Identification and characterization of a novel polysaccharide deacetylase C (PdaC) from Bacillus subtilis

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Background: Peptidoglycan modification is very important process that bacteria use to adjust to various environmental conditions. Results: B. subtilis pdaC was associated with lysozyme sensitivity. Surprisingly PdaC is able to deacetylate N-acetylmuramic acid but not N-acetylglucosamine in peptidoglycan. But chitin oligomers were deacetylated by PdaC. Conclusion: PdaC is a unique enzyme exhibiting two different deacetylase activities. Significance: Novel deacetylase is characterized.

SUMMARY

Cell wall metabolism and cell wall modification are very important processes that bacteria use to adjust to various environmental conditions. One of the main modifications is deacetylation of peptidoglycan. The polysaccharide deacetylase homologue, Bacillus subtilis YjeA (renamed PdaC), was characterized and found to be a unique deacetylase. The pdaC deletion mutant was sensitive to lysozyme treatment, indicating that PdaC acts as a deacetylase. The purified recombinant and truncated PdaC from Escherichia coli deacetylated B. subtilis peptidoglycan and its polymer, (-GlcNAc-MurNAc[1-Asp-D-Glu]-)n. Surprisingly, RP-HPLC and ESI-MS-MS analyses showed that the enzyme deacetylates N-acetylmuramic acid (MurNAc) not GlcNAc from the polymer. Contrary to Streptococcus pneumoniae PgdA which shows high amino acid sequence similarity with PdaC and is a zinc-dependent GlcNAc deacetylase toward peptidoglycan, there was less dependence on zinc ion for deacetylation of peptidoglycan by PdaC than other metal ions (Mn²⁺, Mg²⁺, Ca²⁺). The kinetic values of the activity toward B. subtilis peptidoglycan were $K_m$=4.8 mM and $k_{cat}$=0.32 s⁻¹. PdaC also deacetylated N-acetylglucosamine (GlcNAc) oligomers with a $K_m$=12.3 mM and $k_{cat}$=0.24 s⁻¹ toward
GlcNAc. Therefore, PdaC has GlcNAc deacetylase activity toward GlcNAc oligomers and MurNAc deacetylase activity toward B. subtilis peptidoglycan.

Peptidoglycan consists of GlcNAc-MurNAc glycan strands covalently bound to peptide side chains (1). It is one of the most important cell wall components for many microorganisms as it acts as a protector toward various environmental conditions. Peptidoglycan can be modified by several cell wall hydrolases and deacetylases (2).

Several GlcNAc deacetylases have been characterized including chitin deacetylases that modify chitin (GlcNAc polymer) and/or its derivatives (glycol chitin). Deacetylases from Mucor rouxii (3), Colletotrichum lindemuthianum (4) and Aspergillus nidulans (5) were able to modify not only chitin but also chitin oligomers such as GlcNAc₆. Recently, in several bacteria such as Streptococcus pneumoniae (6, 7), Listeria monocytogenes (8), and Lactococcus lactis (9), GlcNAc deacetylases have been identified, and it is known that S. pneumoniae PgdA can target not only peptidoglycan but also chitin oligomers as a GlcNAc deacetylase (6, 7).

Only one MurNAc deacetylase (B. subtilis PdaA) has been characterized (10). Previously, our group demonstrated that this deacetylase acts as a MurNAc deacetylase toward spore peptidoglycan to produce a muramic-δ-lactam structure in vivo (10), and also that it is active toward a GlcNAc-MurNAc polymer in vitro (11). Blair and van Aalten published the crystal structure of PdaA (12). However, no other MurNAc deacetylases have been identified. Therefore, we have further investigated deacetylase activity to better understand the processes of GlcNAc and/or MurNAc deacetylation.

We have identified a unique polysaccharide deacetylase gene, yjeA (renamed pdaC). It is known that this gene is regulated by an essential two-component system, YycFG (13), which is associated with cell division (14, 15, 16). We demonstrated that the pdaC mutant is sensitive to lysozyme treatment and that PdaC acts as a GlcNAc deacetylase toward chitin oligomers and as a MurNAc deacetylase toward B. subtilis peptidoglycan.

**EXPERIMENTAL PROCEDURES**

Construction of Deacetylase Mutants—The strains, plasmids, and primers used in this study are shown in Supplemental Tables S1 and S2. The pdaC gene fragment was amplified by PCR with YJEASD and YJEARV primers, digested with SalI and MunI and then ligated to the SalI-EcoRI site of pBluescriptII-SK(+), resulting in pBLJE. A plasmid, pDG1515, was digested with XbaI and SalI, and the fragment containing a tetracycline cassette was blunted and then ligated to the EcoRV site of pBluescriptII-SK(+), resulting in pBLTC. The plasmid, pBLTC, was digested with HindIII and the fragment containing the tetracycline cassette was ligated into the HindIII site of pBLJE. A plasmid, pBLJE, was digested with BamHI and HindIII and then digested with HindIII and the fragment containing the tetracycline cassette was ligated into the HindIII site of pBLJE, resulting in pBLJEATC.

The yheN gene fragment was amplified by PCR with YHEN-SD and YHEN-RV primers, digested with BamHI and HindIII and then
ligated to the corresponding sites of pGEM3Zf(+), resulting in pGMHEN. A plasmid, pBCATERV containing a chloramphenicol cassette, was digested with SmaI and HincII, and the fragment containing the cassette was ligated in the Eco47III site of pGMHEN, resulting in pGMHENCM.

The yxkh gene fragment amplified by PCR with YXKH-SD and YXKH-RV primers was blunted and phosphorylated with a BKL kit (Takara) and then ligated to the EcoRV site of pBluescriptII-SK(+), resulting in pBLXKH. To eliminate the ClaI site of pBLXKH, the plasmid was digested with HindIII and HincII, blunted and then self-ligated, resulting in pBLΔXKH. A plasmid, pDG792 containing a kanamycin cassette, was digested with ClaI and the fragment containing the cassette was ligated to the corresponding site of pBLΔXKH, resulting in pBLΔXKHKM.

*Botulinus subtilis* 168 was transformed with the linearized pBJEATC, pGMHENCM and pBLΔXKHKM, resulting in JEATd (pdaC::tet), HENCdd (yheN::cat) and XKHKdd (yxkh::km) strains, respectively.

The truncated ylxY gene fragment amplified by PCR with YLXY-HF and YLXY-BR primers was digested with HindIII and BamHI and ligated to the corresponding sites of pMUTIN4, resulting in pM4ΔLXY. *B. subtilis* 168 was transformed with the concatenated pM4ΔLXY derived from *Escherichia coli* C600 (pM4ΔLXY) by single crossing over recombination, resulting in YLXYd strain (ylxY::erm).

