Mechanistic basis of choline import involved in teichoic acid and lipopolysaccharide modification

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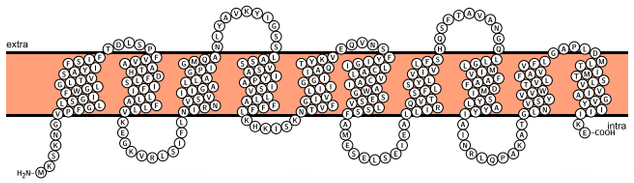
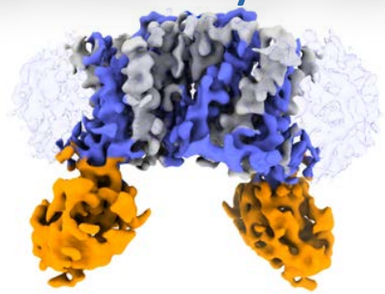
1 – LicB in *S. pneumoniae* is crucial for virulence



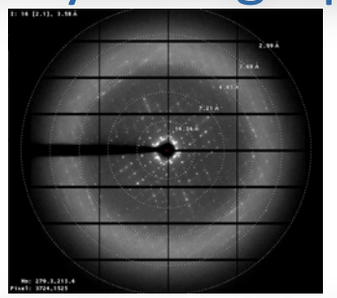
LicB, a 32 kDa choline importer with 10 TM helices from the DMT superfamily

2 – Sybody bound LicB dimer solved with single particle cryo-EM



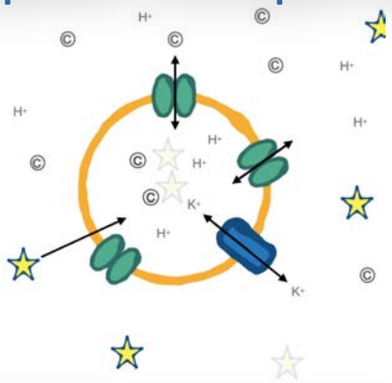
LicB dimer in nanodiscs

3 – Substrate bound structure solved via X-ray crystallography



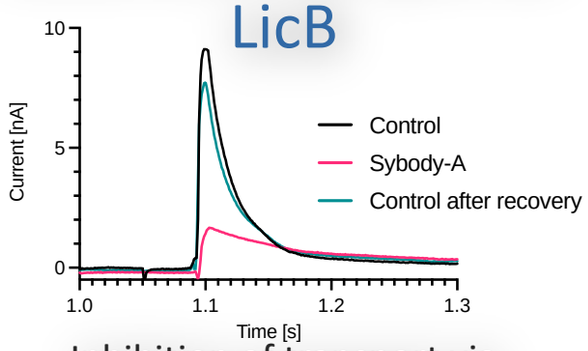
Crystal diffraction after post-crystallization treatment

4 – LicB is a proton coupled transporter



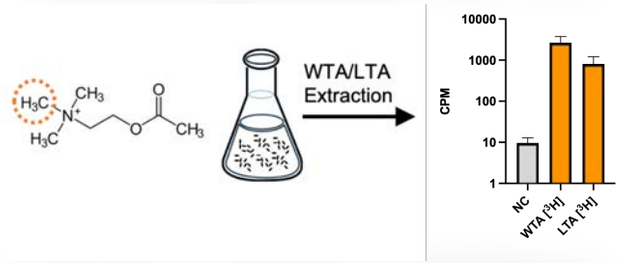
ACMA fluorescence assay

5 – Inhibition of essential transporter LicB



Inhibition of transport via SSM-electrophysiology

6 – LicB can use acetylcholine as a building block for TA



Extraction of teichoic acids from *S. pneumoniae*

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1 – LicB in *Streptococcus pneumoniae* is a choline importer and crucial for virulence

Phosphocholine molecules decorating bacterial cell wall teichoic acids and outer-membrane lipopolysaccharide have significant roles in adhesion to host cells, immune evasion, and persistence[1,2]. Bacteria carrying the operon that performs phosphocholine decoration, synthesize phosphocholine after uptake of the choline precursor by LicB (**Fig.1**), a conserved transporter among divergent species[3,4]. *Streptococcus pneumoniae* is a prominent pathogen where phosphocholine decoration plays a fundamental role in virulence[5].

