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Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms

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Abstract

Bacteria growing in biofilms experience gradients of environmental conditions, including varying levels of nutrients and oxygen. Therefore, bacteria within biofilms may enter distinct physiological states, depending on the surrounding conditions. In this study, *rpoS* expression and RpoS levels were measured as indicators of stationary phase growth within thick continuously-fed *Pseudomonas aeruginosa* biofilms. The level of *rpoS* expression in a 3-day-old biofilm was found to be three-fold higher than the average expression in stationary phase planktonic culture. RpoS levels in biofilms, indicated by immunoblot analysis, were similar to levels in stationary phase planktonic cultures. In planktonic cultures, oxygen limitation did not lead to increased levels of RpoS, suggesting that oxygen limitation was not the environmental signal causing increased expression of *rpoS*. These results suggest that bacteria within *P. aeruginosa* biofilms may exhibit stationary phase characteristics even when cultured in flow conditions that continually replenish nutrients. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: RpoS; Biofilm; Gene expression; Protein level; Stationary phase; Oxygen limitation; *Pseudomonas aeruginosa*

1. Introduction

When microorganisms attach to surfaces and form biofilms, they often acquire remarkable protection from killing by antimicrobial agents [1]. This phenomenon frustrates efficient control of fouling and corrosion problems in industrial settings [2] and contributes to the persistence of many chronic infections [3]. The mechanisms leading to reduced susceptibility of microorganisms to antimicrobials in biofilms are not clear. One hypothesis to explain biofilm resistance is that some of the microorganisms in a biofilm enter a starved or stationary phase state. The slow growth of these bacteria may render them less susceptible to antimicrobial challenges [4–6].

Spatial gradients in physiological status of bacteria within biofilms have been observed using a variety of physiological probes and indicators [7–11]. For example, in a *Pseudomonas aeruginosa* biofilm model system, Xu et al. [10,11] demonstrated that with a 110-μm biofilm, only bacteria in the upper 30 μm exhibited de novo protein synthesis. Oxygen penetration was limited to a zone of similar dimension [11]. These observations led us to hypothesize that thick biofilms likely harbor slower growing bacteria and bacteria that have entered a stationary growth phase. The purpose of this work was to test this hypothesis by investigating gene expression and protein levels of the stationary phase sigma factor, RpoS, in mature continuously-fed *P. aeruginosa* biofilms.

2. Expression of *rpoS* in continuously-fed *P. aeruginosa* biofilms exceeded *rpoS* expression levels in planktonic stationary phase cultures

The primary strain used in this study was the environ-

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mental isolate *P. aeruginosa* ERC1. Plasmid pMAL.S contained the *lacZ* gene under control of the *P. aeruginosa* *rpoS* promoter [12], and plasmid pMP220 contained *lacZ* with no promoter (plasmids were provided by A. Lazdunski). Plasmids were introduced into *P. aeruginosa* ERC1 by electroporation [13]. The resulting strains were maintained on LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g Bacto Agar, per liter) with tetracycline (Tc) at 100 μg ml^{-1}. To determine if *rpoS* expression in *P. aeruginosa* ERC1 was similar to published reports on *P. aeruginosa* PAO1 [12,14], β-galactosidase activity of ERC1 (pMAL.S) was determined at various growth phases. Specific β-galactosidase activity of cell extracts was determined spectrophotometrically (410 nm) by the conversion of the substrate *o*-nitrophenyl β-D-galactopyranoside (Sigma) to *o*-nitrophenol [15]. Activity was normalized to total cell protein as determined by the modified micro-Lowry method (Sigma). The specific activity of β-galactosidase was expressed as ΔA_{410} ml^{-1} (mg protein)$^{-1}$ min^{-1}. Fig. 1 shows the results of *rpoS-lacZ* expression during batch planktonic growth of *P. aeruginosa* (pMAL.S) and *P. aeruginosa* (pMP220) at 37°C in LB broth. The strains grew at similar rates and both strains entered stationary phase approximately 10 h after subculturing. The background level of β-galactosidase activity expressed by the strain harboring the control plasmid, pMP220, was low throughout the experiment. *P. aeruginosa* ERC1 containing the *rpoS-lacZ* fusion, pMAL.S, showed an increase in *rpoS* expression 3 h after subculturing (mid-exponential phase). β-Galactosidase activity increased throughout late exponential phase and peaked at the transition from exponential phase to stationary phase. The peak level of *rpoS* expression, normalized to total cell protein, was six times that of the initial level. Expression decreased in stationary phase to 60% of its peak level before largely disappearing at 32 h. These results were similar to results previously described for *P. aeruginosa* PAO1 [12], and therefore demonstrated that the *rpoS-lacZ* reporter construct can be used as an indicator of the entry of *P. aeruginosa* ERC1 into stationary phase.

