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Arc and Sfr Functions of the *Escherichia coli* K-12 *arcA* Gene Product Are Genetically and Physiologically Separable

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The *Escherichia coli arcA* gene product regulates chromosomal gene expression in response to deprivation of oxygen (Arc function; Arc stands for aerobic respiration control) and is required for expression of the F plasmid DNA transfer (*tra*) genes (Sfr function; Sfr stands for sex factor regulation). Using appropriate *lacZ* fusions, we have examined the relationship between these two genetic regulatory functions. Arc function in vivo was measured by anaerobic repression of a chromosomal *sdh-lacZ* operon fusion (*sdh* stands for succinate dehydrogenase). Sfr function was measured by activation of a plasmid *traY-lacZ* gene fusion. An eight-codon insertion near the 5′ terminus of *arcA*, designated *arcA1*, abolished Arc function, as previously reported by S. Iuchi and E. C. C. Lin (Proc. Natl. Acad. Sci. USA 85:1888–1892, 1988), but left Sfr function largely (≥60%) intact. Similarly, the *arcB1* mutation, which derepressed *sdh* expression and is thought to act by abolishing the signal input that elicits ArcA function, had little effect (≤20%) on the Sfr function of the *arcA*$^+$ gene product. Conversely, a valine-to-methionine mutation at codon 203 (the *sfrA5* allele) essentially abolished Sfr activity without detectably altering Arc activity. These data indicate that Sfr and Arc functions are separately expressed and regulated properties of the same protein.

The *Escherichia coli* gene *arcA* was identified initially in deletion strains as a locus near *trpR* conferring sensitivity to redox dyes such as methylene or toluidine blue (Dye phenotype) and resistance of Hfr strains to donor-specific RNA bacteriophages (Msp phenotype) (8, 27). Lerner and Zinder (20), Beutin and Achtman (4), and Silverman et al. (29) all isolated mutants defective in F-plasmid-dependent DNA donor activity in which the responsible mutation mapped to the *trpR-thr* region; each laboratory assigned a different mnemonic (*fex*, *sfrA*, and *cpxC*, respectively). Most recently, Iuchi and Lin (18) isolated mutants unable to repress the anaerobic expression of genes whose products function in aerobic but not anaerobic growth. The mutations mapped to the *dye/msp/fex/sfrA/cpxC* locus, which they redesignated *arcA*. Combined genetic and molecular biological data indicate that all these loci are in fact one gene, now designated *arcA* (3).

Gene *arcA* was sequenced by Drury and Buxton (9), who noted its similarity to *ompR*. Both the *arcA* and the *ompR* gene products are now regarded as members of a broad family of bacterial polypeptides called response regulators (reviewed in reference 32). Each response regulator is phosphorylated in a reaction catalyzed by a histidine kinase. The histidine kinases constitute a second prokaryotic protein family. Most are transmembrane proteins that function as sensors of environmental change. Histidine kinase-catalyzed phosphorylation alters response regulator function, most frequently DNA binding. Thus, responses elicited by phosphorylation are most often altered patterns of gene expression.

Beutin et al. (5) originally suggested that the *arcA* (then designated *sfrA*) gene product was a transcriptional control factor. In confirmation, we showed that in vivo expression of the F plasmid *traY* promoter is reduced about 10-fold in *sfrA* mutants (30). Conversely, when *tra* gene expression is not under *traY* promoter control, as occurs in plasmid pTG801 (13), *sfrA* mutations have no effect on the level of *tra*-dependent functions (12).

Since the *arc* system was defined on the basis of genetic regulation in response to oxygen deprivation (18), it might be expected that *tra* gene expression should also be significantly altered by anaerobiosis. This, however, was not the case; anaerobiosis only slightly altered *traY* promoter activity in vivo (30). It thus appeared that the *arcA* gene product might have two separately regulated activities, Arc (18) and Sfr (30) (Arc and Sfr stand for aerobic respiration control and sex factor regulation, respectively). The data presented in this communication provide more direct evidence that the Arc and Sfr functions of the *arcA* gene product are in fact independently expressed and regulated.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains used in this study are all derivatives of *E. coli* K-12 (Table 1). The low-copy plasmid pLW403 contains a *traY-lacZ* fusion gene and *lacY*, both driven by the F plasmid *traY* promoter, and the F plasmid *traJ* gene, which is essential for *traY* expression (30). Bacteriophages Qβ and P1*vir* were from laboratory stocks.