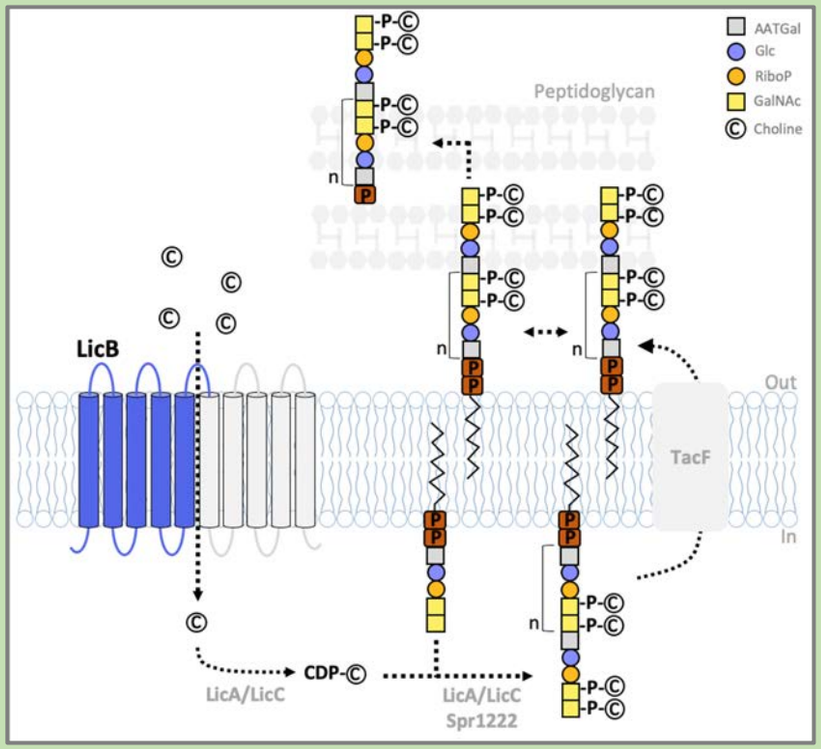

Figure 1: Phosphorylcholine decoration of teichoic acids. *Pathway for phosphocholine decoration of teichoic acids (TA) in S. pneumoniae. Choline is imported by LicB and activated by LicA and LicC, before being covalently attached as phosphocholine to TA, which are later exposed at the cell wall. Inverted repeats are shown in blue and gray.*

2 – Sybody bound LicB-dimer in apo state solved by single-particle cryo-EM

The structure of outward-open LicB in complex with a synthetic nanobody and reconstituted into nanodiscs was determined via single particle cryo-EM (**Fig. 2**). The dimer interface of the cryo-EM structure exhibits a POPG density (**Fig. 2D**).

