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Regulation of Acetyl Coenzyme A Synthetase in *Escherichia coli*

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Cells of *Escherichia coli* growing on sugars that result in catabolite repression or amino acids that feed into glycolysis undergo a metabolic switch associated with the production and utilization of acetate. As they divide exponentially, these cells excrete acetate via the phosphotransacetylase-acetate kinase pathway. As they begin the transition to stationary phase, they instead resorb acetate, activate it to acetyl coenzyme A (acetyl-CoA) by means of the enzyme acetyl-CoA synthetase (Acs) and utilize it to generate energy and biosynthetic components via the tricarboxylic acid cycle and the glyoxylate shunt, respectively. Here, we present evidence that this switch occurs primarily through the induction of *acs* and that the timing and magnitude of this induction depend, in part, on the direct action of the carbon regulator cyclic AMP receptor protein (CRP) and the oxygen regulator FNR. It also depends, probably indirectly, upon the glyoxylate shunt repressor IclR, its activator FadR, and many enzymes involved in acetate metabolism. On the basis of these results, we propose that cells induce *acs*, and thus their ability to assimilate acetate, in response to rising cyclic AMP levels, falling oxygen partial pressure, and the flux of carbon through acetate-associated pathways.

Cells of *Escherichia coli* undergo a metabolic switch associated with the production and utilization of acetate (19, 30). During exponential growth on a mixture of amino acids such as tryptone broth, cells consume first L-serine and then L-aspartate in a strictly preferential order. Simultaneously, they produce and excrete acetate. Once they have consumed both the serine and aspartate, these cells resorb and utilize acetate instead of excreting it. This acetate-associated metabolic switch occurs just as the cells begin to decelerate growth, i.e., just as they begin the transition to stationary phase (30).

Acetate production depends on one acetate activation pathway, while under these growth conditions, utilization requires a second (Fig. 1A). The first pathway, catalyzed by the enzymes acetate kinase (AckA; ATP:acetate phosphotransferase; EC 2.7.2.1) and phosphotransacetylase (Pta; acetyl coenzyme A [acetyl-CoA]:P_i acetyltransferase; EC 2.3.1.8) proceeds through an unstable, high-energy, acetyl phosphate (acetyl-P) intermediate (34). Cells use this low-affinity pathway to activate large concentrations of acetate (4, 21). The second pathway, catalyzed by the enzyme acetyl-CoA synthetase (Acs; acetate:CoA ligase [AMP forming]; EC 6.2.1.1) proceeds through an enzyme-bound acetyladenylate (acetyl-AMP) intermediate (2). Cells use this high-affinity pathway to scavenge for small concentrations of acetate (4, 21).

In vivo, the Acs pathway is irreversible due to intracellular pyrophosphatases that remove pyrophosphate, a critical pathway intermediate. This pathway, therefore, functions only anabolically. In contrast, the Pta-AckA pathway is completely reversible. As such, it plays a critical catabolic role during both mixed acid fermentation and aerobic growth on excess glucose or other glycolytic intermediates (4). Under conditions that result in mixed acid fermentation, acetyl-CoA cannot enter the tricarboxylic acid (TCA) cycle. Thus, the cells convey acetyl-CoA through the Pta-AckA pathway, producing and excreting acetate while generating ATP (10). Similarly, under aerobic conditions, when the carbon flux into cells exceeds the amphibolic capacity of the central metabolic pathways, e.g., the TCA cycle, cells adjust by moving acetyl-CoA through the Pta-AckA pathway, again excreting acetate and generating ATP. As a consequence, such cells also accumulate the intermediate of this pathway, acetyl-P (31). Later, as they begin the transition to stationary phase, cells undergo the metabolic switch. Instead of excreting acetate, they resorb it, activate it to acetyl-CoA by means of Acs (21), and utilize it to generate energy and biosynthetic components via the TCA cycle and the glyoxylate shunt, respectively (4) (Fig. 1B). Simultaneously, the levels of acetyl-P decline (31).

Evidence exists that this metabolic switch can play a significant role in the regulation of certain two-component signal transduction pathways (reviewed in references 25 and 46). By serving as a phosphodonor for the autophosphorylation of the two-component response regulator, OmpR, acetyl-P functions in the control of flagellar synthesis (31, 37), cell division (29), and the expression of outer membrane porins (11). Acetyl-P also seems to play a critical role protecting cells against carbon starvation (27), presumably through some as yet unidentified response regulator. Furthermore, acetylation by the enzyme Acs can activate the chemotaxis response regulator, CheY (1, 32, 47), although the physiological relevance of this modification remains unclear.

