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To cite this version:
Ashwini Chauhan, Chizuko Sakamoto, Jean-Marc Ghigo, Christophe Beloin. Did I Pick the Right Colony? Pitfalls in the Study of Regulation of the Phase Variable Antigen 43 Adhesin. PLoS ONE, Public Library of Science, 2013, 8 (9), pp.e73568. 10.1371/journal.pone.0073568 . pasteur-01385431
Did I Pick the Right Colony? Pitfalls in the Study of Regulation of the Phase Variable Antigen 43 Adhesin

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Abstract

Ag43 is an abundant outer membrane autotransporter adhesin present in most commensal and pathogenic Escherichia coli. Expression of the agn43 gene is characterized by a regulated reversible switch or phase variation between the agn43 ON and agn43 OFF states. Although the agn43 regulatory switch leads to a heterogeneous population of ON and OFF bacteria, studies of Ag43 seldom consider potential biases associated with phase variation. We monitored agn43 ON/OFF phase-variation status genetically and phenotypically and we show that the use of populations with random agn43 ON or OFF status could result in misleading conclusions about Ag43 function or regulation. In particular, we demonstrate that Lrp and MqsR, previously identified as agn43 regulators, do not regulate agn43 expression or ON/OFF switch frequency. We also show that biofilm formation in dynamic flow conditions does not influence agn43 ON/OFF switching but physically selects aggregating agn43 ON cells. This indicates that misinterpretation is possible when studying gene expression within biofilms. Finally, we provide evidence that ignoring the initial agn43 ON/OFF status of the E. coli populations studied is likely to bias analyses of phenotypes associated with other E. coli adhesins. This study therefore emphasizes the importance of monitoring Ag43 phase variation and indicates that caution is required when interpreting experiments using strains that are neither deleted for agn43 nor carefully assessed for agn43 ON/OFF status.

Introduction

Colonization of diverse environments by E. coli requires high adaptation abilities and a variety of colonization factors ensuring successful attachment to various surfaces. Recent post-genomic studies have demonstrated that E. coli indeed possesses a very large arsenal of adhesins with different specificities [1–11]. Two major families of adhesins have been identified in E. coli: adhesins carried by chaperone-usher fimbriae that generally recognize glycosylated proteins or lipids [12–14] and type V secretory autotransporter adhesins, recognizing specific receptors or self-associating, and implicated in bacterium-to-bacterium interactions [15–17]. Prototypical members of this family of self-associating autotransporters (SAATs) are AidA, an adhesin initially characterized in an E. coli O126:H27 strain isolated from a pediatric patient with diarrhea [18], TlbA, first found in the ETEC O78:H11 strain H10407 [19], and the Antigen 43 adhesin (Ag43) one of the most abundant outer membrane proteins in E. coli [20,21].

The gene coding for Ag43 is present in nearly all commensal and pathogenic E. coli and some isolates carry multiple copies of agn43 alleles on pathogenicity islands [15,22]. Whereas eukaryotic receptors specific for AidA and TlbA have been identified, the only identified function of most Ag43 variants is the ability to promote bacterial autoaggregation and biofilm formation in vitro. In vivo, the Ag43 variant Ag43a was found to be involved in long-term persistence of uropathogenic CFT073 within mouse bladder [10]. Consistently, human epidemiological studies have associated the agn43a allele with UPEC persistence in bladder and recurrent infections [23].

Remarkably, expression of agn43 is phase variable and is characterized by ON and OFF states and switching rates of about 10^{-5} per cell per generation. This phase variable expression is due to the concerted action of a repressor, the oxidative stress regulator OxyR, and of an activator, the Dam methylase that methylates GATC sites in the regulatory region of agn43 and overlaps with the OxyR binding site [24–28] (Fig. 1A). Most studies of the functions of Ag43 have been performed using strains overproducing Ag43 or containing mutations locking its expression in either the ON or OFF state therefore ignoring its natural phase variation. Any wild-type E. coli population is likely to be composed of a mixture of Ag43 ON and OFF bacteria, and the characterization of agn43 regulators or studies of agn43 expression using DNA arrays or RT-PCR experiments can be misleading due to absence of information about the Ag43 ON/OFF state of the bacterial populations tested (see results for agn43/flj regulation in GenExdb database - http://genexpdb.ou.edu/main) [29–36]. Indeed, van der Woude and Henderson suggested that differential expression observed in

