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Functional Analysis of Putative Virulence Genes of *Campylobacter fetus*

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ABSTRACT

The *Campylobacter* species *Campylobacter fetus* includes both human and animal pathogens. This species is further divided into the subspecies *C. fetus* subsp. *fetus* (*Cff*) and *C. fetus* subsp. *venerealis (Cfv).* While *Cfv* uniquely infects cattle, *Cff* has a broader host range including human. Although *Cff* and *Cfv* differ clearly in their host range, they show 92.9% sequence identity. The aim of this study is to investigate the function of genes that are unique to either *Cff* or *Cfv* and are putatively involved in virulence.

Serotype A *Cff* strains harbor a putative UDP-Galactosefuranose mutase (*glf*), which is absent in *Cfv* but known in other pathogens (e.g. *Mycobacterium tuberculosis*) to be involved in lipopolysaccharide (LPS) synthesis and therefore contribute to virulence. In this work, a *glf* knockout-strain (C*ff* 82-40 Δ*glf*) was generated which showed grossly normal levels of LPS production and resistance to human serum but reduced acid tolerance compared to the wildtype. Therefore, *glf* might be important for *Cff* during oral infection and passage through the acidic stomach.

Cfv harbors a functional toxin-antitoxin system consisting of Fic1 (antitoxin) and Fic2 (toxin). While Fic1 can compensate the toxicity of Fic2 in *Escherichia coli*, both proteins are toxic in HeLa cells. To better understand the cellular process and protein target of Fic2 in eukaryotes the gene was heterologously overexpressed in *Saccharomyces cerevisiae.* However, ectopic expression of Fic1 and Fic2 in *S. cerevisiae* showed no effect on cell growth or shape. Thus, *S. cerevisiae* lacks homology to the human cellular processes disrupted by *Cfv* Fic proteins. This finding identifies *S. cerevisiae* as a possible host for overexpression and purification of Fic1 and Fic2.

ZUSAMMENFASSUNG

Die Spezies *Campylobacter fetus* kann weiter unterteilt werden in die beiden Subspezies *C. fetus* subsp. *fetus* (*Cff*) und *C. fetus* subsp. *venerealis* (*Cfv*). Während *Cfv* ausschließlich Rinder infiziert, zeigt *Cff* eine weitaus größere Wirtsspezifität und zählt auch zu den humanen Pathogenen. Trotz dieser unterschiedlichen Wirtsanpassung, zeigen *Cff* und *Cfv* eine hohe genomische Sequenzübereinstimmung von 92.9%. Ziel dieser Arbeit ist es, die Funktion von Genen zu untersuchen die entweder für *Cff* oder *Cfv* einzigartig sind.

Serotyp A *Cff* Stämme tragen ein Gen (*glf*), welches vermutlich eine UDP-Galaktosefuranose Mutase kodiert. Dieses Enzym ist in anderen pathogenen Bakterien (z.B.: *Mycobacterium tuberculosis*) an der Bildung von Lipopolysaccharid (LPS) und damit an deren Pathogenität beteiligt. In dieser Arbeit wurde ein *glf* Knockoutstamm (C*ff* 82-40 Δglf) hergestellt. Dieser zeigt gleiche LPS Menge und Serumtoleranz, jedoch verminderte Säuretoleranz verglichen zum Wildtyp. Daher könnte *glf* wichtig sein für die orale Infektion und das Überleben der sauren Magenpassage.

Cfv trägt ein funktionelles Toxin-Antitoxin System bestehend aus Fic1 (Antitoxin) und Fic2 (Toxin). Fic1 reprimiert die Toxizität von Fic2 in *Escherichia coli*, in HeLa Zellen wirken beide Proteine toxisch. Um den zellulären Prozess bzw. die daran beteiligten Proteine zu untersuchen, wurden die beiden Gene in *Saccharomyces cerevisiae* exprimiert. Hier zeigte weder Fic1 noch Fic2 einen Effekt auf Wachstum oder Zellmorphologie. Folglich scheint der molekulare Mechanismus der durch die Fic Proteine von *Cfv* in HeLa Zellen gestört wird, in *S. cerevisiae* nicht konserviert zu sein. *S. cerevisiae* könnte sich jedoch als Expressionsorganismus für die Überexpression und Aufreinigung von Fic1 und Fic2 eignen.

INDEX

1 INTRODUCTION

1.1 *Campylobacter fetus*

The bacterium *C. fetus* is a gram-negative S shaped rod which grows under microaerophilic conditions. It is highly adapted to the intestinal and/or urogenital tracts of different hosts [1], [2]. *C. fetus* can be divided into two subspecies: *C. fetus* subsp. *fetus (Cff)* and *C. fetus* subsp. *venerealis (Cfv),* which differ in epidemiology and in their mode of infection [3]. *Cfv* exclusively colonizes the urogenital tract of cattle and is the causative agent of Bovine Veneral Campylobacterosis (BVC) [3], [4]. *Cff* has a much broader host range including sheep, cattle, swine, poultry, reptiles and humans (reviewed in [5]).

1.1.1 Pathogenesis and medical relevance

The predominant subspecies associated with human infection is Cff [6]*.*
Most cases of confirmed human *Cff* infections happen due to consumption of raw or undercooked food or unpasteurised milk (reviewed in [5]). After oral ingestion the bacteria have to survive passage through the acidic milieu of the stomach to colonize the mucus of the intestine. Intestinal colonization can lead to diarrhea or serve as starting point for portal bacteraemia. Healthy hosts overcome the infection without further symptoms, but in impaired hosts systemic bacteraemia and/or secondary tissue distribution can happen [1] (Figure 1).
Several severe illnesses, such as septicaemia, peritonitis, endo- and pericarditis, cellulitis, meningoencephalitis and osteoarthritis have been reported after *Cff* infection. In contrast to animal infection, human abortion is rare (reviewed in [5]).

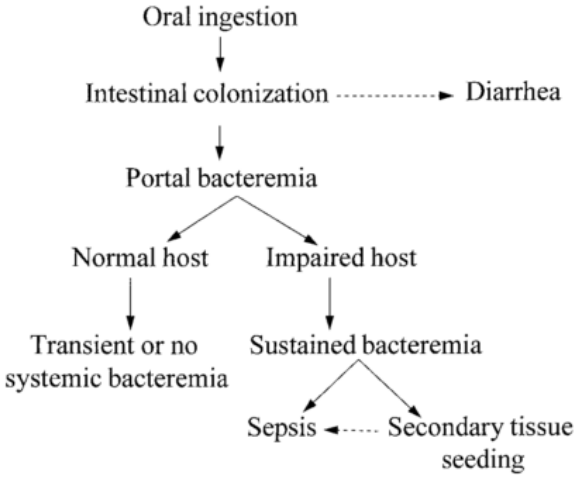

Figure 1: Proposed pathogenesis for *Campylobacter fetus* infections in humans. (figure from [1])

Cff is the *Campylobacter* species most often isolated from patients suffering from bacteraemia [2]. Nevertheless it is believed that the number of *Cff* infections is still underestimated because of three major problems in clinical diagnostics: (I) *C. fetus* grows slowly under *in vitro* conditions; (II) the organism needs microaerophilic atmosphere to grow; (III) most clinical isolates of *C. fetus* are susceptible to cephalothin, which is present in selective media for *Campylobacter* cultivation (reviewed in [1]).

Cfv *infections mainly occur in female cattle.*
Bulls are mostly asymptomatic carriers of *Cfv* but transfer the pathogen into the urogenital tract of female cattle during coitus. Subsequent inflammation causes abortion and infertility [3], [4]. Therefore, BVC is a big economic issue in large cattle herds and in trading of bulls and sperm.

1.1.2 Genome comparison and subspecies definition

Comparative analysis of the first available whole genomes of both subspecies *Cff* 82-40 and *Cfv* 84-112, revealed a sequence identity of 92.9% and a homology of more than 99%. A comparative plot is shown in Figure 2. About 180 kbp are uniquely present in *Cfv*, and 35 kbp in *Cff*. The unique sequences are clustered in hot spots and named variation regions (VRs). This work focuses on genes located on two of these VRs. One VR that co-localises on both genomes encode genes that are putatively involved in surface carbohydrate metabolism and although present on the same location of the genome, their gene content varies. These regions are called venerealis subspecies definition region (VSDR) or fetus subspecies definition region (FSDR) [7]. A second VR contains a genomic island specific for *Cfv* (VGI) harbouring a functional type IV secretion system and two proteins which belong to the Fido protein superfamily, namely Fic1 and Fic2. Furthermore *Cfv* 84-112 has an extra-chromosomal element (ICE_84-112) which encodes two more Fido proteins, Fic3 and Fic4 [7]. Fido motives are known to occur in bacterial effector molecules and toxin-antitoxin systems. The Fido proteins of *Cfv* 84-112 represent a functional toxin-antitoxin system [8].

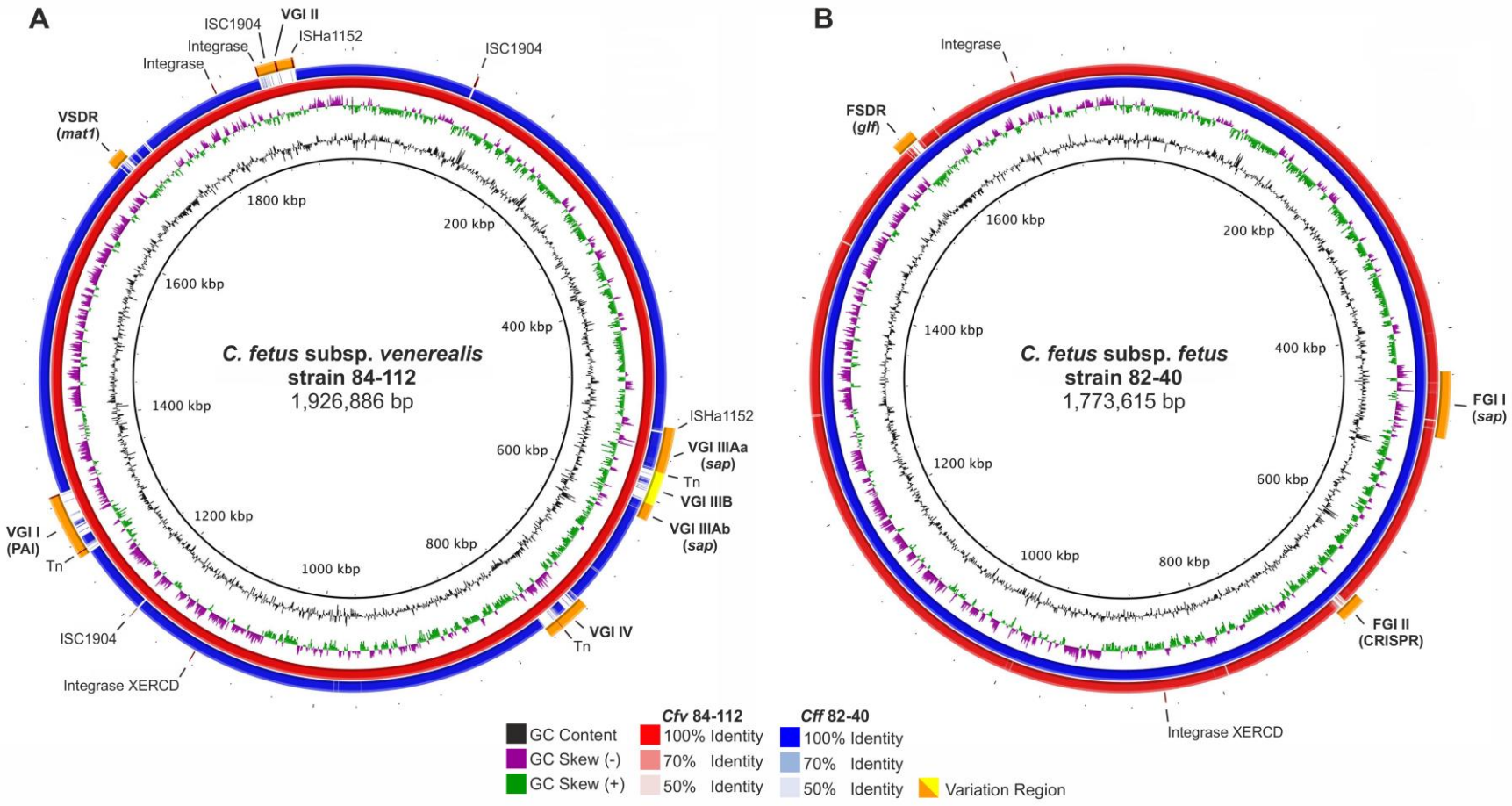

Figure 2: Genome comparison of *C. fetus* subspecies. Plots were generated using *C. fetus* subsp. *venerealis* 84-112 (*Cfv*) as a reference (A) or *C. fetus* subsp. *fetus* 82-40 (*Cff*) (B). Inside tracks represent GC-content (ring 1) and GC-skew (ring 2). *Cff* is shown in blue and *Cfv* in red. Variation regions (VR) relative to the reference genome are indicated in orange/yellow and named according to the corresponding Genomic Island (GI) or the subspecies definition region (SDR). (V) and (F) in the feature names designate the subspecies *venerealis* and *fetus*, respectively. Important genes or features are indicated in parenthesis. Positions of selected mobility genes are indicated. doi:10.1371/journal.pone.0085491.g001. (figure and legend from [7])

1.2 Part 1 – lipopolysaccharide, surface layer and virulence of *Cff*

1.2.1 Lipopolysaccharide (LPS)

General structure of LPS of gram-negative bacteria

LPS is a structure on the surface of most gram-negative bacterial cells and consists mainly of three parts, the lipid A, the core and the O-Antigen (Figure 3). The hydrophobic Lipid A anchors the LPS molecule into the outer membrane of the bacterial cell with its fatty acid chains. Lipid A acts as endotoxin and is recognised by host immune system [9]. The core region can be further separated into inner and outer core. The inner core tends to be conserved within a genus or family and consists of 2-Keto-3-Desoxy-Octonat (KDO) and a heptose [10], [11]. The outer core consists of a greater variety of sugars, but mostly of linear hexoses which can be branched and phosphorylated. The third part is the O-Antigen. This part is very variable. It can be a homo- or heteropolymer chain of sugars which can be linear or branched, modified e.g. by acetylation or methylation and can be built up from up to 164 units in *E. coli* [9], [10]. The O-Antigen contains epitopes for immune recognition by the host immune system. Alteration in the O-Antigen structure or modification can contribute to evasion of host immune system. In *C. fetus* the O-Antigen composition distinguishes between the three serotypes (A, B and AB) [12].

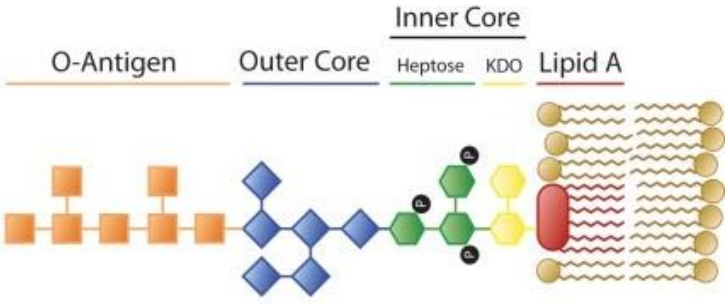

Figure 3: Scheme of LPS structure of gram-negative bacteria; shown are the three main regions: lipid A (red) which anchors LPS molecules in the outer membrane of the bacterium, core region which is attached to lipid A by KDO (yellow) followed by heptose (green) and hexose (blue). The O-Antigen (orange) composition of *C. fetus* causes the three serotypes (A, B and AB) and represents epitopes for host immune system. (figure from [11])

Genomics and characteristics of C. fetus *LPS*

C. fetus is not only divided into subspecies, but also into serotypes or sap-types. There are type A, B and rarely AB which correlate to O-antigen of LPS structure. While *Cfv* always belongs to the type A group, *Cff* can be A, B or AB [12], [13]. Serotypes also correlate with susceptibility to human serum. While type A is resistant, type B is susceptible to normal human serum [12], [14], [15].

