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Molecular cloning and characterization of *Escherichia coli* K12 *ygjG* gene

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Abstract

Background: Putrescine is the intermediate product of arginine decarboxylase pathway in *Escherichia coli* which can be used as an alternative nitrogen source. Transaminase and dehydrogenase enzymes seem to be implicated in the degradative pathway of putrescine, in which this compound is converted into γ-aminobutyrate. But genes coding for these enzymes have not been identified so far.

Results: The 1.8-kbp DNA fragment containing *E. coli* K12 *ygjG* gene with *aer-ygjG* intergenic region was examined. It was found that the fragment contains σ^{54}-depended open reading frame (ORF) of 1,380 nucleotides encoding a 459-amino acid polypeptide of approximately 49.6 kDa. The cytidine (C) residue localized 10 bp downstream of the σ^{54} promoter sequence was identified as the first mRNA base. The UUG translation initiation codon is situated 36 nucleotides downstream of the mRNA start. The YgjG was expressed as a his_6-tag fused protein and purified to homogeneity. The protein catalyzed putrescine:2-oxoglutaric acid (2-OG) aminotransferase reaction (PATase, EC 2.6.1.29). The K_m values for putrescine and 2-OG were found to be 9.2 mM and 19.0 mM, respectively. The recombinant enzyme also was able to transaminate cadaverine and, in lower extent, spermidine, and gave maximum activity at pH 9.0.

Conclusion: Expression of *E. coli* K12 *ygjG* coding region revealed σ^{54}-depended ORF which encodes a 459-amino acid protein with putrescine:2-OG aminotransferase activity. The enzyme also was able to transaminate cadaverine and, in lower extent, spermidine.

Background

Polyamines, such as putrescine and spermidine, are present in virtually all living cells, from bacteria to plant and human cells, with a very important though poorly understood biological role [1,2]. They can bind to nucleic acids, stabilize membrane and stimulate activity of several enzymes [2–5]. Despite the proved necessity of intracellular polyamine for optimal cellular growth, polyamine accumulation can lead to inhibition of cellular growth and protein synthesis [6,7]. Two major metabolic routes of polyamines breakdown have been described, one for the free bases *via* Δ^1-pyrroline (4-aminobutyraldehyde) and the other *via* N-acetyl derivatives. Acetylation pathway serves to prevent polyamine toxicity in prokaryotes and eukaryotes [1,8]. Spermidine and spermine are acetylated and further oxidized by polyamine oxidase or either deacetylated or excreted from the cell. Acetyltransferase

forming exclusively of *N*-acetylspermidine has been described in *E. coli* [9,10], but neither spermidine-deacetylating activity nor *N*-acetylspermidine oxidase activity has been detected, suggesting that the *N*-acetylspermidine is excreted or kept in the inert acetylated form [11]. In the aminotransferase pathway of polyamine degradation, amino groups are converted from polycations to electron acceptors. Polyamine aminotransferase activity was found in some microorganisms [12–14]. In the case of putrescine, the resulting Δ^1-pyrroline is an intermediate product of 4-aminobutyric acid (GABA) metabolism [8,15,16]. The putrescine:2-oxoglutarate (2-OG) aminotransferase enzyme (PATase, EC 2.6.1.29) of *E. coli* was partially purified from the mutant strains [12,17] and characterized. The synthesis of putrescine aminotransferase is controlled by catabolite repression and nitrogen availability [18]. But the corresponding gene remains to be identified. It was speculated that *E. coli* K12 *ygjG* gene (b3073, [19]) specifies the ω-aminotransferase which either removes the amino groups from compounds with terminal primary amines, or adds amino groups to compounds with an aldehyde group [20]. Putrescine and compounds metabolized to putrescine activate *ygjG* expression [21], which suggests a possible role in putrescine catabolism. To elucidate YgjG biochemical functions, we have cloned the *ygjG* gene, expressed, purified an active recombinant protein and investigated some molecular and biochemical properties of the YgjG enzyme.