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Competing Interests: Christophe Beloin is a PLOS ONE Editorial Board member. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

Published: September 5, 2013

Citation: Chauhan A, Sakamoto C, Ghigo J-M, Beloin C (2013) Did I Pick the Right Colony? Pitfalls in the Study of Regulation of the Phase Variable Antigen 43 Adhesin. PLoS ONE 8(9): e73568. doi:10.1371/journal.pone.0073568
global expression analysis for genes subject to phase variation may be due to differences in the distribution (possibly random) of the ON/OFF cell ratio between bacterial populations rather than to genuine, robust regulatory differences [22].

In this study, we reinvestigated agn43 regulation using a genetic approach allowing the agn43 ON/OFF phase-variation status to be monitored while keeping a functional agn43 gene. We confirmed that the agn43 ON/OFF status strongly influences E. coli autoaggregation and biofilm formation, and demonstrate that biofilm formation leads to a physical selection of Ag43 ON and OFF cell populations rather than to differences in the distribution (possibly random) of the ON/OFF cell ratio between bacterial populations. Genome-wide quantification of gene expression by ON/OFF phase-variation status was performed using global microarray analysis. Two approaches were used for the generation of Ag43 ON/OFF phase-variation strains. Strain TG is a top-10ku derivative of MG1655 cured of the F plasmid and cured of the top-10ku marker [38].

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are described in Table 1. All experiments were performed in 0.4% glucose M63 minimal medium (M63B1) [37] or in Lysogeny Broth (LB) medium at 37°C unless specified otherwise. Antibiotics were added as required. All constructions were confirmed by PCR and/or sequence analysis.

The E. coli strains used in this study were constructed by P1vir phage transduction from various strains including mutants from the Keio collection [38], or by using the λ-red linear DNA gene inactivation method [39,40]. For construction of lacZ fusions we used the strain MG1655lacZ-zeo where the gene encoding zeocin resistance was placed after the lacZ gene on its native location [6]. The E. coli K-12 TG strain is a TG1 strain derivative commonly used in biofilm studies which has been cured of the F plasmid. TG possesses only one allele of agn43, located at the same chromosomal position as other K-12 laboratory strains. We constructed strain TG agn43-lacZ by introducing the lacZ gene with its own ribosome binding site (rbs) and the zeocin resistance gene after the stop codon of agn43; and strain TG Δagn43::lacZ-zeo by replacing agn43 (ATG to STOP codons) by lacZ-zeo keeping the rbs of agn43. The constitutive expression of yfaL was obtained by introducing, upstream from the ATG of yfaL, the kmPrLhs cassette containing the constitutive λPr promoter [41]. Primers used in this study are listed in Table 2. All constructions were confirmed by PCR and/or sequence analysis.
Switch frequencies
The Ag43 switch frequencies were calculated as described previously [27,42]. Briefly, five blue or five white colonies were suspended in 1 mL LB medium and dilutions were plated on LB agar plates supplemented with 100 μg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The plates were incubated overnight at 37°C. The blue and white colonies were counted and used to calculate the switch frequencies. Both total counts of viable cells (N) and the number of 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) = \( 1 - \frac{1}{e} \left( 1 - \left( \frac{M}{N} \right)^{g} \right) \) where \( g \) is the number of generations of growth and is calculated as \( g = \log_{N} \log_{2} \).

Autoaggregation Assay
Aggregation assays were performed as described in [8]. Isolated blue or white colonies were picked from LB/X-gal plates, and individual colonies were used to inoculate 5 mL LB medium and grown overnight (16–18 h). The optical density of the culture at 600 nm (OD600) was adjusted to 3.0 by dilution with nutrient-exhausted LB medium (supernatant obtained from respective overnight grown cultures after centrifugation), and 3 mL of each adjusted culture was transferred to 5 mL hemolysis tubes. These tubes were incubated without agitation at room temperature. The OD600 of the upper part of each standing tube culture was determined every hour for 8 h.

