Role of Alkyl Hydroperoxide Reductase (AhpC) in the Biofilm Formation of *Campylobacter jejuni*

Euna Oh, Byeonghwa Jeon*

School of Public Health, University of Alberta, Edmonton, Alberta, Canada

Abstract

Biofilm formation of *Campylobacter jejuni*, a major cause of human gastroenteritis, contributes to the survival of this pathogenic bacterium in different environmental niches; however, molecular mechanisms for its biofilm formation have not been fully understood yet. In this study, the role of oxidative stress resistance in biofilm formation was investigated using mutants defective in catalase (KatA), superoxide dismutase (SodB), and alkyl hydroperoxide reductase (AhpC). Biofilm formation was substantially increased in an ahpC mutant compared to the wild type, and katA and sodB mutants. In contrast to the augmented biofilm formation of the ahpC mutant, a strain overexpressing *ahpC* exhibited reduced biofilm formation. A perR mutant and a CosR-overexpression strain, both of which upregulated *ahpC*, also displayed decreased biofilms. However, the introduction of the *ahpC* mutation to the perR mutant and the CosR-overexpression strain substantially enhanced biofilm formation. The *ahpC* mutant accumulated more total reactive oxygen species and lipid hydroperoxides than the wild type, and the treatment of the *ahpC* mutant with antioxidants reduced biofilm formation to the wild-type level. Confocal microscopy analysis showed more microcolonies were developed in the *ahpC* mutant than the wild type. These results successfully demonstrate that AhpC plays an important role in the biofilm formation of *C. jejuni*.

Introduction

*Campylobacter jejuni* is a leading bacterial cause of human gastroenteritis and accounts for 400 million–500 million cases of diarrhea worldwide per year [1]. *C. jejuni* is also implicated in the onset of approximately a quarter of cases of Guillain–Barré syndrome, an autoimmune disorder characterized by acute and progressive neuromuscular paralysis [2]. While *C. jejuni* is isolated from a variety of domestic animals and wildlife, poultry is considered to be the major reservoir for *C. jejuni* [3]. *Campylobacter* infection is zoonotic and most cases of human *campylobacteriosis* are associated with consumption of undercooked poultry [4]. Although *C. jejuni* is known to be fastidious to culture because of complex nutrient and growth requirements [5], this bacterium is isolated from diverse environmental sources, such as surface water, sewage and farms [6,7], suggesting that *C. jejuni* may possess unique survival mechanisms to persist in the environment. However, mechanisms for stress resistance and survival in the environment have not been well understood in *C. jejuni*.

Oxidative stress resistance is an important defense mechanism. As a microaerophilic bacterium, *C. jejuni* possesses unique oxidative defense systems. *C. jejuni* has a sole catalase (*KatA*) and a sole superoxide dismutase (*SodB*) for the detoxification of H$_2$O$_2$ and superoxide, respectively [8]. In *Escherichia coli*, alkyl hydroperoxide reductase consists of AhpC and AhpF [9] and plays an important role in scavenging endogenous H$_2$O$_2$ [10], whereas an *ahpF* homolog is absent from the *C. jejuni* genome [11]. Although the substrates of AhpC have not yet been identified in *C. jejuni*, an *ahpC* mutation increases susceptibility to aerobic stress and cumene hydroperoxide, but not to H$_2$O$_2$ [11]. As to the regulation of oxidative stress response, *C. jejuni* lacks homology of the oxidative stress regulators OxyR and SoxRS, one or both of which are usually present in many bacterial species [8]. Instead, PerR, Fur and CosR regulate genes of oxidative stress resistance in *C. jejuni* [12–15].

