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Backbone circularization of *Bacillus subtilis* family 11 xylanase increases its thermostability and its resistance against aggregation

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Electronic Supplementary Information

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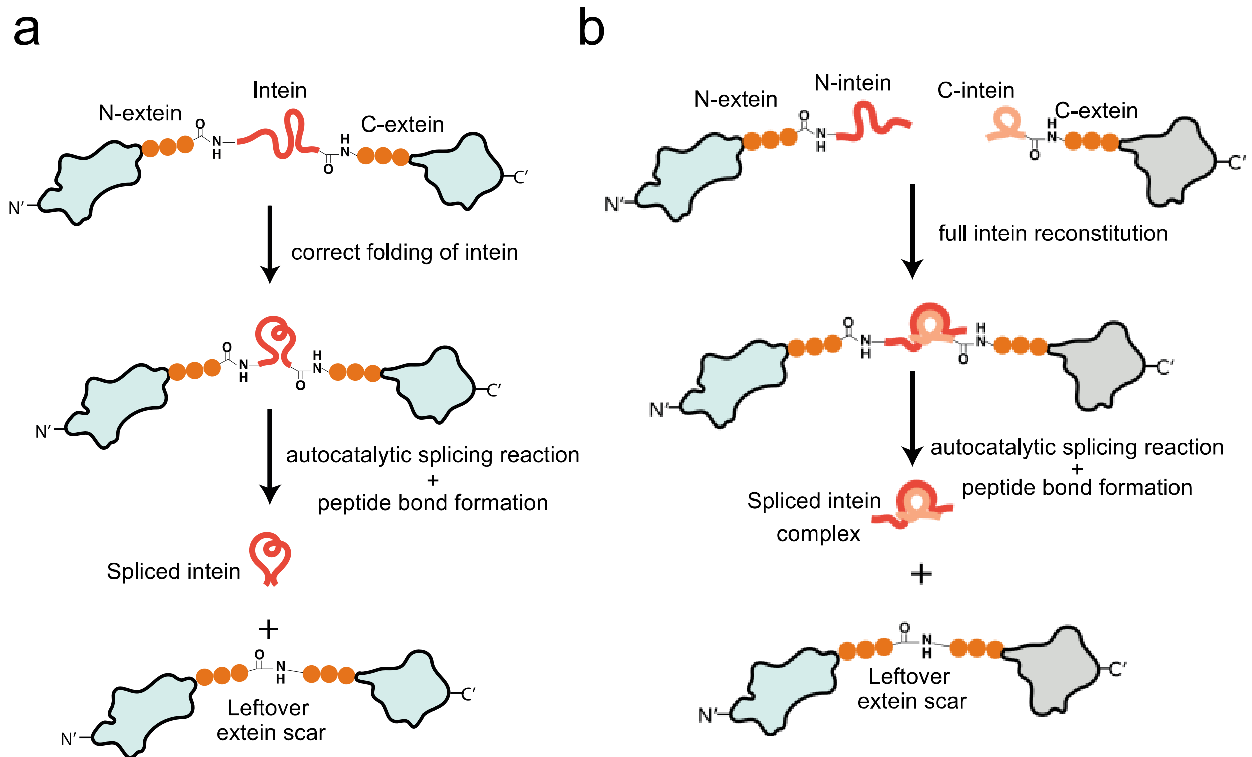

Fig. S1 Schematic overview of intein-mediated protein a) cis- and b) trans-splicing. We depict here inteins artificially included in proteins to control their function. In this case, we indicate with N- and C-extein the residues that need to be introduced for efficient splicing and not the entire fragments that are joined after splicing.

