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Gene Structure and Expression of the *Corynebacterium flavum* N13 *ask-asd* Operon

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Two promoters required for expression of the *ask-asd* genes, encoding aspartokinase (AK) and aspartate-semialdehyde dehydrogenase (ASD), in *Corynebacterium flavum* N13, *ask*P1 and *ask*P2, have been identified by deletion analysis and S1 nuclease mapping. Transcription from *ask*P1 initiates 35 and 38 bp upstream of the *ask* structural gene. A second promoter, *ask*P2, lies within the *ask* coding region, upstream of the translation start site of the AKβ subunit and can direct the expression of AKβ and ASD. Western immunoblot analysis and heterologous expression in *Escherichia coli* demonstrate that two separate polypeptides, a 44.8-kDa α subunit and an 18.5-kDa β subunit, are expressed from the *C. flavum* N13 *ask* gene from distinct, in-frame translation initiation sites. A second AK mutation, G345D, which reduces the sensitivity of AK to concerted feedback inhibition by threonine plus lysine, was identified.

Aspartokinase (AK) and aspartate-semialdehyde dehydrogenase (ASD) catalyze the initial reactions in the amino acid-biosynthetic pathway responsible for the synthesis of L-lysine, L-methionine, L-threonine, L-isoleucine, and the cell wall precursor *meso*-diaminopimelate. Several distinct genomic organizations and a diversity of regulatory mechanisms controlling the metabolic flux through this complex, multibranched pathway have been identified in the bacterial species studied to date.

Regulation of the aspartate amino acid family in *Corynebacterium* spp. apparently does not involve multiple isoenzymes but is instead coordinated by the relative specific activities and patterns of inhibition and repression at key branchpoint enzymes. The 15-fold-higher specific activity of homoserine dehydrogenase than of dihydropicolinate synthetase, which compete for the common intermediate aspartate-semialdehyde, establishes a preferential pathway flux toward the threonine-methionine branch (14). Increased concentrations of methionine repress the expression of homoserine dehydrogenase (7), while threonine inhibits its activity, directing the flow of aspartate-semialdehyde into the lysine branch (14). The overall metabolic flux into the pathway is mediated by the concerted feedback inhibition of AK activity by lysine and threonine (11, 20).

We previously reported on the *Corynebacterium flavum* N13 gene encoding AK, *ask* (5, 6) (GenBank accession number L16848), and this gene has also been described as *lysC* by other authors (9, 10). In the *Corynebacterium* genome, the genes *ask* and *asd* lie adjacent, oriented in the same direction and separated by a 23-bp intercistronic region (5, 6, 9, 10). Analysis of the *ask* sequence identified an internal ribosome-binding site and translation initiation codon corresponding to the N-terminal region of the β subunit of the *Bacillus subtilis* AKII. The *Bacillus lysC* gene encodes the lysine-sensitive AKII, one of three AK isoenzymes, which comprises two nonidentical polypeptides, a 44-kDa α subunit and an 18-kDa β subunit (15, 16). Both AK subunits are encoded by the gene *lysC* (1–4). These observations raised the possibility that the *Corynebacterium ask* gene may also encode two polypeptides, a full-length α subunit and a truncated β subunit, similar to the *B. subtilis lysC* gene (5, 6, 9, 10). Here we report the results of a detailed analysis of the expression of the *C. flavum* N13 *ask-asd* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. All bacterial strains and plasmids used in this study are listed in Table 1. *C. flavum* N13 was provided by Roquette Frères, Lestrem, France. LB (19) or MB (10 g of tryptone, 4 g of yeast extract, 4 g of Soytone, and 5 g of NaCl per liter) was used as a rich growth medium. Minimal medium for *Corynebacterium* spp., MCGC, has been described previously (24). Ampicillin or kanamycin (50 μg/ml) and amino acids (200 mg/liter) were added when appropriate. *C. flavum* N13 transformants were regenerated on SSBK agar plates (47 g of brain heart infusion [Difco], 30 g of sorbitol, 10 g of sucrose, 14 g of agar, and 15 mg of kanamycin per liter). Plasmid pMT1 was derived from plasmid pWST1 (12) by deleting nonessential regions as follows. A 3.6-kb fragment between the *Sal*I site and an *Hae*III site in the pBD10 region was deleted by partial restriction with *Hae*III followed by complete digestion with *Sal*I; following treatment with T4 polymerase, the DNA was treated with T4 DNA ligase and transformed into *C. glutamicum* E12. The smallest Kan^r plasmid identified (6.8 kb) was subsequently digested with *Eco*RV and *Aat*II, treated with T4 DNA polymerase, and religated. Plasmid pMT1 (6.6 kb) was identified as having the 260-bp *Eco*RV-*Aat*II fragment originally derived from pBR322 deleted.

