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Characterization of the *Bacillus subtilis* Penicillin-Binding Protein PBP4*

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Abstract

Purpose: The PBP4* is a Penicillin Binding Protein belonging to the class C of AmpH type whose function remains poorly understood. This study aimed to evaluate the biophysical and enzymatic properties of the *Bacillus subtilis* PBP4* to gain insights into its role in the context of bacterial cell wall recycling. **Methods:** To characterize the PBP4*, the full-length PBP4* and its N-terminal penicillin-binding domain have been produced in *Escherichia coli* and purified. **Results:** A comparison of biophysical properties has shown that both recombinant proteins are monomeric in solution and retain the same thermal stability. On the other hand, the D-alanine methyl esterase activity detected with the full-length PBP4* is impeded by the cleavage of the 92 amino acid C-terminal domain. The esterase activity of the full-length PBP4* demonstrates a clear D-stereospecificity. The PBP4* is also active on *B. subtilis* cell walls bearing teichoic acids, compounds commonly substituted with D-alanine residues. **Conclusions**: Our results are in agreement with the hypothesis that PBP4* could play a role in recycling cell wall components, as previously suggested.

Keywords

B. subtilis PBP4*, Class-C PBP, D-Stereospecific Esterase

1. Introduction

The genome of *Bacillus subtilis* contains many genes coding for Penicillin-Binding Proteins (PBPs) including *pbpE* which is translated into PBP4*, a protein of 451 amino acids (NCBI accession number: CCD10845). The PBP4* belongs to the class C of AmpH type and its function and cellular role remain poorly understood. This PBP is composed of a N-terminal domain which har-

bors the three highly conserved motifs characteristic of PBPs ($S^{61}LSK^{64}$, $Y^{153}SN^{155}$ and $H^{302}SG^{304}$) and is related to the *Ochrobactrum anthropi* D-aminopeptidase. The 92 amino acid C-terminal domain (CTD) consists of a predicted lipocalin-like fold of unknown function. At downstream of *pbpE*, there is a second gene named *racX* since its product presents sequence similarity with aspartate or glutamate racemases. The two genes constitute an operon and are parts of the σ^W regulon. Popham and Setlow [1] have purified multiple PBPs from *B. subtilis* membranes by penicillin affinity chromatography. The N-terminal sequence of an apparent 56 kDa protein was identified as the beginning of the *pbpE* ORF, indicating that its product, named PBP4*, is not processed. This observation is in agreement with the absence of a predicted signal peptide in the PBP4* sequence. The purification method indicates that the PBP4* is associated with the cytoplasmic membrane. The *pbpE* gene is weakly expressed during the phase of vegetative growth; however its expression is induced upon entrance into the phase of stationary growth [1]. Partial or complete deletion of the *pbpE-racX* operon neither resulted in a particular phenotype (at least in growth on rich or minimal media) nor in differences in heat resistance and germination rates of spores, invalidating the hypothesis that PBP4* is a sporulation-specific gene [2]. Using a chromogenic cephalosporin, Popham and Setlow have detected a weak β-lactamase activity in a *B. subtilis* wild-type preparation in contrast to that from the *pbpE* mutant strain [1]. The authors also conducted D-aminopeptidase activity assays with D-alanine-*p*-nitroanilide substrate but obtained negative results. They proposed a role for PBP4* and RacX in the recycling of peptidoglycan components, a process especially important under starvation conditions [1].

The expression of *pbpE* is enhanced in response of *B. subtilis* to different stresses. The extracytoplasmic RNA polymerase σ^W factor activates genes involved in the antimicrobial resistance mechanisms, in the synthesis or secretion of bacteriocins and genes expressed in response to antibiotics that interfere with the biosynthesis and/or in proper functioning of the cell wall (e.g. vancomycin, cephalosporin and D-cycloserine). A role in detoxifying the cell has been proposed for the genes of the *yceC* operon, *ybfO*, *ydjP*, *yfhM* and *pbpE* [3] [4] [5] [6]. Experiments aiming to identify *B. subtilis* genes induced by a sudden increase in the external pH (from pH 6.3 to 8.9) revealed the induction of more than 80 genes including *pbpE* and *racX* [7] [8]. The PBP4* is also listed among the proteins induced by ammonium starvation, mainly during the transition to the phase of stationary growth [9]. Other studies [10] [11] have shown that *pbpE* was overexpressed in response to high salt stress, suggesting that PBP4* could play a role in the cell wall rearrangements that take place during the osmoadaptation of *B. subtilis*. Interestingly, a zymogram analysis demonstrated that PBP4* has a hydrolysis activity on cell walls [11]. Furthermore, a strain in which *pbpE* has been disrupted presents a salt-sensitive phenotype and an increased sensitivity to cell envelope active antibiotics (vancomycin, penicillin and bacitracin) [11]. However, neither precise structural modifications of the cell wall are elucidated nor is the specific contribution of PBP4* to these modifications.

