Enhanced Production of Arginine and Urea by Genetically Engineered *Escherichia coli* K-12 Strains

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Escherichia coli strains capable of enhanced synthesis of arginine and urea were produced by derepression of the arginine regulon and simultaneous overexpression of the *E. coli carAB* and *argI* genes and the *Bacillus subtilis rocF* gene. Plasmids expressing *carAB* driven by their natural promoters were unstable. Therefore, *E. coli carAB* and *argI* genes with and without the *B. subtilis rocF* gene were constructed as a single operon under the regulation of the inducible promoter *ptrc*. Arginine operator sequences (Arg boxes) from *argI* were also cloned into the same plasmids for titration of the arginine repressor. Upon overexpression of these genes in *E. coli* strains, very high carbamyl phosphate synthetase, ornithine transcarbamylase, and arginase catalytic activities were achieved. The biosynthetic capacity of these engineered bacteria when overexpressing the arginine biosynthetic enzymes was 6- to 16-fold higher than that of controls but only if exogenous ornithine was present (ornithine was rate limiting). Overexpression of arginase in bacteria with a derepressed arginine biosynthetic pathway resulted in a 13- to 20-fold increase in urea production over that of controls with the parent vector alone; in this situation, the availability of carbamyl phosphate was rate limiting.

The genes encoding the enzymes of the urea cycle in mammals are derived evolutionarily from bacterial genes for de novo synthesis of arginine (19). The urea cycle is essential in mammals for the detoxification of ammonia by converting it to urea, which is efficiently eliminated in the urine. Patients in whom the urea cycle is insufficiently active develop hyperammonemia, which can cause brain damage and death and for which current available therapy is not satisfactory. The present work was undertaken to determine if *Escherichia coli* strains could be engineered to enhance ammonia consumption by its incorporation into nitrogen-containing organic compounds. The availability of such biological systems could then be used for the development of specific therapies for the removal of ammonia from body fluids such as in hyperammonemic patients with liver disease or inherited metabolic disorders. We report here in vitro studies on the content and biosynthetic capacity for amino acids and urea in *E. coli* strains overexpressing some of the arginine biosynthetic enzymes. Since arginine contains three nitrogen atoms compared with the one atom in glutamate, the flux through this pathway, if enhanced, would result in incorporation of a large number of nitrogen atoms into organic compounds.

In *E. coli*, the arginine biosynthetic genes code for enzymes which catalyze the conversion of glutamate to arginine in multiple steps (3) (Fig. 1). The bacterial arginine biosynthetic genes are all regulated via a common repressor protein encoded by the *argR* gene. Free arginine acts as a corepressor (2, 8). Thus, to enhance the activity of the arginine biosynthetic pathway, strategies must be developed to modify the regulation of these genes and to overcome the rate-limiting step(s). This was partially achieved by derepression of the chromosomal arginine regulon (2, 8) and overexpression of the *E. coli carAB* (encoding carbamyl phosphate synthetase [CPS]) and *argI* (encoding ornithine transcarbamylase [OTC]) genes and the *Ba-*

cillus subtilis rocF (encoding arginase) gene carried on plasmids. These genes are involved in citrulline synthesis from glutamine (or ammonia) and ornithine and in urea production from arginine (Fig. 1).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Plasmids pUC19 and pACYC184 were purchased from New England Biolabs (Beverly, Mass.), and pTRC99A was obtained from Pharmacia Biotech (Piscataway, N.J.). Plasmid pLLK12, a pUC19 derivative containing the *E. coli carAB* operon (5,400 bp) including the *P1* (pyrimidine-dependent) and *P2* (arginine-dependent) promoters (1), was obtained from Carol Lusty (New York Public Health Research Institute). The *carAB* operon encodes the small (glutaminase) and large (carbamyl phosphate synthetase) subunits of *E. coli* CPS (11–13). Plasmid pA11, a pBR322 derivative containing the *E. coli argI* gene coding for OTC with its natural promoter (2,500 bp) cloned into the unique *HindIII* and *EcoRI* sites of the parent plasmid (10), was obtained from Nicolas Glansdorff (University of Brussels). Plasmid pROC10, a pHT315 derivative containing the *B. subtilis rocF* gene coding for arginase (6), was obtained from Michael Debarbouille (Pasteur Institute, Paris, France).

