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Enhanced Surface Colonization by *Escherichia coli* O157:H7 in Biofilms Formed by an *Acinetobacter calcoaceticus* Isolate from Meat-Processing Environments[▽]†

Olivier Habimana,* Even Heir, Solveig Langsrud, Anette Wold Åsli, and Trond Møretrø

Nofima Mat AS, Osloveien 1, NO-1430 Ås, Norway

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A meat factory commensal bacterium, *Acinetobacter calcoaceticus*, affected the spatial distribution of *Escherichia coli* O157:H7 surface colonization. The biovolume of *E. coli* O157:H7 was 400-fold higher (1.2×10^6 μm^3) in a dynamic cocultured biofilm than in a monoculture (3.0×10^3 μm^3), and *E. coli* O157:H7 colonized spaces between *A. calcoaceticus* cell clusters.

Shiga toxin-producing *Escherichia coli* (STEC) is a foodborne human pathogen responsible for severe gastrointestinal disease (16, 17). Processing, handling, and preparation of food may lead to cross-contamination of food and uncontaminated surface areas of the food chain with pathogens from contaminated surfaces (8). Though most processing plants ensure and maintain good manufacturing practices with elaborated sanitary operations, persisting microorganisms may survive well after cleaning and disinfection procedures (1, 9, 12–14), possibly in the form of biofilms (11). A review of the underlying problems caused by biofilms in the food industry was presented by Carpentier and Cerf (4). Several studies have shown that *E. coli*, including STEC strains, has the capacity to attach to and form biofilms on various surface materials (5, 18). However, such studies have mainly used monocultures without considering the possible influence of resident organisms from food-processing environments on the surface colonization of *E. coli*. One recent study showed that resident microflora increased *E. coli* O157:H7 colonization on solid surfaces under static conditions (10). To our knowledge, no studies have investigated the influence of meat industry resident bacteria on surface colonization by *E. coli* under dynamic-flow conditions.

The aim of this study was to investigate how a biofilm-forming isolate of *Acinetobacter calcoaceticus* influences surface colonization by *E. coli* O157:H7. This study focused on the spatial distribution of cells during biofilm formation under static and dynamic growth conditions.

Here we used an *A. calcoaceticus* strain (MF3627) isolated from a clean and disinfected meat-processing environment, as well as Shiga toxin-negative *E. coli* O157:H7 (ATCC 43888) harboring the plasmid pGFP-uv (Clontech Laboratories, Palo Alto, CA). For static growth conditions, mono- and coculture biofilms were harvested at 25°C in Lab-Tek II chamber slide systems (VWR, Oslo, Norway) consisting of miniature polystyrene medium chambers with a sealed cover glass as the growth surface. For dynamic growth conditions, mono- and coculture biofilms were grown at 25°C in three-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm and a sealed glass coverslip substratum (Knittel Glass, Germany). A 1/10 dilution of Luria-Bertani broth was continuously pumped through the sterile flow cell channels at a rate of 0.5 ml/min. In two of the channels, *A. calcoaceticus* and *E. coli* were inoculated individually, while the third channel was reserved for the inoculation of a mixed culture of *A. calcoaceticus* and *E. coli* (1:1). The flow cell channels and Lab-Tek chambers were stained with SYTO 61. Horizontal-plane images of the biofilms were acquired using a Leica SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Lysaker, Norway). Three independent biofilm experiments were performed for each biofilm growth condition. Three-dimensional projections were performed with IMARIS software (Bitplane, Zürich, Switzerland). The structural quantification of biofilms (biovolume in cubic micrometers) was performed using the PHLIP Matlab program (http://www.phlip.org/phlip-ml/).

