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High salt stress in *Bacillus subtilis*: involvement of PBP4* as a peptidoglycan hydrolase

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Abstract

The study was focused on the role of the penicillin binding protein PBP4* of *Bacillus subtilis* during growth in high salinity rich media. Using *pbpE-lacZ* fusion, we found that transcription of the *pbpE* gene is induced in stationary phase and by increased salinity. This increase was also corroborated at the translation level for PBP4* by western blot. Furthermore, we showed that a strain harboring gene disruption in the structural gene (*pbpE*) for the PBP4* endopeptidase resulted in a salt-sensitive phenotype and increased sensitivity to cell envelope active antibiotics (vancomycin, penicillin and bacitracin). Since the *pbpE* gene seems to be part of a two-gene operon with *racX*, a *racX::pRV300* mutant was obtained. This mutant behaved like the wild-type strain with respect to high salt. Electron microscopy showed that high salt and mutation of *pbpE* resulted in cell wall defects. Whole cells or purified peptidoglycan from WT cultures grown in high salt medium showed increased autolysis and susceptibility to mutanolysin. We demonstrate through zymogram analysis that PBP4* has murein hydrolyze activity. All these results support the hypothesis that peptidoglycan is modified in response to high salt and that PBP4* contributes to this modification.

Keywords: *Bacillus subtilis*; High salt; Penicillin binding protein (PBP); *pbpE*; Peptidoglycan; Muramidase

1. Introduction

Bacillus subtilis is a Gram-positive sporulating bacterium able to adapt to wide variations in osmotic and saline strength. We have shown that the osmotic response in this bacterium is mainly a stationary phase-regulated event that shares common regulators with the sporulation process, and is dependent on DegU-P [13,28,30]. Cellular adaptation reactions to prolonged growth at high salinity (range 1–1.5 M NaCl) involved immediate and transient induction of the SigW (an extracytoplasmic sigma factor) regulon following salt shock and strong induction of the SigB regulon [7,8]. Initial adaptation to salt shock and continuous growth at high salinity share only a limited set of induced and repressed genes [33]. These two phases of adaptation require distinctively different physiological adaptation reactions by the *B. subtilis* cell. Prolonged growth at high salinity involved the DegS/DegU two-component system in sensing high salinity. Repression of chemotaxis and motility genes by high salinity [33] as well as the absence of proteases [30] was observed. In our laboratory, we have also found that during growth in hypersaline medium, *B. subtilis* cells develop an asymmetric septum similar to that developed by cells in sporulating stage II. However, in these cells, the sporulation process is inhibited [28,29].

The biochemical and biophysical characteristics of the cellular envelopes of cells adapted to prolonged growth at high salinity from both *B. subtilis* and *Lactobacillus casei* have also been analyzed and great differences have been revealed when compared to controls [15–19,24]. However, those studies essentially focused on membranes. Increased sensitivity of cultures grown in saline media to lysozyme and antibiotics or phages whose target is the wall structure suggests that modifications of the wall may also take place [15,25].

In Gram-positive bacteria, the cell wall consists of several distinctive structures. The murein layer is a rigid, shape-determining structure. It also contains equal masses of

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polysaccharides and peptides and can be classified as peptidoglycan [6]. Peptidoglycan is the essential structural element that provides shape and stability to most bacterial cells. Additionally, the cell wall contains teichoic acids associated with peptidoglycan and lipoteichoic acids associated with the membrane. The synthesis of peptidoglycan is mediated by penicillin binding proteins (PBPs). The high molecular weight PBPs are implicated in transglycosylation and basal peptidoglycan layer synthesis, whereas the low molecular weight (LMW) PBPs are usually monofunctional D,D-endopeptidases that regulate the number of peptide cross-links formed between the peptidoglycan strands. The coding genes of these PBPs are redundant, as deduced from the viability observed with multiple mutants of these genes [27]. PBPs might be important in the adaptation to environmental stress. Claessen et al. [4] have recently shown a role of GpsB (*ypsB*) (a salt-induced protein) in guiding the major transglycosylase/transpeptidase PBP1 to the cell pole. In fact, *gpsB* mutants were shown to have a salt-sensitive phenotype.

