Functional Analysis of the *Bacillus subtilis* Zur Regulon

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The *Bacillus subtilis* zinc uptake repressor (Zur) regulates genes involved in zinc uptake. We have used DNA microarrays to identify genes that are derepressed in a *zur* mutant. In addition to members of the two previously identified Zur-regulated operons (*yciC* and *yedHI-yceA*), we identified two other genes, *yciA* and *yciB*, as targets of Zur regulation. Electrophoretic mobility shift experiments demonstrated that all three operons are direct targets of Zur regulation. Zur binds to an ~28-bp operator upstream of the *yciA* gene, as judged by DNase I footprinting, and similar operator sites are found preceding each of the previously described target operons, *yciC* and *yedHI-yceA*. Analysis of a *yciA-lacZ* fusion indicates that this operon is induced under zinc starvation conditions and derepressed in the *zur* mutant. Phenotypic analyses suggest that the YciA, YciB, and YciC proteins may function as part of the same Zn(II) transport pathway. Mutation of *yciA* or *yciC*, singly or in combination, had little effect on growth of the wild-type strain but significantly impaired the growth of the *yedHI* mutant under conditions of zinc limitation. Since the YciA, YciB, and YciC proteins are not obviously related to any known transporter family, they may define a new class of metal ion uptake system. Mutant strains lacking all three identified zinc uptake systems (*yciABC*, *yedHI-yceA*, and *zosA*) are dependent on micromolar levels of added zinc for optimal growth.

Zinc is an essential element for living organisms. It plays a vital role as a cofactor for numerous enzymes and DNA-binding proteins and serves as a structural scaffold for several proteins (32). Consequently, living cells have developed systems for high-affinity zinc uptake (14). Several zinc uptake systems in bacteria have been shown to be under the control of a zinc-sensing Fur homolog, the zinc uptake repressor (Zur) (8, 10, 20, 25).

Bacillus subtilis Zur regulates the expression of two putative zinc transport systems, an ABC transporter encoded by the *yedHI-yceA* operon and a conserved membrane protein encoded by *yciC* (10). In addition, *B. subtilis* was recently shown to express a third zinc uptake system, ZosA, under conditions of oxidative stress (11). The *zosA* gene is not repressed by zinc and is expressed under the regulation of the peroxide-sensing repressor PerR rather than Zur (11). Increased zinc uptake under oxidative stress conditions is postulated to help protect cells against metal-catalyzed oxidation reactions by displacing redox-active metals from adventitious binding sites (11). *B. subtilis* also expresses a fourth transporter that contributes to zinc homeostasis, CadA. CadA is a CPx-type ATPase efflux pump that is induced by high zinc concentrations and plays a major role in zinc resistance (A.G. and J.D.H., unpublished results).

Microarray analysis is a powerful tool for the study of metal ion metabolism (9). For example, the iron starvation stimulon has been characterized in several organisms (2, 26, 29, 30, 38). Zinc deprivation in *Saccharomyces cerevisiae* alters the expression of about 15% of its genes, several of which are regulated

by the Zap1 transcriptional activator (21). Comparable studies of zinc regulons in bacterial systems have not been reported.

Here, we used DNA microarray analysis, *lacZ* reporter fusions, and DNase I footprinting to characterize the *B. subtilis* Zur regulon. In addition to the previously reported genes, we identified two more genes, *yciA* and *yciB*, as targets of Zur regulation. Mutational analyses suggest that the YciABC proteins may function together as a new type of metal uptake system.

MATERIALS AND METHODS

Media and growth conditions. *B. subtilis* CU1065 (Table 1) was grown on Luria-Bertani (LB) medium or minimal medium (MM) 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), (NH₄)₂SO₄ (2 g/liter), MgSO₄ · 7H₂O (0.2 g/liter), trisodium citrate·2H₂O (1 g/liter), potassium glutamate (1 g/liter), tryptophan (10 mg/liter), 3 mM (NH₄)₆Mo₇O₂₄, 400 nM H₃BO₃, 30 nM CoCl₂, 10 nM CuSO₄, 10 nM ZnSO₄, and 80 nM MnCl₂ (5). Low-zinc MM (LZMM) was prepared by omitting zinc from the standard MM. Metals from filter-sterilized stocks were added before inoculation. *Escherichia coli* DH5α was used for routine DNA cloning (27). Unless indicated otherwise, liquid media were inoculated from an overnight preculture and incubated at 37°C with shaking at 200 rpm. Erythromycin (1 µg/ml) and lincomycin (25 µg/ml) (for testing of macrolide-lincosamide-streptogramin B resistance), 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.

