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Construction and Initial Characterization of *Escherichia coli* Strains with Few or No Intact Chromosomal rRNA Operons

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The *Escherichia coli* genome carries seven rRNA (*rrn*) operons, each containing three rRNA genes. The presence of multiple operons has been an obstacle to many studies of rRNA because the effect of mutations in one operon is diluted by the six remaining wild-type copies. To create a tool useful for manipulating rRNA, we sequentially inactivated from one to all seven of these operons with deletions spanning the 16S and 23S rRNA genes. In the final strain, carrying no intact rRNA operon on the chromosome, rRNA molecules were expressed from a multicopy plasmid containing a single rRNA operon (prrn). Characterization of these *rrn* deletion strains revealed that deletion of two operons was required to observe a reduction in the growth rate and rRNA/protein ratio. When the number of deletions was extended from three to six, the decrease in the growth rate was slightly more than the decrease in the rRNA/protein ratio, suggesting that ribosome efficiency was reduced. This reduction was most pronounced in the Δ7 prrn strain, in which the growth rate, unlike the rRNA/protein ratio, was not completely restored to wild-type levels by a cloned rRNA operon. The decreases in growth rate and rRNA/protein ratio were surprisingly moderate in the *rrn* deletion strains; the presence of even a single operon on the chromosome was able to produce as much as 56% of wild-type levels of rRNA. We discuss possible applications of these strains in rRNA studies.

In *Escherichia coli*, the number of ribosomes per cell is proportional to the growth rate to satisfy the cell's demand for protein synthesis (23). At fast doubling times there are as many as 70,000 ribosomes per *E. coli* cell, while at lower growth rates this number is reduced to 20,000 or less (5). Control of ribosome content is exerted at the level of transcription of the seven rRNA (*rrn*) operons on the chromosome (18, 29). Expression of rRNA is gene dosage independent; when the number of rRNA operons in the cell is increased by the presence of plasmid-borne operons, total rRNA synthesis rates remain constant (feedback control) (10, 19, 21, 23). Conversely, inactivation of up to four *rrn* operons on the chromosome leads to a compensatory increase in expression of the remaining intact copies (7). Regulation of *rrn* expression occurs at the level of transcription initiation (23), and one effector of both growth rate-dependent and feedback control is thought to be the intracellular concentrations of ATP and GTP, the initiating nucleotides of the *rrn* P1 promoters (14).

Although it is generally assumed that the redundancy of rRNA operons in *E. coli* has evolved to support the high levels of ribosome production necessary for rapid growth rates (22, 28), there is also evidence suggesting that *E. coli* requires all of its operons for optimal adaptation to changing physiological conditions (8). rRNA operon multiplicity among the best-studied eubacteria has, however, significantly impeded the genetic study of rRNA structure, function, and evolution in these organisms. Besides the 7 *rrn* operons present in *E. coli* (24), *Bacillus subtilis* (4, 26) and *Clostridium perfringens* (15) have 10 each and *Lactococcus lactis* (2, 34) has 6 copies of each of the rRNA genes. As a means of overcoming the multiplicity problem, we sequentially inactivated *rrn* operons in *E. coli* until we ultimately succeeded in constructing a strain containing a single exchangeable operon on a plasmid.

We had previously inactivated up to four of the rRNA operons by a deletion-insertion mutagenesis scheme in which each deletion site was filled in with a different antibiotic resistance gene (7). While this technique provided a facile means of operon inactivation, there was an insufficient number of suitable antibiotic resistance genes to inactivate all seven operons, and we were concerned that the accumulation of antibiotic resistance mechanisms would influence the physiology of the cell. In the study reported here, we therefore employed a different approach, in which many of the operons were inactivated without the introduction of antibiotic resistance cassettes, and succeeded in inactivating all seven chromosomal rRNA operons. The survival of this strain is ensured by the presence of a plasmid-encoded rRNA operon. In a separate publication (1), we have demonstrated one important use of this strain by successfully exchanging the wild-type plasmid-borne *E. coli rrn* operon for operons from *Salmonella typhimurium* and *Proteus vulgaris* as well as a hybrid operon in which the GTPase center of the *E. coli* 23S rRNA had been replaced by the corresponding domain from *Saccharomyces cerevisiae*. Here we describe in detail the construction of the *rrn* deletion series and an initial study of their physiological properties, as much to answer questions about the effect of *rrn* multiplicity on bacterial physiology as to characterize a set of strains we believe will be useful to the scientific community.

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MATERIALS AND METHODS

Bacterial growth conditions. The bacterial growth conditions were described previously (1).

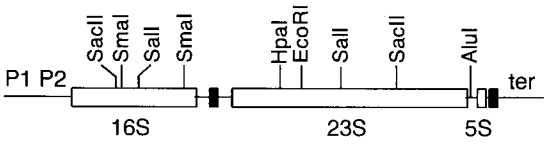

FIG. 1. The common structure of the rRNA operons in *E. coli*. Open and filled rectangles represent rRNA (16S, 23S, and 5S) and tRNA genes, respectively. *rrnB*, *rrnC*, *rrnE*, and *rrnG* contain the spacer tRNA gene for Glu-2, and the other operons (*rrnA*, *rrnD*, and *rrnH*) contain the spacer tRNA genes for Ile-1 and Ala-1B (25). Distal tRNA genes are encoded by only three operons: *rrnC* contains the tRNA genes for Asp-1 and Trp, and *rrnD* and *rrnH* contain the tRNA genes for Thr-1 and Asp-1, respectively. The figure also indicates the relative positions of promoters (P1 P2), terminators (ter), and relevant restriction sites.

