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MOLECULAR PATHOGENESIS

Dual Function of Aar, a Member of the New AraC Negative Regulator Family, in *Escherichia coli* Gene Expression

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ABSTRACT Enteroaggregative *Escherichia coli* (EAEC) is an *E. coli* pathotype associated with diarrhea and growth faltering. EAEC virulence gene expression is controlled by the autoactivated AraC family transcriptional regulator, AggR. AggR activates transcription of a large number of virulence genes, including Aar, which in turn acts as a negative regulator of AggR itself. Aar has also been shown to affect expression of *E. coli* housekeeping genes, including H-NS, a global regulator that acts at multiple promoters and silences AT-rich genes (such as those in the AggR regulon). Although Aar has been shown to bind both AggR and H-NS *in vitro*, functional significance of these interactions has not been shown *in vivo*. In order to dissect this regulatory network, we removed the complex interdependence of *aggR* and *aar* by placing the genes under the control of titratable promoters. We measured phenotypic and genotypic changes on downstream genes in EAEC strain 042 and *E. coli* K-12 strain DH5α, which lacks the AggR regulon. In EAEC, we found that low expression of *aar* increases *aafA* fimbrial gene expression via H-NS; however, when *aar* is more highly expressed, it acts as a negative regulator via AggR. In DH5α, *aar* affected expression of *E. coli* genes in some cases via H-NS and in some cases independent of H-NS. Our data support the model that Aar interacts in concert with AggR, H-NS, and possibly other regulators and that these interactions are likely to be functionally significant *in vivo*.

KEYWORDS ANR, Aar, AggR, enteroaggregative *E. coli*

Enteroaggregative *Escherichia coli* (EAEC) is a common cause of traveler's diarrhea in industrial and developing countries and has been linked to growth failure in children (1–4). Host colonization of EAEC is attributed to the presence of virulence genes that are controlled by AggR, a member of the AraC family of bacterial transcriptional regulators (5–7). A small protein named Aar (AggR activated regulator), whose expression is activated by AggR, has been described as a negative regulator of AggR (5, 8). Further characterization of Aar found that it belongs to a large family of proteins termed AraC negative regulators (ANR). The ANR family is found in hundreds of Gram-negative pathogens, and phylogenetically close homologs are able to complement function in ANR mutants (8).

In addition to regulating AggR expression, Aar has also been found to regulate genes encoding proteins outside of the AggR regulon, such as H-NS (9). H-NS is a global regulatory protein, which usually acts as a repressor at a wide variety of promoters and genes that are AT-rich and therefore intrinsically curved (10, 11). H-NS, AggR, and Aar have a complex dynamic. H-NS transcriptionally silences AraC transcriptional regulators; however, AraC transcriptional regulators may act as antirepressors that counteract H-NS silencing in selected environments (12, 13). It has been hypothesized that regulation of AggR and H-NS by Aar is via Aar binding directly to either AggR or H-NS. Aar has been shown to bind both AggR and H-NS via surface plasmon resonance, the bacterial

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two-hybrid system, pulldown assays, and electrophoretic mobility shift assays (9, 14); however, functional significance of these interactions has not been elucidated.

We have previously postulated that Aar could be acting on AggR through direct formation of Aar/AggR complexes and/or through the formation of Aar/H-NS complexes, which could act to lift H-NS silencing of the regulon (9). The benefit to the bacterium of regulating virulence genes by two different interactions effected by one protein is unclear. In this study, we sought to better understand the mechanism by which Aar downregulates AggR-regulated genes and the functional significance (if any) of the hypothesized Aar/AggR and Aar/H-NS binding events.

RESULTS

Independent expression of *aggR* and *aar* affects biofilm formation. Although we have observed that Aar binds both AggR and H-NS (9, 14), the mutual interdependence of these genes obfuscates the functional implications of these putative protein-protein interactions. Specifically, (i) H-NS has been shown to bind to AT-rich structural genes (10, 11), which include both AggR and Aar; (ii) AggR is the activator of Aar gene expression (5); (iii) Aar has been shown to repress AggR expression (8); and (iv) transcriptomic data suggested that Aar may activate expression of the H-NS-encoding gene (9). Therefore, in order to better dissect the roles and contributions of these interdependent regulators in the control of gene expression in EAEC, we assembled systems in which expression of the genes could be controlled independently. Accordingly, we first constructed a derivative of EAEC strain 042 that harbored mutations in *aar* and *aggR* and then introduced plasmids that carried the structural genes of *aar* and *aggR* under independently controllable promoters.

Plasmid pP*rham-aar* (designated here p*aar*) features the *aar* gene under the control of the rhamnose promoter; the plasmid is built on a pBR322 backbone (pMB1 replicon) and confers resistance to ampicillin. In preliminary experiments, we demonstrated that there were growth differences between LB and LB with rhamnose after 4 h, likely due to rhamnose catabolism, so all experiments were performed at 3 h postinduction unless stated otherwise (see Fig. S1 in the supplemental material). Similarly, plasmid pP*lacz-aggR* (designated here p*aggR*) features *aggR* under the *lacZ* promoter and is built on a pACYC177 backbone (p15A replicon) that confers resistance to kanamycin (km).

In EAEC, AggR production induces the expression of *aafA*, leading to the formation of a bacterial biofilm (5, 15); thus, biofilm formation is a ready phenotypic screen for *aggR* expression. Strain 042Δ*aar* Δ*aggR* did not produce an observable biofilm on a polystyrene substratum after 3 h of incubation at 37°C (data not shown). To assess the effect of p*aggR* expression in strain 042Δ*aar* Δ*aggR*, we subjected the strain to increasing concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG). As predicted, we observed a dose-dependent increase in biofilm formation (Fig. 1A).

Expression of *aar* in 042Δ*aar* Δ*aggR* via introduction of plasmid p*aar* was affected by increasing concentrations of rhamnose; such a construct did not display expression of *aggR*. As predicted from our previous observations that *aafA* and resultant biofilm formation requires AggR (5, 15), we observed no change in biofilm formation in this construct under conditions of increasing rhamnose concentrations (Fig. 1B).

