JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5554–5560 Vol. 177, No. 19
0021-9193/95/$04.00+0

Functional Analysis of the *ffh-trmD* Region of the *Escherichia coli* Chromosome by Using Reverse Genetics

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Received 2 May 1995/Accepted 27 July 1995

We have analyzed the essentiality or contribution to growth of each of four genes in the *Escherichia coli trmD* operon (*rpsP*, *21K*, *trmD*, and *rplS*) and of the flanking genes *ffh* and *16K* by a reverse genetic method. Mutant alleles were constructed in vitro on plasmids and transferred by recombination to the corresponding λ phage clone (λ439) and from the phage clone to the *E. coli* chromosome. An ability to obtain recombinants only in cells carrying a complementing plasmid indicated that the mutated gene was essential, while an ability to obtain recombinants in plasmid-free cells indicated nonessentiality. In this way, Ffh, the *E. coli* homolog to the 54-kDa protein of the signal recognition particle of mammalian cells, and ribosomal proteins S16 and L19 were shown to be essential for viability. A deletion of the second gene, *21K*, of the *trmD* operon reduced the growth rate of the cells fivefold, indicating that the wild-type 21-kDa protein is important for viability. A deletion-insertion in the same gene resulted in the accumulation of an assembly intermediate of the 50S ribosomal subunit, as a result of polar effects on the expression of a downstream gene, *rplS*, which encodes ribosomal protein L19. This finding suggests that L19, previously not considered to be an assembly protein, contributes to the assembly of the 50S ribosomal subunits. Strains deleted for the *trmD* gene, the third gene of the operon, encoding the tRNA(m^1G37)methyltransferase (or TrmD) showed a severalfold reduced growth rate. Since such a strain grew much slower than a strain lacking the tRNA(m^1G37)methyltransferase activity because of a point mutation, the TrmD protein might have a second function in the cell. Finally, a 16-kDa protein encoded by the gene located downstream of, and convergently transcribed to, the *trmD* operon was found to be nonessential and not to contribute to growth.

More than 1,700 gene products have been identified in *Escherichia coli* K-12 (2, 40), and more than half of its 4,700-kbp genome has been sequenced (39, 42). In the past, most genes were identified by selection for mutations that conferred specific phenotypes, followed by mapping of various mutations. However, with the increasing number of open reading frames predicted from DNA sequence analysis, it is often more efficient to inactivate the cloned open reading frames first and then transfer the mutant alleles to the chromosome to study gene function. Previously, we developed a reverse genetic method for distinguishing between essential and nonessential genes (27). The method entails (i) transfer of a plasmid-carried inactivated chromosomal gene marked with a resistance trait to the appropriate λ phage clone of the collection of Kohara et al. (24) by homologous recombination between chromosomal DNA flanking the inactivated gene in the plasmid and the corresponding chromosomal segment in the λ phage clone and (ii) transduction with pure stocks of the resultant recombinant phage, in the presence or absence of a complementing plasmid in the recipient strain. The ability to obtain transductants only in the presence of a complementing plasmid indicates the essentiality of the targeted gene, whereas the ability to obtain transductants in the absence of a complementing plasmid demonstrates nonessentiality of the gene in question. Using this method, we have here examined the essentiality of six genes in the *ffh-trmD* region (56.7 min) of the *E. coli* chromosome (32). A plasmid covering this region contains the four genes of the *trmD* operon and two flanking genes (10). The *ffh* gene, situated upstream from the *trmD* operon, encodes a methionine-rich 48-kDa protein (Ffh) (9, 10) suggested to be the *E. coli* homolog to the 54-kDa protein of the mammalian signal recognition particle (3, 38, 41), which plays a central role in directing the export of nascent proteins from the cytoplasm of mammalian cells (13, 25, 28, 52). The Ffh protein was recently found to be essential in *E. coli* (37) and has been shown to form a complex with 4.5S RNA, which has been suggested to participate in a cotranslational secretory pathway for certain *E. coli* proteins (38). The *trmD* operon contains the genes for ribosomal protein S16, a 21-kDa protein (21K) of unknown function, the tRNA(m^1G37)methyltransferase or TrmD, and ribosomal protein L19, in that order (10). The tRNA-(m^1G37)methyltransferase modifies the guanosine in position 37 next to the anticodon of a subset of the tRNAs in *E. coli* and *Salmonella typhimurium* (5, 6). From studies on a *trmD* mutant of *S. typhimurium*, it was concluded that the modified guanosine was important for the maintenance of the correct reading frame during translation (7, 18). The 21K and TrmD proteins are found in 12- and 40-fold-lower amounts, respectively, than the two ribosomal proteins (54), although different parts of the polycistronic mRNA are equally abundant (11). The expression of the two ribosomal protein genes of the *trmD* operon is regulated differently than that of most other ribosomal protein genes, since *trmD* operon expression seems not to be autoregulated by the translational feedback mechanism that controls the overall synthesis of ribosomes in *E. coli* (35, 56). The gene downstream from the *trmD* operon is transcribed in the orientation opposite that of the *trmD* operon (10) and encodes a 16-kDa protein (16K), as judged by expression in minicells (9). Here we demonstrate that the Ffh protein and the two ribosomal proteins are essential for viability; the

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TABLE 1. Bacterial strains, bacteriophages, and plasmids used

