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Evidence that the PBP 5 Synthesis Repressor (*psr*) of *Enterococcus hirae* Is Also Involved in the Regulation of Cell Wall Composition and Other Cell Wall-Related Properties

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***psr* has been reported by M. Ligozzi, F. Pittaluga, and R. Fontana, (J. Bacteriol. 175:2046–2051, 1993) to be a genetic element located just upstream of the structural gene for the low-affinity penicillin-binding protein 5 (PBP 5) in the chromosome of *Enterococcus hirae* ATCC 9790 and to be involved in the repression of PBP 5 synthesis. By comparing properties of strains of *E. hirae* that contain a full-length, functional *psr* with those of strains that possess a truncated form of the gene, we have obtained data that indicate that *psr* is involved in the regulation of several additional surface-related properties. We observed that cells of strains that possessed a truncated *psr* were more sensitive to lysozyme-catalyzed protoplast formation, autolyzed more rapidly in 10 mM sodium phosphate (pH 6.8), and, in contrast to strains that possess a functional *psr*, retained these characteristics after the cultures entered the stationary growth phase. Cellular lytic properties did not correlate with differences in the cellular contents of muramidase-1 or muramidase-2, with the levels of PBP 5 produced, or with the penicillin susceptibilities of the strains. However, a strong correlation was observed with the amounts of rhamnose present in the cell walls of the various strains. All of the strains examined that possessed a truncated form of *psr* also possessed approximately one-half of the rhamnose content present in the walls of strains that possessed a functional *psr*. These data suggest that *psr* is also involved in the regulation of the synthesis of, or covalent linkage to the cell wall peptidoglycan of, a rhamnose-rich polysaccharide. These differences in cell wall composition could be responsible for the observed phenotypic differences. However, the multiple effects of *psr* suggest that it is part of a global regulatory system that, perhaps independently, affects several cell surface-related properties.**

Fontana et al. (10, 18) observed that both laboratory-generated penicillin-resistant mutants and some clinical isolates of various species of *Enterococci* possessed substantially larger amounts of a penicillin-binding protein (PBP) that characteristically had a low affinity for binding benzylpenicillin (Pen G). In *Enterococcus hirae* ATCC 9790 (formerly called *Streptococcus* or *Enterococcus faecium*), this low-affinity PBP has the electrophoretic mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of a protein with a molecular mass of approximately 71 kDa, corresponding to that of PBP 5 (4, 7). The amounts of PBP 5, as detected by the binding of radioactive Pen G, paralleled the levels of drug resistance. In addition, cultures of a laboratory-derived resistant strain, R40, grew normally in concentrations of Pen G that saturated all of the PBPs except for PBP 5 (1, 19). Increasing the Pen G concentration to that which saturates PBP 5 resulted in growth inhibition. These results, plus other data, were interpreted to indicate that in the resistant strains, the low-affinity PBP (PBP 5 in *E. hirae* ATCC 9790) is the sole target essential for growth and, in the presence of low concentrations of Pen G, takes over the function(s) of the other PBPs (19). Fontana's group also isolated a Pen G-hypersensitive derivative of the R40 strain, Rev14, that fails to produce detectable amounts of PBP 5 (11). Recently, the gene for PBP 5 from the R40 strain has been cloned in *Escherichia coli* and sequenced (7). Cloning, sequencing, and comparing the region upstream of the PBP 5 gene in the 9790, R40, and Rev14 strains revealed the presence of an open reading frame approximately 1 kb upstream of the PBP 5 gene. This genetic element was called *psr* (PBP 5 synthesis repressor [18]). The DNA of the R40 strain had an 87-bp deletion in *psr*, accounting for derepression of PBP 5 synthesis and Pen G resistance. DNA of the Rev14 strain not only possessed the same 87-bp deletion but also possessed a change in the 42nd codon of the PBP 5 gene, from TCA to the stop codon TAA, accounting for the absence of a functional PBP 5 in the Rev14 strain (18).

Despite their divergence in susceptibility to Pen G, the R40 and Rev14 strains were reported to have several cell surface-related properties in common that were different from those of the ATCC 9790 parent strain. For example, exponential-phase cells of both mutant strains have been reported to have smaller diameters than cells of the parent strain and to autolyze more rapidly in 0.3 M sodium phosphate (pH 7.0) (11). Thus, we considered the possibility that *psr* is involved in the regulation of autolysis and of other cell surface-related properties or functions. In the studies reported here, we have compared a number of cell wall-related properties of *E. hirae* strains that possess a truncated *psr* with the same properties of strains that possess an apparently full-length, presumably functional *psr*. Remarkably, the wall-related phenotypic differences observed correlated with the presence or absence of a fully functional *psr* and not with overproduction of PBP 5 or levels of resistance to Pen G.