To confirm that changes seen in biofilm formation correlated with changes in the expression of *aggR*, *aar*, and *aafA*, quantitative reverse transcriptase PCR (qRT-PCR) was performed for these gene transcripts. We discovered that inducer concentrations lower than that which produced observable biofilms were found to maximize mRNA transcript production by qRT-PCR; induction curves for qRT-PCR demonstrated that lower concentrations of the inducers were necessary to detect differences at the RNA level (Fig. 2A and B). The combination of *aar* and *aggR* expression induced by the lower concentrations of rhamnose and IPTG, respectively, leads to measurable changes in *aafA* expression (Fig. 2C). Gene expression of *aggR*, *aar*, and *aafA* confirmed that biofilm formation parallels *aafA* gene expression (Fig. 2A to C).

Aar has a paradoxical effect on *aafA* expression. We have previously observed that Aar serves as a negative regulator of the AggR regulon and that the two proteins

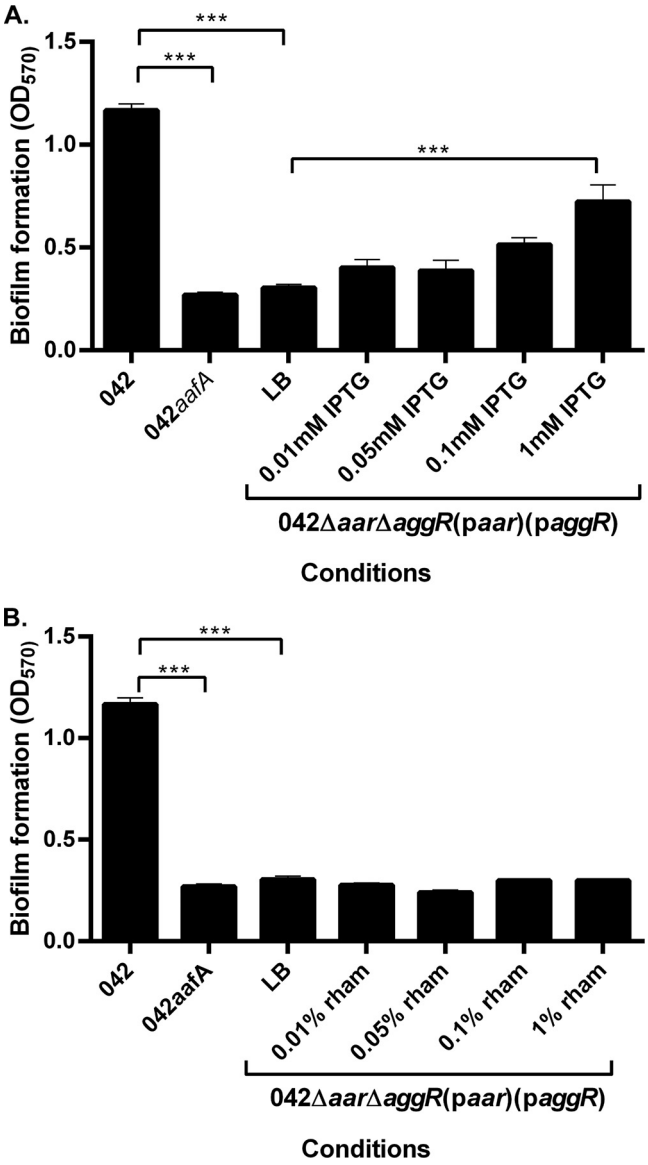

FIG 1 Biofilm formation in the presence and absence of inducer molecules. (A) Biofilm formation was measured using crystal violet staining after 3 h in 042 and 042Δ*aafA* in DMEM high glucose and in 042Δ*aar* Δ*aggR*(p*aar*)(p*aggR*) in LB with varying concentrations of IPTG. (B) Biofilm formation was measured using crystal violet staining after 3 h in 042 and 042Δ*aafA* in DMEM high glucose and in 042Δ*aar* Δ*aggR*(p*aar*)(p*aggR*) in LB with varying concentrations of rhamnose. Biofilm data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

bind to each other *in vitro* (8, 14). As predicted from this model, we observed a dose-dependent decrease in biofilm formation with increasing expression of *aar* (increasing concentrations of rhamnose) under conditions of constant *aggR* expression (Fig. 3A, colored bars with same fill pattern). qRT-PCR measurements of *aafA* transcription support that this decrease in biofilm formation was associated with a decrease in *aafA* expression (Fig. 3B, colored bars with same fill pattern) under constant *aggR* expression and increasing *aar* expression (see Fig. S2A and B, colored bars with same fill pattern, in the supplemental material).

Unexpectedly, given our model, we observed a paradoxical effect: in the presence of *aggR*, low levels of *aar* expression lead first to increased biofilm (Fig. 3A, dark gray bars to blue bars) followed by the expected dose-dependent decrease in biofilm

