Inactivation of chemotaxis-associated genes increases flagellar motility in *Clostridium difficile*

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Background

Flagella are important for motility and the expression of chemotaxis-associated genes enables bacteria to sense their surrounding environment and control the rotation of their flagella to move along a chemical gradient (1). The role of flagella in the pathogenesis of *Clostridium difficile* infection (CDI) is unclear, but current data suggest that non-flagellated mutants are more virulent in hamsters. This could potentially be attributed to an increased toxin production in the absence of flagella (2,3).

In *Escherichia coli* and *Bacillus subtilis*, chemotaxis and flagella rotation is controlled by the concerted action of several proteins, whereby CheA and CheY play central roles. Upon binding of a specific chemical signal by a membrane chemoreceptor, the histidine kinase CheA becomes phosphorylated and then phosphorylates CheY, which acts directly on the flagellar motor, thus altering rotation of flagella. (1)

C. difficile 630 and R20291 strains encode a complete chemotaxis locus comprising at least 10 genes including *cheA* and *cheY* homologs, as well as a putative diguanylate cyclase (DGC) encoded by gene *cd0537*. This DGC is thought to be involved in the synthesis of cyclic-di-GMP (c-di-GMP), a second messenger controlling motility, biofilm formation and virulence in several pathogens. High intracellular c-di-GMP levels were recently shown to reduce motility through repression of flagella genes expression (4).

Objectives

- To determine the role of chemotaxis and c-di-GMP associated genes in *C. difficile* motility.
- To define the involvement of chemotaxis and flagellar motility in virulence and pathogenesis of *C. difficile* infections using a mouse model.

Main conclusions

- Inactivation of *cheA* and *cheY*, but not the DGC CD0537 increases motility on soft agar
- In the mouse model of infection, inactivation of *cheA* leads to:

- Faster colonization vs WT
- Sustained vegetative growth and faster sporulation vs WT

Our data suggest that disregulation of chemotaxis increases motility, which leads to faster colonization of the mouse gut, faster spore production and more persistent infection

Material and methods

Bacterial strains and gene inactivation:

- *C. dificile* strains 630ΔErm was obtained from Nigel P. Minton and strains R20291 and M120 were obtained from Trevor D. Lawley.
- Clostron mutagenesis was done using the pMTL007C-E5 plasmid (5). Intron retargeting was done using the design tool available at www.Clostron.com and constructions were purchased from DNA2.0 Inc (California, USA). The *ΔfliC* was generated using the same Clostron as published before (6)
- Conjugation between *E. coli* strain CA434 and *C. difficile* was done as described before (5).

Spore preparation:

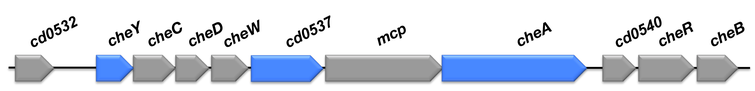
- Spores were grown on TY agar plates for 5 days, then washed 3 times with sterile water and store at 4°C until use. Spores counts were determined after growth on BHI agar containing 0.1% taurocholate and 1mM glycine (BHI-TAG).

Mouse model:

- C57BL/6 female mice (6-8 weeks old) were purchased from Charles River (Kingston, NY)
- Mice were housed in groups of 3 in sterile cages with sterile water, food, and bedding
- Mice were made susceptible to C. difficile infection after antibiotic treatment as described in Fig 2. Infection was initiated after gavage with 105 spores
- Fresh fecal samples were collected at different time intervals and C. difficile spores and vegetative cells were counted after plating dilutions of fecal suspensions on BHI agar containing 8 mg/L cefoxitin, 12 mg/L norfloxacin, 0.1% taurocholate and 1mM glycine (BHI-CN-TAG). An alcool shock was performed to select spores.

Inactivation of chemotaxis genes increases motility

A) Organization of the chemotaxis locus in *C. difficile* 630



B) Motility assay on soft BHI agar (0.3%)

