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Divergent roles of RpoS in *Escherichia coli* under aerobic and anaerobic conditions

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Abstract

Escherichia coli exhibited different levels of *rpoS* expression and general stress resistance under aerobiosis and anaerobiosis. Expression measured using reporter gene fusions and protein levels was lower under anaerobic conditions. Consistent with earlier findings, *rpoS* mutants were selected in aerobic nutrient-limited cultures but *rpoS* mutants were not enriched under anaerobiosis. This result suggested that, despite its decreased level, RpoS had a function under anaerobic conditions not essential under aerobiosis. Competition experiments between *rpoS*$^{+}$ and *rpoS* bacteria confirmed the advantage conferred by RpoS under anaerobiosis. In contrast, stress resistance assays suggested RpoS made a greater contribution to general stress resistance under aerobiosis than anaerobiosis. These results indicate a significant, but different role of RpoS in aerobic and anaerobic environments.

Keywords: Stress resistance; σ^{S}; Anaerobic regulation; Chemostat culture; Oxygen

1. Introduction

The primary habitat of *Escherichia coli* is the gastrointestinal tract, and as such, much of its existence is spent under anaerobiosis. Despite this, stress regulation under anaerobic conditions is poorly understood. The literature contains conflicting reports on the effect of anaerobiosis on the expression of the master regulator of the general stress response, RpoS [1]. Mulvey et al. [2] found that anaerobiosis did not affect the pattern of expression of *rpoS* or the RpoS-dependent gene *katE* in *E. coli*. In contrast, Ševčík et al. [3] reported that *rpoS* transcription in *Salmonella typhimurium* was greater under aerobic than microaerobic conditions. In support of this latter finding, Schellhorn and Hassan [4] reported that in *E. coli katE* was expressed under anaerobic conditions at levels that were approximately one-fourth of those found in aerobically grown cells. Similarly, in a DNA microarray study, Salmon et al. [5] found that there was >1.5-fold reduction in *rpoS* and *katE* expression under anaerobic conditions. To resolve the regulation of the general stress response under anaerobic conditions, an *rpoS* reporter fusion and RpoS protein levels were monitored in this study, as well as stress sensitivities under anaerobic conditions.

Recent results suggest that expression of RpoS is not always beneficial to bacteria and is indeed a selective disadvantage in some situations, such as nutrient limitation [6,7]. This is because RpoS is a sigma factor that steers transcription away from house-keeping genes dependent on another sigma factor (sigma 70 or RpoD [8]) to expression of unneeded stress–response genes [9,10]. Under aerobic conditions, *rpoS* mutations are strongly

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selected in nutrient limited cultures [6,7]. Here, we test whether the same situation arises under anaerobic conditions and investigate the surprising finding that *rpoS* mutants are not enriched under anaerobic conditions. The implications of these findings to our understanding of the role of RpoS under aerobiosis and anaerobiosis are discussed below.

2. Methods

2.1. Bacterial strains

All strains used in this study were derivatives of the *E. coli* K-12 strain MC4100 [11]. Strain BW2952 is an MC4100 derivative containing a *malG–lacZ* transcriptional fusion [7]. BW3709 is an *rpoS*::*Tn* 10 derivative of BW2952 [7]. RO91 has a single copy chromosomal *rpoS–lacZ* translational fusion which contains the turnover element susceptible to proteolysis (MC4100 λ RZ5 *rpoS742*::*lacZ* (hybr) [12]).

2.2. Growth medium and culture conditions

Aerobic chemostats were set up as described previously [7]. For anaerobic chemostats, the sparger was removed and cultures were flushed continuously with 100% N_2 at a flow-rate of 40 cc/min. The minimal medium used in aerobic chemostats [13] and anaerobic chemostats [14] was supplemented with 0.02% (wt/vol) and 0.04% (wt/vol) glucose, respectively. Chemostats were maintained at 37 °C and dilution rates were set at 0.1 h^{-1} (doubling time 6.9 h). The culture densities were between 1.9×10^8 and 2.1×10^8 ml^{-1}. Stationary phase batch cultures were grown in minimal medium A [13] supplemented with 0.2% glucose. Anaerobic batch cultures were maintained by ensuring that the headspace between the media and screw-lid was minimal.

2.3. Growth competition experiments

Chemostats were set up as described above. Independent chemostat cultures of each of the strains to be competed were grown for 16 h. The individual cultures were mixed in a 1:1 ratio prior to monitoring of competitive growth in the same medium at the same dilution rate as that in the inoculum cultures. Mixed cultures were sampled after 1, 6, 15 and 21 h and the number of bacteria was determined by total plate counts on Luria agar and selective media containing tetracycline.

