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rRNA Operon Multiplicity in *Escherichia coli* and the Physiological Implications of *rrn* Inactivation

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Here we present evidence that only five of the seven rRNA operons present in *Escherichia coli* are necessary to support near-optimal growth on complex media. Seven *rrn* operons are necessary, however, for rapid adaptation to nutrient and temperature changes, suggesting it is the ability to adapt quickly to changing environmental conditions that has provided the selective pressure for the persistence of seven *rrn* operons in *E. coli*. We have also found that one consequence of *rrn* operon inactivation is a miscoordination of the concentrations of initiation factor IF3 and ribosomes.

In *Escherichia coli*, the number of ribosomes per cell varies in proportion to the growth rate to meet the cell's changing demand for protein synthesis. At doubling times of around 20 min, there are as many as 70,000 ribosomes per *E. coli* cell, while at lower growth rates there may be only 20,000 ribosomes per cell (5). This control of ribosome content is exerted at the level of transcription of the seven rRNA operons (*rrn*) present on the *E. coli* genome (16, 22, 29). It is generally assumed that this redundancy of rRNA operons in *E. coli* exists to support the high levels of ribosome production necessary for rapid growth rates (21, 27).

Many other bacteria also possess multiple rRNA transcription units. In *Bacillus subtilis* (3, 23) and *Clostridium perfringens* (14), there are 10 *rrn* operons, and in *Lactococcus lactis*, there are 6 (1, 32). There are also examples of organisms with few *rrn* operons: *Mycoplasma capricolum* has two (30), and the archaebacterium *Halobacterium cutirubrum* possesses only one (20). In general, organisms with multiple *rrn* operons are capable of achieving faster doubling times than those with just one or two. In both *E. coli* (9, 11) and *B. subtilis* (34), it has been demonstrated that one operon can be deleted without a major influence on cell growth rate or physiology. Furthermore, we have previously obtained evidence suggesting that, even at optimal growth rates, the seven *E. coli rrn* operons do not function at their maximal potential but are capable of higher levels of expression under certain conditions (8). These observations suggest that neither organism should really require its full complement of *rrn* operons to sustain high growth rates, and yet the persistence of 7 or 10 functional operons on their genomes suggests some selective advantage to the organism of retaining all of its *rrn* genes.

The seven *rrn* operons in *E. coli* are located in several noncontiguous sites on one-half of the chromosome, centered around the origin of replication, *oriC* (12). Like most other highly expressed genes, the *rrn* operons are all transcribed in the same direction in which the chromosome is replicated, which serves to prevent collisions between RNA and DNA polymerases (13). All seven operons have approximately the same organization: tandem promoters, *P1* and *P2-16S*-spacer tRNA(s)-*23S-5S*-distal tRNA(s) (6, 21). Four of the operons contain $tRNA^{Glu-2}$ in their spacer regions, and three contain $tRNA^{Ala-1B}$ plus $tRNA^{Ile-1}$, while at the 3′ end of the operons, a variety of tRNA genes can be found. In addition to differences in types and numbers of tRNAs encoded, the operons also contain sequence heterogeneities within the genes themselves (2, 7, 31) and within the control (21, 28) and spacer regions (18). Although the regulation of these operons is similar under a wide range of conditions (9), it is conceivable that the heterogeneities within the structural genes could cause differences in the function of the stable RNAs produced. However, it is more generally assumed that all of the *rrn* operons are essentially equivalent; if this is so, a precise number of operons must be required to maintain a selective advantage under certain growth conditions.

We have studied the multiplicity of rRNA operons in *E. coli* by using strains with multiple operons inactivated by antibiotic cassettes in an attempt to identify conditions under which the presence of fewer than seven *rrn* operons was detrimental to the cell. We have found that, in general, recovery from stress conditions is more difficult than rapid growth for strains with less than their full complement of operons. Although multiple features of the translation apparatus are likely to be involved in the deficiencies observed in these strains, we have identified that one consequence of ribosomal operon inactivation is a disruption of the balance between the initiation factor IF3 and the ribosome concentration in the cell.

rRNA operon redundancy under steady-state conditions. There are at least two possible reasons for an organism to possess multiple *rrn* operons. Either some of the operons have evolved to carry out specific cellular functions—for example, to translate specific mRNAs or operate under particular physiological conditions—or the actual number of operons gives the organism a selective advantage under some circumstances. Previous experiments with testing of a variety of genetic and physiological perturbations failed to show a unique requirement for any single *rrn* operon (9). Here, we examine the significance of the number of *rrn* operons on the *E. coli* genome by using strains with multiple *rrn* operons inactivated by antibiotic resistance cassettes.

