Investigation on the regulatory roles of a second messenger cyclic diadenosine-monophosphate (C-DI-AMP) in streptococcus gallolyticus subsp. gallolyticus

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**NANYANG
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**INVESTIGATION ON THE REGULATORY ROLES OF A
SECOND MESSENGER CYCLIC DIADENOSINE-
MONOPHOSPHATE (C-DI-AMP) IN *STREPTOCOCCUS*
GALLOLYTICUS SUBSP. *GALLOLYTICUS***

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INTERDISCIPLINARY GRADUATE SCHOOL

SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE SCIENCES

ENGINEERING

2019

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TEH WOUI KEONG

Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

August 5, 2019

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TEH Wooi Keong

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

August 5, 2019

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Date

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LIANG Zhao-xun

Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal where I was the first author.

Chapter 3 is published as W.K. Teh, S. Dramsi, T. Tolker-Nielsen, L. Yang, M. Givskov. Increased intracellular cyclic di-AMP levels sensitize *Streptococcus gallolyticus* subsp. *gallolyticus* to osmotic stress and reduce biofilm formation and adherence on intestinal cells. *Journal of Bacteriology* **201**:e00597-18 (2019). DOI: 10.1128/JB.00597-18.

The contributions of the co-authors are as follows:

- Prof Givskov provided the initial project direction.
- I prepared the manuscript drafts and revised the manuscript together with Prof Dramsi, Prof Tolker-Nielsen, A/P Yang and Prof Givskov.
- I designed the study and performed all the laboratory works at the Singapore Centre for Environmental Life Sciences Engineering. I also analysed the data.
- Quantification of c-di-AMP by liquid chromatography-mass spectrometry was performed in Singapore Phenome Centre as service.
- Prof Dramsi provided *Streptococcus gallolyticus* subsp. *gallolyticus* UCN34 wildtype and the bacteriocin-deletion strain, *Streptococcus gallolyticus* subsp. *macedonicus*, plasmids for gene deletion and complementation, and antibodies for the detection of pilus 3 (used in Western Blot) and *Streptococcus gallolyticus* subsp. *gallolyticus* UCN34 (used in immunofluorescence).
- A/P Yang and Prof Givskov acquired the funding for this project.

Chapter 4 and 5 are unpublished works.

August 5, 2019

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Date

A handwritten signature in black ink, consisting of stylized, cursive letters that appear to read 'TEH' followed by a more complex, flowing script for the surname 'Wooi Keong'.

.....

TEH Wooi Keong

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Summary

Streptococcus gallolyticus subsp. *gallolyticus* is an emerging pathogen responsible for infective endocarditis especially in the elderly. Many infected patients were found to exhibit a condition of concomitant colorectal neoplasia; therefore colonoscopy is now advised for *S. gallolyticus*-infected patients to screen for the presence of early stage colorectal tumours. It has been recently proposed that *S. gallolyticus* subsp. *gallolyticus* plays an auxiliary role in colorectal cancer development, albeit the detailed mechanisms allowing *S. gallolyticus* subsp. *gallolyticus* to promote the tumorous cells progression remains unidentified.

Previous studies suggested that bacterial biofilms residing in the gastrointestinal tract may play a role in the colorectal cancer development. In this dissertation, the primary objective was to understand *S. gallolyticus* subsp. *gallolyticus* biofilm biology, for the interest of further investigations on the roles of *S. gallolyticus* subsp. *gallolyticus* biofilms in colorectal cancer development. I first provide evidence that *S. gallolyticus* subsp. *gallolyticus* biofilm development is controlled by the bacterial second messenger cyclic diadenosine-monophosphate (c-di-AMP). It was found that the deletion mutant ($\Delta gdpP$) of the c-di-AMP specific phosphodiesterase GdpP accumulating higher intracellular level of c-di-AMP displayed defect in biofilm development. Further investigations focusing on the molecular mechanisms of c-di-AMP control on *S. gallolyticus* subsp. *gallolyticus* biofilm development revealed that the expression of the extracellular pullulanase Pula and the extracellular appendage pili Pil3 is responsive to the intracellular c-di-AMP level. Both the

reduced expression of PulA and Pil3 contribute in part to biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant.

In view of the numerous studies that have indicated c-di-AMP to play a primary role in controlling bacterial osmotic homeostasis, the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant was characterised. It was found that the mutant cells were more sensitive to high osmotic stress and were smaller in cell size compared to the parental strain. It was also found that the expression of various transporters involved in controlling the uptake of osmolytes was down-regulated in the $\Delta gdpP$ mutant compared with the parental strain.

Collectively, the present PhD thesis supports the notion that c-di-AMP plays a conserved role in maintaining osmotic homeostasis in many Gram-positive bacteria and establishes that c-di-AMP is a bona fide signalling molecule controlling *S. gallolyticus* subsp. *gallolyticus* biofilm development.

Chapter 1 - Introduction

1.1 Background information

Streptococcus gallolyticus subsp. *gallolyticus*, a Lancefield Group D *Streptococcus*, is an emerging opportunistic pathogen. It is one of the gut microbiota members present in 2.5 to 15% of healthy individuals (1). Upon entering into bloodstream, *S. gallolyticus* subsp. *gallolyticus* can colonise damaged heart valves, causing infective endocarditis. *S. gallolyticus* subsp. *gallolyticus* infections can represent up to 25% of all infective endocarditis cases (2).

S. gallolyticus subsp. *gallolyticus* was previously known as *S. bovis* biotype I, a traditional nomenclature that has been replaced with a system that clearly differentiate *S. gallolyticus* at the subspecies level. The renaming of *S. bovis* is clinically important. Unlike *S. infantarius* subsp. *coli* (biotype II.1) and *S. gallolyticus* subsp. *pasteurianus* (biotype II.2), *S. gallolyticus* subsp. *gallolyticus* is the specific *S. gallolyticus* that exhibits a strong association with the incidence of colorectal cancer among the endocarditis patients infected with *S. gallolyticus* (3-6). The significant enrichment of *S. gallolyticus* subsp. *gallolyticus* in the stools of these patients was reported as early as in 1951, and subsequently supported by many case reports and systemic reviews (1, 6-10). Due to the strong association of *S. gallolyticus* subsp. *gallolyticus* with colorectal cancer, UptoDate database currently lists a follow up colonoscopy as a strong medical recommendation for the endocarditis patients infected with *S. gallolyticus*.

It is unclear whether the increased presence of *S. gallolyticus* subsp. *gallolyticus* in the gastrointestinal tract of colorectal cancer patients triggers cancer development, or is merely a consequence of the disease development. Although it was recently reported that *S. gallolyticus* subsp. *gallolyticus* can promote the proliferation of tumour cells, this was not observed with the normal, healthy cells (11). Current research data suggest that *S. gallolyticus* subsp. *gallolyticus* may be an auxiliary contributing factor, in which the early stage of colorectal cancer microenvironment promotes the colonisation of *S. gallolyticus* subsp. *gallolyticus* in the gastrointestinal tract, and *S. gallolyticus* subsp. *gallolyticus* subsequently promotes cancer progression (12). Sequentially, the change of mammalian cell physiology alters the membrane barrier, and *S. gallolyticus* subsp. *gallolyticus* translocates paracellularly through the colonic epithelial cells into the bloodstream, causing infective endocarditis (13).

To have increased chances of initiating infections, *S. gallolyticus* subsp. *gallolyticus* first need to express colonisation factors to colonise and persists – possibly forming biofilms – at the mucosal layer in the gastrointestinal tract. Identification of these colonisation factors is therefore important to understand the pathogenicity of *S. gallolyticus* subsp. *gallolyticus*. This can potentially provide means for early diagnostics as well as suggest novel treatment targets particularly in cases where *S. gallolyticus* subsp. *gallolyticus* is carcinogenic to the host, with the aim of mitigating disease progression.

1.2 Research gaps and objectives

Although the clinical importance of *S. gallolyticus* subsp. *gallolyticus* has been highlighted in many clinical case reports, not much is known about the pathogenicity of this emerging pathogen. Notably, the diseases that *S. gallolyticus* subsp. *gallolyticus* is associated to, especially endocarditis, is considered biofilm-associated. Even more intriguing is the potential link of *S. gallolyticus* subsp. *gallolyticus* biofilm with the colorectal cancer initiation or progression (14). Therefore, studying *S. gallolyticus* subsp. *gallolyticus* from the biofilm biology perspective may offer interesting insights into the pathogenicity of *S. gallolyticus* subsp. *gallolyticus*. In this thesis, I explore the signal governing the biofilm development of *S. gallolyticus* subsp. *gallolyticus*, and investigate the underlying molecular mechanisms on how is the process regulated. These series of investigations are described in three main chapters:

In **Chapter 3**, I aim to study the roles of the second messenger cyclic diadenosine-monophosphate (c-di-AMP) in *S. gallolyticus* subsp. *gallolyticus*. I established the study on a clinical isolate strain UCN34 that was isolated from an endocarditis patient later diagnosed with colorectal cancer. I created a deletion mutant of c-di-AMP phosphodiesterase in the UCN34 background, and explored the phenotypic alterations of this deletion mutant from various aspects, including the bacterial growth, the cellular morphology, the susceptibility to the beta-lactam antibiotics, the sensitivity to osmotic stress and the biofilm formation and cell adherence. A genome-wide transcriptomic analysis

(RNA-seq) was also performed to have an overview on the cellular processes altered by the intracellular c-di-AMP level.

In the process of generating the deletion mutant of c-di-AMP phosphodiesterase, I co-isolated a deletion mutant of c-di-AMP phosphodiesterase with a point mutation in the genome that partially restores the biofilm developmental defect. It was hypothesised that the mutation may provide clue to understand the molecular mechanism on how c-di-AMP regulates the biofilm development in *S. gallolyticus* subsp. *gallolyticus*. Results of the investigation are presented in **Chapter 4**.

One of the intriguing results of the RNA-seq experiment from Chapter 3 was that c-di-AMP regulates the expression of Pil3. Pil3 is known to be important for *S. gallolyticus* subsp. *gallolyticus* to bind to mucin and to colonise in the murein gastrointestinal tract (15). Although the regulation of the Pil3 expression is known to rely on the number of GCAGA tandem repeats in the promoter region through a transcriptional attenuation-like mechanism, the signal(s) governing the regulation remains unknown. Therefore, the finding from the RNA-seq experiment suggested that c-di-AMP may establish a link with Pil3 expression regulation. To verify this, I used a flow cytometer to study the Pil3 expression at single cell levels in various strains with decreased or increased c-di-AMP levels. The link between the Pil3 expression and the biofilm formation in *S. gallolyticus* subsp. *gallolyticus* was also investigated. Data are presented in **Chapter 5**.

Chapter 2 - Literature Review

2.1 Bacterial physiology in disease progression

Bacterial colonisation on surfaces constitutes the first key step in the establishment of chronic bacterial infection. Once attached to surfaces, bacterial cells can continue to multiply, eventually forming clusters of cells that are structurally encased in a self-produced extracellular matrix, entering into a 'multicellular-like' lifestyle which has been named a 'biofilm'. Bacterial biofilms may account for an estimated 80% of microbial infections in humans, which includes middle ear infection, cystic fibrosis pneumonia, native valve endocarditis, and indwelling device-related infections like urinary catheter cystitis (16, 17).

Microbial biofilms are also present in the lower gastrointestinal tracts that is also known as colon (18, 19), where the surface of the epithelial cells is covered by a layer of mucus produced by the goblet cells. This layer of mucus is permissive to many bacteria to colonise and form biofilms (20). In the early 90s, the presence of this structured microbial communities in the lower gastrointestinal tracts was less well appreciated, largely due to the lack of appropriate techniques to preserve the mucus layer and the biofilm extracellular matrix, as well as the lack of more advanced microscopy technology for better visualisation (21). Indeed, the visualisation of the biofilms formed in the mucosal layer of the lower gastrointestinal tract using fluorescence *in situ* hybridisation (FISH) probes critically relies on the technique to prepare the biopsy specimens. The use of formalin, instead of the Carnoy solution as a fixative agent can destroy the mucous layer and diminish the indigenous

biofilms (22). Recently, Barnes and colleagues used low-voltage scanning electron microscopy to visualise the *Enterococcus faecalis* colonised in the germ-free murein gastrointestinal tracts. They found that *E. faecalis* formed microcolonies encased in sweater-like extracellular matrix, a structure highly resemble to the *in vitro* *E. faecalis* biofilms, providing a clear, direct evidence on the biofilms formed in the gut lining (21).

Biofilms formed at the gut mucosal layer can be associated with diseases. The biopsy specimens from the inflammatory bowel diseases patients were found covered with denser and thicker biofilms than the ones from the healthy individuals (22, 23). Importantly, at the regions where the mucosal layer was covered with prolific biofilms but lacks the signs of cellular inflammations and mucosal destruction, bacteria can also be detected in the crypt, as well as intracellularly in the gut epithelial cells (22, 23). These observations suggest that following prolonged colonisation of the region, some bacteria demonstrate the capability to cross the mucosal barrier prior to the onset of diseases. This permits a close contact of bacteria – potentially the opportunistic pathogens – with the host epithelial cells and may trigger the diseases onset and/or progression. Furthermore, the persistent presence of immunogenic bacteria in the form of biofilms may result in a higher localised concentration of bacteria antigens or secretory products that may result in greater extent of tissue damage (23).

In 2014, Dejea and colleagues investigated the general role of bacterial biofilms residing in the gut. The group defined bacterial biofilms as massive bacterial invasions ($>10^9$ bacteria/mL) of the mucus layer spanning at least a linear distance of 200 μm of the

epithelial surface (14). They compared the normal mucosa samples from colorectal cancer (CRC) patients with and without bacterial biofilms and showed that the biofilm-positive normal mucosa from the CRC patients displayed reduced crypt colonic epithelial cell E-cadherin, increased IL-6 and an activation of Stat3, which are altogether critical in colon mutagenesis (14). Redistribution of crypt colonic epithelial cell E-cadherin and an increased epithelial cell IL-6 were also noticed on the biofilm-positive normal mucosa from healthy individual, demonstrating that the observations were not restricted to the tissues from CRC patients (14). Importantly, the biofilm-positive normal mucosa from both the CRC patients and the healthy individual showed increased epithelial proliferation, while no increased cell death observed (14). Follow-up investigations demonstrated that compared to the biofilm-negative cancerous tissues, the biofilm-positive cancerous tissues have higher content of N¹, N¹²-diacetylspermine (24), a polyamine metabolite that the increased concentration is associated with eukaryotic proliferation and bacterial growth (25). This suggests that biofilm formed on the colonic tissue may increase the host polyamine metabolism, therefore promoting the host cells proliferation. Taken together, these studies argued that biofilm in the lower gastrointestinal tract can play a role in modifying the host epithelial biology, and that may be dependent on the microbiota members residing in the biofilms (14, 26) (Figure 2.1).

Figure 2.1 Bacterial biofilms drive colorectal cancer development. Multispecies bacterial biofilms forming in the inner colonic mucus layer can alter the biology of the colonic epithelial cells (CEC), affecting the E-cadherin distribution, polyamine metabolism, cell proliferation, IL-6 and Stat3 activation, which are altogether critical in colon mutagenesis. Changes of the epithelial biology can in turn promote intestinal dysbiosis, allowing the colonisation and proliferation of opportunistic bacteria that may also drive the colorectal cancer development. Figure reprinted from (27) with permission (Licence number 4694060631130).

2.2 The regulation of bacterial biofilm formation involves second messenger

Biofilm formation is a complex biological process. One of the most well described molecules governing the regulation of biofilm formation and dissolution is a second

messenger cyclic diguanosine-monophosphate (c-di-GMP). C-di-GMP is synthesised by diguanylate cyclases containing GGDEF domains and is hydrolysed by phosphodiesterases containing EAL or HD-GYP domains. In general, high intracellular levels of c-di-GMP promote biofilm formation, whereas low levels of c-di-GMP is associated with free-flowing, planktonic lifestyle (28).

The regulation of c-di-GMP on biofilm formation as well as many other cellular processes relies on various c-di-GMP effectors, including mRNA riboswitches (29, 30), transcriptional regulators (31, 32), PilZ-domain proteins (33, 34), YajQ protein family (35) and ATPase (36, 37). In the context of biofilm formation regulation, upon the increase of c-di-GMP concentrations, c-di-GMP can bind to the *Escherichia coli* YcgR to obstruct the flagellar motor function to promote surface attachment (28, 38-40). Following surface attachment, c-di-GMP at multiple gene regulatory levels activate the surface organelle (e.g. type IV pili) and extracellular matrix production (e.g. cellulose, exopolysaccharide) to facilitate the progression of microcolonies to mature biofilm (28). Binding of c-di-GMP to proteins can also result in the sequestration of proteases to prevent adhesins degradation that is crucial for surface attachment (41). When the c-di-GMP concentration decreases, c-di-GMP dissociates from YcgR to promote motility, or de-sequesters the protease to permit the degradation of adhesins, subsequently dispersing the biofilms (28).

In addition to biofilm formation, c-di-GMP also controls processes of bacterial cellular development including morphogenesis (28). For instance, the binding of c-di-GMP to TipF localises the protein to one of the bacterial poles of *Caulobacter crescentus*, allowing the

protein to bind to its polar receptor and subsequently recruiting flagellar proteins to initiate the flagellar assembly (42). C-di-GMP also affects the bacterial cell cycle. It binds to CckA, a bifunctional cell cycle histidine kinase, to stimulate the CckA phosphatase activity to dephosphorylate a transcriptional regulator CtrA responsible for controlling the cell cycle in *C. crescentus* (43, 44). The dephosphorylated CtrA is then delivered by the c-di-GMP-bound protease adaptor PopA to the ClpXP protease to be degraded, thereby permitting the chromosome replication (45-47).

C-di-GMP also modulates expression of bacterial virulence (28, 48). In addition to its regulatory role in the expression of virulence factors, such as toxins TcdA and TcdB of *Clostridium difficile* (49), c-di-GMP also interfere the bacterial quorum sensing system as well as the secretion systems including Type II, Type III and Type VI (36, 50-52). These multifaceted roles of c-di-GMP encourages further studies especially on the roles of c-di-GMP on the pathology of the new emerging pathogens as well as the roles of the other cyclic dinucleotides.

Genomic search based on the c-d-GMP turnover enzymes suggested that c-di-GMP signalling system is widespread in the phylum Proteobacteria of Gram-negative bacteria and some members of the phyla Firmicutes and Actinobacteria of Gram-positive bacteria such as *Bacillus*, *Clostridium*, *Mycobacterium* and *Streptomyces* spp. (48). However, the genome of other species of the Phyla Firmicutes and Actinobacteria such as *Staphylococcus* and *Streptococcus* spp. do not encode a functional c-di-GMP system, but rather a functional cyclic diadenosine-monophosphate (c-di-AMP) signalling system (53). C-di-AMP is

synthesised from 2 ATP molecules by diadenylate cyclases containing DAC domain, and hydrolysed by phosphodiesterases containing DHH/DHHA1 or HD domains (53, 54). Intriguingly, c-di-AMP can also be hydrolysed by an extracellular ectonucleotidase, which is important for *Streptococcus agalactiae* to control the extracellular levels of c-di-AMP to prevent exciting the host immune response (55).

Similar to c-di-GMP, the level of c-di-AMP can affect biofilm formation. In *Staphylococcus aureus* and *Streptococcus suis*, it was observed that the increased levels of intracellular c-di-AMP is associated with an increased biofilm formation (56, 57). A detailed mechanism on the regulation of biofilm formation by c-di-AMP has been demonstrated by Wu and colleagues in *Streptococcus mutans*. The group showed that the c-di-AMP phosphodiesterase deletion mutant and the c-di-AMP diadenylate cyclase-overexpressing strain, both of which have elevated c-di-AMP levels, formed more biofilms than the parental strain. It was found that in *S. mutans*, the c-di-AMP receptor protein CabPA can bind to a response regulator VicR to down-regulate the expression of a glucosyltransferase GtfB, which is involved in the extracellular polymeric substances production and is critical for the biofilm formation (58). These studies together suggest that c-di-AMP mediates biofilm formation in a similar fashion as c-di-GMP, where increased c-di-AMP levels is associated with biofilm formation, whereas decreased c-di-AMP levels is associated with planktonic lifestyle.

Nevertheless, several works investigating the effect of c-di-AMP levels on biofilm formation and development showed the opposite trend. Stulke and colleagues first

demonstrated in 2016 in *Bacillus subtilis* colony biofilm, in which the deletion of the genes encoding the c-di-AMP phosphodiesterases, resulted in the loss of complex colony formation, a phenotype suggestive of weak biofilm former (59). This regulation likely involves the interaction of c-di-AMP and a *B. subtilis* biofilm master regulator SinR to down-regulate the expression of the genes in the SinR regulon (59). Surprisingly, contradicting data were also observed in *S. mutans* and *S. aureus*. It was found that the *S. mutans* c-di-AMP diadenylate cyclase deletion mutant exhibiting low levels of c-di-AMP produces more extracellular polymeric matrix substances, has increased *gtfB* expression and enhanced glucosyltransferase activities (60). These observations are in sharp contrast to the study by Wu and colleagues (58). Similarly, Losick and colleagues reported that both a *S. aureus* c-di-AMP phosphodiesterase deletion mutant and a transposon-insertional mutant showed reduced biofilm level, as compared to the parental strain (61). Due to the lack of consistency in the observations, more experimental data are required to better understand the effect of c-di-AMP levels on biofilm formation.

2.3 The major roles of c-di-AMP control

Studies investigating the roles of c-di-AMP found that this molecule rather plays a major role in maintaining osmotic homeostasis. This is partially reflected in the c-di-AMP receptor proteins identified thus far. These receptor proteins include the potassium ion transporter KtrA, KtrC, CabP, CabPA, CabPB, TrkH (58, 62, 63), the ATP-binding protein of the osmoprotectant transporter OpuCA (62-66), and the cation/proton antiporter CpaA which likely functions to stimulate the export of sodium or potassium from the bacteria (63).