DNA manipulations. Routine DNA manipulations were performed as described by Sambrook et al. (19). DNA sequence analysis was performed by the dideoxy chain termination method with Sequenase II kits (United States Biochemical). *Corynebacterium* strains were transformed by polyethylene glycol-mediated DNA uptake of spheroplasts (25) or by electroporation. For the latter procedure, *Corynebacterium* cells were grown in MB to an A_{600} of 0.8, 2.5 mg of ampicillin per liter was added, and the cells were incu-

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant genotype or description	Source or reference
C. glutamicum		
AS019	Spontaneous rifampin-resistant mutant of ATCC 13059	25
AS019E12	Restriction-deficient variant of AS019	8
C. flavum N13	Aminoethyl cysteine-resistant, lysine-producing strain	Roquette Frères
E. coli		
DH5α	F⁻ ϕ80d *lac*ΔM15 (*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*	Bethesda Research Laboratories
JM101	Δ(*pro-lac*) *thi supE* F′ (*traD36 proAB lacI lacZ*ΔM15)	23
5080	*hisA323* Δ(*bioH-asd*)*29* λ⁻	*E. coli* Genetic Stock Center
K38/pGP1-2		22
Plasmids		
pWST1	*Corynebacterium*/*E. coli* shuttle vector, Kan^r Amp^r	12
pT7-7	T7 expression vector	22
pGEX2T	GST fusion vector	21
pASDF11	pUC18 containing the *C. flavum* N13 *ask-asd* operon on a 2.9-kb *Sac*I chromosomal fragment	5, 6

[a] All other plasmids used are illustrated in Fig. 1 and described in the text.

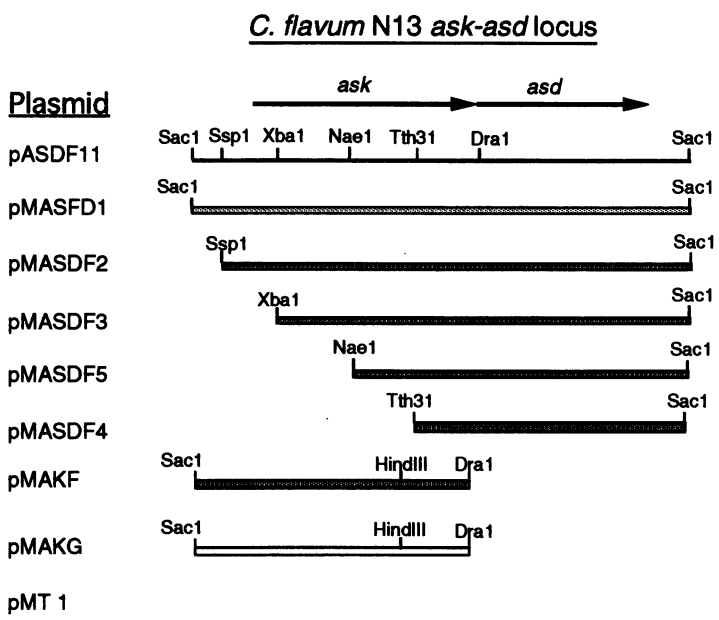
bated for an additional 2 h at 30°C with agitation. The cells were harvested by centrifugation, washed twice with 0.5 volume of sterile 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2)–5% glycerol, and resuspended in 0.02 volume of 5 mM HEPES (pH 7.2)–16% glycerol. Cells (0.1 ml) and DNA were mixed, transferred into a 0.2-cm electroporation cuvette, and pulsed (2.5 kV, 25 μF, 200 ohms) with a Bio-Rad Gene Pulse apparatus. Following electroporation, 0.9 ml of SSB medium (brain heart infusion medium supplemented with 30 g of sorbitol and 10 g of sucrose per liter) was added, and the cells were incubated for 1.5 h at 30°C and subsequently plated on SSBK plates. When introducing recombinant plasmid DNA purified from *Escherichia coli* into *Corynebacterium* strains, the DNA was passed through the restriction-deficient strain ASO19E12 (8) before being used to transform other strains.

RNA analysis. RNA was extracted from *C. flavum* N13 cells grown in MCGC medium by the glass beads-hot phenol extraction procedure (7), and S1 nuclease protection analysis was performed as described by Peoples et al. (18). For promoter P1, 10 μg of pASDF11 DNA was digested to completion with *Rsa*I, and the 5′ phosphate was removed by treatment with calf intestinal phosphatase. 5′-End labeling was achieved by using [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham) and T4 polynucleotide kinase. After labeling, the DNA was digested to completion with *Sac*I, the fragments were separated on a 5% polyacrylamide gel, and the 380-bp probe fragment was excised from the gel and eluted into TE buffer. For promoter P2, pASDF11 was first digested with *Sty*I, and after similar labeling with T4 polynucleotide kinase, a second digestion was made with *Pst*I. Subsequently, the 370-bp probe was recovered as described above. Maxam-Gilbert chemical sequencing was carried out with a kit from DuPont/New England Nuclear.

