

**Structural characterization and directed evolution of a novel acetyl xylan esterase reveals thermostability determinants of the Carbohydrate Esterase 7 family**

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## SUPPLEMENTAL FIGURES AND TABLES

**S1:** Most closely related proteins to the putative novel AcXE-encoding genes (Axe1<sub>NaM1</sub>, Axe1<sub>NaM2</sub> and XynB<sub>NaM3</sub>-like) and their potential source organisms retrieved from the non-redundant database using BLASTX 2.4.0+ (1).

	<b>Protein</b>	<b>Organism</b>	<b>Phylum/Family</b>	<b>Query cover (%)</b>	<b>E-value</b>	<b>Identity (%)</b>	<b>%GC content</b>
Axe1 <sub>NaM1</sub>	Hypothetical	<i>Actinopolymorpha alba</i>	<i>Actinobacteria / Norcardiadiacea</i>	98	5e-135	64	
	AcXE	<i>Saccharothrix espanaensis</i>	<i>Actinobacteria / Pseudonocardia</i>	98	8e-131	61	71.4
	ACE	<i>Paenibacillus ehimensis</i>	<i>Firmicutes / Paenibacillaceae</i>	96	4e-130	56	
Axe1 <sub>NaM2</sub>	AcXE	<i>Arthrobacter</i> sp. 35W	<i>Actinobacteria /</i>	96	2e-151	69	
	AcXE	<i>Microbacterium mangrove</i>	<i>Micrococcaceae</i>	95	9e-129	65	72.5
	AcXE	<i>Georgenia</i> sp. SUBG003	<i>Actinobacteria / Bogoriellaceae</i>	97	3e-125	62	
XynB <sub>NaM3</sub> -like	Lipolytic	<i>Pseudonocardia dioxanivorans</i> CB1190	<i>Actinobacteria /</i>	86	6e-67	58	
	Hypothetical	<i>Pseudonocardia dioxanivorans</i>	<i>Pseudonocardia</i>	86	6e-67	58	74.8
	Hypothetical	<i>Pseudonocardia asaccharolytica</i>		81	8e-64	59	

a. Feature 1

gi 516274	439	IKILPAGDSITNG.	[41].	DTDHAGFSGYQI.	[30].	PDIILLMIGTND.	[8].	ACTADLRSMLDYML.	[3].	PSGGMI	577
query	41	LRVMPPLGASSTVG.	[38].	DRDHEGRSGITL.	[15].	PDVLLHNGTND.	[8].	ETAERLTAVLDAIY.	[2].	SDAHVV	160
gi 87303449	2	LTIMPMGDSITHG.	[36].	DRDHWRPGRWGI.	[48].	INVLLMIGSND.	[32].	ASIDRLKAFLEVN.	[7].	LQLELI	181
gi 5834678	44	IKIMPLGDSITYG.	[45].	DDNHAGYSGYTI.	[28].	PDIILLQIGTND.	[7].	GSEERLHKLLDYLR.	[3].	PSNGKV	183
gi 42551017	30	LRIMPLGDSITKG.	[38].	DNDHEGHSKVL.	[14].	PNVVLVHAGTNN.	[8].	GSPQILESIIIDGLF.	[3].	PDTTVL	149
gi 40747781	291	LRLPLGDSITKG.	[47].	DRDHQGLSGKRI.	[14].	PNVILVHVGTND.	[9].	TAPDRLRALLDGLR.	[3].	PDAAIL	420
gi 111057360	38	LRILPLGDSITWG.	[37].	DKDNQGFPGYTI.	[14].	PNVLLHAGTND.	[12].	QAPQRLGQLIDDLV.	[3].	PDAAVI	160
gi 89241797	30	VRVMPPLGDSITDG.	[36].	DHDHEGHPGWRI.	[15].	PRTILLHIGTND.	[8].	NAPARLSALIDKIR.	[3].	PQVQLF	148
gi 35213333	107	AKVMPPLGDSITEG.	[36].	DKNHEGHPGYFI.	[15].	PETVLLIGTND.	[8].	GAPGRLSALIDQIF.	[3].	SSVKLY	225
gi 4959943	59	LRILPLGASITWG.	[24].	DMVGKAHSGDVI.	[14].	PNVLLINAGTND.	[8].	NAGERMRSLIETLI.	[5].	ANTLIV	166

Feature 1

gi 516274	578	FLCS.	[1].	P.	[13].	NYNNTVKSVAAE.	[7].	VRFADVH.	[12].	DHLHPNGTGYKKMGNF.	[4].	655
query	161	VAGV		W.	[11].	EFTRLRSVVVDE.	[7].	ATFVDT.	[9].	DGLHPNAEGYRRIAAMW.	[4].	232
gi 87303449	182	VATI.	[1].	T.	[23].	EYNDFIKIDLPD.	[7].	LRVVDQF.	[8].	DGLHPSSQAFERMAATW.	[4].	265
gi 5834678	184	FLTT.	[1].	P.	[16].	KYNELIKKVAND.	[5].	VIYADIH.	[10].	DGVHPNAGGYEKMGKYW.	[4].	260
gi 42551017	150	VAPV.	[1].	W.	[11].	RFNPQVIAMIEK.	[5].	KHILEVP.	[9].	DEKHPNDSGYEKMANAW.	[4].	220
gi 40747781	421	VSEV.	[1].	L.	[11].	VFNQALNGIVTE.	[7].	IADVPMG.	[11].	DYKHPGNQGYKKMAGAW.	[4].	495
gi 111057360	161	VAKI.	[1].	Q.	[11].	TFNNAIPAIIVAA.	[7].	VSVVDM.	[9].	DNLHPSVAGYARMGDIW.	[4].	233
gi 89241797	149	VAQI.	[1].	P.	[11].	TFNAALPGIVAQ.	[4].	THLVDQH.	[9].	DGVHPNAGYDRMAARW.	[4].	218
gi 35213333	226	VASI.	[1].	P.	[11].	DYNAAIPGIVNG.	[7].	VVYVDIY.	[9].	DTVHPDAEGYAKIADRW.	[4].	298
gi 4959943	167	LSTL.	[1].	P.	[11].	SVNAQFRELVL.	[8].	IVLADM.	[18].	DNKHPNDYGYSQMAIWI.	[4].	249

