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YcfR (BhsA) Influences *Escherichia coli* Biofilm Formation Through Stress Response and Surface Hydrophobicity

Xue-Song Zhang[1#], Rodolfo García Contreras[1#], and Thomas K. Wood[1,2, 3*]

Artie McFerrin Department of Chemical Engineering[1], the Department of Biology[2], and the Zachry Department of Civil Engineering[3]

*Corresponding author: 220 Jack E. Brown Building, Texas A & M University, College Station, TX 77843-3122, Phone: (979) 862-1588, Fax: (979) 865-6446, Email: Thomas.Wood@chemail.tamu.edu

[#]Both authors contributed equally.

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ABSTRACT

DNA microarrays revealed that expression of *ycfR,* which encodes a putative outer membrane protein, is significantly induced in *E. coli* biofilms and is also induced by several stress conditions. We show that deletion of *ycfR* increased biofilm formation 5-fold in the presence of glucose; the glucose effect was corroborated by showing binding of the cAMP receptor protein to the *ycfR* promoter. It appears that YcfR is a multiple stress resistance protein since deleting *ycfR* also renders the cell more sensitive to acid, heat treatment, hydrogen peroxide, and cadmium. Stress increasing biofilm formation through YcfR appears to be the result of decreasing indole synthesis since a mutation in the *tnaA* gene encoding tryptophanase prevents enhanced biofilm formation upon stress, and adding indole prevents enhanced biofilm upon stress. Deleting *ycfR* also affected outer membrane proteins and converted the cell from hydrophilic to hydrophobic, as well as increased cell aggregation 4-fold. YcfR seems to be involved in the regulation of *E. coli* K-12 biofilm formation by repressing cell aggregation and cell-surface adhesion, by influencing the concentration of signal molecules, and by interfering with stress responses. Based on our findings, we propose this locus to be named *bhsA* for influencing biofilm through hydrophobicity and stress response.

INTRODUCTION

DNA microarrays show hundreds of genes are differently expressed in *Escherichia coli* biofilms (4, 60, 69) and that their expression is temporal (13). These genes are involved in many aspects of cellular physiology, from metabolism to signal transport. Many of these differentially-expressed genes have unknown functions; hence, another round of DNA microarrays to compare differential gene expression in the biofilm for isogenic mutants relative to the wild-type strain has helped to determine some of the molecular roles of the uncharacterized proteins in biofilm formation. For example, differential gene expression was used to determine that MqsR regulates *E. coli* biofilm formation through the quorum-sensing-signal autoinducer-2 (AI-2) (82), TqsA is involved in transporting AI-2 (28), and BssR/BssS regulate biofilms by influencing the biofilm signals AI-2 and indole (14).

Indole is an interspecies extracellular biofilm signal (42) that represses the biofilm formation of *E. coli* (14) through its interaction with SdiA (42). It is generated from tryptophanase (encoded by *tnaA*), which converts tryptophan to indole, ammonia, and pyruvate (32). *tnaA* is induced in the stationary phase (61) and by high pH (7), and has been shown to regulate *gabT, astD,* and *tnaB* (78). Transcription of *tnaA* is under the control of cyclic AMP (cAMP) and the cAMP receptor protein (CRP) (78); hence, catabolite repression as an important regulatory mechanism involved in indole synthesis and biofilm formation.

In addition to the AI-2 and indole signals, bacterial surface components, such as flagella, fimbriae, proteins, and surface hydrophobicity also influence biofilm formation in *E. coli*. Flagella-mediated motility is important for *E. coli* cell-surface contact to initiate biofilm formation and bacteria spreading along surfaces (54). Type 1 fimbriae are required for *E. coli* abiotic surface attachment to initiate biofilm formation (54), and aggregative fimbriae (curli) are required for *E. coli* to form three-dimensional biofilms (39). The prominent surface protein Antigen 43 (Ag43) of *E. coli* is a self-recognizing adhesin which promotes cell aggregation (40) and increases biofilm formation (10). Conjugative plasmid-encoded fimbriae enhance biofilm formation (25) and mask the importance of flagella, type I fimbriae, Ag43, and curli (58). In addition, hydrophobic interactions between cells and the abiotic surface mediate bacterial attachment (16) and thereby may initiate biofilm development. Therefore, changes in cell

surface components caused by mutation or environmental factors influence biofilm development.

YcfR belongs to the YhcN family, which contains nine paralogous low-molecular-weight proteins (YcfR, YahO, YbiJ, YbiM, YdgH, YhcN, YjfN, YjfO, and YjfY) of unknown function in *E. coli* and other bacteria (66). Since most members share a common motif in their N-termini and C-termini and a predicted signal peptide, the YhcN family may have evolved from a common ancestor that was thought to play roles in self-identification or colony organization by cell-cell contacts or intercellular signaling (66). Previously we found that *ycfR* is induced 12-fold in *E. coli* biofilm cells compared to planktonic cells (60). YcfR (85 aa) is also involved in the general cellular stress response since *ycfR* is induced in the presence of heavy metals (25-fold at 25 μM Cd(II)) (18), during drastic pH changes (2.6-fold for pH 8.7 to 5) (45), heat shock (12-fold after 7 minutes at 50°C) (63), sodium salicylate treatment (9-fold at 5 mM of NaSal) (53), and 1 mM hydrogen peroxide (26-fold) (84). This gene has also been linked to the global regulator CRP and cellular catabolite repression (8); however, aside from its identification in these various microarray studies, its relationship to these phenotypes has not been investigated previously. Here, we sought to investigate the relationship between stress response and biofilm formation by comparing differential gene expression within a biofilm upon deleting *ycfR*.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and growth rate assay. The strains and plasmids used in the present study are listed in Table 1. Wild-type *E. coli* K12 BW25113 and the isogenic mutants were obtained from the Genome Analysis Project in Japan for *E. coli* K12 (46). Plasmid pCA24N *ycfR,* carrying *ycfR* under the tight regulation via the $lacI^q$ repressor, was obtained from the Genomic Analysis Project in Japan (46). Expression of *ycfR* was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, Mo.).

Luria-Bertani medium (LB) was used to pre-culture all the *E. coli* cells (67). LB and LB medium supplemented with 0.2 wt/vol% glucose (LB glu) were used for the crystal-violet biofilm experiment, aggregation, indole, and specific growth rate experiments. LB glu medium was also used for the glass

wool biofilm DNA microarray and glucose consumption experiments. Kanamycin (50 μg/mL) was used for pre-culturing the isogenic knock-outs. Chloramphenicol (30 μg/mL) was used for selecting plasmid pCA24N *ycfR*. Cells were pre-cultured at 37ºC with shaking (250 rpm) for the indole assay, growth rate assay, cell aggregation assay, cell surface hydrophobicity, glass wool biofilm DNA microarray, and glucose consumption experiments. The specific growth rates of *E. coli* wild type and the *ycfR* mutant were determined by measuring the cell turbidity at 600 nm of two independent cultures of each strain as a function of time using values less than 0.7.

Crystal-violet biofilm assay. A static biofilm formation assay was performed in 96-well polystyrene plates as reported previously (54). Briefly, cells were inoculated with an initial turbidity at 600 nm of 0.05 at 37°C in LB or LB glu for 24 h without shaking, then cell growth, biofilm at liquid-plastic interface, and total biofilm were measured using crystal violet staining. Each data point was averaged from at least twelve replicate wells (six wells from each of two independent cultures). For evaluating the effects of Cd(II), H_2O_2, and acid on biofilm formation, after inoculation, cells were incubated for 6 h at 37°C, then either Cd(II) (25 μg/mL in the form of $CdCl_2$), H_2O_2 (4 and 20 mM), HCl (10 mM), or Cd(II) (25 μg/mL) plus indole (500 μM, in order to study the effect of indole addition on stress induced biofilm formation) were added, and the plates were incubated for another 18 h at 37°C. For evaluating the effect of low temperature, cells were incubated at 22°C for 24 h. Each data point was averaged from twelve replicate wells (six wells from each of two independent cultures).

Indole, aggregation, and cell-surface hydrophobicity assays. Extracellular and intracellular indole concentrations of the *E. coli* wild type and the *ycfR* mutant cultured in LB and LB glu medium at 37°C with shaking (250 rpm) were measured spectrophotometrically as described previously (14). Extracellular indole concentration of each stationary-phase planktonic culture was measured at 7 h, 15 h, and 24 h. Intracellular indole concentrations in biofilm cells were measured at 7 h and 15 h. Each experiment was performed twice with two independent cultures for each strain.

Cell aggregation was measured as described previously (26). Briefly, each bacterial culture was

incubated for 20 h at 37°C with shaking (250 rpm) to stationary phase and then washed and diluted into 3 mL LB or LB glu medium with the turbidity at 600 nm of 2.5 in 14 mL sterile tubes; after tubes were incubated quiescently at 37°C for 15 h, the absorbance 5 mm beneath the surface was used to gauge aggregation. To detect coaggregation between the wild type strain and the *ycfR* mutant, 1.5 mL of each of the diluted culture was mixed together in one tube followed by brief vortexing. Each experiment was performed twice with two independent cultures for each strain.

