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ppGpp mediates the growth phase dependent regulation of *agn43*, a phase variable gene, by stimulating its promoter activity.

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Running title: ppGpp-mediated regulation of *agn43*

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Originality-Significance statement

Expression studies of phase variable genes are challenging since the switching between the ON- and OFF-states of the individual bacterial cells provides heterogeneity within a clonal population. In this report, the expression of Antigen 43, a biofilm promoting factor in *E. coli*, has been studied. The expression is strictly dependent of the growth phase in a ppGpp-dependent manner. Single cell studies demonstrate that ppGpp does not affect phase variation but the promoter activity of the Antigen 43 expressing cells. Our data let to speculate that the ppGpp-mediated regulation of Antigen 43 may have relevant ecological implications for the adaptation and survival of bacteria to different stress conditions, since changes in the ON/OFF states require DNA replication and cell division.

ABSTRACT

Antigen 43 (Ag43) is a self-recognizing outer membrane protein of *Escherichia coli* expressed during intracellular growth and biofilm formation, suggesting a role in infection. The expression of *agn43* is under phase variation control, meaning that there are regulatory mechanisms adjusting the percentage of *agn43*-expressing cells in the population, in addition to mechanisms modulating the transcriptional expression level in each expressing cell. Phenotypic and transcriptional studies indicate that Ag43 expression is induced upon entry into the stationary phase in a ppGpp-dependent and RpoS-independent manner. The use of single cell approaches and phase variation deficient strains let to conclude that ppGpp stimulates *agn43* promoter activity, rather than affecting the percentage of *agn43*-expressing cells. The data highlight the relevance that promoter activity regulation may have, without any involvement of the phase variation state, in the final Ag43 expression output. The *agn43* promoter of the MG1655 strain carries an AT-rich discriminator between positions -10 and +1, which is highly conserved among the *agn43* genes present in the different pathotypes of *E. coli*. Remarkably, the AT-rich discriminator is required for the positive transcriptional control mediated by ppGpp.

INTRODUCTION

Antigen 43 (Ag43) is a self-recognizing, afimbrial adhesin that promotes cell aggregation, biofilm formation, resistance to oxidative stress and survival in human neutrophils (Diderichsen, 1980; Henderson *et al.*, 1997; Kjaergaard *et al.*, 2000; Schembri *et al.*, 2003; Fexby *et al.*, 2007). Ag43 belongs to the family of autotransporters and it is composed of two subunits present in an equimolar ratio. The α-subunit is exposed in the cell surface through non-covalent binding to the β-subunit, an integral outer membrane component (Kjaergaard *et al.*, 2002; van der Woude and Henderson, 2008). The *agn43* gene has been detected in many, but not all, *E. coli* isolates and in some strains of the related species *Shigella flexneri* and *Citrobacter rodentium*. Interestingly, *agn43* is more frequently found among pathogenic than commensal *E. coli* strains, with higher prevalence in uropathogenic *E. coli* (UPEC), where more than one *agn43* allele coding for highly homologous proteins is often found (Al-Hasani *et al.*, 2001; van der Woude and Henderson, 2008). Ag43 is expressed when UPEC grow intracellularly within uroepithelial cells, contributing to the long-term persistence of urinary tract infections (Anderson *et al.*, 2003; Restieri *et al.*, 2007; Ulett *et al.*, 2007).

The expression of *agn43* is under phase variation control. Thus, in a clonal population both *agn43*-expressing cells (ON-cells) and non-expressing cells (OFF-cells) coexist. The phenotypic diversity generated by phase variation in genetically homogeneous populations is considered an adaptive advantage to survive in challenging conditions, such as living within the host. Phase-variable genes are widely spread among bacteria and most of them encode for bacterial surface structures (van der Woude, 2011).

The phase variation is mediated by two proteins, OxyR (a DNA-binding protein) and Dam (a deoxyadenosine methyltransferase), that target specific sequences of the *agn43* promoter (reviewed by (van der Woude and Henderson, 2008). The binding of OxyR to the *agn43* promoter overlaps with the -10 element and three GATC boxes that are susceptible to be methylated by Dam. If the GATC boxes are not methylated, OxyR

binding will interfere with the RNA polymerase, inhibiting transcription (OFF-phase). But if they are methylated, OxyR cannot bind allowing transcription from the *agn43* promoter (ON-phase). DNA replication is required to switch between phase states.

The secondary messenger ppGpp is involved in bacterial adaptation to diverse environmental stresses (Magnusson *et al.*, 2005; Hauryliuk *et al.*, 2015; Steinchen and Bange, 2016). In *E. coli*, the ppGpp levels dramatically change during the growth curve. Under standard laboratory growth conditions, ppGpp concentration is around 40 μM during exponential growth, increasing up to 800 μM upon entry to stationary phase to later stabilizing at 150 μM (Varik *et al.*, 2017). ppGpp promotes severe physiological changes upon entry into the stationary phase by directly affecting transcription of a broad set of genes or by promoting the activity of RpoS, the stationary phase sigma subunit, at different levels (Aberg *et al.*, 2009; Traxler *et al.*, 2011; Landini *et al.*, 2014). In Gram-negative bacteria, most of the ppGpp regulatory effects are consequence of its interaction with the RNA polymerase, either at site I (Mechold *et al.*, 2013) or together with DksA at site II (Ross *et al.*, 2016; Molodtsov *et al.*, 2018) , although ppGpp can also bind to other specific proteins (Zhang *et al.*, 2018; Wang *et al.*, 2019). In UPEC, ppGpp is involved in the control of biofilm formation and pathogenesis through regulation of type 1 fimbriae expression (Aberg *et al.*, 2006, 2008).

In this report, we show that Ag43 expression is induced upon entry into stationary phase at the transcriptional level by the secondary messenger ppGpp. Using single-cell approaches and *oxyR* strains, which are phase variation deficient, we demonstrate that ppGpp regulates Ag43 expression solely at the promoter activity level. The ppGpp-mediated regulation of *agn43* expression requires the AT-rich discriminator located between the -10 box and the position +1, which is highly conserved among the *agn43* alleles present in the different pathotypes of *E. coli.* Our data highlight the relevance of regulation at the promoter activity level defining the final *agn43* gene expression output, despite being a phase variation-controlled gene.

