THE ROLE OF *rpoS* IN THE FORMATION OF *Escherichia coli* BIOFILMS

THESIS

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DEDICATION

I would like to dedicate this thesis to my family, Cary and Carolyn Barton, Robert L. Adams, and Holly and Laura Adams. Their belief in my abilities, and their dedication to education is a constant inspiration for me.

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ABSTRACT

In their natural environments, bacteria grow predominately as surface-adherent biofilms, a feature of great importance to medicine, industry and the environment. I hypothesize that the potential for biofilm formation may be enhanced by the ability of bacteria to grow slowly in nutrient limited environments. I have tested the hypothesis that *rpoS*, an alternate sigma factor expressed as planktonic cells enter stationary phase is imperative for biofilm formation. Previously, the *rpoS* gene has been demonstrated as necessary for bacterial survival in stationary phase. Using a chemostat and a Modified Robbins Device, I quantified biofilm formation of *rpoS* deleted and *rpoS*$^+$ strains of *Escherichia coli*. With glucose as the limiting nutrient, both strains of *E. coli* were cultured in a chemostat at dilution rates of $0.0083h^{-1}$ and $0.033h^{-1}$. While there were no differences in planktonic cell densities, a significant decrease in biofilm formation was observed by the strain deficient in *rpoS* gene function. This phenomena was observed at both dilution rates. I further compared biofilm formation of *rpoS*$^-$ and *rpoS*$^+$ bacterial strains using Scanning Laser Confocal Microscopy. The results indicate a difference in the biofilm morphology for the two *E. coli* strains. I also attempted to evaluate the production of σ^s (the product of *rpoS*) using a *lacZ* reporter strain. I compared *rpoS* transcription in biofilms as well as the planktonic cultures. There were no significant differences found. Therefore, I conclude that the presence or absence of slow growth genes, regulated by *rpoS*, affects biofilm formation. However, further research is necessary to establish the precise mechanisms by which *rpoS* influences the physiology of cells within a biofilm.

INTRODUCTION

Biofilms are defined as an adherent community of microorganisms attached to a surface and to each other in a complex matrix structure. This structure contains a heterogeneous mixture of cells that cluster together into microcolonies. Biofilms contain regions of low cell density called water channels. These channels allow water to flow around and throughout the microcolonies supplying nutrients to the bacteria and removing waste products. The water channels are said to function analogously to a circulatory system of a higher organism (Costerton et al., 1995, Lawrence et al., 1991). Microcolonies are not fixed, but are dynamic. They have been shown to be influenced by several factors including gene expression (E.P. Greenberg, personal communication) and nutrition (Moller et al., 1997).

Cells within a biofilm possess a different physiology than planktonic, unattached cells. Several studies have shown that bacteria undergo morphological change once they have adhered to a substratum (Costerton and Lappin-Scott, 1995, Fletcher, 1991). When cells enter a biofilm they increase exopolysaccharide (EPS) synthesis and form complex microcolonies (Costerton et al., 1995). Adhesion to surfaces and biofilm growth is correlated with a shift in the physiology of the colonizing organisms. Korber et al. (1994) found that cells inside the biofilm were growing at a slower rate than cells closer to the biofilm surface. Further, James et al. (1995) showed that *Acinetobacter* sp. biofilm cells adopt a coccoid morphology and pack tightly together in response to starvation.

Bacteria within a biofilm have been demonstrated to be as much as 20 - 1000 fold less susceptible to antibiotic treatments than equivalent planktonic cultures (Gilbert et al., 1990, Stickler and McLean, 1995). There are many possible explanations for the antibiotic resistance of biofilms. Brown et al. (1988) suggests two hypotheses. The first hypothesis states that due to the condition of slow growth, the cells are growing at a rate at which they are not susceptible to many antimicrobials. The second hypothesis states that the cells have altered their cell envelope and extracellular enzymes thus influencing the biochemical drug activity with the cell. An alternate hypothesis is that EPS matrices of biofilms act as physical diffusion barriers to antibiotics (Costerton et al., 1987). Moreover, planktonic organisms form a biofilm in an effort to survive times of low nutrients (Camper et al., 1996, Whiteley, 1997a). The biofilm may allow cells to cooperate and form a coordinated community that is metabolically more efficient than the planktonic cells (Costerton et al., 1995).

When batch-grown planktonic cells enter stationary phase, the period in the bacterial growth curve at which cells stop growing due to nutrient starvation, bacteria shift their metabolic activity and overall protein synthesis. Despite these shifts, the expression of 30-50 new proteins is stimulated (Lange and Hengge-Aronis, 1991b, McCann et al., 1991). These proteins play a major role in modulating the physiology of cells in response to slow growth conditions. *rpoS* is an important regulator that is required for the expression of many starvation induced genes including *bolA* (Lange and Hengge-Aronis, 1991a). *bolA* is normally associated with cell septum formation (Loewen and Hengge-Aronis, 1994). Increased expression of this gene produces a morphological change in *E. coli* from the normal rod shape to the coccoid shape associated with

decreased nutrient availability. The production of fibronectin-binding curli in *E. coli* is also regulated by *rpoS* (Olsen et al., 1993).

The gene product of *rpoS* is a sigma factor (σ^s) that acts as a subunit of RNA polymerase (Mulvey and Loewen, 1989, Nguyen et al., 1993). RNA polymerase is an enzyme complex responsible for the transcription of RNA, and it guides the enzyme to the promoter. *rpoS* expression is enhanced during entry into stationary phase since *rpoS* expression is inversely related to growth rate of the cells (Lange and Hengge-Aronis, 1994, Notley and Ferenci, 1996). In addition, *rpoS*$^-$ cells show a decreased ability to develop characteristic stationary phase properties, and demonstrate decreased ability to withstand multiple stresses such as oxidation and low pH (Lange and Hengge-Aronis, 1991b, McCann et al., 1991). As bacteria within biofilms are in a condition of slow growth, the expression of *rpoS* may be essential for their ability to form biofilms.

