Inactivation of the *dnaK* gene in *Clostridium difficile* 630 D*erm* yields a temperature-sensitive phenotype and increases biofilm-forming ability

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ABSTRACT

Clostridium difficile infection is a growing problem in healthcare settings worldwide and results in a considerable socioeconomic impact. We used ClosTron to construct an insertional mutation in the *dnaK* gene of *C. difficile* 630 Δ*erm*. The *dnaK* mutant exhibited temperature sensitivity, grew more slowly than *C. difficile* 630 Δ*erm* and was less thermo tolerant.

INTRODUCTION

Clostridium difficile is recognised as the most common cause of infectious antibiotic-associated bacterial diarrhoea in healthcare settings worldwide. Cases of CDI have been exacerbated by the recent emergence of new, hyper virulent strains of the organism, and are associated with higher recurrence rates and higher mortality. Antibiotic resistance plays an important role in driving these epidemiological changes. However, the precise function of clostridial genes has been difficult to determine considering the lack of genetic manipulation tools. The ClosTron, developed by Heap *et al.*, utilises a retargeted mobile group II intron to allow targeted, permanent gene disruptions and the introduction of an erythromycin resistance gene, *ermB*, that enables positive selection of mutants. The reader is referred to Kuehne and Minton for a comprehensive summary of the ClosTron technology, intron design procedures and mutant nomenclature. We previously demonstrated up-regulation of class I heat shock genes in *C. difficile* strain 630 in response to mild, clinically relevant heat-stress ranging from 37°C to 41°C. To dissect the *C. difficile* heat-stress response in detail, we utilised ClosTron to attempt to create knockout mutants of the class I molecular chaperones *dnaK* and *groEL*, in addition to their negative transcriptional regulator *hrcA*.

RESULTS

We previously reported on the effects of clinically relevant heat-stress on the proteome and transcriptome of *C. difficile* strain 630, showing that a 4°C temperature up shift (37–41°C) resulted in a classical heat-stress response characterised by the up-regulation of various class Iand III chaperones and cell-surface adhesins in addition to increased expression of Fe-only hydrogenases. In the current work, we hypothesised that disruption of key cellular chaperones would lead to pleiotropic changes in the physiology of *C. difficile*.

ClosTron mutant construction

For the *dnaK* gene (target site 722|723a; score 6.925), PCR screening of erythromycin-resistant colonies confirmed the generation of a ClosTron knockout mutant (Figure1a). Southern blot analysis (Figure 1b) using an intron-specific probe for ErmRAM further verified the existence of a single copy of the insertion element. The insertion site was verified by sequencing across intron–exon junctions (Supplementary Data S1), and confirmatory PCR of the ErmRAM region was also performed (Figure 1c). Despite multiple attempts and intensive PCR screening, it was not possible to isolate verifiable disruption mutants of either *groEL* or *hrcA* in *C. difficile* using the ClosTron system. Whether HrcA and GroEL are essential in *C. difficile*—as reported for certain other bacteria —remains unclear, but further attempts to isolate *groEL* or *hrcA* mutants were not pursued.

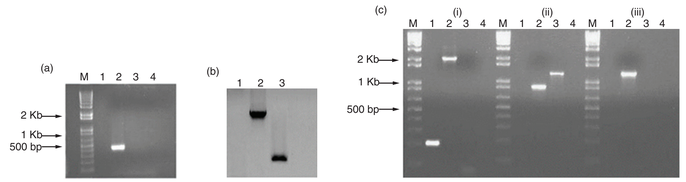

Figure 1: Validation of C. *difficile* 630 Δ*erm*::*dnaK* 723a mutant by PCR screening and Southern blotting Lanes: M, 1 kb Plus DNA ladder (Invitrogen); Lane 1, C. *difficile* 630 Δ*erm*; Lane 2, dnaK mutant; Lane 3, pMTL007C-E2 plasmid DNA; Lane 4; negative control (water).
(a) PCR across the intron-exon junction using EBS universal and Cdi-*dnaK-R* primers generated a 428 bp product from *dnaK* mutant (lane 2) showing presence of the intron; **(b)** Southern blot analysis to confirm single genomic insertion of the intron: An intron-specific probe for the ErmRAM was hybridised to *Hin*dIII-digested: genomic DNA extracted from C. *difficile* 630 D*erm* (Lane 1), pMTL007C-E2 plasmid DNA (Lane 2, positive control), and genomic DNA from the dnaK mutant (Lane 3). **(c)** Additional confirmatory PCR: **(i)** PCR using Cdi-*dnaK*-F and Cdi-*dnaK*-R primers generated a 210 bp product from C. *difficile* 630 Δ*erm* (lane 2), whereas the *dnaK* mutant produced a 2059 bp product, indicating the insertion of the group II intron (lane 3); **(ii)** PCR using ErmRAM-F and ErmRAM-R primers generated a 900 bp product from the *dnaK* mutant (lane 2) indicative of splicing out of the *td* group I intron, whereas unmodified pMTL007C-E2 template generated a 1300 bp product (lane 3), **(iii)** PCR across the other intron-exon junction using ErmRAM-R and Cdi-*dnaK*-F primers generated a 1300 bp from the dnaK mutant only. These experiments confirm insertion of the group II intron into the C. *difficile* 630 Δ*erm* chromosome at the desired site and in the correct orientation, resulting in dnaK inactivation.

