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RegR Virulence Regulon of Rabbit-Specific Enteropathogenic *Escherichia coli* Strain E22

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AraC-like regulators play a key role in the expression of virulence factors in enteric pathogens, such as enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli*, enteroaggregative *E. coli*, and *Citrobacter rodentium*. Bioinformatic analysis of the genome of rabbit-specific EPEC (REPEC) strain E22 (O103:H2) revealed the presence of a gene encoding an AraC-like regulatory protein, RegR, which shares 71% identity to the global virulence regulator, RegA, of *C. rodentium*. Microarray analysis demonstrated that RegR exerts 25- to 400-fold activation on transcription of several genes encoding putative virulence-associated factors, including a fimbrial operon (SEF14), a serine protease, and an autotransporter adhesin. These observations were confirmed by proteomic analysis of secreted and heat-extracted surface-associated proteins. The mechanism of RegR-mediated activation was investigated by using its most highly upregulated gene target, *sefA*. Transcriptional analyses and electrophoretic mobility shift assays showed that RegR activates the expression of *sefA* by binding to a region upstream of the *sefA* promoter, thereby relieving gene silencing by the global regulatory protein H-NS. Moreover, RegR was found to contribute significantly to virulence in a rabbit infection experiment. Taken together, our findings indicate that RegR controls the expression of a series of accessory adhesins that significantly enhance the virulence of REPEC strain E22.

Infectious diarrhea is an important cause of morbidity and death in children in developing countries. Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of acute infantile diarrhea in this setting (1, 2). EPEC can be further categorized into two subgroups, typical EPEC (tEPEC) and atypical EPEC (aEPEC) (3), which differ from each other in terms of their genetic characteristics, serotypes, and virulence factors (4).

The central mechanism of EPEC pathogenesis is colonization of the intestine accompanied by the formation of characteristic "attaching-and-effacing" (A/E) lesions on the surface of intestinal epithelial cells (5, 6). A 35-kb chromosomal pathogenicity island, termed the locus of enterocyte effacement (LEE), contains all of the genes necessary for A/E lesion formation (7–10). All EPEC strains carry the LEE and are Shiga toxin negative, but tEPEC strains also carry an EPEC adherence factor plasmid (EAF), which encodes bundle-forming pili (BFP), an essential colonization factor, and PerA, which directly regulates the expression of these pili and indirectly controls expression of the LEE, via PerC (11). The EAF plasmid is absent from aEPEC.

Humans are the only host and reservoir of tEPEC. In contrast, aEPEC can cause disease in humans and other animals (4, 12, 13). Infection with rabbit-specific EPEC (REPEC) causes the same clinical and pathological features in baby rabbits that human-specific EPEC produces in children (14, 15). Thus, REPEC infection of rabbits serves as a valuable model of human EPEC infection and has been used to establish the contribution of LEE-encoded proteins to virulence (16).

Recent epidemiological studies indicate that aEPEC is more prevalent than tEPEC in both developed and developing countries (17) and is frequently associated with persistent diarrhea (18). The mechanism by which aEPEC infection causes diarrhea is incompletely understood. Although aEPEC strains lack the EAF plasmid, they are able to cause disease; unlike tEPEC strains, which become markedly attenuated when they are "cured" of this plasmid (19). These observations suggest that aEPEC must produce colonization factors that compensate for the absence of BFP and also for PerA, but the identities of these virulence factors are yet to be determined.

Transcriptional control of virulence genes is essential for bacterial survival in their hosts, as it enables bacteria to respond to changing environmental conditions, such as a change in temperature or pH, or to adapt to a niche environment (20, 21). The AraC superfamily is an important group of transcriptional regulators that play a central role in the expression of virulence factors in enteric pathogens (22). Members of this superfamily include PerA of EPEC (23), Rns of enterotoxigenic *E. coli* (ETEC) (24), AggR of enteroaggregative *E. coli* (EAEC) (25), and ToxT of *Vibrio cholerae* (26). In general, AraC-like proteins are composed of 200 to 300 amino acids, comprising two distinct domains: an N-terminal stretch, which can be highly variable, and a C-terminal region harboring a characteristic double helix-turn-helix (HTH) motif

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E22	REPEC serotype O103:H2, Rif^r	14
E22 Δ*regR*	E22 Δ*regR*, Rif^r	This study
E22 Δ2	E22 Δ*regR* ΔEcE22_5293, Kan^r Rif^r	This study
E22 Δ*sefA*::*kan*	E22 Δ*sefA*, Kan^r Rif^r	This study
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thiA*	66
BL21(DE3)	F$^-$ *ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3)	New England BioLabs
TOP10	F$^-$ *mcrA* Δ(*mrr*$^-$ *hsdRMS*$^-$ *mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^r) *endA1* λ$^-$	Invitrogen
Plasmids		
pGEM-T Easy	High-copy-no. vector, Ap^r	Promega
pCR2.1-TOPO	High-copy-no. vector, Ap^r Kan^r	Invitrogen
pACYC184	Medium-copy-no. vector, Cm^r Tc^r	67
pFT-A	Low-copy-no. vector, *flp* Ap^r	68
pKD4	FRT-flanked *kan* gene, Kan^r Ap^r	36
pKD46	Low-copy-no. vector, P_{BAD}-λred Ap^r	36
pMU2385	Single-copy-no. transcriptional fusion vector, Tp^r	69
pMAL-c2x	Expression vector for N-terminal MBP fusion proteins	New England Biolabs
pYS1	pGEM-T Easy+Δ*regR*::*kan*	This study
pYS2	pCR2.1-TOPO+*regR*	This study
pYS3	pACYC184+*regR*	This study
pYS4	pGEM-T Easy+$sefA_1$	This study
pYS5	pGEM-T Easy+$sefA_2$	This study
pYS7	pGEM-T Easy+$sefA_3$	This study
pYS8	pGEM-T Easy+$sefA_4$	This study
pYS9	pMU2385+$sefA_1$	This study
pYS10	pMU2385+$sefA_2$	This study
pYS11	pMU2385+$sefA_3$	This study
pYS12	pMU2385+$sefA_4$	This study
pYS13	pGEM-T Easy+*regR*	This study
pYS14	pMAL-c2x+*regR*	This study
pYS15	pGEM-T Easy+$sefA_{prom}$	This study
pYS16	pGEM-T Easy+$sefA_{control}$	This study
pYS17	pGEM-T Easy+Δ*sefA*::*kan*	This study
pYS21	pGEM-T Easy+ ΔEcE22_5293::*kan*	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

for DNA binding (27). Importantly, AraC-like regulators can directly sense environmental chemicals that are abundant at the sites where the bacterial pathogens colonize and damage their hosts (20).

We recently identified a non-LEE-encoded, AraC-like regulatory protein, RegA, which plays a key role in the ability of *Citrobacter rodentium* to colonize mouse intestine (28). *C. rodentium* is a mouse-specific pathogen that is similar to EPEC in that it possesses the LEE, which allows it to cause A/E lesions in mouse intestine (29, 30). *C. rodentium* infection of mice is also used as a convenient small-animal model to study EPEC virulence (31–33). RegA functions as a global transcriptional regulator in *C. rodentium* by activating the transcription of *grlA* (global regulator of LEE activator) and a number of genes encoding colonization factors, such as fimbriae (*kfc*) and an autotransporter adhesin (*adcA*), while repressing transcription of a series of housekeeping genes (28, 34). Importantly, RegA requires a gut-associated environmental signal, bicarbonate, to exert its effect on gene expression (35). In this study, we characterized an AraC-like transcriptional regulator, RegR, from REPEC strain E22 (O103:H2). Our *in vitro* and *in vivo* analyses demonstrate that RegR, which controls the expression of several putative virulence proteins, is an important virulence determinant of REPEC.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, bacteria were grown at 37°C in Luria-Bertani broth (LB) or on Luria agar (LA) plates supplemented with the appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; and trimethoprim, 40 μg/ml. Primers used in this study are listed in Table 2.