Biofilm formation assay in micro-titer plates
Biofilm formation was assayed by determining the ability of cells to adhere to the wells of 96-well polystyrene plate (PVC) micro-titer plates [43,44]. An overnight culture in M63B1 medium supplemented with 0.4 mg/mL proline was inoculated at a 1/100
Table 2. Primers used in this study.

| Primers | Sequence (5’ to 3’) | Target region/gene |
|---------|---------------------|--------------------|
| end-agn43.lacZ2neo.L-5 | agggtacaaggtcgccacacagacttggtcgctggattcttggtcagctcga | insertion lacZ after agn43 |
| end-agn43.lacZ2neo.L-3 | cgggtcatcagagggccacagacagggcgtctggctctctcgccgac | insertion lacZ after agn43 |
| agn43.ext-3 | atacgtcagaggtcgtgc | agn43 verification |
| end-agn43.ext-5 | aagctcagacgacataaatcttgg | agn43 verification |
| lacZ.ATG+100-3 | gggggatgctgtgcaaggggattaaag | lacZ cassette-gene junction verification |
| zeo.verf | cagaggacgaggtcgccgacacacc | lacZ cassette-gene junction verification |
| agn43.lacZ2neo.L-5 | tacggctttttattacccctccatcggaaagggtgatgagcattgtgtgagctgctgctcttcg | replacement agn43 by lacZ |
| agn43.lacZ2neo.L-3 | tcatatgagcggccacagagggcgtctggctcagttaagctctcttcgccgac | replacement agn43 by lacZ |
| agn43.ext-5 | atacgtcagaggtcgtgc | agn43 deletion verification |
| MqSR-500-5 | gagcagcgttgcacacagcga | mqSR verification |
| MqSR-500-3 | caaacaacaatgcctgtgcatc | mqSR verification |
| LrP-500-5 | gaagctcctattgtgttg | LrP verification |
| LrP-500-3 | agaagcagagggtaaggt | LrP verification |
| yfaL.PclKmrbs-L-3 | tgaaagataaatactccttgcgtagaagataatccgcatgcggtacctttctcctctttaatg | insertion KmPclLrb in front of yfaL |
| yfaL.PclKmrbs-L-5 | ttccccatgtataatgcggataaatcggcctctttaaatggatctgctcttgcttccttcg | insertion KmPclLrb in front of yfaL |
| yfaL.A1.500-5 | ggtcagacaaggtgcctgg | yfaL |
| yfaL.Ext-5 | catatactgtggttaactctgg | yfaL |
| yfaL.B1. PclKmrbs-500-3 | ctgtagtgacagctaaatgc | yfaL |
| yfaL.PclKmrbs-ext-3 | cattattaagtagtaaaattg | yfaL |
| yfaL.ATG+100-3 | caaactcgctttgacatcat | yfaL |
| Pcl-kr.verif-5 | cagagcgagaaggttcttggttg | cassette-gene junction verification |
| Pcl-kr.verif-3 | cttctcgcgtccttacggtatcg | cassette-gene junction verification |

Ag43 Phase Variation Pitfalls

Biological formation assay in microfermentors

All experiments were performed in triplicate in M63B1 Gluc medium supplemented with 0.4 mg mL⁻¹ proline at 37°C. Sixty-milliliter microfermentors containing a removable glass slide were configured as continuous-flow culture bioreactors with a flow rate of 40 mL h⁻¹ [45,46]. Bacterial inocula equivalent to an OD600 of 1 from overnight precultures grown in M63B1 Gluc medium supplemented with 0.4 mg mL⁻¹ proline and appropriate antibiotics were used to inoculate the microfermentors; the cultures were then cultivated for 24 h and 48 h. Images of each removable glass slide were captured at the end of the incubation period. After 24 h or 48 h of growth the biofilm on the slide was resuspended in 10 mL of M63B1 medium and the OD600 of the suspension was determined. The resuspended biofilms were also used to determine percentages of cells in the ON and OFF states, by immunofluorescence in the case of the TG agn43::lacZ::zeo strain or by plating on LB-Xgal agar plates for the TG Δagn43::lacZ::zeo strain.

Immunofluorescence

Immunofluorescence microscopy analysis was performed as previously described [41]. Briefly, strains were cultured overnight at 37°C in LB medium with the appropriate antibiotics. Overnight cultures were diluted to OD600 1 in 1× PBS and aliquots were loaded onto 0.1% poly-L-lysine-treated immunofluorescence microscope slides. A 1:1,000 dilution of primary polyclonal rabbit anti-serum raised against the α-domain of Ag43 was used to label Antigen 43 (antibodies given by P. Owen). A 1:300 dilution of a secondary polyclonal goat anti-rabbit serum coupled to Alexa488 (Molecular Probes-Invitrogen) was used to reveal bound antibody and 10 μg mL⁻¹ 4',6-diamidino-2-phenylindole (DAPI) was used to stain the bacterial DNA nucleoid. The slides were mounted with Mowiol 4088 (Calbiocem) and observed under an epifluorescence microscope with green fluorescent protein and DAPI filters.