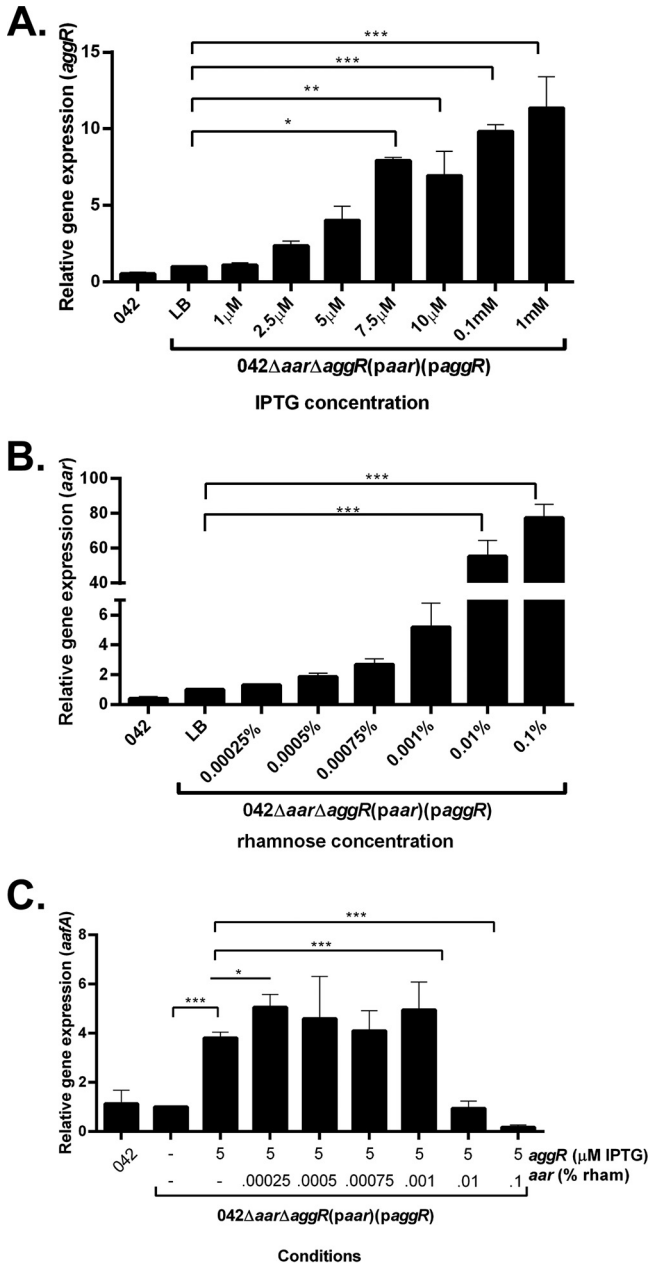

FIG 2 Gene expression after induction of *aggR* by IPTG or *aar* by rhamnose. (A) Three-hour *aggR* expression measured using qRT-PCR on 042 in DMEM high glucose and 042Δ*aar* Δ*aggR*(p*aar*)(p*aggR*) in LB with varying concentrations of IPTG. (B) Three-hour *aar* expression measured using qRT-PCR on 042 in DMEM high glucose and 042Δ*aar* Δ*aggR*(p*aar*)(p*aggR*) in LB with varying concentrations of rhamnose. (C) Three-hour *aafA* expression measured using qRT-PCR on 042 in DMEM high glucose and 042Δ*aar* Δ*aggR*(p*aar*)(p*aggR*) in LB with 0.005 mM IPTG and a range of concentrations of rhamnose. qRT-PCR data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). The difference between *aggR* (5 μM) and *aggR* (5 μM) with *aar* (0.00025%) was found to be significant with a two-tailed paired *t* test.

formation at higher *aar* concentrations. This effect was not due to the effects of the inducers themselves (see Fig. S3A and B in the supplemental material).

The paradoxical effect of Aar requires H-NS. Transcriptome sequencing (RNA-seq) data suggested that Aar has an effect on expression of the histone-like protein H-NS, and binding assays suggested that the two proteins physically interact (9). Like many members of the AraC family of transcriptional activators, AggR and the genes that it regulates (i.e., *aafA*) are repressed by H-NS (12, 13). Members of the AraC family of

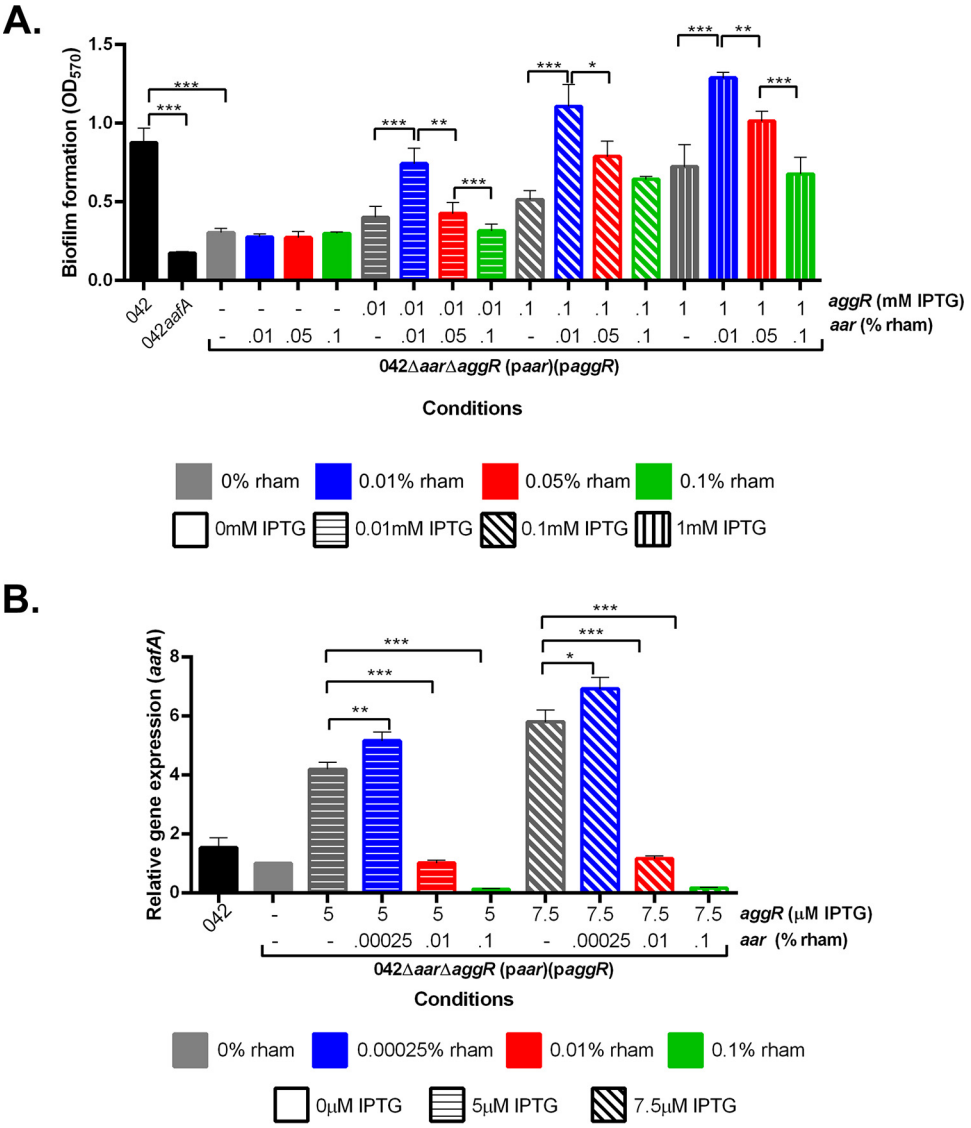

FIG 3 Biofilm formation and gene expression of *aafA* in 042Δ*aar* Δ*aggR* titrated with *aar* and *aggR*. (A) Biofilm growth at 3 h postinduction with increasing concentration of IPTG and rhamnose. *aggR* expression was induced with 0.01 mM IPTG (horizontal fill pattern), 0.1 mM IPTG (diagonal fill pattern), or 1 mM IPTG (vertical fill pattern). *aar* expression was induced with 0.01% rhamnose (rham) (blue), 0.05% rham (red), or 0.1% rham (green). (B) qRT-PCR analysis of *aafA* using titratable *aar* and *aggR*. *aggR* expression was induced with either 5 μM IPTG (horizontal fill pattern) or 7.5 μM IPTG (diagonal fill pattern). *aar* expression was induced with 0.00025% rham (blue), 0.01% rham (red), or 0.1% rham (green). Biofilm data and qRT-PCR data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

transcriptional regulators are thought to counteract H-NS-induced silencing in select environments (12, 13). The paradoxical effect of Aar expression on AggR-dependent *aafA* expression suggested the action of another regulator, and we hypothesized that this regulator was H-NS.