RESULTS

Induction of Ag43 expression upon entry into stationary phase requires the secondary messenger ppGpp

To gain insight into the physiological conditions affecting *agn43* expression in *E. coli*, the levels of Ag43 along the growth curve were monitored by immunodetection of the Ag43 α-subunit in LB cultures grown at logarithmic phase (OD_{600} of 0.4) and after entry into the stationary phase (OD_{600} of 2.0) (Fig. 1A). The amount of Ag43 was up to five-fold higher in early stationary than in logarithmic phase cultures.

ppGpp together with its cofactor DksA and RpoS are global regulators involved in the drastic rearrangement of gene expression that takes place when *E. coli* enter into the stationary phase (Magnusson *et al.*, 2005). Ag43 levels were determined in cultures of mutant strains deficient in ppGpp, DksA and RpoS. No relevant differences in growth kinetics were detected for the different strains in rich medium, as previously observed (Aberg *et al.*, 2008). The levels of Ag43 were strongly reduced in both the ppGpp-deficient ($ppGpp^0$) and the Δ*dksA* mutant strains compared to Wt (Fig. 1B), indicating that the ppGpp/DksA regulatory system is required for the stationary phase induction of the Ag43 expression. Consistent with a previous work, showing a reduction on the *agn43* transcript levels in ppGpp-deficient and Δ*dksA* mutant strains (Aberg *et al.*, 2009). On the contrary, the Ag43 levels were not significantly affected in a Δ*rpoS* mutant strain, suggesting that Ag43 induction was independent of the alternative sigma factor RpoS (Fig. 1B). The unresponsiveness of *agn43* expression to RpoS has been previously reported (Wallecha *et al.*, 2002).

Some *agn43* alleles promote cell-to-cell aggregation supporting both oxidative stress protection and biofilm formation (Klemm *et al.*, 2004). The ability to cell aggregate, measured as a decrease in the turbidity (OD_{600}) of cell suspensions, was monitored (Fig. 1C). The Wt strain forms cell aggregates as indicated by a clear decrease in the turbidity (>60%) after one hour whereas no change in turbidity was observed with cell suspensions of the Δ*agn43* strain. No apparent cell aggregation was detected with

ppGpp^0 cell suspensions, consistent with the low Ag43 expression detected. In further accordance with the levels of Ag43 (Fig. 1B), cell aggregation was observed for the Δ*rpoS* strain, whereas no cell aggregation was detected for the Δ*dksA* strain (Fig. S2, supplementary information). These results strongly suggest that ppGpp is required for the Ag43-mediated cell aggregation in MG1655.

Transcription of *agn43* is stimulated by ppGpp

To further characterize the ppGpp-mediated regulation, the transcriptional expression of *agn43* through the growth curve was monitored in Wt and ppGpp^0 strains (Fig. 1D). A chromosomal *agn43::lacZ* transcriptional fusion at position +98, containing all known regulatory elements required for phase variation, was used. The transcriptional profile of *agn43* was monitored. Although no differences in the growth kinetics (open symbols) were observed between the strains, the *agn43* expression profile (closed symbols) displayed significant differences. In the Wt strain, *agn43* expression was low during the logarithmic growth phase and rapidly increases at the beginning of the stationary phase (OD_{600} of 1.5 - 2.0). No such induction was observed in absence of ppGpp, resulting on a 5-fold difference compared to Wt during the stationary phase.

To corroborate the stimulatory effect of ppGpp, the *agn43* transcriptional expression was monitored when the intracellular levels of ppGpp were ectopically increased in logarithmic-phase cells. Strains carrying the *relA256 spoT203* alleles produce an excess of ppGpp (up to 7-fold above the basal levels) under normal exponential growth conditions (Sarubbi *et al.*, 1988). The expression of *agn43* was induced in *relA256 spoT203* as compared to Wt in logarithmic-phase cells (OD_{600nm} of 0.4), reaching values higher than those observed for Wt in stationary phase (Fig. 1E). ppGpp levels were also increased in logarithmic-phase cells by overexpressing a catalytically active RelA-variant (RelA'), being RelA one of the two ppGpp synthetases present in *E. coli* (Hauryliuk *et al.*, 2015). Upon IPTG induction of the synthesis of the RelA' protein, a robust increase in the *agn43* expression was observed, clearly indicating that ppGpp stimulates *agn43* transcription (Fig. 1F).

ppGpp stimulates transcription from the main promoter of *agn43*

To establish the *agn43* promoter that is affected by ppGpp, primer extension analyses were performed in total RNA samples from early stationary phase cultures of Wt and ppGpp^0. Three different primer extension products were detected in Wt samples and none in ppGpp^0 (Fig. 1G). Consistently, a 5-fold reduction in *agn43* mRNA levels was detected in the ppGpp^0 strain compared to that in the Wt strain, when assayed by semi-quantitative RT-PCR (Fig. S3). The 5'-end of the *agn43* transcript deduced from the longer primer extension product (labeled 1 in Fig. 1G and 1H) coincides with the *agn43* transcriptional start site previously described (Wallecha *et al.*, 2002) and it is located downstream of predicted -35 and -10 elements, highly homologous to the consensus sigma 70 promoter sequence (Fig. 1H). The two short primer extension products mapped at positions +155 and +157 (labeled 2 and 3 in Fig. 1G and 1H). *In silico* analyses did not identify any putative -35 and -10 elements upstream of these locations suggesting that the primer extension products 2 and 3 are generated by mRNA processing from the transcript initiated in the described P_{agn43}. To confirm the *in silico* data, chromosomal *lacZ* fusions with two different fragments of the *agn43* gene were generated (Fig. S4). Fragment I spans from -109 to +248 and fragment II from +44 to +248. Both fragments contain the sequences upstream of the transcriptional start sites 2 and 3, but only fragment I carries the previously described P_{agn43} (responsible for transcriptional start site 1). Fragment I fusion allowed clear detection of *agn43* expression (2500 MU), whereas no significant expression was sensed with fragment II (8 MU) (Fig. S4). These results indicate that there is not an active promoter immediately upstream of the transcriptional starts 2 and 3.

ppGpp stimulates the *agn43* promoter activity

Genes under phase variation control, have two independent levels of transcriptional regulation: i) the percentage of ON-cells (expressing a specific promoter) in the population and ii) the classical regulation at the promoter activity level in each ON-cell. To elucidate at which level ppGpp exerts its effect, single cell studies were performed

using a reporter system based on the green fluorescent protein (GFP). This *agn43-gfp* reporter system was successfully used to monitor the percentage of ON-cells (phase variation regulation) and the expression level in each single bacterial cell (P_{agn43} activity) with the MC4100 *E. coli* strain (Lim and van Oudenaarden, 2007). Since MC4100 is deficient for the ppGpp synthetase RelA (Ferenci *et al.*, 2009), the reporter system was transferred to the MG1655 genetic background (see experimental procedures in supplementary information). Validation assays were performed to corroborate the appropriate monitoring of *agn43* expression in both strains, JDC69 (MG1655 derivative) and CHNL135 (MC4100 derivative). Phase variation can be observed in both strains by detection of fluorescent sectors in colonies (Fig. S5A), a certain percentage of cells emitting fluorescence in liquid cultures as shown by confocal microscopy (Fig. S5B) and two distinct populations on the basis of green fluorescence intensity (GFI) by flow cytometry (Fig. S5C). High GFI population (green) corresponds to ON-cells whereas low GFI population (red) corresponds to OFF-cells. As a control, MG1655 Δ*oxyR* strain (JDC95), where *agn43* expression is locked in the ON-phase (Owen *et al.*, 1996), generates a homogeneous fluorescence-emitting population (Fig. S5). Overall, we conclude that the *agn43-gfp* reporter system monitors efficiently both levels of *agn43* transcriptional control in MG1655 derivatives.

