

Lactococcus lactis ZitR Is a Zinc-Responsive Repressor Active in the Presence of Low, Nontoxic Zinc Concentrations *In Vivo*^{∇†}

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In the family *Streptococcaceae*, the genes encoding zinc ABC uptake systems (called *zit* or *adc*) are regulated by a coencoded MarR family member (i.e., ZitR or AdcR), whereas in the great majority of bacteria, these genes are regulated by Zur, the Fur-like zinc-responsive repressor. We studied the *zit* operon from *Lactococcus lactis* and its regulation in response to Zn(II) *in vivo*. *zit* transcription is repressed by Zn(II) in a wide concentration range starting from nontoxic micromolar levels and is derepressed at nanomolar concentrations. The level of *zit* promoter downregulation by environmental Zn(II) is correlated with the intracellular zinc content. The helix-turn-helix domain of ZitR is required for downregulation. *In vitro*, the purified protein is a dimer that complexes up to two zinc ligands per monomer and specifically binds two intact palindromic operator sites overlapping the –35 and –10 boxes of the *zit* promoter. DNA binding is abolished by the chelator EDTA or TPEN and fully restored by Zn(II) addition, indicating that the active repressor complexes Zn(II) with high affinity. These results suggest that derepression under starvation conditions could be an essential emergency mechanism for preserving Zn(II) homeostasis by uptake; under Zn(II)-replete conditions, the function of ZitR repression could be to help save energy rather than to avoid Zn(II) toxicity. The characterization of a MarR family zinc-responsive repressor in this report gives insight into the way *Streptococcaceae* efficiently adapt to Zn(II) fluctuations in their diverse ecological niches.

Zinc is a transition metal that plays key roles in major cellular functions, such as transcription, translation, replication, resistance to oxidative stress, and virulence, but zinc, like other metals, is both vital in trace amounts and toxic at high concentrations (15). Zinc homeostasis is achieved by the tight control of storage systems and transporters with broad diversity (5, 15, 30, 50). Bacterial responses to zinc have been studied by genome-wide transcriptional analyses in *Escherichia coli* (8, 26), *Bacillus subtilis* (31), and *Streptococcus pneumoniae* (24), showing altered expression of numerous genes implicated in tolerance for and efflux of zinc and other metals, and also in general stress resistance. Conversely, zinc depletion in *E. coli* regulates a small number of genes involved in high-affinity zinc binding

and uptake or encoding zinc-independent ribosomal proteins (18).

The regulation of zinc transport is an essential cellular process achieved by zinc-responsive regulators (30), although some general regulators can be involved independently of zinc levels (17). Regulators specific to zinc resistance genes (encoding metallochaperones or efflux systems), including SmtB/ArsR and MerR family members, act as repressors in apoprotein form, whereas in the presence of zinc, the holoprotein form is either no longer active or becomes a true activator (9). In contrast, Zur repressors (Fur family) specific to zinc ABC uptake systems are active as holoproteins and switch off expression of transporters in the presence of zinc (20, 25). In *E. coli*, the prototypes of regulators for zinc efflux and uptake, ZntR and Zur, behave as efficient zinc sensor proteins whose affinity is in the femtomolar range (35), revealing the absence of free Zn(II) in the cytoplasm and the extraordinary chelation capacity of cytoplasmic proteins (35).

The *Streptococcaceae* (the genera *Streptococcus* and *Lactococcus*) differ from the great majority of other bacterial groups, including Gram-negative proteobacteria and most Gram-positive low-GC-percent bacteria, by their zinc uptake regulator. Rather than Zur proteins, *Streptococcus* and *Lactococcus* genomes encode homologous regulatory proteins named AdcR and ZitR, respectively (10, 30, 38), which do not belong to a well-known metalloregulator family. They form part of the MarR family (10, 38), whose members, in general, control diverse functions, including the transport of antimicrobials or drugs, but not of metals (51). *adcR* and *zitR* genes, in contrast to *zur* genes, belong to putative *adc-zit* operons (29, 38) that

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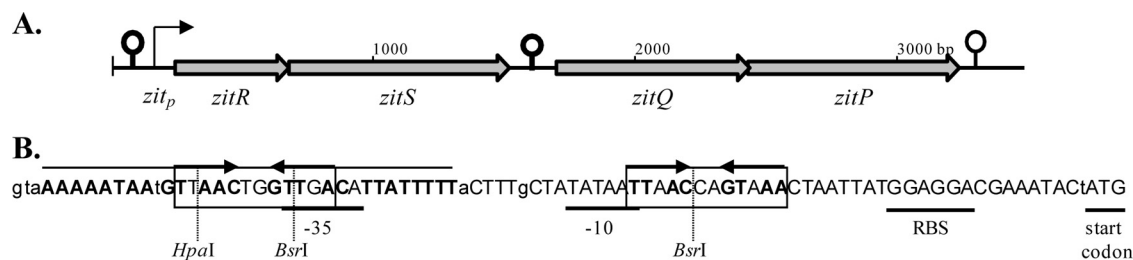


FIG. 1. *L. lactis* *zit* operon. (A) *L. lactis* *zitRSQP* genes (conserved in all four sequenced strains: MG1363, SK11 [both subsp. *cremoris*], IL1403, and KF147 [both subsp. *lactis*], although they are annotated as *LACR_2420* to *LACR_2417* in strain SK11) are represented as large gray arrows. A putative ZitR repressor, ZitS lipoprotein, ZitQ ATP-binding cassette protein, and ZitP permease are homologous to streptococcal AdcR, AdcA, AdcC, and AdcB proteins, respectively (even though the AdcA lipoprotein seems to be a fusion between two zinc-binding proteins, *L. lactis* ZitS [or *E. coli* ZnuA] and *E. coli* ZinT [38]). The predicted *zitp* promoter is shown, and ρ -independent terminators in strains MG1363, SK11, and IL1403 (http://bonsai.hgc.jp/~mdehoon/terminators/NC_009004.trms) are indicated by hairpins. The strongest ρ -independent terminator, perfectly conserved in all three strains, is present between *zitS* and *zitQ* (although there is no consensus promoter sequence upstream of *zitQ*). Downstream of *zit*, ρ -independent terminators of different sequences are present in MG1363 and IL1403 only. (B) The *zitp* sequence from strain MG1363 (perfectly canonical -35 and -10 boxes are properly spaced at 17 bp) is shown, with nucleotides conserved in all four strains in uppercase letters. Putative signals for both transcription (-35 and -10 boxes) and translation (ribosome binding site [RBS] and start codon) are underlined. The two imperfect TTAACYRGTTAA palindromes (38) are boxed, with complementary nucleotides in boldface and marked by thick arrows. The upstream palindrome is embedded in a larger inverted repeat (thin lines) corresponding to the weak ρ -independent terminator for the upstream operon (http://bonsai.hgc.jp/~mdehoon/terminators/NC_009004.trms). Relevant restriction sites are indicated (Fig. 7).

