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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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Summary

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 catalyze 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 is 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 intracellular c-di-GMP concentration, but specifically requires DGC activity by the YddV protein, thus supporting the notion that, in *E. coli*, c-di-GMP biosynthesis by a given DGC protein triggers regulatory events leading 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 to planktonic cells (Costerton et al., 1995; Anderl et al., 2000; Harrison et al., 2007; Harrison et al., 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 diguanylic 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 (Mendez-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 DGC-encoding genes is turned on at 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: diguanylate cyclases (DGCs, c-di-GMP biosynthetic enzymes), also termed GGDEF proteins from the conserved gly-gly-asp-glu-phe motif in their

catalytic domain, 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 much higher numbers in Gram negative than in Gram positive bacteria (Galperin, 2004), where 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 might constitute a mechanism for signal transduction involving c-di-GMP-responsive proteins interacting 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 in Hengge, 2009), including several DNA-binding protein, *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), by-passing the need for c-di-GMP binding regulatory proteins.

The YddV protein is arguably one of the most expressed DGCs in *E. coli* (Sommerfeldt et al., 2009). Recently, we have shown that YddV can affect expression of curli-encoding genes (Tagliabue et al., 2010) that, 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 of 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 M9Glu/sup (M9 inorganic salts (Smith and Levine, 1964), 5 g/L glucose, 0.25 g/L Peptone, 0.125 g/L Yeast Extract). When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 25 μg/ml; rifampicin, 100 μg/ml. For Congo red (CR) or Calcofluor (CF) assays, overnight cultures were spotted, using a replicator, on agar media supplemented with 0.5% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$; either 0.004% Congo red and 0.002% Coomassie blue (for CR medium) or 0.005% Calcofluor (for CF medium) were added after autoclaving. Bacteria were grown for 20 h at 30°C; phenotypes were better detectable after 24-48h incubation at 4°C.

Biofilm formation assays. Biofilm formation in microtiter plates was determined essentially as described (Dorel et al., 1999). Bacterial cultures were grown overnight in M9Glu/sup at 30°C in polystyrene microtiter plates (0.2 ml); cell density of the culture was determined spectrophotometrically at 600nm (OD_{600nm}). Cells attached to the microtiter plates were washed gently with water and stained for 20 min with 1% crystal violet in ethanol (CV), thoroughly washed with water and dried. For semi-quantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 95% ethanol by vigorous pipetting. The OD_{600nm} of each sample was determined and normalized to the OD_{600nm} of the corresponding liquid cultures (Adhesion units). Sensitivity of biofilms to treatment with the PNAG-degrading enzyme Dispersin B (Kaplan et al., 2004; purchased from Kane Biotech Inc., Winnipeg, Canada) was performed by adding 20 μg/ml of the 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 P*lac* promoter) was verified by PCR using primers listed in 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 P*lac* promoter. The pYddV$_{GGAAF}$ plasmid, carrying the *yddV* gene mutated in the DGC catalytic site, was obtained by 3-step PCR mutagenesis (Li & Shapiro, 1993) using the primers listed in 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 Table S1. RNA extraction and further Reverse Transcription and cDNA amplification steps were performed as described (Gualdi et al., 2007), using 16S RNA as reference gene. mRNA stability was measured by Real-Time PCR experiments in the presence of rifampicin as described (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 the AM89 strain (MG1655 *ydaM::Tn5-kan*) obtained in a transposon mutagenesis screening for adhesion-deficient MG1655 mutants using the EZ-Tn5<R6Kγori/KAN-2> transposon (Epicentre; Landini, unpublished data). Primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Table S1. Bacterial cell motility was evaluated as described (Pesavento et al., 2008). Determination of intracellular c-di-GMP concentration was performed as previously described (Antoniani et al., 2010).

Results

Overexpression of diguanylate cyclases (DGCs). In Enterobacteria, production of EPS such as poly-*N*-acetyl-glucosamine (PNAG) and cellulose (Römling et al., 2000; Zogaj et al., 2001; Boehm et al., 2009), and of proteinaceous adhesion factors such as curli fibers (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 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 (Mendez-Ortiz et al., 2006), thus suggesting that *yddV* can induce biofilm formation by acting on additional, not yet identified, targets. In order to study 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 *pgaABCD* 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 ca. 10-fold (Figure 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). Expression of each DGC led to reduction in bacterial mobility (Table S2), in agreement with previous observations (Mendez-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 (Δ*csgA::cat)*, unable to produce curli; LG26, a Δ*bcsA::kan* mutant impaired in cellulose production; AM73, a Δ*csgA*/Δ*bcsA* double mutant, and AM56, a Δ*pgaA::cat* mutant unable to export PNAG and to expose it on the cell surface (Itoh et al., 2008). We expected that phenotypes depending on 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 bacterial cell surface to the dye Congo red (Olsen et al., 1989; Zogaj et al., 2001; Perry et al., 1990; Gualdi et al., 2008), we measured the effects of DGC overexpression on colour phenotype on agar medium supplemented with Congo red (CR medium). In the absence of DGC-overexpressing plasmids, strains carrying mutations in curli-related genes (Δ*csgA* and the Δ*csgA*/Δ*bcsA* double mutant) showed a white phenotype on CR plates (Figure 2). In contrast, inactivation of genes responsible for either cellulose (Δ*bcsA*) or PNAG biosynthesis (Δ*pgaA*) did not affect the red phenotype of the parental strain, consistent with previous observations that in *E. coli* MG1655 Congo red-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 to the *csgA* mutant strain, but not to the Δ*csgA* Δ*bcsA* 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 CR phenotype in MG1655 and in its Δ*pgaA* mutant derivative, but it conferred a

