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The structure of *Clostridioides difficile* SecA2, an ATPase in charge of surface virulence factor export, reveals regions important for differential protein target recognition of the SecA1 and SecA2 export systems

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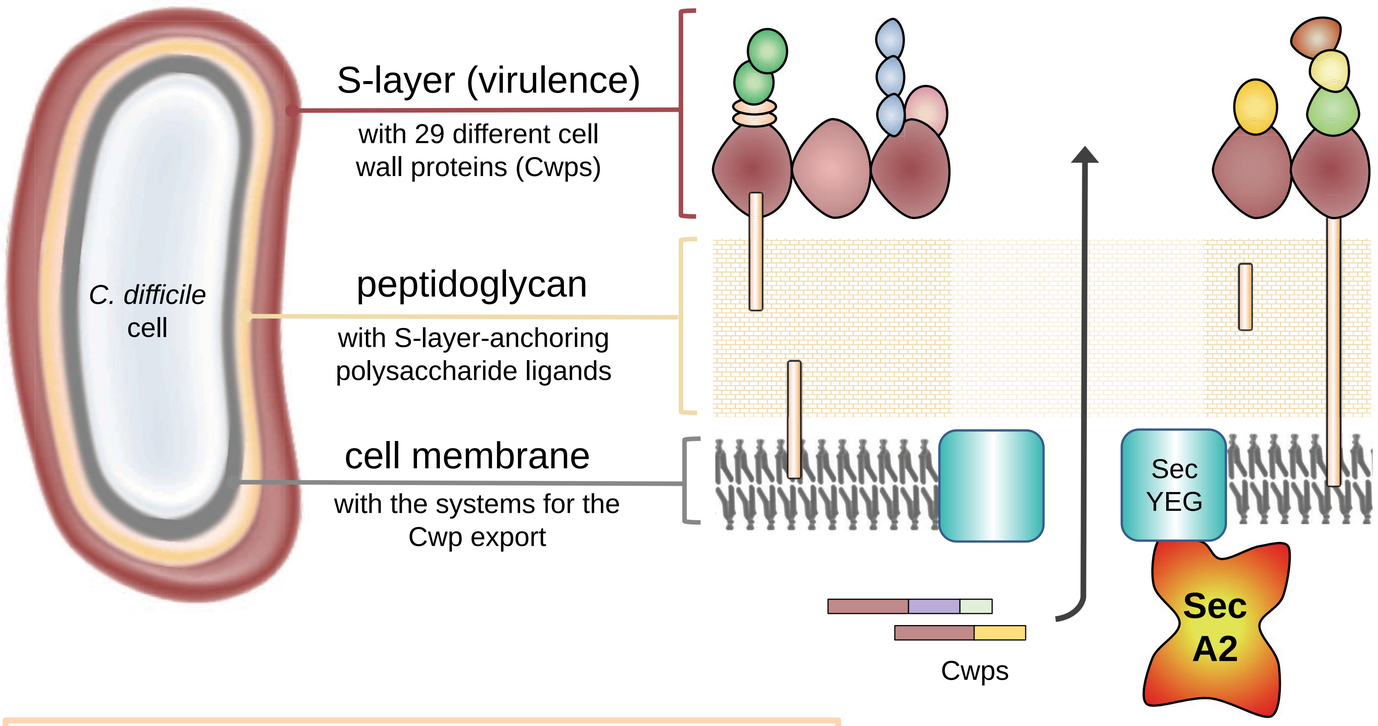
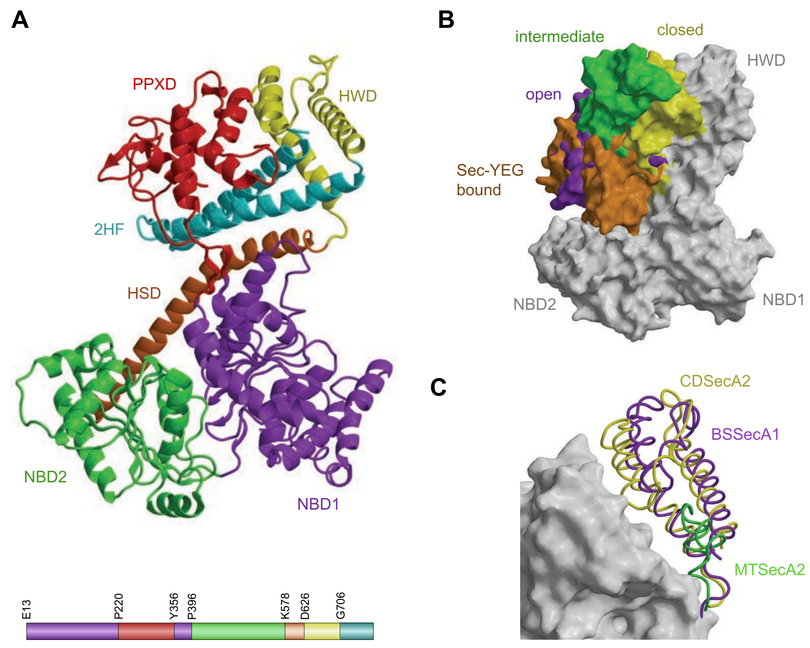
Background and aims: SecA proteins are the driving forces of bacterial protein export systems. They couple ATP hydrolysis with protein translocation through the SecY transmembrane channel. General SecA systems export proteins involved in cell wall maintenance, nutrient acquisition and communication with the environment. In some Gram-positive pathogens, such as *Clostridioides difficile*, a second paralogue, SecA2, exists that is designated for the export of a different set of substrates, usually virulence factors. To get an insight into the structural determinants of SecA(1) and SecA2 systems that enable differential protein recognition, we determined and analyzed the crystal structure of SecA2 from *C. difficile* (CDSecA2).

