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Effects on Transcription of Mutations in *ygjD*, *yeaZ*, and *yjeE* Genes, Which Are Involved in a Universal tRNA Modification in *Escherichia coli*▽†

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The *Escherichia coli ygjD* gene is critical for the universal tRNA modification N^6-threonylcarbamoyladenosine, together with two other essential genes, *yeaZ* and *yjeE*. This study showed that the transcription of the *thr* and *ilv* operons in *ygjD* mutants was increased through the inhibition of transcription attenuation and that *dnaG* transcription was reduced.

The *ygjD* gene is essential in *Escherichia coli* and is conserved among eukaryotes, bacteria, and *Archaea* (5). The functions of YgjD orthologs have been investigated in many microbial species. In *Mannheimia* (formerly *Pasteurella*) *haemolytica*, the YgjD ortholog was initially identified as an *O*-sialoglycoprotein endopeptidase (Gcp), suggesting that YgjD family members function as endopeptidases (1). However, the yeast YgjD ortholog, kinase-associated endopeptidase 1 (Kae1), has been reported to be a component of a complex known as KEOPS (kinase, endopeptidase, and other small proteins), which is involved in the regulation of telomere length (3). Kae1 also forms part of the endopeptidase-like kinase chromatin-associated (EKC) complex, which is involved in the transcription of essential genes (12). The YgjD ortholog in "*Pyrococcus abyssi*" possesses apurinic-endonuclease activity (8), and the YgjD ortholog Qri7 has been implicated in mitochondrial genome maintenance in both *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (17).

Recently, YgjD was shown to be essential for the synthesis of the universal tRNA modification N^6-threonylcarbamoyladenosine (t^6A). This raises the possibility that the previously defined functions of YgjD orthologs may actually be indirect effects of its role in translational regulation (4, 19). t^6A, which is present in all tRNAs that pair with ANN codons, comprises a carbonyl group and a threonine attached to the amino group of adenine at position 37, immediately 3′ of the anticodon. The role of t^6A is to strengthen the A-U codon-anticodon interaction (15), and it is necessary for the decoding of ANN codons.

In *E. coli*, YgjD functionally interacts with two additional essential proteins, YeaZ and YjeE, both of which are conserved in eubacteria (6, 19). YeaZ shows homology with YgjD and interacts physically with both YjeE and YgjD, mediating the proteolysis of the latter (6). It has also been suggested that YeaZ and YjeE regulate YgjD activity (6).

Previous studies characterized genes that are essential in *E. coli* by constructing mutants with large chromosomal deletions (7, 10, 11). The aim of the present study was to identify only genes that are functionally related to *ygjD*, *yeaZ*, and *yjeE*. The *ygjD*(Ts), *yeaZ*(Ts), and *yjeE*(Ts) mutants, which exhibited negligible growth at the nonpermissive temperature of 42°C, were isolated by random mutagenesis (9) (see Fig. S1 in the supplemental material). An *E. coli* chromosomal multicopy plasmid library was then screened for genes able to rescue the cell growth defects observed in the temperature-sensitive mutants. Several plasmids that reversed the growth defect seen in the *yeaZ*(Ts) mutant were isolated, and sequence analysis revealed that the cloned regions showed sequence identity to *yjeE*. Similarly, genes that corrected the growth defect observed in the *ygjD*(Ts) mutant showed homology to *yeaZ* and *yjeE*. Individual plasmids carrying *yeaZ*, *ygjD*, and *yjeE* were then introduced into the *ygjD*(Ts), *yeaZ*(Ts), and *yjeE*(Ts) mutant strains, and cell growth was examined at different temperatures (30°C, 37°C, and 42°C) (Fig. 1). *yjeE* strongly suppressed the growth of both the *yeaZ*(Ts) and *ygjD*(Ts) mutants; however, *yeaZ* weakly rescued the growth defect in the *ygjD*(Ts) mutant, and expression of *ygjD* corrected the growth defect in the *yeaZ*(Ts) mutant (Fig. 1). These results confirm that *yeaZ*, *ygjD*, and *yjeE* functionally interact with each other. Using a bacterial two-hybrid system, Handford et al. showed that YeaZ physically interacts with both YjeE and YgjD. Based on these results, they suggested that YeaZ and YjeE are regulators of YgjD (6). In agreement with this, bioinformatic and structural analyses suggested that YgjD and YeaZ heterodimerize through the same interface, and El Yacoubi et al. postulated that YeaZ is essential for the function of YgjD (4). They also showed that the complementation of *E. coli ygjD* function was dependent on both *ygjD* and *yeaZ* from *Bacillus subtilis*.