Cultures of the Wt and $ppGpp^0$ derivative strains were analyzed for *agn43* expression by flow cytometry (Fig. 2A). In addition to the GFI (P_{agn43} activity), the accumulation of propidium iodide (PI) was also monitored, indicating the presence of dead cells in the population and allowing to distinguish OFF-cells from dead cells. In Wt cultures, two clear populations were detected according to the GFI signal, with low (OFF-cells) and high (ON-cells) intensities, and the average percentage of ON-cells was approximately 6% (Fig. 2B). In $ppGpp^0$ cultures, the well-defined cell population with similar GFI as the Wt ON-cells (220 arbitrary units) was not detected (Fig. 2A). Instead, cells with an intermediate intensity (45 units), significantly higher than that of the well-defined OFF-population, were detected (green dots). To clarify if these cells represent the ON-

population, cultures of the ppGpp^0 strain inoculated with a cell suspension enriched in ON-cells (GFP-emitting colonies) were analyzed (ppGpp^0-ON). Two well-defined populations were detected, strongly suggesting that the ppGpp^0 ON-cells have an average GFI lower than 50 units, about 5-fold lower than that of the ON-cells from the Wt strain (Fig. 2A and 2B). No significant difference was observed in the percentage of ON-cells between the Wt and ppGpp^0 strains (Fig. 2B). Our data suggest that ppGpp notably affects P_{agn43} activity at the promoter output level whereas it has no significant effect on phase variation, under the conditions tested. The number of dead cells in a ppGpp^0 strain is higher than in a Wt strain (Fig. 2A, orange plot), underlining the physiological relevance of ppGpp.

GFI determination in the *oxyR* strain monitors regulatory effects occurring at the P_{agn43} output since P_{agn43} is locked-ON. Consistent with the previous results, ppGpp-deficiency in a Δ*oxyR* genetic background causes a significant drop (5-fold) in the average GFI (Figs. 2C and Fig. S6). Additionally, using Δ*oxyR* derivatives, the *agn43* downregulation in a ppGpp^0 was also detected at the protein level (Fig. 2D) and no significant cell aggregation was observed in the absence of ppGpp, in contrast with the very rapid decrease in turbidity (>80%) in the ppGpp-proficient derivative (Fig. 2E). As controls, cell aggregation profiles of Wt and Δ*oxyR* Δ*agn43* mutant strains were used. Our results suggest that regulation at the level of P_{agn43} activity is the driving force of *the agn43* growth phase dependent regulation. Accordingly, in Δ*oxyR* strains, a substantial induction of P_{agn43} activity occurs in stationary phase as compared to logarithmic phase (up to 5-fold) and the ability to cell aggregate is greater after entering stationary phase (Fig. 2F).

The growth phase control of P_{agn43} activity is abolished by alteration of the AT-rich discriminator

A hallmark of genes directly regulated by ppGpp is the particular composition of their discriminator sequence located between the -10 element and the transcription start (Sanchez-Vazquez *et al.*, 2019). In the presence of a GC-rich discriminator and other

suboptimal transcriptional elements, ppGpp destabilization of the RNA polymerase – promoter open complexes results in direct repression (Haugen *et al.*, 2008). In contrast, ppGpp-stimulated genes are often characterized by having an AT-rich discriminator, as exemplified by several amino acid biosynthetic operons (Sanchez-Vazquez *et al*., 2019). The AT-rich discriminator promotes the formation of stable RNA polymerase-promoter open complexes that are efficiently transcribed even in the presence of ppGpp (Haugen *et al.*, 2008). The promoter sequence of 13 amino acid biosynthetic operons, where an AT-rich discriminator was found, is depicted (Fig. 3A). Nucleotide frequency analysis reveals that in five positions (labelled in bold in Fig. 3A), AT nucleotides were present in more than 90% of those promoters. The *agn43* promoter contains an AT-rich discriminator and AT nucleotides were detected in all five positions considered (Fig. 3A). In a previous report, nucleotide substitutions leading to exchange the AT-rich discriminator of the *uspA* gene for the GC-rich discriminator of the *rrnB* P1 promoter proved that the *uspA* AT-rich discriminator is crucial for the ppGpp-mediated transcriptional direct stimulation (Gummesson *et al.*, 2013). An identical discriminator exchange was performed in the *agn43* promoter (Fig. 3B, upper panel). The *agn43* promoter activity of the P_{agn43}^{Wt} (Wt promoter) and the P_{agn43}^{Mt} (genetic construct with the AT-rich discriminator exchanged for the GC-rich discriminator of the *rrnB* P1 promoter) was monitored in different genetic backgrounds, using chromosomal *lacZ* promoter fusions (Fig. 3B). The *agn43* expression was normalized to 100% ON cells, after determination of the percentage of ON cells on X-gal LB plates. The P_{agn43}^{Wt} activity was induced in the stationary phase, whereas the P_{agn43}^{Mt} activity did not respond to the growth phase stimulus. The unresponsiveness of the P_{agn43}^{Mt} to the growth phase was confirmed using a locked-ON (Δ*oxyR*) strain since apparently identical results were obtained (Fig. 3C). Therefore, the exchange of the discriminator abolishes the induction of the P_{agn43} activity upon entry into the stationary phase. To rule out that the discriminator exchange done causes the inactivation of the promoter, the P_{agn43} activity was analyzed in a Δ*dam* strain, since Dam activity, despite

promoting switching to the ON-state, is required for the full activation of P_{agn43} (Haagmans and van der Woude, 2000; Wallecha *et al.*, 2002). The P_{agn43} activity was further downregulated in a Δ*dam* derivative strain.

Notably, the sequence of the *agn43* discriminator was fully conserved among several *E. coli* strains – including commensals, human pathogenic (both intestinal and extraintestinal) and avian pathogenic isolates – and one strain of *Shigella* (Fig. S7) suggesting a pivotal role of this regulatory motif in the control of *agn43* expression levels by ppGpp in a growth phase dependent manner.

