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A CsgD-Independent Pathway for Cellulose Production and Biofilm Formation in *Escherichia coli*†

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Bacterial growth on a surface often involves the production of a polysaccharide-rich extracellular matrix that provides structural support for the formation of biofilm communities. In *Salmonella*, cellulose is one of the major constituents of the biofilm matrix. Its production is regulated by CsgD and the diguanylate cyclase AdrA that activates cellulose synthesis at a posttranscriptional level. Here, we studied a collection of *Escherichia coli* isolates, and we found that the ability to produce cellulose is a common trait shared by more than 50% of the tested strains. We investigated the genetic determinants of cellulose production and its role in biofilm formation in the commensal strain *E. coli* 1094. By contrast with the *Salmonella* cellulose regulatory cascade, neither CsgD nor AdrA is required in *E. coli* 1094 to regulate cellulose production. In this strain, an alternative cellulose regulatory pathway is used, which involves the GGDEF domain protein, YedQ. Although $AdrA_{1094}$ is functional, it is weakly expressed in *E. coli* 1094 compared to YedQ, which constitutively activates cellulose production under all tested environmental conditions. The study of cellulose regulation in several other *E. coli* isolates showed that, besides the CsgD/AdrA regulatory pathway, both CsgD-independent/YedQ-dependent and CsgD-independent/YedQ-independent pathways are found, indicating that alternative cellulose pathways are common in *E. coli* and possibly in other cellulose-producing *Enterobacteriaceae*.

Bacterial colonization and survival in different ecological niches often involve growth on a surface and formation of multicellular biofilm communities. Whereas bacterial envelope proteins and fimbriae have been shown to play critical roles in this process, the production of an extracellular matrix is also recognized as a key element in determining the mature biofilm architecture (61).

The biofilm matrix is a complex hydrated milieu that contains proteins, DNA, RNA, ions, and polysaccharidic polymers (9, 61, 62). These polymers are very diverse and include components such as β-1,6-*N*-acetyl-D-glucosamine polymer (PIA in *Staphylococcus* spp. or PGA in *Escherichia coli*), colanic acid (*E. coli*), and alginate-, glucose-, and mannose-rich components (*Pseudomonas aeruginosa* and *Bacillus subtilis*), as well as cellulose (*Salmonella*, *E. coli*, and *Pseudomonas fluorescens*) (7, 8, 19, 20, 27, 44, 58, 59, 64, 68). Polysaccharides are often involved in the establishment of productive cell-to-cell contacts that contribute to the formation of biofilms at liquid/solid interfaces, of pellicles at air/liquid interfaces, of cell aggregates and clumps in liquid cultures, and of wrinkled colony morphology on agar plates. Evidence for a structural role of some of these matrix polysaccharides is accumulating, and the regulation of the production of these exopolysaccharides is being actively investigated for different bacteria (9, 33, 55).

Cellulose production is a widespread phenomenon in *Enterobacteriaceae*, including *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* subsp. *enterica* serovar Enteritidis, *E. coli*, *Citrobacter* spp., and *Enterobacter* spp. (49, 58, 67, 68). In association with the production of curli fibers, cellulose synthesis in *Salmonella* has been shown to be a primary cause of biofilm formation and to lead to a distinctive phenotype on agar plates, the red, dry, and rough (rdar) morphotype in LB medium at 28°C (58, 68). The genetic dissection of the rdar morphotype in *Salmonella* showed that both curli fibers and cellulose production are positively regulated by CsgD (AgfD in *Salmonella*), a transcriptional regulator belonging to the LuxR family (47, 51). The roles of CsgD in these two pathways can be monitored by the absorption of the Congo red dye (CR; indicative of curli and cellulose production) or the fixation of calcofluor (CF; indicative of cellulose production). In *Salmonella* serovar Typhimurium and *Salmonella* serovar Enteritidis, the ability to bind CF depends on the expression of two divergent cellulose synthesis operons, *bcsABZC* and *bcsEFG*, that are constitutively expressed (58, 68). The positive regulation of cellulose production by CsgD is mediated through the transcriptional regulation of *adrA*. AdrA, in turn, activates cellulose synthesis at a posttranscriptional level by controlling the synthesis of cyclic diguanylate (c-di-GMP). In *Gluconacetobacter xylinus*, it has been suggested that c-di-GMP binds to BcsB, promoting an allosteric change of the protein conformation that leads to its activation (36). Recently, a PilZ domain proposed to be part of the c-di-GMP binding protein has been identified in several bacterial cellulose synthases, including BscA, although direct evidence for c-di-GMP binding is still missing (2). In addition to being subject to several global regulators, such as OmpR, CpxR, H-NS, and IHF, depending on environmental conditions (25, 31), CsgD expression is also controlled by RpoS through the positive action of the transcriptional regulator MlrA (see Fig. 6A) (11, 46).

Owing to the high genetic conservation of the cellulose synthesis and regulatory genes, it has been suggested that the *Salmonella* cellulose regulatory cascade could be common to

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all cellulose-producing *Enterobacteriaceae*, including *E. coli* (46, 5). However, some deviations from this regulatory network were found in several *Salmonella* strains. Romling et al. observed that in *Salmonella* serovar Enteritidis, *csgD* expression was only partially dependent on RpoS, and in one of the *Salmonella* serovar Enteritidis strains, cellulose synthesis was uncoupled from *csgD* expression in LB medium (49). Similarly, in *Salmonella* serovar Enteritidis, but in minimal ATM medium at 37°C, cellulose production and biofilm formation were not affected by mutations in the regulatory protein RpoS, OmpR, or CsgD (58). Under these conditions, cellulose synthesis has proven to be independent of *adrA* but dependent on a putative diguanylate cyclase, stm1987 (23). These observations suggested the existence of alternative cellulose regulatory pathways in *Salmonella* and other eubacteria.

Here, we have investigated cellulose and biofilm formation in a collection of *E. coli* isolates. We show that cellulose synthesis is the primary cause of biofilm formation and of the expression of multicellular behavior (rdar morphotype) in the commensal *E. coli* strain 1094. In this strain, cellulose synthesis does not require CsgD or AdrA, which is indicative of an alternative CsgD-independent cellulose regulatory pathway. We identified the genetic determinant involved in this pathway and provided evidence of the existence of alternative cellulose regulatory networks in *E. coli*. These findings indicate that the regulation of cellulose production in *E. coli* is more complex than previously recognized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and liquid growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1 (see also Table S1 in the supplemental material). (Supplemental material may also be found at http://www.pasteur.fr/recherche/unites/Ggb/supmet.html.) All experiments were performed in 0.4% glucose M63B1 minimal medium or in LB medium at 30°C or 37°C as specified. All liquid cultures were shaken, agitated cultures. Antibiotics were added when required at the following concentrations: ampicillin, 100 μg ml^{-1}; apramycin, 30 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; zeocin, 30 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

Generation of deletion and *uidA* fusion mutants in *E. coli*. The deletion mutants in the different strains of *E. coli* and the *pyedQ-uidA* fusion mutant in *E. coli* 1094 were generated by the λ-red linear DNA gene inactivation method using the three-step PCR procedure as described previously (12, 17; for details, see http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html). In the *pyedQ-uidA* fusion mutant in *E. coli* 1094, *yedQ* was replaced by *uidA*. The primers used to delete the genes presented in this study are listed in Table S2 in the supplemental material. All constructs were checked by PCR with specific primers (see Table S2 in the supplemental material).

Molecular biology procedures. Standard techniques were used for cloning, DNA analysis, PCR, electroporation, and conjugation, as described previously (54). As the genome sequence for 1094 is currently unavailable, *E. coli* 1094 genomic sequences were determined from PCR products amplified from the bacterial genome with proofreading polymerase. All DNA sequencing was performed by Genome Express SA, France.

Congo red and calcofluor phenotype assays. Two microliters of an overnight (o/n) culture grown at 37°C in LB medium (with added antibiotics when needed) was spotted onto LB plates (without NaCl) containing 0.004% CR and 0.002% brilliant blue (referred to as CR plates) or onto LB or M63B1-0.4% glucose (M63B1-glu) plates containing 0.02% CF (Sigma reference F-3543) and 1 mM HEPES (referred to as CF-LB or CF-M63 plates, respectively). The spotted drops were allowed to dry, and the plates were incubated for 24 to 48 h at 30 or 37°C. Red or pink colonies on CR plates indicated the binding of CR. Fluorescence of a colony under UV light indicated the binding of CF on CF-LB or CF-M63B1 plates.

Plasmid construction. pZE12-*yedQ* and pZE12-*adrA*, which encode YedQ and AdrA, respectively, placed under the control of a synthetic *lacp* promoter (34), were constructed as follows. The *yedQ* and *adrA* open reading frames were amplified from the *E. coli* 1094 chromosome and cloned into pZE12-*gfp* by replacing the *gfp* gene with *yedQ* or *adrA*. A similar procedure was used to clone the promoter region and the entire *adrA* gene from the *E. coli* 1094 chromosome into pZE12-*gfp*. The resulting plasmid, pZE1*Pr-adrA*, encodes *adrA* placed under the control of its own promoter. The second glycine and first glutamic acid from the GGEEF motif of YedQ were both mutated to alanine, leading to a GAAEF motif. First, we amplified from pZE12-*yedQ* the *yedQ* sequence between two FspI sites using the primer FspI-GAEEF-5, which contains two mismatches compared to the wild-type sequence of *yedQ* from *E. coli* 1094 and FspI-GAEEF-3. The amplified PCR products were then used to replace the wild-type GGEEF motif of YedQ with a GAAEF motif in pZE12-*yedQ*, leading to plasmid pZE12-$yedQ_{gaaef}$. DNA fragments corresponding to 247 bp preceding the ATG start codon of *yedQ* or *adrA* of *E. coli* strain 1094 were amplified, and the PCR products were cloned into pRS145 to create pRS*pyedQ*::*lacZ* and pRS*padrA*::*lacZ* plasmids, which carried a transcriptional fusion between the promoterless reporter gene *lacZ* and the *yedQ* or *adrA* promoter, respectively. The integrity of all the cloned fragments was verified by sequencing. The sequences of the primers used for plasmid construction can be found in Table S2 in the supplemental material.

