Supporting Information

Substrate recognition and catalysis by LytB, a pneumococcal peptidoglycan hydrolase involved in virulence

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lhfy	EQLT	KEVFQKLKDLKDY <mark>GG</mark> V	/SLPEWVCTAFHT <mark>SG</mark> YI	D Q A I V E Y <mark>G</mark>		DDLTDD	- IVCAKKILDKVG-			AHKALCSE	KLDQ	GH22 3.	. 6
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Supplementary Figure S1

Supplementary Figure S1. 3D Structure-based sequence alignment of LytB_{GH73} (PDB 4Q2W) with related proteins identified by the Dali server¹. Sequences are sorted by structural similarity with LytB (Z-score). GH families are indicated on the right. Secondary structural elements are indicated at the top of the alignment drawn with JalView². Amino acids are coloured by side chain properties. The star indicates the invariant glutamic acid acting as proton donor and the blue box the conserved YA(S/T)D motif of the GH73 family. Bottom histograms show statistics on alignment quality and consensus annotations, and the consensus sequence.



Supplementary Figure S2. Comparison of the far-UV CD spectra of LytB wild type and the E585A and D657 mutants. Measurements performed at 20°C in Pi buffer (pH 8.0) at 0.16 mg/ml protein. Data were transformed into average molar ellipticities per residue.

1	2	3/3´	4/	′4´	5	6/6	· 7/7′
DS I L-Ala J-iGlu L-Lys	DS* I L-Ala I D-iGIn L-Lys	DS I L-Ala I D-iGln L-Lys	DS I L-Ala I D-iGIn L-Lys-L-Ala	DS I L-Ala I D-iGln L-Lys I D-Ala	DS I L-Ala D-iG L-I L-L D-Al I D-Al	G* DS a L-Ala In D-iGI s L-Lys a D-Ala a Gly	DS I L-Ala D-iGin L-Lys D-Åla D-Åla
Tri[Glu]	Tri[deAc]	Tri	Tri(A)	Tetra	Penta[d	eAc] Penta[(Gly] Penta
10		12ª	12/12′			13	14/14′
DS* I L-Ala D-iGlu L-Lys-L-Ser D-Ala	ב 	S* la Gln ys-L-Ser-L-Ala	DS I L-Ala J D-iGln I L-Lys-L-Ser-L-A	Ala	DS* A D-iGln L-Lys-L-Ala-L-Al	DS I L-Ala I D-iGln I a L-Lys-L-Ser I D-Ala	DS I L-Ala J D-iGIn I L-Lys-L-Ala-L-Ala
Tetra(S)[Glu/de	Ac] Tri	(SA)[deAc]	Tri(SA)		Tri(AA)[deAc]	Tetra(S)	Tri(AA)
15/15´		16/1	6′		17	7/18	19/19′
DS I L-Ala J D-iGIn L-Lys-L-Ser-L-Al J D-Ala Tetra(SA)	DS - L-Ala D-iGIn Ia L-Lys- D-Ála D-Ála Pei	L-Ser-L-Ala	M I L-Ala D-iGln L-Lys-L-Ser-L-A J D-Ala I D-Ala Penta(SA)I-(la i	DS I I L-Ala D-iGIn I D-iGIn L-Lys I I-Lys – D-Ala TetraT	DS* I G-M L-Ala I L-Ala D-iGln I J-iGln L-Lys I L-Lys – D-Ala rri[deAc] [‡]	DS I DS L-Ala I I L-Ala D-iGIn I I D-iGIn L-Lys I I L-Lys – D-Ala TetraTri
22/22/		25 /2	(,/	-1	20		24
23/23	DC	25/2	5		29		31
DS I L-Ala I D-iGln I L-Lys-L-Ser-L-Ala	L-Ala I D-iGln L-Lys D-Åla	DS I L-Ala I D-iGln L-Lys-L-Ala-L-/	L-Ala L-Ala D-iGln L-Lys Ala _ D-Åla	DS* I L-Ala D-iGln L-Lys —	L-Ala L-Ala I D-iGln D-iGln L-Lys L-Lys I D-Ala — D-Ala	DS I L-Ala D-iGln I L-Lys-L-Ala-L-	DS I L-Ala D-iGin L-Lys-L-Ser-L-Ala Ala — D-Ala
Tetra(SA)Tı	ri	Tetra(AA	A)Tri T	etraTet	raTri[deAc],[-	DS] TetraT	ri(AA) [‡] (SA) [‡]