pbpE has been shown to be induced in several situations involving structural changes in the envelope that are essential for growth in the presence of environmental stresses like antibiotics [3,11]. This gene is also induced in a mutant defective for a membrane-bound protease such as *ftsH* [38]. However, to date, there is no clear role for this operon (*pbpE-racX)* or protein products. The product encoded by *racX* shares high homology with the amino acid racemase family (EC 5.1.1.-), having higher identity with aspartate/glutamate/alanine racemase. A supply of D-amino acids is essential for peptidoglycan synthesis; moreover, D-Ala is the main constituent of wall teichoic acids and lipoteichoic acids, which are polyanionic polymers exclusively found in Gram-positive bacteria [37]. The protein encoded by the *pbpE* gene of *B. subtilis* (PBP4*) belongs to the LMW-type class C PBPs and is similar to *Escherichia coli* PBP4, which has been reported to possess D,D-endopeptidase activity capable of cleaving peptidoglycan-peptide cross-links [12]. The exact enzymatic function of these enzymes is generally not determined, except in a few cases [31]. This activity has been proposed to be involved in the insertion of new peptidoglycan strands into already existing peptidoglycan. However, no phenotypic effect is accompanied by loss of its activity in *E. coli* or *B. subtilis*. In the former species, neither during vegetative growth nor during sporulation does the absence of PBP4* result in a significant phenotypic defect [5]. In the Subtilist Database, PBP4* is annotated as a spore-cortex-specific protein by sequence homology assignation; however, no true function has yet been assigned. Its activity is detected during stationary phase and it responds to AbrB and sigma W [3,11,34,38]. AbrB is involved in hyperosmotic stress adaptation [28] and the SigW regulon is induced by high salt [23].

This study focuses on the role of PBP4* of *B. subtilis* during growth in high salinity rich media and investigates the role of the *pbpE-racX* operon and protein products in high salt adaptation, tested as prolonged growth at high salinity.

2. Materials and methods

2.1. Strains

The *B. subtilis* strains used in this study were all derivative and isogenic strains of the wild-type (WT) 168 (*trpC2*). PS1805 (*pbpE::Erm*) [26] was originally constructed in this genetic background and checked for the integrated erythromycin cassette by PCR. *pbpE-lacZ* from strain PS1772 was constructed by D. Popham (personal communication) exactly in the same way as PS1845 [26] and moved into the 168 background by transformation. *pbpE* is the first gene in a two-gene operon with the second gene being *racX* (originally termed *orf2*) of unknown function and sharing high homology with the amino acid racemase family (EC 5.1.1.-). A deletion mutant of this gene was obtained using the SOEING technique [10] and by generating a ClaI site within the *racX* gene. The primers used were RX1 5′GAA*AAGCTT*TATCCTCTATGAGG3′, RX2 5′CG*TTCGAA*TCTATAATCGGAATC3′, XfwCla 5′GC GATT*ATCGAT*CGGAGC3′, XrevCla 5’CGCTCCG*ATCGAT*A ATCGC3′. Restriction sites are italicized. The PCR amplicon was cloned in TOPO TA cloning (Invitrogen). A HindIII-ClaI 247 nt fragment corresponding to the 5′ end of the *racX* gene was transferred to a pRV300 vector that presented compatible sites in the MCS [14]. A disruption mutant *racX::pRV300* was generated by integration upon transformation and erythromycin selection. Further validation by PCR amplification with Fermentas Dream Taq polymerase and restriction mapping confirmed the construct (data not shown).

2.2. Media and assays

Cultures were carried out in Schaeffer sporulation (D) medium [32]. Media containing NaCl were noted DN and the concentration indicated. When sporulation-repressing medium was needed, 1% (w/v) glucose was added (DG). Solid media contained 1.5% (w/v) agar.

2.3. Expression of pbpE-LacZ fusion

ß-galactosidase activity was determined as described by Miller [22] following the procedure described in Ref. [29].

2.4. Sensitivity of cultures to autolysis

Growing cultures in D and DN media were centrifuged, suspended in water and incubated at 37 °C. Relative changes in optical density (OD) at 550 nm were reported. The result shown in Fig. 5A is one of three independent experiments.

