JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 6893–6901 Vol. 187, No. 20
0021-9193/05/$08.00+0 doi:10.1128/JB.187.20.6893–6901.2005
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Genetic Analysis Identifies a Function for the *queC* (*ybaX*) Gene Product at an Initial Step in the Queuosine Biosynthetic Pathway in *Escherichia coli*

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Received 17 May 2005/Accepted 4 August 2005

Queuosine (Q), one of the most complex modifications occurring at the wobble position of tRNAs with GUN anticodons, is implicated in a number of biological activities, including accuracy of decoding, virulence, and cellular differentiation. Despite these important implications, its biosynthetic pathway has remained unresolved. Earlier, we observed that a naturally occurring strain of *Escherichia coli* B105 lacked Q modification in the tRNAs. In the present study, we developed a genetic screen to map the defect in *E. coli* B105 to a single gene, *queC* (renamed from *ybaX*), predicted to code for a 231-amino-acid-long protein with a pI of 5.6. As analyzed by mobility of $tRNA^{Tyr}$ on acid urea gels and two-dimensional thin-layer chromatography of the modified nucleosides, expression of QueC from a plasmid-borne copy confers a Q^+ phenotype to *E. coli* B105. Further, analyses of $tRNA^{Tyr}$ from *E. coli* JE10651 (*queA* mutant), its derivative generated by deletion of chromosomal *queC* (*queA* Δ*queC*), and *E. coli* JE7325, deficient in converting $preQ_0$ to $preQ_1$, have provided the first genetic evidence for the involvement of QueC at a step leading to production of $preQ_0$, the first known intermediate in the generally accepted pathway that utilizes GTP as the starting molecule. In addition, we discuss the possibilities of collaboration of QueC with other cellular proteins in the production of $preQ_0$.

Modified nucleosides found in all tRNAs make important contributions to their structural integrity and biological functions (4). Biochemistry of modified nucleoside biosynthesis ranges from a simple one-step reaction mediated by a single enzyme to a complex multistep pathway involving a battery of enzymes and cofactors. Queuosine (Q), 7-{5-[(4S, 5R-dihydrooxy-2-cyclopenten1S-yl)amino]methyl}-7-deazaguanosine, one of the most complex nucleoside modifications, occurs at the wobble position of GUN anticodons in tRNAs for Asn, Asp, Tyr, and His (14, 18). Queuosine is found in nearly all eukaryotic and eubacterial organisms, with the known exceptions of *Saccharomyces cerevisiae* and *Mycoplasma* spp. (47). Archaea lack Q but possess a related modified nucleoside, archaeosine (12). Biosynthesis of Q is restricted to eubacteria. Eukaryotes obtain queuine, the base of Q, from diet or intestinal microflora (19) and insert it into the tRNAs using the tRNA guanine transglycosylase.

Queuosine modification modulates the codon-anticodon interactions and enhances translational fidelity (3, 25, 44). Interestingly, cells deficient in Q modification downregulate translation of VirF, the most upstream regulator of virulence in *Shigella flexneri* (9). Queuosine deficiency adversely affects the fitness of *Escherichia coli* under limiting nutrient conditions (8, 28) and prevents aggregate formation in *Dictyostelium discoideum* (19). Hypomodification of tRNAs for Q has also been correlated with cellular differentiation (23), malignant tumors, neoplastic cell lines (2, 10, 33), and oncogenic transformation of fibroblasts (27). A surprising aspect of Q biochemistry that was recently revealed is its glutamylation in $tRNA^{Asp}$ by YadB (36). The presence of this "modification of a hypermodification" further deepens the mystery of the physiological roles of Q and its analogs.

Although the Q modification has been known for over three decades, details of its biosynthetic pathway remain unresolved. As shown in Fig. 1, Q is believed to arise from GTP (17, 21) by the action of an unknown cyclohydrolase-like enzyme to form 7-cyano-7-deazaguanine, $preQ_0$ (29), which is then converted to 7-aminomethyl-7-deazaguanine ($preQ_1$) (31). The enzyme Tgt exchanges the base of G34 in the anticodon with $preQ_1$ (30). However, Tgt is also known to utilize $preQ_0$ and guanine as substrates with lower efficiencies (17). $preQ_1$ is further modified at the level of tRNA. The enzyme QueA utilizes *S*-adenosylmethionine (40) to form epoxyqueuosine (oQ) (11, 32). In the final step, an unknown enzyme reduces oQ to Q in a vitamin B12-dependent manner (11).

Genetic studies have played a major role in identification of both the Tgt and the QueA steps in Q biosynthesis (29, 31, 35). However, difficult genetic screens to isolate such strains (28) combined with complex biochemical assays have rendered most other proposed steps in the Q biosynthetic pathway virtually intractable. Recently, a bioinformatics approach led to identification of a tetracistronic operon, *ykvJKLM* (renamed *queCDEF*), in *Bacillus subtilis* for its involvement at uncharacterized steps of Q biosynthesis upstream of Tgt (34). In *E. coli*, orthologs of these cistrons (*ybaX*, *ygcM*, *ygcF*, and *yqcD*, respectively) are found independently in different locations. Further, the product of *queF* thought to be the missing cyclohydrolase turned out to be an enzyme that converts $preQ_0$ to $preQ_1$ (45). Functional details of the products of the other cistrons (*queC*, *queD*, and *queE*) have not been established.