Supplementary Figure S3. Proposed structures of the fragments solubilized by treatment of *S. pneumoniae* **cell wall with the glucosaminidase LytB or the PG with the muramidase cellosyl.** DS (disaccharide unit) stands for GlcNAc-MurNAc(r) (*N*-acetyglucosamine-*N*-acetylmuramitol) in cellosyl products, and MurNAc-GlcNAc(r) (*N*-acetylmuramic acid-*N*-acetylglucosaminitol) in those of LytB (numbers with apostrophe); DS*, disaccharide unit with a deacetylated glucosamine residue (instead of *N*-acetylglucosamine). Peak numbering as in Bui *et al.*, 2012³.



Supplementary Figure S4



Supplementary Figure S4. MS analysis of isomeric PG fragments obtained by Cellosyl or LytB. (a) MS and MS fragmentation spectra of peak 12 (Tri(SA)) obtained by cellosyl digest followed by reduction with NaBH4. The intense ion with m/z = 781, the weak ion with m/z = 576 and the ion with m/z = 504 are all consistent with the presence of a reduced *N*-acetylmuramitol residue [MurNAc(r), corresponding to GlcNAc(r)-D-Lac, grey box in the inset]. (b) LytB released a fragment with identical mass but different MS fragmentation pattern (peak 12'). The intense ion with m/z = 761 and an intense lactoyl-peptide ion (m/z = 576), obtained with high intensity only from fragments containing non-reduced MurNAc (grey box) are consistent with the presence of a reduced GlcNAc(r) residue. This analysis shows that LytB is an *N*-acetylglucosaminidase, and cellosyl is an *N*-acetylmuramidase.



Supplementary Figure S5. Schematic representation of PG analogues used in this work. The numbering of (GMPP)₂ sugar units used in docking studies is indicated in the figure labels.



Supplementary Figure S6. HPLC and MALDI-TOF analysis of (GlcNAc)₅ products upon treatment with LytB. The reaction was carried out at 37°C in Pi buffer, pH 7.0. Aliquots of the reaction sample were withdrawn at different times along the hydrolysis reaction. (a) Incubation of 1.5 mM (GlcNAc)₅ with LytB (8.4 μ M). (b) Deconvolution of the elution profile after 35h of incubation with LytB. (c) Detection of glycosyltransferase products by HPLC and MALDI-TOF after 35h of incubation. Product masses (*m/z*) correspond to the [M+Na]⁺ species of (GlcNAc)₆ together with the hydrolysis products. Monoisotopic masses [M+Na]⁺ for each compound are indicated. C traces correspond to controls without protein at the longest incubation time. Peaks labelled as in Fig. 4 (main text)



Supplementary Figure S7. HPLC analysis of $(GlcNAc)_4$ products upon treatment with LytB wild type and D657A protein mutant. The reaction was carried out at 37°C in Pi buffer, pH 7.0, during 48 hours. Aliquots of the reaction sample were withdrawn at different times along the hydrolysis reaction. (a) Hydrolysis of $(GlcNAc)_4$ (1.5 mM) by LytB wild type (4.0 μ M) and (b) by D657A mutant protein (4.0 μ M). C traces correspond to controls without protein at the longest incubation time. Peaks labelled as in Fig. 4 (main text)



Supplementary Figure S8. HPLC analysis of LytB activity on GMDP and GMPP. The incubation was carried out at 37°C in Pi buffer (pH 7.0) and aliquots of the mixture were withdrawn at different times and analysed by HPLC on a reversed-phase column (*Tracer Excel 120 ODS-B*, 250 × 4 mm, 5 μ m). Samples were eluted at 20°C using a 40 min gradient from 2% to 15% of acetonitrile in 0.1% trifluoroacetic acid. Continuous traces and symbols correspond to controls and samples after 48 h of incubation, respectively. α and β refer to GMDP alpha and beta anomers. (a) Incubation of GMDP (106 μ M) with LytB (5.3 μ M). (b) Incubation of GMPP (200 μ M) with LytB (5.6 μ M).