B. subtilis overproducing PBP4* has a filamentous phenotype. In the absence of the FtsH metalloprotease, an upregulation of the whole σ^W regulon is observed, as well as an enhanced transcription of *pbpE*, causing an accumulation of PBP4* in *B. subtilis.* The inactivation of both *ftsH* and *pbpE* restored the wild-type cell morphology, indicating that the accumulation of PBP4* is responsible for the filamentous phenotype [12]. The way this phenotype is induced by PBP4* remains unclear.

The localization of the Green Fluorescent Protein fused to PBP4* has been monitored [13]. During the phase of vegetative growth, GFP-PBP4* localized in a punctuated pattern along the bacterial rod, similar to those observed with most PBPs [14].

To gain knowledge on the *B. subtilis* PBP4* role, we have purified the full-length protein and its N-terminal domain (PBP4*ΔCTD) and performed biophysical and enzymatic characterizations.

2. Material and Methods

2.1. Cloning Strategies and Plasmids

The pJet1.2/blunt vector (Thermo Scientific) was used to clone the fragments amplified by PCR and to verify their sequences (GIGA sequencing platform, University of Liège, Belgium). The pET28-MHL plasmid (Addgene) was used as expression vector to produce the entire PBP4* or its N-terminal domain (PBP4* ΔCTD) in fusion with a N-terminal 18 amino acid peptide containing six histidine residues followed by a TEV protease cleavage site. In the pET28-MHL plasmid, the translation initiation codon is present in an NdeI restriction site (CATATG). Silent mutations were introduced in *pbpE* to suppress two internal NdeI restriction sites using the following strategy: three fragments of *pbpE* were amplified from the *B. subtilis* ATCC21332 genomic DNA with the Q5 High Fidelity DNA polymerase (Bioke). The first fragment (5' portion of the gene) was obtained with the primers pbpE_frag1_fw and pbpE_frag1_mut1_rev. The second fragment corresponding to the central part of the gene was amplified with the primers pbpE_frag2_mut1_fw and pbpE_frag2_mut2_rev and the third fragment (3' portion of the gene) was obtained with the oligonucleotides pbpE_frag3_mut2_fw and pbpE_frag3_rev. The external primers pbpE_frag1_fw and pbpE_frag3_rev contain an NdeI or XhoI restriction site respectively. The internal oligonucleotides form two pairs of primers with complementary sequences and a single modified nucleotide in the NdeI restriction sites. A final PCR using the mixture of the three fragments as templates and the primers pbpE_frag1_fw and pbpE_frag3_rev allowed to amplify the entire *pbpE* gene without internal NdeI restriction sites. The 1.4 kb PCR product was cloned into the pJet1.2/blunt vector and the insert sequence was completely verified. The modified *pbpE* gene was cloned into pET28-MHL yielding the pET28-MHL-pbpE plasmid. The PBP4* ΔCTD coding sequence (comprising the first 359 residues) was amplified from the pET28-MHL-pbpE plasmid with the pbpENterm-fw and pbpENterm-rev primers. After verification of its sequence, the fragment was

cloned into the pET28-MHL vector yielding the pET28-pbpENterm plasmid. The sequences of the primers are reported in (Table 1).

2.2. Production of PBP4* and PBP4* ΔCTD

E. coli Nico21 (DE3) competent cells (New England Biolabs) were transformed with the pET28-MHL-pbpE or pET28-MHL-pbpENterm plasmids. Bacterial cells were grown in two litres of Terrific Broth medium at 37˚C until an OD_{600nm} of 1.2 and induced with 1 mM IPTG. After 4 hours at 37˚C, the cells were collected by centrifugation at 7000 g for 20 min. The pellet was resuspended in 80 mL of 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM PMSF. After addition of 200 U benzonase (Novagen), the cells were broken in a high-pressure homogenizer (Emulsiflex C3, Avestin Europe GmbH) and debris and intact cells were eliminated by centrifugation at 25,000 g for 30 min. The His-tagged PBP4* or PBP4* ΔCTD were purified using the Profinia™ Affinity Chromatography Protein Purification System (Biorad). The equilibration, washing and elution buffers were supplemented with 5, 10 and 250 mM imidazole respectively. The purified proteins were eluted in desalting buffer (25 mM HEPES pH 7.5, NaCl 100 mM) with a yield of 63 mg of full-length PBP4* and 50 mg of PBP4* ΔCTD.

