University of Lethbridge

Structure-function studies reveal ComEA contains an oligomerization domain essential for transformation in gram-positive bacteria

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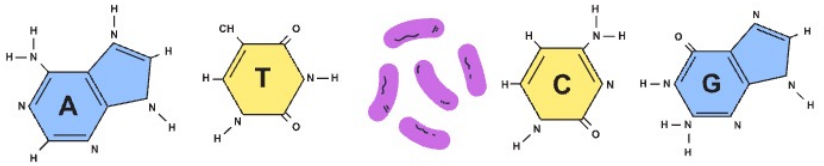
Presentation Format

- **Background**
  - Bacterial Transformation
  - ComEA
  - Objectives of Study
- Methods/Results/Conclusions
  - X-ray structures reveal OD and related behaviour
  - ComEA oligomerizes in solution
  - ComEA oligomerizes on DNA
  - ComEA oligomerization is required for transformation
  - ComEA-DNA interactions are required for transformation
- Summary

Bacterial Transformation



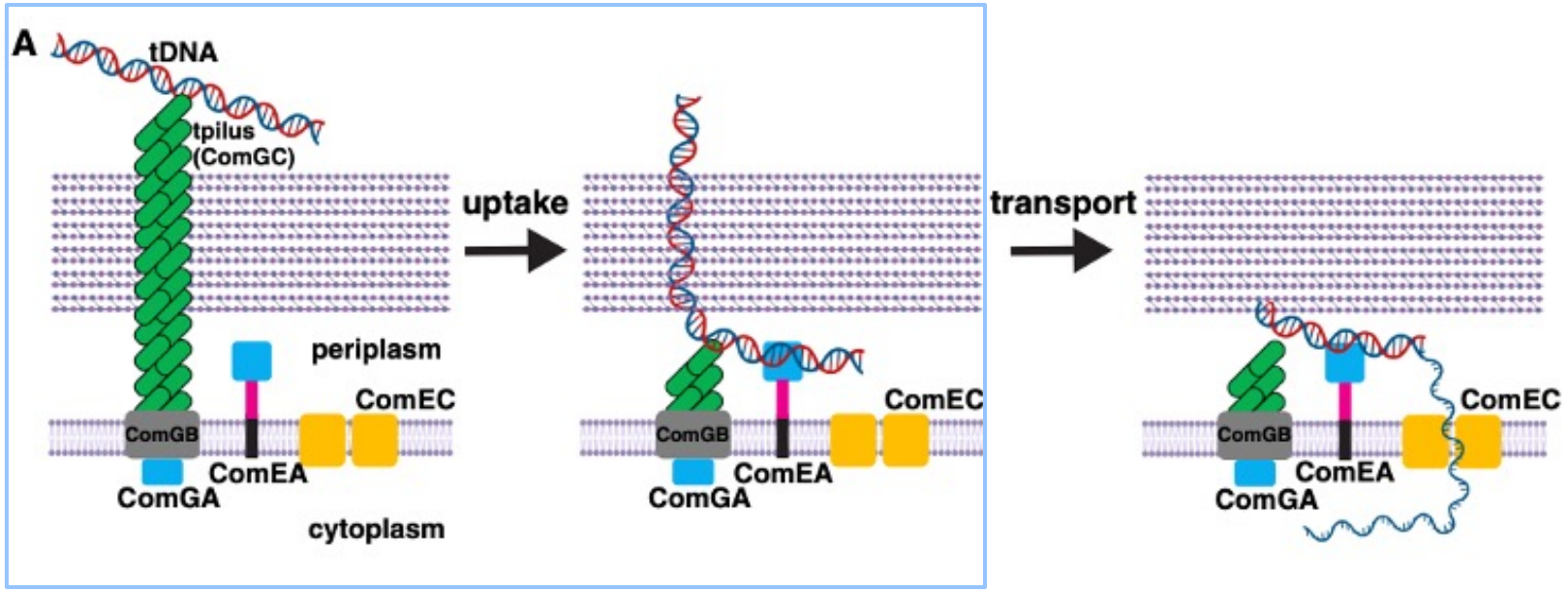
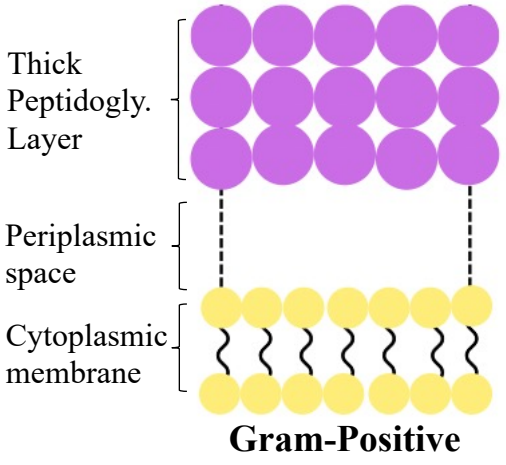
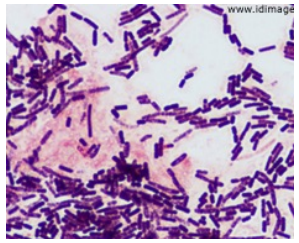


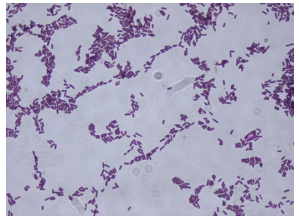
Fig. 1A | Existing model of genetic transformation in Gram-positive bacteria. The tpilus is composed of ComGC, whose assembly requires the ATPase ComGA. The tpilus is believed to be anchored to the membrane protein ComGB. The tpilus binds weakly to DNA and retracts to pull it into the periplasm. Here, the DNA encounters ComEA, which stabilizes binding to the cell and propels continued uptake of the DNA. ComEC is proposed to degrade one strand of DNA and provide the channel for transport of DNA into the cytoplasm.

