Supporting Information

Lytic Transglycosylase MltB of *Escherichia coli* and Its Role in Recycling of Peptidoglycan Strands of Bacterial Cell Wall

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Experimental

Cloning procedures. The general procedure is described in detail below for cloning of *mltA*. This procedure was applied also for the cloning of the genes of the other lytic transglycosylases. A brief description in each case is given.

Lytic Transglycosylase MltA. The *mltA* gene encoding lytic transglycosylase MltA was amplified from *E. coli* K12 chromosome using the high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: MltAD: (CACCATGTCTTCCAAACCAACCGATCGCGGACAGCAAT), and MltAR: (GCCGCTAAAGACGTTACCTGCGCCCGGGGCGG). The resulting PCR fragment contained *mltA* without the first 60 bp encoding the N-terminal signal peptide and the cysteine responsible for membrane anchoring. The PCR product was gel purified and cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system (Invitrogen) according to the manufacturer's protocol. The ligation reaction was performed for 30 minutes at room temperature. A 3-µL aliquot of the ligation mixture was used to transform One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen). Colonies containing the plasmid were selected on LB agar supplemented with 100 µg/mL of ampicillin. Plasmid DNA from a few colonies was isolated, and nucleotide sequences of the inserts were verified by sequencing of both DNA strands. The resulting plasmid containing the lytic transglycosylase gene under the T7lac promoter was used to transform *E. coli* BL21(DE3) cells. Cells containing the plasmid were selected on agar supplemented with 100 µg/mL ampicillin. The resulting recombinant MltA would have a C-terminal His-tag to simplify purification and detection of the protein.

Lytic Transglycosylase MltB. The *mltB* gene encoding lytic transglycosylase MltB was amplified from *E. coli* K12 chromosome using the high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: MltBD: (CACCATGAAGCCAAAACCTACTGAGACTGATACGACC), and MltBR: (CTGTACTCGCGCCAGCGCCACGGCTTG). The resulting PCR fragment contained *mltB* without the first 63 bp encoding the N-terminal signal peptide and the cysteine responsible for membrane anchoring. The PCR product was cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system using the same procedure as described for *mltA*.

Lytic Transglycosylase MltC. The *mltC* gene encoding lytic transglycosylase MltC was amplified from *E. coli* K12 chromosome using the high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: MltCD: (CACCATGTCGACGACCAAAAAAGGCGATACCTATAACG), and MltCR: (TCGGCGGCGGTAAGATTTTTGCGCGGTATTCAC). The resulting PCR fragment contained *mltC* without the first 54 bp encoding the N-terminal signal peptide and the cysteine responsible for membrane anchoring. The PCR product was cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system using the same procedure as described for *mltA*.

Lytic Transglycosylase MltD. The *mltD* gene encoding lytic transglycosylase MltD was amplified from *E. coli* K12 chromosome using the high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: MltDD: (CACCATGAGTACCGGCAACGTTCAACAGCACGCA), and MltDR: (CACCATGAGTACCGGCAACGTTCAACAGCACGCA). The resulting PCR fragment contained *mltD* without the first 51 bp encoding the N-terminal signal peptide and the residues responsible for membrane anchoring. The PCR product was cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system using the same procedure as described for *mltA*.

Lytic Transglycosylase MltE. The *mltE* gene encoding lytic transglycosylase MltE was amplified from *E. coli* K12 chromosome using high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: MltED: (CACCATGATATCATGCCGCTTAGGTGTGCCGTTGTC), and MltER: (CATCGCGTCCAGTGCCTGCTCAAGTTTGTAG). The resulting PCR fragment contained mltE without the first 48 bp encoding the N-terminal signal peptide and the cysteine responsible for membrane

anchoring. The PCR product was cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system using the same procedure as described for *mltA*.

Lytic Transglycosylase Slt70. The *slt* gene encoding lytic transglycosylase Slt70 was amplified from *E. coli* K12 chromosome using the high fidelity *Pfu*Ultra™ II Fusion HS DNA Polymerase with primers: SltD: (CACCATGATATCATGCCGCTTAGGTGTGCCGTTGTC), and SltR: (CATCGCGTCCAGTGCCTGCTCAAGTTTGTAG). The resulting PCR fragment contained *slt* without the first 84 bp encoding the N-terminal signal peptide and the cysteine responsible for membrane anchoring. The PCR product was cloned into pET101-TOPO vector from Champion pET Directional TOPO® expression system using the same procedure as described for *mltA*.