2.3. Cleavage of the N-Terminal Sequence with the TEV Protease

In order to cleave the N-terminal extension containing the polyhistidine sequence, the purified PBP4* and PBP4* ΔCTD were incubated overnight at 4˚C with TEV protease (TEV-recombinant proteins mass ratio 1:50) in 25 mM HEPES pH 7.5, 100 mM NaCl. The cleavage efficiency was verified by SDS-PAGE analysis and the mixture loaded on Ni-NTA agarose to retain the N-terminal peptide. A mass spectrometry measurement (CART-LSM GIGA University of Liège, Belgium) and the N-terminal sequencing by Edman degradation confirmed the complete

Table 1. Names and sequences of primers used for suppressing the *pbpE* internal NdeI restriction sites and for cloning the full-length PBP4* or PBP4* ΔCTD coding sequences into the pET28-MHL expression plasmid. The sequences in bold (CATATG and CTCGAG) correspond to a NdeI and XhoI restriction site respectively. The underlined nucleotides indicate the silent mutations introduced in internal NdeI restriction sites.

Primer name	Sequence (5' to 3')
pbpE_frag1_fw	GCG**CATATG**AAACAGAATAAAAGAAAGCATCTTCAGACA
pbpE_frag1_mut1_rev	GTACATCATACACATATC**CATAAG**CATAATGATCA
pbpE_frag2_mut1_fw	TGATCATTATG**CTTATG**GATATGTGTATGATGTAC
pbpE_frag2_mut2_rev	GGGACCT**CATAAG**GCTGGCCAAATA
pbpE_frag3_mut2_fw	TATTTGGCCAGC**CTTATG**AGGTCCC
pbpE_frag3_rev	ACAA**CTCGAG**ATTAATTTGTACGGACCGCTTCTTCTTCT
pbpENterm-fw	GCG**CATATG**AAACAGAATAAAAGAAAGCATCTTC
pbpENterm-rev	ACAA**CTCGAG**ATTAAGCAGGACGTTCCGGG

elimination of the N-terminal extension leaving only two additional amino acids (Gly and His) before the native sequence of the purified proteins (**Figure 1(a)**).

2.4. Thermal Denaturation Assay

Thermal denaturation analyses of PBP4* and its N-terminal domain (PBP4* ΔCTD) were performed by DSF (Differential Scanning Fluorimetry) using the Prometheus NT.48 (NanoTemper Technologies), a capillary-based instrument measuring microscale thermophoresis. The NanoDSF technology detects very small changes in the fluorescence of tryptophan and tyrosine present in the protein without needing any dye. Experiments were performed as following: protein samples were buffer exchanged against 25 mM HEPES pH 8.0, 100 mM NaCl using Micro Bio-Spin P6 columns (BioRad) and diluted at 30 µM in the same buffer. Ten µL of each sample were injected in a thin capillary (provided with the Prometheus NT48 system) and submitted to a 20˚C - 95˚C temperature gradient with a 1˚ min^{-1} increase rate. The first derivatives of the sigmoid curves provide fusion temperatures (Tm) of the proteins, corresponding to the denaturation midpoint. Experiments were performed 3 times for both proteins.

2.5. Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were carried out in 25 mM HEPES pH 8.0, 100 mM NaCl using a DynaPro NanoStar™ (Wyatt Technologies, 100 mW He-Ne laser, λ_0 = 658 nm, θ = 90˚) spectrophotometer. DLS experiments were performed at 15˚C in disposable Uvette™ cells (Eppendorf) filled with 50 µL of PBP4* or PBP4* ΔCTD protein solution at 0.5 or 1.5 mg mL^{-1}. Measurements, corresponding to 10 readings of 5 seconds, were taken three times with a pause of 5 minutes between them to provide means and square deviations of the parameters. Scattering intensities were analyzed using the Dynamics software (Wyatt Technologies) to calculate the hydrodynamic diameters (Dh), the polydispersity indexes and to estimate the molecular weights.

2.6. Size-Exclusion Chromatography

Proteins were loaded on a Superdex S200 10/300 column (GE Healthcare) pre-equilibrated in 25 mM HEPES pH 8, 100 mM NaCl to determine their elution volumes and calculate their apparent masses based on a calibration curve (BioRad).

2.7. Enzyme Assays

2.7.1. DD-Carboxypeptidase Activity

DD-carboxypeptidase activity was assayed with benzoyl-D-alanyl thioacetyl ester (S2d) as substrate [15]. A sample (150 µL) containing 3 µg PBP4*, 1 mM S2d and 1 mM DTNB in 50 mM HEPES pH 7 and a blank without enzyme were incubated at room temperature for 20 min. The absorbance values were monitored at 412 nm.

2.7.2. Aminopeptidase Activity

Aminopeptidase activity was tested on L or D-amino acids fused to a para(*p*)n-

itroanilide (NA) group. L- and D-Ala-*p*NA, L- and D-Glu-*p*NA, L-Met-*p*NA, L- and D-Phe-*p*NA and L-Pro-*p*NA (Sigma-Aldrich) have been used. Samples (100 µL) containing 500 µM of putative substrate and 50 µg PBP4* in 25 mM HEPES pH 7.5, 100 mM NaCl were incubated at 37˚C for 2 hours. A blank without enzyme allows to determine if the yellow colour is due to the aminopeptidase activity of PBP4*.

