

# Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*

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In enterobacteria, the CsgD protein activates production of two extracellular structures: thin aggregative fimbriae (curli) and cellulose. While curli fibres promote biofilm formation and cell aggregation, the evidence for a direct role of cellulose as an additional determinant for biofilm formation is not as straightforward. The MG1655 laboratory strain of *Escherichia coli* only produces limited amounts of curli and cellulose; however, ectopic *csgD* expression results in strong stimulation of curli and cellulose production. We show that, in a *csgD*-overexpressing derivative of MG1655, cellulose production negatively affects curli-mediated surface adhesion and cell aggregation, thus acting as a negative determinant for biofilm formation. Consistent with this observation, deletion of the *bcsA* gene, necessary for cellulose production, resulted in a significant increase in curli-dependent adhesion. We found that cellulose production increased tolerance to desiccation, suggesting that the function of cellulose might be related to resistance to environmental stresses rather than to biofilm formation. Production of the curli/cellulose network in enterobacteria typically takes place at low growth temperature (<32 °C), but not at 37 °C. We show that CsgD overexpression can overcome temperature-dependent control of the curli-encoding *csgBA* operon, but not of the cellulose-related *adrA* gene, suggesting very tight temperature control of cellulose production in *E. coli* MG1655.

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## INTRODUCTION

In *Escherichia coli* and *Salmonella* spp., curli fibres (also known as Tafi, thin aggregative fimbriae) are a major factor in adhesion to surfaces, cell aggregation and biofilm formation (Doran *et al.*, 1993; Olsen *et al.*, 1993; Hammar *et al.*, 1995; Romling *et al.*, 1998a). Curli-encoding genes are located at a single genetic locus and clustered in the divergently oriented *csgDEFG* and *csgBA* operons (Arnqvist *et al.*, 1994; Hammar *et al.*, 1995). Transcription of the *csgBA* operon, encoding the structural components of curli fibres, strictly depends on the CsgD transcription regulator (Hammar *et al.*, 1995). In addition, expression of both the *csgDEFG* and the *csgBA* operons takes place in response to a combination of environmental conditions, i.e. low growth temperature (<32 °C), low osmolarity and slow growth (Olsen *et al.*, 1993). These environmental signals are mediated at the gene expression level by a number of regulators, such as OmpR, H-NS,

CpxR, Crl, and the alternative sigma factor  $\sigma^S$ , involved in *csg* gene expression regulation (Arnqvist *et al.*, 1994; Romling *et al.*, 1998b; Prigent-Combaret *et al.*, 2001; Gerstel *et al.*, 2003; Bougdour *et al.*, 2004). However, mutations either in regulatory genes (Arnqvist *et al.*, 1994; Vidal *et al.*, 1998) or in the *csgDEFG* promoter (Romling *et al.*, 1998b) can lead to loss of environmental control of curli expression in several enterobacterial isolates. Indeed, temperature-dependent regulation does not take place in several pathogenic *E. coli* strains, in which curli are expressed even at 37 °C and appear to be involved in virulence-associated processes (Ben Nasr *et al.*, 1996; Bian *et al.*, 2000; Persson *et al.*, 2003). 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 (Romling *et al.*, 1998a; Bokranz *et al.*, 2005; Castonguay *et al.*, 2006).

Besides 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 (Latasa *et al.*, 2005; Gibson *et al.*, 2006), as well as genes involved in transport, metabolism and gene regulation (Chirwa &

Abbreviation: CV, crystal violet.

Two supplementary figures are available with the online version of this paper.

Herrington, 2003; Brombacher *et al.*, 2006). Arguably, however, the main function of CsgD besides curli regulation is activation of cellulose production, which results in the formation of a curli/cellulose extracellular matrix (Romling *et al.*, 2000; Zogaj *et al.*, 2001). CsgD stimulates cellulose production indirectly, by activating transcription of the *adrA* gene; in turn, the AdrA protein positively affects the enzymic activity of the cellulose biosynthetic machinery through its diguanylate cyclase activity, i.e. synthesis of the signal molecule cyclic di-GMP (c-di-GMP). c-di-GMP is a widely conserved bacterial second messenger that, in addition to biosynthesis of cellulose and other extracellular polysaccharides (Simm *et al.*, 2004), is involved in various cellular processes, including biofilm formation (Hickman *et al.*, 2005) and virulence (Tischler & Camilli, 2005), and in morphological and physiological differentiation in *Caulobacter crescentus* (Paul *et al.*, 2004).

