Molecular Mechanisms of *Campylobacter* Biofilm Formation and Quorum Sensing

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Abstract Even though *Campylobacter* spp. are known to be fastidious organisms, they can survive within the natural environment. One mechanism to withstand unfavourable conditions is the formation of biofilms, a multicellular structure composed of different bacterial and other microbial species which are embedded in an extracellular matrix. High oxygen levels, low substrate concentrations and the presence of external DNA stimulate the biofilm formation by *C. jejuni*. These

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external factors trigger internal adaptation processes, e.g. via regulating the expression of genes encoding proteins required for surface structure formation, as well as motility, stress response and antimicrobial resistance. Known genes impacting biofilm formation will be summarized in this review. The formation of biofilms as well as the expression of virulence genes is often regulated in a cell density depending manner by quorum sensing, which is mediated via small signalling molecules termed autoinducers. Even though quorum sensing mechanisms of other bacteria are well understood, knowledge on the role of these mechanisms in *C. jejuni* biofilm formation is still scarce. The LuxS enzyme involved in generation of autoinducer-2 is present in *C. jejuni,* but autoinducer receptors have not been identified so far. Phenotypes of *C. jejuni* strains lacking a functional *luxS* like reduced growth, motility, oxygen stress tolerance, biofilm formation, adhesion, invasion and colonization are also summarized within this chapter. However, these phenotypes are highly variable in distinct *C. jejuni* strains and depend on the culture conditions applied.

1 Introduction

Compared to other food-borne pathogenic bacteria, *Campylobacter* spp. are susceptible to various stressors including elevated ambient oxygen concentrations, dehydration and UV-light, which are present in the natural environments and in food processing plants. Nevertheless, *Campylobacter* spp. are widespread in the environment and persist in the food production chain indicating that these bacteria are capable to survive these unfavourable conditions (Boronowsky et al. 2014; Golz et al. 2018; Hansson et al. 2018; Tram et al. 2020a). However, how they regulate their stress responses and environmental adaptation is still not fully understood as campylobacters are lacking several classical regulatory factors. One microbial strategy to survive within hostile surroundings is the formation of biofilms. Biofilms are organized aggregates of microorganisms encased by an extracellular matrix. This extracellular matrix structures the biofilm and also protects microorganisms from stressful conditions present outside of the biofilm (Kostakiotis et al. 2013). The process of biofilm formation as well as the expression of virulence factors is often coordinated at a multicellular stage, which depends on the detection of the cell density via quorum sensing (QS) systems which are present in many bacteria, fungi and parasites (Mukherjee and Bassler 2019). Within this article, we summarize information on external factors and genes involved in biofilm formation and QS of *C. jejuni*.

2 Microbial Biofilm Formation

Bacteria can switch from a planktonic single-cell lifestyle to a multicellular lifestyle, e.g. in biofilms, and back to planktonic style. In biofilms, bacterial species live in close contact with communities which can also contain fungi, algae, protists and archaea

(Flemming et al. 2016). These biofilms can be found either attached to a surface or as free-floating aggregates, which are both surrounded by a matrix of extracellular polymeric substances (EPS) (Joshua et al. 2006; Roy et al. 2018). Depending on the microorganisms within the biofilm, the EPS consists of proteins, nucleic acids, polysaccharides, lipids and other compounds which form part of highly viscous watery solutions (Flemming et al. 2016). Within these biofilms, microorganisms are protected from several external stressors—such as dehydration, and exposure to oxygen radicals, disinfectants or antimicrobial substances—and grow much more slowly compared to planktonic cells, thereby facilitating survival under unfavourable conditions in diverse environmental niches. Furthermore, microorganisms within biofilms can support each other by exchanging of substrates or by degradation of toxigenic substances (Flemming et al. 2016). The ability to form biofilms and to colonize preformed biofilms as well as the specific architecture of biofilms depends on the microbial composition, the genetic background of the individual strains involved and the environmental conditions.

2.1 Building and Dispersion of Microbial Biofilms

Biofilm formation takes place in three major steps: In the first two steps, the microorganisms build up microcolonies by attachment to surfaces and/or to each other, and the production of EPS establishes the biofilm structure, which matures the microcolonies into a three-dimensional architecture. In the third phase, the microorganisms actively or passively detach from the biofilm and are released back to the planktonic lifestyle. In bacterial biofilms, surface or cell-to-cell attachment is mediated by extracellular adhesive appendages, like flagella, pili or outer membrane proteins, secreted adhesins as well as by the molecular structure and adhesive properties of the abiotic surfaces (Kostakioti et al. 2013). Once the microcolonies are built, multiple regulatory networks translate signals to concerted gene expression changes, which lead to building of the extracellular matrix and mediate the spatial and temporal reorganization of the microbial cells within the final biofilm (Petrova and Sauer 2016). The biofilm matures into a well-organized architecture, with intervening water channels for nutrient and waste exchange which is embedded in a viscous EPS matrix (Coughlan et al. 2016). In the final state, biofilms represent highly dynamic structures, in which the bacteria could disperse passively or actively. Passive dispersal is due to external shear forces or abrasion when the biofilm structure grows (Kaplan 2010). Active dispersion of biofilms is triggered by beneficial conditions outside the biofilm or detrimental conditions inside the biofilm. These include scarcity of substrates including carbon and energy sources, accumulation of signalling molecules and in case of *Campylobacter* also elevated oxygen levels (Kostakioti et al. 2013, Petrova and Sauer 2016). The release of microorganisms from biofilms is supported by increased motility. Active dispersion of biofilms can be mediated by bacterial secretion of EPS-degrading enzymes including glycosidases,

lipases, proteases and deoxyribonuleases, as well as by production of surfactants (Kaplan 2010).

2.2 *Methods to Analyse Biofilms*

Analysis of biofilms is focussed on the quantification and successful measurement of several multiple parameters including the biomass and architecture of biofilms, the bacterial viability, attachment and motility within biofilms and the composition of the EPS (reviewed by Azeredo et al. 2017). Briefly, the total amount of the biofilm-mass is commonly quantified by indirect staining methods, e.g. by the Crystal Violet Assay, while the viable cell count can be determined by, e.g. direct plating, flow cytometry or live/dead staining combined with confocal laser scanning microscopy (CLSM). CLSM is further applied to study the spatial structure of biofilms. The metabolic activity of bacteria in biofilms can be measured by colorimetric determination of the conversion of tetrazolium salts to formazan by a spectrophotometer. The amount of initially attached bacteria can be quantified by direct plating or microscopic methods.

