Organization of threonine biosynthesis genes from the obligate methylotroph Methylobacillus flagellatus

George N. Marchenko,1† Natalia D. Marchenko,1‡ Yuriy D. Tsygankov1 and Andrei Y. Chistoserdov2

The genes encoding aspartate kinase (ask), homoserine dehydrogenase (hom), homoserine kinase (thrB) and threonine synthase (thrC) from the obligate methylotroph Methylobacillus flagellatus were cloned. In maxicells hom and thrC directed synthesis of 51 and 48 kDa polypeptides, respectively. The hom, thrB and thrC genes and adjacent DNA areas were sequenced. Of the threonine biosynthesis genes, only hom and thrC were tightly linked in the order hom-thrC. The gene for thymidylate synthase (thyA) followed thrC and the gene for aspartate aminotransferase (aspC) preceded hom. All four genes (aspC-hom-thrC-thyA) were transcribed in the same direction. mRNA analysis indicated that hom-thrC are apparently transcribed in one 7.5 kb transcript in M. flagellatus. Promoter analysis showed the presence of a functional promoter between aspC and hom. No functional promoter was found to be associated with the DNA stretch between hom and thrC. The thrB gene encoded an unusual type of homoserine kinase and was not linked to other threonine biosynthesis genes.

Keywords: Methylobacillus flagellatus, threonine biosynthesis, homoserine dehydrogenase, homoserine kinase, threonine synthase

INTRODUCTION

Methylobacillus flagellatus is an obligate methylotrophic bacterium able to grow only on methanol and methylamine as sources of carbon and energy (Govorukhina et al., 1987). This bacterium has a high maximum growth rate (µ = 0.74) and a high methanol conversion coefficient (50 %, Baev et al., 1992). These properties make M. flagellatus an ideal producer for the biotechnology industry. This bacterium has been shown to have potential to be used for overexpression of heterologous proteins (Chistoserdov et al., 1987), production of amino acids (threonine; Shilova et al., 1989) and vitamins (biotin; Serebriiski & Tsygankov, 1995). Further development of M. flagellatus producing strains is hampered by the absence of basic knowledge about the organization and functioning of its genes. Only a few genes have been cloned from M. flagellatus. These are the gene for the RecA protein (Gomelsky et al., 1993; Malumbres et al., 1993; Malumbres et al., 1994), several species of Corynebacterium (Mateos et al., 1987a, b; Kalinowski et al., 1991; Follette et al., 1993; Malumbres et al., 1994), several species of Mycobacterium (Cirillo et al., 1994), Lactobacillus lactis (Le et al., 1996), Bacillus spp. (Bondaryk & Paulus, 1996).

The pathway of threonine biosynthesis has been shown to be similar in all micro-organisms (see Fig. 1). However, the organization of the genes for threonine biosynthesis enzymes is different in different micro-organisms. Some or all genes for threonine biosynthesis have been cloned from several Gram-positive bacteria, several species of Corynebacterium (Mateos et al., 1987a, b; Kalinowski et al., 1991; Follette et al., 1993; Malumbres et al., 1994), several species of Mycobacterium (Cirillo et al., 1994), Lactobacillus lactis (Le et al., 1996), Bacillus spp. (Bondaryk & Paulus, 1996).
The pathway of threonine biosynthesis in *M. flagellatus*. Numbers refer to the following enzymes: 1, ASK; 2, ASD; 3, HDH; 4, HK; 5, TS. The biosynthetic pathways leading to lysine, methionine and isoleucine are shown by dashed lines. Dotted lines represent feedback inhibition of ASK and HDH (see Results and Discussion sections).