Kienesberger et al [7] showed that there are several genes or rather gene combinations, putatively involved in LPS synthesis, that are unique for each serotype. They screened 102 *C. fetus* strains, 62 *Cfv* and 40 *Cff*, for *glf* (putative UDP-galactopyranose mutase), *mat1* (putative maltose O-acetyltransferase) and *wcbK* (putative GDP-mannose 4,6-dehydratase). They showed that *Cff* type A encodes *glf* but no *mat1* or *wcbK*. 58 *Cfv* strains harboured *mat1*, but no *glf* or *wcbK*. *Cff* type B harbours *wcbK* and *mat1,* but no *glf*. It was shown that a knockout of *wcbK* in *Cff* ATCC 27474 (serotype B) leads to a loss of LPS O-Antigen, increased serum resistance and decreased tolerance to acidic conditions compared to the wildtype [7]. These results confirm that *wcbK* is somehow involved in the synthesis of LPS. Since *wcbK* is not present in type A strains, *glf* might be the corresponding gene there.

Moran et al [13] compared some biological activities (e.g. the ability to induce mitogenicity or lethal toxicity in mice) of *C. fetus* LPS with *Salmonella typhimurium* LPS and found, that *C. fetus* LPS has relatively low activities compared to *S. typhimurium* LPS. The lower toxic activity of Lipid A might be due to longer fatty acids of *C. fetus* LPS. Furthermore *Cff* serotype A strains showed a lower activity than serotype B strains. They suspect that these differences between the two serotypes may be due to different sugar compositions of the O-Antigen [13].
While LPS of *Cff* serotype B strains contains mainly D-rhamnose together with 3-O-methylrhamnose, that of *Cff* serotype A consists mainly of D-mannose. Glucose, D-glycero-D-manno-heptose, L-glycero-D-manno-heptose and trace amounts of galactose occur in the LPS of both serotypes. While type B O-Antigen is a linear D-rhamnan with a terminal 3-O-methyl-D-rhamnose residue, the O-Antigen of type A strains is a D-mannan chain with partial O-acetylation [16] [17]. The serotypes A and B also show different patterns in SDS Page electrophoresis of proteinase K treated whole cell lysate, type AB shows the same pattern as B [12].

LPS is essential for acid resistance of Helicobacter pylori *and* Cff *serotype B*
McGowan et al [18] showed that a knockout of a gene called *wcbJ*, which is homologous to known O-antigen biosynthesis proteins, leads to a loss of O-Antigen of *Helicobacter pylori*. The knockout strain also lacks the ability of the wildtype to survive pH 3.5. It was also shown that a knockout of *wcbK* in *Cff* serotype B has similar effects [7]. So the O-Antigen seems to be essential for the organism to survive the acidic conditions in the host stomach.

1.2.2 Surface layer (S-layer)

Surface layers or S-layers are mononuclear crystalline arrays of one single protein or glycoprotein and represent the outermost layer of many bacteria and archaea. S-layers have a smooth outside and a rougher inner surface. These shield-like structures occur on gram-positive and gram-negative bacteria as well as on archaea. The subunits are non-covalently bound to surface structures of the cell, e.g. LPS in gram-negative bacteria (Figure 4) (reviewed in [19]).

C. fetus can produce several variants of S-layer proteins (slp). All variants show a conserved N-terminal region (184 amino acids) which has been shown to be responsible for the interaction with cell surface [20]. The fact that isolated slps reattach to the bacterial surface in a stereotypic manner (SapA reattaches only to serotype A cells, SapB to serotype B) indicates that slps interact directly with the LPS [21].

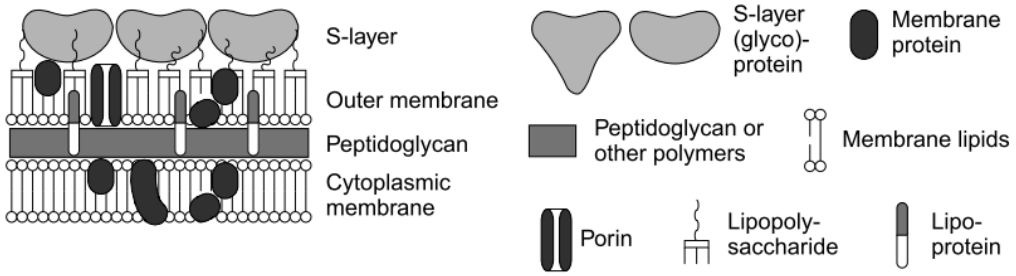

Figure 4: schematic view of a gram-negative cell-envelope with attached S-layer (figure from [22]).

S-layer of C. fetus *and virulence*

It was shown that *C. fetus* strains lacking the S-layer (S^-) are attenuated in virulence. S^- strains lose the ability to cause bacteraemia in mice after oral uptake. S^- strains also fail to cause abortion in pregnant sheep when administered orally, but lead to abortion when inoculated directly into the uterus. So the S-layer contributes importantly to the ability of the pathogen to spread through the bloodstream (reviewed in [23]). This is true because the presence of a S-layer prevents the cells from binding complement mediators (C3b) and therefore from phagocytosis by immune cells [15]. Furthermore, slps of *C. fetus* undergo a fast and efficient phase variation. With this mechanism *C. fetus* is able to evade antibody binding of host immune system (reviewed in [23]). These two effects enable serum-resistant *C. fetus* to spread efficiently through blood system of their hosts.

1.2.3 UDP-Galactopyranose mutase (*glf*)

The gene encoding the flavoenzyme UDP-galactopyranose mutase (UGM), was first identified and described in 1996 in *E. coli* K12 and named *glf* [24]. UGM catalyses the conversion from UDP-galactopyranose to UDP-galactofuranose (UDP-Gal*f*) (schematically shown in Figure 5) [24]–[26].

UDP-Gal*f* represents the precursor of Gal*f*, which is found in the cell wall and cell surface glycoconjugates of different gram-negative bacteria including human pathogens like *Mycobacterium tuberculosis, Klebsiella pneumoniae, Trypanosoma cruzi* and others (reviewed in [25]).

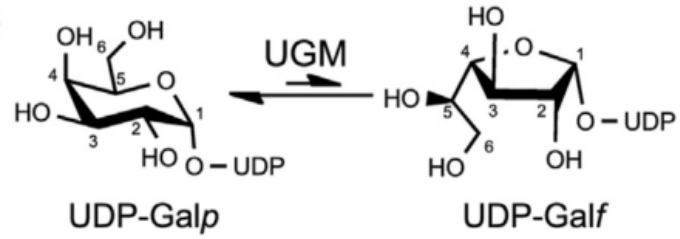

Figure 5: schematic representation of the reaction catalysed by UGM. (figure from [25])

In some pathogens such as *Aspergillus fumigatus* and *Leishmannia major* it was shown that deletion of the genes encoding UGM attenuate virulence [27], [28]. In *Mycobacterium tuberculosis* UGM and a second enzyme (Rv3808c) are essential for growth and viability, because Gal*f* forms an important link between LPS and the mycolic acid layer of the bacterium [29].

1.3 Part 2 - Toxin-Antitoxin system of *Cfv*

1.3.1 Toxin-antitoxin systems

Toxin-antitoxin (TA) systems occur in numerous bacteria and archaea. They play a major role in adaptation to stress conditions, population control, programmed cell death and are suspected to contribute to virulence in some cases. The first identified TA system was encoded on a plasmid, contributing to its maintenance. TA systems consist of a toxin and an antitoxin which are co-transcribed from one TA operon. The toxin always shows higher stability as the antitoxin, so the antitoxin has to be continuously synthesised to repress the toxin. There are several types of TA systems. In type I and III the toxin is repressed by a small antitoxin RNA. The other known systems consist of two proteins where the unstable antitoxin forms a complex with the more stable toxin to inactivate it.

TA systems are widely spread in bacteria. *E. coli* for example has at least 33 TA systems, *M. tuberculosis* has more than 60. Interestingly, the non-pathogenic counterpart of *M. tuberculosis*, *M. smegmatis* only encodes 2 TA systems. This observation led to the speculation that the number of TA systems may be related to pathogenicity, at least in this organism (reviewed in [30]).

1.3.2 Fido protein superfamily

The Fido protein superfamily is characterised by the conserved sequence HPFX[D/E]GN[G/K]R, which can be found in the members of two protein families, Fic (filamentation induced by cAMP) and Doc (death on curing) [31].

Proteins containing the classical Fic motif (HPFX[D/E]GN[G/K]RXXR) show AMPylation activity [32], whereas the degenerated Fic motif of AnkX found in *Legionella pneumophilae* shows phosphocholination activity [33].

While Fic proteins are encoded chromosomally and some are known to be transferred as effector molecules into host cells via type IV secretion systems [34], the Doc protein is derived from the bacteriophage P1. The P1 TA system consists of the toxin Doc and the antitoxin Phd and ensures maintenance of the prophage in the host [30]. Doc phosphorylates its target, the translation elongation factor EF-Tu and inhibits translation [35].

1.3.3 Fic1 and Fic2 – preliminary data

Because for this work only Fic1 and Fic2 are relevant, only the data concerning these two genes will be summarised here.

Sprenger et al. [8] showed that HeLa cells show cell-rounding and increased apoptosis when transfected with *fic1* or *fic2*. Mutation of histidine 184 to alanine abolished these effects (data not shown). This indicates that H184 is crucial for the function of Fic2 in HeLa cells. Heterologous expression of Fic2 in *E. coli* leads to severe filamentation of the cells, while Fic1 and Fic2H184 do not show this effect. Co-expression of Fic1 and Fic2 reduces the filamenting effect of Fic2 (Figure 6 A). It also was shown that expression of Fic2 in *E. coli* leads to a strong delay of growth compared to the empty vector control. Fic1 causes only a moderate growth effect. Co-Expression of Fic1 and Fic2 nearly restores the growth to the level of Fic1 expression (Figure 6 B).

They further found out that Fic2 expression leads to an accumulation of 30S subunits in *E. coli*, indicating that Fic2 has its bacterial target somewhere in translation (data not shown).

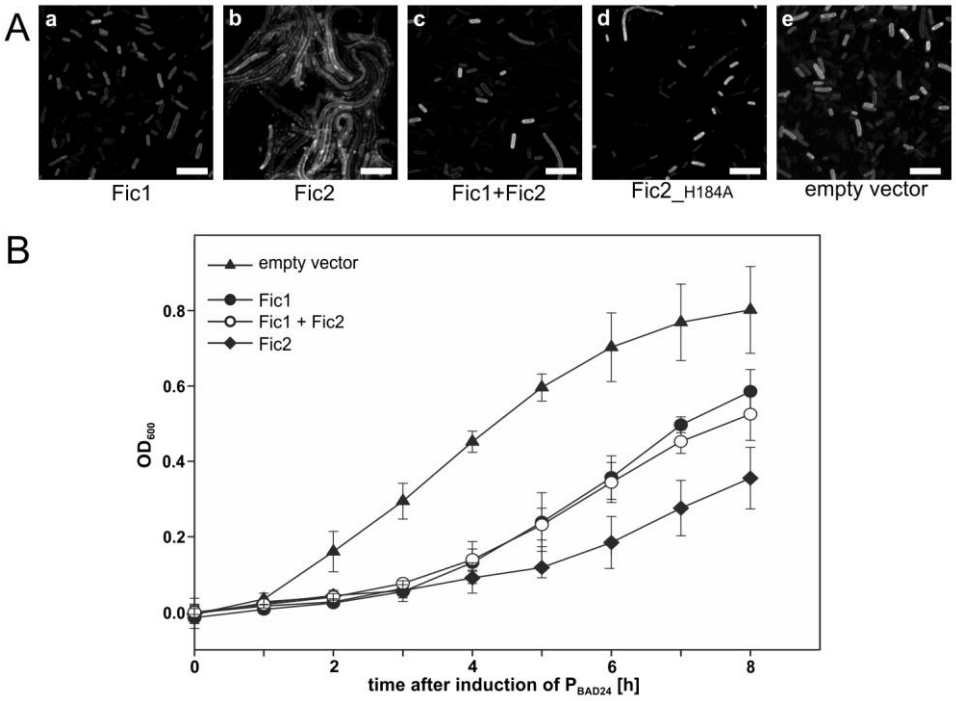

Figure 6: preliminary data of the effects of Fic expression in *E. coli*; A Expression of fic toxin genes in *E. coli* leads to a filamentous phenotype of the cells. Confocal microscopy of Nile red stained *E. coli* expressing *C. fetus* subsp. *venerealis* 84-112 chromosomally encoded *fic* or fic2H184A (a-d), and the empty vector as a control (e). **B** Chromosomally encoded Fic2 is toxic in *E.coli*, while Fic1 reduces toxicity of Fic2. Growth profiles of *E. coli* expressing Fic from pBAD24 or pBADCm at 37°C in LB medium + 0.05% arabinose. Figure and legend adapted with permission of the author from [8].

2 AIM OF THIS WORK

Aim of this work was the characterisation of genes that are unique for each *C. fetus* subspecies and to investigate their contribution to virulence.

As an extension of previous work on *C. fetus* I focused on two important genes subsets.

The first part of this work describes the characterization of *glf*, a gene putatively involved in biosynthesis of the LPS of *Cff* serotype A strains. A *glf* knockout strain was created and phenotypically characterised. The performed assays focused on LPS production and resistance to acid and human serum.

In the second part, the two genes (fic1 and fic2) that build a toxin antitoxin system of *Cfv* were cloned and expressed in *S. cerevisiae* for further investigation of possible eukaryotic targets.

3 MATERIAL AND METHODS

3.1 Media, solutions and reagents

3.1.1 Self-made media

LB (Lysogeny Broth)-Medium

10 g/l	Tryptone
5 g/l	Yeast extract
5 g/l	NaCl

LB-plates

10 g/l	Tryptone
5 g/l	Yeast extract
5 g/l	NaCl
15 g/l	Agar-Agar

CBA (Colombia Blood Agar)

42.5 g/l Colombia-D Medium	Biomérieux
5% defibrinated Sheep Blood [1)]	Biomérieux

YPD (Yeast extract, Peptone, Dextrose)

10 g/l	Yeast extract
20 g/l	Bacto peptone
20 g/l	Glucose
15 g/l	Agar

YNB (Yeast Nitrogen Base – minimal media)

Solution A (100 ml)

0.7 g	Yeast Nitrogen Base
2.5 g	$(NH_4)_2SO_4$

Solution B (400 ml)

10 g	Glucose
10 g	Agar
	Amino acid mix [2)]

All components were solved in VE-water and autoclaved at 121°C for 20-25 minutes. Solution A and B of YNB media were autoclaved separately.

