EFFECT OF *relA* AND *spoT* DELETIONS ON *Escherichia coli* BIOFILM FORMATION

THESIS

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ABSTRACT

Bacteria predominantly grow as surface adherent biofilm communities composed of high cell density aggregates (microcolonies). Within microcolonies, gradients of nutrients and growth rates exist from the outer to inner cells due to diffusion and nutrient uptake. Therefore, in order to grow as a biofilm, cells must possess the capacity to survive under conditions of nutrient depletion. The stringent response is one starvation-survival mechanism that regulates gene expression at the level of RNA transcription. Two major genes whose products control the stringent response in *Escherichia coli* are *relA* and *spoT*. To understand the relationship between the stringent response and biofilm formation, two *E. coli* K-12 derivatives, DS 291 and its isogenic *relA*$^-$ *spoT*$^-$ derivative, DS 293, were grown as biofilms using a chemostat coupled to a modified Robbins device (MRD) containing plugs of silicone rubber catheter material. The strains were grown at two dilution rates (0.025 h^{-1} and 0.25 h^{-1}) in a MOPS minimal medium with serine-limitation. At the low dilution rate, a significant decrease ($P < 0.05$) was noted with the *relA*$^-$ *spoT*$^-$ strain only. When evaluating biofilm density as an adherence ratio, significant differences were noted at both dilution rates. Examination of biofilms by scanning confocal laser microscopy illustrated qualitative differences in biofilm structure in the wild type and *relA*$^-$ *spoT*$^-$ strains. This study indicates that the stringent response is important for *E. coli* biofilm growth.

INTRODUCTION

Bacteria reside predominately as surface-adherent biofilms, a phenomenon observed widely in medicine, industry, and the environment (Costerton et al., 1987). Biofilms form at solid-liquid interfaces. On medical devices such as catheters and prostheses they cause persistent infections (Costerton et al., 1999). In industrial settings they can cause biofouling and clogging (Costerton, 1995). Alternatively, they can be beneficial in mediating pollutant removal (White et al., 2000). Mature biofilm development occurs through a process of primary cell attachment, cell division, microcolony formation, and exopolysaccharide production ultimately leading to the celebrated mushroom/tower formation. These mature biofilms are interspersed with water channels to allow the flow of nutrients and removal of metabolic waste products (Lawrence et al., 1991).

Due to differences in environmental surroundings, bacteria present in biofilms are phenotypically different than those in the planktonic culture. Within a biofilm, bacteria are able to express genes enabling them to become resistant to hostile physical and chemical environments or nutrient depleted conditions (Watnick and Kolter, 2000). Most often, biofilms form in a situation where nutrients are scarce or intermittent. As a result of inadequate nutrients, there exists a gradient of growth rates within the individual microcolonies (Sternberg et al., 1999). Cells at the bulk liquid-biofilm interface are more metabolically active than

those inside the microcolonies. This gradient can be caused by factors such as oxygen and pH, but mainly because of nutrient depletion by other members of the microcolony and production of biopolymers that impede diffusion of nutrients (de Beer et al., 1994). Much work remains to be done on the study of bacterial physiology and gene expression within biofilms. A review of the stringent response follows.

To survive nutritional stresses such as those in a biofilm, bacteria possess several mechanisms. One such mechanism is the stringent response, which is a global regulator of cellular metabolism. The main component of the stringent response is to shut down stable RNA synthesis (tRNA and rRNA) when nutrients such as, amino acids, ammonia, carbon, phosphate, and nitrogen are depleted (Cashel et al., 1996). Once the stringent response has been activated, there are profound effects on the metabolic activity of the cell. These effects include the inhibition of RNA synthesis, protein synthesis, nucleotide metabolism, transport, phospholipid metabolism, peptidoglycan synthesis, and DNA synthesis. In addition, other cellular functions such as, amino acid metabolism, DNA binding proteins, and carbohydrate metabolism are stimulated (Cashel et al., 1996).

The stringent response begins by the production of the transcriptional regulator (p)ppGpp (guanosine 5'-triphosphate-3'-diphosphate and guanosine 5'-diphosphate-3'-diphosphate) when high uncharged/charged tRNA ratios appear (Rojiani et al., 1989). The signal

transducer molecule (p)ppGpp binds with RNA polymerase, which inhibits rRNA and tRNA transcription (Chaloner-Larsson and Yamazaki, 1978). Basically, the effector molecule (p)ppGpp, is the sole regulator for stable RNA gene control through binding to RNA polymerase and changing the promoter selectivity from a high affinity for stable RNA promoters to that of mRNA promoters (Ryals et al., 1982).

Principally, the products of the two genes *relA* and *spoT* are responsible for the stringent response. RelA controls the production of (p)ppGpp under amino acid starved conditions, and SpoT, a bifunctional enzyme possessing both hydrolase and transferase activity, controls the production accumulation by regulating degradation and production during carbon starvation (Xiao et al., 1991). In *relA*$^-$ cells, (p)ppGpp fails to accumulate during aminoacyl-tRNA limitation, and cells with *spoT*$^-$ deletions have been found to have several defects in (p)ppGpp metabolism. Examples of altered physiology include increased basal (p)ppGpp levels at balanced and slow growth rates, higher (p)ppGpp levels during the stringent response, slower turnover of (p)ppGpp when the stringent response is reversed, and the inability to accumulate pppGpp during the stringent response (Cashel et al., 1996).

In stringent wild type cells, (p)ppGpp normally increases during low nutrient conditions leading to an increase in the expression of *rpoS* (Gentry et al., 1993), which is a gene whose expression is induced by slow growth or the onset of stationary phase (Hengge-Aronis, 1993).

RpoS is an alternative sigma factor associated with cell survival during the stationary phase (Lange and Hengge-Aronis, 1991 and McCann et al., 1991). Sigma factor S is responsible for the transcription of a number of genes related to resistance, cell envelope, membrane composition, DNA super coiling, and storage molecules (Hengge-Aronis, 1993). Overall, sigma S is responsible for controlling more than 30 genes or operons involved in starvation and stationary phase (Hengge-Aronis, 1996). However, when *rpoS*$^-$ cells are grown under nutrient limited conditions, the expression of these slow growth genes does not increase (McCann et al., 1991). In addition, when *relA* and *spoT* are deleted, cells loose the ability to produce (p)ppGpp, and *rpoS* expression is reduced from that of wild type cells (Gentry et al., 1993).

In addition, recent studies in this lab, an *rpoS* mutant was found to form reduced biofilm growth under nutrient limited conditions (Adams and McLean, 1999). Based on conclusions from the reviews above an experimental hypothesis can be established. As nutrients are depleted in the biofilm interior, the potential for cell survival within biofilms may be reduced when the stringent response is absent (*relA*$^-$ *spoT*$^-$). This study will lead to a further understanding of starvation in biofilms, and if the starvation response is necessary for biofilm formation.

