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The penicillin resistance of *Enterococcus faecalis* JH2-2r results from an overproduction of the low-affinity penicillin-binding protein PBP4 and does not involve a *psr*-like gene

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A penicillin-resistant mutant, JH2-2r (MIC 75 μg ml^{-1}), was isolated from *Enterococcus faecalis* JH2-2 (MIC 5 μg ml^{-1}) by successive passages on plates containing increasing concentrations of benzylpenicillin. A comparison of the penicillin-binding protein (PBP) profiles in the two strains revealed a more intensely labelled PBP4 in JH2-2r. Because the sequences of the JH2-2 and JH2-2r *pbp4* genes were strictly identical, even in their promoter regions, this intensive labelling could only be associated with an overproduction of the low-affinity PBP4. No *psr* gene analogous to that proposed to act as a regulator of PBP5 synthesis in *Enterococcus hirae* and *Enterococcus faecium* could be identified in the vicinity of *pbp4* in *E. faecalis* JH2-2 and JH2-2r. However, a *psr*-like gene distant from *pbp4* was identified. The cloning and sequencing of that *psr*-like gene from both *E. faecalis* strains indicated that they were identical. It is therefore postulated that the PBP4 overproduction in *E. faecalis* JH2-2r results from the modification of an as yet unidentified factor.

Keywords: β-lactam resistance, low-affinity PBP, PBP-synthesis repressor (*psr*) gene

INTRODUCTION

Enterococci are normal human commensals. However, they are now considered as pathogens responsible for a wide variety of infections in humans (Murray, 1990; Jett *et al*., 1994; Schmit *et al*., 1994). They appeared in second position on a list of the five leading pathogens in a survey made in the USA between 1986 and 1997 (Hancock & Gilmore, 2000). *Enterococcus faecalis* caused 80–90 % of enterococcal infections, while *Enterococcus faecium* accounted for 5–10 % (Murray, 1990; Jett *et al*., 1994; Schmit *et al*., 1994).

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Abbreviations: HMM, high molecular mass; LMM, low molecular mass; PBP, penicillin-binding protein.

The GenBank accession numbers for the sequences reported in this paper are Y17797 for the 8·4 kb segment of pDML521; AJ290435 for *pbp4* of *E. faecalis* JH2-2; AJ276231 and AJ276232 for the *psr*-like gene of *E. faecalis* JH2-2 or JH2-2r, respectively.

Enterococci are intrinsically more resistant to β-lactams than other streptococci, with 10- to 100-fold higher MICs (Gutmann, 1994). The vast majority of *E. faecalis* strains have penicillin MICs ranging between 1 and 4 μg ml^{-1}. Many *E. faecium* strains have a MIC above 8 μg ml^{-1}, with the majority ranging between 16 and 32 μg ml^{-1} (Murray, 1990). The resistance mechanism of enterococci to β-lactams has mainly been studied in two closely related species: *Enterococcus hirae* and *E. faecium*. Both have an essential low-affinity penicillin-binding protein (PBP5) which is directly responsible for the phenomenon (Fontana *et al*., 1983, 1994; Williamson *et al*., 1985; El Kharroubi *et al*., 1991; Piras *et al*., 1993; Ligozzi *et al*., 1996; Zorzi *et al*., 1996). When PBP5 is not synthesized, the cells become susceptible to β-lactams with MICs below 0·2 μg ml^{-1} (Fontana *et al*., 1985). On the other hand, overproduction of PBP5 and/or reduction of its affinity in both species leads to high-level resistance (Fontana *et al*., 1983; Klare *et al*., 1992; Ligozzi *et al*., 1996; Zorzi *et al*., 1996).

In both species, synthesis of PBP5 was reported to be under the control of a repressor-encoding gene, *psr* (for PBP5 synthesis repressor), which is located immediately