2.7.3. Esterase Activity

Esterase activity was tested on D-Alanine methyl ester (D-Ala OMe) hydrochloride (Bachem) or DL-Alanine methyl ester hydrochloride (Sigma-Aldrich) as substrates. The assays were all performed at 24˚C in 50 mM HEPES pH 7.5, 5 mM $MgCl_2$. In these conditions of temperature and pH, the spontaneous hydrolysis of the substrate is less than 2%. Samples (40 µL) containing 1 - 7 mM D-Ala OMe and 4.65 µM full-length PBP4* or the substrate alone were incubated for 15 - 30 min and then transferred on ice. The amounts of D-Alanine resulting from the enzymatic activity or from the substrate instability were measured using the D-Amino Acid Oxidase (DAAO) method [16]. Absorbance values of standards containing 0 - 30 nmoles D-Alanine yielded a straight line that allowed to calculate the esterase activity associated with PBP4*.

The initial rates (v_0) were fitted to the Henri-Michaelis-Menten equation using a nonlinear regression analysis with the help of the GraphPad Prism 5.04 software. The kinetic constants were also determined with the Hanes plot in which the S/v_0 ratios are plotted *versus* the substrate [S] concentrations, which gave a straight line that allowed to calculate the K_m and K_m/V_{max} ratio.

2.7.4. Determination of the Substrate Stereospecificity of PBP4*

Samples (40 µL) containing 4.65 µM of full-length PBP4* and 20 mM of racemic DL-Ala OMe substrate or the substrate alone were prepared in duplicates, incubated for 30 min and then transferred on ice. After addition of a second reaction mixture containing either 50 U DAAO or 12 U LAAO (L-Amino Acid Oxidase from the *Crotalus adamanteus* venom, Sigma-Aldrich), the samples were incubated at 37˚C for 15 or 30 min respectively. Absorbance values of standards containing 0 - 40 nmoles L-Alanine fit to a straight line in the LAAO test.

2.7.5. Enzyme Assay on *Bacillus subtilis* Cell Walls

Suspensions (2 × 100 µL at 10mg mL^{-1}) of cell walls bearing teichoic acids were rinsed three times with 50 mM HEPES pH 7.5 and resuspended in the same buffer at the initial concentration. One sample constituted a blank while the other one was supplemented with 10 µg PBP4*. Both samples were incubated at 24˚C for 16 hours. After centrifugation (at 14,000 g for 10 min) the possible release of D-alanine residues in the supernatants was quantified using the DAAO method.

3. Results and Discussion

The *B. subtilis* PBP4* produced in *E. coli* as a recombinant protein of 453 resi-

dues or its N-terminal domain (PBP4* ΔCTD) lacking the 92 last residues (Figure 1(a)—lanes II and IV) were used in all experiments.

3.1. Physical Characterizations of the Full-Length PBP4* and Its N-Terminal Domain PBP4* ΔCTD

The thermal denaturation curves indicated that the T_m temperatures of PBP4* and PBP4* ΔCTD are 59˚C ± 0.3˚C and 60˚C ± 0.2˚C respectively (Figure 1(b)).