Under static growth conditions, *E. coli* O157:H7 formed a homogeneous flat biofilm yielding biovolumes ranging between 3.3×10^5 and 5.4×10^5 μm^3 after 24 and 72 h of biofilm growth. Although the *E. coli* biovolume revealed no significant differences in monoculture or when cocultured with *A. calcoaceticus*, microscopic analysis revealed how *E. coli* cells were gradually covered by a carpet of *A. calcoaceticus* cells after 72 h of biofilm growth (for visualization, see the supplemental material). *A. calcoaceticus* monospecies biofilms were heterogeneous, highly structured, and channeled under both static and dynamic conditions (Fig. 1A), yielding a biovolume of 1.46×10^6 μm^3 after 72 h of biofilm growth (Fig. 2). *E. coli* O157:H7 did not form monospecies biofilms under dynamic-flow conditions (Fig. 1A), with biovolume values below 3.5×10^4 μm^3 after 72 h (Fig. 2). The presence of *A. calcoaceticus* had a significant impact on *E. coli* O157:H7 surface colonization with a 400-fold increase in the total biovolume of *E. coli* O157:H7 from 3.0×10^3 μm^3 to 1.2×10^6 μm^3 between 24 and 48 h (Fig. 2), as observed from the increase in *E. coli* O157:H7 biomass between *A. calcoaceticus* cell clusters (Fig. 1A and B). After 72 h of development, *E. coli* O157:H7 cell clusters were partially covered by *A. calcoaceticus* cells. The poor settlement and subsequent poor colonization of *E. coli* O157:H7 under

* Corresponding author. Mailing address: Nofima Mat AS, Osloveien 1, NO-1430 Ås, Norway. Phone: 47 64970100. Fax: 47 64970333. E-mail: olivier.habimana@nofima.no.

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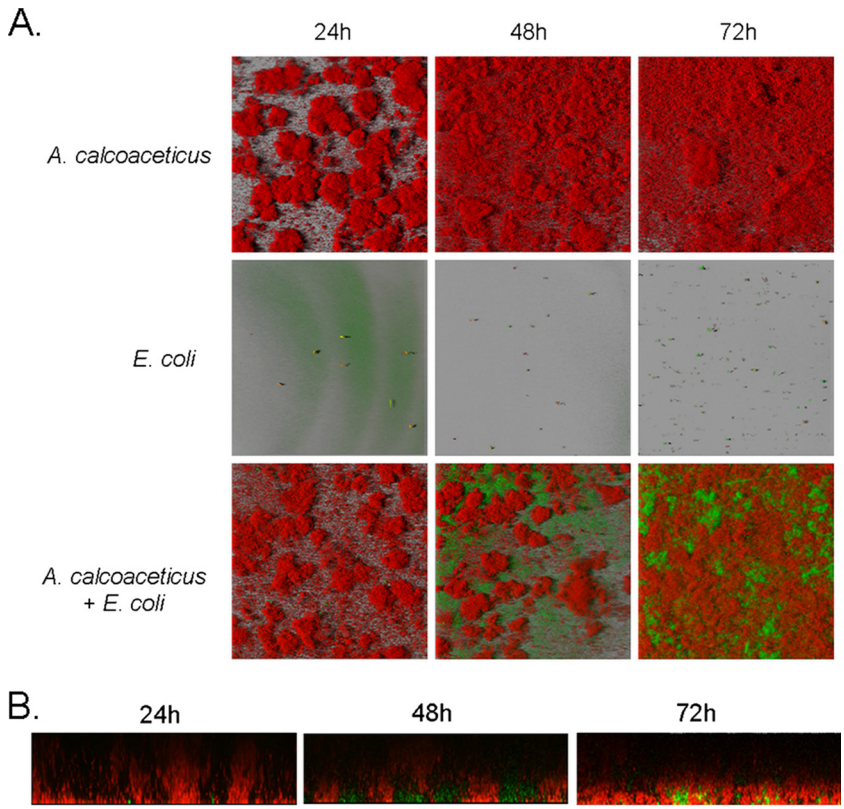

FIG. 1. Structural development of *A. calcoaceticus* and *E. coli* in mono- and dual-species biofilms under dynamic conditions. (A) Representative biofilms of *A. calcoaceticus* and pGFP-uv-tagged *E. coli* O157:H7 grown in flow cells using Luria-Bertani broth as a growth medium at 25°C. The spatial structures in the developing biofilms were studied by laser scanning confocal microscopy. (B) Vertical sections (in the *x-z* plane) representing the spatial distribution of pGFP-uv-tagged *E. coli* O157:H7 in the presence of *A. calcoaceticus* under dynamic-flow conditions after 24, 48, and 72 h of growth. The lower side of each section corresponds to the substratum. Green cells represent pGFP-uv-tagged *E. coli* O157:H7, red cells represent SYTO 61-stained *A. calcoaceticus* cells, and yellow cells represent GFP-tagged *E. coli* O157:H7 marked with SYTO 61.