Results

Cloning and characterization of the 5'-flanking region of the ygjG *gene*

The 1.8-kbp DNA fragment from *E. coli* K12 genomic DNA including *ygjG* and *aer-ygjG* intergenic region (Fig. 1A) was cloned into pUC18 vector and sequenced. The obtained plasmid was designated as pUC18-ygjG. The computer analysis of the *ygjG* upstream regulatory region revealed recognizable consensus sequence of σ^{54}-dependent promoter with score 70.7 [21]. The localization of this promoter is shown on Fig. 1. Likewise, according to DNA array data, the nitrogen limitation results in 3- to 5-fold increase in levels of *ygjG* transcripts [22]. For more detail characterization of the role of the 5'-flanking region in the *ygjG* expression and nitrogen starvation induction we compared the expression levels of three different *ygjG* upstream regions fused to the *lacZ* reporter gene in plasmid pBRP [23] under different growth conditions. pBR-σ^{54}-lac*Z*-1 and pBR-σ^{54}-lac*Z*-2 plasmids carried 343-bp *Kpn*I-*Acc*III and 56-bp *Bgl*I-*Acc*III fragments, respectively, containing predicted σ^{54} promoter sequence, and pBR-lac*Z*-3 plasmid harbored 282-bp *Kpn*I-*Bgl*I fragment upstream of σ^{54} promoter. *E. coli* TG1 cells were transformed by each of plasmids and β-galactosidase productions in three media: LB broth and salt M9 media [24] with ammonium or supplemented with proline as a sole nitrogen source were analyzed (Table 1).

The all three recombinant strains were found to have low β-galactosidase activity (below 200 Miller unites [24]) in LB broth. The strain harboring pBR-lac*Z*-3 also revealed low levels of expression in all media, pointing that the 282-bp fragment doesn't include noticeable promoter sequence. The strain carried plasmid pBR-σ^{54}-lac*Z*-2 containing practically σ^{54} sequence alone, showed 3 – 5 times higher *lacZ* expression levels than pBR-lac*Z*-3 in both variant of minimal media. But *lacZ* activity was dramatically increased in the strain harboring pBR-σ^{54}-lac*Z*-1, especially under nitrogen limited conditions. The increase was about 9-fold in M9 medium with ammonium and 28-fold in M9 with proline in comparison with pBR-σ^{54}-lac*Z*-2. Thus, the 56-bp region contains promoter sequence, and we concluded that σ^{54} promoter localized in the *Bgl*I-*Acc*III fragment is the main promoter of *ygjG*. The *Kpn*I-*Bgl*I fragment is strongly essential for *ygjG* expression in minimal media and activation of *ygjG* expression (up to 6-fold) under nitrogen limitation. Because enhancer proteins are absolutely necessary for σ^{54}-dependent transcription [25] we reanalyzed the sequence of this fragment and found some putative binding sites for *E. coli* activators: two general nitrogen regulatory protein (NR_I) binding sites and binding site for integration host factor (IHF) (Fig. 1B). In addition, existence of factor of inversion stimulation (FIS) binding site was suggested http://www.ncbi.nlm.nih.gov:80/. Thus the activation of *ygj*G expression is complicated, depends on growth conditions and needs further investigations.

TG1 cells harboring pUC18-ygjG were grown in three media (see above), the total RNAs were isolated and used to identify the transcriptional initiation site of *ygjG* mRNA by primer extension experiment. The same cDNA fragment was observed as the major transcript for all three RNA preparations, and the cytidine (C) was identified as the first mRNA base (Fig. 2, also shown as "+1" in Fig. 1). The quantities of *ygjG* cDNA synthesized were estimated using Typhoon imager. The highest level of transcript was observed for RNA preparation from cells grown under nitrogen limitation, while only about 20% and less than 1% of it were detected if cells were grown in M9 and LB media, accordingly. Also, crude protein extracts from these cells were examined for PATase activity with putrescine as amino group donor and with 2-OG as acceptor. Enzyme activity was determined routinely by glutamate formation, which was measured by capillary zone electrophoresis (CE) (Fig. 3). The second product of the reaction, γ-aminobutyraldehyde, was unstable [16] and formed cycled Δ^1-pyrroline at alkaline pH. PATase activity was highest in extracts of cells grown under nitrogen limitation (47 $nmol \cdot min^{-1} \cdot mg^{-1}$). It was significantly reduced in cell