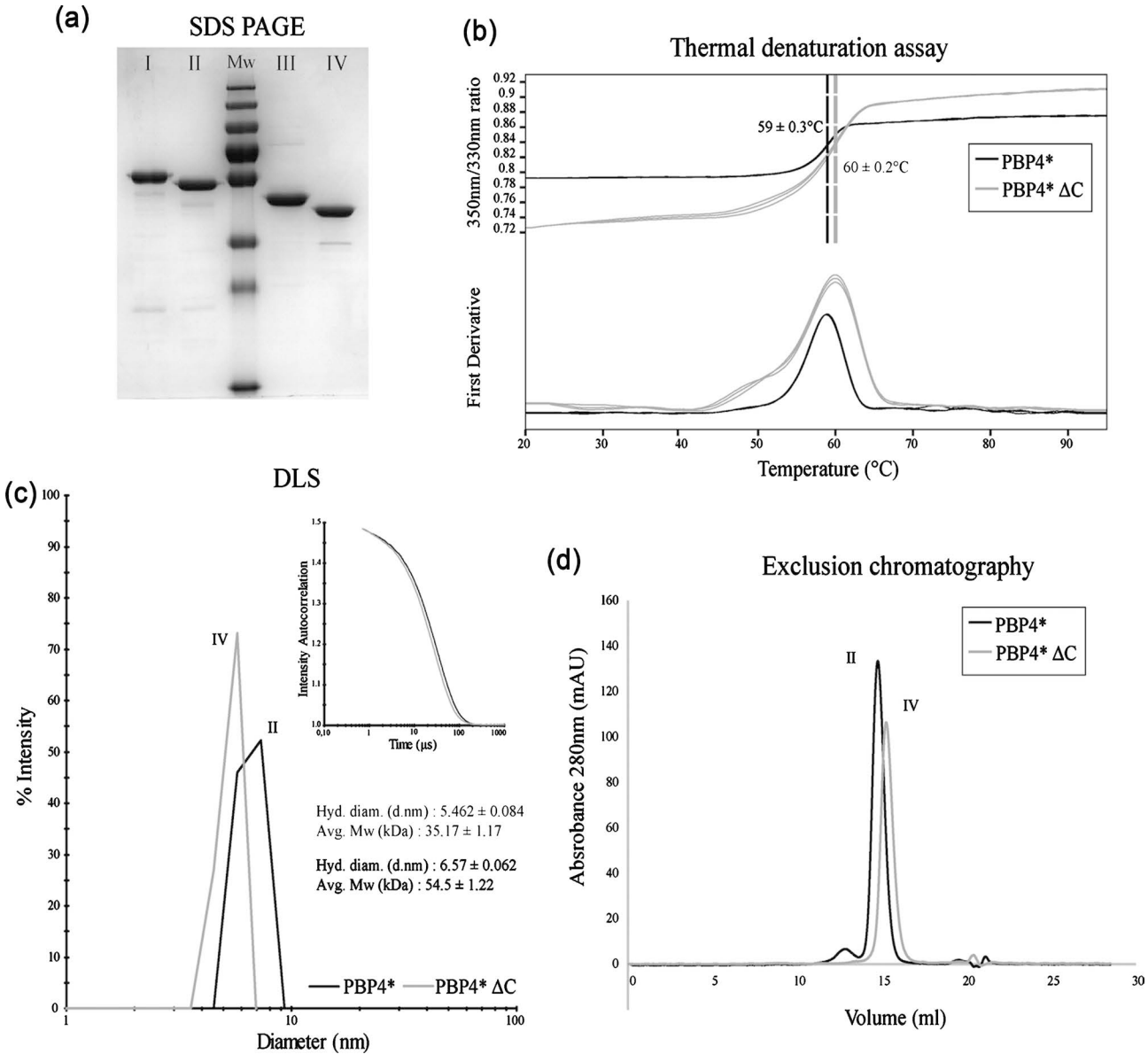

Figure 1. Biophysicals characterizations of the full-length PBP4* and its N-terminal domain (PBP4* ΔCTD). (a) SDS-PAGE analysis on a 12% acrylamide gel of PBP4* and PBP4* ΔCTD before and after treatment with the TEV protease. Lane I: PBP4* with a N-terminal tag, lane II: PBP4* after TEV cleavage, lane III: PBP4* ΔCTD with a N-terminal tag, lane IV: PBP4* ΔCTD after TEV cleavage. (b) Thermal denaturation analyses of PBP4* and its N-terminal domain (PBP4* ΔCTD). The curves from three experiments are presented and the data were treated with the PR control software (NanoTemper Technologies). (c) Dynamic Light Scattering profiles of PBP4* and PBP4* ΔCTD. The intensity autocorrelation curves are shown as an inset. (d) Size-exclusion chromatography profiles of PBP4* and PBP4* ΔCTD.

Deletion of the C-terminal domain did not show a significant impact on the thermal stability of PBP4*. The results of Dynamic Light Scattering experiments performed on PBP4* and PBP4* ΔCTD have shown that both proteins are monomeric in solution, with estimated masses (kDa) of 54.5 ± 1.22 and 35.17 ± 1.17 respectively (**Figure 1(c)**). Compared to the PBP4* ΔCTD, the diffusion properties of the full-length PBP4* were slowed and relaxation of the autocorrelation was longer. The profiles of exclusion size chromatography of PBP4* and PBP4* ΔCTD were also those of monomeric proteins, with estimated masses (kDa) of 55.97 and 45.37 respectively (**Figure 1(d)**). All the results are summarized in (**Table 2**).

3.2. Enzymatic Characterization of the Full Length PBP4*

3.2.1. DD-Carboxypeptidase Activity

The full-length PBP4* exhibits a specific activity of 0.12 μmol·min^{-1}·mg^{-1} on the S2d substrate. In comparison, the *B. subtilis* PBP4a used in the same conditions as a control, hydrolysed S2d with a 60-fold higher specific activity [17].

3.2.2. Aminopeptidase Activity

All the tested compounds gave negative results even after an overnight incubation at 37˚C.

3.2.3. Esterase Activity

The full-length PBP4* did efficiently cleave the D-Ala methyl ester and the kinetic parameters have been determined (**Figure 2** and **Figure 3**). The K_m values calculated with the GraphPad Prism 5.04 software or using the Hanes plot were 2.24 and 2.5 mM respectively.

