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Polymorphisms of the *pbp5* gene and correlation with ampicillin resistance in *Enterococcus faecium* isolates of animal origin

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The C-terminal region of the *pbp5* gene was sequenced in 11 ampicillin-resistant and 5 ampicillin-susceptible *Enterococcus faecium* isolates of animal origin, and compared with a *pbp5* reference sequence (GenBank accession no. X84860). Eight different *pbp5* alleles (designated A–H) were detected when amino acid changes in the region 461–629 were considered. Three of these alleles (A–C) were detected in ampicillin-susceptible isolates (MIC range 1–8 μg ml^{-1}), and included the changes 470H→Q, 471V→I, 487Q→L, 581I→V, 595E→A or 622E→D. The remaining five alleles (D–H) were found in ampicillin-resistant isolates (MIC range 32–256 μg ml^{-1}); three of these alleles (F–H) presented a serine insertion at position 466′, in addition to other important amino acid changes (485M→A, 496N→K, 499A→T, 525E→D, 586V→L or 629E→V). The other two alleles presented the amino acid changes 496N→K and 629E→V (allele D), and 470H→Q (allele F). A correlation between deduced amino acid changes in PBP5 and ampicillin MICs was detected in animal *E. faecium* isolates.

INTRODUCTION

Enterococcus faecium is an important human pathogen whilst also being a commensal bacterium of the intestinal tract of humans and animals. *E. faecium* is intrinsically resistant to moderate levels of ampicillin, with MICs typically ranging from 1 to 16 μg ml^{-1} (Kak & Chow, 2002). This natural ampicillin resistance of *E. faecium* was demonstrated to be associated with expression of penicillin-binding protein 5 (PBP5), which has a low affinity for β-lactams (Fontana *et al.*, 1983, 1994; Rice *et al.*, 2001). The *pbp5* gene is located in the bacterial chromosome and is considered to be intrinsic to the species, although recently it has also been reported as a transferable determinant (Rice *et al.*, 2005). Recently, an increase in ampicillin resistance has been observed in clinical *E. faecium* isolates, which could compromise therapy in life-threatening infections caused by this micro-organism (Klare *et al.*, 2003). The emergence of high-level ampicillin resistance in *E. faecium* may be due to either increased production of PBP5 or mutations in the *pbp5* gene resulting in lower affinities for ampicillin (Kak & Chow, 2002; Rice *et al.*, 2001; Sifaoui *et al.*, 2001; Williamson *et al.*, 1983; Zorzi *et al.*, 1996). Specific amino acid changes in the C-terminal region of PBP5 (especially at aa 466′, 485, 496, 499, 525, 586 and 629, around the active-site region of PBP5) have been associated with β-lactam resistance (Jureen *et al.*, 2003; Ligozzi *et al.*, 1996; Rice *et al.*, 2001, 2004; Rybkine *et al.*, 1998; Sauvage *et al.*, 2002; Zorzi *et al.*, 1996), and 12 different alleles of this gene have previously been demonstrated in clinical *E. faecium* isolates when this C-terminal region of *pbp5* gene was analysed (Jureen *et al.*, 2003).

As far as we know, all studies of the characterization and detection of mutations in *pbp5* have been performed on *E. faecium* isolates of human origin and not on animal isolates. Thus, the *pbp5* gene of ampicillin-resistant (Amp^R) and ampicillin-susceptible (Amp^S) *E. faecium* isolates recovered from healthy animals was sequenced in the present study. The deduced amino acid changes were found to correlate with their specific ampicillin MICs.

Abbreviation: PBP5, penicillin-binding protein 5.

METHODS

Bacterial isolates. Antimicrobial resistance was analysed by the disc-diffusion method in a previous study in enterococcal isolates

recovered from faecal samples of healthy poultry and pets (Poeta *et al.*, 2006). A total of 11 *E. faecium* isolates of that study showed an Amp^R phenotype, comprising 10 faecal samples from poultry and 1 faecal sample from a dog. These 11 Amp^R *E. faecium* isolates, as well as 5 Amp^S *E. faecium* isolates (4 from poultry and 1 from a dog), were included in the present study.

