The diguanylate cyclase YddV controls production of the exopolysaccharide poly-N-acetylglucosamine (PNAG) through regulation of the PNAG biosynthetic pgaABCD operon

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In Gram-negative bacteria, production of adhesion factors and extracellular polysaccharides (EPS) is promoted by the activity of diguanylate cyclases (DGCs), a class of enzymes able to catalyse the synthesis of the signal molecule bis-(3',5')-cyclic di-guanylic acid (c-di-GMP). In this report we show that in Escherichia coli, overexpression of the YddV protein, but not of other DGCs such as AdrA and YcdT, induces the production of the EPS poly-N-acetylglucosamine (PNAG) by stimulating expression of pgaABCD, the PNAG-biosynthetic operon. Stimulation of PNAG production and activation of pgaABCD expression by the YddV protein are abolished by inactivation of its GGDEF motif, responsible for DGC activity. Consistent with the effects of YddV overexpression, inactivation of the yddV gene negatively affects pgaABCD transcription and PNAG-mediated biofilm formation. pgaABCD regulation by the yddV gene also takes place in a mutant carrying a partial deletion of the csrA gene, which encodes the main regulator of pgaABCD expression, suggesting that YddV does not regulate pgaABCD through modulation of CsrA activity. Our results demonstrate that PNAG production does not simply respond to c-di-GMP concentration, but specifically requires the DGC activity of the YddV protein, thus supporting the notion that in E. coli, c-di-GMP biosynthesis by a given DGC protein triggers regulatory events that lead to activation of specific sets of EPS biosynthetic genes or proteins.

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 differ significantly in their physiology, in their gene expression pattern and even in their morphology. In particular, biofilm cells are characterized by production of adhesion factors and extracellular polysaccharides (EPS), resistance to environmental stresses, and lower sensitivity to antibiotics compared with planktonic cells (Costerton et al., 1995; Anderl et al., 2000; Harrison et al., 2007, 2009). Transition from planktonic cells to biofilm is regulated by environmental and physiological cues, relayed to the bacterial cell by signal molecules or ‘second messengers’. A second messenger, bis-(3',5')-cyclic di-guanylic acid, better known as cyclic-di-GMP (c-di-GMP), plays a pivotal role in biofilm formation and maintenance by stimulating production of EPS and adhesion factors (Ross et al., 1991; Simm et al., 2004; Kader et al., 2006; Weber et al., 2006). In addition, c-di-GMP biosynthesis affects important cellular processes, such as morphological differentiation and cell replication in Caulobacter crescentus (Paul et al., 2004), cell motility (Méndez-Ortiz et al., 2006; Jonas et al., 2008) and virulence factor production (Kulasakara et al., 2006; Hammer & Bassler, 2009). In Enterobacteria, c-di-GMP seems to be involved in regulation of adhesion factors, such as curli and cellulose, important for adaptation and survival outside the warm-blooded host (Simm et al.,
2004; Kader et al., 2006; Weber et al., 2006; Solano et al., 2009), as also suggested by the observation that expression of the several diguanylate cyclase (DGC)-encoding genes is turned on at a growth temperature of 30 °C or lower (Weber et al., 2006; Sommerfeldt et al., 2009). Intracellular levels of c-di-GMP are regulated by two classes of isoenzymes: DGCs (c-di-GMP biosynthetic enzymes), also termed GGDEF proteins from the conserved Gly-Gly-Asp-Glu-Phe motif in their catalytic domains, and c-di-GMP phosphodiesterases (PDEs), which degrade c-di-GMP (Cotter & Stibitz, 2007). Genes encoding proteins involved in c-di-GMP biosynthesis and turnover are present in Gram-negative bacteria (Galperin, 2004), in which c-di-GMP does much higher numbers in Gram-negative than in Gram-positive bacteria (Galperin, 2004), in which c-di-GMP does not appear to play a significant role in biofilm-related cell processes (Holland et al., 2008). The high number of DGC- and PDE-encoding genes in Gram-negative bacteria would suggest that c-di-GMP biosynthesis and degradation constitute a mechanism for signal transduction involving the interaction of c-di-GMP-responsive proteins with specific DGCs. Indeed, several c-di-GMP-driven cell processes, such as cellulose production in Salmonella (Zogaj et al., 2001), depend on specific interactions between a given DGC and one or more target proteins. An increasing number of proteins responsive to c-di-GMP has been identified (reviewed by Hengge, 2009), including several DNA-binding proteins, i.e. the FleQ regulator in Pseudomonas aeruginosa (Hickman & Harwood, 2008), the VpsT protein in Vibrio cholerae (Krasteva et al., 2010) and the CLP protein in Xanthomonas campestris (Chin et al., 2010). In addition, c-di-GMP can regulate gene expression through direct binding to riboswitch elements in mRNAs (Sudarsan et al., 2008), bypassing the need for c-di-GMP-binding regulatory proteins.

