R27 pilus biogenesis is promiscuous and electrostatic perturbations impair functionality

Running title: Surface electrostatic potential affects mating pilus functionality

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Abstract

During conjugation, plasmid DNA is transferred from donor to recipient bacteria via the plasmid-encoded mating pilus, built as thin helical assemblies of polymerised pilin subunits. The structure of the IncHI1 R27 plasmid encoded pilus revealed that the mature ThrA pilin is cyclized by a peptide bond formed between Gly1 (G1) and Asp69 (D69). Loss of cyclization abolished pilus biogenesis and conjugation. Here we report that G1 and D69 are completely conserved in all TrhA pilins in the Plascad database. Substituting D69 with N, A, G or R showed that cyclization is a promiscuous process as all mutants cyclised and polymerised into pili. However, the D69R substitution specifically altered the electrostatic potential of the pilus surface, resulting in very low conjugation efficiency. Moreover, conjugation efficiency of the other substitutions into *Escherichia coli* MG1655, Enteropathogenic *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae, Citrobacter rodentium* and *Citrobacter amalonaticus* showed a consistent trend towards correlation with the side chain size: TrhA D69N > D69A > D69G. These results suggest that while cyclisation is promiscuous, there is a strong selective pressure to maintain D as the carboxy terminal residue for TrhA cyclisation.

Introduction

Bacterial evolution is driven by random mutations and horizontal gene transfer (HGT) followed by natural selection. HGT is mediated by transformation, transduction and conjugation [1,2]. Conjugation, first discovered in 1946 using the F plasmid [3], facilitates the acquisition and dissemination of metabolic, virulence and antibiotic resistance genes (ARGs) [4]. Conjugation takes place in a broad range of environments, including soil, aquatic ecosystem and the lumen of the gut. In contrast to transformation and transduction, during conjugation DNA is transferred unidirectionally from one bacterium (donor) to another (recipient) in a contact-dependent manner [5,6].

Conjugative plasmids are found in both Gram-negative and Gram-positive bacteria [5]. Multiple classification systems have been used to categorise different plasmid-encoded conjugation systems, including a relaxase phylogenetic diversity, which defines nine mobility (MOB) groups [7], and mating pair formation (MPF) classification which defines four groups: MPFF, MPFG, MPFI and MPFT [8]. Plasmids in the MPFI group belong to the incompatibility (Inc) groups IncI and IncL/M, plasmids in the MPFT group belong to IncN, IncP and IncW, and those in the MPFF group belong to IncA, IncC, IncF and IncH.

The MPFF conjugation process is divided into three phases [8,9]**.** Phase 1 occurs exclusively within the donor, which assembles the type IV secretion system injectisome [10] and the mating pili, built as a thin helical pilin assembly with phospholipid molecules [11–13]. Phase 2 involves both the donor and recipient, where initial contacts are mediated by the pilus in the donor and an unknown receptor on the recipient, a process called mating pair formation (MPF) [5,6,14]. During MPF plasmid DNA is mobilised from the donor to the recipient in low efficiency [5,15]. MPF-mediated contact is believed to induce retraction of the pilus, bringing the donor and recipient into proximity through a process called mating pair stabilisation (MPS) [5,16]. MPS-mediated intimate contact enables efficient plasmid transfer [14]. Phase 3 occurs exclusively within the recipient, which following plasmid entry starts to express plasmid genes involved in anti-defence systems and exclusion [17,18].

Recently, using a de-repress and chloramphenicol resistant derivative of the reference IncHI plasmid, R27 (drR27), we determined the cryo-EM structure of the H-pilus at 2.2 Å resolution [12]. Like the pOX38/pED208/pKpQIL (IncF) [11–13], R388 (IncW) [19] and pKM101 (IncN) [20], the H-pilin subunits, TrhA, form helical assemblies with phospholipid molecules at a stoichiometric ratio of 1:1 [12]. Uniquely, we found that unlike all other pilin structures, which adopt a hairpin topology, TrhA forms a cyclic structure. We have shown that cyclisation is mediated following cleavage of the N-terminal signal peptide and the five C-terminal residues of the pre-pilin. Cyclization occurs via condensation of the amine and carboxyl residues on Gly1 (G1) and Asp69 (D69) to generate the mature pilin. Conjugation assays and negative staining EM revealed that cyclization is essential for biogenesis of a functional pilus, as the non-cyclic $TrhA_{\Delta 67\text{-}74}$ did not assemble into a pilus [12]. Previous biochemical studies have shown that the RP4 plasmid (IncP) pilin TrbC also forms a cyclic structure [21]. We have recently confirmed the cyclic nature of TrbC by cryo-EM [22]. Cyclisation occurs via formation of a peptide bond between Ser1 (S1) and Gly78 (G78) of the mature pilin [21,22]. Substitutions S1A/G/T or G114A/S did not affect TrbC cyclisation while TrbC G114C/L/T did not cyclise, resulting in loss of pilus biogenesis and DNA transfer [23].

Complementing our previous work probing the effect of loss of cyclisation on pilus biogenesis, the aim of this study was to determine the role of D69 in mediating TrhA cyclisation. Comparing the available 147 TrhA sequences in the Plascad database revealed that G1 and D69 are 100% conserved. We constructed four site directed pilin mutants, D69N, D69A, D69G, and D69R. We show that ThrA cyclisation is promiscuous as all four pilin mutants cyclised and produced a pilus, the structure of which is indistinguishable from the wildtype pilus. Functionally, while the TrhA D69R polymerised into a pilus, it mediated significantly low conjugation frequency. In contrast, pili made by the other TrhA D69 substitutions showed a trend towards a gradient of conjugation efficiency correlated with the amino acid side chain size, N>A>G. Together, these data show that while pilin cyclisation

and pilus biogenesis is promiscuous, the size and charge of the distal amino acid play a significant role in conjunction efficiency.

