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Regulation of Bacterial Conjugation in Microaerobiosis by Host-Encoded Functions ArcAB and SdhABCD

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ABSTRACT

The virulence plasmid of *Salmonella enterica* (pSLT) is an F-like conjugative plasmid. High rates of pSLT transfer occur in the mammalian gut, a microaerobic environment. In this study, we describe genetic screens for host-encoded activators and repressors of the transfer operon (*tra*) of pSLT. We show that the transcription factor ArcA is an activator of conjugation, especially under microaerobiosis. In turn, succinate dehydrogenase (SdhABCD) is a repressor of mating in aerobiosis. ArcA binds upstream of the main *tra* promoter (p_{traY}) and activates *tra* transcription, as previously described in F, R1, and R100. In the absence of ArcA, transfer of pSLT decreased 7-fold in aerobiosis and >100-fold in microaerobiosis. In aerobiosis, ArcA activates the *traY* promoter in an ArcB-independent manner, as described in other F-like plasmids. In microaerobiosis, however, the ArcB sensor is necessary for activation of p_{traY}. Lack of Sdh causes a >20-fold increase in pSLT transfer in aerobiosis, but has little effect under microaerobiosis. Sdh inhibits conjugal transfer by reducing *traJ* transcription, probably in an indirect manner. In turn, the *sdhCDAB* operon is repressed by the ArcAB system under microaerobiosis. Hence, the ArcAB two-component system of *S. enterica* stimulates pSLT transfer under microaerobiosis by two concerted actions: activation of the *tra* operon and repression of the *sdhCDAB* operon.

THE F-like plasmid family includes a large number of conjugative plasmids whose most conspicuous member is the F sex factor (Willetts and Skurray 1980). Plasmids harboring an F-like conjugation system fall into several incompatibility groups and determine a wide range of phenotypes including antibiotic resistance, colicin production, and synthesis of virulence factors such as enterotoxins and hemolysin (Willetts and Skurray 1980). Six decades of research on F-mediated conjugation have provided an exquisite picture of the mating process and a detailed knowledge of many of the gene products involved (Frost *et al.* 1994; Firth *et al.* 1996; Lawley *et al.* 2003).

F relatives such as the antibiotic resistance plasmids R1 (Meynell and Datta 1966) and R100 (Nakaya *et al.* 1960) and the virulence plasmid of *Salmonella enterica* (Smith *et al.* 1973; Spratt and Rowbury 1973) have also played historic roles in the study of bacterial conjugation. The virulence plasmid of *S. enterica* (known as pSLT in serovar Typhimurium) is an F-like plasmid whose conjugation system is closely related to those of F and R100 (Rotger and Casadesus 1999). Transfer of pSLT occurs at low frequency in batch cultures (Ahmer *et al.* 1999; Camacho and Casadesus 2002) and becomes derepressed in the ileum of infected mice (Garcia-Quintanilla *et al.* 2008). High osmolarity and microaerobiosis, two reductionist conditions for imitation of the intestinal environment, derepress pSLT transfer in the laboratory (Garcia-Quintanilla *et al.* 2008).

Regulatory mechanisms that control expression of the conjugal gene cluster in the F episome and other F-like plasmids have been known since the 1970s (Willetts and Skurray 1980). Some such mechanisms rely on regulatory elements encoded on the plasmid itself. For instance, synthesis of TraJ, the main transcriptional activator of the *tra* operon, is controlled by the FinOP system of fertility inhibition (Frost *et al.* 1994; Firth *et al.* 1996). Regulatory feedback loops involving the TraM, TraJ, and TraY plasmid products also contribute to autogenous control of *tra* operon expression (Penfold *et al.* 1996; Polzleitner *et al.* 1997; Stockwell *et al.* 2000).

The circuitry that governs mating involves also host-encoded functions, and the known controls involve both transcriptional and post-transcriptional regulation. Examples of transcriptional regulators are the transcription factors ArcA (Silverman *et al.* 1991; Strohmaier *et al.* 1998), CRP (Starcic *et al.* 2003), and Lrp (Camacho and Casadesus 2002; Starcic-Erjavec *et al.* 2003; Camacho *et al.* 2005a) and the nucleoid protein H-NS (Will *et al.* 2004; Camacho *et al.* 2005b). The DNA binding capacity of some such factors is in turn controlled

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by the DNA methylation state of critical DNA regions (Camacho and Casadesus 2002, 2005). Post-transcriptional control of *tra* operon expression has been shown to involve the RNA chaperone Hfq (Will and Frost 2006), the GroEL heat-shock chaperone (Zahrl *et al.* 2007), and the extracytoplasmic stress CpxAR system (Gubbins *et al.* 2002; Zahrl *et al.* 2006; Lau-Wong *et al.* 2008). Some host-encoded regulators may control conjugal transfer in most (perhaps all) F-like plasmids while others may be plasmid specific. For instance, F transfer undergoes a drastic decrease in stationary phase (Frost and Manchak 1998), a behavior that is not observed in pSLT (Camacho *et al.* 2005b). Another example involves the leucine-responsive regulatory protein, which is an activator of *traJ* transcription in pSLT (Camacho and Casadesus 2002) but not in R100 (Starcic-Erjavec *et al.* 2003). Adaptation to the host lifestyle and adjustment of conjugal transfer to favorable circumstances can be postulated as tentative explanations for these differences and for others that may exist.

The identification of host-encoded regulators of plasmid transfer is amenable to classical genetics, as initially shown for the F sex factor (Silverman *et al.* 1980, 1991) and later for pSLT (Torreblanca and Casadesus 1996; Camacho and Casadesus 2002; Camacho *et al.* 2005b). On the basis of these antecedents, below we describe genetic trials for host-encoded activators and host-encoded repressors of the pSLT *tra* operon. All screens involved visual scrutiny, distinguishing between Lac^+ and Lac^- colonies. The general layout of the screens was that mutations that decreased *tra* operon expression would identify activators, and mutations that increased *tra* expression would identify repressors. The trials were expected to reveal mutations that altered *tra* operon expression in aerobiosis and also in microaerobiosis, because the centers of Salmonella colonies become microaerobic during growth (Aliabadi *et al.* 1986; Wei and Miller 1999). We show that ArcAB, a two-component system that regulates gene expression in response to the availability of oxygen (Lynch and Lin 1996), is a key factor for the activation of pSLT transfer under microaerobiosis. Aerobic transfer of pSLT is also regulated by ArcA but in an ArcB-independent manner, as previously described in F (Beutin and Achtman 1979; Buxton and Drury 1984). We also show that the ArcAB system plays a second role in the activation of pSLT transfer under microaerobiosis: repression of the *sdhCDAB* operon, which encodes succinate dehydrogenase. Succinate dehydrogenase (SdhABCD) turns out to be an inhibitor of conjugation and represses *traJ* expression, probably in an indirect manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and strain construction: The strains of *S. enterica* used in this study (Table 1) belong to serovar Typhimurium and derive from strain LT2. For simplicity, *S. enterica* serovar Typhimurium is often abbreviated as *S. enterica*. The phagemid pBluescript II SK(+) and the *Escherichia coli* B derivative BL21 [F^- *dcm ompT hsdS*(rB^- mB^-) *gal* [$malB^+$]K-12(λS)] are products of Stratagene (La Jolla, CA). Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger 1972; G. Roberts, unpublished data) were used for strain construction operations involving chromosomal markers. The transduction protocol was described elsewhere (Garzon *et al.* 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

Media, chemicals, and growth conditions: E medium (Vogel and Bonner 1956) was used as minimal medium for *S. enterica*. The rich medium was Luria–Bertani (LB). Solid media contained agar at 1.5% final concentration. Green plates were prepared according to the original recipe (Chan *et al.* 1972), except that methyl blue (Sigma-Aldrich, St. Louis) substituted for aniline blue. 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) was also from Sigma-Aldrich. Antibiotics were used at the final concentrations described elsewhere (Garzón *et al.* 1996). YT liquid medium, used for production of recombinant GST-ArcA protein, contained tryptone (16 g/liter), yeast extract (10 g/liter), glucose (5 g/liter), and ampicillin. Microaerobic conditions for culture on solid media were created using GasPak incubation jars (Becton Dickinson Biosciences, San Agustín de Guadalix, Spain). For liquid cultures, microaerobiosis was achieved by incubation without shaking. Neither GasPak jars nor static incubation produce strict anaerobiosis; for this reason, the term "microaerobiosis" is used.

