When *Escherichia coli* cells enter stationary phase due to carbon starvation the synthesis of ribosomal proteins is rapidly repressed. In a ΔrelA ΔspoT mutant, defective in the production of the alarmone guanosine tetraphosphate (ppGpp), this regulation of the levels of the protein synthesizing system is abolished. Using a proteome approach we demonstrate that the production of the vast majority of detected *E. coli* proteins are decontrolled during carbon starvation in the ΔrelA ΔspoT strain and that the starved cells behave as if they were growing exponentially. In addition we show that the inhibition of ribosome synthesis by the stringent response can be qualitatively mimicked by artificially lowering the levels of the housekeeping factor, σ70. In other words, genes encoding the protein-synthesizing system are especially sensitive to reduced availability of σ70 programmed RNA polymerase. This effect is not dependent on ppGpp since lowering the levels of σ70 gives a similar but less pronounced effect in a ppGpp0 strain.

The data is discussed in view of the models advocating for a passive control of gene expression during stringency based on alterations in RNA polymerase availability.

The alarmone guanosine tetraphosphate (ppGpp)1 of the stringent response network in *Escherichia coli* affects ribosome production by specifically lowering the transcription of ribosomal RNA (rRNA) (1) and some of the genes encoding ribosomal proteins (2–4). The production of ribosomal proteins is also post-transcriptionally feedback-regulated to match the rRNA production (reviewed in 5). Two different ppGpp synthetases (PS) exist in *E. coli*, the ribosome-associated PS I, encoded by relA, and the cytoplasmic PS II (6), encoded by spoT (7, 8). The protein PS II is also responsible for ppGpp hydrolysis (1).

Increased levels of ppGpp not only down-regulate ribosome production but also induce transcription from several promoters (9–14). As suggested by Schreiber et al. (9), the *E. coli* promoters can be divided into three groups depending on their response to ppGpp: promoters specifically induced or repressed by the stringent response and promoters that are unaffected by the rise in ppGpp levels. Promoters dependent on the housekeeping σ factor, σ70, exist in at least two of these groups; one group is positively regulated by ppGpp, e.g. *PusPA* (11) and *Phis* (8), whereas the other group is repressed during stringency, e.g. *rrn*P1. Attempts to explain this dual effect of ppGpp have involved considerations of RNAP (RNA polymerase) availability and intrinsic differences in the kinetic properties of the promoters affected (15). Several lines of evidence suggest that the availability of transcriptional and/or translational machinery play a role in global gene regulation in concert with classical activators and repressors (13–17). One well known example of this type of regulation, where the level of RNAP is involved, is σ factor competition in both *Bacillus subtilis* (18) and *E. coli* (19, 20).

Whether or not RNAP availability is involved in the actual mechanism of the stringent response has been discussed, and several models for this have been suggested (13–16, 20). Arguments have been raised both for an increased and diminished availability of RNAP during entry into stationary phase. In addition, different models exist on how changes in the availability of RNAP would affect the stringent promoters. Barker et al. (13, 14) and Zhou and Jin (15) suggest an increased availability of free RNAP in stationary phase since ppGpp destabilizes the open complex resulting in RNAP drop off at stable RNA promoters, which form intrinsically unstable open complexes. As a consequence of the increased availability of RNAP, positively regulated promoters are then induced since they are relatively poor at recruiting RNAP and are subordinated during normal growth according to this model. Thus, this model encompasses a direct and active role for ppGpp on stringently controlled promoters (e.g. *rrn*) and a passive role on the positively regulated promoters (through RNAP availability).

Jensen and Pedersen (16), on the other hand, have argued for a diminished availability of RNAP during stringency. The “stringent” promoters, e.g. stable RNA promoters, are dependent on high concentration of RNAP to transcribe at their maximal rate and are therefore argued to be repressed when the RNAP availability is diminished. Krohn and Wagner (21) showed that ppGpp increases pausing of RNAP during transcription in general but more at the stringently controlled genes, which could be another reason for inhibition of expression of these genes. Jensen and Pedersen (16) suggested that one way through which the RNAP availability could be diminished during stringency is through ppGpp-dependent pausing and therefore sequestering of RNAP in transcription. However, Vogel and Jensen (22) have demonstrated that ppGpp-induced pausing is not required for the stringent response since the stringent response was still observed when ppGpp-induced pausing was abolished and the transcriptional elongation speed was made constant by the introduction of the *nusA*Δ*cs10* allele. Thus, it is presently unclear if and how the availability of RNAP changes during the stringent response.