Genetic methods. Generalized transductions with P1*vir* were as previously described (21). Details of individual selections are described in Results. JCFL0 (F′ *lac* [1]) was introduced into ECL547 derivatives by conjugation. Selection was on minimal lactose plates containing arginine (required by ECL547), tetracycline to counterselect JC3272, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Deep-blue Lac$^+$ colonies were tested for bacteriophage Qβ sensitivity as a measure of Sfr function.

Media and culture conditions. LB and Vogel/Bonner minimal media were as described previously (21). Solid media were prepared with 1.5% Bacto-agar. Nutritional supplements were provided at 40 μg/ml. Antibiotics were added to

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference(s)
NK5148	F⁻ *thr-34*::Tn*10*	CGSC 6166[a]
ECL547	F⁻ *sdh*⁺ Φ(*sdh-lac*)	18
ECL585	F⁻ *sdh*⁺ Φ(*sdh-lac*) *arcA1* *zjj*::Tn*10*	18
ECL594	F⁻ *sdh*⁺ Φ(*sdh-lac*) *arcB1* *zgi*::Tn*10*	15, 19
M1174	F⁻ *sfrA*⁺	4
M1164	F⁻ *sfrA5*	4
AE2366	F⁻ *sfrA5* *thr-34*::Tn*10*	This study[b]
37-1	HfrH *sfrA*⁺	27
122-1	HfrH Δ*sfrA*	27

[a] E. coli Genetic Stock Center, Yale University.
[b] Constructed by P1 transduction of M1164.

10 μg/ml (tetracycline), 50 μg/ml (kanamycin), or 100 μg/ml (ampicillin). X-Gal was added at 40 μg/ml. Where indicated, LB agar plates were supplemented with 200 μg of toluidine blue per ml. Aerated and anaerobic culture conditions were as previously described (30).

Molecular biological methods. Chromosomal DNA was isolated from 1.5 ml of overnight cultures by the sodium dodecyl sulfate (SDS)-proteinase K method (2) and dissolved in 0.1 ml of TE buffer (10 mM Tris HCl [pH 8], 0.1 mM EDTA). Amplification of *arcA* alleles by the polymerase chain reaction (PCR) was done with 1 to 2 μl of DNA, 100 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Perkin-Elmer/Cetus), and other components (28) in a final volume of 0.1 ml. Amplification was for 30 cycles (one cycle was 1 min of denaturation at 94°C, 1 min of annealing at 60°C and 3 min of extension at 72°C). Amplification primer I corresponded to *sfrA* nucleotides 24 to 47 (9) and included an *Sph*I site. Amplification primer II corresponded to nucleotides 993 to 1014; nucleotide 1008 was changed from A to T to create a *Bam*HI site. Before cloning, reaction products were purified by phenol-chloroform extraction and ethanol precipitation and digested with *Sph*I and *Bam*HI. The *sfrA*⁺, *sfrA5*, and *arcA1* genes were cloned in a T7/T3 vector for overproduction of their respective gene products (11).

Single-stranded DNA amplification reaction mixtures contained 0.1 pmol of double-stranded PCR product and 4 pmol of primer. Reactions were run for 40 cycles (one cycle was 35 s of denaturation at 92°C, 35 s of annealing at 60°C, and 45 s of elongation at 77°C). Reaction products were purified by addition of 2 ml of water followed by filtration in a Centricon-30 unit (Amicon Co.) subjected to centrifugation at 4,000 × *g* and 4°C for 50 min (14). The residual reaction mixture (40 μl) was brought to 2 ml with water and concentrated again by filtration. The remaining water was removed in the vacuum centrifuge. The reaction product was dissolved in 0.5 volume of TE buffer. DNA-sequencing reaction mixtures contained 1 pmol of single-stranded DNA and 10 pmol of the appropriate primer.

DNA sequence analyses employing internal oligonucleotide primers were as previously described (30). Except as indicated, the results were identical to those of Drury and Buxton (9).

