Hha, YbaJ, and OmpA Regulate *Escherichia coli* K12 Biofilm Formation and Conjugation Plasmids Abolish Motility

Andrés F. González Barrios, Rongjun Zuo, Dacheng Ren, Thomas K. Wood

Departments of Chemical Engineering and Molecular & Cell Biology, University of Connecticut, 191 Auditorium Road, Storrs, CT, 06269-3222; telephone: (979) 862-1588; fax: (979) 845-6884

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Abstract: *Escherichia coli* Hha is an environmental-response regulator of the pathogenic hemolysin operon, and Hha and the contiguous YbaJ are both induced 30-fold in *E. coli* biofilms (*Appl. Microbiol. Biotechnol.* 64:515, 2004). Here it is shown that Hha and YbaJ regulate biofilm formation since the *hha*/*ybaJ* deletion reduces biofilm mass in microtitre plates (81% in minimal medium, 50% in complex medium) and in flow cells (1,000-fold less surface coverage in minimal medium). The addition of the derepressed conjugative plasmid R1*drd*19, which increases significantly biofilm formation, eliminated motility completely in wild-type *E. coli* K12, promoted cell aggregation 27.18 ± 0.05-fold, and produced a flatter biofilm. Deletion of *hha*/*ybaJ* or *ybaJ* restored motility (this motility phenotype may be complemented by providing hha^+/$ybaJ^+$ or $ybaJ^+$ in trans) and reduced cell aggregation to that of the wild-type strain that lacks the conjugation plasmid. This increase in motility due to deleting *hha*/*ybaJ* was found to be due to 8-fold induction of *fliA* transcription. In addition, deletion of *ompA* reduced biofilm mass by 80% in both LB medium and LB medium with glucose. Also, Hha/YbaJ promotes conjugation since there was five-fold less conjugation in the *hha*/*ybaJ* mutant. It appears that conjugation plasmids promote biofilm formation by promoting cell aggregation, and that Hha and YbaJ increase biofilm formation by increasing conjugation and by decreasing motility when a conjugative plasmid (R1*drd*19) is present (YbaJ plays the most important role in this regulation of motility). When *hha*/*ybaJ* are deleted, there is less conjugation, less aggregation, more motility, and less biofilm.

Keywords: biofilm regulation; *E. coli*; Hha; YbaJ; OmpA; conjugation; motility

INTRODUCTION

Biofilms are ubiquitous and cause serious health problems like lung, eye, and urinary tract infections (Potera, 1999). As estimated by the Centers for Disease Control and Prevention (CDC), biofilms are involved in 65% of human bacterial infections (Potera, 1999). In addition, biofilms cause engineering problems such as corrosion and fouling (Elvers and Lappin-Scott, 2000). Hence, prevention of biofilm formation is important for health care and industry, and a thorough understanding of biofilm formation is critical for such control.

While biofilm formation is subject to many factors such as nutrient concentration, surface condition, and hydrodynamics, it generally occurs in three phases: initial attachment, microcolony formation, and the formation of a stable three-dimensional structure (Branda et al., 2005; Ramsey and Whiteley, 2004) with cells packed in clusters and water channels among the clusters for nutrient transfer and waste removal (Elvers and Lappin-Scott, 2000). Although the genetic basis of biofilm formation has been investigated using several bacterial strains including *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Escherichia coli* (Ghannoum and O'Toole, 2004; O'Toole et al., 2000; Pratt and Kolter, 1999), an understanding of biofilm formation at the genetic level is not complete. Since there is evidence that the expression of as many as 38% of the genes in *E. coli* changes during the transition from the planktonic to biofilm state (Prigent-Combaret et al., 1999), and since these genes must be coordinately regulated, finding the regulatory proteins responsible for these three phases has become a primary goal.

For cell-cell and cell-surface adhesion, genetic approaches indicate the importance of surface appendages like flagella, pili, and other membrane adhesins (Pratt and Kolter, 1998). Plasmid-encoded factors also enhance biofilm formation in *E. coli* such as aggregative adherence fimbriae in *E. coli* (EAEC) (Prigent-Combaret et al., 2001) and conjugative plasmids in *E. coli* K12 (Ghigo, 2001; Reisner et al., 2003). Cells bearing a conjugative plasmid may form a biofilm more efficiently to ensure that antibiotic resistance genes and virulence factors may be spread readily in the population (Ghigo, 2001), and the presence of a conjugative plasmid appears to override the importance of flagella, type I fimbriae, curli, and the outer membrane protein Ag43 in biofilm formation (Reisner et al., 2003). In addition, the formation of aggregates has been shown to regulate biofilm development in *Serratia liquefaciens* MG1 (Labbate et al., 2004).

Correspondence to: T.K. Wood. Current address: Department of Chemical Engineering, Texas A & M University, College Station, TX 77843-3122.

The contiguous genes *hha* and *ybaJ* appear to form an operon (with the promoter upstream of *ybaJ*), and are induced 30-fold in biofilms (Ren et al., 2004b). Little is known about YbaJ and it appears to be a 124 aa regulator protein since *ybaJ* is induced by stationary-phase signals (Ren et al., 2004a), and YbaJ has 64% identity with YmoB of *Yersinia enterolitica* which is a transcriptional regulator for some virulence factors (Ellison et al., 2003). Additionally, *ymoA* has been used to complement *hha* mutations (Balsalobre et al., 1996). Hha is better studied and is a 72 amino acid modulator of environmental stimuli like osmolarity and temperature (Mouriño et al., 1998) and contains a helix-turn-helix DNA-binding motif (Yee et al., 2002). It regulates multiple proteins and therefore causes pleotropic effects (Balsalobre et al., 1999). Hha interacts with the nucleoid-associated protein H-NS (Nieto et al., 2002), which plays an important role in bacterial motility at low pH and low oxygen tension (Landini and Zehnder, 2002; Soutourina et al., 2002). Moreover, Hha negatively regulates virulence factors via its DNA binding and silencing of the hemolysin expression region *hlyC*, designated *hlyM* (Jubete et al., 1995). At high osmolarity, Hha positively regulates the expression of OmpA, one of the most abundant proteins in the *E. coli* envelope (Balsalobre et al., 1999). OmpA maintains the integrity of the *E. coli* outer membrane (Sonntag et al., 1978), functions as a mediator in F-dependent conjugation (Schweizer and Henning, 1977), interacts with solid surfaces (Lower et al., 2005), and plays an important role as a phage receptor (Datta et al., 1977). Because of their relatedness, the goals of this study were to investigate the roles of Hha, YbaJ, and OmpA in biofilm formation. To our knowledge, this is the first report demonstrating Hha, YbaJ, and OmpA play a direct role in biofilm formation and that addition of a conjugation plasmid eliminates motility.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

The *E. coli* strains and plasmids are listed in Table I. Biofilm assays were performed using strains harboring the conjugation plasmid R1*drd*19 (Reisner et al., 2003) to promote biofilm formation in *E. coli* K12 (Ghigo, 2001; Reisner et al., 2003); R1*drd*19 is a permanently derepressed mutant of the F-like natural plasmid R1 that constitutively forms conjugative pili. The GFP plasmid pCM18 (Hansen et al., 2001) was used to visualize the biofilm in the flow cell experiments.