Supplementary Figure S9. LC/MS and LC/MS/MS analysis of LytB activity on (GM)₂. The incubation was carried out at 37°C in Pi buffer (pH 7.0) and aliquots of the mixture were withdrawn at different times and analysed by UPLC on a reversed-phase column (Acquity UPLC HSS T3, 150×2.1 mm, 1.8μ m, Waters). Samples were eluted at 40°C using a 15 min gradient from 0% to 5% of acetonitrile in 0.1% formic acid. (a) Possible reaction products of LytB on (GM)₂. Among three possibilities (I, II or I/II), the reaction of LytB goes by route II. (b) LC/MS total-ion chromatograms of (GM)₂ at time = 0 h and (c) at time = 48 h of incubation of (GM)₂ (1.5 mM) with LytB (4.0 μ M). (d) LC/MS extracted-ion chromatograms of Product 1 at m/z = 700.3 and (e) Product 2 at m/z = 308.1. (f) Collision-induced spectra (MS/MS spectra) of Product 1 and (g) Product 2.



Supplementary Figure S10. Structural comparison of the LytB_{CAT} with related enzymes of the GH23 family. (a) Superimposition of secondary structural elements labelled as in LytB_{CAT} structure. Helices are shown as cylinders. Catalytic proton donor (E585 in LytB) and conserved aromatic residues (W660 and Y664 in LytB) are in stick representation. Colour codes: green, Australian black swan G-type lysozyme (PDB 1LSP); blue, Slt70 from *E. coli* (PDB 1QTE), yellow, MltE from *E. coli* (PDB 4HJV), and red, LytB_{CAT} (PDB 4Q2W). (b) Surface representation of LytB_{CAT} (grey) with different glycan ligands (coloured sticks) docked into the active site by direct superposition with complexes of related GH23 proteins. Left panel, Superimposition with Ra-ChiC chitinase bound to two (GlcNAc)₂ units (blue and green; PDBs 3W6C and 3W6F) or (GlcNAc)₄ (orange; PDB 3W6D). Central panel, superposition with MltC from *E. coli* bound to (GlcNAc-MurNAc)₂ (pink; PDB 4CFP) or (GlcNAc)₅ (blue; PDB 4HJC), and MltE in complex with bulgecin and GMDD (purple; PDB 4HJV). Right panel, superposition with Slt70 in complex with a 1,6-anhydromurotripeptide (magenta; PDB 1QTE), phiKZ transglycosylase bound to (GlcNAc)₄ (light pink; PDB 3BKV); and the catalytic domain of RpfB from *Mycobacterium tuberculosis* bound to (GlcNAc)₃ (orange; PDB 4KPM). Numbering indicates subsites (from –4 to +2) occupied by the saccharide units in the solved complexes.



Supplementary Figure S11. Putative interactions mediated by the stem peptides in the $LytB_{CAT}/(GMPP)_3$ complex model. Contact networks of MurNAc+1 pentapeptide (a) and MurNAc-2 pentapeptide (b). LytB residues making contacts are shown as grey sticks and the stem peptides in green. The protein surface is coloured according to the electrostatic potential and the feasible hydrogen bonds are indicated with yellow dashed lines.

