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Effects of the flrA regulatory locus on biosynthesis and excretion of amino acids in Escherichia coli B/r*

Brian A. Nummer[1], Susan F. Barefoot[1,2]**, and Ellis L. Kline[1]

Departments of [1]Microbiology and [2]Food Science, Clemson University, Clemson, SC 29634

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Abstract: We have partially characterized phenotypic effects of an unusual amino acid regulatory locus, flrA, in E. coli B/r that alters the expression of the ilv and leu operons [Kline, E.L (1972) J. Bacteriol. 110:1127-1134]. This study demonstrated that a primary effect of the flrA7 mutation in haploid strains was overproduction of valine. In diploid strains (F'flrA+/ flrA7) this mutation resulted in excretion of valine, isoleucine, leucine, aspartate, threonine, glutamate, histidine and lysine. Increased excretion of amino acids by mutant strains might be explained by a membrane alteration or by flrA encoding a positive regulatory factor that affects the ilv operon and has pleiotropic effects on other amino acid operons.

Interest in amino acid excretion by Escherichia coli has been renewed in the light of recent advances in recombinant DNA technology. Increasing the gene copy number of enzymes involved in synthesis of a particular metabolite may increase the economic competitiveness of E. coli with industrial amino acid producers such as Corynebacterium spp. Recombinant bacterial strains that overproduce metabolites may be enhanced further by mutations altering the regulation or excretion of metabolites. Combining these two features then might yield increased gene copy numbers, favorable synthesis of the gene products and excretion of amino acids.

One regulatory mutation with potential usefulness in amino acid biosynthesis and excretion by E. coli is the mutation flrA. Spontaneous mutants of E. coli B/r resistant to the leucine analog 5',5',5',-trifluoroleucine (TFL) contain defects in a gene unlinked to the leu operon, designated flrA, that maps by P1 transduction between serB and trpR

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** To whom correspondence should be addressed.

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(1). These mutants possess an unusual phenotype in that the mutation causes low level constitutive expression of the ilv and leu operons in haploid strains. In contrast, extremely high levels of gene expression for these same operons were detected in the diploid strain (F'flrA+/flrA7) (1). The unusual phenotype of the flrA strains warranted further investigation into the effects of the mutation on amino acid biosynthesis and excretion and its possible physiological role in E. coli B/r.

Materials and Methods

Microbial strains and media. Bacteria, phage and plasmid strains, their sources and pertinent markers are listed in Table 1. Minimal salts medium containing 0.2% glucose (GMS) was prepared as described earlier (4) with citrate omitted and ammonium sulfate increased to 0.15%. Media were supplemented with 3 mM amino acids as needed. Complex medium was L broth or agar (8).

Table 1. Escherichia coli Strains, Phages and Plasmids[1]

Strain	Relevant markers	Reference
B/r EK1	flrA+, pyrA1[2]	(1)
B/r EK3	flrA7, pyrA1	(1)
B/r EK1000	F'flrA+/ flrA+	(1)
B/r EK1003	F'flrA+/ flrA7	(1)
B/r SB5022	flrA7, thr1	(1)
B/r FL10	ΔserB-trpR37-1, zjj::Tn10, thr1	This paper
K12 SP516	ΔserB-trpR37-1, zjj::Tn10	(2)
K12 JM101	thi, supE, Δ(lac-pro), F'[traD36, proAB+, lacZΔM15]	ATCC#33876
K12 JM516	ΔserB-trpR37-1, zjj::Tn10 thi, supE, Δ(lac-pro), F'[traD36, proAB+, lacZΔM15]	This paper
K12 CU946	ilvGEDA::lacZ	(3)
NS2114		(7)
Phage		
P1kc		ATCC# 25404-B1
Plasmids		
pHSS8	Km, lacZ, ColE1	(7)
pOX38::m-Cm3	Tn3, cm^r, tra,	(7)
pTCA	tc^r, transposase	(7)
pFL1	serB-flrA7-trpR	This paper
pFL2	serB-flrA7-trpR::Tn3	This paper

[1]Stock cultures were maintained at 4°C. on GMS medium supplemented as required. [2]pyrA1 results in arginine and uracil auxotrophy.