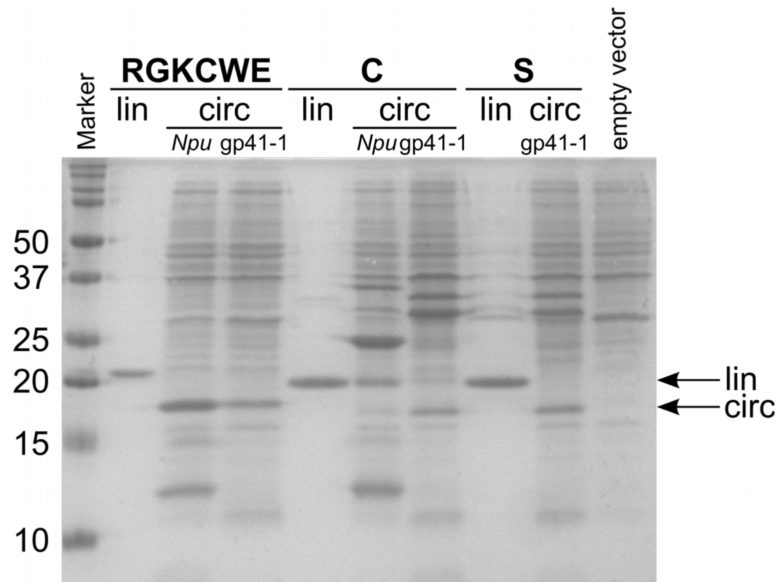

Fig. S2 Analysis of expression of constructs in *E. coli*. SDS-PAGE of crude cell lysates of bacterial cultures expressing the indicated constructs. The different exteins used for circularization are indicated in bold. *Npu*, *Npu* DnaE split intein. Arrows point to the position in the gel where the linear (lin) or circular (circ) xylanases migrated.

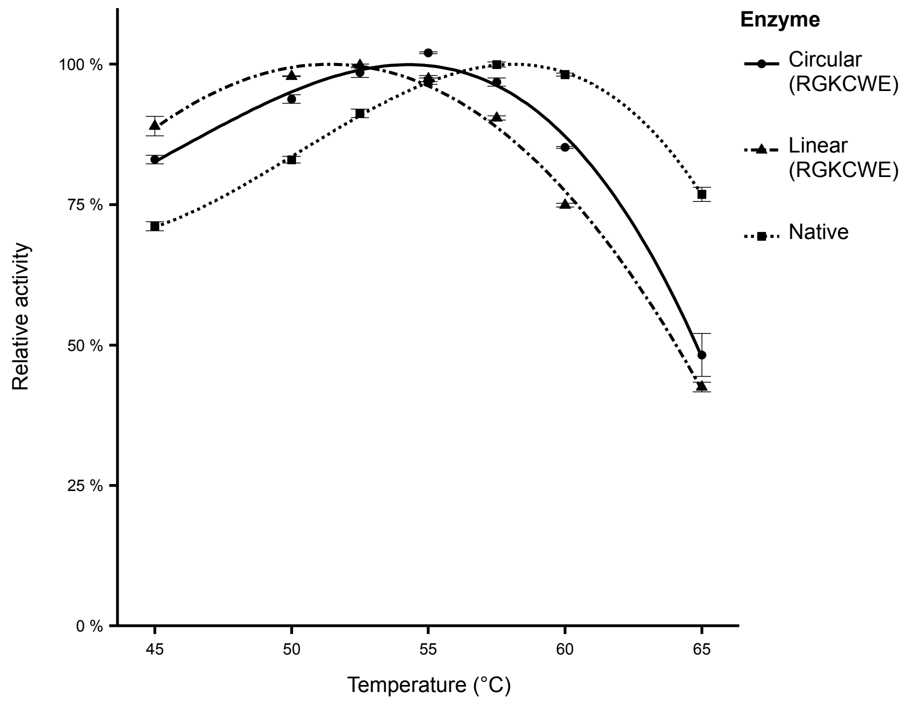

Fig. S3 Temperature dependence of catalytic activities for the circular and linear RGKCWE variants and native xylanase. Enzymes were used at a final concentration of 2 μg/mL in the presence of 1.26 % glycerol and 0.25 mM dithiothreitol. The data points are normalized to the local maximum of a fitted 3rd degree polynomial for each variant. Error bars represent the standard error of the mean for three independent replicates.

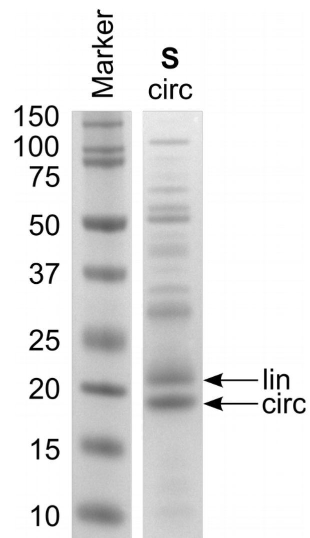

Fig. S4 Impurities present in the circular S variant after purification with CM Sephadex C-50 before incubation at 60 °C. SDS-PAGE of the indicated construct. Other lanes in the gel are not shown and are indicated by a white space. Arrows point to the position in the gel where the linear (lin) or circular (circ) xylanases migrated.

Table S1 Oligonucleotides used for the construction of the plasmids.