Earlier, we reported (8) on the serendipitous observation of an uncharacterized mutation in *E. coli* B105 resulting in the absence of Q (Q^-). Here, we present a genetic analysis of this strain to identify *queC* (*ybaX*) as a new member of the Q

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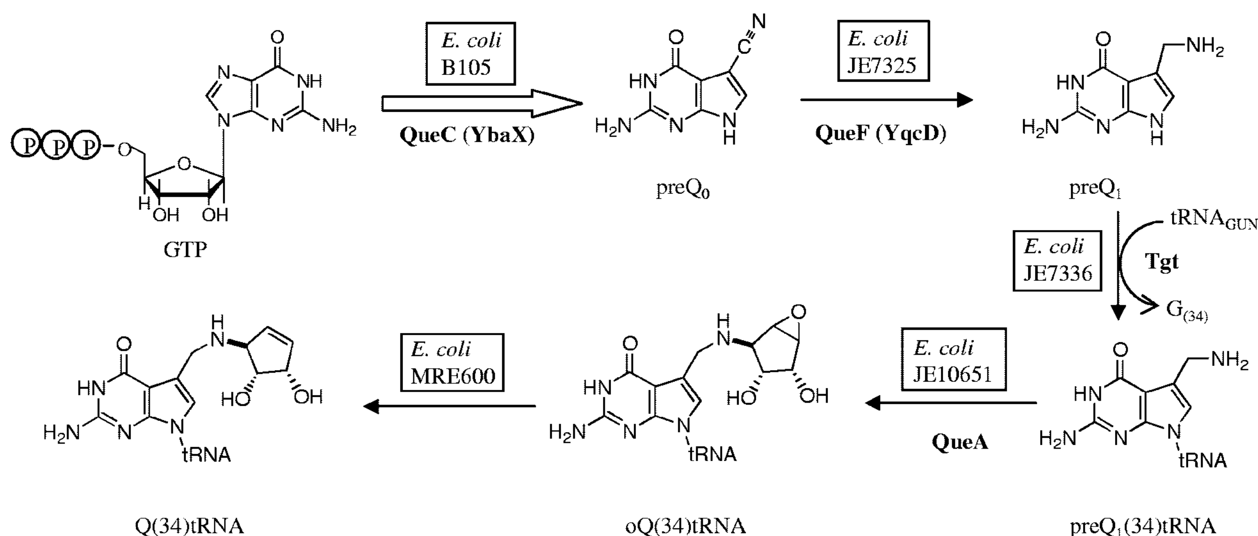

FIG. 1. Current scheme of queuosine incorporation in tRNAs. The involvement of various proteins at different steps in the pathway and the *E. coli* strains deficient at these steps are shown. Transformation of GTP to $preQ_0$ at the first step in the pathway is shown by an open arrow. This transformation may involve additional proteins (such as QueD and QueE [13]) or may represent a multistep process.

biosynthetic pathway whose gene product is essential in biosynthesis of $preQ_0$ in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in LB broth or agar medium (26). When required, ampicillin (Amp, 100 $\mu g\ ml^{-1}$), kanamycin (Kan, 25 $\mu g\ ml^{-1}$) chloramphenicol (Cam, 30 $\mu g\ ml^{-1}$), or tetracycline (Tet, 7.5 $\mu g\ ml^{-1}$) was added to the medium. For growth curve experiments, 0.05% (vol/vol) of saturated cultures was inoculated into 50 ml fresh LB broth with the appropriate antibiotic. Growth was monitored by measuring absorbance (595 nm) at hourly intervals.

Generation of a transposon library in *E. coli* BL21 and isolation of *E. coli* B105 (Q^+). Transposon (Tn*10* Kan^r version) delivery vehicle λ NK1316 was prepared by infecting a permissive host, *E. coli* LE392, and used to generate a transposon insertion library (~26,000 colonies) in *E. coli* BL21 (20). The P1 phage lysate raised on the library (donor) was used for genetic transfer into *E. coli* B105 (recipient) by P1-mediated transduction (26). Transductants (~13,000) thus obtained were pooled and made competent by the $CaCl_2$ method (37) to take up pTZ*queA-tgt* (Amp^r), and the earliest appearing transformants (12 h) were screened for the Q^+ phenotype by analysis of Tyr-$tRNA^{Tyr}$ using acid urea gels (46).

Isolation of tRNA, acid urea polyacrylamide gel electrophoresis, and Northern analysis. Total tRNA was isolated under acidic conditions from log-phase cultures of various strains, fractionated on polyacrylamide (6.5%) acidic (pH 5.0) urea (8 M) gels, and transferred onto a Nytran membrane (46). When needed, the tRNA preparations were subjected to alkaline treatment (0.1 M Tris-HCl, pH 9.0, at 37°C for 30 min) to deacylate the tRNAs prior to electrophoresis. Northern blot analysis was performed using a 5′-^{32}P end-labeled DNA oligomer (5′ TACAGTCTGCTCCCTTTGGCCGCTC 3′) complementary to $tRNA^{Tyr}$ (8).

Mapping of the transposon insertion site in B105 (Q^+). The inverse-PCR approach (15) was used to map the transposon insertion site in B105 (Q^+). Briefly, 1 μg genomic DNA from *E. coli* B105 (Q^+) was treated with 10 U of Sau3AI at 37°C for 5 h in a 30-μl volume, followed by heating at 70°C for 20 min