MATERIALS AND METHODS

Bacterial strains. *E. hirae* ATCC 9790 and the two previously described mutant strains R40 (10), which is resistant to Pen G and which hyperproduces PBP

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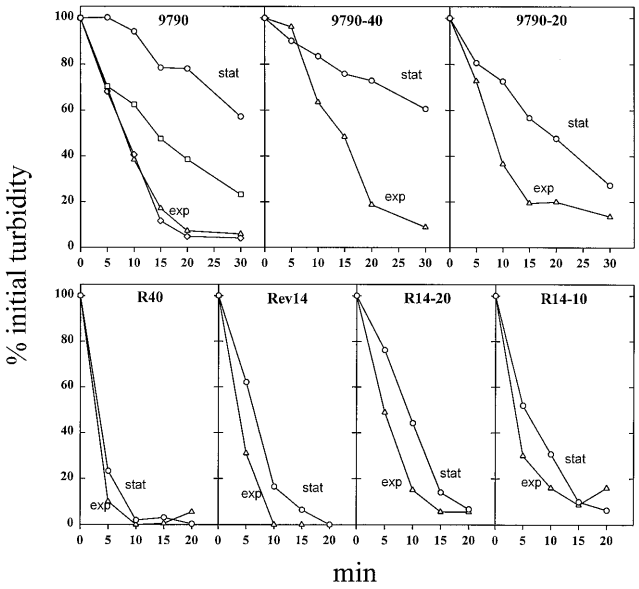

FIG. 1. Osmotic fragility of lysozyme-treated cells of *E. hirae* ATCC 9790, R40, Rev14, and some of their derivative mutants. Samples containing approximately 17 mg (dry weight) of cells were taken from cultures grown to mid-exponential phase (AOD of 430 [◇] and 860 [△]), the beginning of stationary phase (AOD of 1,330 [□]), and later stationary phase (AOD of 1,700 [○]), as shown for ATCC 9790. For the other strains, only results obtained with cells grown to AODs of 860 and 1,700 are shown. Cells were harvested, washed, and then incubated at 37°C in protoplast buffer containing lysozyme. Changes in turbidity of the diluted samples were measured at 450 nm as described in Materials and Methods. exp, exponential; stat, stationary.

5, and its Pen G-hypersusceptible derivative Rev14 (11), which lacks PBP 5, were used in this study. The two mutants were kindly provided by J. Coyette (University of Liège, Liège, Belgium). The R40 strain was plated several times on agar medium containing Pen G (40 μg/ml) to obtain and maintain a uniformly Pen G-resistant strain. On subculturing in the absence of Pen G, the R40 strain exhibits a heterogeneous response with respect to the level of Pen G resistance.

Culture medium and growth conditions. Bacterial strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% glucose (THGB). Alternatively, S broth (3) or chemically defined medium (24) was used when specified. Growth in liquid medium was monitored turbidimetrically at 675 nm as previously described (24). Turbidity measurements were converted into adjusted optical density (AOD; 1 AOD unit = 0.29 μg [dry weight] of cells per ml of culture) to agree with Beer's law. Generally, cultures were grown to the late exponential phase (AOD of 860; 0.25 mg [dry weight] of cells per ml). When specified, cultures were grown to other selected culture turbidities.

Preparation of protoplasts. Bacterial cultures were grown overnight at 37°C in THGB. In the morning, the cultures were diluted 1:100 in prewarmed fresh medium and were incubated at 37°C. Growth was monitored every 30 min. Samples (135 ml) were taken from bacterial cultures when they reached AODs of 430 and 860, in the exponential phase, at the beginning of stationary phase (AOD of 1,330), and well into the stationary phase (AOD of 1,700). The cultures were rapidly chilled in an ice bath and centrifuged (20,000 × *g*, 15 min, 4°C). The bacterial pellets were washed once (28,000 × *g*, 5 min, 4°C) in 18 ml of protoplast buffer (0.05 M Tris-HCl [pH 7.2], 0.01 M magnesium acetate, 0.25 M sucrose) containing lysozyme (1.25 mg/ml) and resuspended in 1.8 ml of the same buffer, and the suspension was incubated at 37°C. At intervals, osmotic fragility was monitored by measuring the turbidity (A_{450}) of 5-μl samples diluted into 1.5 ml of 10 mM sodium phosphate (pH 7.0). After 30 min at 37°C, protoplast suspensions were transferred to microfuge tubes and centrifuged (14,000 × *g*, 15 min, 4°C). The supernatants were carefully decanted into clean tubes, and pellets were resuspended in the same volume (1.8 ml) of 10 mM sodium phosphate (pH 7.0) containing DNase (5.5 μg/ml).

Cellular autolysis. Bacterial cultures were grown as described above. When cultures reached AODs of 470, 750, and 1,700, respectively, 6 ml was rapidly chilled to 0°C and filtered (0.65-μm-pore-size filter; Millipore Corp., Bedford, Mass.), washed three times with distilled water at 4°C, and resuspended in 6 ml of 10 mM sodium phosphate (pH 6.8), and the suspension was incubated in a 37°C water bath (25). Changes in turbidity at 675 nm were monitored at 15-min intervals, and rates of autolysis (*k*) (per hour) were calculated from the exponential portion of lysis curves (22).