The YddV protein is arguably one of the most expressed DGCs in Escherichia coli (Sommerfeldt et al., 2009). Recently, we have shown that YddV can affect the expression of curli-encoding genes (Tagliabue et al., 2010), which, however, are extremely sensitive to perturbations in intracellular c-di-GMP concentrations (Sommerfeldt et al., 2009). In this work, we show that overexpression of YddV, but not of other DGCs, stimulates production of poly-N-acetylglucosamine (PNAG), an EPS able to promote biofilm formation, by triggering expression of pgaABCD, the PNAG biosynthetic operon. Our results identify a specific physiological role for the YddV protein, and suggest that different DGCs can target cognate operons (or proteins) in a specific fashion in order to trigger production of different EPS.

### METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this work are listed in Table 1. When not otherwise stated, bacteria were grown in M9Glutricin and M9Glu/sup [M9 inorganic salts (Smith and Levine, 1964), 5 g glucose 1⁻¹, 0.25 g peptone 1⁻¹, 0.125 g yeast extract 1⁻¹]. When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg ml⁻¹; chloramphenicol, 50 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; tetracycline, 25 μg ml⁻¹; rifampicin, 100 μg ml⁻¹. For Congo red (CR) and Calcofluor (CF) assays, overnight cultures were spotted, using a replicator, on agar media supplemented with 0.5 %

### Table 1. E. coli strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>MG1655</td>
<td>K-12, F⁻ rph-1</td>
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<tr>
<td>AM56</td>
<td>MG1655 ΔpgaA::cat</td>
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<td>AM89</td>
<td>MG1655 ydaM::Tn5 kan</td>
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<td>MG1655ΔyddVCTD::cat (yddVΔ233−1383::cat)</td>
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<td>Promega</td>
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<td>Tagliabue et al. (2010)</td>
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<td>pYddVGGAAF</td>
<td>yddV allele carrying mutation resulting in GGDEF→GGAAF change in the DGC catalytic site of the YddV protein</td>
<td>Tagliabue et al. (2010)</td>
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Casamino acids, 0.15% yeast extract, 0.005% MgSO₄, 0.0005% MnCl₂; either 0.004% CR and 0.002% Coomassie blue (for CR medium) or 0.005% CF (for CF medium) were added after autoclaving. Bacteria were grown for 20 h at 30 °C; phenotypes were better detectable after 24–48 h incubation at 4 °C.

**Biofilm formation assays.** Biofilm formation in microtitre plates was determined essentially as described by Dorel et al. (1999). Bacterial cultures were grown overnight in M9Glu/sup medium at 30 °C in polystyrene microtitre plates (0.2 ml); the cell density of the culture was determined spectrophotometrically at 600 nm (OD₆₀₀). Cells attached to the microtitre plates were washed gently with water and stained for 20 min with 1% crystal violet (CV) in ethanol, thoroughly washed with water and dried. For semiquantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml 95% ethanol by vigorous pipetting. The OD₆₀₀ of each sample was determined and normalized to the OD₆₀₀ of the corresponding liquid cultures (adhesion units). The sensitivity of biofilms to treatment with the PNAG-degrading enzyme Dispersin B (Kaplan et al., 2004; purchased from Kane Biotech) was determined by adding 20 µg ml⁻¹ enzyme to the growth medium.

