Redefining the Role of *psr* in β-Lactam Resistance and Cell Autolysis of *Enterococcus hirae*

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The contribution of penicillin-binding protein 5 (PBP5) and the PBP5 synthesis repressor (Psr) to the β-lactam resistance, growth, and cell autolysis of wild-type strain ATCC 9790 and resistant strain R40 of *Enterococcus hirae* was investigated by disruption or substitution of the corresponding *pbp5* and *psr* genes by Campbell-type recombination. The resulting modifications were confirmed by hybridization and PCR. The low susceptibility of *E. hirae* to β-lactams was confirmed to be largely dependent on the presence of PBP5. However, against all expectations, inactivation of *psr* in ATCC 9790 or complementation of R40 cells with *psr* did not modify the susceptibility to benzylpenicillin or the growth and cell autolysis rates. These results indicated that the *psr* gene does not seem to be involved in the regulation of PBP5 synthesis and consequently in β-lactam resistance or in the regulation of cell autolysis in *E. hirae*.

The natural low susceptibility of enterococci to β-lactams is due to a low-affinity penicillin-binding protein (PBP) that is overproduced in moderately resistant laboratory mutants and some clinical strains (16, 17, 35, 45). Highly resistant clinical strains generally do not overproduce this low-affinity PBP but modify its primary structure to further reduce its binding capacity (21, 24, 35, 45). Conversely, when the low-affinity PBP is not synthesized, the cells become highly susceptible to benzylpenicillin (PenG) (18).

Sequencing of the enterococcal genes encoding low-affinity PBPs showed that these proteins are relatively similar (~75 kDa) (13, 14, 33, 35, 45). Including PBP2′ of methicillin-resistant staphylococci (1) and PBP3 of *Bacillus subtilis* (28), these protein form subgroup B1 of the class B high-molecular-mass PBPs (19). When penicillin is present, they can take over the functions of most if not all the other PBPs. This has not been verified for PBP3 in *B. subtilis*. Thus, in enterococci and staphylococci these proteins appear to be multifunctional PBPs that are not essential for growth under laboratory conditions but synthesize peptidoglycan when the other PBPs are inhibited (5, 6, 7, 17, 18).

The study of *Enterococcus hirae* contributed a great deal to these observations. From wild-type strain ATCC 9790 (MIC of PenG, 0.6 μg/ml), resistant strain R40 (MIC of PenG, 60 μg/ml) was isolated by a four-step selection procedure on plates containing increasing PenG concentrations. The greater resistance of strain R40 was attributed to overproduction of the low-affinity PBP5 (17). However, it also appeared that R40 differed from parent strain ATCC 9790 by slower growth,

faster autolysis, a lower cell wall rhamnose content, and greater susceptibility to lysozyme (27). PenG-hypersusceptible strain Rev14 (MIC of PenG, 0.015 μg/ml), which does not synthesize PBP5, was derived from R40 by chemical mutagenesis (18). Except for its high level of susceptibility to β-lactams, this strain has the properties described above for R40 (27).

Expression of the PBP5-encoding gene, *pbp5*, in *E. hirae* was proposed to be under control of *psr*, a 882-bp gene starting 1 kb upstream of *pbp5*, on the basis of the 87-bp deletion that overlapped the 5′end of *psr* (23, 26). It was also suggested that the *psr* gene has a larger regulatory function in control of the different cell wall-related properties described above (27).

It is also assumed that a similar situation exists in *Enterococcus faecium* strains; all of these strains except D63 have immediately upstream of *pbp5* (*pbp5fm*) a 756-bp *psr* gene (*psrfm*), the product of which exhibits 79.9% identity with the Psr protein of *E. hirae* (P. Brouillard and J. Coyette, unpublished data). In D63, *psrfm* ends prematurely with a 597-bp sequence (45). However, in contrast to previous results, it was recently demonstrated that the amount of the *pbp5fm* transcript in a *psrfm*-deficient clone of *E. faecium* was similar to the amount in the wild-type strain (34). Finally, a *psr*-like gene was also detected in *Enterococcus faecalis* JH2-2, but in contrast to the *psr* genes of the two species described above, this gene appeared to be located several kilobases away from *pbp4*, the low-affinity PBP4-encoding gene. No indication that the *psr*-like gene is involved in the overproduction of PBP4 in a resistant clone derived from *E. faecalis* JH2-2 (13) was found.

As the mutants used in previous studies were not isogenic strains, we could not eliminate the possibility that they contained different unidentified genetic modifications that could have contributed to their different traits, including their higher levels of resistance. The purpose of this study was to reexamine the role of the *psr* gene in cell autolysis and β-lactam resistance

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype[a]	Source or reference
E. coli strains		
Top10F′	F′ [*lac1*q Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80 *lacZ*Δ′*M15* Δ*lacX74 deoR recA1* *araD139* Δ(*ara-leu*)7697 *galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)	F′ [*ompT hsd* S$_B$(r$_B^-$ m$_B^-$) *gal dcm*(DE3)]	Novagen
E. hirae strains		
AS21	Pen^s mutant derived from ATCC 9790 (MIC of PenG, 0.075 μg/ml)	11
AS21R2	Pen^r mutant derived from AS21 (MIC of PenG, 30 μg/ml)	11
ATCC 9790	Wild-type strain (MIC of PenG, 0.6 μg/ml)	ATCC[b]
Rev14	Pen^s mutant derived from R40 (MIC of PenG, 0.015 μg/ml)	18
FS1	R40 *pbp5*::Spc^r	This study
FS2	ATCC 9790 *pbp5*::Km^r	This study
FS3	ATCC 9790 *psr*::Km^r (disruption)	This study
FS4	ATCC 9790 *psr*::Km^r (substitution)	This study
FS5	ATCC 9790 *psr*::Km^r (substitution)	This study
R40	Pen^r mutant derived from ATCC 9790 (MIC of PenG, 60 μg/ml)	17
Plasmids		
pBR322	Ap^r Tet^r *E. coli* cloning vector	Amersham Pharmacia Biotech
pCR2.1	Ap^r Km^r *E. coli* TA cloning vector	Invitrogen
pDG792	*E. coli* vector carrying the 1.5-kb Km^r gene cassette	20
pER924	Ap^r Erm^r *E. coli*–gram-positive shuttle vector(thermosensitive gram-positive replication)	3
pET22b(+)	Km^r *E. coli* expression vector	Novagen
pGEM-3Zf(−)	Ap^r *E. coli* cloning vector	Promega
pHP45Ω	Ap^r *E. coli* vector carrying the 2.0-kb Sm^r-Spc^r gene cassette	32
pIL252	Erm^r gram-positive cloning vector (low copy)	39
pIL253	Erm^r gram-positive cloning vector (high copy)	39
pSL1190	Ap^r *lacZ E. coli* cloning vector	Amersham Pharmacia Biotech
pUC18	Ap^r *lacZ E. coli* cloning vector	Amersham Pharmacia Biotech
pDML529	pGEM-3Zf(−) carrying the 1.43-kb PCR-amplified *psr* gene	This study
pDML530	pBR322 carrying the 6.9-kb *Eco*RI fragment (*psr-pbp5*) of ATCC 9790	This study
pDML531	pDML529-pIL253 fusion	This study
pDML532	pDML530-pIL252 fusion	This study
pDML533	pDML530-pIL253 fusion	This study
pDML534	pET22b(+) carrying truncated *psrN*	This study
pDML535	pUC18 carrying the 4.0-kb *Pst*I *psr*::Km^r fragment	This study
pDML540	pBR322 carrying the 6.9-kb *Eco*RI fragment (deleted *psr-pbp5*) of R40	14
pDML541	pBR322 carrying the 2.6-kb *Eco*RI/*Pvu*I fragment (*pbp5*) derived from the pDML540 insert	29
pDML542	pDML541-pIL252 fusion	29
pDML543	pDML541-pIL253 fusion	29
pDML1600	pUC18 carrying *pbp5* (1.6-kb PCR fragment)	This study
pDML1601	pUC18 carrying *pbp5*::Spc^r	This study
pDML1602	pUC18 carrying *pbp5*::Km^r	This study
pDML1603	pSL1190 carrying *psr* (1.55-kb PCR fragment)	This study
pDML1604	pSL1190 carrying *psr*::Km^r	This study
pDML1605	pUC18 carrying an *ftsW* fragment (0.88-kb PCR fragment)	This study
pDML1606	pSL1190 carrying pDML1605 insert	This study
pDML1607	pUC18 carrying a *pbp5* fragment (0.85-kb PCR fragment)	This study
pDML1608	pSL1190 carrying pDML1605 insert-pDML1607 insert fusion	This study
pDML1609	pSL1190 carrying pDML1608 insert::Km^r (*Kpn*I)	This study
pDML1610	pER924 carrying pDML1601 insert	This study
pDML1611	pER924 carrying pDML1602 insert	This study
pDML1612	pER924 carrying pDML1604 insert	This study
pDML1613	pER924 carrying pDML1609 insert	This study

