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Contributions of Zur-Controlled Ribosomal Proteins to Growth under Zinc Starvation Conditions▽

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Maintaining intracellular zinc levels is critical, because zinc serves as a cofactor for many required enzymes and is toxic in excess. *Bacillus subtilis* Zur, a Fur family repressor, controls the zinc starvation response including two ribosomal proteins (r-proteins) paralogous to L31 and S14. Biochemical analyses suggest that Zur-controlled r-proteins (which lack the two CXXC metal-binding motifs) may functionally replace their cognate zinc-requiring proteins during zinc limitation. We demonstrate here that Zur regulates the expression of an additional r-protein paralog, RpmGC (L33c), and, using strains defective in zinc uptake, we investigate the physiological contributions of all three Zur-regulated r-proteins. In the 168 lineage, *rpmGC* is a pseudogene containing a frameshift mutation. Correction of this mutation allows expression of a functional L33c that can suppress the poor growth phenotype of an *rpmGA rpmGB* (encoding L33a, L33b) double mutant. Similarly, we provide physiological evidence in support of the "failsafe" model (Y. Natori et al., Mol. Microbiol. 63:294–307, 2007) in which the Zur-regulated S14 paralog YhzA allows continued ribosome synthesis when there is insufficient zinc to support S14 function. The L31 paralog YtiA can replace L31 and complement the growth defect of an *rpmE* mutant (Nanamiya et al., Mol. Microbiol. 52:273–283). We show that, under zinc starvation conditions, derepression of YtiA significantly increases the growth of cells in which preexisting ribosomes carry, as the sole L31 protein, RpmE (containing zinc), but not if they carry YtiA (which lacks zinc). These results support a direct and physiologically relevant role for YtiA in mobilizing zinc from ribosomes.

Zinc is a required metal cofactor for many proteins and yet can be toxic in excess (12). Thus, controlling zinc levels within the cell is of utmost importance. *Bacillus subtilis* Zur, a Fur family member, is responsible for controlling cellular adaptation to zinc starvation. Its regulon includes a high-affinity zinc uptake system (an ABC transporter encoded by the *ycdHI-yceA* operon), the complex *yciAB-yciC* operon encoding a putative metallochaperone (YciC), *zinT*, and several ribosomal protein (r-protein) paralogs (7, 19).

As anticipated from their roles in ribosome assembly and function, ribosomal proteins (r-proteins) are highly conserved and usually encoded by essential genes (10). However, bacterial genomes frequently contain duplicate copies of the genes encoding some or all of the r-proteins L36, L33, L31, and S14. Interestingly, the duplicated proteins fall into two groups: those that contain a zinc binding motif (two CxxC motifs; designated as "C+") and those that do not ("C−") (13). Bioinformatic analyses suggest that the non-zinc-containing proteins are preferentially expressed under zinc-limiting conditions: they are associated with predicted binding sites for Zur or functionally analogous zinc-sensing transcription factors (19). Thus, it was hypothesized that the constitutively expressed C+ r-protein paralogs use zinc as a cofactor and, in times of zinc depletion, they are replaced by their Zur-controlled C− counterparts (13, 19). The *B. subtilis* strain 168 genome encodes five duplicated pairs of r-proteins, as judged from the recently corrected DNA sequence (2). Three of the duplicated genes are under the control of Zur and are presumed to facilitate adaptation to zinc-limiting conditions (19). The functions of the others are unknown. The Zur-regulated L31 paralog, YtiA, replaces the C+ protein, RpmE, in ribosomes isolated from cells grown under zinc-limiting conditions (1). YhzA, a Zur-regulated S14 paralog, was postulated to allow continued ribosome synthesis in the absence of available zinc (17). Unlike L31, which is surface exposed and loosely associated with the ribosome, S14 is buried deep within the ribosome and is required for de novo assembly (17). A gene for a third r-protein paralog, *rpmGC*, has been postulated based on its association with a Zur box-like regulatory sequence (19), but the function of this gene is unknown and, in *B. subtilis* 168 strains, *rpmGC* is a pseudogene.

The Zur-regulated C− proteins may function to maintain the proper assembly or functioning of the ribosome during zinc limitation. For example, the zinc-requiring S14 protein (9, 21, 24) is essential for the assembly of the ribosome, and the Zur-regulated paralog (YhzA) may function as a substitute S14 protein. This "protein substitution model" postulates that YhzA provides a mechanism for de novo ribosome synthesis under zinc-limiting conditions, previously referred to as a "failsafe" model (17). In addition, or alternatively, the Zur-regulated r-proteins may function in zinc nutrition. Specifically, it has been speculated that displacement of L31, together with its bound zinc, from the surface of the ribosome by the C− paralog YtiA mobilizes stored zinc (1, 14). According to this "zinc mobilization model," the major function of L31 is to store zinc ions, rather than as an important functional component of the ribosome (1, 15). It has been noted, for example, that L31 (unlike most r-proteins) is not absolutely essential and is only loosely associated with the ribosome (4). The function of L33 paralogs is currently unknown and might, in principle, include

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TABLE 1. *B. subtilis* r-protein characterization strain genotypes