Although cellulose was originally described as an additional determinant for biofilm formation in enterobacteria (Romling *et al.*, 2000), its role as an adhesion factor is not straightforward (Wang *et al.*, 2006). In this report, we show that cellulose negatively affects curli-mediated biofilm formation in a derivative of *E. coli* MG1655 constitutively expressing the CsgD protein, and we propose that cellulose production could be a determinant for resistance to environmental stresses rather than for biofilm formation.

## METHODS

**Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. Bacteria were grown in M9 minimal medium (Smith & Levine, 1964) supplemented with 0.5% glucose and 2.5% Luria broth (M9Glu/sup) either at 30 °C or at 37 °C, as indicated. When needed, antibiotics were used at the following concentrations: ampicillin, 100 µg ml<sup>-1</sup>, chloramphenicol, 35 µg ml<sup>-1</sup>; kanamycin, 50 µg ml<sup>-1</sup>. For growth on Congo-red-supplemented agar medium (CR medium), bacteria were inoculated in M9Glu/sup medium in a microtitre plate, and the cultures were spotted, using a replicator, on CR medium (1% Casamino acids, 0.15% yeast extract, 0.005% MgSO<sub>4</sub>, 2% agar) to which 0.004% Congo red and 0.001% Coomassie blue were added after autoclaving. Both dyes were dissolved in 50% ethanol to a final concentration of 0.2% prior to addition to CR medium. Bacteria were grown for 20 h at either 30 °C or 37 °C; staining by Congo red was better detected after 1–2 days of additional incubation at 4 °C.

**Biofilm formation assays.** Biofilm formation in microtitre plates was determined as described by Dorel *et al.* (1999). Bacterial cultures were grown in M9Glu/sup medium (Brombacher *et al.*, 2006) either at 30 °C or at 37 °C, for 18 h, either in microtitre plates (0.2 ml) or in 12 ml glass tubes (2.5 ml cultures) with vigorous shaking. The liquid culture was removed, and the cell density was determined by determining the OD<sub>600</sub>. Cells attached to the microtitre 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 microtitre plates were scanned. For semiquantitative determination of biofilms, CV-stained cells were resuspended in either 0.2 ml (for microtitre plates) or 2 ml (for glass tubes) of 70% ethanol. The A<sub>600</sub> of the resuspended CV was

**Table 1.** Strains, plasmids, and primers used in this study

Strain, plamid or primer	Relevant genotype or characteristics	Reference or source
<b><i>E. coli</i></b>		
MG1655	Reference strain	Brombacher <i>et al.</i> (2006)
PHL856	MG1655 <i>csgA::uidA-kan</i>	Prigent-Combaret <i>et al.</i> (2001)
LG20	MG1655 <i>crl::920cam</i> ; obtained by transduction from LP468 (Pratt & Silhavy, 1998)	This work
LG26	MG1655 $\Delta$ <i>bcsA::kan</i> ; obtained by P1 <i>vir</i> transduction from MG1655 <i>bcsA</i> (gift from C. Beloin)	This work
<b>Plasmids</b>		
pT7-7	Control vector, ampicillin resistance, T7 RNA polymerase-dependent promoter	S. Tabor, Institute of Cancer Research, London, UK
pT7CsgD	<i>csgD</i> gene cloned into plasmid pT7-7 as a 651 bp <i>NdeI/PstI</i> fragment	Prigent-Combaret <i>et al.</i> (2001)
pTOPO	Control vector allowing direct cloning of PCR products, ampicillin and kanamycin resistance	Invitrogen
pTOPOAdrA	<i>adrA</i> gene cloned as PCR product into pTOPO vector	This work
<b>Primers</b>	<b>Sequence</b>	<b>Utilization</b>
<i>csgB</i> _fw	CATAATTGGTCAAGCTGGGACTAA	RT-PCR experiments
<i>csgB</i> _rev	GCAACAACCGCCAAAAGTTT	RT-PCR experiments
<i>adrA</i> _rt_fw	GGCTGGGTCAGTACCAG	RT-PCR experiments
<i>adrA</i> _rt_rev	CGTCGGTTATACACGCCCG	RT-PCR experiments
<i>bcsA</i> _rt_fw	GACGCTGGTGGCGCTG	RT-PCR experiments
<i>bcsA</i> _rt_rev	GGGCCGCGAGATCACC	RT-PCR experiments
<i>adrA</i> _fw	GCTCCGTCTCTATAATTTGGG	<i>adrA</i> cloning
<i>adrA</i> _rev	ATCCTGATGACTTTCGCCGG	<i>adrA</i> cloning

determined and normalized to the OD<sub>600</sub> of the corresponding grown cell density; this value is defined as 'adhesion unit'. In addition to CV staining, we measured the amount of bacterial cells by direct plating after vigorous resuspension of the biofilm; c.f.u. ml<sup>-1</sup> counts always correlated with the amount of CV staining (data not shown).