It has been possible to calculate, given the elongation rate of

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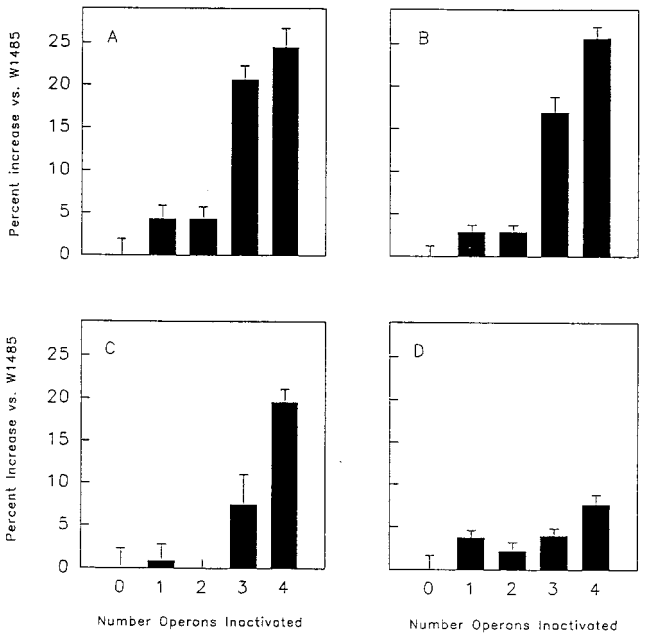

FIG. 1. Percent increase in doubling time of strains carrying multiple *rrn* inactivations. Values are calculated relative to the doubling time of W1485 of 46.9 min on Luria broth glucose at 28°C (A), 24.1 min on Luria broth glucose at 37°C (B), 21.4 min on Luria broth glucose at 42°C (C), and 49.6 min on M9 glucose at 37°C (D). Strains with *rrnA*, *rrnB*, or *rrnD* inactivated are represented by 1; strains with *rrnAG* or *-BA* inactivated are represented by 2; those with *rrnBAG* or *-BAH* inactivated are represented by 3; and those with *rrnBAGH* inactivated are represented by 4. Values are the average of at least six independent measurements for each strain with standard errors as shown. The value for strains with two operons inactivated at 42°C is $-1.9\% \pm 1.4\%$.

RNA polymerase and the length of the operon, that one *rrn* operon could provide only a fraction of the rRNA required for very fast growth rates, even if the operon were transcribed at the maximum density of RNA polymerase that could be accommodated on the DNA (4, 10). Thus, it has been generally hypothesized that the purpose of the redundancy of rRNA operons is to facilitate the high rate of rRNA synthesis in rapidly growing cells (21, 27). However, it has been demonstrated that at least one *rrn* operon can be deleted in *E. coli* without major consequences for the balanced growth of the cell (9, 11). Furthermore, in strains carrying multiply inactivated *rrn* operons, the expression of the remaining copies can increase significantly to compensate for the deficit, suggesting that *E. coli rrn* operons do not operate at maximal capacity (8). Thus, we have suspected that *rrn* operon multiplicity may serve a more important physiological function than to simply provide ample rRNA to the cell to support very short doubling times. To address this issue, we used a set of strains, described elsewhere (8), with as many as four *rrn* operons inactivated by antibiotic resistance cassettes. The strains are named according to the *rrn* operons inactivated; thus, the BA strain has both *rrnB* and *rrnA* inactivated. We measured the growth rates of these strains with different media and under different temperature conditions to assess the minimum number of operons required to sustain doubling times comparable with those of wild-type strains (24.1 min). Although minor increases in doubling time were observed upon inactivation of one or two operons (24.8 min), it was not until at least three *rrn* operons were inactivated that a dramatic increase in doubling time (28.2 min) on complex medium was observed (Fig. 1). This effect was similar for both BAG and BAH strains, and thus the effect is, at least to some extent, independent of the operons inactivated. Inactivation of four *rrn* operons predictably caused yet a further increase in doubling time (30.3 min). Temperature had little effect on the relationship between growth rate and the number of operons inactivated. At 28 and 42°C, the growth rate profiles were similar to that at 37°C in that a significant increase in doubling time was recorded upon inactivation of three *rrn* operons. Thus, it appears that *E. coli* requires only five operons to attain growth rates comparable to those of wild-type strains on complex medium, regardless of the growth temperature.