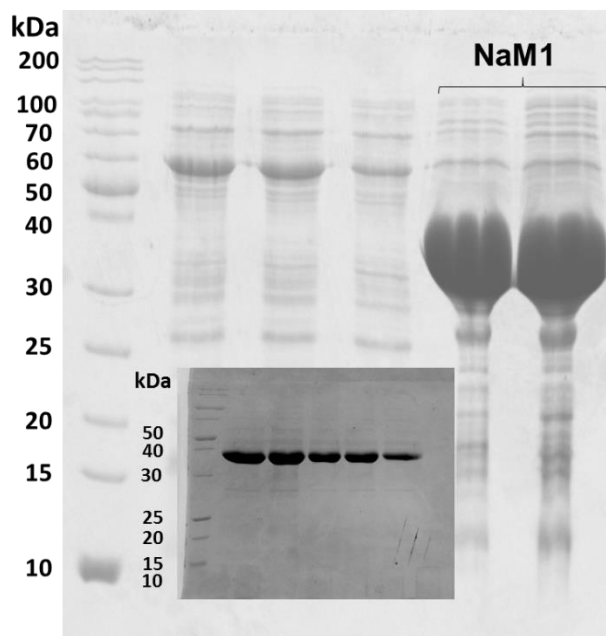
b.

		170	180	190	200	210	220	230	240	
		....*.... ....*.... ....*.... ....*.... ....*.... ....*.... ....*.... ....*....								
1VLQ_H	171	RVFTDAVRAVEAAASFPQVDQERIVIAAGS	QGGGIALAVSALS	KKAKALLCDVPFLCHFRRAVQLVD	TH	PYAEITNFL	248			
query	156	HLFLDARLAQIVLAMDDVDPDRVAATGY	QGGGLTLACAAL	EPRIRLAAPVYPFLCDFRRAWEMDL	EK	GPYNEITTYF	234			
gi 81539232	158	RVFTDAVRAVEAARSHPLTPDSRTVALGAS	QGGGIIAVGGLVRD	LAAPVYPFLCDFPRATTLTD	RH	PYREVGLYL	235			
gi 81855256	157	RLFTDAVRAIDALLGLDFVPERIAVCGDS	QGGGISLAVGGIDPR	KAVMPDPVFLCDFPRAVQTA	RD	PYLEIVRFL	234			
1L7A_B	152	GVYLDVRALEVISSFDEVDERTIGVTGG	QGGGLTIAAALS	SDIPKAAVADYPYLSNFERAIDVAL	EQ	PYLEINSFF	229			
gi 81785830	154	HVFLDTAQLANIVMNLPEVDEERVAVTGW	QGGALAIACAAL	EPKIKKVAPVYPFLSDYQRVWEMDL	aEK	AYDELQTYF	232			
gi 81849206	154	NIFLDTAILARIVMMEWVDEERVATGG	QGGALALACAAL	ENRIKGVYAYYPFLCDYKRIWVMDL	gGD	SYDELIRYF	232			
gi 81845057	152	DVYLDIYQLVEIVASLSQVDEKRLSSYGAS	QGGALALVAAL	NPRIQKTVAIYPFLSDFRRVIEIGNTSE	AYDELFRYF	230				
gi 504571229	153	HIFLDTAQLAGIVMMPVDEDRVGMGSP	QGGGLSLACAAL	EPVRKVVSEYPFLSDYKRVWDL	LaKN	AYQEITDYF	231			
gi 500857360	152	GVYLDVRAALDVIQSFPEVDEHRIGVVG	QGGALAVAAALS	SDIPKVVVADYPYLSNFERAVDVAL	EQ	PYLEINSYF	229			
		250	260	270	280	290	300	310	320	
		....*.... ....*.... ....*.... ....*.... ....*.... ....*.... ....*.... ....*....								
1VLQ_H	249	K---THRDKKEIVFRTL	SYFDGVNFAARAKIPAL	FSVGLX	DNICPPSTVFAAYNYAGP	KEIRIYPYNN	HEGGGSFQAV	324		
query	235	RardPRHLREEEIFSRLGYVDVQHLAPRVRAEVLMTVSLAD	KICPPSTQFAAYNKLGGP	KDYRLPDPFAHETLPG	TDD	312				
gi 81539232	236	K---THRGRSGDALRTL	SYFDGVHFAARGRAPAL	FSAAL	EQTCPSTVFAAFNAWTHEdK	TIEVYDFND	HEGGGPYQEA	312		
gi 81855256	235	A---QHREKKAAVFETL	NYFDCVNFARRSKAPAL	FSAVMD	EVCCPSTVYGAFNAYAGE	KTITEYEFNN	HEGGQYQER	310		
1L7A_B	230	Rr-nGSPETEVQAMKTL	SYFDIMNLADR	VKVPVLSIGLID	KVTPSTVFAAYNHLETK	KELKVRYFG	HEYIPAFQTE	307		
gi 81785830	233	RrfdPQHRREAEIFTK	LYIDIQHLAPLVKGEVLLAVGLMD	TVCCPSTQFAMYNKLT	TT	KSIELYPDFAH	EDLPGH	RD	310	
gi 81849206	233	KfidPNHENEYVFN	TLGYIDIKNMAHRIK	KGKVNMAIGLRD	ICPPSTQFAAYN	NILCE	KELVLYDGGQ	KPYLLNL	KD	310
gi 81845057	231	KfydPFHETEEEMAT	LAYIDVKNLAHRIQ	GEVVMITGLDD	VCYPITQFAIYN	NRLTCD	KTYRIMPEYA	HEAMNVFVND	309	
gi 504571229	232	RlfdPRHERENEVFT	KLGYIDVKNLAKRIK	GDVLMCVGLMD	QVCCPSTVFAAYN	NIQSK	KDIKVYPDYGH	HEPMRGF	GD	309
gi 500857360	230	Rr-nSDPAVEEKAFET	LSYFDLINLAGVWNP	TMAIGLID	QITPPSTVFAAVNHLETD	KDLKVRYFG	HEYISAFQ	T	306	

S2: Multiple sequence alignment of NCBI curated domains a. cd01833: XynB-like, with user query (XynB<sub>NaM3</sub>-like) included. SHD and SGNHD catalytic residues are highlighted in yellow respectively.