[a] Ap^r, ampicillin resistant; Erm^r, erythromycin resistant; Km^r, kanamycin resistant; Pen^r, PenG resistant; Pen^s, PenG sensitive; Sm^r, streptomycin resistant; Spc^r, spectinomycin resistant; Tet^r, tetracycline resistant.
[b] ATCC, American Type Culture Collection.

of *E. hirae* by specifically disrupting or deleting the gene by homologous double-recombination techniques. Isogenic *pbp5*-deficient mutants were also prepared and used as controls.

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MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and MIC determination. The *Escherichia coli* and *E. hirae* strains used in this work are listed in Table 1. *E. coli* Top10F′ was used to generate and maintain recombinant plasmids in Luria broth (Difco, Erembodegem, Belgium) at 37°C (2). *E. hirae* strains were grown in brain heart infusion broth (Difco) or in Todd-Hewitt broth (Difco) supplemented with

2% glucose (THG) at 37°C except for isolation of recombinants as indicated below. Growth curves were established by measuring the optical density at 550 nm (OD$_{550}$) at regular intervals. M17 medium (Difco) was also used to prepare electrocompetent *E. hirae* cells (42).

The antibiotics and concentrations used were as follows. Ampicillin (100 μg/ml; Squibb Bristol Myers, Brussels, Belgium), kanamycin (100 μg/ml; Calbiochem, Bierges, Belgium), and spectinomycin (100 μg/ml; Pharmacia Upjohn, Puurs, Belgium) were used for selection of *E. coli* transformants. Erythromycin (5 or 10 μg/ml; Fluka, Bornem, Belgium) was used for initial selection of *E. hirae* transformants after electroporation. Kanamycin (100 μg/ml) and PenG (0.1 μg/ml; Aventis, Lyon, France) were used to select *E. hirae* recombinants as indicated below. Other selective additives, such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) and isopropyl-β-D-thiogalactopyrano-

side (IPTG) (7 μg/ml) (both obtained from Immunosource, Zoersel-Halle, Belgium), were used or added to the media as required.

MICs were determined in 1 ml of brain heart infusion broth in 24-well culture plates in at least three independent experiments, essentially as previously described (9).

Cell autolysis. Cells grown in THG and collected in the exponential (OD_{550}, ≅1.0) and stationary (OD_{550}, ≅1.7) phases were washed three times in cold water, resuspended in 10 mM sodium phosphate buffer (pH 6.8), and incubated at 37°C as previously described (27). Rates of autolysis were calculated from the exponential portions of lysis curves.

Plasmid construction. Common *E. coli* plasmids (Table 1) were used for cloning purposes. The *E. coli-E. hirae* shuttle vectors were cointegrates of pIL252 or pIL253 (39) on the one side and pBR322 on the other side, usually at the *Eco*RI sites. Disruption or deletion experiments were performed by using the pER924 thermosensitive shuttle vector (3), into which resistance gene cassettes and specific DNA fragments were inserted.

General DNA methods. Standard DNA methods were used (2, 36). Restriction endonucleases from Promega (Leiden, The Netherlands), Life Technologies (Merelbeke, Belgium), and Fermentas (St. Leon-Rot, Germany), calf intestinal alkaline phosphatase from Roche Diagnostics (Brussels, Belgium), T4 DNA polymerase from Life Technologies, and T4 DNA ligase from Boehringer Ingelheim Bioproducts (Verviers, Belgium) were used as recommended by the suppliers.

E. coli plasmids were extracted with a GFX Microplasmid prep kit (Amersham Pharmacia Biotech, Roosendall, The Netherlands). DNA fragments or PCR products were purified with a GeneClean Spin kit from Bio 101 Systems (Polylab, Antwerp, Belgium) or a FREE DNA kit from Millipore (Brussels, Belgium). The *Enterococcus* genomic DNA was purified by using a Wizard genomic DNA extraction kit (Promega) or as previously described (25).

Southern hybridization. Transfer of DNA (4 μg per lane) from 0.8% (wt/vol) agarose gels to Hybond N$^+$ membranes (Amersham Pharmacia Biotech) was performed by using the VacuGeneXL vacuum blotting system (Amersham Pharmacia Biotech) and standard protocols (36). Probe labeling, hybridization, and washing were done by using the AlkPhos direct labeling and detection systems (Amersham Pharmacia Biotech). The sizes of the hybridizing fragments of the chromosomal digestion products were determined by using the Smart ladder marker from Eurogentec (Seraing, Belgium) labeled by the same technique.

PCR. PCR amplification was performed with a TRIO-Thermoblock Biometra thermocycler (Eurogentec). The *Taq*, *Pwo*, Vent, and Biotools DNA polymerases from Amersham Pharmacia Biotech, Eurogentec, New England Biolabs (Westburg, Leusden, The Netherlands), and Labsystem (Brussels, Belgium), respectively, were used according to the manufacturers' recommendations. Amplification was usually carried out by using 25 to 30 cycles. Each cycle consisted of 1 min at 95°C, 30 s at the melting temperature minus 3°C, and 1 min/kb at 72°C. The last cycle was followed by a 10-min elongation step at 72°C.

Nucleotide sequencing. DNA sequencing was performed by using Autoread Thermo Sequenase sequencing kits (Amersham Pharmacia Biotech) with Cy5 primers and an ALF Express DNA sequencer (Amersham Pharmacia Biotech). The primers were chosen so that we obtained sequences that overlapped on both strands.

Transformation of *E. hirae* cells and isolation of recombinants. *E. coli* competent cells were transformed as previously described (36). *E. hirae* electrocompetent cells were transformed by electroporation by using 0.1-cm cuvettes and a Gene Pulser (Bio-Rad, Nazareth-Eke, Belgium). The parameters used were as follows: 1,000 Ω, 25 μF, and 18 to 23 kV/cm (41).

Gene disruptions or deletions in *E. hirae* cells were obtained by double recombination of electroporated integration vectors. The recombinants grown at 28 and 42°C (the permissive and nonpermissive temperatures, respectively) were selected by replica plating (~200 colonies/plate) on the basis of phenotypic susceptibility or resistance to PenG, erythromycin, kanamycin, or spectinomycin (4).

PBP detection by radioactive labeling and immunoblotting. Preparation of the membranes of the different *E. hirae* clones, estimation of the protein contents, [^{14}C]PenG PBP labeling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography were performed as described elsewhere (45). However, in the fluorography procedure, the 2,5-diphenyloxazole scintillant used previously was replaced by a 1 M sodium salicylate solution (8). PBPs were detected by using a phosphorimager screen (Kodak-K screen) after 24 h of exposure at room temperature (FX-Imager; Bio-Rad).

Anti-PBP5 rabbit polyclonal antibodies that were prepared and partially purified by immunoadsorption, as previously described (14, 30), were used for detection of PBP5 by immunoblotting. Anti-Psr rabbit polyclonal antibodies were kindly provided by L. Daneo-Moore for detection of Psr by immunoblotting

(27). Alkaline phosphatase-coupled goat anti-rabbit polyclonal antibodies were used as secondary antibodies (Immun-Blot colorimetric assay kit; Bio-Rad).

