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GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in *Escherichia coli*

Viviana Sanchez-Torres · Hongbo Hu · Thomas K. Wood

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Abstract The second messenger 3′–5′-cyclic diguanylic acid (c-di-GMP) promotes biofilm formation, and c-di-GMP is synthesized by diguanylate cyclases (characterized by a GGDEF domain) and degraded by phosphodiesterases. Here, we evaluated the effect of the 12 *E. coli* GGDEF-only proteins on biofilm formation and motility. Deletions of the genes encoding the GGDEF proteins YeaI, YedQ, YfiN, YeaJ, and YneF increased swimming motility as expected for strains with reduced c-di-GMP. Alanine substitution in the EGEVF motif of YeaI abolished its impact on swimming motility. In addition, extracellular DNA (eDNA) was increased as expected for the deletions of *yeaI* (tenfold), *yedQ* (1.8-fold), and *yfiN* (3.2-fold). As a result of the significantly enhanced motility, but contrary to current models of decreased biofilm formation with decreased diguanylate cyclase activity, early biofilm formation increased dramatically for the deletions of *yeaI* (30-fold), *yedQ* (12-fold), and *yfiN* (18-fold). Our results indicate that YeaI, YedQ, and YfiN are active diguanylate cyclases that reduce motility, eDNA, and early biofilm formation and contrary to the current paradigm, the results indicate that c-di-GMP levels should be reduced, not increased, for initial biofilm formation so c-di-GMP levels must be regulated in a temporal fashion in biofilms.

V. Sanchez-Torres · H. Hu · T. K. Wood (✉)
Department of Chemical Engineering, Texas A & M University,
220 Jack E. Brown Building,
College Station, TX 77843-3122, USA
e-mail: Thomas.Wood@chemail.tamu.edu

H. Hu
Key Laboratory of Microbial Metabolism, Ministry of Education,
College of Life Science and Biotechnology,
Shanghai Jiao Tong University,
Shanghai, China 200240

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Introduction

Bacterial cells can adopt two different lifestyles, the planktonic mode characterized by single motile cells, or the biofilm mode where bacterial cells form sedentary multicellular communities attached to themselves or to a surface (Pesavento et al. 2008). The second messenger, 3′–5′-cyclic diguanylic acid (c-di-GMP), acts as a switch promoting the transition from the planktonic to the biofilm lifestyle (Hengge 2009). The current c-di-GMP paradigm is that high intracellular c-di-GMP levels promote the synthesis of exopolysaccharides which are components of the biofilm matrix, promote the formation of adhesive fimbriae, and inhibit motility (Hengge 2009).

c-di-GMP is synthesized from guanosine-5′-triphosphate by diguanylate cyclases which are characterized by the GGDEF domain (Hengge 2009). Diguanylate cyclases have a conservative GG[D/E]EF motif in their active site (A-site), and some of them also have an inhibitory site for c-di-GMP binding (I-site) (Sommerfeldt et al. 2009). Degradation of c-di-GMP is catalyzed by phosphodiesterases, characterized by EAL or HD-GYP domains (Schirmer and Jenal 2009). Most bacteria have multiple diguanylate cyclases and phosphodiesterases. *Escherichia coli* K-12 has 12 proteins with a GGDEF domain, 10 proteins with an EAL domain, and 7 proteins with both GGDEF and EAL domains in a single polypeptide (Weber et al. 2006).

Among the 12 GGDEF-only proteins in *E. coli*, the diguanylate cyclase activity of AdrA (Antoniani et al. 2009), YdaM (Weber et al. 2006), YddV (Méndez-Ortiz et al. 2006), YcdT (Jonas et al. 2008), YdeH (Jonas et al.

