Electric Supporting Information (ESI)

# Crystal structure and functional analysis of large-terpene synthase belonging to a newly found subclass

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## Experimental Procedures

**General procedure**

NMR spectra were recorded using a Bruker DPX 400 spectrometer at 400 MHz for proton ($^{1}$H) and 100 MHz for carbon ($^{13}$C). GC-MS was performed on a JMS-T100GCV spectrometer (JEOL) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm; J&W Scientific), using the EI mode operated at 70 eV. GC analyses were performed on a Shimadzu GC-2014 chromatograph equipped with a flame ionization detector and a DB-1 capillary column.

**Vector construction, expression and purification of BalTS**

The BalTS expression vectors (wild-type as well as the D85A, D88A, D92A, D249A, D253A, and D257A mutants) were constructed by Genewiz (South Plainfield, NJ, USA) as follows. DNA fragments optimized for BalTS (WP_003323837.1) expression by *E. coli* were synthesized. The DNA fragments contain the codons to express the peptide HMSSGLVPRGSH prior to the BalTS sequence. The first His and the second Met corresponds to the NdeI restriction site (DNA: CATATG). Residues SSGLVPRGSH correspond to the amino-acid residues around the thrombin digestion site appearing in pET14b, pET15b and pET28a-c. The synthesized DNA fragments were inserted between the NdeI and the BamHI sites of the pCold-II plasmid. The constructed vectors express translation enhancing element (TEE), $His_6$-tag and thrombin digestion sites followed by BalTS. After the thrombin-treatment, the recombinant BalTS proteins (wild-type and mutants) possess three amino-acids, GSH, at their N-terminals.

An *E. coli* strain BL21(DE3)pLysS was transformed by the expression vectors. The transformants were cultivated in Luria-Bertani medium with 50 μg/mL ampicillin. When the optical cell density of the culture reached 0.5-0.7, the culture was incubated at 15 °C for 30 min without flask-shaking. After the incubation, isopropyl 1-thio-D-galactopyranoside (IPTG, final concentration 1 mM) was added to the medium and cultured at 15 °C for an additional 24 h. The cells were harvested by centrifugation.

The harvested cells were resuspended in flow buffer composed of 20 mM Tris-HCl (pH 8.0), 300 mM sodium chloride and 5 mM dithiothreitol. The suspension was sonicated and centrifuged (30,000 ×g for 1 h at 4 °C), and the supernatant was applied to a Ni-NTA superflow (QIAGEN) column equilibrated with the flow buffer. The column was washed with binding buffer (flow buffer supplemented with 10 mM imidazole) and wash buffer (flow buffer supplemented with 30 mM imidazole). The bound BalTS was eluted with elution buffer (flow buffer supplemented with 250 mM imidazole). The eluent was used for enzymatic assays, or applied to a Superdex200 increase 10/300 column (GE Healthcare) for further purification for crystallization. The column was equilibrated with a gel filtration buffer composed of 25 mM Tris-HCl (pH 8.0), 25 mM ammonium chloride, 1 mM magnesium chloride, 300 mM sodium chloride and 5 mM dithiothreitol at a flow rate of 0.5 mL/min at 4 °C. Thrombin (GE Healthcare) was added to the fractionized BalTS (10 units thrombin per 1 mg BalTS) and incubated 1 day at 4 °C. The sample was concentrated and re-applied to the Superdex200 increase 10/300 column (GE Healthcare) for the final purification. The protein purity was judged by SDS-PAGE, and the concentration was determined by UV-absorption at 280 nm (1.42 for 1 mg/ml BalTS).

The selenomethionine (Se-Met) derivative of BalTS was prepared as follows. *E. coli* B834(DE3)pLysS cells were transformed by the BalTS expression vector. The cells were cultivated in a Se-Met medium composed of 1 g/L ammonium chloride, 4.5 g/L potassium dihydrogenphosphate, 10.5 g/L dibasic potassium phosphate, 0.5 g/L trisodium citrate, 0.2 g/L magnesium sulfate, 10 g/L D-(+)-glucose, 7 mg/L iron(III) chloride hexahydrate, 0.5 g/L thiamine, 0.5 g/L biotin, 19 kinds of amino acids not including methionine (80 mg/mL each), 4 kinds of nucleotide base (adenine, guanine, thymine and uracil, 0.5 g/L each), and 50 mg/mL selenomethionine. Induction by IPTG, cell harvest and purification were performed as non-derivatized BalTS.

**Crystallization and crystallographic analysis of BalTS**

Both the native and the selenomethionine derivative of the purified BalTS were concentrated to 11 mg/mL. Crystallization was performed with the hanging-drop vapor-diffusion method. The BalTS samples were mixed with an equal amount of the precipitant solution and equilibrated with the precipitant solution at 20 °C. The precipitant solution for the native crystals was composed of 15% (w/v) polyetheleneglycol3350 (PEG3350), 0.1 M N,N-Bis(2-hydroxyethyl)glycine (BICINE, pH 9.0), and 200 mM ammonium phosphate dibasic, whereas that for the selenomethionine derivative crystals was composed of 16% (w/v) PEG3350, 0.1M BICINE (pH 8.5), and 200 mM ammonium phosphate dibasic.

The crystals were dipped in the cryoprotectant solution for several seconds and flash-frozen in a dry nitrogen-stream at 100 K. The components of the cryoprotectant solution for native crystals were 25% (w/v) PEG3350, 0.1M BICINE (pH 9.0), 200 mM ammonium phosphate dibasic, and 10% (v/v) glycerol, whereas those for selenomethionine derivative crystals were 32.5% (w/v) PEG3350, 0.1 M BICINE (pH 8.5), and 50 mM ammonium phosphate dibasic. The data sets were collected on beamline 5A (Se-Met dataset) and beamline NE-3A (Native) at the Photon Factory, KEK, Japan, using X-ray wavelengths of 0.9788 Å and 1.000 Å, respectively. The datasets

were processed with the program XDS.[1] Phasing was performed with the single-anomalous-dispersion (SAD) method using the program SOLVE.[2] The initial model was automatically constructed by the program RESOLVE[3] with the selenomethionine derivative dataset. Then, the model was manually modified using the program COOT[4] and refined using the program REFMAC[5] with the native dataset. The model was refined to $R$ and $R_{free}$ factors of 0.204 and 0.229, respectively, with the translation-libration-screw (TLS) technique. The data and the refinement statistics are shown in Table S1. The Protein Data Bank (PDB) ID for the refined model is 5YO8.

**Oligomeric state analysis**

The molecular weight of BalTS in solution was analyzed using a size exclusion column Superdex 200 increase 10/300. The column was equilibrated with gel filtration buffer prior to the analysis. The purified BalTS (2.9 mg/mL) was applied to the column at a flow rate of 0.55 mL/min. The retention volume was compared with that of the marker proteins applied to the same column under the same conditions. The markers used were 1.0 mg/mL blue dextran (2 MDa), 0.8 mg/mL ferritin (440 kDa), 5.4 mg/mL aldolase (158 kDa), 3.0 mg/mL conalbumin (75 kDa), 3.8 mg/mL ovalbumin (43 kDa), 2.6 mg/mL carbonic anhydrase (29 kDa), 3.2 mg/mL ribonuclease A (13.7 kDa), and 2.6 mg/mL aprotinin (6.5 kDa). All of the markers were purchased from GE Healthcare Science.

**Analysis of the dimer geometry**

The oligomer states of determined terpene synthases in protein data bank (PDB) were estimated by PISA.[6] One subunit of each dimeric enzyme was superposed on a subunit of BalTS. The superposed subunits are shown in white in Fig. 2c, and the other monomers are represented in various colors. The superpoed enzymes are selinadiene synthase from *S. pristinaespiralis* (PDB 4OKM, cyan for the non-superposed subunit),[7] 2-methylisoborneol synthase from *Streptomyces coelicolor* (PDB 3V1X, red),[8] MoeN5 from *Streptomyces ghanaensis* (PDB 5B00, yellow),[9] geosmin synthase from *Streptomyces coelicolor* (PDB 5DZ2, orange),[10] germacradien-4-ol synthase from *Streptomyces citricolor* (PDB 5I1U, yellow-orange),[11] (+)-bornyl diphosphate synthase from *Salvia officinalis* (sage) (PDB 1N1B, magenta),[12] γ-terpinene synthase from *Thymus vulgaris* (PDB 5C05, bright yellow-green),[13] pentalenene synthase from *Streptomyces exfoliates* (PDB 1HM4, pink),[14] ent-kaur-16-ene synthase from *Bradyrhizobium japonicum* (PDB 4XLX, light blue),[15] aristolochene synthase from *Penicillium roqueforti* (PDB 1DGP, pale cyan),[16] cyclooctat-9-en-7-ol synthase from *Streptomyces melanosporofaciens* (PDB 4OMG, brown),[17] and trichodiene synthase from *Fusarium sporotrichioides* (PDB 1JFA, blue-purple).[18]

**Vector construction, expression and purification of BsuTS**

The BsuTS gene was initially inserted into pCold I vector by the same strategy to construct pColdTF-ytpB, which was used for our previous investigation.[19] These vectors were named pColdI-BsuTS-standard and renamed pColdTF-BsuTS-standard (TF+standard in Fig. S6b), respectively. The BsuTS gene on the two BsuTS-standard vectors contains the L258S replacement comparing to the database sequence (Kyoto Encyclopedia of Genes and Genomes (KEGG) entry ID: BSU30500). Also, the gene contains one NdeI digestion site (81st to 86th bases).

The NdeI site in the BsuTS gene in pColdI-BsuTS-standard plasmid was removed by inverse PCR method using primers BsuTS-delNdeI-F and BsuTS-delNdeI-R (5'-TGGACATTTGGAAGCAAAAATCAGAATCGATTC-3' and 5'-GCTTCCAAATGTCCAGCTCCTGATGAACGAGCG-3'). The resulting plasmid pColdI-BsuTS-delNdeI was amplified using primers BsuTS-pCold2-F and BsuTS-pCold2-R (5'-CCAATTCATATGAGCAGTGGTCTGGTGCCGCGTGGTAGCCACTTGACAGTACCGGAACATCCTTTT-3' and 5'-CCAATTGGATCCTCAGCAAATGATTTGAGCTTTTTTCTT-3'; NdeI and BamHI sites are underlined). The amplified DNA fragment was digested by NdeI and BamHI and inserted into pCold II vector. The resulting plasmid pColdII-BsuTS-standard (standard(control) in Fig. S6b) expresses TEE-tag, $His_6$-tag and thrombin digestion sites at their N-terminal as BalTS. Also, the BsuTS coding region on pColdII-BsuTS-standard does not contain NdeI digestion site.

The L258S replacement in pColdTF-BsuTS-standard and pColdII-BsuTS-standard vectors were removed by inverse PCR method using primers BsuTS-S258L-F and BsuTS-S258L-R (5'-GAGGACTTGCTGGAAGGAGACTTGAA-3' and 5'- TTCCAGCAAGTCCTCCTCTTGATCAA -3'). The resultant plasmids were named pColdTF-BsuTS-database and pColdII-BsuTS-database (TF+database and database in Fig. S6b), respectively.