The *E. coli* strains used in this study were the K-12 derivatives DH5 α (Gibco-BRL, Bethesda, Md.), C600 (Gibco-BRL), C600 Δ OTC (lacking *argI* and *argF*; obtained from Nicolas Glansdorff, University of Brussels), and MG1655R, an *argR* strain incapable of producing the arginine repressor (obtained from W. Maas, New York University).

Media and growth conditions. Luria broth (LB) was used routinely for liquid cultures. Bacto Agar (Difco Laboratories, Detroit, Mich.) was added at 1.5% for solid media. The following antibiotics (obtained from Sigma Chemical Co., St. Louis, Mo.) were used at the indicated concentrations (in micrograms per milliliter): ampicillin, 50 to 100; chloramphenicol, 25 to 40.

Recombinant DNA techniques. Recombinant DNA techniques were as described by Sambrook et al. (14). The restriction enzymes were obtained from Promega (Madison, Wis.) and Gibco-BRL and used as suggested by the manufacturers. Following digestion, DNA fragments were separated by horizontal agarose (SeaKem; FMC, Rockland, Maine) gel electrophoresis, and the excised bands were purified with a Qia-Quick kit (Qiagen, Chatsworth, Calif.) or a Wizard PCR kit (Promega). All ligation reactions were performed by mixing linear DNA fragments at a molar ratio of the free ends of 3:1 (insert/vector), and the ligated products were used to transform competent cells of DH5 α . Rapid DNA preparations were made by the alkaline lysis method (14), and after confirmation of the correct structure, plasmids were introduced into competent cells of various *E. coli* strains for further study.

Construction of a pACYC184 derivative containing *argI*. A DNA fragment containing the *argI* gene was excised from the pA11 plasmid by using *HindIII* and *ScaI* (2,988 bp) and inserted into pACYC184 within the tetracycline resistance gene linearized with *HindIII* and *NruI*; the resulting plasmid was resistant only to chloramphenicol. The correct structure of the new plasmid, pACAI, was con-

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FIG. 1. Arginine-urea pathway in engineered *E. coli* described herein. Genes in bold type belong to the arginine regulon and were derepressed in these experiments; (P), overexpression of the genes on engineered plasmids.

firmed by restriction analysis. This plasmid was then used to cotransform suitable *E. coli* cells along with the pLLK12 plasmid containing the *carAB* operon to form a strain that could overexpress both CPS and OTC.

Construction of an inducible system for *carAB*, *argI*, and *rocF*. The *carAB* operon is transcribed from tandem promoters *P1* and *P2* located upstream of the *carA* gene (1, 11). To construct an inducible expression system for these genes, a *NcoI*-*Clal* and *SacI* fragment comprising the translational start of the *carA* gene (+1) through base 338 was amplified by PCR using a forward primer with an *NcoI* site (5'-GTCAGTCTACCATGGTGAAGTCAGCGCTATTGGTTCTGGAA-3'), a reverse primer with *Clal* and *SacI* sites (5'-GTCAGTCTAGAGCTCATCGATATCGGCAATCGCCAC-3'), and the plasmid pLLK12 as the template. The resulting fragment was digested with *NcoI* and *SacI* and cloned into pTRC99A to obtain the plasmid pTRC1. This construct enabled us to position the *carA* gene under the control of the *trc* promoter and to change the translational start from TTG to ATG. We also introduced a base change from A to G

TABLE 2. Expression plasmids used in this study

Designation ^a	Parent vector	Expressed gene(s)	Promoter	Reference or source
pLLK12	pUC19	<i>carAB</i>	Natural	C. J. Lusty
pAII	pBR322	<i>argI</i>	Natural	10
pACAI	pACYC184	<i>argI</i>	Natural	This study
pTRC1	pTRC99A	<i>none</i>	<i>trc</i>	This study
pTRC2	pTRC99A	<i>carA</i>	<i>trc</i>	This study
pTRCAB1	pTRC99A	<i>carAB</i>	<i>trc</i>	This study
pTRCAB2	pTRC99A	<i>carAB</i>	<i>trc</i>	This study
pTRCABI	pTRC99A	<i>carAB</i> , <i>argI</i>	<i>trc</i>	This study
pTRCABI+	pTRC99A	<i>carAB</i> , <i>argI</i>	<i>trc</i>	This study
pUCAB	pUC19	<i>carAB</i>	<i>trc</i>	This study
pUCABI	pUC19	<i>carAB</i> , <i>argI</i>	<i>trc</i>	This study
pUCABI+	pUC19	<i>carAB</i> , <i>argI</i>	<i>trc</i>	This study
pUCABIF	pUC19	<i>carAB</i> , <i>argI</i> , <i>rocF</i>	<i>trc</i>	This study
pUCABIF+	pUC19	<i>carAB</i> , <i>argI</i> , <i>rocF</i>	<i>trc</i>	This study
pUCF	pUC19	<i>rocF</i>	<i>trc</i>	This study
pUCF+	pUC19	<i>rocF</i>	<i>trc</i>	This study
pUCABF	pUC19	<i>carAB</i> , <i>rocF</i>	<i>trc</i>	This study
pUCABF+	pUC19	<i>carAB</i> , <i>rocF</i>	<i>trc</i>	This study