also encode an ABC uptake system (10, 13, 20) that is complete or incomplete (AdcA lipoprotein can be separately encoded) (29, 38). Although AdcA belongs to a cluster of binding proteins specific for either zinc or manganese, it was shown in *S. pneumoniae* that AdcABC/ZitSPQ proteins form an ABC uptake system specific for zinc that is widely conserved in bacteria (10, 20). *adc-zit* promoter regions display a conserved TTAACYRGTTAA palindrome (in one or two copies), proposed to be an AdcR/ZitR binding site (38). In pathogenic species, *adcR* is involved in biofilm formation (29), in the control of immunogenic surface proteins (1, 3, 34) and of zinc transport proteins (3), and in virulence (2). At the beginning of this work, little was known about AdcR/ZitR proteins (10, 30, 38). *S. pneumoniae* and *Streptococcus suis* AdcR proteins were then found to be DNA-binding proteins (1, 3), and *S. suis* AdcR was shown to be a metalloregulator specifically binding the TTAACYRGTTAA palindrome (1). While this work was submitted for publication, the *S. pneumoniae* AdcR protein was characterized in both apo- and Zn(II)-bound forms, and three of its Zn(II)-binding residues were identified (44).

The lactic acid bacterium *Lactococcus lactis* has a conserved chromosomal *zitRSPQ* region (Fig. 1A) that is annotated as being involved in zinc uptake and regulation by homology to streptococcal *adc* operons. The *zit* promoter sequence contains two imperfect TTAACYRGTTAA palindromes (Fig. 1B) (38). In a previous study screening for exported fusions to a signal peptideless reporter in strain MG1363, the *zitR* gene was co-cloned with the 5' part of the *zitS* gene, and the latter was joined to the reporter open reading frame (ORF) (and referred to as *nlp3*, for *new lipoprotein 3*-encoding gene) (41). The *zit* promoter (*zitp*, previously referred to as P_{Zn} [28]), cloned together with *zitR* on a plasmid, was shown to be inducible by EDTA and repressed by zinc and proved to be a useful and efficient tool for heterologous expression in *L. lactis* (28, 32, 49).

In this work, we studied the regulation of the *L. lactis* *zit* operon both *in vivo* and *in vitro*. *In vivo*, we determined the range of Zn(II) concentrations leading to repression and its

low threshold. *In vitro*, a purified recombinant rZitR protein was analyzed: the conditions of its DNA-binding activity and the natures of its metal ligand and of its operator sites were determined. This study is the first biochemical characterization of a lactococcal ZitR/AdcR-type regulator. The *L. lactis* ZitR protein displays high affinity for zinc and is a repressor active in low, nontoxic zinc concentrations *in vivo*.

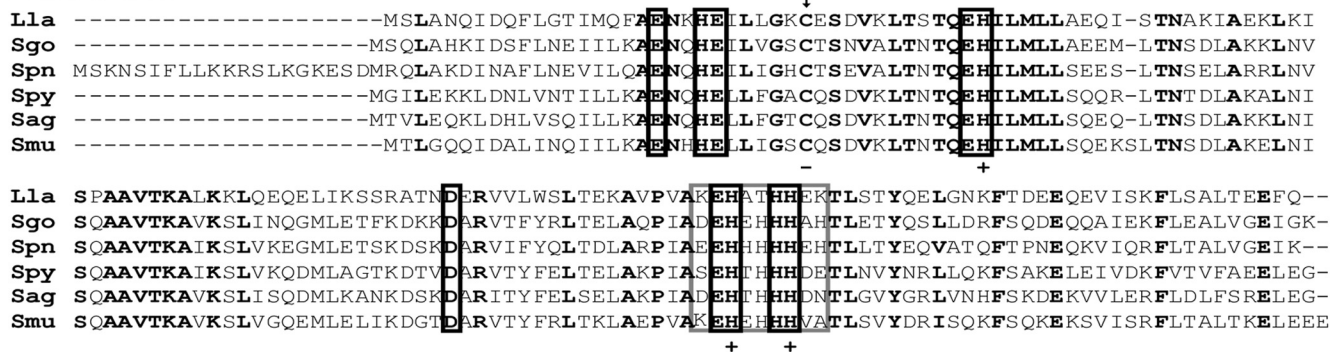
MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study were *E. coli* strain BL21(DE3) [F^- *ompT hsdS_B(r_B⁻ m_B⁻) gal dem* (DE3)] (Novagen) and *Lactococcus lactis* subsp. *cremoris* strain MG1363 (plasmid free). Plasmids (see Table S1 in the supplemental material) were constructed as follows. The *zitp zitR* region was amplified from pVE8064 (28) using MCPz (5'-GCTGATATCGAATTCGAGCTCGGTACCCGGGGATCCTTATCGAAACTCTTCAGTTAGTACTG-3') and M13 Reverse (5'-CAGGAAACAGCTATGACC-3') primers. This PCR fragment was digested with BglII and EcoRI and cloned into pRC2, a medium-copy-number vector digested with BamHI and EcoRI (14), giving pVE8071. For pVE8072, Φ (*usp_{SP}-nuc_B*)(Hyb), a gene fusion encoding a recombinant form of the staphylococcal nuclease reporter (SP_{Usp}-NucB [NucB fused to the Usp45 signal peptide]), was excised from pVE8064 (28) with BamHI and EcoRI and inserted into pVE8071, leading to a *zitR* Φ (*usp_{SP}-nuc_B*) operon under *zitp* control. To delete the *zitR* 3' end (encoding the ZitR helix-turn-helix [HTH] domain), pVE8072 was digested with MluI and BamHI and filled in by T4 DNA polymerase, leading to pVE8074 bearing a *zitR* _{Δ HTH} Φ (*usp_{SP}-nuc_B*) operon. For pVE8073, a *zitR*, allele encoding rZitR was amplified from the MG1363 chromosome using *zitRNco2* (5'-AATACCATGGCTTTACGAAATCAATCGAAC-3') and Stop (5'-GCTCTAGAGCGGGATCCTTACTTCATCGAAACTC-3') (the BamHI restriction site is in boldface) primers, and NcoI-BamHI digestion allowed its cloning into pET-28b(+) (Novagen). The *zitR*, allele was subcloned into pVE8064 instead of *zitR*, leading to pVE8075. *zitR* was also entirely deleted from pVE8064 to produce pVE8076.

