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atp Mutants of *Escherichia coli* Fail To Grow on Succinate Due to a Transport Deficiency†

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Escherichia coli atp **mutants, which lack a functional H^+-ATPase complex, are capable of growth on glucose but not on succinate or other C_4-dicarboxylates (Suc^- phenotype). Suc^+ revertants of an *atp* deletion strain were isolated which were capable of growth on succinate even though they lack the entire H^+-ATPase complex. Complementation in *trans* with the *yhiF* gene suppressed the growth of the Suc^+ mutants on succinate, which implicates the *yhiF* gene product in the regulation of C_4-dicarboxylate metabolism. Indeed, when the *E. coli* C_4-dicarboxylate transporter (encoded by the *dctA* gene) was expressed in *trans*, the Suc^- phenotype of the *atp* deletion strain reverted to Suc^+, which shows that the reason why the *E. coli atp* mutant is unable to grow aerobically on C_4-dicarboxylates is insufficient transport capacity for these substrates.**

The membrane-bound H^+-ATPase plays a central role in free energy transduction in *Escherichia coli*. Under aerobic conditions, the H^+-ATPase catalyzes the phosphorylation of ADP to ATP by use of proton motive force; under fermentative conditions, it energizes the inner membrane by catalyzing the extrusion of protons at the expense of ATP hydrolysis (7).

The H^+-ATPase complex in *E. coli* is encoded by nine genes, located in the *atp* operon and transcribed into a polycistronic messenger (7, 21, 22). Deletion of the entire *atp* operon results in an *E. coli* mutant that is completely devoid of the H^+-ATPase and that therefore lacks any of its associated activities (10). Such strains have to rely solely on substrate-level phosphorylation to produce ATP but grow relatively quickly under aerobic conditions when supplemented with glucose, although the growth rate and growth yield are somewhat decreased compared with those of the wild-type strain. Under these conditions, the respiration rate is increased compared with a normal *E. coli* strain, and it was suggested that the *atp* deletion strain supported uncoupled respiration in order to be able to profit from the increased rate of substrate-level phosphorylation (10).

The C_4-dicarboxylates, succinate, fumarate, and malate, sustain growth of wild-type *E. coli* strains under aerobic conditions. When these substrates are completely dissimilated to carbon dioxide via the tricarboxylic acid (TCA) cycle, ATP is produced mainly via oxidative phosphorylation, while only one ATP arises in the TCA cycle via substrate-level phosphorylation. It is a well-established fact that *atp* mutants of *E. coli* are unable to grow on nonfermentable C_4-dicarboxylates. Indeed, the Suc^- phenotype of *atp* mutants has traditionally been used to distinguish an *atp* mutant from a wild-type strain (3). There is, however, a difference between a substrate being nonfermentable, i.e., unable to support growth under anaerobic conditions, and a substrate which does not support the aerobic growth of an *atp* deletion mutant. This is because the aerobic *atp* mutant has the option to respire away any surplus of reducing equivalents that may be formed in catabolism, whereas the anaerobic *E. coli* cell must rely on the formation of reduced byproducts to get rid of any surplus of reducing equivalents. The *atp* deletion mutants do indeed respire away their excess of redox equivalents produced in the catabolic reactions, even though these mutants are unable to use the generated proton motive force for driving ATP synthesis by the H^+-ATPase (10).

Therefore, the fact that *atp* mutants do not grow on succinate or malate in the first place is somewhat more surprising: malate can be converted into pyruvate, which can in turn be converted either through the TCA cycle to carbon dioxide, yielding one ATP, or to acetate and ATP. In fact, pyruvate is quite a good substrate for growth of the *atp* deletion mutant.