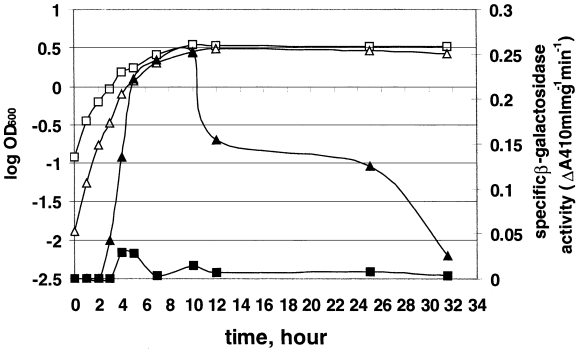

Fig. 1. *rpoS* expression in planktonic *P. aeruginosa* ERC1 (pMP220) and ERC1 (pMAL.S) during growth in full strength LB. The cell density is expressed as log OD_{600}, specific β-galactosidase activity is expressed as (A_{410} ml^{-1} mg^{-1} min^{-1}; (□) and (■) represent log OD_{600} and specific β-galactosidase activity of ERC1 (pMP220), respectively; (△) and (▲) represent log OD_{600} and specific β-galactosidase activity of ERC1 (pMAL.S), respectively.

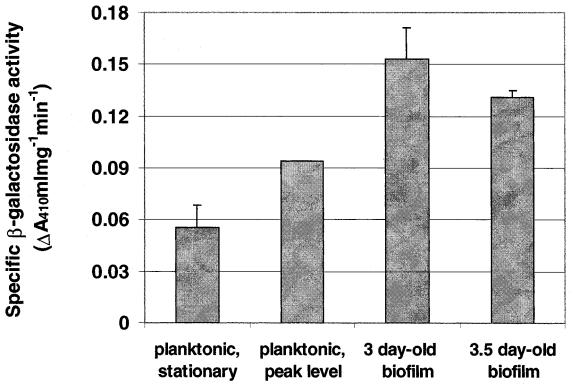

Fig. 2. Comparison of *rpoS* expression in stationary phase planktonic culture and biofilms grown in 1/5-strength LB medium. The first bar at the left is the average level of *rpoS* expression of planktonic culture within 20 h stationary phase. The peak level shown in the second bar from the left was the maximum value measured in all of the planktonic experiments. This peak level occurred at the onset of stationary phase.

To determine *rpoS* expression in biofilms, *P. aeruginosa* ERC1 containing the *rpoS-lacZ* reporter construct was cultured in continuous drip-flow reactors [11]. Medium, 1/5-strength LB broth (2 g tryptone, 1 g yeast extract, 5 g NaCl, per liter) with 100 μg ml^{-1} Tc, flowed over 1.2 by 7.8-cm stainless steel coupon at a rate of 50 ml h^{-1}. Experiments were performed at room temperature (22°C). To assay β-galactosidase activity of biofilm cells, biofilms were scraped into phosphate-buffered saline (PBS) and homogenized. This cell suspension was then disrupted with an ultrasonic cell disruptor and centrifuged. The supernatant was used for enzyme and total protein assays. The results were compared to planktonic cultures also grown in 1/5 l broth at 22°C in baffled flasks with 250 rpm shaking agitation. Biofilms cultured for 72–84 h had *rpoS* expression that exceeded the peak level of stationary phase planktonic culture (Fig. 2). These results demonstrated that *rpoS* is expressed in continuously-fed *P. aeruginosa* biofilms at levels comparable to or exceeding that of stationary phase planktonic cultures.