β-Galactosidase assays. Assays were carried out as described by Miller (22); results are expressed in Miller units.

Immuno-overlay (Western) blots. Cells in 1.3 ml of overnight cultures were sedimented at 13,000 × *g* for 5 min. Pellets were suspended in 0.08 ml of Tris-buffered saline

FIG. 1. *arcA1* and *sfrA5* mutations. The figure shows a linear representation of the *arcA/sfrA* gene product. The stippled region indicates the amino-terminal domain that is structurally homologous to the CheY protein (32). The solid line indicates the carboxyl-terminal domain presumably involved in regulating *tra* gene expression. The sites of the eight-amino-acid insertion between amino acids A-33 and T-34 (*arcA1*) and the V-203-M mutation (*sfrA5*) are indicated.

followed by 0.02 ml of fivefold-concentrated electrophoresis sample buffer. The suspension was heated in a boiling water bath for 5 min. Five microliters of each extract was subjected to SDS-polyacrylamide gel electrophoresis, and proteins were blotted to polyvinylidine difluoride paper (Millipore Corp., Bedford, MA) essentially as described previously (30). Primary antibody was obtained from a goat immunized with SfrA protein purified to apparent homogeneity (11). Immune serum was used at a 1/10,000 dilution. The secondary reagent was alkaline phosphatase-conjugated protein G used at a 1/5,000 dilution. The blot was developed with the NBT-BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium) alkaline phosphatase substrate. Conjugated protein G and substrate were obtained from the Pierce Chemical Co., Rockford, Ill.

RESULTS

Characterization of the *arcA1* and *sfrA5* alleles. We chose to examine the *arcA1* (18) and *sfrA5* (4) mutations. The former was as effective as a deletion in permitting anaerobic expression of *sdh* (18), and the latter was as effective as a deletion in reducing conjugal DNA transfer (4, 7). Amplification of chromosomal *arcA/sfrA* genes by PCR yielded in each case a major component of about 1 kb, as expected from the positions of the primers (see Materials and Methods). However, the mobility of the *arcA1* gene was slightly less than that of the other alleles, suggesting an insertion. The insertion site was localized to the 5′-terminal half of the amplified DNA by analysis of restriction fragment sizes after digestion with *Bgl*I and *Eag*I. Both enzymes cut once in the gene to produce 5′ and 3′ fragments distinguishable by size; in both cases, the 5′ fragment from the *arcA1* gene was the larger (data not shown). The insertion site was determined precisely by sequence analysis of the cloned gene. The *arcA1* allele proved to be a 24-bp duplication (data not shown). The position of the mutation with respect to the primary structure of ArcA is shown in Fig. 1; the mutation added eight codons in frame with the rest of the coding sequence. As expected, the insertion slightly reduced the electrophoretic mobility of the *arcA1* gene product (Fig. 2).

The *sfrA5* gene was sequenced in its entirety from a single clone. That analysis revealed two missense alterations with respect to the published sequence (9). Analysis of a second clone confirmed only one of these, a GC-to-AT transition in codon 203; the corresponding missense mutation is valine to methionine (V-203-M) (Fig. 1). We attribute the second alteration to a mutation introduced during amplification. Sequence analysis of bulk (uncloned) DNA amplified by the asymmetric PCR confirmed the V-203-M mutation (not shown).

That the V-203-M mutation is responsible for the altered properties of the *sfrA5* gene product was confirmed by

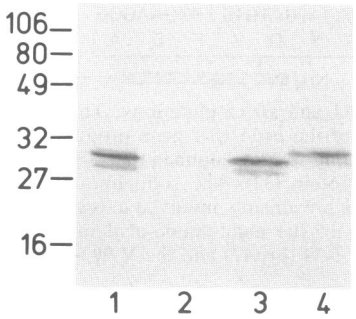

FIG. 2. Analysis of *arcA*/*sfrA* gene products by Western blot. Extracts were prepared and analyzed as described in Materials and Methods. Lane 1, strain 37-1 (*sfrA*$^+$); lane 2, strain 122-1 (Δ*sfrA*); lane 3, strain ECL547 (*sfrA*$^+$); lane 4, strain ECL585 (*arcA1*; note the slightly lower mobility of the *arcA1* gene product). Numbers to the left denote the molecular weights (in thousands) of proteins with the indicated electrophoretic mobilities. The relationship between the two closely spaced immunoreactive species is not presently clear; both are evidently derived from *sfrA*, insofar as they are both absent from the *sfrA* deletion strain 122-1.