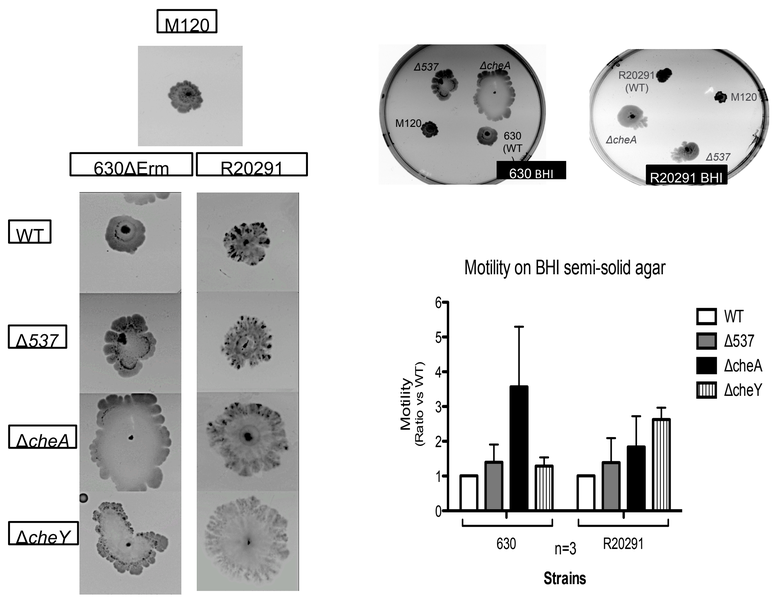
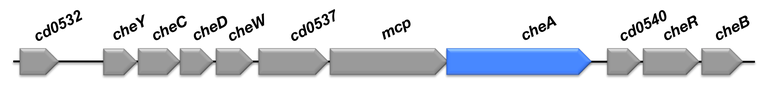
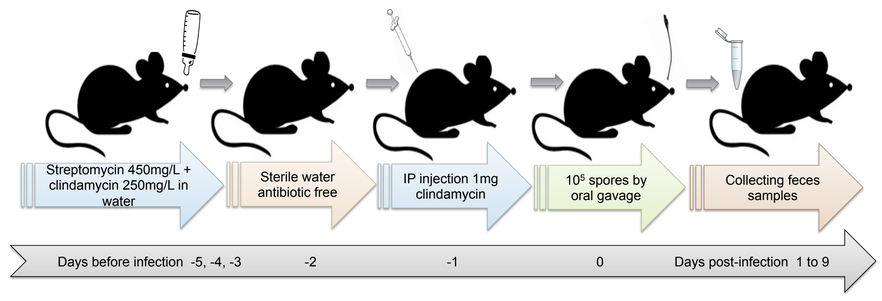


Fig. 1. Gene inactivation and in vitro motility assays. A) Chemotaxis locus in *C. difficile* strain 630 (conserved in R20291); genes in blue are those that were inactivated with the Clostron: cdi-cd0537-88s, cdi-cheA-135a, cdi-cheY-46s). B) Five microliters (5 µL) of log phase cultures of wild type (WT) and mutant strains grown in BHI were spotted on the surface of soft agar (0.3%) and incubated at 37°C for 96h under anaerobic atmosphere. The total growth area was determined for all strains using Image J and the relative motility was plotted on the graph on the right. Values represent the ratio of the growth surface area of the mutant vs the WT. A ratio of >1 means that the mutant was more motile than the WT strain.

Increased virulence of the R20291*ΔcheA* chemotaxis mutant



A) Infection protocol



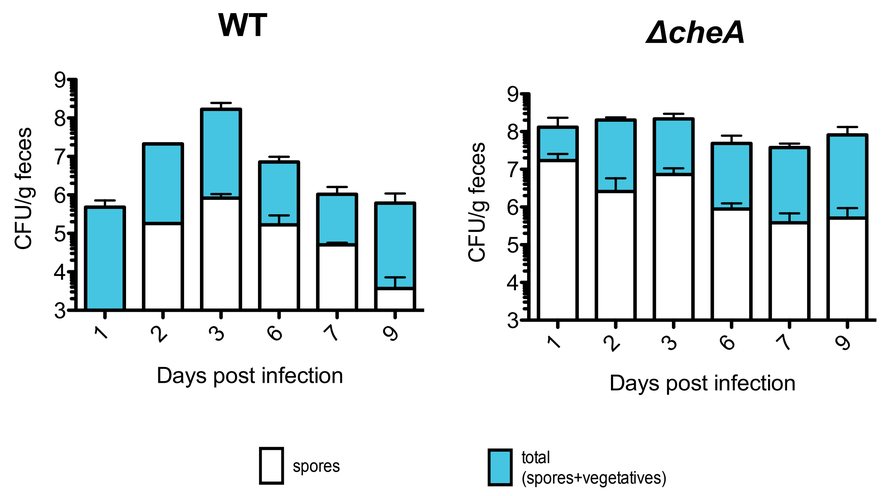


Fig. 2. Mouse infection model. Spores and total bacteria (spores + vegetative cells) were counted in fresh fecal samples collected during 9 days post-infection. Each bar represents the average of three mice!