Ag43 immunodetection

For each culture, the equivalent of 0.2 OD600 units was analyzed by sodium dodecyl sulfate—10% polyacrylamide gel electrophoresis, followed by immunodetection of Ag43. Protein loading accuracy was verified using staining of membrane with Ponceau S. The α-subunit of E. coli RNA polymerase (Neocloste biotech) was used as an internal control. A polyclonal rabbit anti-serum raised against the α-domain of Ag43 was used at a dilution of 1:10,000 for immunodetection and the antibody specific for the α-subunit of E. coli RNA polymerase was used at a dilution of 1:15,000.

RNA isolation and semi-quantitative RT-PCR

Bacterial strains were grown overnight in LB medium and their RNA was extracted using the RNeasy Protect Bacteria Mini-Kit (Qiagen). Extracted RNA was treated with RNase-free DNase, repurified and stored at −80°C. RNA at a concentration of...
500 ng µL⁻¹ was used for cDNA synthesis by Superscript II (Invitrogen Life Technologies) and 150 ng random primers (mostly hexamers). The obtained cDNA was diluted 1:1, 1/10 and 1/100 and the transcripts for the *agn43* and *16S* were amplified (94°C 1 min, 60°C 1 min, 72°C 1 min, for 30 cycles) using Supermix and Ex Taq polymerase (TaKaRa) with 10 mM of the appropriate primers (see Table 2). Non-reverse-transcribed RNA was used as a negative control to confirm the absence of contaminating genomic DNA.

**Statistical analysis**

Results presented are means ± standard deviation. Statistical differences were evaluated using one-way ANOVA (Tukey multiple comparison test) included in Graphpad Prism Version 5.0c. The treatment groups were considered significantly different if p-values were lower than 0.05.

**Results**

**Ignoring Ag43 phase-variation status randomizes analyses of E. coli aggregation phenotypes**

To determine to what extent the naturally occurring *agn43* phase variation in *E. coli* influences its community behavior, we streaked the wild-type *E. coli* strain K-12 TG on LB agar plates from a ~80°C glycerol stock. One hundred isolated colonies were used to inoculate LB and grown overnight at 37°C. We tested the auto-aggregation properties of each individual overnight culture as a marker of *Ag43* expression status. Only five of the 100 colonies displayed an auto-aggregation phenotype (+) (Fig. 1B). Serial dilutions of one of these (+) aggregating cultures was plated on LB agar plates, and 100 isolated colonies were used to inoculate liquid cultures to re-test their aggregation phenotype. This time, 75% percent of these cultures aggregated (+) and 25% did not (–). Immunodetection using Anti-Ag43 antibodies were used to test for Ag43 in one aggregating culture and one non-aggregating culture. This analysis showed that the presence of Ag43 correlated with the aggregation phenotype (Fig. 1C). Moreover, aggregating clones (+), but not non-aggregating clones (–), displayed strong biofilm forming ability in a continuous flow system (Fig. 1D).

Despite the observed correlation between auto-aggregation and Ag43 production, the + and – phenotypes obtain with this wild-type phase variable strain were less marked than those of control strains locked-ON (oxyR) or locked-OFF [5] for *agn43* expression (Fig. 1B). We tested whether mixtures of Ag43+ and Ag43– bacteria, in various proportions, could determine the degree of aggregation of the corresponding culture: locked-OFF *dam* mutant bacteria were mixed with locked-ON oxyR mutant bacteria in various ratios. The degree of aggregation was directly proportional to the number of Ag43+ bacteria (see Fig. S1). These results demonstrate that streaking ~80°C stocks of *E. coli* results in a heterogeneous population of colonies, some expressing and some not expressing Ag43; consequently, picking an ON or OFF colony at random strongly influences the outcome of analyses of bacterium-bacterium interactions.