To test this hypothesis, we constructed 042Δ*aar* Δ*aggR* Δ*hns*, transformed the strain with p*aar* and p*aggR*, and measured biofilm production with varying IPTG and rhamnose concentrations. 042Δ*aar* Δ*aggR* Δ*hns* had a growth defect compared to that of 042Δ*aar* Δ*aggR*; growth of 042Δ*aar* Δ*aggR* Δ*hns* at 5 h produced an optical density at 600 nm (OD_{600}) similar to that observed in 042Δ*aar* Δ*aggR* at 3 h (see Fig. S4 in the supplemental material). Rhamnose catabolism had no effect on the growth phase of 042Δ*aar* Δ*aggR* Δ*hns* at 5 h (Fig. S4). Similar to 042Δ*aar* Δ*aggR*, a low-level increase in

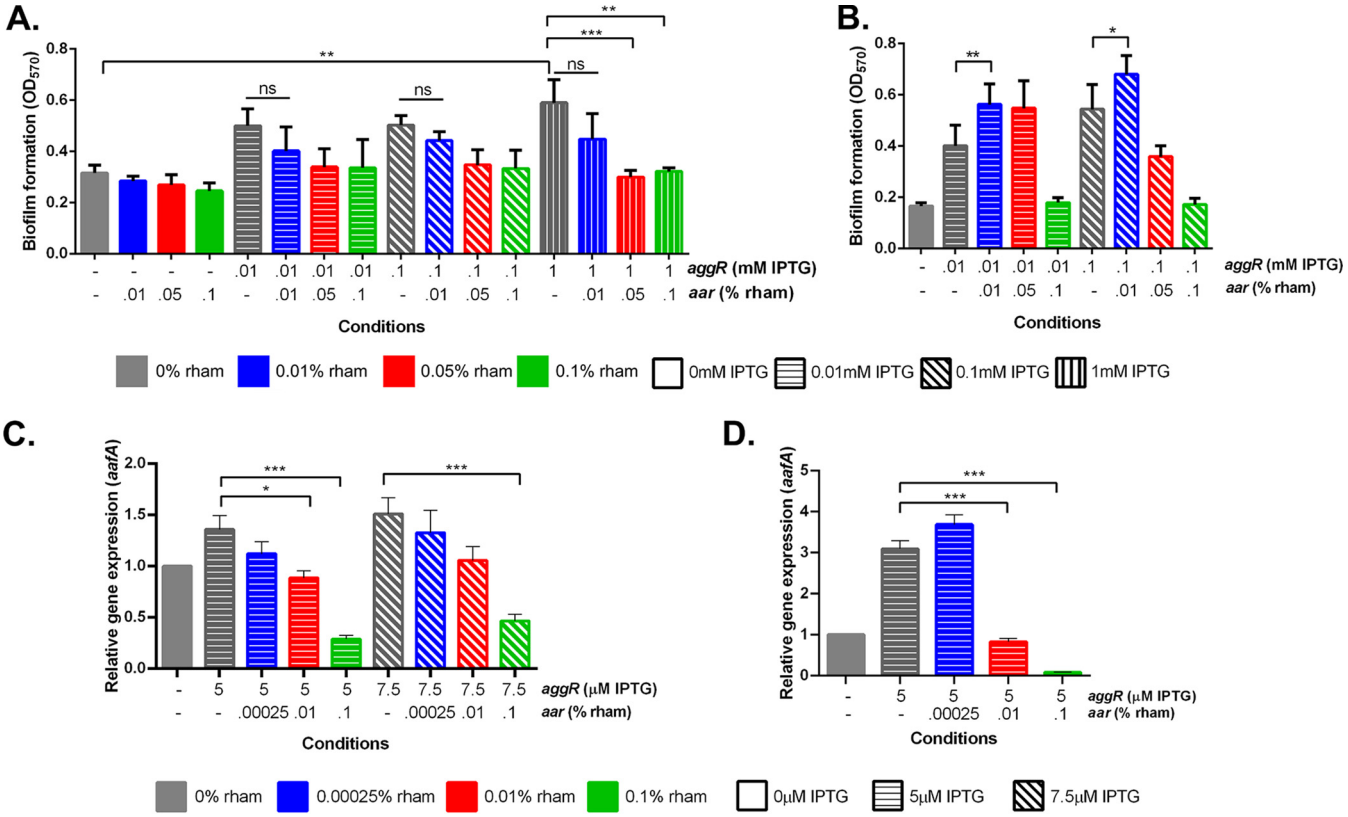

FIG 4 Biofilm formation and expression of *aafA* in 042Δ*aar* Δ*aggR* Δ*hns* and *hns* repair titrated with *aar* and *aggR*. (A) Biofilm growth in 042Δ*aar* Δ*aggR* Δ*hns* at 5 h postinduction with increasing concentration of IPTG and rhamnose. (B) Biofilm growth in *hns* repair at 3 h postinduction with increasing concentration of IPTG and rhamnose. *aggR* expression was induced with 0.01 mM IPTG (horizontal fill pattern), 0.1 mM IPTG (diagonal fill pattern), or 1 mM IPTG (vertical fill pattern). *aar* expression was induced with 0.01% rham (blue), 0.05% rham (red), or 0.1% rham (green). (C) qRT-PCR analysis of *aafA* using titratable *aar* and *aggR* in 042Δ*aar* Δ*aggR* Δ*hns* after 5 h. (D) qRT-PCR analysis of *aafA* using titratable *aar* and *aggR* in the *hns* repaired 042Δ*aar* Δ*aggR* after 3 h. *aggR* expression was induced with either 5 μM IPTG (horizontal fill pattern) or 7.5 μM IPTG (diagonal fill pattern). *aar* expression was induced with 0.00025% rham (blue), 0.01% rham (red), or 0.1% rham (green). Biofilm data and qRT-PCR data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