The cell surface hydrophobicity was measured as published previously by extracting the stationary-phase cells with organics (65), except a mixture of linear hexanes isomers (H302-4, Fisher Scientific Co., Pittsburgh, PA) were used to generate the hydrophobic fractions. The mixtures were vortexed thoroughly for 1 min. After standing 15 min at room temperature for phase separation, the aqueous phase was removed and was measured to determine the cell density in aqueous phase. Each experiment was performed twice with two independent cultures for each strain.

Glucose and curli assay. Glucose concentrations in LB glu planktonic-cell cultures was analyzed enzymatically with a glucose assay kit (GAHK-20, Sigma). Each culture was sampled after 3 h, 7 h, and 15 h of incubation at 37°C with shaking (250 rpm), and two independent experiments were performed (the 7 h and 15 h samples were from the stationary phase). LB agar medium containing 20 μg/mL Congo red (Sigma), 10 μg/mL Coomassie brilliant blue (Sigma), and 15 g/L agar was used as described previously (59) to visualize *E. coli* curli expression by inspecting the red color intensity which is proportional to curli concentration after 16-h incubation at both 37°C and 30°C.

Acid resistance assay. This assay was adapted (44). Overnight cultures grown for 18 h at 37°C in LB were re-grown either to mid-log phase in LB (turbidity at 600 nm of 1) or for 24 h at 37°C in LB glucose (to validate our microarray acid-resistance gene induction data), and then the cultures were diluted 40-fold into phosphate-buffered saline (PBS, pH 7.2) or 37°C LB (pH 2.5). *E. coli* in LB (pH 2.5) was incubated for 1 h at 37°C without shaking. The percentage of cells surviving the acid treatment was calculated as the number of CFU/mL remaining after acid treatment divided by the initial CFU/mL at

time zero. At least two independent experiments were conducted.

Hydrogen peroxide resistance assay. Overnight cultures grown for 18 h at 37°C in LB were re-grown to mid-log phase in LB (turbidity at 600 nm of 1), and 1 mL of each culture was incubated with H_2O_2 at a final concentration of 20 mM at 37°C for 15 min without shaking. The percentage of cells surviving the H_2O_2 treatment was calculated as the number of CFU/mL remaining after H_2O_2 treatment divided by the initial CFU/mL at time zero. At least two independent experiments were conducted.

Cadmium resistance assay. Overnight cultures grown for 18 h at 37°C in LB were re-grown to mid-log phase in LB (turbidity at 600 nm of 1), and 1 mL of each culture was incubated with a final concentration of 200 µg/mL $CdCl_2$ at 37°C for 20 min without shaking. The percentage of cells surviving the Cd(II) treatment was calculated as the number of CFU/mL remaining after Cd(II) treatment divided by the initial CFU/mL at time zero. At least two independent experiments were conducted.

Heat shock resistance assay. Overnight cultures grown for 18 h at 37°C in LB were re-grown to mid-log phase in LB (turbidity at 600 nm of 1); 1 mL of each culture was transferred to a water bath and incubated at 65°C for 20 min without shaking. The percentage of cells surviving the heat treatment was calculated as the number of CFU/mL remaining after the heat treatment divided by the initial CFU/mL at time zero.

Biofilm total RNA isolation for DNA microarrays. Wild-type and the *ycfR* mutant strains were pre-cultured overnight in LB and LB Kan (50 µg/mL), respectively. From each of these cultures, 2.5 mL was used to inoculate 250 mL of fresh LB glu medium with 10 g of submerged glass wool (Corning Glass Works, Corning, NY) for forming biofilm. After incubating for 15 h at 37°C with shaking (250 rpm), the glass wool was carefully and quickly removed and rinsed with 100 mL of sterile 0.85% NaCl solution at 0°C. Biofilm cells were removed by sonicating the glass wool in 200 mL of sterile 0.85% NaCl solution at 0°C, then the total RNA was isolated as described previously (60).

DNA microarrays. The *E. coli* Genechip antisense genome array (Affymetrix, P/N 900381) was used to analyze the complete *E. coli* transcriptome as described previously (82). Based on the manufacturer's guidelines, each array contains probes for more than 4200 open reading frames (ORFs). Each ORF is

covered by 15 probe pairs consisting of a perfect match and a mismatch pair. Expression of each gene is evaluated by comparing intensity of the perfect match probe and the mismatch probe in each of the 15 probe pairs, leading to reliable gene expression profiles (http://www.affymetrix.com/products/arrays/specific/ecoli_antisense.affx). The procedures of DNA microarrays were followed by the description in the *Gene Expression Technical Manual* (Affymetrix). Individual strain reports for both the wild-type strain and the *ycfR* mutant cDNA samples as well as comparison reports of the *ycfR* mutant to wild-type were obtained by using the GeneChip operating software (Affymetrix). The data quality was assessed based on the manufacturer's guidelines (*GeneChip Expression Analysis: Data Analysis Fundamentals*; Affymetrix) and also based on the expected signals of the *E. coli* K-12 BW25113 and the *ycfR* mutant genotypes (e.g., both the wild type and the *ycfR* mutant have a low signal for deleted genes *araA* and *rhaA*, while the *ycfR* mutant had no signal for *ycfR*). Total signal intensity was scaled to an average value of 500. Genes were identified as differentially expressed if both the P value and the corrected P value based on Benjamini and Hochberg False Discovery Rate Method (5) were less than 0.05 (the corrected P value was adapted in this analysis to reduce incorrectly-identified differentially-expressed genes) and if the expression ratio was greater than 3-fold since the standard deviation for the expression ratio for all of genes in the data was 2.3. The gene functions were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) (17), and the SRI International, the Institute for Genomic Research, University of California at San Diego, and UNAM database (http://ecocyc.org/) (38). The expression data have been deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession Number (GSE5904) (17).

Electrophoretic mobility shift assay (EMSA). CRP protein was synthesized using the EasyXpress Linear Template Kit Plus (Qiagen, Valencia, CA). The *ycfR* promoter region (262 bp, consisting of 259 bp of upstream and 3 bp 5' of *ycfR*) was amplified by PCR from genomic DNA of the wild-type strain BW25113 with the primers 5'-GTG TTG AGT CAG TTG CCA-3' and 5'-CAT AAT AGT GGC CTT ATG-3'; the PCR product was gel-purified with QIAquick Gel Extraction Kit (Qiagen) and then labeled

with biotin using the Biotin 3' End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL). After binding reaction, samples were loaded on a 6% DNA retardation gel (Invitrogen, Carlsbad, CA), and electrophoresis was carried out with 100 volts for 2.5 h at 4°C. Samples were transferred to nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). 3'-Biotin-labeled DNA was detected with the LightShift Chemiluminescent EMSA kit (Pierce). In vitro-synthesized CRP protein (1 µL) was incubated with biotin-labeled *ycfR* promoter (8 ng) and nonspecific competitor DNA (poly dI·dC, 1 µg) in a 20 µL binding reaction system supplied by the EMSA kit. A final concentration of 1 mM cAMP (Sigma) was applied to each reaction for CRP-DNA probe binding. For the competition assay, unlabeled *ycfR* promoter from 40 ng to 1200 ng was used to confirm the specificity of protein-DNA binding. As an additional negative control, biotin-labeled *gadA* promoter (8 ng, 294 bp, consisting of 285 bp of upstream and 9 bp 5' of *gadA*) lacking a CRP binding site was also amplified by PCR (primers 5'-GAT GTG GAT GAT ATC GTA-3' and 5'-CTG GTC CAT TTC GAA CTC-3') and incubated with i*n vitro*-synthesized CRP (1 µL) in the presence of 1 mM cAMP.

RESULTS

Deletion of *ycfR* increases biofilm formation in LB glucose medium. To investigate how YcfR controls biofilm formation, biofilm formation of the *ycfR* deletion mutant was measured using the 96-well crystal-violet assay at 37°C. Upon deleting *ycfR,* total biofilm (at both the air-liquid and liquid-solid interfaces) in LB glu medium after 24 h was 5 ± 1-fold greater than that of the wild type strain. A consistent 3- to 4-fold larger cell pellet was observed for the *ycfR* mutant after 15 h of incubation on glass wool during the LB glu microarray assay. In addition, the biofilm at the liquid-solid interface in LB glu medium after 24 h was 3.0 ± 0.6-fold greater than that of the wild type strain (data not shown); hence, the *ycfR* mutation elicited significant biofilm at the liquid-plastic interface. The specific growth rates of the *ycfR* mutant in LB and LB glu media were not significantly different from the wild-type strain (1.42 ± 0.05 vs. 1.4 ± 0.1/h in LB and 1.3 ± 0.2 vs. 1.44 ± 0.03/h in LB glu). These results indicate the presence

of YcfR leads to a decrease in biofilm formation for *E. coli* K12 in LB glu medium after 24 h at 37°C (especially at the liquid-solid interface), and the effect is not due to a difference in growth. The biofilm formation of the *ycfR* mutant could be complemented (diminished) to that of the wild-type strain by expressing YcfR from pCA24N *ycfR* under the induction of 4 mM IPTG; hence, YcfR reduces *E. coli* K12 biofilm formation in LB glu medium.