Chemicals. Amino acids, antibiotics, and 5-methyl-DL-tryptophan (5MT) were from Sigma Chemicals Co. (St. Louis, MO). 5',5',5'-trifluoroleucine (TFL) was from Fairfield Chemicals (Blythfield, SC). All other chemicals were from Fisher (Norcross, GA). Restriction enzymes and T4 ligase were from Promega (Madison, WI).

Genetic techniques. P1 transductions were performed by the methods of Low (5). To isolate the flrA deletion mutant FL10, ΔserB - trpR from SP516 was co-transduced into SB5022 by selecting for tetracycline resistance (12.5 ug/ml) encoded by a nearby transposon, zjj::Tn10. Tetracycline resistant transductants were screened for serine auxotrophy and resistance to 200 μg/ml 5MT. FL10 (ΔflrA) was as sensitive as the wild-type to 100-200 μg/ml TFL in GMS medium. For plasmid selection a host strain was constructed by P1 transduction of ΔserB - trpR from SP516 into the highly transformable strain JM101. The resulting transductant strain, JM516, was a serine auxotroph and resistant to 5MT.

Cloning protocols were those of Sambrook et al (6), unless otherwise indicated. Plasmids containing flrA7 were constructed by partial cleavage of SB5022 genomic DNA with Sau3A. Fragments in the 2-10 Kb range were ligated with T4 ligase into pHSS8 cut with BamHI and transformed into JM516. Transformants were selected for complementation of serB and for resistance to 50 μg/ml kanamycin sulfate, then screened for sensitivity to 200 μg/ml 5MT due to complementation of ΔtrpR to yield the plasmid pFL1.

Transposon mutagenesis was performed by the methods of Seifert et al (7) to obtain the plasmid pFL2. The plasmid PFL1 was transformed into RDP146, that contains a transposase-complementing plasmid, pTCA. Transformants were mated with W3100 containing the transposon pOX38::m-Cm3. Transconjugates were selected that carried all three plasmids, pFL1, pTCA, and pOX38::m-Cm3. Cultures of the transconjugates was grown under conditions allowing transposase activity to form cointegrates between POX38::m-Cm3 and pFL1. The cointegrates then were conjugated into NS2114 and counterselected to remove pTCA. Cointegrates of pFL1 and pOX38::m-Cm3 were then transformed into JM516 and assayed for 5MT resistance indicative of insertional inactivation of trpR.

Excretion of amino acids. Cultures were grown in 500 ml sidearm flasks containing 100 ml GMS medium. Media were inoculated with 0.5% overnight cultures and agitated (200 rpm) at 37°C for up to 36 h. Aliquots were taken at indicated intervals and analyzed for amino acids and residual ammonium by ion exchange chromatography (Dionex 2000i) as described previously (8).

Enzyme assays. The Km for the charging of [^{14}C]-leucine (0.5 μCi/ml; Sigma) by leucyl-tRNA synthetase was determined by the methods of Brown et al (9). ß-galactosidase produced by lac fusion strains was measured by the method of Miller (10). The appropriate plasmid was transformed into CU946, an ilvGEDA::lacZ fusion strain. Cultures were grown to mid-log phase in GMS medium. Cells were made permeable and ß-galactosidase activity was measured colorimetrically using o-nitrophenyl-ß-D-galactopyranoside as the substrate. Activity was expressed in Miller's units (10). Isopropylmalate synthetase (IPMS), the leuA gene product, was assayed by the methods of Kline (1). Protein was determined with a Bio Rad kit as recommended by the manufacturer (Bio Rad, Rockville Center, NY).

