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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, 10^7 to 10^9 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 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 microcopy (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 natural 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 over batch cultures of providing a dynamic 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-

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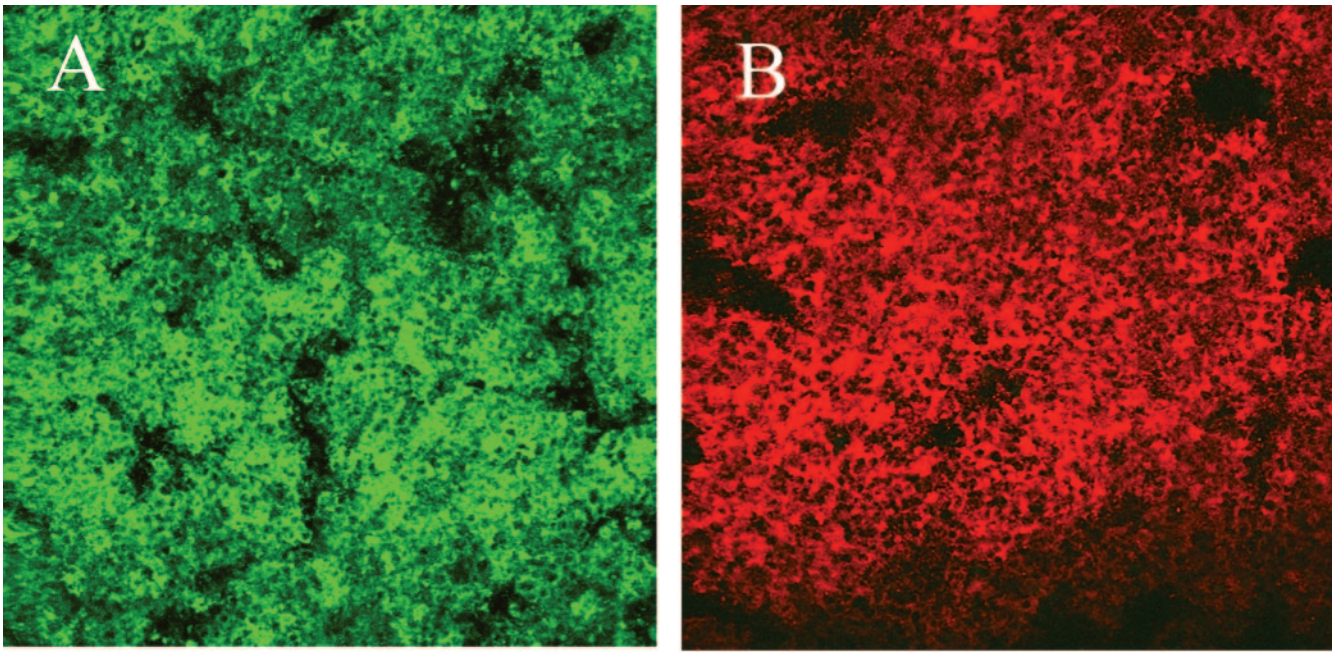

FIG. 1. Biofilms produced by *S. enterica* serovar Typhimurium BJ2710 (A) and *E. coli* 3.14 (B) expressing GFP and RFP, respectively. The CSLM composite images were obtained 12 h after bacterial incubation in the flowthrough chambers coated with HEp-2 cells.

rovar 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 inoculation, 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 ($\approx 5 \times 10^8$ 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 Typhimuri-

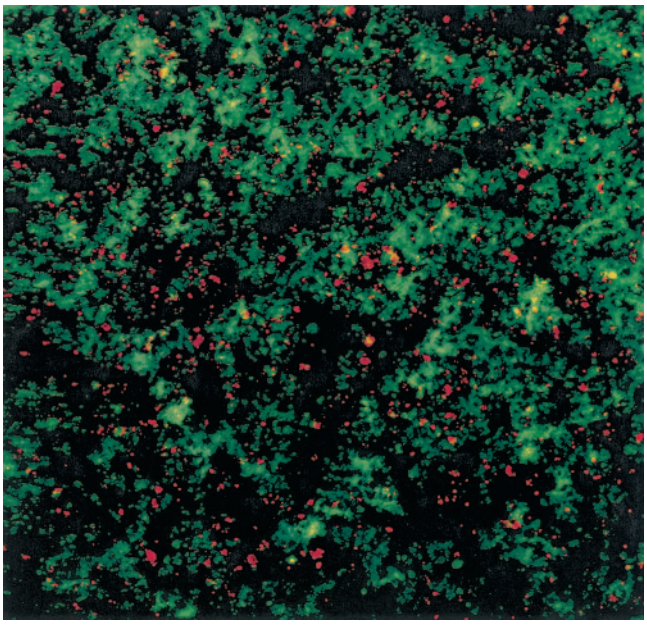

FIG. 2. Biofilm produced by a coinoculum of *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 containing equal numbers of each bacterial species. *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 expressed GFP and RFP, respectively. The CSLM composite images were obtained 24 h after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

