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RESEARCH ARTICLE

Cooperative Function of TraJ and ArcA in Regulating the F Plasmid *tra* Operon

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ABSTRACT The F plasmid *tra* operon encodes most of the proteins required for bacterial conjugation. TraJ and ArcA are known activators of the *tra* operon promoter P_Y, which is subject to H-NS-mediated silencing. Donor ability and promoter activity assays indicated that P_Y is inactivated by silencers and requires both TraJ and ArcA for activation to support efficient F conjugation. The observed low-level, ArcA-independent F conjugation is caused by *tra* expression from upstream alternative promoters. Electrophoretic mobility shift assays showed that TraJ alone weakly binds to P_Y regulatory DNA; however, TraJ binding is significantly enhanced by ArcA binding to the same DNA, indicating cooperativity of the two proteins. Analysis of binding affinities between ArcA and various DNA fragments in the P_Y regulatory region defined a 22-bp tandem repeat sequence (from −76 to −55 of P_Y) sufficient for optimal ArcA binding, which is immediately upstream of the predicted TraJ-binding site (from −54 to −34). Deletion analysis of the P_Y promoter in strains deficient in TraJ, ArcA, and/or H-NS determined that sequences upstream of −103 are required by silencers including H-NS for P_Y silencing, whereas sequences downstream of −77 are targeted by TraJ and ArcA for activation. TraJ and ArcA appear not only to counteract P_Y silencers but also to directly activate P_Y in a cooperative manner. Our data reveal the cooperativity of TraJ and ArcA during P_Y activation and provide insights into the regulatory circuit controlling F-family plasmid-mediated bacterial conjugation.

IMPORTANCE Conjugation is a major mechanism for dissemination of antibiotic resistance and virulence among bacterial populations. The *tra* operon in the F family of conjugative plasmids encodes most of the proteins involved in bacterial conjugation. This work reveals that activation of *tra* operon transcription requires two proteins, TraJ and ArcA, to bind cooperatively to adjacent sites immediately upstream of the major *tra* promoter P_Y. The interaction of TraJ and ArcA with the *tra* operon not only relieves P_Y from silencers but also directly activates it. These findings provide insights into the regulatory circuit of the F-family plasmid-mediated bacterial conjugation.

KEYWORDS ArcA, bacterial conjugation, DNA binding, F plasmid, TraJ, gene regulation

Conjugation is a major mechanism for dissemination of antibiotic resistance and virulence among bacterial populations (1, 2). Members of the F-family conjugative plasmids carry various antibiotic resistance and virulence factors in *Enterobacteriaceae* (3–5). Most of the proteins involved in F-family plasmid-mediated bacterial conjugation are expressed from a polycistronic *tra* operon in the plasmid transfer (*tra*) region that contains essentially identically organized conjugation-related genes (Fig. 1A) (4, 6). The *tra* operon promoter P_Y is responsible for efficient *tra* operon expression and subsequent F conjugation (7, 8). Low levels of read-through transcripts from *traM* and *traJ*

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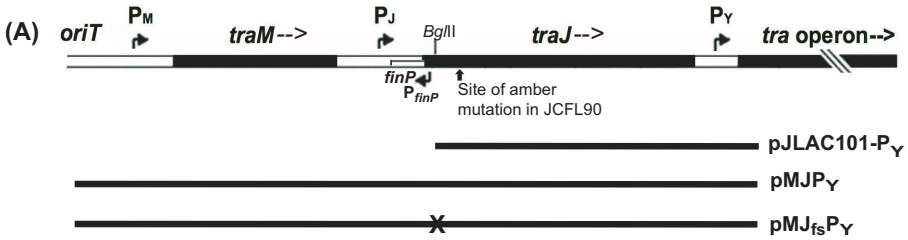

(B)

Plasmids	BW25113 (ArcA$^+$)	JW4364-1 (ArcA$^-$)
JCFL0 (TraJ$^+$) + pACYC184	1 x 10^{-1}	5 x 10^{-6}
JCFL90 (TraJ$^-$) + pACYC184	<1 x 10^{-7}	<1 x 10^{-7}
JCFL90 (TraJ$^-$) + pAC-TraJ	1 x 10^{-1}	<1 x 10^{-7}

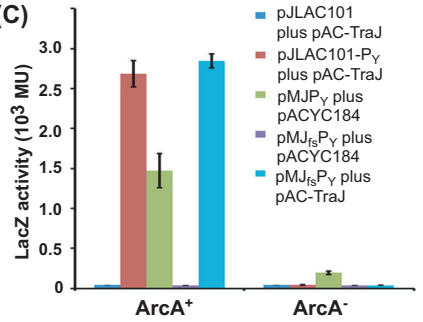

FIG 1 Dependence of *tra* expression and F conjugation on TraJ and ArcA. (A) Organization of the F plasmid (JCFL0) transfer (*tra*) region. The region starts from *oriT* (origin of transfer) and is followed by three transcriptional units: *traM*, *traJ*, and the multicistronic *tra* operon (GenBank accession number U01159). The antisense RNA gene *finP* overlaps the 5′ untranslated region of *traJ* in an opposite direction. The location of the *traJ* amber mutation (GAG to TAG) in the JCFL0 derivative, JCFL90, is indicated by an arrow. The lengths of the F plasmid fragments cloned upstream of the *lacZ* coding region as transcriptional fusions in plasmids pJLAC101-P_Y, pMJP_Y, and pMJ$_{fs}P_Y$ are indicated by black lines, with an X indicating the position of the *traJ* frameshift mutation. (B) Conjugative transfer efficiency of JCFL0 and JCFL90 in ArcA$^+$ and ArcA$^-$ *E. coli* strains. Conjugative transfer efficiency is represented by the donor ability (number of transconjugants per donor cell) of *E. coli* strain BW25113 (or its ArcA knockout derivative, JW4364-1) containing JCFL0 and JCFL90 with or without TraJ supplied in *trans*, as indicated. Each number represents the average obtained from three independent assays, with results being within 1 log unit of each other. (C) A bar graph representation of the promoter activities reported by different P_Y constructs in ArcA$^+$ (BW25113) and ArcA$^-$ (JW4364-1) backgrounds. For cells containing plasmid pJLAC101, pJLAC101-P_Y, or pMJ$_{fs}P_Y$, TraJ was expressed in *trans* by a coresident plasmid pAC-TraJ. For cells containing pMJP_Y, TraJ was expressed in *cis* from the *traJ* gene upstream of P_Y (panel A). The LacZ activity of cells containing the vector pJLAC101 was used as the background control. The mean values of promoter activity obtained from four independent assays ($n = 4$) are presented here, with error bars indicating standard deviations from the means.

into the downstream *tra* operon have been detected by a previous Northern blot analysis (9), but their biological relevance has yet to be determined. P_Y is normally silenced by a nucleoid-associated host protein, H-NS (histone-like nucleoid structuring protein) (10, 11), which shuts down gene expression by forming large, oligomeric filamentous structures on DNA to block transcription (12, 13).

