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Biofilm Formation-Gene Expression Relay System in *Escherichia coli*: Modulation of σ^S-Dependent Gene Expression by the CsgD Regulatory Protein via σ^S Protein Stabilization▽†

Luciana Gualdi, Letizia Tagliabue, and Paolo Landini*

Department of Biomolecular Sciences and Biotechnology, University of Milan, Via Celoria 26, 20133 Milan, Italy

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Bacteria can switch from a single-cell (planktonic) mode to a multicellular community (biofilm) mode via production of cell-cell aggregation and surface adhesion factors. In this report, we present evidence that the CsgD protein, a transcription regulator involved in biofilm formation in *Escherichia coli*, modulates the expression of the *rpoS* (σ^S) regulon. Protein pattern analysis of *E. coli* cells in stationary phase shows that CsgD affects the expression of several proteins encoded by σ^S-dependent genes. CsgD regulation of σ^S-dependent genes takes place at gene transcription level, does not bypass the need for *rpoS*, and is abolished in an *rpoS*-null mutant. Consistent with these results, we find that CsgD expression leads to an increase in σ^S intracellular concentration. Increase in σ^S cellular amount is mediated by CsgD-dependent transcription activation of *iraP*, encoding a factor involved in σ^S protein stabilization. Our results strongly suggest that the CsgD regulatory protein plays a major role as a relay between adhesion factors production and σ^S-dependent gene expression via σ^S protein stabilization. Direct coordination between biofilm formation and expression of the *rpoS* regulon could positively impact important biological processes, such as host colonization or response to environmental stresses.

Transition from the unicellular (planktonic) state to the multicellular community (biofilm) lifestyle can be considered one of the major developmental processes in bacteria. Indeed, most bacteria are capable of surface colonization and biofilm formation through the production of a large number of adhesion factors, whose expression is usually finely regulated in response to both environmental and physiological cues. Adaptation to growth as a biofilm affects cell morphology, physiology, and metabolism via major redirection of gene expression (4, 52, 54, 62); interestingly, biofilm formation appears to induce the expression of different stress responses (34, 54) and even programmed cell death (70).

In enterobacteria, curli fibers (also known as Tafi, thin aggregative fimbriae, in *Salmonella*) are an important factor in adhesion to surfaces, cell aggregation, and biofilm formation (19, 28, 68). Curli-encoding genes are clustered in two operons: *csgBA* encodes the structural components, while the divergently oriented *csgDEFG* operon encodes proteins involved in curli assembly and transport (38), as well as the CsgD transcription factor, which is necessary for *csgBA* transcription (28). Expression of the *csg* operons takes place in response to a combination of environmental conditions, such as low growth temperature (<32°C), low osmolarity, and slow growth (43). Such environmental signals are mediated at the gene expression level by a number of regulators, including, besides CsgD, global regulatory proteins such as OmpR, H-NS, CpxR, and the alternative σ factor σ^S (3, 25, 53, 58). However, this strict environmental control is lost in many bacterial strains due to mutations either in regulatory genes (3, 68) or in the *csgDEFG* promoter (57). Indeed, temperature-dependent regulation does not take place in several *Salmonella* and pathogenic *Escherichia coli* strains, in which curli are also expressed at 37°C and represent an important virulence factor (5, 6, 48). In contrast, curli operons are silent in a large number of laboratory strains, as well as in some clinical and environmental isolates, despite the presence of functional *csg* genes (57).

In addition to its role as activator of the *csgBA* operon, CsgD regulates a number of genes involved in biofilm formation and production of cell surface-associated structures, such as *adrA*, which promotes cellulose production (59, 75); the *yhiU* operon, encoding biosynthetic genes for the O antigen (26); and the *bapA* gene, which encodes a membrane protein acting as a positive determinant for biofilm formation (35). However, gene regulation by CsgD is not strictly limited to adhesion factors but also affects genes involved in transport, metabolism, and gene regulation, whose dependence on CsgD might possibly be linked to adaptation of cell physiology to the biofilm lifestyle (10, 16). Gene regulation by CsgD is tightly connected to production and sensing of cyclic di-GMP, a bacterial second messenger involved in various cellular processes, including biosynthesis of extracellular polysaccharides (63), biofilm formation (30), and virulence (50, 65), as well as morphological and physiological differentiation (47). Indeed, the CsgD-dependent *adrA* gene, involved in cellulose biosynthesis (75), encodes a cyclic di-GMP synthase (63). CsgD can also activate *yoaD*, whose gene product is a cyclic di-GMP phosphodiesterase, suggesting that CsgD is directly involved in feedback regulation of cyclic di-GMP intracellular levels and of cellulose biosynthesis (10). In turn, other proteins potentially involved in

* Corresponding author. Mailing address: Department of Biomolecular Sciences and Biotechnology, University of Milan, Via Celoria 26, 20133 Milan, Italy. Phone: 39-02-50315028. Fax: 39-02-50315044. E-mail: paolo.landini@unimi.it.

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TABLE 1. Strains and plasmids used in the present study

Strain or plasmid	Relevant genotype or characteristics	Source or reference
Strains		
MG1655	*E. coli* K-12 λ^- F^-, *rph-1*	Reference strain
EB1.3	MG1655 *rpoS*::Tn*10*	53
LG03	MG1655 *iraP*::*kan*	This study
LG05	EB1.3 *csgA*::*uidA-kan*	This study
LG07	EB1.3 *csgD*::*uidA-kan*	This study
PHL628	MG1655 *ompR234*	53
PHL856	MG1655 *csgA*::*uidA-kan*	53
PHL1087	PHL628 *csgD*::*uidA-kan*	53
PHL1088	MG1655 *csgD*::*uidA-kan*	53
Plasmids	Ampicillin resistance, T7 RNA polymerase-dependent promoter	
pT7-7	*csgD* gene open reading frame cloned into pT7-7 plasmid as an NdeI/PstI 651-bp fragment	S. Tabor, Institute of Cancer Research, London, United Kingdom
pT7-CsgD	*E. coli* K-12 λ^- F^-, *rph-1*	53

cyclic-di-GMP biosynthesis affect *csgD* expression in *Salmonella* (32).

The σ^S protein is an alternative sigma factor of RNA polymerase which directs transcription of genes involved in adaptation to slow growth and to cellular stresses (the *rpoS* regulon [37]). We show here that CsgD positively controls σ^S expression by activation of the *iraP* gene, a σ^S protein stabilization factor (8). Thus, CsgD can act as a relay between adhesion factor production and σ^S-dependent gene expression, coordinating the expression of biofilm determinants to stress response genes, and possibly virulence factors, belonging to the *rpoS* regulon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in the present study are listed in Table 1. Bacteria were grown in M9Glu/sup medium (M9 minimal medium supplemented with 0.5% glucose and 2.5% Luria broth) at 30°C. When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 25 μg/ml; and kanamycin, 50 μg/ml.

