Gene Expression Regulation by the Curli Activator CsgD Protein: Modulation of Cellulose Biosynthesis and Control of Negative Determinants for Microbial Adhesion

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Curli fibers, encoded by the csgBAC genes, promote biofilm formation in Escherichia coli and other enterobacteria. Curli production is dependent on the CsgD transcription activator, which also promotes cellulose biosynthesis. In this study, we investigated the effects of CsgD expression from a weak constitutive promoter in the biofilm-formation-deficient PHL65 strain of E. coli. We found that despite its function as a transcription activator, the CsgD protein is localized in the cytoplasmic membrane. Constitutive CsgD expression promotes biofilm formation by PHL65 and activates transcription from the csgBAC promoter; however, csgBAC expression remains dependent on temperature and the growth medium. Constitutive expression of the CsgD protein results in altered transcription patterns for at least 24 novel genes, in addition to the previously identified CsgD-dependent genes. The cspA and fecR genes, encoding regulatory proteins responding to cold shock and to iron, respectively, and yoaD, encoding a putative negative regulator of cellulose biosynthesis, were found to be some of the novel CsgD-regulated genes. Consistent with the predicted functional role, increased expression of the yoaD gene negatively affects cell aggregation, while yoaD inactivation results in stimulation of cell aggregation and leads to increased cellulose production. Inactivation of fecR results in significant increases in both cell aggregation and biofilm formation, while the effects of cspA are not as strong in the conditions tested. Our results indicate that CsgD can modulate cellulose biosynthesis through activation of the yoaD gene. In addition, the positive effect of CsgD on biofilm formation might be enhanced by repression of the fecR gene.

Most bacteria are capable of surface colonization and biofilm formation through the production of specific adhesins and extracellular structures. Curli fibers (also known as thin aggregative fimbriae) are a major factor in adhesion to surfaces, cell aggregation, and biofilm formation in many enterobacteria (11, 36, 42, 43, 53). Expression of curli is linked to cellulose biosynthesis, which leads to the production of an extracellular matrix and results in tight cell-cell and cell-surface interactions and in the so-called rdar morphotype in Salmonella (45, 57, 58). Expression of both curli and cellulose depends on the CsgD protein, a putative transcription regulator of the LuxR family, which activates transcription of the csgBAC operon (2), which encodes curli structural subunits, and transcription of the adrA gene, a positive effector of cellulose biosynthesis (45). In addition to csgBAC activation by CsgD, production of curli is subject to complex regulation, which affects both the csgDEFG operon (encoding the CsgD transcription regulator and the CsgEFG curli-specific transport system) and the csgBAC operon (encoding curli structural subunits) (8, 20, 22, 53). Curli expression is dependent on different environmental and physiological cues, such as a low growth temperature (<32°C), low osmolarity, and slow growth or starvation (i.e., conditions usually encountered by the bacteria outside the mammalian host) (19, 22, 31, 36, 44). However, curli are an important virulence factor in some Salmonella and pathogenic Escherichia coli strains, in which temperature-dependent regulation can be bypassed and curli expression can also take place at 37°C (3, 4, 37, 38). In contrast, curli operons are cryptic in a large number of both clinical and environmental E. coli isolates, as well as in laboratory strains, despite the presence of functional csg genes. However, mutations either in the specific promoters (44, 52) or in global regulatory genes, such as hns (2) or ompR (53), can restore the expression of curli-encoding genes.

The product of the CsgD-dependent adrA gene is a member of the GGDEF protein family (16, 50). The AdrA protein can catalyze the synthesis of bis-(3′,5′)-cyclic diguanylic acid (cyclic di-GMP), which in turn stimulates the enzymes responsible for cellulose production (48). In addition to the genes coding for factors directly involved in the curli-cellulose extracellular matrix, the CsgD protein positively regulates glyA, which encodes the glycine biosynthetic enzyme serine hydroxymethyltransferase (10), and represses the dipeptidase-encoding pepD gene (7). The promoters of the csgBAC operon and of the adrA and pepD genes share a conserved 11-bp sequence (CGGGKGAKNAG), which is necessary for CsgD-dependent regulation (7).

