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ORIGINAL PAPER

Alkaline phosphatase as a reporter of σ^S levels and *rpoS* polymorphisms in different *E. coli* strains

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Abstract σ^S is responsible for the transcriptional regulation of genes related to protection against stresses and bacterial survival and it accumulates in the cell under conditions of stress, such as nutrient limitation. An increase in the levels of σ^S causes a reduction in the expression of genes that are transcribed by RNA polymerase associated with the principal sigma factor, σ^{70}. *phoA*, that encodes alkaline phosphatase (AP) is expressed under phosphate shortage conditions, and is also repressed by σ^S. Here we show that in a Pi-limited chemostat, accumulation of *rpoS* mutations is proportional to the intrinsic level of σ^S in the cells. Acquisition of mutations in *rpoS* relieves repression of the PHO genes. We also devised a non-destructive method based on the *rpoS* effect on AP that differentiates between *rpoS*$^+$ and *rpoS* mutants, as well as between high and low-σ^S producers. Using this method, we provide evidence that σ^S contributes to the repression of AP under conditions of Pi excess and that AP variation among different strains is at least partly due to intrinsic variation in σ^S levels. Consequently, a simple and non-destructive AP assay can be employed to differentiate between strains expressing different levels of σ^S on agar plates.

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Abbreviations

AP	Alkaline phosphatase
X-P	5-Bromo-4-chloro-3-indolyl-phosphate
p-NPP	*p*-Nitrophenyl-phosphate

Introduction

The core RNA polymerase of *E. coli* may interact with seven different types of sigma subunits, each responsible for the transcription of a specific set of genes (Maeda et al. 2000). The principal sigma factor is σ^{70}, which recognizes the majority of *E. coli* promoters, is particularly active in exponentially growing cells (Paget and Helmann 2003). The second most important sigma subunit, σ^S, accumulates in the cell during the stationary phase and under diverse stress conditions (Hengge-Aronis 2002). Many genes transcribed by the RNA polymerase holoenzyme coupled to σ^S ($E\sigma^S$) are related to bacterial protection from stresses. Some examples are *katE*, that encodes catalase and the genes *osmB, osmC* and *osmY*, which are involved in the cell response to hyperosmotic shock (Schellhorn and Hassan 1988; Vijayakumar et al. 2004; Weber et al. 2005). σ^S sometimes interacts with other transcription factors, such as cAMP/CRP, Lrp and IHF to control transcription (Colland et al. 2000; Lange et al. 1993; Taschner et al. 2006).

The accumulation of σ^S in the stationary phase and under stress leads to the competition between this sigma factor and σ^{70} for the binding to the core RNA polymerase, which results in partial inhibition of transcription of σ^{70}-dependent genes (Farewell et al. 1998; Maeda et al. 2000). This form of negative regulation is at the core of the SPANC

(*s*elf-*p*reservation and *n*utritional *c*ompetence) equilibrium, a trade-off pattern in which the transcription of genes related to protection against stress comes at the expense of the expression of growth-related genes (Ferenci 2005). Part of the σ^S/σ^{70} trade-off is an inverse relationship between stress resistance and metabolic capability, which varies in strains with different σ^S levels (King et al. 2004). For example, a strain with a high level of σ^S exhibits poorer growth on acetate as well as with many other carbon sources.

Since σ^S negatively affects the expression of σ^{70} and other sigma factors dependent genes, and since the cellular level of σ^S varies considerably among different *E. coli* strains (King et al. 2004), it is likely that the level of expression of a σ^{70}-dependent gene would be a good reporter for σ^S variation among different strains. A good candidate is the gene *phoA*, which encodes the periplasmic enzyme alkaline phosphatase (AP). AP expression is strongly inhibited by the presence of *rpoS* (Taschner et al. 2004) and is sensitively detected using chromogenic substrates.

phoA belongs to the PHO regulon, which comprises more than 40 genes and operons that are induced in response to phosphate (Pi) shortage (Wanner 1996). Genes that belong to the PHO regulon possess one or more PHO-box sequences at their promoter regions instead of the conventional −35 (Wanner 1996). PHO genes are regulated by a two-component system formed by the sensor protein PhoR and the regulator PhoB. When the concentration of Pi in the medium falls below a critical level, PhoR autophosphorylates and transfer its Pi moiety to PhoB, which in turn binds to the PHO-boxes and interacts with $E\sigma^{70}$ initiating the transcription of the PHO genes.

