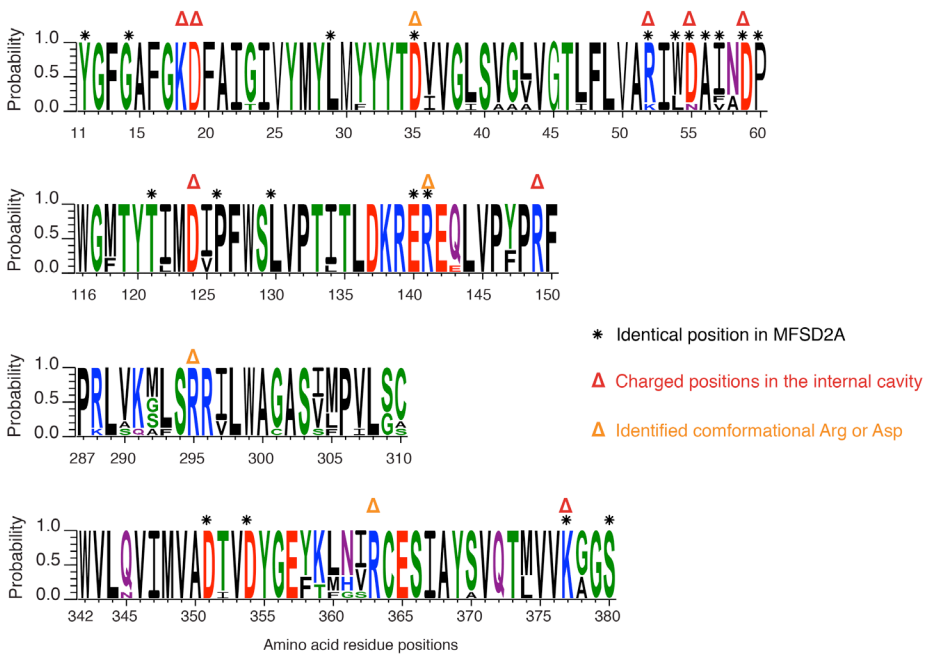
a

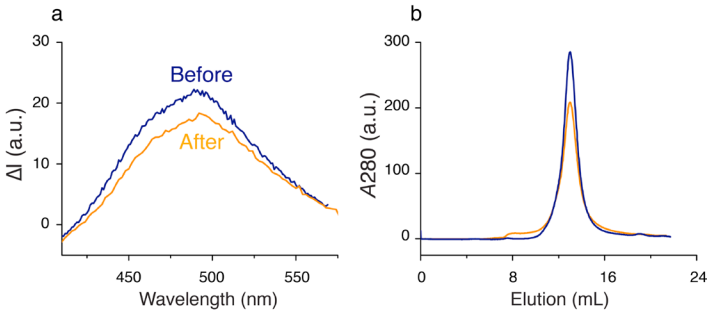


b

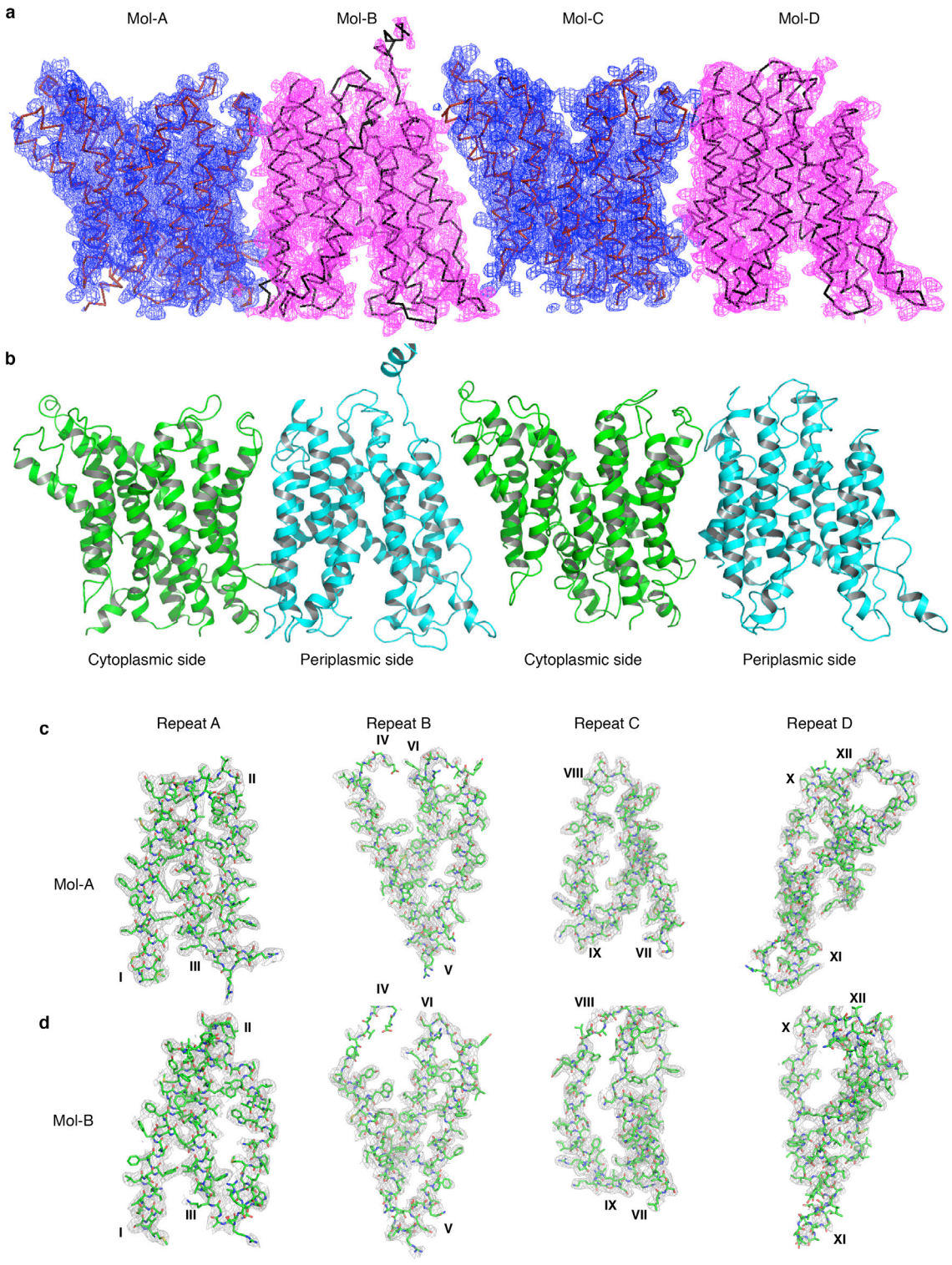
	Cation-binding site	L-2
MelB_*Salmonella typhimurium*	48-FLVARIW**D**AIN**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPFPR-149
MelB_*Klebsiella pneumoniae*	48-FLVARIL**D**AIA**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**Q**LVPYPRFF-149
MelB_*Enterobacter aerogenes*	48-FLVARIL**D**AIA**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPYPR-149
MelB_*E.coli*	48-FLVARIW**D**AIN**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPYPR-149
MelB_*Shigella flexneri*	44-FLVARIW**D**AIN**D**P-56	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPYPR-145
MelB_*Salmonella typhi*	48-FLVARIW**D**AIN**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPFPR-149
MelB_*Salmonella paratyphi*	48-FLVARIW**D**AIN**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPFPR-149
MelB_*Citrobacter freundii*	48-FLVAKIW**N**AFN**D**P-60	120-YTIM**D**IPFWSLVPTITLDKRE**R**EQLVPYPR-148
MelB_*Vibrio shilonii*	48-FLVARIW**D**AVN**D**P-60	120-YTLM**D**IPFWSLVPTLTLDKRE**R**EELVPYPR-149
LacS_*Streptococcus thermophilus*	60-ISILRIL**E**VFI**D**P-72	135-YSIK**D**IGFWSMLPALSLDSHE**R**EKMATFAR-164