C-di-AMP also binds to the components at the upstream of some regulatory pathways, such as the transcriptional repressor BusR to repress the expression of the BusAB glycine betaine transporter (62, 63, 67, 68) and the sensor kinase KdpD of the KdpD-KdpE two component system to inactivate the high-affinity potassium transporter Kdp (63, 69). These transporters are responsible for the transport of osmolytes or compatible solutes such as potassium, carnitine and glycine betaine, which are important in the osmoregulation to maintain the bacterial cellular turgor (70). The binding of c-di-AMP to these receptors either represses the expression, or inhibits the activity of the transporters, rendering the cells unable to rapidly respond to osmotic shift. Consistently, the c-di-AMP phosphodiesterase deletion mutants, which have high cellular c-di-AMP levels, were found unable to grow in the presence of high NaCl concentrations (71, 72).

Figure 2.2 c-di-AMP receptors. A summary of the c-di-AMP receptors currently identified. Figure reprinted from (54) with authorisation from the American Society for Microbiology for use in doctoral thesis/dissertation.

The major role of c-di-AMP in osmoregulation is further supported in two recent studies indicating that the cellular levels of c-di-AMP is responsive to the availability of potassium ions and compatible solutes. In *Lactococcus lactis* and *Lactobacillus reuteri*, the uptake of potassium ion and glycine betaine leads to c-di-AMP accumulation (68). Similarly in *B. subtilis*, c-di-AMP accumulates two-fold when grown in high (5 M) than in low (0.1 M) potassium ion concentration (73). Following the elevated cellular c-di-AMP levels, c-di-AMP binds to the *kimA* (or *ydaO*) riboswitch, turning off the expression of the high-affinity

potassium transporter KimA to reduce the excessive uptake of potassium ions which would otherwise be toxic to bacteria (73). These two studies indicate that upon osmotic shift, bacteria respond by depleting or accumulating c-di-AMP to increase or decrease the uptake of osmolytes to ultimately maintain the cellular water level.

Presumably due to the major role of c-di-AMP in osmoregulation that is critical for the bacterial survival especially upon osmotic shift, c-di-AMP is therefore an essential second messenger in bacteria. This is supported by the observation that the deletion of the sole or all of the diadenylate cyclases is detrimental to the bacterial growth under the standard laboratory growth conditions. Nevertheless, the c-di-AMP diadenylate cyclase is dispensable when the deletion mutant is cultured at low (0.1 M) potassium ion concentration where the uptake of potassium ions does not reach the toxic level, as observed in *B. subtilis* (73). Similarly, when being cultured in minimal medium (74), in medium with high salt (72, 75), medium buffered with sucrose (76), or medium without osmolytes (62), or under anaerobic condition (62, 77), the c-di-AMP diadenylate cyclases deletion mutants can be successfully obtained. All of these conditions likely protect the bacteria from osmotic stressors. In many occasions, when these diadenylate cyclase mutants were subsequently passaged in the conditions not permissive for growth (e.g. rich medium, aerobic conditions, high potassium ions concentrations), the mutants developed suppressor mutations to relieve the survival stress. Many of these suppressor mutations were identified in the genes encoded proteins responsible for, or involved in osmoregulation, such as the oligopeptide transporter OppABCDF (62, 74, 75), the osmoprotectant glycine-betaine transporter GbuABC or BusAB (62, 75), the glutamine ABC transporter GlnPQ (62), the cation antiporter NhaK

(73), as well as the pyruvate carboxylase PycA that can function to accumulate citrate to modulate osmosis (75). Mutation was also found in the guanosine penta- and tetraphosphate ((p)ppGpp) synthase/hydrolase RelA, which led to decreased accumulation of (p)ppGpp, thereby activating the transcriptional regulator CodY that can indirectly repress the osmoprotectant uptake system OpuCA (74, 78).

Given that c-di-AMP functions in controlling the osmotic homeostasis, it is expected that the dysregulation of the cellular levels of c-di-AMP, resulted from the inactivation of either the diadenylate cyclases or the phosphodiesterases, can critically affect the cellular turgor. There is no machinery of active transport of water molecules in bacteria. Therefore, bacteria are required to accumulate or expel osmolytes to direct the water fluxes to prevent cell rupture or dehydration (79). In the context of the role of c-di-AMP in osmoregulation, it is postulated that at high cellular c-di-AMP levels, the uptake of potassium ions is inhibited, leading to water efflux and cell shrinkage, whereas at low cellular c-di-AMP levels, bacteria excessively accumulate osmolytes, causing water influx and increasing the cell tendency to rupture (70).

This cellular turgor model proposed by Commichau and colleagues is indeed supported by many observations. Cell shrinkage has been observed in *S. aureus* and *S. pneumoniae*, where the c-di-AMP phosphodiesterase deletion mutants were found to display a 20% reduction in cell size, and display a shortened and smaller chain morphology compared to the parental strain, respectively (56, 77, 80). Another indirect observation is the hypersensitivity to beta-lactam antibiotic cefuroxime of Firmicutes with a low cellular c-di-

AMP level, either due to the deletion of diadenylate cyclase, or the over-expression of c-di-AMP phosphodiesterase (75, 76, 81). Notably, this observation does not suggest a regulation of c-di-AMP on the cell wall homeostasis, because a significant and a strong beta-lactam antibiotics resistance phenotype was not observed in the c-di-AMP phosphodiesterase deletion mutant in Firmicutes (71, 81, 82). In addition, the protein interaction of the diadenylate cyclase with the peptidoglycan biosynthesis enzyme GlmM that would be expected to alter the cell sensitivity to beta-lactam antibiotics was found to play no role in this phenotype. Rather, the depletion of the cellular c-di-AMP pools was found to be the key determinant (75). Therefore, the sensitivity of the strains to beta-lactam antibiotics can be better explained by the cellular turgor model aforementioned: upon beta-lactam antibiotics treatment, the cells with a low cellular c-di-AMP level that would have already experienced an increased membrane tension are further weakened, rendering the cells unable to resist the osmotic stress and ultimately rupture.

It is worth mentioning that this cellular turgor model can better explain the cell wall-associated phenotype of Firmicutes than of the Actinobacteria. Particularly in *S. aureus*, the c-di-AMP phosphodiesterase deletion mutant is significantly more resistant to the beta-lactam antibiotics than the parental strain, but that is likely due to the increased cross-linked peptidoglycans (56, 83). More studies are required to visit the association of c-di-AMP with cell wall metabolism among the bacteria producing c-di-AMP.

Figure 2.3 The cellular turgor model proposed by Commichau and colleagues. (A) Increased intracellular c-di-AMP concentration inhibits ion and osmolytes uptake for osmotic adjustment, resulting in water efflux and cell shrinkage. On the other hand, decreased intracellular c-di-AMP concentration result in increased ion and osmolytes uptake and excessive water influx, causing cell lysis. (B) Cellular turgor can be re-balanced through adjustments on the intracellular c-di-AMP levels (eg. genetic manipulations of the c-di-AMP synthesising and degrading genes) and the extracellular osmoprotectants concentrations. Figure reprinted from (70) with permission (Licence number 4684680599759).

Given the importance of c-di-AMP in osmoregulation, bacteria are required to fine-tune the cellular c-di-AMP level to ensure survival. It is therefore not surprising that when grown in high osmotic stress conditions, the c-di-AMP phosphodiesterase deletion mutant develops suppressor mutations in the genes responsible for the control of cellular c-di-AMP levels.

To accomplish that, bacteria develop mutations in the c-di-AMP diadenylate cyclase, the c-di-AMP phosphodiesterase, or the c-di-AMP receptor GlmM to control the synthesis and hydrolysis of c-di-AMP, or overexpress multidrug resistance transporters to export c-di-AMP to lower the cellular c-di-AMP levels (68, 72). In the context of host-pathogen interactions, the export of c-di-AMP can be a double-edged sword to the bacteria because the export of c-di-AMP to the host cytosol can induce the host expression of interferon- β (IFN- β) (84-86), which can be detrimental or protective to the host depending on the bacterial types causing the infections (87). To *L. monocytogenes*, the elevated IFN- β levels increases host susceptibility to the infection and promotes host cells apoptosis (87), whereas to *S. agalactiae*, the elevated host IFN- β levels promotes infection clearance (55). Therefore, in the host-pathogen warfare, the export of c-di-AMP seems to be beneficial to *L. monocytogenes*, but detrimental to *S. agalactiae*. It would then be expected that *S. agalactiae* encodes a mechanism to prevent the accumulation of extracellular c-di-AMP. Indeed, *S. agalactiae* expresses an extracellular c-di-AMP ectonucleotidase CndP to hydrolyse the extracellular c-di-AMP to escape from the host immune surveillance (55). These studies demonstrated the delicate modulation of c-di-AMP pools by bacteria at both intracellular and extracellular levels.

Taken together, c-di-AMP is an essential second messenger in bacteria mainly responsible for osmoregulation, and involved in bacterial pathogenesis. While its role in maintaining osmotic homeostasis is clearly supported by many layers of evidences based on the studies in a wide range of bacteria (e.g the c-di-AMP receptors, the responsiveness of cellular levels of c-di-AMP to osmotic shift, the suppressor mutations identified in c-di-AMP diadenylate

cyclase and c-di-AMP phosphodiesterase deletion mutants), its role in biofilm formation remains controversial and should be further studied. It would be expected that c-di-AMP is involved in regulating the expression of colonisation factors to mediate biofilm formation.

2.4 Colonisation factors identified in *S. gallolyticus* subsp. *gallolyticus*

In Gram-positive bacteria, one of the most common colonisation factors is an extracellular surface structure termed a pilus made up by covalently-linked pilin subunits. The protein sequence of pilin subunits typically contains a LPxTG motif, in which x refers to any amino acid, a hydrophobic region, and a charged tail. The LPxTG motif is recognised by sorting enzymes known as Sortase A and C, which are responsible for the anchorage to the peptidoglycan layer and the elongation of pilus, respectively (88). Specifically in *S. gallolyticus* subsp. *gallolyticus* clinical isolate UCN34, three pilus encoding operons have been identified, namely Pil1, Pil2 and Pil3 (89). Each operon carries two major pilin genes, and a gene encoding Sortase C responsible for the elongation of pilus. In addition to this, Pil3 operon carries additional two open reading frames the function of which is uncharacterised (15, 90). Pil1 and Pil3 recognise collagen and mucins respectively, and are responsible for the attachment of *S. gallolyticus* subsp. *gallolyticus* to the damaged heart valves and intestinal tract, respectively (15, 90).

The pilus expression in many Gram-positive bacteria such as *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Enterococcus faecalis* is known to be heterogenous (91-94). The heterogenous expression of pilus results in a mix of pilated and non-piliated cell

populations. While bacteria require pilus to colonise surfaces, excessive expression of pilus can be a reducing factor for bacterial survival in the host because pilus contains highly antigenic determinants (88). While it has been suggested that pilus expression in *S. pneumoniae* involves transcriptional regulators (91), the molecular mechanisms regulating the pilus expression largely remained unknown in many bacteria, although the pilus expression is known to respond to various environmental signals such as temperature, pH, presence of bicarbonate or serum (92, 95-98).

Not unexpectedly, heterogenous expression was also observed on the *S. gallolyticus* subsp. *gallolyticus* Pil1. Using flow cytometry, Dramsi and her team showed that about 67% of the cells in a population are lightly piliated, 28% are highly piliated, whereas the remaining 5% of the cells remained non-piliated (99). In the *S. gallolyticus* subsp. *gallolyticus*, the transcription of the Pil1 operon starts at -390 bp upstream from the start codon of the Pil1 operon open reading frame (99). This transcribed region contains a leader peptide sequence with GCAGA repeats that critically control the Pil1 expression (99). In the clinical isolate UCN34, the Pil1 leader peptide sequence contains 22 GCAGA repeats and is translated into a peptide of 38 amino acids (99). The translation of the leader peptide stops at 39 bp upstream of a stem-loop transcriptional termination structure that signals for a premature transcriptional termination, preventing transcription of the Pil1 operon (99). Notably, the repeat numbers of GCAGA can be altered due to gene addition or deletion events of one or multiples of GCAGA repeats. For cells carrying in-frame mutations of GCAGA repeats (*e.g.* 19 or 25 GCAGA repeats) in the Pil1 promoter region, the translation of the leader peptide remains terminating at 39 bp upstream of the stem-loop structure (99). However,

for cells carrying out-of-frame mutations of GCAGA repeats such as repeat number 17, 18, 20, 21, 23, 24 or 26, the translation of the leader peptide terminates near or at the stem-loop structure (99). The ribosome recruited during the translation process destabilises the stem-loop structure, therefore permitting the transcription of *Pil1*, allowing the *Pil1* overexpression in the *S. gallolyticus* subsp. *gallolyticus* populations (99). The factors governing the alterations of GCAGA repeat numbers remained unidentified, but this event is independent from various environmental signals, including pH, temperature, serum, oxygen concentration, glucose, bicarbonate, plasma and intestinal juice (99).

Figure 2.4 *Streptococcus gallolyticus* subsp. *gallolyticus* UCN34 *pilI* regulation by a transcriptional attenuation-like mechanism. *Pil1* is heterogenously expressed in *S. gallolyticus* subsp. *gallolyticus* UCN34. The promoter region of the *pilI* locus contains a DNA sequence encoding for a leader peptide. This DNA sequence contains 22 GCAGA repeats and the translation of this sequence ends 39 bp upstream of the hairpin transcription terminator. In-frame addition/deletion of the GCAGA repeats preserves the formation of the hairpin transcription terminator, therefore the transcripts initiated at the *pilI* promoter terminate prematurely at this terminator, resulting in low expression of *pilI* as observed on the weakly-piliated cells (*Pil1_{low}*). The hyper-piliated cells (*Pil1_{high}*), on the other hand, contain out-of-frame addition/deletion of the GCAGA repeats, therefore resulting in the translation of longer leader

peptides terminating within or downstream of the hairpin terminator. This translation disrupts the formation of the hairpin transcription terminator, allowing transcriptional read-through and resulting in strong *pil1* expression. Figure reprinted from (99) distributed under the Creative Commons Attribution (CC BY) licence.

Intriguingly, GCAGA repeats are also found in the promoter region of the *S. gallolyticus* subsp. *gallolyticus* UCN34 Pil3 locus. Similar to Pil1, the Pil3 promoter region also contains a leader peptide sequence, but with only 13 GCAGA repeats, and the translation of the leader peptide stops at 33 bp upstream of a stem-loop transcription terminator structure (99). Pil3-overexpressing (Pil3+) variants were found to carry out-of-frame GCAGA repeats (*e.g.* 12 or 14 repeats), in which the translation of the leader peptide is predicted to terminate within or at the downstream of the stem-loop structure (15, 99). Like Pil1, the regulatory mechanisms governing the Pil3 expression remains unknown.

S. gallolyticus subsp. *gallolyticus* was found to colonise more efficiently in the murein gut with tumours (12). The investigation on the underlying molecular mechanisms uncovers the enhanced colonisation of *S. gallolyticus* subsp. *gallolyticus* is at the expense of the commensal enterococci (12). In tumorous conditions, secretion of secondary bile acids is markedly increased, and that signals *S. gallolyticus* subsp. *gallolyticus* for the production of a specific class II bacteriocin termed gallocin to outcompete the indigenous *Enterococcus* species (12). This suggests that gallocin is an important factor for the colonisation of *S. gallolyticus* subsp. *gallolyticus* in the murein intestinal tract. Consistently, a deletion mutant of the genes responsible for the gallocin production displayed a reduced colonisation ability

(12). Altogether, Pil1, Pil3 and gallocin constitute the currently identified colonisation factors in *S. gallolyticus* subsp. *gallolyticus*.

In *Streptococcus* spp., pullulanase is another important adhesin that together with pilus represent the attractive targets for vaccine development to combat Streptococcal infections. Pullulanase is an extracellular protein anchored to the bacterial cell wall via its LPxTG motif, and is mainly responsible for the hydrolysis of pullulan to maltotriose repeating units at the alpha-1,6 glycosidic linkage. Pullulanase can also degrade other carbohydrates with alpha-1,6 glycosidic linkage, such as amylopectin, and arguably starch, amylose and glycogen (100-102). In addition to the alpha-amylase hydrolytic domain responsible for the degradation of these carbohydrates, pullulanase also contains multiple copies of carbohydrate-binding modules belonging to the family 41 for the substrate binding (103). Interestingly, these modules also allow *Streptococci* to bind *in vitro* to various glycoproteins such as thyroglobulin, submaxillary mucin, fetuin, asialofetuin and porcine mucin (100, 104), as well as the host cell surfaces such as porcine tracheal cells, human cervical epithelial cells and alveolar type II cells (102, 104, 105). This suggests that pullulanase is an important surface protein for *Streptococci* *in vivo* colonisation. Indeed, it was found that a pullulanase transposon insertion mutant of *S. pneumoniae* displayed attenuated virulence in murein pneumoniae and bacteraemia model (106). More importantly, antibodies against pullulanase have been detected in the sera of *S. pyogenes*-infected individuals (100, 107), strengthening the expression of pullulanase by bacteria during host infection. Nevertheless, whether pullulanase plays a similar role in *S. gallolyticus* subsp. *gallolyticus* remained unexplored.

Chapter 3 - Increased intracellular c-di-AMP levels sensitise *Streptococcus gallolyticus* subsp. *gallolyticus* to osmotic stress and reduce biofilm formation and adherence on intestinal cells

3.1 Introduction

Streptococcus gallolyticus subsp. *gallolyticus* was previously known as *S. bovis* biotype I. This Gram-positive bacterium belonging to the Group D Streptococcus is an emerging pathogen responsible for septicemia and infective endocarditis (IE) in the elderly. *S. gallolyticus* subsp. *gallolyticus* infections represent up to 25% of all cases of IE (2) and up to 94.5% of IE caused by Group D *Streptococcus* (108). Once established, IE is usually difficult to treat and has an in-hospital mortality of up to 22% (109-111), and high-risk surgery interventions are often needed to resolve the infection (110-112). Importantly, *S. gallolyticus* subsp. *gallolyticus* also has a strong association with the occurrence of colorectal cancer in endocarditis patients (1, 9). Two recent studies indicate that *S. gallolyticus* subsp. *gallolyticus* is both a driver and a passenger in colonic tumorigenesis. On one hand, *S. gallolyticus* subsp. *gallolyticus* can accelerate colorectal cancer development by inducing cell proliferation through the β -catenin pathway (11); on the other hand, *S. gallolyticus* subsp. *gallolyticus* can take advantage of tumoral conditions to outcompete the colonic microbiota members, such as *Enterococcus faecalis*, through the production of a specific bacteriocin named gallocin (12). *S. gallolyticus* subsp. *gallolyticus* was also shown to express two specific pili named Pil1 and Pil3, which are involved in collagen and mucin binding, respectively. These two specific pili allow *S. gallolyticus*

subsp. *gallolyticus* to attach at infection sites (i.e. cardiac valves and colon, respectively) and forming biofilms (15, 90). While these studies provided important clues to understand *S. gallolyticus* subsp. *gallolyticus* pathogenicity, not much is known about the signal(s) governing the induction of virulence.

In bacteria, second messenger signalling molecules are exploited to regulate important physiological functions, including biofilm formation and virulence. Among these molecules, cyclic diadenosine-monophosphate (c-di-AMP) has gained attention due to its widespread presence in Gram-positive bacteria, its essentiality for survival in certain conditions, and its pleiotropic role as both an intracellular and extracellular molecule modulating many biological processes, including the host immune responses (55, 84, 113, 114).

c-di-AMP is synthesised by DisA_N or DAC domain-containing diadenylate cyclases (DACs) and hydrolysed by DHH/DHHA1 or HD-domain-containing phosphodiesterases (PDEs) (53, 54, 115-117). Unlike *Bacillus* and *Clostridium* spp., which carry multiple DACs in their genomes, *Firmicutes*, such as *Streptococcus* and *Staphylococcus* spp., are known to carry only one DAC (commonly encoded by *dacA*) (53, 113, 118). Previous attempts to delete *dacA* from *Listeria monocytogenes* and *Streptococcus pyogenes* and all DACs from *Bacillus subtilis* using conventional gene deletion protocols were unsuccessful (76, 84, 118, 119), suggesting that c-di-AMP is essential for bacterial growth in standard laboratory culturing conditions. It is now known that c-di-AMP is dispensable for growth in specially formulated media and under anaerobic culturing conditions (62, 73, 74, 77).

However, the deletion of *dacA* often results in the occurrence of compensatory mutations (62, 77). Therefore, c-di-AMP PDE deletion mutants were particularly useful to address the regulatory roles of c-di-AMP in Gram-positive bacteria (56-58).

Here, we constructed and characterised a c-di-AMP PDE, *gdpP* deletion mutant in *S. gallolyticus* subsp. *gallolyticus* UCN34. We found that the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant was morphologically smaller than the parental strain UCN34, more sensitive to osmotic stress, formed less biofilm on abiotic surfaces, attached less efficiently and formed less cell aggregates on human intestinal cells. Furthermore, a genome-wide transcriptomic analysis indicated that c-di-AMP regulates many other important biological processes and modulates the expression of a few genes associated with pathogenicity. Overall, our results indicate that c-di-AMP could be an important signalling molecule controlling the ability of *S. gallolyticus* to colonise the host.

3.2 Results

3.2.1 Deletion of *GALLO_2236* results in increased intracellular c-di-AMP levels in *S. gallolyticus* subsp. *gallolyticus* UCN34. In Firmicutes, c-di-AMP is commonly synthesised by diadenylate cyclase DacA and hydrolysed by a specific phosphodiesterase GdpP (54, 56). In many bacterial genomes, *dacA* often co-localises with *ybbR* and *glmM*, whereas *gdpP* often co-localises and is co-expressed with *rplI* and *dnaC* (53, 82). By protein homology search and gene location identification in the genome of *S. gallolyticus* subsp. *gallolyticus* UCN34, we identified *GALLO_1455* (GenBank accession number CBI13946) and *GALLO_2236* (GenBank accession number CBI14727) as the best candidates for *dacA* and *gdpP*, respectively (89). Further analysis of *GALLO_1455* and *GALLO_2236* in the SMART database (<http://smart.embl-heidelberg.de>) confirmed that *GALLO_1455* and *GALLO_2236* contain the typical domain architecture of DacA and GdpP, respectively (53, 117). In particular, *GALLO_1455* contains three transmembrane regions and a DisA_N domain, whereas *GALLO_2236* contains two transmembrane regions, a PAS sensory domain, a GGDEF domain and a DHH/DHHA1 catalytic domain (Figure 3.1A and 3.1B). We therefore renamed *GALLO_1455* and *GALLO_2236* as *dacA* and *gdpP*, respectively.