DISCUSSION

Expression studies of phase variable genes are challenging since the switching between the ON- and OFF-states of the individual bacterial cells provides heterogeneity within a clonal population (Chauhan *et al.*, 2013). Most studies, as exemplified by the *fim* and *pap* operon – coding for type 1 and P fimbriae, respectively – have focused on the impact of phase variation on the gene expression profile, whereas the contribution of promoter output modulation has often been overlooked (Blomfield, 2001; Casadesús and Low, 2006).

The epigenetic switch responsible for the *agn43* phase variation has been extensively studied, resulting in an exhaustive dissection of its molecular mechanisms (Henderson and Owen, 1999; Haagmans and van der Woude, 2000; Waldron *et al.*, 2002; Wallecha *et al.*, 2002; van der Woude and Henderson, 2008). Conversely, very little is known about the factors and environmental conditions that regulate expression at the *agn43* promoter activity level and the relevance of this regulation in defining the final Ag43 expression output in the cell. Different genetic screenings showed that expression of Ag43 is affected by regulatory factors such as RfaH (Beloin *et al.*, 2006), SeqA (Correnti *et al.*, 2002) and YdgG (Herzberg *et al.*, 2006), suggesting that regulation of Ag43 might be influenced by more factors than initially expected.

In this report, we showed that the physiological state of the cell, through the secondary messenger ppGpp, controls the expression of Ag43 (Fig. 4). Previously, it was shown

that cultures of the ppGpp deficient strain did not cell aggregate which was associated with a stimulatory effect of ppGpp in the expression of Ag43 (Magnusson *et al.*, 2007). Here, we demonstrated that ppGpp, in coordination with DksA and independently of RpoS, is required for proper transcriptional expression of *agn43*. The no responsiveness of *agn43* expression to RpoS confirmed previous data showing that *agn43* expression in a locked ON state by overexpressing the Dam methylase was not affected by the presence or absence of RpoS (Wallecha *et al.*, 2002). By using single cell approaches, we showed that ppGpp acts as a positive regulator of P_{agn43} activity and it does not alter the percentage of ON-cells in the population. To the best of our knowledge, this is the first mechanism of *agn43* expression regulation acting at the level of P_{agn43} activity. It highlights the impact of environmental fine-tuning of P_{agn43} activity for the overall regulation of Ag43 expression, which may have relevant ecological implications for the adaptation and survival of bacteria to different stress conditions. Taking into account that switching from OFF- to ON-states requires up to two cycles of chromosome replication (van der Woude and Henderson, 2008), it seems imperative that, under cell arrest, stimulation of *agn43* expression should occur at the level of promoter activity. *E. coli* cells, particularly during host colonization and infection, may encounter conditions of sudden growth arrest. If replication is quickly impaired, the cells will be unable to switch the *agn43* expression phase. Growth arrest causes a concomitant increase in the intracellular levels of ppGpp, which could act as a trigger to the rapid increase in the amount of Ag43 protein expressed on the surface of the ON-cells, promoting cell-to-cell aggregation and biofilm formation. This will endorse adaptation and/or survival of a part of the population in the prevailing harsh conditions. *In vitro* studies identify a putative transcriptional terminator in the 5'-UTR of the *agn43* transcript (Wallecha *et al.*, 2014). The authors suggest that antitermination is required for efficient expression of Ag43 and that it decreases as the culture goes from the logarithmic to the stationary phase. Although these results would suggest a higher level of Ag43 expression in the logarithmic phase, the P_{agn43} activity level in different growth

phases was not monitored. Our data, at both protein and mRNA levels, are highly convincing of the fact that Ag43 expression is promoted at the stationary phase of growth, in agreement with the stimulating role of the regulators, ppGpp and DksA. Moreover, one of the chromosomal *agn43-lacZ* fusions used (CBPU137 and derivatives) lacks the putative terminator but responds to both the growth phase and ppGpp in an apparently identical way as when mRNA levels were monitored (RT-PCR) and the GFP reporter system, which carries the putative terminator, was used.

The *agn43* gene carries an AT-rich discriminator between the -10 and +1 promoter, a common feature among many genes directly stimulated by ppGpp. The relevance of this DNA sequence in the ppGpp-mediated control of *agn43* expression was shown by sequence exchange of the AT-rich discriminator by the GC-rich *rrnB* P1 discriminator as in previous reports (Gummesson *et al.*, 2013).

Our data indicate that the physiological state of the cell, and presumably diverse environmental parameters, are sensed through the secondary messenger ppGpp, causing alteration of Ag43 expression. We previously described that ppGpp-mediated regulation occurs for the phase variable *fim* operon encoding the type 1 fimbriae (Aberg *et al.*, 2006, 2008, 2009). Both Ag43 and type 1 fimbriae are associated with *E. coli* virulence, particularly in the case of uropathogenic isolates (UPEC). Interestingly, it was reported that during cystitis, UPEC cells, following the invasion of umbrella bladder cells, form complex intracellular bacterial communities with an initial stage of rapid intracellular growth followed by a drastic drop in the growth rate and expression of biofilm traits such as Ag43 and type 1 fimbriae (Justice *et al*, 2004). It is tempting to speculate that ppGpp, the general stress response regulator that responds to growth kinetics, plays a crucial role in coordinating the complex process of biofilm formation and intracellular survival.

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REFERENCES

Aberg, A., Fernandez-Vazquez, J., Cabrer-Panes, J.D., Sanchez, A., and Balsalobre, C. (2009) Similar and divergent effects of ppGpp and DksA deficiencies on transcription in *Escherichia coli*. *J Bacteriol* **191**: 3226–3236.

Aberg, A, Shingler, V., and Balsalobre, C. (2006) (p)ppGpp regulates type 1 fimbriation of *Escherichia coli* by modulating the expression of the site-specific recombinase FimB. *Mol Microbiol* **60**: 1520–1533.

Aberg, A., Shingler, V., and Balsalobre, C. (2008) Regulation of the *fimB* promoter: a case of differential regulation by ppGpp and DksA *in vivo*. *Mol Microbiol* **67**: 1223–1241.

Al-Hasani, K., Rajakumar, K., Bulach, D., Robins-Browne, R., Adler, B., and Sakellaris, H. (2001) Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. *Microb Pathog* **30**: 1–8.

Anderson, G.G., Palermo, J.J., Schilling, J.D., Roth, R., Heuser, J., and Hultgren, S.J.

