

Targeted disruption of homoserine dehydrogenase gene and its effect on cephamycin C production in *Streptomyces clavuligerus*

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Abstract The aspartate pathway of *Streptomyces clavuligerus* is an important primary metabolic pathway which provides substrates for β -lactam synthesis. In this study, the *hom* gene which encodes homoserine dehydrogenase was cloned from the cephamycin C producer *S. clavuligerus* NRRL 3585 and characterized. The fully sequenced open reading frame encodes 433 amino acids with a deduced M_r of 44.9 kDa. The gene was heterologously expressed in the auxotroph mutant *Escherichia coli* CGSC 5075 and the recombinant protein was purified. The cloned gene was used to construct a plasmid containing a *hom* disruption cassette which was then transformed into *S. clavuligerus*. A *hom* mutant of *S. clavuligerus* was obtained by insertional inactivation via double crossover, and the effect of *hom* gene disruption on cephamycin C yield was investigated by comparing antibiotic levels in culture broths of this mutant and in the parental strain. Disruption of *hom* gene resulted in up to 4.3-fold and twofold increases in intracellular free L-lysine concentration and specific cephamycin C production, respectively, during stationary phase in chemically defined medium.

Keywords *Streptomyces clavuligerus* · Homoserine dehydrogenase · Cephamycin C · Aspartate pathway · Gene disruption

Introduction

β -lactams constitute a very important class of antibiotics, and improvement of β -lactam yields in the producer microorganisms is of great economical importance. For this purpose, there have been many metabolic engineering attempts dealing with the β -lactam biosynthetic genes and gene clusters as reviewed by Thykaer and Nielsen [27].

Streptomyces clavuligerus is the producer of the medically important β -lactam antibiotics, including cephamycin C and the potent β -lactamase inhibitor clavulanic acid. The aspartate pathway of *S. clavuligerus* is an important primary metabolic pathway which provides substrates for β -lactam synthesis (Fig. 1). The carbon flow through the L-lysine-specific branch of aspartate pathway is a rate-limiting step for the formation of cephamycin C [12, 15, 19]. Since several branch points exist in the aspartate pathway, the flow of carbon from aspartate through L-lysine to α -aminoadipyl side chain of *cephamycins* might be controlled by regulatory mechanisms operating at the initial and branching steps of pathway [11, 19]. Formation of L-homoserine from aspartate- β -semialdehyde is the first step of the other branch of the pathway leading to L-threonine, L-isoleucine and L-methionine syntheses and catalyzed by homoserine dehydrogenase (HSD, EC 1.1.1.3). The gene encoding homoserine dehydrogenase (*hom*) has been cloned and sequenced from diverse bacteria including *Corynebacterium glutamicum* [23], *Lactobacillus lactis* [14], *Bacillus subtilis* [22], *Bacillus* sp. ULM1 [16], *Pseudomonas aeruginosa* [5], *Methylobacillus glycogens* [21], *Methylobacillus flagellatus* [18], *Escherichia coli* and *Salmonella typhimurium* [6] and *Streptomyces* sp. NRRL 5331 [7].

Regulation of the activity or biosynthesis of HSD in *S. clavuligerus* might determine the availability of