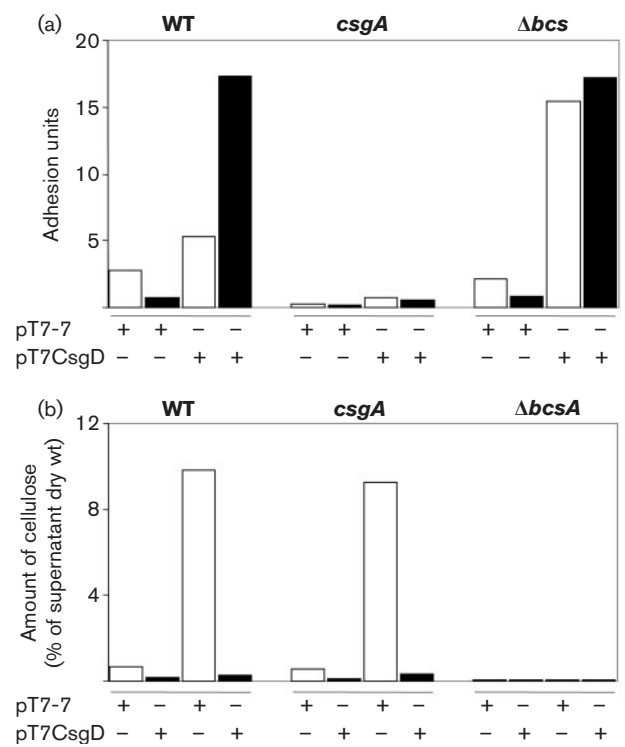
To determine cell aggregation, 2 ml cultures were grown overnight in 15 ml Falcon tubes with vigorous shaking, then left standing at room temperature for 1 h. Cell aggregation was determined by visual estimation of the cell sediment at the bottom of the Falcon tube.

**Extracellular polysaccharide determination.** Cellulose in the growth medium was determined by measuring the glucose reducing units produced after treatment with cellulase. Cultures (50 ml) were grown overnight at either 30 °C or 37 °C in M9Glu/sup medium. The OD<sub>600</sub> of the cultures was very similar for all strains and conditions used. Cells were pelleted by centrifugation, and the culture supernatants were lyophilized. Dried culture supernatants were dissolved in water at 100 mg ml<sup>-1</sup> final concentration, and the resuspended culture supernatants were incubated with cellulase from *Trichoderma reesei* ATCC 26921 (5 mg ml<sup>-1</sup>, 30 U ml<sup>-1</sup>; Sigma) in sodium acetate buffer (pH 5.0) at 37 °C for 16 h. Glucose released from cellulose by cellulase digestion was estimated with the procedure described by Somogyi (1952) for the determination of reducing sugar units. As a standard for cellulose quantitative determination, we used a carboxymethylcellulose solution (5 mg ml<sup>-1</sup>) in sodium acetate buffer. For both culture supernatants and carboxymethylcellulose solution three different volumes (5, 15 and 30 µl) were incubated with cellulase. The amounts of sugar released by carboxymethylcellulose degradation were used as calibration curve. For culture supernatants, the amounts of reducing sugars released by cellulase treatment were expressed as the percentage of total dry mass after lyophilization. In culture supernatants of the *bcs* mutant LG26 strain, used as negative control, the percentage of glucose released by cellulase treatment never exceeded 0.05 % (Fig. 1b).