Growth conditions, metal deprivation, or supplementation. *E. coli* was grown at 37°C with shaking in LB medium supplemented with kanamycin (50 μ g/ml) where appropriate. *L. lactis* strains were grown at 30°C either in rich GM17 medium (48) or in chemically defined SA medium (10 nM ZnSO₄, 80 nM MnCl₂, 10 nM CuSO₄, 30 nM CoCl₂, and 10 μ M FeSO₄ [22]) supplemented with erythromycin (5 μ g/ml) or chloramphenicol (10 μ g/ml) where appropriate.

zitp regulation by a concentration gradient of Zn(II) was studied on plates as described previously (40). Highly concentrated ZnSO₄ solutions (i.e., 1 mM or 1 M) were streaked on SA-agar medium, where lactococcal cells expressing a *zitp*-controlled reporter fusion were then cross-streaked and grown overnight (ON).

A. ZitR / AdcR



B. Zur

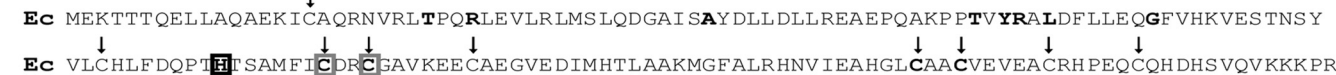


FIG. 2. Streptococcal ZitR/AdcR proteins. (A) Sequences of the ZitR protein from *L. lactis* strain IL1403 (Lla; gb|AAK06214.1|AE006439_11) and of AdcR proteins from *Streptococcus gordonii* strain Challis (Sgo; gb|AAO43167.11), *S. pneumoniae* strain R6 (Spn; gb|AAL00780.11), *S. pyogenes* strain M1 group A streptococcus (GAS) (Spy; gb|AAK33210.11), *Streptococcus agalactiae* strain NEM316 (Sag; emb|CAD45795.11), and *S. mutans* strain UA159 (Smu; gb|AAN59599.1|AE015022_6) are aligned. (B) The sequence of the Zur protein from *E. coli* strain K-12 (Ec; P0AC51) is shown for comparison. Conserved residues are shown in boldface, with histidine and acidic residues boxed in black. Cysteine residues are indicated by arrows. Previously proposed Zn(II) (metal) ligands, i.e., a histidine- and aspartate-rich motif in ZitR/AdcR proteins (29) and the conserved cysteine residues of Zur proteins (39), are boxed in gray. The three recently identified Zn(II) ligands (all histidine residues) and the unique cysteine residue found not to be involved in Zn(II) binding in AdcR from *S. pneumoniae* strain D39 (44) are marked by + and -, respectively.

Northern blotting. An overnight culture of MG1363 in SA medium was diluted in fresh medium, grown to the exponential phase (at an optical density at 600 nm [OD₆₀₀] of 0.2), divided in two for addition or not of 2 μM ZnSO₄, and then allowed to grow to stationary phase (OD₆₀₀ = 0.8). Samples were taken from exponential-phase cultures prior to the addition and from stationary-phase cultures, and their volumes were standardized with respect to the OD₆₀₀. Total RNAs were extracted as described previously (43), and after quantification (OD₂₆₀), 70 μg was loaded on a 1.25% SeaKem GTG agarose gel in 10 mM phosphate buffer, using a 0.24- to 9.50-kb size standard (Promega). Electrophoresis was carried out at 100 V for 3 h (to allow specific *zitRS* mRNAs to migrate slightly faster than 16S rRNA), and ethidium bromide staining was performed. After being blotted on a Nytran-N nylon membrane (Schleicher and Schuell) and methylene blue stained (see Fig. S1 in the supplemental material), RNAs were hybridized at 42°C with an MG1363 *zitRS*' probe (41). This probe was amplified from pVE8020 using the oligonucleotides 5'-AGTCGCAGGTTCTTTATG-3' and 5'-CTAATGAGCGGCTTTTT-3'. Labeling and detection were carried out using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech Europe GmbH).

Staphylococcal nuclease activity test. Nuclease activity was detected as a pink halo on toluidine blue-agar medium (reference 41 and references therein) either overlaid on agar plates or used for dot-blotting of trichloroacetic acid (TCA)-precipitated cultures, using a culture volume standardized with respect to the OD₆₀₀ (41), and the culture aliquot was diluted in SA medium when necessary to spot a constant volume.

Determination of metal content by ICP-MS. Strain MG1363(pVE8072) grown overnight in SA plus chloramphenicol was diluted about 100-fold (according to the OD₆₀₀) in 2.2 liters of SA. In early exponential phase (OD₆₀₀ = 0.1), the culture was divided in two for 2 μM ZnSO₄ addition or no treatment. Culture aliquots standardized according to the OD₆₀₀ were taken from the subculture without ZnSO₄ in the exponential phase (OD₆₀₀ = 0.2) and from both subcultures after overnight treatment (OD₆₀₀, ~1), washed twice in water, and lyophilized. Inductively coupled plasma-mass spectrometry (ICP-MS) analyses of lyophilized cells or protein (see below) were performed by Service Central d'Analyse du CNRS (Vernaison, France) (<http://www.sca.cnrs.fr>).

rZitR overproduction and purification. rZitR, a recombinant untagged form of MG1363 ZitR protein (UniProtKB/TrEMBL no. A2RNS2) was produced in *E. coli* after cloning of *zitR*, into the pET-28b(+) expression vector. rZitR bears three N-terminal substitutions (Ser₂Ala, Ala₄Arg, and Asp₆Glu) at nonconserved positions in ZitR/AdcR proteins (Fig. 2) that have no effect on activity *in vivo*, as rZitR is as efficient as wild-type (WT) ZitR in repressing, in response to ZnSO₄, a *zitP*-controlled and plasmid-borne reporter fusion (see Fig. S2 in the

supplemental material). For overproduction in *E. coli*, an overnight culture of strain BL21(DE3)(pVE8073) was diluted 100-fold in prewarmed medium (1 liter), grown to an OD₆₀₀ of 0.5, induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and then grown for 3 h at 25°C to an OD₆₀₀ of 2.0. Cells were centrifuged; washed twice in a 50 mM Tris-HCl, pH 6.5, 100 mM NaCl, and 1% saccharose buffer; frozen; resuspended in 40 ml of 20 mM Tris-HCl, pH 6.5, and 50 mM NaCl buffer; and disrupted by sonication on ice. The soluble fraction was recovered after 30 min of centrifugation at 20,000 × g and 4°C.