In our attempt to identify the signals that trigger this acetate-associated metabolic switch and to dissect its underlying mechanisms, we focused on the enzyme Acs. We did so because the activity of this enzyme, strictly required for acetate assimilation (21), had been shown previously to vary as much as 22-fold depending on the nature of the available carbon source (4). In contrast, the levels and activities of the enzymes AckA and Pta and the expression of their respective genes, *ackA* and *pta*, vary no more than 2- to 10-fold (4, 22, 27, 43). By performing *acs*::*lacZ* reporter, Northern, and immunoblot analyses, we have learned that cells control Acs activity to a large degree by regulating the induction of its gene *acs* in

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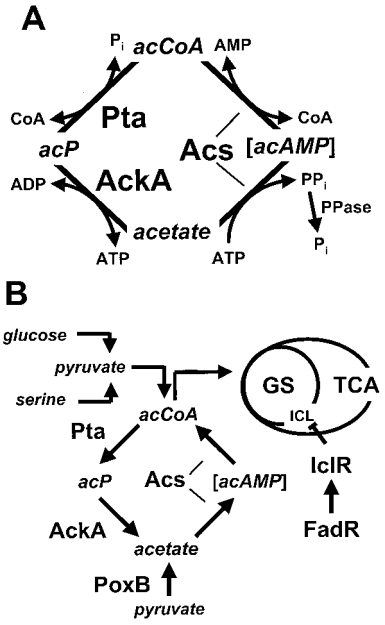

FIG. 1. (A) Pathways of acetate activation in *E. coli*, acAMP, acetyl-AMP; acCoA, acetyl-CoA; AckA, acetate kinase; acP, acetyl P; Acs, acetyl-CoA synthetase; CoA, coenzyme A; P_i, inorganic phosphate; PPi, pyrophosphate; PPase, pyrophosphatase; Pta, phosphotransacetylase. (B) Carbon flux through Pta-AckA, Acs, and associated pathways during growth in TB or on glucose. GS, glyoxylate shunt; TCA, tricarboxylic acid cycle; PoxB, pyruvate oxidase; ICL, *aceA* gene product isocitrate lyase; IclR, repressor of the glyoxylate shunt operon *aceBAK*; FadR, regulator of fatty acid metabolism that also activates *iclR*.

response to both the phase of growth and the nature of the carbon source. We also have found that the timing and/or magnitude of this induction depends, in part, on the carbon regulator cyclic AMP (cAMP) receptor protein (CRP), the oxygen regulator FNR, the glyoxylate shunt repressor IclR and its activator FadR, and several enzymes involved in acetate metabolism. On the basis of these results, we propose that cells induce *acs* transcription, and thus the ability to assimilate acetate, in response to rising cAMP levels, falling oxygen partial pressure, and the flux of carbon through pathways associated with acetate metabolism.

MATERIALS AND METHODS

Chemicals. Enzymes and substrates were obtained from Sigma Chemical Company (St. Louis, Mo.) or Promega (Madison, Wis.). Radiolabeled materials were from Amersham (Arlington Heights, Ill.), and Y-PER was obtained from Pierce Biochemicals (Rockford, Ill.).

Bacterial strains, plasmids, bacteriophage, and alleles. All strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 1 along with plasmids and phage.

The *acs*::*lacZ* transcriptional (operon) fusion λCB7 has been described previously (20). It was constructed by subcloning the *nrfA-acs* intergenic region into the multicopy vector pRS415 followed by recombination into the single-copy vector λRS88 using strain P90C (41). DH5α was used for constructing and propagating plasmids. Single lysogens of strain AJW678 (Δ*lac* Ace^+) were constructed and verified as described previously (41). Generalized transduction was performed using phage P1kc (39).

The *ackA*::Km allele was introduced into the AJW678 chromosome by means of homologous recombination using the temperature-sensitive suicide vector pMAK705 (15). The resultant *ackA* recombinants were verified by their poor ability to grow on 25 mM acetate as the sole carbon source (21) and their lack of motility due to an inability to form flagella at 35°C (31).

Media and growth conditions. Cells were grown at 37°C in tryptone broth (TB; 1% [wt/vol] tryptone, 0.5% [wt/vol] sodium chloride) or in minimal salts medium (M63 [26]) containing either D-glucose (11 mM) or acetate (10 mM). The optical density at 590 nm (OD_{590}) was monitored. For experiments involving a shift from one carbon source to another, cells were grown until the culture reached transition phase (defined as the point at which cells begin to grow at a lower rate), washed, and diluted 1:10 in fresh prewarmed M63 supplemented with acetate or glucose, further incubated as specified, harvested, washed, and resuspended in fresh prewarmed M63 supplemented with glucose or acetate, respectively.

Promoter activity assays. β-Galactosidase activity was determined quantitatively using the Y-PER β-galactosidase assay kit from Pierce Biochemical. Each value is the mean ± standard error of the mean (SEM) of three independent measurements. Each experiment was repeated two to five times.