Strain, phage, or plasmid	Relevant markers	Origin or reference[a]
Strains		
DB1434	*lac-3350 galT2 galK22 rpsL179* (λ*plac5 c*I857 *S*am7)	Strain R594 in reference 1; 46
MW27	Hfr P4X λ$^-$ Δ*rpsP-1*/pMW291	
MW32	Hfr P4X λ$^-$ Δ*trmD-2 16K*::*nptI-1*/pDBMW23 + p*lacI*^{q}	
MW33	Hfr P4X λ$^-$ *trmD*$^+$ *16K*::*nptI-1*/pDBMW23 + p*lacI*^{q}	
MW35	Hfr P4X λ$^-$ Δ*21K-1*/pMW279 + p*lacI*^{q}	
MW37	Hfr P4X λ$^-$ Δ*21K-2 16K*::*nptI-1*	
MW38	Hfr P4X λ$^-$ *21K*$^+$ *16K*::*nptI-1*	
MW40	Hfr P4X λ$^-$ Δ*21K-2 16K*::*nptI-1*/pMW279 + p*lacI*^{q}	
MW41	Hfr P4X λ$^-$ Δ*rplS-1*/pMW271 + pHMG64	
MW42	Hfr P4X λ$^-$ Δ*21K-1*	
MW42/pBY03	Hfr P4X λ$^-$ Δ*21K-1*/pBY03	
MW42/pDBMW9	Hfr P4X λ$^-$ Δ*21K-1*/pDBMW9	
MW44	Hfr P4X λ$^-$ *21K*$^+$ *16K*::*nptI-1*/pMW279 + p*lacI*^{q}	
MW46	Hfr P4X λ$^-$/pMW279 + p*lacI*^{q}	
MW48	Hfr P4X λ$^-$/pMW271 + pHMG64	
MW49	Hfr P4X λ$^-$ Δ*ffh-1*/pMW292	
MW100	Hfr P4X λ$^-$	56
MW100/pBY03	Hfr P4X λ$^-$/pBY03	56
MW100/pMW291	Hfr P4X λ$^-$/pMW291	
MW100/pMW292	Hfr P4X λ$^-$/pMW292	
λ phage clones[b]		
λ439/λ22D7	*ffh*$^+$-*rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$	24
λ439Δ*ffh-1*	Δ*ffh*::*nptI* from plasmid pDBMW24	
λ439Δ*rpsP-1*	Δ*rpsP*::*nptI*-P$_{lac}$ from plasmid pDBMW48	
λ439Δ*21K-1*	Δ*21K*::*nptI*-P$_{tac}$ from plasmid pDBMW5	
λ439Δ*21K-2*	Δ*21K 16K*::*nptI* from plasmid pMW287	
λ439Δ*trmD-1*	Δ*trmD*::*nptI* from plasmid pDBMW53	
λ439Δ*trmD-2*	*'trmD 16K*::*nptI* from plasmid pMW281	
λ439Δ*rplS-1*	Δ*rplS*::*nptI* from plasmid pDBMW31	
λ439*16K*::*nptI-1*	*16K*::*nptI* from plasmid pDBMW20	
Plasmids[c]		
pBY03	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$ *bla*	8
pBY12	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$ *bla*	8
pBY45	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$ *bla*	55
pCL1921	*lacPOZ' str/spc*	31
pDBMW5	*ffh*$^+$ *rpsP*$^+$-Δ*21K*::*nptI*-P$_{tac}$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$ *bla*	
pDBMW9	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*'trmD-rplS*$^+$ *16K*$^+$ *bla*	
pDBMW20	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*+ *16K*::*nptI bla*	
pDBMW23	P$_{lac}$-*21K*$^+$-*trmD*$^+$-*rplS'-cat-rrnB*T$_1$ *bla*	
pDBMW24	Δ*ffh*::*nptI rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$ *bla*	
pDBMW31	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-Δ*rplS*::*nptI 16K*$^+$ *bla*	
pDBMW48	*ffh*$^+$ Δ*rpsP*::*nptI*-P$_{lac}$-*21K*$^+$-*trmD*$^+$-*rplS'-cat-rrnB*T$_1$ *bla*	
pDBMW53	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-Δ*trmD*::*nptI-rplS*$^+$ *16K*$^+$ *bla*	
pHMG64	*lacI*^{q} in pAYCY184	16
p*lacI*^{q}	*lacI*^{q} in pACYC184	12, 58
pMW27	*ffh*$^+$ *rpsP*$^+$-*21K'-lac'Z lacY*$^+$ *lacA*$^+$ *bla*	55
pMW38	*ffh*$^+$ *rpsP*$^+$-Δ*21K-trmD*$^+$-*rplS*$^+$ *16K*$^+$ *bla*	53
pMW146	*ffh*$^+$ *P$_{rpsP}$-lac'Z lacY*$^+$ *lacA*$^+$ *bla*	57
pMW271	P$_{lac}$-*rplS*$^+$ *16K*$^+$ *cat-rrnB*T$_1$ *bla*	
pMW279	P$_{lac}$-*21K*$^+$-*cat-rrnB*T$_1$ *bla*	
pMW281	*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*'trmD-rplS*$^+$ *16K*::*nptI bla*	
pMW287	*ffh*$^+$ *rpsP*$^+$-Δ*21K-trmD*$^+$-*rplS*$^+$ *16K*::*nptI bla*	
pMW291	*ffh*$^+$ *rpsP*$^+$-*21K' spc/str*	
pMW292	*ffh*$^+$ *bla*	
pUC4K	*nptI bla*	50
pUHE21-2	P$_{lac}$-*cat-rrnB*T$_1$ *bla*	30

[a] Unless otherwise noted, the origin was this study.
[b] λ439 corresponds to the systematic numbering of the miniset in Kohara (23), while λ22D7 refers to the designations in Kohara et al. (24).
[c] Plasmid constructions are described in Materials and Methods.