Electrophoretic mobility shift assay (EMSA). Protein samples containing various amounts of PsrN (10 to 250 ng) were incubated with 20 ng of the Cy5-labeled probe in the presence of 2 to 4 μg of double-stranded poly(dI-dC) (Amersham Pharmacia Biotech) in 20 μl of a reaction buffer containing 25 mM HEPES (pH 7.5), 0.1 mM EDTA, 5 mM dithiothreitol, and 50 mM KCl. Incubation was carried out at room temperature or at 30°C for 20 to 60 min, and the reactions were stopped by adding 4 μl of the loading dye fluorescent sample buffer (Amersham Pharmacia Biotech). The samples were resolved on 4% (wt/vol) nondenaturing polyacrylamide gels in 0.89 M Tris-borate–20 mM EDTA buffer (pH 8.2) by using an Alf Express sequencer (Amersham Pharmacia Biotech). After electrophoresis (180 min, 10 W, 800 V, 45 mA), the gels were analyzed by using the Fragment Manager program (Amersham Pharmacia Biotech) (15).

Nucleotide sequence accession number. The sequence of the 6.9-kb *Eco*RI insert of *E. hirae* ATCC 9790 has been deposited in the EMBL database under accession no. AJ429231.

RESULTS

Sequence analysis of the *psr-pbp5* locus of *E. hirae* and its flanking regions. The complete sequence of the 6.9-kb *Eco*RI insert of pDML540 bearing the *psr* and *pbp5* genes, which was isolated previously (14) from the PenG-resistant (Pen^r) *E. hirae* R40 strain, was established for both strands. This sequence was compared to that of the allelic 6.9-kb *Eco*RI fragment of wild-type strain *E. hirae* ATCC 9790 carried by pDML530. This vector was selected from an *Eco*RI gene library made in the pUC18 Ready To Go vector (Amersham Pharmacia Biotech). Positive colonies were identified by PCR in the presence of specific *psr* and *pbp5* primers O1 and O2*, O1 and O4*, and O3 and O4* (Table 2).

No difference except the 87-bp deletion identified previously (23, 26) encompassing the initial *psr* codon of *E. hirae* R40 was found when we compared the two inserts that contained four genes in addition to *psr* and *pbp5* (Fig. 1A).

Upstream of *psr* there was a 1,194-bp gene whose product exhibited 53, 35, 34, and 30.9% identity with the *ftsW* products of *E. faecium*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *E. coli*, respectively.

The three genes located downstream of *pbp5* are the *napA*, *napB*, and *napC* genes identified previously on a 3.7-kb *Sau*IIIA DNA fragment of *E. hirae* ATCC 9790 coding for an Na$^+$/H$^+$ antiporter activity (43). These genes appear to be oriented in the opposite direction compared to the orientation of *pbp5*. The product of the *napC* gene (1,203 bp) exhibits 41% similarity with the tetracycline resistance gene *tet*(H) from *Pasteurella multocida* (G. Guillaume, personal communication). In spite of this similarity, *napC* does not confer a resistant phenotype to the ATCC 9790 and R40 strains as both of these strains are normally susceptible to tetracycline (MIC, 1 μg/ml). The product of the *napB* gene (453 bp) exhibits 25 and 24% identity with two proteins of *B. subtilis*, one involved in secretion and the other related to the MraR regulator, respectively. A 517-bp fragment of *napA* forms the 3′ end of the pDML540 *Eco*RI insert shown in Fig. 1A. The 1,236-bp *napA* gene codes for an Na$^+$/H$^+$ antiporter (43).

Isolation of *pbp5*-deficient *E. hirae* clones. To construct a *pbp5* integration vector, a 1.6-kb *pbp5* fragment extending from position 361 to position 1,929 was amplified by PCR by using the *Taq* polymerase, pDML540 as the template, and the O7 and O8* oligonucleotides (Table 2), which added *Eco*RI and

TABLE 2. Oligonucleotides used in this study

Oligonucleotide[b]	Sequence[a]	Gene	Position from ATG
O1	5′ GGAATCG<u>CATATG</u>AAAACATTTCAAAAAGTG 3′	psr	−10
O2*	5′ AAATAAAATGTAAAT<u>CTCGAG</u>GTCTAAAAACTG 3′	psr	900
O3	5′ CGGTCGACTCTTGGGAAGTATTAAA 3′	psr	669
O4*	5′ AGCATCGGCATCAAGAGTTAATTT 3′	pbp5	1052
O5	5′ GAGTTTGGGTTGATAGGCGCA 3′	psr	431
O6*	5′ TTTGTCCCTCCGCTCACATTTTC 3′	psr	1001
O7	5′ AAAT<u>GAATTC</u>AAGCTTAGGCGAATTAAA 3′	pbp5	361
O8*	5′ ATC<u>AGGATCC</u>ACAGCAAATAGGAAG 3′	pbp5	1928
O9	5′ TAAGAAAATGTGAGCGGAGGG 3′	pbp5	−26
O10*	5′ TCAGGATTCACAGCAAATAGGAAGC 3′	pbp5	1927
O11	5′ ACGCTTAAGACCGAATCGCAATG 3′	ftsW	652
O12*	5′ AAGGATATCGCCTCTTTTAGC 3′	pbp5	531
O13	5′ AAGATCGACTGGTTGATTTTAGGCC 3′	ftsW	15
O14	5′ TTT<u>GAATTC</u>TTTTTTTAGAAATGACCGCAGTGTTC 3′	ftsW	377
O15	5′ CAAGCGCCCTTGTTTTGGTTCTTGCGGC 3′	pbp5	49
O16*	5′ ACC<u>GGGCCC</u>AGGTATATCTACATAAGAATC 3′	psr	303
O17*	5′ CCGAACCTCTTACTTGAAATATAGCCA 3′	ftsW	1253
O21	5′ AAA<u>GGATCC</u>TTAGCAATCAATCAGTTTTTAGA 3′	pbp5	−160
O22*	5′ GGTTGTACCCAACTTTGAGAGATAGCT 3′	pbp5	703
O23	5′ AGGATGAAGAGGATGAGGAGGC 3′	aph3′	122
O24*	5′ CTCGTAGGCGCTCGGGAC 3′	aph3′	1475
O25*	5′ AAATAAAATGTAAAT<u>CTCGAG</u>GTCTAAAAACTG 3′	psr	897
O26	5′ GGTTACAACTCAAG<u>AATTC</u>TAAACTGTTAAA 3′	psr	−123
O27*	5′ AGAATAACCGTGTACTTTTTGGAATGTTTTCATCTTCGAT 3′	psr	−7
O28	5′ AAAACATTCCAAAAAGTCACGGTTATTCTTCAACGAGTC 3′	psr	4
O29*	Cy5-GCAAGAACCAAAACAAGGGCGC 3′	pbp5	74
O30	5′ CGGTCAACTCTTGGGAAGTATTAAAA 3′	psr	669

[a] The underlined sequences are NdeI, AvaI, EcoRI, BamHI, and SmaI restriction sites.
[b] *, complementary.

BamHI restriction sites at the 5′ and 3′ ends of the DNA fragment, respectively (14). The purified PCR fragment was inserted into the EcoRI and BamHI sites of pUC18 to obtain pDML1600. The SstI site of the pDML1600 insert (position 1,144 from the ATG codon of pbp5) was blunt ended with the T4 DNA polymerase and used to introduce the 2.0-kb spectinomycin resistance marker (Spc[r] cassette), which was isolated by SmaI digestion of pHP45Ω. The pDML1600 derivative obtained in this way served as an intermediate for transfer of the disrupted pbp5 fragment into the EcoRI and BamHI sites of pER924. The final construct was designated pDML1610. This plasmid was used only once because the resistance gene did not confer a Spc[r] phenotype to E. hirae R40 recombinants. Nevertheless, one pbp5-deficient mutant designated FS1 (Fig. 1B) was identified by replica plating as a cefotaxime-sensitive clone among cefotaxime-resistant R40 revertants. In contrast to Pen[r] R40 cells but like penicillin-susceptible (Pen[s]) Rev14 cells, FS1 cells elongated when they were treated with 1 μM cefotaxime in liquid medium (as determined by microscopic examination) (10). This was an indication that the pbp5 gene had been interrupted.

To confirm these preliminary results, a second pbp5 integration vector, pDML1611, was constructed, in which a 1.5-kb kanamycin resistance marker (Km[r] cassette) isolated by SmaI/NruI double digestion of pDG792 was inserted at the SstI site of pDML1600 instead of the Spc[r] cassette. Double recombinants derived from ATCC 9790 were selected as Km[r] Erm[s] (erythromycin-sensitive) clones by replica plating. One randomly chosen mutant, designated FS2, was selected for comparison with FS1 (Fig. 1B).