rZitR protein was then purified by a 2-step chromatography procedure: (i) anion-exchange chromatography (Q-Sepharose-Sepharose FF; Amersham) and (ii) heparin affinity chromatography (Heparin-Sepharose FF; Amersham) in the presence of 1 mM ZnCl₂. The eluted sample (0.48 mg/ml protein, eluted by 50 mM Tris-HCl, pH 7, and 600 mM NaCl buffer) was >95% pure. A second batch purification was performed by essentially the same procedure, except that Heparin HyperDM20 (Biosepra) was used, and resulted in a 0.72-mg/ml protein yield (eluted by 20 mM Tris-HCl, pH 7, and 330 mM NaCl) that was >90% pure. The two purified samples (stored at 4°C) gave similar results in electrospray ionization-mass spectrometry (ESI-MS) and electrophoretic mobility shift assay (EMSA) experiments (see Fig. 6A for the first and Fig. 5, 6B, and 7 for the second).

Gel filtration. Five milliliters of rZitR (0.7 mg/ml) was injected on a HiLoad 16/60 (GE Healthcare) Superdex 75 equilibrated in the following buffer: 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol, and 100 μM ZnSO₄ at 8°C. Apparent molecular weights were determined by comparison with a calibration curve using standard molecular weight markers. The two elution peaks were compatible with dimeric and tetrameric species of rZitR.

ESI-MS. Two rZitR aliquots (15 nmol; 240 μg) were diluted in 15 ml of 10 mM ammonium acetate buffer with or without 3.5 mM EDTA. Cycles of protein desalting in 10 mM ammonium acetate buffer were performed using Centrifix YM-3 centrifugal filter devices (3 kDa) from Amicon (Millipore) to dilute initial buffer salts ~10⁶-fold. Lyophilized protein aliquots (2 nmol; 32 μg) were suspended either at 2 μM in a denaturing solvent (50% acetonitrile acidified with 0.1% formic acid) or at 10 μM in a nondenaturing buffer (10 mM ammonium acetate salt, pH 6.8). Mass measurements were performed on a hybrid quadrupole time-of-flight instrument (Q-TOF Ultima Global; Waters) equipped with an electrospray ion source. Under nondenaturing conditions, the instrument parameters (accelerating voltage and pressure in the interface of the mass spectrometer) were carefully controlled to preserve weak noncovalent interactions. In further experiments (collision-induced dissociation in the gas phase), kinetic energy was increased to produce gas phase dissociation of dimeric species into monomers (45, 47).

EMSA. An 811-bp PCR fragment containing *zitp* (and *zitR*) was amplified from pVE8020 (41) or pVE8064 (28) using M13 Reverse and MUT (5'-GCTCTAG AGCGGGATCCTTCATCGAACTCTTCAG-3') primers (28) and purified with a QIAquick Gel Extraction Kit (Qiagen). Its digestion by (i) AluI or (ii) BsrI generates fragments of (i) 277, 248, 115, 69, 37, 33, 22, and 10 bp (HpaI cuts the 277-bp AluI fragment into two fragments of 172 and 105 bp) and (ii) 293, 280, 152, 54, and 32 bp. DNA-binding reactions (8 μ l) were performed in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 μ g/ml bovine serum albumin (BSA), 200 μ M dithiothreitol (DTT), 200 μ M MgCl₂, and 4% glycerol with 50 to 80 ng (13 to 21 nM) of DNA, and the rZitR protein was added at the indicated molar DNA/protein ratios. rZitR was preincubated with ligands for 10 min at room temperature (see Fig. 6A). Binding reactions were carried out at room temperature for 30 min, and the reaction mixtures were then subjected to electrophoresis in 8% polyacrylamide gels using Tris-borate buffer (45 mM Tris, 45 mM boric acid, pH 8.0) that had been supplemented with 0.1 mM ZnSO₄ or 1 mM EDTA in the experiment (see Fig. 6A). Electrophoresis was carried out at 70 V for 1.5 h at 4°C (see Fig. 6A) or at room temperature (see Fig. 7) and at 50V for 10 to 15 min and 80 V for 65 to 70 min at room temperature (see Fig. 6B). The gels were stained with ethidium bromide (5 μ g/ml) for 30 min and rinsed with distilled water.

RESULTS

***L. lactis* ZitR protein.** *L. lactis* was chosen as a *Streptococcaceae* prototype to study the regulation of the *zitRSQP* genes (Fig. 1A) that encode a putative repressor (ZitR) and an ABC uptake system (ZitSQP). Lactococcal ZitR proteins (88% to 99% identity) and streptococcal AdcR proteins share 45% to 50% identity and are rich in acidic and histidine residues (Fig. 2A). Their C-terminal putative HTH domain is typical of the MarR family (smart00347) (10, 30) but is also related to the ArsR (smart00418) and distantly to the DtxR (smart00529) families dedicated to the regulation of metal homeostasis (9, 30). The predicted secondary structure of the *L. lactis* ZitR protein displays the $\alpha\alpha\alpha\beta\beta$ motif (<http://bioinf.cs.ucl.ac.uk/psipred/> and data not shown) shared by MarR regulators (51) and the majority of metalloregulators (30), suggesting that the ZitR protein could fold as a winged-helix protein. Finally, the *L. lactis* ZitR protein is a good model for AdcR regulators (MarR family) and could control both zinc uptake by ZitSQP and its own expression.

Repression of *zit* transcription. The expression of the WT *zit* operon from *L. lactis* subsp. *cremoris* (strain MG1363) and its regulation in response to extracellular Zn(II) was explored by Northern blotting. To accurately test the specific effect of Zn(II), we used the chemically defined and metal-poor SA medium (22). An exponential-phase culture of MG1363 cells grown in SA medium was supplemented or not with a nontoxic concentration (2 μ M) of ZnSO₄, and mRNAs were extracted prior to the addition and after growth until the stationary phase. The exponential-phase extract revealed a major transcript corresponding in size to *zitRS* mRNA and a very weak band corresponding to a larger *zitRSQ(P)* mRNA (Fig. 3A and data not shown). *zitRSQP* genes thus seem to be cotranscribed, even though they are expressed at a low level compared to *zitRS* genes. The existence of *zitRS* mRNA supports the proposal that the strong and conserved stem-loop downstream of *zitS* (Fig. 1A) could be a ρ -independent terminator (12; http://bonsai.hgc.jp/~mdehoon/terminators/NC_009004.trms), although mRNA processing cannot be excluded.