**Plasmid construction.** Plasmids used in this work are listed in Table 1. For overexpression of genes encoding DGCs, genes of interest were amplified by PCR and the corresponding products cloned into the pGEM-T Easy vector. Correct orientation of the inserts (i.e. under the control of the Pₙ₆₆ promoter) was verified by PCR using the primers listed in Supplementary Table S1. For DGC-overproduction studies, strains carrying pGEM-T Easy derivatives were grown at 30 °C in M9Glu/sup medium in the absence of IPTG induction of the Pₙ₆₆ promoter. The pYdV_GGAAT plasmid, carrying the yddV gene mutated in the DGC catalytic site, was obtained by three-step PCR mutagenesis (Li & Shapiro, 1993) using the primers listed in Supplementary Table S1. All constructs were verified by sequencing.

**Gene expression studies.** Real-time PCR for determination of relative expression levels was performed on overnight cultures grown in M9Glu/sup medium at 30 °C. Primers for real-time PCR are listed in Supplementary Table S1. RNA extraction and further reverse transcription and cDNA amplification steps were performed as described by Gualdi et al. (2007), using 16S RNA as a reference gene. mRNA stability was measured by real-time PCR experiments in the presence of rifampicin, as described by Wang et al. (2005).

**Other methods.** E. coli MG1655 mutant derivatives were constructed either using the λ Red technique (Datsenko & Wanner, 2000) or by bacteriophage P1 transduction (Miller, 1972), except for the AM98 strain (MG1655::ydaM<sup>+</sup>; Tn5-kan), which was obtained in a transposon mutagenesis screening for adhesion-deficient MG1655 mutants using the EZ-Tn5 <R6K<sup>ori</sup>/KAN-2> transposon (Epitect; P. Landini, unpublished data). Primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Supplementary Table S1. Bacterial cell motility was evaluated as described by Pesavento et al. (2008). Determination of intracellular c-di-GMP concentration was performed as previously described (Antoniani et al., 2010).

**RESULTS**

**Overexpression of DGCs**

In Enterobacteria, production of EPS such as PNAG and cellulose (Römling et al., 2000; Zogaj et al., 2001; Boehm et al., 2009), and of proteinaceous adhesion factors such as curli fibres (Kader et al., 2006; Weber et al., 2006), is regulated by DGC proteins and c-di-GMP biosynthesis. However, for several genes encoding putative DGCs, their functional role in the production of adhesion factors has not been fully determined. For instance, yddV, arguably the most highly expressed DGC-encoding gene in E. coli (Sommerfeldt et al., 2009), can activate transcription of the csgBA operon, encoding curli subunits (Tagliabue et al., 2010); however, YddV overexpression can stimulate biofilm formation independently of curli production (Méndez-Ortiz et al., 2006), thus suggesting that yddV can induce biofilm formation by acting on additional, not yet identified targets. In order to study the specific effects of YddV on the production of extracellular structures, we cloned the yddV gene into the pGEM-T Easy plasmid, which allows constitutive expression of cloned genes in the absence of IPTG induction. We compared yddV with three different DGC-encoding genes: adrA, encoding an activator of cellulose production (Zogaj et al., 2001); ycdT, located in the pgaABCDD locus and co-regulated with the PNAG-biosynthetic genes (Jonas et al., 2008); and ydaM, required for expression of curli-encoding genes (Weber et al., 2006). Plasmid-driven expression of each of the four genes resulted in a significant increase in intracellular c-di-GMP concentrations, consistent with production of active proteins; however, while overproduction of the AdrA and the YdaM proteins resulted in a more than 150-fold increase in intracellular c-di-GMP, in agreement with previous observations (Antoniani et al., 2010), YcdT and YddV only enhanced c-di-GMP concentration by about 10-fold (Fig. 1). c-di-GMP intracellular concentrations did not strictly correlate with DGC overproduction levels, as judged by SDS-PAGE analysis of cell extracts (data not shown). The expression of each DGC led to a reduction in bacterial mobility (Supplementary Table S2), in agreement with earlier observations (Méndez-Ortiz et al., 2006; Jonas et al., 2008; Pesavento et al., 2008).

