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Tight Modulation of *Escherichia coli* Bacterial Biofilm Formation through Controlled Expression of Adhesion Factors▽†

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Despite the economic and sanitary problems caused by harmful biofilms, biofilms are nonetheless used empirically in industrial environmental and bioremediation processes and may be of potential use in medical settings for interfering with pathogen development. *Escherichia coli* is one of the bacteria with which biofilm formation has been studied in great detail, and it is especially appreciated for biotechnology applications because of its genetic amenability. Here we describe the development of two new genetic tools enabling the constitutive and inducible expression of any gene or operon of interest at its native locus. In addition to providing valuable tools for complementation and overexpression experiments, these two compact genetic cassettes were used to modulate the biofilm formation capacities of *E. coli* by taking control of two biofilm-promoting factors, autotransported antigen 43 adhesin and the *bscABZC* cellulose operon. The modulation of the biofilm formation capacities of *E. coli* or those of other bacteria capable of being genetically manipulated may be of use both for reducing and for improving the impact of biofilms in a number of industrial and medical applications.

Most natural and artificial surfaces available in the environment are prone to bacterial colonization. Following the initial adhesion event, cell-to-cell adhesion and the secretion of an extracellular matrix rapidly lead to the formation of a surface-attached multicellular structure known as a biofilm (3, 43, 75, 84). Besides being resistant to environmental shear forces, biofilm communities are also phenotypically more resistant to antibiotic and biocide treatments, a trait that poses important sanitary and economic problems. Indeed, biofilms formed by bacterial pathogens on medically relevant surfaces are difficult to eradicate and are thus often involved in the development of infections (12, 13, 48). Moreover, industrial biofouling resulting from bacterial biofilm formation is a major cause of pipe biocorrosion and reduces the efficiency of pharmaceutical or food bioprocesses (2, 10, 70, 71).

While recent studies have focused mainly on the negative impact of biofilms, bacterial biofilms can also have valuable applications, including those involving bioremediation and wastewater treatment bioreactor processes and the improvement of biomineralization or plant-bacteria symbiosis (1, 14, 40, 42, 52, 62, 73, 74, 80). Beneficial biofilms may also have medical applications, and the use of protective innocuous bacterial biofilms that interfere with the development of bacterial pathogens is considered a promising approach (19).

Escherichia coli is a gram-negative enterobacterium that has been used extensively as a model to study biofilm development due to its relevance to the human biotic environment and its genetic amenability. In *E. coli*, various cell surface appendages were shown to be necessary to achieve mature biofilm development (78). Flagella, type I fimbriae, and curli are implicated in early adhesion steps, while the production of a polysaccharide-rich matrix (cellulose, colanic acid, and poly-β-1,6-*N*-acetylglucosamine) and of short adhesins such as antigen 43 (Ag43) and conjugative plasmid pili contributes to biofilm maturation (11, 16–18, 30, 32, 59, 60, 63, 67, 79, 83, 85).

We previously showed that the ability of *E. coli* K-12 to form a biofilm could be enhanced by increasing the expression of specific adhesin genes (68). Here we describe the genetic engineering of different *E. coli* strains whose capacity to develop as biofilms was tightly controlled by the modulation of the expression levels of different biofilm-promoting factors. We developed new genetic tools designed to enable the cloning-free, site-directed insertion of either inducible or constitutive promoters in front of genes of interest. We chose genes coding for either adhesin Ag43 or cellulose production to show that this new expression strategy can be used to create plasmid-free *E. coli* strains with defined and tightly controlled biofilm-forming abilities. This approach may be used to improve the biofilm potential of laboratory and natural bacterial strains used in industrial and medical bioprocesses.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were constructed by transformation and the λ red linear DNA gene inactivation method (see below), followed by P1*vir* transduction into a fresh *E. coli* background when possible.

Growth conditions. All experiments were performed in M63B1 0.4% glucose minimal medium (M63B1glu) or in lysogeny broth (LB) medium at 37°C. The following antibiotics at the indicated concentrations were added when required: kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), and ampicillin (100 μg/ml). Repression-expression TetR-controlled (RExTET) cassette constructs were induced with anhydrotetracycline (aTc) at the concentrations indicated in the figures. As previously shown, we observed that aTc did not have any effect on bacterial growth at concentrations below 500 ng/ml (55).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
MG1655	F$^-$ lambda$^-$ *ilvG rfb-50 rph-1*	Laboratory collection
MG1655λ*att-kmgfp*	Insertion, at the λ*att* site, of the *gfp* gene under the control of the constitutive λp_R promoter; source of the *km*PcL and *km*PcLrbs cassettes; Km^r	This study
MG1655λ*att-ampgfp*	Insertion, at the λ*att* site, of the *gfp* gene under the control of the constitutive λp_R promoter; source of the *amp*PcL and *amp*PcLrbs cassettes; Amp^r	This study
MG1655Δ*flu*	Δ*flu*::*cat* Cm^r	68
MG1655Δ*oxyR*	Δ*oxyR*::*aph* Km^r	68
MG1655Δ*oxyR*Δ*flu*	Δ*oxyR*::*aph* Δ*flu*::*cat* Km^r Cm^r	68
MG1655*km*RExTETrbs-*flu*	*flu* placed under the control of the *km*RExTETrbs cassette $P_{LtetO-1}$ promoter; Km^r	This study
MG1655*km*PcL*flu*	*flu* with its own RBS sequence placed under the control of the *km*PcL cassette λp_R promoter; Km^r	This study
MG1655*km*RExTET*lacZ*	*lacZ* with its own RBS sequence placed under the control of the *km*RExTET cassette $P_{LtetO-1}$ promoter; Km^r	This study
MG1655*km*RExTETrbs-*lacZ*	*lacZ* placed under the control of the *km*RExTETrbs cassette $P_{LtetO-1}$ promoter; Km^r	This study
MG1655*km*PcL*lacZ*	*lacZ* with its own RBS sequence placed under the control of the *km*PcL cassette λp_R promoter; Km^r	This study
MG1655*km*PcLrbs-*lacZ*	*lacZ* placed under the control of the *km*PcLrbs cassette λp_R promoter; Km^r	This study
MG1655*amp*PcL*lacZ*	*lacZ* with its own RBS sequence placed under the control of the *amp*PcL cassette λp_R promoter; Amp^r	This study
MG1655*amp*PcLrbs-*lacZ*	*lacZ* placed under the control of the *amp*PcLrbs cassette λp_R promoter; Amp^r	This study
1094	*E. coli* commensal strain	18
1094Δ*bcsABZC*	Δ*bcsABZC*::*aph* Km^r	18
1094*km*RExTETrbs-*bcsA*	*bcsABZC* operon placed under the control of the *km*RExTETrbs cassette $P_{LtetO-1}$ promoter; Km^r	This study
DH5αZ1	DH5α with integrated transcription units encoding LacI and TetR at the λ*att* site	49
Plasmids		
pZE21-gfp	*gfp* under the control of the synthetic $P_{LtetO-1}$ promoter; Km^r	49
pZEtetR21-gfp	Same as pZE21-gfp with an insertion of P_{N25}-*tetR*-T1 between the *nptII* gene and the terminator t_0; Km^r	This study
pZE2R-gfp	*gfp* under the control of the constitutive λp_R promoter; Km^r	Gift from C. C. Guet
pZE1R-gfp	*gfp* under the control of the constitutive λp_R promoter; Amp^r	Gift from C. C. Guet
pAg43	P*flu*::*lacZ* transcriptional fusion in pQF50; Amp^r	81

[a] Km^r, kanamycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

Three-step PCR. In order to place chromosomal target genes (*lacZ*, *flu*, and the *bcsABZC* operon) under the control of the RExTET cassette and the constitutive lambda promoter (PcL) cassettes, we used a three-step PCR procedure as described in references 9, 20, 21, and 47 and detailed at http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html. The primers used to insert the cassettes upstream of the target genes are listed in Table 2.