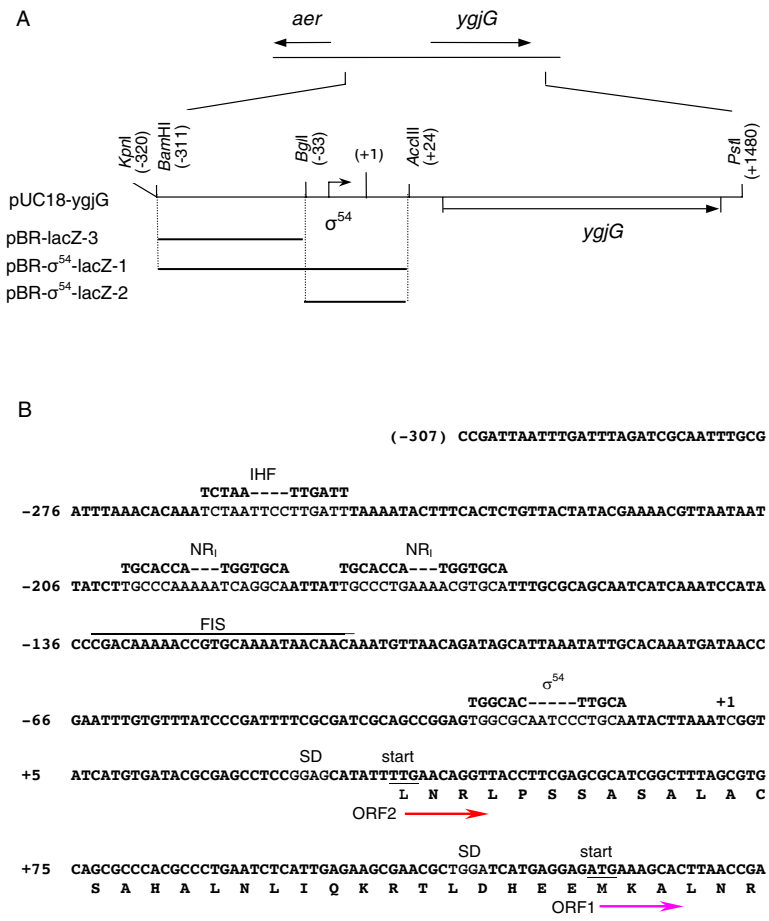

Figure 1

The scheme of the *ygjG* gene and *aer-ygjG* intergenic region. A. The fragment (from -311 to +1480) containing ygjG gene (b3073) with aer-ygjG intergenic region, and sites for restriction enzymes used in cloning procedure are shown. The orientation of genes and location of σ^{54} promoter are represented by arrows. Nucleotides numbering is in relation to *ygjG* mRNA transcription initiation site designated as +1. *ygjG* upstream fragments fused with *lacZ* and subcloned into pBRP [23] vector are shown as bold lines. B. The sequence of *ygjG* upstream region. Nucleotides numbering as in part A. σ^{54} promoter, putative NR_I and IHF binding sites, SD sequences and the first mRNA base are bold. The consensus sequences for IHF, NR_I and σ^{54} promoter are shown above the main sequence. Two possible translation start codons are underlined and corresponding N-terminal amino acids are bold. The putative FIS binding site http://www.ncbi.nlm.nih.gov:80/ is overlined. The directions of ORF1 and ORF2 translation are shown by arrows.

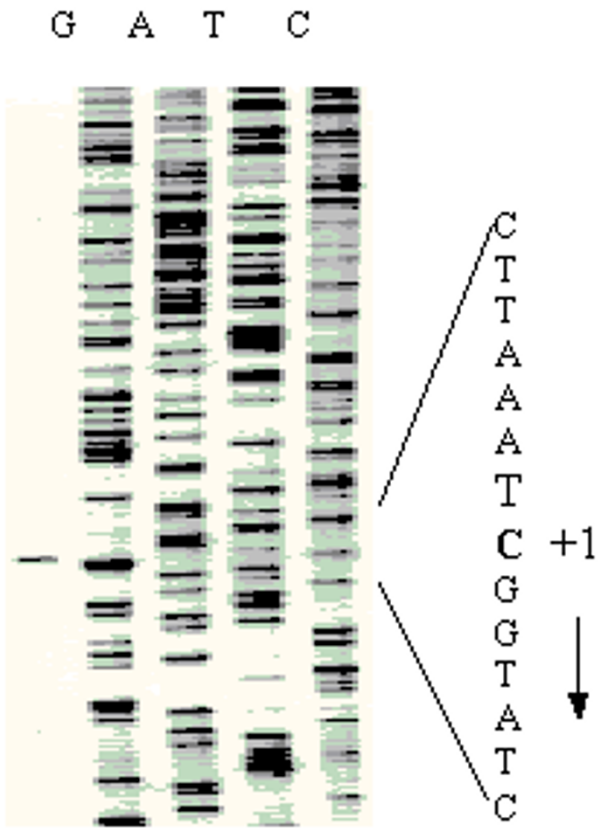

Figure 2
Determination of the *ygjG* transcriptional initiation site. Transcriptional initiation site was determined by primer extension. The reaction was carried out as described in Materials and Methods and cDNA transcript of RNA preparation obtained from cells grown under nitrogen limited conditions was analyzed on 6% PAGE (left track). The corresponding sequencing ladders are also labeled. The first nucleotide of *ygjG* mRNA (C) is bold and shown as "+1". The direction of transcription is shown by the arrow.