Notably, the *S. gallolyticus* subsp. *gallolyticus* UCN34 genome neither encodes a PgpH-type (115) nor a CdnP-type (55) c-di-AMP phosphodiesterase, but it does encode a stand-alone DHH/DHHA1 domain-containing protein (*GALLO_0742*). The stand-alone DHH/DHHA1 domain-containing protein, usually designated as Pde2 (or DhhP), was previously reported to hydrolyse c-di-AMP (80, 120), but has been shown recently to

preferentially act on linear nucleotides, such as pApA and pGpG (120-122). Pde2 also exhibits additional enzymatic properties, such as being a nanoRNA ribonuclease and a 3'-phosphoadenosine 5'-phosphate (pAp) phosphatase (120, 123, 124). Therefore, to investigate the roles of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus*, the function of GALLO_0742 was not further explored in this study.

Our multiple attempts to create a *dacA* deletion mutant in *S. gallolyticus* subsp. *gallolyticus* UCN34 under standard laboratory culturing conditions by employing conventional knock-out protocols were unsuccessful, suggesting that c-di-AMP may be essential for the survival of *S. gallolyticus* subsp. *gallolyticus* under standard culturing conditions, as has been shown for other Gram-positive bacteria (84, 119). Therefore, we focused on the construction of an in-frame *gdpP* deletion mutant to modulate the c-di-AMP levels in *S. gallolyticus* subsp. *gallolyticus* UCN34. We deleted the gene sequence encoding the PAS, the GGDEF and the DHH/DHHA1 domains of GdpP and denoted this mutant strain *S. gallolyticus* subsp. *gallolyticus* UCN34 Δ *gdpP*. Of note, 69bp at the 3' end of *gdpP* was left undeleted to preserve the ribosomal binding site of the downstream gene *rplI* encoding ribosomal subunit L9 (Figure 3.1B).

To verify that *gdpP* truly encodes a c-di-AMP phosphodiesterase in *S. gallolyticus* subsp. *gallolyticus* UCN34, we quantified the intracellular c-di-AMP levels of *S. gallolyticus* subsp. *gallolyticus* UCN34 wildtype, the Δ *gdpP* mutant and the *gdpP* complemented strain (Δ *gdpP/pgdpP*). As expected, the intracellular c-di-AMP levels of the Δ *gdpP* mutant were approximately 1.5-fold higher than in the wildtype strain UCN34. Importantly, the

intracellular c-di-AMP levels in the $\Delta gdpP/pgdpP$ complemented strain were about 80% of the wildtype UCN34 (Figure 3.1C), probably due to the over-expression of *gdpP* in the complemented strain.

Figure 3.1 *GALLO_1455* and *GALLO_2236* encode c-di-AMP diadenylate cyclase and phosphodiesterase, respectively. (A and B) Gene locations and the domain architecture of (A) *GALLO_1455* and *GALLO_2236* (B) resemble the typical properties of DacA and GdpP, respectively. PAS, GGDEF and DHH/DHHA1 domains were deleted to generate *S. gallolyticus* subsp. *gallolyticus*

UCN34 $\Delta gdpP$. 69 bp of the gene at 3' end of *gdpP* was left undeleted to preserve the ribosomal binding site of *rplI*. The arrow at the upstream of *GALLO_2236* marks the putative transcriptional start site of *GALLO_2236* based on *in silico* algorithm-based promoter prediction. (C) Liquid chromatography-mass spectrometry quantification of the intracellular concentration of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Error bars represent the standard deviation of the measurements from three samples. Ordinary one-way ANOVA test, **, $P \leq 0.01$, ***, $P \leq 0.001$, ****, $P \leq 0.0001$.

3.2.2 *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ is morphologically smaller and is more sensitive to high osmotic stress than the parental strain. We next investigated the physiological changes in the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant in comparison to the wildtype and the complemented strain. We first monitored the growth kinetics of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain by measuring the optical density of the bacterial culture inoculated with the same number of cells (approximately 3×10^7 CFU/mL). Throughout the experimental period, the optical density readings of the $\Delta gdpP$ mutant remained lower than that observed for the wildtype and the $\Delta gdpP/pgdpP$ complemented strain (Figure 3.2A). However, the doubling time of the three isogenic strains was similar (28 ± 2 min, 27 ± 2 min and 31 ± 1 min for the wildtype, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain, respectively; $\pm$ values are standard deviations [SD]) (Figure 3.2B). A replotted growth curve based on CFU enumeration further showed that the increased intracellular c-di-AMP levels does not affect the growth of *S. gallolyticus* subsp. *gallolyticus* UCN34 (Figure 3.2C).

When the bacterial cells were observed under a phase contrast microscopy, the UCN34 $\Delta gdpP$ mutant cells appeared clearly smaller than the wildtype and the $\Delta gdpP/pgdpP$ complemented strain (Figure 3.2D). A cell area measurement of 300 cells for each bacterial

strain, using ImageJ software, confirmed that the UCN34 $\Delta gdpP$ mutant cells were about 25% smaller than the wildtype and the $\Delta gdpP/pgdpP$ complemented strain (Figure 3.2E). These data suggest that the lower optical density reading observed for the $\Delta gdpP$ mutant may primarily be due to the reduced cell size.

Increased intracellular c-di-AMP levels were previously reported to affect the bacterial tolerance to high osmotic stress and the sensitivity to β -lactam antibiotics (56, 70). Therefore, we tested the tolerance of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain to high osmotic stress by spotting serially-diluted log-phase culture on brain heart infusion (BHI) agar and BHI agar supplemented with 0.4 M NaCl. As shown in Figure 3.2F, the *S. gallolyticus* UCN34 $\Delta gdpP$ mutant was more sensitive than the wildtype to high osmotic stress. The tolerance to the osmotic stress was restored when *gdpP* was expressed in *trans*. We next tested the sensitivity of these 3 strains to β -lactam antibiotics. Our data did not show any significant differences in the susceptibility of the $\Delta gdpP$ mutant to ampicillin or penicillin G compared with that of the wildtype and the complemented strain (Figure 3.2G). Taken together, our data showed that high intracellular c-di-AMP affects bacterial cell size and tolerance to osmotic stress but not bacterial growth or sensitivity to β -lactam antibiotics in *S. gallolyticus* subsp. *gallolyticus*.

Figure 3.2 Phenotypic changes associated with an increased intracellular c-di-AMP level resulted from the deletion of *gdpP*. (A) Representative anaerobic growth kinetics of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Initial inoculum was prepared from log-phase culture adjusted to approximately 3×10^7 CFU/mL. Growth, reflected in optical density, was measured at 600 nm (OD_{600}) at the indicated time point. The arrow indicates the sample collection time point for biofilm assay and the RNA-seq experiment. Error bars represent the standard deviation of the measurements from three samples. (B) Doubling time of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain was calculated based on the absorbance value of samples from 3 independent experiments. Values are expressed in mean $\pm$ standard deviation. (C) Growth curve of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain plotted based on CFU enumeration at the indicated time point. Error bars represent the standard deviation of the measurements from three samples. (D) Representative phase contrast microscopy images on the stationary-phase culture of the *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Images were acquired with Carl Zeiss Axio Observer.Z1 Inverted Widefield Microscopy fitted with 100X/1.3-numerical aperture objective oil lens. Images were processed using Imaris version 8.2. Scale bar = 3 μ m. (E) Cell area measurement of 300 imaged cells from three independent experiments using ImageJ software. Error bars represent the standard deviation of the 300 measurements. Kruskal-Wallis test, ****, $P \leq 0.0001$. (F) Representative images of 5 μ L of *S. gallolyticus* subsp. *gallolyticus* log-phase culture adjusted to approximately 3×10^7 CFU/mL spotted onto BHI agar and BHI agar supplemented with 0.4 M NaCl. (G) Minimal inhibitory concentrations (MIC) of ampicillin and penicillin G against *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. MIC was determined based on the optical density reading at 600nm on a Tecan Microplate reader, Infinite M200Pro.

3.2.3 Increased intracellular c-di-AMP levels reduce *S. gallolyticus* subsp. *gallolyticus* biofilm formation. c-di-AMP was previously shown to regulate biofilm formation in a number of Gram-positive bacteria (56-59). To test the impact of increased intracellular c-di-AMP levels on *S. gallolyticus* subsp. *gallolyticus* biofilm formation, conventional biofilm assays on microtiter plates were carried out with *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Unexpectedly, we found that the biofilm formed by the $\Delta gdpP$ mutant was only 57% of the wildtype level, whereas the biofilm formed by the $\Delta gdpP/pgdpP$ complemented strain was 160% of the wildtype level (Figure 3.3A). Confocal laser scanning microscopy images of the biofilms suggested that the reduced biofilm formation of the $\Delta gdpP$ mutant may be primarily due to reduced attachment of the $\Delta gdpP$ mutant cells to the surfaces (Figure 3.3B). Taken together, our data indicate that biofilm formation of *S. gallolyticus* subsp. *gallolyticus* is negatively regulated by c-di-AMP, i.e., an increased intracellular c-di-AMP level reduces biofilm formation, whereas a decreased intracellular c-di-AMP level enhances biofilm formation.

Figure 3.3 Accumulation of intracellular c-di-AMP levels inhibits *S. gallolyticus* subsp. *gallolyticus* biofilm formation. (A) Biofilm quantification using conventional microtiter plate biofilm assay. Error bars represent the standard deviation of 12 samples from 3 independent experiments. Ordinary one-way ANOVA test, ****, $P \leq 0.0001$. (B) Representative biofilm images of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain acquired using Carl Zeiss confocal laser scanning microscopy LSM780 fitted with Plan Apochromat 100x/1.4 oil objective lens, with excitation at 488 nm. Scale bar = 10 μm .

3.2.4 Increased intracellular c-di-AMP levels attenuate the ability of *S. gallolyticus* subsp. *gallolyticus* to adhere and to form cell aggregates on human colonic epithelial cells. We next tested whether c-di-AMP can alter the ability of *S. gallolyticus* subsp. *gallolyticus* to adhere to biotic surfaces, such as human colonic cells. We introduced *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain onto a monolayer of human colorectal adenocarcinoma HT-29 cells and quantified the number of

adherent bacterial cells after 1 hour of incubation at 37°C. As for biofilm formation, the $\Delta gdpP$ mutant adhered less efficiently on the monolayer of HT-29 cells than the wildtype, whereas the complemented $\Delta gdpP/pgdpP$ strain adhered more efficiently than the wildtype (Figure 3.4A). Immunofluorescence microscopy showed that *S. gallolyticus* subsp. *gallolyticus* UCN34 formed cell aggregates on HT-29 cells, which was rarely observed for the $\Delta gdpP$ mutant. Interestingly, the complemented $\Delta gdpP/pgdpP$ strain formed larger cell aggregates than the wildtype on HT-29 cells (Figure 3.4B). These data demonstrated that c-di-AMP modulates the adherence of *S. gallolyticus* on both abiotic and biotic surfaces.

Figure 3.4 Increased intracellular c-di-AMP levels attenuates the ability of *S. gallolyticus* subsp. *gallolyticus* to adhere and to form cell aggregates on human colonic epithelial cells. (A) Quantification of the cell number of *S. gallolyticus* subsp. *gallolyticus* attached on a monolayer HT-29 human colorectal adenocarcinoma cells. Error bars represent the standard deviation of 9 samples from 3 independent experiments. Kruskal-Wallis test, **, $P \leq 0.01$, ***, $P \leq 0.001$. (B) Representative immunofluorescence images on the *S. gallolyticus* subsp. *gallolyticus* adhered on a monolayer of HT-29 human colorectal adenocarcinoma cells. Green, *S. gallolyticus* subsp. *gallolyticus* labelled with primary antibody rabbit-UCN34 and secondary antibody goat anti-rabbit conjugated with AlexaFluor 488. Blue, Hoechst 33342-stained DNA of the HT-29 cells. Scale bar = 10 μm (top panel) and 70 μm (bottom panel).

3.2.5 C-di-AMP regulates various biological functions in *S. gallolyticus* subsp. *gallolyticus* UCN34, including the gallocin production and Pil3 pilus biosynthesis. To uncover other regulatory roles of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus*, genome-wide transcriptomic sequencing (RNA-seq) was performed on the stationary phase culture of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Comparative transcriptomic analysis revealed 109 genes whose expression was either up-regulated or down-regulated by ≥ 2 -fold in the $\Delta gdpP$ mutant as compared to the wildtype and the complemented strain. Functional classification of these differentially regulated genes suggests that c-di-AMP regulates various biological functions such as carbohydrate, amino acid, nucleotide and coenzyme transport and metabolism; development of competence for genetic transformation; DNA replication, recombination and repair; translation, ribosomal structure and biogenesis, in *S. gallolyticus* subsp. *gallolyticus*. In addition, several families of transcriptional regulators were found to be differentially expressed, which may serve as the mediators for c-di-AMP to regulate the cognate cellular functions (Supplementary data, Table 3.3).

Interestingly, the RNA-seq data also reflected that an increased intracellular c-di-AMP level was associated with the reduced expression of multiple ABC transporters, including spermidine/putrescine and proline/glycine betaine ABC transporters, which can be linked to osmotic regulation (Supplementary data, Table 3.3). The *S. gallolyticus* subsp. *gallolyticus* UCN34 proline/glycine betaine ABC transporters are the homologs of *B. subtilis* OpuCABCD osmoprotectant ABC transport system. In *S. aureus* and *L. monocytogenes*, OpuCA containing a cystathionine-synthase (CBS) domain is a known c-

di-AMP binding protein (62, 65, 66). Whereas a putative homolog of OpuCA can be identified in *S. gallolyticus* subsp. *gallolyticus* UCN34 (GALLO_1283), GALLO_1283 appears to be a truncated OpuCA containing no CBS domain, which is similar to the OpuCA of *S. pneumoniae* (125). The CBS domain is required for c-di-AMP binding (65). Therefore, it is expected that c-di-AMP will not bind to the *S. gallolyticus* OpuCA to regulate the osmoprotectant transporter activity. Instead, as suggested by the RNA-seq data, c-di-AMP may control the uptake of osmoprotectant by regulating the transcription of the OpuCA transporter.

Intrigued by this observation, we further explored whether the putative homologs of other c-di-AMP binding proteins were also differentially expressed in *S. gallolyticus* subsp. *gallolyticus* UCN34. These homologs include GALLO_2236 (homolog of PdeA (126)), GALLO_1832 (homolog of K⁺ transporter KtrA/KtrC/CabP/CabPA (58, 62-64, 127)), GALLO_1797 (homolog of K⁺ transporter CabPB (58, 62)), GALLO_1748 (homolog of P_{II}-like signal transduction protein PstA (63, 126, 128, 129)), GALLO_1804 (homolog of hypothetical protein CbpB (126)), GALLO_1824 (homolog of transcriptional repressor NrdR (126)) and GALLO_2191 (homolog of recombination protein A RecA (130)). Notably, homologs of many other known c-di-AMP binding proteins, such as cation/proton antiporter CpaA (63, 67, 126), sensor kinase KpdD (63, 69), transcriptional regulator BusR (62, 68) and DarR (131), pyruvate carboxylase PycA (126), hypothetical protein CbpA (126) and Lmo1466 (126) were not found in *S. gallolyticus* subsp. *gallolyticus* UCN34 (Table 3.1). None of these homologs - except the knocked out protein PdeA/GdpP - were strongly differentially expressed in the *S. gallolyticus* subsp. *gallolyticus* Δ *gdpP* mutant

compared with the wildtype UCN34 and the complemented strain (Supplementary data, Table 3.3). This is however not unexpected, given that c-di-AMP commonly interacts with the binding proteins to directly modulate the protein activity.

Table 3.1 Protein homologs of the known c-di-AMP binding proteins in *S. gallolyticus* subsp. *gallolyticus* UCN34.

Known c-di-AMP binding proteins	Homologs in UCN34	Fold change in transcriptional expression	
		$\Delta gdpP$ vs wildtype	$\Delta gdpP$ vs $\Delta gdpP/pgdpP$
Osmoprotectant transport ATP-binding protein OpuCA	GALLO_1283*	-4.4506	-2.0119
DHH subfamily 1 protein PdeA	GALLO_2236	-63.2552	-982.8238
K ⁺ transporter KtrA/KtrC/CabP/CabPA	GALLO_1832	-1.0034^	-1.1513^
K ⁺ transporter CabPB	GALLO_1797	1.2758	1.1956
P _{II} -like signal transduction protein PstA	GALLO_1748	-1.3411^	-1.9281
Hypothetical protein CbpB	GALLO_1804	-1.4435	1.1393^
Transcriptional repressor NrdR	GALLO_1824	1.0667^	1.5798
Recombination protein A RecA	GALLO_2191 [#]	1.4660	1.2683
Cation/Proton antiporter CpaA	Not found	Not applicable	
Sensor kinase KdpD	Not found	Not applicable	
Transcriptional regulator BusR	Not found	Not applicable	
Hypothetical protein Lmo1466	Not found	Not applicable	
Pyruvate carboxylase PycA	Not found	Not applicable	
Hypothetical protein CbpA	Not found	Not applicable	
Transcriptional regulator DarR	Not found	Not applicable	

* GALLO_1283 is a homolog of OpuCA containing no cystathionine-beta-synthase (CBS) domain.

[#] GALLO_2191 does not contain the conserved motif of MsRecA for the binding of c-di-AMP.

^ p-adjusted value > 0.05.

Importantly, the transcription of *blpB* encoding gallocin and *blpC* encoding gallocin immunity protein (12) was found up-regulated (~2 fold), whereas the expression of *pil3A*, *pil3B* and *srtC* of the Pil3 operon (15) was found down-regulated (~4 fold) in the $\Delta gdpP$ mutant compared with the wildtype and the complemented strain (Supplementary data, Table 3.3). Gallocin and Pil3 pilus were previously shown as two important colonisation factors enabling *S. gallolyticus* subsp. *gallolyticus* to persist in the murine colon (12, 15). The RNA-seq data indicating that these colonisation factors are differentially expressed in the $\Delta gdpP$ mutant prompted us to assess the gallocin production and Pil3 pilus biosynthesis in the three isogenic *S. gallolyticus* subsp. *gallolyticus* strains. To measure gallocin production, we spotted approximately 2×10^5 log-phase cells of UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain on BHI agar flooded with the gallocin-sensitive strain *Enterococcus faecalis* OG1RF and *S. gallolyticus* subsp. *macedonicus* (12). *S. gallolyticus* subsp. *gallolyticus* UCN34 Δblp mutant producing no gallocin was used as a negative control (12). After an overnight incubation under anaerobic conditions, the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant created a larger zone of inhibition than the wildtype and the $\Delta gdpP/pgdpP$ complemented strain, demonstrating an increased gallocin production in the $\Delta gdpP$ mutant (Figure 3.5A). To quantify Pil3 biosynthesis, Western blot analysis was carried out using the cell wall proteins from UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. We included the isogenic UCN34 $\Delta pil3$ to check for antibody specificity. Our data showed that Pil3 expression was noticeably decreased in the $\Delta gdpP$ mutant compared with the wildtype and the $\Delta gdpP/pgdpP$ complemented strain (Figure 3.5B). Taken together, these data indicate that c-di-AMP modulates gallocin and Pil3 levels in *S. gallolyticus* subsp. *gallolyticus*.

Figure 3.5 c-di-AMP regulates the gallocin production and Pil3 biosynthesis in *S. gallolyticus* subsp. *gallolyticus*. (A) Gallocin production by *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain under anaerobic condition. 5 μ L of *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain log-phase culture adjusted to approximately 3×10^7 CFU/mL was spotted onto BHI agar flooded with *Enterococcus faecalis* OG1RF or *S. gallolyticus* subsp. *macedonicus*. Zone of clearance reflects the growth inhibition of *E. faecalis* or *S. gallolyticus* subsp. *macedonicus*. The strain deficient in producing gallocin Δblp mutant was used as the negative control. (B) Western blot analysis of the cell wall proteins from *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Equal amounts of the cell wall proteins were loaded (right) and probed with specific polyclonal antibodies against Pil3B (left). Cell wall proteins of the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta pil3$ mutant were used as a negative control. Theoretical position of Pil3B monomers, based on the molecular weights, is indicated (m) and high molecular weight species corresponding to pili polymers are labeled (p).

3.3 Discussion

Streptococcus gallolyticus subsp. *gallolyticus* is an emerging pathogen responsible for septicemia and endocarditis in the elderly, whose presence is strongly associated with the occurrence of colorectal cancer (1, 4, 9, 132-134). Here, we investigated the roles of c-di-AMP in this emerging pathogen. We show that c-di-AMP plays a pleiotropic role in *S. gallolyticus* subsp. *gallolyticus*, controlling the tolerance to osmotic stress, cell size, biofilm formation, adherence to intestinal cells, cell aggregates formation, gallocin production and Pil3 pilus expression.

c-di-AMP has been proposed to regulate several processes in Gram-positive bacteria. One of the conserved roles is to maintain osmotic homeostasis (70). We speculate that c-di-AMP maintains this conserved role in *S. gallolyticus* subsp. *gallolyticus* for two reasons. First, the observation that the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant is more sensitive to osmotic stress and exhibits altered bacterial morphology suggest an underlying perturbation in the cellular turgor to osmotic pressure (70, 77, 135). Second, our RNA-seq data suggests that at high intracellular c-di-AMP levels, the expression of spermidine/putrescine and proline/betaine glycine ABC transporters was down-regulated in *S. gallolyticus* subsp. *gallolyticus*. These transporters are crucial for bacterial cells for rapid osmotic adjustment during osmotic shift (136, 137). Combined, these data point toward a role of c-di-AMP in maintaining osmotic homeostasis in *S. gallolyticus* subsp. *gallolyticus*. Another proposed role of c-di-AMP is to indirectly mediate sensitivity to β -lactam antibiotics (70). However, we did not observe a significant difference in the minimal

inhibitory concentration of β -lactam antibiotics across our wildtype, the $\Delta gdpP$ mutant, and the $\Delta gdpP/pgdpP$ complemented strain. Similar observations were also reported in *Streptococcus suis* (57), suggesting that c-di-AMP may not mediate the sensitivity to β -lactam antibiotics in *Streptococcus* spp.

