IraL Is an RssB Anti-adaptor That Stabilizes RpoS during Logarithmic Phase Growth in *Escherichia coli* and *Shigella*

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ABSTRACT  RpoS (σ^S), the general stress response sigma factor, directs the expression of genes under a variety of stressful conditions. Control of the cellular σ^S concentration is critical for appropriately scaled σ^S-dependent gene expression. One way to maintain appropriate levels of σ^S is to regulate its stability. Indeed, σ^S degradation is catalyzed by the ClpXP protease and the recognition of σ^S by ClpXP depends on the adaptor protein RssB. Three anti-adaptors (IraD, IraM, and IraP) exist in *Escherichia coli* K-12; each interacts with RssB and inhibits RssB activity under different stress conditions, thereby stabilizing σ^S. Unlike K-12, some *E. coli* isolates, including uropathogenic *E. coli* strain CFT073, show comparable cellular levels of σ^S during the logarithmic and stationary growth phases, suggesting that there are differences in the regulation of σ^S levels among *E. coli* strains. Here, we describe IraL, an RssB anti-adaptor that stabilizes σ^S during logarithmic phase growth in CFT073 and other *E. coli* and *Shigella* strains. By immunoblot analyses, we show that IraL affects the levels and stability of σ^S during logarithmic phase growth. By computational and PCR-based analyses, we reveal that *iraL* is found in many *E. coli* pathotypes but not in laboratory-adapted strains. Finally, by bacterial two-hybrid and copurification analyses, we demonstrate that IraL interacts with RssB by a mechanism distinct from that used by other characterized anti-adaptors. We introduce a fourth RssB anti-adaptor found in *E. coli* species and suggest that differences in the regulation of σ^S levels may contribute to host and niche specificity in pathogenic and nonpathogenic *E. coli* strains.

IMPORTANCE  Bacteria must cope with a variety of environmental conditions in order to survive. RpoS (σ^S), the general stress response sigma factor, directs the expression of many genes under stressful conditions in both pathogenic and nonpathogenic *Escherichia coli* strains. The regulation of σ^S levels and activity allows appropriately scaled σ^S-dependent gene expression. Here, we describe IraL, an RssB anti-adaptor that, unlike previously described anti-adaptors, stabilizes σ^S during the logarithmic growth phase in the absence of additional stress. We also demonstrate that *iraL* is found in a large number of *E. coli* and *Shigella* isolates. These data suggest that strains containing *iraL* are able to initiate σ^S-dependent gene expression under conditions under which strains without *iraL* cannot. Therefore, IraL-mediated σ^S stabilization may contribute to host and niche specificity in *E. coli*.

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Transcription in *Escherichia coli* is catalyzed by the RNA polymerase holoenzyme, which is composed of a core polymerase and a dissociable sigma factor. *E. coli* has one housekeeping sigma factor (σ^70) and six alternative sigma factors (1). The core polymerase is unable to initiate transcription alone and requires an associated sigma factor to define promoter specificity and initiate transcription. RpoS (σ^S), the best studied of the alternative sigma factors, affects the expression of ~10% of the genes in *E. coli* K-12 either directly or indirectly (2). Many σ^S-dependent genes help to mitigate the effects of a variety of stressful conditions. To maintain appropriate levels of σ^S-dependent gene expression, the levels of σ^S and its activity are regulated at many levels: transcription, translation, stability, and association with the core polymerase (3). A widely appreciated manifestation of this regulatory network in laboratory-adapted *E. coli* is that the level of σ^S is low during logarithmic phase growth and increases substantially into and during stationary phase growth.

σ^S degradation is catalyzed by the ClpXP protease, and the recognition of σ^S by ClpXP depends on the adaptor protein RssB. A class of proteins known as RssB anti-adaptors was previously described in *E. coli* K-12 (4). These anti-adaptors (IraD, IraM, and IraP) interact with RssB and inhibit RssB activity under different stress conditions, thereby stabilizing σ^S. Interestingly, the anti-adaptors found in K-12 share no sequence identity and differ in the ways that they interact with RssB (5). RssB anti-adaptors occur in *Salmonella* and additional uncharacterized anti-adaptors in K-12 are hypothesized (6, 7).