Results

Growth and amino acid excretion by *flrA* strains. Derivatives EK1003 and EK1000 exhibited doubling times of approximately 70 min. in GMS medium; EK1, EK3

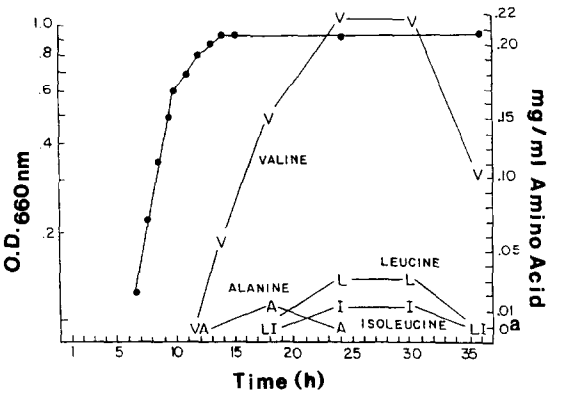

Figure 1. Growth and excretion of amino acids by EK1003. Strain EK1003 was grown in GMS medium (37°C, 200rpm) and tubidity (•) was monitored ($O.D._{660nm}$) Amino acids (mg/ml) were determined by HPLC as described in materials and methods.

and FL10 had doubling times of approximately 60 min. Growth and excretion of amino acids by EK1003 are shown in Fig. 1. In preliminary experiments EK1003 cultures depleted the provided (0.10%) ammonium sulfate; therefore, so it was increased to 0.15% in subsequent experiments. Strain EK1003 excreted primarily valine in stationary phase culture yielding 0.22 mg/ml by 24 h. Addition of 0.1% D-glucose or 0.05% ammonium sulfate did not further increase valine accumulation (data not shown). Cultures (24 h) of EK1003 also contained isoleucine, leucine, aspartate, threonine, glutamate, histidine and lysine (Fig. 2.). Strain EK3 (flrA7) yielded 0.05 mg/ml valine

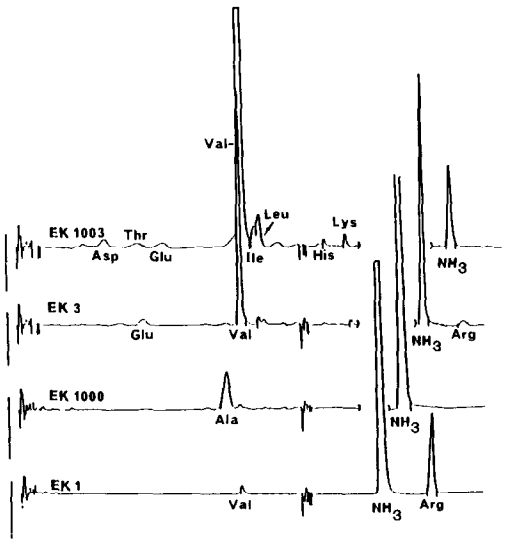

Figure 2. Excretion of amino acids by flrA strains. Aliquots of GMS medium cultures were taken at 24h and amino acids determined by HPLC as described in materials and methods.

and less isoleucine and leucine by 24 h; the homozygous controls, EK1 and EK1000, and the deletion strain FL10 did not excrete detectable amino acids. Ammonium remaining in culture supernates was related inversely to excretion of amino acids suggesting that flrA7 strains utilized the increased nitrogen for amino acid biosynthesis. The possibility that amino acids accumulated intracellularly in EK1003 was assessed in GMS medium grown cultures (37°C, 200 rpm). Duplicate 2 ml. aliquots were removed at 6, 12, 18, and 24 h. At each interval, cells in one aliquot were disrupted by sonication (0°C, 3 x 30s, separated by 30s). Both aliquots were centrifuged and supernates were subjected to amino acid analysis. Quantities and types of amino acids detected did not differ significantly for any culture sample indicating that amino acids did not accumulate intracellularly.