The goal of this study was to determine if expression of *rpoS* was in fact necessary for biofilm formation. To determine this, I cultured and enumerated biofilms of an *rpoS* deleted strain and its isogenic parental strain. Biofilm formation of the two strains were compared as well as the amount of biofilm formation relative to the concentration of planktonic cells. Further, I attempted to determine the distribution of living and dead cells within biofilms with a viability indicating stain and SCLM. Finally, the expression of *rpoS* in both biofilm and planktonic cultures was compared using an *rpoS::lacZ* reporter strain. In wild type cells, I hypothesized that I would find an increase in *rpoS* transcription in the biofilm over that of planktonic cultures.

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* K12 strains used in this study are listed in Table 1. Strains were provided by D.A. Siegele at Texas A&M University. Cells were cultured in minimal media including per liter: 1.5 g nitriloacetic acid, 1.47 g anhydrous magnesium sulfate, 0.1 g $CoCl_2$-$6H_20$, 0.5 g $MnSO_4$, 1.0 g NaCl, 0.1 g $FeSO_4$-$7H_20$, 0.1 g $CaCl_2$, 0.1g $ZnSO_4$-$7H_20$, 0.01g sodium borate, 0.01g $CuSO_4$-$5H_20$, 0.01g Na_2MoO_4-$2H_20$, 0.017g $CaCl_2$, 0.025g magnesium sulfate anhydrous, 1g K_2HPO_4, 0.24g NH_4Cl and 0.25g glucose. The pH of the media was established at 7.2. This recipe was adapted from Whiteley et al. (1997b). The glucose concentration was determined to be limiting for *E. coli* ZK126. The glucose limitation was determined by inoculating the bacteria into a series of tubes containing minimal media and a gradient of 10% glucose. The growth of the bacteria was quantified after 24 hours at OD_{600}. The point at which the increased concentration of glucose no longer impacted the concentration of bacterial cells was determined to be the glucose concentration at which carbon was no longer the limiting nutrient. ZK1000 and ZK126 were evaluated for their ability to form biofilms. This revealed the significance of *rpoS* to biofilm formation. DS526 contains an *rpoS::lacZ* gene fusion which was used to assay σ^s expression. Expression of this fusion protein is correlated with the expression of the σ^s protein (Lange and Hengge-Aronis, 1994).

Biofilm Growth and Enumeration

Bacterial strains were cultured in carbon limited, minimal media in a chemostat apparatus allowing continuous growth (Whiteley et al., 1997b) (Fig. 1). The organisms were inoculated into the aerated chemostat resting in a 37°C water bath. The ZK126 and ZK1000 cultures (Table 1) were each grown in the chemostat at dilution rates (D) of $0.033h^{-1}$ and $0.0083h^{-1}$. The cultures were allowed to equilibrate for one full generation time (30 hours at $D=0.033h^{-1}$ and 121 hours at $D=0.0083h^{-1}$). At that time, a Modified Robbins Device (MRD) (Nickel et al., 1985) was connected by a peristaltic pump at a flow rate of 100ml min^{-1}, biofilms were allowed to develop over a 48h period.

Sampling Methods

Each of the 25 ports on the MRD held an 8mm diameter silicone rubber disk. At sampling, nine disks from across the device were aseptically removed and individually suspended in 2ml of 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. Serial dilutions were performed, and cells were plated on LB agar followed by incubation at 37°C for approximately 24 hours. CFU's were determined to quantify cell density. The CFU counts were calibrated with direct counts using epifluorescent microscopy and 4',6-diamidino-2-phenylindole (DAPI) as the stain.

Both ZK126 and ZK1000 were evaluated for biofilm growth in the chemostat at both the high and low dilution rates stated above. The parental strain grown at the faster dilution rate was replicated three times and the parental strain at the slower dilution rate

was replicated four times. Biofilm enumeration for the mutant strain at both dilution rates was replicated four times. Each replicate consisted of an independent chemostat culture. Nine sample plugs were removed, and CFU values for all of the nine plugs were averaged to determine a mean biofilm cell density for each replicate (Table 2). No significant relationship between plug position and the cellular concentration of the biofilm on the disc was found ($F = 1.49$, $R^2 = 0.004$, $P > 0.2$). The density of planktonic cells were evaluated for each replicate using serial dilutions and plate counts (as above).

Data Analysis

In order to compare biofilm formation of the two bacterial strains, the mean biofilm cell densities were analyzed. However, due to variation in planktonic culture 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. The ratio was calculated by dividing the titer of the biofilm by the titer of the planktonic culture. These ratios were arcsin transformed to account for non-normality in percentile data (Sokal and Rohlf, 1969). Data was analyzed using general linear models (GLM) followed by a comparison of means by Fisher's Least Significant Difference (LSD) (SAS Institute, Inc.). Comparisons of cell density across dilution rates were analyzed with an un-paired t-test.

Scanning Confocal Laser Microscopy

In order to understand the morphological variation of biofilms formed by ZK126 and ZK1000, we examined biofilms grown in a flow cell (Davies et al., 1998) and viewed under SCLM. Similar to biofilm enumeration experiments, the bacteria were cultured in the chemostat at a dilution rate of 0.033 h^{-1}. A flow cell was attached to the chemostat by

a pump at a flow rate of 500ml h^{-1} (flow rate was dictated by the capacity of the flow cell) and the biofilms were allowed to form on the slide for 48 hours. At this time, Molecular Probes Bac Light Live/Dead stain was circulated through the flow cell for fifteen minutes in the dark. The samples were observed using the SCLM at a magnification of 830X.

Reporter Gene Assay

In an effort to compare the level of *rpoS* expression in cells in biofilms with cells in the planktonic cultures, I used the reporter strain, *E. coli* DS526 (Table 1). This strain was cultured exactly as described for biofilm enumeration in the chemostat at a dilution rate of 0.033 h^{-1}. Once again, the MRD was connected with a peristaltic pump at a flow rate of 100ml min^{-1}. Biofilm and planktonic samples were removed from the system at 48 hours and frozen for future β-gal assays.