**Gene expression determination by real-time PCR.** For RNA isolation, strains were grown in M9Glu/sup either at 30 °C or at 37 °C overnight to OD<sub>600</sub> 1.0 (early stationary phase). Cells were harvested by centrifugation at 10 000 g for 5 min at 4 °C, and total RNA was extracted using the RNeasy Mini kit (Qiagen). RNA samples were checked by agarose gel electrophoresis to assess lack of degradation, and quantified spectrophotometrically. Genomic DNA was removed by DNase I treatment. Reverse transcription was performed on 1 µg 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 using the SYBR Green PCR master mix, and the results were determined with an iCycle iQ Real-Time detection system (Bio-Rad). Reaction mixtures (25 µl) included 0.1 µg cDNA and 300 nM primers in the reaction buffer and enzyme supplied by the manufacturer. Primer sequences are listed in Table 1. All reactions were performed in triplicate, including negative control samples, which never showed significant threshold cycles. The relative transcript amounts were determined using 16S rRNA as the reference gene:  $C_{t(\text{Gene of interest})} - C_{t(16S)} = \Delta C_t$  value.

**Other methods.** Bacteriophage P1vir transductions were carried out as described by Miller (1972). For overexpression of the AdrA protein, the *adrA* gene was amplified by PCR and the PCR product directly cloned into the pTOPO vector. The correct orientation of the *adrA* insertion (i.e. under the control of the *lac* promoter) was confirmed by digestion with *EcoRI/EcoRV*, which gives two distinct digestion patterns depending on orientation of the *adrA* gene.

Desiccation experiments were performed as follows. Overnight cultures grown at 30 °C in M9Glu/sup were diluted 1 : 100 in H<sub>2</sub>O.



**Fig. 1.** (a) Biofilm formation on microtitre plates of *E. coli* MG1655 (wild-type, WT), PHL856 (*csgA::kan*) and LG26 ( $\Delta bcs::kan$ ) transformed with either pT7-7 or pT7CsgD, grown either at 30 °C (white bars) or at 37 °C (black bars). Surface-attached cells were quantified by spectrophotometric determination after CV staining and resuspension in ethanol, as described in Methods. Values are the mean of four independent experiments; standard deviation was always less than 15% of the mean. (b) Determination of cellulose production in *E. coli* MG1655 (WT), PHL856 (*csgA::kan*) and LG26 ( $\Delta bcs::kan$ ) transformed with either pT7-7 or pT7CsgD, grown either at 30 °C (white bars) or at 37 °C (black bars). The values indicate the amount of cellulose as percentage of total dry weight of the culture supernatant after lyophilization. Values are the mean of two independent experiments which gave very similar results.

To determine bacterial concentration in the suspension before desiccation, 20 µl was spotted on a glass slide, to which 80 µl H<sub>2</sub>O was added immediately. Serial dilutions (10<sup>-2</sup> to 10<sup>-5</sup>) were plated on LB agar. Typical c.f.u. ml<sup>-1</sup> values for the bacterial suspensions used in the desiccation experiments ranged between 10<sup>7</sup> and 2.5 × 10<sup>7</sup>. For the desiccation assay, 20 µl of the bacterial suspension was spotted on a glass slide and allowed to air-dry at 30 °C for 1 h, a time sufficient for full drying of the suspension drop. The dried suspension was resuspended in 100 µl H<sub>2</sub>O, and serial dilutions (10<sup>-1</sup> to 10<sup>-4</sup>) were plated on LB agar and incubated overnight at 37 °C. The percentage of cells surviving drying was calculated as recovered cells (c.f.u. ml<sup>-1</sup>) divided by the number of cells (c.f.u. ml<sup>-1</sup>) spotted on the glass slide. Efficient recovery of bacterial cells from the glass slide after exposure to dryness was verified by direct microscopic observation (at 100 × magnification).

Curli subunit determination by SDS-PAGE was performed after formic acid solubilization of membrane-associated proteins. Samples

(50 ml) of cultures grown in M9Glu/sup at 30 °C for 18 h were centrifuged at 4000 g for 10 min at 4 °C and washed with 5 ml 0.1 M sodium phosphate buffer pH 7.0 (PB). Cells were resuspended in 1 ml PB with addition of 100 µg lysozyme ml<sup>-1</sup> and 1 mM EDTA pH 8.0 and incubated at room temperature for 10 min. Cells were disintegrated using a French press and centrifuged at 30 000 g for 30 min. The pellet was dissolved in 1 ml PB and treated with formic acid as described by Collinson *et al.* (1991).