^a + indicates *argR*-binding boxes from the *argI* gene cloned into the plasmid to titrate the repressor and derepress the chromosomal arginine biosynthetic genes.

at the +4 position of the coding sequence of *carA*. The remaining portion of *carA* and *carB* was cloned in two steps as follows. An internal *NruI* fragment of pLLK12 was cloned into pTRC1 to obtain pTRC2. After confirming the proper orientation, a *SacII*-*SacI* fragment from pLLK12 was cloned into pTRC2 to obtain pTRCAB1. This plasmid contained the *carA* and *carB* genes under the control of the inducible *trc* promoter and retained the putative transcriptional termination sequence of the *carAB* operon (9).

To express the *argI* gene along with *carA* and *carB*, the purported transcriptional termination sequence was removed and an intercistronic region which contains the ribosome binding site from the *carAB* operon (9) was inserted at the end of the *carB* gene. A 190-bp *SauI*-*KpnI* fragment was amplified by PCR using a forward primer with a *SauI* site (5'-ATCAACACACCTCAGGCCGTCGTGCG-3'), a reverse primer with a *KpnI* site (5'-GCGCTATAAGGTACCTTTATCTCTGATTATTGATCTGTGCGTGCAT-3'), and pLLK12 as the template. The resulting fragment had the translational termination signal (TAA) of *carB* followed by an intercistronic region with a ribosome binding site. This fragment was cut with *SauI* and *KpnI* and cloned into pTRCAB1 to obtain pTRCAB2. The *argI* fragment comprising the ATG initiation codon at position +1 up to the termination signal at position +1004 was amplified by PCR using a forward primer with a *KpnI* site (5'-ATCCTATACGGTACCATGTCCGGGTTTATCATAAGCAT-3'), a reverse primer with a *BamHI* site (5'-GCGATCCAGGATCCTTATTACTGAGCGTCGCGACCAT-3'), and the plasmid pAII as the template. The resulting fragment was cut with *KpnI* and *BamHI* and cloned into pTRCAB2 to obtain pTRCABI.

To clone an *arg* operator capable of binding the arginine repressor (Arg box), the respective sequence from the *argI* gene (10) was amplified with forward and reverse primers containing *XbaI* and *HindIII* sites, respectively (5'-CCAGTGTGTTAGAAATGCTTTAGACTTGCAATGA-3' and 5'-TGTGATCCTAAGCTTATGATCCCTCTGTGGCTAACG-3') and pAII as the template. The resulting fragment (112 bp) was cut with *XbaI* and *HindIII* and cloned into pTRCABI to obtain pTRCABI+.

All of the PCR fragments were confirmed by sequencing with the Sequenase kit (United States Biochemicals, Cleveland, Ohio).

To maximize *carAB* expression and to increase repressor titration in *trans*, we utilized the high-copy-number plasmid pUC19. First, plasmid pTRCAB2 was cut with *NdeI* and *HindIII* and the resulting fragment was cloned into pUC19 at the same sites to obtain the plasmid pUCAB. This plasmid is identical to pTRCAB2 except for the control of copy number. To move the *argI* gene with or without the Arg box into pUCAB, a *KpnI*-*HindIII* fragment from pTRCABI and pTRCABI+ was cloned into pUCAB to obtain pUCABI and pUCABI+, respectively.