Exchange of alleles with a *polA* strain and the *sacB* gene. We have developed an effective method for allele exchange between chromosomal and plasmid-encoded rRNA operons by modifying previously reported techniques (20, 31). DNA fragments containing each of the seven rRNA operons (Fig. 1) and their flanking regions were first cloned into ColE1-type plasmid vectors carrying the ampicillin resistance (Ap^r) gene. Deletion mutations inactivating both the 16S and 23S rRNA genes were then introduced into each operon. A cassette containing the *B. subtilis sacB* gene and the kanamycin resistance marker (*sacB*-Km^r) was then prepared from pBIP3 (31) and inserted into the plasmid within the vector sequence (Fig. 2A). Expression of *sacB* in *E. coli* is lethal in the presence of sucrose (16). Thus, the cassette allows both positive (Km^r) and negative (sucrose-sensitive [Suc^s]) selection of the resulting plasmid. The plasmid was then electroporated into *polA1* (Am) mutant cells in which the corresponding rRNA operon on the chromosome had been inactivated with the chloramphenicol resistance (Cm^r) gene (the *cat* gene [Fig. 2B]). We took advantage of previous work from our laboratory (9) in which each rRNA operon on the chromosome was inactivated by this gene. Initiation of DNA replication from the ColE1-type origin requires the *polA* gene product, DNA polymerase I. Thus, *polA* mutant cells transformed to Ap^r and Km^r are likely to contain the entire plasmid integrated into the chromosome by a single crossover event (Fig. 2C). All integrants showed sucrose sensitivity. Since the rRNA genes encoded in the seven operons have essentially identical primary structures, we relied on flanking sequences to direct recombination with the desired operon, and by Southern blot analysis (*rrnB*) or P1 transduction (*rrnH*, *rrnG*, and *rrnA*) we confirmed that integration had occurred in the correct operon. In the latter case, P1 lysates were prepared on each integrant and the cotransduction frequency of antibiotic resistance markers was analyzed. If the plasmid integrated into the correct operon, the Cm^r marker of the inactivated chromosomal operon and the Ap^r and Km^r markers introduced by the plasmid cotransduced with a high frequency.

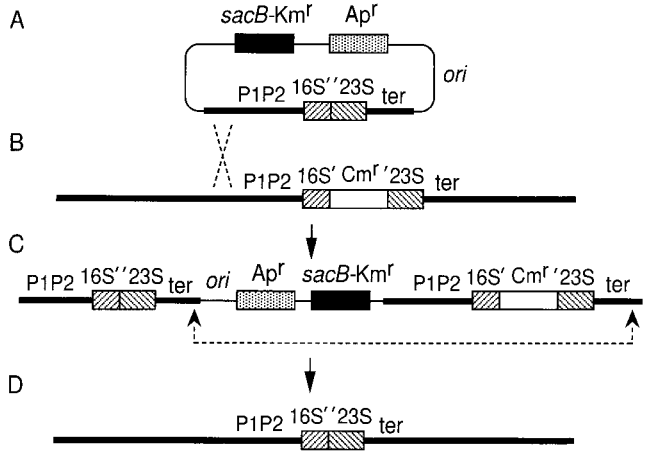

FIG. 2. The basic strategy for allele exchange. Thick and thin lines represent chromosomal and plasmid sequences, respectively. The hatched rectangles indicate the 16S and 23S rRNA genes. The 5S rRNA and tRNA genes are not shown. Stippled and open rectangles represent the ampicillin and chloramphenicol resistance genes, respectively, and closed rectangles the *sacB*-Km^r cassette. *ori* indicates the relative position of the ColE1-type replication origin. Broken lines indicate possible crossover sites for a successful allele exchange. In panels B, C, and D, only a part of the chromosome is shown. See Fig. 1 for definitions of the other symbols.

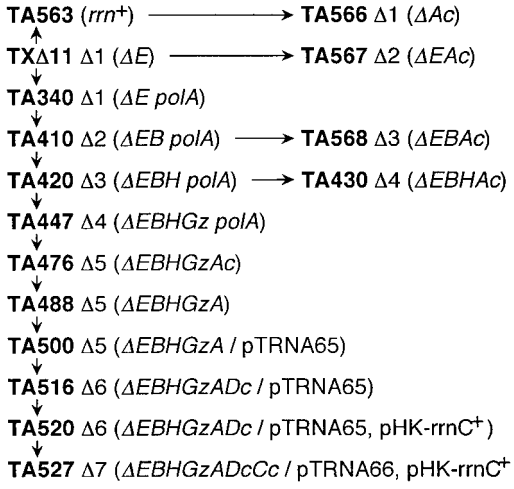

FIG. 3. The pedigree of *rrn* deletion strains. Inactivated rRNA operons are indicated by uppercase letters derived from their specific operon names (for example, *A* for *rrnA*). When the inactivation was carried out by a deletion-insertion mutation, an uppercase letter is followed by a lowercase *c* or *z*, representing the inserted gene *cat*$^+$ or *lacZ*$^+$, respectively (for example, *Ac* for *rrnA*::*cat*$^+$). See Table 1 for precise genotypes. *polA* mutant strains are resistant to tetracycline since these mutations are linked to Tn*10*. pTRNA and pHK-rrnC$^+$ contain spectinomycin (Spc^r) and Km^r markers, respectively.

If a second crossover occurs, as indicated in Fig. 2C, the vector DNA, which includes the Ap^r gene and the *sacB*-Km^r cassette, and the *cat*-containing operon are excised, leaving the operon with the deletion mutation on the chromosome (Fig. 2D). Cells that had undergone such an excision event were effectively isolated by selecting the integrants for sucrose resistance followed by screening the Suc^r cells for sensitivity to ampicillin, kanamycin, and chloramphenicol. In practice, we grew several Suc^s Cm^r integrants independently at 37°C overnight in Luria-Bertani (LB) broth without antibiotics. The cultures were then diluted and plated on salt-free LB plates containing 8% sucrose (3). The plates were incubated at 30°C (for *rrnB*, *rrnH*, and *rrnG*) or 37°C (for *rrnA*) for 20 h, and colonies were picked for a chloramphenicol sensitivity test. Typically ~1% of the cells grown overnight in the absence of antibiotics were Suc^r, and ~5% of these Suc^r cells were Cm^s. All Cm^s cells were sensitive to ampicillin and kanamycin.

