

**Characterization of a Novel β -L-Arabinofuranosidase in *Bifidobacterium longum*:
FUNCTIONAL ELUCIDATION OF A DUF1680 FAMILY MEMBER**

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Running head: Characterization of a novel β -L-arabinofuranosidase.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary FIG. S1. Chemical structures of the β -L-arabinooligosaccharides used in this study. The *arrows* indicate the cleavage sites for HypBA1.

Supplementary FIG. S2. SDS-PAGE analysis of recombinant HypBA1. Purified HypBA1 was electrophoresed on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 2, purified HypBA1; lane 1 and 3, molecular size markers. The *arrow* indicates the band that corresponds to HypBA1.

Supplementary FIG. S3. TLC analysis of HypBA1 reactions in the presence of reducing agents. *Cis*-Ara₃-Hyp-DNS was incubated with the recombinant enzyme in the absence (lane 2) or presence of β -mercaptoethanol (lane 3), dithiothreitol (lane 4), or TCEP (lane 5) at 37°C for 16 h. Lane 1, *cis*-Ara₃-Hyp-DNS.

Supplementary FIG. S4. Effects of pH and temperature on the activity of HypBA1. A, pH dependence of HypBA1 activity in various buffers at 37°C for 10 min. Enzyme activities were expressed as the percentage of activity in sodium acetate buffer at pH 4.5. B, Temperature dependence of HypBA1 activity for 10 min. Enzyme activities were expressed as the percentage of the activity at 35°C. Buffers: sodium acetate (closed square), MES (open circle), sodium phosphate (closed circle).

Supplementary FIG. S5. Comparison of the reactivities of HypBA1 and HypBA2 with glycoproteins. A, TLC analysis of the reaction products. Potato lectin (lane 2), extensin (lane 3), and Ara₃-Hyp (lane 4) were incubated with HypBA2-CΔ486 (lane b) or HypBA1 (lane c) at 37°C for 16 h. Lane a, control without enzyme; lane 1, L-arabinose standard. B, HPAEC-PAD analysis of the reaction products. Potato lectin was reacted without enzyme (2a), with HypBA2-CΔ486 (2b), or with HypBA1 (2c). Extensin was reacted without enzyme (3a), with HypBA2-CΔ486 (3b) or with HypBA1 (3c).

Supplementary FIG. S6. Chemical structure and ¹H- and ¹³C-NMR spectra of Ara-Me. The asterisks indicate peaks from impurity or sideband signals. The chemical shifts are listed in supplemental Table S3.

Supplementary FIG. S7. Phylogenetic relationships between HypBA1 homologs in *Bifidobacterium* strains. The black boxes indicate the DUF1680 conserved region (middle). The lengths of the sequences are shown on the right side of each schematic sequence. The organisms, locus tag, and GenBank accession numbers are as follows: BLL, *B. longum* subsp. *longum* JCM 1217, 1 (BLLJ_0211, BAJ65881), 2 (BLLJ_1826, BAJ67491), 3 (BLLJ_1848, BAJ67512), 4 (BLLJ_0089, BAJ65759); BL, *B. longum* NCC2705, 1 (BL0422, AAN24259), 2 (BL0174, AAN24029); BI, *B. longum* subsp. *infantis* 157F, 1 (BLIF_0192, BAJ70339), 2 (BLIF_1895, BAJ72029); BP, *B. pseudocatenulatum* DSM 20438, 1 (BIFPSEUDO_02879, EEG71985), 2 (BIFPSEUDO_02839, EEG71945); BC, *B. catenulatum* DSM 16992, 1 (BIFCAT_02005, EEB20621), 2 (BIFCAT_00247, EEB22303), 3 (BIFCAT_01782, EEB20699); BD, *B. dentium* ATCC 27678, 1 (BIFDEN_01462, EDT45627), 2 (BIFDEN_00978, EDT45157); BAd, *B. adolescentis* ATCC 15703 (BAD_1529, BAF40310); BAn, *B. animalis* subsp. *lactis* AD011 (BLA_1513, ACL29795); BB, *B. breve* DSM 20213 (BIFBRE_03130, EFE89858). The protein characterized in this study is enclosed in the box. The phylogenetic tree was constructed with the neighbor-joining method using MEGA5 software (left). Comparison of the gene clusters containing GH127, GH121 and GH43 members in *Bifidobacterium* strains (right).

Supplementary FIG. S8. Multiple sequence alignment of HypBA1 and its homologs. The homologous proteins are shown in supplemental Fig. S7. The alignment was created with MUSCLE and BoxShade 3.21. Identical residues and conservative substitutions are highlighted

in black and dark gray, respectively. Asterisks indicate the residues selected for site-directed mutagenesis. The protein characterized in this study is enclosed in the box.

Supplementary FIG. S9. Schematic β -L-arabinooligosaccharides metabolic pathway in *B. longum*. The domain organizations of β -L-arabinooligosaccharides degradation enzymes and the predicted sugar transporters are shown in the upper panel. The gene cluster containing β -L-arabinooligosaccharides degradation enzymes is shown in the lower panel. The annotated gene products are as follows: LacI-type transcriptional regulator (BLLJ_0207) in *black*; putative ABC-type sugar transport system (BLLJ_0208–BLLJ_0210) in *yellow*; HypBA1 (BLLJ_0211) in *green*; HypBA2 (BLLJ_0212) in *red*; and putative GH43 α -L-arabinofuranosidase (BLLJ_0213) in *blue*.

Table S1. The primers used for site-directed mutagenesis.