We assumed production of *lacZ* is proportional to the production of σ^s, I quantified the expression of *rpoS* by measuring cellular production of β-gal with *ortho*-nitrophenyl-galactopyranoside (ONPG; Sigma) using the chloroform and 1% SDS method of cell lysis described by Miller (1972). β-gal specific activity was expressed as nmols ONPG cleaved $cell^{-1}$ $minute^{-1}$. Cell number was determined from direct cell counts of both the biofilm and the planktonic cell suspensions. The cells were stained with DAPI and counts were performed using epifluorescent microscopy and the guidelines described by the Poretics® Corporation (Livermore, CA) in 1992.

RESULTS

Biofilm Growth and Enumeration

In an effort to evaluate the importance of *rpoS* to the formation of biofilms, an *rpoS* deleted strain and its isogenic parental strain were cultured in a chemostat, and. biofilms were enumerated in an MRD. When evaluating biofilm growth independently without reference to the concentration of planktonic bacteria, the parental strain, when grown at a dilution rate of $0.0083h^{-1}$ (generation time of 121hours), formed a significantly less dense biofilm than when grown rapidly at a dilution rate of $0.033h^{-1}$ (generation time of 30 hours) ($p<0.05$). Interestingly, the mutant strain at the slower dilution rate and at a faster dilution rate were not different in cell density within the biofilm (Table 3). Most importantly, I noted there was a significant difference in the cell density of the biofilm of the parent fed more slowly and the mutant fed more slowly ($p<0.05$), and I noted a significant difference in the concentration of cells within the biofilm of the parent fed at the faster rate and the mutant fed at the faster rate ($p<0.05$) (Fig.2).

In an effort to address the impact of differences in planktonic population densities on biofilm populations, I calculated a ratio of the concentration of planktonic cells and the concentration of cells adhered in the biofilm. The results were similar to those of the biofilm studied independently of planktonic cellular concentration. The biofilm ratio of the parental strain fed at the slower rate was significantly larger than that of the mutant fed at the slower rate ($p<0.05$), and the biofilm ratio of the parent fed at the faster rate was significantly larger than the mutant fed at the faster rate ($p<0.05$). Further, the ratios for the *rpoS* deleted strain at both feeding rates were almost identical ($p=0.9244$) (Table 3).

However, the ratio data revealed that the parental strain fed more slowly had a significantly larger ratio of biofilm growth than the parental strain fed more rapidly ($p<0.05$) (Fig.3).

Scanning Confocal Laser Microscopy

The distribution of living and dead cells within the biofilm of the *rpoS* deleted strain and its isogenic parental strain was compared using SCLM. The images created by the SCLM illustrate a qualitative view of the structure of the biofilm. Interestingly, there were similarities in the biofilms of both the *rpoS* mutant and its isogenic parental strain. The vertical image of the parental strain is similar to that of the mutant strain. The image shows a layer of dead cells on the bottom of the film, and accordingly, the live cells are more prevalent on the top layers of the film (Fig. 7). The XY image of the parental strain is interesting. It is a more patchy distribution than the mutant strain. The living cells appear to have lifted away from the bulk of the biofilm to form hydrophilic balls of bacteria (Fig. 6). The micrographs of the mutated strain show an equal distribution of live and dead cells (Fig. 4). The vertical image shows a clear difference in the distribution of live and dead cells throughout the cell layers. There are noticeably more dead cells at the bottom layers of the biofilm, and the live cells are mostly restricted to the upper layers of the film (Fig. 5).

Reporter Gene Assay

To quantify *rpoS* expression, the amount of β-galactosidase production was measured by the amount of ONPG cleaved per minute per cell. β-gal production was determined for biofilm and planktonic cultures. Both planktonic and biofilm cultures demonstrated very similar amounts of β-gal and therefore *rpoS* production. The biofilm cultures cleaved 3.04

x 10^{-6} nmols of ONPG $minute^{-1}$ $cell^{-1}$. The planktonic cultures cleaved 3.08 x 10^{-6} nmols of ONPG $minute^{-1}$ $cell^{-1}$ (Table 4).

DISCUSSION

Moller et al. (1997) examined the impact of nutrient composition on biofilm structure and exopolysacharide chemistry with SCLM. They found that bacteria within a biofilm are growing slowly. I demonstrate that a σ^s is can affect the ability of bacteria to form substantial biofilm. I found a significant decrease in the biofilms cell density formed by the *rpoS* deleted strain compared to the parental strain. This difference was noted at the two dilution rates. Although these results were expected based on the function of *rpoS* this is not the only explanation for the variability in adherence. I have examined one variable in an extremely complex system. Variation in planktonic cell concentration may be expected to result in variation in biofilm formation. Therefore, to account for differences in planktonic cell concentration, I calculated the ratio of the planktonic culture that actually adhered to the surface. Once again, I found a significant decrease in the ratio of biofilm formed by the mutant strain compared to those formed by the parental strain.

Initially, biofilm growth was considered independently, without reference to the cell density of the planktonic cultures. The parental strain, when grown at a lower dilution rate, had only a slightly decreased cell density in the biofilm than at the higher dilution rate. I also discovered, as expected, that the planktonic culture concentration was slightly depressed at the lower dilution rate. Therefore, I evaluated the ratio of planktonic cells forming a biofilm. I found a significant increase in the ratio of biofilm formation of the parental strain at a lower dilution rate. These results agree with work previously

conducted in this laboratory. Whiteley (1997a) found that bacterial cell counts within a biofilm were inversely proportional to growth rate of the planktonic culture. Therefore, because natural populations of bacteria are not commonly found in nutrient rich environments (Ford, 1993), Costerton and Lappin-Scott (1995) describe biofilms as a growth strategy for bacteria in response to oligotrophic and hostile environments.

When evaluating biofilms independently without consideration for the concentration of planktonic cells and with reference to planktonic concentration, I consistently found a decrease in the biofilm production of the *rpoS* deleted strain when compared to that of the parental strain. From this I concluded that *rpoS* and the genes it regulates affect biofilm formation. Moreover, unlike the parental strain, there was a lack of variation in biofilm formation of the mutant strain at low and high dilution rates. This information further validates the importance of *rpoS* to biofilm formation. I conclude that σ^s expression and expression of the genes in the σ^s regulon can limit biofilm formation independent of nutrient limitation (i.e. nutrient was saturating at the low dilution rate).

