JOURNAL OF BACTERIOLOGY, Oct. 2010, p. 5304–5311 Vol. 192, No. 20
0021-9193/10/$12.00 doi:10.1128/JB.00738-10

Genetics and Regulation of the Major Enzymes of Alanine Synthesis in *Escherichia coli*[▽]

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Received 24 June 2010/Accepted 6 August 2010

Genetic analysis of alanine synthesis in the model genetic organism *Escherichia coli* has implicated *avtA*, the still uncharacterized *alaA* and *alaB* genes, and probably other genes. We identified *alaA* as *yfbQ*. We then transferred mutations in several transaminase genes into a *yfbQ* mutant and isolated a mutant that required alanine for optimal growth. For cells grown with carbon sources other than pyruvate, the major alanine-synthesizing transaminases are AvtA, YfbQ (AlaA), and YfdZ (which we designate AlaC). Growth with pyruvate as the carbon source and multicopy suppression suggest that several other transaminases can contribute to alanine synthesis. Expression studies showed that alanine modestly repressed *avtA* and *yfbQ* but had no effect on *yfdZ*. The leucine-responsive regulatory protein (Lrp) mediated control by alanine. We purified YfbQ and YfdZ and showed that both are dimers with K_ms for pyruvate within the intracellular range of pyruvate concentration.

The enzymes and pathway of alanine synthesis in the model organism *Escherichia coli* have not been well characterized (25). The most likely pathway is transamination of pyruvate by glutamate, catalyzed by glutamic-pyruvic transaminase (GPT). However, labeling studies have suggested some unanticipated complexities (7, 25, 26). Claire Berg and colleagues performed the only genetic analysis of alanine synthesis. They identified three genes that participate in alanine synthesis, namely, *avtA*, *alaA*, and *alaB* (1, 2, 36, 40). The activity of AvtA, also called transaminase C, was initially detected as an alanine-synthesizing enzyme with valine, not glutamate, as the nitrogen donor (27). Loss of either *avtA* or *alaA* did not affect growth and was apparent only in an *ilvE* background (2, 36, 40). An *alaA* mutant had normal AvtA and GPT activities, which suggested that AlaA was not a transaminase (1, 36). The *alaA* gene was physically mapped, but its product was not subsequently characterized (1). The *alaB* gene was identified from its partial suppression of the phenotype of an *ilvE alaA* strain (36). Multicopy *alaB* had elevated GPT activity, which suggested that *alaB* specifies a GPT (36). Except for a partial physical map of the *alaB* region, nothing else is known about *alaB* and its product (36).

Our goal in this study was to determine the enzymes of alanine synthesis using current knowledge of known and potential transaminase genes. Our genetic analysis suggests that AvtA, YfbQ, and YfdZ are the major enzymes of alanine synthesis, but eight other transaminases can potentially synthesize alanine. To confirm these conclusions, we also analyzed the regulation of *avtA*, *yfbQ*, and *yfdZ* and purified and partially characterized YfbQ and YfdZ.

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▽ Published ahead of print on 20 August 2010.

MATERIALS AND METHODS

Cell growth. The minimal medium for growth was W salts (10.5 g/liter K_2HPO_4, 4.5 g/liter KH_2PO_4, and 50 mg/liter $MgSO_4$) supplemented with 0.4% of a specified carbon source and 0.2% $(NH_4)_2SO_4$. Overnight cultures contained the following antibiotics: ampicillin (100 μg/ml), kanamycin (25 μg/ml), tetracycline (12.5 μg/ml), or chloramphenicol (10 μg/ml). The cells were harvested by centrifugation, washed and suspended in 150 mM NaCl, and inoculated into fresh medium without antibiotics. The cells were grown at 37°C for growth rate determinations, and cell density was measured using a Klett-Summerson colorimeter with a no. 42 filter.

Bacterial strains and plasmids. All strains used in this study are derivatives of *E. coli* K-12 strain W3110. Strains and plasmids are described in Table 1, except for the unnamed plasmids that contain genes for the *E. coli* transaminases: *argD*, *aspC*, *astC*, *avtA*, *bioA*, *gabT*, *glyA*, *hemL*, *hisC*, *ilvE*, *puuE*, *serC*, *tyrB*, *ybdL*, *yfbQ*, *yfdZ*, and *ygjG*. These plasmids were from the ASKA(-) clone library of the National BioResource Project (National Institute of Genetics [NIG], Japan) for *E. coli* strains (17).

The general procedures for constructing strains include deletions generated by the method of Datsenko and Wanner, transduction with phage P1, using the antibiotic resistance gene which replaced the gene of interest as a selectable marker, and removal of the antibiotic gene as described to produce an in-frame deletion (8). These general procedures are not described for each strain. Gene-specific details are provided below. Oligonucleotide primers used to construct strains are listed in Table 2. Deletions of *yfdZ*, *yfbQ*, *serC*, and *ybdL* were constructed using primers 1 to 8. The deletions removed codons 6 to 379 of the 412-residue *yfdZ*, codons 36 to 387 of the 405-residue *yfbQ*, codons 11 to 347 of the 362-residue *serC*, and codons 22 to 362 of the 386-residue *ybdL*.

E. coli strains SHK202, SHK203, and SHK204 contain *avtA-lacZ*, *yfdZ-lacZ*, and *yfbQ-lacZ* transcriptional fusions, respectively, which were inserted into the chromosomal *att*λ site. DNA for the promoter regions was obtained by PCR amplification of genomic DNA as follows. The *avtA* DNA (from positions −220 to +160 of the *avtA* start codon) was generated with primers 17 and 18 and digested with EcoRI and PstI. The *yfdZ* DNA (from positions −447 to +90 of the *yfdZ* start codon) was generated with primers 13 and 14 and digested with EcoRI and PstI. The *yfbQ* DNA (from −948 to +90 bases of the *yfbQ* start codon) used primers 15 and 16 and was digested with SphI and KpnI. These DNAs were cloned into pAH125's multicloning site, which is just upstream of a promoterless *lacZ* gene. Insertion of the fusion into the chromosome was as described previously (10). Integrant copy number was verified by PCR.