**Effects of DGC overexpression on cell surface-associated structures**

The plasmids carrying DGC-encoding genes were used to transform a set of mutant derivatives of E. coli MG1655 deficient in the production of curli, cellulose or PNAG, namely: AM70 (ΔcsgA::cat), unable to produce curli; LG26, a ΔcbsA::kan mutant impaired in cellulose production; AM73, a ΔcsgA/ΔcbsA double mutant; and AM56, a ΔpgaA::cat mutant unable to export PNAG and to expose it on the cell surface (Itoh et al., 2008). We expected that phenotypes depending on an increase in production of cell surface-associated structures caused by DGC overexpression would be abolished by inactivation of the corresponding target genes. Since curli, cellulose and PNAG affect binding of the bacterial cell surface to the dye CR (Olssén et al., 1989; Zogaj et al., 2001; Perry et al., 1990), we measured the effects of DGC overexpression on the colour phenotype on agar medium supplemented with CR (CR medium). In the absence of DGC-overexpressing plasmids, strains carrying mutations in curli-related genes...
(ΔcsgA and the ΔcsgA/ΔbcsA double mutant) showed a white phenotype on CR plates (Fig. 2). In contrast, inactivation of genes responsible for either cellulose (ΔbcsA) or PNAG biosynthesis (ΔpgaA) did not affect the red phenotype of the parental strain, consistent with previous observations that in *E. coli* MG1655, CR binding mostly depends on curli production (Gualdi *et al.*, 2008; Ma & Wood, 2009). Plasmid-driven expression of DGCs resulted in very different effects on colony phenotype on CR media: expression of the AdrA protein conferred a red phenotype upon the *csgA* mutant strain, but not upon the ΔcsgA ΔbcsA double mutant, consistent with its role as an activator of cellulose production (Zogaj *et al.*, 2001; Antoniani *et al.*, 2010). Overexpression of YdaM did not affect the CR phenotype in MG1655 and in its ΔpgaA mutant derivative, but it conferred a weak red phenotype upon the curli-deficient mutant and the ΔcsgA/ΔbcsA double mutant impaired in both curli and cellulose production. Since YdaM controls the production of both curli and cellulose via expression of the *csgD* gene (Weber *et al.*, 2006), this observation suggests that either YdaM or CsgD triggers the production of yet additional cell surface-associated structures able to bind CR. In contrast to AdrA and YdaM, YcdT expression led to no detectable changes in CR phenotype in any of the strains tested (Fig. 2). However, YcdT overexpression, in addition to increasing c-di-GMP intracellular concentrations (Fig. 1), clearly affected cell motility (Supplementary Table S2) and colony size on LB medium (data not shown), suggesting that YcdT is produced in an active form in strains carrying the pYcdT plasmid. Finally, YddV overexpression led to the loss of the red phenotype on CR medium in curli-producing strains, with the exception of the ppgaA mutant, unable to expose PNAG on the cell surface (Fig. 2).

Although a white CR phenotype could indicate negative regulation of curli production by YddV, the observation that the YddV-dependent white colony phenotype on CR medium requires a functional ppgaA gene suggests that YddV overexpression triggers PNAG overproduction. Indeed, in curli-producing strains of *E. coli*, EPS overproduction can result in the loss of the red colony phenotype on CR medium, possibly due to shielding of curli fibres (Gualdi *et al.*, 2008; Ma & Wood, 2009). To understand whether YddV-dependent loss of the red colony phenotype on CR medium could indeed be due to PNAG overproduction, we verified EPS production in the presence and absence of the pYddV plasmid by plating