GalP_*Lactocaccus lactis*	55-VVIIRLV**E**IIF**D**P-68	133-YSFK**D**IAFWSMIPALSEKNSE**R**ETLGTFAR-162
XylP-*Lactobacillus pentosus*	72-FLVARIV**D**AF**D**GP-84	144-YSAV**N**IPITSILPSLTSNPQE**R**VTLSTIRQ-173
GusB_*E.coli*	50-LLLVRVF**D**AFA**D**V-62	123-YSLV**N**IPYGSLATAMTQQPQS**R**ARLGAARG-152
TogT_*Klbesiella pneumoniae*	45-FSVASII**D**AIS**N**P-57	116-YTSIMVPYETLATEMTDDFSI**R**SKLTGYKA-145
Sucrose transporter 1_*Zea mays*	76-WLCGPIAGLVV**Q**P-88	161-DFSN**N**TVQGPARAMMADLCGH**H**GPSAANSI-190
MFSD2A_*Homosapiens*	99-LFVGRAW**D**AIT**D**P-111	171-VTCF**H**VPYSALTMFISTEQTE**R**DSATAYRM-200
MFSD2B_*Homosapiens*	84-LFGGKVSGAAA**D**P-96	156-ATFF**Q**VPYTALTMLLTPCPRE**R**DSATAYRM-185

	L-1	L-2	L-3
MelB_*Salmonella typhimurium*	287-PRLVKMLS**R**RI-297	351-**D**TV**D**YGEFKLNI**R**CE-365	
MelB_*Klebsiella pneumoniae*	287-PRLVKGLS**R**RI-297	351-**D**TV**D**YGEYTMNI**R**CE-365	
MelB_*Enterobacter aerogenes*	287-PRLVKGLS**R**RI-297	351-**D**TV**D**YGEYTMNI**R**CE-365	
MelB_*E.coli*	287-PRLVKSLS**R**RI-297	351-**D**IV**D**YGEYKLHV**R**CE-365	
MelB_*Shigella flexneri*	283-PRLVKSLS**R**RI-293	347-**D**TV**D**YGEYKLHV**R**CE-361	
MelB_*Salmonella typhi*	287-PRLVKMLS**R**RI-297	351-**D**TV**D**YGEFKLNI**R**CE-365	
MelB_*Salmonella paratyphi*	287-PRLVKMLS**R**RI-297	351-**D**TV**D**YGEFKLNI**R**CE-365	