2008), and YeaP (Ryjenkov et al. 2005) has been confirmed either in vitro, by using purified proteins, or in vivo, by measuring the effect on c-di-GMP intracellular levels after deleting or overexpressing the corresponding genes. Some of these *E. coli* GGDEF-only proteins alter biofilm related phenotypes since YdaM (Weber et al. 2006), YeaP (Sommerfeldt et al. 2009), and YddV (Tagliabue et al. 2010b) promote curli formation, since AdrA (Antoniani et al. 2009) and YedQ (Da Re and Ghigo 2006) activate cellulose production, and since YdeH activates production of the polysaccharide adhesin poly-β-1,6-*N*-acetyl-glucosamine (Boehm et al. 2009). Also, swimming motility decreases by overexpressing the genes encoding YdeH and YcdT (Jonas et al. 2008). Similarly, YeaJ (Pesavento et al. 2008), YedQ (Pesavento et al. 2008), YddV (Boehm et al. 2010), and YfiN (Boehm et al. 2010) negatively regulate motility in a mutant lacking the phosphodiesterase YhjH. The phenotypes controlled by the other GGDEF-only proteins (YeaI, YliF, and YneF) have not been characterized.

Mature *E. coli* biofilm formation increased by overexpressing the genes encoding the GGDEF-only diguanylate cyclases AdrA (Antoniani et al. 2009), and YddV (Méndez-Ortiz et al. 2006). Hence, high c-di-GMP concentrations enhance late biofilm formation while reducing motility. However, mutants with decreased motility have reduced adhesion (Genevaux et al. 1996); hence, we reasoned that decreasing diguanylate cyclase activity should increase motility and lead to increased early biofilm formation, even though diguanylate cyclase activity is known to increase biofilm formation. To address this paradox and to investigate the role of heretofore unstudied *E. coli* diguanylate cyclases on biofilm formation, we compared biofilm formation of the wild-type BW25113 and its isogenic mutants defective in each of the 12 genes encoding GGDEF-only proteins and found knock-outs of the genes encoding the GGDEF proteins YeaI, YedQ, and YfiN enhanced early biofilm formation dramatically. Our results suggest that a reduction in the c-di-GMP levels caused by inactivating *yeaI*, *yedQ*, and *yfiN* enhances swimming motility which contributes to enhanced initial attachment to the polystyrene surface thus promoting early biofilm formation. Therefore, the current paradigm for c-di-GMP should be refined to indicate its inverse relationship to initial biofilm formation and to indicate that the timing of c-di-GMP production is likely more sophisticated than just elevated in biofilms.

Materials and methods

Bacterial strains, media, and growth conditions The *E. coli* strains and plasmids used in this study are listed in Table 1. Single deletion mutants of the parental strain *E. coli* K-12 BW25113 were obtained from the Keio collection (Baba et al. 2006). The double deletion strains BW25113 *yeaI yedQ* and BW25113 *yeaI yfiN* were constructed via P1 transduction (Maeda et al. 2008). The deletions of *yeaI*, *yedQ*, and *yfiN* were verified as described previously (Sanchez-Torres et al. 2009) via polymerase chain reaction (PCR) using primers listed in Table 2. Primers flhDC-F2 and flhDC-R (Table 2) were used to verify via PCR that the wild-type strain and mutants used here *adrA, ydaM*, *ycdT*, *yddV*, *ydeH*, *yeaP*, *yeaI*, *yeaJ*, *yedQ*, *yfiN*, *yliF*, *yneF,* and *ycgR* do not contain IS insertions in the regulatory sequence of *flhDC* that may increase motility (Barker et al. 2004).

Experiments were conducted at 37 °C in either Luria–Bertani (LB; Sambrook et al. 1989) or M9 minimal medium supplemented with 0.4% casamino acids (M9C). Kanamycin (50 μg/mL) was used for pre-culturing the knock-out mutants and chloramphenicol (30 μg/mL) was used for selecting plasmid pCA24N (Kitagawa et al. 2005) and its derivatives. The specific growth rates of BW25113 and the *yeaI, yedQ,* and *yfiN* knock-out mutants were measured in LB using two independent cultures for each strain with the turbidity measured at 600 nm from 0.05 to 0.7.

Crystal violet biofilm assay Biofilm formation was assayed in 96-well polystyrene plates (Corning, Lowell, MA) as described previously (Fletcher 1977). Wells were inoculated with overnight cultures at an initial turbidity at 600 nm of 0.05 in LB and incubated for 7 and 24 h quiescently. Biofilm formation was also assayed in M9C (Sambrook et al. 1989) for 7 h. For each strain, at least two independent cultures were assayed in 12 replicate wells.