Diethyl sulfate mutagenesis: Fifty microliters of diethyl sulfate (DES) were dissolved in 5 ml of liquid E medium without carbon source and kept at 37° during 10 min in a screw-capped tube. One hundred microliters of a bacterial suspension ($\sim 10^8$ bacterial cells) was then added. The treatment was allowed to proceed at 37° during 30 min, without shaking. Two hundred microliters of DES-treated bacterial suspension was then used to start a liquid culture in LB medium. When the culture reached saturation, aliquots were spread on LB medium supplemented with X-gal. Use of the *traB1::lac* fusion permits the detection of mutations affecting the expression of *tra, traJ,* or *finP* (Camacho and Casadesus 2002; Camacho *et al.* 2005b). In fact, amplification down the regulatory cascade facilitates the discrimination of changes, even if subtle, in the expression of either *finP* or *traJ* (Camacho *et al.* 2005b). DES-induced mutations were transferred from strain to strain using a cotransducible Tn*10d*Cm element, as previously described (Camacho *et al.* 2005b). Whenever a Tn*10d*Cm insertion was linked (>60%) to the point mutation, the boundaries of the Tn*10d*Cm element were sequenced (Torreblanca *et al.* 1999). Primers for chromosome walking and serial DNA sequencing were designed on the basis of such sequences. The identification of point mutations was achieved by DNA sequence alignment, using the LT2 genome database (McClelland *et al.* 2001).

Tn10*d*Cm mutagenesis: The pSLT-cured strain SV3081 was mutagenized with Tn10*d*Cm as previously described (Torreblanca and Casadesus 1996). Pools of 5000 colonies, each carrying an independent Tn10*d*Cm insert, were then prepared and lysed with phage P22 HT. The lysates were used to transduce either SV3003 or SV3069, selecting chloramphenicol-resistant transductants on LB plates supplemented with X-gal. Candidates were made phage free and reconstructed by P22 HT transduction (Torreblanca and Casadesus 1996).

Construction of *S. enterica arcA, arcB*, and s*dhA* mutants by gene targeting: Targeted disruption of genes in the *S. enterica* chromosome was achieved by adapting to *S. enterica* a method

TABLE 1

Strains of *Salmonella enterica* serovar Typhimurium

Strain	Genotype	Reference or source
LT2	Wild type	SGSC[a]
SV3003	Φ*traB1*::Mu*dJ*	Torreblanca and Casadesus (1996)
SV3069	*dam-201*::Tn*10d*Tc Φ*traB1*::Mu*dJ*	This study
SV3081	pSLT$^-$	Torreblanca *et al.* (1999)
SV3083	pSLT$^-$ *dam-201*::Tn*10d*Tc	Torreblanca *et al.* (1999)
SV3109	*hisO1242 pdx-543 serC*::Tn*10d*Tc	Mouslim *et al.* (2000)
SV4201	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km	Camacho and Casadesus (2002)
SV4500	*arcA*(G76A) *sthE*::Tn*10d*Cm Φ*traB1*::Mu*dJ*	This study
SV4508	Δ*finO*	Camacho and Casadesus (2002)
SV4509	Δ*finO* Φ*traB1*::Mu*dJ*	Camacho and Casadesus (2002)
SV4519	Δ*finO dam-201*::Tn*10d*Tc Φ*traB1*::Mu*dJ*	Camacho and Casadesus (2002)
SV4522	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km Δ*finO*	Garcia-Quintanilla *et al.* (2008)
SV4761	Φ*traJ::lacZ*	Camacho *et al.* (2005b)
SV4839	Φ*finP::lacZ*	Camacho *et al.* (2005b)
SV4914	Φ*traJ::lacZ dam-201*::Tn*10d*Tc	E. M. Camacho
SV5067	*arcA*::Cm^r	This study
SV5068	*arcB*::Cm^r	This study
SV5608	*sdhA*::Cm^r	This study
SV5867	Φ*sdhB::lacZ*	R. Balbontín
SV5868	*arcA*::Cm^r Φ*sdhB::lacZ*	This study
SV5986	*sdhA*::Cm^r Φ*traB1*::Mu*dJ*	This study
SV5987	*sdhA*::Cm^r Φ*traB1*::Mu*dJ* Δ*finO*	This study
SV6052	Φ*traJ::lacZ* Δ*finO*	This study
SV6053	Φ*traJ::lacZ* Δ*finO* Δ*sdhA*	This study
SV6054	Φ*traJ::lacZ* Δ*finO* Δ*sdhA arcA*::Cm^r	This study
SZ102	*arcA*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ103	*arcB*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ104	*arcA*::Cm^r Φ*traB1*::Mu*dJ dam-201*::Tn*10d*Tc	This study
SZ105	*arcB*::Cm^r Φ*traB1*::Mu*dJ dam-201*::Tn*10d*Tc	This study
SZ106	Δ*finO arcA*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ107	Δ*finO arcB*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ108	*dam-201*::Tn*10d*Tc Δ*finO arcA*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ109	*dam-201*::Tn*10d*Tc Δ*finO arcB*::Cm^r Φ*traB1*::Mu*dJ*	This study
SZ110	*hisI9960*::Mu*d*1-8 *spvA*::Tn5dKm *arcA*::Cm^r	This study
SZ111	*hisI9960*::Mu*d*1-8 *spvA*::Tn5dKm *arcB*::Cm^r	This study
SZ112	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km Δ*finO arcA*::Cm^r	This study
SZ113	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km Δ*finO arcB*::Cm^r	This study
SZ114	Φ*traJ::lacZ arcA*::Cm^r	This study
SZ115	Φ*traJ::lacZ arcB*::Cm^r	This study
SZ116	Φ*traJ::lacZ arcA*::Cm^r *dam-201*::Tn*10d*Tc	This study
SZ117	Φ*traJ::lacZ arcB*::Cm^r *dam-201*::Tn*10d*Tc	This study
SZ118	Φ*finP::lacZ arcA*::Cm^r	This study
SZ119	Φ*finP::lacZ dam-201*::Tn*10d*Tc	This study
SZ120	Φ*finP::lacZ arcA*::Cm^r *dam-201*::Tn*10d*Tc	This study
SZ122	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km *sdhA*::Cm^r	This study
SZ123	*hisI9960*::Mu*d*1-8 *spvA*::Tn5*d*Km Δ*finO sdhA*::Cm^r	This study

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previously described in *E. coli* (Datsenko and Wanner 2000). Primers designed to eliminate specific DNA stretches were based on the LT2 nucleotide sequence (McClelland *et al.* 2001). When necessary, the kanamycin resistance cassette introduced by the gene targeting procedure was eliminated by recombination with plasmid pCP20 (Datsenko and Wanner 2000). Pairs of additional, external PCR primers were used to verify the predicted gene deletions. Gene-specific primers were designed using PRIMER3 software (http://primer3.sourceforge.net). Disruption of *arcA* was achieved with primers 5′ TAA CTT ACC GGC TGT TTT TAC AGT TTG GCG CCT GGG CCG AGT GTA GGC TGG AGC TGC C 3′ and 5′ TTG TAC TTC CTG TTT CGA TTT AGT TGG CAA TTT AGG TAG CCA TAT GAA TAT CCT CCT TAG 3′. Verification was performed with primers 5′ CGC AAG CTG AGA TAA ACA GC 3′ and 5′ GTC ATG TT CGC CGA TCA TG 3′. Primers for *arcB* disruption were 5′ TGG TGT TGG CGC AGT ATT CGC GCA CCC CGG TCA AAC CGG GGT GTA GGC TGG AGC TGC C 3′ and 5′ TAA TTG GGT ATT ATG TGC GAA GTT GTG GTG AAG GAA TCC TCA TAT GAA TAT CCT CCT TAG 3′. Primers for verification of *arcB* disruption were 5′ ACT GCG CCT TTG ACA TCA TC 3′ and 5′ CTG TAG CGT AGC GTG ATG AG 3′.