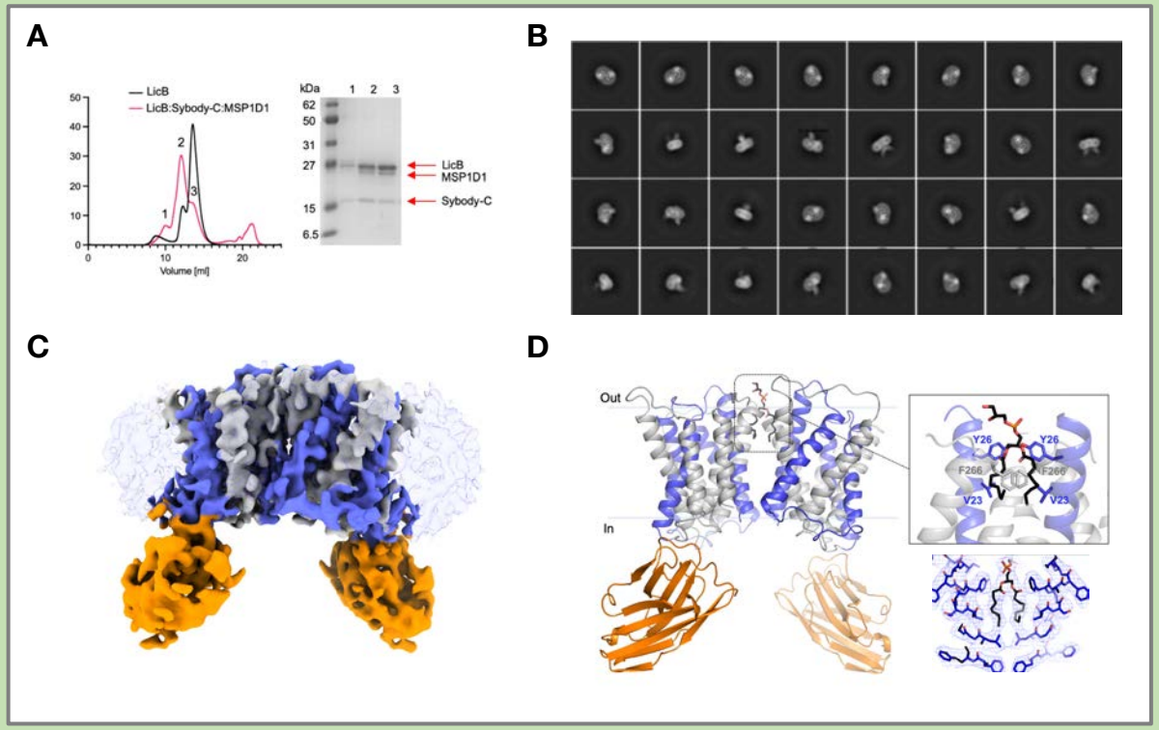

Figure 2. Nanodisc reconstituted LicB in complex with a synthetic nanobody. A. *Size exclusion profiles of LicB and LicB:Sb complex reconstituted in nanodiscs. SDS-PAGE of the three main peaks of the red trace is shown.. **B**. Representative 2D classes of LicB:Sb complex particles. **C.** Cryo-EM density of LicB homodimer (blue and grey) in nanodiscs (light blue) with bound sybody (orange) at 3.75 Å. **D.** Side view of the LicB homodimer and bound Sybodies. Inverted repeats TM1-5 and TM6-10 in blue and grey, respectively. Sybody is shown in orange. The inset shows a POPG lipid molecule at the dimer interface. Surrounding residues are shown. Cryo-EM density (mesh) superimposed on the POPG lipid and surrounding residues is shown at he bottom right.*

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3 – Substrate bound structure solved via X-ray and identification of important residues

LicB in the substrate-bound, occluded state was determined with X-ray crystallography (**Fig. 3**). The substrate binding pocket and putative proton coupling residues were described based on the crystal structure. The conserved residues were mutated to alanine and the activity of LicB variants measured in *E. coli* MKH13 cells (**Fig, 3 B, C**), where mutations in the proton binding residues decreased activity.