MelB_*Citrobacter freundii*	286-PRLSKALS**R**RV-296	350-**D**TV**D**YGEYKFNV**R**CE-364	
MelB_*Vibrio shilonii*	287-PKLAQMFS**R**RV-297	351-**D**TV**D**YGEYKLGS**R**CE-365	
LacS_*Streptococcus thermophilus*	305-PSLAGKFN**R**KR-315	365-**D**SV**E**YGQWKTGH**R**DE-379	
GalP_*Lactocaccus lactis*	300-PTLAKKFG**R**RK-310	359-**D**SV**E**YGQWKNGV**R**NE-373	
XylP_*Lactobacillus pentosus*	308-PWTAKRIG**K**RN-318	370-**D**SV**D**YGEWKNGV**R**AE-384	
GusB_*E.coli*	281-APLVPGMVA**R**I-291	346-**D**TV**E**YGEYLTGV**R**IE-360	
TogT_*Klbesiella pneumoniae*	289-GLCVKKGFS**K**P-299	358-**D**VD**E**IYTGRR**E**EGIY-372	
Sucrose Transporter 1_*Zea mays*	332-QISAFDEGV**R**V-342	398-**D**YHGYVQDAITASTS-412	
MFSD2A_*Homosapiens*	361-QWFLTRFG**K**KT-371	421-**D**VI**D**DFHLKQP**E**FHG-435	
MFSD2B_*Homosapiens*	337-EWVLQRFG**K**KT-346	395-**D**VV**D**DFQLQH**R**HGPG-409	

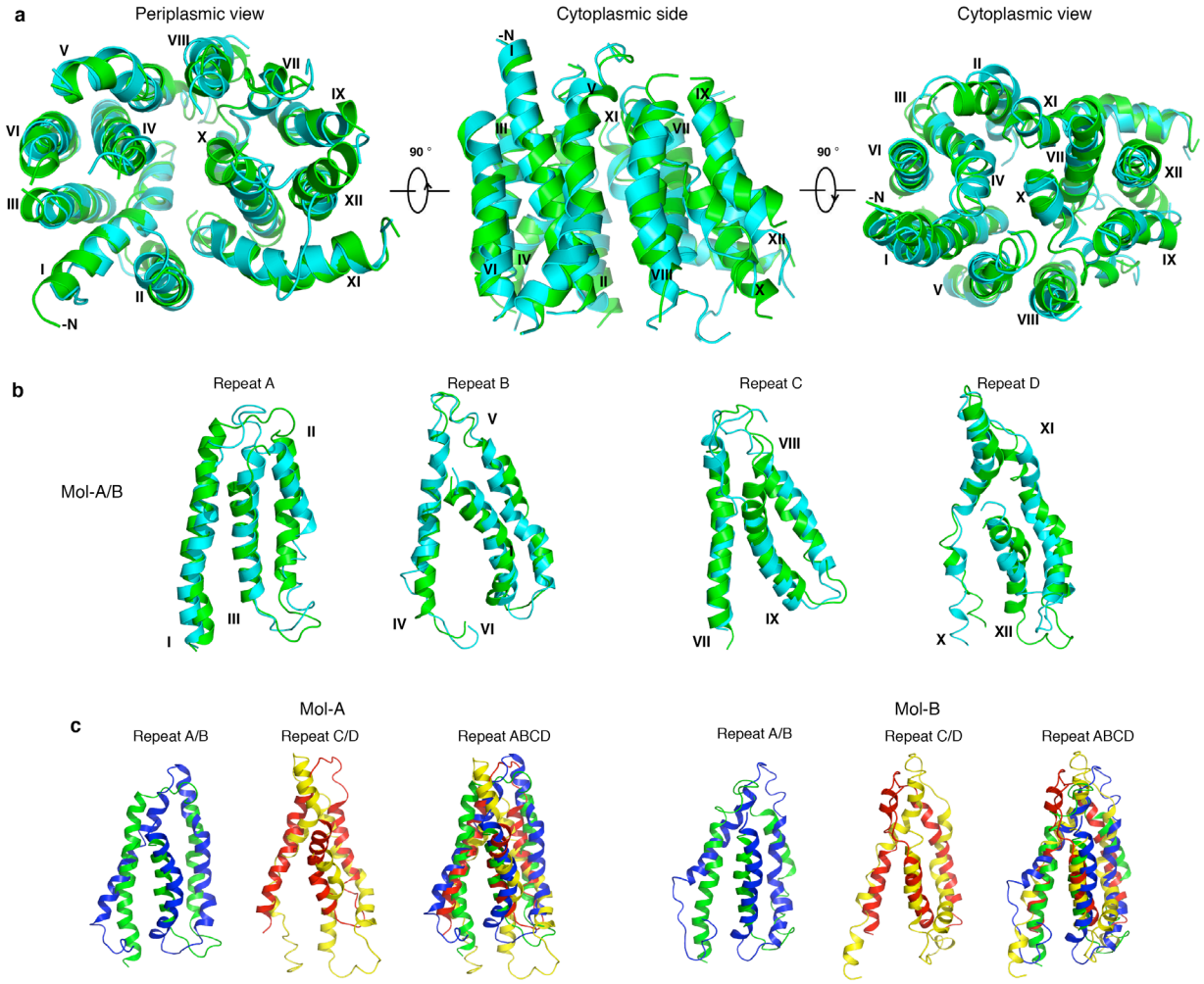
Supplementary Figure S1 | Sequence alignment. **a**, The sequence logos generated by the Weblogo for MelB orthologues with MFSD2A. Four stretches (11-60, 116-150, 287-310, 342-360) of primary-sequence alignments of $MelB_{St}$ (Gene ID: 16767549) with other MelB orthologues from *Klebsiella pneumoniae,* GI:150957831; *Enterobacter aerogenes,* GI:3914018; *E. coli,* GI:353526256; *Salmonella