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C D Archer

J Jin

T Elliott

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Stabilization of a HemA-LacZ Hybrid Protein against Proteolysis during Carbon Starvation in *atp* Mutants of *Salmonella typhimurium*

C. DAWN ARCHER,[1]† JIN JIN,[2]‡ AND THOMAS ELLIOTT[1]*

Department of Microbiology and Immunology, West Virginia University Health Sciences Center, Morgantown, West Virginia 26506,[1] and Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294[2]

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Transposon insertions that stabilize the β-galactosidase activity of a HemA-LacZ hybrid protein following carbon starvation were mapped to the *atp* operon of *Salmonella typhimurium*. This effect is similar to that seen with *nuo* mutants defective in the energy-conserving type I NADH dehydrogenase. Insertions in several other genes, including such highly pleiotropic mutants as *rpoS*, *polA*, and *hfq*, were isolated with the same phenotypic screen, but they do not affect the β-galactosidase activity of HemA-LacZ. All of these mutants act indirectly to alter the colony color of many different fusion strains on indicator plates.

One strategy to recognize *lac* fusions in genes regulated by carbon starvation has been to employ an indicator medium containing a limiting amount of glucose as the carbon and energy source. Minimal agar plates with 0.02% glucose and the *lac* indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used to recover *lac* fusions to several starvation-induced genes including *rpoS* in *Escherichia coli* (9, 15). In our studies of the glutamyl-tRNA reductase (*hemA*) gene of *Salmonella typhimurium*, we used this medium to recover transposon-induced mutants (1, 2) which confer a dark-blue-colony phenotype on a strain carrying a *hemA-lac* protein fusion (Fig. 1). Strain TE2685 carries the promoter and the first 18 codons of *hemA* fused to *lacZ* at codon 10, followed by the rest of the *lac* operon (6, 12). The construct is in single copy at the *put* locus so that a functional copy of *hemA* is also present.

The mutants that were isolated carry insertions of mini-Tn*10* transposons (7, 14) marked with either Tet^r or Cam^r; these were all found to be unlinked to the *hemA-lac* fusion. Transductional crosses were used to place the insertions into linkage groups. One such group which we analyzed previously was shown to affect the *nuo* genes and lack the type I (energy-conserving) NADH dehydrogenase (2). The *nuo* mutants are not obviously defective under standard growth conditions, because a type II NADH dehydrogenase (non-energy-conserving) is also present in enteric bacteria (4, 5).

In previous work (2), it was found that during the growth of an otherwise wild-type *hemA-lac* (protein fusion) strain in medium with a limiting amount of glucose, the β-galactosidase activity of the HemA-LacZ hybrid protein declined rapidly following the onset of carbon starvation (half-life ≈ 30 min). In contrast, enzyme activity in the *nuo* mutants remained unchanged after carbon starvation. Pulse-chase and immunoprecipitation with anti-β-galactosidase antibody demonstrated that the HemA-LacZ hybrid protein was stabilized against proteolysis in *nuo* mutants. In a *lon* mutant, the decrease in β-galactosidase activity after carbon starvation was considerably slower, indicating that Lon is the main protease responsible for the degradation of HemA-LacZ in the wild-type. Since proteolysis by Lon is ATP dependent, we suggested that the proteolysis defect of *nuo* mutants may be due indirectly to a deficit of ATP. Cells grown on glucose as the carbon and energy source excrete acetate which can then be scavenged from the medium after starvation ensues (3, 11). The reduced energetic efficiency of *nuo* mutants, reflected in their very poor growth on acetate as a sole carbon and energy source, might be expected to decrease ATP synthesis.

Here, we report that a second group of mutants defective in the proton-translocating ATPase encoded by the *atp* operon has a phenotype similar to that of the *nuo* mutants in all respects we have examined. Figure 2 shows the results of β-galactosidase assays of a series of different mutants obtained in