(2003) Intracellular bacterial biofilm-like pods in urinary tract infections. *Science (80-.).* **301**: 105–107.

Beloin, C., Michaelis, K., Lindner, K., Landini, P., Hacker, J., Ghigo, J.M., and Dobrindt, U. (2006) The transcriptional antiterminator RfaH represses biofilm formation in *Escherichia coli*. *J Bacteriol* **188**: 1316–1331.

Blomfield, I.C. (2001) The regulation of pap and type 1 fimbriation in *Escherichia coli*. *Adv Microb Physiol* **45**: 1–49.

Casadesús, J. and Low, D. (2006) Epigenetic gene regulation in the bacterial world. *Microbiol. Mol. Biol. Rev.* **70**: 830–856.

Chauhan, A., Sakamoto, C., Ghigo, J.-M., and Beloin, C. (2013) Did I pick the right colony? Pitfalls in the study of regulation of the phase variable antigen 43 adhesin. *PLoS One* **8**: e73568.

Correnti, J., Munster, V., Chan, T., and Woude, M. (2002) Dam-dependent phase variation of Ag43 in *Escherichia coli* is altered in a *seqA* mutant. *Mol Microbiol* **44**: 521–532.

Diderichsen, B. (1980) *flu*, a metastable gene controlling surface properties of *Escherichia coli*. *J Bacteriol* **141**: 858–867.

Ferenci, T., Zhou, Z., Betteridge, T., Ren, Y., Liu, Y., Feng, L., et al. (2009) Genomic sequencing reveals regulatory mutations and recombinational events in the widely used MC4100 lineage of *Escherichia coli* K-12. *J Bacteriol* **191**: 4025–4029.

Fexby, S., Bjarnsholt, T., Jensen, P.O., Roos, V., Hoiby, N., Givskov, M., and Klemm, P. (2007) Biological Trojan horse: Antigen 43 provides specific bacterial uptake and survival in human neutrophils. *Infect Immun* **75**: 30–34.

Gummesson, B., Lovmar, M., and Nyström, T. (2013) A proximal promoter element required for positive transcriptional control by guanosine tetraphosphate and DksA protein during the stringent response. *J. Biol. Chem.* **288**: 21055–21064.

Haagmans, W. and van der Woude, M. (2000) Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated

repression of transcription. *Mol Microbiol* **35**: 877–887.

Haugen, S.P., Ross, W., Manrique, M., and Gourse, R.L. (2008) Fine structure of the promoter-sigma region 1.2 interaction. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 3292–3297.

Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T., and Gerdes, K. (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat. Rev. Microbiol.* **13**: 298–309.

Henderson, I.R., Meehan, M., and Owen, P. (1997) Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in *Escherichia coli* K-12. *FEMS Microbiol Lett* **149**: 115–120.

Henderson, I.R. and Owen, P. (1999) The major phase-variable outer membrane protein of Escherichia coli structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and OxyR. *J Bacteriol* **181**: 2132–2141.

Herzberg, M., Kaye, I.K., Peti, W., and Wood, T.K. (2006) YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. *J Bacteriol* **188**: 587–598.

Justice, S.S., Hung, C., Theriot, J.A., Fletcher, D.A., Anderson, G.G., Footer, M.J., and Hultgren, S.J. (2004) Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Proc Natl Acad Sci U S A* **101**: 1333–1338.

Kjaergaard, K., Hasman, H., Schembri, M.A., and Klemm, P. (2002) Antigen 43-mediated autotransporter display, a versatile bacterial cell surface presentation system. *J Bacteriol* **184**: 4197–4204.

Kjaergaard, K., Schembri, M.A., Ramos, C., Molin, S., and Klemm, P. (2000) Antigen 43 facilitates formation of multispecies biofilms. *Env. Microbiol* **2**: 695–702.

Klemm, P., Hjerrild, L., Gjermansen, M., and Schembri, M.A. (2004) Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from

Escherichia coli. *Mol Microbiol* **51**: 283–296.

Landini, P., Egli, T., Wolf, J., and Lacour, S. (2014) sigmaS, a major player in the response to environmental stresses in *Escherichia coli* : role, regulation and mechanisms of promoter recognition. *Environ. Microbiol. Rep.* **6**: 1–13.

Lim, H.N. and van Oudenaarden, A. (2007) A multistep epigenetic switch enables the stable inheritance of DNA methylation states. *Nat Genet* **39**: 269–275.

Magnusson, L.U., Farewell, A., and Nyström, T. (2005) ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* **13**: 236–242.

Magnusson, L.U., Gummesson, B., Joksimovic, P., Farewell, A., and Nystrom, T. (2007) Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli*. *J Bacteriol* **189**: 5193–5202.

Mechold, U., Potrykus, K., Murphy, H., Murakami, K.S., and Cashel, M. (2013) Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Res.* **41**: 6175–6189.

Molodtsov, V., Sineva, E., Zhang, L., Huang, X., Cashel, M., Ades, S.E., and Murakami, K.S. (2018) Allosteric Effector ppGpp Potentiates the Inhibition of Transcript Initiation by DksA. *Mol. Cell* **69**: 828-839.

Owen, P., Meehan, M., de Loughry-Doherty, H., and Henderson, I. (1996) Phase-variable outer membrane proteins in *Escherichia coli*. *FEMS Immunol. Med. Microbiol.* **16**: 63–76.

Restieri, C., Garriss, G., Locas, M.C., and Dozois, C.M. (2007) Autotransporter-encoding sequences are phylogenetically distributed among *Escherichia coli* clinical isolates and reference strains. *Appl Env. Microbiol* **73**: 1553–1562.

Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.-H., Burgos, H.L., and Gourse, R.L. (2016) ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response. *Mol. Cell* **62**: 811–823.