The *rocF* gene from *B. subtilis* was cloned into the plasmids pUCABI and pUCABI+ as follows. The gene was engineered by PCR to include an intercistronic region with a ribosome binding site and an *NcoI* site near the ATG initiation codon. Forward and reverse primers containing flanking *BamHI* and *XbaI* and *HindIII* sites, respectively (5'-CCCGATCCTCAGGAGTAAAAGAGCCATGGATAAAACGATTTCGGTT-3' and 5'-CCCAAGCTTTCTAGATTACAGCAGCTTCTTCCAAA-3'), were used to amplify the entire gene from pROC10 (6). The resulting fragment (934 bp) was cut with *BamHI* and *XbaI* and cloned into pUCABI and pUCABI+ to obtain pUCABIF and pUCABIF+. The plasmids pUCF and pUCF+ were obtained by linearizing pUCABIF and

TABLE 1. *E. coli* strains used in this study

Strain	Genotype	Reference or source
DH5 α	<i>supE44 lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1 endA1 gyrA96 thi-1 relA1</i>	7
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</i> λ^-	7
C600 Δ OTC	Same as that for C600 but <i>DeltaI</i> <i>DeltaF</i> <i>proA</i>	N. Glansdorff
MG1655R	<i>argR::Tn7</i>	W. Maas

pUCABIF+ with *Nco*I to remove *carAB* and *argI* followed by religation. The resulting plasmids contained the *rocF* gene under the control of the *trc* promoter. The plasmids pUCABIF and pUCABIF+ were digested with *Kpn*I and *Bam*HI (to remove the *argI* fragment), filled in by the Klenow reaction, and ligated. The resulting plasmids were named pUCABF and pUCABF+.

Expression of the plasmid genes was verified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Western blots (immunoblots), and specific enzyme assays as described below.

Enzymatic analyses. A single colony of the desired bacterial strain was inoculated into 5 ml of LB with appropriate antibiotics and grown to saturation at 37°C. Saturated cultures containing plasmids with inducible *trc* promoter were diluted 20-fold into LB and grown to the log phase (optical density at 600 nm [OD₆₀₀], 0.5) at 37°C. The cultures were then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown for another 4 h. Five- to ten-milliliter aliquots of this culture were removed and centrifuged at 3,000 rpm for 5 min in a Beckman TJ-6 centrifuge. The pellet was washed with 1 ml of 100 mM HEPES buffer (pH 7.4) and then resuspended in 0.5 to 1.0 ml of 100 mM HEPES buffer (pH 9.0). The bacterial suspension was sonicated on ice three times for 20 s with a Branson Sonifier 200 (output control 2, continuous pulse, 50% duty cycle) and centrifuged at 4°C for 2 min at 14,000 rpm. The supernatant fluid was used for enzyme and protein analysis.

CPS and OTC activity determinations were performed with a modified radiochromatographic assay as described before (17). For glutamine-dependent CPS activity, the incubation mixture contained 20 mM L-glutamine, 40 mM sodium bicarbonate, 80 mM KCl, 20 mM ATP, 20 mM MgCl₂, and 5 mM L-[1-¹⁴C]ornithine (5 μCi/ml), with or without 5 U of purified OTC enzyme from *Streptococcus faecalis* (Sigma; dependent on the level of bacterial OTC in the extracts) per ml and 100 μl of bacterial sonicate supernatant in 500 μl of 100 mM HEPES buffer (pH 7.4). For ammonia-dependent CPS activity, glutamine and bicarbonate were replaced by 40 mM ammonium bicarbonate. The reaction was started by the addition of the enzyme source and incubated for 10 to 30 min at 37°C. The reaction was terminated by the addition of 40 μl of 30% trichloroacetic acid. The precipitated protein was removed by centrifugation, and the supernatant filtered through a 0.2-μm-pore-size filter was injected into the high-performance liquid chromatography (HPLC) column coupled to a radioactivity flow monitor. The OTC reaction mixtures contained 5 mM carbamyl phosphate, 5 mM L-[1-¹⁴C]ornithine (5 μCi/ml), and the enzyme source. The incubation was terminated after 10 min. In those cases in which it was determined that the OTC activity in the bacteria was in large excess to that of CPS, the addition of exogenous OTC enzyme for coupling of the CPS reaction was omitted. L-Citrulline formed during the reaction was separated from L-ornithine by HPLC, and the radioactive peaks of ¹⁴C-labelled compounds were detected and quantitated by the radioactivity flow monitor (Radiomatic Instruments Co., Tampa, Fla.) connected to the HPLC outflow as described previously (17).

Arginase activity was measured by determination of urea formation from arginine with the blood urea nitrogen kit purchased from Sigma (5, 15).

