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Effect of Inducible *thrB* Expression on Amino Acid Production in *Corynebacterium lactofermentum* ATCC 21799

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Amplification of the operon *hom*^{dr}-*thrB* encoding a feedback-insensitive homoserine dehydrogenase and a wild-type homoserine kinase in a *Corynebacterium lactofermentum* lysine-producing strain resulted in both homoserine and threonine accumulation, with some residual lysine production. A plasmid enabling separate transcriptional control of each gene was constructed to determine the effect of various enzyme activity ratios on metabolite accumulation. By increasing the activity of homoserine kinase relative to homoserine dehydrogenase activity, homoserine accumulation in the medium was essentially eliminated and the final threonine titer was increased by about 120%. Furthermore, a fortuitous result of the cloning strategy was an unexplained increase in homoserine dehydrogenase activity. This resulted in a further decrease in lysine production along with a concomitant increase in threonine accumulation.

In *Corynebacterium* spp., lysine, methionine, threonine, and isoleucine derive part or all of their carbon atoms from aspartate (Fig. 1) (22). (The genus *Corynebacterium* will be used in this article to include *Brevibacterium lactofermentum* and *Brevibacterium flavum*, as stipulated by Liebl et al. [18].) Fluxes in the aspartate amino acid pathway of *Corynebacterium* spp. seem to be controlled by the relative specific activities and regulatory mechanisms of the unique branchpoint enzymes, making this organism an ideal system for studies of metabolic engineering.

The genes encoding the three enzymes required for conversion of DL-aspartic-β-semialdehyde (ASA) to L-threonine, i.e., homoserine dehydrogenase (HD; EC 1.1.1.3; *hom*), homoserine kinase (HK; EC 2.7.1.39; *thrB*), and threonine synthase (TS; EC 4.2.99.2; *thrC*), have been cloned from *Corynebacterium glutamicum* (11, 13, 27). The molecular biology of their expression and regulation has been elucidated (11). The *hom* and *thrB* genes are present in an operon in the *C. glutamicum* genome.

The main control site of the threonine-specific biosynthetic pathway in wild-type *Corynebacterium* spp. is the enzyme homoserine dehydrogenase as a result of the strong feedback inhibition of this enzyme by threonine. Wild-type HD from this organism is completely inhibited at concentrations of 1 to 5 mM threonine (1, 8, 22). A secondary control element in this pathway is HK, which is also inhibited by threonine but to a lesser extent, i.e., by about 60% at 30 mM threonine (22). HKs from *Escherichia coli* and *Saccharomyces cerevisiae* are also inhibited by threonine (45% at 10 mM threonine and 38% at 25 mM threonine, respectively) (35). The same studies have shown little or no inhibition of threonine synthase activity by members of the aspartate amino acid family. In a threonine-producing strain, high intracellular levels of threonine are observed, in some cases up to 100 mM (data not shown) (28). These levels may be sufficient to significantly inhibit the in vivo activity of HK under these conditions.

An obvious strategy for obtaining threonine overproduction in this system would be to abolish threonine inhibition of the first two enzymes in this pathway. Threonine overproduction has been reported by mutants of *Corynebacterium* spp. resistant to DL-α-amino-β-hydroxyvaleric acid (a threonine analog) (15, 26, 30). Amplification of a feedback-insensitive *hom* gene in a *Corynebacterium* lysine producer has resulted in homoserine accumulation in the medium as well as considerable residual lysine accumulation (25, 28). Similarly, the amplification of the *hom*^{dr}-*thrB* operon, by use of a threonine-insensitive DL-α-amino-β-hydroxyvaleric acid allele of the *hom* gene isolated in our laboratory (*hom*^{dr}) (1), resulted in homoserine accumulation. These results suggest that the phosphorylation of homoserine by HK may be limiting threonine biosynthesis under these conditions.