MATERIALS AND METHODS

Strains.

Two isogenic *E. coli* K-12 strains used in this study were provided by D. A. Siegele at Texas A&M University and are listed in Table 1.

Media.

Both strains were grown in a potassium morpholinopropane sulfonate (MOPS) buffered minimal medium with serine as a limited carbon source and the addition of amino acids required by amino acid auxotrophic strains. For amino acid requirements refer to Xiao et al. (1991). The media is prepared as follows: (I) prepare 1 liter 10X concentrate by mixing the following solutions in the given order to prevent precipitation of various salts: MOPS, freshly prepared, 1.0 M, at pH 7.4 using KOH (400 ml); *N*-Tris(hydroxymethyl)-methyl glycine (Tricine), freshly prepared, 1.0 M at pH 7.4 using KOH (40 ml); $FeSO_4$, freshly prepared, 0.01 M (10 ml); NH_4Cl, 1.90 M (50 ml); K_2SO_4, 0.276 M (10 ml); $CaCl_2$, 5.0×10^{-4} M (10 ml); $MgCl_2$, 0.528 M (10 ml); NaCl, 5.0 M (100 ml); micronutrients [a solution containing $(NH_4)_6(MO_7)_{24}$, 3.0×10^{-6} M; H_3BO_3, 4.0×10^{-4} M; $CoCl_2$, 3.0×10^{-5} M; $CuSO_4$, 1.0×10^{-5} M; $MnCl_2$, 8.0×10^{-5} M; $ZnSO_4$, 1.0×10^{-5} M] (10 ml); and glass distilled water (360 ml). Total volume is 1000 ml. (II) Filter sterilize this solution. This medium lacks a carbon source, phosphate source, and the nutrients for the amino acid auxotrophs. (III) For each liter of MOPS medium,

aseptically add 10X MOPS concentrate (100 ml); the carbon source, serine, (1 mg/ml); uridine, (0.01 mg/ml); required amino acids [Ile, Arg, Gly, His, Leu, Met, Phe, Thr], (each at 0.04 mg/ml). The final pH of the medium is approximately 7.2. This recipe was adapted from Neidhardt et al. (1974).

Serine Limitation.

The serine concentration was determined to be limiting for both strains. The limitation was determined by inoculating a series of tubes with serially diluted serine plus the remaining medium components. Growth was measured (OD_{600}) after 24h growth with agitation at 37 °C. See Figure 1. Based on these results a serine concentration of 1 mg/ml was chosen for experimentation.

Biofilm Cultivation.

Bacterial strains were cultured in serine-limited, minimal media in a chemostat apparatus allowing continuous growth (Whiteley et al., 1997b) (Figure 2). The organisms were taken from a subculture on MOPS agar with nutrients identical to the chemostat medium and inoculated into the aerated chemostat resting in a 37° C water bath. The two strains (Table 1) were each grown in the chemostat at dilution rates (D) of $0.025h^{-1}$ and $0.25h^{-1}$. The cultures were allowed to equilibrate for one full generation time (40 hours at D = $0.025h^{-1}$ and 4 hours at D = $0.25h^{-1}$). At that time, a modified Robbins device (MRD) (Nickel et al.,

1985) was connected to the chemostat and the culture was recirculated using a peristaltic pump at a flow rate of 100 ml/hr, biofilms were allowed to develop over a 48h period.

Sampling Methods.

Each of the 25 ports on the MRD held a 0.7 cm^2 diameter silicone rubber disc. At sampling, five discs from across the device were aseptically removed and individually suspended in 2 ml of phosphate buffered saline (PBS). In order to disrupt the microcolonies and separate individual cells, the samples were bath sonicated (Sonicor Instrument Corporation, Copiague, New York) for 5 minutes and vortexed for 2 minutes. The sonication time of 5 minutes was found to separate the greatest number of cells while allowing the greatest viability (Barnes, unpublished data). Serial dilutions were performed in PBS, and cells were plated on LB agar followed by incubation at 37^0 C for approximately 24 hours. Colony forming units (CFU) were determined to quantify biofilm density.

Both DS291 and DS293 were evaluated for biofilm growth in the chemostat at both the high and low dilution rates stated above. All runs were replicated three times (DS293 grown at the low dilution rate was replicated four times). Each replicate consisted of an independent chemostat culture. The CFU values for the five sample plugs were averaged to determine a mean biofilm cell density for each replicate

(Table 2). The density of the planktonic culture was evaluated for each replicate using serial dilutions and plate counts at three different times (after 1 dilution, after 24h and 48h with the MRD). The mean was taken to evaluate the biofilm to planktonic ratio.

Data Analysis.

To compare biofilm formation of the two bacterial strains, the mean biofilm cell densities were analyzed. However, due to variation in planktonic cell density it was important to evaluate biofilm formation relative to the density of planktonic cells. Accordingly, the biofilm population was calculated as a ratio of the planktonic population this will be referred to as the adherence ratio. The ratio was calculated by dividing the quantified density of the biofilm by the mean cell density of the planktonic culture. Comparisons of cell density across dilution rates were analyzed with a one-way analysis of variants.

Microscopy.

To evaluate the morphological variation of biofilms formed by DS 291 and DS293, we examined biofilms grown on the silicone discs and viewed them using scanning confocal laser microscopy (SCLM). Five plugs were stained with Syto 9 (Molecular Probes, Eugene, Oregon) and viewed with the SCLM using a 40X objective to obtain a qualitative view of the biofilm distribution.

RESULTS

Biofilm Density Evaluation.

To evaluate the significance of the stringent response on biofilm formation, the *relA*$^-$ *spoT*$^-$ strain and its isogenic wild type were cultured in a chemostat to allow the formation of biofilms using a MRD. Counts were taken of the biofilms along with counts of the planktonic culture. Means of these values are shown in Table 2. When comparing the density of biofilms formed by the two strains independently of planktonic concentration, there was a significant difference at the low dilution rate only. At a low dilution rate (D = 0.025h^{-1}) there was a large significant difference (P = 1.3 x 10^{-12}), and at a high dilution rate (D = 0.25h^{-1}) there was not a significant difference (P = 0.103). See Figure 3.