Luria-Bertani medium (LB) (Sambrook et al., 1989) containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl was used to pre-culture all the *E. coli* cells involved in the study. LB, LB supplemented with 0.2% glucose (LB glu), and M9 supplemented with 0.4% glucose and 0.4% of casamino acids (M9C glu) (Rodriguez and Tait, 1983) were used to study biofilm formation in the 96-well plate assay. For plasmid selection, antibiotics were added to the cultures at 100 μg/mL for ampicillin, 50 μg/mL for kanamycin, 30 μg/mL for chloramphenicol, and 12 μg/mL for tetracycline; all media had chloramphenicol 30 μg/mL for strains harboring R1*drd*19.

Deletion of *hha*-ybaJ

E. coli gene replacement was performed using the bacteriophage λ *red* recombination system to promote recombina-

Table I. *E. coli* strains and plasmids used. Amp^R, Km^R, Cm^R, Sm^R, Em^R, Rif^R, and Tc^R are ampicillin, kanamycin, chloramphenicol, streptomycin, erythromycin, rifampicin, and tetracycline resistance, respectively.

Strain or plasmid	Genotype	Source
Strains		
K12 ATCC25404	Wild-type *E. coli*	ATCC
K12 ATCC25404 K12 Δ*hha* Δ*ybaJ*	K12 *Δhha ΔybaJ* Ω Km^R	This study
K12 BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ hsdR514 *ΔaraBA*-D$_{AH33}$ *ΔrhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
K12 BW25113 Δ*hha*	K12 *Δhha* Ω Km^R	Baba et al. (2003)
K12 BW25113 Δ*ybaJ*	K12 *ΔybaJ* Ω Km^R	Baba et al. (2003)
K12 MG1655	F^- lambda$^-$ *ilvG- rfb*-50 *rph*-1	Blattner et al. (1997)
K12 MG1655 Δ*ompA*	K12*ΔompA*::Tn5Kan-2	Kang et al. (2004)
K12 MG1655 Δ*motA*	K12*ΔmotA*::Tn5Kan-2	Kang et al. (2004)
Plasmids		
R1*drd*19	*finO*, Amp^R, Km^R, Cm^R, Sm^R, IncFII	Ghigo (2001)
pVLT31	*lacI*, Tc^R	de Lorenzo et al. (1993)
pVLT31 *hha ybaJ*	pVLT31 *plac*::*hha*$^+$ *ybaJ*$^+$	This study
pDG780	Km^R	Guérout-Fleury et al. (1995)
pCM18	Em^R; pTRKL2-P$_{CP25}$RBSII-*gfp*3*-To-T1	Hansen et al. (2001)
pKM208	pAMPts *ptac*::*gam*-red	Murphy et al. (2000)
pVS159	*qseB*::*lacZ* in pRS551	Sperandio et al. (2002)
pVS176	*motA*::*lacZ* in pRS551	Sperandio et al. (2002)
pVS175	*fliC*::*lacZ* in pRS551	Sperandio et al. (2002)
pVS183	*fliAehK*12::*lacZ* in pRS551	Sperandio et al. (2002)
pVS182	*flhD*::*lacZ* in pRS551	Sperandio et al. (2002)

tion (Murphy, 1998). Plasmid pKM208 carrying *ptac*-gam-red was electroporated into *E. coli* K12. The *red* locus consists two genes, *exo* and *bet*; Exo acts on double-stranded DNA with 5′-3′ exonuclease activity, Bet binds to single-stranded DNA and promotes gene replacement, and Gam represses RecBCD activity (Murphy et al., 2000). To delete the *hha*-ybaJ locus, a 3.4 kb PCR fragment containing the kanamycin resistance gene from plasmid pDG780 (Guérout-Fleury et al., 1995) flanked by sequences of 925 bp upstream and 922 bp downstream of *hha*-ybaJ was constructed by performing three PCR reactions (primer sequences are shown in Table II). First, the upstream fragment (1,134 bp, primers L01 and L02) and downstream fragment (1,112 bp, primers L03 and L04) of *hha*-ybaJ were amplified, then the kanamycin gene from strain *Streptococcus faecalis* (GenBank accession number V01547) was amplified from pDG780 to generate a PCR fragment (1,623 bp, primers L05 and L06) having 43 bp and 41 bp at each end that are complementary to the upstream and downstream fragments, respectively. Secondly, nested primers L07–L08 and L09–L10 were used to fuse the upstream and downstream *hha*-ybaJ fragments, respectively, with the kanamycin resistance marker. Finally, the two fragments obtained were fused using primers pA and pB to yield a fragment with the kanamycin resistance gene flanked by 926 bp of the upstream region of *ybaJ* and 922 bp of the downstream region of *hha*. All fragments were purified using QIAquick gel extraction kit. Following final purification, the linear fragment was transformed into *E. coli* K12 carrying pKM208 and a kanamycin-resistant transformant was selected.

To check if the desired *hha*/*ybaJ* deletion mutant was obtained, PCR using primers pA and pB was performed and yielded the desired bands (wild-type gave a PCR product of 2,467 bp and the *hha-ybaJ* double deletion mutant gave a PCR product of 3,387 bp). To further confirm that the recombination events happened at the expected sites, sequencing using primers pC and pD located 100–150 bp away from the recombination sites were conducted. Sequencing showed that the kanamycin resistance gene (1,539 bp) replaced the chromosome fragment (619 bp) which spans from the start codon of *ybaJ* to the stop codon of *hha*. One recombination site is 546 bp away from the stop codon of gene *acrB* (b0462, the gene immediately upstream of *ybaJ*), and the other recombination site is 172 bp away from the start codon of gene *maa* (b0459, the gene immediately downstream of *hha*). Thus, the desired mutant was obtained, that is, only the complete *hha-ybaJ* allele was knocked out without affecting any of the neighboring genes (a 619 bp fragment was deleted from position 479,932 to 479,314 of the GenBank accession # NC_000913).

Microtitre Biofilm Assay

This assay was modified slightly from that reported previously (Li et al., 2001; Pratt and Kolter, 1998). *E. coli* strains were grown in minimal media M9C glu, LB, or LB supplemented with 0.2% glucose in polystyrene 96-well plates at 37°C for 2 days without shaking. Two identical plates were prepared and one plate was processed for measuring biofilm mass at 12 and 48 h. Before measuring the biofilm mass in each well, the optical density at 620 nm (OD) was taken to quantify the growth of *E. coli* K12. To quantify the biofilm mass, the suspension cultures were decanted, the plates were washed 3 times with water, and the biofilms were stained with 0.1% crystal violet (Fisher, Hanover Park, IL) for 20 min. The extra dye was removed by

Table II. Primers used for *hha*/*ybaJ* deletion, for sequencing to confirm the *hha*/*ybaJ* deletion, and for constructing the complementation plasmid.

