

The fish pathogen *Aliivibrio salmonicida* LFI1238 can degrade and metabolize chitin despite major gene loss in the chitinolytic pathway

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Supplementary material

Supplementary figures

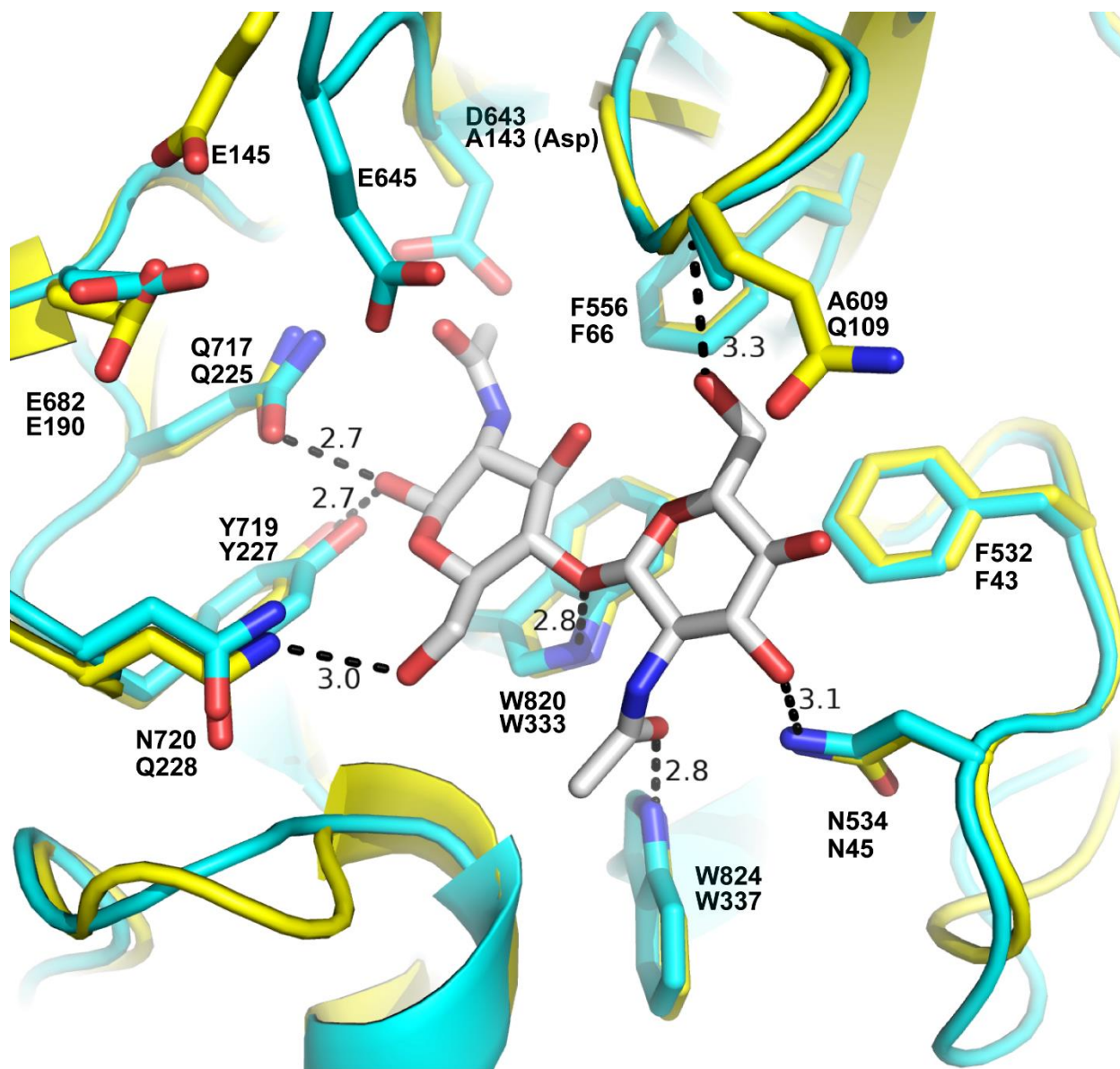


Figure S1. Active site of the AsChi18A homology model superimposed on ChiNCTU2 (D143A variant). Proteins are shown as cartoon representation with side chains shown in stick representation (AsChi18A colored cyan, ChiNCTU2 D143A [PDB id: 3N13] colored yellow). Side chains are labeled showing AsChi18A amino acid numbers above the ChiNCTU2 numbers. The chitobiose ligand bound in the -1 and -2 subsite of the ChiNCTU2 active is shown in stick representation with gray colored carbon atoms. Hydrogen bonds are illustrated by dashed lines and distances (Å) are indicated. It should be noted that the positioning of the ChiNCTU2 catalytic acid, E145, deviates from the position observed in the ChiNCTU2 wild type structure (which is more similar to the positioning of AsChi18A E645) due to the absence of an Asp at position 143.

