Host adaptation mechanisms and transcriptional regulation in *Campylobacter jejuni*

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ISBN 978-90-393-5515-2

Cover design: Gerard Rieuwers, Tiel
Lay-out: Thea Verkerk, Lienden
Printed by Rieuwers Grafisch, Tiel

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Gastheeradaptatie mechanismen en transcriptionele regulatie in *Campylobacter jejuni*

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 22 februari 2011 des middags te 2.30 uur

door

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geboren op 20 september 1979 te Zoelen

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The research described in this thesis was financially supported by NWO VIDI grant 917.66.330.

Printing of this thesis was financially supported by:
Infection and Immunity Center Utrecht

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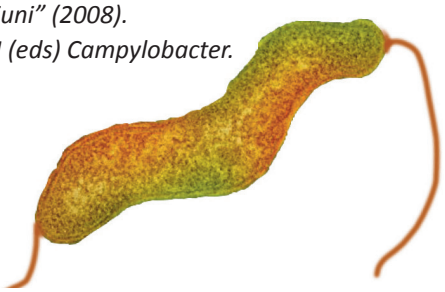
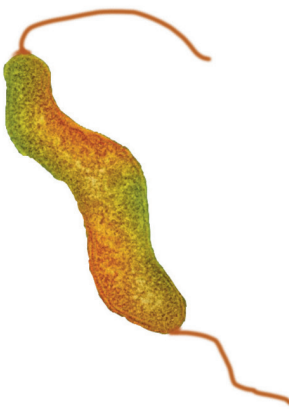
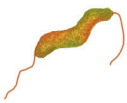
Part of this chapter was published in:
"Regulation of genes in Campylobacter jejuni" (2008).
In: Nachamkin I, Szymanski CM, Blaser MJ (eds) Campylobacter.
ASM press, Washington DC, pp. 611-624







Introduction

Campylobacteriosis is the leading cause of bacterial foodborne disease. The infection is caused by members of the bacterial genus *Campylobacter* and is estimated to cause more than 100 million cases of gastroenteritis per annum (1, 27). The infection may be followed by serious sequelae including the severe paralyzing autoimmune neuropathies Miller-Fisher syndrome (MFS) and Guillain-Barré syndrome (GBS) (136). Recently, *Campylobacter* infection has also be linked to the inflammatory disorder, irritable bowel disease (IBD) (55). In many warm-blooded animals *Campylobacter* is part of the commensal flora (49). The large animal reservoir makes *Campylobacter* a major zoonotic pathogen. As *Campylobacter* infections cause the huge economical burden of up to 8 billion dollars for the US alone (12) the development of vaccines that prevent human disease and/or reduce the colonization of animals is urging.

Campylobacteriosis

The genus *Campylobacter* is composed of 17 species which can be divided into more than 60 Penner serotypes, a typing method based on differences in capsular polysaccharide composition (57). Virtually all human infections are caused by two *Campylobacter* species: *C. jejuni* (90%) and *C. coli* (10%)(10, 64). Despite their global presence, the incidence of *Campylobacter* infections varies greatly between developing- and industrialized countries. In developing countries, often with poor sanitation, the infection rate is estimated at 40.000-60.000/100.000 individuals for children below the age of five (14). Because of a recurrent exposure to contaminated food and water during life, at older ages *Campylobacter* infections are more mild or even asymptomatic due to increasing acquired immunity (37). In industrialized countries both children and adults are at risk for infection with incidence estimates varying from on average 51/100.000 in the European Union, to 12/100.000 in the USA and 396/100.000 in New Zealand (6, 51).

Campylobacter infections can develop after a low infective dose; only ~500-800 bacteria are sufficient to initiate clinical disease (9, 99). The clinical symptoms often include watery to bloody diarrhea, abdominal pain, fever and headache (2), but many infections remain unnoticed which likely results in an underestimation of the true incidence (137). The course of an infection depends on the balance between bacterial viability and the patients' immune status. Most individuals with a *Campylobacter* infection recover without antibiotic treatment although in some cases (i.e. immunosuppressed patients or systemic infection) therapy with macrolides or fluoroquinolones is required to support recovery (19, 106). However, antibiotic resistance is rapidly emerging in *Campylobacter* (19, 23, 45, 97, 102, 103). Because of the rapid spread of *Campylobacter* between animals and humans, the bacterium may even be a reservoir of antibiotic resistance for other bacterial species. Also from this perspective, the availability of an animal or human *Campylobacter* vaccine would be a great asset.

Campylobacter jejuni – the organism

Campylobacter jejuni and *C. coli* belong to the family of *Campylobacteraceae* next to the genera *Arcobacter*, *Sulfurospirillum* and *Thiovulum* altogether placed in the class of epsilon-proteobacteria. The genus *Campylobacter* was first proposed in 1963 by Sebald and Véron, however the first description appeared in 1886 when it was described as a *Vibrio*-like organism. The first isolation of a *C. jejuni* strain from the feces of cattle was reported in 1931 by Jones *et al.* (17). Almost seventy years later the whole genome sequence of a *C. jejuni* strain was determined (85). In the last ten years, improved sequence technologies made it possible that today >100 strains have been completely sequenced (66). This genomic information greatly aids to a better understanding of the bacterial lifestyle and genetic diversity of the *Campylobacter* species. The *C. jejuni* genome contains between 1650 and 1800 coding sequences which seem a relatively small number considering its broad habitat (21, 35, 44, 85, 87, 92).

C. jejuni differs from other enteropathogens like *Salmonella* spp., by its microaerophilic growth as is evident from its inability to grow at atmospheric oxygen concentrations while low oxygen levels are required for growth. The preferred gas composition for *in vitro* culturing of *C. jejuni* is 3-10% oxygen and 5-10% carbon dioxide. The growth temperature of *C. jejuni* ranges between 32°C and 45°C. Perhaps most characteristic of *Campylobacter* is its corkscrew (spiral) shape decorated at one or both ends with a single polar flagellum that is essential for its motility. Under less favourable circumstances the spiral shape of the bacterium can change into a coccoid form, resulting in a viable but non-culturable state (78).

Campylobacter virulence factors

Despite the huge numbers of *Campylobacter* infections worldwide, the virulence mechanisms of *C. jejuni* and *C. coli* including the cause of the diarrhea are still an enigma. In humans *C. jejuni* is able to colonize different parts of the intestine (jejunum, cecum and colon), to penetrate the mucus layer and invade intestinal epithelial cells, and to activate the immune system (48, 53, 118, 120), but the underlying molecular events are largely unknown. Factors that have been associated with pathogenesis include flagella-driven motility, chemotaxis, protein secretion, toxin production, adhesion and invasion of eukaryotic cells and metabolic adaptation, but also the presence of lipooligosaccharide (LOS), capsular polysaccharide (CPS) and a sophisticated glycosylation machinery may contribute to the bacterial infection process. A number of these potential *C. jejuni* infection strategies are detailed below.

Cell-surface glycoconjugates

Lipooligosaccharide (LOS)

Lipopolysaccharide (LPS) is an essential constituent of the outer membrane of most Gram-negative bacteria and often contributes to bacterial virulence. LPS generally consists of two or three different structural domains: the lipid A moiety, the core oligosaccharide and the O-antigen. *Campylobacter* lacks the O-antigen in contrast to most enteric pathogens. In bacteria that lack the O-antigen, LPS is often referred to as LOS (lipooligosaccharide).

Carbohydrate analysis of *C. jejuni* LOS indicates that its core oligosaccharide consists of up to fifteen sugar residues. The nature and linkages of the carbohydrates are highly variable both within and among strains resulting in a high antigen diversity. The antigen variation influences the bacterial interaction with host receptors (33, 94). The LOS variation among strains is related to the presence of variable clusters of LOS biosynthesis genes in different isolates. Intrastrain LOS variation is largely due to the presence of genes with homopolymeric G- and A-tracts that are prone to slipped-strand mispairing resulting in on-off switching of gene function and consequently phase variation of the LOS (26). In addition, gene inactivation by deletion or insertion of single or multiple bases, amino acid substitution in glycosyltransferases and mutations leading to variant glycosyltransferases (25, 26, 83) can generate LOS core variation in *C. jejuni*.

Bacterial lipid A is also subject to variation and this can influence both resistance to antibiotics and the interaction with the immune system (95, 124). The lipid A of *C. jejuni* consists of a glucosamine disaccharide backbone substituted with six fatty acyl chains, linked to the core oligosaccharide via a single KDO group. Chemical analysis indicates that the molecule represents the first so-called mixed lipid A, meaning that it contains D-glucosamine (GlcN) as well as 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) linked fatty acyl chains (74). *C. jejuni* also appears to be able to structurally modify its lipid A structure. The genome of *C. jejuni* contains a homolog of the phosphoethanolamine transferase (Cj0256) (85) that is involved in the variable addition of phosphoethanolamine to the glucosamine backbone. This modification influences the sensitivity of *C. jejuni* to antimicrobial peptides, as reported for its *E. coli* functional homolog EptA (16). As shown in this thesis, *C. jejuni* can also change the ratio between the type of fatty acyl linkages in the lipid A. This modification affects the susceptibility of the bacteria to antimicrobial peptides as well as the activation of the pro-inflammatory TLR4/MD-2 complex, belonging to the family of immune receptors (Chapter 3).

Capsular polysaccharide (CPS)

The existence of a polysaccharide capsule in *C. jejuni* was revealed after sequencing of the *C. jejuni* genome (56, 57, 85). *C. jejuni* CPS was for a long time considered as the high-molecular weight form of LPS. The CPS of *C. jejuni* consists of a polysaccharide chain of a variable number of repeating sugar residues that are linked to a lipid anchor for attachment in the bacterial membrane (98). The composition of the capsule can vary between strains and possibly also within a single strain. This results from genetic rearrangements or phase variation in the capsule locus (98), similar to the mechanisms described for LOS variation. Extensive decoration of the variable sugar residues of the

CPS may further extend capsule diversity between strains and may function as immune evasive strategy (72). The variation in CPS composition is the basis for distinguishing *C. jejuni* isolates, defined in the worldwide Penner serotyping system (57).

The polysaccharide capsule likely serves to protect the bacteria from harmful conditions in the environment and hostile conditions in the host (98, 114). Consistent with this hypothesis, a capsule deficient strain displayed attenuated virulence in a ferret diarrheal disease model (4), reduced colonization of chicken (54), and reduced adhesion and invasion of cultured INT-407 intestinal cells (4).

Glycoproteins

C. jejuni was one of the first bacterial species that were found to be able to synthesize glycoproteins (113, 122) and nowadays *C. jejuni* has still one of the most extensive prokaryotic protein glycosylation machineries that have been discovered. To decorate proteins with different glycosyl residues *C. jejuni* contains two systems, *O*- and *N*-linked glycosylation (112). *O*-linked sugars, mainly pseudaminic acid or legionaminic acid derivatives, are attached to serine and threonine residues of the flagellin subunits and thus decorate the flagellum with a layer of sugar(s) (47, 71, 115). The *N*-linked glycosylation process very much resembles the eukaryotic *N*-linked protein glycosylation machinery and is encoded by the *pgl* locus (113, 122). So far at least 35 proteins have been identified that contain the conserved heptasaccharide, GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac. The oligosaccharide is attached to the asparagine residue present in the glycosylation consensus motif Asp/Glu-Y-Asn-X-Ser/Thr (61, 135).

The function of both glycosylation machineries is still a major point of investigation. *C. jejuni* flagellin *O*-glycosylation influences bacterial autoagglutination (119) known to be involved in bacterial virulence (29), but additional functions may exist. The *N*-linked glycosylation of proteins influences various processes including the invasion of intestinal epithelial cells *in vitro*, chicken colonization, and DNA uptake (58, 63, 111). However, because all *N*-linked glycosylated proteins appear to be located in the periplasm, it is still unclear whether these proteins directly contribute to virulence.

Flagella and protein secretion

C. jejuni contains a single flagellum on each pole. The polar flagellum is essential for bacterial motility and results with animal infection models and human volunteer studies indicate that flagella are important for the virulence properties of the bacterium (75, 123). The assembly of the flagellum requires the synthesis of >40 different flagellar proteins that are expressed in a hierarchical order to ensure correct and efficient construction of a functional flagellum. The transcription regulation of the structural flagellar genes is regulated by two different transcription factors FliA (σ^{28}) and RpoN (σ^{54}) and requires the two-component signal transduction system FlgS/FlgR (50, 131). Flagella are immunodominant antigens during human and animal infection. The antigen properties of the *C. jejuni* flagella are highly variable between strains mainly due to variations in the subunit

protein and its glycan modifications (34, 36, 47). This flagella diversity contributes to evasion of the immune response and is a major drawback in vaccine development.

Beside its role in bacterial motility, the *C. jejuni* flagellum also serves to secrete virulence related proteins as demonstrated for the proteins FlaC, CiaB and FspA2 (59, 93, 105). The mechanism of secretion and the signals that trigger the event are unknown.

Apart from the flagella-mediated secretion of proteins into the extracellular environment, *C. jejuni* contains several other inner and/or outer membrane bound protein secretion systems, including a type I and II secretion system (70, 129). The function of these systems in *C. jejuni* is unknown, although the type II system is required for DNA competence. *C. jejuni* does not have a type III secretion system typically involved in bacterial virulence in many other enteropathogens. Some strains like 81-176 contain a type IV secretion system that is expressed from the pVIR plasmid (5). This plasmid however, is not required for bacterial virulence. Recently some *C. jejuni* isolates were found to express a type VI secretion system (Bleumink-Pluym and van Putten, unpublished results). The function of this potential pathogenicity islet is under investigation.

Adhesins and toxins

Adhesins are most often surface exposed proteins that confer binding to specific host cell receptors which may be essential for colonization. So far there are only a few putative adhesins identified in *C. jejuni*. The so-called CadF adhesin harbors a fibronectin binding domain and promotes bacterial adherence in cell culture systems as well as during chicken colonization (20, 60). For two other proteins that also contain a fibronectin binding domain (Cj1279 and Cj1349) only for Cj1279 (renamed as FlpA) it has been reported that it could contribute to adhesion to cultured cells and chicken colonization. Another potential adhesin is the lipoprotein JlpA. This protein has been reported to mediate the adhesion to human HEp-2 cells which can be inhibited in a dose-dependent manner with anti-JlpA antibodies (52). A role of JlpA in the adhesion to chicken epithelial cells or chicken colonization could not be demonstrated (20). A similar species specific adherence has been found for the Peb1 protein (20, 88). As the main function of Peb1 is to transport amino acids (67, 88), it can be speculated that the effect of Peb1 on *C. jejuni* adherence might be indirect and related to its function in amino acid transport. Except for the fibronectin binding proteins the host cell receptors for the other putative adhesions are unknown.

Thus far, cytolethal distending toxin (CDT) is the best characterized multiprotein toxin in *C. jejuni*. The toxin consists of three different membrane anchored subunits CdtA, CdtB and CdtC with molecular masses of respectively 27, 29 and 20 kDa. CDT is released via outer membrane vesicles (OMVs) (68). The protein complex causes cell cycle arrest, apoptotic processes and induces pro-inflammatory immune responses, like IL-8 secretion and NF-κB activation (22, 39, 40, 62, 90, 128). However, not all *C. jejuni* isolates from patients secrete CDT, suggesting that the toxin is not essential for the establishment of infection.

Metabolism and electron transport in *C. jejuni*

Metabolic properties are essential in all living organisms to generate energy for cellular biosynthesis and replication. Genome analysis of all sequenced *C. jejuni* strains thus far show no possibility to reduce exogenous sugars (glycolysis) due to the lack of a gene encoding a 6-phosphofructokinase enzyme (21, 35, 44, 85, 92). To fulfil the need for cellular biosynthesis the bacterium mainly utilizes amino acids to feed the tricarboxylic acid (TCA) cycle, the major metabolic energy pathway in *C. jejuni*. Running the TCA cycle in an oxidative direction makes it possible for the bacterium to generate the essential carbon molecules. In addition electron flow through the highly branched electron transport chain enables the bacterium to produce the major energy source ATP in an aerobic as well as an anaerobic way (Figure 2).

Amino acid catabolism

Monitoring of *C. jejuni* amino acid utilization during continuous growth in a chemical defined medium identified initially serine, aspartate, glutamate and proline as primary energy sources (65). Later, the consumption of asparagine and glutamine was demonstrated (32). In addition, some *C. jejuni* strains can utilize glutathione (7, 44, 101).

The four main amino acids (serine, aspartate, glutamate and proline) utilized by *C. jejuni* are present in large quantities in the excreta of chicken (86), contributing to an optimal growth environment for the bacterium. Serine is taken up by *C. jejuni* via the (low affinity) serine transporter SdaC and converted by the oxygen-labile iron-sulfur protein SdaA identified as a serine dehydratase. These proteins are located in one operon (121). Transport kinetics studies with a *sdaC* mutant indicated the existence of a second (high affinity) serine transport system (121). So far this high-affinity transporter has not been identified. Once inside the bacterium, serine is converted into pyruvate which can enter the TCA cycle.

The other three utilized amino acids (aspartate, glutamate and proline) are taken up via the Peb1, Cj1192 (a DctA homolog) and PutP transporters, respectively. The metabolism of the various amino acids involves one or more steps that all result in the formation of aspartate. Proline is first oxidized to glutamate by PutA. Glutamine and glutamate are interconverted through glutamate synthase (GltB) and glutamine synthetase (GlnA) wherein glutamate could be sequentially transaminated to aspartate by AspB. Asparagine is deaminated to aspartate by asparaginase (AnsA). The central end product aspartate is in all cases converted into fumarate that can enter the TCA cycle. In a low oxygen environment, aspartate can also be transported via an alternative uptake system consisting of the DcuA and DcuB proteins. This system can also transport fumarate with exchange of succinate. The key steps in the amino acid metabolism of *C. jejuni* is summarized in Figure 1.

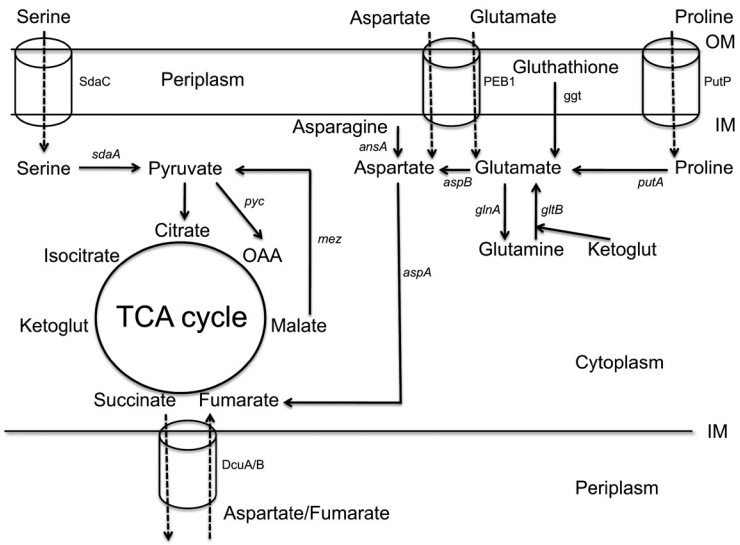


Figure 1. *Schematic overview of the main metabolic pathways involved in amino acid transport and catabolism in C. jejuni functioning under microaerobic and/or oxygen limited conditions* (32)*. The figure is reproduced with permission from John Wiley and Sons.*

Electron transport system

The electron transport chain of *C. jejuni*, characterized by Hoffman and Goodman (42), is complex and highly branched as depicted (Figure 2, adapted from (41)). The preferred electron acceptor is oxygen however alternative molecules can be used. This may be particularly relevant in the low oxygen environment of the gut. Several studies revealed evidence for the utilization of organic and inorganic molecules as electron donor (79, 81, 116, 125) and a variety of molecules that can serve as electron acceptor (31, 91, 100, 126, 127) (Figure 2). The different combination of electron donors and electron acceptors form specific redox couples in which each couple has its characteristic redox potential. Differences in redox potential determine ATP production based on the proton gradient created. The order in which the different redox couples are used in *C. jejuni* is not well defined. In *E. coli* a strict hierarchal transcriptional regulation of usage of electron acceptors in oxygen limited environments is established (28, 117). Electron carriage during transport from electron donor to acceptor is dependent on so-called quinones. *C. jejuni* contains two different kinds of quinones, benzoquinones and napthoquinones (76). Because several proteins involved in the electron transport chain are localized in the periplasm and often need specific cofactor(s) for their active conformation, these proteins are transported over the inner membrane as fully folded complexes. In *E. coli* the twin arginine translocase (Tat) system is responsible for transport of numerous protein complexes involved in the electron transport chain (8). In this thesis we searched for and investigated the function of the *C. jejuni* Tat system (Chapter 2).

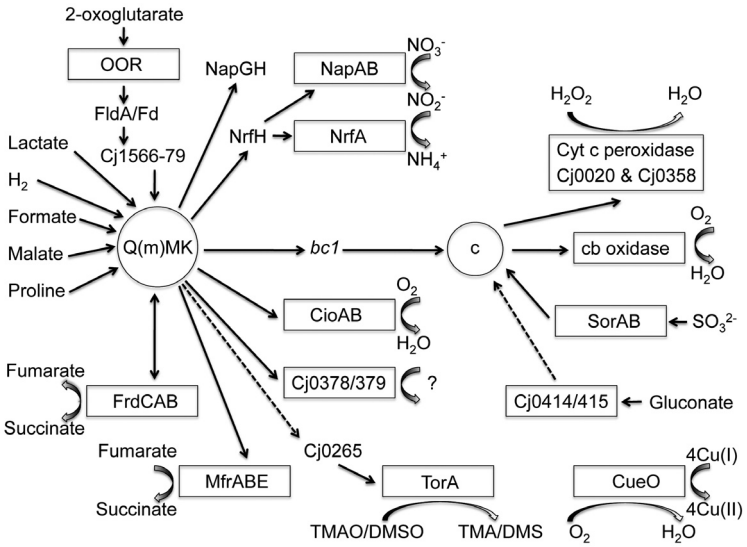


***Figure 2**. Proposed model of the electron transport chain of C. jejuni (41). The figure is reproduced with permission from the American Society for Microbiology.*

Metabolic adaptation

Because *C. jejuni* encounters a variety of natural environments that likely differ in the availability of amino acids and oxygen, the bacterium needs sophisticated metabolic adaptation strategies for survival. Indeed transcriptional regulation of *C. jejuni* genes involved in metabolism and respiration under different environmental conditions has been demonstrated (32, 100, 107, 134). *In vitro* assays as well as chicken colonization experiments indicate that inactivation of the genes *aspA* (80) or *sdaA* (121) reduces the interaction of *C. jejuni* with epithelial cells as well as colonization, indicating the importance of a coordinated amino acid metabolic system. Similarly, Peb1 amino acid transporter and gamma glutamyl transferase (GGT) mutants indicate a role for these proteins in virulence (7, 77). Hofreuter *et al*. demonstrated that the available nutrient composition can determine the ability of *C. jejuni* to colonize specific host tissues (43). The mechanisms that control the metabolic adaption are still unknown, although the two component regulatory system RacS-RacR appears to regulate distinct metabolic genes and is essential for chicken colonization (11, Chapter 4 & 5). Altogether the data indicate that fine regulation of metabolic processes is important to control virulence potential.

Gene regulation in *C. jejuni*

Dissection of regulatory systems that explain the behavior of *C. jejuni* in different environmental niches is accelerating due to the rapid advances in genomics and proteomics. As stated above, ge-

nome-wide analysis of several *C. jejuni* strains indicates that this species contains between approximately 1650 and 1800 genes (84, 44, 85, 87). Transcription profiling on *C. jejuni* maintained under a variety of growth conditions indicates considerable variation in gene expression (3, 13, 18, 24, 38, 46, 70, 73, 82, 96, 107, 108, 130, 132, 133, Chapter 5), supporting the idea that gene regulation is essential for the lifestyle of *C. jejuni*. A search for putative promoters in the *C. jejuni* genome suggests that the genes are regulated by approximately 650-750 promoter elements (13, 89). Computational analysis of sequence similarities with transcriptional regulators in other bacterial species and the presence of conserved DNA binding motifs predict the presence in the *C. jejuni* genome of at least 37 putative transcription regulators (Table 1). The *C. jejuni* genome carries only three sigma factors: RpoD, FliA and RpoN; the remaining 34 regulators belong to the specific transcription factors. In the absence of other major mechanisms of gene regulation, this indicates that all *C. jejuni* biology including bacterial replication, adaptation to environments such as cold, nutrient-poor water and the nutrient-rich habitat of the intestine, and bacterial pathogenicity, are largely determined by the controlled activity of a limited (~2% of the total) number of *C. jejuni* proteins. A major current challenge is to translate the genomic observations to protein function and bacterial adaptation and behavior.

Sigma factors

In many prokaryotes, different environmental cues can result in the activation of specific sigma factors that control specific gene transcription. As mentioned *C. jejuni* contains only three sigma factors RpoD, FliA and RpoN. Both FliA and RpoD belongs to the sigma 70 (σ^{70}) family of which RpoD is the main essential sigma factor responsible for regulation of almost all *C. jejuni* promoters including *fliA* and *rpoN*. Therefore RpoD is considered to comprise the primary sigma factor. FliA regulates the transcription of 14 different genes via at least 10 different promoters (13). These genes encode proteins involved in the assembly of the flagellar apparatus, the glycosylation of the major flagellin subunits (69), as well as virulence-associated proteins secreted through the flagellum, namely FspA and Cj0977 (30, 93). RpoN belongs to the sigma 54 (σ^{54}) family which, in contrast to the σ^{70} family of sigma factors, contains a very well conserved consensus sequence throughout the bacterial kingdom (110). Genome-wide analysis of *C. jejuni* based on this sequence (TGGCAC-N5-TTGC) indicates the existence of 17 putative RpoN promoters. Transcription profiling confirmed that 15 of these indeed regulate the transcription of the downstream gene(s) (13). The 17 identified RpoN promoters of *C. jejuni* control the transcription of 23 genes of which 15 encode proteins that are involved in the assembly of the flagella. Factors regulated by RpoN include components of the basal body, the flagellar hook protein, the filament subunit flagellin B as well as the putative anti-sigma factor FlgM (13, 131). Besides known flagellar genes, RpoN regulates a number of hypothetical genes (Cj0243, Cj0428, Cj1242 and Cj1650), a gene involved in motility but not in assembly (Cj1026) (104), and an UDP-GlcNAc C6 dehydratase (Cj1293) (15). As the known *C. jejuni* sigma factors are mainly involved in the regulation of flagella biosynthesis and function, additional systems must exist to control adaptation-directed gene responses.

Two-component regulatory systems

An important group of proteins that respond to and can translate changes in the environment are the so-called two-component regulatory systems. These systems generally consist of a membrane-bound sensor protein and a cognate response regulator located in the bacterial cytoplasmic compartment. Sensing of environmental cues results in autophosphorylation of the sensor which is followed by transfer of the phosphate to the response regulator altering its DNA binding properties. Two-component signal transduction systems can be distinguished into two functional types: (1) those that negatively regulate gene transcription (repressors), and (2) those that positively regulate gene expression (activators). However some can act both as activator and repressor. Analysis of the *C. jejuni* genome of strain 11168 predicts the presence of 7 putative protein kinase sensor proteins and 11 response regulators. As a regulon of a two-component system consist of an average of about 25 genes (109), it is estimated that approximately 20% of the *C. jejuni* total number of genes is under the control of two-component systems. To date some of these systems has been studied in more detail like PhosR/PhosS, DccS/DccR, RacS/RacR and FlgS/FlgR (11, 70, 132, 133, Chapter 4 & 5, 131), but for the other ones the function and their role in bacterial adaptation and virulence needs to be elucidated.

Aims and outline of this thesis

Because *Campylobacter* is the most important bacterial foodborne pathogen with approximately 400 million cases each year worldwide, thereby exceeding more known enteropathogens like *Salmonella* and *Shigella* spp., development of a proper vaccine against this zoonotic pathogen would solve a major health concern. *C. jejuni* appears to lack typical virulence factors as are present in most other pathogens. Instead metabolic adaptation and changes in bacterial growth and behavior in the different host environments (chicken, human) may trigger and explain differences in pathology. *C. jejuni* appears to have evolved a large repertoire of sophisticated mechanisms that may enable rapid environmental adaptation. The major **goal** of the work described in this thesis was to gain insight in essential steps in the adaptation of *C. jejuni* and to assess the effects of variation in bacterial phenotype on survival and immunity.

First we investigated the Tat export machinery of *C. jejuni* potentially of importance for the secretion of folded proteins required for metabolic activity and bacterial survival. As described in Chapter 2, we successfully identified a functional Tat export machinery and two of its substrates. The alkaline phosphatase PhoX, controlled by the two-component system PhosS/PhosR, and the nitrate reductase (NapA), involved in nitrate respiration under reduced oxygen levels. Identification of the Tat consensus export signal and a series of other putative substrates also involved in alternative respiration led us to hypothesize that the Tat export system of *C. jejuni* has an essential function in metabolic adaptation.

As changes in lipid A may occur under different environmental conditions, we investigated the existence and consequences of a novel lipid A modification strategy in relation to immune evasive properties of *C. jejuni* (Chapter 3). Evidence was obtained that the ratio between *N*- and *O*-linked fatty acyl chains in the lipid A part influences the resistance to antimicrobial peptides but also influences the ability to activate the pro-inflammatory TLR4/MD-2 pathway. The lipid A modification may thus enhance the immune evasive properties of the organism.

In Chapter 4 we demonstrate that the activity of the RacS-RacR signaling complex requires a functional disulfide bridge formation (Dsb) system. A non-functional Dsb system activates RacS, due to the loss of the inhibition of its autokinase activity because of incorrect folding of the signaling domain. Furthermore we describe a direct regulation of the *ggt* enzyme that is required for the degradation of glutamine and glutathione by the RacS-RacR system. To our knowledge, our findings are the first example of the existence of a Dsb sensitive bacterial sensor protein among prokaryotes.

In Chapter 5 we provide evidence that the RacR and RacS proteins form a two-component system and that this system regulates key pathways in *C. jejuni* metabolism. Using complementary transcriptomics, proteomics and metabolomics we discovered that the RacS-RacR system is important to regulate amino acid metabolism and fumarate respiration in oxygen limited environments. We determined part of the regulon of this two-component system and demonstrated that the system controls the switch between pyruvate and fumarate utilization. The key role of the RacS-RacR system in the metabolic adaptation of *C. jejuni* inside the host indicates that the system and/or its regulon may be attractive targets for future infection intervention.

In Chapter 6 the most important results described in this thesis are summarized and discussed.

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Chapter 2

Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery

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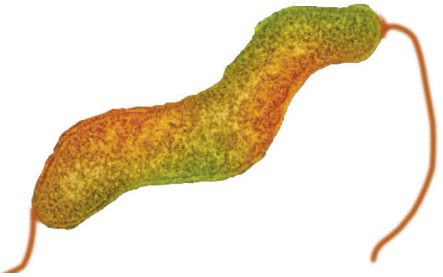
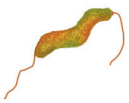
Microbiology 154: 584-592. 2008





Abstract

Bacterial alkaline phosphatases (PhoA) hydrolyse phosphate-containing substrates to provide the preferred phosphorus source inorganic phosphate (P_i). *Campylobacter jejuni* does not contain a typical PhoA homologue but contains a phosphatase that is regulated by the two-component system PhosS/PhosR. Here we describe the characterization of the enzyme, its secretion pathway and its function in the bacterium's biology. Phosphatase assays showed that the enzyme utilizes exclusively phosphomonoesters as a substrate, requires Ca^{2+} for its activity, and displays maximum activity at a pH of 10. Gene disruption revealed that it is the sole alkaline phosphatase in *C. jejuni*. The protein contained a twin-arginine motif (RR) at its N terminus, typical of substrates of the Tat secretion system. Substitution of the twin-arginine residues showed that they are essential for enzyme activity. *C. jejuni* genome analysis indicated the presence of four ubiquitously expressed Tat components that may form a functional Tat secretion system as well as 11 putative Tat substrates, including the alkaline phosphatase ($PhoA^{Cj}$) and the nitrate reductase NapA. Inactivation of *tatC* caused defects in both $PhoA^{Cj}$ and NapA activity as well as a reduction in bacterial growth that were all restored by complementation *in trans* with an intact *tatC* copy. The atypical overall features of the $PhoA^{Cj}$ compared to *Escherichia coli* PhoA support the existence in prokaryotes of a separate group of Tat-dependent alkaline phosphatases, classified as the PhoX family.

Introduction

The human pathogen *Campylobacter jejuni* is one of the most important causes of bacterial gastroenteritis worldwide (1). The natural habitat of *C. jejuni* is the intestine of warm-blooded animals and a wide variety of watery environmental sources (4, 22). This diversity in ecological niche demands that the bacterium is able to adapt rapidly to changing environments, such as alterations in temperature or the availability of oxygen and nutrients. In recent years a number of signal transduction systems have been identified in *C. jejuni* that may contribute to bacterial adaptation (13). One two-component signal transduction system important for bacterial survival is the PhosS-PhosR system, which senses changes in phosphate concentration and responds by altering the expression of a number of genes (the *pho* regulon) involved in phosphate acquisition and diverse metabolic processes (33).

For most bacterial species, inorganic phosphate (P_i) is the preferred source of phosphate. Most bacteria assimilate P_i via specific uptake systems after hydrolysis of phosphate-containing substrates in the periplasm. An important enzyme involved in the hydrolysis of a variety of exogenous phosphate sources is alkaline phosphatase (PhoA). This metalloenzyme catalyses the non-specific hydrolysis of phosphomonoesters to an alcohol and P_i and thus makes P_i available for utilization by the bacterium. The production of PhoA is regulated by the phosphate concentration in the environment and upregulated during phosphate limitation. The enzyme is transported into the periplasm via the general secretory (Sec) system and becomes active after dimerization and binding of two Zn^{2+} and one Mg^{2+} cations (27, 29).

