Identification and Characterization of a Novel Polysaccharide Deacetylase C (PdaC) from *Bacillus subtilis*<sup>‡1</sup>

Received for publication, December 5, 2011, and in revised form, January 23, 2012. Published, JBC Papers in Press, January 25, 2012, DOI 10.1074/jbc.M111.329490

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**Background:** Peptidoglycan modification is a 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.

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[-L-Ala-D-Glu]-)<sub>10</sub>. 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 (Mn<sup>2+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>). The kinetic values of the activity toward *B. subtilis* peptidoglycan were *K<sub>m</sub> = 4.8 mM and *k<sub>cat</sub> = 0.32 s<sup>-1</sup>*. PdaC also deacetylated N-acetylglucosamine (GlcNAc) oligomers with a *K<sub>m</sub> = 12.3 mM and *k<sub>cat</sub> = 0.24 s<sup>-1</sup> toward GlcNAc<sub>4</sub>. 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 GlcNAc<sub>4</sub>. 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 (*Bacillus 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 *vivo* (11). Blair and van Aalten (12) published the crystal structure of PdaA. 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 (14–16). 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 YJEA-SD and YJEA-RV primers, digested with Sall
and MunI, and then ligated to the Sall-EcoRI site of pBlue-scriptII SK(+) resulting in pBLJEA. A plasmid, pDG1515 was digested with XbaI and Sall, and the fragment containing a tetracycline cassette was blunted and then ligated to the EcoRV site of pBlue-scriptII SK(+), resulting in pBLTC. The plasmid, pBLTC, was digested with HindIII and HindIII and the fragment containing the tetracycline cassette was ligated into the HindIII site of pBLJEA, resulting in pBLJEATC.

The yehN 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 Smal and HindIII, and the fragment containing the cassette was ligated in the Eco47III site of pGMHEN, resulting in pGMHENCM.

The ykxH gene fragment amplified by PCR with YYKH-SD and YYKH-RV primers was blunt end phosphorylated with a BLK kit (Takara) and then ligated to the EcoRV site of pBlue-scriptII SK(+), resulting in pBLYKH. To eliminate the Clal site of pBLXKH, the plasmid was digested with HindIII and HindII, blunted, and then self-ligated, resulting in pBLAXKH. A plasmid, pDG792 containing a kanamycin cassette, was digested with Clal and the fragment containing the cassette was ligated to the corresponding site of pBLAXKH, resulting in pBLAXKHM. B. subtilis 168 was transformed with the linearized pBLJEATC, pGMHENCM, and pBLAXKHM, resulting in JEATdd (pdaC::tet), HENCdd (yehN::cat), and XKHdd (ykxH::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 pM4LXY. B. subtilis 168 was transformed with the concatenated pM4LXY derived from Escherichia coli C600 (pM4LXY) by single crossing over recombination, resulting in the 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 pM4LXY. B. subtilis 168 was transformed with the plasmid by single crossing over recombination, resulting in the PdaCp strain (PداCsp-pdaC). PdaC was expressed in the strain by isopropyl β-d-thiogalactopyranoside (IPTG) addition.

Construction of a Plasmid, pQE30AyeA for Overexpressing Truncated PdaC—The truncated pdaC gene fragment amplified by PCR with yjeA+87F and yjeA+1383R primers was digested with SphiI and Sall, and then ligated to the corresponding sites of pQE-30, resulting in pQE30AyeA. This plasmid was utilized to overexpress truncated PdaC (from amino acids 30 to 467).
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 buffer (pH 7.0) containing 5 mM MnCl₂, ZnCl₂, MgCl₂, NiSO₄, or CaCl₂, 5 μM ZnCl₂, or 50 mM ZnCl₂ was digested by 10 μg/ml of PdaC (final concentration) for 1, 2, 3, and 4 h 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 h 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% CH₂CN; flow rate, 0.5 ml/min; monitoring wavelength, 202 nm; Shimadzu LC-10Avp HPLC system), and then the tetrasaccharide peak material was collected and freeze-dried. The sample was solubilized in 0.05% TFA, and then 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% CH₂CN. 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 h 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 by 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 h. The samples were digested by 10 μg of the N-terminal domain of CwlT, which is a muramidase (25), at 37 °C for 4 h, followed by boiling. Borate buffer (pH 9.0) (final concentration, 0.5 μl) 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% CH₂CN. 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.

Decacylation with PdaC toward 4S2P—4S2P reduced with NaBH₄ was deacetylated with PdaC in 50 mM HEPES buffer (pH 7.0) containing 5 mM MnCl₂ at 37 °C for 4 h. Mutanolysin 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). The sample was reduced and separated by RP-HPLC as described above. The deacylated 4S2P was identified by ESI-MS and ESI-MS/MS.