1985; Parsot & Cohen, 1988; Malumbres et al., 1995), *Pseudomonas aeruginosa* (Clepet et al., 1992), *Thermus flavus* (Nishiyama et al., 1995) and enterobacteria (Cohen & Saint-Girons, 1987; Omori et al., 1993). Organization of threonine biosynthesis genes in *Haemophilus influenzae* became known upon completion of its genome sequencing (Fleischmann et al., 1995). In enterobacteria (Cohen & Saint-Girons, 1987; Omori et al., 1993) and in plants (Weisemann & Matthews, 1993) aspartate kinase (ASK) and homoserine dehydrogenase (HDH) activities are present in the same polypeptide, ThrA. The thrA gene is organized in an operon along with the genes for homoserine kinase (HK; thrB) and HDH (thrC), and the gene for aspartate semialdehyde dehydrogenase (asd) is not linked to them. A different type of thr gene organization is found in Gram-positive bacteria. In *L. lactis* (Madsen et al., 1996), *Brevibacterium lactofermentum* (Mateos et al., 1987a, b) and *Corynebacterium glutamicum* (Peoples et al., 1988) the *hom* and *thr* genes are linked and *thrC* is located elsewhere in the chromosome. In *Bacillus* spp. *hom-thrC-thrB* are clustered together (Malumbres et al., 1995). In some Gram-positive bacteria studied so far (Mycobacterium spp., Corynebacterium spp.) the genes for ASK (ask) and aspartate semialdehyde dehydrogenase (ASD) are organized in an operon (Cirillo et al., 1994; Jetten et al., 1995); in others, and in all Gram-negative bacteria studied so far, these genes are not linked (Streptomyces akiyoshiiensis, Le et al., 1996). The third type of threonine biosynthesis gene organization is found in Gram-negative bacteria other than enterobacteria. In these *hom* and *thrC* are clustered together (Clepet et al., 1992; Motoyama et al., 1994) and *thrB* is located elsewhere in the chromosome (Clepet et al., 1992).

There have been only three reports in the literature about cloning genes involved in threonine biosynthesis from methylo trophic bacteria. The gene for ASD has been cloned from *Thiothrix versutus* (Jaguszyn-Krynicki & Malashewska-Keough, 1989), the gene for ASK has been cloned from a methylo trophic *Bacillus* sp. (Shendel & Flickinger, 1992) and the *hom* and *thrC* genes have been cloned from two species of *Methylobacillus glycogenes* (Motoyama et al., 1994). In this article, we report the organization of threonine biosynthesis genes from *M. flagellatus* which appears to be similar to that of *P. aeruginosa* and *M. glycogenes*. Preliminary results of this work have been presented previously (Marchenko & Tsygankov, 1992).

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this work are shown in Table 1. All *Escherichia coli* strains were grown in Luria–Bertani medium in the presence of appropriate antibiotics as described by Maniatis et al. (1982). IPTG and X-Gal were added at 0.04 mg ml⁻¹. *Methylobacillus flagellatus* strains were grown in the minimal medium described previously (Govorukhina et al., 1987). The concentrations of ampicillin (Ap), rifamycin, streptomycin (Sm) and kanamycin (Km) for growing *M. flagellatus* strains were 1, 0.1, 0.15 and 0.15 mg ml⁻¹, respectively. In experiments with the promoter probe vector pACYC37, Sm was used in increasing concentrations up to 4 mg ml⁻¹. Methanol (2%, v/v) was used as carbon source.

**DNA manipulations.** Plasmid isolation, *E. coli* strain transformation, preparative isolation of the DNA fragments from agarose gels, restriction endonuclease digestion, ligation and formation, preparative isolation of the DNA fragments from *E. coli* host is grown at 30 °C or as phage particles when temperature is shifted to 42 °C. Plasmids from the *pSL5* library were transfected into *E. coli* strains with lesions in threonine biosynthesis genes as described by Yankovsky et al. (1989).

**Cloning of threonine biosynthesis genes.** A nearly complete (99.5%) EcoRI-generated gene library of *M. flagellatus* was constructed in the phasmid vector *pSL5* (Yankovsky et al., 1989) in *E. coli* LE392. The *pSL5* vector with 6–25 kb DNA inserts may exist either as a plasmid when the *E. coli* host is grown at 30 °C or as phage particles when temperature is shifted to 42 °C. Plasmids from the *pSL5* library were transfected into *E. coli* strains with lesions in threonine biosynthesis genes as described by Yankovsky et al. (1989).

**RNA isolation.** Total RNA was isolated from *E. coli* TG1 and *M. flagellatus* as follows. Bacteria were grown in 10 ml minimal medium. Cells were collected by centrifugation, resuspended in 1 ml solution A (50 mM Tris/HCl, 50 mM EDTA, 0.1% SDS, pH 8.0) and incubated for 15 min at room temperature. Samples were transferred into a boiling water bath for 2 min, then quickly cooled down and centrifuged for 20 min in an Eppendorf microcentrifuge. The supernatant was extracted subsequently with acidic phenol (pH 5.0), phenol/chloroform and chloroform. RNA was precipitated with 2.5 vols cold ethanol in the presence of 0.2 M sodium acetate buffer (pH 5.0). The RNA precipitate was washed three times.
Table 1. Bacterial strains and plasmids used in this study

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*Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rf, rifamycin; Sm, streptomycin; Su, sulfonamide; Tp, trimethoprim.
with cold 3 M sodium acetate (pH 5.0) to remove contaminating DNA.