In cells grown to the exponential phase in Zn(II)-poor SA medium (10 nM) (22), *zitRS(QP)* genes were expressed, suggesting that *zitp* was turned on. In the stationary phase without Zn(II) addition, *zitRS* mRNA was clearly upregulated com-

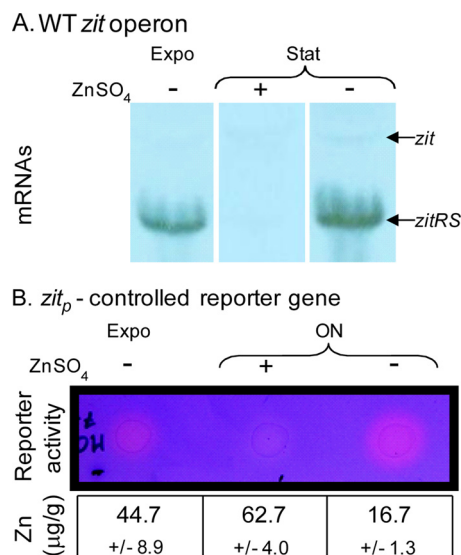


FIG. 3. *zitp* regulation in response to extracellular Zn(II). (A) Regulation of a chromosomal *zit* operon in response to extracellular Zn(II). MG1363 cells were grown in SA medium to the exponential phase (Expo), and after the addition of nontoxic (2 μ M) ZnSO₄ (+) or not (-), growth was continued to the stationary phase (Stat). Equivalent amounts of RNA were loaded on a gel (as measured by the OD₂₆₀). After electrophoresis, blotting, and methylene blue staining of the membrane, Northern blotting was performed with a probe covering the *zitR* gene and the *zitS* 5' end. An mRNA corresponding in size to *zitRS* by comparison to a molecular weight marker and a weak and larger *zit* mRNA are indicated by arrows. (B) Correlation of the *zitp* expression level with the intracellular Zn content. MG1363(pVE8072) cells were grown in SA medium to exponential phase (Expo) or ON (i.e., late stationary phase) in the presence (+) or absence (-) of 2 μ M ZnSO₄, and culture aliquots (adjusted for equivalent OD₆₀₀ values) from three independent experiments were lyophilized or not. Reporter activity was revealed by spotting fresh cells on toluidine blue-agar medium (one representative experiment with cells spotted on the same detection medium is shown). In parallel, ICP-MS was used to measure the Zn contents of all three lyophilized cell aliquots: average values (μ g/g) and standard deviations are indicated. The Cu content, which was measured as a control, was not correlated with the *zitp* expression level, in contrast to the Zn content (not shown).

pared to the exponential phase. However, upregulation could also be achieved in the exponential phase by EDTA addition (data not shown): this result and other data (Fig. 3B) suggested that upregulation during growth could be an indirect effect of a decrease in the extracellular Zn(II) concentration rather than true growth phase-dependent regulation. After ZnSO₄ addition in the stationary phase, no transcript was detectable, showing that *zit* operon transcription is repressed by Zn(II) at a moderate and nontoxic concentration. All these results demonstrate that the WT chromosomal *zit* operon is repressed in response to extracellular Zn(II) and that the repression threshold is low (<2 μ M), i.e., well below the level of Zn(II) toxicity in *L. lactis*. These results are in keeping with our previous data using *zitp*-controlled reporter fusions in an operon with *zitR* (28), which showed that *zitp* regulation is preserved when *zitp* and *zitR* are cocloned on a plasmid. Using those reporter fusions, the *zitp* repression threshold was estimated at around 100 nM Zn(II) (reference 28 and data not shown), which fit the Northern blot results well.

Intracellular effector of *zit* regulation. To gain insight into the intracellular signal for *zitp* regulation by extracellular Zn(II), the cellular metal content was determined by ICP-MS, while *zitp* expression was followed in parallel using a plasmid-borne reporter cassette. In pVE8072, a staphylococcal nuclease reporter fusion (28) is in an operon with the *zitR* gene and is expressed under the control of *zitp*. Strain MG1363(pVE8072) was grown in SA medium to early exponential phase for supplementation or not by ZnSO₄ at 2 μM. Cells from the exponential phase (without addition) or overnight cultures were analyzed for both reporter activity and zinc content (Fig. 3B).

In overnight samples after ZnSO₄ addition, reporter activity was nearly undetectable, as expected (Fig. 3A) (28), and the intracellular Zn content was high: it was increased by ~4 fold compared to ON samples without addition (Fig. 3B). On the other hand, during growth in SA without addition (compare overnight and exponential samples in Fig. 3B), reporter activity was increased, consistent with chromosomal *zit* induction (Fig. 3A), while the intracellular Zn content was significantly decreased. These results were confirmed in another strain context (data not shown), indicating that the *zitp* expression level is inversely correlated with the cellular Zn content, suggesting that cytoplasmic zinc could mediate repression by extracellular Zn(II).

***zitR* with an intact HTH-encoding domain is necessary for *zitp* regulation *in vivo*.** The above-mentioned and previous expression results (28), together with sequence data, all point to ZitR as the likely repressor of *zitp* expression. We tested whether *zitR* could regulate a *zitp*-controlled reporter gene carried on a plasmid in an otherwise WT strain. When *zitR* was deleted from pVE8064 (28), resulting in the transcriptional fusion of the reporter gene to the *zitp* sequence, reporter expression was almost abolished, whatever the conditions (see Fig. S2 in the supplemental material), suggesting a major effect on transcription initiation or the mRNA 5' structure and stability. A partial deletion specifically affecting the DNA-binding domain-encoding region was then designed, as in several other regulator genes (reference 39, and reference 53 and references therein), and it resulted in loss of function (inactivation) without loss of expression.