Intriguingly, search of the whole genome of *C. jejuni* for *phoA* homologues suggested that the pathogen lacks the typical PhoA. Characterization of the *pho* regulon of *C. jejuni*, however, indicated that the bacterium possesses a phosphatase that is involved in phosphate assimilation (33). The corresponding gene (Cj0145) was upregulated during phosphate limitation and under the control of the PhosS-PhosR two-component system (33). To further decipher the nature of this seemingly atypical enzyme, which was provisionally designated $PhoA^{Cj}$, we further investigated the regulation, transport, activation and function of the enzyme. Here we provide evidence that the *C. jejuni* phosphatase deviates from the classical *Escherichia coli* PhoA in that it is transported over the cytoplasmic membrane via a previously unidentified twin-arginine translocation (Tat) secretion system, requires Ca^{2+} for its activity, and exclusively utilizes phosphomonoesters as a substrate.

Materials and methods

Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were routinely cultured under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37°C on plates containing blood agar base II (Oxoid) and 5% horse blood lysed with 0.5% saponin (Sigma) or in Heart Infusion broth (HI) (Oxoid). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB medium at 37°C. When appropriate, media were supplemented with ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (20 μg ml^{-1}).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
***C. jejuni* strains**		
81116	Wildtype	(18)
NCTC 11168	Wildtype	(20)
480	Wildtype	(31)
81116*tatC*::Km	81116 derivative *tatC*::Km	This study
81116*tatC*::Km + pMA2-tatC	81116 derivative *tatC*::Km + pMA2-tatC	This study
81116 *phoA*^{Cj}::Cm	81116 derivative *phoA*^{Cj}::Cm	(33)
81116 *phoA*^{Cj}::Cm + pMA1	81116 derivative *phoA*^{Cj}::Cm + pMA1	This study
81116 *phoA*^{Cj}::Cm + pMA1-phoA^{Cj}	81116 derivative *phoA*^{Cj}::Cm + pMA1- phoA^{Cj}	This study
81116*phoA*^{Cj}::Cm + pMA1-GGphoA^{Cj}	81116 derivative *phoA*^{Cj}::Cm + pMA1-GGphoA^{Cj}	This study
81116*phoA*^{Cj}::Cm + pMA1-phoA^{Ec}	81116 derivative *phoA*^{Cj}::Cm + pMA1- phoA^{Ec}	This study
***E. coli* strains**		
PC2955	*relA*1 ϕ80d*lacZ*ΔM15 *phoA*8 *hsdR17 recA1 endAl gyrA96 thi-1 relA1 luxS glnV44*	NCCB
Plasmids		
pGEM-T easy	PCR cloning vector, Amp^r	Promega Corporation, Madison, WI
pGEM578	pGEM-T easy containing *tatC*	This study
pILL550	Shuttle cloning vector	(9)
pBluescript KS M13+	Cloning vector, Amp^r	Stratagene, La Jolla, CA
pCB267	*lacZ* and *phoA* promoter probe vector	(24)
pGEM145	pGEM-T easy containing *phoA*^{Cj}	This study
pGEM145GG	pGEM145 containing a RR substituted *phoA*^{Cj} gene	This study
pGEMphoA	pGEM-T easy containing *E. coli phoA*	This study
pGEM578-2	pGEM-T easy containing *tatC*	This study
pMA1	*C. jejuni/E. coli* shuttle conjugative expression vector, Km^r	This study
pMA2	*C. jejuni/E. coli* shuttle conjugative expression vector, Cm^r	This study
pWM1007	*C. jejuni/E. coli* shuttle vector	(14)
pWM1007-pr.metK	pWM1007 containing the *metK* promoter	This study
pAV35	pBluescript II SK containing *E. coli* Cm^r cassette	(28)
pMA1-phoA^{Cj}	pMA1 containing *phoA*^{Cj}	This study
pMA1-GGphoA^{Cj}	pMA1 containing a RR substituted *phoA*^{Cj}	This study
pMA1-phoA^{Ec}	pMA1 containing *E. coli phoA*	This study
pMA2-tatC	pMA2 containing *C. jejuni tatC*	This study

Construction of a *C. jejuni tatC* mutant

To inactivate *tatC* (Cj0578), the gene and its flanking genes were amplified with the primers TAT-1 and TAT-2 (Table 2) using *C. jejuni* 81116 chromosomal DNA as template. The resulting 2181 bp PCR product was cloned into the pGEM-T easy vector (Promega) to form plasmid pGEM578. Inverse PCR was performed to introduce a deletion of 424 bp in the *tatC* gene and to create an unique *Bam*HI restriction site by using the primers TAT-3BamHI and TAT-4BamHI (Table 2). After self-ligation, the plasmid was digested with *Bam*HI and ligated to the Km^r cassette gene of pILL550 (9). Natural transformation (31) was used to introduce the resulting knockout plasmid pGEM578::Km into *C. jejuni* to obtain the 81116 *tatC*::Km mutant.

Construction of complementation plasmids

To complement the $phoA^{Cj}$::Cm (33) and *tatC*::Km mutants, two *E. coli-C. jejuni* shuttle conjugative expression plasmids, pMA1 and pMA2, were constructed. To this end, the *C. jejuni metK* (Cj1096) promoter was amplified with the primers metKFSphI and metKRNsiI (Table 2). After digestion with *Sph*I and *Nsi*I, the resulting 115 bp PCR product was ligated into *Sph*I- and *Nsi*I-digested pWM1007 (14), yielding plasmid pWM1007-pr.metK. The *metK* promoter, polylinker and the gene coding for green fluorescent protein (GFP) were amplified from pWM1007-pr.metK with the primers pILL550-pWM1007F and pILL550-pWM1007R (Table 2) and the 1.9 kb product was digested with *Bgl*II and *Eco*RI. The shuttle conjugative expression plasmid pMA1 was obtained by ligation of this 1.9 kb fragment into the *Bgl*II/*Eco*RI-digested plasmid pILL550. Plasmid pMA2 contains a chloramphenicol cassette (Cm^r) instead of the kanamycin resistance gene. For construction of this plasmid, the Cm^r of pAV35 (28) was amplified with the primers T7 and 22CATDO (Table 2). The PCR product of 880 bp containing one *Eco*RI site was digested with *Eco*RI and ligated into an *Eco*RI- and *Swa*I-digested pMA1, resulting in plasmid pMA2.

To complement the *tatC*::Km mutant, the *tatC* gene was amplified with the primers TATcplforw. xbaI and TATcplrev1.sacI (Table 2). The restriction enzymes *Sac*I and *Xba*I were used to clone the 800 bp *tatC* PCR fragment into pMA2, generating the complementation plasmid pMA2-tatC.

Plasmid pMA1 was used to introduce the $phoA^{Cj}$, the GG$phoA^{Cj}$ construct (a gene coding for a $PhoA^{Cj}$ protein where the twin arginine residues are substituted by glycine residues) and an *E. coli phoA* gene into the *C. jejuni phoA*::Cm mutant. By using the primer combinations phoAF1/phoAR and phoAF2/phoAR (Table 2) and *C. jejuni* 81116 chromosomal DNA as template, $phoA^{Cj}$ and GG-$phoA^{Cj}$ PCR products were generated. These PCR products of 1800 bp in size were digested with *Sac*I and *Sac*II and ligated into pMA1, resulting in the plasmids pMA1-$phoA^{Cj}$ and pMA1-GG$phoA^{Cj}$, respectively. The *phoA* gene of *E. coli* was PCR amplified from pCB267 (24) with the primers phoAECF/phoAECR (Table 2). The 1800 bp PCR product was digested with *Sac*I and *Sac*II and introduced into pMA1 to form plasmid pMA1-$phoA^{Ec}$. All PCR products in this study were obtained with the proofreading enzyme *Pfu* (Promega) according to the instructions of the manufacturer. Nucleotide sequences of the cloned PCR products were verified by sequencing both strands. Complementation plasmids were introduced into *C. jejuni* mutants via conjugation (9).

Table 2. Primers used in this study

primer name	DNA sequence (5' → 3')
TAT-1	AAATTTAGAAGGCGGGCGTGTT
TAT-2	GCAAAAATTCTAAAGGCTGTAAA
TAT-3BamHI	AAAGGATCCTTCATTGCGGTAAATAAGGC
TAT-4BamHI	AAAGGATCCTCAATTTTTAATGGCAGGACC
phoAF1	GAGCTCAGGAGAAAAAACAAATGGAAAGAAGA
phoAF2	GAGCTCAGGAGAAAAAACAAATGGAAGGAGGATTGTTT
phoAR	CCGCGGTATTTAGCTTCCTATCACTCCAC
phoAECF	GAGCTCAGGAGAAAATAAAATGAAACAAA
phoAECR	CCGCGGGGTATCAGGCGGCTTTCTTGAGG
TATcplforw.xbaI	TCTAGAGTGTAATCATAGCTTGAAAG
TATcplrev1.sacI	GAGCTCCAAGATAAAAACGGAAAAATAAATG
metKFSphI	GCATGCAGTTGATTTTAACTAACTTTTGCT
metKRNsiI	ATGCATAAAAAGTCCTTTCATTTAAAATG
pILL550-pWM1007F	CCAGATCTGATGATAAGCTGTCAAACATGAG
pILL550-pWM1007R	CCGAATTCAGCGACCGGCGCTCAGCTGG
T7	TAATACGACTCACTATAGGG
22CATDO	CTGGGATTTTATTTATTCAGCAAG

Alkaline phosphatase assay

Alkaline phosphatase activity was determined as previously described (33), except that EDTA was omitted from the lysis buffer. In short, alkaline phosphatase activity of a bacterial culture grown in defined medium (11) containing 1.6 mM P_i (high) or 0.08 mM P_i (low) was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (PNPP) (Sigma). The units of alkaline phosphatase were calculated using the formula $10^3 x[A_{420}-(1.75 x A_{550})]/t x OD_{600} x V$.

Nitrite assay

Nitrate reductase activity was determined as previously described (25). Briefly, *C. jejuni* strains were grown overnight in HI medium containing 50 mM potassium nitrate. To the culture supernatants 1% (w/v) sulphanilamide dissolved in 1 M HCl and 0.02% (w/v) naphthylethylenediamine were added and after 15 min absorbance was measured at 540 nm. Nitrite concentrations, adjusted to the cell density, were determined by reference to a standard curve.

Growth experiments

Overnight cultures grown in HI medium or defined medium were diluted to a starting OD_{600} of 0.05 and grown at 37°C under microaerophilic (5% O_2) or oxygen-limited (0.3% O_2) conditions using the anoxomat system (MART Microbiology BV). Bacterial growth and cell density were monitored by measuring OD_{600} and counting CFU at intervals.

Localization of the alkaline phosphatase activity

A 10 ml *C. jejuni* culture grown with 0.08 mM P_i was subjected to cellular fractionation as described by Myers & Kelly (16).

Results

Regulation of PhoA^{Cj} phosphatase activity

Inactivation of gene Cj0145 encoding PhoA^{Cj} in *C. jejuni* strain 81116 resulted in a complete loss of bacterial phosphatase activity (Fig. 1) (33). To ensure that this phenotype was caused by disruption of PhoA^{Cj}, we complemented the *phoA*^{Cj}::Cm mutant *in trans* with the shuttle plasmid pMA1-phoA^{Cj}. This plasmid constitutively expresses the *C. jejuni phoA* gene via the *metK* promoter. Plasmid pMA1-phoA^{Cj} restored the phosphatase activity when bacteria were grown in low-phosphate medium (Fig. 1, filled bars), indicating that PhoA^{Cj} is responsible for the phosphatase activity. To investigate whether additional components induced under conditions of low P_i were required for PhoA^{Cj} activity, we compared the enzyme activity for the wild-type, mutant and complemented strains after growth in low- and high-phosphate conditions. Whereas for the wild-type enzyme activity clearly varied with the availability of phosphate in the medium, this regulatory effect was lost in the complemented strain in which PhoA^{Cj} was constitutively expressed (Fig. 1). This indicates that, at least when the gene is expressed from a multicopy plasmid, no additional components present under low-phosphate conditions are necessary for the PhoA^{Cj} activity.

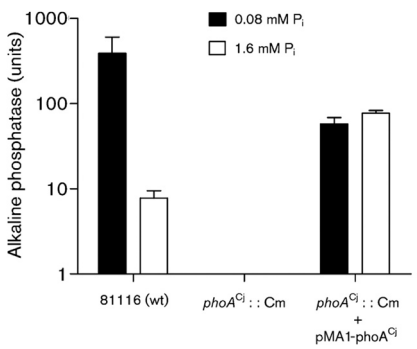


Figure 1. Phosphatase (PhoA^{Cj}) activity of *C. jejuni* strains during phosphate starvation. Alkaline phosphatase activity in bacterial whole-cell lysates was measured for strain 81116 wild-type, the *phoA*^{Cj}::Cm mutant strain, and the *phoA*^{Cj}::Cm mutant strain containing the complementation plasmid pMA1-PhoA^{Cj}. Bacteria were grown in defined medium containing 1.6 or 0.08 mM P_i. Standard errors based on four independent experiments are shown.

pH optimum of *C. jejuni* phosphatase activity

To assess whether PhoA^{Cj} is an alkaline phosphatase, we measured the phosphatase activity at different pH values. Wild-type 81116 and the *phoA*^{Cj}::Cm mutant were grown in defined medium under low-phosphate conditions and lysed in Tris/HCl buffer containing SDS and lysozyme with different pH values. Maximum phosphatase activity was obtained at pH 10 (Fig. 2a). No phosphatase activity was observed for the *phoA*^{Cj}::Cm mutant over the entire pH range (data not shown), indicating that PhoA^{Cj} is the sole phosphomonoesterase of *C. jejuni*.

PhoA^{Cj} activity requires Ca^{2+}

Assessment of the conservation of alkaline phosphatase activity among *C. jejuni* strains revealed considerable variation in enzyme activity (Fig. 2b), although they all contained an intact *phoA*^{Cj} gene (data not shown). One explanation for this diversity may be the variable presence of enzyme cofactors. As alkaline phosphatases of other species require divalent cations for their activity, we tested *C. jejuni* phosphatase activity in bacteria lysed in the absence of the metal chelator EDTA. All *C. jejuni* strains tested showed a strong increase in alkaline phosphatase activity in the absence of EDTA (Fig. 2b), indicating that PhoA^{Cj} needs a cation for its activity.

The nature of the cation that activates the PhoA^{Cj} was sought by selective addition of cations to bacteria grown overnight in the presence of 1 mM EDTA (Fig. 2c). Enzyme activity was measured 30 min after the addition of the cation(s) to the culture. Alkaline phosphatase activity in the EDTA-treated strains was almost fully restored by the addition of 10 mM Ca^{2+}, while other ions including Mn^{2+}, K^{+}, Mg^{2+}, Zn^{2+} (Fig. 2c) or Na^{+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mo^{6+}, VO_4^{3-}, Fe^{2+} or Fe^{3+} or combinations thereof (data not shown), did not restore enzyme activity.

Substrate specificity of *C. jejuni* PhoA^{Cj}

The main function of bacterial alkaline phosphatases is to release P_i from various exogenous organophosphate compounds for use in bacterial growth. To assess which type of compounds may serve as a substrate for PhoA^{Cj}, we tested various potential phosphate donors for their ability to support growth of *C. jejuni* 81116 and the *phoA*^{Cj}::Cm mutant strain. Measurement of the optical density and CFU counting of overnight cultures showed that the wild-type strain but not the mutant grew with 1.6 mM of the organophosphate monoesters, glucose 6-phosphate (G6P) and glycerol 3-phosphate (G3P) as sole phosphate source, while no growth was observed with the phosphodiesters cAMP or DNA (Fig. 2d).

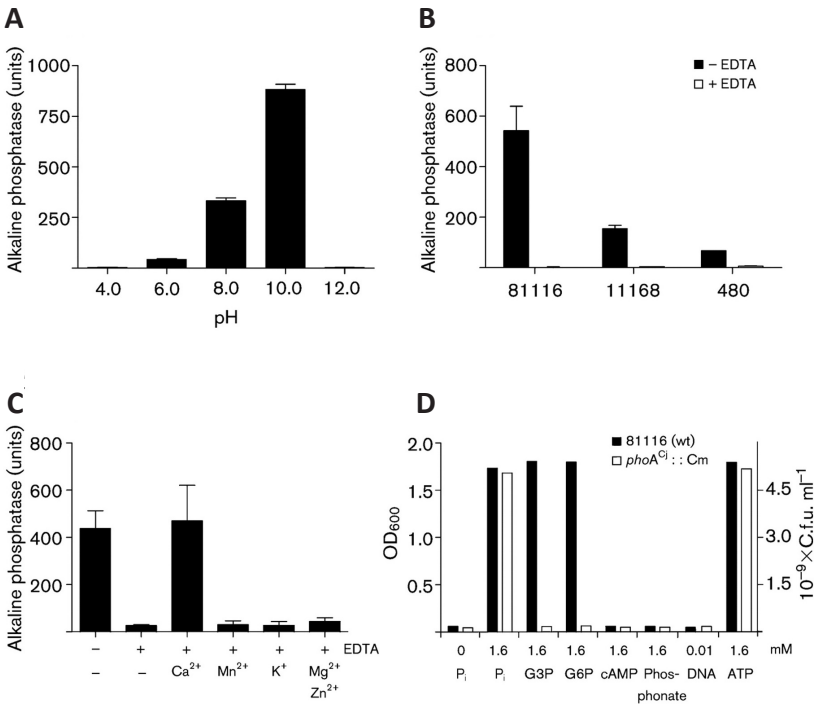


Figure 2. Biochemical characteristics of PhoA^{Cj}. (A) The pH optimum of PhoA^{Cj} activity was determined after lysis of *C. jejuni* 81116 in buffer of the indicated pH using PNPP as substrate. Standard errors based on four independent experiments are shown. (B) EDTA inhibits PhoA^{Cj} activity. PhoA^{Cj} activity was measured for three *C. jejuni* strains grown in defined medium containing 0.08 mM P_i. Bacteria were lysed in the presence or absence of 1 mM EDTA and PhoA^{Cj} activity was measured using PNPP as substrate. Standard errors based on four independent experiments are shown. (C) PhoA^{Cj} requires Ca^{2+} as cofactor. *C. jejuni* strain 480 transformed with a plasmid containing *phoA*^{Cj} downstream of the constitutive *metK* promoter was grown overnight in HI medium in the absence or presence of EDTA. Then, bacteria were incubated with the indicated cation(s) (each 10 mM) for 30 min to monitor their effect on alkaline phosphatase activity. Standard errors based on four independent experiments are shown. (D) Substrate specificity of PhoA^{Cj}. *C. jejuni* strain 81116 and the *phoA*^{Cj}::Cm mutant were grown overnight in defined medium containing the indicated phosphate sources. Optical densities as well as the corresponding CFU ml^{-1} are shown. Bacterial cultures were diluted to OD_{600} between 0.1 and 0.5 to enable accurate measurement of OD_{600}. Data correspond to one representative of three independent experiments.

PhoA^{Cj} requires a twin-arginine sequence for enzyme activity

A large number of bacterial alkaline phosphatases are secreted via the Sec-dependent pathway. Analysis of the PhoA^{Cj} protein revealed that it contains a conserved twin-arginine domain near the N terminus, characteristic of proteins that are exported by the Tat secretion machinery (26). To address whether PhoA^{Cj} might be transported by the *C. jejuni* Tat system, we replaced the coding sequence for two arginine residues with two glycine residues in the *phoA*^{Cj} located on plasmid pMA1-phoA^{Cj}, containing the constitutively expressed *metK* promoter. The resulting plasmid pMA1-GG-phoA^{Cj} was introduced into the *phoA*^{Cj}::Cm mutant strain and alkaline phosphatase activity was

measured under high- and low-phosphate conditions. This strain lacked alkaline phosphatase activity under conditions of phosphate limitation, while minimal activity (less than 5% of wild-type) was observed under high-phosphate conditions (Fig. 3a). For the control strain carrying the plasmid with the original *phoA*^{Cj} (pMA1-PhoA^{Cj}), high levels of enzyme activity were present independent of the phosphate concentration in the medium (Fig. 3a). These results strongly suggest that PhoA^{Cj} is secreted via a thus far unidentified *C. jejuni* Tat secretion machinery.

PhoA^{Cj} is transported via the *C. jejuni* Tat secretion system

Complementary evidence that PhoA^{Cj} is secreted via the Tat pathway was sought by inactivation of the system. Genomic analysis suggests that *C. jejuni* has a Tat system, as four of its putative components, *tatA/E* (Cj1176), *tatB* (Cj0578), *tatC* (Cj0579) and *tatD* (Cj0644), are present in the genome of the sequenced strain NCTC 11168 (7, 20) (Fig. 3b). To test the functionality of the Tat system in *C. jejuni*, we inactivated the putative *tatC* homologue by insertion of a kanamycin-resistance cassette. A severe growth defect as measured by optical density and CFU counting was observed for the *tatC*::Km mutant compared to the wild-type 81116 strain (Fig. 3c). This defect was rectified by introduction of plasmid pMA2-tatC harbouring a constitutively expressed *C. jejuni tatC* gene.

Further evidence of the functionality of the *C. jejuni* Tat system was obtained by measurement of nitrate reductase activity. A search of the *C. jejuni* proteome for putative Tat substrates based on the presence of the specific Tat recognition consensus sequence (RRxFLK) (12) indicated 11 potential Tat substrates (Table 3) (5), including NapA. In other bacterial species this Tat substrate reduces nitrate to nitrite, which may be used by the bacterium as an alternative electron acceptor (21). Analysis of nitrate reductase activity indicated that *C. jejuni* 81116 exhibited this enzyme activity, while the *tatC* : : Km mutant did not (Fig. 3d). This defect was restored by complementation of the *tatC*::Km mutant with pMA2-tatC.

Table 3. Putative Tat substrates of *C. jejuni* NCTC 11168

Gene designations		Predicted function	Position of the Twin-Arginine motif
Cj0005c		putative molybdenum containing oxidoreductase	MKQNDQKENRRDFLKNIG
Cj0145	*phoA*^{Cj}	alkaline phosphatase	MERRLFLKGSA
Cj0264c		molybdopterin-containing oxidoreductase	MLDRRKFLKIGA
Cj0379c		hypothetical protein	MLITPEKLYKQRRNFLKLGA
Cj0414		putative oxidoreductase subunit	MQDNIIDRRSFFKLGL
Cj0437	*sdhA*	succinate dehydrogenase flavoprotein subunit	MGEFSRRDFIKTAC
Cj0780	*napA*	periplasmic nitrate reductase	MNRRDFIKNTA
Cj1358c		putative periplasmic cytochrome C	MIILRRKILKKTS
Cj1511c	*fdhA*	putative formate dehydrogenase large subunit	MSSVGENIKLTRRSFLKMAA
Cj1513c		possible periplasmic protein	MKNRREFLKKSA
Cj1516		putative periplasmic oxidoreductase	MNRRNFLKFNA

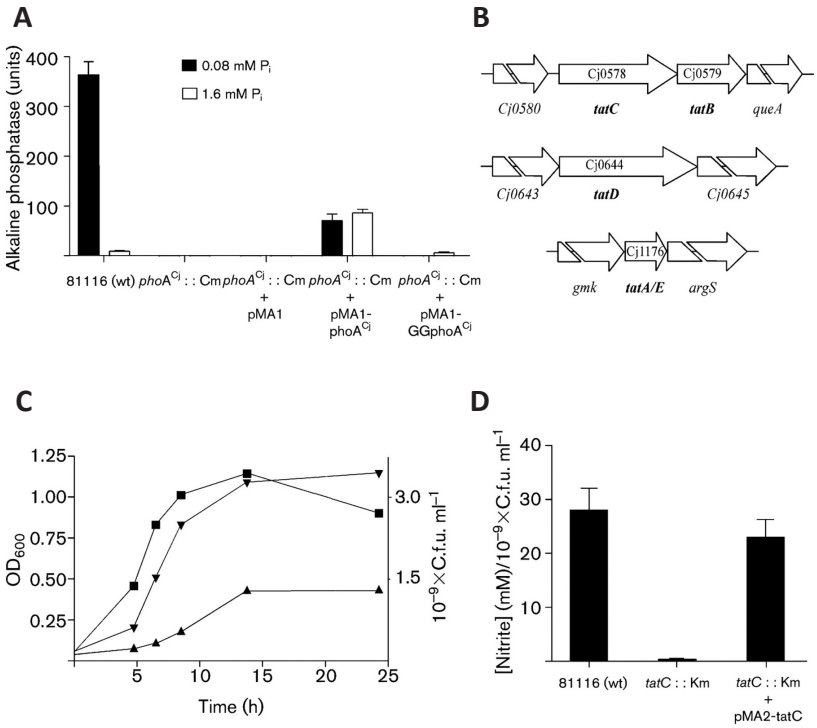


Figure 3. *C. jejuni* possesses a functional Tat secretion system. (A) Alkaline phosphatase assay showing that the twin-arginine residues are essential for PhoA^{Cj} activity. Alkaline phosphatase activity was measured for the *phoA*^{Cj}::Cm mutant, and mutants containing plasmid pMA1 (empty vector), pMA1-phoA^{Cj} (containing intact PhoA^{Cj}) or pMA1-GGphoA^{Cj} (intact PhoA^{Cj} where the twin-arginine residues were replaced by glycine residues) after growth under high- or low-phosphate conditions. (B) Organization of the Tat genes in the genome of *C. jejuni* NCTC 11168. The length of the gene arrows corresponds to the length of the genes. (C) Growth of strain 81116 (■), the *tatC* mutant (*tatC*::Km) (▲) and complemented *tatC* mutant carrying plasmid pMA2-tatC (▼) in HI medium under microaerophilic conditions. Growth curves are from one representative of three independent experiments. The optical densities as well as the corresponding CFU ml^{-1} are presented. Bacterial cultures were diluted to OD_{600} between 0.1 and 0.5 to enable accurate measurement of OD_{600}. The actual CFU counts fully reflected optical densities for each strain for the entire duration of the incubation, indicating that bacterial growth was measured. (D) Nitrite reductase assay demonstrating that the enzyme required a functional TatC. Nitrite accumulation was measured in culture supernatants of 1x10^9 CFU of *C. jejuni* strain 81116, the *tatC*::Km mutant and the mutant containing complementation plasmid pMA2-tatC grown in HI with 50 mM potassium nitrate. Standard errors based on four independent experiments are indicated.

To unequivocally demonstrate that PhoA^{Cj} is transported via the Tat system, we assessed the phosphatase activity under low-phosphate conditions for the parental strain, the *tatC*::Km mutant and the pMA2-tatC complemented *tatC* mutant. Inactivation of TatC resulted in very low alkaline phosphatase activity compared to the parent strain. The defect was fully rectified after introduction of an intact copy of the *tatC* gene (Fig. 4a). To exclude that the lack of enzyme activity in the TatC mutant was caused by a more general protein secretion defect, we introduced into the *tatC*::Km mutant strain a

plasmid carrying the *E. coli* PhoA, which utilizes the Sec secretion pathway (19). This enzyme was active in this background, indicating that the Sec system in the *C. jejuni tatC* mutant was still functional (data not shown). These results, in conjunction with the requirement of the twin-arginine motif, indicate that PhoA^{Cj} is transported across the cytoplasmic membrane by the Tat secretion pathway. Like the *E. coli* PhoA, the *C. jejuni* PhoA^{Cj} remains cell associated, as measurement of enzyme activity in the culture supernatant indicated that virtually all the activity remained associated with the cell fractions (Fig. 4b). The enzyme activity for the *E. coli* PhoA in *C. jejuni* was primarily found in the periplasmic fraction, while active *C. jejuni* PhoA was mostly found in the membrane fraction.

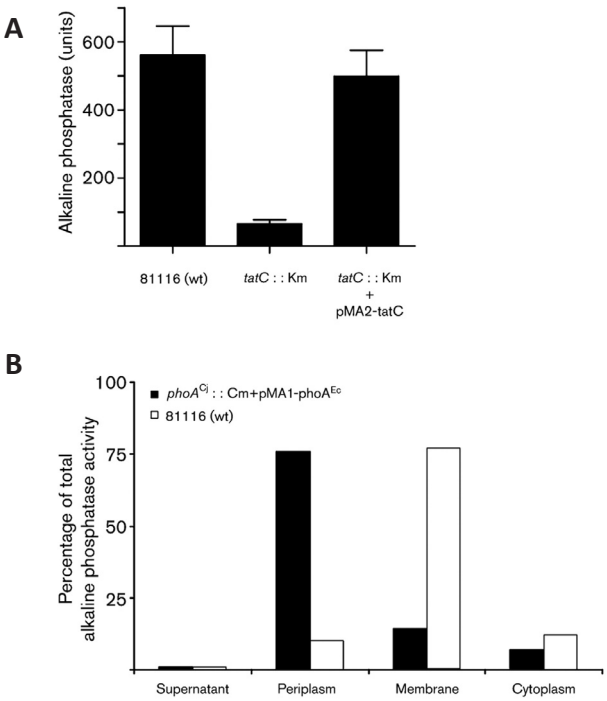


Figure 4. Localization of PhoA^{Cj} and the role of the *C. jejuni* Tat secretion system. (A) Alkaline phosphatase assay demonstrating that PhoA^{Cj} requires a functional TatC. PhoA^{Cj} activities for *C. jejuni* strain 81116, the *tatC*::Km mutant and the mutant complemented with plasmid pMA2-tatC were determined after 8 h growth in defined medium containing 0.08 mM P_i. Standard errors based on four replicates are shown. (B) Alkaline phosphatase assay showing that PhoA^{Cj} is associated with the cellular fraction. PhoA^{Cj} activities for strain 81116 and the *phoA*^{Cj}::Cm mutant complemented with plasmid pMA1-phoA^{Ec} containing the *E. coli phoA* were measured after overnight growth in defined medium containing 0.08 mM P_i. The culture supernatant, periplasm, cytoplasm and membrane were separated to determine the localization of active alkaline phosphatase. The values corresponding to 100 % were 386 units of alkaline phosphatase for 81116 and 120 units for the *phoA*::Cm mutant containing pMA1-phoA^{Ec}. The experiment was repeated four times with similar results.

Discussion

Most bacterial species contain a number of proteins to assimilate the essential element phosphate (P_i) from the environment (30). Here we provide evidence that the *C. jejuni* alkaline phosphatase ($PhoA^{Cj}$) is a key enzyme in the acquisition of P_i. The enzyme differed from the typical PhoA present in most bacterial species as it required calcium as a cofactor and is transported into the periplasm via the Tat secretion pathway. The transport of $PhoA^{Cj}$ via the Tat system is the first functional evidence of the existence of this pathway in *C. jejuni*.

Key evidence that *C. jejuni* Cj0145 encodes the only functional alkaline phosphatase in *C. jejuni* was the successful complementation *in trans* of the mutant strain with an intact copy of this gene. We placed the intact *phoA*^{Cj} gene on a shuttle plasmid under a constitutive promoter rather than its own PhosS-PhosR-regulated promoter to investigate whether additional phosphate-regulated factors were required for the enzyme activity. The strong enzyme activity in the complemented strain measured under high-phosphate conditions (Fig. 1) indicates that the *C. jejuni* phosphatase can act independently of other phosphate-regulated molecules. The enzyme utilizes calcium as a cofactor. This became evident when multiple *C. jejuni* strains were tested for phosphatase activity. In some strains, enzyme activity was only observed when EDTA, which was used to lyse the bacteria, was omitted from the lysis buffer or when an excess of calcium was added to the EDTA-containing buffer. The need for a divalent cation as a cofactor is not unusual, as alkaline phosphatases from other bacterial species also require divalent cation(s) to hydrolyse different types of phosphate compounds (15, 29, 34). *E. coli* PhoA requires Mg^{2+} and Zn^{2+} as cofactors. The requirement of Ca^{2+} as cofactor has thus far only been reported for $PhoA^{Vc}$ of *Vibrio cholerae* (23) and PhoX of *Pasteurella multocida* (34). In *P. multocida* an aspartic acid together with a stretch of hydrophobic amino acids near the C terminus of the protein are essential for Ca^{2+} binding and phosphatase activity (34). Analysis of the $PhoA^{Cj}$ protein sequence indicates the presence of a similar motif.

Bacterial alkaline phosphatase provides bacteria with P_i by reducing different phosphoester compounds available in the environment. The alkaline phosphatase of *P. multocida* (34) can cleave both phosphomonoester and phosphodiester bonds. Our results indicate that the *C. jejuni* enzyme is able to utilize phosphomonoesters such as glucose 6-phosphate and glycerol 3-phosphate, but not phosphodiesters (Fig. 2d). This may limit the ecological niches of the bacterium.

Alkaline phosphatases generally exert the hydrolysis of phosphomonoester-containing substrates in the bacterial periplasm. *E. coli* PhoA and most of its homologues in other bacterial species are transported across the cytoplasmic membrane by the main protein secretion system, the Sec system (19). Analysis of the protein sequence of $PhoA^{Cj}$ indicated the presence of a typical twin-arginine (Tat) consensus motif in the N terminus. This RRxFLK motif, in which the twin-arginine is highly conserved (26), led to the discovery that the *C. jejuni* phosphatase exploits the Tat secretion machinery to gain access to the periplasm. Evidence that the Tat system serves as transport machinery for $PhoA^{Cj}$ includes the lack of enzyme activity after substitution of the twin-arginine residues (Fig. 3a) and after inactivation of the *tatC* gene, which results in dysfunction of the Tat system (Fig. 4a). The absence of enzyme activity in these strains even in the presence of calcium (data not shown) implies that the enzyme is not active in the cytosol, consistent with its assumed function.