Preparation of enzymes and assaying of enzymatic activities. Cultures of the three strains were grown in THGB to AODs of 430 (exponential phase [400 ml]), 860 (exponential phase [200 ml]), 1,330 (immediately after the end of exponential phase [130 ml]), and 1,700 (well into stationary phase [100 ml] and 1.5 h later [100 ml]). Thus, each sample of culture contained approximately 50 mg (dry weight) of cells. The cultures were rapidly chilled in an ice bath and then centrifuged (20,000 × *g*, 10 min, 4°C). The bacterial pellets were suspended in 1.5 ml of ice-cold distilled water, transferred to microcentrifuge tubes, centrifuged (14,000 × *g*, 10 min, 4°C), and washed once with 1.5 ml of ice-cold distilled water. The pelleted samples of intact cells were treated with 1 ml of 6 M guanidine-HCl at 0°C for 10 min and then centrifuged (14,000 × *g*, 10 min, 4°C). Fifteen microliters of each guanidine-HCl extract was diluted to 1:100 into the lytic enzyme assay buffer (1.5 ml of 10 mM sodium phosphate [pH 7.0]). Muramidase-1 and muramidase-2 activities were assayed spectrophotometrically as described previously (14). *E. hirae* and *Micrococcus luteus* cell walls were used as substrates for muramidase-1 and muramidase-2 activities, respectively.

Preparation of cell wall matrices and chemical analyses. Chemically defined medium (24) was used to grow the bacteria for the preparation of cell wall matrices. Bacterial cultures (1 liter) were grown to an AOD of 860 (exponential phase) and for 4 h after the beginning of stationary phase (AOD of ~2,500). The cells were pelleted (16,000 × *g*, 10 min, 4°C), washed twice with ice-cold distilled water, and suspended in 10 or 30 ml of water for the cells grown at an AOD of 860 or ~2,500, respectively. Then, cells were added dropwise to 8% SDS at 100°C and were adjusted to a final concentration of 4% SDS in 200 or 600 ml for cells grown to an AOD of 860 or ~2,500, respectively, and incubated for 30 min at 100°C. The SDS extraction was continued with gentle stirring at room temperature overnight. After centrifugation, the SDS extractions were repeated twice. The pellet was then sedimented and washed with 10 mM Tris-HCl (pH 7.2) until there was no foaming upon shaking (a minimum of 10 times). These preparations were designated crude cell walls. A portion of the *E. hirae* crude cell walls was treated with 0.1 N HCl at 60°C for 16 h (15). The suspension was neutralized with 2.5 N NaOH and centrifuged (20,000 × *g*, 20 min), and the resuspended pellet was washed five times with 10 mM Tris-HCl (pH 7.2). This preparation was designated crude peptidoglycan. A portion of cells, crude cell walls, and crude peptidoglycans was dried for 3 days at 105°C and weighed. The amounts of methylpentose and amino sugars were determined by the method described by Dische and Shettles (29) and by a modified Elson-Morgan method (12), respectively, after acid hydrolysis in 3 N HCl for 4 h at 95°C.

Isolation of Pen G-resistant mutants from strain ATCC 9790. Samples (100 μl; ca. 10^8 cells) of several dilutions of an overnight culture of *E. hirae* ATCC 9790 were plated onto THG agar plates containing concentrations of Pen G ranging from 10 to 40 μg/ml. The 9790 parent grows normally at a Pen G concentration of 5 μg/ml. First-step mutants were isolated at 20 μg/ml, with a frequency of 2.1×10^{-4}. The clones resistant to Pen G at this level were grown without Pen G and were plated again on agar plates containing Pen G (20 μg/ml). One clone (9790-20) was used for the subsequent step. Again, 100 μl of several dilutions of an overnight culture was plated onto THG agar plates containing 40 μg of Pen G per ml. Second-step mutants were isolated at a frequency of approximately 1.5×10^{-7}. These cultures exhibited some heterogeneity in response to Pen G. We failed to obtain any first-step mutants resistant to 40 μg/ml even when 10^{10} cells were plated. In addition, even upon subculturing of strains resistant to 40 μg of Pen G per ml, we failed to obtain any strain resistant to more than 40 μg/ml.

Isolation of Pen G-resistant mutants of the hypersusceptible Rev14 strain. An overnight culture of Rev14 was grown in THGB. Samples (100 μl; ca. 10^8 cells) were plated on THG agar containing 1 μg of Pen G per ml. After overnight incubation at 37°C, mutants were isolated with a frequency of 1.6×10^{-8}. A total of 120 colonies were subcultured to plates containing 1 μg of Pen G per ml. All 120 strains grew well upon subculturing and were again subcultured to plates containing 20 or 40 μg of Pen G per ml. Twenty strains grew on the plates containing 20 μg of Pen G per ml, of which 19 also grew on 40 μg of Pen G per ml. All of the 120 strains that grew on 1 μg of Pen G per ml grew on plates containing 4 μg of Pen G per ml, and 58 of the 120 grew on 10 μg of Pen G per ml. All of the strains that grew on 20 or 40 μg of Pen G per ml retained their resistance even after subculturing in the absence of Pen G.

Amplification by PCR. The nucleotide primers used for amplification by PCR of the *psr* region were the same as those described by Ligozzi et al. (18). In later experiments, the primer pair 5′-CGCGGATCCAAAACATTTCAAAAAGTG ATTTTAG-3′ and 5′-CCGGAATCCCAATCGCTTGTTTCTAACTTC-3′ was also used for PCR. Reaction mixtures (50 μl) were prepared with 1 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 1× *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 50 μM each deoxynucleotide triphosphate, 0.2 μM each primer, and 1.5 μl of cells from overnight cultures as the DNA template. The reaction mixture was cycled 30 times in a PTC-100 (MRJ Research, Waterford, Mass.) thermocycler with the following temperature profile: 94°C for 60 s to denature, 58°C for 90 s for annealing of the primer, and 72°C for 90 s for extension. Chromosomal DNA (0.2 μg) of *E. hirae* ATCC 9790, extracted as described previously (2), was used for all PCRs as a control.