**DNA–DNA and RNA–DNA hybridizations.** These were carried out in accordance with Maniatis et al. (1982). The temperature of hybridization (6 × SSC, 0.1 % SDS) and washes (1 × SSC, 0.1 % SDS) was 42 °C in the presence of 50 % formamide for DNA–DNA hybridization and 65 °C without formamide for RNA–DNA hybridization (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate). The RNA Ladder RNA molecular size standard was obtained from Gibco-BRL.

**DNA sequencing.** DNA sequencing was performed by the dideoxy chain-termination method on both strands on an Applied Biosystems model 370A sequencer.

**Matings.** Matings were conducted as described previously (Tsygankov et al., 1990).

**HDH assay.** M. flagellatus AM1 and E. coli Gif102 cells were broken using an MSE sonicator at 20 kHz in 10 mM sodium phosphate buffer (pH 7.0). HDH activity was assayed in the crude extracts (30 µl) in a 1 ml reaction mix containing 250 mM Tris/HCl buffer (pH 9.1), 100 mM KCl, 0.4 mM NAD(P) and 10 mM homoserine.

**ASK assay.** ASK activity in M. flagellatus AM1 and E. coli GT14 was assayed in crude extracts as described by Stephan & Datta (1973).

**Computer analysis of DNA sequences.** Computer analysis was carried out by using PGENE (Genofit) and the GCG software package (University of Wisconsin Genetic Computer Group). Multiple protein sequences were aligned using the CLUSTAL V program (Higgins et al., 1992) and alignments were refined visually. Phylogenetic analyses were conducted using the Protpars and Neighbors programs from the PHYLIP 3.57c package (Felsenstein, 1989) and the PAUP 3.1.1 program (Swoford, 1991). Phylogenetic trees created by the three different programs were essentially identical.

**Maxicell expression and electrophoresis of proteins.** Plasmid-encoded proteins were labelled by the maxicell procedure described by Sancar et al. (1981) with the following modifications: strains bearing plasmids were E. coli AB2463 derivatives and these strains were irradiated with 40 J short wave UV light m⁻². SDS-PAGE was carried out according to Laemmli (1970). Separations were done in 14 % (w/v) gels. Protein standards for SDS-PAGE were from New England Biolabs.

**RESULTS**

**Cloning of ask, hom, thrB and thrC**

The genes for ASK, HDH, HK and threonine synthase (TS) were cloned by complementation of E. coli strains GT14, Gif102, VL537 and GT25 (see Table 1), which have a lesion in threonine biosynthesis at the levels of ASK, HDH, HK and TS, respectively. These strains were infected with pSL5 phages (plasmids) from a M. flagellatus gene library and plated onto corresponding minimal media without threonine (VL537, Gif102 and GT25) or diaminopimelic acid (GT14).

Plasmids from GT14 clones, able to grow without diaminopimelic acid after infection with the pSL5 clone library, shared the same 10-4 kb EcoRI fragment (shown in Fig. 2). The ask gene was mapped within the 4 kb KpnI–Eco47I fragment. Southern blot hybridization was conducted to confirm that the 10-4 kb EcoRI fragment originates from M. flagellatus (data not shown). ASK activity was measured in crude extracts of M. flagellatus, E. coli GT14 and E. coli GT14(pASK6). No ASK activity was found in E. coli GT14 (Table 2). The higher specific ASK activities in E. coli GT14(pASK6) compared to these of M. flagellatus can be accounted for by the high copy number of the plasmid. The inhibition patterns of ASK activity in crude extracts from M. flagellatus and E. coli GT14(pASK6) were similar and did not resemble inhibition patterns of any of the three ASKs from E. coli. To verify whether the gene for ASK is linked to the gene for ASD, C600∆asd was transformed with pASK1A. None of the 100 analysed transformants was able to grow without diaminopimelic acid. Since the ask gene is located approximately in the middle of the 10-4 kb insert in pASK1A, the asd gene is not immediately adjacent to ask.

Plasmids which are able to complement E. coli VL537 carried two common 6-6 and 3-7 kb fragments (shown in the Fig. 2 as an insert in pTHRC1). pTHRC1 was not only able to complement the thrB mutation of strains VL334 and GT28 but it also conferred the ability to grow without methionine and threonine to E. coli Gif102. However, it did not complement the thrB mutation in E. coli AB2463. The genes for HDH and TS were more precisely localized within pTHRC1 by subcloning (Fig. 2). The smallest fragment able to...
complement the lesion in HDH in *E. coli* Gif102 was the 1·9 kb *PstI* fragment. The gene for TS (*thrC*) was mapped in the 1·6 kb *HindIII–Bpu1102I* fragment.