3. Immunoblot analysis demonstrates similar levels of RpoS in continuously-fed biofilms and stationary phase planktonic cultures

RpoS in *P. aeruginosa* biofilms was detected by immunoblot analysis. *P. aeruginosa* strains PAO1 and SS24 were used as positive and negative control strains for the RpoS immunoblot analyses. SS24 is a PAO1 derivative containing an *rpoS* deletion, with the *rpoS101*::*aacCI* allele [16]. Biofilms and planktonic cultures were grown in 1/5-strength LB at 22°C. Planktonic cells were centrifuged (5000×*g*, 10 min) and resuspended in PBS to yield an optical density (OD) of 2.0 at 600 nm. Biofilm cells were

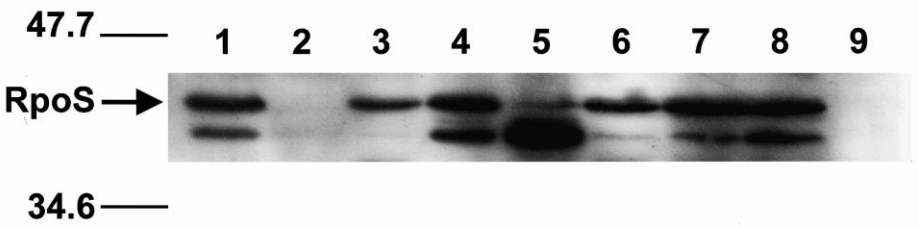

Fig. 3. Immunoblot blot analysis of RpoS levels in 1/5-strength LB grown planktonically and in biofilm cultures. The arrow indicates RpoS. The positions of molecular mass markers (in kDa) are indicated on the left. Lane 1: onset of stationary phase PAO1; lane 2: onset of stationary phase SS24 (*rpoS* deletion mutant); lanes 3–5: mid-exponential, onset of stationary phase, after 6 h stationary phase ERC1; lanes 6–8: 2.5-, 3-, 3.5-day ERC1 biofilms; lane 9: 3.5-day SS24 biofilm.

harvested by scraping cells from the metal coupons into 20 ml of PBS. Biofilm cells were homogenized and diluted in PBS to give ODs of 2.0 at 600 nm. Equal amounts of protein were loaded in each lane of the gel by starting with suspensions of bacteria of equal ODs. An aliquot of cells was assayed for total cellular protein. An equal volume of resuspended cells (1 ml) was pelleted, resuspended in 100 μl Nanopure water, and lysed with 100 μl of protein sample buffer [17]. Ten-μl aliquots of cell lysates were separated by SDS–PAGE using 4% stacking gels and 12% resolving gels [17]. Proteins were transferred to nitrocellulose membranes overnight at 4°C then probed with affinity-purified rabbit anti-RpoS antibodies (1:6000 dilution). Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Bio-Rad) was used as the secondary antibody. HRP was detected by chemiluminescence [18], and exposure of immunoblots to X-ray film for 15 s. X-ray film signal intensities were determined using a Bio-Rad Fluor-S Mulitimager (Bio-Rad Labs, Hercules, CA, USA).

Fig. 3 shows the results of RpoS levels in planktonic cultures and in biofilms. A 43-kDa band and a 39-kDa band were observed in strain PAO1 (lane 1). The 43-kDa band was identified as the *P. aeruginosa* RpoS on the basis of its match to the reported size of RpoS by other researchers [19–21]. This band was not found in the *rpoS* deletion mutant, SS24. In planktonic cultures, the 43-kDa band increased during exponential phase, and peaked at the transition from exponential phase to stationary phase. The 43-kDa band decreased during stationary phase (lanes 3–5, Fig. 3) and could not be detected following 2 h of stationary phase. A lower molecular mass (39 kDa) antibody-reacting band was observed in late stationary phase (lane 5). The 39-kDa band was likely a degradation product of RpoS. This band was not detected in planktonic or biofilm cultures grown in a glucose minimal medium whereas the 43-kDa band was detected in minimal medium-grown bacteria (data not shown). The results demonstrate similarities in *rpoS* expression, using the reporter construct and the levels of RpoS. Both the RpoS band and the putative RpoS degradation product band were detected in *P. aeruginosa* biofilms (lanes 6–8) at levels similar to that of the planktonic cultures at the onset of stationary phase (lane 4). However, in the biofilms the 43-kDa band was maintained longer than in planktonic cultures. One explanation for this pattern is that the biofilm contains some cells that are undergoing the transition from exponential phase into stationary phase and some cells that are in stationary phase. The biofilm may also contain some cells in exponential phase, but it is not possible to infer their presence based on the RpoS immunoblot banding pattern.