Purification of the Lytic Transglycosylases. Cells expressing the desired lytic transglycosylases were incubated overnight in 5 mL of LB medium supplemented with 100 μg/mL of ampicillin. The cells were diluted into 50 mL of fresh ampicillin-supplemented LB medium and growth was continued with agitation (120 rpm) at 37 °C until the culture reach OD_{600} of 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added at this stage to a concentration of 0.4 mM and the cultures were incubated at 15 °C for an additional 12 h. Cells were harvested by centrifugation, resuspended in 10 mL of 20 mM HEPES buffer, pH 7.0, supplemented with 0.5 M NaCl, 10% glycerol, 25 mM imidazole and 0.1% Triton X-100. The proteins were released from the cells by sonication on ice for 10 min. Bacterial debris was removed by centrifugation at 18 000 *g* for 30 min. The supernatant was loaded using a 10 mL syringe onto a 1 mL HiTrap Chelating column (GE). The column was washed with 10 mL of 20 mM HEPES buffer, pH 7.0, supplemented with 0.5 M NaCl, 10% glycerol, 25 mM imidazole and 0.1% Triton X-100. Elution was performed using 20 mM HEPES buffer, pH 7.0, supplemented with 0.25 M NaCl, 10% glycerol, 250 mM imidazole and 0.1% Triton X-100. The first 2 mL of the flow-through were discarded, and the 3rd 1-mL fraction was collected. SDS-PAGE analysis showed that purity of the lytic transglycosylase by this protocol exceeded 90%.

Zymography. In order to assess activity of the recombinant lytic transglycosylases, zymography was used as an assay. The sacculus of *Escherichia coli* K12 was isolated for zymography. Cells were grown overnight in 500 mL of LB media at 37 ºC with agitation. Bacteria were harvested by centrifugation at 4000 *g* for 10 minutes. The pellet was resuspended in 50 mL of Milli-Q water, and the suspension was slowly mixed into 50 mL of boiling 20% sodium dodecylsulfate (SDS). The solution was allowed to boil for 1 h with stirring to dissolve membranes and most of bacterial proteins. The sacculus was pelleted by centrifugation at 18 000 *g* for 50 minutes. The pellet was resuspended in 50 mL of Milli-Q water, and repelleted. The resuspention of the pellet and water washes were performed twice more to remove SDS. Subsequently the pellet was resuspended in 10 mL of 10 mM Tris-HCl buffer, pH 7.6, supplemented with 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and treated with 10 units of RNase A and 10 units of DNase I (New England Biolabs) for 1 h at 37 ºC to remove all nucleic acids. Subsequently, a 1 mL portion of 10X trypsin (Fisher Scientific) was added, and the sacculus suspension was incubated for 1 h at 37 ºC on an orbital shaker. To remove all of the enzymes added to the sacculus suspension, 10 mL of 20% SDS was added, and the mixture was boiled for 10 minutes. Subsequently, the SDS solution was removed by pelleting the sacculus by centrifugation at 18 000 *g* for 50 minutes, resuspending it in 40 mL of Milli-Q water by vortexing, and pelleting again. This washing step was repeated twice more. After the final pelleting, the sacculus was resuspended in 10 mL of Milli-Q water by sonication using Branson Sonifier 450 ultrasonic cell disrupter (VWR) for approximately ten seconds. Longer sonication should be avoided, as it can result in damage to the sacculus, leading to low-contrast zymograms. The resulting *E. coli* sacculus suspension did not contain any visible particles and appeared opalescent. It was stored frozen at -20 ºC.

