A mutation in rpoS enhances biofilm formation in Escherichia coli during exponential phase of growth

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Abstract

Biofilm formation in Escherichia coli is a process that involves slow growth and stress conditions where several molecular signals and growth phase regulated genes are involved. Here we show that rpoS mutant strains (defective in the stress regulator σS) exhibit an increased production of biofilm, especially in the exponential phase of growth. Our results indicate that rpoS mutants produce an extracellular factor that promotes the production of biofilm during the exponential phase of growth. Thus, RpoS plays an important role in the regulation of the amount and initiation of biofilm formation in E. coli. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: RpoS; Stationary phase; Biofilm; Extracellular factor; Escherichia coli

1. Introduction

Bacterial lifestyle in natural environments occurs normally as a complex group of organisms attached to a surface called a biofilm [1]. Analysis of biofilms has revealed that they are relevant in medical, industrial and environmental settings. Biofilm-associated bacteria generally possess increased resistance to antimicrobial agents [2,3]. In addition, biofilms can be formed in industrial settings and could be a latent source of infection or promote the clogging of industrial pipes [4,5]. Biophysical, structural, and chemical studies performed thus far have led to a model where bacteria form microcolonies surrounded by copious amounts of exopolysaccharides. Between the microcolonies are water-filled channels that serve to promote the influx of nutrients and the efflux of waste products [5,6]. Escherichia coli has been a useful model for the study of genetic determinants of biofilm formation [7–9]. Mutant cells lacking flagella (type I pili) (fliC or flhD) or affected in motility (motAB) are severely impaired in the first steps of biofilm formation [8]. However, little is known about the genes and signals involved in the expansion and disruption of biofilms in E. coli. Mutant strains of Vibrio cholerae El Tor, and Staphylococcus epidermidis affected in the synthesis of capsular polysaccharide/adhesins (PS/A) were shown to be defective in biofilm formation [10–12]. Interestingly, the expression of type I pili genes in E. coli and PS/A in V. cholerae El Tor and S. epidermidis is growth phase dependent [13].

The master regulator of stationary phase-specific gene expression in E. coli is the product of the rpoS gene, RpoS (σS), which controls the synthesis of more than 50 proteins during the transition from the exponential to the stationary phase of growth, starvation, and osmotic shock [14]. Increasing evidence has suggested that RpoS may have an important role during the exponential phase of growth and that it may also function as a negative regulator [15–18].

The importance of RpoS in biofilm formation by E. coli has been suggested previously [19,20]; however, the role σS may play in biofilm metabolism is still unclear. One report suggested that rpoS expression is enhanced during biofilm development [20], others found similar levels of expression in planktonic and biofilm-constituent cells [19]. In this communication, we report on the role of...
RpoS in biofilm formation of E. coli using the O’Toole and Kolter protocol [21]. Our results strongly suggest that RpoS plays a key role in controlling the amount of biofilm formed, in determining the initiation of biofilm formation, and in the prevention of the synthesis of an extracellular factor that apparently promotes the formation of biofilm during the exponential phase of growth.

2. Materials and methods

2.1. Bacterial strains

The E. coli K12 strains used in this study are described in Table 1. Genetic crosses used to introduce the rpoS::Tn10 mutation were performed using bacteriophage P1vir-mediated transduction [22].

2.2. Media, growth conditions and biofilm assays

Cells were grown at 37°C in Luria-Bertani (LB) medium [22], with tetracycline (Tc, 25 μg mL⁻¹) when appropriate. Overnight cultures were grown in 10-ml test tubes containing 3 ml LB medium at 200 rpm. The presence of the rpoS::Tn10 allele was confirmed by Southern hybridization [23] (Fig. 1). Growth curves were determined by subculturing an overnight culture of the relevant strain into fresh LB medium (1/100 dilution) and growing the culture at 37°C under the same conditions of biofilm assays. OD₆₀₀ readings were taken over time using a Beckman DU-65 spectrophotometer.