Identification of Separated Peak Materials on RP-HPLC by ESI-MS/MS—The peak materials separated by RP-HPLC were freeze-dried and then solubilized in 50% CH₂CN with or without 0.05% TFA. The samples were identified by ESI-MS or ESI-MS/MS (Agilent 1100 series LC/MSD Trap VL).

Determination of DNase Activity of PdaC—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 MgCl₂) 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 Streptococcus pneumoniae 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 PdB play roles in sporulation (10, 28, 29) but the other gene products, YjeA (renamed PdaC), YlxY, YxxH, 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 because the growth rate was decreased (closed circles in Fig. 2). To confirm the pdaC mutant phenotype, a new strain, PdaCp (p̂_spa-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 because lysozyme cannot digest deacetylated peptidoglycan (30).

PdaC Deacetylates B. subtilis Peptidoglycan—Because 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, PdaC seems to have a transmembrane region (from amino acids 7 to 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 amino acids 30 to 467) lacking the transmembrane region was overexpressed in B. subtilis (wild-type) and then purified by affinity chromatography. As shown in supplemental Fig. S3, the truncated PdaC (53.0 kDa) could be purified containing 2.5 mg/ml of peptidoglycan and 10 mM sodium phosphate buffer (no EDTA treatment).

The deacetylase activity of PdaC toward peptidoglycan was determined in 50 mM HEPES (pH 7.0) for 4 h at 37 °C. Since Co2+ interfered with the assay kit, the released acetic acid in the presence of Co2+ was not determined.

The deacetylase activity of PdaC for peptidoglycan was measured with or without 5 mM cations except Zn2+. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 h) are shown in supplemental Fig. S3. Concentrations are indicated in parentheses.

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 mM Zn2+) was added to EDTA-treated PdaC.

Deacetylation was performed with or without 5 mM cations except Zn2+ (5 mM) containing 2.5 mg/ml of peptidoglycan and 10 mM NaCl of PdaC in 50 mM HEPES buffer (pH 7.0) for 4 h at 37 °C.

Released acetic acid for 4 h incubation was measured with an F-kit. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 h) are shown in supplemental Fig. S3. Concentrations are indicated in parentheses.

Native PdaC indicates that PdaC was dialyzed against 20 mM sodium phosphate buffer (no EDTA treatment).

Divalent cation

| Released acetic acid (μg/ml) |
|-----------------------------|
| Native PdaC | 1.90 (31.5) |
| EDTA-treated PdaC | 0.91 (15.1) |
| + Mn2+ | 5.58 (92.4) |
| + Zn2+ | 1.93 (31.9) |
| + Mg2+ | 2.57 (42.6) |
| + Ni2+ | 1.81 (30.0) |
| + Ca2+ | 3.59 (59.4) |
| + Co2+ | Not determined |

Transmembrane regions of a target protein, PdaC seems to have a transmembrane region (from amino acids 7 to 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 amino acids 30 to 467) lacking the transmembrane region was overexpressed in E. coli and then purified by affinity chromatography. As shown in lane 3 in supplementary 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 1 and

### Table 1

| Divalent cation | Released acetic acid (μg/ml) |
|-----------------|-----------------------------|
| Native PdaC     | 1.90 (31.5) |
| EDTA-treated PdaC | 0.91 (15.1) |
| + Mn2+          | 5.58 (92.4) |
| + Zn2+          | 1.93 (31.9) |
| + Mg2+          | 2.57 (42.6) |
| + Ni2+          | 1.81 (30.0) |
| + Ca2+          | 3.59 (59.4) |
| + Co2+          | Not determined |

Deacetylation was performed with or without 5 mM cations except Zn2+ (5 mM) containing 2.5 mg/ml of peptidoglycan and 10 mM sodium phosphate buffer (pH 7.0) for 4 h at 37 °C. Released acetic acid for 4 h incubation was measured with an F-kit. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 h) are shown in supplemental Fig. S3. Concentrations are indicated in parentheses.

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 mM Zn2+) was added to EDTA-treated PdaC.

Since Co2+ interfered with the assay kit, the released acetic acid in the presence of Co2+ was not determined.

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 because lysozyme cannot digest deacetylated peptidoglycan (30).