The pepD gene was identified as a CsgD-dependent gene using a whole-genome expression approach in which the following two laboratory strains of E. coli were compared: PHL65, which is unable to produce curli, and a spontaneous curli-producing mutant, PHL628 (7). The PHL628 strain has a
mutation in the ompR gene that results in a single leucine-to-arginine substitution (ompR234 allele); the ompR234 mutation increases transcription of ompR-dependent genes, including csgD (53), and stimulates specific DNA binding by the OmpR protein at the csgD promoter (25, 42). However, comparisons of laboratory strains unable to express curli with curli-producing mutants with mutations in global regulatory genes, such as ompR, do not allow precise evaluation of the direct contribution of the CsgD protein to gene regulation. To circumvent this problem, in this work we transformed the curli-negative strain PHL565 (53) and derivatives of this strain (Table 1). Bacterial cells were propagated either in Luria-Bertani broth (LB) or in M9Glu/sup (M9 minimal medium supplemented with 0.4% glucose and 2.5% LB). When necessary, ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (25 μg/ml) was added. For 42°C temperature, and centrifuged at 40,000 g.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** For this study, we used E. coli K-12 laboratory strain PHL565 (53) and derivatives of this strain (Table 1). Bacterial cells were grown either in Luria-Bertani broth (LB) or in M9Glu/sup (M9 minimal medium supplemented with 0.4% glucose and 2.5% LB). When necessary, ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (25 μg/ml) was added. For csgD expression, the csgD gene was cloned from the pCP900 plasmid (42) into the pT7-7 plasmid using the NdeI and PstI sites to obtain the pT7-CsgD plasmid, in which csgD was under the control of a phage T7 RNA polymerase-dependent promoter. For yoaD overexpression studies, the yoaD gene was amplified by PCR using PHL565 genomic DNA as the template and the yoaD-fw and yoaD-rev primers (Table 2). The PCR product was cloned into pGEM-T Easy, in which the yoaD gene was placed under the control of the Plac promoter, using the following primers: yoaDfw (5′-ATGCAAAGACGACAAGCG-3′) and yoaD-rev (5′-GTTCGAAACCGATAATG-3′). MG1655 mutant strains carrying either yoaD, fecR, or cspA regulatory alleles were obtained from the laboratory of F. Blattner, University of Wisconsin (http://www.genome.wisc.edu/functional/tnmutagenesis.htm).

The mutant alleles were transformed into PHL565 by P1 transduction (33).

**CsgD localization experiments.** Cell fractionation was performed as described previously (12). Five hundred-milliliter cultures of PHL565/pT7-7 and PHL565/pT7-CsgD were grown in M9Glu/sup at 30°C for 15 h. The cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C, washed, and resuspended in 20 ml phosphate-buffered saline (PBS). Cells were disintegrated by sonication and centrifuged as described above to remove unbroken cells. The supernatant was transferred into a 15-ml tube, where the low-speed centrifugation was performed at 10,000 g for 1 h at 4°C to separate the cytoplasm (supernatant) and the membrane fraction (pellet). The pellet was washed with 2 ml of 2% Sarkosyl in PBS, left for 20 min at room temperature, and centrifuged at 40,000 g 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, incubated, 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 H2O. Protein concentrations were determined, and either 40 μg (for cytoplasmic fractions) or 20 μg (for membrane fractions) of total proteins was loaded onto a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Specific bands were visualized by mass spectrometry of the peptide products after in-gel tryptic digestion (9).

**TABLE 1. Strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHL65</td>
<td>Reference strain, MG1655</td>
<td>53</td>
</tr>
<tr>
<td>PHL628</td>
<td>ompR234 derivative of PHL65</td>
<td>53</td>
</tr>
<tr>
<td>PHL856</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>PHL857</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>EB9</td>
<td>PHL856, crb920:cam</td>
<td>This study</td>
</tr>
<tr>
<td>EB15</td>
<td>PHL65, yoaD::Tn5kan (transduced from FB22583)</td>
<td>This study</td>
</tr>
<tr>
<td>EB16</td>
<td>PHL65, cspA::Tn5kan (transduced from FB21280)</td>
<td>This study</td>
</tr>
<tr>
<td>EB17</td>
<td>PHL65, fecR::Tn5kan (transduced from FB21762)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

- **pT7-7**: Control vector, ampicillin resistance, T7 RNA polymerase-dependent promoter
- **pT7-CsgD**: pT7-7 carrying csgD gene cloned as an NdeI/PstI 651-bp fragment
- **pGEM-TEasy**: Control vector, ampicillin resistance, Plac promoter
- **pGEMTyoaD**: pGEM-TEasy carrying yoaD gene cloned as a 1.6-kbp fragment