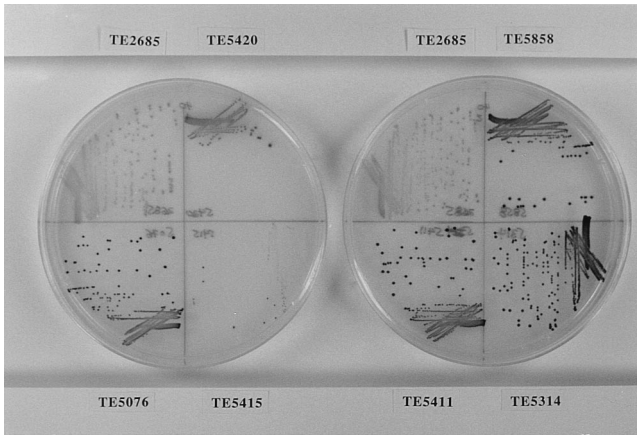

FIG. 1. Dark blue colonies formed by the mutants on minimal X-Gal indicator plates. All strains carry a *hemA-lacZ* protein fusion. Other than the wild-type fusion strain (TE2685), the mutants carry insertions in the following genes: *nuoA* (TE5076), *atpF* (TE5415), unidentified (TE5420), *polA* (TE5411), *hfq* (TE5314), and *rpoS* (TE5858). The plates contained 40 μg of X-Gal per ml with 0.02% glucose as the carbon and energy source (2) and were incubated for 3 days at 37°C.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, P.O. Box 9177, WVU Health Sciences Center, Morgantown, WV 26506-9177. Phone: (304) 293-8637. Fax: (304) 293-4667.

† Present address: Division of Infectious Diseases, Vanderbilt University School of Medicine, Nashville, TN 37232-2605.

‡ Present address: Department of Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35294.

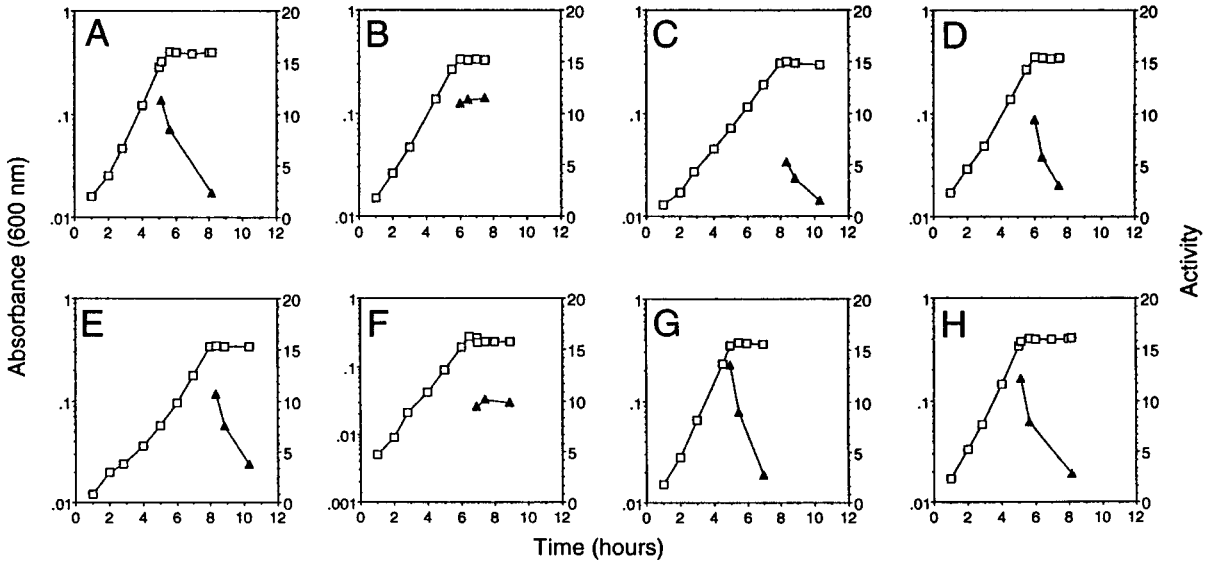

FIG. 2. Assay of β-galactosidase activity in *hemA-lacZ* protein fusion strains after carbon starvation. Growth (A_{600}) is indicated by open squares; β-galactosidase activity is indicated by solid triangles. (A) Wild type (TE2685). (B) *nuoA* (TE5076). (C) *hfq* (TE5314). (D) *zwf* (TE5406). (E) *polA* (TE5411). (F) *atpF* (TE5415). (G) Unidentified (TE5420). (H) *rpoS* (TE5858).

this study. For each strain, the first sample analyzed for enzyme activity was taken about 15 min before the onset of carbon starvation. Two of the mutant strains, *nuo* (Fig. 2B) and *atp* (Fig. 2F), show a stabilization of β-galactosidase activity compared with the decrease seen with the wild-type strain (Fig. 2A). However, the other mutants do not significantly stabilize HemA-LacZ and act in a manner similar to the wild type. A pulse-chase experiment using immunoprecipitation with anti-β-galactosidase antibody shows that the HemA-LacZ hybrid protein is stabilized in *atp* mutants (Fig. 3). The similar effects of *nuo* and *atp* mutants can be explained by their similar difficulty in growth on acetate.