**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 gray rectangles, and a 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-acetylmuramyl-dipeptide deacetylases (6–9) and cosamine deacetylases (6–9) and are polysaccharide deacetylase domains. The PgdA proteins in *C. phytofermentans* Lmo0415; *C. phytofermentans*, and *C. phytofermentans* protein (Cphy_3069); *L. lactis*, *L. lactis* PgdA (XynD); *E. 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 h 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 (jeaA) mutant (JEATdd); diamonds, yheN mutant (HENCdd); triangles, ylyX mutant (YLYYd); squares, yxkH mutant (XKHKdd).
TABLE 2

| Substrate*                                                                 | Released acetic acid† (μg/ml) |
|---------------------------------------------------------------------------|------------------------------|
| Peptidoglycan                                                             |                              |
| (-GlcNAc-MurNAc-L-Ala-D-Glu-A,pm-d-Ala)_n, cross-linked between d-Ala and A,pm |                              |
| Peptidoglycan digested by \(\alpha\)-endopeptidase (CwlK) (23)             |                              |
| (-GlcNAc-MurNAc-L-Ala-D-Glu)_n                                            |                              |
| Peptidoglycan digested by \(\beta\)-endopeptidase (CwlK)                  |                              |
| (-GlcNAc-MurNAc-L-Ala)_n                                                 |                              |
| Peptidoglycan digested by \(\chi\)-alanine amidase (CwlH)                 |                              |
| (-GlcNAc-MurNAc-MurNAc)_n                                               |                              |

*The substrate was prepared from \(B.\) subtilis peptidoglycan digested with 12.5 μg/ml of \(\alpha\)-endopeptidase, \(\beta\)-endopeptidase, \(\chi\)-alanine amidase or with no enzyme (control) at 37 °C overnight, followed by boiling.
†Deacetylation was performed with 5 mM MnCl\(_2\) containing 2.5 mg/ml of peptidoglycan and 10 μg/ml of PdaC in 50 mM HEPES (pH 7.0) for 4 h at 37 °C, and then the released acetic acid was measured with an F-kit. Standard errors were calculated for three independent experiments.

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**Novel MurNAc Deacetylase from \(B.\) subtilis**

**TABLE 3**

| Divalent cation*            | Released acetic acid† (μg/ml) |
|-----------------------------|------------------------------|
| Native PdaC                 | 1.67 (27.7)                  |
| EDTA-treated PdaC\(^{a}\)   | 1.17 (19.4)                  |
| +Mn\(^{2+}\)                | 2.73 (45.1)                  |
| +Zn\(^{2+}\)               | 1.59 (26.3)                  |
| +Mg\(^{2+}\)               | 1.65 (27.3)                  |
| +Ni\(^{2+}\)               | 0.65 (10.8)                  |
| +Ca\(^{2+}\)               | 1.75 (28.9)                  |
| +Co\(^{3+}\)               | Not determined               |

*Deacetylation was performed with or without 5 mM cations except Zn\(^{2+}\) (5 μM) containing 2 mg/ml of GlcNAc\(_n\) and 10 μg/ml of PdaC in 50 mM HEPES (pH 7.0) for 4 h at 37 °C.
†Released acetic acid for 4 h incubation was measured with an F-kit. Released acetic acid amounts for other samples (incubation for 1, 2, and 3 h) are shown in supplemental Fig. S8. Concentrations are indicated in parentheses.
*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\(^{2+}\)) was added to EDTA-treated PdaC.
Since Co\(^{3+}\) interfered with the assay kit, the released acetic acid in the presence of Co\(^{3+}\) was not determined.

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**PdaC Works as a MurNAc Deacetylase toward Glycan Strands Containing \(L\)-Ala-\(\alpha\)-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 (I 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. S4, A 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. S5, A and B, respectively), corresponding to \([M + Na]^+\) and \([M − H]^−\) of reduced tetrasaccharide digieptide (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. S5, C, D, and E, and 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. The peak 2 material in the with PdaC sample gave fragment ions at \(m/z\) 1,358.7 and 1,334.6 in positive and negative modes, respectively, corresponding to \([M + Na]^+\) and \([M − H]^−\) of a deacetylase-combined product of reduced 4S2P (\(M_r\) 1,334.9) (supplemental Fig. S6, A and B, respectively). The peak 2 material was further analyzed by ESI-MS/MS in the positive and negative modes. As shown in Fig. 4, A 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[-L-Ala-D-Glu]) (Fig. 4C and supplemental Table S4), although the possibility that the structure is GlcN-MurNAc(-L-Ala-D-Glu)-GlcNAc-MurNAc(-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

