Identification and characterization of a novel polysaccharide deacetylase C (PdaC) from *Bacillus subtilis*[*] [S]

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Running title: *Novel MurNAc deacetylase from B. 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[-L-Ala-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 (Mn^{2+}, Mg^{2+}, Ca^{2+}). 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

$GlcNAc_4$. 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 $GlcNAc_6$. 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 YJEA-SD and YJEA-RV primers, digested with SalI and MunI and then ligated to the SalI-EcoRI site of pBluescriptII-SK(+), resulting in pBLJEA. 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 pBLJEA, 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.

B. subtilis 168 was transformed with the linearized pBLJEATC, pGMHENCM and pBLΔXKHKM, resulting in JEATdd (*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 (P_{spac}-*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 $MnCl_2$, 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_2pm (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 $GlcNAc_4$, 2 mg/ml $GlcNAc_4$ (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 $MnCl_2$ 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 $GlcNAc_4$ or 2.5 mg/ml of peptidoglycan (final concentration) in 50 mM HEPES (pH 7.0) containing 5 mM $MnCl_2$, $ZnCl_2$, $MgCl_2$, $NiSO_4$, or $CaCl_2$, 5 μM $ZnCl_2$, or 50 nM $ZnCl_2$ was digested by 10 μg/ml of PdaC

(final concentration) for 1, 2, 3 and 4 hours at 37°C. Five mM $CoCl_2$ was also utilized for analysis of deacetylase activity with an F-kit. However, Co^{2+} 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 Co^{2+}. 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 $GlcNAc_4$ by PdaC–After $GlcNAc_4$ (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 $MnCl_2$, the sample was separated by normal-phase HPLC [TSKgel Amide-80 column (TOSOH); flow buffer, 0.02% TFA containing 70% CH_3CN; 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% CH_3CN. 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).

Deacetylation 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 $MnCl_2$. 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 $NaBH_4$ 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_3CN. 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-MurNAcr(-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–4S2P reduced with $NaBH_4$ was deacetylated with PdaC in 50 mM HEPES buffer (pH 7.0) containing 5 mM $MnCl_2$ at 37°C overnight. The sample was digested with a muramidase, mutanolysin (Sigma-Aldrich) at 37°C for 4 hours. 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 deacetylated 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% CH_3CN 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 $MgCl_2$] 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 $(\text{-GlcNAc-MurNAc[-L-Ala-D-Glu]-})_n$ is a suitable substrate for PdaC and that its substrate specificity is very different from PdaA since PdaA can digest only $(\text{-GlcNAc-MurNAc-})_n$ (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 $(\text{-GlcNAc-MurNAc[-L-Ala-D-Glu]-})_n$. 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. 3*A*, 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. 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-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-MurNAcr(-L-Ala-D-Glu) (Supplemental Fig. S5*C*, *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. 3*A*). 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. 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-MurNAcr[-L-Ala-D-Glu]) (Fig. 4*C* and Supplemental Table S4) though the possibility that the structure is GlcN-MurNAc(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-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. 3*A*). Thus it was very likely that PdaC deacetylates 4S2P. Identified *peak 3* material (reduced 4S2P) as shown in Fig. 3*A* was utilized as a substrate for PdaC. As shown in Fig. 3*B*, 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-MurNAcr[-L-Ala-D-Glu]) (Supplemental Fig. S7*A* 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. 3*B*) 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. 3*C*, the *peak 3* material (reduced 4S2P) was completely digested to 2S1P and reduced 2S1P, followed by $NaBH_4$ 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-MurNAcr (-L-Ala-D-Glu). Moreover, when the *peak 2* material in Fig. 3*A* (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 ($GlcNAc_{2\text{-}5}$), 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 $GlcNAc_4$, and $GlcNAc_5$, while it did not deacetylate GlcNAc and had reduced activities toward $GlcNAc_2$, and $GlcNAc_3$. PdaC was essentially not active toward chitosan and had very low activity toward chitin (Fig. 5). These results suggest that PdaC deacetylates chitin oligomers ($GlcNAc_{4,\ 5}$) as an *N*-acetylglucosamine deacetylase.

The deacetylase activity of PdaC toward $GlcNAc_4$ (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 Mn^{2+}. Therefore, the result suggests that PdaC in the presence of Mn^{2+} efficiently deacetylates not only *B. subtilis* peptidoglycan but also GlcNAc oligomer, $GlcNAc_4$.

To identify the position of deacetylated GlcNAc by PdaC toward the chitin oligomer, $GlcNAc_4$ 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. 6*B*) and the deacetylated sample contained an additional peak (*peak 1* in Fig. 6*A*), 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 $[M+H]^+$ of $GlcNAc_4$ (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 α and β anomers at the reducing end as described previously (32). *Peak 1* in Fig. 6*A* had a fragment ion at *m/z* 790.0 by ESI-MS

in the positive mode, corresponding to $[M+H]^+$ of deacetylated $GlcNAc_4$ (M_r, 788.5) (Fig. 6*C*). Moreover, as shown in Fig. 6*D*, the substrate was identified by ESI-MS-MS as GlcNAc-GlcNAc-GlcN-GlcNAc (Fig. 6*E*). 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 $GlcNAc_4$ (M_r, 790.5) (Supplemental Fig. S9*A*), and the fragments (*b2′* and *b3′* in Supplemental Fig. S9*B*) by ESI-MS-MS strongly support that the structure of the reduced compound of deacetylated $GlcNAc_4$ is GlcNAc-GlcNAc-GlcN-GlcNAcr (Fig. 6*E*).