The resulting products were resolved by electrophoresis in 1.2% SeaPlaque GTG agarose (FMC, Rockland, Maine) in 1× TAE (40 mM Tris-acetate, 2 mM EDTA) and were visualized after staining of the gels with ethidium bromide.

TABLE 1. Rates (k) of cellular bacteriolysis[a]

Strain	k (h^{-1})		
	Exponential phase at		Early stationary phase at AOD of 1,700
	AOD of 470	AOD of 750	
ATCC 9790	0.5	0.5	0.03
Mutant 9790-40	1.1	0.8	0.04
Mutant 9790-20	0.9	0.9	0.05
R40	0.9	1.3	0.5
Rev14	1.4	1.5	0.3
Mutant R14-20	1.4	1.4	0.2

[a] k is the first-order reaction rate. Values are the averages of three to five determinations.

Phage λ DNA digested with *Hin*dIII and *Eco*RI (Boehringer, Mannheim, Germany) were used as molecular size standards.

SDS-PAGE and Western blots (immunoblots). The protoplast pellet fractions (bacterial membranes) prepared as described above were separated by SDS-PAGE as previously described (13). After SDS-PAGE, the proteins were transferred (30) to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, Mass.) and the membranes were blocked with 5% nonfat dry milk in 50 mM sodium phosphate (pH 7.4)–150 mM NaCl containing 0.05% (vol/vol) Tween 20 (PBST) as previously described (2, 14). The Immobilon P membranes were incubated with an anti-PBP 5 rabbit polyclonal antibody (kindly provided by J. Coyette) for 2 h at room temperature with gentle shaking. After extensive washing in PBST, the membranes were incubated with anti-rabbit immunoglobulin G peroxidase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). A blue band corresponding to PBP 5 was visualized on the Immobilon P membranes after addition of TMB peroxidase substrate (Kirkegaard & Perry Laboratories).

RESULTS

The relative susceptibilities of the three strains to lysozyme as estimated by the rate of attainment of osmotic fragility in protoplast buffer. While preparing protoplasts of cells from exponential-phase culturing (AOD of 860) of the three strains, we noted that cells of the R40 and Rev14 strains appeared to become osmotically fragile more rapidly than did cells of the parent ATCC 9790 strain (Fig. 1). Cells from exponential-phase cultures (AODs of 430 and 860) of both the R40 and Rev14 strains became virtually completely osmotically fragile in approximately 10 min at 37°C in lysozyme-containing protoplast buffer (Fig. 1), whereas it took approximately 20 min or longer for exponential-phase cells of the parent ATCC 9790 strain to become osmotically fragile (Fig. 1). These observations did not seem to be due to a faster rate of autolysis of cells of the two mutant strains, since, in the absence of lysozyme, all three strains showed little difference in their very low rates of attainment of osmotic fragility in protoplast buffer (data not shown).

In agreement with previous observations (28), cells of the parent strain ATCC 9790 became progressively more resistant to lysozyme-catalyzed osmotic fragility as cultures entered the stationary phase (Fig. 1). In contrast, cells from stationary-phase cultures of both the R40 and Rev14 mutant strains retained much of their sensitivity to lysozyme (Fig. 1). These data suggest that both the R40 and the Rev14 strains differ from the parent strain in some cell wall-related property.

Cellular autolysis in 0.01 M sodium phosphate (pH 6.8). The attainment of osmotic fragility via the action of lysozyme in an osmotically protective environment results from the combined effects of endogenously present autolysins and the hydrolytic action of added lysozyme. In the absence of lysozyme, achievement of osmotic fragility in protoplast buffer was a very slow process. Therefore, we examined the capacity of cells of the three strains taken from cultures at various growth stages to autolyze in 0.01 M sodium phosphate (pH 6.8) (25). Exponential-phase cells (AODs 430 and of 860) of the Rev14, R40, and parental strains autolyzed rapidly (Table 1 and Fig. 2), with cells of the Rev14 strain lysing somewhat faster than cells of the R40 strain and at nearly three times the rate of cells of the parental ATCC 9790 strain (Table 1 and Fig. 2). Perhaps more striking is that cells of the Rev14 and R40 strains failed to develop the high level of resistance to cellular autolysis that is characteristic of the parental strain (25) after entrance into the stationary phase (compare the results shown in Fig. 2 for the ATCC 9790 strain with those for the R40 and Rev14 strains).

Autolytic enzyme-related properties of the various strains of *E. hirae*. The ability of the three strains to attain osmotic fragility in protoplast buffer and to autolyze in 0.01 M sodium phosphate (pH 6.8) could be related to their contents of muramidase-1 and/or muramidase-2 activities. However, the levels of autolytic enzyme activity detected in cells via extraction with 6 M guanidine-HCl were very similar for all three strains (Fig. 3). In cultures of all three strains, the levels of both muramidase-1 activity (measured after trypsin activation) and muramidase-2 activity increased in parallel with, and remained relatively constant during, the exponential phase and decreased somewhat after the cultures reached the stationary phase. The small differences in the levels of activity of either enzyme did not appear to account for the observed differences in the ability of the cells to autolyze or become protoplasts.