The pBLS17 plasmid was used to construct strain SHK502 which contains a *yfdZ-lacZ* translational fusion. pBLS17 is a derivative of pAH125 with the truncated *lacZ* from pUJ9 (12). It contains a multicloning site that can fuse proteins to *lacZ*. The *yfdZ* regulatory region, from positions −447 to +90 of the start codon, was generated with primers 19 and 20. This 537-bp fragment and the vector pBLS17 were cut with EcoRI, filled in with the Klenow fragment of RNA

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+) λ−	Laboratory stock
CBK741	Δ(*proB-lac*) *thyA ilvE12 avtA23*::Tn*5* [Kan^r]	36
CA69	*lrp*::Tn*10 gcv*::*lacZ* Δ*ara714* Δ*lac*	Elaine Newman
JW1178	BW25113 Δ*dadA*::*kan*	Keio collection
BW23474	Δ(*lacZYA-argF*)*U169 rpoS*(Am) *robA1 creC510 hsdR514* Δ*uidA*(MluI)::*pir-116 endA*(*BT333*) *recA1*	Genetic stock center
BL21 (λDE3)/pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Laboratory stock
W3110	*lacL8 lacI*^{q}	Laboratory stock
BLS5	W3110 *avtA23*::Tn*5*	This work
BLS61	W3110 Δ*yfdZ*::*cat*	This work
KE1	W3110 Δ*yfbQ*::*cat*	This work
KE3	W3110 Δ*yfbQ*	This work
KE4	W3110 Δ*yfbQ avtA23*::Tn*5*	This work
KE6	W3110 Δ*yfbQ* Δ*yfdZ*::*cat*	This work
KE7	W3110 *avtA23*::Tn*5* Δ*yfbQ* Δ*ybdL*::*cat*	This work
KE8	W3110 *avtA23*::Tn*5* Δ*yfbQ* Δ*yfdZ*::*cat*	This work
SHK1	W3110 *avtA23*::Tn*5* Δ*yfdZ*::*cat*	This work
AMMH8	W3110 *avtA23*::Tn*5* Δ*yfbQ* Δ*yfdZ* Δ*serC*	This work
SHK201	W3110 [′*lacZ* Kan^r]	This work
SHK202	W3110 [Φ(*avtA-lacZ*) Kan^r]	This work
SHK203	W3110 [Φ(*yfdZ-lacZ*) Kan^r]	This work
SHK204	W3110 [Φ(*yfbQ-lacZ*) Kan^r]	This work
SHK300	W3110 *lrp*::Tn*10*	This work
SHK301	W3110 *lrp*:: Tn*10* [′*lacZ* Kan^r]	This work
SHK302	W3110 *lrp*:: Tn*10* [Φ(*avtA-lacZ*) Kan^r]	This work
SHK303	W3110 *lrp*:: Tn*10* [Φ(*yfdZ-lacZ*) Kan^r]	This work
SHK304	W3110 *lrp*::Tn*10* [Φ(*yfbQ-lacZ*) Kan^r]	This work
SHK400	W3110 Δ*dadA*::*kan*	This work
SHK401	W3110 Δ*dadA* [′*lacZ* Kan^r]	This work
SHK402	W3110 Δ*dadA* [Φ(*avtA-lacZ*) Kan^r]	This work
SHK403	W3110 Δ*dadA* [Φ(*yfdZ-lacZ*) Kan^r]	This work
SHK404	W3110 Δ*dadA* [Φ(*yfbQ-lacZ*) Kan^r]	This work
BLS6	W3110 *att*λ::pBLS17 [′*lacZ* Kan^r] translational fusion control	This work
SHK502	W3110 [Φ(*yfdZ-lacZ*) Kan^r] translational fusion	This work
Plasmids		
pBLS1	Φ(*lacUV5p-glnG*) Amp^r	29
pSHK101	Φ(*lacUV5p-yfdZ*) Amp^r	This work
pSHK102	Φ(*lacUV5p-yfbQ*) Amp^r	This work
pAH125	*lacZ* transcriptional fusion vector; Kan^r	Genetic stock center
pBLS17	*lacZ* translational fusion vector (from pAH125)	Laboratory stock
pRSETC	T7 promoter-based expression vector; Amp^r	Laboratory stock
pRSHKQ	pT7::His$_6$-*yfbQ* Amp^r	This work
pRSHKZ	pT7::His$_6$-*yfdZ* Amp^r	This work

polymerase, and then cut with PstI. The appropriate fragments were ligated, and the translational fusion from the resulting plasmid was moved into the *E. coli* W3110 strain as described previously (10). The corresponding region of the promoterless pBLS17 was also inserted into the chromosome as a negative control.

Strain SHK300 was constructed by P1 transduction of strain CA67 (*lrp*::*tet*) into strain W3110. The transductant required 0.1% glutamate and 0.1% aspartate for optimal growth in minimal medium. Strain SHK400 was constructed by P1 transduction of the *dadA*::*kan* allele from strain JW1178 into strain W3110. The resulting strain could not grow with alanine as the sole carbon source in minimal medium and formed white colonies on TTC indicator plates supplemented with alanine (3). The *lacZ* transcriptional fusions were moved into these two strains with the various pAH125 derivatives (described above for strains SHK201 to SHK204), resulting in strains SHK301 to SHK304 and SHK401 to SHK404.

Plasmids pSHK101 and pSHK102 carried the structural genes for *yfdZ* and *yfbQ*, respectively. For both plasmids, the *lac* promoter precedes the transaminase gene. DNA for *yfdZ* was generated by PCR using primers 9 and 10, digested with BclI and EcoRI, and ligated into pBLS1 after removal of the 1.6-kb BclI-EcoRI fragment of the vector. The 1.2-kb *yfdZ* fragment extends from 98 bases upstream of the *yfdZ* initiation codon to 201 bases downstream of the termination codon. Plasmid pSHK102 expresses *yfbQ*. DNA for *yfbQ* was produced by PCR amplification with primers 11 and 12, digested with KpnI and EcoRI, and ligated into pBLS1 after removal of the 1.7-kb fragment between the KpnI and EcoRI sites. The 1.3-kb *yfbQ* fragment extends from 61 bases upstream of the initiation codon to 85 bases downstream of the termination codon.