**Monitoring the *agn43* expression state using an *agn43*-lacZ operon reporter fusion**

To alleviate the uncertainty about the *agn43* ON/OFF expression status of an *E. coli* inoculum, we created a strain allowing direct distinction between colonies in the *agn43* ON and OFF states. We inserted the β-galactosidase lacZ gene immediately downstream of the *agn43* coding sequence to generate an operon consisting of *agn43* and lacZ in *E. coli* TG. This construction at *agn43* chromosomal locus, allows the production of a functional Ag43 protein and is stable without any antibiotic selection pressure. The *E. coli* *agn43-lacZ* strain generated both blue (ON) and white (OFF) colonies on X-gal plates, and the switching frequency from ON to OFF was ~5.10⁻³ cell/generation and from OFF to ON was ~5.10⁻⁴ cell/generation (Fig. 2A). These frequencies are consistent with previous reports, and indicate that expression of the *agn43-lacZ* operon is subject to *agn43* phase variation [42]. Immunolocalization experiments confirmed the presence of Ag43 at the cell surface of most or few bacteria in blue and white colonies respectively (Fig. 2B): blue colonies were composed of 33+/−2% of ON bacteria whereas white colonies contained 96+/−1% of OFF bacteria. Consistently with results obtained with wild-type *E. coli*, a culture of a blue colony, which does not contain solely *agn43* ON bacteria, aggregated less (Fig. 2C) and produced slightly less Ag43 (Fig. 2D) than cultures originating from an *oxyR* mutant (locked-ON). These results show that this *agn43-lacZ* strain faithfully reproduces both *agn43* phase variation and associated phenotypes.

**Physical selection, and not switch towards *agn43* ON state, results in higher Ag43+ populations in biofilms**

Previous reports suggested that heterogeneity in Ag43-mediated cellular aggregation may constitute a selective bias in certain experimental situations [35]. We studied this possibility by monitoring the evolution of the *agn43* ON/OFF state during biofilm formation in a continuous flow system; in this system, population of Ag43+ cells may have an advantage, therefore introducing potential bias in gene expression analysis. We inoculated continuous flow biofilm microcolonies with bacterial populations grown either from an ON (blue) colony or an OFF (white) colony. In parallel, planktonic cultures were grown from the same inocula for 24 h and 48 h. We used Ag43 immunofluorescence to estimate proportion of ON and OFF cells in the initial inocula, and in 24 h and 48 h planktonic and biofilm populations.

Biofilm formation in microcolonies was greater following inoculation with a culture originating from an ON colony than from an OFF colony (Fig. 3A); this was consistent with the capacity of Ag43 to promote bacterial aggregation. However, biofilm biomass increased between 24 h and 48 h independently of the initial *agn43* ON/OFF state of the inoculum (Fig. 3B). Moreover, the proportion of ON cells increased substantially over time within biofilms regardless of the initial *agn43* ON/OFF state, whereas it changed only moderately in planktonic cultures (Table 3). This was especially striking with *agn43* OFF cells inocula: ON cells made up only 2.3% of the initial population, but were 55% in biofilms after 48 h, and 7.3% in planktonic culture after 48 h. Immunoblot was used to detect Ag43 protein: it was more abundant in biofilms than in the corresponding planktonic cultures, where almost no change compared to inoculum was detected (Fig. 3C). Therefore the proportion of Ag43+ bacteria in biofilm increases, irrespective of the initial *agn43* ON/OFF state.

This positive selection for Ag43+ cells could result from a physical selection of Ag43+ cells or from an increased OFF to ON switching frequency during biofilm formation. To address this issue, we replaced *agn43* in its native chromosomal locus with the lacZ gene, thereby generating strain *E. coli* TG *agn43-lacZ*. In this strain, *agn43* promoter remains subject to phase variation but there is no production of Ag43 such that the strain is non-aggregating (Fig. 2). Because of the *agn43* deletion, the proportion of ON/ OFF Ag43 cells during biofilm formation could not be evaluated by immunofluorescence and was determined by plating and counting blue and white colonies. Surprisingly, we did not observe any difference in the percentages of *agn43* ON/OFF cells between
biofilm and planktonic *E. coli* TG *agn43::lacZ* populations (Table 4). These results demonstrate that the increased Ag43 expression in biofilms is due to physical selection of ON cells in the biofilm, rather than switching towards the *agn43* ON phase.