Notably, an increased intracellular c-di-AMP level reduces biofilm formation in *S. gallolyticus* subsp. *gallolyticus*. Similar finding was reported in *B. subtilis* (59). This is in contrast to several other publications showing the opposite effect of c-di-AMP on the biofilm formation of *S. aureus*, *S. suis* and *S. mutans* (56-58). The detailed mechanisms explaining how c-di-AMP affects the biofilm formation in *S. gallolyticus* subsp. *gallolyticus* are currently being investigated. It is important to emphasise that both *S. gallolyticus* subsp. *gallolyticus* and bacterial biofilms can play a role in the development of colorectal cancer (11, 14, 24, 26, 27). It would be interesting to explore whether *S. gallolyticus* subsp. *gallolyticus* presence as biofilms play a role as the potential promoting factors in the development of colorectal cancer, and this can be approached by manipulating the intracellular c-di-AMP levels to modulate the biofilm formation of *S. gallolyticus*.

It is also worth mentioning that although the Pil3 pilus is an important colonisation factor for *S. gallolyticus* subsp. *gallolyticus* *in vivo* in the murine colon, it plays a minor role in mediating the adherence of *S. gallolyticus* subsp. *gallolyticus* on HT-29 cells *in vitro* (15). Therefore, the reduced expression of Pil3 pilus in the *S. gallolyticus* $\Delta gdpP$ mutant may not solely account for the reduced adherence of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant on HT-29 cells. We speculate that in the $\Delta gdpP$ mutant, the combined effects of the

reduced expression of Pil3 pilus and of other putative adhesins may instead account for the reduced adherence on HT-29 cells and the absence of cell aggregates. Given that cell aggregates may progressively develop into biofilms (138), it awaits further investigation on whether Pil3 pilus and the putative adhesins establish a link between biofilm formation, cell attachment, cell aggregation and potentially host colonisation in *S. gallolyticus* subsp. *gallolyticus*.

In this study, despite a relatively low difference in the intracellular c-di-AMP levels in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant and the *gdpP*-complemented strain compared with the wildtype, significant phenotypic differences were observed especially in terms of biofilm formation and adherence on intestinal cells. Small differences in the intracellular c-di-AMP were also observed for *S. pneumoniae*, *S. suis* and *B. subtilis* and their isogenic *gdpP* mutants (57, 114). In sharp contrast, *S. aureus* and *S. agalactiae* *gdpP* mutants were shown to accumulate up to 38-fold more intracellular c-di-AMP than the parental strains (56, 62). The driving factors for the difference is currently unclear.

In conclusion, we report here that the second messenger signalling molecule c-di-AMP controls osmotic tolerance, biofilm formation on abiotic surfaces, adherence on human intestinal cells, formation of cell aggregates, expression of pilus proteins and production of bacteriocin in *S. gallolyticus* subsp. *gallolyticus*. This study, thus, indicates that c-di-AMP could be an important signalling molecule governing the pathogenicity of *S. gallolyticus* subsp. *gallolyticus*.

3.4 Materials and Methods

3.4.1 Bacterial strains, culturing conditions, plasmids and primers. All bacterial strains, plasmids and primers used in this study are listed in Table 2.2. Unless stated otherwise, an overnight culture of *S. gallolyticus* subsp. *gallolyticus* UCN34 was typically prepared from a single colony in 5 mL of M9YEG broth (1X M9 minimal salts (MP Biomedicals) supplemented with 0.5% of yeast extract (Becton, Dickinson and Company) and 1.0% of glucose (VWR)), and incubated at 37°C under static condition for 12 to 14 hours. The overnight culture was diluted (1:5) in M9YEG the next day and incubated further at 37°C for the preparation of log-phase culture. When necessary, erythromycin was supplemented to a final concentration of 2 µg/mL (Sigma-Aldrich).

Table 3.2 Bacterial strains, plasmids and primers used in this study.

Bacterial strains	Description	Reference
<i>E. coli</i> DH5α	<i>deoR endA1 gyrA96 hsdR17 (Δlac)U169 recA1 relA1 supE44 thi-1 (Φ80 lacZΔM15)</i>	Lab collection
<i>S. gallolyticus</i> subsp. <i>gallolyticus</i> UCN34	a clinical strain isolated from an infective endocarditis patient who later diagnosed with CRC	(89)
UCN34 Δ <i>gdpP</i>	in-frame <i>gdpP</i> (<i>GALLO</i> 2236) knock-out mutant	This study
UCN34 Δ <i>gdpP</i> /p <i>gdpP</i>	<i>gdpP</i> -complemented strain; UCN34 Δ <i>gdpP</i> containing pTCVerm:: <i>gdpP</i>	This study
UCN34 Δ <i>pil3</i>	in-frame <i>pil3</i> (<i>GALLO</i> 2038 to <i>GALLO</i> 2042) knock-out mutant	(15)
UCN34 Δ <i>blp</i>	gallocin-deficient knock-out mutant (<i>GALLO</i> 2021 to <i>GALLO</i> 2020)	(12)
<i>S. agalactiae</i> NEM316	MLST-23, serotype III isolated from neonate blood culture	(139)
<i>E. faecalis</i> OG1RF	derived from a clinical isolate OG1; rifampicin and fusidic acid resistant	From Kimberly Kline
Plasmids		Reference
pG1	Em, <i>oriR</i> pUC, <i>oriR^{ts}</i> pWV01, MCS pUC18	(140)
pG1:: <i>gdpPKO</i>	pG1 containing 2-kb fragment corresponding to the 5' and 3' end of <i>GALLO</i> 2236	This study
pTCVerm- <i>oriT_{TnGBS1}</i>	Em, Km, Mob ⁺ (IncP); <i>oriR</i> pACYC184; <i>oriR</i> pAMβ1; MCS <i>lacZα</i> ⁺	(140)
pTCVerm:: <i>gdpP</i>	pTCVerm- <i>oriT_{TnGBS1}</i> containing promoter and ORF of <i>gdpP</i> (<i>GALLO</i> 2236)	This study
Primers		Reference
<i>gdpP</i> _up_F	GTCAAACCAATGGTACG	This study
<i>gdpP</i> _up_R	TAAGTGTTTCGGCTTGACTCAAGCCTATCATGACTAA	This study
<i>gdpP</i> _dn_F	TTAGTCATGATAGGCTTGAGTCAAGCCGAACACTTA	This study
<i>gdpP</i> _dn_R	CTGCGATTGCTATGTTC	This study
<i>gdpP</i> _F	CACAGGATCCGTTTACCTAGAAAGGCAAG	This study
<i>gdpP</i> _R	CACAGCATGCCATCACTCTACCTCCAT	This study

* Restriction sites are underlined.

3.4.2 Construction of *gdpP* deletion mutant. The construction of *gdpP* in-frame deletion mutant was performed as previously described (140, 141). In brief, two approximately 1 kb DNA fragments corresponding to the 5' and 3' end of *gdpP* was PCR-amplified using primer pairs *gdpP*_up_F/*gdpP*_up_R and *gdpP*_dn_F/*gdpP*_dn_R. The resulting PCR products were purified and further amplified using primer pair *gdpP*_up_F and *gdpP*_dn_R, and subsequently cloned into pG1 plasmid, generating pG1::*gdpPKO*. The pG1::*gdpPKO* was introduced into *S. agalactiae* NEM316 (139) by electroporation, and later into *S. gallolyticus* subsp. *gallolyticus* UCN34 by conjugal transfer. *S. gallolyticus* subsp. *gallolyticus* UCN34 with pG1::*gdpPKO* integrated into the genome was selected by growing the bacteria at 37°C in the presence of erythromycin. Next, integrants were serially passaged at 30°C in BHI broth without antibiotic to facilitate the excision of the plasmid by homologous recombination. In-frame deletion of *gdpP* gene was verified by PCR and Sanger-sequencing of the *gdpP*-chromosomal flanking regions.

3.4.3 Construction of *gdpP*-complemented strain. A DNA fragment containing the putative promoter and the full-length open reading frame of *GALLO_2236* was amplified by PCR using primer pair *gdpP*_F and *gdpP*_R. The PCR product was restricted with *Bam*HI and *Sph*I, and ligated to *Bam*HI/*Sph*I-restricted pTCVerm-ori*T_hGBS1*, generating a *GALLO_2236* complementation plasmid, pTCVerm::*gdpP*. The complementation plasmid was introduced into *E. coli* DH5 α and then extracted and sequenced. Next, it was introduced into *S. agalactiae* NEM316 and later into *S. gallolyticus* subsp. *gallolyticus* UCN34 Δ *gdpP*, as described earlier (140).

3.4.4 Preparation of c-di-AMP extract. Intracellular c-di-AMP was extracted following the published protocol, with minor modifications (56). A total of 6 mL of log-phase culture adjusted to approximately 3×10^7 CFU/mL was added into each well of a 6-well plate. After 5 hours of incubation at 37°C under anaerobic condition (0% O₂, 10% CO₂, AnaeroGen Compact, prepared according to the manufacturer's instruction, Thermo Fisher Scientific), the bacterial culture was well-mixed by using cell scrapers (TPP) and repeated pipetting. A total of 300 µL of the well-mixed bacterial culture was collected, pelleted, lysed in 300 µL of 0.1M of NaOH for 10 minutes at 80°C, and was subjected to protein quantification with a Qubit™ 2.0 Fluorometer (Thermo Fisher Scientific) for normalisation purposes. 5 mL of the well-mixed bacterial culture was transferred to a 15 mL Falcon tube and centrifuged at 8,000 xg for 2 minutes at 4°C. The bacterial pellet was washed once with 0.9% NaCl. 1 mL of ice-cold extraction buffer (40% v/v acetonitrile, 40% v/v methanol and 20% ultrapure water) was added to the pellet, and the suspension was mixed well. The samples were snap-frozen in liquid nitrogen for 30 seconds before being boiled for 10 minutes. The samples were subsequently transferred to Lysing Matrix B tubes (MP Biomedicals) and were homogenised in a FastPrep-24 instrument at setting of 6.0 m/s for 45 seconds (MP Biomedicals), before being separated from the silica beads by centrifugation at 17,000 xg for 5 minutes at 4°C. A total of 600 µL of the top layer was transferred to a new tube. The silica beads/cell debris mixture was added with 1 mL of ice-cold extraction buffer, briefly vortexed, and incubated on ice for 5 minutes, before a second centrifugation at the same setting. The top layer was again collected and combined with the first extract. The samples were dried at 4°C in a CentriVac Centrifugal Vacuum Concentrators (Labconco). Dried samples were stored at -80°C until analysis.

3.4.5 Quantification of c-di-AMP in cellular extracts by liquid chromatography-mass spectrometry analysis. Detection and quantification of c-di-AMP were performed as described previously with modifications (142) in Singapore Phenome Centre. Briefly, it was performed with a Xevo® TQ-S instrument (Waters) with a binary pump, a temperature-controlled autosampler maintained at 4°C, and a column oven compartment maintained at 40°C, interfaced to the electrospray ionisation (ESI) positive ion source. A total of 5 µL of the c-di-AMP extract dissolved in 100 µL of water was injected into a BEH C18 column (1.7 µm; 2.1x50 mm) (Waters). Mobile phase A was 10 mM ammonium formate in water containing 0.1% formic acid, whereas mobile phase B was methanol containing 0.1% formic acid. Samples were run in gradient condition, with 100% mobile phase A from initial to 3 minutes, 80% mobile phase A from 3 to 3.5 minutes, 10% mobile phase A from 3.5 to 6.5 minutes, and 100% mobile phase A from 6.6 to 8 minutes. The total run was 8 minutes, with flow rate of 0.30 mL per minute. Software MassLynx and TargetLynx were used for chromatography and quantification of c-di-AMP, respectively.

3.4.6 Growth curve of *S. gallolyticus* subsp. *gallolyticus* UCN34 and derivatives. Log-phase *S. gallolyticus* subsp. *gallolyticus* culture was diluted to approximately 3×10^7 CFU/mL in M9YEG. 1 mL of the culture was seeded into each well of a 24-well plate, and incubated at 37°C under anaerobic condition. The bacterial growth was monitored at the desired time point by optical density (OD) measurement at 600 nm using a UV spectrophotometer.

3.4.7 Antibiotic susceptibility test. Ampicillin and penicillin G (Sigma-Aldrich) dissolved in water was 2X-serially diluted in M9YEG in a 96-well microtiter plate. 100 µL of log-

phase *S. gallolyticus* subsp. *gallolyticus* culture diluted to approximately 1×10^6 CFU/mL was added into the wells containing antibiotics. After a 20-hour incubation, the plate was measured at wavelength of 600 nm on a microplate reader, Infinite M200Pro (Tecan). The lowest concentration of antibiotics that inhibited the bacterial growth was determined as the minimal inhibitory concentration (MIC).

3.4.8 Microtiter plate biofilm assay. Assay was performed as described with modifications (143). 1 mL of log-phase *S. gallolyticus* subsp. *gallolyticus* culture diluted to approximately 3×10^7 CFU/mL was added into each well of a 24-well plate and incubated at 37°C under anaerobic condition (0% O₂, 10% CO₂, AnaeroGen Compact, prepared according to the manufacturer's instruction, Thermo Fisher Scientific). After 5 hours of incubation, the bacterial culture was removed. The wells were washed twice with 0.9% NaCl, before being stained with 1 mL of 0.1% of crystal violet solution for 15 minutes. Following this step, the crystal violet solution was removed, the wells were washed twice with 0.9% NaCl, and the biofilm was solubilised by 30% acetic acid for 15 minutes. Solubilised biofilm was quantified and measured on a microplate reader, Infinite M200Pro (Tecan), at a wavelength of 550 nm.

3.4.9 Confocal Laser Scanning Microscopy (CLSM) imaging. The 5-hour biofilms formed by *S. gallolyticus* subsp. *gallolyticus* UCN34 and the derivatives under anaerobic condition were washed with 0.9% NaCl twice, fixed with 4% paraformaldehyde for 10 minutes, and stained with SYTO9 (1:500 diluted from stock, ThermoFisher Scientific) for 10 minutes. Biofilm images were acquired using an LSM780 inverted confocal laser scanning

microscopy (Carl Zeiss) fitted with Plan Apochromat 100x/1.4 oil objective lens, with excitation at 488 nm. The images were processed using Imaris version 8.2.0 (Bitplane).

3.4.10 Cell adherence assay. Human colorectal adenocarcinoma cell line ATCC HTB-38 (HT-29) was routinely maintained in DMEM high glucose (with L-glutamine, without sodium pyruvate) (Gibco) supplemented with 10% fetal bovine serum. 1 mL of the log-phase *S. gallolyticus* subsp. *gallolyticus* culture diluted to approximately 5×10^7 CFU/mL was seeded onto a monolayer HT-29 cells cultured in a 24-well plate, with a multiplicity of infection of 20. After 1 hour of incubation at 37°C, 5% CO₂, the monolayer was washed two times with PBS to remove the non-adherent bacteria. The monolayer was re-suspended in 0.05% Triton X-100. The amount of adherent bacteria was determined by CFU count.

3.4.11 Immunofluorescence imaging. A monolayer of HT-29 cells was infected as described in “Cell adherence assay”. Following incubation, the monolayer was washed once with PBS and fixed in 4% paraformaldehyde for 15 minutes. The samples were subsequently incubated for 1 hour in PBS containing rabbit anti-UC34 (1:200), followed by an additional 1 hour incubation in PBS containing Alexafluor-488-conjugated goat anti-rabbit antibody (1:200) (Abcam) and Hoechst 33342 (1:500) (Thermo Fisher Scientific) (12). The samples were imaged using an LSM780 inverted confocal laser scanning microscopy (Carl Zeiss) fitted with Plan Apochromat 40x/1.3 and 63x/1.4 oil objective lens, with excitation at 405 nm and 488 nm. Tile scan images were stitched using Image Stitching plugins on Fiji (144). All images were processed using Imaris version 8.2.0 (Bitplane).

3.4.12 RNA extraction and sequencing. 1 mL of the log-phase *S. gallolyticus* subsp. *gallolyticus* culture diluted to approximately 3×10^7 CFU/mL was added into each well of a 24-well plate and incubated at 37°C under anaerobic condition (0% O₂, 10% CO₂, AnaeroGen Compact, prepared according to the manufacturer's instruction, Thermo Fisher Scientific). After 5 hours of incubation, 300 µL of the non-biofilm fraction of the bacterial culture was collected and preserved in 2 volume of RNeasy Protect Bacterial Reagent (Qiagen) and extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. The extracted RNA was depleted by using the Ribo-Zero rRNA removal kit (Bacteria) (Illumina) and converted to cDNA by using the NEBNext RNA First Strand Synthesis Module and the NEBNext Ultra Directional RNA Second Strand Synthesis Module (New England Biolabs), and subsequently sequenced on the Illumina HiSeq 2500 platform (100bp pair-end reads) in our in-house sequencing facility.

3.4.13 RNA-seq data analysis and functional annotation. The sequencing raw reads from the RNA-seq experiment were trimmed and mapped to *S. gallolyticus* subsp. *gallolyticus* UCN34 genome with CLC Genomics Workbench 8.0. A differential analysis of the *S. gallolyticus* subsp. *gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain was performed using the R/Bioconductor DESeq2 package. Functional annotation on the differentially regulated genes was performed based on Clusters of Orthologous Groups (COG) classification and manually corrected based on published literatures.

3.4.14 Cell wall extracts preparation and immunoblotting. Cell wall extracts were prepared as described earlier (99) and quantified using a Qubit™ 2.0 Fluorometer (Thermo Fisher Scientific). Equal amount of the cell wall extracts were boiled in NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific), separated by SDS-PAGE on a NuPAGE™ 4-12% Bis-Tris Protein gradient gel (Thermo Fisher Scientific) and transferred to a PVDF membrane using an iBlot transfer pack (Thermo Fisher Scientific). The membrane was blocked in casein blocking buffer (Sigma Aldrich) and incubated for 1 hour with rabbit primary Pil3B antibodies (1:1000) and subsequently with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000). The membrane was washed 3 times with PBS + 0.1% Tween 20 between the incubation with antibodies. Chemiluminescence was detected on a ChemiDoc Gel Imaging System (Bio-rad laboratories).

3.4.15 Accession numbers. The raw RNA-sequencing reads were deposited on NCBI Sequence Read Archive (SRA) database under BioProject number PRJNA484077.

3.5 Supplementary data

Table 3.3 Genes differentially expressed in *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$.

Functions	Locus tag	Gene annotation	$\Delta gdpP$ vs UCN34	$\Delta gdpP$ vs $\Delta gdpP/pgdpP$
Up-regulation				
Transcription	Gallo_0126	Putative transcriptional regulator, MutR family	2.2203	2.0186
Transcription	Gallo_0876	Putative transcriptional regulator, GntR family	2.1961	2.1038
Transcription	Gallo_1206	Putative transcriptional regulator, AraC family	2.4988	2.7743
Transcription	Gallo_1524	Putative transcriptional regulator, TetR family	3.7866	4.8320
Transcription	Gallo_2146	Putative phage transcriptional regulator	2.2095	2.1041
Transcription	Gallo_2208	Putative transcriptional regulator	2.3114	2.0343
Gallocin production	Gallo_2019	BlpC, gallocin-immunity protein	2.4953	3.5185
Gallocin production	Gallo_2020	BlpB, gallocin	2.0721	3.4750
Competence for genetic transformation	Gallo_0087	Putative competence protein ComGA	4.6259	3.1731
Competence for genetic transformation	Gallo_0088	Putative competence protein ComGB	3.0641	3.0039
DNA replication, recombination and repair	Gallo_0833	Putative 7,8-dihydro-8-oxoguanine-triphosphatase (MutT)	2.1296	2.0645
DNA replication, recombination and repair	Gallo_0627	Putative exonuclease VII large subunit	2.6346	2.3805

DNA replication, recombination and repair	Gallo_0628	Putative exonuclease VII small subunit	2.4982	2.0377
Translation, ribosomal structure and biogenesis	Gallo_0128	Putative ribosomal large subunit pseudouridine synthase, RluD family	2.4465	2.3700
Translation, ribosomal structure and biogenesis	Gallo_1418	GuaD; Putative guanine deaminase	2.6843	2.4553
Translation, ribosomal structure and biogenesis	Gallo_2260	Conserved hypothetical protein	2.2551	2.8034
Carbohydrate transport and metabolism	Gallo_0153	Putative PTS system, lactose/cellobiose-specific, EIIB component	2.7401	2.1511
Carbohydrate transport and metabolism	Gallo_0961	Putative glycosyl hydrolase	2.3043	2.0720
Carbohydrate transport and metabolism	Gallo_1055	GtfA; Putative glucosyltransferase	2.5654	3.9848
Carbohydrate transport and metabolism	Gallo_0238	Phosphoglycerate mutase family protein	2.3446	2.2999
Carbohydrate transport and metabolism	Gallo_1460	GlxK; Putative glycerate kinase	2.0648	2.0631
Coenzyme transport and metabolism	Gallo_0218	Putative HAD-superfamily hydrolase/phosphatase	2.0432	2.3437
Coenzyme transport and metabolism	Gallo_0362	Conserved hypothetical protein	2.3278	2.0179
Nucleotide transport and metabolism	Gallo_0025	PurC; phosphoribosylaminoimidazole succinocarboxamide synthetase	2.3515	2.3036

Amino acid transport and metabolism	Gallo_1809	LysA; diaminopimelate decarboxylase	2.1265	2.5204
Unknown	Gallo_0577	Putative collagen-binding protein, peptidoglycan linked protein (LPXTG motif)	3.7235	4.4586
Unknown	Gallo_0696	Putative serine-rich lipoprotein	4.9980	3.8973
Unknown	Gallo_1689	Tn916 conserved hypothetical protein	2.0751	2.0279
Unknown	Gallo_1690	Tn916 conserved hypothetical protein	2.3340	2.1500
Unknown	Gallo_1691	Tn916 conserved hypothetical protein	2.0016	2.1037
Unknown	Gallo_1692	Tn916 conserved hypothetical protein	2.0663	2.3064
Unknown	Gallo_1693	Tn916 conserved hypothetical protein	2.0395	2.4673
Unknown	Gallo_1694	Tn916 conserved hypothetical protein	2.2405	2.2777
Unknown	Gallo_1695	Tn916 conserved hypothetical protein	2.5377	2.4170
Unknown	Gallo_1696	Putative transcriptional regulator, Cro/C1 family (Tn916)	2.2828	2.6422
Unknown	Gallo_1697	Tn916 conserved hypothetical protein, putative translocase	2.1169	2.2720
Unknown	Gallo_1698	Tn916 conserved hypothetical protein	2.0943	2.4597
Unknown	Gallo_1701	Conserved hypothetical protein	2.4449	2.5155
Unknown	Gallo_1702	Conserved hypothetical protein	2.3774	2.5283
Unknown	Gallo_0129	Conserved hypothetical protein	3.0382	2.2538
Unknown	Gallo_0275	Conserved hypothetical protein, transposon related	2.5606	2.3988
Unknown	Gallo_0276	Hypothetical protein	2.0167	2.7539