## RESULTS

### Role of curli and cellulose in biofilm formation in *E. coli* MG1655

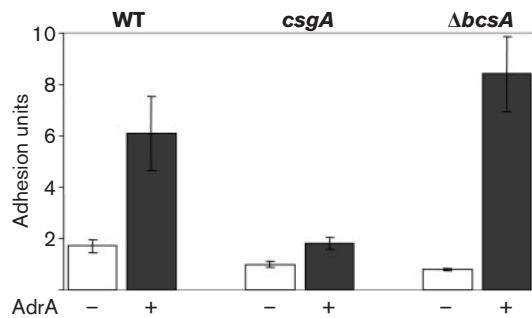
The MG1655 laboratory strain of *E. coli* is proficient in curli production, as determined by its red phenotype on agar medium supplemented with Congo red stain (CR medium); the red phenotype of MG1655 on CR medium is totally abolished by inactivation of the *csgA* gene, encoding the major subunit of curli (see Supplementary Fig. S1, available with the online version of this paper). However, despite efficient curli production, MG1655 only shows limited ability to form biofilm on polypropylene microtitre plates (Fig. 1a), consistent with previous observations (Vidal *et al.*, 1998; Prigent-Combaret *et al.*, 2001; Gualdi *et al.*, 2007). Inactivation of the *csgA* gene, but not of the cellulose biosynthetic *bcsA* gene, results in total loss of biofilm formation (Fig. 1a). Transformation of MG1655 with plasmid pT7CsgD greatly increases biofilm formation by MG1655, and this increase is totally dependent on curli production (Fig. 1a; Gualdi *et al.*, 2007). In plasmid pT7CsgD, the *csgD* gene is under the control of a T7 RNA polymerase-dependent promoter, which is only weakly recognized by *E. coli* RNA polymerase (Brown & Campbell, 1993). Presence of pT7CsgD results in a roughly 60-fold increase of the *csgD* gene transcript (see Table 3).

In *E. coli* and *Salmonella*, expression of the *csgBA* genes, in turn leading to curli production and biofilm formation, is typically temperature-regulated and can take place at ≤30 °C, but not at 37 °C (Olsen *et al.*, 1993; Romling *et al.*, 1998a; Prigent-Combaret *et al.*, 2001; Castonguay *et al.*, 2006). However, transformation of MG1655 with pT7CsgD results in dramatically stronger biofilm formation at 37 °C compared to 30 °C (Fig. 1a). Biofilm formation experiments performed in glass tubes gave very similar results to those in microtitre plates, confirming the increased surface adhesion properties at 37 °C of the MG1655/pT7CsgD strain (data not shown). Biofilm formation at 37 °C is totally dependent on a functional *csgA* gene, suggesting that it is still mediated by curli production (Fig. 1a). However, *csgBA* operon transcription levels at 30 °C and 37 °C are comparable in MG1655/pT7CsgD (Table 3), suggesting that increased biofilm formation at 37 °C in this strain is not mediated by a corresponding increase in curli production. These observations could suggest that curli fibres might synergize with an additional adhesion factor that is only expressed at 37 °C. To identify such a factor, we analysed the membrane

protein expression pattern (to verify temperature-dependent production of proteinaceous adhesion factors), and we determined production of extracellular polysaccharides (EPS) in MG1655/pT7CsgD, both at 30 °C and at 37 °C. While the membrane protein pattern failed to show any significant difference in CsgD-dependent protein expression at either growth temperature (data not shown), analysis of EPS production pointed to a clear temperature dependence in the amount of cellulose production. Indeed, presence of plasmid pT7CsgD resulted in a significant increase in the amount of cellulose found in the culture medium after cell removal by centrifugation (cellulose amounted to 9.8% of culture supernatant dry weight for MG1655/pT7CsgD versus 0.7% for MG1655 harbouring the pT7-7 control vector; Fig. 1b). However, pT7CsgD-mediated increase in cellulose production was not detectable at 37 °C (Fig. 1b), i.e. in the conditions in which stimulation of biofilm formation by pT7CsgD is greater. Thus, cellulose production negatively correlates with surface adhesion in MG1655/pT7CsgD. Cellulose production was not affected by *csgA* inactivation, while, as expected, it was totally abolished in LG26, the *bcsA* mutant derivative of MG1655 (Fig. 1b). Chemical analysis identified glucose as the sole detectable sugar in the EPS recovered from the culture medium, indicating that cellulose is indeed the main component of EPS in the strains and conditions tested (data not shown).