Site-directed mutagenesis The codon encoding the second glutamic acid of the EGEVF motif of YeaI GAG was mutated to the alanine codon GCG to yield a EGAVF motif. Site-directed mutagenesis was performed using pCA24N-*yeaI* as the template with complementary primers containing the target mutation (Table 2) as described previously (Steffens and Williams 2007).

Swimming motility assay Single colonies were inoculated onto motility plates (1% tryptone, 0.25% NaCl, and 0.3% agar; Sperandio et al. 2002) using a toothpick. For cells with pCA24N-based plasmids, the motility plates were supplemented with 30 μg/mL chloramphenicol and 0.1 mM IPTG. The motility halos were measured after a 12-h incubation and at least two independent cultures for each strain were used.

eDNA assay eDNA was assayed as described previously using quantitative PCR (qPCR) (Ueda and Wood 2010). Briefly, LB cultures with an initial turbidity at 600 nm of 0.05 were incubated for 24 h. Supernatants (1 mL) were

Table 1 *E. coli* strains and plasmids used in this study

Strains and plasmids	Genotype/relevant characteristics[a]	Source
Strains		
BW25113	F⁻ *Δ(araD-araB)567 ΔlacZ4787*(::rrnB-3) λ⁻ *rph-1 Δ(rhaD-rhaB) 568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
adrA (*yaiC*)	BW25113 *ΔyaiC750::kan* Km^R	(Baba et al. 2006)
ydaM	BW25113 *ΔydaM778::kan* Km^R	(Baba et al. 2006)
ycdT	BW25113 *ΔycdT771::kan* Km^R	(Baba et al. 2006)
yddV	BW25113 *ΔyddV783::kan* Km^R	(Baba et al. 2006)
ydeH	BW25113 *ΔydeH756::kan* Km^R	(Baba et al. 2006)
yeaP	BW25113 *ΔyeaP790::kan* Km^R	(Baba et al. 2006)
yeaI	BW25113 *ΔyeaI782::kan* Km^R	(Baba et al. 2006)
yeaJ	BW25113 *ΔyeaJ783::kan* Km^R	(Baba et al. 2006)
yedQ	BW25113 *ΔyedQ730::kan* Km^R	(Baba et al. 2006)
yfiN	BW25113 *ΔyfiN767::kan* Km^R	(Baba et al. 2006)
yliF	BW25113 *ΔyliF734::kan* Km^R	(Baba et al. 2006)
yneF	BW25113 *ΔyneF743::kan* Km^R	(Baba et al. 2006)
yeaI yedQ	BW25113 Δ*yeaI882 ΔyedQ730::kan* Km^R	This study
yeaI yfiN	BW25113 Δ*yeaI882 ΔyfiN767::kan* Km^R	This study
Plasmids		
pCA24N	*lacI*^{q}, Cm^R	(Kitagawa et al. 2005)
pCA24N-*yeaI*	pCA24N $P_{T5\text{-}lac}$::*yeaI* Cm^R	
pCA24N-*yeaIE407A*	pCA24N $P_{T5\text{-}lac}$::*yeaI407* Cm^R; encodes YeaI with E407A	This study

[a] Km^R and Cm^R are kanamycin and chloramphenicol resistance, respectively

centrifuged for 10 min at 13 krpm to find eDNA, and the total amount of DNA in the culture (outside and inside the cells) was determined using 1 mL of culture that was sonicated for 45 s at 10 W (60 Sonic Dismembrator, Fisher Scientific Co, Pittsburgh, PA) and centrifuged at 13 krpm for 10 min. eDNA and total DNA were purified using phenol:chloroform:isoamyl alcohol (25:24:1) extraction and sodium acetate and isopropanol precipitation. eDNA and total DNA were quantified by qPCR using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) with primers for the reference gene *purA* (Table 2). At least two independent cultures were used.

Quantification of c-di-GMP c-di-GMP was assayed as described previously using HPLC (Ueda and Wood 2009). Cells from overnight cultures were inoculated into 350 mL of LB medium and cultured for 16 h with shaking (250 rpm). Nucleotides were extracted using 65% ethanol, and c-di-GMP was quantified using HPLC (Waters 515 with photodiode array detector, Milford, MA). Commercial c-di-GMP (BIOLOG Life Science Institute, Bremen, Germany) was used as the standard. The c-di-GMP peak was verified by spiking each sample with the commercial c-di-GMP, and the in vitro degradation of c-di-GMP by purified phosphodiesterase YahA was used as control.