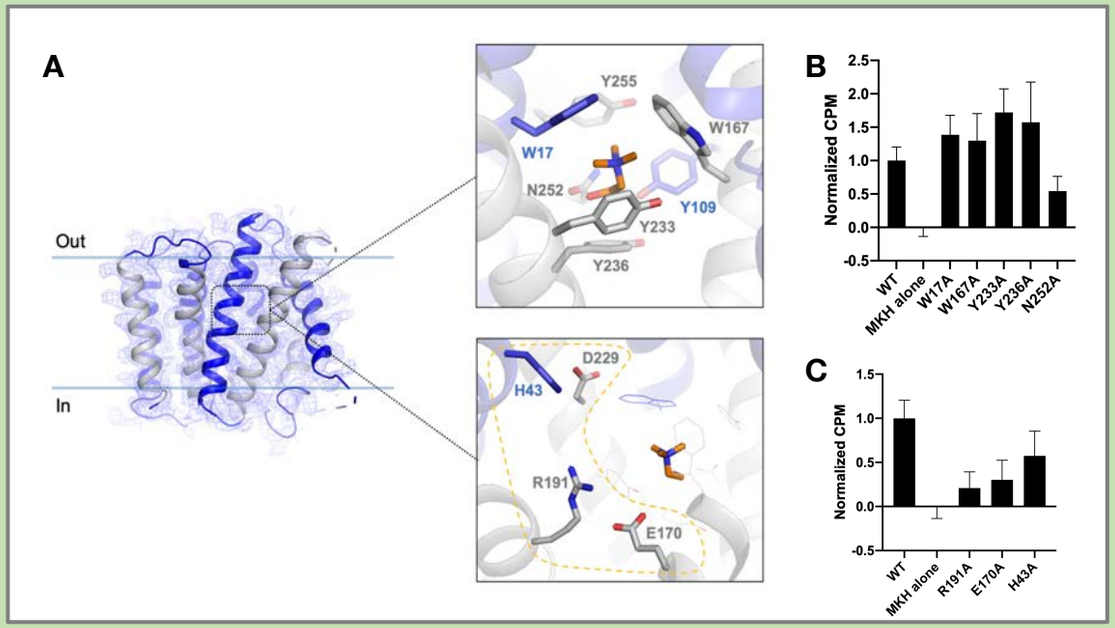

Figure 3. Choline binding pocket and proton coupling residues. A. *LicB and 2Fo-Fc electron density map contoured at 1.0 σ. (Top) Choline is shown in orange, and the coordinating residues in blue and grey, respectively. (Bottom) Charged residues in the vicinity of the choline binding site.* ***B****. Activity of LicB variants with alanine-mutations in the choline binding pocket.* ***C.*** *Activity of LicB variants with alanine-mutations in the putative proton coupling residues.*

4 – Proton coupling of LicB and variants

Proton coupling was confirmed by a fluorescence based assay, using ACMA, a proton sensitive fluorophore that is quenched by proton binding (**Fig. 4**). LicB variants with mutations of the putative proton-coupling residues H43A and R191A exhibit less activity (**Fig. 4C**).