Construct verification. All constructs were checked by PCR with specific primers (Table 2). The integrity of the cassettes was verified by sequencing the junction between the RExTET cassette or the PcL cassette and the target gene by using primers described in Table 2.

β-Galactosidase activity assay. To determine the level of β-galactosidase enzyme activity, the different cultures were grown in LB for 8 h. Cultures were then diluted 1:100 in LB or M63B1glu medium containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) or various amounts of aTc when needed and grown overnight (16 to 18 h) at 37°C. The enzyme activity of each strain was assayed in triplicate as described in reference 50 and expressed in arbitrary Miller units.

Immunodetection of Ag43 and immunofluorescence microscopy. Amounts of overnight cultures equivalent to an optical density at 600 nm (OD_{600}) of 0.2 were analyzed on sodium dodecyl phosphate–10% polyacrylamide gel electrophoresis gel, followed by the immunodetection of Ag43. Equivalent loads of proteins in the lanes were verified by staining the nitrocellulose membranes with Ponceau S. Immunodetection was performed using a 1:10,000 dilution of polyclonal rabbit antiserum raised against the α domain of Ag43, a kind gift of P. Owen.

Immunofluorescence microscopy analysis was performed as follows. Overnight cultures of the different strains were grown at 37°C in LB medium without aTc or, for strain MG1655*km*RExTETrbs-*flu*, with 50 ng of aTc/ml. Cells were diluted to an OD_{600} of 1 in LB medium, and an aliquot was loaded onto 0.1% poly-L-lysine-treated immunofluorescence microscope slides. Slides were washed three times with phosphate-buffered saline (PBS) between each step of this protocol. Cells were fixed with 3% paraformaldehyde for 10 min before quenching with 50 mM NH_4Cl in PBS for 3 min. Slides were then saturated for 15 min with 0.5% bovine serum albumin in PBS before being incubated first for 45 min with a 1:1,000 dilution of primary polyclonal rabbit antiserum raised against the α domain of Ag43 and next with a 1:300 dilution of a secondary polyclonal goat anti-rabbit serum coupled to Alexa488 (Molecular Probes-Invitrogen) along with 10 μg of 4′,6-diamidino-2-phenylindole (DAPI)/ml. Finally, the slides were mounted with Mowiol 4088 (Calbiochem) and observed under an epifluorescence microscope with green fluorescent protein and DAPI filters.

Ag43 switching frequency. The Ag43 switching frequency was calculated as previously described (58, 81). Five white (phase-"off") and five blue (phase-"on") LB-grown colonies of the MG1655/pAg43 strain were serially diluted for examination. Dilutions were plated onto LB agar supplemented with 100 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml, and plates were incubated at 37°C. Both total counts of viable cells (*N*) and the number of

TABLE 2. Primers used in this study

Primer name	Sequence	Target gene
Primers used to generate *km*RExTET and PcL cassette insertions		
lacZACTIV.A1.500-5	CTCAGGTCAAATTCAGACGGC	*lacZ*
lacZACTIV.B1.500-3	CTCAGGTCAAATTCAGACGGC	*lacZ*
lacZ.CATptetO.A2.L-3	GAGAATCCAAGCACTAGTAACCACAATTCCACACAACATA	*lacZ*
lacZ.CATptetO.B2.L-5	GCACATCAGCAGGACGCACTGACAGCGGATAACAATTTCACAC	*lacZ*
lacZ.CATptetOB2LrbstetO5	CATTAAAGAGGAGAAAGGTACCATGACCATGATTACGGATTC	*lacZ*
lacZ.ext-5	CATTGGGTCACCAGCAAATC	*lacZ*
lacZ.CATBAD.ext-3	CCAGATAACTGCCGTCACTC	*lacZ*
flu.KmRExTET.500-5	CCCGAATTCTGCGGTGGACCGGATATTTG	*flu*
flu.KmRExTET.500-3	ATGACGGTTCCTGTGGCTATC	*flu*
flu.KmRExTET.ext-3	GCCCGGTATCACCGTTTTCTG	*flu*
flu.KmRExTET.ext-5	ATACGCTGGTCAGTGCGCTC	*flu*
flu.KmRExTET.Lbrs-5	CATTAAAGAGGAGAAAGGTACCATGAAACGACATCTGAATAC	*flu*
flu.KmRExTET.L-3	GAGAATCCAAGCACTAGTAACCACATTGAGGGTGAATAAAAAAG	*flu*
flu.PcL.A2.L-3	GTGAGAATTACTAACTTGAGCGAATTGAGGGTGAATAAAAAAG	*flu*
flu.PcL.B2.L-5	CGGTGATAATGGTTGCATGTACTATCTAAGGAAAAGCTGATGAAACGA	*flu*
bcsA-ext-5	CGCATTAGCCTGGTCATTAC	*bcsA*
yhjQ.KmRExTET.bcsA.ext-3	AGAAATCAGCGAGAAGGTGAC	*bcsA*
KmRExTET.bcsA.L-3	GAGAATCCAAGCACTAGTAACCACTTATGATGCACTCCCGACTGGCGTTTTC	*bcsA*
yhjQ.KmRExTET.bcsA.Lbrs-5	CATTAAAGAGGAGAAAGGTACCatgaGTATCCTGACCCGGTGG	*bcsA*
yhjQ.KmRExTET.bcsA.ext-5	TTGTCTGATTATCAGTTTAC	*bcsA*
Primers used to verify cassette insertions		
KmRExTET.verif-5	GCGAAACGATCCTCATCCTG	
KmRExTET.verif-3	CATTGCTTATCAATTTGTTGC	
PcL-km-verif-5	CAGAGCAGCCGATTGTCTGTTG	
PcL-km-verif-3	CTTCCTCGTGCTTTACGGTATCG	
PcL-amp-verif-5	CGAAAACTCTCAAGGATCTTAC	
PcL-amp-verif-3	TGGTTTATTGCTGATAAATCTG	
Primers used to sequence the junction between cassettes and the target gene ATG codon		
lacZATG + 100-3	GGGGGATGTGCTGCAAGGCGATTAAG	*lacZ*
fluATG + 100-3	GACGTGACTGCGGCAAGAGACAGTG	*flu*
KmRExTET.bcsA.500-3	GAGGATCAACCGCCGCGCCCCG	*bcsA*
Primers used to amplify *km*RExTET and PcL cassettes		
tetR.ptetOgfp.ampl-5	GAAGATCCTTTGATCTTTTC	
tetR.ptetOgfp.ampl-3	TGCCCATTAACATCACCATC	
km.Rex.Tet.ampli-5	CACTTTATGCTTCCGGCTCGTATG	
km.Rex.Tet.ampli-3	CGCCAGGGTTTTCCCAGTCACGAC	
PcL-ampli-5	CTCTGGCAAGCGCCTCGATTACTG	
PcL-ampli-3	CATCACCTTCACCCTCTCCACTGAC	
Primers used for pZEtet21-gfp plasmid construction		
tetR-T1-sacI-5	CGCGGGGAGCTCGCGCAACGCAATTAATGTAAG	
tetR-T1.sacI-3bis	CAGAACGAGCTCGATTTGTCCTACTCAGGAGAG	

colonies that switched from the phenotype of the original inoculum (*M*) were determined. Based on the assumption that predominantly phase-on and phase-off colonies are derived from phase-on and phase-off cells, respectively, the following equation was used to calculate the frequency of phase switching: switching frequency (per cell per generation) = [1 − *g*th root of (1 − *M*/*N*)], where *g* is the number of growth generations, calculated as $g = (\log N/\log 2)$.