The pRSET C protein expression system (Invitrogen) was employed to construct pRSHKZ and pRSHKQ for overproduction of *yfdZ* and *yfbQ*, respectively. In each case, a protein with a His_6 tag at the amino terminus was generated. The *yfbQ* structural gene was amplified by PCR from the genomic DNA of *E. coli* W3110 using primers 23 and 24. This generates a 1.3-kb fragment that extends from the initiation codon of *yfbQ* to 85 bases downstream from the termination codon. The resulting DNA was digested with BamHI and EcoRI and cloned into the BamHI and EcoRI sites of the expression vector pRSETC. The *yfdZ* gene was cloned into pRSETC using the same method employed for *yfbQ* cloning except with primers 21 and 22. The resulting DNA extends from the initiation codon of *yfdZ* to 201 bases downstream from the termination codon. Plasmids were propagated in *E. coli* DH5α.

Enzyme assays. For β-galactosidase assays, the cells were grown in the indicated minimal medium in 15-ml cultures grown to a density of about 100 Klett

TABLE 2. Oligonucleotide primers used to construct strains in this study

Primer	Use[a]	Sequence
1	Δ*yfdZ*	GGTCCGGGCGCTAACGTTAATCTGAGGAAATTATGGCTGACACTCGGTGTAGGCTGGAGCTGCTTC
2	Δ*yfdZ*	ACATCGCTTTAATCCCTCTGATGGCCTGACGAATACGGTCGCGCATATGAATATCCTCCTTAG
3	Δ*yfbQ*	GCTGAAAGAAGCAAAACGCCTGGAAGAAGAAGGTAACAAGGTACTGGTGTAGGCTGGAGCTGCTTC
4	Δ*yfbQ*	GATAACCAGAAAGGAAACGCGCGAACTTGCTCAAAGACAGCTCGATCATATGAATATCCTCCTTAG
5	Δ*serC*	CAACGCAACGTGGTGAGGGGAAATGGCTCAAATCTTCAATTTTAGTTCTGGTGTGTAGGCTGGAGCTGCTTC
6	Δ*serC*	ATTAACCGTGACGGCGTTCGAACTCAACCATGAAGTCTGTCAGCGCTTTCATATGAATATCCTCCTTA
7	Δ*ybdL*	GATTCCACAAAGCAAACTTCCACAACTTGGCACCACTATTTTCACCCGTGTAGGCTGGAGCTGCTTC
8	Δ*ybdL*	CCAGCAACGTCGATTCCTTCTTGGCAAAACAGAGACGAATCAGTTTCATATGAATATCCTCCTTAG
9	*lacp-yfdZ*	CCCTGATCACTGGACAGACAGAAATTAAT
10	*lacp-yfdZ*	CCGGAATTCAACTCACTGTATCAGGTGTAATGAAGT
11	*lacp-yfbQ*	CGGGGTACCATTGTTCTAAGCTGACTTCCAC
12	*lacp-yfbQ*	CCGGAATTCATAGTGACCTTACGTATGATG
13	*yfdZp-lacZ*	AAAACTGCAGAAACAGGCGGATACGGAT
14	*yfdZp-lacZ*	CCGGAATTCAGCCATTTTCAGTTCAGC
15	*yfbQp-lacZ*	ACATGCATGCTTAATTATCGGACGTTTG
16	*yfbQp-lacZ*	CGGGGTACCTTCTTCCAGGCGTTTTGCT
17	*avtAp-lacZ*	AAAACTGCAGGGCGACGATAAAGTGC
18	*avtAp-lacZ*	CCGGAATTCTAGCGTCTGGAAGTAGTC
19	*yfdZ′-′lacZ*	AAAACTGCAGAAACAGGCGGATACGGAT
20	*yfdZ′-′lacZ*	CCGGAATTCAGCCATTTTCAGTTCAGC
21	T7*p-yfdZ*	CCGGGATCC ATGGCTGACACTCGCCCTGAA
22	T7*p-yfdZ*	CCGGAATTCAACTCACTGTATCAGGTGTAATGAAGT
23	T7*p-yfbQ*	CGCAGGATCCATATG TCCCCCATTGAAAAATCCAGC
24	T7*p-yfbQ*	CCGGAATTCTTACAGCTGATGATAACCAGAAAGGA

[a] The primers were used to delete genes (e.g., *yfdZ*, *serC*, and *ybdL*) and fuse genes (e.g., fuse the *lac* promoter to the *yfdZ* gene [*lacp-yfdZ*] or fuse the T7 promoter to the *yfdZ* gene [T7*p-yfdZ*]).

units (A_{600} of about 0.7). The cells were harvested by centrifugation and washed with 1 ml of 150 mM NaCl, and cell pellets were frozen at −80°C until needed. The cells were thawed on ice, resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 2.7 μl/ml β-mercaptoethanol), and permeabilized by the addition of 50 μl of 0.1% SDS and 33 μl chloroform. The protein concentrations of the extracts were determined with bovine serum albumin as a standard (5). Specific activity is reported as units, which are nanomoles of product per minute per milligram of protein.

Glutamic-pyruvic transaminase (GPT) activity from crude extracts or during purification was followed using the reverse reaction, i.e., pyruvate formation from alanine. Activity was assayed at 37°C in two steps. The 1-ml reaction mixture contained 100 mM glycylglycine (pH 8.5), 1 mM KCl, 0.2 mM pyridoxal 5′-phosphate (PLP), 5 mM α-ketoglutarate, 5 mM alanine, 1 mM dithiothreitol (DTT), and 1 mM EDTA. The reaction mixture was preincubated without alanine at 37°C for 5 min, and the reaction was started by the addition of alanine. The reaction was stopped at 4, 6, 8, 10, 12, and 14 min, which was determined to be the linear portion of the reaction. The reaction was stopped by incubation for 5 min at 90°C. The pyruvate from the six time points was assayed by converting pyruvate to lactate, with the stoichiometric oxidation of NADH. Reaction samples were added to a mixture containing 100 mM glycylglycine (pH 8.5), 0.2 mM NADH, and 7.2 U of rabbit muscle lactate dehydrogenase (Sigma) in a final volume of 1 ml. The A_{340} was monitored. A molar extinction coefficient of 6,300 M^{-1} cm^{-1} for NADH was used for calculation. One unit of enzyme activity is 1 nmol of NADH per min per mg of protein. For determining the K_m for alanine, the alanine concentration was varied from 0.75 to 15 mM in the first reaction, and the proteins were purified through the affinity chromatography step.