Ampicillin-susceptibility testing. The MIC of ampicillin (Eli Lilly) was determined in our *E. faecium* isolates using the agar-dilution method according to the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, 2005). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality-control strains.

Analysis of β-lactamase activity. The production of β-lactamase was tested by streaking the bacterial colonies over nitrocefin discs (Cefinase; Becton Dickinson Microbiology Systems) (Marshall *et al.*, 1995). After 5–10 min at room temperature, a change in colour from yellow to purple was indicative of a positive reaction.

PCR amplification of *pbp5* and DNA sequence analysis. Total DNA from *E. faecium* isolates was obtained using an InstaGene matrix (Bio-Rad) and 10 μl DNA was used in each PCR, with 3.5 mM $MgCl_2$. The C-terminal region of the *pbp5* gene was amplified by PCR in all 16 *E. faecium* isolates included in this study using primers PBP5F-1 (5′-AACAAAATGACAAACGGG-3′) and PBP5R (5′-TATCCTTGGTTATCAGGG-3′), producing an amplicon of 779 bp. PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 2 min, with a final 7 min extension period at 72 °C (Jureen *et al.*, 2003). PCR products were detected on ethidium-bromide-stained agarose gels and purified using a QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. The purified products were sequenced on both strands using an Applied Biosystems 3730 DNA sequencer, using primers PBP5F-2 (5′-GACAAACGGGATCTCACAAG-3′) and PBP5R. Duplicated sequences were obtained from independent PCRs and compared with that of the *pbp5* gene reference sequence (GenBank accession no. X84860).

The complete *pbp5* gene was also amplified by PCR in six of our *E. faecium* isolates (two Amp^S and four Amp^R) using primers PBP5F-3 (5′-AAAAATCGAACAACAGGCGCTTA-3′) and PBP5R (amplicon size 1916 bp), using *TaKaRa* DNA polymerase (Takara Bio) under conditions recommended by the manufacturer. Amplicons were purified and sequenced by primer walking. The sequences obtained from both strands were also compared with that of the reference sequence.

RESULTS AND DISCUSSION

Polymorphisms of the *pbp5* gene were studied in a series of *E. faecium* isolates of animal origin that showed different susceptibilities to ampicillin. None showed β-lactamase activity when nitrocefin discs were used for screening purposes.

C-terminal region of the *pbp5* gene

The sequence of the C-terminal region of *pbp5* was analysed in our 11 Amp^R and 5 Amp^S *E. faecium* isolates and was correlated with their specific ampicillin MIC values. The deduced amino acid changes detected between aa 461 and 634 of these enterococci are shown in Table 1, with amino acid positions considered to be important for ampicillin resistance indicated in bold.

Eight different *pbp5* alleles, designated here as A–H, were detected when amino acid changes in this region were taken into account, none of which showed 100% identity with the reference sequence (GenBank accession no. X84860). Our five Amp^S isolates (MICs 1–8 μg ml^{-1}) were classified as only three of these alleles (A–C) and included changes at positions not previously considered to be important for ampicillin resistance (allele A 470H→Q, 581I→V, 595E→A; allele B 470H→Q, 595E→A; allele C 470H→Q, 471V→I, 487Q→L, 622E→D) (Table 1). The remaining five alleles (D–H) were found in our 11 Amp^R isolates. It is important to stress that three of these alleles (F–H) presented a serine insertion at position 466′ in addition to other important amino acid changes: alleles F and H (485M→A, 496N→K, 499A→T, 525E→D and 629E→V) and allele G (485M→A, 496N→K, 499A→T, 525E→D, 586V→L and 629E→V). Allele F, identified in seven Amp^R isolates (MICs 32–256 μg ml^{-1}), was the most frequent allele in our study. Allele D was found in one Amp^R isolate (MIC 32 μg ml^{-1}) and showed amino acid changes at two principal positions (496N→K and 629E→V). The remaining allele (E) was detected in one Amp^R isolate (MIC 64 μg ml^{-1}), recovered from a dog, and showed only one amino acid change (470H→Q) with respect to the reference sequence. This specific substitution was also found in all of our isolates, both Amp^R and Amp^S, and has not previously been associated with increased ampicillin MICs by other authors (Jureen *et al.*, 2003).