![Fig. 1. HPLC determination of intracellular c-di-GMP concentrations in MG1655 and in MG1655 transformed with the pGEM-T Easy vector or pGEM-T Easy carrying the genes encoding the DGCs AdrA, YcdT, YdaM and YddV. The peaks corresponding to c-di-GMP are marked by arrows; the peak with a retention time of 21.8 min corresponds to NAD, while the peak at 23.5 min was not identified. The c-di-GMP concentrations ([c-di-GMP]) determined are given above each HPLC profile.](image-url)
on agar medium supplemented with CF, a fluorescent dye able to bind EPS. The presence of pYddV promoted CF binding, which was however abolished in the pgaA mutant strain AM56, indicating that YddV overexpression increases EPS production in a manner dependent on the presence of a functional pgaA gene (Fig. 3a). We determined YddV stimulation of surface adhesion in MG1655 and in its mutant derivatives deficient in the production of specific cell surface-associated factors. As shown in Fig. 3(b), YddV overexpression stimulated surface adhesion in the MG1655 strain as well as in mutants unable to synthesize either curli or cellulose, while failing to enhance biofilm formation in a pgaA mutant. Treatment with the PNAG-degrading enzyme Dispersin B abolished YddV-dependent stimulation of surface adhesion in MG1655 (Fig. 3b). In contrast to YddV, overexpression of either AdrA or YcdT resulted in little or no increase in surface adhesion (Supplementary Fig. S1). Finally, YdaM overexpression stimulated PNAG production; indeed, YdaM-dependent biofilm formation was affected (about twofold) by pgaA inactivation and by treatment with Dispersin B. However, unlike YddV, YdaM-mediated biofilm formation was totally abolished in the AM70 csgA mutant, indicating that it mostly depends on curli production (Supplementary Fig. S1).

**Regulation of pgaABCD expression by DGCs**

Regulation of EPS production by DGCs can take place at different levels: cellulose production is stimulated by AdrA through allosteric activation of the cellulose synthase protein machinery (Zogaj et al., 2001; Simm et al., 2004); the YdeH protein affects PNAG production through stabilization of the PgaD protein (Boehm et al., 2009); and finally, the YdaM protein activates curli and cellulose production via upregulation of csgDEFG transcription (Weber et al., 2006). We tested the possibility that the YddV protein regulates PNAG production by affecting transcription of the pgaABCD operon, encoding the proteins involved in PNAG biosynthesis. Towards this aim, we performed quantitative real-time PCR experiments in MG1655 transformed with pYddV and determined the transcript levels of the pgaA gene. As
shown in Fig. 4, pgaA transcript levels were increased by roughly 10-fold by YddV overexpression. In contrast, overexpression of AdrA and YcdT did not lead to a significant increase in pgaA transcript levels. Interestingly, YdaM overexpression also resulted in an increase in pgaA transcript levels, albeit lower than that observed for YddV, consistent with YdaM-dependent stimulation of PNAG production (Supplementary Fig. S1).

To test whether YddV-dependent activation of pgaABCD transcription is mediated by its DGC activity, we constructed a plasmid carrying a mutant yddV allele encoding a protein lacking DGC activity. This mutant allele encoded a YddVGGAAF protein in which the amino acids in the GGDEF catalytic site were changed to GGAAF (YddV GGAAF); this mutation resulted in loss of DGC activity (De et al., 2008; Antoniani et al., 2010; data not shown). Overexpression of the YddVGGAAF protein did not affect pgaA transcript levels in real-time PCR experiments (Fig. 4), suggesting that pgaABCD regulation by YddV requires its DGC activity.

**yddV positively controls pgaABCD expression and PNAG production**

To test whether PNAG production is indeed controlled by the yddV and ydaM genes through pgaABCD regulation, we constructed MG1655yddV and MG1655ydaM mutant derivatives (AM95 and AM89, respectively). In the AM89 strain, the ydaM gene is inactivated by the insertion of the EZ-Tn5 <R6K<ori/KAN-2> transposon at nucleotide 654, i.e. in the central part of the ydaM ORF (1233 bp). The AM95 strain carries a yddV allele in which the portion of the gene encoding the C-terminal domain 150 amino acids of the YddV protein, which includes the GGDEF domain responsible for DGC activity, has been replaced by a chloramphenicol-resistance cassette (AyddVCTD::cat; Table 1). We measured the effects of the AyddVCTD::cat mutation on levels of pgaA transcript by real-time PCR, which showed that partial deletion of the yddV gene resulted in an approximately 3.5-fold reduction in pgaA transcript levels in comparison with MG1655 (Fig. 5). In contrast, no detectable reduction was observed in the MG1655ydaM mutant AM89, suggesting that the ydaM gene is not crucial for pgaABCD expression (Fig. 5).