Unlike LB glu, total biofilm formation of the *ycfR* mutant in LB medium was only 25 to 50% greater than that of the wild-type strain. This suggests that YcfR leads to a decrease in biofilm formation by affecting glucose uptake and metabolism. Measurement of glucose consumption of the *ycfR* mutant and the wild type in LB glucose medium indicated that deleting *ycfR* significantly reduced glucose consumption; after incubating 3 h, the wild type strain consumed 55 ± 5% glucose in the medium, while the *ycfR* mutant consumed only 31 ± 7% glucose (cf., 100 ± 0% glucose consumption of the wild-type vs. 88 ± 2% after 7-h incubation). Glucose consumption of the *ycfR* mutant could be increased to that of the wild-type strain by expressing YcfR from pCA24N *ycfR* (data not shown).

Time, temperature, and medium dependence of YcfR-mediated biofilm formation. Beloin et al. (4) reported that deleting *ycfR* in *E. coli* K-12 TG1 (which carries a conjugation plasmid) resulted in reduced biofilm formation at 30°C in flow cells with minimal glucose medium whereas we found deleting *ycfR* in *E. coli* BW25113 at 37°C in LB glu resulted in large increases in biofilm formation. An obvious difference is the absence of the conjugation plasmid in our study (conjugation plasmids dramatically affect biofilm formation (25)), but we investigated this discrepancy further by examining the effect of time, temperature, and medium on biofilm formation with the *ycfR* mutation. In the absence of glucose at lower temperatures (30°C in LB medium), deleting *ycfR* results in a 16 ± 4-fold decrease in biofilm at 15 h, a 4 ± 2-fold decrease at 24 h, and a 1.4 ± 0.2-fold increase at 43 h; hence, the time of biofilm quantification is important with this mutant. Furthermore, in LB glu after 24 h, there was 2.7 ± 0.1-fold greater biofilm at 30°C but there was 1.7 ± 0.2-fold less biofilm at 22°C; hence, the temperature at which biofilm is measured is also important for this mutation.

It appears the effect of temperature on curli production is part of the reason for these effects since deleting *ycfR* does not affect curli production of BW25113 at 37°C in LB medium (both the *ycfR* mutant and the wild type BW25113 produce curli at low levels as shown in Fig. 1A), but at 30°C, deleting *ycfR* dramatically decreased curli production compared to the wild-type strain (Fig. 1B). So the 16-fold diminished biofilm production of the *ycfR* mutant at 30°C after 15 h may be linked with the dramatic decrease in curli formation at this temperature. Clearly the effect of *ycfR* deletion on biofilm formation is complex and depends on temperature, time, and medium composition.

Deletion of *ycfR* induces acid-response genes in biofilms. To investigate the mechanism of biofilm increase caused by the *ycfR* deletion in glucose-containing medium (LB glu), DNA microarrays were performed to explore differential gene expression in a biofilm as a result of deletion of *ycfR*. At 15 h, 1.8% of the *E. coli* genes were differentially expressed in the glass wool biofilm using a 3-fold cut-off based on the 2.3-fold standard deviation, which includes 48 induced genes (Table 2) and 28 repressed genes (Table 3).

The microarray analysis indicated that deleting *ycfR* induced a group of acid-resistance genes in the biofilm; for example, *gadABC*, which encode two glutamate decarboxylase polypeptides (GadA and GadB) (73) and a putative gamma-aminobutyrate antiporter (GadC) (12), were induced 5- to 6-fold. *gadE*, which encodes a transcriptional activator of GadABC (30), was also induced 3-fold. *hdeABD*, which encode two periplasmic acid-resistance proteins (HdeA and HdeB) (74) and an acid-resistance related protein (HdeD) (44), were induced 4- to 5-fold. *slp*, which encodes an outer membrane starvation-inducible lipoprotein (1), was induced 4-fold. Slp, HdeABD, GadE, and GadABC are involved in the *E. coli* YdeO-induced acid resistance regulatory network (44).

To investigate why the *ycfR* mutation induced acid-resistance genes in the LB glu biofilm, we measured the culture pH. After 7 h, the pH of the cultures of both *ycfR* mutant and wild type dropped to 5. After 15 and 24 h, the pH of the culture of the *ycfR* mutant was significantly lower than the wild-type strain (4.9 vs. 7.6 and 5.0 vs. 8.6, respectively); hence, the acid-resistance genes were identified in the

microarrays as induced by deleting *ycfR* since the *ycfR* cells were experiencing a much lower pH. To further verify our microarray results, the survival of 24-h, LB glu-grown planktonic *ycfR* cells after 1-h incubation at pH 2.5 was investigated; *ycfR* cells were 6 ± 2-fold more resistant to the acid incubation than the wild type strain (due to previous induction of the acid-resistance genes), which validates our microarray results.

Deletion of *ycfR* induces stress-response genes in biofilms and increases sensitivity to stress. Along with the acid-resistance genes, other stress-response genes were induced 3- to 4-fold in the biofilm by the *ycfR* deletion including three osmotic-stress-inducible genes, *osmY,* which encodes a hyperosmotically-inducible periplasmic protein (83), *osmB,* which encodes an osmotically-inducible outer membrane lipoprotein (35), and *ompX,* which encodes an outer membrane protein regulated by osmolarity and pressure (47). Other stress genes that were induced include *sodC,* which encodes a periplasmic superoxide dismutase and protects bacteria from oxidation (27), *uspB*, which encodes an inner membrane ethanol tolerance protein (21), *bssS,* which encodes a global regulator involved in stress response as well as regulation of biofilm formation (14), and *dnaK,* which encodes a heat-shock protein maintaining DNA structure against thermal stress (50). Therefore, acid, osmotic, oxidative, and heat stress-response genes were induced upon deletion of *ycfR* in the biofilm, and taken together with the fact that the transcription of *ycfR* in suspension cells is induced during various stress conditions (e.g., H_2O_2 treatment (84), Cd(II) (18), pH (45), heat shock (63), sodium salicylate treatment (53)), it is likely that YcfR may be involved in a global stress resistance response and that the *ycfR* mutant biofilm is contending with higher stress levels than the wild type biofilm. Therefore, we tested the sensitivity of the *ycfR* mutant to the following stresses in LB: low pH, heat, H_2O_2, and high Cd(II) concentration. For all the tested stress conditions (1 hour at pH 2.5, 20 min at 65°C, 15 min with 20 mM H_2O_2, and 20 min with 200μg/mL Cd(II)), the *ycfR* mutant had diminished survival compared to the wild type strain; deleting *ycfR* caused a 10 ± 2-fold increase in sensitivity to acidic pH, 14 ± 3-fold more sensitivity to the heat, 66 ± 7-fold more sensitivity to the H_2O_2, and 16 ± 4-fold more sensitivity to Cd(II) compared to the wild type strain (Fig. 2). Therefore, YcfR is a multiple stress resistance protein.

Deletion of *ycfR* decreases indole synthesis. In *E. coli*, indole is synthesized from tryptophan by tryptophanase (encoded by *tnaA*) and is exported by multidrug exporters such as ArcEF (37). Our microarray analysis indicated that deleting *ycfR* repressed *tnaA* 5-fold at 15 h in LB glu biofilm; this was validated by the 2.3 ± 0.2-fold reduction in extracellular indole concentration for the stationary-phase planktonic *ycfR* culture at 15 h and 4.9 ± 1.0-fold reduction at 24 h in LB glu medium. The extracellular indole concentrations were corroborated by examining the intracellular indole concentrations of LB glu biofilm cells (on glass wool); as expected, the intracellular indole concentration for the *ycfR* biofilm cells was 4.3 ± 1-fold lower at 7 h lower 4.8 ± 0.6-fold at 15 h. Since indole acts as an extracellular signal that represses biofilm formation in *E. coli* K-12 (14), our finding that deleting *ycfR* decreased indole is consistent with the observation that the *ycfR* mutation increased biofilm formation in LB glu medium. In contrast, in LB medium, the extracellular indole concentrations of the *ycfR* mutant after 15 h and 24 h incubation were 75 ± 32% and 85 ± 32% of that of the wild-type strain, respectively, which is consistent with the observation that deletion of *ycfR* did not significantly affect biofilm formation in LB medium.

Stress increases biofilm formation by decreasing indole. Since we found that YcfR protects suspension cells from different kinds of stresses (low pH, heat, H_2O_2, and Cd(II)) and that the *ycfR* deletion induced stress genes (the acid resistance genes *gadABC*, *gadE*, and *hdeABD,* the DNA-binding heat-shock gene *hspQ*, the starvation lipoprotein *slp*, the osmotic-stress-induced genes *osmBY*, the ethanol-resistance protein *uspB*, the periplasmic superoxide dismutase precursor *sodC*, and the chaperone protein *dnaK* involved in the protection of cells to heat shock and oxidative stress), we hypothesized that the biofilm formed by the *ycfR* mutant would be defective in coping with stress and that this elevated stress level may stimulate biofilm formation in LB glu medium. This hypothesis is corroborated by the fact that the deletion of genes that are involved in stress tolerance increase biofilm 30% to 5-fold (e.g., the stress regulator *oxyR* in *E. coli* (10), *ropA* in *Streptococcus mutans* (80), *spx* in *Staphylococcus aureus* (52) and *soxS* in *E coli* in this work). Further evidence of this relationship is that stress-associated genes are induced in biofilms (4, 13, 60, 69), and that biofilm has been shown to increase in response to stress conditions (e.g., sub-lethal concentrations of aminoglycoside antibiotics in *Pseudomonas aeruginosa* and

E. coli (29), osmotic stress in *S. aureus* (56) and high metal concentration, extreme pH and temperature, the addition of xenobiotics, antibiotics, and oxygen in the archaebacteria *Archaeoglobus fulgidus* (41)).