Biofilm formation assays. Biofilm formation in microtiter plates was determined essentially as described previously (44). Cells were grown in liquid cultures in microtiter plates (0.2 ml) for 18 h in M9Glu/sup at 30°C. The liquid culture was removed, and the cell optical density at 600 nm (OD_{600}) was determined spectrophotometrically. Cells attached to the microtiter plates were washed with 0.1 M phosphate buffer (pH 7.0) and then stained for 20 min with 1% crystal violet (CV) in ethanol. The stained biofilms were thoroughly washed with water and dried. CV staining was visually assessed, and the microtiter plates were scanned. For semiquantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 70% ethanol, and their absorbance was measured at 600 nm and normalized to the OD_{600} of the corresponding liquid culture.

Protein localization experiments. Cell fractionation was performed as described previously (23). Portions (250 ml) of cultures grown in M9Glu/sup at 30°C for 18 h were centrifuged at 4,000 × *g* for 10 min at 4°C and washed with 5 ml of 0.1 M phosphate buffer pH 7.0 (PB). Cells were resuspended in 2 ml of PB with addition of 100 μg of lysozyme/ml and 1 mM EDTA (pH 8.0) and incubated at room temperature for 10 min. Cells were disintegrated by using a French press and centrifuged as described above to remove unbroken cells. The low-speed centrifugation supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C to separate the cytoplasm (supernatant) and the membrane fraction (pellet). The pellet was resuspended in 2 ml of 2% Sarkosyl in phosphate-buffered saline, left for 20 min at room temperature, and centrifuged at 40,000 × *g* at 10°C for 10 min to remove ribosomes and cytoplasmic proteins that were still associated with the membrane fraction. The pellet was resuspended in 1 ml of 1% Sarkosyl, precipitated again 20 min at room temperature, and centrifuged as described above. The supernatant, corresponding to inner membrane proteins, was collected, and the pellet, corresponding to outer membrane proteins, was resuspended in 0.5 ml of H_2O. Protein concentrations were determined, and 20 μg of total proteins was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Specific bands were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis of the peptide products after in-gel trypsin digestion (15; performed by CRIBI, University of Padua, Padua,Italy [http://www.bio.unipd.it/cribi/]).

σ^S determination by Western blotting. Protein amounts in cytoplasmic samples were determined by the Bradford method, and 20-μg portions of total proteins were loaded onto an SDS-PAGE gel (12% acrylamide). Proteins were transferred on Hybond P membranes (Amersham Life Sciences) and incubated with the polyclonal rabbit antibodies against the σ^S protein (55). The anti-σ^S antibodies were detected by using a secondary anti-rabbit antibody conjugated with fluorescein. For σ^S turnover experiments, 50 μg of cell extracts (cytoplasmic fractions) was incubated at 37°C for different times (0, 5, 10, and 20 min). Reactions were stopped by the addition of an equal amount of SDS-PAGE loading buffer, and the samples were used for Western blotting. Bands were quantified by using the ImageQuant 5.2 software (Molecular Dynamics).

RNA isolation, cDNA synthesis, and real-time-PCR analysis. For RNA isolation, strains were grown in M9Glu/sup at 30°C to stationary phase (OD_{600} = 2). The cells were harvested by centrifugation at 13,000 rpm for 5 min at 4°C, and total RNA was extracted by using an RNeasy minikit (QIAGEN). RNA samples were checked by agarose gel electrophoresis to assess the lack of degradation and then quantified spectrophotometrically. Genomic DNA was removed by DNase I treatment. Reverse transcription was performed on 1 μg of total RNA, along with negative control samples incubated without reverse transcriptase. cDNA synthesis efficiency was verified by electrophoresis on agarose gel in comparison to negative controls. Real-time PCR was performed by using the SYBR green PCR master mixture, and the results were determined with an iCycle iQ real-time detection system (Bio-Rad). Reaction mixtures (25 μl) included 0.1 μg of cDNA and 300 nM concentrations of primers in the reaction buffer and enzyme supplied by the manufacturer. The sequences of the primers used are listed (see Table S1 in the supplemental material). All reactions were performed in triplicate, including negative control samples, which never showed significant threshold cycles (C_T). The relative amounts of the transcripts were determined by using 16S rRNA as the reference gene ($[C_{T(\text{gene of interest})} - C_{T(16S)}] = \Delta C_T$).

Other methods. β-Glucuronidase assays were performed as described previously (10). Bacteriophage P1*vir* transductions were carried out as described previously (40). The correctness of transduction was checked by PCR verification of the presence of the antibiotic cassette used for selection in the gene of interest, except for the *rpoS* mutants, which were checked by catalase activity assays as previously described (69).

RESULTS

Ectopic CsgD expression in MG1655. Several *E. coli* laboratory strains, including MG1655, as well as some clinical and environmental isolates, can only produce curli fibers in very limited amounts, insufficient to promote biofilm formation in

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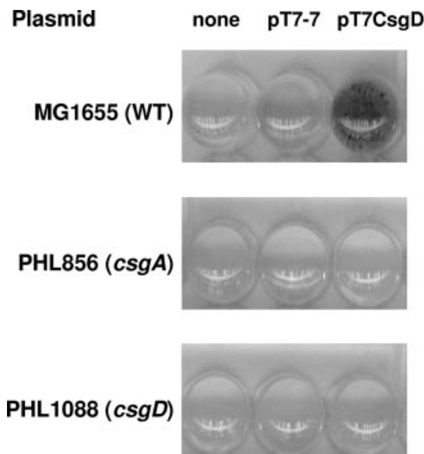

FIG. 1. Surface adhesion by MG1655 (WT), PHL856 (*csgA*), and PHL1088 (*csgD*) transformed with either the pT7-7 or the pT7CsgD plasmids. Surface adhesion was performed in polypropylene microtiter plates as described in Materials and Methods; bacterial biofilm was revealed by CV staining. The images shown are direct scans of microtiter wells.

standard laboratory assays (44), even though the genes necessary for curli biosynthesis and assembly are fully functional (57). However, transformation of MG1655 with the pT7CsgD plasmid results in an ability to form biofilm (Fig. 1). In the pT7CsgD plasmid, the *csgD* gene is under the control of a T7 RNA polymerase-dependent promoter. In *E. coli* strains such as MG1655, which do not carry the T7 RNA polymerase-encoding gene, low-level, constitutive transcription of genes cloned under the control of T7 promoters can still take place due to weak promoter recognition by bacterial RNA polymerase (12). Surface adhesion properties conferred by the pT7CsgD plasmid were totally abolished by inactivation of either the *csgA* gene, whose gene product is the curli major subunit, or the chromosomal *csgD* gene (Fig. 1); inactivation of chromosomal *csgD* has a polar effect on the whole *csgDEFG* operon, thus preventing expression of genes involved in curli assembly and export. These results indicate that, as expected, stimulation of biofilm formation by pT7CsgD is mediated by curli production.