Name	Sequence of oligonucleotide primers
E322A_Forward_Primer	5'-ACCCACGTGGGCG <u>CG</u> TTCACCTACG-3'
E322A_Reverse_Primer	5'-CGTAGGTGAACGAC <u>GCG</u> CCCACGTGGGT -3'
E338A_Forward_Primer	5'-CACGATGTACGGT <u>GCG</u> ACCTGTGCTTCCG-3'
E338A_Reverse_Primer	5'-GAAGCACAGGT <u>CGC</u> ACCGTACATCGTGT <u>CG</u> -3'
E366A_Forward_Primer	5'-CCGACGTGCTGG <u>GCG</u> AAGGAACTGTTCAACG-3'
E366A_Reverse_Primer	5'-CGTTGAACAGTTCCTTC <u>GCG</u> CAGCACGT <u>CG</u> -3'

The positions of the mutated sequences are underlined.

Table S2. Substrate specificity of HypBA1.

Substrates	Activity
β -Ara ₂	+ ^a
Ara ₄ -Hyp	ND ^b
Ara ₃ -Hyp	+
Ara ₂ -Hyp	+
Ara-Hyp	+
Ara ₂ -Me	+
Ara-Me	+
Extensin	ND
Potato lectin	ND
<i>p</i> NP- α -L-arabinofuranoside	ND
<i>p</i> NP- α -L-arabinopyranoside	ND
<i>p</i> NP- β -L-arabinopyranoside	ND
<i>p</i> NP- α -D-xylopyranoside	ND
<i>p</i> NP- β -D-xylopyranoside	ND
<i>p</i> NP- α -D-galactopyranoside	ND
<i>p</i> NP- β -D-galactopyranoside	ND
<i>p</i> NP- α -D-glucopyranoside	ND
<i>p</i> NP- β -D-glucopyranoside	ND

^a Cleavage of substrate was detected.

^b Cleavage of substrate was not detected.

Table S3. Assignments of signals in ^1H and ^{13}C NMR spectra of methyl β -L-arabinofuranoside.

	1	2	3	4	5	Me	
^1H (δ)	4.74	3.99	3.85	3.73	3.61	3.46	3.26
J (Hz)	4.8	8.2, 4.8	7.5	7.1, 3.1	12.2, 3.4	12.2, 7.5	-
^{13}C (δ)	102.72	76.86	75.03	82.54	63.65	55.65	

Table S4. Growth capacity of *Bifidobacterium* strains on carbohydrates.

Carbon source	<i>B. longum</i> JCM 1217	<i>B. adolescentis</i> JCM 1275
Glucose	++	++
L-Arabinose	++	-
β -Ara ₂	++	-

Judgment of bacterial growth: -, $\Delta\text{pH} < 0.5$; \pm , $0.5 \leq \Delta\text{pH} < 1.0$; +, $1.0 \leq \Delta\text{pH} < 1.5$; ++, $1.5 \leq \Delta\text{pH}$. $\Delta\text{pH} = (\text{test pH}) - (\text{control pH})$

Figure S1

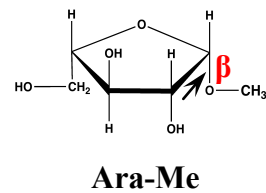
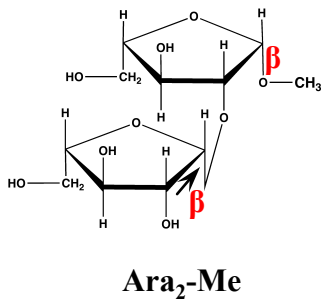
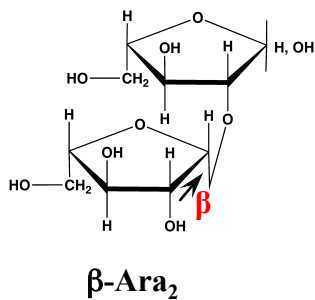
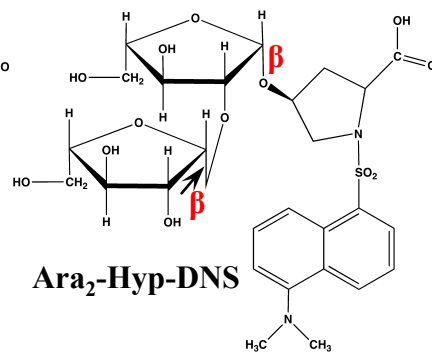
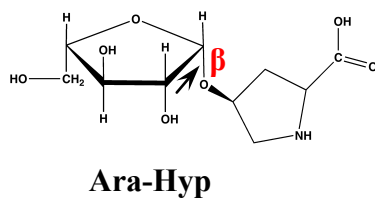
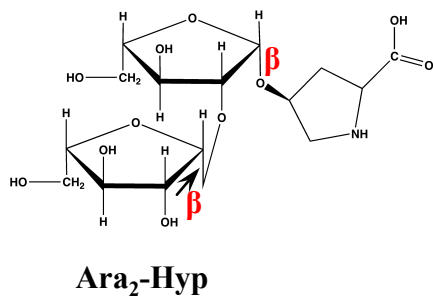
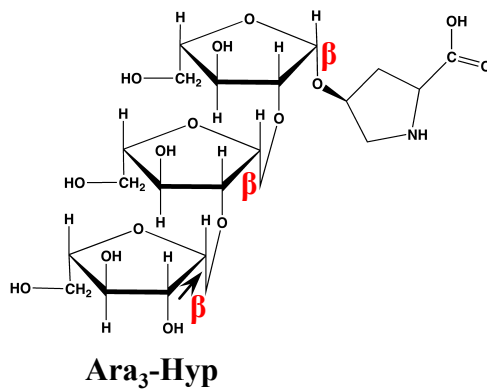
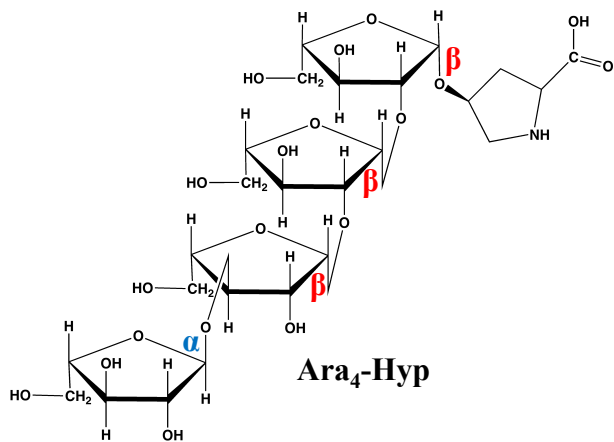