Biofilms are a mode of bacterial growth which is often found in natural settings [16]. High numbers of *Campylobacter* spp. are isolated from biofilms in nature [17], implying that the ability of *Campylobacter* to form biofilms contributes to its prevalence in the environment [17–19]. *C. jejuni* forms biofilms on various abiotic surfaces, such as glass, plastics and stainless steel [20–22]. The biofilm formation of *C. jejuni* is affected by nutritional, and environmental conditions. Cultivation with Mueller Hinton media at 37°C under 10% CO$_2$ enhanced biofilms, whereas nutrient-rich media (e.g., Brucella broth) or high osmolarity (e.g., >0.05M NaCl) decreases biofilm formation of *Campylobacter* [22]. As motility is an important factor of biofilm formation in many bacterial species [23], mutations of genes associated with bacterial motility significantly affect the biofilm formation of *C. jejuni* [20,21,24]. Surface polysaccharides influence biofilm formation as mutations of genes involved in the synthesis of capsular polysaccharide (CPS) or lipooligosaccharide (LOS) increase biofilm formation [20,25]. Interestingly, the *pgp1* gene encoding a peptidoglycan D-lactarboxyypeptidase affects the corkscREW morphology of *C. jejuni*, and a *pgp1* mutation results in defects in motility and biofilms [26]. Quorum sensing is also involved in biofilm formation as a luxS
Table 1. Strains and plasmids used in this study.

| Strains and Plasmids | Description | Sources or references |
|----------------------|-------------|-----------------------|
| E.coli | DH5α | F- ΔMtic Δ(ermC-96) ΔlacZΔM15 ΔlacZYA-argF U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 Δ-thi-1 gyr96 relA1 | Life Technologies |
| C.jejuni | NCTC 11168 | Wild type, a human isolate | [37] |
| ΔahpC | ahpC mutant, ahpC::aphA-3 | This study |
| ΔkatA | katA mutant, katA::aphA-3 | [31] |
| ΔsodB | sodB mutant, sodB::cat | [31] |
| ΔperR | perR mutant, perR::cat | [57] |
| ahpC over | C. jejuni NCTC 11168 harboring an extra copy of ahpC in rRNA region | This study |
| cosR over | C. jejuni NCTC 11168 harboring an extra copy of cosR in rRNA region | [13] |
| ΔperR &ΔahpC | ahpC (ahpC::aphA-3) and perR (perR::cat) double mutant | This study |
| ΔcosR over & ΔahpC | ahpC mutant (ahpC::aphA-3) and CosR- overexpression mutant | This study |
| ahpC comp | ahpC complementation | This study |
| katA comp | katA complementation | [31] |
| sodB comp | sodB complementation | [31] |
| perR comp | perR complementation | [57] |
| Plasmids | pUC19 | Cloning vector used for suicide vector in C. jejuni; Amp’ | New England Biolabs |
| | pFMBcomCM | pUC19 derivative carrying an rRNA gene cluster; Cm’ | [13] |
| | pMW10 | E.coli-C. jejuni shuttle vector | [32] |

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Bacterial Strains and Culture Conditions

Bacterial strains used in this study are listed in Table 1. All C. jejuni strains were grown at 42°C with Mueller-Hinton (MH) media (Oxoid, Canada) under a microaerobic condition (5% O₂, 10% CO₂, and 85% N₂). Occasionally, MH media were supplemented with kanamycin (50mg/L) and/or chloramphenicol (25mg/L). E. coli DH5α harboring plasmids was grown at 37°C with Luria-Bertani (LB) media (Difco, US) that were supplemented with ampicillin (100mg/L), chloramphenicol (25mg/L), or kanamycin (50mg/L), where required.

Materials and Methods

Construction of the ahpC mutant, and the perR&ahpC and cosR over&ahpC double mutants

The ahpC gene and its flanking region were amplified by PCR with DahpC-F and DahpC-R primers (Table 2). After digestion with EcoRI and BamHI, the PCR product was ligated to pUC19 that had been treated with the same enzymes. The pUC19::ahpC plasmid was digested with EcoRV and ligated with the kanamycin resistance cassette which had been amplified from pMW10 [32] with Kan-F and Kan-R primers (Table 2). The constructed suicide plasmid was introduced to C. jejuni NCTC 11168 by electroporation and mutants were selected on MH agar plates supplement-

Table 2. Primers used in this study.

| Primers | Sequences (5’-3’) |
|---------|------------------|
| DahpC-F | GGAATTCCTCCCTCCACCTTGCATATC |
| DahpC-R | GGGATCCCAAAGTCGGCCGATCTTGT |
| Kan-F | GCGATGAAAGTGCGTAAAG |
| Kan-R | CGCGTCCGGTCACTATAG |
| mahpC-F | CATGATAGTACTAAAAAGTTTAG |
| mahpC-R | GTAAAAGTTGACTCGTTTGGCC |
| com_ahpC-F | GTCTCTAGAAGCTGCCGACTCTGGAGACTTGT |
| com_ahpC-R | CGCTCTAGACACCTCTGGGATTATACTCAT |