Construction of EPEC E22 Δ*regR*, E22 Δ2 and E22 Δ*sefA*::*kan* nonpolar mutant strains. The λ Red recombinase system was utilized to construct nonpolar deletion mutants of REPEC E22 (36). Phusion high-fidelity DNA polymerase (Finnzymes), which generates blunt-ended fragments, and primer pairs RegR-F/RegR-kanR and RegR-R/RegR-kanF were used to amplify the DNA sequences flanking the *regR* gene from the E22 chromosome, and primers pKD4F and pKD4R were used to amplify the kanamycin resistance (*kan*) gene, bordered by Flp recombination target (FRT) sites, from plasmid pKD4. The products of these three PCRs (100 ng each) served as templates in overlapping-extension PCR (37) using Platinum *Taq* DNA polymerase (Invitrogen)

TABLE 2 Primers used in this study

Primer	Sequence (5′ → 3′)
RegR-F	GGGACCTGTTAAATGCATATGTAC
RegR-kanR	GAAGCAGCTCCAGCCTACACAGCATTTTGACTATT CATACAGTCACC
RegR-kanF	CTAAGGAGGATATTCATATGGCTTGAAAGTGAAAA GGAAATCATAATATG
RegR-R	CAGAACTCAATGGCGGCGATGTC
RegR-F1	GGCCGAACCTCTGTATATGAG
RegR-R1	CGTATCACCTGACACACTTCTC
RalR-F	GAGAAACGCAGAGCAGCCTAAA
RalR-kanR	GAAGCAGCTCCAGCCTACACAGCCCCTTCGCATTAGCC AAT
RalR-kanF	CTAAGGAGGATATTCATATGGGTCTGGGGTTGAATC AGTGCCT
RalR-R	CATTATCTGGCCATTTGCATCGTC
RalR-F1	ACGCAGAGCAGCCTAAAACG
RalR-R1	CAAGCCCCCCTCAAAAAT
RegR-BamHIF	CGGGATCCCGAGTTTACAGAGTGGATATCC
RegR-SalIR	CGGTCGACCAGTAAGAACTGCTTCTGCC
RegR-BamHIF	GGATCCATGAATAGTCAAAATGCTTTAAGTAATATG AGGCTTG
RegR-HindIIIR	AAGCTTTTATGATTTCCTTTTCACTTTCAAGGAAAACTGT
SefA-PE-F	CTCCCTAAATCCACTCATTCTTACTTA
SefA-PE-R	GCTGTTTCAGCGGCATTTGC
SefA_RegRB1_F	CCAATCAAAATAACAAAAAAACGCGCGTATACAAAACAA
SefA_RegRB1_R	TTGTTTTGTATACGCGCGTTTTTTTGTTATTTTGATTGG
SefA_RegRB2_F	CAAAACCAATAAAACACTCCACGCGCGTAAAAAAATATG
SefA_RegRB2_R	CATATTTTTTTACGCGCGTGGAGTGTTTTATTGGTTTTG
$SefAP_1$-HindIII-F	GCCGAAGCTTTAGGCACTGACAATACGGCTGCAAACTT
SefAP-EcoRI-R	GCCGGAATTCTGGAGCCTGAACAGTAGCAACATCACC TAT
$SefAFP_2$-HindIII-F	GCCGAAGCTTTAATGAAAATTCACTTAACCTAACGA TTAAATTG
SefA-F	AAGTGTATGAGGTGCGTGGTCAGC
SefA-kanR	GAAGCAGCTCCAGCCTACACACCTGAACAGTAGCAAC ATCACCTATAGC
SefA-kanF	CTAAGGAGGATATTCATATGAGCAACATTCTACATTCA ACAATATCAAGATTAA
SefA-R	TGGGGCTTTCGTCTTTTTATCG
SefA-F1	CATCCATCCACAACTGGTTAG
SefA-R1	CCAGACCGAAATCAGTTGAC
SefA-EMSA-F	TAGGCACTGACAATACGGCTGCAAACTT
SefA-EMSA-R	TTACAATGAAATAAAAACAATATAATTCACGCA
SefA-EMSAc-F	CTTATTCTTGCTTTGATGACGTGTG
SefA-EMSAc-R	ATCTCCCGTAACATAGACCCCTGC
pKD4F	TGTGTAGGCTGGAGCTGCTTC
pKD4R	CATATGAATATCCTCCTTAG
pKD4seqF	TGACGAGTTCTTCTGAGCGGGAC
pKD4seqR	TCTAGCTATCGCCATGTAAGCC

and primers RegR-F and RegR-R, to generate a DNA fragment carrying a *kan* gene flanked by ~500-bp regions up- and downstream of *regR*. This DNA fragment was cloned into pGEM-T Easy (Promega), the recombinant plasmid (pYS1) was introduced into *E. coli* K-12 TOP10 cells (Invitrogen), and the insert was then confirmed by sequencing. pYS1 was used as a template in a PCR with primer pair RegR-F/RegR-R to amplify the linear allelic replacement DNA fragment, which was introduced into strain E22 expressing λ Red recombinase from plasmid pKD46. The resultant E22 Δ*regR*::*kan* mutant was confirmed by PCR using primer pairs in which one primer flanked the targeted region and the other primed within the *kan* gene (RegR-F1/pKD4seqR) and (RegR-R1/pKD4seqF). The *kan* gene was then excised from the E22 Δ*regR*::*kan* strain by using Flp recombinase encoded on plasmid pFT-A, generating a marker-free deletion mutant, E22 Δ*regR*, which was confirmed by PCR and sequencing using primers RegR-F1 and RegR-R1.

The same general protocol was employed to delete the EcE22_5293 gene from E22 Δ*regR* using the EcE22_5293-specific primer pairs RalR-F/RalR-kanR and RalR-R/RalR-kanF. The resultant 2.2-kb fragment was cloned into pGEM-T Easy to generate pYS21. The ΔEcE22_5293::*kan* mutation was confirmed by PCR using primer pairs RalR-F1/pKD4seqR and RalR-R1/pKD4seqF, and the PCR products were sequenced by using primers RalR-F1 and RalR-R1. The resultant double mutant strain, E22 Δ*regR* ΔEcE22_5293::*kan*, here referred to as E22 Δ2, was used as the parent strain in microarray analysis.

To generate the E22 Δ*sefA*::*kan* mutant, primer pairs SefA-F/SefA-kanR and SefA-R/SefA-KanF were used. The resultant 2.5-kb fragment was cloned into pGEM-T Easy to obtain pYS17. This construct was used as a template in a PCR with primer pair SefA-F/SefA-R to amplify the linear allelic replacement DNA fragment, which was introduced into strain E22 expressing λ Red recombinase from plasmid pKD46. The E22 Δ*sefA*::*kan* mutant was confirmed by PCR using primer pairs SefA-F1/pKD4seqR and SefA-R1/pKD4seqF, and the PCR products were sequenced using primers SefA-F1 and SefA-R1.

Construction of a *trans*-complementing plasmid, pYS3. For *trans* complementation of the *regR* mutants of E22, a 950-bp fragment containing *regR* was amplified from genomic DNA of EPEC strain E22 using primer pair RegR-BamHIF/RegR-SalIR. The resultant fragment was cloned into pCR2.1-TOPO to yield pCR2.1-TOPO+*regR* (pYS2), which was then confirmed by sequencing. The fragment was excised by BamHI/SalI digestion and ligated into BamHI/SalI of pACYC184 to generate the plasmid pYS3 (pACYC184-*regR*).

Antisense EPEC E22 microarrays. An antisense oligonucleotide microarray was custom designed using the Agilent eArray platform (Agilent Technologies). The array contained representative sequences from the open reading frames (ORFs), representing all gene predictions, for the *E. coli* E22 genome available at NCBI (GenBank accession number AAJV00000000). Each ORF was represented by at least three different oligonucleotides.

RNA isolation and labeling. *E. coli* strains E22 Δ2(pYS3) and E22 Δ2(pACYC184) were cultivated in LB overnight at 37°C. Quadruplicate cultures of a 1:100 dilution were grown to an optical density at 600 nm (OD_{600}) of 0.85. One volume of cells was incubated with two volumes of RNAprotect solution and processed according to the manufacturer's instructions (Qiagen). Cell lysis and RNA preparation were carried out by using the FastRNA Pro Blue kit (Qbiogene Inc.). After a 10-min treatment with RNase-free DNase I (Qiagen), the RNA was further purified utilizing the RNeasy MiniElute kit (Qiagen). A total of 5 μg of RNA was labeled either with Cy5-ULS or Cy3-ULS as described in the Kreatech ULS labeling manual (Kreatech Diagnostics). RNA quality and degree of labeling were determined with an Agilent 2100 bioanalyzer and an ND-1000 spectrophotometer (NanoDrop Technologies). A dye swap was performed for two of the four cultures to minimize the effects of any labeling artifacts.