There are also models for how the stringent response works that do not involve RNAP availability. For example Barrachini and Bremer (23) suggested that RNAP can exist in two forms, one with and one without ppGpp bound to it. The RNAP without ppGpp can only transcribe genes encoding stable RNA, including the ribosomal RNA genes, whereas the RNAP with ppGpp bound can transcribe genes encoding small RNA which are able to inhibit transcription.
while RNAP with ppGpp bound can transcribe from both stable RNA and mRNA promoters but prefers mRNA promoters. The fact that cells totally deficient in making ppGpp can still grow shows that there is no absolute requirement for ppGpp for the transcription of mRNA promoters, but no evidence presented so far discredits the idea that there are direct effects of ppGpp on promoter selection.

The questions that need to be solved are whether variations in the level of free RNAP play a physiologically relevant role in gene regulation and if this mechanism is involved in the stringent response. The specific question we set out to answer in this work was whether variation in the levels of free $\sigma^{70}$-programmed RNAP does affect gene expression and, if so, what genes are sensitive to the altered RNAP availability? We used two-dimensional gel analysis to elucidate the global effects of underproduction of the housekeeping $\sigma$ factor, $\sigma^{70}$, during exponential growth and to study the role of ppGpp in this effect. We found that lowering the amounts of $\sigma^{70}$-programmed RNAP mimics the proteome of stringent cells and that this effect is not ppGpp-dependent. We speculate on the physiological relevance of these results with respect to the availability of RNAP during the stringent response.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The E. coli strains used in this work are listed in Table 1. The markerless $\Delta rplA \Delta spoT$ strain was constructed by K. Kvint and co-workers (20) by the method of Datsonko and Wanner (24). $\sigma^{70}$ levels were manipulated by using a system where $\sigma^{70}$-specific levels were used and the RNAP beta subunit was controlled by the use of macroarrays. Apart from differences between the rate of production of specific proteins in the wild type and relaxed strain, it is clear that the magnitude of the proteome response is much reduced in the $\Delta rplA \Delta spoT$ mutant strain (Figs. 1 and 2). The proteome of the $\Delta rplA \Delta spoT$ mutant strain appears to be locked in a growth mode.

**Measurement of Cellular Components**—Western blot analysis was performed as described (22). Cell sampling and measurement of the cellular level of ppGpp with high pressure liquid chromatography was done according to Neubauer et al. (34). For RNA and protein measurements, bacteria were first incubated in 5% trichloroacetic acid on ice and subsequently washed once with 5% trichloroacetic acid and lysed with 1 N NaOH (30 min at 40 °C). Protein content in the cell extracts was analyzed using the BCA protein assay kit (Pierce). RNA concentration was measured using the UV absorption assay as described (35). Based on the values obtained (normalized to optical density of the culture at the time of sampling), the RNA/protein ratio was calculated for cells underproducing $\sigma^{70}$ and compared with the control.

**RESULTS**

A ppGpp$^{\alpha}$ Mutant Fails to Respond to Carbon Starvation—A proteomic analysis demonstrated that the ribosomal proteins are immediately down-regulated 4- to 6-fold when wild type cells enter stationary phase due to carbon starvation (Fig. 1, B and D). The fold reduction in the synthesis rate is different for each of the ribosomal proteins but eventually the production of all the ribosomal proteins falls below the detection limits (data not shown). As demonstrated previously a set of proteins is induced during entry into stationary phase (Fig. 2A). Among those are a set of $\sigma^{70}$-dependent proteins, e.g. UspA and GlyA (Fig. 1D). In a ppGpp$^{\alpha}$ strain the ribosomal proteins are not down-regulated when the cells enter stationary phase (Fig. 1E), and the protein production pattern, as seen on the two-dimensional gels, looks rather like a growing cell even after the entry into stationary phase (Fig. 1C). It should be noted that relA$^{\alpha}$ and relAI strains behave indistinguishably during the conditions analyzed. The SpoT protein (PS II) is responsible for ppGpp production during the glucose starvation conditions employed (8), and the relAI allele did not affect the proteome during these conditions.

Apart from differences between the rate of production of specific proteins in the wild type and relaxed strain, it is clear that the magnitude of the proteome response is much reduced in the $\Delta rplA \Delta spoT$ mutant strain (Figs. 1 and 2). The proteome of the $\Delta rplA \Delta spoT$ mutant strain appears to be locked in a growth mode.