**TABLE 2. Primers used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rrsB</td>
<td>GAGATCCAGCGGTGAATAGTTT</td>
<td>ACCCACTCCATATGGTGTTGA</td>
</tr>
<tr>
<td>csgA</td>
<td>TGGTCAAGATGACAGTCGAATC</td>
<td>CCGTTTCCACTGTAAGAGTGA</td>
</tr>
<tr>
<td>csgB</td>
<td>CCGGAGCGAGCAAAAGTAA</td>
<td>GCAAACGCCCAAAGTTT</td>
</tr>
<tr>
<td>csgD</td>
<td>CCCGTACCCGAGATTT</td>
<td>AGCTGTTGATCCTCCAGAGG</td>
</tr>
<tr>
<td>cspA</td>
<td>GTTGGCCCGAGAGAAATCT</td>
<td>GATGAAACGAGTCGATGGC</td>
</tr>
<tr>
<td>cspF</td>
<td>GCGCATGGTGACCCACAAGTTA</td>
<td>TGCCGATCTGGTACTGATG</td>
</tr>
<tr>
<td>cspG</td>
<td>TGGTTGGCGGCTATGTACAG</td>
<td>CGGTCTGGGGCTTCATTTA</td>
</tr>
<tr>
<td>yoaC</td>
<td>GGGTGATGAGGAGGCTTATAC</td>
<td>ACCCCCAACTATACCTGGATA</td>
</tr>
<tr>
<td>pepD</td>
<td>TGGTGACCGGTGTTGATTC</td>
<td>CGGACGTCGACGGAGGTTT</td>
</tr>
<tr>
<td>yagS</td>
<td>ATGGTGGCGGCTACGTAC</td>
<td>CAGTCTGCGGACCTTAC</td>
</tr>
<tr>
<td>yoaD</td>
<td>AAGGCACTCCGTTTGGTATC</td>
<td>CATTCCTGACCACGCAT</td>
</tr>
<tr>
<td>cspA</td>
<td>TCCGTTAAGATGACTGGTATCGT</td>
<td>AGCCATCGTACGAGGATGTA</td>
</tr>
<tr>
<td>fecR</td>
<td>CCCCGGAGGAAAAC</td>
<td>AAATCTAGAGGCGCTGACTG</td>
</tr>
<tr>
<td>glyA</td>
<td>GCAGGAAAAGTACGTCAGGAGA</td>
<td>GGGCGGCTGGTGATTG</td>
</tr>
</tbody>
</table>
Biofilm formation and cell aggregation assays. Biofilm formation in microtiter plates was determined essentially as described previously (15). Cells were grown in liquid cultures in microtiter plates (0.2 ml) for 18 to 20 h either in M9Glu/sup at 30°C or in LB at 30°C; the liquid medium was removed, and the cell density was determined spectrophotometrically by determining the optical density at 600 nm (OD600). Cells adhering to the microtiter plates were then washed twice gently with PBS and stained for 20 min with 1% crystal violet (CV) in ethanol. The stained biofilms were washed with tap water and dried. For semiquantitative analysis by using a procedure similar to the one used for adhesion assays, CV-stained cells were resuspended in 0.2 ml of 70% ethanol for semiquantitative analysis by using a procedure similar to the one used for adhesion assays.

RNA isolation, cDNA labeling, and microarray data analysis. Total RNA from E. coli cells that were grown for 15 h in M9Glu/sup at 30°C to the OD600 of the corresponding liquid cultures. For cell aggregation assays, biofilm formation was checked as described in the Materials and Methods section. Overnight cultures were left to stand for 24 h at room temperature to allow sedimentation of cell aggregates. In order to obtain better visualization of biofilms, CV-stained cells were resuspended in 0.2 ml of 70% ethanol for semiquantitative analysis by using a procedure similar to the one used for adhesion assays.

TABLE 3. Results of whole-genome transcription assay and real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of gene product</th>
<th>Mean whole-genome transcription pT7-CsgD/pT7-7 ratio (SD)</th>
<th>Mean real-time PCR pT7-CsgD/pT7-7 ratio (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adrA</td>
<td>Cold shock protein CS 7.4</td>
<td>0.14 (0.1)</td>
<td>0.28 (0.1)</td>
</tr>
<tr>
<td>csgB</td>
<td>Curli minor subunit</td>
<td>0.24 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>csgC</td>
<td>Curli major subunit</td>
<td>0.19 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>csgD</td>
<td>Curli production protein</td>
<td>0.22 (0.1)</td>
<td>0.24 (0.2)</td>
</tr>
<tr>
<td>gsk</td>
<td>Unknown</td>
<td>0.11 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>yaiB</td>
<td>Unknown</td>
<td>0.13 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>ygiW</td>
<td>Unknown</td>
<td>0.17 (0.0)</td>
<td>ND</td>
</tr>
<tr>
<td>ymdA</td>
<td>Unknown, similar to fimA/fapa family of fimbrial proteins</td>
<td>0.25 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>yoaD</td>
<td>Unknown</td>
<td>0.08 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>yfl</td>
<td>Unknown</td>
<td>0.08 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>csgA</td>
<td>Curli minor subunit</td>
<td>0.91 (0.2)</td>
<td>1.21 (0.4)</td>
</tr>
<tr>
<td>csgB</td>
<td>Curli minor subunit</td>
<td>0.63 (0.3)</td>
<td>1.19 (0.5)</td>
</tr>
<tr>
<td>csgC</td>
<td>Curli major subunit</td>
<td>0.45 (0.1)</td>
<td>1.0 (2.0)</td>
</tr>
<tr>
<td>csgD</td>
<td>Curli production/assembly component, CsgE</td>
<td>0.56 (0.4)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>glyA</td>
<td>Serine hydroxymethyltransferase</td>
<td>0.77 (0.1)</td>
<td>ND</td>
</tr>
<tr>
<td>pepD</td>
<td>Aminoacyl-histidine dipeptidase</td>
<td>0.41 (0.1)</td>
<td>0.24 (0.1)</td>
</tr>
</tbody>
</table>