When determining the biofilm density in a system that uses a chemostat with recirculation of the culture through a device used to form the biofilms, it is important to evaluate the biofilm density as a ratio of the mean planktonic density. This takes into account any changes that may have occurred due to an increase in planktonic cell numbers. The results of the adherence ratio analysis showed significant differences at both dilution rates as opposed to the evaluation done independently of the planktonic concentration. When evaluating the adherence ratio data, the low dilution rate (D = 0.025h^{-1}) showed a large significant difference (P = 7.0 x 10^{-13}), and the high dilution rate (D = 0.25h^{-1}) also showed a significant difference (P = 0.00027). See Figure 4. There were also significant differences when comparing the different dilution rates of the

same strain ($P < 0.05$). There was a considerable increase in biofilm densities of both the wild type and the *relA*$^-$ *spoT*$^-$ strain at the high dilution rate.

Scanning Confocal Laser Microscopy.

In order to obtain images of biofilms formed by these two strains at the different dilution rates, plugs were taken from the MRD, stained, and visualized. These micrographs present qualitative data of the biofilm densities. Differences between that of the wild type and *relA*$^-$ *spoT*$^-$ strain grown at the slow growth rate were quite noticeable. In Figures 5 and 6, you can see the biofilms formed by the two strains grown at $D = 0.025h^{-1}$. The wild type is fairly dense (Fig. 5), however, the density of the *relA*$^-$ *spoT*$^-$ strain is quite scattered (Fig 6). In Figures 7 and 8, the biofilms formed by the two strains grown at $D = 0.25h^{-1}$ are shown. Differences between the two strains in Figures 7 and 8 can also be determined. It is evident that there are differences between the biofilm densities of the two strains at the high dilution rate but there is a considerable difference in the density when comparing the micrographs at the low dilution rate. Densities between the two dilution rates of the same strain also show marked differences in biofilm density as shown in Figures 5 and 7 for the wild type and Figures 6 and 8 for the *relA*$^-$ *spoT*$^-$ strain.

DISCUSSION

It has been shown that biofilms exhibit growth gradients within individual microcolonies (Sternberg et al., 1999). These microcolonies contain areas with cellular growth rates increasing from the inner to outer part of the aggregate. Gradients within these microcolonies can be due to the actual nutrient levels in the bulk liquid, nutrient uptake from the outer cells, or the inability of nutrients to diffuse through the exopolymer produced by the biofilm. Therefore in order to survive under nutrient limited conditions, cells in a biofilm must be able to generate survival responses.

In wild type cells with nutrients available for supporting maximal growth rate, (p)ppGpp levels are low, and stable RNA synthesis is high with lower levels of unstable RNA synthesis. However, if one of the many necessary nutrients becomes exhausted, the cell will shut down certain processes to preserve viability. Under stringent conditions, synthesis of proteins for starvation survival is up regulated mainly through changes in promoter selectivity. The stringent response in particular involves the sensing of amino acids, glucose and phosphate depletion. Cells are able to perform this by sensing when high uncharged/charged tRNA levels become present due to low amino acid levels (Rojiani et al., 1989).

In a stringent cell, many cellular functions are affected, most notably, RNA synthesis but also others such as peptidoglycan synthesis. Recently, peptidoglycan was shown to have an affect on the formation of

biofilms and cellular adherence (Loo et al., 2000). Therefore, if the expression regulator for these genes is knocked out it is expected that there would be an effect on biofilm formation also.

The results of this study, indicate that if cells are unable to commit to a stringent response their ability to form biofilms is decreased. This is most likely due to down regulation of starvation genes possibly because stable RNA (tRNA and rRNA) is being expressed and unstable RNA (mRNA) is not expressed as well (Ryals et al., 1982). This study shows that *E. coli* biofilm formation requires a stringent response to develop a biofilm equal to that of the wild type. When comparing the densities of a *relA*$^-$ *spoT*$^-$ strain to that of a wild type at a dilution rate of 0.025h^{-1}, biofilm density is significantly decreased. Densities of biofilms at the higher dilution rate (0.25h^{-1}) showed no significant differences. The difference at the high dilution rate may be a result of starvation less likely to be occurring. Notley and Ferenci (1996) have reported that up-regulation of RpoS-dependent genes occurs at a dilution rate below 0.2 h^{-1} and the high experimental rate is at a level above that induction level. However, in this study, a serine-limited medium was used, and the setup used by Notley and Ferenci was in a glucose-limited system. Similar studies using a serine-limited system would need to be done to confirm the RpoS induction for the experimental set up used in this study.

In this experimental setup, biofilm density is influenced by planktonic cell density in addition to genotype and growth rate. By

expressing biofilms as a ratio of adherent cells to planktonic cells the influences of genotype and dilution rate can be better analyzed. After evaluating the results from the ratio data, I found that there were significant differences at both the low and high dilution rates. Once again the differences at the low dilution rate were much greater than that of the high dilution rate.

Qualitative examination of biofilms formed on the silicone discs using SCLM revealed differences corresponding to that of the quantitative data from density counts. The distribution of the low dilution rate biofilms films revealed significant differences between the two strains. With the *relA*$^-$ *spoT*$^-$ strain the biofilm distribution was quite sporadic with the surface revealing the presence of individual cells and small clusters of cells. These small clusters don not exhibit the three-dimensional biofilm morphology commonly formed by the wild type. The clusters from the *relA*$^-$ *spoT*$^-$ strain appeared to be in the stage of initial attachment. Possibly, without the ability to survive in conditions where the nutrients are not ideal, these cells are unable to continue on to form a mature biofilm. When evaluating micrographs from the high dilution rates there were differences in cell distribution but not as noticeable as with the slow dilution rate micrographs. Once again, quantitative data from the biofilm densities correlates with the qualitative data from the micrographs.

A variety of factors could have influenced the biofilm density differences demonstrated in this study. We have demonstrated that one factor, the global regulator (p)ppGpp, which is a product of the stringent response, has a role in biofilm formation. Nonetheless, factors such as growth media and environmental affects also play an important role in the formation of biofilms. RelA and SpoT definitely play an important role. Ultimately this may be a direct response of the downstream regulation of slow growth induced by RpoS. Even so, RpoS, RelA, and SpoT all control gene expression when cells are limited by metabolic requirements. In order to understand which genes are affecting biofilm formation the most, strains with knock outs of individual metabolically controlled genes will have to be studied. One start would be the gene required for peptidoglycan synthesis.

Once again, this is the study of one of many global regulators responsible for the adaptation of cells to environmental fluctuactions. The global regulator here is (p)ppGpp but in addition there is RpoS and others that are either more or less important in metabolic regulation. With this in mind, future biofilm studies should take into account these metabolic regulators and the genes they affect.

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Table 1. Genetic designations of *E. coli* strains used in study.