Results

Deletions of yeaI, yedQ, and yfiN increase early biofilm formation To investigate whether reductions in diguanylate cyclase activity (which serve to decrease c-di-GMP concentrations) increase initial biofilm formation due to increased cell motility, we assayed biofilm formation after 7 h upon deleting 12 genes encoding GGDEF proteins in *E. coli*. In LB medium, there were not significant differences in biofilm formation between wild-type BW25113 and most of the mutants (Fig. 1a); however, the *yddV* mutant decreased biofilm formation (fourfold), and three mutants increased biofilm formation dramatically: *yeaI* (30-fold), *yedQ* (12-fold), and *yfiN* (18-fold) (Fig. 1a). These same three mutations also increased biofilm formation in minimal medium (Fig. 1b). After 7 h of incubation, biofilm formation increased 10-fold for the *yeaI* mutant, 6-fold for *yedQ* mutant, and 18-fold for the *yfiN* mutant relative to the wild-type (Fig. 1b). Hence, deleting *yeaI, yedQ,* and *yfiN* increase biofilm formation dramatically in both rich and

Table 2 Primers used for site-directed mutagenesis, quantitative real-time PCR (qPCR), DNA sequencing, and verification of the relevant deletions in the strains used

Primer	Sequence[a]
Site-directed mutagenesis at position E407 of YeaI	
yeaIE407-F	5′-ATTTTAGCGCGACTGGAGGGTGCGGTGTTTGGCTTGCTATTTACC-3′
yeaIE407-R	5′-GTAAATAGCAAGCCAAACACCGCACCCTCCAGTCGCGCTAAAAT-3′
qPCR	
purA-f	5′-GGGCCTGCTTATGAAGATAAAGT-3′
purA-r	5′-TCAACCACCATAGAAGTCAGGAT-3′
DNA sequencing of pCA24N-*yeaIE407A*	
hha rear	5′-GAACAAATCCAGATGGAGTTCTGAGGTCATT-3′
Verification of strains	
yeaI front	5′-GTGGCGAGAATATGAGCATCTG-3′
yeaI rev	5′-CTGGATCAGTGTACTGCCGTTA-3′
yedQ front	5′-GAGTGTCGTTGGTATGACGGTTAC-3′
yedQ rev	5′-GTTCCCAGCTAACATAGCGACT-3′
yfiN front	5′-AGTACCGCCCTACAAGAGAATG-3′
yfiN rev	5′-CAGAATACAACCGGTCAGTACG-3′
kanrev	5′-ATCACGGGTAGCCAACGCTATGTC-3′
flhDC-F2	5′-CCTGTTTCATTTTTGCTTGCTAGC-3′
flhDC-R	5′-GGAATGTTGCGCCTCACCG-3′

[a] Underlined text indicates the site-directed mutation for the codon corresponding to E407 (5′-GAG to 5′-GCG for E407A)

minimal medium. Note there was no change in the growth rate in rich medium so these changes in biofilm formation are not related to changes in growth.

Since the double deletion mutants *yeaI yedQ* and *yeaI yfiN* did not increase further biofilm formation (Fig. 1a), YeaI appears to regulate the same process controlled by YedQ and YfiN. In addition, after 24 h of incubation in LB medium, none of the mutants significantly altered biofilm formation (Fig. 1c). These results indicate that the deletions of *yeaI, yedQ*, and *yfiN* mainly alter the initial stages of biofilm formation which are influenced by motility (Pratt and Kolter 1998).

YfiN was first characterized by us in *Pseudomonas aeruginosa* where we showed it was related to rugose colony formation due to its diguanylate cyclase activity that is controlled by a tyrosine phosphatase (Ueda and Wood 2009; Pu and Wood 2010). Since *yfiN* is part of the operon *yfiRNB* in *E. coli*, the biofilm formation of the *yfiB* and *yfiR* mutants was also assayed to evaluate the effect of YfiB and YfiR on the activity of YfiN. While the *yfiR* mutant produces a similar level of biofilm formation compared to the wild-type strain, the *yfiB* mutant increases biofilm formation like the *yfiN* mutant (Fig. 1a). These results indicate that YfiB is a positive regulator of YfiN activity and that the activity of YfiN is not significantly altered by deleting *yfiR*.