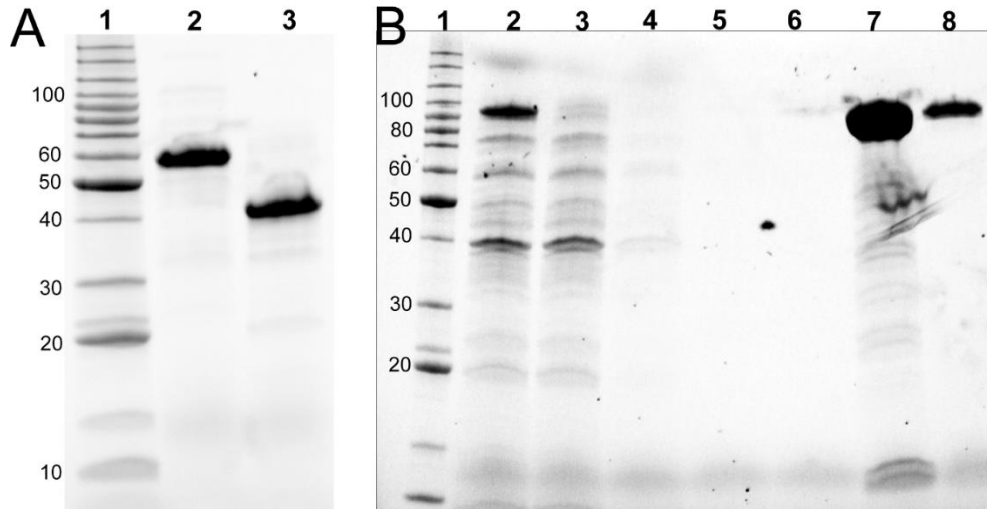


Figure S2. Analysis of protein purity. SDS-PAGE was used to determine protein purity. Panel A shows an SDS-PAGE gel with lanes displaying protein benchmark ladder (Invitrogen) in lane 1, *AsLPMO10A* in lane 2 and *AsLPMO10B* in lane 3. The SDS-PAGE gel in panel B displays the stages of protein purification for *AsChi18A*, showing the protein benchmark ladder in lane 1, cell free extract from an induced culture in lane 2, flow through in lane 3, wash fraction in lane 4-6 and the eluted protein in lanes 7 and 8. Only fractions containing highly pure protein were used in biochemical assays. All proteins used in biochemical assays were estimated to be >95% pure.

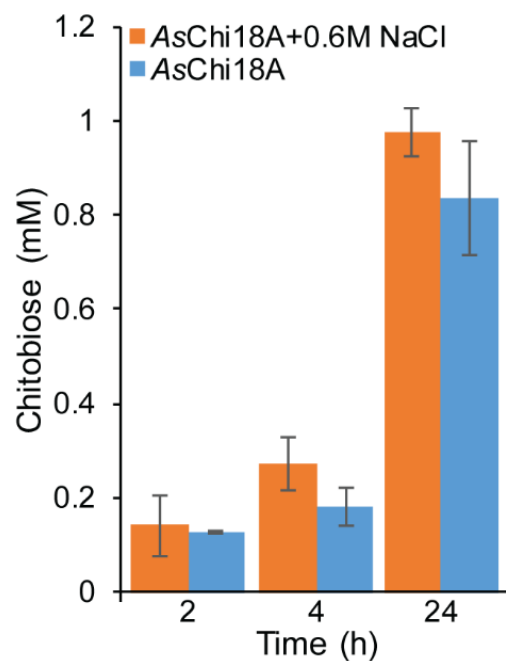


Figure S3. Influence of NaCl on AsChi18A activity. The amount of (GlcNAc)₂ (chitobiose) released from 10 mg/mL β -chitin in Tris-HCl pH 7.5 by 1.0 μ M AsChi18A in the presence and absence of 0.6 M NaCl was evaluated at three time points (n=3). Reactions were incubated at 30°C.

