RESEARCH ARTICLE

The *yddV-dos* operon controls biofilm formation through the regulation of genes encoding curli fibers' subunits in aerobically growing *Escherichia coli*

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Abstract

In bacteria, intracellular amounts of the signal molecule cyclic di-GMP (c-di-GMP) are determined by biosynthetic enzymes, or diguanylate cyclases (DGCs), and degradative enzymes, or c-di-GMP phosphodiesterases (c-PDEs). In *Escherichia coli*, the production of curli fibers, an important adhesion factor, responds to c-di-GMP. The *yddV-dos* operon, which encodes a DGC and a c-PDE acting as a protein complex, is highly expressed at a low growth temperature and in the stationary phase, i.e. conditions that also stimulate curli production. We show that perturbations in the balance between YddV and Dos, obtained either through inactivation of the *yddV* gene or through overproduction of either YddV or Dos, strongly affect curli production. Both YddV and Dos proteins regulate the transcription of the *csgBAC* operon, which encodes curli structural subunits, while not affecting the expression of the regulatory operon *csgDEFG*. Consistent with the role of both YddV and Dos proteins as oxygen sensors, their effects on *csgBAC* gene expression were dramatically reduced in cells grown under anoxic conditions. Our results show that the *yddV-dos* operon plays an important role in the expression of curli-encoding genes in aerobically growing *E. coli*, and suggest that YddV and Dos, through their opposite activities, might finely tune curli production in response to oxygen availability.

Introduction

Most bacteria are able to switch between two different 'lifestyles': single cells (planktonic mode) and biofilm, i.e. a sessile microbial community. Biofilm and planktonic cells can significantly differ in their physiology, morphology and gene expression pattern. An extensive description of the molecular mechanisms regulating biofilm-related cellular processes can be found in a recent review by Karatan & Watnick (2009). Biofilms are characterized by a lower susceptibility to antibiotics compared with planktonic cells (Anderl *et al*., 2000; Harrison *et al*., 2009). The tolerance of biofilm cells to antibiotics is mediated by different mechanisms, such as reduced penetration into bacterial cells and induction of a dormant physiological state, poorly sensitive to antibiotics, in a significant fraction of the biofilm population (reviewed in Lewis, 2008). In addition to tolerance to antibiotics, biofilms show reduced sensitivity to the host immune defenses. These properties contribute to the chronic nature of most biofilm-mediated infections. Finally, some biofilm determinants can also act as virulence factors: for instance, the production of fimbrial structures, such as curli fibers or type 1 fimbriae, allows the adherence of *Escherichia coli* of eukaryotic cells and promotes its internalization (Gophna *et al*., 2001; Wright *et al*., 2007).

The transition from planktonic cells to biofilm is regulated by environmental and physiological cues, relayed to the bacterial cell by signal molecules such as cyclic di-GMP (c-di-GMP). Intracellular levels of c-di-GMP are regulated by two classes of isoenzymes: diguanylate cyclases (DGCs, c-di-GMP biosynthetic enzymes), also termed GGDEF proteins from the conserved amino acid sequence in their catalytic site, and c-di-GMP phosphodiesterases (c-PDEs), which degrade c-di-GMP (Cotter & Stibitz, 2007). While in Gram-negative bacteria genes encoding DGC and c-PDE proteins are present in high numbers, they are much less conserved in Gram-positive bacteria (Galperin, 2004), where c-di-GMP does not appear to play a significant role