Construction of *rrn* deletion strains. As a starting point for *rrn* operon inactivation, we used Ellwood and Nomura's TXΔ11 strain (12), which carries a large chromosomal deletion spanning the *rrnE* operon. Before the first allele exchange, the *polA1* mutation was introduced into TXΔ11 by P1 transduction by virtue of its linkage to *zih*::Tn*10* (encoding tetracycline resistance), generating TA340 (Fig. 3; Table 1). The presence of the mutation in this strain was verified by its sensitivity to methyl methanethiosulfonate.

(i) Inactivation of *rrnB*. The *rrnB* operon in TA340 was first inactivated by introducing the Δ(*rrsB-gltT-rrlB*)*1*::*kan*$^+$ mutation (7). (For *rrnB*, a *cat*-containing operon was not used in this step.) The resulting strain (TA405) was then transformed to ampicillin resistance (TA406) with pMA101 (Table 2), and the Δ(*rrsB-gltT-rrlB*)*1*::*kan*$^+$ allele was removed from the chromosome as described above except that Suc^r cells were screened for sensitivity to ampicillin and kanamycin. The presence of the Δ(*rrsB-gltT-rrlB*)*101* deletion mutation on the chromosome of one of the Ap^s Km^s clones (TA410) was confirmed by PCR amplification of ribosomal DNA followed by agarose gel analysis. The primers used for the PCR were 5′-GGCCTAACACATGCAAGTCGAA-3′ and 5′-GCTTACACACCCGGCCTATCAA-3′, which hybridize near the 5′ end of the 16S gene and the 3′ end of the 23S gene, respectively. With these primers, the *rrnB* operon carrying the Δ(*rrsB-gltT-rrlB*)*101* deletion gives a 2,287-bp PCR fragment whereas the wild-type and *kan*-containing operons give 4,791- and 4,026-bp fragments, respectively.

(ii) Inactivation of *rrnH*. TA410 (Δ*EB polA*) was first transduced to Cm^r (TA415) with Δ(*rrsH-ileV-alaV-rrlH*)*37*::*cat*$^+$ (Δ*rrnH* in reference 9) and then transformed to Ap^r and Km^r (TA418) with pMA103 (Table 2). The Δ(*rrsH-ileV-alaV-rrlH*)*37*::*cat*$^+$ allele was removed from TA418 as described above. The presence of the new deletion mutation in one of the Cm^s Ap^s Km^s clones (TA420) was confirmed by PCR with the primers described above. We detected a PCR fragment of the expected size (1,290 bp).

(iii) Inactivation of *rrnG*. TA420 (Δ*EBH polA*) was first transduced to Cm^r (TA443) with Δ(*rrsG-gltW-rrlG*)*33*::*cat*$^+$ (Δ*rrnG* in reference 9) and then transformed to Ap^r and Km^r (TA445) with pNY30 (Table 2). This plasmid carries an *rrnG* allele inactivated by an internal deletion and the concomitant insertion of the *lacZ* coding region into the site of the deleted operon. The Δ(*rrsG-gltW-rrlG*)*33*::*cat*$^+$ allele was removed from TA445 as described above

TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype	Source, reference, or construction
TXΔ11[b]	Δ(*purDH-rrnE-metA*)	12
Strains of TXΔ11 background		
TA340	TXΔ11 *polA1 zih*::Tn*10*	TXΔ11 × P1.AQ8809, with selection for Tc^r, PolA$^-$
TA405	TA340 Δ(*rrsB-gltT-rrlB*)*1*::*kan*$^+$	TA340 × P1.CC164, with selection for Km^r
TA406	TA340 Δ(*rrsB-gltT-rrlB*)*1*::*kan*$^+$ *zij*::pMA*101*	TA405 transformed with pMA101; selection for Ap^r, Suc^s
TA410	TA340 Δ(*rrsB-gltT-rrlB*)*101*	Suc^r derivative of TA406; Ap^s Km^s
TA415	TA410 Δ(*rrsH-ileV-alaV-rrlH*)*37*::*cat*$^+$	TA410 × P1.JP37, with selection for Cm^r
TA418	TA410 Δ(*rrsH-ileV-alaV-rrlH*)*37*::*cat*$^+$ *zaf*::pMA*103*	TA415 transformed with pMA103; selection for Ap^r, Km^r, Suc^s
TA420	TA410 Δ(*rrsH-ileV-alaV-rrlH*)*103*	Suc^r derivative of TA418; Ap^s Km^s Cm^s
TA430	TA420 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ *polA*$^+$	TA420 × P1.CC164, with selection for Cm^r, Tc^s, PolA$^+$
TA443	TA420 Δ(*rrsG-gltW-rrlG*)*33*::*cat*$^+$	TA420 × P1.JP33, with selection for Cm^r
TA445	TA420 Δ(*rrsG-gltW-rrlG*)*33*::*cat*$^+$ *zfg*::pNY*30*	TA443 transformed with pNY30; selection for Ap^r, Km^r, Suc^s
TA447	TA420 Δ(*rrsG-gltW-rrlG*)*30*::*lacZ*$^+$	Suc^r derivative of TA445; Ap^s Km^s Cm^s
TA472	TA410 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$	TA410 × P1.CC164, with selection for Cm^r, Tc^r, PolA$^-$
TA476	TA447 *polA*$^+$ Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$	TA447 × P1.CC164, with selection for Cm^r, Tc^s, PolA$^+$
TA480	TA410 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ *zih*::pNY*34*	TA472 transformed with pNY34; selection for Ap^r, Km^r, Suc^s
TA485	TA447 *polA*$^+$ Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ *zih*::pNY*34*	TA476 × P1.TA480, with selection for Cm^r, Km^r, Ap^r, Tc^s, PolA$^+$, Suc^s
TA488	TA447 *polA*$^+$ Δ(*rrsA-ileT-alaT-rrlA*)*34*	Suc^r derivative of TA485; Ap^s Km^s Cm^s
TA500	TA488/pTRNA65	TA488 transformed with pTRNA65; selection for Spc^r
TA516	TA488 Δ(*rrsD-ileU-alaU-rrlD*)*25*::*cat*$^+$/pTRNA65	TA500 × P1.JP25, with selection for Cm^r
TA520	TA488 Δ(*rrsD-ileU-alaU-rrlD*)*25*::*cat*$^+$/pTRNA65 pHK-rrnC$^+$	TA516 transformed with pHK-rrnC$^+$; selection for Km^r
TA520.5	TA488 Δ(*rrsD-ileU-alaU-rrlD*)*25*::*cat*$^+$/pTRNA66 pHK-rrnC$^+$	$tRNA_2^{Glu}$ deletion from pTRNA65 in TA520, generating pTRNA66
TA525	TA520.5 Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$ *ilv500*::Tn*10*	TA520.5 × P1.TA575, with selection for Tc^r, *rrnC*::*cat*$^+$
TA527	TA520.5 Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$ *ilv*$^+$	TA525 × P1.JP15, with selection for Ilv$^+$, Tc^s
TA559.5	TXΔ11/pBEU49	TXΔ11 transformed with pBEU49; selection for Ap^r, Km^r, Ts[a]
TA560[c]	*pur*$^+$ rrnE$^+$ metA$^+$ metB1 btuB3191::Tn*10*/pBEU49	TA559.5 × Hfr CAG5052, with selection for Tc^r Ap^r, Km^r, Pur$^+$, *rrnE*$^+$, Met$^-$
TA563[c]	*pur*$^+$ *rrnE*$^+$ *metA*$^+$ *metB1 btuB3191*::Tn*10*	Temperature-resistant derivative of TA560; Ap^s Km^s
TA566	TA563 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$	TA563 × P1.CC164, with selection for Cm^r
TA567	TXΔ11 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$	TXΔ11 × P1.CC164, with selection for Cm^r
TA568	TA410 Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ *polA*$^+$	TA410 × P1.CC164, with selection for Cm^r, Tc^s, PolA$^+$
Intermediates in strain constructions		
AQ8809	*polA1 zih*::Tn*10*	T. Kogoma
CAG5052	KL227 *btuB3191*::Tn*10*	30
CAG18431	MG1655 *ilv500*::Tn*10*	30
CC164	W1485 Δ(*rrsB-gltT-rrlB*)*1*::*kan*$^+$ Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ Δ(*rrsG-gltW-rrlG*)*1*::*spc*$^+$	BAG1 in reference 7
JP15	W1485 Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$	W1485 ΔC in reference 9
JP25	W1485 Δ(*rrsD-ileU-alaU-rrlD*)*25*::*cat*$^+$	W1485 ΔD in reference 9
JP33	W1485 Δ(*rrsG-gltW-rrlG*)*33*::*cat*$^+$	W1485 ΔG in reference 9
JP37	W1485 Δ(*rrsH-ileV-alaV-rrlH*)*37*::*cat*$^+$	W1485 ΔH in reference 9
TA575	W1485 Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$ *ilv500*::Tn*10*	JP15 × P1.CAG18431, with selection for Tc^r, Cm^r

[a] Ts, temperature sensitive.
[b] The remaining genotypes are F$^-$ *ara* Δ*lac thi*.
[c] The presence of *metA*$^+$ *metB1* in these strains has not been verified (see Materials and Methods).

except that 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 60 μg/ml) was added to sucrose-containing plates. Suc^r, blue colonies were then screened for sensitivity to ampicillin, kanamycin, and chloramphenicol. Cells that were sensitive to these antibiotics should have contained only the Δ(*rrsG-gltW-rrlG*)*30*::*lacZ*$^+$ allele on the chromosome and were designated TA447.

(iv) Inactivation of *rrnA*. The standard inactivation procedure was slightly modified as described below to restore the *polA*$^+$ genetic background. The Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ mutation (Δ*rrnA* in reference 9) was first introduced into TA410 (Δ*EB polA*) by P1 transduction. The TA410 transductants were selected for Cm^r and Tc^r and screened for PolA$^-$ by assaying the transformation efficiency of pBR322. This strain was named TA472. Next, pNY34 (Table 2) was integrated into the chromosome of TA472 near *rrnA*. Plasmid pNY34 contains an internally deleted *rrnA* operon. A P1 lysate was prepared on the resulting strain (TA480) and used to transduce integrated pNY34 (*zih*::pNY*34*) into TA476. TA476 was generated by transducing the Δ(*rrsA-ileT-alaT-rrlA*)*1*::*cat*$^+$ mutation into TA447 (Δ*EBHGz polA*) and is *polA*$^+$ due to cotransduction of the *polA*$^+$ allele. The TA476 transductants were selected for resistance to ampicillin, kanamycin, and chloramphenicol and screened for sensitivity to tetracycline (Tc^s). The presence of *polA*$^+$ in Ap^r Km^r Cm^r Tc^s cells was confirmed by assaying the transformation efficiency of pACYC184, which also requires DNA polymerase I for replication. The resulting cells (TA485) were grown to saturation, and Suc^r cells were obtained as described above. Finally, the Suc^r cells