weak red phenotype on CR medium both to the curli-deficient mutant and to the Δ*csgA* Δ*bcsA* 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 might trigger the production of yet additional cell surface-associated structures able to bind Congo red. In contrast to AdrA and YdaM, YcdT expression led to no detectable changes in CR phenotype in any of the strains tested (Figure 2). However, YcdT overexpression, in addition to increasing c-di-GMP intracellular concentrations (Figure 1), clearly affected cell motility (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 *pgaA* mutant unable to expose PNAG on the cell surface (Figure 2, last row).

Although a white CR phenotype could indicate negative regulation of curli production by YddV, the observation that YddV-dependent white colony phenotype on CR medium requires a functional *pgaA* gene suggests that YddV overexpression might trigger 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 fibers (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 absence and in the presence of the pYddV plasmid by plating on agar medium supplemented with Calcofluor, a fluorescent dye able to bind EPS. Presence of pYddV promotes Calcofluor binding, which is 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 (Figure 3A). We determined YddV stimulation of surface adhesion in MG1655 and in its mutant derivatives deficient in

production of specific cell surface-associated factors. As shown in Figure 3B, 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 (Figure 3B). In contrast to YddV, overexpression of either AdrA or YcdT resulted in little or no increase in surface adhesion (Figure S1). Finally, YdaM overexpression stimulated PNAG production: indeed, YdaM-dependent biofilm formation was affected (ca. 2-fold) 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 (Figure 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); finally, the YdaM protein activates curli and cellulose production via up-regulation of *csgDEFG* transcription (Weber et al., 2006). We tested the possibility that the YddV protein might regulate PNAG production by affecting transcription of the *pgaABCD* operon, encoding the proteins involved in PNAG biosynthesis. To this aim, we performed quantitative Real Time PCR experiments in MG1655 transformed with pYddV and determined transcript levels of the *pgaA* gene. As shown in Figure 4, *pgaA* transcript levels were increased by roughly 10-fold by YddV overexpression. In contrast, overexpression of AdrA and YcdT did not lead to any significant increase in *pgaA* transcript levels. Interestingly, YdaM overexpression also resulted in an increase in *pgaA* transcript levels, albeit lower than what observed for YddV, consistent with YdaM-dependent stimulation of PNAG production (Figure S1).

To test if YddV-dependent activation of *pgaABCD* transcription is mediated by its DGC activity, we constructed a plasmid carrying a mutant *yddV* allele encoding a protein in which the amino acids in the GGDEF catalytic site are changed to GGAAF ($YddV_{GGAAF}$); this mutation results in loss of DGC activity (De et al., 2008; Antoniani et al., 2010; data not shown). Overexpression of the $YddV_{GGAAF}$ protein did not affect *pgaA* transcript levels in Real Time PCR experiments (Figure 4), suggesting that *pgaABCD* regulation by YddV requires its DGC activity.

The *yddV* gene positively controls *pgaABCD* expression and PNAG production. To test if PNAG production is indeed controlled by the *yddV* and *ydaM* genes through *pgaABCD* regulation, we constructed MG1655*yddV* and MG1655*ydaM* mutant derivatives (AM95 and AM89, respectively). In the AM89 strain, the *ydaM* gene is inactivated by the insertion of the EZ-Tn5<R6Kγori/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 (Δ*yddV*CTD*::cat*, Table 1). We measured the effects of the Δ*yddV*CTD*::cat* mutation on levels of *pgaA* transcript by Real-Time PCR, which showed that partial deletion of the *yddV* gene resulted in a ca. 3.5-fold reduction in *pgaA* transcript levels in comparison to MG1655 (Figure 5). In contrast, no detectable reduction was observed in the MG1655*ydaM* mutant AM89, suggesting that the *ydaM* gene is not crucial for *pgaABCD* expression (Figure 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 post-transcriptional level, via negative control by the RNA-binding CsrA protein (Wang et al., 2004; Wang et al., 2005; Cerca et al., 2008); CsrA

negatively controls *pgaABCD* expression through binding to a 234-nucleotide untranslated region (UTR) in its mRNA, thus blocking its translation and stimulating its degradation (Wang et al., 2005). To test whether the YddV protein might regulate *pgaABCD* expression by modulating CsrA activity, we constructed AM98, an MG1655*csrA/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 Δ*yddV*CTD*::cat* mutation resulted in a 6-fold reduction in *pgaA* transcript levels in the MG1655*csrA* background (Figure 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 might affect *pgaABCD* mRNA stability via its DGC activity. mRNA decay kinetics experiments showed that the *pgaA* transcript has a half-life of ca. 1.5 minutes 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 microtiter plates is strongly stimulated by inactivation of the *csrA* gene, consistent with higher *pgaABCD* expression in this strain (see Figure 5); disruption of the *pgaA* gene, involved in PNAG biosynthesis, counteracts the effects of the *csrA* mutation (Figure 6A), indicating that increased biofilm formation in the *csrA* derivative of MG1655 depends solely on PNAG production. Partial