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in biofilm-related cell processes (Holland *et al.*, 2008). In contrast, c-di-GMP promotes biofilm formation in Gram-negative bacteria by stimulating the production of adhesion factors, while repressing cell motility (Kader *et al.*, 2006; Méndez-Ortiz *et al.*, 2006; Weber *et al.*, 2006; Jonas *et al.*, 2008), by acting on c-di-GMP-responsive proteins (reviewed in Hengge, 2009). In addition, c-di-GMP can directly affect virulence factor production in pathogenic bacteria (Kulasakara *et al.*, 2006; Hammer & Bassler, 2009). In *E. coli* and other enterobacteria, the production of curli fibers and cellulose, which form an extracellular matrix promoting biofilm formation (Römling *et al.*, 1998a; Prigent-Combaret *et al.*, 2000; Gualdi *et al.*, 2008), is strongly stimulated by c-di-GMP (Zogaj *et al.*, 2001; Kader *et al.*, 2006; Weber *et al.*, 2006). At least six different genes encoding c-di-GMP-related proteins are involved in curli gene regulation (Sommerfeldt *et al.*, 2009). The expression of several DGC-encoding genes, as well as curli-encoding genes, is controlled by σ^S, an alternative σ factor mainly active under slow growth conditions and in response to cellular stresses. The *yddV-dos* operon is the most expressed among σ^S-dependent genes encoding enzymes related to c-di-GMP metabolism (Weber *et al.*, 2006; Sommerfeldt *et al.*, 2009). The *yddV-dos* operon encodes, respectively, a protein with DGC activity and a c-PDE that can degrade c-di-GMP to pGpG (the noncyclic form of diguanylic acid), not known to function as a signal molecule (Schmidt *et al.*, 2005). Dos stands for direct oxygen sensor, because the Dos protein is complexed to a heme prosthetic group that can bind O_2, CO and nitric oxide (NO) (Delgado-Nixon *et al.*, 2000). A recent publication (Tuckerman *et al.*, 2009) has reported that YddV is also a heme-binding oxygen sensor, and that YddV and Dos interact to form a stable protein complex. Although it has been reported that YddV overexpression can stimulate biofilm formation (Méndez-Ortiz *et al.*, 2006), the targets of *yddV*-dependent biofilm induction have not yet been identified. In this work, we have investigated the role of the *yddV-dos* operon in the regulation of curli production.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this work are listed in Table 1. *Escherichia coli* MG1655 mutant derivatives were constructed using either the λ Red technique (Datsenko & Wanner, 2000) or by bacteriophage P1 transduction (Miller, 1972). The primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Supporting Information, Table S1. Bacteria were grown in M9Glu/sup medium (Brombacher *et al.*, 2006), a glucose-based medium supplemented with 2.5% Luria–Bertani medium as a source of amino acids and vitamins. For growth under anoxic conditions, liquid cultures were grown with no shaking in 12-mL glass tubes filled to the top; these conditions are sufficient for the full induction of genes responding to anaerobiosis (Landini *et al.*, 1994). Antibiotics were used at the following concentrations: ampicillin, $100\,\mu g\,mL^{-1}$; chloramphenicol, $50\,\mu g\,mL^{-1}$; tetracycline, $25\,\mu g\,mL^{-1}$; and kanamycin, $50\,\mu g\,mL^{-1}$. For Congo red-binding assays, bacteria grown overnight in a microtiter plate were spotted, using a replicator, on Congo red-supplemented medium (CR medium), composed of 1.5% agar, 1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$ and 0.0005% $MnCl_2$, to which 0.004% Congo red and 0.002% Coomassie blue were added after autoclaving. Bacteria were grown for 20 h at 30 °C; phenotypes were better detectable after 24–48 h of additional incubation at 4 °C. Surface adhesion assays in polystyrene microtiter plates were performed as described (Dorel *et al.*, 1999).

Plasmid construction

The plasmids used in this work are listed in Table 1. For the construction of the pGEM-T Easy (http://www.promega.com/tbs/tm042/tm042.pdf) plasmid derivatives, either the *yddV* or the *dos* genes were amplified by PCR from *E. coli* MG1655 genomic DNA and the PCR product was cloned into the plasmid, producing pGEM-YddV$_{WT}$ and pGEM-Dos$_{WT}$, respectively. The pGEM-YddV$_{GGAAF}$ and pGEM-Dos$_{AAA}$ plasmids were obtained by three-step PCR mutagenesis (Li & Shapiro, 1993) using the primers listed in Table S1. All constructs were verified by sequencing.

Gene expression studies

Quantitative real-time PCR (qRT-PCR) for the determination of the relative expression levels was performed on cultures grown at 30 °C in M9Glu/sup medium. Samples were taken in the early ($OD_{600\,nm} = 0.25$) and late ($OD_{600\,nm} = 0.7$) exponential phase and in the stationary phase ($OD_{600\,nm} \approx 2.5$) for cultures grown aerobically, and in the stationary phase ($OD_{600\,nm} \approx 1.6$) for cultures grown under anoxic conditions. RNA extraction, reverse transcription and cDNA amplification steps were performed as described (Gualdi *et al.*, 2007), using 16S RNA as the reference gene.

Results

Partial deletion of the *yddV* and *dos* genes

We investigated the possibility that the *yddV-dos* operon might affect the production of curli fibers, a major biofilm determinant in *E. coli* (Prigent-Combaret *et al.*, 2000, 2001), by constructing two mutants in either the *yddV* or the *dos* genes. In order to evaluate more precisely the contribution of c-di-GMP synthesis and turnover toward YddV and Dos

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Table 1. *Escherichia coli* strains and plasmids used in this work

Escherichia coli	Relevant genotype or characteristics	References or sources
Strains		
MG1655	K-12, F^{-} λ^{-} *rph-1*	Blattner *et al*. (1997)
EB1.3	MG1655 *rpoS*::*tet*	Prigent-Combaret *et al*. (2001)
PHL856	MG1655 *csgA-uidA*::*kan*	Gualdi *et al*. (2008)
AM75	MG1655 Δ*csgD*::*cat*	This work
AM95	MG1655 $yddV\Delta_{931-1383}$::*cat*	This work
Plasmids		
pGEM-T Easy	Control vector allowing direct cloning of PCR products, ampicillin resistance	Promega
pGEM-$YddV_{WT}$	The *yddV* gene cloned as a PCR product into the pGEM-T Easy vector	This work
pGEM-$YddV_{GGAAF}$	*yddV* allele carrying the mutation resulting in the GGDEF → GGAAF change in the YddV DGC catalytic site	This work
pGEM-Dos_{WT}	The *dos* gene cloned as a PCR product into the pGEM-T Easy vector	This work
pGEM-Dos_{AAA}	*dos* allele carrying the mutation resulting in the EAL → AAA change in the Dos c-PDE catalytic site	This work