To overcome this limitation for the purpose of improved threonine production, a plasmid enabling separate transcriptional control of each gene was constructed to determine the effect of various enzyme activity ratios on metabolite accumulation. A combination of kinetic and thermodynamic effects was taken into account in the analysis of our results.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *Corynebacterium lactofermentum* ATCC 21799 (a lysine producer resistant to *S*-2-aminoethyl-L-cysteine, a lysine analog) was used as the base strain. The complex medium used for all strains was Luria-Bertani broth (17). Cells were cultured at 30°C in *C. glutamicum* minimal medium adapted from von der Osten et al. (34) by Kiss (16). Amino acids were supplemented at 150 mg/liter for assays and at 400 mg/liter for fermentations. Media used for assays contained 20 g of glucose per liter and 5 g of ammonium sulfate per liter. For fermentations, these concentrations were increased to 80 and 50 g/liter, respectively, and calcium carbonate (30 g/liter) was added for buffering. When appropriate, kanamycin (50 mg/liter) and ampicillin (50 mg/liter) were added. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used as an inducer when appropriate. The organisms were cultured aerobically at 30°C as 100-ml cultures in

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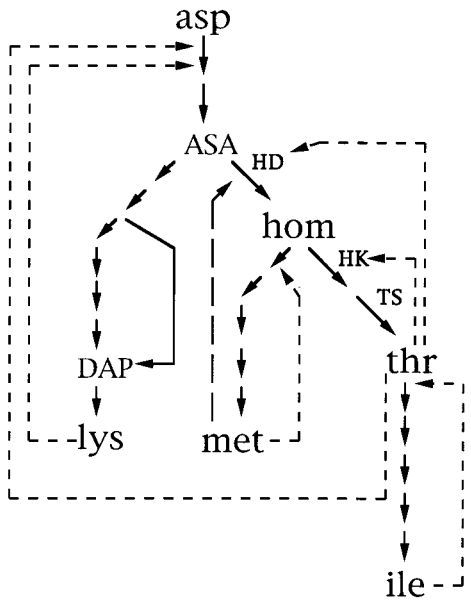

FIG. 1. Scheme for biosynthesis and feedback regulation of aspartate family amino acids in *C. glutamicum*. Abbreviations: asp, aspartate; ASA, DL-aspartic-β-semialdehyde; hom, homoserine; DAP, diaminopimelate; lys, lysine; met, methionine; thr, threonine; ile, isoleucine; TS, threonine synthase. Solid arrows represent direction of preferential synthesis; short dashes represent feedback inhibition; long dashes represent repression.

500-ml baffled Erlenmeyer flasks on a rotary shaker at 250 rpm. For fermentation precultures (50 ml in 100-ml flasks), seed medium containing the following ingredients was used (per liter): sucrose, 50 g; polypeptone, 20 g; urea, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 1.5 g; KH_2PO_4, 0.5 g (pH 7.0).

DNA manipulations. Isolation of plasmid DNA, preparation of DNA fragments, ligations, and other DNA manipulations were performed by established DNA recombinant procedures (4, 29, 36). *Corynebacterium* cells were transformed by electroporation as described by Follettie et al. (10). All restriction enzymes and related reagents for DNA manipulations were obtained from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim and used as described in the instructions of the manufacturer.

Enzyme assays. Cell extracts were prepared as described by Jetten and Sinskey (14) for determination of enzyme activities. For determination of specific activities during shake flask fermentations, a 1- to 2-ml sample of cells was removed from the culture flask and treated as described above. The protein concentration of the extracts was determined by the method of Bradford (6). Enzyme assays were performed at room temperature (22 ± 2°C) with a Hewlett-Packard model 8452A diode-array spectrophotometer. HD activity was determined by the method of Follettie et al. (11) without KCl in a total volume of 1 ml. The absorbance decrease in the absence of the substrate, ASA, was determined and found to be negligible. ASA was synthesized by the ozonolysis of DL-allyl glycine by the procedure of Black and Wright (5). HK activity was measured as described by Follettie et al. (11) with the following modifications: 5 mM ATP, 1 mM NADH, 5 mM phosphoenolpyruvate, and 10 mM L-homoserine were used. The absorbance decrease in the absence of the substrate L-homoserine was determined as a control and subtracted from values obtained with the complete reaction mixture. For the kinetic studies, HK activity as a function of L-homoserine concentration was measured in crude extracts of ATCC 21799(pJD4) in the presence of various concentrations of threonine (0, 10, and 25 mM). The concentration of L-homoserine was varied from 0.1 to 40 mM. The concentration of ATP was maintained at saturating conditions (5 mM). The Michaelis-Menten constant of homoserine for HK was estimated by the nonlinear regression analysis program GraFit from Erithacus Software Ltd.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Source or reference
Strains		
C. glutamicum AS019-E12	Restriction-deficient derivative of *C. glutamicum* AS019	9
C. lactofermentum ATCC 21799	Lysine-producing strain; AEC^R	ATCC
E. coli DH5α	*lacZ*ΔM15 *recA1*	BRL
Plasmids		
pMG108	Km^r Ap^r *lacI*^{q} *tac*	12
pM2	*E. coli-C. glutamicum* shuttle vector	10
pFS3.6	3.6-kb *Sal*I fragment containing *hom-thrB* operon cloned in a *C. glutamicum-E. coli* shuttle vector	11
pJD4	Km^r *hom*^{dr}*-thrB* operon	1
pGC18	Km^r Ap^r *lacI*^{q}; *thrB* under control of *tac* promoter	This study
pGC42	Km^r Ap^r *lacI*^{q} *hom*^{dr}; *thrB* under control of *tac* promoter	This study