Primers 5′ TGT AAC CGA AGT CTT AAG GGA ATA ATA AGA ACA GCATGT GGT GTA GGC TGG AGC TGC TTC 3′ and 5′ AGA CTG TAC GTC GCC ATC CGG CAA CCA CTA CAA CTA CTT ACA TAT GAA TAT CCT CCT TAG 3′ were used for *sdhA* disruption. Strain SV5608 (SdhA$^-$) was verified with primers 5′ TGG CTA CAG GTA GAT TCA CC 3′ and 5′ CAC TTC TAT TGC CTG ATG GC 3′.

β-Galactosidase assays: Levels of β-galactosidase activity were assayed using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure (Miller 1972). To measure β-galactosidase activities below 10 Miller units, bacterial cell lysis was employed instead of permeabilization.

Construction and purification of a GST-ArcA fusion protein: The *S. enterica arcA* gene was PCR amplified using primers 5′ TTT GGA TCC TAT TAG GTG TCC GGT ACG TC 3′ and 5′ CCG GAA TTC CGC AAG CTG AGA TAA ACA GC 3′. The resulting fragment was purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). After digestion with *Bam*HI and *Eco*RI the amplified fragment was cloned onto pGEX4T-1 (GE Healthcare, Little Chalfont, UK) to obtain a fusion protein containing glutathione-S-transferase (GST) at the N terminus (Smith and Johnson 1988) and ArcA at the C terminus. The ligation mixture was used to transform *E. coli* BL21, selecting Ap^r. Candidate clones were analyzed by restriction analysis. An *Escherichia coli* BL21 derivative carrying a plasmid-borne *GST-arcA* gene construct was thus obtained. Expression of the GST-ArcA recombinant protein was induced with 1 mm IPTG. GST-ArcA was purified from cultures grown in YT, at an $OD_{600} = 1$. The culture was centrifuged at 8000 rpm for 10 min, and the pellet was resuspended in 1 ml of lysis buffer (10 mm Tris–HCl, pH 7.4, 150 mm NaCl, 10% glycerol, 1% NP40, 1 mm EDTA, 1 mm dithiothreitol, 1 mm PSMF, and 1 μg/ml commercial protein inhibitors). The mixture was sonicated for 3 min using a Branson Sonifier 2005 (Biogen Cientifica, Madrid), and the resulting lysate was centrifuged at 10,000 rpm at 4° during 30 min. The supernatant and the pellet (resuspendend in lysis buffer) were both immersed in liquid nitrogen. To identify the fraction that contained the GST-ArcA protein, 10-μl aliquots from the supernatant and the pellet were heated at 95° during 5 min and subjected to SDS–PAGE. Electrophoresis was carried out at 175 V for 45–60 min. After drying, gels were stained with Coomassie blue. Because the GST-ArcA protein was found in the supernatant, large-scale purification was carried out on this fraction. Elution from glutathione–agarose was achieved with a solution of 10 mm glutathione, prepared in 50 mm Tris–HCl, pH 8.0. Further work was carried out with a GST-ArcA preparation judged to be ≥95% pure by SDS–PAGE and Coomassie blue staining.

Gel retardation assays with GST-ArcA protein: For gel retardation analysis, a 618-bp DNA fragment encompassing the *traY* upstream activating sequence (UAS), the *traY* promoter, and part of the *traY* coding sequence was end labeled with Klenow DNA polymerase in the presence of [γ-^{32}P]dATP. DNA-binding reactions were prepared to obtain a final volume of 20 μl, as described elsewhere (Camacho and Casadesus 2002). Each binding reaction contained 0.4 pmol of labeled DNA, 4 μl of GST-ArcA protein diluted in binding buffer, and 0.5 μg of competitor DNA [poly(dI-dC)]. The final composition of the binding buffer was 20 mm Tris-HCl, pH 8.0, 75 mm NaCl, 5 mm $MgCl_2$, 1 mm dithiothreithol, 12.5% glycerol, 0.1 mg/ml bovine serum albumin, and 25 μg/ml poly(dI-dC). For a specific competitor, the same DNA fragment that was used as probe (unlabeled) was added in excess. Binding was allowed to proceed for 20 min at room temperature. Five microliters of loading buffer was then added. Samples were subjected to electrophoretic separation in a nondenaturing 5% polyacrylamide gel prepared in 1× TBE. Electrophoresis was carried out at 200 V for 2–3 hr. After drying, gels were analyzed with a Fujifilm FLA-3000 betascope.

Quantitative reverse transcriptase PCR and calculation of relative expression levels: Salmonella RNA was extracted from stationary phase cultures using the SV total RNA isolation system (Promega). The quantity and quality of the extracted RNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To diminish genomic DNA contamination, the preparation was treated with DNase I (Turbo DNA free; Applied Biosystems/Ambion, Austin, TX) as previously described (Beuzon *et al.* 1999). An aliquot of 0.5 μg of DNase I-treated RNA was used for cDNA synthesis, using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System. Each reaction was carried out in a total volume of 15 μl on a 96-well optical reaction plate (Applied Biosystems) containing 7.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems), 6.9 μl cDNA (1/10 dilution), and two gene-specific primers at a final concentration of 0.2 μm each. Real-time cycling conditions were as follows: (i) 95° for 10 min and (ii) 40 cycles at 95° for 15 sec and 60° for 1 min. No-template and no reverse-transcriptase controls were included for each primer set and template. Melting curve analysis verified that each reaction contained a single PCR product. Reported gene expression levels were normalized to transcripts of *ompA*, a housekeeping gene that served as an internal control. Gene-specific primers, designed with PRIMER3 software (http://primer3.sourceforge.net), were as follows: for *traJ*, 5′ TCA GCC TCT TTC GGG AGA TAG T 3′ and 5′ AGC GAC TGA CAT TCA AGT TCC A 3′; for *traY*, 5′ GAG GGA TCA TCT GAA ACG ATA TCC 3′ and 5′ AAT GTG GAC TCT GTT TCT TCA ATT ACC T 3′; for *finP*, 5′ TTC TCA CGA TGC GTC GGA CAC AT 3′ and 5′ TAA ATC GCC GAT ACA GGG AG 3′; for *sdhA*, 5′ TGG CTA CAG GTA GAT TCA CC 3′ and 5′ CAC TTC TAT TGC CTG ATG GC 3′; and for *ompA*, 5′ TGT AAG CGT CAG AAC CGA TAC G 3′, and 5′ GAG CAA CCT GGA TCC GAA AG 3′.

Cloning and molecular characterization of Tn10*d*Cm inserts: Genomic DNA from each Tn10*d*Cm-carrying isolate was digested with *Sma*I and *Pst*I and cloned onto pBluescript II SK(+). Plasmid inserts were sequenced at the facilities of Sistemas Genómicos SL, Parque Tecnológico de Valencia, Paterna, Valencia, Spain, using the M13L and M13R universal primers.

Bacterial matings: Overnight cultures of the donor and the recipient were prepared in LB medium. Aliquots of 500 μl were mixed to obtain a donor/recipient ratio of 1:1. Each mixture was centrifuged 2 min at 13,000 rpm, and the supernatant was discarded. The pellet was resuspended in 50 μl of LB broth. Mating mixtures were incubated at 37° during 4 h. Diluted and undiluted aliquots were then spread on selective plates. Microaerobic conditions were obtained using GasPak microaerobic jars as previously described (Garcia-Quintanilla *et al.* 2008). Irrespective of the conditions in which mating was carried out, all crosses had the same design: the donor strain was a histidine auxotroph whose pSLT plasmid carried a kanamycin resistance tag in the *spv* locus, which is dispensable for conjugation (Garcia-Quintanilla *et al.* 2008), and the recipient was a pSLT$^-$ prototroph. This combination permitted selection of transconjugants on E plates supplemented with kanamycin.