protein activities, our mutagenesis strategy targeted exclusively the region of the gene encoding the domains involved in c-di-GMP metabolism, allowing the production of truncated YddV and Dos proteins carrying functional heme-binding and sensor domains. Because *yddV* and *dos* are part of the same transcriptional unit (Méndez-Ortiz *et al*., 2006), insertions of antibiotic resistance cassettes into the *yddV* gene can result in transcription termination, thus preventing *dos* transcription. However, in the AM95 ($yddV\Delta_{931-1383}$::*cat*) mutant, replacement of the distal part of the *yddV* gene by the chloramphenicol acetyl-transferase (*cat*) gene, placed in the same orientation, results in semi-constitutive transcription of the *dos* gene from the *cat* promoter, as determined by qRT-PCR (data not shown). Because YddV and Dos constitute a highly expressed protein complex possessing both DGC and PDE activity (Sommerfeldt *et al*., 2009; Tuckerman *et al*., 2009), the production of truncated forms of either YddV or Dos should result in the formation of mutant YddV–Dos protein complexes unbalanced either towards accumulation or towards degradation of c-di-GMP. However, we found that mutants in the *dos* gene showed phenotypic instability at the level of cell aggregation in liquid culture and Congo red binding, suggesting that the *dos* mutant strain might accumulate spontaneous mutations suppressing the *dos* defect. Thus, the *dos* mutant strain was not investigated any further, and we focused on the *yddV* mutant AM95 and on MG1655 derivatives overexpressing either the YddV or the Dos proteins from multicopy plasmids.

Effects of the *yddV* and *dos* mutations on Congo red binding and biofilm formation

To determine the possible effects of mutations in the *yddV* gene on curli production, we performed Congo red-binding assays using CR medium. Curli fibers bind with very Congo red high affinity, due to their β-amyloid structure (Olsén *et al*., 1989; Chapman *et al*., 2002). Congo red can bind, albeit with a lower affinity, other cell surface-exposed structures, such as the extracellular polysaccharides cellulose and poly-*N*-acetylglucosamine (Jones *et al*., 1999; Zogaj *et al*., 2001); however, in *E. coli* MG1655, due to the low production of extracellular polysaccharides, the red phenotype on CR medium is totally dependent on curli production (Gualdi *et al*., 2008). Indeed, a mutant carrying a null mutation in the *csgA* gene, encoding the main curli structural subunit, displays a white phenotype on CR medium (Fig. 1a). The $yddV\Delta_{931-1383}$::*cat* mutation resulted in a clear, albeit partial, loss of the red phenotype on CR medium, indicative of a reduction in curli production. To further confirm the effects of the mutation in the *yddV* gene, we cloned either the *yddV* or the *dos* genes into the pGEM-T Easy vector, under the control of the *lac* promoter, producing the pGEM-$YddV_{WT}$ and pGEM-Dos_{WT} plasmids (Table 1). In addition, we constructed plasmids carrying mutant alleles of either gene (pGEM-$YddV_{GGAAF}$ and pGEM-Dos_{AAA}, Table 1), in which the coding sequence for the amino acids responsible for either DGC activity (in the YddV protein) or c-PDE activity (in the Dos protein) had been altered. The substitution of GGDEF motif into the DGC catalytic site to GGAAF results in a drastic loss (> 90%) of DGC activity (De *et al*., 2008; Antoniani *et al*., 2010). In the Dos protein, the glutamic acid and leucine in the EAL motif were changed to alanine residues, giving rise to the Dos_{AAA} mutant; mutations affecting the EAL motif abolish c-PDE activity (Kirillina *et al*., 2004; Bassis & Visick, 2010). Transformation of the *yddV* mutant AM95 strain with pGEM-$YddV_{WT}$, but not with pGEM-$YddV_{GGAAF}$, restored the red phenotype on CR medium (Fig. 1b), indicating that YddV can affect the CR phenotype in a manner dependent on its DGC activity. Transformation of MG1655 with the pGEM-Dos_{WT} plasmid (Fig. 1c) resulted in a white CR phenotype, consistent with a negative role of

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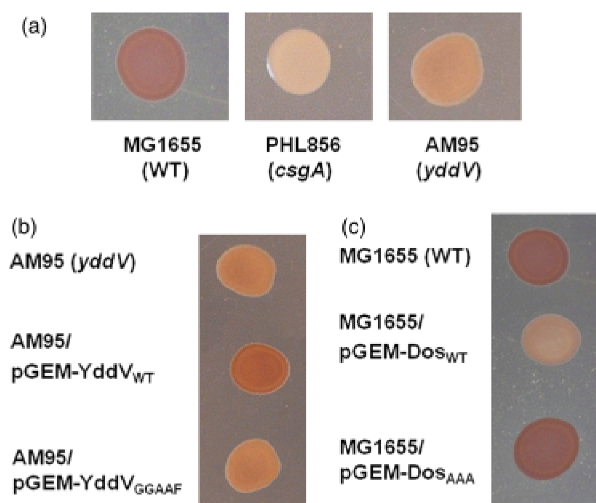

Fig. 1. (a) Congo red phenotype of MG1655 (WT), PHL856 (*csgA*) and AM95 (*yddV*). (b) AM95 (*yddV*) strain transformed with either pGEM-YddV$_{WT}$ or pGEM-YddV$_{GGAAF}$. (c) MG1655 strain transformed with either pGEM-Dos$_{WT}$ or pGEM-Dos$_{AAA}$.