Preparation and analysis of proteins. *Corynebacterium* cells were grown in 800 ml of MCGC medium with appropriate amino acid and antibiotic supplementation at 30°C to the mid-exponential phase (A_{600} = 1.2 to 1.5) and harvested by centrifugation (4,000 rpm, 15 min, 4°C) in a Sorvall GSA rotor. After the cell pellets were washed with 100 ml of 50 mM HEPES (pH 7.8)–0.2 M KCl, crude extracts were prepared by resuspending the cell pellets in 2 ml of assay buffer (100 mM HEPES [pH 7.8], 400 mM KCl, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 5% glycerol). For disruption, cells were passed twice through a French pressure cell (10,000 to 11,000 lb/in^2) and sonicated for 2 min (20 W with 50% pulsing). Cell debris was subsequently removed by centrifugation. Protein concentrations were determined by the Bradford assay with bovine plasma albumin standard 11 (Bio-Rad) as a standard.

ASD (EC 1.2.1.11) was assayed as follows. To 950 μl of assay solution (55 mM sodium arsenate, 300 mM KCl, 100 mM HEPES [pH 7.8], 1.1 mM NADP) was added 10 to 50 μl of enzyme extract; the reaction was initiated by addition of DL-aspartate-semialdehyde to a final concentration of 3 mM. Enzyme activity was monitored by the increase in A_{340} due to the reduction of NADP over a period of 60 s. Nonspecific activity was determined in the absence of substrate aspartate-semialdehyde and subtracted from the values for reactions containing aspartate-semialdehyde, and the ASD specific activity (nanomoles of NADPH formed per milligram per minute) was calculated by using a molar extinction coefficient of 6220.

AK (EC 2.7.2.4) activity was measured by determining the rate of formation of the stable aspartyl-hydroxamate (14). Proteins were precipitated from the crude extract by the addition of 5 volumes of saturated ammonium sulfate, and the precipitate was collected by centrifugation and resuspended in 1 ml of assay buffer. This protein solution was used in the enzyme assays described below. The assay mixture contained 100 mM HEPES (pH 7.8), 400 mM KCl, 12 mM $MgCl_2$, 500 mM hydroxylamine, 10 mM ATP, and 15 mM L-aspartate (pH adjusted to 7.6) in a total volume of 0.5 ml. Crude protein extract was added, the reaction mixture was incubated for 30 or 60 min at 30°C, and the reaction was terminated by the addition of 0.75 ml of ferric chloride solution (10% $FeCl_3 \cdot 6H_2O$, 3.3% trichloroacetic acid, 0.7 N HCl). After centrifugation for 5 min to remove protein debris, the A_{540} of the supernatant was measured in a diode array spectrophotometer (Hewlett Packard, model 8451A). Background activity measured in the absence of added substrate aspartate was subtracted, and AK specific activity is reported as nanomoles of aspartyl-hydroxymate formed



Plasmid	Specific Activity (nmole/mg-min) AK	ASD
pASDF11	22.6	805
pMASFD1	17.9	750
pMASDF2	2.8	309
pMASDF3	ND	87
pMASDF5	2.5	41
pMASDF4	18.4	ND
pMAKF	24.4	ND
pMAKG	2.5	35
pMT 1		

FIG. 1. Restriction map of the 2.9-kb *Sac*I fragment of *C. flavum* N13, which complements the *E. coli asd* mutant, cloned in plasmid pASD11 (5, 6) is shown, with the locations of the two open reading frames indicated. Restriction sites used to construct the subclones in plasmid pMT1 (see Materials and Methods) are also shown, with the origin of each of the specific fragments subcloned indicated by solid vertical bars. The restriction map of plasmid pMAKG (pMT1 containing the *ask* gene from wild-type *C. glutamicum* AS019) is also shown. The results of AK and ASD enzyme assays for recombinant *C. flavum* N13 strains containing each of the pM series of plasmids are presented. The specific activities reported are the averages for assays performed in duplicate from a minimum of three independent cultures. ND, not determined.

per milligram per minute, with a molar extinction coefficient of 600.

Expression of *C. flavum* N13 *ask*-encoded polypeptides in *E. coli*. Recombinant plasmids containing the *C. flavum* N13 *ask* gene sequences under the control of the T7 promoter were constructed in the vector pT7-7 (22). Expression of *ask* gene fragments from the T7 promoter in *E. coli* K38/pGP1-2 was performed essentially as described by Tabor and Richardson (22). Following induction of the T7 polymerase and incubation in the presence of rifampin (200 μg/ml) at 42°C, the cells were incubated for 20 min at 30°C. Then, 2 μl of L-[^{35}S]methionine (specific activity, 1,000 Ci/mol; Amersham) was added, and the cells were incubated at 30°C for 10 min before being harvested, resuspended in 1× sodium dodecyl sulfate (SDS) cracking buffer (Bio-Rad), and boiled for 2 min. Samples were fractionated on a 4 to 20% polyacrylamide gradient gel containing SDS (Bio-Rad), treated with Amplify (Amersham), and autoradiographed. Methyl-^{14}C-labeled protein size markers (lysozyme, 14.3 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin, 69 kDa; and phosphorylase *b*, 97.4 kDa) were obtained from Amersham.