For zymography gels, 3 mL of this sacculus suspension was added to every 10 mL of the acrylamide monomer and the buffer mixture used for the preparation of the stacking gel of SDS-PAGE gel. Depending on the size of the protein that was being analyzed, the gels contained between 10 to 15% of

acrylamide. The samples containing the purified recombinant lytic transglycosylases were mixed with the following 6 × loading buffer:

0.5 M Tris-HCl, pH 6.8, supplemented with 0.4% SDS – 7 mL
Glycerol – 3 mL
SDS – 1 g
Bromphenol blue – 1.2 mg
Water – to 10 mL

The samples were loaded on the gel without boiling. During electrophoresis, which was performed at constant 125 Volt, the electophoretic cell was placed into a container with ice to prevent overheating. Electrophoresis was performed in the XCell SureLock electrophoretic cell (Invitrogen) using 25 mM Tris base, 0.2 M glycine, 0.1% SDS, pH 8.3. The gel was rinsed twice with 200 mL of Milli-Q water, and then it was placed in the renaturing buffer[1] (20 mM sodium phosphate buffer, pH 7.0, supplemented with 10 mM $MgCl_2$ and 0.1% Triton X-100). The renaturation was performed at 37 °C with gentle agitation for 18 h. Then, the gels were boiled for 30 s in the staining solution containing 0.1% methylene blue and 0.01% KOH in water, and placed on an orbital shaker for another 15 min. Destaining of the gel was carried out using Milli-Q water at room temperature for a few hours with gentle agitation on an orbital shaker. Upon destaining, the cleared zones corresponded to the areas where the sacculus was hydrolyzed by lytic transglycosylases (Figure S1). All six lytic transglycosylases were active on zymography.

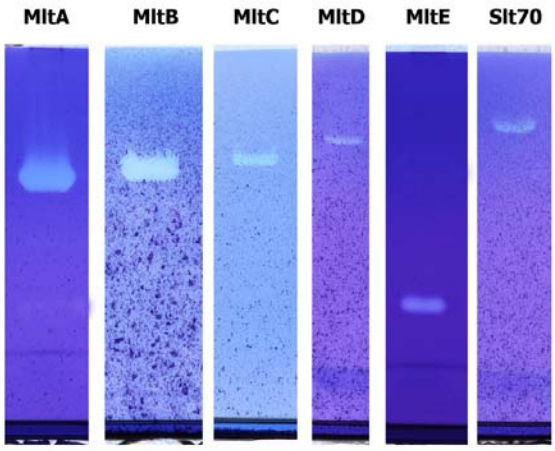

Figure S1: Zymography with lytic transglycosylases. A total of 3 µL of a 1 mg/mL solution of MltA and MltB, and 3 µL of a 0.25 mg/mL solution of MltC, MltD, MltE, Slt70 were loaded on zymography gels. For MltA, MltB, MltC, MltD proteins 10% gel was used, while MltE and Slt70 were analyzed on 15% gels.

pH Profile of MltB. To determine the optimal pH range for MltB, zymography with this enzyme was performed using buffers with pH ranging from 4.0 to 9.0 for renaturation of this protein (see below for buffers). As was done previously for regular zymography, multiple MltB protein samples (1 µL of 5 mg/mL MltB) were loaded onto a zymography gel. Later, the gel was sliced into pieces each containing one MltB sample. These MltB samples were placed in 100 mm Petri dishes, rinsed twice with 40 mL of Milli-Q water, and renatured in the buffers listed below at 37 °C for 18 h:

20 mM malate buffer, pH 4.0, supplemented with 10 mM $MgCl_2$ and 0.1% TritonX-100; 20 mM acetate buffer, pH 5.0, supplemented with 10 mM $MgCl_2$ and 0.1% Triton X-100; 20 mM MES buffer, pH 6.0, supplemented with 10 mM $MgCl_2$ and 0.1% Triton X-100; 20 mM phosphate buffer, pH 7.0,

supplemented with 10 mM $MgCl_2$ and 0.1% Triton X-100; 20 mM Tris buffer, pH 8.0, supplemented with 10 mM $MgCl_2$ and 0.1% TritonX-100; 20 mM glycine buffer, pH 9.0, supplemented with 10 mM $MgCl_2$ and 0.1% Triton X-100.

According to the zymograms, MltB was able to degrade the sacculus, seemingly equally well, in all of the buffers (Figure S2).