The GPT reaction could also be measured in the forward direction, i.e., alanine formation, to study kinetic parameters. This assay measures α-ketoglutarate formation and was also performed in two steps. In the first step, a 1-ml reaction mixture contained 10 mM glutamate, 100 mM HEPES (pH 7.5), 40 mM NH_4Cl, 0.1 mM ADP, and 0.2 mM PLP. This mixture was preincubated at 37°C for 5 min. The reaction was started by the addition of pyruvate at a final concentration of 5 mM, incubated for 10 min at 37°C, and stopped by incubation for 5 min at 90°C. To these reaction mixtures, 0.15 mM NADPH and 3 U of beef liver glutamate dehydrogenase (Roche) were added, and the oxidation of NADPH was monitored at 340 nm. For determination of the K_m, the pyruvate concentration was varied from 0.015 to 20 mM in the first reaction, and the proteins were purified through the affinity chromatography step.

Protein expression and purification. *E. coli* strain BL21(DE3) carrying pLysS was transformed with either pRSHKQ (for YfbQ) or pRSHKZ (for YfdZ). A single colony was picked from L-broth plates supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and incubated into 250 ml of Terrific broth (28) containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The following procedure gave optimal soluble protein. Two-liter flasks were incubated without agitation at 37°C until the A_{600} was about 0.6. The culture was then transferred to room temperature (23°C) for 20 h with agitation. Although the T7 RNA polymerase is induced by isopropyl-β-D-thiogalactopyranoside, it was found that induction resulted in formation of insoluble protein. Therefore, the inducer was not added. The cells were harvested by centrifugation, and the cell pellet was stored at −80°C until further use.

All subsequent steps were done at 4°C. The cell pellets were resuspended in 20 ml of solubilization buffer (pH 7.8) (20 mM sodium phosphate, 0.05 mM PLP, 1 mM phenylmethylsulfonyl fluoride, 500 mM NaCl, and 20 mM imidazole). EDTA-free protease inhibitor cocktail (catalog no. P8840; Sigma) was added. Protein was solubilized through three cycles of freeze-thawing and six to nine cycles of sonication (30-s bursts with 60-s breaks). The cell debris was removed by centrifugation at 20,000 × *g* for 30 min, and the resulting cell-free crude extract was applied to a HisTrap HP column (GE Healthcare) equilibrated with solubilization buffer. After unbound protein was washed away with the same buffer, His-tagged YfdZ and YfbQ were eluted with 150 mM and 250 mM imidazole, respectively, in 20 mM sodium phosphate (pH 7.8) and 500 mM NaCl. Elution fractions were concentrated with 55% ammonium sulfate. The pellets had a light yellowish color. They were dissolved in 50 mM HEPES (pH 7.5) and dialyzed against 4 liters of 20 mM sodium phosphate buffer containing 0.15% β-mercaptoethanol, 0.05 mM PLP, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT and loaded onto an HP Superdex 200 column (GE Healthcare) to remove salts. His-tagged YfdZ formed a white precipitate during dialysis and lost activity upon dilution. The color suggests either loss of or failure to acquire PLP, but PLP in growth media, extraction buffer, dialysis buffer, and all solutions during purification had no effect on precipitation. Among a variety of conditions tested to prevent precipitation, only 100 mM KCl, 10% glycerol, and limiting the dialysis time reduced, but did not eliminate, precipitation (4).

Native molecular weight determination. Native masses of purified proteins were determined by gel filtration chromatography using an HP Superdex 200 column (GE Healthcare) equilibrated with 50 mM sodium phosphate (pH 7.6) containing 150 mM NaCl. Recombinant YfbQ or YfdZ was injected into the column and eluted at a flow rate of 0.5 ml/min in a fast protein liquid chromatograph (FPLC) (AKTA system; GE Healthcare). The molecular mass standard proteins were blue dextran (2,000 kDa), apoferritin (443 kDa), β-amylase (200

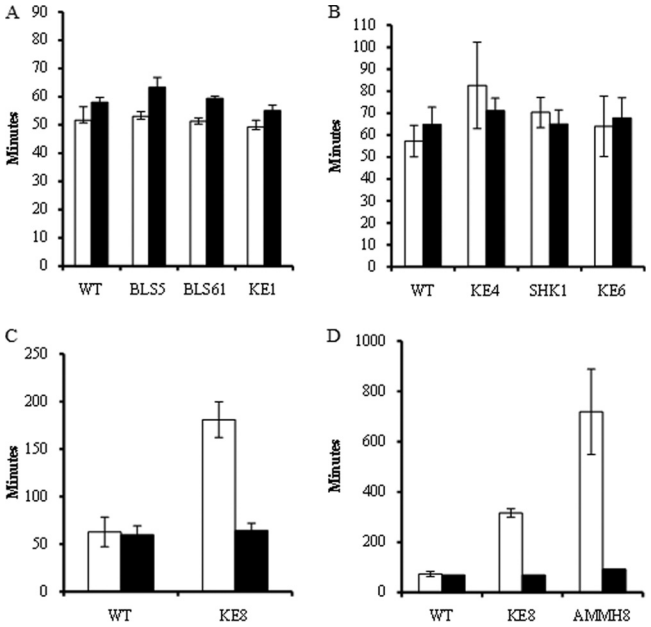

FIG. 1. Doubling times of *E. coli* mutants lacking transaminase genes. Panels A, B, C, and D show the doubling times for mutants lacking one, two, three, and four transaminase genes, respectively. For all growth rates, the medium was minimal medium containing glucose and ammonia supplemented with 0.01% alanine (black bars) or not supplemented with alanine (white bars). For the experiments in panel D, all cultures were supplemented with 0.01% serine and 1 μM pyridoxine, which are required for growth of strain AMMH8. The overnight cultures were the same as the experimental culture, except for strain AMMH8. The overnight culture for strain AMMH8 was supplemented with 0.01% alanine, washed twice with 150 mM NaCl to remove residual alanine, and inoculated into medium with or without alanine. The means ± standard errors of the means (SEMs) (error bars) are shown. WT, wild type.

kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

RESULTS

Identification of *alaA*. Three genes, *alaA*, *alaB*, and *avtA*, have been implicated in alanine synthesis. There is sufficient information to identify *alaA*, but not *alaB*. The midpoint of *alaA* is coordinate 2420450 of the first physical map of *E. coli* (1). To calibrate this location with the current physical map, we used the *nrdAB* locus, which is the closest common marker in both maps. This suggests a 14,000-bp correction, which places the midpoint of *alaA* at coordinate 2406450 of the current map. This is very close to the midpoint of *yfbQ*, which is coordinate 2406190. The *yfbQ* gene specifies a putative transaminase, which we conclude is *alaA*.