a ND, not determined.
RESULTS

CsgD constitutive expression and cell localization. In the pT7-CsgD plasmid, the csgD gene is under the control of a T7 RNA polymerase-dependent promoter. However, in E. coli strains such as PHL565, which do not carry the T7 RNA polymerase-encoding gene, detectable csgD transcription can still take place and most likely depends on recognition by bacterial RNA polymerase of promoter-like sequences upstream of the csgD gene. According to real-time PCR experiments, in PHL565/pT7-CsgD the amount of csgD transcript was roughly 100-fold greater than the amount in the PHL565 strain carrying the control vector pT7-7 (Table 3), in which csgD expression was negligible. The level of the csgD transcript in PHL565/pT7-CsgD was 1.5- to 2-fold higher than the levels in other curli-proficient E. coli strains, such as the ompR234 mutant PHL628 (53) or WK2, a curli-producing environmental derivative, as determined by real-time PCR (Landini, unpublished data). However, unlike curli-producing strains in which csgD expression is driven from its own promoter, the levels of csgD transcription in PHL565/pT7-CsgD do not vary significantly in different growth conditions, again as determined by real-time PCR (data not shown). Thus, we concluded that pT7-CsgD allows constitutive csgD expression totally uncoupled from physiological and environmental signals, such as growth phase and osmolarity, which, in contrast, control the expression of the csgDEFG promoter (2, 8, 42). No band corresponding to the CsgD protein was detectable in crude extracts of PHL565/pT7-CsgD as determined by SDS-polyacrylamide gel electrophoresis (PAGE) (data not shown). However, analysis of the different cell compartments (cytoplasm, inner membrane, and outer membrane) after fractionation of cells grown overnight at 30°C in M9Glu/sup led to identification of a 25-kDa band present only in the cytoplasmic membrane fraction of PHL565/pT7-CsgD, where it accounted for only a small percentage of the total proteins (Fig. 1). In-gel trypsin digestion of the protein followed by mass spectrometry analysis confirmed that this band indeed corresponded to CsgD. Unlike CsgD, proteins that form inclusion bodies, such as green fluorescent protein, are not readily solubilized by Sarkosyl treatment and are not found in the inner membrane after cell fractionation (data not shown). Thus, CsgD localization in the cytoplasmic membrane did not appear to be due to the formation of inclusion bodies or to other artifacts that depended on nonphysiological CsgD expression. Expression of CsgD from the pT7-CsgD plasmid resulted in a fourfold increase in surface attachment by the PHL565 strain in M9Glu/sup and in a roughly twofold increase in LB. A similar degree of surface attachment stimulation was observed previously for PHL628, an ompR234 mutant derivative of PHL565. The PHL565/pT7-CsgD strain formed red colonies when it was plated on growth medium supplemented with the amyloid protein-binding dye Congo red (Fig. 2B), suggesting that curli production was induced in this strain. Increased Congo red binding and surface colonization induced by pT7-CsgD were indeed dependent on curli, since transformation with pT7-CsgD of the PHL856 strain, a PHL565 derivative in which the csgA gene encoding the main curli subunit has been inactivated, did not result in surface attachment (Fig. 2A) or in Congo red binding (Fig. 2B) by this strain. Thus, csgD expression from the pT7-CsgD plasmid led to the production of a functional CsgD protein and conferred an adherent, curli-expressing phenotype to the PHL565 strain.
a CsgD-expressing mutant derivative of PHL565 (data not shown). Recently published observations suggest that in E. coli temperature-dependent regulation of curli is mediated by the product of the \( \text{crl} \) gene, which acts as the temperature sensor at the \( \text{csgBAC} \) promoter (6). To further investigate this possibility, we transformed the EB9 strain, a \( \text{crl920}:\text{xam} \) derivative (40) of PHL856, with either pT7-7 or pT7-CsgD, and we measured \( \text{csgBAC} \) transcription. The \( \text{crl} \) mutation resulted in a clear reduction in \( \text{csgBAC} \) transcription at 30°C, while it did not have any effect at 37°C, in agreement with the proposed role of the \( \text{crl} \) gene (Fig. 3).