The Ag43 state biases phenotypic analysis of the function of *E. coli* adhesins

Our results indicate that the outcome of adhesion and biofilm studies in *E. coli* depends largely on whether *E. coli* colonies originating from Ag43 ON or Ag43 OFF bacteria are used. This raises a question of whether the initial Ag43 ON or OFF state of *E. coli* cultures also biases the analysis of adhesion and biofilm phenotypes mediated by potential uncharacterized adhesins other than Ag43. We therefore investigated the role of Ag43 phase variation status on phenotypes mediated by the potential autotransporter adhesin YfaL, previously shown to increase biofilm formation by *E. coli* studies in *in vitro* biofilm formation by *E. coli* [6]. A genetic construction constitutively expressing yfaL (PcL-yfaL) was introduced into our *agn43-lacZ* reporter strain and we tested the ability of the resulting *E. coli agn43-lacZ* PcL-yfaL blue (ON) and white colonies (OFF) to form biofilm in the widely used microtiter plate assay. This assay has been extensively used as a straightforward assay for evaluating bacterial adhesion properties in most studies related to biofilms. The constitutive expression of yfaL led to significantly more biofilm formation when the inoculum originated from an OFF than ON colony (Fig. 4). Also, yfaL expression from PcL-yfaL did not promote biofilm formation in an *oxyR* mutant, in which cells are 100% ON, whereas deletion of *agn43* in this *oxyR* context restored the enhancement of biofilm formation by YfaL (Fig. 4). This shows that Ag43-mediated aggregation can affect the outcome of biofilm or adhesion experiments; such analyses should always be performed in a genetic background where *agn43* status can be monitored or in a Δ*agn43* background.

Despite previous reports, neither Lrp nor MqsR regulate *agn43*

Ignoring the *agn43* expression status could also skew whole population analyses and lead to erroneous identification of regulators of *agn43* expression or ON/OFF switching frequency. To illustrate this point, we used the *E. coli agn43-lacZ* strain to study the roles of the previously identified *agn43* regulators Lrp and MqsR. These factors were identified as *agn43* regulators by transcriptomic approaches [30,34]. It has been reported that deletion of the *lp* and *mqsR* genes reduce *agn43* expression by almost 5-fold [34] and 17-fold [30], respectively. However, their deletions from TG *agn43-lacZ* had no effect on *agn43* switching frequencies (Fig. 5A) or on the amount of *agn43* transcripts as assessed by RT-PCR with blue and white colonies (Fig. 5B). Ag43 levels in the *lp* and *mqsR* mutants were not different to that in...
wild-type (WT) cells (Fig. 5C), and these mutations did not modify the auto-aggregation properties of blue or white colonies (Fig. 5D).

Thus, neither Lrp nor MqsR regulate agn43 expression or ON/OFF switching frequencies in our genetic background, evidence that ignoring the phase variation status of agn43 in the studied population can result in major biases in whole-population agn43 gene expression analyses.

**Discussion**

There have been numerous studies of Ag43 phase variation, but few investigated how this unusual regulatory process interferes with *E. coli* adhesion to a surface. Here, we demonstrate that ignoring the agn43 ON/OFF status can make studies of both the regulation and function of agn43 extremely difficult to interpret.

We show that the simple and mundane act of picking a colony to start an *E. coli* culture is equivalent to a random choice of an agn43 ON or OFF colony, unless an appropriate detection

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**Table 3.** Percentages of ON and OFF cells in 24 h/48 h-old biofilms or planktonic cultures of strain TG agn43-lacZ.

|                | OFF colony | ON colony |
|----------------|------------|-----------|
|                | % OFF      | % ON      | % OFF      | % ON      |
| Fermentor      |            |           |            |           |
| inoculum*      | 97.6       | 2.4       | 93.5       | 6.5       |
| 24 h           | 73.1       | 26.9      | 96.5       | 3.5       |
| 48 h           | 44.8       | 55.2      | 99.0       | 1.0       |
| Planktonic     |            |           |            |           |
| inoculum*      | 97.6       | 2.4       | 93.5       | 6.5       |
| 24 h           | 93.7       | 6.3       | 82.8       | 17.2      |
| 48 h           | 92.6       | 7.4       | 83.2       | 16.8      |

*ON or OFF colonies were used to grow the inocula. The same inoculum was used for fermentors and planktonic cultures.