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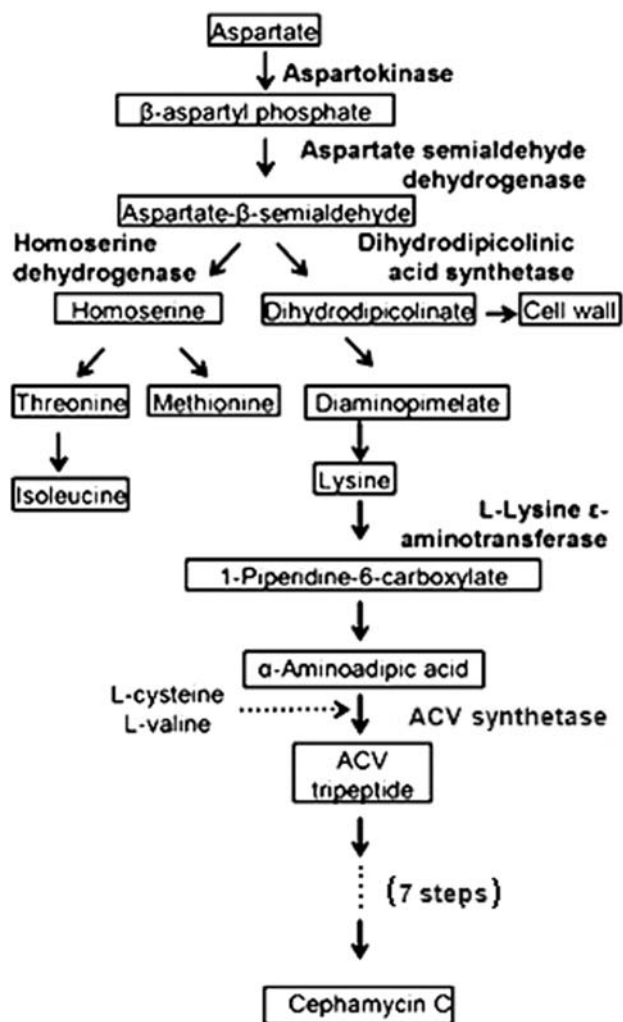


Fig. 1 Aspartate pathway in *S. clavuligerus* [19, 24]

aspartate- β -semialdehyde for the biosynthesis of diaminopimelate, L-lysine and α -amino adipic acid [19]. Such a regulation is expected to control the levels of L-threonine which together with L-lysine inhibits aspartokinase, the key enzyme in the control of carbon flow towards α -amino adipic acid. Hence, the absence of HSD activity in *S. clavuligerus* is expected to lead to an increase in cephamycin C production levels in two ways. Firstly, all the carbon coming from aspartate would be directed to L-lysine rather than being shared between the two branches; and secondly, concerted feedback inhibition of aspartokinase would be relieved.

In this paper, we describe cloning and characterization of the *hom* gene from *S. clavuligerus* NRRL 3585 and some properties of the HSD enzyme expressed in *E. coli*. The cloned gene was used to disrupt the *hom* gene in *S. clavuligerus* via cassette mutagenesis, and the effect of this disruption on cephamycin C yield was investigated.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* cultures were grown in either Luria Broth (LB) or on Luria agar plates at 37 °C. *E. coli* CGSC 5075 was grown on M9 medium [25] supplemented with L-methionine and L-threonine (50 μ g/ml, each).

Streptomyces clavuligerus was maintained on sporulation agar [10] supplemented with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (20 μ g/ml). For isolation of genomic or plasmid DNA and for protoplast preparation, seed culture media containing trypticase soy broth (TSB, Oxoid) supplemented with 0.5% (w/v) maltose were inoculated with either spore or mycelium stocks of *S. clavuligerus* and incubated at 28 °C on a rotary shaker (220 rpm) in baffled flasks for 24 h. About 5 ml of this seed culture was inoculated into 50 ml of 2:3 (v/v) mixture of TSB:YEME [10] supplemented with 0.3% (v/v) glycine and 3 mM MgCl_2 and incubated at 28 °C for 24 h. In the case of plasmid-containing cultures, ampicillin (100 μ g/ml, Sigma), kanamycin (25 μ g/ml for *E. coli* and 200 μ g/ml for *S. clavuligerus*, Sigma) or thiostrepton (50 μ g/ml, Sigma) was added into the medium. For antibiotic selection, *Streptomyces* colonies were replica plated on TSA (3 g TSB; 1.5 g agar per 100 ml) supplemented with 8 μ g/ml thiostrepton or 200 μ g/ml kanamycin. MM [10] containing glycerol and $(\text{NH}_4)_2\text{SO}_4$ was used to test auxotrophy in *S. clavuligerus*. For HSD and cephamycin C assay, 100 ml of chemically defined medium (CDM) [15] was inoculated with a mid-log phase seed culture to give an initial OD_{595} of 0.03–0.04, and the cells were grown for 96 h. MM and CDM were supplemented with L-methionine (50 μ g/ml) and L-threonine (50 μ g/ml) for growth of *S. clavuligerus* AK39.