Although σ^S levels are low during logarithmic phase growth...
and high during the stationary phase in K-12, elevated levels of $\sigma^S$ during log phase growth are observed in some E. coli strains. In 2001, Culham et al. reported that two independent uropathogenic E. coli (UPEC) isolates (CFT073 and GR12) have comparable levels of $\sigma^S$ during logarithmic phase and stationary phase growth (8) and in Shiga toxin-producing E. coli (STEC), there is a positive correlation between log phase $\sigma^S$ levels and stress resistance (9). Additionally, it is appreciated that in K-12 and enterohemorrhagic E. coli (EHEC) strain O157:H7, $\sigma^S$ contributes either directly or indirectly to gene expression during logarithmic phase growth (10, 11). These observations, combined with a larger body of knowledge that $\sigma^S$ is needed for virulence in many, but not all, pathogenic bacterial species (12, 13), suggest that differences in the timing and magnitude of $\sigma^S$ levels and activity may help to define host and niche specificity in both pathogenic and non-pathogenic bacteria.

We recently demonstrated that $\sigma^S$ is needed by UPEC strain CFT073 to cope with phagocyte oxidase-mediated oxidative stress during urinary tract infection (13) and began to study the regulation of $\sigma^S$ in this strain. Using a CFT073-based overexpression library, we identified an RssB anti-adaptor, heretofore named IraL, to reflect that it inhibits RssB activity during logarithmic phase growth, leading to $\sigma^S$ stabilization. Prior to this study, iraL was named ygiW. iraL is found in many Escherichia and Shigella isolates in addition to CFT073. Furthermore, although IraL and IraM, a previously characterized anti-adaptor from K-12, are homologs, they stabilize $\sigma^S$ under different conditions and by different mechanisms.

**RESULTS**

**Identification of a novel RssB anti-adaptor in E. coli CFT073.** $\sigma^S$ levels in CFT073 are comparable during the logarithmic and stationary growth phases, which contrasts with what is known about the regulation of $\sigma^S$ levels in K-12 (8). To identify effectors of $\sigma^S$ levels in CFT073, we constructed a transcriptional fusion of lacZYA to the promoter of a $\sigma^S$-dependent gene, katE, by using pPK7034 (14). $\sigma^S$ was destabilized in CFT073 P$_{katE}$-lacZYA by deleting genes that code for homologs of RssB anti-adaptors that were characterized in K-12 (IraD, IraM, IraP), as determined by amino acid sequence identity. IraD, IraM, and IraP from K-12 share 74.6, 58.9, and 98.9% identity with their homologs in CFT073, YjiD, IraL, and YaiB, respectively. When CFT073 P$_{katE}$-lacZYA $\Delta$yaiB $\Delta$iraL $\Delta$yjiD ($\sigma^S$-destabilized reporter strain) is plated onto MacConkey’s medium plus lactose, the colonies are less red than those of either the parent strain (CFT073 P$_{katE}$-lacZYA) or CFT073 P$_{katE}$-lacZYA $\Delta$srbB but more red than those of CFT073 P$_{katE}$-lacZYA $\Delta$proS (see Fig. S1 in the supplemental material). This indicates that there is less lacZ expression from the katE promoter in the $\sigma^S$ destabilized reporter strain and that katE is $\sigma^S$ dependent during growth under these conditions.

To identify regulators of $\sigma^S$ levels in CFT073, the $\sigma^S$-destabilized reporter strain was transformed with a pACYC184-based CFT073 genomic library and plated onto either MacConkey’s medium plus lactose or urine indicator agar. Urine indicator agar was designed for this study and was used as a surrogate for nutrient limitation and other stressful conditions present in the urinary tract (see Materials and Methods). A combined ~22,000 transformants were analyzed on these media. Transformants with LacZ activity higher or lower than that of pACYC184-containing transformants on either of these media were studied further. Among the transformants were clones containing homologs of genes known to regulate levels of $\sigma^S$ in K-12, including genomic fragments with yaiB, iraL, and yjiD (see Table S2 in the supplemental material). We then noticed that the genomic context of iraL in CFT073 is different from that of its homolog in K-12, iraM, and that these two genes share 67.0 and 58.9% sequence identity at the nucleotide and amino acid levels, respectively (Fig. 1). We reasoned that IraL might act as an RssB anti-adaptor in CFT073 on the basis of sequence identity with IraM and its identification in our screen. We also hypothesized that the differences in sequence identity and genetic context between iraM and iraL contribute to differences under conditions under which these anti-adaptors stabilize $\sigma^S$.