To test the hypothesis that cellulose might act as a negative determinant for biofilm formation, we constructed an MG1655 derivative in which the *bcsA* gene, necessary for cellulose biosynthesis (Zogaj *et al.*, 2001), is deleted. To this end, we transferred a  $\Delta bcsA::kan$  cassette to MG1655 by P1vir phage transduction, obtaining strain LG26. In contrast to the effects of *csgA* inactivation, which totally abolishes biofilm formation by MG1655 even in the presence of plasmid pT7CsgD, deletion of the *bcsA* gene had very little effect on MG1655, but it strongly stimulated adhesion to microtitre plates at 30 °C in the presence of pT7CsgD, suggesting that cellulose can negatively affect curli-mediated biofilm formation (Fig. 1a). At 37 °C, deletion of the *bcsA* gene had very little effect on biofilm formation, consistent with lack of cellulose production at this temperature.

To further test the role of cellulose in biofilm formation, we overexpressed the AdrA protein, which stimulates cellulose production via the c-di-GMP signal molecule (Simm *et al.*, 2004). As shown in Fig. 2, AdrA overexpression resulted in a significant increase in surface adhesion by MG1655 at 30 °C; this increase, however, was totally dependent on a functional *csgA* gene, thus suggesting that AdrA-induced adhesion properties are mediated by curli production, in agreement with recent results showing feedback regulation of the CsgD regulon by the AdrA protein (Kader *et al.*, 2006). In contrast, deletion of the cellulose biosynthesis *bcsA* gene led to an increase in surface adhesion, consistent with cellulose being a negative determinant for biofilm formation. At 37 °C, no detectable



**Fig. 2.** Effect of AdrA overexpression on surface adhesion. Strains MG1655 (WT), PHL856 (*csgA::kan*) and LG26 ( $\Delta bcsA::kan$ ) were transformed either with the pTOPO vector (indicated as AdrA '-'), white bars) or with pTOPOAdrA (AdrA '+', black bars) and grown overnight in M9Glu/sup at 30 °C prior to determination of surface adhesion by CV staining. Values were calculated as indicated in Methods and are the mean  $\pm$  SD of three independent experiments.

effects of AdrA overexpression on biofilm formation could be detected (data not shown).

Our results strongly suggest that, despite being produced in a co-ordinated fashion with curli fibres, cellulose counteracts curli-mediated biofilm formation on polypropylene (microtitre plates) and glass tubes. Thus, in order to understand the role of cellulose in *E. coli* MG1655, we performed bacterial cell aggregation (a biofilm-related process) tests and also tested resistance to desiccation. Several reports point to a role for cellulose in resistance to environmental stresses such as desiccation (Gibson *et al.*, 2006) or resistance to chlorine treatment (Solano *et al.*, 2002) in *Salmonella* species. As shown in Table 2, MG1655 grown in M9Glu/sup did not form any detectable cell

**Table 2.** Cell aggregation properties and resistance to desiccation

Strain	Cell aggregation		Cells surviving exposure to dry conditions (%; $\pm$ SD)*
	30 °C	37 °C	
MG1655/pT7-7	-	-	1.8 ( $\pm$ 0.14)
MG1655/pT7CsgD	-	++	7.6 ( $\pm$ 0.25)
PHL856 ( <i>csgA::kan</i> )/pT7-7	-	-	0.5 ( $\pm$ 0.07)
PHL856 ( <i>csgA::kan</i> )/pT7CsgD	-	-	3.5 ( $\pm$ 0.17)
LG26 ( $\Delta bcsA::kan$ )/pT7-7	-	-	0.3 ( $\pm$ 0.06)
LG26 ( $\Delta bcsA::kan$ )/pT7CsgD	++	++	1.6 ( $\pm$ 0.11)

\*Cells were incubated for 1 h at 30 °C, and values (mean of three experiments) were determined as described in Methods.

**Table 3.** Relative expression of curli- and cellulose-related genes

	<i>csgB</i>	<i>csgD</i>	<i>csgG</i>	<i>adrA</i>
MG1655/pT7-7 30 °C	1*	1*	1*	1*
MG1655/pT7-7 37 °C	0.014	0.11	0.032	0.42
MG1655/pT7CsgD 30 °C	2430	60.8	15.3	337
MG1655/pT7CsgD 37 °C	1518	33.5	2.25	1.23

\* $\Delta C_t$  between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655/pT7-7 grown at 30 °C and expressed as relative values for other strains or growth conditions. Actual  $\Delta C_t$  values for MG1655/pT7-7 were: *csgB*, 12.1; *csgD*, 9.2; *csgG*, 12.4; *adrA*, 16.5.