The pgaABCD operon is regulated at the transcription initiation level by the NhaR protein, which responds to Na+ ions (Goller et al., 2006). However, the main mechanism of pgaABCD regulation takes place at the post-transcriptional level, via negative control by the RNA-binding CsrA protein (Wang et al., 2004, 2005; Cerca & Jefferson, 2008); CsrA negatively controls pgaABCD expression through binding to a 234 nt untranslated region (UTR) in its mRNA, thus blocking its translation and stimulating its degradation (Wang et al., 2005). To test whether the YddV protein regulates pgaABCD expression by modulating CsrA activity, we constructed AM98, an MG1655csrA/yddV double mutant (Table 1); the csrA mutant allele carried by this strain produces a truncated CsrA protein impaired in its RNA-binding ability, and thus unable to repress pgaABCD translation (Mercante et al., 2006). As expected, pgaA transcript levels were increased by more than 12-fold in the csrA mutant strain LT24; the

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**Fig. 4.** Effects of DGC overexpression on pgaA transcript levels. The MG1655 strain was transformed with the pGEM-T Easy vector or with the following plasmids: pYddV, pYddVGGAAF, pAdrA, pYcdT and pYdaM. The pYddV plasmid carries a copy of the wild-type yddV allele, while pYddVGGAAF carries a mutant yddV allele encoding a protein lacking DGC activity. pgaA expression values in MG1655 transformed with pGEM-T Easy (corresponding to a ΔCt relative to 16S rRNA of 15.7) were set to 1. The strains were grown overnight in M9Glu/sup medium at 30 °C in the absence of IPTG. Results are the mean of three independent experiments performed in duplicate; error bars, SD.

**Fig. 5.** Relative expression levels of the pgaA gene in strains MG1655 (WT), AM95 (yddV), AM89 (ydaM), LT24 (csrA) and AM98 (csrA/yddV), as measured by real-time PCR. pgaA expression values in MG1655 (corresponding to a ΔCt relative to 16S rRNA of 15.7) were set to 1. Data are the mean of three independent experiments, each performed in triplicate. SDs (error bars) were calculated from the mean value of each independent experiment.
ΔyddVCTD::cat mutation resulted in a sixfold reduction in pgaA transcript levels in the MG1655 csrA background (Fig. 5), indicating that the yddV gene positively controls levels of pgaABCD transcripts even in a mutant csrA background. Thus, YddV does not seem to regulate pgaABCD expression by modulating CsrA activity. Since c-di-GMP has been shown to act as a riboswitch, and to be able to increase the chemical and functional half-life of mRNA carrying c-di-GMP-responding elements (Sudarsan et al., 2008), we tested the possibility that the yddV gene affects pgaABCD mRNA stability via its DGC activity. mRNA decay kinetics experiments showed that the pgaA transcript has a half-life of ~1.5 min in the MG1655 strain; yddV inactivation did not affect pgaABCD mRNA stability in the MG1655 background (data not shown), suggesting that yddV-dependent pgaABCD regulation is not mediated by mRNA stabilization.

We investigated the effects of partial deletion of the yddV gene on PNAG production by surface adhesion experiments. Surface adhesion to polystyrene microtitre plates is strongly stimulated by inactivation of the csrA gene, consistent with higher pgaABCD expression in this strain (see Fig. 5); disruption of the pgaA gene, involved in PNAG biosynthesis, counteracts the effects of the csrA mutation (Fig. 6a), indicating that the increased biofilm formation in the csrA derivative of MG1655 depends solely on PNAG production. Partial deletion of the yddV gene abolished surface adhesion in MG1655csrA (Fig. 6a), consistent with reduced pgaABCD expression in the MG1655csrA/yddV mutant (Fig. 5). Mutations in either the pgaA or the yddV gene resulted in a 2.5-fold reduction in surface adhesion in the MG1655 background, in agreement with previous observations (Wang et al., 2004; Tagliabue et al., 2010).