Unknown	Gallo_0277	Hypothetical protein	2.0118	2.3777
Unknown	Gallo_0298	Hypothetical protein	2.5655	2.8552
Unknown	Gallo_0401	Conserved hypothetical protein	2.2383	2.1381
Unknown	Gallo_0553	Conserved hypothetical protein	2.2815	2.3283
Unknown	Gallo_0578	Conserved hypothetical protein	2.7291	2.3280
Unknown	Gallo_0579	Conserved hypothetical protein	2.2829	3.3717
Unknown	Gallo_0580	Conserved hypothetical protein	7.9744	6.4795
Unknown	Gallo_0610	Conserved hypothetical protein	2.6511	2.0823
Unknown	Gallo_0611	Conserved hypothetical protein, MutT/nudix family	2.4904	3.8769
Unknown	Gallo_0647	Conserved hypothetical protein	2.4801	2.5021
Unknown	Gallo_1066	Conserved hypothetical protein	2.3822	2.3172
Unknown	Gallo_1199	Hypothetical protein	2.1046	2.6831
Unknown	Gallo_1526	Conserved hypothetical protein	2.4440	3.4303
Unknown	Gallo_1704	Conserved hypothetical protein	2.1550	2.5287
Unknown	Gallo_1705	Conserved hypothetical protein	2.4121	2.0303
Unknown	Gallo_1943	Conserved hypothetical protein	2.2780	2.0434
Unknown	Gallo_1951	Conserved hypothetical protein	2.0056	2.1246
Unknown	Gallo_1955	Conserved hypothetical protein	2.4103	2.0387
Unknown	Gallo_2215	Conserved hypothetical protein	2.3301	2.6427

Down-regulation				
Signal transduction mechanisms	Gallo_2236	GdpP; c-di-AMP phosphodiesterase	-63.2552	-982.8238
Ligands transportation across membrane	Gallo_1041	Putative ABC-type multidrug transporters, ATP-binding/permease protein	-3.1381	-2.9258
Ligands transportation across membrane	Gallo_1042	Putative ABC-type multidrug transporters, ATP-binding/permease protein	-2.9779	-2.5898
Ligands transportation across membrane	Gallo_1172	Putative ABC transporters, permease protein	-4.1248	-2.1596
Ligands transportation across membrane	Gallo_1224	Putative spermidine/putrescine ABC transporters, spermidine/putrescine-binding protein	-3.7523	-2.9422
Ligands transportation across membrane	Gallo_1225	Putative spermidine/putrescine ABC transporters, permease protein	-2.6229	-2.4697
Ligands transportation across membrane	Gallo_1226	Putative spermidine/putrescine ABC transporters, permease protein	-2.2420	-2.3960
Ligands transportation across membrane	Gallo_1282	Putative proline/glycine betaine ABC transporters, permease protein	-3.8579	-2.2637
Ligands transportation across membrane	Gallo_1283	Putative proline/glycine betaine ABC transporters, ATP-binding protein	-4.4506	-2.0119
Ligands transportation across membrane	Gallo_1284	Putative proline/glycine betaine ABC transporters, substrate-binding protein	-5.0981	-2.4639
Ligands transportation across membrane	Gallo_1299	Putative ABC-type multidrug transporters, ATP-binding/permease protein	-2.5724	-2.6940
Ligands transportation across membrane	Gallo_1300	Putative ABC-type multidrug transporters, ATP-binding/permease protein	-2.6572	-2.0567

Ligands transportation across membrane	Gallo_1919	Putative sulfonate ABC transporters, ATP-binding protein	-2.5969	-4.0894
Ligands transportation across membrane	Gallo_1920	Putative sulfonate ABC transporters, permease protein	-2.5035	-4.1822
Ligands transportation across membrane	Gallo_1921	Putative sulfonate ABC transporters, substrate-binding protein	-2.0983	-3.3076
Pilus 3 biosynthesis	Gallo_2038	SrtC	-5.2687	-4.7045
Pilus 3 biosynthesis	Gallo_2039	Pil3B	-3.6501	-3.5732
Pilus 3 biosynthesis	Gallo_2040	Pil3A	-4.7754	-2.7881
Transcription	Gallo_0210	Transcriptional regulator, MarR family	-2.4851	-3.9567
Transcription	Gallo_1212	Putative transcription anti terminator BglG family	-2.1111	-2.0056
Transcription	Gallo_1286	Putative transcriptional regulator, MarR family	-3.9529	-2.2224
DNA replication, recombination and repair	Gallo_0978	ParC; DNA topoisomerase IV subunit A	-2.3698	-2.6929
DNA replication, recombination and repair	Gallo_0007	Mfd; transcription repair coupling factor	-2.7378	-3.6222
DNA replication, recombination and repair	Gallo_1925	Putative A/G-specific adenine glycosylase	-3.3180	-2.0135
Nucleotide transport and metabolism	Gallo_0704	NrdF; Ribonucleoside-diphosphate reductase (minor subunit)	-10.0501	-2.3125
Nucleotide transport and metabolism	Gallo_1449	Putative hydrolase	-2.6656	-2.0092

Nucleotide transport and metabolism	Gallo_1474	Putative 5'-Methylthioadenosine/S-adenosylhomocysteine nucleosidase	-3.8207	-2.0309
Translation, ribosomal structure and biogenesis	Gallo_0006	Pth; Peptidyl-tRNA hydrolase	-3.1115	-3.9031
Translation, ribosomal structure and biogenesis	Gallo_0373	RbfA; ribosome binding factor A	-4.5914	-2.4593
Carbohydrate transport and metabolism	Gallo_0183	Putative PTS system, mannitol/fructose-specific, IIC component	-2.0946	-2.6001
Amino acid transport and metabolism	Gallo_1468	HisB; Imidazoleglycerol-phosphate dehydratase	-2.0256	-2.8221
Amino acid transport and metabolism	Gallo_1469	SerB; phosphoserine phosphatase	-2.0495	-2.5757
Coenzyme transport and metabolism	Gallo_1230	FolB; Putative dihydroneopterin aldolase	-2.2070	-2.3828
Coenzyme transport and metabolism	Gallo_0211	Putative NADPH-dependent FMN reductase	-2.5166	-2.7520
Coenzyme transport and metabolism	Gallo_1160	RibF; Putative riboflavin kinase/FAD synthetase	-2.3192	-2.0414
Energy production and conversion	Gallo_0870	Putative Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component	-3.5310	-2.3151
Cell wall/membrane/envelope biogenesis	Gallo_1371	Putative glycosyltransferase	-2.5386	-3.6903
Unknown	Gallo_0703	Conserved hypothetical membrane protein	-3.4322	-2.4818
Unknown	Gallo_0720	Conserved hypothetical protein	-5.4100	-2.1619

Unknown	Gallo_0739	Conserved hypothetical protein	-2.0009	-3.0087
Unknown	Gallo_0815	Conserved hypothetical protein	-2.7158	-6.5851
Unknown	Gallo_1039	Conserved hypothetical protein	-2.7108	-2.5887
Unknown	Gallo_1091	Conserved hypothetical protein	-2.9080	-3.1903
Unknown	Gallo_1369	Conserved hypothetical protein	-2.0555	-3.0494
Unknown	Gallo_1408	Conserved hypothetical protein	-3.2086	-2.9453
Unknown	Gallo_1926	Conserved hypothetical protein	-2.5550	-2.1053
Unknown	Gallo_2206	Conserved hypothetical protein	-2.0666	-2.5841
Unknown	Gallo_2229	Conserved hypothetical membrane protein	-9.6665	-2.2782

Chapter 4 : The extracellular pullulanase PulA is responsible in part for the c-di-AMP control on *S. gallolyticus* subsp. *gallolyticus* biofilm development

4.1 Introduction

It has been recently proposed that *S. gallolyticus* subsp. *gallolyticus* may be an auxiliary factor in colorectal cancer development (134). This likely requires *S. gallolyticus* subsp. *gallolyticus* to stably colonise the mucosal layer of the gastrointestinal tract in order to have prolonged contact with the host cells. I have shown in **Chapter 3** that the adherence of *S. gallolyticus* subsp. *gallolyticus* on human colorectal adenocarcinoma cells *in vitro* is modulated by the second messenger cyclic diadenosine-monophosphate (c-di-AMP). I have also shown that c-di-AMP is involved in the *S. gallolyticus* subsp. *gallolyticus* biofilm development, a factor that has been found to promote colorectal cancer progression (14, 26, 145).

Although c-di-AMP mediates biofilm formation in a number of Gram-positive bacteria, it is less clear on how changes in the intracellular c-di-AMP level impact biofilm development, largely due to a lack of consistency in the observations reported (56, 58-61). Whereas the molecular mechanisms concerning the positive impacts of high levels c-di-AMP on *Streptococcus mutans* biofilm development have been unravelled, the findings are found to be in sharp contrast to the study by Li and colleagues demonstrating that low levels of c-di-AMP promotes *S. mutans* extracellular polymeric substances production and subsequent

biofilm development (60). Further studies will therefore allow better understanding on the regulatory roles of c-di-AMP in bacterial biofilm development.

In this study, I focused on a *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant that displays a partial restoration in biofilm developmental defect of the $\Delta gdpP$ mutant. This strain was found to carry a point mutation in the maltose operon repressor MalR, producing a truncated, loss-of-function MalR. Due to the mutation, this $\Delta gdpP$ mutant, denoted as $\Delta gdpP^{MalR^*}$, overexpresses the extracellular pullulanase PulA. Both the complementation with the wildtype *malR* in *trans* and the deletion of *pulA* in the $\Delta gdpP^{MalR^*}$ mutant reverted the biofilm levels back to the parental $\Delta gdpP$ mutant levels. I further showed that MalR can bind to the *pulA* putative promoter region, confirming that MalR is a bona fide transcriptional regulator that modulates the expression of PulA. Importantly, I found that the *pulA* expression was down-regulated in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant compared to the UCN34 wildtype strain and the $\Delta gdpP/pgdpP$ complementation strain. Combined, these data suggest that the *pulA* expression is responsive to the intracellular c-di-AMP level in *S. gallolyticus* subsp. *gallolyticus*, and contribute in part to the biofilm development.

4.2 Results

4.2.1 Point mutation in MalR at the amino acid position 258 partially restored the biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant. We have previously reported the pleiotropic roles of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus* UCN34 by characterising a deletion mutant of *gdpP* encoding the c-di-AMP phosphodiesterase (145). At the final step of the deletion mutant generation procedure, we isolated a few *gdpP* deletion mutants and tested the biofilm formation capability of these mutants. One of the *gdpP* deletion mutants displayed clearly reduced biofilm formation, and was later whole-genome sequenced and found to be a clean deletion mutant with no additional non-synonymous point mutations in the genome (Supplementary data, Table 4.2). This mutant was used in the serial investigations in our previous work (145).

Surprisingly, a second *gdpP* deletion mutant appeared to have less impact on the biofilm formation capability compared with the UCN34 wildtype (Figure 4.1A). Whole genome sequencing on this strain revealed a point mutation at the nucleotide position 722 (C>T) of an open reading frame encoding a protein annotated as a putative maltose operon repressor MalR. This point mutation altered the coding sequence CAG to TAG, and therefore introduced a translational stop codon, substituting the glutamine residue at the amino acid position 258. This mutation resulted in the expression of a C-terminal truncated MalR. The truncated MalR contains only a partial sequence encoding signal sensing domain, therefore, the mutation likely renders the protein loss its function to sense or bind to the cognate

signalling molecules (Figure 4.1B). From now onwards, we denote this $\Delta gdpP$ mutant as $\Delta gdpP^{MalR^*}$.

To confirm that the MalR mutation is responsible for the partial restoration of the biofilm developmental defect of the $\Delta gdpP$ mutant, *malR* was overexpressed in *trans* with a constitutive promoter *ptet* in the $\Delta gdpP^{MalR^*}$ mutant ($\Delta gdpP^{MalR^*}/ptet::malR$). As expected, the $\Delta gdpP^{MalR^*}/ptet::malR$ strain formed biofilms comparable to the $\Delta gdpP$ mutant levels, confirming that the loss-of-function MalR partially restored the biofilm development defect of the $\Delta gdpP$ mutant (Figure 4.1A).

Figure 4.1 Point mutation in MalR at the amino acid position 258 partially restored the biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant. (A) The *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant expressing C-terminal truncated MalR (denoted as $\Delta gdpP^{MalR^*}$ mutant) showed a partial restoration of the biofilm developmental defect of the parental $\Delta gdpP$ mutant. An *in trans* overexpression of the wildtype *malR* with the *ptet* constitutive promoter reverted the biofilm levels of the $\Delta gdpP^{MalR^*}$ mutant back to the $\Delta gdpP$ mutant levels. Scattered data points represent every single measurement. Bar graph represents the data mean value from three independent experiments. Error bars represent the standard deviation. Ordinary one-way ANOVA test, ****, P-value ≤ 0.0001 ; ns, P-value > 0.05 . (B) A single nucleotide mutation in MalR at the amino acid position 258 resulted the translation of a stop codon rather than a glutamine in the $\Delta gdpP^{MalR^*}$ mutant.

4.2.2 The partial restoration of the biofilm developmental defect in the $\Delta gdpP^{MalR^*}$ mutant is likely not due to restoration of the intracellular c-di-AMP level. Our previous findings indicated that the *S. gallolyticus* subsp. *gallolyticus* biofilm development is mediated by the intracellular c-di-AMP level (145). Given that c-di-AMP can be exported from bacteria

through efflux pumps (84), and MalR has been previously reported to regulate the efflux pumps expression (146), we therefore hypothesised that MalR may control the intracellular c-di-AMP levels. To test this, we quantified the intracellular c-di-AMP level of the UCN34 wildtype, the $\Delta gdpP$ mutant, and the $\Delta gdpP^{MalR^*}$ mutant. As shown in Figure 4.2A, the intracellular c-di-AMP level in the $\Delta gdpP$ mutant was approximately 1.4-fold higher than the wildtype, a similar difference level we have previously observed (145). The intracellular c-di-AMP level of the $\Delta gdpP^{MalR^*}$ mutant was not significantly altered than the $\Delta gdpP$ mutant level (Figure 4.2A). Further phenotypic characterisation of the $\Delta gdpP^{MalR^*}$ mutant revealed that the strain remained smaller than the wildtype (even smaller than the $\Delta gdpP$ mutant), and remained sensitive to high osmotic stress (Figure 4.2 B-D). These two phenotypic alterations are the characteristics of an increased c-di-AMP level in *S. gallolyticus* subsp. *gallolyticus* as well as in other bacteria such as *Staphylococcus aureus* (56, 77). Taken together, these results indicate that the partial restoration of the biofilm developmental defect in the $\Delta gdpP^{MalR^*}$ mutant is likely not due to the restoration of the intracellular c-di-AMP level.

Figure 4.2 The partial restoration of the biofilm developmental defect in the $\Delta gdpP^{MalR^*}$ mutant is unlikely due to the restoration of the intracellular c-di-AMP level. (A) Quantification of the intracellular c-di-AMP level in *S. gallolyticus* subsp. *gallolyticus* and the derivatives using high-liquid performance chromatography. Scattered data points represent measurements from four biological replicates. Bar graph represents the data mean value. Error bars represent the standard deviation. Ordinary one-way ANOVA test, *, P-value ≤ 0.05 ; **, P-value ≤ 0.01 ; ns, P-value > 0.05 . (B) Representative phase contrast microscopy images of *S. gallolyticus* subsp. *gallolyticus* and the derivatives. Scale bar = 10 μm . (C) Cell area measurement on 150 cells of *S. gallolyticus* subsp. *gallolyticus* and the derivatives from three independent experiments using ImageJ software. Scattered

data points represent every single measurement. Bars represent the data mean value $\pm$ standard deviation. Ordinary one-way ANOVA test, ****, P-value $\leq$ 0.0001. (D) Representative images of 5 μ L *S. gallolyticus* subsp. *gallolyticus* log-phase culture adjusted to approximately 3×10^7 CFU/mL spotted onto BHI agar and BHI agar supplemented with 0.4 M NaCl, and incubated at 37°C for up to 2 days.

4.2.3 Proteomic approach revealed a Pula overexpression in the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP^{MalR^*}$ mutant. We next hypothesised that MalR may be regulating the expression of surface proteins or adhesins that are responsible for *S. gallolyticus* subsp. *gallolyticus* surface attachment or biofilm formation. To test this, we employed a proteomics approach by identifying and quantifying the proteins localised in the cell wall and the cell membrane fraction (collectively known as surface proteins) (Figure 4.3A). This approach identified a significant overexpression of GALLO_1462 in the $\Delta gdpP^{MalR^*}$ mutant compared to the $\Delta gdpP$ mutant (Figure 4.3B).

GALLO_1462 encodes a putative pullulanase that shares 59% similarity to the *Streptococcus pyogenes* Pula (Spy1972) and *Streptococcus pneumoniae* SpuA (SP0268). We renamed GALLO_1462 as Pula in this study. The protein sequence of Pula contains a YSIRK signal peptide sequence in a region overlapped with a transmembrane region, an alpha-amylase domain flanked by four carbohydrate-binding module belonging to the Family 41 (Figure 4.4A). The C-terminal part of Pula contains an LPxTG motif, a hydrophobic region and a charged tail, suggesting that it is a LPxTG family surface protein.

To verify that a Pula overexpression restores the biofilm developmental defect of the $\Delta gdpP$ mutant, we replaced the genetic region encoding Pula with a kanamycin resistance cassette in the $\Delta gdpP^{MalR^*}$ mutant. This strain, denoted as $\Delta gdpP^{MalR^*} \Delta pula::kanR$ mutant, formed less biofilm compared to the $\Delta gdpP^{MalR^*}$ mutant, to a level comparable to the $\Delta gdpP$

mutant (Figure 4.3C). Combined, these data indicate that the loss-of-function MalR resulted in a PulA overexpression to partially restore the biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant. These data also suggest that MalR and PulA are involved in *S. gallolyticus* subsp. *gallolyticus* biofilm development.

Figure 4.3 Proteomic approach revealed PulA overexpression in the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP^{MalR^*}$ mutant. (A) Overview of the surfomics workflow. *S. gallolyticus* subsp. *gallolyticus* surface proteins were trypsinised for an hour at 37°C. The fraction containing the trypsinised surface proteins (surfomes) was collected and subjected to liquid chromatography coupled with tandem mass spectrometry for protein identification and quantification. (B) Surface proteins identified in the surfomes of the $\Delta gdpP$ mutant and the $\Delta gdpP^{MalR^*}$ mutant. Proteins were shortlisted based on the abundance difference of > 2 fold in relative to the mean value of the $\Delta gdpP^{MalR^*}$ mutant. NF, not found. N.A., not applicable. (C) The replacement of *pulA* with a kanamycin resistance cassette in the $\Delta gdpP^{MalR^*}$ mutant ($\Delta gdpP^{MalR^*} \Delta pulA::kanR$) restored the biofilm back to the $\Delta gdpP$ mutant levels. Scattered data points represent every single measurement. Bar graph represents the data mean value from three biological replicates. Error bars represent the standard deviation. Ordinary one-way ANOVA test, ***, P-value ≤ 0.001 ; ****, P-value ≤ 0.0001 ; ns, P-value > 0.05 .

4.2.4 Recombinant MalR binds to the putative *pulA* promoter region. We next wondered how did the production of a loss-of-function MalR contribute to the overexpression of PulA. Since MalR is a transcriptional regulator, therefore it is likely that MalR binds to the *pulA* promoter region to inhibit the *pulA* expression. Inspection on the 5' untranslated region of *pulA* revealed a short imperfect palindrome repeat sequence highly similar to the previously reported consensus MalR binding site (AAGCAATCGCTTGCGC, compared with the consensus sequence NNGCAARCGNTTGCRY) (146, 147) (Figure 4.4B). Our electromobility shift assay confirmed that the recombinant MalR specifically binds to a 35-bp DNA sequence containing the putative MalR binding sequence found in the promoter region of *pulA*, but not to the scrambled DNA sequence (Figure 4.4C). This result indicates that MalR recognises and binds to a specific DNA sequence present in the putative *pulA* promoter region to represses *pulA* transcription.

Figure 4.4 Recombinant MalR binds to the *pulA* promoter region to regulate the *pulA* expression. (A) *In silico* analysis on the domain architecture of GALLO_1462 (renamed Pula in this study) revealed that Pula contains a YSIRK signal peptide sequence in a region overlapped with a transmembrane region (indicated in gray), an alpha-amylase domain flanked by 4 carbohydrate-binding modules belonging to the Family 41. (B) The 5' untranslated end of *pulA* contains a sequence highly similar to the previously reported MalR consensus binding sequence. The base pair different to the consensus sequence was in bold and underlined. (C) Representative images of the electromobility shift assay performed with the recombinant MalR and a 35-bp DNA sequence with various protein:DNA molar ratio. The DNA

sequence used in the assay was indicated at the bottom of the images. Scrambled DNA sequence (left) was used as a negative control.

4.2.5 The *pulA* expression is responsive to the intracellular c-di-AMP level and is in part responsible for the biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant. Our results suggest that MalR/PulA pathway may involve in *S. gallolyticus* subsp. *gallolyticus* biofilm development. Given that MalR can repress *pulA* expression, we therefore hypothesised that the up-regulated *malR* expression – therefore the down-regulated *pulA* expression – can limit *S. gallolyticus* subsp. *gallolyticus* biofilm development. To test this, we transformed the UCN34 wildtype with a plasmid to induce *malR* overexpression. This strain, denoted as UCN34/*ptet::malR*, as expected displayed a 20% reduction in the biofilm level compared to the parental strain UCN34 (Figure 4.5A). We next compared the biofilm levels of the $\Delta pul::kanR$ mutant to the back-to-wildtype (bWT) strain and found that the deletion of *pulA* resulted in 18% reduction in the biofilm level (Figure 4.5B). Notably, the bWT strain, which had undergone the same gene replacement procedure as the $\Delta pul::kanR$ mutant but remained carrying the wildtype *pulA* gene, but not the UCN34 wildtype was used for the comparison. This is due to the observations that both the $\Delta pul::kanR$ mutant and the bWT strain unexpectedly overproduced biofilms than the UCN34 wildtype (Figure 4.5B), probably caused by the unknown factors introduced during the gene replacement procedure.