We then considered the possibility that the substrate properties of the cell wall or the cell wall peptidoglycan of the mutant strains became modified in such a way that they became more rapidly hydrolyzable substrates of either or both of the muramidases. Examination of the rates of hydrolysis of SDS walls and the acid-insoluble peptidoglycan fractions of stationary-phase cells of each of the three strains (Table 2) failed to account for the increased ability to autolyze. In fact, the data shown in Table 2 indicate that the peptidoglycan fractions of the Rev14 and R40 strains are not as good substrates for purified muramidase-2 as is the peptidoglycan fraction of the parental ATCC 9790 strain. Similarly, the action of a guanidine-HCl extract of the ATCC 9790 strain hydrolyzed the SDS walls of the Rev14 and R40 strains a little less rapidly than it did the SDS walls of the parental strains either in the absence or in the presence of trypsin to activate the latent form of muramidase-1.

The generation of Pen G-resistant mutants of the parent strain and of the Pen G-hypersensitive Rev14 strain. Because the Rev14 strain was reported to have an 87-bp deletion at the

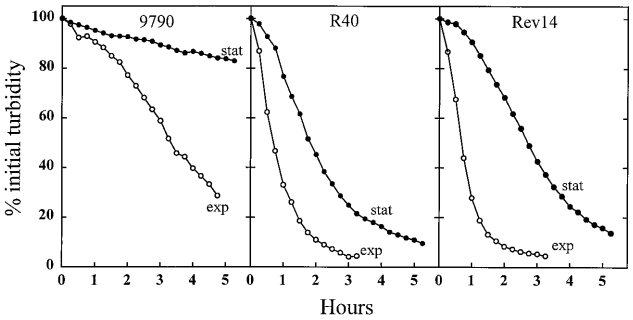

FIG. 2. Cellular autolysis of *E. hirae* ATCC 9790, R40, and Rev14 strains. Bacterial cultures (6 ml) of exponential-phase (exp; AOD of 670 [○]) and stationary-phase (stat; AOD of 1,700 [●]) cells, grown in THGB, were rapidly filtered, washed, and resuspended in 10 mM sodium phosphate (pH 6.8). At intervals, changes in turbidity were monitored spectrophotometrically at 675 nm.

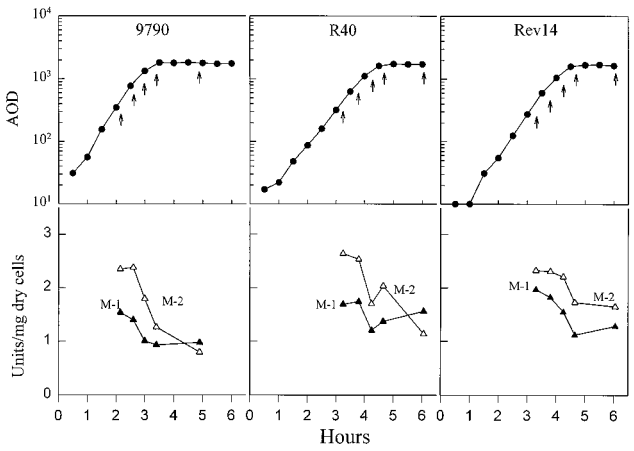

FIG. 3. The production of muramidase-1 and -2 activities (M-1 and M-2, respectively) during growth of the ATCC 9790, R40, and Rev14 strains. Cultures of the three strains were grown in THGB, and culture turbidity (●) was monitored and is indicated in AOD units. Samples were taken at the times indicated by the arrows, centrifuged, and extracted with 6 M guanidine-HCl as described in Materials and Methods. Muramidase-1 activity was measured after the addition of 0.04 μg of trypsin per ml, with *E. hirae* cell walls as the substrate (14). Muramidase-2 activity was measured with *M. luteus* as the substrate (14).

5′ end of its *psr* (18), we decided to obtain Pen G-resistant mutants of Rev14 and, for comparison, resistant mutants of the parental ATCC 9790 strain. The mutants were obtained by growing the two strains in increasing concentrations of Pen G (see Materials and Methods). We obtained strains resistant to 10, 20, and 40 μg of Pen G per ml. We then analyzed some of these strains for the presence and molecular sizes of their *psr* genes, using PCR, and the primers described by Ligozzi et al. (18). As shown in Fig. 4, the Pen G-resistant strains derived from the Rev14 strain possess a *psr* that is slightly but significantly smaller than the *psr* present in the parent strain. (For example, compare the bands in Fig. 4, lanes 6 and 7, with the *psr* band in lane 2.) In contrast, the resistant strains derived from the ATCC 9790 strain possessed a *psr* band indistinguishable in size from that present in the ATCC 9790 strain. Recently, we found that the *psr* gene is longer than originally thought (reference 20; also see GenBank accession no. U42211). In view of these data, a set of primers able to amplify the full-length *psr* were designed to check whether detectable deletions other than the 87-bp deletion at the 5′ end may have occurred in the Pen G-resistant derivatives of ATCC 9790. Again, the Pen G-resistant strains derived from the ATCC 9790 strain possessed a *psr* band indistinguishable in size from that present in the ATCC 9790 strain (data not shown). Thus, we obtained two different classes of Pen G-resistant strains, one of which possesses a presumably functional *psr* and the other of which possesses a truncated, inactive *psr*.

