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In Vivo Synthesis of Histidine by a Cloned Histidine Ammonia-Lyase in *Escherichia coli*

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Histidine ammonia-lyase catalyzes the first step in histidine catabolism, the deamination of histidine to urocanate and ammonia. In vitro experiments have shown that histidine ammonia-lyase also can catalyze the reverse (amination) reaction, histidine synthesis, relatively efficiently under extreme reaction conditions (4 M NH_4OH, pH 10). An *Escherichia coli hisB* deletion strain was transformed with a pBR322 derivative plasmid (pCB101) containing the entire *Klebsiella aerogenes* histidine utilization (*hut*) operon to determine whether the catabolic histidine ammonia-lyase could function biosynthetically in vivo to satisfy the histidine auxotrophy. Although the initial construct did not grow on media containing urocanate and ammonia as a source of histidine, spontaneous mutants possessing this ability were isolated. Four mutants characterized grew at doubling times of 4 h compared with 1 h when histidine was present, suggesting that histidine synthesis, although unequivocally present, remained growth limiting. Each mutant contained a plasmid-encoded mutation which eliminated urocanase activity, the second enzyme in the Hut catabolic pathway. This genetic block led to the accumulation of high intracellular levels of urocanate, which was subsequently converted to histidine via histidine ammonia-lyase, thus satisfying the histidine auxotrophic requirement.

The specific amination of the α-carbon of the appropriate α,β-unsaturated carboxylic acid represents one way to synthesize selected amino acids. Unfortunately, this approach is not amenable to chemical synthesis because this amination reaction is nonspecific at the α-carbon. Ammonia-lyases, in contrast, have been shown to catalyze this type of reaction with complete specificity. For example, histidine ammonia-lyase (HAL) and phenylalanine ammonia-lyase, which function physiologically in the nonoxidative deamination of their respective amino acids, can catalyze the amination of their α,β-unsaturated carboxylic acids, albeit at a very slow rate (17, 18). Previous results suggest that the equilibrium constant for these enzymes approaches 4 (17, 18; R. L. Fuchs, unpublished data). In fact, it has been reported that HAL does not function biosynthetically under physiological conditions to catalyze the amination of urocanate to histidine at a rate sufficient to support the growth of a histidine auxotroph of *Klebsiella aerogenes* (11).

We have observed that the rates of amination for HAL from different microbial sources vary significantly (Fuchs, unpublished data), suggesting that evolutionary changes in this enzyme have occurred altering the relative rates of amination and deamination. Based upon these observations we sought to isolate specific structural gene mutations in HAL that would enable this catabolic enzyme to function biosynthetically in vivo. This objective was pursued by placing the cloned *K. aerogenes hutH* gene, which specifies HAL, into a His^- *Escherichia coli* and selecting for His^+ derivatives. *E. coli* does not contain the *hut* operon (13); therefore, strong selective pressure could be exerted to obtain these mutants. This report describes the physiological and genetic changes required to enable this host-vector system to synthesize histidine via HAL.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the strains used in this study were derivatives of *E. coli* K-12; descriptions and origins are shown in Table 1. Relevant descriptions and origins of plasmids also are listed in Table 1. Plasmid pCB101, constructed by Boylan et al. (2) and kindly supplied by R. A. Bender, contains a 7.9-kilobase *Hin*dIII insert carrying the entire wild-type *hut* operon from *K. aerogenes* cloned into the *Hin*dIII site of pBR322.

Media. Luria broth and M9 minimal medium (medium A) were prepared as described previously (14). Medium B contained M9 salts, 0.4% glucose, and 0.2% histidine in place of $(NH_4)_2SO_4$. Mutants capable of catalyzing the in vivo synthesis of histidine from urocanate and ammonia were selected on medium C, which contained M9 salts, 0.4% glucose, 1.0% urocanate, and 2.0% $(NH_4)_2SO_4$ at a pH of 7.5. Urocanate uptake mutants were isolated on solid medium containing M9 salts, 0.4% urocanate, 0.2% $(NH_4)_2SO_4$, and 0.005% histidine (medium D). All plasmid-containing strains were grown in media containing 0.005% ampicillin.