Results and Discussion

Distribution of TrhA variants amongst the MPFF plasmids

We assessed the sequence diversity of TrhA proteins among 148 plasmids carrying IncH replicons from the Plascad database [24]. Some of these plasmids represent "fusion" plasmids in which IncH replicons were found together with replicons from different incompatibility groups (e.g. IncF). Using a tBLASTn approach with TrhA from R27 as the query sequence, we identified a single copy of TrhA in each of the plasmids, except for one plasmid in which the gene was found fragmented (accession NZ_CP021209.1).

We next used the alignment to construct a phylogenetic tree of the 147 TrhA protein sequences (Fig. 1A; https://microreact.org/project/trhA). The tree shows that TrhA sequences are divided into three main homology groupings, each of which are associated with distinct IncH replicon subtypes. The first group, which contains TrhA(R27), are largely from IncHI1A/IncHI1B(R27) plasmids found among *Escherichia coli* and *Salmonella enterica*, most of which possess ≥98% protein similarity to TrhA(R27). Another group contains TrhA from IncHI2/IncHI2A plasmids that are associated with diverse Enterobacteriaceae species and share ~66% protein similarity with TrhA(R27). The final group contains TrhA sequences that are almost exclusively from *Klebsiella pneumoniae* plasmids with IncHI1B (and IncFIB(Mar)) replicons and which share ~55% protein similarity with TrhA(R27). An alignment of the 147 TrhA pilin showed high level of similarity, including 100% conservation of the Gly1 and Asp69 residues within the mature protein sequence (Fig. 1B).

H pilus cyclisation is promiscuous

As G1 and D69 are 100% conserved amongst the 147 TrhA pilins, we hypothesised that any substitution of D69 will interfere with cyclisation. To test this, we made four site directed TrhA C-terminal substitution mutants: D69N, D69A, D69G and D69R. The pili were purified and cryo-EM analyses confirmed that similarly to the wildtype ThrA, all mutant pilins cyclised and assembled into indistinguishable pili structures (Fig. 2). All the mutants display the same pilus inner diameter, 22 Å, which is identical to the wildtype pilus.

This suggests that cyclisation of the TrhA pilin is a promiscuous process, similarly to that shown for the cyclic RP4 TrbC pilin [21]. As cyclisation of ThrA involves cleaving the carboxy terminal five amino acids and bringing the N- and C- termini of the mature pilin into proximity, the promiscuous nature of the process implies low specificity of protease/cyclase involves recognition of N- and C- binding sites in TrhA. While TrbF is the protease/cyclase involves cyclisation of TrbC [23], the identity of the protease/cyclase involved in cyclisation of TrhA is not yet known.

The D69 substitutions did not alter the pilin:lipid ratio, which was maintained at 1:1, or the conformation of the bound lipid (Fig. 2). Electrostatic analysis revealed that loss of the negative charge from the D69 N/A/G substitutions specifically alters the electrostatic potential of the pilus surface, but did not affect the lumen. Similarly, the D69R substitution did not alter the overall lumen electrostatic potential, but it introduces a strong positive charge on the pilus exterior (Fig. 3). Mapping the side chains onto the pilus structure, it is apparent that the side chain of D69 projects away from the surface, explaining why the mutants do not alter the electrostatic potential of the lumen (Fig. 4 and Supplementary Figure 1).

Carboxy terminal amino acid sidechain size and charge affect conjugation efficiency

To determine the functional consequences of the amino acid substitutions, we performed conjugation assays using an auxotrophic *E. coli* MG1655 Δ*trp* harbouring wildtype drR27 ($R27_{WT}$), $R27_{D69N}$, $R27_{D69A}$, $R27_{D69G}$ or $R27_{D69R}$ as donors and *E. coli* MG1655 (Δ*fim*) [12], enteropathogenic *E. coli* (EPEC O127:H7) [25], *Klebsiella pneumoniae* (ICC8001) [26,27], *Enterobacter cloacae* (ATCC 13047) [27], *Citrobacter rodentium* (ICC169) [28] and *C. amalonaticus* (ICC3000) [29] as recipients, as described [12]. As expression of the conjugation operon in IncH plasmid is thermoregulated [30], we performed conjugation assays at 25 ℃. The frequency of $R27_{D69R}$ conjugation was significantly lower than $R27_{WT}$. We hypothesise that the positively charged $R27_{D69R}$ pilus surface forms electrostatic interactions with the

negatively charged outer membrane of the recipient, which disturb placing the tip into a conjugation permissive position.

In contrast to $R27_{D69R}$, the conjugation efficiency of $R27_{D69N}$, $R27_{D69A}$ and $R27_{D69G}$ into *E. coli* MG1655 and EPEC was equivalent. In contrast, conjugation efficiency into *K. pneumoniae, E. cloacae, C. rodentium* and *C. amalonaticus* recipients showed a trend towards direct correlation between the side chain size (i.e. the bigger the side chain the greater the conjugation efficiently), which was most prominently seen in *C. amalonaticus* (Fig. 5, Table 1).

While most conjugation pilus structures reported today showed that the pilin has a hairpin topology, we recently reported that ThrA encoded by R27 and TrbC encoded by RP4 plasmids are cyclic [12,21,22]. Importantly, while TrbC G114A/S pilins cyclised and mediated conjugation as efficiently as wild type TrbC, TrbC G114C/L/T did not cyclase or polymerised into a pilus [20]. As such, it is not known if the side chain size rule, we observed in TrhA, is also applicable to TrbC.

Our, as well as published [20,23,31], data suggest that the TrhA and TrbC proteases/cyclases are to some degree promiscuous, as a functional mating pilus could be formed when the proximal or distal amino acids forming the peptide bond are replaced. However, analysis of 147 TrhA proteins from IncH plasmids has shown that while they are divided into three distinct sequence homology groups, D69 is fully conserved. This suggests that selective pressure maintains D69, despite amino acid substitutions in this position resulting in functional pili.