C-terminal truncation mutants were constructed by inverse PCR method using pColdII-BsuTS-standard as the template. The K349ter, F350ter, Q351ter, K352ter, and M353ter mutants, whose C-terminal residues are K349, F350, Q351, K352, and M353, are constructed using the primer pairs of K349ter-F/K349ter-R (5'-CGGAAATGATTTCAAAAAATGTCCTGG-3'/5'-TTGAAATCATTTCCGGTAGGCTCTGCC-3'), F350ter-F/F350ter-R (5'-AAATTTTGACAAAAAATGTCCTGG-3'/5'-TTTTTGTCAAAATTTCCGGTAGGCTCTGCCGTT-3'), Q351ter-F/Q351ter-R (5'-TTTCAATGAAAAATGTCCTGGATG-3'/5'-CATTTTTCATTGAAATTTCCGGTAGGCTCTGCC-3'), K352ter-F/K352ter-R (5'-CAAAAATGAATGTCCTGGATGTCC-3'/5'-GGACATTCATTTTTGAAATTTCCGGTAGGCTCT-3'), and M353ter-F/M353ter-R (5'-AAAATGTGATCCTGGATGAAAAATTCA-3'/5'- CCAGGATCACATTTTTTGAAATTTCCG-3'), respectively. The sequences of the all plasmids were confirmed by DNA sequencing (Hokkaido System Science Co., Ltd., Sapporo, Japan or Macrogen Japan Corp., Kyoto, Japan).

An *E. coli* strain Novablue was transformed by the vectors, and cultivated in Luria-Bertani medium with 50 μg/mL ampicillin. Induction of BsuTS overexpression and harvesting of the cells were performed as BalTS. Resuspension and sonication of the harvested cells, and purification using the Ni-NTA resin of BsuTS were also performed as for BalTS. The eluent from Ni-NTA resin with elution buffer was dialyzed against the gel filtration buffer overnight at 4 °C. The samples were concentrated to the appropriate concentration and used for the enzymatic assays.

**Homology modeling of BsuTS**

Homology model of BsuTS was constructed using swiss-model[20] server using the refined crystal structure of BalTS as a template. The BsuTS model structure was constructed as a monomer. The monomeric model was dimerized based on the dimeric assembly of BalTS. It should be noted that no information is available to date regarding the oligomeric state of BsuTS.

**Enzymatic assays**

The substrates (**1**-**4**) for the enzymatic assays of BalTS and BsuTS were prepared as previously described.[21, 22] For the product analysis (shown in Figs. 1 and 4), the reaction mixture was composed of 25 mM Tris-HCl buffer (pH 8.5), 10 mM dithiothreitol, 1 mM $MgCl_2$, 130 μM substrate (**1**-**4**), and 0.93 μM purified enzyme (BalTS or BsuTS) in a total volume of 2 mL. The reaction was carried out at 37 °C for 16 h. A 15% KOH/MeOH solution (3 mL) was added to terminate the reaction. The lipophilic product was extracted from the reaction mixture by using *n*-hexane (3 × 5 mL) and analyzed by GC-MS (injection temperature, 300 °C; oven temperature, 60**–**280 °C in increments of 10 °C $min^{-1}$). The products were identified both by comparisons of the GC-MS spectrum and by co-injection experiments with authentic prenes **6**-**9**. The authentic **7**-**9** were isolated in previous studies [21, 22], and **6** was obtained and analyzed in this study (Figs. S7-S11). The GC-MS analysis of *n*-hexane extracts from *B. alcalophilus* cells was also performed under the same conditions.

For the activity measurements of BalTS (Fig. 3c), reactions were performed with substrate **1** in the same manner as for the product analysis, except that the incubation time was 1 h. The amount of product was quantified with GC (injection temperature, 300 °C; oven temperature, 220**–**300 °C in increments of 3 °C $min^{-1}$).

**Isolation and identification of β-springen (6)**

The reaction mixture contained 25 mM Tris-HCl buffer (pH 8.5), 10 mM dithiothreitol, 1 mM $MgCl_2$, 130 μM (11.7 mg) **1**, and 7.5 μM purified BalTS in a total volume of 200 mL. The reaction was carried out at 37°C for 16 h. After 15% KOH/MeOH solution (240 mL) was added to the reaction mixture, the lipophilic products were extracted with *n*-hexane (440 mL × 3) and concentrated. The *n*-hexane extract (28.6 mg) was partially purified by silica gel (3 g) column chromatography with *n*-hexane and *n*-hexane/EtOAc (100:20). The fraction (3.4 mg) eluted with *n*-hexane containing the product **6** was subjected to $SiO_2$ HPLC (Inertsil PREP-SIL, 6.0 × 250 mm; GL Science) with *n*-hexane, and pure **6** (colorless oil; 1.8 mg) was obtained. The structure of **6** was determined to be β-springene by MS (Fig. S7) and NMR (Figs. S8-S11). HR-EI-MS: *m/z* calculated for $C_{20}H_{32}$ $[M]^+$ 272.2504; found 272.2509.

## Movie Captions

**Movie S1.**
Surface representation of the physiological dimer of BalTS. White and gray molecules indicate the respective monomers. Carbons, nitrogens, and oxygens of highly conserved residues in homologue proteins are colored green, blue, and red, respectively.

**Movie S2.**
Surface representation of BalTS. The six characteristic aspartates and hydrophobic residues conserved in homologous enzymes are colored in magenta and yellow, respectively.

**Movie S3.**
Surface representation of BalTS showing distribution of conserved residues between BalTS and BsuTS. Yellow and green parts represent identical and similar residues shown in Fig. S5, respectively. Brighter and darker colors represent each subunit of BalTS. The BalTS molecules in Movies S1-S3 are rotated around the same axis. Only the color scheme is different from each other.

## Author Contributions

M.F. and T.Sa designed the whole project, analyzed the results. M.F. and Y.T. determined the crystal structure of BalTS. D.Y., T.N., D.U and M.M. analyzed the reactions of BalTS and BsuTS. Y.Y., A.S., K.F., and T.Sh synthesized the substrates of BalTS and BsuTS. K.M. administrated and supervised the project. The manuscript was written by M.F. and T.Sa, edited by T.Sh and K.M., and approved by all authors.

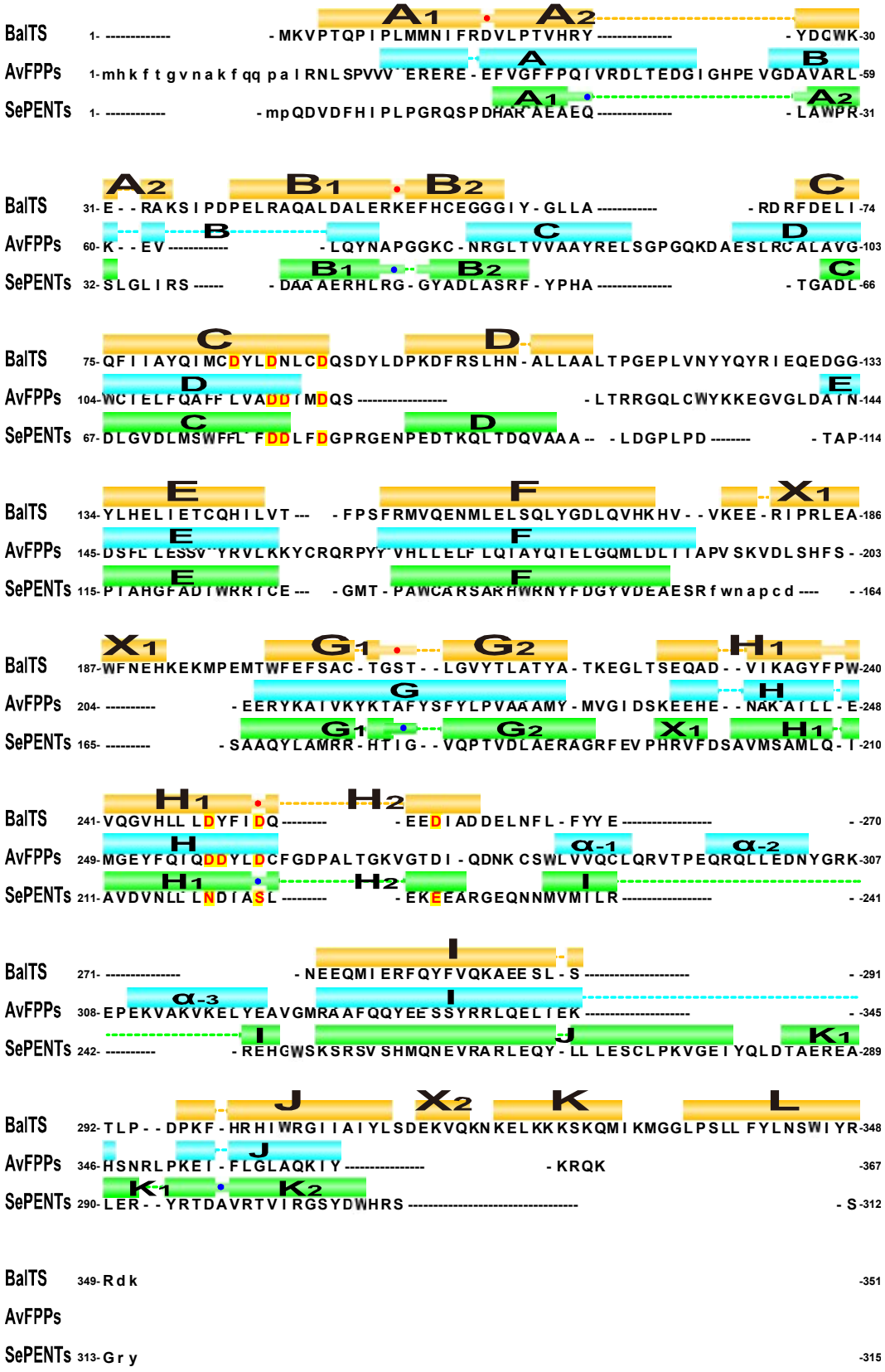



**Fig. S1.** Structural sequence alignment among BalTS, farnesyl diphosphate synthase from avian (AvFPPs, a prenyltransferase),[23] pentalenene synthase from *Streptomyces exfoliates* (SePENTs, a Class I terpene synthase).[24] Cartoon over the sequences show helix names for each structure. Small red and blue closed circles represent the helix kink on BalTS and SePENTs, respectively. Residues (potentially) involved in the binding of pyrophosphate part of the ligands are highlighted in red font with yellow background.