21K and TrmD proteins must have important functions in the cell, since strains lacking either of the corresponding genes (*21K* and *trmD*) had a fivefold-reduced growth rate; and the 16K protein does not contribute significantly to growth rate, at least not under the conditions examined.

MATERIALS AND METHODS

Strains, phages, and plasmids. The strains, phages, and plasmids used are listed in Table 1.

Media and growth conditions. Rich medium was LB (4) supplemented with 0.4% glucose and E+B$_1$ (51). Cultures were grown at 37°C, and growth was

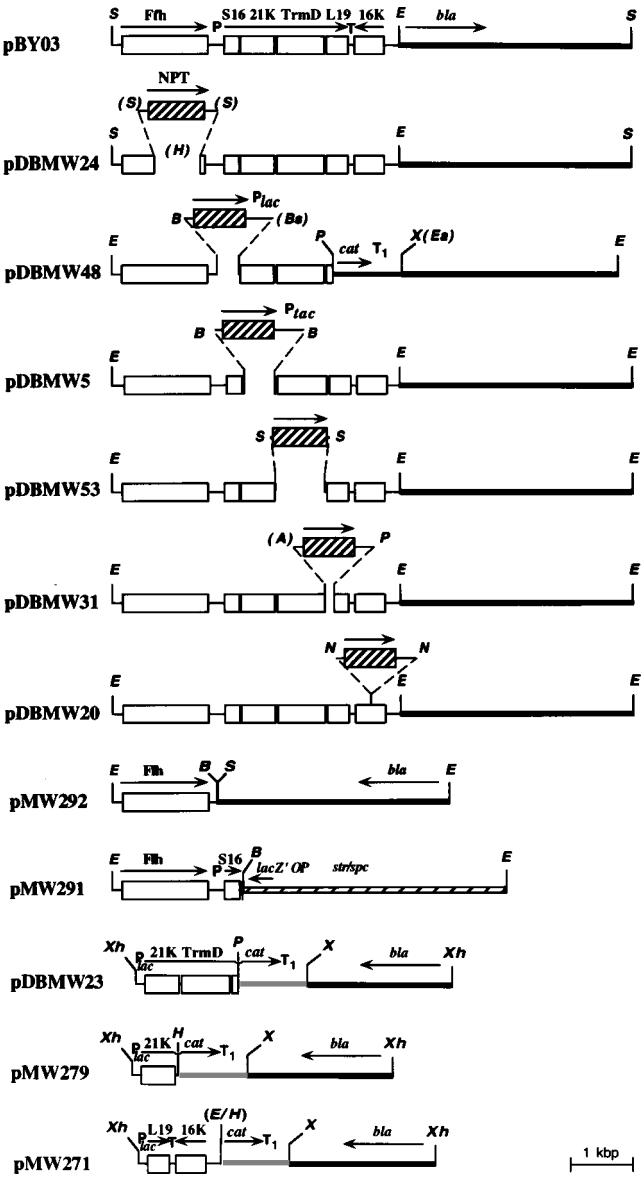

FIG. 1. The most important plasmids used in this study. Open boxes indicate genes with chromosomal origin, and a hatched box indicates the *nptI* gene. Relevant restriction sites: *A*, *Ava*I; *B*, *Bam*HI; *Bs*, *Bsp*HI; *E*, *Eco*RI; *Ea*, *Eag*I; *H*, *Hpa*I; *N*, *Nco*I; *P*, *Pst*I; *S*, *Sal*I; *X*, *Xba*I; *Xh*, *Xho*I. Those within parentheses were destroyed as a result of the cloning procedure. The arrows above the *16K*, *bla*, *cat*, *ffh*, and *nptI* genes show their 5′-to-3′ orientation, while the arrow above the *trmD* operon symbolizes the *trmD* operon mRNA. P and T indicate the promoter and terminator, respectively, of the *trmD* operon. P_{tac} is the *tac* promoter (15, 43), and P_{lac} indicates the strong inducible *lac* promoter $P_{A1/04/03}$ (30).

monitored on a Zeiss PMQ3 spectrophotometer at 420 nm. Tryptone broth, tryptone top agar, and tryptone agar plates were used for preparation of λ lysates.