A variety of physiological and environmental factors can influence adherence of bacteria to a surface. We have demonstrated the importance of the *rpoS* gene function in this capacity. However, environmental effects such as ion species and concentration within the culture (Fletcher, 1988), pH (McEldowney and Fletcher, 1986), and temperature (Fletcher, 1977) have all been shown to affect the ability of the bacteria to adhere. Dexter et al. (1975) determined that there is a significant difference in bacterial adhesion to various substrates in marine communities. Surface properties such as wettability influenced bacterial cell ability to adhere to the substrate. In our efforts to examine experimental genetics of biofilm formation, environmental parameters may have

affected the results. An inconsistency in biofilm formation in the MRD and in the flow cell may be due to the variation of substrates. The cultural biofilms developed on silicone rubber discs in the MRD whereas the SCLM biofilms developed on glass slides in the flow cell.

Davies et al. (1993) reported that alginate or capsule production was up-regulated by *Pseudomonas aeruginosa* cells within a biofilm compared with planktonic cells in liquid media. However, Davies and Geesey (1995) found that the increased production of capsule was only initiated after attachment in a flow cell chamber similar to the one used in this experiment. I thus, hypothesize that the delayed ability of a cell to form a capsule on the glass slide of the flow cell may have impacted cellular ability to form a biofilm. Again, this may have affected my interpretation of the SCLM work.

Examination of the biofilms under SCLM revealed morphological differences in the biofilms formed by the mutant strain and its parent. A hypothesis that may explain this discrepancy is that the microcolonies of the parental strain were more evenly spaced across the slide whereas the microcolonies of the *rpoS* deleted strain were sparse and exhibited a patchy distribution. The biofilms grown in the flow cell on glass slides were very small and only a few cells deep. A variety of physiological and environmental factors can influence the adherence of bacteria to a surface.

The distribution differences of the biofilms of the two strains were interesting. Although both organisms appeared to have similar cell densities attached to the slide, the distribution of cells was unique to each strain. The parental strain appeared to form cellular micelles when exposed to the immersion oil. The *rpoS* deleted strain remained more closely adhered to the slide. The gene products of *rpoS* may influence cell surface

hydrophobicity and/or cell to cell interactions of the cultures causing the formation of the cellular micelles. It is also interesting to note that the cellular micelles formed by the parental strain were almost completely composed of living cells. There was a small inner core of non-living cells within each cellular micelle.

The results of the reporter gene assay were not as expected. Biofilm and planktonic cultures both cleaved almost the exact number of nmoles of ONPG min^{-1} $cell^{-1}$. This cleavage of ONPG directly correlates with the β-galactosidase production and the *rpoS* expression. From the results of the CFU determination, I hypothesized that I would find more σ^s production in the biofilm than in planktonic cells. Having not observed this, it appears that *rpoS* expression is the same in adhered and unattached communities, but the expression of *rpoS* dependent genes may vary with the environment of the cells. The genes under the regulation of *rpoS* have been shown to respond to different environmental signals (Hengge-Aronis, 1996). That is, *rpoS* has been expressed in both planktonic and biofilm culture, however, the gene products and their activities may vary with the cultural environment. Further, in minimal media such as that used in these experiments, σ^S concentration reaches its peak a few hours into stationary phase, and in rich media such as LB, the content of σ^s reaches its maximum at the onset of stationary phase (Lange and Hengge-Aronis, 1994).

The importance of *rpoS* to bacterial and especially biofilm physiology is complex and widespread. As σ^s increases in cells growing slowly, the physiological changes associated with slow growth and biofilm formation could in fact be due to any one gene or combination of many genes under the direction of *rpoS*. Further, more specific studies of the cascade of gene regulation initiated by *rpoS* controlled genes such as *bolA* (Lange and

Hengge-Aronis, 1991a) and *appY* (Atlung et al., 1989) may give insight into the mechanisms associated with physiological changes at the individual gene level. Yet another avenue of research will include the study of other global regulatory genes such as *relA* and *spoT* and their impact on the formation of biofilms. During amino acid starvation, *relA* codes for a protein called stringent factor. Stringent factor is involved in the synthesis of ppGpp. The production of ppGpp results in increased amino acid biosynthesis and inhibited protein translation, the stringent response (Cashel and Rudd, 1987). *spoT* is a gene that has been associated with carbon starvation and the stringent response. The gene products of *spoT* catalyzes the degradation as well as the production of ppGpp and the subsequent stringent response (Cashel and Rudd, 1987).

Previous research has tried to explain the variability in bacterial responses associated with biofilms. There has been a surge of recent research into the effects of quorum sensing genes associated with biofilm formation. Production of *N*-Acyl homoserine lactones (AHL), the signaling molecule associated with quorum sensing, was found to be enhanced in naturally occurring biofilms (McLean et al., 1997). Davies et al. (1998) found that deletion of a *lasI* signaling gene associated with quorum sensing in *Pseudomonas aeruginosa* resulted in flat biofilms lacking the structural complexity of their wild type counterparts.

This work is an examination of the affects of one global regulator on biofilm formation. The exact genetic and biological cause of the discrepancy of adherence of the two bacterial strains was not studied. Future studies should include research of the products regulated by *rpoS* as well as other global regulators and their impact on biofilm formation.

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Table 1. Listing and genetic designations of *E. coli* strains used in study.

Strain Name	Genotype
ZK126[a]	W3110 *ΔlacU169*tna-2
ZK1000[b]	ZK126 Δ*rpoS::kan*
DS526[cd]	ZK126[λRZ5:*rpoS*742::*lacZ* (hybr.)]

[a] Connell et al. (1987)

[b] Bohannon et al. (1991)

[c] D.A. Siegele, Texas A&M University College Station, TX

[d] The λ phage that carries the *rpoS::lacZ* fusion was described by Lange and Hengge-Aronis (1994).