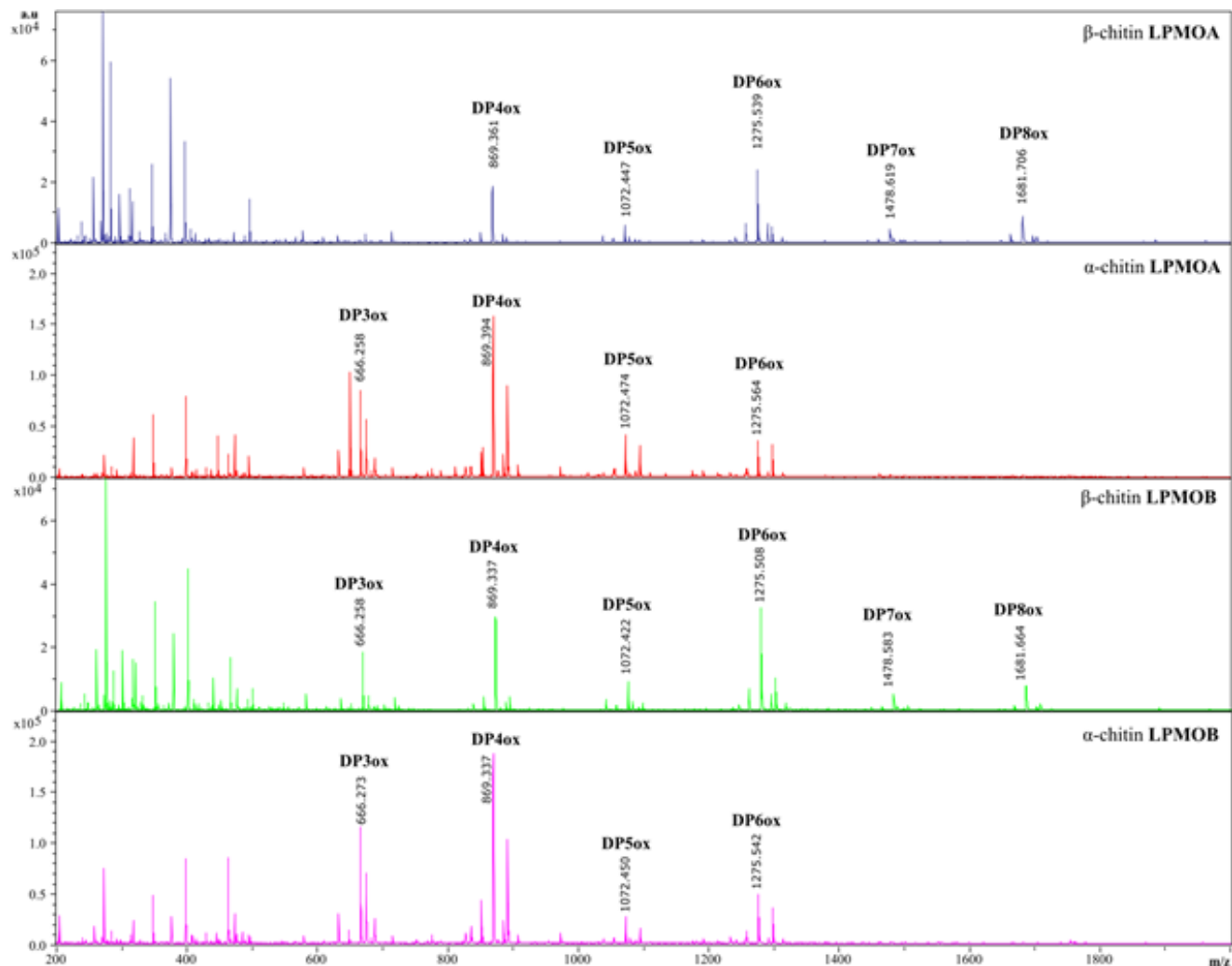


Figure S4. MALDI-TOF MS analysis of oxidized products generated by *AsLPMO10A* and **-B** from *A. salmonicida* on chitin (α and β). The MS spectra show soluble C1 oxidized chito-oligosaccharides, i.e. aldonic acids. The degree of polymerization of each product is indicated by “DPn ox”, where n equals the number of monosaccharides in the chain. The main peaks are labelled with the respective masses.