Protein electrophoresis and Western blots. The bacterial proteins in the sonicates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie blue or transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.) and probed with rabbit antibacterial CPS and OTC polyclonal antibodies by use of the alkaline phosphatase Immun-Blot assay kit (Bio-Rad) as described before (14, 16).

Amino acid studies. A single colony of cells transformed with the engineered plasmid or parent vector was inoculated into 50 ml of LB with the appropriate antibiotic and grown to saturation at 37°C. The saturated culture was diluted 10- or 20-fold, grown to the log phase (OD₆₀₀, 0.5), and then induced with 1 mM IPTG at 37°C for 4 h. The saturated cultures containing the plasmids with their native promoters were diluted 10-fold into LB and grown to an OD₆₀₀ of 1.0. An aliquot of the cultures was removed for enzyme analysis, four 45-ml aliquots of the cultures were centrifuged for 10 min at 3,000 rpm in a Beckman TJ-6 centrifuge, and the bacterial pellets were washed three times with 10 ml of M9 minimal medium without a nitrogen source. The pellets were then resuspended in 1 ml of M9 minimal medium containing one of the following supplements: 20 mM L-glutamine, 20 mM L-glutamine-5 mM L-ornithine, 20 mM ammonium chloride, or 20 mM ammonium chloride-5 mM L-ornithine. In a similar experiment using glutamine as the nitrogen source, L-ornithine was replaced by either *N*-acetylornithine or *N*-acetylglutamate at a concentration of 5 mM each. Following incubation for 3 h at 37°C, the cells were centrifuged for 2 min at 4°C and the supernatant media was removed. In the experiment reported in Fig. 5, samples were removed every hour to determine the proportionality of amino acid synthesis with time. The cell pellets were then washed twice with 100 mM HEPES buffer (pH 7.4), resuspended in 1 ml of Li-S buffer (pH 2.2; high-performance amino acid analysis buffer; Beckman Instruments, Palo Alto, Calif.), and sonicated three times for 20 s. The disrupted cells were removed by centrifugation, and the supernatant fluid was used for amino acid and polyamine determinations. Amino acid analysis was performed with a Beckman automatic amino acid analyzer. When necessary, the identification of significant amino acid and polyamine peaks was confirmed by spiking the sample with known standards. Separation and quantification of putrescine and spermidine were performed by HPLC and fluorescence detection following dansyl derivatization and extraction with ethyl acetate as described before (4).

FIG. 2. pUCABIF+ vector engineered for this study. All inducible vectors contained the expressed genes linked as an operon downstream from the *trc* promoter and a *lac* operator. This construct also contained an Arg box cloned from the *argI* gene for binding and titration of the arginine repressor.

Urea formation study. Bacterial cultures for urea formation studies were grown as described above. The cultures were induced with 1 mM IPTG at 37°C for 1 h. An aliquot was removed for enzyme analysis, two 45-ml aliquots of the cultures were centrifuged for 10 min at 3,000 rpm, and the bacterial pellets were then resuspended in 1 ml of M9 minimal medium containing either 5 mM L-glutamine or 5 mM ammonium chloride. After incubation for 3 h, the cells were sonicated and centrifuged to remove membranes and cell debris. After precipitation of the soluble proteins with 50% trichloroacetic acid, the urea concentration was determined as described above. In a separate experiment, samples were removed every hour to determine the proportionality of urea formation with time.

RESULTS AND DISCUSSION

Cloning and enzyme expression. The high-copy-number plasmids expressing the *E. coli carAB* genes under the control of their natural promoters were unstable in our *E. coli* strains; a significant proportion of bacteria lost these plasmids without constant antibiotic pressure (data not shown). No significant plasmid loss occurred with *argI*-containing vectors. To overcome the plasmid instability problem, we developed an inducible expression system so that overexpression of the cloned arginine biosynthetic genes could be specifically achieved without loss of cell viability or plasmid content.