Preparation of cell extracts and enzyme assays. Cultures of *E. coli* were grown by the following protocol. An isolated colony of the appropriate strain was inoculated into Luria broth and grown for 6 to 7 h at 37°C. A 1-ml sample of this culture was used to inoculate a 50-ml overnight culture of the minimal medium that was to be used in the final growth stage. On the next day the appropriate volume of the overnight culture was used as an inoculum to give an A_{600} of 0.1. Cultures were grown to an A_{600} of 1.0, harvested by centrifugation at 4°C, washed with cold saline (4°C), and frozen at −70°C. Cells were thawed in an ice bath, suspended in 20 mM potassium phosphate buffer (pH 7.2) containing 25% glycerol and 0.1 mM $MnCl_2$, and lysed by two passes through an Aminco French pressure cell at 20,000 lb/in^2. Lysates were centrifuged at 20,000 × *g* for 20 min. The supernatant fraction was removed and centrifuged again for 20 min at 20,000 × *g*. The resultant supernatant was used as the source of enzyme to measure HAL and urocanase activities.

HAL activity was determined spectrophotometrically by measuring the rate of urocanate formation at an A_{277} as described by Magasanik et al. (9). Urocanase activity was

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TABLE 1. Description and origin of bacterial strains and plasmids

Strain or plasmid	Description	Origin
RH202	*thi hss lacY1 tonA21 supE44*	R. A. Bender
294	*endA thi hsr pro*	ATCC 31446
SB3930	*thi ΔhisB463* λ^-	P. E. Hartman
pBR322	*tet*$^+$ *amp*$^+$	F. Bolivar
pCB101	pBR322 containing the *K. aerogenes hut* operon	R. A. Bender
pMON260	*hutU701* derivative of pCB101	This paper
pMON261	*hutU702* derivative of pCB101	This paper
pMON262	*hutU703* derivative of PCB101	This paper
pMON263	*hutU705* derivative of pCB101	This paper

determined by measuring the disappearance of urocanate at an A_{277} as previously described (10). Specific activities were expressed as micromoles of product formed or substrate utilized per minute per milligram of protein. Protein was measured by the method of Bradford (3), with bovine serum albumin as the standard.

Intracellular pools of urocanate. Intracellular urocanate concentrations were determined in cells from 100 ml of an appropriately grown culture harvested on a 0.45-μm membrane filter (diameter, 90 mm; Millipore Corp., Bedford, Mass.). The filter was washed with 75 ml of M9 salts and placed in ice-cold perchloric acid for 20 min to lyse the cells. The perchlorate was precipitated with potassium carbonate as described by Deshpande et al. (7) to provide a cell-free supernatant for quantification of intracellular components. Urocanate concentrations were determined by high-pressure liquid chromatography analysis on an Altex Ultrasphere-ODS reverse-phase column. The column eluate was passed through a Beckman detector (254 nm) equipped with a Spectra Physics 4270 computing integrator. Elution was carried out with a 0.1 M potassium phosphate buffer (pH 7.5) with 0.1% tetrabutyl-ammonium hydroxide at a rate of 1.5 ml min^{-1}.

Selection of mutants capable of the in vivo synthesis of histidine from urocanate and ammonia. Cultures of histidine-requiring strain SB3930(pCB101) were grown to an A_{600} of 0.6 to 0.9 in either medium A containing 0.005% histidine or medium B. Cells were collected by centrifugation, washed with sterile saline, and suspended to the original volume with sterile saline. A 100-μl sample from these cultures was applied onto duplicate plates of medium C and incubated at 37°C until colonies developed.

Isolation of urocanate uptake mutants. A culture of SB3930(pCB101) was grown on Luria broth-ampicillin to late-log phase, harvested by centrifugation, washed twice with sterile saline, and suspended to the original volume with sterile saline. An 100-μl sample of the washed cell suspension was applied to plates containing medium D and incubated at 37°C for 5 days after which large colony mutants were isolated.

Plasmid isolation, transformation, and curing. Plasmids were isolated from cultures grown and amplified as described by Clewell and Helinski (5). Plasmid DNA was extracted by the alkaline extraction method of Birnboim and Doly (1), phenol-chloroform extracted, and further purified by the hydroxyapatite method of Coleman et al. (6). Calcium chloride-treated cells were transformed with plasmid DNA (10 to 100 ng) as described by Mandel and Higa (12). Plasmid-containing strains were cured by acridine orange treatment by the procedure of Miller (14) with 50 μg of acridine orange ml^{-1}.