Overexpression and purification of CRP and FNR DA154. CRP was overexpressed and purified as described previously (12). Purified FNR DA154 was generously provided by Helen Wing (University of Birmingham). FNR DA154, a constitutively active mutant of FNR that dimerizes stably in the presence of oxygen (48), was purified as a His_6-tagged protein from strain M15 by a new method described by Wing et al. (H. Wing, J. Green, J. Guest, and S. Busby, submitted for publication). Strain M15 (Qiagen) carries plasmid pREP4 (derived from pACYC) that encodes constitutively expressed LacI (Qiagen). M15 cells were transformed with pQE60, encoding FNR DA154 His_6 tagged at its C terminus. Transformants were grown at 37°C in 100 ml of L broth with appropriate antibiotics until cultures reached an OD_{600} of 0.5 to 0.6. Overexpression of the His_6-tagged FNR DA154 protein was induced by the addition of 0.1 M isopropyl-β-D-thiogalactopyranoside for 1 h. Cells were harvested, and pellets were sonicated in 10 ml of lysis buffer at 4°C (1 mg of lysozyme per ml, 50 mM NaH_2PO_4-Na_2HPO_4 [pH 8.0], 750 mM $NaNO_3$, 10 mM imidazole, 10 mM benzamidine). Sonicates were centrifuged at 10,000 × *g* and the amount of His_6-tagged FNR DA154 was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Supernatants were applied to nickel-nitrilotriacetic acid agarose (Qiagen) columns at 4°C so that the binding capacity of the agarose (5 to 10 mg/ml) was exceeded (typical column volumes were 0.75 to 1.0 ml). Columns then were washed with 50 column volumes of wash buffer (50 mM NaH_2PO_4-Na_2HPO_4 [pH 8.0], 750 mM $NaNO_3$, 20 mM imidazole), and FNR DA154 was eluted with elution buffer (50 mM NaH_2PO_4-Na_2HPO_4 [pH 8.0], 750 mM $NaNO_3$, 250 mM imidazole). Protein was stored at 4°C in elution buffer and remained stable for up to 6 months.

EMSA. Electrophoretic mobility shift assays (EMSA) using purified CRP were performed as described previously (35). EMSA using purified His_6-tagged FNR DA154 protein were carried out essentially as detailed by Ziegelhoffer and Kiley (48). Purified *nrfA-acs* intergenic region fragments were end labeled with [γ-^{32}P]ATP, and 2.5 to 0.5 ng of each fragment was incubated with various amounts of purified FNR DA154. The reaction buffer contained 10 mM potassium phosphate (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 50 μM dithiothreitol, 5% glycerol, and 25 μg of herring sperm DNA per ml. The final reaction volume was 10 μl. After incubation at 37°C for 10 min, samples were run in 0.25× Tris-borate-EDTA on a 6% polyacrylamide gel (12 V/cm) containing 2% glycerol and analyzed by autoradiography.

RESULTS

Regulation of *acs* transcription. To determine whether cells regulate *acs* primarily at the level of transcription, we used the single-copy *acs*::*lacZ* transcriptional fusion carried by phage λCB7 (Fig. 2A). With λCB7, we lysogenized cells wild type for acetate metabolism but deleted for the *lac* locus (strain AJW678) and monitored the growth and β-galactosidase activity of the resultant lysogen (strain AJW1786 [Fig. 2B]). Immediately following resuspension of the overnight inoculum into fresh TB, *acs* transcription was low (~200 Miller units [MU]). Within about 1 h, transcription began to rise, continued to increase throughout exponential growth, reached a maximum (~5,000 MU) during transition, and decreased substantially as the culture approached stationary phase. Northern hybridization studies yielded similar results, while immunoblot analyses showed that Acs protein levels parallel transcription (data not shown).

To investigate the transcriptional response to specific carbon sources, we grew cells of the *acs*::*lacZ* fusion strain (AJW1786) in M63 minimal medium supplemented with acetate or glucose and monitored their β-galactosidase activity. Cells grown on acetate as the sole carbon source (Fig. 3A) grew slowly (t_D ~210 min) and yielded high activity (~8,000 MU). In contrast, those grown on glucose (Fig. 3B) grew rapidly (time of doubling [t_D] of ~50 min) and produced low activity (~1,000 MU). Whereas cells grown initially on acetate and then exposed to glucose quickly shut off *acs* transcription (Fig. 3A), those grown first on glucose quickly induced *acs* transcription when