ComEA

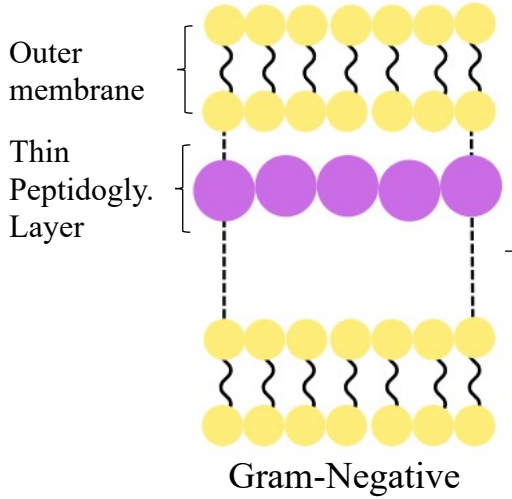


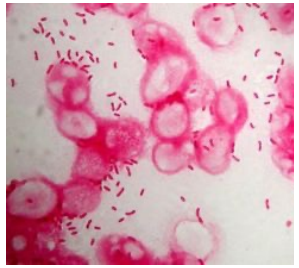
G. stearothermophilus





B. subtilis





V. cholerae

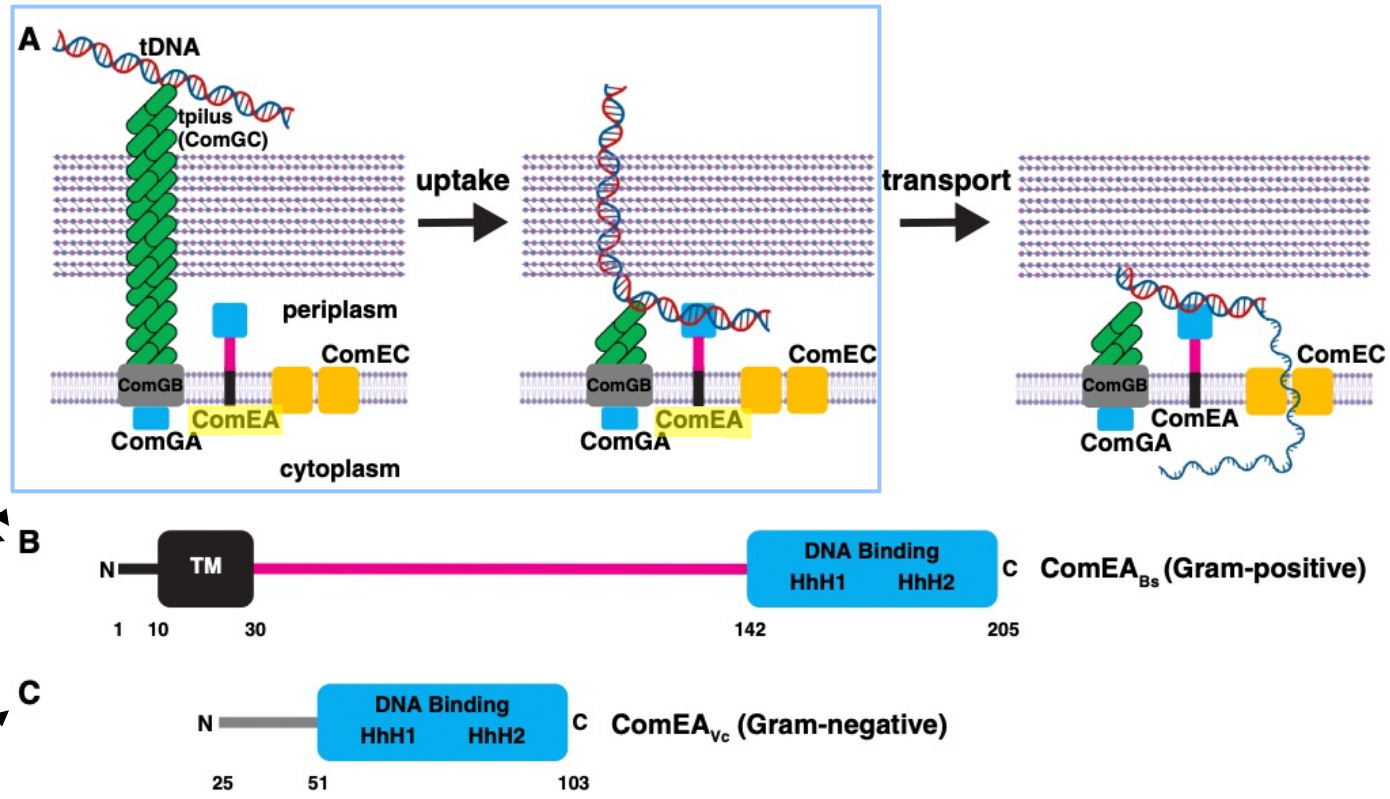


Fig. 1 | Existing model of genetic transformation in Gram-positive bacteria, and ComEA domain architecture in Gram-positive and Gram-negative bacteria. **A** The tpilus is composed of ComGC, whose assembly requires the ATPase ComGA. The tpilus is believed to be anchored to the membrane protein ComGB. The tpilus binds weakly to DNA and retracts to pull it into the periplasm. Here, the DNA encounters ComEA, which stabilizes binding to the cell and propels continued uptake of the DNA. ComEC is proposed to degrade one strand of DNA and provide the channel for transport of DNA into the cytoplasm. **B** Domain architecture of ComEA from a representative Gram-positive bacterium, *B. subtilis*. **C** Domain architecture of ComEA from a representative Gram-negative bacterium, *V. cholerae*. Residues 1–24 are not shown in order to highlight the fact that they comprise a predicted secretion signal that is cleaved to generate mature ComEA$_{Vc}$, which diffuses freely in the periplasm. TM, predicted transmembrane region. The magenta line denotes a region of unknown function, which is addressed in this study. HhH, helix-hairpin-helix motifs. Residue numbering corresponds to ComEA$_{Bs}$ or ComEA$_{Vc}$. Elements of the figure were created with BioRender.com.

Study Objectives

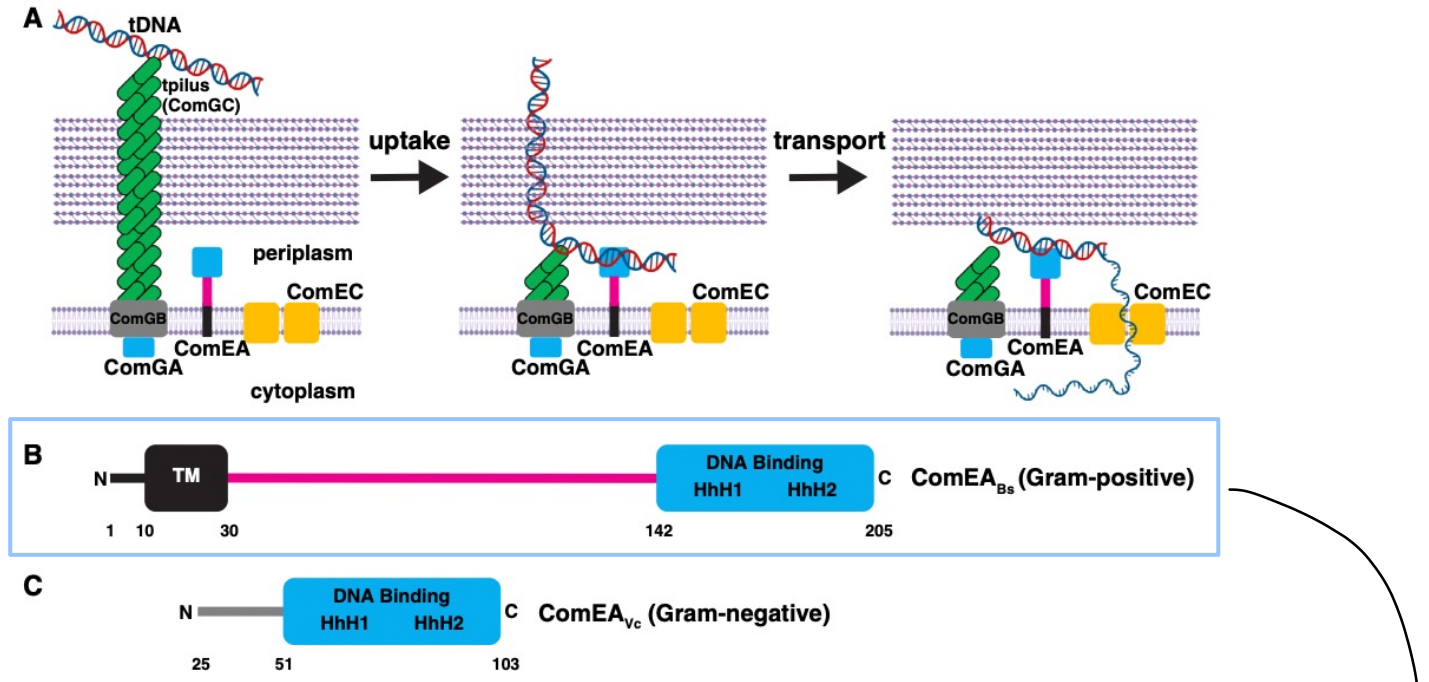


Fig. 1 | Existing model of genetic transformation in Gram-positive bacteria, and ComEA domain architecture in Gram-positive and Gram-negative bacteria. **A** The tpilus is composed of ComGC, whose assembly requires the ATPase ComGA. The tpilus is believed to be anchored to the membrane protein ComGB. The tpilus binds weakly to DNA and retracts to pull it into the periplasm. Here, the DNA encounters ComEA, which stabilizes binding to the cell and propels continued uptake of the DNA. ComEC is proposed to degrade one strand of DNA and provide the channel for transport of DNA into the cytoplasm. **B** Domain architecture of ComEA from a representative Gram-positive bacterium, *B. subtilis*. **C** Domain architecture of ComEA from a representative Gram-negative bacterium, *V. cholerae*. Residues 1–24 are not shown in order to highlight the fact that they comprise a predicted secretion signal that is cleaved to generate mature ComEA$_{Vc}$, which diffuses freely in the periplasm. TM, predicted transmembrane region. The magenta line denotes a region of unknown function, which is addressed in this study. HhH, helix-hairpin-helix motifs. Residue numbering corresponds to ComEA$_{Bs}$ or ComEA$_{Vc}$. Elements of the figure were created with BioRender.com.

