Table S1. List of strains used in this study.

Strain	Description	Designation	Source
USA300 LAC	*S. aureus* USA300 Strain LAC (AH-1263). Plasmid cured.	AH-LAC (WT)	(1)
DH5α	*E. coli* strain for recombinant plasmids	DH5α	(2)
lysY/I^{q}	*E. coli* strain for expression of LipL, LplA1, LplA2, lipoyl-H and lipoyl-E2 proteins	*lysY/I*^{q}	NEB
RN4220	Restriction-deficient *S. aureus*	RN4220	(3)
RN9011	RN4220 + pRN7203 expressing SaPI integrase	RN9011	(4)
FA-E1344	*E. coli lysY/I*^{q} Δ*lipA*::*kan* for expression of apo-H and apo-E2 proteins	*lysY/I*^{q} Δ*lipA*	(5)
FA-S1176	AH-LAC Δ*lipL*	Δ*lipL*	(6)
FA-S1691	AH-LAC Δ*lipL* containing pJC1111-*lipL*$_{(ATG-1)}$	Δ*lipL*+*lipL*$_{(ATG-1)}$	This work
FA-S1190	AH-LAC Δ*lipL* containing pJC1111-*lipL*$_{(ATG-2)}$	Δ*lipL*+*lipL*$_{(ATG-2)}$	(6)
FA-S1038	AH-LAC Δ*gcvH*::*kan*	Δ*gcvH*	(6)
FA-S1637	AH-LAC Δ*gcvH*::*kan* containing pJC1111-*gcvH*	Δ*gcvH* + *gcvH*	(7)
FA-S1041	AH-LAC Δ*pdhC*::*kan*	Δ*pdhC*	(6)
FA-S2088	AH-LAC Δ*pdhC*::*kan* containing pOS1-*pdhC-6x-His*	Δ*pdhC* + *pdhC*	This work
FA-S1042	AH-LAC Δ*odhB*::*kan*	Δ*odhB*	(6)
FA-S2079	AH-LAC Δ*odhB*::*kan* containing pOS1-*odhB-6x-His*	Δ*odhB* + *odhB*	This work
FA-S1646	AH-LAC Δ*lipL* Δ*gcvH*	Δ*lipL* Δ*gcvH*	This work
FA-E1357	*E. coli lysY/I*^{q} Δ*lipA*::*kan* + pET15b-*gcvH*	6x-His-GcvH	(7)
FA-E1383	*E. coli lysY/I*^{q} Δ*lipA*::*kan* + pET15b-*gcvH-L*	6x-His-GcvH-L	(7)
FA-E1359	*E. coli lysY/I*^{q} Δ*lipA*::*kan* + pET15b-*pdhC*	6x-His-E2-PDH	(7)
FA-E1363	*E. coli lysY/I*^{q} Δ*lipA*::*kan* + pET15b-*odhB*	6x-His-E2-OGDH	(7)
FA-E1367	*E. coli lysY/I*^{q} Δ*lipA*::*kan* + pET15b-*bmbBB*	6x-His-E2-BCODH	(7)
FA-E1284	*E. coli lysY/I*^{q} + pET15b-*lplA1*	6x-His-LplA1	(7)
FA-E1278	*E. coli lysY/I*^{q} + pET15b-*lplA2*	6x-His-LplA2	(7)
FA-E1349	*E. coli lysY/I*^{q} + pET15b-*gcvH*	6x-His-LA-GcvH	(7)
FA-E1373	*E. coli lysY/I*^{q} + pET15b-*gcvH-L*	6x-His-LA-GcvH-L	(7)
FA-E1350	*E. coli lysY/I*^{q} + pET15b-*pdhC*	6x-His-LA-E2-PDH	(7)
FA-E1351	*E. coli lysY/I*^{q} + pET15b-*odhB*	6x-His-LA-E2-OGDH	(7)
FA-E1352	*E. coli lysY/I*^{q} + pET15b-*bmbBB*	6x-His-LA-E2-BCODH	(7)
FA-E1547	*E. coli lysY/I*^{q} + pET15b-*lipL*	6x-His-LipL	This work

Table S2. List of oligonucleotides used in this study.