Evidence for three major enzymes of alanine synthesis. A strain with a deletion of *yfbQ* or with an insertion in *avtA* had no growth defect in liquid medium (Fig. 1A) or on solid medium (not shown). Unlike strains with only one lesion, *E. coli* strain KE4 (*avtA yfbQ*) formed smaller colonies on a glucose-ammonia agar plate, and alanine increased the colony size (not shown). There was no obvious defect for strain KE4 grown in liquid medium (Fig. 1B).

We then tested whether additional deletion of genes homologous to *avtA* and *yfbQ* further impaired alanine synthesis. The *avtA* gene is its own homology group, while *yfbQ* is a member of a group that contains *yfdZ*, *ybdL*, and *hisC* (16). Strains with a deletion of either *yfdZ* or *ybdL* were constructed and then transduced into strain KE4. Strain KE7 (*avtA yfbQ ybdL*) grew as well as strain KE4 did and was not examined further. Strain KE8 (*avtA yfbQ yfdZ*) grew three times slower than the wild type in liquid medium, and alanine restored normal growth (Fig. 1C and Table 3). Strain KE8 also grew poorly with glycerol, succinate, or acetate as the carbon source but grew normally with supplemental alanine (Table 3). The phenotype on agar plates was more pronounced. Strain KE8 formed colonies only after 5 to 7 days on glucose minimal medium plates without alanine, whereas strain W3110 (wild type) formed large colonies within 2 days (not shown). Strain KE8 containing a multicopy plasmid with either *yfbQ* or *yfdZ* grew as well as strain W3110 did in liquid or solid medium without supplemental alanine (not shown).

To determine whether deletion of all three genes is required for the growth defect, we completed construction and characterization of strains missing only one or two of the three defective genes in strain KE8. All single and double mutants grew normally in liquid medium without alanine, and alanine did not stimulate growth (Fig. 1A and B). Similarly, all single and double mutants grew normally on plates, except for strain KE4 (*avtA yfbQ*), which formed smaller colonies (not shown). These results show the following. (i) Significant impairment of alanine synthesis requires loss of three enzymes, AvtA, YfbQ (AlaA), and YfdZ. (ii) Any one of these enzymes is sufficient for optimal growth. (iii) No single enzyme or combination of enzymes in KE8 is sufficient for optimal growth. We conclude that AvtA, YfbQ, and YfdZ are the major enzymes of alanine synthesis.

Evidence for several minor alanine-synthesizing transaminases. At least one more enzyme can synthesize alanine because pyruvate as a carbon source supported near-normal growth of *E. coli* KE8 (Table 3). Phosphoserine aminotransferase, the product of *serC* in *E. coli*, has been shown in other organisms to synthesize alanine or use alanine as a nitrogen donor (11, 13). Therefore, we examined whether *serC* contrib-

TABLE 3. Growth of *E. coli* strains W3110 (wild-type) and KE8 (*avtA yfdZ yfbQ*) on various carbon sources[a]

Carbon source	Doubling time (min) (mean ± SEM)[b]			
	W3110 (wild type)		KE8 *(avtA yfbQ yfdZ)*	
	Without alanine	With alanine	Without alanine	With alanine
Pyruvate	106 ± 5	109 ± 2	132 ± 7	137 ± 10
Glucose	63 ± 16	60 ± 10	181 ± 19	64 ± 8
Glycerol	71 ± 3.5	74.6 ± 2.9	206 ± 22[c]	83 ± 8.7[d]
Succinate	72 ± 1.4	70 ± 5	433 ± 99	120 ± 18[d]
Acetate	121 ± 19	144 ± 11	1352 ± 187	188 ± 2[d]

[a] Cells were grown with the indicated carbon source at 0.4% with and without 0.01% alanine.

[b] The mean doubling times ± standard errors of the means (SEMs) of three determinations are shown.

[c] There was a 5- to 8-h initial lag, following by the doubling time shown from 10 to 50 Klett units. After this growth, the doubling time was 72 min ± 21 min.

[d] Results are for the initial phase of growth, until the alanine was presumably depleted.

TABLE 4. GPT activity from various mutants[a]

Strain	Presence or absence of enzyme[b]			GPT activity[c]
	AvtA	YfbQ	YfdZ	
W3110	+	+	+	5.07 ± 1.47 (5)
BLS5	−	+	+	6.84 ± 0.66 (5)
BLS61	+	+	−	5.72 ± 1.84 (5)
KE1	+	−	+	4.79 ± 1.00 (4)
KE4	−	−	+	5.55 ± 2.50 (3)
KE6	+	−	−	0.40 ± 0.36 (2)
SHK1	−	+	−	6.51 ± 1.08 (4)
KE8	−	−	−	0.69 ± 0.05 (2)
KE8/pSHK101	−	−	+++[d]	15 (1)
KE8/pSHK102	−	+++[d]	−	600 (1)

[a] Cells were grown with glucose as the carbon source.
[b] The presence (+) or absence (−) of AvtA, YfbQ, and YfdZ enzymes in *E. coli* strains is shown.
[c] GPT activity is shown in nanomoles per minute per milligram of protein. The means ± standard errors of the means are shown. The number of determinations is shown in parentheses for each value.
[d] The corresponding gene was carried on a high-copy plasmid.

utes to alanine synthesis in a mutant lacking the major alanine transaminases. *E. coli* AMMH8 (*avtA yfbQ yfdZ serC*) grew 10 times slower than the wild type in glucose minimal medium without alanine, and alanine restored growth (Fig. 1D). With pyruvate as the carbon source without supplemental alanine, strain AMMH8 (195-min doubling time) grew 2.3 times slower than the wild type did (85-min doubling time). These defects are worse than those for strain KE8, which suggests that SerC is a minor alanine transaminase. The growth of strain AMMH8 without supplemental alanine suggests at least one more minor alanine-synthesizing enzyme.