[1)] Sheep blood was added after autoclaving into cooled medium.

[2)] MP biomedicals, amount differs according to amino acid mix.

3.1.2 Ready-to-use media

CBA – plates	Biomérieux
MEM (minimal essential medium)	Gibco® by life technologies

3.1.3 Buffers and Solutions

Table 1: Buffers and solutions used in this study.

Name of solution	Components	Amount/Concentration
1x PBS buffer (Phosphate Buffer Saline)	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4 x 7 H_2O	4.3 mM
	KH_2PO_4	1.4 mM
	in $_{dd}H_2O$; pH 7.3	
50x TAE buffer (Tris-Acetate-EDTA-buffer)	Tris-base	242 g
	Acetic acid	57.1 ml
	0.5 M EDTA pH 8.0	100 ml
	filled to 1 l with $_{dd}H_2O$	
10 mM Tris-buffer	Tris-base	1.2 g/l
	in $_{dd}H_2O$; pH 7.5	
10x DNA loading dye	SDS 20%	0.1 ml
	Glycerol	5.7 ml
	1x TAE buffer	4.2 ml
	Bromophenol blue	5 mg
	Xylenecyanol	5 mg
2x Taq buffer	10x Thermo-Pol reaction buffer	200 µl
	dNTP-mix (NEB)	20 µl
	nuclease free water (Fresenius)	780 µl
LPS isolation and staining		
Lysis buffer	$_{dd}H_2O$	4.5 ml
	Glycerol	800 µl
	10% SDS	1.6 ml
	β-Mercaptoethanol	400 µl
	1 M Tris/HCl pH 6.8	500 µl
	Bromphenol blue	
Fixation solution	Isopropanol	50 ml
	Acetic acid	14 ml
	$_{dd}H_2O$	136 ml
Dying solution	$_{dd}H_2O$	70 ml
	1 M NaOH	1.4 ml
	NH_3	1 ml
	20% $AgNO_3$	1.25 ml
	keep order when preparing!	
Developer	2.5% Na_2CO_3	200 ml
	Formaldehyde (add directly before use!)	30 µl

Table 1: Buffers and solutions used in this study - continuing

Polyacrylamide gel staining		
Coomassie staining solution	Coomassie brilliant blue Acetic acid Ethanol filled to 100% with $_{dd}H_2O$	0.25% 10% 50%
Coomassie destaining solution	Ethanol Acetic acid filled to 100% with $_{dd}H_2O$	30% 10%
Kang-solution	CBB G-250 Aluminiumsulfate (14-18)- hydrate Ethanol (96%) ortho-Phosphoric acid (85%)	0.2 g 50 g 100 ml 23.5 ml $_{dd}H_2O$ to 2'000 ml
Yeast disintegration		
2x FSB (Final Sample buffer)	Tris/HCl pH 6.8 DTT SDS Glycerol Bromphenol blue	40 mM 3% 2% 6% 0.02%
50% TCA	Trichloroacetic acid	50 g/ 100 ml $_{dd}H_2O$
SDS-Gels		
10x SDS running buffer	Tris-base Glycin SDS in $_{dd}H_2O$; pH 8.3	30 g/l 144 g/l 10 g/l
Lower Gel Buffer	0.5 M Tris/HCl pH 6.8	
Upper Gel Buffer	1.5 M Tris/HCl pH 8.8	
Western blotting		
CAPS buffer	3-(Cyclohexylamino)-1-Poroanesulfonic - acid Methanol in $_{dd}H_2O$; pH 11	2.21 g/l 100 ml
10x TBS buffer (Tris buffer saline)	Tris/HCl NaCl in $_{dd}H_2O$; pH 7.4	50 mM (60.6 g/l) 150 mM (87.6 g/l)
Solution A (Western blot detection)	0.1 M Tris/HCl pH 8.6 Luminol sonicate for 10 min	200 ml 50 mg
Solution B (Western blot detection)	Hydroxychumarinsäure	11 mg in 10 ml DMSO

3.1.4 Enzymes, Antibodies and Kits

Table 2: Enzymes, antibodies and kits used in this study.

Name	Concentration/Description	Company/Reference
Taq-DNA-Polymerase	5 U/µl	New England Biolabs
RQ1 RNase-Free DNase	1 U/µl	Promega
Restriction enzymes NdeI BamHI-HF®	 20 U/µl 20 U/µl	New England Biolabs
T4 DNA Ligase	1 U/µl	Thermo Scientific
Phusion® High-Fidelity DNA Polymerase		New England Biolabs
anti Flag antibody	monoclonal ANTI-FLAG(R) M2-Peroxidase HRP	Sigma-Aldrich
QIAprep Spin Miniprep Kit	isolation of Plasmid-DNA	Qiagen
DNeasy Blood & Tissue Kit	isolation of Chromosomal-DNA	Qiagen
QIAquick Gel Extraction Kit	elution of DNA out of agarose gel and purification of PCR reaction products	Qiagen
GeneJET RNA Purification Kit	RNA isolation	Thermo Scientific
RevertAid RT Kit	reverse transcription	Thermo Scientific
Proteinase K	15 mg/ml	Qiagen

All enzymes and kits were stored and used as described in the manual provided by the manufacturer.

3.1.5 Antibiotics

Table 3: Used antibiotics; stock- and used concentrations for media and plates.

Antibiotic	Stock-solution	used concentration
Ampicillin (amp)	100 mg/ml	100 µg/ml
Kanamycin (km)	100 mg/ml	30 µg/ml
Nalidixic acid (nal)	100 mg/ml	75 µg/ml
Chloramphenicol (cm)	100 mg/ml	25 µg/ml

Concentrations shown in Table 3 were used throughout whole experiments.

Stocks were stored at -20°C and vortexed prior to use.

3.2 Primer

Table 4: Used primers, their sequences and binding sites.

#	Name	Sequence	Binding site
1	glf_BamHI_fwd	TAAGGATCCATGAAAAAGGCTATTATAATA GGTG	pTG2: 1540-1564 glf_up_overlap: 1-25
2	glf_PstI_rev	TAACTGCAGTCAAATTTGATTCATAATTTCT TTTATA	pTG2: 2972-2999 glf_down_overlap: 237-264
3	Km_screen_rev	GATCTTTAAATGGAGTGT	gatC-aphA-3: 736-753
4	Fw_mob_KpnI	TTGGTACCGTTGGCTTGGTTTCATCAGC	pTG2: 3948-3967
5	Rv_mob_KpnI	TTGGTACCTTCCGTGCATAACCCTGCTT	pTG2: 3670-3689
6	AphA3_forw	GAGGATCCGCTAAAATGAGAATATCACCG	*gatC-aphA3*: 255-275
7	glfko_down_ EcoRI_fwd	TAAGAATTCGCCGGAGACGAGCCATATAC TAG	pTG2: 2736-2758 glf_down_overlap: 1-23
8	GatC_PstI_fwd	ATTCTGCAGAATAGTATCCTTAACATAAAA TTTT	pRYTG1: 5277-5301
9	Glfup_KO-Screen_F	GCTGATATAGAAAATGTTAGCAG	*Cff* 82-40 genome: 1567081-1567103; NC_008599.1
10	gatCaphA3_S-fwd	CTGGATGAATTGTTTTAGTACC	*gatC-aphA3*: 1029-1050
11	Glfdown_ KOScreenR	GCTTTTCTTTGTCCTTTATTTGG	*Cff* 82-40 genome: 1569738-1569760; NC_008599.1
12	M13_fwd	TGTAAAACGACGGCCAG	pGAL plasmids, see Figure 8
13	M13_rev	CAGGAAACAGCTATGAC	pGAL plasmids, see Figure 8
14	NdeI_flag_Y_fwd	AAACATATGGATTACAAGGATGACGAC	*flag*: 1-18
15	NdeI_fic2_Y_fwd	GGACATATGCAAGAACAATATACGGAA	*fic2*: 1-18
16	BamHI_fic2_Y_rev	AAAGGATCCTTATCTTTCCTTTTCTTTTG	*fic2*: 899-918
17	BamHI_Flag-Y-rev	AAAGGATCCTTACTTATCGTCGTCATCC	*flag*: 9-24
18	NdeI_fic1_Y_fwd	AAACATATGGATGGCGGTGTAAATTTAGG	*fic1*: 1-20
19	BamHI_Y_fic1_ rev	TTTGGATCCTCTCTCCTTTTCCTTTGAATT TG	*fic1*: 809-831
20	pGAL-seq_fwd	CTTAACTGCTCATTGCTATATTG	pGAL plasmids: $P_{GAL1-10}$ 193-215, see Figure 8
21	pGAL-seq_rev	GACCTCATGCTATACCTGAG	pGAL plasmids: ADH1 terminator 143-162, see Figure 8
22	RT_glf_fwd	CAAAGGGCATCCTTACACATTTG	*glf*: 135-157
23	RT_glf_rev	GTCGATATCGCTTTTAACTGGTGG	*glf*: 289-312
24	RT-gapDH_ 82-40_F	GCGACGAAAATCACGGCA	$gapDH_{Cff\ 82\text{-}40}$: 412-429
25	RT_gapDH 82-40_R	GAGTGCACCTGCAAAAGACGA	$gapDH_{Cff\ 82\text{-}40}$: 620-640

Restriction sites are highlighted underlined.

Primer stocks were stored at -20°C.

3.3 Strains

Table 5: Used strains

Strain	Description/ Genotype	Reference
Escherichia coli		
DH5α	*endA1 recA1 gyrA96 thi-l hsdR17 supE44 λ- relA1 deoR Δ(lacZYA- argF)- U169 φ80dlacZΔ(M15)*	[36]
S17 *λpir*	*Tp^r Sm^r; recA thi pro hsdR$^-$M$^+$ RP4:2-Tc::Mu::Km Tn7λpir*	[37]
Campylobacter fetus subsp. fetus		
82-40	Human isolate, GenBank acc. no. NC_008599, *Nal^r*	[12]
Δglf	*Cff* 82-40 glf::P_{gatC}-*aphA3*, *Nal^r, Km^r*	this study (F78)
Δglf [pRYTG1]	*Cff* 82-40 glf::P_{gatC}-*aphA3*, trans complemented with *glf* wildtype, *Nal^r, Km^r, Cm^r*	this study (F79)
Δglf [pRYBM5]	*Cff* 82-40 glf::P_{gatC}-*aphA3*, empty complementation vector, *Nal^r, Km^r, Cm^r*	this study (F81)
Saccharomyces cerevisiae		
W303α	*MATα, his3, leu2, ura3, ade2, trp1, can1-100*	lab strain
W303α [pGAL111-*flag-fic2*]	*MATα, his3, leu2, ura3, ade2, trp1* [flag-*fic2*; *LEU2*]	this study (Y4)
W303α [pGAL111-HA-*fic2H184A*]	*MATα, his3, leu2, ura3, ade2, trp1* [*fic2H184A*; *LEU2*]	this study (Y5)
W303 α [pGAL22-*fic1-flag*]	*MATα, his3, leu2, ura3, ade2, trp1* [*fic1-flag*; *TRP1*]	this study (Y3)
W303α [pGAL111-*flag-fic2*] + [pGAL22-*fic1-flag*]	*MATα, his3, leu2, ura3, ade2, trp1* [*fic1-flag*; *TRP1*] [*flag-fic2; LEU2*]	this study (Y7)
W303 Rps3-Flag	*MATα, his3, leu2, ura3, ade2, trp1, can1-100, RPS3-Flag::natNT2*	[not published]

3.3.1 Growth and storage conditions

E. coli strains were cultivated on LB-plates or in liquid LB media with suitable antibiotics, respectively and incubated at 37°C.

Campylobacter strains were cultivated on CBA plates with suitable antibiotics and incubated at 37°C under microaerophilic conditions using the GENbag/box microaer system (Biomérieux). Frozen *Campylobacter* strains were thawed and sub cultivated on CBA plates three times for 24 h before use.

Saccharomyces cerevisiae strains were cultivated on YPD or YNB plates or in liquid media at 30°C.

All strains were stored in 1:1 mixture with 40% Glycerol at -80°C.

3.1 Plasmids

3.1.1 knockout of *glf*

Figure 7 shows schemes of the used plasmids in this part of the study. pTG2 and pRYTG1 were cloned by Tanja Gumpenberger [38]. The cloning strategy for pRYBM5 can be found in [39].

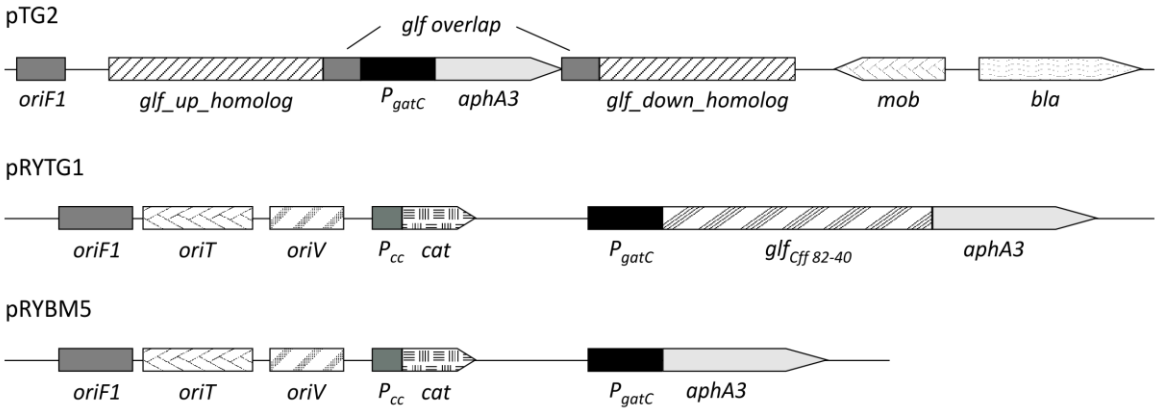

Figure 7: Plasmids used for the knockout of *glf* [pTG2], the trans complementation of *glf* [pRYTG1] and the empty vector control [pRYBM5]; *oriF1*: F1 origin of replication for *E. coli*, glf_up_homolog and glf_down_homolog: homologous regions upstream and downstream of *glf* in *Cff 82-40*, glf overlap: homologous regions of the edges of *glf*, P_{gatC} promoter recognized by *Campylobacter*, *aphA*3: kanamycin resistance, *mob*: minimal sequence for mobilization via conjugation, *bla*: ampicillin resistance, *oriT*: origin of transfer, *oriV*: universal origin of replication, *cat*: chloramphenicol resistance

3.2 General methods

3.2.1 PCR-protocols

To identify colonies that carry the complementation vector pRYTG1 or the empty vector pRYBM5 a colony PCR was performed. In brief, colonies were picked, suspended in 20 µl nuclease free water and incubated at 95°C for 10 min. Cell debris were removed by centrifugation (30 sec. at 13'000 rpm) and the supernatant was used as template for the PCR. As a positive control the respective isolated vector was used, negative control was nuclease free water. Composition of the PCR reaction and protocol are listed in Table 6, Table 7 and Table 8.

Table 6: Components and volumes of typical PCR reactions.