It is important to note that the insertion of serine at position 466′ was detected in most of our Amp^R animal *E. faecium* isolates, and was generally associated with other amino acid changes. Insertions of aspartic acid or serine at position 466′ have also been detected previously in strains with an increased level of ampicillin resistance (Jureen *et al.*, 2003; Rybkine *et al.*, 1998; Zorzi *et al.*, 1996). According to the previously reported PBP5 crystal structure (Sauvage *et al.*, 2002), when PBP5 protein of *E. faecium* (PBP5fm) forms a complex with benzylpenicillin, the active-site cavity of this protein is delimited by the β_{C3} strand on one side, by residues 461–465 on the opposite side and by residues 537–541 on the bottom of the cavity. Residues 461–465 form part of a loop (aa 451–465) that is well conserved in the B1 subgroup of class B high-molecular-mass PBPs. Thus, the substitution 461Q→K found in most of our Amp^R strains could be implicated in lower affinity for the antibiotic. The residue V465 points into the active site, being close to the β-lactam ring; therefore, the insertion of a residue at position 466′ may slightly displace V465 inside the active site, reducing its accessibility for ampicillin (Rice *et al.*, 2004; Sauvage *et al.*, 2002). The serine insertion was found in the strains with high-level ampicillin resistance and seems to be an essential determinant of resistance, affecting antibiotic recognition.

As in the findings of other authors (Rybkine *et al.*, 1998; Zorzi *et al.*, 1996), the change of methionine to alanine at aa 485 was observed in isolates with higher MICs

Table 1. Polymorphisms in the C-terminal region of *pbp5* in 16 *E. faecium* isolates of animal origin and correlation with their specific ampicillin MIC values

E. faecium isolate	*pbp5* allele*	Amino acid change at position:†															Ampicillin MIC (μg ml^{-1})
		461	466′	470	471	485	487	496	497	499	525	581	586	595	622	629	
X84860		Q	–	H	V	M	Q	N	F	A	E	I	V	E	E	E	
P4	A	Q	–	**Q**	V	M	Q	N	F	A	E	**V**	V	**A**	E	E	1
P44	B	Q	–	**Q**	V	M	Q	N	F	A	E	I	V	**A**	E	E	4
P47	B	Q	–	**Q**	V	M	Q	N	F	A	E	I	V	**A**	E	E	4
P43	B	Q	–	**Q**	V	M	Q	N	F	A	E	I	V	**A**	E	E	8
P54	C	Q	–	**Q**	**I**	M	**L**	N	F	A	E	I	V	E	**D**	E	4
P12	D	Q	–	**Q**	V	M	Q	**K**	F	A	E	I	V	E	E	**V**	32
P42‡	E	Q	–	**Q**	V	M	Q	N	F	A	E	I	V	E	E	E	64
P67	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	32
P76	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	32
P13	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	128
P1	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	256
P3	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	256
P4	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	256
P6	F	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	V	E	E	**V**	256
P2	G	**K**	**S**	**Q**	V	**A**	Q	**K**	F	**T**	**D**	I	**L**	E	E	**V**	128
P11	H	**K**	**S**	**Q**	V	**A**	Q	**K**	**L**	**T**	**D**	I	V	E	E	**V**	256

*Alleles were designated A–H based on amino acid substitutions.

†Important amino acid positions and amino acid changes with respect to the reference sequence (GenBank accession no. X84860) are indicated in bold.