Because the time taken to replicate the chromosome can be greater than the rate of cell division, *E. coli* resolves this apparent paradox by initiating replication many times in one cell cycle. Once the leading replication fork reaches the terminus of replication, the cell divides and each daughter cell inherits an already partially replicated chromosome. For this reason, genes close to the origin of replication (*oriC* [84 min]) are present in a higher copy number than genes close to the terminus, and the actual gene dosage can be calculated from the doubling time and map locus as described in reference 8. Thus, for example, an *E. coli* strain with a doubling time of 24 min would be predicted to have about 6.5 copies of *rrnC* (84.5 min) but only 3.5 copies of *rrnG* (56.1 min) and a total of about 36 *rrn* equivalents per cell. Since the *rrnG* and *rrnH* operons are furthest from *oriC*, and are thus the two minor contributors of *rrn* gene dosage, this increases the significance of the growth rate decrease seen in cells with these operons inactivated. We have calculated that in the strains represented in this study, inactivation of a single operon removes, on average, about 15% of the total *rrn* equivalents; strains with two operons inactivated have about 30% fewer *rrn* operons; BAG and BAH strains have about 43% of their *rrn* capacity inactivated; and BAGH has 53% of its *rrn* capacity inactivated.

On minimal medium, deletion of four operons was required to see any significant alteration in growth rate, and even then, the increase in doubling time was only 8% greater than that of the wild-type strain, W1485 (Fig. 1). Thus, it appears that *E. coli* strains have many more *rrn* operons than are necessary for near-maximal growth rates on minimal media; just four operons seems to be sufficient.

The genes for spacer $tRNA^{Glu-2}$ and $tRNA^{Ala-1B}$ plus $tRNA^{Ile-1}$ are deleted in the inactivation of *rrnB*, *-G* and *rrnA*, *-H*, respectively. It is possible that the loss of these genes contributes to the growth rate defects observed in these strains. If this is the case, then the minimum number of operons required to sustain near-optimal growth rates may actually be less than five in rich media. However, since the growth rates of BAG and BAH strains are similar, despite the fact that different proportions of each of the sets of spacer tRNA genes are present in these strains, the defects observed are probably primarily caused by rRNA, rather than tRNA, gene deficiency. This is not to say, however, that deletion of the spacer tRNA genes cannot eventually become limiting.

rRNA redundancy under conditions of adaptation. In nature, *E. coli* strains experience constantly changing environments in the host intestine and extreme starvation conditions in the water system and probably achieve balanced growth for only short periods at a time. It is in this context that *E. coli* has evolved to carry seven rRNA operons. Because of the physical restraint on the number of RNA polymerase molecules that can fit on an rRNA operon, strains with fewer operons might be predicted to have problems coping with a sudden escalation in the number of RNA polymerases attempting to initiate *rrn* transcription upon a change to a favorable growth environment. While there is some indication that RNA polymerase

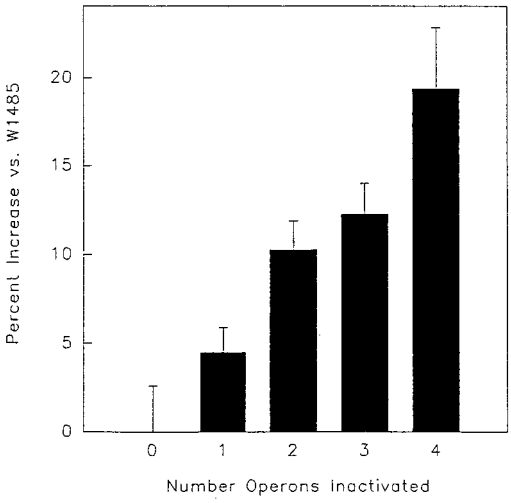

FIG. 2. Percent increase in nutritional shift up time of strains carrying multiple *rrn* inactivations. Values are calculated relative to the shift up time of W1485 of 46.5 min *rrnA*, *-B*, *-D*, *-G*, and *-H*-inactivated strains are represented by 1; *rrnAG*, *-BA*, and -inactivated-*AH* strains are represented by 2; *rrnBAG* and *-BAH* inactivated strains are represented by 3; and the *rrnBAGH*-inactivated strain is represented by 4. Values are the average of at least six independent measurements with standard errors as indicated.