Primers	Nucleotide sequence
Deletion	
L01	5′-CGGTGTTGGCTATGACTGGACG-3′
L02	5′-GCGTCCCCTTCTTAGCGG-3′
L03	5′-TAAATAATTCGCTTTCGGAGC-3′
L04	5′-CGTCGATATCTTCATAGCGG-3′
L05	5′-CGAAAGGTGTCCGTTAGTTCAACCGCTA AGAAGGGGACGCGTTCTCTAGAACTAGTGGATCCC-3′
L06	5′-TTAATAAACAGCCGGTTATAGCTCCGAAA GCGAATTATTTACGATACAAATTCCTCGTAGGCGC-3′
L07	5′-TCCGTTATGCTGGTCGTTCCGC-3′
L08	5′-CGATACAAATTCCTCGTAGGCGCTC-3′
L09	5′-CCAGCGAACCATTTGAGGTGATAGG-3′
L10	5′-GCGTTTCGGACTGCAACATCACC-3′
pA	5′-GACGTTTACTTCCAGGTAGGCC-3′
pB	5′-ACTAAAGCGGGGTTTGTTGTCG-3′
Sequencing	
pC	5′-AAAAGTGAGTCCTGTCCCGCTTCC-3′
pD	5′-AATGCGGCAACACAGTAAGACG-3′
Complementation	
pE	5′-GGTGTCCGTGAATTCAACCGCTAAGAAGGGGACG-3′
pF	5′-CAGCCGGTCTAGAATCCGAAAGCG-3′
pG	5′-GAGCGGATAACAATTTCACACAGG-3′

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washing three times with water. The OD reading at 540 nm was used to quantify the biofilms at the bottom of each well. Then the remaining dye staining the air–liquid interface and liquid–solid biofilms was dissolved with 300 μL 95% ethanol, and OD reading at 540 nm was measured to quantify the total biofilm mass (both for the biofilms at the bottom and those at the air–liquid interface). Each data point was averaged from four replicate wells.

Flow Cell Biofilm Experiments and Image Analysis

M9C glu medium supplemented with 30 μg/mL chloramphenicol and 150 μg/mL erythromycin to maintain plasmids R1*drd*19 and pCM18, respectively, was used to grow biofilm at 37°C in a continuous flow cell (BST model FC81, Biosurface Technologies Corp., Bozeman, MT). The flow cell contains a standard glass microscope slide on one side and a glass cover slip on the other side, and the flow channel has dimensions of 47.5×12.7 mm with a 1.6 mm gap between the glass plates. An overnight culture was centrifuged and resuspended in the M9C glu medium. This diluted culture (OD 0.05) was pumped into the flow cell for 2 h at 10 mL/h before fresh medium was added at the same flow rate. The biofilm development in flow cell was monitored using a TCS SP2 scanning confocal laser microscope (Leica Microsystems, Heidelberg, Germany) at 24 h (and 48 h for some experiments).

Color confocal flow cell images were converted to gray scale using Image Converter (Neomesh Microsystems, Wainuiomata, Wellington, New Zealand). Biomass, substratum coverage, surface roughness, and mean thickness were determined using COMSTAT image-processing software (Heydorn et al., 2000) written as a script in Matlab 5.1 (The MathWorks) equipped with the Image Processing Toolbox. Thresholding was fixed for all images stacks, and between 25 and 50 planar images were processed for each position; in total, 4 positions with 200 images were obtained for the wild-type K12 biofilm, and 6 positions with 150 images were obtained for the biofilm formed by the *hha*/*ybaJ* mutant. Values are means of data from the different positions at the same time point, and standard deviations were calculated based on these mean values for each position. Simulated three-dimensional images were obtained using IMARIS (Bitplane, Zurich, Switzerland). Twenty-five planar images for the *hha*/*ybaJ* mutant and fifty planar images for wild-type strain were processed for each three-dimensional image.

Motility Assay and Growth Rate Measurement

LB overnight cultures were used to assay motility in plates containing 1% tryptone, 0.25% NaCl, and 0.3% agar. The motility halos were measured at 5 h and 8 h (Sperandio et al., 2002). Between 3 and 25 plates were used to evaluate motility in each strain.

The specific growth rates of *E. coli* K12 and its mutants carrying the conjugation plasmid R1*drd*19 in LB or M9C glu medium were determined using the optical density at 600 nm (OD_{600}) and calculated using the linear portion of the natural logarithm of OD_{600} versus time (OD_{600} from 0.08 to 0.5). All the growth rates were measured twice.

Construction of the Complementation Plasmid

To show that the *hha*-*ybaJ* gene cluster is responsible for the altered motility phenotype, a complementation plasmid was constructed using pVLT31 (de Lorenzo et al., 1993). The whole coding sequence of *hha*-ybaJ was amplified using the primer pair pE (front primer) and pF (rear primer) (Table II). The PCR product was digested with *EcoR* I and *Xba* I and cloned into the multiple cloning site of pVLT31 that was digested with the same two enzymes to create the complementation plasmid pVLT31 hha^+ $ybaJ^+$. This recombinant plasmid was confirmed through DNA sequencing using primer pG, which is part of the *ptac* promoter upstream of the multiple cloning site in pVLT31. Since pVLT31 carries $lacI^q$ for tight regulation of the hha^+ $ybaJ^+$ locus, the expression of the *hha*-ybaJ allele gene requires isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis), which was added at 0.25, 0.50, 0.75, and 1 mM.

Conjugation Rate

In order to investigate the effect of the *hha* deletion on bacterial conjugation in a biofilm, the method of Ghigo (2001) was adapted. The recipient *E. coli* K12 was grown as a biofilm in 96-well polystyrene plates in 250 μL of M9C glu, and after 24 h, 50 μL of an equal concentration (OD_{600} 3) of the donor strains (*E. coli* K12/R1*drd*19 or *E. coli* K12 *Δhha* Δ*ybaJ*/R1*drd*19) were added to the 96-well plates; these cells were from an overnight LB chloramphenicol culture that was washed and resuspended in phosphate buffer solution (PBS, 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KCl, 0.2 g/L KH_2PO_4). To quantify the number of cells that received plasmid R1*drd*19 in the biofilm, the suspension cultures were decanted after an additional 24 h, and the biofilm cells were detached with shaking into 300 μL of PBS. The number of conjugations was estimated by counting the number of biofilm colonies with chloramphenicol resistance using LB chloramphenicol (30 μg/mL) plates. Data were averaged from four replicate wells. The negative control was the K12 biofilm contacted with PBS. To determine the number of chloramphenicol-resistant cells that arise from the addition of the donor to each well, K12/R1*drd*19 cells were added to wells that lacked a K12 biofilm (to eliminate conjugation) and contained spent M9C glu medium and were allowed to form a biofilm; these cells were enumerated after 24 h and subtracted from the total number of chloramphenicol colonies. This experiment was performed twice.

Promoter Transcriptional Assays

E. coli strains with the *flhD*::*lacZ*, *fliA*::*lacZ*, *fliC*::*lacZ*, *motA*::*lacZ*, and *qseB*::*lacZ* fusions were cultured overnight in LB ampicillin (100 μg/mL) then diluted 1:100 in LB and M9C glu media (both supplemented with 100 μg/mL ampicillin) to create exponentially-growing cells that were harvested at OD_{600} 1. Cells were centrifuged 5 min at 4°C at 13,800*g*, resuspended in 750 μL of Tris-ethyl diamine tetracetic acid-phenylmethyl sulphonyl fluoride buffer, and sonicated in two intervals of 15 s at 10 Watts. These samples were assayed for β-galactosidase activity using *o*-nitrophenyl-β galactopyranoside (Sigma, St. Louis, MO) as a substrate based on method described by Miller (1972). All activities were calculated based on a protein concentration of 0.24 mg protein/mL/$O.D_{600}$ unit (Fishman et al., 2005). Each experiment was performed twice with two different cultures for each strain in minimal medium M9C glu and LB.

Aggregation Assay

This assay was adapted from Roux et al. (2005). *E. coli* K12 ATCC25404, *E. coli* ATCC25404/R1*drd*19, and *E. coli* ATCC25404 *Δhha ybaJ*/R1*drd*19 were cultured overnight in LB medium (16 h) with 30 μg/mL chloramphenicol for strains harboring R1*drd*19, diluted in 3 mL of LB with the OD_{600} adjusted to 2.5 without antibiotics, and placed in 10 mL tubes. These tubes were incubated quiescently at 37°C for 24 h, and the OD_{600} was measured 5 mm underneath the surface to determine the cell concentration for an indirect measure of the cell aggregation. This experiment was repeated twice with two independent cultures.

