Mutagenic strategies against *luxS* gene affect the early stage of biofilm formation of *Campylobacter jejuni*

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Abstract

Currently, it is clear that the *luxS* gene has an impact on the process of biofilm formation in *Campylobacter jejuni*. However, even within the species, naturally occurring strains of *Campylobacter* lacking the *luxS* gene exist, which can form biofilms. In order to better understand the genetic determinants and the role of quorum sensing through the LuxS/AI-2 pathway in biofilm formation, a set of mutant/complemented strains of *C. jejuni* 81–176 were prepared. Additionally, the impact of the mutagenic strategy used against the *luxS* gene was investigated. Biofilm formation was affected by both the presence and absence of the *luxS* gene, and by the mutagenic strategy used. Analysis by CLSM showed that all mutant strains formed significantly less biofilm mass when compared to the wild-type. Interestingly, the deletion mutant (∆*luxS*) showed a larger decrease in biofilm mass than the substitution (∙*luxS*) and insertional inactivated (∶∶*luxS*) mutants, even though all the mutant strains lost the ability to produce autoinducer-2 molecules. Moreover, the biofilm of the ∆*luxS* mutant lacked the characteristic microcolonies observed in all other strains. The complementation of all mutant strains resulted in restored ability to produce AI-2, to form a complex biofilm, and to develop microcolonies at the level of the wild-type.

Keywords Quorum sensing · *Campylobacter jejuni* · AI-2 · Signal molecules · Biofilm formation · Mutagenesis

Introduction

*Campylobacter jejuni* is a microaerophilic food-borne pathogen capable of entering the human gastrointestinal tract (GIT), particularly after consumption of undercooked meat. After reaching the target area, usually the lower GIT, *Campylobacter* is able to induce the gastroenteritis known as campylobacteriosis, found world-wide (European Food Safety Authority 2019; Bolton 2015; Vondrakova et al. 2015). During transfer along the food chain, *Campylobacter* cells have to overcome the hostile conditions of the external environment, including temperature changes and the presence of reactive oxygen species (ROS) formed in aerobic conditions (Gambino and Cappitelli 2016). Additionally, in the host, bacteria must survive the impact of acidic pH in the stomach, which ranges between 1.5 and 5.5, depending on many factors including food intake (Askoura et al. 2016; Varsaki et al. 2015). It is therefore surprising that such fastidious and sensitive bacterium like *Campylobacter* can endure this spectrum of harsh conditions. However, in its small genome of approximately 1.7 Mbp, a wide range of mechanisms are encoded, allowing
Campylobacter to survive, persist, and ultimately induce the disease within a host (Bronowski et al. 2014). One of the survival mechanisms is the ability to form a biofilm, where cells are enclosed and protected in an extracellular matrix (Turonova et al. 2016). The extracellular matrix consists mostly of proteins, polysaccharides, and extracellular DNA (Bae et al. 2014; Brown et al. 2015). Biofilms can be attached on different biotic and abiotic surfaces and represent a reservoir of viable cells, which may be released during the dispersion process and serve as a source of contamination of food products (Reuter et al. 2010). In the structure of the biofilm, cells behave as a multicellular organism via coordinated changes in gene expression. This ability is mediated by the presence of signalling molecules used in the cell-to-cell communication system also known as quorum sensing (QSemerenini et al. 2015; Li and Tian 2012).

Quorum sensing is based on the production, release, and detection of signalling molecules known as autoinducers (AIs). The production and accumulation of AIs allow the population of bacteria to regulate their behaviour and thus affect important processes, including biofilm formation, motility, or expression of virulence factors (Plummer 2012). In gram-negative bacteria, two predominant types of AIs have been previously described. The first type consists of highly species-specific acyl-homoserine lactones (AHLs), also known as autoinducers-1 (AI-1), produced by AHL synthases (Rickard et al. 2010). The second type of signalling molecule, autoinducer-2 (AI-2), contains a characteristic furanone ring and is produced by S-ribosylhomocysteine lyase (LuxS) within the activated methyl cycle (AMC). This molecule is recognised by both gram-positive and gram-negative bacteria (Xavier and Bassler 2003).

Currently, only the LuxS/AI-2 pathway has been identified in C. jejuni (Elvers and Park 2002). However, unlike Escherichia coli and Vibrio harveyi, C. jejuni is not able to internalize a synthetic AI-2 (Adler et al. 2015). It is therefore possible that C. jejuni may produce and recognise a chemically distinct form of AI-2, or it possesses a specific receptor protein for AI-2 recognition (Adler et al. 2015; Miller et al. 2004).