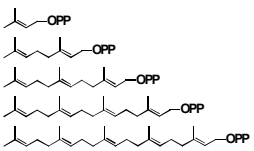
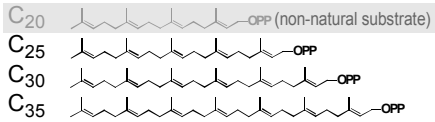
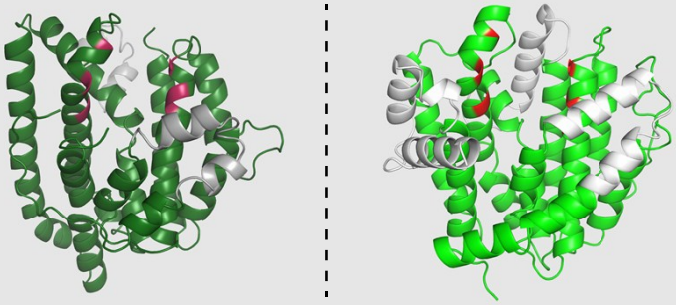
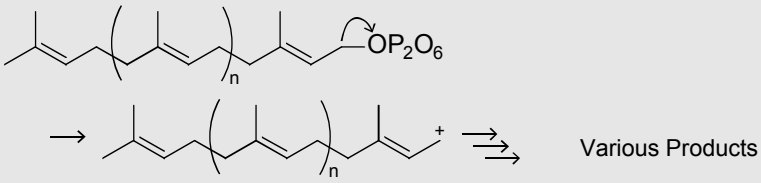
| | Class I enzymes | Class IB enzymes (large terpene synthase) |
|---|---|---|
| Substrates | prenyl-pyrophosphates<br>$C_5$ (rare)<br>$C_{10}$ (major)<br>$C_{15}$ (major)<br>$C_{20}$ (major)<br>$C_{25}$ (rare)<br>  |  |
| Cleft size | shallow | large penetrating hole |
| Primary structure | no similarity | |
| 3D structure | categolized in the same group<br>  | |
| Motif sequence | NSE/DTE<br>**DD**xx**D** | **D**YL**D**NLx**D**<br>**D**Y(F,L,W)I**D**xxE**D** |
| Reaction trigger | leaving pyrophosphate from the substrate<br>  | |

**Fig. S2.** Summary of the differences between Class I and Class IB enzymes.

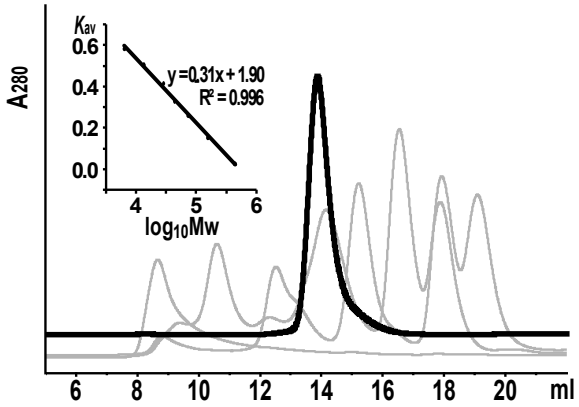



**Fig. S3.** Oligomer state analysis of BalTS by size-exclusion chromatography. Gray lines represent the peaks for marker proteins, whereas thick black line shows that for BalTS. The estimated molecular size of BalTS is 89,000 Da, which is very close to twice the calculated molecular size of a BalTS subunit (43,189 Da). The estimated oligomeric state suggests that the two subunit in an asymmetric unit of the crystal is the physiological dimer.