While the prevailing model suggests that the lipid moiety is essential for pili biogenesis and for repelling the negatively charged DNA during transfer of the relaxase-ssDNA complex [11,32,33], we have recently reported that the RP4 pilus, made by a cyclic pilin in the absence of lipid, is functional [22]. This raises the possibility that coating the lumen in negative charge might not be essential for conjugation. Indeed, in the case of the T pilus, encoded by the Ti plasmids of *Agrobacterium tumefaciens*, the lumen is coated by a positive charge due to the

presence of a PE phospholipid [21,23,31]. In fact, here we report that the exterior charge of the pilus affects conjugation as the ThrA D69R substitution, which resulted in pilus biogenesis, mediated conjugation at frequency of ca 10^6 6 lower than D69R.

We found that both amino acids implicated in cyclisation, G1 and D69, are surface exposed and that the positively charged R69 project further from the pilus shaft. We hypothesise that the positively charged surface forms electrostatic interactions with the negatively charged outer membrane of the recipient. This interrupts the process of bringing the pilus tip into a conjugation permissive position for canonical MPF and subsequent plasmid transfer. While the initial stages of pilus driven MPF formation is unclear, our observation suggests that the pilus surface charge and the size of the exposed side chains play central role.

Declaration of interests

The authors declare no competing interests.

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Author contributions

KB and GF conceived and supervised the study. SH performed conjugation assays. JLCW performed mutagenesis. NI purified the RP4 pilus, determined the cryo-EM structure and

performed model building and refinement. SD perfomed bioinformatic analysis. NI, GF and KB analyzed the structures. KB and GF wrote the manuscript with input from all authors.

Data availability

The cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession code EMD-65235 (D69N), EMD-65234 (D69A), EMD-65250 (D69G) and EMD-65236 (D69R). The structural coordinates have been deposited to the RCSB Protein Data Bank (PDB) under the accession code 9VP3 (D69N), 9VP2 (D69A), 9VPE (D69G) and 9VP4 (D69R).

Methods

Identification and phylogenetic analysis of TrhA homologues

We first identified publicly-available plasmids from the mating pair formation "F" (MPFF) group in the Plascad database [24]. We then used Plasmidfinder (database version 29/11/2021) [34] to identify the subset of these that carry IncH replicons. The TrhA protein sequence from the R27 plasmid (accession AF250878.1) was used as a query in a tBLASTn with BLAST+ v2.14.1 [35] in order to identify homologues among the IncH plasmids. The resulting hits were aligned using MUSCLE v5.1.0 [36]. RAxML-NG v1.2.0 [37] was used to generate a maximum likelihood phylogenetic tree from the alignment. The phylogenetic tree was visualised together with metadata using Microreact [38].

Site directed mutagenesis

We previously constructed an open reading frame deletion of *thrA* on an R27 background (R27Δ*trhA*) [12]. Here we generated cloned the wild-type *trhA* gene into a mutagenesis plasmid (pSEVA612) and using site directed mutagenesis to change the D69 codon to that encoding N, A and R. These were inserted into the R27Δ*trhA* plasmid by homologous recombination [14]. Plasmids were constructed by Gibson Assembly (NEB) and a one-step KLD reaction used to introduce mutations. All mutations were confirmed by Sanger sequencing (Eurofins genomics).

Primers

F_WT_trhA gttatccctaagtgacttttacgataagc
R_WT_trhA ctctattcttgatggagctagggataac
F_D69_SDM gctggtattcctctgtga
R_69N_SDM GTTcaggaacgagctga
R_69R_SDM GCGcaggaacgagctga
R_D69A_SDM CGCcaggaacgagctg
R_D69G_SDM GCCcaggaacgagctgata

Bacterial Conjugation

E. coli MG1655 Δ*trp* containing the derepressed *htdA* R27 mutant (drR27) [12], drR27$trhA_{D69A}$, drR27$trhA_{D69G}$, drR27$trhA_{D69N}$, drR27$trhA_{D69R}$, were used as donor; multiple bacterial species

were used as recipients. The donor and recipients were grown overnight in LB at 37°C, mixed in a ratio of 10:1, diluted 1 in 25 in PBS and 40 µL spotted onto LB agar plates (no selection). After incubation at 37°C for 3 h, the plates were moved to 25°C and incubated overnight. The conjugation mixture was then resuspended in 1 mL of PBS and serial dilution were plated on a M9 salts minimal media containing 4% glucose plates for selection of recipients and M9 salts minimal media containing 4% glucose and 30 µg/mL chloramphenicol selection plate for selection of transconjugants before incubating at 37°C for 24 h. Data are presented as proportions of transconjugants/recipients. All conjugation experiments were done in three technical repeats and at least three biological repeats. The conjugation data were analysed by one-way Anova.

Expression and Purification of H-Pilus mutants

The H-pilus mutants were purified as we recently described [12]. Briefly, *E. coli* MG1655Δ*fimA* containing the R27 mutants was grown in 10 to 20 mL LB media with 10 µg/mL Chloramphenicol at 37 °C overnight. 10 mL were used to inoculate 1 L LB media, which was incubated with shaking overnight at 25°C. The following steps were performed at 4 °C. The bacterial cells were harvested by centrifugation at 7,000×*g* for 20 min and the pellet was resuspended in 20 mL PBS. The resuspended bacterial cells were shaved through 25 G needles 25~30 times and centrifuged at 50,000×g for 1 h. The supernatant was collected, and PEG 6,000 was added at a final concentration of 5% with stirring for 1 h, which was followed by centrifugation at 50,000×*g* for 30 min. The supernatant was poured, and the pellet resuspended in 200 µL imaging buffer (50 mM Tris-HCL pH 8, 200 mM NaCl). The solution containing the filaments was dialyzed overnight in imaging buffer. Sample purity was judged by SDS-PAGE.