Aggregation assay. Aggregation assays were performed as described in reference 68. Briefly, overnight cultures were adjusted to an OD_{600} of 2.5 by dilution with LB medium. Three-milliliter aliquots of cultures were incubated in 5-ml standing tubes at room temperature, and the OD_{600} of the upper parts of the cultures were measured every hour for 6 h and after 24 h before image capture. MG1655*km*RExTETrbs-*flu* cultures were grown in the presence of various concentrations of aTc.

Calcofluor phenotype assays. Two-microliter aliquots of overnight cultures grown at 37°C in LB medium (with added antibiotics and aTc when needed) were spotted onto LB plates containing 0.02% calcofluor (Sigma; reference no. F-3543) and 1 mM HEPES with or without 200 ng of aTc/ml. The spotted drops were allowed to dry, and the plates were incubated for 24 h at 30 or 37°C. The fluorescence of a spot under UV light revealed the binding of calcofluor, indicating cellulose production.

Biofilm formation assay in microfermentors. All biofilm formation experiments were performed in triplicate with M63B1glu minimal medium supplemented or not with 20 ng of aTc/ml at 37°C. Sixty-milliliter microfermentors containing a removable glass slide were configured as continuous-flow culture bioreactors with a 40-ml h^{-1} flow rate as described in references 5 and 30 and at http://www.pasteur.fr/recherche/unites/Ggb/biofilmfermenter.html. Because of

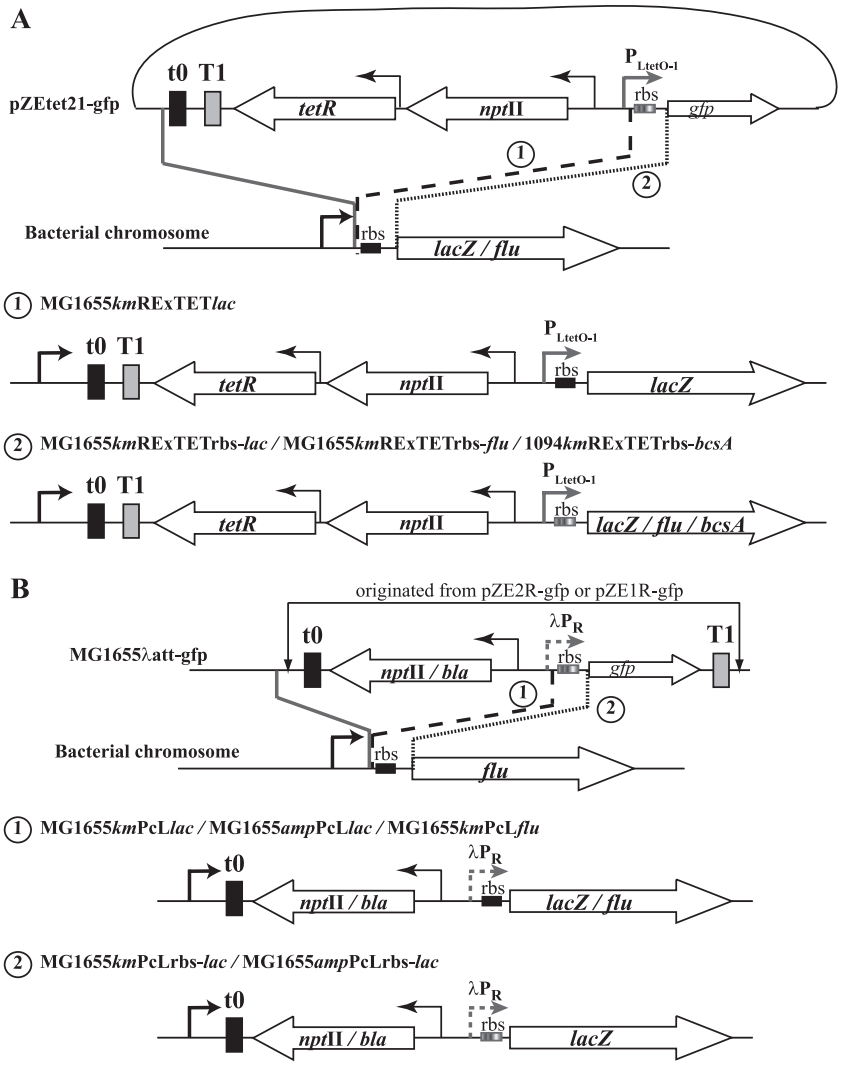

FIG. 1. Construction of the RExTET and PcL transcriptional fusions. (A) RExTET fusions were constructed in three steps, as follows: (i) cloning of the *tetR* gene with its own constitutive promoter and terminator T1 downstream of the *nptII* gene encoding resistance to kanamycin and upstream of the t_0 terminator, on the pZE21-gfp plasmid; (ii) amplification of the 2,537-bp *km*RExTET or the 2,555-bp *km*RExTETrbs cassette by using primers annealing to the 3′ end of the t_0 terminator and just upstream from the 5′ end of the RBS sequence (1; *km*RExTET; no RBS) or just upstream from the 5′ end of the *gfp* gene (2; *km*RExTETrbs; RBS present in the cassette); (iii) insertion of the *km*RExTET or *km*RExTETrbs cassette by three-step PCR upstream of the RBS sequence or at the start codon of the target gene (*lacZ*, *flu*, or *bcsA*, as indicated), respectively. (B) Construction of constitutive PcL fusions by (i) amplification of the 1,222-bp *km*PcL, 1,281-bp *amp*PcL, 1,254 *km*PcLrbs, or 1,313-bp *amp*PcLrbs cassette by using primers annealing to the 3′ end of the t_0 terminator and just upstream from the 5′ end of the RBS sequence (1; *km*PcL and *amp*PcL; no RBS) or just upstream from the 5′ end of the *gfp* gene (2; *km*PcLrbs and *amp*PcLrbs; RBS present in the cassette) and (ii) insertion of the PcL or PcLrbs cassette by three-step PCR upstream of the RBS sequence or at the start codon of the target gene (*lacZ* or *flu*, as indicated), respectively.

the different natures of the biofilms, for the study of *flu* expression or cellulose production, two distinct protocols were used to determine biofilm biomasses. In the case of the *flu* gene, overnight cultures grown in M63B1glu supplemented with appropriate antibiotics and 20 ng of aTc/ml when required were diluted to an OD_{600} of 2 in M63B1 medium. Microfermentors were inoculated by dipping the removable glass slides into 15 ml of the diluted cultures for 1 min, followed by a brief rinsing in M63B1 medium before insertion into the microfermentor. Biofilms were grown for 30 h under nonbubbling conditions, and pictures were taken before the resuspension of the biofilms in the microfermentors for OD_{600} measurements. In the case of the *bcsABZC* operon, microfermentors were inoculated by direct injection with an amount equivalent to an OD_{600} of 1 of overnight cultures grown in M63B1glu supplemented with appropriate antibiotics and 20 ng of aTc/ml when required. Biofilms were grown for 26 h under bubbling conditions before the resuspension of the biofilms in the microfermentors for OD_{600} measurements.