Most of the mutants recovered have been identified (Table 1). Of 47 mutants characterized, 43 lie in five genes: *nuo*, *atp*, *rpoS* (stationary phase sigma factor), *polA* (DNA polymerase I), and *zwf* (glucose-6-phosphate dehydrogenase). Several of these mutants are pleiotropic, especially *polA* (11a), *nuo* (2), *hfq* (13), and *rpoS* (10, 15). The *hfq* locus (represented by a single insertion mutant) encodes an RNA-binding protein of unknown function for the bacterial cell that is required for RNA phage Qβ replication in *E. coli* (13). Also represented are two previously unsequenced genes and one unsequenced insertion that is unlinked to any other mutant. For the *polA* and *rpoS* mutants, complementation tests show that the plate phenotype is due to a loss of function of the respective gene.

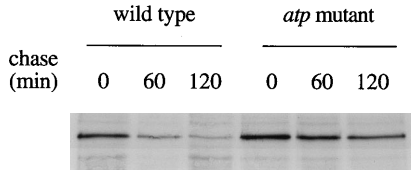

FIG. 3. Stabilization of the HemA-LacZ hybrid protein in an *atp* mutant after carbon starvation. Cultures were grown in minimal medium with 2 mM glucose as the carbon and energy source, labeled with [^{35}S]methionine before carbon starvation, chased with cold methionine, and processed for immunoprecipitation exactly as described previously (2). The wild-type strain was TE2685 *hemA-lac* [pr], and the mutant strain was TE5415 *atpF121*::Tn*10d*-Tet *hemA-lac* [pr].

Interestingly, the *polA* defect (caused by an insertion at codon 394) could be complemented by F′ plasmids producing either the N-terminal 5′-to-3′ exonuclease domain or the C-terminal DNA polymerase and 3′-to-5′ exonuclease domain. This unusual complementation behavior is reminiscent of the ability of either PolA domain to suffice for growth on rich medium (8).

Because the plate phenotype led to the discovery of the *nuo* mutants' defect in HemA-LacZ degradation, we expected that the other mutants would also show a change in β-galactosidase activity if an appropriate condition were tested. However, we found subsequently that none of the mutants (even *nuo* or *atp*) has a plate phenotype specific to the *hemA-lac* protein fusion employed in the original screen. Rather, *atp*, *nuo*, *polA*, *hfq*, and *rpoS* insertions each confer a dark-blue-colony phenotype on a number of *lac* operon and protein fusions which are otherwise very light blue on X-Gal plates. Similar findings were made for a limited set of fusions with all of the mutants. In fact,

TABLE 1. Mutants isolated in this study

Representative strain	Mutant allele	No. of isolates	Other drug resistance[a]	Method(s) of identification
TE5076	*nuoA2*::Tn*10d*-Tet	18	Cam^r	DNA sequence
TE5858	*rpoS1071*::Tn*10d*-Cam	13	Tet^r	DNA sequence
TE5406	*zwf-71*::Tn*10d*-Tet	5	No	PCR
TE5415	*atpF121*::Tn*10d*-Tet	4	No	PCR; DNA sequence
TE5411	*polA101*::Tn*10d*-Cam	3	Tet^r	DNA sequence
TE5314	*hfq-1*::Mud-Cam	1	No	DNA sequence
TE5420	*zxx-6823*::Tn*10d*-Cam	1	No	DNA sequence[b]; linkage tests
TE5421	*zxx-6824*::Tn*10d*-Cam	1	No	DNA sequence[c]; linkage tests
TE5419	Unknown	1	No	Linkage tests
Total		47		

[a] Indicates the drug resistance of other insertion mutants in the same locus.
[b] GenBank accession number U28166.
[c] GenBank accession number U28167.

an operon fusion that was constructed without a promoter-bearing insert and that has very low β-galactosidase levels and forms white colonies on indicator plates is affected by these mutations. The strain forms light blue colonies if a mutation in *nuo*, *polA*, *hfq*, or *rpoS* is also present. Thus, the discovery of the proteolysis defect in *nuo* and *atp* mutants was apparently accidental. As one possible explanation for the X-Gal indicator plate phenotype, perhaps these mutations accentuate the carbon limitation of strains growing with a very low concentration of glucose. If this limitation leads to the increased expression of a permease for X-Gal, then increased transport of the indicator dye could explain our results.

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