2.5. Wall isolation, fractionation and modifications

Early stationary phase cultures were harvested by centrifugation and washed with water at room temperature. The cells were resuspended in 1/10 volume of water. Purified peptidoglycan was prepared as described in Ref. [25]

2.6. Susceptibility of peptidoglycan fractions to mutanolysin

Purified peptidoglycan fractions were resuspended at an OD of 1 at 550 nm in 100 mM Tris–HCl buffer pH 8.5; mutanolysin (Sigma Chemical Co., St. Louis, USA) was added at 25 U/ml final concentrations and samples were incubated at 37 °C with constant rotation to keep the contents in suspension. OD at 550 nm was measured throughout the experiment and results are expressed relative to the initial OD. Result (in Fig. 5B) is one of three independent experiments.

2.7. Disk diffusion assays of antibiotic sensitivity

The assay was performed on solid media as described in Ref. [21]. The diameters of the inhibition zones were measured using Fuji software Image Gauge 3.122 (Fuji Film, Tokyo, Japan) from the images obtained. Three assays were performed with independent cultures of each strain and three diameters measured for each inhibition zone. Standard deviation was calculated.

2.8. MIC determination

The broth dilution assay was performed in a 1.5 ml final volume of D or DN media as described in Ref. [25]. Serially diluted antibiotics vancomycin (0.15–0.5 μg ml^{-1}), bacitracin (0.1–0.7 mg ml^{-1}) and penicillin G (10–150 μg ml^{-1}) either with or without 1 M NaCl were inoculated with 10^5 CFU of each strain. After incubation at 37 °C for 18 h with aeration, OD_{600nm} was scored. MICs were calculated as the lowest concentration at which growth was inhibited. Testing was performed in duplicate for each drug medium combination studied. The mean and standard deviations of three independent experiments are shown.

2.9. Electron microscopy

Samples for transmission electron microscopy (TEM) were prepared according to Ref. [25] from cultures grown until early stationary phase.

2.10. Detection of PBP4* from bacterial membrane preparations

Cultures in D and DN media were collected at early stationary phase, centrifuged and resuspended in 50 mM NaH_2PO_4 buffer (pH 6.8) and 1 mM PMSF. The membrane was prepared by cell disruption as described in Ref. [25]. The protein concentration of each membrane sample was estimated using the Bio-Rad protein assay with bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, VA, USA).

2.11. Western blot experiments

Samples were prepared for sodium dodecyl sulfate (SDS)-PAGE and immunoblot analysis after electrotransfer to parablot PVDF according to the manufacturer's instructions (Macherey-Nagel, Germany). Polyclonal antibody anti-PBP4*, kindly provided by W. Schumann, was used at 1:10,000 dilution. Biotin-conjugated anti-rabbit was detected by chemiluminescence using CDP-Star (GE-Biosciences).

2.12. Zymogram analysis

Protein samples (5–10 μg) were boiled for 3 min in Laemmli sample buffer and loaded onto 12% (w/v) polyacrylamide gels containing 0.1% (w/v) *Salmonella newport* peptidoglycan prepared according to Ref. [2], since this was evaluated as the best peptidoglycan substrate for the assay. Gels were cast, but with only 0.01% SDS. After running, the gel was incubated at 37 °C for 16 h in 1% Triton X-100, 25 mM Tris–HCl pH 8.5; washed once in water, stained for 3 h with 0.5% methylene blue in 0.01% KOH and destained with water. Peptidoglycan hydrolase activity was detected as a clear zone in a blue background of stained peptidoglycan. Gels not containing peptidoglycan were stained with Coomassie Brilliant Blue. The lysozyme used as control was purchased from Sigma.