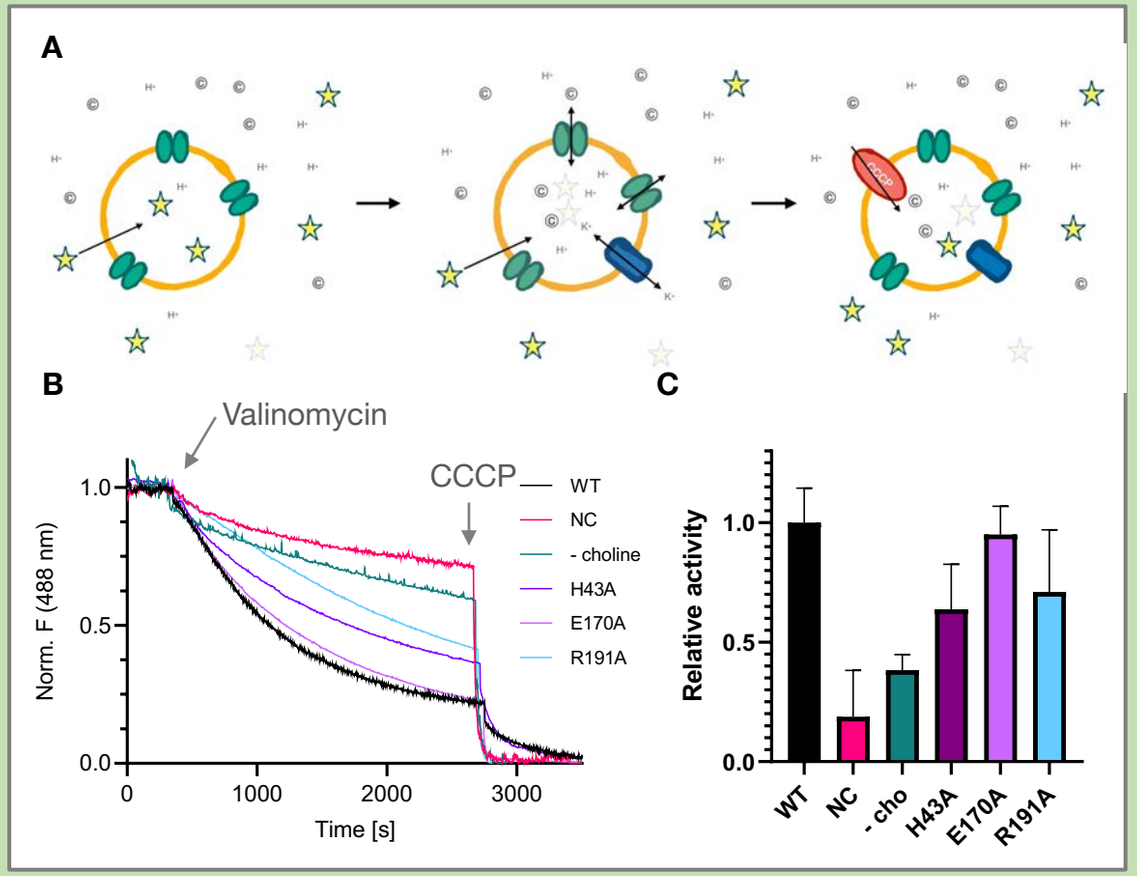

Figure 4. Fluorescence based proton coupling assay. A. *Schematic representation of fluorescence based ACMA assay.* ***B****. Proton transport assay with WT LicB and variants in proteoliposomes. Representative time courses are shown. H^+ influx was initiated by establishing a membrane potential by addition of the potassium ionophore valinomycin.* ***C.*** *Normalized fluorescence at equilibrium after addition of valinomycin.*

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5 – Choline import and its inhibition via synthetic sybodies or hemicholinium-3

Transient currents of substrate import were measured with solid supported membrane electrophysiology (SSM), where proteoliposomes are adsorbed on a SSM surface (**Fig. 5**). Choline import and import of similar substrates was measured to determine affinity and saturation concentrations (**Fig. 6**). Inhibition of LicB was possible with the chemical compound hemicholinium-3 (**Fig. 6 B, C**) and with synthetic nanobodies, specifically selected against LicB (**Fig. 6D**).

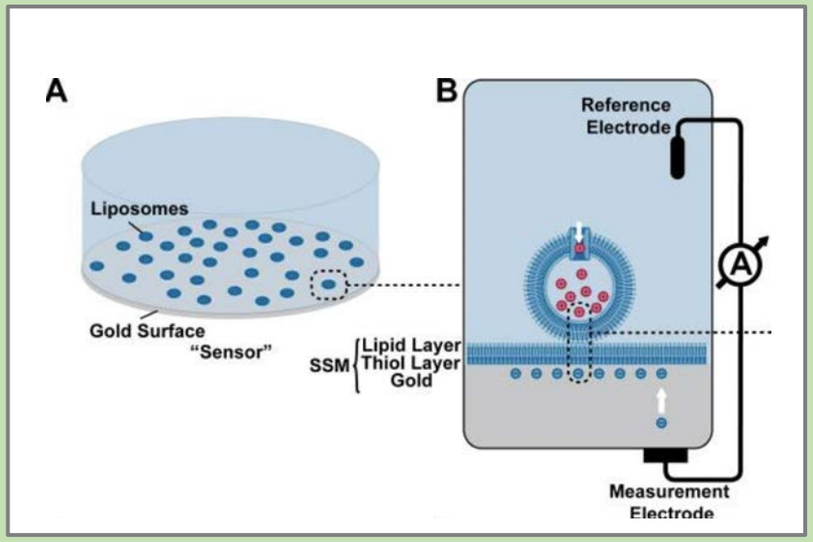

Figure 5. SSM electrophysiology setup. A. *Liposomes with reconstituted protein are adsorbed on the thiolized gold sensor.* ***B.*** *Changes in electrical potential are detected that are generated by electrogenic transport. Figure adapted from Barthmes et al., 2016*[5]