We then wondered whether c-di-AMP mediates *S. gallolyticus* subsp. *gallolyticus* biofilm development in part through the regulation on *malR/pulA* expression. To test this, we compared the *malR* and *pulA* transcript levels among the UCN34 wildtype, the $\Delta gdpP$ mutant, and the *gdpP*-complemented strain ($\Delta gdpP/pgdpP$). While it is inconclusive

whether the *malR* expression was differentially regulated, the *pulA* expression was found down-regulated by 2-fold in the $\Delta gdpP$ mutant compared to the other two strains (Figure 4.5C). This result suggests that *pulA* expression is responsive to the intracellular c-di-AMP level, in which high intracellular c-di-AMP represses *pulA* expression, and the reduced expression of *pulA* subsequently limits biofilm development in *S. gallolyticus* subsp. *gallolyticus*.

Figure 4.5 The expression of *pulA* is responsive to the intracellular c-di-AMP level and is in part responsible for the biofilm developmental defect of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant. (A) An overexpression of *malR* driven by the constitutive promoter *Ptet* in *S. gallolyticus* subsp. *gallolyticus* limits the biofilm development compared to the UCN34 wildtype. Scattered data points represent every single measurement. Bar graph represents the data mean value from three biological replicates. Error bars represent the standard deviation. Two-tailed T-test, *, P-value ≤ 0.05 . (B) The gene replacement procedure of *pulA* with a kanamycin resistance cassette has unexpectedly resulted in the generation of both the $\Delta pul::kanR$ and the back-to-wildtype (bWT) strains that overproduce biofilms compared to the UCN34 wildtype. Scattered data points represent every single measurement. Bar graph represents the data mean value from at least two biological replicates. Two-tailed T-test, *, p-value ≤ 0.05 , ****, p-value < 0.0001 . (C) Quantitative PCR measuring the *malR* and *pulA* transcript levels in the *S. gallolyticus* subsp. *gallolyticus* UCN34 wildtype, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$. Whereas the *malR* expression is differentially regulated in the $\Delta gdpP$ mutant, the *pulA* expression is down-regulated in the $\Delta gdpP$ mutant compared to the UCN34 wildtype and *gdpP*-complemented strain ($\Delta gdpP/pgdpP$). Scattered data points represent the measurements of each biological replicate. Bar graph represents the data mean value from three biological replicates. Ordinary ANOVA, *, p-value ≤ 0.05 , ns, p-value > 0.05 .

4.3 Discussion

C-di-AMP is a second messenger molecule produced in a wide-range of bacteria including Firmicutes. The notion that c-di-AMP plays a major role in osmotic regulation is well supported in the published literature (70). However, the role of c-di-AMP in biofilm formation is relatively less well defined. We have previously shown that an increased intracellular c-di-AMP level due to the deletion of c-di-AMP phosphodiesterase *gdpP* resulted in reduced biofilm development in *S. gallolyticus* subsp. *gallolyticus* (145). In this study, we took the advantage of a spontaneously isolated *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant that produces a C-terminal truncated maltose operon repressor MalR. The mutant, denoted as $\Delta gdpP^{MalR^*}$, overexpresses the extracellular pullulanase Pula to partially restore the biofilm developmental defect of the $\Delta gdpP$ mutant. This partial biofilm developmental defect restoration was lost when the wildtype *malR* was expressed in *trans* and when *pula* was deleted in the $\Delta gdpP^{MalR^*}$ mutant. We further verified that MalR can bind to the 5' untranslated region of *pula* through its recognition of a specific DNA sequence that has previously reported to be the MalR consensus binding sequence, suggesting that *malR* is a transcriptional repressor of *pula*. Taken together with the observations that *malR* overexpression in the UCN34 wildtype limits biofilm development, and that *pula* expression was down-regulated in the $\Delta gdpP$ mutant compared to the UCN34 wildtype, our data suggest that c-di-AMP controls *S. gallolyticus* subsp. *gallolyticus* biofilm formation in part through the regulation on *pula* expression.

PulA is a LPxTG-family surface protein primarily functions as an extracellular enzyme to hydrolyse pullulan to maltotriose that is subsequently imported into bacterial cells as a carbon source. The hydrolysis takes place at the alpha-amylase domain, with the carbohydrate-binding modules aids in the targeting of the substrate (148). Previous studies have shown that the carbohydrate-binding modules of the *Streptococcus pneumoniae* and *Streptococcus pyogenes* PulA/SpuA can bind to various alpha-glucans including amylose (pure alpha-(1,4)-linked glucose), amylopectin (alpha-(1,4)-linked glucose with alpha-(1,6) branch), pullulan (linear polymer of mixed alpha-(1,4)- and alpha-(1,6)-linked glucose), glycogen (alpha-(1,4)-linked glucose with alpha-(1,6) branch) and maltotetraose (alpha-(1,4)-glucotetraose) (102, 146). Another study indicated that the full-length *S. pyogenes* PulA can in addition bind to glycoproteins including thyroglobulin, submaxillary mucin, fetuin and asialofetuin that are present on host cell surfaces (100). Therefore, the presence of PulA at the bacterial cell surface would be expected to function as an adhesin to facilitate bacterial surface attachment.

Indeed, PulA has been identified as a streptadhesin (*Streptococcus* adhesin) (100). The purified carbohydrate-binding modules of the PulA/SpuA can bind to the murine alveolar type II cells that are rich in glycogen (102), whereas the full-length PulA can bind *in vitro* to the human cervical epithelial cells (105). Consistently, SpuA was found essential for *S. pneumoniae* to establish infection in the murein lung infection model, and a *S. pyogenes* $\Delta pulA$ mutant was found to colonise less efficiently in the mouse oropharynx (106, 146). These studies suggested that pullulanase is an important colonisation factor for *Streptococcus* spp. and is most likely important for establishment of an infection. In support

of this, antibodies against the bacterial pullulanase can be detected in the human convalescent-phase serum (149). Intriguingly, mice subjected to immunisation with pullulanase prolonged the mean survival time, suggesting that pullulanase can be an attractive vaccine target (150). Nevertheless, while it is established that PulA can facilitate the bacterial binding to host cells, how does PulA contribute to *S. gallolyticus* subsp. *gallolyticus* on abiotic surfaces in this study awaits further investigation.

Notably, the arisen of MalR truncation in the $\Delta gdpP$ mutant appears to be a result of random events. Repeated passaging of *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant did not result in further isolation of strains with restored biofilm developmental defect due to MalR mutations or PulA overexpression (**Chapter 5**, Figure 5.1, Table 5.2). Neither maltose nor pullulan was present in the culturing medium during the selection of the $\Delta gdpP$ mutant. Therefore, it is unlikely that maltose catabolism poses as a selective pressure for the MalR truncation to arise.

In summary, we have demonstrated that c-di-AMP controls the *S. gallolyticus* subsp. *gallolyticus* biofilm development partially by regulating the expression of the extracellular pullulanase PulA. Our data established a link between c-di-AMP signalling and surface protein expression in *S. gallolyticus* subsp. *gallolyticus*, and advanced our understandings on the c-di-AMP control on bacterial biofilm development.

4.4 Materials and methods

4.4.1 Bacterial strains, primers and plasmids. All bacterial strains, primers and plasmids used in this study were listed in Table 3.1. Overnight cultures of *S. gallolyticus* subsp. *gallolyticus* were typically prepared from single colonies in 2 mL of M9YEG (1X M9 minimal salts supplemented with 0.5% yeast extract and 1.0% glucose). The overnight cultures were 5X diluted the next day and subsequently grown to mid-log phase for all the experiments. When appropriate, erythromycin was supplemented at a final concentration of 2 µg/mL for *S. gallolyticus* subsp. *gallolyticus* or 500 µg/mL for *Escherichia coli* DH5α.

Table 4.1 Bacterial strains, plasmids and primers used in this study.

Bacterial strains	Description	Reference
<i>E. coli</i> DH5α	<i>deoR endA1 gyrA96 hsdR17 (Δlac)U169 recA1 relA1 supE44 thi-1 (Φ80 lacZΔM15)</i>	Lab collection
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B (r_B⁻ m_B⁻) λ</i> (DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>]) [<i>malB</i> ⁺] _{K-12} (λ ^S)	Lab collection
<i>S. gallolyticus</i> subsp. <i>gallolyticus</i> UCN34	a clinical strain isolated from an infective endocarditis patient who later diagnosed with CRC	(89)
UCN34 Δ <i>gdpP</i>	in-frame <i>gdpP</i> (<i>GALLO_2236</i>) knock-out mutant	(145)
UCN34 Δ <i>gdpP</i> ^{MalR*}	in-frame <i>gdpP</i> (<i>GALLO_2236</i>) knock-out mutant with a point mutation in <i>malR</i> (<i>GALLO_1398</i>) at nucleotide pos.1468237	This study
UCN34 Δ <i>gdpP</i> ^{MalR*} / <i>ptet::malR</i>	UCN34 Δ <i>gdpP</i> ^{MalR*} carrying the plasmid pTCVerm-Ptet:: <i>malR</i>	This study
UCN34 Δ <i>gdpP</i> ^{MalR*} Δ <i>pulA::kanR</i>	UCN34 Δ <i>gdpP</i> ^{MalR*} with <i>pulA</i> (<i>GALLO_1462</i>) replaced with kanamycin resistance cassette	This study
<i>E. coli</i> BL21(DE3)/pET28b:: <i>rMalR</i>	<i>E. coli</i> BL21(DE3) carrying the plasmid pET28b:: <i>rMalR</i> for recombinant MalR purification	This study
UCN34/ <i>ptet::malR</i>	UCN34 carrying the plasmid pTCVerm-Ptet:: <i>malR</i>	
UCN34 Δ <i>pulA::kanR</i>	UCN34 with <i>pulA</i> (<i>GALLO_1462</i>) replaced with kanamycin resistance cassette	This study
UCN34 Δ <i>gdpP</i> /pg <i>dpP</i>	<i>gdpP</i> -complemented strain; UCN34 Δ <i>gdpP</i> containing pTCVerm:: <i>gdpP</i>	(145)
<i>S. agalactiae</i> NEM316	MLST-23, serotype III isolated from neonate blood culture	(139)
Plasmids		Reference
pG1	Em, <i>oriR</i> pUC, <i>oriR</i> ^{ts} pWV01, MCS pUC18	(140)
pG1:: <i>pulA-kanRKO</i>	pG1 containing a kanamycin resistance cassette flanked by a 0.5-kb fragment corresponding to the 5' and 3' end of <i>GALLO_1462</i>	This study
pGCP123	Kan, <i>oriR</i> ^{ts} pABG5, MCS pJSR233	(151); from Kimberly Kline
pTCVerm-Ptet	Em, Km, Mob ⁺ (IncP); <i>oriR</i> pACYC184; <i>oriR</i> pAMβ1; MCS <i>lacZα</i> ⁺ ; <i>oriT_{TnGBS1}</i> ; Ptet constitutive promoter	(140)
pTCVerm-Ptet:: <i>malR</i>	pTCVerm-Ptet containing the native ribosomal binding site and the open reading frame of <i>malR</i> (<i>GALLO_1398</i>)	This study
pET28b	Kan, <i>ori</i> pBR322, <i>ori</i> f1, MCS (XhoI, NotI, EagI, HindIII, SalI, SacI, EcoRI, BamHI, NheI, NdeI, NcoI),	Lab collection
pET28b:: <i>rMalR</i>	pET28b containing the open reading frame of <i>malR</i> (<i>GALLO_1398</i>) without the sequence encoding the native start codon	This study

Primers	Sequence (5' to 3')	Reference
<i>malR</i> overexpression plasmid		
malR_F	TATAGGATCCAGGAGACTTAGATGGTTAC	This study
malR_R	GCTACTGCAGTAGTTAATCATTGACGTG	This study
<i>pulA</i> replacement with kanamycin resistance cassette		
pulA_up_F	GCAACATGTTGACAATG	This study
pulA_up_R	CAACTGTCAGAATAACTTACAGTAGG	This study
Kan_F	TTCTGACAGTTGCGGATGTACTTC	This study
Kan_R	CTATATGGCAAGGAACAGTGAATTGG	This study
pulA_down_F	CCTTGCCATATAGAACCTCCGTATC	This study
pulA_down_R	GGAAGAAGCGACAGAAG	This study
Recombinant MalR expression		
rMalR_F	GCATCATATGGTTACAATAAAAGATGTAGC	This study
rMalR_R	GCATGGATCCTAGTTAATCATTGACGTG	This study
Oligonucleotides for EMSA		
pulA_EMSA_F	CCTTTCAAAGCAATCGCTTGCCTTAAAATAACGC	This study
pulA_EMSA_R	GCGTTATTTTAAGCGCAAGCGATTGCTTTGAAAGG	This study
pulA_EMSA scrambled_F	AACACTACTGGCTACATATTACTGCAGCCATGCAT	This study
pulA_EMSA scrambled_R	ATGCATGGCTGCAGTAATATGTAGCCAGTAGTGTT	This study
Quantitative PCR primers		
16s_qPCR_F	CAGGTCTTGACATCCCGATGCTAT	(99)
16s_qPCR_R	CGCTAGAGTGCCCAACTGAATGAT	(99)
malR_qPCR_F	CGTCATCCCTGTTGCGATTG	This study
malR_qPCR_R	CGCAAATGTTAGCAAGCGGT	This study
pulA_qPCR_F	TGCAATGGCAGGTGTCGTAT	This study
pulA_qPCR_R	GCCTTGAGCAGTTAGCAACG	This study

* Restriction sites are underlined.

4.4.2 Molecular cloning. Molecular cloning was performed according to the standard protocols described in (152). For the construction of the *malR*-overexpression plasmid (pTCVerm-ptet::*malR*), the *malR* open reading frame with the native ribosomal binding site were amplified from the *S. gallolyticus* subsp. *gallolyticus* UCN34 genome with the primer pair malR_F and malR_R. The resulted PCR product was restricted with *Bam*HI and *Pst*II, ligated into the *Bam*HI/*Pst*I-restricted pTCVerm-Ptet, and cloned into *E. coli* DH5 α .

For the construction of the *pulA* gene-replacement knock-out plasmid (pG1::*pulA*-kanRKO), two fragments with the size of an approximate 450 bp corresponding to the 5' and the 3' end of *pulA* were PCR-amplified from the *S. gallolyticus* subsp. *gallolyticus* UCN34 genome using the primer pairs pulA_up_F and pulA_up_R, and pulA_down_F and pulA_down_R, respectively. A kanamycin resistance cassette (*aph3'*-IIIa) was amplified from the plasmid pGCP123 (151) using the primer pair Kan_F and Kan_R. The PCR fragments generated were subsequently subjected to overlapped PCR extension, and the PCR product was ligated into the *Sma*I-restricted pG1 and cloned into *E. coli* DH5 α .

For the construction of a plasmid for recombinant MalR purification, the open reading frame of *malR* without the start codon was amplified from the *S. gallolyticus* subsp. *gallolyticus* UCN34 genome using the primer pair rMalR_F and rMalR_R. The PCR fragment was restricted with *Nde*I and *Bam*HI, ligated into the *Nde*I/*Bam*HI-restricted pET28b and cloned into *E. coli* DH5 α . Subsequently, the plasmid pET28b::*malR* was extracted, Sanger sequenced verified and electroporated into *E. coli* BL21(DE3).

4.4.3 Construction of *pulA* gene replacement mutants. The pG1::*pulA*-*kanRKO* was introduced into *S. agalactiae* NEM316 (139) by electroporation, and later into *S. gallolyticus* subsp. *gallolyticus* UCN34 or UCN34 $\Delta gdpP^{MalR^*}$ by conjugal transfer. *S. gallolyticus* subsp. *gallolyticus* UCN34 or UCN34 $\Delta gdpP^{MalR^*}$ with the pG1::*pulA*-*kanRKO* integrated into the genome was selected by growing the bacteria at 37°C in the presence of erythromycin. Next, the integrants were serially passaged at 30°C in BHI broth without antibiotic to facilitate the excision of the plasmid by homologous recombination. The erythromycin-sensitive but kanamycin-resistant colonies were screened with PCR by using the primer pair *pulA*_up_F and *pulA*_down_R. The replacement of *pulA* by the kanamycin resistance cassette was confirmed by Sanger-sequencing of the original *pulA* chromosomal region.

4.4.4 Microtiter plate biofilm assay. Assay was performed as described in Chapter 3.

4.4.5 Genomic DNA extraction and sequencing. A total of 500 μ L of overnight *S. gallolyticus* subsp. *gallolyticus* culture was pelleted at 10,000 xg for 2 minutes. The cell pellets were washed once with 0.9% NaCl, and re-suspended in 600 μ L of buffer containing 480 μ L of 50 mM EDTA (pH 8.0) and 120 μ L of 20 mg/mL lysozyme (Sigma Aldrich) with 20 U of mutanolysin (Sigma Aldrich). The mixture was incubated at 37°C for 30 minutes, and pelleted. The genomic DNA was extracted from the cell pellet using Promega Wizard SV Genomic DNA Purification System, by following the manufacturer's protocol. The extracted genomic DNA was sequenced on an Illumina Miseq platform (300 bp pair-end) in our in house sequencing facility.

4.4.6 Bioinformatics analysis. The sequencing raw reads were trimmed and mapped to the *S. gallolyticus* subsp. *gallolyticus* UCN34 genomic sequence with the CLC Genomics Workbench 10.0 (Qiagen). Single nucleotide polymorphisms were identified using the workbench variant detectors suite. The trimmed sequencing reads were also assembled *de novo* into contigs and aligned with the *S. gallolyticus* subsp. *gallolyticus* UCN34 genomic sequence with the Mauve Genome Alignment (153) to identify any large scale mutations.

4.4.7 Preparation of c-di-AMP extract. Intracellular c-di-AMP was extracted as described in Chapter 3.

4.4.8 Intracellular c-di-AMP quantification using high liquid performance chromatography (HPLC). The quantification of the intracellular c-di-AMP level was performed by following the published protocol with minor modifications (154). The dried c-di-AMP crude extract was reconstituted in 200 μ L of ultrapure water and centrifuged at 10,000 xg for 10 minutes at 4°C. The supernatant was collected and analysed on a Shimazu HPLC system equipped with a LC-20AD dual-plunger parallel-flow pumps, a SPD-M20A photo-diode array detector, a SIL-20A autosampler and a CTO-20A column oven maintained at 30°C. A total of 10 μ L of the reconstituted c-di-AMP extract was injected into an EC 50/3 Nucleodur C18 pyramid column (3.0 μ m; 3x50 mm) (Macherey-Nagel). Mobile phase A was 10 mM ammonium acetate in water containing 0.1% acetic acid, whereas mobile phase B was pure methanol. Samples were run in gradient condition, with 100% mobile phase A from initial to 5 minutes, decreased to 90% mobile phase A from 5 to 6.3 minutes, maintained at 90%

mobile phase A from 6.3 to 8.3 minutes, decreased to 70% mobile phase A from 8.3 to 11.0 minutes, and increased to 100% mobile phase A from 11.0 to 15 minutes. The total run was 15 minutes, with flow rate of 0.40 mL per minute. Purified c-di-AMP standard (Biolog) of various concentrations were included for c-di-AMP identification and quantification.

4.4.9 Phase contrast microscopy. A total of 5 μ L of stationary phase *S. gallolyticus* subsp. *gallolyticus* cultures was mixed with 5 μ L molten low-melting agarose, and was fixed onto a glass slide. Images were acquired with Carl Zeiss Axio Observer.Z1 Inverted Widefield Microscopy fitted with 100X/1.3-numerical aperture objective oil lens, and were further processed using Imaris version 8.2.

4.4.10 Surface proteins extraction and data analysis. Surface proteins were extracted as previously described with minor modifications (155). Briefly, a total of 50 mL of *S. gallolyticus* subsp. *gallolyticus* cultures was prepared by diluting (1:50) the overnight culture in M9YEG. The culture was grown to OD₆₀₀ of 2 and harvested at 6,000 rpm for 10 minutes at 4°C. The bacterial pellet was washed once with 1X PBS and was then gently re-suspended in 500 μ L of PBS buffer containing 40% sucrose and 5 μ g of sequencing grade trypsin (Promega). The re-suspended culture was incubated at 37°C for 60 minutes with gentle agitation. Following the incubation, the culture was centrifuged at 4,600 xg for 5 minutes at 4°C. The supernatant was collected, filtered through pre-wetted 0.22 μ m filter unit and was further incubated with 2 μ g of sequencing grade trypsin (Promega) at 37°C for 2 hours with gentle agitation. The extracted surface proteins were subsequently purified through Oasis® HLB extraction cartridges (Waters) as described in (155), and dried on a

SpeedVac Concentrator (Thermo Fisher Scientific). These samples were analysed on an Orbitrap mass spectrometers (Thermo Fisher Scientific) at the Taplin Mass Spectrometry Facility. Proteins that were not consistently detected among the three biological replicates and that have low spectral count ($n = 1$) were discarded for data analysis.