DNA manipulations. Routine molecular biology procedures were done as described by Sambrook et al. (27). Isolation of *B. subtilis* chromosomal DNA, transformation, and specialized SPβ transduction were done as described by Cutting and Vander Horn (7). Restriction enzymes, DNA ligase, Klenow fragment, and DNA polymerase were used in accordance with the manufacturer's (New England Biolabs) instructions.

Microarray analysis: RNA isolation, cDNA synthesis, and slide hybridization. For RNA isolation, 100 µl of an overnight cell culture of the wild type or the isogenic *zur* mutant was diluted into 6 ml of LB medium (in tubes measuring 18 by 150 mm) and grown at 37°C with shaking at 250 rpm. When the cells reached an optical density at 600 nm of 0.6, the tubes were transferred to an ice-water bath and the cells were collected by centrifugation for 1 min at 5,000 rpm. The pellets were frozen in a dry-ice-methanol bath and resuspended in 10 mM Tris

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Source or reference
<i>B. subtilis</i>		
CU1065	W168 <i>att</i> SP β <i>trpC2</i>	33
HB1000	ZB307A <i>att</i> SP β	6
ZB307A	W168 SP β c2 Δ 2::Tn917::pSK10 Δ 6 (MLS ^r)	40
HB8132	CU1065 <i>zur::spc</i>	4
HB8072	HB1000 <i>yciC::kan</i>	10
HB8073	HB1000 <i>ycdH::cat</i>	10
HB8074	HB1000 <i>yciC::kan ycdH::cat</i>	10
HB8182	CU1065 SP β c2 Δ 2::Tn917:: ϕ (<i>yciA'</i> - <i>cat-lacZ</i>) (MLS ^r Neo ^r)	This work
HB8183	HB8132 SP β c2 Δ 2::Tn917:: ϕ (<i>yciA'</i> - <i>cat-lacZ</i>) (MLS ^r Neo ^r)	This work
HB8184	CU1065 SP β c2 Δ 2::Tn917:: ϕ (<i>sboA'</i> - <i>cat-lacZ</i>) (MLS ^r Neo ^r)	This work
HB8185	HB8132 SP β c2 Δ 2::Tn917:: ϕ (<i>sboA'</i> - <i>cat-lacZ</i>) (MLS ^r Neo ^r)	This work
HB8190	HB1000 <i>yciA::spc</i>	This work
HB8191	HB1000 <i>zosA::kan</i>	11
HB8192	HB1000 <i>yciC::kan yciA::spc</i>	This work
HB8193	HB1000 <i>yciC::kan yciA::spc ycdH::cat</i>	This work
HB8194	HB1000 <i>zosA::kan ycdH::cat</i>	This work
HB8195	HB1000 <i>ycdH::cat zosA::kan</i>	This work
HB8196	HB1000 <i>yciA::spc zosA::kan</i>	This work
HB8197	HB1000 <i>yciA::spc zosA::kan ycdH::cat</i>	This work
<i>E. coli</i> DH5 α	ϕ 80 Δ (<i>lacZ</i>)M15 Δ (<i>argF-lac</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 gyrA96 relA1</i>	27

HCl (pH 8.0)–1 mM EDTA buffer containing 15 mg of lysozyme per ml and incubated for 5 min at room temperature. Total RNA was extracted with RNeasy Minikit (Qiagen), contaminating DNA was removed with a DNA-free kit (Ambion), and the resulting RNA preparation was frozen on dry ice and stored at –80°C until further use. Microarrays contained 4,020 PCR products spotted in duplicate on each glass slide and were prepared as described previously (36). The protocol for labeling utilized random hexamers and was performed as described previously (36).