Taq Polymerase			Phusion Polymerase	
Component	**colony PCR**	**isolated DNA**	**Component**	**Volume**
2x Taq buffer	12.5 µl	12.5 µl	Phusion buffer (5x)	8.0 µl
Primer fwd.(10 µM)	1.25 µl	1.25 µl	Primer fwd. (10 µM)	2.0 µl
Primer rev. (10 µM)	1.25 µl	1.25 µl	Primer rev. (10 µM)	2.0 µl
nuclease free H_2O	7.6 µl	depending	nuclease free H_2O	depending
Taq-polymerase	0.4 µl	0.4 µl	Phusion HF polymerase	0.4 µl
DNA template	2.0 µl	40 - 60 ng	dNTP mix (10 mM each)	0.4 µl
	25 µl	**25 µl**	DNA template	40 - 60 ng
				40 µl

Table 7: Components and volumes for colony PCR in *S. cerevisiae.*

Component	**Volume**
10x Thermo Pol Reaction Buffer	2.0 µl
Primer fwd (10 µM)	2.0 µl
Primer rev (10 µM)	2.0 µl
nuclease free H_2O	4.0 µl
dNTP mix (2.5 mM each)	2.0 µl
Taq-polymerase (5 U/µl)	0.5 µl
DNA template	7.5 µl
	20 µl

Table 8: Time and temperature protocols for PCRs.

		colony PCR *E. coli*, *Cff* and isolated DNA	***S. cerevisiae* colony PCR**	
Taq Polymerase PCR				
Initial denaturation	95°C	3:00	5:00	
Denaturation	95°C	0:30	1:00	35x
Primer annealing	*)	0:30	1:00	
Elongation	72°C	1:00/ kb	1:00/ kb	
Final elongation	72°C	5:00	5:00	
Cooling	4/12°C	∞	∞	
Phusion Polymerase PCR				
Initial denaturation	98°C	1:00		
Denaturation	98°C	0:10		35x
Primer annealing	*)	1:00		
Elongation	72°C	1:00/ kb		
Final elongation	72°C	5:00		
Cooling	4/12°C	∞		

*) Temperature depending on the used primer set.

3.3 Agarose gel electrophoresis

For the gels peqGold Universal Agarose (peqlab) was dissolved in 1x TEA buffer by heating in the microwave. The percentage of the agarose was adapted to the expected size of the DNA fragments (1% agarose for fragments 500 – 1'500 bp, 0.8% for fragments >1'500 bp, 1.2% for fragments <500 bp). Ethidium bromide (1 µg/ 100 ml) was added to gels for visualization of the DNA. Gels were run between 80 and 100 V for 30 to 60 minutes. Detection was performed via UV radiation.

3.3.1 SDS-page

Separating gels (15% polyacrylamide) and the stacking gels (4.5% polyacrylamide) were prepared as described in Table 9. Separating gel was prepared up to 5 days prior to use and stored covered with wet paper in the fridge. Stacking gels were always freshly prepared before use. Gels were run at 15 mA per gel.

Table 9: Composition of separating and stacking gel for SDS page.

Component	Stacking gel	Separating gel
$_{dd}H_2O$	1.18 ml	7.2 ml
4x Upper Gel Buffer	0.5 ml	7.5 ml
30% Acrylamide	0.3 ml	15 ml
10% SDS	20 µl	300 µl
10% APS	8.0 µl	81 µl
TEMED	2.7 µl	26.1 µl
0.5% Bromphenol blue	5.0 µl	-
	2 ml	**30 ml**

3.3.2 Protein staining

Polyacrylamide gels were rinsed two times in VE-H_2O and incubated in Kang dying solution overnight, or in Coomassie staining solution for 30 minutes and destained 4-times with destaining solution for about 30 minutes. Pictures were taken with ChemiDoc™ MP Imaging System (BIO RAD).

3.4 Western blotting

Nitrocellulose protein membrane (Immobilon) was activated in Methanol for 2 s, washed in VE-H_2O for 2 min and equilibrated in CAPS buffer for at least 5 min. SDS gels were also equilibrated in CAPS buffer.

Proteins were transferred onto the membrane with Trans-Blot® Turbo™ Transfer Starter System (BioRad) at 220 mA for 1.5 h.

For the detection of the Flag-tagged Fic proteins the membrane was blocked (TBS buffer + 3% milk powder) at 4°C overnight. After washing the membrane (2x, 10 min, TBE buffer) the Anti-Flag Antibody (ANTI-FLAG® M2-Peroxidase HRP (Sigma-Aldrich)) was diluted 1:15'000 in TBS buffer with 1% milk powder and the membrane was incubated under gentile shaking for 1.5 h. The membrane was washed 3 times with TBE buffer and signals were detected as follows.

Detection:

Membrane was rinsed in detection solution (4 ml Solution A plus 400 µl Solution B and 1.2 µl H_2O_2 (30%), mixed immediately before use - solutions see Table 1) for about 2 minutes.

The signals were detected in ChemiDoc™ MP Imaging System (BIO RAD).

3.5 Methods for the characterization of the *glf*-knockout

3.5.1 Conjugation

For the genetic modification of *Cff* the used plasmids were brought in via conjugation. As donor *E. coli* SM10λ*pir* with the respective plasmid was used. *Cff* strains were thawed as described previously and suspended in 1 ml 1x PBS. Three hours prior the conjugation a main culture of *E. coli* SM10λpir [respective plasmid] was inoculated (1 ml ONC in 4 ml fresh media). For conjugation 1x 10^7 *E. coli* cells were mixed with 1x 10^9 *Cff* cells (*E. coli* OD_{600} 0.1 corresponds to 5x 10^7 cells/ml, *Cff* OD_{600} 0.1 corresponds 5x 10^8 cells/ml). The mixture was centrifuged (5'000 rpm, 2 min) and the pellet was resuspended in 30 µl 1x PBS. This suspension was put onto a nitrocellulose filter (25 mm, 0.45 µm, Millipore) which was placed on a CBA plate. This plate was incubated at 37°C, microaerophilic for at least 1 hour. Cell material was suspended by vortexing the filter in 1 ml 1x PBS. The suspension was plated on CBA plates with respective selective antibiotics and incubated for 2 to 4 days.

3.5.2 LPS isolation and staining

LPS isolation

Campylobacter cells (OD_{600} of 0.5, approx. 5x 10^7 cfu) were suspended in 1x PBS (pH 7.3) and harvested by centrifugation (5'000 rpm, 5 min). Cells were washed 1 time in 1x PBS. The pellet was resuspended in 100 µl lysis buffer (Table 1) and incubated at 100°C for 10 min. 54 µl proteinase K solution (15 mg/ml) were added and samples were incubated at 55°C overnight. The next day 10 µl of the samples were loaded on two 15% polyacrylamide gels. SDS pages were run at 20 mA for about 35 min and at 40 mA for about 20 min. Gels were rinsed with $_{dd}H_2O$.
One gel was used for protein staining with Kang solution (see 3.3.2), one for silver staining.

Silver staining:

The gels were incubated in 100 ml fixation solution (Table 1) overnight. The next day the gels were incubated 10 min in 100 ml fixation solution with 0.87 g Sodiumperiodat, washed 3 times in $_{dd}H_2O$ (30 min each) and incubated in dying solution (Table 1) for 10 min. After washing 3 times (15 min each) in $_{dd}H_2O$ the gels were incubated in 200 ml developer (Table 1) until the bands appeared (about 5 min). The reaction was stopped by adding 100 ml 50 mM EDTA (pH 8.0) and incubating for at least one hour. Pictures were taken with ChemiDoc™ MP Imaging System (BIO RAD).

3.5.3 Serum resistance assay

Campylobacter cell material was suspended in MEM (pre-warmed to 37°C) and 1.0x 10^7 cells (OD_{600} of 1.0 corresponds to 5.0x 10^9 cfu/ml) were transferred into a fresh tube and brought to a final volume of 50 µl with MEM. Active human serum (thawed on ice) or heat inactivated human serum (56°C, 30 min) was added to a final concentration of 10%, respectively. After an incubation of 1 h at 37°C (static incubator), serial dilutions were plated on CBA plates. Plates were incubated for 3 days. The assay was performed in triplets to equalize variations in pipetting.

3.5.4 Acid survival Assay

Campylobacter cells were suspended in 1x PBS (pH 7.3, pre-warmed to 37°C). 1.0x 10^8 cells (OD_{600} of 1.0 corresponds to 5.0x 10^9 cfu/ml) were added to 1 ml 1x PBS with different pH levels (7.3, 4.0, 3.5 and 3.4). Bacterial suspensions were incubated at 37°C for 30 min in the incubator. Serial dilutions (1x PBS, pH 7.3) were plated on CBA plates. Plates were incubated for 3 days. The assay was performed in triplets to equalize variations in pipetting.

3.5.5 RT-PCR

RNA from the investigated *Campylobacter* strains was isolated using GeneJET RNA Purification Kit (Thermo Scientific). DNA was removed by adding RQ1 RNase-Free DNase (Promega). Reverse transcription was performed with the RevertAid RT Kit (Thermo Scientific). All steps were performed according to manufacturer's recommendations.

3.6 Methods for the expression of Fic proteins in *S. cerevisiae*

3.6.1 Cloning

The Fic proteins had to be cloned in a shuttle vector for *S. cerevisiae* which has an adjustable promoter for expression of the Fic proteins. Therefore, *fic* genes were cloned into vectors with $Gal_{1\text{-}10}$ promotors. It was necessary to use two different vectors with different selection markers for *fic1* and *fic2* to enable co-transformation of the two genes. Selection of *S. cerevisiae* is realised with selection plates that lack a certain amino acid. The strain lacks one gene of the biochemical pathway for the synthesis of this amino acid which is then complemented by the plasmid. Therefore the vectors pGAL22 (tryptophan as selection marker), pGAL111 and pGAL111-HA (leucine as selection marker) were used. The vectors were sent by Dieter Kressler, University Fribourg. These plasmids carried a gene between NdeI and BamHI restriction sites.

For cloning the *fic* genes into these vectors, primer pairs 18/17, 14/16, 15/16 and 18/19 were used for *fic1-flag*, *flag-fic2*, *fic2H184A and fic1*, respectively to amplify the desired genes and add the necessary restriction sites. As templates pBAD-Cm-fic1-flag (wildtype *fic1*), pBAD24-flag-fic2 (wildtype *fic2*) and pBAD24-fic2H184A (*fic2* with mutated fic-motif) [8] were used.

Vectors and inserts were digested with NdeI and BamHI (NEB), purified and ligated. The generated vectors are shown in Figure 8. Ligation mixes for pGAL22-fic1-flag and pGAL22-fic1 were used to transform *E. coli* DH5α and those for pGAL111-flag-fic2 and pGAL111-HA-fic2H184A were introduced directly into *S. cerevisiae* W303α.

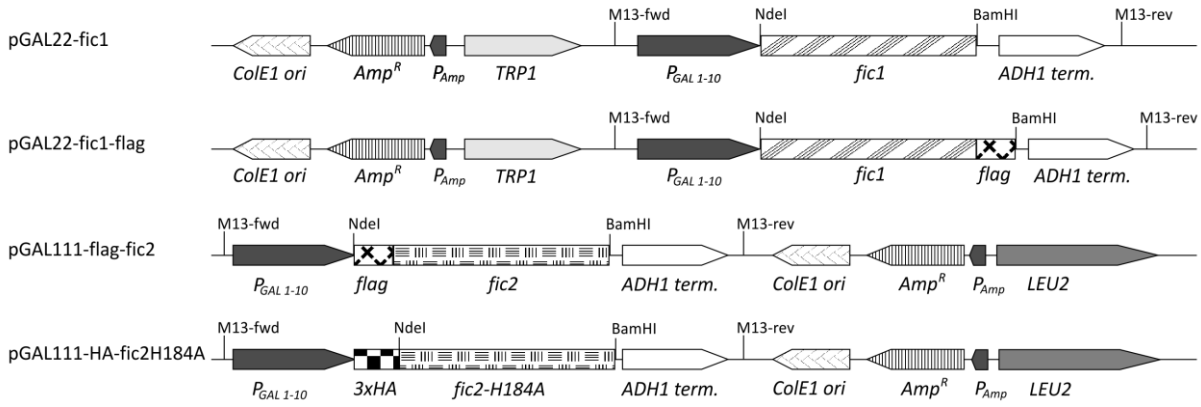

Figure 8: Plasmids used for the expression of Fic1-Flag, Flag-Fic2, HA-Fic2H184A and Fic1 in *S. cerevisiae*; *ColE1 ori*: origin of replication, *Amp*^{R}: ampicillin resistance (for selection in *E. coli*), TRP1: tryptophan metabolism gene 1 (for selection in *S. cerevisiae*), $P_{GAL1-10}$: galactose promoter, ADH1 term.: ADH1 terminator, *flag*: flag-tag, LEU2: leucine metabolism gene 2 (for selection in *S. cerevisiae*), 2xHA: 3 times his-tag

3.6.2 Yeast transformation

3.6.2.1 Competent yeast cells

A 50 ml main culture was inoculated with an ONC to an OD_{600} 0.2 and incubated at 30°C, 180 rpm to a final OD_{600} of 0.6 – 0.8. Cells were harvested (3'500 rpm, 7 min) and two times washed with 10 ml 0.1 M Lithium acetate solution (Table 1).

The supernatant was removed thoroughly and the pellet was suspended in 300 µl 0.1 M Lithium acetate solution. After incubating at 30°C for 20 min the suspension was divided into 50 µl aliquots and stored on ice until use.

3.6.2.2 Transformation

The carrier DNA (Herring sperm DNA) was denatured at 95°C for 10 min and cooled on ice. The used transformation mixture is shown in Table 10. The mixture was vortexed to get a homologous suspension, incubated at 30°C (heating block) for 30 min and then heat shocked at 42°C for 20 min. As control one mixture was made without plasmid DNA/ligation reaction product and one contained an empty plasmid with the same selection marker as the desired DNA. Transformation mixtures were plated completely onto respective selection plates and incubated at 30°C 3 to 4 days.

Table 10: Components and volumes for one yeast transformation mixture.

Component	Volume
Competent cell suspension	50 µl
Plasmid DNA/ ligation reaction product	3 - 6 µl/ 20 µl
Carrier DNA (herring sperm DNA) (10 mg/ml)	5 µl
PEG (40%)	300 µl

3.6.3 Spot test

Investigated strains were cultured on respective selection media containing glucose. Cell material was suspended in sterile 1x PBS. 300 µl 1x PBS were inoculated with an OD_{600} of 1. Dilutions to $1.0x10^{-3}$ were made in a 96 well plate. Suspension was dropped three times on selective media containing glucose (repressed) and three times on selection media containing galactose (induced). Plates were incubated at either 25°C, 30°C or 37°C for 3 days.

3.6.4 Yeast disintegration

ONCs of the investigated strains were prepared in respective selection media. An OD_{600} of 1.0 was harvested (3'500 rpm, 7 min) and suspended in 200 µl 1.85 M NaOH with 7.5% β-Mercaptoethanol. After incubation on ice for 10 min 200 µl 50% TCA were added, samples were mixed by pipetting and incubated on ice for 10 min. Proteins were spun down (13'200 rpm, 15 min, 4°C) and the supernatant was removed properly. The resulting pellets were washed two times in $_{dd}H_2O$ and resuspended in 80 µl 2x FSB (Table 1). Proteins were denatured at 95°C for 10 min, cell debris were removed by centrifugation (10'000 rpm, 10 min) and the supernatant was used for SDS page.

3.6.5 Microscopy

ONCs of the investigated strains were prepared in respective selection media, one time with induced (galactose) and one time repressed (glucose) expression. 1 ml of the ONC was spun down (2 min, 2'000 rpm, Eppendorf table top centrifuge) and 2 µl of the pelleted cells were taken for microscopy.