[a] Km^r and Ap^r indicate resistance to kanamycin and ampicillin, respectively. AEC^R indicates resistance to *S*-2-aminoethyl-L-cysteine, a lysine analog.

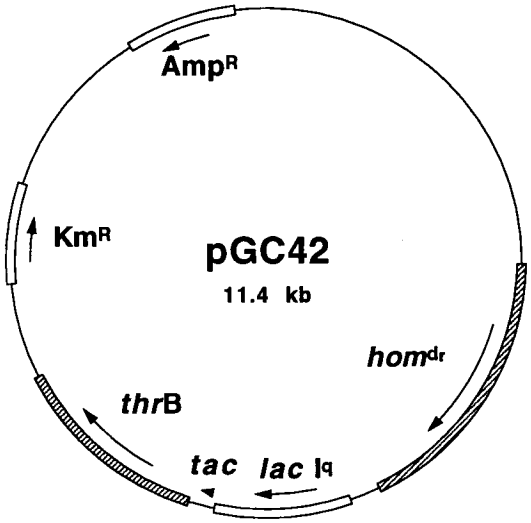

FIG. 2. Plasmid pGC42 containing the *hom*^{dr} gene under the control of its native promoter and the *thrB* gene under control of the *tac* promoter. Km^R and Ap^R indicate resistance to kanamycin and ampicillin, respectively.

Construction of pGC42. For construction of pGC42, the *E. coli-C. glutamicum* shuttle vector pMG108 (12) was restriction digested with *Sma*I and *Sal*I and subsequently treated with calf intestine phosphatase. The plasmid pFS3.6 containing the *hom-thrB* operon (11) was restriction digested with *Bpu*1102I, treated with the Klenow fragment of DNA polymerase I, and digested with *Sal*I. The 1.4-kb *Bpu*1102I-*Sal*I fragment was isolated from a 1% agarose gel by the QIAEX gel extraction kit (QIAGEN, Chatsworth, Calif.) after size fractionation by electrophoresis. The *Bpu*1102I site is located about 50 bp upstream of the ATG start codon of the *thrB* gene (27). The fragment was ligated into pMG108, placing the gene downstream of the *tac* promoter in this vector and creating the plasmid pGC18. The *tac* promoter is a hybrid of the *E. coli trp* and *lac* promoters which has been shown to function efficiently in *Corynebacterium* strains (7, 12, 24). To add the feedback-insensitive HD gene, pGC18 was restriction digested with *Xho*I, treated with the Klenow fragment of DNA polymerase I, and subsequently treated with calf intestine phosphatase. The *hom*^{dr} gene (under control of its native promoter) was isolated as a 2.0-kb *Sal*I-*Mun*I fragment from pJD4 (1) and ligated into pGC18 after treatment with the Klenow fragment of DNA polymerase I, creating the 11.4-kb plasmid pGC42 (Fig. 2).

