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The Legacy of HfrH: Mutations in the Two-Component System CreBC Are Responsible for the Unusual Phenotype of an *Escherichia coli arcA* Mutant▽†

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Strains derived from HfrH carrying the *arcA2* null mutation exhibit a higher respiratory rate, enhanced glucose consumption, and a more-reduced intracellular redox state than *arcA* deletion mutants of a different lineage. The phenotype of the *arcA2* mutants was due to the presence of a *creC* constitutive mutation introduced by P1 transduction.

Escherichia coli and other facultative aerobes can adapt their metabolism according to O_2 availability by means of aerobic and anaerobic respiration and fermentation. ArcAB is a two-component signal transduction system that controls, at the transcriptional level, the choice of energy generation pathways according to the intracellular redox state (13, 16, 22). *E. coli arc* mutants are unregulated for aerobic respiration in microaerobic conditions, and so the enzymes of the tricarboxylic acid cycle are not repressed, and the pool of reducing equivalents (such as NADH or NADPH) is elevated (1, 24, 25). As a result, large amounts of reducing equivalents are available to be used in the synthesis of reduced compounds. Based on these facts, we studied the synthesis of two reduced bioproducts, poly(3-hydroxybutyrate) (18), which has the potential to be used as a biodegradable plastic, and ethanol (19), in a modified *arcA* genetic background. Two different mutations were analyzed: Δ*arcA* and *arcA2* (12), carried by strains CT1062 and CT1061, respectively, which are derivatives of *E. coli* K1060 [F^- *fadE62 lacI60 tyrT58* (AS) *fabB5 mel-1*] (20). Some Δ*arcA* mutants are unable to grow in minimal medium (15), whereas *arcA2* strains can grow in synthetic medium if it is supplemented with a low amount of casein amino acids to prime growth (9). *arcA2* and Δ*arcA* mutants were also observed to differ in other traits: *arcA* deletion mutants are very sensitive to redox dyes, such as toluidine blue (4), while strains bearing the *arcA2* mutation produce medium-sized colonies on toluidine blue agar. Besides, *arcA2* strains show a higher respiratory capacity (18, 21) and show a higher NADH/NAD^+ ratio when glycerol is used as a carbon substrate (19); as a consequence, enhanced yields of poly(3-hydroxybutyrate) and ethanol were observed. The different behaviors of these mutants were intriguing, and a deeper analysis was necessary in order to explain the molecular basis for the differences in phenotypes.

***arcA2* is a null mutation.** Partial sequencing of the *arcA2* allele had shown an IS*10* insertion element in codon 170 (10). A more complete sequencing performed over an amplification fragment obtained from strain CT1061 revealed an IS*10*-L insertion. Crude extracts of K1060, CT1062, and CT1061 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot experiments using a polyclonal rabbit antiserum raised against ArcA (Fig. 1A). No reactive bands were found for CT1062, while a 29-kDa band (corresponding to native ArcA) was detected for K1060. CT1061 showed only a 19.4-kDa band, which corresponded to the truncated protein inferred from the DNA sequence. An in silico analysis revealed no DNA binding domains in the incomplete ArcA2 protein. These results suggested that CT1061 is a null mutant whose genotype is *arcA*::IS*10*-L.

A constitutive *creC* mutation is present in CT1061. Strain CT1062 is a K1060 Δ*arcA* mutant constructed by allelic replacement, while CT1061 was obtained through P1 transduction using *E. coli* ECL618 [*arcA2 zij*::Tn*10* Δ(*lac-proAB*) *X111 supE44 thiA*/F′ *proAB*$^+$ *lacI*^{q} Δ*lacZ* (*M15*)] (12) as the donor and K1060 as the recipient (18). Because of this, it was possible that its unusual phenotype could be due to other genes, apart from *arcA*::IS*10*-L, transferred from the parental strain. A search of genes near *arcA* in the *E. coli* chromosome revealed the presence of the *cre* genomic region, which includes *creABCD*, situated between 60 and 4,069 bp from *arcA*. CreBC is a two-component signal transduction pair, where CreB is the regulator and CreC the sensor kinase (2, 28). The *cre* regulon includes *ack-pta*, *talA*, *trgB*, and *malE*, which are involved in carbon metabolism. These genes are activated upon transfer to minimal medium, with the exception of *malE*, which is repressed (2). The different growth behaviors of the two *arcA* mutants under study in semisynthetic medium focused our attention on these particular genes. A constitutive *creC* mutation, which increases the expression of CreBC-regulated genes, is widespread in *E. coli* strains. This mutation, formerly known as *creC510*, was originally present in HfrH and transferred to its derivative Hfr 3000 *thi* (3, 27). Strain ECL618 carries the