Construction of mutant alleles. The *ffh* gene, encoding a 48-kDa protein, was inactivated by replacing the region between codons 172 and 414 with the *nptI* (kanamycin resistance [Km^r]) gene from pUC4K (Fig. 1, plasmid pDBMW24). The first gene in the *trmD* operon, *rpsP*, encoding ribosomal protein S16, was replaced in its entirety by the *nptI* gene (Fig. 1, plasmid pDBMW48). To overcome polar effects on expression of the downstream genes in the operon, the strong inducible *lac* promoter $P_{A1/04/03}$ (30) was placed upstream of the *21K* gene, and a new translation initiation region was created to ensure efficient translation of *21K*. All but the first five and last five codons of *21K* were replaced by a fragment carrying *nptI* upstream of the *tac* promoter joined to the translation initiation region of the *tnpA* gene (49) from transposon γδ (Fig. 1, plasmid pDBMW5). The *tac* promoter was included to relieve possible polar effects of *nptI* on transcription of the downstream *trmD* and *rplS* genes. Since the *tnpA* gene (second codon) was fused in frame with the 3′ end of *21K*, the intercistronic region between *tnpA′-′21K* and *trmD* is identical to that between *21K* and *trmD* in wild-type cells, which should ensure proper translation of *trmD*. The entire *trmD* gene was replaced by a PCR-amplified fragment only containing the *nptI* structural gene with its translational initiation region (Fig. 1, plasmid pDBMW53). To inactivate the *rplS* gene, the translation initiation region and the first 39 codons were replaced by the *nptI* gene from pUC4K (Fig. 1, plasmid pDBMW31). The *16K* gene located just downstream of and transcribed in the orientation opposite that of the *trmD* operon was inactivated by inserting the *nptI* gene in the 75th of its 160 codons (Fig. 1, plasmid pDBMW20). To obtain a selectable marker tightly linked to nonpolar deletions of *21K* and *trmD*, the *nptI* fragment from plasmid pDBMW20 was inserted into the *Nco*I site of *16K* of plasmids pMW38 (Δ*21K*) and pDBMW9 (Δ*trmD*). The resulting plasmids with the *nptI* gene in the same orientation as the *trmD* operon were named pMW287 (Δ*21K*) and pMW281 (Δ*trmD*).

Construction of complementing plasmids. The 1,190-bp *Eco*RI-*Eco*RV fragment carrying *rplS* and *16K* from plasmid pBY45 was cloned into the *lac* promoter vector pUHE21-2 (Fig. 1, plasmid pMW271). The *21K* gene with a new translation initiation region was cloned into pUHE21-2 (Fig. 1, plasmid pMW279). The *ffh* and *rpsP* genes were cloned on an *Eco*RI-*Bam*HI fragment from plasmid pMW27 into the low-copy-number vector pCL1921 (Fig. 1, plasmid pMW291). Plasmid pMW292 (*ffh*$^+$; Fig. 1) was constructed by replacing the *lac* operon on a *Bam*HI-*Sal*I fragment in plasmid pMW146 (*ffh*$^+$ P_{S16}-*lacZYA*) by the adaptor oligonucleotide 5′-GATCGTCGAC-3′.

Transfer of the inactivated genes to λ439 by homologous recombination. The mutations shown in Fig. 1 were transferred to phage λ439 from the collection of Kohara et al. (24) by recombination between the mutated DNAs cloned in plasmids and the homologous region in the λ439 phage clone. The recombinant clones were recovered by transducing strain DB1434 and selecting for Km^r at 30°C as described previously (27).

PCR amplification and DNA sequencing. Bacterial colonies were resuspended in H_2O and PCR amplified as described elsewhere (33, 45), using either Amplitaq DNA polymerase from Perkin-Elmer Cetus, Norwalk, Conn., or *Taq* DNA polymerase from Boehringer Mannheim. To verify that the chromosomal copies of the studied genes had been inactivated in plasmid-free as well as plasmid-containing cells, PCR analyses were designed so that at least one of the primers in each test could not bind to the complementing plasmid, thereby ensuring that the PCR product size would reflect the status of the chromosomal allele. The postulated mutant strains gave PCR products of expected sizes, which were different from those of the corresponding wild-type strains (data not shown).

Linear amplification cycle sequencing of double-stranded DNA with Amplitaq DNA polymerase, using primers end labeled with ^{32}P, was performed as previously described (26).

Sucrose gradients. Preparation of S30 extracts and separation of ribosomal subunits by sucrose gradient centrifugation were done as described by Rydén-Aulin et al. (44).

RESULTS

Replacement of chromosomal genes by inactivated alleles in λ phage clones. The mutations shown in Fig. 1 were transferred to phage λ439 from the collection of Kohara et al. (24) by homologous recombination (see Materials and Methods). To test if any of the six genes that we inactivated is essential for viability, strain MW100 with or without plasmid pBY03 (Fig. 1) was transduced by the appropriate λ phage stocks. Km^r transductants were obtained at frequencies of at least 10^{-6} in all but two cases: transduction of strain MW100 with phage clones carrying mutations in *ffh* or *rpsP* (frequencies of $\leq 3 \times 10^{-8}$; Table 2). In contrast, the frequencies of Km^r transductants, using these phage clones and strain MW100/pBY03, were $>10^{-6}$ in each case. The rare Km^r transductants of strain MW100, using λ439Δ*ffh-1* or λ439Δ*rpsP-1*, segregated the Km^r marker if grown without selection for kanamycin (data not shown), indicating that the inactivated genes had been integrated into the chromosome of cells containing preexisting duplications of the targeted region. That such transductants contained both a wild-type and a mutant copy of the targeted gene was confirmed by PCR analysis (data not shown). Thus, these results suggest that *ffh* and *rpsP* are essential genes.