- Characterize ComEA's region of unknown function (magenta region in Fig 1A)
- Elucidate the mechanism for bacterial transformation with respect to ComEA's elucidated role

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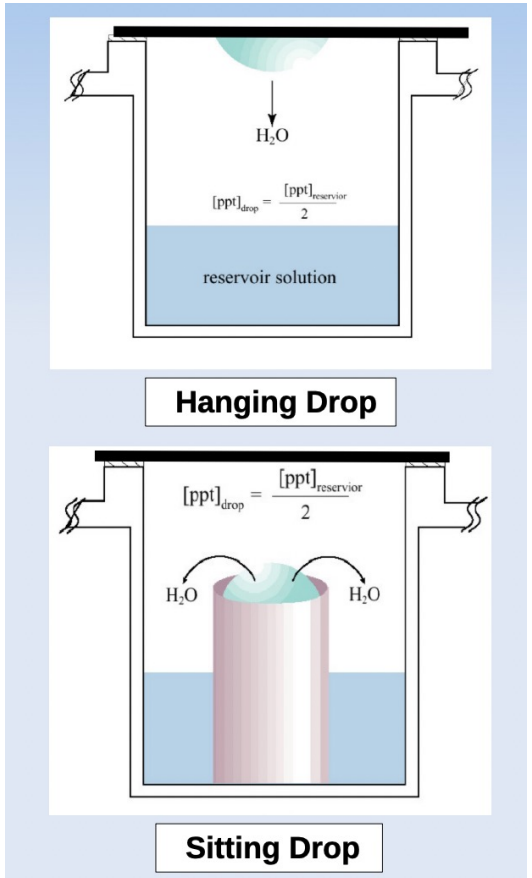
5 major conclusions

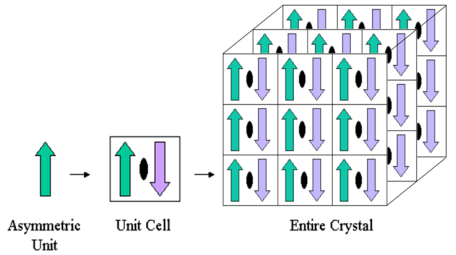
- [ ] Summary

Methods #1

LEGEND
$ComEA_{Bs}$ = ComEA from *B. subtilis*
$ComEA_{Gs}$ = ComEA from *G. stearothermophilus*

General Methods	More Detail
$ComEA_{Bs}$ production	PCR → vector → Transform E. coli → expression induced → pellet and resuspend in lysis buffer with DNase → French Press → centrifugation → His-60 column (low imidazole) → washed (low imidazole)→ protein eluted with high imidazole → SDS-PAGE → protein dialyzed → Source 15Q column → proteins pooled and concentrated twice
$ComEA_{Gs}$ production	
$ComEA_{Bs}$ SeMet derivative production	ComEABs synthesized → pET28b → pComEABs-A101M,T126M with same tag as wild type and two mutations → grown and resultant cells pelleted via wild-type protocol → pellet washed with SeMET base media → resuspended in media with SeMet → secondary inoculation grown to 0.6 OD → expression induced → grown → centrifuged → purified the same
$ComEA_{Bs}$-A101M,T126M and $ComEA_{Bs}$ crystallization and X-ray crystallography	Vapour diffusion (sitting drop) → data collected using SSRL beamline 14-1 and a CCD detector.
$ComEA_{Gs}$ crystallization and X-ray crystallography	





Results and Conclusions #1

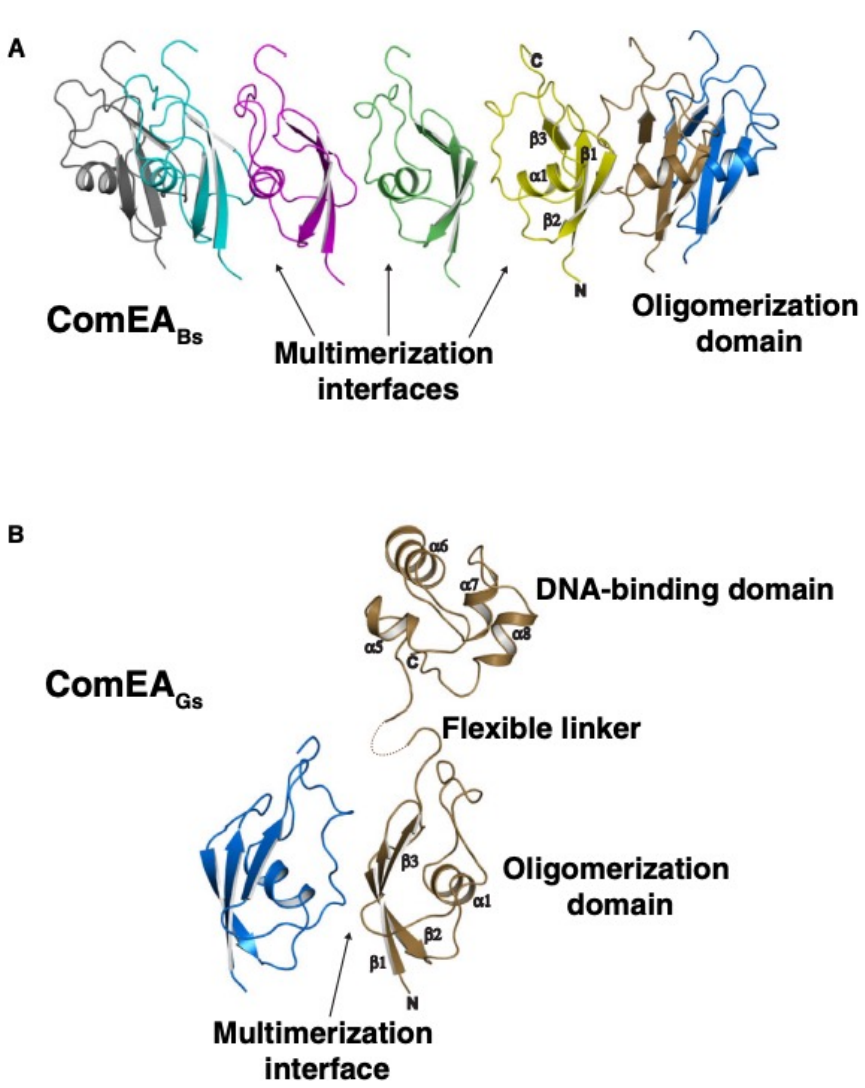


Fig. 2 | ComEA X-ray crystal structures. A) X-ray crystal structure of ComEA$_{Bs}$. One asymmetric unit containing seven protomers is shown. Arrows here point to only three of the six multimerization interfaces in the asymmetric unit. B) X-ray crystal structure of ComEA$_{Gs}$. One asymmetric unit containing two protomers is shown.