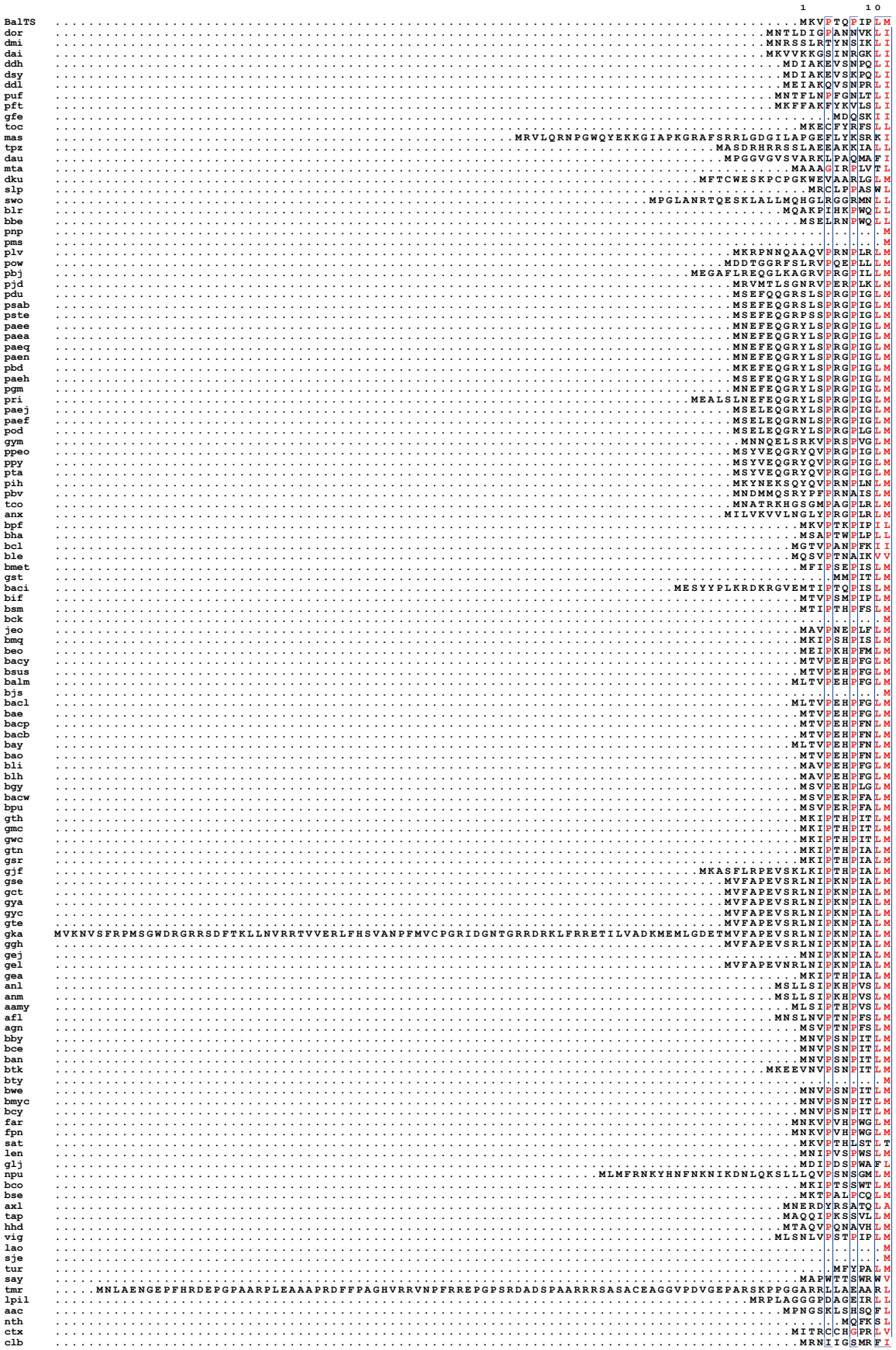


**Fig. S4.** (1/6)

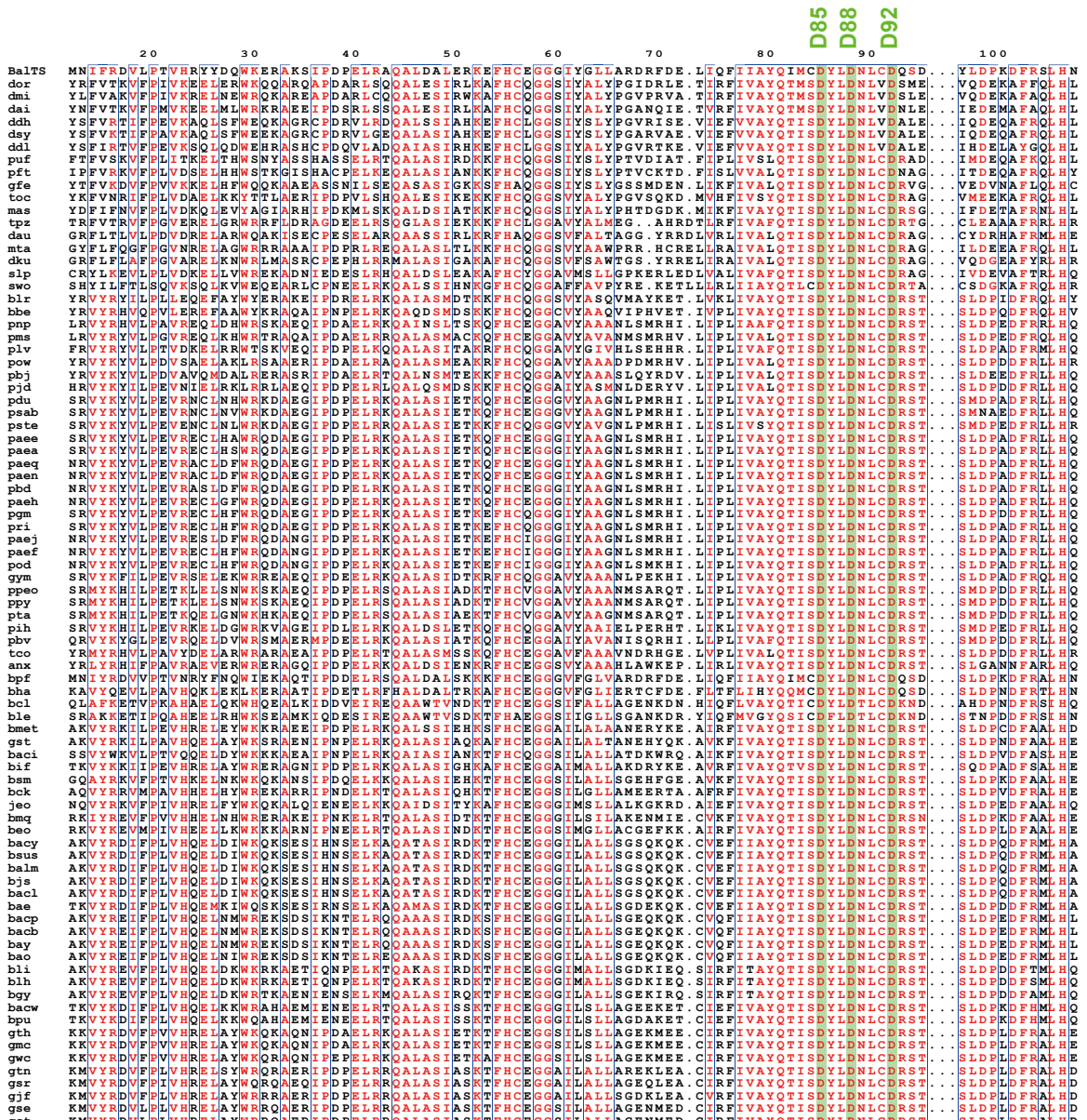



**Fig. S4.** (2/6)

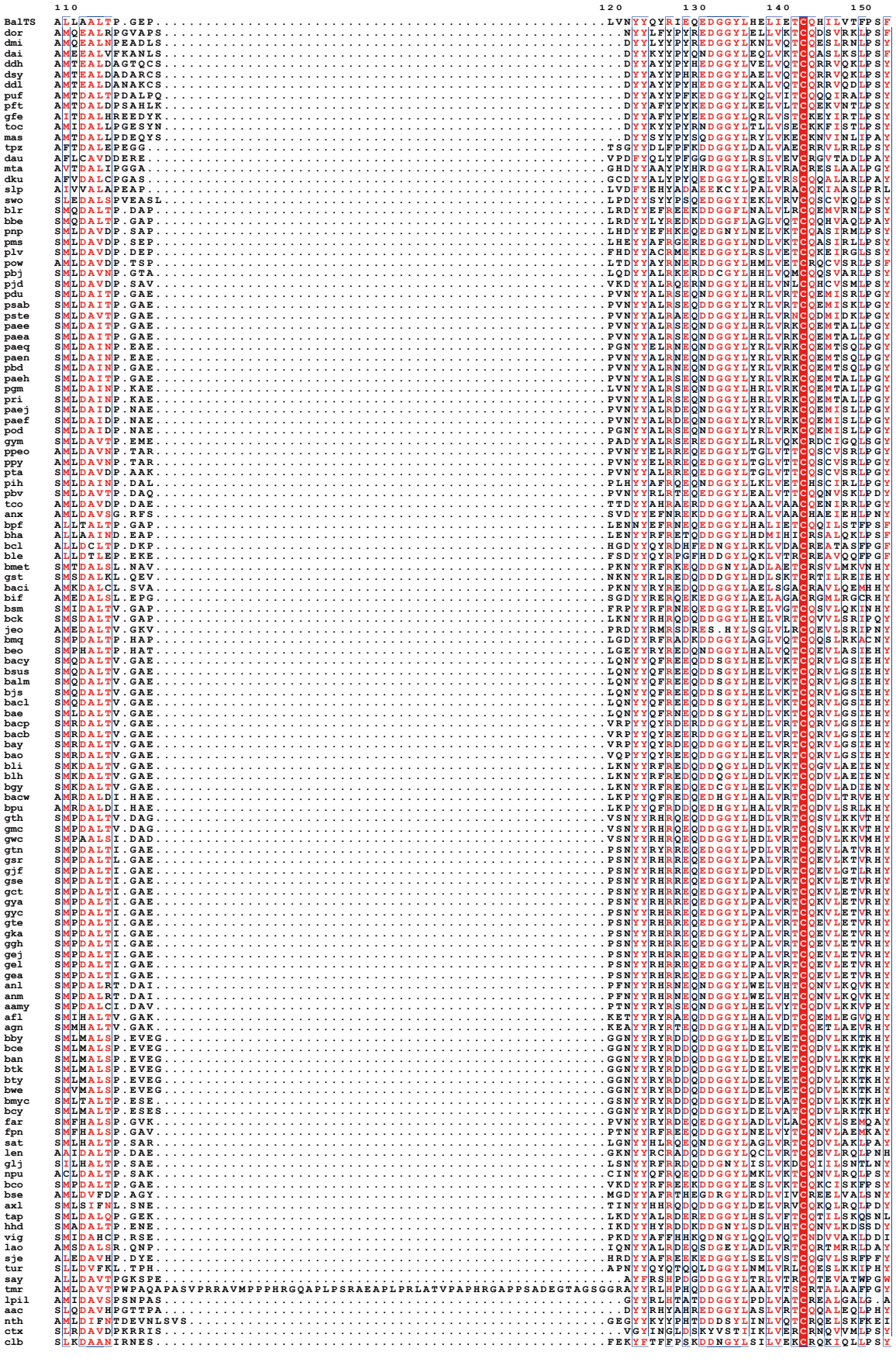


**Fig. S4.** (3/6)

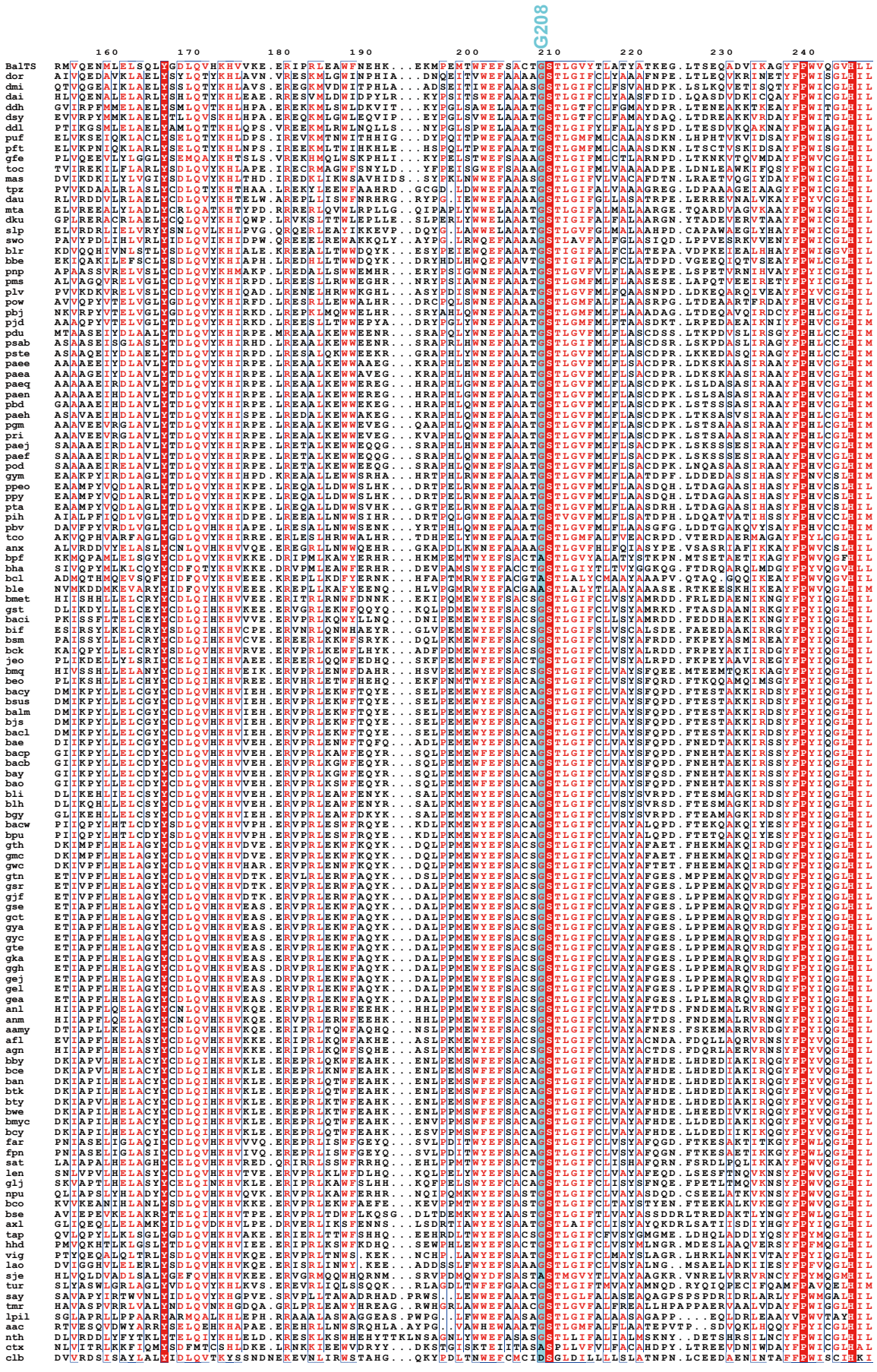

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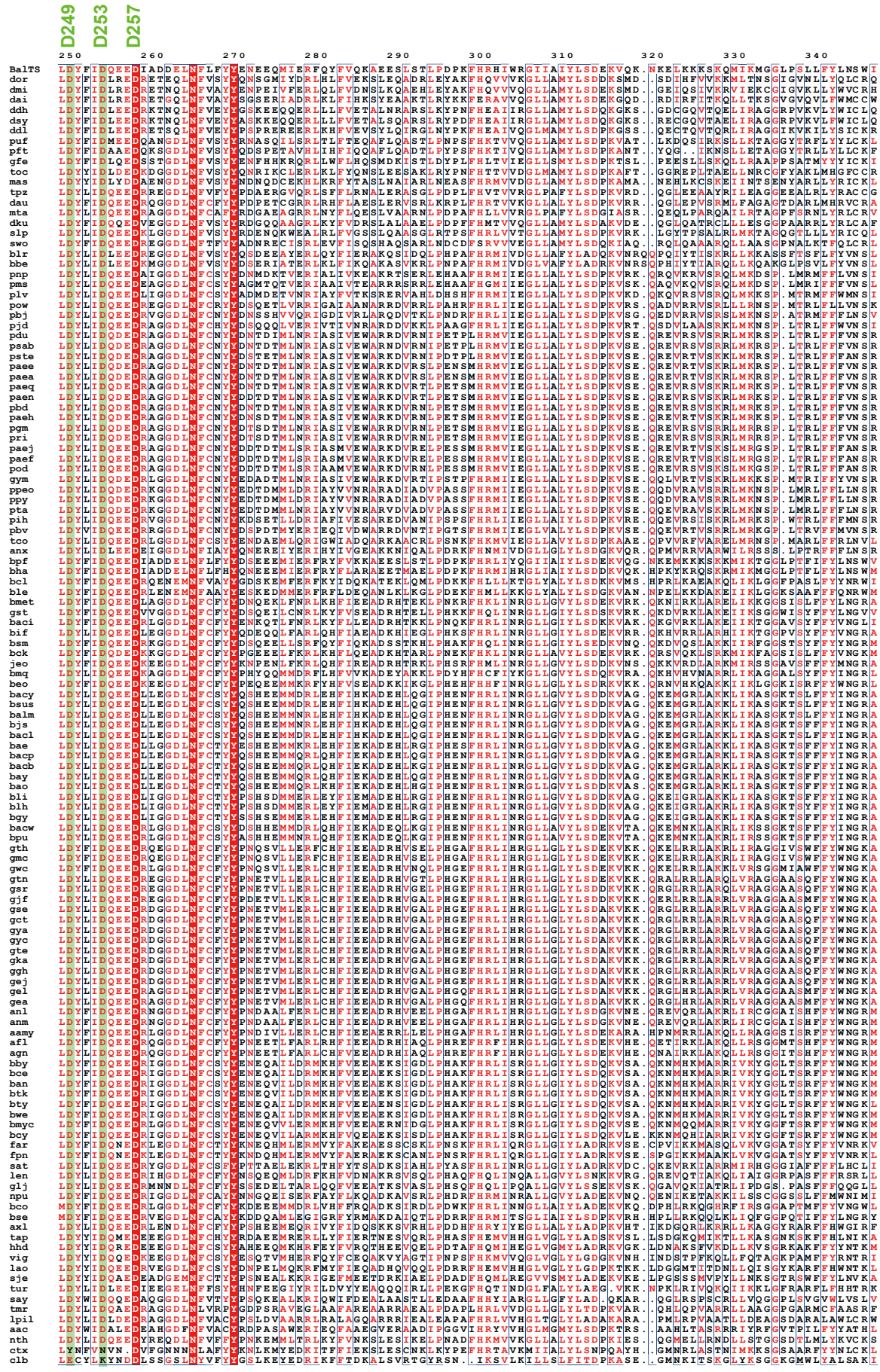
**Fig. S4.** (4/6)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anm
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afl
agn
bby
bce
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bmyc
bcy
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fpn
sat
len
glj
npu
bco
bse
axl
tap
hhd
vig
lao
sje
tur
say
tmr
lpil
aac
nth
ctx
clb

**Fig. S4.** (5/6)


350
BalTS YRRDK
dor LRRGKVI
dmi LRKRKII
dai LRKRNLL
ddh LRKKGRL
dsy LRKKGKL
ddl LRGKGML
puf LRFKKKL
pft LRFRKKL
gfe LRRNNII
toc LRKTARL
mas IRRRGSL
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paej WIRNRFL
paef WIRNRFL
pod WIRKRFL
gym WIRKHKHE
ppeo WIRRLI
ppy WIRRLI
pta WIRRLL
pih WIRKHLYK
pbv WIRKYMYEH
tco WIRRRAKPPR
anx GYRGGKAVLQAPPSGK
bpf FRRS
bha FRRKSGEQPAMDSM
bcl FKRKIS
ble FKRAI
bmet YRAMQKWMPLGLMK
gst YRSLQKSVPSLMERMLITK
baci FRRVEKLVPAGVIRALVK
bif YRTVQKWVPAGLARLLF
bsm YRKFRQHVSK
bck YRAIKNMIPASLAKTIVK
jeo YRNVMRFLSGNPYIKSQV
bmq YRMLKR
beo YRILQKSTPH
bacy YRKFQKMSWMKNSKKKAQIIC
bsus YRKFQKMSWMKNSKKKAQIIC
balm YRKFQKMSWMKNSKKKAQIIC
bjs YRKFQKMSWMKNSKKKAQIIC
bacl YRKFQKMSWMKNSKKKAQIIC
bae YRKFQKMPWMRSS
bacp YRKFQKMAWTKSS
bacb YRKFQKMAWTKSS
bay YRKFQKMAWTKSS
bao YRKFQKMAWTKSS
bli YRKIQKMPWMKSS
blh YRKIQKMPWMKSS
bgy YRKIQKMPWMKSS
bacw YRTYQKMPWMKSS
bpu YRTYQKMPWMKSS
gth YRLWQKKRSSIAK
gmc YRLWQKKRSSIAK
gwc YRLWQKKFSVIAK
gtn YRFWQEKQKRLSFS
gsr YRFWQEKQKRLSFS
gjf YRFWQEQQKRLSLS
gse YRFWQERQKRLSFS
gct YRFWQERQKRLSFS
gya YRFWQERQKRLSFS
gyc YRFWQERQKRLSFS
gte YRFWQERQKRLSFS
gka YRFWQERQKRLSFS
ggh YRFWQERQKRLSFS
gej YRFWQERQKRLSFS
gel YRFWQERQKRLSFS
gea YRFWQEQQKRLSFS
anl YRAWQKWAK
anm YRAWQKWAK
aamy YRAWQKWA
afl YRKWQRWMYA
agn YRKWQKWMYA
bby YRKKTAQ
bce YRKKTAQ
ban YRKKTAQ
btk YRKKTAQ
bty YRKKMAQ
bwe YRKKMAQ
bmyc YRKKMAQ
bcy YRKKMAQ
far YQKLKPSLS
fpn YQRLKPSLS
sat YRRIHEKQDPVPPSDS
len LSHH
glj ASHSTQ
npu MAQIRYRKILA
bco YRRASGT
bse IHKK
axl LNRHIKTTDQIF
tap YNKMKEARTPVS
hhd YHKLKPGRPL
vig YYFFKQFIH
lao YHRMTKTRN
sje YHKLKA
tur FVK
say GRAP
tmr VRRWRGNAG
lpil RNPFGQVAEPQPPPAR
aac YRRMA
nth LRRVGVI
ctx LRLSGKF
clb LRFLNKF

