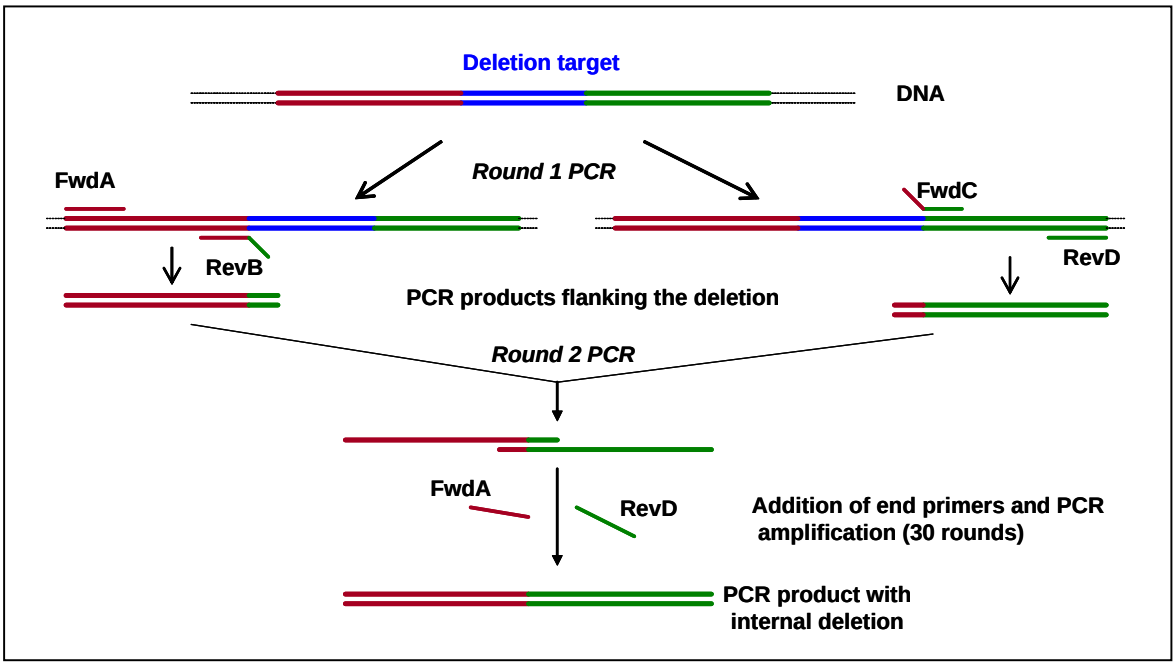
Construction of B and A' mutants with site directed mutagenesis

To study the function of specific amino acids in the largest subunits B (fork 1 and fork 2 domains and R445 in fork2) and A' (rudder and lid domains and switch 2 amino acids R313 and K306), internal deletions were introduced by PCR-fusion (see Figure below).



Schematic representation of the strategy for deletion mutagenesis by PCR-fusion. The sequence to be deleted is shown in blue. FwdA and RevD are end primers; RevB and FwdC represent primers flanking the deletion.

For construction of BΔFork1, BΔFork2, A'ΔRudder and A'ΔLid, deletion mutagenesis was performed by overlapping extension PCR using a two-round, four-primer technique. In *round 1*, two PCR products were generated in separate reactions. Each PCR reaction was carried out using genomic DNA as template, an end primer (FwdA or RevD) and a primer flanking the internal deletion sequence (RevB or FwdC). For point mutations the primers containing the amino acid substitution were complementary.
The resulting products were analyzed by agarose gel electrophoresis and purified using the QIAquick® spin Gel Extraction Kit.
Purified segments were added jointly for *round 2* of PCR. Fusion of the two intermediate segments was achieved as a result of overlapping complementary regions in the products produced in *round 1*. These products paired during the annealing phase of PCR *round 2* and were amplified by addition of end primers (primers FwdA and RevD). After analyzing PCR products by agarose gel electrophoresis, the resulting mutant insert was purified with the QIAquick® spin Gel Extraction Kit, and ligated into pET151/D-TOPO.

Primers for generation of internal deletions and point mutations

End primers for subunit A'

A'-FwdA: 5'-CACCATGAAAAAAGTTATTGGAAGTATTGAGTTTGGC-3'
A'-RevD: 5'-TCACACCTTCGCCTTGTTATTTCCTCTCATCTTTA-3'

End primers for subunit B

B-FwdA: 5'-CACCATGAGAGGTCCGACTGTTGTAGATGTTACTCCCG-3'
B-RevD: 5'-TCACACCCTCTCTGAGAGGTTTAACTTAGGTCTAATAACC-3'

BΔFork1

Fork1-RevB: 5'-GCAACTGACTAGCTCCAGTTGCAAGAGCATGTTCAATTCT-3'
Fork1-FwdC: 5'-AACTGGAGCTAGTCAGTTGCTAGATAGAACTAACTACATG-3'

BΔFork2

Fork2-RevB: 5'-GAAGGTCTCTAGACGTGACTCTTCTAAGGTGGGATAATGT-3'
Fork2-FwdC: 5'-AGTCACGTCTAGAGACCTTCACGGAACTCACTGGGGAAGA-3'

A'ΔRudder

Rudder-RevB: 5'-TCTGAGCTAGAATGTAAGTTGTAACGTGATACTGCAAGAG-3'
Rudder-FwdC: 5'-AACTTACATTCTAGCTCAGAGACTTAAAGGAAAAGAAGGT-3'

A'ΔLid

Lid-RevB: 5'-AAGTCTCTAGCAGTTACTGGGGGAACAGGCAAAACT-3'
Lid-FwdC: 5'-CTAGAGACTTAACTCATAAACTTGTTGACATAATAAGGATAA-3'

Primers for generation ofsingle point mutations

B R445A

B R445A-RevB: AGTGGGGCTGTTCAGCGTCTAGTGGAGAC
B R445A-FwdC: GTCTCCACTAGACGCTGAACAGCCCCACT

A'K306A

A'K306A-RevB CTACCTTCTTTTCCAGCAAGTCTCTGAGCTA
A'K306A-FwdC TAGCTCAGAGACTTGCTGGAAAAGAAGGTAG

A'R313A

A'R313A-RevB CCGCTCAGGTTTCCAGCAAATCTACCTTCTT
A'R313A-FwdC AAGAAGGTAGATTTGCTGGAAACCTGAGCGG