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
Enterococcus faecalis		
JH2-2	Rif^r Fus^r	Williamson *et al.* (1986)
JH2-2r	Rif^r Fus^r Pen^r	This study
Escherichia coli		
RR1	*supE44 hsdS20 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	United States Biochemical
Top10F′	F′ [*lac*I^q Tn*10*(Tet^R)] *mcrA* Δ(*mrr–hs*dRMS–mcrBC) *ϕ*80*lacZ*ΔM15 Δ*lacX74 dcoR recA1 araD139* Δ(*ara–leu*)7697 *galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
INVαF′	F′ *endA1 recA1 hsdR17*(r_λ^- m_λ^+) *supE44 thi-1 gyrA96 relA1 ϕ*80*lacZ*ΔM15 Δ(*lacZYA–argF*)U169	Invitrogen
Plasmids		
pBR322	Low-copy vector, Ap^r, Tet^r	Promega
pUC18	High-copy vector, Ap^r	Amersham Pharmacia Biotech
pUCBM20	High-copy vector, Ap^r	Boehringer
pGEM-T Easy	PCR fragment cloning vector, Ap^r	Promega
pCR2.1	PCR fragment cloning vector, Ap^r Kan^r	Invitrogen
pMCL210	Low-copy vector derived from pACYC184, Cm^r	Nakano *et al.* (1995)
pDML521	pBR322 carrying a ∼ 11 kb *pbp4*-containing *Eco*RI fragment of *E. faecalis* JH2-2r	This study
pDML522	pBR322 carrying a ∼ 11 kb *pbp4*-containing *Eco*RI fragment of *E. faecalis* JH2-2r	This study
pDML523	pMCL210 carrying a 2·2 kb fragment containing the *pbp4* gene of *E. faecalis* JH2-2	This study
pDML524	pMCL210 carrying a 2·2 kb fragment containing the *pbp4* gene of *E. faecalis* JH2-2r	This study
pDML525	pGEM-T Easy carrying a 1·6 kb PCR fragment containing the *psr*-like gene of *E. faecalis* JH2-2	This study
pDML526	pGEM-T Easy carrying a 1·6 kb PCR fragment containing the *psr*-like gene of *E. faecalis* JH2-2r	This study

*Rif^r, rifampicin-resistant; Fus^r, fusidic acid resistant; Pen^r, penicillin resistant; Ap^r, ampicillin resistant; Tet^t, tetracycline resistant; Kan^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

upstream from the *pbp5* gene (Ligozzi *et al.*, 1993; Massidda *et al.*, 1998). Inactivation of *psr* by a point mutation or a deletion seemed to result in the full expression of *pbp5* and increased resistance of the cells. In contrast, normal activity of *psr* in wild-type strains apparently reduced the expression of *pbp5* to a low level and induced a higher susceptibility of the cells to penicillin (Ligozzi *et al.*, 1993).

E. faecalis also has a low-affinity PBP (Signoretto *et al.*, 1994). Analysis of clinical and laboratory resistant strains has shown that it is involved in *β*-lactam resistance exactly as PBP5 is in *E. hirae* and *E. faecium* (Williamson *et al.*, 1985; Fontana *et al.*, 1994; Signoretto *et al.*, 1994). However, information concerning that PBP, previously designated as PBP5 (Signoretto *et al.*, 1994), mainly concerns its cloning, sequencing and expression in *Escherichia coli*.

In this study we could not identify a *psr*-like gene in the immediate vicinity of the *E. faecalis* low-affinity PBP4-encoding gene as observed in *E. hirae* or *E. faecium*. A *psr*-like gene was however found elsewhere in the genome, but it appeared not to be involved in the overproduction of the *E. faecalis* low-affinity PBP4.

METHODS

Bacterial strains, isolation of plasmids and membranes. *Enterococcus faecalis* and *Escherichia coli* cells were grown in Brain Heart infusion broth (BHI) and Luria–Bertani (LB) medium, respectively. Resistant clones of *E. faecalis* JH2-2 (benzylpenicillin MIC value: 5 μg ml^{-1}) were selected by successive growths on BHI plates containing increasing concentrations of benzylpenicillin (PenG). One clone was randomly chosen and designated as JH2-2r (PenG MIC value: 75 μg ml^{-1}). *E. coli* strains Top 10 F′ and INVαF′ were used to replicate the different *E. coli* plasmids described in Table 1. *E. coli* plasmids were extracted by the Wizard Plus Minipreps DNA purification system (Promega) or with the GFX Micro-

plasmid prep kit (Amersham Pharmacia Biotech). Membranes of the enterococcal strains and *E. coli* K-12 RR1 were isolated as described previously (Lindström *et al.*, 1970; Coyette *et al.*, 1978). Membrane-bound PBPs (150 μg total protein) were labelled with [^{14}C]PenG or [^{125}I]PenG and detected by fluorography or a phosphorus storage K screen (FX-imager apparatus, Bio-Rad) (Laskey, 1980; Masson & Labia, 1983; Zorzi *et al.*, 1996).

Molecular biology techniques. *Enterococcus* genomic DNA was isolated as reported previously (Loureiro Dos Santos & Chopin, 1987) from cells grown unshaken at 37 °C in BH medium and collected at the end of the exponential phase.

PCR amplifications were made as described previously (Innis *et al.*, 1990) using the Biotools DNA polymerase (Life Sciences International). The DNA fragments or PCR products were purified with the help of the GeneClean spin kit (Bio 101) or the Wizard PCR preps DNA purification system (Promega).

Hybridizations using oligonucleotides or DNA fragments labelled with digoxigenin were prepared according to the instructions of the DIG system user's guide from Boehringer Mannheim.