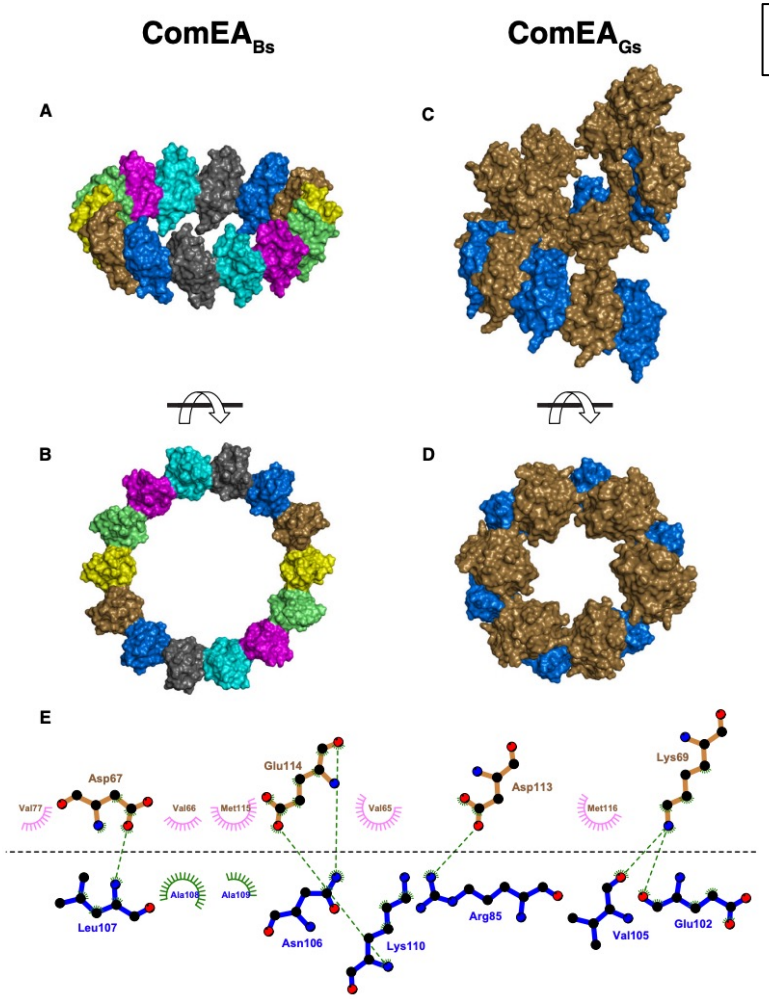
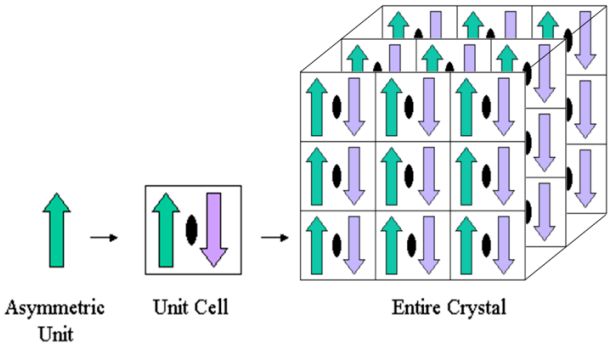


Fig. 3 | ComEA multimerization as observed in the X-ray crystal structures. **A** and **B** Front and side views of the ComEA$_{Bs}$ crystallographic ring, respectively. **C** and **D** Front and side views of the ComEA$_{Gs}$ crystallographic kinked ring, respectively. **E** Schematic representation of the ComEA$_{Gs}$ multimerization interface. ComEA chains A and B are depicted as blue and brown bonds, respectively. Hydrogen bonds are depicted as green dashed lines. Hydrophobic contacts are depicted as lines radiating from the semicircles and spheres. The schematic was produced with LigPlot+[50].

Methods #1 + Results #1 = Conclusion #1

Conclusion(s) #1

- Linker domain is flexible
- Minimal contacts between OD and DNABDs
- ODs may form multimers in solution
- ComEA may oligomerize in solution…?



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- [ ] Summary

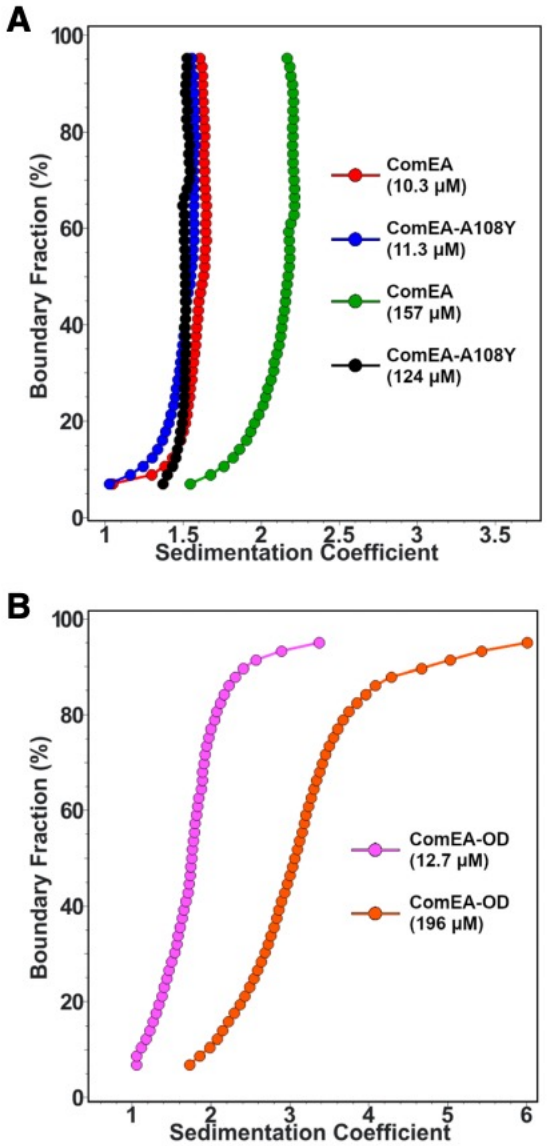
Methods #2

LEGEND
$ComEA_{Bs}$ = ComEA from *B. subtilis*
$ComEA_{Gs}$ = ComEA from *G. stearothermophilus*

General Methods	More Detail
Generation and purification of $ComEA_{Gs}$-OD	PCR → Gibson Assembly method → pTB146 vector → pComEAGs-OD construct with His-Sumo tag at N-terminus → clones verified by DNA seq → same purification protocol as wild type
Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)	▪ ComEAGs-OD measured at 37 krpm ▪ ComEAGs and DNA measured at 60 krpm ▪ 14-bp DNA sequence MW-AUC data collected at 55 krpm ▪ 40-bp DNA seq MW-AUC data collected at 43 krpm ▪ MW-AUC data recorded between 235–285 nm with 2 nm increments = 26 datasets/sample ▪ Extinction coefficient of 7,450 M-1 cm-1 at 280 nm for ComEAGs-A108Y; 5,960 M-1 cm-1 at 280 nm for ComEAGs
X-ray crystallography structure examination	

$$\left(\frac{\partial C}{\partial t}\right)_r = \frac{-1}{r}\frac{\partial}{\partial r}\left[s\,\omega^2 r^2 C - D\,r\,\frac{\partial C}{\partial r}\right]_t$$ **(Lamm Equation)**