Construction of a conditional-null mutant of *pdaC*, PdaCp–A fragment containing the SD sequence of *pdaC* and truncated *pdaC* was amplified by PCR with PC-SD-HinF and PC-D-BamR primers. The amplified fragment was digested with HindIII and BamHI and ligated to the corresponding sites of pMUTIN4, resulting in pM4SD-PdaC. After concatenated pM4SD-PdaC derived from *E. coli* C600 (pM4SD-PdaC) strain had been purified, *B. subtilis* 168 was transformed with the plasmid by single crossing over recombination, resulting in PdaCp strain (Pspac-pdaC). PdaC was expressed in the strain by IPTG addition.

**Construction of a Plasmid, pQE30ΔYjeA for Overexpressing Truncated PdaC**—The truncated *pdaC* gene fragment amplified by PCR with yjeA+87F and yjeA+1383R primers was digested with SphI and SalI, and then ligated to the corresponding sites of pQE-30, resulting in pQE30ΔYjeA. This plasmid was utilized to overexpress truncated PdaC (from a.a. 30 to a.a. 467).

Transformation of *E. coli* and *B. subtilis*—*E. coli* and *B. subtilis* transformations were performed as described by Sambrook *et al.* (17) and Anagnostopoulos and Spizizen (18), respectively.

**SDS-PAGE and Purification of Cell Wall and Peptidoglycan from *B. subtilis***—SDS-PAGE was performed as described by Sambrook *et al.* (17). *B. subtilis* was grown at early stationary phase, and the cells were utilized for preparation of cell wall and peptidoglycan as described previously (19, 20, 21). Purified peptidoglycan was N-acetylated as described previously (11).

Overexpression and Purification of Truncated PdaC—Truncated PdaC (30-467 a.a.) was overexpressed in *E. coli* JM109 harboring
pQE30ΔYjeA. The strain was incubated at 37°C in LB medium containing 100 μg/ml ampicillin. When the absorbance reached 0.5 at 600 nm, 1 mM IPTG (final concentration) was added to the culture, and then the cells were further incubated for 1 hour. The cells were harvested and suspended in 10 mM imidazole NPB buffer [10 mM imidazole, 1 M NaCl, 20 mM sodium phosphate (pH 7.4)], and then disrupted by sonication. After centrifugation, the supernatant was utilized for purification of the protein with a HiTrap Chelating HP column (GE Healthcare), according to the manufacturer’s instructions. The purified truncated PdaC was dialyzed against 20 mM phosphate buffer (pH 7.4).

Overexpression and Purification of CwlH, CwlK and LytF (CwlE)–CwlH (L-alanine amidase), CwlK (L,D-endopeptidase) and LytF (CwlE) (D,L-endopeptidase) were overexpressed and purified from E. coli cells as described previously (20, 22, 23).

Measurement of deacetylase activity–Deacetylase activity toward several substrates was measured using an F-kit for determination of released acetic acid (Roche). The sample preparation was described previously (11). The substrates, GlcNAc oligomers, chitin (from crab, Seikagaku Biobusiness), chitosan (Wako [deacetylation rate: 40-50%]), and B. subtilis peptidoglycan (final concentrations, 2 mg/ml), were deacetylated by PdaC (final concentration, 10 μg/ml [188.7 nM]) for 4 hours at 37°C in 50 mM HEPES buffer (pH 7.0) containing 5 mM MnCl₂ and then the sample was boiled for 10 min to denature the enzyme.

For purpose of kinetics for peptidoglycan, the molarity was calculated based on the assumption that peptidoglycan consists of a repeating unit of GlcNAc-MurNAc-L-Ala-D-Glu-A:pm (M_r, 868.8) because that is the main unit of B. subtilis peptidoglycan (24). As a result, we defined that 2.5 mg/ml peptidoglycan is equal to 2.88 mM. For kinetics analysis of GlcNAc₄, 2 mg/ml GlcNAc₄ (2.41 mM) was used. For the assay 188.7 nM PdaC was also utilized.

For the other substrates derived from B. subtilis peptidoglycan, peptidoglycan (final concentration, 2.5 mg/ml) was suspended in 50 mM HEPES buffer (pH 7.0) and then digested without or with 12.5 μg/ml (final concentration) of cell wall hydrolases, an L-alanine amidase (CwlH) (22), L,D-endopeptidase (CwlK) (23) and D,L-endopeptidase [LytF (CwlE)] (20) at 37°C overnight. After the sample had been boiled for 10 min to denature the hydrolases and centrifuged, the supernatant was collected. Ten μg/ml PdaC (final concentration) and 50 mM HEPES buffer (pH 7.0) containing 5 mM MnCl₂ were added to the supernatant, followed by incubation for 4 hours at 37°C.

For determination of a divalent cation effect, PdaC was dialyzed against 20 mM EDTA (pH 7.4) twice and then against 20 mM sodium phosphate buffer (pH 7.4) (“EDTA-treated PdaC”). On the other hand, PdaC was dialyzed against 20 mM sodium phosphate buffer (pH 7.4) (no EDTA treatment; “Native PdaC”). Two mg/ml of GlcNAc₄ or 2.5 mg/ml of peptidoglycan (final concentration) in 50 mM HEPES (pH 7.0) containing 5 mM MnCl₂, ZnCl₂, MgCl₂, NiSO₄, or CaCl₂, 5 μM ZnCl₂, or 50 nM ZnCl₂ was digested by 10 μg/ml of PdaC.
(final concentration) for 1, 2, 3 and 4 hours at 37°C. Five mM CoCl₂ was also utilized for analysis of deacetylase activity with an F-kit. However, Co²⁺ interfered with the assay kit [drastic increase in absorbance without any acetic acid because of changing solution color from crystal clear to yellow during the assay], thus the released acetic acid was not able to be determined in the presence of Co²⁺. After the reaction by PdaC had been performed, all samples were boiled for 10 min and centrifuged, and then the supernatant was utilized for measurement of deacetylase activity with an F-kit.

Identification of deacetylation of GlcNAC₄ by PdaC—After GlcNAC₄ (final concentration, 2.5 mg/ml) had been deacetylated by PdaC (final concentration, 10 µg/ml) for 4 hours at 37°C in 50 mM HEPES buffer (pH 7.0) containing 5 mM MnCl₂, the sample was separated by normal-phase HPLC [TSKgel Amide-80 column (TOSOH); flow buffer, 0.02% TFA containing 70% CH₃CN; flow rate, 0.5 ml/min; monitoring wavelength, 202 nm; Shimadzu LC-10Avp HPLC system], and then tetrasaccharide peak material was collected and freeze-dried. The sample was solubilized in 0.05% TFA, and then it was analyzed by RP-HPLC with a Symmetry Shield RP18 column (Waters) (flow rate, 0.3 ml/min; monitoring wavelength, 202 nm; Shimadzu LC-10Avp HPLC system). Elution buffer A contained 0.05% TFA, and buffer B contained 0.05% TFA with 40% CH₃CN. Elution was performed for 50 min with a linear gradient of buffer B (from 0 to 50%).