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 Typhimurium BJ2710 and UK-1 and *E. coli* 3.14 and IA52 were dual-inoculated in the ratio 1:1 (*S. enterica* serovar Typhimurium:*E. coli*) on the HEp-2 cells. The flowthrough chambers were analyzed by CSLM 24 h after inoculation, and in all assays *S. enterica* serovar Typhimurium formed extensive biofilm, whereas *E. coli* growth was very limited and scattered (Fig. 3). These results demonstrate that, when equal amounts of each bacterium was used in the assay, the outgrowth of *S. enterica* serovar Typhimurium over *E. coli* was strain independent.

TABLE 1. Coinoculation of *S. enterica* Serovar Typhimurium BJ2710 and *E. coli* 3.14 in batch culture and in flowthrough chambers coated with HEp-2 cells[a]

Inoculum	Viable counts	
	Batch culture	Flow chamber
1:1	1:1	700:1
1:10	n.d.	7.9:1

[a] Values are expressed as a proportion of CFU/ml of *S. entericaz*:*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.

In the biofilm experiments described above, both *S. enterica* serovar Typhimurium and *E. coli* strains were transformed with plasmids encoding fluorescent proteins so that the bacterial strains could be visualized by CSLM. These plasmids also conferred resistance to ampicillin, and to maintain the transformants in the biofilm assays, this antibiotic was used in the flow medium. When *S. enterica* serovar Typhimurium interacts with *E. coli* in the intestine, the bacteria are not normally exposed to antibiotics. Therefore, the mixed-inoculum experiments were repeated using the plasmidless *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 in the absence of antibiotic, and viable plate counts alone were used to determine the number of bacteria growing on the HEp-2 cells. The results, expressed as the proportion of CFU/ml of *S. enterica* serovar Typhimurium to *E. coli*, were 770:1 and were consistent with the fluorescence experiments, as *S. enterica* serovar Typhimurium BJ2710 significantly outcompeted *E. coli* 3.14, confirming that *S. enterica* serovar Typhimurium BJ2710 could effectively compete with and outgrow *E. coli* 3.14 on epithelial surfaces in mixed inoculations.

In order to demonstrate that the results obtained using mixed inoculations were not simply due to the ability of *S. enterica* serovar Typhimurium to replicate more rapidly than *E. coli*, we grew *S. enterica* serovar Typhimurium and *E. coli* strains in batch culture in the same medium as used in the biofilm assays. After 24 h of incubation in RPMI broth, viable counts of *S. enterica* serovar Typhimurium and *E. coli* were obtained by plating on MacConkey agar to differentiate between the two species. The numbers of *S. enterica* serovar Typhimurium and *E. coli* in mixed cultures were equivalent, and Table 1 shows the results for *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14. In these assays, the *S. enterica* serovar Typhimurium strains displayed the same growth rate as the *E. coli* strains. The ability of *S. enterica* serovar Typhimurium to outgrow *E. coli* during biofilm formation on HEp-2 cells may be due to several factors involving the attachment and subsequent growth by each bacterial species. However, the molecular mechanism(s) of this observed phenomenon has yet to be elucidated.

Growth and biofilm formation by *S. enterica* serovar Typhimurium BJ2710 on an established *E. coli* 3.14 biofilm on HEp-2 cells. When *S. enterica* serovar Typhimurium reaches the intestine after being ingested with contaminated food, it encounters an established natural flora. Under these conditions, *S. enterica* serovar Typhimurium would have to establish itself in the presence of the resident *E. coli* in order to colonize the intestine. In the experiments presented above, *S. enterica* serovar Typhimurium and *E. coli* strains were mixed in the inoculum and then allowed to compete to form a biofilm on the HEp-2 cells. To further explore the interaction of those two bacterial species growing on HEp-2 cells in vitro, we per-

