



# 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 ( $\Delta luxS$ ) showed a larger decrease in biofilm mass than the substitution ( $\bullet luxS$ ) and insertional inactivated ( $: luxS$ ) mutants, even though all the mutant strains lost the ability to produce autoinducer-2 molecules. Moreover, the biofilm of the  $\Delta luxS$  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

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*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 ( $\Delta luxS$ ), substitution ( $\bullet luxS$ ), 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  $-80\text{ }^{\circ}\text{C}$  in 20% glycerol stocks. During genetic manipulations, *C. jejuni* 81–176 (ATCC® BAA-2151™) was routinely cultivated at  $42\text{ }^{\circ}\text{C}$  under microaerobic conditions (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , 85%  $\text{N}_2$ ) 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  $\mu\text{g}/\text{mL}$ , Merck, USA) for the cultivation of *luxS* deletion, substitution and insertion inactivated mutant strains, or kanamycin (50  $\mu\text{g}/\text{mL}$ , Merck, USA) for the cultivation of *luxS* complemented strains. For biofilm formation, *C. jejuni* was pre-cultivated on Karmali agar (Oxoid, UK). *Escherichia coli* DH5 $\alpha$  (Q5® Site-Direct Mutagenesis Kit, NEB, USA) was cultivated aerobically at  $37\text{ }^{\circ}\text{C}$  on the Lysogeny agar (LB-A) or broth (LB; Hi-Media, India), eventually supplemented either with kanamycin (50  $\mu\text{g}/\text{mL}$ ; bacteria carrying a plasmid with the complementation cassette), chloramphenicol (20  $\mu\text{g}/\text{mL}$ ; bacteria carrying plasmids containing the cassettes for deletion, substitution or insertion inactivation) or ampicillin (100  $\mu\text{g}/\text{mL}$ ; 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  $30\text{ }^{\circ}\text{C}$ .

### Genetic engineering of *Escherichia coli* DH $\alpha$ 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 (Kalendar 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* DH5 $\alpha$  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.

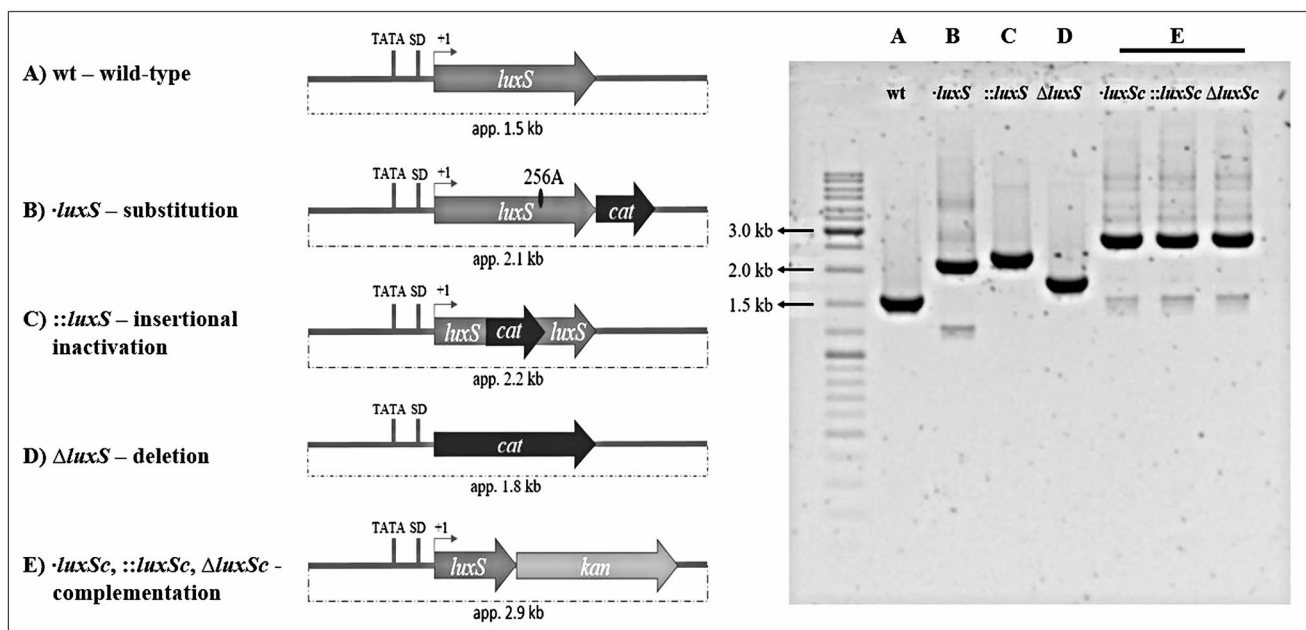
### Construction of the *luxS* deletion cassette