The transduction frequency obtained by using the recombinant phages carrying the other four inactivated genes was independent of whether the recipient strain was MW100 or MW100/pBY03 (Table 2). Transductants of MW100 obtained

TABLE 2. Transduction of strains either haploid or polyploid for the 56.7-min region of the chromosome with pure λ phages carrying the inactivated genes

λ phage	Transduction frequency, growth characteristics[a]	
	MW100[b]	MW100/pBY03
λ439Δ*ffh-1*	2×10^{-8}, ++	5×10^{-6}, ++
λ439Δ*rpsP-1*	8×10^{-9}, +	5×10^{-6}, ++
λ439Δ*21K-1*	2×10^{-5}, (+)	7×10^{-6}, ++
λ439Δ*trmD-1*	3×10^{-6}, (+)	2×10^{-6}, ++
λ439Δ*rplS-1*	8×10^{-6}, (+)	7×10^{-6}, ++
λ439*16K*::*nptI-1*	4×10^{-6}, ++	1×10^{-6}, +

[a] Transduction frequency is expressed as number of transductants per added phage. The growth characteristics are those observed on the plates with the original transductants: ++, normal growth after 16 h of incubation; +, reduced growth rate but colonies visible after 16 h; (+), small colonies visible after 48 h.
[b] Recipient strain.

by using λ439*16K*::*nptI* showed no significant growth impairment on rich or minimal medium under aerobic or anaerobic conditions compared with strain MW100, indicating that *16K* is a nonessential gene. However, when a phage that carried inactivated *21K*, *trmD*, or *rplS* was used, the transductants of strain MW100 formed small colonies only after about 48 h, in contrast to transductants of strain MW100/pBY03, which formed normal-size colonies after 16 h (Table 2). Upon restreaking, the small colonies of the MW100 derivatives with inactivated *21K* or *trmD* showed a stable phenotype, whereas MW100 with inactivated *rplS* barely grew and formed minute colonies after 72 h. When again restreaked, only a few cells from each of these minute colonies were again able to form colonies, and most of them had become faster growing, probably as a result of secondary mutations (data not shown). These results indicated that mutants lacking *21K* or *trmD* were severely affected in growth and that the *rplS* gene was essential for viability in the absence of compensatory mutations.

Complementation of inactivated genes by plasmids containing different parts of the *ffh-trmD* chromosomal region. To test if the observed complementation by plasmid pBY03 (Table 2) was due to the wild-type copy of the inactivated gene, strain MW100 harboring plasmids with different segments of the region cloned in plasmid pBY03 was transduced with the different λ phage clones. Transductants were obtained at frequencies similar to those in Table 2 (data not shown). The Δ*ffh-1* and Δ*rpsP-1* mutations were fully complemented by the plasmids pMW292 (*ffh*$^+$) and pMW291 (*ffh*$^+$ *rpsP*$^+$), respectively (Table 3).

The strain carrying the Δ*21K-1* mutation (Δ*21K*::*nptI*-P_{tac}) showed a fivefold-reduced growth rate, and the induction of expression of *trmD* and *rplS* from the *tac* promoter on the chromosome did not significantly increase the growth rate (Table 3), suggesting that the demand for the TrmD and L19 proteins can be satisfied by transcription from the *tac* promoter even in the absence of isopropylthio-β-D-galactopyranoside (IPTG) (probably as a result of the leakiness of P_{tac} [29]). However, if the *21K* and *trmD* genes were supplied in *trans* on plasmid pBY12 (*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$), then faster growth became IPTG dependent, i.e., requiring expression of *rplS* on the chromosome (data not shown). This finding is in agreement with the need for (and synthesis of) higher amounts of ribosomal proteins at higher growth rates in wild-type *E. coli* cells (35). Plasmid pMW279 ($P_{A1/04/03}$-*21K*$^+$; Fig. 1) did not fully complement the slow growth of the Δ*21K-1* mutant even in the presence of IPTG (Table 3). Thus, the expression of *trmD* and *rplS* from P_{tac} on the chromosome may not be sufficient to support normal growth when the *21K* gene was provided in *trans* on a plasmid. We emphasize, however, that in cells not synthesizing the 21K protein, the expression of *trmD* and *rplS* does not limit the growth rate, since growth was not enhanced by the presence of plasmid pMW38 (data not shown), which is deleted for *21K* but expresses *ffh*, *rpsP*, *trmD*, *rplS*, and *16K* (56). These findings suggest that the fivefold-reduced growth rate of the Δ*21K-1* mutant is due to the lack of the 21K protein, not to limiting amounts of the TrmD and L19 proteins.

The slow growth of clones that carried the Δ*trmD-1* (Δ*trmD*::*nptI*) mutation was complemented only by plasmid pBY03 carrying the intact *trmD* operon and not by plasmid pDBMW23 ($P_{A1/04/03}$-*21K*$^+$-*trmD*$^+$; data not shown), suggesting that the *nptI* gene was polar on expression of *rplS*.

The Δ*rplS-1* mutation on the chromosome was complemented by plasmid pMW271 ($P_{A1/04/03}$-*rplS*$^+$ *16K*$^+$), although not completely (Table 3). The growth rate of the wild-type strain containing plasmid pMW271 was decreased by addition of IPTG (Table 3). On the other hand, the growth rate of the Δ*rplS-1* mutant containing pMW271 increased with increasing concentrations of IPTG up to 0.25 mM, while at higher concentrations it decreased (data not shown), showing that both too low and too high levels of expression of *rplS*, reduce the growth rate. Probably, plasmid pMW271 complemented the Δ*rplS-1* mutation only partially as a result of a nonoptimal IPTG-induced expression of *rplS*.

