**Supplemental Information**

**Chitin, chitin oligosaccharide and chitin disaccharide metabolism of *Escherichia coli* revisited: reassignment of the roles of ChiA, ChbR, ChbF and ChbG**

**Axel Waltera, Simon Friza, and Christoph Mayera\***

aMicrobiology/Glycobiology, Interfaculty Institute of Microbiology and Infection Medicine,
University of Tübingen, Germany

Short title: chitin metabolism of *E. coli*

**Supplemental Materials: 2 Tables, 1 Figure**

**Supplemental Tables**

**Table S1. Strains, plasmids and primers used in the study**

|  |  |  |
| --- | --- | --- |
| **Strain** | **Characteristics**  | **References**  |
| *E. coli* |  |  |
| K12, Strain BW25113“wild-type” (wt) | *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3); λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Yale Genetic Stock Center |
| JW1723-1Δ*chbF::kan* | *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔchbF733::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Yale Coli Genetic Stock Center |
| JW1722-2Δ*chbG::kan* | *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔchbG733::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514* | Yale Coli Genetic Stock Center |
| DH5α | *SupE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | [Hanahan, 1983] |
| BL21 (DE3) | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* | New England Biolabs |
| BL21(DE3) pET28a-ChbF | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5,* pET28a-ChbF | this work |
| BL21(DE3) pET22b-ChiA | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5,* pET22b-ChiA | this work |
| BL21(DE3) pET28a-Csn | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5,* pET28a-Csn | this work |
| Δ*chbG::kan*pQE32-ChbBCA | *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔchbG733::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514,*pQE32-*chbBCA* | this work |
| BL21(DE3) pET28a-CwlC | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1)**i21 Δnin5,* pET28a-CwlC | [Müller et al., 2021] |
| BL21(DE3) pET16b-NagZ | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5,* pET16b-NagZ | [Litzinger et al., 2010a;Litzinger et al., 2010b] |
| BL21(DE3) pET28a-Atl(Glc) | *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λDE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5,* pET28a-Atl(Glc) | [Müller et al., 2021] |
| *B. subtilis* |  |  |
| strain 168(wild-type) | *trpC2*; genome sequenced *B. subtilis* type strain | *Bacillus* Genetic Stock Center |
| **plasmid** |  |  |
| pET28a(+) | KanR, T7 promoter, ori pBR322, lacI, C/N-terminal His6-tag | Novagen |
| pET22b(+) | AmpR, T7 promotor, lacI, C-terminal His6-tag | Novagen |
| pQE32 | AmpR, T5 promoter/lac operator, N-terminal His6-tag | Qiagen |
| pET28a-ChbF | KanR, T7 promoter, ori, pBR322, lacI, adds C-terminal His6-tag to *chbF* | this work |
| pET22b(+)-ChiA | AmpR, T7 promotor, lacI, adds C-terminal His6-tag to *chiA* | this work |
| pET28a-Csn | KanR, T7 promoter, ori, pBR322, lacI, adds C-terminal His6-tag to *csn* | this work |
| pET28a-CwlC | KanR, T7 promoter, ori, pBR322, lacI, adds C-terminal His6-tag to *cwlC* | [Müller et al., 2021] |
| pET16b-NagZ | AmpR, T7 promotor, lacI, adds N-terminal His10-tag to *nagZ* | [Litzinger et al., 2010a;Litzinger et al., 2010b] |
| pET28a-Atl(Glc) | KanR, T7 promoter, ori, pBR322, lacI, adds C-terminal His6-tag to *atl(glc)* | [Müller et al., 2021] |
| pQE32-ChbBCA | AmpR, T5 promoter/lac operator, *chbBCA* without N-terminal His6-tag | this work |
| **primer** |  |  |
| pET28a-*chbF*-NcoI-for | CACCATGGGAAGCCAGAAATTAAAAGTCGT | this work |
| pET28a-*chbF*-XhoI-rev | CACTCGAGATGTGCTTTTTTAAGCTCTG | this work |
| pET22b-*chiA*-NdeI-for | GGCCCCCATATGAAATTAAATATATTTACTAAATC | this work |
| pET22b-*chiA*-HindIII-rev | CCAAGCTTTTGCTTAGTAAACGGCGCGA | this work |
| pET28a-*csn*-NcoI-for | CCACCATGGGAGCGGGACTGAATAAAGATCA | this work |
| pET28a-*csn*-XhoI-rev | CCACTCGAGTTTGATTACAAAATTACCGT | this work |
| pQE32-*chBCA*-GA-for | CAGAATTCATTAAAGAGGAGAAATTAACTATGGAAAAGAAACACATTTATCTGTTTTGT | this work |
| pQE32-*chBCA*-GA-rev | TCTATCAACAGGAGTCCAAGCTCAGCTAATTATGCCTTCAGTTTTTCATGAAGC | this work |
| pET28a-*cwlC*-NcoI-for | GCTCCATGGCGGTTAAAATTTTTATTGATCCTGGACAT | [Müller et al., 2021] |
| pET28a-*cwlC*-XhoI-rev | GCTAGCTCGAGTGATTCTAGGATCACAATAGC | [Müller et al., 2021] |
| pET16b-*nagZ*-NdeI-for | AAAACCATGGGCCATATGTTTTTCGGGGCCAGACAGAC | [Litzinger et al., 2010a;Litzinger et al., 2010b] |
| pET16b-*nagZ*-XhoI-rev | TTTTCTCGAGTTAAAGCGGTCTTCCCGTTTTG | [Litzinger et al., 2010a;Litzinger et al., 2010b] |
| pET28a-*atl(glc)*-NcoI-for | CAGCTCCATGGCTTATACTGTTACTAAACCAC | [Müller et al., 2021] |
| pET28a-*atl(glc)*-XhoI-rev | GCGCCTCGAGTTTATATTGTGGGATGTCGAAG | [Müller et al., 2021] |

**Table S2. Exact monoisotopic masses of chitin, chitosan and PGN metabolites**

Overview of chitin, chitosan and PGN metabolites and their exact neutral masses and masses with proton adducts in positive ionization mode.