aggregates; however, MG1655/pT7CsgD displayed a strong aggregative phenotype at 37 °C, but not at 30 °C. Deletion of the *bcsA* gene resulted in pT7CsgD-dependent cell aggregation even at 30 °C, suggesting that cellulose plays a negative role in cell aggregation as well as in biofilm formation. In contrast, lack of cellulose biosynthetic genes resulted in a four- to fivefold decrease in sensitivity to desiccation (Table 2). Thus, our results would suggest that, in MG1655, cellulose function might be more related to resistance to environmental stresses rather than to biofilm formation and cell aggregation.

### Temperature-dependent regulation of curli- and cellulose-related genes

Our results suggest that, despite constitutive expression of the CsgD protein in MG1655/pT7CsgD, cellulose biosynthesis does not take place at 37 °C, in contrast to curli production (Fig. 1b). To better understand the mechanism of temperature regulation of both curli- and cellulose-related genes, we performed gene expression experiments on the cellulose-related *bcsA* (cellulose biosynthesis) and *adrA* (regulator of cellulose production) genes, as well as on the *csgG* and *csgB* genes, which were chosen as representative of the two operons involved in curli biosynthesis (*csgDEFG* and *csgBA*), and on the *csgD* gene, to estimate the extent of its overexpression in MG1655/pT7CsgD (Table 3). In order to evaluate the specific effects of CsgD overexpression, we compared MG1655/pT7CsgD to MG1655 transformed with the pT7-7 vector. Transcription of the *bcsA* gene was not affected by CsgD overexpression (data not shown), suggesting that expression of the *bcs* operon is independent of CsgD, as previously reported for *Salmonella* (Romling *et al.*, 2000). At 30 °C, CsgD overexpression from pT7CsgD results in increased transcription of both the curli-encoding *csgBA* operon and the *adrA* gene. In addition, CsgD can stimulate transcription from its own promoter, as indicated by *csgG* transcription levels (Table 3), in agreement with previous observations (Gualdi *et al.*, 2007). In contrast, CsgD overexpression at 37 °C results in different levels of transcription activation at the promoters tested. Indeed, while CsgD can still activate the *csgB* promoter, indicating

that its overexpression can bypass *csgB* temperature regulation, it fails to stimulate *adrA* expression (Table 3), suggesting tight temperature control at the *adrA* promoter. Finally, autoregulation of the *csgDEFG* operon by CsgD appears to still take place at 37 °C, although overall *csgDEFG* transcription levels are much lower at 37 °C than at 30 °C even in MG1655/pT7CsgD (Table 3).

We also tested transcription levels of curli and cellulose-biosynthetic genes in LG20, a *crl::cam920* derivative of MG1655, and we found that Crl is absolutely necessary for their transcription, at either growth temperature and regardless of the presence of pT7CsgD (data not shown).

## DISCUSSION

The CsgD regulatory protein activates transcription of the *csgBA* operon and the *adrA* gene, thus leading to production of two extracellular structures: curli fibres and cellulose. Co-ordinated production of both curli and cellulose was initially proposed to be functional in cell adhesion and biofilm formation (Romling *et al.*, 2000). However, a more recent report pointed to a negative role for cellulose in curli-mediated interaction of a commensal isolate of *E. coli* with the human epithelial cell line HT-29 (Wang *et al.*, 2006). This observation would suggest that cellulose can inhibit, rather than stimulate, the function of curli as cell adhesion determinants. The results in our report are consistent with such a notion, strongly suggesting that cellulose overproduction in a *csgD*-overexpressing derivative of the MG1655 laboratory strain of *E. coli* negatively affects both biofilm formation (Fig. 1) and cell aggregation (Table 2). Cellulose can stimulate, albeit weakly, biofilm formation in MG1655 (Fig. 1); however, in *adrA*- or *csgD*-overexpressing MG1655 derivatives, cellulose either fails to significantly affect or strongly impairs curli-dependent biofilm formation (Fig. 2). These results indicate that cellulose might act as a weak adhesion factor in strains poorly efficient in curli production; however, its production is detrimental for biofilm formation in bacterial strains that strongly produce curli (Figs 1 and 2), thus suggesting that cellulose modulates the cell's adhesion properties. Cellulose production does not inhibit curli-dependent biofilm formation through negative regulation of curli-encoding genes (data not shown), nor does it appear to prevent curli assembling on the bacterial cell surface, since similar amounts of formic-acid-soluble curli fibres were recovered from the cell membrane-associated protein fraction regardless of cellulose production (Supplementary Fig. S2). Thus, a possible mechanism for inhibition of curli-mediated adhesion by cellulose might be physical masking of curli fibres by excessive cellulose production, similar to the effect of other capsular polysaccharides on proteinaceous adhesion factors described by Schembri *et al.* (2004). Shielding of curli fibres by cellulose might be even more pronounced in cells growing on plates, as suggested by the observation that cellulose production in the MG1655/pT7CsgD at 30 °C