Supplementary Table S1. Primers used in this study for construction of alanine mutants and							
truncated variants of LytB							
DNA Oligo	Oligonucleotide sequence ^a						
E585A Fw	5'-CCATAGTGCGCTAG <u>CG</u> AGTAACTGGGGAAG-3'						
E585A Rv	5'-CTTCCCCAGTTACT <u>CG</u> CTAGCGCACTATGG-3'						
D596A Fw	5'-GGGGAAGAAGTAAAATTGCCAAAG <u>CG</u> AAGAATAATTTCTTTGG-3'						
D596A Rv	5'-CCAAAGAAATTATTCTTCCCC-3'						
D607A Fw	5'-TTTGGCATTACAGCCTATG CG ACGACCCCTTACC-3'						
D607A Rv	5'-GGTAAGGGGTCGT <u>CG</u> CATAGGCTGTAATGCCAAA-3'						
D618A Fw	5'-CTGCCAAGACATTTG <u>CG</u> GACGTGGATAAGGG-3'						
D618A Rv	5'-CCCTTATCCACGTC C GCAAATGTCTTGGCAG-3'						
D619A Fw	5'-CCTTTCTGCCAAGACATTTGATG <u>CC</u> GTGGATAAGGGAATTTTAGG						
D619A Rv	5'-CCTAAAATTCCCTTATCCAC CG CATCAAATGTCTTGGCAGAAAGG-3'						
D621A Fw	5'-CCAAGACATTTGATGATGTGGCCAACC-3'						
D621A Rv	5'-GGTTGCACCTAAAATTCCCTTCCCACATCATCAAATGTCTTGG-3'						
E633A Fw	5'-GGTGCAACCAAGTGGATTAAGG <u>CG</u> AATTATATCGATAGGGGAAG-3'						
E633A Rv	5'-CTTCCCCTATCGATATAATTCCACTTGGTTGCACC-3'						
D637A Fw	5'-GGATTAAGGAAAATTATATCG CG AGGGGAAGAACTTTCCTTGG-3'						
D637A Rv	5'-CCAAGGAAAGTTCTTCCCCT <u>CC</u> CGATATAATTTTCCTTAATCC-3'						
D653A Fw	5'-GGCTTCTGGTATGAATGTGG <u>CG</u> TATGCTTCAGACCCTTATTGGG-3'						
D653A Rv	5'-CCCAATAAGGGTCTGAAGCATA <u>CG</u> CCACATTCATACCAGAAGCC-3'						
D657A Fw	5'-GGTATGAATGTGGAATATGCTTCAG <u>CC</u> CCTTATTGGGGC-3'						
D657A Rv	5'-GCCCCAATAAGG <u>CG</u> CTGAAGCATATTCCACATTCATACC-3'						
E662A Fw	5'-GCTTCAGACCCTTATTGGGGCGCGCGCGCGAAAATTGCTAGTGTGATGATG-3'						
E662A Rv	5'-CATCATCACACTAGCAATTTTCCGCGCCCCAATAAGGGTCTGAAGC-3'						
E673A Fw	5'-GTGTGATGATGAAAATCAATG C GAAGCTAGGTGGCAAAGATTAG-3'						
E673A Rv	5'-CTAATCTTTGCCACCTAGCTTC G CATTGATTTTCATCATCACAC-3'						
LytB∆1	5'- CG <u>GGATCC</u> GGGAATGAGTGGGTTTTTGATAC-3'						
LytB∆2	5'- CG <u>GGATCC</u> AAATCAGAATGGGTAGAAGACAAG-3'						
LytB∆3	5'- CG <u>GGATCC</u> CACTATTATTATCTAAAATCCGGTG-3'						
LytB∆4	5'- CG <u>GGATCC</u> GCCAATGAATGGATTTG-3'						
LytB∆5	5'- CG <u>GGATCC</u> AATGAGACAGTAGATGGTTATCAG-3'						
LytB∆1Ndel	5'- GTAAATAT <u>CATATG</u> GCGAATGAGTGGGTTTTTGATAC-3'						
LytB∆2Ndel	5'- GTAAATAT <u>CATATG</u> AAATCAGAATGGGTAGAAGACAAG-3'						
LytB∆3Ndel	5'- GTAAATAT <u>CATATG</u> CACTATTATTATCTAAAATCCGGT-3'						
LytB∆4Ndel	5'- GTGATTAT <u>CATATG</u> GCCAATGAATGGATTTG-3'						
LytBΔ5Ndel 5'- GTAAATAT <u>CATATG</u> AATGAGACAGTAGATGGTTATCAG-3'							
LytB52 5'- AA <u>CTGCAG</u> TTAATCTTTGCCACCTAGCTTC-3'							
^a Bold and underlining indicate mutated nucleotides or the restriction sites introduced for cloning							
purposes, respectively.							