To confirm that initial biofilm formation is enhanced when motility is not inhibited by c-di-GMP, we investigated biofilm formation with a *ycgR* deletion. YcgR with bound c-di-GMP inhibits swimming motility by reducing the flagella motor speed through its interaction with MotA (Boehm et al. 2010), FliG, and FliM (Paul et al. 2010). We found the *ycgR* mutant, like the *yeaI, yedQ*, and *yfiN* mutants, increases biofilm formation (23-fold) after 7 h (Fig. 1a) but not after 24 h (Fig. 1c). These results suggest that the *yeaI, yedQ*, and *yfiN* deletions decrease the levels of c-di-GMP which promotes motility (since c-di-GMP bound to YcgR decreases motility) thus increasing early biofilm formation.

Deletions of yeaI, yedQ, and yfiN increase swimming motility and the EGEVF motif of YeaI is necessary to reduce swimming motility Low levels of c-di-GMP promote swimming and swarming motility (Römling and Amikam 2006); hence, we investigated swimming with the *yeaI, yedQ,* and *yfiN* mutants since inactivation of these genes should decrease c-di-GMP levels by inactivating diguanylate cyclase activity. A large increase in swimming motility was observed for the single deletions in *yeaI* (fourfold), *yedQ* (sixfold), and *yfiN* (tenfold; Fig. 2a); therefore, inactivating the diguanylate cyclase activity of these three mutants consistently increases motility. We also evaluated swimming motility for the remaining nine mutants encoding GGDEF-only proteins (Fig. 2b) and found that *yeaJ* (twofold), and *yneF* (fourfold) also have increased motility, suggesting that the GGDEF proteins encoded by these genes are diguanylate cyclases that control swimming motility.

The increase in swimming motility phenotype caused by the *yeaI* deletion was complemented by plasmid pCA24N-*yeaI* (encoding YeaI with EGEVF; Fig. 2a). Since YeaI has a EGEVF motif instead of the conserved GG[D/E]EF motif characteristic of active diguanylate cyclases, a single amino acid change of the second glutamic acid of EGEVF (corresponding to the catalytic residue of GGDEF (Chan

