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Architectural adaptation and protein expression patterns of *Salmonella enterica* serovar Enteritidis biofilms under laminar flow conditions

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Abstract

Salmonella enterica serovar Enteritidis is a significant biofilm-forming pathogen. The influence of a 10-fold difference in nutrient laminar flow velocity on the dynamics of *Salmonella* Enteritidis biofilm formation and protein expression profiles were compared in order to ascertain how flow velocity influenced biofilm structure and function. Low-flow (0.007 cm s^{-1}) biofilms consisted of diffusely-arranged microcolonies which grew until merging by ~72 h. High-flow (0.07 cm s^{-1}) biofilms were significantly thicker (36±3 μm (arithmetic mean±standard error; n=225) versus 16±2 μm for low-flow biofilms at 120 h) and consisted of large bacterial mounds interspersed by water channels. Lectin-binding analysis of biofilm exopolymers revealed a significantly higher (P<0.05) proportion of *N*-acetylgalactosamine (GalNAc) in low-flow biofilms (55.2%), relative to only 1.2% in high-flow biofilms. Alternatively, the proportions of α-L-fucose and *N*-acetylglucosamine (GlcNAc2)–*N*-acetylneuraminic acid (NeuNAc) polymer-conjugates were significantly higher (P<0.05) in high-flow biofilms (69.1% and 29.6%, respectively) than low-flow biofilms (33.1% and 11.7%, respectively). Despite an apparent flow rate-based physiologic effect on biofilm structure and exopolymer composition, no major shift in whole-cell protein expression patterns was seen between 168 h-old low-flow and high-flow biofilms, and notably did not include any response involving the stress response proteins, DnaK, SodB, and Tpx. Proteins involved in degradation and energy metabolism (PduA, GapA, GpmA, Pgk, and RpiA), RNA and protein biosynthesis (Tsf, TufA, and RpoZ), cell processes (Crr, MalE, and PtsH), and adaptation (GrcA), and some hypothetical proteins (YcbL and YnaF) became up-regulated in both biofilm systems relative to a 168 h-old planktonic cell control. Our results indicate that *Salmonella* Enteritidis biofilms altered their structure and extracellular glycoconjugate composition in response to flow and this response is suggested to be significant in the survival of this pathogen as biofilms.

Keywords: *Salmonella* Enteritidis; Biofilms; Laminar flow; Architecture; Adaptation; Protein expression

1. Introduction

Salmonella enterica serovar Enteritidis has emerged as one of the most important of all the food-borne pathogens, causing major outbreaks, especially in developed countries over the past 30 years (Patrick et al., 2004; Guerin et al., 2006). *Salmonella* Enteritidis results in losses of more than 500 million dollars annually due to associated medical costs of infections in humans, as well as lost productivity and revenue to the egg and meat industries (Frenzen et al., 1999; Food and Drug Administration, 2004).

Biofilm formation by *Salmonella* Enteritidis has been documented (Korber et al., 1997; Stepanović et al., 2003; Mangalappalli-Illathu and Korber, 2006) and it has been reported by Solano et al. (2002) that nearly all (97%) of 204 *Salmonella* Enteritidis isolates tested from various sources may form biofilms. These biofilms may serve as a reservoir of pathogens during the production of food products (Stepanović et al., 2003). Thus, attachment of *Salmonella* to food processing surfaces and subsequent development of biofilms may have significant economic and public health consequences. In systems dominated by laminar flow, molecular diffusion is the primary mechanism whereby nutrients and wastes are transported into

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and out of the biofilm matrix (Bryers, 1987; Davey and O'Toole, 2000). While there have been recent attempts to generalize the effects of fluid flow velocity on biofilm developmental processes, it should be noted that considerable variability in biofilm formation has been reported for different bacteria grown under various hydrodynamic regimens. It has further been hypothesized that cellular automaton models are applicable for describing the development and architecture of microbial biofilms; the interaction between substrate concentration, substrate gradient, and detachment forces might significantly influence the biofilm structure resulting in a system with overall reduced stress (Van Loosdrecht et al., 1997; Wimpenny and Colasanti, 1997).