RESULTS

Genetic trials for chromosomal regulators of the pSLT *tra* operon: Tn10*d*Cm mutagenesis: Screens performed

in this study made use of the *traB1::lac* transcriptional fusion (Torreblanca and Casadesus 1996) and involved discrimination of Lac$^+$ and Lac$^-$ colonies on indicator plates (Camacho and Casadesus 2002; Camacho *et al.* 2005b). All trials were carried out in aerobiosis. However, because the centers of Salmonella colonies become microaerobic or even anaerobic (especially when the colony diameter is >1 mm), colony trials can also detect changes in gene expression in response to oxygen availability (Aliabadi *et al.* 1986; Wei and Miller 1999). Two types of screens were carried out:

i. Searches for *tra* operon repressors, seeking Tn10*d*Cm insertions that derepressed the *traB1::lac* fusion: Tn10*d*Cm pools were used to transduce SV3003, selecting chloramphenicol resistance in the presence of X-gal. Candidates were detected by the formation of Lac$^+$ (blue) colonies. The Tn10*d*Cm pools used in this screen had been prepared in SV3083 (pSLT$^-$ Dam$^-$). Use of a pSLT$^-$ strain prevented the isolation of pSLT-borne insertions (*e.g.*, in *finO*). In turn, the fact that the strain was Dam$^-$ prevented the isolation of insertions in *dam*, which are a common class of mutations that derepress *tra* (Camacho and Casadesus 2002).
ii. Trials for *tra* operon activators, seeking Tn10*d*Cm insertions that prevented pSLT *tra* operon expression: Genetic screens for mutations that disrupt chromosomal *tra* activators cannot be performed in the wild type, because the *tra* operon is tightly repressed in pSLT (Camacho and Casadesus 2002), as in other FinOP$^+$ F-like plasmids (Yoshioka *et al.* 1987; Koraimann *et al.* 1996). However, *tra* derepression occurs in *S. enterica* mutants lacking Dam methylase (Torreblanca and Casadesus 1996; Camacho and Casadesus 2002). As a consequence, *S. enterica* strains carrying the *traB1::lac* fusion are Lac$^+$ in a Dam$^-$ background (Torreblanca and Casadesus 1996; Camacho and Casadesus 2002). Isolates carrying Tn10*d*Cm insertions that prevented pSLT *tra* operon expression were thus sought in a Dam$^-$ host (SV3069), and candidates were detected by the formation of Lac$^-$ (white) colonies. A variant of this trial involved diethyl sulfate mutagenesis instead of Tn*10d*Cm mutagenesis (see below).

Characterization of mutants lacking putative *tra* repressors: Analysis of 45,000 Tn*10d*Cm-containing isolates yielded 22 independent Lac$^+$ transductants whose Lac$^+$ phenotype was 100% linked to the Tn10*d*Cm insertion. Nearby DNA sequencing with a Tn*10* primer (Way and Kleckner 1984) indicated that more than half of the insertions (14/22) were in the *sdh* operon: 3 in *sdhA*, 6 in *sdhC*, and 5 in *sdhD*. The remaining insertions were in *flhC* (3 insertions), *flhD* (3 insertions), *gcvA* (1 insertion), and *fruR* (1 insertion).

Characterization of mutants lacking putative *tra* activators: Analysis of 38,000 Tn*10d*Cm-containing isolates yielded 30 independent Lac$^-$ transductants whose Lac$^-$ phenotype was 100% linked to the Tn10*d*Cm insertion. Eight isolates of this class were Tc^s, indicating that the Tn10*d*Cm element was linked to *dam* and thus suggesting that their Lac$^-$ phenotype was due to cotransduction of the wild-type *dam* allele. DNA from 3 Tc^r isolates was sequenced using a Tn*10* primer (Way and Kleckner 1984), as above. All three insertions were in *lrp*. Genetic mapping of additional isolates was performed in transductional crosses using SV3109 as donor. Appearance of Tc^r Cm^s transductants indicated that all Tn10*d*Cm insertions might be in *lrp* (cotransducible with *serC*). Tn*10* insertion is known to have preferential DNA targets (Kleckner *et al.* 1979), even if the ATS transposase is used to generate the pool of inserts (Kleckner *et al.* 1991). The failure of the screen to provide knockouts in *tra* operon activators other than Lrp, a well known *traJ* activator (Camacho and Casadesus 2002), led us to use chemical mutagenesis by DES.

DES mutagenesis was carried out on strain SV3069 and provided us with ~60 independent Lac$^-$ isolates that were not in *lrp* or linked to *dam*. Around one-third of the isolates analyzed (14/38) were ascribed to a single phenotypic class on the basis of their *tra* expression pattern, which showed a mild decrease in aerobiosis and a stronger decrease under microaerobiosis (see below). One such isolate was propagated as strain SZ101. The point mutation carried by this isolate was identified as follows:

i. The isolate was transduced with a pool of Tn10*d*Cm insertions, prepared in a pSLT$^-$ strain (SV3081). Transductants were selected on plates containing chloramphenicol and X-gal.
ii. Several Lac$^+$ transductants were purified, lysed with P22, and used to transduce the original isolate. Transductants were selected on LB plates containing chloramphenicol and X-gal. Occurrence of both Lac$^+$ and Lac$^-$ transductants provided evidence for linkage between the Tn10*d*Cm element and the chromosomal locus containing the mutation that affected *tra* expression.
iii. A Tn*10d*Cm insertion 66% linked to the chromosomal mutation was chosen for further study. A 66% linkage upon P22 transduction can be expected to be indicative of an ~5- to 6-kb distance (Wu 1966). Genomic DNA from this isolate (propagated as strain SV4500) was extracted, digested with *Pst*I, and cloned on pBluescript SKII(+). DNA sequencing with T1 and T7 primers indicated that the Tn10*d*Cm element was inserted in the *sthE* gene (McClelland *et al.* 2001). Additional sequencing reactions using primers *ad hoc* revealed a GC → AT transition in the coding sequence of the *arcA* gene. This change is predicted to cause an amino acid substitution (Glu → Lys). Six additional isolates of

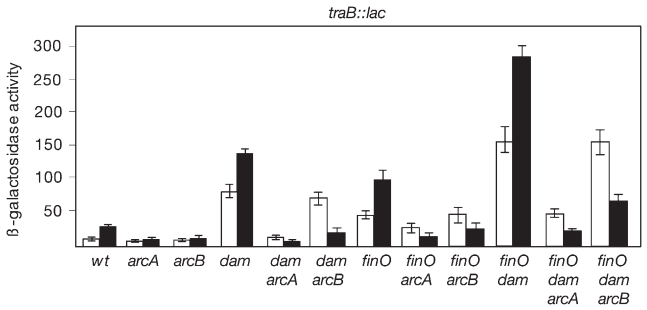

FIGURE 1.—β-Galactosidase activity of the *traB1::lac* transcriptional fusion in different genetic backgrounds, monitored in aerobic cultures (open histograms) and microaerobic cultures (solid histograms). Strains and relevant genotypes were as follows (from left to right): SV3003 (wild type), SZ102 (ArcA$^-$), SZ103 (ArcB$^-$), SV3069 (Dam$^-$), SZ104 (Dam$^-$ ArcA$^-$), SZ105 (Dam$^-$ ArcB$^-$), SV4509 (FinO$^-$), SZ106 (FinO$^-$ ArcA$^-$), SZ107 (FinO$^-$ ArcB$^-$), SV4519 (FinO$^-$ Dam$^-$), SZ108 (FinO$^-$ Dam$^-$ ArcA$^-$), and SZ109 (FinO$^-$ Dam$^-$ ArcB$^-$). Enzymatic activities are averages and standard deviations from four to six independent experiments.

the same phenotypic class as SZ101 carried point mutations 65–67% linked to *sthE*, suggesting the occurrence of *arcA* mutations.