In this paper we describe the isolation and characterization of mutants of an *E. coli atp* deletion strain that are capable of growth on the nonfermentable C_4-dicarboxylates. We demonstrate that the expression of the *dctA* gene, the structural gene encoding the transporter for C_4-dicarboxylates, enables an *atp* deletion strain to grow on the C_4-dicarboxylates. Furthermore, our data suggest that the product of the *yhiF* gene may act as a transcriptional regulator of the *dctA* gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The *E. coli* K-12 strain BOE270 is highly competent with respect to transformation and was derived from strain MT102, which in turn is an *hsdR* derivative of strain MC1000 (4). BOE270 was used for cloning purposes and for propagation of plasmid DNA in *E. coli*. Plasmid pFH2106 is a pBR322-derived expression vector that harbors a synthetic *lacUV5* promoter, two *lacO* operator sites, a poly-linker region, and a strong transcriptional terminator. Furthermore, it carries the *lacI* gene, encoding the *lac* repressor protein that binds to both *lacO* operators, conferring a tight uninduced repression of the cloned gene of interest in addition to the *bla* gene, conferring ampicillin resistance to the transformants. pFH2106 was kindly provided by F. G. Hansen (Technical University of Denmark) and will be described in detail elsewhere. pUN121 (18) was used for preparing gene libraries of genomic DNA.

Growth of bacteria. Luria-Bertani broth (LB) (15) was used as a rich medium, supplemented with antibiotics as required for the cloning experiments. Complementation tests were performed on AB minimal medium (5), pH 7.0, supplemented with thiamine (2.5 mg/liter) and the indicated carbon source. Plates were incubated at 37°C and contained 2% agarose in place of the usual (impure) agar.

Enzymes. Restriction enzymes and T4 DNA ligase were obtained from and used as recommended by Pharmacia and New England Biolabs.

Oligonucleotides. Oligonucleotides were obtained from Hobolth DNA synthesis (Hillerød, Denmark).

PCR amplification. A 1 μM concentration of each primer was combined with approximately 30 ng of genomic DNA isolated from LM1237 in a 100-μl PCR mixture. Thirty cycles, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 60 s of elongation at 72°C, were carried out with the

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† This paper is dedicated to the memory of Lars Boe.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference
N43	*ara-14* Δ*lac-85 acrA1 supE44 galK2 rpsL197 malA1 xyl-5 mtc-1*	17
LM1237	F^+ *asnB32 thi-1 relA1 spoT1*	10
LM2800	LM1237, but Δ*atpIBEFHAGDC750*	10
LM3115	LM1237, but Δ*atpIBEFHAGDC750 lacUV5 lacY*	11
LM3305	Spontaneous Suc^+ mutant of LM2800	This study
LM3559	LM2800, but *yhiD*::mini-Tn*5*	This study
BOE270	MC1000, but *hsdR* highly competent for Ca^{2+}-competent cells	This study
MC1000	*araD139* Δ(*ara-leu*)*7697 galU galK* Δ(*lacIPOZY*)*X74 rpsL thi-1*	4
pOMC3694	pBR322, carrying a mini-Tn*5* transposon plus flanking DNA cloned from LM3559 chromosomal DNA	This study
pFH2106	Expression vector derived from pBR322, carrying the *lacUV5* promoter, the *lacI* gene, and a multiple cloning site	F. G. Hansen, personal communication
pLAC-*yhiD*	pFH2106, carrying the *yhiD* gene from *E. coli*	This study
pLAC-*yhiF*	pFH2106, carrying the *yhiF* gene from *E. coli*	This study
pLAC-*dctA*	pFH2106, carrying the *dctA* gene from pKAT204	This study
pKAT204	pBR322, carrying the *dctA* gene from *E. coli*	1

AmpliTaq DNA polymerase, obtained from and used as recommended by Perkin Elmer.

Cloning of the *yhiD* and *yhiF* genes into pFH2106. Primers complementary to DNA sequences upstream and downstream of the *yhiD* gene and the *yhiF* gene were designed on the basis of the genomic sequence of *E. coli* (2) in order to amplify the two genes individually, including their ribosome binding site, from chromosomal DNA isolated from strain LM1237. The primers used were 5′-G CTCTAGACTTGCCGAATTAATGAGGTGC and 5′-CGGAATTCGTGTGA ATTTCAGGCTTACGG for amplification of the *yhiD* gene and 5′-GCTCTAG AGTCCTGTTAATTACCTTTGGC and 5′-CGGAATTCGTCGATAGAAGA CCTGTTGCG for amplification of the *yhiF* gene. In both cases the forward primer was extended at the 5′ end with an *Eco*RI site and the reverse primer was extended with an *Xba*I site in order to allow for proper insertion into the multiple cloning site of pFH2106. The fragments were ligated and transformed into *E. coli* by standard ligation and transformation procedures (15) and were plated with selection for ampicillin resistance, resulting in the plasmids pLAC-*yhiD* and pLAC-*yhiF*, in which the expression of the *yhiD* and *yhiF* genes has been placed under the control of the *lacUV5* promoter.