Strain Name	**Genotype**
DS291[a]	Wildtype = MG 1655 = CF 1648
DS293[a]	DS 291 ΔrelA::kanΔspoT207::cam = CF 1693

[a] Strains obtained from D.A. Siegele, Texas A&M University, College Station, TX. Construction of strains described by Xiao et al. 1991.

Table 2. Summary of means from planktonic and biofilm densities, adherence ratio, and standard error data from the different treatments. The mean for DS293 at D = $0.025h^{-1}$ was calculated with n = 4. All other data was calculated with n = 3.

Treatment	**Mean Planktonic CFU/ml**	**Mean Biofilm CFU/ml**	**Adherence Ratio**
DS291 D = $0.025h^{-1}$	$1.41 \pm 0.13 \times 10^9$	$7.78 \pm 1.67 \times 10^6$	0.731 ± 0.0147
DS293 D = $0.025h^{-1}$	$1.96 \pm 0.46 \times 10^9$	$0.11 \pm 0.015 \times 10^6$	0.534 ± 0.0102
DS291 D = $0.25h^{-1}$	$5.89 \pm 2.92 \times 10^9$	$49.0 \pm 12.2 \times 10^6$	0.792 ± 0.0134
DS293 D = $0.25h^{-1}$	$15.2 \pm 3.57 \times 10^9$	$26.3 \pm 7.82 \times 10^6$	0.720 ± 0.0107

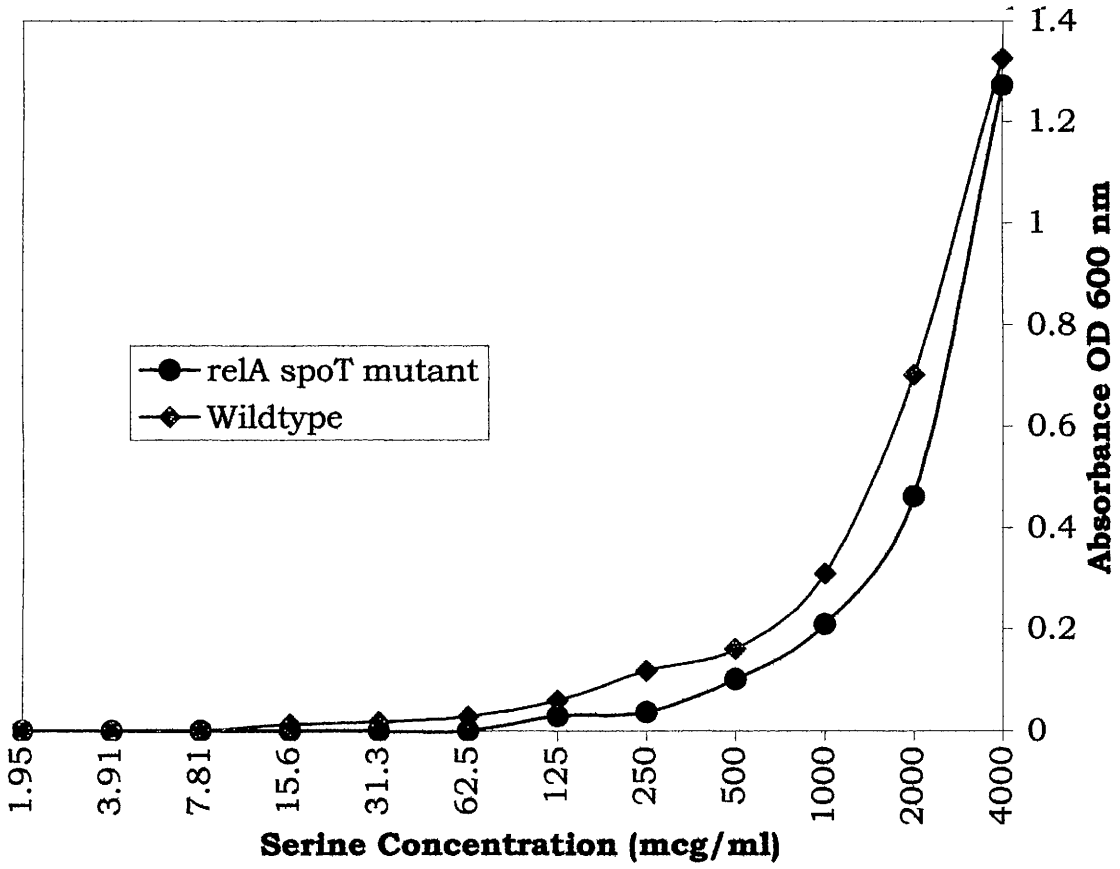

Fig. 1. Growth curve of the wild type and *relA*$^-$ *spoT*$^-$ strains to obtain limiting concentrations of serine in the growth medium. The limiting concentration is shown with an arrow (1000 µg/ml).