Altogether, these experiments suggested that ArcA might be an activator of the *tra* operon in the Salmonella virulence plasmid, as previously described for F, R100, and R1 (SILVERMAN *et al.* 1991; STROHMAIER *et al.* 1998; TAKI *et al.* 1998).

Activation of *tra* in the Salmonella virulence plasmid requires both ArcA and ArcB: In the absence of proof that the GC → AT transition of strain SV4500 (and the additional alleles of the same class) caused loss of *arcA* function, we constructed a null *arcA* allele in *S. enterica*. Targeted disruption of the *S. enterica arcA* gene was achieved by the λ Red recombination method (DATSENKO and WANNER 2000) to generate strain SV5067. Even though ArcB is not involved in regulation of the *tra* operon in the F sex factor (SILVERMAN *et al.* 1991), an *arcB* null allele was also constructed (strain SV5068). The effect of *arcA* and *arcB* mutations on pSLT *tra* operon expression was then tested in the wild type and in strains carrying mutations that derepress the pSLT *tra* operon (*finO* and *dam*, alone and combined). The activity of the *traB1::lac* fusion was measured in shaken (aerobic) LB cultures and static (microaerobic) LB cultures. A summary of these experiments, shown in Figure 1, is as follows:

i. Lack of ArcA caused a decrease in *tra* operon expression, which was best observed under microaerobiosis, probably because expression of the *tra* operon is higher under such conditions. When *tra* expression was derepressed by *dam* and *finO* mutations, alone or combined, the *arcA* mutation was epistatic over both *dam* and *finO* (Figure 1). These observations provided evidence that ArcA is a *tra* activator.
ii. Lack of ArcB had little or no effect in aerobiosis but caused a significant reduction of *tra* operon expression in microaerobiosis (Figure 1), suggesting that both ArcA and ArcB are necessary to activate the pSLT *tra* operon under microaerobiosis. The latter conclusion is consistent with the physiological activity of the ArcAB system in response to the redox state of the bacterial cell (LYNCH and LIN 1996).
iii. When both *arcA* and *arcB* mutations were present, the β-galactosidase activity of the *traB1::lac* fusion was similar to that detected in the ArcA$^-$ background (data not shown), indicating that the *arcA* mutation was epistatic over *arcB*. The latter observation is consistent with the known workings of the ArcAB signal transduction system (IUCHI and LIN 1991).

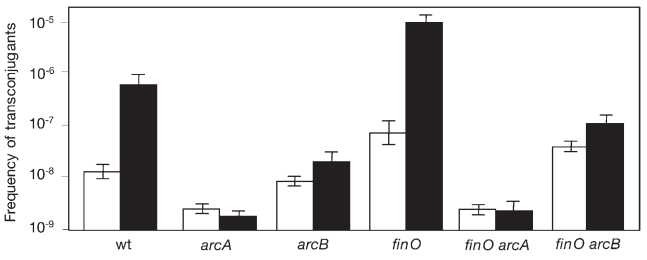

FIGURE 2.—Effect of *arcA* and *arcB* mutations on conjugal transfer of pSLT in aerobiosis (open histograms) and in microaerobiosis (solid histograms). The recipient was SV3081 in all matings. Donors were SV4201 (relevant genotype: wild type), SZ110 (ArcA$^-$), SZ111 (ArcB$^-$), SV4522 (FinO$^-$), SZ112 (FinO$^-$ ArcA$^-$), and SZ113 (FinO$^-$ ArcB$^-$). Frequencies are averages and standard deviations from six independent matings.

Effects of *arcA* and *arcB* mutations on conjugal transfer of pSLT: *S. enterica* strains carrying *arcA* or *arcB* mutations were used as donors in mating experiments carried out under either aerobiosis or microaerobiosis. Mating conditions were as previously described (GARCIA-QUINTANILLA *et al.* 2008). Because the effect of *arcA* and *arcB* mutations on *tra* operon expression is better observed in strains derepressed for conjugation (Figure 1), a pair of isogenic FinO$^-$ and FinO$^-$ ArcA$^-$ donors was included in the study (SV4522 and SZ112, respectively). Results from these experiments can be summarized as follows:

i. Under aerobiosis, lack of ArcA caused a significant reduction in the frequency of transconjugants: 7-fold when the donor was FinO$^+$ and 50-fold when the donor was FinO$^-$ (Figure 2). In contrast, lack of ArcB did not alter the frequency of conjugal transfer upon aerobic mating (Figure 2). These results are strongly reminiscent of classical studies of the F sex factor, where ArcA regulates p_{traY} in an ArcB-independent manner (SILVERMAN *et al.* 1991).

ii. Both *arcA* and *arcB* mutations drastically reduced the frequencies of transconjugants under microaerobiosis: in the presence of an *arcA* mutation, pSLT transfer decreased two orders of magnitude in a FinO$^+$ background and nearly three orders of magnitude in a FinO$^-$ background. The reduction in the frequency of transconjugants caused by a null *arcB* mutation under microaerobiosis was ~50-fold (Figure 2). Hence, a functional ArcAB signal transduction system is necessary to activate microaerobic transfer of pSLT.

Identification of the pSLT promoter under ArcAB control: experiments using gene fusions: The effect of *arcA* and *arcB* mutations on the expression of the *traJ* and *finP* genes of pSLT was examined using *traJ::lac* and *finP::lac* transcriptional fusions (Camacho and Casadesus 2005; Camacho *et al.* 2005b). β-Galactosidase activities were measured in LB cultures grown under aerobiosis and under microaerobiosis. The main observations in these experiments were as follows:

i. In a repressed background, an *arcA* mutation did not alter *traJ* expression when the culture was grown under aerobiosis but caused a one-half reduction in *traJ* expression under microaerobiosis (Figure 3). The sensitivity of this assay was judged to be limited, given the low level of *traJ* expression in a FinOP$^+$ strain (Camacho and Casadesus 2002). When the same experiments were carried out in a derepressed (Dam$^-$) background, no difference in *traJ* expression was observed between ArcA$^+$ and ArcA$^-$ hosts grown under aerobiosis (Figure 3). In contrast, the activity of the *traJ::lac* fusion decreased under microaerobiosis. Albeit modest, this reduction was surprising, since ArcA is not known regulate *traJ* in other F-like plasmids. However, regulation of *traJ* by ArcA appears to be an indirect effect (see below).
ii. A *finP::lac* transcriptional fusion was expressed at similar levels in ArcA$^+$ and ArcA$^-$ hosts, under both aerobiosis and microaerobiosis (Figure 3). A *dam* mutation decreased *finP::lac* activity as previously described (Torreblanca *et al.* 1999), but similar expression levels were detected in Dam$^-$ ArcA$^+$ and Dam$^-$ArcA$^-$ isogenic hosts (Figure 3).