**Fig. S4.** (6/6)

**Fig. S4.** Sequence alignment of BalTS homologue proteins. Several residues including the characteristic aspartates in the two motifs are indicated over the alignment. These panels were prepared using ESPript.[25] Abbreviations; dor: *Desulfosporosinus orientis*, dmi: *Desulfosporosinus meridiei*, dai: *Desulfosporosinus acidiphilus*, ddh: *Desulfitobacterium dehalogenans*, dsy: *Desulfitobacterium hafniense* Y51, ddl: *Desulfitobacterium dichloroeliminans*, puf: *Pelosinus* sp. UFO1, pft: *Pelosinus fermentans*, gfe: *Geosporobacter ferrireducens, Thermosediminibacter oceani*, mas: *Mahella australiensis*, tpz: *Thermacetogenium phaeum*, dau: *Candidatus Desulforudis audaxviator,* mta: *Moorella thermoacetica*, dku: *Desulfotomaculum kuznetsovii*, slp: *Syntrophothermus lipocalidus*, swo: *Syntrophomonas wolfei*, blr: *Brevibacillus laterosporus*, bbe: *Brevibacillus brevis*, pnp: *Paenibacillus naphthalenovorans*, pms: *Paenibacillus mucilaginosus* KNP414, plv: *Paenibacillus larvae*, pow: *Paenibacillus* sp. 32O-W, pbj: *Paenibacillus beijingensis*, pjd: *Paenibacillus* sp. JDR-2, pdu: *Paenibacillus durus*, psab: *Paenibacillus sabinae*, pste: *Paenibacillus stellifer*, paee: *Paenibacillus* sp. FSL R7-0331, paea: *Paenibacillus* sp. FSL R7-0273, paeq: *Paenibacillus* sp. FSL R5-0912, paen: *Paenibacillus* sp. FSL P4-0081, pbd: *Paenibacillus borealis*, paeh: *Paenibacillus* sp. FSL H7-0357, pgm: *Paenibacillus graminis*, pri: *Paenibacillus riograndensis*, paej: *Paenibacillus* sp. FSL H7-0737, paef: *Paenibacillus* sp. FSL R5-0345, pod: *Paenibacillus odorifer*, gym: *Paenibacillus* sp. Y412MC10, ppeo: *Paenibacillus peoriae*, ppy: *Paenibacillus polymyxa* E681, pta: *Paenibacillus terrae*, pih: *Paenibacillus* sp. IHBB 10380, pbv: *Paenibacillus bovis*, tco: *Thermobacillus composti*, anx: *Aneurinibacillus* sp. XH2, bpf: *Bacillus pseudofirmus*, bha: *Bacillus halodurans*, bcl: *Bacillus clausii*, ble: *Bacillus lehensis*, bmet: *Bacillus methanolicus*, gst: *Bacillus* sp. X1(2014), baci: *Bacillus* sp. 1NLA3E, bif: *Bacillus infantis*, bsm: *Bacillus smithii*, bck: *Bacillus coagulans* 2-6, jeo: *Jeotgalibacillus malaysiensis*, bmq: *Bacillus megaterium* QM B1551, beo: *Bacillus endophyticus*, bacy: *Bacillus* sp. YP1, bsus: *Bacillus subtilis* subsp. subtilis 168, balm: *Bacillus* sp. LM 4-2, bjs: *Bacillus* sp. JS, bacl: *Bacillus* sp. BS34A, bae: Bacillus atrophaeus 1942, bacp: *Bacillus* sp. Pc3, bacb: *Bacillus* sp. BH072, bay: *Bacillus* velezensis FZB42, bao: *Bacillus amyloliquefaciens* DSM 7, bli: *Bacillus licheniformis* ATCC 14580, blh: *Bacillus paralicheniformis*, bgy: *Bacillus glycinifermentans*, bacw: *Bacillus* sp. WP8, bpu: *Bacillus pumilus* SAFR-032, gth: *Parageobacillus thermoglucosidasius*, gmc: *Geobacillus* sp. Y4.1MC1, gwc: *Geobacillus* sp. WCH70, gtn: *Geobacillus thermodenitrificans*, gsr: *Geobacillus subterraneus*, gjf: *Geobacillus* genomosp. 3, gse: *Geobacillus stearothermophilus*, gct: *Geobacillus* sp. C56-T3, gya: *Geobacillus* sp. Y412MC52, gyc: *Geobacillus* sp. Y412MC61, gte: *Geobacillus thermoleovorans*, gka: *Geobacillus kaustophilus*, ggh: *Geobacillus* sp. GHH01, gej: *Geobacillus* sp. JS12, gel: *Geobacillus* sp. LC300, gea: *Geobacillus* sp. 12AMOR1, anl: *Anoxybacillus* sp. B7M1, anm: *Anoxybacillus* sp. B2M1, aamy: *Anoxybacillus amylolyticus*, afl: *Anoxybacillus flavithermus*, agn: *Anoxybacillus gonensis*, bby: *Bacillus bombysepticus*, bce: *Bacillus cereus* ATCC 14579, ban: *Bacillus anthracis* Ames, btk: *Bacillus thuringiensis* 97-27, bty: *Bacillus toyonensis*, bwe: *Bacillus weihenstephanensis* KBAB4, bmyc: *Bacillus mycoides* 219298, bcy: *Bacillus cytotoxicus*, far: *Fictibacillus arsenicus*, fpn: *Fictibacillus phosphorivorans*, sat: *Syntrophus aciditrophicus*, len: *Leptolyngbya* sp. NIES-3755, glj: *Gloeobacter kilaueensis*, npu: *Nostoc punctiforme*, bco: *Bacillus cellulosilyticus*, bse: *Bacillus selenitireducens*, axl: *Amphibacillus xylanus*, tap: *Terribacillus aidingensis*, hhd: *Halobacillus halophilus*, vig: *Virgibacillus* sp. 6R, lao: *Lentibacillus amyloliquefaciens*, sje: *Salimicrobium jeotgali*, tur: *Turicibacter* sp. H121, say: *Sulfobacillus acidophilus* TPY, tmr: *Thermaerobacter marianensis*, lpil: *Limnochorda pilosa*, aac: *Alicyclobacillus acidocaldarius* subsp. acidocaldarius DSM 446, nth: *Natranaerobius thermophilus*, ctx: *Ruminiclostridium thermocellum* DSM 1313, clb: *Clostridium* sp. BNL1100.

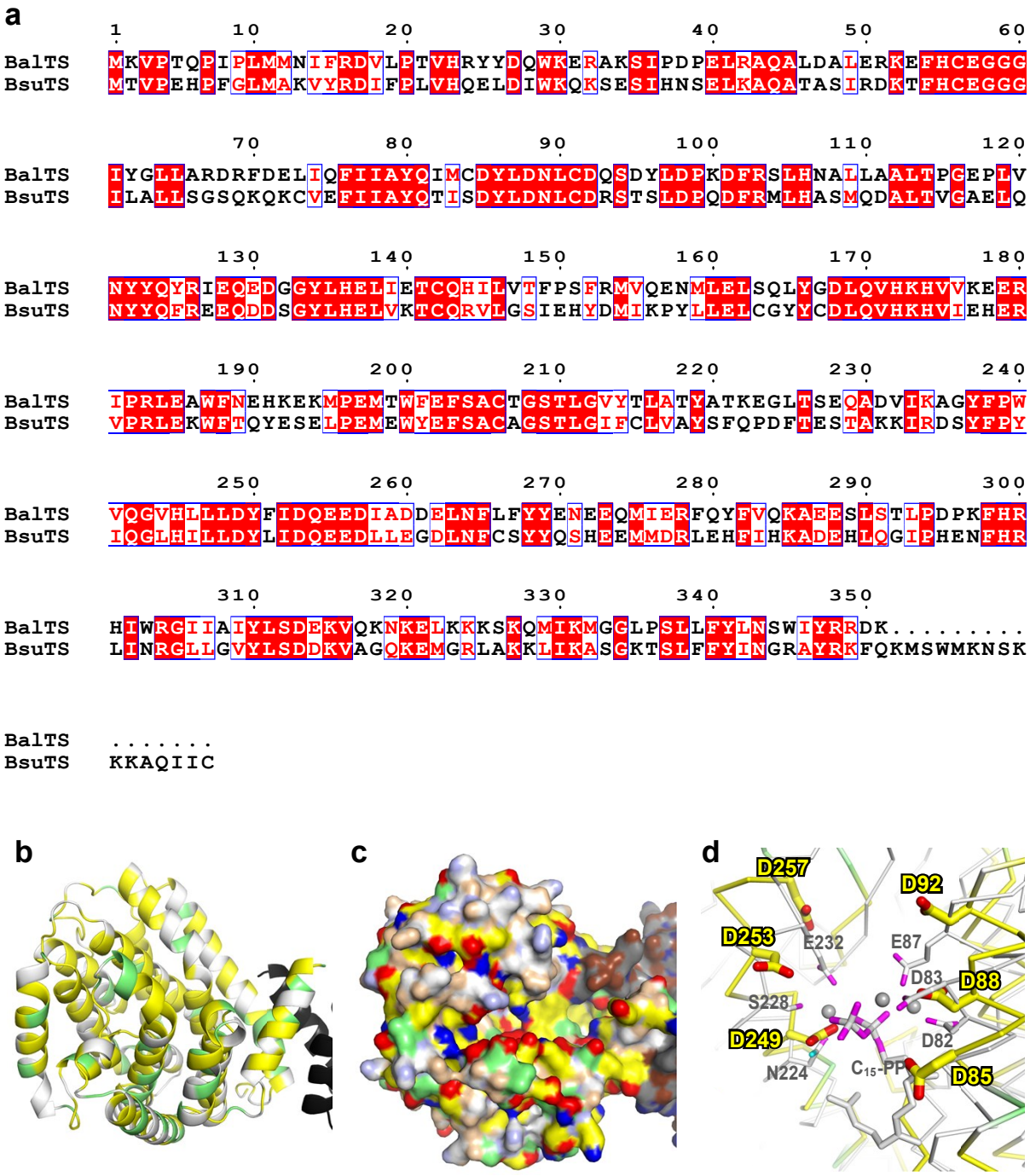



**Fig. S5.** Comparison between BalTS and BsuTS. (a) Primary sequence alignment of the two proteins. This panel was prepared using ESPript.[25] Distribution of the conserved residues on BalTS is shown in Movie S3. (b,c) Distribution on conserved residues on BsuTS structure shown by Cartoon (b) and Surface (c) models. The BsuTS structure was constructed by homology modelling using swiss-model server.[20] Yellow and green residues are identical and similar residues as presented in panel (a). Dark gray molecule is the other molecule of the dimer. Note that no information is available regarding the oligomeric state of BsuTS. (d) Assumption of the ligand binding on BsuTS. The model is constructed as Fig. 3d with the homology modelling structure of BsuTS. Selinadiene synthase from *S. pristinaespiralis* bound with the ligand-analogue molecule (PDB: 4OKM, white)[7] is superposed on the BsuTS structure. NSE/DTE and DDxx(D,E) motifs on selinadiene synthase, and the six characteristic aspartates of BsuTS are represented. Gray spheres represent the $Mg^{2+}$ cation found between the pyrophosphate part of the ligand-analogue and selinadiene synthase.