Amino acid analysis. Amino acids were analyzed as *ortho*-phthaldialdehyde derivatives by reversed-phase chromatography using an AminoQuant column with a Hewlett-Packard series 1050 high-pressure liquid chromatography (HPLC) system (Hewlett Packard, Waldbronn, Germany). HPLC-grade solvents were obtained from Mallinckrodt Specialty Chemicals, Paris, Ky.

RESULTS

Comparison of expression of *hom-thrB* and *hom*^{dr}*-thrB* in *Corynebacterium* spp. The specific activities of HD and HK in strains ATCC 21799(pFS3.6) and ATCC 21799(pJD4) grown

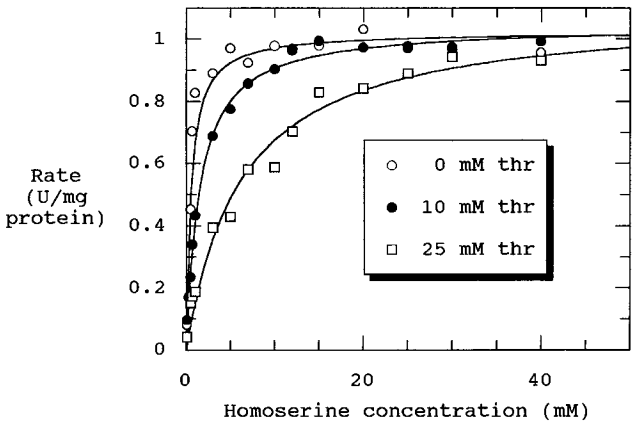

FIG. 3. Initial HK reation rate as a function of homoserine concentration in the presence of 0, 10, and 25 mM threonine. The concentration of ATP was maintained at 5 mM. One unit of enzyme activity corresponds to the oxidation of 1 μmol of NADH per minute. Curves shown are a nonlinear regression fit to Michaelis-Menten parameters calculated by the program GraFit.

in minimal medium were measured. HD activities in ATCC 21799(pFS3.6) and ATCC 21799(pJD4) were 5.8 and 6.1 μmol min^{-1} mg of $protein^{-1}$, respectively, whereas HK activities in the same strains were 0.7 and 0.8 μmol min^{-1} mg of $protein^{-1}$, respectively.

HK kinetics. Threonine inhibition of HK has been observed in several species (22, 32, 35). Figure 3 shows a plot of initial enzyme reaction rate as a function of homoserine at 0, 10, and 25 mM threonine. The concentration of the second substrate, ATP, was maintained at saturating conditions (5 mM). It can be seen that increasing concentrations of threonine do indeed inhibit the activity of HK and that this inhibition is relieved as the homoserine concentration is increased. This behavior is characteristic of competitive inhibition. Parameters for a competitive inhibition model were determined by a nonlinear regression fit (GraFit, Erithacus Software Ltd.) as follows: K_m for homoserine = 0.7 mM; K_i for threonine = 7.7 mM; V_{max} = 1.1 μmol min^{-1} mg of $protein^{-1}$.

Expression of genes in pGC42. By performing inducibility studies with pGC42 in ATCC 21799, it was determined that the HK activity could be increased to an average of 6.0 to 6.2 μmol min^{-1} mg of $protein^{-1}$. This range of values is approximately 10- to 20-fold higher than the activities observed in cells harboring plasmids containing the *hom-thrB* operon under the control of its native promoter (8, 11). Although there was some slight variation depending on the levels of IPTG added to the medium, HD activity was on average about 80 to 100% higher in ATCC 21799(pGC42) than in either ATCC 21799(pJD4) or ATCC 21799(pFS3.6).