RESULTS

Deletion of *hha* and *ybaJ* Decreases Biofilm Formation in 96-Well Plates and Flow Cells

Based on the DNA microarrays that indicated the contiguous genes *hha* and *ybaJ* are induced 25-fold and 36-fold, respectively, in rich medium during biofilm formation (Ren et al., 2004b), we deleted the *hha*/*ybaJ* locus while inserting a kanamycin resistance gene. Both PCR and sequencing verified these two genes were precisely excised. The *hha*/*ybaJ* mutation did not affect growth as there was no significant difference in the specific growth rate for any of the mutants used here relative to the wild-type strain in both minimal and complex medium (Table III). Also, for the *hha*, *ybaJ*, *hha*/*ybaJ*, and *ompA* deletions there was no impact on growth (Table III).

To enhance biofilm formation in K12, the derepressed conjugation plasmid R1*drd*19 was used; the addition of R1*drd*19 increases biofilm formation significantly since K12 ATCC25404 formed 90% less biofilm without this plasmid (Fig. 1). Using flow cells we found that the presence of R1*drd*19 leads to a flatter biofilm in both K12 ATCC25404 and K12 MG1655 (Fig. 2A vs. B, D vs. E) which lacks the typical flow channels previously reported (Ghannoum and O'Toole, 2004). The effect of adding R1*drd*19 was quantified with COMSTAT (Table III), and substratum coverage at 24 h was found to increase by 3.5-fold for K12 ATCC25404 and 1.8-fold for K12 MG1655, respectively. Moreover, biomass increased 2.2-fold for both strains. K12 ATCC25404 and K12 MG1655 biofilms with R1*drd*19 plasmid were also monitored at 48 h; however, since there was a reduction in thickness (28%), biomass (35%), and substratum coverage (26%) compared with 24 h, we considered the 24 h evaluations more representative.

The isogenic K12 ATCC25404 *Δhha ΔybaJ* mutant, with plasmid R1*drd*19 to promote biofilm formation, was then tested for its ability to form biofilms in minimal medium supplemented with glucose and casamino acids (M9C glu). Deleting both *hha* and *ybaJ* resulted in an 81% reduction in biofilm mass compared to the wild-type strain in M9C glu (Fig. 1). Also, in both LB and LB supplemented with 0.2% glucose, biofilm formation was reduced with the *Δhha ΔybaJ* mutant by 50% (after 24 h) relative to wild-type K12.

We also tested the ability of the double mutant with R1*drd*19 to form a biofilm in a continuous flow system so that the effect on the biofilm architecture could be studied (Fig. 2B vs. C). The *hha*/*ybaJ* deletion dramatically affects biofilm formation because significantly fewer microcolonies were observed compared with the wild-type. Using COMSTAT, these changes in biofilm architecture were quantified (Table III). After 24 h, the *hha*/*ybaJ* deletion reduced biomass by four-fold, substratum coverage by 1,460-fold,

Table III. Specific growth rates of strains in LB and M9C glu media and COMSTAT analysis from the flow cell using M9C glu (after 24 h).

Strain	Growth rate in LB, 1/h	Growth rate in M9C glu, 1/h	Biomass	Substratum coverage, %	Mean thickness, μm	Roughness coefficient
K12 ATCC25404	1.66 ± 0.02	1.07 ± 0.02	24 ± 4	21 ± 8	42 ± 14	0.51 ± 0.12
K12 ATCC25404/R1*drd*19	1.440 ± 0.004	1.19 ± 0.01	54 ± 20	73.10 ± 0.1	75 ± 28	0.4 ± 0.4
K12 ATCC25404 *Δhha ΔybaJ*/R1*drd*19	1.49 ± 0.05	1.04 ± 0.01	14 ± 9	0.05 ± 0.05	30 ± 15	2.11 ± 0.03
K12 MG1655	1.64 ± 0.02	0.990 ± 0.004	11 ± 8	34 ± 13	44 ± 6	0.20 ± 0.09
K12 MG1655/R1*drd*19	1.48 ± 0.04	1.3 ± 0.1	25 ± 7	61 ± 10	47 ± 15	0.57 ± 0.14
K12 MG1655 *ΔompA*/R1*drd*19	1.31 ± 0.02	1.15 ± 0.04	5 ± 5	4 ± 5	7 ± 5	2.9 ± 0.8
K12 BW25113/R1*drd*19	1.7 ± 0.1	1.5 ± 0.1	12 ± 11	53 ± 14	18 ± 13	0.4 ± 0.2
K12 BW25113 *Δhha*/R1*drd*19	1.4 ± 0.1	1.490 ± 0.005	1.45 ± 0.05	0.9 ± 0.1	1.1 ± 0.1	2.4 ± 0.1
K12 BW25113 *ΔybaJ*/R1*drd*19	1.7 ± 0.1	1.27 ± 0.03	N/M	N/M	N/M	N/M

N/M, not measured.

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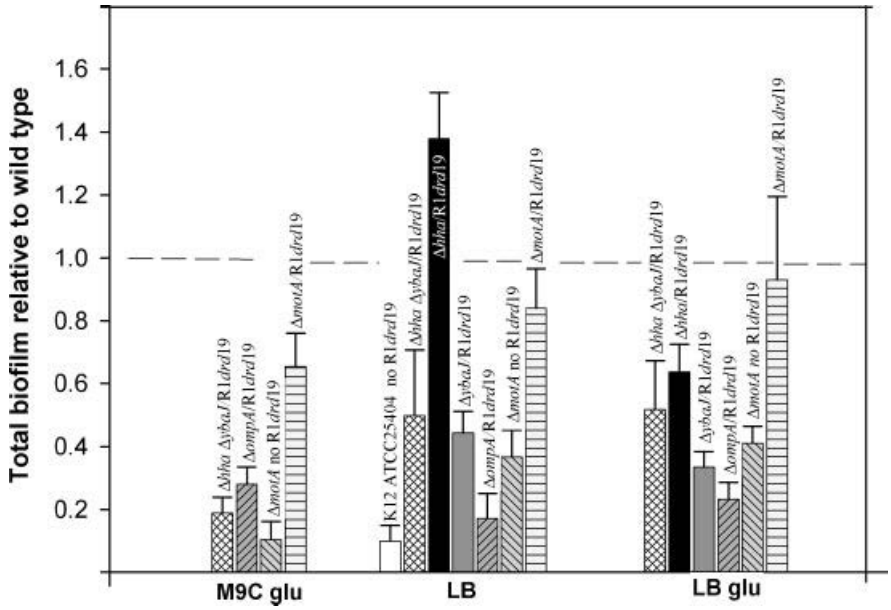

Figure 1. Effect of Hha, YbaJ, OmpA, and MotA on biofilm formation of *E. coli* K12/R1*drd*19 in microtiter plates in M9C glu medium, LB, and LB glu. Biomass measured after 24 h. Each experiment was performed in duplicate and one standard deviation is shown. Each mutant is compared with its respective wild-type strain (K12 ATCC25404 for K12 *Δhha ΔybaJ*, K12 BW25113 for K12 *Δhha* and K12 *ΔybaJ*, and K12 MG1655 for K12 *ΔompA* and K12 *ΔmotA*). To see the impact of adding plasmid R1*drd*19, biofilm formation of K12 ATCC25404 (first bar on left in the LB group) was compared with K12 ATCC25404/R1*drd*19.

and mean thickness by 2.5-fold, and the roughness coefficient was increased 5.1-fold compared to the wild-type strain.