Sanchez-Vazquez, P., Dewey, C.N., Kitten, N., Ross, W., and Gourse, R.L. (2019)

476 Genome-wide effects on *Escherichia coli* transcription from ppGpp binding to its
477 two sites on RNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **116**: 8310–8319.
478 Sarubbi, E., Rudd, K.E., and Cashel, M. (1988) Basal ppGpp level adjustment shown
479 by new *spoT* mutants affect steady state growth rates and *rrnA* ribosomal
480 promoter regulation in *Escherichia coli*. *MGG Mol. Gen. Genet.* **213**: 214–222.
481 Schembri, M.A., Hjerrild, L., Gjermansen, M., and Klemm, P. (2003) Differential
482 expression of the *Escherichia coli* autoaggregation factor antigen 43. *J Bacteriol*
483 **185**: 2236–2242.
484 Steinchen, W. and Bange, G. (2016) The magic dance of the alarmones (p)ppGpp.
485 *Mol. Microbiol.* **101**: 531–544.
486 Traxler, M.F., Zacharia, V.M., Marquardt, S., Summers, S.M., Nguyen, H.-T., Stark,
487 S.E., and Conway, T. (2011) Discretely calibrated regulatory loops controlled by
488 ppGpp partition gene induction across the “feast to famine” gradient in *Escherichia*
489 *coli*. *Mol. Microbiol.* **79**: 830–845.
490 Ulett, G.C., Valle, J., Beloin, C., Sherlock, O., Ghigo, J.M., and Schembri, M.A. (2007)
491 Functional analysis of antigen 43 in uropathogenic *Escherichia coli* reveals a role
492 in long-term persistence in the urinary tract. *Infect Immun* **75**: 3233–3244.
493 Varik, V., Oliveira, S.R.A., Hauryliuk, V., and Tenson, T. (2017) HPLC-based
494 quantification of bacterial housekeeping nucleotides and alarmone messengers
495 ppGpp and pppGpp. *Sci. Rep.* **7**: 11022.
496 Waldron, D.E., Owen, P., and Dorman, C.J. (2002) Competitive interaction of the OxyR
497 DNA-binding protein and the Dam methylase at the antigen 43 gene regulatory
498 region in *Escherichia coli*. *Mol Microbiol* **44**: 509–520.
499 Wallecha, A., Munster, V., Correnti, J., Chan, T., and van der Woude, M. (2002) Dam-
500 and OxyR-dependent phase variation of *agn43*: essential elements and evidence
501 for a new role of DNA methylation. *J Bacteriol* **184**: 3338–3347.
502 Wallecha, A., Oreh, H., van der Woude, M.W., and deHaseth, P.L. (2014) Control of
503 gene expression at a bacterial leader RNA, the *agn43* gene encoding outer

membrane protein Ag43 of *Escherichia coli*. *J. Bacteriol.* **196**: 2728–2735.

Wang, B., Dai, P., Ding, D., Del Rosario, A., Grant, R.A., Pentelute, B.L., and Laub, M.T. (2019) Affinity-based capture and identification of protein effectors of the growth regulator ppGpp. *Nat. Chem. Biol.* **15**: 141–150.

van der Woude, M.W. (2011) Phase variation: how to create and coordinate population diversity. *Curr. Opin. Microbiol.* **14**: 205–211.

van der Woude, M.W. and Henderson, I.R. (2008) Regulation and function of Ag43 (*flu*). *Annu Rev Microbiol* **62**: 153–169.

Zhang, Y., Zborníková, E., Rejman, D., and Gerdes, K. (2018) Novel (p)ppGpp Binding and Metabolizing Proteins of *Escherichia coli*. *mBio* **9**: e02188-17.

FIGURE LEGENDS

FIGURE 1. Ag43 expression is induced during entry into stationary phase in a ppGpp-dependent manner. A and B. Immunodetection of Ag43 in whole cell lysates using a polyclonal serum raised against the α-subunit of Ag43. In A, lysates from cultures of the MG1655 strain grown in LB at 37ºC up to logarithmic phase (OD_{600} of 0.4) and early stationary phase (OD_{600} of 2.0). In B, lysates from cultures of the strains: Wt (MG1655), $ppGpp^0$ (AAG93), Δ*dksA* (TE8114) and Δ*rpoS* (JDC111) grown to early stationary phase. Two independent cultures were analyzed, numbers indicate the average ± standard deviation of the relative signal intensity. The band corresponding to Ag43 α - subunit is indicated. The amount of protein loaded was normalized by biomass (OD_{600}) and verified by Coomassie blue-stained SDS-PAGE of the same extracts (Fig. S1). C, cell aggregation assays of cell suspensions from overnight cultures of the strains Wt (MG1655), Δ*agn43* (JDC100) and $ppGpp^0$ (AAG93). Percentage of the initial turbidity (OD_{600}) at the indicated time points. Values are the average and standard deviations of three independent cultures. D, expression profile of *agn43* assayed at different time-points through the growth curve in cultures of Wt (CBPU137, triangles) and $ppGpp^0$ (CBPU158, squares) strains carrying a chromosomal P_{agn43}*::lacZ* fusion. The growth curve (empty symbols) was monitored by measurements of the OD_{600}. The *agn43* expression (filled symbols) was monitored by determination of the β-galactosidase activity (Miller units). E and F, effect of ectopic production of ppGpp on *agn43* expression. E, *agn43* transcriptional expression in cultures of Wt and the *relA256 spoT203* derivative grown up to logarithmic phase (OD_{600} of 0.4) and early stationary phase (OD_{600} of 2.0). F, *agn43* transcriptional expression in cultures of the CBPU137 strain carrying the pVI751 plasmid (open bars), coding a RelA' protein, after induction of ppGpp production by adding IPTG (0.02 mM) at an OD_{600} of 0.1. As control, cultures of the same strain carrying the vector pMMB66HE (filled bars) were also tested. β-galactosidase activity was monitored at different time points after IPTG addition in. In

D, E and F, data are mean values of duplicate determinations in at least three independent experiments plotted with standard errors. G, primer extension analyses using the primer Ag43-7 (see location in panel H) of total RNA samples from cultures of Wt (AAG1) and ppGpp^0 (AAG3) strains grown up to early stationary phase. Two independent cultures were analyzed. Sequencing reactions used as size markers are shown to the right. The primer extension products detected are labeled from 1 to 3. H, promoter sequence of *agn43*. In bold are indicated the -35 and -10 elements and the ATG codon. The three Dam-methylation sites (GATC) are underlined. The OxyR binding site is labeled with a discontinuous line. The position of the Ag43-7 primer is indicated by underlined italics characters. The nucleotides corresponding to the 5' start of the primer extension products detected are labeled 1, 2 and 3. All cultures were grown in LB at 37ºC.