**iraL TSS identification.** We determined the transcription start site (TSS) of iraL via 5’ rapid amplification of cDNA ends (RACE) as described in Materials and Methods. Briefly, CFT073 was grown to mid-log phase and RNA was isolated. cDNA was made from this RNA sample with an iraL-specific primer (iraL-gsp1), the RNA was degraded, and a poly(C) tail was added to the 3’ end of the iraL cDNA that remained. By using another iraL-specific primer (iraL-gsp2) and a primer that anneals to the poly(C) tail, the cDNA was amplified. Finally, a Sanger sequencing reaction was carried out with this amplified cDNA and iraL-gsp2. The 3’-most end of the reverse strand of iraL cDNA contains the poly(C) tail (see Fig. S2A in the supplemental material), indicating that the 5’ end of the iraL mRNA starts with the sequence 5’ ACA TCA CCA GCA AGG CAT AAA CAA GGA AAC CA 3’. On the basis of the similarity of the predicted –10 and –35 elements to the $\sigma^S$-consensus promoter sequence (15), we suggest that the transcription of iraL is dependent on $\sigma^S$ (Fig. 1A).

**iraL is found in many E. coli and Shigella isolates.** Knowing that iraL is found in CFT073 but not K-12 and that iraM is found in K-12 but not CFT073, we decided to characterize the distribution of these genes in a larger population of isolates. To do this, we queried the NCBI GenBank database for fully sequenced genomes containing nucleotide sequences that match iraL or iraM and we developed a PCR-based assay to identify strains that have iraL or iraM. Primers were designed to regions of sequence divergence within the coding region of each of these genes (see Table S1 in the supplemental material) and were target specific (see Fig. S3 in the supplemental material). We assayed four available, previously described strain collections, the ECOR (16), Andreu prostatitis (17), Johnson urosepsis (18), and Mand STEC (9) collections. On the basis of these analyses, we observed that some strains have iraL alone (like CFT073), some have iraM alone (like K-12), some have both iraL and iraM, and others have neither iraL nor iraM (Fig. 2; see Tables S3 and S4 in the supplemental material). None of the laboratory-adapted strains tested have iraL. Additionally, as determined by Fisher’s exact test, iraL is found more frequently in UPEC ($P = 0.0163$), prostatitis ($P = 0.0083$), and Shigella ($P = 0.0001$) isolates than in commensals and is found less frequently in EHEC or STEC isolates than in commensals ($P = 0.0323$). Furthermore, the iraL promoter and its upstream regulatory region are present and well conserved among all of the fully sequenced iraL-containing isolates, all sharing ≥96% identity with the homologous region in CFT073 (see Fig. S2B in the supplemental material).

**iraL affects the levels and stability of $\sigma^S$.** To determine whether IraL affects levels of $\sigma^S$ during growth in Luria-Bertani (LB) broth, immunoblot assays for $\sigma^S$ were done with the total
FIG 1  \(\text{iraL}\) shares sequence identity with \(\text{iraM}\) from \(E.\ coli\) K-12 but differs in genetic context. (A) Visualization of regions of the K-12 and CFT073 chromosomes containing \(\text{iraM}\) and \(\text{iraL}\), respectively. Block arrows represent annotated open reading frames from these two strains, and the number below each region indicates the chromosomal location. The region immediately upstream and containing the \(\text{iraL}\) start codon is detailed. The TSS of \(\text{iraL}\) (indicated by \(\text{T}\) and a leftward-pointing arrow) was determined by 5' = RACE. The predicted \(\text{T}\) sites are in bold, and the \(\text{iraL}\) start codon is underlined. (B) Nucleotide sequence alignment of the coding regions of \(\text{iraL}\) and \(\text{iraM}\). Alignment was carried out with the ClustalW feature and the default parameters in MacVector 9.0.2. It was determined that these genes share 67% nucleotide sequence identity. Stars below the alignment denote regions of sequence identity. (C) Alignment of the amino acid sequences of IraL and IraM. Alignment was carried out with ClustalW as described above. It was determined that these polypeptides share 58.9% amino acid sequence identity. Stars below the alignment denote regions of sequence identity, while periods represent regions of weak similarity.