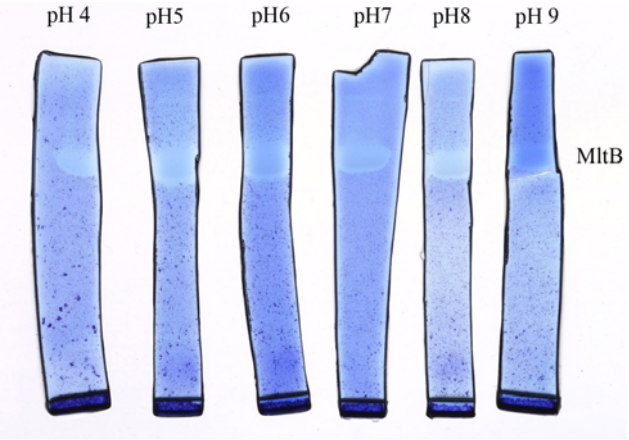

Figure S2: Hydrolysis of *E. coli* sacculus by MltB at different pH values. The gel slices were stained and destained using the same procedure as described for regular zymogram.

Screening Against Compound 1. Solutions of all lytic transglycosylases were prepared in 20 mM HEPES buffer, pH 7.0, containing 0.1 M NaCl and 0.1% Triton X-100. Concentration of each protein was measured using the BCA kit from Pierce, and was adjusted to 5 µM. Compound **1** was dissolved in water to give a stock solution of 10.0 mM, and combined in Eppendof tubes with the lytic transglycosylases to give mixtures of 20 µL total containing 2.5 µM of enzyme and 1 mM of the synthetic substrate in 20 mM HEPES, pH 7.0, containing 0.1 M NaCl and 0.1% Triton X-100. Contents of each tube were analyzed by HPLC periodically during three days of incubation at 37 ºC. Before injecting into the HPLC system, reactions were stopped by the addition of 20 µL of 10% acetic acid in acetonitrile to each mixture. The products of the reaction were analyzed using a Perkin Elmer Series 200 analytical HPLC system, including a Series 200 autosampler, pump, and UV/Vis detector with SunFire C18 reversed-phase column (Waters). The separation protocol was based on the method reported by Glauner[2] with some modifications. The column was equilibrated with 2% acetonitrile in water containing 0.1% of trifluoroacetic acid (TFA) for 40 minutes, then 10 µL of the reaction mixture was injected. The reaction products were resolved using a linear gradient of 2-15% acetonitrile in water supplemented with 0.1% TFA over 40 min at 0.5 mL/min. Detection of the samples was by UV/Vis at 205 nm (Figure S3). Chemical composition of the reaction products was verified by electrospray ionization (ESI) mass spectrometery, as described below.