TABLE 1. List of *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype/characteristic	Reference and/or source
Strains		
B105	An *E. coli* B strain, r_B^- m_B^- Gal^- *queC*	24, this study
B105 (Q^+)	B105 Tn*10* (Kan^r) Q^+	This study
BL21	F^- *hsdS gal*	41
HB101	*supE44 hsdS20* (r_B^- r_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	5
JE7325	Incorporates $preQ_0$ in $tRNA^{Tyr}$ at 43°C	29
JE10651	*queA* mutant	35
JE10651Δ*queC*	*queA* Δ*queC*	This study
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	37
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	37
Plasmids		
pACDH	Tet^r	8
pTZ*queA-tgt*	Amp^r, contains *queA* and *tgt* genes from *E. coli* cloned into pTZN (a pTZ19R-derived vector)	8
pTZ*queA*Δ*tgt*	Same as pTZ*queA-tgt*, except for an ~200-bp deletion in the *tgt* reading frame	8
pTrc*queC*	Amp^r, *queC* amplified from *E. coli* HB101 cloned into pTrc99C	This study
pKD3		7
pKD46	Amp^r Cam^r, *cat* is flanked by FRT sequences	7
pCP20	Amp^r, harbors λ Red recombination genes (γ, β, and *exo*) and expresses FLP recombinase	7

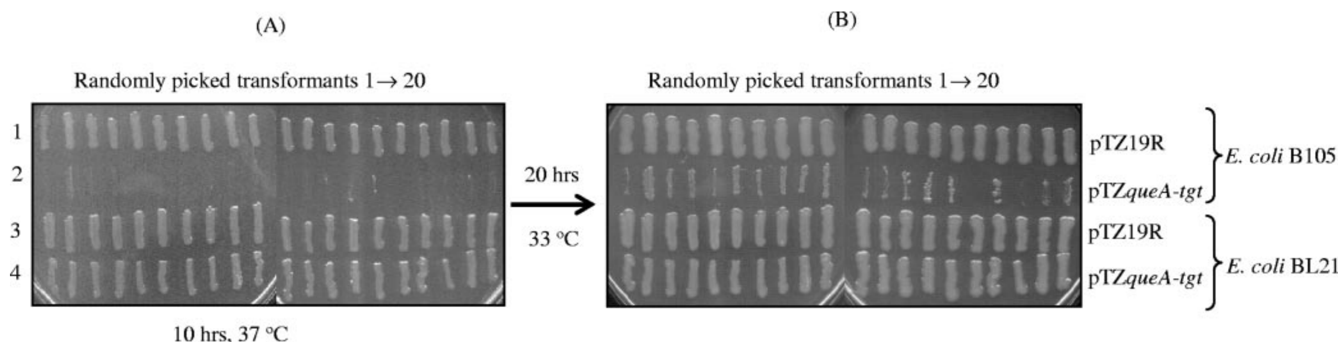

FIG. 2. Growth of *E. coli* B105 (rows 1 and 2) and *E. coli* BL21 (rows 3 and 4) transformants on LB agar (Amp) plates. Transformants in rows 1 and 3 harbored pTZ19R vector, whereas those in rows 2 and 4 harbored the pTZ*queA-tgt* plasmid. Randomly picked transformants (20 each) were patched and grown at 37°C for 10 h, photographed (A), and incubated further for 20 h at 33°C (B).

to inactivate the enzyme. The reaction was diluted to 300 μl, and a 150-μl aliquot was supplemented with 16.6 μl of 10× ligase buffer and 5 U of T4 DNA ligase and incubated at 16°C for 12 h. The DNA was ethanol precipitated and taken up in 10 μl water, and a 5-μl aliquot was used for PCR in a 50-μl reaction containing 200 μM deoxynucleoside triphosphates (dNTPs), 20 pmol each of forward and reverse primers (15), 5 μl of 10× *Taq* buffer, and 1 U *Taq* DNA polymerase. The PCR conditions were as follows: 94°C for 4 min, followed by 29 cycles of 94°C for 1 min, 53°C for 30 s, and 70°C for 1 min, followed by 70°C for 10 min and 4°C for 5 min. The product (~600 bp) was cloned into pGEMTeasy and sequenced by Sanger's dideoxy method using the vector-specific reverse primer 5′ CAGG AAACAGCTATGAC 3′.

Cloning of *queC* (*ybaX*). *queC* was amplified from *E. coli* HB101 DNA using *Pfu* DNA polymerase in a 50-μl PCR containing 20 pmol each of forward (5′ AAACGTGCTGTCGTTGTGTTC 3′) and reverse (5′ GTGGATCCGGATGC TCAAGCCG 3′) primers, as well as 200 μM dNTPs in the supplied buffer. Initial denaturation was done at 95°C for 4 min, followed by 30 cycles of 94°C for 1 min, 46°C for 30 s, and 70°C for 1 min 35 s, and a final extension of 10 min at 70°C. For cloning, the vector pTrc99C was digested with NcoI, supplemented with dNTPs (final concentration of 200 μM), and end filled with the Klenow fragment of DNA polymerase I. The vector was then digested with BamHI, eluted after agarose gel electrophoresis, and ligated to a BamHI-digested *queC* amplicon.

In vivo labeling of tRNA and enrichment of tRNA^{Tyr}. Exponentially growing cells of *E. coli* B105 harboring vector (pTrc99C) or pTrc*queC* were harvested, metabolically labeled with [^{32}P]orthophosphate in low-phosphate medium, and used to isolate total tRNA by phenol extraction of the cells (38, 42). Approximately 75 ng of a biotinylated DNA oligomer (5′ Biotin-GTCTGCTCCCTTTG GCCGCTCGGGAA 3′) complementary to tRNA^{Tyr} was mixed with total tRNA preparation in 2× SET buffer (0.3 M NaCl, 50 mM Tris-HCl [pH 8.0], and 2 mM EDTA), heated at 95°C for 5 min, and allowed to return to room temperature over 3 h. A 50-μl aliquot of streptavidin-iron oxide resin (Sigma) was added to