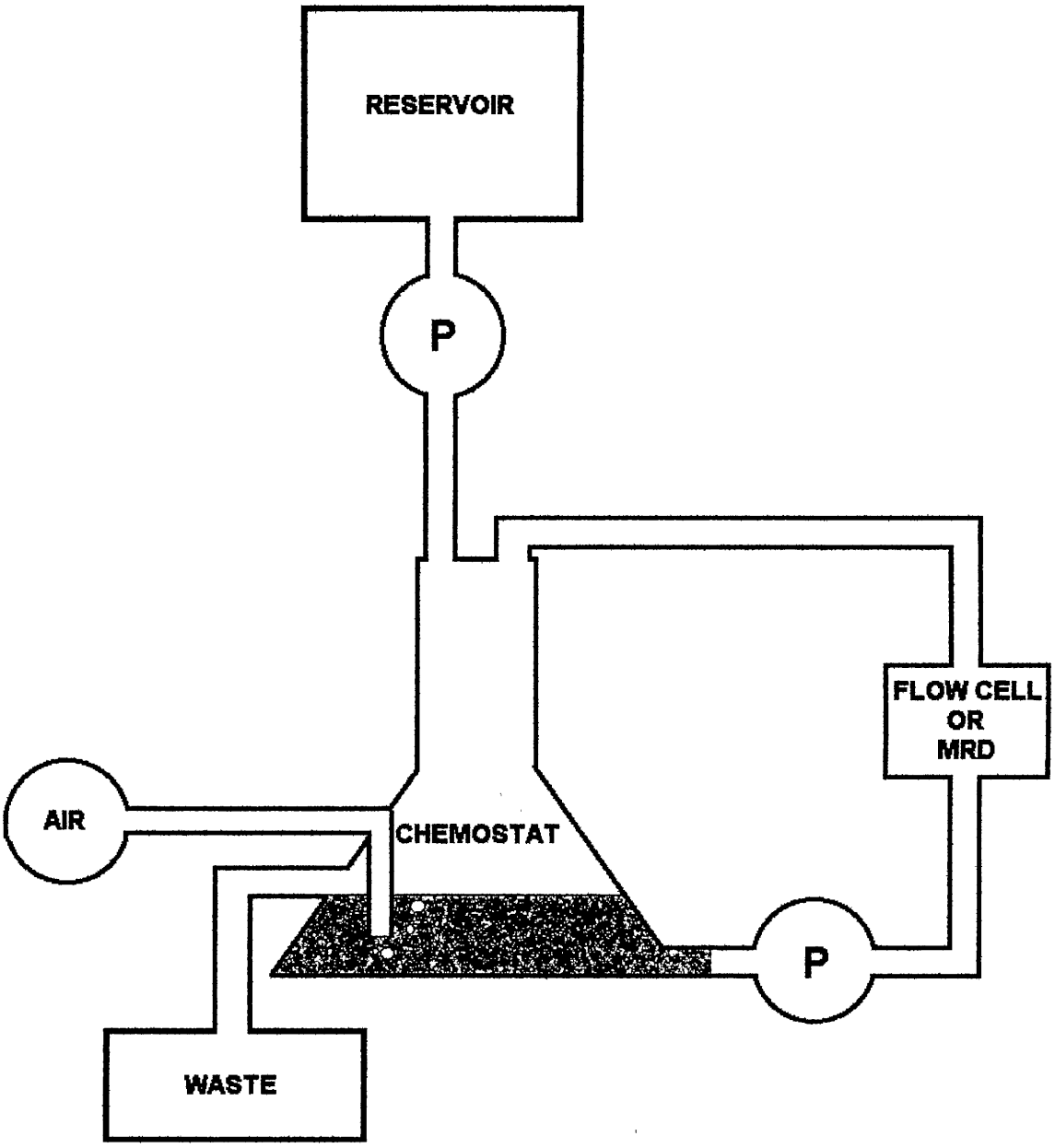

Fig. 2. Schematic of chemostat apparatus used in study.

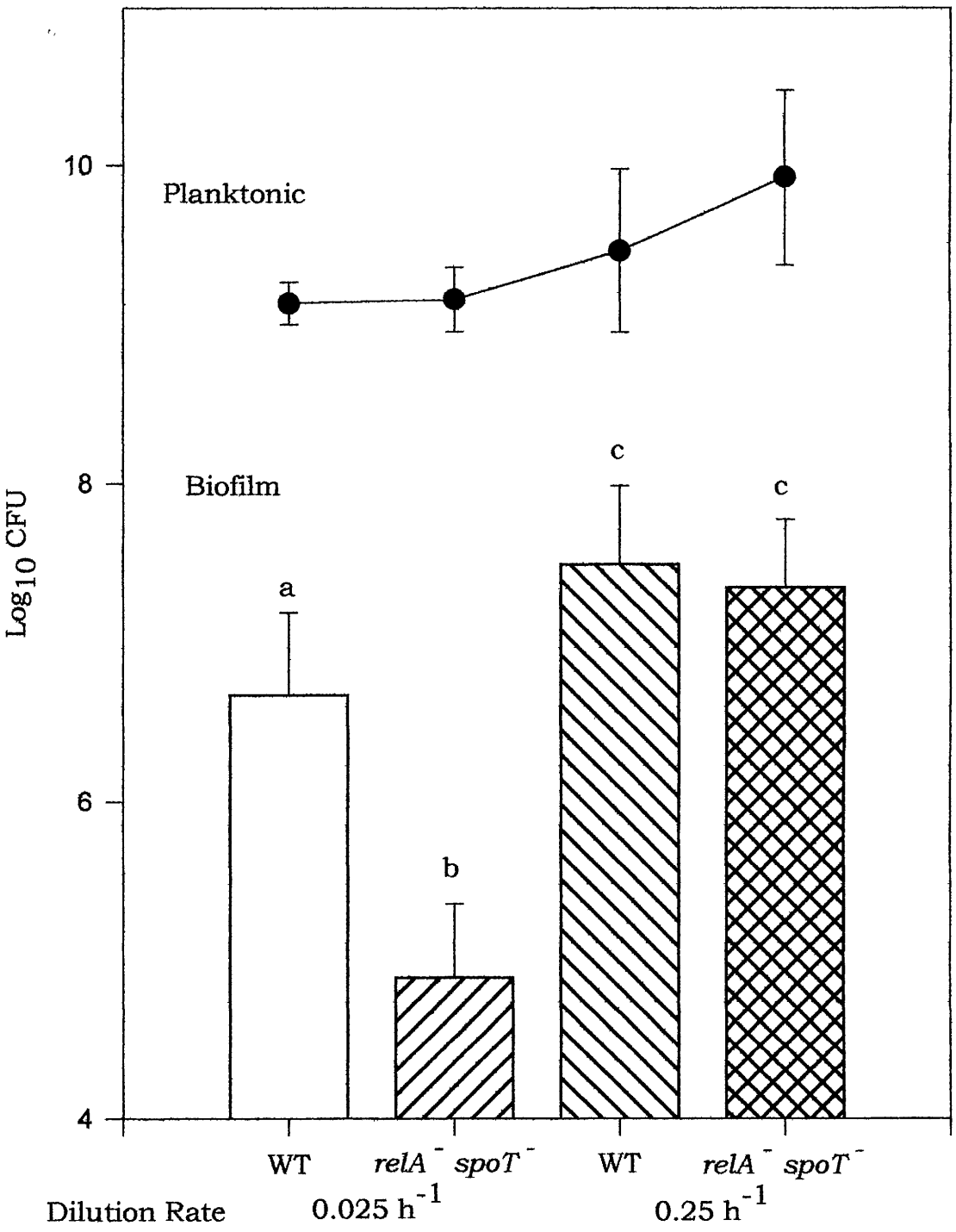

Fig 3. Graph showing effects of *relA spoT* deletions on planktonic density (log_{10}CFU/ml) and biofilm density (log_{10} CFU/disc). Error bars represent standard deviations. Values with the same letter are not significantly different ($P = 0.05$).