Results and Conclusions #2



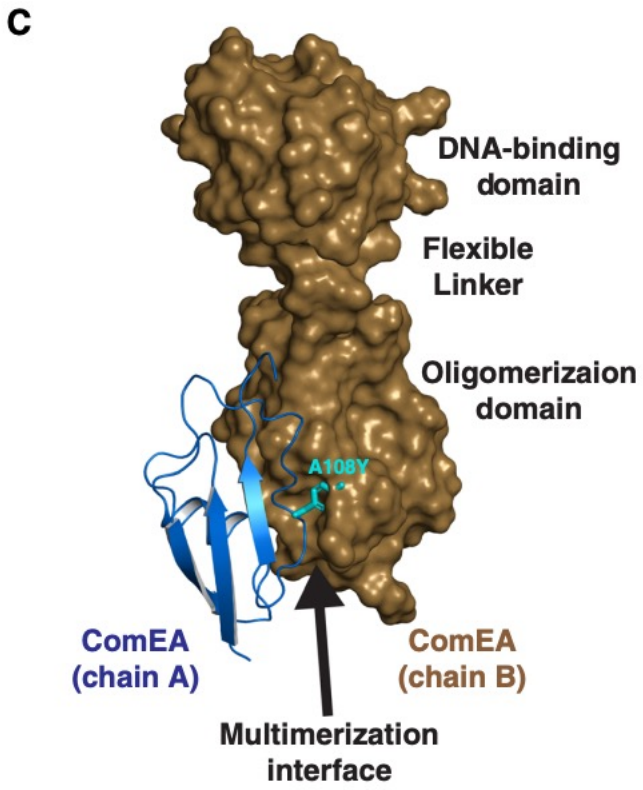


Fig. 4 | AUC analysis of ComEA, ComEA-A108Y, and the ComEA oligomerization domain. A Integral sedimentation coefficient distribution overlays comparing the dimerization potential of $ComEA_{Gs}$ at 10.3 μM (red) and 157 μM (green) and $ComEA_{Gs}$-A108Y at 11.3 μM (blue) and 124 μM (black). Only $ComEA_{Gs}$ dimerizes at higher concentration, while $ComEA_{Gs}$-A108Y remains monomeric. **B** Integral sedimentation coefficient distribution overlays of the $ComEA_{Gs}$ OD at 12.7 μM (magenta) and 196 μM (orange), showing reversible self-association. **C** Structure of $ComEA_{Gs}$ with chain A depicted as a cartoon and chain B depicted as a surface. $ComEA_{Gs}$ chain A Ala108 was mutated to Tyr and is depicted as magenta sticks.

Cyan? Not magenta?

Methods #2 + Results #2 = Conclusion #2

Conclusion #2

The OD drives ComEA multimerization in solution as predicted previously

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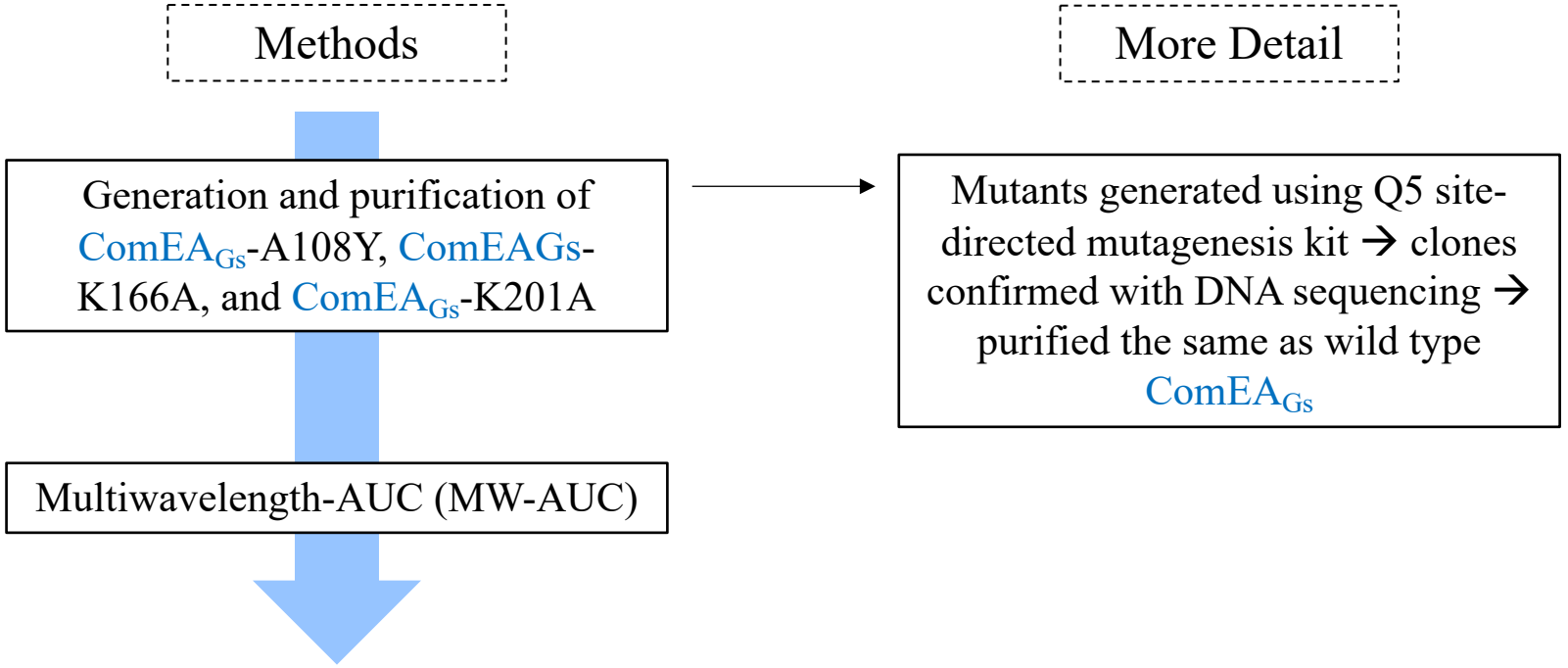