4.4.11 Recombinant MalR protein purification. Recombinant MalR was purified at the Protein Production Platform at Nanyang Technological University Singapore using the in-house developed protocol. Briefly, the cell pellet of *E. coli* BL21(DE3)/pET28b::*malR* was re-suspended and sonicated (Sonics Vibra-cell) for 3 minutes at 70% amplitude with 3-second on/off cycles. The lysate was centrifuged at 47,000 xg for 25 minutes at 4°C. The supernatant was collected, filtered through 1.2 µm syringe filters and loaded onto an immobilised metal affinity chromatography (IMAC) column packed with Ni-NTA Superflow (Qiagen) on the AKTApurify System (GE Healthcare). The IMAC column was washed with 20 column volume of wash buffer 1 (20 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 0.5M TCEP), followed by 20 column volume of wash buffer 2 (20 mM HEPES pH 7.5, 500 mM NaCl, 25 mM imidazole, 10% glycerol, 0.5M TCEP). The His-tagged proteins eluted with 5 column volume of elution buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 10% glycerol, 0.5M TCEP) were collected and injected into a HiLoad 16/60 Superdex 75 prep grade gel filtration column (GE Healthcare). Proteins were eluted with 20mM HEPES pH 7.5, 300mM NaCl, 10% (v/v) glycerol, collected in 2 mL fractions and analysed on SDS-PAGE. Relevant peaks were pooled and added with Tris(2-carboxyethyl)phosphine (TCEP) to a final concentration of 2

mM. The purified protein was concentrated in Vivaspin 20 filter concentrators (VivaScience), frozen in liquid nitrogen and stored at -80°C until use.

4.4.12 Electromobility shift assay (EMSA). EMSA was performed as previously described with slight modifications (156). Duplex DNA was prepared by incubating equal molar amount of pulA_EMSA_F and pulA_EMSA_R in IDT annealing buffer (100 mM potassium acetate; 30 mM HEPES pH 7.5) at 94°C for 2 minutes and slowly cool down to room temperature over 45 minutes. 8 µM of the duplex DNA was allowed to bind with various amount of rMalR in the EMSA binding buffer (20 mM Tris, pH 8; 0.1 mM EDTA; 10 mM magnesium chloride; 200 mM sodium chloride; and 5% glycerol) at 37°C for 20 minutes in a reaction volume of 10 µL. The reactions were resolved in a 6% native polyacrylamide gel (prepared as described in (157)) with 0.5X Tris/Borate/EDTA (TBE) buffer for an hour, at 100V, 4°C. The polyacrylamide gel was stained with 2X SYBR Green EMSA nucleic acid stain (Thermo Fisher Scientific) for 15 minutes and imaged with GelDoc XR+ Imaging System (Biorad).

4.4.13 RNA extraction. RNA was extracted from *S. gallolyticus* subsp. *gallolyticus* as described in Chapter 3.

4.4.14 Quantitative PCR (qPCR). A total of 1 µg of the purified RNA was primed with random hexamers and reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific). The resulted cDNA was subjected to qPCR using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) on a StepOnePlus Real

Time PCR system (Applied Biosystem). The 16S rRNA transcript was used as the endogenous control.

4.5 Supplementary data

Table 4.2 Mutations in the genomes of various *S. gallolyticus* subsp. *gallolyticus* strains.

Nucleotide position	Gene	Annotation	Type	Mutation	Frequency (%)		
					UCN34	$\Delta gdpP$	$\Delta gdpP_{MalR^*}$
2911	-	Intergenic region	SNV	T > C	100	99.32	99.3
349705	Gallo_0324	ABC transporter substrate-binding protein	SNV	A > C	98.97	100	97.87
378443^378444	Gallo_0349	PTS mannose transporter subunit IIC	Insertion	- > T	94.17	93	95.96
378456	Gallo_0349	PTS mannose transporter subunit IIC	SNV	G > A	99.01	98.1	96.04
378459..378460	Gallo_0349	PTS mannose transporter subunit IIC	MNV	GC > CA	97.46	95.37	90.1
378466	Gallo_0349	PTS mannose transporter subunit IIC	SNV	C > A	100	100	100
378498	Gallo_0349	PTS mannose transporter subunit IIC	SNV	A > G	100	97.12	100
410320	Gallo_0381	membrane protein	SNV	A > G	100	99.15	100
508104	Gallo_0485	acetoin reductase	SNV	G > A	100	99.17	100
551746	Gallo_0528	valine--tRNA ligase	SNV	G > C	99.45	100	98.97
714648	Gallo_0688	MerR family transcriptional regulator	SNV	C > A	100	98.91	99.03
715324	Gallo_0689	membrane protein	SNV	C > T	99.33	97.2	97.47
1063901	Gallo_1007	AI-2E family transporter	SNV	T > G	100	100	98.61
1468237	Gallo_1398	LacI family transcriptional regulator	SNV	G > A	-	-	98.25
1818314	Gallo_1738	oxidoreductase	SNV	G > A	100	99.12	98.37
2149527	Gallo_2068	MFS transporter permease	SNV	G > A	99.12	100	97.83

SNV, single nucleotide variants. MNV, multiple nucleotide variants.

Chapter 5 : C-di-AMP controls *Streptococcus gallolyticus* subsp. *gallolyticus* pilus expression to mediate biofilm development

5.1 Introduction

S. gallolyticus subsp. *gallolyticus* genome encodes three pilus operons, namely Pil1, Pil2 and Pil3 (89). Previous studies have shown that Pil1 can recognise collagen I, and is important for heart valve colonisation which can cause the development of endocarditis (90, 99). Meanwhile, Pil3 can recognise various host matrices, such as fibrinogen and intestinal mucins, and is important for the colonisation in the murein gastrointestinal tract (15, 158). Pil1 and Pil2 is highly specific to *S. gallolyticus* subsp. *gallolyticus*, whereas Pil3 can be found in the other subspecies of *S. gallolyticus*, such as *S. gallolyticus* subsp. *pasteurianus* and *S. gallolyticus* susp. *macedonicus* (159).

Both the Pil1 and Pil3 pilus were heterogeneously expressed in *S. gallolyticus* subsp. *gallolyticus*, of which the expression was determined by the numbers of the GCAGA tandem repeats in the promoter region of the operons, through a transcriptional attenuation-like mechanism (99). In the *S. gallolyticus* subsp. *gallolyticus* clinical isolate UCN34, there are 22 and 13 GCAGA tandem repeats in the promoter region of the *pil1* and *pil3* operons, respectively (99). Intriguingly, these tandem repeats resides in a short open reading frame consisting of 117 and 75 bp nucleic acids, which are translated into 38 and 24 amino acid peptides, respectively (99). The addition or deletion of GCAGA tandem repeats number not in a multiple of 3 results in a frame-shift mutation, and the translation of the open

reading frame therefore terminates close to or at a region downstream that has high tendency to form a stem-loop structure that signals for pre-mature transcriptional termination (99). Due to the frame-shift mutation, the ribosomes recruited during the translational process prevent the formation of the stem-loop structure, permitting higher levels of transcription, therefore increased Pil1 and Pil3 expression, giving rise to populations termed as Pil1+ or Pil3+ (99). The switch of *S. gallolyticus* subsp. *gallolyticus* populations to Pil1+ or Pil3+ populations was reported not be affected by external factors such as oxygen concentration and serum (99), but it is found that the Pil3 expression can be modulated by the intracellular c-di-AMP levels (145).

It is shown in **Chapter 3** that *S. gallolyticus* subsp. *gallolyticus* c-di-AMP phosphodiesterase deletion mutant $\Delta gdpP$ accumulates 1.5 fold higher intracellular c-di-AMP levels than the wildtype, and displayed reduced biofilm formation ability (145). This biofilm defect can be partially restored by the overexpression of extracellular pullulanase Pula due to the expression of a loss-of-function maltose operon repressor MalR (**Chapter 4**). In this study, we showed that the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutants expressing high levels of Pil3 also showed significant restoration of the biofilm developmental defect. The contribution of Pil3 to the biofilm formation of *S. gallolyticus* subsp. *gallolyticus* was further confirmed by employing a Pil3 deletion mutant. Finally, we provide evidence that *gdpP* overexpression – with consequently reduced intracellular c-di-AMP levels – leads to increased expression of both Pil1 and Pil3 pili, confirming the role of c-di-AMP control on *S. gallolyticus* subsp. *gallolyticus* pilus expression.

5.2 Results

5.2.1 Serial passaging of the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant gave rise to mutants with restored biofilm developmental defect. We have previously shown that a nonsense mutation in the maltose operon repressor MalR in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant partially restored the biofilm development defect (**Chapter 4**). In order to identify more factors that could be responsible for the biofilm development defect of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant, we serially passaged the mutant for four days in brain-heart infusion broth with no selective pressure, which was the condition of which the MalR mutation arose (Figure 5.1A).

A total of 42 $\Delta gdpP$ mutant derivatives were isolated (denoted as $\Delta gdpP$ E1 to E42) and screened for their biofilm formation capability. As shown in Figure 5.1B, most of the $\Delta gdpP$ mutant derivatives formed biofilms at a similar level as the parental strain. However, 8 of the 42 $\Delta gdpP$ mutant derivatives displayed a clear restoration of the biofilm developmental defect. Whole genome sequencing of these 8 $\Delta gdpP$ mutant derivatives revealed alterations in the numbers of the GCAGA tandem repeats in the promoter region of the Pil3 pilus operon. Compared to the UCN34 wildtype and $\Delta gdpP$ parental strain, both of which carries 13 GCAGA tandem repeats in the Pil3 promoter region, these 8 $\Delta gdpP$ mutant derivatives carry 9, 12, 14 or 15 GCAGA tandem repeats (Figure 5.2B, Supplementary data, Table 5.2).

Another 8 of the $\Delta gdpP$ mutant derivatives that remained displaying biofilm development defect as the parental strain $\Delta gdpP$ mutant were also included for whole genome sequencing. As expected, all of these 8 $\Delta gdpP$ mutant derivatives remained carrying 13 GCAGA tandem repeats in the Pil3 promoter region (Figure 5.2B, Supplementary data, Table 5.2). Notably, neither large scale insertion/deletion, nor other mutations were found in the genomes of these sequenced $\Delta gdpP$ mutant derivatives.

Figure 5.1 Serial passing of the *S. gallolyticus* subsp. *gallolyticus* UCN34 $\Delta gdpP$ mutant gave rise to mutants displaying restored biofilm development defects. (A) Schematic diagram on the overview of the serial passing. A single colony of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ was picked and subjected to serial passing for consecutive four days. Single colonies from the forth-day culture were randomly selected and screened for biofilm formation capability. (B) Biofilm formation capability of the serially-passaged *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutants (denoted as $\Delta gdpP$ E1 to E42) was screened using the conventional crystal violet microtiter biofilm assay. Scattered data plots represent

every single measurements. Bar graphs represent the mean value of three biological replicates. Error bars represent the standard deviation of the values. Red arrow pointed the samples subjected to whole genome sequencing. Dotted line marks the mean value of the $\Delta gdpP$ mutants biofilm levels.

5.2.2 Altered numbers of the GCAGA tandem repeats in the Pil3 promoter region resulted in Pil3 overexpression. A previous study has shown that the GCAGA tandem repeats in the Pil3 promoter region dictate the Pil3 pilus expression through an transcriptional attenuation-like mechanism (15, 99) (Figure 5.2A). Out-of-frame mutations in the numbers of the GCAGA tandem repeats such as 9, 11, 12, 14 and 15 gave rise to the *S. gallolyticus* subsp. *gallolyticus* cell populations overexpressing Pil3 pilus. These populations were denoted as Pil3+ variants (15). Therefore, we hypothesised that the eight $\Delta gdpP$ mutant derivatives we isolated were Pil3+ variants. To verify this, we examined the Pil3 expression of these strains at single cell level using a flow cytometer that allows the analysis on Pil3+ variants. As expected, the eight $\Delta gdpP$ mutant derivatives overexpresses Pil3 pilus, evidenced by a marked increase of the Pil3-piliated cells in the populations compared to the UCN34 wildtype and the $\Delta gdpP$ mutant (Figure 5.2C).

Diagram illustrating the genomic organization and predicted structure of the *Gallo_2042* gene. The gene is located on the *Gallo_2040* *pii3A* locus, flanked by *Gallo_2041* and *Gallo_2039* *pii3B*. The gene structure shows a single coding sequence (CDS) with a predicted start site (ATG) and a predicted stop site (TGA). The CDS is flanked by inverted repeat stem-loop termination sites. The gene is 1.0 kb in length.

Genomic organization and predicted structure of the *Gallo_2042* gene:

Genomic context: *Gallo_2042* is located on the *Gallo_2040* *pii3A* locus, flanked by *Gallo_2041* and *Gallo_2039* *pii3B*. The scale bar indicates 1.0 kb.

Gene structure: The gene is represented by a black arrow indicating the direction of transcription. The predicted start site (ATG) and predicted stop site (TGA) are marked. The CDS is flanked by inverted repeat stem-loop termination sites.

Sequence analysis: The sequence is shown in the following lines, with the predicted start site (ATG) and predicted stop site (TGA) highlighted. The inverted repeat stem-loop termination site is also indicated.

Sequence (5' to 3'): ATTATTGACATTAATTTTAATTAGTTTAGAATGAATCAGTATTATCTTAAAAATTGTAAATAAGTTATAAAGTTA

Sequence (3' to 5'): TTTTGGCGTACAGTTATTTATTTTAAAGTCACTTTTTCGTGTATCTATATAATAGTTATACGAAAAAGTGACTT

Sequence (5' to 3') (continued): TTTGTGTCTCTTAAATAGCAATGATTTTATATGAAAGGTAAGAACTCTATG

Key features:

- predicted -35
- predicted -10
- ATG (start site)
- STOP (stop site)
- inverted repeat stem-loop termination site
- ATG GALLO_2042

Pil3 GCAGA repeat number	Mutation types	Strains
13	wildtype	UCN34, $\Delta gdpP$, $\Delta gdpP$ E7, E8, E12, E16, E26, E34, E39, E42
9	out-of-frame	$\Delta gdpP$ E1
12	out-of-frame	$\Delta gdpP$ E13, E33
14	out-of-frame	$\Delta gdpP$ E21, E25, E41
15	out-of-frame	$\Delta gdpP$ E20, E28

A bar graph comparing the absorbance (OD_{550nm}) of two bacterial strains, UCN34 and $\Delta pil3$. The y-axis is labeled 'Absorbance (OD_{550nm})' and ranges from 0.0 to 1.0. The x-axis has two categories: 'UCN34' and ' $\Delta pil3$ '. For UCN34, the mean absorbance is approximately 0.75, with individual data points ranging from about 0.70 to 0.85. For $\Delta pil3$, the mean absorbance is approximately 0.65, with individual data points ranging from about 0.60 to 0.70. A horizontal bar with two asterisks (**) is placed above the bars, indicating a statistically significant difference between the two groups.

Strain	Absorbance (OD _{550nm})
UCN34	0.72
UCN34	0.73
UCN34	0.74
UCN34	0.75
UCN34	0.76
UCN34	0.77
UCN34	0.78
UCN34	0.80
UCN34	0.82
UCN34	0.85
$\Delta pil3$	0.60
$\Delta pil3$	0.61
$\Delta pil3$	0.62
$\Delta pil3$	0.63
$\Delta pil3$	0.64
$\Delta pil3$	0.65
$\Delta pil3$	0.66
$\Delta pil3$	0.67
$\Delta pil3$	0.68
$\Delta pil3$	0.70

Figure 5.2 Altered numbers of the GCAGA tandem repeats in the *pil3* promoter region resulted in Pil3 overexpression. (A) Schematic representation of the *pil3* operon consisted of five genes: the unknown function open reading frame *Gallo_2042* and *Gallo_2041*, the pilin subunits *Gallo_2040* and *Gallo_2039*, and the sortase required for pilin polymerisation *Gallo_2038*. The nucleotide sequence (lower panel) of the upstream region of *Gallo_2042* shows the predicted -35 and -10 promoter boxes, a short leader peptide containing 13 GCAGA tandem repeats (boxed), an inverted repeat stem-loop transcriptional termination site, and an initiator codon for *Gallo_2042*. The start codon and the stop codon of the leader peptide was highlighted as ‘ATG’ and ‘TAA’ respectively. Alternative stop codons of the leader peptide due to out-of-frame mutations of the GCAGA tandem repeat number were in bold and underlined. (B) Summary of the Pil3 GCAGA repeat numbers of various $\Delta gdpP$ mutant strains isolated in this study. (C) Flow cytometry analysing the Pil3 expression of various *S. gallolyticus* subsp. *gallolyticus* strains. The Pil3 negative (cells that do not express Pil3) and the Pil3 positive (cells that express Pil3) cell populations were as indicated. The $\Delta gdpP$ derived mutants carrying out-of-frame mutations in the Pil3 GCAGA tandem repeat number have marked increased in the Pil3 expressing populations. (D) The conventional crystal violet microtiter biofilm assay showed that the UCN34 $\Delta pil3$ mutant developed less biofilms than the UCN34 wildtype. Scattered data plots represent every single measurements. Bar graphs represent the mean value of three biological replicates. Error bars represent the standard deviation of the values. Two-tailed T-test, **, P-value < 0.01.

5.2.3 c-di-AMP controls Pil3 pilus expression to mediate *S. gallolyticus* subsp. *gallolyticus* biofilm formation. Given that the out-of-frame mutations in the numbers of the GCAGA tandem repeats in the *pil3* promoter region gave rise to the Pil3+ variants, and that the $\Delta gdpP$ Pil3+ variants displayed restored biofilm developmental defect of the $\Delta gdpP$ mutant, our results suggested that Pil3 expression may control *S. gallolyticus* subsp. *gallolyticus* biofilm formation. To verify this, we deleted the Pil3 pilus operon of *S. gallolyticus* subsp. *gallolyticus*, and found that the $\Delta pil3$ mutant formed less biofilm than the wildtype parental strain (Figure 5.2D). We therefore concluded that Pil3 is an important factor for *S. gallolyticus* subsp. *gallolyticus* biofilm development.

Our previous Western Blot analysis showed that the Pil3 expression was down-regulated in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant compared to the wildtype (145). In this study, we further analysed the Pil3 pilus expression in *S. gallolyticus* subsp. *gallolyticus* at single cell level using a flow cytometer. Consistent with our previous findings, the Pil3 expression is down-regulated in the $\Delta gdpP$ mutant and the reduced Pil3

expression can be complemented when *gdpP* was expressed *in trans* in the $\Delta gdpP$ mutant (Figure 5.3A). Notably, none of the strains analysed were Pil3⁺ variants (Figure 5.2C). These data confirmed that the Pil3 expression is down-regulated in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant, and that Pil3 pilus constitutes as another pathway regulated by c-di-AMP to mediate *S. gallolyticus* subsp. *gallolyticus* biofilm development.

A

B

Figure 5.3 c-di-AMP controls Pil3 pilus expression. (A) Flow cytometry analysis on the Pil3 expression of various *S. gallolyticus* subsp. *gallolyticus* strains. The Pil3 expression was measured in fluorescence intensity. Scattered data plots represent every single measurements. Bar graphs represent the mean value of two biological replicates. (B) Flow cytometry analysis on the Pil3 expression of the *S. gallolyticus* subsp. *gallolyticus* strains overexpressing *gdpP*. Scattered data plots represent every single measurements. Bar graphs represent the mean value of two biological replicates.

5.2.4 c-di-AMP controls both the *S. gallolyticus* subsp. *gallolyticus* Pil1 and Pil3 pili expression.

To demonstrate further that the Pil3 expression is responsive to the intracellular c-di-AMP level, we introduced a plasmid (*ptet::gdpP*) into the UCN34 wildtype which would allow for constitutive *gdpP* expression (UCN34/*ptet::gdpP*). As expected, we observed an increased Pil3 expression in the UCN34/*ptet::gdpP* strain than the UCN34 wildtype (Figure 5.3B). We next wondered will the overexpression of *gdpP* further enhance the Pil3 expression in strains already expressing high level of Pil3. To test this, we introduced the plasmid *ptet::gdpP* into a $\Delta gdpP$ Pil3⁺ variant, and found that *gdpP* overexpression similarly induced the Pil3 expression (Figure 5.3B). These data indicate that c-di-AMP is the bona fide signalling molecule regulating the Pil3 expression in *S. gallolyticus* subsp. *gallolyticus*.

Notably, in *S. gallolyticus* subsp. *gallolyticus*, the GCAGA tandem repeats are also present in the promoter region of the Pil1 pilus operon (99). We therefore wondered whether the Pil1 pilus expression is similarly controlled by the intracellular c-di-AMP level. As shown in Figure 5.4A-C, c-di-AMP controls the Pil1 pilus expression in a manner similar to Pil3, *i.e.* a decreased intracellular c-di-AMP level induces Pil1 expression, whereas an increased intracellular c-di-AMP level reduces Pil1 expression. Taken together, we showed that c-di-AMP controls both the Pil1 and Pil3 expression in *S. gallolyticus* subsp. *gallolyticus*.

Figure 5.4 c-di-AMP also controls the *S. gallolyticus* subsp. *gallolyticus* Pil1 expression. (A) Western blot analysis of the cell wall proteins from *S. gallolyticus* UCN34, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain. Equal amounts of the cell wall proteins were loaded (left) and probed with specific polyclonal antibodies against Pil1B (right). (B)(C) Flow cytometry analysis on the Pil3 expression of various *S. gallolyticus* subsp. *gallolyticus* strains. The Pil3 expression was measured in fluorescence intensity. Scattered data plots represent every single measurements. Bar graphs represent the mean value of two biological replicates.

5.3 Discussion

Pili are important appendages that contribute to surface motility and attachment. In Gram-positive bacteria such as *S. gallolyticus* subsp. *gallolyticus*, pilus is made up of covalently-linked pilin subunits (88). The pilin elongation requires Sortase A (SrtA) and the pilus anchorage to the bacterial peptidoglycan layer is facilitated by Sortase C (SrtC) through the enzyme's recognition on the LPxTG motif at the C-terminal of pilin subunits (88). Similar to many other bacteria, the pili Pil1 and Pil3 are heterogeneously expressed in the *S. gallolyticus* subsp. *gallolyticus* cell populations (15, 99). This heterogenous pilus expression is regulated by a transcriptional attenuation-like mechanism, mainly controlled by the number of GCAGA tandem repeats in the respective promoter region (15, 99).

Here, we showed that Pil3 is an important factor for *S. gallolyticus* subsp. *gallolyticus* biofilm formation and is in part responsible for the biofilm developmental defect of the c-di-AMP phosphodiesterase deletion mutant $\Delta gdpP$. We further demonstrated that Pil3 expression is controlled by the intracellular c-di-AMP level. This is supported by the observations that the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant displayed reduced Pil3 expression compared with the wildtype, and the overexpression of *gdpP* in *trans* in various *S. gallolyticus* subsp. *gallolyticus* strains background increases Pil3 expression. Given that GCAGA tandem repeats can also be found in the Pil1 promoter region, we therefore extended our investigation and found that c-di-AMP also controls the Pil1 expression.