2.4. Detection of rpoS mutants

rpoS partial and null mutants were distinguished from the wild type by staining colonies on Luria agar plates as described previously [7]. Plates were inoculated overnight at 37 °C and then left at 4 °C for 24 h before being flooded with concentrated iodine. Dark brown colonies were wild type, while pale brown or white colonies indicated partial or null mutants with different levels of glycogen.

2.5. β-Galactosidase assay

The β-galactosidase activity of the *rpoS–lacZ* translational fusion in RO91 was assayed by the method of Miller [13] using sodium dodecyl sulfate- and chloroform-treated cells. Assays were performed on chemostat and batch cultures 24 h after inoculation.

2.6. Catalase assay

The specific activity of KatE/HPII in day one glucose-limited chemostat cultures of BW2952 was determined by the method of Visick and Clarke [15].

2.7. Tolerance to external stress

Assays were conducted with 1-day old chemostat cultures. Resistance to high osmolarity was tested by determining the percentage of survivors after 3 h exposure to 2.5 M NaCl in MMA. Bacteria were plated directly onto nutrient agar plates, and dilutions were counted after overnight incubation at 37 °C. Starvation survival of bacteria in water was assessed after 26 h incubation at 25 °C.

3. Results

3.1. Expression of rpoS under aerobic and anaerobic conditions

The β-galactosidase activity of an *rpoS* fusion containing the posttranslational element subject to proteolysis (RO91, [12]) was determined under anaerobic and aerobic conditions. Previously, strain variation was observed in endogenous RpoS protein levels amongst *E. coli* K-12 lineages and non-domesticated isolates [16]. RO91 and other strains used in this study are derivatives of a strain (MC4100) that has high RpoS levels under aerobiosis [16]. In stationary phase batch culture of RO91, the β-galactosidase activity was 2-fold lower under anaerobic conditions compared to aerobiosis (Fig. 1(a)). The same trend was observed, but with a greater difference, between glucose-limited continuous cultures with the same controlled growth rate under aerobic and anaerobic conditions (Fig. 1(a)). RpoS protein levels were also determined in continuous culture bacteria by a quantitative immunoblot technique [17]. RpoS levels were more than 2-fold lower under anaerobiosis compared to aerobic conditions whereas the housekeeping

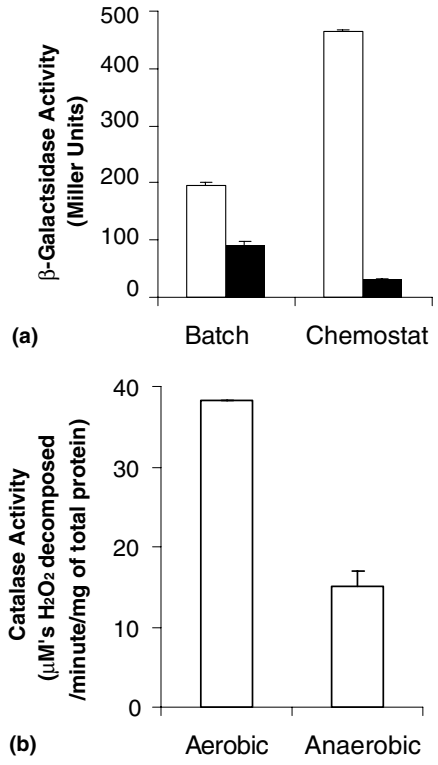

Fig. 1. RpoS expression and the specific activity of the RpoS-dependent catalase KatE under aerobic and anaerobic conditions. (a) The β-galactosidase activity of the *rpoS–lacZ* fusion RO91 was measured under aerobic (open bars) and anaerobic conditions (black bars). (b) The specific activity of KatE/HPII in BW2952. In (a) and (b) error bars represent the standard deviation according to 2 replicates.