Name	Sequence
UniCompSOE1-**PstI**	ATAT**CTGCAG**ATCCCATTATGCTTTGGCA
LipLCompSOE2	ATTTACTCGCTAAATCCATGGGTTTCACTCTCCTTCTA
LipLCompSOE3	TAGAAGGAGAGTGAAACCCATGGATTTAGCGAGTAAAT
LipLCompSOE4-**SalI**	ATA**GTCGAC**CTATTGCATTTGATCTATCATT
LipL-**NdeI**	ATAT**CATATG**GATTTAGCGAGTAAATATTTTA
LipL-**BamHI**	ATAT**GGATCC**CTATTGCATTTGATCTATCATT
pOS1-pdhCSOE1-**EcoRI**	ATAT**GAATTC**CTGATATTTTTGACTAAACCA
pOS1-pdhCSOE2	TAATCTAAATTCAAATGCCAC-AAATAATCATCCTCCTAAGGT
pOS1-pdhCSOE3	ACCTTAGGAGGATGATTATTTGTGGCATTTGAATTTAGATTA
pOS1-pdhCSOE4-**SalI**	ATAT**GTCGAC**TTAGTGATGGTGATGGTGATGGCCACCCCCCTCCATTA ATAATAATTC
pOS1-odhBSOE1-**EcoRI**	ATAT**GAATTC**CTGATATTTTTGACT
pOS1-odhBSOE2	GAACTTTAACCTCGGCATAAATAATCATCCTCCTAAGGT
pOS1-odhBSOE3	ACCTTAGGAGGATGATTATTTATGCCAGAGGTTAAAGTTC
pOS1-odhBSOE4-**SalI**	ATAT**GTCGAC**TTAGTGATGGTGATGGTGATGAGATTCTAATAATAAGTC TTCT