Table 2. Summary of CFU/ml means, sample size and standard deviation for each treatment and replicates for the biofilm growth.

Treatment	Replicate	n	Mean CFU/ml	Standard Dev.
	1	8	2.53×10^6	1.17×10^6
Parent	2	6	2.77×10^6	8.73×10^5
D=0.033h^{-1}	3	9	2.36×10^6	1.05×10^6
	4	9	2.64×10^6	1.73×10^6
	1	7	1.96×10^6	1.91×10^6
Parent	2	9	2.52×10^6	2.15×10^6
D=0.0083h^{-1}	3	8	1.26×10^6	5.39×10^5
	4	8	1.31×10^6	3.05×10^5
	1	7	1.96×10^6	1.29×10^6
rpoS$^-$	2	8	1.94×10^5	1.41×10^5
D=0.033h^{-1}	3	8	1.45×10^6	2.54×10^6
	4	8	1.14×10^6	2.23×10^6
	1	9	7.81×10^5	1.08×10^6
rpoS$^-$	2	8	1.94×10^5	2.57×10^5
D=0.0083h^{-1}	3	8	7.95×10^5	8.41×10^5
	4	7	1.43×10^6	1.70×10^6

Table 3. Summary of biofilm and planktonic means, ratio of adherence and standard errors. The mean for ZK126 at D = 0.033h^{-1} for the planktonic cell concentration and the ratio of the adherence ratio were calculated with n = 3. All other data was calculated with n = 4.

Treatment	Mean Planktonic CFU/ml	Mean Biofilm CFU/ml	Adherence Ratio biofilm/planktonic CFU/ml
ZK126	1.62×10^8	2.57×10^6	0.0168
D=0.033h^{-1}	$\pm 9.33 \times 10^7$	$\pm 1.28 \times 10^6$	$\pm$0.00910
ZK126	5.6×10^7	1.74×10^6	0.0363
D=0.008h^{-1}	$\pm 2.88 \times 10^7$	$\pm 8.68 \times 10^5$	$\pm$0.00133
ZK1000	1.76×10^8	8.94×10^5	0.0054
D=0.033h^{-1}	$\pm 8.81 \times 10^7$	$\pm 4.47 \times 10^5$	$\pm$0.00245
ZK1000	1.66×10^8	8.00×10^5	0.0051
D=0.008h^{-1}	$\pm 8.3 \times 10^7$	$\pm 4.0 \times 10^5$	$\pm$0.00152

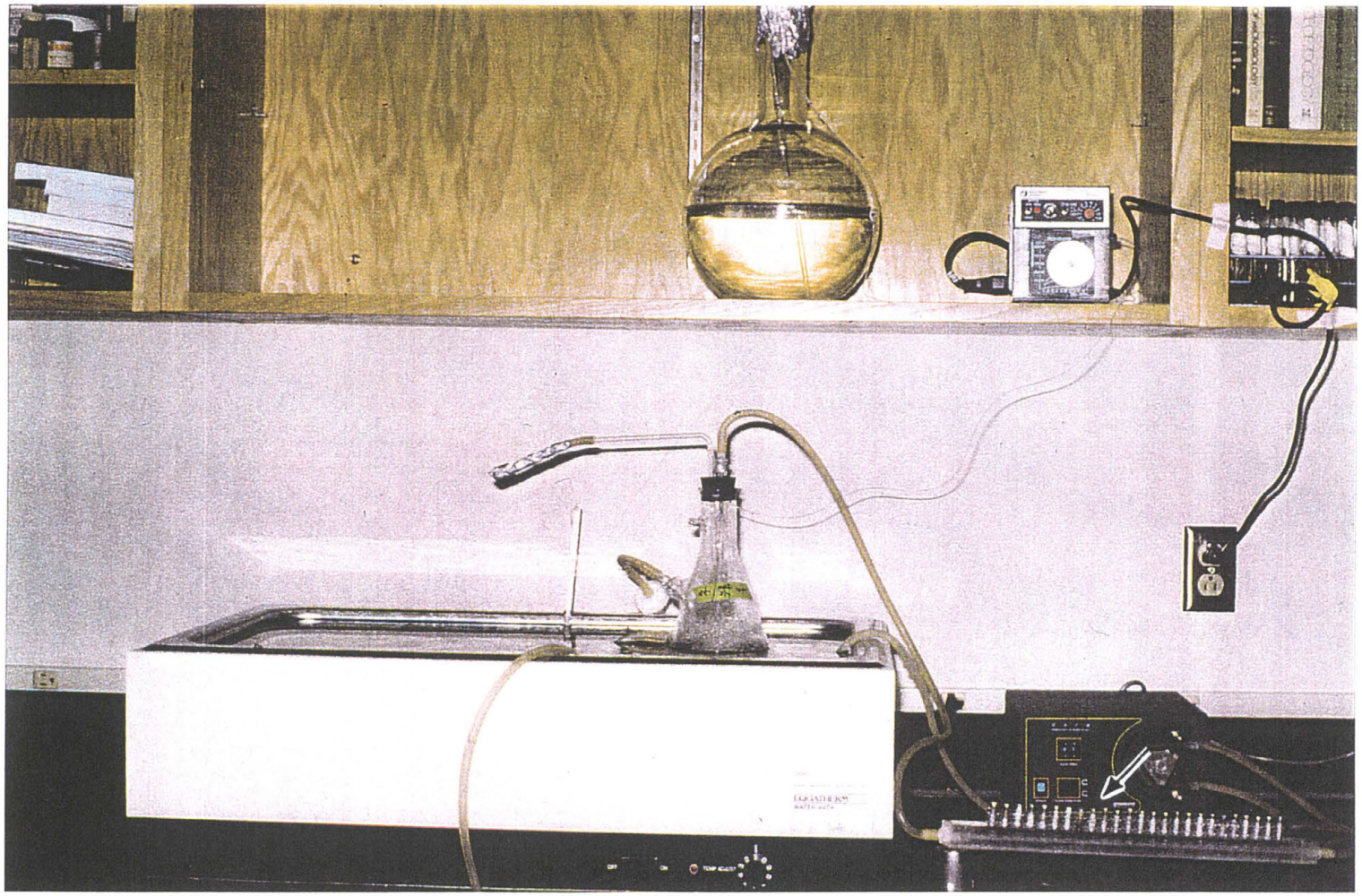

Fig. 1 Photograph of experimental apparatus including: fresh media flowing through pump to chemostat, chemostat in 37°C water bath, aeration from the rear, waste flowing to receptacle below, and Modified Robbins Device connected by peristaltic pump marked with an arrow.