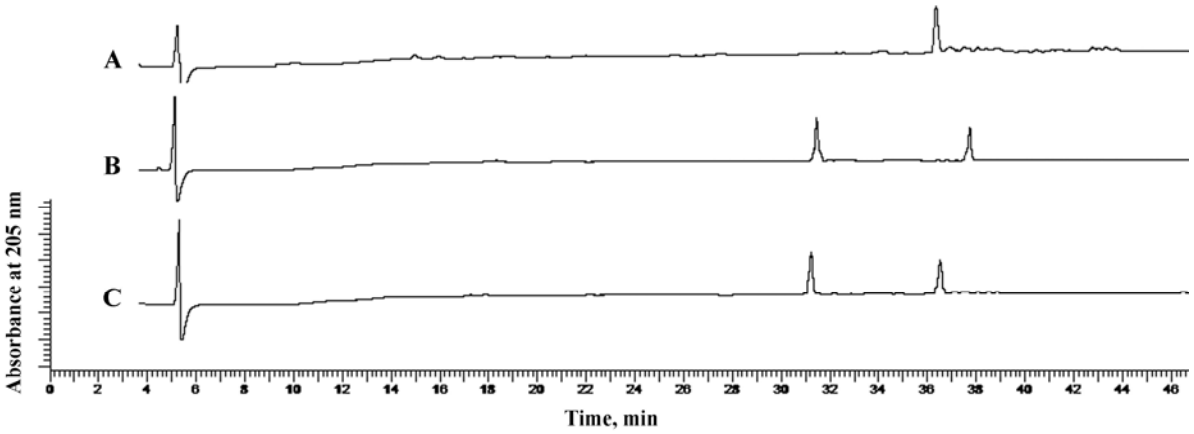

Figure S3: Screening of lytic transglycosylases against compound **1**. (A) No hydrolysis. The only peak observed corresponds to **1**. The reaction mixture of 20 µL total contained 2.5 µM of MltD and 1 mM of compound **1** in 20 mM HEPES, pH 7.0, supplemented with 0.1 M NaCl and 0.1% Triton X-100. (B) Substrate **1** was hydrolyzed by MltB, and the products were formed (retention time 31.0 and 37.5 min). The reaction mixture of 20 µL total contained 2.5 µM of MltB and 1 mM of **1** in 20 mM HEPES, pH 7.0, supplemented with 0.1 M NaCl and 0.1% Triton X-100. (C) A mixture of compound **3** (at 31.0 min) and compound **1** (at 36.5 min). The mixture of 20 µL total contained 0.5 mM of compound **1** and 1 mM compound **3** in 20 mM HEPES, pH 7.0, supplemented with 0.1 M NaCl and 0.1% Triton X-100.

ESI-MS. Characterization of the reaction products was performed using a Waters Alliance 2690 Separations Module coupled with a Waters 996 Photodiode Array detector, and MicroMass Quattro-LC Triple Quad electrospray ionization (ESI) mass spectrometer (FigureS4). The peaks were initially analyzed using positive ionization mode throughout the *m*/*z* of 50–2000. The charge states of the major ions were determined as the reciprocal of the spacing between two adjacent isotopic peaks differing in mass by 1 Da.[3] The chemical structures of the reaction products were elucidated using positive-ion electrospray tandem mass spectrometry (MS/MS). Fragmentation of the molecular ions was evaluated at different collision energy levels ranging from 20 to 40 eV (Figures S4 and S5). Analysis of the MS data and fragmentation pattern of the reaction products and of compounds **1**, **2**, and **3** allowed us to confirm chemical structure of the reaction products.