Name	Sequence (5'− 3')	Target	Amplicon usage
pSBX_F_taataa	TAATAAATCGGTGAAATGCACGACTGATAG	pSB1K30-T7RBS-IGEMHD	Vector fragment for pCIRC$_{Npu}$ and pCIRC$_{gp41\text{-}1}$
pSBX_R_atg	CATCTAGTATCTCCTTCTTAAAGTTAAACAAAATTATTCTC		
NpuDnaEC_F_pSB-T7RBS	TCTAGAGAATAATTTTGTTTAACTTTAAGAAGGAGATACTAGATGATCAAAATAGCCACACGTAAATATTTAGGC	pVS41[1]	*Npu* DnaE^{C} insert for pCIRC$_{Npu}$
NpuDnaEC_R	GAGAGGCGGTTTGCGTATTGGGTCTCTGTTGGAAGCTATGAAGCCATTTTTGAGTGC		
mRFP_F_BsaI	AGAGACCCAATACGCAAACCGCCTCTC	pSB1C3-BBa_J04450	mRFP insert for pCIRC$_{Npu}$ and pCIRC$_{gp41\text{-}1}$
mRFP_R_BsaI	TGAGACCTATAAACGCAGAAAGGCCCACC		
NpuDnaEN_F	GTGGGCCTTTCTGCGTTTATAGGTCTCATGCTTAAGCTATGAAACGGAAATATTGACAG	pVS07[2]	*Npu* DnaE^{N} insert for pCIRC$_{Npu}$
NpuDnaEN_R_taataa-pSBX	CTAGTACTATCAGTCGTGCATTTCACCGATTTATTAATTCGGCAAATTATCAACCCGCATC		
gp41-1(C)_F_T7RBS_v2	CTTTAAGAAGGAGATACTAGATGATGCTGAAAAAAATCC	pSB4C50-BBa_K1362160	gp41-1^{C}+mRFP insert for pCIRC$_{gp41\text{-}1}$
RedGate_R	GACCTATAAACGCAGAAAGGC		
RedGate_F	CCCAATACGCAAACCG	pSB4C50-BBa_K1362161	mRFP+gp41-1^{N} insert for pCIRC$_{gp41\text{-}1}$
gp41-1(N)_R_IGEMHD	GTGCATTTCACCGATTTATTATTCTTTAACATACAGACACATACC		
Xyla_F_BsaI_C-ext	CTGGTCTCACAACTGCTGGGAAGCTAGCACAGACTACTGGCAAAATTG	*B. subtilis* 168 genome or pNAT-xynA	Xylanase insert for pCIRC$_{Npu}$-^{CWE}xynA^{RGK} and pCIRC$_{gp41\text{-}1}$-^{CWE}xynA^{RGK}
Xyla_R_BsaI_N-ext	CTGGTCTCTAGCATTTACCACGCCACACTGTTACGTTAGAACTTCCAC		
Xyla_F_BsaI_C	CTGGTCTCACAACTGCGCTAGCACAGACTACTGGCAAAATTG	pNAT-xynA	Xylanase insert for pCIRC$_{Npu}$-^{C}xynA and pCIRC$_{gp41\text{-}1}$-^{C}xynA
Xyla_R_BsaI	CTGGTCTCTAGCACCACACTGTTACGTTAGAACTTCCAC	pNAT-xynA	Xylanase inserts for pCIRC$_{Npu}$-^{C}xynA, pCIRC$_{gp41\text{-}1}$-^{C}xynA, and pCIRC$_{gp41\text{-}1}$-^{S}xynA
xynA_F_CC-S	GTTTCTGGTCTCACAACAGCGCTAGCACAGACTACTGGC	pNAT-xynA	Xylanase insert for pCIRC$_{gp41\text{-}1}$-^{S}xynA
Xyla_BB_F_RBS34_X	GCCGCTTCTAGAGAAAGAGGAGAAATACTAGATGGCTAGCACAGACTACTGGCAAAATTG	pCIRC$_{Npu}$-^{CWE}xynA^{RGK}	Xylanase inserts for pNAT-xynA, pLIN-xynA^{S}, and pLIN-xynA$^{\Delta}$
Xyla_BB_R_SP	GGAAGCCTGCAGCGGCCGCTACTAGTATTATTACCACACTGTTACGTTAGAACTTCCAC	pCIRC$_{Npu}$-^{CWE}xynA^{RGK}	Xylanase inserts for pNAT-xynA, pLIN-^{C}xynA, pLIN-^{S}xynA, and pLIN-$^{\Delta}$xynA
ExteinInsert_F	GTGGCGTGGTAAATGCTGGGAA	(Annealing yields exteins insert for pLIN-xynA^{RGKCWE})	
ExteinInsert_R	ATTATTCCCAGCATTTACCACG		
XylaLin_F_BsmBI	CTCGTCTCATAATAATACTAGTAGCGGCCGCTG	pNAT-xynA	Vector fragment for pLIN-xynA^{RGKCWE}
XylaLin_R_IBmsB	CTCGTCTCTCCACACTGTTACGTTAGAACTTCCAC		
xynA_F_X-RBS34-C	CGCTTCTAGAGAAAGAGGAGAAATACTAGATGTGCGCTAGCACAGACTACTGGC	pNAT-xynA	Xylanase insert for pLIN-^{C}xynA
xynA_F_X-RBS34-S	CGCTTCTAGAGAAAGAGGAGAAATACTAGATGAGCGCTAGCACAGACTACTGGC	pNAT-xynA	Xylanase insert for pLIN-^{S}xynA
xynA_F_X-RBS34-del	CGCTTCTAGAGAAAGAGGAGAAATACTAGATGAGCACAGACTACTGGCAAAATTG	pNAT-xynA	Xylanase insert for pLIN-$^{\Delta}$xynA
xynA_R_S-S	GTTTCTACTAGTATTATTAGCTCCACACTGTTACGTTAGAACTTC	pNAT-xynA	Xylanase insert for pLIN-xynA^{S}
xynA_R_del-S	GTTTCTACTAGTATTATTACACTGTTACGTTAGAACTTCCAC	pNAT-xynA	Xylanase insert for pLIN-xynA$^{\Delta}$