Results and Conclusions #3

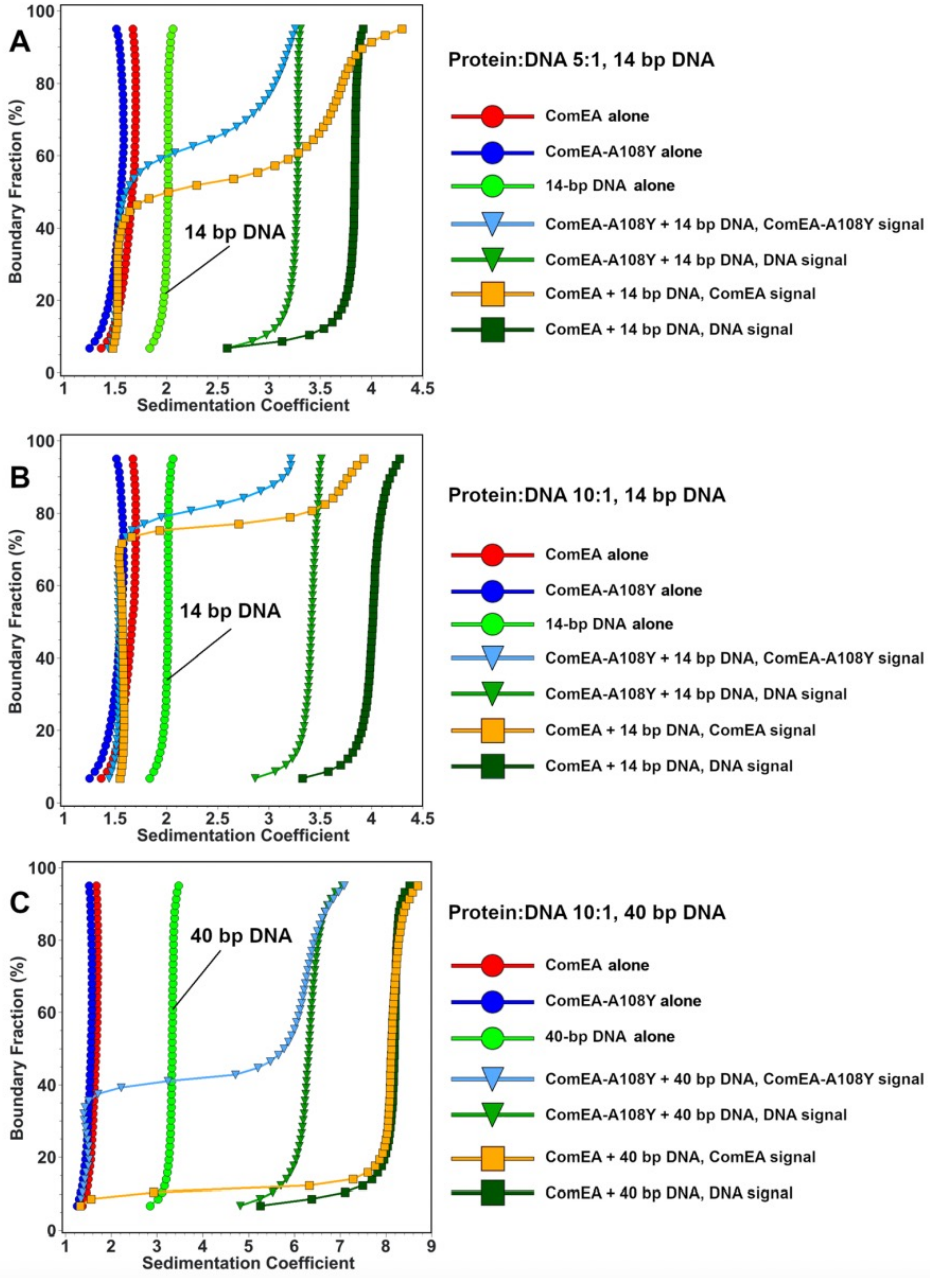

Fig. 5 | AUC analysis of ComEA and ComEA-A108Y, and their interactions with 1.5 µM DNA. A and **B** Mixtures 5:1 and 10:1 molar ratios of wild-type $ComEA_{Gs}$ and $ComEA_{Gs}$-A108Y with the 14-bp DNA molecule, respectively. Here, the DNA signals still suggest full saturation with protein, but the protein signals show more heterogeneous sedimentation coefficient distributions, consistent with more rapid exchange with the protein-DNA complex, which suggests a faster k_{off} rate. More than 90% of the DNA is complexed with protein, shifting the 14 bp DNA distribution from 2.0 S for the control by itself to 3.3 S for the mutant, and 3.9 S for the wild-type in panel A. In panel B, increasing the protein concentration to 10:1, marginally shifts the DNA sedimentation for the wild-type further to about 4.0 S, while barely affecting the DNA sedimentation when mixed with the mutant. **C** Integral sedimentation coefficient distribution overlays for the deconvoluted protein and DNA signals from the 10:1 mixture of $ComEA_{Gs}$ and $ComEA_{Gs}$-A108Y with 1.5 µM of the 40-bp DNA molecule. Unbound ComEA in the presence of DNA co-sediments with ComA in the absence of DNA. Again, more than 90% of the DNA signal shifts from the position of free 40 bp DNA at 3.3 s to a homogeneous composition at 6.3 S for the mutant, and 8.1 S for the wild-type, suggesting saturation of the DNA with ComEA. The ComEA signal closely tracks the DNA signal, suggesting a tight complex formation with a slow k_{off} rate. For all plots, reference controls of each molecule by itself are shown as circles (ComEA: red circles, ComEA-A108Y: blue circles, 14 or 40 bp DNA, as indicated: green circles), symbols for interactions between DNA and wildtype protein are shown as squares (ComEA signal: orange squares, DNA signal: dark green squares) and interactions between ComEA-A108Y and DNA are shown in triangles (ComEA-A108Y signal: olive triangles, DNA signal: light blue triangles).

Methods #3 + Results #3 = Conclusion #3

Conclusion #3

Oligomerization facilitates efficient and cooperative packing of ComEA on DNA

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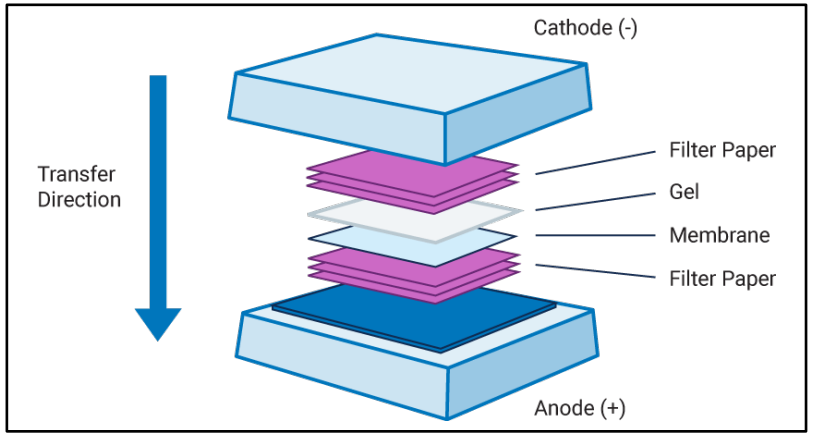
- [ ] Summary

Methods #4

5′... G▼A A T T C ...3′
3′... C T T A A▲G ...5′

EcoRI recognition sequence (palindromic RE)

Methods	More Detail
B. subtilis strain construction and transformation	▪ pED2232 cut with EcoRI → 3045 bp fragment with a YFP-comEA construct → cloned into pDR1664 with EcoRI → linearized by PvuI → transformed into IS75 → ORF deleted by insertion ▪ ComEA mutations constructed using pED2401 and Q5S-DM kit → success verified by sequencing RF → linearized by PvuI → transformed into BD5810-→ native ComEA RF inactivated again
Preparation of rhodamine-labeled DNA	Bacteriophage lambda DNA was labelled with a rhodamine TM reagent and put through a G50 spin coloumn to remove excess label
Semi-dry Western Blotting	