4 RESULTS

4.1 Part1 - Characterization of a *Cff 82-40 glf* knockout strain

4.1.1 Verification of *glf*-knockout

My goal was to knock out (KO) the *glf* gene of *Cff* 82-40 by insertion of a kanamycin resistance cassette (P_{gatC}-*aphA3*) via homologous recombination and remove parts of the gene. A suicide plasmid (pTG2) carrying the insertion cassette and *glf* targeting DNA [38] was transferred from *E. coli* S17 λpir via conjugation into *Cff* 82-40. Obtained colonies were screened for presence of the *aphA3* cassette and candidate *glf* knock outs were tested with a set of PCR primers (1/2, 9/3, 6/3, 10/11, 9/11) to verify the correct position of the resistance cassette in the genome. Figure 9 A shows a schematic picture of the wildtype and KO genome with the binding position of the primers used and the expected PCR product sizes. Additionally, a PCR for the pTG2 backbone was performed (primers 4/5) to verify that no plasmid DNA was left in the KO-strain.

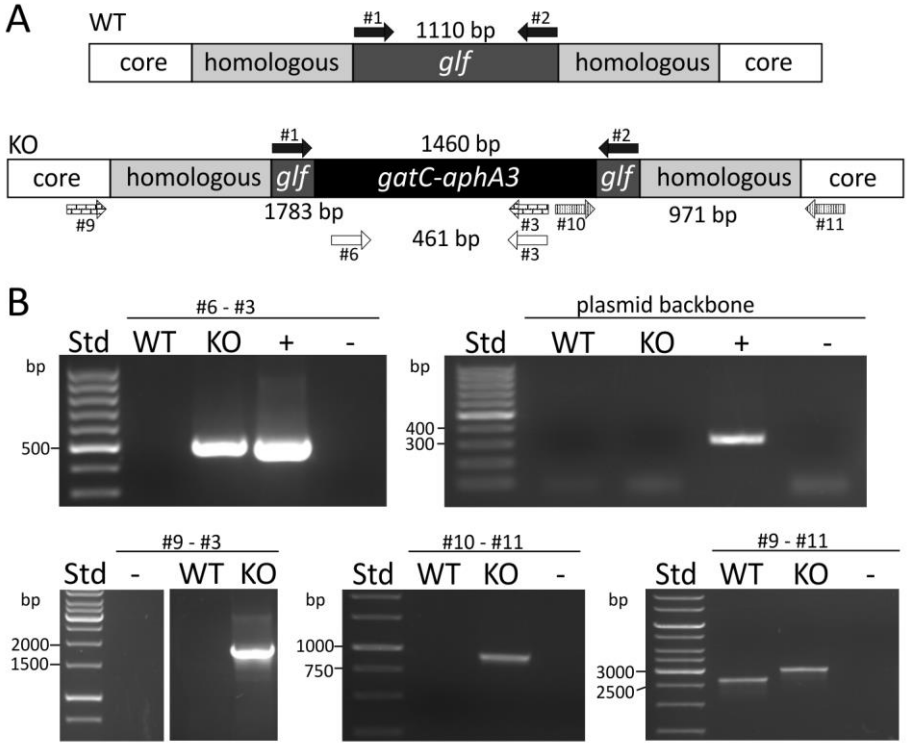

Figure 9: Verification of the *Cff* 82-40 *glf* knockout strain Δglf; (A) Schematic representation of the wildtype (WT) *glf* genome region and its isogenic knockout. Primers used for the verification of correct integration of *aphA3* and expected fragment sizes are indicated. **(B)** Gel electrophoresis of the amplified fragments shown in A. As positive control (+) the knockout plasmid was used, as negative control (-) H_2O. To screen for presence of the plasmid backbone a 298 bp fragment of the *mob* gene was amplified. All pictures show bands in the expected lanes and at the expected size and no bands in the negative controls.

As all PCRs yielded expected fragment sizes (Figure 9 B). I conclude that the *aphA3*-cassette has been integrated into *glf* and a *glf*-knockout strain (Δglf) was created successfully.

4.1.2 Trans complementation of *glf* with pRYTG1 leads to an overexpression of *glf* - RT-PCR

To complement the *glf*-knockout, a complementation vector (pRYTG1) was introduced into Δglf via conjugation. To verify the expression of *glf* from pRYTG1 a reverse transcription PCR analysis was performed. RNA was isolated from *Cff* 82-40, Δglf and Δglf [pRYTG1] (comp). An equal amount of RNA of each sample was transcribed into cDNA. One set of samples was prepared without reverse transcriptase (RT-) to check for DNA contaminations. Two sets of PCR-primers were used to amplify a 178 bp fragment of *glf* (target gene, primer 22/23) and a 229 bp fragment of *gapDH* (reference gene, primer 24/25) (Figure 10).

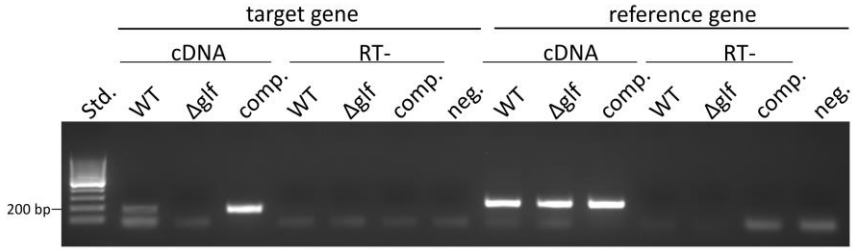

Figure 10: Complementation of *glf* in trans leads to a higher mRNA level of *glf* compared to *Cff* 82-40 (WT). RNA of *Cff* 82-40 (WT), Δglf and Δglf [pRYTG1] (comp.) was isolated, reverse transcribed and amplified with two sets of primers (target gene *glf* (178 bp) and reference gene *gapDH* (229 bp). RT- samples were not reverse transcribed and serve as a negative control for DNA contamination.

RT-PCR results (Figure 10) showed that *glf* mRNA is detectable in the WT but not in Δglf. Furthermore there are higher mRNA levels for *glf* in the complemented strain (comp.) whereas mRNA levels for the reference gene were the same for all tested strains. The complementation strain was used in the phenotypic assays to evaluate possible polar effects of the *glf* knockout.

4.1.3 Knockout of *glf* does not influence the amount of LPS O-Antigen - LPS staining

To find out, whether Glf is involved in the synthesis of LPS silver staining of isolated LPS was performed. LPS was isolated from WT, Δglf and the complemented strain as well as from *Cff* ATCC K19 (negative control) and Δglf with a vector control. This vector control stain (Δglf with an empty complementation plasmid ([pRYBM5]) was used to analyse potential effects due to the used vector backbone. After cell lysis proteins were digested with proteinase K. The complete digestion was verified with a SDS-page and Kang staining (Figure 11 A). LPS parts were separated with a SDS-page and visualized via silver staining (Figure 11 B).

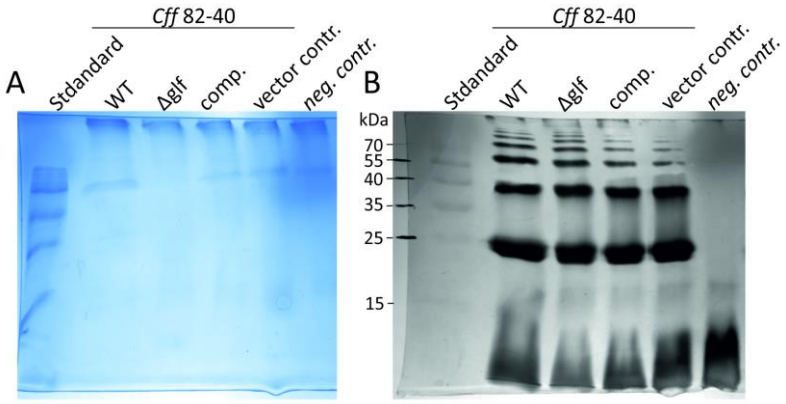

Figure 11: Glf does not influence the amount of LPS in *Cff* 82-40. (A) SDS-page and protein staining after LPS isolation as control for protein digestion **(B)** SDS-page pattern of purified LPS after silver staining. Samples were isolated from *Cff* 82-40 (WT), Δglf, Δglf [pRYTG1] (*glf* in trans, comp.), Δglf [pRYBM5] (vector contr.) and *Cff* ATCC 27374 wcbK::aphA3 (neg. contr.).

Kang staining of the LPS samples showed that there was hardly any protein left after proteinase K digestion (Figure 11 A). This indicates that the various bands on the silver stained SDS-page (Figure 11 B) are not proteins but LPS as described in literature [40], [41]. Furthermore there was no difference in the LPS pattern between the investigated strains except the negative control, which has been shown to lack LPS O-Antigen [7]. In conclusion, it was not possible to detect any obvious differences in LPS synthesis between the Δglf strain and the WT strain.

4.1.4 Knockout of *glf* leads to increased acid sensitivity at pH 3.5 and 3.4 compared to the wildtype - acid survival assay

Because it was known that genes involved in LPS synthesis influence acid tolerance in other *Cff* strains [7], acid survival assays were performed. Equivalent numbers of cells of the compared strains (WT, Δglf, comp. vector contr.) were exposed to buffers with different pH values (7.3, 4.0, 3.5, 3.4) for equivalent times, then diluted and plated. The surviving cfu were counted and plotted (Figure 12).

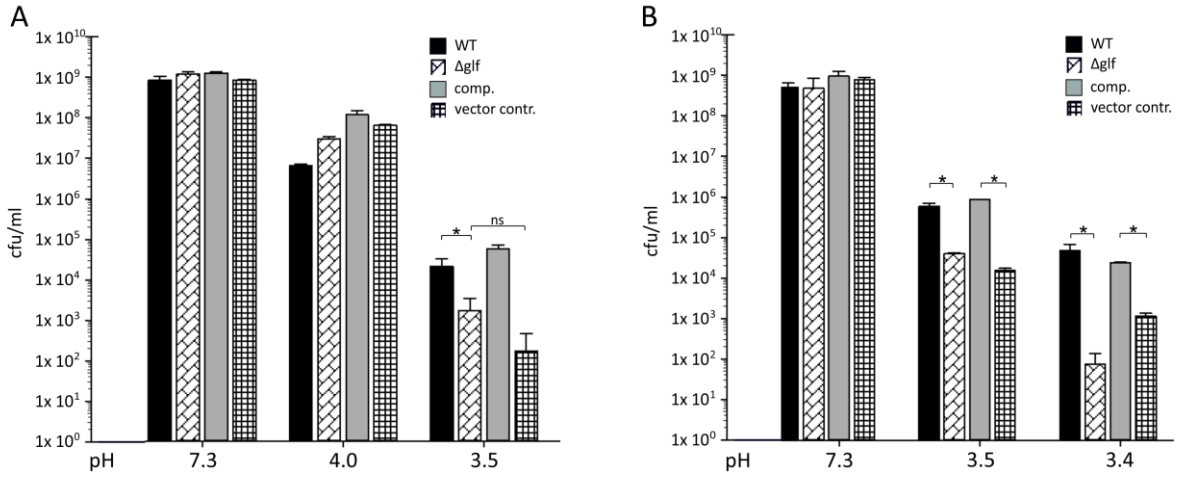

Figure 12: Glf is important for acid tolerance in *Cff* 82-40. Acid survival assay. (A) *Cff* strains were incubated in PBS within a pH range of 7.3 to 3.5, or **(B)** within a pH range of 7.3 to 3.4. Colony forming units (cfu) were counted after plating on CBA plates. The survival of the strains *Cff* 82-40 (WT), Δglf, Δglf [pRYTG1] (glf in trans, comp.), Δglf [pRYBM5] (vector contr.) was compared. The assay was performed in triplets.

There were significantly less surviving colonies of Δglf compared to *Cff* 82-40 (WT) when bacteria were incubated at pH 3.5 and 3.4. Reduced acid tolerance could be complemented by providing *glf* in trans (comp.) but not by the empty complementation vector (vector contr.) (Figure 12).

In summary, I conclude that Glf is involved in acid tolerance of *Cff* 82-40.

4.1.5 Knockout of *glf* does not alter serum resistance compared to the wildtype - serum resistance assay

The loss of acid tolerance exhibited by Δglf is consistent with an alteration in surface structure as we hypothesized for this mutant. We now tested whether another surface related phenotype, resistance to host serum, was also affected.

Equivalent numbers of cells of each strain (WT, Δglf, comp., vector control, *Cff* ATCC) were exposed to MEM, active or inactive human serum for equal times, then diluted and plated. The surviving cfu were counted and plotted (Figure 13 A). The serum sensitive strain *Cff* ATCC was added as control. For better visualization the active serum treated surviving cfu were normalized against cfu of MEM media treated cfu (Figure 13 B).

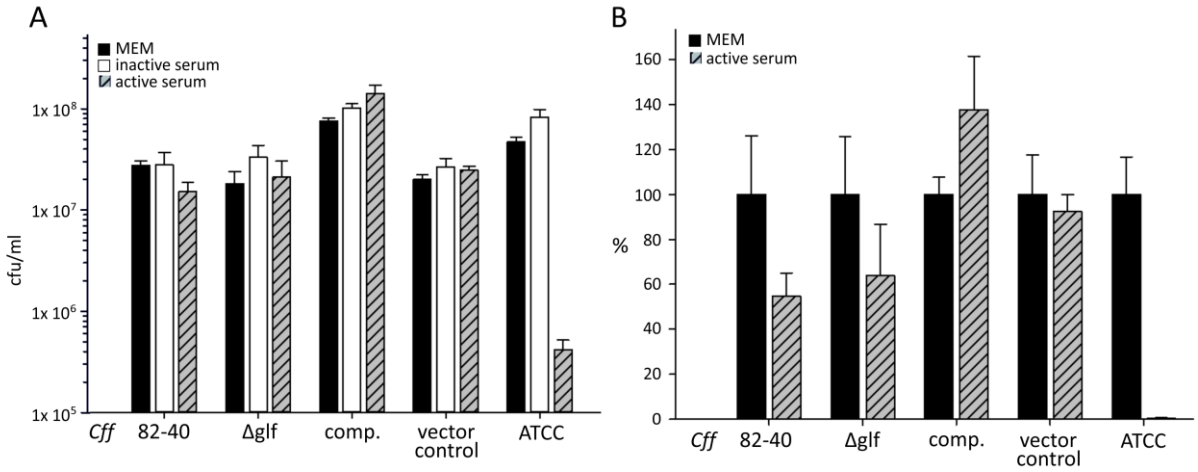

Figure 13: Glf is not involved in serum resistance of *Cff* 82-40. Serum survival assay; (A) Strains were incubated either with MEM, heat-inactivated or active human serum. Colony forming units (cfu) were counted after plating on CBA plates. Compared are the strains *Cff* 82-40, Δglf, Δglf [pRYTG1] (*glf* in trans; comp.), Δglf [pRYBM5] (vector control) and *Cff* ATCC 27374 as a serum sensitive control. **(B)** Same setup as (A) but values normalized to MEM cfu/ml for better visualisation. The assay was performed in triplets.

There was no significant difference in surviving colonies for Δglf compared to *Cff* 82-40 (WT) Figure 13 A) but a better survival of the trans complemented Δglf (comp.) (Figure 13 B) was observed. From these results I conclude that knock out of *glf* does not interfere with serum resistance of *Cff* 82-40.