‡Allele E, detected in the *pbp5* gene of the P42 isolate, was equivalent to allele 3 described by Jureen *et al.* (2003).

(128–256 μg ml^{-1}), although two isolates showed a MIC of 32 μg ml^{-1}. The bulky side chain of M485 located behind the K425 side chain is substituted by the small alanine molecule, obtaining more conformational space for lysine and resulting in a lower affinity for the β-lactam antibiotic. Although the substitution 485M→A seems to be important for ampicillin resistance, it has been reported that this change by itself does not confer high-level ampicillin resistance (Sifaoui *et al.*, 2001), as observed in our study.

We identified only one Amp^R isolate (MIC 128 μg ml^{-1}) that showed the 586V→L substitution. However, Ligozzi *et al.* (1996) suggested that the region from aa 558 to 586 might play an important role in the β-lactam-binding site of PBP5, as changes in this region occurred in the highly resistant strains. Residue 629 is close to one edge of the active site and the 629E→V substitution causes a change of a hydrophilic charged residue for a hydrophobic one, which is unfavourable for the overall stability of the protein and may decrease the ability of the protein to bind to β-lactam by restricting mobility (Leszczynski & Rose, 1986; Rice *et al.*, 2004).

In a comparison of the 8 alleles of the *pbp5* gene detected in our animal isolates with the 12 alleles reported by Jureen *et al.* (2003) in human isolates, it is important to note that only one of our alleles (allele E with the single change 470H→Q) corresponded to one of those determined by Jureen and co-workers (designated allele 3). Allele 3 of *pbp5* was detected particularly among Amp^S human isolates and also in a few Amp^R isolates (Jureen *et al.*, 2003). In our study, allele E was found in one Amp^R *E. faecium* isolate (P42, MIC 64 μg ml^{-1}) recovered from a dog faecal sample.

If only the potentially significant amino acid positions (466′, 485, 496, 499, 525, 586 and 629) of the C-terminal region of PBP5 were considered, all of our Amp^S isolates presented the same amino acid pattern as the reference sequence. In the case of Amp^R isolates, four different patterns could be identified: (i) amino acid changes at the above seven positions in one isolate (MIC 128 μg ml^{-1}, allele G); (ii) changes at six positions in eight isolates (MICs 32–256 μg ml^{-1}, alleles F and H); (iii) changes at two positions in one isolate (MIC 32 μg ml^{-1}, allele D); and (iv) no changes at important positions in one isolate (P42, MIC 64 μg ml^{-1}, allele E) (Table 1). Thus, it seems that specific alterations in the C-terminal part of the *pbp5* gene alone could not entirely be correlated with the different levels of ampicillin resistance among our strains and other factors are probably implicated (Jureen *et al.*, 2004; Rice *et al.*, 2001; Sifaoui *et al.*, 2001).

N-terminal region of the *pbp5* gene

In order to determine whether the ampicillin resistance phenotype detected in *E. faecium* P42 (allele E) was due to amino acid changes in positions outside the C-terminal

region of *pbp5*, the complete *pbp5* gene was amplified and sequenced for this isolate, as well as for five additional Amp^R and Amp^S *E. faecium* isolates (P44, P54, P13, P2 and P11, belonging to alleles B, C, F, G and H, respectively). The amino acid changes detected in the N-terminal region of the *pbp5* gene of these isolates with respect to the reference sequence are indicated in Table 2. It was found that *E. faecium* P42 and one of the Amp^S isolates showed no amino acid changes with respect to the reference sequence, whilst four changes were detected in the other Amp^S isolate. Nevertheless, a large number (up to 12) and variety of amino acid changes were demonstrated in this region in the three Amp^R isolates (P2, P11 and P13). Zorzi *et al.* (1996) described most of these amino acid changes in the N-terminal domain of their studied strains, with the 68A→T and 85E→D substitutions only detected in the highly resistant penicillin strains (EFM-1 and H80721, with MICs of 90 and 512 μg ml^{-1}, respectively). These two changes were also observed in our high-level Amp^R isolates. The function of the N-terminal domain is still unclear. It has been hypothesized that it is necessary for correct folding of the C-terminal module and both domains seem to be interdependent (Sauvage *et al.*, 2002). In fact, deletion of some segments of the N-terminal domain of PBP5 from *Enterococcus hirae* resulted in proteins that were unable to bind penicillin (Mollerach *et al.*, 1996). Future studies should be carried out to determine whether the amino acid changes in the N-terminal region of PBP5 could be associated with changes in *β*-lactam susceptibility in *E. faecium* species.