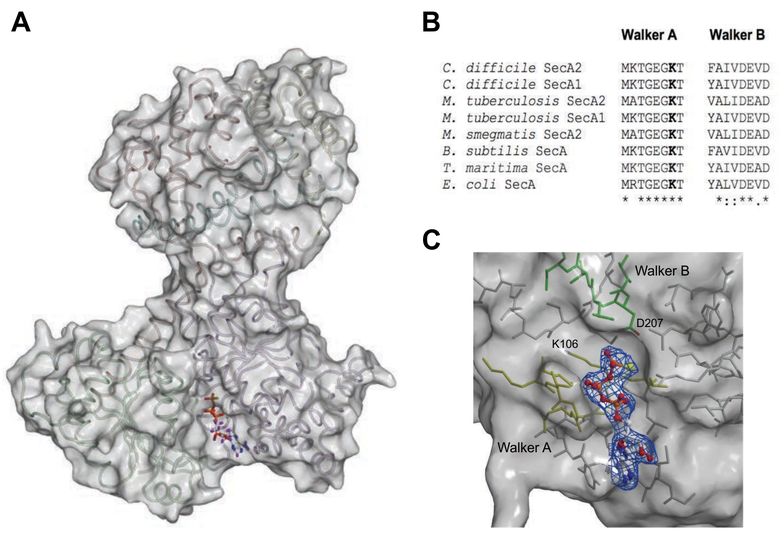


The crystal structure of apo CDSecA2 and conformational variability of SecA homologues



A. The CDSecA2 crystal structure in closed conformation. Domains are color coded. **B**, **C.** The structures of different SecA molecules, superimposed onto CDSecA2 shown as white surface. **B.** The colored surface of PPXDs in different conformations.Closed, intermediate and open conformations of CDSecA2, *M. tuberculosis* SecA2 and *B. subtilis* SecA are shown in yellow, green and violet, respectively. Sec-Y(EG) bound conformation of *T. maritima* SecA is shown in orange. **C.** Ribbon representation of CDSecA2, *B. subtilis* SecA and *M. tuberculosis* SecA2 HWDs shown in yellow, violet and green, respectively.