According to the method used for calculating the parameters, the enzyme turnover (k_{cat}) given by the V/E_0 ratio (E_0 being the enzyme molarity in the assay) was comprised between 129 and 133 s^{-1} yielding K_{cat}/K_m ratios of 58 and 53 mM^{-1}·s^{-1} respectively. The V_{max} values of the PBP4* with this substrate were comprised between 0.6 to 0.62 mM^{-1}·s^{-1}. Surprisingly, the esterase activity of the N-terminal domain (PBP4* ΔCTD) was only 20% of that obtained with the full-length PBP4*, indicating that the C-terminal domain is important for the hydrolysis of D-Ala methyl ester.

3.2.4. Determination of the Substrate Stereospecificity of PBP4*

To know if the full-length PBP4* hydrolyses only the substrate in the D conformation, the racemic DL-Ala OMe substrate was used at the final concentrations of 10 mM for each form (a concentration corresponding to 4-fold the K_m value determined with the pure D-substrate) and the release of D- or L-alanine was quantified using the DAAO or LAAO enzymes. The small amounts of L-alanine measured in the blank or in the sample were equal and it appeared clearly that only the D conformation of the substrate was recognized and hydrolyzed. The PBP4* esterase activity is therefore D-stereospecific. Since teichoic acids attached to the peptidoglycan of Gram positive bacteria are partly substituted with

Table 2. Conditions and results of biophysical characterizations of the *B. subtilis* PBP4* and PBP4* ΔCTD.

Thermal Denaturation Assay		
Instrument	Prometheus NT48 (Nano Temper)	
Employed software	PR. control (NanoTemper)	
Wavelength (nm)	ratio 350 - 330	
Temperature gradient (˚C)	20 - 95	
Heating rate (˚C min^{-1})	1	
Protein sample	**PBP4***	**PBP4* ΔCTD**
Concentration (μM)	30	30
Buffer condition	25 mM HEPES, 100 mM NaCl, pH 8.0	
Volume (μL)	10	10
Results		
Fusion temperature Tm (˚C)	59˚C ± 0.3˚C	60˚C ± 0.2˚C
Dynamic Light Scattering Assay		
Instrument	DynaPro NanoStarTM (Wyatt Technologies)	
Employed software	Dynamics 7.1.9. (Wyatt Technology)	
Laser	100 mW He-Ne	
Wavelength (nm)	658	
Angle (˚)	90	
Exposure time (sec)	50 (10 frames × 5 sec)	
Protein sample	**PBP4***	**PBP4* ΔCTD**
Concentration (μM)	30	30
Buffer condition	25 mM HEPES, 100 mM NaCl, pH 8.0	
Temperature (˚C)	10	10
Volume (μL)	50	50
Results		
Hydrodynamic diameter (nm)	6.57 ± 0.062	5.462 ± 0.084
Polydispersity index (PDI)	0.016 ± 0.004	0.009 ± 0.002
Estimated molecular weight (kDa)	54.5 ± 1.22	35.17 ± 1.17
Exclusion Chromatography		
Column	Superdex S200 10/30 GL (GE Healthcare)	
Instrument	Åkta Purifier (GE Healthcare)	
Employed software	Unicorn (GE Healthcare Life Sciences)	
Wavelength (nm)	280	
Flow (mL min^{-1})	0.5	
Injection loop volume (μL)	100	
Protein sample	**PBP4***	**PBP4* ΔCTD**
Injected mass (μg)	100	100

Continued

Buffer condition	25 mM HEPES, 100 mM NaCl, pH 8.0	
Temperature (˚C)	4	4
Results		
Elution volume (mL)	14.75	15.24
Estimated mass (kDa)	55.97	45.37
Mass Determination		
Protein sample	**PBP4***	**PBP4* ΔCTD**
Calculated monomeric mass(kDa) (from the sequence, using Expasy software)	~51	~41
Mass (kDa) (from DLS)	54.5 ± 1.22	35.17 ± 1.17
Mass (kDa) (from size-exclusion chromatography)	55.97	45.37
Oligomeric state in solution	Monomeric	Monomeric

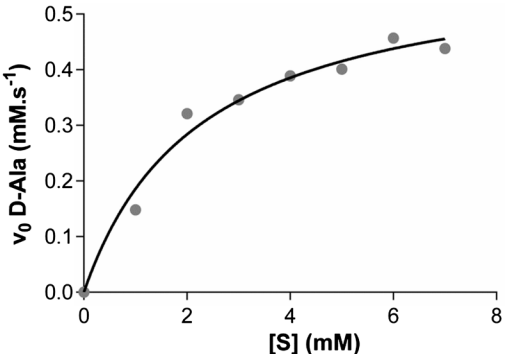

Figure 2. Initial rates (v_0) of the PBP4* esterase activity in function of the D-Ala methyl ester substrate [S] concentrations. After subtraction of the values given by a blank without enzyme to deduce the spontaneous hydrolysis of the substrate, the graph presents the initial rates of D-Ala methyl ester hydrolysis by the PBP4* in mM·s^{-1}. In all cases, the values of substrate hydrolysis by the enzyme was less than 20%.