Two different RNA preparations (from cells grown on different days) were prepared for both the wild-type and *zur* mutant strains. Each RNA preparation was used to make both Cy3- and Cy5-labeled cDNAs, and all competitive hybridizations were done twice, once with each cDNA preparation, to control for any differences in labeling between the two fluorophores. Since all PCR products are spotted twice on each slide, all signal intensities and calculated ratios are the averages of four values.

Microarray data analysis. Signal intensities were detected and quantified with ArrayVision software (Molecular Dynamics) and assembled into EXCEL spreadsheets. Expression ratios were calculated as *zur* mutant/wild type (*zur*/WT) for each experiment, and the mean and standard deviation were calculated. The data were filtered to remove measurements with high variability (those where the standard deviation was equal to or larger than the mean) or those where valid ratios were found for only one of the two replicates.

Protein purification and DNA-binding assays. *B. subtilis* Zur, overexpressed in *E. coli* and purified as described previously (10), was used in electrophoretic mobility shift assays (EMSA) and DNase I footprinting experiments performed essentially as previously described (10, 11). For EMSA experiments, the gel was prerun for 30 min and samples were electrophoresed at 4°C.

Construction of *yciA* and *sboA* transcriptional fusions. Promoter regions were amplified from the *B. subtilis* genome by PCR with primers 5'-GCGAAGCTT GCCGAACATCTTAGGATCT-3' and 5'-TGACGGATCCAAAGTATTGAA GAC-3' for *yciA* and primers 5'-GCGAAGCTTTTCATCATTCCACTTTGAC T-3' and 5'-GCGGGATCCAAAGAGGTAGATTTTAGTTA-3' for *sboA*. The

resulting PCR products were cloned as *Hind*III-*Bam*HI fragments (sites are underlined) into pJPM122 (28) to generate the corresponding *lacZ* operon fusions. The resulting plasmids were linearized with *Sca*I and introduced by transformation into ZB307A (Table 1) with selection for neomycin resistance. SP β transducing lysates were prepared by heat induction and used to lysogenize *B. subtilis* CU1065.

Construction of a *yciA* mutant. The *yciA* region was amplified from chromosomal DNA with the primers 5'-GCGAAGCTT GCCGAACATCTTAGGATC T-3' and 5'-GCGGAATTCTTCTGCATGATCGGAGCA-3' and digested with *Hind*III (site underlined) and *Eco*RI (at a site internal to *yciA*), and the resulting fragment was cloned in pGEM3Zf(+)-cat-1 (37). A gene cassette coding for spectinomycin resistance from pDG1726 (13) was isolated as a *Pst*I fragment and cloned into an internal *Pst*I site in the *yciA* gene. The resulting construct was linearized with *Sca*I and introduced by transformation into *B. subtilis* CU1065 with selection for spectinomycin resistance, and the transformants were screened for loss of the plasmid-borne chloramphenicol resistance to ensure the double-crossover event. Genomic DNA was isolated from selected transformants, and the mutation was confirmed by PCR.

Primer extension assays. Total RNA was isolated from wild-type or *zur* mutant cells with the RNeasy RNA isolation kit (Qiagen). For primer extension analysis, 100 μ g of total RNA was precipitated with 4 pmol of 5'-end ³²P-labeled reverse primer and the reverse transcripts were generated as previously described (18, 19). Reverse transcripts were analyzed by 8 M urea–6% polyacrylamide gel electrophoresis. The PCR product was sequenced with the same primer, and the resulting sequence ladder was used to identify the 3' end of the reverse transcript (corresponding to the 5' end of the RNA).

β -Galactosidase assays. Overnight cultures were diluted 1:100 in LZMM medium containing different concentrations of metal ions (as indicated) and grown to mid-logarithmic phase. Cells were collected and assayed for β -galactosidase as previously described (4, 22).

RESULTS AND DISCUSSION

The zinc uptake repressor Zur was originally discovered in *B. subtilis* (10) and *E. coli* (25), and homologs have been identified in *Listeria monocytogenes* (8) and *Staphylococcus aureus* (20). *B. subtilis* Zur regulates the expression of two proposed zinc uptake systems, YcdHI-YceA and YciC, and purified Zur binds, in a zinc-dependent manner, to the promoter region of both *yciC* and *ycdH* in vitro (10).