CDSecA2 ATP binding

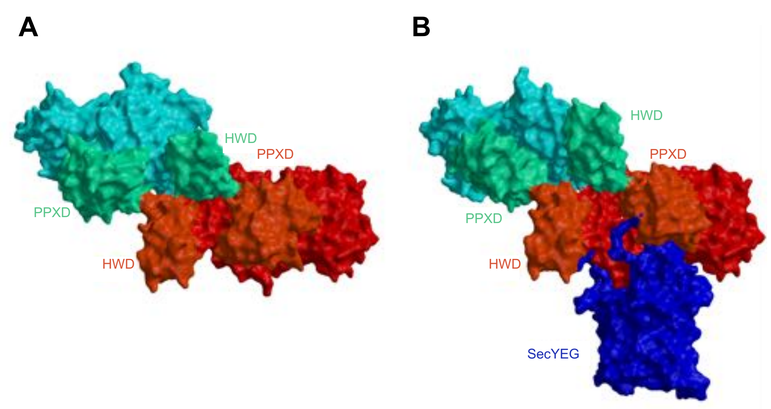


A. The structure of CDSecA2 (transparent surface with ribbons) in complex with ATP-γ-S (sticks). **B.** The conservation of Walker motifs in the SecA1 and A2 proteins shown in sequence alignment. **C.** The CDSecA2 surface region that binds ATP-γ-S (stick and ball representation).

Methods: Structures of the whole length CDSecA2 in apo and ATP-bound form were determined by X-ray crystallography at 2.3 Å and 2.6 Å, respectively. ATPase activity was determined using a commercial kit. Modeling combined with structure-based sequence alignments and conservational analysis was used to identify similarities and differences between the SecA(1) and SecA2 systems and to determine the regions involved in target protein binding, SecY channel interaction and dimerization.

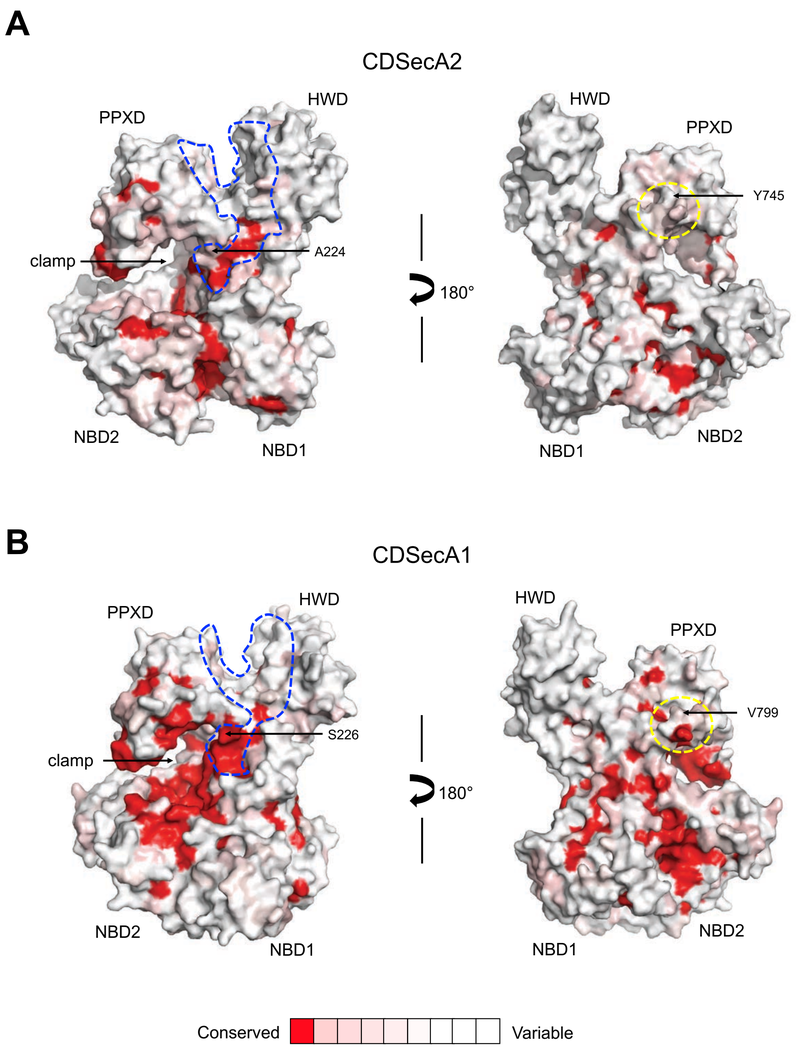
Results: The structures revealed a closed monomer lacking the C-terminal tail with otherwise similar multidomain organization to its SecA(1) homologues and conserved regions for nucleotide binding. We confirmed that the CDSecA2 is an active ATPase with an average in vitro activity rate of 2.6 ± 0.1 μmolPi/min/μmol. We found that the movement of CDSecA2 domains affects the conformation of the target protein binding site and that an open conformation is necessary for CDSecA2 dimerization and SecY channel binding. Finally, our conservational analysis indicated several sites responsible for similar and different interactions of SecA1 and SecA2 with their substrate and the SecY channel.