\[\begin{array}{l}
\text{iraL} \\
1 \text{ ATGAGTAGTGATGTGATTGATAGTGATTATAACCTCCATCTGCTGGCTATGTCATATTTTTGAATGTAAACCATAGAGC} \\
75
\\
\text{iraM} \\
1 \text{ ATGAGTAGTGATGTGATTGATAGTGATTATAACCTCCATCTGCTGGCTATGTCATATTTTTGAATGTAAACCATAGAGC} \\
75
\\
\end{array}\]
soluble protein isolated from logarithmic and stationary phase cells. In CFT073 ΔiraL and a Shigella sonnei ΔiraL mutant, the levels of $\sigma^7$ were lower than in the wild type during logarithmic phase growth (Fig. 3A). To determine if IraL affects $\sigma^7$ stability, cultures were grown to mid-log phase, protein synthesis was stopped by chloramphenicol (Cm) addition, and the total protein was sampled over time. The half-life of $\sigma^7$ during the log phase in CFT073 is ~5 min, but its half-life is reduced to ~2.5 min in CFT073 ΔiraL. When iraL, under the control of its native promoter, is reintroduced into CFT073 ΔiraL at the att Tn7 site, the stability of $\sigma^7$ is increased (Fig. 3B).

Additionally, when a plasmid containing iraL under the control of its native promoter (region containing the sequence corresponding to position −100 relative to the iraL TSS through the entire iraL coding region) is introduced into K-12, the levels of $\sigma^7$ during logarithmic phase growth are higher than those in vector-only controls (Fig. 3A). For accompanying Coomassie blue-stained gels that illustrate the consistent loading of the protein samples examined in Fig. 3A, see Fig. S4 in the supplemental material.

IraL interacts with RssB. We hypothesized that IraL, as a suspected RssB anti-adaptor, should interact with RssB to stabilize RpoS. To test this hypothesis, we first performed copurification experiments (Fig. 4A; see Fig. S5A in the supplemental material). In these assays, an N-terminal His$_6$ tag was added to IraL and an N-terminal calmodulin binding peptide (CBP) tag was added to RssB. The purification of His$_6$-IraL on cobalt beads led to the copurification of CBP-RssB (Fig. 4A). Similarly, the purification of CBP-RssB on calmodulin beads led to His$_6$-IraL copurification (see Fig. S5A). By this approach, we obtained evidence that, as expected, IraL interacts with RssB.

To further characterize the interaction between IraL and RssB, we aimed to define the subdomain(s) of RssB that interacts with IraL. To this end, we used the bacterial two-hybrid method, as

![Graph showing distribution of iraL and iraM among selected E. coli pathotypes](image)

**FIG 2** Distribution of iraL and iraM among selected E. coli pathotypes. The coding sequences of iraL and iraM were queried against complete bacterial genomes in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and several unsequenced strain collections were subjected to a PCR-based assay for iraL and iraM. Pathotypes with eight or more representatives are shown, with strains represented more than once consolidated into one entry. For the names and sources of all of the strains subjected to these analyses, including pathotypes with fewer than eight representatives, see Tables S3 and S4 in the supplemental material.

![Graph showing percent of strains tested](image)

**FIG 3** IraL affects the levels and stability of $\sigma^7$ during log phase growth. (A) To assess the effects of IraL on $\sigma^7$ levels, CFT073, CFT073 ΔiraL, S. sonnei, S. sonnei ΔiraL, K-12/pACYC184 (vector only), and K-12/piraL (pACYC with iraL under the control of its native promoter) were grown to the log phase (LOG) or the stationary phase (STAT) and immunoblot assays for $\sigma^7$ were carried out with 10 μg of total soluble protein from these samples. For accompanying Coomassie blue-stained gels that illustrate consistent loading of the protein samples, see Fig. S4 in the supplemental material. Marker (M) lanes contain the Precision Plus Protein Dual Color Standard that cross-reacts with the detection reagents used. (B) To assess the effects of IraL on $\sigma^7$ stability, CFT073 and isogenic mutants were grown to the mid-log phase as described for panel A, protein synthesis was stopped with Cm, and samples of total protein were precipitated in 10% TCA at 3-min intervals after Cm treatment. Immunoblot assays were carried out with total protein normalized to the OD$_{600}$, and $\sigma^7$ levels were measured by densitometry. Data points represent the mean percentages of $\sigma^7$ remaining relative to those at t = 0, and error bars represent ± the standard error of the mean of three replicates.
IraL-Mediated RpoS Stabilization