The *zitR* 3' end coding for the predicted ZitR HTH domain was deleted from pVE8072, leading to pVE8074, which carries the reporter gene in an operon with a truncated *zitR*_{ΔHTH} allele under the control of the *zitp* promoter. In an otherwise WT strain, the abilities of plasmid-borne intact *zitR* and truncated *zitR*_{ΔHTH} alleles to regulate reporter expression on plates in the presence of a Zn(II) concentration gradient were compared, as described previously (40). At high Zn(II) concentrations (millimolar range and below), whereas the reporter was repressed in the presence of intact *zitR*, it was derepressed at an intermediate level in the presence of the *zitR*_{ΔHTH} allele (see Fig. S3 in the supplemental material), indicating that in contrast to *zitR*, *zitR*_{ΔHTH} fails to fully repress *zitp* expression. At higher Zn(II) concentrations (molar range and below), the reporter in an operon with *zitR*_{ΔHTH} was fully induced (Fig. 4). *zitp* derepression in the presence of both plasmid-borne truncated *zitR*_{ΔHTH} and chromosomal WT *zitR* copies strongly suggests that *zitR*_{ΔHTH} in multicopy is a *trans*-dominant-negative allele. As proposed for other regulators (53), including the *E.*

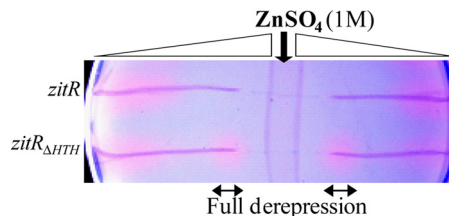


FIG. 4. Derepression of the *zitp* promoter in the presence of both plasmid-borne truncated *zitR*_{ΔHTH} and chromosomal WT *zitR* alleles at sublethal Zn(II) concentrations. The *zitp* expression level was followed using the staphylococcal nuclease reporter. WT intact *zitR* or truncated *zitR*_{ΔHTH} alleles are in an operon with the reporter gene on pVE8072 and pVE8074 plasmids, respectively, and their effects on reporter expression are compared in the otherwise WT strain MG1363 (carrying a WT *zitR* allele). Twenty microliters of ZnSO₄ at 1 M (a lethal concentration) were streaked (vertical arrow) on chemically defined SA-agar medium to create a concentration gradient by diffusion (triangles). Lactococcal cells [MG1363(pVE8072) at the top and MG1363(pVE8074) at the bottom] were then cross-streaked on the same plate. After overnight growth, nuclease activity was revealed by a toluidine blue-agar overlay. In the presence of a truncated *zitR*_{ΔHTH} allele, the reporter gene is specifically derepressed near the deposit streak, and the full derepression area is indicated by arrows.

coli Zur protein (39), plasmid-encoded ZitR_{ΔHTH} could sequester chromosome-encoded WT ZitR into inactive heteromultimers (multimerization was indeed observed *in vitro* [see below]). These results suggest that *in vivo*, WT ZitR with an intact HTH domain is necessary for *zitp* repression by Zn(II).

Purified rZitR protein is a dimer that complexes divalent cations. We chose to purify a recombinant untagged ZitR form (rZitR), as the possibility that the His₆ tags of most purified AdcR proteins (1, 3, 34) could interfere with metal binding cannot be excluded. After overexpression in *E. coli* and purification in the presence of ZnCl₂, rZitR was first analyzed by gel filtration, revealing a major dimeric form and a minor tetrameric form (see Fig. S4 in the supplemental material). The rZitR protein was then subjected to ESI-MS analysis under denaturing or nondenaturing conditions (16, 42). Under denaturing conditions, the measured protein mass, 16,286.5 ± 0.5 Da, fit well with the predicted mass of the monomer (145 amino acids) after methionine deletion (16,285.6 Da). Under nondenaturing conditions, carefully controlled to preserve specific noncovalent interactions (i.e., by lowering the voltage of ion extraction and by increasing the pressure in the source-analyzer interface of the mass spectrometer to ~400 Pa), untreated rZitR was observed mainly as a mixture of three main dimeric forms, species C, D, and, in the majority, E (Fig. 5A), and also as minor tetrameric forms (data not shown). EDTA treatment was found to shift the spectrum toward slightly lighter forms, comprising uncomplexed dimer (species A at 32,569.9 ± 1 Da) and species B, in addition to the previous species D, E, and, in the majority, C (Fig. 5A). These results indicated that divalent cations were noncovalently bound to purified rZitR protein and could be displaced by EDTA, leading to less coordinated complexes. Interestingly, the presence of the uncomplexed dimer (species A) showed that divalent cation binding is not required for protein dimerization, although it could help the process (as *zitp* derepression in the presence of both *zitR*_{ΔHTH} and WT *zitR* alleles was observed only at high Zn(II) concentrations).