The finding that the "*P. abyssi*" ortholog of YgjD possesses apurinic-endonuclease activity raised the possibility that YgjD also interacts with DNA or RNA. A stock of multicopy plas-

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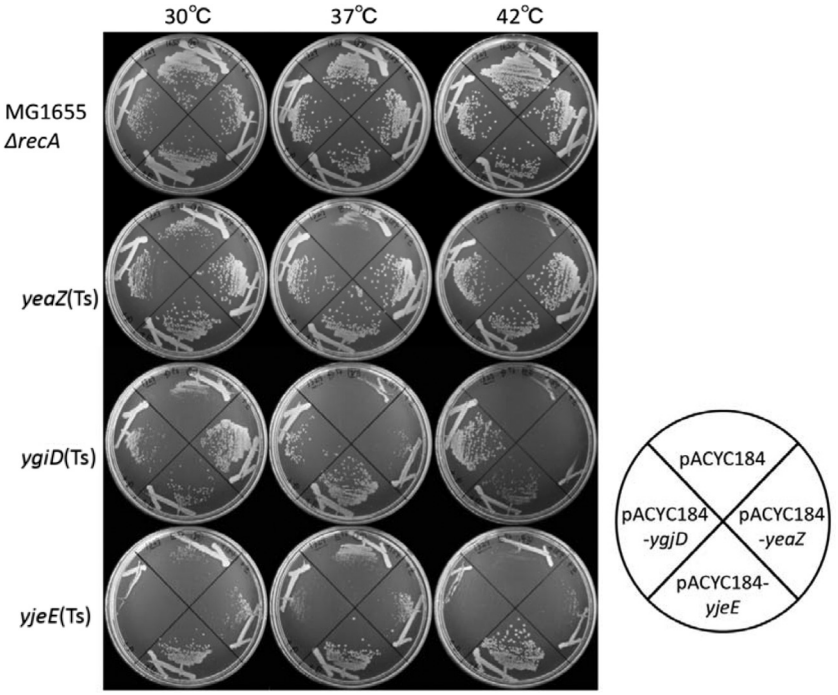


FIG. 1. Multicopy suppression of the *yeaZ*(Ts), *ygjD*(Ts), and *yjeE*(Ts) mutant phenotypes. pACYC184 or one of its derivatives carrying *yeaZ*, *ygjD*, or *yjeE* was introduced into *yeaZ*(Ts), *ygjD*(Ts), and *yjeE*(Ts) mutant strains. These *yeaZ*, *ygjD*, and *yjeE* plasmids carry the *E. coli* chromosomal regions 1888076 to 1890587, 3203778 to 3208659 (the same result was obtained with the *ygjD* plasmid carrying the 3206931-to-3208659 region), and 4392209 to 4394317, respectively. Transformants were streaked on Antibiotic Medium 3 and incubated at 30°C, 37°C, or 42°C. The genotypes of the temperature-sensitive mutants are as follows: *yeaZ*(Ts), Δ*yeaZ*::Km^r Δ*recA*::Tc^r/mini-F (Sm^r)-*yeaZ*(Ts); *ygjD*(Ts), Δ*ygjD*::Sm^r Δ*recA*::Tc^r/mini-F (Ap^r)-*ygjD*(Ts); *yjeE*(Ts), Δ*yjeE*::Sm^r Δ*recA*::Tc^r/mini-F (Ap^r)-*yjeE*(Ts).