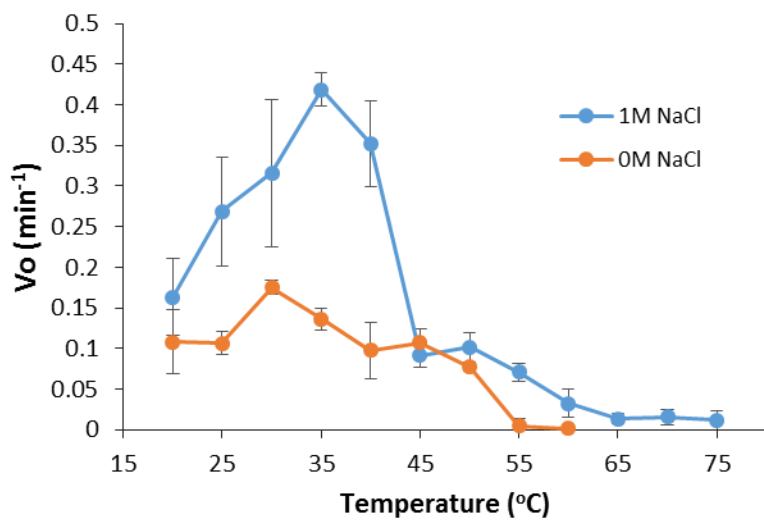
b. pfam05448: Axe1 with user query (Axe1<sub>NaM1</sub>) included. Conserved residues are displayed in red letters (1). Axe1 domain was curated with esterases from *Thermotoga maritima* (1VLQ\_H), *Streptomyces coelicolor* (gi 81539232), *Mesorhizobium loti* (gi 81855256), *Bacillus subtilis* (1L7A\_B),

*Bacillus halodurans* (gi 81785830), *Clostridium perfringens* (gi 81849206), *Streptococcus pneumoniae* R6 (gi 81845057), *Thermoanaerobacterium aotearoense* (gi 504571229) and *Bacillus pumilus* (gi 500857360). XynB\_like domain was curated with esterases from *Ruminococcus flavefaciens* (gi 516274), *Synechococcus* sp. WH 5701 (gi 87303449), *Ruminococcus flavefaciens* 17 (gi 5834678), *Gibberella zeae* PH-1 (gi 42551017), *Aspergillus nidulans* FGSC A4 (gi 40747781), *Phaeosphaeria nodorum* SN15 (gi 111057360), *Actinoplanes* sp. SE50/110 (gi 89241797), *Gloeobacter violaceus* PCC 7421 (gi 35213333) and *Aspergillus terreus* (gi 4959943).

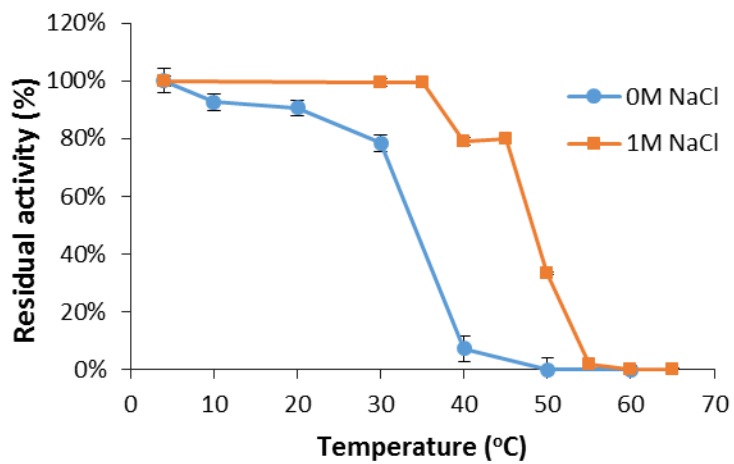


**Figure S3:** SDS-PAGE of ~36 kDa NaM1 production (lanes 5 and 6, pET28a-NaM1 construct) and controls (lanes 2-4, empty vector), as well as, IMAC cobalt-affinity purification (inset).