Western blot analysis of *C. flavum* N13 *ask*-encoded polypeptides. The 536-bp *Nae*I-*Dra*I-generated fragment encoding the C-terminal 179 residues of the *C. glutamicum* AS019 AK (Fig. 1) was inserted into the vector pGEX2T (21) to create an in-frame fusion to glutathione-*S*-transferase (GST). The construction was confirmed by nucleotide sequence analysis. *E. coli* JM101 cells harboring the recombinant plasmid were grown in LB containing ampicillin and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the 46-kDa GST-AK fusion protein was purified and cleaved with thrombin as described by Smith and Johnson (21) to recover the AK polypeptide. The purified protein was subsequently used to immunize BALB/c mice with alum (Pierce) as an adjuvant. Anti-AK serum was collected and used without further purification.

Proteins from crude extracts of *C. flavum* N13 were fractionated on 4 to 20% polyacrylamide–SDS gels (Bio-Rad) and transferred to nitrocellulose filters (0.4 μm; BA 85; Schleicher & Schuell) with a Mini-Transblot electrophoretic transfer cell (Bio-Rad). Nitrocellulose filters were blocked with TBS (10 mM Tris [pH 8.0], 150 mM NaCl) containing 3% nonfat dry milk and 0.05% Tween-20, washed with TBS, and incubated with anti-AK serum diluted 1:200 in TBS. The filters were washed in TBS, and bound antibodies were visualized with a goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate detection system (Bio-Rad).

RESULTS AND DISCUSSION

Location of promoters in the *C. flavum* N13 *ask-asd* locus. The proximity and common orientation of the *Corynebacterium ask* and *asd* genes (5, 6, 9, 10), illustrated in Fig. 1, suggested that the two genes may be expressed coordinately as an operon. In order to investigate the expression of these genes in *C. flavum* N13, a series of plasmids, pMASDF1 through pMASDF5 and pMAKF (Fig. 1), were constructed in the vector pMT1. Each of these constructs was reintroduced into *C. flavum* N13, and the AK and ASD enzyme activities were measured. A seven- to ninefold increase in AK activity was observed in *C. flavum* N13 harboring pMASDF1, pMASDF2, or pMAKF over the activity in the vector pMT1 (Fig. 1). A similar level of overproduction of AK was observed with plasmid pMAKG, which contains the wild-type *C. glutamicum* AS019 *ask* gene. Cloned fragments which do not contain the entire AK sequence, pMASDF3 and pMASDF4, exhibit no increased AK activity relative to

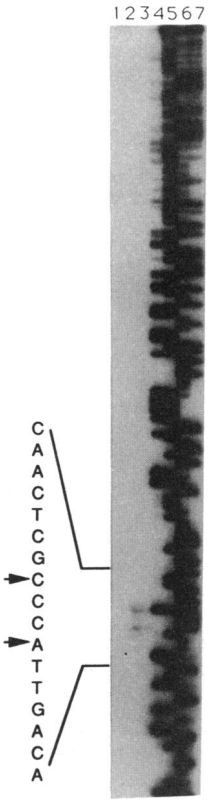


FIG. 2. S1 nuclease mapping of *ask*P1 transcription start. The results of S1 nuclease mapping studies of the transcription initiation sites for *ask*P1 are shown. Experiments were performed as described in Materials and Methods. In this autoradiograph of the S1 map of promoter *ask*P1, the lanes contained the following: lane 1, S1-digested sample containing probe plus 30 μg of yeast tRNA; lane 2, S1-digested sample containing probe plus 30 μg of *C. flavum* N13 RNA; lane 3, S1 digest of sample containing probe plus 15 μg of *C. flavum* N13 RNA and 15 μg of yeast tRNA; lanes 4 to 7, Maxam and Gilbert sequencing reactions: lane 4, G reaction; lane 5, G+A reaction; lane 6, C+T reaction; lane 7, C reaction. The DNA sequence in the region of the transcription start site is shown, and the transcription start sites are indicated by arrows.

control cultures harboring the parental vector pMT1. As deletion up to the *Ssp*I site (pMASDF2) had no significant effect on AK expression, the *ask* promoter was localized to within the 184 bp immediately upstream of the *ask* structural gene.

S1 nuclease mapping was used to locate the transcription start sites for *ask*P1 as described in Materials and Methods, and the results are shown in Fig. 2. For *ask*P1, these results reveal *C. flavum* N13 S1-protected RNA fragments (Fig. 2, lanes 2 and 3) which are absent from the control reaction containing yeast tRNA (Fig. 2, lane 1). These data identify transcription start sites for the *C. flavum* N13 *ask*P1 at 38 bp (G) and 35 bp (T) upstream of the predicted translation start site of the *C. flavum* N13 *ask* gene (Fig. 3). Unlike the *B. subtilis lysC* gene (4, 13), no attenuator-like sequences were identified in the *C. flavum* N13 *ask*P1 region.

A 23-fold increase in ASD activity was observed in crude extracts of *C. flavum* N13 harboring pMASDF1 over that in cells harboring pMT1 (Fig. 1). As for *ask* gene expression, deletion to the *Ssp*I site (pMASDF2) had no effect on the expression of the *asd*-encoded activity. Plasmid pMASDF3, which has the entire *ask* upstream region and 150 bp of the N-terminal coding region of *ask* deleted, increases ASD activity 8.8-fold. These data demonstrate that the two genes are expressed from promoter *ask*P1. Plasmid pMASDF5 increases ASD activity only twofold, and plasmid pMASDF4, which retains 256 bp upstream of the *asd* structural gene, has only background (chromosomal) levels of ASD activity. These data indicate that a second promoter, designated *ask*P2, is located between the *Xba*I and *Nae*I restriction sites within the *ask* structural gene. Additional evidence for this second promoter is provided by the overproduction of the ASKβ polypeptide in *C. flavum* N13 carrying plasmid pMASDF3 (see Fig. 5, lanes 4 and 5).