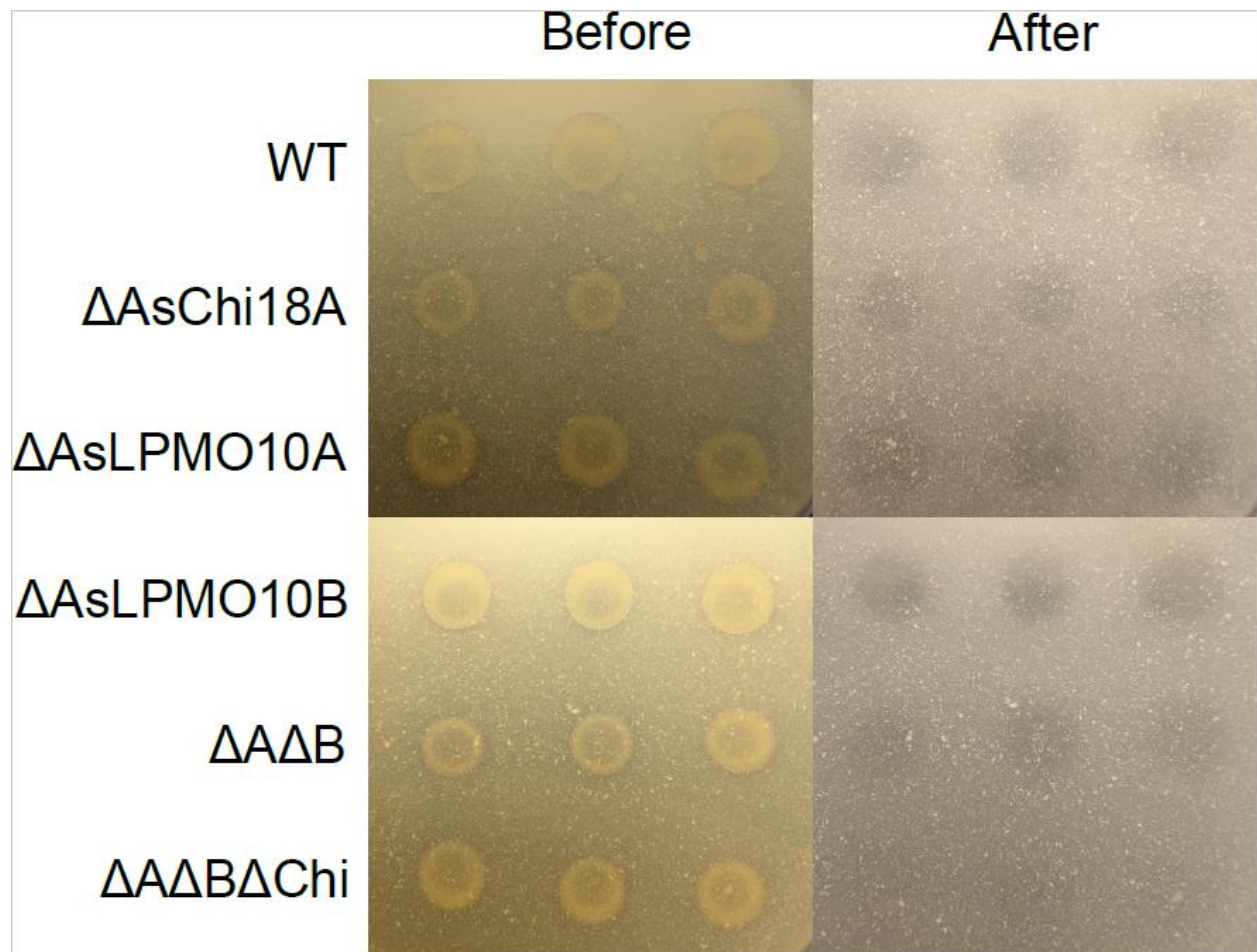


Figure S5. Chitin degradation assay. Images show photographs of agar plates containing LB25 supplemented with 2% colloidal chitin with *Al. salmonicida* variants (indicated on the left side of the image) spotted in triplicate and allowed to grow at 12 °C for 20 days. The photographs show the agar plates before (left) and after (right) the colonies had been removed by gentle washing. Halos indicate chitin degradation.

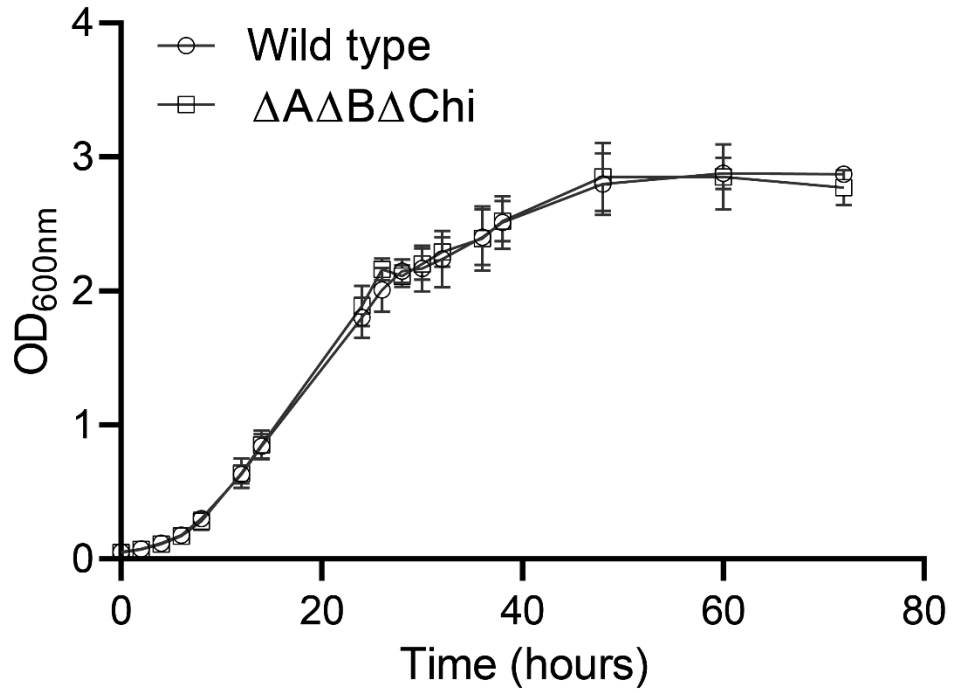


Figure S6. Growth of *Al. salmonicida* LFI1238 variants. Growth of the wild type *Al. salmonicida* LFI1238 strain compared to the triple knock-out strain ($\Delta A\Delta B\Delta Chi$) in LB25 broth. Standard deviation is indicated by error bars (n=3).