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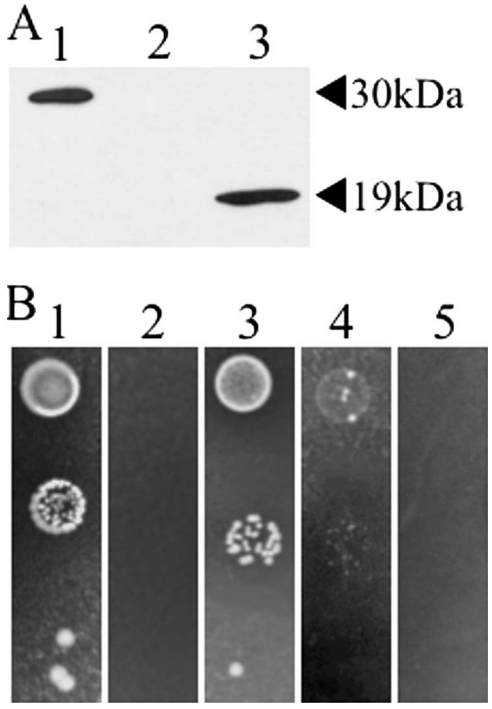

FIG. 1. (A) Western blot analysis of crude bacterial extracts. Equal amounts of protein (20 μg total protein per lane) were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose using a Trans-Blot semidry system (Bio-Rad), and probed with a rabbit anti-ArcA serum diluted 1:10,000. Detection was performed by using an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) diluted 1:3,000 and revealed by chemiluminescence. (B) Dye phenotype. Serial dilutions of overnight cultures on LB medium were plated on toluidine blue agar medium. CT1062/pWKS30 did not grow on this medium, and all the strains grew equally on LB agar plates (data not shown). Plates were incubated for 24 h at 37°C. The numbers correspond to the following strains: 1, K1060; 2, CT1062; 3, CT1061; 4, CT1062/pWKS30-*arcA*; 5, CT1062/pWKS30-*arcA*::IS*10*-L.

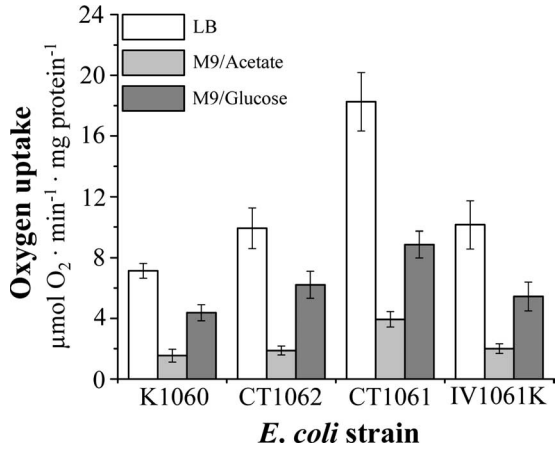

FIG. 2. Specific oxygen consumption rates. Strains were grown in LB medium for 24 h. Cells were spun at 10,000 × *g* for 10 min and washed twice by resuspension in LB medium or in the salt medium without a carbon source. A 100-μl aliquot of the bacterial suspension was used for the assay, which was performed essentially as described in reference 21. For the measurements in minimal medium, M9 (23) containing either 20 mM glucose or 60 mM sodium acetate was used. The results are mean values of duplicate measurements from two independent cultures ± standard deviations.

deletion spanning *lac-pro* [Δ(*lac-proAB*)*X111*], originally constructed in Hfr 3000 (3), so it was likely that ECL618 carried the *creC*(Con) mutation. This constitutive genotype is due to an R77P amino acid substitution in CreC (28). The sequence of a *creC* fragment obtained by PCR amplification (for primers, see Table S1 in the supplemental material) from K1060, CT1062, CT1061, and ECL618 revealed that strains ECL618 and CT1061 carry the constitutive *creC* mutation. Accordingly, these strains did not grow on minimal maltose medium plates. Some revertants which appeared after a 48-h incubation on this medium showed the dye-sensitive phenotype associated with *arcA* deletions, suggesting that in *cre* revertants, *arcA*::IS*10*-L and Δ*arcA* mutants have the same phenotype.