Although *phoA*^{Cj} was transcribed, PhoA^{Cj} is probably not transported through the *E. coli* Tat system, because no difference was observed in alkaline phosphatase activity between *E. coli* wild-type and a *tatC* mutant (data not shown). Furthermore, the *E. coli tatC* mutant could not be complemented with an intact *C. jejuni tatC* (data not shown). This may indicate that, in contrast to the Sec systems, at least some components of the Tat systems of *C. jejuni* and *E. coli* are not compatible.

The transport of PhoA^{Cj} via the Tat system is the first reported evidence that this secretion pathway is functional in *C. jejuni*. Genome analysis predicts at least 11 putative Tat substrates in *C. jejuni* strain NCTC 11168 (Table 3) (5). Besides PhoA^{Cj}, we provide evidence that nitrate reductase NapA, a well-documented Tat substrate in other bacterial species (6, 10, 32), requires the Tat system of *C. jejuni* (Fig. 3d). The lack of nitrate reductase activity in the TatC mutant but gain-of-function after introduction of an intact copy of the gene clearly demonstrates that TatC, which is an essential component of the Tat system (3, 8), is required for NapA function. Considering the nature of most predicted Tat substrates, which are mostly cofactor requiring enzymes involved in electron transport (Table 3), it seems plausible to assume that the main function of the Tat secretion system in *C. jejuni* is to translocate redox cofactor-containing proteins that contribute to the assembly of the electron transport chain and energy conservation under oxygen-limited conditions (19). Why the alkaline phosphatase of *C. jejuni* also exploits this secretion pathway, rather than the classical Sec system often used by PhoA of other species, awaits further investigation.

The atypical use of the Tat system to transport alkaline phosphatase across the cytoplasmic membrane has also been demonstrated for *Pseudomonas fluorescens* and *Thermus thermophilus* (15, 2) and is assumed for *V. cholerae* and *P. multocida* (34). *P. fluorescens*, however, also possesses Tat-independent alkaline phosphatases (15). Interestingly, the sole alkaline phosphatase PhoA^{Cj} of *C. jejuni* is 52% identical to the *V. cholerae* PhoA^{Vc} protein, and 49% and 41% identical to the PhoX proteins of *P. fluorescens* and *P. multocida*, respectively. Furthermore, PhoA^{Vc} of *V. cholerae* and PhoX of *P. multocida* are activated by Ca^{2+}, as we found for the *C. jejuni* enzyme. Similarly, the pH optimum of PhoA^{Cj} (~pH 10, Fig. 2a) is comparable to that of the *P. multocida* homologue (34), which is higher than that for *E. coli* PhoA (pH 8.5) (17). Because of their shared dependence on the Tat secretion system, the alkaline phosphatases of *V. cholerae*, *P. fluorescens* and *P. multocida* have been proposed to form a separate group of enzymes, designated the PhoX family (34). The sequence similarity and functional characteristics of the *C. jejuni* PhoA^{Cj} indicate that PhoA^{Cj} also belongs to this group and therefore we propose to rename PhoA^{Cj} as PhoX. This further supports the designation of a novel PhoX family of Tat-dependent alkaline phosphatases.

Acknowledgements

We thank Prof. Dr. T. Palmer for providing us the *E. coli* strains MC4100 and B1LKO (MC4100Δ*tatC*). This work was supported by NWO-VIDI grant 917.66.330 to M.M.S.M. Wösten.

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Chapter 3

Altered linkage of hydroxyacyl chains in lipid A of *Campylobacter jejuni* reduces TLR4 activation and antimicrobial resistance

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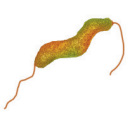
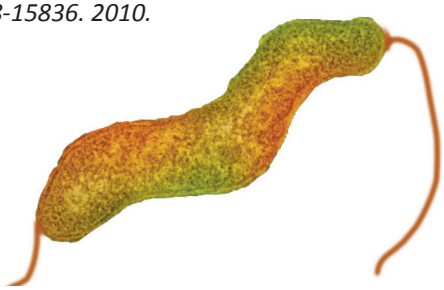
Journal of Biological Chemistry 285:15828-15836. 2010.





Abstract

Modification of the lipid A moiety of bacterial lipopolysaccharide influences cell wall properties, endotoxic activity, and bacterial resistance to antimicrobial peptides. Known modifications are variation in the number or length of acyl chains and/or attached phosphoryl groups. Here we identified two genes (*gnnA* and *gnnB*) in the major foodborne pathogen *Campylobacter jejuni* that enable the synthesis of a GlcN3N precursor UDP 2-acetamido-3-amino-2,3-dideoxy-α-d-glucopyranose (UDP-GlcNAc3N) in the lipid A backbone. Mass spectrometry of purified lipooligosaccharide verified that the gene products facilitate the formation of a 2,3-diamino-2,3-dideoxy-d-glucose (GlcN3N) disaccharide lipid A backbone when compared with the β-1'-6-linked D-glucosamine (GlcN) disaccharide observed in *Escherichia coli* lipid A. Functional assays showed that inactivation of the *gnnA* or *gnnB* gene enhanced the TLR4/MD2-mediated NF-kB activation. The mutants also displayed increased susceptibility to killing by the antimicrobial peptides polymyxin B, colistin, and the chicken cathelicidin-1. The *gnnA* and *gnnB* genes are organized in one operon with *hemH*, encoding a ferrochelatase catalyzing the last step in heme biosynthesis. These results indicate that lipid A modification resulting in amide-linked acyl chains in the lipid A is an effective mechanism to evade activation of the innate host defense and killing by antimicrobial peptides.

Introduction

Lipopolysaccharide (LPS) is an amphipathic molecule that is an essential component of the outer membrane of most Gram-negative bacteria. LPS generally consists of three distinct structural domains: the lipid A moiety that anchors the molecule in the membrane, the core oligosaccharide, and a variable number of repeating oligosaccharide units, the O-antigen (25). LPS is not only critical for cell wall integrity, it is also one of the most potent activators of the innate immune system. LPS can activate the Toll-like receptor 4 (TLR4)/MD-2 receptor complex which triggers the production of pro-inflammatory mediators and antimicrobial peptides and indirectly steers the adaptive immune response (11, 18, 21).

To escape recognition by the innate immune system, Gram-negative bacteria have evolved several mechanisms to modify the structure of lipid A. Most lipid A modifying enzymes are known to be regulated in response to changes in environmental conditions (26). The modifications can alter recognition by the TLR4 complex and/or promote resistance of the bacterial cell wall to host cationic antimicrobial peptides. The addition of polar groups, such as phosphoethanolamine (pEtN), 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or palmitate, to the lipid A reduces its negative charge and limits its interaction with the cationic antibiotic polymyxin B or other antimicrobial peptides (AMP) (8, 14, 38, 45). Another mechanism of lipid A modification is to change the number of acyl chains attached to the disaccharide backbone. Hereto *Salmonella* has the outer membrane enzymes PagP and PagL. The altered number of acyl chains increases the resistance to AMP's and decreases the cellular signaling through TLR4 (3, 37). This indicates that lipid A modifications can have immune evasive as well as immune modulating effects.

Biologically potent lipid A usually consists of a conserved β-1'-6-linked glucosamine (GlcN) disaccharide backbone with two ester- and two amide-linked acyl chains (30). Some bacterial species, including *Leptospira interrogans* and *Acidithiobacillus ferrooxidans*, can synthesize lipid A with only amide linked acyl chains (GlcN3N). In *A. ferrooxidans* the dehydrogenase (GnnA) and the transaminase (GnnB) convert UDP-GlcNAc to the analogue UDP-GlcNAc3N, in which the GlcNAc 3-OH group is replaced with an amine (33). Recombinant GnnA and GnnB from *A. ferrooxidans,* expressed in *Escherichia coli,* also yield large quantities of UDP-GlcNAc3N lipid A molecules. The possible immune modulating or immune evasive role of this type of modification of lipid A has thus far not been investigated (34).

Campylobacter jejuni is a Gram-negative spiral-shaped bacterium that causes gastrointestinal illness in humans. The LPS of *C. jejuni* lacks the O-antigen and therefore is often referred to as lipooligosaccharide (LOS). The structure of the *Campylobacter* LOS is highly variable, and this may contribute to modulation of the host innate immune response. Structural analysis of *C. jejuni* lipid A indicates that this bacterium displays heterogeneity in its lipid A (Fig.1). The major structure of the *C. jejuni* lipid A contains a hybrid backbone of a β-1'-6-linked GlcN3N-GlcN disaccharide with two phosphate groups and six saturated fatty acids (20). *C. jejuni* has mixed lipid A with mainly three amide-linked acyl chains and only one ester-linked acyl chains instead of two ester- and two amide-linked acyl chains, present in most Enterobacteriaceae (19). It is proposed that lipid A with more hydroxyl-bound acyl chains is more biologically active toward TLR4 than lipid A with more amide-

bound acyl chains (19, 28, 30). This might explain the reported difference in biological activity between *E. coli* and *C. jejuni* lipid A (30).

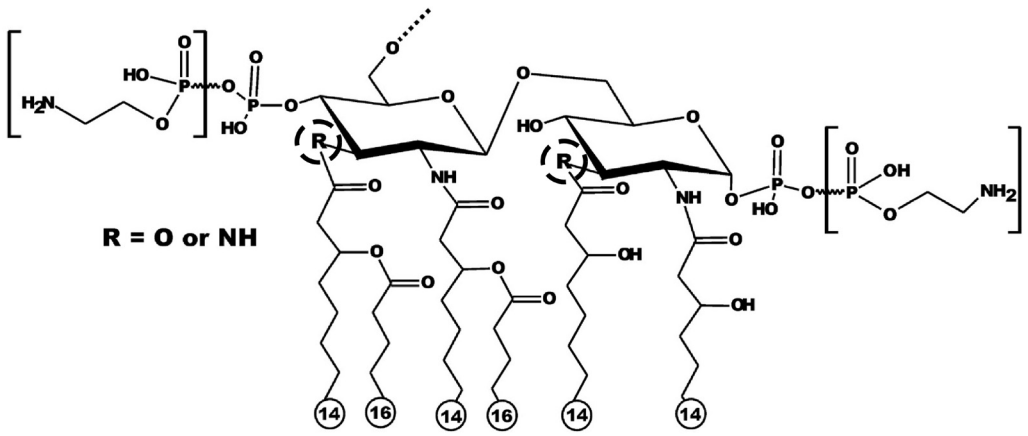


Figure 1. Schematic structure of the lipid A of *C. jejuni* wild-type 11168. The variable acyl linkages are indicated by R (encircled by a dotted line), which can be either O or NH. Variable phosphoethanolamine group(s) are indicated between brackets (35).

In this study we identified the *C. jejuni* genes responsible for adding amide-linked acyl chains in the lipid A and demonstrated that this modification influences the biological activity of lipid A by altering the TLR4 response and the bacterial resistance to antimicrobial peptides.

Materials and Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were routinely grown on plates containing blood agar base II (Oxoid, London, UK) supplemented with 5% horse blood (Biotrading Benelux b.v., Mijdrecht, The Netherlands) lysed with 0.5% saponin (Sigma, St Louis, MO) or in Heart Infusion broth (HI) (Oxoid) at 37°C or 42°C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). *E. coli* strains were grown in Luria-Bertani medium (Biotrading) at 37°C. When appropriate, medium was supplemented with chloramphenicol (20 µg/ml), kanamycin (50 µg/ml) and/or ampicillin (100 µg/ml).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
***C. jejuni* strains**		
11168 (H1)	Wild-type	(7, 23)
11168 *gnnA*::Cm	11168 derivative *gnnA*::Cm	This study
11168 *gnnB*::Cm	11168 derivative *gnnB*::Cm	This study
11168 *gnnA*::Cm^R	11168 derivative *gnnA*::Cm (reverse)	This study
11168 *gnnB*::Cm^R	11168 derivative *gnnB*::Cm (reverse)	This study
11168 *gnnB*::Cm + pMA1-504-505	11168 derivative *gnnB*::Cm + pMA1-504-505	This study
***E. coli* strains**		
PC2955	*relA*1 ϕ80d*lacZ*ΔM15 *phoA*8 *hsdR17 recA1 endAl gyrA96 thi-1 relA1 luxS glnV44*	NCCB
Plasmids		
pGEM-T easy	PCR cloning vector, Amp^R	Promega Corporation, Madison, WI
pGEMhemH-504-505	pGEM-T easy containing *hemH, gnnA* and *gnnB*	This study
pGEM504	pGEM-T easy containing *gnnA*	This study
pGEM505	pGEM-T easy containing *gnnB*	This study
pGEM504::Cm	pGEM-T easy containing *gnnA*::Cm	This study
pGEM505::Cm	pGEM-T easy containing *gnnB*::Cm	This study
pGEM504-505	pGEM-T easy containing *gnnA* and *gnnB*	This study
pMA1	suitable for conjugation	(41)
pMA1-504-505	pMA1 containing *C. jejuni gnnA* and *gnnB*	This study
pAV35	pBluescript II SK containing *E. coli* Cm^r cassette	(42)

Construction of *C. jejuni gnnA*::Cm and *gnnB*::Cm mutants

The *Campylobacter* genes *hemH*, Cj0504c (*gnnA*) and Cj0505c (*gnnB*) were amplified by PCR using the primers HemHRev and 505Fwd (Table 2). The resulting fragment of 1912-bp was cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI) to form plasmid pGEMhemH-504-505. Inverse PCR using the primers Cj504BamHIR and Cj504BamHIF was performed on pGEMhemH-504-505 to replace 811-bp of the Cj0504 gene by an unique *Bam*HI site. The inverse PCR product was self-ligated to form plasmid pGEMΔ504. The primers Cj505BamHIR and Cj505BamHIF were used in a similar procedure to replace 441-bp of the Cj0505 gene by a unique *Bam*HI site, yielding pGEMΔ505. The pGEMΔ504 and pGEMΔ505 plasmids were digested with *Bam*HI to insert a 0.7-kb *Bam*HI chloramphenicol cassette (Cm) isolated from plasmid pAV35 (42). Both Cm orientations were selected resulting in the plasmids pGEMΔ504::Cm, pGEMΔ504::Cm^R, pGEMΔ505::Cm and pGEMΔ505::Cm^R. The plasmids isolated from *E. coli* DH5α were introduced into *C. jejuni* 11168H1 via electroporation. Homologous recombination resulting in double-crossover events was verified by PCR.

Table 2. Primers used in this study

primer name	DNA sequence (5' → 3')
505Fwd	GCAACAGCTGAAGTTGTGG
HemHRev	CACAGGCTTTGAGTAAGTTC
Cj504stopXbaI	AGATCTTTCACGCAAACCTTTCTAAAATTT
Cj505startSacI	GAGCTCATTTCTCTAAGGTTTTTTATGAATTT
21Fwd (CmFwd)	GGAGGATAAATGATGCAATTCAC
22Rev (CmRev)	CTGGGATTTTATTTATTCAGCAAG
HemHR	CTTAGTTAAGATCAGATAAATAC
Cj505F	ATGAATTTTATCAATCTTCAAG
Cj504BamHIR	AGGATCCGACCTATGATACCTATTTTC
Cj504BamHIF	TGGATCCACAAAGTTCAAGAAATTTTA
Cj505BamHIR	TGGATCCATCATCATGGCAAAAAATAG
Cj505BamHiF	AGGATCCGCAAGAGCAAGTTATTTGTATTTTT
pmetKsphI	GCATGCAGTTGATTTTAACTAACTTTTGCT
GFPrev	ACAAGTGTTGGCCATGGAACA

Complementation of *C. jejuni gnnA*::Cm and *gnnB*::Cm mutants

To complement the *C. jejuni gnnA*::Cm and *gnnB*::Cm mutants, a shuttle plasmid was constructed with intact copies of Cj0504c and Cj0505c as insert. Therefore, the genes were amplified from the chromosome of *C. jejuni* strain 11168H1 by PCR with the primers Cj504stop*Xba*I and Cj505start-*Sac*I and the proofreading enzyme platinum *Pfx* DNA polymerase (Invitrogen). After the addition of a 5'A-overhang to the PCR product with *Taq* polymerase (Invitrogen) the product was ligated into pGEM-T easy to obtain plasmid pGEM504-505. Finally, pGEM504-505 and pMA1 (41) were digested with *Sac*I and *Sac*II and the resulting 1986-bp fragment of pGEM504-505 was ligated into plasmid pMA1 behind the *C. jejuni metK* promoter to yield plasmid pMAI-504-505. *E. coli* S17 was used to conjugate plasmid pMAI-504-505 into *C. jejuni gnnB*::Cm, following described procedures (12).

Protoporphyrin IX (PPIX) detection

For the detection of PPIX, *Campylobacter* grown overnight in HI were harvested by centrifugation and resuspended in TEN buffer (40 mM Tris/HCl pH 7.5, 1 mM EDTA and 150 mM NaCl). Fluorescence of bacterial cells from 2×10^9 CFU/ml was measured using a LS50B luminescence spectrometer (Perkin Elmer) (excitation at 405 nm, emission at 630 nm) (1).

Isolation of LOS

C. jejuni LOS was isolated by the hot-phenol extraction method as described (10). In brief, bacteria grown on blood agar plates for 20 h at 42°C under microaerophilic conditions were harvested in 5 ml of water, pelleted by centrifugation (5,500 × g, 30 min, 4°C), and resuspended in 10 volumes of bacterial wet weight in distilled water and an equal volume of hot phenol (70°C). After 2 h of incubation (70°C) with intermittently vortexing and centrifugation (18,000 x g, 20 min, 4°C), the water phase containing the LOS was collected. To increase the yield of LOS a second extraction was done by adding an equal volume of distilled water to the phenol phase. LOS was precipitated from the water phase by

adding sodium acetate (0.1 g/g of wet weight of bacteria) and two volumes of cold acetone (-20°C). After incubation for 16 h at -20°C, LOS was collected by centrifugation (18,000 x g, 30 min, 4°C), washed in cold acetone, and dissolved in 3.5 ml of distilled water. Centrifugation (100,000 x g, 2 h, 4°C) was performed to remove DNA. For further purification the pellet was resuspended in 250 µl of water and treated overnight with 1 U of DNase and 0.25 mg of proteinase K. Finally, the LOS was collected by centrifugation (100,000 x g, 2 h, 4°C) and dissolved in distilled water up to a final concentration of 1 mg/ml. Purified LOS of *C. jejuni* was quantified by the purpald assay (13).

Tricine-SDS-PAGE

C. jejuni LOS samples were analyzed by tricine-SDS-PAGE (15). Each lane was loaded with 100 ng of purified *C. jejuni* LOS. The gels were run at a constant current of 20 mA for about 4 h and silver stained to visualize the LOS (39).

Mass spectrometry analysis

C. jejuni lipid A analyses were done as described by Geurtsen *et. al*. (6) with a modification in the LOS hydrolysis. The LOS sample was dissolved in 500 µl of 10 mM sodium acetate pH 4.5 containing 1% SDS and placed in an ultrasonic bath until the sample was completely dissolved. After 1 h of incubation (100°C) the sample was dried by vacuum centrifugation. To remove the SDS the sample was washed with 100 µl of distilled water and 500 µl of acidified ethanol (100 µl of 4 M HCl with 20 ml 95% ethanol) followed by centrifugation (2,000 x g, 10 min). To remove the acid the sample was washed twice with non-acidified 95% of ethanol, and centrifuged (2,000 x g, 10 min). The sample was lyophilized to yield solid lipid A. Just prior to the analysis, the sample was reconstituted in 200 µl of a mixture of chloroform-methanol-water (3:1.5:0.25, v/v) and a 10-µl aliquot of the clear supernatant was pipetted into a gold-coated borosilicate glass nanospray needle (manufactured in-house, essentially as described by Wilm and Mann (43). A static nanoelectrospray source (built in-house) was mounted onto a LCQ Classic mass spectrometer (Thermo Scientific, San Jose, USA), operated in negative ion mode at mass unit resolution. The heated capillary was set at 200°C, and the atmosphericpressure ionizationsource collision-induced dissociation energy (SID) was enabled at 15V. Automatic Gain Control (AGC) was used to control the filling of the ion trap at a Full MS target of 1 x 10^7 ions. Scans were acquired from 400-2,000 Da with 3 µscans and a maximum inject time of 150 msec.

Cell culture, transfection and TLR activation

The HeLa 57A cell line stably transfected with an NF-κβ luciferase reporter construct (29) was routinely cultured in 25-cm^2 tissue culture flasks (Corning) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 5% FCS in a humidified incubator at 37°C and 10% CO_2. Transfection of the cells with plasmids encoding TLR4, MD2 and CD14, was essentially performed as described (40). After 48 h of incubation the medium was replaced with fresh medium and cells were stimulated (5 h) with the indicated concentrations of purified LOS. After stimulation with LOS the cells were rinsed twice with 500 µl Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4) and lysed in 100 µl of Reporter Lysis Buffer (Promega) for 30 min at -80°C. Firefly luciferase activity was measured

with the Luciferase Assay System (Promega) using a luminometer (TD-20/20, Turner Designs, Sunnyvale, USA). The transfection efficiency was normalized using the β-galactosidase assay (Promega) as described (40).

Antimicrobial peptide susceptibility

Log phase bacteria grown at 42°C in HI were resuspended to a concentration of 10^6 Colony Forming Units (CFU)/ml in 10 mM of sodium phosphate buffer, pH 7.0 supplemented with 1/100 volume HI medium (minimal HI medium) in 96-well polypropylene microtiter plates. After the addition of the indicated concentrations of polymyxin B, colistin E or cathelicidin-1 and 1 h of incubation (37°C, microaerophilic conditions), serial dilutions were plated and incubated 24 h at 42°C under microaerophilic conditions on charchoal plates to enable counting of CFU's.

Statistical analysis

Data were analyzed with a two-tailed paired t-test (Graphpad prism). A *p*-value of <0.05 was considered to be significant.

Results

Identification of the putative *C. jejuni gnnA* and *gnnB* homologs

C. jejuni lipid A contains both GlcN-saccharides and GlcN3N-saccharides in contrast to *E. coli* LPS (28, 30). In *A. ferrooxidans* the genes *gnnA* and *gnnB* are responsible for the synthesis of GlcN3N-containing lipid A (34). Bioinformatics revealed that the genome of *C. jejuni* strain 11168 contains two genes, Cj0504c and Cj0505c, which are respectively 40% and 55% similar at the amino acid level to the GnnA and GnnB proteins of *A. ferrooxidans*. The *C. jejuni gnnA* and *gnnB* gene and the downstream gene *hemH,* putatively involved in the protoheme biosynthesis, have a similar gene orientation and overlapping start and stop codons, suggesting that they may form an operon (Fig. 2A). The upstream gene *alaS* coding for an alanyl-tRNA synthetase has an opposite orientation, indicating that the promoter of the putative *gnnB-gnnA-hemH* operon may be located directly in front of *gnnB*. Genomic analysis of all *C. jejuni* genome sequences available in the public databases revealed a similar organization of the *gnnB-gnnA-hemH* genes in all strains.

Fluorescent phenotype of *C. jejuni gnnA* and *gnnB* mutants

To investigate the function of the putative *gnnA* and *gnnB* homologs in *C. jejuni* strain 11168, both genes were disrupted by insertion of a chloramphenicol resistance (Cm^R) cassette in either orientation. PCR analysis verified that the desired four types of transformants (*gnnA*::Cm and *gnnB*::Cm with Cm^R in two orientations) were obtained. Unexpectedly, the pellets of the mutants that contained the Cm^R cassette in the opposite direction as the *gnnA* or *gnnB* genes, appeared red. This red color was also visible when these mutants were grown on a transparent thioglycollate plate (Fig. 2B). Fluorescence microscopy revealed that these bacteria were intensely red fluorescent (data not shown). In other bacterial species the HemH protein is involved in the protoheme biosynthesis and catalyzes the insertion of a ferrous ion into protoporphyrin IX to synthesize protoheme. Disruption

of *hemH* in *Pseudomonas fluorescens* causes the accumulation of protoporphyrin resulting in typical reddish colonies which are highly fluorescent under UV light (1). To investigate whether the red fluorescence observed for the *C. jejuni gnnA*::Cm^R and *gnnB*::Cm^R mutants is due to the accumulation of protoporphyrin, we performed fluorescence spectrum analysis (Fig. 2C). This yielded a spectrum with a peak at 630 nm only for the mutants with the Cm^R cassette in a polar orientation, which corresponds to that of protoporphyrin (1).

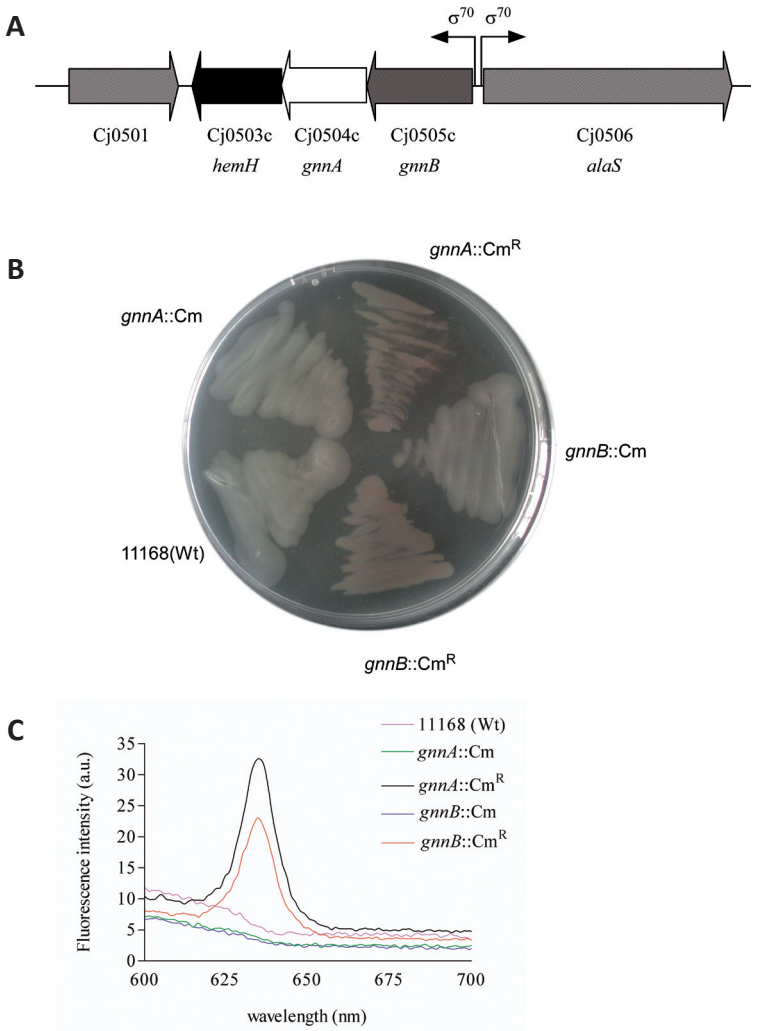


Figure 2. The *gnnA* and *gnnB* genes are organized in one operon with *hemH*. (A) Chromosomal arrangement of the *hemH*-Cj0504(*gnnA*)-Cj0505(*gnnB*) operon and its flanking regions in wild-type *C. jejuni* 11168. The position of the presumed sigma 70 (σ^{70}) promoter is indicated. (B) Thioglycollate agar plate inoculated with wild type strain 11168 and the mutants *gnnA*::Cm, *gnnA*::Cm^R, *gnnB*::Cm and *gnnB*::Cm^R illustrating the red colonies caused by the accumulation of protoporphyrin. (C) Spectrofluorometry of the strains mentioned in B after resuspension in TEN buffer to verify the presence of protoporphyrin. a.u., arbitrary units

We hypothesized that the fluorescent bacterial phenotype may have been caused by a dampening effect of the Cm^R promoter on the transcription of the *hemH* gene. Real-time RT-PCR analysis confirmed the strong reduction of *hemH* transcript in these *gnnA* and *gnnB* mutants. These results confirm that the genes *gnnA*, *gnnB* and *hemH* are transcribed from a promotor in front of *gnnB*. To investigate whether the transcription of the *gnnB-gnnA-hemH* operon is altered in response to heme or oxidative stress, quantitative RT-PCR analysis was performed on RNA isolated from bacteria grown in the presence of the heme precursor d-aminolevulinic acid (ALA), iron, dipyridyl or H_2O_2. None of these conditions significantly changed the *gnnB-gnnA-hemH* transcript levels.

Further analysis of the transformants demonstrated that the *gnnA* and *gnnB* mutants that carried the Cm^R cassette in the non-polar orientation were not fluorescent and showed parental growth rates and motility in semi-solid agar (data not shown). These mutants were used for further structural analysis of lipid A and in the biological assays described below.

LOS structural analysis.

The main function of the GnnA and GnnB proteins in *A. ferrooxidans* is in the biosynthesis of GlcN3N-substituted lipid A (33, 34). To analyze the linkage of the acyl chains in the lipid A of the *C. jejuni gnnA*::Cm and *gnnB*::Cm mutants, LOS was isolated from the mutants and parental strain after growth at the optimal *C. jejuni* growth temperature of 42°C. Tricine-SDS-PAGE of these samples demonstrated similar electrophoretic mobility and staining of the LOS (Fig. 3A), indicating no large structural differences. To enable more detailed analysis of the lipid A, the different LOS samples were hydrolyzed and analyzed by static nanoelectrospray ionization mass spectrometry (nano-ESI-MS) in the negative ion mode. This showed similar relative abundances of peaks for the lipid A of the wildtype and the mutant strains (Fig. 3B), indicating a similar distribution of lipid A isoforms. The lipid A of the wildtype (Fig. 3B, *upper trace*) showed an abundant peak at a monoisotopic molecular ion of *m/z* 1877.9 Da, representing the hexa-acylated diphosphate containing lipid A, whereas the peak at *m/z* 1798.0 Da corresponds to a hexa-acylated monophosphate species (16). Because of the heterogeneity of the constituents, we have confirmed these monoisotopic *m/z*-values with higher mass resolution data acquisitions (data not shown). The remaining minor peaks at *m/z* 1770.2 Da, 1849.9 Da and 1921.0 Da represent respectively hexa-acylated monophosphate species with a C14 instead of a C16 acyl chain, hexa-acylated diphosphate species with a C14 instead of a C16 acyl chain and hexa-acylated monophosphate species with a phosphoethanolamine. As expected, all peaks observed for the lipid A of the parent strain had shifted by two mass units in the *gnnA*::Cm and *gnnB*::Cm mutants, indicating substitution of one *N*-linked acyl chain by an *O*-linked acyl chain. To verify that this difference was solely due to disruption of the *gnnA* or *gnnB* gene, we analyzed the LOS of the *gnnB* mutant complemented *in trans* with plasmid pMA1-504-505. This plasmid contains intact copies of the *gnnA* and *gnnB* gene placed under the control of a constitutive (*metK*) promoter. Mass spectrometry demonstrated a decrease in lipid A masses to wildtype levels for the complemented *gnnB* mutant strain, although some heterogeneity in mass was observed (Fig. 3B, *bottom panel*). Overall, these results demonstrate that inactivation of either *gnnA* or *gnnB* results in replacement of one *N*-linked by an *O*-linked acyl chain in the lipid A.

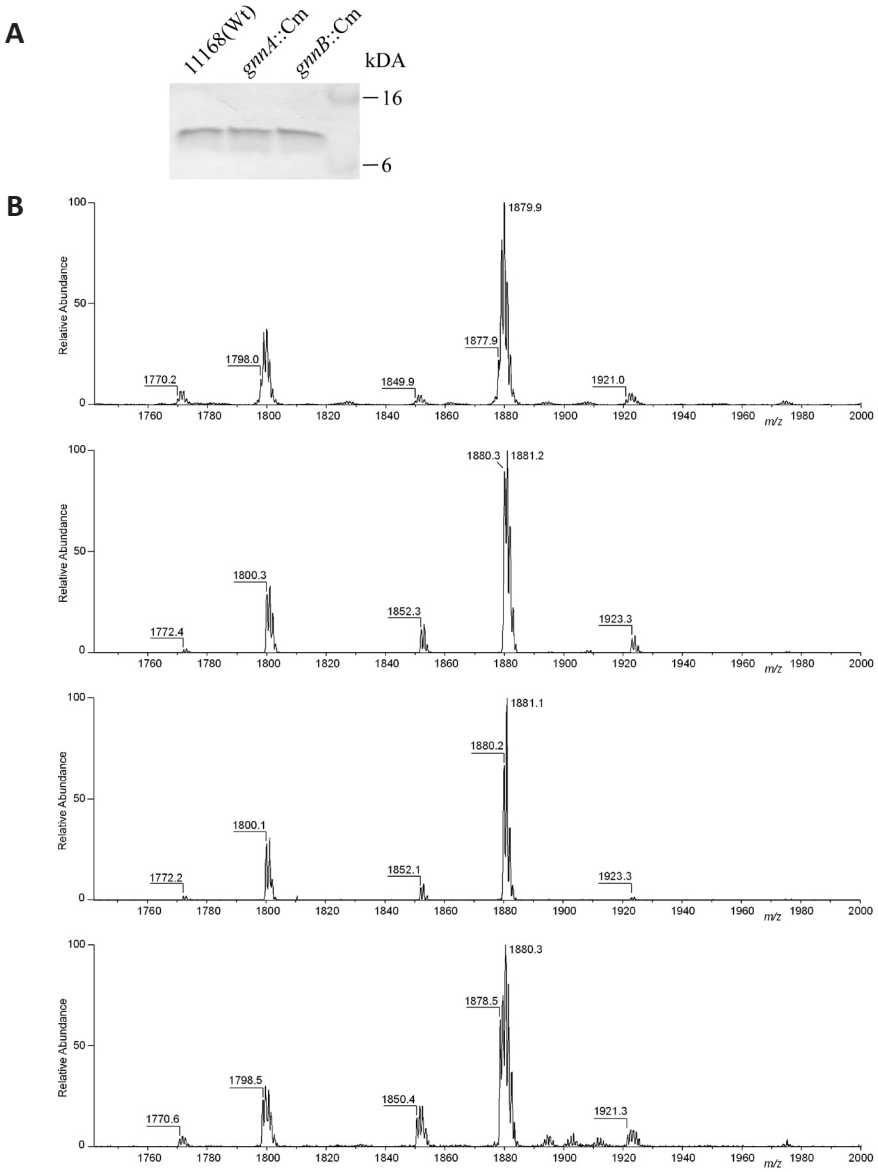


Figure 3. LOS structural analysis. (A) Silver-stained tricine-SDS-PAGE gel loaded with equal amounts of LOS isolated from *C. jejuni* strain 11168 and its *gnnA*::Cm and *gnnB*::Cm derivatives grown at 42°C. (B) Negative ion electrospray mass spectra of purified lipid A obtained from the wildtype strain, the *gnnA*::Cm mutant, the *gnnB*::Cm mutant, and the *gnnB*::Cm mutant strain containing the complementation plasmid pMA1-504-505 (*top to bottom*). Monoisotopic peaks at *m/z* 1798.0 Da and 1877.9 Da represent a hexa-acylated monophosphate species and the characteristic hexa-acylated biphosphate species, respectively. Three additional minor peaks were observed in all strains, the monoisotopic molecular ions corresponding to penta-acylated monophosphate (*m/z* 1770.2 Da), a hexa-acylated biphosphate species substituted with a C14 instead of a C16 acyl chain (*m/z* 1849.9 Da) and hexa-acylated lipid A with one phosphate molecule and one phosphoethanolamine group attached to the lipid A (*m/z* 1921.0 Da).