Chemicals. All chemicals were reagent grade and commercially available. DNA grade Bio-Gel HTP hydroxyapatite was purchased from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Isolation of mutants capable of the in vivo synthesis of histidine by HAL. *E. coli* SB3930 (Hsr$^+$ Hsm$^+$ *ΔhisB463*) contains a deletion that removes the entire *hisB* gene of the histidine biosynthetic operon (8). Plasmid pCB101, which contains the entire *K. aerogenes hut* operon (histidine catabolic pathway) cloned into the *Hin*dIII site of pBR322 (2), was isolated from *E. coli* 294 (Hsr$^-$ Hsm$^+$) for proper methylation before transformation into strain SB3930. The resulting transformant SB3930(pCB101) was unable to use urocanate and NH_3 as a source of histidine, despite the presence of the histidine-deaminating enzyme HAL. This result suggests that, although HAL can synthesize histidine in vitro (17; Fuchs, unpublished data), the physiological conditions within the cell do not permit the efficient reversal of HAL (see also reference 11). Therefore, strain SB3930(pCB101) was plated on a medium lacking histidine but containing high ammonium (2%) and urocanate (1%) levels and incubated at 37°C until histidine prototrophic mutants developed. Eight spontaneous mutants were isolated in two independent selections at a frequency of approximately 2×10^{-8} to 3×10^{-8}. These mutants were isolated only in pCB101-containing strains and were dependent on high levels of urocanate and $(NH_4)_2SO_4$ and the prior induction of HAL in the parental strain. We propose that prior induction of HAL is required for the isolation of urocanase-deficient mutants for the following reasons. Cells induced with histidine have both high HAL activity, providing an immediate, albeit low, potential for histidine synthesis from urocanate and $(NH_4)_2SO_4$, and residual histidine pools, allowing for initial growth and an increased potential to accumulate urocanate. In contrast, uninduced cells contain little or no HAL and a very inefficient urocanate transport system (4). It is likely, therefore, that in the latter case the uninduced cells die of histidine starvation before selection of urocanase-deficient mutants can occur.

Each of these eight mutations was localized to the plasmid, since transformation of strain SB3930 with any one of the eight plasmids conferred histidine prototrophy. Biochemical characterization of four of the original mutants [SB3930 (pMON260), SB3930(pMON261), SB3930(pMON262), and SB3930(pMON263)] was carried out on extracts prepared from cultures grown with glucose and histidine as the carbon and nitrogen sources, respectively (Table 2). In each case, HAL activity was significantly induced but urocanase, the second enzyme of the histidine catabolic pathway, was not detected. As a result, urocanate accumulated within the cells containing these plasmids (Table 2). Since Schlesinger et al. (16) reported that urocanate was the inducer of the *K. aerogenes hut* operon, it was not surprising that the specific activity of HAL in strain SB3930 carrying plasmids pMON260, pMON261, pMON262, and pMON263 was 5- to 6-fold higher than that of the fully induced HAL in SB3930(pCB101) and 100-fold higher than the uninduced level (specific activity, 0.03). Similar results were found when these same plasmid-containing strains were grown in

medium C (Table 3), although growth rates and intracellular urocanate levels were decreased, probably due to the limited capacity of *E. coli* to transport urocanate.

Isolation of mutants with enhanced uptake of urocanate. Strain SB3930(pMON263) grew in medium C with a doubling time of 4 h, significantly longer than the 1-h doubling time found when histidine was added to this medium. This result suggests that histidine biosynthesis is rate limiting for growth despite the increased pool level of urocanate and the hyper-induced level of HAL. Nevertheless, it was suspected that the urocanate pool was the cause of reduced growth for the following reasons. First, urocanate is about 100-fold less effective than histidine in inducing HAL in *E. coli* (Fuchs, unpublished data). Second, urocanate was transported very slowly, if at all, in the closely related microorganisms *Salmonella typhimurium* (4) and *K. aerogenes* (4, 15, 16). Third, chromosomal mutations were isolated in *S. typhimurium* and *K. aerogenes* that specifically enhanced the transport of urocanate as the sole carbon or nitrogen source or both.