In contrast to the substantial changes induced by media and temperature, the effects of growth medium osmolarity on \( \text{csgBAC} \) transcription in PHL565/pT7-CsgD were modest. Addition of up to 0.25 M NaCl to M9Glu/sup resulted in a less-than-twofold reduction in \( \text{csgBAC} \) transcription, which was, in contrast, totally abolished in the PHL857 strain, in which CsgD was expressed from its own promoter (Fig. 4).

**Effects of constitutive CsgD expression on whole-genome transcription.** The results of both adhesion and \( \text{csgBAC} \) transcription experiments showed that constitutive expression from pT7-CsgD led to production of an active CsgD protein. Thus, we performed a whole-genome transcription assay in which we compared PHL565 strains that were transformed with either pT7-7 or pT7-CsgD and were grown in M9Glu/sup at 30°C (i.e., the optimal conditions for curli expression). We considered an average difference in gene expression that was equal to or greater than fourfold significant (see Materials and Methods). Ten genes were found to be up-regulated and 14 genes were found to be down-regulated in the PHL565 strain transformed with pT7-CsgD (Table 3). Among the up-regulated genes we found, as expected, \( \text{csgBAC} \) and \( \text{adrA} \), which are known to be CsgD regulated, and CsgD itself. Increased CsgD transcription was exclusively due to the presence of the pT7-CsgD expression vector and was independent of the \( \text{csgDEFG} \) promoter, as indicated by a lack of any increase in \( \text{csgEF} \) transcripts (see the results for real-time PCR experiments).

In addition to known CsgD-dependent genes, we found three genes with as-yet-unknown functions (\( \text{yaiB}, \text{yaiW}, \) and \( \text{yfrF} \)) and two genes (\( \text{ymdA} \) and \( \text{yoaD} \)) whose putative functions can be predicted based on the amino acid sequences of their products. The \( \text{ymdA} \) gene encodes a hypothetical protein similar to proteins in the \( \text{fimA/papA} \) fimbrial protein family and is located 120 bp downstream of the \( \text{csgBAC} \) operon. The \( \text{yoaD} \) gene encodes a member of the EAL protein family, which is thought to be responsible for degradation of cyclic di-GMP, a signal molecule able to trigger cellulose biosynthesis, biofilm formation, and different cellular processes in several gram-negative bacteria (23). Interestingly, the CsgD-activated \( \text{adrA} \) gene encodes a diguanylate cyclase, the biosynthetic enzyme for cyclic di-GMP (45, 48, 57), suggesting that both intracellular accumulation and degradation of cyclic di-GMP are mediated by CsgD-regulated genes. In addition to \( \text{adrA} \) and \( \text{yoaD} \), a third GMP-related gene, \( \text{gsk} \), was found to be more highly expressed in PHL565/pT7-CsgD. The Gsk protein is a GMP synthase belonging to the nucleoside salvage pathway (24, 27) and might be involved in either repletion or maintenance of the GMP cellular pool.

All 14 genes that were down-regulated in PHL565/pT7-CsgD have known functions, and none of them has yet been

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**FIG. 2.** Effect of CsgD on biofilm development on polystyrene microtiter plates and Congo red binding. (A) Surface attachment by strain PHL565 (wild type) and strain PHL856 (csgD) transformed with either pT7-7 or pT7-CsgD. Details are described in Materials and Methods. Open bars, M9Glu/sup; gray bars, LB. The bars indicate the averages for at least three experiments, and the error bars indicate standard deviations. (B) Congo red binding by PHL565 and PHL856 transformed with either pT7-7 or pT7-CsgD.
shown to be regulated by CsgD. The *pyrBI* operon encodes the two subunits of aspartate carbamoyl transferase, an enzyme that is part of the pyrimidine biosynthetic pathway (28, 55). The *gatA*, *gatC*, and *gatZ* genes listed in Table 3 belong to the *gatYZABCDR* operon, encoding a phosphoenolpyruvate-dependent phosphotransferase system transporter specific for the sugar galactitol (35, 39). Although the *gatY, gatB, gatD*, and *gatR* genes are not listed in Table 3, they were all down-