To identify potential alanine-synthesizing enzymes, we complemented the defect in a mutant lacking the three major alanine transaminases with plasmids containing all 16 transaminase genes in *E. coli*. The cells were grown with glucose and succinate as the sole carbon source. In addition to the plasmids with genes for the three major alanine transaminases, plasmids with *argD*, *astC*, and *serC* almost completely suppressed the growth defect, and plasmids with *aspC*, *gabT*, *puuE* (also called *goaG*), *tyrB*, and *ygjG* partially suppressed the growth defect (results not shown). Eight transaminases, when expressed from multicopy plasmids, appear to synthesize alanine.

Assay of GPT activity. To test whether growth rates corresponded to loss of detectable alanine-synthesizing transaminases, we assayed GPT activity from crude extracts. The assay for GPT activity does not measure AvtA activity, which we did not assay, since a mutation in *avtA* reduces AvtA activity at least 60-fold (36). All three strains that had lost only one transaminase (BLS5, BLS61, and KE1) and two of the double mutant strains, strains KE4 (*avtA yfbQ*) and SHK1 (*avtA yfdZ*), had a wild-type level of GPT activity (Table 4). In contrast, strains KE6 (*yfdZ yfbQ*) and KE8 (*yfdZ yfbQ avtA*) retained about 10% of wild-type GPT activity. The sum of YfdZ and YfbQ activities accounts for 90% of GPT activity in *E. coli*, and the residual GPT activity presumably results from minor alanine-synthesizing enzymes. Since loss of either YfdZ or YfbQ does not affect total activity, it appears that loss of one results in an increase in the activity of the other. However, as expected, loss of activity below a certain level does impair growth.

Regulation of *yfbQ*, *yfdZ*, and *avtA* expression. For wild-type cells grown in minimal medium containing glucose and ammonia, total GPT activity from crude extracts, with and without alanine, was 5.0 ± 0.9 and 5.1 ± 1.5 units per mg of protein, respectively. This activity is largely the sum of YfbQ and YfdZ activities. Alanine appears to exert little regulation on total GPT activity.

We also examined the gene-specific regulation of the three major genes of alanine synthesis with *lacZ* transcriptional and translational fusions in a wild-type background. The fusions were inserted in the λ attachment site and were therefore single copy. The results with *yfbQ* and *yfdZ* translational and transcriptional fusions gave identical results, and therefore, the former are not shown. First, we tested whether the end product, alanine, repressed expression. In minimal medium, 1 mM exogenous alanine repressed β-galactosidase 2-fold in strains with *avtA-lacZ* and *yfbQ-lacZ* transcriptional fusions (Fig. 2A). Alanine (10 mM) and the dipeptide L-alanine–L-alanine repressed *yfbQ* and *avtA* as well as 1 mM alanine did (results not shown). We tested whether alanine degradation prevents significant alanine accumulation and obscures a larger repression. L-Alanine is degraded by racemization to D-alanine, followed by oxidation by D-amino acid dehydrogenase (24). However, introduction of a deletion of *dadA*, which codes for a subunit of the dehydrogenase, had no effect on repression (Fig. 2C). Second, we tested whether alanine starvation, imposed by the alanine analog, β-chloro-L-alanine (41), activated expression. The analog at 1 mM increased the lag phase before exponential growth but did not affect the exponential growth rate. Higher concentrations prevented growth. The analog (1 mM) had no effect on expression of any of the three genes examined (data not shown). Third, we tested a result from a gene profiling analysis that suggested that high pH induced *avtA* and *yfbQ* (21). However, pH 8.7 did not affect expression of any of the three transaminase genes for cells grown in L broth (results not shown). Finally, anomalous labeling of alanine was observed for acetate-grown cells (26), and we tested whether such growth altered regulation. However, growth with acetate as the carbon source had no effect on *yfbQ* and *yfdZ* expression (results not shown). The only regulation found was modest repression by alanine.

The leucine-responsive regulatory protein (Lrp) binds alanine (25), although it is most often associated with the ligand leucine (6, 22). We tested whether Lrp regulated *avtA* or *yfbQ*. In a *lrp* background, alanine repressed neither *avtA* nor *yfbQ*, which implies that Lrp mediates alanine repression (Fig. 2B). The ligand leucine modestly represses *avtA* and *yfbQ*, although not as well as alanine does (Fig. 2A). Lrp is not synthesized when cells are grown in broth (19), which predicts that alanine will not repress for cells grown in broth. Consistent with this prediction, alanine had no effect on either gene (data not shown). We conclude that Lrp mediates repression of both *avtA* and *yfbQ* by alanine and leucine.

Alanine (1 or 10 mM) did not affect *yfdZ* expression in strains with a *yfdZ-lacZ* fusion (Fig. 2A). Furthermore, no condition tested affected *yfdZ* expression (results not shown). Vanderpool and Gottesman have reported that SgrR stimulates *yfdZ* expression 2-fold and mediates activation by sugar-phosphate stress (35). Mutants lacking *yfdZ* did not have al-