$\sigma^{70}$ Underproduction Qualitatively Mimics a Stringent Response—When $\sigma^{70}$ was underproduced in a wild type strain the ribosomal proteins were found to be specifically affected. When the $\sigma^{70}$ levels were lowered to half the level of a normal strain, the ribosomal proteins were down-regulated 2- to 3-fold (Fig. 3A). This effect was not as great as the effect of carbon starvation, but this was expected since the cells are still growing in the $\sigma^{70}$ underproduction experiment. The effect of $\sigma^{70}$ underproduction on ribosomal proteins has been confirmed on the transcriptional level by the use of macroarrays.$^2$

The ppGpp levels were not affected by the $\sigma^{70}$ underproduction (Fig. 3B), which means that the down-regulation of ribosomal protein production is not caused by elevated levels of ppGpp in this experiment. In agreement with the effect on ribosomal proteins the RNA/protein ratio was reduced (Fig. 3C). This measurement can be used as a rough measure of stable RNA since total RNA consists of 98% stable RNA (36). In addition, the levels of RNA $\alpha$-subunit were not affected by the reduced $\sigma^{70}$ levels (data not shown).

Lowering the levels of Er$^{70}$ also affected the growth rate (Fig. 4A), as has previously been shown for ppGpp overproduction (9, 10). In the case of $\sigma^{70}$ underproduction no steady state of growth could be obtained, but rather the growth rate decreased gradually over time (Fig. 4A). The $\sigma^{70}$ underproduction levels were therefore checked at later times in exponential phase and showed that the level of underproduction did not

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2 Miki Jishage, unpublished results.

*Table 1*

| Strain | Relevant genotype | Source/reference |
|--------|------------------|-----------------|
| MC4100 | F $\Delta araD139 \Delta xylA169 \Delta rpsL$ | M. Giskov |
| 150 relA1 fllB5301 deoC1 ptsP25 | rbB | |
| K2356 | MC4100 $\Delta rplA \Delta spoT$ | 20 |
| AF1000 | MC4100 relA$^{\alpha}$ | A. Farewell |
| LM100 | MC1000 P$_{\Delta rpoD}$:cam | This work |
| LM102 | KK356 P$_{\Delta rpoD}$:cam | This work |
| LM128 | AF1000 P$_{\Delta rpoD}$:cam | This work |

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$^2$ Miki Jishage, unpublished results.
The effects of \(H9268\) underproduction were studied at two different absorbance values during growth \(A_{420} = 0.5\) and 0.7, and no significant differences in the protein expression between the two absorbance values were found (data not shown). Finally, the \(H9268\) underproduction was performed in both a \(relA1\) and a \(relA\) strain with the same result (data not shown).

**DISCUSSION**

In this work we demonstrate that a strain unable to produce ppGpp is largely decontrolled upon the entry to stationary phase. The production of all these proteins depends on the housekeeping \(\sigma\) factor, \(\sigma^{70}\). The global effects of \(\sigma^{70}\) underproduction were less pronounced, but the same trend was seen as in the wild type strain (comparison of Fig. 5A with 3A). In addition, the effect of \(\sigma^{70}\) underproduction on growth rate (Fig. 4B) was not ppGpp-dependent. Moreover, the level of underproduction of \(\sigma^{70}\) was the same in the ppGpp0 as in the wild type (data not shown), and the levels of RNAP \(\omega\)-subunit were not affected in the ppGpp0 strain. In agreement with the effect on ribosomal proteins the RNA/protein ratio was reduced (Fig. 5B).
phase and its proteome appears “locked” in a growth mode. By comparing the effects of a wild type and a ppGpp strain going into stationary phase (Fig. 1) we can define the significant changes in the proteome mediated by the stringent response under these conditions. The ribosomal proteins are, as expected, down-regulated by the entry into stationary phase, and this effect is dependent on ppGpp (Fig. 1, C and D). Some proteins, e.g. UspA, are induced in a ppGpp-dependent manner as has been shown previously (10, 11). A few amino acid biosynthesis proteins (e.g. the proteins encoded by carB, gldD, ilvE, metC, metH, and tyrB) are identified on NEPHE two-dimensional gels (33), but those were not among the proteins significantly affected by the stringent response to carbon starvation. In a recently published study using macroarrays, the only genes encoding amino acid biosynthesis proteins that were induced by the stringent response were the ones involved in the histidine and arginine biosynthetic pathways (37). Thus, it appears that promoters requiring ppGpp do not necessarily behave identically during a stringent response as demonstrated by the fact that the production of amino acid biosynthetic gene products, in contrast to the Usp family proteins, are not induced during carbon starvation induced stringency.