References

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(3) Aubry A. et al (2012). Infect Immun 80(10), 3521–3532.
(4) Purcell, E. B. (2012) J Bacteriol 194 (13), 3307-3316
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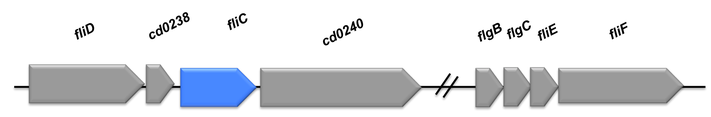
(6) Twine S, et al. (2009) J Bacteriol 191(22):7050–7062.

The R20291*ΔfliC* mutant is non-motile



A) Transmission electron microscopy

B) Motility in 0.175% BHI agar

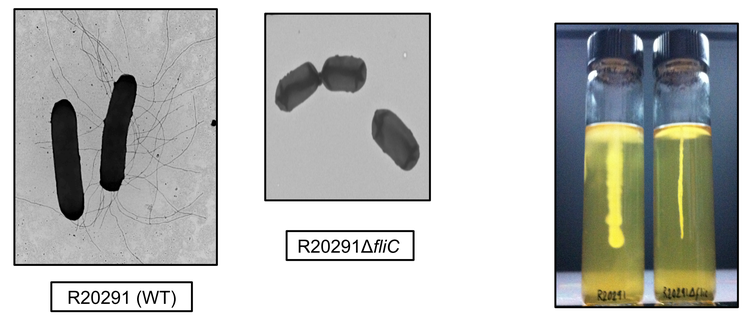


Fig. 3. Inactivation of fliC in *C. difficile* strain R20291. A) Transmission electron microscopy of the vwild type (wt) and *ΔfliC* mutant. Bacteria were grown overnight in BHI, and then washed with 0.1% formaldehyde. Bacteria were deposited onto formvar/carbon copper grids and stained with 1.5% phosphotungstic acid. B) Motility assay in semi-solid agar. Bacteria from overnight cultures in BHI were stabbed into 0.175% BHI agar tubes and incubated overnight at 37°C under anaerobic atmosphere.

Hypothetical model for chemotaxis and motility in *Clostridium difficile*

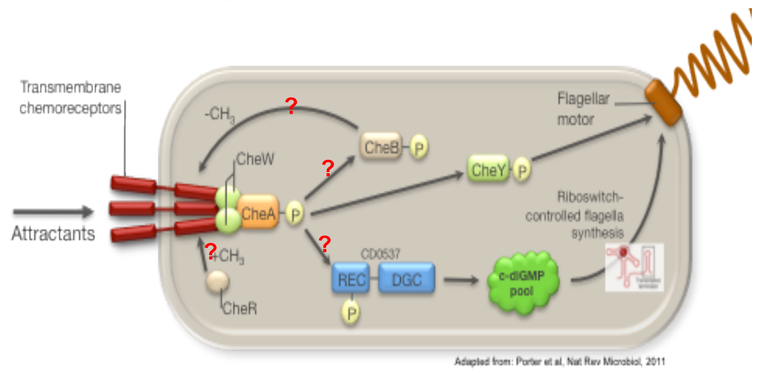


Fig. 4. Hypothetical model for chemotaxis and motility in *C. difficile*. This hypothetical model is based on the putative function of the genes encoded in the chemotaxis locus of *C. difficile* as well as the established function of homologs and current models in other bacteria (1). According to this model, *C. difficile* flagella would rotate continuously until CheY becomes phosphorylated by the activated CheA-P, upon sensing of a chemical signal. Interaction of CheY-P with the flagella motor would lead to complete arrest of flagella rotation, or make the rotation to reverse or alternate from clokkwise to counterclockwise. In a *ΔcheA* or a *ΔcheY* mutant, this interaction would be prevented, and the flagella would thus be continuously rotating, irrespective of the outside chemical signals. The phenotype would thus be hyper motility. In the conditions tested, deletion of the CD0537 DGC had little effect on the overall expression of the flagella and on motility, but acording to a recent report, an increase in c-di-GMP levels through DGC activity would lead to a decreased expression of flagella and hence, reduced motility (4).

Perspectives

- Infection experiments with the *ΔcheY*, *Δcd0537* and *ΔfliC* mutants
- Sporulation assays with the chemotaxis mutants
- Creation of a *ΔcheA* + *ΔfliC* double knockout

Aknowledgements

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