S1 mapping tentatively identified the transcription start site of *ask*P2 47 bp upstream of the GTG translation initiation codon of the ASK β subunit (data not shown). Although reproducible, the signals in these experiments were very weak, possibly due partly to hybridization to the full-length mRNA1. Alternatively, mRNA2 may not be expressed appreciably under the growth conditions used when *ask*P1 is active. The sequence of the region of the *ask* gene containing the putative translation start site and upstream region of AKβ is shown in Fig. 3. A mutation in the *C. glutamicum* ATCC 13032 AK (a change of C to A at nucleotide 1118 of the reported DNA sequence results in the S301Y mutation) which confers a loss of sensitivity to feedback inhibition by threonine and lysine and a concomitant increase in expression of the downstream ASD activity has been reported (10). Comparison with *E. coli* promoter sequences suggested (10) that the latter effect reflects conversion from a weak to a strong promoter. We do not agree, since the region is not significant in wild-type expression of ASD activity (Fig. 1); we have commented previously on the differences between *E. coli* and *Corynebacterium* promoters (5).

Analysis of proteins expressed from *C. flavum* N13 *ask*. Amino acid sequence comparisons of *Corynebacterium* AK with *Bacillus* AK11 indicated that the *Corynebacterium ask* gene may also encode two subunits from the same reading frame (5, 6, 9, 10). To investigate the expression of the *C. flavum* N13 *ask* gene in the heterologous *E. coli* genetic background, either the complete *ask* coding sequence (*Sph*I-*Dra*I fragment) or the C-terminal *ask* sequence (*Hae*II-*Dra*I fragment) was inserted into the *E. coli* expression vector pT7-7 (22). The resulting plasmids, pT7AK1 (full length) and pT7AK2 (C-terminal part), are illustrated in Fig. 4a. Each of these plasmids and the vector pT7-7 were introduced into *E. coli* K38/pGP1-2, and [^{35}S]methionine labeling studies were performed as described in Materials and Methods. Plasmid pT7AK1 (Fig. 4b, lane 3) encodes two polypeptides with approximate M_rs of 48,000 and 18,000 which are not present in the control sample (Fig. 4b, lane 2). The M_r 34,000 band in lanes 3 and 4 is also present in the control lane 2 on longer exposure and represents the plasmid-encoded β-lactamase. Plasmid pT7AK2, which contains only the C-terminal portion of the *ask* gene under the control of the T7 promoter, does not encode the M_r 48,000 protein but still expresses the M_r 18,000 protein (Fig. 4b, lane 4).

These data demonstrate that in the heterologous *E. coli* genetic background, the *C. flavum* N13 *ask* gene expresses two distinct proteins, the full-length M_r 48,000 polypeptide (44.8 kDa predicted from the gene sequence) and a second M_r 18,000 polypeptide (18.5 kDa predicted from the gene sequence), the latter localized to the C-terminal region of the *C. flavum* N13 *ask* structural gene. Although a 30-min exposure of the autoradiogram was sufficient to demonstrate a distinct M_r 18,000 band, overnight exposure was required