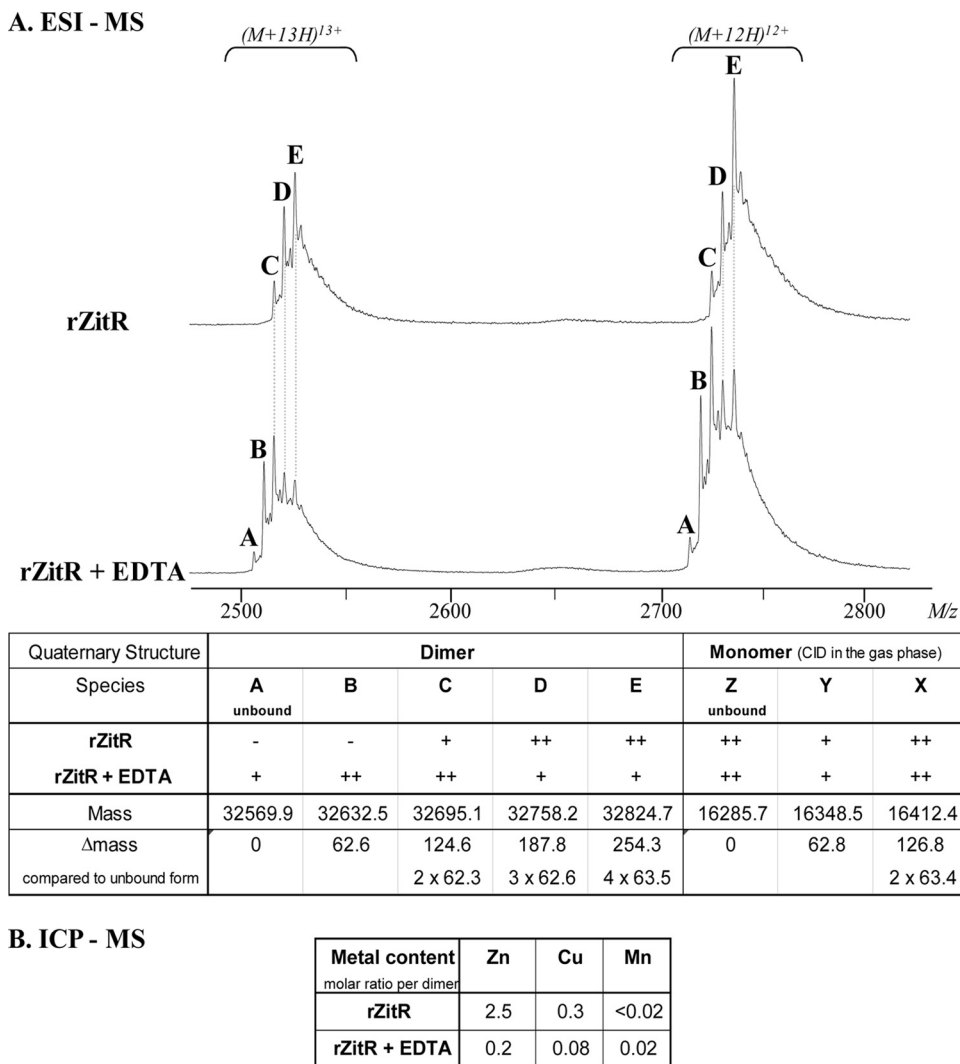


FIG. 5. Purified rZitR protein analysis: oligomerization and ligand binding. (A) ESI-MS analysis. At the top, only a window on the mass spectra obtained under nondenaturing conditions is presented, showing the 12 $[(M + 12H)^{12+}]$ and 13 $[(M + 13H)^{13+}]$ charge states of both untreated and EDTA-treated rZitR dimers. Up to five species (A to E, with species A being the uncomplexed dimer) could be detected, two of which (the lightest species, A and B) were specific to the EDTA-treated rZitR protein. The table summarizes all ESI-MS measurements, including collision-induced dissociation (CID) in the gas phase that allowed the detection of three monomeric forms (species Z, Y, and X). According to their masses, species A and Z are the uncomplexed dimer and monomer, respectively. The mass of each species (the average of the masses measured for both EDTA-treated and untreated proteins) is indicated, together with its apparent abundance in the spectrum (–, absent; +, present; ++, major peak). (B) ICP-MS analysis. Zinc, copper, and manganese (as a negative control) contents were measured for both untreated and EDTA-treated proteins, and the molecular ratios of metal per dimer were calculated.

Zinc cation is the metal ligand of rZitR protein. The mass differences between uncomplexed dimer (species A) on one hand and species B, C, D, or E on the other hand correspond to 1, 2, 3, or 4 times ~ 63 Da (Fig. 5A), suggesting that a unique divalent cation of ~ 63 Da can bind to the rZitR protein in up to four cations per dimer. In further experiments, gas phase dissociation of dimeric species (collision-induced dissociation measurement [Fig. 5A]) (45, 47) generated three monomeric species from both treated and untreated rZitR; compared to uncomplexed monomer (Z species), species Y and X display increased masses of 1 or 2 times ~ 63 Da, confirming the noncovalent binding of up to two divalent cations per monomer. The cation mass can be estimated from the average mass difference between all (dimeric and monomeric) consecutive

species (62.9 Da). Taking into account measurement precision (0.005%, i.e., 1.6 Da for the dimer) and the possibility of proton loss upon metal binding, this value fits the atomic mass of either Zn (65.4 Da) or Cu (63.5 Da), whereas the nearest neighbors in the periodic table, Ni (58.7 Da) and Ga (69.7 Da), are clearly excluded. All these results indicate that the rZitR protein was purified as a mixture of dimeric forms noncovalently bound to two to four Zn(II) or Cu(II) cations.

ICP-MS analysis was performed to determine the rZitR ligand, and Zn, Cu, and Mn contents were measured in the protein samples (treated or not with EDTA) used for ESI-MS analysis. The results are shown as molar ratios per dimer of purified protein (Fig. 5B). The Mn content remained at a background level, and a low Cu content could be detected in