4. Increased RpoS was not due to anaerobic growth within biofilms

In earlier work, using similar *P. aeruginosa* biofilms, we

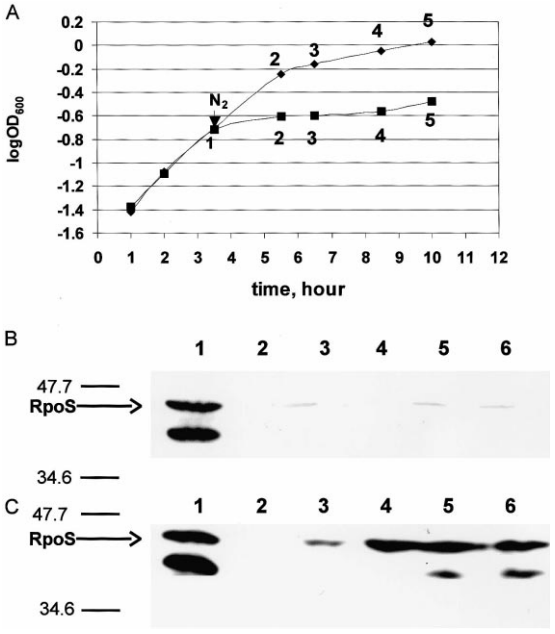

Fig. 4. Effect of oxygen limitation on growth and RpoS accumulation. A shows growth curves in LB medium under ambient air or nitrogen. The growth is expressed as log OD_{600}; (♦) represents the growth of ERC1 in LB under ambient air condition without interruption (control culture); (■) represents the growth of ERC1 in LB under ambient air condition for 3.5 h followed by continuous introduction of pure nitrogen. The arrow indicates when pure nitrogen was introduced. B and C are immunoblots of samples taken from the cultures above: (B) lane 1: stationary phase PAO1 as positive control, lanes 2–6 corresponding to samples 1–5 from experimental culture; (C) lane 1: stationary phase PAO1 as positive control, lanes 2–6 corresponding to samples 1–5 from control culture.

demonstrated that oxygen only penetrates into the upper 30–40 μm of the 100–110-μm biofilms [11]. This suggested the possibility that oxygen limitation could have led to the *rpoS* induction observed in the present study. To test this hypothesis, nitrogen was sparged into a mid-exponential phase culture of *P. aeruginosa* growing in LB medium at 22°C. Growth was quickly arrested in the nitrogen-sparged culture (Fig. 4), but not in a control culture exposed to atmospheric oxygen (Fig. 4). There was no loss of viability in the nitrogen-sparged culture during 6 h. In the control culture, RpoS accumulated as described previously. However, in the nitrogen-sparged culture, RpoS accumulation was not observed (Fig. 4). These results showed that oxygen limitation was not the environmental signal leading to the expression of *rpoS*. Oxygen is the only electron acceptor in this system. When oxygen is removed, bacteria no longer have means to generate ATP and are unable to synthesize new protein.

Bacteria are subject to an array of stresses within their natural environment, and it has been demonstrated previously that stationary phase (starving) cells survive these insults better than their exponential phase counterparts [22]. RpoS is thought to play a central role in development of starvation-mediated general resistance in *Escherichia coli* [23,24] and to contribute to *P. aeruginosa* resistance to some antimicrobial agents [16,25]. Deletion of *rpoS* altered the structural properties of biofilms formed by *E. coli*, although that same study did not detect differences in *rpoS* expression between planktonic and biofilm bacteria [26]. Quorum sensing was thought to control *rpoS* expression [12], though a recent report refutes this result [27]. It is interesting to note that *rpoS* was strongly expressed from cystic fibrosis patients with chronic *P. aeruginosa* lung infection [28]. Our demonstration of *rpoS* gene expression and RpoS accumulation in *P. aeruginosa* biofilms may aid in explaining why these biofilms are so resistant to antimicrobial agents and can establish persistent infections.

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