Strain	Genotype
HB6865	CU1065 *ycdH*::*cm citM*::*tet*
HB6866	CU1065 *ycdH*::*cm yciC*::*kan*
HB6867	CU1065 *ycdH*::*cm zinT*::*spc*
HB6868	CU1065 *ycdH*::*cm yciC*::*kan citM*::*tet*
HB6869	CU1065 *ycdH*::*cm zinT*::*spc citM*::*tet*
HB6870	CU1065 *ycdH*::*cm yciC*::*kan zinT*::*spc*
HB6871	CU1065 *ycdH*::*cm yciC*::*kan citM*::*tet yciC*::*kan zinT*::*spc*
HB6880	CU1065 *ycdH*::*cm yciC*::*kan yhzA*::*spc*
HB6882	CU1065 *ycdH*::*cm yciC*::*kan ytiA*::*tet*
HB6883	CU1065 *ycdH*::*cm yciC*::*kan ytiA*::*tet yhzA*::*spc*
HB6888	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls*
HB6889	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls ytiA*::*tet*
HB6916	CU1065 *rpmGA*::*tet rpmGB*::*cm rpmE*::*spc*
HB6918	CU1065 *ycdH*::*cm yciC*::*kan thrC*::*rpmGC-mls*
HB6919	CU1065 *ycdH*::*cm yciC*::*kan ytiA*::*tet thrC*::*rpmGC-mls*
HB6920	CU1065 *ycdH*::*cm yciC*::*kan yhzA*::*spc thrC*::*rpmGC-mls*
HB6921	CU1065 *ycdH*::*cm yciC*::*kan ytiA*::*tet yhzA*::*spc thrC*::*rpmGC-mls*
HB6972	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls zur*::*spc*
HB6975	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls ytiA*::*tet zur*::*spc*
HB6976	CU1065 *rpmGA*::*tet rpmGB*::*cm zur*::*kan*
HB6983	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls ytiA*::*tet amyE*::*rpmE-spc*
HB6984	CU1065 *ycdH*::*cm yciC*::*kan rpmE*::*mls ytiA*::*tet amyE*::P_{rpmE} *ytiA-spc*
HB8250	CU1065 *rpmGA*::*tet rpmGB*::*cm thrC*::*rpmGC-mls*
HB8251	CU1065 *rpmGA*::*tet rpmGB*::*cm zur*::*kan thrC*::*rpmGC-mls*
HB8252	CU1065 *rpmGA*::*tet rpmGB*::*cm rpmE*::*spc thrC*::*rpmGC-mls*
HB8253	CU1065 *rpmGA*::*tet rpmGB*::*cm rpmE*::*spc zur*::*kan thrC*::*rpmGC-mls*
HB8608	CU1065 *rpmE*::*mls*
HB8644	CU1065 *rpmGA*::*tet rpmGB*::*cm*

either or both "protein substitution" or "zinc mobilization" functions.

Here, we have used strains deficient in high-affinity zinc uptake to test the contributions of the Zur-controlled r-proteins to zinc nutrition. We demonstrate that *rpmGC* (encoding the L33 paralog designated L33c) is Zur regulated and that derepression of L33c suppresses the growth defect of an *rpmGA rpmGB* double mutant, but only if the frameshift mutation in *rpmGC* is corrected. Expression of L33c does not confer a significant growth advantage in zinc-limiting conditions, which is consistent with a primary role as a substitute L33 protein. In contrast, derepression of the L31 paralog, YtiA, does confer a significant growth advantage under zinc limitation, but only if the preexisting ribosomes contain RpmE (containing zinc) and not if they contain YtiA (lacking zinc). These results provide physiological evidence that YtiA mobilizes a physiologically relevant pool of zinc from the ribosome to facilitate growth under conditions of severe zinc deprivation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains (Table 1) are derivatives of the wild-type CU1065 (*trpC2 att*SPβ). *B. subtilis* was grown in LB or in a defined minimal medium as previously described (7). Erythromycin (1 μg/ml), lincomycin (25 μg/ml), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), neomycin (10 μg/ml), and chloramphenicol (5 μg/ml) were used for the selection of various *B. subtilis* strains. Zinc starvation minimal medium (ZSMM) was prepared with Chelex-treated and filter-sterilized stocks of all non-metal-containing components and ultrapure filter-sterilized metal stocks containing 40 mM potassium morpholinepropanesulfonate (MOPS; adjusted to pH 7.4 with KOH), 2 mM potassium phosphate buffer (pH 7.0), glucose (2% [wt/vol]), $(NH_4)_2SO_4$ (2 g/liter), $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), trisodium citrate $2H_2O$ (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), and 80 nM $MnCl_2$ (5). To more readily generate zinc starvation conditions in this medium, we also used null mutant strains of both the high-affinity zinc uptake ABC transporter (encoded by *ycdHI-yceA*) and the proposed metallochaperone (encoded by *yciC*). Strains were precultured overnight in LB, followed by a 1/100 dilution into ZSMM. Once cultures reached mid-log phase, they were spun down, resuspended in 10 mM EDTA to remove any loosely associated metals from the cell wall, and then rinsed twice with fresh ZSMM to remove the EDTA. All growth curves were determined by using a Bioscreen C Machine (Growth Curves USA). Cultures were grown at 37°C with shaking and normalized to a given starting optical density (in the experiments described here, the starting optical densities ranged from 0.005 to 0.03) after treatment with EDTA and ZSMM washings. Zinc limitation during growth is correlated with both an increase in the lag phase and a slower growth rate. Although variability in the duration of the lag phase is often observed between experiments (presumably reflecting the efficiency of removal of zinc and other cations from the cell wall by EDTA), the relative behavior of strains grown in parallel was highly reproducible, and the presented growth curves are representative of data obtained from at least three independent trials.