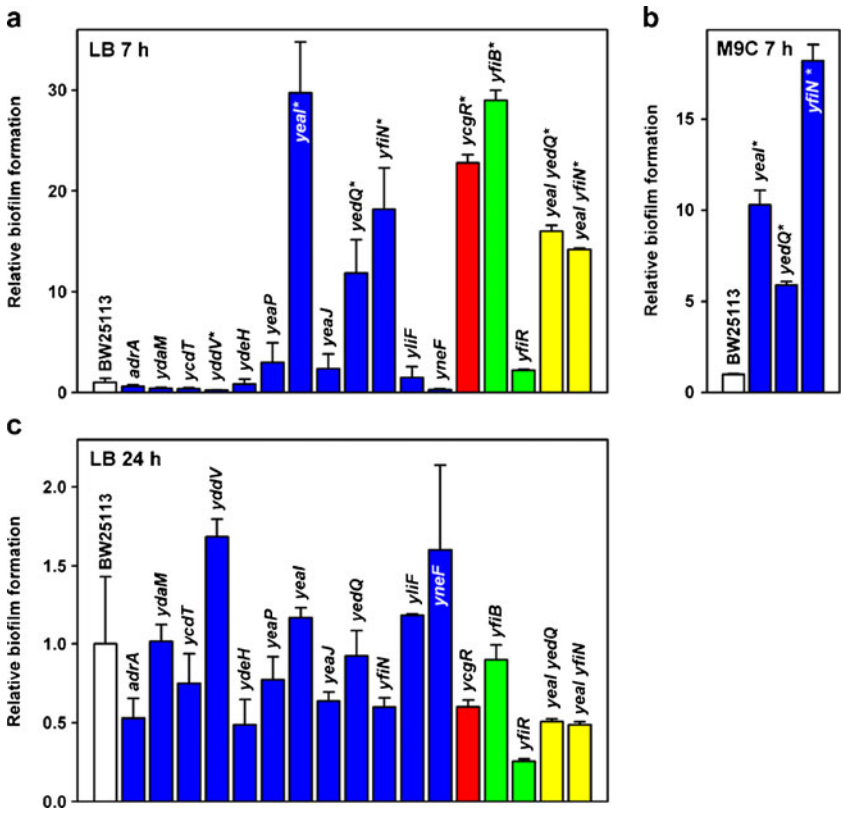

Fig. 1 Relative normalized biofilm formation in polystyrene microtitre plates at 37 °C after 7 h in LB (**a**), after 7 h in M9C (**b**), and after 24 h in LB (**c**). Wild-type BW25113 is shown in *white*; single mutants defective in genes encoding GGDEF-only proteins are in *blue*, the *ycgR* mutant, which is defective in the gene encoding the c-di-GMP regulated flagellar velocity braking protein, is *red*, the *yfiB* and *yfiR* mutants which lack genes in the same operon as *yfiN* are in *green*, and the double deletion mutants *yeaI yedQ* and *yeaI yfiN* are *yellow*. Biofilm formation (turbidity at 540 nm) was normalized by the amount of planktonic growth (turbidity at 620 nm) and is shown relative to the BW25113 normalized biofilm value. Each data point is the average of at least 12 replicate wells from two independent cultures. The *error bars* correspond to the standard deviation, and an *asterisk* indicates p values<0.05 using a Student's T test

et al. 2004)) to alanine was introduced via site-directed mutagenesis to show that YeaI is an active diguanylate cyclase. Since motility was not complemented by pCA24N-*yeaIE407A* (encoding YeaI with EGAVF; Fig. 2a), the EGEVF domain of YeaI is necessary to reduce swimming motility which provides additional evidence that YeaI increases c-di-GMP as a diguanylate cyclase.

Deletions of yeaI, yedQ, and yfiN increase eDNA eDNA is an important component of the bacterial biofilm matrix (Whitchurch et al. 2002). c-di-GMP is inversely proportional to eDNA in *P. aeruginosa* cultures (Ueda and Wood 2010); therefore, we investigated if the deletions of *yeaI*, *yedQ*, and *yfiN* alter eDNA in *E. coli* with the expectation that deleting these genes would reduce c-di-GMP and thereby increase eDNA. For planktonic cells cultured for 24 h in LB medium, the percentage of eDNA relative to the total amount of DNA in the cultures (eDNA + genomic DNA) was 0.09±0.02 for the wild-type strain, 0.9±0.4 for the *yeaI* mutant (a 10±4-fold increase), 0.16±0.02 for the *yedQ* mutant (a 1.8±0.3-fold increase), and 0.282±0.004 for the *yfiN* mutant (a 3.2±0.1-fold increase). These results suggest that the mutations in *yeaI, yedQ,* and *yfiN* decrease c-di-GMP which results in increased eDNA.

Deletions of yeaI, yedQ, and yfiN do not alter the total concentration of c-di-GMP We evaluated the c-di-GMP concentrations from cell extracts of the wild-type strain and the *yeaI*, *yedQ*, and *yfiN* mutants from planktonic cultures in LB medium and did not find significant differences in the total concentration of c-di-GMP inside the *yeaI*, *yedQ*, and *yfiN* mutants vs. wild-type BW25113. These results suggest that the effect of YeaI, YedQ, and YfiN on the c-di-GMP levels may occur locally, close to the inner membrane, since YeaI, YedQ, and YfiN are integral inner membrane proteins (Misra et al. 2005).

Discussion

Biofilm formation occurs following a developmental sequence (Petrova and Sauer 2009). First, bacteria move