Biofilm formation assay. All experiments were performed at least in triplicate in M63B1 minimal medium with 0.4% glucose at 37°C. Sixty-milliliter microfermentors containing a removable glass slide were configured as continuous-flow culture bioreactors with a 40-ml h^{-1} flow rate, which minimizes planktonic growth of the bacteria as described previously (26; http://www.pasteur.fr/recherche/unites/Ggb/matmet.html). The equivalent of 1 optical density at 600 nm (OD_{600}) unit from o/n bacterial cultures grown in 0.4% glucose M63B1 minimal medium supplemented with the appropriate antibiotics was used to inoculate the microfermentors. The bacteria were then cultivated for 24 h. Pictures of the microfermentors were taken before resuspension of the bacterial biofilm. The biomass was estimated by measuring the OD_{600} of the biofilm after resuspension in the microfermentor by vigorous shaking (the biofilm is weakly attached and easily detached from the surface) and further vortexing once the resuspended biofilm was transferred in a 15-ml tube.

Mutagenesis. CF-negative mutants were screened after mutagenesis with the ori6K plasmid pSC189 carrying the kanamycin-resistant Mariner transposon described previously (13). pSC189 was conjugated from strain S17-1 λpir (pSC189) into strain 1094*csgD*. The loss of CF-binding ability was screened under UV light at 30°C on CF-LB plates containing kanamycin (selection of the transposon insertion) and spectinomycin (selection for 1094*csgD*).

Multiplex PCR. Two PCRs were performed for each mutant: one used the primers pairs yhjL.ext5/yhjL.500-3 and cell.2-3/yhjO.ext5, leading to the amplification in the wild-type strain 1094 of 4,266-bp and 3,161-bp fragments, respectively, while the second PCR used the primers pairs cell. 1-3/cell.1-5, yhjO.int3/cell.3-5, and cell.4-3/cell.4-5, leading to the amplification of 3,180-bp, 1,692-bp, and 3,456-bp PCR products, respectively. The primer sequences are listed in Table S2 in the supplemental material.

Arbitrary PCR. To determine the transposon insertion sites in the studied mutants, an arbitrary PCR was used as described previously (14, 18). This method involves a first round of PCR using a primer specific for the right end of the transposon (IR2) and an arbitrary primer (ARB1 or ARB6). A second PCR is then performed on the product from the first PCR, using a primer specific for the rightmost end of the transposon (IR2-60-5) and a primer that is identical to the 5′ end of the arbitrary primer (ARB2). Arbitrary PCR primer sequences are listed in Table S2 in the supplemental material.

β-Galactosidase assays. To determine the β-galactosidase enzyme activities of strains carrying transcriptional *lacZ* fusions, pRSp*yedQ-lacZ* and pRSp*adrA-lacZ*, cultures were grown o/n (stationary-phase samples) or until the OD_{600} reached 0.3 to 0.5 (exponential-phase samples) in LB or M63B1-glu medium at 37°C or 30°C. To avoid formation of clumps in the cultures grown in M63B1 medium, 3 μg ml^{-1} of purified endo-β-1,4-glucanase from *Clostridium thermocellum* (30) was added to the medium. Biofilms were grown for 24 h in M63B1-glu plus 50 μg ml^{-1} ampicillin medium. The enzyme activity was assayed at least in triplicate for each strain under each condition as described previously (37).

Sequence analysis. Amino acid sequence comparisons were performed using the Clustal program package (28). Homology searches were performed using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?). DNA sequence manipulations were done with DNA Strider 1.3 (35).

RESULTS

Calcofluor binding is a widespread phenotype in natural *E. coli* isolates. In order to investigate the occurrence of cellulose,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
1094	*E. coli* commensal strain isolated from a healthy male adult; phylogenetic group A as detected by PCR	Gift; C. Le Bouguenec
1094*bcsC*	Δ*bcsC*::Km Km^r	This study
1094*bcsA-C*	Δ*bcsA-C*::Km Km^r	This study
1094*bcsEFG*	Δ*bcsE-G*::Km Km^r	This study
1094*csgD*	Δ*csgD*::*aadA* Spec^r	This study
1094*adrA*	Δ*adrA*::*aadA* Spec^r	This study
1094*mlrA*	Δ*mlrA*::*aac* Apra^r	This study
1094*yedQ*	Δ*yedQ*::*zeo* Zeocin^r	This study
1094*rpoS*	Δ*rpoS*::Km Km^r	This study
1094*yedQ-uidA*	*uidA* transcriptional fusion placed under the control of the *yedQ* promoter activity; Zeocin^r	This study
1094*csgD-pgi*	Δ*csgD*::*aadA*; Spec^r with a transposon inserted into *pgi*; Km^r	This study
1125	*E. coli* commensal strain	Gift; C. Le Bouguenec
1125*bcsC*	Δ*bcsC*::*aac* Apra^r	This study
1125*csgD*	Δ*csgD*::*aadA* Spec^r	This study
1125*yedQ*	Δ*yedQ*::*zeo* Zeocin^r	This study
1125*adrA*	Δ*adrA*::*aadA* Spec^r	This study
1125*yedQ adrA*	Δ*adrA*::*aadA* Spec^r Δ*yedQ*::*zeo* Zeocin^r	This study
DSM6601	*E. coli* commensal strain	Laboratory collection
DSM6601*bcsC*	Δ*bcsC*::Km Km^r	This study
DSM6601*csgD*	Δ*csgD*::*aadA* Spec^r	This study
DSM6601*yedQ*	Δ*yedQ*::*zeo* Zeocin^r	This study
55989	*E. coli* entero-aggregative pathogenic strain	Gift; C. Le Bouguenec
55989*bcsC*	Δ*bcsC*::Km Km^r	This study
55989*csgD*	Δ*csgD*::*aadA* Spec^r	This study
55989*yedQ*	Δ*yedQ*::*zeo* Zeocin^r	This study
55989*adrA*	Δ*adrA*::*aadA* Spec^r	This study
MG1655	λ$^-$ rph-1	Laboratory collection
MG1655*csgD*	Δ*csgD*::*aadA* Spec^r	This study
PHL818	MG1655*ompR234 malT*::Tn*10* Tet^r	43
PHL818*csgD*	Δ*csgD*::*aadA* Spec^r	This study
3934	*Salmonella* serovar Enteritidis clinical isolate	58
3934*adrA*	Δ*adrA*::Km	23
S17-1λpir	RP4-2Tc::mu km::Tn7 λpir; Pir-dependent replication	Laboratory collection
EcoR collection	*E. coli* strains listed in Table S1 in the supplemental material	41
Plasmids		
pSC189	Amp^r Km^r	13
pKOBEGA	Arabinose-inducible λ Red recombinase expression plasmid [*oriR101 repA101*(Ts) P_{araB}-*gam-bet-exo* Amp^r]	12
pKOBEG	Arabinose-inducible λ Red recombinase expression plasmid [*oriR101 repA101*(Ts) P_{araB}-*gam-bet-exo* Cm^r]	12
pRS415	Amp^r pMB1 ori; promoterless-*lacZ* plasmid	57
pRS*pyedQ*::*lacZ*	*lacZ* fusion placed under the transcriptional control of the *yedQ* promoter from strain 1094 in pRS415; pMB1 *ori*	This study
pRS*padrA*::*lacZ*	*lacZ* fusion placed under the transcriptional control of the *adrA* promoter from strain 1094 in pRS415; pMB1 *ori*	This study
pZE12-*gfp*	Green fluorescent protein placed under the control of the synthetic *lacp* promoter on the pZE vector used as a cloning vector; Amp^r ColE1 *ori*	Gift; C. C. Guet
pZE12-*yedQ*	*yedQ* of strain 1094 placed under the control of the synthetic *lacp* promoter on the pZE vector; Amp^r ColE1 *ori*	This study
pZE12-$yedQ_{gaaef}$	$yedQ_{gaaef}$ (mutation in the GGDEF domain) placed under the control of the synthetic *lacp* promoter on the pZE vector; Amp^r ColE1 *ori*	This study
pZE12-*adrA*	*adrA* of strain 1094 placed under the control of the synthetic *lacp* promoter on the pZE vector; Amp^r ColE1 *ori*	This study
pZE1*Pr-adrA*	*adrA* placed under the control of its own promoter on the pZE vector; Amp^r ColE1 *ori*	This study
pSTC14	*rpoS* placed under the control of the *lacp* promoter on the pACYC177 vector; Amp^r p15A *ori*	40

or cellulose-like, extracellular material production in *E. coli*, we analyzed the phenotypes of 87 natural isolates, including the pathogenic and commensal strains from human and animal sources of the EcoR reference collection (41). These strains were grown on agar plates containing CF, a fluorochrome that binds to (1-3)-β- and (1-4)-β-D-glucopyranosides, such as cellulose, chitin, and succinoglycans (65). Although *E. coli* K-12 strains (such as MG1655 and TG1) do not bind CF, CF binding

is a common phenotype in *E. coli* strains, since 47 (52.8%) of the tested strains displayed some fluorescence on CF agar plates under at least one of the tested conditions (rich or minimal medium; 30 or 37°C) (see Table S1 in the supplemental material). As extracellular cellulose synthesis plays an important role in biofilm formation in *Salmonella*, we investigated whether the biofilm phenotype could be correlated with the ability to bind CF in *E. coli*. Biofilm formation was monitored in continuous-flow culture microfermentors for 17 of the most CF-positive strains and 18 of the CF-negative strains. Eleven out of the 17 CF-positive strains showed a significant biofilm phenotype, and 8 out of 18 of the CF-negative strains showed no biofilm biomass after 48 h (see Table S1 in the supplemental material).

These results indicate that the ability to bind CF is a widespread phenotype among natural *E. coli* isolates. However, this phenotype does not strictly correlate with the capacity to form a biofilm under our experimental conditions.