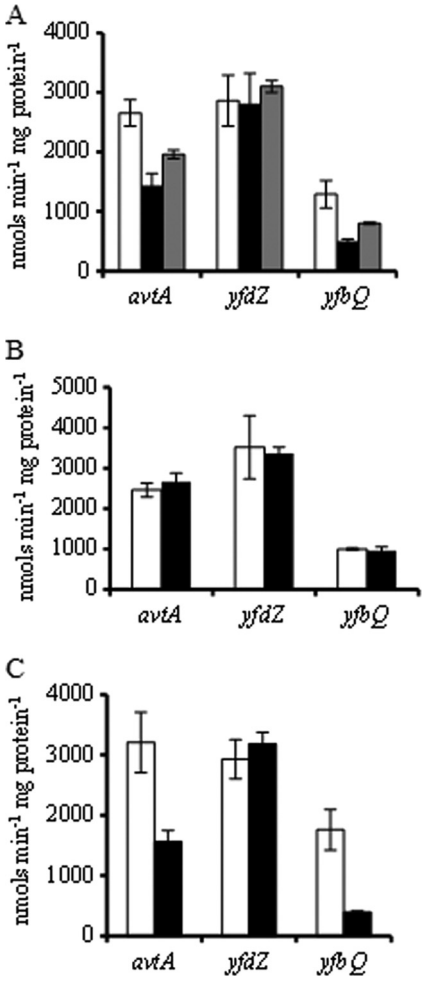

FIG. 2. β-Galactosidase activity from reporter strains with *avtA-lacZ*, *yfdZ-lacZ*, and *yfbQ-lac*Z transcriptional fusions. All β-galactosidase activities are the averages ± SEMs (error bars) for three independent determinations. Panels A, B, and C show results from W3110 (wild type), SHK300 (*lrp*), and SHK400 (*dadA*) backgrounds, respectively. Cells were grown in minimal medium containing glucose and ammonia without supplementation (white bars) or supplemented with 0.01% alanine (black bars) or with 1 mM leucine (gray bars). Growth of the *lrp* mutants was supplemented with 0.1% glutamate and 0.1% aspartate. A strain with a promoterless *lacZ* fusion gave ≤100 units of activity.

tered sensitivity to α-methylglucoside, which induces sugar-phosphate stress (results not shown).

The genes adjacent to *yfbQ* and *yfdZ* code for proteins whose functions have not been established, including some potential transcriptional regulators. The genes divergently transcribed from *yfdZ* are *ypdA*, *ypdB*, and *ypdC*, and they specify a putative membrane-bound sensor kinase, a proposed response regulator, and a proposed AraC-like regulator, respectively. Deletions of these genes in a mutant lacking both *avtA* and *yfbQ* had no effect on growth, which implies that synthesis of the remaining major alanine transaminase, YfdZ, does not require these genes (results not shown). The gene downstream from *yfbQ* is *yfbR*, which specifies a putative deoxyribonucleoside 5′-monophosphatase. An in-frame deletion of *yfbR* had no effect on growth in an *avtA yfdZ* background (results not shown), which implies that *yfbR* is not required for *yfbQ* expression. However, an insertion in *yfbR* severely impaired growth in an *avtA yfdZ* strain (results not shown), which implies that the insertion eliminated *yfbQ* expression. We suspect that *yfbQ* and *yfbR* are members of an operon and that the insertion affected the stability of the *yfbQ* mRNA. Even though the genes adjacent to *yfbQ* and *yfdZ* are not required for their expression, we cannot exclude, nor did we test for, more subtle regulatory roles.

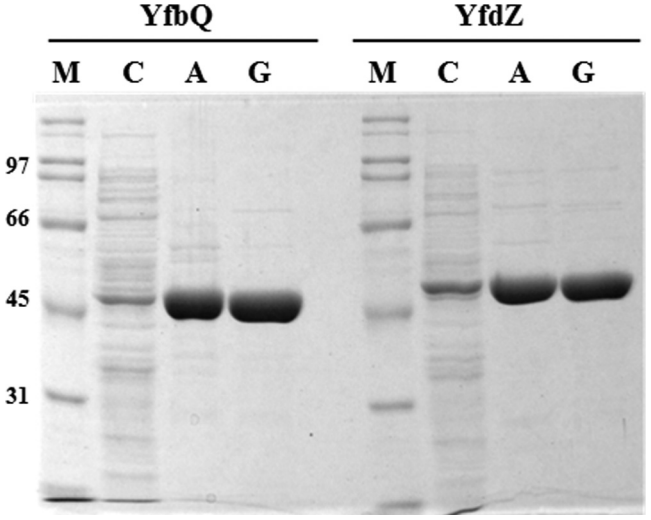

FIG. 3. Purification of His_6-tagged YfbQ and His_6-tagged YfdZ. Protein samples (20 μg) were separated on a 10% SDS-polyacrylamide gel. Lanes: M, molecular mass markers; C, crude extract; A, protein after affinity purification; G, protein after size exclusion chromatography. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel.

Purification and preliminary characterization of YfdZ and YfbQ. The genes for these two enzymes were initially cloned into a multicopy plasmid under the control of the *lac* promoter. *E. coli* KE8/pSHK102 (*lacp-yfbQ*) and KE8/pSHK101 (*lacp-yfdZ*) generated 600 and 15 units of GPT activity, respectively, upon induction (Table 4). Both proteins were difficult to purify from these extracts, especially YfdZ, which tended to precipitate (described in Materials and Methods). Because of these problems, we switched to His-tagged derivatives expressed from a phage T7 promoter, which allowed more rapid purification. Both enzymes were greater than 90% pure after a one-step affinity purification (Fig. 3). YfbQ had a final specific activity of 4,530 units per mg of protein, which represents an 18-fold purification. Comparable estimates of YfdZ activity were not possible, since it lost activity in the reverse direction (which is generally more convenient for measurements).

A thorough comparison of these two enzymes will require solving some of these technical issues. Therefore, we only partially characterized their properties. The apparent K_ms for pyruvate were 0.55 ± 0.06 mM and 0.94 ± 0.19 mM for YfbQ and YfdZ, respectively. These values are close to the apparent intracellular concentration of pyruvate (9, 20, 31). The apparent K_m for alanine in the reverse direction was 4.9 ± 1.2 mM for YfbQ. Purified YfbQ and YfdZ had subunits with an estimated M_rs of 46,000 and 47,000, respectively (data not shown), which is close to the predicted size of the gene products. The

masses of both native proteins were about 87,000 as determined by size exclusion chromatography (data not shown), suggesting that both are homodimers.

DISCUSSION

Alanine synthesis genes. Our results suggest that the major genes of alanine synthesis are *avtA*, *yfbQ*, and *yfdZ*. Any one of these three genes is sufficient for optimal growth without alanine. Eight other transaminase genes on multicopy plasmids suppressed the alanine limitation of a mutant lacking the major alanine transaminases. All eight are enzymes in other pathways, and alanine synthesis would appear to be a secondary activity. It is not known whether these enzymes contribute to alanine synthesis in a wild-type strain.