DNA isolation and manipulations

Genomic DNA was isolated from *S. clavuligerus* as described by Keiser et al. [10]. Plasmid DNA was isolated using Plasmid Midi Kits (Qiagen). DNA fragments were isolated from agarose gels using the Quickgel Extraction Kits (Qiagen). Restriction enzyme digestion of DNA was carried out as specified by the manufacturers. PCR products were cloned into pGEM-T vector prior to cloning into the desired plasmid vector. DNA manipulations in *E. coli* were performed as described by Sambrook et al. [25]. *E. coli* plasmids that were used in construction of recombinant *S. clavuligerus* plasmids were propagated in *E. coli* ET12567 to avoid restriction barriers. Southern blot hybridization analysis was performed by standard procedures using AlkPhos Direct labeling and detection kit (Amersham).

Table 1 Bacterial strains and plasmids used in the study

Strains and plasmids	Description	Source or reference
Strains		
<i>S. clavuligerus</i>		
NRRL 3585	Wild type, cephamycin C and clavulanic acid producer	Prof. J. Piret, Northeastern University, USA
AK39	Null mutant of <i>S. clavuligerus</i> NRRL 3585, <i>hom::kan</i>	This study
<i>E. coli</i>		
CGSC 5075	K + thrA1015 metLM1005 lysC1004	<i>E. coli</i> Genetic Stock Center
ESS	β -lactam supersensitive <i>E. coli</i> strain	Prof. J. Piret, Northeastern University, USA
DH5- α	F ⁻ , general cloning host	<i>E. coli</i> Genetic Stock Center
ET12567	F ⁻ , <i>dam</i> 13::Tn9 <i>dcm</i> -6 <i>hsdM hsdR lacYI</i>	Prof. K. Chater, John Innes Centre, Colney, Norwich, UK
Plasmids		
pGEM-T	Amp ^R , <i>lacZ'</i>	Promega
pQE-30	Expression vector, Amp ^R	Qiagen
pK19	<i>E. coli</i> plasmid vector, Kan ^R	Dr. D. Pridmore Nestle Research Center, Lausanne, Switzerland [24]
pIJ486	<i>Streptomyces</i> plasmid vector, PIJ101 replicon, Thio ^R	Prof. K Chater, John Innes Centre, Colney, Norwich, UK [10]
pEBH1	pQE-30 derivative with <i>hom</i> cloned as <i>Bam</i> HI– <i>Hind</i> III fragment	This study
pEBHK	pEBH1 derivative with <i>kan</i> cloned into the <i>Sac</i> II site	This study
pAEHK	pIJ486 derivative with <i>hom::kan</i> cloned as <i>Bam</i> HI– <i>Hind</i> III fragment	This study

Transformation of *S. clavuligerus*

PEG-mediated protoplast transformation technique [10] with slight modifications was used to transform *S. clavuligerus*. Transformants were regenerated on R2YE medium [10] at 26 °C for 48 h, and then each plate was overlaid with soft nutrient agar [10] containing antibiotics.

Cloning of *S. clavuligerus hom* gene

A 1.3 kb DNA fragment containing the *hom* gene was amplified by PCR using the nucleotide primers 5'-AGGATCCATGATGCTGACGCGTCCG-3' and 5'-TAAGCTTTTACTCCCCTTCAACACG-3' and *S. clavuligerus* genomic DNA as a template. Primer design was based on homologous sequences from other known *hom* genes. The PCR amplification condition was as follows: 95 °C (5 min) and 30 cycles of 95 °C (1 min), 63 °C (1 min) and 72 °C (2 min). The amplified DNA was cloned into pGEM-T, digested with *Bam*HI and *Hind*III enzymes and inserted into *Bam*HI–*Hind*III digested pQE-30 expression vector. This allowed the expression of the gene under the control of T5 promoter and subsequent purification of 6×His-tagged protein. The resulting recombinant plasmid, designated pEBH1, was used to transform *E. coli* CGSC 5075 which lacks HSD activity. *hom* complementation was assessed on

M9 medium containing 100 µg/ml ampicillin in the absence of L-methionine and L-threonine.