Northern blot. Portions (7 μg) of RNA from wild-type and HB8604 strains were run on a 1% agarose gel in the presence of formaldehyde and blotted on Zeta-Probe membrane (Bio-Rad). To construct the *rpmGC* probe, the primers 1798 (5′-CGGCAAGCTTTGCACTGAAACGG-3′) and 1799 (5′-TGTTTCAC GGTGAAGGG-3′) were used to create a 150-bp PCR fragment that was subsequently end labeled with α-^{32}P by polynucleotide kinase (Epicenter) and purified by using NucAway columns (Ambion) according to the manufacturer's instructions. Membranes were prehybridized in ULTRAhyp buffer (Ambion) at 42°C for 1 h and then hybridized overnight at 42°C. Membranes were washed twice with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) for 5 min at room temperature and then washed twice with 0.1× SSPE at 42°C for 15 min. Membranes were visualized on a Storm 840 PhosphorImager (Molecular Dynamics).

EMSA of *rpmGC* promoter fragment. PCR fragments containing the *rpmGC* promoter region and a promoter region not known to bind Zur were created by PCR, end labeled with α-^{32}P by polynucleotide kinase (Epicenter), purified by using NucAway columns (Ambion) according to the manufacturer's instructions, and used in electrophoretic mobility shift assay (EMSA) experiments as previously described (6).

Determining the zinc content of the ribosomes. Ribosomes were purified as previously described (16). Briefly, 750-ml portions of mid-log LB cultures were spun down and resuspended in buffer I (10 mM Tris [pH 7.6], 10 mM magnesium acetate, 100 mM ammonium acetate, 6 mM β-mercaptoethanol (BME), 2 mM phenylmethylsulfonyl fluoride [PMSF]). After disruption by a French press, the supernatant was spun at 45,000 rpm for 100 min in a Beckman TLA 100.3 rotor. The pellet was then dissolved in buffer II (20 mM Tris [pH 7.6], 15 mM magnesium acetate, 1 M ammonium acetate, 6 mM BME, 2 mM PMSF) and spun at 18,000 rpm for 60 min in a Beckman TLA 100.3 rotor. The supernatant was layered on a 30% (wt/vol) sucrose bed and spun for 3.5 h at 45,000 rpm in a Beckman TLA 100.3 rotor. The pellet was resuspended in buffer III (50 mM Tris [pH 8.0], 6 mM BME, 2 mM PMSF) and quantified by absorbance (1 A_{260} = a 26 nM concentration of 70S ribosomes). 4-(2-Pyridylazo)resorcinol (PAR) was used to determine zinc content of the preparations. Under our experimental buffer conditions (40 mM Tris [pH 8.0], 5% glycerol, 0.5% sodium dodecyl sulfate) the absorption maximum of the Zn^{2+}-PAR complex was observed at 494 nM and had a linear relationship to a zinc concentration from 0 to 8 μM. Concentrations (3 μM) of purified ribosomes were placed in our experimental buffer conditions with 0.1 mM PAR, followed by boiling for 15 min. After boiling, the absorbance at 494 nM was read, and the total amount of released zinc calculated.

RESULTS AND DISCUSSION

Zur regulation of *rpmGC*. Bioinformatics studies have revealed that a small subset of r-proteins are duplicated in many bacterial species and that one of the pair invariably contain a Zn ribbon motif (13). In a subsequent study, Panina et al. (19) extended this initial observation by showing that genes encoding non-zinc-containing r-proteins contained binding sites in

TABLE 2. *B. subtilis* r-protein ribosomal gene duplications

Protein	Gene duplication(s)		
	L31	L33	S14
C+	*rpmE*	*rpmGA*, *rpmGB*	*rpsN*
C−[a]	*ytiA*	*rpmGC*	*yhzA*

[a] Zur controlled.

their promoter regions for Zur or a related zinc-sensing regulator. This led them to hypothesize that Zur-regulated r-proteins allow the cell to respond to zinc limitation by synthesizing alternative r-proteins that lack a zinc metal cofactor requirement (19). In *B. subtilis*, they identified three Zur-controlled r-proteins (Table 2), two of which have been further characterized (1, 16, 17).