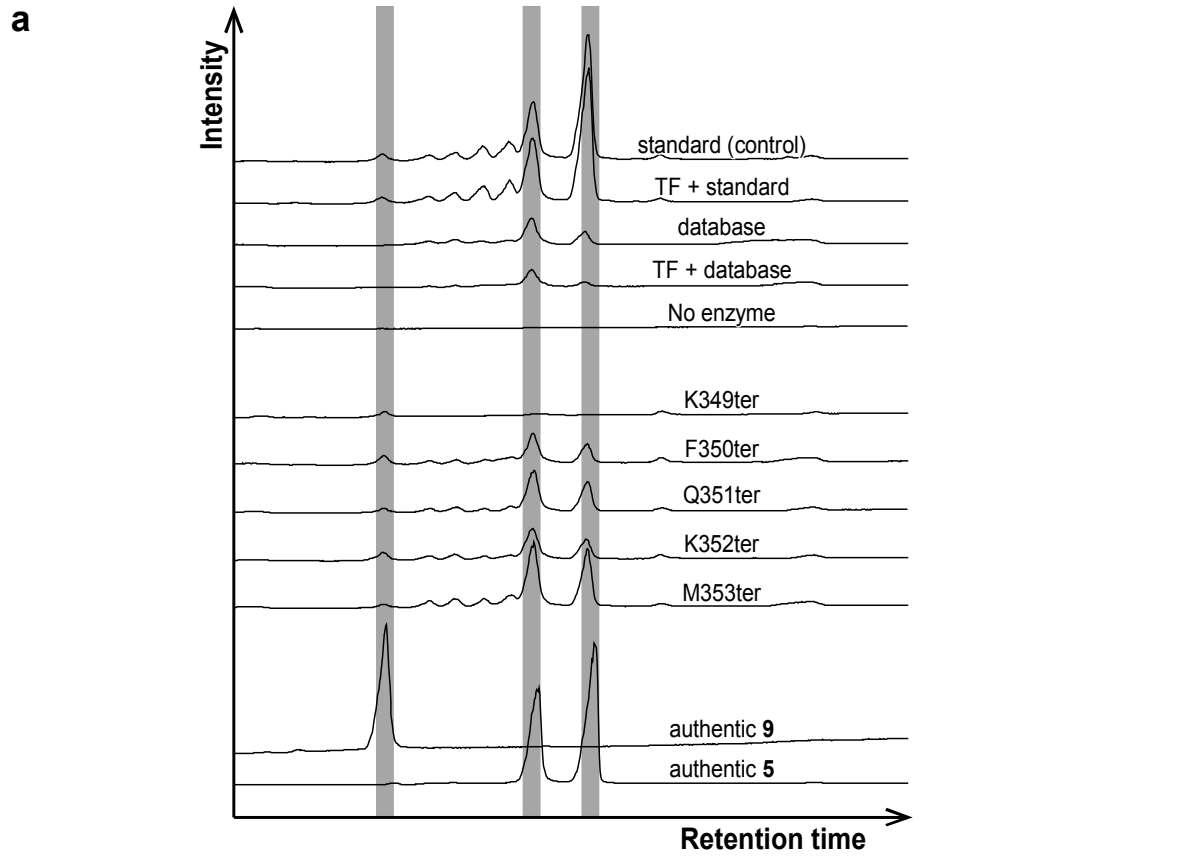



**Fig. S6.** Product analyses of BsuTS variants. (a) GC-MS analyses of products. The vertical gray bars represent the retention times for the authentic **5** and **9**. (b) Schematic drawing of primary structures of recombinant BsuTS proteins used for this analyses. TF is Trigger Factor tag and "TF+standard" is the recombinant used for our previous work.[19] Tag1 is composed of translation enhancing element (TEE), $His_6$-tag and thrombin digestion site.

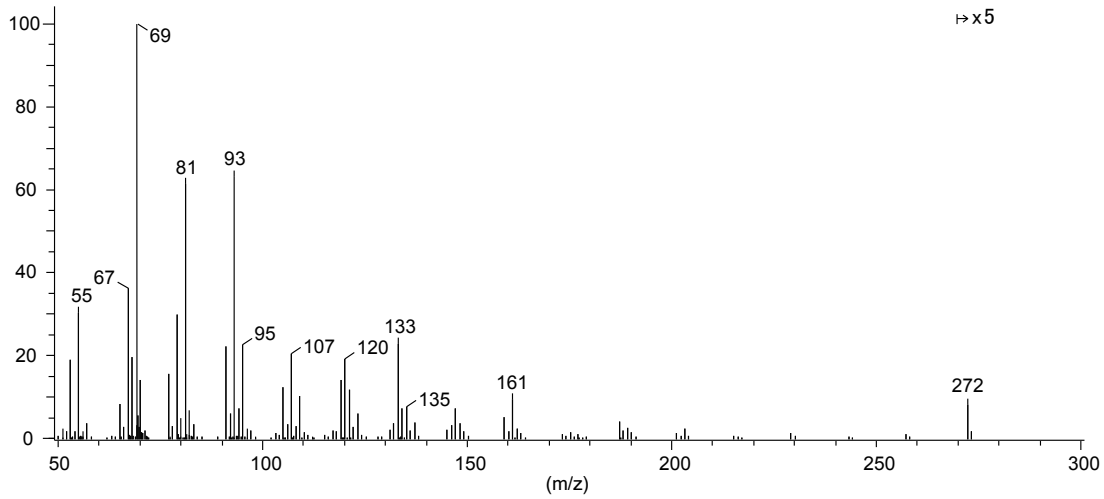
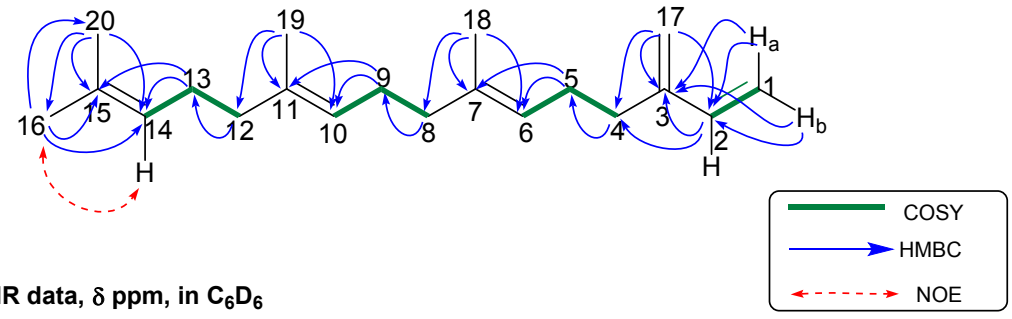



**Fig. S7.** Mass spectrum (EI) of **6**.




**NMR data, δ ppm, in $C_6D_6$**

| NO. | $^1$H | $^{13}$C | NO. | $^1$H | $^{13}$C | NO. | $^1$H | $^{13}$C |
|---|---|---|---|---|---|---|---|---|
| 1 | Ha: 5.34 (1H, d, 17.6)<br>Hb: 5.10 (1H, d, 12.4) | 113.09 (t) | 9 | 2.22 (m) | 27.06 (t) [a] | 17 | 5.12 (2H, s) | 115.97 (t) |
| 2 | 6.49 (1H, dd, 17.6, 10.8) | 139.42 (d) | 10 | 5.40 (m) | 124.78 (d) [b] | 18 | 1.69 (3H, s) | 16.10 (q) |
| 3 | —— | 146.44 (s) | 11 | —— | 134.99 (s) | 19 | 1.72 (3H, s) | 16.10 (q) |
| 4 | 2.38 (m) | 31.85 (t) | 12 | 2.30 (m); 2.22 (m) | 40.20 (t) [c] | 20 | 1.69 (3H, s) | 17.72 (q) |
| 5 | 2.37 (m) | 25.82 (t) [a] | 13 | 2.30 (m) | 27.24 (t) [a] | | | |
| 6 | 5.40 (m) | 124.06 (d) [b] | 14 | 5.40 (m) | 124.94 (d) [b] | | | |
| 7 | —— | 135.38 (s) | 15 | —— | 131.10 (s) | | | |
| 8 | 2.30 (m); 2.22 (m) | 40.13 (t) [c] | 16 | 1.80 (m) | 25.82 (q) | | | |

[a - c] The assignments may be exchangeable.