DNA sequencing was performed on both strands using the T7 sequencing kit with [^{35}S]dATPαS labelling, and the Autoread or ThermoSequenase sequencing kits with 5′-fluorescein or Cy5 primers, in which case the electrophoresis was performed on an ALF express DNA sequencer (Amersham Pharmacia Biotech). The nucleotide sequences were introduced in GELASSEMBLE (Pearson & Lipman, 1988), the ORFs were identified with CODONREFERENCE (Devereux *et al.*, 1984) and homology searches (SWISS-PROT, PIR, Genpept) were made using FASTA or BLASTP (Altschul *et al.*, 1990). The EMBL accession numbers for the different sequences are as follows: 8·4 kb segment of pDML521, Y17797; *pbp4* of *E. faecalis* JH2-2, AJ290435; and *psr*-like of *E. faecalis* JH2-2 or JH2-2r, AJ276231 and AJ276232, respectively.

Construction of plasmids pDML523 to pDML526. The JH2-2 and JH2-2r *pbp4* genes were amplified by PCR using the oligonucleotides O16 and O17. The purified 2·2 kb fragments thus obtained were digested with *Bam*HI and *Eco*RI and cloned into pMCL210 between the *Bam*HI and *Eco*RI sites to yield pDML523 and pDML524, respectively.

The complete JH2-2 and JH2-2r *psr*-like genes were amplified by PCR using oligonucleotides O14 and O15 and cloned into the pGEM-T Easy vector to yield pDML525 and pDML526, respectively.

RESULTS

PBP profile in *E. faecalis*

In the penicillin-resistant *E. faecalis* 56R (Signoretto *et al.*, 1994), five high-molecular-mass (HMM) PBPs, namely PBPs 1 to 5, and a low-molecular-mass (LMM) PBP, designated PBP6, were proposed as membrane-bound PBPs. In that profile, however, PBP4 and PBP5 co-migrated as a single thick band, probably due to an overloading of membrane proteins on the gel. Another more recent study (Mainardi *et al.*, 1998) proposed only four HMM PBPs, PBP1 to 4, in *E. faecalis* JH2-2. When we compared the PBP profiles of *E. faecalis* JH2-2 and JH2-2r, we also detected only four HMM PBPs and a barely visible LMM PBP (probably a DD-carboxy-peptidase), designated in our study as PBP5 (Fig. 1, lanes 3–6). Generally, PBP2 appeared as a thin band in contrast with the three other HMM PBPs. Occasionally, PBP3 and PBP4 co-migrated and formed one very thick band. However, because of its low affinity, PBP4 could easily be distinguished from PBP3 by direct labelling and prelabelling fluorography experiments. Some membrane preparations seemed to show an additional PBP that migrated just below PBP4. However, freshly prepared membrane samples did not show this additional band, which could derive from another PBP by a spontaneous breakdown phenomenon analogous to that observed for PBP4* in membrane preparations of *E. hirae* ATCC 9790 (Coyette *et al.*, 1980). Nevertheless, from the protein sequence comparisons, it is clear that the PBP, named PBP5 by Signoretto *et al.* (1994) corresponds to the PBP4 studied in this work.