2.3 *Environmental Conditions Influencing* Campylobacter jejuni *Biofilm Formation*

Investigations focused on the biofilm formation capacity of *C. jejuni* were mostly conducted under laboratory conditions with well-defined reference strains of the pathogen as monospecies biofilms, which do not reflect the situation outside the laboratory (Lamas et al. 2018; Teh et al. 2014). The resulting data demonstrate that *C. jejuni* bacteria are able to form biofilms on glass, polystyrene and stainless steel surfaces (Joshua et al. 2006; Li et al. 2017; Oh et al. 2016; Teh et al. 2016; Wagle et al. 2019). However, the whole biofilm formation process of *C. jejuni* is modulated by many extrinsic and intrinsic factors which will be discussed in more detail.

2.3.1 Substrate Availability and Oxygen

Distinct external stress conditions which all depend on the specific metabolic properties of *C. jejuni* have been found to regulate the biofilm formation and lifestyle of the pathogen (Fig. 1). Corresponding results confirmed that biofilm formation enables *C. jejuni* to survive hostile environmental conditions. Nutrient availability is a key factor in the regulation of biofilm formation by *C. jejuni*. Starvation induces biofilm formation by *C. jejuni* which was indicated by significantly higher biofilm production by bacteria grown in less nutrient-rich Mueller–Hinton medium as compared to bacteria grown in Brucella or Bolton broth (Reeser et al. 2007). Similarly, addition of fucose

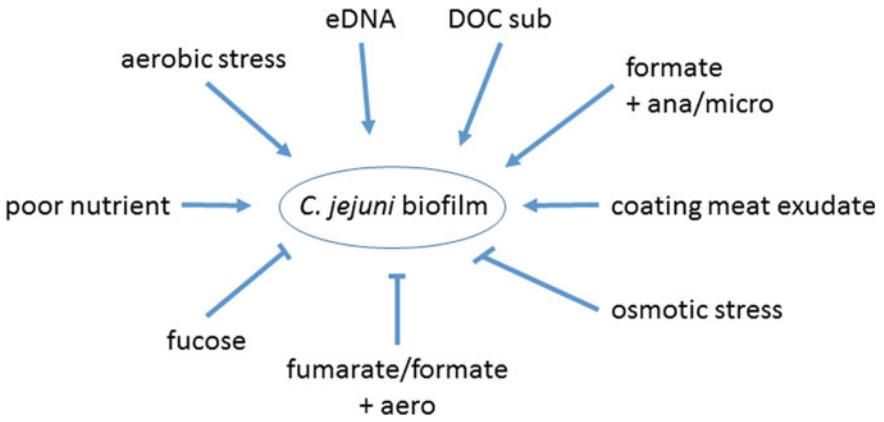

Fig. 1 Impact of environmental conditions relevant to formation and survival of *C. jejuni* biofilms. The biofilm formation by *C. jejuni* is enhanced by starvation, aerobic stress, extracellular DNA (eDNA), sublethal bile salt (desoxycholate, DOC sub) concentrations, formate at microaerobic (micro) and anaerobic (ana) conditions, as well as surface coating with meat exudates. Osmotic stress (induced by NaCl, glucose and sucrose), fumarate and formate at aerobic conditions and fucose decreased biofilm formation

inhibited biofilm formation of *C. jejuni* strains encoding enzymes required for fucose utilization (Dwivedi et al. 2016). In contrast, meat exudate significantly enhanced biofilm formation of *C. jejuni* grown on surfaces or in liquid media. However, this might be rather due to enhanced attachment than to active biofilm formation (Brown et al. 2014; Li et al. 2017; Wagle et al. 2019). In further support of the role of nutrients in biofilm formation, a recent study demonstrated that addition of energy sources such as fumarate and formate enhanced biofilm formation in a microaerobic atmosphere, but reduced biofilm formation under aerobic conditions (Kassem et al. 2017). Supplementation of growth media with formate additionally enhanced biofilm formation under anaerobic conditions (Kassem et al. 2017). In most studies, aerobic atmosphere enhanced the biofilm formation of several *C. jejuni* strains (Feng et al. 2018; Pascoe et al. 2015; Reuter et al. 2010; Stetsenko et al. 2019; Turonova et al. 2015; Zhong et al. 2020). Results from a recent study revealed that extracellular DNA (eDNA) enhances biofilm formation by *C. jejuni* (Feng et al. 2018). Interestingly, release of eDNA was induced by exposure of *C. jejuni* to aerobic conditions. In other studies, however, biofilm formation was similar or even lower if *C. jejuni* were incubated under aerobic conditions (Kassem et al. 2017; Reeser et al. 2007; Teh et al. 2017). These conflicting results might be explained by the different strains and methods used (see also Section Genetic Background and Genes Impacting Biofilm Formation of *C. jejuni*). Besides that, also small genomic variations within clones of one strain might influence investigated phenotypes, as recently shown for several clones of the reference strain NCTC11168 by Pascoe and co-workers (2019). Furthermore, the application of the bile salt deoxycholate in sublethal concentrations enhanced the biofilm formation of *C. jejuni*, while no differences in biofilm formation have

been observed by the addition of sublethal concentrations of other detergents, such as Triton X-100, Tween-20 or sodium dodecyl sulphate (Svensson et al. 2009). In contrast, osmotic stress generated by NaCl, glucose or sucrose inhibited biofilm formation of *C. jejuni* (Reeser et al. 2007). The knowledge about the influence of temperature on *C. jejuni* biofilm production is still scarce. In two studies, biofilm production was higher if *C. jejuni* was incubated at 37 °C as compared to 25 °C or 20 °C, respectively (Reeser et al. 2007; Wagle et al. 2019). Taken together, biofilm formation of *C. jejuni* is influenced by multiple factors. Under laboratory conditions, biofilm formation was induced by nutrient starvation and oxygen stress, while osmotic stress rather reduced the biofilm formation. However, as the results obtained by the studies described above were generated in artificial systems, the transferability of these results to the real world is only limited. The multitude of conflicting results obtained in this highly innovative field of research underlines the urgent need for standardization and better control of future studies on factors influencing *C. jejuni* biofilm formation as a major mechanism to survive outside the vertebrate hosts.

2.3.2 Other Bacterial Species in Multispecies Biofilms

Even though *C. jejuni* forms biofilms in monocultures, the biomass of these monospecies biofilms is much lower as compared to biofilms formed by monocultures of *Pseudomonas aeruginosa* or *Escherichia coli*. If *C. jejuni* were co-cultivated in biofilms with *E. coli*, *P. aeruginosa*, *Enterococcus faecalis*, *Salmonella enterica* or *Staphylococcus simulans*, the survival of *C. jejuni* was prolonged as compared to monocultured cells, and the biofilm-mass was increased to levels produced by the co-cultured species (Feng et al. 2016; Indikova et al. 2015; Teh et al. 2019, 2010; Zhong et al. 2020). Furthermore, it has been demonstrated that in *C. jejuni-Salmonella* dual-species biofilms *C. jejuni* is located at the bottom of the biofilms in areas with high eDNA concentrations, while *Salmonella* is located at the top of the biofilm and in areas where less eDNA is present (Feng et al. 2018). It was assumed that other bacteria in co-cultures establish a more favourable environment, e.g. by lowering the oxygen level, providing CO_2 and alteration of metabolite concentrations (Zhong et al. 2020). Taken together, these results indicate that *C. jejuni* is able to colonize multispecies biofilms but the use of multispecies biofilms as a target for pathogen control via biosafety measures awaits further investigations.