Grid Preparation and Cryo-EM Data Collection

Before preparing the cryoEM grid, the purified sample was centrifuged at 41,000 rpm for 30 min, and the pellet was resuspended with imaging buffer. 3 µL of the purified sample at ~4 mg/mL was applied to a glow-discharged carbon grid (Quantifoil R1.2/1.3, Cu, 300 mesh).

The grid was blotted with a blotting force of 0 for 3 s at 4 °C and 100% humidity, and flash-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific). The frozen grids were stored in liquid nitrogen until data collection. Cryo-EM data were collected on 200kV Glacios 2 (Thermo Fisher Scientific) with a Falcon 4 detector. A total of 200 – 711 movies were collected for each mutant dataset at a magnification of ×120,000 (pixel size of 1.192 Å/pixel) with a total electron dose of 46-48 e/Å2. The defocus range was set between −0.8 and −1.8 µm. All data were automatically acquired using EPU software. The data collection parameters are summarised in Table S1.

Cryo-EM Data Processing and Model Building

The data were processed using cryoSPARC (v.4.6.1) [39]. Each mutant dataset was collected and analysed independently. Every dataset was processed following similar steps (Supplementary Figure 2). Motion correction was performed using Patch Motion Correction, and CTF estimation for micrographs was done by Patch CTF estimation [40]. Particles were automatically picked using a Filament tracer without templates and extracted in a binned state at 4.76 Å/pix. The extracted particles were classified in 2D, and selected high-quality particles were used as references for the Filament tracer. 2D classification of particles from each dataset was selected. Particles from a suitable 2D class were used for Helical refinement without helical parameters. Then, using the initial helical 3D map, the average power spectrum was calculated by helical symmetrical search. Symmetrical search job suggested an axial rise of ~12.2 Å and a twist of ~28.9°. These symmetry parameters were used as starting parameters for helical reconstruction using Helical refinement. The final H-pilus mutant helical reconstructions were shown 2.8-3.1 Å resolution with a rise of ~12.2 Å and a twist of ~29.0°.

The initial model of every mutant was based on PDBID: 9VHC. Real space refinement was performed in PHENIX [41]. The model was subjected to iterative cycles of refinement and manual rebuilding in COOT [42]. The structural model was validated using MolProbity [43]. The processing parameters and refinement statistics are summarized in in Table S1.

Figure legends

Fig. 1. A. Midpoint-rooted phylogenetic tree of 147 TrhA homologues from IncH plasmids. The tree tips show the percent identity of each TrhA sequence with respect to TrhA(R27). Metadata columns show the host genus of the associated plasmid and the presence/absence of matches to individual IncH replicons. The scale bar shows the number of substitutions per site. A similar visualisation can be accessed via Microreact: https://microreact.org/project/trha. B. Conservation of the TrhA pilin amino acids from the phylogenetic tree were mapped onto the cryo-EM structure (PDB ID: 9HVC). There is a high degree of conservation amongst the different pilins. G1 and D69 are indicated.

Fig. 2. Cryo-EM structure of the D69 N/A/G/R ThrA substitutions. The four mutants can cyclise and assemble into a pilus. The cryo-EM maps of the reconstructed pilus are shown in grey with a single pilin subunit shown in different colours for each of the mutants. The maps unambiguously show that the mutants can cyclise. A ThrA mutant pilin subunit (shown in sticks) is placed in the map and the cyclization residues labelled. Each pilin is associated with one PG lipid (shown in grey sticks).

Fig. 3. Electrostatic potential of the ThrA D69 N/A/G/R ThrA substitutions. The substitutions alter the charge profile of the pilus surface (left panel) but not its lumen (middle panel). The D69R results in a strong positive charge between pilin subunits. A top view (right panel) highlights how loss of the negative or gain of a positive charge specifically impacts the exterior of the pilus. The coulombic electrostatic potential is displayed as red and blue isosurfaces at levels -10 and +10, respectively; the potential is in units of kcal/(mol·*e*) at 298 K.

Fig. 4. The side chain of the cyclised C-terminal residue points on the pilus surface. Upon cyclisation, the side chain of D69 in both the (a) $ThrA_{D69}$ and (b) $ThrA_{D69R}$, is pointing on the

surface of the pilus that significantly changes the surface electrostatic potential but not of the lumen (see Fig. 3). The PG lipid (shown in blue) headgroup is pointing in the interior. The ThrA pilin is shown in grey. The key residues of a single ThrA pilin are labelled.

Fig. 5. Conjugation via TrhA WT and D69 substations into various recipients. Compared to wildtype ThrA, conjugation efficiency via the mutant TrhAs follows a gradient paralleling the sidechain size (N>A>G). Minimal conjugation was seen via TrhA D69R.

Table 1. Summary of the effect of the D69 substitutions on pilus biogenesis and conjugation efficiency.