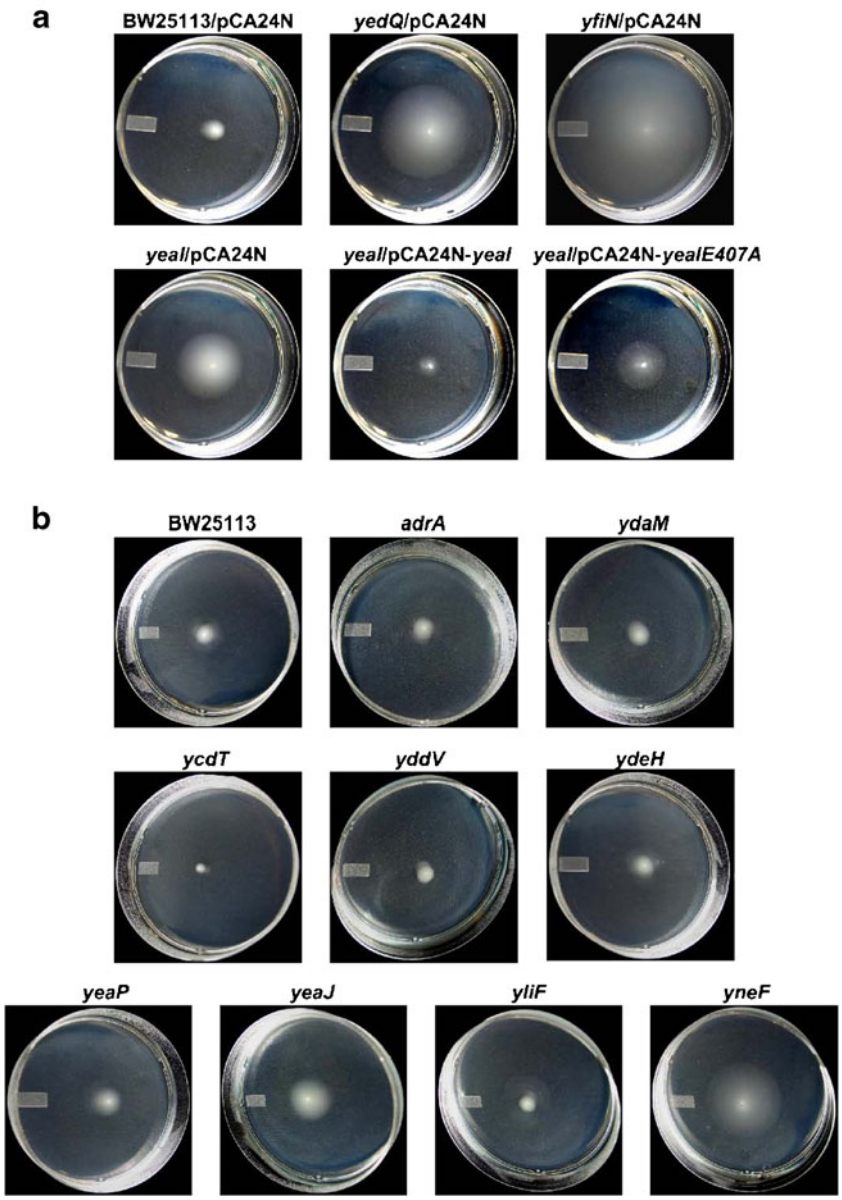

Fig. 2 Swimming motility for strains with mutations in the genes encoding diguanylate cyclases YedQ (GGEEF), YfiN (GGDEF), and YeaI (EGEVF), and complementation studies for the *yeaI* mutant using pCA24N-*yeaI* producing YeaI and pCA24N-*yeaIE407A* that produces YeaI E407A (EGAVF) (**a**). Swimming motility for BW25113 and its isogenic mutants of genes encoding diguanylate cyclases AdrA, YdaM, YcdT, YddV, YdeH, YeaP, YeaJ, YliF, and YneF (**b**). Swimming motility was assayed after 12 h at 37 °C; motility plates were supplemented with 30 μg/mL Cm and 0.1 mM IPTG to induce diguanylate cyclase production from the pCA24N-based plasmids

in the liquid culture and reach a surface where cells are reversibly attached, and some cells have a strong adhesion to the surface and become sessile (Hall-Stoodley et al. 2004). These cells replicate and aggregate in a self-produced polymeric matrix thus forming a mature biofilm (Hall-Stoodley et al. 2004). Finally cells disperse from the biofilm and return to a motile state (Kaplan 2010). For all of these steps, c-di-GMP plays a role (Kaplan 2010).