2.3.3 Antimicrobial Substances

Within biofilms, microorganisms are protected against the antimicrobial activities of various substances including well-established antibiotics (Sharma et al. 2019). The molecular mechanisms by which biofilms protect bacteria from antimicrobial activity are multifactorial. The EPS structure hampers penetration of distinct antibiotics and can contain enzymes which actively inactivate antibiotics by molecular modifications (Hall and Mah 2017). In addition, the dormant state of bacteria in

biofilms may passively enhance the tolerance to antimicrobial substances (Petrova and Sauer 2016). On the other hand, the close cell proximity within biofilms and the eDNA in the EPS structure support horizontal gene transfer. In accordance, *C. jejuni* transfers chromosomally encoded antibiotic resistance genes more frequently in biofilms as compared to bacteria in the planktonic lifestyle (Bae et al. 2014). Furthermore, antibiotic resistance is also influencing the biofilm formation ability of *C. jejuni* strains. Of 206 *C. jejuni* and *C. coli* strains isolated from poultry products, biofilm-producing strains possessed a significantly higher resistance to ampicillin, neomycin, sulfamethoxazole, amikacin, clindamycin and erythromycin as compared to strains unable to form biofilms (Zhang et al. 2017). Another study reported that fluoroquinolone resistance of *C. jejuni* is associated with an increased ability to form biofilms in oxygen-rich environments (Whelan et al. 2019). These aspects of enhanced antimicrobial resistance gene transfer within biofilms and higher biofilm formation in antibiotic resistant strains indicate the necessity to control and reduce *C. jejuni* biofilms.

2.4 *Genetic Background and Genes Impacting Biofilm Formation of* C. jejuni

The transition from planktonic lifestyle to the embedding of bacterial cells in the biofilm matrices goes along with substantial alterations in gene expression, which result in the production of adhesive surface molecules and in a comprehensive metabolic reprogramming (Kostakiotis et al. 2013). Recently, it has been reported that the expression of approx. 600 genes was differentially regulated during the biofilm formation of *C. jejuni*, with increased expression of genes involved in iron metabolism and acquisition, cell division, glycan production and attachment and reduced expression of genes involved in energy metabolism, amino acid catabolism and chemotaxis (Tram et al. 2020b). However, which of these changes are responsible for biofilm formation itself or which are going along with altered lifestyle in the established biofilm have to be determined. Nevertheless, several genes, impacting the biofilm formation capacity of *C. jejuni*, are summarized in Table 1, and their putative involvement in the biofilm formation process is described in more detail below.

2.4.1 Genetic Background of Individual *C. jejuni* Strains

The composition of genes differentially regulated during biofilm formation and genes directly involved in the synthesis of biofilm matrix molecules is highly variable in genomes of individual *C. jejuni* strains. These differences are suspected to be responsible for the fact that some strains of the pathogen form only weak or nearly no biofilm-mass and others produce biofilm-mass in large amounts (Bronnec et al.

Table 1 *C. jejuni* genes impacting the biofilm formation

Gene	Function	Mutation[a]	Biofilm formation	Reference
Stress response				
ahpC	Alkyl hydroperoxide reductase	lack	increased	Oh and Jeon (2014)
		over	reduced	Oh and Jeon (2014)
katA	Catalase A	lack	reduced	Oh and Jeon (2014)
perR	Peroxide stress response regulator	lack	reduced	Oh and Jeon (2014)
cosR	*Campylobacter* oxidative stress regulator	over	reduced	Oh and Jeon (2014)
cprS	*Campylobacter* planktonic growth regulation sensor	lack	increased	Svensson et al. (2009)
csrA	Carbon-starvation regulator	lack	reduced	Fields and Thompson (2008)
pta	Polyphosphate acetyltransferase Pta	lack	reduced	Joshua et al. (2006)
dps	Iron-binding protein	lack	reduced	Theoret et al. (2012)
spoT	Guanosine-3′,5′-bis(Diphosphate) 3′-pyrophosphohydrolase	lack	increased	Svensson et al. (2009)
recA	Recombinase A	lack	increased	Feng et al. (2018)
ppk-1	Polyphosphate kinase	lack	increased	Drozd et al. (2014)
ppk-2	Polyphosphate kinase	lack	increased	Drozd et al. (2014)
phoX	Alkaline phosphatase	lack	increased	Drozd et al. (2014)
Surface structures				
peb-4	Adhesion	lack	reduced	Asakura et al. (2007)
		lack	increased	Rathbun et al. (2009)
pglB	Oligosaccharyltransferase	lack	increased	Cain et al. (2019)
eptC	Phosphoethanolamine transferase	lack	reduced	Lim & Kim (2017)
waaF	Heptosyltransferase II	lack	increased	Naito et al. (2010)
lgtF	Glycosyltransferase	lack	increased	Naito et al. (2010)

(continued)

Table 1 (continued)

Gene	Function	Mutation[a]	Biofilm formation	Reference
Flagella				
flhA	Flagellar biosynthesis protein	lack	reduced	Kalmokoff et al. (2006)
fliA	Sigma factor 28	lack	reduced	Kalmokoff et al. (2006)
flaA	Major flagellin A	lack	reduced	Li et al. (2017)
flaB	Minor flagellin B	lack	reduced	Li et al. (2017)
flaC	Secreted flagellin	lack	reduced	Kalmokoff et al. (2006)
flaG	Flagellar filament length control	lack	reduced	Kalmokoff et al. (2006)
flgA	Flagella basal body *p*-ring formation protein	lack	reduced	Kim et al. (2015)
fliS	Flagellar secretion chaperon	lack	reduced	Joshua et al. (2006)
pflA	Paralyzed flagellum protein	lack	reduced	Svensson et al. (2014)
cj1324	Flagellar glycosylation protein	lack	reduced	Howard et al. (2009)
Chemotaxis				
tlp3	Transducer-like protein-3	lack	increased	Rahman et al. (2014)
tlp8	Transducer-like protein-8	lack	reduced	Chandrashekhar et al. (2015)
cheA	Histidine kinase sensor	lack	reduced	Reuter et al. (2020)
cheY	Cytoplasmic response regulator	lack	reduced	Reuter et al. (2020)
		lack	increased	Tram et al. (2020b)
cheW	Phosphotransferase	lack	reduced	Reuter et al. (2020)
		lack	increased	Tram et al. (2020b)
cheV	Phosphotransferase	lack	reduced	Reuter et al. (2020)
Others				
cje1441	Extracellular DNase	lack	increased	Brown et al. (2015)

[a]Lack: lack of function, over: overexpression

2016; Feng et al. 2018; Joshua et al. 2006; Melo et al. 2017). For example, *C. jejuni* strains encoding for extracellular DNases, mostly located on the mobile elements CJIE1, CJIE2 and CJIE4, are unable or only poor biofilm producer and are further able to remove pre-established biofilms of other *C. jejuni* strains (Brown et al. 2015).