Transcriptome analyses. To identify other genes regulated by Zur, we used DNA microarray analysis of RNA isolated from both wild-type and *zur* mutant cells grown under zinc-replete conditions [in LB medium estimated to contain ca. 15 μ M Zn(II)] (24). The *zur*/WT expression ratios in two independent experiments were compared. Overall, 97% of the expressed genes varied less than twofold in expression level between the wild-type and *zur* mutant cells.

To identify those genes whose expression was most significantly (and reproducibly) altered in the *zur* mutant, we used a two-dimensional graphical display of the two sets of measurements (Fig. 1A). Ten genes were found to reproducibly differ more than threefold between the two samples (Fig. 1B). By far, the most dramatic change is the >300-fold derepression of the *yciC* gene in the *zur* mutant strain. This is consistent with the original identification of the corresponding gene product as an abundant membrane protein in the *zur* mutant but not in the isogenic wild type (10). Three other strongly derepressed genes correspond to the *ycdHI-yceA* operon, which was previously shown to be under Zur control (10). Unexpectedly, we also identified the *yciA* and *yciB* genes as strongly derepressed in the *zur* mutant. These genes are immediately upstream of and codirectional with *yciC*. Since the *yciC* promoter region has already been identified as a target of Zur-mediated regulation (10), this suggests that the *yciC* gene can be transcribed inde-

FIG. 3. Primer extension mapping of the transcription start sites of *yciA* (A) and *ycdH* (B). The primer extension product was generated with RNA isolated from either wild type (WT) or *zur* mutant cells grown in LB medium. The sequence ladders were generated by PCR cycle sequencing with the same primer as in the primer extension reaction.

inhibit enzymes in the respiratory chain and may thereby mimic anaerobiosis (3). Indeed, in the experiment in which *sbo* induction was most pronounced, other anaerobically induced, ResDE-regulated genes (e.g., *hmp* and *nasDE*) were also up-regulated.

Only two genes, *rocE* and *rocD*, were repressed more than threefold in the *zur* mutant (Fig. 1). These genes encode an arginine and ornithine amino acid permease (12). The σ^{54} -dependent *rocDE* operon is under the control of *rocR* and is induced by the presence of arginine, ornithine, or proline in

FIG. 4. The *yciABC* and *ycdHI-yceA* regions of *B. subtilis*. Open reading frames are indicated by open arrows, promoter sites are indicated by bent arrows, and Zur boxes are indicated by closed circles. Sequences of the three Zur-controlled promoters are shown with σ^A -dependent recognition sites (bold), and the residue corresponding to the predominant transcription start site is indicated by +1. Note that the *yciC* transcript initiates more than 200 bases upstream of the *yciC* start codon. Regions of Zur protection in the operators of *yciA*, *yciC*, and *ycdH* are underlined (see Fig. 5; data not shown).

the growth medium (12). The relationship between *rocDE* expression and *zur* is not clear. There are no obvious candidates for Zur box-like elements in the *rocDE* regulatory region, and we favor the idea that the decreased expression in the *zur* mutant is an indirect effect of altered zinc homeostasis.

Regulation of *yciA* by zinc and Zur. To investigate the role of Zur in the regulation of *yciA* and *yciB*, we constructed a P_{yciA} -*cat-lacZ* fusion. Expression from the *yciA* promoter region was repressed by zinc at concentrations above 1 μ M and completely derepressed in the *zur* mutant background (Fig. 2B). This regulation precisely parallels that shown previously for the Zur-regulated *yciC* and *ycdH* promoter regions (10).

To identify the promoter element for *yciA*, we used primer extension analysis with RNA extracted from wild-type and *zur* mutant cells grown in LB medium. Transcription of the *yciA* gene starts at or near a G residue 37 bases upstream of the translation start site (Fig. 3A). The *yciA* transcript could only be detected with RNA from the *zur* mutant, consistent with the microarray and *lacZ* fusion data (Fig. 3A).