Activation of P_Y requires TraJ, a transcriptional activator encoded by the gene immediately upstream of the *tra* operon (14), and the activity of P_Y is a function of the TraJ level (8). TraJ has an N-terminal PAS (Per-Arnt-Sim) domain responsible for dimerization and a C-terminal helix-turn-helix (HTH) motif that carries allelic specificity in recognizing its cognate plasmid DNA (11, 15–17). A previous DNase I footprinting experiment indicated that TraJ specifically binds to an inverted repeat sequence immediately upstream of the −35 region of P_Y in the F-like plasmid R100 (18). It has been proposed that the F plasmid TraJ activates P_Y through relieving P_Y from H-NS-mediated silencing (9, 10). However, removal of H-NS only partially restored conjugative transfer of the *traJ*-deficient F plasmid, and supplying TraJ in *trans* could further increase conjugative transfer of the F plasmid to maximal levels (9), suggesting that TraJ can not only relieve H-NS-mediated silencing but also activate P_Y through additional mechanisms. As a central nexus in regulation of plasmid conjugation, TraJ itself is regulated by different host and plasmid-encoded factors (3). Most F-family plasmids are naturally transfer repressed due to FinO-FinP antisense RNA inhibition of *traJ* mRNA translation (19–21). Transfer-repressed plasmids can be derepressed for two to seven generations immediately after the transfer of the plasmid into the host through a

phenomenon called "high frequency of transfer" or "epidemic (or infectious) spread" (22, 23); however, the mechanism underlying this initial derepression remains unclear (24).

A host-encoded protein, ArcA (aerobic respiration control or anoxic redox control), originally called SfrA (sex factor regulation), has also been found to activate P_Y in the presence of TraJ (8, 25). Knockout mutations in the host gene *arcA* could not completely abolish conjugative transfer of the F and F-like plasmids, suggesting the existence of low-level, ArcA-independent *tra* expression (14, 25, 26). In contrast, an amber mutation of *traJ* (*traJ90*) in the F plasmid derivative JCFL90 completely abolishes F conjugation, implying different levels of importance of TraJ and ArcA during P_Y activation. An *hns* knockout mutation in the ArcA$^-$ host strain can significantly increase conjugative transfer of the F-like plasmid R1-16, but maximal transfer could be achieved only in the ArcA$^+$ strain, leading to the hypothesis that ArcA works together with TraJ to play a direct role to synergistically activate *tra* operon transcription (11). ArcA typically serves as the response regulator in the ArcAB two-component system that regulates gene expression in response to limited oxygen availability (27). Upon shifting from aerobiosis to anaerobiosis, the sensor histidine kinase (ArcB) phosphorylates the N-terminal regulatory domain of ArcA to promote its dimerization, resulting in enhanced DNA binding affinity. Under aerobic conditions, ArcB promotes the dephosphorylation of ArcA (28–30). Intriguingly, ArcB-mediated ArcA phosphorylation appears to be dispensable for ArcA to activate P_Y and F conjugation under aerobic conditions; however, it has been reported that ArcB is important for P_Y activation and conjugative transfer of the F-like virulence plasmid pSLT of *Salmonella enterica* under microaerobic conditions (31, 32). As a transcription factor in the OmpR/PhoB subfamily, ArcA has an N-terminal regulatory domain mediating dimerization and a C-terminal helix-turn-helix DNA-binding domain (30, 33). Early DNase I footprinting analysis revealed that all phospho-ArcA (ArcA-P) target sequences contain a 10-bp motif with a consensus sequence (A/T)GTTAATTA(A/T) (34). DNase I footprinting analysis of the F-like plasmid R1 revealed an ArcA-P protection pattern of approximately 30 bp upstream of P_Y, containing a tandem repeat of the 10-bp motif (25). More recently, analysis based on chromatin immunoprecipitation-DNA sequencing revealed that most ArcA-P binding sequences contain a so-called ArcA-P box, which consists of two direct repeat elements with a center-to-center spacing of 11 bp (35). Unphosphorylated ArcA has been found to bind the same DNA fragments as ArcA-P, albeit with 3- to 10-fold-lower affinity (25, 36).

To characterize the relationship of TraJ and ArcA in P_Y activation, we analyzed F conjugation efficiency and F plasmid P_Y activities in backgrounds with or without ArcA or TraJ. Our results indicate that both TraJ and ArcA are required to activate P_Y for high-level *tra* expression and F conjugation. Existence of low-level, ArcA-independent *tra* expression and F conjugation is due to the presence of alternative promoters upstream of P_Y. We further defined the minimal target sequence of ArcA as a 22-bp tandem repeat sequence from -76 to -55 of P_Y. Promoter activity analysis of a set of P_Y constructs with gradually truncated upstream regulatory regions revealed that sequences upstream of -103 of P_Y are important for P_Y inactivation by multiple silencers, including H-NS, whereas TraJ and ArcA target sequences downstream of -77. These results, together with electrophoretic mobility shift assays (EMSA) and previous footprinting data (18), indicate that TraJ and ArcA cooperatively bind adjacent DNA sites to activate transcription. The roles of TraJ and ArcA during P_Y activation and the mechanism of ArcA-independent *tra* expression and its implications in epidemic spread of the naturally transfer-repressed F-family plasmids are further discussed.

RESULTS

Both TraJ and ArcA are required for activation of the major *tra* operon promoter P_Y. The F plasmid-mediated bacterial conjugation requires expression of the F *tra* operon that encodes most of the conjugation-related proteins (3, 4). To evaluate effects of the two known activators (TraJ and ArcA) of the *tra* operon promoter (P_Y) on

F conjugation, we assayed conjugative transfer of an F plasmid derivative, JCFL0, and its TraJ$^-$ mutant, JCFL90 (with an amber mutation in *traJ*), in two *Escherichia coli* strains: BW25113 (ArcA$^+$) and its ArcA$^-$ derivative JW4364-1 (Fig. 1A). In the ArcA$^+$ strain, JCFL0 transfers at 1×10^{-1} efficiency (calculated based on the number of transconjugants per donor cell). JCFL90 has no detectable conjugative transfer in the same ArcA$^+$ strain but can resume transfer at levels comparable to those of JCFL0 when it is complemented by a coresident plasmid, pAC-TraJ (supplying TraJ in *trans*) (Fig. 1B). These results are consistent with previous findings that the TraJ protein is essential for relieving P_Y from silencing and that the *tra* expression originating from P_Y supports efficient F conjugation (4, 9). In the ArcA$^-$ strain, JCFL0, transfer decreased 20,000-fold to 5×10^{-6} efficiency, agreeing with previous results that ArcA is important for P_Y activation (8, 25). This level of transfer, while low, was still significantly higher than the level of JCFL90 conjugative transfer in the ArcA$^-$ strain. Supplying TraJ in *trans* did not enhance the transfer of JCFL90 from the background levels in the ArcA$^-$ strain, suggesting that the ArcA-independent *tra* expression in JCFL0 requires an intact *traJ* sequence upstream of the *tra* operon rather than the TraJ protein.