Isolation of psr-deficient E. hirae clones. To obtain a psr integration vector, a 1.55-kb psr fragment, extending from position 652 in the ftsW gene and from position 882 in the psr gene, was amplified by PCR by using the Taq polymerase, pDML540 as the template, and the O2* and O11 primers (Table 2). The purified PCR fragment was first inserted into pCR2.1 and then transferred after purification into the EcoRI site of pSL1190 from which the NdeI site was first removed by EcoRV/NruI double digestion. The resulting construct, designated pDML1603, was used to insert the 1.5-kb Km[r] cassette into the NdeI site (at position 864, almost in the middle of the PCR fragment) after the overhangs were blunt ended with the T4 DNA polymerase. The disrupted psr fragment obtained in this way (pDML1604) was then transferred into the EcoRI site of pER924 to generate pDML1612. ATCC 9790/pDML1612 transformants were isolated and used to obtain psr-inactivated mutants that were selected as Km[r] Erm[s] clones by replica plating. No Pen[r] clones were identified on PenG-containing replica plates. One clone was randomly chosen for further analysis and was designated FS3 (Fig. 1B).

To confirm the results obtained with the FS3 mutant, a psr-deleting vector was constructed by inserting the Km[r] cassette between a 0.88-kb ftsW fragment and a 0.86-kb pbp5 fragment into pER924. The ftsW fragment extending from position 377 to position 1,253 from the ATG site was amplified by PCR by using the Vent polymerase, pDML540 as the template, and the O14 and O17* primers (Table 2). The O14 primer added an EcoRI site at the 5′ end of the PCR fragment, which was cloned into the EcoRI and HincII sites of pUC18 to obtain pDML1605. Then the ftsW fragment was excised
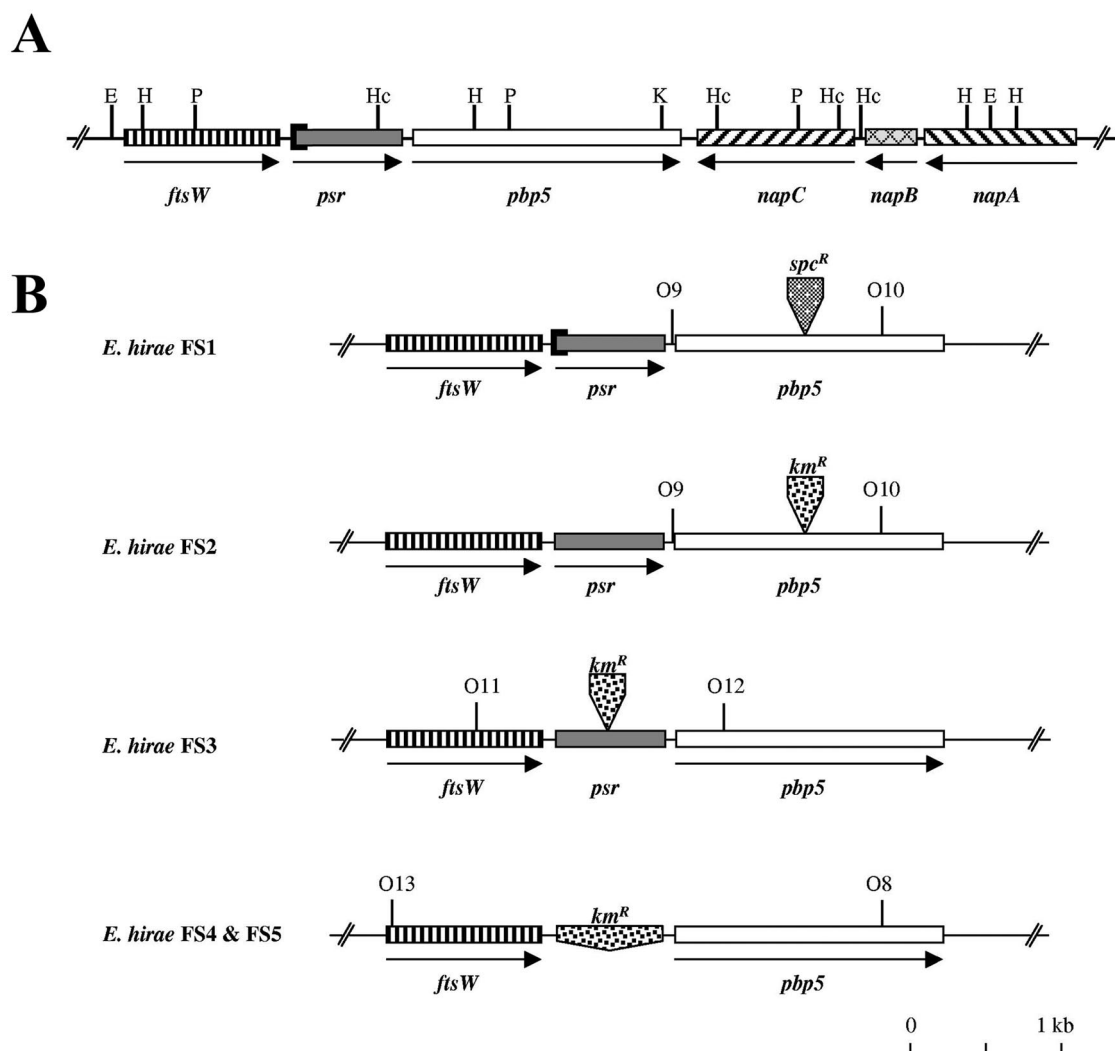
FIG. 1. (A) Map of the *ftsW-psr-pbp5* locus of *E. hirae*. (B) *ftsW-psr-pbp5* loci of the different *E. hirae* recombinants. The arrows indicate the orientation of transcription. The solid rectangle at the origin of *psr* corresponds to the 87-bp deletion present in R40 and its FS1 derivative. The spc^R and km^R inserts indicate the positions of the resistance cassettes. The hybridization sites of the O8 to O13 probes used in PCR are also shown. The restriction sites are indicated as follows: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Hc, *Hin*cII; K, *Kpn*I.

by *Eco*RI/*Sph*I double digestion from pDML1605 and inserted into the corresponding sites of pSL1190 to generate pDML1606.

The 0.86-kb *pbp5* fragment extending from position −160 to position 690 from the initial ATG site was amplified by PCR by using the Vent polymerase, pDML540 as the template, and oligonucleotides O21 and O22* (Table 2). The PCR fragment was cloned into the *Sma*I site of pUC18 (SURECLONE kit; Amersham Pharmacia Biotech) to obtain pDML1607, from which it was excised with the *Kpn*I and *Hin*cII enzymes; it was then inserted into the *Kpn*I and *Stu*I sites of pDML1606 to generate pDML1608. The 1.5-kb *Sma*I/*Stu*I-digested Km^r cassette (from pDG792) was then introduced into the *Kpn*I site of pDML1608 after the *Kpn*I overhangs were blunt ended with the T4 DNA polymerase. This construct, designated pDML1609, was digested with the *Eco*RI and *Sst*I enzymes to isolate a 3.2-kb insert that was blunt ended with the T4 DNA polymerase and then ligated into the *Sma*I site of pER924 to

form pDML1613. In this construct, the Km^r cassette started 60 bp downstream of the *ftsW* stop codon and ended 160 bp upstream of the *pbp5* ATG codon.

With pDML1613, mutants of ATCC 9790 could be selected as Km^r Erm^s clones by *psr*, replica plating. Similar to the strains with an interrupted *psr*, none of the mutants with a deleted *psr* appeared to be resistant to PenG. Two of these mutants were randomly chosen for further analysis and designated FS4 and FS5.