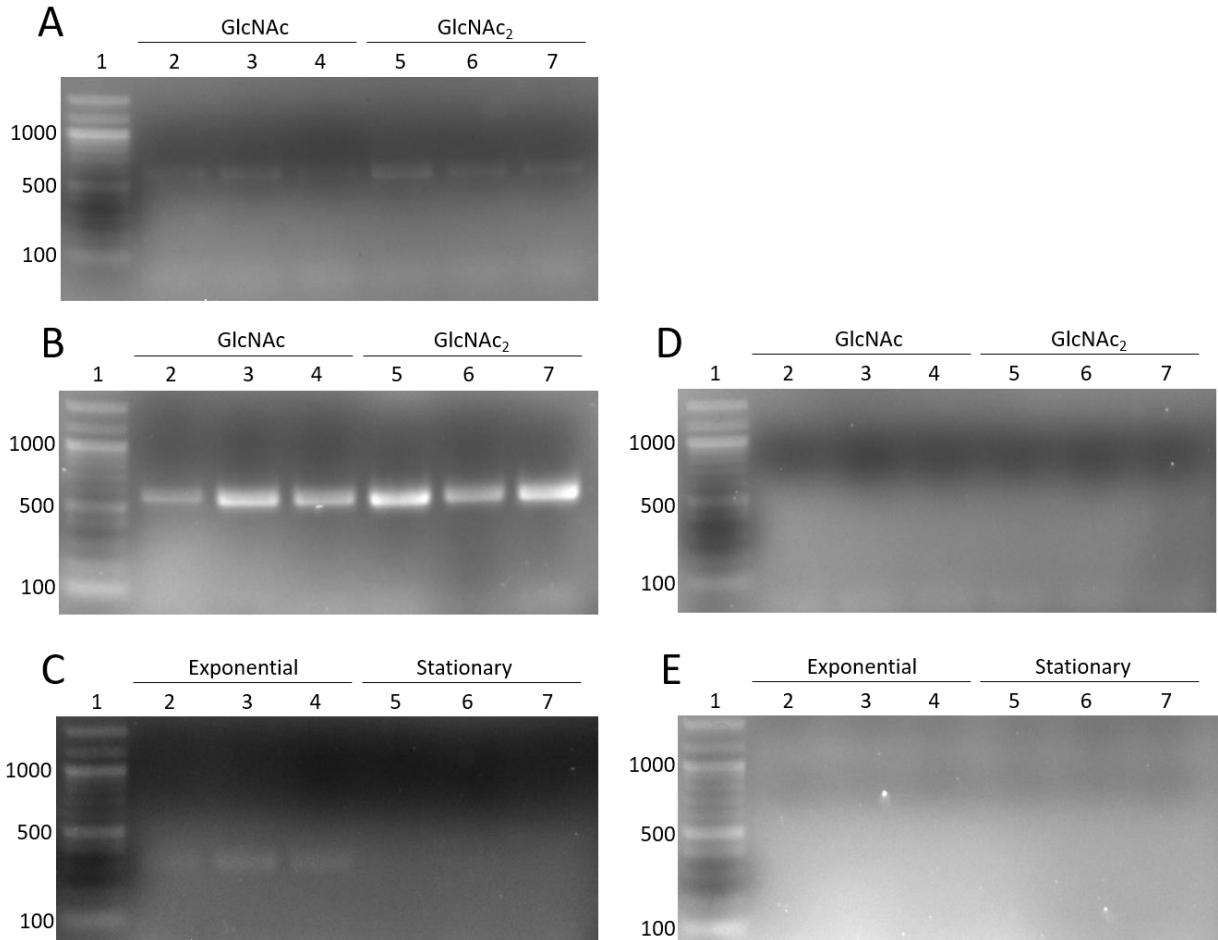


Figure S7. Gene expression analysis by PCR amplification of cDNA. Panel A and B shows the products formed in PCR experiments using cDNA from samples obtained during exponential growth in GlcNAc and GlcNAc₂ combined with primer pairs *GH18Expression* and *10AExpression*, respectively. Panel D shows the PCR experiments using -RT controls as template combined with primer pair *10AExpression*. The -RT templates used in Panel D corresponds to the cDNA applied to Panel A and B. Panel C shows the products formed in PCR experiments using cDNA from samples obtained during exponential and stationary growth in glucose combined with primer pair *I0902Expression*. In this case gene expression was evaluated as positive (+) during exponential growth (lane 2-4) and negative (-) during stationary growth (lane 5-7). Panel E shows the products formed in PCR experiments using the -RT samples corresponding to the cDNA template used in Panel C combined with primer pair *I0902Expression*. Lane 1; 100 bp DNA ladder, lanes 2-4 or 5-7; biological replicates within the same condition.