Fragmentation, microarray hybridization, scanning, and analysis. Fragmentation, hybridization, and scanning were performed at the Australian Genome Research Facility Ltd. (AGRF), Melbourne, Australia. Normalization and data analysis were performed using the limma package in Bioconductor (38–40). Genes were considered differentially expressed if they showed an average change of ≥2-fold with an adjusted *P* value of ≤0.05.

Primer extension. Primer extension was performed as described previously (41). Briefly, total cellular RNA was purified from *E. coli* MC4100(pYS9, pYS3) and MC4100(pMU2385, pYS3). Cells were grown to mid-log phase (OD_{600} = 0.8), and RNA was isolated by using the FastRNA Pro kit. Primer SefA-PE-R was labeled at its 5′ end with ^{32}P by T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled primer was coprecipitated with 5 μg of total RNA. Hybridization was carried out at 45°C for 15 min in 10 μl of Tris-EDTA (TE) buffer containing 150 mM KCl. Primer extension reactions were started by the addition of 24 μl of extension solution (20 mM Tris-HCl [pH 8.4], 10 mM $MgCl_2$, 10 mM dithiothreitol [DTT], 2 mM deoxynucleoside triphosphates [dNTPs], 1 U/ml avian myeloblastosis virus [AMV] reverse transcriptase) and were carried out at 42°C for 60 min. Samples were then precipitated and analyzed on a sequencing gel. A GA ladder was made by using the method of Maxam and Gilbert (42) to sequence a *sefA* fragment that was generated by PCR using primers SefA-PE-F and ^{32}P-SefA-PE-R.

Construction of *lacZ* fusion plasmids. The *sefA-lacZ* transcriptional fusions were constructed by PCR amplification of the regulatory region of *sefA* by using primer pairs $SefAP_1$-HindIII-F/SefAP-EcoRI-R and $SefAP_2$-HindIII-F/SefAP-EcoRI-R. The PCR fragments were cloned into

pGEM-T Easy to generate plasmids pYS4 and pYS5, and these fragments were sequenced. The fragments were then excised from the pGEM-T Easy derivatives by HindIII/EcoRI digestion and cloned into the same sites of the single-copy transcriptional fusion vector pMU2385 to create the *sefA-lacZ* fusions *sefA-lacZ*$_1$ (pYS9) and *sefA-lacZ*$_2$ (pYS10).

sefA-lacZ$_3$ (pYS11) carrying a RegR-Box1 mutation was generated by using a multiple-step overlapping PCR method. The first PCR involved the use of primer pairs SefAP$_1$-HindIII-F/SefA_RegRB1_R and SefA_RegRB1_F/SefAP-EcoRI-R, template *sefA-lacZ*$_1$ (pYS9), and Phusion high-fidelity DNA polymerase. The two resulting PCR fragments (100 ng each) were mixed together with Platinum *Taq* DNA polymerase master mix. After seven cycles of extension, primers SefAP$_1$-HindIII-F and SefAP-EcoRI-R were added to the mixture. The sample was then subjected to 30 cycles of PCR, which resulted in the formation of a 700-bp DNA fragment. This fragment was cloned into pGEM-T Easy to generate pYS7, and the mutation was confirmed by sequencing. The mutant *sefA* fragment was excised by HindIII/EcoRI digestion and cloned into the same sites of the single-copy transcriptional fusion vector pMU2385 to create the *sefA-lacZ*$_3$ (pYS11). *sefA-lacZ*$_4$ (pYS12) was constructed in the same manner, except that primers pairs SefAP$_1$-HindIII-F/SefA_RegRB2_R and SefA_RegRB2_F/SefAP-EcoRI-R were used in the Phusion PCR and the resultant fragment generated from the Platinum *Taq* PCR was cloned into pGEM-T Easy to generate pYS8.

β-Galactosidase assay. Bacteria were grown to mid-log phase (OD_{600} ~ 0.6). β-Galactosidase activity was assayed as described by Miller (43), and the specific activity was expressed in Miller units (MU). The data shown are the results of at least three independent assays.

Expression and purification of MBP::RegR. The coding sequence of *regR* was amplified from E22 genomic DNA by using primer pair RegR-BamHIF/RegR-HindIIIR and then cloned into pGEM-T Easy to obtain plasmid pYS13. *regR* was excised by BamHI/HindIII digestion and inserted into expression vector pMAL-c2 (New England BioLabs) for N-terminal fusion to *malE*. The resulting vector, pYS14, was transformed into *E. coli* strain BL21(DE3). Overnight cultures of transformants were diluted 1:100 into fresh LB and grown at 30°C with shaking at 200 oscillations per min to an OD_{600} of 0.9. Induction of gene expression was carried out for 19 h at 16°C by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Afterwards, bacterial cells were harvested (15 min, 3,000 × *g*, 4°C) and disrupted by the addition of lysozyme (100 μg/ml) and subsequent sonication in column buffer (20 mM Tris-HCl [pH 7.4], 1 M NaCl, 1 mM EDTA). Purification of RegR was achieved through binding of the fusion protein to an amylose resin as recommended by the manufacturer (New England BioLabs). All steps were carried out at 4°C. The concentration and purity of eluted maltose-binding protein (MBP)::RegR were determined by using an ND-1000 spectrophotometer and by SDS-PAGE of the fusion protein.

EMSA. Labeling of DNA fragments with ^{32}P was performed as follows. Primers SefA-EMSA-F and SefA-EMSAc-F were labeled at their 5′ ends by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The DNA fragments to be analyzed for RegR binding were generated by PCR using primer pairs SefA-EMSA-F/SefA-EMSA-R (for fragment *sefA*$_{prom}$) and SefA-EMSAc-F/SefA-EMSAc-R (for fragment *sefA*$_{control}$) from plasmids carrying either the entire promoter region of *sefA* (pYS15, for *sefA*$_{prom}$) or part of the downstream coding sequences for the control experiment (pYS16, for *sefA*$_{control}$). Electrophoretic mobility shift assay (EMSA) was carried out as published previously (35). Briefly, each fragment was incubated with various amounts of purified MBP::RegR protein at 25°C for 30 min in binding buffer [10 mM Tris-HCl (pH 7.4), 100 mM KCl, 0.1 mM DTT, 0.01% (vol/vol) Triton X-100, 1 mM EDTA, and 100 μg/ml bovine serum albumin (BSA), 5 ng/μl poly(dI-dC), 10% (vol/vol) glycerol]. DNA and DNA-protein complexes were then separated on 5% native polyacrylamide gels (37.5:1) for approximately 12 h at 10 V/cm and 4°C.

Preparation of surface-associated proteins and immunoblotting. Bacterial strains were grown overnight in 10 ml of LB with and without 45 mM sodium bicarbonate at 37°C. Cells were harvested by centrifugation at 3,000 × *g* for 10 min, resuspended in 160 μl of phosphate-buffered saline (PBS) (pH 7.4), vortexed at high speed for 1 min, and subsequently incubated at 60°C for 30 min with intermittent vortexing. The samples were then pelleted by centrifugation at 3,000 × *g* for 10 min, and the supernatant was transferred to a fresh tube, where it was mixed with NuPAGE lithium dodecyl sulfate (LDS) sample reducing buffer and boiled at 100°C for 5 min. The samples were separated by SDS-PAGE using 4 to 12% Bis-Tris NuPAGE gels (Invitrogen), and the proteins were stained with Coomassie brilliant blue R-250. For immunoblotting, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 1% casein in PBS. The membrane was probed with 1-in-600 dilution of mouse anti-SefA serum followed by 1-in-10,000 goat anti-mouse horseradish peroxidase (HRP)-labeled secondary antibody (Bio-Rad). Immunopositive bands were developed with tetramethylbenzidine (TMB) substrate (KPL).

Preparation of secreted proteins. Bacterial strains were grown in Dulbecco modified Eagle medium (DMEM) (Invitrogen). Cells were harvested by centrifugation (5,000 × *g*, 10 min), and the supernatant, containing the extracellular proteins, was passed through a 0.20-μm-pore-size filter (Sartorius). Proteins in the supernatants were precipitated with 10% (vol/vol) trichloroacetic acid on ice for 1 h, washed in 25% (vol/vol) acetone, separated by SDS-PAGE using 4 to 12% Bis-Tris NuPAGE gels, and stained with Coomassie brilliant blue R250.