To test the roles of upstream sequences in ArcA-independent *tra* expression, we compared the LacZ activities reported by pJLAC101-P_Y and pMJP$_Y$ in the ArcA$^+$ and ArcA$^-$ strains (Fig. 1A). The P_Y reporter plasmid pJLAC101-P_Y contains a partial *traJ* coding region upstream of P_Y fused with the *lacZ* coding region (15), whereas pMJP$_Y$ contains the complete *traM* and *traJ* transcriptional units upstream of P_Y fused with the same *lacZ*. With TraJ supplied in *trans*, pJLAC101-P_Y reported ~2,700 Miller units (MU) of promoter activity in the ArcA$^+$ strain but only ~40 MU in the ArcA$^-$ strain, the same as the background-level activity reported for the vector control pJLAC101 (Fig. 1C). These results indicate that P_Y is completely inactive in the absence of ArcA. In comparison, pMJP$_Y$ reported ~200 MU of LacZ activity in the same ArcA$^-$ strain, approximately 5-fold higher than that of either pJLAC101-P_Y or the vector control, indicating that sequences upstream of P_Y are responsible for the ArcA-independent promoter activity. Consistent with the idea that read-through transcription from upstream promoters accounts for the ArcA-independent promoter activity, a polar +1 frameshift mutation at the beginning of the *traJ* coding region (pMJ$_{fs}$P$_Y$) (Fig. 1A) reduced promoter activity in the ArcA$^-$ strain to background levels (~40 MU) even when TraJ was supplied by a coresident plasmid (Fig. 1C). Combined with findings from donor ability assays (Fig. 1B), our results together suggest that both TraJ and ArcA are required for activation of the F plasmid P_Y, which leads to high-level *tra* expression and F conjugation. The low-level, ArcA-independent transcription of the *tra* operon appears due to read-through transcription originating from promoters upstream of P_Y. The ArcA$^+$ strains containing pJLAC101-P_Y plus pAC-TraJ or pMJ$_{fs}$P$_Y$ plus pAC-TraJ have much higher promoter activities than those containing pMJP$_Y$, presumably because expression of TraJ from pACYC184, which has an ~5-fold-higher copy number than the pJLAC101-based plasmid pMJP$_Y$ (10, 37), yielded more TraJ and subsequently enhanced P_Y promoter activity. This agrees with a previous finding that steady-state abundance of TraJ positively correlates with P_Y activity (8).

ArcA promotes TraJ binding to P_Y regulatory DNA. Previous chromatin immunoprecipitation assays suggested that the F plasmid TraJ binds to a 200-bp DNA molecule at the P_Y region *in vivo*; however, *in vitro* EMSA could not detect specific TraJ binding to the corresponding DNA (17). As both ArcA and TraJ are required for P_Y activation, we purified the F plasmid TraJ and *E. coli* ArcA proteins to test their ability to specifically bind to this ^{32}P-labeled 200-bp DNA in the presence of an excess of nonspecific unlabeled DNA [poly(dI-dC)] by EMSA (Fig. 2). Consistent with previous observations (17), only a small fraction of DNA was shifted to a lower-mobility band at the highest TraJ concentration tested (6 μM), indicating that TraJ binds to this DNA weakly, with an apparent dissociation constant (K_d) well above 6 μM (Fig. 2A). In contrast, ArcA binds much better than TraJ to this 200-bp DNA, with an apparent K_d between 0.6 and 1.0 μM (Fig. 2B), comparable to results previously observed with the

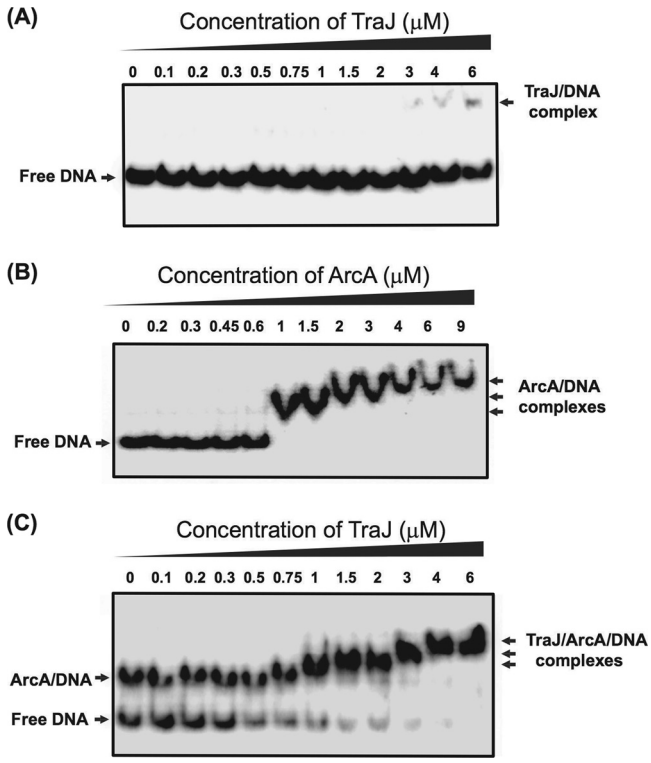

FIG 2 ArcA and TraJ binding to the regulatory region of the F plasmid P_Y. A ^{32}P-labeled 200-bp dsDNA containing the F plasmid P_Y and its regulatory region up to position −179 was used for EMSA. Each binding mixture contained 1 μg of poly(dI-dC) as the nonspecific DNA competitor. Arrows indicate different DNA or DNA-protein species. TraJ and ArcA concentrations are shown above the gels. (A) EMSA of TraJ binding to P_Y regulatory DNA. (B) EMSA of ArcA binding to P_Y regulatory DNA. (C) EMSA of TraJ binding to P_Y regulatory DNA that was prebound by 0.7 μM ArcA.

F-like R1 plasmid DNA (38). Addition of ArcA at concentrations up to 9 μM increasingly retarded mobility of the DNA substrate, suggestive of a concentration dependence of ArcA occupancy at multiple binding sites or of ArcA oligomerization on the DNA substrate.

To understand how TraJ and ArcA together bind to this 200-bp region, we preincubated the same ^{32}P-labeled P_Y DNA with 0.7 μM (near the K_d level) of ArcA in a set of binding mixtures. Increasing concentrations of TraJ were added to each ArcA-DNA binding mixture, and the mixtures were separated by EMSA. Without TraJ, approximately half of the labeled DNA was free whereas the other half was bound by ArcA and shifted to a lower-mobility band (Fig. 2C). With TraJ at concentrations of 1 μM and higher, the ArcA/DNA complex band was completely supershifted to progressively lower-mobility species. Since TraJ and ArcA proteins do not appear to bind each other directly, as indicated by pulldown and chemical cross-linking assays (J. Lu and J. N. M. Glover, unpublished data), this supershift of ArcA/DNA complexes is likely due to TraJ binding to the labeled DNA. Therefore, TraJ can specifically bind to the P_Y regulatory DNA with significantly higher affinity (K_d between 0.75 and 1 μM) in the presence of ArcA than when binding DNA alone (K_d over 6 μM), suggesting that ArcA and TraJ together cooperatively recognize P_Y in a sequence-specific manner.