extracts from M9 medium (nearly proportionally to decreasing of *ygjG* transcript level), and was practically undetectable in cell extracts from LB medium (near to the limit of accuracy for the CE method). Thus, the level of *ygjG* transcription and PATase activity correlated in cells grown in each of three media used.

The predicted AUG initiation codon for the *ygjG* gene of 1290 bp (denoted downstream as ORF1) which codes 429-amino acid polypeptide (SWISS-PROT Database, P42588) is situated 126 nucleotides downstream of the mRNA start. Analysis of the nucleotide sequence revealed the other ORF (denoted as ORF2) encoding 459-amino acid protein with UUG initiation codon (Fig. 1B), which is 30 amino acids upstream from an in frame ORF1 start codon. Because both putative ORFs have the potential Shine-Dalgarno (SD) sequence [26], we decided to express each of them as his_6-tag fused recombinant protein to determine if these recombinant constructs actually yield a catalytically active enzyme.

Cloning and expression of the ORF1 and OFR2 coding proteins

The 1.29-kbp (ORF1) and 1.38-kbp (ORF2) DNA fragments of pUC18-ygjG were cloned in translation fusion with N-terminal his_6-tag sequence under the control of the T7 promoter in pET15b(+) vector. The resulting plasmids, pET-Ht-ORF1 and pET-Ht-ORF2, were expressed in *E. coli* BL21(DE3) cells. SDS-PAGE of cellular extracts demonstrated a high level of expression of these proteins. To elucidate which of polypeptides encodes an active enzyme, crude extracts were examined for PATase activity. The essential level of glutamate formation activity was observed in crude cell extracts of the BL21(DE3) harboring pET-Ht-ORF2. It was equal to 2.16 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The BL21(DE3)(pET-Ht-ORF1) strain exhibited only traces of PATase activity. The BL21(DE3) strain transformed with pET15b(+) as a control revealed no activity. Obviously, the UUG is the real initiation codon of the *ygjG* encoding 459-amino acid protein with a calculated molecular mass (M_r) of 49,644 Da.

Purification and characterization of the ygjG *gene recombinant product*

In order to confirm the identity of ORF2 as the gene encoding PATase, we purified recombinant protein from the BL21(DE3)(pET-Ht-ORF2) strain by immobilized-metal-affinity chromatography (IMAC) to give a final preparation having a specific activity of 11.68 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with yield of 81%. The purity of Ht-YgjG recombinant protein was about 95%. SDS-PAGE showed one distinctive band with a relative M_r of about 52 kDa. This value well corresponds to the sum of mass of 49.6 kDa for ORF2 coding protein and 2.1 kDa for his_6-tag leader peptide.

Some parameters of PATase reaction were determined. The enzyme was active at the alkaline pH, with the maximum activity at pH 9.0 in Tris-HCl buffer and displayed a broad temperature optimum between 20°C and 80°C with maximum activity at 60°C. The K_m values for putrescine and 2-OG were determined to be 9.2 mM and 19.0 mM, respectively.

Substrate specificity of purified Ht-YgjG was measured with different donors and acceptors of amino group (Table 2). Several compounds (putrescine, cadaverine, spermidine, agmatine, and ornithine) were tested for their ability to function as amino donors for 2-OG. Among them putrescine was found to be the best amino group

