International Journal of Food Microbiology 238 (2016) 172–182

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Chemical, physical and morphological properties of bacterial biofilms affect survival of encased *Campylobacter jejuni* F38011 under aerobic stress

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ARTICLE INFO

Article history:
Received 25 January 2016
Received in revised form 22 June 2016
Accepted 11 September 2016
Available online 13 September 2016

Keywords:
Raman spectroscopy
Atomic force microscopy
Confocal laser scanning microscopy
Contact angle measurement

ABSTRACT

Campylobacter jejuni is a microaerophilic pathogen and leading cause of human gastroenteritis. The presence of *C. jejuni* encased in biofilms found in meat and poultry processing facilities may be the major strategy for its survival and dissemination in aerobic environment. In this study, *Staphylococcus aureus*, *Salmonella enterica*, or *Pseudomonas aeruginosa* was mixed with *C. jejuni* F38011 as a culture to form dual-species biofilms. After 4 days' exposure to aerobic stress, no viable *C. jejuni* cells could be detected from mono-species *C. jejuni* biofilm. In contrast, at least 4.7 log CFU/cm^2 of viable *C. jejuni* cells existed in some dual-species biofilms. To elucidate the mechanism of protection mode, chemical, physical and morphological features of biofilms were characterized. Dual-species biofilms contained a higher level of extracellular polymeric substances with a more diversified chemical composition, especially for polysaccharides and proteins, than mono-species *C. jejuni* biofilm. Structure of dual-species biofilms was more compact and their surface was >8 times smoother than mono-species *C. jejuni* biofilm, as indicated by atomic force microscopy. Under desiccation stress, water content of dual-species biofilms decreased slowly and remained at higher levels for a longer time than mono-species *C. jejuni* biofilm. The surface of all biofilms was hydrophilic, but total surface energy of dual-species biofilms (ranging from 52.5 to 56.2 mJ/m^2) was lower than that of mono-species *C. jejuni* biofilm, leading to more resistance to wetting by polar liquids. This knowledge can aid in developing intervention strategies to decrease the survival and dispersal of *C. jejuni* into foods or environment.

1. Introduction

Campylobacter jejuni is a Gram-negative, microaerophilic bacterium and is one of the leading causes of foodborne gastrointestinal diseases worldwide. *Campylobacter* infection causes an acute gastroenteritis characterized by inflammation, abdominal pain, fever and diarrhea (Young et al., 2007). Previous reports indicated that *C. jejuni* infection cases in Canada outnumbered reported cases of *Escherichia*, *Listeria*, *Shigella* and *Salmonella* infections combined (Kalmokoff et al., 2006). The paradox associated with *C. jejuni* is that this bacterium is prevalent in the environment and difficult to eliminate from the food chain; however, as a microaerophile, *C. jejuni* is sensitive to aerobic stress and does not multiply in the aerobic environment. Studies have confirmed that bacteria shed from biofilms could continue to contaminate foods, potentially leading to food poisoning (Donlan and Costerton, 2002; Kumar and Anand, 1998). Under this condition, *C. jejuni* may survive within a biofilm microenvironment and further lead to food contamination (Ica et al., 2012), even though there remain controversies about how *C. jejuni* can resist environmental stress (*e.g.*, temperature fluctuation, aerobic, or shear stress) and form biofilms alone (Teh et al., 2014). In the natural environment, bacterial cells mainly reside in a multispecies culture. According to the previous reports, *C. jejuni* biofilms are present in the gastrointestinal tract of poultry, in water supply and plumbing systems in animal husbandry facilities and food processing plants (Hermans et al., 2011; Newell and Fearnley, 2003; Siringan et al., 2011; Trachoo et al., 2002) along with other foodborne pathogens including *Staphylococcus aureus*, *Salmonella enterica*, and *Pseudomonas aeruginosa*. The biofilms formed by these microorganisms are believed to provide protection to *C. jejuni* against antimicrobial treatments and aerobic stress (Ica et al., 2012; Joshua et al., 2006).