mids that carry different genes involved in DNA repair/recombination, replication, and transcription were introduced into the *yeaZ*, *ygjD*, and *yjeE* temperature-sensitive mutant strains, and cell growth was examined at semipermissive temperatures. In all three mutants, growth was inhibited by plasmids carrying *nusB* or *rnhA* (see Fig. S2 in the supplemental material). NusB is an essential component of the RNA polymerase antitermination complex and is also involved in the transcription termination process at certain sites during normal bacterial growth (18). The *rnhA* gene encodes RNase HI, one of the RNase H proteins that specifically degrade the RNA component of RNA-DNA hybrids. In *E. coli*, RNase H removes RNA primers from the Okazaki fragments during the synthesis of the lagging strand and helps to suppress initiation at origins other than the *oriC* locus (14). The effects of the overexpression of *nusB* and *rnhA* in *E. coli* suggest that *yeaZ*, *ygjD*, and *yjeE* are involved in transcriptional regulation and/or DNA replication.

To examine the transcriptional profile of the *ygjD*(Ts) mutant, cells were incubated at 30°C to avoid inducing a heat shock response. The *ygjD*(Ts) mutant grew more slowly at 30°C than the isogenic *ygjD*$^+$ strain, indicating that this temperature was semipermissive for growth. Genes exhibiting altered transcription in the *ygjD*(Ts) strain compared to the wild-type strain at 30°C are shown in Table 1 (see Table S1 in the supplemental material). Expression of the *thr* operon was markedly increased in the *ygjD*(Ts) strain at 30°C (see Fig. S3A in the supplemental material). A similar analysis at 37°C (Table 2; see Table S2 in the supplemental material) showed that the expression of the *ilv* operon was also increased in the mutant strain (see Fig. S3B in the supplemental material). To confirm these results, the expression of *thrA* and *ilvG*, the upstream genes of those operons, was examined using a *lacZ* reporter gene assay (see Materials and Methods in the supplemental material for details). Expression of *thrA* was significantly increased in *yeaZ*(Ts), *ygjD*(Ts), and *yjeE*(Ts) mutants

TABLE 1. Up-regulated genes in the *ygjD*(Ts) mutant (30°C)

Gene	*ygjD*(Ts) signal intensity	Wild-type signal intensity	Ratio
thrC	2,320.2	472.5	4.9105
thrB	2,495.2	536.5	4.6509
thrA	3,494.8	871.2	4.0115
cirA	499.6	152.6	3.2739
ilvA	431.0	138.3	3.1164
ygdI	373.5	129.3	2.8886
osmE	342.3	126.9	2.6974
grpE	1,967.9	800.5	2.4583
fimA	1,710.6	708.4	2.4147
fimC	401.2	175.5	2.2860
rpiB	582.9	263.5	2.2121
ygjD	803.3	371.1	2.1646
rmf	240.5	113.4	2.1208
elaB	314.6	148.9	2.1128
ilvE	950.1	450.8	2.1076
cspD	280.2	134.1	2.0895
yiaG	467.3	224.0	2.0862
ybfH	288.6	140.4	2.0556
ryfA	713.3	348.8	2.0450
nlpA	985.2	483.0	2.0398