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** 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 ( $\bullet luxS$  256A); (C) insertional inactivation ( $::luxS$ ); (D) deletion ( $\Delta luxS$ ); (E) com-

plementation ( $\bullet luxSc, ::luxSc, \Delta luxSc$ ). 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  $\Delta luxS$  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	CACCATGGACCTGCATCTTCTTCTAAGTGTGCT GCCGTCTCGGATCCCTCCTTAAATATAAATTGTCT AGAATTATAG	Border sequence of <i>luxS</i> (in front)	NcoI BamHI
2	TTACCGAGGGATCCGAGAATGCTTAAAAAGAATC GTGAATTCCTGTGCCTATATCCAGTTGTTCTC	Border sequence of <i>luxS</i> (behind)	BamHI EcoRI
3	TGCGGATCCCTGCTCGGCGGTGTTCTTTCCAAG TCAGGATCCCTGCGCCCTTTAGTTCCTAAAGGGT	<i>cat</i> cassette	BamHI BamHI
4	ATCTTCTTCCAAGTGCGCTC GATCCTGCTGTGTATTCA	Border sequence of deletion cassette	Sequencing
5	TCGAGCTCAGGCTCACTGACAATCTCAAGC GTGGATCCACATCTCGCACATCAGTT	<i>luxS</i> and border sequences	SacI BamHI
6	CTGTGCTACGAGTTTTTATATGAG CCCATAGGTGAAATATCAATG	<i>luxS</i>	Forward contains 256A mutation
7	CGCCATGGGAGCATGAACTTCAAGACCT ACGAATTCCAAAGGACGCACTAGATACT	<i>luxS</i> and border sequences	NcoI EcoRI
8	TTACCGAGGGATCCGAGAATGCTTAAAAAGAATC GTCTTAGATTCTGTGCCTATATCCAGTTGTTCTC	Border sequence of <i>luxS</i> (behind)	BamHI XbaI
9	CACCATGGACCTGCATCTTCTTCTAAGTGTGCT GTGAATTCCTGTGCCTATATCCAGTTGTTCTC	<i>luxS</i> and border sequences	NcoI EcoRI
10	TCTTAATTCAGGATCCGTTGAAATCATTGATATTTAC TGATCTCTCATAAATCCTG	<i>luxS</i>	Forward with substitution
11	CACCATGGACCTGCATCTTCTTCTAAGTGTGCT CGTGGATCCCAAAGGACGCACTAGATACT	<i>luxS</i> and border sequences	NcoI BamHI
12	TGCGGATCCCGCTTATCAATATATCTATAGAATG TCAGGATCCGATAATGCTAAGACAATCACTAAA	<i>kan</i> cassette	BamHI BamHI
13	ACCTGCATCTTCTTCTAAGTGTGCT TCTGTGCCTATATCCAGTTGTTCTC	<i>luxS</i> and border sequences	Cassette amplification
14	TTGATTTGCGTTTTTTCGTA CTTTCATGGCTGCTCCCAA	<i>luxS</i>	PCR verification
SubF	GAGCATGAACTTCAAGACCT	Border sequence of <i>luxS</i> (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 the 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 *luxS*, requiring substitution of three nucleotides. Using the primer Set 10 (Table 1), where the forward primer carried the required substitution, and Q5® Site-Direct Mutagenesis Kit (NEB, USA), the substitution was introduced and the BamHI site was created and confirmed by the restriction analysis. Into the BamHI unique restriction site in the middle of *luxS*, 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 characteristic band of the pGEM-T easy vector containing the fragment F2 was cut out and purified (Fig. S4b, Online Resource 1), and was subsequently ligated with the functional copy of the *luxS* gene. Positive colonies were confirmed by colony PCR using primer Set 11 (Table 1). For complementation, the kanamycin resistance cassette from plasmid pRY107 was amplified using the modified primer Set 12 (Table 1; Shang et al. 2016) and was cloned between both fragments into the BamHI site (Fig. S4a, Online Resource 1). Positive colonies were selected on

the LB-A plate with kanamycin (50 µg/mL). The correct assembly of the complementation cassette was confirmed through the Sanger sequencing (Fig. S4c, Fig. S4d, and Fig. S4e, Online Resource 1) using commercially available primers M13 and primer SubF (Table 1) to assure a precise sequencing of the long construct.