Biofilm assays were carried out using 96-well, non-tissue culture-treated polystyrene chloride dishes (Falcon 3911 microtest III flexible assay plates) using the O’Toole and Kolter protocol described previously [21]. Every experiment was performed in triplicate at least three times to confirm the results. Microtiter dishes were covered with a plastic box to prevent evaporation. Quantiﬁcation of attached cells to the wells was performed as described in the Kolter protocol described previously [21]. Every experiment was performed in triplicate; results were conﬁrmed in at least three independent experiments.

2.3. β-Galactosidase assays

β-Galactosidase activity speciﬁed by the chromosomal Φ(rpoS::lacZ) translational fusion of strain RO91 (Table 1) was measured as described by Miller [22]. Samples of cultures at different times were harvested and suspended in 2.5-4 ml of Z buffer [22] and stored on ice. β-Galactosidase activity in CHCl₃ and sodium dodecyl sulfate-permeabilized cells was measured by monitoring the hydrolysis of o-nitrophenyl β-d-galactopyranoside. Activities are expressed in terms of the OD₆₀₀ of cell suspensions by the formula of Miller [18]. Each culture was assayed in triplicate; results were conﬁrmed in at least three independent experiments.

2.4. Purification of chromosomal DNA and Southern hybridizations

Chromosomal DNA from bacterial cells of different strains was purified using the chromosomal DNA purification kit from Promega. Quantiﬁcation of DNA was carried out spectrophotometrically at 260 nm. Twenty micrograms of DNA were then digested with BamHI for 6 h at 37°C. Digested DNA was run on a 1% agarose TBE 1× gel, transferred to a Hybond N+ membrane (Amersham) by capillary blotting [23] and then hybridized with a PCR-generated 700-bp DNA probe containing 500 bp of the 5’ end of rpoS and 200 bp upstream from the translational start site (ATG) (Fig. 1). Hybridization was carried out using the ECL direct nucleic acid detection system from Amersham.

3. Results and discussion

3.1. E. coli rpoS mutants form higher amounts of biofilm

The formation of biofilm in E. coli has been described as a metabolic state where many stresses are present [6]. In order to determine whether or not the global stress regulator RpoS plays a key role in the biofilm formation in E. coli, we tested the ability of strain MC4100 and its isogenic rpoS::Tn10 mutant counterpart (strain JMH0039, Table 1; Fig. 1A,B) to form a biofilm on PVC microtiter dishes following the standard O’Toole and Kolter protocol [21].

As shown in Fig. 1C, strain JMH0039 formed 3–5-fold greater amounts of biofilm than strain MC4100 after 48 h of incubation at 37°C. This was in contrast to a previous report where an rpoS mutant was shown to form less biofilm than its wild-type counterpart [19]; however, it is important to note that those experiments were carried out in cells grown in chemostats and the biofilm formation was tested on glass, additionally, the time of biofilm formation was not reported [19]. To try to clarify this phenomenon, and to test if our observations were true for
other *E. coli* strains, we used two additional pairs of strains to test for their ability to form biofilms. The strains W3110 and CF1648, and their *rpoS*::Tn10 corresponding mutants (WG01 and CFG02 respectively, 1; Fig. 1A,B) showed a similar pattern of biofilm formation to that of the MC4100 and JMH0039 strains (their respective *rpoS* mutant produced approximately 3-6-fold more biofilm; data not shown). These results confirmed that, under our experimental conditions, an *rpoS* mutant produces more biofilm after 48 h of incubation than its wild-type counterpart. It should be noted that Jishague and Ishihama [24] have reported variations in the σ5 subunit of the RNA polymerase in stocks of strain W3110; therefore, we tested several W3110 strains from 10 different laboratory stocks and found that seven of them are indeed *rpoS* mutants due to a deletion event (data not shown). However, the other three were *rpoS*+. The functionality of this gene was confirmed by monitoring a *bolA*-*lacZ* fusion [25]. The W3110 strain used in this study was confirmed to be *rpoS*+. As expected, W3110 strains carrying an *rpoS* mu-

