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Biofilm Formation by *Salmonella enterica* Serovar Typhimurium and *Escherichia coli* on Epithelial Cells following Mixed Inoculations

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Biofilms were formed by inoculations of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* on HEp-2 cells. Inoculations of *S. enterica* serovar Typhimurium and *E. coli* resulted in the formation of an extensive biofilm of *S. enterica* serovar Typhimurium. In experiments where an *E. coli* biofilm was first formed followed by challenge with *S. enterica* serovar Typhimurium, there was significant biofilm formation by *S. enterica* serovar Typhimurium. The results of this study indicate that *S. enterica* serovar Typhimurium can outgrow *E. coli* in heterologous infections and displace *E. coli* when it forms a biofilm on HEp-2 cells.

*Salmonella enterica* serovar Typhimurium is a frequent cause of gastroenteritis in humans. It is estimated that every year, in the United States alone, there are 2 to 4 million cases of salmonellosis, resulting in a loss of over $2 billion to the economy (13). The bacteria are usually acquired by ingestion of contaminated food, commonly beef, poultry, or eggs (9).

Salmonellosis is associated with the small intestine, although some studies report colonic involvement during the acute phases of the disease (7, 8, 12). Both the distal small intestine and the large intestine have diverse, abundant normal flora populations that provide a barrier to infection by pathogens. Large numbers of *S. enterica* serovar Typhimurium, 10⁷ to 10⁸ organisms, are required to cause clinical illness in humans, and in order to colonize the intestine, *S. enterica* serovar Typhimurium has to compete with the natural flora (13). Indeed, both in humans and in mice, it has been shown that treatment with antibiotics, which reduces the natural flora of the intestine, resulted in higher susceptibility to salmonellosis (2, 11). Therefore, in order to establish infection, *S. enterica* serovar Typhimurium has to effectively compete with the resident bacteria during the process of attachment to the intestinal epithelial cells and subsequent growth on those cells.

In some instances, biofilm formation seems to be associated with the ability to cause disease, and it has been suggested that biofilms play a role in the pathogenesis of numerous bacterial species (4, 5, 14). The most abundant gram-negative facultative anaerobe in the colon is *Escherichia coli*. In an effort to establish a system for studying the interactions of pathogens with normal flora organisms, we describe herein investigations to establish a system for studying the interactions of pathogens with normal flora organisms, we describe herein investigations to assess the ability of *S. enterica* serovar Typhimurium to compete with the resident *E. coli* during colonization of and growth on epithelial cells in biofilm development.

In the present work, we examined the competition between *S. enterica* serovar Typhimurium BJ2710, UK-1, and 986 and the gastrointestinal *E. coli* isolates 3.14 and IA52 while forming biofilms on the HEp-2 epithelial cells. For this purpose a flowthrough continuous culture system was used, as described previously by our group (1). The use of this system has the advantage of allowing the biofilm to form in a dynamic rather than static environment that can be directly visualized by confocal scanning laser microscopy (CSLM). To the best of our knowledge, the present study is the first report on mixed bacterial biofilm formation, using this system, by *S. enterica* serovar Typhimurium and *E. coli* on eukaryotic cells.

**Growth and biofilm formation by *S. enterica* serovar Typhimurium and *E. coli* strains on HEp-2 cells.** The *S. enterica* serovar Typhimurium strains used in this study were BJ2710, UK-1 (6), and 986. *S. enterica* serovar Typhimurium BJ2710 is a derivative of strain SL1344 (15) possessing the fimH gene isolated from the LT2 fim gene cluster that mediates binding to HEp-2 cells (1). The *E. coli* strains 3.14 and IA52 were both isolated from the normal flora of the gastrointestinal tract. Growth and biofilm formation by *S. enterica* serovar Typhimurium and *E. coli* strains on HEp-2 cells were assayed using a flowthrough continuous culture system that has the advantage of allowing the biofilm to form in a dynamic rather than static environment, where bacteria grow attached to eukaryotic cells, compared to the static conditions of batch cultures.

In these experiments, HEp-2 cells were grown as a confluent layer attached to a glass coverslip in the flow chambers and were then inoculated with bacteria. *S. enterica* serovar Typhimurium and *E. coli* were transformed with plasmids expressing green fluorescent protein (GFP) and red fluorescent protein (RFP). The flow chambers used allowed the direct visualization of bacterial growth on HEp-2 cells by CSLM, thus minimizing any disturbance of the biofilm formed.

Initially, we investigated the ability of *S. enterica* serovar Typhimurium BJ2710 or *E. coli* 3.14 alone to grow and form biofilms on the HEp-2 cells over a 48-h period. When inoculated alone, either *S. enterica* serovar Typhimurium BJ2710 or *E. coli* 3.14 grew and formed extensive biofilms on HEp-2 cells, and by 12 h of incubation, each bacterial strain had already established a biofilm on the HEp-2 cells (Fig. 1A and B). *S. enterica* serovar Typhimurium UK-1 and 986 and *E. coli* IA52 were also grown on HEp-2 cells in the flow chambers, and after 24 h incubation of monoinoculated chambers, each strain formed an extensive biofilm on the HEp-2 cells (data not shown). We have previously demonstrated that *S. enterica* se-
S. enterica serovar Typhimurium efficiently colonizes and grows on HEp-2 cells using this system (1).