ArcA requires a tandem repeat from −76 to −55 of P_Y for optimal binding. A 10-bp ArcA-P binding motif with a consensus sequence (A/T)GTTAATTA(A/T) has previously been proposed based on DNase I footprinting data (34). There are four such motifs (highlighted by boxes in Fig. 3A) with at least 7 identities to the consensus 10-bp motif within position −200 of the F plasmid P_Y. To locate the ArcA-binding site in the F plasmid P_Y regulatory region, we used EMSA to analyze a series of ^{32}P-labeled 40-bp double-stranded DNA (dsDNA) fragments (Fig. 3A, fragments 1 to 8) that span this

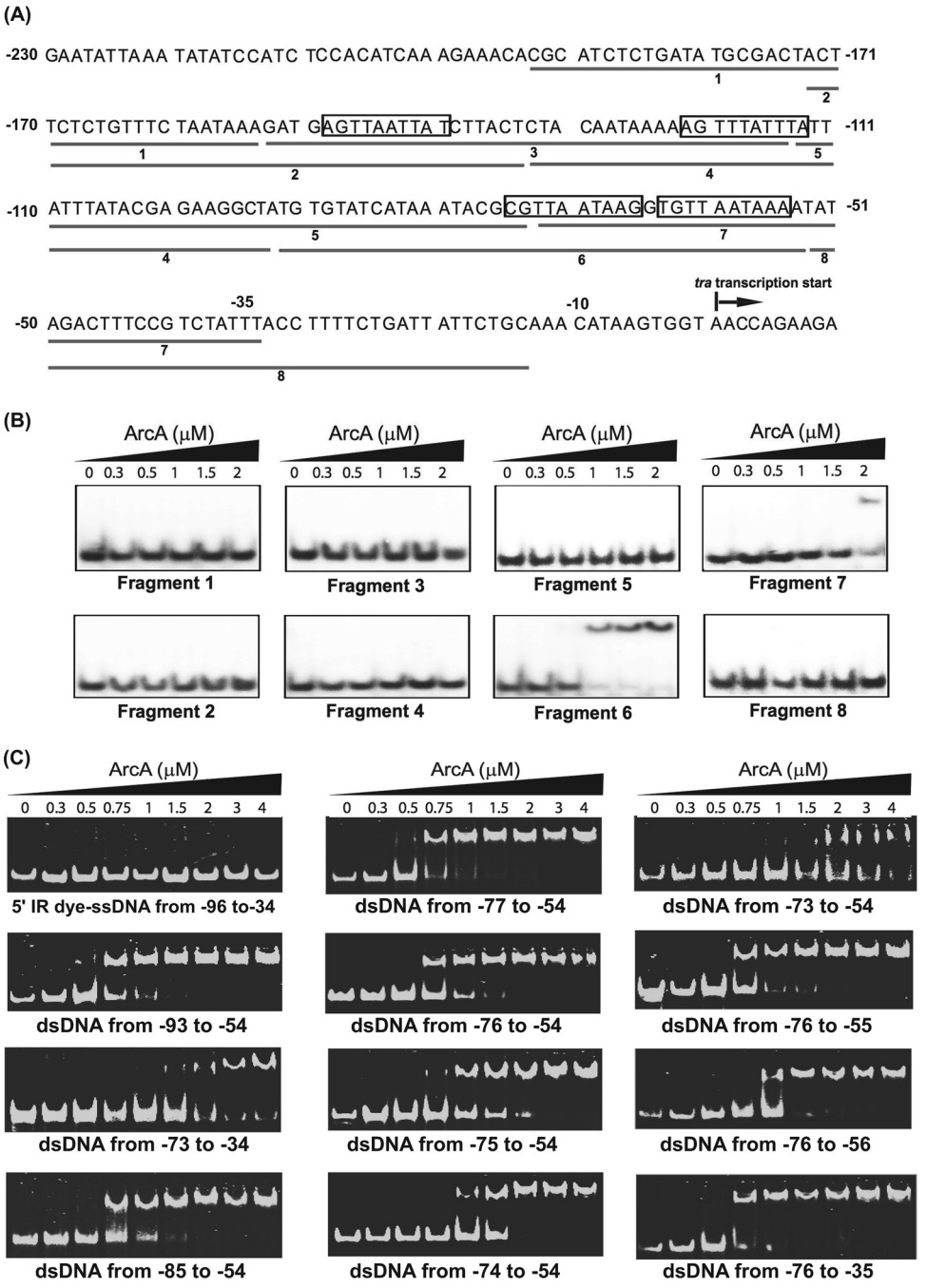

FIG 3 Location of ArcA-binding sequences in the regulatory region of the F plasmid P_Y. (A) Sequence of the F plasmid *tra* operon regulatory region. The rectangles highlight the sequences with ≥7-bp matches to the consensus 10-bp ArcA-binding motif (T/A)GTTAATTA(A/T). The bars under the sequence represent the eight 40-bp DNA fragments (1 to 8) scanned through the regulatory region for EMSA of ArcA binding. (B) Locating the ArcA-binding sequence in the F plasmid P_Y regulatory region by EMSA. Increasing ArcA protein concentrations are shown above each gel. Eight ^{32}P-labeled 40-bp dsDNA fragments (fragments 1 to 8) covering the P_Y regulatory region up to position −193 were used in EMSA, and the corresponding DNA fragments used are indicated below each gel. (C) Defining the minimal dsDNA in the P_Y regulatory region for optimal ArcA binding by EMSA. Various ssDNA fragments were annealed to a 5′ infrared (IR) dye-labeled 63-bp ssDNA corresponding to positions −96 to −34 of the P_Y regulatory region (as in panel A) to generate partially double-stranded DNA molecules (with the dsDNA region indicated under each gel) for EMSA. Increasing ArcA protein concentrations are indicated at the top.

region. Only fragment 6 (from −93 to −54 of P_Y), which contains a tandem repeat of two 10-bp ArcA-binding motifs separated by a single base pair, bound ArcA efficiently, with an apparent K_d between 0.5 and 1.0 μM. The ArcA-binding affinity of fragment 6 is comparable to that of the 200-bp P_Y DNA containing all four predicted ArcA-binding

motifs (Fig. 3B), indicating that the fragment 6 contains the complete sequence sufficient for ArcA binding. Fragment 7 is the only other DNA fragment that bound to ArcA but at a significantly lower affinity, with a K_d of over 2 μM. Fragment 7 lacks the first 2 bp of the repeat, suggesting that a tandem repeat of the 10-bp DNA motif is important for ArcA binding.

To further define the minimal ArcA-binding sequence, we used EMSA to test ArcA binding to DNA with gradual truncations from either end of fragment 6 (Fig. 3C). We synthesized a 63-bp 5′ infrared (IR) dye-labeled single-stranded DNA (ssDNA) corresponding to positions −96 to −34 of P_Y, and annealed it with various lengths of unlabeled complementary ssDNA fragments to generate partially double-stranded DNA with a 5′ IR dye label. These DNA fragments with partially double-stranded regions were used for EMSA. ArcA does not bind the 63-bp IR dye-labeled ssDNA (Fig. 3C); therefore, the ability of the annealed partially double-stranded DNA fragments to bind ArcA would be determined by their double-stranded regions. The IR dye-labeled DNA that contains the same 40-bp dsDNA region as fragment 6 (positions −93 to −54) (Fig. 3B) bound to ArcA with a K_d at ~0.75 μM whereas the DNA that contains the fragment 7 dsDNA region (positions −73 to −34) bound to ArcA with a K_d between 2.0 and 3.0 μM. These tests indicated that results of EMSAs using the IR dye-labeled dsDNA substrate paralleled those using the radioactive dsDNA substrate (Fig. 3B and C). Gradual reductions of the 5′ dsDNA regions from −93 to −76 did not affect ArcA binding; however, further 5′-end progressive reductions of dsDNA regions from −76 to −73 resulted in progressively lower ArcA-binding affinity. On the 3′ end, reducing the dsDNA region from −54 to −55 did not impair ArcA binding; however, removal of one more base pair to −56 resulted in decreased ArcA-binding affinity. Extending the double-stranded region on the 3′ end to −35 did not increase the ArcA-binding affinity. These results indicate that ArcA requires a minimal 22-bp direct repeat dsDNA sequence (−76 to −55) in the P_Y regulatory region for optimal DNA binding.