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**FIG. 3.** β-Glucuronidase activity for a *csgA:uidA* chromosomal fusion either in PHL856 (PHL565 *csgA:uidA*) or in EB9 (PHL856 *crl920:cam*) transformed with pT7-CsgD. (A) β-Glucuronidase activity in cells grown in M9Glu/sup. (B) β-Glucuronidase activity in cells grown in LB. Open symbols, cells grown at 30°C; solid symbols, cells grown at 37°C; squares, PHL856; triangles, EB9. The β-glucuronidase activities in strains PHL856 and EB9 transformed with the pT7-7 vector or not carrying any plasmid were less than 50 U, and the corresponding curves are hardly distinguishable from the x axis. The data are averages for three independent experiments. The standard deviations were always less than 15% and are not shown for reasons of clarity.
regulated in PHL565/pT7-CsgD by factors ranging from 2.7- to 3.3-fold, suggesting that the whole *gat* transcription unit is indeed repressed in a CsgD-dependent fashion (data not shown). Two genes encoding outer membrane proteins, the main OmpF porin and the OmpT protease, as well as the methionine biosynthesis *metA* gene, were also down-regulated in PHL565/pT7-CsgD (Table 3).

The five remaining genes which are down-regulated by constitutively expressed CsgD belong to the following two functional groups: iron-sensing genes and cold shock-responding genes. Our assays showed that there was 4.5-fold repression of transcription of both the *fecR* and *fhuE* genes in PHL565/pT7-CsgD (Table 3). The outer membrane FhuE protein serves as a receptor for ferric coprogen and ferric rhodotorulic acid, which upon binding by FhuE can be taken up via the TonB system (47). The periplasmic FecR protein plays a role in iron sensing and in regulation of the alternative sigma factor FecC. The two genes are cotranscribed in the *fecIR* operon (41, 49), and the level of the *fecI* transcript is 2.5-fold lower in PHL565/pT7-CsgD (data not shown), which is consistent with the possibility that there is either direct or indirect transcriptional repression of the whole *fecIR* operon by CsgD. Finally, three of the main cold shock-induced genes in *E. coli* (*cspA*, *cspB*, and *cspG*) (29, 54) appeared to be down-regulated by factors of four- to sevenfold in PHL565/pT7-CsgD. In addition, the level of the transcript of the *infA* gene, which is known to respond to cold shock (21), was also fourfold lower in the CsgD-expressing strain.

Real-time PCR analysis of genes differentially expressed in PHL565/pT7-CsgD. A selection of genes that were found to be differentially expressed in the whole-genome transcription assay were tested in real-time PCR experiments. First, we tested genes belonging to the *csgABC* and *csgDEFG* operons and genes proposed to be CsgD dependent (*adaA, glyA*, and *pepD*). Real-time PCR experiments showed that the levels of expression of the *csg* genes were 10- to 100-fold-higher than the levels in the whole-genome transcription experiment (Table 3). The large differences were probably due to the difficulty of evaluating precisely the very low levels of expression of *csg* genes in the PHL565 strain. In contrast, real-time PCR and whole-genome transcription assays yielded very similar results for all other genes tested (Table 3).

Unlike the levels of transcription of the *csgBAC* operon, the levels of transcription of the *csgE*, *csgF* and *csgG* genes (i.e., the *csgDEFG* operon) were not altered by the presence of the pT7-CsgD plasmid, strongly suggesting that the CsgD protein does not regulate its own gene and indicating that, despite the presence of a functional chromosomal copy of *csgD* in the PHL565/pT7-CsgD strain, *csgD* expression is solely dependent on the plasmid copy of the gene.

Transcription of the *glyA* gene was only weakly stimulated (1.3-fold) by the CsgD protein according to the results of our real-time experiments (Table 3). Although CsgD has been shown to suppress glycine autotrophy and to stimulate serine hydroxymethyltransferase activity via the *glyA* gene (10), little CsgD-dependent stimulation (1.2- to 2-fold) of *glyA* transcription was detectable when cells were grown in minimal medium supplemented with amino acids (10), which is consistent with our results. In contrast, the *pepD* gene appeared to be 4-fold negatively regulated by CsgD in real-time PCR experiments, similar to the 4-fold repression observed for the curli-expressing PHL628 derivative of PHL565 (7) and to the 2.5-fold repression observed for the PHL565/pT7-CsgD gene array compared with the PHL565/pT7-7 gene array (Table 3).

Among the novel identified genes whose expression was affected by CsgD, we tested the EAL protein-encoding *yoaD* gene, as well as the *fecR* and *cspA* genes, in real-time PCR experiments (Table 3). For these genes, the real-time PCR results closely reflected the results of the whole-genome transcription assays, showing that there was an eightfold increase for the *yoaD* transcript and a roughly fourfold decrease for both the *cspA* and *fecR* transcripts in PHL565/pT7-CsgD.