Both the *hha* and *ybaJ* mutations were also evaluated independently for biofilm formation using *E. coli* BW25113. Since *E. coli* BW25113/R1*drd*19 formed poor biofilms in M9C glu, it was difficult to determine the impact of the single mutations in this medium; hence, we measured biofilm formation in LB and LB glu. Deleting *ybaJ* (which results in inactivation of both loci due to polar effects) decreased biofilm mass by 56% in LB and 67% in LB glu in agreement

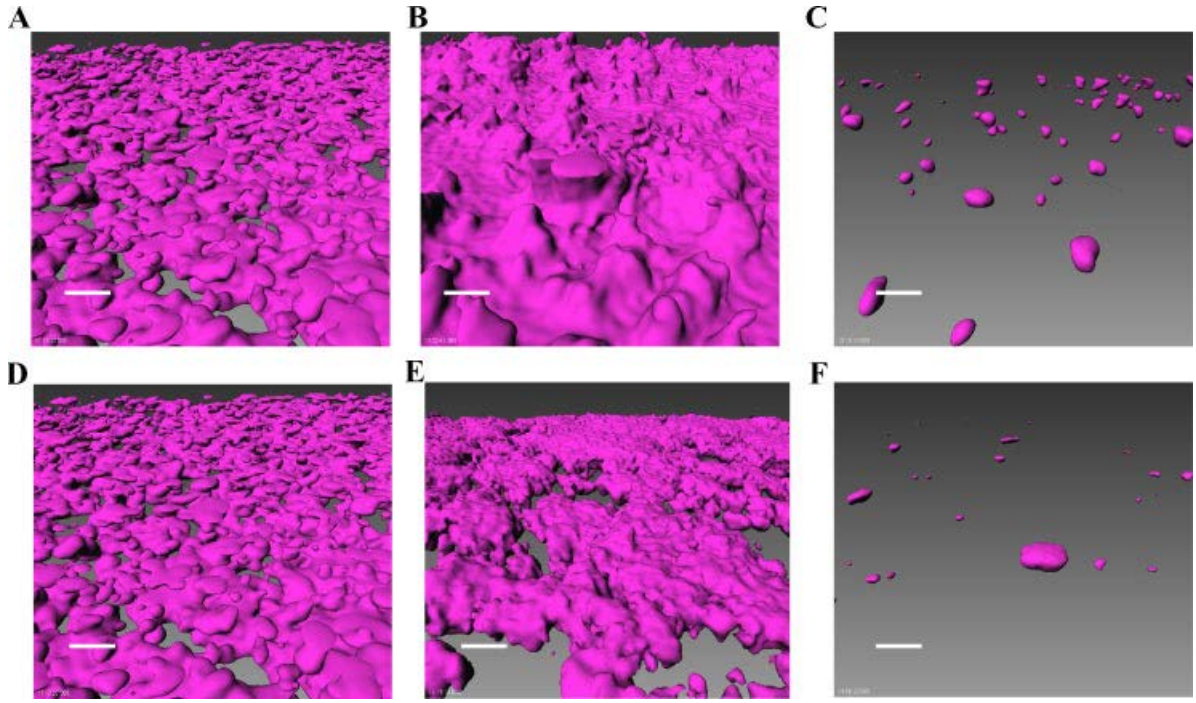

Figure 2. Effect of the conjugation plasmid R1*drd*19, Hha/YbaJ, and OmpA and on biofilm formation in flow cells with M9C glu medium. **A**: K12 ATCC25404, (**B**) K12 ATCC25404/R1*drd*19, (**C**) K12 ATCC25404 *Δhha ΔybaJ*/R1*drd*19, (**D**) K12 MG1655, (**E**) K12 MG1655/R1*drd*19, and (**F**) K12 MG1655 *ΔompA*/R1*drd*19. Biomass measured after 24 h, and images were analyzed with IMARIS. Scale bar is 5 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

with the *Δhha ΔybaJ* mutant while the *hha* single deletion reduced biofilm mass by 37% in LB glu but actually increased biomass by 36% in LB medium (Fig. 1).

E. coli BW25113/R1*drd*19 and *E. coli* BW25113 *Δhha*/R1*drd*19 were also tested for biofilm formation in the continuous flow system; the deletion of *hha* corroborates the results obtained with the double mutation since this deletion caused a 8.5-fold decrease in biomass, 60-fold decrease in substratum coverage, and 16-fold decrease in biofilm thickness (Fig. 3 and Table III). Hence, we were able to determine the effect of the single *hha* mutation in flow cells.

Effect of R1*drd*19, Hha, and YbaJ on Motility and Aggregation

We found that the addition of the constitutive conjugation plasmid to wild-type K12 to promote biofilm formation completely abolishes motility in K12 ATCC25404 (Fig. 4A). This result was corroborated by the 90% reduction in motility upon the addition of R1*drd*19 to K12 BW25113 (Fig. 4B).

To determine if this difference in motility is due to a change in the regulation of flagella or motility genes, we used *lacZ* transcriptional fusions with the promoters of *flhD, fliA, fliC, flhD*, and *qseB* (Sperandio et al., 2002) which are known to encode critical proteins in the synthesis and motion of flagella in *E. coli* (Chilcott and Hughes, 2000). No significant differences were found in the expression of those genes between K12 ATCC25404 and K12 ATCC25404/R1*drd*19 in both M9Cglu and LB medium (data not shown). Hence, we investigated the effect of adding the conjugation plasmid on K12 ATCC25404 aggregation; the addition of the conjugation plasmid was found to increase cell aggregation by 27.18 ± 0.05-fold. Furthermore, deletion of *hha*/*ybaJ* decreases cell aggregation and makes the cells behave just like the wild-type K12 ATCC25404 that lacks the conjugation plasmid. Therefore, the addition of the conjugation plasmid makes the cells aggregate but deleting *hha*/*ybaJ* restores the non-aggregation phenotype.

Deleting both *hha* and *ybaJ* or deleting solely *ybaJ* restores motility (in the presence of R1*drd*19) to levels almost equal to that of the wild-type strain that lacks R1*drd*19 (Fig. 4). This increase in motility is caused by a twofold increase in expression of the flagella master regulator *flhD* (Chilcott and Hughes, 2000) which activated expression of sigma factor *fliA* (eightfold), *fliC* (2.3-fold), and *motA* (threefold) in minimal media (Fig. 5A). These results were corroborated in LB medium (Fig. 5B) since *flhD, fliA, fliC*, and *motA* were also induced 2-, 4-, 2-, and 3.5-fold, respectively. Hence, deleting *hha* and *ybaJ* increases motility via flagella and motility genes expression.

To complement the *hha ybaJ* mutation at low gene dosage, we constructed the low-copy number plasmid pVLT31 which carries hha^+ $ybaJ^+$ under control of the ptac promoter. Increasing expression of hha^+ $ybaJ^+$ via IPTG reduced motility to match that of wild-type K12/R1*drd*19; therefore, both the *hha*/*ybaJ* and *ybaJ* mutations may be complemented (Fig. 4). Note there was an insignificant effect on motility by adding pVLT31 to K12.