TABLE 2. Plasmids used in this study

Plasmid designation	Relevant characteristics	Source or reference
Plasmids used for inactivation of *rrnB*		
pSTL102	pBR322 carrying a mutant (Spc^r Ery^r) *rrnB* operon and its flanking regions in the *tet* gene	33
pMA100	pSTL102 carrying the *Sal*I-*Sal*I deletion in *rrnB*	This work
pMA101	pMA100 carrying the *sacB*-Km^r cassette in the *Bam*HI site	This work
Plasmids used for inactivation of *rrnH*		
pLC7-21	A ColE1 plasmid carrying the *rrnH* operon and its flanking regions	6
pC5	pBR322 carrying $rrnH^+$ (*Eco*RV fragment of pLC7-21) in the *Eco*RV site	This work
pMA102	pC5 carrying the *Sac*II-*Sac*II deletion in *rrnH*	This work
pMA103	pMA102 carrying the *sacB*-Km^r cassette in the *Sal*I site	This work
Plasmids used for inactivation of *rrnG*		
pLC23-30	A ColE1 plasmid carrying the *rrnG* operon and its flanking regions	6
pC14	pBR322 carrying $rrnG^+$ (*Bam*HI fragment of pLC23-30)	7
pNY2	The *Sma*I-*Hpa*I region of *rrnG* in pC14 was replaced with a $lacZ^+$ fragment	This work[a]
pNY30	pNY2 carrying the *sacB*-Km^r cassette in the *Hin*dIII site	This work[a]
Plasmids used for inactivation of *rrnA*		
pLC19-3	A ColE1 plasmid carrying the *rrnA* operon and its flanking regions	6
pC1	pBR322 carrying $rrnA^+$ (*Bam*HI fragment of pLC19-3) in the *Bam*HI site	This work
pC1ΔSacII	pC1 carrying the *Sac*II-*Sac*II deletion in *rrnA*	This work
pNY34	pC1ΔSacII carrying the *sacB*-Km^r cassette in the *Eco*RV site	This work
Other plasmids		
pBEU49	A runaway-replication mutant plasmid carrying the *recAo281* gene (Ap^r Km^r)	35
pBIP3	A source of the *sacB*-Km^r cassette (4.7-kb *Bam*HI fragment)	31
pC4	pBR322 carrying $rrnC^+$ (*Eco*RV fragment of pLC22-36) in the *Eco*RV site	This work
pC8	pBR322 carrying $rrnD^+$ (*Afl*II-*Pst*I fragment of pLC16-6) in the *Eco*RV site	This work

[a] All cloning experiments for these plasmids were carried out at 30°C with M9-glycerol medium.

were screened for sensitivity to ampicillin, kanamycin, and chloramphenicol, thereby obtaining TA488. The presence of the *rrnA* deletion in this strain was verified by Southern blot analysis.

(v) Inactivation of *rrnD*. TA500 is the same as TA488 (Δ*EBHGzA*) but carries the tRNA-containing plasmid pTRNA65 (1). This strain was transduced to Cm^r with Δ(*rrsD-ileU-alaU-rrlD*)*25*::*cat*$^+$ (Δ*rrnD* in reference 9), generating TA516.

(vi) Inactivation of *rrnC*. pHK-rrnC^+ (Km^r) (1) was first introduced into TA516 (Δ*EBHGzADc*/pTRNA65), generating TA520. A spontaneous deletion of the gene for $tRNA_2^{Glu}$ from pTRNA65 in TA520 resulted in TA520.5 carrying pTRNA66 (1). Restriction mapping indicated that pTRNA65 suffered a spontaneous deletion, likely by recombination between redundant parts of the 23S gene surrounding $tRNA_2^{Glu}$. The Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$ mutation (Δ*rrnC* in reference 9) was then introduced into TA520.5 by P1 transduction by virtue of its linkage to *ilv500*::Tn*10* in strain TA575. The presence of Δ(*rrsC-gltU-rrlC*)*15*::*cat*$^+$ in Tc^r transductants (TA525) was verified by PCR with the following primers: 5′-CTTCCATGTCGGCAGAATGCTT-3′ and 5′-GCCTGCATACCG TTGTCGATAG-3′. These primers hybridize near the ends of the *cat* gene and the *rrnC* operon, respectively, and amplify an 850-bp fragment. Finally, the ilv^+ allele was introduced into TA525 (Δ*EBHGzADcCc ilv*/pTRNA66/pHK-rrnC^+) by P1 transduction, generating TA527. The lack of intact 16S and 23S rRNA genes on the chromosome of this strain was confirmed by Southern blot analysis (1).

Construction of an rrn^+ strain, TA563. An rrn^+ strain was constructed from TXΔ11 by introducing $rrnE^+$ by Hfr mating. The donor and recipient strains were CAG5052 and TA559.5, respectively. TA559.5 was constructed from TXΔ11 by introducing pBEU49 (35). This plasmid was used only to provide convenient counterselection (Ap^r Km^r) of the donor strain. The mating was carried out at 30°C for 20 min, and the cells were plated on LB plates containing tetracycline, ampicillin, and kanamycin. The plates were incubated at 30°C for 16 h, and the exconjugants were screened for Pur^+ and $rrnE^+$. The screening for $rrnE^+$ was carried out by PCR. The primers used for the reaction were 5′-GA ATTCGACGATACCGGCTTTG-3′ and 5′-CCACTCGTCAGCAAAGAAGC AA-3′, which hybridize to the *purH* and 16S sequences, respectively. These primers amplify a 787-bp fragment from the wild-type *rrnE* region. Finally, pBEU49 was removed from one of the Pur^+ $rrnE^+$ exconjugants (TA560) by using its runaway replication property, generating TA563. Although $metA^+$ was most likely introduced into TA560 with pur^+-$rrnE^+$ by Hfr mating, the strain remains Met^- since the *metB1* mutation is located close to *btuB*::Tn*10* in the donor chromosome. The presence of all seven rRNA operons in TA563 was confirmed by Southern blot analysis (1).