We also show that it is possible to mimic the down-regulation of ribosomal proteins seen in the stringent response by underproducing the housekeeping σ factor, σ^70 (Fig. 3A). The expression of ribosomal proteins has been shown to depend on the availability of rRNA, and the ribosomal protein promoters have also been shown to be under direct stringent control (2-4). Thus, the effects seen on ribosomal proteins in the σ^70 underproduction experiment could be due to effects on rRNA production via transcription of rrr promoters causing a subsequent feedback control of the expression of genes encoding ribosomal proteins. However, we can not rule out the possibility of direct effects on the transcription from ribosomal protein promoters. The effects seen are most likely not post-transcriptional since the ribosomal protein transcripts were similarly affected by a reduction in σ^70-programmed RNAP. It should also be noted that the total RNA/total protein ratio decreased during σ^70 underproduction demonstrating that rRNA levels decreased. The data indicate that among the genes expressed during exponential growth of E. coli the expression of rRNA and ribosomal protein genes is more sensitive than the average to a reduction in RNAP availability. The results support the idea of stringent promoters requiring high levels of free RNAP, as suggested by Jensen and Pedersen (16). In fact, σ^70 promoters may not work at their maximal capacity even during logarithmic growth since data from Squires and co-workers (38) showed that a cell containing only one rrn operon instead of seven was still able to produce 56% of the wild type rRNA.

Our results of σ^70 underproduction also go in line with the model of Jishage et al. (20) who suggested that one mechanism of ppGpp-dependent gene regulation is to affect the relative competitiveness of σ factors, possibly by affecting the affinity of σ^70 to the core enzyme. The lowering of σ^70 interaction with RNAP increases the opportunities for alternative σ factors to bind RNAP core enzyme and redirect RNAP to their respective promoters, and at the same time it lowers the availability of RNAP holoenzyme for the σ^70-dependent promoters. If the mechanism behind the stringent control is RNAP availability, either by changing the pool of free RNAP or by affecting σ factor competition or both, the constitutively stringent mutations in RNAP (e.g. (15)) should also affect these parameters. One interesting feature of the stringent rpoB mutants characterized by Zhou and Jin (15, 39) is that they express lower levels of σ^70. The stringent rpoB alleles had effects on transcription in vitro so the lower level of σ^70 expressed by these cells is not the only explanation for their action. But, considering the results presented here, it is possible that the lower σ^70 levels of the mutants fortify the stringent phenotype in vivo.

The fact that lowering the availability of σ^70-programmed
RNAP has effects that mimic the stringent response does not, per se, prove that a reduction of σ70-programmed RNAP is part of a bona fide stringent response, but it is possible to speculate on such a mechanism. Even if changing the availability of σ70-programmed RNAP is one effect of ppGpp in the cell, direct positive effects of ppGpp on expression of specific genes/proteins has been shown previously by Choy (40) using a coupled in vitro transcription-translation system. It is possible that the RNAP availability works together with more direct effects of ppGpp on specific promoters.

Lowering the levels of σ70-programmed RNAP also affects growth rate (Fig. 4, A and B), as has previously been shown for cells overproducing RelA and consequently ppGpp (9, 10). It could be argued that the effects seen on the protein synthesizing system are merely an effect of the growth rate-dependent regulation of these particular genes. But, then the question remains how σ70 underproduction mechanistically causes a reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate, since most gene products, with the exception of for example the ribosomal proteins, were unaffected by reduced growth rate.

Moreover, there are two key differences between the effects seen in growth on minimal medium and under carbon starvation. We also show that it is possible to mimic a stringent down-regulation of ribosome production by underproducing σ70. We suggest that the effects on expression of ribosomal proteins by lowering the levels of σ70 indicate that the ribosomal protein promoters are sensitive to the availability of RNAP. Thus, it is formally possible that the stringent response could encompass a mechanism of lowered availability of σ70-programmed RNAP. Further experiments will address the question of whether available, transcription-competent, RNAP levels change significantly in response to ppGpp accumulation.

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