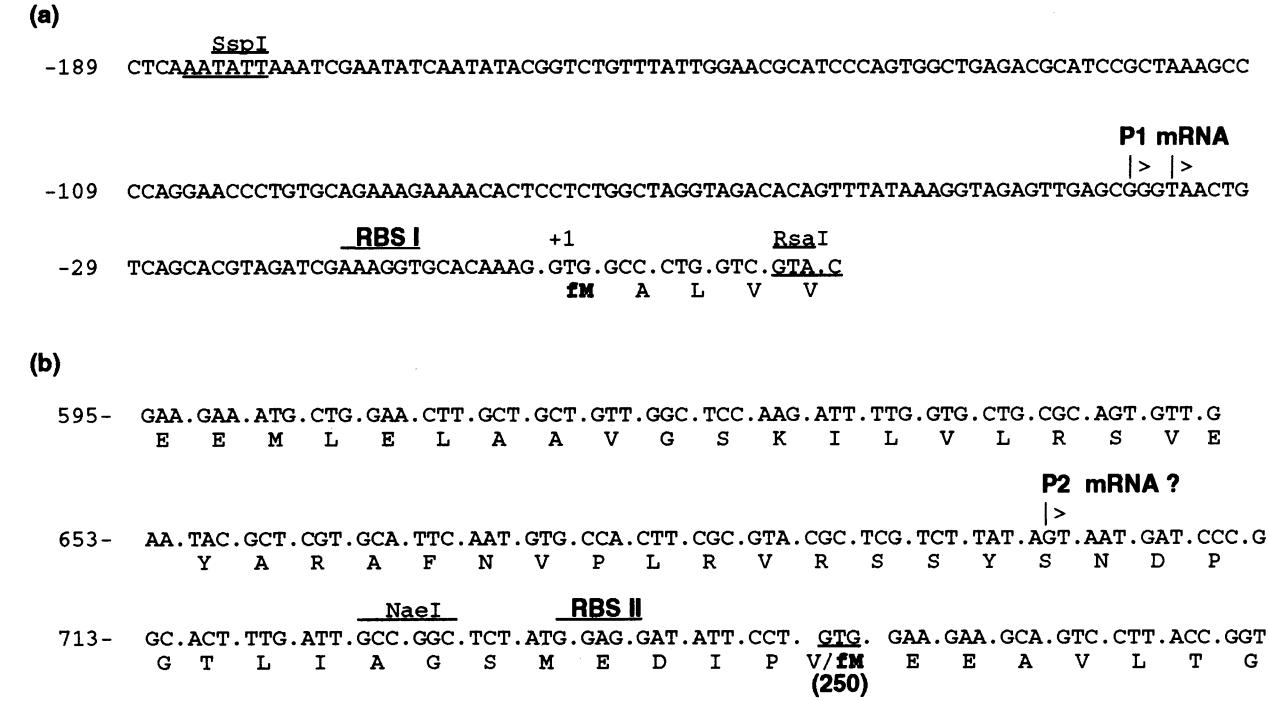


FIG. 3. Nucleotide sequence of the regions of the *C. flavum ask* locus containing promoters *ask*P1 (a) and *ask*P2 (b). The transcription start sites for *ask*P1 are indicated, and the putative transcription start for *ask*P2 is shown. Potential ribosome-binding regions (RBS) for the AKα and AKβ polypeptides are overlined.

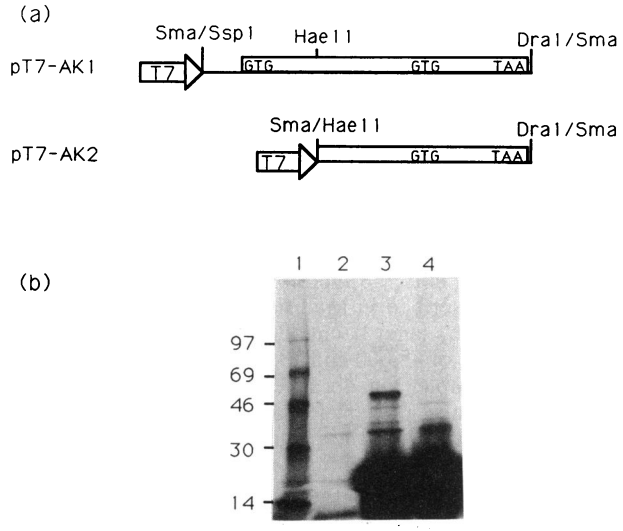


FIG. 4. Expression of *C. flavum* N13 *ask*-encoded polypeptides in *E. coli*. (a) Restriction maps of plasmids pT7AK1 and pT7AK2 used for this analysis. (b) Autoradiograph of SDS-PAGE (4 to 20% polyacrylamide gradient gel) used to analyze ^{35}S-labeled polypeptides expressed by pT7AK1 and pT7AK2. Samples were prepared as described in Materials and Methods. Lanes: 1, ^{14}C-labeled size markers (in kilodaltons); 2, 20 μl of extract prepared from cells containing the vector pT7-7; 3, 20 μl of extract prepared from cells containing plasmid pT7AK1; 4, 20 μl of extract prepared from cells containing plasmid pT7AK2. Shorter exposures of the gel clearly identified the M_r 18,000 signal as a unique band.

to visualize the M_r 48,000 protein, resulting in the overexposure of the M_r 18,000 signal seen in Fig. 4.

To examine the expression of the postulated AK α and β subunits in *C. flavum*, murine polyclonal antibodies were generated to the C-terminal 179 amino acid residues of the *C. glutamicum ask* gene product as described in Materials and Methods. Crude extracts from *C. flavum* N13 cells harboring plasmid pMT1, pMASDF1, or pMASDF3 (Fig. 1) were prepared, and the resulting proteins were size fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to nitrocellulose paper and probed with the anti-AK C terminus antibodies. The antibodies identified two protein bands with approximate M_rs of 47,000 and 19,000, expressed from *C. flavum* N13 harboring the parental vector pMT1 (Fig. 5B, lanes 2 and 8), both of which are overproduced in cells harboring pMASDF1, which contains the cloned *C. flavum* N13 *ask* gene (Fig. 5B, lanes 3 and 6). Cells harboring plasmid pMASDF3, which contains a cloned *C. flavum* N13 *ask* gene fragment minus promoter *ask*P1 and the initial 150 bp of the AKα N-terminal coding region, expressed only the background level of AKα encoded by the chromosome. The 18.5-kDa β subunit, however, is still overproduced from pMASDF3 (Fig. 5B, lanes 4 and 5). Unlike the vast overexpression of AKβ observed in *E. coli* (Fig. 4), approximately equivalent amounts of AKα and AKβ are detected in *C. glutamicum* crude extracts for both the chromosome- and plasmid-encoded gene products. The fact that the antibodies detect both polypeptides clearly demonstrates that AKα and AKβ are translated from the same reading frame, as predicted previously (5, 6, 9, 10).