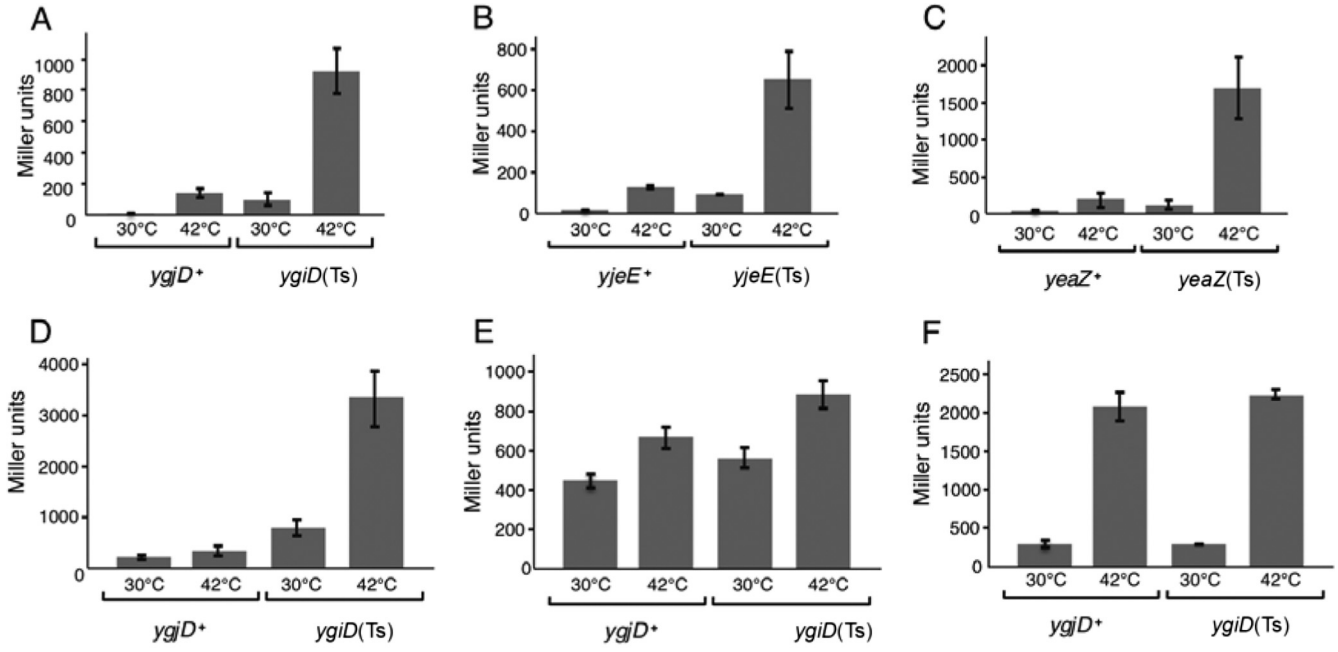
TABLE 2. Up-regulated genes in the *ygjD*(Ts) mutant (37°C)

Gene	*ygjD*(Ts) signal	Wild-type signal	Ratio
ilvG_2	1,023.5	137.2	7.4599
ilvG_1	2,380.2	471.7	5.0460
ilvE	632.4	203.0	3.1153
thrB	3,726.3	1,255.7	2.9675
dadA	487.7	171.4	2.8454
gcvT	670.5	239.6	2.7984
ybiJ	301.5	111.0	2.7162
ilvD	452.2	173.5	2.6063
rof	385.6	153.4	2.5137
lysA	333.8	133.0	2.5098
pheA	1,249.6	499.2	2.5032
thrC	3,913.0	1,587.1	2.4655
yjjJ	246.9	101.0	2.4446
aceB	443.9	188.8	2.3512
gdhA	514.9	219.8	2.3426
dadX	419.7	179.2	2.3421
thrA	5,104.3	2,184.8	2.3363
yicH	242.3	104.6	2.3164
putA	367.7	160.5	2.2910
yfdZ	248.7	109.8	2.2650

grown at 42°C (Fig. 2A, B, and C). The relative changes at 30°C were similar to those at 42°C, although there was a significant difference between the total expression levels at 30°C and 42°C. In addition, the expression of *ilvG* was increased in the *ygjD*(Ts) mutant (Fig. 2D).

Both the *thr* operon (containing *thrL*, *thrA*, *thrB*, and *thrC*) and the *ilv* operon (containing *ilvL*, *ilvG*, *ilvM*, *ilvE*, *ilvD*, and *ilvA*) are regulated by attenuation (13). The *thrL* and *ilvL* genes encode the leader peptides. This mechanism controls the ability of RNA polymerase to read through an attenuator, which is an intrinsic terminator located at the end of the leader region. Attenuation is controlled by the position of the ribosome on the leader transcripts. When the ribosome is prevented from translating the leader (*thrL* and *ilvL*), the terminator is inactive and downstream genes are transcribed.