In order to test this hypothesis, we made a series of crystal-violet biofilm assays under stress conditions, initiated after 6 hours of normal growing at 37°C in LB medium, by adding 25 µg/mL Cd(II), 20 mM H_2O_2, or 10 mM HCl; also low temperature stress was evaluated by incubating cells continuously at 22°C (Fig. 3). Except for 22°C, these conditions only affected slightly the total growth of both the wild-type strain and the *ycfR* mutant; therefore, the results were not due to differences in growth. These stresses stimulated biofilm formation of the wild-type strain by 0.50 ± 0.02-fold for Cd(II) addition, 3.0 ± 0.2-fold for the H_2O_2 treatment, 3.0 ± 0.3-fold for the HCl treatment, and 2.0 ± 0.8-fold for low temperature incubation. Under the same conditions, these stresses did not stimulate biofilm formation of the *ycfR* mutant except for the addition of Cd(II) that increased its biofilm slightly (0.3-fold, Fig. 3). Hence, biofilm formation in *E. coli* is induced by oxidative, acid, low temperature, and heavy metal stress and YcfR is required for this response.

Based in the observation that the induction of sigma E factor (*rpoE*), which controls the expression of an important stress resistance regulon, drastically represses the transcription of the *tnaA* gene (7-fold in stationary phase cells and 33-fold in exponential cells) (36), we hypothesized that the induction of *rpoE* by the accumulation of unfolded proteins produced by stress (36) probably reduces the indole production via *tnaA* repression and that the decreased indole concentration enhances biofilm formation. To further explore this proposed mechanism, we tested the Cd(II) biofilm induction phenotype of the *tnaA* and *trpE* deletion mutants (each has 10-fold less indole than the wild-type strain (42)) and found that both mutants repressed their biofilm formation upon the Cd(II)addition (*tnaA* 0.8 ± 0.07-fold and *trpE* 0.5 ± 0.14-fold) rather than increasing it as the wild-type strain and other non-biofilm related deletion mutants (e.g., *melR*). Additionally deleting *tnaA* prevented the increase in biofilm formation upon H_2O_2 and HCl addition, but surprisingly not upon low temperature (22°C) (data not shown). These results indicate that decreasing indole could be also a mechanism that enhances biofilm formation in response to Cd(II), H_2O_2 and acidification. Furthermore, adding 500 µM indole to the wild-type strain simultaneously with Cd(II),

H_2O_2, HCl, or during the 22°C incubation rendered the cells incapable of increasing biofilm formation (Fig. 3); hence, indole concentrations are important for controlling biofilm formation upon stress in *E. coli*.

Deletion of *ycfR* increases aggregation and increases cell-surface hydrophobicity. Deleting *ycfR* caused 4 ± 1-fold greater aggregation than the wild-type strain in LB medium (Fig. 4A/B) and 5.1 ± 0.1-fold greater aggregation in LB glu medium (data not shown). Furthermore, this phenotype could be partially complemented by expressing *ycfR* from a multi-copy plasmid (Fig. 4B). These results indicate that YcfR impedes cell aggregation. To further examine the mechanism how YcfR influences cell aggregation, a coaggregation test was performed by mixing the wild type strain with the *ycfR* mutant; no apparent coaggregation was observed (data not shown). Resuspending wild type bacterial cells in the supernatant of the *ycfR* mutant LB or LB glu culture did not increase aggregation of the wild type cells either (data not shown).

Deleting *ycfR* also caused the cell to become dramatically more hydrophobic (Fig. 5) which explains the increased cell aggregation. The increase in hydrophobicity also explains our observation that deleting *ycfR* increased significantly biofilm formation at the liquid-solid interface in 96-well plates since hydrophobic interactions between the hydrophobic bacterial cell surface and the hydrophobic surface of the plastic well mediates attachment to plastic (16), which appears to enhance biofilm formation. It was also noticed that although deleting *ycfR* increased cell aggregation in both LB and LB glu media in a similar way, the increase of biofilm by deleting *ycfR* in LB glu is much more than that in LB medium. This suggests that increase in cell-surface hydrophobicity is only one of several effects on biofilm formation caused by this mutation.

The *ycfR* biofilm microarray data also indicated that deleting *ycfR* differentially induced a large group of genes encoding cell-surface proteins (33% of the induced genes), including outer membrane proteins (e.g., OmpX, OsmB, Slp, YbaY), periplasmic proteins (e.g., OsmY, SodC, HdeA, HdeB, YbiM), and some inner membrane-associated proteins (e.g., GadC, HdeD, NhaA, YfeP, YgaM, YhiO, YohC). Additionally, 60% of the repressed genes encoded proteins on the cell surface including the outer

membrane proteins OmpW and NmpC, and membrane-associated transporters, such as AtpBEF and RrbsDAC. Hence, deletion of *ycfR* critically affects bacterial cell surface properties including cell-surface hydrophobicity, aggregation, and ultimately, biofilm formation.

Differentially expressed genes due to *ycfR* deletion are biofilm related. In order to further explore the importance of *ycfR* for biofilm formation, we assayed biofilm formation for six isogenic mutants based on their differential gene expression (*hspQ*, *gatB, cspBG*, and *ompWX*) in both LB and LB glu media after 24 h at 37°C. Deletion of all the genes but *hspQ* and *ompW* significantly altered biofilm formation in LB (*cspBG* and *ompX* reduced biofilm formation by 2 to 3-fold whereas *gatB* increased biofilm 2-fold) and in LB glu (*cspBG, gatB,* and *ompX* increased biofilm formation 2-fold). These results are consistent with our conclusion that *ycfR* plays an important role in *E. coli* biofilm formation and also show that the previously-identified biofilm genes *cspBG* (13) control biofilm formation.

YcfR is a putative membrane protein and *ycfR* promoter has CRP and SoxS binding sites. We investigated the possibility that YcfR is a membrane protein using multiple protein analysis programs such as ExPASy server (23), SignalP (49) and PSORTb v.2.0 (62), all of which predict YcfR (85 aa) contains a N-terminal signal peptide with cleavage between Ala22 and Ala23; therefore, YcfR may have multiple localization sites, in the membrane, in the periplasmic space, or in the extracellular space. Consistently, expression of YcfR protein from pCA24N *ycfR* and consequent analysis by SDS-PAGE indicated that the YcfR protein was from the insoluble membrane fraction not from soluble cytosolic fraction (data not shown).

We hypothesized that the remarkably enhanced biofilm formation of the *ycfR* mutant when cultured in LB glu but not in LB may be due to regulation by CRP, and a putative CRP binding site (8) was identified between positions -70 and -83 in the *ycfR* promoter. Hence, we tested CRP binding to the *ycfR* promoter using an EMSA assay. We found that CRP binds upstream region of the *ycfR* gene (Fig. 6 ~~9~~). This binding is specific since applying excess nonspecific DNA (poly dI·dC or the *gadA* promoter) does not affect CRP-*ycfR* promoter region binding while increasing unlabeled *ycfR* promoter DNA reverses the binding.

Further analysis of the *ycfR* promoter with Bprom (http://www.softberry.com/) showed the presence of a putative SoxS binding site between positions -59 and -66. Hence, *ycfR* may be also part of the stress response *soxRS* regulon which protects the cell against superoxide (2) and H_2O_2 stress (70). Moreover, *soxS* is one of the most induced genes in *E. coli* K12 biofilms (50-fold) (60), so it may be responsible for the concomitant *ycfR* induction. However, EMSA to confirm protein SoxS binding *ycfR* was unsuccessful (data not shown) which was probably caused by the extremely short half life of the SoxS protein (~2 min) (71).

DISCUSSION

In this study, by investigating the profound changes induced by deletion of *ycfR*, we demonstrate that YcfR inhibits *E. coli* K-12 biofilm formation by repressing cell aggregation, by increasing the biofilm signal indole, and by interfering with acid-stress response. We also show that YcfR increases viability during stress conditions and that stress in general increases biofilm formation.