PCR and hybridization analyses of the *pbp5*- and *psr*-deficient *E. hirae* mutants. Insertion of the resistance cassettes into the different recombinants was verified by PCR by using primers (*pbp5* primers O9 and O10*, "*psr* primers O11 and O12*," and *ftsW* primer O13 and *pbp5* primer O8*) that hybridized upstream and downstream of the insertion positions in both the *pbp5* and *psr* genes (Fig. 1B). Comparison of the sizes of the PCR fragments indicated that for each recombinant an insertion or a deletion had occurred, as expected (results not shown).
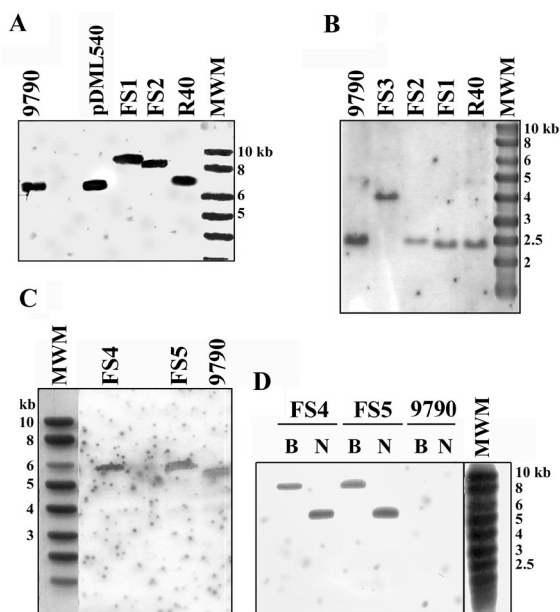
FIG. 2. Southern blots of restriction enzyme digestion products of the DNA of the *E. hirae* recombinants. The *E. hirae* ATCC 9790 (9790) and R40 strains, as well as pDML540 (6.9-kb *Eco*RI insert from R40) and the Smart ladder (MWM), were used as controls. (A) *Eco*RI digestion products probed with O15 (*pbp5*). (B) *Pst*I digestion products probed with O16* (*psr*). (C) *Pvu*II digestion products probed with O15 (*pbp5*). (D) *Bgl*II (lanes B) and *Nco*I (lanes N) digestion products hybridized with the Km[r] probe.

The presence of exogenous DNA inserts in the *pbp5* and *psr* genes of the *E. hirae* deficient mutants was also confirmed by hybridization analysis. Southern blot analyses of restriction enzyme digestion products of the DNA of these mutants and their controls were performed with the specific *pbp5* O15 and *psr* O16* probes or with a specific 1.2-kb probe derived by PCR (the O23 and O24* primers) from the Km[r] cassette (from pDG792) and labeled with the AlkPhos Direct system.

Both the O15 and O16* probes revealed that the fragments originating from the FS1, FS2, and FS3 recombinants were systematically larger than those of the parental strains (Fig. 2). As shown in Fig. 2, the *pbp5* O15 probe hybridized on one side with a 6.9-kb *Eco*RI fragment of R40 and on the other side with 8.9- and 8.4-kb *Eco*RI fragments of the FS1 and FS2 strains, respectively (Fig. 2A). These sizes were in agreement with insertion of the 2.0-kb Spc[r] and 1.5-kb Km[r] cassettes into the 6.9-kb fragment, respectively. Similarly, the *psr* O16* probe hybridized with 2.5- and 4.0-kb *Pst*I fragments of the ATCC 9790 and FS3 DNA digests, respectively (Fig. 2B). Again, this indicated that the 2.5-kb *Pst*I fragment had integrated the 1.5-kb Km[r] cassette. In addition, *E. coli* Top10F′ cells transformed with pDML535 (pUC18 bearing the 4.0-kb FS3 *Pst*I fragment) were selected on plates supplemented with kanamycin. Sequencing of the insert confirmed that the *psr* gene was disrupted and that the Km[r] gene was oriented in the direction opposite to that of *psr*.

Finally, the *Pvu*II digestion products of FS4 and FS5 DNA that responded to the *pbp5* O15 probe were 0.6 kb larger than the positive *Pvu*II DNA fragments of ATCC 9790 (Fig. 2C). This size was expected if the 0.9-kb *psr* was replaced by the 1.5-kb Km[r] cassette. Confirmation that the Km[r] cassette had been inserted into the genome of the FS4 and FS5 recombinants was provided by the presence of one *Bgl*II fragment and one *Nco*I fragment in the digestion products of the FS4 and FS5 DNA that hybridized with the Km[r] probe, while no hybridization was detected in ATCC 9790 (Fig. 2D).

PBP5 detection in the *pbp5*- and *psr*-deficient *E. hirae* mutants. In order to determine whether inactivation of *pbp5* or *psr* could modify the PBP profiles of *E. hirae*, membranes of the mutants were prepared, labeled with radioactive PenG, and compared with membranes of the ATCC 9790, R40, and Rev14 strains. As shown in Fig. 3 and in agreement with previous results (14, 17), the Pen[r] R40 strain produced a larger amount of PBP5 than ATCC 9790 produced. In contrast, the Pen[s] Rev14 strain did not synthesize PBP5, confirming the importance of this protein for β-lactam resistance (18).

As expected, the *pbp5*-deficient FS1 and FS2 mutants behaved like Rev14, since they were unable to synthesize PBP5. Synthesis of PBP5 was restored in these mutants by complementation with the *pbp5*-bearing pDML532, pDML533, pDML542, and pDML543 shuttle vectors. The protein was produced in amounts that were more or less related to the plasmid copy number, as pDML532 and pDML542 are low-copy-number vectors and pDML533 and pDML543 are high-copy-number vectors. Interestingly, membranes of the *psr*-deficient FS3, FS4, and FS5 mutants contained the same amount of PBP5 as membranes of the parental ATCC 9790 strain, indicating that neither the lack of Psr nor the presence of a resistance cassette inside or substituting for the *psr* gene modified PBP5 synthesis. In addition, when several copies of *psr* were reintroduced into R40 cells, PBP5 was still overproduced. The R40 transformants that were used in the latter experiment contained the pDML531 shuttle vector (Table 1) bearing a 1.4-kb *psr* fragment amplified from ATCC 9790 (primers O5 and O6* [Table 2]) and extending between position −431 and position 1,001 from the ATG site.

Western blot analysis with anti-PBP5 antibodies corroborated the results obtained by fluorography.

Penicillin susceptibility of the *pbp5*- and *psr*-deficient *E. hirae* mutants. The results shown in Table 3 confirmed that the MICs of PenG for cells lacking PBP5, such as the cells of Rev14 (23) or the cells of FS1 and FS2, were markedly lower (20-fold lower for FS2, an ATCC 9790 derivative, and 4,000-fold lower for Rev14 and FS1, two R40 derivatives). When complemented with several copies of *pbp5*, these hypersusceptible strains regained a resistant phenotype, but at different levels. If the MIC for the Rev14 transformant (which should not have produced Psr) was the same as the MIC for R40, the FS1 and FS2 transformants were 6- to 12-fold less resistant. The FS3 clone with *psr* disrupted or the FS4 and FS5 clones with *psr* deleted were as susceptible as the parental ATCC 9790 strain. Moreover, in spite of a complementing *psr* gene (pDML531), the PenG resistance of R40 cells was not diminished. Thus, whether it was absent or present, the *psr* gene did not affect *E. hirae* susceptibility or resistance to PenG.