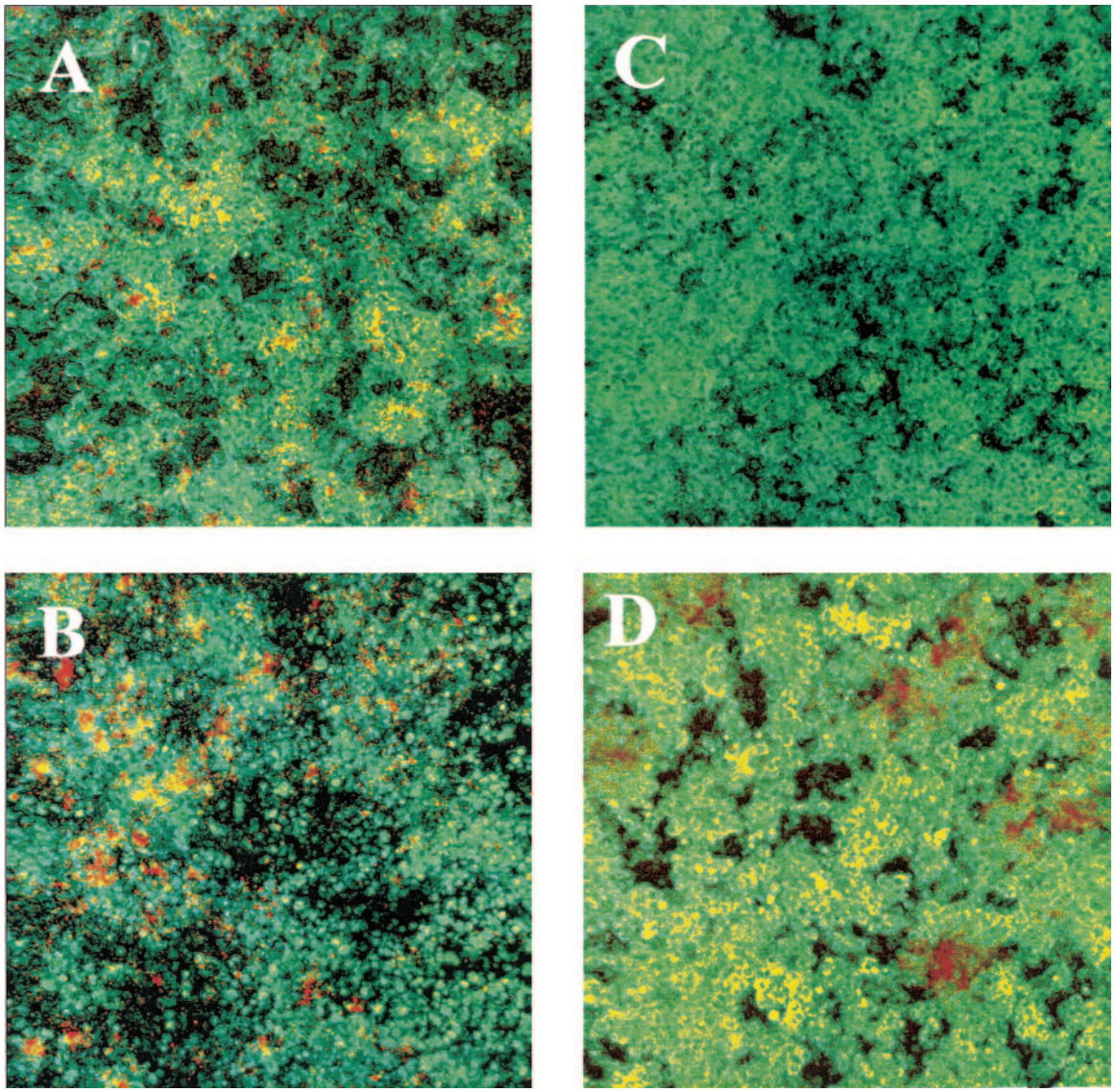

FIG. 3. Biofilms produced by a coinoculum of *S. enterica* serovar Typhimurium and *E. coli* strains containing equal numbers of each bacterial species and expressing GFP and RFP, respectively. The CSLM composite images show the growth of *S. enterica* serovar Typhimurium UK-1 and *E. coli* 3.14 (A), *S. enterica* serovar Typhimurium 986 and *E. coli* 3.14 (B), *S. enterica* serovar Typhimurium BJ2710 and *E. coli* IA52 (C), and *S. enterica* serovar Typhimurium UK-1 and *E. coli* IA52 (D) 24 h after bacterial incubation in the flowthrough chambers coated with HEp-2 cells.