OmpA and MotA Affect Biofilm Formation in *E. coli* K12

Since Hha positively regulates the expression of OmpA under high osmolarity (Balsalobre et al., 1999), we hypothesized that OmpA could play a role in biofilm formation. In contrast to the results reported by Ghigo (2001), who did not find an effect for OmpA in *E. coli* K12 biofilm formation, it was found here using an isogenic *ompA* strain that deletion of OmpA decreased biofilm formation significantly and consistently by 72% in M9Cglu, 83% in LB, and 77% in LB glu media (Fig. 1). Flow cells with K12 MG1655 *ΔompA*/R1*drd*19 and its isogenic wild-type K12 MG1655/R1*drd*19 corroborated the 96-well experiments; when *ompA* is deleted, the biofilm thickness, substratum coverage, and biomass were reduced by 6.7-, 16-, and 5-fold, respectively (Table III, Fig. 2E vs. F). Therefore, OmpA promotes biofilm formation in *E. coli*.

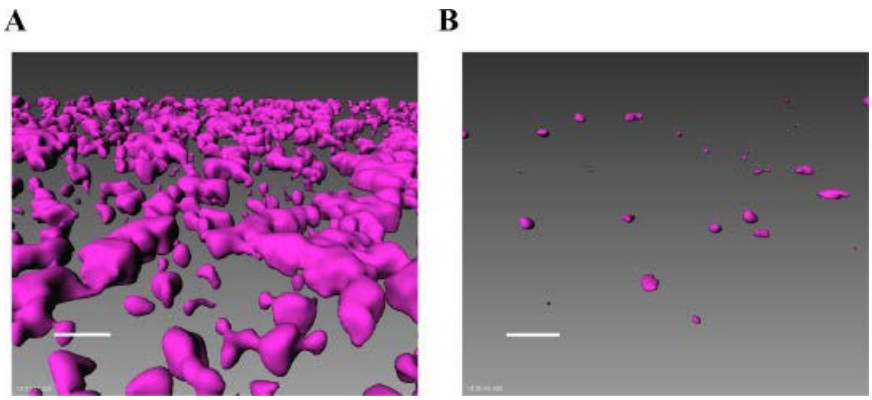

Figure 3. Effect of Hha on biofilm formation in flow cells. **A**: *E. coli* K12 BW25113/R1*drd*19, (**B**) *E. coli* K12 BW25113 *Δhha*/R1*drd*19. Biomass measured after 24 h, and images analyzed with IMARIS. Scale bar is 5 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

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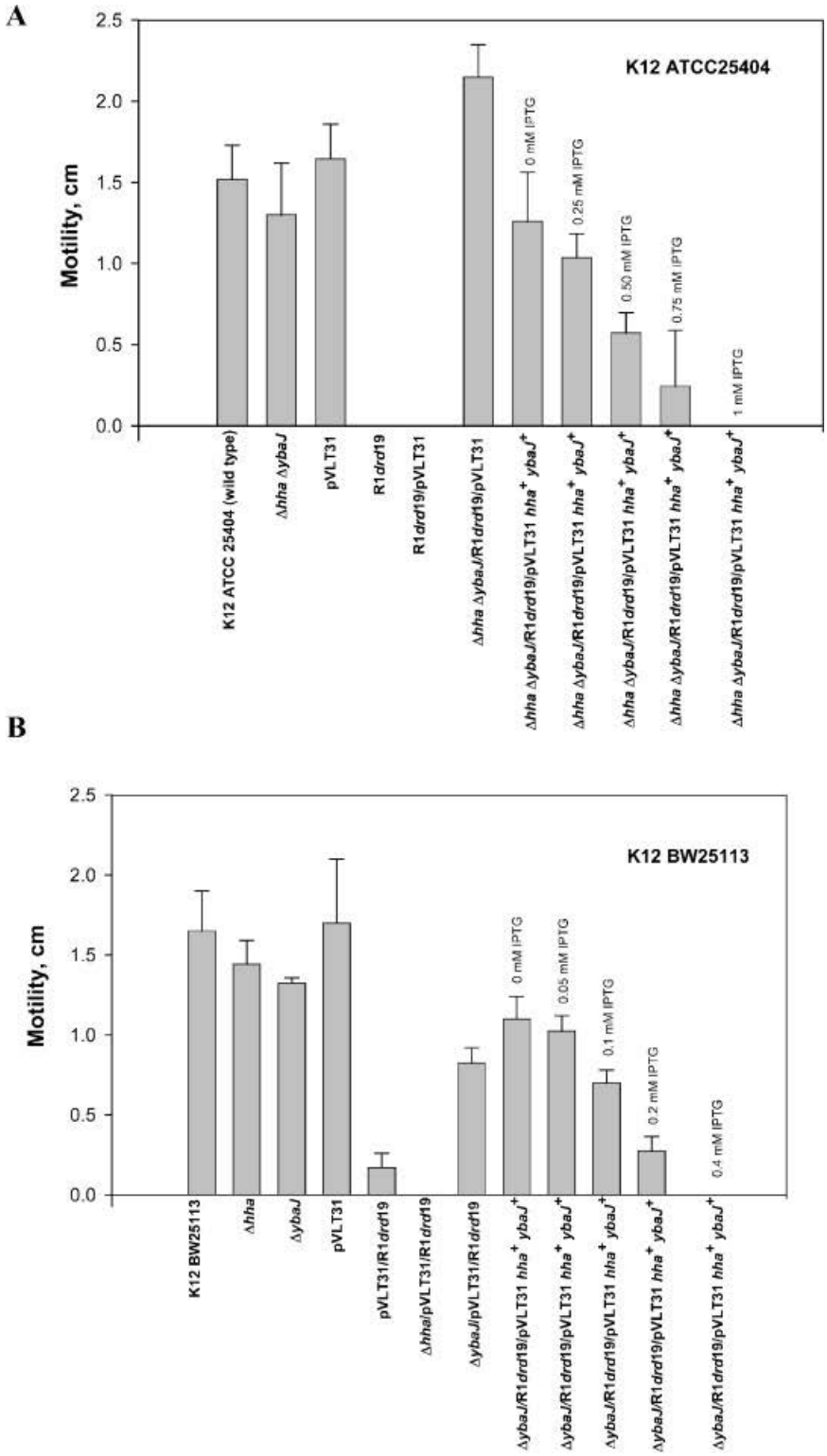

Figure 4. Effect of the deletion of both Hha/YbaJ and R1*drd*19 on the motility of *E. coli* K12 ATCC25404 (**A**), and the effect of Hha and YbaJ (independently) on the motility of *E. coli* K12 BW25113 (**B**). Motility diameter measured at 8 h. For the complementation experiments, IPTG was added to all cultures; no effect of IPTG addition was found with the negative controls *E. coli* K12 *Δhha ΔybaJ*/R1*drd*19/pVLT31, *E. coli* K12 *Δhha* R1*drd*19/pVLT31, and *E. coli* K12 *ΔybaJ* R1*drd*19/pVLT31. Each experiment was done in duplicate, and one standard deviation shown.