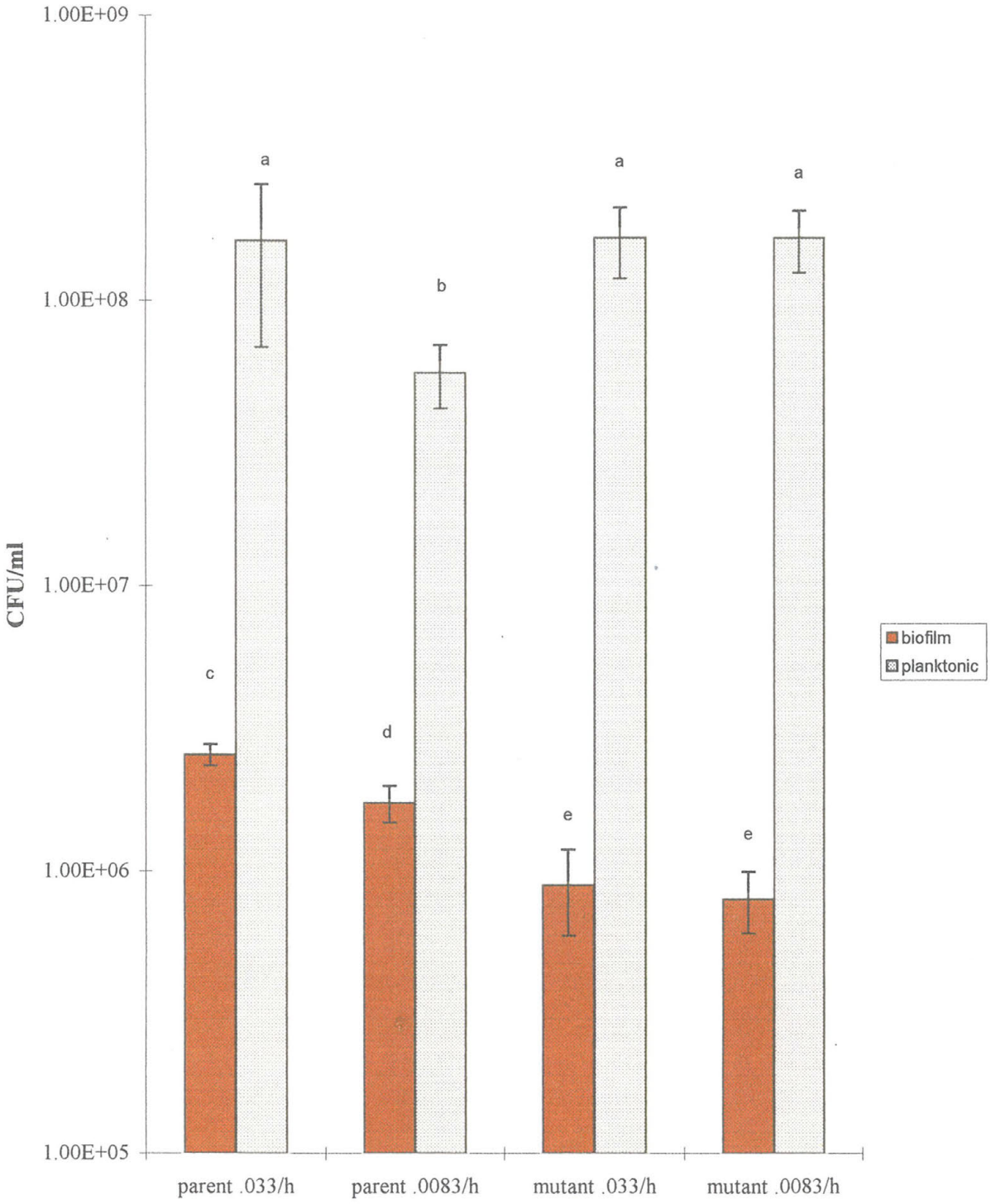

Fig. 2. The mean CFU/ml of the planktonic and adhered cells for *E. coli* ZK126 and *E. coli* ZK1000. Both dilution rates are illustrated for each strain of bacteria. Different letters represent significantly different means by Fisher's PLSD test ($p < 0.05$). Error bars represent standard error.