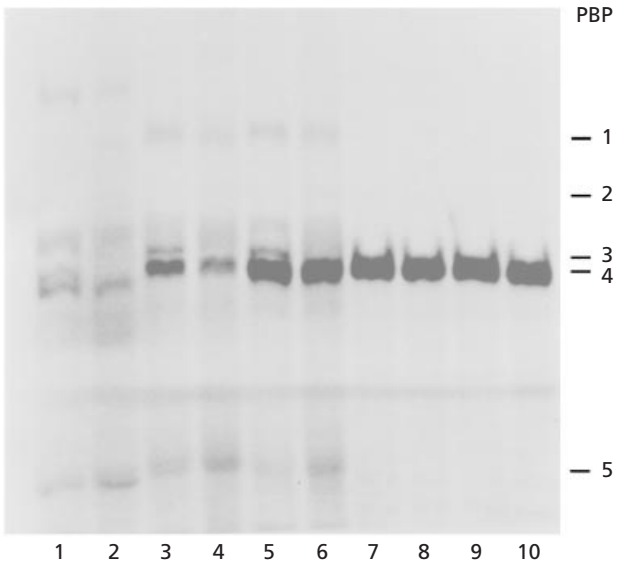

Fig. 1. SDS-PAGE and fluorogram of the membrane-bound PBPs from *E. hirae* ATCC 9790, *E. faecalis* JH2-2, JH2-2r and *Escherichia coli* overproducing the *E. faecalis* PBP4. Conditions of electrophoresis: 7·2 % (w/v) acrylamide; 16 h; gel length 24 cm. Membranes (150 μg protein) of *E. hirae* and *E. faecalis* and membranes (15 μg protein) of recombinant *E. coli* were labelled with 60 μM benzyl[^{125}I]penicillin for 30 min either directly (odd-numbered lanes) or with 90 μM benzyl[^{125}I]penicillin after previous treatment with 15 μM non-radioactive benzylpenicillin for 30 min (even-numbered lanes). Exposure conditions: 16 h with a phosphorus storage K screen (FX-imager apparatus, Bio-Rad). Lanes 1 and 2, *E. hirae* ATCC 9790; lanes 3 and 4, *E. faecalis* JH2-2; lanes 5 and 6, *E. faecalis* JH2-2r; lanes 7 and 8, *E. coli* overproducing the PBP4 of *E. faecalis* JH2-2; lanes 9 and 10, *E. coli* overproducing the PBP4 of *E. faecalis* JH2-2r.

PBP4 was the only PBP in *E. faecalis* JH2-2 and JH2-2r membrane preparations that reacted on a Western blot with polyclonal antibodies raised against *E. hirae* PBP5 (El Kharroubi *et al.*, 1991) or PBP3r (Piras *et al.*, 1990). As already previously reported (Signoretto *et al.*, 1994), the *E. faecalis* PBP4 reacted with the antibodies much

Table 2. Oligonucleotides designed for hybridization or DNA amplifications by PCR

	Sequence*	Positions
O1	5′TGGCAAAAAGA(T,C)(A,C)(G,A)(T,A)TC(T,A)TGGGG3′	7078–7099†
O2	5′T(C,T)(T,C)T(C,T)(A,G)TT(A,T)GA(A,G)ATTTG(A,T)GC3′	7301–7282†
O3	5′TATTTTGCCCAACAAACATTAC3′	7177–7198†
O4	5′GGTTGTGTCCCCCATATTATCAGG3′	5419–5442†
O5	5′CCAAGGCAGAAACACCAAGAATAAG3′	5788–5764†
O6	5′GACATATTATATAGTTAGGGGATTATTGAATGGAA3′	5693–5727†
O7	5′CCAGAACCGAGTTTGCCAGGCACTAC3′	6335–6310†
O8	5′GGTAGTGGGCTAGCAATTAACAAAGTG3′	6268–6294†
O9	5′GCACTACCAGGGCGTTTGTCAAATATAGC3′	6824–6796†
O10	5′CGATTGACAGTGGCGTACAACAACAAGC3′	6764–6791†
O11	5′GAAATTTGAGCAGGCGTCATGGCAATTGG3′	7292–7264†
O12	5′GGGGGAAGACAAATTTAGAGCGGG3′	7203–7226†
O13	5′CGCTTCATTGTAGCACACTTTCCTTTTTC3′	7851–7823†
O14	5′GAAGCAGCCTTAACAGAACTGGAACTC3′	4102–4128‡
O15	5′CTAAAATGACTGGGATGGGAATTACAAAA3′	5676–5648‡
O16	5′GAGGGATCCAAGCCGCCGCCAACTGAAC3′	5469–5489†
O17	5′GCTCTAGACGCACACGAATCC CTTTCCTTCTAC3′	7884–7860†

* Underlined sequences are *Bam*HI and *Xba*I restriction sites.

† The indicated numbers correspond to positions in the EMBL Y17797 sequence (8·5 kb insert of pDML521).

‡ The indicated numbers correspond to positions in contig 6330 of the partially sequenced genome of *E. faecalis* V583 (TIGR database).

more weakly than *E. hirae* PBP5 or PBP3r (data not shown).

PenG acylation and deacylation rates of the PBPs were estimated by 5 min labelling of JH2-2 and JH2-2r membrane preparations with increasing concentrations of [^{14}C]PenG (ranging from 1 to 500 μM). The concentrations yielding a 50% saturation (ID_{50}) of PBP3 and PBP4 were $6{\cdot}7 \pm 3$ and 46 ± 34 μM, respectively (which are equivalent to $2{\cdot}4 \pm 1{\cdot}1$ and $16 \pm 11{\cdot}8$ μg ml^{-1}; mean value of three different experiments). The second-order rate constants of acylation (k_{+2}/K) of these PBPs calculated from these values were 340 ± 150 and 50 ± 40 M^{-1} s^{-1}, respectively (Ghuysen *et al*., 1986). The deacylation constant k_{+3} values of both PBP3 and PBP4 complexes were close to 5×10^{-6} s^{-1} indicating a very slow breakdown of the complexes, which could thus be neglected for calculation of the k_{+2}/K acylation constant. It is interesting to note that no difference could be seen in the affinity of PBP4 in JH2-2 and JH2-2r membranes. However, it was obvious that PBP4 was more intensely labelled in JH2-2r (Fig. 1, lanes 5 and 6) than in JH2-2 membrane samples (Fig. 1, lanes 3 and 4). When the amount of PBP4 was related to the amount of total membrane protein or to the amount of PBP1, used as an internal standard (lanes 3 and 5), it clearly appeared that the production of PBP4 was 3·5-fold (mean value) higher in JH2-2r than in JH2-2 while that of the other PBPs remained constant. This level of overproduction is much lower than the increase of the MIC value (15-fold factor when JH2-2r and JH2-2 are compared). However, as mentioned for *E. faecium* (Rice *et al*., 2001) and as we have observed with *E. hirae* transformants (unpublished results), there is no direct correlation between the MIC values and the quantities of low-affinity PBP overproduced in the resistant strains.