deletion of the *yddV* gene abolished surface adhesion in MG1655*csrA* (Figure 6A), consistent with reduced *pgaABCD* expression in the MG1655*csrA*/*yddV* mutant (Figure 5). Mutations either in the *pgaA* or the *yddV* genes 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 MG1655*csrA*/*yddV* background are indeed due to reduced PNAG production, we transformed the AM98 strain with either pYddV, carrying the wild type copy of the *yddV* gene, or pYddV$_{GGAAF}$, expressing the YddV$_{GGAAF}$ 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 ability to form biofilm to AM98 (Figure 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 (Figure 6B), strongly suggesting that YddV-dependent increase in biofilm formation depends on PNAG production.

Effects of the c-di-GMP phosphodiesterase Dos on *pgaABCD* expression. The *yddV* gene is transcribed in an operon with the *dos* (*yddU*) gene (Mendez-Ortiz et al., 2006); the product of the *dos* gene is a heme-binding oxygen sensor (Delgado-Nixon et al., 2000), which possesses putative domains for both DGC and c-di-GMP phosphodiesterase (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 genes coding for a DGC and a PDE suggests that Dos might modulate YddV DGC activity. Indeed, a recent report shows

that the two proteins co-purify and form a complex in solution (Tuckerman et al., 2009), suggesting that the YddV-Dos protein complex might exist in a stable form in the bacterial cell. Environmental signals might modulate either the DGC activity of YddV or the PDE activity of Dos. Since the insertion of the cloramphenicol resistance cassette into the *yddV* gene could result in polar effects on *dos* expression, we compared *dos* transcript levels in the MG1655Δ*yddV*CTD*::cat* strain to MG1655 by Real Time-PCR. Transcription of the *dos* gene was only reduced by ca. 2.5-fold in the MG1655*yddV* 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 both in the MG1655 strain and in its *csrA* mutant derivative. Real-Time PCR experiments confirmed that *dos* inactivation increased *pgaA* transcript levels both in the MG1655 (ca. 4-fold) and in the MG1655*csrA* strains (ca. 2-fold; Figure 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 diguanylate cyclases (DGCs) stimulates the transition from planktonic to biofilm cell, repressing flagellar synthesis and cell motility while promoting production of adhesion factors (Mendez-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 poly-β-1,6-*N*-acetylglucosamine (PNAG; Figures 2-3) by activating expression of *pgaABCD*, the PNAG biosynthetic operon (Figure 4). *pgaABCD* activation and consequent stimulation of PNAG biosynthesis requires DGC

activity by the YddV protein (Figures 4, 6B); however, increase of intracellular c-di-GMP due to overexpression of other DGCs, such as AdrA and YcdT, is not sufficient to activate PNAG production (Figures 1-2; Figure S1). In contrast, overexpression of YdaM, a cytoplasmic DGC, resulted in increased PNAG production (Figure S1) and *pgaABCD* expression (Figure 4), although to a lesser degree than YddV. However, unlike *yddV*, *ydaM* inactivation did not affect *pgaABCD* expression (Figure 5), suggesting specific dependence of this process on the YddV protein. Specificity of DGCs-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 could act as an activating step in signal transduction pathways leading to regulation of gene expression and of protein activity.

Dependence of PNAG production on c-di-GMP biosynthesis has already been described both 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* (Figures 4-5), 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 gene expression and 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 (Romling 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 Figure 8.

Recent observations indicate that c-di-GMP can act as a riboswitch, binding specific elements (aptamers) in the untranslated regions (UTR) in some mRNAs and affecting their stability (Sudarsan et al., 2008). The *pgaABCD* transcript is characterized by a rather long UTR (234nt, 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 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 (Figure 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 but it might affect transcription initiation at the *pgaABCD* promoter, as proposed in Figure 8. Future work will focus on the identification of additional factors involved in YddV-dependent regulation of *pgaABCD* expression.

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

Escherichia coli **Strains**	**Relevant genotype or characteristics**	**Reference or source**
MG1655	K-12, $F^- \lambda^-$ *rph-1*	Blattner et al., 1997
AM56	MG1655 *ΔpgaA::cat*	This work
AM70	MG1655 *ΔcsgA::cat*	This work
LG26	MG1655 *ΔbcsA::kan*	Gualdi et al., 2008
AM73	MG1655 *ΔcsgA::cat*, *ΔbcsA::kan*	This work
AM89	MG1655 *ydaM::Tn5-kat*	This work
AM95	MG1655Δ*yddV*CTD*::cat* (*yddV*$\Delta_{931-1383}$*::cat*)	Tagliabue et al., 2010
AM98	MG1655 *csrA::kan*, *yddV*$\Delta_{931-1383}$*::cat*	This work
AM109	MG1655 *dos::tet*$\Delta_{1200-2400}$	This work
LT24	MG1655 *csrA::kan*	This work. Obtained by bacteriophage P1 transduction from TRMG1655 (Romeo et al., 1993)
LT108	MG1655 *csrA::kan ΔpgaA::cat*	This work
LT110	MG1655 *csrA::kan*, *dos::tet*$\Delta_{1200-2400}$	This work
Plasmids		
pGEM-T Easy	Control vector allowing direct cloning of PCR products, ampicillin resistance	Promega
PAdrA	*adrA* gene cloned as PCR product into pGEM-T vector	This work
PYcdT	*ycdT* gene cloned as PCR product into pGEM-T vector	This work
PYdaM	*ydaM* gene cloned as PCR product into pGEM-T vector	This work
PYddV	*yddV* gene cloned as PCR product into pGEM-T vector	Tagliabue et al., 2010
$pYddV_{GGAAF}$	*yddV* allele carrying mutation resulting in GGDEF→GGAAF change in the DGC catalytic site of the YddV protein	Tagliabue et al., 2010