Protein identification. Tandem mass spectrometry was performed at the Walter and Eliza Hall Institute for Medical Research, Proteomics Laboratory, Melbourne, Australia.

Immunoelectron microscopy. Immune serum was obtained by immunizing mice with purified SefA protein obtained from homogenized gel slices via three subcutaneous injections at 2-week intervals. Specific anti-SefA serum was obtained by absorbing the immune sera with strain E22 Δ*sefA*::*kan* bacteria. The absorbed serum was then diluted for use in Western blotting or immunogold labeling as required.

Strains were grown overnight at 37°C on LA supplemented with 45 mM bicarbonate and appropriate antibiotics. Colonies were suspended in PBS using a swab, and 10 μl of the suspension was applied onto 200-mesh carbon-coated copper grids, left for 5 min, and then negatively stained with 1% (wt/vol) ammonium molybdate for 10 s. For immunoelectron microscopy, the bacteria were reacted for 15 min with mouse anti-SefA serum (diluted 1:10 in PBS), followed by a 15-min incubation with goat anti-mouse IgG(Fc) conjugated to 10-nm gold particles (diluted 1:20 in PBS containing 1% bovine serum albumin) (Amersham). A Philips CM120 BioTwin transmission electron microscope operated at a voltage of 120 kV was used to examine the grids.

Infection of rabbits. For *in vivo* assays of virulence, 4- to 5-week-old New Zealand White rabbits were inoculated with the wild-type REPEC strain, E22, or an isogenic mutant. Rabbits were weighed daily and examined for clinical signs of illness, including weight loss and evidence of diarrhea, as described previously (44). Fecal shedding of the infecting strains was determined by culture of rectal swabs on MacConkey agar supplemented with rifampin (50 μg/ml) to distinguish them from the rabbits' microbiota. Animals were euthanized if they lost more than 15% of their body weight or demonstrated severe diarrhea.

Statistical analysis. All analyses of quantitative data were performed by using Student's *t* test. A two-tailed *P* value of <0.05 was taken to indicate statistical significance.

Microarray data accession number. The microarray data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE41093.

RESULTS

Identification of a putative virulence AraC-like regulator and its gene targets in *E. coli* E22. We screened the entire genome of REPEC strain E22 (GenBank accession number AAJV02000000) for open reading frames (ORFs) homologous to the *C. rodentium* RegA protein by performing a BLASTp search (National Center

TABLE 3 Summary of genes that were strongly differentially regulated by RegR as identified by microarray analysis[a]

ORF	Gene	Predicted function	Avg fold regulation[b]	Adjusted *P* value < 0.05[c]
EcE22_2922	*glcA*	Glycolate permease	−6.3	1.77E−10
EcE22_2924	*glcB*	Malate synthase G	−11.1	1.05E−10
EcE22_2925	*glcG*	Glyoxylate	−10.0	1.05E−10
EcE22_2926	*glcF*	Glycolate oxidase, iron-sulfur subunit	−8.9	1.57E−08
EcE22_2927	*glcE*	Glycolate oxidase, subunit	−10.8	8.69E−10
EcE22_2928	*glcD*	Glycolate oxidase, subunit	−12.9	8.51E−10
EcE22_5321	*espC*	Putative serine protease	22.6	1.05E−10
EcE22_5333	*sefA*	Fimbrin subunit	390.3	1.74E−07
EcE22_5334	*sefB*	Fimbrial chaperone	39.4	1.53E−11
EcE22_5335	*sefC*	Fimbrial usher	35.3	2.90E−11
EcE22_5336	*sefD*	Fimbrin subunit	49.3	4.23E−10
EcE22_5340	*adcA*/*tsh*	Putative autotransporter adhesin	17.3	5.76E−11

[a] See Table S1 in the supplemental material for the complete list of ORFs regulated by RegR.
[b] The negative and positive values represent the degrees of down- and upregulation, respectively.
[c] Only the most significant *P* values are shown.

for Biotechnology Information [http://www.ncbi.nlm.nih.gov/]). A protein encoded by ORF EcE22_5323 which exhibited 71% identity with the RegA protein was identified, and this ORF was designated *regR*. Interestingly, *regR* is located on an ~62-kb, E22-specific genetic island (GenBank accession number AAJV02000028.1), which also includes an ORF, EcE22_5293, that encodes a protein homologous to the LdaA regulator (48% identity, 68% similarity) responsible for the expression of an afimbrial adhesin in EPEC (45).

To identify those genes whose expression is controlled by RegR, we carried out a microarray analysis. Because RegR and the LdaA-like protein share significant homology within their HTH DNA-binding motifs and to avoid any complication arising from possible redundancy and interplay between the two proteins in the microarray assay, we knocked out both loci in the E22 genome to generate strain E22 Δ2 (E22 Δ*regR* ΔEcE22_5293). This strain was used as the host strain in the microarray assay.

The microarray data showed that 270 genes represented by 663 probes were significantly differentially expressed (adjusted *P* value of <0.05) (see Table S1 in the supplemental material). However, only six of these genes, which are located adjacent to the *regR* locus, were strongly upregulated (>15-fold) by the RegR protein. Four of these genes (*sefABCD*) appear to form one operon, encoding proteins with similarity, ranging from 59 to 74%, to the fimbrins chaperone and usher of SEF14 fimbriae of *Salmonella enterica* serovar Enteritidis (Table 3) (46). Strikingly, transcription of *sefA*, the first gene of the *sef* operon, was activated 390-fold by RegR.

The other two genes significantly upregulated by RegR encode a homolog of the autotransporters AdcA of *C. rodentium* (90% similarity) and Tsh of avian-pathogenic *E. coli* (APEC) (63% similarity) and a homolog of the serine protease EspC of enteropathogenic *E. coli* (75% similarity).

In addition, RegR downregulated the expression of six genes (>6-fold) (Table 3), including members of the glycolate and glyoxylate degradation pathway (*glcDEFGB*) and its associated glycolate permease gene, *glcA*.

Mapping of the *sefA* promoter. To characterize the molecular interaction of RegR with its gene targets, we investigated the *sefA* promoter, as *sefA* is the most upregulated gene target identified for RegR (Table 3). To identify the start site of transcription of *sefA* we performed a primer extension experiment as follows. A ^{32}P-labeled primer (Sef14-PE-R) was hybridized with total RNA isolated from strain MC4100 carrying a pMU2385 derivative containing the *sefA-lacZ*$_1$ transcriptional fusion (see below) and pACYC184+*regR* (pYS3). Only one major extension product was seen in the sample, indicating the presence of a single promoter for the *sef* operon (Fig. 1A). The start site of transcription was mapped to a guanine residue located 53 nucleotides (nt) upstream of the start codon for SefA (Fig. 1B). Based on this start site, a putative *sefA* promoter was identified (Fig. 1B). Its putative core elements included an extended −10 region (TGtTATAAA), a −35 region (TTAACC), and a spacer of 15 bp (Fig. 1B).

Analysis of RegR-mediated activation of the *sefA* promoter. To examine RegR-mediated activation of *sefA* expression, we carried out transcriptional analyses using a *lacZ* reporter system. Two promoter-*lacZ* transcriptional fusions were constructed by cloning DNA fragments, encompassing positions −309 to +155 and −51 to +155 (relative to the transcriptional start site of *sefA*), in front of the *lacZ* structural gene of the single-copy vector pMU2385. The resulting plasmids, *sefA-lacZ*$_1$ (pYS9) and *sefA-lacZ*$_2$ (pYS10), were each transformed into *E. coli* MC4100 RegR$^+$ [MC4100(pYS3)] and RegR$^-$ [MC4100(pACYC184)]. Because the RegA protein from *C. rodentium* (a homolog of RegR) requires sodium bicarbonate as a cofactor to exert maximal regulatory effects (35), we also tested whether this chemical is involved in RegR-mediated activation of *sefA* transcription. These strains were grown to mid-log phase in the absence or presence of 45 mM bicarbonate, and promoter activity was measured by assaying for β-galactosidase activity.

Although extremely low levels of β-galactosidase activities (11 to 12 MU) were detected for the *sefA-lacZ*$_1$ construct in the RegR$^-$ background, high levels of expression were observed in the RegR$^+$ background, where the *sefA* promoter activity increased from around 230 times in the absence of bicarbonate to around 700 times in the presence of bicarbonate, respectively (Fig. 2A). These data clearly demonstrate that RegR strongly activates *sefA* transcription and that bicarbonate positively influences the RegR-mediated effect.