Figure S2

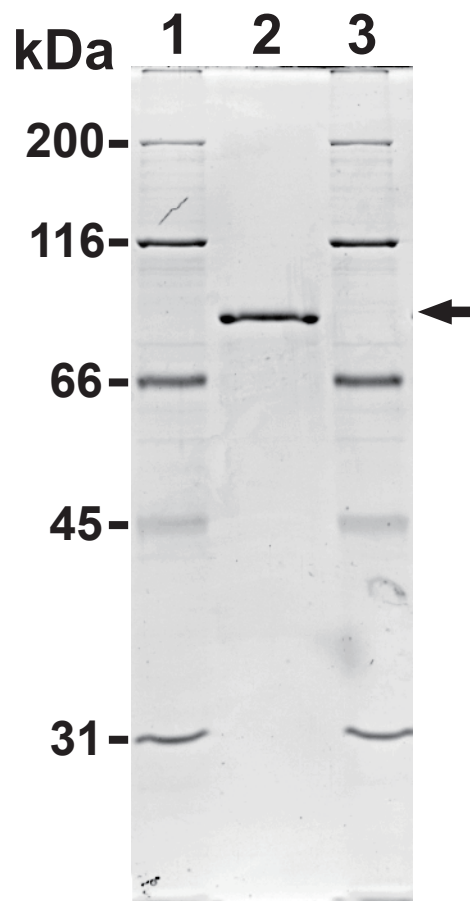


Figure S3

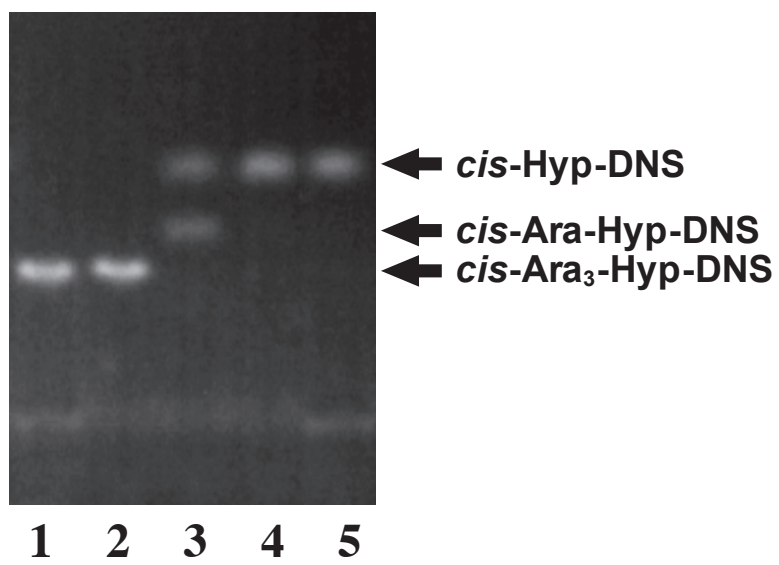


Figure S4

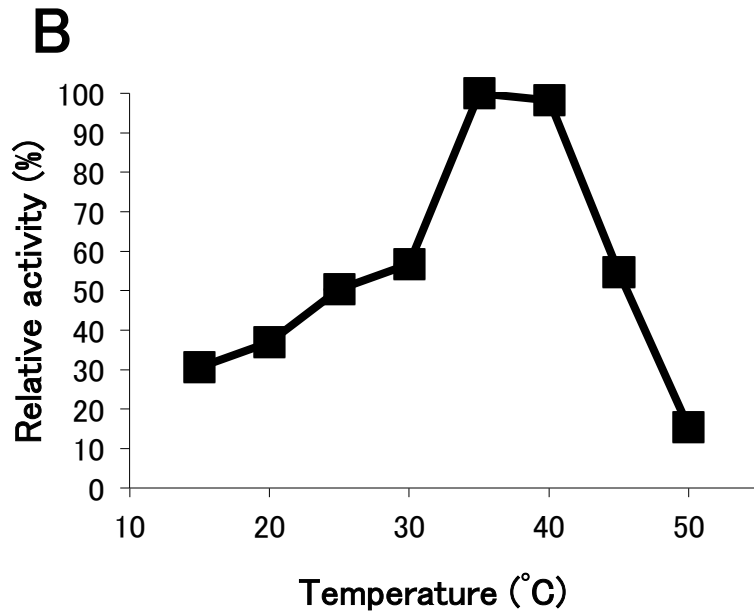
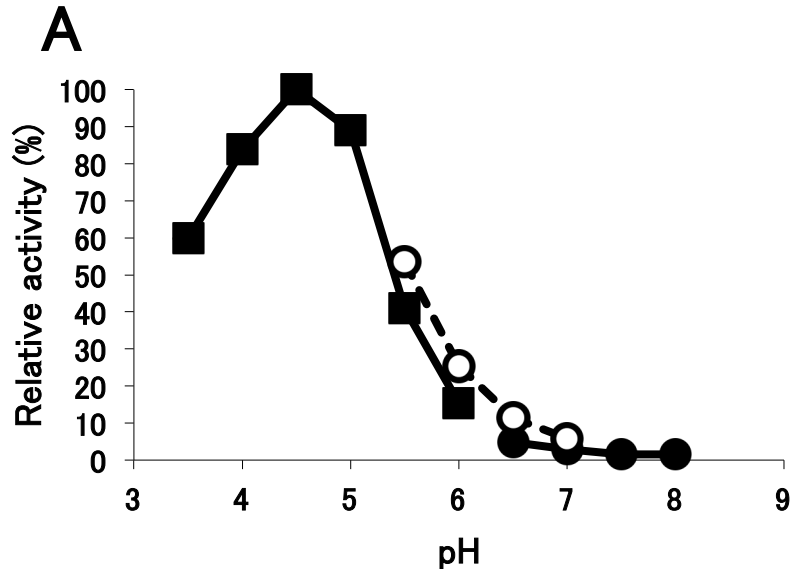