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**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), \(\beta\)-endopeptidase (CwlK) (23), or \(\chi\)-alanine amidase (CwlF (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 \(\chi\)-endopeptidase (10.7 ± 0.17 μg/ml). PdaC exhibited less deacetylase activity toward peptidoglycan digested with \(\beta\)-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. 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 because PdaA can digest only (-GlcNAc-MurNAc\(_n\)) \(_n\).
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, -GlcN-MurNAc - GlcN-MurNAc, but not -GlcN-MurNAc-MurNAc (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-MurNAc(-L-Ala-D-Glu). Moreover, when the *peak 2* material in Fig. 3A (predicted GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAc(-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 (GlcNAc₉₋₅), 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 GlcNAc₅ and GlcNAc₆, whereas it did not deacetylate GlcNAc and had reduced activities toward GlcNAc₇ and GlcNAc₈. PdaC was essentially not active toward chitosan and had very low activity toward chitin (Fig. 5). These results suggest that PdaC deacetylates chitin oligomers (GlcNAc₉₋₅) 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 3 and supplemental Fig. S8, PdaC showed the maximum activity with Mn²⁺. Therefore, the result suggests that PdaC in the presence of Mn²⁺ efficiently deacylates not only *B. subtilis* peptidoglycan but also the 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. 6, A 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

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**FIGURE 3.** Deacetylation of glycan strands consisting of (-GlcNAc-MurNAc(-L-Ala-D-Glu))- (panel A) and reduced tetrasaccharide dipetides (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 CwlT, which is a muramidase (25). Both samples were reduced by NaBH₄, 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. S4, A 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. S5, A–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-MurNAc(-L-Ala-D-Glu) by ESI-MS (supplemental Fig. S6, A and B) and ESI-MS/MS analyses (see Fig. 4, A and B). B, the reduced substrate, tetrasaccharide dipetides (*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-MurNAc(-L-Ala-D-Glu) by ESI-MS and ESI-MS/MS analyses in the negative mode (supplemental Fig. S7, A 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-MurNAc(-L-Ala-D-Glu). The arrow indicates the elution position of reduced 4S2P.
In Fig. 6A, in addition to peaks 2 and 3. Peaks 2 and 3 in Fig. 6, A and B, had a fragment ion at \( m/z \) 832.1 by ESI-MS in the positive mode, corresponding to \([M + H]^+\) \( M_r 830.5 \) (data not shown), and ESI-MS/MS analysis indicated that the sample was actually GlcNAc\(_4\) (data not shown). The reason for the different elution times (peaks 2 and 3) is unknown, but it may reflect the difference of \( \alpha \) and \( \beta \) anomers at the reducing end as described previously (32).

Figure 4. ESI-MS and ESI-MS/MS analyses of GlcNAc-Mur(-L-Ala-D-Glu)-GlcNAc-MurNAc(-L-Ala-D-Glu). A and B, peak 2 material was analyzed by ESI-MS (supplemental Fig. S6, A and B) and ESI-MS/MS in positive and negative modes (panels A and B, respectively). The fragment peaks b2, b3, and y2 correspond to the structure of the 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 \( \mu \)g/ml) was added into 50 mM HEPES buffer (pH 7.0) with 5 mM MnCl\(_2\) containing various substrates (final concentration, 2 mg/ml), and then the sample was incubated for 4 h at 37 °C. The released acetic acid was measured with an F-kit. Standard error bars are calculated for three independent experiments.
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(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
corresponding to [M + H]+ of a reduced compound of deacetylated GlcNAC4 (M, 790.5) (supplemental Fig. S9A), and the fragments (b22 and b33 in supplemental Fig. S9B) by ESI-MS/MS strongly support that the structure of the reduced compound of deacetylated GlcNAC4 is GlcNAC-GlcNAC-GlcNAc-GlcNAc (Fig. 6E).