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<th>Strain</th>
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<tr>
<td>CF1648</td>
<td>MG1655 (prototroph)</td>
<td>[17]</td>
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<tr>
<td>CFG02</td>
<td>CF1648 <em>rpoS</em>::Tn10 P1(JMH0039)X CF1648</td>
<td>This work</td>
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<tr>
<td>MC4100</td>
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<td>Michael Casadaban</td>
</tr>
<tr>
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<td>MC4100 <em>rpoS</em>::Tn10</td>
<td>Laboratory collection</td>
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<tr>
<td>W3110</td>
<td>F− *λaw-ir(rrnD-rrnE)1 rph-1</td>
<td>Genetic Stock Center</td>
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<tr>
<td>WG01</td>
<td>W3110 <em>rpoS</em>::Tn10 P1(JMH0039)X W3110</td>
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<td>R091</td>
<td>MC4100 Φ(<em>rpoS</em>−-<em>lacZ</em>)</td>
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![Fig. 1. Construction of *rpoS*::Tn10 mutants and biofilm formation by strains MC4100 and JMH0039 (*rpoS*::Tn10) after 48 h of incubation in LB medium. Chromosomal DNA from different strains and their *rpoS*::Tn10 derivatives was digested with BamHI (Bm), run on a 1% TBE agarose gel and then hybridized with a 700-bp probe containing 500 bp of the 5'-end of RpoS (A, Fragment 1). The presence of a Tn10 element was confirmed by a change in the hybridization pattern (B) indicating an insertion event (A, Fragment 2). Biofilm formation of strain MC4100 and its *rpoS*::Tn10 derivative was quantified by measuring the amount of CV attached to the surface of the PVC wells (C). One arbitrary unit corresponds to the biofilm formed by strain MC4100. The experiments were carried out in triplicate at least three times; the standard deviation (S.D.) is indicated. Wells from a typical experiment are shown.](image-url)
3.2. An rpoS mutant forms biofilm earlier than the wild-type strain

It has been suggested that the main role of RpoS is the regulation of many genes at the onset of the stationary phase of growth [14]; however, growing evidence indicates that RpoS has a broader role in bacterial metabolism and that its regulatory role is not limited to the stationary phase [14,18]. In order to determine if the increased amounts of biofilm seen in an rpoS mutant was present only during the stationary phase of growth (48 h of incubation) we tested strains MC4100 and JM0039 for their ability to form biofilm throughout the growth cycle. As shown in Fig. 2, growth curves of both strains were indistinguishable (Fig. 2C).

Strain MC4100 exhibited a significant increase in biofilm formation after 14 h of growth (15 times higher than the amount seen after 2 h of incubation; Fig. 2A). The corresponding OD_{600nm} was 2.5 (Fig. 2C). By contrast, strain JM0039 displayed detectable biofilm formation after 6–8 h of incubation (4–8 times higher than the amount seen after 2 h of incubation; Fig. 2A). The corresponding OD_{600nm} was 0.8–1.0 (Fig. 2C). This suggests that the presence of RpoS could be preventing biofilm formation during the exponential phase of growth. Negative regulation by RpoS has already been reported [16]. These results were later confirmed using different strains (Table 1). It is important to note that the starting time for biofilm formation differed in the several strains tested. However, in all cases, the rpoS mutant strains produced biofilm earlier (and always in the exponential phase of growth) than their parental wild-type strains. Additionally, in all cases rpoS mutant strains made more biofilm than their isogenic wild-type parental strain (data not shown). The variability of biofilm formation in different E. coli strains has already been reported [8].