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 μM from peptidoglycan and 23.1 μM from $GlcNAc_4$). To determine the kinetics for GlcNAc and MurNAc deacetylation, $GlcNAc_4$ 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-A_2pm (M_r, 868.8), since this is the main unit contained in *B. subtilis* peptidoglycan (24). Initial velocities for $GlcNAc_4$ 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. 7*A* and *B*), and the measured initial velocities fit Michaelis-Menten kinetics. The K_m for $GlcNAc_4$ and peptidoglycan were 12.3±1.84 mM and 4.8±0.30 mM, respectively, and the values of k_{cat} for $GlcNAc_4$ and peptidoglycan were 0.24±0.031 s^{-1} and 0.32±0.010 s^{-1}, respectively (Fig. 7*C*). The value of K_m/k_{cat} for peptidoglycan (0.067 $mM^{-1}s^{-1}$) is much larger than the value for $GlcNAc_4$ (0.020 $mM^{-1}s^{-1}$), and non-treated peptidoglycan was utilized instead of peptidoglycan treated with D,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 $GlcNAc_3$ 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. S10*A*). 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. pneumonia* 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 deacetylated 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 deacetylated muramic acid residues (33% per total muramic acid) and deacetylated 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.

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REFERENCES

1. Vollmer, W., Blanot, D., and de Pedro, M. A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol. Rev.* **32,** 149-167
2. Vollmer, W. (2008) Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiol. Rev.* **32,** 287-306
3. Kafetzopoulos, D., Martinou, A., and Bouriotis, V. (1993) Bioconversion of chitin to chitosan: purification and characterization of chitin deacetylase from *Mucor rouxii*. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2564-2568
4. Tsigos, I. and Bouriotis, V. (1995) Purification and characterization of chitin deacetylase from *Colletotrichum lindemuthianum. J. Biol. Chem.* **270,** 26286-26291
5. Alfonso, C., Nuero, O. M., Santamaría, F., and Reyes, F. (1995) Purification of a heat-stable chitin deacetylase from *Aspergillus nidulans* and its role in cell wall degradation. *Curr. Microbiol.* **30,** 49-54
6. Vollmer, W. and Tomasz, A. (2000) The *pgdA* gene encodes for a peptidoglycan *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. *J. Biol. Chem.* **275,** 20496-20501
7. Blair, D. E., Schüttelkopf, A. W., MacRae, J. I., and van Aalten, D. M. (2005) Structure and metal-dependent mechanism of peptidoglycan deacetylase, a streptococcal virulence factor. *Proc.*

Natl. Acad. Sci, U. S. A. **102,** 15429-15434

8. Boneca, I. G., Dussurget, O., Cabanes, D., Nahori, M. A., Sousa, S., Lecuit, M., Psylinakis, E., Bouriotis, V., Hugot, J. P., Giovannini, M., Coyle, A., Bertin, J., Namane, A., Rousselle, J. C., Cayet, N., Prévost, M. C., Balloy, V., Chignard, M., Philpott, D. J., Cossart, P., and Girardin, S. E. (2007) A critical role for peptidoglycan *N*-deacetylation in *Listeria evasion* from the host innate immune system. *Proc. Natl. Acad. Sci, U. S. A.* **104,** 997-1002
9. Meyrand, M., Boughammoura, A., Courtin, P., Mézange, C., Guillot, A., and Chapot-Chartier, M. P. (2007) Peptidoglycan *N*-acetylglucosamine deacetylation decreases autolysis in *Lactococcus lactis*. *Microbiology* **153,** 3275-3285
10. Fukushima, T., Yamamoto, H., Atrih, A., Foster, S. J., and Sekiguchi, J. (2002) A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis*. *J. Bacteriol.* **184,** 6007-6015
11. Fukushima, T., Kitajima, T., and Sekiguchi, J. (2005) A polysaccharide deacetylase homologue, PdaA, in *Bacillus subtilis* acts as an *N*-acetylmuramic acid deacetylase *in vitro*. *J. Bacteriol.* **187,** 1287-1292
12. Blair, D. E. and van Aalten, D. M. (2004) Structures of *Bacillus subtilis* PdaA, a family 4 carbohydrate esterase, and a complex with *N*-acetyl-glucosamine. *FEBS Lett.* **570,** 13-19
13. Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., Jarmer, H., and Devine, K. M. (2007) The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol. Microbiol.* **65,** 180-200
14. Fukushima, T., Szurmant, H., Kim, E. J., Perego, M., and Hoch, J. A. (2008) A sensor histidine kinase co-ordinates cell wall architecture with cell division in *Bacillus subtilis*. *Mol. Microbiol.* **69,** 621-632
15. Fukushima, T., Furihata, I., Emmins, R., Daniel, R. A., Hoch, J. A., and Szurmant, H. (2011) A role for the essential YycG sensor histidine kinase in sensing cell division. *Mol Microbiol.* **79,** 503-522
16. Szurmant, H., Fukushima, T., and Hoch, J. A. (2007) The essential YycFG two-component system of *Bacillus subtilis*. *Methods Enzymol.* **422,** 396-417
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Anagnostopoulos, C. and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81,** 741–746
19. Fein, J. E. and Rogers, H. J. (1976) Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. *J. Bacteriol.* **127,** 1427–1442
20. Ohnishi, R., Ishikawa, S., and Sekiguchi J. (1999) Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of *Bacillus subtilis*. *J. Bacteriol.* **181,** 3178-3184