4.2 Part 2 - Expression of Fic proteins in *S. cerevisiae*

4.2.1 Verification of *fic*-containing plasmids- colony PCR and Sequencing

To investigate possible targets of the Fic proteins from *Cfv* in *S. cerevisiae* it was necessary to clone the respective genes into a vector system which is suitable for protein expression in *S. cerevisiae*. The used vectors provided the adjustable $Gal_{1\text{-}10}$ promotor which can be repressed by offering glucose and induced by offering galactose in the cultivation media. Furthermore the vectors used for *fic1* and *fic2* variants harboured different selection markers respectively to enable co-transformation and co-expression.

After amplification of the *fic* genes, restriction and ligation with the respective vectors the ligation mixes were used to transform *E. coli* DH5α. To confirm the successful vector to insert ligations, colony PCRs were performed. For the screening the respective insert primers, which also were used for the cloning (see section 3.6.1), were used. Positive colonies for pGAL22-fic1-flag and pGAL22-fic1 could be identified (Figure 14), but several attempts to transform *E. coli* with the ligation mixes of pGAL111-flag-fic2 and pGAL111-HA-fic2H184A failed. Therefore these two ligation mixes were transformed directly into *S. cerevisiae*. Colony PCR and agarose gel electrophoresis were performed to identify positive clones (Figure 14).

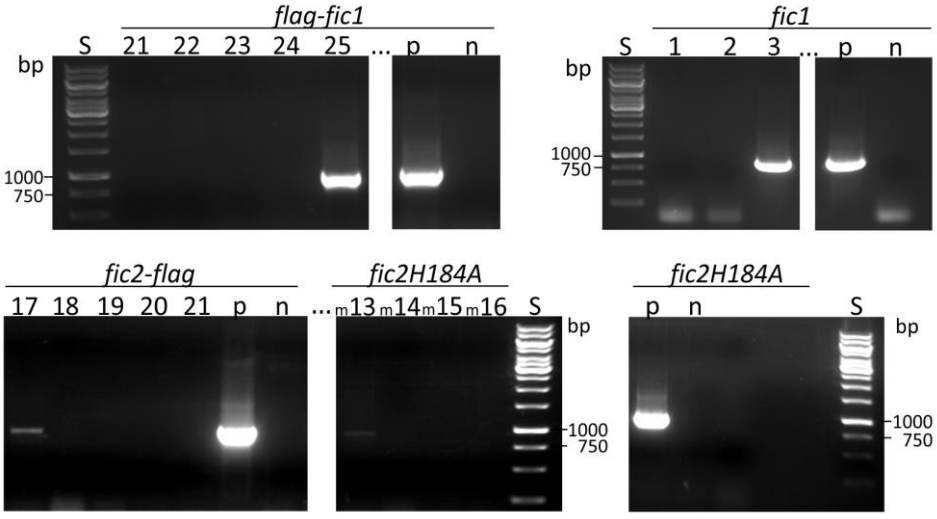

Figure 14: Screen for hosts transformed with *fic*-containing plasmids. Colonies were picked from selection plates and screened with colony PCR using *fic*-specific primers. Colonies 25, 3, 17 and m13 showed bands for *flag-fic1, fic1, fic2-flag* and HA-*fic2H184A*, respectively.

Plasmids of positive *E. coli* clones (pGAL22-fic1-flag, pGAL22-fic1) were isolated and partly sequenced with primers binding to the vector up- and downstream of the insert (primer 20/21, sequencing by Microsynth AG). Comparison with the wildtype sequences showed that both, *fic1-flag* and *fic1*, matched 100%. The plasmids were used for all further experiments.

As it is more difficult to isolate plasmid DNA from *S. cerevisiae*, the yield for pGAL111-flag-fic2, pGAL111-HA-fic2H184A was too low for sequencing. Therefore, PCR amplification of the *fic*-containing region of the plasmids was performed (primer 12/13, shown in Figure 8). The resulting PCR products were purified and sequenced (primer 20/21, sequencing by Microsynth AG). Comparison with the expected sequences showed that both, *flag-fic2* and *HA-fic2H184A*, matched 100%. The plasmids were used for all further experiments. The sequence comparisons are shown in appendix section 7.2.

4.2.2 Microscopy of *S. cerevisiae* expressing Fic1-Flag and Flag-Fic2 showed normal cell-shape

In previous experiments HeLa cells showed cell-rounding due to Fic1 and Fic2 expression and *E. coli* showed filamentation due to Fic2 expression [8]. If the target of Fic2 is conserved in *S. cerevisiae* alteration in cell morphology could be expected.

S. cerevisiae strains with the different fic expression vectors were grown in ONCs, either under repressed (glucose present) or under induced (galactose present) conditions. Cells were harvested and examined under the microscope. The resulting pictures are shown in Figure 15.

There was no difference in cell shape observed in repressed and induced samples and also no difference to the control strains which harboured an empty plasmid that enables growth in the same selection media ([pRS314] and [pRS315]).

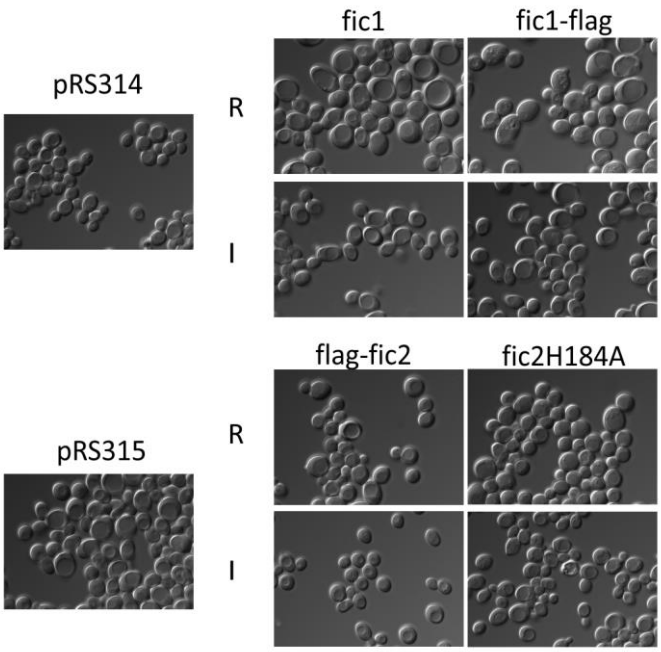

Figure 15: *S. cerevisiae* showed no visible effect on cell shape due to expression of Fic1, Fic1-Flag, Flag-Fic2 or HA-Fic2H184A. microscopy; *S. cerevisiae* strains with an empty vector or the respective plasmid were grown under induced (I) and repressed (R) conditions over night and examined under the microscope (40x magnification, 1.6x secondary magnification).

4.2.3 *S. cerevisiae* with Fic1-Flag and Flag-Fic2 showed no difference in growth - spot tests

Because it was shown that Fic2 has an effect on growth of *E. coli* cells as well as on survival of human cells, the target for this effect seems to be well conserved. I expected that there also would be an effect on growth of *S. cerevisiae*.

To test this, *S. cerevisiae* expressing either Fic1-Flag, Flag-Fic2 or HA-Fic2H184A was investigated via spot test.

In a first experiment each tested clone harboured two plasmids. On one hand to be compared on the same selection media plate and on the other hand to test combinations of *fic1* and *fic2*, because it was known that Fic1 can repress the toxicity of Fic2 in *E. coli*. As empty plasmids for the combination either pRS314 or pRS315 were used, pRS314 + pRS315 served as growth control for wildtype growth.

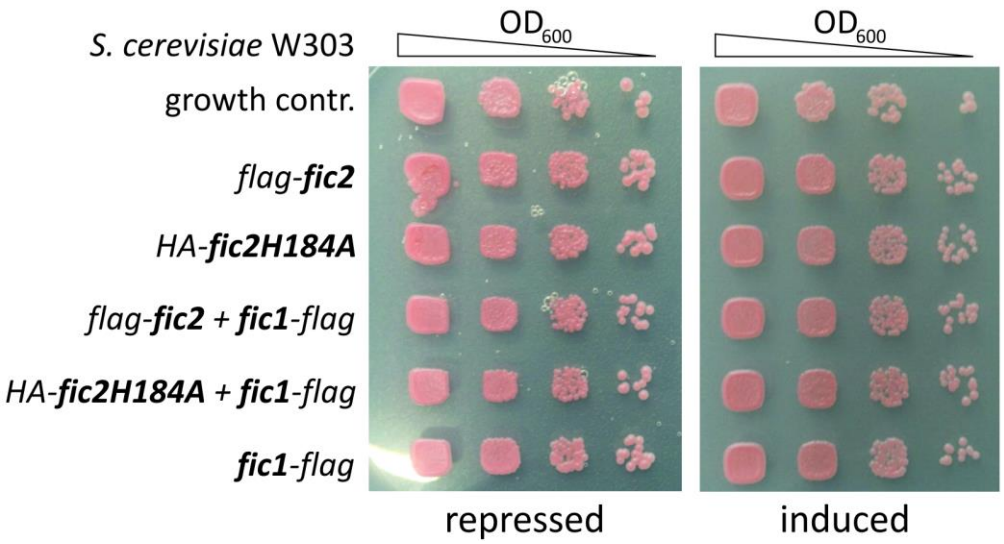

Figure 16: *S. cerevisiae* showed no visible growth defect due to expression of Fic1-Flag, Flag-Fic2 or HA-Fic2H184A. Spot test; Strains were grown on repressing media, suspended in PBS, diluted and spotted on selection plates, induced and repressed, and incubated at 30°C for three days. Colony size can be compared to growth control (pRS314 + pRS315).

As Figure 16 shows, there was no difference in colony size for the clones harbouring different fic genes, alone or in combination neither when the expression was repressed (left) nor when it was induced (right). Colony size was compared to control strains that harbour empty plasmids ([pRS314] and [pRS315]) that enable them to grow on the same selection media.

In a second experiment *fic1* without any tag was added to the set of strains, because it could be possible that the tag influences toxicity. *Fic1* was used because it also shows toxic activity in cell culture experiments [8] and was easier to clone than *fic2*.
Furthermore the plates were incubated at different temperatures to find out whether there is a temperature dependent effect of the Fics. Figure 17 shows that there was hardly any difference in colony size for all tested strains.
In summary, there was no clearly visible growth defect of *S. cerevisiae* expressing different Fic proteins compared to growth control strains ([pRS314] and [pRS315]).

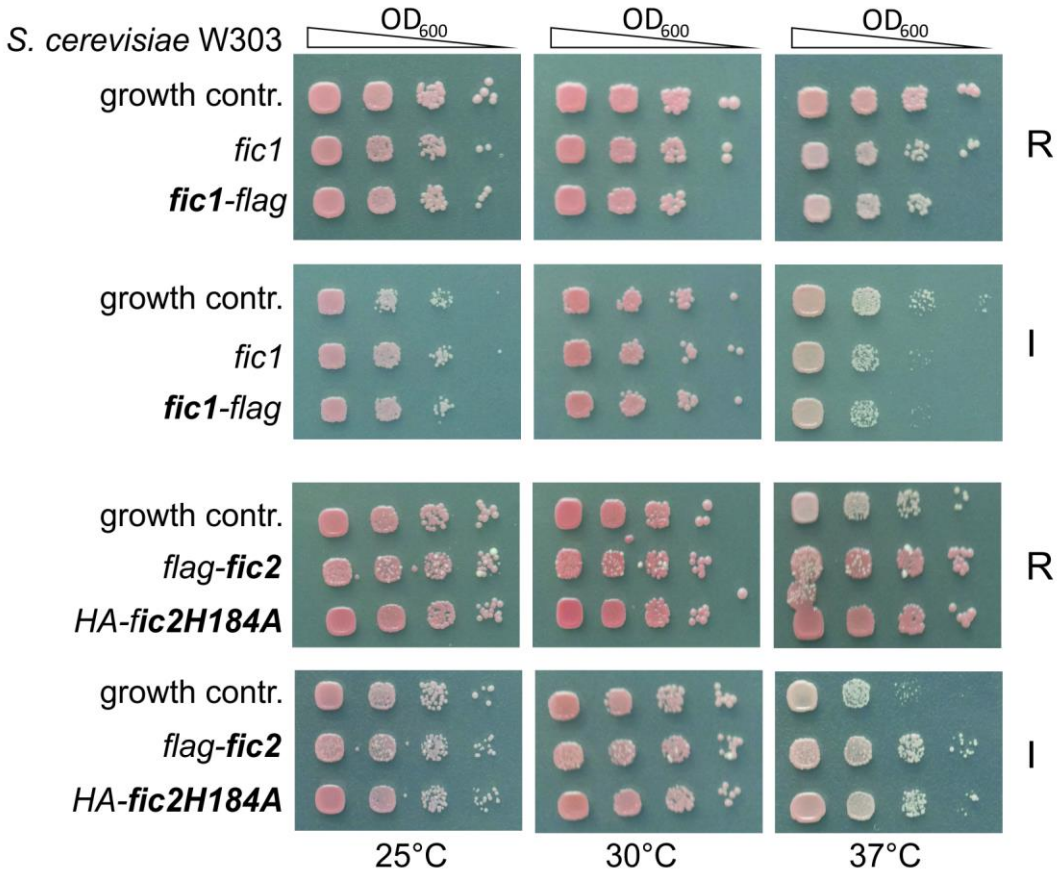

Figure 17: *S. cerevisiae* showed no temperature dependent growth defect due to expression of Fic1, Fic1-Flag or Flag-Fic2. Spot test; Strains were grown on repressing media, suspended in PBS, diluted and spotted on selection plates, induced (I) and repressed (R), and incubated at 25°C, 30°C and 37°C for three days. Colony size can be compared to growth control (pRS314 or pRS315).

4.2.4 Fic1-Flag and Flag-Fic2 are expressed in *S. cerevisiae*

- Anti-Flag western blot

Because of the lack of any growth phenotype of the *fic* containing *S. cerevisiae* cells, the expression of Fic1-Flag and Flag-Fic2 was verified with western blot. The strains were grown in inducing media (with galactose) overnight, harvested by centrifugation and lysed as described (3.3.1 SDS-page). As controls *S. cerevisiae* W303 wildtype (negative control) and *S. cerevisiae* W303 Rps3-Flag (positive control) were taken. Because it was not known how strong the signals from the western blot would be, different volumes (10 µl, 5 µl, 2.5 µl) of the whole cell lysate were analysed. As the amount of protein was not normalized, one SDS-PAGE was stained with Kang dying solution to visualize the total amount of loaded protein (Figure 18 A). A second SDS-PAGE gel was blotted onto nylon membrane and Flag tags were detected with Anti-Flag-HRP® antibody (Sigma Aldrich, Figure 18 B).