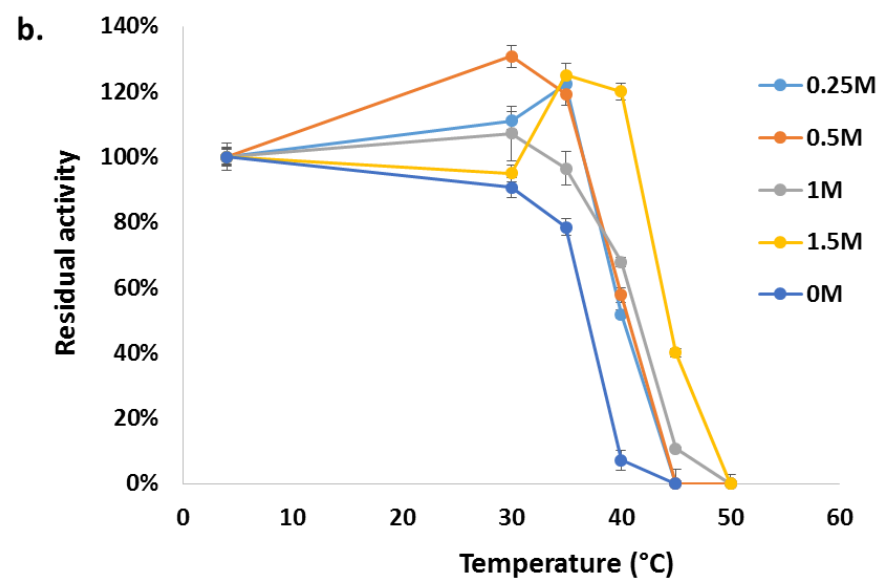
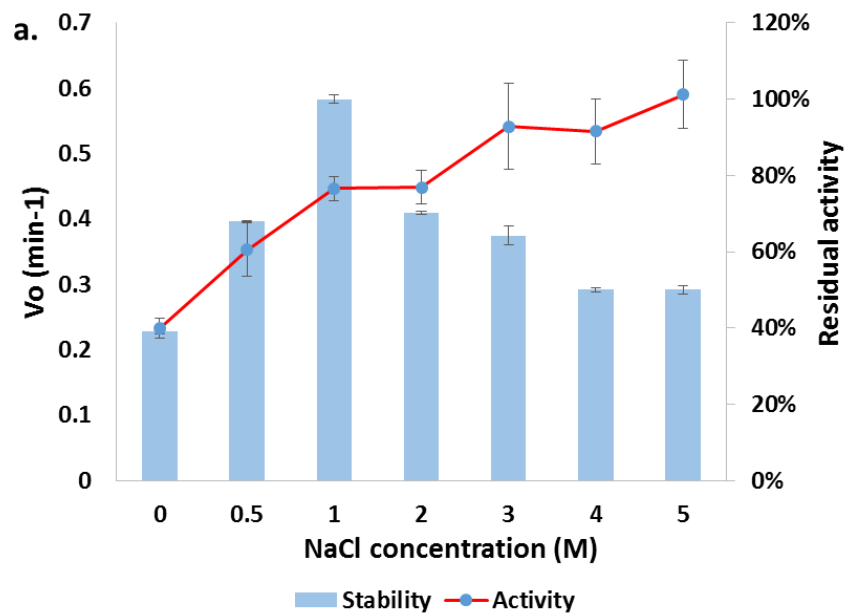
**a.**



**b.**



**Figure S4: a.** Effect of temperature (10-75 $^{\circ}\text{C}$ ) on NaM1 activity with and without the addition of NaCl in assay buffer. **b.** Residual NaM1 acetyl esterase activity after incubation at various temperatures (4-60 $^{\circ}\text{C}$ ) for 1 h.

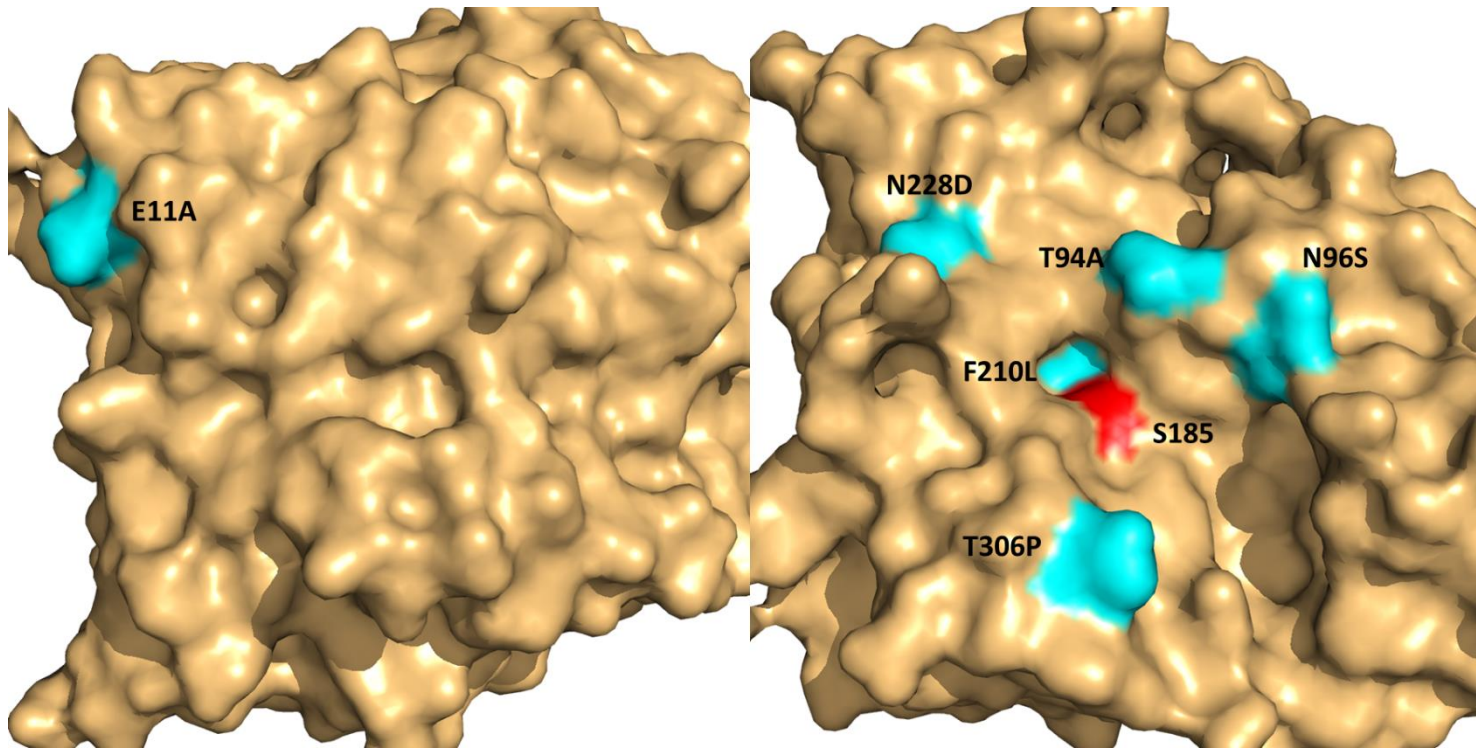


**S5:** Effects of **a.** increasing NaCl concentration on NaM1 activity (primary y-axis) and stability (secondary y-axis) at 40°C. Residual activity was determined following incubation of NaM1 for 1 h in NaCl of specified concentration. **b.** various trehalose concentrations on NaM1 thermal stability after incubation for 1 h at various temperatures (4-50°C).