Identification of the pSLT promoter under ArcAB control: quantitative reverse transcriptase–PCR analysis of *traY* mRNA: The product of the first gene of the *tra* operon (*traY*) has been shown to undergo autogenous control of *tra* operon transcription in the F sex factor (Silverman and Sholl 1996; Lum *et al.* 2002) and in R100 (Stockwell *et al.* 2000). Evidence that TraY is an autogenous activator of the *tra* operon also exists in pSLT (unpublished data from our laboratory). For this reason, we avoided the use of *traY::lac* fusions and employed quantitative reverse transcriptase (RT)–PCR to monitor the effect of *arcA* and *arcB* mutations on *traY*

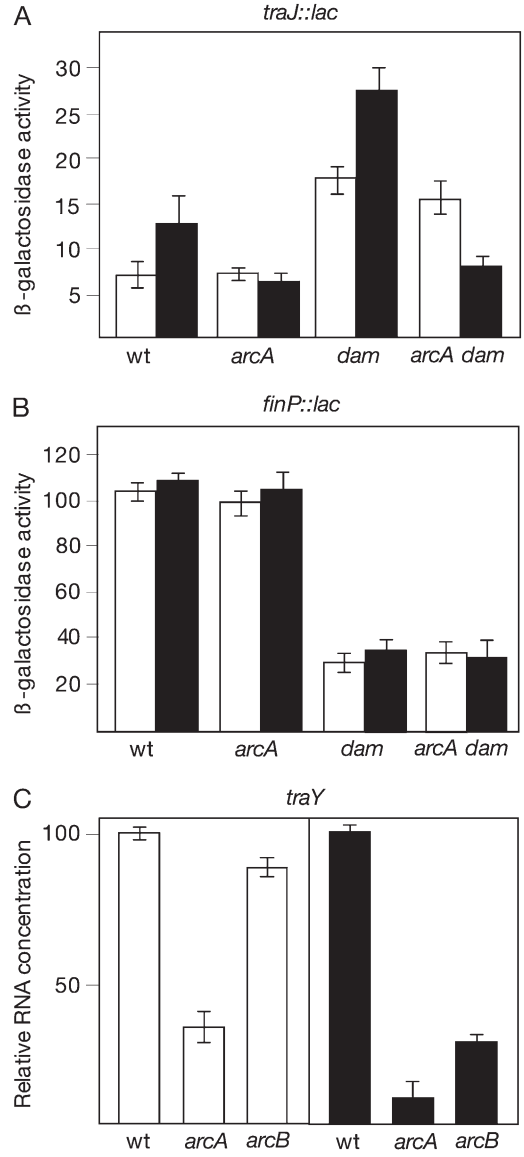

Figure 3.—(A) Activity of a *traJ::lac* transcriptional fusion in a wild-type background (strain SV4761) and in ArcA$^-$, Dam$^-$, and ArcA$^-$ Dam$^-$ mutants (SZ114, SV4914, and SZ116, respectively). β-Galactosidase activities are averages from five independent experiments. (B) Activity of a *finP::lac* transcriptional fusion in a wild-type background (strain SV4839) and in ArcA$^-$, Dam$^-$, and ArcA$^-$ Dam$^-$ strains (SZ118, SZ119, and SZ120, respectively). β-Galactosidase activities are averages and standard deviations from four to six independent experiments. (C) Relative amounts of *traY* mRNA, normalized to *ompA* mRNA, in the wild type (LT2) and in ArcA$^-$ and ArcB$^-$ mutant derivatives (SV5067 and SV5068, respectively). Data are averages from three independent experiments. In A–C, histograms are as follows: open, aerobiosis; solid, microaerobiosis.

expression. The results were clear cut: in aerobiosis, an *arcA* mutation caused a 3-fold decrease in *traY* mRNA content, while an *arcB* mutation had little or no effect (Figure 3). Microaerobic conditions amplified up to 10-fold the difference in *traY* mRNA content between ArcA$^+$ and ArcA$^-$ hosts and revealed a 3-fold difference between ArcB$^+$ and ArcB$^-$ hosts (Figure 3). Together with

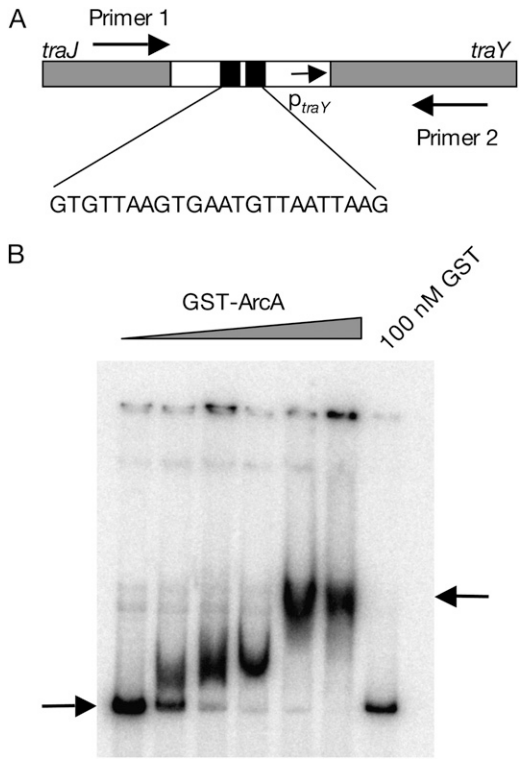

FIGURE 4.—(A) Diagram of the *traJ-traY* border in the pSLT plasmid, indicating the position of p_{traY}, the region homologous to ArcA binding sites of other F-like plasmids, and the locations of the primers used for PCR amplification. (B) Gel retardation analysis of GST-ArcA binding to the *traY* UAS. GST-ArcA concentrations were, from left to right, 0, 5, 20, 40, 80, and 160 nM.

results described in the former section, these observations suggest that the promoter under ArcA control is mainly p_{traY}. However, ArcA appears to regulate p_{traY} in two distinct ways: (i) in an ArcB-independent manner under aerobiosis, as in the F sex factor (SILVERMAN *et al.* 1991), and (ii) in an ArcB-dependent manner under microaerobiosis. ArcA function is modulated by phosphorylation mediated by ArcB (IUCHI and LIN 1991). Hence, the ArcAB system may activate the pSLT p_{traY} promoter in response to low oxygen concentration, thus explaining the high rates of pSLT transfer detected in microaerobiosis (GARCIA-QUINTANILLA *et al.* 2008).

Binding of ArcA to the upstream activating sequence of the pSLT *tra* operon: Computer analysis of DNA sequences upstream of the *traY* promoter of plasmid pSLT was performed using Clustal W, in a search for regions homologous to the consensus sequence for ArcA binding found in other F-like plasmids. The database DNA sequences used were NC002483 (F), NC00234 (R100), M19710 (R1), and NC003277 (pSLT). A region containing two overlapping ArcA binding motifs was found (Figure 4A), as in other F-like plasmids (SILVERMAN *et al.* 1991; STROHMAIER *et al.* 1998). To investigate whether the ArcA protein was able to bind this DNA region, gel retardation assays were carried out. A 0.6-kb fragment of pSLT containing the *traY* promoter and the *traY* UAS was PCR amplified, purified, and end labeled with [γ-^{32}P]dATP. This labeled DNA was mixed with aliquots containing increasing concentrations of GST-ArcA protein. Binding reactions were allowed to proceed for 20 min at room temperature. Electrophoretic separation was then carried out in an 8% polyacrylamide gel. A representative experiment is shown in Figure 4B. Retardation of the DNA fragment is clearly observed as the GST-ArcA protein concentration increases. As a control, GST alone did not cause DNA retardation (Figure 4B). Excess nonspecific competitor DNA [poly(dI-dC)] did not alter retardation. In contrast, addition of unlabeled *traY* UAS caused a decrease in the amount of retarded DNA (data not shown). These observations indicate that ArcA specifically binds the *traY* UAS in the Salmonella virulence plasmid and suggest that ArcA may activate transcription of the pSLT *tra* operon by a mechanism similar to those described in F (SILVERMAN *et al.* 1991) and R1 (STROHMAIER *et al.* 1998).

Effect of *sdh* mutations on the expression of the pSLT *tra* operon: Tn*10d*Cm insertions in *sdhA*, *sdhC*, and *sdhD* caused a 4- to 5-fold increase in the expression of the *traB1::lac* fusion under aerobiosis but had little effect on the expression of the fusion under microaerobiosis (data not shown). An *sdhA* deletion constructed by gene targeting conferred an identical phenotype (Figure 5A). These observations provided evidence that loss of function of the *sdhCDAB* operon derepressed *tra* operon expression under aerobiosis. In *E. coli*, *sdhCDAB* encodes succinate dehydrogenase, a membrane-bound enzymatic complex composed of four subunits (CECCHINI *et al.* 2002).