aar led to a decrease in biofilm growth (Fig. 4A, colored bars with same fill pattern). However, in the absence of *hns*, the increase of biofilm formation at low levels of *aar* was no longer observed (Fig. 4A, dark gray bars to blue bars). The effect of low levels of Aar on biofilm production was rescued when *hns* was restored (Fig. 4B). qRT-PCR analysis confirmed that in 042Δ*aar* Δ*aggR* Δ*hns*, under conditions of constant *aggR* expression (see Fig. S5A in the supplemental material) but increasing *aar* expression (Fig. S5B), the direct dose-dependent expression of *aafA* by *aggR* is maintained as is the negative effect by *aar* (Fig. 4C); however, the paradoxical effect of *aar* on *aafA* is lost in the absence of H-NS (Fig. 4C). As seen in the biofilm assay (Fig. 4B), qRT-PCR supported that the restoration of *hns* rescued the paradoxical effect (Fig. 4D) under conditions of constant *aggR* expression (Fig. S5C) but increasing *aar* expression (Fig. S5D). The paradoxical effect was only seen in the presence of AggR production; i.e., *aar* expression by itself did not affect *aafA* expression regardless of the presence of *hns*. Taken together, these data suggest a tripartite model of AggR/Aar/H-NS interaction, consistent with our *in vitro* observation that Aar binds to both AggR and H-NS.

***aggR* diminishes the *aar*-induced upregulation of non-AggR-regulated genes in *E. coli* K-12 via *aar*.** The results of our AggR and Aar controllable gene expression studies reveal dose-dependent effects of the two regulators consistent with the previously published model of AggR/Aar protein-protein binding (14), i.e., that *aafA* expression may depend on the concentration of AggR unbound to Aar. If the mechanism is in fact due to protein-protein interaction, then binding of AggR to Aar might also reduce the activity of the latter protein. We sought to utilize a simplified system

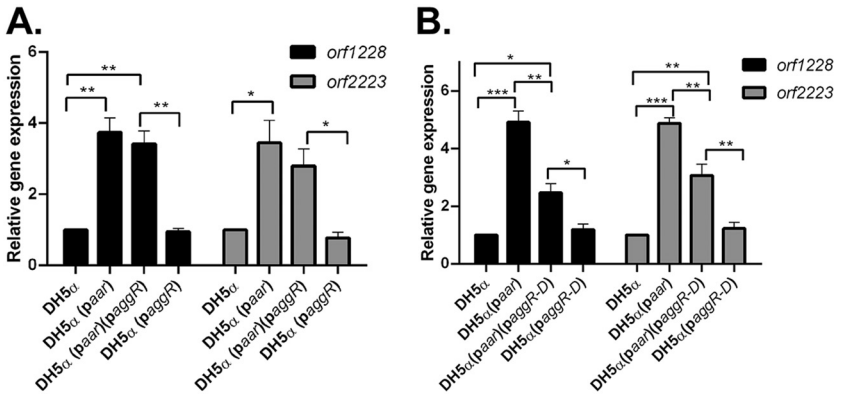

FIG 5 The effect of *aar* and *aggR* on gene expression in DH5α transformed with p*aar* and/or p*aggR*/p*aggR-D*. (A) DH5α was transformed with p*aar* and p*aggR* expressing full-length *aggR* or their corresponding empty vectors pBR322 and pACYC177, respectively. Transcriptional levels of *E. coli* chromosomal genes *orf1228* and *orf2223* were analyzed by qRT-PCR. (B) DH5α was transformed with p*aar* and p*aggR-D* expressing the AggR dimerization domain or their corresponding empty vectors. Transcriptional levels of *E. coli* chromosomal genes *orf1228* and *orf2223* were analyzed by qRT-PCR. RT-PCR data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

with which to probe hypothetical interference of Aar activity by AggR. Such an effect would add another regulatory dimension to the tripartite protein-protein interaction system.

We have previously observed in strain 042 that Aar activates housekeeping genes that are AggR independent (9); if true, we would hypothesize that expression of such genes would similarly be affected in a K-12 background. Interrogating this effect in K-12 would eliminate the effect of AggR on other genes of the AggR regulon, which does not exist in K-12.

We transformed *E. coli* DH5α separately with p*aar* and p*aggR* or their corresponding empty vector controls (pBR322 and pACYC177, respectively). To interrogate a possible inhibitory effect of AggR on Aar, we chose to study two chromosomal *E. coli* core genes previously shown to be affected by *aar* in strain 042 (*orf1228* and *orf2223*) (9). As predicted, we observed that in K-12 strain DH5α, when *aar* was expressed in p*aar*, the expression levels of *orf1228* and *orf2223* were increased (Fig. 5A). The expression of *aggR* alone in DH5α had no effect on the expression of either target gene in the absence of *aar* (Fig. 5A).

The expression of *aggR* simultaneously with *aar* caused only a small reduction to the observed increase in gene expression (Fig. 5A). Given that AggR is a DNA-binding protein, we repeated this experiment using an *aggR* construct comprising only the AggR dimerization domain (amino acids 69 to 181), therefore, lacking the DNA binding helix-turn-helix C-terminal region (pP*lacZ-aggR-D* is referred to as p*aggR-D*) (14); we previously reported that the AggR dimerization domain binds Aar in the bacterial two-hybrid system (14). As seen with the full-length *aggR*, the expression of the *aggR-D* in DH5α had no effect on the expression of *orf1228* and *orf2223* (Fig. 5B); when *aggR-D* and *aar* were both expressed in DH5α, the expression levels of the two queried genes were both significantly decreased compared to the level of expression when *aar* was expressed alone (Fig. 5B), suggesting that the expression of *aggR-D* in this system affects the expression of *orf1228* and *orf2223* via *aar*, consistent with protein-protein interaction of the two proteins. The expression levels of *aar* and *aggR* were similar whether expressed alone or together (see Fig. S6A in the supplemental material); the same was true for *aar* and *aggR-D* (Fig. S6B).