**Table S6:** Analysis of mutations from the NaM1 epPCR library.

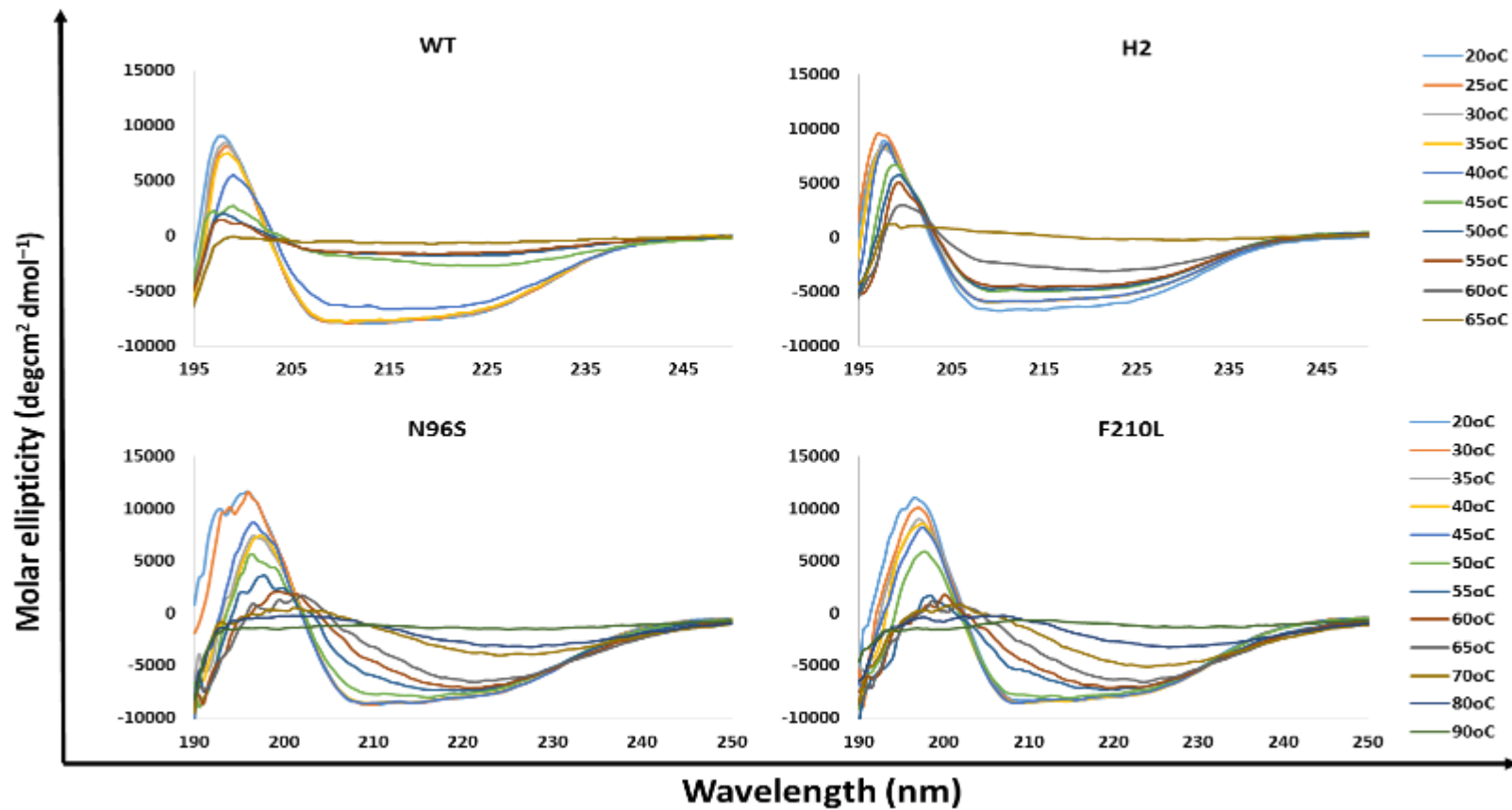
<b>Mutant</b>	<b>DNA mutation</b>	<b>Substitution type</b>	<b>Amino acid substitution</b>	<b>Mutation type</b>	<b>Distance from S185 (Å) by C<math>\alpha</math></b>
<b>H2</b>	A→G	Transition	N96S	Missense	12.51
	T→C	Transition	F210L	Missense	4.84
<b>E1</b>	A→C	Transversion	T306P	Missense	11.43
<b>F9</b>	A→C	Transversion	E11A	Missense	22.08
<b>D8</b>	A→G	Transition	T94A	Missense	10.92
	C→T	Transition	-	Silent	
<b>B4</b>	A→G	Transition	N228D	Missense	15.08
	T→A	Transversion	-	Silent	
<b>H1</b>	A→T	Transversion	T232S	Missense	20.41
	A→T	Transversion	-	Silent	
<b>B10</b>	A→T	Transversion	T232S	Missense	20.41
<b>H10</b>	A→G	Transition	-	Silent	
	A→C	Transversion	D147A	Missense	30.12
<b>E5N</b>	T→C	Transition	-	Silent	
	T→C	Transition	-	Silent	
<b>4-3</b>	A→C	Transversion	M220L	Missense	18.23
	A→G	Transition	Q319R	Missense	22.16
<b>4-4</b>	T→A	Transversion	L190Q	Missense	8.63
<b>4-11</b>	T→A	Transversion	V39E	Missense	28.76
	A→G	Transition	Y106C	Missense	20.82
	T→A	Transversion	I167N	Missense	23.77
<b>4e</b>	T→C	Transition	F214L	Missense	14.17





**S7: NaM1 mutation map:** All mutations selected are located in the core except one located on the outer surface, close to the N-terminus of NaM1.