Cloning of the *E. coli dctA* gene in the expression vector pFH2106. Plasmid pKAT204 carries a 2.5-kb *Pvu*I fragment of *E. coli* chromosomal DNA encoding the *E. coli dctA* gene (1). pKAT204 was digested with *Kpn*I and *Fsp*I, resulting in a 1.4-kb fragment which harbors the intact *dctA* gene without its native promoter. This fragment was then cloned into pUC19, digested with *Kpn*I and *Hin*cII, resulting in the plasmid pUC-*dctA*. Subsequently, the *dctA* gene was cut out with *Hin*dIII and *Sac*I and inserted into pFH2106, which was also digested with *Hin*dIII and *Sac*I. This resulted in the plasmid pLAC-*dctA*, in which the expression of the *dctA* gene has been placed under the control of the *lacUV5* promoter.

Preparation of gene libraries. A preparation of chromosomal DNA from strain CSH11 (16) was digested with *Eco*RI, *Bam*HI, or *Hin*dIII. The resulting fragments were then cloned into the *Eco*RI, *Bcl*I, or *Hin*dIII sites, respectively, all located within the repressor cI gene of plasmid pUN121 carrying a tetracycline resistance gene and an ampicillin resistance gene. Successful insertions in pUN121 were selected after transformation into strain LM1237 by growth on LB plates containing ampicillin (100 μg/ml) and tetracycline (4 μg/ml). Each of the three gene libraries consisted of more than 10,000 transformants.

Transposon mutagenesis. A library of transposon-induced mutations was prepared in strain N43 (17). The method uses a Tn*5*-derived mini-transposon that specifies resistance to kanamycin as a selection marker (6); the transposon is located on a suicide delivery plasmid, pUT (9), that provides the transposase gene in *cis* orientation, but external to the mobile element, so as to generate stable insertion mutants after transposition. The library of transposon insertions was transferred into the recipient strain LM2800 by P1 transduction.

P1 transduction. Generalized P1 transduction was carried out by the standard protocol of Miller (16).

RESULTS AND DISCUSSION

Isolation of a spontaneous mutant of an *E. coli atp* deletion strain that grows on C_4-dicarboxylates. The *E. coli atp* deletion strain LM2800 is unable to grow on minimal medium supplemented with succinate as the sole carbon and energy source. However, when this strain was incubated on this medium, colonies began to appear after one week of incubation at 37°C and turned out to be mutants able to grow on succinate (Suc^+ phenotype), although at a much lower growth rate than an atp^+ strain. One of these colonies was restreaked to obtain a pure culture (LM3305) and used to investigate what kind of mutation had occurred to enable the *atp* deletion strain to grow on succinate.

In principle, the Suc^+ mutants could have activated an enzyme activity that would somehow allow these strains to benefit from oxidative phosphorylation in the absence of the H^+-ATPase complex (although this is not very likely in view of the complexity of this huge enzyme complex). If this was the explanation, then the Suc^+ mutants should grow also with acetate as the sole energy source. Table 2 shows the relative aerobic growth of LM3305 (Suc^+) and LM2800 (Suc^-) on agarose plates with various substrates. The growth of the two strains on the glycolytic substrates glucose, pyruvate, and lactate is similar, whereas neither strain could grow on acetate. These data show that the Suc^+ phenotype does not result from oxidative phosphorylation being reestablished. The Suc^+ mutant grew on all the C_4-dicarboxylates tested, whereas the Suc^- mutant did not. Thus, the Suc^+ phenotype includes growth on all of the C_4-dicarboxylates that can easily serve as carbon and energy sources for aerobic growth of wild-type *E. coli* strains. Interestingly, the data revealed quantitative differences in the growth of the Suc^+ mutant on the various substrates: strain LM3305 grew on succinate but grew even better on the two other C_4-dicarboxylates, fumarate and malate.