The mechanisms that underlie the increased expression of the *thr* and *ilv* operons may be either increased transcription initiation or decreased efficiency of transcription attenuation. To discriminate between these two possibilities, the expression of *thrL* was examined using a *lacZ* reporter gene assay. The upstream region of *thrA* was inserted into the chromosomal *lac*



G

< *thrL* >

ATG AAA CGC ATT AGC ACC ACC ATT ACC ACC ACC ATC ACC ATT ACC ACA GGT AAC GGT GCG GGC TGA

< *ilvL* >

ATG ACA GCC CTT CTA CGA GTG ATT AGC CTG GTC GTG ATT AGC GTG GTG GTG ATT ATT ATC CCA CCG TGC GGG GCT GCA CTT GGA CGA GGA AAG GCT TAG

FIG. 2. Enhanced expression of *thr* and *ilv* operons in *yeaZ*(Ts), *ygjD*(Ts), and *yjeE*(Ts) mutant strains. β-Galactosidase activity was measured in temperature-sensitive mutants and their isogenic strains in which the *thrA* and *ilvG* upstream regions containing the promoter-leader peptide coding regions were inserted into the chromosomal *lac* operon to generate *thrA′-lacZ* and *ilvG′-lacZ* gene fusions, respectively. The fusion junctions are the initiation codons of the genes. See Materials and Methods and Fig. S5 to S7 in the supplemental material for details. (A) *thrA ygjD*(Ts). (B) *thrA yjeE*(Ts). (C) *thrA yeaZ*(Ts). (D) *ilvG ygjD*(Ts). β-Galactosidase activity in the *ygjD*(Ts) mutant and the parental strains carrying a *thrL′-lacZ* gene fusion (E) or a *thrA′-lacZ* terminator deletion mutant gene fusion (F). The amino acid sequences of the leader peptides of the *thr* and *ilv* operons (G). The codons for Thr, Ile, and Val are underlined, and the ANN codons are in bold font. Cells were grown overnight in Antibiotic Medium 3 and subcultured in fresh medium at a dilution of 1:100 at 30°C. Cells were incubated for 4 h, and 5 ml of the culture was used for the β-galactosidase assay. Cells were also incubated at 42°C for 2 h and assayed.

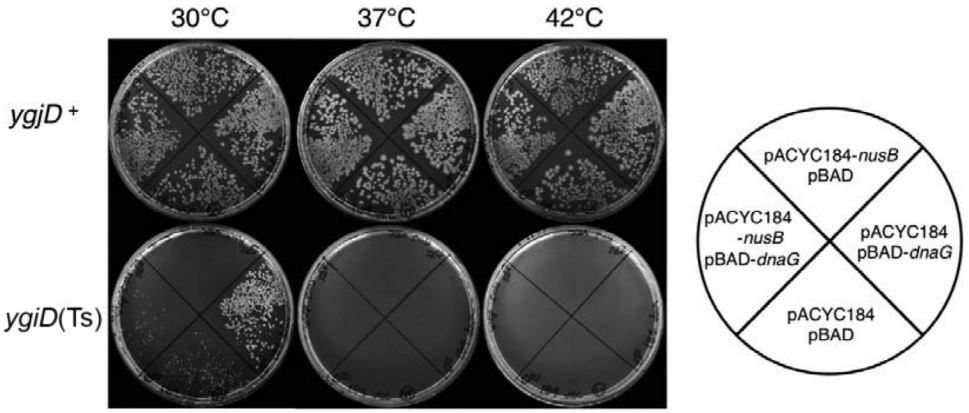


FIG. 3. Suppression of the *ygjD*(Ts) mutant phenotype by introduction of a *dnaG* multicopy plasmid. The *ygjD*(Ts) mutant and the isogenic parental strain (*ygjD*$^+$) were transformed with pACYC184-*nusB* or an empty vector, together with pBAD-*dnaG* (or an empty vector as a control). Transformants were plated on Antibiotic Medium 3 containing chloramphenicol and ampicillin and grown at 30°C, 37°C, or 42°C.

operon to generate a fused gene in which the *lacZ* initiation codon was replaced with that of *thrL*. No difference in expression at 30°C and 42°C was observed, and the expression of *thrL* did not differ between the *ygjD*(Ts) and *ygjD*$^+$ strains, indicating that transcriptional initiation is not affected by the *ygjD* mutation (Fig. 2E). To investigate the effects on attenuation, a *thr* mutant was constructed in which the Rho-independent terminator involved in attenuation was deleted (see Materials and Methods in the supplemental material for details). The effect of the expression of this gene fusion in the *ygjD*(Ts) mutant was similar to that in *ygjD*$^+$, which suggests that attenuation at the *thr* operon is inhibited by the *ygjD* mutation (Fig. 2F).