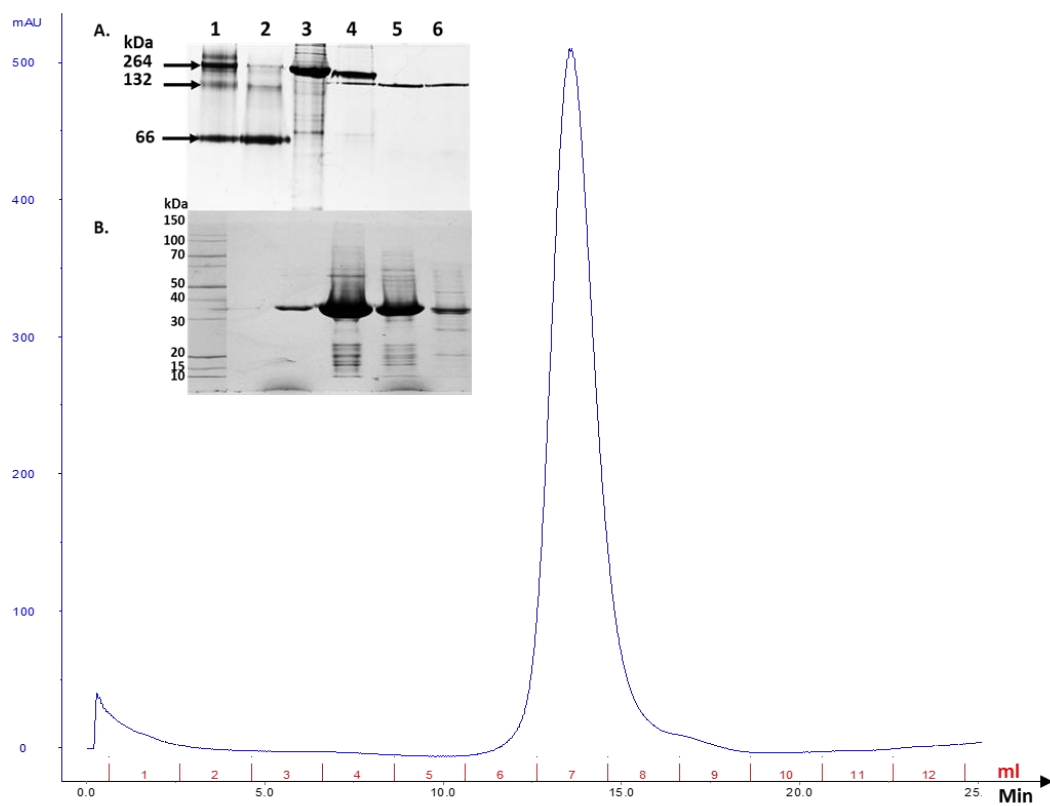




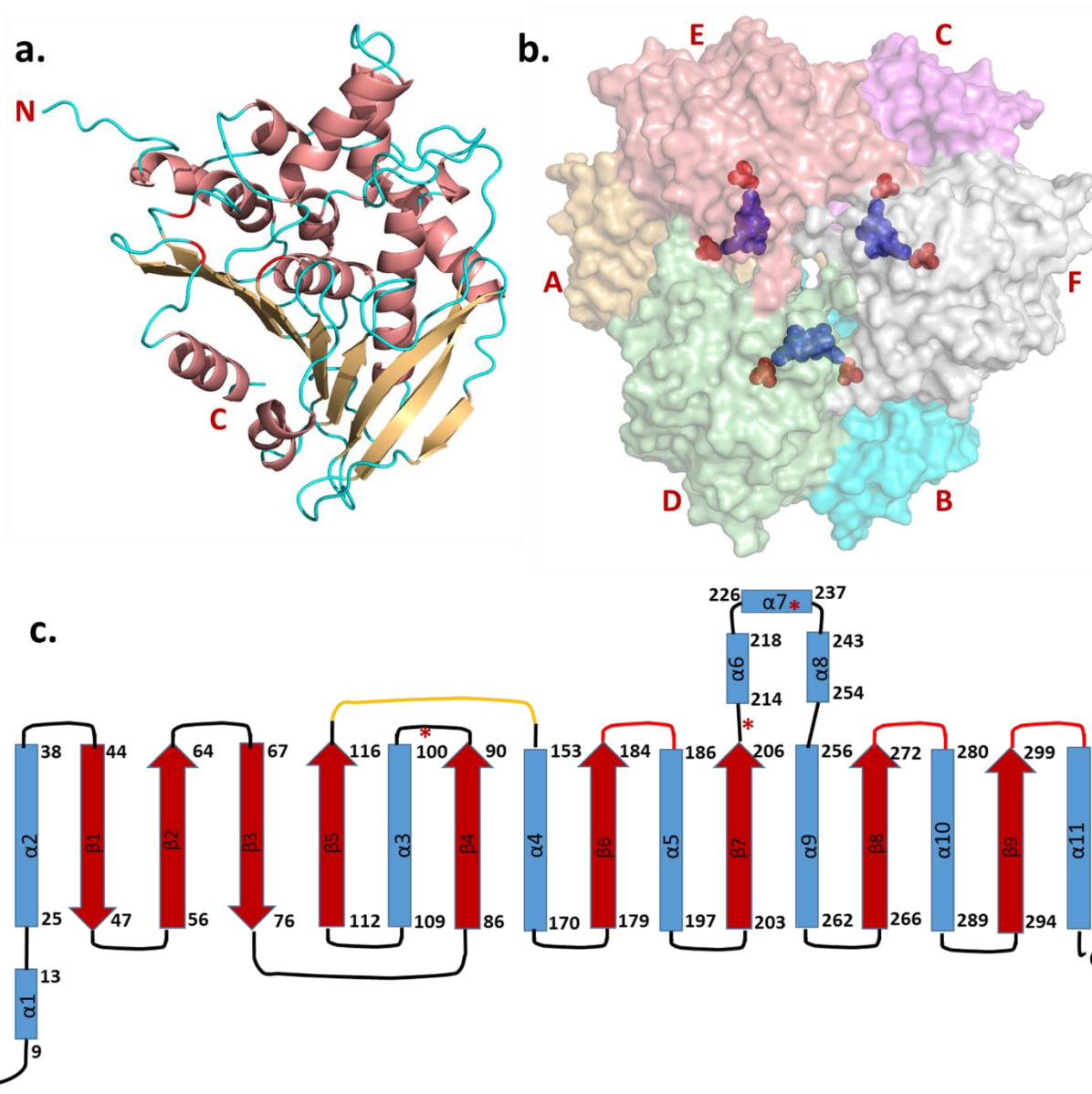
**S8:** Far-uv CD spectrum of wildtype (WT) NaM1 showing denaturation at temperatures >40°C; mutant H2 showing denaturation only at temperatures >60°C and indicating >10°C increase in thermal stability of the wildtype; and mutants NaM1<sub>N96S</sub> and NaM1<sub>F210L</sub> showing gradual denaturation from 55°C, indicating co-contribution to thermal stability of H2.