21. DeHart, H. P., Heath, H. E., Heath, L. S., LeBlanc, P. A., and Sloan, G. L. (1995) The lysostaphin endopeptidase resistance gene (*epr*) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and S*taphylococcus aureus*. *Appl. Environ. Microbiol.* **61,** 1475–1479
22. Nugroho, F. A., Yamamoto, H., Kobayashi, Y., and Sekiguchi, J. (1999) Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. *J. Bacteriol.* **181,** 6230-6237
23. Fukushima, T., Yao, Y., Kitajima, T., Yamamoto, H., and Sekiguchi, J. (2007) Characterization of new L,D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in *Bacillus subtilis. Mol. Genet. Genomics* **278,** 371-383
24. Atrih, A., Bacher, G., Allmaier, G., Williamson, M. P., and Foster, S. J (1999). Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *J. Bacteriol.* **181,** 3956-3966.
25. Fukushima, T., Kitajima, T., Yamaguchi, H., Ouyang, Q., Furuhata, K., Yamamoto, H., Shida, T., and Sekiguchi, J. (2008) Identification and characterization of novel cell wall hydrolase CwlT: a two-domain autolysin exhibiting *N*-acetylmuramidase and DL-endopeptidase activities. *J. Biol. Chem.* **283,** 11117-11125
26. Gilmore, M. E., Bandyopadhyay, D., Dean, A. M., Linnstaedt, S. D., and Popham, D. L. (2004) Production of muramic δ-lactam in *Bacillus subtilis* spore peptidoglycan. *J. Bacteriol.* **186,** 80-89
27. Budzik, J. M., Oh, S. Y., and Schneewind, O. (2008) Cell wall anchor structure of BcpA pili in *Bacillus anthracis*. *J. Biol. Chem.* **283,** 36676-36686
28. Fukushima, T., Tanabe, T., Yamamoto, H., Hosoya, S., Sato, T., Yoshikawa, H., and Sekiguchi, J. (2004) Characterization of a polysaccharide deacetylase gene homologue (*pdaB*) on sporulation of *Bacillus subtilis*. *J. Biochem.* **136,** 283-291
29. Silvaggi, J. M., Popham, D. L., Driks, A., Eichenberger, P., and Losick, R. (2004) Unmasking novel sporulation genes in *Bacillus subtilis*. *J. Bacteriol.* **186,** 8089-8095
30. Hayashi, H., Amano, K., Araki, Y., and Ito, E. (1973) Action of lysozyme on oligosaccharides from peptidoglycan N-unacetylated at glucosamine residues. *Biochem. Biophys. Res. Commun.* **50,** 641-648
31. Eymann, C., Dreisbach, A., Albrecht, D., Bernhardt, J., Becher, D., Gentner, S., Tam, L. T., Büttner, K., Buurman, G., Scharf, C., Venz, S., Völker, U., and Hecker, M. (2004) A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics* **4,** 2849–2876
32. Sudiarta, IP., Fukushima, T., and Sekiguchi, J. (2010) *Bacillus subtilis* CwlQ (previously YjbJ) is a bifunctional enzyme exhibiting muramidase and soluble-lytic transglycosylase activities. *Biochem. Biophys. Res. Commun.* **398,** 606-612
33. Ng, K. L., Lam, C. C., Fu, Z., Han, Y. F., Tsim, K. W., and Wong, W. K. (2007) Cloning and characterization of the *yjeA* gene, encoding a novel deoxyribonuclease, from *Bacillus subtilis*. *J. Biochem.* **142,** 647-654