Growth characteristics of the dnaK mutant

Raw attenuance data for these experiments can be found online in Supplementary Data S2. We previously determined using *C. difficile* strain 630 that there was no statistically significant difference in either growth rate or biomass production when the growth temperature was shifted from 37°C to 41°C, indicating a certain robustness of this strain to temperature up shift. In the current work, however, we observed a significant difference in the growth rate of the *dnaK* mutant as compared to the parental Δ*erm* strain following the induction of heat stress (Figure 2c, 2d). This altered growth behaviour and thermo sensitivity of the *dnaK* mutant could be interpreted as a direct consequence of *dnaK* inactivation.

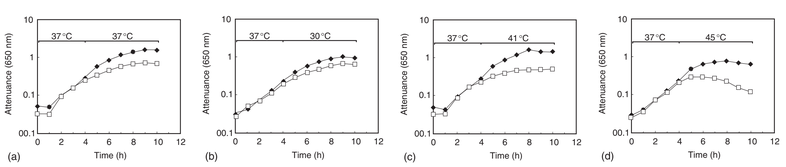

Figure 2: Growth of C. *difficile* 630 Δ*erm* (♦) and C. *difficile* 630 Δ*erm*::*dnaK* 723a mutant (□) in BHIS broth at different temperatures. Temperature shifts were induced at early exponential phase, 4 h.
(a) When grown at 37 °C, the *dnaK* mutant exhibited a temperature-sensitive phenotype, growing more slowly than C. *difficile* 630 Δ*erm*. (b) Cells grown to early exponential phase at 37 °C and then transferred to 30 °C grew in a comparable manner. Cells grown to early exponential phase at 37 °C were challenged by transfer to temperatures of (c) 41 °C and (d) 45 °C, respectively, where temperature sensitivity of the *dnaK* mutant was more pronounced. D_{650nm} values are plotted on a logarithmic scale and are averages of D_{650nm} measurements from biological triplicate cultures; error bars represent the standard error of mean.

Disruption of dnaK results in impaired motility due to a FliC-deficient phenotype

Thus, we assessed cellular motility by stab inoculating *C. difficile* strains into motility agar tubes (in three replicates) and assessing growth following anaerobic incubation at 37°C for 48h. The parental *C. difficile* 630 Δ*erm* strain displayed a diffuse spreading pattern, with clear evidence of growth away from the inoculum stab,indicative of a motile phenotype (Figure 3a). In contrast, the *dnaK* mutant (Figure 3b) failed to produce the spreading pattern typical of motile organisms. We hypothesised that this lowered motility could be due to the reduced expression of *fliC* or the lack of flagella on the *dnaK* mutant cell surface. This hypothesis was tested using both transmission and scanning electron microscopy (TEM and SEM, respectively) on cells grown at 37°C. TEM images indicated that *dnaK* disruption also resulted in a filamentous phenotype in the mutant (Figure 4b), an observation further investigated using SEM, which clearly showed that cells of the *dnaK* mutant (Figure 4d) were longer than those of the Δ*erm* strain [mutant cells, 9.04 ± 1.42 µm in length; wild-type cells, 6.72 ± 1.28 µm in length; 12 cells of each strain were measured].

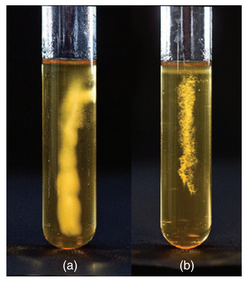

Figure 3: Motility of C. *difficile* strains in BHIS agar (0.175%).
(a) C. *difficile* 630 Δ*erm*, (b) *dnaK* mutant. Motility was visualised as a diffuse spreading pattern from the point of stab inoculation.