RESULTS

Construction of chromosomal inducible-promoter insertion cassettes. We recently described a selectable repression-expression cassette that places target genes under the control of the inducible P_{BAD} promoter directly at their native chromosomal loci (the RExBAD cassette) (68). While this cassette proved to be an appropriate tool in many applications, the use of a potential carbon source (here, the sugar arabinose) as an inducer may introduce a phenotypic bias. This issue is of particular relevance in the study of biofilms, in which the production of a polysaccharide-based matrix is strongly dependent on

bacterial sugar metabolism (see below). As an alternative to the arabinose-inducible RExBAD cassette, we chose to create a new repression-expression cassette based on a TetR-controlled system. TetR binds very tightly to the *tet* operators (*tetO*) of the *tetA* promoter of the Tn*10* tetracycline resistance operon (38). TetR-controlled expression systems have been used efficiently in different bacteria (25, 29, 41, 49, 61). This system is induced either by tetracycline, which displays a high affinity for the TetR repressor, or by the tetracycline derivative aTc, a gratuitous nonmetabolizable inducer. To generate the RExTET cassette, we first amplified the sequence encompassing the *tetR* gene controlled by the constitutive promoter P_{N25} up to transcription terminator T1 of the *rrnB* operon from strain DH5αZ1 (49). The PCR fragment was then cloned between the kanamycin resistance gene and the t_0 terminator from phage lambda into the pZE21-gfp plasmid, where the *gfp* gene is under the control of the $P_{LtetO-1}$ promoter (49), to create pZEtetR21-gfp (see Fig. 1A). pZEtetR21-gfp carries the resulting RExTET cassette, a compact genetic element composed of (i) the tandem and constitutively expressed *nptII* selectable marker gene (Km^r) and the TetR repressor-encoding gene and, (ii) in the opposite direction, the synthetic TetR-regulated and aTc-inducible $P_{LtetO-1}$ promoter. Terminators t_0 and T1 were placed downstream of the *tetR* gene to prevent read-through transcription from potential external promoters. The presence of the *tetR* gene in the RExTET cassette overcomes the need for a specific bacterial background that already expresses this gene. Moreover, the direct insertion of the RExTET cassette-controlled $P_{LtetO-1}$ promoter by using λ red-mediated homologous recombination can be done either with the original ribosome binding site (RBS) sequence of the chromosomal target gene (*km*RExTET cassette) (Fig. 1A, step 1) or with the RBS included in the cassette (*km*RExTETrbs) (Fig. 1A, step 2).

To test the functionality of the cassette in the pZEtetR21-gfp plasmid, *E. coli* strain MG1655 was transformed with the plasmid and the expression of the *gfp* gene was evaluated via the monitoring of fluorescence. In the absence of the aTc inducer, none of the cells were green, whereas in the presence of 1 μg of aTc/ml, all the cells were fluorescent (data not shown). This result indicated that a large amount of the TetR repressor was produced from the multicopy plasmid and that it could, in the absence of aTc, repress the $P_{LtetO-1}$ promoter, blocking *gfp* transcription. Plasmid pZEtetR21-gfp thus served as a template to amplify the 2,527-bp *km*RExTET and 2,555-bp *km*RExTETrbs cassettes.

Construction of chromosomal constitutive-promoter insertion cassettes. In order to enable the constitutive expression of chromosomal target genes at their original loci, we took advantage of the pZE2R-gfp and pZE1R-gfp plasmids, which carry *gfp* under the control of the constitutive λp_R promoter (Table 1).

To create the chromosomal *km*PcL and *amp*PcL cassettes, fragments containing the plasmid region from terminator t_0 to terminator T1 were amplified and inserted using λ red-mediated homologous recombination at the λ*att* site in strain MG1655. The resulting strains, MG1655λ*att-kmgfp* and MG1655λ*att-ampgfp*, constitutively expressed the green fluorescent protein and fluoresced (data not shown). They served as templates for the amplification of the 1,222-bp *km*PcL, 1,254-bp *km*PcLrbs, 1,281-bp *amp*PcL, and 1,313-bp *amp*PcLrbs cassettes (Fig. 1B). These cassettes comprise (i) a constitutively expressed selectable marker (kanamycin or ampicillin resistance) terminating with the t_0 terminator sequence and (ii) the constitutive λp_R promoter oriented in the opposite direction (Fig. 1B).

Inducible or constitutive expression of chromosomal target genes. To test the functionality of both the inducible RExTET and the constitutive PcL cassettes, we inserted the different cassettes upstream of the *lacZ* gene, between the native *lacZ* promoter and the *lacZ* RBS or start codon, in the *E. coli* K-12 MG1655 chromosome (Fig. 1). These events replaced the native *lacZ* promoter with the aTc-inducible promoter $P_L tet_{O-1}$ or with the constitutive λp_R promoter, creating strains MG1655*km*RExTET*lacZ* (with the native *lacZ* RBS sequence) or MG1655*km*RExTETrbs-*lacZ* (with the cassette's RBS sequence) and MG1655*km*PcL*lacZ* and MG1655*amp*PcL*lacZ* (with the native *lacZ* RBS sequence) or MG1655*km*PcLrbs-*lacZ* and MG1655*amp*PcLrbs-*lacZ* (with the cassette's RBS sequence), respectively.

All site-directed promoter replacements resulted in functional reporter transcriptional *lacZ* fusions, as shown in Fig. 2. PcL cassette-*lacZ* fusions containing the *lacZ* RBS (PcL*lacZ* fusions), associated with either ampicillin or kanamycin resistance, displayed a level of constitutive activation of *lac*Z comparable to that of the naturally occurring induction of *lacZ* expression by IPTG in a wild-type background. Interestingly, the transcriptional activation of PcL cassette-*lacZ* fusions containing the cassette's RBS (PcLrbs-*lacZ* fusions) resulted in β-galactosidase activities that were at least threefold higher than those resulting from the transcriptional activation of PcL*lacZ* fusions (Fig. 2). This finding suggests that the RBS sequence of the PcL cassette (present in PcLrbs-*lacZ* fusions) promotes more efficient translation than the native *lacZ* RBS.

To evaluate the extent of the modulation of target gene expression by the *km*RExTET cassette, we assessed the range of expression of the *lacZ* gene in cultures supplemented with various concentrations of aTc (Fig. 2). Both *km*RExTET and *km*RExTETrbs cassettes displayed a strongly repressed state in the absence of an inducer, with no detectable β-galactosidase activity. Progressive induction was achieved with increasing aTc concentrations of up to 10 ng/ml; higher concentrations led to a steep induction that quickly reached a plateau at around 20 ng of aTc/ml for the *lacZ* RBS-containing cassette (*km*RExTET) and around 35 ng of aTc/ml for the cassette containing the cassette's RBS (*km*RExTETrbs). A concentration as low as 10 ng of aTc/ml was sufficient to induce a wild-type level of transcriptional activation (with IPTG) of the *km*RExTETrbs-*lacZ* fusion.

As observed for the constitutive λp_R promoter of the PcL cassettes, the origin of the RBS had an influence on the final level of induction of the inducible $P_{LtetO-1}$ promoter of the RExTET cassette. The same activity was exhibited by the fully induced *km*RExTETrbs-*lacZ* and *km*PcLrbs-*lacZ* and *amp*PcLrbs-*lacZ* fusions, which was approximately 1.5-fold the maximal activity of the *km*RExTET-*lacZ* fusion (Fig. 2). In the case of *lacZ*, the RBS from the cassettes seemed better suited to achieving high levels of activity.