Cellulose synthesis is necessary for *E. coli* 1094 biofilm formation. Although *E. coli* K-12 does not bind CF, it has the homologous genes of the two divergent operons, *bcsABZC* (formerly *yhjONML*) and *bcsEFG* (formerly *yhjSTU*), which are involved in the synthesis of cellulose in *Salmonella* (see Fig. 6) (58, 68). These genes could also be amplified by PCR in *E. coli* strain 1094, an *E. coli* commensal strain that exhibited strong fluorescence on CF agar plates, a typical rdar morphotype on CR plates, and a strong biofilm phenotype in a microfermentor under all tested conditions (LB; M63B1-glu; 30 or 37°C) (Fig. 1A and B). To test the roles of these genes in the different phenotypes displayed by strain 1094, we deleted *bcsC*, *bcsA-C*, or *bcsE-G* genes in *E. coli* 1094. These deletions abolished CF binding and led to smooth and red (sar) colonies on CR plates under all tested conditions (Fig. 1A and data not shown). Furthermore, the 1094*bcsC* mutant lost its ability to form biofilm in microfermentors (Fig. 1B).

E. coli 1094 also makes small clumps in liquid minimal medium, leading to rapid bacterial aggregation in standing tube cultures. Neither clump formation nor aggregation could be observed with the 1094*bcsC* mutant (see Fig. S1A in the supplemental material) or when either commercially available cellulase or purified endo-β-1,4-glucanase from *C. thermocellum* was added to a liquid culture of *E. coli* 1094 (see Fig. S1B in the supplemental material). Consistent with this, a mature biofilm of strain 1094 was completely disrupted after 16 h of incubation in a buffer containing commercial cellulase (see Fig. S1C in the supplemental material). However, the same treatment had no effect on the biofilm of *E. coli* K-12 TG1, a strain that forms a strong biofilm due to the expression of the F conjugative pilus and does not produce cellulose (see Fig. S1C in the supplemental material) (26).

Taken together, these results indicate that the extracellular material produced by *E. coli* 1094 is cellulose and that it depends on the expression of the *bcs* operons. Furthermore, cellulose production is absolutely required for the expression of mature biofilm and multicellular behavior in *E. coli* 1094.

CsgD does not regulate cellulose synthesis in *E. coli* 1094. In *Salmonella*, cellulose synthesis generally occurs at 28°C in LB medium and is regulated by CsgD via the MlrA-CsgD-AdrA pathway (hereafter referred to as the CsgD-dependent pathway) (see Fig. 6A). In *E. coli* 1094, however, cellulose production (determined by CF binding on agar plates) occurs under all tested conditions (30 and 37°C; LB; M63B1-glu), suggesting that the regulation of cellulose synthesis may be different in *Salmonella* and *E. coli* 1094. In order to test the contribution of CsgD to cellulose regulation in *E. coli* 1094, a 1094*csgD* mutant was grown on CF and CR agar plates. Surprisingly, 1094*csgD* displayed a wild-type fluorescent phenotype on both LB and minimal-medium agar plates containing CF at both 30°C and 37°C (Fig. 1A), suggesting that CsgD is not involved in the regulation of cellulose synthesis in *E. coli* 1094. Consistent with this, in *E. coli* 1094, the deletion of regulatory genes acting either upstream or downstream of *csgD* (*mlrA*/*yehV* and *adrA*/*yaiC*, respectively) led to the same phenotype as a *csgD* mutant on CF plates (Fig. 1A). Moreover, all three mutants exhibited a rough and dry morphotype on CR plates and an almost wild-type biofilm phenotype in microfermentors (Fig. 1A and B). As expected from the loss of curli fiber (which bind CR) production in both 1094*csgD* and 1094*mlrA* strains, colonies formed by these strains appeared pink instead of red on CR plates compared to the wild-type 1094 or the 1094*adrA* strain (Fig. 1A). This phenotype, comparable to the one displayed by a 1094*csgA* strain (Fig. 1A), is indicative of a reduced ability to bind CR. It also indicates that both the *mlrA* and *csgD* genes are expressed and functional in *E. coli* 1094.

These results demonstrate that *mlrA*, *csgD*, and *adrA* do not regulate cellulose production in *E. coli* 1094, suggesting the existence of an alternative and CsgD-independent cellulose regulation pathway in this strain.

RpoS participates in both CsgD-dependent and CsgD-independent cellulose pathways. In *Salmonella*, RpoS, the main sigma factor of the stationary growth phase, is involved in the CsgD-dependent cellulose regulation pathway via the transcriptional control of MlrA (11). Although neither *mlrA*, *csgD*, nor *adrA* mutants have any impact on cellulose production in *E. coli* 1094, the deletion of the *rpoS* gene in *E. coli* 1094 led to a CF-negative phenotype on LB medium at both 30 and 37°C, as well as a smooth and pink (sap) morphotype on CR plates (Fig. 1A). In minimal medium, *E. coli* 1094*rpoS* still displayed a low and inconsistent CF-positive phenotype associated with a reduced but significant biofilm formation phenotype (Fig. 1B). The introduction of a wild-type *rpoS* allele on the plasmid pSTC14 complemented both the CF-negative phenotype and the sap morphotype (data not shown). Since the pink phenotype is indicative of a lack of curli expression, these results indicate that, in LB medium, RpoS both regulates curli expression and contributes to the CsgD-independent cellulose synthesis regulation pathway. Therefore, although cellulose synthesis in *E. coli* 1094 is independent of CsgD, it still depends on RpoS.

Screening for *E. coli* 1094 mutants impaired in cellulose production. In order to identify the genetic determinants of the production of cellulose in *E. coli* 1094, we performed a Mariner transposon mutagenesis on 1094*csgD*. We screened for CF-negative mutants on CF-LB agar plates at 30°C and identified 17 independent nonfluorescent mutants out of 7,000 clones.

To directly identify expected CF-negative mutants due to the insertion of the transposon in the cellulose genes, we performed a multiplex PCR on the two divergent *bcsABZC* and *bcsEFG* cellulose operons. A transposon mutant exhibiting a

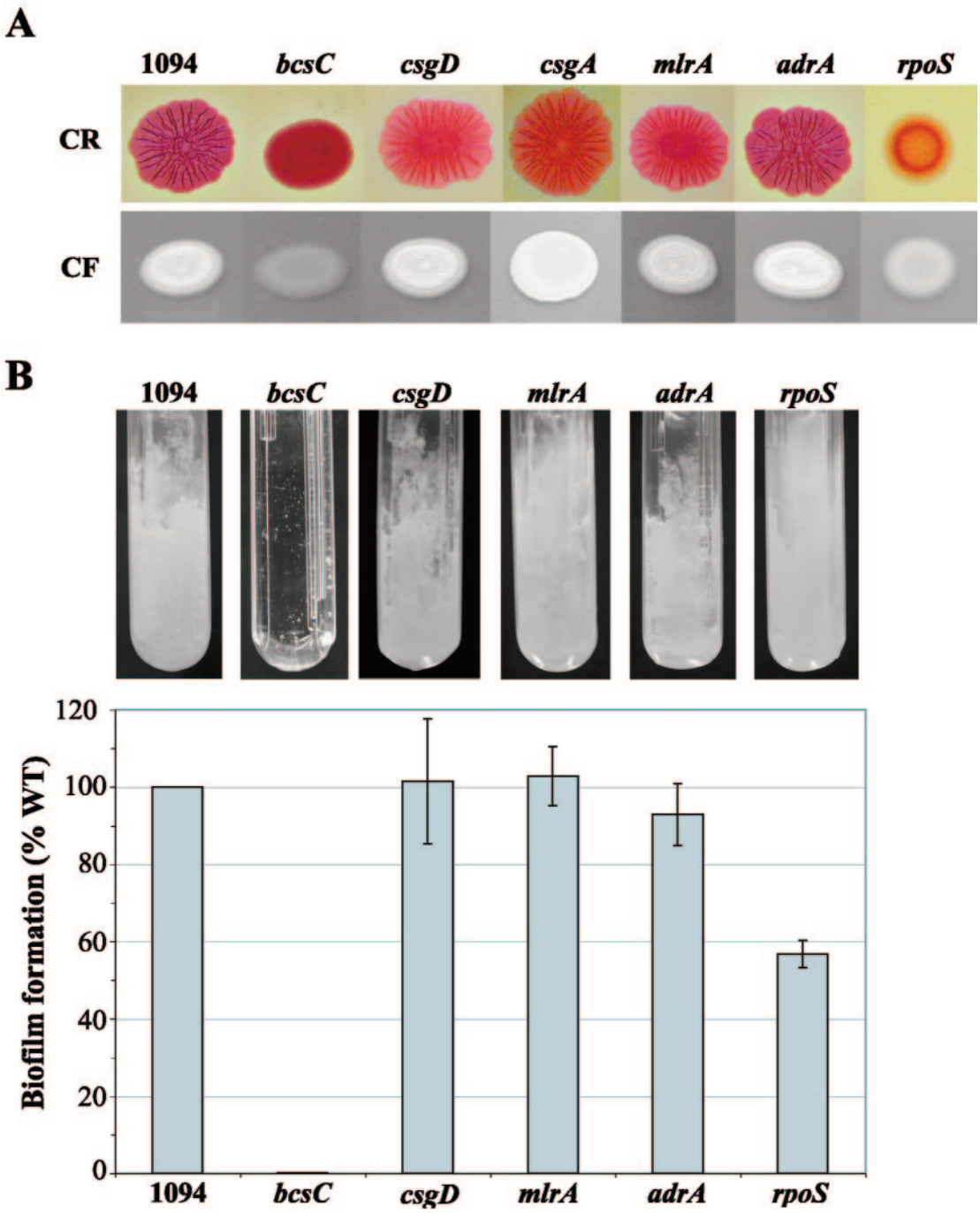

FIG. 1. Cellulose production and biofilm formation in *E. coli* strain 1094. (A) CR- and CF-binding phenotypes of strains 1094, 1094*bcsC*, 1094*csgD*, 1094*csgA*, 1094*mlrA*, 1094*adrA*, and 1094*rpoS*. Two microliters of an o/n culture was spotted onto CR-LB plates (upper row) and CF-LB plates (lower row) and incubated for 48 h at 30°C (CR) or 37°C (CF). (B) Biofilm formation in microfermentors. The biofilms were grown for 24 h at 37°C in M63B1-glu. The biofilm formation ability of the mutant strains is expressed as a percentage of the 1094 wild-type biofilm, set to 100%. The error bars represent standard errors of the means.

non-wild-type multiplex PCR profile indicates that, in this mutant, the transposon was inserted in one of the cellulose genes. We found that 11 out of 17 independent mutations were indeed located in the *bcsABZC* operon and 1 in the *bcsEFG* operon. These mutants displayed a CF-negative phenotype and a white and smooth phenotype on Congo red plates (data not shown). The natures of the five remaining CF-negative mutants carrying a transposon insertion located outside of the cellulose operons were determined by sequencing the regions adjacent to the transposon insertion site. The five mutants corresponded to insertions in four different loci belonging to three functional classes and are presented in Table 2.