The development of biofilm is a complex and dynamic process that couples a cascade of responses to a variety of environmental signals in bacterial cells (13, 55). Cell-surface adhesion and cell aggregation initiate bacterial biofilm formation (68). Our results demonstrate that deleting *ycfR* induces significant cell aggregation and also increases liquid/solid biofilm formation. This suggests that YcfR decreases biofilm formation by repressing cell-cell interaction and cell-surface interaction. It is intriguing that it is the absence of this small membrane protein that induces rather than represses aggregation and shows YcfR itself is not the cell surface protein which is directly involved in cell-cell adhesion. Further evidence of this is that there is no coaggregation between wild-type cells and the *ycfR* mutant, and since coaggregation is mediated by specific interactions between cell surface lectin-like adhesin proteins and receptors on the surface of other cells (64), the aggregation caused by deleting *ycfR* should not be a consequence of changes in cell surface adhesins or receptors. Furthermore, resuspending wild type cells in the supernatant of the *ycfR* mutant LB or LB glu cultures did not increase aggregation of the wild type cells, which suggests that the aggregation of the *ycfR* mutant is not caused by extracellular signals

secreted by the mutant. In *E. coli*, the adhesion protein Ag43 encoded by the *flu* gene is a self-recognizing surface adhesin which confers cells aggregation (40) and is subject to phase variation (10). The fact that *flu* is not induced in the *ycfR* mutant in the LB glu biofilm implies that the aggregation is not mediated by overexpression of Ag43. Our hydrocarbon-extraction experiment (Fig. 5) clearly demonstrates the dramatic change of *E. coli* K12 cell surface from hydrophilic to hydrophobic is caused by the *ycfR* deletion. This is consistent with the observation that *ycfR* mutant has increased bacterial aggregation and increased liquid-solid interface biofilm formation. However, this hydrophobicity change cannot be simply explained by loss of the YcfR protein since the predicted isoelectric point (pI) of YcfR is 9 yet when the culture pH was 9, the wild type strain did not show increased aggregation (data not shown). Therefore, most likely YcfR modulates bacterial cell surface hydrophobic properties by affecting the expression of other surface proteins.

Our differential gene expression analysis demonstrated that a previously identified aggregation-related gene, *osmB*, was significantly induced by deletion of *ycfR*. *osmB* encodes an osmotically-inducible outer membrane lipoprotein (34). Interestingly, *E. coli* cells aggregate upon elevated osmolarity in an OsmB-dependent manner (34). Moreover, we demonstrated deleting *ycfR* induces and represses about 30 outer membrane-associated, periplasmic, or inner membrane-associated protein-encoding genes. The differential expression of these genes may critically change bacterial cell surface properties. Taken together, these results suggest that YcfR affects bacterial cell surface structures and properties by affecting cell surface protein gene expression, which further affects cell aggregation and consequent biofilm formation. Further research on these differentially-expressed genes may reveal the molecular mechanism for the change of the cell surface hydrophobicity and their roles in biofilm development.

Another part of the mechanism by which YcfR decreases biofilm formation is through its induction of indole. Indole is an extracellular signal (42) that represses biofilm formation (14). Our analysis clearly demonstrates that deleting *ycfR* significantly reduces both extracellular and intracellular indole concentrations, and this result is consistent with our DNA microarray analysis that shows *tnaA* is repressed by 5-fold in the *ycfR* mutant. The expression of *tnaA* is under the cAMP and CRP regulation

(78) and is also inducible by high pH (75). Indole production in the wild-type strain increased 10-fold from 7 h to 15 h after glucose in the medium was depleted and increased further when the pH turned alkaline. However, since the culture of the *ycfR* mutant culture remained acidic even after the glucose was consumed, the expression of the *tnaA* gene was repressed. Therefore, deletion of *ycfR* decreases indole generation by reducing glucose uptake and metabolism and by maintaining an acidic pH, which together leads to greater biofilm formation.

That YcfR protein may be directly involved in the cellular transport of metabolites is suggested by its periplasmic or outer membrane nature, by the fact that glucose remains in the culture media of the *ycfR* mutant (which suggests a slower glucose transport rate than the wild type), by the switch from acid to alkaline pH in the wild-type strain when grown in LB glucose (the transmembrane H^+ gradient and metabolite transport are interdependent (48)), by the fact that transport of several amino acids like phenylalanine, tyrosine, and tryptophan depend on the H^+ gradient (9), and by the fact that acidification of the external media in the *ycfR* mutant may be both cause and consequence of a different electrochemical potential between the cell interior and the external medium. The induction and repression of many transporter encoding genes in our microarray and the capacity of YcfR protein to provide the cells with multiple stress resistances also suggest that YcfR is involved in the transport of metabolites since some stress resistance proteins function as efflux systems and pumps like the MtsABC transporter in *S. pyogenes* (33) and the heavy-metal transport ATPases of several species of bacteria (72). Moreover, a protein BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) of YcfR shows that this protein has high identity with predicted cation transport ATPases (important in stress response) of *Shigella boydii* and several *E. coli* strains; however, due the small size of YcfR (85 aa) and the apparent absence of transmembrane segments suggest YcfR is part of a multimeric complex.

Besides the acid-response genes, our differential gene expression analysis demonstrates that other stress-related genes, such as *ompXY*, *omsB*, *sodC*, *yhiO*, *bssS,* and *dnaK*, were induced by deletion of *ycfR*. The expression of *ompX* is induced by both acidic and basic conditions (75), and deletion of *ompX* increases cell-surface adhesion of fimbriated strains of *E. coli*, and decreases cell surface adhesion of non-

fimbriated strains (51). Our observation that deletion of *ycfR* significantly increases biofilm at the liquid-solid interface in the crystal-violet biofilm assay may be related to a YcfR-OmpX-adhesion feedback. Both the lipoprotein OmsB (35) and the predicted periplasmic OsmY (83) are thought to function in osmotic stress response, since their expression was induced by osmotic stress. Interestingly, the expression of *dnaK*, *omsBY,* and *ompX* responds to a global signal, acetyl phosphate, which functions during biofilm development (81). Whether YcfR influences biofilm development through acetyl phosphate requires further research. Recently our lab identified a negative regulator of *E. coli* K12 biofilm formation, *bssS*, which influences cell signaling (14). Our finding of induction of *bssS* in the *ycfR* mutant biofilm might indicate the bacterial cells try to repress the abnormal increase of biofilm caused by deletion of *ycfR*.

Our study demonstrates that *ycfR* gene, which is strongly induced upon stress conditions, confers resistance to several kinds of stress including low pH, heat shock, H_2O_2, and high Cd(II) concentration. Hence, *ycfR* induction may be related to a general effect of all the stresses or related to the common mechanisms that the cells use to protect themselves against stress as seen in plants (22), yeast (76), and bacteria (31); for example, it has been shown that both heavy metal stress (22) and heat shock stress (76) converge in the generation of oxygen reactive species.

ycfR is one of the most induced genes in *E. coli* biofilms since it is induced 12-fold in *E. coli* biofilms at 7 hours as compared to planktonic cells (60) and 6.4-fold in mature (8 days) biofilm (4). These data suggest that *ycfR* helps cells to contend with the stress generated in the biofilm, and we have shown that YcfR is required for survival under some stress conditions (Fig. 2). The fact that stress genes are induced in biofilms is established; for example, (i) *ycfR,* the transcriptional regulator *soxS*, and the small heat shock proteins genes *ibpAB* are among the most induced genes in biofilms (60), (ii) genes involved in cellular processes such as envelope stress responses like *pspABCDE*, *cpxP, spy, rpoE, rseA,* and other stress-associated genes like *recA* and *dinI* are induced in mature biofilms (4), and (iii) the cold shock proteins *cspABFGI* are induced in *E. coli* K12 young biofilms (4 and 7 hours) (14). Moreover it has been proposed that the Cpx regulon is a strategic signaling pathway for coping with adverse conditions

necessary for biofilm communities (15). Therefore a strong cellular response against stress is being developed in biofilms. In fact, high stress levels are normal in microbial biofilms since there is a large proportion of cells with injured membranes as in *Streptococci* oral biofilm (24), and there are reports about elevated cell death in the biofilm of *P. aeruginosa* (79) and *P. tunicate* (43).

The relationship between stress and bacterial biofilm formation has been studied to some extent in eubacteria, particularly in *S. aureus* (56) and *S. mutans* (80), where it is established that osmotic and acid/oxidative stress, respectively, are related to biofilm induction. In *E. coli*, biofilm induction in response of sub-inhibitory concentrations of aminoglycoside antibiotics has been shown (29); but, there is only indirect evidence about the general relationship between stress and biofilm formation in this organism (6). In this work, we show that *E. coli* K12 produces more biofilm as a defensive response against several stresses including acidic pH, oxidative stress (H_2O_2), heavy metals (Cd(II)), and cold shock (22°C). In addition, we show that this stress induction in the biofilm is related to a diminution of indole concentrations. In Archea, it has been demonstrated that the hyperthermophile *Archaeoglobus fulgidus* can produce enhanced biofilm in response to nonphysiological extremes (e.g., pH , temperature, metals, antibiotics, xenobiotics, oxygen), and these biofilm cells show an increased tolerance to adverse environmental conditions (41). Several other works show that biofilm cells resist better than planktonic cells stresses like H_2O_2 (19), heavy metals (77), and antibiotics (29). Our data also suggest that the *E. coli* biofilm tolerates H_2O_2 stress better than suspension cells, and the enhanced biofilm tolerance to stress is not surprising since biofilm cells form a barrier that protects inner cells from the hazardous environment via a polysaccharide matrix capable of sequestering metals and dangerous compounds (41). The gradient in concentrations found in biofilms (57) is also expected to protect inner biofilm cells, and only the most metabolically-active cells are more susceptible to antimicrobial agents (20); therefore, internal biofilm cells are less exposed to toxins. Taking into account all of the above, it is reasonable to surmise that biofilm induction in response to stress is a general strategy that prokaryotes (eubacteria and archeobacteria) evolved in order to protect themselves against unfavorable environmental conditions. Although we are just beginning to explore the mechanism by which stress induces biofilm formation, at

least for *E. coli,* our findings suggest that reducing the concentration of the biofilm inhibitor indole (14) is linked with increases in biofilm formation as a result of stress.