These results show that the RExTET cassette is tightly repressed in the absence of aTc and can be used to modulate target gene expression levels, from no expression to a level

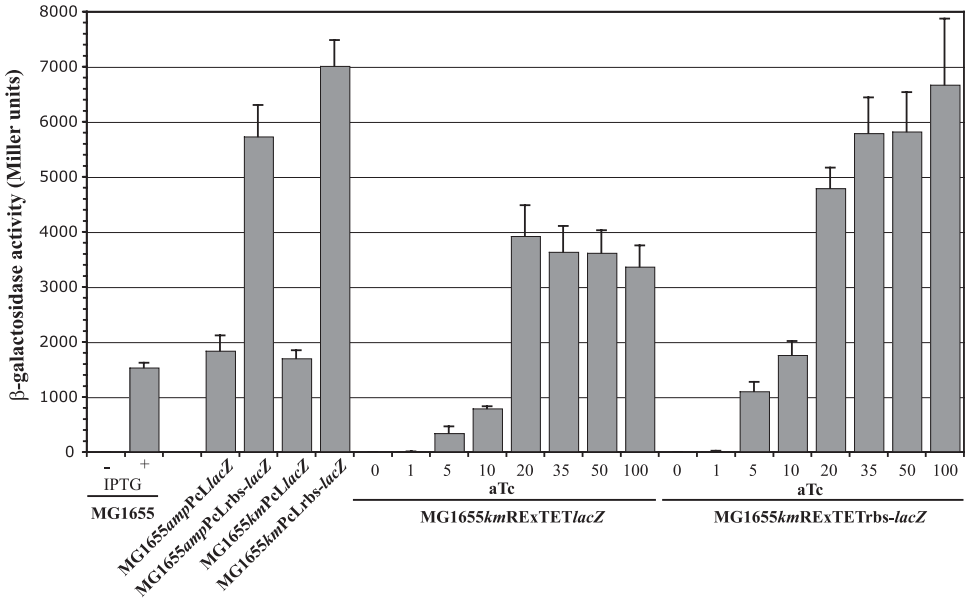

FIG. 2. Gene expression can be modulated by RExTET cassettes and can be taken over by PcL cassettes. β-Galactosidase activity measurements of RExTET and PcL*lacZ* fusions. Strains were grown up until stationary phase in M63B1glu medium at 37°C. Concentrations (nanograms per milliliter) of aTc, when added, are indicated below the bars. MG1655 grown in the presence of 1 mM IPTG was considered to demonstrate the wild-type expression of the *lac* operon. The experiments were performed in triplicate; error bars represent standard deviations of the means. All the results were qualitatively the same with cultures grown in LB medium (data not shown), with slight changes in the levels of enzymatic activity and aTc concentrations needed to reach the plateau. +, present; −, absent.

approximately fourfold higher than the wild-type level of activation.

Modulation of autotransported Ag43 adhesin production. To demonstrate that RExTET and PcL expression systems can be used to control biofilm-promoting factors in *E. coli*, we introduced into the *E. coli* K-12 strain MG1655 the *km*RExTETrbs and *km*PcL cassettes upstream of *flu* (3.120 kb), the gene encoding the self-recognizing autotransported adhesin Ag43 (23, 57). Autotransporter proteins possess a modular structure with a C-terminal β domain allowing the insertion of the protein into the outer membrane and an N-terminal α passenger domain, exposed at the cell surface, which carries the activity of the protein (35, 36, 56). Ag43 is a major surface protein of *E. coli* that promotes biofilm formation through cell-to-cell interaction and microcolony formation (16, 44). The expression of Ag43 is phase variable, and the shift from the $Ag43^+$ to the $Ag43^-$ phenotype is governed by a mechanism involving the concerted action of both Dam, the GATC site DNA-methylating enzyme deoxyadenosine methylase (activation), and the transcriptional regulator OxyR (repression) (34, 58, 82). In a wild-type situation, each bacterium is either in an Ag43-off situation (if, after DNA replication, the OxyR protein manages to bind to its consensus site before DNA methylation, thus stopping RNA polymerase progression) or in an Ag43-on situation (if DNA methylation occurs before OxyR can bind to DNA). To check that *km*RExTETrbs-*flu* and *km*PcL*flu* constructions could take full control of Ag43 production while maintaining the localization properties of the protein, we monitored the quantities of the α domain of Ag43 in these strains by immunodetection (Fig. 3A) and performed immunofluorescence experiments to locate the protein at the single-cell level (Fig. 3B and C). As shown in Fig. 3B, the on or off state of each wild-type MG1655 bacterium was reflected, respectively, by the presence or absence of Ag43 at the cell surface, and wild-type cells were predominantly in the Ag43-off state. We calculated that in LB medium, the rate of switching from on to off in strain MG1655 bearing pAg43, a plasmid containing the *lacZ* gene under the control of the *flu* promoter (81), is ca. 7×10^{-3} switches per cell per generation and the rate of switching from off to on is ca. 10^{-3} switches per cell per generation. These data are in good agreement with switching frequencies observed previously for chromosomal Ag43 expression in other *E. coli* strains (58, 66). This finding explains why most MG1655 wild-type cells are in the Ag43-off state (Fig. 3B) and is consistent with the detection of low levels of Ag43 in wild-type MG1655 cells (Fig. 3A). As expected, the deletion of *flu* or of *oxyR* resulted, respectively, in the absence of Ag43 or in the detection of high levels of Ag43, which localized at the cell surface (Fig. 3A and B).

Consistent with the previously observed *km*PcL-mediated activation of *lacZ*, large amounts of Ag43 were produced when the PcL cassette was introduced in front of the *flu* gene (Fig. 3A), and all bacteria in the culture exposed Ag43 at the cell surface (Fig. 3B). In the absence of aTc, no Ag43 could be detected in cell extracts from strain MG1655*km*RExTETrbs-*flu* (Fig. 3A) and no bacterium displayed detectable Ag43 at the cell surface (Fig. 3B). These results confirmed that expression from the $P_{L\,tetO\text{-}1}$ promoter in the RExTET cassette was tightly repressed in the absence of an inducer, therefore mimicking the phenotype of a strain carrying a deletion of *flu*. They also demonstrated that, in this situation, the natural phase variation of the intact *flu* promoter located upstream of RExTET does not interfere with the repression process, probably because of the presence of transcriptional terminators in the RExTET