TABLE 2. Phenotypes and molecular analysis of *E. coli* 1094*csgD* CF-binding-deficient mutants

COG group or strain	Insertion site[a]	Gene	BLAST description	Phenotype[e]						
				M63B1-glu + CF[b]		LB + CF[b]		LB + CR[c]		Biofilm[d]
				30°C	37°C	30°C	37°C	30°C	37°C	
Information storage/processing	2865100	*rpoS*	RNA polymerase sigma factor	+/−	+/−	−	−	sap	sap	+
Metabolism	4232307	*pgi*	Phosphoglucose isomerase	+++	+++	−	−	sar	sar	ND
	1291485	*galU*	UDP-glucose-1-P uridylyltransferase	−	−	−	−	sar	sar	−
	1290696	*galU*	UDP-glucose-1-P uridylyltransferase	−	−	−	−	sar	sar	−
Cellular processes	2024925	*yedQ*	Unknown	−	−	−	−	cdar	cdar	−
E. coli 1094*csgD*				+++	+++	+++	+++	rdar	rdar	++

[a] Insertion site in bp compared to MG1655 sequence.
[b] Fluorescence under UV light was examined after growth on minimal (M63B1-glu) or rich (LB) agar plates containing CF at 30°C or 37°C for 48 h. The intensity of the fluorescence was estimated visually. The highest fluorescence is expressed as +++; no fluorescence as −.
[c] Morphotype on LB-CR plates without NaCl. cdar, circular, dry, and red.
[d] Biofilm in microfermentor at 37°C in M63B1-glu medium. The biofilm was grown for 24 hours. Strong biofilm, ++; medium biofilm, +; poor biofilm, −; ND, not determined.
[e] Phenotypes compared to 1094*csgD* levels.

Unsurprisingly, one mutant exhibiting a CF-negative phenotype on LB medium was identified in the *rpoS* gene. Two insertions were in *galU* (encoding a glucose-1-P-uridylyltransferase that catalyzes the conversion of glucose-1-P into UDP-glucose [UDP-Glc]) and one in *pgi* (encoding a phosphoglucose isomerase that catalyzes the interconversion of glucose-6-P and fructose-6-P). These mutants are involved in the synthesis of UDP-Glc, the building block of the cellulose polymer. The *pgi* mutant led to a CF-binding-deficient mutant on CF-LB plates but displayed a highly CF-positive phenotype on CF-M63-glucose plates. The latter phenotype is probably due to enhanced production of UDP-Glc from the conversion of glucose-6-P into glucose-1-P by the phosphoglucomutase Pgm. This correlates with the production of cellulase-sensitive bacterial aggregates when the *pgi* insertion mutant is grown in liquid M63B1-glucose culture, suggesting that it overproduces cellulose (see Fig. S1B in the supplemental material).

Finally, one insertion was found in the *yedQ* gene. This uncharacterized gene (1,695 bp) is predicted to encode a putative protein of 564 amino acids (aa) (64.11 kDa) with an unknown function. Domain analysis using the protein family (Pfam) database indicated the presence of a signal peptide (aa 1 to 45), two potential transmembrane domains (aa 20 to 42 and 358 to 380), and, most interestingly, a carboxy-terminal GGDEF domain (aa 394 to 557), shared by proteins that display proven or suspected c-di-GMP synthase activity, such as AdrA in *Salmonella* (Fig. 2A) or PleD of *Caulobacter crescentus* (42, 56). Interestingly, *yedQ* is highly similar (64.7% amino acid

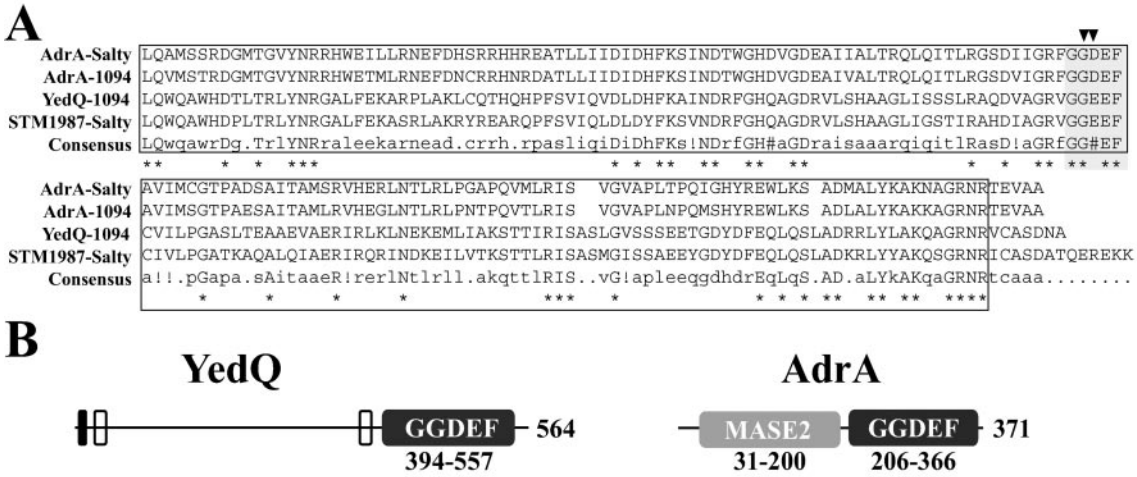

FIG. 2. YedQ contains a GGDEF domain. (A) Sequence alignment of the GGDEF domains of AdrA-Salty and AdrA-1094 from *Salmonella* and *E. coli* 1094, respectively, YedQ-1094 from *E. coli* 1094, and STM1987-Salty (GcpA) from *Salmonella*. Stars under the sequences identify residues conserved among all the aligned sequences. The GGDEF domain is boxed, and the GGDEF motif is shaded. The two black arrowheads show the positions of the mutated residues in the $YedQ_{gaaef}$ mutant. The alignment was produced using the program Multalin version 5.4.1 (15). # indicates conservation of a charged amino acid; ! indicates conservation of hydrophobic amino acids. (B) Schematic domain structures of YedQ and AdrA proteins from domain analysis using the Pfam database. Black box, signal sequence; white boxes, potential transmembrane domains; dark-gray boxes, GGDEF domains; light-gray box, MASE2 domain. The size of the protein is indicated in residue numbers; the sizes of the GGDEF and MASE2 domains are indicated under the domains by residue numbers in the protein.

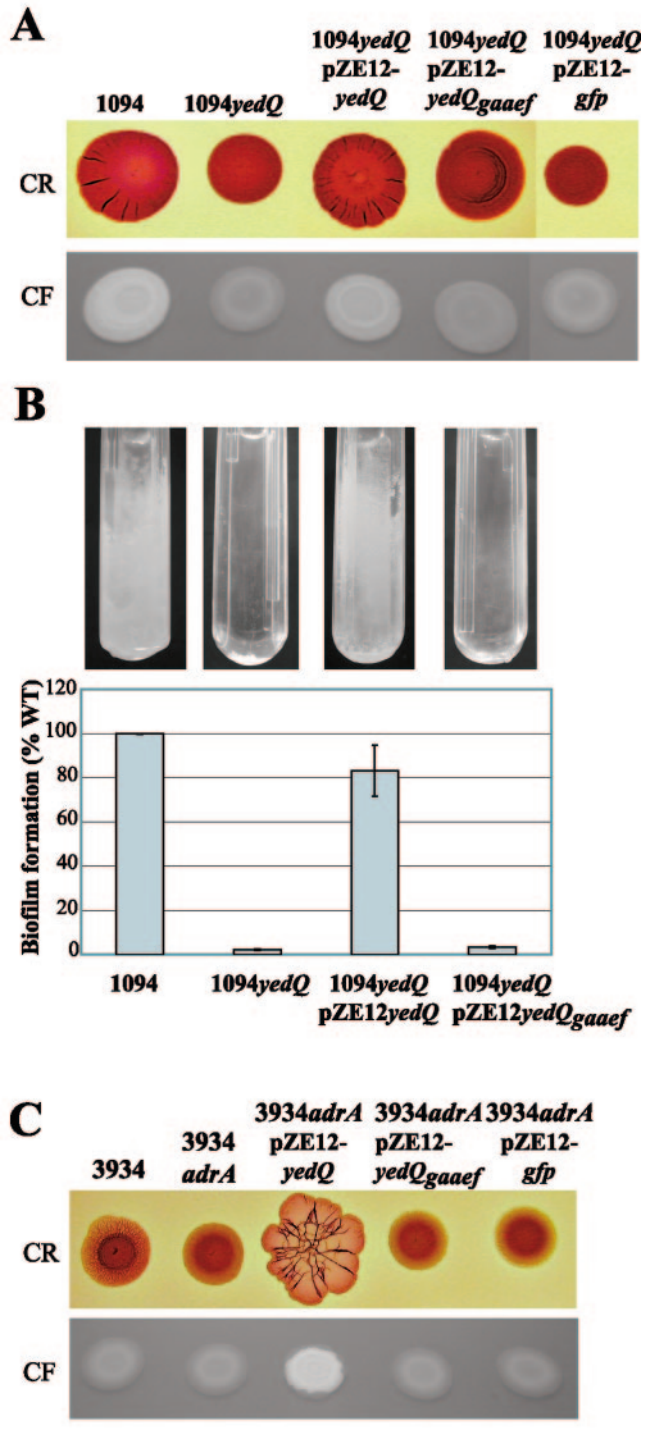

FIG. 3. YedQ is the regulator for cellulose production in *E. coli* 1094. (A) CR- and CF-binding phenotypes of strains 1094, 1094*yedQ*, 1094*yedQ*(pZE12-*yedQ*), 1094*yedQ*(pZE12-$yedQ_{gaaef}$), and 1094*yedQ* (pZE12-*gfp*). Two microliters of an o/n culture was spotted onto CR-LB (upper row) and CF-LB (lower row) plates and incubated for 24 h at 30°C. Similar phenotypes were obtained at 37°C and on CF-M63B1 plates. (B) Biofilm formation in microfermentors. The biofilms were grown for 24 h at 37°C in M63B1-glu. The biofilm formation ability of 1094*yedQ* and complemented mutants is expressed as a percentage of the 1094 wild-type biofilm, set to 100%. The error bars represent standard errors of the means. (C) Complementation of the CR- and CF-binding phenotypes of the *Salmonella* serovar Enteritidis *adrA* (3934*adrA*) mutant by pZE12-*yedQ*. Two microliters of o/n cultures was spotted onto CR-LB (upper row) and CF-LB (lower row) plates and incubated for 24 h at 30°C.

identity) to GcpA (STM1987), a regulator of cellulose synthesis in minimal media at 37°C identified recently in *Salmonella* serovar Typhimurium (23).