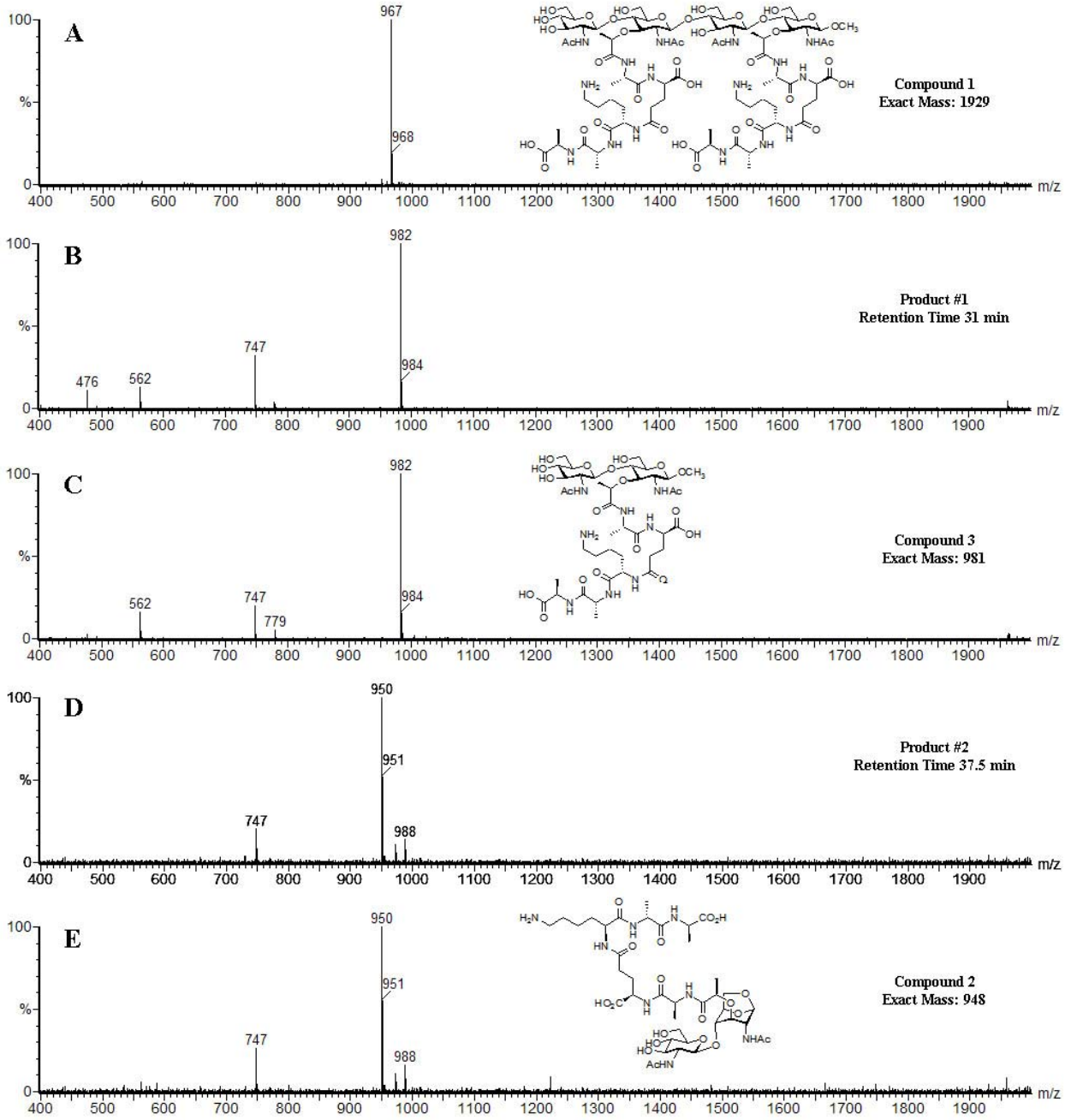

Figure S4: ESI-LC-MS analysis of the reaction products and comparison to authentic synthetic samples. The spectrum of the synthetic compound **1** (A), of reaction product #1 (B), authentic compound **3** (C), of reaction product #2 (D), and of authentic compound **2** (E).

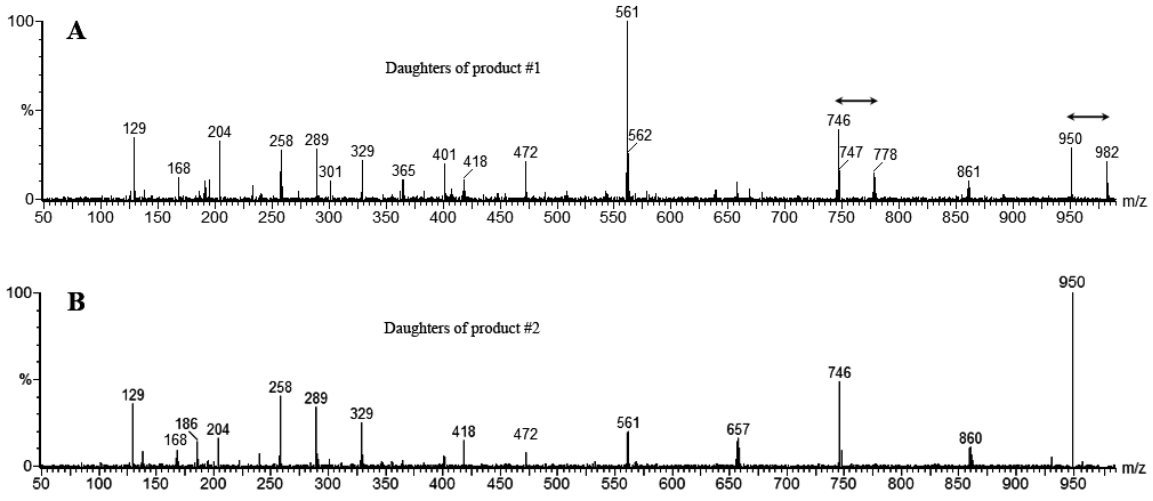

Figure S5: Tandem mass spectrometry of the reaction products. (A) Daughter ion mass spectrum of product #1 (retention time 31.0 min). Arrows mark the peaks revealing the loss of neutral CH_4O fragment, corresponding to the loss of the methoxy group and a proton from the product #1. (B) Fragmentation spectrum of product #2 (retention time 37.5 min).