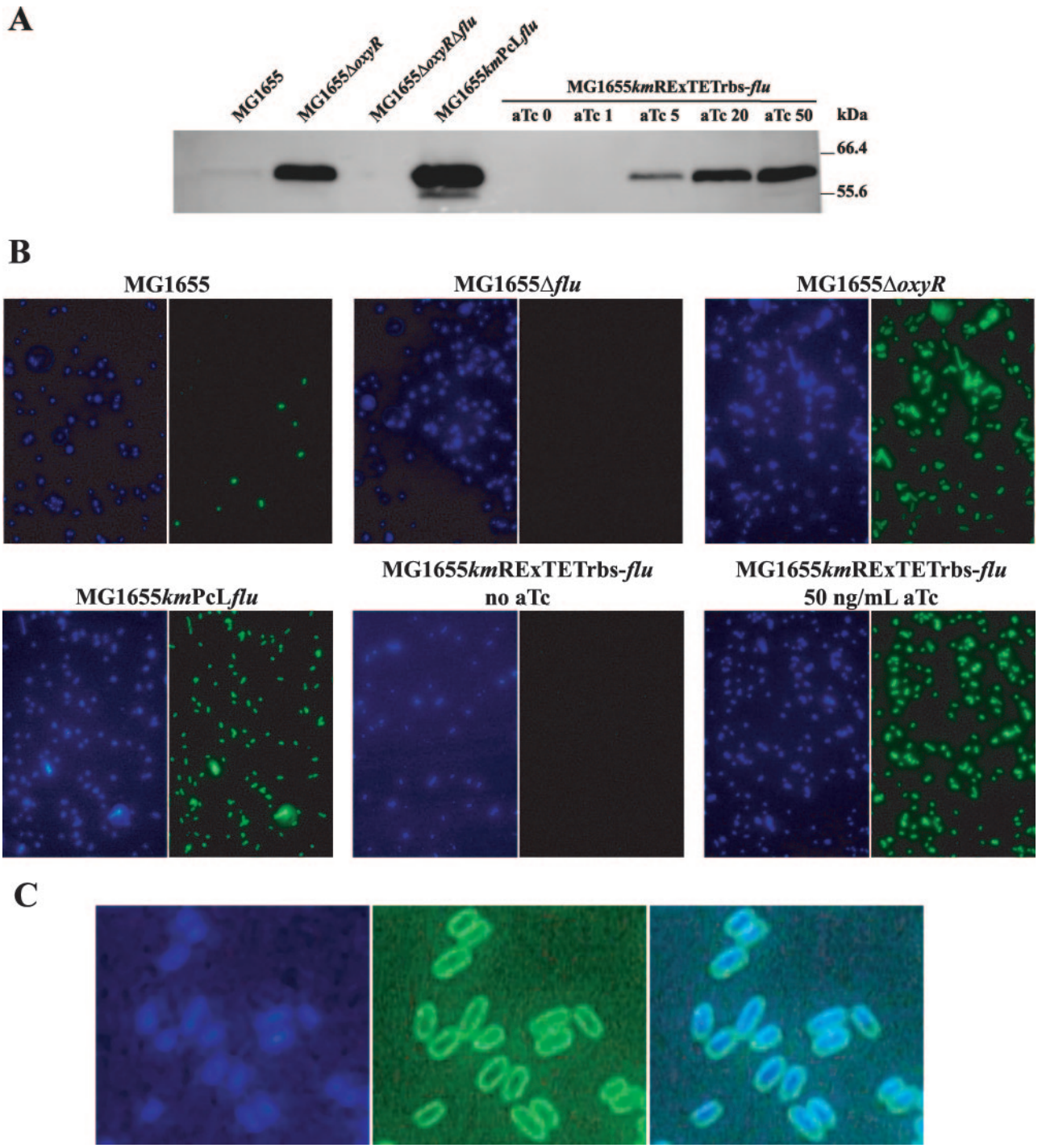

FIG. 3. Modulation of Ag43 production by using PcL and RExTET cassettes. (A) RExTET and PcL cassettes can modulate Ag43 production. An amount of each culture equivalent to an OD_{600} of 0.2 was loaded onto a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gel. Immunodetection was performed using a polyclonal rabbit antiserum raised against the α domain of Ag43. Concentrations (in nanograms per milliliter) of aTc, when added, are indicated above the lanes. (B) Overnight cultures of the different strains were grown at 37°C in LB medium without aTc or, for strain MG1655*km*RExTETrbs-*flu*, with 50 ng of aTc/ml. After fixation and successive incubations with a 1:1,000 dilution of a primary polyclonal rabbit antiserum raised against the α domain of Ag43 and with a 1:300 dilution of a secondary polyclonal goat anti-rabbit serum coupled to Alexa488 along with DAPI, cells were observed with a 600× objective under oil immersion. A DAPI filter was used to reveal the bacterial nucleoid (in blue), and a green fluorescent protein filter was use to reveal the presence of Ag43 (in green) at the cell surface. (C) Closeup of a culture of MG1655*km*RExTETrbs-*flu* in which the Ag43 production had been induced with 50 ng of aTc/ml. The cell surface localization of Ag43 is clearly demonstrated, as is the integrity of the bacterial membrane. The right panel shows the superimposition of the two preceding images.

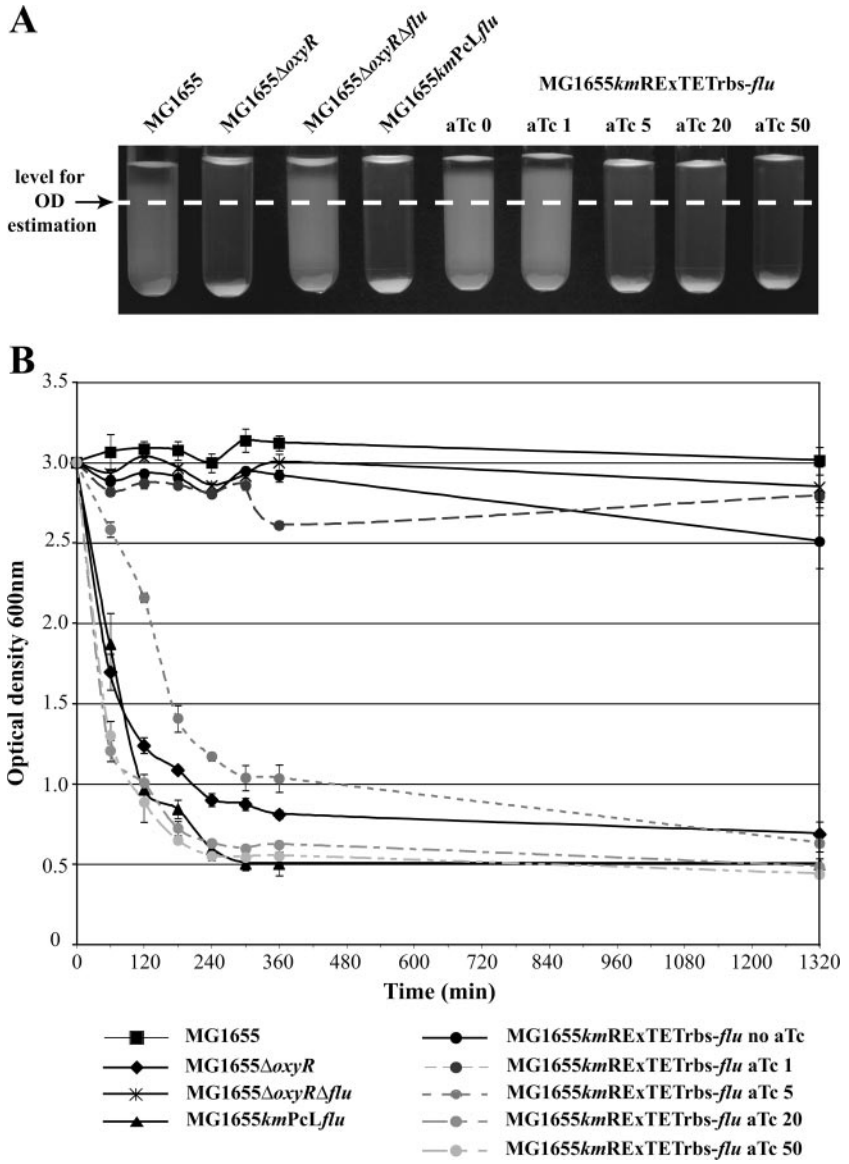

FIG. 4. Modulation of bacterial autoaggregation via controlled production of the Ag43 protein. The different strains were grown overnight in LB with increasing concentrations of aTc ranging from 1 to 50 ng/ml when indicated. After growth, cells were diluted to an OD_{600} of 2.5 in a 3-ml volume and the autoaggregation of each strain over 24 h at room temperature was assessed by capturing images of the tubes at 24 h (A) and determining the OD_{600} of the upper part of the standing culture tubes (white dashed line) at the indicated time (B). Aggregation tests were performed in triplicate; error bars represent the standard deviations of the means.

cassette. Upon the addition of aTc, a strong correlation between the aTc concentration and the level of Ag43 production from the RExTET cassette was observed (Fig. 3A). Maximal Ag43 production was achieved with 50 ng of aTc/ml, with all bacteria expressing Ag43 at the cell surface (Fig. 3B).

These results demonstrate that PcL and RExTET cassettes can be used to tightly regulate the expression of a naturally phase-variable adhesin-encoding gene such as that for autotransporter Ag43 without affecting the surface localization of the adhesin or the cell membrane integrity, as shown by the regular shapes of the bacteria revealed by the detection of Ag43 (Fig. 3C).