3. Results

3.1. Transcription analysis of pbpE

The transcriptional activity of *pbpE-lacZ* fusion (constructed by Campbell recombination [26]; retained WT phenotype for *pbpE*) was analyzed during the entire growth phase in either the presence or absence of high NaCl concentrations. The strain showed a fivefold transient increase in transcriptional activity during the stationary phase and a long-lasting level of induction in high salt medium (25-fold induction) (Fig. 1). Expression in

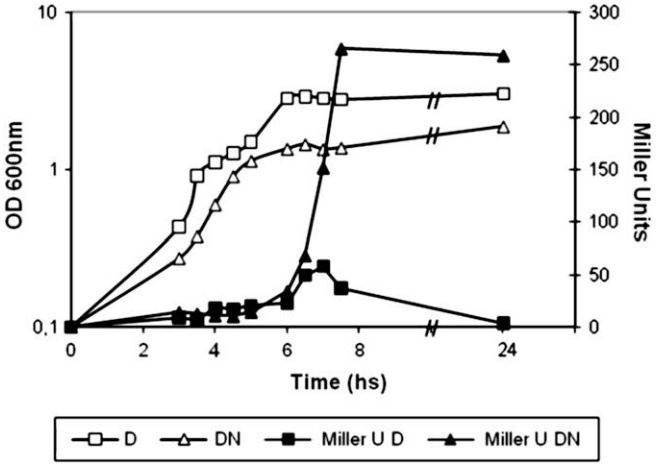

Fig. 1. Expression of *pbpE-lacZ* fusion either with or without NaCl. Strain was grown in D or DN medium with aeration at 37 °C and samples taken for determination of ß-galactosidase specific activity as described in Section 2. Filled squares (■ ß-galactosidase-specific activity) and empty squares (□ OD 600 nm) for the D condition. Filled triangles (▲ ß-galactosidase-specific activity) and empty triangles (△ OD 600 nm) for the DN condition (1.2 M NaCl).

high salt medium persisted for 24 h of incubation (data not shown). In order to determine whether this effect was due to osmolarity or to sporulation, cultures in sporulation-repressing media (presence of 1% glucose) were analyzed. The same upper level of induction by the presence of NaCl was observed, eliminating a sporulation effect (data not shown). In addition, increased expression was confirmed at the translational level by western blot analysis (Fig. 6B).

3.2. High salt-sensitive phenotype

In order to further investigate the role of *pbpE::Erm* and *racX::pRV300* in the high salt response, strains were cultivated in variable NaCl concentrations and compared to their WT controls. In contrast with the WT and *racX::pRV300* mutant strains, the *pbpE::Erm* mutant grew poorly at high NaCl concentrations (Fig. 2), showing a salt-sensitive phenotype. In fact, the ratio between growth in 1.5 M NaCl and the basal D medium was similar for WT and *racX* mutant strains (0.48 and 0.5), and significantly lower for the *pbpE* mutant (0.09). Therefore, we concluded that PBP4*, but not RacX, is important for adaptation to high salt.

As reported by Popham and Setlow [26], we did not observe any defect in sporulation or spore properties for *pbpE::Erm*, suggesting that PBP4* might play a role in adaptive stress responses such as high salt tolerance, and probably in other stresses such as alkaline, oxidative and cell envelope stressors, such as antibiotics, as was suggested in Ref. [39], but not in the sporulation process.

3.3. Whole cell analysis: sensitivity to antibiotics

As previously described, *pbpE* is part of the SigW regulon, one of the extracytoplasmic sigma factors (ECF) involved in the maintenance of cell envelope integrity and regulatory functions that contribute to the innate resistance of *B. subtilis* to antibacterial compounds [21]. To provide evidence of structural changes or modifications taking place in the cell wall during high salt adaptation involving the function of PBP4*, we analyzed the effect of antibiotics known to interact

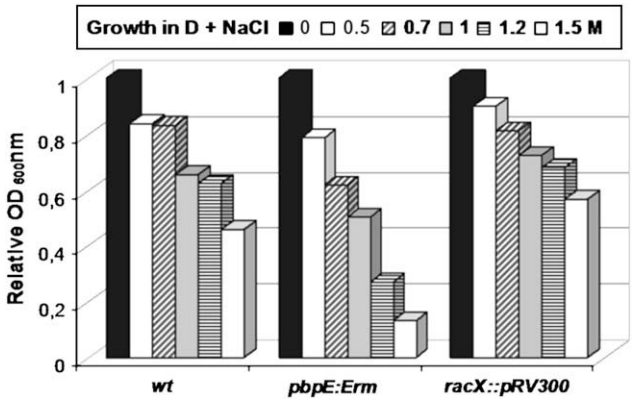

Fig. 2. Extent of growth in media either with or without NaCl. Concentrations of NaCl added to D medium are expressed on the top legend.