Figure S5

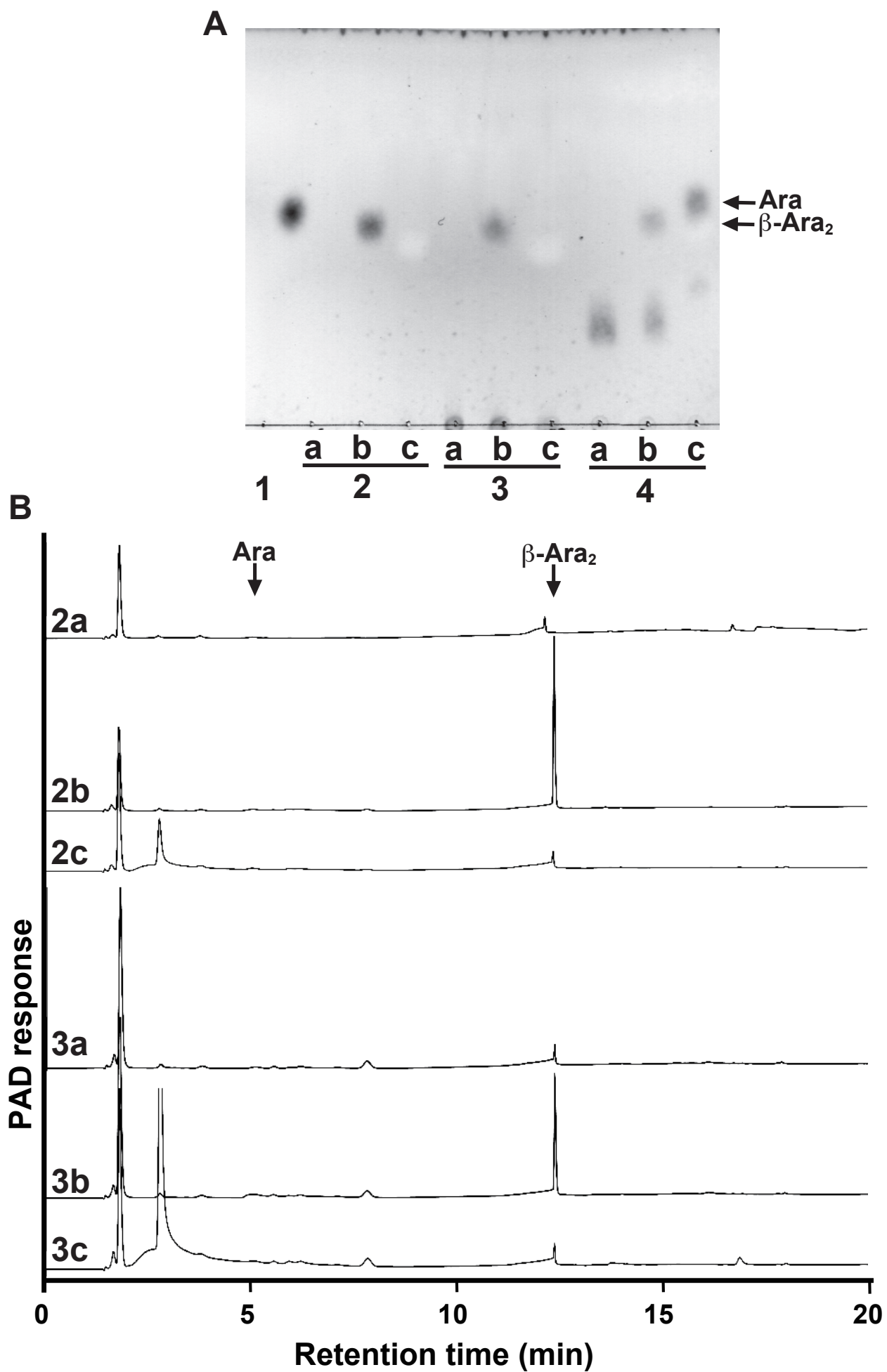


Figure S6

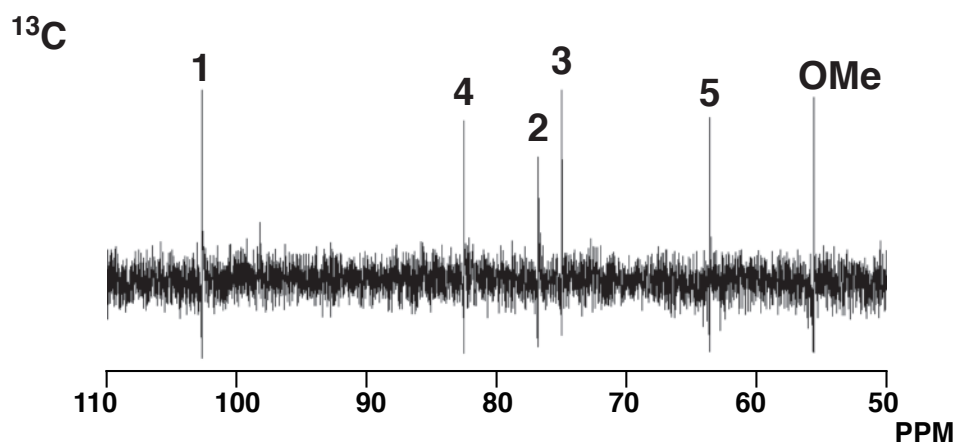