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

The transformation was performed as described by Wasseenaar 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 MicroPulser™ (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.

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.

## 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 OD<sub>600</sub> 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 OD<sub>600</sub> 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 (RLU) 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 OD<sub>600</sub> 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 10× 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

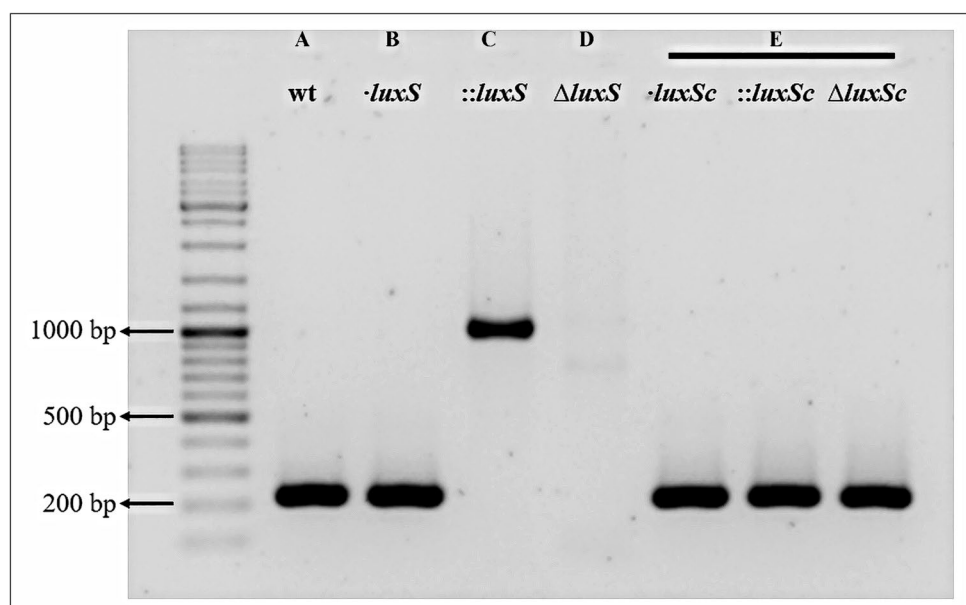
To evaluate the effect of S-ribosylhomocysteine lyase (LuxS) and the mutagenic strategy on the production of AI-2 and biofilm formation of *C. jejuni* 81–176, several mutants were prepared. The *luxS* gene was inactivated by deletion ( $\Delta luxS$ ), substitution ( $\bullet luxS$ ), and insertion ( $: : luxS$ ). 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  $\Delta luxS$  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  $\bullet 256A$  ( $A \rightarrow G$  in the 256<sup>th</sup> 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

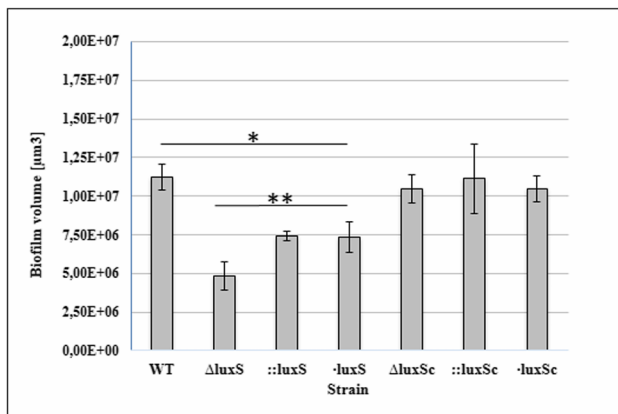
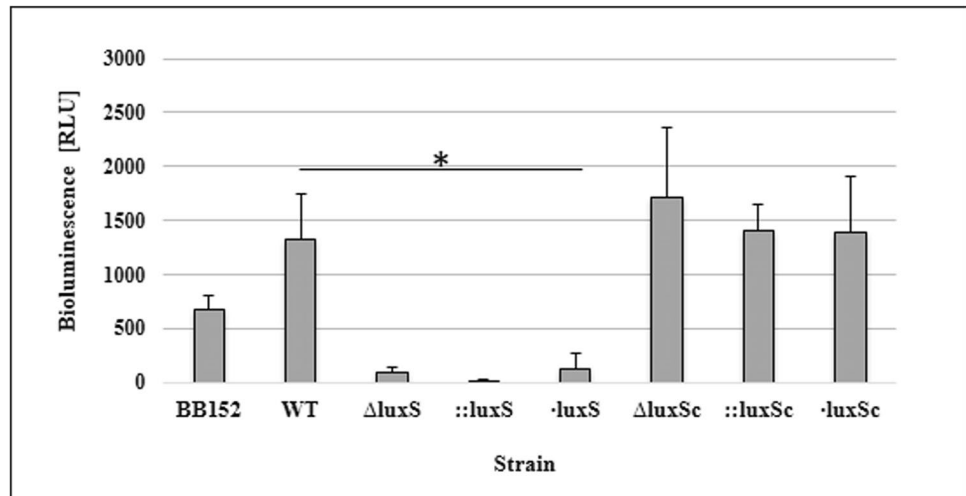
The presence of AI-2 in the tested cell-free supernatant (CFS) was determined by the bioluminescence of the *Vibrio harveyi* BB170 (reporter strain). The bioluminescence was evaluated as relative light units obtained from the tested sample of CFS in the moment of maximal stimulation of the reporter strain, 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 ( $\Delta luxS$ ), substitution ( $\bullet luxS$ ), 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  $9.71 \pm 0.07 \log_{10}$  CFU/mL for the wild-type to  $9.87 \pm 0.11 \log_{10}$  CFU/mL for the deletion