Comparison of Zur-regulated promoter regions. In parallel with the above studies, we have also determined the transcription start site for the *ycdH*-*yceA* operon (Fig. 3B) and the *yciC* gene (F. Miyagi, A. Gaballa, and J. D. Helmann, unpublished data). The *ycdH* transcript originates from an A residue 60 bases upstream of the translation start site (Fig. 3B) and could only be detected in the RNA extracted from *zur* mutant cells. The *yciC* regulatory region is especially complex and includes a long untranslated leader region and two Zur boxes. The promoter-proximal Zur box (Fig. 4) is primarily responsible for Zn-dependent repression (Miyagi et al., unpublished). Note that this site differs from that previously shown to bind Zur (10). All three promoters conform to the consensus sequences expected for recognition by σ^A holoenzyme (15). On the basis of these DNA microarray analysis results, we propose that these three transcription units constitute the *zur* regulon (Fig. 4).

Zur interaction with Zur boxes. EMSA showed that purified Zur protein binds to the promoter regions of *yciC*, *yciA*, and *ycdH* with high affinity (Fig. 5A). By DNase I footprinting, we demonstrated that purified Zur protein bound to an extended region of ~28 bases overlapping the *yciA* promoter (Fig. 5B). This region contains an imperfect inverted repeat similar to those noted previously for the *yciC* and *ycdH* operators (10). Alignment of the four known Zur boxes with CLUSTAL W (31) reveals a conserved sequence of aatttAAatcGTaAT-ATTacgTTTaa (Fig. 5C and data not shown). This sequence contains a core element of a 7-1-7 inverted repeat similar to the known binding sites for other Fur homologs in *B. subtilis* (1, 2, 16).

A *yciA* mutation affects zinc-limited growth. It has been shown previously that a mutation in *ycdH* affected the ability of the cell to grow in a zinc-deficient medium (LZMM) (10). While a *yciC* mutation had no effect on cell growth in LZMM, it clearly exacerbated the growth defect of the *ycdH* single mutant in LZMM and this defect could be suppressed by added zinc (10). To determine the role of YciA in zinc uptake, we constructed a *yciA::spc* mutant by allelic replacement. While this mutation is likely to be polar on *yciB*, *yciC* is expressed from its own promoter and should not be affected. As noted for the *yciC* mutant, a *yciA* mutant is unaffected in growth under conditions of zinc limitation induced by EDTA

FIG. 5. Interaction of Zur with its target operons. (A) EMSA of Zur binding to the promoter regions of *yciC*, *yciA*, and *ycdH*. Zur protein (concentrations indicated in nanomolar monomer) was incubated with 32 P-end-labeled fragments and analyzed by 6% polyacrylamide gel electrophoresis. (B) DNase I footprinting analysis of Zur binding to the *yciA* promoter region. Zur protein was incubated with a 32 P-end-labeled DNA fragment, and a G+A chemical cleavage ladder was included for localization of the binding sites. The concentration of Zur (monomer) in each reaction was (from left to right) 0, 20, 40, or 100 nM. (C) Alignment of the Zur-protected DNA regions in the promoters of *yciA*, *yciC*, and *ycdH*. The bottom part of panel C compares the core region protected by Zur (a 7-1-7 inverted repeat motif designated a Zur box) with similar operator sites recognized by the Fur homolog PerR and by Fur (1, 17).

addition to MM (Fig. 6). However, the *yciA* mutation increased the growth defect of the *ycdH* single mutant. This is indistinguishable from the phenotype of a *yciC* mutant studied previously (10). A *yciA yciC* double mutant is phenotypically iden-

FIG. 6. Effects of EDTA on the growth of the wild type and zinc uptake mutants. Inhibition of the growth of the wild type ($\blacklozenge$) and the *ycdH* ($\triangle$), *ycdH yciA* ($\circ$), *ycdH yciC* ($\bullet$), and *yciA ycdH zosA* ($\diamond$) mutants by EDTA is shown. The *zosA*, *yciA*, and *yciC* single mutants and the *yciA-yciC* double mutant were indistinguishable from the wild type. For each strain, the diameter of the zone of growth inhibition was determined on MM plates overlaid with a 0.6-cm-diameter filter paper disk containing 10 μ l of EDTA at either 10, 100, or 500 mM. Values represent the average of three independent measurements with an average error of ± 0.1 cm.

tical to either single mutant (Fig. 6 and data not shown). Both YciC and YciB are predicted membrane proteins with at least one membrane-spanning segment (and YciC fractionates with the membrane; 10). Together, these results suggest that YciAB and YciC may function in the same pathway for zinc uptake, presumably as subunits of a low-affinity Zn(II) uptake system or permease. The lack of similarity to known zinc uptake or metal transport systems suggests that these conserved proteins may define a new family of transporter.