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B6EGV7_ALISL      1 MKKTLALTVSSII LSTPSLASAPNTNLNLMYPYPQSVELKSGKLPIDNNFSIYIDGYKSE
D9ISD9_VIBHA      1 -----MGGSGQGKITLDKSFSIYIKGYDSP

B6EGV7_ALISL     61 RIQQLAQRIIKRIEAQTGLPLLSPFNSSEKNATLIIRVNKAAKKEIQDNHIDESYQLSVN
D9ISD9_VIBHA     26 RVQFNARKTMDRLYRQTGLPMLNWHAESEKDATLVIDIRNAPKSEVQDIHSDESYLESR

B6EGV7_ALISL    121 QKQIILQAERPYPGIRGAETFLQLITTSKNQYSVPQIITIEDQPRFPWRGASFDSSRHFVVS
D9ISD9_VIBHA     86 NGQIIRSERPYGAFHGLETFLLQVTTDATGYFVPAVSIQDEPRFPWRGVSYDTSRHFIE

B6EGV7_ALISL    181 IETIKRQIDGFASAKMNVFHWHLWDDQAIRIQIESYPKLWQKTADGDVYTKKEIKDVIIEY
D9ISD9_VIBHA    146 LDVILRQLDAMASAKMNVFHWHLWDDQAIRIQLDNYQSLWQDTADGDYVTKDEIRYVVNY

B6EGV7_ALISL    241 ARLRGIRVIPEISLPGHASGVAHAYPELMSGEGKQSYEQQRAWGVFVPLMNPPLNPELYIF
D9ISD9_VIBHA    206 ARNLGIRVIPEISLPGHASAVAHAYPELMSGMGEGQSYPHQRCGWGVFEPLMDPTNPELYKM

B6EGV7_ALISL    301 FDNVFSSEVTDLFPDEYIHIIGGDEPNYQQWSNNKKIQAFIKENNDGNRGLQSYLNARIEK
D9ISD9_VIBHA    266 LASVFEDEVVELFPDEYFHIIGGDEPNYQQWKDNPKIQQFIKDNNDGERGLQSYLNTKVEQ

B6EGV7_ALISL    361 MINDKGGKIMGWDEIWHKDLPTSIVIQSWRGHDSIGQAAKEGYAGLILSTGFYLDQPQPTS
D9ISD9_VIBHA    326 MLEQRGGKMTGWDEIWHKDLPTSIVIQSWRGHDSIGRAAKEGYQGILSTGYYLDQPQPTS

B6EGV7_ALISL    421 YHYRNDPMPKGPQVDDQHQGESFETYTWQKPRGKGGPRKGTLTIIITSKEGNARAFTDYN
D9ISD9_VIBHA    386 YHYRNDPMPKGITVDDQLHQGEKFATYDWWKPRNKGGPRTGNLTIIEATDGSYRAFTDYN

B6EGV7_ALISL    481 GKSRAKVDIIEYTKGESFRGHFDNFMSYTEFNLTMDNRQFS DGSYQLIGNVRWPTTGELT
D9ISD9_VIBHA    446 GKSREEVFIIEYVPGVKFKGHFDNFMSYTEFNDFAGGKLDSSYQLIGNVRWPATGELV

B6EGV7_ALISL    541 ASSSMKGTKIEKPTGGYPVALNEKEQVLILGGEAAIWAENYDDLTVEARIWPRTYAVGER
D9ISD9_VIBHA    506 ASSDMEGGSVIPEPNGGYPALTEKEQPLILGGEITIWGENLDSMTIEQRLWPRSYAVAER

B6EGV7_ALISL    601 LWSAESLTDEDSMYKRLEVMNWNATISVGLQHQANSYKQYLRLA-----SFANYAE
D9ISD9_VIBHA    566 LWSSQDLTDRERSMYKRMKVMDTWSEISLGLRHHADANIMLKRLANGADETPLQTLAKYIE

B6EGV7_ALISL    652 PAQYYARNWAKFNATDPKGELYNQYERLNRFDVAVPVESLAVLKMMSL-ATTATNTPSDL
D9ISD9_VIBHA    626 PAQYYARHWKWKWISTPNEGDLNQYERLNRFDALPVESTAVYEMQDLVAAYALGDKTAL

B6EGV7_ALISL    711 QELKRHYQTVLDSAIKSKPIFENNIASIDTAPLAEKHKEVATAALELIAI LEKGNKPTST
D9ISD9_VIBHA    686 DALAMHYQSVKLAQQAKPIFAANVASVETVPVAEAAIKVADLGLTLIELAKQGSDISQS

B6EGV7_ALISL                                771 QLRHVISVTTTASGMYDEMIVAIVRPTEELVRQLRKQ
D9ISD9_VIBHA    746 DAEAYQRIINENAIIFDETIVAIIVVPTTEQLLHTLTD-

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Figure S8. Pairwise sequence alignment of the *Al. salmonicida* GH20 (UniProt ID B6EGV7) and *Vibrio harvey* GH20 *VhNAG1* (UniProt ID D9ISD9). The alignment was made using the EMBOSS pairwise sequence alignment tool using default parameters. The Alignment was formatted using BoxShade. Based on data from W. Suginta et al. (1), catalytic amino acids are shown in red shading and amino acids involved in substrate binding are shown in blue shading.