CsgD effects on MG1655 protein production. In order to investigate the possible effects of CsgD on global gene expression in the MG1655 strain, we carried out protein analysis of fractionated cell extracts on monodimensional SDS-PAGE, comparing MG1655 transformed either with pT7CsgD or with the pT7-7 control vector. We have performed a similar approach in another laboratory strain, PHL565 (10), which, however, is impaired in expression of the *rpoS* gene (P. Landini, unpublished observation), a feature common to several laboratory isolates (31). Unlike PHL565, in the *rpoS*-proficient MG1655 strain CsgD expression from pT7CsgD significantly affects protein production pattern (Fig. 2). We excised the bands differentially expressed in MG1655/pT7CsgD and identified the corresponding proteins by MALDI-TOF after in-gel trypsin digestion (Table 2). We found that CsgD positively affects expression of the PflB, GadA, WrbA, and Dps proteins in the cytoplasmic fraction and of the Dps, CsgG, and OmpW

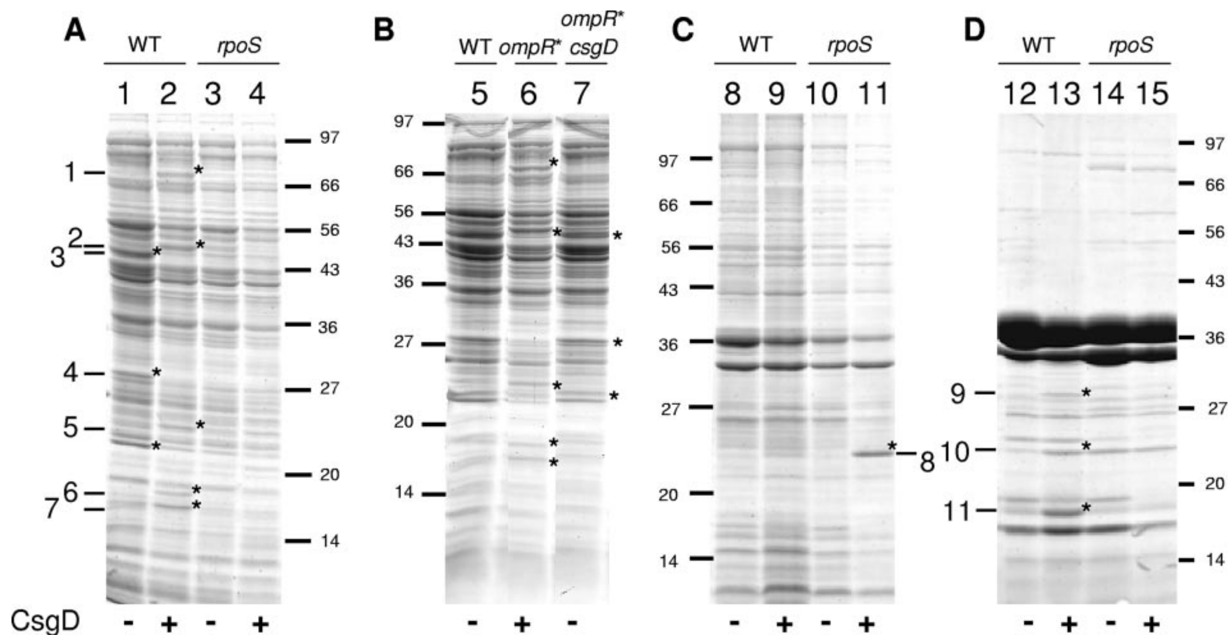

FIG. 2. SDS-PAGE of fractionated cell extracts. (A) Cytoplasmic proteins. Lane 1, MG1655/pT7-7; lane 2, MG1655/pT7CsgD; lane 3, EB1.3/pT7-7; lane 4, EB1.3/pT7CsgD. (B) Cytoplasmic proteins. Lane 5, MG1655; lane 6, PHL628; lane 7, PHL1087. (C) Inner membrane proteins. Lane 8, MG1655/pT7-7; lane 9, MG1655/pT7CsgD; lane 10, EB1.3/pT7-7; lane 11, EB1.3/pT7CsgD. (D) Outer membrane proteins. Lane 12, MG1655/pT7-7; lane 13, MG1655/pT7CsgD; lane 14, EB1.3/pT7-7; lane 15, EB1.3/pT7CsgD. The relevant genotype of the different bacterial strains is indicated in the figure. *ompR** stands for the *ompR234* mutation resulting in increased *csgD* expression (68). The position of molecular mass markers is shown (numbers indicate molecular masses in kilodaltons). Asterisks indicate bands differentially expressed in a CsgD-dependent manner that were excised and identified by MALDI-TOF (numbered from 1 to 11).

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TABLE 2. Gene characteristics

Band no.[a]	Gene product[b]	Predicted molecular mass (Da)[c]	Gene function (reference)	Regulation of corresponding gene (reference)
1	PflB*	85,357	Pyruvate formate lyase I (56)	Induced by ArcA and FNR (61)
2	GadA*	52,685	Glutamate decarboxylase (64)	Positively regulated by *rpoS*, CRP, *gadE*, *gadW*, and *gadX*; negatively regulated by H-NS (21, 66)
3	TnaA†	52,773	Tryptophan deaminase (tryptophanase) (22)	Positively activated by *rpoS* and by CRP (33, 74)
3	GatZ†	47,109	Subunit of tagatose-1,6-bisphosphate aldolase 2 (42)	Positively activated by CRP (74); repressed by ArcA (60)
4	GatY†	30,812	Subunit of tagatose-1,6-bisphosphate aldolase 2 (42)	Same transcription unit as *gatZ*
5	WrbA*	20,846	Quinone reductase; response to oxidative stress (45)	Positively regulated by *rpoS* (33, 46, 71)
6, 7, and 11	Dps*	18,695	Bacterial ferritin, DNA protection during stationary phase (39, 73)	Positively regulated by *rpoS* (2, 33, 46, 71)
8	CsgD*	24,935	Transcriptional regulator (28)	Positively regulated by *rpoS*, *hns*, and *ompR* (25, 58)
9	CsgG*	30,557	Outer membrane protein; curli transport component (38)	Same transcription unit as *csgD*
10	OmpW*	22,928	Outer membrane protein; receptor for colicin S4 (49)	Positively regulated by FNR (18)