We and others observed that levels of $\sigma^5$ in the logarithmic and stationary growth phases are comparable when CFT073 is grown in LB medium, which contrasts with what is known about the regulation of $\sigma^5$ levels in laboratory-adapted strains of E. coli (8) (Fig. 3A). We rationalized that there may be differences in the regulation of $\sigma^5$ between laboratory-adapted E. coli K-12 and UPEC strain CFT073 and sought to determine the genetic basis for this phenotype. We identified iraL in CFT073 after screening to change the levels of $\sigma^5$ in a $\sigma^5$-destabilized reporter strain. We determined that IraL affects the levels and stability of $\sigma^5$, that IraL interacts with RssB in a manner that is different from that of other characterized anti-adaptors, and that iraL is found in many Escherichia and Shigella isolates.

IraL and a related gene, iraM, are located in different genetic contexts in CFT073 and K-12, respectively. In K-12, iraM is found on the e14 prophage; in CFT073, iraL is found on the potB genomic island; and neither of them is present in the other strain. The differences in location and sequence identity of these genes prompted us to examine other bacterial strains for the presence of iraL and iraM. By NCBI BLAST analysis, we determined that iraL and iraM are present in many fully sequenced Escherichia and Shigella strains (see Table S4 in the supplemental material) but are not found in the sequenced strains of other genera. Interestingly, the matches to iraL share 95% sequence identity over 100% of the coding region queried, with E values of $\leq 1 \times 10^{-74}$. All matches to iraM are also conserved to this extent. Furthermore, there is no overlap between iraL or iraM matches. This observation of anti-adaptor sequence conservation led us to develop a PCR-based assay to detect iraL and iraM in unsequenced strain collections. By our BLAST- and PCR-based analyses, we found that many Escherichia and Shigella isolates have iraL. Additionally, iraL is significantly associated with some pathotypes of E. coli relative to commensal isolates (Fig. 2), suggesting that iraL-mediated $\sigma^5$ stabilization may provide a fitness advantage in some pathotypes (UPEC, prostatitis, and Shigella isolates) but not others (EHEC or STEC isolates).

Previously, anti-adaptors were shown to be expressed under stressful conditions in K-12 and Salmonella (4). IraL is unique among the characterized anti-adaptors because it elevates $\sigma^5$ during logarithmic growth in the absence of any additional stress. Some EHEC or STEC isolates have elevated levels of $\sigma^5$ during logarithmic phase growth (9). Surprisingly, none of the strains described in this study by Mand et al. have iraL (Fig. 2; see Table S3 in the supplemental material). This supports the idea that there are one or more additional mechanisms by which to elevate the levels of $\sigma^5$ during logarithmic phase growth and that these mechanisms, along with the specific environmental conditions that lead to their expression and activity, may be favored under conditions encountered by some pathotypes and not others.

FIG 4 IraL interacts with RssB. (A) IraL and RssB copurify. Strain AB054 was cotransformed with plasmids encoding CBP-RssB and His6-IraL, and cell lysates were prepared as described in Materials and Methods. Cobalt beads were used to precipitate CBP-RssB and its interacting partner(s), and immunoblot assays were carried out with protein and anti-CBP or anti-His6-peroxidase antiserum and visualized as described in Materials and Methods. (B) IraL interacts with full-length RssB and subdomains of RssB, as visualized on MacConkey’s medium. Overnight cultures of BTH101 coexpressing either T18-IraL or T18-IraM fusion protein and the T25-RssB or T25-RssB subdomain were spotted onto MacConkey’s medium plus maltose. Interaction of the proteins of interest is qualitatively shown as red coloration. (C) IraL interacts with full-length RssB and subdomains of RssB, as determined by $\beta$-galactosidase assay. Overnight cultures of BTH101 coexpressing either T25-IraL or T25-IraM and the T25-RssB or T25-RssB subdomain were subjected to a $\beta$-galactosidase assay as previously described. Bars on the graph represent mean $\beta$-galactosidase activities (Miller units [M.U.]), and error bars represent the standard error of the mean of three replicates. WT, wild type.