In both the homologous (Fig. 5) and heterologous *E. coli* (Fig. 4) genetic backgrounds, the *C. flavum* N13 *ask* gene expresses two polypeptides (44.7 and 18.5 kDa) corresponding to the AKα and AKβ subunits, respectively, predicted

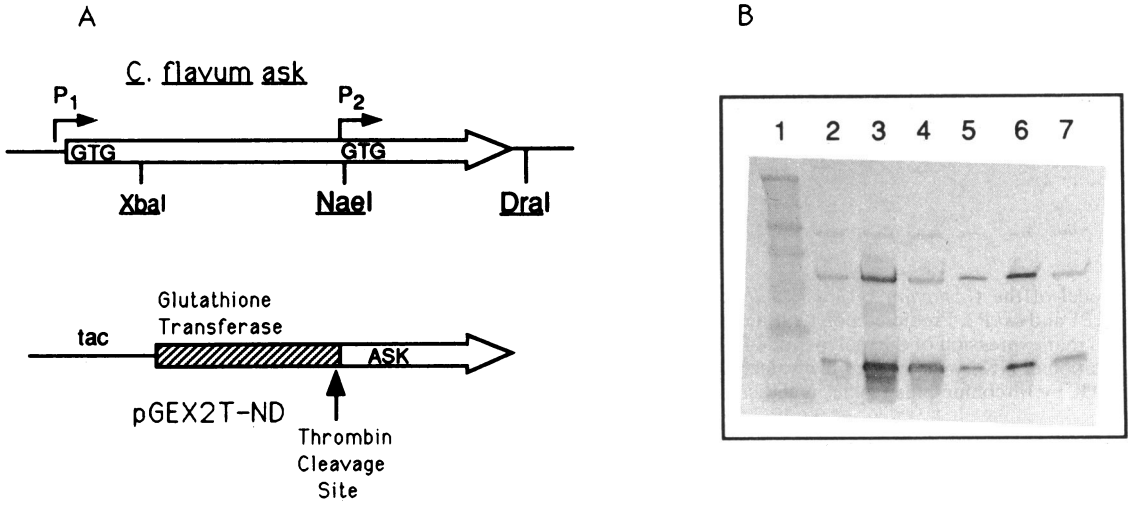


FIG. 5. (A) Graphic depiction of *C. flavum* N13 *ask* gene (open box) including relative positions of promoters P_1 and P_{24} GTG translation initiation sites for the AK α and β subunits, and relative restriction enzyme cleavage sites. Hybrid gene contained in recombinant plasmid pGEX2T-ND (constructed as described in text) encoding glutathione-S-transferase (shaded box)/*C. flavum* AK fusion protein under the control of the *E. coli tac* promoter is depicted below. The relative position of the thrombin proteolytic cleavage site used to cleave carboxy-terminal AK protein fragment from the hybrid protein is indicated. (B) Western blot analysis of AK polypeptides in *C. flavum* N13 harboring pMT1 (lanes 2 and 7; 30 μg of protein), pMASDF1 (lanes 3 and 6; 30 and 3 μg of protein, respectively), and pMASDF3 (lanes 4 and 5; 30 and 3 μg, respectively). Size standards (Bethesda Research Laboratories) in lane 1 were myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

from the gene sequence. SDS-PAGE analysis of partially purified material has been interpreted similarly (10). Deletion of the upstream and N-terminal coding regions of the *ask* gene results in overexpression of the AKβ polypeptide (plasmid pMASDF3, Fig. 4 and 5), yet disrupts the plasmid-encoded expression of AK activity (Fig. 1). These results clearly demonstrate that AKα is required for enzymatic activity and that AKβ is a separate translation product of the *C. flavum* N13 *ask* gene, not derived by proteolysis of the larger AK α subunit. In the heterologous *E. coli* genetic background, with a bacteriophage T7 promoter used to express the *C. flavum* N13 *ask* gene, a vast overexpression of AKβ relative to AKα is observed (Fig. 4). In *C. flavum* N13, however, the levels of the AKα and AKβ proteins are the same (Fig. 5). Similar findings have been reported for the expression of the *B. subtilis lysC* gene (4).

Identification of AEC^r mutation(s) in the *C. flavum* N13 *ask* gene. In order to determine the mutation responsible for the AEC^r mutation(s) in the *C. flavum* N13 *ask* gene, we isolated the *ask-asd* locus from *C. glutamicum* AS019 on a 2.7-kb *Sac*I fragment as described for the *C. flavum ask-asd* locus (5). The restriction map of the *C. glutamicum* AS019 *ask* gene cloned in the vector pMT1 is shown in Fig. 1 (plasmid pMAKG). The complete nucleotide sequence of the insert in plasmid pMAKG was determined as described in Materials and Methods (4a) and compared with the *C. flavum* N13 (6) and *C. glutamicum* ATCC 13032 (10) *ask* sequences. The two independently determined *C. glutamicum ask* sequences are identical. A total of 18 base pair differences were observed between the *C. glutamicum* wild-type and the *C. flavum* N13 *ask* structural genes. Only two of the mutations, T to G at nucleotide 1317 and G to A at nucleotide 1402, alter the AK primary structure, resulting in S317A and G345D mutations, respectively.