**Fig. 2** Confirmation of *C. jejuni* 81–176 mutant/complementation strains by amplification of a characteristic 222 bp product formed within the inner region of the *luxS* by primer Set 12. (A) wild-type (wt); (B) substitution ( $\bullet luxS$  256A); (C) insertional inactivation ( $: : luxS$ ); (D) deletion ( $\Delta luxS$ ); (E) complementation ( $\bullet luxSc$ ;  $: : luxSc$ ,  $\Delta luxSc$ )



**Fig. 3** Bioluminescence emitted by *V. harveyi* BB170 after stimulation with CFSs obtained from cultivation of the tested strains in MH broth. WT – *C. jejuni* 81–176 (wild-type); BB152 – *V. harveyi* BB152 (positive control);  $\Delta luxS$  – deletion mutant;  $::luxS$  – insertional inactivation;  $\bullet luxS$  256A – substitution;  $\Delta luxSc$  – deletion mutant complemented;  $::luxSc$  – insertional inactivation mutant complemented;  $\bullet luxSc$  – substitution complemented. \*statistically significant difference when compared to WT ( $p < 0.05$ )



**Fig. 4** Biofilm volume quantified by CLSM after the Syto-9 staining. WT – *C. jejuni* 81–176 (wild-type);  $\Delta luxS$  – deletion mutant;  $::luxS$  – insertional inactivation;  $\bullet luxS$  256A – substitution;  $\Delta luxSc$  – deletion mutant complemented;  $::luxSc$  – insertional inactivation mutant complemented;  $\bullet luxSc$  – substitution complemented. \*statistically significant difference when compared to WT ( $p < 0.01$ ). \*\*statistically significant difference when compared to  $\Delta luxS$  ( $p < 0.01$ )

*luxS* mutant, and  $8.25 \pm 0.06 \log_{10}$  CFU/mL for the positive control).