***aar* upregulates gene expression in *E. coli* K-12 via *hns*.** Several of the Aar-controlled genes affected in 042 that are AggR independent are thought to be under H-NS control based on previous literature (9, 16–18). We hypothesize that the effect of Aar on these genes is via the proposed model of Aar/H-NS protein-protein binding.

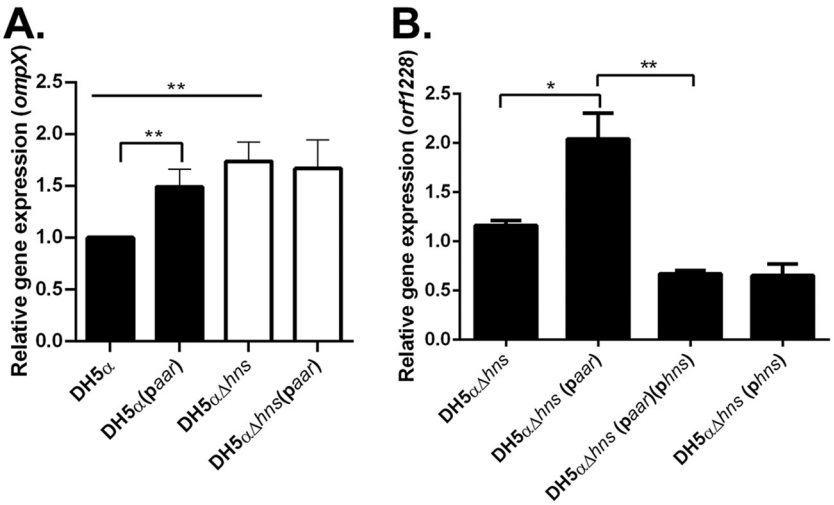

FIG 6 The effect of *aar* on gene expression in the presence or absence of *hns* in DH5α. (A) DH5α and DH5$\alpha\Delta$*hns* were transformed with p*aar* or its corresponding empty vector pBR322. Transcriptional levels of *ompX* were analyzed by qRT-PCR. (B) DH5$\alpha\Delta$*hns* was transformed with p*aar* and p*hns* or their corresponding empty vectors pBR322 and pKNT25, respectively. Transcriptional levels of *orf1228* were analyzed by qRT-PCR. RT-PCR data are representative of at least three independent experiments. Asterisks indicate significant differences by ANOVA (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

We used DH5α and DH5$\alpha\Delta$*hns* transformed with p*aar* to probe the expression of *ompX*, a gene known to be under the regulation of H-NS (17). The expression of *ompX* was increased in the absence of *hns*, confirming that *ompX* expression in DH5α is regulated by *hns* (Fig. 6A). In DH5α, we found that the expression of *ompX* was significantly increased when *aar* was induced (Fig. 6A). However, when *aar* was induced in DH5$\alpha\Delta$*hns*, there was no change in the expression of *ompX* (Fig. 6A), suggesting that *hns* is required for the effect of *aar* on *ompX*.

***hns* diminishes the *aar*-induced upregulation of non-H-NS-regulated genes in *E. coli* K-12 via *aar*.** We reasoned that if Aar bound H-NS in the bacterium, the interactions of the two genes may be mutually interfering, and the activity of Aar in the bacterium may be diminished by expression of *hns*, similarly to what was demonstrated with AggR (Fig. 5A and B). We, therefore, sought to use the DH5α system to probe hypothetical interference of Aar activity by H-NS.

We transformed *E. coli* DH5$\alpha\Delta$*hns* separately with p*aar* and our previously published pKNTHNS (designated here p*hns*) (9) or their corresponding empty vector controls pBR322 and pKNT25, respectively. We determined that *orf1228* was not affected by H-NS expression (see Fig. S7 in the supplemental material); therefore, we chose to probe the expression of this gene to interrogate a possible inhibitory effect of H-NS on Aar activity. We performed the experiment in DH5$\alpha\Delta$*hns* to remove any effects that the native *hns* could have on the system. When *aar* was expressed in DH5$\alpha\Delta$*hns*, the expression of *orf1228* was increased (Fig. 6B). The expression of *hns* alone had no effect on the expression of *orf1228* in the absence of *aar* (Fig. 6B). As suspected, when *hns* and *aar* are expressed together, the effect of *aar* on *orf1228* is significantly reduced (Fig. 6B). This suggests that the expression of *hns* is affecting the expression of *orf1228* via *aar*, which is consistent with the proposed model of a direct interaction of the two proteins.

DISCUSSION

Although binding of Aar to AggR and H-NS has been previously demonstrated in artificial systems, we have not yet provided evidence that either of these binding phenomena have a functional role in the bacterium. In this work, we constructed a series of experimental systems to probe potential interrelationships among Aar, AggR, and H-NS, regulators that are expected to have mutual interdependence. By using a system in which we remove the transcriptional interdependence of *aar* and *aggR*, our

data suggest functional roles for Aar binding to both AggR and H-NS in EAEC 042. In addition, employing *E. coli* DH5α, we observe evidence for Aar function in the absence of AggR, both via H-NS and potentially other regulators of the core *E. coli* genome.

As predicted, increasing *aar* expression led to a dose-dependent decrease in *aggR*-regulated *aafA* expression and biofilm formation. This relationship was inversely reciprocal. Expression of *aafA* was increased by increasing *aggR* expression but decreased by *aar*; *aafA* expression seemed to correlate best with excess *aggR* abundance over the level of *aar* expression. Previously published data failed to reveal evidence that Aar binds to DNA (9, 14), and because we removed the transcriptional dependence of *aggR* and *aar* on one another, our data best support the model that Aar binds and sequesters AggR and that *aafA* expression levels would be determined by free AggR protein concentrations.

In titration experiments, we were surprised to observe a phenotypic effect on biofilm formation with low expression of *aar* that was contrary to our hypothesis regarding how Aar would affect the expression of AggR-regulated genes. At the lowest levels of *aar* expression, we observed a paradoxical increase in *aafA* expression, independent of *aggR* expression. This effect was abrogated in an *hns* mutant. These data suggest a potential role for dual binding of both AggR and H-NS by Aar. It is tempting to speculate that Aar has a higher affinity for H-NS than for AggR, given that *aar* has a positive effect on *aafA* through *hns* first and then a negative effect via *aggR*. Such a nuanced effect could permit early expression of *aafA in vivo* before the time required for cycles of *aggR* transcription and translation and subsequent binding to the *aafA* promoter. Given that there is evidence for EAEC infection of both the duodenal (19) and the colonic mucosae (20), this dual regulation could provide distinct pathogenic timing.