We first sought to explore the possible role(s) of the third postulated Zur-controlled r-protein, L33c, encoded by the *rpmGC* gene (Fig. 1B). However, a frameshift mutation is present in the putative *rpmGC* gene of *B. subtilis*, including strains W168, JH642, and the "undomesticated" strain NCIB3610. In contrast, in closely related *Bacillus* strains (e.g., *B. amyloliquefaciens* and *B. licheniformis*) *rpmGC* encodes a full-length L33 protein. This suggests that this mutation occurred early in the lineage leading to current laboratory strains of *B. subtilis* (25). The *B. subtilis rpmGC* pseudogene is located between *yqjK* and *yqjL* (Fig. 1A). Correction of the frameshift mutation reveals a gene encoding a 49-amino-acid protein (L33c), which is more than 90% identical to the corresponding *B. licheniformis* L33 C− protein (Fig. 2A). L33c is also highly similar to the two C+ L33 proteins of *B. subtilis* (RpmGA and RpmGB) (Fig. 2B), although it lacks the two CxxC zinc-binding motifs, as noted by Panina et al. (19).

To test whether Zur regulates *rpmGC*, we analyzed RNA from wild-type and *zur* mutant cells in a Northern blot analysis. A transcript corresponding in size to the predicted *rpmGC* transcription unit was only detected in the *zur* mutant strain (Fig. 2C). Using the same RNA samples, we determined the transcription start site of *rpmGC* by 5′RACE (rapid amplification of cDNA ends) (Fig. 1B). As expected for a Zur-regulated gene, purified Zur bound with high affinity to the *rpmGC* promoter (K_d < 5 nM) in an EMSA analysis (Fig. 2D).

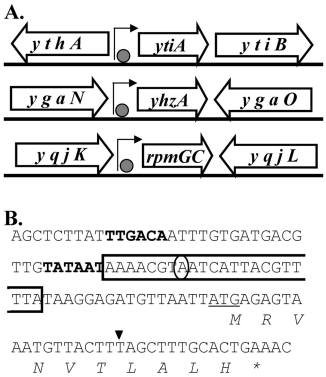

FIG. 1. (A) Genomic context of Zur-controlled C− proteins. The bent arrow and filled circle show the promoter and Zur box, respectively. (B) Detailed view of *rpmGC* promoter region (for *ytiA* and *yhzA* promoter information, see references 16 and 17, respectively). The −35 and −10 regions are in boldface, the Zur operator is depicted by a box, and the start site of transcription (as determined by 5′RACE) is circled. The initiating codon is underlined, and the site of the frameshift mutation is noted by the filled triangle. The translated message produces a truncated product that terminates four amino acids downstream from the frameshift mutation (in italics).

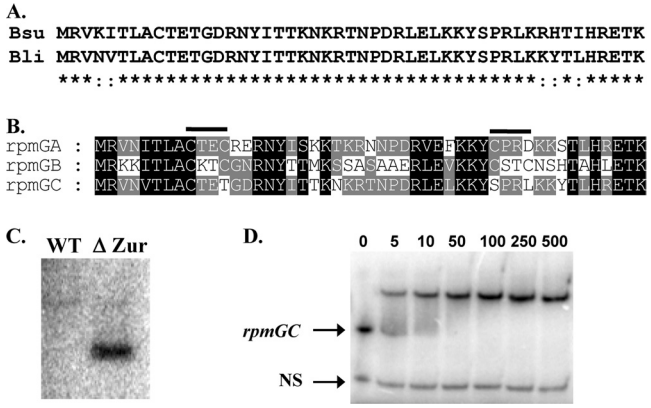

FIG. 2. (A) Protein sequence alignment of the L33 C− proteins of *B. subtilis* (with the frameshift mutation corrected) and *B. licheniformis* (Bli). (B) Amino acid conservation of *B. subtilis* L33 C+ and C− paralogs. Black bars highlight the CXXC motif which binds a structurally important zinc cofactor in C+ proteins. (C) Northern blot of RNA isolated from CU1065 (wild type) and a *zur* mutant showing that the *rpmGC* transcript is only produced when Zur is absent. (D) Zur binds with high affinity to the *rpmGC* promoter in this EMSA using purified ZurBS protein, the *rpmGC* promoter, and a nonspecific (NS) promoter region with protein concentrations (in nM) increasing from left to right.

L33c can complement cells deficient for L33a/L33b. To test whether L33c (encoded by *rpmGC*) is a functional protein, we repaired the frameshift mutation (denoted by "fs" in the figure) and integrated the resulting *rpmGC* (denoted as *rpmGC*$^+$) at an ectopic locus. When grown in LB, cells lacking L33 proteins (an *rpmGA rpmGB* double mutant) displayed a reproducible growth lag (Fig. 3, filled circles), similar to what was observed upon deletion of the L31 C+ protein (1). This phenotype was not observed in either single mutant, indicating that *rpmGA* and *rpmGB* encode functionally redundant proteins and both are expressed under these conditions. However, when neither L33 C+ protein is expressed, this double mutant presumably displays a growth lag due to nonoptimal ribosome function. Introduction of a *zur* mutation suppresses the poor-growth phenotype of the *rpmGA rpmGB* double mutant, but only in the strain in which the frameshift mutation in *rpmGC* has been corrected (Fig. 3, open triangles). Thus, *rpmGC* encodes a functional L33 protein. This also raises the possibility that L33c might function, as also postulated for the L31 paralog YtiA, in mobilization of zinc as part of the zinc starvation response.