It is well known that both LuxS and AI-2 have a critical impact on the biofilm formation of C. jejuni (Reeser et al. 2007). However, their exact role in this process has not yet been fully investigated. Therefore, a set of C. jejuni 81–176 luxS mutant strains (deletion (∆luxS), substitution (+luxS), and insertion (: luxS)), and their complements, were created using cassette mutagenesis. The disruption of AI-2 production was confirmed by bioluminescence assays, and the impact of the LuxS/AI-2 QS pathway and mutagenic strategy used on the process of biofilm formation was investigated.

### Material and methods

#### Bacterial strains and cultivation

All strains used in this work were stored at −80°C in 20% glycerol stocks. During genetic manipulations, C. jejuni 81–176 (ATCC® BAA-2151™) was routinely cultivated at 42°C under microaerobic conditions (5% O2, 10% CO2, 85% N2) in a multi-gas incubator (MCO-18 M, Schoeller, Germany) on the Mueller–Hinton (MH) agar or broth (Hi-Media, India), eventually supplemented either with chloramphenicol (20 µg/mL, Merck, USA) for the cultivation of luxS deletion, substitution and insertion inactivated mutant strains, or kanamycin (50 µg/mL, Merck, USA) for the cultivation of luxS complemented strains. For biofilm formation, C. jejuni was pre-cultivated on Karmali agar (Oxoid, UK). Escherichia coli DH5α (Q5® Site-Direct Mutagenesis Kit, NEB, USA) was cultivated aerobically at 37°C on the Lysogeny agar (LB-A) or broth (LB; Hi-Media, India), eventually supplemented either with kanamycin (50 µg/mL; bacteria carrying a plasmid with the complementation cassette), chloramphenicol (20 µg/mL; bacteria carrying plasmids containing the cassettes for deletion, substitution or insertion inactivation) or ampicillin (100 µg/mL; bacteria carrying plasmids with partially assembled cassettes before the insertion of chloramphenicol or kanamycin selection marker. For the bioluminescence assay, Vibrio campbellii ATCC® BAA-1117™, designated as Vibrio harveyi BB170 (AI-2 sensitive reporter strain), and V. campbellii ATCC® BAA-1119™ designated as Vibrio harveyi BB152 (AI-2 positive control) were cultivated on the Autoinducer bioassay (AB) agar or broth (ATCC Medium: 2746) aerobically at 30°C.

#### Genetic engineering of Escherichia coli DHα and Campylobacter jejuni 81–176

For all cloning experiments, amplification parameters of the specific regions of C. jejuni 81–176 were designed using the FastPCR software (Kalander et al. 2017). Primers for site-specific mutagenesis with substitution and insertion inactivation cassettes were designed in the NEBaseChanger® (NEB, USA). Genomic DNA of C. jejuni 81–176 was isolated according to the protocol described by He (2011). Competent E. coli DH5α cells were prepared and transformed by routine heat-shock transformation according to the protocol described by Sambrook and Russell (2006). The electro-competent cells of C. jejuni were prepared according to the protocol described by Wassenaar et al. (1993) with the modification of replacing the saponin agar plates with MH agar without...
supplement. All plasmids used in this study were isolated by the GenElute HP Plasmid Miniprep Kit (Merck, USA). The restriction enzymes used in this study (XbaI, BamHI, NcoI, EcoRI and SacI) were purchased from NEB, USA. Ligase T4 DNA was used for ligations, and Q5® High-Fidelity DNA Polymerase was used for all PCRs (both NEB, USA). The accuracy of assembled cassettes in both *E. coli* and transformed *C. jejuni* strains was confirmed by PCR and Sanger sequencing. All PCR programs used in this study are available in Online Resource 1.