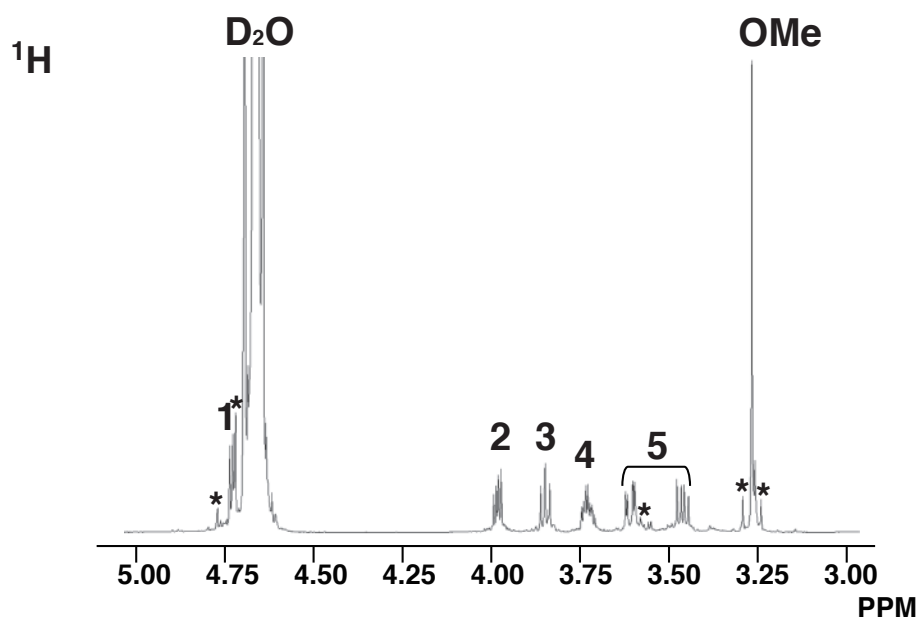
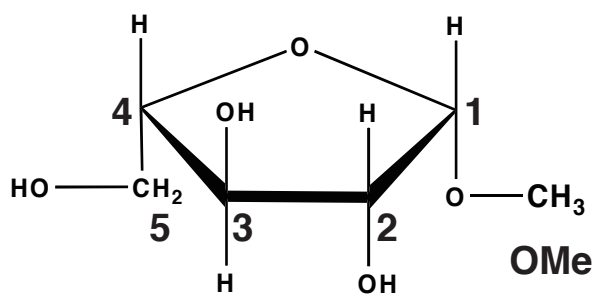
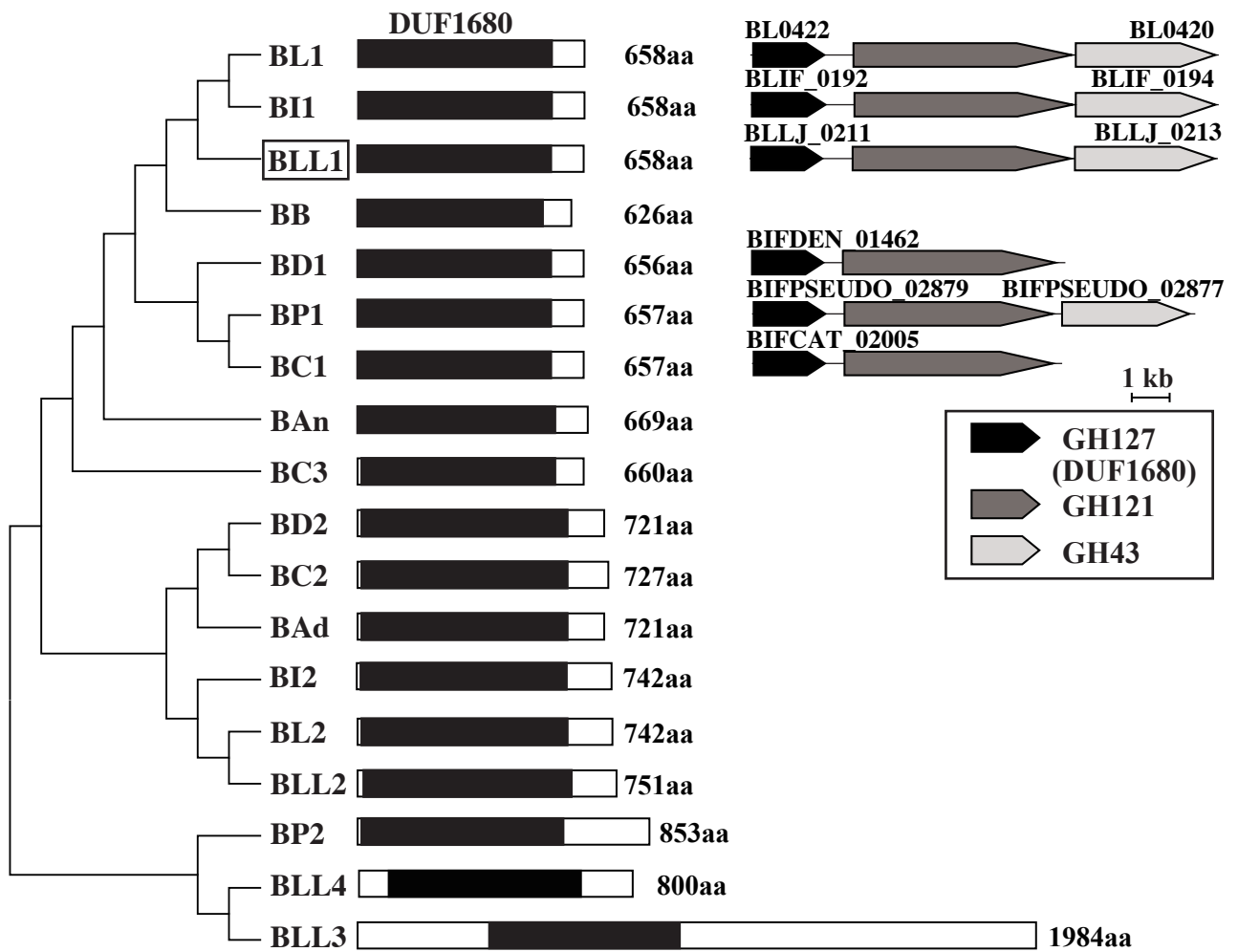