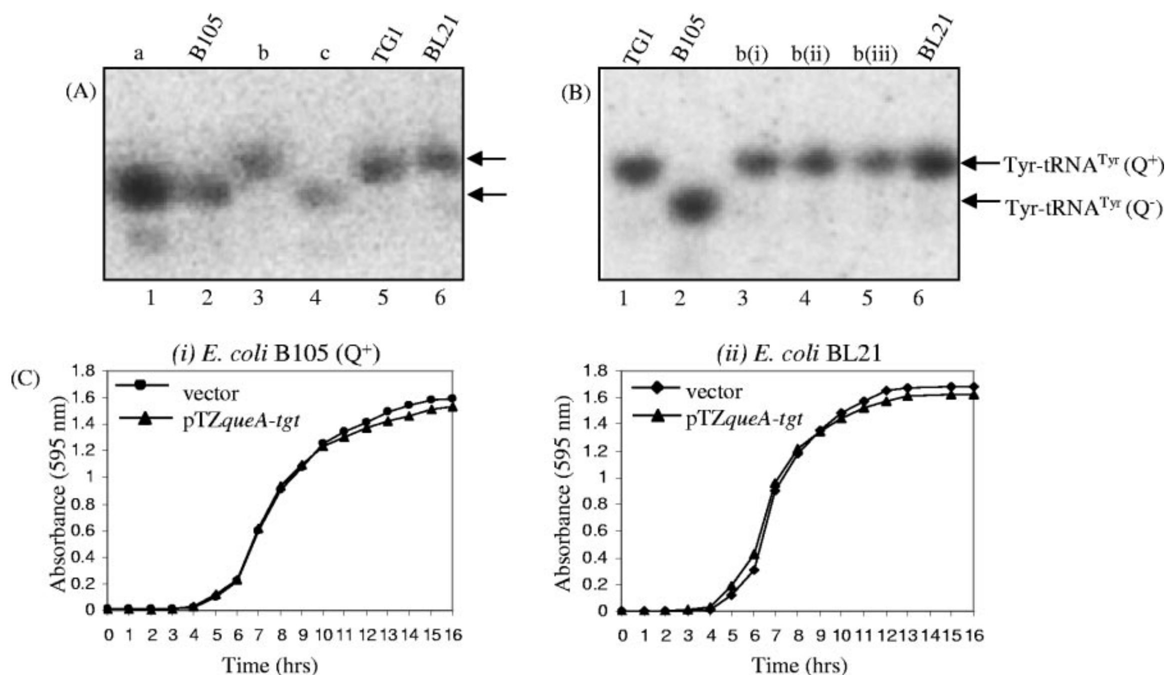

FIG. 3. Analysis of tRNA^{Tyr} in *E. coli* B105 transductants (A and B) and growth of the *E. coli* B105 (Q$^+$) transductant and the *E. coli* BL21 control (C). Total tRNA was fractionated on an acid urea gel, transferred to a nylon membrane, hybridized to ^{32}P-labeled probe against tRNA^{Tyr}, and subjected to BioImage analysis (FLA2000; Fuji). (A) Analysis of tRNA from the putative transductants (lanes 1, 3, and 4); the Q$^+$ control strains, *E. coli* TG1 and *E. coli* BL21 (lanes 5 and 6, respectively); and the recipient strain, *E. coli* B105 (Q$^-$) (lane 2). (B) The positive transductant b (shown in lane 3 of panel A) was streaked, and three daughter colonies [b(i), b(ii), and b(iii)] were analyzed (lanes 3 to 5) along with positive (lanes 1 and 6) and negative (lane 2) controls. (C) Growth of the *E. coli* B105 (Q$^+$) transductant (i) and *E. coli* BL21 (ii), harboring vector (pTZ19R) or pTZ*queA-tgt*, as indicated.