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.09.008
0168-1605/

Biofilms have a complex chemical composition. In the particulate fraction of a biofilm, up to 90% is composed by extracellular polymeric substances (EPS) including polysaccharides, proteins, nucleic acids, lipids, and humic-like substances. Specific chemical components of a biofilm may contribute to its resistance to exogenous stress. For example, hydrophilic polysaccharides and proteins in EPS can hold water and keep entrained microbial cells hydrated limiting the impact of desiccation stress (Roberson and Firestone, 1992; Tamaru et al., 2005). Enzymes within biofilms could inactivate stress inducers and neutralize these in a biofilm microenvironment (Davies, 2003). Unfortunately, it has been difficult to characterize the chemical profiles of biofilms because common methods, such as crystal violet (Reeser et al., 2007) and Congo red staining (Reuter et al., 2010), are destructive and can only be used to evaluate the biofilm formation level. Innovative spectroscopic methods, particularly Raman spectroscopy coupled with confocal technique can provide *in situ* and nondestructive determination of the chemical composition of bacterial biofilms and changes in the composition of biofilms in response to various forms of stress (Ivleva et al., 2008; Ivleva et al., 2010; Lu et al., 2012a).

Besides chemical composition, morphological properties of bacterial biofilms are also important in determining their resistance to the environmental stress. Joshua and coauthors compared biofilms produced by wild-type and mutant *C. jejuni* strains using scanning electron microscopy (SEM) (Joshua et al., 2006). Reuter and coworkers evaluated surface adhesion and microstructure of *C. jejuni* mono-species biofilm formed under microaerobic and aerobic environment using light microscopy after staining (Reuter et al., 2010). Both studies confirmed that the assemblage structure of biofilms were associated with the survival of encased sessile cells under environmental stress. Due to the destructive sample preparation process (*e.g.*, chemical fixation for SEM, staining for light microscopy), artifacts may be introduced that affect accurate characterization of biofilms. Atomic force microscopy (AFM) offers an alternative characterization methodology. By recording interaction signals between the probing tip and biofilm surface, AFM can generate high-resolution topographic images that accurately reflect the structural details of morphological information of a biofilm in a nano-scale without sample preparation (Ivanov et al., 2011; La Storia et al., 2011; Lim et al., 2011; Scheuring and Dufrêne, 2010).

Physical properties of biofilms, such as surface wettability (hydrophobicity/hydrophilicity), surface roughness, surface free energy, and water holding capability, play a role in the response of bacterial biofilms to a variety of stresses, such as desiccation and shear stress (Bove et al., 2012; Ng and Kidd, 2013). Wettability is related to the surface area of biofilm that could contact water while a high water holding capability maintains a high relative humidity in biofilms, both of which are important to protect encased cells from desiccation (Allison et al., 1990). Surface roughness predicts the susceptibility of biofilms to shear force, thus the smoother the biofilm surface the less it is influenced by mechanical shearing forces (Beech et al., 2002; Li and Logan, 2004).

Few studies have been conducted to investigate the effect of mixed bacterial culture on *C. jejuni*-containing biofilms and the susceptibility of *C. jejuni* cells in these multispecies biofilms. Therefore, the aim of this study is to characterize chemical, physical and morphological properties of dual-species *C. jejuni*-containing biofilms and correlate these to the stress resistance of encased *C. jejuni* cells compared to that of mono-species *C. jejuni* biofilm. The knowledge will be important to further understand the ecology of *C. jejuni* and its survival in the environment and subsequently develop innovative mitigation strategies to more successfully eliminate biofilms and reduce public health risk associated with this microbe.