		*Conjugation efficiency					
$ThrA_{D69X}$	pilus	Ec	Ep	Kp	Ecl	Cr	Ca
WT	+	-0.34031	-0.50265	-0.37063	-2.94605	-0.69875	-1.32275
N	+	-0.36666	-0.31927	-0.56222	-2.87298	-0.94061	-1.83475
A	+	-0.35164	-0.34794	-0.61797	-3.72521	-1.32666	-2.5947
G	+	-0.37401	-0.68352	-1.01001	-4.11923	-1.87622	-2.78699
R	+	-4.53783	-4.91391	-4.47619	-5.37201	-5.33167	-6.30431

*Log_{10} transformed conjugation efficiency

Ec-*E. coli*, Ep-EPEC, Kp-*K. pneumoniae*, Ecl-*E. cloacae*, Cr-*C. rodentium*, Ca-*C. amalonaticus*

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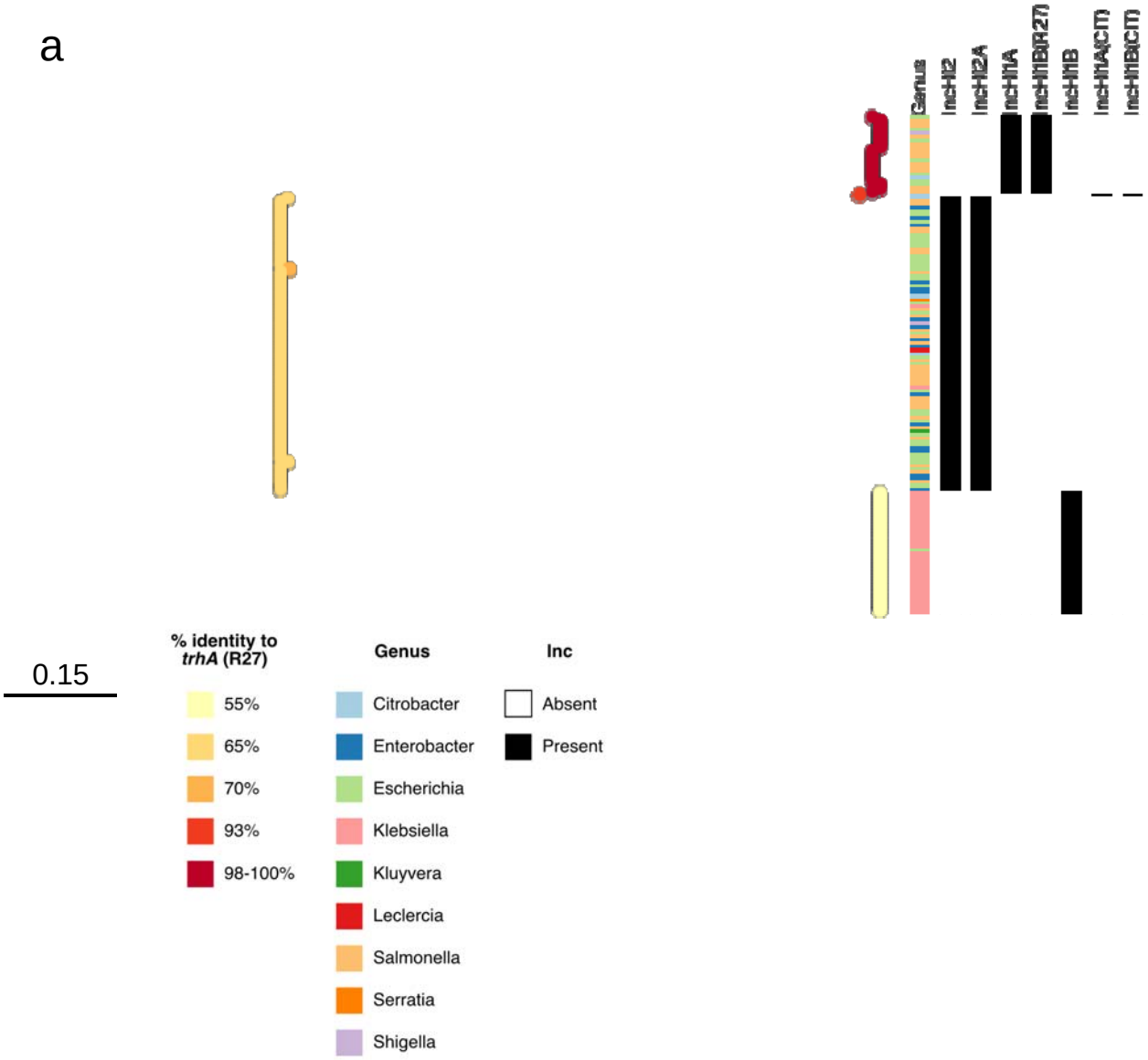
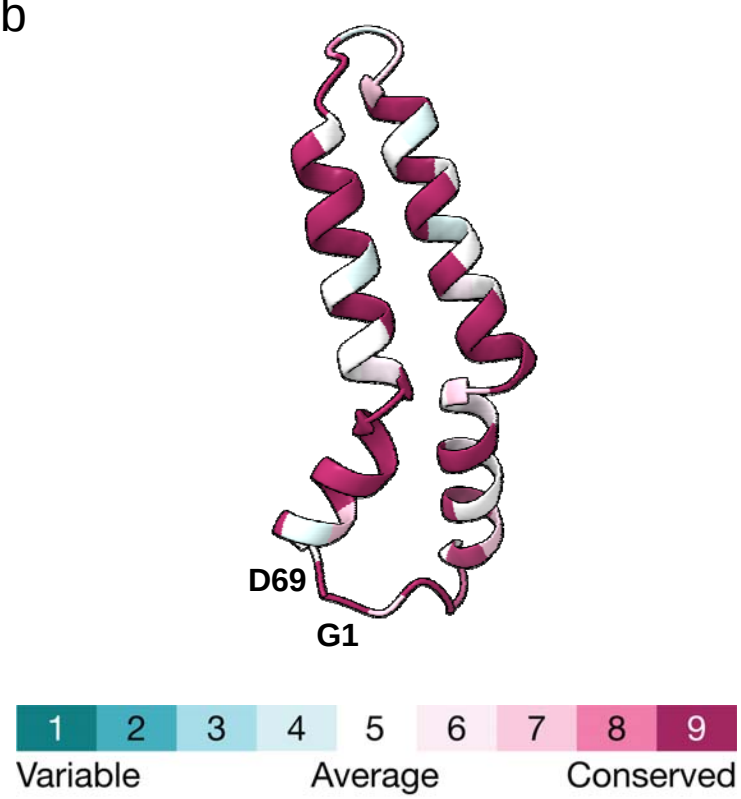
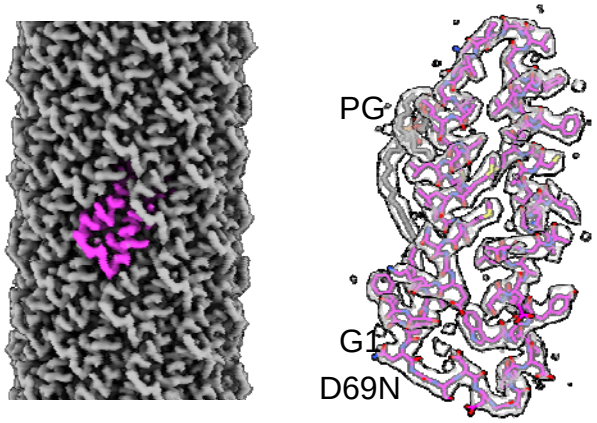
a