typhi,* GI:20141536; *Shigella flexneri,* GI:81723953; *Salmonella paratyphi*, GI:81360816; *Citrobacter freundii,* GI:75416057; *Vibrio shilonii,* Uniprot:A6CV97; MFSD2A, GI: 74751132 (Weblogos3.0). **b**, MelB with other GPH members. $MelB_{St}$ (GI: 16767549) orthologues include *Klebsiella pneumoniae,* GI:150957831; *Enterobacter aerogenes,* GI:3914018; *E. coli,* GI:353526256; *Shigella flexneri,* GI:81723953; *Salmonella typhi,* GI:20141536; *Salmonella paratyphi*, GI:81360816; *Citrobacter freundii,* GI:75416057; *and Vibrio shilonii,* Uniprot:A6CV97. MelB homologues of GPH family include LacS of *Streptococcus thermophiles,* GI:386345083; GalP of *Lactococcus lactis,* GI:4995688; XylP of *Lactobacillus pentosus,* GI:3688059; GusB of *E. coli*, GI:292630909; Oligogalacturonide transporter (TogT) of *Klebsiella pneumonia,* GI:206580788; Sucrose transporter 1 of *Zea mays*, GI:162463612; human MFSD2A, GI:74751132; and human MFSD2B, GI:298286913. Paired alignment of four stretches of primary sequences between $MelB_{St}$ and each protein were done manually. The three Asp residues (55, 59 and 124) important for Na^+ binding and three Arg (295, 141 and 363) and two Asp residues (351 and 354) important for lock interactions in $MelB_{St}$ are highlighted.