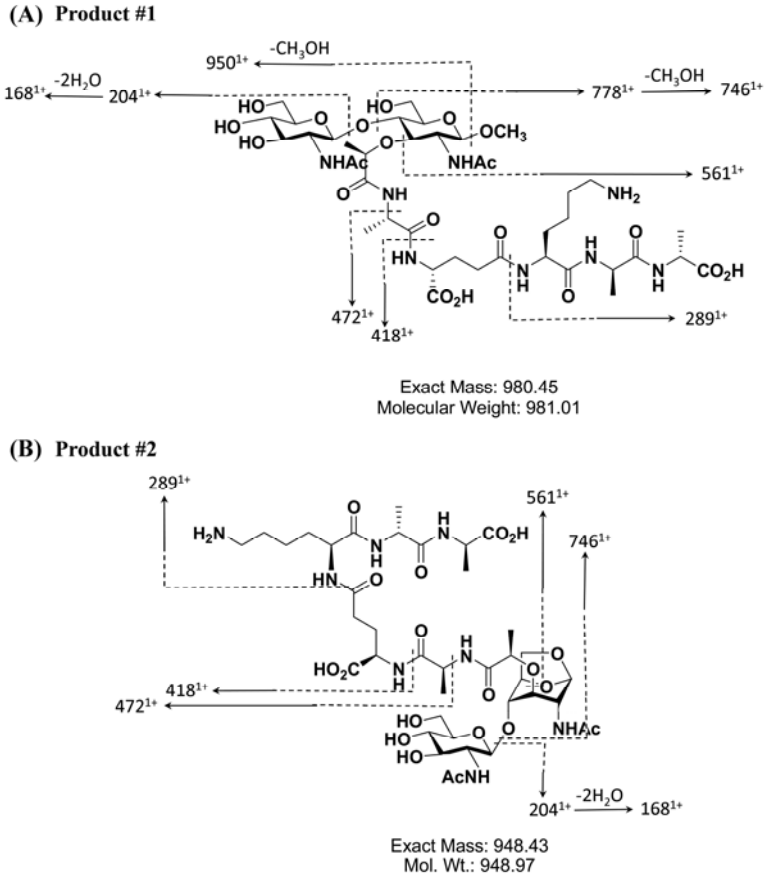

Figure S6. Fragmentation of the reaction product #1 (A) and reaction product #2 (B) at collision energy of 40 eV (positive ion mode).