Comparison of metabolite accumulation in various ATCC 21799 recombinants. *C. lactofermentum* ATCC 21799(pM2) was used as a lysine-producing control. ATCC 21799(pM2), ATCC 21799(pJD4), and ATCC 21799(pGC42) were precultured in 50 ml of seed medium. One milliliter of exponentially growing cells was inoculated into 100 ml of mineral salts medium containing 80 g of glucose per liter. After approximately 30 h, and twice more at about 30-h intervals, either 0, 1.5, or 5.0 μmol of IPTG was added to the ATCC 21799(pGC42) cells. The duration of a typical fermentation was approximately 100 h, with the cells reaching a typical optical density at 600 nm of about 50. The specific activities of HD and HK were monitored throughout the fermentation (Table 2). In ATCC 21799 (pGC42), the HD activity decreased slightly with IPTG addition, whereas the HK activity was greatly induced by the addition of 1.5 μmol of IPTG to the flask. The addition of higher concentrations of IPTG did not significantly increase the HK activity. The culture fluid was analyzed for amino acid content at the point of glucose exhaustion (Table 3). ATCC 21799 (pM2) produced 22 g of lysine per liter, whereas ATCC 21799 (pJD4) produced 4.5 g of lysine, 5.4 g of threonine, and 2.0 g of homoserine (each per liter) as well as some glycine and isoleucine. In ATCC 21799(pGC42), for all cases irrespective of IPTG addition, less than 1 g of lysine per liter was produced. In the case of no induction, significant amounts of threonine and homoserine were produced. In the case of low induction, accumulation of homoserine was not observed, but up to 11.8 g of threonine per liter was produced. Higher concentrations of IPTG did not significantly increase the amount of HK and in fact resulted in a lower final threonine titer. In all cases, there was significant accumulation of glycine and isoleucine. Because of concerns about possible plasmid instability (28), the structural stability of the plasmid pJD4 was investigated by isolating several colonies from the ATCC 21799(pJD4) flask and treatment with the restriction enzyme *Eco*RI. None of the plasmids showed signs of major deletions (data not shown). In addition, the activity of HD was also measured in the presence of 50 mM threonine to determine if any structural instability had occurred in the plasmids containing the *hom*^{dr} gene. After 80 h, the enzyme still retained from 75 to 95% of its activity in the presence of 50 mM threonine.

TABLE 2. Average specific activities of HD and HK during shake flask fermentations of various ATCC 21799 recombinants

C. lactofermentum strain[a]	Sp act (μmol min^{-1} mg of $protein^{-1}$)[b]	
	HD	HK
ATCC 21799(pM2)	0.2	0.1
ATCC 21799(pJD4)	3.6	2.0
ATCC 21799(pGC42) with:		
No induction	8.9	0.5
1.5 μmol of IPTG	8.0	3.8
5.0 μmol of IPTG	7.3	4.6

[a] ATCC 21799(pGC42) cultures were induced by the addition of IPTG in the amount shown to the 100-ml culture at approximately 30-h intervals during the course of the fermentation.
[b] Average specific activity during fermentation.

DISCUSSION

To effect threonine accumulation in a lysine producer, carbon flux must be redistributed away from lysine biosynthesis at the ASA branchpoint. Furthermore, the activities of enzymes in the threonine pathway must be coordinated to minimize accumulation of intermediates such as homoserine. Thus, in our case, the simple overexpression of the *hom*^{dr}*-thrB* operon resulted in the expected reduction in lysine and increase in threonine production, along with accumulation of homoserine (25, 28), because of an apparent imbalance in enzyme activities under these conditions.

Our initial assumption was that the frameshift mutation in the *hom* gene which gave rise to *hom*^{dr} also exerted a polar effect on the expression of the downstream *thrB* gene, thus reducing the intracellular specific activity of HK. No significant difference in HK activity between ATCC 21799(pFS3.6) and ATCC 21799(pJD4) was observed, indicating that a disruption of HK transcription due to the *hom* deletion is highly unlikely. Therefore, the homoserine accumulation is probably due to

TABLE 3. Amino acids accumulated in culture medium by various ATCC 21799 recombinants

C. glutamicum strain[a]	Amino acid concn in supernatant (g/liter)[b]				
	Lysine	Threonine	Homoserine	Glycine	Isoleucine
ATCC 21799(pM2)	22.0 ± 1.0	<0.1	<0.1	<0.1	<0.1
ATCC 21799(pJD4)	4.5 ± 0.2	5.4 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	1.3 ± 0.1
ATCC 21799(pGC42) with:					
No induction	0.9 ± 0.1	5.6 ± 0.3	6.7 ± 0.3	1.3 ± 0.1	1.0 ± 0.1
1.5 μmol of IPTG	0.8 ± 0.1	11.8 ± 0.6	<0.1	4.6 ± 0.2	1.9 ± 0.2
5.0 μmol of IPTG	0.9 ± 0.1	9.3 ± 0.1	<0.1	3.4 ± 0.2	0.9 ± 0.1