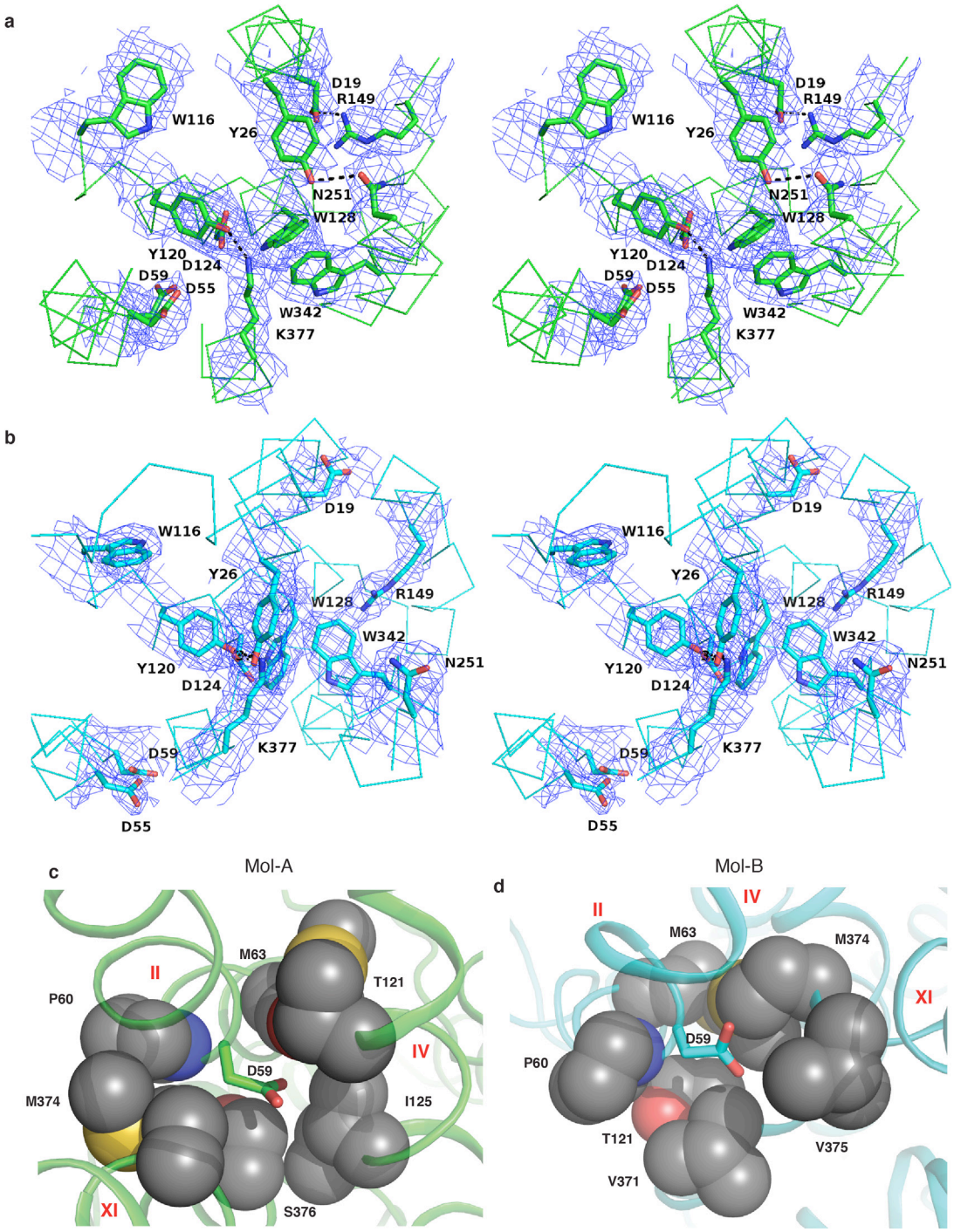
Supplementary Figure S2 | Stability of $MelB_{St}$. Purified $MelB_{St}$ proteins were incubated at 23°C for five days, and the samples before (blue) and after (orange) incubation were tested for stability. **a**, D^2G binding. D^2G FRET emission spectra (ΔI) obtained by displacement of D^2G with 50 mM melibiose. **b**, Gel filtration chromatography profiles (100 μg $MelB_{St}$).