Moreover, biofilm formation capacities of individual *C. jejuni* isolates are significantly associated with distinct multilocus sequence types (MLST) and with several clonal complexes, which display specific features concerning host adaptation, termed host-generalists and host-specialists, respectively (see also Pascoe et al. 2015 and Chapters "Population Biology and Comparative Genomics of *Campylobacter* Species" and "Emission Sources of *Campylobacter* from Agricultural Farms, Impact on Environmental Contamination and Intervention Strategies" in this book). It is of note that a strong biofilm formation capacity of *C. jejuni* isolates is correlated with the absence of specific host adaptation, leading to the fact that the host-generalist group of *C. jejuni* isolates displays an enhanced capacity for biofilm formation. Furthermore, nearly 2/3 of the *C. jejuni* isolates belonging to the chicken-specialists belonged to the group of weak biofilm producers (Pascoe et al. 2015). Even though genes with a robust association to biofilm formation differed between the isolates of the host-generalist group, most of these genes are involved in adhesion, motility, glycosylation, capsular polysaccharides and oxidative stress response (Pascoe et al. 2015). Taken together, these findings provide evidence that the genomic repertoire necessary for biofilm formation is highly variable within *C. jejuni* isolates and that biofilm formation is more important for isolates that are not adapted to specific vertebrate hosts.

2.4.2 Flagella-Associated Genes and Motility

Besides the involvement in motility and chemotaxis, the flagella of *C. jejuni* is also crucial for secretion of proteins, autoagglutination, microcolony formation and avoidance of the innate immune response (Guerry 2007), indicating that mutation of the flagella might have multifactorial effects. Generally, motility mediated by flagella is essential for the biofilm formation capacity of *C. jejuni*. Loss of motility caused by targeted mutation of flagella-associated *C. jejuni* genes *flhA, fliA, flaA, flaB, flaC*, *flaG, flgA* and *fliS*, resulted in impaired biofilm formation (Feng et al. 2018; Joshua et al. 2006; Kalmokoff et al. 2006; Kim et al. 2015; Li et al. 2017; Reuter et al, 2010; Turonova et al. 2015). Besides the fact that flagella-associated motility is essential to reach substrates where biofilms can be formed, also flagella-associated attachment seems to impact *C. jejuni* biofilm formation. This was supported by the observation that aflagellated *C. jejuni* mutants (mutation of *flhA*) formed less biofilm-mass as compared to *pflA* mutants with paralyzed flagella only (Svensson et al. 2014). Furthermore, the biofilm formation capacity of *C. jejuni* depends on flagellar *O*-linked glycan modifications. This was shown by targeted deletion of the *cj1324* gene, which resulted in the loss of flagellar sugar modifications and reduced biofilm formation but does not alter the motility (Howard et al. 2009). Additionally,

the reduced biofilm-mass formation of a *flaA/flaB* mutant could be restored by addition of chicken meat exudate (Li et al. 2017). Taken together, these findings indicate that surface attachment mediated by the flagella is essential for *C. jejuni* biofilm formation.

2.4.3 Chemotaxis-Associated Genes

Directed movement of bacteria is interactively controlled and directed by the sensing of attractants or repellents by transducer-like proteins (Tlp). The activation of Tlp results in a signalling cascade mediated by the Che proteins, which modulate flagellar rotation (Tram et al. 2020b). Deletion of *cheY* and *cheW* genes in *C. jejuni* enhanced the formation of biofilm-mass, even though motility of both mutants was significantly reduced in the planktonic state (Tram et al. 2020b). The authors suggested that the enhanced biofilm-mass production could be due to the higher autoagglutination displayed by these mutants. In contrast, defects in robust biofilm formation at the air-media interface were reported for *C. jejuni* mutants lacking functional *cheA*, *cheY*, *cheW* or *cheV* genes (Reuter et al. 2020). The authors concluded that the chemotaxis signalling system is rather necessary for organized biofilm formation at the air-media interface than for biofilm formation per se. The contradicting findings described in these studies might also be due to differences in the experimental conditions or biofilm detection assays applied. Moreover, deletion of the chemoreceptor Tlp3 resulted in enhanced biofilm formation, while deletion of Tlp8 resulted in reduced biofilm formation rates by respective *C. jejuni* mutants (Chandrashekhar et al. 2015; Rahman et al. 2014). These data indicate that distinct chemotactic compounds as well as chemotaxis signalling pathway are essentially involved in biofilm formation by *C. jejuni*.

2.4.4 Stress Response-Associated Genes

The influence of oxidative stress on the biofilm formation capacity of *C. jejuni* has been intensively investigated at the molecular level. Deletion of alkyl hydroperoxide reductase (*ahpC*) and catalase A (*katA*) genes increased biofilm formation by the respective mutant strains (Oh and Jeon 2014). Results from confocal laser scanning microscopy support the assumption that AhpC is involved in the development of *C. jejuni* microcolonies at the early stages of biofilm formation. This role of *ahpC* was further confirmed elegantly by genetic manipulation of *perR* and *cosR* genes encoding positive and negative regulators of *ahpC*, respectively (Oh and Jeon 2014; Turonova et al. 2015). The important role of oxygen stress responses in biofilm formation of *C. jejuni* was further confirmed by the finding that deletion of the sensor for the *Campylobacter* planktonic growth regulation system (*cprS*) reduced oxidative stress resistance, but enhanced biofilm formation in respective mutants (Svensson et al. 2009). However, deletion of the gene encoding the major carbon-starvation regulator *csrA* also rendered *C. jejuni* more prone to aerobic stress but reduced the

biofilm formation capacity, which is in contrast to many other bacteria in which *csrA* represses the biofilm formation (Fields and Thompson 2008). However, given that the translation of more than 100 genes is dysregulated in a *csrA* mutant, it is difficult to determine which of them are responsible for the observed phenotype (Fields et al. 2016; El Abbar et al. 2019). The role of *csrA* in biofilm formation of *C. jejuni* is further supported by the fact that deletion of the gene encoding polyphosphate acetyltransferase Pta (Cj0688), also under post-transcriptional control of *csrA*, resulted in reduced biofilm formation (Joshua et al. 2006). Additionally, a *C. jejuni* mutant lacking the gene for the iron-binding protein Dps displayed increased susceptibility to H_2O_2 but reduced biofilm formation (Theoret et al. 2012). Deletion of *spoT* (involved in the stringent stress response) and recombinase A (*recA*) enhanced biofilm formation especially at aerobic conditions (Feng et al. 2018; Svensson et al. 2009). In addition, results from both studies demonstrated that the lack of *spoT* and *recA* enhanced lysis of the bacteria thereby releasing high molecular DNA, which is one of the prerequisites for bacterial biofilm production.