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant genotype	Source or reference(s)
AJW647	CP750 *proC*::Tn*5*-132	(P1)CP942→CP750
AJW678	AJW647 *proC*$^+$ Δ*lacX74* = *thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL136* Δ*lacX74*	(P1)BW13711→AJW647
AJW1074	CP875 *fadR*::Km	(P1)RS3040→CP875
AJW1075	CP875 *iclR*::Km	(P1)ERL5R→CP875
AJW1292	CP875 *crp*::Tn*5*	(P1)IT1133→CP875
AJW1706	AJW678 *aceA1 zja*::Tn*10*	(P1)SM6009→AJW678
AJW1727	AJW678 *fadR*::Km	(P1)AJW1074→AJW678
AJW1728	AJW678 *iclR*::Km	(P1)AJW1075→AJW678
AJW1729	AJW678 *poxB*::Km	(P1)YYC877→AJW678
AJW1730	AJW678 *fnr*::Tn*10*	(P1)M182*fnr*→AJW678
AJW1786	AJW678 λCB7	λCB7 lysogeny of AJW678
AJW1794	AJW678 *iclR*::Km λCB7	λCB7 lysogeny of AJW1728
AJW1807	AJW678 *fadR*::Km λCB7	λCB7 lysogeny of AJW1727
AJW1818	AJW678 *poxB*::Km λCB7	λCB7 lysogeny of AJW1729
AJW1868	AJW678 Δ(*ackA pta hisJ hisP dhu*) λCB7	(P1)CP911→AJW1786
AJW1876	AJW678 *aceA1 zja*::Tn*10* λCB7	λCB7 lysogeny of AJW1706
AJW1884	AJW678 *crp*::Tn*5* λCB7	(P1)AJW1292→AJW1786
AJW1938	AJW678 *fnr*::Tn*10* λCB7	λCB7 lysogeny of AJW1730
AJW1939	AJW678 *ackA*::Km	This study
AJW1940	AJW678 *ackA*::Km λCB7	λCB7 lysogeny of AJW1939
BW13711	Δ*lacX74*	B. Wanner
CP750	*thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL136*	30
CP875	*thi-1 thr-1*(Am) *leuB6 metF159*(Am) *rpsL136* Δ*lacX74* λ*lacY*	30
CP911	CP875 Δ(*ackA pta hisJ hisP dhu*)	30
CP942	*proC*::Tn*5*-132	C. Park
DH5α	*lacZ*Δ*M15 recA*	Bethesda Research Laboratories
ERL5R	*iclR*::Km	14, 33
IT1133	W3110 *crp*::Tn*5*	H. Aiba
M182*fnr*	*fnr*::Tn*10*	17
P90C	*ara* Δ(*pro-lac*) *thi*	41
RS3040	*fadR*::Tn*10*	40
SM6009	*aceA1 zja*::Tn*10*	23
YYC877	KL333 *poxB*::Km	7
pAA121/*pnrf53*	*E. coli nrfA-acs* intergenic region fragment carrying nucleotides −71 to −411 and −209 to +131 relative to the *acs* and *nrfA* promoters, respectively	18, 45
pCB26	+130 *nrfA* to the 3′ end of *acs* open reading frame subcloned into pGEM-T	This study
pGEM-T	General cloning vector for PCR-amplified products	Promega
pMAK705	*rep*(Ts) *cam*	15
pQE60	*fnrDA154-his*$_6$	Wing et al., submitted
pREP4	*lacI*$^+$(Con)	Qiagen
pRS415	*bla′ lacZ*$^+$ (transcriptional fusion vector)	41
λCB7	*bla′ acs*::*lacZ imm*^{434} *ind* (*acs* transcriptional fusion)	This study
λRS88	*bla′ lacZ imm*^{434} *ind* (transcriptional fusion vector)	41

shifted to acetate (Fig. 3B). Both Northern hybridization and immunoblot analyses yielded similar results (data not shown).

Involvement of CRP. To determine whether the acetate-dependent increase in *acs* transcription depends on CRP, we constructed a *crp* derivative of the λCB7 lysogen AJW1786, grew the resultant strain and its parent in TB, and monitored their growth and β-galactosidase activity (Fig. 4A and 5). Whereas wild-type cells induced *acs* transcription reproducibly to about 5,000 MU, those that lacked CRP exhibited activity that was barely detectable. We observed similar results at the protein level. In contrast to wild-type cells, those lacking CRP did not induce Acs protein synthesis when shifted from glucose to acetate (data not shown).

To determine whether CRP acts directly to facilitate *acs* transcription initiation, we performed EMSA (Fig. 6). We incubated fragments corresponding to the entire *acs-nrfA* intergenic region, its 5′ portion, or its 3′ portion with increasing concentrations of purified CRP protein in the presence of cAMP (0.2 mM). On the basis of these assays, we conclude that CRP binds to a single site located in the 3′ portion of the *acs-nrfA* intergenic region proximal to the putative *acs* promoter.