**S9: Chemical composition of NaM1**

<b>Chain</b>	<b>Component</b>	<b>Quantity</b>	<b>Resolved Histidine(s) of His<sub>6</sub>-tag</b>
<b>A</b>	Amino acids	324	3
<b>B</b>	Amino acids	322	1
<b>C</b>	Amino acids	319	-
<b>D</b>	Amino acids	322	1
<b>E</b>	Amino acids	322	1
<b>F</b>	Amino acids	323	1
<b>G</b>	Na <sup>+</sup>	6	-
<b>H</b>	SO <sub>4</sub> <sup>2-</sup>	1	-
<b>K</b>	MES (head group)	13	-
<b>L</b>	CHOO <sup>-</sup>	1	-
<b>S</b>	H <sub>2</sub> O	2264	-



**S10:** Size exclusion chromatogram of NaM1<sub>WT</sub>. Inset A. Native PAGE showing BSA marker (lanes 1&2), NaM1 hexamer eluted at 6 ml (Lane 6) and 7-9 ml (lanes 3-5) respectively. B. SDS-PAGE showing protein ruler (10-150 kDa) and fractions eluted at 5-9 ml (lanes 2-6).



**S11:** **a.** Tertiary structure of NaM1:  $\alpha$ -helices,  $\beta$ -strand and loops are shown in salmon tan and cyan, respectively. Catalytic residues are in red. **b.** NaM1 quaternary structure: Surface representation of the flattened spherical hexamer (top-view) showing monomers A to F. Active site residues shown as spheres located around the three-fold rotational axes. Catalytic Asp275 and His304 are in blue, while Ser185 is in red. **c.** NaM1 Topology:  $\alpha$ -Helices ( $\alpha$ 1-11) are represented by blue cylinders,  $\beta$ -strands ( $\beta$ 1-9) by red arrows with relative orientation, and connecting loops by black lines. Loops carrying active site residues (Ser185, His304, Asp275) and the  $\beta$ -interface loop are coloured red and yellow, respectively. The red

asterisks mark secondary elements that harboured thermostabilizing substitutions. The typical  $\alpha/\beta$  hydrolase fold begins with a  $\beta$ -hairpin (Arg56 – Arg76) and ends at the C-terminus, excluding the triple  $\alpha$ -helix extension (Phe214 - Val254).

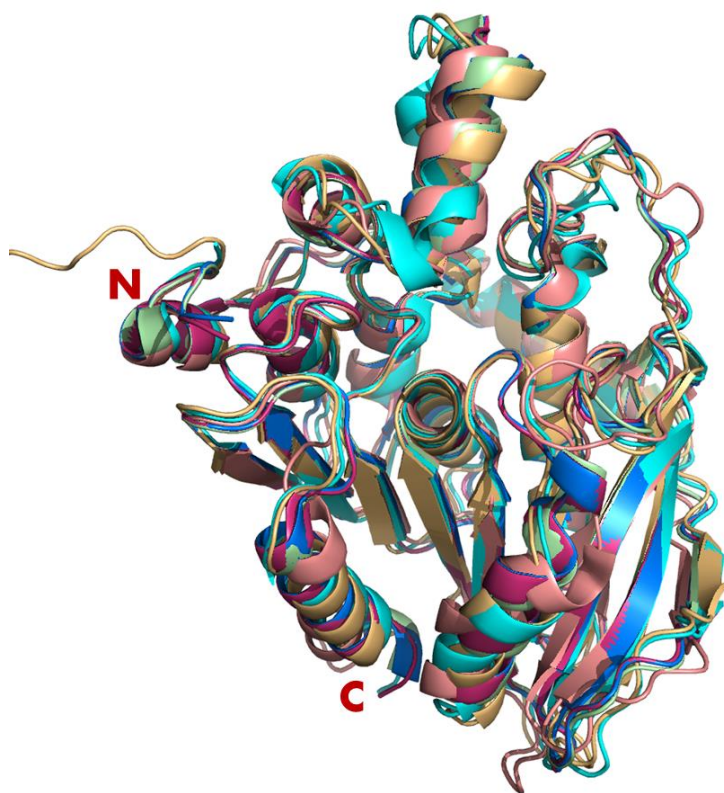
**S12:** Monomer-monomer H-bonds, typical of each subunit, involving subunit A of NaM1 (PDBsum analysis).