Purification of glycan strands consisting of (-GlcNAc-MurNAc-[L-Ala-D-Glu]-)ₙ

Polymer—Purification of glycan strands from B. subtilis peptidoglycan was performed as described previously (11). After 1 mg of peptidoglycan had been digested with 5 µg of LytF (CwlE) in 20 mM HEPES buffer (pH 7.0) at 37°C for 14 hours and then boiled for 10 min, it was centrifuged and the supernatant was collected. The supernatant components (containing the glycan strands and peptides) were separated by size exclusion chromatography. After the purified glycan strands (-GlcNAc-MurNAc-[L-Ala-D-Glu]-)ₙ had been freeze-dried, they were N-acetylated as described previously (11).

Decacylation of glycan strands containing L-Ala-D-Glu side chains (-GlcNAc-MurNAc-[L-Ala-D-Glu]-)ₙ with PdaC—Two mg of purified glycan strands containing L-Ala-D-Glu side chains were deacetylated at 37°C overnight with (deacetylated sample) or without (non-deacetylated sample) 20 µg of PdaC in 50 mM HEPES buffer (pH 7.0) containing 5 mM MnCl₂. To further deacetylate the strands, 20 µg of PdaC was added to the deacetylated sample, and then incubation was performed at 37°C for 4 hours. The samples were digested by 10 µg of the N-terminal domain of CwlT, which is a muramidase (25), at 37°C for 4 hours, followed by boiling. Borate buffer (pH 9.0) (final concentration, 0.5 M) was added to the samples, and then the reducing ends of MurNAc were reduced with NaBH₄ as described previously (11). The reduced samples were separated by RP-HPLC with a Symmetry Shield RP18 column (Waters) (flow rate, 1 ml/min; monitoring wavelength, 202 nm; column oven
temperature, 40°C; Shimadzu LC-10AD HPLC system). Elution buffer A contained 0.05% TFA, and buffer B contained 0.05% TFA with 40% CH$_3$CN. Elution was performed for 10 min with buffer A only with an isocratic gradient and then for 60 min with a linear gradient of buffer B (from 0 to 50%).

**Purification of reduced tetrasaccharide-dipeptides (4S2P)**—To purify reduced tetrasaccharide-dipeptide, GlcNAc-MurNAc(-L-Ala-D-Glu)-GlcNAc-MurNAc(-L-Ala-D-Glu), purified glycan strands containing L-Ala-D-Glu side chains were digested with a muramidase, CwlT, and then the sample was reduced and separated by RP-HPLC as described above. The purified 4S2P was identified by ESI-MS and ESI-MS-MS.

**Deacetylation with PdaC toward 4S2P**—Five hundred ng of pBR322 plasmid DNA was digested with 2 μg of PdaC or DNase I (TaKaRa) in reaction buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl$_2$] at 37°C for 1, 5, 10, 20, 30, 60, 90 and 120 min. After the reaction was complete, the sample was boiled for 10 min to inactivate the enzyme. The sample was centrifuged and then the supernatant was applied to a 0.7% agarose gel.

**RESULTS**

*B. subtilis* *pdaC* Mutant is Sensitive toward Lysozyme—Many microorganisms have polysaccharide deacetylases such as *S. aureus* PgdA (6, 7) and *B. subtilis* PdaA (10, 11, 26). Fig. 1 shows identified or predicted polysaccharide deacetylases in *B. subtilis* and some other Gram-positive bacteria. It is known that PdaA and PdaB play roles in sporulation (10, 28, 29) but the other gene products, YjeA (renamed PdaC), YlxY, YxkH and YheN, are not associated with sporulation and germination (28). Thus, these disruptants were created, and the sensitivity of these strains toward lysozyme (muramidase that digests a linkage of MurNAc-GlcNAc in peptidoglycan) was determined. As shown in Fig. 2, only the *pdaC* mutant was sensitive toward lysozyme since the growth rate was decreased (closed circles in Fig. 2). To confirm the *pdaC* mutant phenotype, a new strain, PdaCp (P$_{spac}$-pdaC) was constructed. When the strain was incubated without IPTG
(weak PdaC expression), it was sensitive toward lysozyme (closed squares in supplemental Fig. S1). The strain with 1 mM IPTG (PdaC expression) was resistant toward lysozyme (closed diamonds in supplemental Fig. S1), suggesting that PdaC is necessary for protection of peptidoglycan toward lysozyme since lysozyme cannot digest deacetylated peptidoglycan (30).

**PdaC Deacetylates B. subtilis Peptidoglycan**—Since the pdaC deletion mutant was sensitive toward lysozyme (Fig. 2), it was predicted that PdaC deacetylates B. subtilis peptidoglycan. From the SOSUI algorithm that can be used to predict the membrane regions of a target protein (http://bp.nuap.nagoya-u.ac.jp/sosui/), PdaC seems to have a transmembrane (TM) region (from a.a. 7 to a.a. 29). Moreover, ~50 kDa PdaC is detected from the membrane fraction by proteome analysis described by Eymann et al. (31). Therefore, the truncated PdaC (from a.a. 30 to a.a. 467) lacking the TM was overexpressed in E. coli and then purified by affinity chromatography. As shown in lane 3 in Supplemental Fig. S2, the truncated PdaC (53.0 kDa) could be purified (one band on SDS-PAGE).

The deacetylase activity of PdaC toward peptidoglycan was measured by released acetic acid. As shown in Table 1A and Supplemental Fig. S3, PdaC dialyzed against EDTA weakly deacetylated the peptidoglycan without addition of a divalent cation (“EDTA-treated PdaC”). Moreover, PdaC had the maximum activity with Mn$^{2+}$. Therefore, these results suggest that PdaC in the presence of Mn$^{2+}$ efficiently deacetylates *B. subtilis* peptidoglycan.

To further characterize the deacetylase activity toward *B. subtilis* peptidoglycan, peptidoglycan was digested with L-alanine amidase (CwlH) (22), L,D-endopeptidase (CwlK) (23) or D,L-endopeptidase (LytF [CwlE]) (20), and then the deacetylase activity was measured by quantitating the released acetic acid. As shown in Table 2, PdaC exhibited the strongest deacetylase activity toward peptidoglycan digested with D,L-endopeptidase (10.7±0.17 μg/ml). PdaC exhibited less deacetylase activity toward peptidoglycan digested with L,D-endopeptidase (3.25±0.35 μg/ml) compared with the activity toward peptidoglycan alone (6.0±1.3 μg/ml). The enzyme showed no activity toward peptidoglycan digested with L-alanine amidase (< 0.03 μg/ml). This suggested that (-GlcNAc-MurNAc[-L-Ala-D-Glu]-)$_n$ is a suitable substrate for PdaC and that its substrate specificity is very different from PdaA since PdaA can digest only (-GlcNAc-MurNAc-)$_n$ (11).