Involvement of FNR. An FNR binding site, identified genetically and shown previously to be required for the anaerobic induction of the *nrfA* promoter, resides within the putative *acs-nrfA* intergenic region included in the *acs*::*lacZ* fusion carried by λCB7 (45). To determine whether Fnr actually binds this site, we performed an EMSA. We incubated a fragment of the *acs-nrfA* intergenic region with increasing amounts of purified FNR DA154 (FNR*). Surprisingly, we observed two FNR-dependent shifts, suggesting that FNR binds a second, previously unknown, site within this region. To determine whether the binding of FNR to either of these sites exerts any influence on *acs* transcription, we tested an *fnr* derivative of AJW1786 during growth in TB and found that this mutant transcribed *acs* at about half the level achieved by its wild-type parent (Fig. 7). Other evidence supports the hypothesis that FNR affects *acs* transcription through its ability to bind to this second FNR site and that this site is located 3′ of the intergenic *Nsi*I site, i.e., proximal to *acs*: (i) a mutation in the *nrf*-proximal

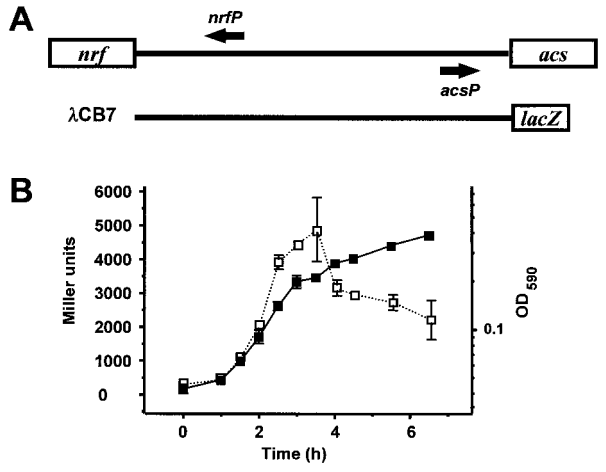

FIG. 2. (A) Schematic representation of the *nrfA-acs* intergenic region and the *acs*::*lacZ* operon fusion carried by λCB7. (B) OD_{590} (closed squares) and β-galactosidase activity in Miller units (open squares) for cells of strain AJW1786 (a λCB7 lysogen of the wild-type strain AJW678) grown at 37°C in TB. The SEM is shown only when it exceeded the size of the symbol.

FNR site (p46A; a GC-to-AT mutation at positions −46 and −234 relative to the *nrf* and putative *acs* transcription initiation sites, respectively) that completely eliminates *nrfA* transcription (45) had no effect on *acs* transcription during growth on TB (data not shown) and (ii) an *acs*::*lacZ* fusion that does not include the 5′ region behaved transcriptionally in an FNR-dependent manner (data not shown). To date, we have not identified this second FNR binding site; however, several potential sites are located within the *acs*-proximal region.

Involvement of the glyoxylate shunt. To determine whether carbon flux through the glyoxylate shunt influences *acs* transcription, we tested *iclR*, *fadR*, and *aceA* derivatives of AJW1786. Intriguingly, null mutations (in *iclR* or *fadR*) that cause the glyoxylate shunt to operate constitutively yielded results similar to that of a null mutation (*aceA*) that incapacitates the shunt by eliminating synthesis of the first shunt enzyme, isocitrate lyase (23) (Fig. 4B and 5). Although all three strains exhibited a pattern of *acs* transcription whose timing resembled that of their wild-type parent, their peak expression reached less than 40% that of the parent. Surprisingly, *poxB* mutant cells lacking pyruvate oxidase, the enzyme that oxidizes pyruvate directly to acetate (7), exhibited very similar behavior (Fig. 5).

Involvement of acetate production. To determine whether the ability to produce acetate influences *acs* transcription, we monitored *ackA* and *ackA pta* derivatives of AJW1786 (Fig. 4C and 5). Cells lacking only AckA or both AckA and Pta exhibited reduced *acs* transcription, a result consistent with immunoblot analyses that showed decreased steady-state levels of Acs protein in these mutants (data not shown). The exogenous addition of acetate to *ackA pta* mutant cells had no effect on the timing or levels of *acs* transcription (data not shown).

DISCUSSION

Observations made during these studies permit us to define the timing of *acs* induction (Fig. 8). During growth in TB, induction occurs at or near the conclusion of serine consumption and the beginning of aspartate consumption. This corresponds approximately to the time at which acetyl-CoA and acetyl-P pools reach their maximum (31). With the induction of Acs, both acetyl-CoA and acetyl-P pools decrease. The increasing presence of Acs likely contributes to this decrease by siphoning acetate, ATP, and CoA, the products of the Pta-AckA pathway. As long as acetyl-CoA remains in excess, such siphoning should continue to favor the generation of acetate and ATP. Indeed, the concentration of extracellular acetate continues to rise until the cells exhaust the supply of aspartate (31). At this time, the net acetate flux reaches zero (31) and the culture completes the metabolic switch. The extracellular acetate concentration starts to fall, the growth rate

