct tandem duplication of 109 bp of the translational attenuator was observed in a constitutive mutant selected *in vitro* [23]. Other tandem duplications were seen *in vivo* in epidemiologically unrelated staphylococcal strains. Thus, duplication of a 28-bp fragment which contained parts of the inverted repeats 1 and 2 was detected in plasmid pRJ5 from *S. aureus* [12] while Lodder *et al.* [9] described the duplication of a 23-bp fragment which included the inverted repeat 4 and the first 5 bp of the *ermC* structural gene to be present in plasmid pSES6 from *S. equorum*.

First indications for the in-vitro selection of uninducible/constitutive mutants by point mutation of important positions within the translational attenuator were obtained from analysis of the *tyc-1* mutant of plasmid pE194 [23]. The conversion from A to C

within the translational start codon of the reading frame for the small peptide deleted this start codon and was considered to be responsible for constitutive *ermC* gene expression [23]. The assumption that translation of the small peptide is required for *ermC* induction was confirmed by Dubnau [39] who obtained an uninducible mutant by replacing the second codon of the 19 amino-acid peptide by a stop codon. While these mutations were selected randomly under laboratory conditions [23] or introduced artificially [39], data have not been available to confirm the occurrence of similar mutations *in vivo*. The data obtained from the naturally occurring plasmid pSES21, however, might fill this gap. Multiple base-pair exchanges and insertions were detected in the pSES21-encoded translational attenuator, several of which affected structures of importance for the regulation of the *ermC* gene. Deletion of the translational start codon for the small regulatory peptide in combination with frame-shift mutations in the reading frame destroyed this regulatory element. As the first nine codons of this small 19 amino-acid peptide are crucial for stalling a ribosome during induction [35, 40, 41], deletion of the reading frame for this small peptide might explain the uninducibility of the pSES21-encoded *ermC* gene. However, lack of inducibility does not consequently result in constitutiveness. As long as segments 1:2 and 3:4 form stable mRNA secondary structures and no ribosome is stalled in the region of segment 1, the *ermC*-associated ribosomal binding site and the start codon for the *ermC* methylase gene will be buried within the stem-loop structure 3:4 and therefore remain inaccessible to ribosomes. In pSES21, the base-pair substitutions within the inverted repeated segments mainly affected segments 1 and 2 and distinctly decreased the stability of mRNA secondary structures formed by pairing of 1:2 as well as 2:3. Pairing of the newly generated inverted repeats 5:6, which include part of segment 2 and the entire segment 3, might explain the constitutive type of *ermC* gene expression. Pairing of segments 5:6 will lead to the energetically most stable stem-loop structure and will leave segment 4 unpaired. Thus, the *ermC*-associated ribosome-binding site and the start codon for the *ermC* methylase gene will become accessible to ribosomes.

Previous studies have shown that an inducibly *ermC* resistant strain is able to mutate to constitutive *ermC* gene expression with a high probability [35]. The corresponding mutations were all located in the *ermC* translational attenuator and similar if not identical mutations were found under laboratory conditions as well as *in vivo*. The rapid development of constitutive resistance by sequence deletions or sequence duplications might be explained by an apparent single mutational step [35]. The observation that the corresponding ErmC methylases encoded by plasmids such as pSES6, pSES4a, pNE131 or pIM13 were either identical to that of pE194 or differed from it by

4, 5 or 6 amino acids confirmed that mutation to constitutive resistance was not accompanied by extensive alterations in the *ermC* structural gene, but remained mainly limited to the translational attenuator. Comparison of the deduced amino-acid sequences of the sequenced *ermC* genes of staphylococci and bacilli showed that the ErmC methylases are very closely related (Fig. 3a, b), however, with the pSES21-encoded ErmC methylase differing the most from all other adenine methylases of this hybridisation class. It is not known how many mutational steps during which time have been necessary to change the pSES21-encoded *ermC* translational attenuator from an inducible type to its present constitutive type and to account for the 16-amino-acid exchanges within the *ermC* methylase gene.

From the clinical point of view, mutation from inducible to constitutive resistance is accompanied by a widening of the spectrum of antibiotics to which resistance is expressed, which means from limited resistance to 14- and 15-membered macrolides in inducibly resistant strains to overall resistance to 14-, 15-, and 16-membered macrolides, lincosamides and streptogramin B antibiotics in constitutively resistant strains. The use of non-inducing MLS antibiotics as growth promoters in livestock might favour the occurrence of mutations to constitutive resistance. A recent study [10] revealed that randomly selected *ermC*-carrying strains from various domestic animals showed a strong correlation between the type of *ermC* gene expression and the previous contact of the animal to non-inducing MLS antibiotics. While none of the animals which carried inducibly *ermC* resistant staphylococcal strains had any previous contact with 16-membered macrolides, lincosamides or streptogramins, most of the constitutively *ermC* resistant strains were obtained from animals that had received 16-membered macrolides as growth promoters or lincosamides for therapeutic purposes. Knowledge of the mutational events that cause a change in the type of *ermC* gene expression and of the consequences for the effectiveness of MLS antibiotics to control macrolide-resistant staphylococci demand a careful and judicious use of non-inducing MLS antibiotics. This may include a restriction of the use of 16-membered macrolides as growth promoters in livestock.

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