Cloning of *pbp4*, the PBP4-encoding gene of the resistant *E. faecalis* strain JH2-2r

The cloning and sequencing of *pbp4* was undertaken well before the sequencing data of the *E. faecalis* V583 genome were released. The sequences of two degenerate oligonucleotides, O1 and O2 (Table 2), were based on the amino acid sequences of two peptides, WQKD(S,Q,K)SWG and AQSN(K,E)(E,D,N), relatively well conserved in the low-affinity PBP3r and PBP5 of *E. hirae* (El Kharroubi *et al*., 1991; Piras *et al*., 1993) and PBP2′ of *Staphylococcus aureus* (Song *et al*., 1987). Both oligonucleotides were used as primers in an amplification experiment with *Taq* DNA polymerase and 2 μg of *E. faecalis* JH2-2r genomic DNA. The expected 223 bp amplified fragment was cloned into the PCR2.1 plasmid (Table 1) and sequenced. Translation of its sequence yielded the typical SDN motif conserved in penicillin-binding modules of PBPs (Goffin & Ghuysen, 1998).

*Eco*RI, *Hin*dIII or *Nco*I genomic DNA fragments of JH2-2r were probed by Southern blotting with the digoxigenin-labelled oligonucleotide O3 derived from the sequence of the 223 bp PCR fragment (Table 2). A single positive signal was obtained in each digest at the level of an 11 kb *Eco*RI fragment, a 2·5 kb *Hin*dIII

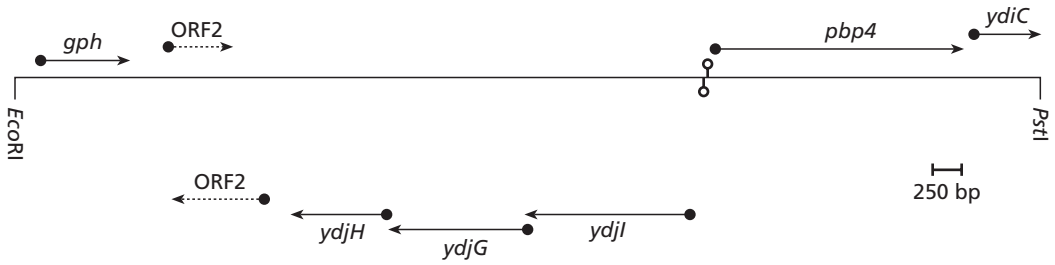

Fig. 2. Organization of the 8456 bp *Eco*RI–*Pst*I fragment of the 11 kb *Eco*RI insert contained in pDML521. The genes are represented by arrows corresponding to the orientation of their transcription; names of genes beginning with *y* were given by analogy with homologous genes of *B. subtilis* (Kunst *et al.*, 1997). The open circle symbol indicates a potential promoter; *gph*, phosphoglycolate phosphatase gene.

fragment and a > 23 kb *Nco*I fragment. After electrophoresis, the 9–15 kb *Eco*RI DNA fragments were eluted and ligated to *Eco*RI-linearized pBR322 plasmid. Transformants of *E. coli* K-12 RR1 were screened by Southern blotting with the same hybridization probe as above. Two positive clones, bearing pDML521 and pDML522, respectively (Table 1), were selected out of 840 transformants. Each clone overproduced an additional membrane-bound PBP which migrated by SDS-PAGE as *E. faecalis* PBP4. This meant that the cloned 11 kb fragment contained all the signals required for transcription and translation of the PBP4-encoding gene.

Sequence of an 8·4 kb segment of the 11 kb *Eco*RI insert of pDML521

The sequencing of the 11 kb *Eco*RI insert of pDML521 was undertaken to explore the genic environment of *pbp4* and to search for a gene which would function as a regulator of PBP4 synthesis, as proposed for *psr* in *E. hirae* (Ligozzi *et al.*, 1993).