with peptidoglycan synthesis in the absence and presence of high salt stress. For this purpose, cultures from both the WT and mutant strains in media with or without NaCl (DN and D conditions) were assayed by disk diffusion assays (Fig. 3) and MIC determined (Table 1). WT was significantly more sensitive to vancomycin, bacitracin and penicillin G in DN medium than in D medium. The increase in the inhibition halo and decreased MIC observed suggested that peptidoglycan was less strongly cross-linked or that fewer peptidoglycan layers were present. *pbpE::Erm* mutant was significantly more sensitive than the WT to vancomycin, bacitracin and penicillin G in DN medium, and to vancomycin and penicillin G in D medium when compared to WT (Table 1 and Fig. 3). Vancomycin was chosen because it binds to the D-alanyl-D-alanine peptide terminus of the nascent peptidoglycan-lipid carrier normally incorporated into the nascent peptidoglycan polymer, and prevents full cross-linking by transpeptidases. The *pbpE::Erm* mutant's higher sensitivity under the D condition was an unexpected result (Table 1 and Fig. 3), since the mutant did not show any defective phenotype under this condition. This result is better understood by the vancomycin induction effect on sigma W and thus *pbpE*, which seems to magnify the difference between the WT and the *pbpE::Erm* mutant. Penicillin G and bacitracin were assayed in similar experiments. Pencillin G, an analog of D-ala-D-ala, acts as a structural inhibitor of the transpeptidation reaction. Therefore, the peptidoglycan structure of the WT strain in DN medium would be less strongly cross-linked; however, under this condition, the *pbpE::Erm* mutant was particularly sensitive to the antibiotic, probably due to a weak wall. Such increased sensitivity observed for the *pbpE::Erm* mutant in DN medium when compared to WT confirms the role of *pbpE* under high salt conditions. Bacitracin, an inducer of sigma M, another ECF involved in osmosensitivity [3,9,20], prevents the peptidoglycan monomers synthesized in the cytoplasm from being transported across the cytoplasmic membrane, thus leaving no building blocks available for peptidoglycan synthesis. The presence of bacitracin in our experiments resulted in increased sensitivity under DN conditions as compared to D conditions, for both strains (Table 1 and Fig. 3); this result may indicate that de novo synthesis and/or increased turnover of peptidoglycan are required for high salt adaptation.

3.4. TEM observation and sensitivity to lysis

In order to further investigate the modifications that took place in the cell wall, cultures grown in DN and D conditions were collected at the early stationary phase and observed in thin section by electron microscopy. Under this phase condition, less than 1% of spores were observed in D medium, whereas, as already reported, a few stage II–III prespores were observed in the DN condition [17]. TEM examination in D medium with a 100,000-fold magnification showed the typical trilaminar structure reported by Foster and Popham [5] with a more electron-dense inner layer, probably corresponding to peptidoglycan (Fig. 4). In DN medium, however, only two layers were observed and the structure was irregular,

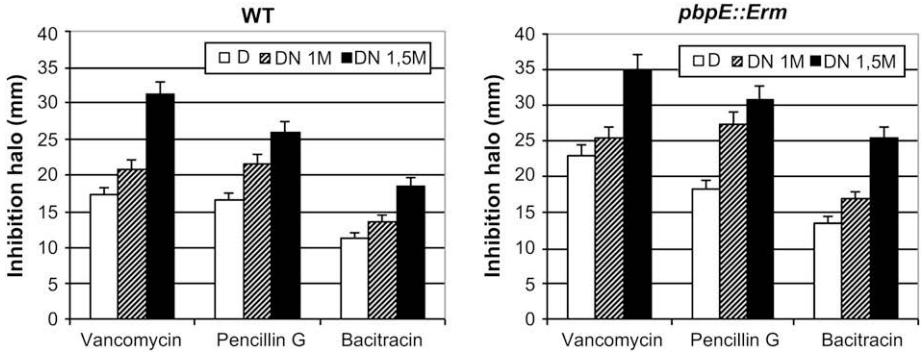

Fig. 3. Disk diffusion assays of antibiotic sensitivity. Each bar represents average zone of inhibition of at least three assays performed with independent cultures of each strain. Each filling pattern represents a different NaCl concentration as expressed in the footnote. The *y*-axis shows the zone of inhibition (in millimeters), expressed as total diameter.