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Table 1. Strains and plasmids used. Km^r, and Cm^r are kanamycin and chloramphenicol resistance, respectively.

Strains and plasmids	Genotype/relevant characteristics	Source
Strain		
E. coli K-12 BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	(11)
E. coli K-12 BW25113 Δ*ycfR*	K-12 BW25113 Δ*ycfR* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*melR*	K-12 BW25113 Δ*melR* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*soxS*	K-12 BW25113 Δ*soxS* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*tnaA*	K-12 BW25113 Δ*tnaA* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*trpE*	K-12 BW25113 Δ*trpE* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*hspQ*	K-12 BW25113 Δ*hspQ* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*gatB*	K-12 BW25113 Δ*gatB* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*cspB*	K-12 BW25113 Δ*cspB* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*cspG*	K-12 BW25113 Δ*cspG* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*ompW*	K-12 BW25113 Δ*ompW* Ω Km^r	(3)
E. coli K-12 BW25113 Δ*ompX*	K-12 BW25113 Δ*ompX* Ω Km^r	(3)
Plasmid		
pCA24N *ycfR*	Cm^r; *lacI*^{q}, pCA24N $P_{T5\text{-}lac}$::*ycfR*$^+$	(3)

Table 2. *E. coli* genes induced more than threefold (P < 0.05) in LB glu biofilm after 15 h at 37°C upon deleting *ycfR*

Group and gene	b number	Description	Expression ratio
tRNA			
metW	*b2815*	tRNA	3.2
leuX	*b4270*	tRNA	3.0
metY	*b3171*	tRNA	3.0
metZ	*b2814*	tRNA	3.0
RNA related			
rnpB	*b3123*	RnpB RNA, catalytic subunit of RNAse P	3.7
rrlC	*b3758*	23S rRNA	3.5
rrlD	*b3275*	23S rRNA	3.5
rrfH	*b0205*	5S rRNA	3.0
Regulator			
ybgS	*b0753*	Putative regulator, not classified, putative homeobox protein	4.9
yiaG	*b3555*	Putative transcriptional regulator	3.2
Stress-related			
gadA	*b3517*	Glutamate decarboxylase A subunit, acid-resistance protein	6.5
gadB	*b1493*	Glutamate decarboxylase B subunit, acid-resistance protein	4.9
gadC	*b1492*	Putative transporter, acid-resistance protein	6.5
gadE	*b3512*	GadE transcriptional activator, acid-resistance protein	3.0
slp	*b3506*	Outer membrane constituents, starvation lipoprotein	4.3
nhaA	*b0019*	Na^+/H antiporter, pH dependent	5.7
hdeB	*b3509*	Acid-resistance protein	4.0
hdeA	*b3510*	Acid-resistance protein, possible chaperone, subunit of HdeA dimer	3.5
hdeD	*b3511*	Protein involved in acid resistance	5.3
osmY	*b4376*	Hyperosmotically inducible periplasmic protein	4.3
osmB	*b1283*	Osmotically inducible lipoprotein, adaptation to osmotic pressure	3.5
bssS (*yceP*)	*b1060*	Regulator of biofilm through signal secretion	3.5
uspB	*b3494*	Ethanol tolerance protein	3.2
dnaK	*b0014*	Chaperone Hsp70, autoregulated heat shock proteins	3.0
sodC	*b1646*	Superoxide dismutase precursor (Cu-Zn), detoxification	3.0

ompX	*b0814*	Outer membrane protein X, adhesion	3.7
hspQ	*b0966*	Hemimethylated DNA-binding protein	3.5
Metabolism			
yohC	*b2135*	Predicted GTP-binding transport protein, essential for *E. coli* growth	5.3
prpB	*b0331*	Putative carboxyphosphonoenolpyruvate mutase	4.6
prpD	*b0334*	2-methyl citrate dehydratase	3.0
ybaY	*b0453*	Glycoprotein/polysaccharide metabolism, predicted outer membrane lipoprotein	3.7
yfeP	*b2392*	High-affinity manganese transporter	3.5
ynhG	*b1678*	Putative ATP synthase subunit	4.0
pykF	*b1676*	Pyruvate kinase I monomer, subunit of pyruvate kinase I	3.5
yjgA	*b4234*	Putative ABC superfamily transport protein	3.5
deoA	*b4382*	Thymidine phosphorylase	3.2
deoC	*b4381*	Deoxyribose-phosphate aldolase	3.2
deoD	*b4384*	Purine-nucleoside phosphorylase	3.0
pyrG	*b2780*	Subunit of CTP synthetase	3.2
yfhN	*b2529*	Scaffold protein involved in iron-sulfur cluster assembly	3.0
yfhO	*b2530*	Cysteine desulfurase	3.0
Unknown function			
ybiM	*b0806*	Hypothetical protein	5.7
ybaA	*b0456*	Hypothetical protein	4.6
yceK	*b1050*	Hypothetical protein	4.6
yjbJ	*b4045*	Highly abundant nonessential protein	4.3
yjdN	*b4107*	Hypothetical protein	3.7
ygaM	*b2672*	Conserved hypothetical protein	3.2
ymgE	*b1195*	Predicted inner membrane protein	3.0

Table 3. *E. coli* genes repressed more than threefold (P<0.05) in LB glu biofilm after 15 h at 37°C upon deleting *ycfR*

Group and gene	b number	Description	Expression ratio
tRNA			
thrT	*b3979*	tRNA	-3.0
valX	*b2402*	tRNA	-3.2
valZ	*b0746*	tRNA	-3.0
Regulator			
cspG	*b0990*	Homolog of Salmonella cold shock protein	-3.0
lrhA	*b2289*	NADH dehydrogenase transcriptional regulator, LysR family	-3.2
marA	*b1531*	Regulator, drug/analog sensitivity	-3.0
putA	*b1014*	Bifunctional enzyme as well as a transcriptional repressor of the put (proline utilization) regulon	-4.0
Transport and metabolism			
atpB	*b3738*	Membrane-bound ATP synthase, F_0 sector, subunit A	-3.5
atpE	*b3737*	Membrane-bound ATP synthase, F_0 sector, subunit C	-3.0
atpF	*b3736*	ATP synthase, F_0 complex, subunit B	-3.0
atpH	*b3735*	Membrane-bound ATP synthase, F_1 sector, delta-subunit	-3.2
cspB	*b1557*	CspA family of cold-shock protein	-5.7
feoA	*b3408*	Ferrous iron transport protein A	-3.2
gatA	*b2094*	GatA, subunit of EIIGat, galactitol PTS permease	-3.0
gatB	*b2093*	Transport, transport of small molecules: carbohydrates, organic acids, alcohols	-4.9
gatC	*b2092*	Transport, transport of small molecules: carbohydrates, organic acids, alcohols	-3.7
gatD	*b2091*	Enzyme, degradation of small molecules: carbon compounds	-4.0
nmpC	*b0553*	Outer membrane porin	-6.5
putP	*b1015*	Sodium/proline symporter responsible for the uptake of proline	-4.3
rbsD	*b3748*	D-ribose high-affinity transport system; membrane-associated protein	-3.5
rbsA	*b3749*	ATP-binding component of D-ribose high-affinity transport system	-3.5
rbsC	*b3750*	D-ribose high-affinity transport system	-3.5
sdaC	*b2796*	Probable serine transporter	-4.0
ompW	*b1256*	Outer membrane protein W; colicin S4 receptor; putative transport protein	-6.0
Metabolism			
tnaA	*b3708*	Tryptophanase	-4.6
tnaL (tnaC)	*b3707*	Tryptophanase leader peptide	-4.9
yfiD	*b2579*	Putative formate acetyltransferase	-4.3
Unknown function			
yeeI	*b1976*	Conserved hypothetical protein	-3.2

Figure Captions

Fig. 1. Curli production of wild type *E. coli* BW25113 and the *ycfR* mutant after 16 h in LB medium at 37°C (A) and at 30°C (B) as indicated by Congo red staining.

Fig. 2. Survival percentages of the wild type strain and the *ycfR* mutant in LB medium after addition of hydrogen peroxide (A), heat (B), acid (C), and cadmium (D). The experiments were repeated at least 2 times (one representative data set shown), and one standard deviation is shown.

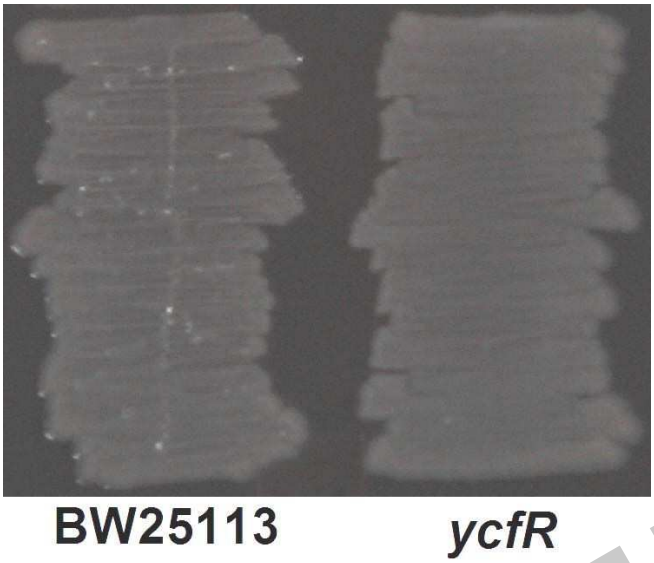
Fig. 3. Relative biofilm formation of the wild type strain and the *ycfR* mutant after addition of 25 μg/mL of cadmium, 20 mM hydrogen peroxide, and 10 mM hydrochloric acid to LB medium at 37°C, and after incubation at 22°C. The effect of the addition of 500 μM indole on wild type biofilm formation under the same conditions is also shown. All the biofilm formation values were normalized to that of the wild type at 37°C. The experiments were repeated at least 2 times (one representative data set shown), and one standard deviation is shown.