HDH from *M. flagellatus* is unusual in that it can use both NAD and NADP as electron acceptors (Table 3). Moreover, HDH activity with NAD is approximately five times higher than with NADP and it is not inhibited by threonine or methionine, or a combination of the two. The NADP-dependent activity of HDH is inhibited by methionine and threonine, and a combination of the two. NAD and NADP-dependent HDH activities expressed by pHOM12B in *E. coli* Gif102(pHOM12B) had similar ratio and inhibition patterns.

All plasmids complementing the *thrB* mutation of *E. coli* GT25 contained a common 4·3 kb *EcoRI* fragment (Fig. 2). The *thrB* gene was more precisely mapped within this fragment by subcloning. The smallest fragment of DNA containing the *thrB* gene was approximately 1·2 kb in size.

### Sequencing of *hom*, *thrB* and *thrC* genes and adjacent areas

A 5·6 kb *PstI–ClaI* fragment from pTHRC1 containing the *thrC* and *hom* genes was sequenced. Five ORFs were identified (Fig. 2). A search in GenBank revealed that a polypeptide encoded by *orfX* (13 463 Da, 122 aa) shares 28% similarity with the 13 kDa major membrane protein of *Francisella tularensis* (Sjoestedt et al., 1990) and 23% similarity with a hypothetical 12·7 kDa protein of *Rhodobacter capsulatus* (Beckman et al., 1992). The second ORF encoded a polypeptide (47 790 Da, 429 aa) which is similar to various aspartate aminotransferases from bacteria and yeasts (the highest identity of 65·8% was observed with AspC from *Haemophilus influenzae*;
The 1094 bp fragment from pTHR5 (Fig. 2), containing the entire gene for HK, was sequenced. Analysis of the sequence revealed that it encoded a polypeptide (35530 Da, 319 aa) which is 23% identical to one of the HKs described in *P. aeruginosa* (Clepet et al., 1992).

**Complementation of the thrB mutation in *M. flagellatus* MFK11 by the cloned thrB gene**

To prove that this gene is able to complement the thrB1 mutation in *M. flagellatus* MFK11, the thrB gene from pTHR5 was cloned in the broad-host-range vector pNGM130K and introduced into *M. flagellatus* MFK11 by conjugation. Out of 120 independent transconjugants analysed, all regained their ability to grow without supplemented threonine.

**Expression of hom and thrC in *E. coli* maxicells**

The *recA* strain *E. coli* AB2463 was used for maxicell expression of the cloned *hom* and *thrC* genes. Plasmids pTHRC1, pTHRC6 (contains the 3 kb *HindIII–Kpnl* subfragment of pTHRC1) and pHOM12B (pUC19 with a 1·9 kb *PstI* fragment containing intact *hom*) were expressed in this strain. The results are shown in Fig. 2. pTHRC1 directs synthesis of four polypeptides of 52, 47, 38 and 28 kDa. The 28 kDa polypeptide apparently belongs to the mature form of \( \beta \)-lactamase (Fig. 3, lane D, pUC19). pTHRC6 directs synthesis of two polypeptides with molecular masses of 52 and 31 kDa. These correspond in molecular mass to TS (52153 Da) and thymidylate synthase (30131 Da), respectively. Although pTHRC1 and pTHRC6 directed synthesis of the HDH polypeptide at similar levels, synthesis of thymidylate synthase in cells containing pTHRC1 was very poor. pHOM12B directs synthesis of a polypeptide with a molecular mass of 47 kDa. Synthesis of the same polypeptide is directed by pTHRC1. This molecular mass estimate coincides with 46620 Da predicted for HDH from the sequence data. Sequence data also confirm that pHOM12B contains only one intact ORF, encoding HDH. The intensity of the 47 kDa band is much higher in the lane with the protein extract from cells containing pTHRC1 (Fig. 3). Apparently, the polypeptides of HDH and aspartate aminotransferase did not resolve well on the gel and this is consistent with their predicted molecular masses which are similar (46620 and 47790 Da, respectively). The 38 kDa polypeptide cannot be ascribed to any sequenced gene and it is apparently encoded by the DNA region downstream of the *thyA* gene.