[a] As in Fig. 2. Band 3 was identified as a mixture of two proteins and thus appears twice, once for each protein.
[b] *, Increased expression in MG1655/pT7CsgD; †, decreased expression in MG1655/pT7CsgD.
[c] Predicted molecular masses were obtained from the EcoCyc database (http://www.ecocyc.org/).

proteins in the outer membrane fraction. The CsgB and CsgA proteins, i.e., the structural components of curli fibers, which are expressed in a CsgD-dependent fashion, cannot be visualized by SDS-PAGE since, once assembled into curli fibers, they form an extremely tight structure that cannot enter the polyacrylamide gel (28). However, evidence of curli production in the MG1655/pT7CsgD strain comes from functional assays (Fig. 1) and from *csg* gene expression experiments (Fig. 4). Interestingly, four of the six proteins produced at higher level in the presence of CsgD, namely, GadA (band 2 in Fig. 2), WrbA (band 5), Dps (bands 6, 7, and 11), and CsgG (band 9), are encoded by genes known to belong to the *rpoS* regulon, i.e., their transcription is dependent upon the alternative sigma factor σ^S. The GadA, WrbA, and Dps proteins are, respectively, a glutamate decarboxylase involved in resistance to acid stress (21, 64), a quinone reductase part of a response to oxidative stress (41, 45), and a bacterial ferritin able to protect DNA from iron-mediated hydroxyl-radical formation (39, 73). Interestingly, two bands corresponding to the Dps protein were found in the cytoplasm fraction of MG1655/pT7CsgD (bands 6 and 7, Fig. 2A); this would suggest the existence of different Dps isoforms, as also observed in recent two-dimensional gel analysis of the MG1655 proteome (36). Despite Dps being a cytoplasmic protein, we also detected its presence in the outer membrane fraction (band 11, Fig. 2C), as already reported for other biofilm-forming *E. coli* strains (34), thus suggesting that a fraction of the Dps protein might be associated with the outer membrane. Unlike Dps, CsgG and OmpW are bona fide outer membrane proteins (38, 49). The CsgG protein is a component of the curli transport system and is encoded by a gene belonging to the *csgDEFG* operon, which also encodes CsgD (28, 38). Consistent with their being part of the *rpoS* regulon, no CsgD-dependent increase in protein expression of GadA, WrbA, Dps, and CsgG could be detected in EB1.3/pT7CsgD, an *rpoS* mutant of MG1655 transformed with the pT7CsgD plasmid (Fig. 2, lanes 4 and 10), thus suggesting that regulation by CsgD does not bypass the need for σ^S. In addition to known members of the *rpoS* regulon, we find that expression of the PflB protein (band 1), encoded by a gene thus far never described as σ^S dependent, is stimulated by CsgD in the MG1655 background only, suggesting that its CsgD-dependent expression requires a functional *rpoS* gene (Fig. 2A). In contrast, expression of the OmpW protein (band 10), although stimulated by CsgD, appears to be negatively regulated by σ^S (Fig. 2, compare lanes 11 and 13).

Among the proteins negatively regulated by CsgD, we could identify band 5 as a mixture of TnaA (tryptophanase [22]) and GatZ (involved in galactitol catabolism). Identification of band 4, probably a complex of different proteins, was inconclusive, although GatY, also involved in galactitol catabolism, appears to be present. The *gatY* gene, together with *gatZ*, is part of the *gatZYABC* operon (42), which, as we already showed, is negatively regulated by CsgD (10). Neither TnaA nor the GatZ/GatY complex could be detected in cell extracts from the *rpoS* mutant EB1.3 strain (Fig. 2A), suggesting that expression of the corresponding genes is σ^S dependent. Indeed, at least for the *tnaA* gene, dependence on *rpoS* has already been reported (33, 36). Negative regulation of *rpoS*-dependent genes by the CsgD protein might be due to direct interaction with their promoters: indeed, at least for the *tnaA* gene, an imperfect inverted repeat with high similarity to the putative CsgD binding site (9) overlaps the start site of the *tnaLAB* transcript, suggesting possible direct control of the *tnaA* gene by CsgD.

The only major difference observed in the inner membrane compartment was the presence of a rather large band of ca. 25 kDa in EB1.3/pT7CsgD, identified as the CsgD protein. This result suggests that the *rpoS* mutation might either stimulate ectopic CsgD expression from the pT7CsgD plasmid or promote CsgD protein association to the cytoplasmic membrane.

The induction of proteins belonging to the *rpoS* regulon in MG1655/pT7CsgD is not due to changes in growth rate (see Fig. 4), nor does it depend on a stress response elicited by nonphysiological protein overexpression from the pT7CsgD plasmid. Indeed, ectopic expression of the AdrA protein (a

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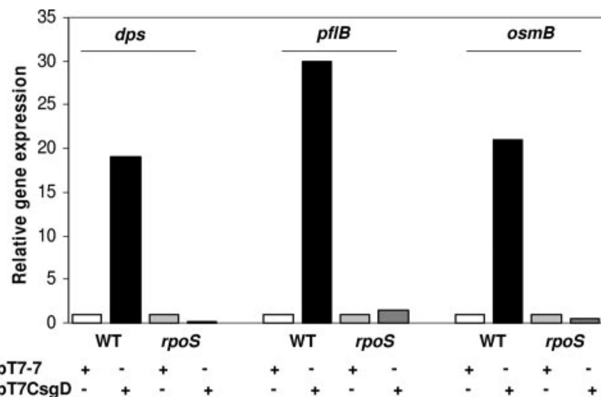

FIG. 3. Relative transcription of the *dps*, *pflB*, and *osmB* genes in MG1655 (WT) and EB1.3 (isogenic *rpoS* mutant), as determined by real-time PCR. Bars: □, strains transformed with pT7-7; ■, strains transformed with pT7CsgD. Relative transcription values were set to 1 for both MG1655/pT7-7 and EB1.3/pT7-7 for better comparison of the CsgD-dependent effects in either strain.