**Effects of novel identified CsgD-regulated genes on surface attachment and cell aggregation.** Since the CsgD protein activates curli and cellulose production, which are factors that are involved in biofilm formation and in cell-cell interaction, we tested the possibility that the *yoaD*, *cspA*, and *fecR* genes could also play a role in these processes. Biofilm formation was measured by determining the ability to attach to a solid surface, while cell-cell interaction was determined by a cell aggregate sedimentation test, as described in Materials and Methods. In aggregation tests, sedimented pellets were fixed and stained with 1% crystal violet, which allowed semiquantitative measurement of cell aggregation. As shown in Fig. 5A, inactivation of either *yoaD*, *cspA*, or *fecR* led to a significant increase in cell aggregation, and the results for dissolution of crystal violet-stained pellets in ethanol ranged from a 5-fold increase for the *cspA* mutant strain to an almost 12-fold increase for the *yoaD* derivative of PHL565. Neither the growth rates nor the viabilities of the mutant strains differed significantly from the value for PHL565, suggesting that increased cell aggregation does not depend on cellular stress. Strong stimulation of cell aggregation following inactivation of *yoaD* would be consistent with the putative role of the YoaD protein as a negative regulator of cellulose biosynthesis (48, 57). Indeed, PHL565::*yoaD* cells grown on calcium agar plates exhibited a 3.5-fold increase in absorbance at 366 nm, which indicated that there was increased cellulose production, while neither the
The positive effect of yoaD inactivation on cell aggregation does indeed depend on the YoaD protein, since introduction of a functional yoaD gene on the pGEMTyoaD plasmid in the PHL565 yoaD strain totally abolished both cell sedimentation (Fig. 5A) and calcofluor binding (Fig. 5B). Despite the stimulatory effect of yoaD or cspA on cell aggregation, inactivation of either yoaD or cspA did not result in a significant increase in surface attachment (Fig. 5C). In contrast, inactivation of the fecR gene positively affected cell adhesion; the effects of the fecR mutation were more pronounced when cells were grown in LB, in which this mutation led to a six- to eightfold increase (Fig. 5C). The PHL628 strain formed cell aggregates that were clearly detectable in our sedimentation assays (Fig. 6). Transformation of PHL628 either with a control vector or with pGEMTyoaD, in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) induction, did not affect cell aggregation. However, upon full P_{lac} induction by addition of 0.5 mM IPTG, which maximized yoaD expression from pGEMTyoaD, PHL628 cell aggregation was completely inhibited (Fig. 6). In contrast, surface adhesion to microtiter plates by PHL628 was not affected by the presence of the pGEMTyoaD plasmid, even in the presence of IPTG (data not shown).

Expression of the yoaD gene in PHL628. Whole-genome transcription analysis experiments suggested that the yoaD gene is activated in a CsgD-dependent fashion. To confirm this, we measured yoaD expression, using real-time PCR, in the CsgD-expressing PHL628 strain and compared it to the expression in PHL565. Samples were taken in different growth phases, and, as a control, we determined the expression of the CsgD-dependent adrA (yaiC) gene in the same conditions. The levels of yoaD transcripts were 10- to 12-fold higher in PHL628 than in PHL565 in stationary-phase cells, while the differences in expression levels were less than 2-fold in exponentially growing cells (Fig. 7). In contrast, growth phase-dependent expression could not be detected for adrA, whose PHL628/PHL565 expression ratio only increased from 15 to 22.
DISCUSSION

In this work, we tested the effects of low-level, constitutive expression of the CsgD protein, a positive regulator of curli and cellulose, on cell adhesion, transcription of curli genes, and global gene expression in the nonadherent PHL565 strain of E. coli. The CsgD protein was found to be associated with the cytoplasmic membrane after cell fractionation (Fig. 1). Although localization of CsgD in the cytoplasmic membrane may be unusual for a transcription factor, other membrane-associated proteins, such as the ToxR protein of Vibrio cholerae (14) and CadC in E. coli (13), can act as transcription regulators. Alternatively, some regulatory proteins, such as Mlc (30, 51), can be found in an active conformation in the cytoplasm and can be temporarily inactivated by sequestration to the inner membrane. In future experiments we will define the nature of the interaction between the cytoplasmic membrane and CsgD.