TABLE 3. Complementation of inactivated genes on the chromosome by different plasmids expressing the corresponding wild-type genes

Chromosomal genotype[a]	Plasmid(s) (relevant genotype)	IPTG[b]	Growth rate (k)[c]
ffh$^+$	pMW292 (*ffh*$^+$)	−	1.58
Δ*ffh-1*	pMW292 (*ffh*$^+$)	−	1.63
rpsP$^+$	pMW291 (*ffh*$^+$ *rpsP*$^+$)	+	1.98
Δ*rpsP-1*	pMW291 (*ffh*$^+$ *rpsP*$^+$)	+	2.02
21K$^+$	None	−	1.73
21K$^+$	None	+	1.81
Δ*21K-1*	None	−	0.34
Δ*21K-1*	None	+	0.37
21K$^+$	pMW279 (P_{lac}-*21K*$^+$) + p*lacI*^{q} (*lacI*^{q})	+	1.73
Δ*21K-1*	pMW279 (P_{lac}-*21K*$^+$) + p*lacI*^{q} (*lacI*^{q})	+	0.77
21K$^+$	None	−	1.89
Δ*21K-2*	None	−	0.40
21K$^+$	pMW279 (P_{lac}-*21K*$^+$) + p*lacI*^{q} (*lacI*^{q})	+	1.70
Δ*21K-2*	pMW279 (P_{lac}-*21K*$^+$) + p*lacI*^{q} (*lacI*^{q})	+	1.63
trmD$^+$	pDBMW23 (P_{lac}-*21K*$^+$-*trmD*$^+$-*rplS′*) + p*lacI*^{q} (*lacI*^{q})	+	1.54
Δ*trmD-2*	pDBMW23 (P_{lac}-*21K*$^+$-*trmD*$^+$-*rplS′*) + p*lacI*^{q} (*lacI*^{q})	+	1.51
rplS$^+$	pMW271 (P_{lac}-*rplS*$^+$ *16K*$^+$) + pHMG64 (*lacI*^{q})	−	1.51
rplS$^+$	pMW271 (P_{lac}-*rplS*$^+$ *16K*$^+$) + pHMG64 (*lacI*^{q})	+	0.56
Δ*rplS-1*	pMW271 (P_{lac}-*rplS*$^+$ *16K*$^+$) + pHMG64 (*lacI*^{q})	−	NP
Δ*rplS-1*	pMW271 (P_{lac}-*rplS*$^+$ *16K*$^+$) + pHMG64 (*lacI*^{q})	+	1.01

[a] The *21K*$^+$ strains isogenic to the Δ*21K-1* strains are all derivatives of strain MW100, while the *21K*$^+$ strains isogenic to the Δ*21K-2* strains are derivatives of strain MW38 (MW100 *16K*::*nptI*).
[b] The concentration of IPTG used was that supporting optimal growth: 0.25 mM for Δ*rplS-1*, 0.1 mM for Δ*rpsP-1* or Δ*21K-1*, and 1 mM when the strains contained plasmid pDBMW23 or pMW279.
[c] The strains were grown in LB supplemented with 0.4% glucose and E+B_1. For pMW291- or p*lacI*^{q}-containing strains, 50 μg of spectinomycin per ml or 20 μg of tetracycline per ml, respectively, was added to the medium. For all other plasmid-containing strains, 50 μg of carbenicillin per ml was used. $k = \ln 2/g$, where g is the mass doubling time in hours. NP, not possible.

Strains containing nonpolar mutations in *21K* and *trmD* have a fivefold-reduced growth rate. As shown above, the Δ*21K-1* and Δ*trmD-1* mutations on the chromosome were not fully complemented by plasmids pMW279 ($P_{A1/04/03}$-*21K*$^+$) and pDBMW23 ($P_{A1/04/03}$-*21K*$^+$-*trmD*$^+$), respectively. The deletion of *21K* as such is not polar on the expression of *trmD* and *rplS* when carried on plasmid pMW38 (56). Therefore, the deletion of *21K* (Δ*21K-2*) on plasmid pMW38 was transferred to the chromosome by using *16K*::*nptI* as a linked selectable marker. Similarly, a deletion of the 5′ one-third of *trmD* (Δ*trmD-2*) was combined with *16K*::*nptI* and transferred to the chromosome. Both the Δ*21K-2*- and Δ*trmD-2*-containing transductants were as slowly growing as the Δ*21K-1* and Δ*trmD-1* mutants described above. The Δ*trmD-2*-containing clones were, however, unstable and mutated at a high frequency to slightly faster-growing derivatives, which made it impossible to determine accurately the growth rate of the original mutant. However, since the mutant formed colonies of approximately the same size as did strains containing the more stable Δ*21K-2* mutation, the growth rate reduction caused by the Δ*trmD-2* mutation is inferred to be approximately fivefold. To examine if the Δ*21K-2* and Δ*trmD-2* mutations were polar on the expression of the respective downstream genes, the deletions were introduced by transduction into strain MW100 containing complementing plasmids. That the deletions had replaced the respective wild-type gene on the chromosome was confirmed by PCR analysis (data not shown). The slow growth of the Δ*21K-2* and Δ*trmD-2* mutants was fully complemented by plasmids pMW279 ($P_{A1/04/03}$-*21K*$^+$) and pDBMW23 ($P_{A1/04/03}$-*21K*$^+$-*trmD*$^+$), respectively (Table 3). This finding indicated that the two mutations were not polar on expression of downstream genes. In summary, the results from the complementation experiments suggest that the Ffh protein and ribosomal proteins S16 and L19 are essential for viability and that the 21K and TrmD proteins have important roles at least at high growth rates.