Conclusions

The specific alterations in the C-terminal part of the *pbp5* gene could be used as markers for ampicillin resistance (at least the insertion at aa 466′ and the changes at position 485), as they are present in most of the Amp^R but not in the Amp^S strains, although, as observed by other researchers (Jureen *et al.*, 2004; Rice *et al.*, 2001; Sifaoui *et al.*, 2001), it seems that other factors could be necessary for a complete explanation of the differences in levels of ampicillin resistance. High levels of resistance could be observed in strains that either overexpress PBP5 and/or present multiple mutations that decrease the affinity of PBP5 for the antibiotic. This possibility could explain the different levels of ampicillin resistance among strains classified as the same allele (allele F) or the P42 strains whose MIC values could be due simply to protein overexpression. Zorzi *et al.* (1996) suggested that only a single copy of the *pbp5* gene was present in their analysed strains. Thus, the variations seen in the amount of PBP5 could not be due to a gene dosage effect, although the detection of *pbp5* located within transferable elements could change this suggestion (Rice *et al.*, 2005). More recently, it has been reported that expression of ampicillin resistance was higher when the *pbp5* gene was located downstream of the $ftsW_{Efm}$ and *psr* genes (Rice *et al.*, 2001). The role of the putative $ftsW_{Efm}$ gene product is unknown, although it has been suggested that its homologue in *Escherichia coli* may serve as a chaperone protein for PBP3 (Eberhardt *et al.*, 2003). In addition, it has been proposed that overproduction of PBP5 could result from either an alteration in the *psr* promoter or a modification of the target site of Psr, probably located between the *psr* and *pbp5* genes (Zorzi *et al.*, 1996).

In conclusion, the C-terminal region of the *pbp5* gene was found to be highly polymorphic among our Amp^R and Amp^S *E. faecium* isolates of animal origin and there seemed to be a correlation between specific changes located at positions close to the active site of PBP5 and the ampicillin MIC, although further studies of the mechanisms of ampicillin resistance in *E. faecium* are necessary.

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Table 2. Amino acid changes detected in the N-terminal region of *pbp5* in six of the *E. faecium* isolates of this study

E. faecium	Amino acid change at position:*													Ampicillin MIC (μg ml^{-1})
	27	34	66	68	73	85	100	144	172	177	204	216	324	
X84860	S	R	G	A	A	E	E	K	T	L	D	A	T	
P44	S	R	G	A	A	E	E	K	T	L	D	A	T	4
P54	**G**	R	G	A	**T**	E	E	**Q**	T	L	D	A	**A**	4
P42	S	R	G	A	A	E	E	K	T	L	D	A	T	64
P13	NA	NA	**E**	**T**	A	E	**Q**	**Q**	**A**	**I**	**G**	**S**	**A**	128
P2	NA	**Q**	**E**	**T**	A	**D**	**Q**	**Q**	**A**	**I**	**G**	**S**	**A**	128
P11	**G**	**Q**	**E**	**T**	A	**D**	**Q**	**Q**	**A**	**I**	**G**	**S**	**A**	256

NA, The sequence at this position was not available.

*Amino acid changes with respect to the reference sequence (GenBank accession no. X84860) are indicated in bold.

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