Genus
IncHI2
IncHI2A
IncHI1A
IncHI1B(R27)
IncHI1B
IncHI1A(CM)
IncHI1B(CM)
0.15
% identity to trhA (R27)
55%
65%
70%
93%
98-100%
Genus
Citrobacter
Enterobacter
Escherichia
Klebsiella
Kluyvera
Leclercia
Salmonella
Serratia
Shigella
Inc
Absent
Present

b


D69
G1
1
2
3
4
5
6
7
8
9
Variable
Average
Conserved

D69N



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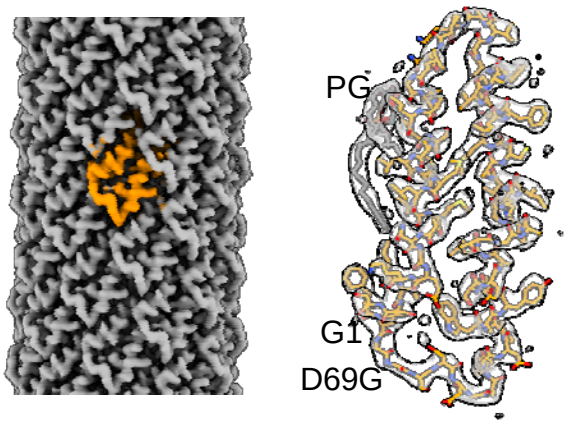