Biological activity of the *gnnA*::Cm and *gnnB*::Cm LOS.

To investigate whether the type of linkage of the acyl chain is important for activation of the innate immune response by lipid A, we tested the effect of isolated *gnnA* and *gnnB* LOS on the activation of HeLa 57A cells transfected with plasmids containing TLR4, MD2 and CD14. Cellular activation was measured using the NF-κB luciferase reporter construct that is stably expressed by HeLa 57A cells (40). Incubation of the cells expressing the human TLR4/MD2 complex with the different purified LOS samples showed significant enhanced cellular activation for the mutant strains when compared with the parent strain at 1 ng/ml purified LOS and for the *gnnB*::Cm mutant also at 10 ng/ml ($P<0.05$) (Fig. 4A). As TLR4 ligand specificity varies between species and chickens are a natural habitat for *C. jejuni*, we also evaluated the effect of the different LOS types on the activation of the chicken TLR4/MD2 complex. HeLa 57A cells transfected with chTLR4/chMD2, stimulated with the different *C.jejuni* LOS showed no difference in NF-κB activation (Fig. 4B), verifying that the effect of the lipid A modification varies between species. The increased activation of the human TLR4/MD2 complex by the mutant LOS was partially restored to wildtype levels after complementation of the *gnnB* mutant with pMA1-504-505. Overall, our results indicate that the substitution of an *N*-linked with an *O*-linked acyl chain in the lipid A results in a more biologically active LOS, at least in humans.

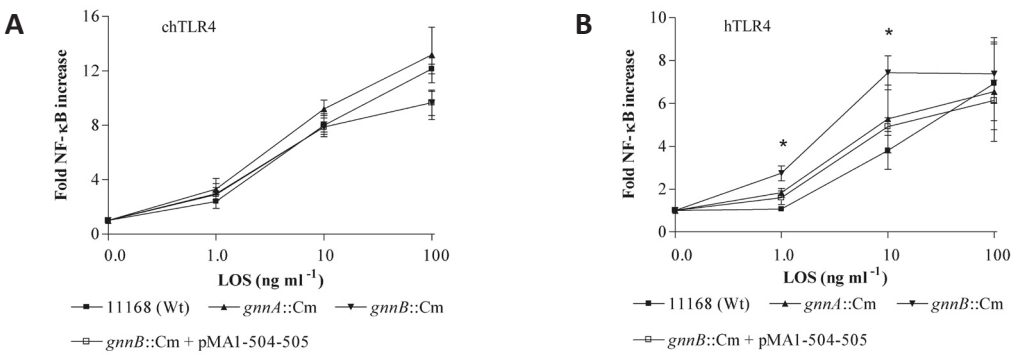


Figure 4. Species specific recognition of *C. jejuni* GnnA and GnnB LPS. NF-κB-luciferase activity in HeLa 57A cells transfected with (A) chTLR4/chMD-2/hCD14 or (B) hTLR4/hMD-2/hCD14 after 5 h of stimulation with LOS purified from wildtype strain 11168, the *gnnA*::Cm mutant, *gnnB*::Cm mutant and the *gnnB*::Cm mutant complemented with pMA1-504-505. Values are indicated as fold NF-κB increase and are the mean ± SD of four independent experiments. *, *p<0.05*.

Modification of the lipid A with GlcN3N influences resistance to antimicrobial peptides.

Modification of lipid A can alter the resistance to AMP's (24). To investigate whether the lipid A modification caused by *C. jejuni* GnnA and GnnB influenced the susceptibility to AMP's, we incubated log-phase grown wildtype strain 11168, the *gnnA* and *gnnB* mutants, and the complemented *gnnB* mutant strain with increasing concentrations of polymyxin B, colistin E, or chicken cathelicidin-1. After 1 h of incubation, bacterial viability was determined by CFU counting. The *gnnA* and *gnnB* mutants showed at least 50% less survival to all three antimicrobial peptides when compared

with the parental strain (Fig. 5). Equal survival percentages for the wildtype and mutants were seen when antibiotics with a different bacterial target were used such as kanamycin (data not shown). The increased sensitivity of the *gnnB* mutant to AMP's was partially abolished in the complemented *gnnB* mutant strain. These data clearly demonstrate that the lipid A modification not only inhibits the activation of the innate immunity, but also decreases the susceptibility to AMP's.

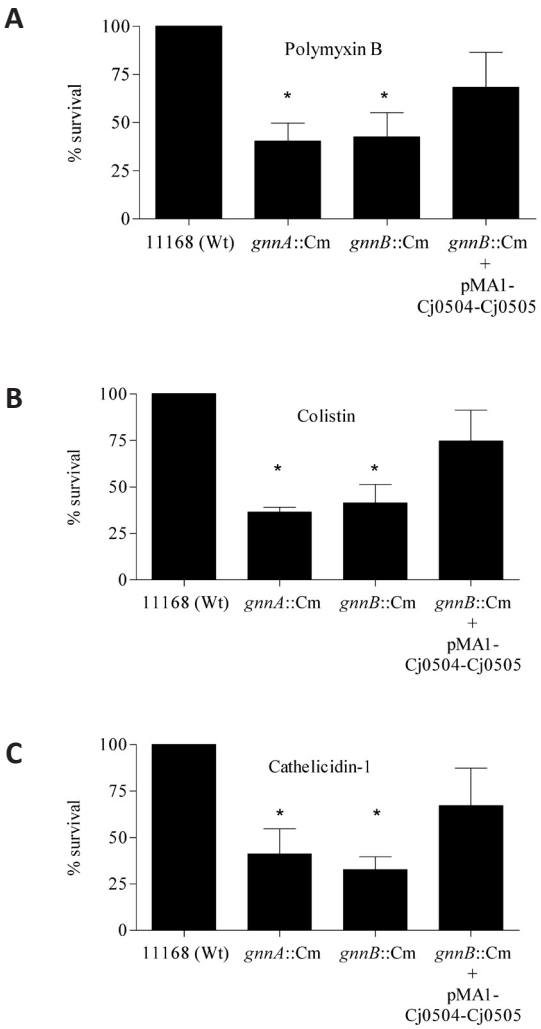


Figure 5. Susceptibility of *gnnA*::Cm and *gnnB*::Cm mutant bacteria to AMP's. Resistance to antimicrobial peptides of the *C. jejuni* strain 11168 wildtype (Wt), the *gnnA*::Cm and *gnnB*::Cm mutants, and the *gnnB*::Cm mutant containing the complementation plasmid pMA1-504-505 is shown. Logarithmic phase bacteria were incubated for 1 h with 5 µg/ml of polymyxin B (A), 5 µg/ml of colistin E (B), and 0.1 µM of chicken cathelicidin-1 (C). Bacterial survival was determined 24 h after plating of the suspensions on charcoal plates. Results are expressed as the percentage of the inoculum that survives the exposure to the antimicrobial agents and are the mean ± SD of three independent experiments. *, *p<0.05*.

Discussion

Lipid A is the main constituent of the outer membrane of Gram-negative microorganisms and is the principal ligand of the innate immune receptor TLR4. During evolution many bacteria have evolved enzymes that modify the lipid A structure and thus alter the physico-chemical and biological properties of the molecule. Here we identified two *C. jejuni* genes, *gnnA* and *gnnB,* that enable the addition of an *N*-linked acyl chain instead of an *O*-linked acyl chain to the disaccharide lipid A backbone. This modification decreases the potency of lipid A as a TLR4 agonist as demonstrated by the enhanced activation of the human TLR4/MD2 complex by purified LOS from the mutant microbes. In addition, these mutants are more susceptible to the antimicrobial peptides polymyxin B, colistin E, and chicken cathelicidin-1. The altered linkage of the acyl chain in the lipid A conferred by GnnA and GnnB may thus have evolved to enable the bacterium to resist the innate host defense.

The *C. jejuni* genes *gnnA* and *gnnB* are located in a single operon with *hemH.* This gene organization is conserved in all sequenced *C. jejuni* strains and *Campylobacter lari* but not in other *Campylobacter* strains or different bacterial species (33). The potential significance of the coordinated transcription of the *gnnA*/*B* and *hemH* genes, is unknown. HemH is a protein that catalyzes the insertion of a ferrous ion into protoporphyrin IX to synthesize protoheme. In *E. coli, hemH* transcription is modestly increased in response to heme limitation (17). Furthermore, high heme concentrations cause lipid A modifications in the oral pathogen *Porphyromonas gingivalis* (4). The *hemH* gene in *E. coli* and *Salmonella Enteritica* is also activated in response to hydrogen peroxide (5, 44). We investigated whether the transcription of the *gnnB-gnnA-hemH* operon is altered in response to heme, the heme precursor d-aminolevulinic acid (ALA), iron, or H_2O_2, but none of these conditions significantly changed the *gnnB-gnnA-hemH* transcript levels.

The function of *C. jejuni gnnA* and *gnnB* in lipid A modification was established by mass spectrometry analyses of the isolated LOS of the wildtype and mutant strains. The observed two mass units increase of the lipid A isoforms for the mutants, which was reversed after introduction of an intact copy of the defective gene, indicates that inactivation of either gene caused the addition of an ester-linked acyl chain instead of an amide-linked acyl chain. This is fully consistent with the assumed function of GnnA and GnnB as lipid A modifying enzymes as determined for *A. ferrooxidans* GnnA and GnnB (33, 34).

Our primary goal was to investigate the impact of variation in the number of amide-bound acyl chains on the biological properties of the lipid A. This was tested using the activation of NF-κB in HeLa cells transfected with genes encoding human or chicken TLR4/MD2 as a read-out system. This assay has previously been shown to be instrumental in demonstrating differences in TLR4 recognition of LOS species (40). Our finding that in this assay the purified LOS of the *gnnA*::Cm and *gnnB*::Cm mutants is more potent than wildtype LOS (Fig. 4A), provides for the first time an experimental basis for the hypothesis that lipid A containing GlcN3N-GlcN is less biologically active than lipid A that has a GlcN disaccharide backbone (19). The altered potency of the LOS was only detected toward the human TLR4/MD2 complex and not for cells expressing the chicken TLR4/MD2 receptor

complex (Fig. 4B). These results are in agreement with previous work that indicates differences in ligand specificity of human, murine and chicken TLR4 (9, 32). The altered potency of the LOS with variable acyl chain linkages extends the known repertoire of immune modulating lipid A modifications such as variation in the length and number of acyl chains, the addition of polar groups, and variation in the number of phosphate molecules attached to the lipid A (26, 27).

The molecular basis for the variable potency of lipid A may be related to a decreased flexibility of the *N*-linked acyl chains when compared with the *O*-linked acyl chains (19). Crystal structures of the interaction between LPS and the TLR4/MD2 complex (PDB code 3FXI) have revealed that the number and the flexibility of the acyl chains might influence the interaction between lipid A and the TLR4/MD2 complex (22). We speculate that because the *gnnA*::Cm and *gnnB*::Cm mutants contain more flexible acyl chains in their LOS, the lipid A may fit more easily into the grove of the TLR4/MD2 complex resulting in enhanced hTLR4 activation.

The composition of LPS may also have major effects on the bacterial resistance to antimicrobial agents. *C. jejuni* is very resistant to the AMP polymyxin B, a compound often added to media to select for *Campylobacter* species (31). We observed that wildtype *C. jejuni* strain 11168 has a lower susceptibility to polymyxin B, colistin E, and cathelicidin-1 when compared with the *gnnA*::Cm and *gnnB*::Cm mutants. This suggests that the reduced number of *N*-linked acyl chains in the lipid A of the mutants causes increased sensitivity to AMP's. Bacterial killing by AMP's supposedly involves interaction of the small amphipathic peptides with the negatively charged LPS. A number of Gram-negative pathogens resist AMP's by modifying their LPS, especially the lipid A part (36). For example, the incorporation of phosphoethanolamine in the lipid A of *Salmonella* strongly reduces the susceptibility toward polymyxin B, whereas phosphate molecules attached to the lipid A backbone appear important for cathelicidin-1 binding (2). Our mass spectrometry results indicate that, apart from adding phosphate or phosphoethanolamine (20), *C. jejuni* exploits the *gnnA* and *gnnB* modification to increase its resistance to polymyxin B, colistin E, and cathelicidin-1 (Fig. 5). The altered resistance may be attributed to increased membrane stability and permeability caused by the amide linkage when compared with ester linked acyl chains.

In conclusion, our results indicate a novel mechanism of lipid A modification in *C. jejuni* that involves GnnA and GnnB-mediated replacement of an ester-linked by an amide-linked acyl chain in the lipid A. This results in a lipid A species with reduced endotoxin activity but increased bacterial resistance to antimicrobial peptides.

Acknowledgements

We thank Dr. A. van Dijk for providing AMP cathelicidin-1. This work was supported by NWO-VIDI grant 917.66.330 to M.W. and ZonMW grant 9120-6150 to J.v.P.

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Chapter 4

The Dsb system of *Campylobacter jejuni* influences γ-glutamyltranspeptidase activity by altering the status of the RacRS two-component system

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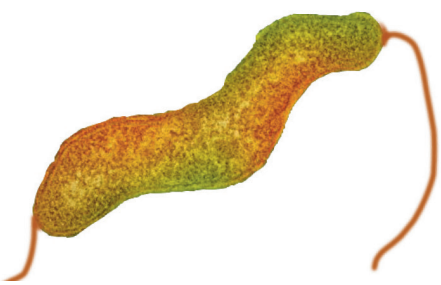
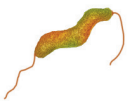
**These authors contributed equally to this work*

Manuscript in preparation





Abstract

The enzyme γ-glutamyltranspeptidase (GGT) of *Campylobacter jejuni* contributes to persistent colonization of mice and the chicken gut. Regulation of GGT activity in prokaryotes is generally poorly understood. Here we report that *C. jejuni* GGT activity requires a functional disulfide bridge formation system (Dsb), although the enzyme does not contain any cysteine amino acid residues. *In silico* analysis to identify a Dsb depending transcriptional unit regulating GGT revealed that the sensor of the two-component system RacS-RacR contains two periplasmic cysteine amino acid residues. Real-time RT-PCR and electromobility shift assays showed that the *C. jejuni* GGT is activated and directly regulated by the RacS-RacR two-component system. Disulfide bridge formation of RacS appears crucial to keep the sensor protein in an inactive state. Overall, our results indicate that the *C. jejuni* two-component system RacS is a major substrate of the Dsb system and demonstrate that RacR is an important regulator of *ggt* transcription. To our knowledge, RacS is the first identified Dsb substrate in *C. jejuni* and the first Dsb dependent bacterial sensor protein.

Introduction

The enzyme γ-glutamyltranspeptidase (GGT, EC 2.3.2.2) is highly conserved among eukaryotic and prokaryotic organisms (19) and has a key function in glutathione metabolism. In prokaryotes GGT is produced as a proenzyme in the cytosol and translocated into the periplasm where it undergoes autocatalytic cleavage. This proteolysis yields a mature dimer which transfers the γ-glutamyl moieties from extracellular glutathione and related compounds to amino acids or peptides (8). GGT activity in *Escherichia coli* and *Bacillus subtilis* is maximal at stationary growth phase (24, 29). In *B. subtilis* GGT is indirectly transcriptionally regulated in response to a low L-glutamate concentration via the quorum sensing two-component system ComP/ComA (15).

The bacterium *Campylobacter jejuni* is a major foodborne pathogen in humans and colonizes the intestinal tract of many warm-blooded animals (3). *C. jejuni* lacks the glycolytic enzyme phosphofructokinase and therefore is not able to use exogenous sugars as a carbon source. Hence, amino acids (i.e. aspartate, glutamate, proline and serine) are likely to sustain the growth of *Campylobacter* in the intestine (7). Some *C. jejuni* isolates are also able to utilize glutamine and glutathione due to the presence of GGT (11). In the periplasm the enzyme converts glutamine and glutathione to glutamate, which is subsequently taken up via the aspartate/glutamate-binding protein PEB1A (18). The presence of GGT allows these *C. jejuni* strains to enhance their colonization persistence in the avian gut (2, 12) and to colonize the intestine of mice (11).

Many periplasmic and secreted proteins are subjected to post-translational modification to obtain their correctly folded functional conformation. One important mechanism influencing protein folding is the formation of disulfide bridges between cysteine residues (17). In Gram-negative bacteria, disulfide bond formation occurs in the oxidative environment of the periplasm and is catalyzed by Dsb (*disulfide bond formation*) proteins (13). All *C. jejuni* strains possess a Dsb oxidative pathway consisting of four thiol oxidoreductases (DsbB, DsbI, DsbA1 and DsbA2). Comparative proteomics on *C. jejuni* strain 81-176 and a Dsb-defective derivative showed altered GTT protein levels in the mutant (Grabowska *et al.*, manuscript in preparation). So far the Dsb system has not been reported to influence GGT activity. As *C. jejuni* GGT lacks cysteine amino acid residues, we hypothesized that GGT expression is regulated by a Dsb-sensitive regulatory system. Here we provide evidence that the two-component system RacS-RacR regulates GGT in a Dsb-sensitive fashion. The sensor RacS is sensitive for disulfide bridge formation and RacR directly activates the *ggt* promoter.

Materials and methods

Bacterial strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* was cultured under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) or under oxygen limited conditions (0.3% O_2, 12% CO_2, 88% N_2) at 37°C on Blood Agar Base No. 2 (BA) medium containing 5% horse blood or in Heart Infusion broth (HI) (Oxoid). Kanamycin (25 μg ml^{-1}) and/or chloramphenicol (15 μg ml^{-1}) were added when appropriate. *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth or

on LB agar plates supplemented with ampicillin (100 µg ml^{-1}), kanamycin (30 µg ml^{-1}) or chloramphenicol (34 µg ml^{-1}).

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Origin or reference
Strains		
E. coli TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) *F'* (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15); used for general cloning	(23)
E. coli BL21(DE3)	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3); used for protein overexpression	Novagen
E. coli S17	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* (Tmp^r Str^r); used for conjugation	(21)
C. jejuni 81-176	wild type	M.J.Blaser, NYU, USA
81-176 Δ*dsbI*	81-176 derivative *dsbI*::Cm	This study
81-176 Δ*dsbB*	81-176 derivative *dsbB*::Km	This study
81-176 Δ*dsbB/dsbI*	81-176 derivative *dsbB*::Km *dsbI*::Cm	This study
C. jejuni 81116	wild type	(20)
81116 Δ*dsbI*	81116 derivative *dsbI*::Cm	This study
81116 Δ*dsbB*	81116 derivative *dsbB*::Km	This study
81116 Δ*dsbB/dsbI*	81116 derivative *dsbB*::Km *dsbI*::Cm	This study
81116 Δ*ggt*	81116 derivative *ggt*::Cm	This study
81116 Δ*racR*	81116 derivative *racR*::Cm	This study
Plasmids		
pBluescript II KS	Ap^R; 3.0 kb; LacZα	Stratagene
pGEM-T Easy	Ap^R; 3.0 kb; LacZα; TA cloning vector	Promega
pT7.7	Ap^R; 2.5 kb; expression vector	(25)
pMA1	Km^R; 10 kb; *E.coli/C.jejuni* shuttle vector	(26)
pMA2	Cm^R; 10 kb; *E.coli/C.jejuni* shuttle vector	(26)
pRY109	Ap^R, Cm^R; 3.5 kb; Cm cassette origin (*cat* gene)	(31)
Plasmids constructed for mutagenesis of *dsb, ggt* and *racR* genes		
pUWM607	Ap^R Km^R; 6.0 kb; pGEM-T Easy/*C. jejuni dsbB*::Km	(22)
pUWM711	Ap^R; 4.5 kb; pGEM-T Easy/internal fragment of *C. jejuni dsbI* (*dsbI'*)	This study
pUWM713	Ap^R Cm^R; 5.0 kb; pGEM-T Easy/*C. jejuni dsbI'*::Cm	This study
pUWM799	Ap^R; 4.2 kb; pBluescript II KS/internal fragment of *C. jejuni ggt (ggt')*	This study
pUWM804	Ap^R Cm^R; 4.9 kb; pBluescript II KS/*ggt'*::Cm	This study
pGEM-1261-1263	Ap^R; 5.5 kb; pGEM-T Easy/*C. jejuni 1261-1263*	This study
pGEM1261::Cm	Ap^R Cm^R; 6.3 kb; pGEM-T Easy/*racR'*::Cm	This study
Plasmids constructed for complementation assays		
pGEM-dsbB	Ap^R; 3.9 kb; pGEM-T Easy/*C. jejuni dsbB*	This study
pMA2-dsbB	Cm^R; 10.9 kb; pMA2/*C. jejuni dsbB*	This study
pMA1-1261-1263	Km^R;12.5 kb; pMA1/*C. jejuni 1261-1263*	This study

Continued table 1

Bacterial strains and plasmids	Relevant characteristics	Origin or reference
Plasmids constructed for RacR and RacS overexpression		
pGEM-RacR(N-his)	Ap^R; 3.7 kb; pGEM-T easy/*C. jejuni racR-N-his*	This study
pGEM-RacS(N-his)	Ap^R; 4.2 kb; pGEM-T easy/*C. jejuni fulllength racS*	This study
pGEM-RacScyto(N-his)	Ap^R; 3.8 kb; pGEM-T easy/*C. jejuni racS*-truncated	This study
pT7.7-RacR(N-his)	Cm^R; 3.2 kb; pT7.7/*C. jejuni* RacR with N-terminal His-tag	This study
pT7.7-RacS(N-His)	Cm^R; 3.7 kb; pT7.7/*C. jejuni* full-length RacS with N-terminal His-tag	This study
pT7.7-RacScyto(N-His)	Cm^R; 3.3 kb; pT7.7/*C. jejuni* truncated RacS with N-terminal His-tag	This study

Construction of *C. jejuni dsbI*, *dsbB*, *dsbI/dsbB*, *ggt* and *racR* mutants

A 1.5 kb DNA fragment containing the *dsbI* gene was amplified of the chromosome of *C. jejuni* strain 81-176 with the primers Cj17RBgl and Cj17LSal (Table 2). The PCR product was cloned into the pGEM-T Easy vector (Promega), resulting in plasmid pUWM711. A 0.3 kb fragment was removed from the central region of the cloned *dsbI* gene by digestion of pUWM711 with EcoRV. The linearized pUWM711 was ligated to a 0.8 kb SmaI fragment containing a chloramphenicol resistance cassette (Cm^R) of pRY109 (31), leading to the *dsbI* knockout construct pUWM713. PCR analysis showed that the inserted *cat* cassette had an orientation opposite to the *dsbI* gene.

To inactivate the *ggt* gene a 2.1 kb fragment containing the *ggt* gene and its flanking regions was amplified by PCR using the oligonucleotides Cjj67Sac and Cjj67Xba (Table 2). After digestion with SacI and XbaI the 2.1-kb PCR fragment was ligated into pBluescript II KS to give plasmid pUWM799. Plasmid pUWM799 was digested with BglII to remove a 0.1-kb internal *ggt* fragment and ligated to a 0.8-kb BamHI fragment containing the *cat* cassette (0.8 kb) of pRY109. The resulting *ggt* knockout construct pUWM804 contained the *cat* cassette in the same orientation as the *ggt* gene.

To disrupt the *racR* gene, a 2540-bp DNA fragment, containing the complete *racR*, *racS* and *recR* genes, was amplified from the *C. jejuni* 81116 chromosome using the primers CJ1261F and CJ1261R (Table 2). The PCR product was tailed with a 5'-A nucleotide using *Taq* polymerase (Invitrogen) and ligated into the pGEM-T Easy vector to obtain pGEM1261. Plasmid pGEM1261 was subsequently amplified with primers Cj1261FBamHI and Cj1261RBamHI to introduce a BamHI restriction site. The PCR product was digested with BamHI and ligated to a 0.7-kb BamHI fragment containing the chloramphenicol resistance gene of pAV35, resulting in the knockout construct pGEM1261::Cm. The *racR* gene in this knockout construct contains a deletion of 7-bp and the Cm^R gene in the same orientation as the *racR* gene.

For inactivation of the *dsbI*, *dsbB* genes in *C. jejuni* 81-176 and 81116, plasmid pUWM713, pUWM607 (containing a disrupted *dsbB gene* (22)) was introduced by natural transformation into *C. jejuni* strain 81-176 and 81116 (27). To create a *dsbI/dsbB* double mutant plasmid pUWM607

was introduced into the obtained *C. jejuni* 81-176 or 81116 *dsbI*::Cm mutant. The genes *ggt* and *racR* were disrupted in *C. jejuni* strain 81116 by natural transformation using the plasmids pUWM804 and pGEM1261::Cm, respectively. Double cross-over recombination events were confirmed by PCR.

Construction of the *racR* complementation plasmid

To complement the *racR* mutant, plasmid pGEM1261 was digested with PstI and SacII. The acquired 2607 bp fragment containing the *racR*, *racS* and *recR* genes was ligated into the shuttle plasmid pMA1 (26), which was digested with NsiI and SacII. The resulting complementation plasmid pMA1-1261-1263 was first transformed into *E. coli* S17 and then conjugated (16) to the *racR* mutant.

Construction and purification of recombinant RacR, cytoplasmic RacS and full length RacS

To obtain recombinant RacR, cytoplasmic RacS and full length RacS the *Campylobacter* genes *racR* and *racS* were amplified by PCR using the primer combinations RacRPstI/RacRNHISNdeI, RacSPstI/RacScytoNdeI and RacSPstI/RacSF-2 (Table 2) and the *Pfu* proofreading enzyme (Promega) according to the instructions of the manufacturer. The resulting PCR fragments of 670, 786 and 1254-bp were cloned into the pGEM-T Easy vector (Promega) to form pGEM-RacR(N-his), pGEM-RacScyto(N-his) and pGEM-RacS(N-his), respectively. After verification by sequence analysis, the plasmids were digested with PstI and NdeI and the PCR fragments were cloned into NdeI and PstI sites of expression plasmid pT7.7 (25) to form pT7.7-RacR(N-his), pT7.7-RacScyto(N-his) and pT7.7-RacS(N-his). Finally, the protein expression plasmids were transformed into *E. coli* BL21(DE3) and expression and purification of histidine-tagged RacR and RacS proteins was performed as described previously (28) except that solutions without DTT and β-mercaptoethanol were used. Protein concentrations were determined using the BCA protein assay kit (Pierce).

Western blotting

Equal amounts of purified native recombinant full length His-tagged RacS as well as a truncated His-tagged RacS were resuspended in Laemmli sample buffer with or without β-mercaptoethanol, electrophoresed on a 12% SDS-polyacrylamide gel, and analyzed by Western immunoblotting. Proteins were detected using anti-histidine horseradish peroxidase-linked antibody (GE Healthcare) and Supersignal® West Pico chemiluminescent substrate (Pierce).

Autophosphorylation assay

Equal amounts (50 pmol) of full length RacS or cytoplasmic RacS recombinant proteins were incubated (15 min, 20°C) with 10 μCi of [γ-^{32}P]ATP in phosphorylation buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 2 mM $MgCl_2$). The reaction was stopped by adding SDS-loading buffer with or without β-mercaptoethanol. Samples were run on 12.5% SDS-polyacrylamide gels. After electrophoresis the gel was dried and autoradiographed.

Table 2. Primers used in this study

Name	Sequence (5'-3')[a]
Cj17RBgl	TTCAGATCTCTAATGTGTTTAGCAGGC
Cj17LSal	GCTGTCGACTGATAAGAAAGAATATTG
Cj1261R	ACTAAATTTTTGTTCTAAAAT
Cj1261F	TAGCAAAAATAAAATCAATAA
Cj1261FBamHI	CAAGGATCCTGAGTCTTATTCGTCGCAC
Cj1261RBamHI	TCAGGATCCTTGCATACATTTCTTTTGGA
Cjj67Sac	CGCGAGCTCGCTTTTTGCGGTGGTAGG
Cjj67Xba	AGTTCTAGAGGAGATCCTGTGCCTGTG
Primers used for construction of expression vectors	
RacRPstI	TTCTGCAGTCATCCTATCAGTTTATAT
RacRNHISNdeI	TACATATGCATCACCATCACCATCACATTAATGTGTTGATGATAGA
RacSpstI	TTTCTGCAGTTATTTTTCTTTATCTCCAAAGAAA
RacScytoNdeI	ATTCATATGCACCATCACCATCATCACTCTTTAGAGCCTTTAAAAAAATT
RacSF-2	CATATGACAAAAAATTATTCTATTC
Primers for gel mobility shift assay	
GGTpromrev	GCTTCAAATTTCATATTGCACTT
GGTprom	TTGAAATCGCAAATATAGCT
Cj200R	GTTTTAGACTATCTGCAAAA
Cj201F	TTTCATCTTCAATATACTCTAA
CJ0145R	TTAAAAACAATCTTCTTTCCAT
Primers used for real-time RT-PCR	
ggttaq2f	AAGCCACCACCACCTATGTTTC
ggttaq2r	CATAGATGCAGCTGTAGCAGTAGGTT
corAftaq	TATGGGATAAGCATAGTGAAGTTCAAG
corArtaq	GCCATGATGCCACCTACTTTAAT
rpoAftaq	ATCAGTGCTTGGCCTTTTGAG
rpoArtaq	TGCTAGTGTAAAGTAAACGGCGTAA

[a] Restriction sites introduced for cloning purposes are underlined

Gel mobility shift assay

The promoter regions upstream of *ggt, phoX* (Cj0145) and Cj0200c were amplified by PCR using the primers sets GGTpromrev/GGTprom, Cj144F/CJ0145R and Cj200R/Cj201F, respectively (Table 2) and *C. jejuni* 81116 chromosomal DNA as a template. Prior to the PCR, primers GGTprom, Cj0144F and Cj200R were radiolabeled with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]ATP. Approximately, 25 pmol ^{32}P-labeled PCR fragments were incubated with 75 pmol His-tagged RacR for 30 min at 4°C. The binding buffer used for protein-DNA incubations was 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 50 µg/ml bovine serum albumin, 10 µg/ml poly(dI-dC) and 10% glycerol. Samples (10 µl) were run on a 6% non-denaturing Tris glycine polyacrylamide gel at 4°C. After electrophoresis the gel was dried and autoradiographed.

Real-time RT-PCR

Total RNA was extracted from oxygen limited (0.3% O_2) stationary phase (16 h) at 42°C grown *C. jejuni* cultures using RNA-Bee™ kit (Tel-Test, Inc) according to the manufacturer's specifications. Real-time RT-PCR analysis was performed as previously described (28). Primers used in this assay are listed in Table 2. Each sample was examined in four replicates and experiments were repeated with at least two independent preparations of RNA. Standard deviations were calculated and displayed as error bars.

GGT activity assay

To assay the GGT activity we followed the production of 3-carboxy-4-nitroaniline by measuring the absorbance at 405 nm according to a modified procedure described by (5). Briefly, 20 µl aliquots of whole bacterial cells were mixed with 180 µl of prewarmed reagent containing 2.9 mM L-γ-glutamyl-3-carboxy-4-nitroanilide, 100 mM glycylglycine and 100 mM Tris-HCl (pH 8.2) and measured every 2' during an incubation period of 60 min at 37°C. From these graphs the slope of all values in a linear range was calculated and corrected for the number of bacterial cells used in the assay as measured at 550 nm. Triplicate HI cultures at 42°C were grown for each assay and the assays were repeated at least twice. The results are presented as a percentage of GGT activity compared to the wild type strain.

Bioinformatic analysis

Prediction of transmembrane regions in protein sequences was carried out using HMMTOP and TMPRED. Prediction of conserved cytoplasmic domains was performed using the Prosite server.

Results

Inactivation of the *C. jejuni dsbI* and *dsbB* genes influences GGT activity

Previous comparative proteomics for strain 81-176 and a *dsbB* mutant demonstrated altered levels of GGT for the mutant (Grabowska *et al.*, manuscript in preparation). To confirm these results, we compared in a colorimetric assay the GGT activity in logarithmic growing (8 h) *C. jejuni* cultures of strain 81-176 and the isogenic *dsbI, dsbB and dsbI/dsbB* mutants (Fig.1). Compared to the parent strain the GGT activity was strongly enhanced in *C. jejuni* 81-176 Δ*dsbB* (120%) and 81-176 Δ*dsbI* (284%) and 81-176 Δ*dsbI/dsbB* (334%). Similar results were obtained for the *dsbI*, *dsbB*, *dsbI/dsbB* mutants of strain 81116. Complementation of the *dsb* mutants in 81116 resulted in an even higher GGT activity (data not shown), suggesting that the function of the Dsb multiprotein system requires a fine Dsb protein balance. Complete loss of enzyme activity was seen in the 81116 Δ*ggt* (Fig. 1).