These strains were compared for PBP 5 production by Western blots. PBP 5 was detected in all of the strains tested, with the exception of the hypersensitive Rev14 strain (Fig. 5, lane 3), as shown by the presence of one band at approximately 71 kDa, that reacted with an anti-PBP 5 antiserum. The Pen G-resistant mutants derived from the ATCC 9790 strain showed an amount of PBP 5 comparable to that of their parent strain, despite their level of resistance to Pen G. These results suggest that a deletion in the *psr* gene, and thus a presumably nonfunctional *psr*, is not required for Pen G resistance in *E. hirae*. These data agree with data reported for some clinical isolates of enterococci (5, 9, 16), in which Pen G resistance without overproduction of PBP 5 was observed. The Pen G-resistant mutants derived from Rev14 (shown in Fig. 5, lane 5, for a representative strain of this class) produced an amount of PBP 5 comparable to that of the R40 strain, indicating possible reversion of the point mutation in the *pbp-5* structural gene of Rev14. These results correlate with the presence of apparently the same 87-bp deletion in the *psr* gene of the R40 and Rev14 strains (Fig. 4).

Cell wall-related properties. Bacteria from exponential-phase cultures of the R40 and Rev14 strains autolyzed more rapidly in 0.01 M sodium phosphate (pH 6.8) than did cells of the parent strain (Fig. 2 and Table 1) and are more sensitive to the action of lysozyme than are exponential-phase cells of the parental ATCC 9790 strain (Fig. 1). These results also indicated that, in contrast to the parent strain, cells of the two mutant strains retained much of their autolytic capacity (Fig. 2) and sensitivity to lysozyme (Fig. 1) after entering the stationary phase. These differences in properties suggested differences in cell wall compositions and/or amounts of cell wall. Since the methylpentose rhamnose is a major component of the nonpeptidoglycan polymer of the wall of the parent strain and is found virtually exclusively in the cell wall fraction of *E. hirae* (23, 27), we determined the amount of methylpentose present in the cells of all three strains. Quantification of the methylpentose contents of cells of the 9790, R40, and Rev14 strains, grown in three different culture media (Todd-Hewitt plus glucose, S broth, and a chemically defined medium) to a variety of turbidities and growth phases, showed that cells of both the R40 and the Rev14 strains contained significantly lower amounts of methylpentose (0.13 ± 0.03 μmol/mg [dry weight] of cells and 0.17 ± 0.03 μmol/mg [dry weight] of cells, respectively) than did cells of the parental strain (0.26 ± 0.04 μmol/mg [dry weight] of cells).

The observation of only 50 to 65% of the methylpentose content of the parent strain in the two mutants could be due to the presence of (i) thinner walls, (ii) less surface area per unit of cell mass (e.g., larger cells), or (iii) a substantial change in cell wall composition (e.g., a lower ratio of rhamnose polysaccharide to peptidoglycan in the walls).

To further examine these possibilities, we prepared and analyzed the methylpentose contents and, as an index of peptidoglycan, the amino sugar contents of crude cell wall fractions of exponential- and stationary-phase cells of the 9790, R40, and Rev14 strains and of the peptidoglycan fractions prepared

TABLE 2. Hydrolysis of stationary-phase cell wall substrates by autolysin preparations[a]

Substrate	Enzyme U of GuHCl extract[b]		Enzyme U of purified extracellular muramidase-2
	No trypsin	With trypsin[c]	
SDS walls			
ATCC 9790	2.0	6.3	
R40	1.8	4.3	
Rev14	1.7	3.6	
Acid-treated peptidoglycan			
ATCC 9790			14.8
R40			9.8
Rev14			9.3

[a] Dissolution of substrate was monitored by decrease in A_{450} values. One unit of muramidase activity is defined as the amount of enzyme that results in a decrease of 0.001 A_{450} U/min (14).

[b] Guanidine HCl extract of ATCC 9790 cells contains both muramidase-1 and -2 activities.

[c] Muramidase-1 was activated by the addition of 0.04 μg of trypsin per ml.

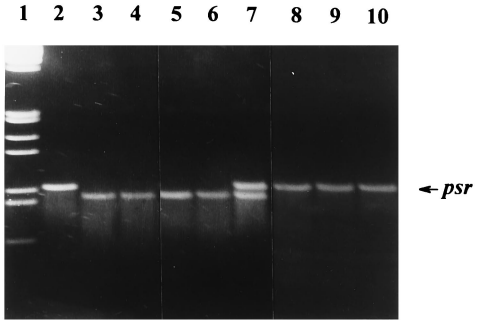

FIG. 4. PCR analysis of the *psr* region of *E. hirae* ATCC 9790, R40, and Rev14 and some of their derived mutants. Samples (1.5 μl) of bacterial cells from overnight cultures (DNA templates) were amplified using the primers described by Ligozzi et al. (18). PCR products were analyzed in a 1.2% low-melting-point agarose gel and were visualized after the gel was stained with ethidium bromide. Mobility in agarose gels showed a difference in the molecular lengths of the *psr* genes from the parental strain (lanes 2, 7, and 10) and its Pen G-resistant derivatives (lanes 8 and 9) from those of the R40 strain (lanes 3 and 7) and the Rev14 strain (lane 4) and its Pen G-resistant derivatives (lanes 5 and 6). Lane 7 contains the PCR products present in lane 2 (from the ATCC 9790 strain) plus the PCR product in lane 3 (from the R40 strain). Lane 1 contains *Hin*dIII-*Eco*RI-digested λ phage DNA.