Dos in curli production. In contrast, no effects were observed on the CR phenotype in the MG1655 strain harboring the pGEM-Dos$_{AAA}$ plasmid, carrying the mutant Dos protein impaired in its c-PDE activity.

In *E. coli* MG1655, curli fibers are the main determinant for adhesion to abiotic surfaces (Prigent-Combaret *et al*., 2000). Thus, we confirmed the results of Congo red-binding assays by biofilm formation experiments on polystyrene microtiter plates (Fig. 2). Consistent with the pivotal role of curli in adhesion to abiotic surfaces, biofilm formation on microtiter plates was reduced by about 10-fold by the inactivation of the *csgA* gene, encoding the major curli subunit (Fig. 2), as well as by growth at 37 °C (data not shown), the temperature at which curli fibers are not produced in most enterobacteria (Römling *et al*., 1998a). Inactivation of the *yddV* gene resulted in a *c*. 3.5-fold reduction in biofilm formation. Overexpression of YddV$_{WT}$, but not of the YddV$_{GGAAF}$ protein, results in strong biofilm stimulation, in agreement with CR phenotypes (Fig. 1b). Overexpression of the Dos protein mimicked the effects of the *yddV* mutation, resulting in decreased biofilm production; however, no effect was detected for overexpression of the Dos mutant protein impaired in c-PDE activity (Fig. 2). Thus, the results of Congo red binding studies and biofilm formation experiments strongly support the hypothesis that the YddV and Dos proteins control curli production through the modulation of intracellular c-di-GMP concentrations.

Effect of the *yddV* and *dos* mutations on curli gene expression

The regulation of adhesion factors' production by DGCs can take place at different levels, such as allosteric activation, as in the stimulation of cellulose biosynthesis by AdrA (Zogaj *et al*., 2001), or gene regulation, such as in the transcription regulation of the *csgDEFG* operon by YdaM and YegE (Sommerfeldt *et al*., 2009). We tested the possibility that the *yddV* gene might affect the CR phenotype and adhesion to polystyrene through gene expression regulation of the curli-encoding operons. Curli production and assembly is mediated by two divergent operons; *csgDEFG* encodes the transport and assembly proteins and the CsgD regulator, which in turn activates the *csgBAC* operon, encoding curli structural subunits (Römling *et al*., 1998b). Because curli genes are subject to growth phase-dependent regulation mediated by the *rpoS* gene (Römling *et al*., 1998b), we assessed the effects of the *yddV* mutation at different growth stages: early exponential phase ($OD_{600\,nm} = 0.25$), late exponential phase ($OD_{600\,nm} = 0.7$) and stationary phase (overnight cultures, $OD_{600\,nm} \geq 2.5$). Transcription levels of the *csgB* and *csgD* genes in M9Glu/sup medium at 30 °C were determined by qRT-PCR (Table 2). Interestingly, the expression of *csgD* and *csgB* follows different kinetics: while *csgB* is only induced in the late stationary phase, *csgD* transcription levels are very similar both in the exponential and in the stationary phase. A different timing between *csgD* and *csgB* transcription in *E. coli* MG1655 has already been reported (Prigent-Combaret *et al*., 2001). Although the lack of stationary-phase-dependent-activation of the *csgD* gene might appear to be surprising, *rpoS*-dependent gene expression during the exponential phase is rather common (Dong *et al*., 2008); indeed, the expression of both *csgB* and *csgD* is totally abolished in the *rpoS*-deficient EB1.3 mutant derivative of MG1655 (data not shown). *yddV* inactivation caused a drastic decrease in *csgB* expression (*c*. 400-fold reduction, Table 2), while showed a much more reduced effect on *csgD* transcription (*c*. 2.5-fold), suggesting that the YddV protein specifically regulates the transcription of the *csgBAC* operon. Overexpression of either the YddV or the Dos protein confirmed this result, showing *csgBAC* upregulation by YddV and downregulation by Dos, in a manner dependent on their DGC and c-PDE activities, respectively (Table 3). The observation that YddV regulates *csgBAC* transcription, which is also dependent on the CsgD protein, may suggest that c-di-GMP synthesis by YddV might trigger CsgD activity as a transcription regulator. To test this hypothesis, we studied the effect of the *yddV* mutation on the expression of *adrA*, a CsgD-dependent gene involved in the regulation of cellulose production (Zogaj *et al*., 2001): as shown in Table 2, *adrA* transcript levels were not significantly affected by *yddV* inactivation, suggesting that the CsgD protein can function as a transcription activator in the *yddV* mutant strain AM95.