Finally, Pen[r] clones could be isolated from FS4 by four passages on plates containing increasing PenG concentrations. After the fourth step the mutant produced large amounts of PBP5 and was even more resistant than R40 (MIC, >70 μg/ml).
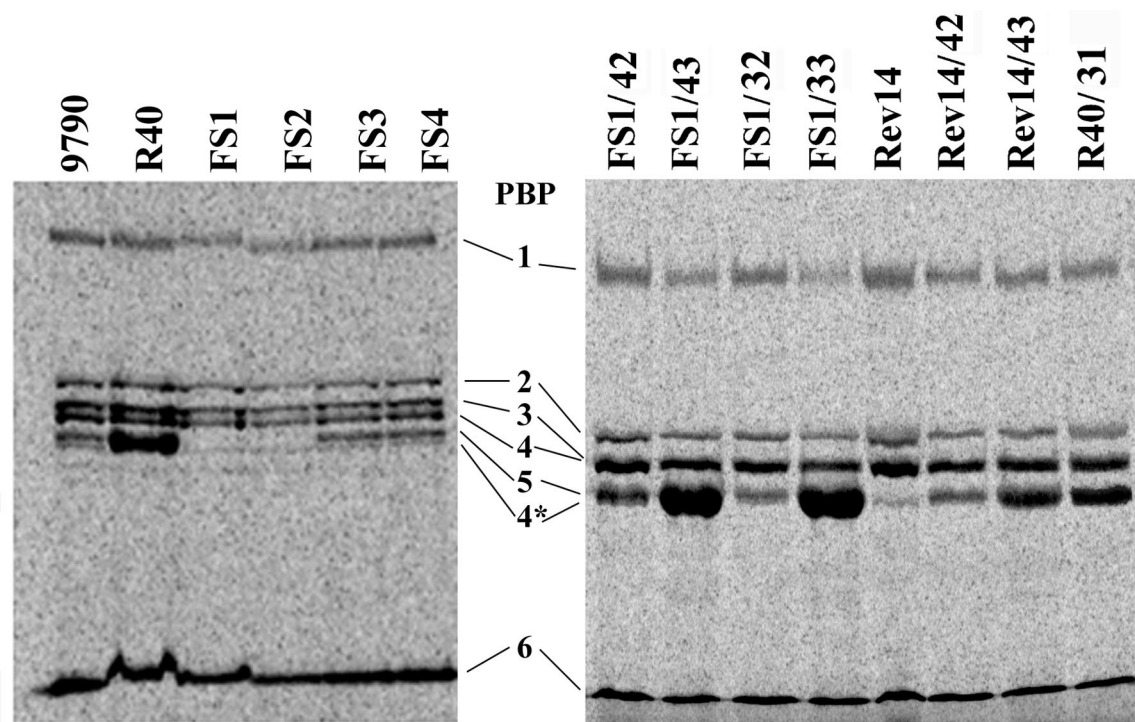
FIG. 3. PBP patterns of the *pbp5*- and *psr*-deficient *E. hirae* mutants. Membrane preparations (200 μg) were labeled for 60 min with 100 μM [¹⁴C]PenG and subjected to gel electrophoresis and fluorography. For simplicity, the last two digits of the plasmid designations are used to identify the different transformants (see Table 1). 9790, ATCC 9790.

The resistant phenotypes could not be attributed to a β-lactamase activity encoded by the complementing vector as none of the extracts of the different clones could hydrolyze the chromogenic nitrocefin compound. In addition, Rev14 cells transformed with a pBR322/pIL252 fusion vector were as susceptible as the parental Rev14 cells.

Cell growth and autolysis of the *psr*-deficient *E. hirae* mutants. The *psr*- and *pbp5*-deficient mutants isolated in this study were also tested to determine their growth rates in THG and their autolytic activities. Table 4 shows the mean generation

times based on two or three independent cultures. On the basis of these results, we subdivided the clones into two groups, one containing ATCC 9790 and its *psr*- and *pbp5*-deficient FS2, FS3, and FS4 derivatives and one containing R40 and its FS1 and Rev14 derivatives. The strains of the first group grew faster than those of the second group.

The autolysis rates determined in three separate experiments (Table 4) confirmed that exponential-phase cells of the different clones autolyzed more rapidly than stationary-phase cells. However, entrance into the stationary phase did not seem to reduce the autolysis rate of R40 cells (1.4-fold) as much as it reduced the autolysis rates of the other strains (3.6- to 6-fold). The results also indicated that the same two groups described above could be distinguished. ATCC 9790 and its derivatives autolyzed more slowly than FS1, Rev14, and R40; the rates were 2- to 2.5-fold and 3- to 9-fold lower than the

TABLE 3. Susceptibility of *E. hirae* strains to PenG

Strain	MIC (μg/ml)
ATCC 9790	0.6
R40	60
Rev14	0.015
FS1	0.015
FS2	0.025
FS3	0.6
FS4	0.6
FS5	0.6
R40/pDML531	60
FS1/pDML532	10
FS1/pDML533	20
FS1/pDML542	5
FS1/pDML543	10
FS2/pDML542	5
FS2/pDML543	10
Rev14/pDML542	60
Rev14/pDML543	60

TABLE 4. Rates of cell growth and cell autolysis of the different *E. hirae* strains

Strain	Generation time (min)	Autolysis rate k (h⁻¹)	
		Exponential-phase cells	Stationary-phase cells
R40	51 ± 1.12	1.25 ± 0.16	0.9 ± 0.08
Rev14	45 ± 1.25	1.52 ± 0.17	0.28 ± 0.05
FS1	48 ± 2.61	1.1 ± 0.17	0.27 ± 0.02
ATCC 9790	27 ± 0.00	0.60 ± 0.28	0.10 ± 0.01
FS2	29 ± 2.14	0.56 ± 0.21	0.14 ± 0.02
FS3	23 ± 1.21	0.61 ± 0.27	0.17 ± 0.01
FS4	28 ± 1.13	0.65 ± 0.39	0.16 ± 0.03

rates for the exponential- and stationary-phase cells, respectively. Thus, in spite of an inactivated *psr* gene, the FS3 and FS4 mutants behaved more like FS2 and the parental ATCC 9790 strain than the Rev14 and R40 strains. We concluded that the inactivation of *psr* could not be responsible for the higher autolysis rates measured with R40.

Analysis of the *psr-pbp5* and *ftsW-psr* intergenic regions. As shown in Fig. 4, direct repeats (DR) and inverted repeats (IR) are present upstream of *pbp5* in *E. hirae* ATCC 9790. Three perfect DR, designated DR_1, DR_2, and DR_3, and a perfect IR, designated IR_1, are present in this region. The right arm of the perfect 9-bp IR_1 is only 28 bp away from the *pbp5* initial ATG codon. The 10-bp DR_1 found at the 3′ end of *psr* terminates 12 bp upstream of its TAG stop codon. DR_2 begins 20 bp downstream of the *psr* stop codon, and there is no separation between its 7-bp repeated sequences. In contrast, 53 nucleotides separate the left and right 8-bp sequences of DR_3, which includes most of DR_2 and the left IR_1 sequence.

In addition, an imperfect 9-bp DR that exhibits some similarity with the DR proposed to be part of the regulating mechanism of the divergent *lytR* gene and *lytABC* operon of *B. subtilis* (22) appears to be present in the same region. The AAAATGAAA sequence overlapping the *pbp5* initial ATG codon is identical to the sequence designated S_1 in *B. subtilis* (Table 5). Two sequences (TGTAAGAAG and AGTTAG AAA) similar to the sequence designated S_2 in *B. subtilis* are 90 and 83 bp, respectively, upstream of S_1 and could form the other arm of the imperfect DR in *E. hirae*. Interestingly, each putative S_2 sequence overlaps one of the DR_2 arms.

Figure 4 also shows that the region upstream of *psr* has several putative regulatory sequences. An almost perfect 10-bp IR (IR_1) overlaps the 3′ end of *ftsW*, and its right sequence starts 1 bp downstream of the *ftsW* stop codon. IR_1 could serve as an atypical *ftsW* *rho*-independent terminator. Between IR_1 and the *psr* initial codon, there are also two 9-bp perfect repeats, which are designated DR_1 and DR_2. The right DR_1 arm ends only 8 bp upstream of the *psr* initial ATG codon. In addition, DR_1 is contained in the DNA segment separating the two DR_2 arms. The left sequence of DR_2 overlaps DR_1 by one nucleotide, and its right sequence forms the third, fourth, and fifth *psr* codons.

Like *pbp5*, *psr* appears to have an imperfect 9-bp DR recalling the DR of the *lytRABC* divergon in *B. subtilis* (22). The same S_1 sequence as in *B. subtilis* overlaps the *psr* initial ATG codon, and two sequences (CTCAAGAAA and AACAAG AAC) found 103 and 83 bp, respectively, upstream of S_1, at the 3′ end of *ftsW*, could play the role of the S_2 sequence (Table 5).