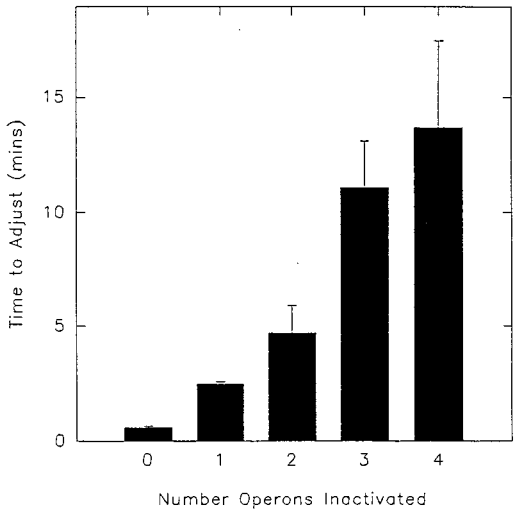

FIG. 3. Time taken to adapt to a temperature shift (28 to 42°C) by strains carrying multiple *rrn* inactivations. *rrnA*, *-B*, *-D*, *-E*, *-G*, and *-H*-inactivated strains are represented by 1; *rrnAG* and *-BA*-inactivated strains are represented by 2; *rrnBAG* and *BAH*-inactivated strains are represented by 3; and the *rrnBAGH*-inactivated strain is represented by 4. Values are the average of at least five independent experiments with standard errors as shown.

can cope with such situations by increasing the rate of initiation and by transcribing faster (8, 33), there is evidence that transcribing too fast may lead to incorrect rRNA folding and subsequently deficient ribosome assembly (24). Therefore, one might speculate that in situations that require a sudden increase in the rate of rRNA production, such as recovery from amino acid starvation or a shift to a more favorable temperature, *E. coli* might benefit from having its full complement of operons.

To assess the ability of *rrn*-deficient strains to adapt to a nutritional shift up, we measured the length of time taken for cells growing exponentially in minimal medium to reachieve logarithmic growth after nutrient addition (i.e., the lag time). Cells were grown to early log phase on M9 glucose medium at 37°C and shifted to complex medium by addition of a 10× solution of Luria broth nutrients. The shift up, or lag, time was defined as the time required to reach the first point of intersection of an extrapolation of the linear portion of the new growth curve with the new growth curve itself. In contrast to the relationship seen under steady-state conditions, the time taken to shift up increased almost linearly with the number of operons inactivated (Fig. 2), suggesting that the persistence of seven rRNA operons in *E. coli* facilitates rapid adaptation from one nutrient environment to the next. It is also interesting to note that if these strains are allowed enter stationary phase, upon shifting up to a fresh medium, they experience extremely long lag times which are directly related to the number of operons inactivated and the length of time in stationary phase (data not shown). Indeed, some strains, e.g., BAGH, cannot be resuscitated after even relatively short periods in stationary phase.

Another condition in which one might predict that inactivation of functional *rrn* operons would cause a delay in the ability of strains to reach balanced growth is after a temperature shift, in which cells growing slowly at a low temperature are suddenly moved to a temperature at which they can grow much faster. We, therefore, measured the lag time of each of the deletion strains after a shift from 28°C to 42°C. This situation is also of potential interest in *rrn* expression because interdigitated with each of the seven $rrnP_1$ promoters, which are sites for sigma-70 recognition, is a potential heat shock promoter. In the case of *rrnB*, this heat shock promoter has been shown to be capable of sigma-32 binding (26). As predicted, the time taken to adapt to the new temperature increased steadily with decreasing numbers of intact operons (Fig. 3), providing indirect evidence consistent with the notion that the role of the seven *rrn* operons is to allow rapid adaptation from one physiological condition to another.

From a hypothetical standpoint, it also makes sense for *E. coli* to have a number of *rrn* operons rather than a few, very highly transcribed operons. In addition to the capability of being turned on more rapidly, multiple operons allow for greater sensitivity of control by virtue of the fact that the effect of small signals is amplified by the number of operons. Multiple operons also provide the potential to evolve independent functions for each of the rRNAs produced. Although no evidence of this has yet been found in *E. coli*, there is precedence for distinct ribosomes in the parasite *Plasmodium* sp., in which one variant of the *18S* gene is expressed predominantly in the mammalian host and another is expressed predominately in the mosquito (17).