The ArcA-TraJ binding site is critical for P_Y activation and release from silencing. H-NS is a transcriptional silencer of the F *tra* operon whereas TraJ has been suggested to activate P_Y by serving as a desilencer (9, 10). To evaluate regulation of P_Y activity by TraJ, ArcA, and H-NS, we assayed the β-galactosidase (LacZ) activities of a P_Y activity reporter plasmid, pJLAC101-P_Y, and its derivative plasmids with gradual truncations of the upstream regulatory region of P_Y (Fig. 4A). Plasmid pJLAC101-P_Y is renamed as pP_{Y715} here to indicate that it contains P_Y and its upstream regulatory region to position −715 (the BglII site at the beginning of the *traJ* coding region) (Fig. 1A).

In a TraJ$^-$ and/or ArcA$^-$ strain (without TraJ- and ArcA-dependent P_Y activation), each construct from pP_{Y715} to pP_{Y120} reported significantly lower P_Y activity in the presence of H-NS than in the absence of H-NS. Further truncations to −103 and beyond (pP_{Y103} to pP_{Y36}) eliminated this apparently H-NS-dependent difference (Fig. 4B), indicating that H-NS targets sequences upstream of −103 for P_Y silencing. Four truncations, from positions −715 to −250, −250 to −200, −164 to −141, and −120 to −103, resulted in stepwise increases of P_Y activity in the H-NS$^+$ strains, suggesting that these four regions function additively for P_Y silencing in an H-NS-dependent mechanism. In the absence of both H-NS-dependent P_Y silencing and TraJ/ArcA-dependent P_Y activation (TraJ$^-$ ArcA$^+$ H-NS$^-$), removal of the P_Y regulatory region from −715 to −103 still resulted in a nearly 10-fold increase of promoter activity. This observation suggests that the region targeted by H-NS is also acted on by some H-NS-independent P_Y-silencing mechanisms.

P_Y is fully activated only when both TraJ and ArcA are expressed (TraJ$^+$ ArcA$^+$), regardless of H-NS expression (Fig. 4B). Consistently, constructs pP_{Y103} and pP_{Y77}, which lack all sequences required for P_Y silencing, still reported ~2-fold-higher promoter activity with TraJ and ArcA present than without TraJ and/or ArcA. These results demonstrate that TraJ and ArcA together can not only relieve P_Y from silencing but also directly activate P_Y. Gradual truncations of the P_Y regulatory region from −715 (pP_{Y715}) to −77 (pP_{Y77}) did not affect P_Y activation, but a further truncation to −64 (pP_{Y64})

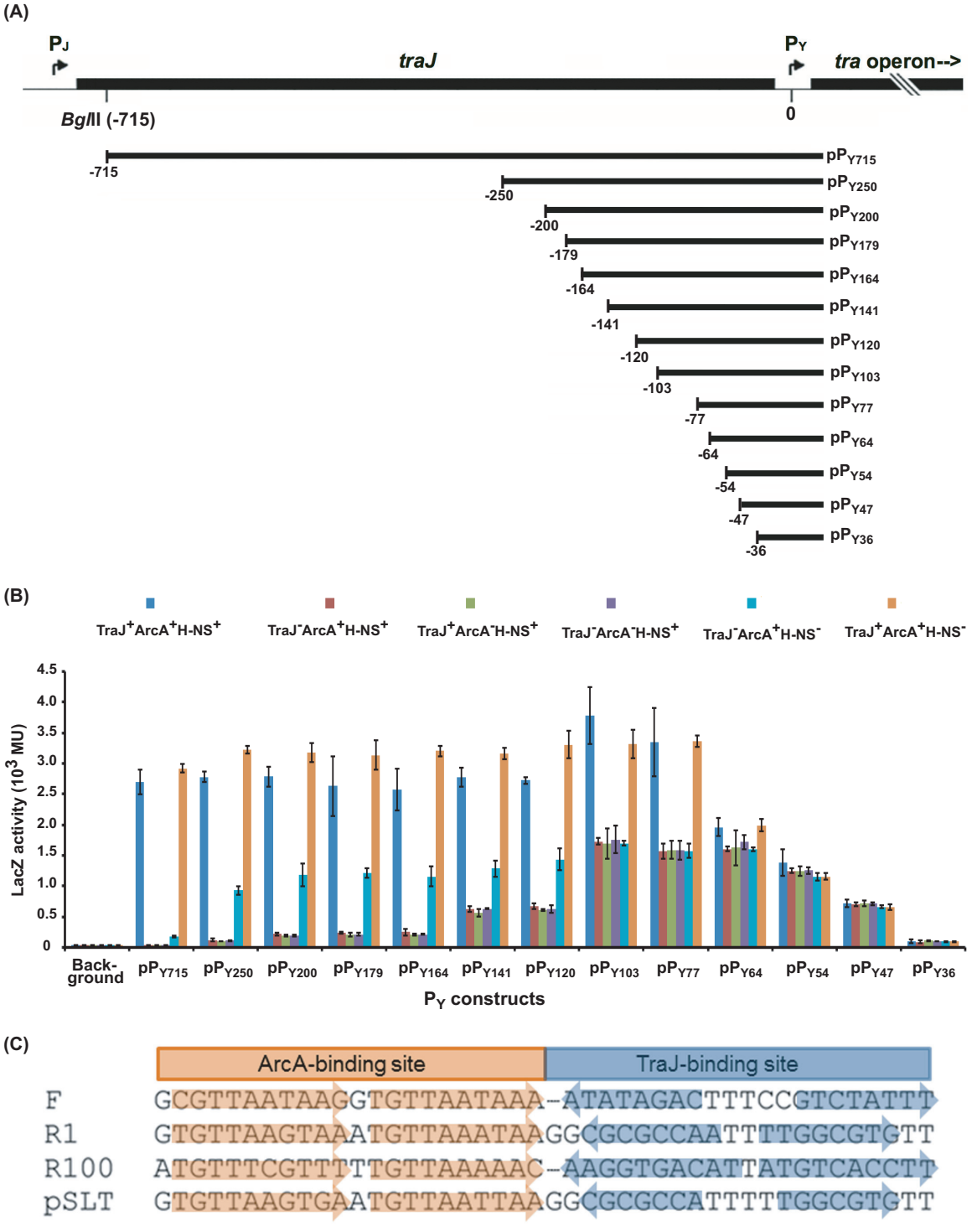

FIG 4 Effects of TraJ, ArcA, and H-NS on the activity of the *tra* operon promoter P_Y. (A) A set of promoter activity reporter plasmids for the F plasmid P_Y with various lengths of its upstream regulatory regions. (B) A bar graph representation of the promoter activities of the F plasmid P_Y with various lengths of its regulatory regions in strains, with TraJ, ArcA, and/or H-NS selectively absent. The activity of P_Y is reported as the β-galactosidase (LacZ) activity in Miller units (MU), measured in various *E. coli* strains containing different P_Y reporter plasmids, as indicated. The LacZ activity of cells containing the vector pJLAC101 is shown as the background control. The mean values of promoter activity obtained from three independent assays ($n = 3$) are presented here, with error bars indicating standard deviations from the means. (C) Alignment of ArcA and TraJ binding sites in the P_Y regulatory regions of the F-family plasmids. The ArcA-binding site alignment is based on the minimal sequence of the F plasmid ArcA-binding site obtained through EMSA in this work (Fig. 3C). The TraJ-binding site alignment is based on the previous DNase I footprinting data of the F-like R100 plasmid TraJ (18). The bars with arrowheads indicate lengths and directions of repeat sequences within the TraJ- and ArcA-binding sites. The DNA sequences are from the F plasmid (GenBank accession number NC_002483) and the F-like plasmids R1 (GenBank accession number X13681), R100 (GenBank accession number NC_002134), and pSLT (GenBank accession number NC_003277).

mostly eliminated this TraJ- and ArcA-dependent effect. These observations suggest that TraJ and ArcA function cooperatively through sequences downstream of −77 for P_Y activation. Additional truncations beyond position −64 resulted in further decreases in P_Y activity and a complete loss of its dependence on the presence of TraJ, ArcA, or H-NS. These data indicate that the sequence downstream of −64 is important for P_Y activity through a mechanism unrelated to the one used by ArcA and TraJ.