It has previously been shown that Aar acts upon H-NS-regulated promoters differentially (9). Due to the low expression of *aar* that is necessary to observe changes in *aafA* through *hns*, our data suggest that Aar may remove H-NS from the *aafA* gene (possibly the structural gene itself), thereby permitting AggR to upregulate expression. This affinity for removing H-NS may extend to other AggR-regulated promoters. It is possible that Aar could add specificity to the removal of H-NS at AggR-regulated genes over other H-NS regulated genes, thus allowing for a timed derepression of those specific genes. The underlying mechanism of how Aar is leading to differential expression of various genes is unclear.

H-NS is a global regulator of *E. coli* gene expression (10, 11), and the putative binding of Aar to H-NS suggested that Aar may have global effects on EAEC gene expression beyond the AggR regulon. For this to prevail *in vivo*, one would expect effects of Aar on gene expression in *E. coli* K-12, which is devoid of the AggR regulon; we not only observed such effects in a K-12 system, but our data suggest still more global complexity accompanying *aar* expression. Our observations in a K-12 system rule out the need for a pathogen-specific intermediary protein.

Although we posit that Aar acts via protein-protein interaction, demonstration of protein-protein binding is not definitive evidence that this phenomenon occurs *in vivo*. The use of multiple assays suggested AggR/Aar binding: surface plasmon resonance, bacterial two-hybrid system, and electrophoretic mobility shift assay (EMSA) (14). The *in vivo* data presented here confirm interrelationships among these regulatory proteins in ways that would be difficult to ascribe to alternate mechanisms. Importantly, the expression of *aggR* alone had no effect on *orf1228* and *orf2223*; therefore, the decrease in expression of these two genes in the presence of both *aar* and *aggR* suggests that AggR may be binding free Aar and preventing Aar from activating the genes. By demonstrating an *aar* effect on gene expression through *aggR* (in EAEC 042) and an *aggR* effect on gene expression through *aar* (in DH5α), our data support the previously published model that the effect of *aar* and *aggR* is through protein-protein binding.

Targeting the *E. coli* gene *ompX*, previously reported to be under H-NS control (17), we confirmed that expression of *aar* induced expression of *ompX* in an *hns*-dependent manner. Surprisingly, however, our data suggest that the Aar effect on *orf1228* expres-

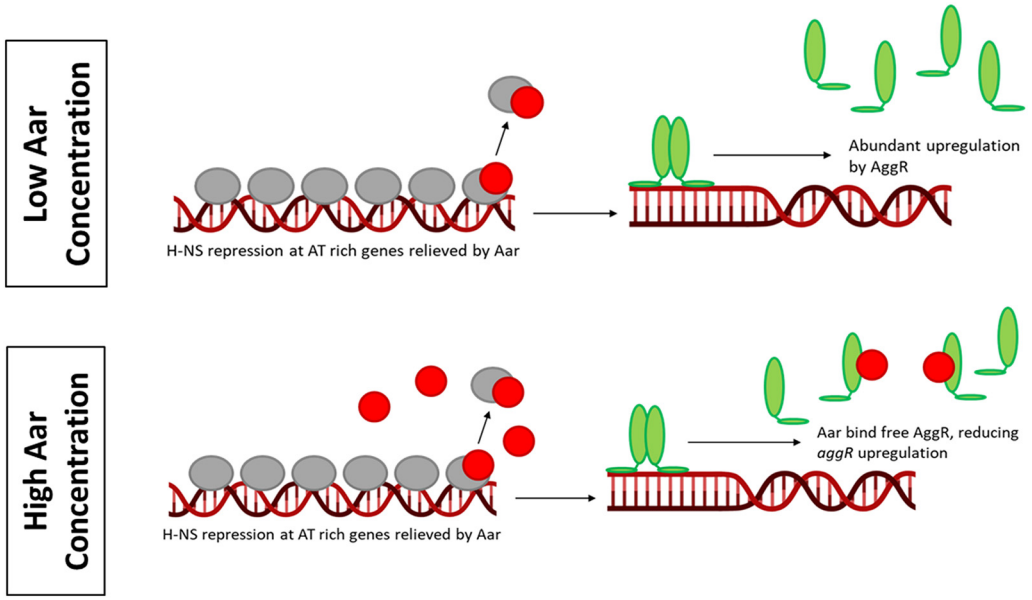

FIG 7 Proposed mechanism of AggR-Aar-Hns interaction *in vivo*. When the concentration of Aar (red circles) is low, Aar removes H-NS (gray ovals) repression at AT-rich genes. This allows AggR (green ovals) to abundantly upregulate gene expression. When the concentration of Aar is high, Aar removes H-NS repression but also binds to AggR. AggR is still able to upregulate gene expression but not as abundantly.

sion persists even in an *hns* mutant, suggesting that Aar may act in concert with still another regulator beyond AggR and H-NS. *E. coli* possesses additional histone-like proteins, which may be responsible for the effect of *aar* on *orf1228* expression, and these are the targets of ongoing research in our laboratory. As we predicted, expression of the AggR dimerization domain (which does not occur naturally in K-12 and does not bind DNA but has been shown to bind Aar *in vitro*) demonstrated an Aar inhibitory effect in a K-12 background. These data strongly support the hypothetical model wherein AggR and Aar bind directly, thereby inhibiting activity of both proteins. The effects we observed show interdependence of AggR, Aar, and H-NS; however, the data do not prove that the mechanism is direct protein-protein interactions.

Based on our data and previous studies (8, 9, 14), we propose a model to illustrate a dual function of Aar in EAEC virulence gene expression (Fig. 7). In abiotic environments, H-NS binds AT-rich genes and silences their expression (i.e., *aggR* and *aggR*-regulated genes). When the bacteria reach the host, temperature change and inducer molecules induce *aggR* expression. AggR upregulates *aar* expression, and at early stages of induction when *aar* expression is low but detectable, Aar binds and relieves H-NS silencing from AggR-regulated genes. This results in immediate upregulation of previously silenced genes by AggR. As the expression of *aar* increases, Aar begins to bind AggR in addition to H-NS, preventing AggR dimerization and therefore reducing activation of *aggR*-regulated genes.