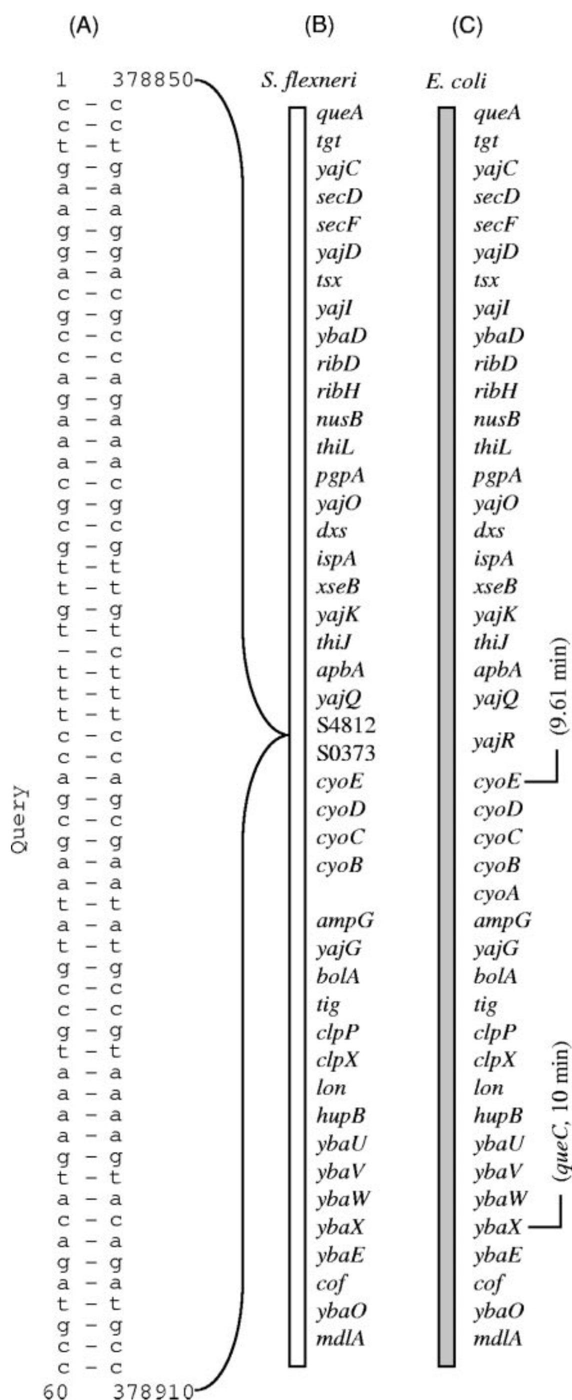

FIG. 4. Mapping of transposon insertion site in *E. coli* BL21 (Q^+) transductant. (A) Alignment of the sequence flanking the transposon (Query) with the homologous sequence found between the S4812 and S0372 genes in *S. flexneri*. (B) Gene map of *S. flexneri* in the vicinity of S4812 and S0372 (as annotated for *S. flexneri* 2a strain 2457T, GenBank accession no. AE014073, version AE014073.1). (C) Corresponding genome region in *E. coli* (as annotated for *E. coli* K-12 MG1655, GenBank accession no. U00096, version U00096.1).

the mix, allowed to bind for 30 min, and pulled down under a magnetic field. The pellet was washed with 100 μl water, resuspended in 75 μl water, heated to 95°C for 5 min, and quick-chilled on ice, and the particles were repelleted with a magnet. The supernatant was used for base analysis (8, 28).

Generation of *E. coli* JE10651Δ*queC* (*queA* Δ*queC*). The *queC* (*ybaX*) knockout in *E. coli* JE10651 was generated as described by Datsenko and Wanner (7). Briefly, 10 pmol each of the *ybaX*ko_fp (5′ GCTGTCGTTGTGTTCAGTGGA GGTCAGGATTCCACCGTGTA-GGCTGGAGCTGCTTCG 3′) and *ybaX*ko_ rp (5′ CCTCAACCCGGTTTTCTGCTTCAT-CGCTGCCATCACCATATGA ATATCCTCCTTA 3′) primers was used to amplify a Cam^r cassette from pKD3 with *Pfu* DNA polymerase. The linear DNA was purified and electroporated into *E. coli* JE10651 harboring pKD46. The transformants were selected on Cam, and the allelic exchange (*queC*::*Cam*) was confirmed by PCR amplification of the *queC* locus with an upstream *ybaX*upfp primer (5′ ATGAATAGCTGGTC CGGG 3′) and the downstream cloning primer, *ybaX*rp. Subsequently, the Cam cassette was excised by introduction of the plasmid pCP20 (Amp^r), and the culture was grown at 42°C to lose pCP20 (7). The *queA* Δ*queC* strain thus generated was sensitive to the antibiotics used and suitable for the introduction of plasmids harboring the *queA* or *queC* gene.

RESULTS

Development of a genetic screen to identify defect in queuosine biosynthetic pathway in *E. coli* B105. By making use of the acid urea gels, we identified a Q^- strain of *E. coli* B105 (8). Detailed analyses revealed that both cistrons of the *queA-tgt* operon in the strain coded for functional products. Interestingly, multicopy presence of the *queA-tgt* plasmid, but not of a *queA*Δ*tgt* plasmid, was found to be toxic to *E. coli* B105. Expectedly, as shown in Fig. 2, when transformants of *E. coli* B105 harboring either the vector or the *queA-tgt* plasmid (which appear upon prolonged incubation) were patched, the transformants harboring the vector but not the *queA-tgt* plasmid grew prominently in 10 h (Fig. 2A, rows 1 and 2). The transformants harboring the *queA-tgt* plasmid revealed weak growth when the plates were left further for 20 h (Fig. 2B, row 2). On the contrary, growth of *E. coli* BL21, a common laboratory B strain (Q^+, but not isogenic with *E. coli* B105), containing either the vector or the *queA-tgt* plasmid was indistinguishable at any time (Fig. 2A and B, rows 3 and 4). While the reasons behind the toxicity of the *queA-tgt* plasmid in *E. coli* B105 are unclear, the observations suggested that a genetic transfer converting *E. coli* B105 Q^- to Q^+ could alleviate the toxicity caused by the *queA-tgt* plasmid and allow for a simple genetic screen to identify the missing genetic information.

Transductional crosses between *E. coli* BL21 (Q^+) and *E. coli* B105 (Q^-). Using standard genetic methods, we first prepared a library of randomly inserted transposon (Kan^r) in the chromosome of *E. coli* BL21 (Q^+). The P1 phage raised on this library was then used to transduce *E. coli* B105. A pool of ~13,000 transductants (Kan^r) was made competent to take up the *queA-tgt* plasmid, and the status of Tyr-$tRNA^{Tyr}$ from the earliest-appearing transformants was analyzed by acid urea gels. This analysis resulted in isolation of one Q^+ transductant (Fig. 3A, lane 3). The stability of the Q^+ phenotype and its linkage with the Kan^r transposon were further confirmed by its growth on a Kan plate and analysis of the tRNA from the daughter colonies. The Tyr-$tRNA^{Tyr}$ from all daughter colonies (Fig. 3B, lanes 3 to 5) migrated with the same mobility as that from the Q^+ strains such as *E. coli* TG1 (Fig. 3B, lane 1) and donor *E. coli* BL21 (Fig. 3B, lane 6). In contrast, Tyr-$tRNA^{Tyr}$ from parent *E. coli* B105 (Q^-) showed the characteristic faster mobility (Fig. 3B, lane 2). Back transductions using *E. coli* B105 (Q^+) as the donor and parent *E. coli* B105 harboring pACDH (to mark it with Tet^r) as the recipient showed a cotransduction of the Kan^r transposon and the ac-