Insertional inactivation of *hom* gene via double crossing over

Kanamycin resistance cassette was obtained from the plasmid pK19 by PCR amplification of *kan* gene with *Sac*II restriction site-tagged primers; 5'-GCCGCGGGAA CACGTAGAAAGCCAGT-3' and 5'-CCCCGCGGTCAG AAGAACTCGTCAAGA-3'. The resulting 1.1 kb *kan* gene was inserted into the 1.3 kb *hom* gene contained in plasmid pEBH1 at unique *Sac*II site giving rise to plasmid pEBHK. 2.4 kb *Bam*HI–*Hind*III fragment of pEBHK was ligated to *Bam*HI–*Hind*III digested plasmid pIJ486 which was then used for the transformation of *S. clavuligerus* protoplasts. Transformants selected for kanamycin resistance were replica plated on sporulation agar. Following five rounds of sporulation, Thio^S–Kan^R colonies were selected. Such cells were next tested on MM for methionine–threonine auxotrophy.

HSD extraction, purification and enzyme assay

In *E. coli* cells containing recombinant plasmid pEBH1, *hom* gene was expressed as follows: The cells were grown

in LB at 37 °C until OD₆₀₀ reached 0.4–0.5. Induction was performed by the addition of 1 mM isopropyl-L-thiogalactopyranoside (IPTG, Sigma), and the cells were grown for three more hours at 37 °C to reach an OD₆₀₀ of 1.5. Cells were centrifuged at 7,000g for 10 min (Sigma) and washed with cold sodium-buffer (50 mM, pH 7.5). The cell pellet was resuspended in 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0. The cells were lysed by ultrasonication (Ultrasonic Processor, Cole Parmer) and following centrifugation at 25,000g for 30 min, the pellet was discarded. Native His-tagged protein was purified using Ni-NTA agarose column (Qiagen) according to the manufacturer's instructions. Purity of the protein was visualized by SDS-PAGE [13] after Coomassie Blue staining of the gels. To test HSD activity in *S. clavuligerus*, cell-free extracts were prepared as mentioned above.

The HSD activity in both *E. coli* and *S. clavuligerus* cells was measured in the reverse reaction by determining the initial rate of increase of A₃₄₀ at room temperature. Standard assay mixture contained 0.2 ml of 500 mM Tris–HCl buffer containing 5 mM EDTA, pH 8.4; 0.1 ml of 100 mM L-homoserine, pH 7.0; 0.1 ml of 4 mM NADP solution, 0.5 ml distilled water and 0.1 ml of cell-free extract or purified enzyme. For crude extracts, absorbance at 340 nm was measured against a blank containing all the components except the substrate. Specific activity of HSD was expressed as micromoles of NADPH formed per min per mg of protein. Protein concentration was measured by the method of Bradford [4] with BSA as the standard.

Determination of cultural growth

Cultural growth was monitored by dry cell weight (DCW) determination. For this, a 5 ml of culture was passed through a cellulose nitrate membrane filter (0.2 µm pore size, Whatman) under vacuum. Filters were dried at 80 °C for 24 h and weighed. DCW values tabulated herein represented the mean of three independent measurements.

Bioassay of β-lactam antibiotics

β-lactam antibiotic bioassay was conducted by using agar plate diffusion method with *E. coli* ESS as the indicator organism [1]. Zones of inhibition were measured, and the amount of cephamycin C in samples was calculated by using the standard curve constructed with cephalosporin C (Sigma) as the standard. One unit of β-lactam produces an inhibition zone equivalent to that formed by 1 g of cephalosporin C.

Determination of intracellular-free L-lysine concentration

Cells were grown in CDM, and samples were collected at intervals during cultivation. Intracellular-free amino acids

were extracted as described by Betancort Rodriguez et al. [3]. Following automated pre-column derivatization with *o*-phthalaldehyde (OPA), amino acid analysis was performed by conventional reverse-phase HPLC (Shimadzu VP series) on a Novapac C18 column (Waters) followed by fluorescence detection. L-lysine concentration was expressed as micromoles of L-lysine extracted from 1 g wet weight of bacteria.