analysis of revertants. We isolated Lac$^+$ derivatives of an *sfrA5* strain carrying pLW403; the parent strain was Lac$^-$ because of the *sfrA5* allele (31). Preliminary analyses identified a set of 45 independently isolated strains in which the mutations to the Lac$^+$ phenotype were chromosomal (31). Further studies of 19 of these showed that in all cases the mutation was ≥90% cotransduced with the *thr-34*::Tn*10* allele, as is *sfrA* (see below). Furthermore, measurements of β-galactosidase activity showed that all 19 mutations had restored wild-type levels of *traY* promoter expression (data not shown). Gene *sfrA* was amplified from eight Lac$^+$ mutants by the asymmetric PCR, and the nucleotide sequences around codon 203 were determined. In all cases, codon 203 was restored to the wild type (data not shown); there was a perfect correlation between restoration of Sfr function and reversion of codon 203.

Sfr activity of the *arcA1* gene product. Sfr function was tested by activation of the *traY* promoter driving the *traY-lacZ* fusion gene of pLW403 (30). The *arcA1* allele of ECL585 was introduced into M1174/pLW403 by cotransduction with the linked *zjj*::Tn*10* allele (18). Tetracycline-resistant transductants were screened for their ability to grow on toluidine blue plates; poor growth indicates presence of the *arcA1* allele (18). Eight of 44 Tet^r transductants were Dye^s (*arcA1*). These and four Tet^r Dye^r (*arcA*$^+$) transductants were tested for *tra* gene expression. The data indicated that the *arcA1* allele reduced *tra* gene expression no more than 40%, in contrast to the sevenfold reduction observed with the *sfrA5* allele (Table 2).

Arc activity of the *sfrA5* gene product. The *sfrA5* allele was introduced into the *sdh-lacZ* strain ECL547 by cotransduction with the *thr-34*::Tn*10* allele. Since *sfrA5* mutants are not obviously toluidine blue sensitive (12), 10 Tet^r transductants were screened for *sfrA5* cotransduction by donor-specific bacteriophage sensitivity after introduction of F′ *lac* and for Arc function by anaerobic derepression of the *sdh-lacZ* operon fusion. Nine of the 10 transductants were resistant to Qβ, indicating they received the *sfrA5* allele. These transductants, however, retained a level of Arc function indistinguishable from that of the *sfrA*$^+$ transductant and readily distinguishable from that of the *arcA1* mutant (Table 2). These experiments confirm that Arc and Sfr functions are separable by mutation.

TABLE 2. Sfr and Arc activities of the *arcA1* gene product are genetically and physiologically separable

Strain (genotype)[a]	β-Galactosidase activity[b]
M1174 (*zjj*::Tn*10* *arcA1*/ pLW403)[c] (8)	1,265 ± 231 (982–1,561)
M1174 (*zjj*::Tn*10* *arcA*$^+$/ pLW403)[c] (4)	2,028 ± 158 (1,926–2,263)
M1164 (*sfrA5*/pLW403) (4)	320 ± 38 (263–343)
ECL547 [*sfrA5* *thr-43*::Tn*10* Φ(*sdh-lac*)][d] (9)	39 ± 3 (35–42)
ECL547 [*sfrA*$^+$ *thr-43*::Tn*10* Φ(*sdh-lac*)][d] (1)	41
ECL585 [*arcA1* Φ(*sdh-lac*)] (1)	438
M1174 (*arcB1* *zgi*::Tn*10*/pLW403)[c] (11)	1,481 ± 148 (1,162–1,712)
M1174 (*arcB*$^+$ *zgi*::Tn*10*/pLW403)[c] (1)	1,862

[a] All cultures except the first three were grown anaerobically. The number of cultures analyzed is indicated in parentheses.

[b] In Miller units. The data are expressed as the mean ± standard deviation and, in parentheses, the range of values.