showing a layer detached from the cytoplasmic membrane. The *pbpE::Erm* mutant strain showed a thinner wall under both conditions (29 ± 3 nm for WT vs. 23 ± 3 nm for *pbpE::Erm*). In addition, whole cells from *B. subtilis* cultures presented higher autolysis when cultured in DN rather than in D medium (Fig. 5A). The mutant strain under both conditions was more resistant to autolysis. In order to evaluate the contribution of peptidoglycan itself to lysis, peptidoglycan preparations devoid of additional polymers, as indicated in Section 2, were subjected to mutanolysin treatment. Two-slope kinetics was observed with the most sensitive fraction (first slope), representing up to 60% in DN, while this was only 35% in D. The peptidoglycan of the mutant was more sensitive to mutanolysin than the WT (Fig. 5B). Thus, the peptidoglycan structure was different in all cases.

3.5. Analysis of the PBP4* function

Our results indicate that the peptidoglycan structure was modified when cells grew in high salt media. The rebuilding of this structure might require the activity of several autolysins (some of which have been shown to be regulated by osmotic stress) [7,33,35]. To this end, we sought to test the autolysin activity of these cultures. For that purpose, proteins were separated by SDS gel electrophoresis through a matrix containing peptidoglycan, renatured and allowed to degrade the peptidoglycan. When such zymograms were compared, an intense hydrolytic band corresponding to that of PBP4* migration was detected with the WT cultured in DN (Fig. 6C); this band was absent from the *pbpE* mutant under both conditions (D and DN) and ran as a 50 kDa protein, coincident with the western blot against PBP4* (Fig. 6B). Western blot analysis of the *pbpE* mutant strain gave no detectable band under either condition (D and DN). In addition, other peptidoglycan hydrolases were overinduced under DN conditions and these were also present in the *pbpE::Erm* mutant.

Table 1
MIC of D and DN cultures for antibiotics acting on peptidoglycan.

MIC[a]	WT		*pbpE::Erm*	
Antibiotic	D	DN	D	DN
Vancomycin (μg/ml)	0.59 ± 0.05	0.29 ± 0.02	0.35 ± 0.01	0.26 ± 0.01
Penicillin G (μg/ml)	105 ± 7	82 ± 3	110 ± 10	40 ± 10
Bacitracin (μg/ml)	650 ± 60	370 ± 20	650 ± 45	310 ± 15

Data are mean and standard deviation of three independent experiments.
DN = 1 M NaCl.
[a] Minimal inhibitory concentration.

4. Discussion

In the present work, we have shown that gene disruption in the structural gene (*pbpE*) for the PBP4* endopeptidase is sensitive to high salt (Fig. 2). Also, we showed through zymogram analysis (Fig. 6) that PBP4* has murein hydrolysis activity. Furthermore, using a *pbpE-lacZ* fusion (Fig. 1), we showed that transcription of the *pbpE* gene is induced in stationary phase by increased salinity.

Since *pbpE* is likely to be part of a two-gene operon with *racX*, a mutant of this second gene was constructed, but it did not show any particular deficiency in terms of growth in high salt (Fig. 2). *pbpE-racX* might not be a typical operon. In fact, Zellmeier et al. [38] reported that in a Δ*ftsH* mutant background, transcription of *racX* and *pbpE* genes showed a very different induction factor (9X and 2.5X, respectively) which was not compatible with an operon. This might imply the existence of a not yet described internal promoter independent of the *pbpE* gene and would need to be further investigated.