Fig. 4. Cell aggregation in LB medium at 37°C after 15 h upon deleting *ycfR* (A). Relative aggregation of the wild type, *ycfR*, and *ycfR*/pCA24N *ycfR* cultures in LB medium (B) (1 mM IPTG added to *ycfR*/pCA24N *ycfR* to induce expression of YcfR). Two to ten replicates were used, and one standard deviation is shown.

Fig. 5. Hydrophobicity of the wild type strain and the *ycfR* mutant after growth in LB medium at 37°C. The experiments were repeated twice, and one standard deviation is shown.

Fig. 6. Electrophoretic mobility shift assay (EMSA) to test the binding of cAMP-CRP to the *ycfR* promoter. Lane 1: labeled *ycfR* promoter (p_{ycfR}), Lane 2: cAMP-CRP protein and labeled p_{ycfR}, and Lanes 3 to 7: cAMP-CRP protein, labeled p_{ycfR}, and nonspecific competitor unlabeled p_{ycfR} promoter.

A



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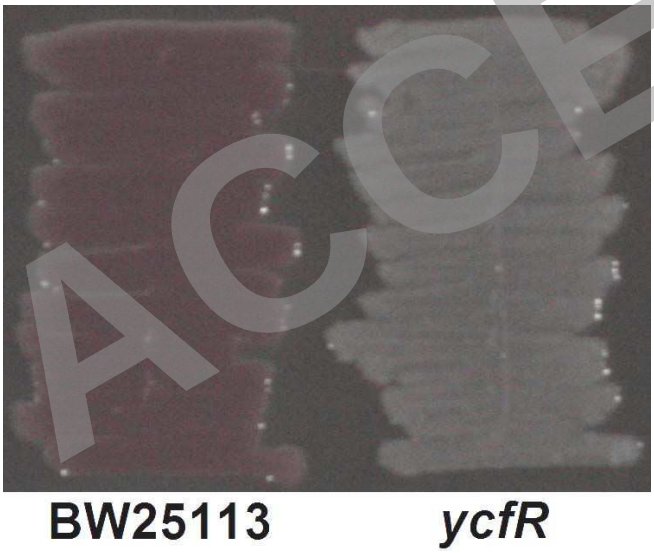


Figure 1-

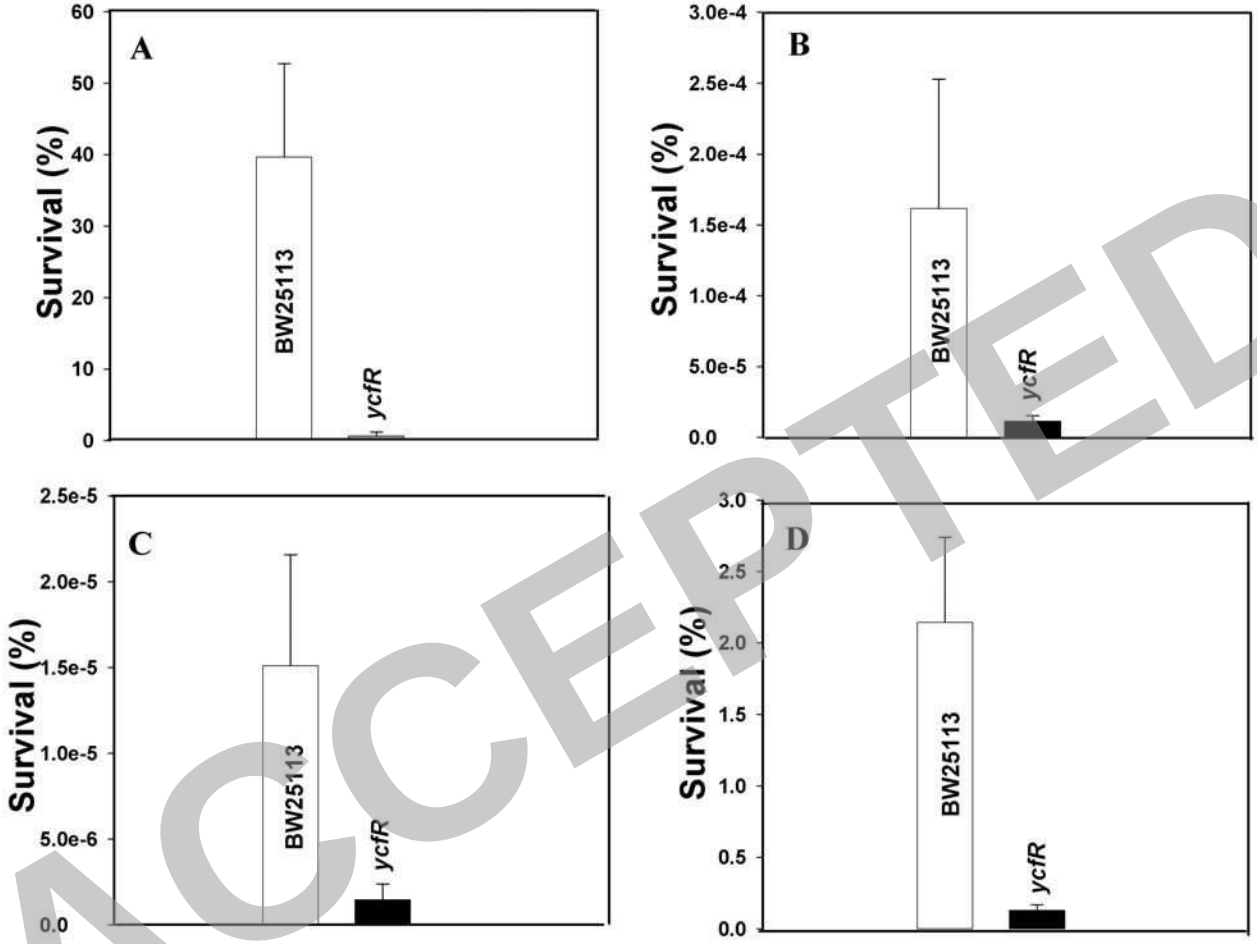


Figure 2

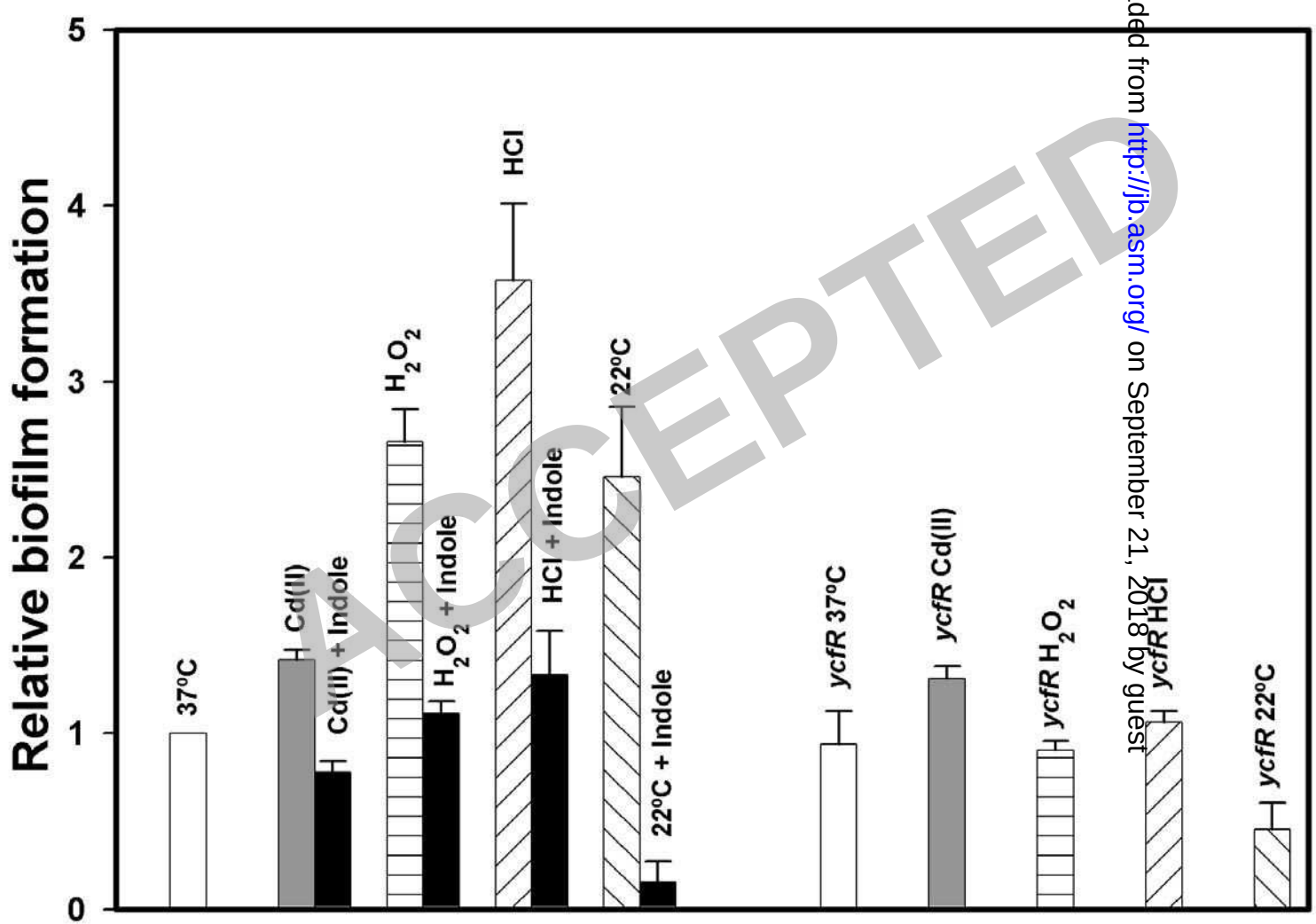
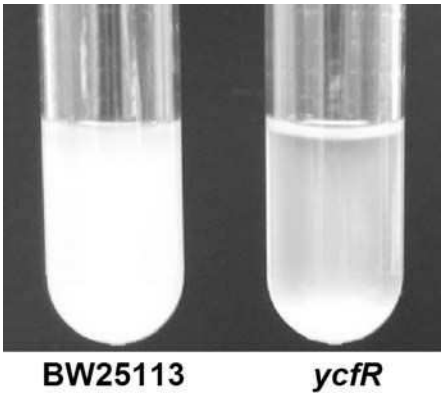