Here we show that both the basal and induced levels of AP vary according to the level of σ^S in different *E. coli* strains. That variation in the levels of σ^S among different strains is easily detected by plating the bacteria on plates containing the AP chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (X-P). We also show that cells growing in a chemostat under Pi-limiting conditions accumulate mutations in *rpoS* and that the rate of acquisition of these mutations is dependent on the intrinsic σ^S level of each strain.

Materials and methods

Strains and growth conditions

The strains used in this study were MC4100 (F^- *araD*139 (*argF-lac*)*U*169 *rpsL*150 *deoC*1 *relA*1 *thiA ptsF*25 *flbB*5301 *rbsR*) and its *rpoS*::Tn*10* mutant BW3323; MG1655 (*E. coli* K-12 wild-type) and its *rpoS*::Tn*10* mutant BW3708; M534 (EHEC isolate) and its *rpoS* mutant; ZK126 (W3110 Δ*lacU*169 *tna*2) and its *rpoS*::Tn10 mutant ZK1171. BW2952 is a derivative of strain MC4100 containing a *malG*::λp*lac*Mu55 insertion, which was used in chemostat evolution experiments (Maharjan et al. 2006). Strains BW3767, BW4002, BW4001, BW4003 and BW4029 evolved from the starting strain BW2952 in a glucose-limited chemostat. The former two are *rpoS* mutants and the three other strains are *rpoS*$^+$. ECOR 13, 14, 24, 25, 64 and 70 are *E. coli* isolates from different locations (Ochman and Selander 1984).

T-salts (Echols et al. 1961) plates contained 0.2% glucose, 1 mM KH_2PO_4 and 40 μg/ml X-P. LB plates were as described (Miller 1992). For the batch cultures, cells were grown overnight in liquid T-salts supplemented with 0.2% glucose and 5 mM KH_2PO_4 for the Pi-excess medium or 0.2 mM KH_2PO_4 for the Pi-limited medium at 37°C. Liquid T-salts supplemented with 0.2% glucose and 30 μM KH_2PO_4 were used to set up an 80 ml chemostat culture as described (Notley-McRobb et al. 2002). The dilution rate was set to 0.1 h^{-1}. Daily samples were taken for AP activity measurements and also for *rpoS* status of members of the population. AP activity was assayed directly on the chemostat sample. The *rpoS* status was determined by diluting the culture, spreading on LB plates and staining with iodine (see below).

AP assays

For the quantitative AP assay *p*-nitrophenyl-phosphate (*p*-NPP) was used as substrate as described (Spira et al. 1995). Briefly, overnight cells were washed and resuspended in unsupplemented T-salts; 0.1 ml of the cell suspension was mixed with 0.9 ml *p*-NPP (1 mg/ml in Tris pH 8) and incubated at 37°C until a yellow colour developed; the reaction was then stopped by the addition of 1 M Na_2HPO_4. The reaction products were briefly centrifuged to pellet cell remains and read in a spectrophotometer at 410 nm. AP activity units are defined as the increase in absorbance at 410 nm min^{-1} optical cell density at 600 nm^{-1}.

Alkaline phosphatase was qualitatively assayed by observing the colour development of bacterial patches growing on T-salts plates supplemented with 40 μg/ml X-P.

Detection of *rpoS* status

The level of *rpoS* was qualitatively assessed by staining glycogen with iodine solution as described (Hengge-Aronis and Fischer 1992). Patches of bacteria or diluted chemostat samples were grown overnight on LB plates, stored at 4°C for 24 h and then flooded with iodine. The intensity of the brown colour varies according to the level of σ^S in the cell (Notley-McRobb et al. 2002). *rpoS*$^+$ strains, show, therefore,

different intensities of brown, while *rpoS* mutants always remain very light.