used previously to visualize interactions between RssB and the anti-adaptor proteins from E. coli K-12 (5). In this assay, the proteins of interest are fused to the two domains of adenylate cyclase (Cya) from Bordetella pertussis, T18 and T25. A positive interaction restores Cya activity in an E. coli cya mutant, and it can be detected on MacConkey’s medium or by $\beta$-galactosidase measurement (19, 20). By using this technique, we confirmed that IraL interacts with RssB (Fig. 4B and C; see Fig. S5B and C in the supplemental material). Interestingly, IraL interacts only with the N-terminal domain of RssB, which is an interaction pattern distinct from that of its homolog, IraM, which interacts with both the N- and C-terminal domains (Fig. 4B and C; see Fig. S5B and C in the supplemental material) (5). These data suggest that although IraL and IraM are homologs, they have diverged through evolution to stabilize RpoS under different conditions and by different mechanisms.
We observed that when \textit{iraL} and its upstream regulatory region are introduced into the att \textit{Tn}7 site of CFT073 \textit{\Delta iraL}, an \sigma^6 stability is increased (Fig. 3B). Consistent with its role as an RssB anti-adaptor, we demonstrate that RssB-IraL interaction occurs and we provide evidence that IraL interacts with RssB differently than does IraM (Fig. 4; see Fig. S5 in the supplemental material). The observation that IraL and IraM interact differently with RssB will serve as the basis for future work in determining the residues that are important for anti-adaptor-mediated \sigma^6 stabilization. Furthermore, differences in the modes of anti-adaptor-RssB interaction may provide for more finely tuned anti-adaptor-mediated regulation of \sigma^6 levels under multiple stresses.

In \textit{Salmonella}, an IraL- and IraM-like protein, RssC, has been identified (7). \textit{rssC} and \textit{iraL} share 49.3\% identity and \textit{rssC} and \textit{iraM} share 47.4\% identity, as determined by ClustalW alignment. On the basis of previous work in the Gottesman laboratory and BLAST analysis of sequenced bacterial strains, we determined that \textit{rssC} is found only in \textit{Salmonella} and that no \textit{Salmonella} isolates have \textit{iraL} or \textit{iraM} (7) (see Table S4 in the supplemental material). This suggests that the \textit{iraL}-\textit{iraM}-\textit{rssC} family of anti-adaptors may be evolutionarily labile to adapt to diverse bacterial hosts.

Our \textit{in silico} analysis of \textit{iraL} and its promoter revealed that the recently sequenced fatal urosepsis isolate JJ1886 (21) has two copies of \textit{iraL} and its associated regulatory region (see Fig. S2B and Table S4 in the supplemental material). Given that there is a point mutation in the predicted 
~35 sequence of the JJ1886 \textit{iraL} promoter (see Fig. S2B), it is not known if this copy of \textit{iraL} is expressed in JJ1886 or in the other strains containing this mutation. Regardless, JJ1886 is the first example of a strain containing two copies of the same anti-adaptor, which further suggests that \textit{iraL} may provide a fitness advantage under some of the environmental conditions encountered by UPEC.