**Mapping of promoters and characterization of RNA transcripts for hom and thrC**

The complementation analysis of *E. coli* Gif102 and GT28 has shown that the *thrC* and *hom* genes from *M. flagellatus* were expressed in *E. coli* under their own promoter(s), since expression of *hom* and *thrC* occurred independently of their orientation relative to the P\(_{lac}\) promoter. The 426 bp *Sau3A* fragment, which includes the 5′ terminus of *hom* and 216 bp upstream, was cloned into the pAYC37 promoter probe vector (pPHD37). The *HindIII–EcoRV* fragment from pHOM12B which was
Threonine biosynthesis genes in *M. flagellatus*

**DISCUSSION**

**Organization of threonine biosynthesis genes in *M. flagellatus***

The genes encoding enzymes involved in threonine biosynthesis, ASK (ask), HDH (bom), HK(*thrB*) and TS (*thrC*), were cloned from the methylo trophic bacterium *M. flagellatus*. Thus the only remaining gene required for threonine biosynthesis, which has not been cloned so far from this bacterium, is the gene for ASD. *bom* and *thrC* are linked in the chromosome of *M. flagellatus* and they are also linked to the *aspC* and *thyA* genes in the order *aspC*-bom-*thrC*-thyA. Our data indicate that *bom* and *thrC* are transcribed in *M. flagellatus* from a promoter located between *aspC* and *bom* as part of a single 7-5 kb mRNA transcript and, therefore, *bom* and *thrC* are organized in an operon. We expected that the hairpin structure identified between *bom* and *thrC* may serve as a transcriptional terminator. However, repeated Northern blot experiments clearly demonstrated the absence of an additional, smaller transcript which might contain *bom* mRNA only. Motoyama *et al.* (1994) have found that the two *bom*-*thrC* transcripts in *M. glycogenes* ATCC 21276 and ATCC 21371 are much smaller, only 1-4 and 2-4 kb in size, respectively. Despite the fact that the 1-4 kb transcript hybridized with both *bom* and *thrC* gene probes, it is not large enough to encode intact products of both genes.

Organization of the threonine biosynthesis genes similar to that of *M. flagellatus* was found in *M. glycogenes* (Motoyama *et al.*, 1994) and *P. aeruginosa* (Clepet *et al.*, 1992), although the cloning of *aspC* or *thyA* has not been reported in the two latter cases. The DNA sequence upstream of *bom* from *M. glycogenes* (Motoyama *et al.*, 1994) shows substantial homology with DNA sequence upstream of *bom* from *M. flagellatus*. In addition, TFASTA analysis (GCC package) indicated that AspC from *M. flagellatus* shares up to 90% identity with some, but not all, ORFs encoded by the upstream DNA sequence from *M. glycogenes*; however no ORF encoding the whole *aspC* gene could be found in the latter.

Three different arrangements of threonine biosynthesis genes have been found so far in bacteria (see Introduction). The organization of threonine biosynthesis genes found in *M. flagellatus* resembles that of non-enteric Gram-negative bacteria (Clepet *et al.*, 1992; Motoyama *et al.*, 1994). In these, *bom* and *thrC* and, at least in *Methylobacillus* spp., *aspC* are clustered together. In all studied Gram-negative bacteria, including *M. flagellatus*, the genes for ASK and ASD are not linked to each other or to other threonine biosynthesis genes.

**Biochemical properties of ASK and HDH from *M. flagellatus***

Based on the inhibition pattern of ASK from *M. flagellatus* it can be assumed that this bacterium encodes only one ASK which is inhibited in concert by threonine.

**Fig. 4.** Northern blot of total mRNA from *E. coli* and *M. flagellatus* hybridized with *bom* and *thrC* probes. Lanes: A and D, total mRNA isolated from *E. coli* TG1; B and C, total mRNA isolated from *M. flagellatus*. Lanes A and B were hybridized with a 665 bp *Hin*II internal fragment of the *hom* gene. Lanes C and D were hybridized with a 1002 bp *PaeI* internal fragment of the *thrC* gene. Approximately 1 µg mRNA was loaded in each lane.
and lysine, but not methionine. This inhibition pattern has been found for ASKs from bacteria which do not have ASK isoenzymes, for example C. glutamicum (Nakayama et al., 1966) and Pseudomonas spp. (Ruban, 1986).

HDH from M. flagellatus is a very unusual NAD(P)-dependent dehydrogenase. It has activity with both NAD and NADP, and the activity with NAD is approximately five times higher. The most striking feature of HDH from M. flagellatus is that its NAD activity is not inhibited by methionine and threonine, whereas its NADP activity is inhibited by methionine and threonine. To our knowledge, no other HDH with such properties has been described.