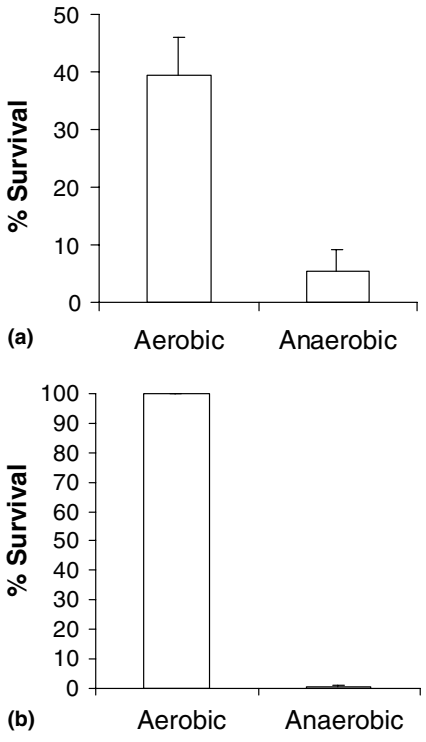

Fig. 2. Resistance of aerobic and anaerobic glucose-limited chemostat cultures of BW2952 to high osmolarity and starvation. (a) Survival after 3 h exposure to 2.5 M NaCl in MMA. (b) Starvation survival after 26 h of incubation in water at 25 °C. Survival was measured in plate counts and error bars represent the standard deviation according to 2 replicates.

sigma factor, RpoD, was unchanged (unpublished results). Another *E. coli* K-12 strain, MG1655, which previously displayed relatively low RpoS levels under aerobiosis [16], also displayed a decrease in RpoS protein levels under anaerobiosis (results not shown). Lowered RpoS expression under anaerobiosis is therefore not unique to MC4100. RpoS-regulated KatE/hydroperoxidase II catalase was also assayed by the method of Visick and Clarke [15] and anaerobic cultures displayed 2.5-fold lower expression than aerobic cultures (Fig. 1(b)). Altogether, these findings support the notion that anaerobiosis results in a decreased level of RpoS and a concomitant decrease in the expression of RpoS-dependent genes.

3.2. Stress resistance under anaerobic conditions

The results on RpoS levels led to the question of whether the general stress resistance of bacteria decreased under anaerobiosis. In this study, we find that resistance to high osmolarity (Fig. 2(a)) and starvation survival (Fig. 2(b)) was significantly lower under anaerobic conditions in cells growing at the same growth rate.

3.3. Is RpoS useful under anaerobic conditions?

RpoS expression is not always beneficial to bacteria because it reduces house-keeping gene expression through competition of σ factors for core RNA polymerase [9]. For example, RpoS expression is a disadvantage in aerobic glucose-limited chemostat cultures and *rpoS* mutants are fitter than *rpoS*$^+$ in this environment [7]. This was because decreased competition between RpoS and RpoD in *rpoS* mutants allowed increased expression of genes enabling higher rates of glucose transport from RpoD-dependent genes (14). To test whether the same situation arose under anaerobic conditions, anaerobic glucose-limited chemostat cultures were monitored for mutational sweeps to determine if RpoS loss was a selective advantage under anaerobic conditions. The anaerobic chemostat population remained 100% *rpoS*$^+$ compared to the aerobic population, which was 98% *rpoS* by day 4 (Fig. 3). Hence RpoS is not a hindrance to growth under anaerobic conditions.

To further determine if RpoS is an advantage or disadvantage under anaerobiosis, competition experiments were conducted between *rpoS* wild-type and null mutants mixed in the same culture under aerobic and anaerobic conditions. The *rpoS* wild-type strain rapidly

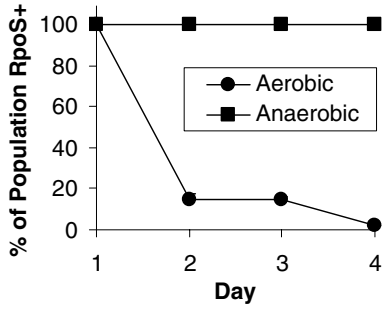

Fig. 3. Appearance of *rpoS* mutations in glucose-limited chemostat cultures of BW2952 grown under aerobic and anaerobic conditions. Culture samples were tested for RpoS phenotype by staining for glycogen. Error bars represent the standard deviation according to 3 replicates.