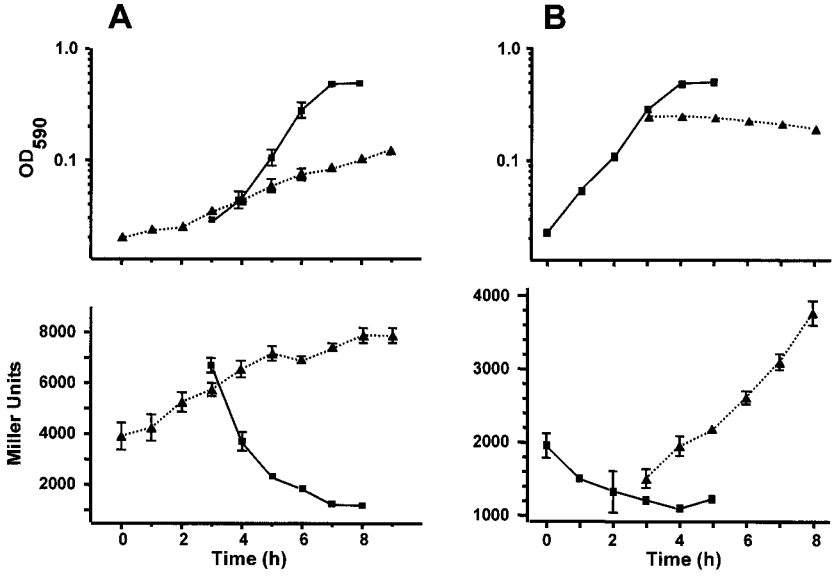

FIG. 3. OD_{590} (top) and β-galactosidase activity in Miller units (bottom) for cells of the wild-type strain AJW1786 subjected to an acetate-to-glucose nutritional shift (A) or to a glucose-to-acetate nutritional shift (B). Cells were grown at 37°C in M63 supplemented either with 10 mM acetate (triangles) or with 11 mM glucose (squares). After 3 h of incubation, each culture was split. One half retained the original medium composition, while the other half was washed and resuspended in fresh, prewarmed medium supplemented with the other carbon source. The SEM is shown only when it exceeded the size of the symbol.

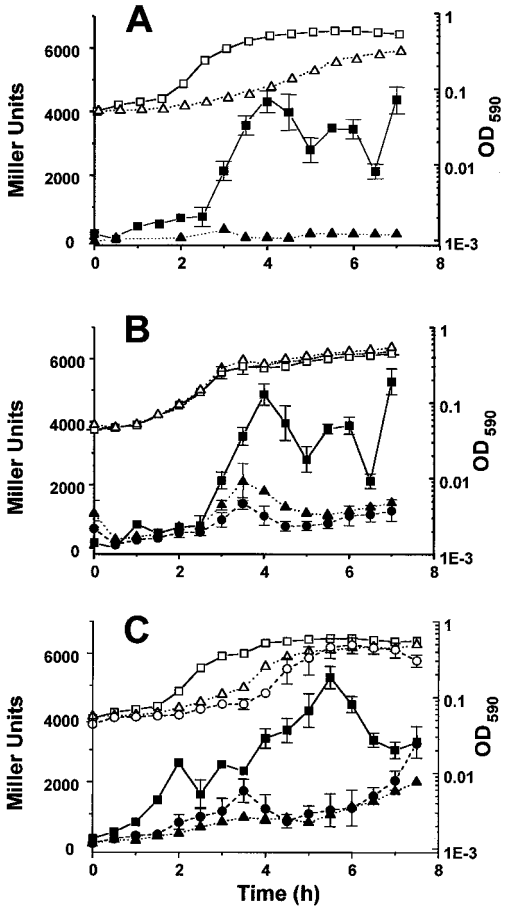

FIG. 4. OD_{590} (open symbols) and β-galactosidase activity in Miller units (closed symbols) for the wild-type strain AJW1786 and derivatives grown at 37°C in TB. (A) Strain AJW1786 (squares) and derivative lacking CRP (AJW1884; triangles); (B) strain AJW1786 (squares) and derivatives lacking IclR (AJW1794; circles) and FadR (AJW1807; triangles); (C) strain AJW1786 (squares) and derivatives lacking AckA (AJW1940; circles) and both AckA and Pta (AJW1868; triangles). The SEM is shown only when it exceeded the size of the symbol.