The Δ*21K-1* mutant is defective in the assembly of the 50S ribosomal subunit as a result of polar effects on *rplS* expression. The Δ*21K-1* mutant strain MW42 grew extremely poorly at 30°C, indicating that it was cold sensitive for growth. Since cold-sensitive strains often are defective in ribosome assembly (17, 48), we examined ribosomal subunits from the mutant and the parental strain by sucrose gradient centrifugation. Logarithmically growing cells of the mutant strain MW42 had a reduced ratio of 50S to 30S subunits (Fig. 2B). In addition, there was an extra peak between the 50S and 30S subunit peaks from the mutant but not from the wild-type strain. Two-dimensional ribosomal protein gel analysis indicated that fractions corresponding to this extra peak contained mainly 50S ribosomal proteins (data not shown). Interestingly, the sedimentation profiles for ribosomes from mutant cells in stationary phase indicated a normal 50S-to-30S subunit ratio and revealed no extra peak between the 50S and 30S subunit peaks (Fig. 2C). These findings suggest that the mutant has a lower rate of 50S subunit assembly than the wild-type strain, which results in accumulation of an assembly intermediate. Since the slow growth of the Δ*21K-1* mutant MW42 was not fully complemented by plasmid pMW279 ($P_{A1/04/03}$-*21K*$^+$; Fig. 1), we examined if the ribosome assembly defect was due to polar effects of *nptI*::P_{tac} on the expression of *trmD* or *rplS* when P_{tac} was not induced, although the growth defect of the mutant was not relieved by addition of IPTG. No assembly intermediate was detected when strain MW42 (Δ*21K-1*) was grown with IPTG (Fig. 2D). Moreover, strain MW37 containing the nonpolar mutation Δ*21K-2* did not show any assembly defect (Fig. 2F). These findings indicate that the assembly defect was indeed caused by limiting amounts of TrmD or L19. To establish which protein was limiting for ribosome assembly, we introduced the TrmD$^-$ L19$^+$ plasmid pDBMW9 (*ffh*$^+$ *rpsP*$^+$ *21K*$^+$-*'trmD-rplS*$^+$ *16K*$^+$) and the TrmD$^+$ L19$^+$ plasmid pBY03 (*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS*$^+$ *16K*$^+$) into strain MW42 and examined ribosome assembly. No 50S assembly intermediate was observed from either strain (Fig. 2E), suggesting that the ribosome assembly defect of plasmid-free MW42 under noninducing conditions was due to limiting amounts of ribosomal protein L19.

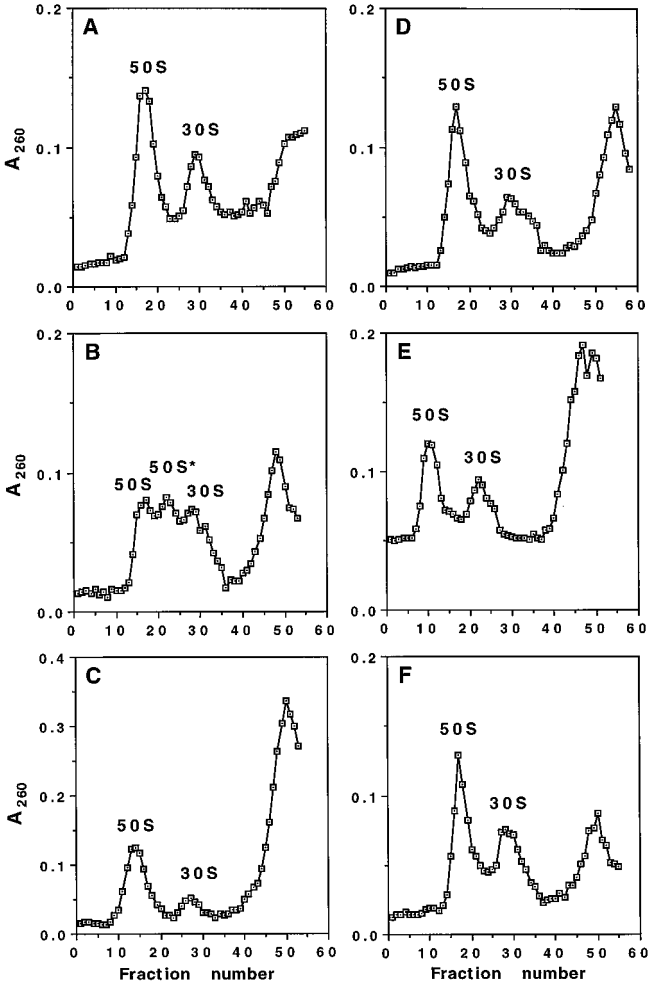

FIG. 2. Separation of ribosomal subunits by sucrose gradient centrifugation. Shown are extracts of log-phase cells of the parental strain MW100 (A), log-phase cells of the Δ*21K-1* mutant MW42 (B), stationary-phase cells of strain MW42 (C), log-phase cells of strain MW42 grown in the presence of 0.5 mM IPTG (D), log-phase cells of strain MW42 containing plasmid pDBMW9 (*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*'trmD-rplS*$^+$ *16K*$^+$) (E) (a similar sedimentation profile was obtained for strain MW42 containing plasmid pBY03 [*ffh*$^+$ *rpsP*$^+$-*21K*$^+$-*trmD*$^+$-*rplS 16K*]), and log-phase cells of strain MW37 (Δ*21K-2*) (F). 50S* indicates a 50S assembly intermediate.