Identical or very similar sequences are found at the initial ends of *pbp5fm* and *psrfm* in *E. faecium* (24, 34, 45; and Brouillard and Coyette, unpublished data) and of *pbp4* in *E. faecalis* (23) (Table 5). S_2 sequences seem not to be as well conserved as S_1 sequences, but in every case except *pbp5fm* in *E. faecium* there is a 9-bp sequence that recalls an S_2 sequence about 80 bp upstream of S_1. For *pbp5fm*, the only 9-bp sequence that exhibits similarity with S_2 is 101 bp upstream of S_1. *E. faecium* D63 differs from the other *E. faecium* strains not only by its shorter *psrfm* but also by the presence of a 202-bp insert 67 nucleotides upstream of *pbp5fm* (45; Brouillard and Coyette, unpublished data). Because of this insert, the putative S_2 sequence, which is 101 bp away from S_1 in the other strains, is

shifted 202 bp farther away in D63. However, at the 3′ end of the 202-bp insert and 108 and 118 bp upstream of S_1 are TAAAAGAAA and GTAAAGAAA sequences, respectively, which are very similar to S_2 sequences.

EMSA with the truncated PsrN protein. As the *psr* gene product was suspected to function as a regulator (23) and as the region separating the *psr* and *pbp5* genes probably contains different signaling sequences (see above), attempts were made to verify that the Psr protein could interact with a 407-bp probe obtained from this region. The sequence extended from position −333 to position 74 from the initial ATG codon of *pbp5* and was amplified by PCR by using *E. hirae* ATCC 9790 chromosomal DNA as the template and Cy5-labeled primers O29* and O30. Prediction analysis of the Psr protein sequence indicated that it has an N-terminal hydrophobic peptide that could serve as a membrane anchoring system. Thus, this protein was overproduced as a soluble derivative, designated PsrN, from which residues 7 to 26 were deleted. The 822-bp truncated *psrN* gene that was used for this purpose was derived from ATCC 9790 and had a 60-bp deletion (from position 19 to position 78) in the region coding for the hydrophobic peptide of Psr. It was amplified by PCR from the genomic DNA by using a mixture of the *Taq* and *Pwo* DNA polymerases and the O1 and O25* to O28 oligonucleotides combined to form three pairs of primers (Table 2). The first six *psr* codons were conserved, and two restriction sites (*Nde*I and *Ava*I) were introduced to facilitate insertion into the pET22b(+) expression vector. The recombinant vector obtained was designated pDML534.

Synthesis of PsrN with a polyhistidine peptide at the C-terminal end was induced by 1 mM IPTG in a 500-ml culture of *E. coli* BL21/pDML534 cells (OD_{550}, ≅0.8) at 37°C. After 3 h of growth, the cells were harvested by centrifugation (6,000 × *g* for 10 min), washed in a 150 mM NaCl solution, and resuspended in 15 ml of conservation buffer containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 5 μM β-mercaptoethanol, 10% (wt/vol) glycerol, and 1 mM Pefabloc (Boehringer Ingelheim Bioproducts). They were then disintegrated at $1.35 × 10^8$ Pa with cell disruption equipment (Constant Systems Ltd., Inceltech, Toulouse, France). Cellular debris was removed by centrifugation (17,000 × *g* for 25 min), and the clarified supernatant was used for an EMSA. The 32-kDa PsrN protein that was detected by immunoblotting and was produced only under inductive conditions represented about 20% of the cytoplasmic protein.

Partially purified PsrN preparations were eluted with an imidazole solution from an Ni^{2+}-nitrilotriacetic acid agarose column (Ni-PDC kit; Affiland, Liège, Belgium) on which crude extracts of PsrN-overproducing *E. coli* cells were filtered. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the PsrN protein detected by Coomassie blue staining and Western blotting was the major component of the preparation (data not shown). However, no EMSA could be conducted with the partially purified PsrN as it had a strong propensity to precipitate. Thus, crude cytoplasmic extracts were used for the EMSA. Various experimental conditions were tested to detect a potential interaction between PsrN and the 407-bp Cy5 probe. The quantity of PsrN (as estimated by densitometry) varied from 10 to 250 ng, and two temperatures (room temperature and 30°C) and different incubation times
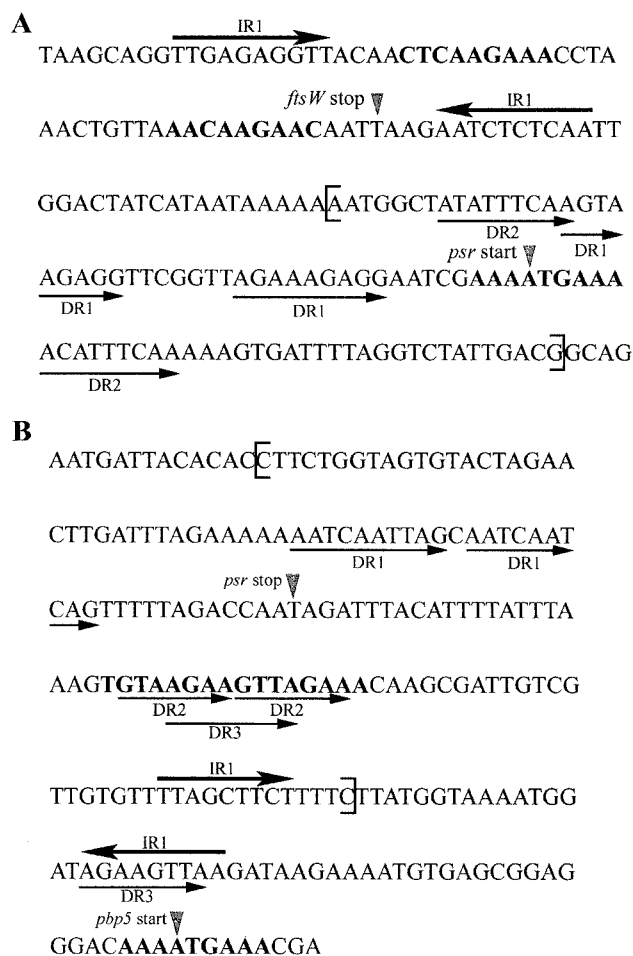
A

IR1

TAAGCAGGTTGAGAGG**TTACAAC**TCAAGAAACCTA

ftsW stop / IR1

AACTGTTA**AACAAGAAC**AATTAAGAATCTCTCAATT

GGACTATCATAATAAAAA[AATGGCTATATTTCAAGTA

DR2 / *psr* start / DR1
AGAGGTTCGGTTAGAAAGAGGAATCGAAAATGAAA
DR1 / DR1

ACATTTCAAAAAGTGATTTTAGGTCTATTGACG]GCAG
DR2

B

AATGATTACACAC[TTCTGGTAGTGTACTAGAA

CTTGATTTAGAAAAAAAATCAATTAGCAATCAAT
DR1 / DR1

psr stop
CAGTTTTTAGACCAATAGATTTACATTTTATTTA

AAGT**GTAAGAAGTTAGAAA**CAAGCGATTGTCG
DR2 / DR2
DR3

IR1
TTGTGTTTTAGCTTCTTTTC]TTATGGTAAAATGG

IR1
ATAGAAGTTAAGATAAGAAAATGTGAGCGGAG
DR3

pbp5 start
GGAC**AAAATGAAA**CGA

FIG. 4. Sequences of the regions separating the 3′ end of *ftsW* and the 5′ end of *psr* (A) and the 3′ end of *psr* and the 5′ end of *pbp5* (B). The initial codons and stop codons of the different genes are indicated by grey arrowheads. The thick and thin arrows indicate the IR and DR, respectively. The putative S_1 and S_2 sequences are indicated by bold-face type. The brackets indicate the beginning and end of the deletion observed in R40 (A) and AS21R$_2$ (B).

(20 to 45 min), as well as the influence of β-mercaptoethanol, were tested. None of these conditions promoted any interaction between PsrN and the *psr-pbp5* intervening sequence.

DISCUSSION

Enterococci have a low natural susceptibility to β-lactam antibiotics attributed to the constitutive synthesis of a low-affinity PBP usually designated PBP5, which is able to take over most functions of other PBPs when they are inhibited by β-lactams (17, 18). The genes of different strains of three species coding for these PBPs have been cloned and sequenced (13, 14, 31, 33, 38, 45). Moreover, with the DNA sequencing data collected recently from clones of *E. hirae*, *E. faecalis*, and *E. faecium* (13, 45; this study) and the determination of the genomic sequences of *E. faecalis* V583 (http://tigr.org) and *E. faecium* DO (http://genome.ornl.gov/microbial/efae/), it is possible to establish the genetic context of these genes.