Finally, the *C. jejuni* biofilm production is linked to intracellular levels of inorganic polyphosphates, which play crucial roles in stress tolerance and virulence of the pathogen (Kumar et al. 2016). Deletion of genes coding for both polyphosphate kinases Pkk 1 and Pkk 2 as well as for the alkaline phosphatase PhoX (Cj0145) resulted in enhanced *C. jejuni* biofilm production and surface attachment. (Drozd et al. 2014; Gangaiah et al. 2009, 2010). Taken together these data demonstrate that various stressors induce biofilm formation of *C. jejuni* via activation of the major stress response regulons known to date.

2.4.5 Surface Structure-Associated Genes

The production of the peptidyl prolyl cis–trans isomerase Peb4, involved in folding of integral outer membrane proteins, is increased in *C. jejuni* cells living in biofilms (Kalmokoff et al. 2006). Mutational analysis of the corresponding gene revealed that Peb4 is required for both adhesion and attachment of *C. jejuni* to host cells in vitro and for biofilm-mass formation (Asakura et al. 2007). In contrast, deletion of this gene in another *C. jejuni* strain resulted in enhanced biofilm-mass formation (Rathbun et al. 2009). These conflicting results might be due to strain-specific variations in the genetic background or by polar effects of the mutation strategy, but this awaits further evaluation. In addition, protein glycosylation is essentially involved in *C. jejuni* biofilm formation. Mutational analysis of the *pglB* gene by targeted deletion revealed that *N*-linked protein glycosylation reduces the biofilm formation capacity of *C. jejuni*, is required for resistance to heat and salt but decreases the resistance to peroxide (Cain et al. 2019). In contrast, *N*-linked protein glycosylation mediated by EptC enhances biofilm formation, indicating that the modulation of biofilm formation by *N*-linked glycosylation is highly dependent on the glycosylated proteins involved (Cullen et al. 2013; Lim and Kim 2017; Scott et al. 2012). Finally, *C. jejuni* lipooligosaccharide (LOS) structures influence the biofilm formation capacity as indicated by enhanced biofilm formation in *C. jejuni waaF* or *lgtF* deletion mutants

with truncated LOS. However, mutational analysis by targeted deletion revealed that LOS modifications by GalT or CstII enzymes did not influence the biofilm-mass, which was comparable in deletion mutants and the wild-type strain (Naito et al. 2010). Besides the LOS surface structure, *C. jejuni* has the ability to coat its surface with a polysaccharide capsule (CPS), being the major serodeterminant of the Penner scheme (Karlyshev et al. 2000). Given that polysaccharides are a common component in the EPS, the knowledge about the impact of CPS on the biofilm formation capacity of *C. jejuni* is still scarce. Deletion of the gene *kpsM*, involved in the transport of capsular polysaccharides across the inner membrane, resulted in enhanced biofilm formation of this uncapsulated *C. jejuni* mutant (Joshua et al. 2006). However, the mechanisms responsible for this phenotype have to be elucidated in future studies. In conclusion, these observations indicate that glycosylation state of surface molecules is essentially involved in *C. jejuni* biofilm formation.

2.5 *Control Strategies Targeting* C. jejuni *Biofilms*

Given that the EPS structure of biofilms protects the microorganisms from physical, chemical and environmental stresses, disruption of the EPS structure is a favoured strategy to combat bacterial pathogens in biofilms (Devaraj et al. 2019). Since eDNA is an essential component of the EPS produced by many bacteria, DNase treatment is a promising measure for inhibition of biofilm formation and for the degradation of established biofilms which has been also successfully proven for *C. jejuni* biofilms (Brown et al. 2015; Feng et al. 2018; Sharma and Pagedar Singh 2018; Svensson et al. 2014). In addition, treatment of *C. jejuni* with the phytochemicals trans-cinnamonaldehyde, eugenol and carvacrol before and after biofilm formation reduced the biofilm-mass (Wagle et al. 2019). Application of all three substances at bactericidal concentrations killed the majority of bacterial cells also in mature biofilms within 10 min (Wagle et al. 2019). Notably, sublethal concentrations of these phytochemicals downregulated periplasmic nitrate reductase NapA involved in energy generation and the chaperon DnaK involved in stress responses by *C. jejuni* cells in the biofilms (Wagle et al. 2019). While the mechanisms by which phytochemicals reduce *C. jejuni* biofilm formation capacity await further investigation, it seems noteworthy that citrus extracts reduced the biofilm-mass of *C. jejuni* (Castillo et al. 2014), most likely by reduction of AI-2 activity (as described in Section Phenotypes of *C. jejuni luxS* Mutants). Finally, biofilm-mass formation by *C. jejuni* in mono- and multispecies cultures was significantly inhibited by zinc oxide nanoparticles, which are small and have a high oxidative potential (Melo et al. 2017; Zhong et al. 2020). In summary, even though several strategies to inhibit *C. jejuni* biofilm formation or to eliminate *C. jejuni* in mature biofilms have been developed, their efficacy as hygiene measures under practical conditions still needs to be investigated in detail.

3 Quorum Sensing

Bacteria adapt their metabolism according to the surrounding environment not only within single cells but also at a multicellular level (Miller et al. 2002). Several processes such as biofilm formation, expression of virulence factors, competence for DNA-uptake or bioluminescence are of particular benefit in multicellular communities (Mukherjee and Bassler 2019). To collectively regulate these processes, bacteria use a cell-to-cell communication system known as quorum sensing (QS). QS is mediated by small signalling molecules, termed autoinducers (AIs), which accumulate in the environment in a cell density dependent manner. The AIs bind to specific bacterial receptors and induce the expression of distinct target genes. Depending on the signalling molecule produced and the presence of appropriate receptors, bacteria can communicate on intra-species, inter-species, inter-genera as well as inter-kingdom levels. The regulation by QS is assumed to be a highly complex process since many QS processes involve more than one signal-receptor combination, exerting their functions in a hierarchical cascade (Abisado et al. 2018). For example, four different QS-pathways are known in *P. aeruginosa*, namely the Las-, Rhl-, Pqs- and IQS-systems. Expression of virulence genes is regulated by AI-RhlR complex, and for the induction of RhlR-system, one of the other three QS-pathways is required (Papenfort and Bassler 2016). Furthermore, it has been described that some bacteria might only sense an AI without the ability to produce it. This is also true for *P. aeruginosa*, which does not produce AI-2, whereas AI-2 molecules generated by other bacteria alter the gene expression in this pathogen (Duan et al. 2003).