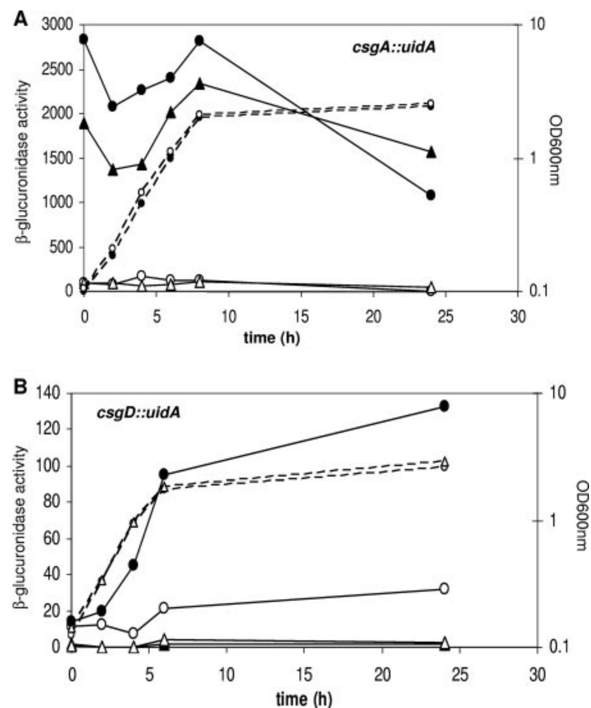

FIG. 4. β-Glucuronidase activity measured from either *csgA*::*uidA* (A) or from *csgD*::*uidA* (B) chromosomal fusions. Cultures were grown in M9Glu/sup at 30°C. (A) Reporter gene expression from the *csgB* promoter was measured either in PHL856 (MG1655 *csgA*::*uidA*; circles) or in LG05 (MG1655 *rpoS csgA*::*uidA*, triangles) transformed either with pT7-7 (open symbols) or pT7CsgD (closed symbols). Dashed lines indicate growth curves of MG1655/pT7-7 (○) and MG1655/pT7CsgD (●) but are representative for all of the strains tested. (B) Reporter gene expression from the *csgD* promoter was measured either in PHL1088 (MG1655 *csgD*::*uidA*; circles) or in LG07 (MG1655 *rpoS csgD*::*uidA*, triangles) transformed either with pT7-7 (open symbols) or with pT7CsgD (closed symbols). Dashed lines indicate growth curves of MG1655/pT7-7 (○) and EB1.3/pT7-7 (●) but are representative for all of the strains tested. The data are an average of four independent experiments. Standard deviations were always lower than 15% and are not shown for clarity.

cyclic-di-GMP synthase), as well as truncated (inactive) forms of CsgD, did not lead to any detectable changes in protein expression (data not shown). In addition, the same pattern of protein regulation was observed when we compared MG1655/pT7CsgD to PHL628, an *ompR234* mutant of MG1655 showing increased transcription of the *csgD* gene (53), both in the cytoplasm (Fig. 2B) and in the membrane compartments (data not shown). Proteins whose production is increased in the cytoplasmic fraction of PHL628 correspond to those identified in MG1655/pT7CsgD, as determined MALDI-TOF analysis, and their expression was totally abolished in PHL1087, a *csgD*::*kan* derivative of PHL628 (Fig. 2B).

Transcription activation of *rpoS*-dependent genes by CsgD. It is likely that the CsgD-dependent effects on protein expression in MG1655 take place via transcription regulation of the corresponding genes. Although monodimensional SDS-PAGE can only provide an incomplete view of CsgD-mediated effects on global protein production in MG1655, our results (Fig. 2 and Table 2) suggest that CsgD might somehow affect the expression of the *rpoS* regulon. In order to confirm this possibility, we determined CsgD effects on transcription of the *dps*, *pflB*, and *osmB* genes by real-time PCR, both in the MG1655 strain and in its *rpoS* derivative EB1.3. These genes were chosen as representatives of known *rpoS*-dependent genes encoding proteins whose production is stimulated by CsgD (*dps*), genes encoding proteins stimulated by CsgD not assigned to the *rpoS* regulon (*pflB*), and *rpoS*-dependent genes encoding proteins for which no stimulation by CsgD could be detected in our SDS-PAGE experiments (*osmB*). As shown in Fig. 3, CsgD activates transcription of *dps*, *pflB*, and *osmB* genes by a similar extent (19- to 30-fold); however, CsgD transcription activation can only occur in the MG1655 strain, and it is totally abolished in its *rpoS* derivative EB1.3. These results confirm that CsgD-mediated effects on protein expression take place at gene transcription level and suggest that CsgD can activate expression of the *rpoS* regulon, or at least of a subset of σ^S-dependent promoters. Interestingly, both the CsgD-dependent *csgB* and the *adrA* promoters, as well as the *csgD* promoter itself, have been proposed to be under the control of σ^S and regulated by the Crl protein, a specific modulator of σ^S activity (7, 51, 55, 57). Thus, it might be possible that CsgD could act as a specific activator for the σ^S-associated form of RNA polymerase ($E\sigma^S$). In order to better understand the interplay between σ^S and CsgD, we compared the effect of *rpoS* inactivation on CsgD-dependent transcription at either the *csgB* or the *csgD* promoter. Activation of its own promoter by CsgD is suggested by increased production of the CsgG protein (Fig. 2), encoded by a gene which is part of the *csgDEFG* operon. Thus, we transformed with either the pT7CsgD or the pT7-7 control vector two derivatives of the MG1655 strain carrying, respectively, a *csgA*::*uidA* (transcription of the *csgA* gene is directed by the *csgB* promoter) or a *csgD*::*uidA* chromosomal transcriptional fusion. The results shown in Fig. 4 indicate that CsgD expression results in increased transcription levels for both the *csgB* and the *csgD* promoters. However, CsgD-dependent transcription activation at the *csgB* and *csgD* promoters greatly differs both in timing and in the extent of dependence on the *rpoS* gene: transcription from the *csgB* promoter (Fig. 4A) is activated by CsgD regardless of growth phase, and *rpoS* inac-

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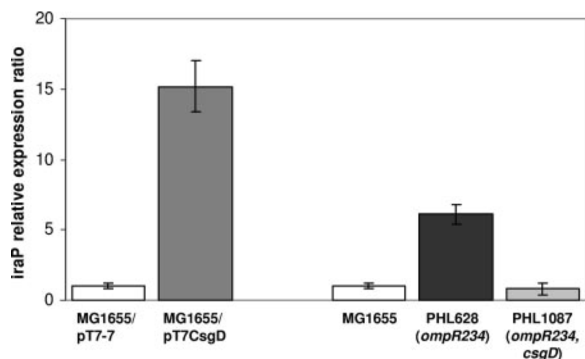