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ed with kanamycin (50 mg/L). The ahpC mutation was confirmed by PCR with mahpC-F and mahpC-R primers (Table 2). The ahpC complementation strain was constructed by chromosomal integration of ahpC as described previously [33]. Briefly, ahpC was PCR-amplified with com_ahpC-F and com_ahpC-R primers (Table 2). After digestion with XbaI, ahpC was ligated with pMBcomCM [13]. The pMBcomCM::ahpC plasmid was transformed into the ahpC mutant strain by electroporation and a complementation strain was selected by growing on MH agar plates containing kanamycin (50 mg/L) and chloramphenicol (25 mg/L). In addition, pMBcomCM::ahpC was introduced into C. jejuni NCTC 11168 as described above to construct an ahpC-overexpression strain. Increased transcription of ahpC in the ahpC-overexpression strain was confirmed by qRT-PCR (Fig. S1). Chromosomal DNA extracted from the ahpC mutant strain was introduced into the perR mutant and the CosR-overexpression strain by electroporation to construct the perR::ahpC and cosR over::ahpC double mutants. Transformants were selected on MH agar plates supplemented with kanamycin (50 mg/L) and chloramphenicol (25 mg/L). Transfer of the ahpC mutation was confirmed by PCR.

Biofilm Assay
Biofilm assay was performed as described previously [25] with some modifications. Briefly, overnight cultures of C. jejuni strains were harvested from MH agar plates and resuspended in MH broth to an OD600 of 0.07. After culturing 5 h at 42°C with shaking (200 rpm) under a microaerobic condition, the bacterial suspension was diluted with fresh MH broth to an OD600 of 0.07 and inoculated into 96-well plates (Nunc, US). Biofilms were cultivated under the same growth condition without shaking. The 96-well plates were washed twice with PBS (pH 7.4) and then treated with 100 μl PBS (pH 7.4). Confocal microscopy was performed with an epifluorescence microscope (Thermo Scientific, US). Occasionally, biofilm assay was carried out in the presence of 1 μM CosR-PNA [34], which was commercially synthesized by PANAGENE (Daejeon, South Korea), or antioxidants, including L-proline, L-cysteine, β-carotene and N-acetyl cysteine, which were purchased from Sigma (St. Louis, US).

Measurement of Total Reactive Oxygen Species (ROS)
The ROS level in biofilms was measured using CM-H2DCFDA (Life Technologies, US), a general oxidative stress indicator. After discarding supernatants from 96-well plates, biofilms were washed with PBS (pH 7.4) and then treated with 100 μl of 1% crystal violet solution at room temperature for 15 min at room temperature. After washing 15 min at room temperature, crystal violet solution was removed and the plates were washed 3 times with PBS (pH 7.4). Stained biofilm was eluted with 10% acetic acid that was provided by the manufacturer.

Figure 1. Biofilm formation of oxidative stress resistance mutants. Biofilm formation was measured using crystal violet staining. The results show the means and standard deviations of a representative assay with triplicate samples. The experiment was repeated six times and all produced similar results. Statistical significance was analyzed using one-way analysis of variance (ANOVA). *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

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Lipid Hydroperoxide (LPO) Assay
LPO levels were measured using a commercial kit (Cayman Chemical Co., US) according to the manufacturer’s instructions. Briefly, LPOs were extracted from biofilms with chloroform and methanol. The LPO extract (500 μl) was mixed with 50 μl of Chromogen reagent (4.5 mM ferrous sulfate in 0.1M HCl and 3% methanolic solution of ammonium thiosulfate) and incubated at room temperature for 5 min. A portion (300 μl) of each sample was transferred into a 96-well glass plate to measure OD500. A standard curve was generated with 13-hydroperoxy-octadecadienoic acid that was provided by the manufacturer.

Confocal Microscopy Analysis
Biofilms were fixed with 4% paraformaldehyde and stained with SYTO9 and propidium iodide from the LIVE/DEAD Biofilm Viability kit (Life Technologies, US) according to the manufacturer’s instructions. Confocal microscopy was performed with an
statistical significance was analyzed with three times and similar results were reproducible in all the experiments. The assays were repeated at least two-way ANOVA (Fig. 3C).