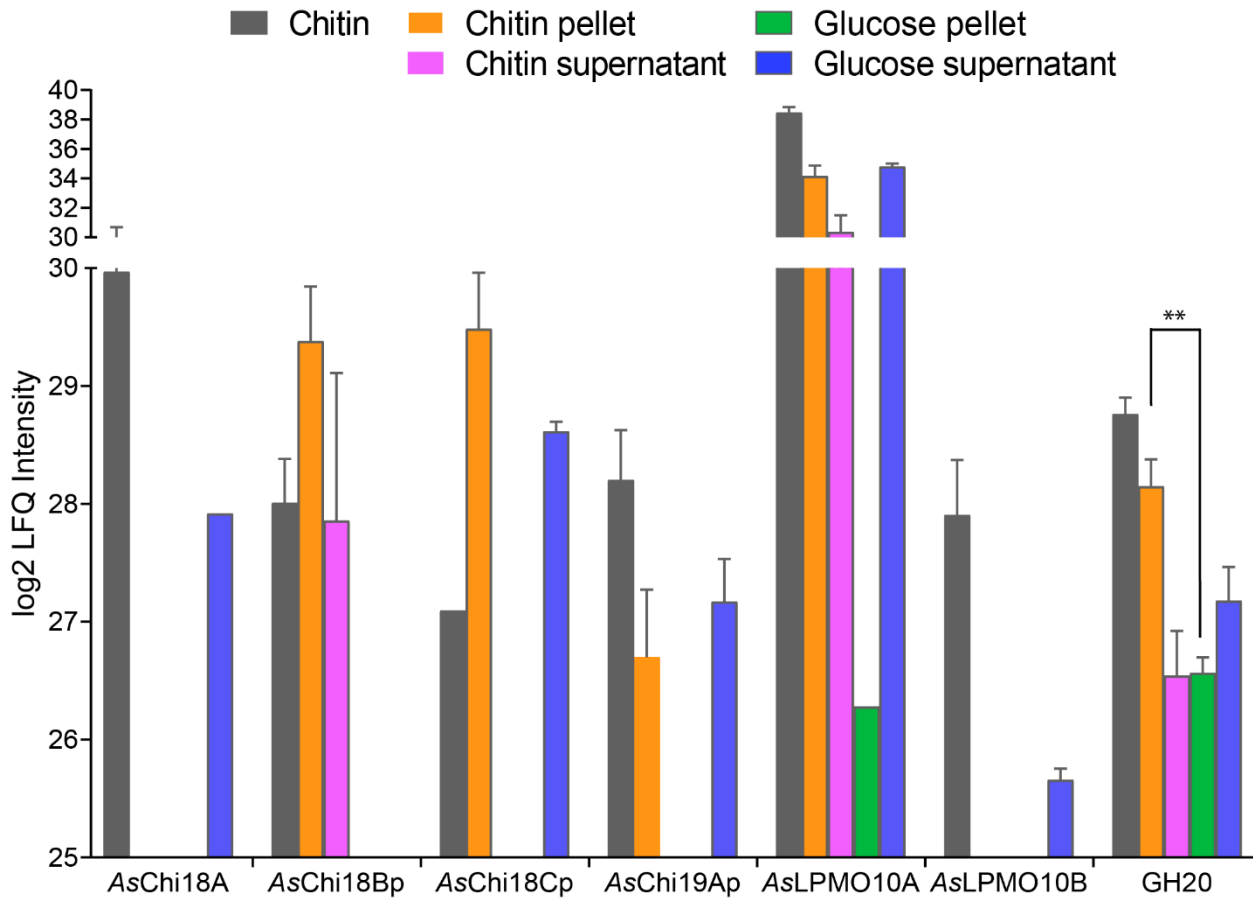


Figure S9. *Al. salmonicida* protein abundances. The abundance of selected *Al. salmonicida* proteins related to chitin catabolism displayed in bar chart format as a supplement to Figure 9. Log₂ LFQ values shown represent the average values obtained from the label free proteomics data (Supplementary Dataset 1). Error bars are shown for proteins that were detected in two or three biological replicates. The GH20 β -*N*-acetylhexosaminidase was calculated to have a statistically significant 1.58 log₂ fold higher abundance during growth on chitin compared to glucose ($p=0.0082$; paired two-tailed t test).