Figure legends

Figure 1. HPLC determination of intracellular c-di-GMP concentrations in MG1655 and in MG1655 transformed with either the pGEM-T Easy vector or pGEM-T Easy carrying the DGC-encoding genes AdrA, YcdT, YdaM and YddV. The peak corresponding to c-di-GMP is marked by an arrow; the peak with a retention time of 21.8 minutes corresponds to NAD, while the peak at 23.5 minutes was not identified.

Figure 2. Congo red binding assay. The MG1655 strain and isogenic mutants deficient in production of cell surface-associated structures were transformed with either the pGEM-T Easy vector or the vector carrying the DGC-encoding genes AdrA, YcdT, YdaM and YddV. Strains tested were: MG1655 (WT); Δ*csgA*: AM70 (curli-deficient mutant); Δ*bcsA*: LG26 (cellulose-deficient mutant); Δ*csgA*/Δ*bcsA*: AM73 (curli- and cellulose-deficient mutant); Δ*pgaA*: AM56 (PNAG-deficient mutant).

Figure 3A. Effects of YddV overexpression on EPS production determined by Calcofluor binding assay. The following strains: MG1655 (WT); Δ*csgA*: AM70 (curli-deficient mutant); Δ*bcsA*: LG26 (cellulose-deficient mutant); Δ*pgaA*: AM56 (PNAG-deficient mutant) were transformed either with the control vector (panel above) or with pYddV (panel below).

Figure 3B. Surface adhesion on polystyrene microtiter plates by strains carrying either pGEM-T Easy (light grey bars) or pYddV (dark grey bars). Surface adhesion values are set to 1 for strains transformed with pGEM-T Easy. Actual Adhesion units values were: MG1655 (WT)= 5.6; AM70 (*csgA*)= 1.1; LG26 (*bcsA*)= 5.4; AM 73 (*csgA/bcsA*)= 1.2; AM56 (*pgaA*)=

3,8, WT+Dispersin B= 4.4. Experiments were repeated three times and standard deviations are shown.

Figure 4. Effects of DGC overexpression on *pgaA* transcript levels. The MG1655 strain was transformed either with the pGEM-T Easy vector or with the following plasmids: pYddV, $pYddV_{GGAAF}$, pAdrA, pYcdT, and pYdaM. The pYddV plasmid carries a copy of the wild type *yddV* allele, while $pYddV_{GGAAF}$ 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=15.7) was set to 1. The strains were grown overnight in M9Glu/sup medium at 30°C in the absence of IPTG. Results are the average of three independent experiments performed in duplicate.

Figure 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 experiments. *pgaA* expression values in MG1655 (corresponding to a ΔCt relative to 16S rRNA=15.7) was set to 1. Data are the average of three independent experiments, each performed in triplicate. Standard deviations were calculated on the average value of each independent experiment.

Figure 6A. Surface adhesion on polystyrene microtiter plates of strains MG1655 (WT), AM95 (*yddV*), AM56 (*pgaA*), LT24 (*csrA*), AM98 (*csrA/yddV*) and LT108 (*csrA/pgaA*). Surface adhesion value for MG1655 (4.9 in this set of experiments) was set to 1. Results are the average of three independent experiments and standard deviations are shown.

Figure 6B. Surface adhesion on polystyrene microtiter plates of strains AM98 (*csrA/yddV*) transformed either with pGEM-T Easy (control vector) or with plasmids carrying *yddV*

alleles. The pYddV plasmid carries a copy of the wild type *yddV* allele, while pYddV$_{GGAAF}$ carries a mutant *yddV* allele encoding a protein lacking DGC activity. For full expression, IPTG was added to growth medium at 0.5 mM. When present, Dispersin B was added to the growth medium at a final concentration of 20 μg/ml. Data are the average of two independent experiments with very similar results.

Figure 7. Relative expression levels of the *pgaA* gene in strains MG1655 (WT), LT24 (*csrA*), AM109 (*dos*), and LT110 (*csrA/dos*), as measured by Real-Time PCR experiments. *pgaA* expression values in MG1655 (corresponding to a ΔCt relative to 16S rRNA=15.5) was set to 1. Results are the average of three independent experiments, each performed in triplicate.