DNA sequencing and sequence analysis

DNA sequencing was performed by the dideoxy chain-termination method using an ABI Prism 377 instrument (Perkin–Elmer). The data were analyzed using the DNASTar software package (Madison, WI, USA). Deduced polypeptide sequences were compared with sequences in the GenBank database using BLAST. The GenBank accession no. for the sequence reported is AY802988.

Results and discussion

Cloning of the *hom* gene from *S. clavuligerus*

The cloning and sequencing showed that the *hom* coding region is 1,302 bp in length and encodes 433 amino acids with a predicted M_r of about 44.9 kDa. The predicted M_r is consistent with the observed M_r of most homoserine dehydrogenase enzymes reported in the GenBank. The G + C content of the nucleotide sequence is 72.1 mol%, well within the range of the reference values for *Streptomyces* DNA [29] and extremely biased towards codons containing G or C in the third position. This position-specific preference for G and C bases is characteristic of *Streptomyces* coding regions and thought to reflect the adaptations to available tRNA pools for the regulation of gene expression.

Comparison with the predicted amino acid sequences of the *hom* genes

The predicted amino acid sequence of *S. clavuligerus hom* gene was compared with those of other bacteria. The deduced amino acid sequence showed 89.8% identity to HSD from aminoethoxyvinylglycine-producing *Streptomyces* sp. NRRL 5331 [7]. Comparison of the sequence of *S. clavuligerus* with those of *S. avermitilis*, *S. coelicolor*, *C. glutamicum*, *B. subtilis*, *P. aeruginosa* and *M. glycogens* revealed identities of 89, 87.9, 54.4, 38.4, 36.6 and 31.1%, respectively.

Several well-conserved domains were found in the amino acid sequences compared. The motif at positions 13 to 18, G-X-G-X-X-G, surrounded by small hydrophobic amino acid residues is the characteristic of the

NAD(P)-binding site of HSDs [7, 21–23]. Like other HSDs, NAD(P), the cofactor of the enzyme, should bind this region. The most conserved region among the HSD sequences lies between the positions 199 and 228 in *S. clavuligerus* HSD, where 21 out of 29 amino acid residues are strictly conserved, suggesting the importance of this region in substrate binding or catalytic activity. Parsot and Cohen [22] showed that the amino acid sequences between positions 200 and 221 of *B. subtilis* are well conserved and suggested that this region could be involved in substrate binding or catalysis. The regulatory domains involved in feedback inhibition by L-threonine were postulated to reside in the C-terminal regions in *B. subtilis* [22], *C. glutamicum* [2], *M. flagellatus* [17] and *M. glycogens* [21]. The C-terminal region of the *S. clavuligerus* HSD show some homology with those of other HSD.

Expression of the *S. clavuligerus* *hom* gene in *E. coli* and the properties of the recombinant enzyme

When introduced into *E. coli* CGSC 5075 (*hom*[−]), pEBH1 restored the ability of the mutant to grow in the absence of L-methionine and L-threonine, suggesting that the cloned gene was functionally expressed in *E. coli*. Specific activities of the recombinant (in the presence of IPTG) and parental HSD were 0.019 and 0.013 μmol min^{−1} mg^{−1} of proteins in the cell-free extracts, respectively. Recombinant HSD was next purified, and its specific activity was shown to be about 70-fold higher than that of the crude extract of the recombinant organism. By performing SDS-PAGE, the molecular mass of the purified HSD expressed by *E. coli* CGSC 5075 was estimated as 45 kDa (data not shown),

almost equal to the value deduced from the nucleotide sequence.