The organization of the 8456 bp segment sequenced from the 11 kb *Eco*RI insert of pDML521 is presented in Fig. 2. The first ORF (890 bp) seemed to be preceded by a Shine–Dalgarno sequence and to start at position 180 from the 5′ end. The translation product was weakly homologous (24–28 % identity and 32–43 % similarity) to those of several bacterial *gph* genes which were considered to code for phosphoglycolate phosphatases (e.g. *Synechocystis* sp., *Alcaligenes eutrophus*, *Haemophilus influenzae*, *Aquifex aeolicus*, *Borrelia burgdorferi*, *Escherichia coli*). It was also weakly but significantly (46 % similarity and 23 % identity) homologous to the product of the *yvoE* gene of *Bacillus subtilis* (Kunst *et al.*, 1997).

A putative 488 bp *orf2* devoid of a Shine–Dalgarno sequence was found beginning 1256 bp downstream from the 5′ end of the 11 kb insert. Its product showed no homology with any other protein available in the databases. *orf3* was preceded by a Shine–Dalgarno sequence and detected on the complementary strand between positions 1273 and 2013. It coded for a putative 247 aa protein homologous (52 % similarity and 31 % identity) to a small protein of unknown function in the temperate lactococcal bacteriophages r1t (Van Sinderen *et al.*, 1996) and ϕLC3 (Lillehang *et al.*, 1997).

Three overlapping genes were also located on the complementary strand (from positions 2036 to 5514). They were named *ydjI*, *ydjG* and *ydjH* by analogy with related genes of unknown functions present in the genome of *B. subtilis* and clustered in one transcriptional unit but in the logical alphabetical order (Kunst *et al.*, 1997). The homology scores determined by the BESTFIT program between each pair of gene products were the following: *ydjG*, 57 % similarity and 34 % identity; *ydjH*, 53 % similarity and 26 % identity; *ydjI*, 55 % similarity and 26 % identity. The *E. faecalis* YdjI protein was also highly similar (71 % identity and 78 % similarity) to the *Lactococcus lactis* ORFE (Andersen *et al.*, 1996) of unknown function. In *L. lactis*, *orfE* is adjacent to the *pyr* operon but transcribed in an orientation opposite to that of the genes involved in the biosynthesis of pyrimidines.

A 207 bp sequence separated the initial ATG codon of *ydjI* from that of the following *pbp4* gene oriented in the opposite direction. This intervening region containing the putative *pbp4* promoter was amplified from the genomic DNA of both the resistant JH2-2r strain and the sensitive JH2-2 strain, using oligonucleotides O4 and O5 (Table 2). After cloning and sequencing, both fragments appeared identical.

The *pbp4* gene was followed by a partial ORF, named *ydiC* for its high similarity with the *ydiC* gene, of unknown function in *B. subtilis* (67 % similarity and 40 % identity on 204 amino acids) (Kunst *et al.*, 1997). YdiC is also related to a hypothetical protein widely conserved in bacteria (e.g. *Escherichia coli*, *Streptomyces coelicolor*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *H. influenzae*, *Neisseria meningitidis*, *Aquifex aeolicus*, *Synechocystis* sp.).

It is interesting to note that a gene organization identical to that of the 8·45 kb segment of JH2-2r (Fig. 2) was found in the genome of *E. faecalis* V583 (TIGR database).

The last part of the 11 kb *Eco*RI insert of pDML521, a 2·5 kb segment located downstream of the 8·45 kb

segment described above, was not sequenced further as subclones appeared to be unstable.

Analysis of the JH2-2 and JH2-2r *pbp4* sequences and expression in *E. coli*

The PBP4-encoding gene of the *E. faecalis* JH2-2 parental strain was completely sequenced. For this purpose, four pairs of oligonucleotides derived from the *pbp4* sequence of *E. faecalis* JH2-2r – O6 and O7, O8 and O9, O10 and O11, O12 and O13 (Table 2) – were used to amplify overlapping fragments of the gene by PCR. The PCR products were cloned into the pGEM-T Easy vector and sequenced on both strands, using the universal and reverse primers.

Alignment of the translation products of the two *pbp4* revealed that they were identical. They were also identical to the product of the *pbp4* gene found in contig 6237 during the sequencing of the *E. faecalis* V583 genome (TIGR database). These results explained why the PBP4 affinities were identical when they were estimated above, on the membranes of the JH2-2 and JH2-2r strains and the *Escherichia coli* Top10 F′ cells transformed with pDML523 and pDML524 (Table 1) carrying the JH2-2 and JH2-2r *pbp4* PCR fragments, respectively. In this latter case, the membranes of the *E. coli* transformants were labelled with [^{125}I]PenG and analysed by the Bio-Rad FX-imager apparatus after electrophoresis. They produced the same amount of PBP4 (Fig. 1, lanes 7–10). Membranes of *E. hirae* ATCC 9790 (Fig. 1, lanes 1 and 2), *E. faecalis* JH2-2 (Fig. 1, lanes 3 and 4) and JH2-2r (Fig. 1, lanes 5 and 6) were used in parallel as controls.