doi:10.1371/journal.pone.0073568.t003

**Table 4.** Percentages of ON and OFF cells in 24 h/48 h-old biofilms or planktonic cultures of strain TG Aagn43::lacZ.

|                | OFF colony | ON colony |
|----------------|------------|-----------|
|                | % OFF      | % ON      | % OFF      | % ON      |
| Fermentor      |            |           |            |           |
| inoculum*      | 98.6       | 1.4       | 89.4       | 10.6      |
| 48 h           | 94.3       | 5.7       | 81.7       | 18.3      |
| Planktonic     |            |           |            |           |
| inoculum*      | 98.6       | 1.4       | 89.4       | 10.6      |
| 48 h           | 91.8       | 8.2       | 90.9       | 9.1       |

*ON or OFF colonies were used to grow the inocula. The same inoculum was used for fermentors and planktonic cultures.

doi:10.1371/journal.pone.0073568.t004
approach is used. As there is a higher probability of switching from ON to OFF than OFF to ON, the odds of working with an OFF colony for Ag43 are higher than those of working with an ON colony. This difference in switching explains why an OFF colony colony for Ag43 are higher than those of working with an ON colony. This difference in switching explains why an OFF colony of the E. coli TG strain used in our study gave rise to a population of 95% of OFF and 5% of ON whereas an ON colony gave rise to a population of 75% of ON and 25% of OFF. The phenotypic consequences of this are such that the initial agn43 phase variation status should be determined for all phenotypic analyses of E. coli adhesion and biofilm formation.

We developed a genetic tool for monitoring the Ag43 status in an E. coli background that is wild-type with respect to Ag43 production. Our agn43-lacZ fusion could be used to study agn43 regulation and Ag43 function, taking Ag43 phase variation into account. This approach could also easily be applied to pathogenic E. coli and it would be possible to construct different reporter fusions if multiple agn43 variants are expressed.

Using the agn43-lacZ reporter strain, we demonstrated a strong correlation between the ON or OFF colony status and aggregation phenotype. Nevertheless, ON state colonies aggregated less than oxyR mutant colonies locked in the ON state. Using various proportion of ON and OFF bacteria we showed that this is probably due to the proportion of ON bacteria being lower in an ON colony than in an oxyR colony, where 100% of the bacteria are in the ON state. Intriguingly, although wild-type aggregating clones aggregated less than an oxyR mutant, they formed as much biofilm as the oxyR mutant in continuous flow biofilm fermentors. Conversely, and albeit to a lesser extent, wild-type non-aggregating clones formed better biofilms than either dam or agn43 mutants. This indicates either an imperfect direct correlation between Ag43-mediated auto-aggregation and biofilm formation, or a phenomenon of positive selection of Ag43+ bacteria during biofilm formation.

Our results are consistent with the second of these two possibilities; we found that, although planktonic and biofilm cultures displayed similar agn43 switching frequencies, Ag43+ bacteria were positively selected in continuous flow biofilm fermentors. We therefore concluded that the high Ag43 level in biofilms is due to physical selection of ON cells rather than increased switching towards the agn43 ON phase. Possibly Ag43+ cells contribute less to biofilm formation than Ag43+ cells and are less well integrated and more easily washed out in experimental systems in which biofilms are formed in dynamic flow conditions. Our results constitute proof of principle in a specific situation in which Ag43+ cells can be enriched and become a potential source of bias in an analysis of Ag43-related function or agn43 regulation. They are also consistent with the idea advanced by Tree et al. that the phenotypic heterogeneity generated by agn43 phase variation could bias studies due to selective advantage for Ag43-mediated cellular aggregation [35]; the authors suggested that the deletion of the cueO gene, encoding a multicopper oxidase, derepressed the expression of agn43 indirectly by a natural selection of ON cells in the population without affecting the level of agn43 expression per ON cell.

The positive selection of Ag43+ cells that we observed in biofilm in vitro can presumably also occur in vivo, for example, in intracellular bacterial communities (IBCs) formed in bladder. The initial colonization of bladder epithelium, mediated by type 1 fimbriae, results in exfoliation of superficial cells, causing many bacteria to be shed in urine, but numerous bacteria remain attached to the urothelium [47]. This phenomenon may result in selective retention of Ag43+ cells: Ag43 may allow initial tighter adherence of bacteria followed by autoaggregation. Indeed,
different variants of Ag43 have been shown to mediate adhesion to renal proximal tubular cells and kidney cell line [48] and a positive selection of Ag43+ cells would explain the strong expression of Ag43 described in intracellular biofilm communities (IBCs) formed by uropathogenic E. coli within murine bladder cells [49].