**Fig. S8.** NMR assignment of **6** measured in $C_6D$.

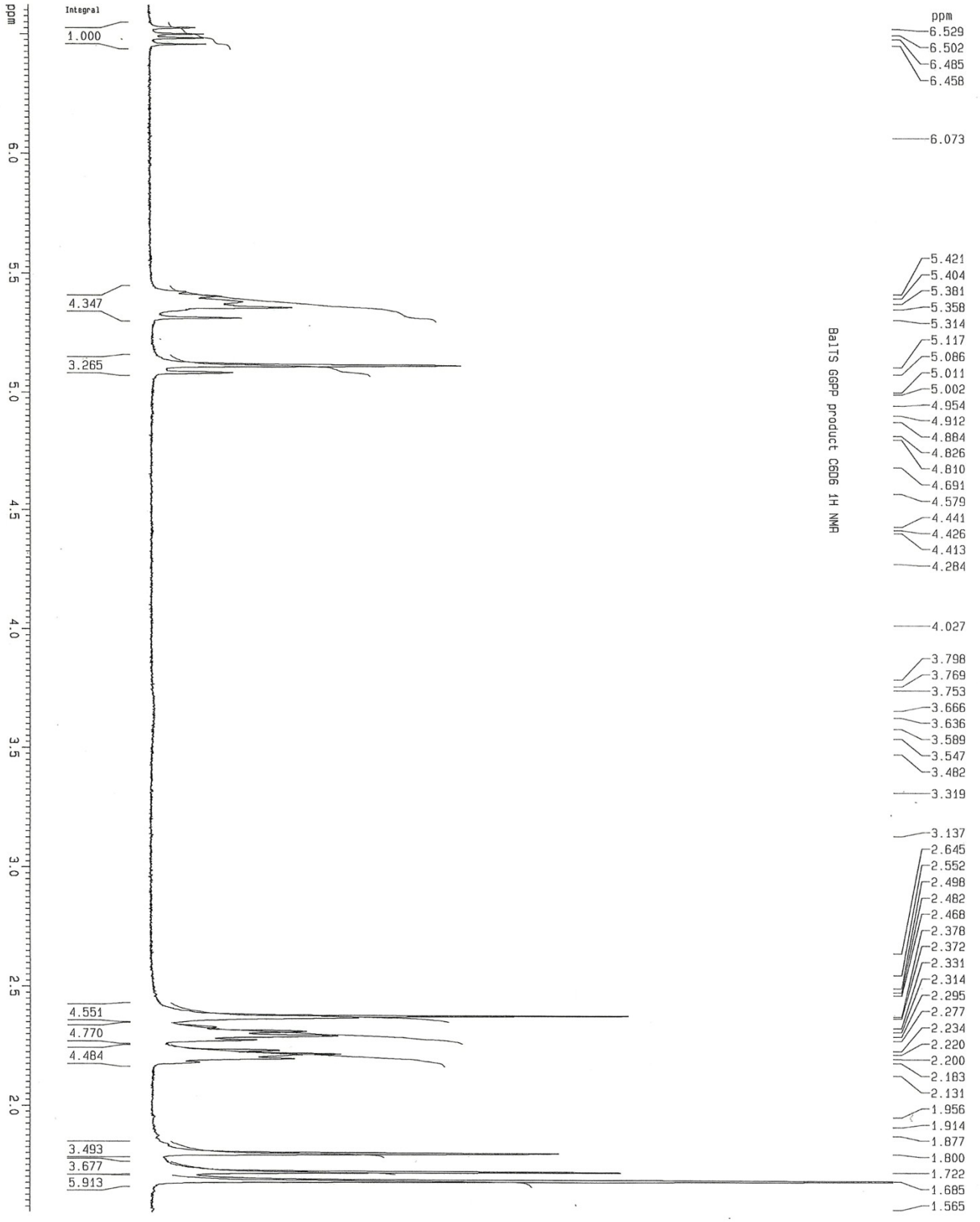



**Fig. S9.** $^{1}$H NMR spectrum of **6** measured in $C_6D_6$.

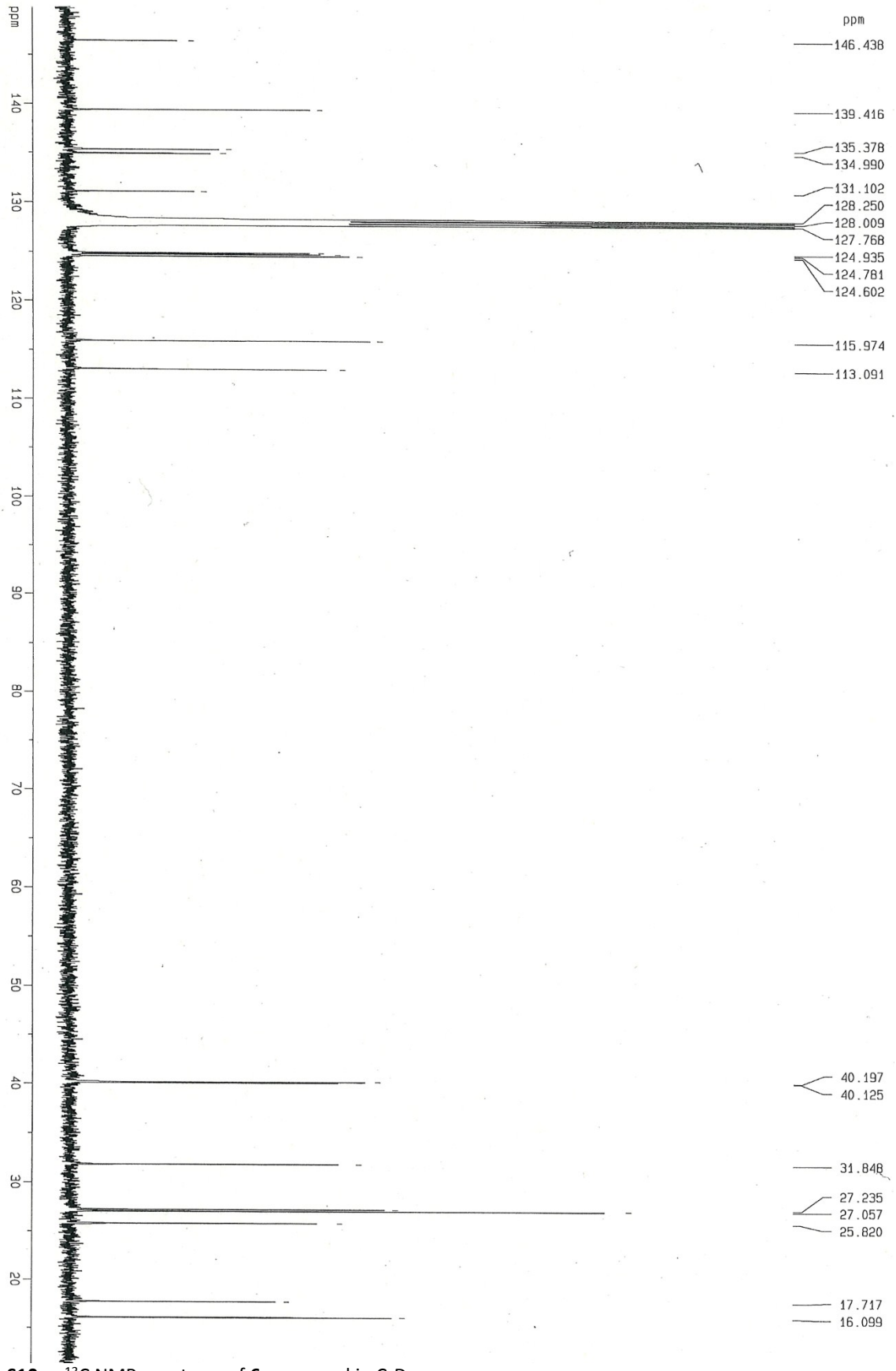



**Fig. S10.** $^{13}C$ NMR spectrum of **6** measured in $C_6D_6$.

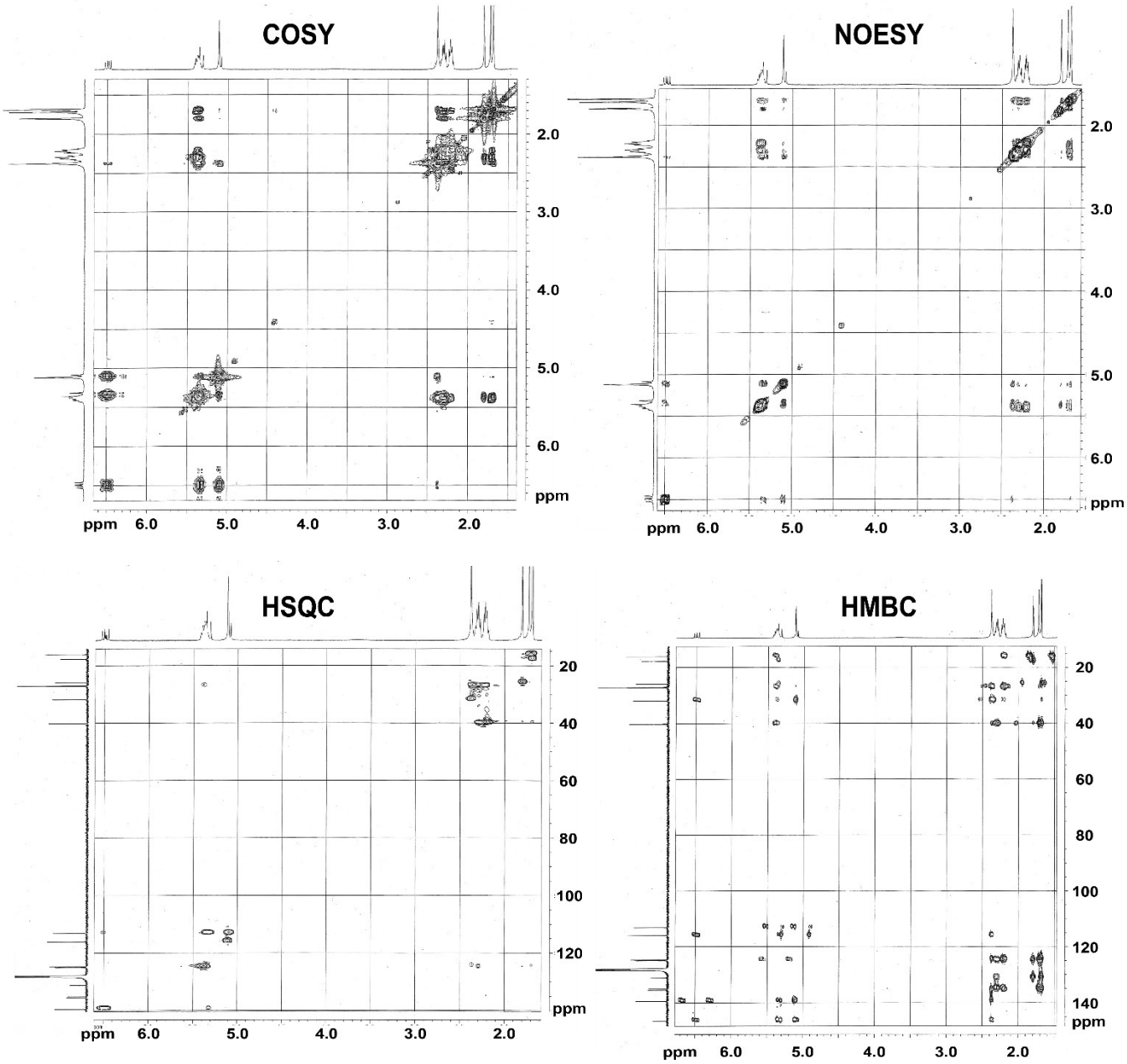



**Fig. S11.** COSY, NOESY, HSQC and HMBC spectrums of **6** measured in $C_6D_6$.

**Table S1.** Crystallographic statistics of BalTS.

| | | Se-Met | Native |
|---|---|---|---|
| **Data statistics** | | | |
| Space group | | $P2_1$ | $P2_1$ |
| Cell constant | *a*/*b*/*c* (Å) | 53.08 / 151.51 / 53.81 | 53.35, 151.67, 53.84 |
| | β(°) | 110.3 | 110.0 |
| Resolution[a] | | 50-1.85 (1.92-1.85) | 50-1.64 (1.68-1.64) |
| # Reflections observed[a] | | 1,025,009 (107,023) | 300,403 (19,390) |
| # Reflections uniq[a,b] | | 134,161 (14,104) | 92,698 (6,015) |
| # Anomalous pairs[a] | | 66,437 (6,003) | n/a[c] |
| Completeness[a] | | 99.7% (99.7%) | 94.4% (87.7%) |
| $R_{merge}$[a] | | 6.6% (114.6%) | 5.8% (67.8%) |
| $I/\sigma(I)$ [a] | | 21.6 (2.0) | 11.3 (2.0) |
| $CC_{1/2}$[a] | | 99.9 (75.5) | 99.6 (69.1) |
| **Refinement statistics** | | | |
| $R$ / $R_{free}$ | | | 0.204 / 0.229 |
| RMS bond (Å) /angle (º) | | | 0.0067 / 1.1187 |
| Ramachandran favored/allowed/outlier | | | 98.3% / 1.7% / 0% |
| Rotamers favored/allowed/outlier | | | 98.1% / 1.8% / 0.16% |
| PDB ID | | | 5YO8 |

[a] Values in parenthesis are those for the highest resolution shells. [b] Reflections of anomalous pairs are independently and non-independently counted for Se-Met and Native datasets, respectively. [c] not applicable