The metabolism of malate will generate one additional molecule of NADH compared with the metabolism of pyruvate, but it is not likely that this excess of redox equivalents makes up the whole difference between those two substrates, since lactate, which produces a similar amount of redox energy, does support the growth of the *atp* deletion mutant (although at a somewhat lower growth rate compared with pyruvate).

The growth rate of LM3305, in liquid minimal medium supplemented with succinate as the sole carbon and energy source, was 33% relative to the wild-type (atp^+) control strain, while the yield of biomass on succinate was 29% of the wild-type yield. The rate of succinate consumption and the rate of oxygen consumption were slightly increased, to 115% and 132%, respectively, compared with the wild type, and the Suc^+ mutant excreted 15% of the succinate input as acetate into the growth medium, which amounts to more than three times as much as the amount excreted by the wild-type (atp^+) strain.

TABLE 2. Growth of an *atp* deletion mutant, a Suc$^+$ mutant, and the wild-type strain of *E. coli* on solid medium and various carbon and energy sources

Strain	Relevant genotype or phenotype	Colony diam[a] (mm) on:						
		Glycolytic substrates[c]				TCA substrates[c]		
		Glucose	Pyruvate	Lactate	Acetate	Succinate	Fumarate	Malate
LM1237	*atp*$^+$ Suc$^+$	1	1.5	0.7	0.7	0.7	0.4	0.8
LM2800	*atp* Suc$^-$	1	1	0.5	—[b]	—	—	—
LM3305	*atp* Suc$^+$	1	1	0.5	—	0.1	0.2	0.3

[a] Diameter of single colonies, measured with a microscope and scored after 4 days of incubation at 37°C. The standard error on the determination of colony sizes is less than 15%.
[b] —, colony diameter smaller than 0.03 mm.
[c] Carbon sources: pyruvate, lactate, acetate, succinate, fumarate, and malate (5 g/liter) and glucose (2 g/liter).

Isolation of a transposon-induced Suc$^+$ mutant. The relatively high frequency at which the spontaneous Suc$^+$ mutant occurred indicated that the Suc$^+$ phenotype could be the result of a gene inactivation; it should be possible, then, to obtain the Suc$^+$ mutant through transposon mutagenesis. Indeed, the screening of a mini-Tn*5* transposon library in strain LM2800 resulted in a transposon-induced Suc$^+$ mutant, LM3559. The phenotype of transposon-induced Suc$^+$ mutant LM3559 was similar to that of the spontaneous mutant, LM3305, except that LM3305 yielded somewhat larger colonies on plates supplemented with succinate.

Mapping the mutation that leads to the Suc$^+$ phenotype. First, in order to ascertain that the Suc$^+$ phenotype of LM3559 was not due to multiple mutations, we performed generalized transduction of the Suc$^+$ mutation with phage P1. Indeed, it was possible to transfer the Suc$^+$ phenotype together with kanamycin resistance (encoded by the transposon) from LM3559 to LM2800, suggesting that only a single mutation (or two closely linked mutations) is involved in the Suc$^+$ phenotype of LM3559.

We then tried, unsuccessfully, to convert the Suc$^+$ phenotype of LM3559 to a Suc$^-$ phenotype by complementation in *trans* with libraries of wild-type *E. coli* chromosomal genes. Also unsuccessful was the reverse complementation test, i.e., conversion of the *atp* deletion strain, LM2800 (Suc$^-$), into a Suc$^+$ strain by complementation in *trans* with *E. coli* genomic libraries.