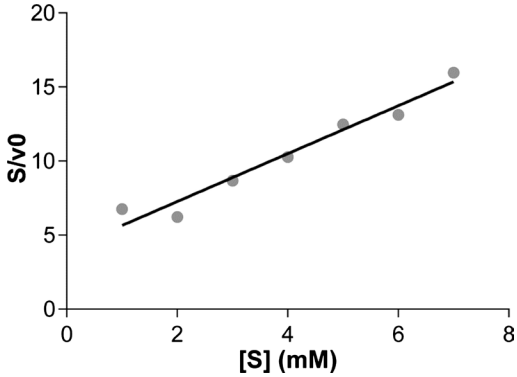

Figure 3. Hanes plot. The mM concentrations of D-Ala methyl ester used as substrate [S] to determine the initial rates (v_0) of the PBP4* esterase activity are indicated on the x axis. The ratio S/v_0 of hydrolysis (mM freed D-Ala s^{-1}) is indicated on the y axis. Equation of the linear interpolation is $y = 1.616x + 4.037$ with $R^2 = 0.957$. The intercept value with the ordinate (4.037) corresponds to the K_m/V ratio and the one with the x axis (−2.5) to $-K_m$. The calculated V_{max} value of D-Ala methyl ester hydrolysis by the full length PBP4* is 0.62 mM·s^{-1}.

glucose and D-alanine, it was tempting to search for an esterase activity of the full length PBP4* on these compounds. Indeed, the PBP4* is active on suspensions of *B. subtilis* cell walls bearing teichoic acids [18] and after subtraction of the amount of D-alanine measured in the blank, 6.2 nmoles of D-alanine were found to be released from 1 mg of cell walls in the conditions described in Material and Methods.

4. Conclusion

The entire *B. subtilis* PBP4* has been produced in *E. coli* as a recombinant protein of 453 residues after successful cleavage of the N-terminal extension. To detect a possible influence of the C-terminal domain (comprising 92 residues) on the folding, stability or enzymatic activity of PBP4*, the recombinant N-terminal (PBP) domain of 361 residues was also produced (**Figure 1(a)**) and some properties of both proteins have been characterized and compared (**Table 2**). The thermal stability of the full length PBP4* protein was not significantly modified by the C-terminal domain deletion (**Figure 1(b)**). The results of Dynamic Light Scattering experiments (**Figure 1(c)**) and the size-exclusion chromatography profiles of PBP4* and PBP4* ΔCTD (**Figure 1(d)**) indicated that both proteins are monomeric in solution, excluding a possible oligomerization of the protein harboring the C-terminal extension. For the first time, a clear esterase enzymatic activity has been detected with the full length PBP4* and the kinetic parameters have been determined (**Figure 2** and **Figure 3**). This activity was D-stereospecific and was significantly lower with the protein lacking the C-terminal domain. A possible role of the C-terminal extension could be its participation in the productive positioning of the substrate in the enzymatic cleft. Structural data should provide information about this hypothesis. The full length PBP4* was also active on *B. subtilis* cell walls containing teichoic acids but however this result does not mean that teichoic acids are physiological substrates for the native PBP4*. This protein has been shown to be associated with the cytoplasmic membrane [1]. To influence the amount of D-alanine-substituted teichoic acids and thus their net charge, the active site of PBP4* must be located on the outer face of the cytoplasmic membrane. How the PBP4* is embedded into the membrane remains to elucidate. Such an esterase activity is compatible with a role for the PBP4* in recycling cell wall components as previously proposed [1].

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Conflicts of Interest

The authors declare that no competing interests exist.

References

[1] Popham, D.L. and Setlow, P. (1993) Cloning, Nucleotide Sequence, and Regulation of the *Bacillus subtilis pbpE* Operon, Which Codes for Penicillin-Binding Protein 4* and an Apparent Amino Acid Racemase. *Journal of bacteriology*, **175**, 2917-2925. https://doi.org/10.1128/jb.175.10.2917-2925.1993

[2] Sowell, M.O. and Buchanan, C.E. (1983) Changes in Penicillin-Binding Proteins during Sporulation of *Bacillus subtilis*. *Journal of Bacteriology*, **153**, 1331-1337.