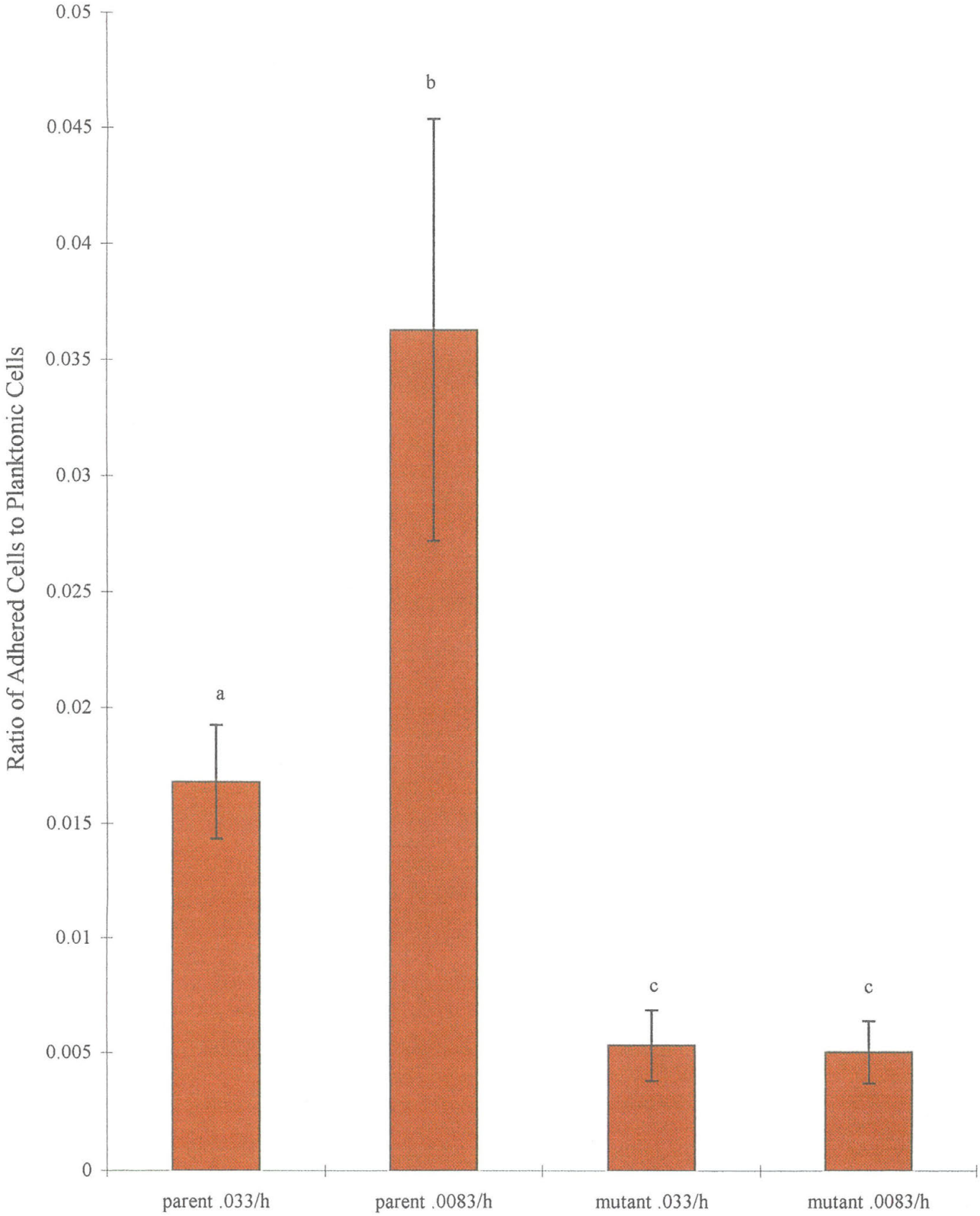

Fig. 3. The mean ratio of cellular density of the biofilm over the planktonic culture for both *E. coli* ZK126 and *E. coli* ZK1000 at the high and low dilution rates. Different letters indicate significantly different means by Fisher's PLSD test ($p < 0.05$). The error bars represent standard error.