To further confirm that the effects of yddV inactivation on surface adhesion in the MG1655csrA/yddV background are indeed due to reduced PNAG production, we transformed the AM98 strain with either pYddV, carrying a wild-type copy of the yddV gene, or pYddVGGAAF, expressing a YddVGGAAF protein lacking DGC activity. Expression of genes cloned into pGEM-T Easy occurs at lower levels in strains carrying a csrA mutation, possibly due to reduced plasmid copy number in the csrA mutant strain (data not shown); thus, in the absence of IPTG induction, no plasmid was able to restore the ability to form biofilm to

Fig. 6. (a) Surface adhesion to polystyrene microtitre plates of strains MG1655 (WT), AM95 (yddV), AM56 (pgaA), LT24 (csrA), AM98 (csrA/yddV) and LT108 (csrA pgaA). The surface adhesion value for MG1655 (4.9 in this set of experiments) was set to 1. Results are the mean of three independent experiments and SDs (error bars) are shown. (b) Surface adhesion to polystyrene microtitre plates of strain AM98 (csrA/yddV) transformed either with the pGEM-T Easy (control vector) or with plasmids carrying yddV alleles. Plasmid pYddV carries a copy of the wild-type yddV allele, while pYddVGGAAF carries a mutant yddV allele encoding a protein lacking DGC activity. For full expression, IPTG was added to the growth medium at 0.5 mM. Where employed, Dispersin B (Disp.B) was added to the growth medium at a final concentration of 20 μg ml⁻¹. Data are the average of two independent experiments with very similar results.
AM98 (Fig. 6b). In contrast, upon IPTG induction, production of YddV, but not of the mutant YddV_GGAAF protein lacking DGC activity, clearly stimulated surface adhesion. Treatment with the PNAG-degrading enzyme Dispersin B led to complete loss of biofilm stimulation by the YddV protein (Fig. 6b), strongly suggesting that the YddV-dependent increase in biofilm formation depends on PNAG production.

Effects of the PDE Dos on pgaABCD expression

The yddV gene is transcribed in an operon with the dos (yddU) gene (Méndez-Ortiz et al., 2006); the product of the dos gene is a haem-binding oxygen sensor (Delgado-Nixon et al., 2000), which possesses putative domains for both DGC and PDE activity (Schmidt et al., 2005). However, due to degeneration of the GGDEF motif responsible for DGC catalytic activity, Dos can only function as a PDE (Schmidt et al., 2005; Tuckerman et al., 2009). The presence in the same transcriptional unit of the insertion of the cloramphenicol-resistance cassette into the dos gene could result in polar effects on dos expression, we compared dos transcript levels in the MG1655ΔyddVCTD::cat strain with those of MG1655 by real-time-PCR. Transcription of the dos gene was only reduced by about 2.5-fold in the MG1655yddV strain (data not shown), suggesting that in this strain the dos gene is still expressed at significant levels, probably due to transcription readthrough from the promoter of the cloramphenicol-resistance cassette upstream of the dos gene. To investigate the possible role of dos in pgaABCD regulation, we inactivated the dos gene in both the MG1655 strain and its csrA mutant derivative. Real-time PCR experiments confirmed that dos inactivation increased pgaA transcript levels in both the MG1655 (about fourfold) and the MG1655csrA strains (about twofold; Fig. 7), consistent with the hypothesis that Dos modulates DGC activity by the YddV protein.