Figure 8. Model summarizing transcriptional and post-transcriptional regulation of EPS biosynthesis by DGC proteins. Proteins with DGC activity are indicated in shaded 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 (Romling et al., 2000; Zogaj et al., 2001). PNAG production is positively affected by YddV through activation of *pgaABCD* transcription (see Figures 4-5) and by YdeH-dependent stabilization of the PgaD protein (Boehm et al., 2009) at post-transcriptional level.

Figure S1. Surface adhesion on polystyrene microtiter plates by strains carrying the pGEM-T Easy control vector (white bars), pAdrA (light grey bars), pYcdT (dark grey bars), and pYdaM (black bars). Surface adhesion values are set to 1 for strains transformed with the control vector. Actual values were: MG1655 (WT)= 5.6; AM70 (*csgA*)= 1.1; LG26 (*bcsA*)=

5.4; AM 73 (*csgA/bcsA*)= 1.2; AM56 (*pgaA*)= 3,8, WT+Dispersin B= 4.4. Experiments were repeated three times and standard deviations are shown.

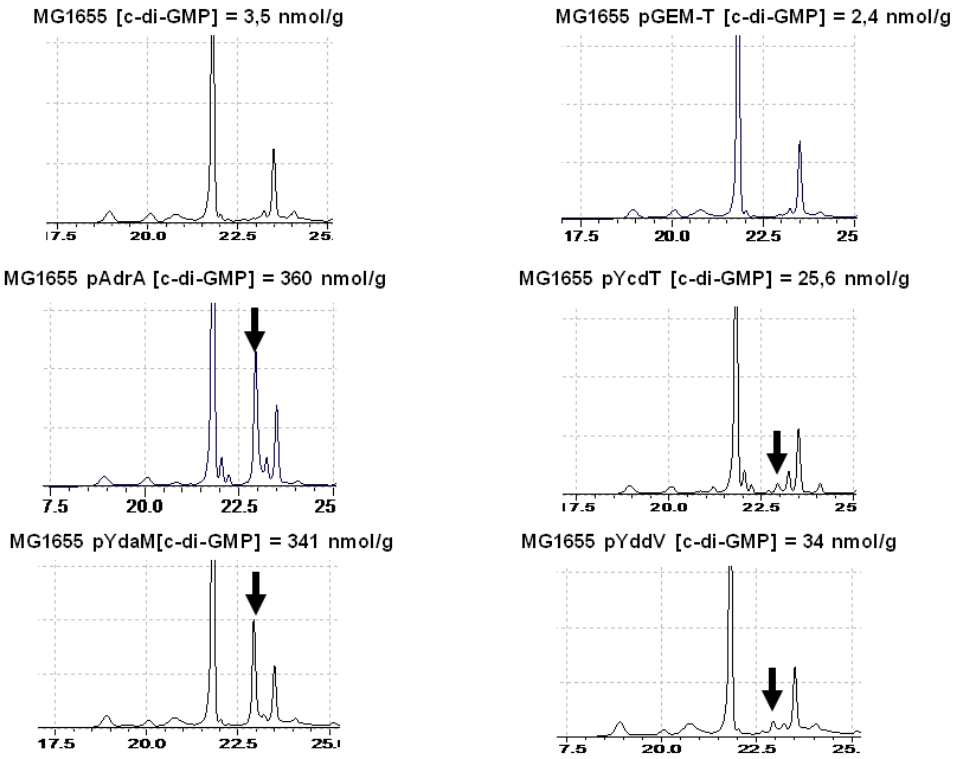

Figure 1

1

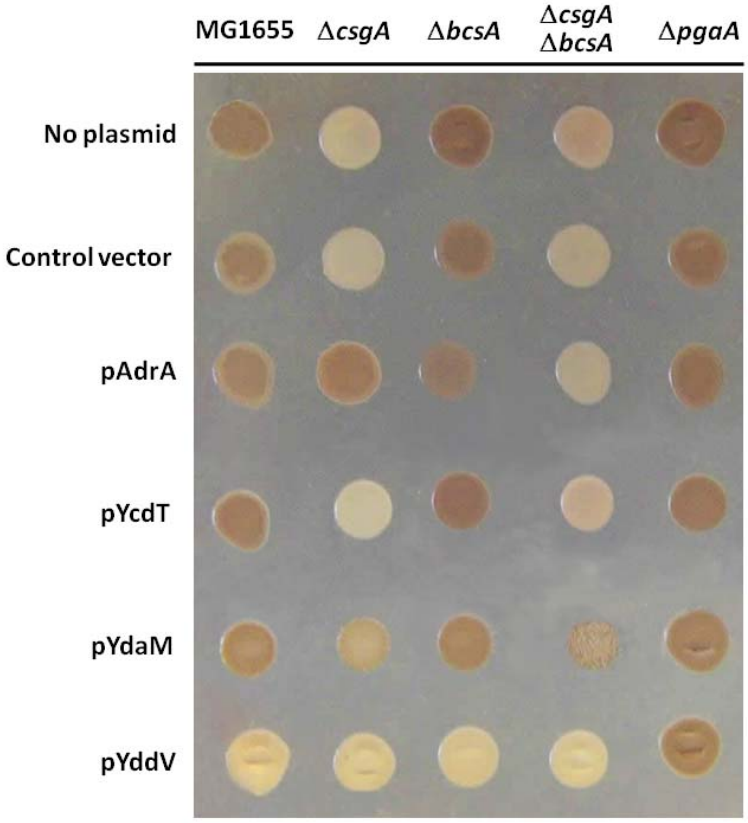

Figure 2

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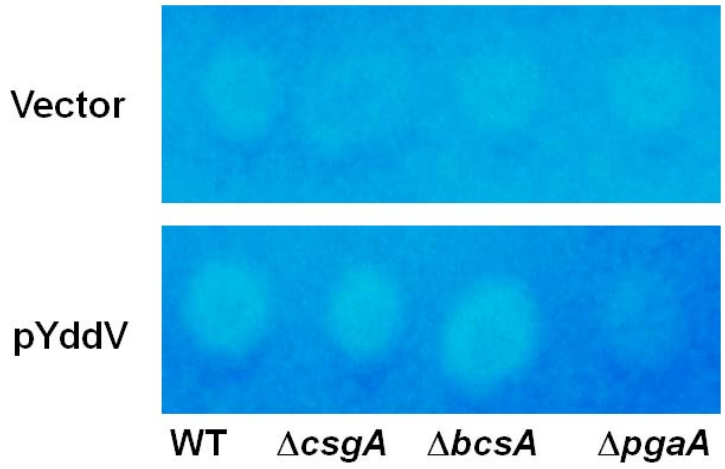

Figure 3A

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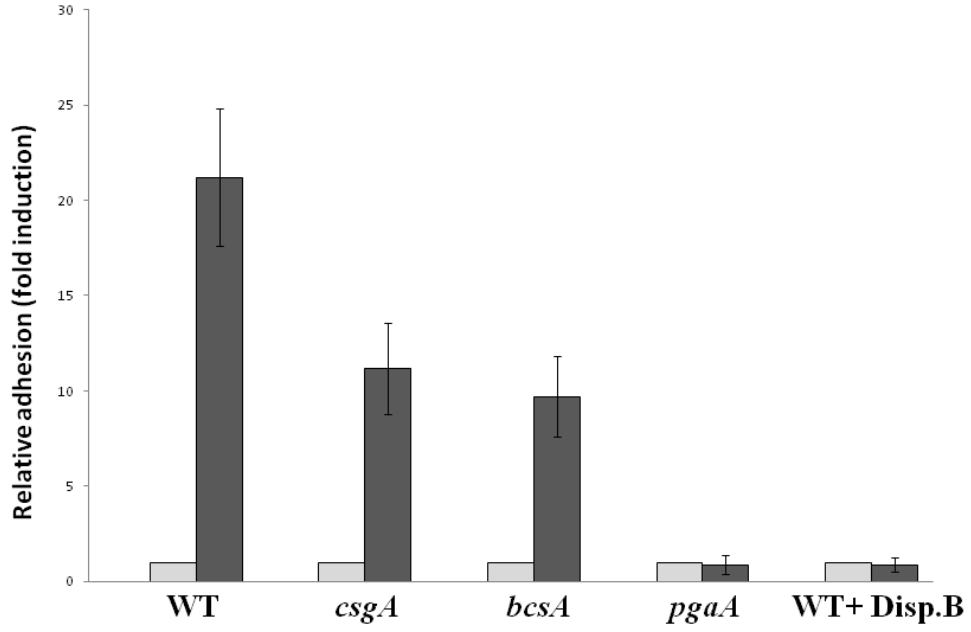

Figure 3B

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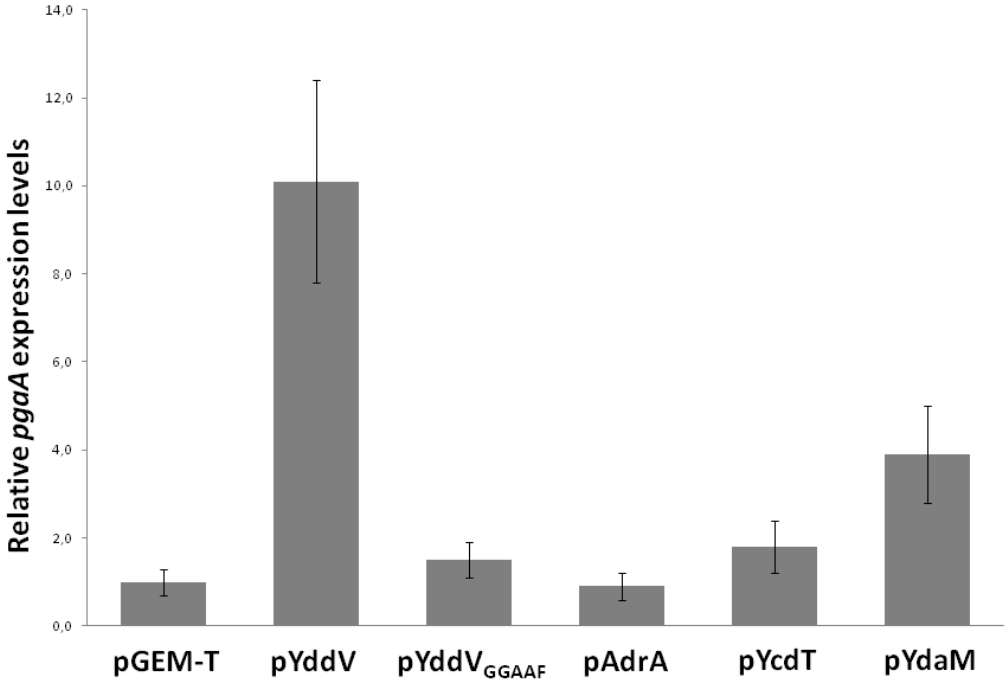