YedQ is a key GGDEF regulator of cellulose synthesis in *E. coli* 1094. In order to confirm the role of YedQ in cellulose regulation, we performed the deletion of the *yedQ* gene in *E. coli* 1094. 1094*yedQ* displayed a CF-negative phenotype on LB and M63-CF plates at both 30 and 37°C (Fig. 3A) and was also severely impaired in biofilm formation in microfermentors (Fig. 3B). The *yedQ* knockout in *E. coli* 1094 was successfully complemented by the introduction of plasmid-borne *yedQ* under the control of an inducible *lac* promoter (pZE12-*yedQ*) but not by the parental vector (Fig. 3A). These results demonstrate that YedQ regulates the production of cellulose under all tested conditions in strain *E. coli* 1094.

The presence of a GGEEF motif in the C-terminal part of YedQ (Fig. 2) is strongly indicative of c-di-GMP synthase activity, common to known regulators of cellulose synthesis (50). This suggested that the motif might be critical for YedQ function. To test this hypothesis, the *yedQ* GGEEF motif was mutagenized to GAAEF in pZE12-*yedQ*. When introduced into *E. coli* 1094*yedQ*, the resulting plasmid-borne $yedQ_{gaaef}$ failed to restore any of the CF- or CR-binding and biofilm formation phenotypes (Fig. 3A and B). These results indicate that the YedQ GGEEF motif is essential for the function of the protein. Moreover, pZE12-*yedQ*, but not pZE12-$yedQ_{gaaef}$, could complement the rdar and CF-binding defects of a *Salmonella* serovar Enteritidis *adrA* mutant (Fig. 3C), suggesting that, like *Salmonella* AdrA, YedQ has c-di-GMP synthase activity.

Altogether, these results indicate that YedQ is a new GGDEF regulator of cellulose synthesis in *E. coli*.

RpoS does not regulate *yedQ* expression in *E. coli* 1094. Because of the functional similarity between YedQ and AdrA, we tested whether they could share an RpoS transcriptional control and if the CF- and CR-negative phenotypes displayed by 1094*rpoS* in LB could be due to the lack of *yedQ* expression. Neither CF nor CR binding could be restored in 1094*rpoS* (pZE12*yedQ*) in LB medium, and the introduction of *yedQ* in a 1094*rpoS* strain led to irregular levels of CF binding in minimal medium (data not shown).

To further study the regulation of *yedQ* transcription, the activity of a plasmid-borne *yedQ-lacZ* transcriptional fusion (pRSp*yedQ*::*lacZ*) introduced in either *E. coli* 1094 or 1094*rpoS* was tested under various conditions, including 30°C and 37°C and exponential and stationary phases in liquid rich and minimal media, as well as biofilm. These experiments showed that the transcription of *yedQ* is only moderately affected by the tested environmental conditions (twofold increase in minimal medium at stationary phase compared to LB) (Fig. 4A; see also Fig. S2A and B in the supplemental material). Moreover, the *rpoS* mutation did not significantly affect the expression of the *yedQ-lacZ* fusion (Fig. 4A; see also Fig. S2A and B in the supplemental material). Therefore, in

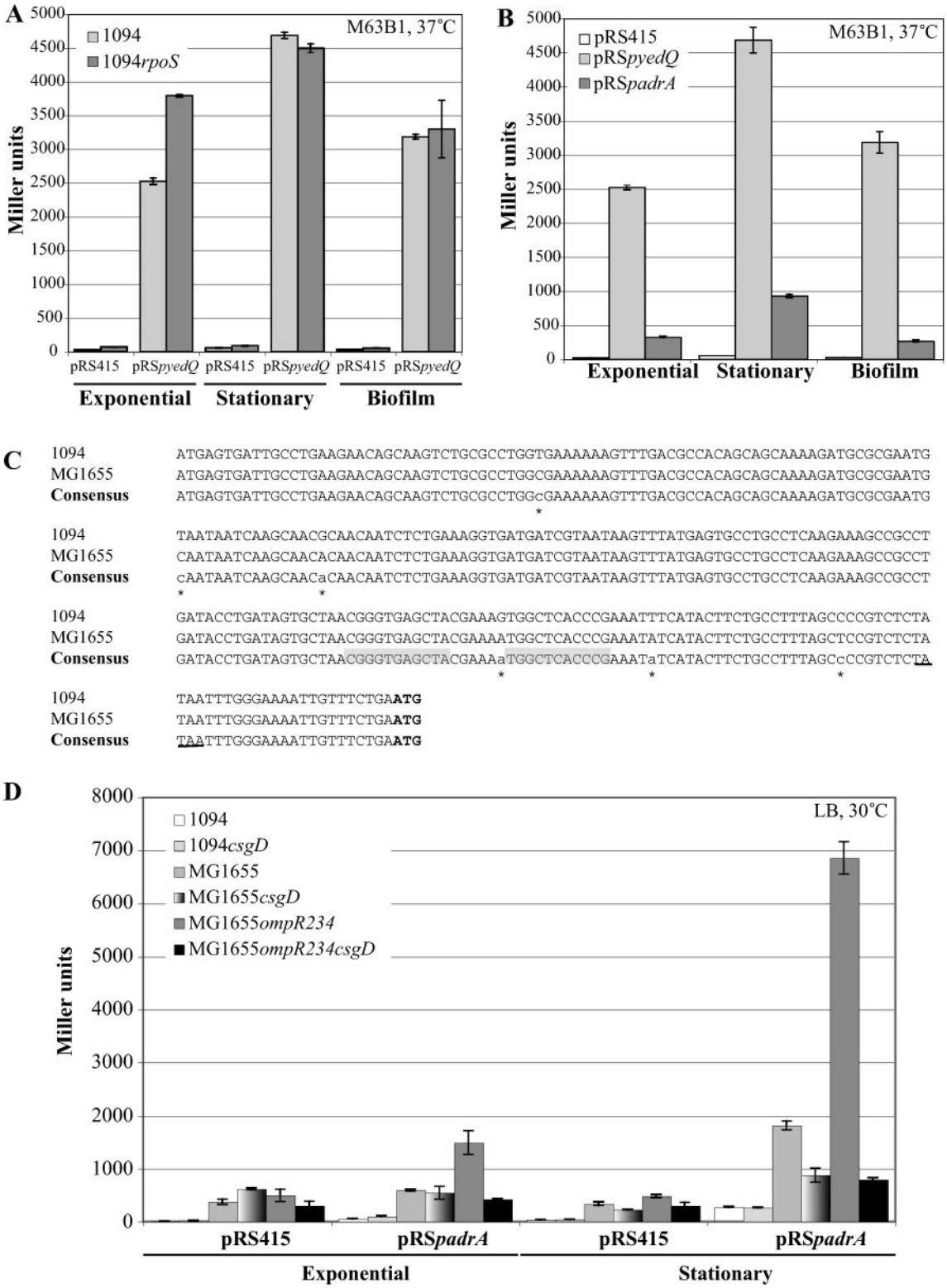

FIG. 4. Transcriptional regulation of *yedQ* and *adrA* in strain 1094. (A) *yedQ* expression was studied in wild-type *E. coli* 1094 and the 1094*rpoS* mutant by measuring the β-galactosidase activity of a *yedQ*::*lacZ* fusion carried on the pRSp*yedQ*::*lacZ* plasmid. The cloning vector pRS415 was used as a negative control in both strains. Light-gray bars, β-galactosidase activity in 1094; dark-gray bars, β-galactosidase activity in 1094*rpoS*. The error bars represent standard errors of the means. (B) *yedQ* and *adrA* expression levels were compared in strain *E. coli* 1094 by measuring the β-galactosidase activities of a *yedQ*::*lacZ* and an *adrA*::*lacZ* fusion carried, respectively, on the pRSp*yedQ*::*lacZ* and pRSp*adrA*::*lacZ* plasmids. The cloning vector pRS415 was used as a negative control. White bars, pRS415 β-galactosidase activity; light-gray bars, pRS*pyedQ*::*lacZ*

contrast to *Salmonella adrA* expression, neither RpoS nor CsgD directly regulates the expression of *yedQ*. In an attempt to identify regulators of *yedQ* activity, we also mutagenized the strain 1094Δ*yedQ*::*uidA*. This strain carries a chromosomal p*yedQ-uidA* (β-glucuronidase) transcriptional fusion and is pale blue to white on LB plates containing 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (a chromogenic substrate for UidA) using the plasmid pSC189 bearing a Mariner-based transposon. Since the extremities of the transposon have strong constitutive promoter-out activity (A. Roux and J. M. Ghigo, unpublished data), this mutagenesis allowed us to screen both for the inactivation of *yedQ* repressor and for the promoter-out-mediated activation of *yedQ* activators by looking for blue colonies due to p*yedQ* derepression or activation on LB–5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid at 30°C. This new screen failed to identify any regulators of *yedQ* expression. Altogether, these results suggest that *yedQ* is constitutively expressed under all tested conditions in *E. coli* 1094.