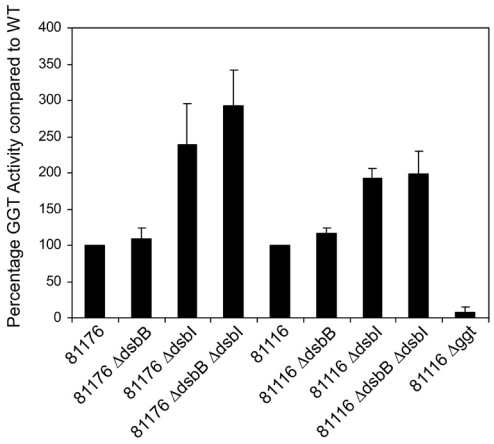


Figure 1. Inactivation of *C. jejuni* Dsb system influences GGT activity. GGT activity in whole bacterial cells from logarithmic phase (8 h) cultures in HI was measured for the wild type strains 81-176 and 81116 and the isogenic *dsbI*, *dsbB*, *dsbI/dsbB* mutants as well in the 81116 *ggt* mutant. Data are presented as percent of GGT activity compared to the wild type strain. Standard deviations of four independent experiments are indicated.

RacR regulates the level of *ggt* transcription

As *C. jejuni* GGT, unlike its eukaryotic homologs, does not contain cysteine residues, we assumed that the Dsb system has an indirect effect on the GGT activity. As only extracytoplasmic proteins are substrates for the Dsb system, we first focused on transcription factors that are under the control of periplasmic protein(s). One candidate group of proteins are two-component systems. Sensory proteins of these systems that contain at least two cysteine residues in their periplasmic region might potentially be direct substrates of the Dsb proteins. Periplasmic loop and cytoplasmic conserved domain predictions of the seven *C. jejuni* sensory proteins revealed that only RacS contains two cysteine residues in its periplasmic domain (at position 110 and 117). These cysteine residues potentially might form a disulfide bond. To investigate whether the two-component system RacS-RacR indeed influences the GGT activity we measured the production of 3-carboxy-4-nitroaniline by stationary phase cultures (20 h) grown in HI as we observed that the *C. jejuni* GGT activity is maximal under these conditions (data not shown). A strong reduction (40%) of GGT activity was observed in the *racR* mutant compared to the wild type strain (Fig. 2A). As the RacS-RacR two-component system is important for *C. jejuni* colonization in the gut of chickens (4), we also measured the GGT activity of cultures grown under low oxygen conditions. In the presence of 0.3% O_2, GGT activity was 70% higher than under microaerobic (5% O_2) conditions (Fig. 2A). This effect was not observed for the 81116 Δ*racR* mutant (Fig. 2A). When the *racR* mutant was complemented with plasmid pMA1-1261-1263 GGT activity was restored to wild type levels.

To investigate whether the two-component system RacS-RacR influences the *ggt* transcription *ggt* mRNA levels were determined for *C. jejuni* strain 81116, the *racR* mutant and the complemented *racR* mutant grown under oxygen limited conditions. Inactivation of *racR* resulted in a drop in *ggt* mRNA level, which was largely restored by introducing complementation plasmid pMA1 1261-1263 (Fig. 2B). These results suggest that RacR activates *ggt* transcription.

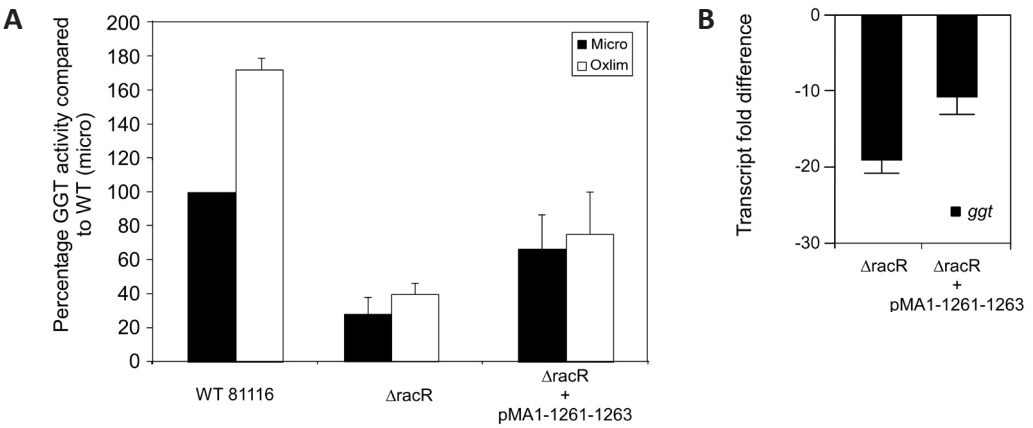


Figure 2. RacR regulates GGT. (A) The GGT activity in whole bacterial cells from stationary phase (20 h) cultures in HI was measured for strain 81116 wild type, the *racR* mutant strain, and in the *racR* mutant strain containing the complementation plasmid pMA1-1261-1263 under microaerobic or oxygen limited conditions. Data are presented as a percent of GGT activity compared to the wild type strain grown at microaerophilic conditions. Standard deviation of four independent experiments is indicated. (B) Real-time RT-PCR data showing the transcript fold difference of the *ggt* mRNA in the *C. jejuni* wild type strain compared to the *racR* mutant or to the *racR* mutant complemented with plasmid pMA1-1261-1263. Data represent the mean values and standard deviation of four independent experiments with two independent preparations of RNA.

RacR protein binds to the promoter region of the *ggt* gene

To ascertain RacR as a transcriptional regulator of GGT activity, binding of recombinant RacR protein to the *ggt* promoter was determined by gel mobility shift assay. Hereto the RacR response regulator was isolated as His-tagged recombinant protein and incubated with a radioactive DNA fragment containing the promoter region of the *ggt* gene or, as a control, the *phoX* or Cj0200c genes (Fig. 3). The *phoX* and Cj0200c promoter fragments did not shift. A clear DNA mobility shift was seen for the *ggt* promoter fragment, consistent with direct regulation of the *ggt* promoter by RacR.

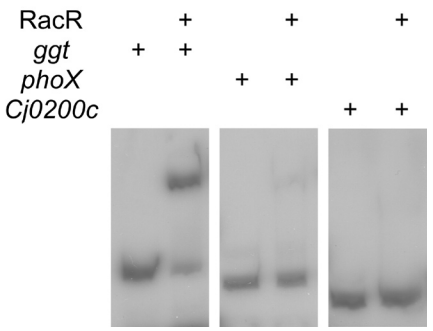


Figure 3. RacR binds to the *ggt* promoter region. Electrophoretic mobility shift assays of the *ggt, phoX* and Cj0200c promoter regions bound by 6xHis-RacR protein. ^{32}P-labeled PCR fragments (~25 pmol) containing the *ggt, phoX* or Cj0200c promoter regions were incubated with 75 pmol RacR as indicated. The *phoX* and Cj0200c promoter regions were used as negative controls.

RacS is sensitive to disulfide bridge formation

To address whether the RacS protein might be a substrate for the *C. jejuni* Dsb system, we first examined by real-time RT-PCR if the *racR* transcript changed after disruption of the Dsb system. No differences in *racR* transcript levels were found between the *C. jejuni* wild type strain and the *dsbI*, *dsbB* or *dsbB/dsbI* mutants (data not shown).

To prove that disulfide bridges play an important role in the function of the RacS sensor kinase, the full-length RacS protein (containing 9 cysteine residues) as well as the cytoplasmic portion of the RacS protein (containing 7 cysteine residues) were isolated and analyzed by Western blotting under reducing and non-reducing conditions. The electrophoretic mobility of the truncated RacS protein (26.5 kDa) was similar under both conditions. In contrast, full-length RacS migrated under reducing conditions as a single band with an apparent mass of 48.5 kDa and under non-reducing conditions as two bands with masses of 48.5 kDa and 97 kDa, respectively. The latter likely represents the functional RacS dimer (Fig. 4). These results indicate that the full length RacS contains disulfide bridges.

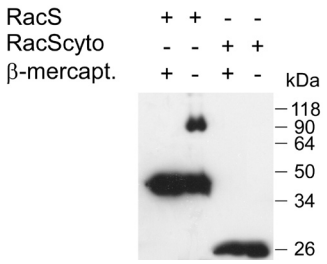


Figure 4. RacS is sensitive for disulfide bridge formation. Western blot showing cytoplasmic RacS as well as full-length RacS protein which were taken up in Laemmli sample buffer with or without β-mercaptoethanol. Proteins were detected by anti-histidine horseradish peroxidase-linked antibody. Molecular mass markers are noted on the right in kilodaltons.

RacS function depends on disulfide bond formation

The functional form of a two-component sensor is a dimer (30). *In vivo* in the absence of a specific stimulus the sensing domain of a two-component sensor inhibits the autophosphorylation of the cytoplasmic kinase domain. Truncated sensor proteins that contain only the kinase domain are constitutively active. We assumed that the recombinant full length RacS dimer resembles more the *in vivo* status of RacS. To determine whether the disulfide bond dependent dimeric state of RacS was important for the sensor function, we performed RacS autophosphorylation experiments with full length and truncated recombinant RacS in the absence and presence of β-mercaptoethanol. Incubation of full length protein with [γ-^{32}P]ATP with or without β-mercaptoethanol yielded one intense band on SDS-PAGE corresponding to monomeric full length RacS, indicating that in the absence of an appropriate stimulus the RacS dimer was unable to autophosphorylate and that the formation of disulfide bonds resulting in the dimer RacS prevents autophosphorylation of RacS (Fig. 5). As expected, β-mercaptoethanol had no effect on the autophosphorylation of the truncated constitutively active cytoplasmic RacS. Overall, the data indicate that full length RacS is sensitive to disulfide bridge formation.

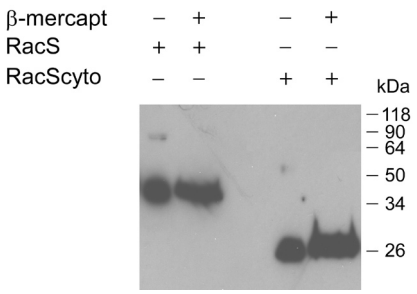


Figure 5. Autophosphorylation assays of the cytoplasmic as well as full length RacS *in vitro*. Autophosphorylation of the RacS proteins was accomplished by incubation of the proteins with [γ-^{32}P]ATP for 15 min at RT. Samples were taken up in Laemmli sample buffer with or without β-mercaptoethanol (as indicated) and run on a 12% SDS-polyacrylamide gel. Autophosphosphorylation was visualized by autoradiography. Molecular mass markers are noted on the right in kilodaltons.

Discussion

The formation of intra- or intermolecular disulfide bond formation by the Dsb system is crucial for the structure and stability of many secreted bacterial proteins. The *E. coli* Dsb machinery is best characterized and may affect more than 300 proteins in *E. coli* (9, 10). *C. jejuni* possesses a functional Dsb system however its targets are unknown (22). Searching for *C. jejuni* Dsb targets using a proteomics approach learned that GGT expression is Dsb-sensitive although the protein lacks cysteine residues. This triggered us to explore the relation between the Dsb system and GTT protein that is important for bacterial colonization of the intestine. In the present study we provide evidence that disruption of the Dsb system increases the GGT activity in the *C. jejuni* strains 81116 and 81-176. Furthermore *ggt* transcription in *C. jejuni* is regulated by the two-component system RacS-RacR and that RacS is a target of the Dsb system. To our knowledge RacS is the first two-component sensor protein that is dependent on a functional Dsb system.

Although present in many bacterial species knowledge regarding the regulation of the γ-glutamyltranspeptidase is limited. In *E. coli* and *B. subtilus* the GGT activity is maximal in the stationary growth phase (24, 29). In *C. jejuni* the highest GTT activity is seen on plates in the logarithmic growth phase (2), however when we used HI medium the highest GGT activity was observed in the stationary growth phase. Nutrient availability might explain this difference as a low concentration of L-glutamate has been shown to activate the *B. subtilis* GGT (15). So far only in *B. subtilis* a transcription factor ComA of the quorum sensing two-component system ComP-ComA has been identified to be able to activate *ggt* transcription (15). This together with the Dsb sensitivity of GGT in *C. jejuni* led us hypothesize that the *ggt* gene of *C. jejuni* might be regulated by a two-component system that is Dsb sensitive. The periplasmic domain of the RacS sensor a component of the RacS-RacR two-component system contains two cysteine residues. As RacS is the only *C. jejuni* sensor protein which contains periplasmic located cysteine residues and both GGT and RacR have been shown to be important for host colonization (2, 4, 12), we assumed that the RacS-RacR system would be the likely candidate to regulate the *ggt* gene. GGT assays, real-time RT-PCR and EMSA showed that RacR directly activates the *ggt* promoter.

Thus far, the knowledge regarding the *C. jejuni* RacS-RacR two-component system has been limited. The system is important for bacterial colonization of chickens and activates or represses at least 11 different proteins of which one is a cytochrome c peroxidase homolog (4). Bacterial cytochrome c peroxidases are able to reduce potentially toxic hydrogen peroxide compounds (1). Interestingly, *C. jejuni* GGT decreases the resistance to hydrogen peroxide stress (2). As the cytochrome c peroxidase is repressed and GGT is activated by RacR, it can be speculated that the RacS-RacR system is regulating the oxidative stress resistance. The increased RacR dependent GGT activity observed when *C. jejuni* was grown under low oxygen vs microaerobic conditions supports this hypothesis. However, detailed analysis of the RacS/RacR regulon is needed to support this hypothesis.

A key question spawn by our findings is how disulfide bond formation influences the function of RacS. We addressed this question by measuring the autophosphorylation of the RacS sensor under non-reducing and reducing conditions. All sensors of two-component systems act as dimers, although signaling does not occur through signal-mediated dimerization of kinase domains. Activation of the dimer is believed to occur by altering the conformation of the surface domain of the sensor by specific stimuli which causes the autokinase domain to become active (6). Removal of the signaling domain strongly enhances the autophosphorylation of the sensor kinase domain (14). Autophosphorylation of the recombinant full-length RacS in the presence of β-mercaptoethanol was only slightly reduced compared to the truncated RacS indicating that the signal domain without disulfide bonds induces autophosphorylation of the RacS kinase domain. A large fraction of the recombinant full-length RacS protein in the absence of β-mercaptoethanol migrated as a dimer during electrophoresis and was hardly able to autophosphorylate, resembling the conformation off state of bacterial sensors due to the inhibition of the signal domain in the absence of their specific stimulus. The enhanced GGT activity observed in the *dsb* mutants is likely due to the lack of disulfide bonds in RacS which activates the autophosphorylation of the sensor. This suggests that the disulfide bonds are important to keep the dimer in a non-functional state, awaiting activation by a specific stimulus. Whether changes of disulfide bonds are related to activation of RacS is unknown and awaits identification of the natural stimulus for this sensor.

In conclusion, our results provide evidence that *C. jejuni* RacS is a substrate for the Dsb system, and that the Dsb activity is important to keep the sensor in an inactive resting state. Activation of RacS results in binding of RacR to the *ggt* promoter region, resulting in activation of *ggt* transcription in a direct fashion, which is most strongly under restricted oxygen levels. To our knowledge *C. jejuni* RacS is the first Dsb dependent bacterial sensor protein that is identified.

Acknowledgements

This work was supported by a fellowship of the Royal Netherlands Academy of Arts and Science, NWO-VIDI grant 917.66.330 to M.M.S.M. Wösten and a grant of the University of Warsaw (BW179134) to P. Łaniewski.

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Chapter 5

The *Campylobacter jejuni* RacRS two-component system regulates fumarate catabolism and respiration in response to oxygen

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Manuscript in preparation

Abstract

The natural environment of the human pathogen *Campylobacter jejuni* is the gastrointestinal tract of warm blooded animals. *C. jejuni* preferentially utilizes amino acids as a carbon source and has all the enzymes for a complete tricarboxylic acid cycle. In the gut, the availability of oxygen is limited therefore less efficient electron acceptors such as nitrate or fumarate are used by *C. jejuni* as an alternative for oxygen. To enable this *C. jejuni* has a highly branched respiratory chain. Gene expression profiling indicates that a large number of *C. jejuni* genes are differentially expressed *in vivo* compared to *in vitro* growth conditions. The regulation mechanisms behind these alterations in gene expression are unknown. Here we demonstrate using a combined transcriptomics, proteomics and metabolomics approach that the response regulator RacR is regulating a number of genes involved in energy generation. The activity of RacR is regulated by, the cognate sensor RacS as demonstrated by phosphorylation studies and is dependent on the available carbon source and electron acceptor. Electrophoretic mobility shift assays showed that RacR binds directly to promoter elements located upstream of genes involved in energy metabolism. Our results indicate that the failure of the *racR*::Cm mutant to colonize chickens may be due to the inability to fine-regulate its metabolic properties by means of fumarate generation in response to low oxygen conditions as are present in the chicken gut.

Introduction

Metabolic activity is essential in all living organisms to generate energy for cellular biosynthesis. A central pathway for biosynthetic processes is the tricarboxylic acid (TCA) cycle. During the main route of the TCA cycle, the oxidative direction, acetyl-CoA is converted to CO_2, NADH and intracellular intermediates. The intermediate chemical transformations allow the TCA cycle to connect with the electron transport chain, a respiratory-driven electron transport system beneficial for cells to produce the major energy source ATP. Bacteria control their energy generating processes at different levels including via transcriptional regulatory systems that respond to the available nutrients and oxygen levels (49, 51, 3, 21). In *Escherichia coli* several metabolic genes are regulated by the two-component system DcuS/DcuR, activated by extracellular C4-dicarboxylate molecules like fumarate (11). But also in other bacterial species two-component systems, like DctB/DctD and CbrA/CbrB, regulate the sensing and utilization of catabolic molecules to optimize metabolic processes (63, 37, 27). The ArcA/ArcB two-component system and the transcription factor FNR are both activated by changes in the oxygen concentration. These systems activate hundreds of genes, including genes involved in carbon- and electron-flow, that control the switch between anaerobic and aerobic growth (23, 17). To regulate the order of alternative electron acceptors in oxygen restricted environments, *E. coli* and *Pseudomonas aeruginosa* exploit the nitrate responsive NarX/NarL or nitrite responsive NarQ/NarP two-component system (52, 42). Several bacterial species possess the two-component system TorS/TorR to sense trimethylamine oxide (TMAO) and regulates transcription of genes for TMAO respiration during oxygen limitation (2, 26).

Campylobacter jejuni is an oxygen-sensitive spiral shaped, microaerophilic bacterial pathogen that shows a high metabolic versatility. The main natural habitat of *C. jejuni* is the oxygen limited gastrointestinal tract of poultry, but the bacterium also survives in surface water and several steps in the food chain. Nevertheless, based on sequence homology, none of the above mentioned two-component systems or transcription factors involved in metabolic adaptation, are encoded in the *C. jejuni* genome (33, 34). The *C. jejuni* metabolic versatility includes a highly branched electron transport chain which allows both aerobic and anaerobic respiration. *C. jejuni* seems to prefer aerobic respiration however it is also able to use alternative electron acceptors like nitrate, TMAO/DMSO and fumarate (43). In addition *C. jejuni* contains all the enzymes for a complete oxidative TCA cycle, central to different metabolic energy pathways. As the bacterium cannot ferment carbohydrates (33, 34) it utilizes amino acids as primary energy source. Serine and aspartate are the most preferred amino acids but also glutamate, proline, asparagine and glutamine can be utilized and some strains also have the ability to reduce glutathione (15, 18).

Transcriptome analyses of *C. jejuni* grown under different environmental conditions indicate that the metabolic gene expression is probably tightly regulated both *in vitro* (9) and *in vivo* (47, 60). Genes involved in the TCA cycle, the electron transport chain and intermediary metabolism are transcribed at higher levels in the chicken cecum (60). Genes highly expressed in this low oxygen environment encode two different fumarate reductases (encoded by *frd* and the recently renamed *mfr* (58)), an aspartase (encoded by *aspA*), the antiport system DcuB which takes up fumarate and secretes succinate (14, 15), and the alternative electron acceptors nitrate and nitrite (respectively

reduced by the *nap*- and *nrf*-complex). Taken together these results suggest that *C. jejuni* must possess regulatory mechanism(s) that control the switch between metabolic pathways in response to nutrient and/or oxygen limitation.

The *C. jejuni* RacS-RacR two-component system has been reported to be important in the ability to colonize chickens and to support optimal growth rates at 42°C (4), suggesting that the system regulates genes important for *in vivo* colonization in a temperature dependent manner. So far two genes are known to be regulated by RacR, i.e. an oxygen sensitive cytochrome peroxidase homolog Cj0358 (4) and *ggt* coding for the enzyme γ-glutamyltranspeptidase (Łaniewski *et al.*, in preparation) which converts glutamine and glutathione to glutamate. Here we report that the two-component system RacS-RacR plays a key role in the metabolic regulation of the aspartate-fumarate pathway and that the system is essential for growth under oxygen limited conditions as is present in its intestinal niche.

Materials and Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were grown on plates containing blood agar base II (Oxoid, London, UK) and 5% horse blood (Biotrading, Mijdrecht, The Netherlands) lysed with 0.5% saponin (Sigma, St Louis, MO), in Heart Infusion broth (HI) (Biotrading), or in chemical defined media (DM) (Leach *et al.* 1997) at 37°C or 42°C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) or oxygen limited (0.3% O_2, 12% CO_2 and 88% N_2) conditions using the anoxomat system (MART Microbiology, Lichtenvoorde, the Netherlands). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth (Biotrading) at 37°C. When appropriate, media were supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml) and/or ampicillin (100 μg/ml).

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Origin or reference
Strains		
E. coli PC2955	*relA1* _80d*lacZ*_M15 *phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44*; used for cloning	NCCB
E. coli BL21(DE3)	F^- *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3); used for protein overexpression	Novagen
E. coli S17	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* (Tmp^r Str^r); used for conjugation	(32)
C. jejuni 81116	Wildtype	(30, 34)
C. jejuni	*C. jejuni* 81116 *ggt*::Cm	Łaniewski *et al.*
C. jejuni	*C. jejuni* 81116 *racR*::Cm	Łaniewski *et al.*
C. jejuni	*C. jejuni* 81116 *aspA*::Km	This study
Plasmids		
pGEM-T Easy	Ap^R; 3.0 kb; LacZα; TA cloning vector	Promega
pBluescript II KS	Ap^R; 3.0 kb; LacZα	Stratagene
pT7.7	Ap^R; 2.5 kb; expression vector	(48)
pAV35	pBluescript II SK containing *Campylobacter coli* Cm^R cassette	(54)
pMA1	Km^R; 10 kb; *E.coli/C. jejuni* shuttle vector	(53)
Plasmids constructed for mutagenesis		
pUWM799	Ap^R; 4.2 kb; pBluescript II KS/internal fragment of *C. jejuni ggt (ggt')*	Łaniewski *et al.*
pUWM804	Ap^R Cm^R; 4.9 kb; pBluescript II KS/*ggt'::*Cm	Łaniewski *et al.*
pGEM1261-1263	Ap^R; 5.5 kb; pGEM-T easy/*C. jejuni* 1261-1263	Łaniewski *et al.*
pGEM1261::Cm	Ap^R Cm^R; 6.3 kb; pGEM-T easy/*C. jejuni racR*::Cm	Łaniewski *et al.*
pEJG2	Ap^R Km^R; 6.3 kb; pGEM-T easy/*C. jejuni aspA*::Km	(15)
Plasmids constructed for complementation assays		
pMA1-1261-1263	Km^R;12.5 kb; pMA1/*C. jejuni 1261-1263*	Łaniewski *et al.*
Plasmids constructed for RacR and RacS overexpression		
pGEM-RacR(N-his)	Ap^R; 3.7 kb; pGEM-T easy/*C. jejuni racR-N-his*	Łaniewski *et al.*
pGEM-RacStrunc.	Ap^R; 3.8 kb; pGEM-T easy/*C. jejuni racS*-truncated	Łaniewski *et al.*
pT7.7-RacR(N-his)	Cm^R; 3.2 kb; pT7.7/*C. jejuni* RacR with N-terminal His-tag	Łaniewski *et al.*
pT7.7-RacStrunc.	Cm^R; 3.3 kb; pT7.7/*C. jejuni* truncated RacS with N-terminal His-tag	Łaniewski *et al.*

Growth experiments

To obtain growth curves for the *C. jejuni* 81116 Wt, *racR*::Cm mutant strain, and a *racR*::Cm mutant strain complemented with the shuttle plasmid pMA1-1261-1263, overnight cultures in DM were diluted to OD_{550nm} of 0.05 in fresh DM media. The cultures were grown under microaerophilic or oxygen limited conditions for 24 h at 37°C or 42°C with or without the addition of 50 mM fumarate or nitrate or 20 mM aspartate or serine. Cell density was measured at OD 550 nm at the indicated time points.

Protein purification

The histidine-tagged proteins RacR and the cytoplasmic part of the RacS were isolated of *E. coli* BL21 (DE3) containing expression plasmids pT7.7-RacR(N-his) or pT7.7-RacScyto(N-his) as described in Łaniewski *et al.* (manuscript in preparation). Protein concentrations were determined using the BCA protein assay kit (Pierce).

Phosphorylation assay

In vitro autophosphorylation of purified recombinant his-tagged RacS (50 pmol) was performed at room temperature for 15 min in the presence of 10 µCi [γ-^{32}P]ATP (MP Biomedicals Netherlands) and 100 µl phosphorylation buffer (50 mM Tris-HCl, 75 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, pH 8.3). After 15 min recombinant RacR (50 pmol) was added to the mixture, in a molar ratio of 1:1. The reaction was stopped by adding SDS loading buffer to 10 µl samples taken after 0.25, 0.5, 1, 2, 4, 8 or 16 min of incubation. Samples were run on a 12% SDS-polyacrylamide gel. After electrophoresis the gel was dried and autoradiographed.

RNA isolation

RNA was extracted from the Wt, *racR*::Cm mutant strain, and the complemented *racR*::Cm mutant grown under low oxygen concentrations in DM with 100 mM of $NaNO_3$ until late logarithmic (log) phase (16 h). RNA samples were treated with RNAse-free DNAse I (Invitrogen) according to the manufacturer's manual.

Microarray hybridization and analysis

Microarray hybridization and analysis was performed as previously described (62). Microarray data have been included as supplementary data.

Real-time RT-PCR

Real-time RT-PCR analysis was performed as previously described (61). Primers used in this study are listed in Table 2. The calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct value for the genes *rpoA, gyrA, corA, fliA* and *Cj0147* amplified of the corresponding sample before calculating fold change using the arithmetic formula ($2^{-\Delta\Delta Ct}$) (40). Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and displayed as error bars.

Electrophoretic mobility shift assay (EMSA)

The promoter regions upstream of the genes *aspA, frdC, ansA, Cj0358, mfrX, dcuB, racR, gltB and glnA* were amplified by PCR using the primer pairs listed in Table 2 and *C. jejuni* 81116 genomic DNA as template. To obtain radioactive labeled PCR fragments one of the primers from each primer pair was first labeled by [γ-^{32}P]ATP and T4 polynucleotide kinase (Invitrogen) for 30 min at 37°C according to the manufacturer's manual. The radioactive labeled PCR products, approximately 25 pmol, were incubated with 50 pmol RacR for 20 min at RT in binding buffer containing 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 50 µg/ml bovine serum albumin, 10 µg/ml poly-(dI-dC) and 10% glycerol. For competition assays RacR was pre-incubated for 15 min with 10 times excess of unlabeled PCR fragment. Samples were run on 6% non-denaturing Tris-glycine polyacrylamide gels at 4°C. After gelelectrophoresis, gels were dried and autoradiographed.

Table 2. Primer sequences used in this study

Name	Sequence (5'-3')
Primers for gel mobility shift assay	
aspAFprom	AGCTTGCAAAAATATATTAATTT
aspARpromprex	TAATAAACCTCATCAGAGATTTC
gltB-F	TTTAGGTAGTATTATCGCCATGTT
gltBrtaq	TACCACAGGCATCGTGTTCG
Cj0176c	AGTAAAAATAAAATTATTTTTTTCATAGAC
Cj0177	CTTGCAAAAAATGAAGAAAAC
Cj1614	TTCATATGAGAAATAATGCTTTCAAAATTC
chuA	AAGCTTTTTTATTTGGGTGC
Cj0449c	TCTCTATATTCATGTAGCAT
Cj0450	CGCAGCTTCTACTCTAAGAA
Cj436fabF	AGAATGCAATTTATGAATGGAGAATT
Cj437mfrx	AATCAAAGAAGATAGATGAATATAAT
Cj1493F	CTCCTGAGTAGGACAAATCC
Cj1493R	CATAGAATAGCAGTGTCCAAAA
Cj1384c	GCACCAATAACCAAAACTGA
katA4	CCAAAATCGTTAGTTAATTTTTTC
Cj144FDig	GATGTTAAGAAGAAGAGGTT
CJ0145R	TTAAAAACAATCTTCTTTCCAT
Cj0358F	CATAATATCCTTTTCAAATCAT
Cj0358Rdig	AATGATTTTACTTTCATTTATAAAC
Primers for real-time RT-PCR	
Cj0087Ftaq	GGTCCAAAATGTGGTCTTAATGAGAT
Cj0087Rtaq	TTCAGGAATAACAGGATTTACCTTACC
sdhAFtaq	TGCAAATGGTGGAACTCTTATTACA
sdhARtaq	GCTCACCACGATTATTTAAAAGATAT
ansAtaqF	AAGGGTGTTATGGTAGCTATGAATGA
ansAtaqR	TCAGGAGAAGAGAAAGCATCAACAT
Cj0358taqF2	TGCTGAAACTGCTCCATATTTTCA
Cj0358taqR2	GCCAAGTTGCACACTACCCATT

Continued table 2

Name	Sequence (5'-3')
ggt-RT-Ftaq	TGCGAGTTATGGTTCAGGTG
ggt-RT-Rtaq	TTAGCTTCTCCGCCTACAAG
gltBFtaq	GTGCATGGCTGTGAATATATGACA
gltBRtaq	AACAACGCCCCCACTCATAC
pspAFtaq	GCTGGAACTTTTGCAAGAGCTT
pspARtaq	GCATTTGCCCATTGATCTTTTT
ungFtaq	CAAGGAGTTTTGCTCTTAAATTCTATTCT
ungRtaq	TCTTGCCAACCCCACGAA
Cj449Ftaq	TTCCATGCTGCTAAGAGAAGTTCTAC
Cj449Rtaq	AATTAATGTCAGAGCTAAAAGGTAAAGATG
Cj1491Ftaq	TTTTTTAAGCCCCTCATCTCCAT
Cj1491Rtaq	GAGGTTAAGGCTAGAGAATCAATGATTAAT
Cj175Ftaq	GCTAATGACGGAAATAAAGAGGCTAA
Cj175Rtaq	GATCTCTATCTCCACCCTTTGGATT
chuBFtaq	GCGGTTTTGGCGTATTTGTTA
chuBRtaq	AGTCGCTATAGAAGCTTTAGAAGAAATTTT
Cj1384Ftaq	CTTGCGATTAACTCTATTTTCATTACGT
Cj1384Rtaq	GATGAAATCACTCCTATTAGAGCTGTTTT
katAFtaq	CTCCTCTTTTCCTACGCTTTTCTACT
katARtaq	TCTCACATCGCGTTCAGCAT
hipOFtaq	CCAGAAAAGGCAAAAGATCCTATTT
hipORtaq	ACATTGCGAGATACTATGCTTTGTAAA
corAftaq	TATGGGATAAGCATAGTGAAGTTCAAG
corArtaq	GCCATGATGCCACCTACTTTAAT
rpoAftaq	ATCAGTGCTTGGCCTTTTGAG
rpoArtaq	TGCTAGTGTAAAGTAAACGGCGTAA
fliAftaq	GCCTAAAGCTTATGCACAAATGC
fliArtaq	CGTTCTTTTAGTCTAAAAGCCATAGCA
gyrAftaq	GAACTCATGGCTAAATTCATACAAACA
gyrArtaq	GCTTTATTTCCGCTTTGCACTT
Cj0147Ftaq	CATCAATAACTGGAGCAAGCATTC
Cj0147Rtaq	GCAAAAGAAGGTGTAGCTTTAGTTGATT

2D-gel electrophoresis and Enzyme assays

2D-gel electrophoresis was performed as described by (15). The γ-glutamyltranspeptidase (GGT) activity was assayed as described (Łaniewski *et al.*, in preparation). The aspartase (AspA) activity and the malic enzyme (MEZ) activity were measured as described by (15) and by (55), respectively. In brief bacterial cultures of Wt, *racR*::Cm mutant strain and the complemented *racR*::Cm mutant strain were grown O/N at 37°C in DM under microaerophilic conditions. These pre-cultures were diluted to OD_{600} of 0.05 in DM with 20 mM serine or aspartate as major carbon source or in MEMα-medium containing 20 mM serine and 20 mM aspartate and/or 50 mM nitrate and incubated under low oxygen conditions. Logarithmic phase bacteria (OD_{600} nm 0.2-0.7) were lysed by sonication (6 × 5 sec.) on ice. Protein concentration was determined using the BCA protein assay kit. Enzyme activ-

ity was measured at 240 nm, corresponding to the absorbance of fumarate, by using the Omega FLUOstar (Isogen, BMG labtech). Enzyme activity is presented as µmol min^{-1} mg $protein^{-1}$.