Supplementary tables

Table S1. Growth rate measurements and max cell density of *Al. salmonicida* cultivated in glucose, GlcNAc and GlcNAc₂

Carbon source	Rate constant μ (hours ⁻¹)	Generation time (hours)	Max cell density (OD ₆₀₀)
Glucose	0.065 ± 0.025	5.36 ± 2.17	2.63 ± 0.094
GlcNAc	0.069 ± 0.029	5.16 ± 2.00	1.31 ± 0.022
GlcNAc ₂	0.055 ± 0.021	4.95 ± 0.89	1.58 ± 0.145

Mean ± SD of three biological replicates.

Table S2. Identified CAZymes sorted by their putative biological processes, according to gene ontology (GO) annotations.

CAZy	Uniprot	Biological process
Carbohydrate metabolic process [GO:0005975]		
AA10	B6EQJ6	AsLPMO10B
CBM73;GH18	B6EH15	AsChi18A
GH13_19	B6EGT4	Alpha-amylase
GH20	B6EGV7	Putative beta-N-acetylhexosaminidase
GT35	B6EQ29	Alpha-1,4 glucan phosphorylase
GH77	B6EQ30	4-alpha-glucanotransferase
GH3	B6ERJ6	Beta-hexosaminidase*
Chitin binding [GO:0008061]		
AA10;CBM73	B6EQB6	AsLPMO10A
Cell cycle, cell division, protein import [GO:0007049/ 0051301/ 0017038]		
PL22	B6EGK3	Tol-Pal system protein TolB
Formaldehyde catabolic process [GO:0046294]		
CE1	B6EH03	S-formylglutathione hydrolase
Glycogen biosynthetic process [GO:0005978]		
GT5	B6EQL7	Glycogen synthase
Lipid A biosynthetic process [GO:0009245]		
CE11	B6ELH0	UDP-3-O-acyl-N-acetylglucosamine deacetylase
GT19	B6EJW7	Lipid-A-disaccharide synthase
Lipopolysaccharide biosynthetic process [GO:0009103]		
GT9	B6EPB8	ADP-heptose-LPS heptosyltransferase II
Peptidoglycan metabolic process [GO:0000270]		
GH23	B6EJV5	Membrane-bound lytic murein transglycosylase D
GH23	B6EGC8	Soluble lytic murein transglycosylase
Trehalose catabolic process [GO:0005993]		
GH13_29	B6ERJ9	Trehalose-6-phosphate hydrolase
Not assigned		
GH103	B6EIW0	Putative exported protein
GT2	B6EKR9	Putative glycosyl transferase
GT51	B6EM36	Penicillin-binding protein 1A

*Also, Cell cycle [GO:0007049];cell division [GO:0051301];cell wall organization [GO:0071555];peptidoglycan biosynthetic process [GO:0009252];peptidoglycan turnover [GO:0009254];regulation of cell shape [GO:0008360]

Table S3. Primers designed for construction of flanking regions and fusion product, sequencing and selection/verification of transconjugates and mutants.

Primer	Sequence 5'-3'
<i>AsGH18_For</i>	GCTGATGGCGTGATCAAC
<i>AsGH18_Rev</i>	GGCGCGTGCTAATTTCAA
<i>AsLPMO10A_For</i>	GGCTGCTATTGTACAGAAATA
<i>AsLPMO10A_Rev</i>	AAGCCTAATAAAGCACACCCA
<i>AsLPMO10B_For</i>	GATGAGGTGTACCATCTTGAA
<i>AsLPMO10B_Rev</i>	TGTAATAGAATGTCACCAGCA
pDM4_Seq_F	CGGGAGAGCTCAGGTTAC
pDM4_Seq_R	GGCTTCTGTTTCTATCAGCT

Table S4. Primers applied for amplification of target genes using cDNA.

<i>Primer</i>	<i>Sequence (5' - 3')</i>	<i>Product size (bp)</i>
<i>GH18Expression_F</i>	AGTCAAGCATCAGCCAAGAAAG	566
<i>GH18Expression_R</i>	TAAGGCAAGGCTCGATCCAG	
<i>10AExpression_F</i>	ATTCGGTCCTGCTGATGG	565
<i>10AExpression_R</i>	ATTTGCTTGACCTTGTGTTGC	
<i>10BExpression_F</i>	TCAAGCGTGTCAGTCTGC	441
<i>10BExpression_R</i>	TGCCAACGAGTGTAGAGC	
<i>I0902Expression_F</i>	ATGCACAAGGTCGATCTG	297
<i>I0902Expression_R</i>	ATGGGATGTACTTGTCGC	

Supplementary references

1. Suginta W, Chuenark D, Mizuhara M, Fukamizo T. 2010. Novel beta-N-acetylglucosaminidases from *Vibrio harveyi* 650: cloning, expression, enzymatic properties, and subsite identification. *BMC Biochem* 11:40.