Both the YddV and the Dos protein require binding of their heme prosthetic groups to O_2, or alternatively to NO, in order to trigger either DGC or c-PDE activity (Taguchi

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et al., 2004; Tuckerman *et al.*, 2009). Thus, we measured *csgB* and *csgD* expression levels in bacteria grown in oxygen limitation, comparing MG1655 with its $yddV\Delta_{931-1383}$::*cat* mutant derivative. Growth under anoxic conditions did not affect *csgD* transcript levels, while reducing *csgB* expression by *c.* 7-fold; *yddV* inactivation resulted only in a *c.* 2.5-fold reduction in *csgB* transcript levels, vs. the *c.* 400-fold reduction in aerobic growth (Table 2), suggesting that YddV-dependent regulation of the *csgBAC* operon is bypassed under oxygen-limiting conditions. Consistent with this observation, no effect on *csgBAC* expression by either YddV or Dos overexpression could be detected in MG1655 grown in oxygen limitation (Table 3).

Growth-phase dependent regulation of the *yddV-dos* operon

Our results clearly indicate that a functional *yddV* gene is required for *csgBAC*, but not *csgDEFG*, expression (Table 2), suggesting that the YddV protein acts downstream of CsgD in the regulatory cascade leading to curli production. It is thus possible that the CsgD protein might activate the transcription of the *yddV-dos* operon and, in turn, YddV might trigger *csgBAC* expression in the stationary phase of growth. However, cotranscription of the *yddV* and the *dos* genes also raises the question of how the opposite activities of the YddV and Dos proteins are modulated. We investigated the possibility that the *yddV-dos* transcript might be processed in the stationary phase of growth, resulting in the accumulation of the YddV protein, with consequent activation of *csgBAC* expression. To address these questions, we determined both *yddV* and *dos* transcripts at different growth stages, and we tested the possible dependence of *yddV-dos* transcription on the CsgD protein by comparing MG1655 with its *csgD* mutant derivative AM75. In addition, because transcription of the *yddV-dos* operon is controlled by the *rpoS* gene (Weber *et al.*, 2006; Sommerfeldt *et al.*, 2009), which also regulates curli-encoding genes (Römling *et al.*, 1998b), we also determined gene expression kinetics of the *yddV-dos* operon in the *rpoS* mutant derivative EB1.3. As shown in Fig. 3, transcription of the *yddV* gene was induced in an *rpoS*-dependent manner in the late exponential phase, reaching maximal induction in overnight cultures; in contrast, *csgD* inactivation did not affect *yddV* expression. Transcription of the *yddV* and of the *dos* genes followed a very similar pattern (Fig. 3) and the overall ratio between *yddV* and *dos* transcripts remained constant in different growth phases, suggesting that neither *yddV* nor *dos* is subject to specific regulation at the level of mRNA processing, at least under the conditions tested.

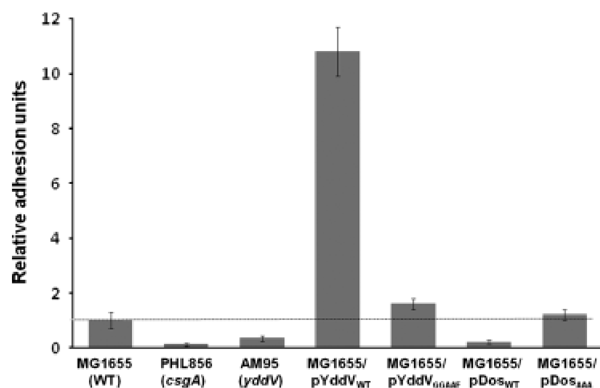

Fig. 2. Surface adhesion on polystyrene microtiter plates by strains: MG1655 (WT), PHL856 (*csgA*), AM95 (*yddV*) and MG1655 transformed with pGEM-YddV$_{WT}$, pGEM-YddV$_{GGAAF}$, pGEM-Dos$_{WT}$ and pGEM-Dos$_{AAA}$. The relative adhesion value was set to 1 for MG1655 (indicated by the dotted line); the actual adhesion unit value for MG1655 was 3.1. Results are the average of three independent experiments, and SEs are shown.

Discussion

In this work, we have shown that the production of curli fibers, a major adhesion factor in *E. coli*, is affected by the *yddV-dos* operon, encoding a YddV–Dos protein complex involved in c-di-GMP biosynthesis and turnover. Control of curli production by *yddV-dos* takes place at the level of transcription regulation of the *csgBAC* operon, encoding curli structural subunits (Table 2), and is mediated by the

Table 2. Relative expression of *csgB* and *csgD* genes in MG1655 vs. AM95 (*yddV*::*cat*)

Genes	*csgB*		*csgD*		*adrA*	
Strains	MG1655 (WT)	AM95 (*yddV*::*cat*)	MG1655 (WT)	AM95 (*yddV*::*cat*)	MG1655 (WT)	AM95 (*yddV*::*cat*)
Growth conditions						
Early exponential ($OD_{600\,nm}$ = 0.25)	1*	0.7	1*	0.6	ND	ND
Late exponential ($OD_{600\,nm}$ = 0.7)	0.8	0.9	1.5	0.7	ND	ND
Stationary ($OD_{600\,nm}$ ≥ 2.5)	391	0.9	1.4	0.6	1*	0.74
Stationary, anoxic ($OD_{600\,nm}$ ≥ 1.6)	57.2	22.4	1.6	1.4	ND	ND

*ΔC_t between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655 in the early exponential growth phase ($OD_{600\,nm}$ = 0.25) for *csgB* and *csgD* genes, and in the stationary phase for *adrA*. The actual ΔC_t values were: *csgD* = 15.0; *csgB* = 21.7; *adrA* = 22.4. ΔC_t between the gene of interest and the 16S gene for different growth phases and for mutant strains are expressed as relative values. Values are the average of two independent experiments performed in duplicate. ND, not determined.