Total RNA/total protein and tRNA/rRNA ratios. Total RNA/total protein and tRNA/rRNA ratios were determined as described previously (1).

RESULTS AND DISCUSSION

Construction of *rrn* deletion strains. The starting material for the construction of our *rrn* deletion series was TXΔ11, an *E. coli* strain constructed by Ellwood and Nomura (12), which contains a deletion mutation encompassing the entire *rrnE* operon (Table 1). The six remaining *rrn* operons in this strain were sequentially inactivated by deletion mutations spanning the 16S and 23S rRNA genes as summarized in Fig. 3. The details of the inactivation procedures are described in Materials and Methods. Briefly, the *rrnB* and *rrnH* operons were inactivated by removal of internal *Sal*I-*Sal*I and *Sac*II-*Sac*II fragments, respectively (Fig. 1). The *rrnG* operon was inactivated by a deletion-insertion mutation (Δ*Gz* in Fig. 3) in which the *Sma*I-*Hpa*I region of the operon (Fig. 1) was replaced by the *lacZ* coding region. The *rrnA* operon was initially inactivated with one of the deletion-*cat* insertion mutations constructed previously in our laboratory (9). This deletion-*cat* insertion mutation in *rrnA* (Δ*Ac* in TA476 [Fig. 3]) was then replaced by a simple deletion mutation (Δ*A* in TA488) that removed the *Sac*II-*Sac*II region of the operon. The *rrnD* and *rrnC* operons were also inactivated with deletion-*cat* insertion mutations (Δ*Dc* and Δ*Cc*, respectively) (9). In the final strain (TA527), carrying no intact rRNA operons on the chromosome (Δ7 prrn), rRNA molecules were expressed from a multicopy plasmid, pHK-rrnC^+ (prrn). This plasmid is a derivative of pSC101 containing only the wild-type *rrnC* operon (1).

Each rRNA operon contains at least one tRNA gene between the 16S and 23S rRNA genes (Fig. 1); *rrnA*, *rrnD*, and

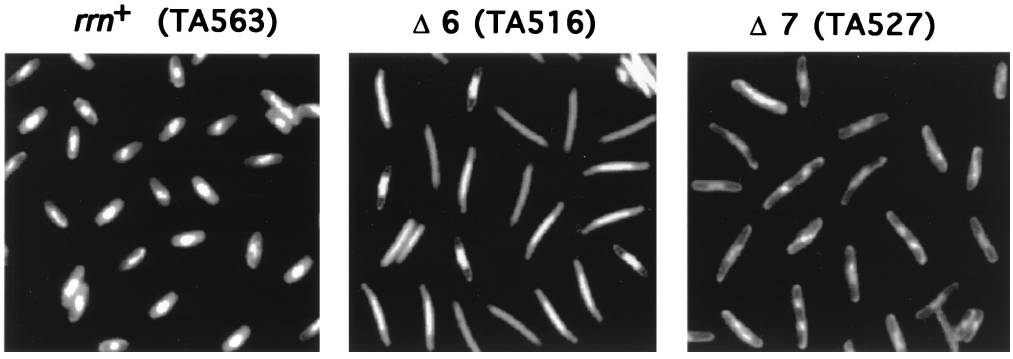

FIG. 4. Microscopic examination. Cells from exponential-phase cultures were stained with BacLight (Molecular Probes Inc., Eugene, Oreg.) and analyzed by fluorescence microscopy.

rrnH contain the tRNA genes for Ile-1 and Ala-1B, and the rest (*rrnB*, *rrnC*, *rrnE*, and *rrnG*) contain the tRNA gene for Glu-2 (25). These tRNA (spacer tRNA) genes are encoded only in the rRNA operons. Since the introduction of our *rrn* deletions ultimately removes all spacer tRNA genes, we have cloned these genes into a derivative of pACYC184, resulting in pTRNA65 and -66 (1) (Fig. 3). In these plasmids, the tRNA genes are transcribed from the *tac* promoter. Expression of tRNAs for Ile-1 and Ala-1B from the cloned genes was essential for the viability of Δ6 strains, in which only the *rrnC* operon carrying the gene for $tRNA_2^{Glu}$ was left on the chromosome. The same tRNA genes were also required in the Δ7 prrn strain that contained pHK-rrnC$^+$.

In addition to the spacer tRNA genes, *rrnC*, *rrnD*, and *rrnH* encode different tRNA genes near the end of the operons (distal tRNA genes) (Fig. 1). Although none of the above-described *rrn* deletions blocks the expression of distal tRNAs, their genes were also cloned in pTRNA65 and -66. This was done to ensure sufficient expression of the distal tRNAs in Δ7 prrn strains carrying a high-copy-number rRNA plasmid (1), in which the chromosomal *rrn* operons are likely to be severely feedback repressed.

Cell morphology. We first carried out a microscopic examination of cells from *rrn*$^+$ (TA563) and *rrn* deletion strains. We found that cells with inactivated rRNA operons showed a pronounced morphological change during exponential growth; the cells became more and more elongated, with this change being very apparent in a Δ6 strain (TA516 [Fig. 4]). Using a vital stain, we saw no indication that dead cells accumulated in the deletion strains (data not shown). The elongated cell morphology was not completely reversed in a Δ7 prrn strain (TA527) containing the rRNA and tRNA plasmids (Fig. 4), suggesting that cellular processes other than *rrn* gene dosage are still perturbed in this strain (see below).