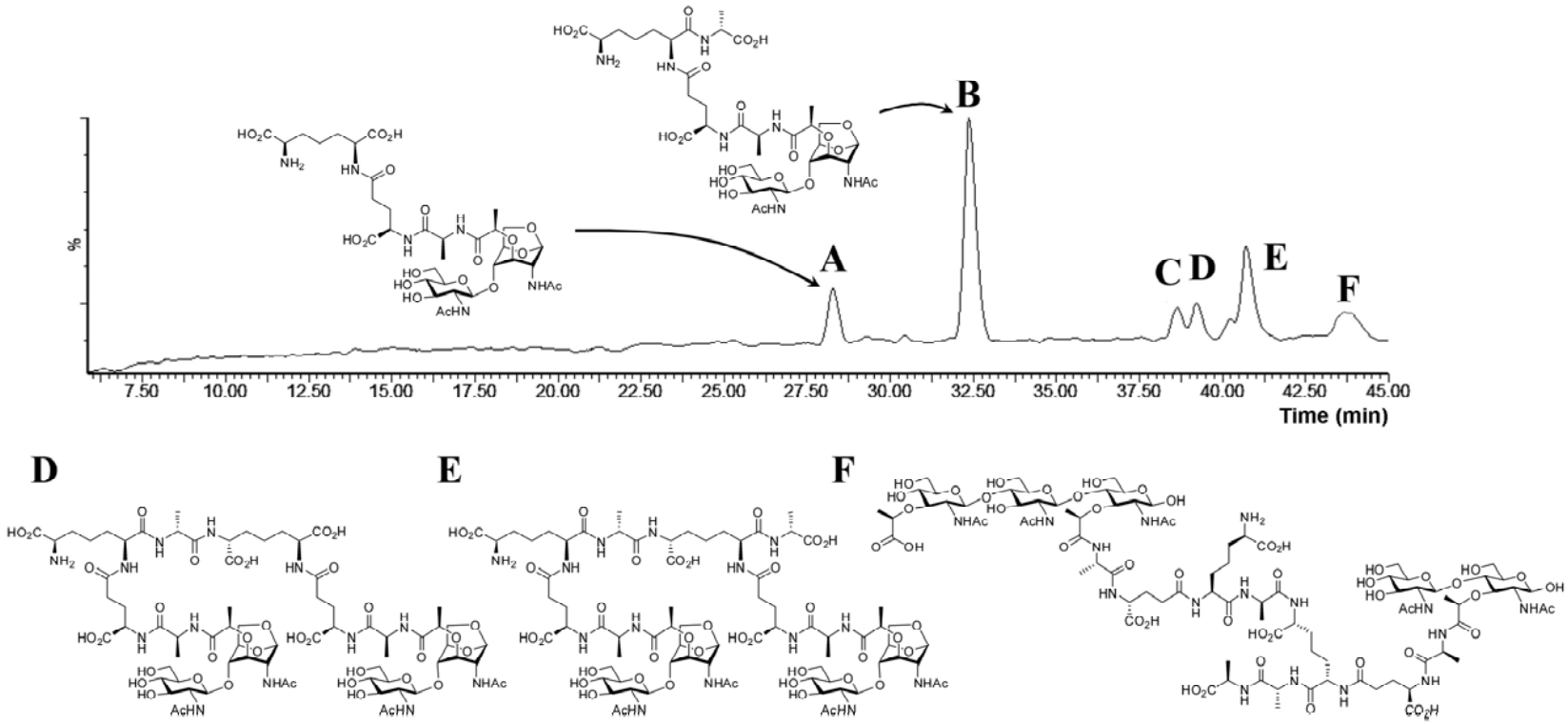

Figure S7. Reverse-phase HPLC profile of the products of sacculus fragmentation by the action of MltB and the structure assignment by ESI-MS.

Degradation of the Bacterial Sacculus by MltB. Multiple reaction mixtures each containing 350 μL of the sacculus preparation and 1 μM MltB (total final volume of 500 μL) in 20 mM HEPES, pH 7.0, were set up in 1.5 mL Eppendorf tubes at at 37 °C. The mixtures were agitated on an orbital shaker. The reactions were stopped after 0.5, 1, 2, 4, and 7 h of incubation by boiling of the mixture for 2 minutes. Each reaction mixture was centrifuged for 20 min at 14 000 *g* and the solution was lyophilized. The residues were dissolved in 100 μL of Milli-Q water for analysis by LC/MS. Reverse-phase HPLC profile of the products of sacculus fragmentation by the action of MltB is given in Figure S7.

Kinetics with MltB. All kinetic measurements were performed by HPLC, utilizing the same hardware and HPLC protocol used for screening of lytic transglycosylases against **1**. Multiple reactions were set up with MltB and compound **1** in 20 mM HEPES, buffer, pH 7.0. Concentration of the substrate was varied from 500 μM to 3 mM, and concentration of the enzyme was kept at 100 nM, with each measurement made in triplicate. The reactions were stopped by the addition of 10% acetic acid in acetonitrile and flash freezing. These data were analyzed by nonlinear regression using the GraFit 4 software (Erithacus Software Ltd) (Figure S8).

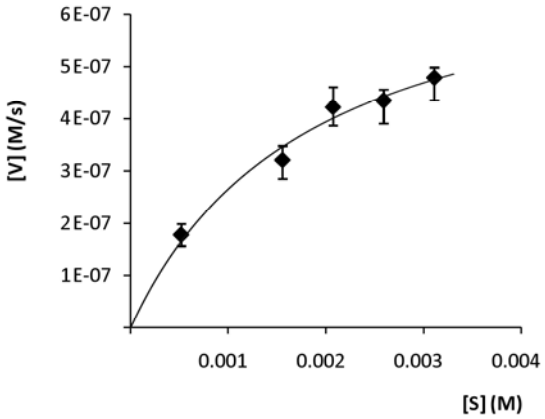

Figure S8: Determination of K_m and V_{max} for turnover of **1** by MltB by nonlinear regression. The K_m and V_{max} values were calculated by fitting the experimental data to the following equation: $V = V_{max}S/(K_m + S)$ where V is reaction rate at concentration of substrate S.

References

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2. Glauner, B. *Anal. Biochem.* **1988,** *172*, 451-464.
3. Henry, K. D.; McLafferty, F. W. *Org. Mass Spectrom.* **1990,** *25*, 490-492.