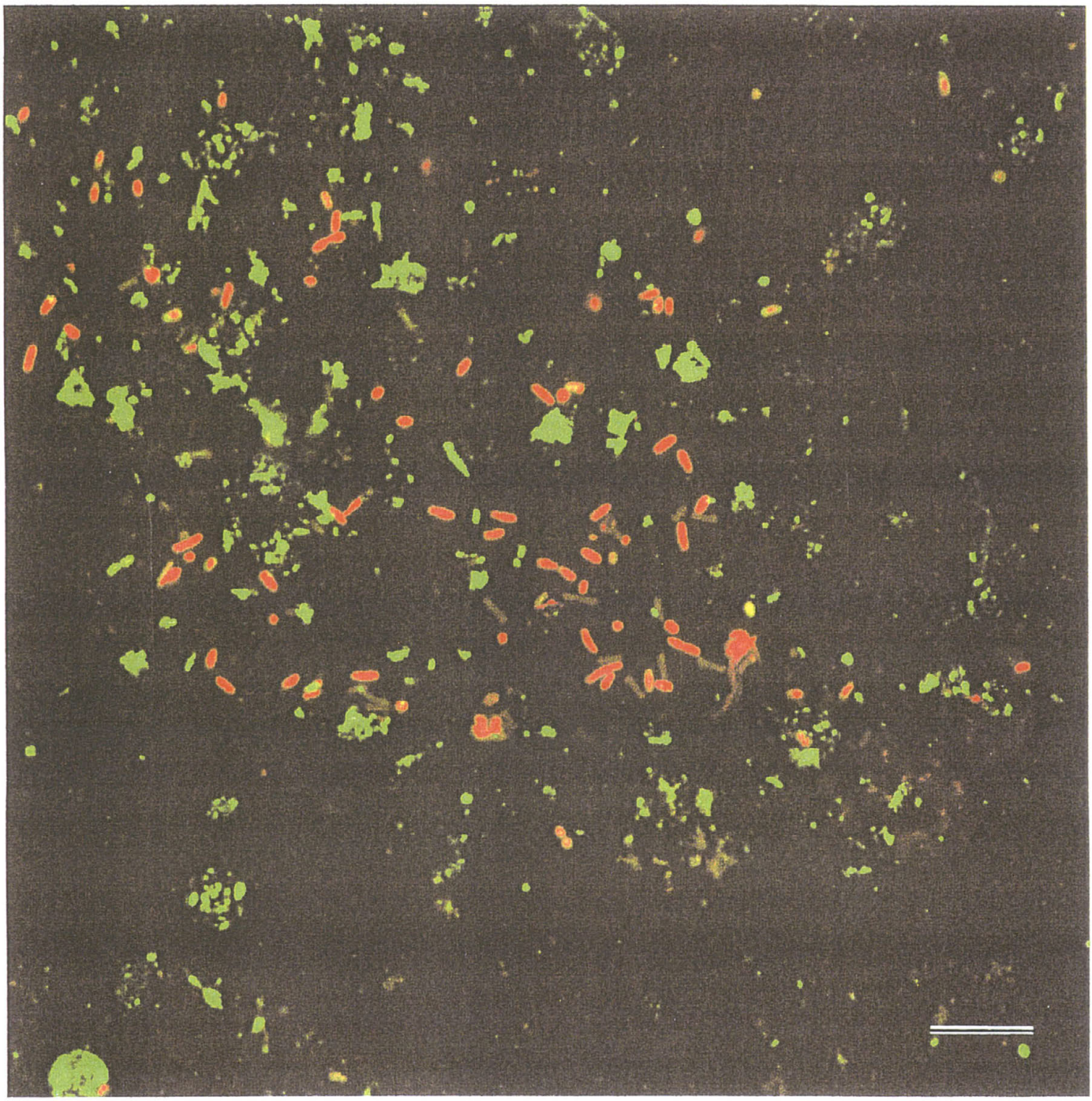

Fig. 4. SCLM image of biofilm formed by *rpoS* deleted strain. Biofilm grown in flow cell on glass slide. Scale bar represents 2μm.

Fig. 5. Vertical image of biofilm grown by *rpoS* deleted strain. Biofilm grown in flow cell on glass slide. Scale bar represents 2μm.

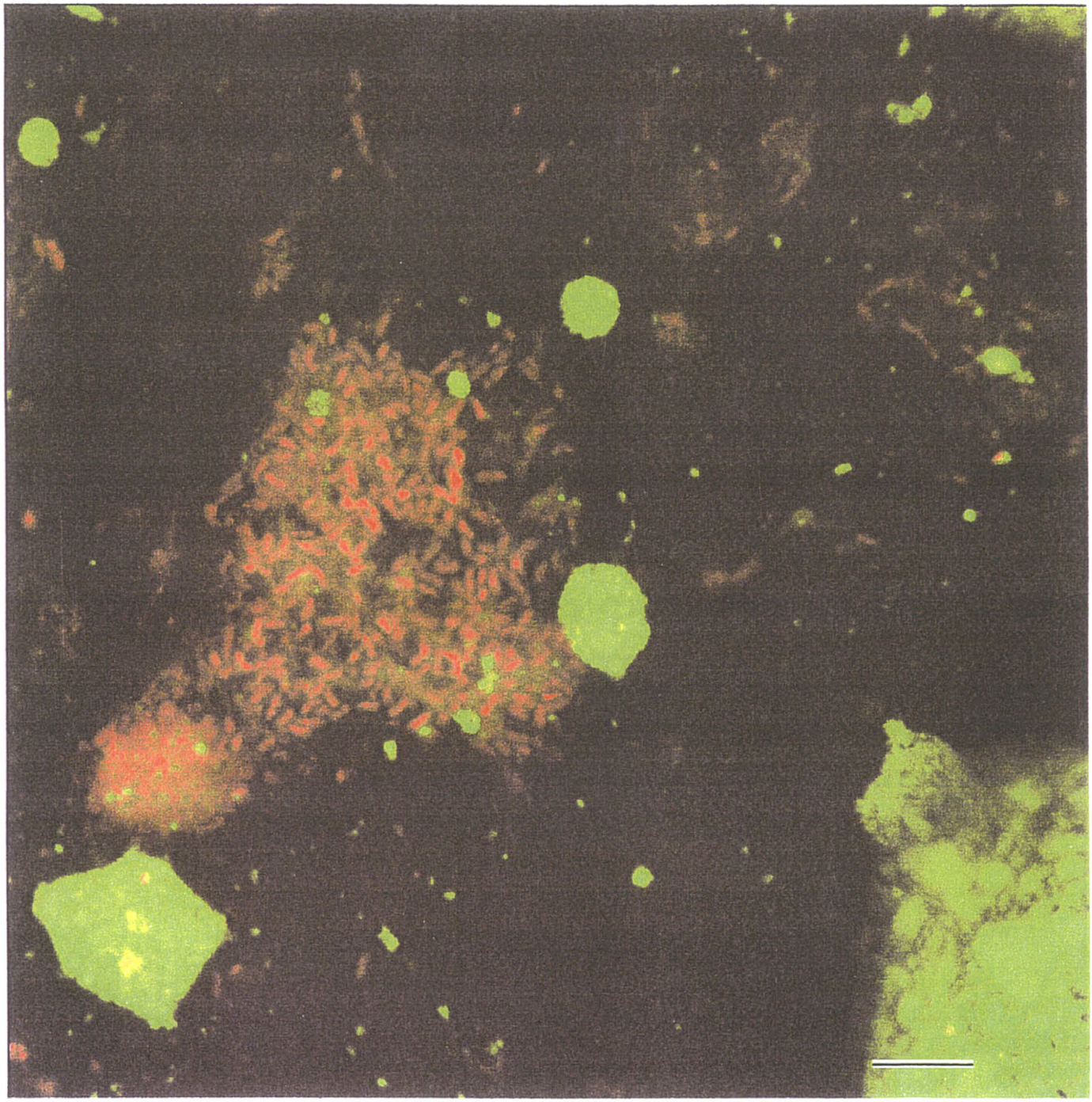

Fig. 6. SCLM image of biofilm formed by parental strain. Biofilm grown on glass slide in flow cell. Scale bar represents 2μm.

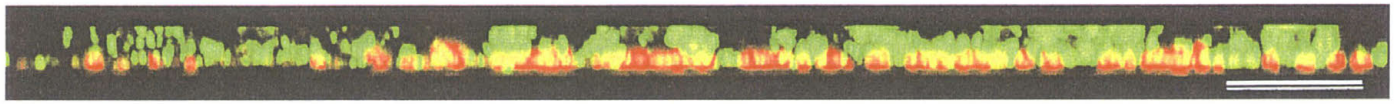

Fig. 7. SCLM vertical image of biofilm formed by parental strain. Biofilm formed on glass slide in flow cell. Scale bar represents 2μm.

Table 4. Nanomols of ONPG cleaved min^{-1} $cell^{-1}$.

Growth Environment	nmol ONPG/ min cell
Planktonic	3.08×10^{-6}
Biofilm	3.04×10^{-6}