The *carAB* and *argI* protein coding sequences were separated from their promoters and cloned to form a single operon downstream of the inducible *trc* promoter in pTRC99A and pUC19 plasmids to form pTRCABI and pUCABI, respectively. Arg boxes, i.e., specific sequences that bind the ArgR regulatory protein (obtained from the region upstream of the *argI* gene), were then added to the plasmids described above to form pTRCABI+ and pUCABI+, respectively. The arginase gene (*rocF*) from *B. subtilis* was also added to this operon to produce pTRCABIF+ (plasmid not shown) and pUCABIF+ (Fig. 2). Several *E. coli* strains were transformed with these constructs to study the expression of the arginine biosynthetic genes. These plasmids were stable with and without induction in all of the *E. coli* strains used in this study (data not shown). Figure 3 shows Western blots and SDS-PAGE gels obtained

FIG. 3. Western blots probed with anti-CPS and anti-OTC (A) and Coomassie blue-stained SDS-PAGE gel (B) of soluble proteins from *E. coli* C600ΔOTC overexpressing the *carAB* (CPS), *argI* (OTC), and *rocF* (arginase) genes and from controls containing the respective parent vectors. (A) Lanes: 1, pTRC99A; 2, pTRCAB2; 3, pTRCABI; 4, pTRCABI+; 5, pUCABI; 6, pUCABI; 7, pUCABI+; 8, molecular mass standards; 9, pLLK12 and pACAI; 10, pUC19 and pACYC184. (B) Lanes: 1, pUC19; 2, pUCAB; 3, pUCABI; 4, pUCABIF; 5, molecular mass standards; 6, pUCF; 7, pUCABF. kd, kilodaltons.

from bacteria overexpressing *carAB*, *argI*, and *rocF*. It is clear that the bacteria containing the engineered plasmids synthesized large amounts of these proteins when compared with the almost undetectable levels in controls.

Table 3 reports CPS, OTC, and arginase activities obtained in different *E. coli* strains, some of which contained the engineered plasmids; very high CPS, OTC, and arginase activities were detected upon overexpression. Strain MG1655R, which carries an *argR* mutation and is derepressed for the arginine regulon, showed at least a 20- to 30-fold-higher CPS specific activity (0.02 to 0.03 $\mu\text{mol}/\text{mg}/\text{min}$) compared with that of *argR*⁺ bacteria with an intact repressor (<0.001 $\mu\text{mol}/\text{mg}/\text{min}$)

TABLE 3. CPS, OTC, and arginase (ARG) activities in bacterial extracts^a

Strain	Vector	Enzyme sp act ($\mu\text{mol}/\text{mg}$ of protein/min)		
		CPS	OTC	ARG
C600ΔOTC	None	<0.001 (1) ^b	<0.001 (1)	<0.003 (1)
C600ΔOTC	pUC19	<0.001 (1)	<0.001 (1)	<0.003 (1)
C600ΔOTC	pUC19-pACYC184	<0.001 (1)	<0.001 (1)	
C600ΔOTC	pLLK12-pACAI	0.2–0.4 (2)	0.43–0.49 (4)	
C600ΔOTC	pUCABI+	0.6–0.8 (3)	19.4–30.4 (3)	<0.003 (1)
C600ΔOTC	pTRCABI+	1.2 (1)	28.7 (1)	
C600ΔOTC	pUCABIF+	0.4 (1)	ND ^c	7.6 (1)
MG1655R	None	0.02–0.03 (2)	0.50–0.56 (3)	
MG1655R	pUCABI+	0.8 (1)	27.0 (1)	
MG1655R	pTRCABI+	0.7–0.8 (2)	6.9 (1)	

^a For all experiments with plasmids containing the *trc* promoter, the cells were induced with 1 mM IPTG for 4 h. Assay conditions were as described in Materials and Methods. CPS activity coupled with endogenous OTC was measured when *argI* was simultaneously overexpressed; otherwise, exogenous OTC enzyme was added.

^b The values in parentheses indicate the numbers of determinations.

^c ND, not determined.

TABLE 4. Intracellular arginine concentrations in *E. coli* C600ΔOTC transformed with pLLK12 and pACAI, CPS- and OTC-expressing plasmids (E), and in control bacteria transformed with the respective parent vectors (C)

Strain	Substrate	Arginine concn (nmol/mg [dry wt]) ^a
E	Gln	1.7
	Gln+Orn	9.3
	NH ₃	3.5
	NH ₃ +Orn	9.9
C	Gln	1.1
	Gln+Orn	0.5
	NH ₃	2.1
	NH ₃ +Orn	1.6

^a All concentrations were determined after 3 h of incubation with various substrates at 37°C.