**PdaC Works as a MurNAc Deacetylase toward Glycan Strands containing L-Ala-D-Glu**—As shown in Table 2, PdaC deacetylates glycan strands consisting of (-GlcNAc-MurNAc[-L-Ala-D-Glu]-)$_n$. The purified glycan strands were deacetylated with or without PdaC, and then the samples were treated with a muramidase, followed by reduction and separation of the sample by RP-HPLC. In Fig. 3A, the “Without PdaC” sample contained two major peaks (1 and 3). Both materials were collected and analyzed by ESI-MS. The peak 1 material in the “Without
PdaC” sample showed fragment ions at m/z 721.5 and 697.3 in the positive and negative modes, respectively (Supplemental Fig. S4A and B, respectively), corresponding to [M+Na]+ and [M-H] of reduced disaccharide-peptide (2S1P) (M_r, 698.5). The peak 3 material in the “Without PdaC” sample showed the fragment ions at m/z 1,400.9 and 1,376.7 in the positive and negative modes, respectively (Supplemental Fig. S5A and B, respectively), corresponding to [M+Na]+ and [M-H] of reduced tetrasaccharide-dipeptides (4S2P) (M_r, 1,376.9). Moreover, ESI-MS-MS analysis strongly indicated that the peak 3 material is reduced 4S2P, GlcNAc-MurNAc(-L-Ala-d-Glu)-GlcNAc-MurNAc(-L-Ala-d-Glu) (Supplemental Fig. S5C, D and E, and Supplemental Table S3).

The “With PdaC” sample contained not only two materials (peaks 1 and 3), but also additional material (peak 2) (Fig. 3A). The peak 1 and 3 materials were the same as those materials in the “Without PdaC” sample, judging from ESI-MS and -MS-MS analyses (data not shown). The additional material (peak 2) was collected and analyzed by ESI-MS and ESI-MS-MS analyses. The peak 2 material in the “With PdaC” sample gave fragment ions at m/z 1,358.7 and 1,334.6 in the positive and negative modes, respectively, corresponding to [M+Na]+ and [M-H] of a deacetylated compound of reduced 4S2P (M_r, 1,334.9) (Supplemental Fig. S6A and B, respectively). The peak 2 material was further analyzed by ESI-MS-MS in the positive and negative modes. As shown in Fig. 4A and B, each fragment peak corresponded to a fragment of substrate, a deacetylated compound of reduced 4S2P (GlcNAc-Mur[-L-Ala-d-Glu]-GlcNAc-MurNAc r[-L-Ala-d-Glu]) (Fig. 4C and Supplemental Table S4) though the possibility that the structure is GlcN-MurNAc(-L-Ala-d-Glu)-GlcNAc-MurNAc(r-L-Ala-d-Glu) cannot be completely eliminated.

PdaC also Deacetylates Reduced 4S2P—After glycan strands containing L-Ala-d-Glu had been treated with PdaC followed by muramidase digestion, the main product was deacetylated 4S2P (Fig. 3A). Thus it was very likely that PdaC deacetylates 4S2P. Identified peak 3 material (reduced 4S2P) as shown in Fig. 3A was utilized as a substrate for PdaC. As shown in Fig. 3B, the “With PdaC” sample contained an additional peak (peak 2) that was identified by ESI-MS and -MS-MS as a deacetylated compound of reduced 4S2P (GlcNAc-Mur[-L-Ala-d-Glu]-GlcNAc-MurNAc r[-L-Ala-d-Glu]) (Supplemental Fig. S7A and B). Thus, PdaC was able to deacetylate 4S2P derived from B. subtilis peptidoglycan.

Confirmation of MurNAc Deacetylation Activity of PdaC with Mutanolysin—From the results of Figs. 3, 4, and Supplemental Fig. S7, the amino sugar deacetylated by PdaC seems to be MurNAc. To confirm this result, reduced 4S2P was deacetylated by PdaC (this sample is exactly the same as the “With PdaC” sample in Fig. 3B) and digested with mutanolysin, which can digest glycosidic linkages such as -GlcN-MurNAc↓-GlcNAc-MurNAc-, -GlcNAc-MurNAc↓-GlcN-MurNAc- and -GlcN-MurNAc↓-GlcN-MurNAc- but not -GlcNAc-Mur-GlcNAc-MurNAc- (arrows indicate the cleavage sites) (26, 27). After the sample had been reduced, it was separated by
RP-HPLC. As shown in Fig. 3C, the peak 3 material (reduced 4S2P) was completely digested to 2S1P and reduced 2S1P, followed by NaBH₄ reduction to yield only reduced 2S1P (the peak 1 material), indicating the mutanolysin works very well in this reaction. However, the peak 2 material (deacetylated compound of reduced 4S2P) was not digested. Therefore, this suggested that the deacetylated product is GlcNAc-Mur(-L-Ala-d-Glu)-GlcNAc-MurNAcrl(-L-Ala-d-Glu). Moreover, when the peak 2 material in Fig. 3A (predicted GlcNAc-Mur[-L-Ala-d-Glu]-GlcNAc-MurNAcr [-L-Ala-d-Glu]) was subjected to mutanolysin, it was not digested (data not shown). Therefore, these results strongly suggest that PdaC is a MurNAc deacetylase.

**PdaC also Deacetylates N-acetylglucosamine (GlcNAc) Oligomers**—From Figs. 3 and 4 it is clear that PdaC is a MurNAc deacetylase. However, some deacetylase homologues of PdaC such as *S. pneumoniae* (6, 7), *L. monocytogenes* (8), and *L. lactis* (9) enzymes are GlcNAc deacetylases (Fig. 1). Thus, it is possible that PdaC may have GlcNAc deacetylase activity.

The deacetylase activities of PdaC toward GlcNAc, several chitin oligomers (GlcNAc₂₋₅), chitin (GlcNAc polymer) and chitosan (partially deacetylated GlcNAc polymer) were measured by quantitating the released acetic acid. As shown in Fig. 5, PdaC displayed deacetylase activities toward GlcNAc₄, and GlcNAc₅, while it did not deacetylate GlcNAc and had reduced activities toward GlcNAc₂, and GlcNAc₃. PdaC was essentially not active toward chitosan and had very low activity toward chitin (Fig. 5).

These results suggest that PdaC deacetylates chitin oligomers (GlcNAc₄₋₅) as an *N*-acetylglucosamine deacetylase.

The deacetylase activity of PdaC toward GlcNAc₄ (a suitable substrate for PdaC) with divalent cations was measured by released acetic acid. As shown in Table 1B and Supplemental Fig. S8, PdaC showed the maximum activity with Mn²⁺. Therefore, the result suggests that PdaC in the presence of Mn²⁺ efficiently deacetylates not only *B. subtilis* peptidoglycan but also GlcNAc oligomer, GlcNAc₄.