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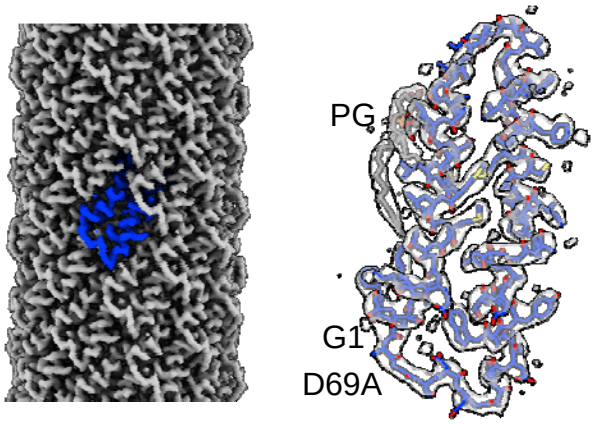
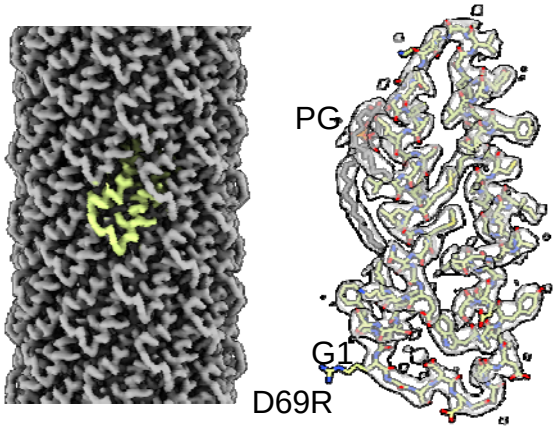
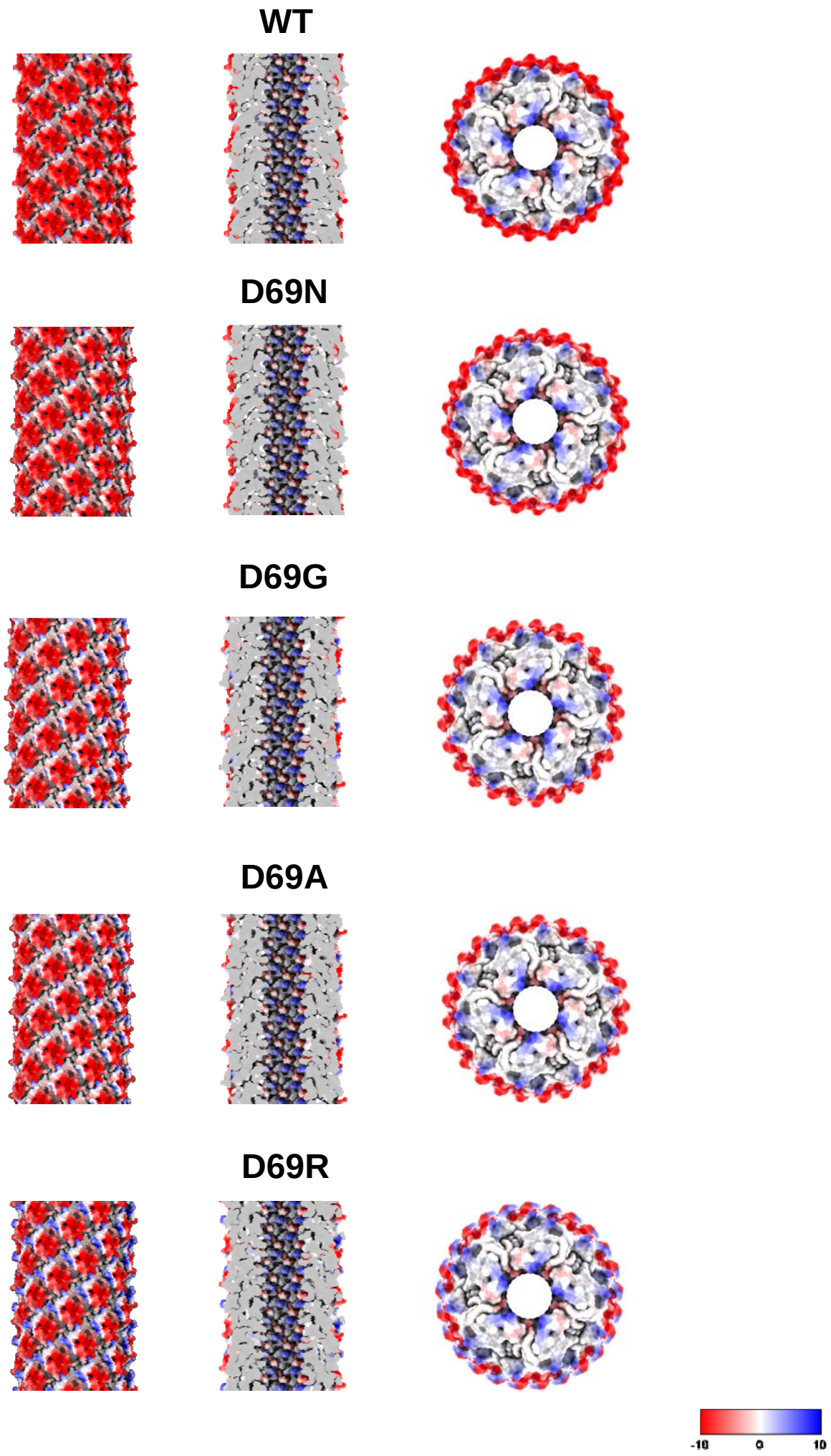
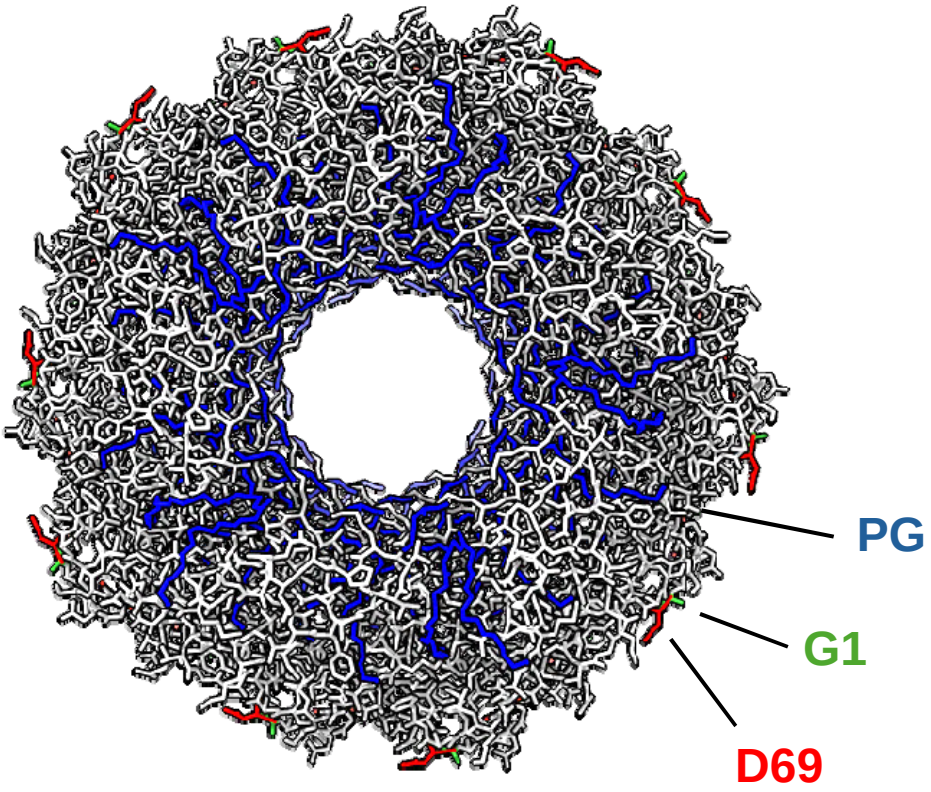