Application of RExTET and PcL cassettes to the study of biofilm phenotypes of *E. coli* K-12. Immunodetection and immunofluorescence experiments (Fig. 3) showed that the expression of Ag43 could be finely regulated under the control of the RExTET and PcL cassettes. To further investigate the phenotype associated with *flu* expression, we studied the capacities of strains MG1655kmPcL-*flu* and MG1655*km*RExTETrbs-*flu* to aggregate. As shown in Fig. 4, the constitutive expression of *flu* led to rapid cell aggregation in standing tubes, and increasing amounts of aTc correlated with increasing bacterial aggregation in tubes. Although the amount of Ag43 in MG1655*km*RExTETrbs-*flu* cells induced with 20 ng of aTc/ml

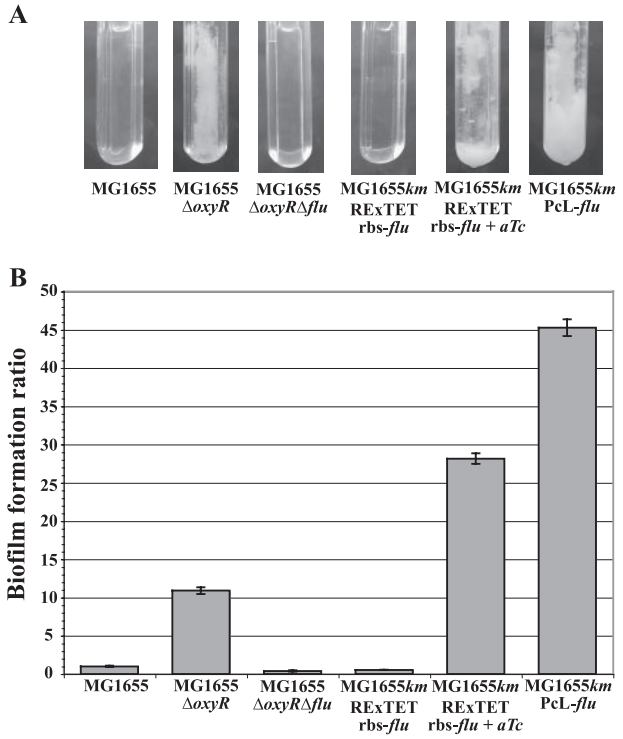

FIG. 5. Modulation of biofilm formation via REXTET- and PcL-mediated control of Ag43 production. The ability of the different strains to form a biofilm in a microfermentor was tested in M63B1glu medium. aTc at 20 ng/ml was added to the medium where indicated. Images of the 30-h-old biofilms in microfermentors (A) were captured before the biofilms were resuspended and OD_{600} measurements were taken (B). The histogram shows the ratios of the biofilm biomasses of the different strains to that of wild-type strain MG1655. Data are the averages of results from three independent experiments; error bars represent standard deviations of the means.

was clearly smaller than that in MG1655*km*PcL-*flu* cells (Fig. 3A), the cells of the two strains aggregated at the same rate (Fig. 4). This suggests that a maximal aggregation pattern is reached at a certain Ag43 concentration, after which an increase in the protein amount does not significantly influence the aggregative phenotype.

Ag43 has been shown to play a role in biofilm formation and structure (16, 44). Indeed, the derepression of *flu* in strain MG1655Δ*oxyR* resulted in a 10-fold increase in the level of biofilm formation compared to that by the wild-type strain in a continuous-flow culture system (Fig. 5) (4). We thus tested the effects of the modulation of the expression of the *flu* adhesin-encoding gene on strain biofilm ability. As shown in Fig. 5, strain MG1655*km*PcL-*flu* displayed the greatest biofilm capacity of the tested strains, with a 45-fold increase in biofilm biomass compared to that of MG1655. In the absence of an inducer, strain MG1655*km*RExTETrbs-*flu* formed a small biofilm comparable to that formed by strain MG1655Δ*oxyR*Δ*flu* (Fig. 5). However, upon aTc induction, the MG1655*km*RExTETrbs-*flu* biofilm biomass increased approximately 30-fold compared to that of wild-type MG1655 and 3-fold compared to that of MG1655Δ*oxyR*.

Consequently, the controlled expression of Ag43 through the RExTET and PcL cassettes enabled the modulation of *E. coli* K-12 biofilm formation abilities.

Modulation of biofilm formation in a natural *E. coli* isolate by using the RExTET cassette. One of the rationales behind creating a new repression-expression cassette lies in the impossibility of using arabinose as an inducer to study biofilm matrix-encoding genes (see above). The biofilm matrix is a complex hydrated milieu that contains proteins, DNA, RNA, ions, and polysaccharide polymers (7). In *E. coli*, three types of polysaccharides in the biofilm matrix have been detected and demonstrated to be important for biofilm formation. We recently showed that the nonsequenced commensal *E. coli* 1094 strain possesses genes involved in cellulose production (*bcsABZC* and *bcsEFG* operons) and that, in this strain, cellulose production is required for biofilm formation (18). Cellulose has the property of binding calcofluor, which then fluoresces under UV light, thereby facilitating the monitoring of cellulose production in bacterial colonies. We had previously observed that the presence of arabinose on LB plates containing calcofluor modifies the fluorescence phenotype of the *E. coli* cellulose-producing strain 1094 (S. Da Re, unpublished results). In order to test whether the promoter cassettes described in this study could be used to modulate the expression of biofilm matrix-encoding genes, we introduced the RExTETrbs cassette in front of the *bcsABZC* cellulose operon. First, we showed that the presence of aTc influenced neither 1094's cellulose production on LB-calcofluor plates nor its capacity for biofilm formation in a microfermentor (Fig. 6). In the absence of aTc, the 1094*km*RExTETrbs-*bcsA* strain did not fluoresce nor form a biofilm and it exhibited the same phenotype as a *bcsABZC* deletion mutant (Fig. 6). In contrast, aTc could induce the expression of the *bcsABZC* operon in the 1094*km*RExTETrbs-*bcsA* strain, as demonstrated by fluorescence on the plate (Fig. 6A), and the induction with 20 ng of aTc/ml was sufficient to restore the strain's capacity for biofilm formation to a level close to that of the wild type (Fig. 6B).

These results show that the use of the gratuitous inducer aTc is an appropriate alternative for matrix polysaccharide gene induction under conditions in which arabinose cannot be used, and they also highlight the possibility provided by our promoter cassettes to modulate the biofilm formation capacities of natural isolates.

DISCUSSION

In the present study, we chose two previously described biofilm-promoting factors, autotransporter adhesin Ag43 and extracellular matrix cellulose, to demonstrate that precise genetic control of the corresponding genes enables the fine modulation of *E. coli* biofilm formation. Ag43 belongs to a family of self-associating autotransporters comprising other adhesins like AidA-I and TibA that can interact with one another and have been shown to play a role in interspecies contacts (44, 45). Cellulose production is associated with biofilm formation, root colonization, and the persistence of many gram-negative bacteria in natural environments (6, 46).