All these results, together with the demonstration that the 207 bp regions located upstream of *pbp4* in both strains were identical, indicated that the differences seen in JH2-2 and JH2-2r in terms of penicillin MIC and PBP4 production could not be attributed to a modification in the coding region of the *pbp4* gene nor in its putative promoter.

Identification of a *psr* gene in *E. faecalis* JH2-2 and JH2-2r

The sequence of the *psr* gene of *E. hirae* ATCC 9790 (Massidda *et al*., 1998; accession number U42211 in the EMBL database) was used to search for a homologous gene in the TIGR database, collecting the sequencing data of the genome of *E. faecalis* V583.

The BLAST (Pearson & Lipman, 1988) search server identified a nucleotide sequence encoding a protein in contig 6330 that had 63% similarity and 46% identity with the *psr* gene product of *E. hirae*. This sequence was considered as the *psr* gene of *E. faecalis*. The complete *psr* genes were amplified from the genomic DNA of *E. faecalis* JH2-2 and JH2-2r, cloned into pGEM-T Easy and sequenced. Comparison of the two sequences indicated that they were identical in the sensitive and resistant strains even in the putative promoter region. They were also identical to the sequence identified in contig 6330 of *E. faecalis* V583 (except that residue 348 is a threonine in JH2-2 or JH2-2r sequences and an alanine in the V583 sequence).

The *E. faecalis psr*-like gene encodes a 390-residue protein that has a 100 aa N-terminal extension without any significant homology in the protein databases and is absent in the 293-residue Psr of *E. hirae* (Massidda *et al*., 1998). Another peculiarity of the structure of the *E. faecalis* Psr-like protein concerns the presence of a hydrophobic peptide that seems to extend from positions 101 to 117 and separate the N-terminal extension from the typical Psr module. Such a structure stands in contrast with that of the other Psr-like proteins, which all appear to have a putative N-terminal hydrophobic membrane-anchoring peptide.

In the sequence of the *E. faecalis* Psr-like protein, one can find amino acid motifs almost identical to those conserved in the Psr and Psr-like proteins (Massidda *et al*., 1998). A pairing comparison within this family of proteins (using the BESTFIT program of the GCG package) gave the highest scores of identity between the *E. faecalis* Psr-like protein and the enterococcal Psr proteins (Ligozzi *et al*., 1993; Zorzi *et al*., 1996; Massidda *et al*., 1998).

DISCUSSION

E. faecalis shows only four HMM PBPs and one LMM PBP when membranes are labelled with radioactive penicillin. This is in agreement with the five PBP genes found in the genome of *E. faecalis* V583 when the sequences available in the TIGR database were analysed. Four of these genes coded for HMM PBPs (one belonging to class A and three to class B). The last one coded for a LMM PBP, presumably a DD-carboxypeptidase.

PBP4 in *E. faecalis* is a low-affinity PBP closely related to other enterococcal low-affinity PBPs described previously in *E. hirae* and *E. faecium* (El Kharroubi *et al*., 1991; Piras *et al*., 1993; Signoretto *et al*., 1994; Zorzi *et al*., 1996). It reacts poorly with anti-*E. hirae* PBP5 antibodies and it has a very low estimated PenG second-order acylation constant value ($k_{+2}/K = 50\ M^{-1}\ s^{-1}$) comparable to those of *E. hirae* PBP5 and PBP3r ($k_{+2}/K = 5$ to $20\ M^{-1}\ s^{-1}$) (Piras *et al*., 1990; El Kharroubi *et al*., 1991). These enterococcal PBPs, PBP2′ in *S. aureus* (Song *et al*., 1987) and PBP3 in *B. subtilis* (Murray *et al*., 1996) form subgroup B1 of class B PBPs (Goffin & Ghuysen, 1998). With the exception of PBP3 of *B. subtilis*, which is not yet well characterized, the members of this subgroup B1 have a low affinity for β-lactams and are thus involved in β-lactam resistance. They all possess a 120–135 aa polypeptide inserted between the N-terminal hydrophobic anchoring peptide and the non-penicillin-binding module (Goffin & Ghuysen, 1998). The exact function of that polypeptide is still unknown. It could be essential for the folding and/or the functioning of the PBP in the cell as different partial deletions produced in it abolished the β-lactam binding activity

(Mollerach *et al.*, 1996). It is however certainly not required for the β-lactam binding on the folded PBP as tryptic fragments of membrane-bound PBP5 of *E. hirae* are still able to bind [^{14}C]PenG (Piras *et al.*, 1990; El Kharroubi *et al.*, 1991).