A previous DNase I footprinting analysis located the F-like R100 plasmid TraJ-binding site to an inverted repeat upstream of the P_Y promoter (18), which happens to be immediately downstream of the predicated ArcA-binding site in the R100 plasmid, based on its DNA sequence homology to the 22-bp ArcA-binding site (from −76 to −55 of P_Y) in the F plasmid (Fig. 4C). Coincidently, there is also an inverted repeat sequence immediately downstream of the ArcA-binding site in other F-family plasmids (Fig. 4C), agreeing with our finding that both the ArcA- and TraJ-binding sites are located within position −77 of P_Y in the F plasmid (Fig. 4B). The putative TraJ-binding inverted repeats in the F, R1, and R100 plasmids share little sequence homology, consistent with the previous finding that TraJ exclusively acts on its cognate P_Y among these three plasmids. In contrast, the putative TraJ-binding repeats in R1 and pSLT plasmids are quite similar, explaining the cross-activity of TraJ between these two F-like plasmids (11, 16).

DISCUSSION

Both TraJ and ArcA are required for P_Y activation through cooperative binding at its upstream regulatory sequences. Expression of the *tra* operon is a prerequisite for conjugative transfer of the F and F-like plasmids (3, 4). In this work, we used a combination of mating assays and promoter activity analysis to study regulation of F plasmid *tra* operon expression by the two known activators, plasmid-encoded TraJ and host factor ArcA, and a known host-encoded silencer, H-NS (8, 10, 14). The native *tra* operon promoter P_Y is responsible for a majority of the *tra* operon expression and subsequent high-level F conjugation (Fig. 1B and C). Our study indicated that P_Y is inactivated by silencers, including H-NS, and that activation of P_Y requires not only TraJ but also ArcA (Fig. 1C). This stringent dependence of P_Y activation on both TraJ and ArcA apparently results from cooperative binding of the two proteins at adjacent sequences downstream of position −77 in the P_Y regulatory region (Fig. 2C, 3C, and 4B and C). This explains why removal of the ArcA-binding site (in P_{Y64} and P_{Y54}) is enough to abolish P_Y activation even when the TraJ-binding site is intact (Fig. 4B). Since TraJ and ArcA do not appear to have direct, stable protein-protein interactions (J. Lu and J. N. M. Glover, unpublished observations), they might achieve cooperative binding either through weak protein-protein interactions that occur only when the proteins are bound to DNA or through alterations in DNA structure, similar to how pairs of F and F-like pED208 plasmid TraM tetramers cooperatively bind to adjacent target DNA elements (39, 40).

Our *in vivo* analysis of the P_Y regulatory region defined several short sequences (positions −250 to −200, −164 to −141, and −120 to −103) required for H-NS-mediated P_Y silencing in an additive manner (Fig. 4B). These sequences are highly AT rich (Fig. 3A) and might serve as initial H-NS-binding sites for further nucleation downstream to block transcription initiation from P_Y. Such a mechanism was observed in the LEE5 promoter of enteropathogenic *Escherichia coli*, in which H-NS binds to AT-rich sequences upstream of −114 and nucleates to the promoter region through oligomerization (41).

Cooperative binding of ArcA and TraJ immediately upstream (from −76 to −34) of the P_Y promoter could certainly prevent extension of H-NS-nucleation into the P_Y promoter, resulting in desilencing effects. However, previous studies and our results have suggested that TraJ and ArcA also activate P_Y directly through a mechanism other than antagonism of P_Y silencers (Fig. 4B) (9, 11). Truncations from position −64 resulted in gradual decreases of P_Y activity irrespective of the presence of TraJ, ArcA, or H-NS (Fig. 4B), suggesting that this region is intrinsically important for P_Y activity and likely

to be specifically bound by the transcription initiation complex. TraJ is also predicted to bind within this region (from −54 to −34) (Fig. 4C), similar to the positioning of the catabolite activator protein (CAP) binding site within the *gal* promoter (42). Recent structural studies revealed that the close interaction of *Thermus thermophilus* transcription activator protein (TAP; a homolog of *Escherichia coli* CAP) with the RNA polymerase (RNAP) holoenzyme allows contacts between the activator and multiple regions of RNAP that drive transcriptional activation (43). It seems most likely that the role of ArcA might be indirect, whereby it acts to stabilize TraJ binding near the P_Y promoter. Such a dependency of the activation of the major *tra* operon promoter on both ArcA and TraJ potentially allows effective regulation of bacterial conjugation by various host and plasmid-encoded factors acting on either of these two proteins.

Implications of ArcA-independent *tra* transcription from promoters upstream of P_Y. Our genetic analysis revealed that the ArcA-independent *tra* expression originates from alternative promoters over 700 bp upstream of P_Y, which leads to low-level F conjugation in the absence of ArcA- and TraJ-mediated P_Y activation (Fig. 1B and C). This upstream read-through transcription is likely terminated before the *tra* operon by polar mutations such as the *traJ* amber mutation in JCFL90 or the *traJ* frameshift mutation in $pMJ_{fs}P_Y$ (Fig. 1B and C) through polar effects (44). This would explain the observation of ArcA-independent conjugative transfer only in strains carrying JCFL0 but not in strains carrying the JCFL0 derivative, JCFL90 (Fig. 1B). Read-through transcripts from *traM* and *traJ* into the *tra* operon have been detected by a previous Northern blot analysis (9), suggesting that the two upstream gene promoters P_M and P_J, which are known to be ArcA independent (18, 38), are likely responsible for the observed ArcA-independent *tra* expression.

Read-through transcription from upstream P_M and P_J may not contribute significantly to F plasmid-mediated bacterial conjugation when the major *tra* promoter P_Y is fully activated by TraJ and ArcA. However, this mechanism could potentially play a significant role in the dissemination of naturally transfer-repressed F-family plasmids via so-called high-frequency transfer or epidemic spread when a new recipient population is encountered, as previously observed (22, 23). In the F-family plasmids, *traM* expression is maintained at low levels due to TraM-mediated feedback repression of P_M (38, 45); however, P_M activity is extremely strong in the absence of TraM (46). When a plasmid is newly transferred into a bacterium, temporary lack of TraM could result in high levels of read-through transcripts from *traM* into *traJ* and the downstream *tra* operon. This could lead to transient overexpression of TraJ and the *tra* operon proteins before establishment of TraM auto-repression and FinOP inhibition in the new transconjugant. This temporary high-level expression of *tra* proteins in the new transconjugant might be enough to support several generations of high-frequency conjugative transfer. Such a mechanism of epidemic spread could allow naturally transfer-repressed plasmids to quickly disseminate throughout a new recipient population. After this initial spread, the establishment of the *tra* repression system would reduce *tra* expression and thereby reduce the metabolic burden of the plasmid on the host cell.