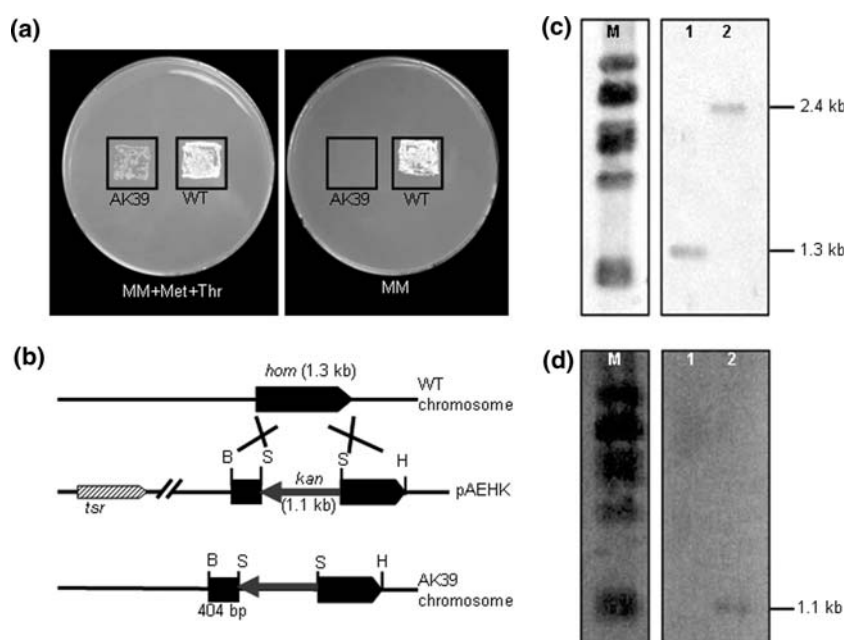
Disruption of the *hom* gene in *S. clavuligerus*

Streptomyces clavuligerus was transformed with the plasmid construct pAEHK containing a *hom* disruption cassette. After plasmid curing, kanamycin-resistant and thiostrepton-sensitive colonies were selected. One putative mutant that did not grow on MM, but grew when supplemented with L-methionine and L-threonine was selected and named AK39 (Fig. 2a). HSD activities of wild type and mutant *S. clavuligerus* were determined in CDM at time intervals, and no activity was detected in the *hom* mutant (data not shown). Insertional inactivation of *hom* via double crossing over (Fig. 2b) was verified by Southern blot hybridization by using the 1,300 bp *hom* (Fig. 2c) and 1,100 bp *kan* (Fig. 2d) gene fragments as the probes.

Effect of *hom* gene disruption on cephamycin C production

The effect of *hom* gene disruption on cephamycin C production was determined by growing the wild type and mutant *S. clavuligerus* in CDM (Fig. 3). *hom* gene disruption in AK39 resulted in a poorer growth in CDM supplemented with L-methionine and L-threonine; however, the organism produced 1.7-fold to twofold higher specific yields of cephamycin C between 48 and 72 h. It is to be noted that the entire experiment was repeated three times with similar results. Mendelovitz and Aharonowitz [19] studied the effects of amino acids of the aspartic acid pathway on cephamycin C production by *S. clavuligerus* NRRL

Fig. 2 a *hom*-Disrupted mutant AK39 exhibiting methionine and threonine auxothrophy. Identical volumes of the exponentially growing wild and mutant strains were streaked onto MM plates and incubated at 28 °C for 72 h. b Diagram of the *hom* gene disruption via double crossing over. Restriction enzyme sites are shown as *B* BamHI, *S* SacII, *H* HindIII. c *hom* Gene amplified by PCR from the chromosome of the wild type (lane 1) and mutant (lane 2) strains, blotted and hybridized to *hom* probe. d *Sac*II-digested chromosomal DNA of the wild type (lane 1) and the mutant (lane 2), blotted and hybridized to *kan* probe. Size marker (*M*) in c and d: *Pst*I-digested lambda DNA



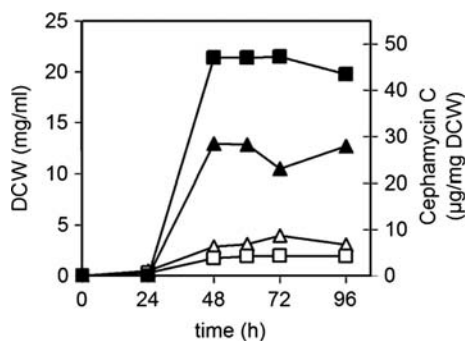


Fig. 3 Growth and specific cephamycin C production in CDM. *Open symbols* represent dry cell weight (DCW) and *closed symbols* represent specific cephamycin C production in wild type *S. clavuligerus* (triangles) and its *hom*-disrupted mutant AK39 (squares)