FIG. 5. Expression levels of the *iraP* genes, normalized to 16S rRNA, are shown as relative values in MG1655/pT7CsgD in comparison to MG1655/pT7-7, as well as in PHL628 (*ompR234*) and PHL1087 (*ompR234 csgD*::*kan*) in comparison to MG1655. Samples were taken from cultures grown overnight in M9Glu/sup at 30°C. Values shown are the average of at least three experiments, and standard deviations are shown.

tivation only results in a slight reduction (down to ca. 65%) in promoter activity. Thus, although σ^S is required for optimal transcription levels, the *csgB* promoter is activated by CsgD in a manner largely independent of σ^S, strongly suggesting that σ^S is not directly involved in protein-protein interaction between CsgD and RNA polymerase leading to transcription activation. CsgD activation of *csgB* transcription in the EB1.3 strain also indicates that the CsgD protein is fully competent in transcription activation even in an *rpoS* mutant, despite its localization in the inner membrane compartment (Fig. 2B), which is unusual for a transcription regulator. In contrast to *csgB*, CsgD-mediated stimulation of its own promoter only takes place in late-log and stationary phase and is totally abolished by inactivation of the *rpoS* gene (Fig. 4B).

Effect of CsgD on σ^S intracellular concentrations. Results shown in Fig. 4, indicating that CsgD does not function as an $E\sigma^S$-specific transcription activator, and the observation that *rpoS*-dependent promoters stimulated by CsgD (Fig. 3) lack a putative CsgD binding site would suggest that the CsgD protein might affect expression of the *rpoS* regulon by altering either σ^S activity or its intracellular concentration. Indeed, in a previous report, we have shown by gene array experiments that CsgD can activate the *yaiB* gene (10). The product of the *yaiB* (now *iraP*) gene has recently been shown to be a stabilization factor for the σ^S protein, which acts by inhibiting RssB-mediated degradation of σ^S in response to phosphate starvation (8). Thus, CsgD activation of the *iraP* gene should result in improved σ^S stability and possibly increase σ^S intracellular concentrations, resulting in increased transcription of σ^S-dependent genes. In order to confirm our previous results, we compared *iraP* expression in both the MG1655/pT7-7 and the MG1655/pT7CsgD strains by real-time PCR experiments. As shown in Fig. 5, CsgD can increase transcription levels of the *iraP* gene by ~15-fold. Stimulation of *iraP* transcription by ~6-fold was also observed when we compared MG1655 to its *ompR234* mutant derivative PHL628 (Fig. 5), which expresses the *csgDEFG* operon at higher levels than MG1655 (53). Activation of *iraP* transcription in the *ompR234* mutant strain was totally abolished by *csgD* inactivation (Fig. 5), thus confirming that *iraP* activation observed in the PHL628 strain completely depends on CsgD. CsgD-induced *iraP* expression in either MG1655/pT7CsgD or PHL628 is consistent with increased production of proteins encoded by *rpoS*-dependent genes observed in both strains (Fig. 2).

In order to prove the possibility that CsgD expression might indeed stimulate the *rpoS* regulon by IraP-dependent stabilization of σ^S, we compared the levels of σ^S intracellular amounts in both MG1655/pT7-7 and MG1655/pT7CsgD by Western blotting experiments (Fig. 6A). Expression of CsgD from the pT7CsgD plasmid resulted in a clearly detectable increase in σ^S intracellular amounts in the MG1655 strain. CsgD-dependent stimulation of σ^S intracellular amounts was also observed in *E. coli* strains with different genetic backgrounds and at different antibody dilutions (data not shown). As expected, no bands reacting with the anti-σ^S antibody were detected in the *rpoS* mutant EB1.3, regardless of the presence of pT7CsgD (lanes 5 and 6). Inactivation of the *iraP* gene completely abolishes CsgD-dependent increase in σ^S intracellular concentration (Fig. 6A, lane 4), strongly suggesting that this effect indeed takes place through IraP-mediated stabilization of the σ^S protein. In the growth conditions used in our experiments, σ^S cellular levels do not differ significantly in MG1655 compared to its *iraP* mutant derivative (Fig. 6A, compare lanes 1 and 3), a finding in agreement with previous results indicating that the IraP protein is only essential for σ^S stability under phosphate starvation conditions (8). In order to correlate σ^S intracellular concentration and expression of σ^S-dependent genes, cell extracts from the MG1655 strain and in its EB1.3 (*rpoS*) and LG03 (*iraP*) derivatives were compared by SDS-PAGE. As shown in Fig. 6B, a strict correlation exists between σ^S intracellular concentration and expression of proteins encoded by σ^S-dependent genes. Consistent with the lack of CsgD-dependent increase in σ^S intracellular concentration in LG03 (*iraP* mutant; Fig. 6A, lane 4), the CsgD protein failed to stimulate expression of the GadA, WrbA and Dps proteins in this strain (Fig. 6B, lane 4). The band running with electrophoretic mobility similar to WrbA in cell extracts of the EB1.3 and LG03 was identified as adenosine phosphoribosyltransferase (data not shown), and its expression appears to be CsgD independent (Fig. 6B).

To confirm that CsgD-dependent accumulation of σ^S is indeed mediated by σ^S stabilization via the IraP protein, we performed σ^S protein turnover assays in cell extracts of either MG1655 or its *iraP* mutant derivative (LG03), grown both in the presence or in the absence of the pT7CsgD plasmid. As shown in Fig. 7, the stability of the σ^S protein is increased in cell extracts of MG1655/pT7CsgD compared to MG1655/pT7-7; however, no CsgD-dependent σ^S stabilization could be detected in cell extracts of the LG03/pT7CsgD strain, a finding consistent with a direct role of the IraP protein in CsgD-mediated stabilization of σ^S. In the absence of CsgD expression, the σ^S half-life in cell extracts of the *iraP* mutant is similar to MG1655 (14 versus 12 min, respectively), as detected by image analysis.