MATERIALS AND METHODS

Growth media and bacterial strains. Cells were grown in LB (Luria-Bertani) broth or on LB solid medium unless otherwise specified. Antibiotics were used at the following final concentrations: ampicillin (Amp), 50 μg/ml; kanamycin (Km), 25 μg/ml; streptomycin (Sm), 200 μg/ml; spectinomycin (Spc), 100 μg/ml. The following *Escherichia coli* strains were used: MC4100 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150* (Sm^r) *relA1 flb5301 deoC1 ptsF25 rbsR*] (47), ED24 (F$^-$ Lac$^-$ Spc^r) (48), BW25113 [F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*] (49), JW4364 (F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514* Δ*arcA726*::Kan] (49), JW1225 [F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*hns-746*::Kan *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*] (49), DH5α [F$^-$Δ*lacU169* (ϕ80d*lacZ*ΔM15) *supE44 hsdR17 recA1 endA1 gyrA96* (Nal^r) *thi-1 relA1*] (50), NEB 10-β [Δ(*ara-leu*)*7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14*− ϕ80d*lacZ*ΔM15 *recA1 relA1 endA1 nupG rpsL* (Str^r) *rph spoT1* Δ(*mrr-hsdRMS-mcrBC*)] (New England Biolabs), and BL21(DE3) [F$^-$ *ompT hsdSB*(r_B^- m_B^-) *gal dcm*] (Invitrogen).

Plasmid construction. All plasmids and PCR primers used for plasmid construction and the resulting constructs are listed in Table 1. The F plasmid *traJ* was PCR amplified from pRS27 (51) by using primers JLU307 and JLU308B or primers JLU356B and JLU308. An 0.8-kb EcoRI-NcoI fragment of *traJ* PCR

TABLE 1 Plasmids and oligonucleotides used for plasmid construction

Plasmid or oligonucleotide	Description or sequence	Reference or source
Plasmids		
JCFL0	A wild-type F plasmid derivative, F′ *lac*	54
JCFL90	A *traJ*-defective JCFL0 derivative with an amber mutation in *traJ*	54
pAC-TraJ	The F plasmid *traJ* coding region and its own ribosome binding site cloned downstream of the *cmlR* gene promoter in pACYC184	This work
pACYC184	Tc^r; cloning vector	37
pJLAC101	A transcriptional fusion-based promoter assessment plasmid; Cm^r Amp^r	10
pJLAC101-P_Y	P_Y with its upstream regulatory region to −715 cloned as a transcriptional fusion to *lacZ* in pJLAC101; also called pP_{Y715} in this work	15
pJLJ356	His_6-tagged F plasmid *traJ* cloned in pK184	This work
pJLOY401	pBluescript KS^+ with an F fragment from *oriT* to P_{traY}	10
pK184	A P15a replicon-based cloning vector; Km^r	52
$pMJP_Y$	The F plasmid fragment from *oriT* to the beginning of *traY* cloned as a transcriptional fusion to *lacZ* in pJLAC101	This work
$pMJ_{fs}P_Y$	A pJLAC101-MJP_Y derivative with a +1 frameshift mutation at the BglII site in the *traJ* coding region	This work
pP_{Y250}	P_Y with its upstream regulatory region to −250 cloned in pJLAC101	This work
pP_{Y200}	P_Y with its upstream regulatory region to −200 cloned in pJLAC101	This work
pP_{Y179}	P_Y with its upstream regulatory region to −179 cloned in pJLAC101	This work
pP_{Y164}	P_Y with its upstream regulatory region to −164 cloned in pJLAC101	This work
pP_{Y141}	P_Y with its upstream regulatory region to −141 cloned in pJLAC101	This work
pP_{Y120}	P_Y with its upstream regulatory region to −120 cloned in pJLAC101	This work
pP_{Y103}	P_Y with its upstream regulatory region to −103 cloned in pJLAC101	This work
pP_{Y77}	P_Y with its upstream regulatory region to −77 cloned in pJLAC101	This work
pP_{Y64}	P_Y with its upstream regulatory region to −64 cloned in pJLAC101	This work
pP_{Y54}	P_Y with its upstream regulatory region to −54 cloned in pJLAC101	This work
pP_{Y47}	P_Y with its upstream regulatory region to −47 cloned in pJLAC101	This work
pP_{Y36}	P_Y with its upstream regulatory region to −36 cloned in pJLAC101	This work
pRS27	An F plasmid fragment from *oriT* to *traV* cloned in pSC101	51
pT7-7	Protein expression vector. pMB1 replicon; Amp^r	53
pT7-ArcA	*E. coli arcA* cloned in pT7-7	This work
Oligonucleotides		
JLU307	ATAGAATTCGTGAGGAGGTTCCTATGTATCCG	
JLU308	TATGGATCCCTTCTGGTTACCACTTATGTTTGCAG	
JLU308B	TATCCATGGCTTCTGGTTACCACTTATGTTTGCAG	
JLU338	TAGAATTCACCATCACCATCACCATGAGAACCTGTACTTCCAAGGACAGACCCCGCACATTCTTATCGTTG	
JLU339	TAGAATTCACCATCACCATCACCATGAGAACCTGTACTTCCAAGGACAGACCCCGCACATTCTTATCGTTG	
JLU356B	ATAGAATTCGAGGAGGTTCCTATGCACCATCACCATCACCATGAGAACCTGTACTTCCAAGGAATAGATCT GCTGGAAAATCTGACGGC	
JLU366	TAAGATCTCGCGTTAATAAGGTGTTAATAAAATATAGACTTTCCG	
JLU367	ATGGTACCTGTTGCAGAACGTGTACCAAATCTTTTCAATAACAC	
JLU368	AAAGATCTTTCCGTCTATTTACCTTTTCTGATTATTCTGC	
JLU371	ATATAGATCTCGAGAAGGCTATGTGTATCATAAATACGCG	
JLU371B	ATATAGATCTTACTCTACAATAAAAAGTTTATTTATTATTTATACGAGAAGGCTATGTGTATCATAAATACGCG	
JLU371C	ATATAGATCTTTTATTTATTATTTATACGAGAAGGCTATGTGTATCATAAATACGCG	
JLU372	ATATAGATCTGCGACTACTTCTCTGTTTCTAATAAAGATGAG	
JLU372B	ATATAGATCTTTCTAATAAAGATGAGTTAATTATCTTACTCTACAATAAAAAGTTTATTTATTATTTATACGAG AAGGC	
JLU373	ATATAGATCTATATAGACTTTCCGTCTATTTACCTTTTCTGATTATTCTGC	
JLU374	ATATAGATCTTTTACCTTTTCTGATTATTCTGCAAACATAAGTGGTAACC	
JLU375	ATATAGATCTGTTAATAAAATATAGACTTTCCGTCTATTTACCTTTTCTGATTATTCTGC	
JLU385	ATATAGATCTAGAAACACGCATCTCTGATATGCGAC	
JLU386	ATATAGATCTAGTCATAATGCTATAGCAAGAATATTAAATATATCCATCTCC	