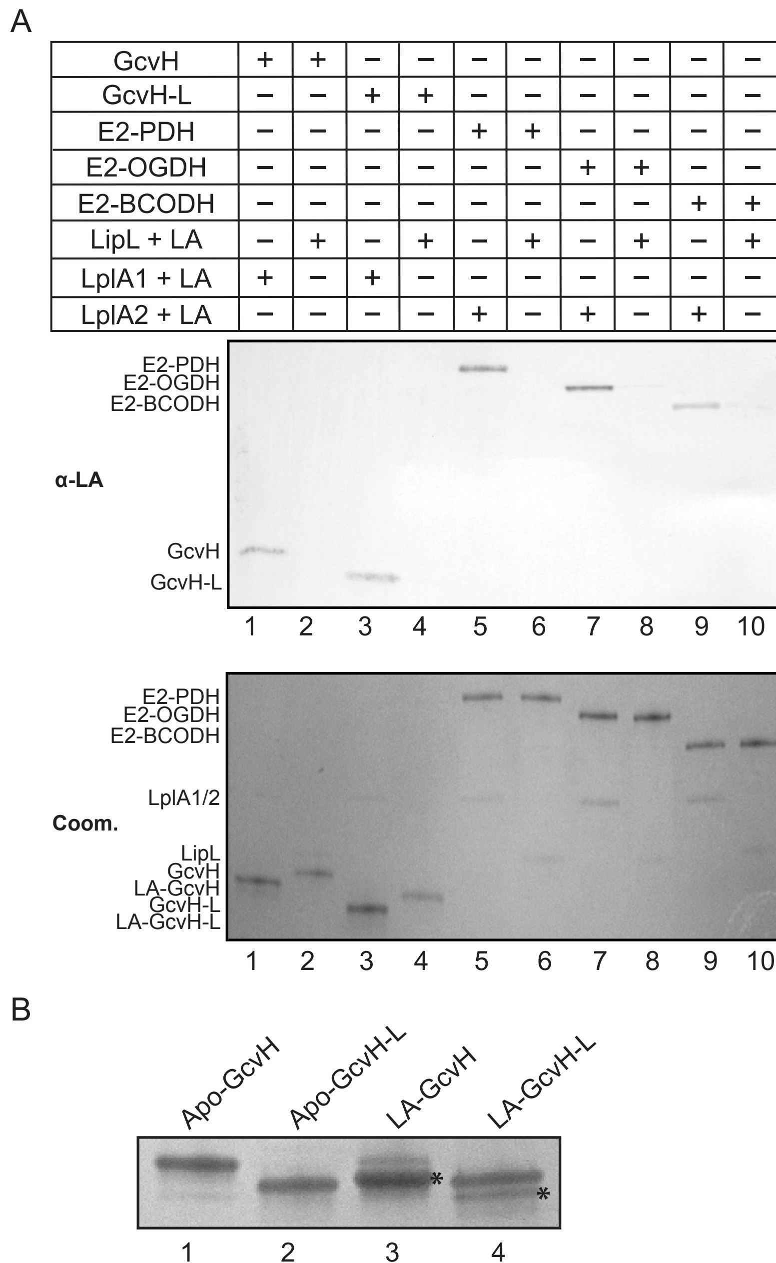


Figure S1. Testing LipL use of free lipoic acid for protein lipoylation and purification of lipoyl-GcvH and lipoyl-GcvH-L. Attachment of free lipoic acid (2.4 mM) to apo-lipoyl domain-containing proteins [20 µM each of GcvH (lane 1 and 2), GcvH-L (lane 3 and 4), E2-PDH (lane 5 and 6), E2-OGDH (lane 7 and 8), and E2-BCODH (lane 9 and 10)] by LipL (1 µM, even-numbered lanes), LplA1 (1 µM, lanes 1 and 3), and LplA2 (1 µM, lanes 5, 7, and 9) was assessed by immunoblot with rabbit α-lipoic acid antibody. 2.5 µL of a 50 µL reaction was loaded into each lane. Presented blot is representative of at least three independent experiments. Parallel 4-15% SDS-PAGE gels were stained with GelCode Blue Stain Reagent (Coom.). (B) GelCode Blue stained SDS-PAGE gel of purified apo-GcvH, apo-GcvH-L, lipoyl-GcvH, and lipoyl-GcvH-L. 2.5 µL of dialyzed protein was loaded into each lane. Band corresponding to lipoylated species, which migrates at a smaller molecular weight than the apo-form, is denoted by (*).

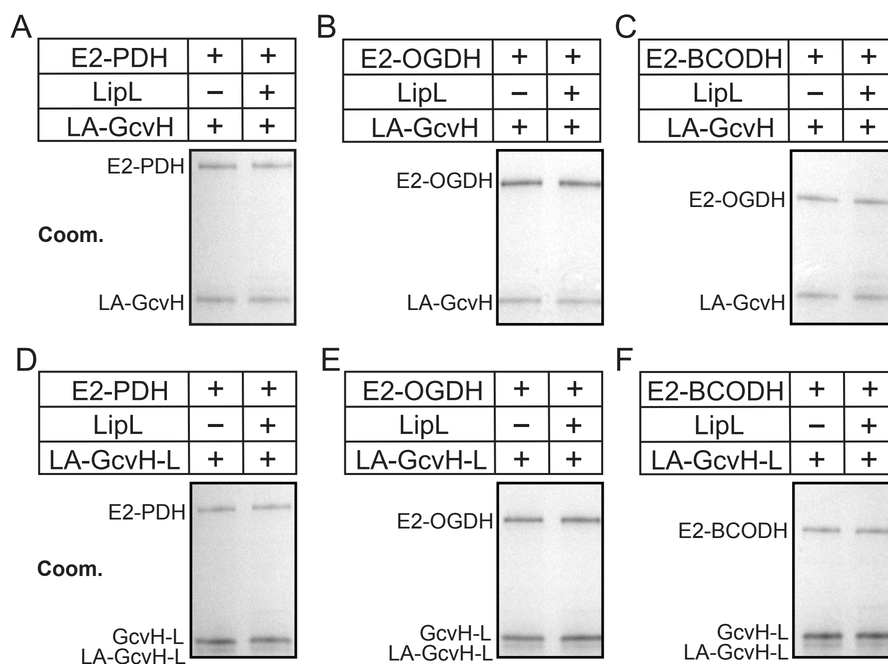


Figure S2. Loading controls related to Figure 2. GelCode Blue stained SDS-PAGE gel of reactions to assess lipoyl transfer from LA-GcvH (10 μM) (A-C) and LA-GcvH-L (40 μM) (D-F) to E2-PDH (A and D), E2-OGDH (B and E), and E2-BCODH (C and F), with LipL (1 μM, even-numbered lanes) and without (odd-numbered lanes). 2.5 μL of a 50 μL reaction was loaded into each lane.