DISCUSSION

The CsgD protein, a transcription regulator responsible for the production of adhesion factors (curli fibers) and biofilm

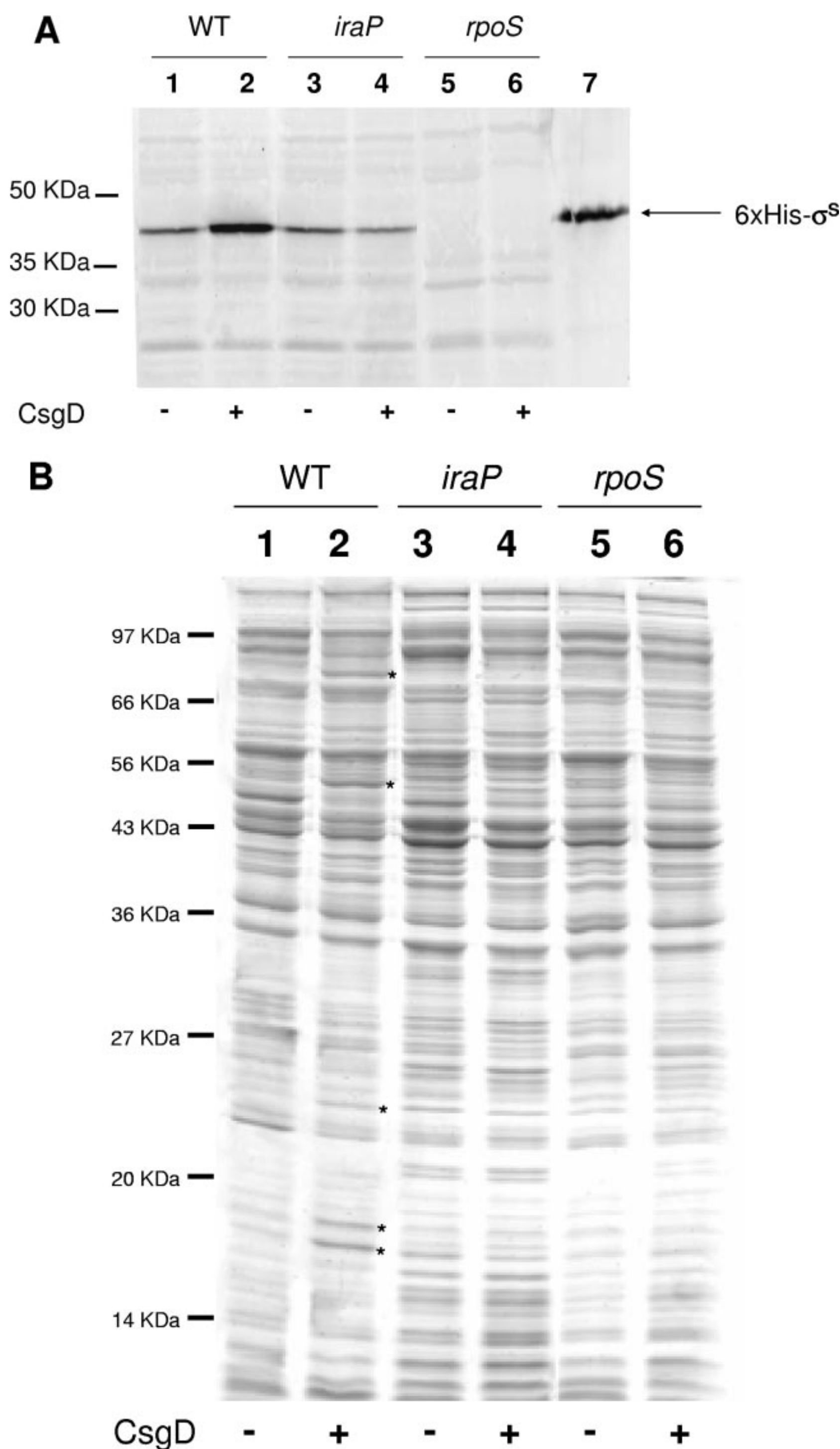

FIG. 6. (A) Western blotting with anti-σ^S antibodies. Cell extracts were prepared from overnight cultures grown in M9Glu/sup at 30°C, and 20 μg of total proteins was loaded onto a SDS-polyacrylamide gel. Lane 1, MG1655/pT7-7; lane 2, MG1655/pT7CsgD; lane 3, LG03/pT7-7; lane 4, LG03/pT7CsgD; lane 5, EB1.3/pT7-7; lane 6, EB1.3/pT7CsgD; lane 7, purified His_6-tagged σ^S. The positions of the 50-, 35-, and 30-kDa molecular mass markers are shown. (B) SDS-PAGE analysis of cytoplasmic proteins (20 μg of total proteins). Lane order is as described in panel A. Differently expressed proteins are indicated by an asterisk.

formation in enterobacteria, can activate expression of the *iraP* gene (10) (Fig. 5). The IraP protein acts as a stabilization factor for the alternative σ factor σ^S by binding to the RssB protein and thus preventing σ^S proteolysis by the RssB-ClpXP protein complex. IraP-dependent stabilization of σ^S takes place in response to phosphate starvation (8). We report here that CsgD transcription activation of the *iraP* gene does indeed result in a significant increase of σ^S intracellular concentration

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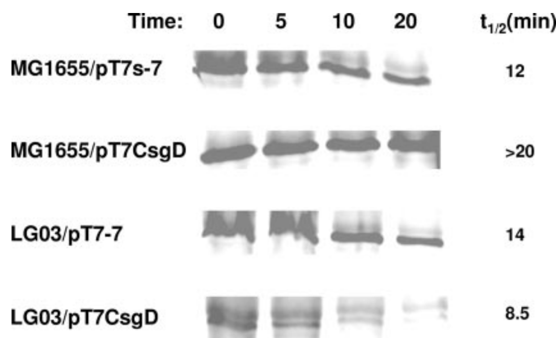

FIG. 7. σ^S turnover experiments. Cell extracts of MG1655/pT7-7, MG1655/pT7CsgD, LG03/pT7-7, and LG03/pT7CsgD were incubated at 37°C. Samples (50 μg of proteins) were taken at the indicated times and loaded onto an SDS-polyacrylamide gel. The amount of σ^S protein was determined by Western blotting. σ^S half-life values were obtained by quantification of the σ^S-corresponding bands using the ImageQuant 5.2 image analysis program. The experiment was performed twice with very similar results.