Proton nuclear magnetic resonance (^{1}H-NMR)

C. jejuni was grown in DM with 20 mM serine or 20 mM aspartate as major carbon source under oxygen limited conditions. At 4, 8, 16 and 24 h two ml of the culture was centrifuged at 5,000 rpm for 15 min. The obtained supernatant (1.5 ml) was centrifuged again for 5 min at 14,000 rpm and then filtered by passing it through a 0.22 µm pore-size syringe filter unit (Millipore). NMR was performed as previously described (41).

Statistical analysis

Data were analyzed by two-tailed paired *t*-tests (Graphpad prism). A P-value of <0.05 was considered to be significant.

Results

The RacS and RacR proteins represent a two-component system

To investigate the function of RacR in *C. jejuni* in more detail we first investigated whether the putative response regulator RacR and the sensor histidine kinase RacS form a true two-component system. Members of two-component systems communicate with each other by phosphate transfer. From this perspective RacR and RacS were purified as recombinant proteins and incubated with radioactive [γ-^{32}P]ATP. Only RacS could rapidly autophosphorylate itself (Fig. 1). When RacR was incubated with autophosphorylated RacS most of the radioactive phosphate residues were transferred to RacR within minutes. This phosphotransfer shows that RacR and RacS function as a two-component system.

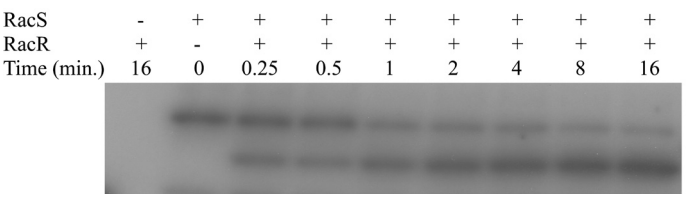


Figure 1. Phosphate transfer between the cytoplasmic domain of the sensor RacS and its cognate regulator protein RacR. *In vitro* phosphate transfer assay between the recombinant truncated Histidine-tagged RacS and recombinant Histidine-tagged RacR protein was visualized using 12% SDS-polyacrylamide gels and autoradiography. Autophosphorylation of the RacS protein (50 pmol) was observed after 15 min of incubation with [γ-^{32}P]ATP at room temperature (not shown). Phosphotransfer from ^{32}P-RacS to RacR (50 pmol) was followed in time at the indicated time points.

The RacS-RacR system is required for growth under reduced oxygen levels

Inactivation of *racR* in strain 81116 has previously been reported to result in a decreased growth rate at 42°C but not at 37°C (4). To confirm these results we inactivated the *racR* gene of 81116 by

insertion of an antibiotic resistance cassette and compared the growth rate of the mutant and parent strain at different temperatures. To our surprise no growth rate differences between the *racR*::Cm mutant and the parent strain were observed when the strains were grown under microaerophilic conditions in chemical defined medium (DM) or Heart Infusion (HI) medium either at 42°C or at 37°C (Fig. 2A). As RacR is important for *C. jejuni* to colonize the (oxygen limited) chicken gut and one of the two genes regulated by the RacS-RacR two-component system is an oxygen sensitive cytochrome peroxidase homolog Cj0358 (4), we hypothesized that instead of temperature, the concentration of oxygen may influence the activity of the RacS-RacR regulon. Therefore we measured the optical density of the Wildtype (Wt) strain, the *racR*::Cm mutant and a complemented *racR*::Cm mutant grown in DM under reduced oxygen (0.3% O_2) conditions (Fig. 2B). A reduced grow rate was seen for the *racR*::Cm mutant compared to the parent strain. This growth defect was restored after complementation of the mutant *in trans* with an intact copy of the *racR* and *racS* genes, indicating that the RacS-RacR system is important for *C. jejuni* to grow in oxygen limited environments.

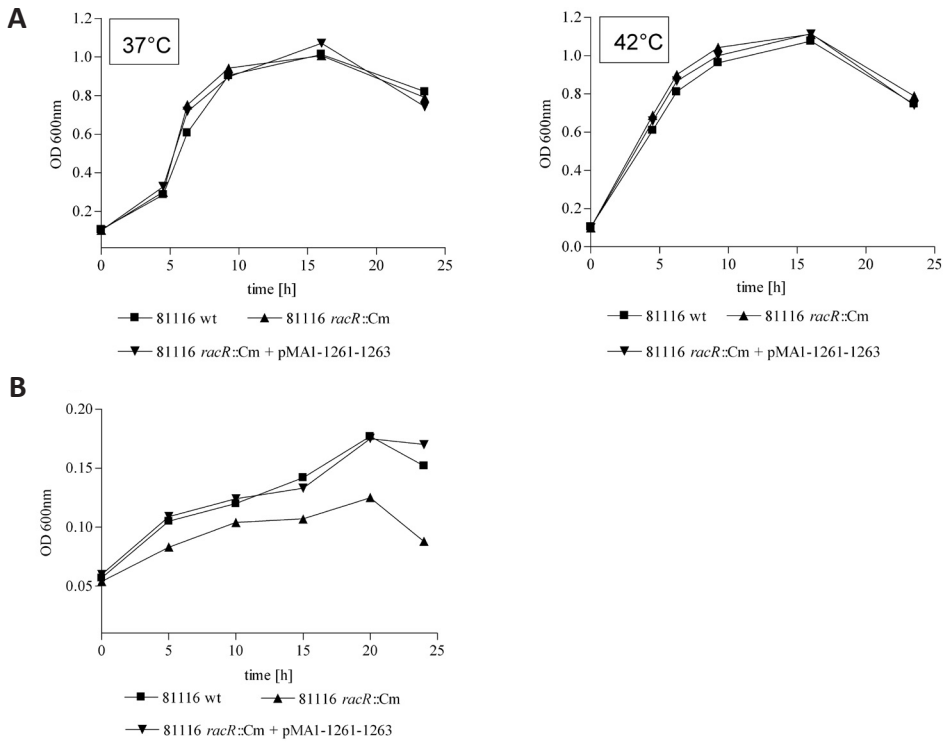


Figure 2. Growth curves of *C. jejuni* Wt, *racR*::Cm mutant and the *racR*::Cm mutant complemented with pMA1-1261-1263 under microaerophilic and oxygen limited conditions. *C. jejuni* 81116 Wt (filled squares), *racR*::Cm (filled triangle up) and the complemented *racR*::Cm mutant (filled triangle down) were grown in DM under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37°C or 42°C (A) or under oxygen limited conditions (0.3% O_2, 12% CO_2 and 88% N_2) at 42°C (B). Growth was determined by monitoring optical density at 600 nm. The results shown are representative of at least three independent experiments.

Identification of the genes dependent on a functional RacS-RacR system

To identify the RacR regulon, gene expression profiles of the Wt and the *racR*::Cm mutant were compared by microarray analysis. For this purpose, total RNA was isolated from stationary phase cultures grown under oxygen limited conditions at 42°C. These experiments revealed that 11 genes are activated and 17 genes are repressed more than 4-fold by the RacS-RacR system (Fig. 3A). To verify the microarray results the transcription levels of 14 of the 28 up or down regulated genes comprising all single genes (except two ribosomal genes) and one gene of each putative operon were determined by real-time RT PCR. The number of transcripts of 10 of the selected genes differed more than 5-fold between the Wt and the *racR*::Cm mutant (Fig. 3B). Collectively, the microarray and real-time RT PCR data indicate that the RacR protein activates the *gltB* gene and four putative operons; Cj0175c-Cj0176c, *fldA*-Cj1384c, *katA*-Cj1386 and *chuABCD*. Beside hypothetical genes these genes code for a glutamate synthase (*gltB*), a Fe^{3+} ABC transporter (Cj0175c), flavodoxin (Cj1382c) and a catalase (*katA*).

In addition activation of RacR repressed the transcription of 4 putative operons; *aspA-dcuA*-Cj0089, *sdhABC* (renamed *mfrXABE* (58)), Cj448c-Cj0449c and Cj1491c-Cj1493c. The *aspA* gene codes for an enzyme which converts aspartate to fumarate. This gene is located in a putative operon together with *dcuA* (an anaerobic C4-dicarboxylate transporter) and Cj0089 coding for a hypothetical protein. The *mfrXABE* genes are coding for a fumarate reductase enzyme complex which converts fumarate to succinate. Cj0448c is predicted to encode an accessory colonization factor (*acfB*) as identified in *Vibrio cholerae* (7) while Cj0449c codes for a hypothetical protein. The proteins translated from the Cj1491c, Cj1492c and Cj1493c genes might form a putative two-component system and a putative membrane protein, respectively. We could confirm that the cytochrome peroxidase homolog Cj0358 identified by Bras et al (4) was indeed repressed by RacR. We did identify the *ggt* gene to be dependent on a functional RacR only by real-time RT PCR as this gene was not present on the used microarray slides. Overall, the data indicate that the RacS-RacR system is mainly regulating genes important for amino acids consumption, iron acquisition and oxidative stress responses.

A Genes upregulated in the Wt compared to *racR*::Cm mutant

Gene designation	Putative function	fold induction
Cj0007	glutamate synthase, GltB	4.3
Cj0175	iron (III) ABC transporter	5.2
Cj0176	conserved hypothetical protein	4.7
Cj0985	hippurate hydrolase, HipO	4.4
Cj1382	flavodoxin A, FldA	5.2
Cj1383	conserved hypothetical protein	5.1
Cj1384	conserved hypothetical protein	5.7
Cj1385	catalase, KatA	6.2
Cj1386	ank repeat domain	5.1
Cj1615	hemin ABC transporter, ChuB	4.9
Cj1617	iron compound ABC transporter, ChuD	4.4

A Genes downregulated in the Wt compared to *racR*::Cm mutant

Gene designation	Putative function	fold induction
Cj0029	L-asparaginase, AnsA	5.0
Cj0068	putative protease IV, PspA	4.0
Cj0069	conserved hypothetical protein	4.8
Cj0086	uracil-DNA glycosylase, Ung	7.3
Cj0087	aspartate ammonia-lyase, AspA	78.2
Cj0088	anaerobic C4-dicarboxylate transporter, DcuA	12.5
Cj0089	conserved hypothetical protein	4.3
Cj0244	ribosomal protein, RpmI	4.0
Cj0358	putative cytochrome peroxidase	8.2
Cj0437	periplasmic fumarate reductase, MfrA	4.0
Cj0438	iron sulfur protein, MfrB	4.2
Cj0448	accessory colonization factor, AcfB	5.1
Cj0449	conserved hypothetical protein	6.9
Cj0961	ribosomal protein, RpmH	4.3
Cj1491	putative response regulator,	4.2
Cj1492	histidine kinase	4.1
Cj1493	putative membrane protein	8.1

B

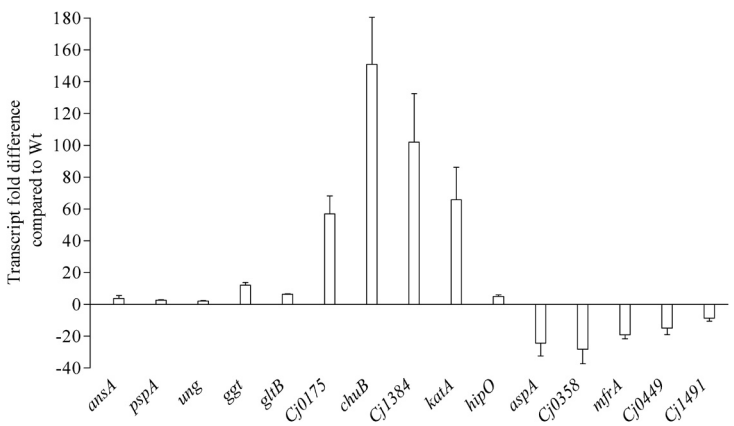


Figure 3. Identification of RacR dependent genes.(A) Microarray results obtained by comparing mRNA profiles of the Wt and the *racR*::Cm mutant strain grown under oxygen limited conditions. Shown are genes upregulated or downregulated more than 4 fold in the Wt strain compared to the *racR*::Cm mutant (upper and lower column, respectively). (B) Transcript fold differences between Wt and *racR*::Cm mutant grown under oxygen limited conditions of the *ansA, pspA, ung, ggt, gltB*, Cj0175c, *chuB,* Cj1384c, *katA*, *hipO*, *aspA*, Cj0358, *mfrA,* Cj0449c and Cj1491c genes as estimated by Real-time RT PCR. Each sample was examined in four replicates and experiments were repeated with at least two independent preparations of RNA. Standard deviations were calculated and displayed as error bars.

DNA binding of RacR as determined by EMSA

To investigate whether the identified genes are regulated directly by RacR, electrophoretic mobility shift assays (EMSA) were performed. Hereto recombinant RacR was incubated with [γ-^{32}P]ATP labeled DNA fragments containing the promoter regions of *gltB,* Cj0176c*, chuA, aspA,* Cj0358*, mfrX,* Cj0449c, Cj1493c, the intergenic region between Cj1384c and *katA* genes or the RacR independent *phoX* gene. A band shift was observed for the *gltB*, *aspA*, Cj0358 and the *mfrX* promoter region (Fig.4) but not for the other promoter elements. The band shifts were not present or only a part of the ^{32}P-labeled DNA fragments shifted when an excess of unlabeled DNA was added. Less RacR protein was needed to observe a band shift when RacR was first phosphorylated by RacS (data not shown). These results show that the *aspA, mfrX,* Cj0358, and *gltB* promoter elements are regulated directly by RacR while the other promoter elements are probably indirectly dependent on RacR.

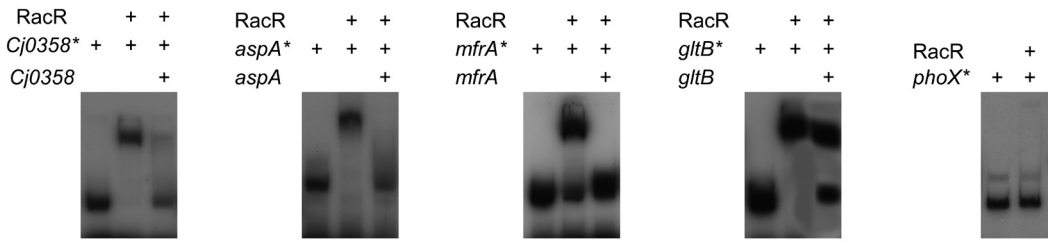


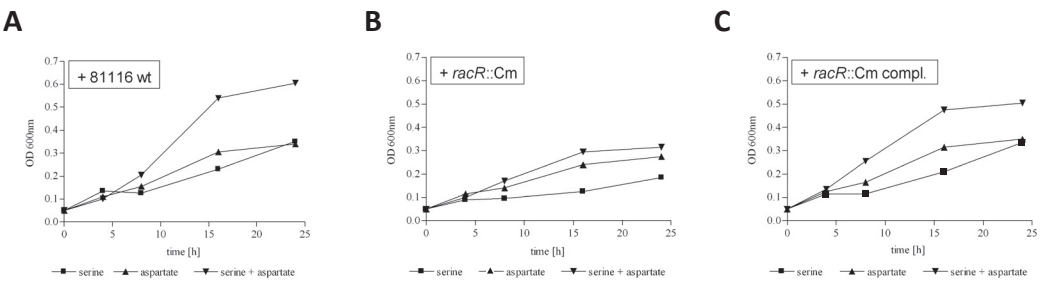
Figure 4. Identification of genes directly regulated by RacR. Electrophoretic mobility shift assays showing that recombinant Histidine-tagged RacR binds to the [γ-^{32}P]ATP labeled promoter regions (~25 pmol) upstream the *gltB*, *aspA*, Cj0358 and *mfrX* genes but not to the negative control, the *phoX* promoter region (gene names with asterisks). The concentration of RacR in each reaction is 50 pmol, indicated at the top of each lane (+)**.** The specificity of the protein-DNA interaction was determined by the addition of a ten-fold excess of unlabeled competition DNA (gene names without asterisks).

RacR controls amino acid utilization by regulation of the aspartase enzyme (AspA)

C. jejuni is unable to ferment exogenous sugars and largely relies on amino acid catabolism, especially the conversion of serine and aspartate into pyruvate and fumarate, respectively (15). The identification of genes regulated directly by RacR indicates that inactivation of *racR* must have an impact on the amino acid utilization and fumarate respiration. To verify whether the RacS-RacR system regulates the utilization of amino acids in an oxygen dependent way, we followed the growth rate of Wt, the *racR*::Cm mutant and the complemented mutant strain in DM (Fig. 5A, B and C) and MEMα medium (data not shown) with serine or aspartate or both as major carbon source(s) under low oxygen concentrations. Although the growth rate of the *racR*::Cm mutant compared to the Wt was reduced in both media, the *racR*::Cm mutant grew much better in media containing aspartate than serine. The growth rate of the Wt but not the *racR*::Cm mutant was almost doubled when both carbon sources were present, again indicating that the mutant strain almost exclusively uses aspartate for growth. These results are in line with the elevated transcripts of the aspartate-fumarate metabolic pathway in the *racR*::Cm mutant.

To verify that the amino acid catabolism changes after inactivation of *racR*, we grew the Wt and *racR*::Cm mutant in DM with serine or aspartate as major carbon source and analyzed at different growth phases the composition of the culture supernatants by proton Nuclear Magnetic Resonance (^{1}H-NMR) (Fig. 5D). Aspartate was utilized faster by the *racR*::Cm mutant than by the Wt. The mutant secreted also more succinate into the medium indicating an enhanced aspartate-fumarate utilization in the mutant. The production of acetate (from serine) was decreased in the *racR*::Cm mutant strain consistent with the proposed inhibition of aspartate-fumarate metabolism by an active RacS-RacR system. To our surprise, when aspartate was used as the major carbon source, we measured relative high acetate concentrations in the supernatant of stationary phase grown cultures of the *racR*::Cm mutant. This may indicate that the anaplerotic route, a mechanism used during scarcity of TCA cycle intermediates (55), is more active in the *racR*::Cm mutant under these conditions. Furthermore we measured ethanol in the stationary phase (16 h) for the *racR*::Cm mutant strain, independent of the carbon source. The source of the produced ethanol is unknown.

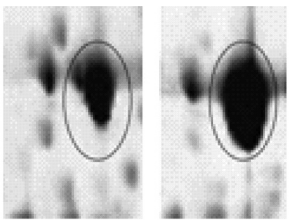
The increased *aspA* transcription and enhanced utilization of aspartate in the *racR*::Cm mutant suggest that in the mutant AspA expression is increased. Two-dimensional gel electrophoresis of whole bacterial lysates of Wt and *racR*::Cm bacteria grown under oxygen limited conditions clearly showed increased amounts of AspA in the mutant strain (Fig. 5E). Measuring the AspA enzyme activity in exponentially growing bacteria in media with serine or aspartate as major C-source revealed that under both conditions the *racR*::Cm mutant displayed a significant higher aspartase activity than the Wt (Fig. 5F) ($P<0.05$). The aspartase activity was restored to almost Wt levels after introduction of the complementation plasmid pMA1-1261-1263 into the *racR*::Cm mutant, while complete loss of aspartase activity was seen in the *aspA*::Km mutant strain. Thus AspA activity is not only dependent on the RacR system but is also dependent on the growth phase. The highest aspartase activity in Wt bacteria was observed in the stationary phase (16 h) (Fig. 5G). During the stationary phase with aspartate as major carbon source the difference in aspartase activity observed between Wt and the *racR*::Cm mutant strain was minimized, indicating that in the stationary phase when aspartate is the major carbon source, the aspartase enzyme is highly active. Together these results indicate that the RacS-RacR two-component system regulates cellular amino acid metabolism mainly in the logarithmic growth phase by repressing the less favourable aspartate-fumarate metabolizing pathway of the TCA cycle.



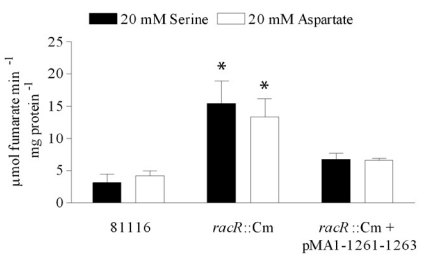
D

		mM Serine				mM Aspartate			
		Serine	**Acetate**	**Succinate**	**Ethanol**	**Aspartate**	**Succinate**	**Acetate**	**Ethanol**
0 hr	81116	17,5	0,9	ND	1,6	11,8	ND	0,8	1,6
	racR::Cm	17,5	0,9	ND	1,6	11,8	ND	0,8	1,6
4 hr	81116	18,6	1,9	1,4	1,6	13,8	1,7	1,5	1,5
	racR::Cm	18,7	1,2	1,1	1,6	12,6	2,1	1,3	1,5
8 hr	81116	17,0	3,1	1,9	1,5	10,3	5,0	1,6	1,4
	racR::Cm	17,7	1,9	1,5	1,5	9,8	5,4	1,7	1,6
16 hr	81116	13,5	7,2	2,0	1,6	ND	14,1	2,8	1,4
	racR::Cm	17,5	2,7	1,5	3,4	ND	12,1	4,5	3,3
24 hr	81116	8,2	11,6	3,0	1,4	ND	15,0	2,6	1,3
	racR::Cm	14,6	6,1	2,2	1,4	ND	15,8	2,5	1,4

E



F



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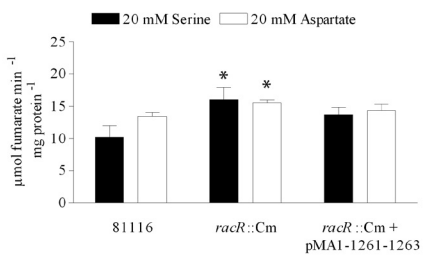

Figure 5. RacR influences the utilization of amino acids by *C. jejuni* (A-C). Growth of *C. jejuni* 81116 Wt (filled squares), *racR*::Cm (filled triangle up) and the complemented *racR*::Cm mutant (filled triangle down) at 42°C under oxygen limited conditions (0.3% O_2, 12% CO_2 and 88% N_2) in DM in the presence of (A) 20 mM serine or (B) 20 mM aspartate or both (C). The results are representative of at least three independent experiments. (D) Metabolomic analysis of culture supernatants of the 81116 Wt and *racR*::Cm mutant strain grown in DM with serine (20 mM) or aspartate (20 mM) at low oxygen after 4, 8, 16 and 24 h of growth. The data are representative of two independent ^{1}H-NMR analysis. (E) 2D-gel electrophoresis of the Wt (left panel) and *racR*::Cm mutant strain (right panel) showing altered expression levels of AspA (encircled) as verfied by mass spectroscopy. Only the area surrounding AspA spot is shown. *C. jejuni* aspartase activity was measured from logarithmic (8 h) (F) or stationary (20 h) (G) phase cultures grown in DM with aspartate (20 mM) or serine (20 mM) at low oxygen. Aspartase activity was followed at 240 nm corresponding to the production of fumarate. Data are represented as mean of at least three independent experiments. *, $p<0.05$.

RacR is involved in the use of electron acceptors

Our results indicate that the RacS-RacR system is also involved in the production of fumarate. Fumarate can be used as a carbon source and as an alternative electron acceptor in oxygen limited environments. In other bacterial species the use of alternative electron acceptors is regulated by different transcriptional factors (10, 50, 51, 52). From this perspective we investigated whether the RacS-RacR system is important to regulate the use of alternative electron acceptors. Growth curves in rich medium under low oxygen concentrations (0.3% O_2) with 50 mM nitrate as alternative electron acceptor showed increased growth of the Wt compared to the *racR*::Cm mutant strain (Fig. 6A). A similar pattern was observed for the less efficient electron acceptor TMAO (data not shown). However when the less preferred compound fumarate (50 mM) was used as alternative electron acceptor in rich medium under low oxygen conditions an opposite effect was observed. Especially in the early logarithmic phase, the mutant strain grew faster than the Wt (Fig. 6B). Differences in growth were nullified after introduction of an intact copy of the *racS* and *racR* genes on the shuttle plasmid pMA1. The reduced growth of the *racR*::Cm mutant strain in the presence of nitrate was not observed under microaerophilic conditions (5% O_2) (data not shown). These results show that the *racR*::Cm mutant has severe difficulties to adapt to the available electron acceptors in response to oxygen concentrations.

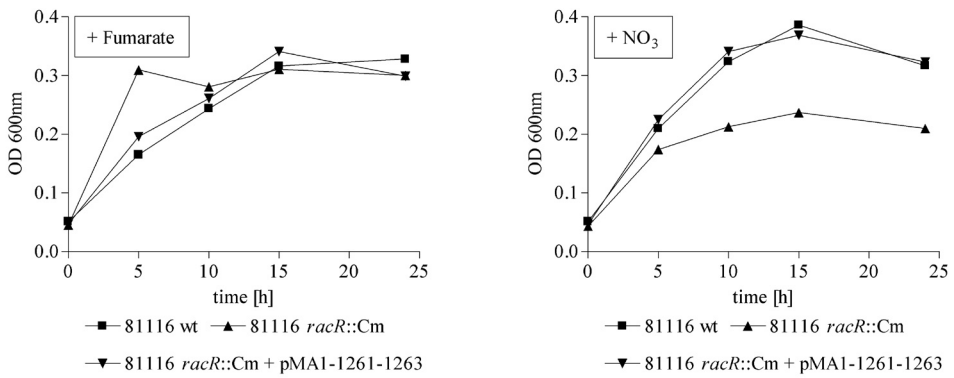

Figure 6. Effect of alternative electron acceptors on the growth of *C. jejuni* Wt, *racR*::Cm and the complemented *racR*::Cm mutant strain. Growth of *C. jejuni* 81116 Wt (filled squares), *racR*::Cm (filled triangle up) and the complemented *racR*::Cm mutant (filled triangle down) in HI medium at 42°C under oxygen limited conditions (0.3% O_2 12% CO_2 and 88% N_2) with (A) 50 mM fumarate or (B) 50 mM $NaNO_3$. The data are representatives of at least three independent experiments.

Activation of the RacS-RacR system

Because both the available electron acceptors and the C-sources influence the growth of the *racR*::Cm mutant, we investigated whether these compounds play a role in the activity of this two-component system. Hereto we measured the bacterial γ-glutamyltranspeptidase (GGT) activity which we recently showed to be directly dependent on the RacS-RacR system (Łaniewski *et al.*, in preparation). The addition of 20 mM serine to defined MEMα-medium significantly decreased the GGT activity to 20% ($P<0.05$), while 20 mM aspartate significantly increased the GGT levels to 140%

compared to plain MEMα-medium (Fig. 7) (P<0.05). A similar significant increase of the GGT activity was observed when 50 mM nitrate was added to the DM (P<0.05). The addition of both aspartate and nitrate significantly enhanced the GGT activity to ~225% (P<0.05), indicating that both the C-source and electron acceptor influence GGT activity. The GGT activity in the *racR*::Cm mutant was similar under these conditions, indicating that the increase in GGT activity in the Wt strain is due to activation of the RacS-RacR system. This means that the activation of the RacS-RacR two-component system depends on the presence of carbon source(s) in combination with alternative electron acceptor(s). Complete loss of enzyme activity was observed for the *ggt*::Cm mutant strain.

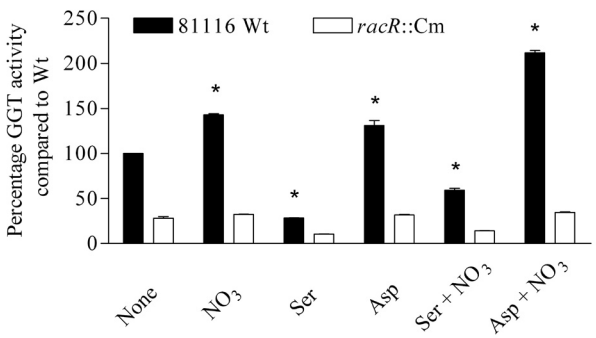

Figure 7. Signals which influence the activity of the RacR-RacS system. GGT activity was used as a measure for RacR-RacS activity. GGT activity was determined for stationary phase bacterial cultures grown in MEMα-medium with or without the addition of 20 mM aspartate, 20 mM serine, 50 mM nitrate, 20 mM aspartate + 50 mM nitrate or 20 mM serine + 50 mM nitrate. GGT activity was followed at 405 nm corresponding the production of 3-carboxy-4-nitroaniline. All data is shown as percentage activity compared to Wt without additions. Data represent the mean of four independent experiments. *, *p<0.05*.

Discussion

In all living organisms energy generating processes are essential to stay alive. The energy metabolism is strictly regulated according to the energy requirements and availability of nutrients. Transcriptional regulation of the energy generating processes in *C. jejuni* has not been studied so far. In this study we show that the RacS-RacR two-component system plays an important role in amino acid utilization and electron acceptor usage. Transcriptome analysis, EMSA and AspA enzyme assays revealed that the RacR protein directly regulates energy generating processes by repressing the aspartate-fumarate route. The available carbon source and electron acceptor determine the activity of the RacS-RacR system. The activating conditions for this system are present in the gut of chickens, explaining the failure of the *racR*::Cm mutant to colonize chickens (4).

A previous study has demonstrated that the RacS-RacR two-component system is necessary to support optimal growth at 42°C and the ability to colonize chickens (4). Although temperature dependent transcription is a well known feature in several other bacterial species (1, 5, 12, 13), we were not able to confirm the temperature-sensitive phenotype for the RacS-RacR system under the conditions employed (Fig. 2A + B). The putative function of the two genes known to be regulated

by RacR; the γ-glutamyltranspeptidase (GGT) (Łaniewski *et al.*, in preparation), an enzyme involved in oxidative stress and amino acid metabolism and Cj0358 an oxygen sensitive cytochrome peroxidase homolog (4), gave us the idea that the RacS-RacR system might be responding to different oxygen concentrations. The *racR*::Cm mutant showed a reduced growth under restricted oxygen conditions (Fig. 2C), which might explain the inability of the *racR*::Cm mutant to colonize the oxygen restricted environment of the chicken gut (4).

Microarray analysis and real-time RT PCR revealed that the *gltB* gene and four putative operons; Cj0175c-Cj0176c, *fldA*-Cj1384c, *katA*-Cj1386 and *chuABCD* are activated while four putative operons; *aspA*-*dcuA*-Cj0089, *mfrXABE*, Cj448c-Cj0449c and Cj1491c-Cj1493c are repressed when RacR is active. Interestingly the operons which are activated by RacR are repressed by the iron-responsive transcription factors Fur (31) or PerR (54). The Fur repressed operons Cj0175-Cj0176, *chu*ABCD and *fldA*-Cj1384c encode respectively for a Fe^{3+}- and hemin ABC transport system and a flavodoxin. The *C. jejuni* flavodoxin is an efficient electron acceptor of OOR, a complex which plays a vital role in central carbon metabolism by decarboxylation of 2-oxoglutarate in the presence of Coenzyme-A (57). PerR represses the *katA*-Cj1386 operon which is coding for a catalase which degrades hydrogen peroxide to prevent the formation of highly reactive oxygen species (ROS). Why the iron regulated genes are activated by the RacSRacR system needs further research. Other genes activated by the RacS-RacR system are the *gltB* and the previously identified *ggt* gene (Łaniewski *et al.*, in preparation). Both genes are involved in the generation of glutamate. Glutamate is by far the most important natural source of nitrogen for bacteria and it can be used to produce aspartate (15, 38). RacR however represses the aspartate-fumarate pathway as the transcription of genes which are involved in uptake (*dcuA*), generation (*aspA*) and conversion (*mfrXABE*) of fumarate, is upregulated in the *racR*::Cm mutant. This indicates that the RacS-RacR system might be important for catabolism or balancing the carbon:nitrogen ratio (22). The repressed genes are known to be regulated in *E. coli* via the transcription factor FNR and/or the two-component systems DcuS/R, NarX/L and/or NarQ/P (10, 29). In *Neisseria gonorrhoeae* the oxygen sensitive transcription factor FNR also regulates the homolog of the RacR dependent cytochrome peroxidase Cj0358 (59). The identification of the RacR dependent genes shows that this system is important to control energy generating processes under restricted oxygen levels. A part of RacR dependent genes may be directly regulated by another two-component system encoded by the Cj1491 and Cj1492 genes as the transcription for these genes is increased in a *racR*::Cm mutant.

The RacR protein contains a clear helix-turn-helix motif, characteristic for DNA binding proteins. We previously showed by EMSA that recombinant RacR binds to the *ggt* promoter region (Łaniewski *et al.*, in preparation). Here we showed that recombinant RacR also binds to the promoter regions located upstream of *gltB*, *aspA*, Cj0358 and *mfrX* genes (Fig. 4). Although some sequence conservation exists between these promoter regions, no clear RacR consensus motif could be identified. This may indicate that RacR binds to a specific DNA structure (39) rather than a conserved nucleotide sequence as exists for the *C. jejuni* two-component systems PhosS/PhosR and DccS/DccR (24, 62). Future research is needed to identify how the DNA (motif) is recognized by RacR.

The major biosynthetic pathway in many microorganisms is the TCA cycle, in which glucose is the most important carbon source. At excess levels of glucose the activity of the TCA cycle is inhib-

ited due to catabolic repression (35). However *C. jejuni* has to rely on amino acids to feed its TCA cycle because of its inability to utilize exogenous sugars (33, 34). *C. jejuni* has the capacity for the complete catabolism of only a limited range of amino acids, mainly serine, aspartate, glutamate and proline. Glutamate and proline are converted to aspartate and finally to fumarate before they are catabolised in the TCA cycle while serine enters the TCA cycle when it is converted to pyruvate (15). The *C. jejuni* genome encodes a complete oxidative TCA cycle, which converts TCA intermediates (carboxylic acids) to CO_2, ATP, and reducing equivalents (8, 16, 19, 33, 36). In *Escherichia coli*, TCA cycle enzyme activities are affected both by the medium composition and by the level of oxygen in the medium. Here we report a clear role for the RacS-RacR system in repression of the aspartate-fumarate route under oxygen restricted conditions. The activity of the RacS-RacR system was strongly inhibited in the presence of serine and active in the presence of aspartate (Fig. 7). The reason why high levels of serine enhanced the activation of the aspartate-fumarate route might be to maintain an optimal balance of the carbon:nitrogen ratio in *C. jejuni* (22).