by acid extraction of each of the wall fractions. In order to recover the maximum amount of wall of each strain, with minimum losses from autolysis, crude wall fractions were prepared by hot SDS extraction of fresh whole cells (see Materials and Methods for details). As summarized in Table 3, it is clear that the crude cell wall fractions of the R40 and Rev14 strains contained 43 to 64% of the methylpentose contents of the crude walls of the parent strain. However, the crude wall (and peptidoglycan) fractions of all three strains contained the same amounts of amino sugar. Thus, it appears that the ratio of rhamnose-containing nonpeptidoglycan wall polysaccharide(s) to peptidoglycan is lower in the two mutant strains than it is in the parental strain.

The Pen G-resistant R40 strain and all of the Pen G-resistant strains derived from the hypersensitive Rev14 strain contained an amount of methylpentose very similar to that of the Rev14 strain and significantly lower than that of the ATCC 9790 strain (Table 4). In contrast, the Pen G-resistant strains derived from the ATCC 9790 strain all had methylpentose contents indistinguishable from that of the ATCC 9790 strain.

Stationary-phase cells of all three strains and their Pen G-resistant derivatives contained slightly more rhamnose than did cells from exponential-phase cells (Tables 3 and 4). However, in all cases stationary-phase cells of the R40 and Rev14 strains and Pen G-resistant mutants of Rev14 contained significantly less methylpentose than did cells of the parental ATCC 9790 strain.

DISCUSSION

The data presented here clearly show that in addition to the previous demonstration of the association of overproduction of PBP 5 with an 87-bp deletion in *psr* (18), this deletion is associated with significant changes in other cell wall-related properties. These properties include (i) increased sensitivity of exponential-phase cells to lysozyme-catalyzed protoplast formation (Fig. 1), (ii) an increased rate of cellular autolysis of exponential-phase cells in 0.01 M sodium phosphate (pH 6.8) (Table 1 and Fig. 2), (iii) retention of lysozyme sensitivity after the cells reach the stationary phase (Fig. 1), and (iv) retention of a substantial portion of the capacity of cells to autolyze after cultures reach the stationary phase of growth (Fig. 2). Furthermore, the strains carrying this 87-bp deletion possessed cell walls that contained approximately one-half of the amount of the methylpentose rhamnose in the cell walls of cells from both exponential- and stationary-phase cultures (Tables 3 and 4).

These differences do not appear to be related to overproduction of PBP 5 or to Pen G resistance, since they were also present in the Pen G-hypersensitive Rev14 strain that fails to produce detectable PBP 5 (11) and that possesses a mutation that resulted in a base change that converted the 42nd codon of the gene that encodes PBP 5 to a TAA stop codon. In addition, Pen G-resistant revertants of the Rev14 strain, which produced PBP 5 (Fig. 5), and of the ATCC 9790 strain, which showed a full-length *psr* gene and which did not appear to overproduce PBP 5 (Fig. 4 and 5), retained the relevant phenotype of their parent strains regarding cellular autolysis, lysozyme sensitivity, and decreased rhamnose contents in their cell walls (Fig. 1 and 2; Table 4).

The presence of significantly less rhamnose in the cells and wall fraction of all of the strains examined that possess a truncated form of the *psr* gene was a most striking observation. These differences in rhamnose contents were not due to differences in the contents of the cell wall, since cellular amino sugar contents, an indicator of wall peptidoglycan amount, were not significantly different (Table 3). The presence of equivalent amounts of peptidoglycan in all strains examined was confirmed by the analysis of the amino sugar contents of cell wall and wall peptidoglycan fractions (Table 3). Furthermore, preliminary data obtained by B. de Jonge (6) failed to show significant differences in the high-performance liquid chromatography (HPLC) profiles of peptidoglycan fragments derived from walls of *psr*-containing and *psr*-defective strains, which was consistent with the absence of major differences in cell wall peptidoglycan structure.

The change in composition of the nonpeptidoglycan portion of the walls of the strains that possess a truncated *psr* gene is reminiscent of the shift in wall composition observed when cultures of *Bacillus subtilis* are starved for phosphate. Phosphate starvation of *B. subtilis* results in a shift from the presence of phosphate-rich teichoic acid in the walls to its replacement by a teichuronic acid (8). This shift in wall composition

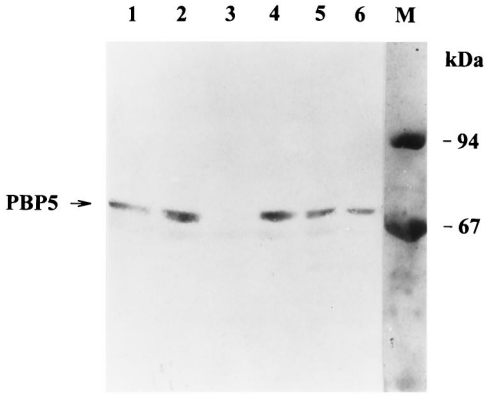

FIG. 5. Western blot analysis of PBP 5 in *E. hirae* ATCC 9790, R40, and Rev14 and some of their derived mutants. Samples of protoplast pellet (bacterial membranes), each equivalent to 1.25 ml of the original culture, were separated by SDS-PAGE and transferred to an Immobilon P membrane. The membrane was incubated with the polyclonal antiserum to PBP 5 as described in Materials and Methods. Lanes 1 to 6 are samples prepared from the ATCC 9790, R40, Rev14, R14-40, 9790-20, and 9790-40 strains, respectively. Lane M contains amido black-stained low-molecular-mass standards (Pharmacia Biotec, Uppsala, Sweden).