Figure S7



BL1 242 ---NIFHDLGFYKPTYFQ---AAEPVRDQQTADGHAVR-----VGYLCTGVAHVGRLL
 BI1 242 ---NIFHDLGFYKPTYFQ---AAEPVRDQQTADGHAVR-----VGYLCTGVAHVGRLL
 BLL1 242 ---NIFHDLGFYKPTYFQ---AAEPVRDQQTADGHAVR-----VGYLCTGVAHVGRLL
 BB 214 ---NIFPDLGFYKPTYFQ---AAEPVRDQQTADGHAVR-----VGYLCTGVAHVGRLL
 BD1 242 ---YIFRDLGFYKPEYFQ---AAEPVRDQQTADGHAVR-----VGYLCTGVAHVGRLL
 BP1 242 ---YIFRDLGFYKPTYFQ---AAQPVREQQTADGHAVR-----VAYLCTGIAHVARI
 BC1 242 ---YIFRDLGFYKPTYFQ---AAQPVREQQTADGHAVR-----VAYLCTGIAHVARI
 BAn 250 GLPAIFPAMETWSHEYTL---TARPIRDQQTAVGHAVR-----VAYLLAGVMQVGRLL
 BC3 250 ----FYTDLHM-PLKYEYV---QDEPILDKQHAEGHAVR-----LLYLAAAVSKVGRLL
 BD2 271 DGRNYEPREQ--NYAYYQ---ADKPVTEQTEALGHAVR-----AAYFYSGVADVARI
 BC2 277 DGRNYEPREQ--NYTYYYQ---ADKPVTEQTEALGHAVR-----AAYFYSGVADVARI
 BAd 270 DGRNYAPREQ--NYAYYQ---ADKPVTEQTEALGHAVR-----AAYFYAGADVARI
 BI2 269 ADANYKPNTDPSRYAYHQ---ANKPVTEQDEAVGHAVR-----AGYFYSGIADVARI
 BL2 269 ADANYKPNTDPNRYAYHQ---ANKPVTEQDEAVGHAVR-----AGYFYSGIADVARI
 BLL2 269 ADANYKPNTDPNRYAYHQ---ANKPVTEQDEAVGHAVR-----AGYFYSGIADVARI
 BP2 233 ERYHVMADR-FKDHAIEDPLAQGEDVLTGMHANTQIPK-----VLCWERLIGAI
 BLL4 337 RSEFLKASAFEDTDKLDNCGAGVDLNNLHANQHHPQ-----FVGYAKDAAMGDADI
 BLL3 598 KQTVLTAAHLEDETALFQKLANGQDPLNGLHANTTIPKLTGAMQRYVAYTEDEDLYNS-L