YedQ and AdrA are both potentially functional in *E. coli* 1094. We hypothesized that the reason why *E. coli* 1094 uses YedQ rather than AdrA to regulate cellulose production could be either a lack of expression or functionality of AdrA or because YedQ fulfills specific functions in *E. coli* 1094. To investigate this, we first tested the expression and functionality of the *E. coli* 1094 *adrA* gene by cloning it into pZE1*Pr-adrA* (expressing *adrA* under the control of its own promoter) and pZE12-*adrA* (expressing *adrA* under the control of an inducible *lac* promoter). These two constructs were tested in complementation experiments with the CF-negative *Salmonella* serovar Enteritidis *adrA* mutant as a heterologous host. As shown in Table 3, $adrA_{1094}$ expressed from both plasmids could restore the CF phenotype of *Salmonella* serovar Enteritidis *adrA*, showing that both the $adrA_{1094}$ promoter and the protein $AdrA_{1094}$ are functional. $AdrA_{1094}$ is likely to display a diguanylate synthase activity in this strain.

Therefore, we tested whether $adrA_{1094}$ could restore the ability of *E. coli* 1094*yedQ* to bind CF and CR. Both pZE12-*adrA* and pZE1*Pr-adrA* complemented the *yedQ* defect, showing that AdrA is also functional in 1094 when expressed from a multicopy plasmid (Table 3).

We then compared the expression levels of *yedQ* and *adrA* in *E. coli* 1094 using a plasmid-borne transcriptional *lacZ* fusion to either the *yedQ* or the *adrA* promoter (pRSp*yedQ*::*lacZ* and pRSp*adrA*::*lacZ*, respectively). These comparisons were performed in minimal or rich medium at 30°C or 37°C in exponential- and stationary-phase cultures or biofilm. In strain 1094, *adrA* is 5- to 16-fold less expressed than *yedQ*, depending on the environmental conditions (Fig. 4B; see also Fig. S2C and D in the supplemental material).

We did not observe a decrease in the expression of the *padrA-lacZ* fusion in the 1094*csgD* mutant, suggesting that under these conditions, *adrA* expression does not depend on CsgD (Fig. 4D). Since *csgD* is expressed and functional in *E. coli* 1094 (see above), the low level of $adrA_{1094}$ expression could result from the inability of its promoter to be recognized and activated by CsgD. The comparison of the *adrA* promoter sequences of *E. coli* strains MG1655 and 1094 showed only a few differences (Fig. 4C). To assess if these differences could affect the capacity of the $adrA_{1094}$ promoter to be activated by CsgD, we introduced the *adrA-lacZ* fusion in the strains MG1655 and MG1655*ompR234* (which up-regulates *csgD*) and their respective *csgD* mutants (10). As shown in Fig. 4D, *adrA* expression was reduced in an MG1655*csgD* mutant but was significantly increased by the *ompR234* mutation in MG1655 (Fig. 4D). The $adrA_{1094}$-*lacZ* fusion activity was also severely reduced in the double *ompR234-csgD* mutant (Fig. 4D), showing that the *adrA* promoter of strain 1094 can be recognized and activated by the CsgD protein of MG1655.

Taken together, these results demonstrated that AdrA is potentially functional in strain 1094. Moreover, although it can be activated by CsgD in *E. coli* MG1655, the $adrA_{1094}$ promoter is not regulated by CsgD in *E. coli* 1094, and its expression level is low compared to YedQ expression.

CsgD-independent cellulose production is found in several natural *E. coli* isolates. To investigate whether *E. coli* isolates other than 1094 may also display a CsgD-independent cellulose regulation pathway, we studied three other CF-positive *E. coli* strains from our collection: strains 1125, DSM6601, and 55989 (Table 1; see also Table S1 in the supplemental material). These three strains appear to produce cellulose, as a deletion

TABLE 3. Complementation by AdrA

Strain	Phenotype/morphotype					
	M63B1-glu + CF[a]		LB + CF[a]		LB − NaCl + CR[b]	
	30°C	37°C	30°C	37°C	30°C	37°C
1094	+++	+++	+++	+++	rdar	rdar
1094*yedQ*	−	−	−	+/−	cdar	cdar
1094*yedQ*(pZE1*Pr-adrA*)[c]	+++	+++	++	++	rdar	rdar
1094*yedQ*(pZE12-*adrA*)[c]	+++	+++	++	++	rdar	rdar
3934[d]	+/−		+/−		rdar	
3934*adrA*	−		−		sar	
3934*adrA*(pZE1*Pr-adrA*)	+++		+++		rdar	
3934*adrA*(pZE12-*adrA*)	+++		+++		rdar	

[a] Fluorescence under UV light was examined visually after growth on minimal or rich medium agar plates containing CF at 30°C or 37°C for 48 h. The highest fluorescence is expressed as +++; no fluorescence as −.

[b] Morphotype on CR plates. cdar, circular, dry and red.

[c] pZE1*Pr-adrA*, expression of $adrA_{1094}$ under the control of its own promoter; pZE12-*adrA*, expression of $adrA_{1094}$ under the control of the synthetic *lac* promoter.

[d] *Salmonella* serovar Enteritidis strain 3934.

β-galactosidase activity; dark-gray bars, pRS*padrA*::*lacZ* β-galactosidase activity. (C) Sequence alignment of the *adrA* promoters of *E. coli* strains 1094 and MG1655. The putative CsgD-binding sequences are shaded, and the −10 box is underlined on the consensus sequence. The sequence differences among *E. coli* strains are indicated by stars. The ATG start codon is in boldface letters. (D) $adrA_{1094}$ expression was studied in the wild-type *E. coli* strains 1094, MG1655, and MG1655*ompR234* (PHL818) and their respective *csgD* mutants by measuring the β-galactosidase activity of an *adrA*::*lacZ* fusion carried on the pRS*padrA*::*lacZ* plasmid. The bar shades for β-galactosidase activities in the different strains are as indicated in the graph legend. All the measurements were done at least in triplicate at 37°C in M63B1 medium supplemented with 0.4% glucose (A and B) or at 30°C in LB medium (D).

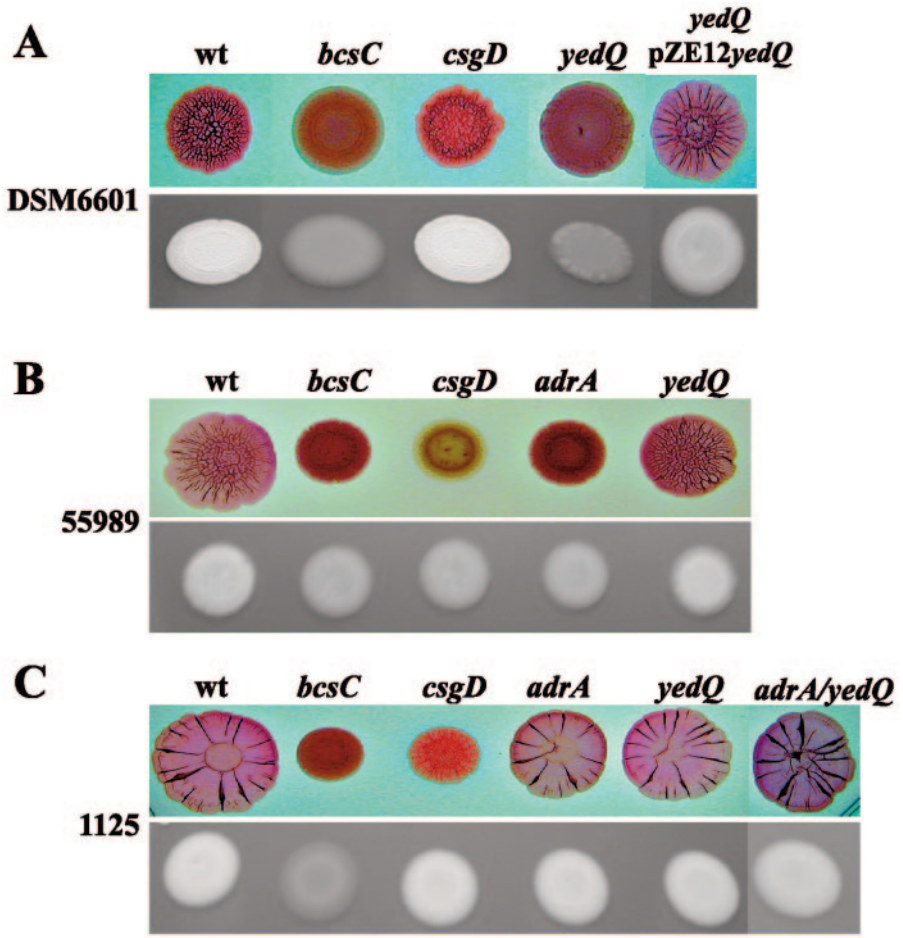

FIG. 5. CsgD-independent and -dependent cellulose synthesis regulation in *E. coli* strains. CR-binding (upper rows) and CF-binding (lower rows) phenotypes of the wild type and deletion mutants of strains DSM6601 (A), 55989 (B), and 1125 (C). Two microliters of an o/n culture was spotted onto CR-LB and CF-LB plates and incubated for 48 h at 30°C.

of the *bcsC* gene led to a CF-negative phenotype on CF agar plates and a sar phenotype on CR plates (Fig. 5).