For *sefA-lacZ*$_2$, no difference of transcriptional activity was seen in the RegR$^-$ and RegR$^+$ backgrounds with or without bicarbonate (Fig. 2A). Moreover, compared to that of *sefA-lacZ*$_1$, the basal level of promoter activity of *sefA-lacZ*$_2$ in the RegR$^-$ background increased approximately 100 times (Fig. 2A). The lack of

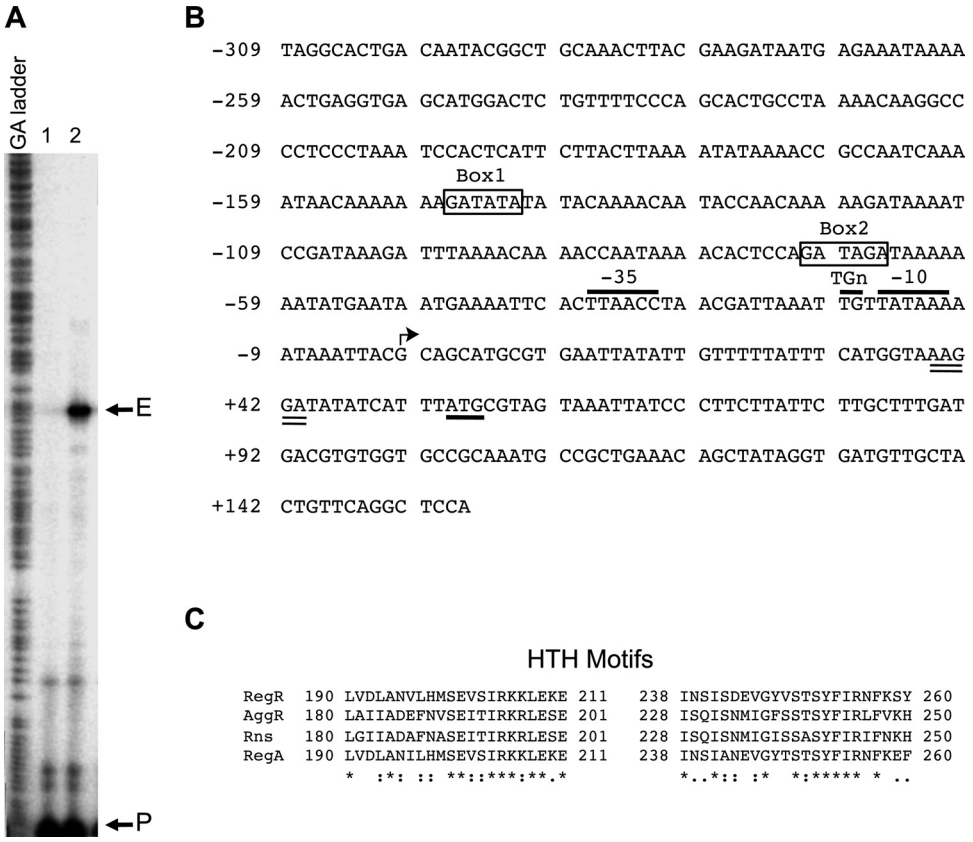

FIG 1 The *sefA* regulatory region and the double helix-turn-helix DNA-binding motifs of RegR and its homologs. (A) The start site of transcription of the *sefA* promoter was mapped by primer extension using RNA isolated from *E. coli* MC4100 strains containing pACYC184-*regR* (pYS3) with either pMU2385 (control) or *sefA-lacZ*$_1$ (pYS9). Lane 1, control experiment using RNA from *E. coli* MC4100 containing pACYC184-*regR* and pMU2385. Lane 2, experiment using RNA from *E. coli* MC4100 containing pACYC184-*regR* and *sefA-lacZ*$_1$. The positions corresponding to the primer and the extension product are marked with P and E, respectively. (B) Nucleotide sequence of the *sefA* regulatory region. The numbering on the left of the sequence is relative to the transcriptional start site of *sefA*, which is indicated by an angled arrow. The putative −10 region, TGn motif, and −35 region are indicated and overlined. The putative start codon and ribosome-binding site are underlined and double underlined, respectively. The putative RegR-binding sites, Box1 and Box2, are boxed and labeled. (C) Alignment of helix-turn-helix (HTH) motifs of RegR, AggR, Rns, and RegA was done using the PS01124 profile defined in PROSITE (65). The numbers flanking the sequences indicate the positions of amino acid residues of the regulatory proteins. Identical and conserved amino acids are indicated with asterisks and colons, respectively.

RegR activation and the strong increase in basal-level transcription of this construct could be attributed to the absence of *cis*-acting elements required for the negative and positive controls of the *sefA* promoter.

The transcriptional analysis of the *sefA-lacZ*$_1$ and *sefA-lacZ*$_2$ constructs was also carried out in E22 using RegR$^-$ [Δ*regR*(pACYC834)] and RegR$^+$ [Δ*regR*(pYS3)] strains. As shown in Fig. 2B, the transcriptional activity of *sefA-lacZ*$_1$ was activated 79- and 356-fold by RegR in the absence and presence of sodium bicarbonate, respectively. In contrast, no significant effect of RegR on transcription of *sefA-lacZ*$_2$ was seen. These results are in agreement with those observed in the *E. coli* MC4100 backgrounds, indicating that only *sefA-lacZ*$_1$ carries an operator sequence responsible for RegR-mediated activation.

Identification of the RegR-binding sites. The helix-turn-helix DNA-binding motif of RegR is highly homologous to that of RegA from *C. rodentium* (86.6% identity and 100% similarity) (Fig. 1C), suggesting that the two regulatory proteins may recognize similar DNA-binding sites. Indeed, a search using the RegA consensus sequence GATATA (47) identified two possible RegR-binding sites, GATATA (Box1) and GATAGA (Box2), which are centered at −144.5 and −68.5, respectively, relative to the start site of *sefA* transcription (Fig. 1B). To test whether these sequences are involved in the positive control of *sefA* by RegR, we made changes to bases within the two sites, from GATATA to CGCGCG and from GATAGA to CGCGCG (Fig. 1B). The two constructs, *sefA-lacZ*$_3$ (pYS11) and *sefA-lacZ*$_4$ (pYS12), containing the mutant *sefA* fragments were assessed for their ability to be activated by RegR. The data in Fig. 2C showed that while neither mutation had any effect on the basal levels of *sefA* promoter activities in the RegR$^-$ background, the mutations in Box1 and Box2 caused a significant and marked reduction in *sefA* expression in the RegR$^+$ background (Fig. 2C). These results indicate that although both boxes are required for maximal levels of expression, Box2 plays a more significant role than Box1 in RegR-mediated activation of the *sefA* promoter.

RegR binds directly to the *sefA* promoter region. To provide further evidence that RegR interacts directly with the *sefA* regulatory region, we performed an electrophoretic mobility shift assay (EMSA). For this assay we used a purified fusion protein (MBP::RegR), because RegR, like many other AraC-like proteins, is insoluble in water. A 348-bp *sefA* promoter fragment (*sefA*$_{prom}$)

		Luria broth			Luria broth + $NaHCO_3$		
		RegR⁻	RegR⁺	Fold act.	RegR⁻	RegR⁺	Fold act.
A	MC4100 *lacZ*	0.2	0.2		0.2	0.2	
	sefA-lacZ$_1$ −309 +155	12	2793	233	11	7649	695
	sefA-lacZ$_2$ −51 +155	1388	1319	1.0	1280	1245	1.0
B	E22 *lacZ*	2.1	2.3		2.2	2.4	
	sefA-lacZ$_1$ −309 +155	21	1661	79	36	12803	356
	sefA-lacZ$_2$ −51 +155	1560	1443	1.0	1774	2072	1.4
C	MC4100 *lacZ*	0.2	0.2		0.2	0.2	
	sefA-lacZ$_3$ −309 +155 (−147 GATATA −142)	11	497	45	12	2526	210
	sefA-lacZ$_4$ −309 +155 (−71 GATAGA −66)	10	79	7.9	13	913	70