In order to design strains with high or controlled biofilm formation capacities, we developed two compact promoter insertion cassettes containing either an inducible (RExTET cassette) or a constitutive (PcL cassette) expression system that

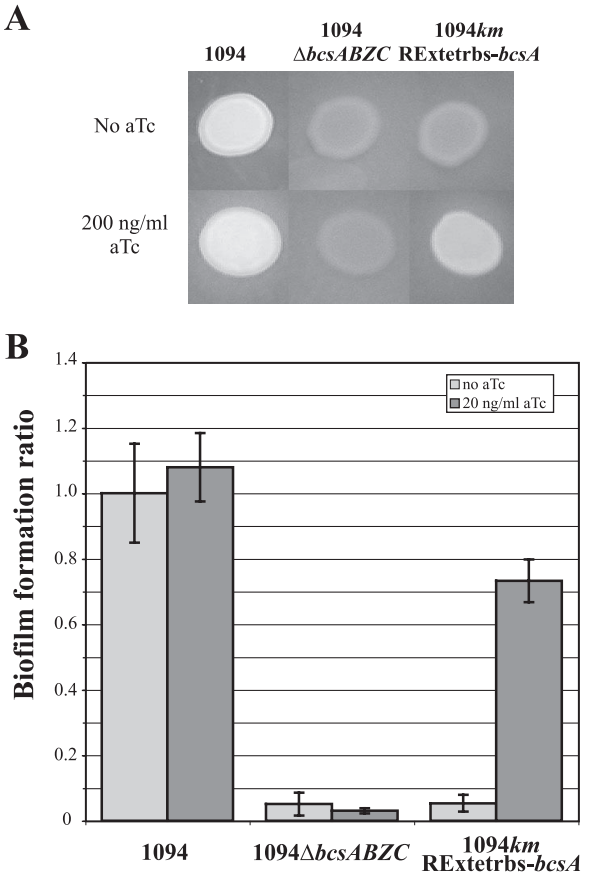

FIG. 6. Modulation by the RExTETrbs cassette of cellulose production and biofilm formation by an *E. coli* natural isolate. The ability of the different 1094-derivative strains to produce cellulose was tested. (A) Calcofluor binding and fluorescence under UV light. Two-microliter volumes of overnight cultures were spotted onto LB-calcofluor (upper row) and LB-calcofluor-aTc (lower row) plates and incubated for 24 h at 37°C; the aTc concentration on the plate was 200 ng/ml. (B) Capacities for the formation of biofilms in microfermentors. Biofilms were grown for 26 h at 37°C in M63B1glu supplemented or not with 20 ng of aTc/ml. The levels of biofilm formation by the different strains are expressed as the ratios of the OD readings for the resuspended biofilms of the different strains to that for the 1094 biofilm in the absence of aTc. Data are averages of results from three independent experiments; error bars represent standard deviations of the means.

can be easily introduced upstream of any genes on the *E. coli* chromosome.

The RExTET cassette was first validated by using transcriptional fusions with the *lacZ* and *flu* genes, and in the absence of the inducer aTc, strong repression of the expression of both genes was observed: neither Ag43 production nor β-galactosidase activity associated with the RExTETrbs-*flu* and RExTETrbs-*lacZ* or RExTET*lacZ* fusions could be detected. Under noninducing conditions, the RExTET cassettes therefore mimic the phenotype of a bona fide deletion. Compared to the RExBAD approach that we developed previously, the RExTET construction effectively led to better repression in the absence of an inducer (data not shown) (68). On the other hand, in the presence of increasing concentrations of the aTc inducer, both *flu* expression and β-galactosidase activity could be induced to increase from levels below those in the wild type to even higher levels. The RExTET cassettes can thus be used to study the functions of the genes of *E. coli* and any related eubacteria at the genes' native loci. These genetic tools allow for both deletion-like (medium without inducer) and complementation (addition of the gratuitous aTc inducer) conditions and therefore constitute a valuable alternative to conditional-mutant construction. Our approach also provides the advantage of using a gratuitous, nonmetabolizable inducer, thus alleviating the need for the creation of a nonmetabolizing mutant (i.e., one carrying the Δ*ara* mutation when the inducer is arabinose), necessary when long-term and homogeneous regulation is required (51, 68). As shown in Fig. 3B, all cells presented Ag43 at the surface when RExTET constructs and the aTc inducer were used, thus demonstrating that the heterogeneity due to *lac* and P_{BAD} promoter autocatalytic induction phenomena was not observed with this expression system (51).

Recent works demonstrated that the use of antibiotics in biofilm experiments can affect or even induce bacterial biofilm formation (39, 69). The RExTET and PcL expression systems do not require the use of antibiotic selective pressure for stable insertion into the chromosome (data not shown), and this feature is well adapted to the study of multicellular complex communities such as biofilms.

The design of both RExTET and PcL cassettes enables the construction of transcriptional fusions carrying either the RBS of the target gene or the RBS of the cassette. In the case of *lacZ*, we showed that the choice between the RExTET or PcL and the RExTETrbs or PcLrbs expression systems provides an additional possibility for modulating the production of the protein(s) of interest through translation efficiency.

Our data also showed that, although the strong promoter λp_R included in the PcL cassette drove the constitutive expression of the outer membrane protein Ag43, this constitutive expression did not perturb cell growth or cell membrane shape. Nevertheless, in certain cases, it is possible that too-strong expression will perturb cell physiology and morphology. The construction of a set of cassettes with known promoters of different strengths could then be considered for constitutively expressing target genes at different levels.

In addition to the functionality of the RExTET cassette, PcL cassettes enable constitutive gene expression, thus alleviating the need for an external inducer and its potentially associated pleiotropic metabolic effects. The use of such a constitutive cassette is therefore particularly indicated in performing in vivo assays in which plasmids are difficult to maintain over a long period of time and the inducer cannot be regularly and homogeneously provided over the course of the in vivo test. The PcL cassettes described here were consistently and successfully used to constitutively express different adhesin genes in a uropathogenic *E. coli* strain and to analyze the effects of the overexpression of these genes on early and long-term colonization of the bladder in a mouse model of urinary-tract infection (77a). Such strategies could thus be envisaged for in vivo studies with different pathogens that are genetically amenable.

Beyond the creation of new tools to facilitate the functional study of genes with unknown expression conditions, we propose that the PcL and RExTET expression strategies, or those of other promoter insertion cassettes optimized for gram-pos-

itive bacteria, could also be used to engineer a wide range of bacterial strains with high or controlled biofilm formation capacities. Indeed, although many efforts are being directed toward fighting biofilm formation or engineering strains with reduced biofilm formation abilities for biotechnological utilization (76), applications which exploit the biofilm formation abilities of harmless bacteria in a medical environment are presently being considered (19). A recent study demonstrating that, compared to planktonic cells, *E. coli* biofilms display enhanced high-copy-number-plasmid maintenance and heterologous protein production is also encouraging the use of biofilms in industrial applications (54).

We showed in an in vitro experiment that a commensal MG1655 strain that constitutively expresses the Ag43 autotransporter adhesin (MG1655*km*PcL*flu*) was more efficient than the isogenic MG1655Δ*flu* strain at competing for biofilm formation with a pathogenic enteroaggregative *E. coli* strain (Fig. S1 in the supplemental material). In vivo approaches using the controlled biofilm capacities of innocuous bacteria could thus be envisaged (i) to challenge deleterious biofilms found in both industrial and medical settings through bacterial interference or competitive adhesion to the surface (77) or through the production of toxic or matrix-dissolving compounds such as cellulase and dispersins, (ii) to optimize the persistence of probiotic strains (15, 19, 24, 27, 28, 31, 33, 37, 64), and (iii) to improve—via stronger heterologous bacterial interactions—the establishment, in nonsterile soil microcosms or in mixed bioreactors, of strains with desirable or genetically engineered features and their development as biosensors or agents in bioremediation processes (8, 22, 26, 53, 65, 72).

In conclusion, our study demonstrates that improved knowledge of bacterial biofilm formation at the molecular level can be used to control cell surface adhesion in bacterial strains of interest. This fine-tuning of cell-to-surface and cell-to-cell interactions of all sorts of bacteria may contribute to the opening up of new perspectives in situations of industrial and medical relevance.

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