when cultured in rich medium. Transformation of MG1655R with plasmids expressing CPS increased the maximal activity of this enzyme to 0.8 to 1.7 $\mu\text{mol}/\text{mg}/\text{min}$. The OTC activities expressed by plasmids pTRCABI, pTRCABI+, pUCABI, and pUCABI+ listed in Table 3 are an underestimate since more than 50% of the assay substrate was consumed during the reaction. Derepression of the chromosomal *argI* gene occurred (increased OTC activity, 0.2 $\mu\text{mol}/\text{mg}/\text{min}$, versus <0.001 $\mu\text{mol}/\text{mg}/\text{min}$ in nontransformed controls) when *argR*⁺ bacteria were transformed with plasmid pLLK12, which contains the *carAB* operon with its natural promoters. This was most likely caused by titration of the *argR* repressor by the many copies of the arginine operator (Arg box) present in the plasmids within the transformed cell. Based on the experiments reported here, there was evidence of derepression of the chromosomal arginine regulon by the presence of Arg boxes cloned into the plasmids since only bacteria containing a plasmid with Arg boxes (or those with an insertion in the chromosomal *argR* gene) produced elevated amounts of arginine or urea (see below). Thus, titration of the arginine repressor rendered repression of the chromosomal arginine regulon inefficient despite the presence of high concentrations of its corepressor, arginine, in the bacterial cells. The amino acid profile in bacteria transformed with the engineered plasmids also reflects this derepression of the arginine regulon (see below). *argR*⁺ strains containing the respective parent cloning vectors had undetectable activities of CPS or OTC in all experiments, confirming the highly repressed state of the chromosomal arginine biosynthetic genes in cells grown in rich media. *E. coli* does not possess an arginase gene (3); therefore, all the measured arginase activity was derived from expression of the *rocF* gene carried on the plasmids.

Arginine synthetic capacity in engineered bacteria. We measured the arginine concentration in several *E. coli* strains containing the parental or engineered plasmids. For these experiments, bacteria were grown to the mid-log phase, induced with 1 mM IPTG for 4 h, centrifuged, and resuspended in M9 minimal medium with ammonium chloride or glutamine in the presence and absence of ornithine.

The arginine synthetic capacity of plasmid-carrying derivatives of C600ΔOTC and MG1655R are illustrated in Table 4 and Fig. 4. The arginine synthesis rate measured as the intracellular arginine level in C600ΔOTC(pUCABI+) was approximately 4.3 nmol/mg (dry weight)/h as compared with 0.2 nmol/mg (dry weight)/h in cells containing pUC19. These rates were calculated from the total amount of arginine produced in 3 h by a known amount of cells. The increase in intracellular

FIG. 4. Intracellular arginine concentrations in *E. coli* C600ΔOTC (*argI argF*) and MG1655R (derepressed arginine regulon) containing the engineered plasmids or the parent vectors. Vectors are described in Table 2. The induced cultures were incubated with glutamine (20 mM) or glutamine (20 mM) plus ornithine (5 mM) for 3 h, and intracellular arginine concentrations were determined.

arginine concentrations following incubation with glutamine and ornithine was linear up to at least 3 h (Fig. 5). Exogenous ornithine was required to achieve increased arginine biosynthesis, since without it, no change in arginine levels was observed in the engineered bacteria compared with that of controls. Substitution with the ornithine precursor *N*-acetylornithine had a similar effect, whereas *N*-acetylglutamate failed to provide substrate for enhanced arginine production (data not shown). This was not unexpected since *E. coli* K-12 is known to transport *N*-acetylornithine but not *N*-acetylglutamate into the cell (3). In the presence of exogenous ornithine, the formation of arginine was 6- to 16-fold higher in bacteria transformed with plasmids expressing *carAB* and *argI* with natural promoters or the inducible *trc* promoter compared with that of controls (Table 4; Fig. 4). In the *argR* strain MG1655R (Fig. 4), massive overexpression of *carAB* and *argI* (Fig. 3) augmented the formation of arginine by approximately 50% only; in these cells, the intracellular ornithine concentration must be limiting as well. As is demonstrated below (see urea experiments), when the supply of endogenous ornithine is replenished by expression of arginase, which hydrolyzes arginine to urea and ornithine, then overexpression of *carAB* from the engineered plasmids enhances the production of urea beyond the effect seen with derepression alone. Concentrations of citrulline or ornithine did not increase, and only traces were measurable (data not shown) in all experiments in which increased arginine was observed. This indicates that citrulline formation was rate limiting in spite of the overexpression of CPS and OTC, reinforcing the conclusion that ornithine was rate limiting even when supplied exogenously. In spite of the expected higher copy number of pUC19-derived plasmids