Results and Conclusions #4

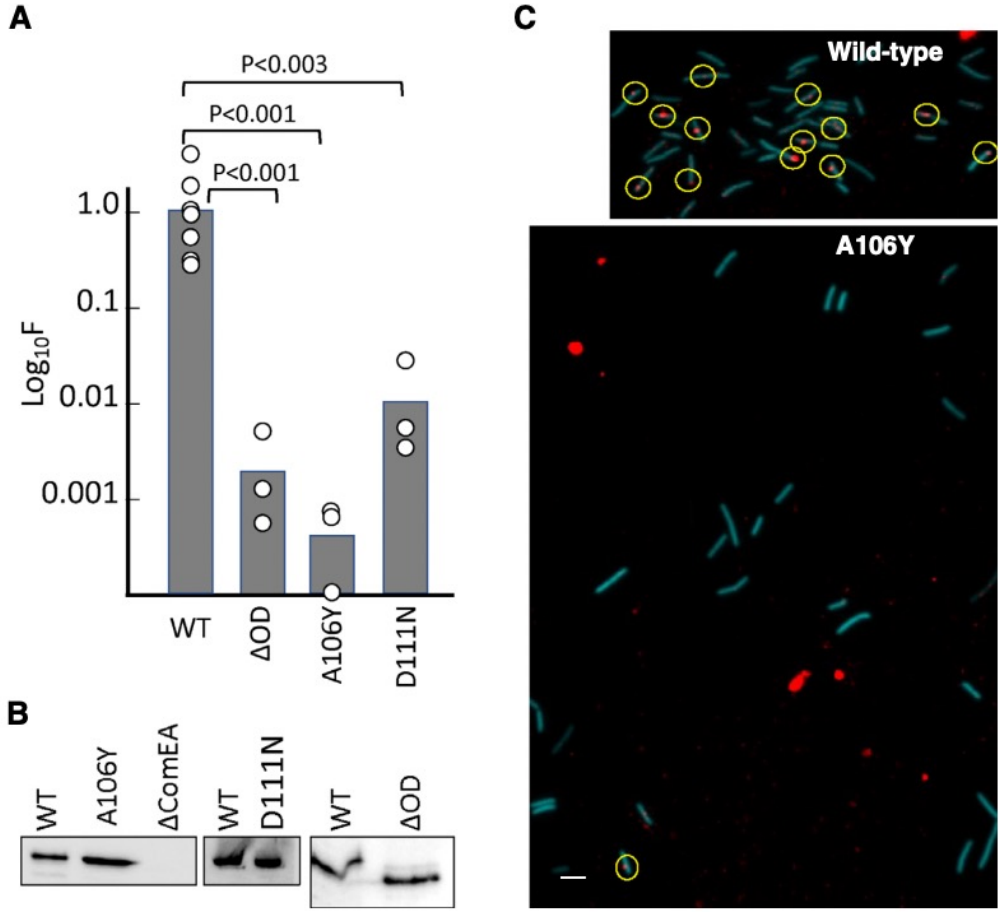


Fig. 6 | Self-association of ComEA is required for transformation. All mutations were introduced into the chromosome of BD9007, which carries a competence-specific fusion of a sequence encoding cyan fluorescent protein (CFP) expressed from the promoter of *comG*, placed at the ectopic *amyE* locus to enable the identification of competence-expressing cells by epifluorescence microscopy. Transformation experiments were performed as biological triplicates and average frequencies, normalized to the wild-type values are plotted as bar graphs in panel **A** with the data points included. The larger number of data points for the wild-type control reflects the inclusion of this strain in all three biological replicates. The mean normalized values with standard deviations for the mutants were 0.0012 ± 0.00047 (ΔOD), 0.0.0005 ± 0.00024 (A106Y), 0.11 ± 0.03 (D111N). The *p* values were determined using two-sided t-tests. Panel **B** shows Western blots for the corresponding wild-type and mutant extracts using anti-GFP antiserum. *ΔcomEA* extracts were included as controls for the identity of the ComEA signals. Panel **C** shows typical epifluorescent images from the wild-type (BD9007) and its isogenic A106Y mutant equivalent, after transformation with rhodamine-labeled lambda bacteriophage DNA for 45-min. Competence-expressing cells were identified by CFP fluorescence (pseudocolored cyan) and detectable cell-associated rhodamine-tDNA signals are circled. For the wild type, 13 out of 42 cells exhibited at least one red dot, while for the 23 mutant cells only one barely detectable cell-associated dot was observed. The large red blotches in the lower panel are not associated with cells and are due to contaminating fluorescent material of unknown origin. The scale bar on the lower left of the image corresponds to 1 micron. The microscopy and Western blotting experiments in panels **B** and **C** were each repeated three times with closely similar results. Source data are provided as a Source Data file.

Methods #4 + Results #4 = Conclusion #4

Conclusion #4

ComEA oligomerization plays a critical role in the transformation of *B. subtilis* and likely other Gram-positives

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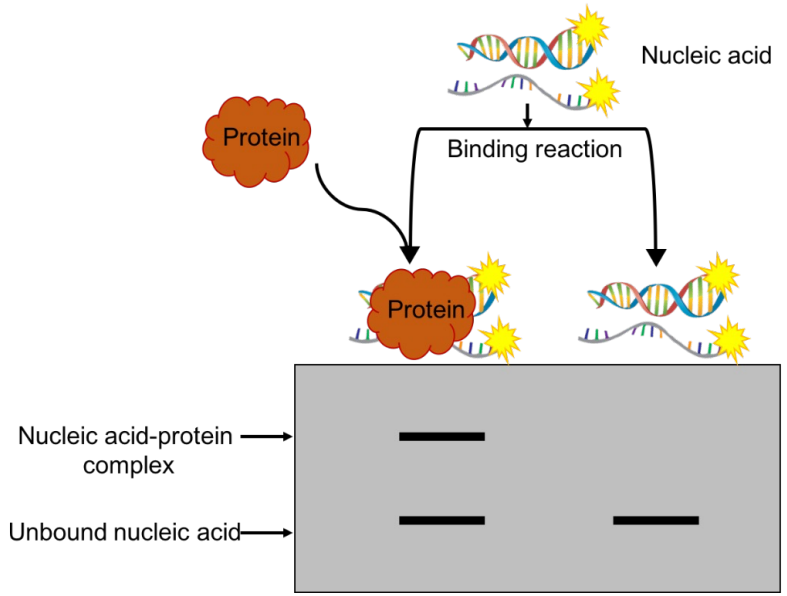
Methods #5

LEGEND
$ComEA_{Bs}$ = ComEA from *B. subtilis*
$ComEA_{Gs}$ = ComEA from *G. stearothermophilus*

Methods	More Detail
Computational modelling of $ComEA_{Gs}$-DNA	▪ Dali Server20 to ID the DNA bound portion of XPF as similar to that of $ComEA_{Gs}$ ▪ HADDOCK to refine the aligned model
Western Blot	
Electrophoretic mobility shift assay (EMSA)	$ComEA_{Gs}$ and 30bp DNA mixed in a 10mM Tris, pH 8, 100 mM KCl, 2.5% glycerol, 0.5 mM EDTA, and 1mM $MgCl_2$ buffer → 0.5 volumes of loading dye with bromophenol blue and 2.5% glycerol added → 8% native polyacrylamide get prerun at 4 ºC in 0.5X TBE → ethidium bromide stained → scanned using BioRad MP imager