To identify the position of deacetylated GlcNAc by PdaC toward the chitin oligomer, GlcNAc₄ was deacetylated. Then the samples were separated by normal-phase HPLC with a TSKgel Amide-80 column to remove salts and buffer, followed by collection of the peaks containing the tetrasaccharide materials (data not shown). These peaks were further separated by RP-HPLC. As shown in Fig. 6A and B, the non-deacetylated sample contained two major peaks (peaks 2 and 3 in Fig. 6B) and the deacetylated sample contained an additional peak (peak 1 in Fig. 6A), in addition to peaks 2 and 3. Peaks 2 and 3 in Fig. 6A and B had a fragment ion at m/z 832.1 by ESI-MS in the positive mode, corresponding to [M+H]⁺ of GlcNAc₄ (Mᵣ, 830.5) (data not shown) and ESI-MS-MS analysis indicated that the sample was actually GlcNAc₄ (data not shown). The reason for the different elution times (peaks 2 and 3) is unknown, but it may reflect the difference of α and β anomers at the reducing end as described previously (32). Peak 1 in Fig. 6A had a fragment ion at m/z 790.0 by ESI-MS
in the positive mode, corresponding to \([M+H]^+\)

of deacetylated GlcNAc, \((M_r, 788.5)\) (Fig. 6C). Moreover, as shown in Fig. 6D, the substrate

was identified by ESI-MS-MS as GlcNAc-GlcNAc-GlcN-GlcNAc (Fig. 6E). To confirm the structure, the peak 1 material was reduced, and then analyzed by ESI-MS and ESI-MS-MS. The reduced material had a fragment ion at \(m/z\) 792.1 by ESI-MS in the positive mode, corresponding to \([M+H]^+\) of a reduced compound of deacetylated GlcNAc, \((M_r, 790.5)\) (Supplemental Fig. S9A), and the fragments \((b2'\) and \(b3'\) in Supplemental Fig. S9B) by ESI-MS-MS strongly support that the structure of the reduced compound of deacetylated GlcNAc is GlcNAc-GlcNAc-GlcN-GlcNAc (Fig. 6E).

MurNAc and GlcNAc Deacetylation by PdaC–PdaC has both MurNAc and GlcNAc deacetylase activities; however, Fig. 5 shows that the activity toward MurNAc seems to be higher than toward GlcNAc, since the released acetic acid from peptidoglycan and GlcNAc oligomers are different (released acetic acid, 83.4 \(\mu\)M from peptidoglycan and 23.1 \(\mu\)M from GlcNAc). To determine the kinetics for GlcNAc and MurNAc deacetylation, GlcNAc, and peptidoglycan were utilized as substrates, and the released acetic acid was measured. For purpose of the kinetics for peptidoglycan, the molarity was calculated using the assumption that peptidoglycan consists of a repeating unit, GlcNAc-MurNAc-L-Ala-D-Glu-A2pm \((M_r, 868.8)\), since this is the main unit contained in \(B.\ subtilis\) peptidoglycan (24). Initial velocities for GlcNAc, and peptidoglycan were measured after 30 min incubation at different substrate concentrations since the rates of released acetic acid from both substrates were constant within at least 30 min (Fig. 7A and B), and the measured initial velocities fit Michaelis-Menten kinetics. The \(K_m\) for GlcNAc and peptidoglycan were 12.3±1.84 mM and 4.8±0.30 mM, respectively, and the values of \(k_{cat}\) for GlcNAc and peptidoglycan were 0.24±0.031 s\(^{-1}\) and 0.32±0.010 s\(^{-1}\), respectively (Fig. 7C). The value of \(K_m/k_{cat}\) for peptidoglycan (0.067 mM\(^{-1}\)s\(^{-1}\)) is much larger than the value for GlcNAc (0.020 mM\(^{-1}\)s\(^{-1}\)), and non-treated peptidoglycan was utilized instead of peptidoglycan treated with di-L-endopeptidase though the treated peptidoglycan (-GlcNAc-MurNAc[L-Ala-D-Glu]-) is a more suitable substrate for PdaC (Table 2). Thus, PdaC seems to prefer to deacetylate MurNAc from peptidoglycan. Moreover, since GlcNAc, deacetylation by \(S.\ pneumoniae\) PgdA had values of \(K_m\)=3.8 mM, \(k_{cat}=0.55\) s\(^{-1}\), and \(k_{cat}/K_m=0.15\) mM\(^{-1}\)s\(^{-1}\) (7), the deacetylation toward GlcNAc by PdaC was much weaker than that by PgdA.

No nuclease activity of PdaC–Ng et al. described that PdaC (YjeA) has DNase activity (33). Our results indicate that PdaC is both a GlcNAc and MurNAc deacetylase. Thus, DNase activity by PdaC was determined under the same conditions as described by Ng et al. Results indicated that PdaC had no DNase activity at all (Supplemental Fig. S10A). This may be because Ng et al. overexpressed the enzyme in \(E.\ coli\) and purified the protein from the culture (not cytoplasm). As well, the region of PdaC expressed by Ng et al. (from a.a. 50 to the end) was slightly different from the region in this
study (from a.a. 30 to the end). Another reason may be that the longer incubation of DNA with the crude enzyme solution described by Ng et al. may easily lead to DNA digestion.

**DISCUSSION**

Surprisingly, PdaC from *B. subtilis* exhibited MurNAc deacetylase activity toward *B. subtilis* peptidoglycan. Moreover, PdaC also had GlcNAc deacetylase activity toward GlcNAc oligomers. Therefore, potentially PdaC can deacetylate both amino sugars, GlcNAc and MurNAc. According to the classification of Henrissat (34), PdaA, PgdA and PdaC belong to the carbohydrate esterase 4 family, which includes peptidoglycan GlcNAc deacetylases, rhizobial NodB chito-oligosaccharide deacetylases, chitin deacetylases, acetyl xylan esterases, and xylanases (2). Thus, potentially these enzymes may be able to exhibit very wide substrate specificities.

**Deacetylation toward GlcNAc oligomer**—As shown in Fig. 6, the product of deacetylated GlcNAc$_4$ by PdaC was only GlcNAc-GlcNAc-GlcN-GlcNAc under these experimental conditions. Since some chitin deacetylases from *Mucor rouxii* (35) and *Colletotrichum lindemuthianum* (36) fully deacetylate GlcNAc$_4$, the enzymatic reaction mechanism of PdaC seems to differ.

Divalent cations (Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$) except Mn$^{2+}$ and Ni$^{2+}$ did not affect on deacetylase activity by PdaC toward GlcNAc$_4$ (Table 1B and Supplemental Fig. S8). Especially PdaC dialyzed against EDTA still has deacetylase activity toward GlcNAc$_4$. *S. pneumoniae* R6 PgdA and *S. mutants* PgdA (homologue of *S. pneumoniae*) require divalent cations such as Zn$^{2+}$ for deacetylation of GlcNAc oligomers (7, 37). The activities of *B. cereus* GlcNAc deacetylases, BC_1960 and BC_3618, are inhibited by 1 mM Zn$^{2+}$ (38). Thus, PdaC deacetylase activity toward GlcNAc oligomers seems to be different from them.