Growth rate. We next measured the growth rates of *rrn*$^+$ and *rrn* deletion strains in a rich nutrient medium (1), in which a large number of ribosomes are needed for short cell division times and in which the effects of *rrn* inactivation should be most pronounced (7, 8). For this and the other physiological studies described below, we constructed new Δ1 to Δ4 strains (TA566, -567, -568, and -430, respectively) (Fig. 3; Table 1) that contained the *polA*$^+$ allele and the deletion-*cat* insertion mutation in *rrnA*) (Δ*Ac*). The Δ5 strain used for physiological studies was TA476, which also contained *polA*$^+$ and Δ*Ac*. For a Δ6 strain, we used TA516, which contained the deletion-*cat* insertion mutation in *rrnD* but not in *rrnA*. Unlike these strains, the Δ7 prrn strain, TA527, contains two deletion-*cat* insertion mutations on the chromosome, within *rrnD* and *rrnC*.

As shown in Fig. 5A, a Δ1 strain, TA566, grew at a rate that was indistinguishable from that of the *rrn*$^+$ strain, TA563. Normal growth of Δ1 strains has been previously reported by us and others (8, 12). Inactivation of two operons, however, significantly reduced the growth rate (Fig. 5A). In a previous study, in which up to four rRNA operons were inactivated, a significant growth rate decrease was observed when the third operon was inactivated (8). This difference is likely because of the different *E. coli* strains used in the previous and present studies. In the present study, the growth rate continued to decrease gradually as the number of deletions was extended from three to six. The Δ7 prrn strain, TA527, grew slower than the *rrn*$^+$ and Δ1 strains, which, like the elongated cell morphology, may reflect the persistence of defects in other cellular processes.

It was originally proposed that multiple rRNA operons are necessary to support the high levels of ribosome production required for rapid bacterial growth (22, 27, 28). However, more-recent data suggest that the role of high-level ribosome

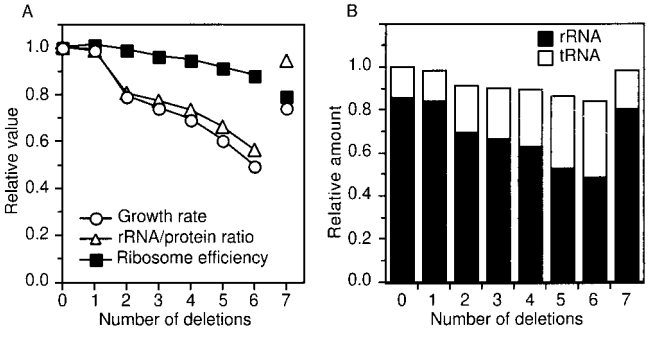

FIG. 5. Physiological effects of *rrn* inactivation. (A) Relative growth rates, rRNA/protein ratios, and ribosome efficiencies. Growth rates (doublings per hour) were determined by monitoring the turbidity of each culture with a Klett-Summerson photoelectric colorimeter and are presented relative to the *rrn*$^+$ strain values. The maximum standard error of growth rate measurements was 0.07. The actual growth rate of the *rrn*$^+$ strain, TA563, was 2.0 doublings/h. rRNA/protein ratios were determined from the data presented in panel B. Ribosome efficiencies were calculated from growth rates and rRNA/protein ratios as described by Bremer and Dennis (5). (B) Relative total RNA/total protein and tRNA/rRNA ratios. The total RNA/total protein and tRNA/rRNA ratios in each total RNA sample were determined as described previously (1). These parameters were normalized to the total amount of RNA in the *rrn*$^+$ strain. Closed and open bars represent the amounts of rRNA and tRNA, respectively. The maximum standard error of RNA measurements was 0.05. Note that the Δ6 strain contains only the tRNA plasmid while the Δ7 strain contains both the tRNA and rRNA plasmids.

production in achieving a maximum growth rate is not as important as the ability to rapidly adapt to better nutritional environments by having a substantial burst in ribosome synthesis (8). The rate of rRNA synthesis in exponentially growing *rrn*$^+$ cells in rich medium is considerably lower than the maximum attainable rate (perhaps only one-sixth of the maximum rate [see below]). Furthermore, strains lacking multiple *rrn* operons require considerably longer periods of time to respond to environmental shifts that demand an upshift in the growth rate (8). It is therefore likely that the higher multiplicity of operons in *rrn*$^+$ cells is necessary for rapid recovery when environmental conditions vary (8).

RNA/protein and tRNA/rRNA ratios. Ninety-eight percent of the total RNA is stable RNA in wild-type *E. coli* (5). We therefore expected that the total RNA/total protein ratio would be one of the physiological parameters most profoundly affected in the *rrn* deletion strains. We measured total RNA and total protein from rapidly growing cells and determined the RNA/protein ratio. Contrary to our expectations, the ratio remained relatively constant, with the Δ6 strain's RNA/protein ratio being approximately 84% of that of the *rrn*$^+$ strain (Fig. 5B).