FIG 2 Expression of β-galactosidase by various *sefA-lacZ* fusions in both *E. coli* and E22 strains. The *E. coli* and E22 derivatives were grown in LB in the absence or presence of sodium bicarbonate (45 mM). The numbering of the *sefA* fragments is relative to the start site of transcription of *sefA*. The promoter activities of the various constructs are shown as specific activities of β-galactosidase (Miller units), which are the mean values from three independent assays, with variation of less than 15%. Fold activation (Fold act.) is the specific activity of β-galactosidase of the RegR$^+$ strain divided by that of the RegR$^-$ strain. (A) The analysis of *sefA-lacZ*$_1$ and *sefA-lacZ*$_2$ was performed in *E. coli* RegR$^-$ [MC4100(pACYC184)] and RegR$^+$ [MC4100(pYS3)] strains. (B) The analysis of *sefA-lacZ*$_1$ and *sefA-lacZ*$_2$ was performed in E22 RegR$^-$ [Δ*regR*(pACYC184)] and RegR$^+$ [Δ*regR*(pYS3)] strains. (C) The analysis of *sefA-lacZ*$_3$ and *sefA-lacZ*$_4$ was performed in *E. coli* RegR$^-$ [MC4100(pACYC184)] and RegR$^+$ [MC4100(pYS3)] strains.

which spanned positions −309 to +38, relative to the start site of transcription, was end-labeled with ^{32}P and incubated with various amounts of MBP::RegR. In the absence of MBP::RegR, the $sefA_{prom}$ fragment migrated to the bottom of the gel during electrophoresis to form a free DNA band (Fig. 3A, lane 1). However, after the addition of increasing amounts of MBP::RegR to the reaction mixture, this band gradually shifted (Fig. 3A, lanes 2 to 7). A major retarded band (C), representing a protein-DNA com-

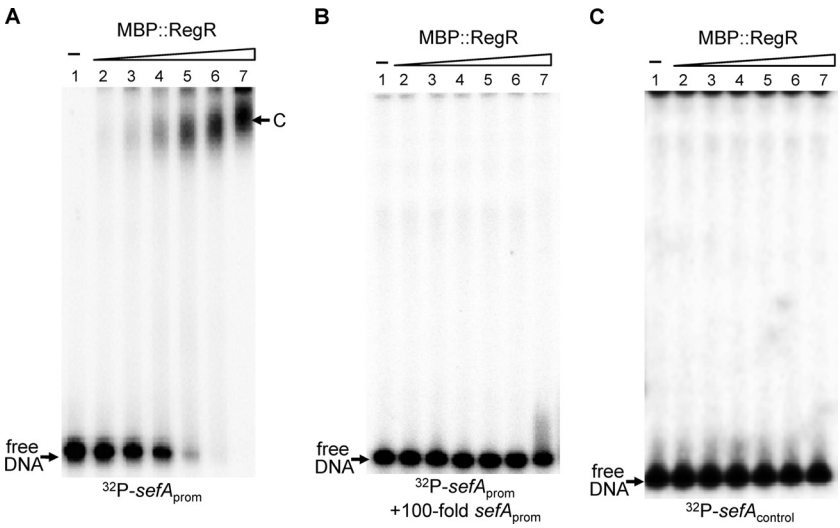

FIG 3 Electrophoretic mobility shift analysis of the binding of the recombinant RegR protein to the *sefA* regulatory region. The ^{32}P-labeled PCR fragments containing the *sefA* regulatory region, $sefA_{prom}$ (A and B), and the coding region, $sefA_{control}$ (C), were each mixed with 0, 15.5, 31.3, 62.5, 125, 250, and 500 nM MBP::RegR protein (lanes 1 to 7, respectively) in the presence of 45 mM sodium bicarbonate. A 100-fold molar excess of the unlabeled $sefA_{prom}$ fragment was added into the reaction mixture to demonstrate RegR specificity (B). Following incubation at 30°C for 20 min, the samples were analyzed on native polyacrylamide gels. The unbound DNA (free DNA) and protein-DNA complexes (band C) are marked.

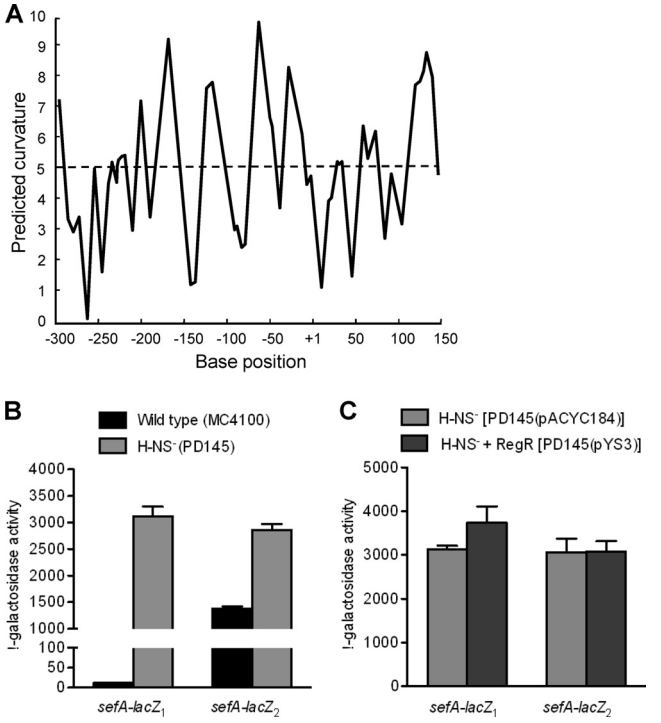

FIG 4 Analysis of H-NS-mediated repression of the *sefA* promoter. (A) *In silico* analysis of intrinsic curvature of the *sefA* regulatory region, using the bend.it program (http://hydra.icgeb.trieste.it/dna/index.php). The regions with >5 degrees per helical turn of DNA (dashed line) represent curved sequences. The base position is relative to the transcriptional start site of *sefA*. (B) Effects of H-NS on the expression of *sefA*. *E. coli* strains MC4100 (H-NS$^+$) and PD145 (H-NS$^-$) which contained either *sefA-lacZ*$_1$ (−309 to +155) or *sefA-lacZ*$_2$ (−51 to +155) were assayed for β-galactosidase activity. (C) Effects of RegR on the expression of *sefA* in the H-NS$^-$ background. *E. coli* strains PD145(pACYC184) (RegR$^-$) and PD145(pYS3) (RegR$^+$) which contained either *sefA-lacZ*$_1$ (−309 to +155) or *sefA-lacZ*$_2$ (−51 to +155) were assayed for β-galactosidase activity. The β-galactosidase activities (Miller units) shown are the means (± standard deviations [SD]) of results from three independent experiments.

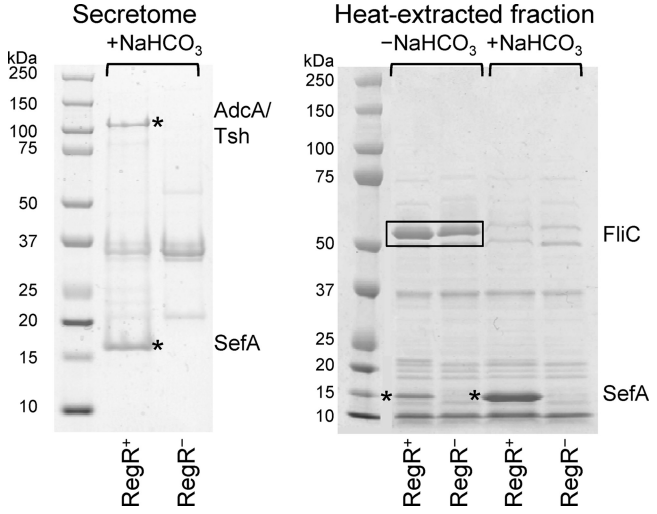

FIG 5 Effects of RegR on the expression of secreted and surface-associated proteins. RegR$^+$ [E22 Δ*regR*(pYS3)] and RegR$^-$ (E22 Δ*regR*) strains of E22 were grown in DMEM (for secretome analysis) or in Luria broth in the absence or presence of 45 mM bicarbonate (for analysis of heat-extracted fractions). Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R250. Four protein bands (two from the secretome fractions and two from the heat-extracted fractions) which were present in the RegR$^+$ but not in the RegR$^-$ strain (indicated by asterisks) were excised and analyzed by tandem mass spectrometry. In the heat-extracted fractions, two bands which are abundant in the absence of bicarbonate (boxed) were analyzed by tandem mass spectrometry. The identities of these proteins are shown at the right of the gels.

plex, was seen when MBP::RegR was added at final concentrations of between 65 and 500 nM (Fig. 3A, lanes 4 to 7). However, addition of excess amounts of cold *sefA* promoter fragment (*sefA*$_{prom}$) into the reaction mixes outcompeted the binding of RegR to the labeled probe (Fig. 3B). An additional control experiment using a ^{32}P-labeled DNA fragment (*sefA*$_{control}$) covering a sequence within the coding region of the *sefA* gene (between positions +75 and +302, relative to the start site of transcription) was also carried out. As shown in Fig. 3C, MBP::RegR failed to bind to this DNA fragment, further confirming the binding specificity of RegR for the *sefA* regulatory region.