3.1 Quorum Sensing Signalling Mechanisms

Three major categories of signalling molecules, namely AI-1, AI oligopeptides (AIP) and AI-2, have been described. AI-1 are used by Gram-negative bacteria, while AIP serve as signalling molecules in Gram-positive bacteria. Both Gram-positive and Gram-negative bacteria utilize AI-2 (a furanone) as signalling molecules. To date, additional AI molecules were identified such as the *Pseudomonas* quinolone signal, diffusible signal factors and AI-3. It is reasonable to postulate that additional AI molecules exist (LaSarre and Federle 2013; Papenfort and Bassler 2016).

AI-1 molecules are acylated homoserine lactones (AHL) composed of an invariant homoserine lactone ring attached to an acyl chain, which can vary in the length of carbon atoms, in saturation and in the oxidation state (LaSarre and Federle 2013). These AHLs are synthesized from S-adenosylmethionine (SAM) by concerted action of the LuxI enzyme family members and acylated acyl carrier proteins. Notably, AI-2 is a by-product of the activated methyl cycle (AMC). Within the AMC, LuxS catalyzes the cleavage of S-ribosylhomocysteine (SRH) to homocysteine and 4,5-dihydroxyl-2,3-pentanedion (DPD), which spontaneously cyclize into AI-2 (Winzer et al. 2002). While *Vibrio harveyi* recognizes the borated form of AI-2, *E. coli*

and other *Enterobacteriaceae* sense the borate-free form of AI-2 (Chen et al. 2002; Miller et al. 2002). Even though the knowledge about QS mechanisms in other bacterial species is constantly growing, information regarding QS in bacteria of the genus *Campylobacter* is rather limited. In 2002, the presence of a *luxS* gene homolog and active production of AI-2 by *C. jejuni* was reported for the first time (Elvers and Park 2002). Whereas several other *Campylobacter* species also produce AI-2, no AI-2 production could be determined in *C. lari*, *C. insulanigrae* and *C. peloridis* (Golz et al. 2012; Tazumi et al. 2011). So far, no AI-1 synthase has been identified in the *C. jejuni* genome. Only one publication described the production of a putative AI-1 molecule (cjA) by *C. jejuni* (Moorhead and Griffiths 2011). The structure of cjA could not be determined, but it was demonstrated that addition of exogenous AI-1 compounds induced the expression of the *C. jejuni* virulence genes *cadF*, *ciaB*, *cdtB* and *flaA* and supported the transition of the pathogen to the dormant—so-called viable but not culturable (VBNC)—state. To date, no additional *C. jejuni* QS signalling molecules have been identified. While most AI-1 molecules can diffuse freely across bacterial membrane, several AI-1 as well as hydrophilic AI-2 molecules might require active transport across the cell membrane (LaSarre and Federle 2013; Pereira et al. 2013). In *E. coli*, AI-2 export is mediated by YdgG, a transmembrane protein belonging to the large group of the so-called AI-2 exporter superfamily (Herzberg et al. 2006; Rettner and Saier 2010). So far, no further AI export systems have been described. However, AI-2 export in *C. jejuni* is modulated by a small non-coding RNA (CjNC110). Mutational analysis by targeted deletion of the CjNC110 sequence revealed decreased extracellular AI-2 levels but increased intracellular levels of AI-2, suggesting that CjNC110 is required for modulation of the AI-2 transport to the extracellular space (Kreuder et al. 2020).

Gram-negative bacteria commonly sense AI-1 molecules by cytoplasmic LuxR-Type receptors, which act as transcription factors or by two-component membrane-bound histidine kinases (Papenfort and Bassler 2016). For detection of AI-2, different receptor types have been described so far. *Vibrionaceae* sense AI-2 by a transmembrane receptor, thereby inducing an intracellular signalling cascade. In contrast, AI-2 is imported and phosphorylated via ABC-transporters by several *Enterobacteriaceae, Bacillaceae* and *Rhizobiaceae* (Pereira et al. 2013). The phosphorylated AI-2 stabilizes transcription factors, which in turn enable the regulation of target gene expression. For *E. coli* and *Helicobacter pylori*, chemoreceptors have been identified sensing AI-2 as chemoattractant and chemorepellent, respectively (Hegde et al. 2011; Rader et al. 2011). However, the low sequence homologies of the AI-2 receptors led to the postulation that additional receptor types may exist (Papenfort and Bassler 2016; Pereira et al. 2013). No AI-2 receptor homolog has been identified in *Campylobacter* yet. However, the results obtained from an AI-2 uptake assay prompted us to speculate that *C. jejuni* might perceive AI-2 by a two-component regulatory system rather than by an ABC-transporter system (Adler et al. 2015).

3.2 *Phenotypes of* C. jejuni luxS *Mutants*

It is still under debate whether *C. jejuni* is using AI-2 to regulate their behaviour as mostly conflicting results were reported. Whether these conflicting results depend on strain variation, culture conditions, methods and/or mutation strategies applied has to be elucidated in the future. Nevertheless, we tried to summarize the findings on putative QS-related *C. jejuni* phenotypes published so far. Since no specific AI-2 receptor of *Campylobacter* is known so far, AI-2-dependent phenotypes have primarily been investigated using *luxS* mutants of various *C. jejuni* strains. Given that LuxS is required for the AMC, it is necessary to complement all experimental assays including a *luxS* mutant by the addition of exogenous AI-2 to determine whether the phenotypes observed are due to interrupted metabolism or lack of AI-2. Recent investigations confirmed that homocysteine and SHR concentrations were significantly reduced or enhanced in a *C. jejuni luxS* mutant compared to the parental strain, respectively (Mou and Plummer 2016). However, reduction of the methionine and SAM concentrations as a result of the *luxS* deletion was less pronounced as expected. Furthermore, the methylome profile of this *luxS* mutant was comparable to that of the wild-type (Mou et al. 2014), indicating that the observed phenotypes of *luxS* mutants are not due to a complete lack of methionine or SAM metabolites (Mou and Plummer 2016). Furthermore, no morphological changes in cell shape or flagella morphology have been determined for *luxS* mutants of *C. jejuni* strains 81116, NCTC11168 or IA3902 (Jeon et al. 2003; Mou and Plummer 2016). The phenotypes of *C. jejuni luxS* mutants are summarized in Fig. 2.