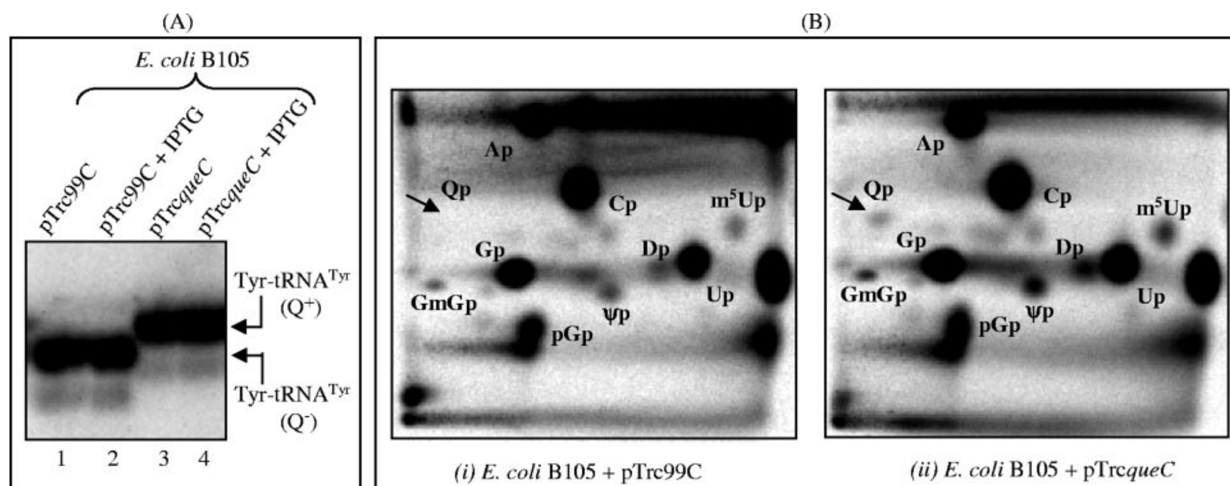

FIG. 5. Analysis of the effect of QueC expression in *E. coli* B105. (A) Northern blot analysis for Tyr-tRNA^{Tyr} from *E. coli* B105 harboring pTrc99C vector (lanes 1 and 2) or *E. coli* B105 harboring pTrc*queC* (lanes 3 and 4), not induced (lanes 1 and 3) or induced (lanes 2 and 4) with 0.5 mM IPTG. (B) Modified base analysis of tRNA^{Tyr} enriched from *E. coli* B105 harboring the pTrc99C vector (i) or pTrc*queC* (ii) by two-dimensional thin-layer chromatography on cellulose F. The spot corresponding to queuosine (Qp), marked by the arrow, is seen in panel ii but not in panel i.

quired marker(s) conferring the Q$^+$ phenotype, with a frequency of ~50% (out of 18 transductants analyzed, 9 were Q$^+$ [data not shown]) suggesting a close linkage between the two. Furthermore, as with *E. coli* BL21, when the *queA-tgt* plasmid was introduced into the *E. coli* B105 (Q$^+$) transductant, its growth was no longer discriminated from that harboring the vector (Fig. 3C, panels i and ii).

Mapping of the transposon insertion site in *E. coli* B105 (Q$^+$). To characterize the site of transposon insertion, we used a divergent set of primers at the ends of the known sequences of the transposon to amplify the flanking DNA from *E. coli* B105 (Q$^+$) by inverse PCR and to sequence it. Use of the 60-nucleotide sequence obtained for the DNA flanking the transposon, as a query for BLAST, revealed no significant matches with any of the presently available *E. coli* genome sequences (none of which are from B strains). However, this analysis showed a remarkably good match (98%) with *Shigella flexneri* 2a strain 2457T (Fig. 4A) and the unfinished genome of *Shigella sonnei*. The region of match in *S. flexneri* lies between hypothetical genes S4812 and S0372 (Fig. 4B), about 700 bp away from *cyoE*. Interestingly, the genome organizations in the vicinity of *cyoE* for *E. coli* K-12 and *S. flexneri* are very similar (compare Fig. 4B and C), allowing us to deduce that the Kan^r transposon in the *E. coli* B105 (Q$^+$) transductant landed in the vicinity of *cyoE* (~9.61 min). At this juncture, a report (34) showed that the genes of the *ykvJKLM* operon are involved in Q biosynthesis in *B. subtilis*. One of the genes at the 10-min locus in the *E. coli* genome, *queC* (renamed from *ybaX*), showed 57% sequence identity and 75% sequence similarity to *ykvJ* at the amino acid level. These observations, and the linkage of ~50% between the Q$^+$ and Kan^r (at 9.61 min) markers in P1-mediated transductions, encouraged us to investigate a possible role of QueC in conferring the Q$^+$ phenotype to *E. coli* B105.