34. Cantarel, B. L, Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* **37** (Database issue), D233-238
35. Tsigos, I., Zydowicz, N., Martinou, A., Domard, A., and Bouriotis, V. (1999) Mode of action of chitin deacetylase from *Mucor rouxii* on *N*-acetylchitooligosaccharides. *Eur. J. Biochem.* **261,** 698-705
36. Tokuyasu, K., Mitsutomi, M., Yamaguchi, I., Hayashi, K., and Mori, Y. (2000) Recognition of chitooligosaccharides and their nacetyl groups by putative subsites of chitin deacetylase from a deuteromycete, *Colletotrichum lindemuthianum. Biochemistry* **39,** 8837-8843
37. Deng, D. M., Urch, J. E., ten Cate, J. M., Rao, V. A., van Aalten, D. M., and Crielaard, W. (2009) *Streptococcus* mutans SMU.623c codes for a functional, metal-dependent polysaccharide deacetylase that modulates interactions with salivary agglutinin. *J. Bacteriol.* **191,** 394-402
38. Psylinakis, E., Boneca, I. G., Mavromatis, K., Deli, A., Hayhurst, E., Foster, S. J., Vårum, K. M., and Bouriotis, V. (2005) Peptidoglycan *N*-acetylglucosamine deacetylases from *Bacillus cereus*, highly conserved proteins in *Bacillus anthracis*. *J. Biol. Chem.* **280,** 30856-30863
39. Pfeffer, J. M., Strating, H., Weadge, J. T., and Clarke, A. J. (2006) Peptidoglycan O acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. *J. Bacteriol.* **188,** 902-908
40. Zipperle, G. F. Jr, Ezzell, J. W. Jr, and Doyle, R. J. (1984) Glucosamine substitution and muramidase susceptibility in *Bacillus anthracis*. *Can. J. Microbiol.* **30,** 553-559
41. Antelmann, H., Yamamoto, H., Sekiguchi, J., and Hecker, M. (2002) Stabilization of cell wall proteins in *Bacillus subtilis*: a proteomic approach. *Proteomics*. 5, 591-602
42. Tjalsma, H., Antelmann, H., Jongbloed, J. D., Braun, P. G., Darmon, E., Dorenbos, R., Dubois, J. Y., Westers, H., Zanen, G., Quax, W. J., Kuipers, O. P., Bron, S., Hecker, M., and van Dijl, J. M. (2004) Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. *Microbiol. Mol. Biol. Rev.* **68,** 207-233
43. Vollmer, W. and Tomasz, A. (2002) Peptidoglycan *N*-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. *Infect. Immun.* **70,** 7176-7178
44. Popowska, M., Kusio, M., Szymanska, P., Markiewicz, Z. (2009) Inactivation of the wall-associated de-*N*-acetylase (PgdA) of *Listeria monocytogenes* results in greater susceptibility of the cells to induced autolysis. *J. Microbiol. Biotechnol.* **19,** 932-945
45. Hebert, L., Courtin, P., Torelli, R., Sanguinetti, M., Chapot-Chartier, M. P., Auffray, Y., and Benachour, A. (2007) *Enterococcus faecalis* constitutes an unusual bacterial model in lysozyme resistance. *Infect. Immun.* **75,** 5390-5398
46. Severin, A., Tabei, K., and Tomasz, A. (2004) The structure of the cell wall peptidoglycan of *Bacillus cereus* RSVF1, a strain closely related to *Bacillus anthracis*. *Microb. Drug Resist.* **10,** 77-82

47. Wang, G., Olczak, A., Forsberg, L. S., and Maier, R. J. (2009) Oxidative stress-induced peptidoglycan deacetylase in *Helicobacter pylori*. *J. Biol. Chem.* **284,** 6790-6800
48. Wang, G., Maier, S. E., Lo, L. F., Maier, G., Dosi, S., and Maier, R. J. (2010) Peptidoglycan deacetylation in *Helicobacter pylori* contributes to bacterial survival by mitigating host immune responses. *Infect. Immun.* **78,** 4660-4666

FOOTNOTES

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[3]Abbreviations 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 legends)

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 (-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 CwlT, 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-MurNAcr(-L-Ala-D-Glu) (reduced 2S1P) by ESI-MS analysis (Supplemental Fig. S4*A* and *B*) and GlcNAc-MurNAc(-L-Ala-D-Glu)-GlcNAc-MurNAcr(-L-Ala-D-Glu) (reduced 4S2P) by ESI-MS and ESI-MS-MS analyses (Supplemental Fig. S5*A* 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. S6*A* and *B*) and ESI-MS-MS analyses (Fig. 4*A* 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. 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 $NaBH_4$. *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. S6*A* 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. 3*A*.

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 $MnCl_2$ 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 $GlcNAc_4$.** (*A* and *B*) $GlcNAc_4$ 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 (*M*r, 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 $GlcNAc_4$ 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. S9*B*), respectively.

Figure 7. **Kinetics with $GlcNAc_4$ and *B. subtilis* peptidoglycan as substrates.** (*A* and *B*) Released acetic acid by PdaC for 2 mg/ml $GlcNAc_4$ (*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*, $GlcNAc_4$ substrate.

TABLE 1A Effect of divalent cations on the deacetylase activity of PdaC for peptidoglycan

Divalent cation[a]	Released acetic acid (μg/ml) [μM][b]
Native PdaC[c]	1.90 [31.5]
EDTA-treated PdaC[d]	0.91 [15.1]
+ Mn^{2+}[e]	5.58 [92.4]
+ Zn^{2+}[e]	1.93 [31.9]
+ Mg^{2+}[e]	2.57 [42.6]
+ Ni^{2+}[e]	1.81 [30.0]
+ Ca^{2+}[e]	3.59 [59.4]
+ Co^{2+}[e]	Not determined[f]

[a]Deacetylation was performed with or without 5 mM cations except Zn^{2+} (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. [b]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. [c]"Native PdaC" indicates that PdaC was dialyzed against 20 mM sodium phosphate buffer (no EDTA treatment). [d]"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. [e] Each cation (5 mM except 5 μM Zn^{2+}) was added to EDTA-treated PdaC. [f]Since Co^{2+} interfered with the assay kit, the released acetic acid in the presence of Co^{2+} was not determined.