DISCUSSION

This communication describes a functional analysis of six genes in the *ffh-trmD* region (56.7 min) of the *E. coli* chromosome. Our results indicate that the Ffh protein and the two ribosomal proteins S16 and L19 encoded by the *trmD* operon are essential in *E. coli* and that the 21K and TrmD proteins have important roles at high growth rates. In contrast, the

16-kDa protein encoded by the gene downstream from, and convergently transcribed to, the *trmD* operon, seems nonessential, at least under the conditions examined here.

The Δ*trmD-1* and Δ*trmD-2* mutants described in this report grew severalfold more slowly than an *S. typhimurium trmD* mutant (data not shown) which lacks tRNA(m^1G37)methyltransferase activity (7). This finding indicates that the TrmD protein might have two functions. Interestingly, another tRNA-modifying enzyme, the tRNA(m^5U54)methyltransferase, seems to have two functions: an insertion in the 3′ part of its gene destroys the tRNA-modifying activity but results in only a 4% growth rate reduction, whereas an insertion further upstream is lethal (36).

A large number of mutants for which the ribosomes contain altered, or even lack, ribosomal proteins have been isolated after heavy chemical mutagenesis (21, 22) or as antibiotic-independent revertants of mutant strains that depended on antibiotics for growth (14, 19). Among mutants that are temperature sensitive for growth, some have altered forms of S16 and L19 (22). It was assumed that the temperature sensitivity was due to alterations in the ribosomal proteins, indicating their essentiality in *E. coli*, although the possibility that the temperature sensitivity was due to mutations in other genes was not excluded. Using reverse genetics, we have here demonstrated that protein S16 is essential for growth. Previously it was found that the assembly rate in vitro of 30S subunits is dramatically reduced if protein S16 is omitted, although ribosomes lacking S16 are still functional in vitro (20). These findings suggest that the inability of cells lacking S16 to survive is due to a reduced assembly rate of 30S subunits. We also show that protein L19 is essential, since transductants lacking L19 did not survive restreaking. Further, reduced amounts of L19 due to polar effects of a substitution of *nptI*::P_{tac} for *21K* (Δ*21K-1*) resulted in a deficiency in the assembly of the 50S subunit of the ribosome. Three precursor particles have been identified in the 50S subunit assembly, $p_1$50S, $p_2$50S and $p_3$50S, sedimenting at 34S, 43S, and "near 50S," respectively (34). We propose that the assembly intermediate, 50S*, observed in our experiments corresponds to the 43S precursor particle, with the difference that 50S* lacks protein L19. Evidently, L19 is limiting for the conversion of the 43S precursor particles to "near 50S" particles in the Δ*21K-1* mutant even though L19 has not usually been regarded as an assembly protein (34). We also note that since the addition of IPTG relieved the assembly defect but did not increase the growth rate of the Δ*21K-1* mutant, the growth rate was probably not limited by the number of mature 50S subunits. That is, there may be a surplus of ribosomes in the mutant at the low growth rate imposed by the lack of the 21K protein. On the other hand, the growth rate of the Δ*rplS-1* mutant expressing only L19 from plasmid pMW271 (P_{tac}-*rplS*$^+$ *16K*$^+$) is strictly dependent on the amount of IPTG added (data not shown). Previously, slowly growing mutants completely lacking protein L19 have been isolated (47). However, because these L19-lacking strains were isolated as antibiotic-independent revertants of an erythromycin-dependent strain, it cannot be excluded that other compensatory mutations were present in the L19-deficient mutants (47). Interestingly, we found that our L19-lacking transductants reverted at a low frequency to clones that survived restreaking. We speculate that the compensatory mutations allowing viability of L19-lacking cells might act by correcting the observed assembly defect.

We have shown that it is possible to systematically inactivate, and distinguish between essential and nonessential, genes on the *E. coli* chromosome by using reverse genetics, and we are currently investigating the molecular basis for the slow growth of the 21K-deficient strain and the possibility that the TrmD protein has two different functions.

ACKNOWLEDGMENTS

Hermann Bujard and Bernt Eric Uhlin are gratefully acknowledged for providing plasmids pUHE21-2 and pHMG64, respectively. We gratefully acknowledge Kristina Nilsson for outstanding technical assistance, Åsa Magnusson for running sucrose gradients, and Lisbet Lind for help with the computer graphics. Glenn Björk and Tord Hagervall are acknowledged for helpful comments on an earlier version of this report.

During part of this work, P.M.W. was the recipient of a postdoctoral fellowship from the Swedish Natural Science Research Council (B-PD 9911). This project was supported by a grant from the Swedish Natural Science Research Council (B-BU 9911) to P.M.W. and by grants to D.E.B. from the National Institutes of Health (HG-00563 and DK 48029). B.C.P. was supported by grants to Glenn R. Björk from the Swedish Cancer Society (project 680) and the Swedish Natural Science Research Council (B-BU 2930).

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