Protocol S1 Subcloning into $pCIRC_{Intein}$.

I. PCR-amplify the coding sequence of your protein of interest

1. Design primers for the coding sequence of your protein of interest using standard primer design guidelines, so that the amplicon starts at the codon of the first amino acid and ends at the codon of the last amino acid to be included in the circular protein. (This assumes that the coding sequence is read in the forward direction; otherwise, the roles of forward and reverse primer are interchanged.) The reading frame of the amplicon must not contain a stop codon.
2. Depending on the application, add extein residues to the 5' end of your primers. For the splicing reaction to succeed, the first amino acid must be a Cys, Ser, or Thr (but, depending on the intein, not all of them may work). When adding to the reverse primer, make sure to add the reverse complement of the sequence coding for the extein residues.
3. Further add the following sequence to the 5' end of your forward primer:

   `CTGGTCTCACAAC`

   and the following sequence to the 5' end of your reverse primer:

   `CTGGTCTCTAGCA`
4. Order the primers and perform the PCR reaction with a polymerase of your choice. Refer to the manufacturer's instructions to determine the conditions of the amplification.
5. Purify the amplified DNA, e. g. by gel extraction, and determine the concentration, e. g. by spectrophotometric measurement.

II. Insert the coding sequence into $pCIRC_{Intein}$ (where 'intein' stands for the selected split intein to be used) via golden gate assembly[3]

1. In a PCR tube, mix:
   - 100 ng $pCIRC_{Intein}$ DNA
   - An equimolar amount of the insert DNA. It can be calculated from the length of the insert amplicon and the length of the vector ($pCIRC_{Npu}$: 3817 bp, $pCIRC_{gp41\text{-}1}$: 3778 bp) as follows:

     $$100\,\text{ng} \cdot \frac{\text{length of insert}}{\text{length of vector}}$$
   - 1.5 µL 10X CutSmart Buffer (New England Biolabs, Ipswich, MA, USA)
   - ATP to a final concentration of 1 mM
   - 1 µL BsaI (New England Biolabs, Ipswich, MA, USA)
   - 1 µL T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA)
   - H_2O ad 15 µL

2. Place the tube into a thermocycler and run the following program[4]:

37 °C	3 min	25 cycles
16 °C	4 min	
50 °C	5 min	
80 °C	5 min	

3. Transform suitable competent *E. coli* with 5 µL of the reaction mixture

The procedure will work in a variety of buffers, including e. g. T4 DNA Ligase buffers (New England Biolabs, Ipswich, MA, USA or Thermo Fisher Scientific, Waltham, MA, USA), as long as sufficient amounts of both ATP (required by T4 DNA Ligase) and BSA (required by BsaI for optimal activity at 37 °C) are present.

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