Results and Discussion

Enhanced Biofilm Formation in the ahpC Mutant

To investigate the role of oxidative stress defense in the biofilm formation of C. jejuni, we measured the biofilm formation levels of three oxidative stress resistance mutants defective in AhpC, SodB and KatA. These enzymes were chosen because of their critical roles in oxidative stress resistance, and C. jejuni possesses only a single gene copy encoding each enzyme. The ahpC mutant displayed most significant increases in the level of biofilm formation compared with the wild type and katA and sodB mutants, and complementation of the ahpC mutant restored biofilm formation to the wild-type level (Fig. 1). The katA mutation slightly increased biofilm formation, whereas the sodB mutant produced biofilms as comparably as the wild type (Fig. 1). Extended (i.e., 48 h) incubation of biofilms increased the levels of biofilm formation in all the tested strains; however, the ahpC mutant developed biofilms to a greater extent than the wild type and katA and sodB mutants both 24 h and 48 h (Fig. 1). SodB is known to be important for Campylobacter’s survival in environmental conditions, such as freeze-thaw stress in foods [35,36]; however, the sodB mutation did not affect biofilm formation (Fig. 1). Bacterial growth is not likely to be associated with the enhanced biofilm formation of the ahpC mutant because the ahpC, katA, and sodB mutants grew as comparably as the wild type (data not shown). The ahpC mutation slightly reduced the motility (Fig. S2); however, this does not account for the substantial increase in biofilms observed in the ahpC mutant, because mutations resulting in a motility defect impair the biofilm formation of C. jejuni [20,21,24], whereas the ahpC mutation substantially increased biofilm formation despite the partial reduction in motility. The results show that AhpC significantly affects the biofilm formation of C. jejuni.

Overexpression of ahpC Reduced Biofilm Formation

Since AhpC is an enzyme detoxifying ROS, we hypothesized that ahpC-overexpression would reverse the phenotype observed in the ahpC mutant if biofilm formation is directly associated with the enzymatic function of AhpC. The ahpC-overexpression was achieved in two different ways by: 1) incorporating an extra copy of ahpC to the chromosome of C. jejuni, and 2) modulating the expression of AhpC-controlling regulators, such as PerR and CosR [15,34]. C. jejuni lacks homologs of OxyR and RpoS [37], which regulate AhpC in E. coli and Salmonella [38,39]. Instead, PerR negatively regulates AhpC in C. jejuni [15]. CosR is an essential response regulator and positively regulates AhpC in C. jejuni [34]. Thus, ahpC-overexpression was alternatively achieved using a perR knockout mutant and a CosR-overexpression strain. In contrast to the stimulated biofilm formation of the ahpC mutant (Fig. 1), interestingly, the ahpC-overexpression strain exhibited significantly reduced biofilm levels compared to the wild type (Fig. 2), and the perR mutant and the CosR-overexpression strain also displayed substantial reductions in the level of biofilm formation (Fig. 2). The perR complementation restored biofilm formation to the wild-type level (Fig. 2). CosR is an essential regulator and its knockout mutant cannot be constructed because of bacterial lethality [40,41]. Thus, a gene knockdown strategy was used to reduce CosR expression with CosR-specific PNA as described in our previous studies [13,34]. Interestingly, CosR knockdown with CosR-PNA significantly increased biofilm formation to the level of the ahpC mutant (Fig. 2).

PerR and CosR are involved in the regulation of genes other than ahpC [13,14,34]. At least 104 genes belong to the PerR regulon [14], and CosR regulates 93 genes involved in various cellular functions, such as energy production, motility, drug efflux, and oxidative stress resistance [13]. An ahpC mutation was introduced to the perR mutant and the CosR-overexpression strain to clarify the role of AhpC in the reduced biofilm formation of the perR mutant and the CosR-overexpression strain. Interestingly, harboring the ahpC mutation abrogated the inhibitory effect of the perR mutation and the CosR-overexpression on biofilm formation and substantially increased biofilm formation similar to the ahpC mutant (Fig. 2), suggesting that CosR and PerR affect biofilms mainly through ahpC.