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Table 3. Relative expression of *csgB* and *csgD* genes in response to either YddV or Dos overexpression

Strains	*csgB* expression (aerobic conditions)	*csgB* expression (anoxic conditions)	*csgD* expression (aerobic conditions)
MG1655/pGEM-T	1*	0.38	1*
MG1655/pYddV$_{WT}$	31.2	0.32	2.1
MG1655/pYddV$_{GGAAF}$	2.3	0.45	1.6
MG1655/pDos$_{WT}$	0.06	0.34	ND
MG1655/pDos$_{AAA}$	1.04	0.37	ND

*ΔC_t between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655/pGEM-T under aerobic conditions. Actual ΔC_t values in MG1655/pGEM-T: *csgB* = 15.9; *csgD* = 14.6. Values are the average of two independent experiments performed in duplicate.

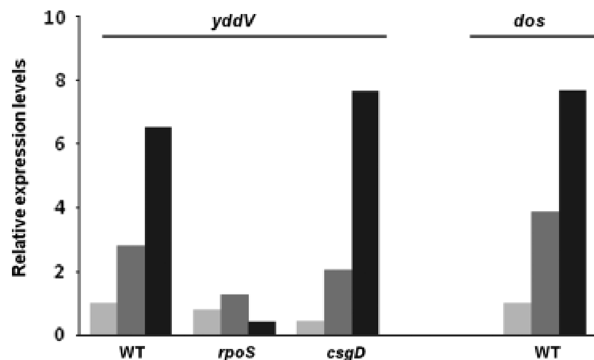

Fig. 3. Relative expression levels of the *yddV* gene in strains MG1655 (WT), EB1.3 (*rpoS*) and AM75 (*csgD*), and of the *dos* gene in strain MG1655, as measured by real-time PCR experiments. Expression values in MG1655 in the early exponential growth phase ($OD_{600\,nm}$ = 0.25; light gray bars) (corresponding to a ΔC_t relative to 16S rRNA = 16.3 for *yddV*, and 15.8 for *dos*) were set to 1. The other samples were taken in late exponential phase ($OD_{600\,nm}$ = 0.7; gray bars) and stationary phase ($OD_{600\,nm} \geq 2.5$; black bars). Data are the average of two independent experiments, each performed in triplicate, with very similar results.

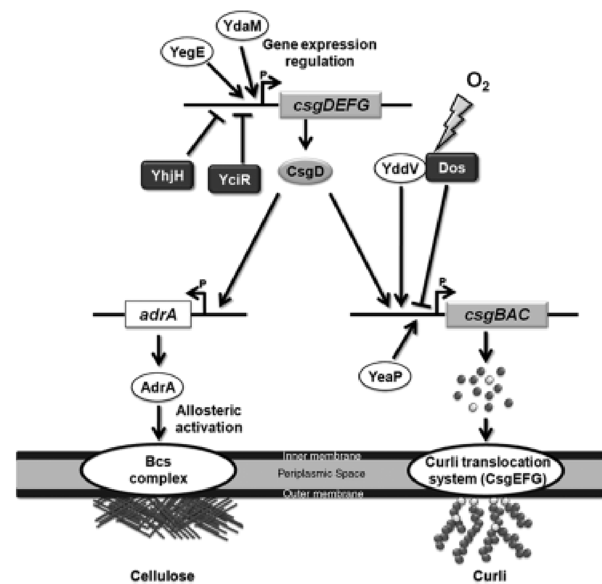

Fig. 4. Model summarizing gene expression regulation of curli- and cellulose-related genes by DGC and c-PDE proteins. Proteins with DGC activity are indicated by open ellipses; c-PDEs are shown as dark squares. The CsgD protein, which activates both the *csgBAC* operon encoding curli structural subunits and *adrA*, encoding a DGC acting as a positive effector of cellulose biosynthesis, is indicated in the gray ellipse. See text for further details.

DGC and PDE activities of YddV and Dos (Fig. 1b and c; Table 3). In contrast, the YddV–Dos protein complex does not strongly influence *csgDEFG* expression, nor does it affect the expression of the CsgD-dependent *adrA* gene, encoding a positive effector for cellulose biosynthesis (Table 2). Regulation of the *csgBAC* operon, but not of *csgDEFG*, has already been described for another DGC, the product of the *yeaP* gene (Sommerfeldt *et al.*, 2009). Thus, in *E. coli*, the production of curli and cellulose involves DGC and c-PDE proteins at various levels (summarized in Fig. 4). Indeed, *csgDEFG* transcription is regulated by the DGC YdaM (Weber *et al.*, 2006) and by the PDEs YciR and YhjH (Pesavento *et al.*, 2008), *csgBAC* transcription by YeaP and by the YddV–Dos complex (Sommerfeldt *et al.*, 2009; this work), while the AdrA protein activates cellulose production (Zogaj *et al.*, 2001). The involvement of such a large number of c-di-GMP-related proteins might depend on the need to relay different environmental signals to the activation of the *csgD* regulon. Indeed, curli and cellulose production responds to a variety of environmental cues, including low temperature, slow growth and low osmolarity (Römling *et al.*, 1998b). In addition, devoted DGCs and PDEs can trigger the expression of individual CsgD-dependent genes (e.g. *csgBAC*) in response to specific environmental signals, thus altering the relative expression of genes belonging to the CsgD regulon and, in particular, the balance between curli and cellulose production.