Characterization of strains deficient in three zinc uptake systems. *B. subtilis* encodes three known zinc uptake systems: an ABC transporter encoded by the Zur-regulated *ycdHI-yceA* operon, a postulated low-affinity transport system encoded by the *yciABC* operon (10), and the P-type ATPase ZosA regulated by the PerR protein (11). We constructed single, double, and triple mutant strains affected in these systems and tested their abilities to grow under conditions of zinc limitation. As expected, the triple mutant was sensitive to zinc limitation (induced by EDTA) in comparison to the single and double mutants (Fig. 6). In experiments with LZMM supplemented with 500 nM Zn(II), no dramatic differences were found between the single and double *yciA*, *yciC*, *ycdH*, and *zosA* mutants (Fig. 7A). However, the *yciA ycdH zosA* triple mutant grew poorly under these same conditions (a doubling time of 68 min versus 49 min for the wild type) and showed an extended lag phase (Fig. 7A). A direct comparison of the growth curves of the wild type and the isogenic triple mutant strain as a function of the zinc concentration revealed that the growth rate and yield of the triple mutant are highly sensitive to the amount of zinc added to the LZMM, with a concentration of greater than

FIG. 7. Effect of zinc limitation on the growth of the wild type and zinc uptake mutants. (A) Growth curves of the wild type ($\blacklozenge$) and the *yciA* ($\blacksquare$), *ycdH* ($\blacktriangle$), *ycdH yciA* ($\circ$), *yciA zosA* ($\square$), *ycdH zosA* ($\triangle$), and *yciA ycdH zosA* ($\diamond$) mutants in LZMM with 500 nM Zn(II). (B) Effect of zinc concentration on the growth yield (10 h after 1:100 dilution into LZMM supplemented as indicated) of the wild type ($\blacklozenge$) and the *yciA ycdH zosA* mutant ($\diamond$). OD₆₀₀, optical density at 600 nm.

1 μ M zinc required to achieve an optical density at 600 nm of greater than 1.0 (Fig. 7B). These data indicate that YciABC, YcdHI-YceA, and ZosA constitute the major zinc uptake pathways in *B. subtilis*. Indeed, under zinc-replete conditions, the *yciABC* and *ycdHI-yceA* systems are repressed and ZosA provides a major conduit for zinc influx (11).

The ability of *B. subtilis* to grow, albeit at a reduced rate and with a reduced yield, even in the absence of these three systems indicates that there are other low-affinity pathways that allow the import of zinc. It has been suggested that the CitM transporter may help supply zinc in a medium that contains citrate (14). While LZMM does contain citrate, it also contains a high glucose concentration, which completely represses the expres-

sion of *citM* (34, 35). More work is needed to identify the transporter(s) that allows zinc uptake in the triple-mutant strain.

Summary. We have used DNA microarray analyses to identify genes whose expression is affected by a *zur* mutation. Remarkably, the most strongly derepressed gene was *yciC*, corresponding to an abundant membrane protein that was first identified as a target for Zur-mediated repression by sodium dodecyl sulfate-polyacrylamide gel analysis of protein profiles (10). We also identified the upstream genes *yciA* and *yciB* as targets of Zur-mediated repression. This regulation is mediated by Zur binding to a high-affinity binding site overlapping a σ^A -type promoter element for the *yciABC* operon. Sequence comparisons of known Zur-regulated genes identified a conserved motif, the Zur box, that is associated with Zur binding. Genetic and physiological studies suggest that *yciA*, *yciB*, and *yciC* likely function in the same pathway for zinc uptake. Since these genes encode proteins conserved in many bacterial species, and at least YciC is membrane localized (10), we suggest that the YciABC system may define a new family of metal ion transporters.

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