In order to study the expression of rpoS under the biofilm-forming conditions tested here, we used merodiplid strain RO91 [26] that bears a chromosomal \( \Phi(rpoS'\text{-}lacZ) \) translational fusion to monitor rpoS expression throughout the growth cycle under our experimental conditions. As shown in Fig. 2B, most \( \beta \)-galactosidase activity was observed at the onset of the stationary phase followed by an abrupt decrease. This is in good agreement with previous reports [26]. Taken together, these results suggest that the negative role of RpoS on biofilm formation is more evident during the exponential phase of growth and that other factors are involved in the increase of biofilm formation after 20 h of growth since the expression of rpoS is extremely low after this period of incubation (Fig. 2B).
Fig. 3. Biofilm formation of strains MC4100 and JMH0039 growing under different culture conditions. Cells were cultivated with spent media from the indicated cultures. Biofilm formation was determined after 48 h of incubation. In this figure, one arbitrary unit corresponds to the amount of biofilm formed by strain MC4100 in LB medium (A). The experiments were carried out in triplicate at least three times with a variation of less than 20%. Results shown are those of a typical experiment.

3.3. An extracellular factor is produced in the rpoS mutant that increases the formation of biofilm in the exponential phase of growth

It is known that biofilm maturation can be regulated by molecular signals (quorum sensing) that regulate gene expression by a number of mechanisms, including modulating the activity of members of the LuxR family, interacting with two-component systems or inhibiting phosphatases [27,28]. In the light of our results, we wondered if the reason for the earlier production of biofilm in the rpoS mutants was due to the production of an extracellular factor or molecular signal that could be enhancing the production of biofilm. As shown in Fig. 3, after 48 h of incubation, the total amount of biofilm formed by the MC4100 strain was 5-fold less than that produced by JMH0039 (A and B respectively). When the spent medium from an overnight culture of the MC4100 strain was used, there was a fall in the amount of biofilm formed (compare D and E with A and B); by contrast, when the spent medium from an overnight culture of the JMH0039 strain was used, there was a 3-fold increase in biofilm formation for MC4100 and 1.6-fold for JMH0039 cells (compare C and F with A and B). These results were also tested in the two pairs of strains described in Table 1. In all cases, the spent medium from an overnight culture of the rpoS mutant strains increased the amount of biofilm formation (data not shown). Our results strongly suggest that the rpoS mutant strains produce an extracellular factor that promotes the formation of biofilm. In order to test whether or not this factor was produced during the exponential phase of growth, we used spent medium from cultures of MC4100 and JMH0039 growing exponentially. As shown in Fig. 3, the spent medium from exponentially growing MC4100 cells showed an inhibitory effect on the formation of biofilm (compare H and I with A and B); stunningly, when the supernatant from JMH0039 cells growing exponentially was used, there was an important increase in biofilm formation (G and J). These results support the idea that an rpoS mutant is producing an extracellular factor even during the exponential phase of growth that promotes the formation of biofilm.

3.4. Conclusions

We have shown that an rpoS mutant produces higher amounts of biofilm than its wild-type counterpart. This could be due to a cumulative effect since we noted that an rpoS mutant starts to produce biofilms earlier in the growth curve. We still do not know the reason or the mechanism for negative regulation of biofilm formation exerted by RpoS during exponential growth; however, we note that RpoS has already been reported to be involved in negative regulatory processes [16]. A clue to the mechanism may be that the increased production of biofilm by the rpoS mutant seems to be mediated by an extracellular factor that is produced during the exponential phase of growth in an rpoS mutant. Several genes have been identified that are strongly induced by the accumulation of self-produced extracellular signals; interestingly, two of them were dependent on RpoS for growth phase expression and response to extracellular factors [29]. rpoS expression is itself stimulated by the presence of a factor in conditioned medium [30,31]. So far, we do not know the identity of the extracellular factor that increases the production of biofilm. Recent studies by Surette and Bassler [32] have revealed that E. coli produces a signal that can substitute for AI-2, one of two Vibrio harveyi signals that control luminescence gene expression. The E. coli signal was heat labile, produced at mid-exponential phase of growth, and degraded at stationary phase. Our results cannot rule out this possibility. In any case, our results suggest that RpoS prevents the production of a factor needed for the formation of biofilm during the exponential phase of growth.

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