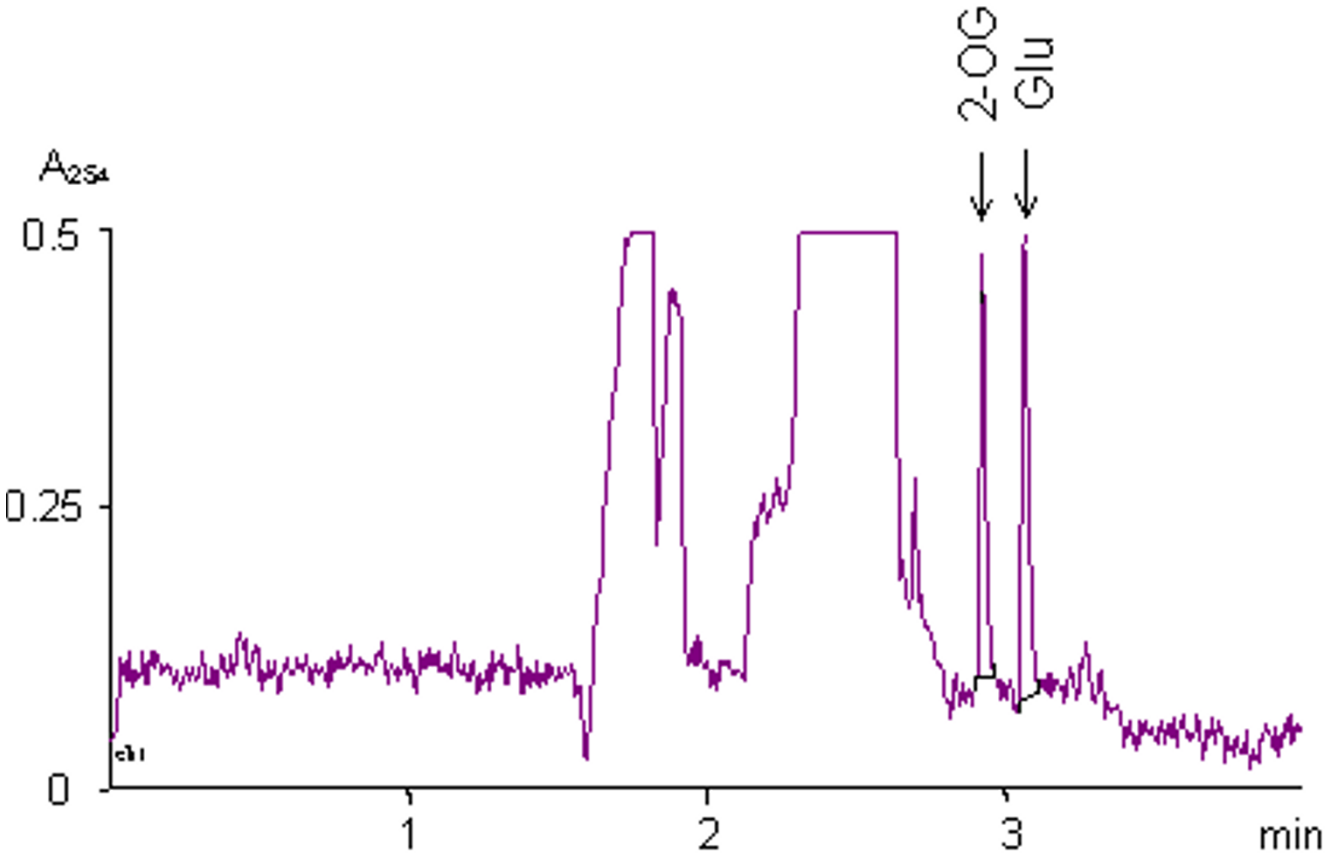

Figure 3
Assay of PATase activity by capillary zone electrophoresis. Capillary zone electrophoresis of PATase reaction mixture. For assay conditions see Materials and Methods. Picks of 2-OG and glutamate are shown by arrows.

donor for the aminotransferase activity of Ht-YgjG. Slightly lower activity with cadaverine (97%) and significantly lower with spermidine (32%) was detected. Also, three keto acids: 2-OG, α-ketobutyrate and pyruvate were used as amino acceptors for putrescine. The enzyme did exhibit significant activity toward α-ketobutyrate (38%) and, to less extent, pyruvate (12%) with putrescine. Almost no activity was detected with ornithine as amino acceptor, while ornithine aminotransferase activity was annotated for *ygjG* in GeneBank Database. GABA, diaminopimelic acid, acetylornithine were completely inactive as amino group donors.