FIGURE 2. A *gfp* reporter system reveals that ppGpp affects *agn43* transcription at the level of promoter activity. A, flow cytometry analyses of cultures of the strains Wt (JDC69) and ppGpp^0 (JDC94). ppGpp^0-ON indicates cultures enriched in ON-cells. The upper plots depict distribution of live/dead cells (PI, Y-axis) depending on P_{agn43} activity (GFI, X-axis). The lower plots depict cell count vs. P_{agn43} activity of the upper plots. B, summary of flow cytometry analyses of the Wt and ppGpp^0 strains shown in A. C, summary of flow cytometry analyses of Δ*oxyR* and Δ*oxyR* ppGpp^0 shown in Fig. S6. D, immunodetection of Ag43 in whole cell lysates from cultures of the Wt (MG1655), ppGpp^0 (AAG93), Δ*oxyR* (JDC99) and Δ*oxyR* ppGpp^0 (JDC98) strains grown up to an OD_{600} of 2.0. The amount of protein loaded was normalized by biomass (OD_{600}) and verified by Coomassie blue-stained SDS-PAGE of the same extracts (Fig. S1). E, cell aggregation assays of cell suspensions of Wt (MG1655), Δ*oxyR* (JDC99), Δ*oxyR* *Δagn43* (CBPU1262) and Δ*oxyR* ppGpp^0 (JDC98) strains. Percentage of the initial turbidity (OD_{600}) at the specified time points is indicated. F. Ag43 is induced in stationary phase in a locked-ON phase genetic background. P_{agn43} activity (*agn43* transcriptional expression, light grey bars) in cultures of the Δ*oxyR* strain (CBPU140),

grown in LB at 37ºC up to logarithmic (log, OD_{600} of 0.4) and early stationary phase (stat, OD_{600} of 2.0), monitored by determination of the β-galactosidase activity (Miller units). Cell aggregation (% of initial OD_{600nm} after 1 hour incubation, dark grey bars) of cultures of the Δ*oxyR* strain (JDC99) grown as indicated. In all cases, values are the average and standard deviations of three independent cultures. All cultures were grown in LB at 37ºC.

FIGURE 3. The AT-rich discriminator of *agn43* is crucial for the growth dependent regulation. A, partial sequence of the promoter region of the indicated genes. The location of the -10 element is shown. With asterisk are labeled the bases that are either A or T in more than 90 % of the genes analyzed. B, upper panel, schematic representation of the promoter including the discriminator sequence of the *rrnB* P1, the native *agn43* (Wt) and the hybrid *agn43* (Mt). Boxes represent the -35 and -10 hexamers (white *rrnB* P1 and light grey *agn43*). Lower panel, transcriptional expression in logarithmic (OD_{600} of 0.4, grey bars) and stationary phase (OD_{600} of 2.0, black bars) using the indicated promoter *lacZ* fusions either in Wt (LFC101 and LFC102) or its Δ*dam* derivative (LFC105 and LFC106) strains. The transcriptional expression was corrected to 100 % cells (see materials and methods). C, transcriptional expression studies as in B in locked ON-phase (Δ*oxyR*) strains (LFC103 and LFC104). Values are the average and standard deviations of three independent cultures.

FIGURE 4. Proposed model of regulation of *agn43* expression in logarithmic and stationary phase of growth.