TABLE 1B Effect of divalent cations on the deacetylase activity of PdaC for $GlcNAc_4$

Divalent cation[a]	Released acetic acid (μg/ml) [μM][b]
Native PdaC[c]	1.67 [27.7]
EDTA-treated PdaC[d]	1.17 [19.4]
+ Mn^{2+}[e]	2.73 [45.1]
+ Zn^{2+}[e]	1.59 [26.3]
+ Mg^{2+}[e]	1.65 [27.3]
+ Ni^{2+}[e]	0.65 [10.8]
+ Ca^{2+}[e]	1.75 [28.9]
+ Co^{2+}[e]	Not determined[f]

[a]Deacetylation was performed with or without 5 mM cations except Zn^{2+} (5 μM) containing 2 mg/ml $GlcNAc_4$ and 10 μg/ml PdaC in 50 mM HEPES (pH 7.0) for 4 hours at 37°C. [b]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 [c, d, e, f] are shown in the footnote of Table 1A.

TABLE 2 Deacetylase activity toward peptidoglycan digested by cell wall hydrolases

Substrate[a]	Released acetic acid (μg/ml) [μM][b]
Peptidoglycan (-GlcNAc-MurNAc[L-Ala-D-Glu-A_2pm-D-Ala]-)$_n$ cross-linked between D-Ala and A_2pm	6.0±1.3 [99.9±21.6]
Peptidoglycan digested by D,L-endopeptidase (LytF [CwlE]) (-GlcNAc-MurNAc[L-Ala-D-Glu]-)$_n$	10.7±0.17 [178.2±2.8]
Peptidoglycan digested by L,D-endopeptidase (CwlK) (-GlcNAc-MurNAc[L-Ala]-)$_n$	3.25±0.35 [54.1±5.8]
Peptidoglycan digested by L-alanine amidase (CwlH) (-GlcNAc-MurNAc-)$_n$	< 0.03 [< 0.5]

[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.