We used a rationale similar to that of Brill and Magasanik (4) to isolate spontaneous mutants with enhanced transport of urocanate. Strain SB3930(pCB101) was plated on a medium containing urocanate as the sole carbon source, and larger colonies, indicative of fast-growing mutants, were isolated. Characterization of several of these permeability mutants showed that the increased urocanate uptake had no discernible effect on the effectiveness of external urocanate as a carbon source or an inducer of HAL activity (data not shown). Although the explanation of this observation is unclear, we suggest that factors other than internal urocanate concentration (e.g., ammonia concentration) might be involved in limiting histidine biosynthesis via HAL.

DISCUSSION

Although several ammonia-lyases have been reported to catalyze the synthetic reaction [RCH = CHCOOH + NH_3 → $RCH_2CH(NH_2)COOH$] in vitro, no ammonia-lyase has been shown to function anabolically with respect to amino acid synthesis in vivo. This can be attributed at least in part to generally high K_m values, high pH optima, and unfavorable equilibrium (Keq) constants. In the case of HAL from *K. aerogenes* the K_m values were found to be 8.5 M NH_3 and 0.1 M urocanate when assayed at the optimal pH of 10 (Fuchs, unpublished data). When these conditions are coupled with a Keq of 4, it is not surprising to find that a cloned HAL could not produce enough histidine to support the growth of a His^- strain of *E. coli* on a growth medium of pH 7.5 containing 0.07 M urocanate and 0.15 M $(NH_4)_2SO_4$. We anticipated, however, that these conditions would allow us to select for mutations in HAL that affected either the K_m values or the rate of amination. Instead, mutants were obtained that had lesions in *hutU*, the gene that codes for urocanase, the second enzyme in the histidine utilization pathway. These mutations caused an increased intracellular pool of urocanate, the HAL substrate-inducer, and, subsequently, the highest levels of HAL yet reported. This combination produced enough histidine to support the growth of the histidine auxotroph at a doubling time equal to twice that observed when histidine was exogenously supplied. Since subsequent attempts to increase the urocanate pools had no affect on the growth rate, some factor(s) other than urocanate must become limiting. It is not unreasonable to propose that this factor is the second substrate NH_3. If faster-growing derivatives of SB3930(pMON260) were selected, it should be possible to obtain mutations that either decrease the K_m for NH_3 or increase the rate of amination and thus evolve a more efficient synthetic enzyme. Analysis of such changes should provide pertinent information on the structural-functional relationships of ammonia-lyases and indicate which specific amino acid alterations are necessary to exploit the synthetic potential of this enzyme, despite apparently unfavorable thermodynamic constraints.

TABLE 2. Biochemical characterization of the plasmid-containing His^+ derivatives grown with histidine as the nitrogen source[a]

Plasmid	Sp act of:		Intracellular urocanate (μg/100 ml)[b]
	HAL	Urocanase	
pCB101	0.5	0.14	<1
pMON260 (*hutU701*)	2.2	<0.01	16
pMON261 (*hutU702*)	2.6	<0.01	14
pMON262 (*hutU703*)	2.1	<0.01	23
pMON263 (*hutU705*)	2.2	<0.01	18

[a] All plasmid-containing cultures of strain SB3930 were grown in medium B.

[b] Intracellular urocanate concentrations were measured as described in the text and are presented as micrograms of urocanate per 100 ml of culture harvested at an A_{600} of 0.3.

TABLE 3. Characterization of the His^+ mutants grown with urocanate as a source of histidine[a]

Plasmid	Generation time (min)	Sp act of HAL	Intracellular urocanate (μg/100 ml)[b]
pMON260	250	3.0	3.5
pMON261	250	2.9	NA
pMON262	250	2.8	5.7
pMON263	250	3.5	NA

[a] All plasmid-containing cultures of strain SB3930 were grown in medium C.

[b] Intracellular urocanate concentrations were measured as described in the text and are presented as micrograms of urocanate per 100 ml of culture harvested at an A_{600} of 1.0. NA, Not assayed.

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