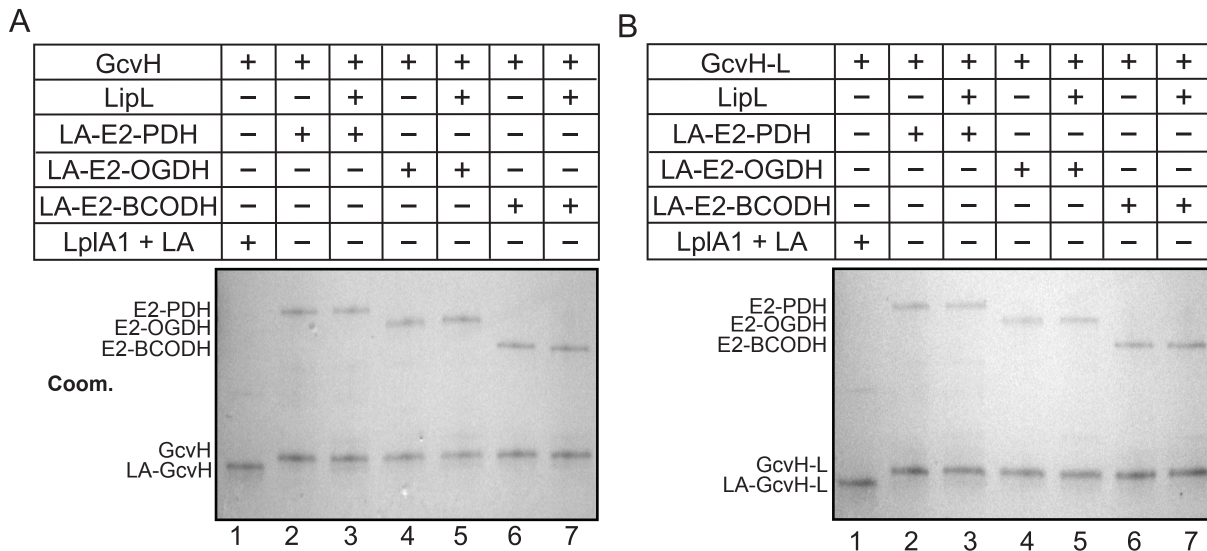


Figure S3. Loading controls related to Figure 3. GelCode Blue stained SDS-PAGE gel of reactions to assess lipoyl transfer from LA-E2-PDH (10 μM, lanes 2 and 3), LA-E2-OGDH (10 μM, lanes 4 and 5), and LA-E2-BCODH (10 μM, lanes 6 and 7) with LipL (1 μM, lanes 3, 5, and 7) and without (even-numbered lanes) to GcvH (20 μM) (A) and GcvH-L (20 μM) (B). For positive controls, GcvH (20 μM) and GcvH-L (20 μM) were lipoylated with free lipoic acid (2.4 mM) by LplA1 (1 μM) [Lane 1 of (A) and (B)]. 2.5 μL of a 50 μL reaction was loaded into each lane.