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.*

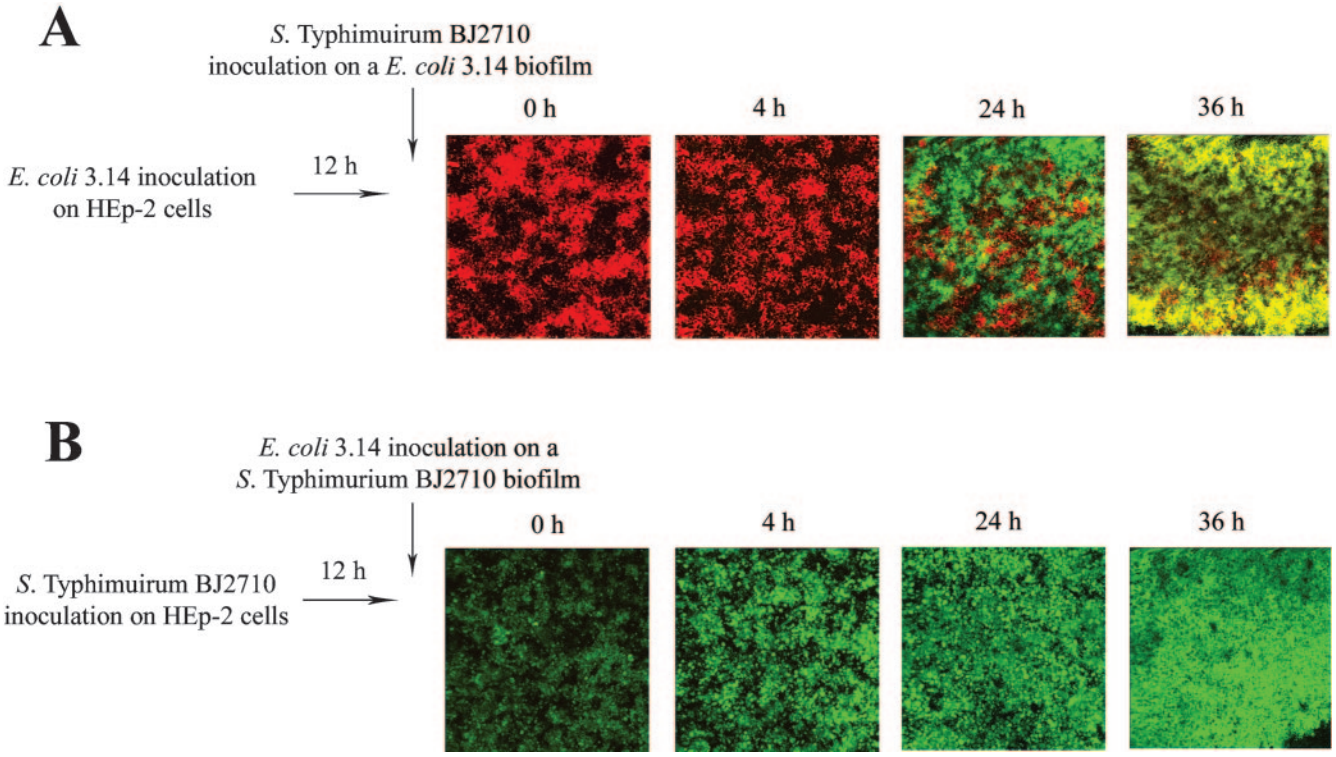

FIG. 4. Growth of *S. enterica* serovar Typhimurium BJ2710 on a preformed *E. coli* 3.14 biofilm (A) and growth of *E. coli* 3.14 on a preformed *S. enterica* serovar Typhimurium BJ2710 biofilm (B). Flowthrough chambers coated with HEp-2 cells were inoculated with one of the bacterial species for 12 h, followed by inoculation of the other bacterial species for 36 h. The biofilms were monitored by CSLM immediately after the second bacterial inoculation and then at 4, 24, and 36 h following the second bacterial inoculation. *S. enterica* serovar Typhimurium BJ2710 and *E. coli* 3.14 expressed GFP and RFP, respectively.

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.

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

Sequential bacterial inoculation	Bacteria harvested from chambers
E. coli 3.14 followed by *S. enterica* BJ2710	0.4:1*
S. enterica BJ2710 followed by *E. coli* 3.14	525:1*

[a] 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.

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