a
Mol-A
Mol-B
Mol-C
Mol-D
b
Cytoplasmic side
Periplasmic side
Cytoplasmic side
Periplasmic side
c
Repeat A
Repeat B
Repeat C
Repeat D
Mol-A
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
d
Mol-B
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

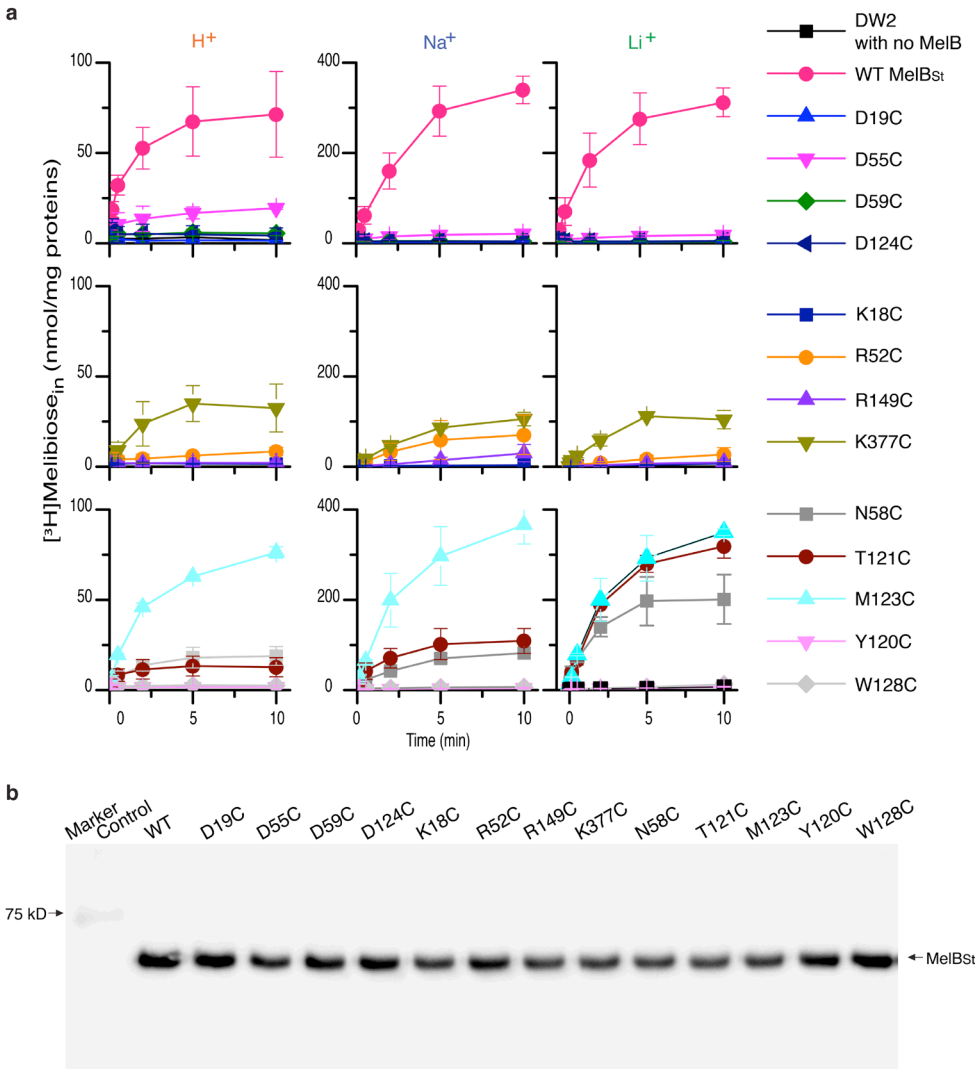
Supplementary Figure S3 | Electron density maps of $MelB_{St}$. **a**, The 2Fo-Fc maps of four molecules of $MelB_{st}$ contoured at 1.0 σ. Mol-A and Mol-C (blue mesh) and Mol-B and Mol-D (pink mesh) are related by 0.5 pseudo-translational symmetry along the *c* axis. **b**, The overall fold of Mol-A and Mol-C (green), Mol-B and Mol-D (cyan) in the asymmetric unit are shown as cartoon models. The N-terminal domain is on the left side of each molecule. **c and d,** The 2Fo-Fc electron density maps contoured at 1.0 σ for helices in each repeats. The helices are oriented for clear views of the density maps.