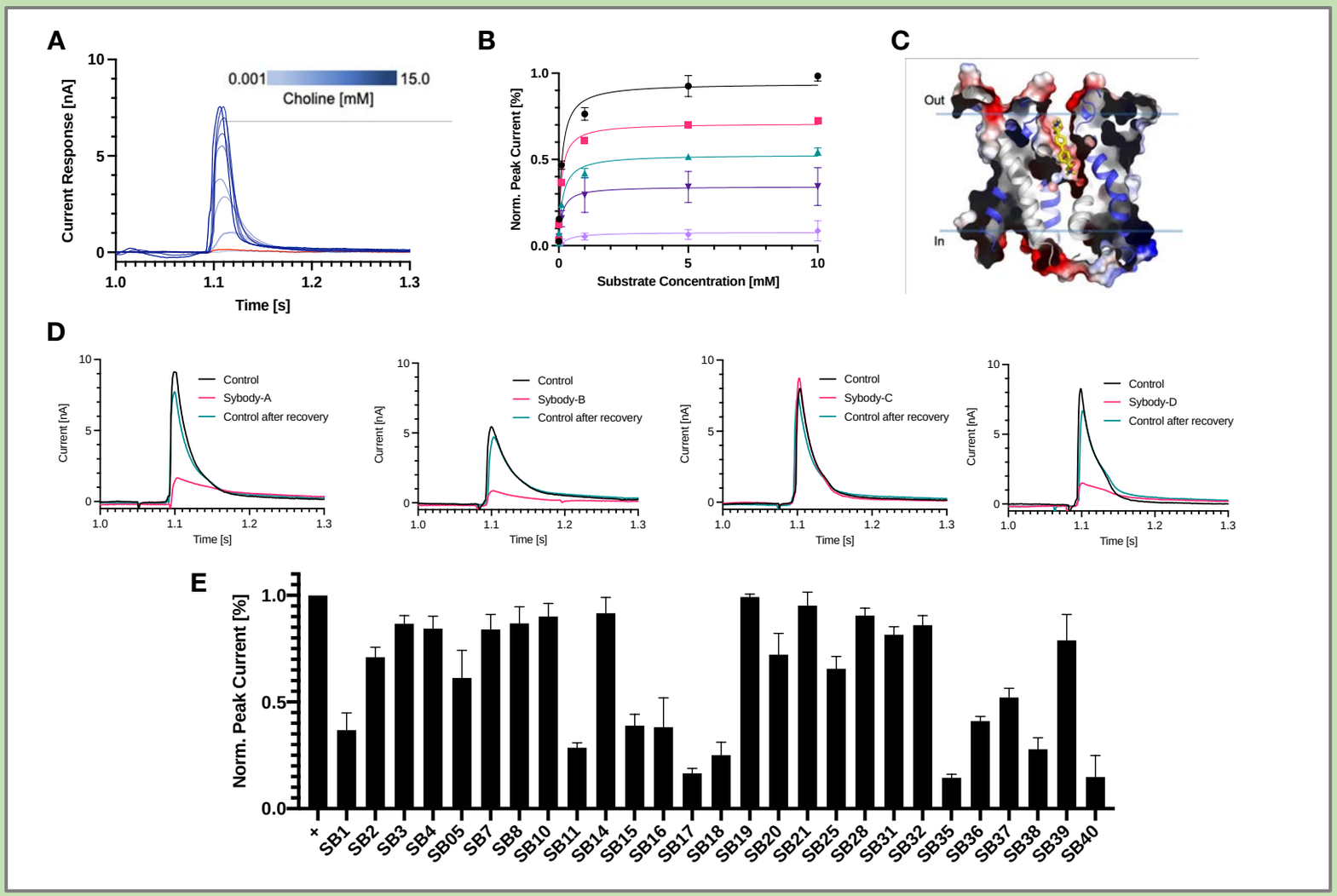

Figure 6. Inhibition of choline import. A. C*urrent response of choline transport at different concentrations* ***B.*** *Inhibition of LicB by HC-3 measured by SSM-electrophysiology (K_i=518 ± 31 µM).* ***C.*** *Surface electrostatic potential representation of outward-open LicB showing a HC-3 molecule docked in the extracellular entry pathway.* ***D.*** *Representative SSM-electrophysiology recordings of LicB choline transport in presence of sybodies. Representative recordings of currents measured during application of 5 mM choline in absence of sybodies in black (control), in presence of 500 nM of sybodies shown in pink and recordings after unbinding of sybodies in green.* ***E.*** *Normalized amplitudes of currents measured in presence of sybodies at 500 nM, under 5 mM choline transport conditions.*

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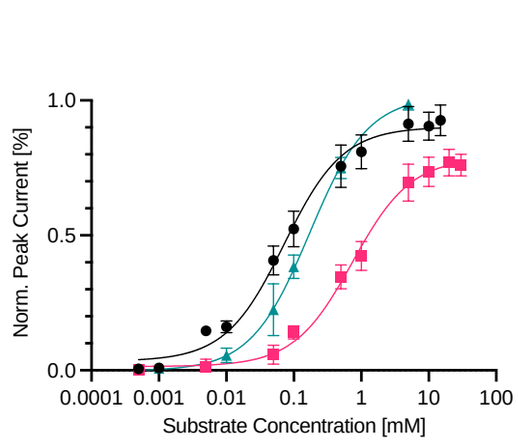
6 – LicB is a promiscuous transporter that can use acetylcholine as an alternative source for teichoic acid modification