Question concerning the mechanism of c-di-AMP control on *S. gallolyticus* subsp. *gallolyticus* Pil1 and Pil3 pili expression remained unanswered in this study. The mutations lead to the altered numbers of GCAGA tandem repeats likely occur during DNA replication due to slipped strand mispairing (also known as replication slippage), which is a mutation that has been previously reported to occur at the direct repeat sites (160). During DNA replication, when the DNA polymerase complex encounters direct repeat sequence, it suspends the replication and is temporarily released from the template strand. The newly synthesised DNA strand subsequently pairs with another direct repeats in the region. When the replication resumes, the newly synthesised DNA strand therefore contains altered numbers of the direct repeats (160). We speculate that during DNA replication in *S. gallolyticus* subsp. *gallolyticus*, c-di-AMP may directly or indirectly stabilise the DNA polymerase complex to prevent the complex from being dissociated from the template strand. This may allow the newly synthesised DNA strand to have the exact GCAGA repeat numbers as the template strand. Coupled with the transcriptional attenuation-like mechanism, c-di-AMP is therefore controlling the Pil1 and Pil3 expression. This proposed model suggests that c-di-AMP might have a role in maintaining the DNA integrity in *S. gallolyticus* subsp. *gallolyticus*.

Intriguingly, the role of c-di-AMP in maintaining the bacterial DNA integrity has been previously reported. It was found that in *Bacillus subtilis*, the c-di-AMP diadenylate cyclase, also known as the DNA integrity scanning protein DisA, synthesises c-di-AMP while scanning the bacterial chromosome prior to sporulation (161). Upon the detection of DNA double-stranded breaks, DisA temporarily stops at the DNA damage site and suspends its

diadenylate cyclase activity (161). During the process, the expression of the c-di-AMP phosphodiesterase *gdpP* is also up-regulated to lower the intracellular c-di-AMP level (162). DNA repair is subsequently initiated (161, 162). Notably, DisA and its homologs are not found in *S. gallolyticus* subsp. *gallolyticus* genomes. Therefore, further investigation on the mechanism of c-di-AMP control on *S. gallolyticus* subsp. *gallolyticus* Pil1 and Pil3 expression may offer interesting insights towards the understanding of the potential roles of c-di-AMP in DNA integrity especially in the microorganisms not producing DisA.

In conclusion, we show here that c-di-AMP controls the Pil3 pilus expression to mediate *S. gallolyticus* subsp. *gallolyticus* biofilm formation. We also demonstrate that c-di-AMP is a bona fide signalling molecule controlling both the *S. gallolyticus* subsp. *gallolyticus* Pil1 and Pil3 pili expression.

5.4 Materials and methods

5.4.1 Bacterial strains, plasmids and primers. All bacterial strains, primers and plasmids used in this study were listed in Table 4.1. Overnight cultures of *S. gallolyticus* subsp. *gallolyticus* were typically prepared from single colonies in 2 mL of M9YEG (1X M9 minimal salts supplemented with 0.5% yeast extract and 1.0% glucose). The overnight cultures were 5X diluted the next day and subsequently grown to mid-log phase for all the experiments. When appropriate, erythromycin was supplemented at a final concentration of 2 µg/mL for *S. gallolyticus* subsp. *gallolyticus* or 500 µg/mL for *Escherichia coli* DH5α.

Table 5.1 Bacterial strains, plasmids and primers used in this study.

Bacterial strains	Description	Reference
<i>E. coli</i> DH5α	<i>deoR endA1 gyrA96 hsdR17 (Δlac)U169 recA1 relA1 supE44 thi-1 (Φ80 lacZΔM15)</i>	Lab collection
<i>S. gallolyticus</i> subsp. <i>gallolyticus</i> UCN34	a clinical strain isolated from an infective endocarditis patient who later diagnosed with CRC	(89)
UCN34 Δ <i>gdpP</i>	in-frame <i>gdpP</i> (<i>GALLO</i> 2236) knock-out mutant	(145)
UCN34 Δ <i>gdpP</i> /p <i>gdpP</i>	<i>gdpP</i> -complemented strain; UCN34 Δ <i>gdpP</i> containing pTCVerm:: <i>gdpP</i>	(145)
UCN34 Δ <i>gdpP</i> Pil3+	UCN34 Δ <i>gdpP</i> overexpressing <i>pil3</i> pilus strain due to the presence of 15 GCAGA tandem repeats in the <i>pil3</i> promoter region	This study
UCN34 Δ <i>gdpP</i> Pil3+/ptet	UCN34 Δ <i>gdpP</i> Pil3+ containing the pTCVerm-Ptet empty plasmid	This study
UCN34 Δ <i>gdpP</i> Pil3+/ptet:: <i>gdpP</i>	UCN34 Δ <i>gdpP</i> Pil3+ containing the pTCVerm-Ptet:: <i>gdpP</i> plasmid	This study
UCN34/ptet	UCN34 containing the pTCVerm-Ptet empty plasmid	This study
UCN34/ptet:: <i>gdpP</i>	UCN34 containing the pTCVerm-Ptet:: <i>gdpP</i> plasmid	This study
UCN34 Δ <i>pil3</i>	In frame <i>pil3</i> (<i>GALLO</i> 2038 to <i>GALLO</i> 2042) knockout mutant	(15)
<i>S. agalactiae</i> NEM316	MLST-23, serotype III isolated from neonate blood culture	(139)
Plasmids		Reference
pTCVerm-ptet	Em, Km, Mob+ (IncP); <i>oriR</i> pACYC184; <i>oriR</i> pAMβ1; MCS <i>lacZα</i> +; <i>oriT_{TnGBS1}</i> ; <i>Ptet</i> constitutive promoter	(140)
pTCVerm-ptet:: <i>gdpP</i>	pTCVerm-Ptet containing the native ribosomal binding site and the open reading frame of <i>gdpP</i> (<i>GALLO</i> 2236)	This study
pTCVerm:: <i>gdpP</i>	pTCVerm containing the native ribosomal binding site and the open reading frame of <i>gdpP</i> (<i>GALLO</i> 2236)	(145)
Primers	Sequence (5' to 3')	Reference
<i>gdpP</i> overexpression plasmid		
<i>gdpP</i> _F	CACAGGATCCGCTAAGAGGTTAACCAATG	This study
<i>gdpP</i> _R	CACAGCATGCCATCACTCTACCTCCAT	This study

* Restriction sites are underlined

5.4.2 Molecular cloning. Molecular cloning was performed according to the standard protocols described in (152). For the construction of the *gdpP*-overexpression plasmid (pTCVerm-ptet::*gdpP*), the *gdpP* open reading frame with the native ribosomal binding site were amplified from the *S. gallolyticus* subsp. *gallolyticus* UCN34 genome with the primer pair *gdpP_F* and *gdpP_R*. The resulted PCR product was restricted with *BamHI* and *SphI*, ligated into the *BamHI/SphI*-restricted pTCVerm-Ptet, and cloned into *E. coli* DH5 α .

5.4.3 *S. gallolyticus* subsp. *gallolyticus* UCN34 Δ *gdpP* Pil3⁺ variants isolation. A single colony of the *S. gallolyticus* subsp. *gallolyticus* Δ *gdpP* mutant was inoculated into 2 mL of brain-heart infusion (BHI) broth, and incubated at 37°C overnight. The overnight culture was diluted (1:200) into a tube of sterile BHI broth the next day. This serial passaging was repeated for a total of 4 passages. A total of 10 μ L of the resulted culture was streaked on a BHI plate. A total of 42 single colonies were randomly picked and screened for the biofilm formation capability using the crystal violet biofilm microtiter assay.

5.4.4 Microtiter plate biofilm assay. Assay was performed as described in Chapter 3.

5.4.5 Genomic DNA extraction and sequencing. Genomic DNA was extracted from *S. gallolyticus* subsp. *gallolyticus* as described in Chapter 4.

5.4.6 Bioinformatics analysis. The genomic DNA sequences were analysed as described in Chapter 4.

5.4.7 Flow cytometry analysis. Bacterial cells were prepared for flow cytometry analysis as previously described with slight modifications (99). Briefly, the overnight *S. gallolyticus* subsp. *gallolyticus* cultures were diluted (1:5) in M9YEG and grown for 4 to 5 hours at 37°C for the preparation of fresh stationary bacterial cultures. A total of 500 µL of the culture was pelleted at 6,000 xg for 3 minutes, washed once with 0.9% NaCl, and re-suspended in 3% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS). After 30 minutes of incubation, the mixture was pelleted and re-suspended in rabbit primary antibodies (anti-Pil1B or anti-Pil3B) diluted (1:800) in 0.5% PBS-BSA. The mixture was incubated on ice for 45 minutes, before being washed three times with PBS. The bacterial cells were subsequently incubated with AlexaFluor 488-conjugated goat antirabbit diluted (1:2000) in 0.5% PBS-BSA. The mixture was again incubated on ice for 30 minutes, before being washed three times with PBS. The bacterial cells were stained with nucleic acid stain SYTO62 (1:1000) (Thermo Fisher Scientific) for 10 minutes, and fixed in freshly prepared 1% paraformaldehyde for 20 minutes. Finally, the cells were washed once, re-suspended in PBS and analysed on Accuri C6 Flow Cytometer System (BD Biosciences).

5.4.8 Cell wall extracts preparation and immunoblotting. Cell wall extracts were prepared as described in Chapter 3.

5.5 Supplementary data

Table 5.2 Non-synonymous mutations in the genomes of various *S. gallolyticus* subsp. *gallolyticus* strains.

Nucleotide position	Gene	Type	Mutation	UCN34	Δ gdpp	Δ gdpp E1	Δ gdpp E7	Δ gdpp E8	Δ gdpp E12	Δ gdpp E13	Δ gdpp E16	Δ gdpp E20	Δ gdpp E21	Δ gdpp E25	Δ gdpp E26	Δ gdpp E28	Δ gdpp E33	Δ gdpp E34	Δ gdpp E39	Δ gdpp E41	Δ gdpp E42
2911	intergenic	SNV	T > C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
349705	Gallo_0324	SNV	A > C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
378443-378444	Gallo_0349	Insertion	- > T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
378456	Gallo_0349	SNV	G > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
378459-378460	Gallo_0349	MNV	GC > CA	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
378466	Gallo_0349	SNV	C > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
378498	Gallo_0349	SNV	A > G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
410320	Gallo_0381	SNV	A > G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
508104	Gallo_0485	SNV	G > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
551746	Gallo_0528	SNV	G > C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
714648	Gallo_0688	SNV	C > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
715324	Gallo_0689	SNV	C > T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
1063901	Gallo_1007	SNV	T > G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
1247010	Gallo_1177	SNV	T > C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
1818314	Gallo_1738	SNV	G > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2149527	Gallo_2068	SNV	G > A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2121365-2121384	Gallo_2038 promotor region	Deletion	CTCTGC TCTGCT CTGCTC TG > -			#															
2121365-2121369	Gallo_2038 promotor region	Deletion	CTCTG > -							#								#			

[illegible]

Chapter 6 : Concluding remarks

6.1 Conclusions

For a long time, *S. gallolyticus* subsp. *gallolyticus* has been associated with the incidence of colorectal cancer among infective endocarditis patients. The strong association of *S. gallolyticus* subsp. *gallolyticus* with the disease has attracted attention and lead to investigation of whether *S. gallolyticus* subsp. *gallolyticus* drives colorectal cancer development. Current investigations suggest that *S. gallolyticus* subsp. *gallolyticus* plays an auxiliary role in further promoting the colorectal cancer development in the patients who are predisposed of the disease (11, 134). Although the direct mechanistic patterns remain unidentified, it is known that the cancer promoting effect requires close contact of *S. gallolyticus* subsp. *gallolyticus* with the colonic epithelial cells (11).

Bacterial contacts and attachments to both abiotic and biotic surfaces are facilitated by bacterial adhesins and are commonly followed by biofilm development. Given that it has been recently suggested that bacterial biofilms are involved in human colorectal cancer development (14, 24, 26), understanding the largely unknown biofilm biology of *S. gallolyticus* subsp. *gallolyticus* may provide clues to understand the correlation of *S. gallolyticus* subsp. *gallolyticus* with colorectal cancer incidence. One of the key molecules governing the biofilm development process is a second messenger c-di-GMP that widely produced by Gram-negative bacteria (28). In recent years, another second messenger c-di-AMP is found to be produced by bacteria, many of them are the Gram-positive bacteria,

including the *Streptococcus* spp. belonging to the phylum Firmicutes. Compared to c-di-GMP, the c-di-AMP regulation on the bacterial biofilm development is less well understood, and the observations reported by many previous studies are in contradiction, and therefore failed to provide a clear pattern of the c-di-AMP regulation on bacterial biofilm development (56-59). In addition, the detailed regulatory mechanisms have only been studied in *Streptococcus mutans* (58) but remained unknown for most of the bacteria producing c-di-AMP, further indicating a lack of knowledge in the field.

To fill these knowledge gaps, I investigated the roles of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus* and presented the findings in this thesis. I first provide evidence in **Chapter 3** that the *S. gallolyticus* subsp. *gallolyticus* c-di-AMP phosphodiesterase mutant ($\Delta gdpP$) that accumulates approximately 1.5-fold more intracellular c-di-AMP than the wildtype displayed defects in biofilm development. The genome-wide transcriptomic analysis comparing the transcriptomes of the *S. gallolyticus* subsp. *gallolyticus* UCN34 wildtype, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain suggests that the biofilm developmental defect observed on the $\Delta gdpP$ mutant may be partly due to the reduced expression of a surface appendage, Pil3 pilus. Whereas the role of Pil3 in *S. gallolyticus* subsp. *gallolyticus* biofilm development was investigated and confirmed (**Chapter 5**), the low and heterogenous expression of Pil3 in the *S. gallolyticus* subsp. *gallolyticus* cell populations suggests that Pil3, however, is not a major determinant in *S. gallolyticus* subsp. *gallolyticus* biofilm development. Largely due to the limited understandings on the biofilm biology of *S. gallolyticus* subsp. *gallolyticus*, especially on the bacterial adhesins and biofilm matrix components involved, analysis the transcriptomic data did not provide

further clues to understand the c-di-AMP regulation on *S. gallolyticus* subsp. *gallolyticus* biofilm development.

I next reasoned that an alternative approach to further study the c-di-AMP regulation on *S. gallolyticus* subsp. *gallolyticus* biofilm development would be to focus on the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutants that display restored biofilm developmental defect. As described in **Chapter 4**, a mutant with such phenotype was isolated and denoted as $\Delta gdpP^{MalR^*}$ due to the presence of a nonsense mutation in a gene encoding for the transcriptional regulator MalR. Further investigations on the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP^{MalR^*}$ mutant revealed that the biofilm developmental defect of the $\Delta gdpP$ mutant can be restored by an overexpression of the extracellular pullulanase Pula, indicating that Pula is involved in *S. gallolyticus* subsp. *gallolyticus* biofilm development. Real-time PCR analysis on the *pula* expression in the UCN34 wildtype, the $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain showed that the *pula* expression is responsive to the intracellular c-di-AMP level, establishing that Pula is part of the c-di-AMP regulons to control *S. gallolyticus* subsp. *gallolyticus* biofilm development. Taken together, the data presented in Chapter 4 and 5 demonstrate that both Pula and Pil3 contribute in part to the *S. gallolyticus* subsp. *gallolyticus* biofilm development, and established for the first time that Pula and Pil3 expression is responsive to the intracellular c-di-AMP level.

The reduced expression of Pula and Pil3 in the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant suggests that c-di-AMP may also play a role in *S. gallolyticus* subsp. *gallolyticus* interactions with the host, particularly in the host intestinal tract colonisation. The

mammalian intestinal tract is covered by dense layers of mucin, which is a type of glycoprotein recognisable by Pil3, and also very likely by PulA given that PulA expressed by other *Streptococi* can recognise various glycoproteins including mucins (100, 102, 146). The previous study showing that the *S. gallolyticus* subsp. *gallolyticus* $\Delta pil3$ mutant was less efficient in colonising the murine intestinal tract demonstrated that the recognition of mucins is essential for bacterial colonisation. Therefore, the $\Delta gdpP$ mutant with reduced expression of both PulA and Pil3 is expected to have a disadvantage when it comes to colonisation of the host intestinal tract. This assumption remains to be corroborated experimentally once a working *in vivo* mouse infection model is established.

The investigations of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus* also uncovered its pleiotropic role in regulating important cellular responses. Characterisation of the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant revealed that the cells appear smaller than the wildtype and are more sensitive to high osmotic stress. The genome-wide transcriptomic analysis on the transcriptomes of the UCN34 wildtype, $\Delta gdpP$ mutant and the $\Delta gdpP/pgdpP$ complemented strain further showed a down-regulated expression of various transporters responsible for osmotic regulation in the $\Delta gdpP$ mutant (**Chapter 3**). These data are in agreement with previous studies demonstrating that c-di-AMP primarily regulates osmotic homeostasis in the bacteria producing c-di-AMP. Importantly, these data also support the cellular turgor model proposed by Commichau and colleagues (70) suggesting that an elevated intracellular c-di-AMP pool limits bacterial import of osmolytes and osmoprotectants, resulting in water efflux and cell shrinkage. Meanwhile, a low intracellular c-di-AMP pool promotes water influx, resulting in swelling that increases

the tendency of cell rupture. The model suggests that the hypersensitivity of the Firmicutes producing low level of c-di-AMP in response to exposure to beta-lactam antibiotics as reported in previous studies (75, 76, 81) may not be due to c-di-AMP regulation on cell wall homeostasis, but could rather fundamentally due to the changes in cellular turgor. Along with this, the Firmicutes accumulating high levels of c-di-AMP are expected not to be more resistant to the beta-lactam antibiotics compared to the parental strain, which was observed herein on the *S. gallolyticus* subsp. *gallolyticus* $\Delta gdpP$ mutant (**Chapter 3**). Collectively, these observations suggest that c-di-AMP maintains its conserved role in regulating the osmotic homeostasis in *S. gallolyticus* subsp. *gallolyticus*.

In summary, the works presented in this thesis offer mechanistic insights into the roles of c-di-AMP in *S. gallolyticus* subsp. *gallolyticus* in biofilm development and strengthens that c-di-AMP is essential in maintaining bacterial osmotic homeostasis in the c-di-AMP-producing bacteria.

6.2 Limitations and future directions

The work presented in the present thesis is centred on *in vitro* investigations, and suffered from the lack of *in vivo* investigations. This is largely due to the time consuming processes of establishing genetic manipulation methods, constructing mutants, and developing functional biofilm models and gene expression analysis. It is however also largely due to the lack of proficiency in establishing a working mouse infection model. Therefore, follow-up investigations may focus on the establishment of a mouse intestinal tract colonisation model, which can advance the understanding of the *S. gallolyticus* subsp. *gallolyticus* infections. As shown in **Chapter 3**, the $\Delta gdpP$ mutant that has increased intracellular c-di-AMP level is deficient in forming *in vitro* biofilms and attachment on both abiotic and biotic surfaces. This mutant is therefore expected to exhibit reduced colonisation *in vivo*. Subsequently, the role of pullulanase PulA as investigated in **Chapter 4** can be further examined *in vivo*. More importantly, the possible reduced colonisation of the $\Delta gdpP$ mutant in the intestinal tract also calls for investigations of the etiological roles of *S. gallolyticus* subsp. *gallolyticus* in colorectal cancer development, which can be done by comparing the colonisation outcomes in particular the presence or increased number of tumour in the intestines after long-term colonisation by the UCN34 wildtype and the $\Delta gdpP$ mutant. Subsequent mechanistic studies may then advance our understandings on the contribution of *S. gallolyticus* subsp. *gallolyticus* colonisation to the development of colorectal cancer.

With an *in vivo* infection model established, a transposon library of UCN34 containing a large pool of mutants can also be introduced into the intestinal tract and allow the

identification, by means of transposon-sequencing, of many more colonisation factors essential for *S. gallolyticus* subsp. *gallolyticus* *in vivo* colonisation. Identification of these factors may offer drug targets for specific removal of *S. gallolyticus* subsp. *gallolyticus* particularly in cases where *S. gallolyticus* subsp. *gallolyticus* is carcinogenic to the host, with the aim of mitigating disease progression.

In **Chapter 3** and **5**, I have provided evidence that c-di-AMP, in addition to controlling biofilm formation and osmotic homeostasis, also regulating the expression of Pil1 and Pil3 pili. Pil1 and Pil3 pili expression has been previously shown to be subjected to phase variation and is regulated by a transcriptional attenuation-like mechanism which is based on the number of GCAGA tandem repeats in the promoter region of the pilus gene locus. The data presented in **Chapter 5** suggested that in addition to the transcriptional attenuation-like mechanism, Pil1 and Pil3 expression is also subjected to a secondary regulation by c-di-AMP. The exact mechanism is currently unclear. As discussed and speculated in **Chapter 5**, c-di-AMP may involve in the DNA replication process, possibly interacting with the DNA polymerase complex. This hypothesis can be tested by performing an *in vitro* replication assay using radiolabelled nucleotides with and without the addition of c-di-AMP, followed by analysis on the DNA sequence. Alternatively, a systematic identification of the c-di-AMP effectors by means of pull down in *S. gallolyticus* subsp. *gallolyticus* can be performed. This robust method may uncover not only the proteins involved in DNA replication or repair, but also other novel c-di-AMP binding proteins that may advance the current understanding on the regulatory roles of c-di-AMP in bacteria.

Lastly, in view that GdpP is a cytoplasmic protein which hydrolyses c-di-AMP inside the cells, the serial investigations on the c-di-AMP roles in *S. gallolyticus* subsp. *gallolyticus* present in thesis focused primarily on the perturbations resulted from altered intracellular c-di-AMP level. This however, leaves the extracellular signalling of c-di-AMP unexplored. Notably, *S. gallolyticus* subsp. *gallolyticus* neither has an extracellular c-di-AMP phosphodiesterase (the *S. agalactiae* ectonucleosidase CdnT) (55), nor it has the *Listeria monocytogenes* efflux pump responsible for c-di-AMP secretion encoded in the genome (84). Whether *S. gallolyticus* subsp. *gallolyticus* also secretes c-di-AMP to the milieu and the underlying mechanisms for the secretion, degradation, and sensing are the few interesting and important aspects for future studies.

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