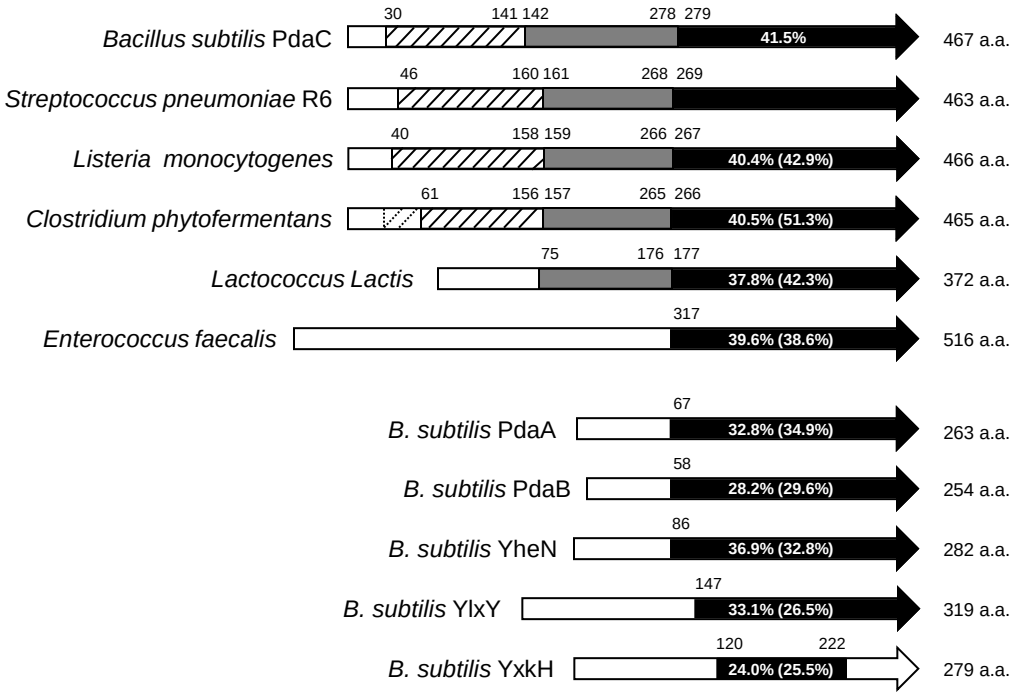


Fig. 1. Kobayashi *et al.*

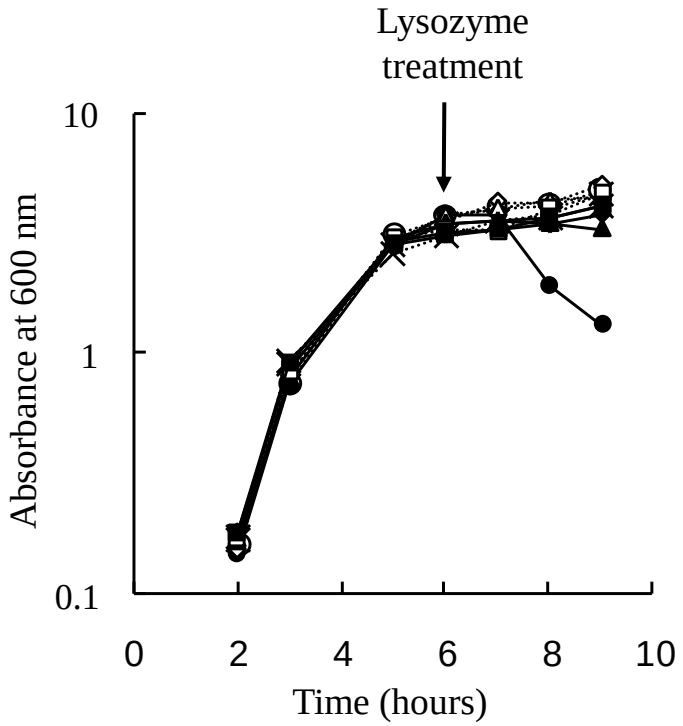


Fig. 2. Kobayashi *et al.*

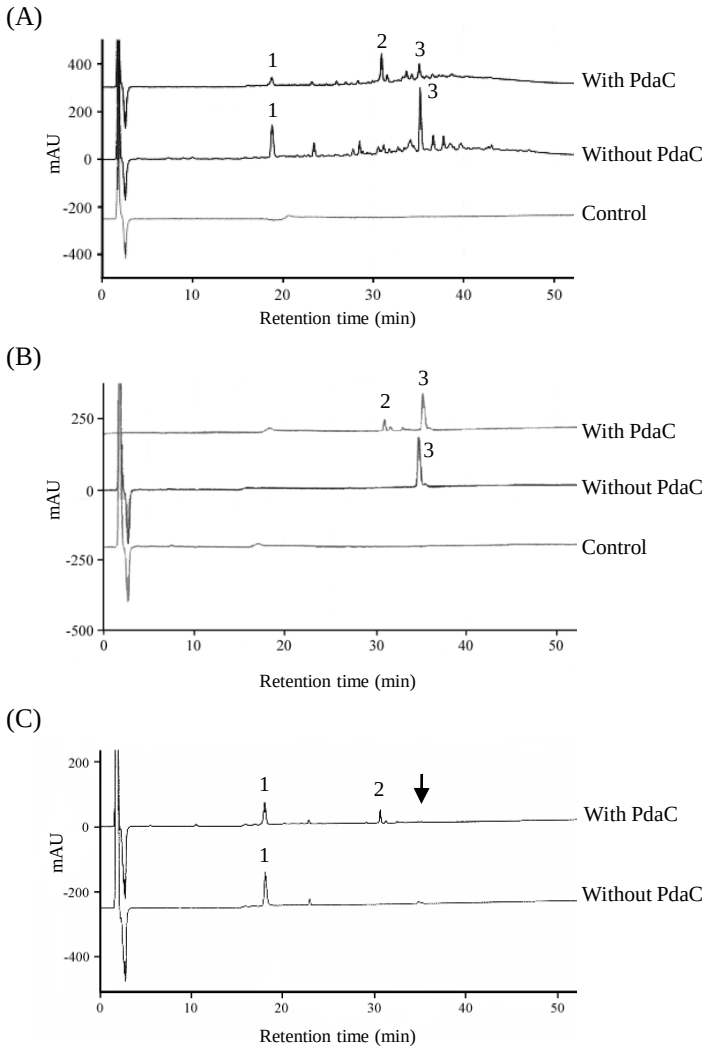
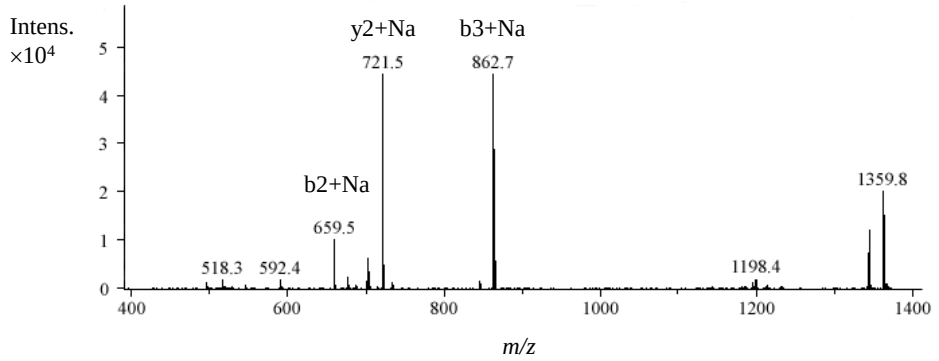
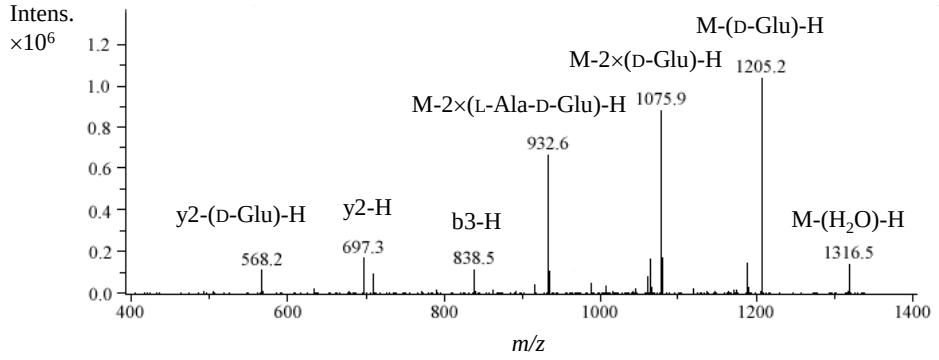