In the genomes of *E. hirae* ATCC 9790 and *E. faecium*

D63R, C68, and DO, genes similar to the *ftsW* gene in the *dcw* cluster of *E. coli* (23, 26, 34, 44, 45) are found upstream of *psr* genes, which in turn precede *pbp5* genes. It can be presumed that in these organisms, these *ftsW* genes are required for cell division as, in contrast to *E. coli*, the organisms have no *ftsW* gene in their *dcw* clusters (12).

PBP5 functions in β-lactam resistance. A point mutation introducing a stop codon in *pbp5* was responsible for the lack of PBP5 in *E. hirae* Rev14 and for the 40- and 4,000-fold-higher susceptibility to PenG of this strain than of strains ATCC 9790 and R40, respectively (23). As expected, similar results were obtained with the FS1 and FS2 mutants (derived from R40 and ATCC 9790, respectively), in which *pbp5* was inactivated by a Campbell-type recombination, as shown by PCR and hybridization analyses. Fluorography and Western blotting analyses confirmed that these mutants were unable to synthesize PBP5.

Resistance to PenG could be restored in Rev14, as well as in the FS1 and FS2 mutants, by complementation with a multi-copy shuttle vector carrying *pbp5*. Whatever the copy number of *psr* (pDML532 or pDML533 and pDML542 or pDML543), the MICs of PenG for the FS1 transformants were not much different (there was a twofold difference). Similar results were obtained with complemented FS2 clones. Interestingly, after complementation, none of the FS1 and FS2 strains was as resistant as Rev14, although they seemed to produce as much, if not more, PBP5. Thus, we concluded that even if PBP5 is an essential element for substantially increasing the resistance to PenG, it is not sufficient to obtain optimal resistance. Similar conclusions were recently drawn from a comparison of different clones of *E. faecium* (37). Also, the *pbp5* gene borne by pDML542 and pDML543 must have its own promoter as large amounts of PBP5 are synthesized in complemented FS2 strains. This is in agreement with the results previously obtained for *E. coli* (23).

Implication of Psr in cell growth and autolysis. Psr, for which a repressor activity was proposed by Ligozzi et al. (23), exhibits 35.3% identity with the LytR protein of *B. subtilis* (26, 40). It has been suggested that LytR behaves like an attenuator of its own expression and expression of the *N*-acetylmuramoyl-L-alanine amidase (*lytC*) gene of *B. subtilis*. LytC is responsible for the bulk of the amidase activity during vegetative growth. However, a *lytR*-deficient mutant of *B. subtilis* showed the same turnover kinetics and cell wall autolysis as the parental strain (22).

Two types of *psr*-deficient mutants were obtained from the wild-type ATCC 9790 strain; FS3 was obtained by disruption, and FS4 and FS5 were obtained by deletion. The alterations were verified by PCR and hybridization analyses.

In spite of the *psr* deficiency, FS3, FS4, and FS5 grew and autolyzed at the same rates as ATCC 9790. This tells us that the *psr* gene product has no regulatory effect on these phenomena. Thus, we concluded that the slower growth and higher autolysis rates of R40, Rev14, and FS1 very likely do not result from the partial *psr* deletion but instead result from at least one other modification in the genome.

Implication of Psr in β-lactam resistance. In contrast to R40, whose *psr* gene was inactivated by a short deletion, the three *psr* mutants constructed in this work synthesized the same amount of PBP5 as the wild-type ATCC 9790 strain. This

TABLE 5. Putative regulatory imperfect DR located upstream of the *psr* and *pbp5* genes of three enterococcal species

Species	Gene	S2	S2–S1 distance (bp)	S1[a]
E. hirae	*pbp5*	AGTTAGAAA	83	AAA**ATG**AAA
		TGTAAGAAG	90	AAA**ATG**AAA
	psr	AACAAGAAC	83	AAA**ATG**AAA
		CTCAAGAAA	103	AAA**ATG**AAA
E. faecium	*pbp5fm*	TTCCAGAAA	101	AAA**ATG**AAA
	psrfm	AAAGAGAAA	96	GCA**ATG**AAA
E. faecalis	*pbp4*	ATTAAGAAA	80	TGA**ATG**AAA
B. subtilis	*lytR*	ACAATGAAA	78	ATA**ATG**AAA
	lytABC	AGAATGAAA	98	AAA**ATG**AAA
Consensus		----aGAAa		--AATGAAA

[a] The initial ATG codon of each gene is indicated by boldface type.

demonstrated that neither of the two modifications in *psr* in FS3, FS4, and FS5 had a polar effect on expression of *pbp5*, which, as indicated above, appears to have its own promoter. When high-copy-number plasmids complementing *psr* were introduced into R40 cells, the level of synthesis of PBP5 was not reduced. In addition, the chromosomal *psr* gene of the *pbp5*-deficient FS2 mutant did not prevent overproduction of PBP5 when this strain was complemented with pDML542 or pDML543. It could be argued that in this case the gene could not act in *trans*. However, it cannot act in *cis* either. Indeed, the *psr* gene that is borne with *pbp5* on the 6.9-kb fragment complementing FS1(pDML532 or pDML533) did not seem to prevent PBP5 overproduction. These different results are thus inconsistent with Psr regulation of PBP5 synthesis and consequently of penicillin resistance.

However, we point out that the impact of the 87-bp *psr* deletion in R40 on expression of *pbp5* is different from the impact of the insertion or deletion in FS3, FS4, or FS5. This means that it is not the *psr* gene per se or its product that is important but that the region including the 87-bp sequence is involved in one way or another in the regulation of the *psr-pbp5* locus. At this point, we have to consider that most probably *pbp5* is regulated by one or several genes found elsewhere in the genome. This hypothesis explains why it was still possible to select PBP5-overproducing resistant clones from FS4 in spite of a deleted *psr* and why the moderately resistant *E. faecium* D344 strain overproduces PBP5fm even though no mutation could be detected in the *psr-pbp5fm* locus (35, 45; Brouillard and Coyette, unpublished).

In the search for a potential regulatory activity for Psr, EMSAs were performed to examine DNA-protein interactions between PsrN, a soluble Psr derivative, and a 407-bp probe corresponding to the DNA region located upstream of *pbp5*. These assays were unsuccessful in spite of the potential DR, IR, S_1, and S_2 regulatory sequences that were identified in that area.

Interestingly, in the *E. hirae* R40 and AS21R$_2$ and *E. faecium* D63R Pen^r mutants (23, 45), overproduction of PBP5 or PBP5fm seems to be related to a deletion or an insertion that occurred in the upstream region of *pbp5*. In R40, the 87-bp deletion removed DR$_1$ and DR$_2$, as well as the S$_1$ sequence

overlapping the *psr* 5′ end. In AS21R$_2$, the 137-bp deletion not only shortened the *psr* open reading frame at its 3′ end but also deleted DR$_2$ and the left DR$_3$, IR$_1$, and S$_2$ sequences that precede *pbp5*. Finally, in the resistant D63R strain derived from D63, an additional 1,330-bp DNA fragment was inserted 10 bp upstream of the 3′ end of the 202-bp insert that precedes *pbp5fm* in D63. As a result, the putative S$_2$ sequences described above were moved farther away from S$_1$ and no new potential S$_2$ sequences were found in the −100 to −80 region.

The S$_1$ and S$_2$ sequences, as well as the IR and DR sequences, might thus be important for regulation of the *psr* and *pbp5* genes in these organisms, but their significance remains to be determined.

Conclusion. Because no change in PBP5 synthesis or cell autolysis occurred when the *psr* gene was inactivated, because the overproduction of PBP5 was not reduced when several *psr* copies were introduced into R40 cells, because the Psr protein did not interact with a DNA fragment amplified from the *pbp5* promoter region, and because mutants as resistant as R40 could be selected from the *psr*-deficient FS4 strains, we concluded that the Psr protein has no regulatory effect either on the transcription of *pbp5* or on the autolysis of *E. hirae* cells. Another regulatory mechanism of PBP5 and probably Psr synthesis should be considered and remains to be identified. The data could implicate one or several of the putative signaling sequences found upstream of *pbp5* and *psr*. Also, functions complementary to synthesis of the low-affinity PBP5 protein are needed for optimal phenotypic resistance to PenG in *E. hirae*.

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