Figure 4

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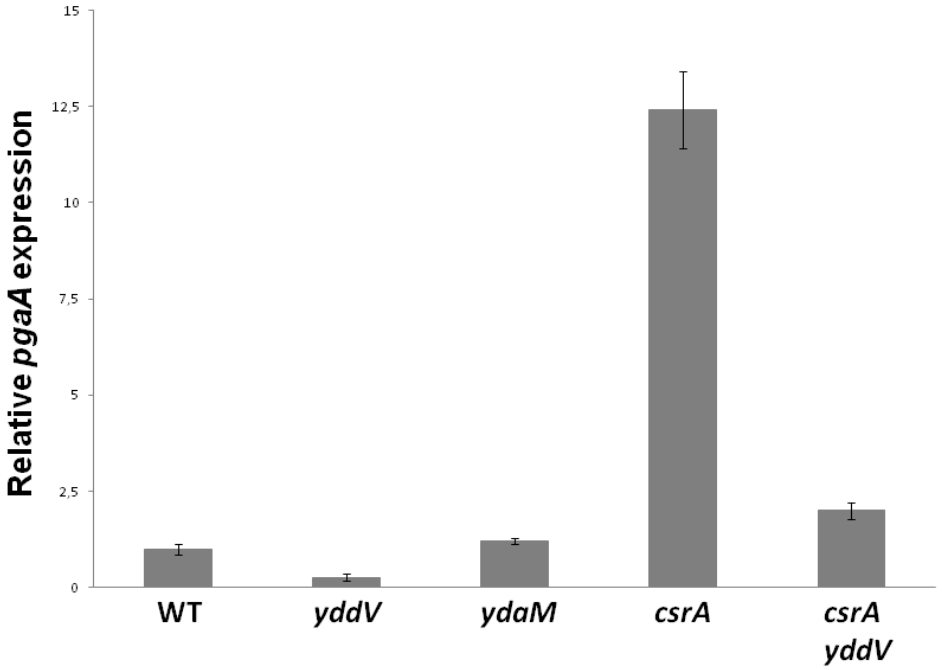

Figure 5

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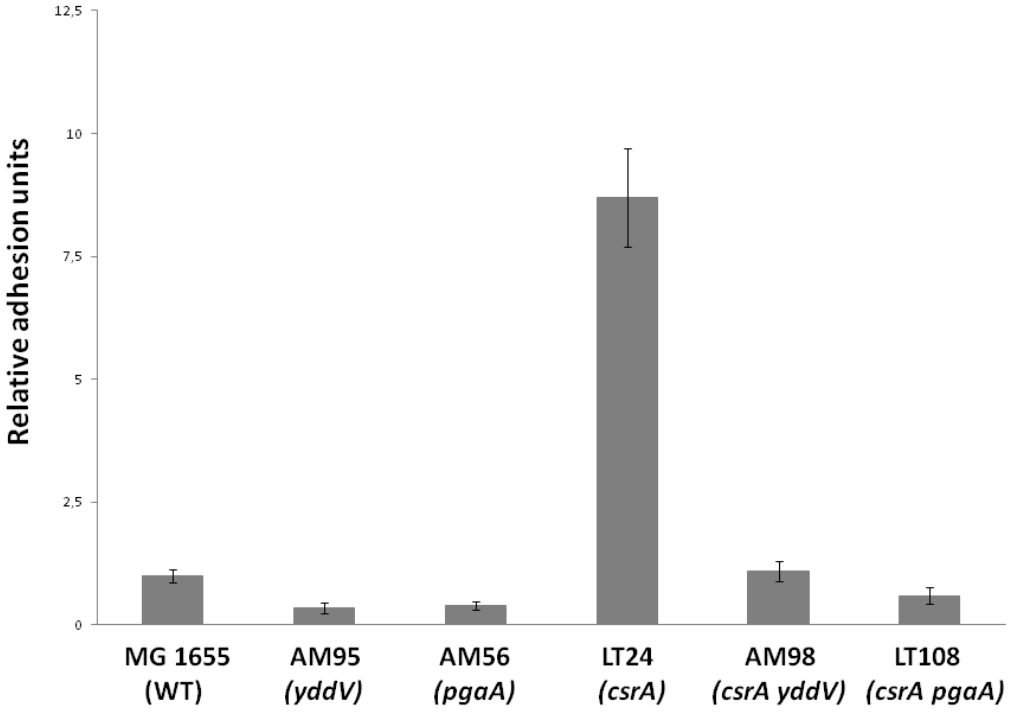