DISCUSSION

In Enterobacteria, biosynthesis of the c-di-GMP signal molecule by DGCs stimulates the transition from planktonic to biofilm cells, repressing flagellar synthesis and cell motility, while promoting production of adhesion factors (Méndez-Ortiz et al., 2006; Pesavento et al., 2008). In this report, we have shown that overexpression of YddV, a DGC protein, promotes production of the EPS PNAG (Figs 2 and 3) by activating the expression of pgaABCD, the PNAG biosynthetic operon (Fig. 4). pgaABCD activation

and the consequent stimulation of PNAG biosynthesis require the DGC activity of the YddV protein (Figs 4 and 6b); however, the increase of intracellular c-di-GMP due to overexpression of other DGCs, such as AdrA and YcdT, is not sufficient to activate PNAG production (Figs 1 and 2, Supplementary Fig. S1). In contrast, overexpression of YdaM, a cytoplasmic DGC, resulted in increased PNAG production (Supplementary Fig. S1) and pgaABCD expression (Fig. 4), although to a lesser degree than YddV. However, unlike yddV, ydaM inactivation did not affect pgaABCD expression (Fig. 5), suggesting a specific dependence of this process on the YddV protein. The specificity of DGC-mediated regulation might indicate that c-di-GMP biosynthesis is needed to trigger specific protein–protein (or protein–DNA, or protein–RNA) interactions between DGCs and their targets (Hengge, 2009). Thus, it can be speculated that c-di-GMP biosynthesis acts as an activating step in signal transduction pathways leading to regulation of gene expression and of protein activity.

The dependence of PNAG production on c-di-GMP biosynthesis has already been described in Yersinia pestis, where the HmsT protein activates PNAG production by allosteric activation of its biosynthetic machinery (Kirillina et al., 2004). In contrast, our results suggest that the YddV protein promotes PNAG production by activating the expression of the PNAG biosynthetic operon pgaABCD (Figs 4 and 5), possibly via interaction with a c-di-GMP-responsive regulatory protein. In addition to YddV, PNAG production is controlled by another DGC, YdeH, which positively affects PgaD protein stability via a yet unknown mechanism (Boehm et al., 2009). Similarly, cellulose biosynthesis is regulated by DGC proteins at both the gene expression and the protein activity levels: the YdaM protein positively regulates csgDEFG transcription (Weber
et al., 2006); the CsgD protein, in turn, activates adrA transcription. The adrA gene encodes another DGC that stimulates cellulose production through allosteric activation of the cellulose synthase machinery (Römling et al., 2000; Zogaj et al., 2001). Thus, it appears that DGC-dependent control at multiple levels is a common mechanism for EPS biosynthesis regulation in E. coli. A model summarizing multiple level EPS regulation by DGCs is summarized in Fig. 8.

Recent observations indicate that c-di-GMP can act as a riboswitch, binding specific elements (aptamers) in the UTRs of some mRNAs and affecting their stability (Sudarsan et al., 2008). The pgaABCD transcript is characterized by a rather long UTR (234 nt; Wang et al., 2005) and is regulated at the level of mRNA stability by the CsrA protein. Effects on pgaABCD expression have already been shown for another protein carrying protein domains associated to c-di-GMP metabolism, CsrD, which negatively affects the stability of small RNAs controlling CsrA activity (Suzuki et al., 2006). We speculated that YddV might stabilize pgaABCD mRNA, possibly counteracting CsrA activity; however, pgaABCD expression is affected by yddV inactivation even in a csrA mutant background (Fig. 5), suggesting that YddV does not act via modulation of CsrA translational repression. In addition, the yddV mutation did not result in destabilization of pgaABCD transcripts, as determined by an mRNA decay assay (data not shown), suggesting that YddV does not affect pgaABCD mRNA stability, although it might affect transcription initiation at the pgaABCD promoter, as proposed in Fig. 8. Future work will focus on the identification of additional factors involved in YddV-dependent regulation of pgaABCD expression.

Fig. 8. Model summarizing transcriptional and post-transcriptional regulation of EPS biosynthesis by DGC proteins. Proteins with DGC activity are indicated by ellipses. Cellulose biosynthesis, represented on the left-hand side of the figure, is regulated by YdaM, promoting transcription of the csgD gene (Weber et al., 2006), and AdrA, which activates cellulose synthase activity by the cellulose synthase (Bcs) complex through its DGC activity (Römling et al., 2000; Zogaj et al., 2001). PNAG production is positively affected by YddV through activation of pgaABCD transcription (see Figs 4 and 5) and by YdeH-dependent stabilization of the PgaD protein (Boehm et al., 2009) at the post-transcriptional level.

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