However, the fact that this strong expression was localized is also consistent with clonal expansion from a small number of original Ag43 ON cells rather than a positive selection for an Ag43+ population. These observations further illustrate the existence of such Ag43 phase variation mechanisms in vivo, and also show that more work is needed to elucidate the behavior of Ag43 ON and OFF cells in vivo.

Our study also suggests that global gene expression analysis is not appropriate for the analysis of Ag43 regulation. For instance, it has been suggested that the 14-fold increase in expression of agn43 associated with the mutation of cueO (the gene for the periplasmic multicopper oxidase) is not a direct regulatory effect but the consequence of the selection of cells with ON phenotype within the population [35]. Here, we show that lrp and mqsR mutants, previously identified by transcriptomic methodology as potential agn43 activators, did not affect either agn43 expression or Ag43 function. Although we cannot exclude that the reported regulations could be strain specific as they were done in W3110 or MG1655 genetic backgrounds that do not markedly differ from TG background, it seems very likely that the earlier identification of these proteins as regulators might have been artifacts of the random picking of colonies expressing or not expressing agn43. This clearly illustrates how ignoring phase variation of agn43, or its associated phenotypes, can introduce a strong bias into analyses of its expression in whole populations.

Finally, we investigated YfaL-mediated biofilm production. We found that Ag43-mediated aggregation can modulate the outcome of biofilm experiments designed to study other adhesins, and in particular experiments involving the popular microtiter plate assay. Although the mechanism by which Ag43 interferes with YfaL-mediated adhesion remains unclear, it is possible that the aggregation property of Ag43 is responsible for this interference. When growing biofilms in microtiter plates, large Ag43-mediated aggregates may be detached by washing procedures thereby reducing the number of attached bacteria and minimizing the effect of other adhesins. Our results are coherent with those observed previously for Ag43 interference with motility [50], thus suggesting that the presence/absence of Ag43 may modulate diverse cell surface structures. It is therefore possible that microtiter plate assays fail to identify all adhesins due to the random picking of Ag43+ ON populations interfering with the results. Inversely, care must be taken not to attribute autoaggregation properties to a protein without checking that it is not due to...
background expression of Ag43. Interestingly, physical interference by surface structures, including various pili, capsule or LPS, reciprocally affect Ag43 [51–53]. E. coli may have therefore evolved multiple mechanisms by which cell surface appendages can interfere or interact with each other, in a network at a higher order of regulation, overlapping with the known transcriptional regulatory network.

Our study clearly shows that careful assessment and monitoring the ON/OFF state of Ag43 in bacterial populations studied are required to avoid both misleading conclusions about agr43 regulation, and misinterpretation of the adhesion and biofilm properties of other surface appendages.

Supporting Information

Figure S1 The quantity of ON cells in a wild-type (WT) culture determines its degree of aggregation. Different amounts of a locked-ON (AssyR) culture were mixed with a locked-OFF (Adam) culture such that there were 0 to 100% ON cells; the mixtures were left to aggregate for 7 h at room temperature. A. Pictures of the settling cultures as with a WT ON (B) colony for reference, and the corresponding immunodetection using anti-Agr43 antibodies. B. Kinetics of aggregation of the same cultures. The degree of auto-aggregation is linearly correlated with the percentage of ON bacteria present in the culture. A threshold of ON bacteria (≥25%) has to be reached before the auto-aggregation phenotype becomes visible and measurable. A WT (ON) colony, grown overnight in liquid LB medium, aggregates like a 75% ON culture, reflecting its natural mixed composition of Ag43+ and Ag43− cells. (TIF)

Acknowledgments

We thank Prof. Peter Owen for kindly providing Ag43 antibodies.

Author Contributions

Conceived and designed the experiments: AC CS JMG CB. Performed the experiments: AC CS CB. Analyzed the data: AC CS CB. Contributed reagents/materials/analysis tools: AC CS JMG CB. Wrote the paper: AC CS JMG CB.

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