leads to partial loss of red phenotype on Congo-red-supplemented medium (Supplementary Fig. S1).

In contrast to the effect on biofilm formation, cellulose appears to play a role in protection against environmental stresses such as desiccation (Table 2), in agreement with previous observations in *Salmonella* spp. (White *et al.*, 2006), and as also reported for other capsular polysaccharides and extracellular structures (Hagiwara *et al.*, 2003; Gibson *et al.*, 2006). Interestingly, even *csgA* inactivation results in a decreased resistance to desiccation (Table 2), suggesting that the curli/cellulose network might confer better protection against desiccation than cellulose alone. While our results confirm the role of cellulose in resistance to environmental stresses, they suggest that cellulose acts as a negative determinant for biofilm formation, in contrast to previous observations in *Salmonella* (Romling *et al.*, 2000; Solano *et al.*, 2002). Such discrepancies could depend on the pattern of adhesion factors produced by different bacterial species: indeed, it is possible that cellulose, rather than acting directly as a biofilm determinant, might modulate either expression or surface exposure of adhesion factors, thus affecting adhesion properties in a species-specific, and even strain-specific, fashion. Finally, it must be stressed that we only tested one experimental system for biofilm formation, and we cannot rule out that cellulose might positively contribute to biofilm formation in other conditions or model systems.

Interestingly, in contrast to the curli-related *csg* operons, the *adrA* gene, encoding a diguanylate cyclase regulating cellulose production, is not activated by constitutive expression of CsgD at 37 °C (Table 3), thus suggesting tighter temperature-dependent control of its transcription. Similar to *csgBA*, *adrA* requires a functional *crl* gene for its expression (data not shown), consistent with previous observations in *Salmonella enterica* (Robbe-Saule *et al.*, 2006).

Our results suggest that, when expressed at 37 °C, CsgD would activate transcription of the curli-encoding *csgBA* operon, but not of the cellulose biosynthetic *adrA* and *bcs* genes. Although in our experiments we used a strain in which CsgD is expressed ectopically, i.e. from plasmid pT7CsgD, our results are consistent with previous observations on *E. coli* isolates expressing curli-encoding genes in a temperature-independent manner. Indeed, in enterohaemorrhagic strains of *E. coli* (Uhlich *et al.*, 2001, 2006), mutations in the *csgDEFG* promoter leading to increased CsgD expression result in increased expression of curli-encoding genes at 37 °C, but fail to stimulate cellulose production. However, ability to produce either curli or cellulose, or both, at 37 °C is likely to be influenced by specific genetic backgrounds. Indeed, in a survey of *E. coli* isolates from humans, it was reported that, when grown at 37 °C, 21 % of the isolates were able to express both curli and cellulose, while 23 % were only able to produce curli (Bokranz *et al.*, 2005). Thus, mutations resulting in sole expression of curli, but not cellulose, at 37 °C, are rather

common in *E. coli* strains isolated from humans. These results seem to suggest that mutations leading to increased curli production at 37 °C might be well tolerated, or even advantageous for some *E. coli* isolates, possibly due to the role of curli in internalization of bacteria by eukaryotic cells (Gophna *et al.*, 2001). In contrast, mutations leading to production of only cellulose, but not curli, at 37 °C are highly unusual, perhaps suggesting that they might be unfavourable in the mammalian host. Finally, inactivation of the *csgD* gene by insertion sequences is a common trait of *Shigella* spp., and loss of expression of *csgD*-dependent genes is considered a pathoadaptive mutation (Sakellaris *et al.*, 2000). These different genetic and mutational patterns leading to different expression patterns of *csgD*-dependent genes at 37 °C probably reflect their highly complex regulation, as well as the multifaceted role played by curli and cellulose in the interaction between bacterial cells and the host environment.

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