A penicillin-resistant clone, JH2-2r (MIC 75 μg ml^{-1}), was derived from the wild-type strain JH2-2 (MIC 5 μg ml^{-1}) which apparently overproduced PBP4. The *pbp4* genes from both strains were cloned and sequenced in this work. According to the *pbp4* gene orientation, opposite to that of the three preceding genes (Fig. 2), it would be surprising if *pbp4* did not possess its own promoter. One should also keep in mind that when cloned into pMCL210 and introduced into *Escherichia coli*, the *pbp4* genes were able to direct the synthesis of large amounts of PBP4. Enterococcal genes are known to be transcribed in *E. coli* cells (Courvalin, 1994). As both *pbp4* genes are identical even in their putative promoter region, one can exclude the possibility that the overproduction of PBP4 in JH2-2r is due to a modification of the strength of the *pbp4* promoter and also rule out a possible modification of the PBP4 affinity for PenG.

The sequences of *pbp5* of *E. faecalis* 56R (Signoretto *et al.*, 1994) and *pbp4* of JH2-2 are identical except in four positions. There are three additional nucleotides in *pbp4* which modify the protein sequence over a short distance and introduce an additional amino acid residue. The peptide Ala-272-Cys-Ala-Ile-Asn-Arg-Val-Tyr-Gly-280 in PBP5 is replaced in PBP4 by the peptide Ala-272-Ala-Ala-Glu-Leu-Ile-Gly-Tyr-Thr-Gly-281. PBP4 of JH2-2 or JH2-2r are thus longer by one residue, with 680 aa. In addition, two residues both at the N-terminal end of the non-penicillin-binding module (Asn-13 vs Lys-13 and Val-29 vs Gly-29 in PBP5 and PBP4, respectively) and one immediately following Lys-424 in the conserved active-site motif SxxK (Ile-424 vs Thr-425 in PBP5 and PBP4, respectively) are different. This last modification, a threonine changed into an isoleucine, could affect the PBP5 affinity in the laboratory resistant *E. faecalis* strain 56R. As information concerning the parental strain 56 is not available, it is difficult to speculate on the importance of such a modification.

The genic environment of *pbp4* was also examined in an attempt to localize a *psr* repressor gene that could perhaps be related to the overproduction of PBP4 in *E. faecalis* JH2-2r. Different genes, most of unknown functions, presenting similarities with *B. subtilis* genes were identified. None however showed similarities with the *psr* genes found in *E. hirae* and *E. faecium* strains (Ligozzi *et al.*, 1993; Zorzi *et al.*, 1996; Massidda *et al.*, 1998). These genes coded for proteins different from those determined by the genes present in the vicinity of *pbp5* in *E. hirae* (O. Dardenne, unpublished results).

A *psr*-like gene was identified in the complete genome of *E. faecalis* V583 by homology search in the TIGR database. It is present on contig 6330 whereas *pbp4* is on contig 6237. A *psr*-like gene was cloned and sequenced from the DNA of both *E. faecalis* JH2-2 and JH2-2r strains. From the analysis of the genic environments of both *pbp4* and *psr*-like genes of *E. faecalis* V583, JH2-2 and JH2-2r strains, one can conclude that both genes are several kilobases away from each other. This organization is very different from that in *E. hirae* and *E. faecium*, where both genes are adjacent (Ligozzi *et al.*, 1993; Zorzi *et al.*, 1996; Massidda *et al.*, 1998).

The Psr-like proteins include the enterococcal Psr (Ligozzi *et al.*, 1993; Zorzi *et al.*, 1996; Massidda *et al.*, 1998), the *B. subtilis* putative regulators LytR (Lazarevic *et al.*, 1992) and YvhJ (Soldo *et al.*, 1996) as well as proteins required for capsular polysaccharide synthesis, such as CpsA of *Streptococcus pneumoniae* (Morona *et al.*, 1997), CpsA of *Streptococcus thermophilus* (Griffin *et al.*, 1996) or EpsA of *Streptococcus thermophilus* Sfi6 (Stingele *et al.*, 1996).

Since the sequences of the *psr*-like and the *pbp4* genes in JH2-2 and JH2-2r are identical, even in their putative promoter regions, one can exclude the possibility that overproduction of PBP4 in JH2-2r is due to the Psr-like protein. Another regulatory mechanism that remains to be identified and investigated should be involved. The function(s) of the Psr-like protein also remain(s) to be elucidated.

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