tRNA accounts for about 14% of the stable RNA, and the tRNA/rRNA ratio is essentially invariant and growth rate independent (5). We wondered, however, whether the *rrn* deletion strains might deviate from this generalization. We have previously shown that inactivation of rRNA operons on the chromosome leads to increased expression of the remaining intact copies (7). While this phenomenon could account for the relatively constant RNA/protein ratio, it might also lead to an increase in the amount of tRNA in the cell, since the control of many tRNA operons is thought to be similar to that of rRNA (23). We therefore measured the proportion of tRNA in the stable RNA of the deletion strains (Fig. 5B). The amounts of tRNA relative to rRNA were similar in the *rrn*$^+$ and Δ1 (TA566) strains, and a substantial increase in the relative amount of tRNA occurred as the number of deletions increased from two to six. This overproduction of tRNA contributes significantly to the maintenance of a relatively constant level of total RNA in the *rrn* deletion strains and supports a model in which tRNA derepression occurs similarly to that of rRNA (11, 21). This result also shows that the reduction in the amount of rRNA in the *rrn* deletion strains is surprisingly moderate. The total rRNA/total protein ratio in the Δ6 strain, in which all of the rRNA is provided by one operon, *rrnC*, is approximately 56% of that measured in the *rrn*$^+$ strain (Fig. 5A). This represents an approximately sixfold increase in the amount of rRNA produced by *rrnC* if one takes into account gene dosage effects contributed by the distance from the origin of replication. (Gene dosage was calculated as described in reference 7, assuming that DNA replication and cell division are not disturbed in the Δ6 strain. The number of *rrn* operons per cell in the Δ6 strain at a doubling time of 61 min is approximately 2.4, versus 27.1 in *rrn*$^+$ cells with a doubling time of 30 min. Since the amount of rRNA in Δ6 strains is 56% of that in *rrn*$^+$ cells, the amount of rRNA produced per operon is increased about sixfold in strains containing only *rrnC*.)

The total RNA/total protein and tRNA/rRNA ratios in the Δ7 prrn strain (TA527) carrying pHK-rrnC$^+$ were similar to those in the *rrn*$^+$ strain (Fig. 5B), indicating that the single rRNA operon (*rrnC*) on this pSC101-based plasmid was able to supply sufficient rRNA to restore these balances.

Ribosome efficiency. The rRNA/protein ratio is proportional to the number of ribosomes per protein, i.e., the ribosome concentration (5). In the Δ2 strain, the percent decrease in the growth rate was similar to the decrease in the rRNA/protein ratio (Fig. 5A), suggesting that the reduced growth rate of this strain was caused by a reduced ribosome concentration. When the number of *rrn* deletions was extended from three to six, however, the decrease in the growth rate was greater than that of the rRNA/protein ratio. This difference between the decreases in the growth rate and the rRNA/protein ratio was most pronounced in the Δ7 prrn strain, in which the rRNA/protein ratio was completely restored to wild-type levels but the growth rate was not. These results suggest that the reduced growth rates of the multiply deleted strains are not simply due to reduced ribosome concentrations. It has been proposed that ribosome concentration and efficiency are growth limiting in any living cell whose protein turnover is negligible and that the growth rate of an exponential-phase culture is proportional to the multiplication product of these two values (5). According to this theory, the reduced growth rates of the Δ3 to Δ7 strains can be attributed, at least in part, to reduced ribosome efficiencies. We have calculated the theoretical ribosome efficiency in each of the *rrn* deletion strains by using the equation presented in reference 5. By these calculations, the ribosome efficiency of Δ7 prrn strains falls to below 80% of wild-type levels (Fig. 5A).

There are several ways in which the ribosome efficiency could be affected by *rrn* inactivation. Ribosome efficiency is determined by the fraction of active ribosomes (i.e., the fraction of ribosomes that are actively engaged in peptide chain elongation) and the rate of peptide chain elongation per ribosome (5). The fraction of active ribosomes is thought to be constant (~80%) at a wide range of growth rates in wild-type *E. coli* (13). This value may not apply to *rrn* deletion strains, however. We have previously demonstrated, for example, that the number of molecules of translation initiation factor IF3 per ribosome is significantly decreased in *rrn* deletion strains (8). Since IF3 stimulates the dissociation of vacant 70S ribosomes and thereby promotes the recycling of the ribosomal subunits for new initiation events (17), the reduced ribosome efficiency that we observe in Δ3 to Δ6 strains may be due to a decrease in the active ribosome fraction.

The other determinant of ribosome efficiency, the rate of peptide chain elongation, is also likely to be affected by the *rrn* deletions. The chain elongation rate is influenced by the concentrations of several factors, such as tRNA, GTP, and elongation factors. We suspect that the concentrations of at least some tRNA molecules may be inadequate in *rrn* deletion strains. In Δ6 and Δ7 prrn strains, for example, the spacer tRNAs for Ile-1 and Ala-1B are expressed from a plasmid and their quantities may differ from those present in the *rrn*$^+$ strain. Alternatively, since derepression of *rrn* expression leads to the overexpression of distal tRNAs and other tRNAs encoded outside of the rRNA operons (Fig. 5B) (11), titration of the tRNA modification machinery by these tRNAs could lead to a reduced chain elongation rate.

In addition to the quantitative differences discussed above, ribosomes in *rrn* deletion strains may exhibit qualitative differences. Since IF3 ensures the accuracy of translation initiation by preventing initiation at codons other than AUG, GUG, or UUG (32), a reduced IF3/ribosome ratio in *rrn* deletion strains could result in the overproduction of certain proteins that are normally poorly expressed. Such an unbalanced expression of proteins might also be predicted to have consequences for the growth rate.

Applications of *rrn* deletion strains. Using the Δ7 prrn strain, we have successfully constructed strains containing an *rrn* operon from a foreign microorganism, such as *Salmonella typhimurium* and *P. vulgaris* (1). Characterization of these strains with hybrid ribosomes emphasizes the usefulness of this

system for evolutionary studies of the translation machinery. The deletion strains can also be used to examine current models of *rrn* regulation and to answer questions about the evolution of bacteria with multiple *rrn* operons. The Δ7 prrn strain should be especially useful for in vitro analysis of ribosome functions, since pure mutant ribosome populations from this strain are available (1). In addition, Δ7 prrn provides a powerful method for the isolation of new rRNA mutations, including conditionally lethal mutations for examining essential functions of rRNA. Questions concerning specific rRNA domains or sequences, modified bases, particular structures, long-range interactions, changes leading to drug resistance, and interaction with other components of the translation apparatus should all be more readily addressed by using the deletion strains described here.

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