Despite that fact that besides AI-2, also the disruption of the AMC may influence bacterial growth, the multiplication of *C. jejuni luxS* mutants has been extensively

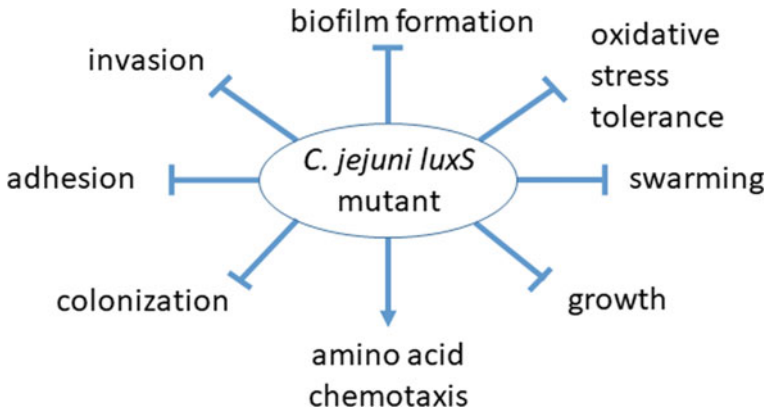

Fig. 2 Overview of *C. jejuni luxS* mutant phenotypes. Besides enhanced chemotaxis towards amino acids, reduced colonization, adhesion, invasion, biofilm formation and swarming abilities as well as reduced oxidative stress tolerance and growth kinetics have been described for *C. jejuni luxS* mutants. However, several phenotypes were only observed for different *C. jejuni luxS* mutants or under specific culture conditions (for details see text)

investigated. Remarkably, reduced growth rates were reported for the *C. jejuni* strain 81–176 with inactivated *luxS* gene, but not for the strains NCTC11168, 81116 and M129 (Elvers and Park 2002; He et al. 2008; Holmes et al. 2009; Jeon et al. 2003; Mou and Plummer 2016; Plummer 2012; Quinones et al. 2009; Reeser et al. 2007). Strain-specific differences in growth-related phenotypes of *C. jejuni luxS* mutants were confirmed by a detailed analysis of various strains grown under different conditions (Adler et al. 2014). These results indicated that the NCTC11168Δ*luxS* mutant in which the *luxS* gene is replaced by an antibiotic resistance gene showed reduced growth in comparison with the wild-type strain both under substrate limited and nutrient-rich conditions. In contrast, two different *luxS* mutants of *C. jejuni* strain 81–176 exhibited growth defects under substrate limited conditions only. Genetic complementation restored the growth kinetics of both mutants of strain 81–176, while the chemical complementation by AI-2 only partially restored growth of the Δ*luxS* mutant of the *C. jejuni* NCTC11168 strain. These data indicate that *C. jejuni* growth might be influenced by *luxS* and AI-2 but in a strain-dependent manner and under certain nutritional conditions only.

Results from a majority of studies showed that motility of *C. jejuni luxS* mutants on swarming plates is strongly reduced, which was independent of strain background or culture conditions (Adler et al. 2014; Elvers and Park 2002; Holmes et al. 2009; Jeon et al. 2003; Plummer et al. 2011; Quinones et al. 2009; Simunovic et al. 2020). However, for the 81–176Δ*luxS* mutant constructed by He and colleagues (2008), reduced motility was only detected if bacteria were incubated on Mueller–Hinton medium-based swarming plates at 37 °C. In Brucella broth, however, the motility of this mutant was neither reduced at 37 °C nor at 42 °C (Adler et al. 2014; He et al. 2008). In contrast, the motility of the 81–176::*luxS* mutant (insertion of antibiotic resistance cassette within the *luxS* gene) constructed by Quinones and co-workers (2009) was reduced in both media and at both temperatures (Adler et al. 2014; Quinones et al. 2009). These results suggest that differences in some strain-specific phenotypic properties of *C. jejuni luxS* mutants are indeed caused by polar effects generated by the genetic manipulations applied. Even though the motility of the NCTC11168Δ*luxS* mutant was not restored by the addition of exogenous AI-2 in the study of Holmes and colleagues (2009), the motility of other *C. jejuni luxS* mutants was at least partially restored by genetic complementation or upon the addition of exogenous AI-2 (Adler et al. 2014; Plummer et al. 2011; Quinones et al. 2009). The latter studies revealed that AI-2 influences the motility of *C. jejuni* on swarming agar. So far, the mechanisms of AI-2-dependent regulation are not understood. Several studies investigating gene expression patterns of *luxS* mutants revealed conflicting results. While reduced *flaA* gene expression was reported for a 81116*luxS* mutant, no difference on protein level nor in flagellar morphology was observed (Jeon et al. 2003). Several flagellar assembly/regulation genes were differentially expressed in 81–176Δ*luxS* cultivated at 42 °C, even though under these conditions the swarming ability of the mutant was comparable to the wild-type (He et al. 2008). Furthermore, Holmes and colleagues (2009) determined the downregulation of several flagellar-associated genes and subsequently reduced swarming capabilities, but the authors could neither restore the gene expression pattern nor the

phenotype by adding exogenous AI-2. Therefore, the confusing and in part contradicting results obtained by mutational analysis of the *C. jejuni luxS* locus indicate that further work under standardized and better controlled experimental conditions is essential for the investigation of the complex mechanisms underlying the interactions of *C. jejuni* LuxS and/or AI-2 with motility.

Whether AI-2 exhibits a direct chemotactic function in *C. jejuni* has not been determined yet. Nevertheless, when compared to the wild-type strain, a Δ*luxS* mutant of the 81–176 strain displayed enhanced chemotactic behaviour towards amino acids (Quinones et al. 2009). Holmes and co-workers (2009) reported reduced mRNA-levels of the genes encoding *cheA* and the chemoreceptors Tlp1, -2 and -4 (Cj1506, Cj0144, Cj0262) in a NCTC11168Δ*luxS* mutant. However, no significantly different regulation in expression of the *cheA, cheB, cheR, cheV* and *cheW* genes has been observed for the Δ*luxS* mutant of *C. jejuni* strain 81–176 (He et al. 2008).

Molecular mechanisms related to host-interactions like adhesion, invasion, cytotoxicity and intestinal colonization are basic to *C. jejuni* pathogenicity (see Chapter "*Campylobacter* Virulence Factors and Molecular Host–pathogen Interactions" of this book). Expression of *Pseudomonas*, *Vibrio cholerae* and *E. coli* virulence factors is regulated by QS (Furniss and Clements 2018; Jiang et al. 2019; Papenfort and Bassler 2016). However, studies investigating the AI-2-dependent regulation of pathogenicity in *Campylobacter* are still scarce. *C. jejuni* LuxS was essential for adhesion of *C. jejuni* 81–176 as demonstrated with a *luxS* mutant of this strain and cultured LMH cells in vitro (Quinones et al. 2009). In contrast, deletion of *luxS* did not alter adhesion of *C. jejuni* strain NCTC11168 on INT-407 cells, while the invasion rate of the Δ*luxS* mutant used in this study was reduced (Simunovic et al. 2020). Interestingly, the invasion rate of a NCTC11168Δ*luxS* mutant was only slightly reduced in Caco-2 cells (Elvers and Park 2002). Whether these highly varying and confusing observations were due to different properties of *luxS* mutants or of the different cell lines remains open. However, complementation with exogenous AI-2 is needed to prove that all these phenotypes were caused by the lack of AI-2 and did not result from disruption of the AMC.