In many bacteria including *E. coli*, high concentrations of c-di-GMP promote biofilm formation (Dow et al. 2007). For example, overexpression of the genes encoding the diguanylate cyclases AdrA (Antoniani et al. 2009) and YddV (Méndez-Ortiz et al. 2006) increase *E. coli* mature biofilm formation. c-di-GMP also inversely regulates motility (Méndez-Ortiz et al. 2006), and motility is important for initial attachment to a surface; non-flagellated cells or cells with paralyzed flagella have reduced initial biofilm formation (Pratt and Kolter 1998), and *E. coli* strains with high motility make more biofilm than strains with poor motility (Wood et al. 2006). Motility also affects biofilm architecture since biofilms of strains with high motility make vertical structures while strains with poor motility form flat biofilms (Wood et al. 2006). Hence, opposite to the current understanding that c-di-GMP promotes biofilm formation, we hypothesized that deletion of the genes encoding diguanylate cyclases should decrease c-di-GMP levels thus increasing motility and early biofilm formation.

Deletions of *yeaI*, *yedQ*, and *yfiN* increased dramatically biofilm formation after 7 h of incubation in LB at 37 °C (Fig. 1a) while increasing motility (Fig. 2a). These results

suggest that the *yeaI*, *yedQ*, and *yfiN* mutations affect the initial steps of biofilm formation by decreasing c-di-GMP which results in higher motility. Hence, c-di-GMP levels should be low for initial biofilm formation. Furthermore, our results with the predicted inner membrane proteins (Misra et al. 2005) YeaI, YedQ, and YfiN suggest these three proteins function as active diguanylate cyclases producing c-di-GMP. Although we found a significant reduction in early biofilm formation for the *yddV* mutant (fourfold), this result does not contradict our hypothesis since deletion of *yddV* did not significantly alter swimming motility (Fig. 2b). The reduction of early biofilm formation by the *yddV* mutant may be due to a decreased production of the exopolysaccharide PNAG as reported previously (Tagliabue et al. 2010a).

Previous reports indicated that single deletions of genes encoding *E. coli* GGDEF proteins do not have a much of an effect on motility (Jonas et al. 2008; Boehm et al. 2010) unless the gene encoding the phosphodiesterase YhjH is inactivated (Pesavento et al. 2008; Boehm et al. 2010). In contrast, we found a dramatic increase in motility after 12 h of incubation at 37 °C for the knock-out mutants of genes encoding the GGDEF proteins YeaI, YedQ, YfiN (Fig. 2a), YeaJ, and YneF (Fig. 2b). These increases in motility combined with an increase in initial biofilm formation for YeaI, YedQ, and YfiN were corroborated by an increase in biofilm formation by the *ycgR* strain which lacks the YcgR motility brake that is activated by c-di-GMP (Fig. 1a).

eDNA is required for initial attachment to a surface and has a structural role connecting the cells in the biofilms (Rice et al. 2007). Previously, we reported that in *P. aeruginosa* eDNA is inversely regulated by c-di-GMP (Ueda and Wood 2010). To determine if the same eDNA regulation occurs in *E. coli*, we evaluated eDNA for the *yeaI*, *yedQ*, and *yfiN* mutants and found that eDNA increases as expected for low c-di-GMP levels. Recently, we reported that deletion of *hns* (encoding the global regulator H-NS) abolished eDNA production in *E. coli* (Sanchez-Torres et al. 2010). The *hns* mutant is also defective in swimming motility (Ko and Park 2000). Since inactivation of *ycgR* (encodes a motility brake) and overexpression of *yhjH* (encodes a phosphodiesterase) restore the motility defect of the *hns* mutant (Ko and Park 2000), the *hns* deletion may increase c-di-GMP levels thus inhibiting motility via YcgR. Hence, our results for the *yeaI*, *yedQ*, and *yfiN* mutants, and our previously reported decrease in eDNA by the *hns* mutant (Sanchez-Torres et al. 2010) suggest that c-di-GMP negatively regulates eDNA production in *E. coli*.

Our results suggest that the network of diguanylate cyclases and phosphodiesterases in *E. coli* tune the c-di-GMP concentrations according to the developmental sequence of biofilm formation. Initially, c-di-GMP concentrations should be low to promote early biofilm through increased swimming motility and increased eDNA as reported here for *yeaI*, *yedQ*, and *yfiN*, then c-di-GMP should be increased to promote biofilm maturation by inducing exopolysaccharide production, formation of adhesive fimbriae, and sessility. Finally, c-di-GMP should decrease to produce biofilm dispersal through higher motility as we reported recently (Ma et al. 2010).

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