Zinc content of the ribosome. Critical to the hypothesis that Zur-regulated r-proteins act to mobilize zinc from the ribosome is the presence, on the ribosome, of C+ proteins, which have bound zinc. Although the previous characterization of RpmE and YtiA showed zinc occupancy of only RpmE, these experiments relied on protein expression in *Escherichia coli* and use of an epitope-tagged protein and column purification

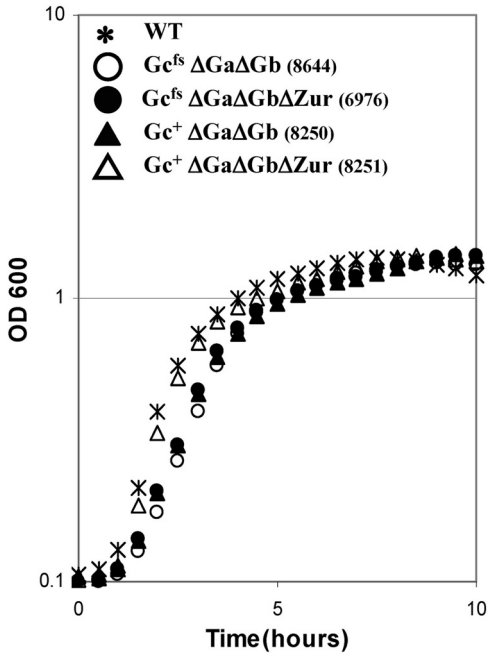

FIG. 3. A *zur* mutation restores a wild-type growth rate to a strain lacking RpmGA and RpmGB only if the gene encoding RpmGC is corrected so as to encode a complete protein product (HB8251 [△]). All other disruption combinations that do not restore the wild-type growth rate are shown as controls, with their strain number listed in parentheses (see Materials and Methods for genotypes). The *rpmGC* frameshift mutation is denoted "fs," and the corrected *rpmGC* is denoted by "+".

(16). Here, we sought to measure total ribosome-bound zinc. We reasoned that a ratio of at least three zinc atoms per ribosome would be expected due to the presence of three known C+ ribosomal proteins (S14, L31, L33). However, L32 (*rpmF*) and L36 (*rpmJ*) of *B. subtilis* also have CxxC motifs and it is possible that they also bind zinc.

Using a PAR-based assay, we monitored zinc release from ribosomes upon denaturation with boiling and SDS. Our wild-type strain was found to have ~2.5 Zn atoms/ribosome (Table 3), in close agreement with our expectation and in the same range as similar measurement of the zinc content of rat liver and *E. coli* ribosomes (3). This should be considered as a lower limit for the Zn/ribosome ratio since loosely associated r-proteins (including, for example, L31) are easily lost during purification (4) and, conversely, even these harsh conditions may not fully denature the ribosome and release all of the bound zinc. As expected, the Zn/ribosome ratio was reduced in strains missing one or more of the C+ r-proteins. Loss of L31 (and possibly L33) from the ribosome during purification may explain why ribosomes from strains missing the C+ L31 or L33 proteins each display a decrease of only ~0.5 Zn/ribosome compared to the wild type (Table 3). Alternatively, these proteins may be stoichiometrically associated with the ribosomes but not fully saturated with zinc. It is difficult to quantify the stoichiometry of r-proteins in purified ribosome preparations with sufficient precision to distinguish between these two hypotheses. Nevertheless, we do consistently observe a decrease in ribosome-associated zinc in cells lacking one or more zinc-containing (C+) r-protein.

Since ribosomes are the most abundant macromolecular complex in the cell (with >50,000 copies in a rapidly growing cell), even two or three zinc atoms per ribosome represents an enormous reservoir of zinc (>10^5 zinc atoms). Previous estimates place the total zinc content of logarithmically growing *E. coli* cells in the vicinity of 2×10^5 atoms per cell (18), and this value seems to be a relatively constant function of cell size. Thus, ribosomes may easily account for the majority of the zinc in the cell. Mobilization of this stored zinc might provide a significant growth advantage under zinc-limiting conditions.

Creation of zinc starvation conditions. We next aimed to test whether the induction of Zur controlled r-proteins can "mobilize" stored zinc from the ribosome by monitoring their effect on growth under conditions of severe zinc limitation. Because bacteria have extremely effective zinc uptake systems, they are able to grow well in media to which no zinc has been added, presumably due to trace contamination of other reagents with zinc. Indeed, most studies of zinc starvation have used strong metal ion chelators to impose zinc limitation. Unfortunately, this approach introduces additional complexities since chelators often impose limitations for multiple required metal ions. In *E. coli*, extraordinary efforts have been required to generate reproducible zinc-limiting conditions (<60 nM zinc) in chemostats (8). In preliminary studies, we also found that *B. subtilis* grew to high cell densities, even upon repeated subculturing in a defined minimal medium containing no added zinc, even when all reagents were of the highest available purity and were treated with Chelex to reduce trace metal contamination.