Although molecular mechanisms mediating cell attachment and biofilm formation in *Salmonella* spp. are not completely understood, it is recognized that various genes and their products are either up-or down-regulated in response to biofilm formation. For example, the *agf*D promoter, encoding a putative response regulator of thin aggregative fimbriae expression, is involved in biofilm formation by *Salmonella* Typhimurium (Römling et al., 2000; Gerstel and Römling, 2001; Stepanović et al., 2003). Information on the regulatory mechanisms controlling the expression of *agf*D, and therefore biofilm formation, is limited even though multicellular behavior has been shown to be positively regulated at the onset of the stationary phase by *agf*D (Römling et al., 1998, 2000; Gerstel and Römling, 2001). Two genetic operons, namely *bcs*ABZC and *bcs*EFG, are also required for the biosynthesis of cellulose which is a constituent of *Salmonella* Enteritidis EPS, suggesting that cellulose production is a factor in the survival and proliferation of *Salmonella* Enteritidis biofilms associated with surface environments (Solano et al., 2002). It has also been reported in *Escherichia coli* that 22% of genes were up-regulated in the biofilm state, and 16% were down-regulated (Prigent-Combaret et al., 1999). Evidence exists that up- and down-regulation of a number of genes occur in surface colonizing cells following initial interaction with the substratum (Donlan, 2002). Notably, despite hypotheses that the biofilm contains zones where conditions would be presumed to be stressful (e.g., cells in the stationary phase), no work has demonstrated large-scale expression of stress genes.

The influence of hydrodynamic conditions on the formation and maintenance of biofilms formed by *Salmonella* spp. is not well understood (Stepanović et al., 2003), and could be significant for the development of resistant phenotypes responsible for resistance to antimicrobial agents on food processing surfaces. In this study we used nutrient flow velocity as a tool to simulate the effect of flow conditions that occur in routine food processing environments, on: (i) development of biofilm architecture, (ii) biofilm EPS composition, and (iii) protein expression profiles of *Salmonella* Enteritidis biofilms.

2. Materials and methods

2.1. Media and chemicals

Tryptic Soy Agar (TSA), Standard Plate Count Agar (SPCA), and Trypticase Soy Broth (TSB) were purchased from BBL (Becton Dickinson, Cockeysville, MD); magnesium chloride ($MgCl_2$), phenylmethylsulphonyl fluoride (PMSF), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), DNase, RNase A, fluorescein sodium salt, bromophenol blue, DL-dithiothreitol (DTT), and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO); sodium chloride was from EM Science (Gibbstown, NJ); EDTA was from J. T. Baker Chemical Co. (Philipsburg, NJ); glycerol, sodium dodecyl sulphate (SDS), tris base, and urea were purchased from Life Technologies (Grand Island, NY); *Bac*Light™ Live/Dead Viability Probe was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada); and immobilized pH gradient (IPG) buffer (pH 4.0 to 7.0), Immobiline DryStrip gels and PlusOne™ Protein Silver Staining Kit were purchased from GE Healthcare Bio-Sciences Inc. (Baie d'Urfé, QC, Canada). The plant lectins *Ulex europaeus* and *Triticum vulgaris* conjugated with fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), respectively, were purchased from Sigma Chemical Co., as was the lectin *Glycine max*. The cyanine dye (CY5) labeling for *G. max* was performed using a commercial labeling kit according to the manufacturer's instructions (Research Organics, Cleveland, OH).

2.2. Bacteria and culture conditions

Salmonella Enteritidis ATCC 4931 was cultured from frozen stock on TSA plates overnight at 37 °C. Cells in the mid-log phase of growth were obtained by transferring a loopful of colony material from TSA plates to 50 ml of 10% [wt/vol] TSB in an Erlenmeyer flask and incubating on a gyratory shaker (150±5 rpm) held at room temperature (RT; 21±2 °C) as batch culture for approximately 12 h. These cells, which were previously determined to be in the mid-log phase of growth, were used to inoculate flow cells. Planktonic cells for control experiments were grown in continuous culture in an Erlenmeyer flask held on a gyratory shaker (150±5 rpm) and incubated at RT for 168 h; the total culture volume was set at 175 ml. Nutrient medium (10% [wt/vol] TSB) was added and removed continuously at the rate of 25 ml h^{-1} resulting in a dilution rate of 0.14 h^{-1}. The medium was pumped into and out of the Erlenmeyer flask via silicone tubing using two Watson–Marlow peristaltic pumps (Model 202U; Watson–Marlow, Cornwall, UK), with each one set to control the input of the medium to the flask and removal of the effluent to a waste reservoir.

2.3. Flow cells, inoculation, and flow velocity

Multi-channel flow cells were constructed using 5 mm thick sheets of polycarbonate plastic into which were milled channels that were covered with glass coverslips, on which the biofilms were analyzed (Korber et al., 1994). Due to optical limitations, flow cells for use in dark-field microscopy were constructed entirely out of glass slides and coverslips. Flow cell channels were sterilized by flushing with 5.25% [wt/vol] sodium hypochlorite solution for 10 min. Reservoirs of sterile nutrient medium (10% [wt/vol] TSB) were connected via silicone tubing to the flow cell channels and subsequently connected to the