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Formula** | **exact monoisotopic mass [M]** | **exact monisotopic mass of the proton adduct [M+H]+** |
| chitobiose (GlcN-GlcN) | C12H24N2O9 | 340.1482 | 341.1555 |
| chitotriose(GlcN-GlcN-GlcN) | C18H35N3O13 | 501.2170 | 502.2243 |
| chitotetraose(GlcN-GlcN-GlcN-GlcN) | C24H46N4O17 | 662.2858 | 663.2931 |
| diacetyl-chitobiose(GlcNAc-GlcNAc) | C16H28N2O11 | 424.1693 | 425.1766 |
| triacetyl-chitotriose(GlcNAc-GlcNAc-GlcNAc) | C24H41N3O16 | 627.2487 | 628.2560 |
| monoacetyl-chitotriose(GlcN-GlcNAc-GlcN) | C20H37N3O14 | 543.2276 | 544.2348 |
| MurNAc-GlcNAc | C19H32N2O13 | 496.1904 | 497.1977 |
| (MurNAc-GlcNAc)-4P | C37H61N7O21 | 939.3921 | 940.3993 |
| (GlcNAc-MurNAc)-3P\*(MurNAc-GlcNAc)-3P\* | C34H57N7O19 | 867.3709 | 868.3782 |
| 3P\*tri-peptide (1x amidation) | C15H27N5O7 | 389.1910 | 390.1983 |
| 3Ptri-peptide | C15H26N4O8 | 390.1751 | 391.1823 |
| 4Ptetra-peptide | C18H31N5O9 | 461.2122 | 462.2195 |
| 3P-4Ptri-tetra-peptide | C33H55N9O16 | 833.3767 | 834.3840 |
| 4P-4Ptetra-tetra-peptide | C36H60N10O17 | 904.4138 | 905.4211 |
| 3P\*-4P\*tri-tetra-peptide (2x amidations) | C33H57N11O14 | 831.4086 | 832.4159 |
| 3P-4P\*tri-tetra-peptide (1x amidation) | C33H56N10O15 | 832.3927 | 833.3999 |

**Supplemental Figures**

**Fig. S1. ChiA does not cleave *B. subtilis* peptidoglycan.** Intact peptidoglycan, derived from *B. subtilis* (left panels), and denuded peptidoglycan (right panels), i.e. treated with the amidase CwlC to remove the peptide stems, were incubated with ChiA, the endo-glucosaminidase Atl (Glc) of *S. aureus* or Mutanolysin*.* The formation of reaction products was analyzed by LC-MS. (***A***) Uncleaved *B. subtilis* peptidoglycan (control). (***B***)Incubation of *B. subtilis* peptidoglycan with ChiA (+ChiA), which does not release any soluble products. (***C***) Incubation of *B. subtilis* peptidoglycan with Atl (Glc) (-Atl (Glc)) releases large amounts of (MurNAc-GlcNAc)-tripeptide (amidated) (M+H)+ m/z = 868.3701 (MurNAc-GlcNAc-3P\*; blue) and to a lesser extent MurNAc-GlcNAc (M+H)+ m/z = 497.1902 (red). (***D***)Mutanolysin (+Mut) exclusively releases (GlcNAc-MurNAc)-3P\* (M+H)+ m/z = 868.3675 (blue). (***E***)CwlC (+CwlC) removes amidated tri- (M+H)+ m/z = 390.1978 (3P\*, light blue), tri-tetra (M+H)+ m/z = 833.3943 (3P-4P\*, purple) and double amidated tri-tetra peptides (M+H)+ m/z = 832.4122 (3P\*-4P\*, orange) from *B. subtilis* peptidoglycan. (***F***)From (*E*)ChiA (+CwlC+ChiA) is unable to cleave denuded peptidoglycan. (***G***)From (*E*), *A*tl (Glc) (+CwlC+Atl (Glc)) is able to release large amounts of MurNAc-GlcNAc (M+H)+ m/z = 497.1954 (red). Intriguing, no MurNAc-GlcNAc-4P could be detected, indicating the complete removal of the stem peptides from the peptidoglycan by CwlC. (***H***) From (*E*), Mutanolysin (+CwlC+Mut), on the other hand, does not release disaccharides from the glycan chain, which indicates that this enzyme is not able to cleave denuded peptidoglycan. Shown are the base peak chromatogram (BPC) mass range (M+H)+ m/z = 200 – 2000 (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (Table S2).

References

Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol. 1983 Jun 5;166(4):557-80.

Litzinger S, Duckworth A, Nitzsche K, Risinger C, Wittmann V, Mayer C. Muropeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase. J Bacteriol. 2010a Jun;192(12):3132-43.

Litzinger S, Fischer S, Polzer P, Diederichs K, Welte W, Mayer C. Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. J Biol Chem. 2010b Nov 12;285(46):35675-3584.

Müller M, Calvert M, Hottmann I, Kluj RM, Teufel T, Balbuchta K, et al. Exo-β-*N*-acetylmuramidase NamZ of *Bacillus subtilis* is the founding member of a family of exo-lytic peptidoglycan hexosaminidases. bioRxiv 2021:2021.01.10.425899.