By starting with plasmids pMAKF and pMAKG and utilizing the unique *Hin*dIII site (Fig. 1), hybrid genes between the wild-type *C. glutamicum* AS019 (S317 and G345) and desensitized *C. flavum* N13 (A317 and D345) *ask* genes were constructed in vector pMT1. These plasmids were introduced into wild-type *C. glutamicum* AS019 for analysis of the encoded AK activity. The sensitivity of the AK activity encoded by these constructs to lysine-threonine feedback inhibition is shown in Table 2. From these experiments, we conclude that only the mutation G345D is functionally important to the decreased sensitivity of *C. flavum* N13 AK activity to concerted feedback inhibition by lysine and threonine. Both AEC^r mutations so far identified in *Corynebacterium* AK are due to amino acid substitutions in the AKβ region of the protein, whereas *Bacillus* AEC^r mutations are found in the untranslated region of the *lysC* operon (13).

Conclusion. The role of the *Bacillus* AKII β subunit is not presently clear, as the AK α subunit has apparently normal catalytic and regulatory properties both in vitro (15) and in vivo (4) in *E. coli*. Yet the similar AK architecture in

TABLE 2. Identification of AEC^r mutations in *C. flavum* N13 *ask* gene[a]

Plasmid	Relevant amino acids	AK sp act (nmol/min/mg of protein) at threonine plus lysine concn:		
		0	1 mM	10 mM
pMAKG	S317, G345	24.4	9.1	2.3
pMAKF	A317, D345	22.6	16.0	6.4
pMAKF-G	A317, G345	18.3	7.1	2.6
pMAKG-F	S317, D345	19.1	14.9	6.3

[a] AK specific activity was determined in *C. glutamicum* AS019 containing each of the recombinant plasmids at different threonine plus lysine concentrations. Values are the averages for three independent assays.

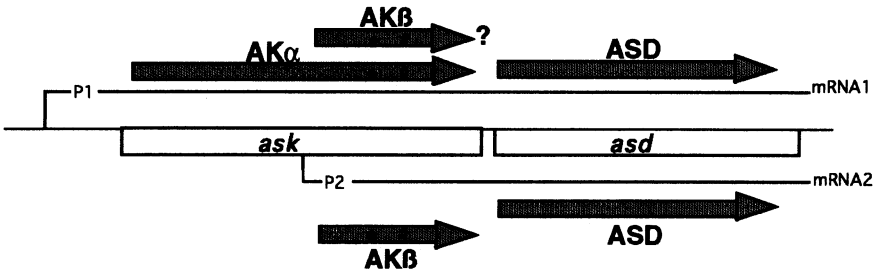


FIG. 6. Model of the *C. flavum* N13 *ask-asd* operon, showing the structural genes (open boxes) and mRNA transcripts initiated at promoters *ask*P1 and *ask*P2. Predicted polypeptides encoded by the two transcripts are shown as shaded boxes. AKβ shown with a question mark indicates that expression of this polypeptide from mRNA1 has not been confirmed. The location of the G345D mutation, which reduces feedback inhibition of AK by threonine plus lysine, is indicated by *, and the location of the S301Y mutation (10), which eliminates feedback inhibition of AK by threonine plus lysine, is indicated by #.

Corynebacterium spp. (Fig. 6) strongly suggests that the β subunit is not superfluous and must play a role in AK function. In *Corynebacterium* spp., experimental evidence points to a role in the feedback regulation of AK activity. Both desensitized *Corynebacterium* AKs thus far characterized are the result of single missense mutations, S301Y (11) and G345D, present in both the AKα and AKβ polypeptides (Fig. 6). In both cases, overproduction of the $AK\beta^{DR}$ in wild-type *C. glutamicum* is sufficient to confer the AEC^r phenotype (4a, 10), demonstrating that the AKα and $AK\beta^{DR}$ subunits interact in vivo to form hybrid AKα-$AK\beta^{DR}$ proteins which are insensitive to feedback inhibition. In this regard, it is worth noting that the ASKβ amino acid sequence aligns with the interdomain region of the bifunctional AK-homoserine dehydrogenase I isoenzymes from *Serratia marcescens* and *E. coli*, which has also been implicated in feedback inhibition (reference 18 and references therein). At present, it is not known whether mRNA2 is expressed in *Corynebacterium* spp. when transcription is proceeding from *ask*P1, what fraction of AKβ expression is mediated by mRNA1 versus mRNA2, and how the balance of AKα and AKβ expression is maintained in *Corynebacterium* spp. (Fig. 6). The physiological significance of establishing two independent transcripts is also unknown; however, by evolving an *ask-asd* operon with two promoters, *Corynebacterium* spp. are clearly capable of separately controlling the expression of AKβ and ASD.

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