The next part of our strategy was to determine the insertion point of the mini-Tn*5* transposon in the *E. coli* chromosome in strain LM3559. Chromosomal DNA from strain LM3559 was digested with *Eco*RI, which will release a DNA fragment carrying the mini-transposon plus one of the regions flanking the insertion point on the *E. coli* chromosome. The DNA fragments were cloned into the *Eco*RI site on pBR322 with selection for kanamycin resistance encoded by the transposon. This resulted in a plasmid, pSUC-Tn*5*, carrying an insert of 1.8 kb. DNA sequencing revealed the insertion point of the mini-Tn*5* transposon in strain LM3559: the transposon is integrated in the C-terminal part of the *yhiD* gene, in the *slp-hdeB* intergenic region (section 317, complement 3652655 to 3653302 bp on the *E. coli* chromosome [2]). The function of the 23.2-kDa polypeptide encoded by the *yhiD* gene is unknown, but the polypeptide shows a relatively weak homology to the MgtC helper proteins involved in high-affinity Mg^{2+} transport in other bacteria (19).

Complementation with the *yhiD* gene in *trans* in the Suc$^+$ mutants does not restore the Suc$^-$ phenotype. The insertion of the transposon probably leads to inactivation of the *yhiD* gene product, and it was therefore of interest to see if we could suppress the Suc$^+$ phenotype by complementation with the wild-type *yhiD* gene in *trans*. We therefore cloned the *yhiD* gene into an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression vector, resulting in plasmid pLAC-*yhiD*. The growth of the Suc$^+$ mutants, LM3305 and LM3559, harboring pLAC-*yhiD* was then compared on succinate plates in the presence and absence of IPTG, but the plasmid had no suppressive effect on growth on succinate medium.

Complementation with the *yhiF* gene in *trans* in the Suc$^+$ mutants restores the Suc$^-$ phenotype. The negative outcome of the *yhiD* complementation test (see above) prompted us to look more closely at the genes flanking the *yhiD* gene on the *E. coli* chromosome. The open reading frames next to the *yhiD* gene, *yhiF* and *hdeB*, encode a hypothetical transcriptional regulator and a protein with unknown function, respectively. The *yhiD* and *yhiF* genes are convergently transcribed, and the transposon insertion in the *yhiD* gene in strain LM3559 may then somehow affect the expression of the *yhiF* gene. Among the proteins which are homologous to the *yhiF* gene product was DctR, the transcriptional regulator of dicarboxylate transport in *Rhodobacter capsulatus* (8).

We therefore cloned the *yhiF* gene into the expression vector pFH2106, yielding plasmid pLAC-*yhiF*, and tested for suppression of the Suc$^+$ phenotype in strain LM3305 and strain LM3559. A typical experiment is shown for LM3305 in Table 3. Clearly, a strong decrease in colony size was observed on the IPTG-containing plates. With malate as the substrate a similar result was obtained, but with pyruvate no suppression of growth occurred on IPTG-containing plates. We therefore conclude that expression of the *yhiF* gene in *trans* can suppress the Suc$^+$ phenotype.

The suppression of the Suc$^+$ phenotype found upon introduction of the wild-type *yhiF* gene in *trans* in the Suc$^+$ mutants strongly suggests that this gene is no longer expressed in strains with the Suc$^+$ phenotype. But it is unclear how the insertion of

TABLE 3. Complementation with the *yhiF* gene in *trans* in the Suc$^+$ mutant LM3305

Strain	Plasmid	Colony diam[a] (mm)		Carbon source[c]
		−IPTG	+IPTG[b]	
LM3305	pFH2106	0.12	0.12	Malate
LM3305	pLAC-*yhiF*	0.12	0.03	Malate
LM3305	pFH2106	0.06	0.06	Succinate
LM3305	pLAC-*yhiF*	0.06	0.01	Succinate

[a] Diameter of single colonies, measured with a microscope and scored after 4 days of incubation at 37°C. The standard error on the determination of colony sizes is less than 15%.
[b] 100 μM IPTG.
[c] Carbon sources at 15 mM.

TABLE 4. Complementation with the *dctA* gene in *trans* in the *atp* mutants, LM2800 and LM3115

Strain	Plasmid	Colony diam[a] (mm)		Carbon source[c]
		−IPTG	+IPTG[b]	
LM2800	pFH2106	—[d]	—	Malate
LM2800	pLAC-*dctA*	—	0.04	Malate
LM3115	pFH2106	—	—	Malate
LM3115	pLAC-*dctA*	—	0.06	Malate

[a] Diameter of single colonies, measured with a microscope and scored after 60 h of incubation at 37°C. The standard error on the determination of colony sizes is less than 15%.
[b] 3 μM for LM2800 and 6 μM for LM3115.
[c] Carbon sources at 15 mM.
[d] —, colonies smaller than 0.02 mm.

the mini-Tn*5* transposon in the *yhiD* gene might have affected the expression of the adjacent *yhiF* gene. One possibility is that the insertion of the transposon affects the degree of negative supercoiling in the vicinity of the *yhiF* gene and thereby the expression of this gene.