C. jejuni contains a complex network of alternative electron donors and acceptors, permitting growth and energy conservation in the low oxygen environment of the poultry gut. Due to differences in reduction potential between redox couples there is a preference in the usage of these molecules (20, 46). In *E. coli* the usage of alternative electron acceptors is under strict hierarchal control of different transcriptional regulators (10, 52). As the generation of fumarate, a low redox potential acceptor, is repressed by RacR, an additional function of the *C. jejuni* RacS-RacR system might be to reduce the production of fumarate when molecules with higher redox potential are available. The alternative electron acceptor nitrate, a molecule with a high redox potential, increased the activity of RacS-RacR system as measured by GGT activity. These results indicate that this system next to amino acid catabolism is also important in usage of alternative electron acceptors.

To investigate the effect caused by inactivation of *racR* on *C. jejuni* metabolism, we measured the aspartase activity and the organic products in the medium by NMR. Expression of aspartase (AspA) is oxygen (15) and RacR dependent as shown by 2D-gelelectrophoresis (fig. 5E). Differences in aspartase activity for the Wt strain between logarithmic phase and stationary phase cultures indicate a RacR-dependent switch to aspartate utilization at the end of the logarithmic phase. Interestingly, our results obtained by ^{1}H-NMR indicated the secretion of ethanol and increased secretion of acetate in stationary phase (16 h) cultures for the *racR*::Cm mutant grown with aspartate as sole carbon source (fig. 5D). In *C. jejuni* the most common route of acetate production is during the conversion of acetyl-CoA to citrate when serine is used as carbon source (25). When the most preferred amino acid, serine, is absent or becomes limited *Campylobacter* switches to aspartate catabolism. An alternative mechanism of acetate production, when aspartate is used as carbon source, is via the malic enzyme (MEZ) used in the anaplerotic route (55). We have measured the MEZ activity, an essential enzyme in this pathway and observed a decrease of MEZ activity in the *racR*::Cm mutant instead of an increase (data not shown). This indicates that the enhanced ethanol and acetate secretion is not due to elevated activity of the alternative anaplerotic route, but suggests the existence of an as yet undiscovered metabolic route repressed by the RacS-RacR system, which needs further investigation.

Several bacterial two-component systems are activated by the availability of oxygen and nutrients consisting of the essential element C, N, O or P. The *E. coli* DcuS/R and *Vibrio cholera* DctB/D responds to C4-dicarboxylates such as aspartate, fumarate and succinate, compounds used as carbon source and to activate anaerobic respiratory pathways (6, 11, 37). In addition, during oxygen restriction bacteria rely on the usage of alternative electron acceptors regulated through the ArcA/B system or the transcription factor FNR (21, 27, 44, 51, 52). To control the nitrate, nitrite and nitrogen availability bacteria use the two component system(s) NarX/L, NarQ/P and/or NtrC/B respectively and bacterial phosphate acquisition is regulated through PhoB/R (45, 56, 64), but also cross-regulation between these systems to maintain a correct ratio between these elements is described (10, 28). So far in *C. jejuni* there is only a functional homolog identified for the phosphate sensitive two component system PhoB/R, named PhosS/PhosR (62). The RacS-RacR system is not a homolog of one of the above described systems as this system is involved in amino acid metabolism and electron acceptor usage and is activated by aspartate by the electron acceptor nitrogen but also by the level of oxygen (Fig. 7). It seems that due to the limited number of transcription factors present in *C. jejuni* the remaining factors like the RacS-RacR two-component system obtained a broader function to maintain the regulation of energy metabolic processes in this organism.

In conclusion we show that the RacS-RacR system is the first *C. jejuni* transcription regulatory system identified to play an important role in the energy metabolism. We show that this system controls the switch between metabolic pathways, mainly by repression of the aspartate-fumarate route in response to the available C-source and electron acceptors in order to fine tune the bacterium metabolic behaviour during restricted oxygen conditions, as present in its primary natural niche the poultry gut.

Acknowledgement

This work was supported by NWO-VIDI grant 917.66.330 to M.M.S.M. Wösten.

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Supplementary data

2 fold difference racR::Cm mutant over Wt

Gene Name	Fold Change	Common	Product
Cj0029	5,001	ansA	L-asparaginase (ansA)
Cj0040	2,306	Cj0040	conserved hypothetical protein
Cj0045c	2,186	Cj0045c	Hemerythrin domain protein
Cj0068	4,037	pspA	protease IV, putative
Cj0069	4,769	Cj0069	conserved hypothetical protein
Cj0070c	2,098		
Cj0075c	2,493	Cj0075c	Domain of unknown function (DUF224) family
Cj0076c	2,495	lctP	L-lactate permease
Cj0079c	2,156	cdtA	CdtA protein
Cj0080	2,389	Cj0080	conserved hypothetical protein
Cj0081	2,6	cydA	cytochrome d ubiquinol oxidase, subunit I (cydA)
Cj0082	2,704	cydB	cytochrome d ubiquinol oxidase, subunit II (cydB)
Cj0086c	7,28	ung	uracil-DNA glycosylase (ung)
Cj0087	78,15	aspA	aspartate ammonia-lyase (aspA)
Cj0088	12,49	dcuA	C4-dicarboxylate transporter, anaerobic (dcuA)
Cj0089	4,281	Cj0089	conserved hypothetical protein
Cj0091	3,07	Cj0091	conserved hypothetical protein
Cj0092	3,264	Cj0092	conserved hypothetical protein

Continued

Gene Name	Fold Change	Common	Product
Cj0095	3,547	rpmA	ribosomal protein L27 (rpmA)
Cj0096	2,158	Cj0096	GTP-binding protein Obg (obg)
Cj0100	2,134	Cj0100	ATPase, ParA family (soj)
Cj0101	2,273	Cj0101	chromosome partioning protein, ParB family (spo0J)
Cj0102	2,053	atpF'	ATP synthase B/B' CF(0) domain protein
Cj0103	2,77	atpF	ATP synthase B/B' CF(0) domain protein
Cj0104	2,197	atpH	ATP synthase F1, delta subunit (atpH)
Cj0144	3,395		
Cj0244	4,048	rpmI	
Cj0245	3,534	rplT	
Cj0289c	2,526	peb3	accessory colonization factor AcfC (acfC)
Cj0290c	2,09		
Cj0291c	2,988	glpT	glycerol-3-phosphate transporter (glpT)
Cj0293	2,081	surE	stationary-phase survival protein SurE (surE)
Cj0296c	2,402	panD	aspartate 1-decarboxylase (panD)
Cj0297c	2,625	panC	pantoate--beta-alanine ligase (panC)
Cj0298c	3,146	panB	3-methyl-2-oxobutanoate hydroxyl-methyltransferase (panB)
Cj0313	2,895	Cj0313	membrane protein, putative
Cj0350	2,785	Cj0350	conserved hypothetical protein
Cj0354c	3,97	fdxB	ferredoxin
Cj0357c	2,849	Cj0357c	conserved hypothetical protein TIGR00023
Cj0358	8,192	Cj0358	cytochrome c551 peroxidase (yhjA)
Cj0369c	2,714	Cj0369c	ferredoxin, putative
Cj0391c	2,008	Cj0391c	conserved hypothetical protein
Cj0408	3,434	frdC	Fumarate reductase respiratory complex, transmembrane subunit
Cj0410	2,862	frdB	fumarate reductase, iron-sulfur protein, putative
Cj0437	4,014	sdhA	succinate dehydrogenase, flavoprotein subunit (sdhA)
Cj0438	4,24	sdhB	fumarate reductase, iron-sulfur protein, putative
Cj0439	2,507	sdhC	Domain of unknown function (DUF224) family
Cj0441	2,16	acpP	acyl carrier protein (acpP)
Cj0442	2,004	fabF	Beta-ketoacyl synthase domain protein
Cj0448c	5,119	Cj0448c	accessory colonization factor AcfB, putative
Cj0449c	6,947	Cj0449c	conserved hypothetical protein
Cj0450c	2,614	rpmB	ribosomal protein L28 (rpmB)
Cj0451	2,348	rpe	ribulose-phosphate 3-epimerase (rpe)
Cj0458c	2,001	miaB	
Cj0459c	3,458	Cj0459c	conserved hypothetical protein
Cj0470	2,366	tuf	translation elongation factor Tu (tuf)
Cj0471	2,364	rpmG	ribosomal protein L33 (rpmG)
Cj0473	2,528	nusG	transcription antitermination protein NusG (nusG)
Cj0493	2,898	fusA	translation elongation factor G (fusA)
Cj0604	2,006	Cj0604	conserved hypothetical protein
Cj0643	2,555	Cj0643	GGDEF domain protein
Cj0671	3,162	dcuB	C4-dicarboxylate transporter, anaerobic (dcuB)
Cj0697	2,008	flgG2	Flagella basal body rod protein domain protein
Cj0853c	2,13	hemL	glutamate-1-semialdehyde-2,1-aminomutase (hemL)
Cj0864	2,213	dsbA	
Cj0884	3,387	rpsO	ribosomal protein S15 (rpsO)
Cj0887c	3,89	flaD	Bacterial flagellin C-terminus domain protein
Cj0893c	3,456	rpsA	ribosomal protein S1 (rpsA)

Continued

Gene Name	Fold Change	Common	Product
Cj0906c	2,448	Cj0906c	conserved hypothetical protein
Cj0909	3,696	Cj0909	conserved hypothetical protein
Cj0921c	2,56	peb1b	major cell-binding factor precursor
Cj0922c	3,418	peb1c	probable abc transporter atp-binding protein peb1c. {campylobacter}
Cj0936	3,616	atpE	ATP synthase F0, C subunit, putative
Cj0951c	2,29	Cj0951c	methyl-accepting chemotaxis protein, putative
Cj0952c	3,585		
Cj0961c	4,296	rpmH	ribosomal protein L34 (rpmH)
Cj0965c	2,488	Cj0965c	conserved hypothetical protein TIGR00051
Cj0977	2,852	Cj0977	conserved hypothetical protein
Cj1026c	3,66	Cj1026c	conserved hypothetical protein
Cj1034c	2,348	Cj1034c	conserved hypothetical protein
Cj1070	2,194	rpsF	ribosomal protein S6, putative
Cj1071	3,121	ssb	single-strand binding protein (ssb)
Cj1096c	2,275	metK	S-adenosylmethionine synthetase (metK)
Cj1170c	2,156	Cj1170c	conserved hypothetical protein
Cj1189c	2,483	Cj1189c	methyl-accepting chemotaxis protein
Cj1190c	2,949	Cj1190c	accessory colonization factor AcfB (acfB)
Cj1191c	2,578	Cj1191c	methyl-accepting chemotaxis protein
Cj1242	5,59	Cj1242	conserved hypothetical protein
Cj1261	2,055	racR	response regulator (regX3)
Cj1359	3,177	ppk	polyphosphate kinase (ppk)
Cj1364c	3,05	fumC	fumarate hydratase, class II (fumC)
Cj1489c	2,171	ccoO	cytochrome c oxidase, cbb3-type, subunit II (ccoO)
Cj1490c	2,712	ccoN	cytochrome c oxidase, cbb3-type, subunit I (ccoN)
Cj1491c	4,167	Cj1491c	DNA-binding response regulator, putative
Cj1492c	4,089	Cj1492c	Histidine kinase-, DNA gyrase B- phytochrome-like ATPase domain
Cj1493c	8,08	Cj1493c	membrane protein, putative
Cj1540	2,185	Cj1540	conserved hypothetical protein
Cj1586	2,381	Cj1586	ferrisiderophore reductase (hmpA)
Cj1592	2,795	rpsM	ribosomal protein S13/S18 (rpsM)
Cj1631c	2,15	Cj1631c	conserved hypothetical protein
Cj1632c	2,331		
Cj1656c	2,98	Cj1656c	
Cj1658	2,459	Cj1658	hypothetical protein
Cj1701c	2,011	rpsC	ribosomal protein S3 (rpsC)
Cj1702c	2,252	rplV	ribosomal protein L22 (rplV)
Cj1703c	2,511	rpsS	ribosomal protein S19 (rpsS)
Cj1704c	2,516	rplB	ribosomal protein L2 (rplB)
Cj1705c	2,783	rplW	ribosomal protein L23 (rplW)
Cj1706c	3,293	rplD	ribosomal protein L4/L1 family (rplD)
Cj1707c	3,583	rplC	hypothetical protein
Cj1708c	3,26	rpsJ	ribosomal protein S10 (rpsJ)
HS41ORF26	2,569		
ORF00311	2,168	ORF00311	methyl-accepting chemotaxis protein, putative
ORF01561	2,021	ORF01561	vacuolating cytotoxin precursor, putative

2 fold difference Wt over racR::Cm mutant

Gene Name	Fold Change	Common	Product
Cj0007	4,282	gltB	glutamate synthase, large subunit (gltB-1)
Cj0009	3,466	rnhB	ribonuclease HII (rnhB)
Cj0034c	2,06	Cj0034c	conserved hypothetical protein
Cj0036	2,065	Cj0036	conserved hypothetical protein
Cj0037c	3,352	Cj0037c	Cytochrome c subfamily, putative
Cj0146c	2,108	trxB	thioredoxin reductase (trxB)
Cj0168c	2,381	Cj0168c	conserved hypothetical protein
Cj0173c	2,107	Cj0173c	spermidine/putrescine ABC transporter, ATP binding subunit
Cj0174c	2,611	Cj0174c	iron(III) ABC transporter, permease protein, putative
Cj0175c	5,154	Cj0175c	iron(III) ABC transporter, periplasmic iron-compound binding protein
Cj0176c	4,735	Cj0176c	conserved hypothetical protein
Cj0182	2,406	Cj0182	membrane protein, putative
Cj0185c	2,044	Cj0185c	alkylphosphonate utilization operon protein PhnA (phnA)
Cj0316	2,16	pheA	chorismate mutase/prephenate dehydratase, putative
Cj0334	3,691	ahpC	antioxidant, AhpC/Tsa family
Cj0389	2,073	serS	seryl-tRNA synthetase (serS)
Cj0403	2,016	Cj0403	conserved hypothetical protein
Cj0483	2,257	uxaA'	conserved hypothetical protein
Cj0531	2,465	icd	isocitrate dehydrogenase, NADP-dependent
Cj0532	2,683	mdh	L-lactate dehydrogenase, putative
Cj0533	2,775	sucC	succinyl-CoA synthase, beta subunit (sucC)
Cj0534	2,07	sucD	succinyl-CoA synthase, alpha subunit (sucD)
Cj0535	2,088	oorD	ferredoxin, 4Fe-4S-related protein
Cj0559	2,052	Cj0559	pyridine nucleotide-disulfide oxidoreductase, putative
Cj0569	2,12		
Cj0596	2,308	peb4	cell binding factor 2 precursor
Cj0613	3,239	pstS	phosphate ABC transporter, periplasmic phosphate-binding protein
Cj0615	2,011	pstA	phosphate ABC transporter, permease protein (pstA)
Cj0623	2,049	hypB	hydrogenase accessory protein HypB (hypB)
Cj0624	2,355	hypC	hydrogenase assembly chaperone hypC/hupF (hypC)
Cj0625	2,643	hypD	hydrogenase expression/formation protein HypD (hypD)
Cj0626	2,354	hypE	AIR synthase related protein, C-terminal domain protein
Cj0699c	2,601	glnA	glutamine synthetase, type I (glnA)
Cj0702	2,242	purE	phosphoribosylaminoimidazole carboxylase, catalytic subunit (purE)
Cj0715	2,477	Cj0715	Transthyretin precursor (formerly prealbumin) domain protein
Cj0716	2,854	Cj0716	phospho-2-dehydro-3-deoxyheptonate aldolase
Cj0717	2,511	Cj0717	conserved hypothetical protein
Cj0719c	2,063	Cj0719c	conserved hypothetical protein TIGR00044
Cj0721c	2,583	Cj0721c	conserved hypothetical protein
Cj0839c	2,266	Cj0839c	conserved hypothetical protein
Cj0840c	2,581	fbp	fructose-1,6-bisphosphatase (fbp)
Cj0984	2,24	Cj0984	conserved hypothetical protein
Cj0985c	4,411	hipO	hippurate hydrolase
Cj0986c	3,388		
Cj0987c	3,142		
Cj1023c	2,142	asd	aspartate-semialdehyde dehydrogenase (asd)
Cj1044c	2,025	thiH	thiH protein (thiH)
Cj1161c	2,032	Cj1161c	cation-transporting ATPase, E1-E2 family, putative

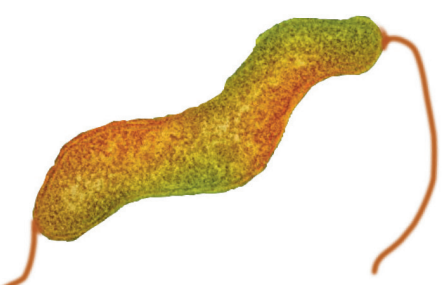
Continued

Gene Name	Fold Change	Common	Product
Cj1162c	2,687	Cj1162c	
Cj1265c	2,158	hydC	Nickel-dependent hydrogenases b-type cytochrome subunit superfamily
Cj1266c	2,09	hydB	hydrogenase-2 large chain precursor
Cj1286c	2,116	upp	uracil phosphoribosyltransferase (upp)
Cj1360c	2,472	Cj1360c	
Cj1381	2,61	Cj1381	conserved hypothetical protein
Cj1382c	5,249	fldA	flavodoxin
Cj1383c	5,065	Cj1383c	conserved hypothetical protein
Cj1384c	5,714	Cj1384c	conserved hypothetical protein
Cj1385	6,23	katA	catalase
Cj1386	5,147	Cj1386	Ank repeat domain protein
Cj1466	2,019	flgK	flagellar hook-associated protein FlgM, putative
Cj1567c	3,453	nuoM	NADH dehydrogenase I, M subunit (nuoM)
Cj1568c	3,367	nuoL	NADH dehydrogenase I, L subunit (nuoL)
Cj1569c	2,88	nuoK	NADH dehydrogenase I, K subunit (nuoK)
Cj1570c	3,681	nuoJ	NADH dehydrogenase I, J subunit, putative
Cj1571c	3,125	nuoI	NADH dehydrogenase I, I subunit (nuoI)
Cj1573c	3,403	nuoG	formate dehydrogenase, alpha subunit, putative
Cj1613c	3,785	Cj1613c	conserved hypothetical protein
Cj1615	4,896	chuB	hemin ABC transporter, permease protein, putative
Cj1616	3,391	chuC	iron compound ABC transporter, ATP-binding protein, putative
Cj1617	4,358	chuD	iron compound ABC transporter, substrate-binding protein, putative
Cj1643	2,083	Cj1643	PDZ domain (Also known as DHR or GLGF). protein
Cj1645	2,626	tkt	transketolase (tkt)

Chapter 6

Summary and General Discussion

Andries van Mourik



1. Aim of the study

Bacterial pathogens often contain multiple virulence factors that enable infection of distinct anatomical niches in different host species. The expression of virulence factors is usually tightly regulated probably to prevent their unnecessary production and to escape neutralization by the host immune system. Classical virulence factors as present in the common enteropathogens such as *Salmonella* and *Shigella* spp. are bacterial adhesins, invasins, type III secretion systems, and secreted toxins (10, 15, 18, 22, 49). *Campylobacter jejuni* is the most common cause of bacterial foodborne illness causing >100 million human cases each year (3, 19). In contrast to other enteropathogens, *C. jejuni* appears to lack the set of classical virulence traits but is equipped with a large repertoire of adaptation skills that influence bacterial surface properties, metabolism and behavior. This environmental adaptation may contribute to bacterial virulence by enabling the bacteria to rapidly alter their phenotype and metabolism in response to changes in host environment. The well developed adaptation of *C. jejuni* is illustrated by the presence of a highly branched electron transport system that is important for the ability of *C. jejuni* to colonize chickens (25, 50, 52). Transcriptome analysis further confirmed large differences in *C. jejuni* gene transcription under *in vitro* and *in vivo* growth conditions (46, 53). Despite their importance the knowledge regarding the adaptation and signal transduction system(s) that direct the environmental adaptation in *C. jejuni* is limited. In this thesis three novel *C. jejuni* adaptation mechanisms are described and we investigated their function in the regulation of bacterial behavior.

In Chapter 2 the discovery and characterization of a functional *C. jejuni* Tat secretion machinery is described. This system is necessary for the transport of different proteins essential for adaptation to nutrient and oxygen limited conditions. In Chapter 3 the existence in *C. jejuni* of two novel complementary lipid A modifying enzymes and their function as an immune modulatory system are described. Analysis of the function of two-component systems as regulators of bacterial metabolism revealed that the Dsb system of *C. jejuni* is important for the function of the RacS sensor protein (Chapter 4) and that the two-component system RacS-RacR regulates metabolic processes during conditions of oxygen limitation (Chapter 5). In this chapter (Chapter 6) the most important findings are summarized and discussed.

2. Functional identification of the Twin Arginine Translocation (Tat) machinery

Bacteria have several types of secretion machineries that allow macromolecules to pass the bacterial membrane(s) and to enable their function outside the cytoplasm. The twin arginine translocation (Tat) system is, next to the general secretory pathway (Sec), essential to translocate proteins into the periplasmic compartment. The Tat system transports fully folded proteins across the inner membrane (5, 6). Identification of a functional Tat export machinery in *C. jejuni* was considered important as the majority of substrates transported by this secretion apparatus are part of alternative respiratory pathways necessary for bacterial adaptation and survival in its most favourable environment, the poultry gut. Although the number of Tat substrates is relatively low compared to

the substrates secreted by the Sec system, for several pathogens the Tat system is important for bacterial virulence (12). Analysis of the *C. jejuni* genome indicated the presence of genes that may encode all putative components for a Tat export machinery (17, 21, 26, 38, 40, 41).

As described in **Chapter 2**, inactivation of the *C. jejuni tatC* gene which encodes an essential component of the Tat complex, revealed that the enzymes alkaline phosphatase (PhoX) and the nitrate reductase (NapA) are transported over the inner membrane by this system. In addition, based on the presence of the characteristic conserved Tat signal motif RRxFLK, we proposed that also several redox cofactor-containing proteins essential for respiration under reduced oxygen tension are likely translocated by the Tat-system (**Chapter 2**, Table 3). Based on the function of these substrates, we assume that the Tat system of *C. jejuni* is important to allow the bacteria to survive and colonize its host and hence may influence bacterial virulence. A recent study by Rajashekara *et al*. (43) showed that a *C. jejuni tatC* mutant exhibited defects in different virulence-associated factors like biofilm formation, motility, flagellation, resistance to antimicrobial compounds, survival of osmotic, oxidative and nutrient stress, and colonization of chicken (43). Individual mutations of Tat substrates like Nap and methylmenaquinol:fumarate reductase (Mfr) and recently Cj0379 also caused a reduced ability to colonize chickens (25, 51, 52). Together these results show that the *C. jejuni* Tat secretion system is crucial for colonization of the chicken gut. Why *C. jejuni* uses the Tat system to especially transport this category of proteins is not clear, although a reasonable hypothesis might be that the (folded) proteins can directly fulfill their function in times that rapid adjustments to changing environmental conditions are necessary.

3. Modification of Lipid A and host immune evasion

The innate immune system is the first line of host defense, displaying pro-inflammatory and antimicrobial activities, but it is also essential in steering the adaptive immune response. It was a bit surprising that the genome sequence of *C. jejuni* presented in 2000 (38) did not reveal the existence of typical virulence factors as are present in many other diarrhea causing enteropathogens. Because *C. jejuni* is able to activate the immune system and causes clinical symptoms, this organism is regarded as a pathogen and therefore must have alternative atypical virulence factors, some of which may modulate the innate host defense (8, 24, 28).

To activate the innate immune system, the host immune receptor repertoire should recognize and respond to the presence of xenobiotic molecules. Toll like receptors (TLRs), a well known group of innate immune receptors, activate the innate immune system by recognizing different conserved bacterial components (2). Lipopolysaccharide (LPS), also named endotoxin, is a conserved membrane anchored glycolipid of Gram-negative bacteria that is able to interact with the TLR4-MD2 complex (29). *C. jejuni* LPS is often referred to as LOS due to its short terminal oligosaccharide chain. The *C. jejuni* lipid A, the bioactive component of the LOS molecule, is composed of a disaccharide backbone carrying ester (*O*)- and amide (*N*)-linked fatty acyl chains and phosphate groups (35). Structural variations of LOS can occur both in the oligosaccharide core and in the lipid A moiety, the part that interacts with the TLR4-MD2 complex (37). In recent years several bacterial lipid

A modifying enzymes have been described that are often activated in response to changes in environmental conditions (42). Variation in the ratio of fatty acyl chain type linkages have also been described (35, 45, 47), but functional assays to assess the result of this variation on bacterial survival are lacking so far.

In this study (**Chapter 3**) we provide evidence that the ratio between *O*- and *N*-linked fatty acyl chains in the lipid A influences the immune activating properties of *C. jejuni*. First we showed by mutagenesis that two genes encoding the enzymes GnnA and GnnB are able to change the ratio of *O*- and *N*-linked fatty acyl chains in the lipid A. These genes are located in one operon together with the upstream located *hemH*, responsible for the final step in heme biosynthesis. However heme nor iron concentrations influenced the transcription of the *gnnA-gnnB-hemH* operon. Functional assays showed a significant increase in human TLR4 (hTLR4) activation for LOS purified from the mutant strains compared to the response towards LOS from the parent strain. The enhanced hTLR4 activation was not observed when LOS was used isolated from the mutants complemented with a plasmid expressing both the *gnnA* and *gnnB* genes, attributing the altered immune response to the bacterial gene defects. Interestingly, the LOS of the mutants and the parent strain induced similar activation of the chicken TLR4-MD2 complex, indicating that the difference in LOS response for the mutants and parent strain is species specific. This result may indicate a fundamental difference in recognition of LOS variants in human and chicken. However, experiments with chTLR4 expressed in a homologous background are necessary to confirm this result. Whether the change in the ratio between *O*- and *N*-linked fatty acyl chains in the lipid A may directly affects host cell function or results from secondary effects like changes in number of phosphate groups in the lipid A could not be determined due to heterogeneity of the molecule.

Intestinal epithelial cells secrete several antimicrobial peptides as a line of defense against bacterial pathogens (16). These peptides often interact with the negatively charged bacterial membrane resulting in destruction or opsonization of the microbes (14). Antimicrobial peptide assays revealed that both *C. jejuni* lipid A mutants were more susceptible to different antimicrobial peptides than the parent strain (**Chapter 3**). The reduced bacterial survival was restored by introduction into the mutants of a plasmid expressing both *gnnA* and *gnnB* genes. We hypothesize that the altered resistance in the mutants may be caused by increased membrane stability and permeability caused by the altered linkage of the acyl chains of the lipid A. However, we were unable to determine whether the charge of the bacterial membrane changed due to indirect effects of the *gnnA* or *gnnB* mutation. The existence of a heterogeneous population of lipid A molecules as demonstrated by mass spectrometry suggests that the lipid A variation is somehow regulated by environmental factors. Clearly, further research is needed to elucidate the regulatory mechanisms. Altogether the results described in Chapter 3 indicate that *C. jejuni* is able to change the ratio of its type of fatty acyl chain linkages in its lipid A and that this influences the activation of and resistance against the innate host defense.

4. The role of the RacS-RacR two-component system during metabolic adaptation

C. jejuni is present in different environments including water, amoebas, birds and mammals that all may show differences in e.g. temperature, humidity, nutrients and/or oxygen. *C. jejuni* thus requires metabolic adaptation machineries that rapidly respond to environmental changes. Two-component regulatory systems are bacterial signal transduction systems that allow bacteria to quickly adapt their gene expression profile to the environment. In **Chapters 4** and **5**, the role of the *C. jejuni* two-component system RacS-RacR is described. The results for the first time show that the sensor protein RacS is active at low oxygen concentrations and that its function requires the presence of disulfide bonds (dsb) and a functional Dsb system. Additionally, we identified a number of genes that are regulated by the cognate effector protein RacR and showed the function of RacS-RacR as transcription regulatory apparatus in *C. jejuni.*

The role of the Dsb system on RacS-RacR activity

The RacS-RacR two-component system is present in all sequenced *Campylobacter* strains (17, 21, 26, 38, 40, 41). RacS consists of a periplasmic sensor domain and a cytosolic histidine kinase domain responsible for autophosphorylation of the sensor in the presence of the appropriate stimulus. The periplasmic region of the RacS sensor contains two cysteine residues which are conserved among all RacS proteins. Cysteine residues are known to be essential for disulfide bridge formation in the oxidizing environment of the periplasm (31). Proteome analysis of a *C. jejuni dsbA* mutant strain, which is unable to make disulfide bonds, showed that the expression of the enzyme γ-glutamyltranspeptidase (GGT) was strongly reduced. This observation was unexpected as the amino acid sequence of GGT does not contain cysteine residues. Because GGT performs its function in the periplasmic compartment, we hypothesized that the lack of expression of GGT was indirect and caused by a non-functional Dsb-sensitive two-component regulatory system that regulates *ggt* transcription. In **Chapter 4**, we describe that the histidine kinase RacS is the only sensor protein of *C. jejuni* that contains periplasmic localized cysteine residues. The proteins can form dimers and its periplasmic domain indeed shows disulfide bridge formation. Autophosphorylation experiments with full length RacS in the absence and presence of the reducing compound beta-mercaptoethanol revealed that the natural dimeric form of RacS does not autophosphorylate without the RacS specific activating signal. This in contrast to the monomeric form of RacS that showed autophosphorylation. These results suggest that the disulfide bridges in the periplasmic part of RacS are essential to stabilize its conformation and thereby keep the protein in an inactive state awaiting stimulation by a specific environmental cue. In the *dsbA* mutant strain, the RacS is constitutively active and shows autophosphorylation, resulting in increased *ggt* transcription which is otherwise only seen in the presence of an activating signal. Regulation of *C. jejuni* RacS and GGT are important *in vivo* as both a *racR* mutant and *ggt* mutant show a reduced ability to colonize chicken (4, 7). To our knowledge the results described for RacS in Chapter 4 are the first example of a bacterial histidine kinase protein that is dependent on a functional Dsb system.

The regulatory function of the RacS-RacR two-component system

Despite the observations that the expression of at least 11 proteins is regulated by the RacS-RacR two-component system and that the system is important for colonization of chickens (7), the regulatory function of this system was unknown. Previous studies demonstrated that transcription of *C. jejuni racR* is enhanced during co-cultivation with chicken embryonic intestinal cells and that transcription of different RacR repressed genes is decreased in a rabbit ileal loop model (11, 30, 33, 46, 48, **Chapter 5**). We discovered, as described in **Chapter 4** that the RacR protein regulates the transcription and activity of the virulence related gene *ggt* (4). However, the major question remained as to which other genes are regulated by the RacS-RacR two-component system?

We were successful in elucidating (part of) the regulon and function of the RacS-RacR two-component system by detailed genetic and phenotype analysis of *racR* knock-out strain (**Chapter 5)**. Phosphorylation assays with recombinant proteins showed that RacS and RacR form a true two-component system. Kinetics studies revealed that the two-component system is essential for growth during oxygen limitation. By combining complementary transcriptomics, proteomics and metabolomics we demonstrated that the RacS-RacR system is able to fine-tune the metabolic behavior of *C. jejuni* by repressing the aspartate-fumarate pathway. One of RacR dependent enzymes is aspartase (AspA). This enzyme is responsible for converting aspartate into fumarate and was recently identified as a factor involved in the adhesion of *C. jejuni* to epithelial cells (36). The fumarate that can be produced once the RacS-RacR system is activated can be used as a carbon source to feed the TCA cycle or used as an alternative electron acceptor in oxygen limited environments. The TCA cycle is essential for cellular biosynthesis but is also important to produce energy (ATP) via connection with the electron transport chain. Recent studies with *Salmonella enteritica* and *Yersinia pseudotuberculosis* identified the transcriptional regulation of metabolic genes including glycolytic genes and respiratory genes as important for bacterial virulence (9, 44). As *C. jejuni* lacks a glycolytic pathway it must rely on amino acids as carbon source to feed the TCA cycle. They enter the TCA cycle via the serine-pyruvate route or the aspartate-fumarate route (20). Only a few amino acids namely serine, aspartate, glutamate and proline were the major source for *C. jejuni* to feed the TCA cycle. The utilization of the amino acids is in a hierarchal order as measured by NMR using a chemical defined medium (32). The present results indicate that the RacS-RacR system controls the activity of the different metabolic pathways. Future studies should reveal whether this switch in metabolic activity occurs *in vivo* and contributes to bacterial virulence.

So far we have only investigated a subset of genes regulated via the RacS-RacR two-component system. In parallel to the genes involved in metabolism we also discovered a strong transcriptional repression in the *racR*::Cm mutant strain for genes involved in Fe^{3+} transport and storage, and in oxidative stress (Cj0175-176, *chu*ABCD, *kat*A and *fld*A respectively). In other bacterial species these genes are regulated by the iron sensitive transcription factor Fur (23). One explanation for controlled transport and storage of iron might be to combat oxidative and nitrosative stress (23, 34). Second, several metabolic related genes, like Mfr and oxidoreductases, are only active when they contain Fe-S clusters and therefore RacR might activate iron uptake systems. Interestingly, many genes involved in iron storage and transport are also upregulated in an environment mimicking the blood stage of the infectious process of *Y. pseudotuberculosis* (44). The regulation (direct or indi-

rect) and importance for adaptation and virulence of the entire set of genes that is regulated by the RacS-RacR system is subject of ongoing research.