***queC* complements *E. coli* B105 Q$^-$ phenotype.** The *queC* open reading frame was cloned into pTrc99C to yield the pTrc*queC* expression construct and introduced into *E. coli* B105. As shown in Fig. 5A, the presence of pTrc*queC* in *E. coli* B105, irrespective of induction by IPTG (isopropyl-β-D-thiogalactopyranoside), conferred slower mobility to Tyr-tRNA^{Tyr}, which corresponded to the Q$^+$ phenotype (Fig. 5A, compare lanes 3 and 4 with lanes 1 and 2). However, to further confirm the role of QueC, ^{32}P-body-labeled tRNA from *E. coli* strain B105 harboring either the vector (pTrc99C) or the expression construct (pTrc*queC*) was enriched for tRNA^{Tyr} and subjected to modified base analysis (8, 28). As shown in Fig. 5B, the tRNA^{Tyr} from *E. coli* B105 containing pTrc*queC* (Fig. 5B, panel ii) revealed a spot corresponding to queuosine (Qp) which was absent from the tRNA^{Tyr} prepared from *E. coli* B105 with vector control (Fig. 5B, panel i). These data demonstrate that a lack of functional QueC in *E. coli* B105 led to its Q$^-$ phenotype and that *queC* codes for a function in the Q biosynthesis pathway in *E. coli*.

***queC* gene product functions at an initial step in Q biosynthesis pathway.** The penultimate step in Q biosynthesis is the conversion of preQ$_1$ to oQ, which is carried out by QueA (Fig. 1). To map the step at which QueC participates, we generated a knockout of *queC* in *E. coli* JE10651 (*queA* mutant). The tRNA from *E. coli* JE10651, a *queA* mutant strain, migrates as a diffuse band between Q$^+$ and Q$^-$ tRNAs (Fig. 6A, compare lane 3 with lanes 1 and 2). Expectedly, when the strain was provided with a plasmid-borne copy of *queA*, mobility of the tRNA was rescued to that of the Q$^+$ form (Fig. 6A, compare lane 3 with lanes 2 and 4). On the other hand, introduction of a plasmid-borne copy of *queC* did not alter the mobility of the tRNA (Fig. 6A, compare lanes 3 and 5). However, deletion of the *queC* gene from the strain resulted in a faster mobility of the tRNA, corresponding to the Q$^-$ form (Fig. 6A, compare lanes 3 and 6), which could not be rescued by the presence of plasmid-borne *queA* (Fig. 6A, compare lanes 4 and 7). As a control, plasmid-borne *queC* restored its mobility to that of the tRNA from *E. coli* JE10651 (Fig. 6A, compare lanes 3 and 8). Similar observations were made when the analysis was repeated using deacylated preparations of tRNA (Fig. 6B).

FIG. 6. Analysis of the effect of deletion of *queC* on tRNA^{Tyr} in *E. coli* JE1065. Total tRNA from the indicated strains was prepared under acidic conditions (A) or deacylated by treatment under alkaline conditions (B) and subjected to Northern blot analysis to probe for tRNA^{Tyr}, followed by electronic autoradiography on a BioImage analyzer (FLA2000; Fuji). Details of tRNA samples are indicated above each lane.

Taken together, these observations clearly suggest that QueC functions upstream of QueA (Fig. 1). Since Tgt is known to be involved immediately upstream of QueA, the function of *queC* can be unambiguously placed at a step prior to that of Tgt in the generally accepted scheme of Q biosynthesis (Fig. 1).

To fine map the step of QueC function, we exploited yet another strain, *E. coli* JE7325, deficient in converting preQ$_0$ to preQ$_1$. Although the site of mutation responsible for this deficiency has not been characterized, its phenotype is more severe at a nonpermissive temperature of 43°C (29). As seen in Fig. 7, when *E. coli* JE7325 is grown at 30°C, Tyr-tRNA^{Tyr} bands corresponding to Q$^+$ and Q$^-$ forms are present. The faster-migrating band in the *E. coli* JE7325 sample most likely represents a population of tRNAs containing both G and preQ$_0$ at position 34 (29). Nevertheless, it is clear that the presence of pTrc*queC* does not result in any increase in Q$^+$ tRNA^{Tyr} or a change in the relative abundance of the two bands in *E. coli* JE7325 (Fig. 7, compare lanes 2 and 3). Expectedly, when the samples were subcultured at 43°C, the amount of tRNA in the Q$^+$ form decreased. At this temperature also, the presence of *queC* in *E. coli* JE7325 did not rescue the mobility of Tyr-tRNA^{Tyr} (Fig. 7, compare lanes 4 and 5). These observations clearly show that introduction of the *queC* plasmid in *E. coli* JE7325 does not complement the defect in the Q biosynthesis pathway and thus makes it unlikely that *queC* codes for a function involved in the conversion of preQ$_0$ to preQ$_1$. In fact, a very recent report has now shown that conversion of preQ$_0$ to preQ$_1$ is carried out by QueF (YkvM in

	1	2	3	4	5
% (Q$^+$)/(Q$^-$ + Q$^+$)	-	38	40	27	26

FIG. 7. Analysis of the effect of QueC expression on tRNA^{Tyr} in *E. coli* JE7325. Total tRNA was isolated from *E. coli* JE7325 harboring the indicated plasmids, grown at 30°C (lanes 2 and 3) or 43°C (lanes 4 and 5), and subjected to Northern blot analysis to probe for the status of tRNA^{Tyr}, followed by BioImage analysis (FLA2000; Fuji). Relative ratios of Q$^+$ and Q$^-$ forms of Tyr-tRNA^{Tyr} shown as percent values were calculated from pixel values in the corresponding bands by the following equation: $100 \times [Q^+/(Q^- + Q^+)]$. The dash indicates that no quantitation was carried out for the sample in lane 1 (a marker for Q$^+$ Tyr-tRNA^{Tyr}).

FIG. 8. Sequence alignment of QueC orthologs from archaeal and eubacterial species. Simel, *Sinorhizobium meliloti*; Memar, *Methanococcus maripaludis*; Feaci, *Ferroplasma acidiphilum*; Thvol, *Thermoplasma volcanium*; Anvar, *Anabaena varibilis*; Hamar, *Haloarcula marismortui*; Negon, *Neisseria gonorrhoeae* FA 1090; Basub, *B. subtilis*; Eco, *E. coli*; Buaph, *Buchnera aphidicola* strain Sg; Hepyl, *Helicobacter pylori*. The alignments were done with ClustalW, and the most-conserved residues were manually shaded.