To more effectively create zinc-limiting growth conditions and to increase the cell's dependence on the hypothesized ability to mobilize zinc stored internally, we genetically inactivated high-affinity zinc uptake. First, we deleted the Zur-regulated *ycdHIyceA* operon, encoding the high-affinity zinc transport system. The *ycdHI yceA* mutant strain displayed a decrease in both growth rate and yield compared to the wild type in our ZSMM. In this genetic background, the additional disruption of *citM*, a transporter of metal-citrate complexes, or *zinT*, a candidate zinc chaperone (8), did not further decrease growth (Fig. 4). In contrast, when *yciC*, a proposed metallochaperone, was also deleted, a more severe growth defect was observed (Fig. 4). These growth deficiencies are seen in ZSMM, but not during growth in rich media such as LB, a finding consistent with the notion that they are due to zinc starvation. Moreover, growth can be completely restored by

TABLE 3. Zinc content of ribosomes purified from cells containing disruptions of genes encoding nonessential C+ ribosomal proteins

Strain	Genotype	No. of Zn atoms/ribosome[a]	
		Avg	SD
CU1065	Wild type	2.5	0.1
HB8608	*rpmE*	2.0	0.4
HB8644	*rpmGA rpmGB*	1.9	0.2
HB6916	*rpmE rpmGA rpmGB*	1.5	0.3

[a] Values are averages of the zinc content as determined by PAR assay normalized to the ribosome number from at least three independent purifications.

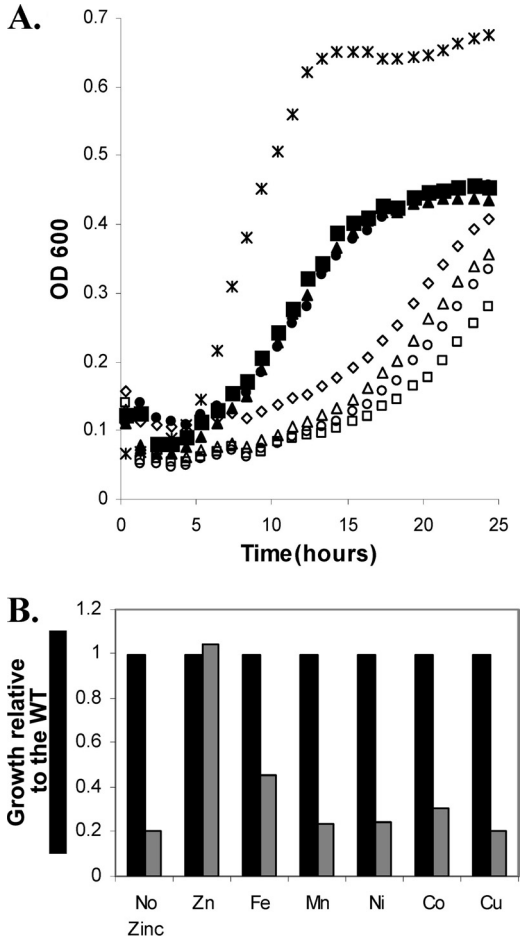

FIG. 4. Mutations in zinc uptake and homeostasis proteins leads to growth defects in ZSMM. (A) Wild-type (✳) (WT) cells grow well in this medium. Cells defective for the *ycdH*-encoded ABC transporter grow more poorly (solid shapes), but there is no additional defect due to deletion of *citM* (6865 [●]), *zinT* (6867 [▲]), or both *citM* and *zinT* (6869 [■]). In contrast, a *ycdH yciC* double mutant (HC) is significantly growth impaired (6866 [◇]), and with the additional mutation of *citM* (6868 [○]), *zinT* (6890 [△]), or both *citM* and *zinT* (6871 [□]) the growth defect phenotype now increases. (B) Only zinc is able to fully restore the growth defect of the HC double mutant observed in panel A. Growth at 12 h in ZSMM (see Materials and Methods) with 1 μM concentrations of added metals for the wild type (■) and the HC double mutant strain (▨) was determined. The values shown are relative to the wild-type growth in each condition.

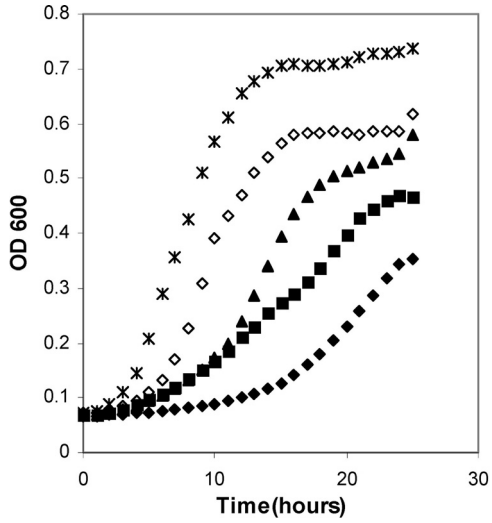

FIG. 5. Zur-regulated r-protein paralogs contribute to growth under severe zinc starvation conditions (in the HC double-mutant background growing in ZSMM). Whereas the wild type (✳) grows well, *ycdH yciC* (6866 [◇]) shows a reproducible lag and decrease in yield. This growth defect is magnified in the *ycdH yciC ytiA* (6882 [▲]), *ycdH yciC yhzA* (6880 [■]), and *ycdH yciC ytiA yhzA* (6883 [◆]) mutant strains.

the addition of zinc (Fig. 4B) but not other metals. For further studies, we have focused on the roles of r-proteins in the double-mutant background lacking high-affinity zinc uptake (a *ycdH* mutation) and the *yciC* metallochaperone, which we hereafter refer to as the HC mutant strain.