We tested whether LicB can transport alternative compounds with choline-like characteristics, using SSM electrophysiology (**Fig. 7**).
Acetylcholine is a neurotransmitter with function in the central and peripheral nervous system, where it is produced in cholinergic neurons and activates acetylcholine receptors before being processed by acetylcholinesterase. During invasion of the central nervous system, *S. pneumoniae* can be exposed to acetylcholine. Thus, in light of the promiscuous transport activity of LicB towards this molecule, we tested whether *S. pneumoniae* can make use of acetylcholine to supply the pathway that carries out decoration of TA (**Fig. 8**).
The mechanism of the pathogenic choline importer and the function can be described with the structural and functional characterization (**Fig. 9**). The movements of TM3 and TM6 close the extracellular entry pathway, whereas the predicted movements of TM1 and TM8 open the cytoplasmic exit pathway.



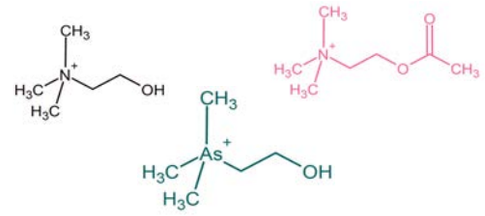


Figure 7. Choline import and promiscuous activity. *Determination of EC_{50} values for choline (black), arsenocholine (turquoise) and acetylcholine (pink).*

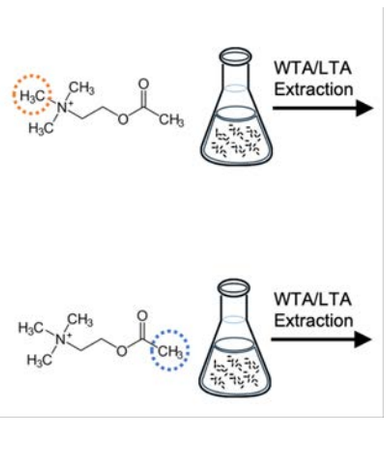


Figure 8. Acetylcholine for the modification of TAs. *Wall teichoic acids (WTA) and lipoteichoic acids (LTA) extraction from S. pneumoniae cells grown in presence of [3H]-acetylcholine labeled at different chemical groups. Histograms show the [3H] radioactivity from extracted WTA and LTA.*

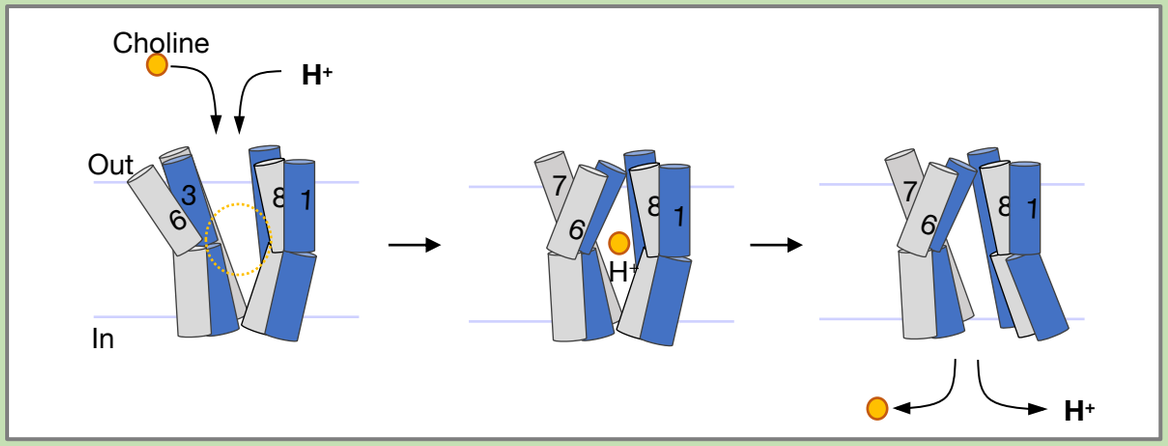


Figure 9. Alternating access model of choline import. *Schematic of the proposed transport cycle. Choline (orange) and protons enter the binding cavity in the outward-open state. LicB changes conformation to the occluded state before opening up to the cytoplasm for choline and release of protons.*

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