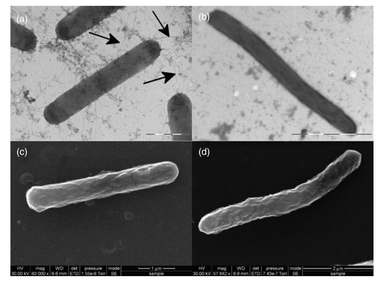

Figure 4: Electron microscopic analysis of C. *difficile* 630 Δ*erm* and C. *difficile* 630 Δ*erm*::*dnak* 723a mutant.
(a) TEM C. *difficile* 630 Δ*erm*. **(b)** TEM *dnaK* mutant. Arrows indicate flagellar filaments. **(c)** SEM C. *difficile* 630 Δ*erm*. (**d**) SEM *dnaK* mutant. The images depict the filamentous phenotype of the dnaK mutant in comparison to the wild-type.

Chaperone genes and fliC are significantly altered in the dnaK mutant

If, as we hypothesised, the *dnaK* mutant was in the 'heat-stress mode', then it was to be expected that expression of other chaperones would be increased as well. Independent biological duplicate cultures of *C. difficile* 630 Δ*erm* and the *dnaK* mutant were grown at 37°C and total RNA was isolated from cells harvested at the late-log phase, reverse transcribed to cDNA and relative expression of chaperone genes and *fliC* was analysed, with *tpi* as reference (see Supplementary Data S3 online for ratios). Expression of *groEL*, *groES*, and *grpE* was significantly increased in the *dnaK* mutant, whereas expression of *dnaJ* was decreased by more than 4-fold (Figure 5). In addition, expression of *fliC* was 4-fold lower in the *dnaK* mutant, confirming that lower level of *fliC* transcript, as opposed to some defect in either translation or in the export of FliC monomers, was the primary reason for lack of flagellar motility.

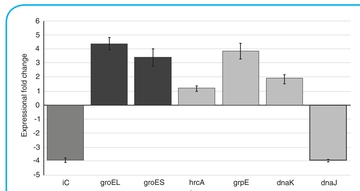

Figure 5: Expressional changes in class 1 chaperone genes and the flagellar filament gene *fliC* in C. *difficile* 630 Δ*erm*::*dnak* 723a mutant.
RNA was extracted and reverse transcribed from biological duplicate cultures and cDNA was quantified in technical triplicate qPCR reactions. The 'calibrator normalised relative quantification including efficiency correction' experimental mode assessed gene expression using the *tpi* gene, whose expression did not change, as a reference. Bars represent average fold-changes in gene expression in the *dnaK* mutant compared with the Δ*erm* parental strain. Error bars represent standard deviation of mean.

dnaK mutant exhibits an increased biofilm-forming phenotype

To assess biofilm development, assays were performed in 96-well polystyrene microtiter plates (Orange Scientific, Alpha Technologies, UK) with measurements at 24, 48 and 72h (see Supplementary Data S4 online). We observed that the *C. difficile* 630 Δ*erm* strain formed weak biofilms (A_{570} < 0.5, per the classification of Varga *et al.*) (Figure 6). By contrast, the effect of glucose addition on biofilm production by *C. difficile* 630 Δ*erm* was insignificant, although biofilm biomass increased after glucose addition.

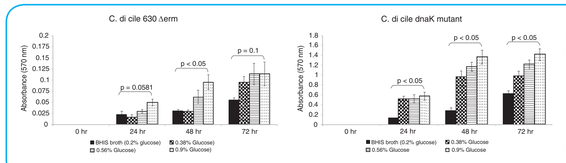

Figure 6: Biofilm-forming ability of *C. difficile* 630 Δ*erm* and *C. difficile* 630 Δ*erm*::*dnak* 723a mutant
Biofilm assays were performed in biological triplicates, each with 6 independent technical replicates. Strains were classified as strong- (A_{570} > 1), moderate- (A_{570} = 0.5-1), or weak- (A_{570} < 0.5) biofilm producers. P values represent statistical comparison (Student's *t*-test) between BHIS broth and BHIS broth with 0.9% (w/v) glucose.