*E322

BL1 288 LGDQG-----LIDTAKREWKNIIVTFRMYVTGAIGSTHV---GESFTYDYDLPN--DTM
 BI1 288 LGDQG-----LIDTAKREWKNIIVTFRMYVTGAIGSTHV---GESFTYDYDLPN--DTM
 BLL1 288 LGDQG-----LIDTAKREWKNIIVTFRMYVTGAIGSTHV---GESFTYDYDLPN--DTM
 BB 260 LGDQG-----LIDTAKREWKNIIVTFRMYVTGAIGSTHV---GESFTYDYDLPN--DTM
 BD1 288 TGDRC-----LLDAVHRMWNIVGKRMVVTGAVGSTHV---GESFTYDYDLPN--DTM
 BP1 288 TGDQG-----LLDAAHREFWNNIVSKRMVVTGAIGSTHV---GESFTYDYDLPN--DTM
 BC1 288 TGDQG-----LLDAAHREFWNNIVSKRMVVTGAIGSTHV---GESFTYDYDLPN--DTM
 BAn 299 TNDEG-----LLRTGERLWNNIVHKRMYITGGIGSTHV---GEAFTYDYDLPN--DTM
 BC3 294 LNDQK-----MLDTAERLWNNIVKRMVITGAVGSCQV---GESFSFDDDLPN--DLV
 BD2 318 TGEAT-----LLESCETLWRNIVDRKLYITGGIGATHM---GEAFSFDYDLPN--DTA
 BC2 324 TGEAA-----LLESCETLWRNIVDRKLYITGGIGATHM---GEAFSFDYDLPN--DTA
 BAd 317 TGDSD-----LLASCERLWRNIVDRKIYITGGIGATHM---GEAFSFDYDLPN--DTA
 BI2 318 ADDQD-----LADAAERLWRNIVDKKLYVTGGIGGTVD---GEAFSYNYDLPN--DSA
 BL2 318 ADDQD-----LADAAERLWRNIVDKKLYVTGGIGGTVD---GEAFSYNYDLPN--DSA
 BLL2 318 ADDQD-----LADAAERLWRNIVDKKLYVTGGIGGTVD---GEAFSYNYDLPN--DSA
 BP2 280 CNDEQ-----ADAATNTFWDSVVEHRS---VSICAHSV---SEHFHPTDDFSSMIESR
 BLL4 390 DADARAR---YLKAVEGYWGMIVPGRMYAHGGTGE---GEMWGAHTVAG--DI-
 BLL3 657 SADERGKLTSLYLKAAQNFEDIIVVKDHTYVNGGNSQSEHFHVAGELWKDA-TQNG--DQN

*E338

*E366

BL1 336 YG-----ETCASVAMSMFAQQMLDLEPKGEYADVLEKELFNCSIAGISLDGKQYYYVN
 BI1 336 YG-----ETCASVAMSMFAQQMLDLEPKGEYADVLEKELFNCSIAGISLDGKQYYYVN
 BLL1 336 YG-----ETCASVAMSMFAQQMLDLEPKGEYADVLEKELFNCSIAGISLDGKQYYYVN
 BB 308 YG-----ETCASVAMSMFAQQMLDLEPKGEYADVLEKELFNCSIAGISLDGKQYYYVN
 BD1 336 YG-----ETCASVCM SMLSRQMLDLEPKGEYADVLERELFNCAIAGISLDGKQYYYVN
 BP1 336 YG-----ETCASVAMSMFARQMLDLEPNGEYADVLERELFNCAIAGISLDGKQYYYVN
 BC1 336 YG-----ETCASVAMSMFARQMLDLEPNGEYADVLERELFNCAIAGISLDGKQYYYVN
 BAn 347 YG-----ESCASVCMCFVARQMLEHELRGEYADVLEKELFNCAIAGIALDGGKFFFYVN
 BC3 342 YG-----ETCASVAMLFYGKSLMETKPRGSVADVMEKELFNGLVLSGVQLDGTTRYFYVN
 BD2 366 YS-----ESCAAIATAFFARRMLEIQPKSEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BC2 372 YS-----ESCAAIATAFFARRMLEIQPKSEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BAd 365 YS-----ESCAAIATAFFARRMLEIQPKSEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BI2 366 YS-----ETCAAIATAFFARRMLEIAPKAEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BL2 366 YS-----ETCAAIATAFFARRMLEIAPKAEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BLL2 366 YS-----ETCAAIATAFFARRMLEIAPKAEYADVME S A L Y N T T L A G M A L D G K S F F Y V N
 BP2 327 EG-----PETCNSYNMSKLAERLWLRSGSADYINFYERVLENHLLSTINPKQPGFVYFT
 BLL4 436 -GKRN---AESCAAYNMLKVARYLFFTEQKPAYMDYYERTILNHLGGKSRDLDSGTALT
 BLL3 714 GGYRNFSTVETCNEYNMLKLARILFQVTKDSKYSEYYEHTFINATVASONPETGMTTYFQ

Fig. S8a

BL1 389 ALETTDPDGLD-NPDRHHVLSHRVDWFGCA-----CCPANIARLIASVDRIYTER-----
 BI1 389 ALETTDPDGLD-NPDRHHVLSHRVDWFGCA-----CCPANIARLIASVDRIYTER-----
 BLL1 389 ALETTDPDGLD-NPDRHHVLSHRVDWFGCA-----CCPANIARLIASVDRIYTER-----
 BB 361 ALETTDPDGLA-NPDRHHVLSHRVDWFGCA-----CCPTNIAQLIASVDRIYTER-----
 BD1 389 ALESTPDGLD-NPDRHHVLSHRVDWFGCA-----CCPANIARLIASVDRIYTER-----
 BP1 389 ALETSPDGLD-NPDRHHVLSHRVDWFGCA-----CCPANVARLIASVDRIYTER-----
 BC1 389 ALETSPDGSD-NPDRHHVLSHRVDWFGCA-----CCPANVARLIASVDRIYTER-----
 BAn 400 PLEADVQATENNPDRRHVLLERAQWFGCA-----CCPSNIARLIASVDRIYTER-----
 BC3 395 PLEADPAASKGNPTKAHILTRRAGWEDCA-----CCPANLGRLLTSLDQYLYTVS-----
 BD2 419 PLEVVPEACHRDERKEHVKPVRQKWFCA-----CCPPNIARMVESVQOYAYTVA-----
 BC2 425 PLEVVPEACHRDERKEHVKPVRQKWFCA-----CCPPNIARMVESVQOYAYTVA-----
 BAd 418 PLEVVPEACHRDERKAHVKPVRQKWFCA-----CCPPNIARIVEDVQOYAYTIG-----
 BI2 419 PLEVNEYACHKDSRLRHVKPVRQKWFCA-----CCPPNIARIVESVQOYAYTVA-----
 BL2 419 PLEVNEYACHKDSRLRHVKPVRQKWFCA-----CCPPNIARIVESVQOYAYTVA-----
 BLL2 419 PLEVNEYACHKDSRLRHVKPVRQKWFCA-----CCPPNIARIVESVQOYAYTVA-----
 BP2 381 PMRSQHYRAYSTPEQECF-----W-----CCVSGLENHARYGRLLIYALQRPAAQ
 BLL4 492 PGNCYMPVNPATQKEY-----GDGNICT-----CCGGTALESHSKYQDSIYFHS-----
 BLL3 774 PMKAGYPKVFGITGTDY-----DADWFGGAIGEYWC-----CCQGTGIENFAKLNDSFYF-----