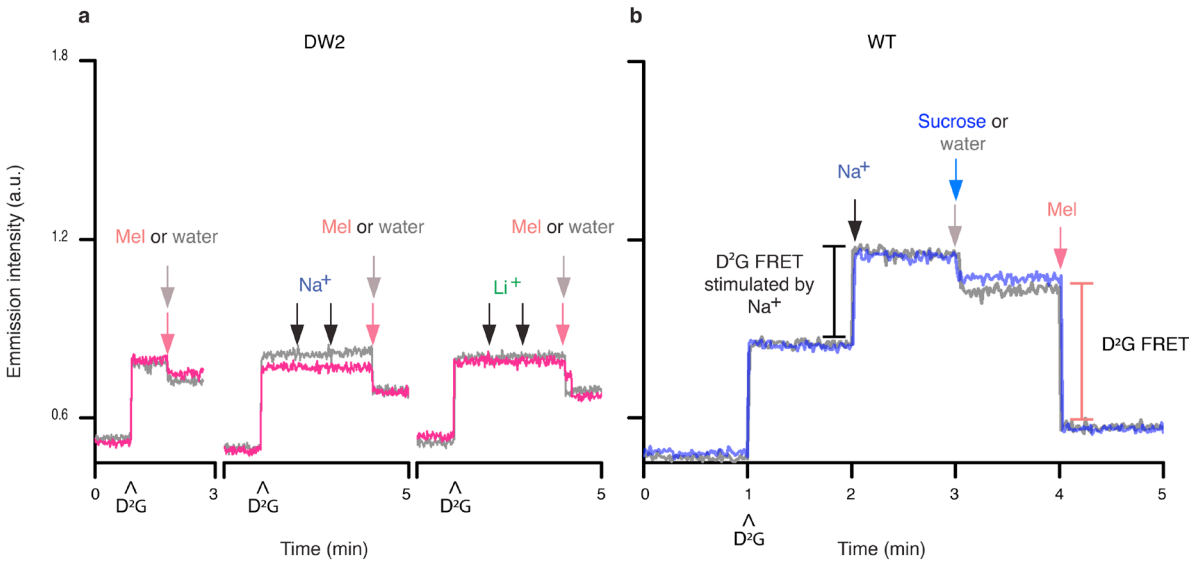
Supplementary Figure S4 | Repeats of MelB$_{St}$. **a**, Superposition of the overall structure of Mol-B (cyan) on Mol-A (green) viewed parallel or perpendicular to the membrane plane. **b**, Superposition of the overall structure of Mol-B (cyan) on Mol-A (green) is presented by four separate repeats. **c**, Superposition of repeats.



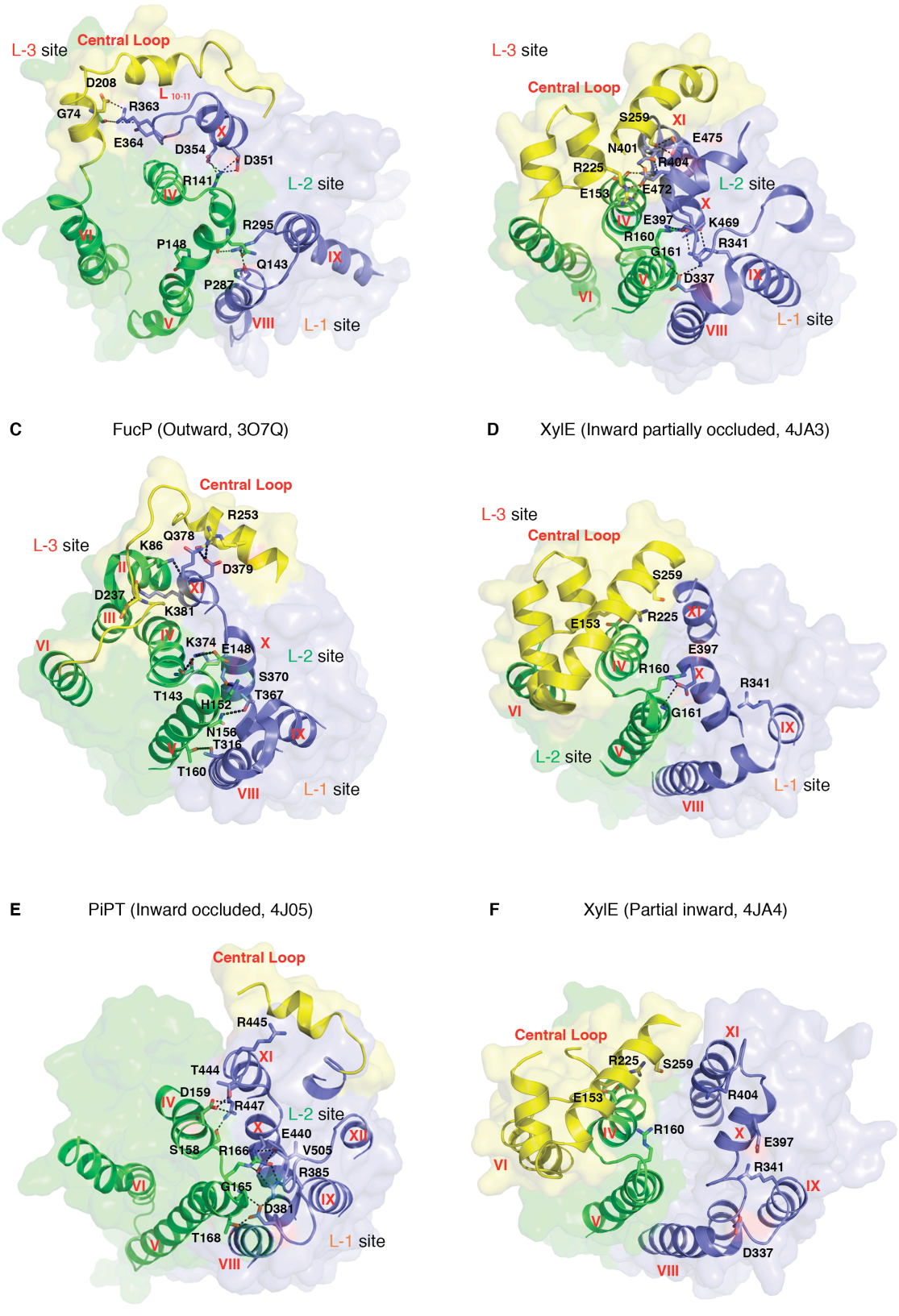
Supplementary Figure S5 | The internal cavity. Wall-eyed stereo view of the 2Fo-Fc maps of the cation- and sugar-binding residues in Mol-A (**a**) and Mol-B (**b**) contoured at 1.0 σ. Asp^{59} is surrounded by hydrophobic residues in Mol-A (**c**) and in Mol-B (**d**).