Depending on the prevalence of either its DGC or its c-PDE activities, the YddV–Dos complex can either activate or repress *csgBAC* expression. In the bacterial cell, this could be achieved by changing the relative intracellular concentrations of either YddV or Dos proteins, for instance through post-transcriptional regulation. However, our results seem to rule out the possible regulation of either *yddV* or *dos* at the level of mRNA stability (Fig. 3), suggesting that YddV and Dos might be regulated either in their protein stability level or through modulation of their enzymatic activities. Both DGC activity by YddV and c-PDE activity by Dos are inhibited in the absence of oxygen; however, YddV and Dos respond differently

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to oxygen concentrations, which can thus affect the overall balance between DGC and c-PDE activities in the YddV–Dos protein complex (Tuckerman *et al*., 2009). Thus, oxygen availability in the bacterial cell might function as an environmental signal for the modulation of intracellular c-di-GMP concentrations via the YddV–Dos complex.

Oxygen tension is known to regulate curli production in *Salmonella* (Gerstel & Römling, 2001). It is conceivable that oxygen sensing can be important for curli expression in relation to biofilm growth: indeed, with the exception of cells in the external layers, bacteria growing in biofilms are exposed to a gradient in oxygen availability that leads to a switch to anaerobic metabolism in the innermost biofilm layers (Borriello *et al*., 2004; Rani *et al*., 2007). The growth of *E. coli* MG1655 in oxygen limitation results in a sevenfold decrease in *csgBAC*, but not *csgDEFG*, expression (Table 2); under these growth conditions, overexpression of neither YddV nor Dos affects *csgBAC* expression (Table 3), consistent with the inhibition of both DGC and c-PDE activities of the YddV and Dos proteins in the absence of oxygen. This result would suggest that in the innermost biofilm layers, oxygen limitation might lead to reduced *csgBAC* expression and curli production, without, however, affecting the expression of *csgDEFG* and of other genes belonging to the CsgD regulon. We conclude that relay of oxygen sensing to curli production is mediated by c-di-GMP signalling involving the YddV–Dos complex.

References

Anderl JN, Franklin MJ & Stewart PS (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Ch* **44**: 1818–1824.

Antoniani D, Bocci P, Maciąg A, Raffaelli N & Landini P (2010) Monitoring of di-guanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl Microbiol Biot* **85**: 1095–1104.

Bassis CM & Visick KL (2010) The cyclic-di-GMP phosphodiesterase BinA negatively regulates cellulose-containing biofilms in *Vibrio fischeri*. *J Bacteriol* **192**: 1269–1278.

Blattner FR, Plunkett G III, Bloch CA *et al*. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1474.

Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD & Stewart PS (2004) Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Ch* **48**: 2659–2664.

Brombacher E, Baratto A, Dorel C & Landini P (2006) Gene expression regulation by the curli activator CsgD protein: modulation of cellulose biosynthesis and control of negative determinants for microbial adhesion. *J Bacteriol* **188**: 2027–2037.

Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, Normark S & Hultgren SJ (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* **295**: 851–855.

Cotter PA & Stibitz S (2007) C-di-GMP-mediated regulation of virulence and biofilm formation. *Curr Opin Microbiol* **10**: 17–23.

Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *P Natl Acad Sci USA* **97**: 6640–6645.

De N, Pirruccello M, Krasteva PV, Bae N, Raghavan RV & Sondermann H (2008) Phosphorylation-independent regulation of the diguanylate cyclase WspR. *PLoS Biol* **6**: e67.

Delgado-Nixon VM, Gonzalez G & Gilles-Gonzalez MA (2000) Dos, a heme-binding PAS protein from *Escherichia coli*, is a direct oxygen sensor. *Biochemistry* **39**: 2685–2691.

Dong T, Kirchhoff MG & Schellhorn HE (2008) RpoS regulation of gene expression during exponential growth of *Escherichia coli* K12. *Mol Genet Genomics* **279**: 267–277.

Dorel C, Vidal O, Prigent-Combaret C, Vallet I & Lejeune P (1999) Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiol Lett* **178**: 169–175.

Galperin MY (2004) Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* **6**: 552–567.

Gerstel U & Römling U (2001) Oxygen tension and nutrient starvation are major signals that regulate *agfD* promoter activity and expression of the multicellular morphotype in *Salmonella typhimurium*. *Environ Microbiol* **3**: 638–648.

Gophna U, Barlev M, Seijffers R, Oelschlager TA, Hacker J & Ron EZ (2001) Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect Immun* **69**: 2659–2665.

Gualdi L, Tagliabue L & Landini P (2007) Biofilm formation-gene expression relay system in *Escherichia coli*: Modulation of sigmaS-dependent gene expression by the CsgD regulatory protein via sigmaS protein stabilization. *J Bacteriol* **189**: 8034–8043.