Subunits	Corresponding Residues	Atoms	Interaction type	Bond length (Å)*	
<b>A – B</b>	Arg 18 – His 0	NH2 – ND1	H-bond	2.93	
	Trp 218 – Asp 301	NE1 – OD1	H-bond	2.83	
	Arg 235 – Glu 305	NH2 – OE1	Salt bridges	2.80	
		NH1 – OE2		3.00	
	Arg 235 – Asp 301	NH1 - O	Salt bridge	2.77	
<b>A – D</b>	Arg 240 – Leu 298	NE – O	H-bond	2.96	
	Gly 120 – Met 136	O – N	H-bond	2.79	
		O – NE	H-bond	3.09	
		O – NH2	H-bond	2.84	
	Val 126 – Trp 134	O – N	H-bond	2.87	
	Glu 128 – Arg 142	OE1 –NH2	Salt bridge	2.98	
	Glu 128 – Glu 128	OE2 - OE2	H-bond	3.07	
	Trp 134 – Val 126	N – O	H-bond	2.89	
	Met 136 – Gly 120	N - O	H-bond	2.90	
	Arg 142 – Glu 128	NH2 – OE1	Salt bridge	2.94	
	Arg 237 – Gln 121	NH2 – O	H-bond	2.87	
	<b>A – C</b>	Leu 298 – Arg 240	O – NE	H-bond	3.02
		Asp 301 – Trp 218	OD1 – NE1	H-bond	2.98
		Asp 301 – Arg 235	O –NH1	Salt bridge	2.97
		Glu 305 – Arg 235	OE2 – NH1	Salt bridges	3.09
OE1 – NH2				2.94	
<b>A – E</b>	Asp 99 – Asp 312	OD2 – N	H-bond	2.95	
	Trp 100 – Asp 312	N – OD2	H-bond	2.88	
	Ser 101 – Asp 312	OG – OD1	H-bond	2.74	
	Ser 101 – Asp 312	N – OD1	H-bond	2.77	
	Asp 312 – Asp 99	N – OD2	H-bond	2.82	
	Asp 312 – Ser 101	OD1 –N	H-bond	2.82	
		OD1 – OG	H-bond	2.69	
	Asp 312 – Trp 100	OD2 – N	H-bond	2.88	

\*Only interactions with inter-atomic distances between 2.6-3.1Å are listed.

**S13: Structural homologs of NaM1**

Organism	Protein	PDB ID	Number of residues	Identity to NaM1 (%)	Z-score	RMSD (Å)
<i>T. saccharolyticum</i> JW/SL-YS485	AcXE	3FCY	317	50	49.6	1.2
<i>B. subtilis</i> 168	CAH	1ODS	316	33	42.8	1.7
<i>B. pumilus</i> PS213	AcXE	3FVT	317	33	42.3	1.8
<i>B. pumilus</i> CECT5072	AcXE	2XLB	317	32	42.3	1.8
<i>T. maritima</i> TM007	AcE/CAH	3M81	322	35	40.6	2.1



**S14:** Superposition of NaM1 (light brown) with CE7 homologs from *Bacillus subtilis* 168 (PDB: 1ODS, pink), *B. pumilus* CECT5072 (2XLB, blue), *Thermoanaerobacterium* sp. (3FCY, cyan), *B. pumilus* PS213 (3FVT, pink), and *Thermotoga maritima* (3M81, salmon) showing structural conservation. N and C-termini are indicated.



**S15:** Specific activities ( $\text{Umg}^{-1}$ ) of functionally characterized 7-ACA deacetylases.

Specific Activity ( $\text{Umg}^{-1}$ )	AX	7-ACA	CPC	<i>p</i> -NPA	Butyrate	$\alpha$ -NA	4-MUA	pH	T <sub>opt</sub> (°C)	References
<i>B. pumilus</i> PS213 (rAXE)	13	179	26	88	NS	144	67	8.5	45	(2, 3)
<i>B. subtilis</i> CICC 20034	-	888	484	2949	2.72	741	1086	7.0	50	(4)
<i>B. subtilis</i> SHS 0133	NS	12.4*	7.0*	201*	NS	NS	NS	7.0	55	(5)
<i>B. subtilis</i> 168 <sup>‡</sup>	-	NS	1.18*	266.7*	NS	NS	NS	6.5	50	(6)
<i>B. pumilus</i> CECT5072 <sup>‡</sup>	NS	23.35	81	16.5	-	NS	NS	7.0	45	(7, 8)
<i>A. tengchongensis</i> (EstD1)	-	2.71	NS	NS	7.4*	79.6*	NS	8.5	65	(9)
<i>T. maritima</i> TM0077 <sup>‡</sup>	-	1140 <sup>a</sup>	376 <sup>a</sup>	310.8*	NS	NS	NS	7.5	90	(10)
<i>Thermoanaerobacterium</i> sp. JW/SL YS485 (rAXE) <sup>‡</sup>	-	41	91	NS	NS	NS	47	7.0	75	(11)
<i>T. maritima</i> (EstA)	-	80	80	1095*	239*	NS	NS	8.5	95	(12)
<i>B. subtilis</i> (YesT)	0.02	1235	356	1580	NS	217	NS	8.5	35	(13)
<b>NaM1</b>	6	200	NS	488.9	12.96	222	277.8	8.5	30	This study

+ active, - inactive, *p*-NPB: para-nitrophenol butyrate, AX: polymeric acetylated xylan, 7-ACA: 7-aminocephalosporanic acid, CPC: cephalosporin C deacetylase,  $\alpha$ -NA:  $\alpha$ -naphthyl acetate, 4-MUA: 4-methylumbelliferryl acetate, \*catalytic efficiency  $k_{\text{cat}}/K_{\text{M}}$  ( $\text{s}^{-1}\text{mM}^{-1}$ ), <sup>a</sup>turnover rate ( $\text{s}^{-1}$ ), NS: not stated, <sup>‡</sup>structurally characterized. References 1 – 12 respectively: (2-13).

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