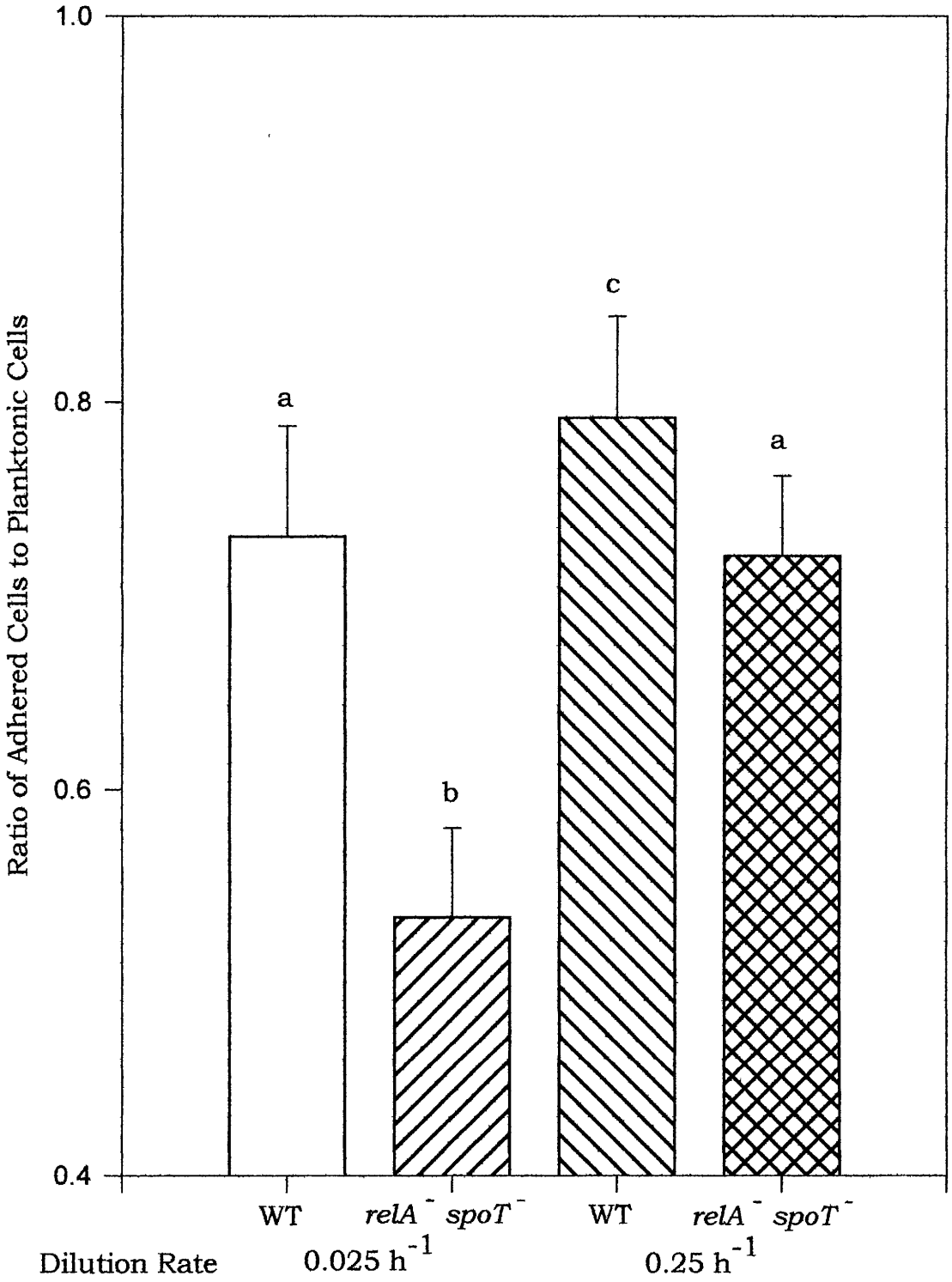

Fig. 4. Graph showing the ratio of biofilm density over the planktonic mean for both strains at the low and high dilution rates. Error bars represent standard deviations. Values with the same letters are not significantly different ($P = 0.05$).

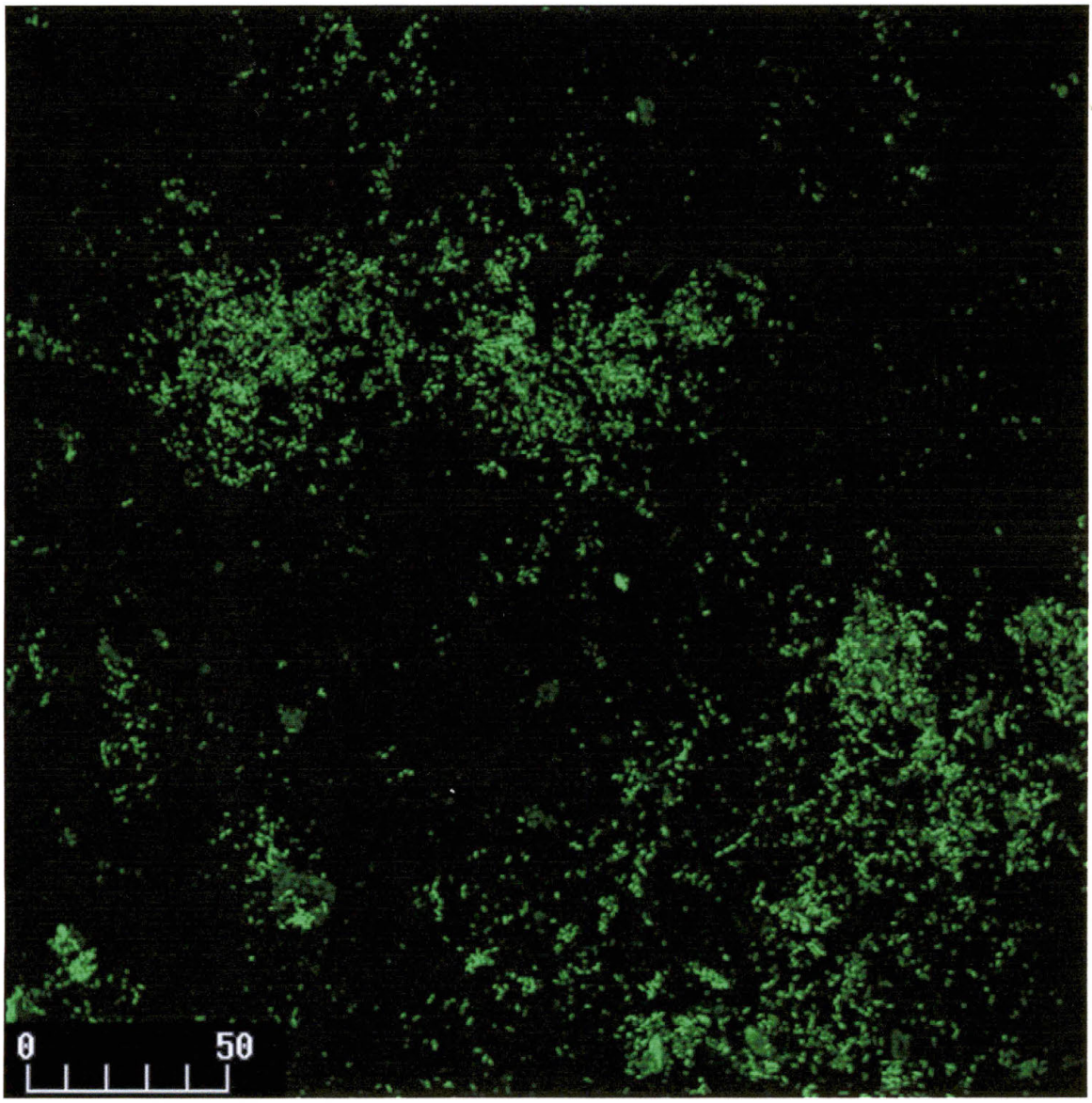

Fig. 5. SCLM micrograph of a 48h *E. coli* biofilm formed by the wild type strain at D = 0.025h^{-1}. Scale bar is in micrometers.