[c] *arcA1* and *arcB1* transductants were identified as Tet^r Dye^s colonies; *arcA*$^+$ *and arcB*$^+$ transductants were Tet^r Dye^r (15, 18).

[d] *sfrA5* transductants were identified by resistance to donor-specific bacteriophage Qβ after introduction of F′ *lac*; the *sfrA*$^+$ transductant was Qβ sensitive.

Sfr function in an *arcB* mutant strain. Epistasis experiments have shown that Arc function can be restored to *arcB* mutants by overproduction of the *arcA* gene product (15). That result suggests that ArcB and ArcA are functionally related, presumably as histidine kinase and response regulator, respectively (15, 16). We therefore tested the effect of an *arcB* mutation on Sfr function in vivo. The mutant allele was cotransduced into a pLW403 strain with the *zgi*::Tn*10* insertion, to which it is 85% linked (15). The Arc phenotype of Tet^r isolates was determined by dye sensitivity, as described for the *arcA* experiment, and Sfr function was assayed by β-galactosidase activity. As shown (Table 2), the *arcB1* allele reduced *tra* gene expression no more than 20%. Hence, Arc and Sfr functions of the *arcA* gene product could be differentiated by physiologic as well as genetic criteria.

DISCUSSION

Iuchi and Lin used the term modulon to denote the set of promoters whose activities are regulated in part by a common regulatory protein (18). According to the definition, the F plasmid *traY* promoter is part of the *arc* modulon, since it clearly requires the *arcA* gene product for maximal activity (30). The signal for Arc regulation is thought to be the accumulation of reduced components of the electron transport chain (16). In the current model, the signal is acquired by the integral membrane protein ArcB, which then transduces the information to a form that can be read by the ArcA protein, which is the actual genetic regulator (15, 16, 19). We show here that *tra* gene expression is only slightly dependent on ArcB function(s), in agreement with earlier, qualitative observations by Iuchi et al. (17). If ArcB is required to elicit ArcA activity, this result implies that the Arc and Sfr functions of the *arcA* gene product are separately regulated. This outcome would be somewhat surprising because generally each response regulator receives a primary regulatory input (32), as recognized in the original "two-component" designation (25). Secondary inputs are evident in vivo when the primary input is absent (32). They may in some cases represent system redundancy (6), but in most cases their

physiological significance is not clear. For ArcA/SfrA, however, it appears that qualitatively different signal inputs regulate the respective activities of the protein. Independent evidence for the same conclusion derived from the slight effect of anaerobiosis on *traY* promoter activity (30) and, as we describe here, from analysis of the *arcA1* and *sfrA5* mutations. Thus, while the modulon designation may be useful in denoting promoters regulated by the same protein (18, 24), it need not imply a common physiological basis for the regulation.

Judged by the structure of homologous response regulators (32), the *arcA* gene product is probably organized into two major domains, an amino-terminal domain that is involved in signal transduction and a carboxy-terminal regulatory domain. The *arcA1* mutation falls within the amino-terminal domain. The mutation is fairly drastic, and it is not hard to see how it could prevent information transfer to ArcA, presumably from ArcB (15, 16, 19). If that is the effect of the *arcA1* mutation, the Arc^- Sfr^+ property of *arcA1* and *arcB1* mutant cells also indicates that Sfr and Arc activities are separately regulated.

The V-203-M mutation occurs in the presumptive regulatory domain of the SfrA protein. In the OmpR response regulator, the same mutation alters the DNA binding properties of that protein (10, 23, 32). It may be argued by analogy that the *sfrA5* gene product does not bind low-affinity F plasmid sites required for *traY* promoter function, while retaining its ability to bind high-affinity chromosomal DNA sites involved in *arc* regulation. Another possibility is that the *sfrA5* mutation alters interactions between the *arcA* gene product and other proteins required for *tra* gene expression. We suggested elsewhere that the F-encoded, positive control protein TraJ is required in vivo for Sfr function at the *traY* promoter (31). Perego et al. (26) have suggested altered protein-protein interactions to explain how a mutation (*spo0A9V*) near the carboxy terminus of the *Bacillus subtilis spo0A* gene product dissociates the genetic repressor and activator functions of that protein. In vitro tests of these hypotheses are in progress (11).

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