We also examined biofilm formation in the *ΔmotA* mutant in order to link motility and biofilm formation. First we tested the *motA* mutant in the absence of R1*drd*19. As expected, the deletion of *motA* decreases biofilm mass 60% as reported previously by Pratt and Kolter (1998) who found a decrease in biofilm mass by deleting *motAB* in *E. coli* K12. In contrast, when cells were transformed with the R1*drd*19 conjugation plasmid, biofilm formation of the *ΔmotA* mutant was re-

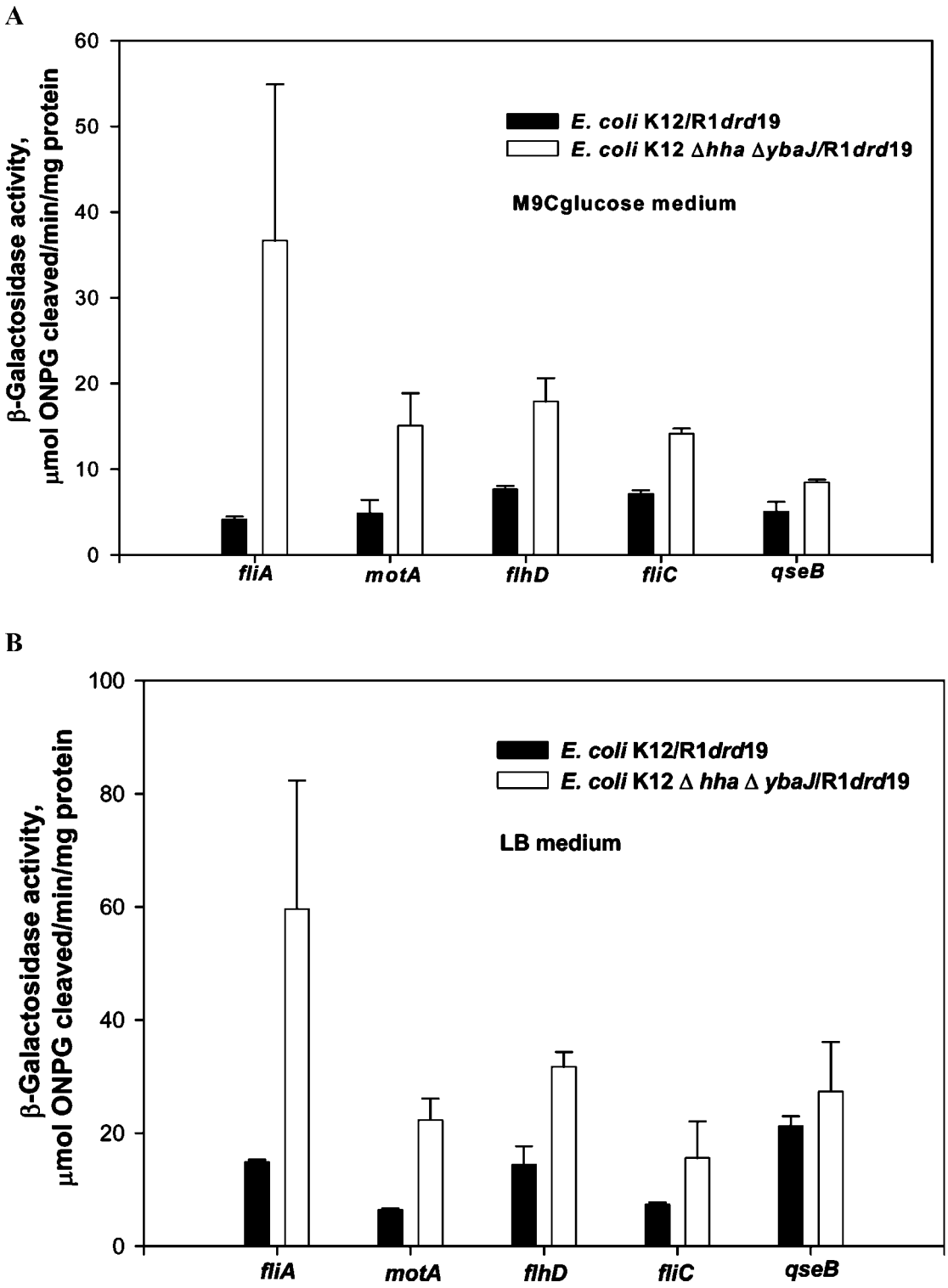

Figure 5. Comparison of transcription of *flhD*::*lacZ*, *fliA*::*lacZ*, *fliC*::*lacZ*, *motA*::*lacZ*, and *qseB*::*lacZ* between *E. coli* K12 ATCC25404/R1*drd*19 and K12 ATCC25404 *Δhha ΔybaJ*/R1*drd*19 in minimal media M9C + 0.4% glucose (**A**) and LB (**B**). The experiment was done in duplicate, and one standard is deviation shown.

established (Fig. 1). Both *E. coli* K12 *ΔmotA* and *ΔmotA*/R1*drd*19 were found to be non-motile, as expected.

Hha Affects Conjugation in *E. coli* K12

To probe further the connection between Hha and conjugation we measured the conjugation rate of R1*drd*19 in our biofilm 96-well system. We hypothesized that deleting *hha* would decrease the conjugation frequency if Hha positively regulates conjugation in *E. coli*. If conjugation promotes biofilm formation, then deleting *hha* should also decrease gene transfer leading to a reduction in biofilm formation. It was found that the conjugation rate of plasmid R1*drd*19 using

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donor K12 *Δhha ΔybaJ*/R1*drd*19 and K12 as a recipient was fivefold lower than the conjugation rate using K12/R1*drd*19 as the donor (13.5×10^5 conjugants/mL for K12/R1*drd*19 vs. 2.47×10^5 conjugants/mL for K12 *Δhha ΔybaJ*/R1*drd*19). No colonies were detected when no donor was added (K12 contacted with phosphate buffer solution).

DISCUSSION

We focused on *E. coli* biofilms since this strain is the most thoroughly-studied bacterium (Blattner et al., 1997) with many isogenic mutants available (Kang et al., 2004) and because the interconnectedness of many of the pathways is known. Also, our group has experience in determining the genetic basis of *E. coli* biofilm formation (Ren et al., 2004b) and biofilm inhibition with the natural plant-derived antagonists furanone (Ren et al., 2001, 2004c) and ursolic acid (Ren et al., 2005). Furthermore, although *E. coli* is well studied, its biofilm has not received the same scrutiny since K12 makes a poor biofilm if it lacks a conjugative plasmid (Ghigo, 2001; Reisner et al., 2003). Hence, the results of our study are helpful for understanding and preventing the biofilm of the archetypal strain as well as helpful for combating pathogenic strains such as *E. coli* O157:H7 (Perna et al., 2001).

Our DNA microarray experiments indicated for the first time that Hha and YbaJ have a role in *E. coli* biofilm formation (Ren et al., 2004b), and we have confirmed this by deleting *hha* and *ybaJ* and showing this deletion results in a dramatic decrease (81% in M9Cglu and 50% in LB) in biofilm formation (Fig. 1 and 2). It is interesting in that both genes have to be deleted to see the greatest effect and without the microarrays to show their interrelatedness, the impact of these two genes on biofilm formation may have been overlooked.

We observed that the presence of glucose made the *hha* deletion more effective in inhibiting biofilm formation (Fig. 1) since there is 35% more biofilm compared with its isogenic wild-type without the presence of glucose. Similar results have been seen by us with other biofilm regulator proteins obtained from our DNA microarray experiments and these results are quorum-sensing related. Hha is induced 11-fold by autoinducer-2 (AI-2) (DeLisa et al., 2001), and AI-2 uptake and synthesis are subject to catabolic repression through the cyclic AMP (cAMP)-CRP complex (Wang et al., 2005; Xavier and Bassler, 2005). Hence the presence of glucose may prevent the uptake and synthesis of AI-2 and introduce another layer of control related to the Hha/YbaJ-cascade and biofilm regulation.