amplified using primers JLU307 and JLU308B was ligated to the 3.9-kb EcoRI-NcoI fragment of pACYC184 (37), resulting in plasmid pAC-TraJ. An 0.8-kb EcoRI-BamHI fragment of *traJ* PCR amplified using primers JLU356B and JLU308 was ligated to the 2.4-kb EcoRI-BamHI fragment of pK184 (52), resulting in plasmid pJLJ356. The 1.8-kb BamHI-KpnI fragment from pJLOY401 (10) was ligated to the 9.5-kb BglII-KpnI fragment of pJLAC101 (10), resulting in plasmid $pMJP_Y$. The plasmid $pMJ_{fs}P_Y$ was constructed by BglII digestion of $pMJP_Y$, blunting the sticky ends with Klenow fragments (Roche Diagnostics), and religation of the blunted fragment. The primers used to amplify the F plasmid DNA to be cloned in different P_Y constructs were the following: JLU386 and JLU367 for pP_{Y250}, JLU385 and JLU367 for pP_{Y200}, JLU372 and JLU367 for pP_{Y179}, JLU372B and JLU367 for pP_{Y164}, JLU371B and JLU367 for pP_{Y141}, JLU371C and JLU367 for pP_{Y120}, JLU371 and JLU367 for pP_{Y103}, JLU366 and JLU367 for pP_{Y77}, JLU375 and JLU367 for pP_{Y64}, JLU373 and JLU367 for pP_{Y54}, JLU368B and JLU367 for pP_{Y47}, and JLU374 and JLU367 for pP_{Y36}. *E. coli arcA* was PCR amplified from *E. coli* 10-β chromosomal DNA by using primers JLU338 and JLU339. A 0.7-kb EcoRI-BamHI fragment of *arcA* PCR amplified using primers JLU338 and JLU339 was ligated to the 2.5-kb EcoRI-BamHI fragment of pT7-7 (53), resulting in plasmid pT7-ArcA.

Donor ability assays. *E. coli* MC4100 and ED24 were used as donor and recipient strains, respectively. MacConkey agar plates were used to detect cells containing F′ *lac* plasmids (JCFL0 and JCFL90) (54). The mating experiments were performed as previously described (55). Donor ability was calculated as the number of transconjugants divided by the number of donors. All mating assays were repeated three times, with the results being within 1 log unit of each other.

β-Galactosidase assays. A fresh, single colony was inoculated into 2 ml of LB broth containing appropriate antibiotics and grown at 37 °C with shaking for 3 h. A 50- to 200-μl sample was used for determining β-galactosidase activity as described by Miller (56), and activity was reported as Miller units (MU). For each bacterial stain, three to four colonies were assayed independently.

Protein expression and purification. As TraJ forms inclusion bodies when overexpressed in *E. coli* cells (57), we expressed TraJ in *E. coli* at low levels close to its physiological concentrations as previously described (16). Briefly, DH5α cells containing pJLJ356 were grown in 6 liters of LB broth at 37°C for 5 h with vigorous shaking before being harvested by centrifugation. Purification of the His_6-tagged TraJ and further cleaving of the His_6 tag were performed as previously described for purification of the TraJ N-terminal domain (16). Protein concentrations were determined by using bicinchoninic acid (BCA) protein assays (Pierce) according to the manufacturer's instructions.

To overexpress His_6-tagged ArcA, BL21(DE3) cells containing pT7-ArcA were grown in 2 liters of LB broth at 37°C with vigorous shaking. After 3 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was grown for another 16 h at 20°C before being harvested by centrifugation. The cell pellet was suspended in 80 ml of lysis buffer (50 mM Tris-HCl, 10 mM imidazole, 250 mM NaCl, 10% glycerol, pH 8.0) with one tablet of complete, EDTA-free, protease inhibitor cocktail (Roche Applied Science). The suspension was lysed by sonication on ice for 3 min (30 s with a 30-s break, repeated 6 times) at maximum output. After centrifugation at 27,000 × *g* for 60 min, the supernatant was loaded on a column with 2 ml of Ni-nitrilotriacetic acid (Ni-NTA)–agarose (Qiagen) preequilibrated with 20 ml of lysis buffer. After a washing step with 30 ml of wash buffer (50 mM Tris-HCl, 20 mM imidazole, 250 mM NaCl, 10% glycerol, pH 8.0), the protein bound to the Ni-NTA–agarose was eluted with 10 ml of elution buffer (50 mM Tris-HCl, 250 mM imidazole, 250 mM NaCl, 10% glycerol, pH 8.0) in 2-ml fractions. The fractions containing His-tagged ArcA were loaded onto a 25-ml cation exchange column (SP Sepharose Fast Flow; GE Healthcare Life Sciences) and eluted with 50 mM malonic acid, pH 5.5, with a NaCl concentration gradient from 0 to 1 M. The ArcA fractions were further loaded onto size exclusion chromatography columns (Hiload 26/60 Superdex 75 prep-grade column; Amersham Biosciences), and proteins were eluted with SEC buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 8.0). The fractions containing His-tagged ArcA were pooled and mixed with 300 units of AcTEV protease (Invitrogen) at room temperature for 24 h to cleave the His_6 tag. The digested mixture was loaded on a column with 2 ml of Ni-NTA–agarose (Qiagen) preequilibrated with 20 ml of lysis buffer. The flowthrough and the wash flowthrough with 10 ml of wash buffer, which contained tagless ArcA, were collected and buffer was changed to 0.5 M ammonium acetate, 10% glycerol, and 5 mM dithiothreitol (DTT) by using an Amicon ultracentrifuge filter (Millipore). ArcA concentration was determined by using BCA protein assays (Pierce) according to the manufacturer's instructions. ArcA-K89E:E94K and ArcA-I90D:L93D were purified similarly.

Preparation of DNA for EMSA. Chemically synthesized or PCR-amplified DNA fragments were used for EMSA in this work. All oligonucleotides used for preparing various dsDNA fragments are available on request. The DNA fragment containing P_Y and its upstream regulatory region to −179 was PCR amplified by using primers JLU336 (CAAAGAAACACGCATCTCTGATATGCG) and JLU337 (CCGCTGTTTATCTTCTGGT TACCAC). The amplified DNA was run on an agarose gel and purified by using a QIAquick Gel Extraction kit (Qiagen). Synthetic DNA oligonucleotides were purified and annealed into dsDNA as previously described (58). The DNA fragments were radiolabeled as previously described (39). IR dye-labeled partially double-stranded DNA fragments were generated by annealing corresponding oligonucleotides to the 5′ IR dye-labeled oligonucleotide 3763.

Electrophoretic mobility shift assays (EMSAs). The binding reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1 μg of poly(dI–dC) (Roche Diagnostics), and 1 nM ^{32}P-labeled DNA or 5 nM IR dye-labeled DNA, with a final volume of 9 μl. After addition of 1 μl of a specified amount of purified ArcA and/or TraJ protein, each reaction mixture was incubated at 30°C for 30 min. The resulting mixture was loaded onto a 12% polyacrylamide gel (buffered with 90 mM Tris-borate, 1 mM EDTA [TBE]) and run in TBE buffer at 4°C and 40 volts for 3 h. The radiolabeled DNA and DNA-protein complexes were visualized and quantified by using phosphorimaging plates and the program ImageQuant (Molecular Dynamics). The IR dye-labeled DNA and DNA-protein complexes were visualized by using an Odyssey imaging system (Li-Cor Biosciences). Apparent K_d was estimated as the concentration of protein added when there was 50% free DNA left.

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