**Construction of the luxS deletion cassette**

To prepare the deletion cassette (Fig. 1; Fig. S1a, Online Resource 1), border sequences of approx. 600 bp in front of and behind the *luxS* gene were amplified by two primer sets containing specific restriction sites (Set 1 and Set 2; Table 1). The fragments from PCR reactions were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Subsequently, approximately 1 µg of each product was digested with 10 units (U) of appropriate restriction enzymes, together with 1 µg of plasmid pGEM®-T easy vector (Promega, USA). Digested fragments and plasmids were electrophoretically separated in 1% agarose gel, cut out, and purified using the Wizard kit. The concentration of all purified fragments was measured using Nanophotometer™ (Implen, Germany) and the appropriate ligation ratio (F1:F2:plasmid = 3:3:1) was calculated in silico using NEBioCalculator® (NEB, USA). Ligation was performed according to a protocol provided by the manufacturer of T4 ligase. After an overnight ligation at 16 °C, 5 µl of the ligation mixture was introduced into competent *E. coli* DH5α by heat shock as described by Sambrook and Russell (2006). Transformed cells were cultivated 24 h at 37 °C on LB agar containing 100 µg/mL of ampicillin. Resulting plasmids were isolated from 10 randomly selected colonies. The assembly of plasmids was verified by restriction analysis with BamHI, as a unique BamHI site was created between the F1 and F2 fragments (Fig. S1a, Online Resource 1). Finally, chloramphenicol acetyltransferase (*cat*, GenBank: M35190.1) was amplified from plasmid pRY111 (Yao et al. 1993) by primer Set 3 (Table 1). After its purification and BamHI treatment, the cassette was ligated into the created BamHI site, replacing the *luxS* gene. The final plasmid was transformed into *E. coli* DH5α as mentioned above. Colonies containing the deletion cassette were selected on the LB-A plate with chloramphenicol (20 µg/mL). The sequence of the deletion cassette was verified by Sanger sequencing using primer Set 4 (Table 1). Data from sequencing are available in Online Resource 1 (Fig. S1b and Fig. S1c).

**Construction of the luxS substitution cassette**

Substitution cassette (Fig. 1; Fig. S2a, Online Resource 1) was created as follows. A fragment of 1.2 kbp containing the *luxS* gene with approx. 600 bp in front of and 100 bp

![Fig. 1](image_url) Design of the mutagenic and complementation cassettes of *C. jejuni* 81–176 and the electropherogram of the confirmation PCR with primer Set 11. (A) wild-type (wt); (B) substitution (*luxS* 256A); (C) insertional inactivation (*::luxS*); (D) deletion (*ΔluxS*); (E) complementation (*luxSc*, *::luxSc*, *ΔluxSc*). The putative regulation promoter sequence (TATA), the Shine-Dalgarno (SD) sequence, and +1 as start codon were left intact in all mutants, except for the *ΔluxS* where the whole *luxS* gene was removed including the start codon
Table 1 Primers used in this study with highlighted modified regions for restriction sites and mutagenesis

| Set no | Primer sequence 5’ - 3’ (modification in Bold) | Target area | Specification |
|--------|-----------------------------------------------|-------------|---------------|
| 1      | CACCATGGAGCCTGATCATCTTCTTTAGTGTGCT            | Border sequence of luxS (in front) | NcoI, BamHI |
|        | GCCGTCTGGATCCCTCTTCTAAATATAATTGTCT           |             |               |
|        | AAGAATTTAG                                |             |               |
| 2      | TTACCGAGGGATCCCGAGAAGTAGCCTTAAAGAATCTC      | Border sequence of luxS (behind) | BamHI |
|        | GTGAACTTCATGTGTCCATATCCAGTTGTTCCT           |             |               |
| 3      | TGCGGATCCCCTGGCGGCGTCGGTGCCTCAAGCAGT      | cat cassette | BamHI |
|        | TGAGGATCCCTGGCCCTTATGTACCTCTGGG           |             |               |
| 4      | ATCTTTTCAAGTGGCTTC             | Border sequence of deletion cassette | Sequencing |
|        | GATCTCTGTGTATTTCA            |             |               |
| 5      | TCGAGCTCAGGGCTACTGCAATCTCAAGCCT  | luxS and border sequences | SacI, BamHI |
|        | AGAATTTCCAAAGGACGCTAGATCTAT |             |               |
| 6      | CTGTCGATACGATTTCATATGAG          | luxS        | Forward contains 256A mutation |
|        | CCCCAGGTGAAATATCAGTG |             |               |
| 7      | CGCCCAGGAGCCTAGAACTTCAAGACCT      | luxS and border sequences | NcoI, BamHI, EcoRI |
|        | AGAATTTCCAAAGGACGCTAGATCTAT |             |               |
| 8      | TTACCGAGGGATCCCGAGAAGTAGCCTTAAAGAATCTC      | Border sequence of luxS (behind) | BamHI |
|        | GTGAACTTCATGTGTCCATATCCAGTTGTTCCT           |             |               |
| 9      | CACCATGGACCTGCATCTTCTTCTAGTGTGCT | luxS and border sequences | NcoI |
|        | TGAGGATCCCTGGCCCTTATGTACCTCTGGG           |             |               |
| 10     | TCTTAAATCCAGATCGGCGGAAATACCATATTTTCAC      | luxS        | Forward with substitution |
|        | TGCAGGCTTCAAAATCTCGT |             |               |
| 11     | CACCATGGACCTGCATCTTCTTCTAGTGTGCT            | luxS and border sequences | NcoI |
|        | CTGGATCCCTAAAGGACGCTAGATCTAT |             |               |
| 12     | TGCGGATCCCGGATTATAATATCTATAGAATC           | kan cassette | BamHI |
|        | TGCGGATCCCTGGCCCTTATGTACCTCTGGG           |             |               |
| 13     | ACCTGCATCTTCTTCTAAGTGTGCT                 | luxS and border sequences | Cassette amplification |
|        | TCCTGTGCTATATCGGCAATACCTAAA |             |               |
| 14     | TTATTTGCGGTTTTTGCGTA                | luxS        | PCR verification |
|        | CTTCATGGCTGCTTCCCAA                  |             |               |
| SubF   | GAGCATGAACCTTCAAGACCT | Border sequence of luxS (in front) | Sequencing |