**Table S2** (1/3)**.** Sequence identities between BalTS and its homologue proteins.

| code[a] | strain | #ident.[b] | #res.[c] | %ident.[d] |
|---|---|---|---|---|
| dor | *Desulfosporosinus orientis* | 140 | 356 | 39.3% |
| dmi | *Desulfosporosinus meridiei* | 138 | 356 | 38.8% |
| dai | *Desulfosporosinus acidiphilus* | 138 | 356 | 38.8% |
| ddh | *Desulfitobacterium dehalogenans* | 136 | 354 | 38.4% |
| dsy | *Desulfitobacterium hafniense* Y51 | 132 | 354 | 37.3% |
| ddl | *Desulfitobacterium dichloroeliminans* | 129 | 354 | 36.4% |
| puf | *Pelosinus* sp. UFO1 | 134 | 355 | 37.7% |
| pft | *Pelosinus fermentans* | 130 | 353 | 36.8% |
| gfe | *Geosporobacter ferrireducens* | 127 | 348 | 36.5% |
| toc | *Thermosediminibacter oceani* | 127 | 352 | 36.1% |
| mas | *Mahella australiensis* | 124 | 387 | 32.0% |
| tpz | *Thermacetogenium phaeum* | 141 | 361 | 39.1% |
| dau | *Candidatus Desulforudis audaxviator* | 129 | 361 | 35.7% |
| mta | *Moorella thermoacetica* | 130 | 354 | 36.7% |
| dku | *Desulfotomaculum kuznetsovii* | 124 | 365 | 34.0% |
| slp | *Syntrophothermus lipocalidus* | 134 | 352 | 38.1% |
| swo | *Syntrophomonas wolfei* | 126 | 370 | 34.1% |
| blr | *Brevibacillus laterosporus* | 147 | 356 | 41.3% |
| bbe | *Brevibacillus brevis* | 140 | 351 | 39.9% |
| pnp | *Paenibacillus naphthalenovorans* | 149 | 341 | 43.7% |
| pms | *Paenibacillus mucilaginosus* KNP414 | 145 | 343 | 42.3% |
| plv | *Paenibacillus larvae* | 153 | 359 | 42.6% |
| pow | *Paenibacillus* sp. 32O-W | 153 | 361 | 42.4% |
| pbj | *Paenibacillus beijingensis* | 150 | 365 | 41.1% |
| pjd | *Paenibacillus* sp. JDR-2 | 149 | 360 | 41.4% |
| pdu | *Paenibacillus durus* | 147 | 360 | 40.8% |
| psab | *Paenibacillus sabinae* | 143 | 360 | 39.7% |
| pste | *Paenibacillus stellifer* | 147 | 360 | 40.8% |
| paee | *Paenibacillus sp.* FSL R7-0331 | 153 | 360 | 42.5% |
| paea | *Paenibacillus sp.* FSL R7-0273 | 152 | 360 | 42.2% |
| paeq | *Paenibacillus sp.* FSL R5-0912 | 147 | 360 | 40.8% |
| paen | *Paenibacillus sp.* FSL P4-0081 | 147 | 360 | 40.8% |
| pbd | *Paenibacillus borealis* | 148 | 360 | 41.1% |
| paeh | *Paenibacillus* sp. FSL H7-0357 | 148 | 360 | 41.1% |
| pgm | *Paenibacillus graminis* | 147 | 360 | 40.8% |
| pri | *Paenibacillus riograndensis* | 146 | 365 | 40.0% |
| paej | *Paenibacillus* sp. FSL H7-0737 | 148 | 360 | 41.1% |
| paef | *Paenibacillus* sp. FSL R5-0345 | 147 | 360 | 40.8% |
| pod | *Paenibacillus odorifer* | 150 | 360 | 41.7% |
| gym | *Paenibacillus* sp. Y412MC10 | 141 | 360 | 39.2% |
| ppeo | *Paenibacillus peoriae* | 151 | 359 | 42.1% |
| ppy | *Paenibacillus polymyxa E681* | 151 | 359 | 42.1% |
| pta | *Paenibacillus terrae* | 151 | 359 | 42.1% |
| pih | *Paenibacillus* sp. IHBB 10380 | 145 | 361 | 40.2% |
| pbv | *Paenibacillus bovis* | 146 | 362 | 40.3% |

**Table S2** (2/3)**.** Sequence identities between BalTS and its homologue proteins.

| code[a] | strain | #ident.[b] | #res.[c] | %ident.[d] |
|---|---|---|---|---|
| tco | *Thermobacillus composti* | 143 | 363 | 39.4% |
| anx | *Aneurinibacillus* sp. XH2 | 146 | 370 | 39.5% |
| bpf | *Bacillus pseudofirmus* | 270 | 350 | 77.1% |
| bha | *Bacillus halodurans* | 220 | 360 | 61.1% |
| bcl | *Bacillus clausii* | 168 | 352 | 47.7% |
| ble | *Bacillus lehensis* | 156 | 352 | 44.3% |
| bmet | *Bacillus methanolicus* | 180 | 360 | 50.0% |
| gst | *Bacillus* sp. X1(2014) | 169 | 361 | 46.8% |
| baci | *Bacillus* sp. 1NLA3E | 176 | 379 | 46.4% |
| bif | *Bacillus infantis* | 169 | 363 | 46.6% |
| bsm | *Bacillus smithii* | 178 | 356 | 50.0% |
| bck | *Bacillus coagulans* 2-6 | 175 | 354 | 49.4% |
| jeo | *Jeotgalibacillus malaysiensis* | 166 | 363 | 45.7% |
| bmq | *Bacillus megaterium* QM B1551 | 180 | 352 | 51.1% |
| beo | *Bacillus endophyticus* | 176 | 356 | 49.4% |
| bacy | *Bacillus* sp. YP1 | 173 | 370 | 46.8% |
| bsus | *Bacillus subtilis* subsp. *subtilis* 168 | 173 | 370 | 46.8% |
| balm | *Bacillus* sp. LM 4-2 | 172 | 371 | 46.4% |
| bjs | *Bacillus* sp. JS | 166 | 360 | 46.1% |
| bacl | *Bacillus* sp. BS34A | 172 | 371 | 46.4% |
| bae | *Bacillus atrophaeus* 1942 | 171 | 359 | 47.6% |
| bacp | *Bacillus* sp. Pc3 | 169 | 359 | 47.1% |
| bacb | *Bacillus* sp. BH072 | 168 | 359 | 46.8% |
| bay | *Bacillus velezensis* FZB42 | 168 | 360 | 46.7% |
| bao | *Bacillus amyloliquefaciens* DSM 7 | 168 | 359 | 46.8% |
| bli | *Bacillus licheniformis* ATCC 14580 | 170 | 359 | 47.4% |
| blh | *Bacillus paralicheniformis* | 169 | 359 | 47.1% |
| bgy | *Bacillus glycinifermentans* | 170 | 359 | 47.4% |
| bacw | *Bacillus* sp. WP8 | 170 | 359 | 47.4% |
| bpu | *Bacillus pumilus SAFR-032* | 170 | 359 | 47.4% |
| gth | *Parageobacillus thermoglucosidasius* | 183 | 359 | 51.0% |
| gmc | *Geobacillus* sp. Y4.1MC1 | 183 | 359 | 51.0% |
| gwc | *Geobacillus* sp. WCH70 | 180 | 359 | 50.1% |
| gtn | *Geobacillus thermodenitrificans* | 181 | 360 | 50.3% |
| gsr | *Geobacillus subterraneus* | 177 | 360 | 49.2% |
| gjf | *Geobacillus genomosp.* 3 | 173 | 372 | 46.5% |
| gse | *Geobacillus stearothermophilus* | 175 | 369 | 47.4% |
| gct | *Geobacillus* sp. C56-T3 | 175 | 369 | 47.4% |
| gya | *Geobacillus* sp. Y412MC52 | 175 | 369 | 47.4% |
| gyc | *Geobacillus* sp. Y412MC61 | 175 | 369 | 47.4% |
| gte | *Geobacillus thermoleovorans* | 175 | 369 | 47.4% |
| gka | *Geobacillus kaustophilus* | 175 | 449 | 39.0% |
| ggh | *Geobacillus* sp. GHH01 | 174 | 369 | 47.2% |
| gej | *Geobacillus* sp. JS12 | 175 | 360 | 48.6% |

**Table S2** (3/3)**.** Sequence identities between BalTS and its homologue proteins.

| code[a] | strain | #ident.[b] | #res.[c] | %ident.[d] |
|---|---|---|---|---|
| gej | *Geobacillus* sp. JS12 | 175 | 360 | 48.6% |
| gel | *Geobacillus* sp. LC300 | 176 | 369 | 47.7% |
| gea | *Geobacillus* sp. 12AMOR1 | 178 | 360 | 49.4% |
| anl | *Anoxybacillus* sp. B7M1 | 171 | 358 | 47.8% |
| anm | *Anoxybacillus* sp. B2M1 | 171 | 358 | 47.8% |
| aamy | *Anoxybacillus amylolyticus* | 176 | 355 | 49.6% |
| afl | *Anoxybacillus flavithermus* | 175 | 359 | 48.7% |
| agn | *Anoxybacillus gonensis* | 175 | 356 | 49.2% |
| bby | *Bacillus bombysepticus* | 185 | 354 | 52.3% |
| bce | *Bacillus cereus* ATCC 14579 | 185 | 354 | 52.3% |
| ban | *Bacillus anthracis* Ames | 185 | 354 | 52.3% |
| btk | *Bacillus thuringiensis* 97-27 | 184 | 358 | 51.4% |
| bty | *Bacillus toyonensis* | 179 | 344 | 52.0% |
| bwe | *Bacillus weihenstephanensis* KBAB4 | 184 | 354 | 52.0% |
| bmyc | *Bacillus mycoides* 219298 | 183 | 353 | 51.8% |
| bcy | *Bacillus cytotoxicus* | 186 | 354 | 52.5% |
| far | *Fictibacillus arsenicus* | 177 | 356 | 49.7% |
| fpn | *Fictibacillus phosphorivorans* | 169 | 356 | 47.5% |
| sat | *Syntrophus aciditrophicus* | 175 | 362 | 48.3% |
| len | *Leptolyngbya* sp. NIES-3755 | 169 | 349 | 48.4% |
| glj | *Gloeobacter kilaueensis* | 160 | 351 | 45.6% |
| npu | *Nostoc punctiforme* | 175 | 381 | 45.9% |
| bco | *Bacillus cellulosilyticus* | 190 | 353 | 53.8% |
| bse | *Bacillus selenitireducens* | 168 | 352 | 47.7% |
| axl | *Amphibacillus xylanus* | 150 | 361 | 41.6% |
| tap | *Terribacillus aidingensis* | 170 | 360 | 47.2% |
| hhd | *Halobacillus halophilus* | 165 | 358 | 46.1% |
| vig | *Virgibacillus* sp. 6R | 153 | 356 | 43.0% |
| lao | *Lentibacillus amyloliquefaciens* | 150 | 343 | 43.7% |
| sje | *Salimicrobium jeotgali* | 158 | 342 | 46.2% |
| tur | *Turicibacter* sp. H121 | 127 | 345 | 36.8% |
| say | *Sulfobacillus acidophilus* TPY | 131 | 349 | 37.5% |
| tmr | *Thermaerobacter marianensis* | 134 | 496 | 27.0% |
| lpil | *Limnochorda pilosa* | 111 | 365 | 30.4% |
| aac | *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* DSM 446 | 125 | 351 | 35.6% |
| nth | *Natranaerobius thermophilus* | 113 | 354 | 31.9% |
| ctx | *Ruminiclostridium thermocellum* DSM 1313 | 85 | 347 | 24.5% |
| clb | *Clostridium* sp. BNL1100 | 80 | 352 | 22.7% |

[a] Strain codes identical with Fig. S4. [b] Number of identical residues with BalTS. [c] Number of residues. [d] Identity with BalTS calculated by [b]/[c].

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