Results and discussion

AP activity in different *E. coli* strains

To test if differences in the AP level found among *E. coli* strains are related to the intrinsic σ^S concentration in these bacteria, we assayed the AP activity of strains MC4100, MG1655, ZK126 and M534 and their isogenic *rpoS*::Tn10 derivatives. These strains were chosen despite having identical *rpoS* sequences, as they display significant differences in σ^S concentration; in both acetate and glucose-limited chemostats the level of σ^S was the highest in the strains M534 and MC4100, followed by ZK126 and MG1655 (King et al. 2004). The cells were grown overnight in minimal medium under Pi-limited and Pi-excess conditions and assayed for AP. Under Pi starvation conditions, the highest AP levels were observed for MG1655 (0.53 ± 0.06 units of activity) and ZK126 (0.56 ± 0.04), followed by M534 (0.33 ± 0.02) and MC4100 (0.18 ± 0.01). Disruption of the *rpoS* gene increased the level of AP in all four strains to similar levels (2.11 ± 0.23 for MG1655 *rpoS*; 1.77 ± 0.14 for ZK126 *rpoS*; 1.9 ± 0.03 for M534 *rpoS* and 1.92 ± 0.1 for MC4100 *rpoS*). However, the net increase in AP due to *rpoS* inactivation was 10.8-fold for strain MC4100, 6.7-fold for M534, fourfold for MG1655 and 3.2-fold for ZK126. Therefore, strains that naturally produce high levels of σ^S showed a stronger effect on AP expression, as high levels of σ^S strongly compete with σ^{70}, thus producing less AP (Taschner et al. 2004).

In the cells growing under Pi abundance conditions (AP basal level), no clear effect of *rpoS* on the level of AP was observed (not shown), with the possible exception of MC4100 in which disruption of *rpoS* increased the AP level by 2.2-fold (from 0.004 ± 0.001 to 0.009 ± 0.0007). The lack of a negative effect of *rpoS* on the non-starved cells could be due to the fact that expression of *phoA* under these growth conditions is so low that it is barely influenced by the σ^S/σ^{70} competition or because the assay with *p*-NPP is not sensitive enough to detect discrepancies in the basal level of AP.

Qualitative measurement of AP level in minimal plates

The wild-type strains and their *rpoS*::Tn10 mutants were patched on minimal medium plates supplemented with excess Pi and the highly sensitive AP-specific chromogenic substrate X-P. Figure 1a shows that the colour intensity developed by MC4100 was the weakest followed by M534, MG1655 and ZK126. At least for M534 and MC4100 the *rpoS::*Tn*10* mutants displayed a stronger blue colour than their wild-type parents. This implies that even under PHO-repressing conditions the negative effect of *rpoS* on AP synthesis can be discerned and that this effect was not noticed previously because the methods employed, such as AP assay with *p*-NPP or northern hybridization (Taschner et al. 2004), were not sufficiently sensitive to observe the effect of *rpoS* under Pi-excess.

To confirm the *rpoS* status of the strains, colonies were patched on LB plates and stained with iodine, which indicates *rpoS*-dependent glycogen accumulation in the cells (Hengge-Aronis and Fischer 1992; Notley-McRobb et al. 2002) (Fig. 1b). The pattern of colour intensity confirmed previous results (King et al. 2004), i.e., glycogen accumulation was higher in strains MC4100 and M534 than in MG1655 and ZK126, confirming that the former strains produce more σ^S than the others. Furthermore, all wild-type strains developed a significantly stronger brown colour than the *rpoS::*Tn*10* mutants.

X-P plates can also be used to differentiate between natural *E. coli* isolates regarding their *rpoS* status. Patches of ECOR collection strains representing diverse sources of *E. coli* (Ochman and Selander 1984) were grown on X-P plates (Fig. 1c). Strains ECOR13, 14 and 24 displayed a