In another series of experiments, S. enterica serovar Typhimurium BJ2710 and E. coli 3.14 were individually inoculated in chambers not containing HEp-2 cells and with glass coverslips as the substrate for bacterial attachment. By 12 h of incubation, no growth was detected for either S. enterica serovar Typhimurium BJ2710 or E. coli 3.14, and after 24 h of incubation a limited patchy growth on the coverslips was observed for S. enterica serovar Typhimurium BJ2710. No growth was observed in the chambers inoculated with E. coli 3.14. Consequently, S. enterica serovar Typhimurium BJ2710 and E. coli 3.14 were unable to form a biofilm when cultured on the glass coverslips of the chambers not possessing HEp-2 cells. Thus, the tissue culture cells are necessary as a substrate in the chambers to support bacterial biofilm growth and were used in all subsequent experiments.

In the experiments described above, the HEp-2 cells were not stained but cell confluence was confirmed using the transmitted light mode of the microscope. However, in preliminary assays, biofilms of S. enterica serovar Typhimurium BJ2710 and E. coli 3.14 were allowed to form on HEp-2 cells prestained with a fluorescent probe requiring cell viability. In those experiments, all bacteria were labeled with GFP. Both S. enterica serovar Typhimurium BJ2710 and E. coli 3.14 formed extensive biofilms on HEp-2 cells either in the presence or absence of the cell tracker probe. In all subsequent experiments, therefore, the use of cell tracker probe was discontinued to avoid cross fluorescence signals between the probe and RFP expressed by E. coli strains.

Bacterial biofilms were formed on HEp-2 epithelial cells, a cell line previously used as the substratum in studies of biofilm formation by enteric bacteria (1). In a second series of experiments, the intestinal epithelial cell line Int 407 was used to coat the flowthrough chambers. However, Int 407 cells attached poorly to the glass coverslips of the flow chamber and were, therefore, not used in subsequent experiments.

**Effect of mixed bacterial inoculation on biofilm formation by S. enterica serovar Typhimurium and E. coli strains on HEp-2 cells.** Since S. enterica serovar Typhimurium has to compete with host flora, including E. coli, during intestinal colonization, we investigated the ability of S. enterica serovar Typhimurium and E. coli to form biofilms on HEp-2 cells using mixed inoculations, to begin to investigate the interactions between these two organisms.

Equal numbers (≈5 × 10⁸ CFU/ml) of S. enterica serovar Typhimurium BJ2710 and E. coli 3.14 were used in a mixture to inoculate the biofilm chambers. After 24 h of inoculation, S. enterica serovar Typhimurium BJ2710 was able to establish a biofilm on the HEp-2 cells, whereas only very limited attachment and growth of E. coli 3.14 was observed (Fig. 2). In addition to visualization of fluorescent bacteria, viable counts of harvested bacteria from the chambers were obtained by plating on differential MacConkey agar, and those counts consistently indicated that significantly higher numbers of S. enterica serovar Typhimurium BJ2710 than E. coli 3.14 were growing on the cells in the chambers (Table 1).

Under these experimental conditions, E. coli 3.14 was not able to develop a biofilm in the presence of S. enterica serovar Typhimurium BJ2710. This was not due to the inability of the E. coli strain to form a biofilm on the HEp-2 cells since, as demonstrated earlier, chambers inoculated with only E. coli 3.14 were rapidly colonized and an extensive biofilm was observed. In additional experiments, the number of S. enterica serovar Typhimurium BJ2710 was decreased so that inoculation ratios of 1:10 and 1:1,000 (S. enterica serovar Typhimurium BJ2710: E. coli 3.14) were used to investigate the interactions between these two organisms.
um: *E. coli*) were tested. At the inoculation ratio of 1:10, both bacterial species showed considerable growth when analyzed by microscopy (data not shown), and the numbers of *S. enterica* serovar Typhimurium BJ2710 were slightly higher than those observed for *E. coli* 3.14 growing on the cells (Table 1). At a ratio of 1:1,000 (*S. enterica* serovar Typhimurium:*E. coli*) *E. coli* 3.14 outgrew *S. enterica* serovar Typhimurium BJ2710, although confocal micrographs indicated that the *S. enterica* serovar Typhimurium strain could still form small patches of growth on the HEp-2 cells. Therefore, even when the inoculum consisted of higher numbers of *E. coli* 3.14 (1:1,000, *S. enterica* serovar Typhimurium:*E. coli*), *S. enterica* serovar Typhimurium BJ2710 could establish growth on limited areas of the HEp-2 cells (data not shown). However, when equal numbers of *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 were present in the inoculum, the growth of *E. coli* 3.14 was significantly reduced on the HEp-2 cells.