The L31 ribosome protein contributes a physiological relevant source of zinc. To begin to characterize the contribution of each of the Zur-controlled r-proteins to growth under zinc-limiting conditions, we disrupted *ytiA* and *yhzA* individually and together in the HC mutant background (Fig. 5). Deletion mutations in either or both *ytiA* and *yhzA* clearly exacerbate the growth defect in ZSMM (Fig. 5). It should be noted that in this background *rpmGC* contains a frameshift mutation. However, similar results were seen in strains in which this frameshift mutation was corrected (data not shown and see below).

The growth defect due to the disruption of *ytiA* (encoding the C− L31 paralog) may result from either of two scenarios or from both scenarios. First, it is possible that the lack of available zinc prevents the C+ L31 (*rpmE* gene product) from folding properly and the lack of the Zur-regulated paralog leads to a situation in which ribosomes no longer have a functional L31 protein. As noted previously (1) and confirmed here (data not shown), an *rpmE ytiA* double mutant does have a modest growth defect in rich medium, which is consistent with the hypothesis that L31, while not an essential protein, contributes to ribosome function even under non-zinc-limiting conditions. Second, it is possible that the growth defect observed in the ZSMM is due to an inability of the cells to mobilize zinc stored in the ribosome (in the L31 C+ protein) by displacement by newly synthesized C− L31 (YtiA). It has previously been shown that the addition of purified YtiA to ribosomes displaces L31 (16).

To distinguish between these two scenarios, we created a strain in which both L31 genes (*ytiA* and *rpmE*) were deleted in an HC double-mutant background. We then placed each gene back into this strain, at an ectopic locus, under the control of the constitutive *rpmE* promoter, thus removing Zur control over *ytiA*. We reasoned that in these strains the cells would contain ribosomes complete with one or the other L31 protein. Upon shifting to ZSMM, the induction of YtiA would displace the C+ L31 (in the HC P_{rpmE}-*rpmE* strain) and thereby mobilize zinc. In contrast, if the preexisting ribosomes were assembled with the C− L31 (in the HC P_{rpmE}-*ytiA* strain), there would be no advantage gained by the derepression of the Zur-regulated *ytiA* gene. Neither would there be a defect in the ribosomes due to a lack of L31 since they are provided with a constitutively expressed and functional L31 encoded by the

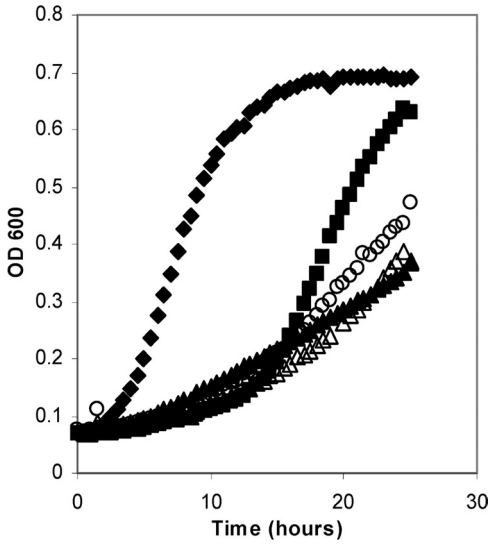

FIG. 6. L31 deletion growth defect is primarily due to the inability to mobilize stored zinc from the ribosome. The wild type (◆) grows well in ZSMM and is shown as a reference. The strain that has only the L31 C− ribosomal protein YtiA being expressed (6984 [○]) has a growth rate and lag similar to that of the *ycdH yciC ytiA* strain (6882 [▲]) and not the *ycdH yciC* strain (6866 [■]), suggesting that zinc mobilization is responsible for the observed growth defect. A strain expressing only the L31 C+ protein RpmE (6983 [△]) is shown as a control.

P_{rpmE}-*ytiA* gene. Indeed, the strain constitutively expressing only *ytiA* grew more like the *ytiA* mutant than the HC double mutant (Fig. 6). These results provide strong support for the inference that Zur-regulated expression of YtiA mobilizes zinc from the ribosome by displacement of L31. Whether zinc is spontaneously released from the small L31 peptide (66 amino acids) or whether this requires an unfoldase or proteolysis is currently unknown.

Production of a C− S14 paralog is important for growth under zinc limitation. It has been previously proposed that the Zur regulation of the S14 paralog, YhzA, allows continued ribosome synthesis even when cells are deprived of zinc. Since S14 is an essential protein (17), this is difficult to verify genetically. However, this model predicts that *yhzA* mutants should be growth impaired upon transfer to ZSMM since newly synthesized S14 (RpsN) will be deprived of its needed zinc cofactor, and the cells must therefore rely on previously assembled ribosomes for growth. Indeed, the HC *yhzA* mutant cells grew much more poorly in ZSMM than the HC parent strain. It is also interesting that the growth of this strain appears to be linear with time, rather than exponential. This is consistent with an inability of the cells to synthesize new ribosomes. Under this condition, the number of ribosomes per cell will decrease with each cell division. As a result, it can be predicted that growth will be linear as a function of time rather than exponential. Specifically, linear growth is postulated to reflect the balance between increasing cell numbers (which double with each cell division) and a decreasing growth rate (as the ribosome content per cell declines with each division). Further studies will be required to test this model.