Kalmokoff et al. showed that proteins of oxidative stress resistance, including AhpC, Tpx (thiol peroxidase), and CosR (Cj0353c), are upregulated in C. jejuni biofilms [21]. Consistently, in this study, we demonstrated that both AhpC and CosR significantly affect the biofilm development of C. jejuni (Figs. 1 and 2). Given the positive regulation of AhpC by CosR [34], upregulated CosR will increase AhpC expression, consequently

In Figure 3, the total ROS level (A) and lipid hydroperoxide levels (B) were determined in the ahpC mutant and reduced biofilm formation in the ahpC mutant by treatment with antioxidants (C). The assays were carried out with 24 h old samples. Antioxidants were treated to a final concentration of 1 nM. The results show the means and standard deviations of a representative experiment with triplicate samples. The assays were repeated at least three times and similar results were reproducible in all the experiments. Statistical significance was analyzed with t-test (Fig. 3A and 3B) and two-way ANOVA (Fig. 3C). *P<0.05, ****P<0.0001.

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inverted confocal microscope (IX-81, Olympus, Japan) and Volocity 3D Image Analysis Software (PerkinElmer Inc., US).

Statistical Analysis

Data analysis was performed with GraphPad Prism 6 (GraphPad Software Inc., US).

Results and Discussion

Enhanced Biofilm Formation in the ahpC Mutant

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promoting the detoxification of ROS in biofilms. Since AhpC overexpression reduced the biofilm formation (Fig. 2), upregulated AhpC will reduce biofilm formation presumably to alleviate oxidative stress in aggregated bacterial cells by decreasing 

**Effect of ROS Accumulation on Biofilm Formation in the ahpC Mutant**

AhpC scavenges endogenous H$_2$O$_2$ at the physiological level in *E. coli* [10]. The substrates of the *Salmonella* Typhimurium AhpC are small hydroperoxides [44] and organic hydroperoxides, including alkyl hydroperoxides which can be produced intracellularly from unsaturated fatty acids and nucleic acids [9]. However, the enzymatic function and substrate of AhpC have not been defined in *C. jejuni*. In this study, total ROS and lipid hydroperoxide (LPO) were measured and compared between the *ahpC* mutant and the wild type (Fig. 3A and B). Consistent with the enzymatic function of AhpC in ROS detoxification, the *ahpC* mutant showed increased accumulation of total ROS and LPO compared to the wild type (Fig. 3A and B). In particular, the LPO level of the *ahpC* mutant was 2-fold higher than that of the wild type (Fig. 3B), suggesting that AhpC may be involved in the detoxification of LPO in *C. jejuni*. The results prompted us to hypothesize that the accumulation of endogenous ROS may be involved in the augmented biofilm formation of the *ahpC* mutant. To examine this possibility, biofilm assay was performed in the presence of antioxidants to reduce the ROS level. Interestingly, addition of antioxidants reduced the biofilm of the *ahpC* mutant to the wild-type level (Fig. 3C). Bacterial growth of the wild type and the *ahpC* mutant was not affected by the treatment with antioxidants (data not shown). The results strongly suggest that the accumulation of ROS enhanced biofilm formation in the *ahpC* mutant.

Bacterial cells in biofilms are physiologically different from planktonic cells and often exhibit increased resistance to environmental stress and antimicrobials [45]. The aggregate growth of bacteria in biofilms reduces the penetration of nutrients into inner layers and limits the diffusion of metabolic wastes, resulting in nutritional limitation and physiological stress [46]. The oxidative stress generated from endogenous ROS in biofilm cells enhances bacterial mutability and diversity [47,48], demonstrating that ROS substantially impacts bacterial physiology in biofilms. Genes involved in general and oxidative stress response are often induced in biofilms, possibly to alleviate the stress generated in biofilms [21,49–50]. The nutritional starvation of *Pseudomonas aeruginosa* in biofilms confers increased antibiotic tolerance in association with oxidative stress defense as the inactivation of stringent response significantly impaired the activities of catalase and superoxide dismutase [51]. The expression levels of Zn-superoxide dismutase (SodC) and thiol peroxidase (Tpx) are increased in biofilms of *E. coli* O157:H7, and *sodC* and *tpx* mutations impaired biofilm formation [52]. Iron stimulates the formation of rugose biofilms in *E. coli* in connection with ROS. Generation of superoxide stress by adding a superoxide generator or mutating *sodA* and *sodB* promotes the development of rugose biofilms [53]. The biofilm formation of *S. aureus* is stimulated by exposure to cigarette smoke that contains bioactive compounds such as free radicals and ROS. Addition of antioxidant (e.g., N-acetyl cysteine) eliminated the enhanced biofilm formation caused by cigarette smoke exposure [54].