[3] Cao, M., Kobel, A., Morshedi, M.M. and Wu, M.F. (2002) Defining the *Bacillus subtilis* σ^W Regulon: A Comparative Analysis of Promoter Consensus Search, Run-Off Transcription/Macroarray Analysis (ROMA), and Transcriptional Profiling Approaches. *Journal of Molecular Biology*, **316**, 443-457. https://doi.org/10.1006/jmbi.2001.5372

[4] Cao, M., Wang, T., Ye, R. and Helmann, J.D. (2002) Antibiotics That Inhibit Cell Wall Biosynthesis Induce Expression of the *Bacillus subtilis* σ^W and σ^M Regulons. *Molecular Microbiology*, 4**5**, 1267-1276. https://doi.org/10.1046/j.1365-2958.2002.03050.x

[5] Butcher, B.G. and Helmann, J.D. (2006) Identification of *Bacillus subtilis* σ^W-Dependent Genes That Provide Intrinsic Resistance to Antimicrobial Compounds Produced by *Bacilli*. *Molecular Microbiology*, **60**, 765-782. https://doi.org/10.1111/j.1365-2958.2006.05131.x

[6] Jordan, S., Hutchings, M.I. and Mascher, T. (2008) Cell Envelope Stress Response in Gram-Positive Bacteria. *FEMS Microbiology Reviews*, **32**, 107-146. https://doi.org/10.1111/j.1574-6976.2007.00091.x

[7] Wiegert, T., Homuth, G., Versteeg, S. and Schumann, W. (2001) Alkaline Shock Induces the *Bacillus subtilis* σ^W Regulon. *Molecular Microbiology*, **41**, 59-71. https://doi.org/10.1046/j.1365-2958.2001.02489.x

[8] Heinrich, J. and Wiegert, T. (2006) YpdC Determines Site-1 Degradation in Regulated Intramembrane Proteolysis of the RsiW Anti-Sigma Factor of *Bacillus subtilis*. *Molecular Microbiology*, **62**, 566-579. https://doi.org/10.1111/j.1365-2958.2006.05391.x

[9] Tam, L.T., Antelmann, H., Eymann, C., Albrecht, D., Bernhardt, J. and Hecker, M. (2006) Proteome Signatures for Stress and Starvation in *Bacillus subtilis* as Revealed by a 2-D Gel Image Color Coding Approach. *Proteomics*, **6**, 4565-4585. https://doi.org/10.1002/pmic.200600100

[10] Hahne, H., Mäder, U., Otto, A., Bonn, F., Steil, L., Bremer, E., Hecker, M. and Becher, D. (2010) A Comprehensive Proteomics and Transcriptomics Analysis of *Bacillus subtilis* Salt Stress Adaptation. *Journal of Bacteriology*, **192**, 870-882. https://doi.org/10.1128/JB.01106-09

[11] Palomino, M.M., Sanchez-Rivas, C. and Ruzal, S.M. (2009) High Salt Stress in *Bacillus subtilis*: Involvement of PBP4* as a Peptidoglycan Hydrolase. *Research in Mi-*

crobiology, **160**, 117-124. https://doi.org/10.1016/j.resmic.2008.10.011

[12] Zellmeier, S., Zuber, U., Schumann, W. and Wiegert, T. (2003) The Absence of FtsH Metalloprotease Activity Causes Overexpression of the σ^W-Controlled *pbpE* Gene, Resulting in Filamentous Growth of *Bacillus subtilis*. *Journal of Bacteriology*, **185**, 973-982. https://doi.org/10.1128/JB.185.3.973-982.2003

[13] Scheffers, D.-J. (2005) Dynamic Localization of Penicillin-Binding Proteins during Spore Development in *Bacillus subtilis*. *Microbiology*, **151**, 999-1012. https://doi.org/10.1099/mic.0.27692-0

[14] Scheffers, D.-J., Jones, L.J. and Errington, J. (2003) Several Distinct Localization Patterns for Penicillin-Binding Proteins in *Bacillus subtilis*. *Molecular Microbiology*, **51**, 749-764. https://doi.org/10.1046/j.1365-2958.2003.03854.x

[15] Adam, M., Damblon, C., Jamin, M., Zorzi, W., Dusart, V., Galleni, M., El Kharroubi, A., Piras, G., Spratt, B.G. and Keck, W. (1991) Acyltransferase Activities of the High-Molecular-Mass Essential Penicillin-Binding Proteins. *Biochemical Journal*, **279**, 601-604. https://doi.org/10.1042/bj2790601

[16] Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., Nieto, M. and Perkins, H.R. (1976) Exocellular DD-Carboxypeptidases-Transpeptidases from *Streptomyces*. *Methods in Enzymology*, **45**, 610-636. https://doi.org/10.1016/S0076-6879(76)45054-1

[17] Duez, C., Vanhove, M., Gallet, X., Bouillenne, F., Docquier, J.-D., Brans, A. and Frère, J.-M. (2001) Purification and Characterization of PBP4a, a New Low-Molecular-Weight Penicillin-Binding Protein from *Bacillus subtilis*. *Journal of Bacteriology*, **183**, 1595-1599. https://doi.org/10.1128/JB.183.5.1595-1599.2001

[18] Margot, P. and Karamata, D. (1996) The *wprA* Gene of *Bacillus subtilis* 168, Expressed during Exponential Growth, Encodes a Cell-Wall-Associated Protease. *Microbiology*, **142**, 3437-3444. https://doi.org/10.1099/13500872-142-12-3437