**Deacetylation of *B. subtilis* peptidoglycan**—PdaC deacetylates MurNAc from *B. subtilis* peptidoglycan (Figs. 3 and 4) though the peptidoglycan contains not only MurNAc but also GlcNAc. *S. pneumoniae* R6 PgdA and *L. monocytogenes* PgdA are similar to the entire region of PdaC (Fig. 1) and both are identified as GlcNAc deacetylases in vivo (6, 7, 8). The other homologue, *L. lactis* PgdA (Fig. 1) is also identified as a GlcNAc deacetylase in vivo (9). Moreover, *S. pneumoniae* R6 PgdA also deacetylates a GlcNAc oligomer, GlcNAc$_3$, as a GlcNAc deacetylase (7). Thus, PdaC is a unique enzyme.

Interestingly, divalent cations strongly affected MurNAc deacetylase (Table 1A and Supplemental Fig. S3). PdaC dialyzed against EDTA showed weak deacetylase activity (15.1 µM). On the other hand, PdaC with 5 mM Mn$^{2+}$ displayed strong activity (92.4 µM). Since divalent cations are not required for GlcNAc$_4$ deacetylation, the deacetylation by PdaC toward peptidoglycan seems to be different from that toward GlcNAc oligomer.

PdaC had no deacetylase activity toward the -GlcNAc-MurNAc- polymer (Table 2). The difference between GlcNAc and MurNAc is the C3 position (MurNAc has a propionic acid group instead of a hydroxyl group). Moreover,
when the substrate had peptide side chains (L-Ala-D-Glu) covalently bound to the glycan strand, the deacetylase activity by PdaC increased compared with the substrate with no peptide side chains (Table 2). Thus, the C3 position of the amino sugar is very important for the deacetylation reaction and/or substrate recognition by PdaC.

It has been reported that two of peptidoglycan modifications, O-acetylation on the C-6 hydroxyl moiety of MurNAc residues and N-deacetylation of GlcNAc and MurNAc residues, are involved in lysozyme resistance of peptidoglycan (2, 6, 39). Since the pdaC mutant is sensitive to lysozyme treatment (Fig. 2) and PdaC is a deacetylase (Figs. 3 to 7), PdaC deacetylates MurNAc and/or GlcNAc in vivo. Atrih et al. reported that B. subtilis 168 peptidoglycan contained deacylated residues of GlcNAc at 17.3% (per muropeptides prepared from peptidoglycan digested with a muramidase) (24). In contrast, Zipperle et al. reported that B. subtilis peptidoglycan contained both deacylated muramic acid residues (33% per total muramic acid) and deacylated glucosamine residues (19% per total glucosamine) (40). Muramic acid residues have also been identified in peptidoglycans from B. cereus, B. anthracis and B. thuringiensis (40) and S. pneumoniae (6). Since PdaC is found in B. subtilis membrane by proteome analysis (31) and is not detected in the cell surface and extracellular fractions (41, 42), at least PdaC seems to work on the membrane as a MurNAc deacetylase.

**Structural comparison among PdaC, PdaA and PgdA**—The polysaccharide deacetylase domains of PdaC and PdaA in *B. subtilis* and PgdA in *S. pneumoniae* R6 are similar (Fig. 1). Moreover, the other domains of PdaC are conserved in PgdA (Fig. 1) though these domains have unknown function(s). The predicted secondary and tertiary structures of PdaC were created using the ESyPred3D algorithm. The results indicated that the secondary and tertiary structures of PdaC were very similar to PdaA and PgdA (Supplemental Fig. S11 and data not shown). The catalytic residues in PgdA (7) are conserved in PdaC and PdaA (in Supplemental Fig. S11). Blair et al. described that the Asp-276, His-326 and His-330 in PgdA interact with a zinc-ligand, and a zinc ion is necessary for the deacetylase activity of PgdA (7). These amino acid residues are also conserved in *B. subtilis* PdaC and PdaA (closed triangles in Supplemental Fig. S11). Very recently *S. mutants* PgdA (homologue of *S. pneumoniae*) was reported to only digest a hexamer of N-acetylglucosamine, but not peptidoglycan, and required zinc ions for its activity (37). Therefore, the various deacetylase activities are regulated by ion type and substrate though their amino acid similarities are high.

**Peptidoglycan deacetylation by GlcNAc deacetylases**—Pathogenic Gram-positive bacteria, *S. pneumoniae* and *L. monocytogenes*, have the PgdA proteins consisting of the three domains like PdaC (Fig. 1) that are associated with virulence (8, 43, 44). *C. phytofermentas* also has the protein similar to PdaC (Cphy_3069) consisting of the three domains (Fig. 1), however the function is unknown. *E. faecalis* PgdA, EF_0108, lacks the three domain structure (Fig. 1) and is not characterized,
though an other deacetylase, EF_1843 has been studied (45).

It has also been elucidated that several pathogenic bacteria contain deacetylases, though the enzymes do not contain the three domain structure. Severin et al. demonstrated that a pathogenic bacterium, *B. cereus* has the deacetylated peptidoglycan (46). Psylinakis et al. described that the BC_1960 and BC_3618 proteins in *B. cereus* are peptidoglycan GlcNAc deacetylases, and these enzymes contribute to increasing resistance to lysozyme digestion (38). Interestingly, Gram-negative pathogenic bacterium, *Helicobacter pylori*, induces a peptidoglycan deacetylase, HP310 (PgdA) under oxidative stress condition (47) and the deacetylation contributes to the bacterial survival by mitigating host immune responses (48). However, no MurNAc deacetylase activities have been characterized in these bacteria.

This paper describes for the first time a unique enzyme that has both GlcNAc and MurNAc deacetylase activities. Moreover, this is only the second MurNAc deacetylase to be reported aside from *B. subtilis* PdaA, which exhibits very narrow substrate specificity.

Acknowledgments—We thank Dr. H. Karasawa (Nagano Prefecture General Technology Center, Nagano, Japan) for helping with determination of the molecular weights by ESI-MS and ESI-MS-MS. We also thank Ms. M. Sakai for researching several deacetylase activities.

REFERENCES


**FOOTNOTES**

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1on-line version of this article (available at hyyp://www.jbc.org) contains Supplemental Tables S1-S4 and Figs. S1-S11.