Transcription analysis of *pbpE-lacZ* fusion indicates that in sporulation D medium, low induction which quickly disappeared took place upon initiation of sporulation (T0); this did not seem to represent an activity linked to modifications of the spore peptidoglycan structure, but rather, a consequence of overall derepression mediated by the Spo0A/AbrB regulatory network taking place at this period of growth. In addition, induction in high salt media was independent of sporulation, since the addition of glucose, which normally represses sporulation, failed to diminish the induction of *pbpE*.

pbpE belongs to the *sigW* regulon controlling wall stress functions [3]. Recently, this gene has been shown to respond to alkaline stress [39]. The mechanism that induces the *sigW* regulon is unknown, but it has been shown that it is activated early in the stationary phase, and is strongly induced by the

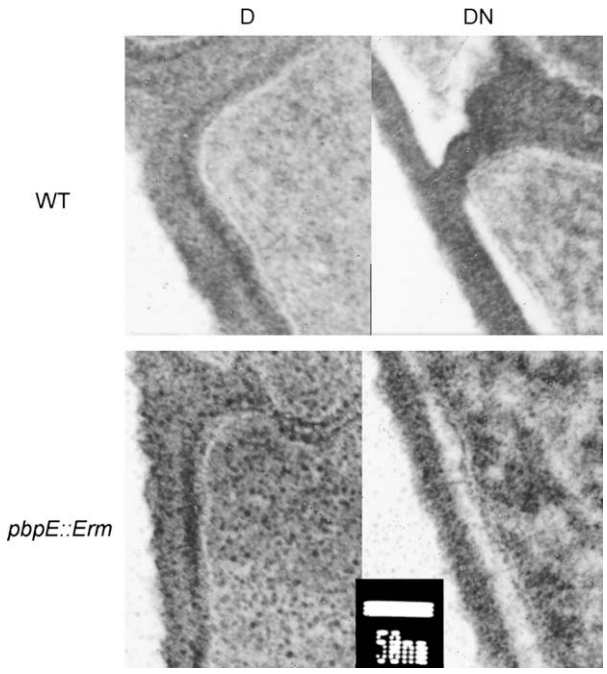

Fig. 4. Transmission electron microscopy (TEMs). TEM of thin sections of stationary phase cultures grown in D or DN medium containing 1.2 M NaCl. Magnification of micrographs on the left is 100,000× (white bars below 50 nm). Names of the strains are indicated.

sudden presence of alkali or salt [23] and by antibiotics such as vancomycin which inhibit peptidoglycan biosynthesis [3]. Accumulation of a sigma W-controlled protein like PBP4* has already been reported in a Δ*ftsH* mutant [38]. This induction is the consequence of SigW overexpression of this mutant. Whether other functions involved in envelope modifications observed during hyperosmotic adaptation (such as fatty acids and phospholipids [7,15,18]) are also sigma W-regulated remains to be determined.

TEM observation of cultures from the WT strain showed that growth in DN medium resulted in different cell walls (Fig. 4). This structure would also lead to the observed increased sensitivity to penicillin G and vancomycin (Table 1 and Fig. 3), suggesting that cross-linking might be modified. Also, DN cells showed a wall detached from the cytoplasmic membrane, indicating plasmolysis. This higher sensitivity to lysis was corroborated by the increased autolysis of DN cultures (Fig. 5A) and sensitivity to mutanolysin of peptidoglycan preparations (Fig. 5B). Overall, these results points to differences in peptidoglycan structure resulting from high salt stress and from the *pbpE::Erm* mutation, without eliminating the possibility that additional envelope modification might occur. The exact nature of such changes remains to be defined and is part of our future work.

The zymogram shown in Fig. 6C indicates that several peptidoglycan hydrolases were induced by salt in both strains. However, the band corresponding to PBP4* from DN cultures presented an upper hydrolase activity absent from the mutant. In this sense, it would be worthwhile to remember that LytF, the main autolysin, has been shown to be repressed by salts, while other putative hydrolases are induced [33]. In fact, autolysis of whole cells from the WT in DN conditions reached 60% after 24 h, while in D conditions it did not greatly progress from 8%.

B. subtilis contains several hydrolases, but none of them is indispensable [1]. This is also true for the PBPs. The redundancy of these activities points to the remarkable capacity of this bacterium to deal with different environmental conditions not necessarily observed in laboratory conditions. Sporulation, stationary phase and stress situations enable mimicking some of them. The high diversity of hydrolases and patterns of activities showed intricate regulation necessary for maintaining viability and growth [36].