We first introduced a *csgD* mutation in the 1125, DSM6601, and 55989 strains and tested the abilities of the mutant strains to bind CF and CR. As shown in Fig. 5, only 55989*csgD* displayed a CF-negative phenotype and completely lost its ability to bind CR. Moreover, the CF-negative phenotype and sar morphotype exhibited by the 55989*adrA* mutant indicated that 55989 shows a CsgD/AdrA-dependent cellulose synthesis regulation (Fig. 5B). By contrast, DSM6601*csgD* and 1125*csgD* remained CF positive and exhibited the rough morphotype on CR-LB plates, suggesting that their cellulose regulation pathway could be YedQ dependent. We therefore introduced a *yedQ* mutation in the 1125, DSM6601, and 55989 strains and tested the abilities of these mutants to bind CF and CR. As expected from a CsgD-dependent strain, 55989*yedQ* remained CF positive and exhibited the rdar morphotype on CR-LB plates. The CsgD-independent strain, DSM6601*yedQ*, however, lost its ability to bind CF and exhibited a smooth morphotype. This defect could be complemented by the introduction of pZE12-*yedQ* (Fig. 5A).

Interestingly, 1125*yedQ* remained CF positive and exhibited an rdar morphotype on CR-LB plates (Fig. 5C). To exclude the possibility that AdrA could regulate cellulose synthesis independently of CsgD in this strain, the *adrA* mutation was introduced in 1125. This mutant displayed wild-type phenotypes on CF and CR plates (Fig. 5C). Finally, in order to check that the CF-positive phenotype exhibited by either the 1125*adrA* or 1125*yedQ* mutant did not result from a cross-complementation by *yedQ* or *adrA*, respectively, the double mutant 1125*adrA-yedQ* was created. It exhibited the same phenotypes as the wild type (Fig. 5C).

These results demonstrate that CsgD-independent and YedQ-dependent cellulose synthesis is not restricted to the *E. coli* 1094 strain and also operates in DSM6601. Moreover, since neither YedQ, CsgD, nor AdrA is required to bind CF or CR in *E. coli* 1125, this suggests that the regulation of the cellulose production in this strain uses a different genetic pathway, possibly involving one of the 17 other GGDEF proteins encoded by genes identified in the *E. coli* genome (21).

DISCUSSION

The production of polysaccharide polymers in the extracellular biofilm matrix is recognized as central in the development of bacterial biofilms (9). Among the identified polysaccharide

components of the biofilm matrix, cellulose, a β-1-4-D-glucose polymer, has been shown to contribute to the formation of biofilm in *Salmonella* and *Pseudomonas* by promoting cell contacts at both liquid and solid interfaces (58, 59, 68).

Although laboratory *E. coli* K-12 derivative strains do not produce cellulose, the polymer has been detected in a few natural *E. coli* isolates (68). Our analysis of a collection of 87 *E. coli* strains that did not undergo significant laboratory subcultivation showed that cellulose production is a common phenotype in *E. coli*. This trait was not, however, strictly associated with strong biofilm formation capacities, suggesting that cellulose synthesis is a biofilm determinant of some but not all *E. coli* strains. Nevertheless, as CF is also a nonspecific indicator of chitin and succinoglycan polymers, we cannot exclude the possibility that some of these biofilm-negative but CF-positive strains do not produce bona fide cellulose, a hypothesis that could be tested by the systematic introduction of mutations in the cellulose operons of these strains. We also observed that half of the non-CF-binding strains that were assessed for biofilm formation were indeed able to form a biofilm. This suggests that, in these strains, other proteinaceous components (such as pili or curli) or other exopolysaccharides, such as colanic acid or poly-1,6-GlcNAc (PGA), could be involved in biofilm formation (4, 16, 44, 64).

To investigate the link between cellulose production and biofilm formation in *E. coli*, the commensal *E. coli* strain 1094, which exhibits both rdar and strong CF-binding phenotypes and forms a biofilm, was studied in more detail. As observed with *Salmonella* (23, 48, 58, 68), cellulose is required for biofilm formation in *E. coli* strain 1094. However, unlike in *Salmonella*, curli production is not necessary for the expression of a mature biofilm and multicellular behavior. Indeed, whereas the deletion of the cellulose operons abolished these phenotypes, the 1094*csgD* or 1094*csgA* mutant behaved like the wild-type strain.

Considering the conservation of the genes involved in cellulose production and regulation in *Salmonella* and *E. coli*, it was reasonable to assume that CsgD and AdrA could play roles in cellulose production in *E. coli* (46). Indeed, during the course of this work, a study of cellulose and curli expression in *E. coli* showed that a representative fecal isolate of *E. coli* regulates cellulose synthesis via CsgD (5). However, the analysis of the genetic basis of cellulose synthesis in the commensal *E. coli* strain 1094 allowed us to show that neither CsgD, AdrA, nor MlrA is involved in this process in *E. coli* 1094.

Using a genetic screen based on the ability of the cellulose-producing strain 1094*csgD* to bind the fluorescent dye CF, we identified five mutants defective for CF binding located outside of the cellulose operons. Three of these transposition mutants corresponded to insertions in the glucose-1-P-uridylyltransferase (*galU*) and phosphoglucose isomerase (*pgi*) genes. Because these enzymes play a direct or indirect role in the synthesis of UDP-glucose, the sugar subunit of cellulose, both *galU* and *pgi* are likely to have an effect on cellulose synthesis per se, but not on its regulation. *galU* has been shown to be necessary for biofilm formation and biosynthesis of the exopolysaccharide of a phage-resistant rugose variant of *Vibrio cholerae* and to affect *E. coli* early adhesion (24, 38). A *pgi* mutant has been isolated from a mutagenesis screen performed on a CF-positive *Salmonella* serovar Enteritidis strain, along with other genes involved in different sugar metabolic pathways (58).

One of the five CF-negative mutants identified in our screen corresponds to an insertion in the *yedQ* gene. The protein with an unknown function, YedQ, is predicted to be a membrane-associated protein that contains a GGDEF (DUF1) domain. Proteins displaying a GGDEF domain are widespread, are present in most bacterial genomes, and form a large family of proteins that have been associated with diguanylate cyclase activity and c-di-GMP production (22, 29). The discovery of c-di-GMP and the control of its intracellular level by proteins with diguanylate cyclase (GGDEF domain proteins) and/or phosphodiesterase (EAL domain proteins) activities originated from work on cellulose production in *G. xylinus* (52, 63). In *Salmonella*, the GGDEF domain protein AdrA has been shown to be involved in cellulose synthesis regulation and its c-di-GMP synthase activity has been recently demonstrated (45, 56). Although most of the GGDEF domain proteins have not yet been experimentally characterized, it is now considered that c-di-GMP, in addition to its role as an allosteric activator of cellulose synthesis, is a second messenger involved in a number of cellular and behavioral functions, such as the transition from sessility to motility, cell cycle-dependent localization of proteins, and cellulose and biofilm formation (3, 6, 22, 23, 29, 33, 42, 45, 50, 51, 56, 58).

The construction of a null *yedQ* mutant in strain 1094 and its plasmid complementation confirmed that, under all tested conditions, YedQ is required for cellulose synthesis and biofilm formation in this strain. A site-directed mutagenesis in the GGEEF motif of YedQ showed that this motif is essential for cellulose synthesis in *E. coli* 1094, as well as for $YedQ_{1094}$ to complement an AdrA defect in *Salmonella* serovar Enteritidis. Altogether, these results suggest that, like AdrA in *Salmonella*, YedQ contains a functional GGDEF domain and that we have identified a new cellulose regulatory protein in *E. coli* 1094 that is probably involved in the regulation of the level of intracellular c-di-GMP through its GGDEF domain activity (56). During the course of this study, *gcpA* (STM1987), a gene encoding a GGDEF protein sharing 64.7% identity with YedQ, was shown to be critical for cellulose and biofilm formation in *Salmonella* serovar Enteritidis (23). However, unlike YedQ, which is required for *E. coli* 1094 cellulose synthesis in rich and minimal media at both 30 and 37°C, in *Salmonella* serovar Enteritidis, GcpA regulates cellulose production under specific culture conditions in a CsgD-independent manner. Indeed, while in *Salmonella* serovar Enteritidis cellulose production depends on CsgD and AdrA in LB medium at 28°C, it depends only on GcpA in ATM at 37°C (23). These differences suggest that *yedQ* and *gcpA* may not be similarly regulated in *E. coli* 1094 and *Salmonella* serovar Enteritidis, as suggested by the differences observed between the two promoters. Therefore, while the roles of YedQ/GcpA could be different in *E. coli* and *Salmonella* serovar Enteritidis, they are both involved in CsgD- and AdrA-independent regulation of cellulose production.

Our finding that an *rpoS* insertion mutant leads to a CF-negative phenotype in *E. coli* 1094 suggested that the CsgD-dependent and -independent pathways, depicted in Fig. 6, may share a common root, i.e., that like *adrA*, *yedQ* expression could also be regulated by RpoS. However, the expression of an inducible overexpressed copy of *yedQ* in a 1094*rpoS* mutant