Discussion

In addition to being involved in polyamine breakdown, the reaction catalysed by PATase appears to be the fourth reaction of the arginine decarboxylase pathway of arginine degradation to succinate via putrescine and GABA in *E. coli* [15,27]. We have found that *E. coli* K-12 *ygjG* gene (denoted above as ORF2) encodes for enzyme catalyzing PATase reaction. According to published data [21,22] and our experimental data, *ygjG* expression is under the control of the σ^{54} promoter and is induced at minimal salt medium and under nitrogen starvation. Two putative NR_I binding sites and IHF binding site in *ygjG* upstream region proposed to be involved into transcription regulation. The cytidine localized 10 bp downstream from the σ^{54} promoter sequence was identified as the first mRNA base. The potential SD site (GGAG) is located 6 nucleotides upstream from the UUG initiation codon of *ygjG* and this distance is functional for *E. coli* genes [28]. The UUG is initiation codon for about 3% of *E. coli* proteins [19] but it is less effective at promoting translation [29] than is AUG codon. Thus the initiation of translation from UUG codon could be a way to confine the synthesis of the YgjG protein.

Table 2: Substrate specificity of purified recombinant Ht-YgjG

Substrate	Relative activity (%)[a]
Amino group donor[b]	
Putrescine	100
Cadaverine	97
Spermidine	32
Agmatine	2
L-Ornithine	2
Amino group acceptor[b]	
2-OG	100
α-ketobutyric acid	38
Pyruvic acid	12

[a] The PATase activity with 2-OG as amino group acceptor was arbitrary defined as 100% relative activity. [b] 2-OG was used as amino acceptor with different polyamines and amino acid as amino group donors, and putrescine was used as amino group donor with different amino acceptors at final concentrations of 10 mM, respectively.

BLAST [30] analysis of the translated *ygjG* sequence revealed moderate identity to the aminotransferases belonging to the class III of pyridoxal phosphate (PLP) dependent aminotransferase family, which includes *E. coli* acetylornithine delta-aminotransferase (36% identity), GABA aminotransferase (34% identity), 7,8-diaminopelargonic acid synthetase (33% identity). In addition, the significant level of identity, 27%, was found for *Pseudomonas aeruginosa* SpuC protein catalyzing putrescine:pyruvate aminotransferase reaction [13]. The multiple sequence alignment of these enzymes (Fig. 4) showed that YgjG contains the conserved sequence segments with four invariant amino acid residues (i.e. Gly-245, Asp-271, Lys-300, and Arg-426) known to be involved in binding of either the substrate or the coenzyme PLP [20]. Interestingly, the N-terminal sequence of the YgjG is the longest among them, and we believe that this sequence may be essential for substrate specificity and enzyme activity. YgjG showed the greatest identity (94%) with a probable aminotransferase encoded by *STY3396* gene from *Salmonella enterica serovar Typhi CT18* (NP 457608) suggesting that STY3396 is likely to be PATase.

We have purified Ht-YgjG recombinant protein and found that pH optimum and substrate specificity of the enzyme, except for GABA, was in a good accordance with data of Kim [12] obtained for partially purified PATase enzyme from mutant *E. coli* strain, while the K_m value for 2-OG of Ht-YgjG is higher. The K_m values of enzymes belonging to the class III aminotransferases are significantly different, from micromolar to millimolar levels, for different substrates (see, for example, http://www.brenda.uni-koeln.de). As mentioned above, the N-terminal sequence of YgjG may be essential for substrate specificity and enzyme activity. According to our preliminary data, K_m values for putrescine and 2-OG of unfused YgjG enzyme are 3–4 times lower than those for the fused Ht-YgjG protein (data not shown). Thus, the N-end modification of YgjG by his_6-tag leader fusion alters kinetic constants of the native enzyme. Therefore further investigations are needed to more detail characterization of YgjG protein.

Conclusions

We found that *E. coli* K12 *ygjG* σ^{54}-depended ORF encodes the 459-amino acid protein. The recombinant enzyme, purified as a fuse with his_6-tag leader, possessed the putrescine:2-OG aminotransferase activity. The enzyme also was able to transaminate cadaverine, spermidine and utilized α-ketobutyrate and pyruvate as amino acceptors.

Methods

Bacterial strains and plasmids

E. coli strains TG1(K12 *supE hsd*Δ5 *thi* Δ (*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q}*lacZ*ΔM15]) and BL21(DE3)(B F$^-$ *dcm ompT hsdS*(r_B- m_B-) *gal λ* (DE3)) were used as a recipients, MG1655 (K12 λ^-, F$^-$) [31] was used for gene amplification. Plasmids pUC18 (Fermentas), pBRP [23] and pET15b(+) (Novagen) were used as a vectors for cloning.