Supplementary Figure S6 | Mutational analysis. a, Melibiose transport time course. Intact *E. coli* DW2 cells (*mel*A$^+$, Δ*mel*B, Δ*lac*ZY), over-expressing WT $MelB_{St}$ or a given single-site Cys mutant, were assayed with [^{3}H]melibiose transport (0.4 mM) in the absence or presence of 20 mM NaCl or LiCl. Error bar, s.e.m., n = 2 for mutants and n = 10 for the WT and DW2. **b, Western blotting**. 25 μg of RSO membranes were loaded onto each well of SDS-16% PAGE. $MelB_{St}$ proteins were detected with anti-His tag antibody, and imaged by the *ImageQuant LAS 4000 Biomolecular Imager*. Protein marker with 75 kD is loaded on the same well with the negative control sample that contains the RSO vesicles prepared from DW2 cells without MelB.



Supplementary Figure S7 | Control experiments for Trp→D^{2}G FRET. Experimental setup was identical as described in Fig. 3d. **a, The negative control experiments.** RSO vesicles prepared with DW2 cells in the absence of MelB were used as the negative control. The assay was carried out under identical protocol as described in Fig. 3d. **b, Specificity**. RSO vesicles prepared with DW2 cells in the presence of $MelB_{St}$ were used as the positive control. After the addition of 10 μM D^{2}G, 20 mM NaCl, 120 mM sucrose or the same volume of water were added step by step. For both traces, 120 mM melibiose was added at the last step. The Na^+ stimulation of D^{2}G FRET and melibiose displacement of D^{2}G bound with $MelB_{St}$ are illustrated by black and pink blunt-ended bars, respectively.


A
MelB (4M64-A)
L-3 site
Central Loop
D208
L 10-11
R363
G74
E364
D354
X
D351
R141
IV
L-2 site
VII
R295
P148
Q143
IX
P287
V
VIII
L-1 site
B
XylE (Outward occluded, 4GBY)
L-3 site
Central Loop
S259
XI
E475
N401
R404
R225
E153
E472
L-2 site
X
IV
E397
K469
R160
R341
G161
V
D337
IX
VI
VIII
L-1 site
C
FucP (Outward, 3O7Q)
Central Loop
R253
L-3 site
Q378
K86
II
D379
XI
D237
K381
III
IV
K374
E148
X
VI
L-2 site
S370
T143
T367
H152
N156
IX
V
T316
T160
VIII
L-1 site
D
XylE (Inward partially occluded, 4JA3)
L-3 site
Central Loop
S259
E153
R225
XI
E397
V
R160
X
R341
VI
G161
IX
L-2 site
V
L-1 site
VIII
E
PiPT (Inward occluded, 4J05)
Central Loop
R445
XI
T444
D159
IV
R447
L-2 site
X
E440
XII
S158
R166
V505
R385
VI
G165
D381
IX
V
T168
L-1 site
VIII
F
XylE (Partial inward, 4JA4)
Central Loop
XI
R225
S259
E153
R404
IV
R160
X
E397
VI
R341
IX
V
D337
VIII

Supplementary Figure S8 | Locks in MFS crystal structures. Surface presentation of all structures with an aligned origin: the N- and C-terminal domains are shown in green and blue, respectively, with the central loop in yellow. The helices are labeled with Roman numerals. Lock interactions are shown as broken lines. All structures are viewed from the cytoplasmic side.