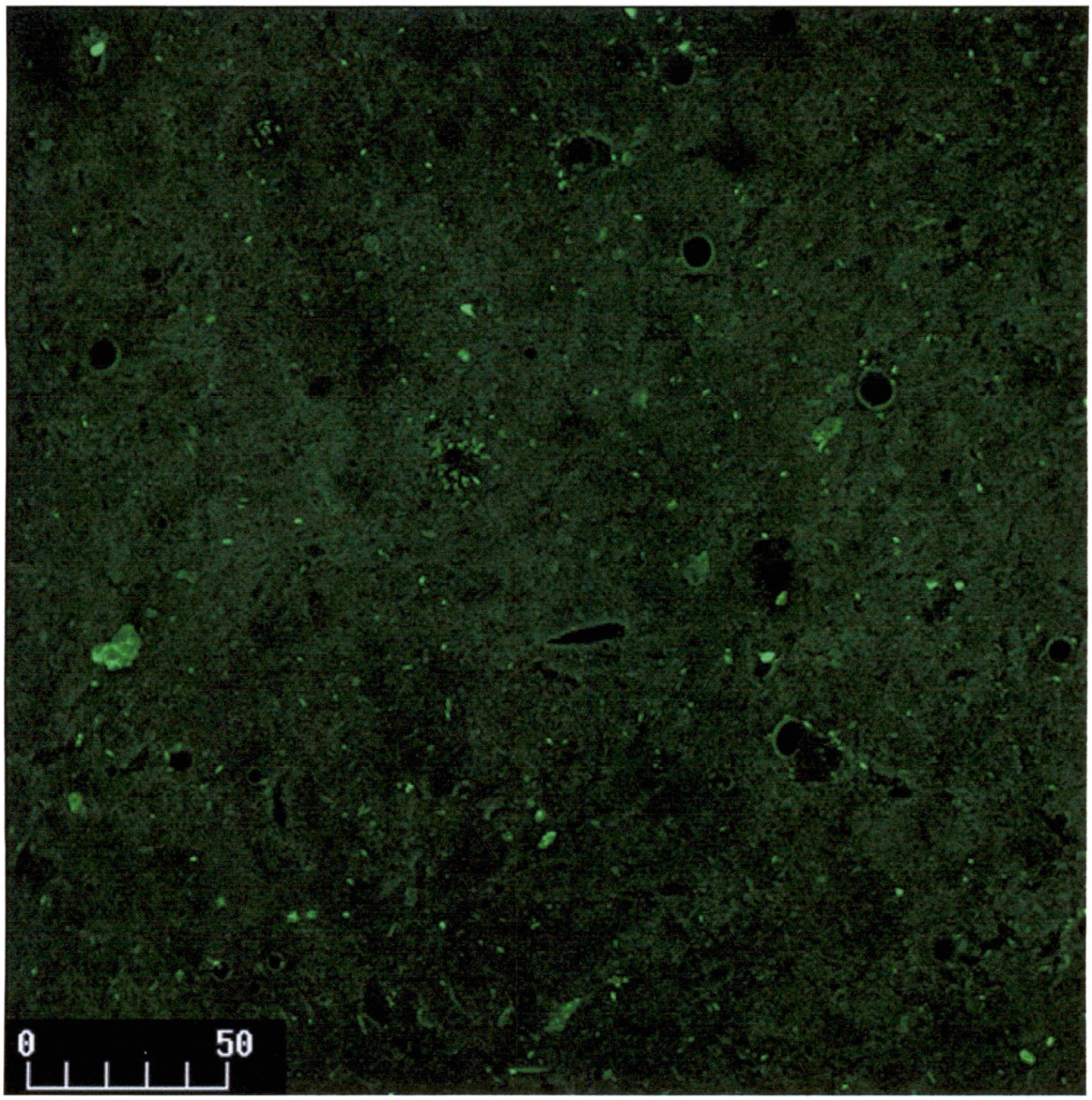

Fig. 6. SCLM micrograph of a 48h *E. coli* biofilm formed by the *relA*$^{-}$ *spoT*$^{-}$ strain at D = 0.025h^{-1}. Scale bar is in micrometers.