Regarding its function, it is important to note that involvement of PBP4* appears late in osmotic adaptation; at

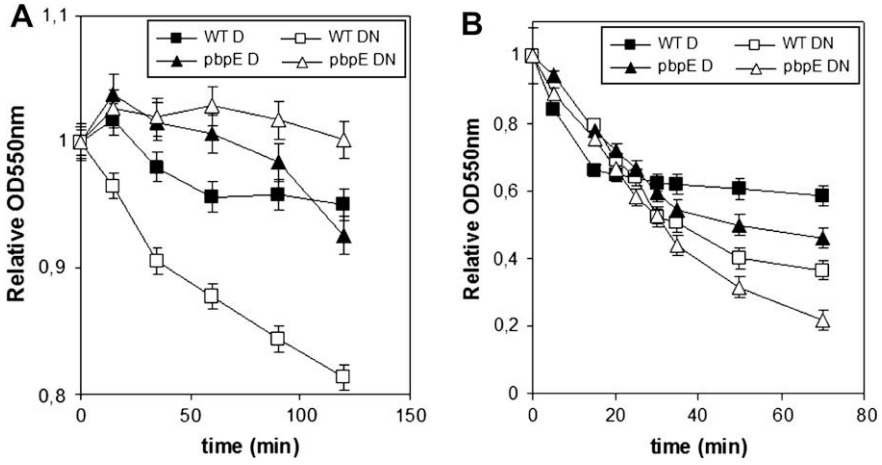

Fig. 5. Sensitivity to lysis. (A) Sensitivity of cultures to autolysis: growing cultures in D and DN media were centrifuged, suspended in water and incubated at 37 °C. Relative changes in OD at 550 nm are reported. (B) Sensitivity of purified peptidoglycan to mutanolysin: Peptidoglycan fractions obtained as described in Section 2 were resuspended at an OD of 1 at 550 nm in 100 mM Tris–HCl buffer pH 8.5 and mutanolysin added at 25 U/ml final concentrations. Full symbols: D condition; empty symbols: DN condition (1.2 M NaCl). Squares: WT and triangles PS1805 (*pbpE::Erm*).

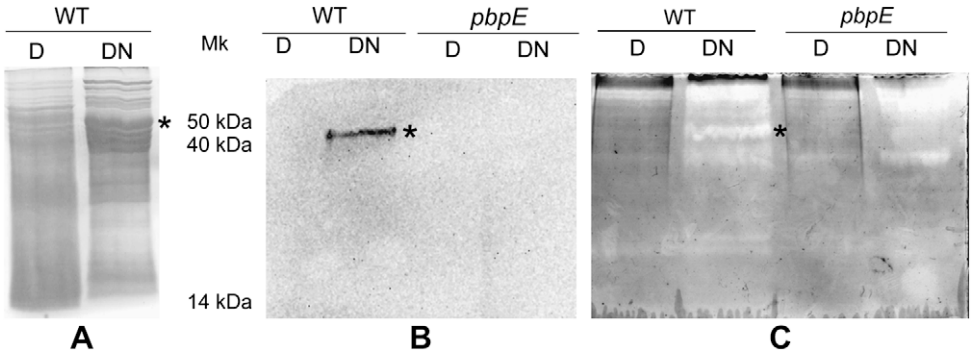

Fig. 6. Western blot and zymogram analysis. See details in Section 2. (A) SDS-PAGE from membrane preparations and Coomassie blue stain; (B) western blot; (C) zymogram detection; * indicates position of PBP4* protein. DN = 1.2 M NaCl.

that point, several envelope components like phospholipids, fatty acids [16–18] and perhaps teichoic acids have already been modified. Peptidoglycan synthesis seems to require active substrate recycling, as suggested by the increased sensitivity to bacitracin under DN conditions. Only then is PBP4* necessary (and induced) and its activity required in order to rebuild peptidoglycan according to the new situation.

Precise structural modifications of the cell wall (peptidoglycan and associated polymers) that takes place during hyperosmotic stress and the specific murein hydrolase activities of PBP4* will be the aim of our future investigations.

Acknowledgments

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