behind the gene was amplified by primer Set 5 containing modified regions with restriction sites (Table 1). The PCR product was purified and 1 µg was digested with 10 U of SacI and BamHI, together with plasmid pUC19 (NEB, USA). Products of digestion were then treated as described in the previous chapter, and they were subsequently ligated and transformed into competent E. coli DH5α by heat shock. Transformed cells were cultivated 24 h at 37 °C on LB agar containing 100 µg/mL of ampicillin, 50 µg/mL of IPTG, and 40 µg/mL of X-Gal, which allows the blue-white screening as described by Juers et al. (2012). After cultivation, 10 random white colonies were selected to confirm the presence of the fragment of interest by colony PCR using primer Set 5 (Table 1). A point substitution 256A previously identified by Plummer et al. (2011) was incorporated into the luxS gene using specifically designed primer Set 6 (Table 1) and Site-Direct Mutagenesis Kit (NEB, USA). To confirm the successful substitution, a small region containing the luxS gene was amplified with modified primer Set 7 (Shagieva et al. 2020) and subsequently cloned between the NcoI and EcoRI restriction sites of the pGEM-T easy vector. The substitution was confirmed by Sanger sequencing using the commercially available M13/forward primer (Fig. S2b, Online Resource 1). Finally, the complementary border sequence (approx. 500 bp) located behind the luxS gene was amplified (primer Set 8, Table 1), purified, and digested by BamHI and XbaI. The products of digestion were ligated at a standard ratio of 3:1, incorporating the border sequence behind the first fragment containing the point mutation 256A (Fig. S2a, Online Resource 1). After transformation into E. coli, positive colonies were confirmed by restriction analysis with BamHI and XbaI enzymes. Finally, the cat cassette was cloned between both fragments, into the
BamHI site, and positive colonies were selected on the LB-A plates with chloramphenicol (20 µg/mL).

**Construction of the cassette for insertion inactivation of luxS**

The modified primer Set 9 containing restriction sites for NcoI and EcoRI (Table 1) was used for amplification of the fragment of approx. 1.5 kbp containing the luxS gene with border sequences on both sides. This fragment was purified from the PCR reaction and subsequently digested with NcoI and EcoRI, together with the plasmid pGEM-T easy vector. The products of digestion were then electrophoretically separated, purified, ligated, and transformed into the E. coli DH5α as described in the sections above. Transformed cells were cultivated for 24 h at 37 °C on LB agar containing 100 µg/mL of ampicillin, 50 µg/mL of IPTG, and 40 µg/mL of X-Gal, which allows the blue-white screening. Positive (white) colonies were confirmed by colony PCR using primer Set 9 (Table 1). In silico analysis of the luxS gene revealed a nucleotide sequence similar to the BamHI restriction site, suitably situated close to the middle of the gene. Using primer set 9 (Table 1), the amplified luxS gene with the required substitution, and Q5® Site-Direct Mutagenesis Kit (NEB, USA), the substitution was introduced and the amplified cat cassette was cloned as described above, disrupting the luxS gene (Fig. S3a, Online Resource 1). Positive colonies were selected on the LB-A plates with chloramphenicol (20 µg/mL). All modifications were confirmed using Sanger sequencing (Fig. S3b and Fig. S3c, Online Resource 1).

**Construction of the luxS complementation cassette**

A fragment containing a functional copy of the luxS gene (approx. 1.2 kbp) was amplified (primer Set 11, Table 1), purified, and digested with NcoI and BamHI. Plasmid pGEM-T easy vector containing the complete deletion cassette was digested in the same way as the fragment. After electrophoretic separation in 1% agarose gel, the remaining cells of positive cultures were collected with pre-warmed MHB containing 20% glycerol and were incubated microaerobically at 42 °C overnight. Subsequently, the culture was harvested with 1 mL of the pre-warmed MH broth and 100 µL aliquots were spread on MH agar plates containing 20 µg/mL of chloramphenicol and incubated for 3–7 days. After the incubation, colonies grown on the selective agar plate were individually inoculated on the surface of fresh antibiotic-supplemented plates and were incubated microaerobically at 42 °C overnight. To confirm the recombination, a part of each grown overnight culture was harvested with a sterile inoculation loop, and its DNA was isolated by thermal lysis. The presence of mutagenic cassettes within the genome was confirmed by PCR (primer Set 13, Table 1). After visualization of products in 1% agarose gel, the remaining cells of positive cultures were collected with pre-warmed MHB containing 20% glycerol and stored at −80 °C.