Model of the CDSecA2 dimer



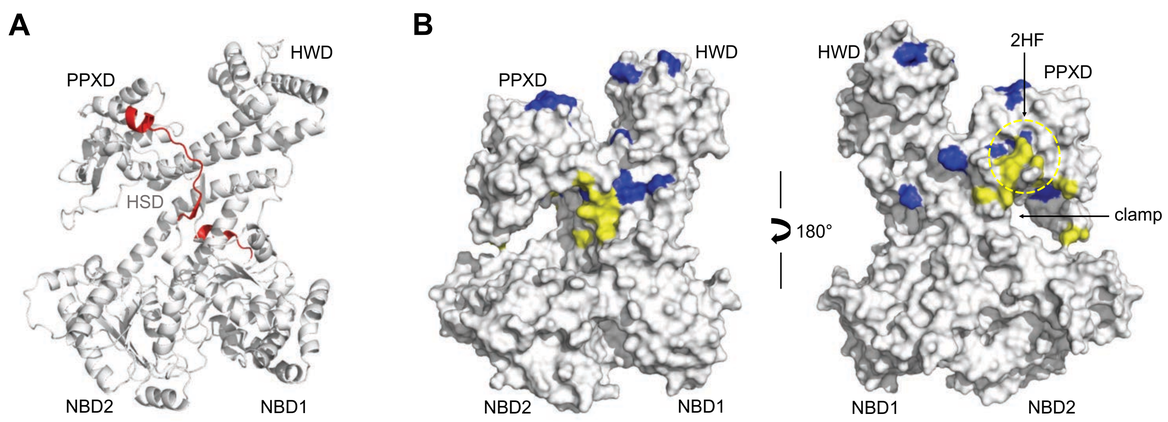
A. CDSecA2 dimer in the open conformation modeled based on *B. subtilis* SecA (PDB ID 3JV2) with one CDSecA2 molecule shown in red and the other in cyan. Interacting PPXDs and HWDs are highlighted. **B.** CDSecA2 dimer in the SecY-bound conformation modeled based on *T. maritima* SecYEG (blue)-bound SecA dimer (PDB ID 3DIN). CDSecA2 dimer color coding is the same as in A.

Surface conservation of SecA proteins in SecA2-encoding organisms



A. Conservation of the SecA2 surface based on the *C. difficile* SecA2 structure and 30 of its orthologs. **B.** Conservation of SecA1 surface based on the *C. difficile* SecA1 model and 30 of its orthologs **A, B.** Both models are shown in "open" conformation and were built using *B. subtilis* SecA (PDB ID 3JV2) as a template. The clamp enclosing the protein target is depicted with an arrow, the signal-peptide binding groove circled with a blue line, and 2HF with the yellow line. Residues with the highest conservational scores are shown in red and the color coding is shown at the bottom of the figure.

Modeling of CDSecA2 domain positioning exposes differences in target recognition area



A. CDSecA2 model of the open conformation was made using *B. subtilis* SecA with a bound peptide as a template (PDB ID 3JV2). Regions that we had trouble modeling for the SecY-bound CDSecA2 model are depicted with red. **B.** The surface of the CDSecA2 model in an open conformation with surface residues previously shown to be involved in signal and nascent protein interaction in other SecA proteins is presented in two different orientations. Structure based sequence alignment (PROMALS3D) was used to expose the conserved residues previously implicated in the signal peptide interaction (blue) and the nascent protein interaction (yellow). The clamp that encloses the protein target is depicted with an arrow, and 2HF encircled with a yellow dotted line.

Conclusions: Our study supports a model whereby an open conformation of the CDSecA2 protein is required for target protein binding, thereby ensuring its movement through the SecY channel. We suggest that dimerization of monomers plays a specific role in this process. The identified differences between SecA(1) and SecA2 homologues are in agreement with the unique adaptation of SecA2 proteins to a specific type of substrate. These exposed differences will be addressed experimentally in further mechanistic studies.

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