FIGURE 1

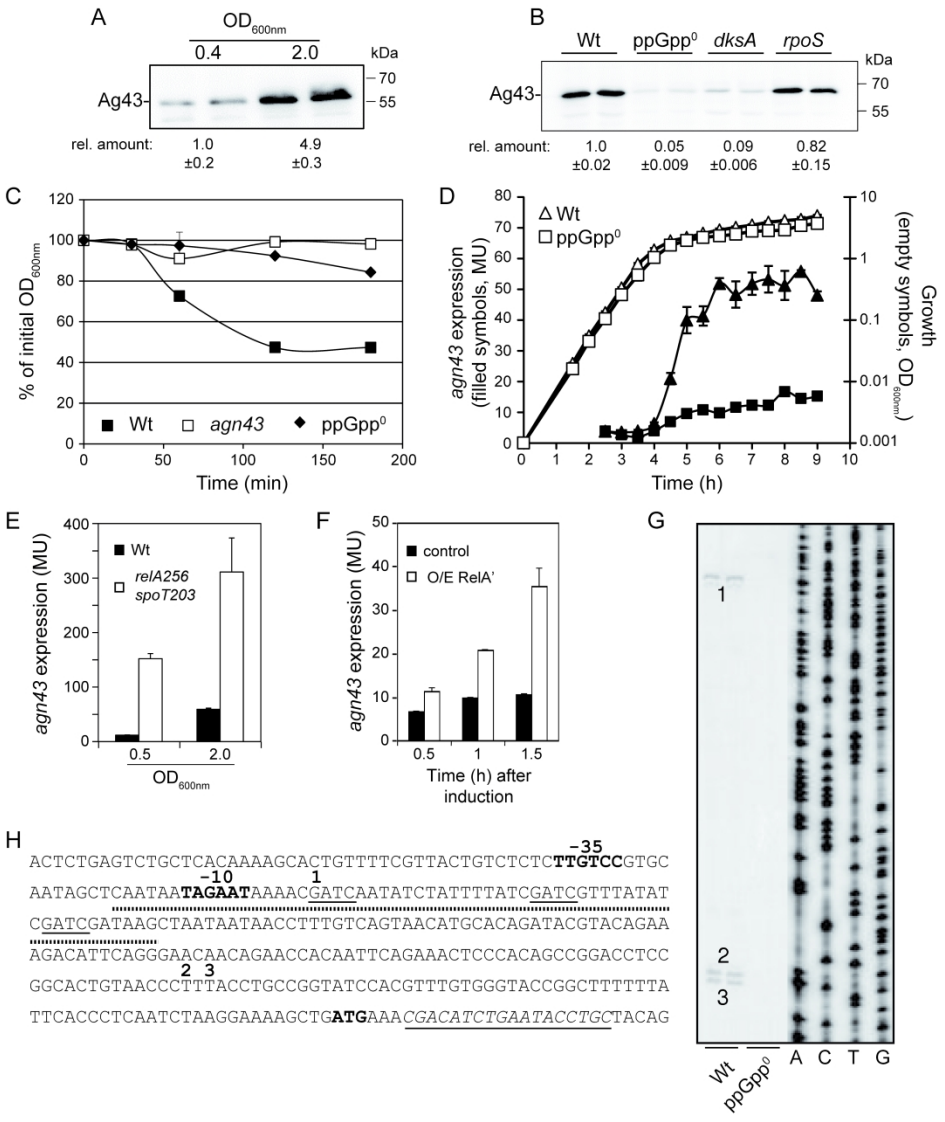

Figure 1

FIGURE 2

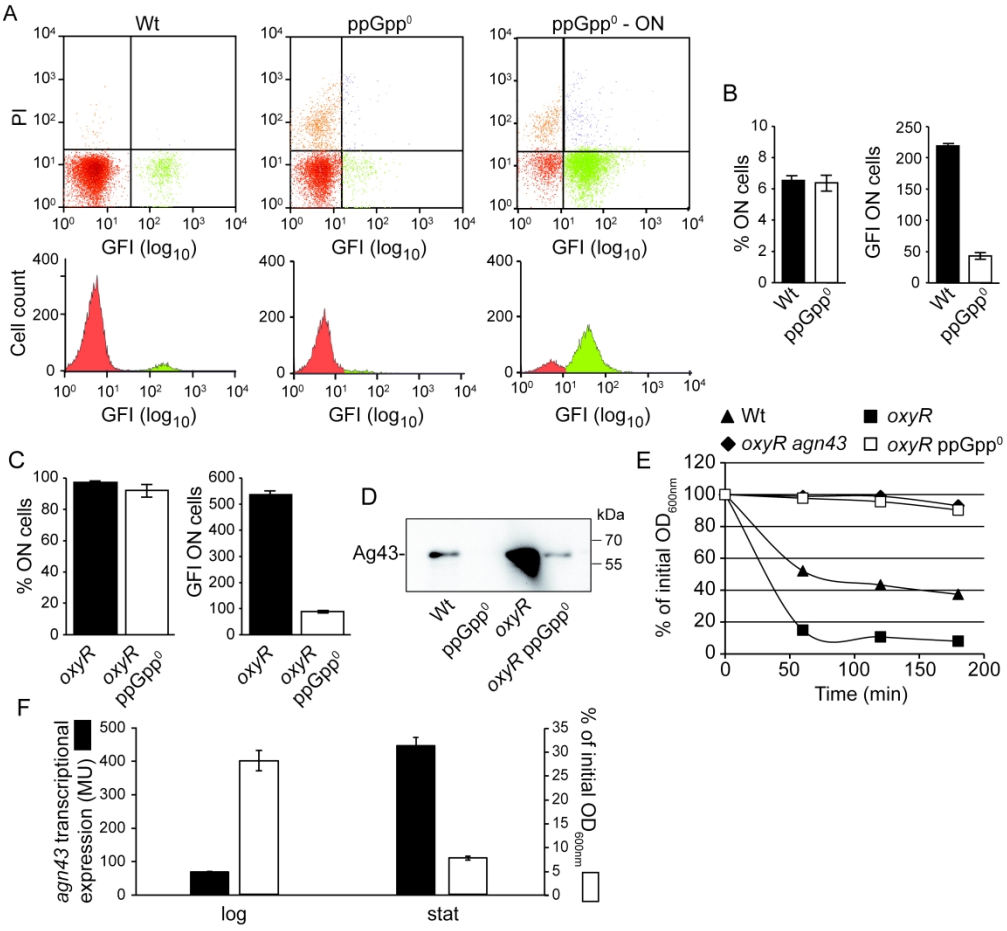

Figure 2

FIGURE 3

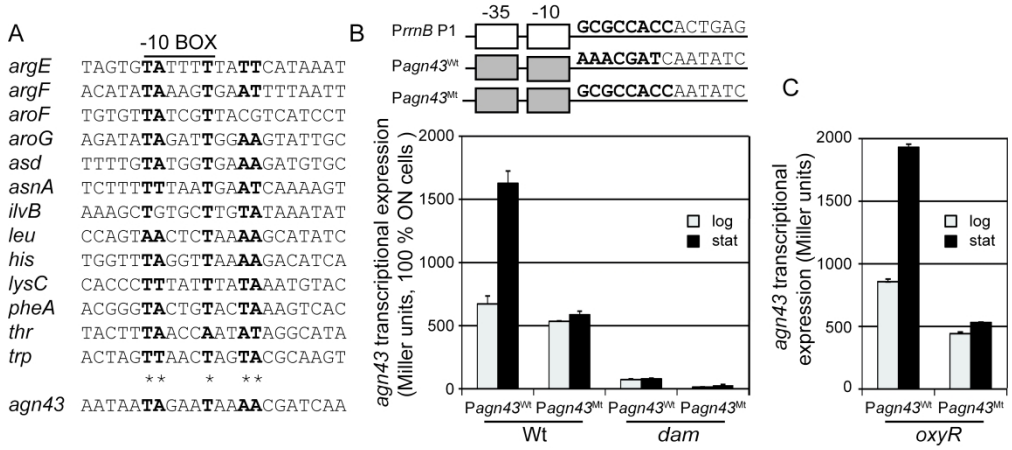

Figure 3

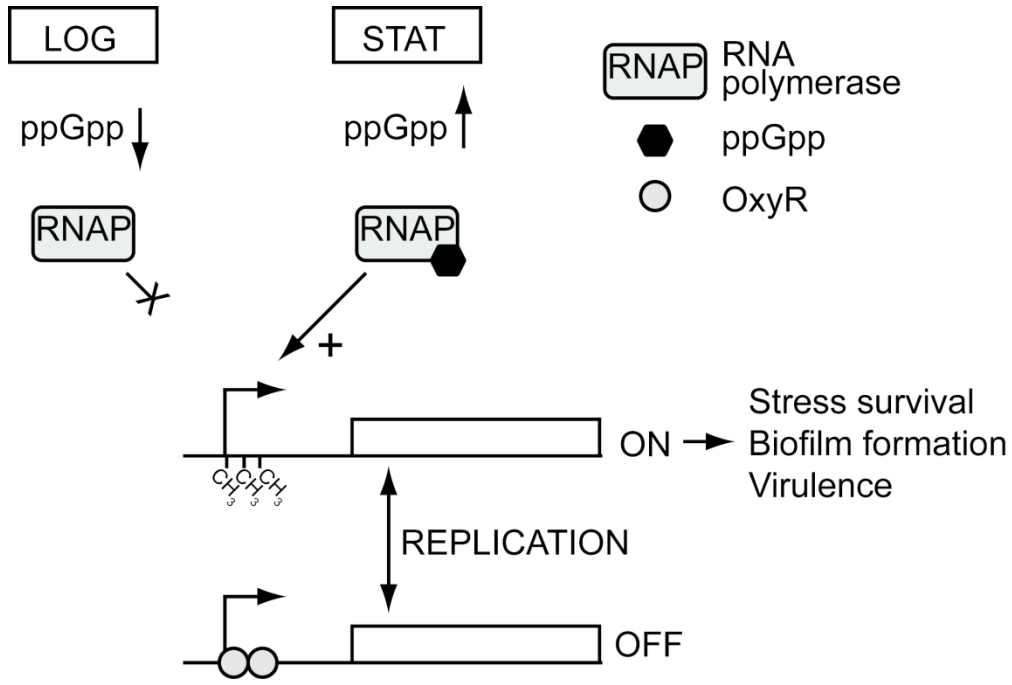
LOG
STAT
RNAP
RNA polymerase
ppGpp
ppGpp
ppGpp
OxyR
RNAP
RNAP
+
Stress survival
Biofilm formation
Virulence
ON
REPLICATION
OFF

Figure 4