D69R




WT
D69N
D69G
D69A
D69R
-10
0
10

a



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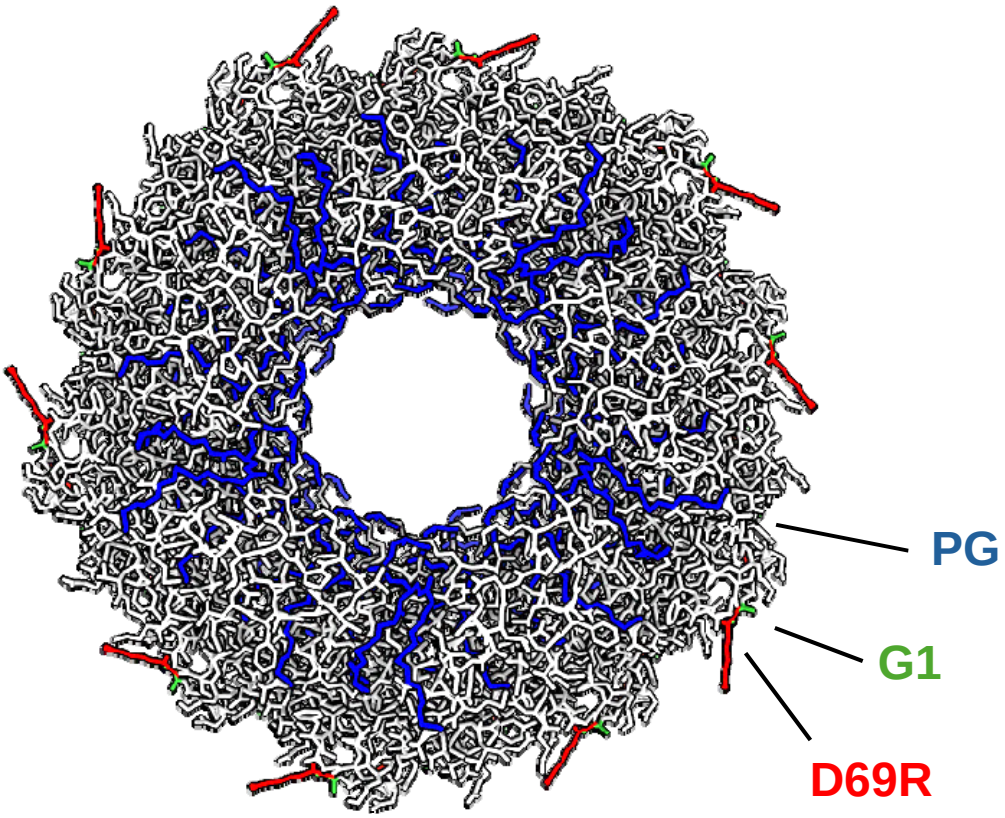

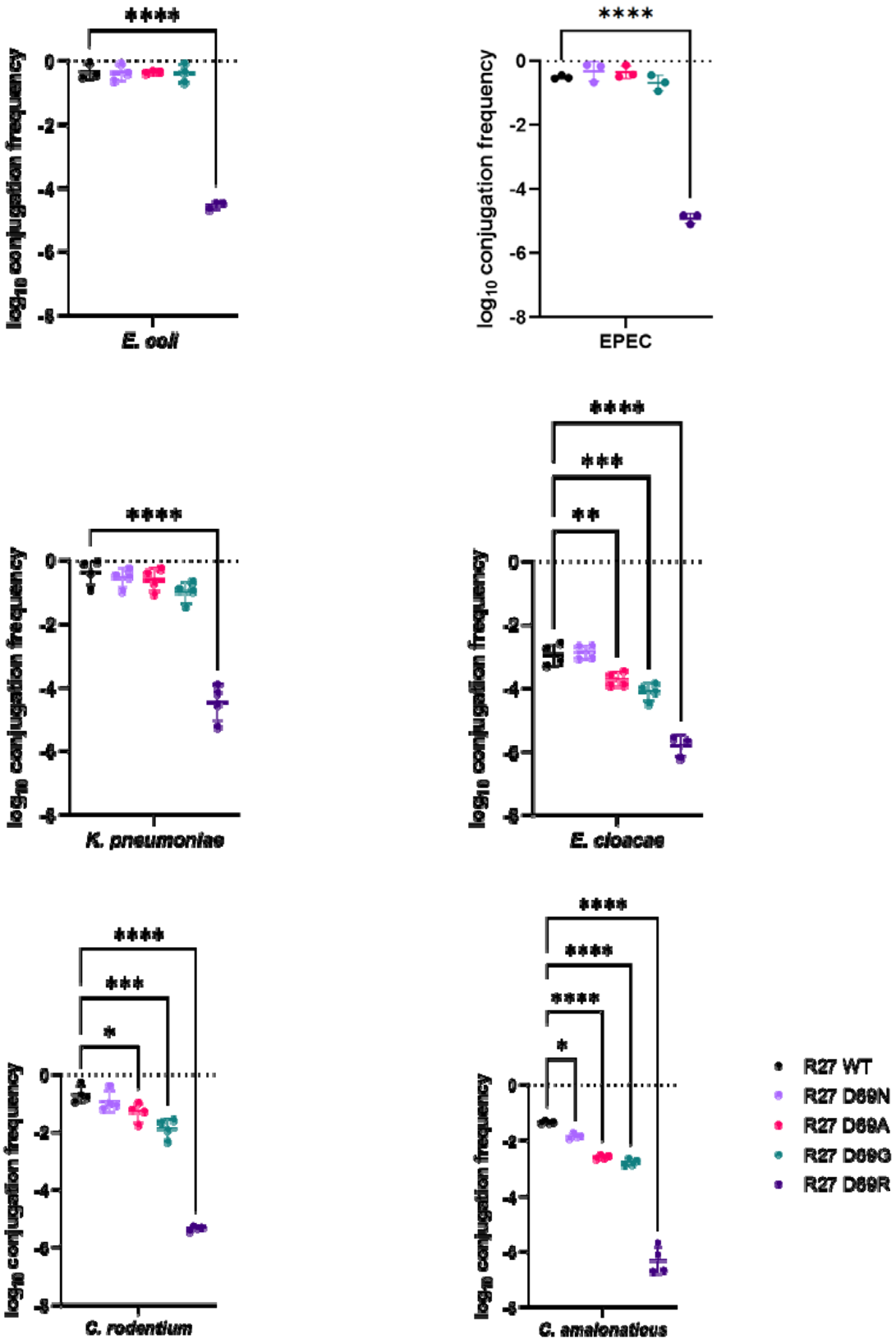
****
log10 conjugation frequency
0
-2
-4
-6
-8
E. coli
EPEC
K. pneumoniae
E. cloacae
C. rodentium
C. amalonaticus
***
**
*
R27 WT
R27 D69N
R27 D69A
R27 D69G
R27 D69R