ArcA and CreC contribute to some of the CT1061 phenotypic traits. Complementation studies were performed to confirm the role of *creC* in the Dye phenotype (Fig. 1B). Strain CT1062 (Δ*arcA*) was complemented by wild-type *arcA* but not by the *arcA*::IS*10*-L allele, both of which are carried in the low-copy-number vector pWKS30 (26) (see Table S1 in the supplemental material). CT1061 [*arcA*::IS*10*-L *creC*(Con)] was the only *arcA* mutant strain which showed resistance to toluidine blue. A Δ*creB*::*kan* derivative of CT1061, IV1061K, was obtained by the method described by Datsenko and Wanner (7) (for primers, see Table S1 in the supplemental material). This strain presented the Dye phenotype expected for *arcA* mutants (data not shown). These results confirm that the *arcA*::IS*10*-L mutation confers a null-mutant phenotype and that the unusual Dye phenotype observed for CT1061 is a result of the contributions of both *arc* and *cre* mutations.

Another characteristic that distinguished CT1061 from CT1062 was respiratory capacity (18, 21). The constitutive *creC* allows the expression of the *cre* regulon even in complex LB medium (2). For the sake of comparison, and taking into account the fact that CT1062 and IV1061K do not grow in minimal medium, O_2 consumption was measured by using 24-h cultures grown in LB medium and transferred to the same medium or minimal medium containing either glucose or acetate. It was expected that the effect of CreBC on *ack-pta* genes would lead to a high level of O_2 consumption of CT1061 on acetate compared to that for the other strains. As expected, CT1061 [*arcA*::IS*10*-L *creC*(Con)] had the highest respiration capacity on the three substrates. In contrast, CT1062 (Δ*arcA*) and IV1061K [*arcA*::IS*10*-L *creC*(Con) Δ*creB*::*kan*] had higher levels of O_2 consumption in LB and minimal glucose media than those measured for the wild-type strain, but the level of O_2 consumption was not higher than that for the wild-type strain when acetate was used as a substrate (Fig. 2).

CreBC and the enzymatic and metabolic differences observed for CT1061. The differences in O_2 consumption rates when acetate was used as a substrate could reflect an increased acetate kinase activity in mutant and wild-type strains. Table 1 shows the results obtained when acetate kinase activity was determined for exponential LB medium cultures according to the method described by Dittrich et al. (8). AckA activity was fourfold higher for CT1061 than for the strains harboring wild-type *creC*. Our results agree with the effect of the constitutive *creC* mutation on genes controlling carbon metabolism as described previously (2). When the metabolic profile of the four

TABLE 1. Phenotypic characterization of the strains studied[a]

Strain	Relevant genotype	Ethanol/acetate ratio[b] (mol · mol^{-1})	Glucose consumption[c] (mM)	AckA[d] (U · mg protein^{-1})	Cytochrome content (pmol · mg protein^{-1})	
					Cytochrome *bd*	Cytochrome *bo*
K1060	Wild type	0.29 ± 0.05	14.67 ± 0.28	2.49 ± 0.67	223 ± 67	115 ± 21
CT1061	*arcA*::IS*10*-L *creC*(Con)	1.65 ± 0.53	19.38 ± 0.39	10.71 ± 2.14	78 ± 11	332 ± 53
CT1062	Δ*arcA*	0.57 ± 0.14	6.02 ± 0.61	2.98 ± 0.68	69 ± 18	308 ± 42
IV1061K	*arcA*::IS*10*-L *creC*(Con) Δ*creB*::*kan*	0.41 ± 0.11	12.06 ± 0.49	3.15 ± 0.97	ND[e]	ND

[a] Means ± standard deviations are shown for each parameter, corresponding to triplicate determinations performed for at least two independent cultures. Strains were grown in LB medium containing 20 mM glucose at 37°C in flasks shaken at 250 rpm for 24 h. Acetate kinase was measured in exponential cultures.
[b] Ethanol and acetate concentrations were analyzed as described in Table S2 in the supplemental material.
[c] Glucose was measured by using a commercial kit (Wiener Laboratorios, Rosario, Argentina) based on glucose oxidase/peroxidase levels.
[d] One unit is defined as the amount of enzyme capable of catalyzing the formation of 1 μmol of acetylhydroxamic acid per minute under the assay conditions described in reference 8.
[e] ND, not determined.

strains in 24-h cultures grown in LB medium supplemented with glucose was analyzed, the ethanol/acetate ratio for CT1061 was found to be higher than that for wild-type and mutant strains (Table 1; see Table S2 in the supplemental material). These results reflect a highly reduced intracellular redox state (24). The high glucose consumption observed for strain CT1061 compared to that for CT1062 can also be attributed to the mutated *creC* (Table 1). These experiments confirm that the different phenotypes observed are the result of the combined effects of an *arcA* null mutation and a constitutive *creC* mutation.