Viable *C. jejuni* barely activate the innate immune system via TLRs, although strong activation is observed when the bacteria disintegrate (13). This observation implies that bacterial integrity plays an important role to prevent stimulation of the innate immune response. The integrity of bacteria depends e.g. on nutrient availability (27). Amino acids are the major nutrient/carbon source for *C. jejuni*. In the chicken gut serine concentrations are much higher than in human gut (1, 39). It is tempting to speculate that this difference in nutrient availability results in various levels of bacterial disintegration and thus variable activation of the innate immune response in humans and chicken. In this way the fine-tuning of amino acid utilization may contribute to silence the hosts' immune response against *C. jejuni* in specific niches.

5. Concluding remarks and future perspectives

The food chain is the major transmission route for *Campylobacter* infection in humans. So far there is no clue as to why this bacterium behaves like a pathogen in humans and is a commensal in poultry. Known variables between both hosts are differences in body temperature, nutrient availability and composition of the gut flora, but whether these factors contribute to the difference in pathogenicity remains unknown. Understanding the bacterial adaptation strategies can lead to a better control of the pathogen. The results described in this thesis contribute to this understanding by providing novel molecular and cellular insights in the *C. jejuni* repertoire of environmental adaptation. The functional characterization of the Tat export machinery led to the identification of secreted proteins that are needed for adaptation to changes in nutrient or electron acceptor availability. The identified lipid A modifying enzymes may enable evasion of the host immune defense. Finally, our research on the RacS-RacR two-component system shows that this transcriptional regulatory system fine-tunes the metabolic pathways of *C. jejuni* so that it can survive and quickly respond to changes in nutrient and oxygen availability as likely occur in the gastrointestinal tract of humans and chicken. The knowledge on the bacterial adaptation and survival mechanisms may provide an important basis for novel strategies as to how to reduce the presence of this pathogen in the human food chain. Future research can focus on the development of such intervention methods.

Another major future challenge is to further dissect the relationship between metabolic adaptation and the virulence of *C. jejuni*. The RacS-RacR two-component system and the *in vitro* and *in vivo* behavior of mutants defective in various metabolic pathways may proof important at this point. First the complete RacR regulon needs to be addressed. Using footprinting assays the DNA binding sequence of the RacR protein can be identified which may help to elucidate the entire RacR regulon. The next challenge will be to discover the specific signal that activates the RacS sensor protein. For this purpose, the RacR dependent *ggt* and *aspA* genes may be suitable as reporter genes to address the activity of the RacR-RacS system. Direct binding of the specific signal to the RacS sensor protein might be shown by using radioactive labeled compounds. In addition crystal-

lography may applied to map the signal binding site and to identify by mutagenesis the crucial amino acids e.g. the cysteine residues which directly binds the specific activation molecule. At the level of *C. jejuni* metabolic activity, determination of the utilization and production of metabolic compounds at the scale of intact bacteria using NMR and mass spectrometry may be instrumental in deciphering the dynamics of *C. jejuni* metabolism. The ultimate goal should be to reduce the colonization and survival of the bacterium *in vivo*. This may be achieved by combining gained knowledge about the critical metabolic pathways and the *in vivo* growth environment in humans and chicken, the identification of protection-inducing antigens as a basis for a vaccine, and, perhaps most important, finding the mechanisms by which the *C. jejuni* causes diarrheal disease in humans.

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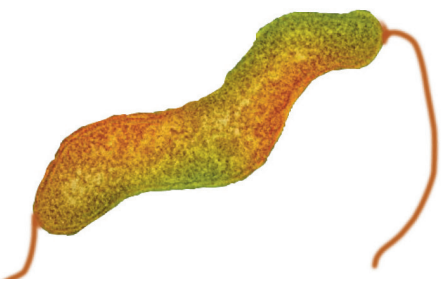
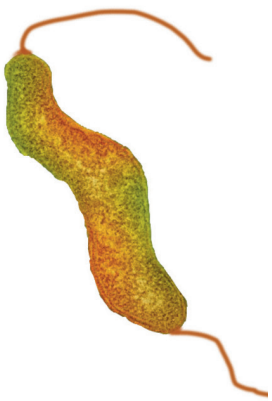
Nederlandse samenvatting





Introductie

De eerste beschrijving van *Campylobacter*, als een "vibrioachtig" organisme, dateert al vanaf het eind van de 19e eeuw. Echter door de specifieke vereisten voor isolatie van dit organisme heeft het tot het einde van de 20e eeuw geduurd voordat de bacterie frequent uit de ontlasting kon worden geïsoleerd als veroorzaker van infecties van de darm. Het complete genus *Campylobacter* omvat 18 soorten die allemaal zoönotisch zijn, dit betekent dat ze overdraagbaar zijn van dier op mens.

Deze Gram-negatieve spiraalvormige *Campylobacter* komt voor in oppervlaktewateren en diverse warmbloedige dieren zoals pluimvee, runderen, varkens, honden en katten. Hoewel de bacterie bij deze organismen in grote aantallen voorkomt in de slijmlaag van de darm, veroorzaakt dit vrijwel geen problemen. Echter is het bij de mens de belangrijkste veroorzaker van bacteriële darminfecties. Waarom de bacterie *Campylobacter*, in het bijzonder *Campylobacter jejuni*, de belangrijkste oorzaak is van bacteriële maag-darminfecties bij de mens, maar niet bij dieren is tot op heden onduidelijk.

Er zijn maar weinig bacteriën nodig om een *Campylobacter* infectie (campylobacteriosis) te veroorzaken. Klinische symptomen van campylobacteriosis zijn heftige (bloederige) diarree, buikkrampen en koorts. In de meeste gevallen wordt de infectie door het immuunsysteem van het lichaam zelf opgeruimd. Niettemin, in ongeveer 1 op de 1000 gevallen wordt een infectie gevolgd door ernstige neurologische complicaties, zoals het Guillain-Barré syndroom. Het gevolg van dit syndroom zijn verlammingsverschijnselen die bij tijdige behandeling grotendeels of zelfs geheel genezen. Verder blijkt uit recent onderzoek dat er een relatie bestaat tussen campylobacteriosis en de ontwikkeling van chronische maag-darmziekten (irritable bowel disease, IBD). Naast deze serieuze complicaties brengt campylobacteriosis ook nog een grote economische schadepost met zich mee waardoor de ontwikkeling van nieuwe bestrijdingsstrategieën ter reductie van het aantal infecties met deze bacterie een hoge prioriteit heeft.

Adaptatie mechanismen

De meeste pathogene bacteriën bezitten diverse vaak strikt gereguleerde virulentiefactoren die alleen worden aangemaakt als ze ook daadwerkelijk nodig zijn. In *Campylobacter* ontbreken de virulentiefactoren zoals die aanwezig zijn in andere darmpathogenen zoals *Salmonella* en *Shigella*. Het immuunsysteem van de gastheer wordt wel door *Campylobacter* geactiveerd, wat kan duiden op de aanwezig van *Campylobacter* unieke virulentie mechanisme. Deze zijn echter tot op heden niet geïdentificeerd. Wel bezit *Campylobacter* een scala aan adaptatiemechanismen die het mogelijk maken om adequaat noodzakelijke aanpassingen aan te brengen in de oppervlaktestructuur, metabolisme of gedrag als reactie op veranderingen die hebben plaatsgevonden in de omgeving. De aanwezigheid van *Campylobacter* in totaal verschillende leefomgevingen geeft aan dat deze bacterie zich succesvol weet aan te passen aan zijn omgeving. Deze adaptatiemechanismen zouden een essentiële bijdrage kunnen leveren aan het virulentieproces van *Campylobacter.*

Kenmerkend voor het uitgebreide aanpassingsrepertoire van *Campylobacter* is de omvangrijke elektron transport keten. Dit energie leverende proces speelt een belangrijke rol bij de kolonisatie van pluimvee door *Campylobacter*. Transcriptoom analyse van *Campylobacter* opgegroeid onder laboratoriumomstandigheden of in de gastheer hebben aangetoond dat er grote verschillen zijn in de transcriptie van genen die een rol spelen in de energie huishouding en elektron transport. Ondanks het belang van deze aanpassingen voor *Campylobacter* is hierover verder nog weinig bekend, ondanks dat deze aanpassingsmechanismen een zeer belangrijke rol kunnen spelen bij het ontstaan van een infectie door deze bacterie.

Doel van dit onderzoek

De belangrijkste bron van *Campylobacter* infecties bij de mens is de voedselketen, in het bijzonder vlees afkomstig van de kip. Tot dusver is het niet duidelijk waarom deze bacterie een pathogeen is voor de mens maar een normale darmbacterie is bij pluimvee. Bekende verschillen tussen beide gastheren zijn de lichaamstemperatuur, de beschikbaarheid van voedingsstoffen, en de samenstelling van de darmflora. Of deze factoren bijdragen aan het verschil in het ontstaan van infecties bij mens en kip, is onbekend. Het doel van dit onderzoek is om inzicht te krijgen in hoe *Campylobacter* zich kan aanpassen aan de omgeving. Kennis van deze adaptatiemechanismen kan een eerste stap zijn tot een betere controle van deze ziekteverwekker.

Functionele identificatie van het Tat secretie systeem

Bacteriën bezitten verschillende secretiesystemen om eiwitten te kunnen transporteren over de celmembraan zodat die een functie buiten het cytoplasma kunnen vervullen. Het Tat secretie systeem is, in tegenstelling tot het meer algemene Sec systeem, in staat om volledig gevouwen eiwitten te transporteren over het cytoplasma membraan.

In hoofdstuk 2 hebben we aangetoond dat *C. jejuni* een functioneel Tat secretie systeem bezit. Eiwitten die via dit secretie systeem worden getransporteerd bezitten een signaal peptide dat een motief bevat bestaande uit twee geconserveerde arginine amino zuren. Computer analyse naar het voorkomen van dit motief in alle *Campylobacter* eiwitten leidde tot de identificatie van 11 potentiële Tat substraten. De meeste van deze substraten zijn betrokken bij de elektron transport keten. In de elektron transport keten kunnen verschillende moleculen dienen als elektron donor of acceptor, echter de geproduceerde energie is molecuul afhankelijk. *Campylobacter* gebruikt bij voorkeur zuurstof als elektron acceptor, omdat dit de meeste energie oplevert. AIs de beschikbaarheid van zuurstof onvoldoende is worden minder energetische elektron acceptoren gebruikt zoals nitraat en fumaraat. Lage zuurstof concentraties zijn aanwezig in belangrijkste natuurlijke leefomgeving van *Campylobacter*, de darm. Het Tat secretie systeem kan hierdoor een belangrijke rol spelen in de overleving van *Campylobacter* in deze omgeving. Recent onderzoek heeft aangetoond dat een *C. jejuni tatC* knock-out mutant niet meer in staat is om kippen te koloniseren en ook dat het Tat systeem betrokken is bij de motiliteit, flagel vorming, biofilm formatie, en resistentie tegen antimicrobiële middelen. Ook is aangetoond dat mutagenese van individuele *Campylobacter* Tat substraten

leidt tot gereduceerde kolonisatie van kippen.

Deze resultaten geven aan dat het *Campylobacter* Tat secretie systeem een essentiële rol speelt in de kolonisatie van de kippen darm. Waarom deze groep eiwitten door het Tat systeem worden getransporteerd is onduidelijk. Een mogelijke verklaring zou kunnen zijn dat deze eiwitten direct inzetbaar moeten zijn wanneer de samenstelling van de darminhoud drastische verandert.

Lipid A modificatie en immuun evasie

Het immuunsysteem kan grofweg worden onderverdeeld in twee delen, het "aangeboren" immuunsysteem en het "verworven" immuunsysteem. Het aangeboren immuunsysteem is verantwoordelijk voor de herkenning van specifieke componenten die voorkomen bij micro-organismen maar niet bij de mens. Deze specifieke componenten worden pathogeen geassocieerde moleculaire patronen genoemd, of te wel PAMP's. Herkenning van een PAMP door het aangeboren immuunsysteem leidt o.a. tot de productie van antimicrobiële eiwitten, cytokinen en chemokinen, maar ook tot het aansturen van het verworven immuunsysteem. Het verworven immuunsysteem produceert vervolgens antilichamen die binden aan de micro-organismen om ze vervolgens te kunnen neutraliseren. PAMP's worden herkend door sensoren van het aangeboren immuunsysteem. Deze worden pattern recognition receptors (PRRs) worden genoemd. Een belangrijke groep binnen de PRRs zijn de Toll-like receptors (TLRs).

Lipopolysaccharide (LPS) is een PAMP dat van nature aanwezig in de buitenmembraan van Gram-negatieve bacteriën. LPS is opgebouwd uit 3 delen; het O-antigeen, de core oligosaccharide, en het lipid A. Het LPS van *Campylobacter* bestaat uit alleen het core oligosaccharide en het lipid A, waardoor dit lipooligosaccharide (LOS) wordt genoemd. Het lipid A gedeelte is opgebouwd uit twee suiker moleculen met elk drie vetzuurstaarten die verbonden zijn met een N- of O-verbinding. Het lipid A is het meest biologische actieve gedeelte van het LPS. Modificaties aan het lipid A kunnen er voor zorgen dat de interactie met het TLR4-MD2 receptor complex vermindert waardoor het immuun systeem niet meer of minder wordt geactiveerd. Diverse bacteriële lipid A modificerende enzymen zijn beschreven die vaak gereguleerd worden door omgevingsfactoren.

In Hoofdstuk 3 hebben we aangetoond dat *Campylobacter* de verhouding van N- en O-gebonden vetzuurstaarten in het lipid A kan variëren door de aanwezigheid van twee enzymen GnnA en GnnB. De verhouding 1 O- en 3 N-gebonden vetzuurstaarten in het lipid A van wildtype *Campylobacter* veranderde in 2 O- en 2 N-gebonden vetzuurstaarten als het gen coderend voor GnnA of GnnB was geïnactiveerd. LOS dat was gezuiverd van de *gnnA* of *gnnB* mutant activeerde sterker het mensen TLR4-MD2 receptor complex dan het LOS afkomstig van de wildtype stam. Echter dit verschil in activiteit werd niet waargenomen indien het kippen TLR4-MD2 complex werd getest. Een duidelijk verklaring is hier niet voor te geven maar dit verschil zou wel kunnen bijdragen aan het verschil in de immuunreactie van *Campylobacter* in de mens t.o.v. de kip.

Activatie van het aangeboren immuunsysteem leidt tot de productie van antimicrobiële peptiden die aan de negatief geladen membraan van bacteriën kunnen binden. Hierdoor ontstaan poriën in de bacteriële celwand wat resulteert in de dood van de organismen. In hoofdstuk 3 hebben we aangetoond dat de *gnnA* en *gnnB* mutanten gevoeliger zijn voor antimicrobiële middelen. Een mogelijk verklaring hiervoor kan zijn dat de stabiliteit van de membraan is veranderd. O-

verbindingen zijn minder stug dan N-verbindingen waardoor de antimicrobiële moleculen makkelijker de bacterie kunnen binnen dringen. Echter secundaire effecten veroorzaakt door mutaties in *gnnA* en *gnnB*, zoals verandering van de membraan lading, kunnen niet worden uitgesloten. Massa spectrometrie toonde een heterogene populatie aan van de lipid A moleculen, wat suggereert dat deze verandering in vetzuurstaart verbinding reguleerbaar is. Nader onderzoek kan hier uitsluitsel over kunnen geven.

De functie van het twee-component systeem RacS-RacR in *Campylobacter*

Campylobacter komt voor in milieus waar factoren als temperatuur, nutriënten en zuurstofspanning sterk kunnen variëren. Om te overleven moet dit organisme zich snel kunnen aanpassen aan zijn omgeving. Twee-component systemen zijn de belangrijkste signaal transductie systemen in bacteriën die in staat zijn veranderingen in de omgeving waar te nemen en hier adequaat op te reageren. *C. jejuni* bezit 7 twee-component systemen waarvan voor 3 alle genen bekend zijn die hierdoor worden gereguleerd. Ondanks dat het specifieke signaal voor deze systemen onbekend zijn wel de condities bekend waardoor deze systemen worden geactiveerd. In hoofdstuk 4 en 5 hebben we de rol van het RacS-RacR systeem in *Campylobacter* uiteengezet.

In hoofdstuk 4 beschrijven we dat de activiteit van het gamma glutamyl transferase (GGT) afhankelijk is van een functioneel disulfide brug system (dsb). Het dsb systeem vormt zwavelbruggen tussen cysteine aminozuren in de periplasmatische ruimte. GGT bevat echter geen cysteine aminozuren waardoor we op zoek zijn gegaan naar een regulatie systeem wat gevoelig kon zijn voor dsb. Het periplasmatisch gedeelte van de sensor RacS bevat twee geconserveerde cysteine aminozuren waardoor het een substraat kon zijn voor het dsb systeem. Het RacS-RacR systeem bleek direct het gen coderend voor GGT te activeren. Gezuiverd recombinant RacS was in tegenstelling tot het geïsoleerde cytoplasmatisch gedeelte van RacS is gevoelig voor beta-mercapthanol, een verbinding die zwavelbruggen verbreekt. De zwavelbrug is belangrijk voor de instandhouding van de RacS dimeer. Twee-componenten sensoren komen altijd voor als dimeer. Autofosforylatie experimenten met het RacS eiwit, in aan- of afwezigheid van beta-mercapthanol lieten zien dat alleen de monomeer autofosforyleert. De dimeer vorm heeft waarschijnlijk een specifiek signaal nodig voordat het autofosforyleert. Deze resultaten laten zien dat RacS een dimeer vormt en een zwavelbrug bevat die gevormd wordt door het dsb systeem. Tot nu toe is RacS de enige bekende bacteriële sensor die afhankelijk is van een functioneel dsb systeem.

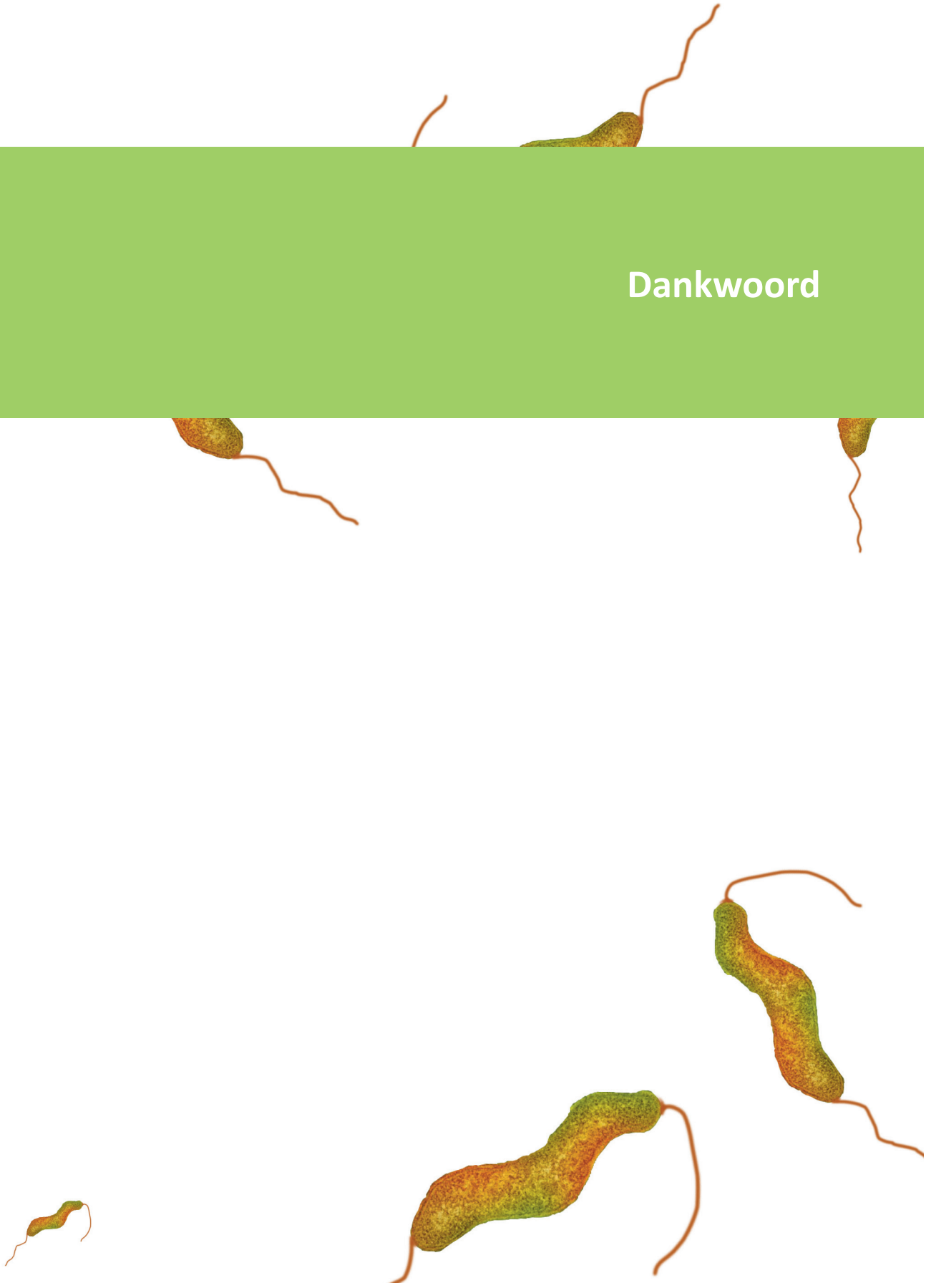
In hoofdstuk 5 hebben we het regulon en de functie van het RacS-RacR systeem bestudeerd. Fosforylatie experimenten met recombinant RacR en RacS lieten zien dat deze twee eiwitten inderdaad een functioneel twee-component systeem vormen. De groeisnelheid van de *C. jejuni racR* mutant onder lage zuurstof concentraties (0.3%) is sterk vertraagd t.o.v. de wildtype. Transcriptoom analyse toonde aan dat onder deze condities het RacS-RacR systeem de 4 operonen activeert; Cj0175c-Cj0176c, *fldA*-Cj1384c, *katA*-Cj1386 en *chuABCD* en ook 4 operonen remt, namelijk *aspA-dcuA*-Cj0089, *mfrXAB*, Cj448c-Cj0449c en Cj1491c-Cj1493c. Bindingsstudies met gezuiverd eiwit en DNA lieten zien dat het gen *aspA*, waarvan het product verantwoordelijk is voor de omzetting van aspartaat naar fumaraat, direct gereguleerd wordt door RacR. Verder bleek uit transcriptie-, eiwit-

en substraatanalyses dat de belangrijkste functie van het RacS-RacR systeem is het onderdrukken van het aspartaat-fumaraat verbruik. Fumaraat kan als koolstofbron dienen, maar ook als elektron acceptor. Dit maakt dat het RacS-RacR systeem een belangrijk regulatie mechanisme is in de energie en aminozuur metabolisme van *Campylobacter* en grote invloed heeft op de overlevingskans van dit organisme in lage zuurstof milieus zoals in zijn de belangrijkste natuurlijke leefomgeving, de darm.

Conclusie

Dit proefschrift beschrijft niet eerder beschreven aanpassingsmechanismen van de darmpathogeen *C. jejuni* die van belang zijn voor de bacterie om te overleven in de gastheer. *C. jejuni* bezit een functioneel Tat secretie mechanisme dat verantwoordelijk is voor het transport van diverse eiwitten die betrokken zijn bij de elektron transport keten en dat essentieel is voor de kolonisatie van kippen. Tevens heeft de bacterie lipid A modificerende enzymen die, naast de lengte en de hoeveelheid vetzuurstaarten van het lipid A, ook het type verbinding (N of O-verbinding) reguleert wat leidt tot een verandering in de activatie van het TLR4-MD2 immuunreceptor complex. De karakterisatie van het RacS-RacR twee-component systeem geeft aan dat diverse genen die betrokken zijn bij metabolische processen onder invloed staan van omgevingsfactoren, waardoor de bacterie zich optimaal kan aanpassen aan de omgevingsomstandigheden. De kennis opgedaan tijdens dit promotieonderzoek kan een belangrijke basis vormen om de aanwezigheid van *Campylobacter* in de voedselketen te reduceren en zodoende het aantal *Campylobacter* infecties te verminderen.

Dankwoord



Dankwoord

Het is écht klaar, vanaf nu geen “bijna” meer! Daarom wil ik graag gebruik maken van de gelegenheid om een aantal mensen te bedanken want, alleen had ik dit niet voor elkaar gekregen.

Allereerst wil ik graag mijn promotor Jos van Putten bedanken. Jos, jouw positieve begeleiding gedurende het onderzoek heeft mij altijd gestimuleerd, vooral in de fases dat het even niet lukte. Zeker in de afrondende fase van dit onderzoek kon ik altijd op een snelle en kritische reactie op mijn manuscripten rekenen waardoor ik lekker door kon gaan.

Marc, mijn co-promotor, waarschijnlijk was ik zonder jou nooit AIO geworden. Bedankt dat jij mij de gelegenheid en het vertrouwen hebt geboden om als AIO aan de slag te gaan. Bedankt voor al je goede ideeën en praktische tips die je mij hebt gegeven. Ik hoop dat je in de toekomst nog veel artikelen publiceert en beurzen binnen haalt zodat je hoofdzakelijk met het geliefde onderzoek bezig kunt blijven.

Liana bij jou ben ik begonnen met werken. Ik denk dat we een goed team waren! Door de zelfstandigheid en ruimte die jij me gaf in het werk heb ik me kunnen vormen als analist, hierdoor ben ik uiteindelijk als AIO weer verder gegaan. Jij hebt de gave om mensen te motiveren door je enthousiasme maar ook door kritische vragen te stellen op het juiste moment. Zonder jouw inbreng was dit proefschrift minder compleet geweest, bedankt voor alles.

De volgende andere collega’s; Wim, zonder jou kritische blik op dit werk had mijn proefschrift er toch iets minder fraai uit gezien. Bedankt hiervoor. Mijn laatste kamergenoot Marcel (ik heb er trouwens wel veel gehad, zou dit aan mij liggen?). Marcel, volgens mij ken ik niemand die in zoveel dingen een potentieel Nature artikel ziet als jij. Bedankt voor alle keren dat je weer eens mee “moest” denken over hoe het RacR systeem nu eigenlijk werkt, ook al dachten sommige mensen dan dat we aan het roddelen waren! Succes met de volgende stap in je carrière, op naar die Nature artikelen! Collega Aio’s Lieneke en Medi, veel succes met jullie onderzoek, het gaat allemaal wel lukken! May-Young, je bent altijd oprecht geïnteresseerd in hoe dingen gaan en lopen, zowel op het lab als er buiten. Bedankt en succes met je carrièreplanning! Geb, ik heb bewondering voor de enorme kennis en je passie voor het onderzoek. De mensen van de moleculaire afweer; Henk, Edwin, Albert, Martin, Hanne, Yib en Herfita bedankt voor de suggesties tijdens werkbesprekingen en de gezellige lunches en koffiepauzes. Van de klinische infectiologie wil ik in het bijzonder Jaap bedanken voor zijn oprechte interesse in zaken op maar ook buiten het lab. En verder nog Mirjam, Niels, Rolf, Lea, Deborah en Esther voor de gesprekken tijdens de koffiepauzes. De oud collega’s Lieke en Marijke, helaas is het ook mij niet gelukt in 4 jaar! Zorica, volgens mij wacht ik nog steeds op die eerste Dutch Friday! Andreas, Nina en Stephanie bedankt voor alle ideeën en suggesties in de afgelopen jaren.

Een belangrijke groep mensen met wie ik heb samengewerkt. De mensen van het NVI; Bert, Hugo en Hendrik-Jan. Bert je hulp bij de NMR analyses is onmisbaar geweest. Hendrik-Jan en Hugo voor jullie hulp bij de LPS analyses. Paweł Łaniewski, thanks for the cooperation on the Dsb system and the RacS-RacR system. Thea zonder jouw inspanning was ik nu waarschijnlijk nog niet klaar geweest met de lay-out. Alle studenten die ik in de afgelopen jaren heb mogen begeleiden; Luis,

Martha, David, Jacoline, Marjolein en Evelien. Zonder jullie bijdrage was dit proefschrift zeker minder compleet geweest.

Natuurlijk gaat er ook nog een bedankje uit naar iedereen die ik ken uit de voetballerij. Met jullie kan ik altijd kletsen of bezig zijn met mijn grootste passie waardoor ik vervolgens ook weer de energie had om met labwerk bezig te gaan. Een aantal mede-verslaafde wil ik in het bijzonder bedanken. Hans, Cees en Geert bedankt voor de extra “bagage” die jullie mij de afgelopen jaren hebben meegegeven. Ik hoop dat we nog vaak mogen samenwerken.

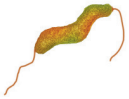
Mijn paranimfen Linda en Nancy, ik ben blij dat jullie mijn paranimfen zijn! Linda, ik denk dat wij het allerbeste Campy-team zijn samen, je enthousiasme en vakmanschap ga ik zeker missen! En bedankt voor alle goede adviezen over baby’s, met jouw raad ging ik toch vaak weer iets geruster naar huis! Nancy, zonder jou zou het heel wat minder flexibel lopen op het lab! Je enorme ervaring met Campylobacter en de discussies die we de laatste tijd hadden over het metabolisme van deze bacterie vond ik erg leuk, maar zijn ook zeker waardevol geweest voor dit onderzoek.

Gerard, ondanks dat het volledig onduidelijk is wat ik nu eigenlijk uit spook met die “beestjes” op het lab en dat je van mijn planning al helemaal niets begrijpt, was je tijdens het maken van dit boekje altijd flexibel en bereidt om te helpen. Bedankt!

Pa en Ma, zonder jullie steun en vertrouwen was ik waarschijnlijk nooit zo ver gekomen. Goed voorbeeld doet goed volgen!

En dan tenslotte de grootste drijfveer, het thuisfront! Esmee en Lars hoewel jullie nu nog veel te jong zijn om dit boekje te lezen, laat staan begrijpen, zullen jullie dit boekje hopelijk toch ooit een keer doorkijken. Carin, de vrijheid en het vertrouwen dat jij mij geeft om dingen te (gaan) doen waardeer ik enorm. Als ik van iemand geleerd heb om ergens echt voor te gaan en door te zetten als het even tegenzit ben jij het, bedankt hiervoor! Zonder de energie die jullie me geven was dit resultaat er niet geweest, jullie zijn geweldig! Op naar een nieuwe uitdaging!

Curriculum Vitae and List of Publications



Curriculum Vitae

Andries van Mourik werd geboren op 20 september 1979 te Zoelen. Na het behalen van het HAVO diploma aan het GSG Lingecollege in Tiel, werd met het Hoger Laboratorium Onderwijs (HLO) begonnen. In 2003 werd het diploma hiervoor behaald met als studierichting Moleculaire Biologie. Tijdens deze opleiding werd stage gelopen op de afdeling Celbiologie van het Universitair Medisch Centrum (UMC) Utrecht onder begeleiding van Prof. Dr. Strouss. Van 2003 tot 2005 was hij werkzaam als research analist onder begeleiding van Dr. Steeghs op de afdeling Immunotherapie van het Wilhelmina Kinder Ziekenhuis (WKZ), onderdeel van het UMC Utrecht. In 2005 werd hij aangesteld als assistent in opleiding. Onder begeleiding van Dr. Wösten en Prof. Dr. van Putten is het in dit proefschrift beschreven onderzoek verricht.

List of Publications

1. **Steeghs L, van Vliet SJ, Uronen-Hansson H, van Mourik A, Engering A, Sanchez-Hernandez M, Klein N, Callard R, van Putten JP, van der Ley P, van Kooyk Y, van de Winkel JG (2006).** *Neisseria meningitidis* expressing *lgtB* lipopolysaccharide targets DC-SIGN and modulates dendritic cell function. *Cell Microbiol* **8(2),** 316-25.

2. **Wösten MM, Parker CT, van Mourik A, Guilhabert MR, van Dijk L, van Putten JP (2006).** The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system. *Mol Microbiol* **62**, 278-291.

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4. **van Mourik A, Bleumink-Pluym NM, van Dijk L, van Putten JP, Wösten MM (2008).** Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery. *Microbiology* **154**, 584-92.

5. **Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R, Klein N, van Putten JP (2008).** Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of *Neisseria meningitidis*. *Infect Immun* **76(8)**, 3801-7.

6. **van Mourik A, Steeghs L, van Laar J, Meiring HD, Hamstra HJ, van Putten JP, Wösten MM (2010).** Altered linkage of hydroxyacyl chains in lipid a of *Campylobacter jejuni* reduces TLR4 activation and antimicrobial resistance. *J Biol Chem* **285(21)**, 15828-36.

7. **van Mourik A*, Łaniewski P*, van Putten JP, Jagusztyn-Krynicka EK and Wösten MM (2010).** The Dsb system of *Campylobacter jejuni* influences γ-glutamyltranspeptidase activity by altering the status of the RacRS two-component system. *Manuscript in preparation.*

8. **van Mourik A, Heijmen-van Dijk L, Guilhabert MR, Zomer B, Kelly D, Parker CT, van Putten JP and Wösten MM (2010).** The *Campylobacter jejuni* RacRS two-component system regulates fumarate catabolism and respiration in response to oxygen. *Manuscript in preparation.*

Book chapter

1. **Wösten MM, van Mourik A, van Putten JP** (2008). Regulation of genes in *Campylobacter jejuni.* In Nachamkin I, Szymanski CM, Blaser MJ (eds). *Campylobacter*. Washington, DC: American Society for Microbiology Press, pp. 611-624.