Recently, it was demonstrated that changes in the cellular energy state (the ATP/ADP ratio) do affect the level of DNA supercoiling in vivo (13, 20), and with succinate as the substrate it is conceivable that the intracellular ATP/ADP ratio reaches such a low level in the *atp* deletion strain (12) that the supercoiling in the *yhiF* locus is affected. One could then speculate that the following scenario is taking place: the expression of the *yhiF* gene is enhanced by the low level of negative DNA supercoiling in the *atp* mutant, and the *yhiF* gene product then represses the expression of the dicarboxylate transporter and prevents the *atp* mutant from growing on these substrates.

Complementation with the *dctA* gene in *trans* transforms the *atp* deletion mutant to a Suc$^+$ strain. The fact that the expression of the *yhiF* gene from the expression vector inhibited the growth of the Suc$^+$ mutants on C_4-dicarboxylates indicated that the *yhiF* gene product might function as a negative transcriptional regulator involved in C_4-dicarboxylate metabolism. Furthermore, since the *atp* deletion mutant grows well on pyruvate but not on malate plates, it is likely that growth of the *atp* deletion mutant on malate is limited either by transport of the C_4-dicarboxylates into the cell or by the conversion of malate into pyruvate. Together, these indications prompted us to analyze how the expression of the *dctA* gene (encoding the C_4-dicarboxylate transporter) might affect the growth of the *atp* deletion strain, LM2800. For this purpose, we inserted the *dctA* gene into the expression vector pFH2106, yielding pLAC-*dctA* (see Materials and Methods). As was noticed by Baker et al. (1), massive overexpression of the C_4-dicarboxylate transporter is detrimental to the cells. This was also the case in our experimental system, and we therefore also used another *E. coli atp* deletion mutant, LM3115, lacking the lactose carrier (*lacY*) in order to allow us to fine-tune the expression of the *dctA* gene, as was previously reported for other systems (11). Indeed, at intermediate concentrations of IPTG, the Suc$^-$ phenotype of strains LM2800 and LM3115 was converted to the Suc$^+$ phenotype (Table 4), whereas concentrations of IPTG that were too low or too high resulted in the Suc$^-$ phenotype. This demonstrates that the reason why the *atp* deletion strains fail to grow on the C_4-dicarboxylates is a lack of transport capacity for these compounds.

The expression in *trans* of the *dctA* gene, the structural gene encoding the C_4-dicarboxylate transporter, enabled the *atp* deletion strain to grow on the C_4-dicarboxylates, which suggests that the transport of succinate across the cytoplasmic membrane has a significant control on the growth (the biomass flux) of the *atp* deletion mutant on these substrates. Metabolic control analysis (14) predicts that the sum of the control coefficients, with respect to a flux, for all the enzymes in a system should add up to one, and experimental determination of control (particularly the control on growth rate) has indicated that the control is probably distributed among the many enzymes in the living cell. Therefore, the finding of a controlling step on the growth of *E. coli* is indeed an interesting result, but one should keep in mind that the *atp* deletion mutant is crippled by a lack of H$^+$-ATPase, which is a very unusual situation for an *E. coli* cell.

In summary, we conclude that growth of *E. coli atp* deletion mutants on C_4-dicarboxylates is limited by the activity of the C_4-dicarboxylate transporter. The results also point towards the *yhiF* gene product as a negative transcriptional regulator of the *dctA* gene in *E. coli*, and our current working hypothesis is that the *atp* deletion strain cannot grow on C_4-dicarboxylates, because the negative transcriptional regulator prevents sufficient expression of the C_4-dicarboxylate transporter. We therefore suggest that the *yhiF* gene be renamed *dctR*, for dicarboxylate transport regulator.

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