Secondary effects of *rrn* inactivation. As a preliminary step to address the issue of what causes the growth rate and adaptive response deficiencies in these strains, we have studied the effect of *rrn* inactivation on some aspects of the translation machinery. We have previously demonstrated that inactivation of *rrn* operons leads to increased expression of the remaining copies, but that a significant ribosome deficiency occurs in strains with three operons inactivated and that a 24% deficiency in the number of ribosomes per cell occurs in BAGH strains (8). Here we show that, in addition to the deficiency in the number of ribosomes, the number of translation initiation factor IF3 molecules per ribosome is also significantly decreased in these strains (Fig. 4). The number of IF3 molecules per ribosome in these strains was calculated by quantitative Western blotting (immunoblotting) as described by Liveris et al. (25). In wild-type cells there were about 0.2 IF3 molecule

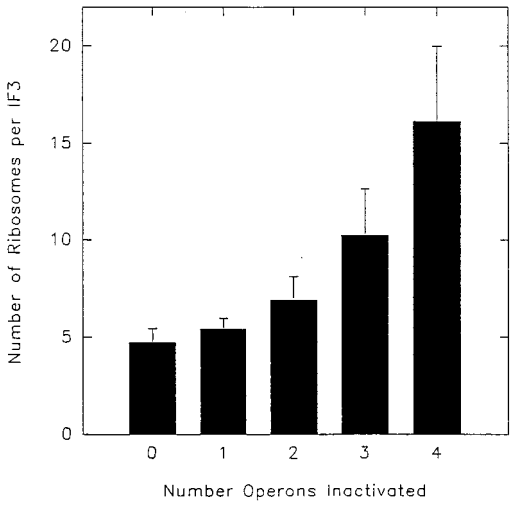

FIG. 4. Ribosome/IF3 ratio in *rrn* inactivation strains. *rrnA* and *-B*-inactivated strains are represented by 1, *rrnAG* and *-BA*-inactivated strains are represented by 2, *rrnBAG* and *-BAH*-inactivated strains are represented by 3, and the *rrnBAGH*-inactivated strain is represented by 4. Values are the average of at least four independent experiments with standard errors as shown.

per ribosome, or 1 IF3 molecule per five ribosomes. However, in BAGH cells, there were only 0.06 IF3 molecule per ribosome, or 1 IF3 for every 16 ribosomes. Thus, in addition to having fewer ribosomes, strains carrying multiple inactivated operons may also be impaired in their ability to initiate translation.

Normally, the amount of IF3 is tightly coupled to the number of ribosomes, such that there is about one molecule of IF3 per five or six ribosomes, regardless of the growth rate (19). This control of IF3 (*infC*) expression is thought to be at the translational level and to be mediated by an autogenous feedback mechanism (15). One of the roles of IF3 is to ensure the accuracy of translation initiation by preventing initiation at codons other than AUG, GUG, or UUG. Because the *infC* mRNA has an AUU initiation codon, low levels of IF3 are thought to permit translation of its own message because of the decreased stringency, whereas high levels repress *infC* translation because of the increased fidelity of the ribosomes. In this way, the ribosome/IF3 ratio is maintained constant, despite the fluctuations of ribosome number with growth rate. Since there appears to be only one IF3 molecule per 16 ribosomes in BAGH cells, these strains appear to have uncoupled the regulation of IF3 from the number of ribosomes by a mechanism that is not yet understood but that appears, at face value, to be inconsistent with the model proposed above. At this ribosome/IF3 ratio, translation initiation would be predicted to be very sloppy and should tend to lead to increased *infC* translation to restore the balance to one IF3 molecule per six ribosomes, but for some reason it does not. It is possible that because the number of ribosomes may be limiting in these strains, preferential translation of messages with good initiation regions occurs, resulting in an IF3 deficit. In any event, for the purposes of this study, these strains are not only deficient in the number of ribosomes but are also presumably deficient in their ability to accurately initiate translation.

In conclusion, although we have found that seven *rrn* operons may appear to be superfluous for near-optimal steady-state growth (five is sufficient), *E. coli* benefits significantly from this redundancy under conditions in which it encounters a sudden, favorable change in environment. We believe this is because multiple operons better facilitate the surge in rRNA production induced by the new conditions. Thus, the significance of seven *rrn* operons in *E. coli* is probably not so much to support very fast growth rates but rather to allow ease of adaptation from one growth environment to the next.

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