[a] ATCC 21799(pGC42) in 100-ml cultures was induced by the addition of the given amount of IPTG at approximately 30-h intervals during the course of the fermentation (total of three times).
[b] Amino acid concentrations are given at the point of glucose exhaustion from the medium (90 to 100 h).

threonine inhibition of HK that is of a competitive nature, as in the case of *E. coli* HK (Fig. 3) (35). In the case of competitive inhibition, it is unlikely that a mutation decreasing inhibitor binding without affecting substrate binding can be found. Therefore, rather than attempting to isolate a threonine-deregulated HK, a strategy involving modulation of HK activity was attempted.

Table 2 shows that under optimal induction, the activities of HD and HK in ATCC 21799(pGC42) are approximately doubled with respect to the base case of ATCC 21799(pJD4). The higher final threonine titer and lack of homoserine accumulation obtained (Table 3) result from (i) further enhancement of carbon flux towards the threonine branch and (ii) reduced in vivo threonine inhibition of HK due to higher intracellular enzyme levels. The combination of these effects apparently results in optimal intracellular conditions for smooth carbon flow to threonine.

Although, under standard conditions, the reduction of ASA to homoserine is thermodynamically favorable ($\Delta G^{\circ\prime}$ of reaction = −21 kJ/mol; calculated by use of references 19, 20, and 31), a high enough accumulation of homoserine may reverse the direction of the reaction. In strains with an amplified *hom*^{dr}-*thrB* operon, the intracellular concentration of homoserine may reach 74 mM during periods of the fermentation (28). Preliminary analysis using the method of Mavrovouniotis (21) shows that taking into account the permissible concentration ranges of the metabolites involved, this is well within the range necessary to drive the reaction towards formation of ASA. This, in turn, may allow some of the ASA to be captured for the lysine pathway (Fig. 1). In our strain, the high HK activity may have contributed to a reduced intracellular level of homoserine and allowed the flux of carbon to proceed through to threonine.

The increased activity of feedback-insensitive homoserine dehydrogenase in ATCC 21799(pGC42) relative to that of ATCC 21799(pJD4) is likely to be important for diverting a higher percentage of the carbon flow at the ASA branchpoint for threonine production. In fact, one study in which a *hom*^{dr} gene was amplified in an *S*-2-aminoethyl-L-cysteine- and DL-α-amino-β-hydroxyvaleric acid-resistant strain, *C. glutamicum* DM368-3, HD activities were comparable to those found in ATCC 21799(pGC42) (8). In this case, residual lysine production in this strain was nearly negligible.

There was considerable accumulation of glycine and isoleucine in fermentations with all threonine producers. There are at least three routes to threonine degradation in *Corynebacterium* spp.: threonine dehydrogenase (EC 1.1.1.129; *tdh*), serine hydroxymethyltransferase (EC 2.1.2.1; *glyA*), and threonine dehydratase (EC 4.2.1.16; *ilvA*) (2). The first two lead to production of glycine. Threonine dehydratase initiates a five-step pathway from threonine to isoleucine and is feedback inhibited by isoleucine (23). Obviously, this inhibition did not suffice to prevent isoleucine accumulation in our threonine producers. Further studies investigating the kinetic parameters of these enzymes are under way.

The induction of cloned genes by IPTG has been shown to have an inhibitory effect on cell growth and metabolite production in both coryneform and enteric bacteria due to overexpression of the cloned gene product (3, 33). We observed that there was an optimal level of IPTG addition at which HK activity sufficed to minimize homoserine accumulation and beyond which threonine production was even hampered. At these levels, growth was not significantly affected.

In summary, two main ideas are demonstrated by our results. First, carbon flux distribution at the ASA branchpoint (and, consequently, the relative amount of lysine or threonine synthesized) is completely controlled by the activity of a feedback-insensitive HD enzyme. Second, HK activity relative to feedback-insensitive HD activity determines the level of homoserine accumulation in the medium and the final titer of threonine.

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