Fig. 3*A*, *B*, and *C*. Kobayashi *et al.*

(A) MS-MS analysis in the positive mode



(B) MS-MS analysis in the negative mode



(C)

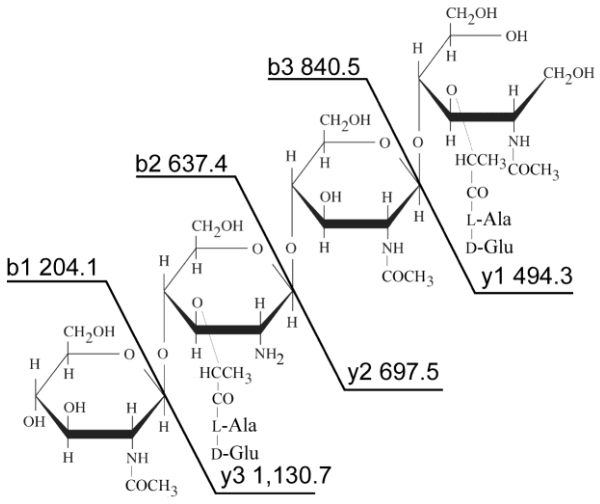


Fig. 4*A*, *B* and *C*. Kobayashi *et al.*

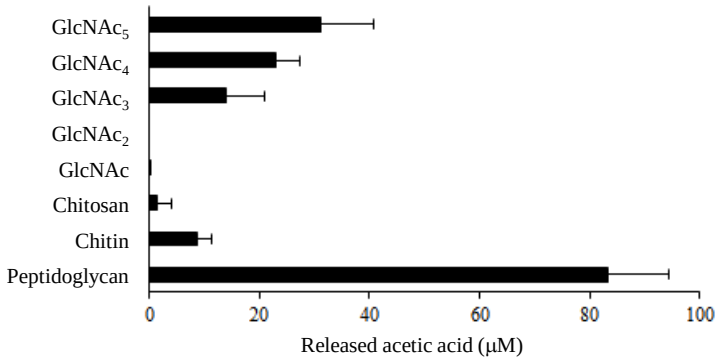
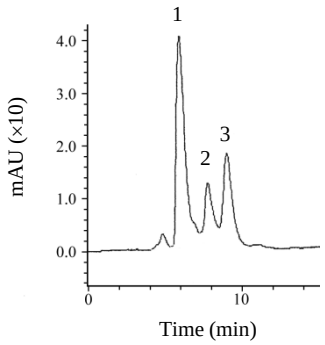


Fig. 5. Kobayashi *et al.*

(A)



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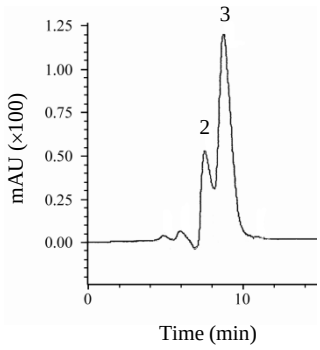
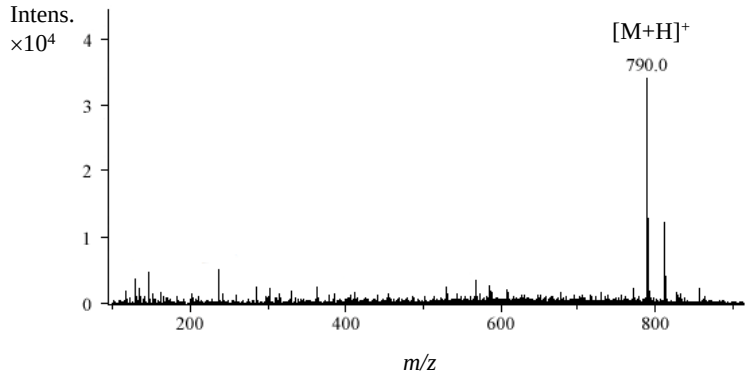
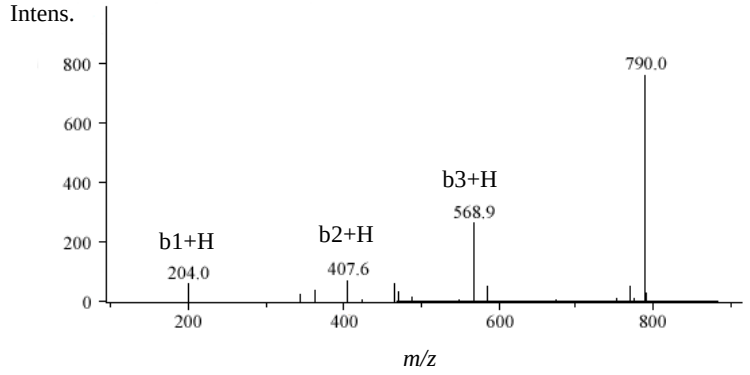