compared with that of pTRC99A plasmids, the rates of arginine biosynthesis were comparable for the two expression systems. We conclude that in these engineered expression systems, the biosynthesis of ornithine was the rate-limiting factor rather than the expression of chromosomal or plasmid-carried biosynthetic genes which convert ornithine to arginine. The rate of ornithine uptake seemed to be unable to saturate the capacity of the cells to incorporate it into arginine. In these experiments, endogenous formation of ornithine from glutamate (Fig. 1) was likely severely limited due to feedback inhibition by arginine of the first enzyme in the pathway (*N*-acetylglutamate synthase, *argA*) required for ornithine synthesis (3, 18).

Polyamine concentrations were determined and found to change only minimally even in cells with high levels of arginine (results not shown).

Urea synthesis in engineered bacteria. To evaluate the maximal rate of ammonia incorporation into arginine in the engineered plasmid-containing cells, attempts were made to maintain high levels of endogenous ornithine. The *B. subtilis* *rocF* gene, which codes for arginase, was cloned into the engineered plasmids in such a way that it was expressed together with *carAB* and *argI* in a polycistronic operon whose expression was inducible by the addition of IPTG (Fig. 2). This expression vector was then used to transform *E. coli* C600 and C600ΔOTC, which lacks the OTC genes *argI* and *argF*. Interestingly, we found low concentrations of urea in control bacteria containing the pUC19 parent vector (Fig. 6). Possibly, it was derived from the degradation of arginine via the decarboxylase and/or oxidase pathways which can form urea (3). Overexpression of *carAB*, *argI*, and *rocF* from pUCABIF+ in an *argR* host resulted in a 13- to 20-fold increase in urea production compared with that of the same cells carrying pUC19. Introduction of pUCABIF+ (which lacks a plasmid copy of *argI*) gave similar results in cells expressing the chromosomal OTC gene (C600) but not in C600ΔOTC. This finding suggests that OTC activity, whether plasmid encoded or chromosomally derived, is not rate limiting in this expression system. In addition, it shows that almost all urea produced by these bacteria derived from biosynthesis of arginine (Fig. 6). The rate of urea formation measured as the sum of intracellular and extracellular levels in C600ΔOTC(pUCABIF+) was approximately 28 nmol/mg (dry weight) per h and was linear over at least 3 h (Fig. 5). This rate was sixfold higher than the rate of arginine biosynthesis described above. This result was expected since the addition of arginase to this system creates an ornithine cycle producing large amounts of urea (whereas

FIG. 5. Intracellular arginine concentrations and urea formation as a function of time in C600ΔOTC (*argI argF*) transformed with pUCABIF+ (arginine) and pUCABIF+ (urea), induced with IPTG, and incubated with glutamine plus ornithine (pUCABIF+) or glutamine (pUCABIF+). Time zero indicates the start of incubation following induction. Each time point represents the average of two experiments.

FIG. 6. Urea production by *E. coli* C600 and C600ΔOTC transformed with different plasmids expressing CPS, OTC, and arginase enzyme activities. Vectors are described in Table 2. The induced cultures were incubated with 5 mM L-glutamine or ammonium chloride for 3 h, and total urea (intracellular and extracellular) was determined.

without arginase, arginine production was limited by the availability of ornithine). Measurement of amino acids in the arginase-expressing bacteria after 3 h of incubation documented that ornithine accumulated to levels of 3.6 to 7.3 nmol/mg (dry weight) while the levels were only 0.3 nmol/mg (dry weight) in cells devoid of arginase. Thus, the availability of ornithine was not rate limiting and the addition of arginase exposed a second rate-limiting step, most likely the formation of carbamyl phosphate. Since CPS activity is high and two molecules of ATP are consumed to produce one molecule of carbamyl phosphate, it is likely that the availability of ATP is the rate-limiting factor.

The studies described herein demonstrate that *E. coli* can be engineered to produce increased amounts of arginine in vitro and that arginine production is limited by the availability of ornithine. The addition of arginase completes the urea cycle in these bacteria but exposes another rate-limiting step, the synthesis of carbamyl phosphate. Optimization of the degree of overexpression of CPS, OTC, and arginase in a continuous culture system designed to remove urea and other byproducts from the medium, while continuously supplying oxygen and

nutrients, may further enhance the efficiency of ammonia assimilation by this biological system.

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