To complement the mutant strains, the complementation cassettes were electroporated into electro-competent cells of the respective luxS mutant strains. Positive colonies were selected on the MH agar plates with kanamycin (50 µg/mL). The presence/absence of the luxS gene was confirmed by PCR (primer Set 14, Table 1). For final sequencing of the incorporated cassettes (mutagenic/complementation), these were amplified from DNA of positive colonies (primer Set 9, Table 1) and cloned into the pGEM®-T easy vector as described above. All sequences were deposited to the GenBank under the following numbers: deletion MN640707; substitution MN738698; insertional inactivation MN738697; and complementation MN711445.

**Transformation of Campylobacter jejuni 81–176 by cassette mutagenesis**

The transformation was performed as described by Wasse-naar et al. (1993). Briefly, the PCR with primer set 13 was performed for amplification of deletion, substitution, insertion inactivation, and complementation cassettes (1 µg of DNA), which were subsequently electroporated into freshly prepared electro-competent cells of C. jejuni 81–176 using MicroPulsedom® (Bio-Rad, Hercules, California, USA) and pre-cooled 0.2 cm cuvettes (Bio-Rad, Hercules, California, USA). For each electroporation experiment, exactly one pulse of 2500 V was used. After electroporation, the cuvette was rinsed two times with 100 µL of pre-warmed MH broth, and each suspension was transferred (without spreading) on a surface of pre-warmed MH agar (without antibiotics) and incubated microaerobically at 42 °C overnight. Subsequently, the culture was harvested with 1 mL of the pre-warmed MH broth and 100 µL aliquots were spread on MH agar plates containing 20 µg/mL of chloramphenicol and incubated for 3–7 days. After the incubation, colonies grown on the selective agar plate were individually inoculated on the surface of fresh antibiotic-supplemented plates and were incubated microaerobically at 42 °C overnight. To confirm the recombination, a part of each grown overnight culture was harvested with a sterile inoculation loop, and its DNA was isolated by thermal lysis. The presence of mutagenic cassettes within the genome was confirmed by PCR (primer Set 13, Table 1). After visualization of products in 1% agarose gel, the remaining cells of positive cultures were collected with pre-warmed MHB containing 20% glycerol and stored at −80 °C.
**Bioluminescence assay for AI-2**

Bioluminescence assay was performed according to the protocol described by Rickard et al. (2006) with some modifications. To prepare the cell-free supernatants (CFSs), one colony of an overnight culture of tested strain was inoculated on a fresh pre-warmed Karmali with appropriate antibiotics (C. jejuni strains) or AB agar (V. harveyi BB152, positive control), and was incubated for 24 h either microaerobically at 42 °C for C. jejuni strains, or aerobically at 30 °C for V. harveyi strain. The cultures were then harvested from plates with 1 mL of pre-warmed MH or AB broth. All cultures were diluted to the OD₆₀₀ 0.1 ± 0.005 and were used to inoculate the MH/AB broth in a ratio of 1:30. The resulting suspensions were then incubated for 24 h either microaerobically at 42 °C for C. jejuni strains, or aerobically at 30 °C for V. harveyi strain. After the incubation, grown cultures were transferred into 50 mL plastic centrifugation tubes and centrifuged at 9000 × g for 10 min in a pre-cooled centrifuge (4 °C). The supernatant was subsequently filtered through a 0.22 µm PES filter (P-Lab, Czech Republic).

The overnight culture of the V. harveyi BB170 (AI-2 sensitive reporter strain) was harvested with 2 mL of AB medium and diluted to the OD₆₀₀ 1.0 ± 0.005. The diluted culture was mixed with the sterile AB medium in a ratio 1:500. Subsequently, 900 µL of the suspension was mixed with 100 µL (10% (v/v)) of the CFS. A parallel with a sterile MH/AB broth instead of CFS served as a negative control, which was used for the normalisation of the background signal. The suspension was briefly homogenised and 100 µL of each sample was transferred to the Corning® Costar 96 Well White Plate (Merck, USA) in triplicate. The bioluminescence was measured in 30-min intervals, 10 s per well, by SpectraMax® i3x Multi-Mode Detection Platform (Molecular Devices, UK) in the kinetic mode for 16 h. The resulting bioluminescence of one biological replicate was interpreted as an average of the relative light units (RLUs) of the three technical replicates normalised by subtracting the negative control with sterile MH/AB broth. To avoid the possibility, that the resulted bioluminescence could be influenced by a different growth rates of the tested C. jejuni strains and V. harveyi BB152, colonies were counted according to a protocol described by Miles et al. (1938). Only the data obtained from cultures with comparable growth rates were used for the interpretation of the bioluminescence assay results. The experiment was performed in three independent biological replicates. The statistical significance of the generated numerical data was determined by paired T-test. Results with a p-value lower than 0.05 were considered statistically significant.