We were unable to complement the *Δhha ΔybaJ* mutations in regard to biofilm formation due to difficulties previously reported (Roux et al., 2005) such as non-physiological levels of expression or requirements for antibiotics as plasmid selection pressure. We also investigated if the reduction in biofilm from the *hha* and *ybaJ* mutations was related to motility because of the link of this phenotype to biofilm formation from our own *E. coli* microarray results (e.g., *fimG* was induced) (Ren et al., 2004b) and the experiments of Pratt and Kolter (1998) that showed motility (normal flagellar motion from genes like *motA*) but not chemotaxis is essential for biofilm formation. We discovered that the addition of the conjugative plasmid R1*drd*19 eliminates motility in *E. coli* K12; but, we found no difference in the expression of key flagellar and motility genes. Instead we found the formation of cell aggregation is the cause of the dramatic reduction in motility and the dramatic increase in biofilm. When both *hha*/*ybaJ* alone are deleted, motility increases dramatically in the presence of R1*drd*19 (Fig. 4), and the biofilm-deficient mutant *Δhha*/*ΔybaJ* has similar swimming motility as the motile wild-type strain that lacks the conjugation plasmid and makes a poor biofilm (Fig. 1). All previous results indicate motility-deficient strains have reduced biofilm formation, whereas here the biofilm-deficient strains have enhanced motility. Note the Hha/YbaJ-controlled motility phenotype may be complemented by expressing Hha and YbaJ from an inducible promoter (*plac*) in the *ybaJ* and *hha*/*ybaJ* deletion mutant (Fig. 4). The effect of Hha/YbaJ on motility was also seen via the increase in expression of *flhD, fliA, motA*, and *fliC* so these proteins play an important role in the regulation of motility.

Our hypothesis (Fig. 6) is that conjugation (via R1*drd*19) positively regulates biofilm formation through Hha/YbaJ by decreasing motility and increasing cell aggregation; therefore, if Hha/YbaJ are deleted, then there is more motility, less aggregation, less conjugation, and less biofilm formation. The conjugation plasmid works through Hha/YbaJ since there is little effect on biofilm formation (results not shown) and motility (Fig. 4) upon deletion of *hha*/*ybaJ* in the absence of the conjugation plasmid. The link between motility and biofilm formation in our system is established based on the decrease in biofilm we observed when *motA* is deleted in the absence of R1*drd*19 (Fig. 1). Our results suggest that when R1*drd*19 is present, conjugation is dominant over motility since flagella motion is not necessary for the biofilm formed here (Fig. 1); when R1*drd*19 is added to the *motA* mutant, there is a significant increase in biofilm mass relative to the *motA* strain that lacks the conjugation plasmid (Fig. 1) even though the cells are completely non-motile. We found the presence of Hha/YbaJ decreases motility by repressing transcription of *flhD* (Fig. 5A and B) and by increasing conjugation 5-fold; hence, conjugation becomes important for biofilm formation if the cell is less motile. When Hha/YbaJ are absent, the bacterium is more motile, conjugates less, aggregates less, and forms less biofilm.

Furthermore, OmpA maintains the integrity of the *E. coli* outer membrane (Sonntag et al., 1978) and functions as a mediator in F-dependent conjugation (Schweizer and Henning, 1977). Also, it has been shown that at high osmolarity, Hha regulates the expression of OmpA, one of the most abundant proteins of the *E. coli* envelope (Balsalobre et al., 1999), so the link between Hha and OmpA is established. This is also supported by the fact that homologues of Hha are found on conjugative plasmids (Nieto et al., 2002). To support our hypothesis, we have studied here the role of

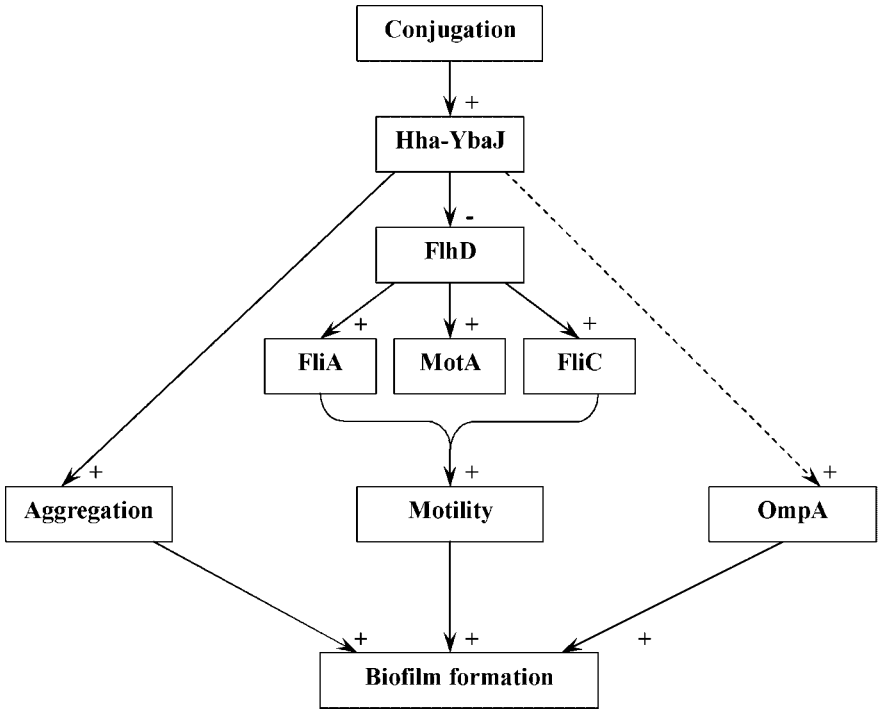

Figure 6. Model of biofilm formation indicating regulation by Hha-YbaJ, conjugation, motility, and cell aggregation. Plus indicates positive regulation and minus is negative regulation. The dotted line suggests a hypothesized connection, which needs to be probed by further experimentation.

OmpA in biofilm formation and found that an isogenic *ompA* mutant produces 59 to 89% less biofilm in LB, LB glu, and M9C glu (96 well assay) and does not form a robust biofilm in flow cells (Table III); hence, OmpA is required for biofilm formation just like Hha/YbaJ (this has not been shown previously), and the difference with the results of Ghigo (2001) may be caused by the different media used (M9C glu vs. M63B1) or the different hydrodynamic conditions (40 ml/h vs. 10 mL/h used here).

In addition, we measured the conjugation rate of the strain with the *hha*/*ybaJ* deletion and found it was reduced; hence, again it is shown Hha and OmpA are related and that there is less conjugation in the absence of Hha, however more studies should be pursued in order to find a clear link between OmpA and Hha under the biofilm conditions we evaluated, as factors such as osmolarity and temperature may have to be considered.

In summary, our results indicate that the reason biofilm formation is induced when a conjugative plasmid is added is that the cells clump which prevents normal motility; motility is not important (as verified by the good biofilms formed by the paralyzed *motA* strain in the presence of the conjugation plasmid in all media, Fig. 1) and is actually decreased due to cell aggregation (no motility in the wild-type strains with the conjugation plasmid, Fig. 4). However, if Hha and YbaJ are absent and the conjugation plasmid is present, the cells have 5-fold less conjugation and become more motile (Fig. 4A), and this decreased conjugation and increased motility (which is shown to be due to overexpression of the flagellar genes, Fig. 5) leads to less biofilm (in the presence of the conjugation plasmid). So loss of motility is not important for biofilm formation but greater motility and decreased conjugation are detrimental. Our work also suggests that specific cell-cell conjugation interactions through OmpA also play a significant role in biofilm formation.

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