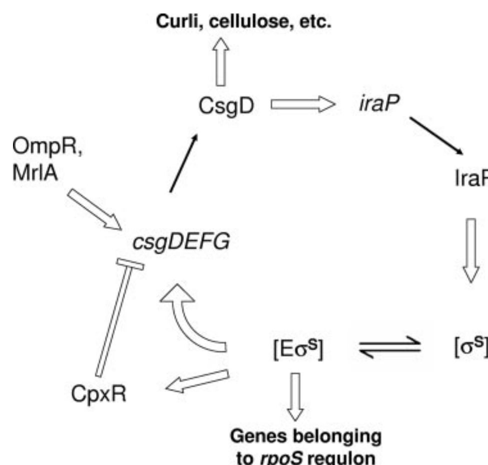

FIG. 8. Model for the CsgD/σ^S autoactivation loop. Thin black arrows indicate gene expression; block white arrows indicate positive regulatory interactions; white lines indicate negative interactions. See the text for details.

by positively affecting σ^S protein stability (Fig. 6 and 7), thus leading to altered expression of σ^S-dependent genes (Fig. 2, 3, 4B, and 6B and Table 1). CsgD modulation of the *rpoS* regulon (or at least of a subset of σ^S-dependent genes) via *iraP* activation was observed both in MG1655 transformed with the pT7CsgD plasmid, allowing low-level, constitutive expression of the CsgD protein, and in the curli-proficient PHL628 strain (53), in which the *csgD* gene is expressed from its own promoter (Fig. 2). Thus, CsgD-mediated stabilization of σ^S, and its consequent effect on the expression of the *rpoS* regulon, do not depend on nonphysiological CsgD expression from the pT7CsgD plasmid. Our experiments were performed in phosphate-rich medium, suggesting that IraP-mediated σ^S stabilization might take place in response to environmental cues other than phosphate starvation; this would be in agreement with a recent report that, in *Salmonella*, *iraP* transcription is activated during growth at low Mg^{2+} concentration, resulting in IraP-mediated σ^S stabilization (67). In the *csgD*-expressing PHL628 strain of *E. coli*, CsgD-dependent *iraP* transcription can only take place in response to environmental signals leading to *csgD* expression and curli production, i.e., low temperature and osmolarity (data not shown). These observations would be consistent with a role for IraP in σ^S stabilization in a broad range of physiological conditions. From our results, we cannot establish whether CsgD activates *iraP* expression via direct interaction with the *iraP* promoter or through additional factors: we could not find in the *iraP* promoter region any sequence closely resembling the putative binding site for CsgD identified in the CsgD-dependent *csgB*, *adrA*, and *pepD* promoters (9). However, this does not rule out the possibility of CsgD binding to less conserved sites in the *iraP* promoter; despite various attempts, we have never succeeded in purifying active CsgD protein for in vitro DNA-binding assays.

CsgD-mediated increase of σ^S cellular concentrations via the *iraP* gene would trigger an autoactivation loop, as summarized in Fig. 8, leading to an increased production of CsgD-dependent adhesion determinants such as curli fibers and cellulose. Indeed, in most curli-producing strains, transcription of the *csgDEFG* operon requires a functional *rpoS* gene (58), although it is not clear whether dependence on the *rpoS* gene is due to direct recognition of the *csgD* promoter by the Eσ^S form of RNA polymerase or whether it is mediated by *rpoS*-dependent expression of the *mrlA* gene, encoding a transcription factor which, in turn, activates *csgDEFG* transcription (11). Either way, an increase in the cellular σ^S pool in conditions permitting *csg* gene expression (i.e., low osmolarity) leads to increased transcription from the *csgD* promoter; in turn, production of the CsgD protein results in *iraP* activation and consequent further stabilization of σ^S. Evidence for this autoregulatory mechanism in the MG1655 strain is provided by σ^S-dependent activation of the *csgD* promoter in the presence of pT7CsgD (Fig. 4B) and by consequent production of proteins encoded by the *csgDEFG* operon, such as CsgG (Fig. 2C), in response to CsgD-dependent accumulation of σ^S. Indeed, σ^S-dependent activation of the *csgDEFG* operon observed in Fig. 4B is impaired in an *iraP* mutant strain (data not shown). This autoregulatory circuitry might be further fueled by σ^S-dependent induction of genes encoding di-guanylate cyclases, i.e., proteins able to synthesize the second messenger di-cyclic-GMP, which, in turn, can positively affect *csg* gene expression (32, 72). A possible feedback control for this regulation loop could be provided by the CpxA/CpxR two-component regulatory system, whose activity is triggered by curli overexpression and results in repression of both the *csgD* and the *csgB* promoters (53). Consistent with this model, transcription of the *cpxRA* operon is itself positively controlled by σ^S (24).

Besides more efficient expression of the *csgDEFG* operon, however, it is likely that CsgD-σ^S interaction plays a more general role, i.e., to coordinate the production of adhesion and cell aggregation factor (curli fibers and cellulose) to σ^S-dependent gene expression. Such coordination would relay the transition from single cell to biofilm to expression of the *rpoS* regulon, i.e., one of the main stress responses in bacteria (29). Interestingly, biofilm formation in *E. coli*, even when independent of CsgD and of curli production, appears to be tightly connected to σ^S-dependent gene expression (1, 4, 17, 34, 62) and to general induction of stress responses (4, 54), possibly

suggesting that biofilm formation might represent a switch to a "resistance form" better equipped to withstand environmental stresses. However, to our knowledge, our work is the first demonstration of a mechanism for activation of a stress response (the *rpoS* regulon) by a positive regulator of biofilm formation.

In addition to resistance to environmental stresses, production of adhesion factors (curli and fimbriae) and σ^S-dependent gene expression play a role in pathogenic enterobacteria in adaptation and survival in the host and even in virulence: the σ^S-dependent *gad* genes allow survival of bacteria to acid stress in the gastric tract (20), whereas curli fibers are involved in bacterial internalization in epithelial host cells (27). Finally, σ^S controls the *spvR* virulence system in *Salmonella* (14). Thus, correlation between adhesion factor production and the *rpoS* regulon could constitute an important regulation mechanism of virulence in enterobacteria.

Other than its effect on the *rpoS* regulon, CsgD expression appears to regulate a subset of anaerobiosis-dependent genes, such as *ompW* and *pflB*, both induced in anaerobic conditions by FNR, either alone or in concert with ArcA (18, 61). In contrast, the *gatZYABC* operon, negatively controlled by CsgD (10) (Table 2) is repressed by ArcA in anaerobic conditions (60). It is thus possible that CsgD-mediated cell aggregation might, to some extent, mimic a switch to anaerobic conditions. Finally, CsgD expression also appears to affect expression of the *tnaA* gene (Fig. 2A), whose product catalyzes tryptophan degradation to indole and pyruvate. Indole acts as a signal molecule, and its accumulation results in inhibition of cell division (13), and repression of *tnaA* might prevent untimely indole accumulation leading to growth arrest. The impact of CsgD on expression of genes not related to adhesion factors' production, such as *tnaA* and anaerobic genes, as well as the *rpoS* regulon, is likely to reflect the cell's need to reprogram its global gene expression in response to transition from planktonic cells to biofilm.

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