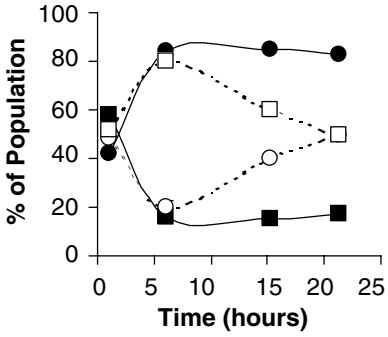

Fig. 4. Competitive fitness of *rpoS*$^+$ and *rpoS*$^-$ strains under aerobic and anaerobic conditions. Chemostat cultures were inoculated with an equal proportion of one day grown cultures of BW2952 (*rpoS*$^+$) (circles) and BW3709 (squares) under aerobic (---) and anaerobic (——) conditions.

out-competed the *rpoS* null mutant under anaerobic conditions, comprising 84% of the population within 6 h (Fig. 4). This was in contrast to aerobic cultures, which exhibited the opposite initial fitness pattern, with *rpoS* bacteria out-competing wild-type [7]. The transient trend in the aerobic cultures is because, as shown in Fig. 3, the starting *rpoS*$^+$ sub-population starts to acquire detectable *rpoS* mutations after about 24 h. The competitions were initiated after 16 h of equilibration in the chemostat, so the *rpoS* mutants beginning to accumulate in the wild-type background have the same fitness as the initial *rpoS* mutants and change the competitive pattern. Altogether, these results suggest that RpoS expression, although low, still confers an advantage under anaerobic conditions.

4. Discussion

Our results on the increased stress sensitivity under anaerobic conditions complement studies by Ganzle et al. [18] and Small et al. [19], who found anaerobically grown bacteria to be less resistant to acid than aerobically grown cells. The difference in stress resistance can be at least partly ascribed to the lower levels of RpoS under anaerobic conditions, as confirmed in this study. So how does this reconcile with the efficient survival of *E. coli* in the anaerobic regions of the gastrointestinal tract? Speculatively, the simplest answer may be that the level of homeostasis offered by the host in the colon does not require the same level of stress resistance as needed in an open environment, or with the more extreme acid challenge in the aerobic upper intestinal tract.

Our competition results between *rpoS*$^+$ and *rpoS* bacteria indicate that RpoS confers an advantage to growth under anaerobic conditions. It is not clear what functions are dependent on RpoS, but RpoS does regulate the expression of a pyruvate oxidase (PoxB) which has been suggested to play a role in the transition between aerobic and anaerobic conditions [20]. In fact, microarray results show an increase in PoxB expression under anaerobic compared to aerobic conditions [5]. Salmon et al. [5] also found an increase in expression of *hdeA*, which encodes the RpoS-dependent acid resistance system. This may contribute to the resistance of anaerobically-grown batch cultures to acids [18] and products of anaerobic fermentation. However, pH changes are not significant in buffered chemostat media and therefore this would not explain the maintenance of RpoS under the anaerobic competition conditions in Fig. 4. Nevertheless, the data suggest that RpoS has an unidentified beneficial role in anaerobic gene regulation.

Mutations in *rpoS* are not selected in nutrient-limited cultures under anaerobic conditions, in stark contrast to aerobic cultures growing at the same growth rate and with the same substrate. The simplest explanation of this finding is that RpoS has a physiological role in anaerobic gene expression, as considered above. It should also be noted that the lower level of expression of RpoS under anaerobiosis could alleviate the competition between RpoS and RpoD for RNA polymerase [8,9] and therefore reduce the selective pressure to lose RpoS. Beneficial mutations may also be occurring in other genes which might contribute to the fitness of the population under anaerobic conditions and offer a level of fitness that surpasses the advantage conferred by mutations in *rpoS*. Indeed, mutational adaptation of *E. coli* to glucose limitation has been shown to involve distinct transport changes in aerobic and oxygen-limited chemostats [21]. The PtsG transport pathway was particularly significant under O_2-limiting conditions and anaerobic cultures may not be under a selective pressure to lose RpoS if mutations in *ptsG* or other genes confer a higher rate of fitness to the bacterium than mutations in *rpoS*.

If indeed RpoS and RpoS-dependent gene expression is decreased in the anaerobic environment of the colon there may also be a concomitant decrease in virulence gene expression, since RpoS also influences virulence in several organisms (*Salmonella typhimurium*, [22];

Yersinia enterolitica, [23]; *Pseudomonas aeruginosa*, [24]; *Vibrio cholerae*, [25]). Although there are many inputs into regulation of these genes besides RpoS, the possible impact of low RpoS on virulence induction under anaerobic conditions is worth considering. In addition, the level of RpoS in anaerobic environments may have relevance to the food industry, as RpoS contributes to susceptibility in food treatments [26]. Modified atmosphere packaged (MAP) foods rely on anoxic gas mixtures and may result in a reduction in RpoS levels in contaminating organisms, and hence increase sensitivity to secondary stresses.

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