In another set of experiments *S. enterica* serovar Typhi-

![FIG. 2. Biofilm produced by a coinoculum of *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 containing equal numbers of each bacterial species. *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 expressed GFP and RFP, respectively. The CSLM composite images were obtained 24 h after bacterial incubation in the flowthrough chambers coated with HEp-2 cells.](image)

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Viable counts</th>
<th>Batch culture</th>
<th>Flow chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>1:1</td>
<td>n.d.</td>
<td>79:1</td>
</tr>
<tr>
<td>1:10</td>
<td>n.d.</td>
<td>700:1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Values are expressed as a proportion of CFU/ml of *S. enterica*: *E. coli* and are the average of three experiments. Viable counts were determined after 24 h of incubation at 37°C. n.d., Not determined.
formed experiments in which *S. enterica* serovar Typhimurium BJ2710 was inoculated to a chamber possessing a preformed *E. coli* 3.14 biofilm.

In this series of experiments, the *E. coli* strain was cultured in the biofilm chambers on the HEp-2 cells for 12 h prior to inoculation with *S. enterica* serovar Typhimurium BJ2710. Under these conditions, an extensive *E. coli* 3.14 biofilm is present (Fig. 4A). The biofilms were subsequently monitored immediately after *S. enterica* serovar Typhimurium BJ2710 inoculation and then 4, 24, and 36 h following *S. enterica* serovar Typhimurium BJ2710 inoculation. By 4 h of *S. enterica* serovar Typhimurium BJ2710 inoculation on the *E. coli* 3.14 biofilm, incipient and random *S. enterica* serovar Typhimurium BJ2710 growth was already detectable by CSLM. As shown in Fig. 4A, *S. enterica* serovar Typhimurium BJ2710 was able to establish itself in the presence of an *E. coli* 3.14 biofilm. Similar results were obtained with plasmid-free bacteria, in which plate counts of the harvested bacteria from the chambers confirmed the ability of *S. enterica* serovar Typhimurium BJ2710 to grow on a fully developed *E. coli* 3.14 biofilm (Table 2).

In additional experiments, *S. enterica* serovar Typhimurium BJ2710 was inoculated on an *E. coli* 3.14 biofilm that had developed for 24 h, and the biofilm was then monitored 12 and 24 h after *S. enterica* serovar Typhimurium inoculation (data not shown). These results were similar to those obtained when *S. enterica* serovar Typhimurium BJ2710 was inoculated on an *E. coli* 3.14 biofilm grown for 12 h.

Next we tested the growth of *E. coli* 3.14 on an established *S. enterica* serovar Typhimurium BJ2710 biofilm of 12 h (Fig. 4B). The biofilm was observed at 0, 4, 24, and 36 h following *E.
coli 3.14 inoculation, and the results showed no evident growth of the E. coli strain and an extensive S. enterica serovar Typhimurium BJ2710 biofilm. These results were confirmed by bacterial plate counts of biofilms formed with plasmid-free bacteria, which indicated S. enterica serovar Typhimurium BJ2710 was present in significantly higher numbers than E. coli 3.14 (Table 2). These results again suggested that in the presence of E. coli 3.14, the S. enterica serovar Typhimurium strain could effectively compete with E. coli 3.14. Furthermore, if S. enterica serovar Typhimurium BJ2710 is established on the HEp-2 cells, the E. coli 3.14 strain is a poor colonizer of the surfaces.

There are indications that multispecies biofilm development depends on the bacterial species involved, the surface composition, and the sequence of attachment (10). Several factors may contribute to the ability of S. enterica serovar Typhimurium to grow and establish a niche on an E. coli biofilm. In the beginning of infection, S. enterica serovar Typhimurium may have the ability to simply displace E. coli or attach to locations not fully occupied by the E. coli biofilm on the HEp-2 cells. We hypothesize that S. enterica serovar Typhimurium adheres to the HEp-2 cells and increases in number, displacing E. coli and resulting in partial replacement of the E. coli biofilm by an S. enterica serovar Typhimurium biofilm. A similar phenomenon may occur when S. enterica serovar Typhimurium colonizes a host intestinal tract prior to causing infection.

We thank J. Jagnow for help in establishing the biofilm chambers. During this research C. L. C. Esteves was supported by Fundação para a Ciência e a Tecnologia, Portugal. The research was supported, in part, by grant AI50011 from the NIH to S. Clegg.

REFERENCES

TABLE 2. Growth of one bacterial species on an established biofilm of the other bacterium in flowthrough chambers coated with HEp-2 cells

<table>
<thead>
<tr>
<th>Sequential bacterial inoculation</th>
<th>Bacteria harvested from chambers</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 3.14 followed by S. enterica BJ2710</td>
<td>0.4:1*</td>
</tr>
<tr>
<td>S. enterica BJ2710 followed by E. coli 3.14</td>
<td>525:1*</td>
</tr>
</tbody>
</table>

* Values are expressed as a proportion of CFU/ml of S. enterica:E. coli and are the average of three experiments. *, means are different (P < 0.005). Student's t test was used for comparison between means.