Additional contradicting results concerning the influence of *luxS* on *C. jejuni* colonization capacity were obtained by the analysis of *C. jejuni luxS* mutant strains in animal models in vivo. While the *luxS* mutant of *C. jejuni* strain IA3902 displayed a loss of chicken colonization, this ability was only reduced in a *luxS* mutant of *C. jejuni* strain 81–176, whereas the NCTC11168 *luxS* mutant colonized chickens with similar rates compared to the wild-type strain (Plummer et al. 2012; Quinones et al. 2009). It is not clear yet if these contradicting findings are the result of real strain-dependent differences or are caused by the mutational strategy applied. No general conclusion regarding the impact of AI-2 on the *C. jejuni* colonization capabilities could be drawn. Given that AI-2 produced by commensal microbiota could also have an impact on the phenotype of *C. jejuni luxS* mutants, and despite the difficulties in determining whether altered phenotypes were due to lack of AI-2 or disrupted AMC, the results summarized here should be interpreted with caution.

The NCTC11168 *luxS* mutant constructed by Elvers and Park (2002) did not show altered hydrogen peroxide or paraquat susceptibility, while the 81–176 *luxS* mutant

was less resistant to cumene hydroperoxide and hydrogen peroxide as compared to the wild-type (He et al. 2008). Gene expression analysis revealed that expression of the peroxide stress defence-related genes *ahpC* (encoding an alkyl hydroxide reductase) and *tpx* (encoding a thiol peroxidase) was reduced in the 81–176 *luxS* mutant after oxidative stress treatment as compared to the wild-type strain (He et al. 2008). The important role of *LuxS* in the *C. jejuni* oxidative stress response is further underlined by the fact that the *Campylobacter* oxidative stress regulator (CosR) negatively regulates the expression of *luxS* (Hwang et al. 2011). Furthermore, a *C. jejuni* NCTC11168 *luxS* mutant displayed lower survival rates as compared to the wild-type strain at cold stress (Ligowska et al. 2011). However, whether these stress responses are directly modulated via AI-2-dependant QS remains to be elucidated.

The biofilm-mass developed by a Δ*luxS* mutant of *C. jejuni* strain M129 was significantly reduced compared to the wild-type and could be partially restored by the addition of cell free supernatants of the wild-type strain (Reeser et al. 2007). Furthermore, reduced adhesion on polystyrene surfaces was reported for a NCTC11168 *luxS* mutant (Simunovic et al. 2020). In contrast, the attachment on stainless steel coupons was comparable for both the NCTC11168 *luxS* mutant and the wild-type strain (Bezek et al. 2016). In addition, biofilm formation of the *C. jejuni* strain 81–176 was reduced by the application of AI-1 molecule cjA (Moorhead and Griffiths 2011). Taken together, these data suggest that the process of *C. jejuni* biofilm formation is regulated by concerted action of several QS systems like in other bacteria (Paluch et al. 2020; Papenfort and Bassler 2016).

3.3 Quorum Quenching

The inhibition of QS, also termed quorum quenching (QQ), has raised much attention in recent years (Paluch et al. 2020). QQ could be implemented as a preventive or therapeutic approach to combat pathogenic bacteria and could be achieved at different stages, e.g. by inhibition of signalling molecule production, degradation of signalling molecules or blockage of the receptor. This is underlined by increasing numbers of patents and applications for QQ compounds and their functions (reviewed by Chen et al. 2018). Even though the exact role of AI-2-mediated QS has not been elucidated for *C. jejuni*, several authors investigated putative agents that can interrupt QS mechanisms. For example, the application of citrus extract nearly eliminated AI-2 activity in cell-free supernatant of several *C. jejuni* strains and further reduced their motility, biofilm formation, adhesion and invasion of HeLa cells as well as the expression of *cadF* and *ciaB* virulence genes (Castillo et al. 2014, 2015). In support, nearly all the 20 natural plant extracts investigated by Simunovic and co-workers (2020) altered several phenotypes of *C. jejuni*. The ethanolic extract of *Rhodiola rosea* (roseroot) had the greatest potential to inhibit AI-2 production, motility, adhesion to polystyrene surface and invasion into INT-407 cells by strain NCTC11168, which were comparably to phenotypes observed for the *C. jejuni* Δ*luxS* mutant. Furthermore, none of the tested compounds exerted a synergistic effect on the phenotype of the *C. jejuni*

Δ*luxS* mutant. These data implicate that these compounds could inhibit AI-2 QS circuit and thereby alter the behaviour of *C. jejuni*. Furthermore, quinolinone alkaloid mixture extracts from the tree *Euodia ruticarpa* reduced AI-2 production and number of attached bacteria on a polystyrene surface by *C. jejuni* NCTC11168 as well as by the Δ*luxS* and Δ*cmeB* mutants (Bezek et al. 2016). However, as attachment of the parental strain and the Δ*luxS* mutant were comparable, the authors concluded that the effects of the extract are not related to AI-2-dependent QQ.

4 Concluding Remarks

The comprehensive review of the literature documented in this book chapter indicates that some aspects of QS and biofilm formation by *C. jejuni* have been investigated to date, but both processes are still not well understood at the molecular level. Obvious shortcomings in these important fields of research are caused by lack of precise genetic analysis of the biological systems involved and by the extensive genetic variation of *C. jejuni* at the isolate level. These limitations should be overcome in the future by standardized and complete genetic analysis including the mutation strategies applied and by whole genome analysis of *C. jejuni* at the strain level. The facts that *C. jejuni* produces AI-2 and that some phenotypes of *luxS* mutants could be partially restored by exogenous AI-2 point towards regulatory functions of AI-2 in *C. jejuni*. Therefore, regarding the QS system, it seems highly recommended that future research should focus on the identification and biochemical characterization of a possible AI-2 receptor including complementation of manipulated pathways by exogenous AI molecules to finally prove the QS-dependent phenotypes of artificially generated *C. jejuni* mutants. The promising results obtained by AI-2-dependent QS-signalling should strengthen intensive research on potential additional AI molecules and their regulatory functions in *C. jejuni* and other *Campylobacter* species.

The manifold environmental and intrinsic conditions affecting *C. jejuni* biofilm formation provide strong evidence that *C. jejuni* actively produces biofilms to survive unfavourable conditions outside vertebrate hosts. Therefore, a deeper understanding of *C. jejuni* biofilm formation is a key to direct future research for improvement of biosafety and hygiene in slaughter and food processing lines. Altogether, biochemical properties of *C. jejuni* QS and biofilms will guide the development of innovative and novel strategies to diminish the entry or cross-contamination of *Campylobacter* in livestock and the food processing chain.

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