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3Abbreviations used are: PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high performance liquid chromatography; aa, amino acid(s); LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; NPB, NaCl-contained phosphate buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; GlcNAcr, GlcNAc with a reduced end; MurNAcr, MurNAc with a reduced end; GlcN, glucosamine; Mur, muramic acid; ESI-MS, electrospray ionization-mass spectrometry; TFA, trifluoroacetic acid; 2S1P, disaccharide-peptide; 4S2P, tetrasaccharide-dipeptide; TM, transmembrane.
Figure 1. **Domain structures of* B. subtilis* PdaC and its similar proteins containing a polysaccharide deacetylase domain.** PgdA in *S. pneumoniae* R6 consists of three domains that are shown in *shadow* and *grey rectangles*, and *black arrow*, judged from the protein structure (7). *Black rectangles* with or without *arrows* are polysaccharide deacetylase domains. The PgdA proteins in *S. pneumoniae* R6, *L. monocytogenes* and *L. lactis* are identified as *N*-acetylglucosamine deacetylases (6, 7, 8, 9) and *B. subtilis* PdaA is identified as an *N*-acetylmuramic acid deacetylase (11, 26). The numbers indicate positions of amino acid residues with respect to the first amino acid of those proteins. The *percentages* without or with *parentheses* are amino acid similarities compared with *S. pneumoniae* R6 PgdA and *B. subtilis* PdaC, respectively. *Streptococcus pneumoniae* R6, *S. pneumoniae* R6 PgdA (Q8DP63); *Listeria monocytogenes*, *L. monocytogenes* PgdA (lmo0415); *Clostridium phytofermentans*, *C. phytofermentans* protein (Cphy_3069); *Lactococcus Lactis*, *L. Lactis* PgdA (XynD); *Enterococcus faecalis*, *E. faecalis* protein (EF_0108).

Figure 2. **Growth curve of deacetylase mutants toward lysozyme.** The growth rates of these mutants in LB medium were measured with a spectrophotometer, and after 6 hours of incubation (arrow), 10 µg/ml of lysozyme (final concentration) was added into the cultures. *Open symbols* and × with *broken lines*, and *closed symbols* and × with *normal lines*, are growth curves without and with lysozyme treatment, respectively. Symbol ×, 168 strain (wild-type); circles, pdaC (yjeA) mutant (JEATdd); diamonds, yheN mutant (HENCdd); triangles, ylxY mutant (YLXYd); squares, yxkH mutant (XKHKdd).

Figure 3. **Deacetylation of glycan strands consisting of \((-\text{GlcNAc-MurNAc-}[L-Ala-D-Glu])\)\(_n\) (panel A) and reduced tetrasaccharide-dipeptides (panel B) by PdaC.** (A) After the purified glycan strands were deacetylated with PdaC or without PdaC (control), they were digested with the N-terminal domain of CwIT, which is a muramidase (25). Both samples were reduced by NaBH\(_4\) and then separated by RP-HPLC as described under “Experimental procedures”. “Control” sample contains PdaC only. The peak 1 (retention time is 19 min) and peak 3 (retention time is 36 min) materials were identified as GlcNAc-MurNAc(-L-Ala-D-Glu) (reduced 2S1P) by ESI-MS analysis (Supplemental Fig. S4A and B) and GlcNAc-MurNAc(-L-Ala-D-Glu)-GlcNAc-MurNAc(-L-Ala-D-Glu) (reduced 4S2P) by ESI-MS and ESI-MS-MS analyses (Supplemental Fig. S5A to D), respectively. The
peak 2 material (retention time is 31 min) exhibited in the “With PdaC” sample was a new product by PdaC, and the material was identified as GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-L-Ala-D-Glu) by ESI-MS (Supplemental Fig. S6A and B) and ESI-MS-MS analyses (Fig. 4A and B). (B) The reduced substrate, tetrasaccharide-dipeptides (peak 3 material) was deacetylated with PdaC or without PdaC, followed by separation by RP-HPLC. “Control” sample contains PdaC only. The peak 3 materials in the “With PdaC” and “Without PdaC” are the same although their retention times are slightly different. The produced peak 2 material in the “With PdaC” sample was identified as GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-L-Ala-D-Glu) by ESI-MS and ESI-MS-MS analyses in the negative mode (Supplemental Fig. S7A and B). (C) RP-HPLC of the reduced fragments of mutanolysin digests. Deacetylated or non-deacetylated reduced-4S2P material was digested with a muramidase, mutanolysin, followed by reduction with NaBH₄. Peak 1, reduced 2S1P; peak 2, GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-L-Ala-D-Glu). The arrow indicates the elution position of reduced 4S2P.

Figure 4. ESI-MS and ESI-MS-MS analyses of GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-L-Ala-D-Glu). (A and B) The peak 2 material was analyzed by ESI-MS (Supplemental Fig. S6A and B) and ESI-MS-MS in the positive and negative modes (panels A and B, respectively). The fragment peaks b2, b3, and y2 correspond to the structure of deacetylated compound of reduced 4S2P (panel C). (C) Identified structure and molecular weight of each fragment in the peak 2 material in Fig. 3A.

Figure 5. PdaC deacetylase activities with GlcNAc oligomers, chitin, chitosan, and B. subtilis peptidoglycan. Purified PdaC (final concentration, 10 µg/ml) was added into 50 mM HEPES buffer (pH 7.0) with 5 mM MnCl₂ containing various substrates (final concentration, 2 mg/ml), and then the sample was incubated for 4 hours at 37°C. The released acetic acid was measured with an F-kit. Standard error bars are calculated for three independent experiments.

Figure 6. PdaC catalyzed deacetylation of GlcNAc₄. (A and B) GlcNAc₄ was deacetylated with (panel A) or without (panel B) PdaC, and then the samples were separated using a TSKgel Amide-80 column by normal-phase HPLC. After the tetrasaccharide fraction had been collected and freeze dried, the sample was separated by RP-HPLC. The peak 1 (retention time is 6 min) material was identified as GlcNAc-GlcNAc-GlcN-GlcNAc (Mr, 788.5) by ESI-MS (panel C) and ESI-MS-MS (panel D) analyses. The peak 2 (retention time is 7.5 min) and peak
3 (retention time is 8.5 min) materials were identified as GlcNAc₄ by ESI-MS and ESI-MS-MS analyses (data not shown). (C and D) ESI-MS (panel C) and ESI-MS-MS (panel D) analyses of the peak 1 material in the positive mode. The fragment peaks b1, b2 and b3 in panel D correspond with the GlcNAc-GlcNAc-GlcN-GlcNAc structure (panel E). (E) Identified structure and molecular weight of each fragment in the peak 1 material. Ion series b and y, and b' and y' are corresponding to non-reduced fragment peak 1 material (panel D) and reduced fragment peak 1 material (Supplemental Fig. S9B), respectively.