Figure 3

A



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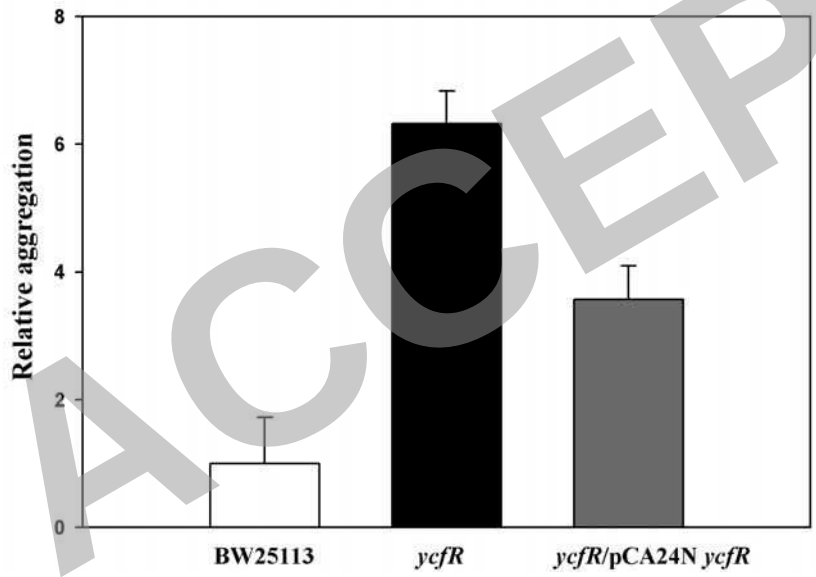

Figure 4

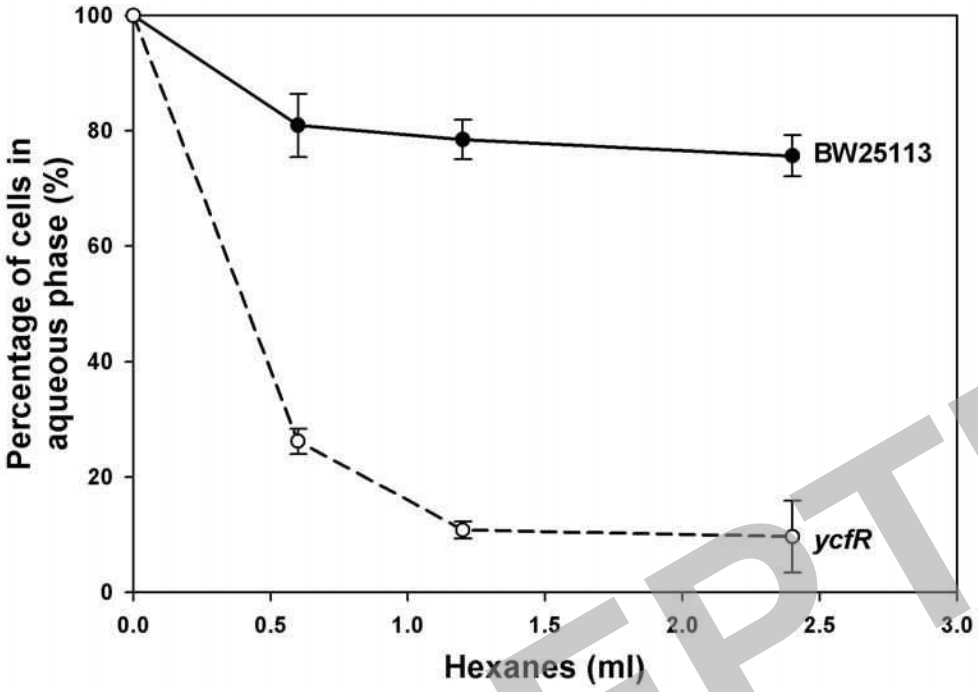

Figure 5

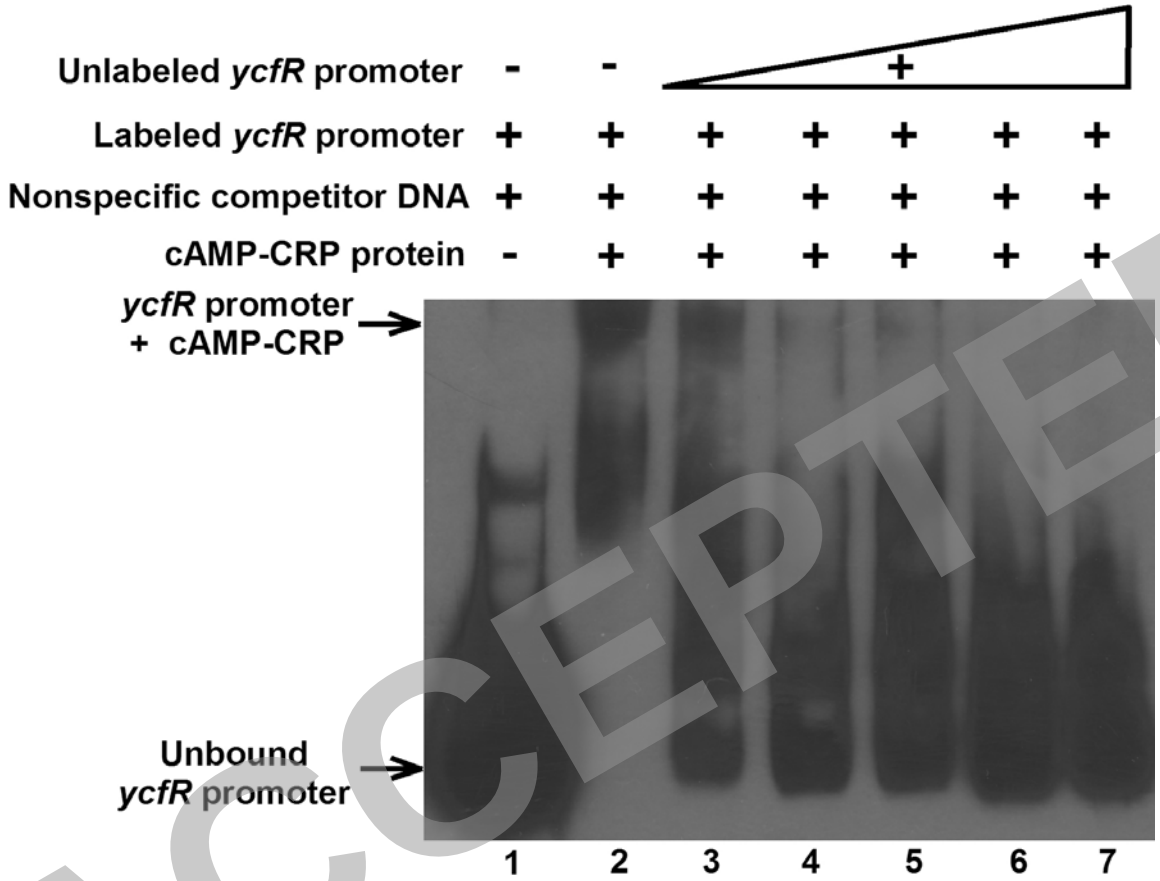

Figure 6