Construction of the recombinant plasmids

The *E. coli* K12 *ygjG* gene with upstream *aer-ygjG* intergenic region was amplified from MG1655 genomic DNA using two oligonucleotides 5'-TTGGATCCGATTAATTTGATTTAGATCGCA-3' and 5'-TTCTGCAGCCTGCGGGCGTACGCGTCG-3' as primers. The 1,8 kb PCR product was digested by *Bam*HI and *Pst*I

Figure 4

Multiple sequence alignment of YgjG to known class III PLP-dependent aminotransferases. The alignment was performed with ClustalW http://www.ebi.ac.uk/clustalw The residues that are identical in all the displayed sequences are marked by asterisks, conserved residues are marked by dots and the four invariant residues (G, D, K and R) proposed for aminotransferases are bold and boxed. Abbreviations are: ArgD, acetylornithine delta-aminotransferase (ATase) (AAC76384); GoaG, 4-aminobutyric acid ATase (AAC74384); SpuC, putrescine:pyruvate ATase [13]; BioA, 7,8-diaminopelargonic acid synthetase (AAC73861).

Table 1: Expression of the *lacZ* fusions under different growth conditions

Plasmid[a]	β-Galactosidase activity, MU[b]		
	LB[c]	M9, Nitrogen Sufficient[c]	M9, Nitrogen Limited[c]
pBR-σ^{54}-lacZ-1	190	3,500	22,500
pBR-σ^{54}-lacZ-2	140	400	800
pBR-lacZ-3	110	130	170

[a] Plasmids were transformed into *E. coli* TG1 strain. [b] In Miller units [24]. [c] For growth conditions and β-galactosidase assay see Materials and Methods.

and cloned into pUC18 vector, yielding pUC18-ygjG plasmid. The insertion in pUC18-ygjG was sequenced for frame verification.

The 338-bp *Kpn*I-*Acc*I, 56-bp *Bgl*I-*Acc*III and 282-bp *Kpn*I-*Bgl*I fragments were excised from pUC18-ygjG, fused with *lacZ* reporter gene and cloned into pBRP vector (pBR322-based plasmid containing pUC18 polylinker region), yielding pBR-σ^{54}-lac*Z*-1, pBR-σ^{54}-lac*Z*-2 and pBR-lac*Z*-3 plasmids, respectively.

The *ygjG* ORF1 and ORF2 sequences were amplified by PCR from pUC18-ygjG using phosphorilated downstream primers 5'-CCGATATCATGAAAGCACTTAACCGAGAG-3' and 5'-CCGATATCTTGAACAGGTTACCTTCGAG-3', respectively, designed so as to provide translation fusion between his_6-tag leader sequence and the gene of interest, and upstream primer 5'-TTCTGCAGCCTGCGGGCG-TACGCGTCG-3'. PCR products were cloned into blunted vector pET15b(+)/NdeI-BamHI to construct plasmids pET-Ht-ORF1 and pET-Ht-ORF2, respectively, and insertions were sequenced.

Growth, induction conditions and preparation of cell-free extracts

Strains were routinely grown in LB broth or M9 medium [24]. Nitrogen limited conditions were provided using M9 medium lacking ammonium and supplemented with 2 mM of a proline as nitrogen source. All media contained 100 μg/ml ampiciline. Strains were grown aerobically at 37°C.

For expression of *lacZ* fusions, *E. coli* TG1 harboring pBR-σ^{54}-lac*Z*-1, pBR-σ^{54}-lac*Z*-2 or pBR-lac*Z*-3 plasmids were grown overnight in M9 medium, washed by 15 mM NaCl, diluted to optical density at 600 nm (OD_{600}) of 0.07 by LB, M9 or M9 nitrogen limited medium. The resulting cultures were grown until reaching on OD_{600} of about 3.0 (stationary phase).

The synthesis of his_6-tag fused recombinant proteins was induced in BL21(DE3) harboring pET-Ht-ORF1 or pET-Ht-ORF2 plasmid. When the cell density in LB medium had reached OD_{600} of 1.0, 1 mM isopropyl-β-D-thiogalactopyranoside was added followed by 2 h incubation.

For preparation of cell-free extracts bacteria were harvested by centrifugation and washed twice with TE buffer. The cell pellets were stored frozen for several days at -70°C without significant loss of enzyme activity. Frozen cells were thawed, suspended to 0,025 g (wet weight) per ml in buffer A (20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM PLP), and disrupted by sonication followed by centrifugation to remove debris.

RNA isolation and primer extension start site analysis

The TG1 harboring pUC18-ygjG has been grown in LB, M9 media and under nitrogen limited conditions, and the total RNAs were isolated using RNAsy MiniKit (Sigma) according to the manufacturer's recommendations.