DISCUSSION

The *C. difficile dnaK* mutant had a lower growth rate and produced less biomass at temperatures between 30 and 45°C (Figure 2) and in addition was also less able to tolerate heat stress (Figure 2), emphasising the importance of the DnaK chaperone in protein folding, especially in relation to core cellular housekeeping functions. In *Escherichia coli*, *dnaK* mutants reportedly grow more slowly and exhibit lower viability than the wild-type, exhibiting severe defects in DNA and RNA synthesis that account for the inhibited growth and reduced viability. Accordingly, we noted that following lethal stress (2min at 64°C) and plating on BHIS agar, no *C. difficile dnaK* mutant cells were recovered, suggesting that *dnaK* mutation is deleterious to sporulation. Our motility experiments (Figure 3) showed that the *C. difficile dnaK* mutant was less motile than the parental *Δerm* strain. Electron microscopy revealed for the first time in C. difficile that the *C. difficile dnaK* mutant had no flagella (Figure 4b) and that the mutant cells were approximately 50% longer than the parental *Δerm* cells (Figure 4d). The *C. difficile dnaK* mutant exhibited 3- to 4-fold increases in the expression of all class I heat shock genes (Figure 5),with the exception of *dnaJ*, the expressions of which was 4-fold lower. We therefore indicate that intracellular concentrations of the molecular chaperones encoded by the *dnaK* operon may directly influence the activity and localisation of FtsZ (encoded by CD2646) in *C. difficile*. Consequently, dnaK disruption may have wider effects on *C.* difficile transcription factors or mRNA processing. The *C. difficile dnaK* mutant exhibited 3-to 4-fold increased expression of the *groESL* operon at 37°C. The absence of a functional DnaK protein leads to σ overproduction and thus *E. coli dnaK* mutants exhibit increased expression of molecular chaperones even at optimal growth temperatures. Transcription of class I heat-inducible genes encoded by the *groE* and *dnaK* operon genes is negatively regulated by the HrcA repressor protein in conjunction with the CIRCE element, a palindromic sequence present in the promoter region of these operons. During stress, accumulation of unfolded proteins sequesters the activity of GroEL, causing inactivation of HrcA and allowing active transcription of the *groE* and *dnaK* operons. The *C. botulinumhrcA* mutant was reported to overexpressall six class I heat shock genes, as would be expected. Our observation that expression of the *groESL* operon was 4-fold higher in the *C. difficile dnaK* mutant indicates that in this organism, DnaK, rather than GroES/GroEL, might have a role to play in the stabilisation of HrcA and thus in the correct regulation of class I heat shock operons. In the current work, the *C. difficile dnaK* mutant strain was non-motile, lacked surface flagella and *fliC* expression was 4-fold lower than that in the parental Δ*erm* strain. It could be hypothesised that non-flagellated *C. difficile* cells would adhere weakly and thus be less virulent. To summarise, this study reports for the first time the construction and characterisation of a ClosTron *dnaK* mutant in *C. difficile*. Our phenotypic characterisation clearly demonstrates that while DnaK is not essential for the viability of the organism, defects in DnaK functionality lead to altered expression of class I heat shock and motility genes, perturbations to the cell surface and adhesion and considerable disruption of global cellular physiology and homeostasis.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Table 1. *C. difficile* strains were anaerobically grown on BHIS agar or broth, as previously described. For heat-stress experiments, liquid cultures growing at 37°C were transferred to a recirculating 41°C water-bath set at the appropriate temperature, as per Jain *et al.*. *C. difficile* 630 Δ*erm* was employed to allow selection of ClosTron mutants, *E. coli* TOP10 was used as the cloning host and *E. coli* CA434 strain was the donor for conjugative transfer of plasmids to *C. difficile* 630 Δ*erm*.

Table 1: Strains/Plasmids used in this work

Strain or Plasmid	Description	Source/Reference
Strains		
CD630	Wild-type (WT) strain	ATCC BAA-1382
CD630 Δ*erm*	Erm sensitive WT strain	Hussein *et al.*
CD630 Δ*erm*::*dnaK* 723a	Strain with insertional inactivation of *dnaK*	This work
E. coli TOP10	Electrocompetent cloning strain	Invitrogen
E. coli CA434	Conjugation donor strain	Heap *et al.*
Plasmids		
pMTL007-CE2	ClosTron mutagenesis vector	Heap *et al.*
pMTL007-CE2::dnaK-722|723a score 6.925	ClosTron mutagenesis vector, intron retargeted to *dnaK*	This work
pMTL007-CE2::hrcA-285|286s score 7.971	ClosTron mutagenesis vector, intron retargeted to *hrcA*	This work
pMTL007-CE2::hrcA-199|200a score 4.19	ClosTron mutagenesis vector, intron retargeted to *hrcA*	This work
pMTL007-CE2::GroeL-600|601s score 8.766	ClosTron mutagenesis vector, intron retargeted to *groEL*	This work
pMTL007-CE2::groEL-688|689a score 6.18	ClosTron mutagenesis vector, intron retargeted to *groEL*	This work