Though roles for \sigma^6 during logarithmic phase growth are appreciated and it is suggested that elevated levels of \sigma^6 during this growth phase may lead to an increase in stress resistance, this is the first example of an RssB anti-adaptor that elevates \sigma^6 during logarithmic phase growth, as all other characterized anti-adaptors are induced under stress. We posit that IraL, a horizontally acquired anti-adaptor, shares active areas of investigation in our laboratories.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The strains, plasmids, and oligonucleotides used in this study are listed in Tables S1 and S3. Cloning procedures were done with GoTaq and T4 DNA ligase from Promega and restriction endonucleases from New England Biolabs according to the manufacturers’ specifications. In-frame deletion mutants of \textit{E. coli} CFT073 were constructed by using the Lambda Red mutagenesis protocol (23), which was modified to incorporate a generalized transduction step with \textit{\PhiEB}49 prior to pCP20-mediated antibiotic resistance cassette removal (24). Attempts to use this method to create the in-frame \textit{iraL} deletion mutant of \textit{S. sonnei} were unsuccessful. Therefore, the \textit{S. sonnei} \textit{\Delta iraL} mutant strain was constructed by Lambda Red mutagenesis, as facilitated by \textit{psIM6} (25), followed by pCP20-mediated antibiotic resistance cassette removal (23). This method of Lambda Red mutagenesis is suggested to be more effective than that of Datsenko and Wanner for recombining recently sequenced fatal urosepsis isolate JJ1886 from K-12 (25). In support of this, our results suggest that pKDa6 is ineffective as a tool in \textit{S. sonnei}. The chromosomal \textit{P}_{\text{att}}-\textit{lacZYA} fusion was constructed by cloning \textit{P}_{\text{att}} downstream of \textit{lacZYA} with \textit{pPK7035} as described previously (14).

Single-copy complementation of CFT073 \textit{\Delta iraL} was carried out by using a modification of the method of Bao et al. (26), which was used to insert \textit{iraL} under the control of its native promoter into the chromosomal att \textit{Tn}7 locus in CFT073. The region containing \textit{iraL} and its promoter region (\textit{iraL} plus the upstream region from \textit{~100 to +33} relative to the \textit{iraL} \textit{TSS}) was cloned into the plCITn7K-\textit{a} carrier plasmid (27) prior to conjugation and \textit{att} \textit{Tn7} insertion into CFT073. The control strain (CFT073 \textit{\Delta iraL} \textit{Tn7:Kan}) contains an insertion of a kanamycin (\textit{Kan}) resistance cassette at the att \textit{Tn}7 site and was constructed as described above, except that no fragment was cloned into plCITn7K-\textit{a} prior to conjugation.

Media and growth conditions. All strains were grown in LB broth, on LB agar, on MacConkey’s medium (all from Difco), or on urine indicator agar. Urine indicator agar plates were made by supplementation of human urine plates (as described in reference 28) with filter-sterilized lactose and neutral red solutions added to final concentrations of 1 and 0.003%, respectively. These supplements were mixed into the urine-agar mixture immediately before it was poured. All strains were grown aerobically at 37°C and supplemented with the antibiotics Kan (40 \mu g/ml), carbenicillin (250 \mu g/ml), Cm (20 \mu g/ml), and ampicillin (Ap; 100 \mu g/ml), as applicable.

Overexpression library and screening for effectors of \sigma^6 levels. Genomic DNA was isolated from cultures of \textit{E. coli} CFT073 with the Promega Wizard Genomic DNA purification kit in accordance with the manufacturer’s instructions. The DNA was hydrodynamically sheared and size selected to approximately 5-kb fragments. The sheared DNA was filled in to create blunt ends with T4 DNA polymerase (New England Biolabs), and the fragments were then treated with T4 polynucleotide kinase in preparation for ligation. pACYC184 was isolated with the Qia-gen Midiprep kit and digested with EcoRV (New England Biolabs). The sheared and blunted genomic DNA fragments were ligated into the digested vectors, transformed into NEB 5-alpha competent \textit{E. coli}, and plated on LB broth plus Cm. Colonies were collected by flooding and swabbing the plates with LB broth plus 50% glycerol. The library contains 
~40,000 clones (~40 \times coverage), 24 of which were sequenced to ensure that random fragments were present in each library (data not shown).

Identification of \textit{iraL} TSS in CFT073. CFT073 was grown overnight in LB broth, subcultured (1:1,000) into 3 ml of fresh LB broth, and then grown to an optical density at 600 nm (OD_{600}) of 0.3. The transcriptional profile of this culture was stabilized by adding 1 ml of the culture to an equal volume of RNAlater (Life Technologies). The mixture was vortexed and placed on ice. Cells were pelleted by centrifugation at 20,000 \times g for 20 min at 4°C, and RNA was extracted from these cells with the TRIzol Plus RNA purification kit with on-column DNase treatment, according to the manufacturer’s specifications (Life Technologies). The Agilent Bioanalyzer 2100 and an RNA Pico chip were used to determine that the RNA was of adequate quality, according to the manufacturer’s specifications (Invitrogen) to map the TSS of \textit{iraL}. cDNA was amplified with \textit{Taq} polymerase (New England Biolabs). For the gene-specific primers (\textit{iraL}-gsp1, \textit{iraL}-gsp2, and \textit{iraL}-gsp3) used in this protocol, see Table S1 in the supplemental material.