Figure 7. Kinetics with GlcNAc₄ and B. subtilis peptidoglycan as substrates. (A and B) Released acetic acid by PdaC for 2 mg/ml GlcNAc₄ (panel A) and 2.5 mg/ml peptidoglycan (panel B) substrates from 0 to 90 min was measured with an F-kit. Standard error bars are calculated for three independent experiments. (C) Initial velocities were measured after 30 min, and the rates with different concentration of substrates were fit to Michaelis-Menten kinetics. The released acetic acid was measured with an F-kit. Standard error bars are calculated for three independent experiments. Open circles, peptidoglycan substrate; closed squares, GlcNAc₄ substrate.
### TABLE 1A Effect of divalent cations on the deacetylase activity of PdaC for peptidoglycan

<table>
<thead>
<tr>
<th>Divalent cation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Released acetic acid (μg/ml) [μM]&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native PdaC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.90 [31.5]</td>
</tr>
<tr>
<td>EDTA-treated PdaC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.91 [15.1]</td>
</tr>
<tr>
<td>+ Mn&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>5.58 [92.4]</td>
</tr>
<tr>
<td>+ Zn&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>1.93 [31.9]</td>
</tr>
<tr>
<td>+ Mg&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>2.57 [42.6]</td>
</tr>
<tr>
<td>+ Ni&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>1.81 [30.0]</td>
</tr>
<tr>
<td>+ Ca&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>3.59 [59.4]</td>
</tr>
<tr>
<td>+ Co&lt;sup&gt;2⁺&lt;/sup&gt;</td>
<td>Not determined&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Deacetylation was performed with or without 5 mM cations except Zn<sup>2⁺</sup> (5 μM) containing 2.5 mg/ml peptidoglycan and 10 μg/ml PdaC in 50 mM HEPES (pH 7.0) for 4 hours at 37°C. Released acetic acid for 4 hours incubation was measured with an F-kit. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 hours) are shown in Supplemental Fig. S3. **Native PdaC** indicates that PdaC was dialyzed against 20 mM sodium phosphate buffer (no EDTA treatment). **EDTA-treated PdaC** indicates that PdaC was dialyzed against 20 mM EDTA twice and then dialyzed against 20 mM sodium phosphate buffer. Thus, the solution does not contain divalent cations. Each cation (5 mM except 5 μM Zn<sup>2⁺</sup>) was added to EDTA-treated PdaC. **Since Co<sup>2⁺</sup> interfered with the assay kit, the released acetic acid in the presence of Co<sup>2⁺</sup> was not determined.**
<table>
<thead>
<tr>
<th>Divalent cation&lt;sup&gt;α&lt;/sup&gt;</th>
<th>Released acetic acid (µg/ml) [µM]&lt;sup&gt;β&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native PdaC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.67 [27.7]</td>
</tr>
<tr>
<td>EDTA-treated PdaC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.17 [19.4]</td>
</tr>
<tr>
<td>+ Mn&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>2.73 [45.1]</td>
</tr>
<tr>
<td>+ Zn&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>1.59 [26.3]</td>
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<tr>
<td>+ Mg&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>1.65 [27.3]</td>
</tr>
<tr>
<td>+ Ni&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>0.65 [10.8]</td>
</tr>
<tr>
<td>+ Ca&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>1.75 [28.9]</td>
</tr>
<tr>
<td>+ Co&lt;sup&gt;2+&lt;/sup&gt;e</td>
<td>Not determined&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
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</table>

<sup>α</sup>Deacetylation was performed with or without 5 mM cations except Zn<sup>2+</sup> (5 µM) containing 2 mg/ml GlcNAc<sub>4</sub> and 10 µg/ml PdaC in 50 mM HEPES (pH 7.0) for 4 hours at 37°C. <sup>β</sup>Released acetic acid for 4 hours incubation was measured with an F-kit. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 hours) are shown in Supplemental Fig. S8. Explanations for <sup>c,d,e,f</sup> are shown in the footnote of Table 1A.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Released acetic acid (µg/ml) [µM]</th>
</tr>
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<tbody>
<tr>
<td><strong>Peptidoglycan</strong></td>
<td></td>
</tr>
<tr>
<td>(-GlcNAc-MurNAc[L-Ala-D-Glu-A2pm-D-Ala]-3)_n cross-linked between D-Ala and A2pm</td>
<td>6.0±1.3 [99.9±21.6]</td>
</tr>
<tr>
<td><strong>Peptidoglycan digested by D,L-endopeptidase (LytF [CwlE])</strong></td>
<td>10.7±0.17 [178.2±2.8]</td>
</tr>
<tr>
<td>(-GlcNAc-MurNAc[L-Ala-D-Glu]-)_n</td>
<td></td>
</tr>
<tr>
<td><strong>Peptidoglycan digested by L,D-endopeptidase (CwlK)</strong></td>
<td></td>
</tr>
<tr>
<td>(-GlcNAc-MurNAc[L-Ala]-)_n</td>
<td>3.25±0.35 [54.1±5.8]</td>
</tr>
<tr>
<td><strong>Peptidoglycan digested by L-alanine amidase (CwlH)</strong></td>
<td></td>
</tr>
<tr>
<td>(-GlcNAc-MurNAc)-_n</td>
<td>&lt; 0.03 [&lt; 0.5]</td>
</tr>
</tbody>
</table>

`a`The substrate was prepared from *B. subtilis* peptidoglycan digested with 12.5 µg/ml D,L-endopeptidase, L,D-endopeptidase, L-alanine amidase or no enzyme (control) at 37°C overnight, followed by boiling. `b`Deacetylation was performed with 5 mM MnCl$_2$ containing 2.5 mg/ml peptidoglycan and 10 µg/ml PdaC in 50 mM HEPES (pH 7.0) for 4 hours at 37°C, and then the released acetic acid was measured with an F-kit. Standard errors were calculated for three independent experiments.
Fig. 1. Kobayashi et al.

- **Bacillus subtilis PdaC**: 467 a.a.
- **Streptococcus pneumoniae R6**: 463 a.a.
- **Listeria monocytogenes**: 466 a.a.
- **Clostridium phytofermentans**: 465 a.a.
- **Lactococcus Lactis**: 372 a.a.
- **Enterococcus faecalis**: 516 a.a.

**Similarity Percentages**:
- Bacillus subtilis Pdam: 41.5%
- Streptococcus pneumoniae R6: 40.4% (42.9%)
- Listeria monocytogenes: 40.5% (51.3%)
- Clostridium phytofermentans: 37.8% (42.3%)
- Lactococcus Lactis: 39.6% (38.6%)
- Enterococcus faecalis: 32.8% (34.9%)

**Additional Proteins**:
- B. subtilis PdaB: 254 a.a.
- B. subtilis YheN: 282 a.a.
- B. subtilis YxkH: 279 a.a.
Fig. 2. Kobayashi et al.
Fig. 3A, B, and C. Kobayashi et al.
Fig. 4A, B and C. Kobayashi et al.
Fig. 5. Kobayashi et al.
Fig. 6A and B. Kobayashi et al.
(C) MS analysis of peak 1 material in the positive mode

(D) MS-MS analysis of peak 1 material in the positive mode

(E) Reduced end

Non-reduced end

Fig. 6C, D and E. Kobayashi et al.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µM)</th>
<th>$k_{cat}$ (S$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$S$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc$_4$</td>
<td>12.3±1.84</td>
<td>0.046±0.0059</td>
<td>0.24±0.031</td>
<td>0.020±0.0010</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>4.8±0.30</td>
<td>0.060±0.0020</td>
<td>0.32±0.010</td>
<td>0.067±0.0032</td>
</tr>
</tbody>
</table>