To identify the pSLT promoter under SdhABCD control, levels of the *traJ*, *finP*, and *traY* transcripts were compared in the wild type and in an SdhA$^-$ mutant (SV5608), using quantitative RT–PCR. A housekeeping transcript, *ompA*, was used as a loading control in all cases. In the results shown in Figure 5B, the absolute mRNA content found in the wild type for each individual promoter has been normalized to "1". Normalization makes the figure simpler given the disparate levels of *finP* and *traJ* mRNAs typically found in *S. enterica* (*finP* mRNA is >50-fold more abundant than *traJ* mRNA; data not shown). Data in Figure 5 rule out the possibility that SdhABCD might control *finP* transcription. In contrast, higher amounts of both *traJ* and *traY* mRNAs were found in the SdhA$^-$ mutant. These experiments indicate that Sdh represses *traJ* transcription and leave open the possibility that repression might also occur at the P_{traY} promoter. However, because TraJ is required to activate p_{traY}, repression of *traJ* transcription seems *a priori* sufficient to explain Sdh-mediated inhibition of *tra* operon expression.

Effects of *sdh* mutations on conjugal transfer of pSLT: Sdh$^+$ FinO$^+$, Sdh$^+$ FinO$^-$, Sdh$^-$ FinO$^+$, and Sdh$^-$ FinO$^-$ isogenic strains were used as donors in matings with a pSLT$^-$ recipient. Matings were carried out under

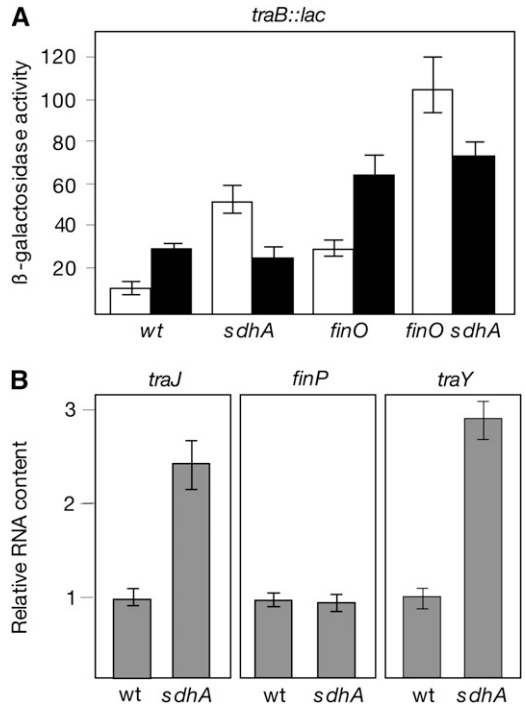

FIGURE 5.—(A) Activity of the *traB1::lac* transcriptional fusion in SdhA$^+$ and SdhA$^-$ hosts. Experiments were carried out in FinO$^+$ and FinO$^-$ backgrounds. Strains were SV3003 (wild-type background), SV5986 (SdhA$^-$), SV4509 (FinO$^-$), and SV5987 (FinO$^-$ SdhA$^-$). Histograms represent averages and standard deviations from three experiments. Open histograms correspond to β-galactosidase activities under aerobiosis. Solid histograms correspond to β-galactosidase activities under microaerobiosis. (B) Pairwise comparisons of the relative amounts of *traJ* mRNA, FinP RNA, and *traY* mRNA in SdhA$^+$ and SdhA$^-$ strains (LT2 and SV5608, respectively). Data for each transcript were normalized to *ompA* mRNA. To avoid disparate histogram sizes, the absolute amount of each individual transcript in the wild type is represented as "1". Data are averages and standard deviations from four experiments.

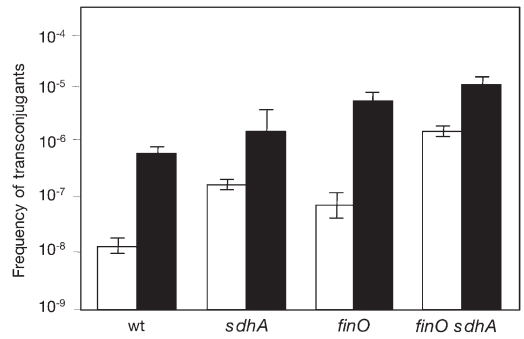

FIGURE 6.—Effect of Sdh absence on conjugal transfer of pSLT under aerobiosis (open histograms) and under microaerobiosis (solid histograms). Donors were SV4201 (wild-type background), SZ122 (SdhA$^-$), SV4522 (FinO$^-$), and SZ123 (FinO$^-$ SdhA$^-$). The recipient was SV3081 in all cases. Frequencies are averages and standard deviations from eight independent matings.

both aerobiosis and microaerobiosis, and their results are summarized in Figure 6. Under aerobiosis, lack of SdhA increased pSLT transfer more than one order of magnitude both from FinO$^+$ and from FinO$^-$ donors. Under microaerobiosis, lack of SdhA had a much smaller effect, with differences near the limit of significance (Figure 6). We thus concluded that succinate dehydrogenase is a repressor of pSLT transfer, mainly (perhaps only) in aerobiosis.

Regulation of *sdhABCD* by the ArcAB system: Studies in *E. coli* have shown that ArcA is a repressor of the *sdhCDAB* operon (PARK *et al.* 1995). To investigate whether ArcA plays an analogous role in *S. enterica*, we examined the effect of an *arcA* mutation on *sdh* expression. Expression of *sdh* in ArcA$^+$ and ArcA$^-$ strains of *S. enterica* was monitored using two procedures: calculation of β-galactosidase activities using an *sdhB::lac* translational fusion and comparison of *sdhA* mRNA levels by quantitative RT–PCR. Data shown in Figure 7 can be summarized as follows: (i) In the wild type, the *sdh* operon was expressed at a lower level under microaerobiosis than under aerobiosis, as previously described in *E. coli* (PARK *et al.* 1995), and (ii) lack of ArcA increased both the activity of the *sdhB::lac* fusion and the level of *sdhA* mRNA under microaerobiosis (Figure 7). The conclusion from these experiments was that ArcA is a repressor of *sdh* expression in *S. enterica*, especially in the absence of oxygen. This conclusion is in agreement with *E. coli* studies (PARK *et al.* 1995). Repression of *sdh* by ArcAB also explains why microaerobic expression of *traJ* decreases in an ArcA$^-$ background (Figure 3): In the absence of ArcAB, SdhABCD represses microaerobic *traJ* expression down to levels similar to those found in aerobiosis. The evidence that ArcA activates *traJ* transcription indirectly (by inhibiting SdhABCD synthesis) is supported by epistasis analysis: A *traJ::lac* transcriptional fusion is expressed at similar levels in ArcA$^+$ SdhA$^-$ and ArcA$^-$ SdhA$^-$ hosts (Figure 7C). ArcA thus plays a dual role in microaerobic activation of pSLT transfer, as a direct activator of the *tra* operon and as an indirect activator of *traJ* (by repressing *sdhCDAB*).