Results and Conclusions #5

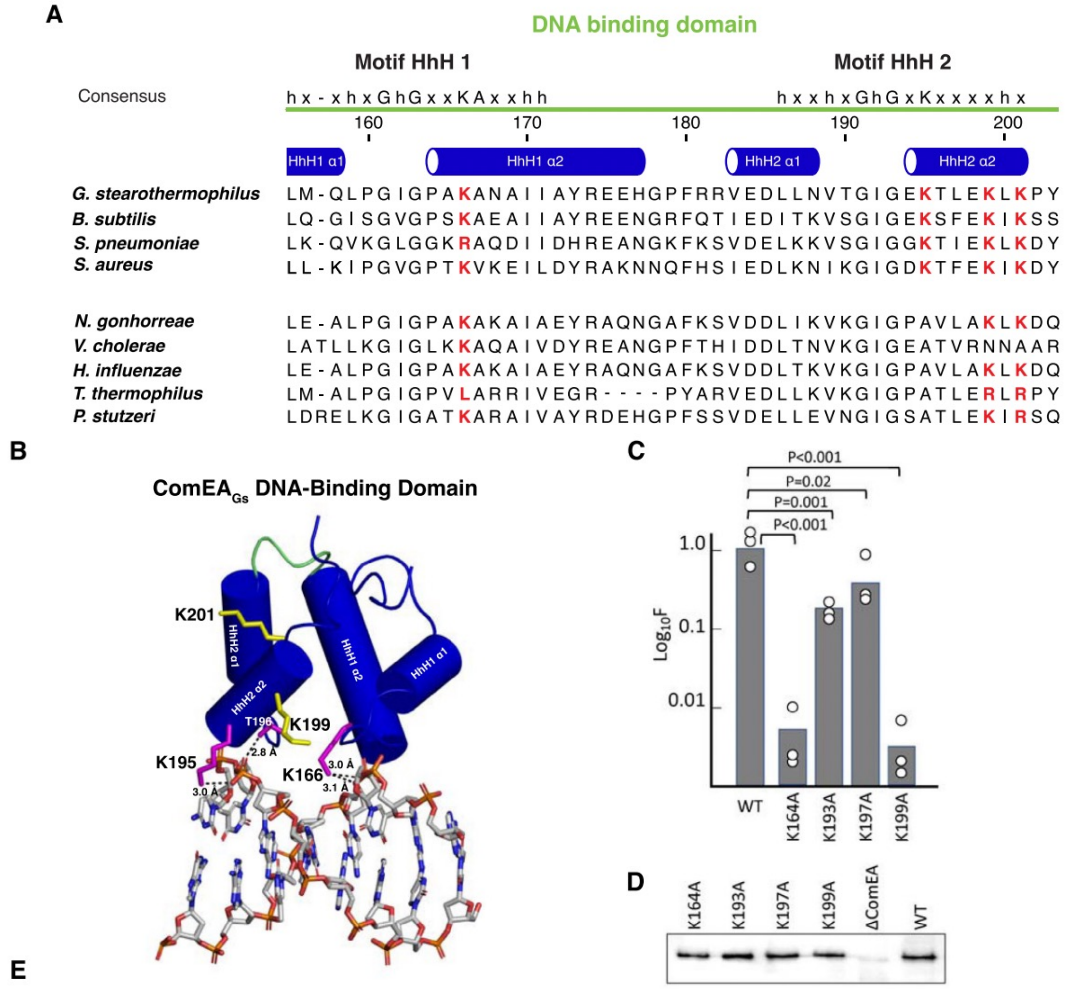


Fig. 7 | DNA binding to ComEA for transformation. A Alignments of the HhH motifs from ComEA proteins expressed by four Gram-positive (top) and Gram-negative (bottom) bacteria that have been used for transformation studies. The four lysine residues chosen for mutagenesis are highlighted in red. Secondary structure assignments were derived from the crystal structure of ComEA$_{Gs}$. **B** Model of the ComEA$_{Gs}$ DNA-binding domain (α-helices depicted as cylinders) in complex with dsDNA (depicted as sticks). The loop connecting the HhH1 and HhH2 is colored green. Hydrogen bonds are depicted as black dashed lines. For clarity, ComEA$_{Gs}$ is depicted in complex with 8 bp central to the complex rather than the 15 bp used in refinement. **C** Transformation frequencies for the HhH motif mutants. Transformation experiments were performed as biological triplicates and average frequencies, normalized to the wild-type (BD9007) values are plotted as bar graphs with the data points included. The mean normalized values with standard deviations for the mutants were 0.0054 ± 0.004 (K164A), 0.23 ± 0.066 (K193A), 0.54 ± 0.22 (K197A), 0.0031 ± 0.0025 (K199A). The p values were determined using two-sided t-tests. **D** shows Western blots for the wild-type and mutant ComEA proteins obtained using anti-GFP antibody. This Western blot experiment was repeated a total of three times with nearly identical results. **E** EMSA analysis of wild-type ComEA$_{Gs}$, ComEA-K166A, and ComEA-K201A. The EMSA analysis was repeated at least two times with nearly identical results. Source data are provided as a Source Data file.

Methods #5 + Results #5 = Conclusion #5

Conclusion(s) #5

ComEA-DNA interactions are cooperative in binding behaviour and are required for transformation

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Summary

Conclusion(s) #1

- Linker domain is flexible
- Minimal contacts between OD and DNABDs
- ODs may form multimers in solution
- ComEA may oligomerize in solution…?

Conclusion #2

The OD drives ComEA multimerization in solution as predicted previously

Conclusion #3

Oligomerization facilitates efficient and cooperative packing of ComEA on DNA

Conclusion #4

ComEA oligomerization plays a critical role in the transformation of *B. subtilis* and likely other Gram-positives

Conclusion(s) #5

ComEA-DNA interactions are cooperative in binding behaviour and are required for transformation

Final Proposition

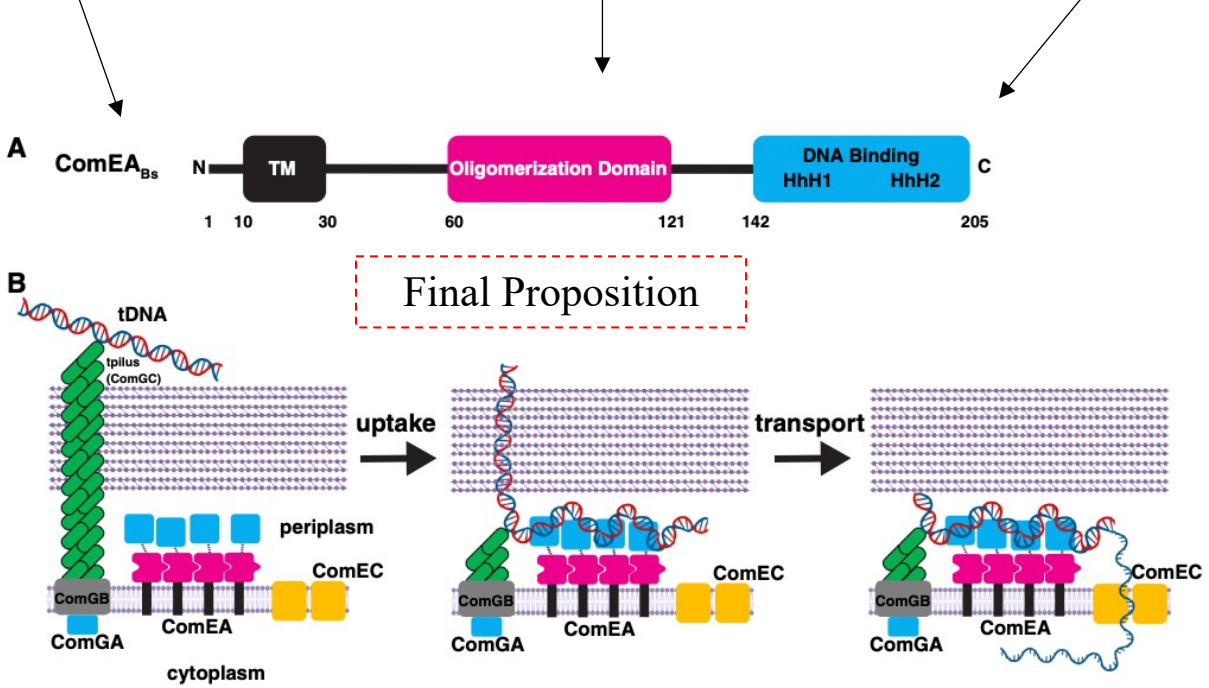

Fig. 8 | ComEA$_{Bs}$ domain architecture, and proposed model of genetic transformation in Gram-positive bacteria. A ComEA$_{Bs}$ contains an N-terminal transmembrane region, a previously unidentified OD, and a C-terminal DNA-binding domain. **B** We have shown that ComEA self-associates using contacts in its ODs. After retraction of the tpilus to bring a loop of DNA into the periplasm, binding of tDNA to the ComEA DNA-binding domains followed by uptake stabilize cell association, while cross-linking of distal DNA segments by binding to adjacent ComEA molecules condenses the incoming tDNA, exerting a pulling force to bring tDNA into the periplasm. Elements of the figure were created with BioRender.com.

Presentation Format

- [x] Background
  - Bacterial Transformation
  - ComEA
  - Objectives of Study
- [x] Methods/Results/Co[illegible]ions
  - X-ray structures [illegible]OD
  - [illegible]mEA oligom[illegible]s in solution
  - [illegible]oligo[illegible]zes on DNA
  - Co[illegible]rization is required for transfo[illegible]
  - ComEA-DN[illegible]nteractions are required for transformation
- [x] Summary

#1
#2
#3
#4
#5

Works Cited

Citation for Main Article

- Ahmed, I., Hahn, J., Henrickson, A., Khaja, F. T., Demeler, B., Dubnau, D., & Neiditch, M. B. (2022). Structure-function studies reveal ComEA contains an oligomerization domain essential for transformation in gram-positive bacteria. *Nature communications*, *13*(1), 7724.

Other Sources Referred to for Background

- Provvedi, R. & Dubnau, D. (1999). ComEA is a DNA receptor for transformation of competent Bacillus subtilis. Mol. Microbiol *31*, 271–280.
- Steven Mosimann Biochemistry 4000 Lecture Notes (Fall 2022)
- https://pdb101.rcsb.org/learn/guide-to-understanding-pdb-data/biological-assemblies
- Borries Demeler Biophysics 4850 Lecture Notes (Spring 2023)

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Questions