MurNAc and GlcNAC Deacetylation by PdA—PdA has both MurNAc and GlcNAC deacetylase activities; however, Fig. 5 shows that the activity toward MurNAc seems to be higher than toward GlcNAc, because the released acetic acid from peptidoglycan and GlcNAC oligomers are different (released acetic acid, 83.4 μM from peptidoglycan and 23.1 μM from GlcNAC4). To determine the kinetics for GlcNAC and MurNAc deacetylation, GlcNAC4 and peptidoglycan were utilized as substrates, and the released acetic acid was measured. For the purpose of the kinetics for peptidoglycan, the molarity was calculated using the assumption that peptidoglycan consists of a repeating unit, GlcNAC-MurNAc-t-Ala-d-Glu-A2pm (Mr 868.8), because this is the main unit contained in B. subtilis peptidoglycan (24). Initial velocities for GlcNAC4 and peptidoglycan were measured after a 30-min incubation at different substrate concentrations because the rates of released acetic acid from both substrates were constant within at least 30 min (Fig. 7, A and B), and the measured initial velocities fit Michaelis-Menten kinetics. The Km for GlcNAC4 and peptidoglycan were 12.3 ± 1.84 and 4.8 ± 0.30 mM, respectively, and the values of kcat for GlcNAC4 and peptidoglycan were 0.24 ± 0.031 and 0.32 ± 0.010 s−1, respectively (Fig. 7C). The value of Km/kcat for peptidoglycan (0.067 mm−1 s−1) is much larger than the value for GlcNAC4 (0.020 mm−1 s−1), and non-treated peptidoglycan was utilized instead of peptidoglycan treated with dl-endopeptidase, although the treated peptidoglycan (−GlcNAC-MurNAc[-t-Ala-d-Glu]-) is a more suitable substrate for PdA (Table 2). Thus, PdA seems to prefer to deacetylate MurNAc from peptidoglycan. Moreover, because GlcNAC3 deacetylation by S. pneumoniae PgdA had values of Km = 3.8 mm, kcat = 0.55 s−1, and kcat/Km = 0.15 mm−1 s−1 (7), the deacetylation toward GlcNAC by PdA was much weaker than that by PgdA.

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

DISCUSSION

Surprisingly, PdA from B. subtilis exhibited MurNAc deacetylase activity toward B. subtilis peptidoglycan. Moreover, PdA also had GlcNAC deacetylase activity toward GlcNAC oligomers. Therefore, potentially PdA can deacetylate both amino sugars, GlcNAC and MurNAc. According to the classification of Henrissat and co-workers (34), PdA, PgdA, and PdA belong to the carbohydrate esterase 4 family, which includes peptidoglycan GlcNAC deacetylases, rhizobial NodB chito-oligosaccharide deacetylases, chitin deacetylases, acetyl-xylan

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FIGURE 6. PdA catalyzed deacetylation of GlcNAC4. A and B, GlcNAC4 was deacetylated with (panel A) or without (panel B) PdA, 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 (M, 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 the 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 PdA for 2 mg/ml GlcNAC4 (panel A) and 2.5 mg/ml of 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 concentrations of substrate 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.
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esters, 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$_4$. PdaC has also been identified as GlcNAc deacetylase (GlcNAc$_{4-}$) (35) and Conalbumin (36) fully deacetyl 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 3 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$_{5,618}$, 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), although 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 (6–8). 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 1 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). Because divalent cations are not required for GlcNAc$_4$ deacetylation, the deacetylation by PdaC toward peptidoglycan seems to be different from that toward the 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 propionyl 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 was 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-acetyltransfer on the C-6 hydroxyl moiety of MurNAc residues and O-deacetylation of GlcNAc and MurNAc residues, are involved in lysozyme resistance of peptidoglycan (2, 6, 39). Because the pdaC mutant is sensitive to lysozyme treatment (Fig. 2) and PdaC is a deacetylase (Figs. 3–7), PdaC deacetylates MurNAc and/or GlcNAc in vivo. Atri et al. (24) reported that B. subtilis 168 peptidoglycan contained deacetylated residues of GlcNAc at 17.3% (per muropeptides prepared from peptidoglycan digested with a muramidase). In contrast, Zipperle et al. (40) reported that B. subtilis peptidoglycan contained both deacetylated muramic acid residues (33% per total muramic acid) and deacetylated glucosamine residues (19% per total glucosamine). Muramic acid residues have also been identified in peptidoglycans from Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis (40). Because PdaC is found in the 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) although 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 sites of PgdA (7) are conserved in PdaC and PgdA (in supplemental Fig. S11). Blair et al. (7) 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. 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-acetylg glucosamine, but not peptidoglycan, and required zinc ions for its activity (37). Therefore, the various deacetylase activities are regulated by ion type and substrate although 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). Clostridium phytofermentas also has the protein similar to PdaC (Cphy_3069) consisting of the three domains (Fig. 1), however, the function is unknown. Enterococcus faecalis PgdA, EF_0108, lacks the three-domain structure (Fig. 1) and is not characterized, although another deacetylase, EF_1843, has been studied (45).

It has also been elucidated that several pathogenic bacteria contain deacetylases, although the enzymes do not contain the three-domain structure. Severin et al. (46) demonstrated that a pathogenic bacterium, B. cereus has the deacetylpeptidoglycan. Psylinakis et al. (38) 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. Interestingly, the Gram-negative pathogenic bacterium, Helicobacter pylori, induces a peptidoglycan deacetylase, HP310 (PgdA) under oxidative stress conditions (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 M. Sakai for researching several deacetylation activities.

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