Supplementary Table S2. MS analysis of HPLC fractions from cell wall						
or PG degradation with LytB or cellosyl						
Peak No. ¹	Proposed structure ²	Theoretical neutral mass (Da)	Measured neutral mass (Da)			
2	Tri[deAc]	783.386	783.44			
3	Tri	825.397	825.45			
3'	Tri	825.397	825.41			
7'	Penta	967.471	967.50			
10	Tetra(S)[Glu/deAc]	942.435	942.47			
12 ^a	Tri(SA) [deAc]	941.455	941.46			
12	Tri(SA)	983.466	983.48			
12'	Tri(SA)	983.466	983.49			
13	Tri(AA)[deAc]	925.460	925.36			
14	Tri(AA)	967.471	967.48			
14'	Tri(AA)	967.471	967.59			
15	Tetra(SA)	1054.503	1054.51			
15'	Tetra(SA)	1054.503	1054.60			
16'	Penta(SA)	1125.540	1125.60			
19	TetraTri	1703.820	1703.96			
19'	TetraTri	1703.820	1703.71			
23	Tetra(SA)Tri	1861.889	1861.85			
23'	Tetra(SA)Tri	1861.889	1861.70			
29	TetraTri(SA) ₂	2019.958	2019.94			
31	TetraTri(AA) [‡] (SA) [‡]	2003.963	2003.97			
¹ The numbers correspond to the peaks in the chromatograms shown in Fig. 3						
(main text).						
² Modificacions: [Glu], presence of isoglutamic acid instead of isoglutamine;						
[deAc], deacetylation of GlcNAc; [‡] , position of modifications not known.						

Table S3. Polar LytB _{CAT} /(GMPP) ₃		interactions	of LytB _{CAT}	discerned	d from the model of			
Subsite Residue		Atom	Residue	Atom	Localization ¹			
+3	MurNAc	O6	Asn587	Νδ2	LytB _{GH73} (α-3)			
10	GlcNAc	O2N	Ser591	Ογ	LytB _{GH73} (α-4)			
+2	GlcNAc	O3	Ser591	Ογ	LytB _{GH73} (<i>α</i> -3)			
	MurNAc	O2N	Asn587	Νδ 2	LytB _{GH73} (α-3)			
	L-Ala	NH	Asp657	Οδ2	LytB _{GH73} (loop <i>α</i> -3/ <i>η</i> -2)			
ــــــــــــــــــــــــــــــــــــــ	L-Ala	CO	Lys663	Nζ	LytB _{GH73} (<i>α</i> -3)			
ΤI	D-γ-Glu	Οδ2	Ser433	Ογ	LytB _{SH3b} (<i>β</i> -4)			
	D-γ-Glu	Οδ2	Ser431	Ογ	LytB _{SH3b} (<i>β</i> -3)			
	D-Ala2	0	Gln490	Νδ2	LytB _{ww} (loop <i>β</i> -2/ <i>β</i> -3)			
	D-Ala2	OXT	Gln490	Νδ2	LytB _{ww} (loop <i>β</i> -2/ <i>β</i> -3)			
	GlcNAc	O3	Ser656	Ογ	LytB _{GH73} (loop <i>α</i> -3/ <i>η</i> -2)			
_1	GlcNAc	O5	Glu585	Οε2	LytB _{GH73} (<i>α</i> -6)			
	GlcNAc	O6	Glu585	Οε2	LytB _{GH73} (<i>α</i> -6)			
	GlcNAc	O6	Phe601	CO	LytB _{GH73} (loop α -4/flexible region)			
	MurNAc	N2	Gly602	CO	LytB _{GH73} (loop α -4/ flexible region)			
	MurNAc	N2	Tyr429	OH	LytB _{SH3b} (<i>β</i> -4)			
-2	L-Ala	NH	Gly602	CO	LytB _{GH73} (loop α -4/ flexible region)			
	D-γ-Glu	Οδ1	His581	Νε2	LytB _{GH73} (<i>α</i> -3)			
	D-γ-Glu	Οδ1	Tyr654	OH	LytB _{GH73} (3 ₁₀ - η2)			
	D-γ-Glu	Οδ2	Tyr654	OH	LytB _{GH73} (α-3)			
	D-Ala2	OXT	Lys629	Νζ	LytB _{GH73} (α-5)			
_3	GIcNAc	N2	Glu653	CO	LytB _{GH73} (3 ₁₀ - η2)			
-5	GlcNAc	O4	Lys427	Nζ	LytB _{SH3b} (loop <i>β</i> -3/ <i>β</i> -4)			
¹ LytB domain (secondary structure element)								

Supplementary methods

The Dali server¹ was used for 3D structural comparison and Jalview2² to represent structure-based sequence alignments. Circular dichroism spectra (average of 4 consecutive scans) were recorded at 20 °C using a JASCO-J810 spectropolarimeter (Jasco Corp.) equipped with a Peltier cell holder. Measurements were performed at a protein concentration of 0.16 mg/ml as reported elsewhere⁴.

Supplementary References

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