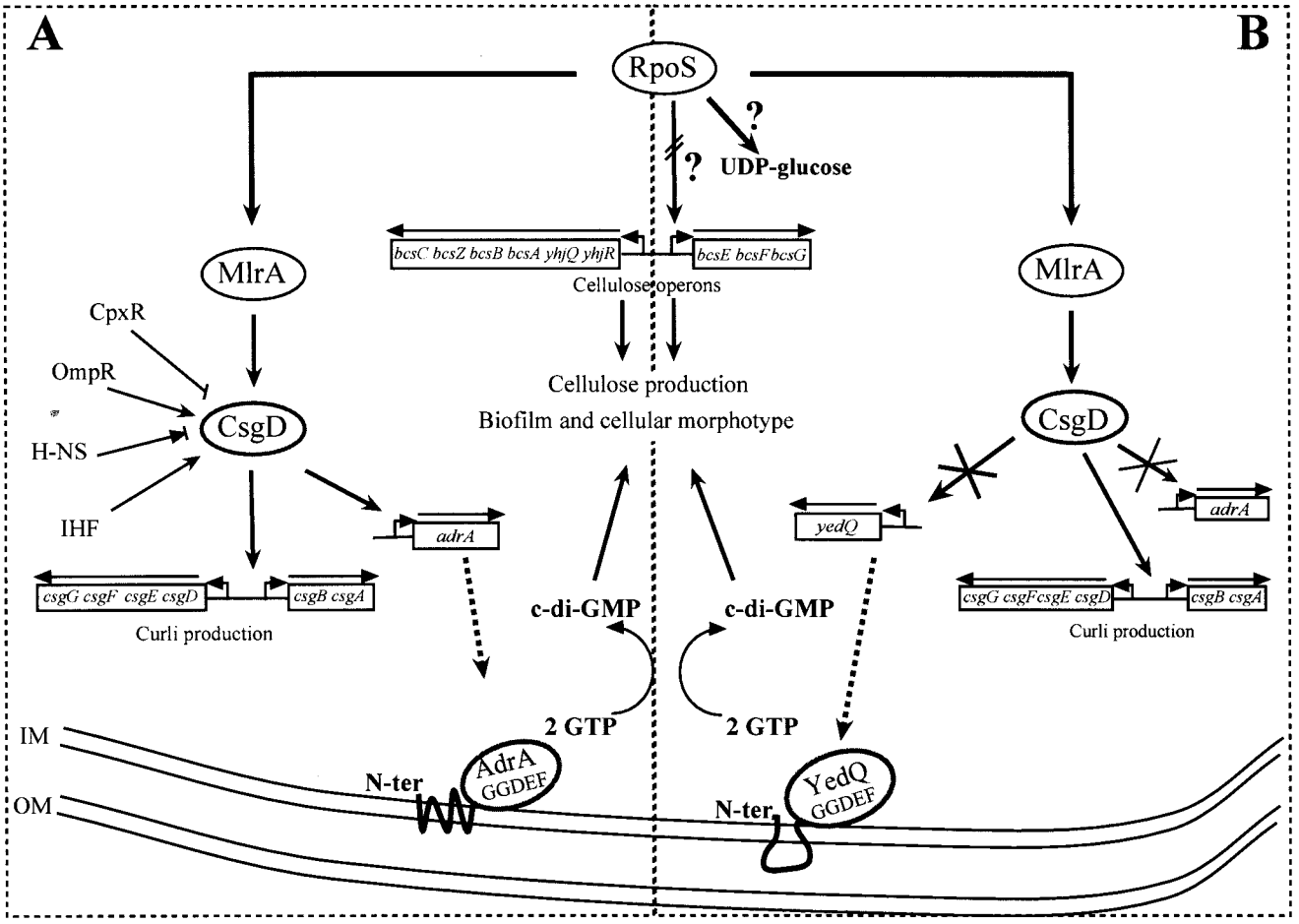

FIG. 6. Models for cellulose production regulation in *Salmonella* and *E. coli*. Schematic regulation of cellulose production via a CsgD/AdrA pathway in *Salmonella* and some *E. coli* strains (A, in LB at 28°C) and via a CsgD-independent and YedQ-dependent pathway in *E. coli* 1094 (B, minimal or LB medium, 30 or 37°C). In both cases, the RpoS sigma factor regulates *csgD* expression via MlrA. Although CsgD is the central regulator for curli and cellulose production in *Salmonella* (A), it regulates only curli synthesis in *E. coli* 1094 (B). In both pathways, the cellulose operons *bcsA-C* and *bcsE-G*, as well as the production of c-di-GMP, are required for cellulose synthesis, biofilm formation, and expression of the cellular morphotype. In the case of *E. coli* 1094, RpoS regulates cellulose production via a yet-uncharacterized pathway. Production of c-di-GMP depends on the diguanylate cyclase activity of the GGDEF domain of the AdrA (A) or the YedQ (B) protein. Both proteins are membrane proteins, but they differ in their N-terminal domains. Both proteins are schematically represented with their N-terminal domains anchored at the membrane (IM, inner membrane; OM, outer membrane).

did not reverse the CF-negative phenotype of a 1094*rpoS* mutant on LB-CF plates (data not shown). Moreover, a p*yedQ-lacZ* transcriptional fusion was still expressed in a 1094*rpoS* background, showing that RpoS is not required for the expression of *yedQ* (Fig. 4; see also Fig. S2 in the supplemental material). Although we did not obtain evidence that *yedQ* could be subjected to any significant transcriptional regulation under our experimental conditions, we cannot exclude *yedQ* regulation at either a transcriptional or a posttranscriptional level. Nevertheless, RpoS is required for the CF- and CR-binding abilities of strain 1094, suggesting that in this strain, RpoS could regulate the transcription of the cellulose *bcs* operons or a metabolic pathway important for cellulose biosynthesis, such as UDP-glucose synthesis, depending on environmental conditions (Fig. 6).

The functional specificity of the multiple GGDEF domain proteins producing freely diffusible c-di-GMP and present in a single bacterium is an important and currently unresolved question (29, 50). Different mechanisms have been proposed, involving either the temporal and environmental transcriptional regulation of GGDEF and EAL domain protein expression, the spatial control of the production of c-di-GMP near its cellular target proteins, or the fine tuning of the activity of GGDEF and EAL domain proteins by a specific ligand or by c-di-GMP itself (1, 29, 33, 42, 50, 59, 60). In *E. coli* 1094, whereas complementation experiments showed that AdrA is functional when overexpressed, transcription analysis using multicopy p*adrA-lacZ* and p*yedQ-lacZ* fusions showed that *adrA* is weakly expressed compared to *yedQ*. These observations support the hypothesis that the specific use of the *yedQ* over the *adrA* cellulose pathway is not due to a defect in AdrA protein activity but may be due in part to substantial differences in gene expression. Indeed the physiological level of expression of AdrA was not sufficient to circumvent *yedQ* de-

fects in *E. coli* 1094, whereas its expression from a high-copy-number plasmid could complement 1094*yedQ*. The level of protein expression has been shown to be important for the complementation of the *Salmonella* serovar Typhimurium *adrA* mutant by the *Yersinia pestis* GGDEF domain protein HmsT (55).

The alignment of the sequences of the *adrA* promoters from *E. coli* strains 1094 and MG1655 shows very few differences (97.7% identity) (Fig. 4C). It is not yet clear if the identified differences in the promoter sequence could explain the lack of CsgD activity on the $adrA_{1094}$ promoter, thus leading to a low level of expression of *adrA* in *E. coli* 1094. Interestingly, however, CsgD can activate the $adrA_{1094}$ promoter in *E. coli* MG1655 (Fig. 4D), suggesting that the $adrA_{1094}$ promoter is functional but is independent of CsgD in *E. coli* 1094. It was previously suggested that CsgD could activate the *csgB* (curli expression) and *adrA* promoters by different mechanisms, since the location of the putative CsgD-binding sites are different in the two promoters (10). Our results raise the possibility that in *E. coli* 1094, CsgD has diverged compared to $CsgD_{MG1655}$ and has lost its ability to regulate *adrA* while still regulating the *csgB* promoter. This hypothesis is under investigation.

The regulation of the level of *adrA* expression does not, however, formally exclude the existence of functional specificities of YedQ and AdrA, possibly related to structural differences outside of their GGDEF domains. Indeed, although YedQ and AdrA are predicted to be membrane proteins and share a GGDEF domain (with 32.9% identity and 48.8% similarity), they have distinct N-terminal domains (Fig. 2B). The N-terminal domain of YedQ is predicted by Phobius software analysis to contain two transmembrane domains and to be mainly periplasmic (32). It does not match any conserved domains in the Pfam database. On the other hand, the N-terminal domain of AdrA is a MASE2 domain, which is predicted to be an integral membrane sensory domain found in many diguanylate cyclases (39, 66). These structural differences in the N-terminal domains of the YedQ and AdrA proteins could lead to distinct localizations and interactions with potential partners and diverse abilities to sense signals for protein activation or repression, all leading to synthesis of c-di-GMP at a specific place and time.

Based on nucleotide sequence homologies, it has been proposed that the cellulose biosynthesis genes could constitute a functional module acquired by horizontal gene transfer under some environmental conditions in which the expression of the rdar morphotype could constitute a selective advantage (53). The acquisition of the capacity to activate cellulose biosynthesis through the production of c-di-GMP would have been acquired after the establishment of the cellulose genes in the genome (46). If so, owing to the large number of proteins potentially involved in the process (11 proteins with a GGDEF domain in *Salmonella* serovar Typhimurium and 19 in *E. coli*), it is possible that, depending on the strain, a GGDEF protein different from AdrA, and possibly more highly expressed due to simple activating mutations, might have fulfilled this regulatory function. In agreement with this hypothesis, *E. coli* strain 1094 displays a CsgD- and AdrA-independent CF-positive phenotype, which involves YedQ. Furthermore, we found that such a CsgD-independent, YedQ-dependent cellulose pathway could also be found in another commensal *E. coli* strain, DSM6601. Additionally, in the case of *E. coli* 1125, an as-yet-uncharacterized GGDEF protein, distinct from both AdrA and YedQ, may have acquired this function, as the *adrA* or *yedQ* mutants, as well as the *adrA*/*yedQ* double mutant in strain 1125, retain their CF-binding abilities (Fig. 5C). Interestingly, experimental data that do not fit the cellulose regulatory pathway schematized in Fig. 6A have been reported (see the introduction and references 23, 48, and 58). This suggests that alternative CsgD-independent pathways may also exist in these *Salmonella* strains and possibly in other *Enterobacteriaceae*. Further studies are under way to test this hypothesis. Owing to the roles of CsgD in different biological processes subject to a complex network of regulatory proteins, the appearance of CsgD-independent cellulose regulation pathways provides a way to uncouple CsgD-regulated processes (e.g., curli regulation) from cellulose synthesis. Such an uncoupling may be favored when the expression of cellulose would confer a selective advantage under conditions in which *adrA* is usually not expressed, such as the high temperature in the human gastrointestinal tract. This may provide a selective pressure leading to the emergence of CsgD-independent cellulose pathways involving regulated diguanylate cyclases, such as YedQ.

In conclusion, the regulation of enterobacterial cellulose production is more complex than previously recognized and, in *E. coli*, can depend on alternative, and possibly widespread, cellulose regulatory pathways. The characterization of an alternative pathway for the regulation of cellulose production in *E. coli* illustrates the diversity of the regulatory arsenal used by bacteria to control the synthesis of polysaccharidic components of the extracellular matrix.

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