Effect of H-NS on expression of the *sefA* promoter. As shown in the previous section, the transcription of the *sefA* promoter is subject to negative regulation by an unknown protein. As the DNA sequence surrounding the *sefA* promoter region is highly AT rich and is predicted by the bend.it program (http://hydra.icgeb.trieste.it/dna/index.php) to be highly curved (Fig. 4A), we hypothesized that H-NS is responsible for the repression. To test this, *sefA-lacZ*$_1$ and *sefA-lacZ*$_2$, were analyzed in strains MC4100 (wild type) and PD145 (*hns*). Relative to the promoter activities in MC4100, both constructs showed enhanced *sefA* expression in PD145 (*hns*) (Fig. 4B), confirming the involvement of H-NS in the negative regulation of *sefA* expression. Furthermore, the fact that deletion of the upstream sequence in the *sefA-lacZ*$_2$ construct resulted in a large increase in promoter activity in *E. coli* MC4100 is consistent with the possibility that the region involved in H-NS interaction is partially removed (Fig. 4B).

We next analyzed the effect of RegR on transcription of the *sefA-lacZ*$_1$ and *sefA-lacZ*$_2$ constructs in the H-NS$^-$ backgrounds PD145(pACYC184) and PD145(pYS3). The results in Fig. 4C showed that in the absence of H-NS, the transcriptional activities of both constructs were essentially the same either with or without the expression of RegR. This suggests that the primary function of RegR at the *sefA* promoter is to overcome H-NS-mediated repression.

Effect of RegR on the synthesis of surface-associated and secreted proteins. To identify secreted and surface-associated proteins whose synthesis is upregulated by RegR we carried out proteomic analyses of the secretomes and heat-extracted fractions of RegR$^+$ [E22 Δ*regR*(pYS3)] and RegR$^-$ (E22 Δ*regR*) E22 strains using SDS-PAGE. Bacterial strains were grown in the presence and absence of bicarbonate, as our transcriptional analysis of RegR showed that bicarbonate enhances its activity. Comparison of the protein profiles revealed two protein bands in the secretome (~135 and ~15 kDa) and two in the heat-extracted fractions (~17 kDa) of the RegR$^+$ strain that were absent from the RegR$^-$ strain (Fig. 5). Tandem mass spectrometric analysis showed that the high-molecular-weight protein band consisted of two proteins: EcE22_5321, a homolog of EspC, and EcE22_5340, a homolog of AdcA. The low-molecular-weight proteins were SefA (EcE22_5333). A surface-associated protein of ~ 52 kDa that was identified as FliC was seen only in the absence of bicarbonate,

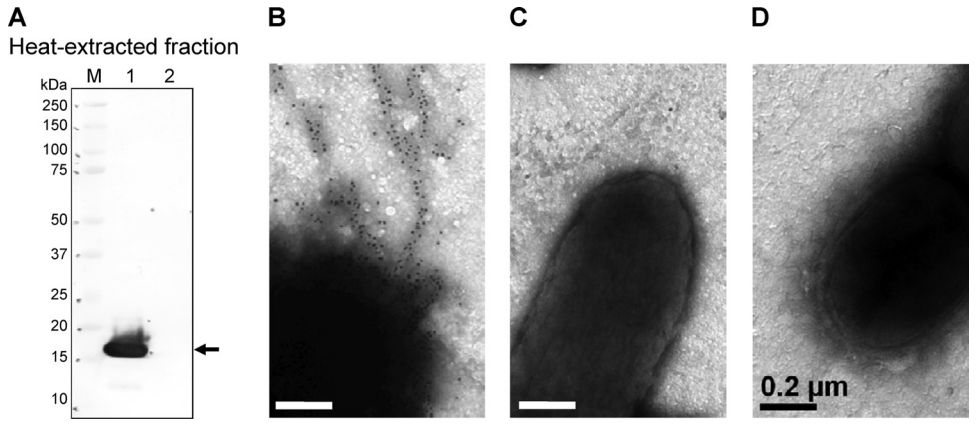

FIG 6 Localization of SefA on the cell surface. (A) Immunoblot of heat-extracted fractions from SefA$^+$ [E22(pYS3)] and SefA$^-$ [E22 Δ*sefA*::*kan*(pYS3)] strains probed with mouse anti-SefA serum. The arrow indicates a protein of approximately 17 kDa produced by the SefA$^+$ strain which was absent from the SefA$^-$ strain. Lanes: 1, E22(pYS3); 2, E22 Δ*sefA*(pYS3). M, protein molecular mass standards. (B to D) Immunoelectron microscopy of SefA$^+$ [E22(pYS3)] (B), SefA$^-$ [E22 Δ*sefA*::*kan*(pYS3)] (C), and SefA$^+$ RegR$^-$ (E22 Δ*regR*) (D) strains probed with mouse anti-SefA serum visualized with goat anti-mouse IgG(Fc) conjugated to 10-nm gold particles. Bars represent 0.2 μm.

either with or without RegR. Because the expression of this protein is not under RegR regulation, it was not studied further.

Visualization of SefA on the surface of E22 by immunoelectron microscopy. To confirm the presence of SefA on the surface of E22, we generated a *sefA* mutant, E22 Δ*sefA*::*kan*, and carried out electron microscopic examination of immunogold-labeled SefA$^+$ [E22(pYS3)], SefA$^-$ [E22 Δ*sefA*::*kan*(pYS3)] and SefA$^+$ RegR$^-$ (E22 Δ*regR*) strains. To ascertain the specificity of the antiserum, we probed heat-extracted fractions of the strains by immunoblotting using the SefA antiserum. This analysis revealed a protein of approximately 17 kDa in extracts of the *sefA*-expressing strain but not in the *sefA* mutant (Fig. 6A). When used for immunogold electron microscopy, the same antiserum revealed fimbria-like structures on the surface of the SefA$^+$ strain (Fig. 6B), but not on the SefA$^-$ strain (Fig. 6C). Fimbria-like structures on the surface of the *regR* mutant strain also were not recognized by the antiserum (Fig. 6D), demonstrating that RegR is required for SefA expression and its presence on the cell surface.

RegR is a virulence determinant of REPEC strain E22. To investigate the effect of RegR on the virulence of EPEC, we infected infant rabbits with wild-type REPEC strain E22 or its isogenic *regR* mutant, E22 Δ*regR*. Following inoculation of six rabbits with 5×10^5 CFU of wild-type E22, large numbers of REPEC organisms were shed in stools, such that $>10^5$ CFU were recovered from rectal swabs from all rabbits within 2 days of inoculation (Fig. 7A). Loss of body weight began within 48 h of infection and continued until animals were euthanized (Fig. 7B). All of the rabbits developed clinical illness, characterized by diarrhea with weight loss, requiring euthanasia. Five rabbits were euthanized on day 5 and one on day 7. These results were consistent with previous reports of infection of rabbits with E22 (14). Following inoculation with 8×10^5 CFU of the *regR* mutant strain, rabbits shed the challenge strain from the second day after inoculation to 7 days postinoculation but yielded significantly lower numbers of bacteria in rectal swabs ($P < 0.0005$ on day 2, $P < 0.005$ on days 3 and 5, and $P < 0.05$ on day 4) (Fig. 7A). One rabbit inoculated with the *regR* mutant strain began to lose weight on day 2, developed diarrhea on day 3, and was subsequently euthanized on day 4.