Assay for relative levels of \sigma^6. To assess levels of \sigma^6, the strains shown in Fig. 3A were grown overnight in LB broth, subcultured 1:1,000 in fresh LB broth, and grown to the mid-logarithmic phase (OD_{600} of 0.3 to 0.4) or for 24 h (stationary phase), and then total soluble protein was collected.
and subjected to Western blot analysis. Cells were collected in plastic bottles on ice containing phenylmethylsulfonyl fluoride (PMSF) and Cm or spectinomycin (final concentrations of 0.1 mM and 200 μg/ml in the cell suspension, respectively). Spectinomycin was used for K-12 strains containing pACYC184 and pWAM5035, which confer Cm resistance. After cells were pelleted by centrifugation at 6,700 × g for 15 min at 4°C, they were resuspended and washed once in resuspension buffer (10 mM Tris [pH 7.9], 1 mM EDTA, 5% glycerol, 0.1 mM PMSF). Cells were sonicated twice for 20 s each to lyse them, and total soluble protein was collected after collection of the insoluble fraction by centrifugation at 20,000 × g for 20 min at 4°C. Levels of protein were measured in triplicate by the Bio-Rad protein assay, which is based on the Bradford method for protein quantification (29). Ten micrograms of total soluble protein was subjected to SDS-PAGE on a 4 to 20% Ready Gel Tris-HCl precast gel (Bio-Rad) in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). Protein was electrophoretically transferred to a Hybond-ECL membrane (Amersham) at 40 V for 50 min in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). The blot was blocked with 5% skim milk in TBST (30 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.6]) for 1 h, and then a 1:1,000 dilution of anti-αs monoclonal antibody (Neorecon) was added directly to the blocking solution and allowed to incubate on a rocker for 1 h. The membrane was then washed three times in TBST for 5 min at each washing. Subsequently, a 1:3,000 dilution of goat anti-mouse IgG antibody (Bio-Rad) was added and allowed to incubate for 30 min. The membrane was then washed with TBST as described above. Next, the ECL Prime Western blot detection reagent (Amersham) was applied to the membrane for 5 min of incubation, according to the manufacturer’s specifications. The blot was then removed from the detection reagent and imaged with Blue Ultra Autoradiography Film (ISC Bioexpress) and an Lumi-Phos WB chemiluminescent substrate (Thermo Scientific) with the LAS-4000 Mini luminescent-image analyzer (Fujifilm).

**Surveys for irlA and irlM in E. coli strains.** To examine the distribution of irlA and irlM among E. coli isolates, we designed oligonucleotides to target dissimilar regions of these genes (see irlA/irlM survey primers listed in Table S1 in the supplemental material). These oligonucleotides were validated as target specific via colony PCR on CFT073, CFT073 ΔiraL, K-12, and K-12 irlA::Tet’ (see Fig. S3 in the supplemental material) and subsequently used to survey the E. coli isolates listed in Table S3 in the supplemental material. After overnight growth on LB agar, isolated colonies were subjected to colony PCR with GoTaq according to the manufacturer’s specifications (Promega). PCR products were visualized via UV illumination after electrophoresis on 2% agarose gels and ethidium bromide staining.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.01043-14/-/DCSupplemental.

**Figure S1, EPS file, 7.8 MB.**

**Figure S2, EPS file, 1.6 MB.**

**Figure S3, EPS file, 3.4 MB.**

**Figure S4, EPS file, 3.6 MB.**

**Figure S5, EPS file, 1.7 MB.**

**Table S1, DOCX file, 0.1 MB.**

**Table S2, DOCX file, 0.1 MB.**

**Table S3, DOCX file, 0.1 MB.**

**Table S4, DOCX file, 0.1 MB.**

**Figure S3, EPS file, 3.4 MB.**

**Figure S2, EPS file, 1.6 MB.**

**Figure S1, EPS file, 7.8 MB.**

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