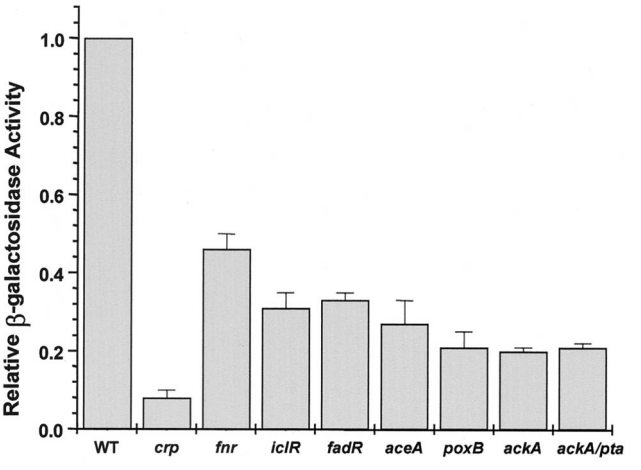

FIG. 5. β-Galactosidase activity monitored at transition phase during growth in TB of the wild-type strain AJW1786 or isogenic derivatives lacking CRP (AJW1884), FNR (AJW1938), IclR (AJW1794), FadR (AJW1807), AceA (AJW1876), PoxB (AJW1818), AckA (AJW1940), or AckA and Pta (AJW1868). Activity is expressed as a percentage of the mean activity of the wild-type strain AJW1786 (4,993 ± 179; $n = 7$). Each value represents the mean ± SEM of at least three independent measurements.

begins to decrease, and the culture commences its transition to stationary phase (30).

On the basis of reporter, Northern, and immunoblot analyses, we conclude that wild-type *E. coli* cells regulate this metabolic switch by inducing *acs*. This regulation occurs, in part, through the actions of the transcriptional regulators CRP, FNR, IclR, and FadR. Several acetate metabolic enzymes, including Pta, AckA, PoxB, and AceA, also contribute.

Evidence exists supporting direct roles for both CRP and FNR in regulating *acs* transcription initiation. A sequence that bears significant similarity to the CRP consensus binding site (5, 9) resides about 70 bp upstream of the proposed *acs* promoter (3, 44). EMSA and preliminary reporter analyses using selected CRP mutants support the relevance of this site (C. M. Beatty, D. Browning, S. Busby, and A. J. Wolfe, unpublished data). Thus, it seems likely that CRP acts directly to activate *acs* transcription and that rising cAMP levels help to trigger that transcription. EMSA data clearly support the existence of two FNR sites within the *acs-nrfA* intergenic region. One site has been identified previously by genetic means as required for the activation of the divergently transcribed *nrfA* promoter (45). We do not believe, however, that this site directly affects the transcription of *acs*. Instead, reporter analyses using mutant and deletion variants of the *acs*::*lacZ* fusion implicate a second site, which must reside considerably closer to the proposed *acs* promoter (Beatty et al., unpublished). Thus, it seems likely that FNR also acts directly to activate *acs* transcription and that falling oxygen partial pressure signals that transcription. If so, then *acs* would fall into the category of promoters controlled by tandem dimers of CRP or FNR or both (36).

Others previously reported the involvement of the glyoxylate shunt repressor IclR in *acs* transcription (38). We verified this

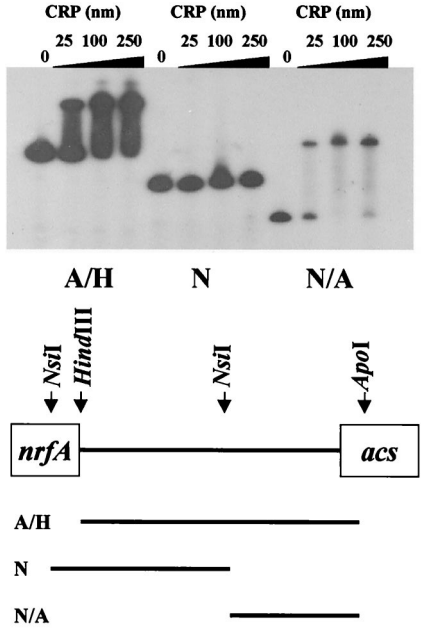

FIG. 6. Autoradiogram of EMSA, analyzing the binding of purified CRP to end-labeled fragments of the *acs-nrfA* intergenic region. The fragments are depicted in the schematic below. These fragments were generated by *Nsi*I, *Nsi*I/*Apo*I, and *Apo*I/*Hin*dIII digestions of pCB26. A/H, entire intergenic region; N, 5′ portion; A/N, 3′ portion.

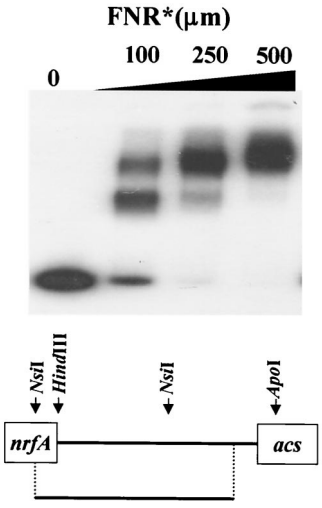

FIG. 7. Autoradiogram of EMSA, analyzing the binding of purified FNR DA154 (FNR*) to an end-labeled fragment of the *acs-nrfA* intergenic region. The fragment extends from +130 to −209 relative to *nrfA* and was generated by an *Eco*RI/*Hin*dIII digest of pAA121/*nrf53* as depicted in the schematic below.