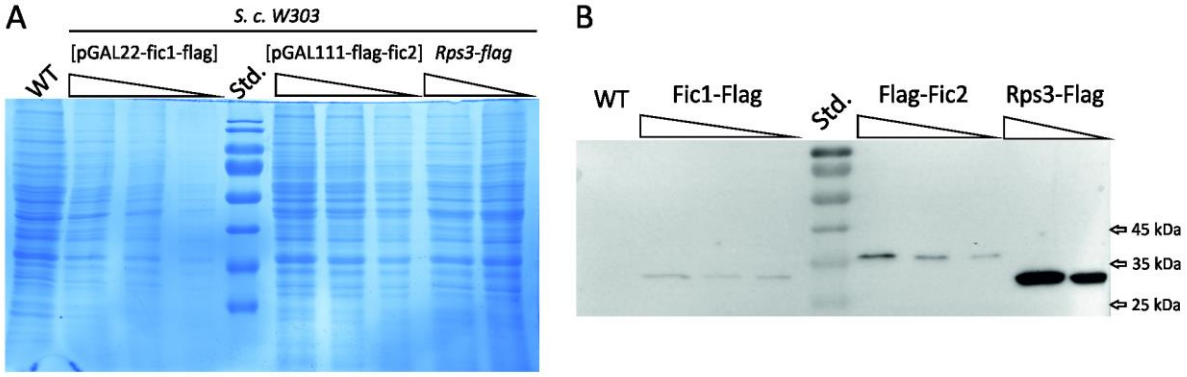

Figure 18: Fic1-Flag and Flag-Fic2 were expressed in *S. cerevisiae*. (A) Kang-stained SDS-PAGE (B) western blot membrane. Samples loaded were whole cell extracts (10 to 2.5 µl) of *S. c.* W303 (WT), *S. c.* [pGA22-fic1-flag], *S. c*. [pGAL11-flag-fic2] and *S. c*. Rps3-flag as pos. control. Std.: PageRuler Prestained Protein Ladder (Thermo). Detection of Flag was performed via Anti-Flag-HRP antibody.

The result of the western blot (Figure 18 B) showed that both Fic1-Flag and Flag-Fic2 were expressed. The negative control showed no band, all Fic1-Flag samples showed a band a bit lower than 35 kDa and all Flag-Fic2 samples showed a band a bit above 35 kDa. Both results fit the expected protein sizes of 32.7 kDa and 36.3 kDa, respectively. The strength of the bands for Fic1-Flag and Flag-Fic2 could not be compared with each other because, as it can be seen in Figure 18 A, the loaded amount of total protein was not equivalent. A direct comparison with the strength of the Rps3-Flag bands is further not possible because *rps3* is a ribosomal protein under its natural promotor whereas *fic1-flag* and *flag-fic2* were under the $Gal_{1\text{-}10}$ promotor.

5 DISCUSSION AND OUTLOOK

Since the niche preference of the two *C. fetus* subspecies is still poorly understood, it is necessary to characterise genes that are different between them [7]. This work gives insight in the role of *glf* in acid and serum resistance of *Cff* serotype A. Furthermore, the successful expression of Fic1 and Fic2 in *S. cerevisiae* will aid further investigation of the TA system of *Cfv* and its contribution to virulence.

5.1 Characterization of a *Cff 82-40 glf* knockout strain

It was shown that in *Cff* serotype B strains, *wcbK* (a putative GDP-mannose 4,6-dehydratase) plays a major role in resistance to acid and human serum. In serotype A strains *glf* (a putative UDP-galactopyranose mutase) is present instead of *wcbK*. As both enzymes are involved in the transformation of sugar molecules that occur in the LPS structure but are unique for either the one or the other serotype it is hypothesized that *glf* may have a similar role as *wcbK*.

Glf *is involved in acid tolerance of* Cff *serotype A*

Cff wildtype shows tolerance to acidic conditions. This feature is very important since *Cff* enters its host orally and needs to survive the passage through the acidic stomach to reach the mucus of the intestine, where it can colonize [1]. The results of this work show that inactivation of *glf* leads to a significant decrease of acid tolerance at pH levels of 3.5 and 3.4. Acid resistance was restored by offering *glf* in trans. The vector backbone alone did not influence the observed phenotypes. These results show that *glf* is involved in acid resistance of *Cff* serotype A in vitro and therefore is expected to contribute to its virulence.

Glf *does not influence serum resistance of* Cff *serotype A*

Cff serotype A is the *Campylobacter* species most frequently found in patients suffering from bacteraemia [2]. It was shown that the S-layer of *Cff* serotype A strains prevents complement factor C3b from binding to the bacterium and therefore leads to serum resistance [15]. This work shows that the knockout of *glf* has no influence on serum resistance of *Cff* 84-40 ($\log_{10}$ kill Δglf 0.21 ± 0.18, Wt 0.26 ± 0.05). It was remarkable, however, that the complementation strain showed an even higher resistance ($\log_{10}$ kill -0.13 ± 0.10) than the wildtype. Inactivation of *glf* had no influence on serum-resistance of *Cff* 82-40. Since the S-layer is known to be the most important component in antibody independent serum resistance [15] this result suggest that the S-layer proteins (slp) attach to the LPS in *glf* independent way.

Trans complementation with pRYTG1 leads to an overexpression of glf

RT-PCR results showed that the mRNA levels of *glf* are higher in the complementation strain than in the wildtype strain. This result is expected given that *glf* transcription is under the control of a strong promoter on the complementation vector. This overexpression in the complementation strain may be the explanation for higher serum-resistance. The product of Glf may thus have effects on the LPS structure that enhance slp binding.

Knockout of glf *does not influence the amount of LPS O-Antigen*

SDS-Page analyses of the protein K treated whole cell lysate revealed that there is no apparent change in the LPS pattern exhibited by the knockout strain compared to the wildtype. The O-Antigen pattern is comparable to the previously described one for *Cff* 82-40 [41]. This result suggests that (UDP)-Galactofuranose, the product of *glf* is not a determinant for the structure of the main chain of the O-Antigen sugar.

These results suggest a model for Cff *serotype A LPS structure and the role of* glf

The fact that there is no visible alteration in LPS O-Antigen pattern in SDS-Page analysis suggests that galactofuranose (Gal*f*) is not an essential part of the main chain of the LPS. Comparison with the LPS O-Antigen structure of other organisms led to two possible explanations. In *M. tuberculosis* Gal*f* is known to build the outermost part of the O-Antigen [29]. If this is also true for *Cff*, a loss of the outermost part of the O-Antigen might be not visible on the SDS gel. A second explanation would be that the Gal*f* is a side chain of the core oligosaccharide as it was shown for *Salmonella enterica* [42].

Since Senchenkova et al. [17] reported that the LPS of *Cff* serotype A contains trace amounts of galactose and moreover determined the structure of the O-Antigen to be a linear D-mannan chain with partial O-acetylation the second explanation seems to be the more likely one.

Based on this knowledge I propose a model of the *Cff* serotype A LPS (Figure 19).

Gal*f* has been reported to form different types of linkages and branched Gal*f* residues were found in *Penicillium* and *Aspergillus* [43], [44]. Therefore, I hypothesize that Gal*f* is a side chain of the core oligosaccharide and is involved in linkage of the single LPS molecules. If this is true, the LPS is still intact in the absense of *glf* which fits the results of the LPS SDS-Page.

Furthermore, the currently unknown attachment site for the slps would probably also stay intact, which explains the remaining serum-resistance of Δglf.

Due to the proposed loss of the linker function of Gal*f* the LPS would be less compact and the outer membrane would be more accessible for acidic media which explains the susceptibility to acidic conditions of Δglf.

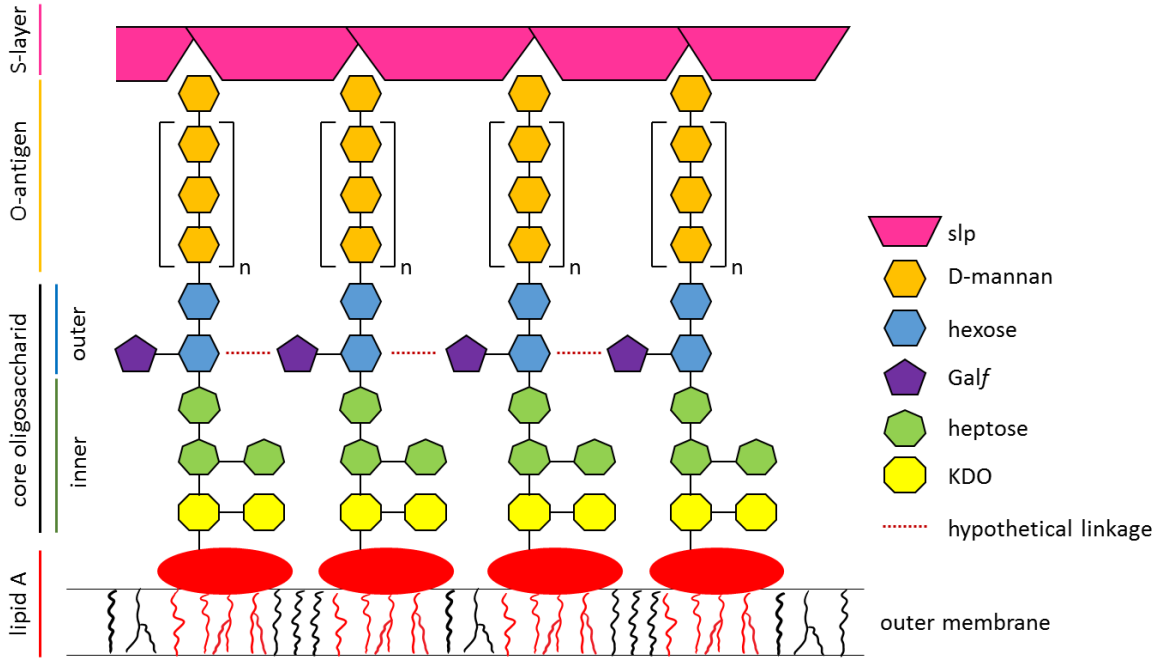

Figure 19: model for *Cff* serotype A LPS structure; Lipid A (red) anchors the LPS into the outer membrane, core consists of KDO (yellow), heptose (green), yet undefined hexose (blue) and Gal*f* (violet) which is supposed to connect the LPS molecules (red dotted line), O-antigen (orange) consists of linear D-mannan [17] and attached S-layer proteins (pink) cover the LPS providing serum resistance.

Outlook

One of the next steps has to be a western blot with antibody against SapA to find out whether it is true that the S-layer is still present. This analysis may provide some insight in the involved mechanisms and could be followed by electron microscopy to visualise the structure of the *Cff* specific S-layer in the wildtype, knockout and complementation strains.

Another interesting experiment would be the heterologous expression of Glf in *E. coli* to purify the protein. With the purified Glf it should be possible to test its predicted UDP-galactopyranose mutase activity. In the best case it would be also possible to analyse the structure of the enzyme. This structure could then be compared with the known structure of UGM of other organisms [25], [45], which may contribute to knowledge about 3D protein structures and protein modifications of *C. fetus*.

5.2 Expression of Fic proteins in *S. cerevisiae*

It was shown that the Fic proteins of *Cfv* 84-112 have toxic effect on human (HeLa) cells and in *E. coli* Fic2 was identified to interfere with translation [8]. The expression of these proteins in *S. cerevisiae* as eukaryotic model organism was the next logical step to find out more about the possible eukaryotic targets.

Fic proteins showed no alteration in cell shape of S. cerevisiae

Fic2 leads to a severe cell elongation in *E. coli* and Fic1 and Fic2 provoke apoptotic phenotypes in HeLa cells [8]. Therefore, the *S. cerevisiae* strains harbouring the Fic proteins were examined under the microscope for alterations in cell shape. There were no differences visible between the repressed and induced samples.

Expression of Fic1 and Fic2 did not affect growth of S. cerevisiae

As it has been shown for *E. coli* that Fic2 leads to significantly reduced growth [8], a spot test was performed with the *S. cerevisiae* strains harbouring the Fic proteins to investigate possible growth phenotypes. The results of the spot test revealed that none of the investigated Fics (Fic1, Fic2, Fic2H184A, or the combinations) led to a growth defect in *S. cerevisiae*. After this first test several possibilities seemed to be likely to cause these results: (I) there is only a temperature dependent phenotype, which is known to occur in *S. cerevisiae*; (II) the tags of the Fic proteins disturb their correct folding and therefore inhibit their function; (III) the Fic proteins are not expressed in *S. cerevisiae*; (IV) the fic proteins do not have an effect on *S. cerevisiae*.
A second spot test, incubated at different temperatures was performed to investigate possibility (I). Furthermore, in this spot test a strain harbouring untagged Fic1 was added, referring to possibility (II). The results showed that the tag of Fic1 is not the reason for the absent effect of the protein as untagged Fic1 did not show any effect either. Furthermore, there was no temperature dependent phenotype detectable.

Fic1-Flag and Flag-Fic2 are expressed in S. cerevisiae

To detect whether the Fic proteins are expressed in *S. cerevisiae* a western blot analysis was performed. The tagged proteins were detected via anti-flag tag antibody. Although the detected protein amount was lower than expected for $Gal_{1\text{-}10}$ promotor driven expression the blot showed that Fic1-Flag and Flag-Fic2 were expressed in *S. cerevisiae*.

It is impossible to clone *fic2* in *E. coli* without constant strict repression which indicates that at least in *E. coli* also very small amounts of Fic2 are sufficient for its toxic effect. So the detected expression of the Fic proteins in *S. cerevisiae* should be enough to show an effect. These results exclude possibility (III).

Conclusion and Outlook

The results of this work suggest that the Fic proteins of *Cfv* 84-112 have no effect on *S. cerevisiae* W303. Maybe there are slight growth defects which are not visible in the colony size of the spot test. To exclude this possibility a growth curve could give more precise information. But also the microscopy showed no effect on cell morphology, this indicates that there is really no effect.

The Fido motif of Fic2 is a non-canonical one, because the conserved glycine (position 191) and arginine (position 195) are exchanged to tyrosine and alanine, respectively [8]. Therefore, it is not clear which catalytic activity Fic2 has. This fact makes it difficult to suggest the eukaryotic target of the toxin. It is likely that *S. cerevisiae* lacks a functional homologue of this target.

Although the results did not fulfil the ambitions to supply an organism for further investigation of the eukaryotic target of the fic proteins they open another interesting possibility. Since *S. cerevisiae* showed no growth defect while expressing the Fic proteins, it should be possible to use yeast for overexpression and protein purification. Because of the severe toxicity it is impossible to purify the protein from *E. coli* and *Cfv* hardly grows in liquid culture, which also makes it very difficult to use for overexpression. With further optimization of the cultivation and expression conditions of Fic1 and Fic2 it could be possible to reach this aim in *S. cerevisiae*.

If the purification succeeds a great opportunity for new experiments arises. Using the purified protein it might be possible to reveal the protein structure. This might give an explanation of the interaction between toxin and antitoxin and/or the possible protein targets. Furthermore, a screening for the enzymatic activity of Fic2 could be performed. In summary, these results open up lots of interesting possibilities for further investigation and understanding of the TA system of *Cfv* 84-112.

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7 APPENDIX

7.1 Notes and Abbreviations

Statistical evaluations were done with SigmaPlot 12.3.