RpmGC does not contribute significantly to the zinc starvation response. It is clear that both YtiA and YhzA contribute to the cell's ability to adapt to zinc deprivation as monitored by growth of the transport-defective HC strain in ZSMM. Next, we sought to determine whether L33c (encoded by *rpmGC*) provides a significant growth advantage and, further, whether it might function to mobilize zinc from the constitutively expressed C+ L33 proteins. The same corrected *rpmGC* construct shown previously to complement an L33 disrupted strain (Fig. 3) was integrated into the HC double mutant, the single *ytiA* and *yhzA* strains, and the double *ytiA yhzA* mutant strains (all in an HC mutant background). We hypothesized that if any of the growth phenotypes we had observed previously with *ytiA* or *yhzA* disruptions were due, in whole or in part, to the lack of L33c, introduction of a functional *rpmGC* gene would increase fitness. However, in all cases strains containing the corrected *rpmGC* gene showed no significant growth improvement compared to strains containing the original frameshift mutation (data not shown). Thus, L33c provides no significant advantage to the cell under these growth conditions, even though it can complement the *rpmGA rpmGB* double mutant for growth in rich medium (Fig. 3). This suggests either that L33 proteins do not provide a mobilizable pool of zinc or that, under these conditions, the L33a and L33b proteins are still able to obtain sufficient zinc for function.

Concluding comments. *B. subtilis* encodes at least three sets of paralogous r-proteins: S14 (*rpsN* and *yhzA*), L31 (*rpmE* and *ytiA*), and L33 (*rpmGA*, *rpmGB*, and *rpmGC*). Previous work led to the proposal of two distinct Zur-mediated mechanisms for responding to zinc limitation (1, 16). The Zur-regulated L31 paralog, YtiA, was shown to actively displace the loosely associated C+ protein, RpmE, from the surface of the ribosome (1). It was speculated that YtiA-mediated release of L31 from the ribosome, followed perhaps by proteolysis, would mobilize zinc for use as a cofactor for essential proteins. It is possible that the paralogous L33 proteins have a similar function. In contrast, the essential zinc-requiring S14 protein (RpsN) is buried deep within the ribosome structure and is required for assembly. Therefore, induction of the Zur-regulated C− S14 paralog (YhzA) was postulated to provide a "failsafe" mechanism for continued ribosome assembly under zinc-limiting conditions (17). Although these are reasonable models, the challenges of limiting cells for zinc had precluded detailed physiological tests of these ideas.

We have explored here the functions of the Zur-regulated C− paralogs in adaptation to conditions of severe zinc deprivation. Since *B. subtilis*, like many bacteria, has extremely efficient zinc-scavenging mechanisms, it is difficult to deprive cells of zinc by simple omission from the growth medium. We have increased zinc stress by genetic ablation of both high-affinity zinc uptake (the *ycdHI-yceA* operon) and the YciC metallochaperone. Using this HC strain, we demonstrate that both the Zur-regulated L31 (YtiA) and S14 (YhzA) paralogs contribute to adaptation to zinc deprivation. In the case of L31, our growth studies indicate that this is due to an inability of YtiA to mobilize zinc. Conversely, the growth properties of the *yhzA* mutant strain are consistent with, and provide support for, the previously proposed "failsafe" mechanism for continued ribosome assembly under zinc limitation. Finally, we have provided evidence that the previously noted *rpmGC* gene can

encode (once the frameshift is corrected) a functional L33 protein, but we find no evidence, under the growth conditions used here, to suggest that this protein provides an advantage for growth under zinc-limiting conditions.

In general, the ribosome is a highly conserved structure, and most r-proteins are encoded by essential genes. However, some r-proteins are not highly conserved, appear to be dispensable for growth, and are variably associated with ribosomes during purification. Whether these are bona fide r-proteins or merely proteins with other functions that associate with the ribosome is not always clear. The finding that L31 functions in storage and mobilization of zinc is one such example. Since cells lacking any L31 are reduced in growth even in rich medium, its seems likely that this protein does play some role in ribosome function. However, it also appears to play a role in storing and mobilizing zinc, in which case L31 can be considered as a dual-function protein.

The extent to which ribosomal proteins may have extraribosomal functions has recently become better appreciated (22). From bacteria to humans, r-proteins are involved in a variety of functions, including roles as DNA endonucleases (23), regulators of macrophage migration inhibitory factor (5), modulators of RNase E activity (20), and factors in DNA regulation (11), just to name a few. The finding of paralogous pairs of r-proteins with one partner regulated by Zur (or other zinc-sensing transcription factors) suggests that zinc mobilization might represent another extraribosomal function for r-proteins. The physiological studies reported here, specifically for L31, provide evidence that this mechanism is indeed operative. Since zinc-regulated, C− r-proteins are widespread in the bacteria, it is likely that this represents a widespread adaptation to conditions of zinc limitation.

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