Gualdi L, Tagliabue L, Bertagnoli S, Ieranò T, De Castro C & Landini P (2008) Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. *Microbiology* **154**: 2017–2024.

Hammer BK & Bassler BL (2009) Distinct sensory pathways in *Vibrio cholerae* El Tor and classical biotypes modulate cyclic dimeric GMP levels to control biofilm formation. *J Bacteriol* **191**: 169–177.

Harrison JJ, Wade WD, Akierman S, Vacchi-Suzzi C, Stremick CA, Turner RJ & Ceri H (2009) The chromosomal toxin gene *yafQ* is a determinant of multidrug tolerance for *Escherichia coli* growing in a biofilm. *Antimicrob Agents Ch* **53**: 2253–2258.

Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**: 263–273.

Holland LM, O'Donnell ST, Ryjenkov DA, Gomelsky L, Slater SR, Fey PD, Gomelsky M & O'Gara JP (2008) A staphylococcal

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GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. *J Bacteriol* **190**: 5178–5189.

Jonas KA, Edwards N, Simm R, Romeo T, Römling U & Melefors O (2008) The RNA binding protein CsrA controls cyclic di-GMP metabolism by directly regulating the expression of GGDEF proteins. *Mol Microbiol* **70**: 236–257.

Jones HA, Lillard JW Jr & Perry RD (1999) HmsT, a protein essential for expression of the haemin storage (Hms^+) phenotype of *Yersinia pestis*. *Microbiology* **145**: 2117–2128.

Kader A, Simm R, Gerstel U, Morr M & Römling U (2006) Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **60**: 602–616.

Karatan E & Watnick P (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol R* **73**: 310–347.

Kirillina O, Fetherston JD, Bobrov AG, Abney J & Perry RD (2004) HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. *Mol Microbiol* **54**: 75–88.

Kulasakara H, Lee V, Brencic A *et al.* (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *P Natl Acad Sci USA* **103**: 2839–2844.

Landini P, Hajec LI & Volkert MR (1994) Structure and transcriptional regulation of the *Escherichia coli* adaptive response gene *aidB*. *J Bacteriol* **176**: 6583–6589.

Lewis K (2008) Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol* **322**: 107–131.

Li XM & Shapiro LJ (1993) Three step PCR mutagenesis for 'linker scanning'. *Nucleic Acids Res* **21**: 3745–3748.

Méndez-Ortiz MM, Hyodo M, Hayakawa Y & Membrillo-Hernández J (2006) Genome-wide transcriptional profile of *Escherichia coli* in response to high levels of the second messenger 3′,5′-cyclic diguanylic acid. *J Biol Chem* **281**: 8090–8099.

Miller JH (ed) (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Olsén A, Jonsson A & Normark S (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* **338**: 652–655.

Pesavento C, Becker G, Sommerfeldt N, Possling A, Tschowri N, Mehlis A & Hengge R (2008) Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli*. *Gene Dev* **22**: 2434–2446.

Prigent-Combaret C, Prensier G, Le Thi TT, Vidal O, Lejeune P & Dorel C (2000) Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. *Environ Microbiol* **2**: 450–464.

Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P & Dorel C (2001) Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. *J Bacteriol* **183**: 7213–7223.

Rani SA, Pitts B, Beyenal H, Veluchamy RA, Lewandowski Z, Davison WM, Buckingham-Meyer K & Stewart PS (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol* **189**: 4223–4233.

Römling U, Bian Z, Hammar M, Sierralta WD & Normark S (1998a) Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J Bacteriol* **180**: 722–731.

Römling U, Sierralta WD, Eriksson K & Normark S (1998b) Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. *Mol Microbiol* **28**: 249–264.

Schmidt AJ, Ryjenkov DA & Gomelsky M (2005) The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J Bacteriol* **187**: 4774–4781.

Sommerfeldt N, Possling A, Becker G, Pesavento C, Tschowri N & Hengge R (2009) Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in *Escherichia coli*. *Microbiology* **155**: 1318–1331.

Taguchi S, Matsui T, Igarashi J, Sasakura Y, Araki Y, Ito O, Sugiyama S, Sagami I & Shimizu T (2004) Binding of oxygen and carbon monoxide to a heme-regulated phosphodiesterase from *Escherichia coli*. Kinetics and infrared spectra of the full-length wild-type enzyme, isolated PAS domain, and Met-95 mutants. *J Biol Chem* **279**: 3340–3347.

Tuckerman JR, Gonzalez G, Sousa EH, Wan X, Saito JA, Alam M & Gilles-Gonzalez MA (2009) An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry* **48**: 9764–9774.

Weber H, Pesavento C, Possling A, Tischendorf G & Hengge R (2006) Cyclic-di-GMP-mediated signalling within the sigma network of *Escherichia coli*. *Mol Microbiol* **62**: 1014–1034.

Wright KJ, Seed PC & Hultgren SJ (2007) Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. *Cell Microbiol* **9**: 2230–2241.

Zogaj X, Nimtz M, Rohde M, Bokranz W & Römling U (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol Microbiol* **39**: 1452–1463.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in this work.

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