**Biofilm formation**

Biofilms were prepared as described by Turonova et al. (2015) with some modifications. Campylobacters were resuscitated from stock on Karmali agar (with chloramphenicol 20 µg/mL for mutant and kanamycin 50 µg/mL for complemented strains) and incubated microaerobically for 24 h at 42 °C. After cultivation, a single colony of each strain was selected and inoculated on a fresh Karmali plate with the appropriate antibiotics and incubated again for 24 h at 42 °C. The culture was then harvested with 1 mL of pre-warmed MH broth and diluted to the OD₆₀₀ 0.8 ± 0.005. Each well of a 96-well clear-bottom plate (thickness of 190 ± 5 µm; Greiner Bio-one, Germany)) was inoculated with 250 µL of suspension in triplicates and incubated for 5 h at 42 °C. Then, the suspension was carefully replaced with 250 µL of a sterile pre-warmed MH broth and cultivated for another 24 h at 42 °C. After the cultivation, the wells were manually washed by gently removing 150 µL of the suspension and replacing it with 150 µL of sterile and filtrated pre-warmed physiological solution. The washing was repeated 5 times for each well. After the final aspiration of the well content, 50 µL of a physiological solution containing Syto-9 (Thermo Scientific, USA) in a concentration of 1 µL/mL was added to each well. Biofilms were stained for 15 min and subsequently scanned using Olympus IX81F- ZDC2 (Olympus, Japan) confocal scanning laser microscope with a spinning disc (CLSM), equipped with Andor IQ software (Andor, Belfast, UK). The signal was recorded in the green channel (excitation at 488 nm, emission 525 nm) using an objective Clara 10x as series of z-stacks of horizontal planar images with a z-step selected according to the Nyquist sampling (3.57 µm). The experiment was performed in three independent biological replicates.

**Image and statistical analysis**

All scans obtained by CLSM were analysed by the software Imaris 7.6.4 (Bitplane, UK). The biofilm structure was rendered using the Easy 3D view with auto-adjustment function to correct pixel intensities. Numerical data and 3D models of the biofilms were generated using the surface generator function. The quantity of the biofilm was evaluated as a volume of biomass stained by Syto-9. The statistical significance of the generated numerical data obtained from the Imaris software was determined by paired T-test. Results with a p-value lower than 0.01 were considered statistically significant.
Results

Preparation of Campylobacter jejuni 81–176 luxS mutants

To evaluate the effect of S-ribosylhomocysteine lyase (LuxS) and the mutagenic strategy on the production of AI-2 and biofilm formation of _C. jejuni_ 81–176, several mutants were prepared. The _luxS_ gene was inactivated by deletion (Δ_luxS_), substitution (∙_luxS_), and insertion (∶∶_luxS_). All the mutants were complemented with a functional copy of the _luxS_ gene carried on a complementation cassette and confirmed by PCR. Electropherogram confirmed that the sizes of all amplified cassettes in the _luxS_ mutants are as predicted (Fig. 1). PCR specific for the inner sequence of the _luxS_, clearly distinguished the mutant with deleted Δ_luxS_ gene and mutant with an enlarged PCR product with _cat_ cassette in the middle of the ∶∶_luxS_ (insertion inactivation; Fig. 2). The results of sequencing showed that all cassettes were incorporated correctly, without unwanted mutations. Moreover, the sequencing directly confirmed the absence of the _luxS_ gene in the deletion mutant, the presence of the _cat_ in the middle of the _luxS_ gene in the insertion mutant, and the point mutation ∙256A (A → G in the 256th nucleotide of _luxS_) in the substitution mutant (Fig. S2b, Online Resource 1). All sequences were deposited to the GenBank database (deletion cassette MN640707; substitution cassette MN738698; insertional inactivation cassette MN738697; complementation cassette MN711445).