BL1 438 -----DGGKTVLSHQFIAN--KADF--ASGLTVEQR---S
 BI1 438 -----DGGKIVLSHQFIAN--KADF--ASGLTVEQR---S
 BLL1 438 -----DGGKTVLSHQFIAN--TAEF--ASGLTVEQR---S
 BB 410 -----DGGKTVLSHQFIIN--KAEF--ASGLTVEQR---S
 BD1 438 -----DGGKTVLSHQFIAN--EATF--DSGLYVVQR---S
 BP1 438 -----DGGRTVLAHQFIAN--QASF--DSGLHVEQR---S
 BC1 438 -----DGGRTVLAHQFIAN--QASF--DSGLHVEQR---S
 BAn 450 -----EDERMIAAHQFIAN--DAEF--FDDVRVKQE---S
 BC3 445 -----NDGKTVYAHQFVAN--KTEF--EDGFTIEQTQAGD
 BD2 469 -----DDASTLYVHLYMGGVVS AKLGGSDVSLEVR---A
 BC2 475 -----DDASTLYVHLYMGGVVS AKLGGSDVSLEVR---A
 BAd 468 -----DSSSTLYVHLYMGGVHARLSGTDVRLDVM---S
 BI2 469 -----EDGGTLFTHLYMGGVAKAELNGTAVELDVT---A
 BL2 469 -----EDGGTLFTHLYMGGVAKAELNGTAVELDVT---A
 BLL2 469 -----EDGGTLFTHLYMGGVAKAELNGTAVELDVT---A
 BP2 425 DSADSAAAGFASSAAETGNTVSNNAEAEATRLLVNLVYIDS--TFDCPEQCLRTTORAARI
 BLL4 537 -----TDNKELYVNLFTAS--TLDWTDITGLKIAQE---T
 BLL3 823 -----TDENNVYVNMFWSS--TYTDTRHNLITITQT---A

BL1 466 DFPWDSHVEYTVSLPASA-----ADSSVRFGLRIPGW--SLGS----YTTLTVNGKPAVG
 BI1 466 DFPWDSHVEYTVSLPASA-----ADSSVRFGLRIPGW--SLGS----YTTLTVNGKPAVG
 BLL1 466 NFPWDGHVEYTVSLPASA-----TDSSVRFGLRIPGW--SRGS----YTTLTVNGKPAVG
 BB 438 DFPWNCHVEYTVSLPASA-----TDSSVRFGLRIPGW--SLGS----YALTVNGKSAVA
 BD1 466 DMPWSGHVEFEVNLAEGA-----QPVRFGVRIPSW--SANA----YALAVDGEPECK
 BP1 466 DFPWNCHIEYMVELPAEA-----AD-SVRFVRIPTW--SADS----YALTCDGVAVKT
 BC1 466 DFPWNCHIEYMVELPAEA-----AD-SVRFVRIPTW--SADS----YALTCDGVAVKT
 BAn 478 DFPRECVVRFVTVDVPEGA-----DPVIEKVRIPSW--SPE----YRLTVDGVDTVG
 BC3 476 EYPWSCDLTFHVSNEGL-----DKKVAVRIPQW--SKD----YTLEVNGEAVEL
 BD2 500 GMPWNGAGAITVTLPESSD---EGQVPEPFALALRIPAW--AGGESAA-DSIHAAGEKDSR
 BC2 506 GMPWNGAGAITVTLPESSD---EGQVPESFALALRIPAW--AGGESAA-DSIHATGEKDSR
 BAd 499 DMPWSCKGSAVAVGFDAAGDSASDASKDAVFTIALRIPAW--AGGETAS-DAVTVRGRDDIS
 BI2 500 NLPWYGDGKAVVRLGNDAAAGASAQAPARFTLAERLIPGW--VGEESAAAAAITATGEPESG
 BL2 500 NLPWQGDGKAVVRLGDAAGTSAQAPARFTLAERLIPGW--VGDESAAAAAITATGESESG
 BLL2 500 NLPWQGDGKAVVRLGDAAGTSAQAPARFTLAERLIPGW--VGDESAAAAAITATGESESG
 BP2 483 EDGVDYTVTFITLESTAEHVPDTPGGLRETTFLFRPWWAEHYGVMEATCAVCTLDPARTN
 BLL4 566 NYPEEETSTISITAAPKS-----AVTERIRIPAW--SKG----AKIEVNGKAIDG
 BLL3 852 NVPKTEDVTFEVSGETCSA-----NLKLRVPDWAITNG-----VKLVVDGTEQAL

Fig. S8b

Figure S9