Body weight is another sensitive indicator of illness in the

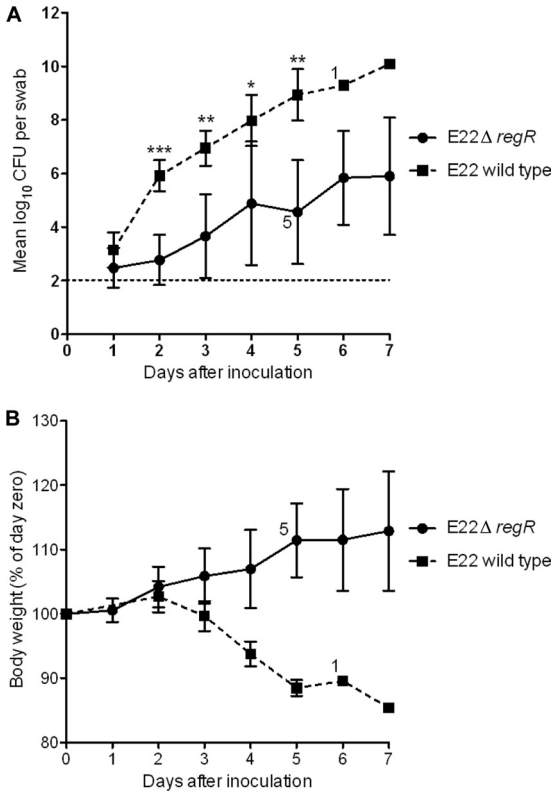

FIG 7 Effect of *regR* on virulence of REPEC strain E22. Six infant rabbits were inoculated with 5×10^5 CFU of wild-type E22 or 8×10^5 CFU of its isogenic *regR* mutant (E22 Δ*regR*). When some rabbits in a group were euthanized, due to loss of body weight of greater than 15% or the presence of severe diarrhea, the surviving numbers of rabbits are shown adjacent to the data points. (A) Colonization of rabbits by REPEC strain E22 and the *regR* mutant as measured by quantitative culture of rectal swabs. Data are the geometric mean CFU per swab (***, $P < 0.0005$; **, $P < 0.005$; and *, $P < 0.05$). The detection limit of the culture method is indicated by the horizontal line. (B) Body weight of rabbits. Values are the mean (± SD) for each group expressed as a percentage of the weight on the day of infection (day zero).

REPEC/rabbit infection model (16, 48), and during the course of infection, rabbits infected with the mutant strain showed weight gain, in contrast to those infected with the wild type (Fig. 7B). Taken together, these results indicated that RegR is required for the full virulence of REPEC strain E22.

DISCUSSION

In this study, we demonstrated that REPEC strain E22 possesses a RegR regulon, which is required for efficient infection of rabbits. The RegR regulon includes the master regulatory gene *regR* and its cognate gene targets *adcA*/*tsh*, *espC*, and *sefABCD*. Although these genes are carried on separate operons, they are colocated in the E22 genome, and their encoded proteins could function cooperatively in pathogenesis. The *adcA* and *espC* loci encode homologs of the autotransporter proteins AdcA/Tsh and EspC, respectively. These proteins are members of the SPATE (serine protease autotransporters of *Enterobacteriaceae*) subfamily of proteins (49, 50). The AdcA protein of *C. rodentium* is responsible for bacterial aggregation as well as for binding to cultured epithelial cells (28). Interestingly, *adcA* is also a member of the RegA regulon of *C. rodentium*, which is required for the intestinal colonization of mice. The Tsh protein of avian-pathogenic *E. coli* (APEC) has agglutinin and mucinolytic activities, which may facilitate colonization of the avian mucus by APEC (51). Other studies have shown that Tsh contributes to the pathogenesis of avian colibacillosis and the early stages of APEC infection of chickens (52, 53).

The EspC protein from EPEC mediates cell adhesiveness and causes cytoskeletal damage to epithelial cells following its internalization by the host cells (54, 55). Both the type III secretion system, which is located on the LEE, and type V secretion system are involved in secretion and translocation of EspC (56).

The E22 *sefABCD* cluster is homologous to that found in group D *Salmonella*. It consists of four cotranscribed genes encoding the major subunit (SefA), chaperone (SefB), usher (SefC), and minor subunit (SefD) of the SEF14 fimbriae (46). The E22 fimbria-like structures that we identified by immunogold electron microscopy using anti-SefA serum have a morphology similar to that of SEF14 fimbriae produced by *S.* Enteritidis, which play a role in colonization of epithelial cell surfaces (57). SEF14 fimbriae are required for binding of *S.* Enteritidis to macrophages and are a virulence determinant of *Salmonella* (58). Interestingly, the expression of *Salmonella sefABCD* is subject to positive control by the AraC-like transcriptional activator SefR (46).

Our microarray analysis showed that expression of the three operons *adcA*/*tsh*, *espC*, and *sefABCD* was strongly upregulated by RegR (Table 3). This finding was supported by proteomic analyses which revealed the abundant presence of the products of all three operons, either in secretomes or on the bacterial cell surface, in the $RegR^+$ but not the $RegR^-$ background (Fig. 5). RegR-mediated activation was enhanced in the presence of the gut-specific environmental factor bicarbonate, which is also the cofactor for the virulence regulator RegA of *C. rodentium* and ToxT of *V. cholerae* (20, 59).

The mechanism of RegR-mediated activation was examined by using the promoter region of the *sefA* gene. A primer extension experiment indicated that transcription of the E22 *sefABCD* operon is driven by a single σ^{70} promoter which contains a well conserved −10 region (TATAAA versus the consensus TATAAT), a TGn motif, a less conserved −35 sequence (TTAACC versus the consensus TTGACA), and a shorter spacer (15 bp versus 17 bp) (Fig. 1B). Data from transcriptional assays using *lacZ* reporters indicated that all of the *cis* elements required for the control of the *sefA* promoter are contained within a region of 464 bp between positions −309 and +155, relative to the start site of transcription (Fig. 1B), as the construct *sefA-lacZ*$_1$ exhibited a degree of maximal activation by RegR similar to that seen in the microarray assay (Fig. 2 and Table 3). The extremely high levels of regulatory outcome of *sefA* transcription are determined by the global negative regulatory protein H-NS and the REPEC-specific activator RegR, which exert opposing effects on *sefA* expression (Fig. 2). The entire regulatory region of *sefA* is highly AT rich and was predicted to form strong DNA curvatures, a general signature of H-NS-binding sites (Fig. 4A). Indeed, deletion of a region between positions −309 and −52 resulted in a pronounced loss of H-NS-mediated repression (Fig. 4).

Two putative RegR-binding sites were identified upstream of the promoter core sequences (Fig. 1B). These sites are similar to the operator sequences of the virulence operons controlled by the Rns, AggR, and RegA proteins (47, 60, 61). This homology is most likely due to the high degrees of similarity between the HTH DNA-binding motifs of RegR, Rns, AggR, and RegA (Fig. 1C), suggesting that these regulatory systems may have evolved from the same ancestry.

In addition to its ability to activate virulence gene expression, RegR also repressed expression of the *glcDEFGBA* operon, whose products are involved in the transport and utilization of glycolate (Table 3). Transcription of the *glcDEFGBA* operon is activated by GlcC (62), but the mode of action of RegR at the *glcD* promoter is unknown.

Although all EPEC strains carry the outer membrane adhesin protein intimin, which is encoded on the LEE, they require other adhesins for their initial attachment to the host intestinal epithelium (63) and regulators that control the expression of these adhesins (11). For tEPEC, these additional virulence determinants are BFP and its associated regulator, PerA (11). The virulence of aEPEC strains suggests that these pathogens also express one or more surface-located factors that compensate for the absence of BFP and a protein which regulates their expression. Our findings that aEPEC strain E22 carries the three operons *adcA*/*tsh*, *espC*, and *sefABCD*, which are regulated by RegR (a homolog of PerA), were highly induced and expressed under conditions similar to those found in the gut, and were required for full virulence in the rabbit model of infection (Fig. 7), support our hypothesis.

We have previously shown that aEPEC strains are a heterogeneous group of bacteria that exhibit considerable variations in terms of the serotype, pattern of adherence to mammalian cells, and carriage of known virulence determinants (64). This group of A/E pathogens carry the LEE and most likely carry AraC-like regulators, such as RegR of E22 and RegA of *C. rodentium*, which are able to sense the environment and regulate the expression of a variety of adherence factors that act cooperatively with intimin and are required for virulence. These AraC-like regulators and accessory adherence factors may have been acquired together via horizontal gene transfer. Alternatively, the regulatory protein may have been acquired independently and subsequently adapted to regulate existing adherence factors.

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