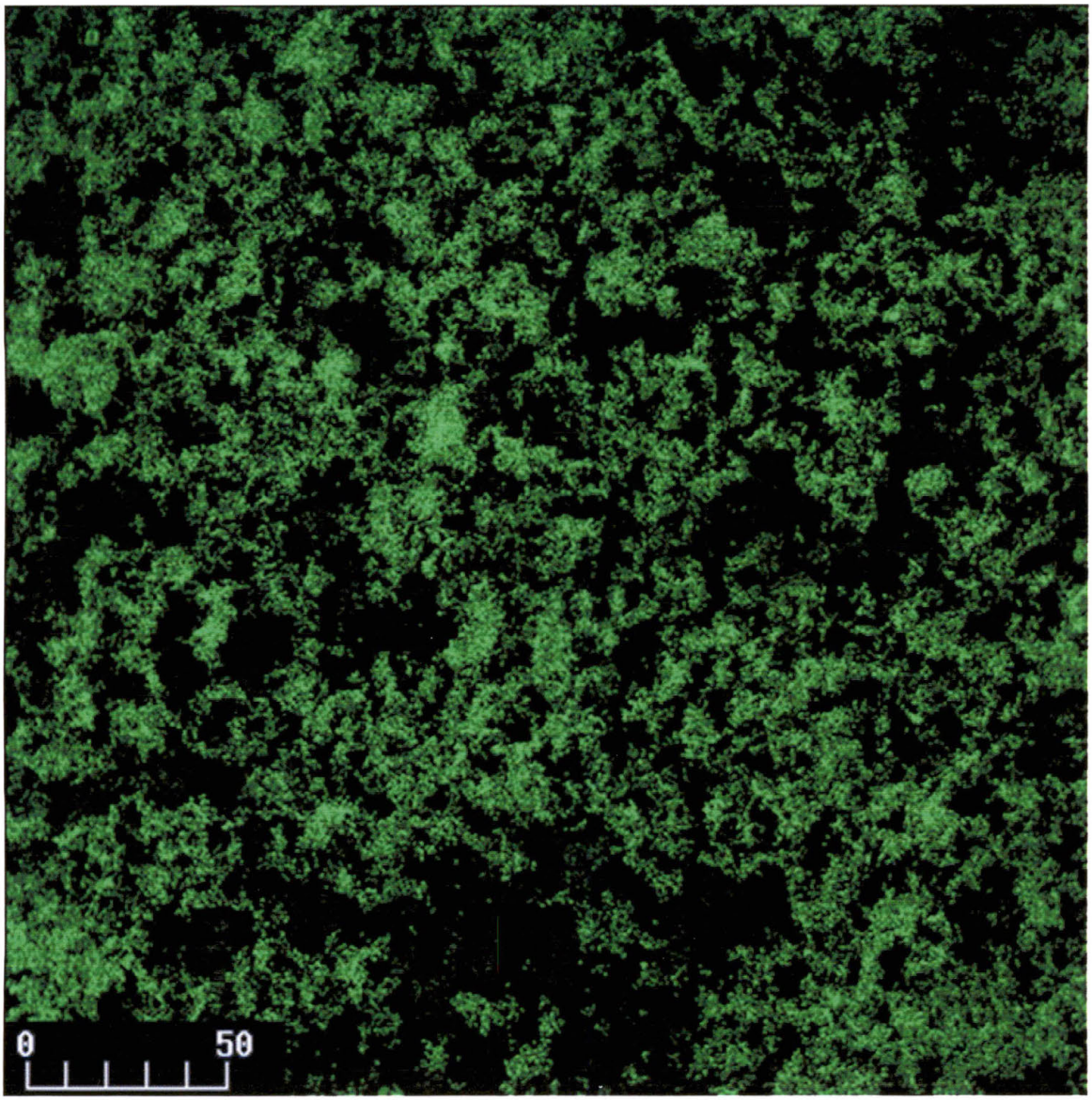

Fig. 7. SCLM micrograph of a 48h *E. coli* biofilm formed by the wild type strain at D = 0.25h^{-1}. Scale bar is in micrometers.

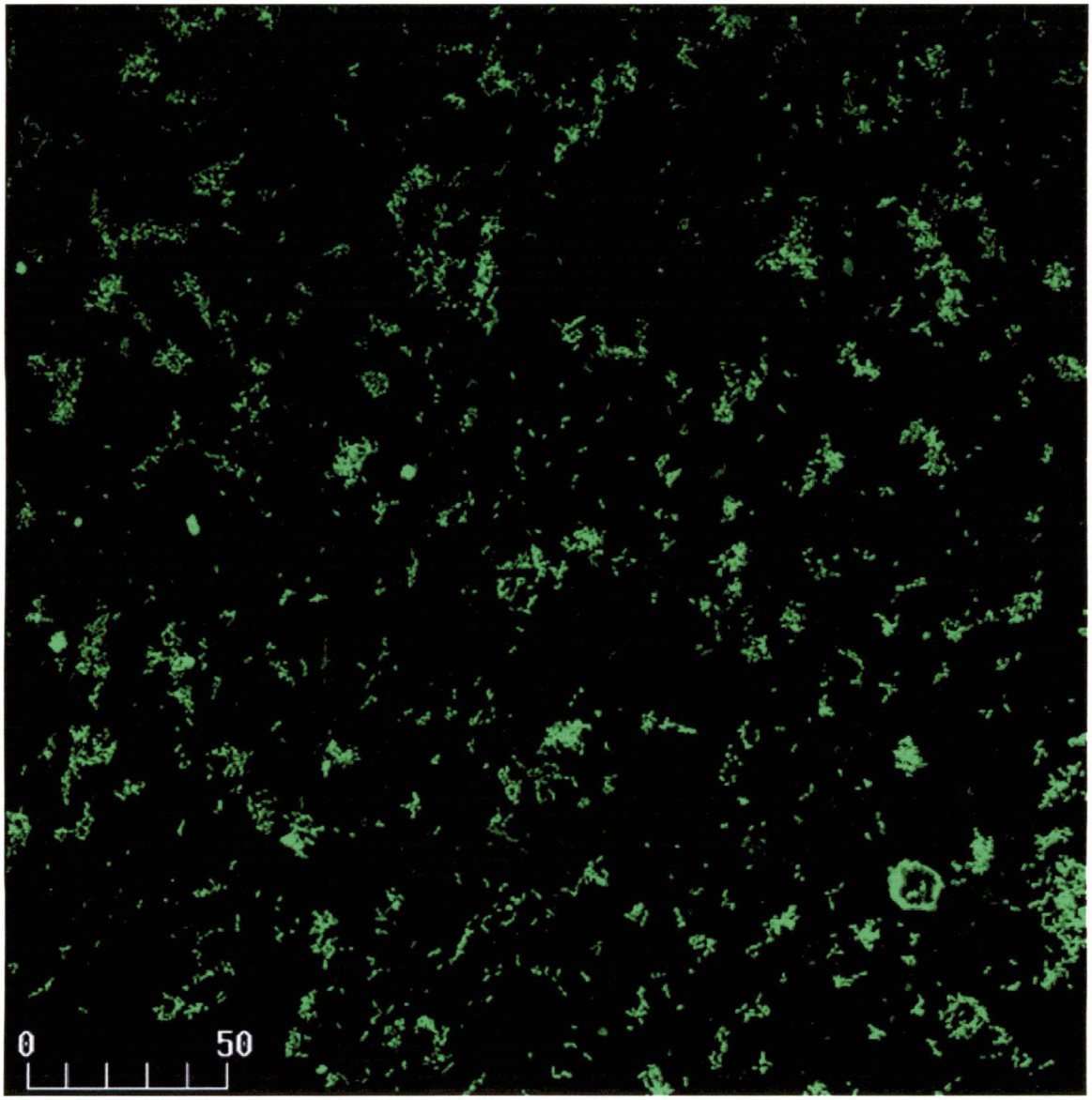

Fig. 8. SCLM micrograph of a 48h *E. coli* biofilm formed by the *relA*$^{-}$ *spoT*$^{-}$ strain at D = 0.25h^{-1}. Scale bar is in micrometers.