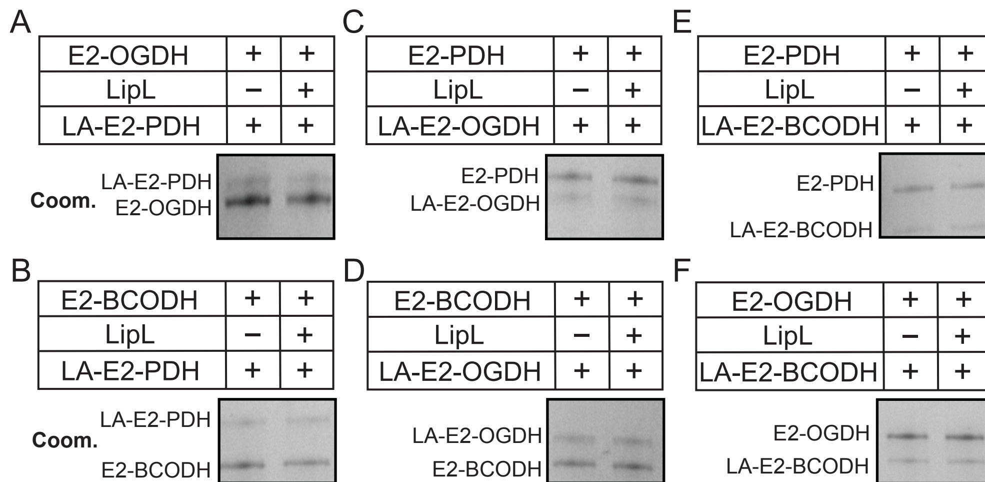


Figure S4. Loading controls related to Figure 4. GelCode Blue stained SDS-PAGE gel of reactions to assess lipoyl transfer from LA-E2-PDH (10 μM) (A and B), LA-E2-OGDH (10 μM) (C and D), and LA-E2-BCODH (10 μM) (E and F), to E2-OGDH (20 μM) (A and F), E2-BCODH (20 μM) (B and D), and E2-PDH (20 μM) (C and E), with LipL (1 μM, lane 1) and without (lane 2). 2.5 μL of a 50 μL reaction was loaded into each lane.

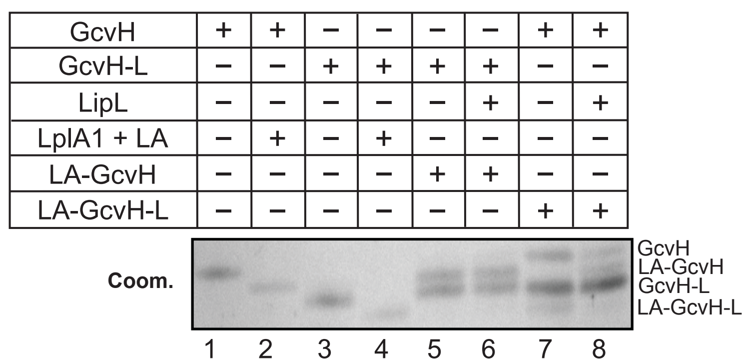
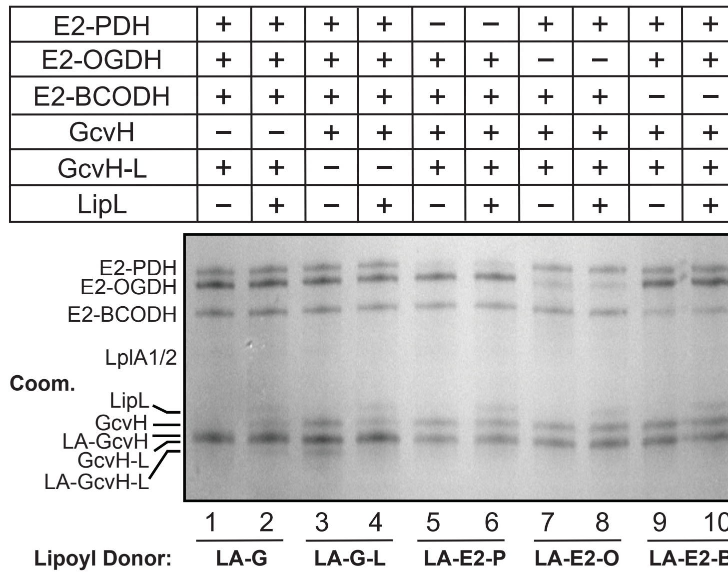


Figure S5. Loading controls related to Figure 5. GelCode Blue stained SDS-PAGE gel of reactions to assess lipoyl transfer from LA-GcvH (10 μM) to GcvH-L (20 μM) (lanes 5 and 6) and from LA-GcvH-L (40 μM) to GcvH (20 μM) (lanes 7 and 8), with LipL (1 μM, lanes 6 and 8) and without (lanes 5 and 7). Apo- (lanes 1 and 3) and LplA1-lipoylated GcvH and GcvH-L (20 μM) (lanes 2 and 4) were included as controls. 2.5 μL of a 50 μL reaction was loaded into each lane.