Bioluminescence assay for AI-2

The presence of AI-2 in the tested cell-free supernatant (CFS) was determined by the bioluminescence of the _Vibrio harveyi_ BB170 (reporter strain). The bioluminescence was evaluated as relative light units obtained from the tested sample of CFS in the moment of maximal stimulation of the reporter strain, with a subtraction of background bioluminescence obtained from the negative control (use of sterile cultivation medium instead of CFSs). CFSs prepared from the cultivation of the wild-type, mutant, and complementation strains of _C. jejuni_ 81–176 in MH broth were tested for the presence of AI-2 and compared with a positive control (CFS from AI-2 producing strain _Vibrio harveyi_ BB152). The results of the bioluminescence assay showed that the wild-type of _C. jejuni_ produced significantly more AI-2 molecules (p < 0.05; Fig. 3) in comparison to the deletion (Δ_luxS_), substitution (∙_luxS_), and insertion (∶∶_luxS_) mutants, which were not able to stimulate the reporter strain. However, after complementation of the _luxS_ mutants with the functional copy of the _luxS_ gene, the bioluminescence signal of the reporter strain was restored at least to the level of the wild-type (Fig. 3). These results suggest that the prepared _luxS_ mutants of _C. jejuni_ 81–176 are not able to produce the signal molecule AI-2.

As the quantity of produced QS molecules correlates with the cell density of the bacterial population, the _C. jejuni_ strains were subjected to colony counting, in order to eliminate the influence of differential growth. The results clearly showed that all tested strains had comparable counts of colony-forming units per milliliter (CFU/mL) at the end of the cultivation time (from 9.71 ± 0.07 log_{10} CFU/mL for the wild-type to 9.87 ± 0.11 log_{10} CFU/mL for the deletion...
Biofilm formation

The biofilms formed by all tested strains were stained with Syto-9 and the biofilm mass was quantified by CLSM. The results clearly showed that ∆luxS, ·luxS, and ::luxS formed significantly lower amounts of biofilm mass when compared to the wild-type (p < 0.01; Fig. 4). Surprisingly, the ∆luxS mutant formed less biomass than both ·luxS, and ::luxS mutants (p < 0.01; Fig. 4). Additionally, differences in the biofilm structure were observed between the strains. The wild-type C. jejuni 81–176 was able to form clusters of biofilm (Fig. 5). The ∆luxS strain formed only thin and sparse biofilms without the visible clusters observed in the ·luxS and ::luxS mutants (Fig. 6). The complementation of all mutants, in particular the ∆luxSc, restored the production of the biofilm mass to the level of the wild-type. Moreover, after the complementation, increased biofilm mass containing small and medium clusters was observed in the ∆luxSc mutant (Fig. 6). These results suggest that, apart from the presence or absence of the luxS gene, the mutagenic strategy employed against the luxS has a critical impact on the formation of biofilm mass and the formation of microcolonies of C. jejuni 81–176.

Discussion

The common strategy of C. jejuni for survival and persistence in the external environment is the ability to form a biofilm. Within the biofilm, QS represents an effective way to coordinate the behaviour of the whole population of bacteria via gene expression regulation (Indikova et al. 2015). Since Elvers and Park (2002) described the existence of the luxS gene and AI-2 in C. jejuni, many studies were focused on the investigation of quorum-sensing system impact on different cell processes including biofilm formation, virulence, motility, and survival in the external environment (He et al. 2008; Ligowska et al. 2011; Plummer et al. 2012; Reeser et al. 2007). To explain discrepancies in the results provided by these studies, our work focused on two aspects that influence QS and therefore could affect biofilm formation — the presence/absence of the luxS gene, and the type of mutagenic strategy used to produce the luxS mutant strains. As it was previously suggested that the mutagenic strategy could have an impact on the results of the experiments (Adler et al. 2014), three different methods of genetic engineering were used to inactivate the luxS gene — deletion, substitution, and insertion.
Indeed, even though none of the prepared luxS mutants was able to produce the signalling molecule AI-2, the deletion mutant formed significantly less biofilm than the substitution and insertion mutants. Also, the biofilm volume of all three mutants was significantly lower compared to the wild-type. A similar discrepancy was observed in the biofilm architecture, as biofilms of the deletion mutant lacked the visible clusters observed in other strains. These results suggest that apart from the presence/absence of the luxS gene itself, the mutagenic strategy used can also impact the biofilm formation and clustering ability of C. jejuni 81–176. In regards to biofilm structure, similar results were published by Balestrino et al. (2005), where the deletion luxS mutant of Klebsiella pneumoniae showed reduced ability to form microcolonies in the early stages of the biofilm formation. In addition, the luxS deletion mutant of Shewanella oneidensis formed an undifferentiated biofilm compared to the wild-type, which formed compact aggregates on the glass surface (Bodor et al. 2011). On the other hand, the deletion luxS mutant of Enterococcus faecalis formed distinct and complex clusters, opposite to the wild-type whose biofilm was more confluent (He et al. 2016). Insertion-inactivated luxS in Streptococcus oralis led to disruption of mutualistic interrelation with Actinomyces naeslundii resulting in a significant decrease of biofilm formation, which was restored after genetic or chemical (AI-2) complementation (Rickard et al. 2006). As described in the work of Golz et al. (2012), not all Campylobacter species encode the luxS gene, and the ability to form a biofilm differs between the strains of C. jejuni also due to different genetic backgrounds (Pascoe et al. 2015). Similarly, Shagieva et al. (2020) demonstrated that two water isolates of C. jejuni missing luxS gene formed significantly lower levels of biofilm biomass in comparison to other water isolates where the luxS gene was detected. In a different study, the deletion luxS mutant of C. jejuni