Amp	Ampicillin
Amp^r	Ampicillin resistance
APS	Ammoniumperoxo-disulfat
BVC	Bovine Veneral Campylobacterosis
CAPS	3-(Cyclohexylamino)-1-Poroanesulfonic - acid
CBA	Colombia blood Agar
Cff	*Campylobacter fetus* subsp*. fetus*
Cfu	colony forming units
Cfv	*Campylobacter fetus* subsp*. venerealis*
Cm	Chloramphenicol
Cm^r	Chloramphenicol resistance
dNTP	desoxy Nucleotide-Phosphate
Doc	death on curing
E. coli	*Escharichia coli*
Fic	filamentation induced by cAMP
FSB	final sample buffer
FSDR	fetus subspecies definition region
Fwd	forward
Gal*f*	Galactofuranose
ICE	Integrative conjugative element
KDO	2-Keto-3-Desoxy-Octonat
Km	Kanamycin
Km^r	Kanamycin resistance
LB	Lysogeny broth
LPS	Lipopolysaccharide
MEM	minimal essential medium
Nal	Nalidixic Acid
Nal^r	Nalidixic acid resistance
NEB	New England Bioloabs
OD	optical density
ONC	overnight culture
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylenglycole
Rev	reverse
Rpm	rounds per minute
RT	reverse transcription
S. c.	*Saccharomyces cerevisiae*
S-Layer	Surface layer
SDS	Sodium dodecyl sulfate
slp	S-Layer protein
Sm^r	Streptomycin resistance
TA system	Toxin-antitoxin system
TAE	Tris Acetat EDTA
TBS	Tris buffer saline
TCA	Trichloracetic acid
TEMED	Tetramethylethylen-diamin
Tp^r	Trimethoprim phenotype
UDP	Uridindiphosphat
UGM	UDP-galacopyranose mutase
VE	voll entsalzt (fully desalted)
VR	variation region
VSDR	venerealis subspecies definition region
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose

7.2 Index of figures

7.3 Sequencing Alignments

pGAL22-fic1-flag

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fic2       1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~caaga>5
Seq-fwd  301>AAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACCCCCATATATGGATTACAAGGATGACGACGATAAGCAAGA>400
Seq-rev    0>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>0

Contig     6>acaatatacggaaatcaaagatagcaatattattaataaacaaaacatagatattgcagtagggcttgggcttgtagatgatctgaagccttctgagtat>105
fic2       6>acaatatacggaaatcaaagatagcaatattattaataaacaaaacatagatattgcagtagggcttgggcttgtagatgatctgaagccttctgagtat>105
Seq-fwd  401>ACAATATACGGAAATCAAAGATAGCAATATTATTAATAAACAAAACATAGATATTGCAGTAGGGCTTGGGCTTGTAGATGATCTGAAGCCTTCTGAGTAT>500
Seq-rev    0>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>0

Contig   106>ttttataaggctgttgaaaaatcgcgaacatacaacgaacttgaagacaatgtaaagaaatattatgatggcaaaaagctagataaaaaagaggtcggcg>205
fic2     106>ttttataaggctgttgaaaaatcgcgaacatacaacgaacttgaagacaatgtaaagaaatattatgatggcaaaaagctagataaaaaagaggtcggcg>205
Seq-fwd  501>TTTTATAAGGCTGTTGAAAAATCGCGAACATACAACGAACTTGAAGACAATGTAAAGAAATATTATGATGGCAAAAAGCTAGATAAAAAAGAGGTCGGCG>600
Seq-rev    1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TGAAGACAATGTAAAGAAATATTATGATGGCAAAAAGCTAGATAAAAAAGAGGTCGGCG>59

Contig   206>aaaaagagtgcgatatagtttcagtaaatatcgccaagtatttagaaaaaagtggctttacgctatctcctgttacattgaagactatacataaaaattt>305
fic2     206>aaaaagagtgcgatatagtttcagtaaatatcgccaagtatttagaaaaaagtggctttacgctatctcctgttacattgaagactatacataaaaattt>305
Seq-fwd  601>AAAAAGAGTGCGATATAGTTTCAGTAAATATCGCCAAGTATTTAGAAAAAAGTGGCTTTACGCTATCTCCTGTTACATTGAAGACTATACATAAAAATTT>700
Seq-rev   60>AAAAAGAGTGCGATATAGTTTCAGTAAATATCGCCAAGTATTTAGAAAAAAGTGGCTTTACGCTATCTCCTGTTACATTGAAGACTATACATAAAAATTT>159

Contig   306>atttgggatgcctttccacaagggcttgaaaaatatgttggagtttttagggatgtaaatatttcaaaaaaagaagaagtcttgggtggcgaaaaaagc>405
fic2     306>atttgggatgcctttccacaagggcttgaaaaatatgttggagtttttagggatgtaaatatttcaaaaaaagaagaagtcttgggtggcgaaaaaagc>405
Seq-fwd  701>ATTTGGGATGCCTTTCCACAAGGGCTTGAAAAATATGTTGGAGTTTTTAGGGATGTAAATATTTCAAAAAAAGAAGAAGTCTTGGGTGGCGAAAAAAGC>800
Seq-rev  160>ATTTGGGATGCCTTTCCACAAGGGCTTGAAAAATATGTTGGAGTTTTTAGGGATGTAAATATTTCAAAAAAAGAAGAAGTCTTGGGTGGCGAAAAAAGC>259

Contig   406>gtagagtatggtaattatgatgagcttggcgatatgctggattatgattttgatagagaaaaacgcaaagattacgccaaaatgactaggcaagaacagg>505
fic2     406>gtagagtatggtaattatgatgagcttggcgatatgctggattatgattttgatagagaaaaacgcaaagattacgccaaaatgactaggcaagaacagg>505
Seq-fwd  801>GTAGAGTATGGTAATTATGATGAGCTTGGCGA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  260>GTAGAGTATGGTAATTATGATGAGCTTGGCGATATGCTGGATTATGATTTTGATAGAGAAAAACGCAAAGATTACGCCAAAATGACTAGGCAAGAACAGG>359

Contig   506>ctttaaatgtcggcaaatttgtaagcggggtatggcaaattcatccgtttcgagaaggcaatactaggacaattgcggtttttactattaagtatctgca>605
fic2     506>ctttaaatgtcggcaaatttgtaagcggggtatggcaaattcatccgtttcgagaaggcaatactaggacaattgcggtttttactattaagtatctgca>605
Seq-fwd  832>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  360>CTTTAAATGTCGGCAAATTTGTAAGCGGGGTATGGCAAATTCATCCGTTTCGAGAAGGCAATACTAGGACAATTGCGGTTTTTACTATTAAGTATCTGCA>459

Contig   606>aagtaaaggctttgaagcaaacaacgatatctttaaagaaaactcaaaatatttcagggatgctttagtgttagcaaattacgacaatatgaaagaaaat>705
fic2     606>aagtaaaggctttgaagcaaacaacgatatctttaaagaaaactcaaaatatttcagggatgctttagtgttagcaaattacgacaatatgaaagaaaat>705
Seq-fwd  832>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  460>AAGTAAAGGCTTTGAAGCAAACAACGATATCTTTAAAGAAAACTCAAAATATTTCAGGGATGCTTTAGTGTTAGCAAATTACGACAATATGAAAGAAAAT>559

Contig   706>ataaaaagtgatttttcatacctagagagtttttttaacaaatttattctaaataaaaatatagagttaaaactgctaccaaacagcccaaaaggtcaca>805
fic2     706>ataaaaagtgatttttcatacctagagagtttttttaacaaatttattctaaataaaaatatagagttaaaactgctaccaaacagcccaaaaggtcaca>805
Seq-fwd  832>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  560>ATAAAAAGTGATTTTTCATACCTAGAGAGTTTTTTTAACAAATTTATTCTAAATAAAAATATAGAGTTAAAACTGCTACCAAACAGCCCAAAAGGTCACA>659

Contig   806>aaagtgagaaatcagtcctatctagacttggtgcagcctatgacaaaaaggtgcaaagtggagcgatcaaacaaccacaaataagcaaaaaatcaaaaga>905
fic2     806>aaagtgagaaatcagtcctatctagacttggtgcagcctatgacaaaaaggtgcaaagtggagcgatcaaacaaccacaaataagcaaaaaatcaaaaga>905
Seq-fwd  832>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  660>AAAGTGAGAAATCAGTCCTATCTAGACTTGGTGCAGCCTATGACAAAAAGGTGCAAAGTGGAGCGATCAAACAACCACAAATAAGCAAAAAATCAAAAGA>759

Contig   906>aaaggaaagataa~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>918
fic2     906>aaaggaaagataa~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>918
Seq-fwd  832>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>832
Seq-rev  760>AAAGGAAAGATAAGGATCCTAACTCGAGCGGCCGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAAATAAGTGTATACAAAT>859
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Figure 22: Contig of sequencing pGAL111-flag-fic2; sequence was performed by Microsynth AG and analyzed with Lasergene SeqMan; Flag-Tag sequence is highlighted and underlined

pGAL111-HA-fic2H184A

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Contig       1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~at>2
Seq-fwd    401>TTAACGTCAAGGAGAAAAAACCCCCCATGGCATACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGGACTATGCATCTAGACATAT>500
Seq-rev      0>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>0
fic2H184A    1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~at>2

Contig       3>gcaagaacaatatacggaaatcaaagatagcaatattattaataaacaaaacatagatattgcagtagggcttgggcttgtagatgatctgaagccttct>102
Seq-fwd    501>GCAAGAACAATATACGGAAATCAAAGATAGCAATATTATTAATAAACAAAACATAGATATTGCAGTAGGGCTTGGGCTTGTAGATGATCTGAAGCCTTCT>600
Seq-rev      0>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>0
fic2H184A    3>GCAAGAACAATATACGGAAATCAAAGATAGCAATATTATTAATAAACAAAACATAGATATTGCAGTAGGGCTTGGGCTTGTAGATGATCTGAAGCCTTCT>102

Contig     103>gagtatttttataaggctgttgaaaaatcgcgaacatacaacgaacttgaagacaatgtaaagaaatattatgatggcaaaaagctagataaaaaagagg>202
Seq-fwd    601>GAGTATTTTTATAAGGCTGTTGAAAAATCGCGAACATACAACGAACTTGAAGACAATGTAAAGAAATATTATGATGGCAAAAAGCTAGATAAAAAAGAGG>700
Seq-rev      1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TTGAAGACAATGTAAAGAAATATTATGATGGCAAAAAGCTAGATAAAAAAGAGG>54
fic2H184A  103>gagtatttttataaggctgttgaaaaatcgcgaacatacaacgaacttgaagacaatgtaaagaaatattatgatggcaaaaagctagataaaaaagagg>202

Contig     203>tcggcgaaaaagagtgcgatatagtttcagtaaatatcgccaagtatttagaaaaaagtggctttacgctatctcctgttacattgaagactatacataa>302
Seq-fwd    701>TCGGCGAAAAAGAGTGCGATATAGTTTCAGTAAATATCGCCAAGTATTTAGAAAAAAGTGGCTTTACGCTATCTCCTGTTACATTGAAGACTATACATAA>800
Seq-rev     55>TCGGCGAAAAAGAGTGCGATATAGTTTCAGTAAATATCGCCAAGTATTTAGAAAAAAGTGGCTTTACGCTATCTCCTGTTACATTGAAGACTATACATAA>154
fic2H184A  203>tcggcgaaaaagagtgcgatatagtttcagtaaatatcgccaagtatttagaaaaaagtggctttacgctatctcctgttacattgaagactatacataa>302

Contig     303>aaatttattttgggatgcctttccacaagggcttgaaaaatatgttggagtttttagggatgtaaatatttcaaaaaaagaagaagtcttgggtggcgaa>402
Seq-fwd    801>AAATTTATTTTGGGATGCCTTTCCACAAGGGCTTGAAAAATATGTTGGAGTTTTTAGGGATGTAAATATTTCAAAAAAAGAAGAAGTCTT~~~~~~~~~~>890
Seq-rev    155>AAATTTATTTTGGGATGCCTTTCCACAAGGGCTTGAAAAATATGTTGGAGTTTTTAGGGATGTAAATATTTCAAAAAAAGAAGAAGTCTTGGGTGGCGAA>254
fic2H184A  303>aaatttattttgggatgcctttccacaagggcttgaaaaatatgttggagtttttagggatgtaaatatttcaaaaaaagaagaagtcttgggtggcgaa>402

Contig     403>aaaagcgtagagtatggtaattatgatgagcttggcgatatgctggattatgattttgatagagaaaaacgcaaagattacgccaaaatgactaggcaag>502
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    255>AAAAGCGTAGAGTATGGTAATTATGATGAGCTTGGCGATATGCTGGATTATGATTTTGATAGAGAAAAACGCAAAGATTACGCCAAAATGACTAGGCAAG>354
fic2H184A  403>aaaagcgtagagtatggtaattatgatgagcttggcgatatgctggattatgattttgatagagaaaaacgcaaagattacgccaaaatgactaggcaag>502

Contig     503>aacaggctttaaatgtcggcaaatttgtaagcggggtatggcaaattgcaccgtttcgagaaggcaatactaggacaattgcggtttttactattaagta>602
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    355>AACAGGCTTTAAATGTCGGCAAATTTGTAAGCGGGGTATGGCAAATTGCACCGTTTCGAGAAGGCAATACTAGGACAATTGCGGTTTTTACTATTAAGTA>454
fic2H184A  503>aacaggctttaaatgtcggcaaatttgtaagcggggtatggcaaattgcaccgtttcgagaaggcaatactaggacaattgcggtttttactattaagta>602

Contig     603>tctgcaaagtaaaggctttgaagcaaacaacgatatctttaaagaaaactcaaaatatttcagggatgctttagtgttagcaaattacgacaatatgaaa>702
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    455>TCTGCAAAGTAAAGGCTTTGAAGCAAACAACGATATCTTTAAAGAAAACTCAAAATATTTCAGGGATGCTTTAGTGTTAGCAAATTACGACAATATGAAA>554
fic2H184A  603>tctgcaaagtaaaggctttgaagcaaacaacgatatctttaaagaaaactcaaaatatttcagggatgctttagtgttagcaaattacgacaatatgaaa>702

Contig     703>gaaaatataaaaagtgatttttcatacctagagagtttttttaacaaatttattctaaataaaaatatagagttaaaactgctaccaaacagcccaaaag>802
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    555>GAAAATATAAAAAGTGATTTTTCATACCTAGAGAGTTTTTTTAACAAATTTATTCTAAATAAAAATATAGAGTTAAAACTGCTACCAAACAGCCCAAAAG>654
fic2H184A  703>gaaaatataaaaagtgatttttcatacctagagagtttttttaacaaatttattctaaataaaaatatagagttaaaactgctaccaaacagcccaaaag>802

Contig     803>gtcacaaaagtgagaaatcagtcctatctagacttggtgcagcctatgacaaaaaggtgcaaagtggagcgatcaaacaaccacaaataagcaaaaaatc>902
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    655>GTCACAAAAGTGAGAAATCAGTCCTATCTAGACTTGGTGCAGCCTATGACAAAAAGGTGCAAAGTGGAGCGATCAAACAACCACAAATAAGCAAAAAATC>754
fic2H184A  803>gtcacaaaagtgagaaatcagtcctatctagacttggtgcagcctatgacaaaaaggtgcaaagtggagcgatcaaacaaccacaaataagcaaaaaatc>902

Contig     903>aaaagaaaaggaaagataa~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>921
Seq-fwd    890>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>890
Seq-rev    755>AAAAGAAAAGGAAAGATAAGGATCCTAACTCGAGCGGCCGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAAATAAGTGTAT>854
fic2H184A  903>aaaagaaaaggaaagataa~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>921
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Figure 23: Contig of sequencing pGAL111-HA-fic2H184A; sequence was performed by Microsynth AG and analyzed with Lasergene SeqMan; 3xHA-tag sequence is highlighted and underlined