B. subtilis) (45). Taken together, these observations strongly suggest that the function of QueC is upstream of preQ_0.

DISCUSSION

Queuosine is one of the most complex modifications occurring in the tRNAs. This modification influences such crucial functions as accuracy of decoding, virulence, and cellular differentiation. Furthermore, recently it was discovered that Q modification in $tRNA^{Asp}$ served as an unusual site for glutamylation by YadB, a paralog of GluRS. Such significant biological properties of the Q modification have made it engaging to study its biosynthetic pathway (13, 16, 36).

In an earlier study, we observed that a naturally occurring strain of *E. coli* B105 lacked Q modification in the tRNAs because of a defect in an uncharacterized gene(s). In the present study, we have genetically mapped the defect in *E. coli* B105 to a single gene, *queC* (*ybaX*), predicted to code for a 231-amino-acid-long protein with a pI of 5.6 and a molecular mass of 25.36 kDa (http://genolist.pasteur.fr/Colibri/). This study provides the first genetic evidence for the involvement of QueC at a step upstream of preQ_0, the first known intermediate in the generally accepted pathway that utilizes GTP as the starting molecule. Our findings may appear to contradict the predictions of this step being carried out by QueF (34). However, in a very recent report, QueF has been demonstrated to convert preQ_0 to preQ_1 (45), further supporting our analysis of the involvement of QueC in the generation of preQ_0.

The distribution of *queC* orthologs is another important indication of the role of *queC* in Q biosynthesis. Archaea are known to synthesize preQ_0 but not preQ_1 (17). As expected, *queC* orthologs are present in archaea (Fig. 8) (34). In the case of mammals, the situation is reversed: mammals obtain the free base queuine and insert it into tRNA. This obviates a requirement for the *queC* ortholog, and in fact the *queC* ortholog is not seen in a BLAST search with the mammalian genomes at the NCBI server. Only seven sequences relating to argininosuccinate or asparagine synthetases come up in such an analysis. Some classes of bacteria, viz., actinobacteria (*Mycobacterium* spp. and *Streptomyces* spp.) and mollicutes (*Mycoplasma* spp. and *Ureaplasma* spp.), also do not seem to possess a QueC-like sequence, and their genomes present argininosuccinate synthases as the best matches to QueC.

An alignment of several QueC orthologs (Fig. 8) from diverse species of archaea and eubacteria reveals the conserved motifs of this protein. The N terminus contains the SGGXDS motif that matches the SXGXDS signature motif of PP-loop ATPases (1). In addition, the comparison reveals the presence of four conserved cysteines in QueC towards the C terminus. Similar motifs have been found in the iron-sulfur cluster-containing and zinc-containing proteins involved in tRNA modifications (17, 22). The way in which these predicted biochemical properties may be related to a role for QueC at the initial step in the accepted Q biosynthetic pathway, wherein queuosine is believed to arise from GTP (reviewed in reference 17), remains to be investigated. The formation of preQ_0 was found to be similar to the biosynthesis of toyocamycin (21), and a GTP cyclohydrolase-like enzyme was proposed to catalyze this step. However, later studies ruled out the involvement of GTP cyclohydrolases I and II in this reaction (39). Considering that complex chemical transformations are needed for the conversion of GTP to preQ_0, such a role for QueC may well necessitate its functional interaction with other cellular proteins. Notably, QueD (*ykvK*, *ygcM*), QueE (*ykvL*, *ygcF*), and QueF (*ykvM*, *yqcD*) have also been suggested to function upstream of Tgt (13, 34). With the recent biochemical characterization that QueF carries out preQ_0-to-preQ_1 conversion (45), it is quite likely that QueD and QueE collaborate with QueC in the production of preQ_0, which may well be a multistep reaction. On the other hand, according to a recent large-scale protein-protein interaction study (6), QueC has been shown to interact with components of translation machinery (*rpsJ*, *tufA*), putative enzymes (*aidB*, *yfiD*), a transporter (*yegT*), and a chaperone (*dnaK*). However, the implications of these interactions for Q biosynthesis are not clear. Furthermore, although toyocamycin production has been studied for *Streptomyces rimosus* (43), its close relatives *Streptomyces avermitilis* MA4680 and *Streptomyces coelicolor* A3 (and actinobacteria in general) do not reveal a QueC ortholog in a protein-protein BLAST, which casts doubt on the proposal of similarity between toyocamycin and queuosine biosynthesis at this step.

Interestingly, with the characterization of the genetic defect in *E. coli* B105, we now have each of the steps in Q biosynthesis identified by a well-defined mutation in *E. coli* (Fig. 1). These strains, and the genetic screen developed in this study, could prove to be crucial in the biochemical characterization of the various proteins involved in this important pathway. Especially, the identification of the genetic defect in *E. coli* B105 at the initial step(s) in Q biosynthesis will be instrumental in detailed biochemical analysis of the reaction mediated by the *queC* gene product.

ACKNOWLEDGMENTS

We thank our laboratory colleagues and the anonymous reviewers for their suggestions and Akiko Nishimura, National Institute of Genetics, Mishima, Shizuoka-ken, 411-8540, Japan, for providing us with various JE series strains.

This work was supported by grants from the Department of Science and Technology, Department of Biotechnology, and Indian Council of Medical Research, New Delhi, India. R.G. is a senior research fellow of the Council of Scientific and Industrial Research, New Delhi, India.

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