TABLE 3. The contents of methylpentose and amino sugars in cells and cell wall and peptidoglycan fractions of the ATCC 9790, R40, and Rev14 strains

Source	Methylpentose content (μmol/mg)[a]		Amino sugar content (μmol/mg)[a]	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
Cells				
ATCC 9790	0.25	0.29		
R40	0.10	0.16		
Rev14	0.12	0.18		
Cell wall fraction				
ATCC 9790	0.35	0.39	0.28	0.23
R40	0.15	0.21	0.29	0.25
Rev14	0.20	0.25	0.28	0.26
Peptidoglycan fraction				
ATCC 9790	<0.02	<0.02	0.37	0.40
R40	<0.02	<0.02	0.36	0.38
Rev14	<0.02	<0.02	0.35	0.43

[a] Bacteria were grown in chemically defined medium to an AOD of 860 for exponential-phase cells and for 4 h after the beginning of stationary phase.

was accompanied by changes in the binding of the *N*-acetylmuramic acid L-alanine amidase to the walls and susceptibility of the walls to hydrolysis by the autolytic amidase (21). Thus, a similar event could occur in *E. hirae*. That is, replacement of a rhamnose-containing polymer with one that lacks rhamnose or possesses a much lower amount of rhamnose could result in a change in affinity of the walls for one or both muramidases and, therefore, in changes in the rates of autolysis of exponential- and stationary-phase cells and in lysozyme sensitivity.

Thus, unpublished observations concerning the wall polysaccharide composition of *E. hirae* which were made by Reiko Kariyama in this laboratory several years ago may be relevant. She obtained data that indicated the presence of two rhamnose-containing polymers covalently linked to the peptidoglycan of walls of *E. hirae*. One polymer is a neutral polysaccharide containing rhamnose and glucose in a molar ratio of approximately four to one. The other polymer is a phosphate-containing, complex teichoic acid containing phosphorus, ribitol, *N*-acetylgalactosamine, glucose, and rhamnose in a molar ratio of approximately 1:1:1.5:4:1. At this time, we do not know if the *psr* deletion is accompanied by smaller amounts of both rhamnose-containing polymers or by less of one or the other polysaccharide. A possible interpretation is that the strains from which *psr* is deleted possess less of the neutral polysaccharide, which contains a large amount of rhamnose, and that the neutral polysaccharide is replaced by the teichoic acid, which contains a much smaller amount of rhamnose.

TABLE 4. Methylpentose contents of cells of several strains of *E. hirae*

Strain	Inhibitory concn of Pen G (μg/ml)[a]	Methylpentose content (μmol/mg [dry wt] of cells)[b]	
		AOD of 860	AOD of 3,670
ATCC 9790	10	0.31	0.32
Mutant 9790-20	40	0.24	0.24
Mutant 9790-40	>40	0.30	0.29
Mutant 9790-40-1	>40	0.30	0.29
Mutant 9790-40-2	>40	0.28	0.36
Mutant 9790-40-3	>40	0.28	0.38
R40	>40	0.15	0.19
Rev14	0.015	0.18	0.21
Mutant R14-4	8	0.23	0.25
Mutant R14-10	40	0.20	0.26
Mutant R14-20	>40	0.16	0.24
Mutant R14-40	>40	0.19	0.24

[a] Inhibitory concentration as determined by complete inhibition of growth on Todd-Hewitt agar plates.
[b] Bacteria were grown in S broth.

We considered the possibility that the decrease in the rhamnose contents of the walls of strains that contained a truncated *psr* is primarily responsible for the observed multiple phenotypic changes. In view of data from several laboratories that indicate that changes in the nonpeptidoglycan portion of walls of gram-positive species can alter their rates of hydrolysis by peptidoglycan hydrolase activities (see reference 26 for a recent review), this concept remains viable. However, the data showing that the walls of stationary-phase cells of all of the strains examined possess approximately the same amount of rhamnose as do the walls of exponential-phase cells are not completely consistent with this hypothesis. Thus, it is possible that *psr* is a component of some stationary-phase, global regulatory system that independently regulates several different wall-related properties. Whether the changes in lytic phenotype are directly related to wall rhamnose content, the data presented strongly indicate that the *psr* genetic element is somehow involved in the regulation of more than one cell wall-related property in addition to, and unrelated to, its role in regulating PBP 5 synthesis. There is considerable derived amino acid sequence similarity (31.5% identity, 43.8% similarity) between *psr* and the *lytR* sequence of *B. subtilis* (17, 20). *lytR* has been reported to be part of a divergon containing the structural genes for an *N*-acetylmuramoyl-L-alanine-amidase and its modifier and is considered to act as an attenuator of expression of the entire divergon. Thus, it would be of considerable interest to investigate the nucleotide sequences of the *E. hirae* chromosome before *psr* and after the PBP 5 gene.

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