**Fig. 5** Early staged biofilm formed by the wild-type of C. jejuni 81–176 with differentiated clusters after 24 h of cultivation in Mueller Hinton medium (MH) statically in microaerobic conditions at 42°C. Biofilm was stained with Syto-9, visualized by the CLSM, and processed in Imaris 7.6.4 software.
Fig. 6 Early staged biofilm structure of the luxS mutant/complementation strains after 24 h of cultivation in Mueller Hinton medium (MH) statically in microaerobic conditions at 42 °C. Biofilm was analysed by CLSM after Syto-9 staining. Images of biofilm of ΔluxS C. jejuni 81–176 (deletion)/ΔluxSc (complementation)::luxS C. jejuni 81–176 (insertional inactivation)::luxSc (complementation); ∙luxS C. jejuni 81–176 (substitution)/∙luxSc (complementation) were processed in Imaris 7.6.4 software.
M129 formed less biofilm mass than the wild-type, but its biofilm formation capacity was increased by the addition of prepared CFS containing the signal molecule AI-2. A similar effect was observed after treatment with CFS prepared from Pseudomonas aeruginosa and Arcanobacterium pyogenes BBR1, while the addition of CFS prepared from Clostridium perfringens and Chromobacterium violaceum did not affect biofilm formation of C. jejuni M129 (Reeser et al. 2007). The molecule of AI-2 is known as a universal signalling molecule (Bassler 2002), but at this time only a few forms of this molecule were identified and described (Chen et al. 2002; Miller et al. 2004). It seems that in the case of C. jejuni, the recognition of AI-2 can be dependent on a specific form of this molecule, as C. jejuni was not able to internalise commercially purchased AI-2 (Adler et al. 2015). According to a mathematical model provided by Emerenini et al. (2015), the translocation of QS molecules within the biofilm allows bacteria to control its size, structure, and dispersion of cells from the matured biofilm. The model was supported by a study performed by Auger et al. (2006), who treated Bacillus cereus with AI-2 extracted from Photorhabdus luminescens. Co-cultivation of Bacillus cells with the AI-2 inhibited its biofilm formation, while the addition of AI-2 to preformed biofilms led to the dispersion of cells from the structure of the biofilm (Auger et al. 2006).

It is important to mention that the proper functionality of quorum sensing is not solely based on the production of autoinducers but requires an appropriate sensor and transport mechanisms allowing the excretion of synthesised molecules to the extracellular space. In C. jejuni, a small non-coding RNA (CjNC110) located downstream of the luxS gene is described. Deletion of CjNC110 resulted in decreased levels of extracellular AI-2, while intracellular levels of AI-2 were significantly increased. Results suggest that CjNC110 may participate in the transport of AI-2 in C. jejuni (Kreuder et al. 2020). However, further research is needed to explain the details of quorum sensing machinery in C. jejuni.

**Conclusion**

This study clearly shows that the mutagenic strategy affects the results of experiments focused on the functionality of the luxS gene and should therefore be taken into consideration in any further research. Even though no mutant strain was able to produce a detectable amount of AI-2 and all formed lower levels of biofilm than wild-type, the volume and ultrastructure of biofilms differed between the particular mutants. As the deletion mutant formed meagre biofilm without distinct microcolonies, the process of biofilm formation is likely affected not only by the concentration of AI-2, but also by the presence of the gene in the genome. However, further research is needed to explain this phenomenon.

**Data and materials availability**

Datasets generated during the current study are available from the corresponding author on request.

**Ethics approval and consent to participate**

This article does not contain any studies with human participants or animals performed by any of the authors.

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**Data Availability** Datasets generated during the current study are available from the corresponding author on request.

**Code Availability** This article does not contain any studies with human participants or animals performed by any of the authors.

**Declarations**

**Conflict of interest** The authors declare no competing interests.

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