3585. It was reported that externally added DL-meso-diaminopimelate and L-lysine exerted precursor effect, increasing specific cephamycin C production by 1.9- and 1.7-fold, respectively. In our study, it was demonstrated that the intracellular free L-lysine levels increased by 1.6- to 4.3-fold upon *hom*-disruption (Fig. 4). Besides the positive impact of elevated L-lysine levels on cephamycin C production, the prevention of concerted feedback inhibition of aspartokinase in our threonine-minus mutant is also thought to make contribution to increased specific production of the antibiotic.

In *Streptomyces*, threonine biosynthetic genes *hom*, *thrC* (threonine synthase) and *thrB* (homoserine kinase) are clustered; *hom* and *thrC* being organized as a single transcriptional unit while *thrB* is transcribed as a monocistronic transcript under a strong promoter [7]. Hence, *hom* disruption in *S. clavuligerus* was not expected to have any polar effects other than that on *thrC*. It was therefore interesting to observe some pleiotropic effects of *hom* disruption in our mutant. Besides the aforementioned growth reduction, AK39 was defective in sporulation on MM supplemented with L-methionine and L-threonine, but not on sporulation agar. This finding accorded well with that of Gehring et al. [9] who reported that an L-methionine auxotroph of

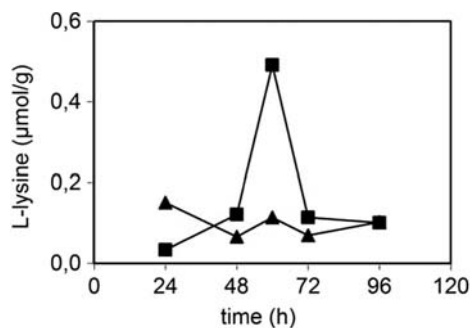


Fig. 4 Intracellular free L-lysine concentration in the cells of wild type (triangles) and *hom*-mutant AK39 (squares) grown in CDM

S. coelicolor had developmental defects which also increased secondary metabolite production. A functional *metH* (methionine synthase gene) was required for conversion of aerial hyphae into chains of spores in this mutant.

When AK39 was grown in CDM, the color of the culture fluid turned into faint red after 48 h. A comparison of the absorption spectra (200–800 nm) of 60 h culture supernatants of the wild and mutant strains grown in CDM revealed the existence of a major peak at 265 nm in the mutant (data not shown). Red pigment accumulation by the blocked mutant was reminiscent of characteristic red pigment formation in certain adenine auxotrophs of *Schizosacharomyces pombe* due to the accumulation of two intermediates (phosphoribosyl aminoimidazole and phosphoribosyl aminoimidazole carboxylate) of purine biosynthetic pathway which was relieved by intracellular homocysteine accumulation due to a methionine synthase mutation [8]. Yet, the structure of the colored substance produced by AK39 and its relation to *hom* mutation remains to be elucidated.

There have been only a limited number of studies focusing on improvement of cephamycin C yields by manipulating aspartate pathway in *S. clavuligerus*. Malmberg et al. [15] was able to elevate cephamycin C yields twofold to fivefold by inserting an additional copy of L-lysine ϵ -aminotransferase gene into the chromosome of *S. clavuligerus* NRRL 3585. In another study, aspartokinase deregulated mutants (AEC-resistant strains) produced 2 to 7 times more cephamycin C than the wild type [20]. Very recently, we accomplished the expression of *ask* (aspartokinase) gene in a multi-copy plasmid in *S. clavuligerus* with a twofold to threefold increase in specific cephamycin C yields (unpublished), and the studies are under way to express multiple copies of *ask* gene in AK39. In view of specific cephamycin C titers, the present study demonstrated a beneficial effect of closing L-threonine branch of aspartate biosynthetic pathway. Further work will focus on determination of the impact of *hom* inactivation on pathway flux distributions and optimization of the nutritional composition of CDM to improve the growth of the disrupted mutant which may lead to a further increase in cephamycin C production.

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