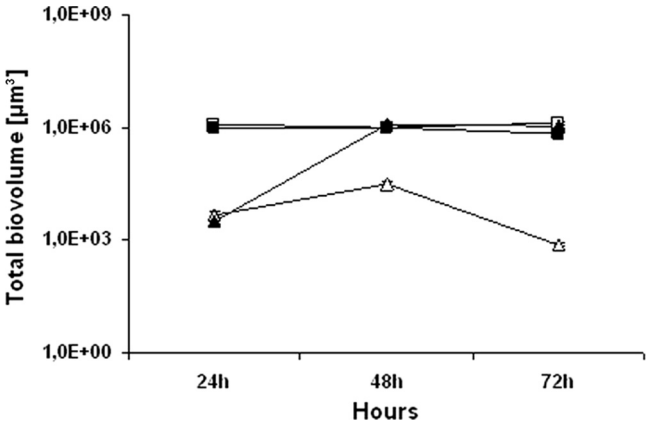

FIG. 2. Biovolume of *A. calcoaceticus* and *E. coli* O157:H7 biofilm development after 24, 48, and 72 h of growth under dynamic conditions. *A. calcoaceticus* in monospecies biofilms is represented by the symbol □, *A. calcoaceticus* in dual-species biofilms is represented by the symbol ■, *E. coli* O157:H7 in mono-species biofilms is represented by the symbol Δ, and *E. coli* O157:H7 in dual-species biofilms is represented by the symbol ▲. Mean values of at least 30 individual images ± the standard errors from three independent experiments are shown.

dynamic-flow conditions could have been attributed to shear forces, which made it difficult for *E. coli* O157:H7 cells to establish colonies on the substratum. The observed spatial distribution of *A. calcoaceticus* cells at the liquid-biofilm interface may offer *E. coli* O157:H7 cells better protection from shear stress and could potentially provide additional protection against disinfectants, as has been observed in other multispecies biofilm studies (2, 21). Whether *E. coli* cells had increased resistance to antimicrobial agents in our experimental setup as a result of being at the bottom layers of mixed-species biofilms will be the subject of further investigations. Biofilm formation of meat industry surface bacteria can enhance *E. coli* surface colonization and thereby increase the risk of persistence of and food contamination by potential pathogens. The occurrence of *Acinetobacter* in food-processing environments is well documented (1, 9, 15), and it has also been isolated from spoiled food products (3, 6, 7). Furthermore, a recent study showed that *A. calcoaceticus* biofilms are able to interact and coaggregate with other bacteria (19). Cleaning and disinfection procedures used in food industries should thus take into account the risks involved in ignoring the presence of resident flora biofilms.

Cleaning and disinfection procedures are employed by the food industry to ensure clean and hygienic surfaces for food

production. However, due to the ubiquitous nature of biofilms and their potential to resist antimicrobial treatments (21), new strategies based on preventive actions to reduce the incidence of biofilm formation on food-processing surfaces should be employed (20). In light of the results obtained in this study, combining curative actions with preventive actions based on the use of surface materials with antiadhesive or antifouling surfaces could enhance the hygienic standards of food-processing surfaces.

In conclusion, we have shown that under both static and dynamic growth conditions, *E. coli* cells were found embedded and covered by *A. calcoaceticus* cells in mixed-species biofilms. Moreover, the presence of an *A. calcoaceticus* biofilm structure favored *E. coli* O157:H7 colonization and biofilm formation under dynamic-flow conditions. These results offer new insights into the spatial distribution of pathogenic bacteria and resident flora during cocultured biofilm formation. Conditions allowing active biofilm formation of resident microflora may provide increased opportunities for pathogens to thrive in food-processing environments. The hazardous influences of resident biofilms should therefore not be ignored, since improper cleaning procedures in food-processing environments could potentially increase the risk of food contamination by spoilage and pathogenic bacteria.

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