Equipment and settings

PCR gels were imagedunder UV light in an AlphaImager™ 2200 (Alpha Innotech, CA, US)equipped with a 1.4 megapixel camera with 12 bit A/D, using default AlphaView Image Analysis Software settings, and exported in jpg format. The jpg images were imported into the GNU Image Manipulation Program (GiMP) 2.8 for generation of Figure 1. Growth curve graphs shown in Figure 2 were produced using MS Excel, individually exported in PDF and imported into GiMP 2.8 for construction and final labelling. Motility agar tubes were photographed using a Nikon Coolpix camera using standard settings, and the resultant jpg images were imported into GiMP 2.8 for construction and labelling of Figure3. Electron microscope images (tiff format) were imported into GiMP 2.8 for construction and labelling of Figure 4. Gene expression data was used to construct a bar chart in MS Excel, prior to chart export in PDF; this was imported into GiMP 2.8 for final labelling of Figure 5. Biofilm assay data was used to construct bar charts in MS Excel. The charts were individually exported in PDF and imported into GiMP for construction of Figure 6. No alterations to brightness or contrast were made to any of the images during figure construction.

Table 2: Oligonucleotides used in this work

Strain or Plasmid	Description
Intron retargeting*	
Cdi-*dnaK*-722a -IBS	AAAAAAGCTTATAATTATCCTTAAATTCCTTCTTAGTGCGCCCAGATAGGGTG
Cdi-*dnaK*-722a -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTCTTAGCTAACTTACCTTTCTTTGT
Cdi-*dnaK*-722a -EBS2	TGAACGCAAGTTTCTAATTTCGATTGAATTTCGATAGAGGAAAGTGTCT
Cdi-*hrcA*-285s -IBS	AAAAAAGCTTATAATTATCCTTACTTATCGAACAAGTGCGCCCAGATAGGGTG
Cdi-*hrcA*-285s -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGAACAATGTAACTTACCTTTCTTTGT
Cdi-*hrcA*-285s -EBS2	TGAACGCAAGTTTCTAATTTCGATTATAAGTCGATAGAGGAAAGTGTCT
Cdi-*hrcA*-200a -IBS	AAAAAAGCTTATAATTATCCTTACTTTTCCAGATGGTGCGCCCAGATAGGGTG
Cdi-*hrcA*-200a -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAGATGGATAACTTACCTTTCTTTGT
Cdi-*hrcA*-200a -EBS2	TGAACGCAAGTTTCTAATTTCGGTTAAAAGTCGATAGAGGAAAGTGTCT
Cdi-*groEL*-601s -IBS	AAAAAAGCTTATAATTATCCTTATTTGTCTCTGCAGTGCGCCCAGATAGGGTG
Cdi-*groEL*-601s -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTCTGCATATAACTTACCTTTCTTTGT
Cdi-*groEL*-601s -EBS2	TGAACGCAAGTTTCTAATTTCGATTACAAATCGATAGAGGAAAGTGTCT
Cdi-*groEL*-689a -IBS	AAAAAAGCTTATAATTATCCTTACTGGTCATAATTGTGCGCCCAGATAGGGTG
Cdi-*groEL*-689a -EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATAATTCTTAACTTACCTTTCTTTGT
Cdi-*groEL*-689a -EBS2	TGAACGCAAGTTTCTAATTTCGGTTACCAGTCGATAGAGGAAAGTGTCT
EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
ClosTron sequencing	
cspfdx-seq-F1	GATGTAGATAGGATAATAGAATCCATAGAAAATATAGG
pMTL007-R1	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG
Screening of clones	
Cdi-*dnaK*-F	CTACAGCTGGTAACAATAGATTAGGT
Cdi-*dnaK*-R	CTGTAGCAGTTATGAAAGGTAAGTT
Cdi-*groEL*-F	AGTCTCAAACTATGAATACTGAATTAGATG
Cdi-*groEL*-R	GCTTTTTACCTTGTTGAACTATTTGT
Cdi-*hrcA*-F	TAGGGTATTTAATTCAGCCTCATACTTC
Cdi-*hrcA*-R	TGCTACAGTTGTATAGTTTGTTAGTTGC
ErmRAM-F	ACGCGTTATATTGATAAAAAATAATAATAGTGGG
ErmRAM-R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG

*Introns were inserted after the indicated number of bases in the sense (s) or the antisense (a) orientation from the start of the open reading frame (ORF) of the target gene. Cdi, *C. difficile*; IBS, intron-binding sites; EBS, exon-binding sites; ErmRAM, erythromycin retrotransposition-activated selectable marker.

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