2. Materials and methods

2.1. Bacterial strains and cultivation

C. jejuni F38011 (human clinical isolate), *Staphylococcus aureus* (a clinical isolate used in our previous study) (Lu et al., 2013), *Salmonella enterica* serovar Enteritidis FDA 3512H, and *Pseudomonas aeruginosa* PAO1 were used in this study. *C. jejuni* strain was stored at −80 °C in Mueller-Hinton (MH) broth (BD Difco) containing 12% glycerol and 75% defibrinated sheep blood. Routine cultivation was conducted either on *Campylobacter* agar (OXOID) supplemented with 5% defibrinated sheep blood or in MH broth with constant shaking at 37 °C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2). *S. aureus*, *S. enterica*, and *P. aeruginosa* were individually cultivated overnight in 5 ml tryptic soy broth (TSB) (BD Difco) at 37 °C to achieve a concentration of *ca.* 9 log CFU/ml.

2.2. Biofilm cultivation

One milliliter of overnight bacterial culture was centrifuged at 8000 × *g* for 10 min at 22 °C. The supernatant was discarded and the bacterial pellets were washed twice and resuspended in sterile phosphate buffered saline (PBS) (pH = 7.0). The resuspended culture was then diluted to ~10^7 CFU/ml. For dual-species biofilm formation, the mixed culture of *C. jejuni* F38011 was generated by addition of a second bacterial strain listed above on the basis of the same volume and concentration. Biofilms were cultivated at both solid-air interface and solid-liquid interface. Nitrocellulose membrane (0.45 mm pore size, 47 mm diameter; Sartorius Stedim-type filters) was used as substrate for biofilm formation at solid-air interface, as described elsewhere (Lu et al., 2012a, 2012b). *C. jejuni* monoculture or mixed culture (100 μl) was deposited onto the surface of a sterile nitrocellulose membrane with a surface area of ~3 × 3 cm^2, which was placed onto an agar plate supplemented with 5% defibrinated sheep blood and incubated under microaerobic environment at 37 °C. The membrane was aseptically transferred to a fresh agar plate every 24 h for up to 72 h. To cultivate biofilms at liquid-solid interface, 0.2 ml of *C. jejuni* monoculture or mixed culture was added into each well of sterile 96-well polystyrene plate. The plate was incubated under microaerobic environment at 37 °C for up to 72 h.

2.3. Survival of *C. jejuni* F38011 and co-cultured bacterial cells in biofilms under aerobic stress

C. jejuni and co-cultured bacterial cell survival in mono-species and dual-species biofilms under aerobic stress were determined by selective agar. Briefly, mature biofilms (cultivated under microaerobic condition for 72 h) formed on nitrocellulose membrane were placed under aerobic environment at 22 °C for up to 5 days. Every 24 h, biofilms were detached from nitrocellulose membrane using 0.1% trypsin solution (20 ml) for incubation at 22 °C for 20 min. This treatment did not affect bacterial cell viability (data not shown). Following detachment, the bacterial suspension was serially diluted and spread onto selective agar plate. Campy Cefex agar is used for enumeration of viable *C. jejuni* cells (Neal-McKinney et al., 2012). Campy-Cefex agar contains 43 g/l Brucella agar, 0.5 g/l ferrous sulfate, 0.2 g/l sodium bisulfite, 0.5 g/l sodium pyruvate, 33 mg/l cefoperazone, and 0.2 g/l cycloheximide, with a supplement of 5% defibrinated sheep blood (Oyarzabal et al., 2005). Mannitol salt agar (BD BBL) is used for the enumeration of viable *S. aureus* cells. Xylose lysine deoxycholate (XLD) agar (BD Difco) is used for enumeration of viable *S. enterica* and *P. aeruginosa* cells. The selective agar for *C. jejuni* was placed under microaerobic environment at 37 °C, while selective agars for co-cultured bacterial strains were placed under aerobic environment at 37 °C.

2.4. Confocal laser scanning microscopy (CLSM)

The survival state of *C. jejuni* cells within a biofilm was further confirmed using CLSM. The SYTO 9 dye (with a green color for live cells) and propidium iodide dye (with a red color for non-viable cells) were