Oligonucleotide 5'-CGCTTCTCAATGAGATTCAG-GGCGTGG-3' which is complementary to the region from +44 to +71 relatively to the *ygj*G ORF2 initiation codon was radiolabeled by T4 polynucleotide kinase (Pharmacia) with γ-[^{33}P]ATP (6000 Ci/mmol).

Aliquots of mRNA (5 μg) were denaturated at 65°C for 10 min, incubated in reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT) containing 0.5 ng of the labeled oligonucleotide, r*Tth* reverse transcriptase (5 u) (Perkin Elmer) and 1 mM deoxyribonucleotide triphosphates at 37°C for 1 h. The synthesized cDNAs were denaturated at 65°C for 10 min and analyzed by electrophoresis on 6% acrylamide gel. The same primer was used for the DNA sequence ladder run on the same gel. The quantities of synthesized cDNAs were estimated using Typhoon 9210 imager and ImageQuant version 5.2 software (Amersham, Molecular Dynamics).

Purification of Ht-YgjG

The Ht-YgjG was purified from 150 ml of BL21(DE3)(pET-Ht-ORF2) induced culture. Frozen cells

(60 mg) were thawed, suspended in 75 ml of buffer B (20 mM Tris-HCl, pH 8.0, 1 mM PMSF) and disrupted by sonication followed by centrifugation to remove debris (10 000 g, 20 min, 4°C). The imidazol and NaCl were added to supernatant up to final concentrations of 50 mM and 500 mM, respectively. The supernatant was applied to a His-Trap column (1 ml) (Pharmacia) equilibrated with the same buffer. The protein was eluted with 10 ml of buffer C (20 mM Tris-HCl, pH 8.0, 400 mM imidazol, 500 mM NaCl). The Ht-YgjG containing fractions were applied on Sephadex G-25 column (Pharmacia) equilibrated with buffer D (20 mM potassium phosphate, pH 7.4, 15% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μM PLP) and eluted in the same buffer. The Ht-YgjG containing fractions were combined, aliquoted and stored at -70°C until required.

The protein concentration was estimated following the method described by Bradford with bovine serum albumin as the standard [32]. Proteins were separated by SDS-PAGE (10%) [33].

Enzyme assays and analytical methods

β-Galactosidase assay was performed in cell-free extracts of TG1 cells harboring pBR-σ^{54}-lacZ-1, pBR-σ^{54}-lacZ-2 and pBR-lacZ-3 plasmids according to Miller [24].

PATase activity was routinely assayed as the formation of L-glutamic acid from putrescine and 2-OG at 37°C. The assay mixture contained (in 0.1 ml of total volume) 100 mM Tris-HCl, pH 9.0, 25 μM PLP, 10 mM putrescine, 10 mM 2-OG. The reaction mixture was preincubated at 37°C for 5 min, and the reaction was started by the addition of cell extract or enzyme solution (5 μl). The reaction was stopped by adding 15 μl of HCl (10%). The analysis of glutamic acid formation was carried out using a Quanta 4000E Capillary Electrophoresis System (Waters) with an uncoated fused-silica capillary (75 μm inner diam. × 60 cm) at 25 kV potential. The injection was performed hydrostatically for 25 s. The separation buffer consisted of 50 mM Tris-base, 25 mM benzoic acid, pH 8.5, 0.25 mM tetradecyl-trimethyl-ammonium bromide (for indirect UV detection at 254 nm). The calibration at three concentrations of the L-glutamic acid (0.025 mM, 0.05 mM, 0.1 mM) and three concentrations of the 2-OG (0.05 mM, 0.1 mM, 0.2 mM) was carried out.

The kinetic constants of the Ht-YgjG were evaluated by varying the concentration of putrescine at a 2-OG concentration of 20 mM or by varying the concentration of 2-OG at a putrescine concentration of 15 mM. The enzyme kinetics data were fitted to Michaelis-Menten kinetics and K_m values were calculated. The substrate specificity of Ht-YgjG was determined using assay as described above with different polyamines and amino acids as amino group donors and 2-OG as acceptor, or with different acceptors and putrescine as amino group donor.

Authors' contributions

N.N.S. carried out the experimental part of the study and drafted the manuscript. S.V.S. conceived of the study, participated in its design and coordination, and performed capillary electrophoresis. I.B.A. carried out the primer extension study. L.R.P. conceived of the study, supervised the work, and edited the manuscript. All authors have read and approved the final manuscript.

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