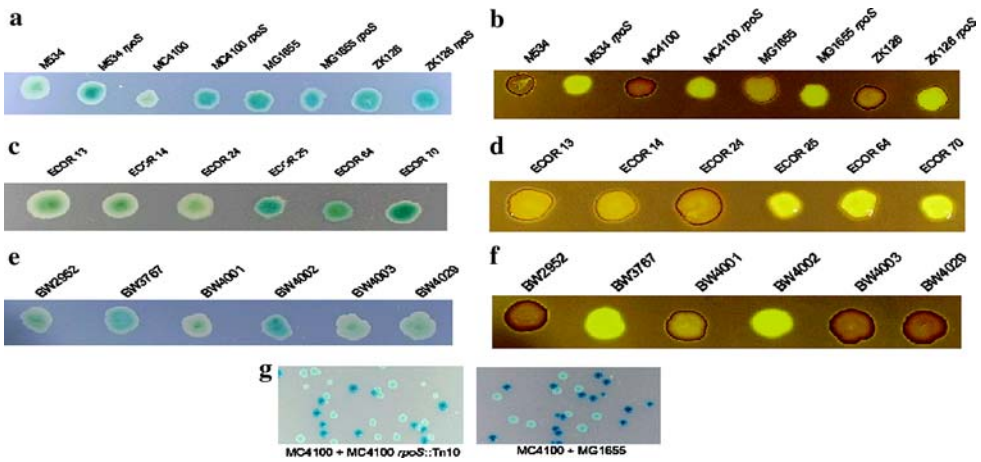

Fig. 1 AP and glycogen variation in patches of bacteria growing on solid media. **a**, **c** and **e** Patches of bacteria on T-salts plates supplemented with X-P. **b**, **d** and **f** Patches of bacteria on LB plates stained with iodine. **g** Cultures of different strains mixed together and plated on T-salts supplemented with X-P

less intense colour when compared to the other three strains. On the other hand, these same strains stained darker with iodine than strains ECOR25, 64 and 70 (Fig. 1d), suggesting that the latter strains produced less or no σ^S. The *rpoS* status of the ECOR strains was confirmed by observing the catalase activity due to the addition of H_2O_2 to the colonies. While ECOR13, 14 and 24 displayed a strong bubbling, the other three strains did not bubble at all (not shown). This suggests that X-P staining can be used to differentiate between *rpoS*$^+$ and *rpoS*$^-$ phenotypes even in strains of very different genetic backgrounds. It is important to note, however, that other properties besides the *rpoS* level, such as mutations in the PHO regulatory genes may influence the level of AP in the cells. Therefore, care must be taken when comparing the AP activity of strains from different backgrounds and whenever possible other *rpoS*-related tests should be performed.

We then tested if plating on X-P plates could be used to follow the evolution of heterogeneity in a glucose-limited chemostat, which selects for *rpoS* mutants in the population (Maharjan et al. 2006; Notley-McRobb et al. 2002); 6 out of 41 individual clones isolated after 26 days of growth under glucose limitation (Maharjan et al. 2006) were patched on an X-P plate (Fig. 1e). Strains BW2952 (parent strain), BW4001, BW4003 and BW4029 contained no *rpoS* mutations and stained lightly, while strains BW3767 (stop codon at position 315 of the σ^S protein) and BW4002 (stop codon at position 247) displayed a considerably darker blue colour. Accordingly, the iodine staining showed an inverse relationship with the AP activity on the plates, as the *rpoS* mutants were lighter than the *rpoS*$^+$ strains (Fig. 1f).

When the cultures of strains exhibiting different levels of σ^S were mixed and plated on X-P, it was easy to discern the high σ^S producers from the other strains as shown in Fig. 1g, where strain MC4100 (light blue colonies) was mixed with its *rpoS*::Tn10 mutant or with MG1655. Therefore, at least for derivatives of strain MC4100, which expresses high amounts of σ^S and is used as the starting strain in evolution experiments in this lab, the *rpoS*$^-$ evolved isolates are easily discernible by streaking or patching the colonies on X-P plates. This technique is better than iodine staining because the cells are not killed and can, therefore, be directly isolated from the plates.

Rate of *rpoS* loss in a Pi-limited chemostat

As noted above, MC4100 bacteria growing in a chemostat under glucose limitation accumulate mutations in *rpoS*, which sweep the population leading to the elimination of the *rpoS*$^+$ parent (Maharjan et al. 2006; Notley-McRobb et al. 2002). This radical change in the population profile occurs because σ^S is a burden for bacteria growing in a carbon-limited environment. In *rpoS*$^+$ bacteria, most of the growth-related genes, such as the ones that encode glucose transporters have their expression negatively affected by σ^S, due to the competition between σ^S and σ^{70} (Farewell et al. 1998; Maeda et al. 2000). A strong selective pressure for the elimination of *rpoS*$^+$ results in the optimisation of the expression of growth-related genes, and these *rpoS* mutants eventually sweep the population (Ferenci 2005). However, this is not the case for MG1655 populations in a glucose-limited chemostat, which do not enrich *rpoS* mutations, probably because the relatively low level of σ^S expressed in these cells does not give rise to a strong selective pressure against *rpoS* (King et al. 2004).