### Biofilm formation

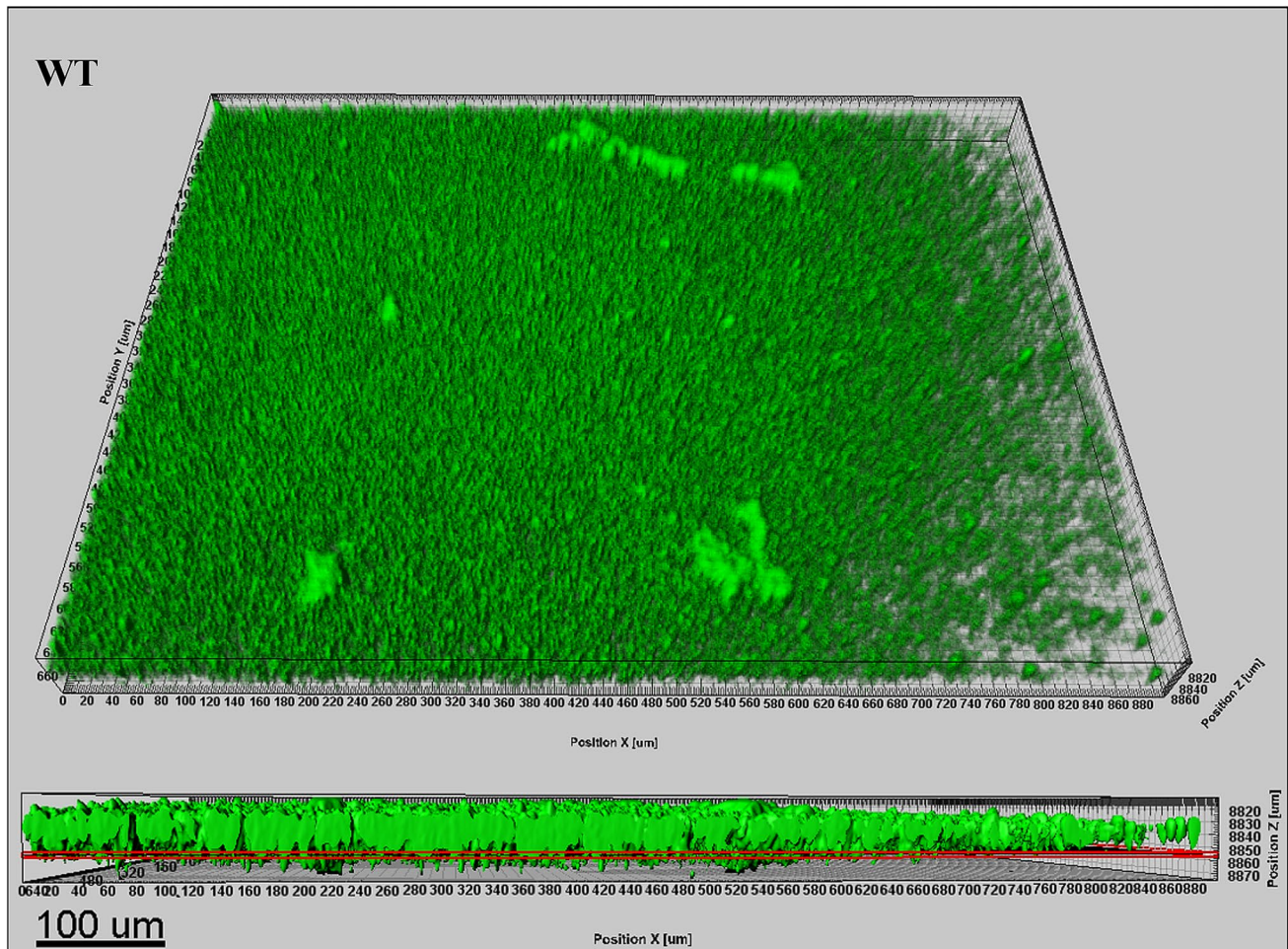
The biofilms formed by all tested strains were stained with Syto-9 and the biofilm mass was quantified by CLSM. The results clearly showed that  $\Delta luxS$ ,  $\bullet luxS$ , and  $::luxS$  formed significantly lower amounts of biofilm mass when compared to the wild-type ( $p < 0.01$ ; Fig. 4). Surprisingly, the  $\Delta luxS$  mutant formed less biomass than both  $\bullet luxS$ , 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  $\Delta luxS$  strain formed only thin and sparse

biofilms without the visible clusters observed in the  $\bullet luxS$  and  $::luxS$  mutants (Fig. 6). The complementation of all mutants, in particular the  $\Delta luxSc$ , 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  $\Delta luxSc$  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.