Cytochromes *bd* and *bo* are under ArcA regulation in CT1061. *E. coli* has two terminal oxidases, cytochrome *o* and cytochrome *d*, with low and high O_2 affinities, respectively (6). The analysis of *lacZ* fusions to *cyo* and *cyd* operons, which encode these cytochromes, showed that ArcA represses *cyo* and activates *cyd* in conditions of low O_2 availability (11). The cytochrome content of strains under study was measured according to previously described techniques (1, 17) in 24-h cultures grown in LB medium, and the results demonstrated that, as expected, ArcA controls their synthesis (Table 1). No activation of cytochrome *bd* and no repression of cytochrome *o* was detected in either *arcA* mutant compared to wild-type K1060. These results indicated that CreC is not involved in the regulation of electron transfer to a terminal acceptor in *arcA* mutant strains.

Conclusions. This is the first report to describe the contribution of CreC to phenotypic traits related to redox control that are normally attributed to ArcA mutants. When *E. coli* cultures are subjected to conditions of low O_2 availability, products with different degrees of oxidation, including acetate and ethanol, are synthesized in order to meet redox balance (5). The mutations in ArcA are characterized by the derepression of tricarboxylic acid cycle enzymes and cytochrome *o*, resulting in higher amounts of reducing equivalents and higher O_2 uptake. Part of the reducing equivalents produced are funneled into reduced compounds, and as a result, carbon flux is directed toward the synthesis of more-reduced products, among them ethanol, and so the ethanol/acetate ratio is higher in ArcA mutants. On the other hand, *creC* enhances carbon catabolism, as was confirmed by increased glucose consumption (Table 1). Thus, part of the excess reducing power generated by the mutants is consumed by the augmented amount of carbon intermediates due to *creC*^{c}, further increasing the synthesis of ethanol and other reduced products.

The Dye phenotype is due to the high respiration rate of *arcA* mutants (21). The particular phenotype of CT1061 on toluidine agar plates (Fig. 1B) can be explained by taking into account the highly reduced redox state in this strain (Table 1), which could be capable of neutralizing the deleterious effects caused by the high O_2 uptake of the null mutants. Moreover, IV1061K, the Δ*creB*::*kan* mutant derived from CT1061, recovers the Dye phenotype characteristic of *arcA* deletion mutants.

The size and stability of the truncated ArcA2 protein could allow its interaction with other regulators, but the complementation experiments and the synthesis of cytochromes *o* and *d* confirmed *arcA*::IS*10*-L as a null mutation. The possibility that another unexpected mutation(s) could have been introduced by P1 transduction cannot be ruled out. However, if any other genes have been cotransferred, their contribution to the phenotypic characteristics analyzed in this work was undetectable.

Avison et al. (2) mention that the wide distribution of *E. coli* strains harboring the unnoticed *creC*(Con) mutation could have an effect on previous results obtained using these strains. It is also worth noting that the (*lac-proAB*)*X111* deletion from HfrH was used to construct cloning hosts (29), and this could eventually lead to the subsequent and unknown propagation of the *creC*(Con) allele.

Kang et al. (14) pointed out the lack of a complete view of *E. coli* physiology in conditions of low oxygen availability, and since then, considerable work has been done based principally on a few well-known redox regulators (22, 24, 25). The role of CreBC in the metabolic response to different levels of oxygen availability may remain unnoticed in the wild-type genetic background, in which a stronger regulator, ArcA, is in control, but when this regulator is inactive, the influence of other regulatory systems gains importance, and their participation becomes relevant. The results presented in this study are a contribution to the understanding of the role of carbon metabolism in redox control.

Finally, the *creC* constitutive mutation plus the null ArcA phenotype may have a considerable impact on the optimization of *E. coli* strains for the synthesis of reduced bioproducts of biotechnological interest.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the EMBL database under accession number AM269884.

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