The only other genetic analysis of alanine synthesis implicated *avtA*, *alaA*, and *alaB*. We confirmed the function of *avtA* in alanine synthesis. We showed that *alaA* is *yfbQ*, and we propose the former designation for *yfbQ*. A comparison of the partial physical map of the *alaB* region with a physical map of *yfdZ* calculated from the genome sequence indicates that *alaB* cannot be *yfdZ* (results not shown). We propose that *yfdZ* should be renamed *alaC*.

The *alaB* gene was identified from its partial suppression of an *ilvE alaA* strain, when *alaB* is on a multicopy plasmid. A comparison of the physical maps of *alaB* and the eight genes of the minor alanine transaminases shows a good, but not precise, match only with *argD*. All the restriction endonuclease sites are in the correct sequence, but the measured distances between sites in the *alaB* region do not exactly correspond with the calculated distances in the *argD* region. In any case, *alaB* codes for a minor alanine transaminase, probably with a different primary activity.

Unexpected results from labeling studies. A pioneering general study of amino acid biosynthesis suggested that pyruvate might not be the substrate for 60% of alanine synthesis with acetate as the carbon source (26). Another general study of amino acid biosynthesis showed a 15% discrepancy of the ratio of [^{14}C]glucose and [^{3}H]glucose incorporation into two pyruvate family amino acids, valine and alanine (7). A possible explanation is that pyruvate is not the only precursor for alanine synthesis. However, pyruvate is not only the precursor for the major alanine transaminases, but because pyruvate supports the best growth of a mutant lacking these enzymes, pyruvate is undoubtedly the precursor for the minor alanine transaminases. To explain the labeling studies, we note that cells for both studies had been grown to stationary phase. The possible significance of stationary-phase metabolism has only recently been appreciated. A more recent study has shown that during stationary phase, serine, alanine, glutamate/glutamine, and aspartate/asparagine are actively synthesized but that other amino acids are instead derived from degraded proteins (30). Therefore, isotopic dilution of pyruvate during stationary phase is sufficient to account for the unusual labeling of alanine compared to labeling of other pyruvate family amino acids. Such dilution may occur by incorporation of unlabeled CO_2 into some intermediates and perhaps by carbon rearrangements during stationary-phase metabolism.

Regulation of alanine synthesis. Excessive alanine synthesis would divert pyruvate from its many essential functions. High levels of alanine reduce the glutamate pool in *Salmonella enterica* (14) and interfere with the synthesis of glutamate, a major biosynthetic nitrogen donor (15). Alanine synthesis must be regulated. Alanine accumulation can cause further metabolic changes. We propose that alanine synthesis is controlled by modest Lrp-mediated alanine repression, pyruvate availability, and possibly other factors.

We showed that alanine or leucine via Lrp modestly represses YfbQ/AlaA and AvtA. This is consistent with a previous study of *avtA* expression which showed that alanine or leucine repressed AvtA (39). (Lrp had not been identified when this study was performed.) Starvation for any single amino acid also represses AvtA (39). It was proposed that amino acid starvation results in an accumulation of alanine or leucine. However, an alternate explanation is that such starvation results in accumulation of guanosine tetraphosphate, which induces Lrp (19). The 2-fold repression by Lrp may not seem significant. However, leucine reduces alanine pools about 2-fold (23), which suggests that steady-state alanine may be proportional to the level of alanine-synthesizing enzymes.

The concentrations of the substrates pyruvate and glutamate could modulate alanine synthesis. The concentrations of pyruvate, alanine, and glutamate vary with environmental changes and growth rate (9, 14, 20, 31, 32, 34, 37, 38). Glutamate is usually the most abundant amino acid in pools, and alanine the second most abundant (23, 32, 42), although for phosphate limitation, the alanine pool appears to exceed the glutamate pool (32). It seems likely that pyruvate concentration affects alanine synthesis for several reasons. First, the apparent K_ms for pyruvate of YfbQ/AlaA and YfdZ/AlaC reported in this paper are close to the estimated physiological level of pyruvate (9, 20, 31). Second, pyruvate as a carbon source induces the enzymes of alanine catabolism (18). Since this induction requires high intracellular alanine (24), it implies that pyruvate increases intracellular alanine. Third, a folic acid antagonist results in accumulation of pyruvate and alanine in *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes* and *Klebsiella aerogenes*), and these studies were extended to show that several antibiotics and a variety of growth transitions also result in accumulation of pyruvate, and possibly alanine (37, 38). If pyruvate accumulation increases the concentrations of alanine and possibly valine (via AvtA), then changes in pyruvate concentration might account for reports of alanine and valine accumulation (33, 34).

Discrepancies between gene-specific transcription and transaminase activity suggest an additional control mechanism. The most major discrepancy is that loss of either YfbQ or YfdZ had no effect on total GPT activity, but loss of both reduced activity by 90%. Another possible discrepancy is that alanine did not reduce total GPT activity, although alanine modestly repressed *yfbQ* expression. Both results suggest that loss or repression of one enzyme increased activity of the other. However, apparent alanine starvation did not obviously affect transcription of either *yfdZ* or *yfbQ*. This argues against compensatory gene transcription and may suggest a posttranscriptional compensation. One possible posttranscriptional mechanism is based on the observation that alanine substantially, but not completely, suppresses the growth defect of a *pdxK* mutant, which contains 20% of the wild-type level of B_6 vitamers (18). If PLP availability limits alanine synthesis, then

the alanine transaminases may compete for an effectively small pool of PLP. Regardless of the explanation, it seems likely that additional mechanisms of regulation will emerge from further study.

ACKNOWLEDGMENTS

This work was supported in part by grant MCB-0323931 from the National Science Foundation and grant GM085536 from the National Institutes of Health.

We thank Elaine Newman and the National BioResource Project (NIG, Japan) for *E. coli* strains and plasmids. We also thank Kristen Evaul, Aurelia A. Moore, and Michael P. Hayes for constructing some strains.

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