Figure 6A

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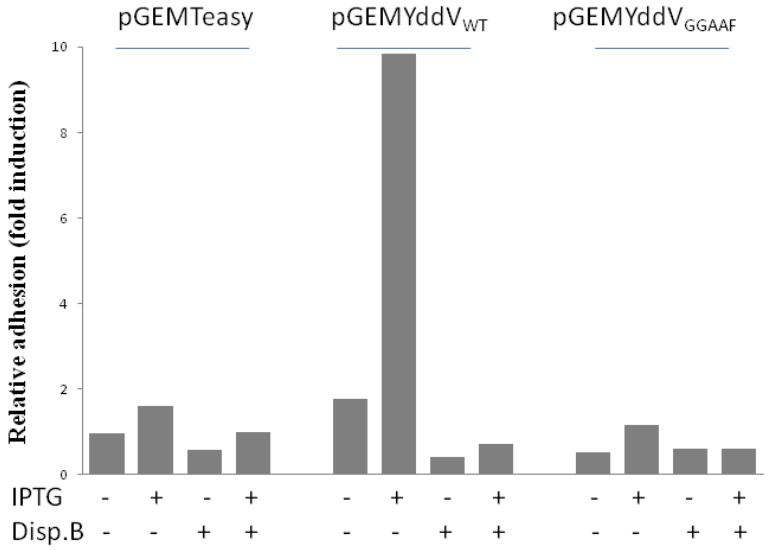

Figure 6B

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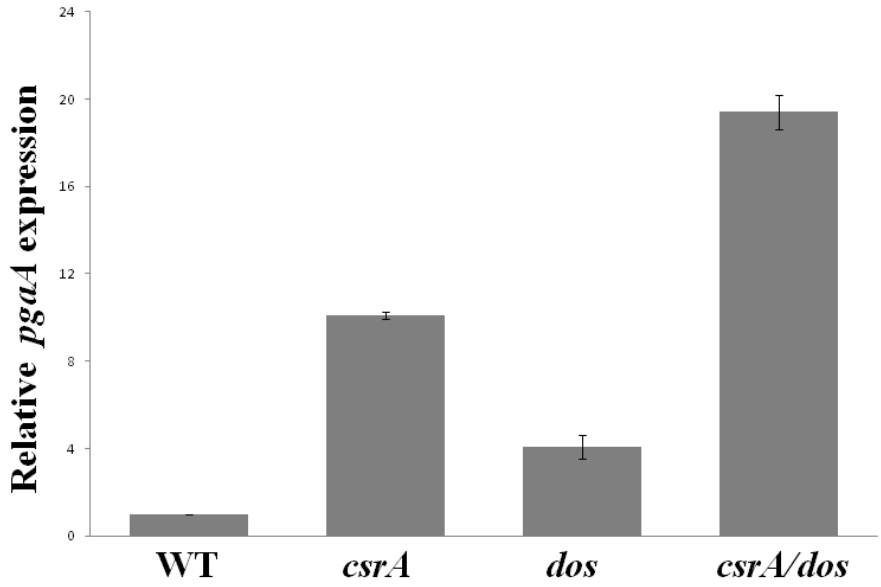

Figure 7

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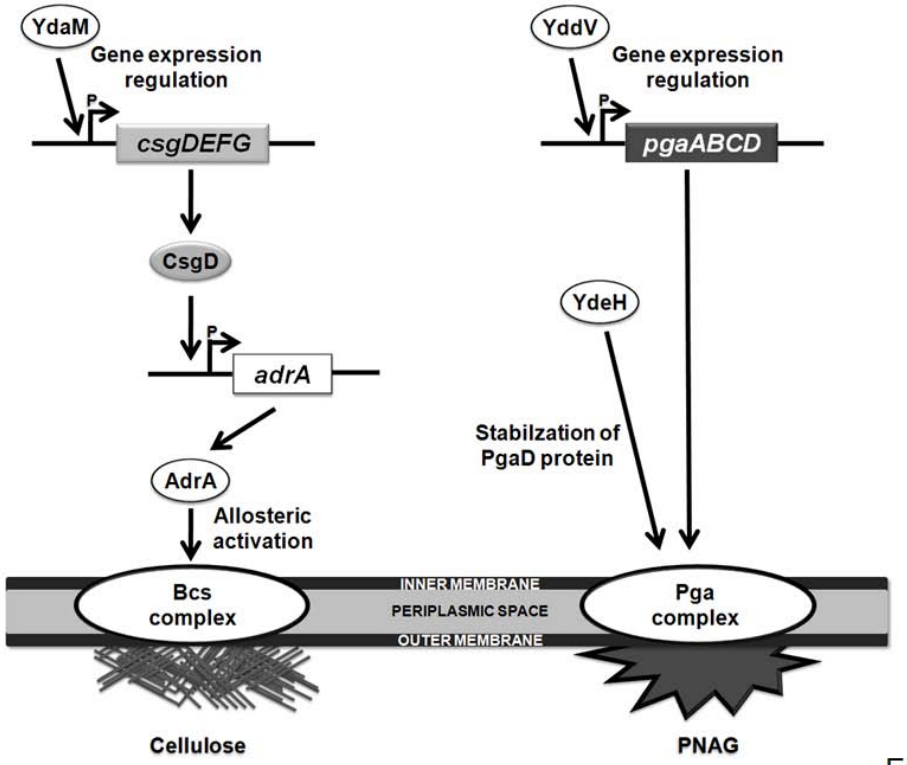

Figure 8

2