To test if *rpoS* also poses a disadvantage to bacteria growing under steady-state Pi-limitation, a short-term evolution experiment using separately both MC4100 and MG1655 as the starting strains was conducted. The cells were inoculated in minimal medium supplemented with excess glucose and 30 μM Pi and grown for 4 days. The proportion of *rpoS*$^+$ cells and the level of AP in the population were followed throughout this period (Fig. 2). In the evolving MC4100 population, after 2 days there was already a 25% decrease in the number of *rpoS*$^+$ cells along with a 2.5-fold increase in the level of AP. On day 4, the *rpoS*$^+$ cells represented only 5% of the population, while the level of AP was fivefold higher than on day 1. For the evolving MG1655, the first *rpoS* mutants appeared only on day 3, comprising just 11% the population. The AP level barely increased at that point. On day 4, the proportion of *rpoS* mutants was 64% and the AP level increased 3.3 times as compared to the day 1 population. This demonstrates that the presence of *rpoS*$^+$ is disadvantageous to cells growing in a Pi-limited environment, and that the proportion and rate of *rpoS* loss is directly related to the intrinsic level of σ^S. Acquisition of mutations in the *rpoS* gene is apparently

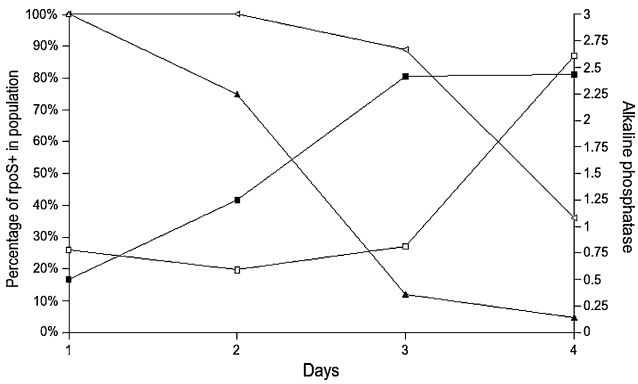

Fig. 2 AP activity and *rpoS* loss in MC4100 and MG1655 cells cultivated in a Pi-limited chemostat. MC4100 (*closed symbols*) and MG1655 (*open symbols*) cells were grown for 4 days in a chemostat containing T-salts supplemented with 0.2% glucose and 30 μM Pi at a dilution rate of 0.1 h^{-1}. Total AP activity (*squares*) in the population and glycogen staining (*rpoS* status) (*triangles*) of isolated colonies were measured daily

the simplest way to enable an increase in the expression of the PHO genes and in this way to improve the fitness under these conditions. This finding is in agreement with the SPANC trade-off hypothesis (Ferenci 2005) and extends to inorganic nutrients. Therefore, bacteria that express high levels of σ^S are subjected to similar selection pressures under steady-state Pi-limitation as those growing in a glucose-limited chemostat.

The reason why MG1655 did not acquire *rpoS* mutations in a glucose-limited chemostat under the same dilution rate as employed here (King et al. 2004) is probably related to the fact that the negative effect of *rpoS* on the expression of glucose transport genes is milder than its effect on PHO genes expression. For instance, MC4100 *rpoS*::Tn*10* showed a fourfold increase in *malG* expression when compared to its *rpoS*$^+$ parent (Notley-McRobb et al. 2002). In contrast, the AP level of MC4100 *rpoS*::Tn10 mutant was 10.8-fold higher than that of its parent and even the MG1655 *rpoS*::Tn10 mutant showed a fourfold increase in comparison to its *rpoS*$^+$ parent. Therefore, it seems that the PHO genes are more strongly repressed by σ^S under Pi-limiting conditions than are the glucose transport related genes in bacteria growing in a glucose-limiting chemostat.

In conclusion, it was shown that the activity of AP is inversely related to the intrinsic σ^S level of individual *E. coli* strains. The negative effect of *rpoS* on PHO expression is proportional to the relative amounts of σ^S in the bacteria, probably because in cells that produce more σ^S there is more competition with σ^{70}. The use of a newly devised non-destructive method enabled us to find out that inhibition of AP by σ^S occurs even under conditions of PHO repression, when only basal levels of AP are synthesised. This agrees with the model that sigma factor competition is at the core of the inhibitory effect of *rpoS*. Under steady-state growth in a Pi-limited medium, high σ^S producers, such as strain MC4100 rapidly accumulate *rpoS* mutations as a way of relieving the repression of the PHO genes. Strains that produce lesser amounts of σ^S, such as MG1655, still acquire mutations in *rpoS*, but more slowly.

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