**Analysis of the AspC, Hom, ThrC, ThyA and ThrB polypeptides from M. flagellatus**

A GenBank search and consequent alignment indicated that the polypeptide most closely related to AspC from M. flagellatus is a putative AspC polypeptide from H. influenzae. It is interesting to note that AspC from H. influenzae is a much shorter polypeptide (251 aa) than that from M. flagellatus (437 aa). They share 66% identity along the first 243 aa. Aspartate aminotransferases from other sources have approximately the same number of amino acids as AspC from M. flagellatus but they share substantially lower levels of identity [from 14% for AspC from Saccharomyces cerevisiae (GenBank accession no. P47039) to 28% for AspC from Bacillus sp., (Sung et al., 1991)]. Alignment of several aspartate aminotransferases indicated that Lys240 in AspC from M. flagellatus is a putative pyridoxal-phosphate-binding amino acid. The sequence NGXS (where X is leucine, valine or phenylalanine) immediately upstream of this amino acid seems to be fairly conserved in sequenced bacterial AspC polypeptides.

The gene downstream of aspC encodes HDH. Phylogenetic analysis of the Hom polypeptides confirms that they can be divided into three groups, with enterobacteria, H. influenzae and eukaryotes (carrot) in the first group, Gram-positive bacteria in the second group and non-enteric Gram-negative bacteria in the third group (data not shown). Results of phylogenetic analyses indicate that HDHs with one activity (HDH) are more closely related to each other than to HDHs with two activities (ASK and HDH). The NAD binding signature, GXGXGG, can be found in all HDHs, including M. flagellatus (aa 10-15). The Hom polypeptide from M. glycolgenes ATCC 21371 is the most closely related to Hom from M. flagellatus, sharing 78.2% identical amino acids. Surprisingly, HDH from M. glycolgenes ATCC 21276 shares only 43.2 and 42.1% identity with M. glycolgenes ATCC 21371 and M. flagellatus, respectively.

The third gene in the threonine biosynthesis gene cluster from M. flagellatus is thrC. Phylogenetically, TSs from different sources are clustered into three groups: enterobacteria, eukaryotes and non-enteric bacteria. As expected, TS from M. glycolgenes is the most closely related to ThrC from M. glycolgenes ATCC 21371; they share 91.2% identical amino acids. An alignment of TSs from different sources shows their high overall levels of similarity (not shown). The conserved region, 119-FKD-121, which binds the pyridoxal-phosphate co-enzyme of TS ( Parsot, 1986) is present in all TSs, including that from M. flagellatus.

The thyA gene is the third gene of the hom-thrC operon. It encodes a typical thymidylate synthase which has 68.2 and 67.8% identity with thymidylate synthases from E. coli (Belfort et al., 1983) and B. subtilis (Montorsi & Lorenzetti, 1993), respectively. Biosynthesis of pyrimidines and threonine requires the same metabolic ancestor, aspartate, and therefore it is reasonable to assume that some of the reactions of pyrimidines and threonine biosynthesis may be co-regulated. Based on our mRNA analysis, the 7.5 kb transcript, which encodes Hom, ThrC and ThyA, is large enough to encode three additional polypeptides with a mean size of ~50 kDa. Some of these polypeptides might be involved in earlier steps of pyrimidine biosynthesis.

The gene for HK is not clustered with other threonine biosynthesis genes in the M. flagellatus chromosome. A search of GenBank confirmed that this ORF is similar to one of the HKs described in P. aeruginosa and they are both dissimilar to HKs from all other bacteria. The ThrB polypeptides from P. aeruginosa and M. flagellatus share 23% identity, with highest overall similarity in the N-terminal regions of the polypeptides. It is likely that the unusual thrB gene found only in M. flagellatus and P. aeruginosa is the only HK-encoding gene in M. flagellatus. The thrB1 mutation in M. flagellatus MKF11 has been generated via chemical mutagenesis (Tsygankov et al., 1990) and it is highly unlikely that this lesion in HK is the result of a double mutation (in two HK genes).

In summary, we cloned and identified four out of five genes involved in threonine biosynthesis in M. flagellatus chromosome. These genes (except hom and thrC) are not linked to each other. Further analysis will be required to determine their relative location on the M. flagellatus chromosome and whether these genes are jointly regulated. Such knowledge can be used in the construction of threonine and other amino acid producers based on methylotrophic bacteria.

**REFERENCES**


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