Fig. 6*A* and *B*. Kobayashi *et al.*

(C) MS analysis of *peak 1* material in the positive mode



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



(E)

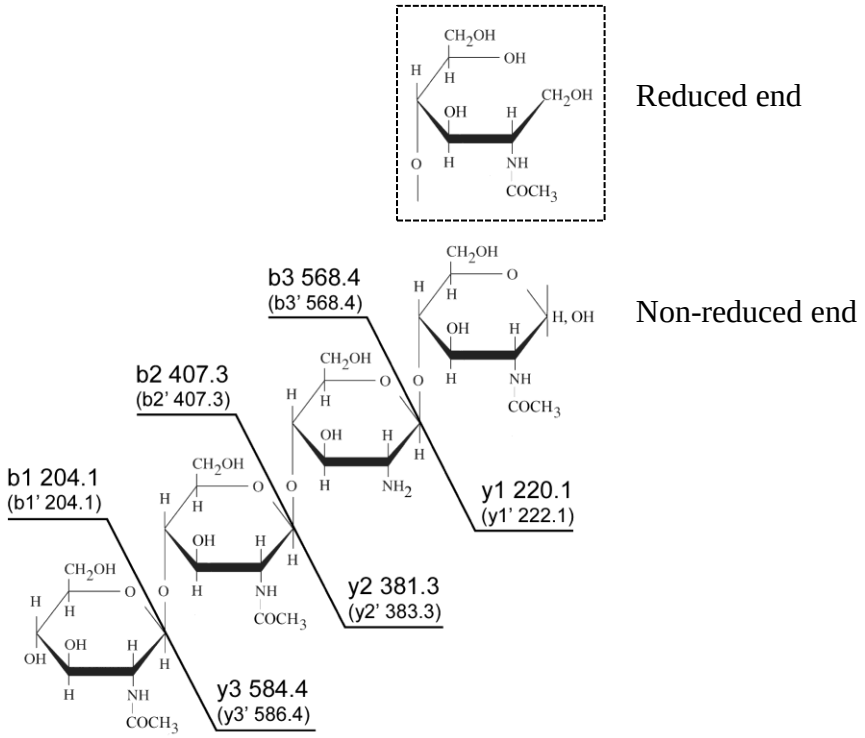
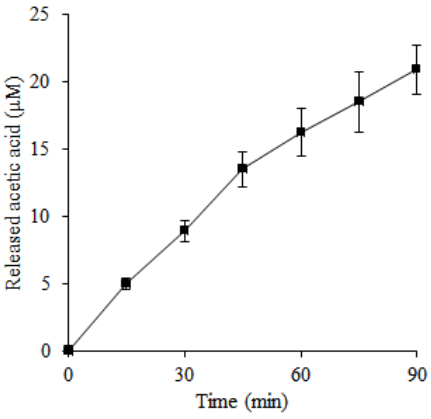


Fig. 6*C*, *D* and *E*. Kobayashi *et al.*

(A)



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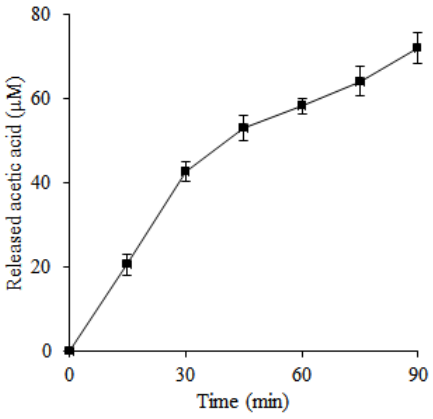

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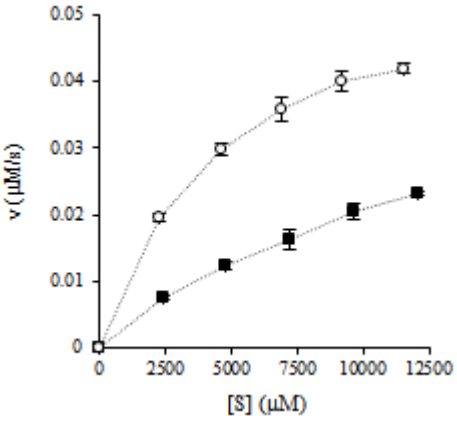

Substrate	K_m (mM)	V_{max} (μMs)	k_{cat} (S^{-1})	k_{cat}/K_m ($mM^{-1}S^{-1}$)
$GlcNAc_4$	12.3±1.84	0.046±0.0059	0.24±0.031	0.020±0.0010
Peptidoglycan	4.8±0.30	0.060±0.0020	0.32±0.010	0.067±0.0032

Fig. 7. Kobayashi *et al.*