E2-PDH	+	+	+	+	−	−	+	+	+	+
E2-OGDH	+	+	+	+	+	+	−	−	+	+
E2-BCODH	+	+	+	+	+	+	+	+	−	−
GcvH	−	−	+	+	+	+	+	+	+	+
GcvH-L	+	+	−	−	+	+	+	+	+	+
LipL	−	+	−	+	−	+	−	+	−	+

Figure S6. Loading controls related to Figure 6. Lipoyl transfer from LA-GcvH (5 μM, lanes 1 and 2), LA-GcvH-L (20 μM, lanes 3 and 4), LA-E2-PDH (5 μM, lanes 5 and 6), LA-E2-OGDH (5 μM, lanes 7 and 8), and LA-E2-BCODH (5 μM, lanes 9 and 10) to the other four apo-lipoyl domain-containing proteins (10 μM of each) with LipL (2 μM, even-numbered lanes) and without (odd-numbered lanes). 5 μL of a 100 μL reaction was loaded into each lane.

```
GcvH   LD   ------VAIIGITEYAQSELGDIVFVELPETDDEINEGDTFGSVESVKTVSELYAPISGKVVEVNEELEDSPEFVNESPYEKAWMVKVE
                  :.:..:|...|.::|.:.:||. .:.||:...|...|:|:.|||.::..|:||.::|.|.:.|:.|..:|....|:.|:.|::
GcvH-L LD   VEKVGDLYVFSMTPELQDDIGTVGYVEF-VSPDEVKVDDEIVSIEASKTVIDVQTPLSGTIIERNTKAEEEPTILNSEKPEENWLFKLD
```

Figure S7. Amino acid sequence alignment of lipoyl domains from *S. aureus* strain USA300 GcvH and GcvH-L. Lipoyl domain of GcvH (GcvH LD) was aligned with lipoyl domain of GcvH-L (GcvH-L LD) using EMBOSS Needle. Identical residues are denoted as (|) while similar residues are denoted as (:). Lipoyl-lysine is highlighted in black, while the residue that determines rate of lipoylation and/or substrate specificity is highlighted in grey.

```
LIPT1     1 MLIPFSMKNCFQLLCNCQVPAAGFKKTVKNGLILQSISNDVYQNLAVEDW 50
                              :.:..|..|     ..:|:..|:..|: |..|
LipL      1 ------------------MQSFAFDDT-----FCESVGKDISDNV-VRTW 26

LIPT1    51 IHDHMNLEGKPILFFWQNSPSVVIGRHQN--PWQECNLN-LMREEGIKLA 97
            ||.|                :|::|.|.:  |:.:..:: |..|.|....
LipL     27 IHQH----------------TVILGIHDSRLPFLKDGIDYLTNEIGYNAI 60

LIPT1    98 RRRSGGGTVYHDMGNINLTFF---TTKKKYDRMENLKLIVRALNAVQPQL 144
            .|.|||..|..|.|.:|::..   .|:...|....:..::.:.......:
LipL     61 VRNSGGLGVVLDQGVLNISLMFKGQTETTIDEAFTVMYLLISKMFENENV 110

LIPT1   145 DVQATK--------RFDLLLDGQFKISGTASKIGRTTAYHHCTLLCSTDG 186
            |:...:        :|||.:||: |.:|.:.:..|........|.....|
LipL    111 DIDTMEIEHSYCPGKFDLSIDGK-KFAGISQRRVRGGIAVQIYLCVEGSG 159

LIPT1   187 --------TFLSSLLKS-----PYQGIRSNATASIPSLVKNLLEKDPTLT 223
                    ||....||.     .|..|..:..||:.:|:      :.|:|
LipL    160 SERALMMQTFYEHALKGEVTKFKYPEIEPSCMASLETLL------NKTIT 203

LIPT1   224 CEVLMNAVATEYAAYHQIDNHIHLINPTD---------ETLFPGINSKAK 264
            .:.:|      :...:.|.:...::|.|.         :|.|..:..:.|
LipL    204 VQDVM------FLLLYAIKDLGGVLNMTPITQEEWQRYDTYFDKMIERNK 247

LIPT1   265 ----ELQTWEWIYGKTPKFSINTSFHVLYEQSHLEIKVFIDIKNGRIEIC 310
                ::|
LipL    248 KMIDQMQ------------------------------------------- 254

LIPT1   311 NIEAPDHWLPLEIRDKLNSSLIGSKFCPTETTMLTNILLRTCPQDHKLNS 360

LipL    255 -------------------------------------------------- 254

LIPT1   361 KWNILCEKIKGIM 373

LipL    255 ------------- 254
```

Figure S8. Amino acid sequence alignment of *Homo sapiens* LIPT1 and *S. aureus* strain USA300 LipL. LIPT1 was aligned with LipL using EMBOSS Needle. Identical residues are denoted as (|) while similar residues are denoted as (:).

Supplementary References

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