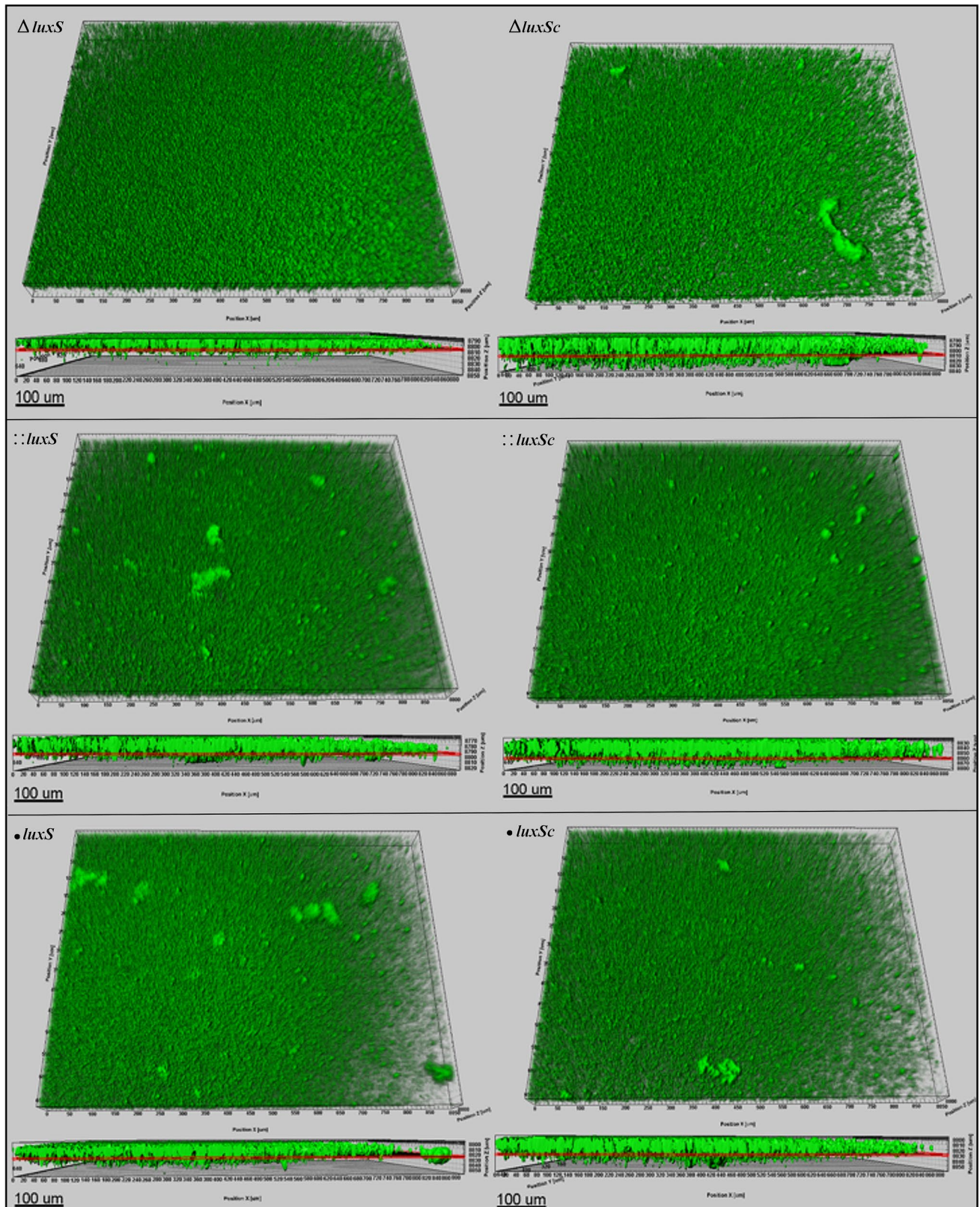
**Fig. 5** Early staged biofilm formed by the wild-type of *C. jejuni* 81–176 with differentiated clusters after 24 h of cultivation in Mueller Hinton medium (MH) statically in microaerobic conditions at

42 °C. Biofilm was stained with Syto-9, visualized by the CLSM, and processed in Imaris 7.6.4 software

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. 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  $\Delta luxS$  *C.*

*jejuni* 81–176 (deletion)/  $\Delta luxSc$  (complementation);  $::luxS$  *C.* *jejuni* 81–176 (insertional inactivation)/  $::luxSc$  (complementation);  $\bullet luxS$  *C.* *jejuni* 81–176 (substitution)/  $\bullet luxSc$  (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 *Photobacterium 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.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s13353-021-00655-y>.

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**Author contribution** Conceptualization: [Martin Teren]; Methodology: [Martin Teren]; Formal analysis and investigation: [Martin Teren, Ekaterina Shagieva, Viviana Svarcova]; Writing – original draft preparation: [Martin Teren]; Writing – review and editing: [Hana T. Michova, Lucie Vondrakova, Jitka Viktorova, Katerina Demnerova]; Funding acquisition: [Katerina Demnerova]; Resources: [Hana T. Michova, Lucie Vondrakova, Jitka Viktorova, Katerina Demnerova]; Supervision: [Katerina Demnerova].

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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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