DISCUSSION

Expression of mating functions in the *S. enterica* virulence plasmid (pSLT) is tightly repressed by a functional FinOP system of fertility inhibition (SMITH *et al.* 1973; CAMACHO and CASADESUS 2002). However, mating conditions have a strong influence on the frequency of pSLT transfer in the laboratory, suggesting the existence of controls that regulate conjugation in response to environmental cues. In LB and other rich media, pSLT transconjugants appear at a frequency ~10^{-8} per donor bacterium, a frequency so low that can be easily overlooked (AHMER *et al.* 1999). Higher frequencies of mating are obtained in minimal medium (AHMER *et al.* 1999; CAMACHO and CASADESUS 2002). Incubation of the mating mixture in LB medium under microaerobiosis also yields relatively high mating frequencies, ~10^{-5} transconjugants per donor (GARCIA-QUINTANILLA *et al.*

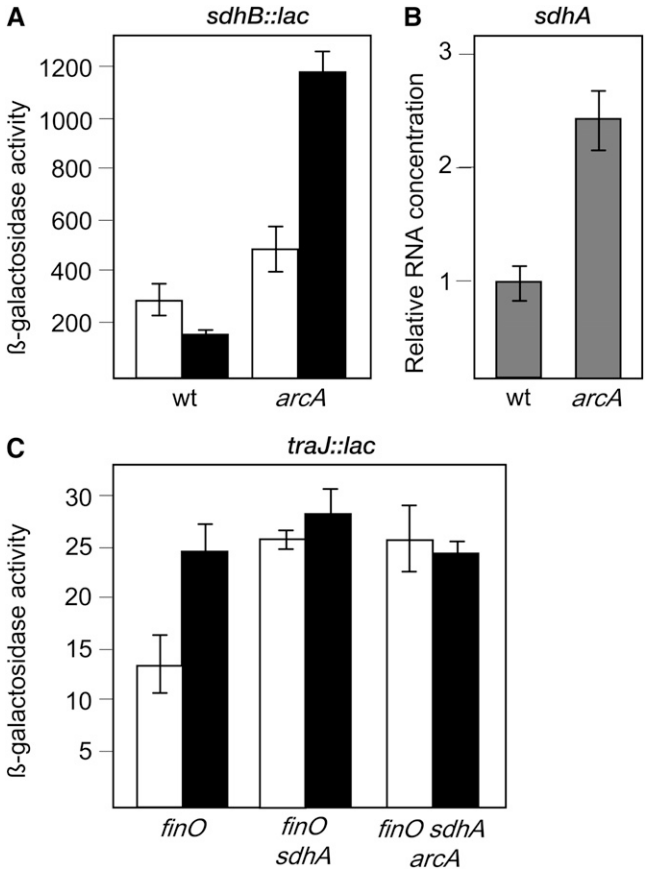

Figure 7.—(A) Activity of an *sdhB::lac* translational fusion in the wild type (SV5867) and in an ArcA$^-$ derivative (SV5868), grown under aerobiosis (open histograms) and under microaerobiosis (solid histograms). β-Galactosidase activities are averages from five independent experiments. (B) Relative amounts of *sdhA* mRNA, normalized to *ompA* mRNA, in the wild type (LT2) and in an isogenic ArcA$^-$ mutant (SV5067) grown under microaerobiosis. Data are averages and standard deviations from three independent experiments. (C) Activity of a *traJ::lac* transcriptional fusion in FinO$^-$, FinO$^-$ SdhA$^-$, and FinO$^-$ SdhA$^-$ ArcA$^-$ backgrounds (SZ114, SV4914, and SZ116, respectively). β-Galactosidase activities are averages from five independent experiments. In A and C, histograms are as follows: open, aerobiosis; solid, microaerobiosis.

2008). Slightly alkaline pH and high osmolarity also increase pSLT transfer, albeit mildly if compared with the effect of microaerobiosis (Garcia-Quintanilla *et al.* 2008). In analogy with studies of Salmonella pathogenesis that employ high osmolarity and microaerobiosis to mimic the environment of the animal intestine (Ohl and Miller 2001), high rates of pSLT transfer under such conditions may reflect the high frequency of matings that occur in the gut of infected mice (Garcia-Quintanilla *et al.* 2008). In murine ileal loops, frequencies can be as high as 10^{-3} transconjugants per donor (Garcia-Quintanilla *et al.* 2008).

Conjugal transfer of pSLT in microaerobiosis is under the control of ArcAB, a signal transduction system responsive to the oxygen level (Iuchi and Lin 1991). ArcA

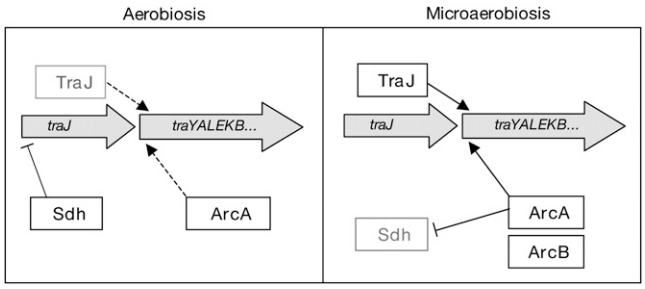

Figure 8.—Model for the regulation of pSLT mating functions in response to oxygen availability. Under aerobiosis, low TraJ level may be a limiting factor for *tra* operon expression, even if ArcA (SfrA) is abundant. A factor that contributes to *traJ* repression is SdhABCD. Under microaerobiosis, however, ArcAB-mediated repression of the *sdhCDAB* operon may indirectly increase TraJ synthesis. As a consequence, TraJ and ArcA may efficiently activate transcription from the p_{traY} promoter.

binds the upstream activating sequence of the pSLT main *tra* promoter (Figure 4), as previously described in other F-like plasmids (Silverman *et al.* 1991; Strohmaier *et al.* 1998). ArcA is a typical response regulator, whose activity is modulated by phosphorylation by the cognate microaerobic sensor histidine kinase, ArcB (Cecchini *et al.* 2002). Hence, the need of both ArcA and ArcB to activate the pSLT p_{traY} promoter under microaerobiosis (Figure 1) makes sense from a physiological point of view. ArcA also activates aerobic expression of the pSLT *tra* operon, albeit at a lower level than in microaerobiosis (Figure 1). ArcA-mediated aerobic activation of *tra* is ArcB independent, as previously described in the F sex factor (Buxton and Drury 1984; Silverman *et al.* 1991). Like pSLT, F is transferred in the absence of oxygen (Stallions and Curtiss 1972). Hence, signal transduction by the ArcAB system might control microaerobic transfer of F as it does in pSLT. To our knowledge, this possibility has not been examined.

Besides p_{traY} activation, the *S. enterica* ArcAB system plays a second role in the regulation of pSLT transfer: repression of the *sdhCDAB* operon, which encodes succinate dehydrogenase, an enzyme identified in this study as a repressor of pSLT transfer in aerobiosis. Preliminary evidence suggests that Sdh may be repressor of *traJ* transcription (Figure 5). Because succinate dehydrogenase is a membrane-bound protein complex (Cecchini *et al.* 2002), it seems *a priori* unlikely that the SdhABCD complex may repress *traJ* transcription directly (*e.g.*, binding to the *traJ* promoter region). An indirect effect may thus be postulated, via a hitherto unknown transcriptional regulator responsive to signals produced by central metabolism. ArcA-mediated repression of *sdh* is mainly observed in microaerobiosis (Figure 7), as previously described in *E. coli* (Park *et al.* 1995).

The model outlined in Figure 8 summarizes the mechanisms of conjugation control discussed in this study. In aerobiosis, ArcA-mediated activation of *tra* has low efficiency, and TraJ is scarce because *traJ* expression is directly or indirectly repressed by SdhABCD. In

microaerobiosis, the ArcB oxygen sensor triggers activation of *tra* operon transcription mediated by ArcA. In addition, *sdhCDAB* expression is repressed by ArcAB, thus relieving *traJ* repression. Maximal activation of p_{traY} in other F-like plasmids requires both ArcA and TraJ (Strohmaier *et al.* 1998). Repression of *sdhCDAB* may therefore be crucial to increase the TraJ level and to boost *tra* expression under microaerobiosis. The level of ArcA transcription factor is less likely to be limiting, because ArcA is abundant in the cell (Salmon *et al.* 2005).

The regulators of pSLT conjugal transfer included in Figure 8 are part of a wider regulatory network whose dimensions we may know only partially (Torreblanca *et al.* 1999; Camacho and Casadesus 2002; Camacho *et al.* 2005b). Host-encoded regulators may adjust conjugal transfer to favorable circumstances, optimizing the balance between its cost and its benefits (Bingle and Thomas 2001). In the case of the ArcAB signal transduction system, control of *tra* transcription under microaerobiosis may be viewed as an adaptation to the animal gut, an environment where the density of potential pSLT recipients is high (Garcia-Quintanilla *et al.* 2008).

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