**Enhanced Development of Microcolonies in the ahpc Mutant**

Biofilm development consists of multiple stages, starting with the attachment of bacteria, formation of microcolonies, maturation of biofilms forming mushroom-like structures and water channels, and dispersion of cells from biofilms [55]. *C. jejuni* forms microcolonies prior to the development of biofilms, and flagellar mutants are defective in forming microcolonies and biofilms [56]. Biofilms were observed with a confocal microscope to investigate the biofilm formation stage that is affected by the *ahpC* mutation. According to the results of confocal microscopy analysis, the *ahpC* mutant showed increased development of microcolonies compared...
to the wild type (Fig. 4). This is consistent with the results of biofilm assays, showing that the difference in biofilm formation between the wild type and the *ahpC* mutant was obvious even after one day (Fig. 1). The results suggest that AhpC is involved in the development of microcolonies at the early stage of biofilm formation.

Physiological heterogeneity exits in biofilms due to the concentration gradients between layers inside and outside [46]. Reduced diffusion of oxygen into biofilms will expose cells in outside layers to more oxygen and oxidative stress compared to bacteria inside biofilms. The impact of heterogeneity in biofilms would not be significant in the *ahpC* mutant because AhpC affects biofilm formation even at an early stage when biofilms are not fully developed (Fig. 4).

To the best of our knowledge, this is the first report showing that AhpC affects biofilm formation. The kind of ROS responsible for the enhanced biofilm formation of the *ahpC* mutant is still unknown. We speculate that the ROS would be neither H$_2$O$_2$ nor superoxide, because mutations of *C. jejuni*’s sole catalase and superoxide dismutase did not affect biofilm formation significantly, albeit the *katD* mutant increased biofilm formation at substantially lower levels than the *ahpC* mutant (Fig. 1). Although AhpC scavenges endogenous H$_2$O$_2$ at physiological levels in *E. coli* [10], such a function has not been demonstrated in the *C. jejuni* AhpC, and addition of exogenous H$_2$O$_2$ rather reduced the biofilm formation (data not shown). Based on the extrapolation of the enzymatic function of AhpC in other bacteria and the increased accumulation of LPO in the *ahpC* mutant, the ROS involved in biofilm formation in the *ahpC* mutant would be an organic peroxyde(s) endogenously generated within the cell. Validation of this possibility first of all requires the identification of ROS substrates of AhpC in *C. jejuni* and still awaits future studies.

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**Supporting Information**

**Figure S1** Increased levels of *ahpC* transcription in the *ahpC* overexpression strain. qRT-PCR was carried out with primer pairs: qPCR_ahpC-F (GGTAATTTGTCAGGTTAAATCC) and qPCR_ahpC-R (GGTTAATCATTAACCAGATG). The results were normalized to the expression level of 16S rRNA (Cj01) as described previously [34]. The assay was repeated three times. **p≤0.01. (TIF)

**Figure S2** Motility of the wild type (WT), the *ahpC* mutant (Δ*ahpC*), the *ahpC* complementation strain (*ahpC* comp), and the *ahpC* overexpression strain (*ahpC* over). (A) The *ahpC* mutation resulted in a slight reduction in motility with a full restoration to the wild-type level by complementation. The assay was performed with MH medium containing 0.4% agar, and the motility agar plate was incubated microaerobically at 42°C for 2 days. The result is a representative of three independent experiments with similar results. (B) Comparison of the size of motility zones. The results show the means and standard deviations. NS: non-significant. (TIF)

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**Author Contributions**

Conceived and designed the experiments: BJ. Performed the experiments: EO. Analyzed the data: EO BJ. Contributed reagents/materials/analysis tools: BJ. Wrote the paper: BJ.
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