The data presented in this paper support the model that Aar is binding to both AggR and H-NS and that both interactions have functional significance. In EAEC strain 042, Aar has a dual function in virulence gene expression. First, when present at low concentrations, Aar removes the inhibitory effect of H-NS on fimbrial gene expression; and then when the concentration of Aar increases, Aar acts as a negative regulator, turning off AggR-activated virulence genes. Our data suggest that not only is Aar an antiactivator, but it can also act as an antirepressor. The role of Aar on genes of the core *E. coli* genome is more difficult to decipher but could play a role in the switch from the nonpathogenic to the pathogenic lifestyle. Further research will address the concerted action of this complex regulatory circuitry.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study can be found in Table S1 in the supplemental material. Bacteria were grown in Luria Broth (LB) and Dulbecco's modified Eagle's medium with 0.4% glucose (DMEM high glucose) (Gibco, Grand Island, NY) as previously described (5). When indicated, medium was supplemented with carbenicillin (100 μg/ml) and/or kanamycin (50 μg/ml). For phenotypic titration studies, 0.01 mM or 1 mM IPTG and 0.01%, 0.05%, or 0.1% rhamnose were added as indicated below. For transcriptional studies, 5 μM or 7.5 μM IPTG and 0.00025%, 0.01%, or 0.1% rhamnose were added as indicated below. Inducer concentrations were selected after a range of concentrations was screened to determine which had detectable effects on the expression of *aggR* or *aar*.

Mutagenesis of *aggR* and *hns* in 042Δ*aar* was accomplished by using lambda red technology (21). The loci (41,080 to 41,877 and 1,376,831 to 1,377,244; GenBank accession number FN554767.1) in 042Δ*aar* were replaced with the kanamycin (km) resistance marker as previously reported (21). The 042Δ*aar* Δ*aggR* and 042Δ*aar* Δ*aggR* Δ*hns* strains were identified by PCR using specific primers for *aggR*, *hns*, and a km resistance marker (see Table S2 in the supplemental material). Deletion strains were cured of the km resistance using pCP20 as previously reported (21). Repair of *hns* in 042Δ*aar* Δ*aggR* Δ*hns* was done by using the lambda red recombination protocol to recombine a PCR product of *hns* with large flanking regions from 042 with the 042Δ*aar* Δ*aggR* Δ*hns* km-resistant strain and testing for recombination via km sensitivity. Primers for recombination of *hns* and screening for the repair are shown in Table S2.

Mutagenesis of *hns* in DH5α was accomplished by using lambda red technology, and mutants were PCR screened and cured of km resistance as stated above.

Titratable expression of *aggR* and *aar*. For the independent expression of *aggR* and *aar*, plasmids pPlacZ-*aggR*, pPrham-*aar*, and pPlacZ-*aggR-D* were generated in this study (Table S1). Briefly, 1,086-bp fragments containing the *lacZ* promoter region, the entire *aggR* gene, a hemagglutination (HA) tag, a termination sequence, and flanked by restriction enzyme sites were synthesized by Genewiz Inc. by fragmentGENE synthesis. The synthesized fragment was inserted into BamHI and PstI sites in pACYC177; the resulting plasmid was designated pPlacZ-*aggR*. pPlacz-*aggR-D* was similar to pPlacZ-*aggR* but only contains the dimerization site of *aggR*, comprising amino acid residues 69 to 181. pPrham-*aar* was generated similarly but harbored a 548-bp fragment containing the rhamnose promoter region, the entire *aar* gene, a 6×histidine tag, a termination sequence, and was flanked by restriction enzyme sites. The synthesized fragment was inserted into the BamHI and HindIII sites in pBR322.

Biofilm production. The biofilm assay previously described by Sheikh et al. (15) was modified. Briefly, bacterial strains were grown in LB overnight at 37°C shaking. Overnight cultures of wild-type (WT) 042 and 042Δ*aafA* were diluted 1:20 in DMEM high glucose, and titration constructs were diluted 1:20 in LB with or without IPTG and rhamnose and inoculated into a 24-well polystyrene plate (Sigma-Aldrich). Bacteria were incubated for 3 h at 37°C. After incubation, plates were washed two times with phosphate-buffered saline (PBS) and fixed with 75% ethanol. The fixed biofilms were dried and stained with 0.5% crystal violet (Sigma). Biofilms were washed 4 times with PBS after staining and solubilized in 95% ethanol. The absorbance was determined at 570 nm. Biofilms for 042Δ*aar* Δ*aggR* Δ*hns* were incubated for 5 h at 37°C due to impaired growth.

RNA extraction and qRT-PCR. For quantitative reverse transcriptase PCR (qRT-PCR), EAEC strain 042, 042Δ*aar* Δ*aggR*, and 042Δ*aar* Δ*aggR* Δ*hns* titration strains were grown aerobically in LB overnight at 37°C with shaking and then diluted 1:100 in DMEM high glucose or LB supplemented with IPTG and rhamnose concentrations as indicated and grown at 37°C. RNA from three biological replicates of each condition was extracted after 3 h or 5 h for the 042Δ*aar* Δ*aggR* Δ*hns* titration strain. RNA was extracted using RNAprotect bacteria reagent (Qiagen) followed by an RNeasy minikit (Qiagen). Primers used were previously published for EAEC (8, 9). qRT-PCR was performed using a one-step reaction in an ABI 7500 Fast sequence detection system (Applied Biosystems). All data were normalized to the levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method (22). The relative quantification method was used to determine the expression levels of target genes. Statistical significance was determined by analysis of variance (ANOVA) with *post hoc* Tukey, and a P value of ≤ 0.05 was considered significant.

For qRT-PCR on *aar* and *aggR* in *E. coli* K-12 strain DH5α was transformed with titratable *aar*, *aggR*, and their corresponding empty vector plasmids and was grown aerobically in LB overnight at 37°C with shaking. A 1:100 dilution was made in LB with 0.1 mM IPTG and 0.1% rhamnose and grown shaking at 37°C for 3 h. RNA was extracted and qRT-PCR performed as above. Primers used were previously published for EAEC (8, 9).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

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