Effects of the flrA alleles on aminoacylation of leucyl-TRNA and activity of the *leuA*-encoded enzyme. Strain FL10 (ΔflrA) exhibited leuA activity comparable to the wild-type, EK1; IPMS activities for EK1003 and EK3 agreed with those published previously (1; Table 2.) Since tRNA synthetases have been implicated in the deregulation of leucine and other branched-chain amino acids (11), comparisons were made of leucyl-tRNA charging in parental and mutant strains. The Km for [^{14}-C]leucine was 2.5 x 10^{-5} M for EK1 (flrA+); it was 1.2 x 10^{-5} M for EK3 (flrA7). These data indicate that charging of tRNA-leu in the flrA7 mutant was at least as efficient as in the wild-type.

Cloning and Characterization of flrA7. The flrA7 locus was cloned onto a multicopy plasmid (pFL1). Strains harboring pFL1 unexpectedly were more sensitive to TFL than the haploid flrA7 mutant. Plasmid encoded trp repressor represses leu enzyme synthesis (12). Trp repressor activity was removed by insertionally inactivating the plasmid-encoded trpR gene with transposon mutagenesis and generating plasmid pFL2.

Table 2. Isopropylmalate synthetase (IPMS) activity in flrA strains

Strain	flrA allele	IPMS (mm/h/mg protein)
EK1003	F'flrA+/flrA7	4.2
EK1000	F'flrA+/flrA+	0.3
EK3	flrA7	0.4
EK1	flrA+	0.1
FL10	ΔflrA	0.1

Table 3. Ilv operon expression in strains harboring the flrA7 plasmids, pFL1 and pFL2

Plasmid	Isopropylmalate synthetase (mm/h/mg protein)	Expression of ilvGEDA in lacZ fusion strains[1]
pFL1	0.1	110
pFL2	0.1	130
control	0.1	100

[1]Expressed in Miller's Units (10).

However, strains harboring the plasmid pFL2 were as sensitive to TFL as wild-type strains. The effects of pFL1 and pFL2 on the activity of IPMS and expression of the ilvGEDA operon were assayed (Table 3.). No discernable flrA phenotype was found in strains harboring flrA7 on these multicopy plasmids.

Discussion

A primary effect of the flrA7 mutation in haploid strains is excretion of valine. In F'flrA/ flrA7 strains, the complexing of wild-type and mutant alleles led to excretion of valine, isoleucine, leucine, aspartate, threonine, glutamate, histidine, and lysine. That the flrA mutant strains overproduced and excreted amino acids suggests that they may be of use in the production of certain amino acids. For example, genetic blocks in the ilv operon of these strains led to an increase in lysine and glutamate excretion (8). Therefore, further genetic tailoring of amino acid operons might permit excretion of economically competitive concentrations of amino acids.

Unfortunately, isolating the mutant allele on a multicopy plasmid did not result in a phenotype of industrial significance. Lac fusion strains harboring the initial plasmid containing both flrA7 and trpR exhibited reduced synthesis of the leuA enzyme and ilv enzymes. Furthermore, strains harboring the mutagenized plasmid containing only flrA7 showed no significant difference in ilv enzyme synthesis. A possible explanation for the lack of a flrA phenotype is that a stoichiometric relationship, perhaps 1:1, of flrA mutant and wild-type alleles may be required; the differences in excretion patterns in haploid versus heterozygous merodiploid strains support this possibility.

Phenotypic data in mutant, deletion and wild type alleles do not support the possibility that flrA encodes attenuation-related enzymes. It is possible that the flrA

gene product may be a positive control element, such as an activator similar to ilvR (13). The pleiotropy of flrA mutants then would be explained by the capacity of this hybrid protein to activate other amino acid operons in a manner similar to ilvR (13). An alternative explanation would be that flrA7 results in membrane changes that cause release of endogenous amino acids and result in reduced feedback inhibition of enzyme expression. Further studies on the physiological role of the flrA locus are required to precisely define its regulatory role in E. coli B/r.

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