Constitutive expression of the csgD gene from the pT7-CsgD plasmid results in a fully active CsgD protein that is able to promote surface colonization by PHL565 in a curli-dependent fashion (Fig. 2) and to activate transcription at the csgBAC promoter (Fig. 3). By expressing the CsgD protein independent of its own promoter we could determine if important environmental signals in curli regulation, such as osmolarity, temperature, and growth medium, target either the csgDEFG promoter or subsequent steps in the curli regulation cascade. Constitutively expressed CsgD could still efficiently activate csgBAC transcription at high osmolarity (Fig. 4), in agreement with previous data showing that osmolarity control of curli production occurs mainly at the csgDEFG promoter via OmpR-dependent regulation (19, 42, 53). Unlike osmolarity-dependent regulation, temperature-dependent regulation of curli seems to take place at a step later than csgD transcription, as indicated by the observation that activation of csgBAC transcription by constitutively expressed CsgD is strongly inhibited at 37°C. This suggests that curli temperature regulation involves different mechanisms in E. coli and in Salmonella. In Salmonella, no csgD transcription takes place at 37°C; however, mutations in the csgDEFG promoter region can restore both csgD transcription and curli production at nonpermissive temperatures (44). In contrast, our observations suggest that temperature regulation comes into play at the csgBAC promoter (Fig. 3). This result is consistent with the hypothesis that the product of the crl gene (40) is the main temperature sensor for curli expression in E. coli (6). Indeed, crl mutations strongly impaired CsgD-dependent csgBAC transcription at 30°C, although our results suggest that additional temperature-dependent regulatory mechanisms could also affect csgBAC transcription (Fig. 3). Finally, growth medium-dependent curli regulation also takes place at a step later than csgD transcription (Fig. 3), suggesting that reduced csgBAC transcription in LB does not depend on the higher osmolarity of the LB but is due to yet another mechanism for environmental control of csgBAC transcription.

From the results of the whole-genome transcription assays we concluded that constitutive CsgD expression might affect the expression of about 30 genes in the conditions that we tested. The results of our experiments did not allow us to conclude that all genes that showed differential expression in PHL565/pT7-CsgD are indeed directly regulated by the CsgD protein. However, the altered expression in PHL565/pT7-CsgD of several genes involved in the modulation of intracellular nucleotide and nucleoside pools (gsk, in addition to genes involved in curli and cellulose biosynthesis (csgBAC, adxA) and in their modulation (youAD). Negative regulation of gene expression by CsgD can further stimulate adhesion and induce metabolic adaptation to growth as a biofilm or as cell aggregates. Novel CsgD-regulated genes that have been found to affect adhesion properties are indicated by boldface type.

FIG. 8. Model for the role of CsgD in gene expression and induction of the adhering phenotype in E. coli. Membrane-associated CsgD can activate GMP-related genes (gsk), in addition to genes involved in curli and cellulose biosynthesis (csgBAC, adxA) and in their modulation (youAD). Negative regulation of gene expression by CsgD can further stimulate adhesion and induce metabolic adaptation to growth as a biofilm or as cell aggregates. Novel CsgD-regulated genes that have been found to affect adhesion properties are indicated by boldface type.

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CsgD strain (data not shown), suggesting that there is possible negative control of the dps gene by CsgD. Dps is a bacterial ferritin that is important for iron storage, particularly in slowly growing cells (34, 56). The main location of Dps is in the cytoplasm, but the Dps protein can also be found in the outer membrane fraction in several E. coli strains (Landini, unpublished data). We are currently investigating the mechanisms of control of surface attachment and Dps regulation by fecR.

Our data suggest that constitutive CsgD expression has a strong effect on the cold shock regulon (Table 3) and that cspA, which encodes the major cold adaptation protein in E. coli, may be involved in the negative regulation of cell aggregation (Fig. 5A). The role of cspA in cell aggregation and adhesion will be evaluated after a temperature downshift (i.e., in conditions in which the cold shock response protein is fully activated).

In contrast to fecR and cspA, the yoaD gene is activated by the CsgD protein (Table 3), although it also negatively affects cell aggregation (Fig. 5A). The yoaD gene belongs to a single-gene transcription unit and encodes a putative 59.5-kDa protein carrying the cyclic d-GMP phosphodiesterase EAL domain. Proteins belonging to the EAL family are involved in the degradation of cyclic d-GMP, a signal molecule that triggers several cellular processes, including cellulose biosynthesis (17, 48). Consistent with the putative role of the yoaD gene, inactivation of this gene stimulates cell aggregation (Fig. 5A) and results in increased cellulose biosynthesis (Fig. 5B), while overexpression of the gene negatively affects cell aggregation in a curli-producing strain of E. coli (Fig. 6). The YoaD protein might be expressed in a CsgD-dependent fashion in order to modulate cellulose biosynthesis by counteracting the positive effect of the adrR gene, which is also controlled by CsgD and encodes a GGDEF protein responsible for cyclic di-GMP biosynthesis and activation of cellulose production (48). YoaD-mediated modulation of cellulose biosynthesis may depend on the cell’s need to prevent excessive consumption of glucose and/or GMP. Consistent with this hypothesis, the timing of yoaD expression is delayed with respect to adrA expression and is limited to the stationary phase of growth in the CsgD-expressing PHL628 strain (Fig. 7). Since yoaD, like adrA (45), plays a role in the regulation of cellulose production and is regulated in a CsgD-dependent fashion, we propose that yoaD should be reannotated as adrB.

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