report, showing that IclR affects the level but not the timing of *acs* promoter activity. We extended this observation to FadR, which activates *iclR* transcription directly (13) in addition to its role as the regulator of fatty acid metabolism genes (8). During growth in TB, which contains no fatty acids, FadR probably operates through IclR. Since we can find no sequences within the *acs-nrfA* intergenic region that closely resemble consensus binding sites for either IclR (28) or FadR (8), we believe it most likely that IclR operates upon *acs* transcription indirectly through its ability to control the synthesis of the glyoxylate shunt enzymes (Fig. 1B). Of course, we cannot rule out a direct effect until we test the ability of either protein to bind to this region. In fact, FadR binds to several sites that bear little resemblance to the reported consensus binding site (42). Because Acs produces the acetyl-CoA that functions as the glyoxylate shunt substrate, it seems reasonable that cells would

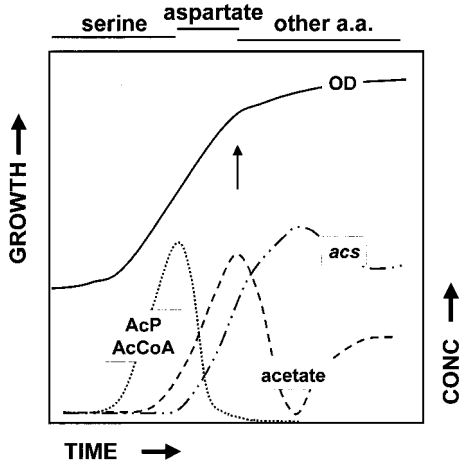

FIG. 8. Schematic showing the relative timing of events during growth of wild-type *E. coli* cells at 37°C in TB. The arrow highlights the timing of the acetate-associated metabolic switch. Lines at the top delineate the duration of amino acid (a.a.) consumption. AcP, acetyl-P; AcCoA, acetyl-CoA; OD, optical density; *acs*, β-galactosidase activity observed from *acs*::*lacZ* fusion carried by λCB7; acetate, extracellular acetate concentration.

coordinate the synthesis of Acs and the shunt enzymes. The fact that cells lacking the first shunt enzyme isocitrate lyase (*aceA*) displayed an *acs* transcription pattern almost indistinguishable from that of *iclR* and *fadR* mutants suggests that the feedback mechanism to *acs* senses both high and low glyoxylate shunt activity. Curiously, a *poxB* mutant that can no longer oxidize pyruvate directly to acetate transcribed *acs* in a manner strongly resembling that exhibited by *iclR*, *fadR*, and *aceA* mutants. This observation suggests that the mechanism that feeds back to *acs* senses some change in carbon flux.

We do not believe that either acetyl-P or acetyl-CoA functions as that feedback signal, despite the fact that both pools peak about the time that cells induce *acs* (31). We rule out acetyl-P because mutants lacking either AckA alone or both Pta and AckA behaved similarly with respect to *acs* transcription. Since the former accumulates acetyl-P and the latter fails to synthesize it at all, this observation argues strongly against any regulatory role for this intermediate of the Pta-AckA pathway. We also rule out acetyl-CoA because mutants lacking FadR display a pattern of *acs* transcription significantly different from that exhibited by wild-type cells. Since both cell types maintain acetyl-CoA pools at very similar levels (16), this observation argues that acetyl-CoA also cannot function in a regulatory capacity.

We are less certain concerning the regulatory role of acetate. Several observations support the hypothesis that acetate participates in the induction of *acs*. First, *acs* transcription correlates with extracellular acetate concentration. Second, cells inoculated into defined medium supplemented with acetate as the sole carbon source induce *acs* transcription rapidly. Third, the FadR-deficient mutant, which transcribes *acs* at reduced levels, utilizes acetate about five times faster than its wild-type parent (24). Such rapid utilization of acetate should keep the extracellular acetate pool low. If acetate functions as an inducing signal, however, then we must explain its failure to improve *acs* transcription by *ackA pta* cells that cannot excrete their own acetate. Such cells compensate for their inability to produce acetate by excreting nonacetate fermentation by-products, e.g., succinate and lactate (6). Perhaps one of these alternative products inhibits the response to acetate. If so, then this inhibitor does not affect the acetate-independent component of *acs* transcription. Alternatively, acetate itself may not signal *acs* induction.

Overall, the observations reported here suggest that the mechanisms used by *E. coli* cells to regulate *acs* expression are varied and complex. These seemingly include direct interactions by CRP and FNR to activate transcription initiation and indirect effects by acetate metabolic enzymes and transcription factors that control carbon flux. If so, then *acs* induction responds, in part, to rising cAMP levels, falling oxygen partial pressure, and changes in carbon flux. Such complexity should not be too surprising in light of the pivotal nature of this acetate-associated metabolic switch.

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