The leader peptides of the *thr* and *ilv* operons are rich in threonine and isoleucine, respectively, which are encoded by the ANN codons (Fig. 2G). A deficiency in t^6A modification in the *yeaZ*, *ygjD*, and *yjeE* temperature-sensitive mutants would slow the translation of the leader peptides, resulting in readthrough and the subsequent expression of the downstream genes responsible for the biosynthesis of Thr, Ile, and Val. If the cellular concentration of Thr is sensed via the modification of t^6A, then this mechanism for regulating the expression of the *thr* and *ilv* operons represents a feedback regulation loop. Since Ile is synthesized, at least in part, from Thr, low concentrations of Thr would result in the increased expression of the *ilv* operon. High levels of Ile would also lead to increased levels of Thr because the Thr-degradative enzyme threonine deaminase is inhibited by Ile (2).

Expression profiling of the *ygjD*(Ts) mutant identified the effects of the mutation on transcription but did not explain why growth was inhibited by the overexpression of *nusB* and *rnhA*. To probe the mechanisms underlying *nusB*-mediated growth inhibition, the expression profile of a *nusB* deletion mutant was compared to that of the *ygjD*(Ts) mutant (see Tables S3 and S4 in the supplemental material). The results showed very little overall correlation between the two strains. However, the expression of one essential gene, *dnaG*, was decreased in the *ygjD*(Ts) mutant and increased in the *nusB* mutant (see Table S5 and Fig. S3C in the supplemental material). To test whether *dnaG* expression is involved in the growth inhibition mediated by the overexpression of *nusB*, individual multicopy plasmids encoding *nusB* or *dnaG* were introduced into the *ygjD*(Ts) mutant strain and cell growth was examined. The growth of the *ygjD*(Ts) mutant was inhibited by the overexpression of *nusB*, but this effect was abrogated by the cointroduction of *nusB* and *dnaG* (Fig. 3). Interestingly, the introduction of *dnaG* alone into the *ygjD*(Ts) strain suppressed the slow-growth phenotype at the semipermissive temperature (30°C), suggesting that the growth defect in the *ygjD*(Ts) mutant may be due, in part, to decreased levels of DnaG primase (Fig. 3). Growth of the *yjeE*(Ts) mutant (but not the *yeaZ*(Ts) mutant) was slightly, but significantly, suppressed by the expression of *dnaG* alone (see Fig. S4 in the supplemental material).

A lack of DnaG primase causes not only defects in DNA replication but also defects in chromosomal segregation (16, 20). Loss of nucleoids in the absence of *ygjD* expression was observed in a conditional *ygjD* mutant (17). Interestingly, cells with irregular chromosomes were also observed when *yeaZ*, *ygjD*, and *yjeE* temperature-sensitive mutants were incubated at nonpermissive temperatures (data not shown). A yeast mutant lacking the YgjD ortholog, Qri7, which localizes to the mitochondria, exhibited abnormal mitochondrial morphology and no detectable DNA. Some of these phenotypes can be attributed to decreased levels of primase.

Although growth suppression was observed at 30°C, it was not observed at 37°C or 42°C (Fig. 3) and the introduction of *dnaG* did not suppress the growth defect observed in the *yeaZ*, *ygjD*, and *yjeE* deletion mutants (data not shown). These results suggest that the growth defects seen in these temperature-sensitive mutant strains cannot be explained entirely by the decreased expression of *dnaG*.

Microarray data accession numbers. Data for experiments with *E. coli ygjD*(Ts) at 30°C, *E. coli ygjD*(Ts) at 37°C, and *E. coli nusB* have been deposited in Array Express under accession numbers E-MEXP-3333, E-MEXP-3334, and E-MEXP-3335.

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