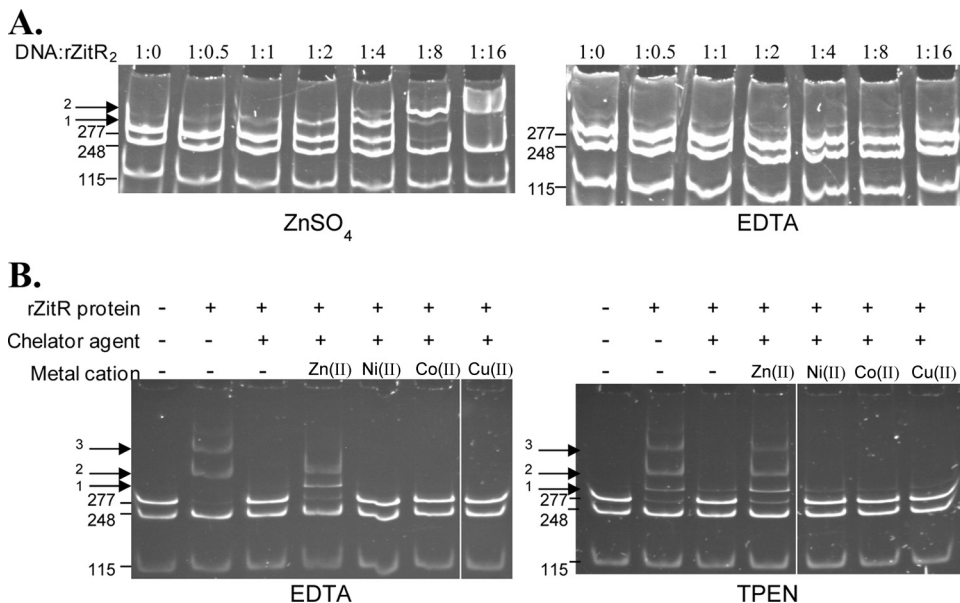


FIG. 6. rZitR DNA-binding activity and metal corepressor. A PCR product containing the *zitp* promoter was digested by AluI and incubated with purified rZitR protein. The sizes of restriction fragments according to a molecular size marker are indicated next to the gels. Only the 277-bp fragment containing intact *zitp* and the 248-bp and 115-bp fragments (negative controls) are shown (Fig. 7B shows smaller fragments). (A) DNA binding activity. ZnSO₄ (0.1 mM) (left) or EDTA (1 mM) (right) was preincubated with purified rZitR protein before the addition of AluI-digested *zitp*-containing DNA (80 ng; 21 nM) and was also added to the electrophoresis buffer. rZitR protein was added in doubling amounts (in a dimer concentration range from 10.5 nM to 336 nM), and molar ratios between DNA and a protein dimer (rZitR₂) are indicated. On the left, two shifted bands are marked by solid arrows and numbered (1 and 2). For the highest rZitR quantity (a ratio of 1:16), the observed smear starting from the second shifted band could be due to protein oligomerization. (B) rZitR corepressor. EMSA was essentially as for Fig. 6A, except that (i) ZnSO₄ was not added during the incubation or electrophoresis step, (ii) less DNA (50 ng; 13 nM) was used, (iii) purified rZitR protein was added at a unique molar ratio between DNA and a protein dimer of 1:8 (i.e., 104 nM dimer), and (iv) slightly different migration conditions were used. Before being added to DNA for the binding assay, the purified rZitR protein was first pretreated or not with 1 mM EDTA (left) or 0.1 mM TPEN (right) to inactivate it; the presence (+) or absence (-) of rZitR protein and of the chelator agent in the binding reactions is indicated. Inactivated rZitR protein was then incubated or not (-) with the following metal cation salt: ZnSO₄ (Zn(II)), CoCl₂ (Co(II)), CuSO₄ (Cu(II)), or NiCl₂ (Ni(II)). The metal cation salts were added in slightly limiting amounts compared to the chelator agent, i.e., 0.9 mM and 90 μM for EDTA and TPEN, respectively (similar results were also obtained when metal cation salts were added at 0.8 mM and 80 μM, respectively). All shifted bands are marked by solid arrows and numbered (a third shifted band was observed here in contrast to panel A under slightly different migration conditions).

untreated rZitR. Zn was clearly the most abundant metal atom of untreated rZitR, with more than two Zn atoms per untreated rZitR dimer, in agreement with the ESI-MS results (Fig. 5A), and its level was drastically (>12-fold) reduced by EDTA treatment. These results demonstrate that purified rZitR dimer is mainly bound to zinc atoms.

rZitR DNA-binding activity. An EMSA was performed on *zitp*-containing PCR DNA digested by AluI (Fig. 6A). In the presence (Fig. 6A, left) or absence (Fig. 6B, second lane from the left in both gels) of added ZnSO₄, rZitR specifically affected the migration of the 277-bp fragment containing *zitp*, in contrast to all other AluI fragments (Fig. 6 and 7). Thus, rZitR had been purified as an active DNA-binding protein able to specifically recognize the *zitp*-containing fragment, and as purified rZitR was shown to be a mixture of three dimeric forms complexing two to four Zn(II) [species C to E, i.e., rZitR₂-Zn(II)₂ to rZitR₂-Zn(II)₄], at least one of them should be endowed with DNA-binding activity.

A first faint shifted band appeared as soon as rZitR was added to *zitp*-containing DNA and increased in intensity as the quantity of rZitR dimer compared to DNA was progressively increased up to 4-fold (Fig. 6A, left). At that point, a second shifted band appeared. The presence of two shifted bands suggested that rZitR bound two operator sites in the *zitp* re-

gion. When the rZitR quantity was further doubled to reach an 8-fold excess compared to DNA, the second shifted band became dominant, and the 277-bp fragment almost completely disappeared. This profile suggested that 50% of *zitp*-containing DNA could be bound by the rZitR dimer when it was present in ~3- to 4-fold excess, allowing the apparent dissociation constant for the DNA-binding reaction to be estimated at ~50 to 70 nM. Finally, EDTA completely inactivated rZitR (Fig. 6A, right), showing that rZitR DNA-binding activity required divalent cations.

The metal ligand necessary for rZitR DNA-binding activity. The nature of the metal ligand necessary for rZitR activity was examined. Four metal cations, Zn(II), Cu(II), Ni(II), and Co(II), were tested for the ability to restore the DNA-binding activity of rZitR that had been pretreated by EDTA or TPEN [*N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine], another chelating agent (6) widely used in studies of zinc-binding regulators (36, 37), but not in the case of the *S. pneumoniae* AdcR protein (1). Both EDTA (Fig. 6B, left) and TPEN (at a lower concentration) (Fig. 6B, right) were able to abolish rZitR DNA-binding activity. Metal cations were subsequently added to inactivated rZitR. When the metal cations were added in limiting amounts compared to the chelator agent (Fig. 6B), full rZitR activity could be restored only in the

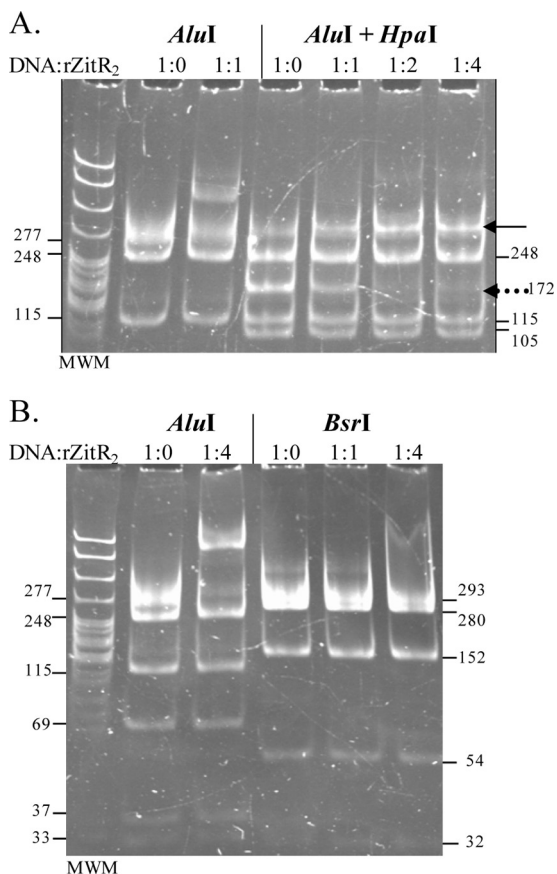


FIG. 7. rZitR operator sites. The role of TTAACYRGTTAA palindromes was tested using HpaI and BsrI restriction enzymes that cut inside one or both of them, respectively (Fig. 1). *zitp*-containing DNA was digested by both HpaI and AluI (A) or by BsrI alone (B), using AluI simple digestion as a control. EMSA was essentially as for Fig. 6A, left, except that (i) ZnSO₄ was not added during the incubation or electrophoresis step and (ii) 60 ng of DNA (15 nM) was used. On the right, the 172-bp AluI HpaI fragment is indicated by a dotted arrow, while the single shifted band is shown by a solid arrow. No shift was observed for the 105-bp AluI HpaI fragment and the BsrI fragments. MWM, molecular weight marker.

case of Zn(II), confirming that Zn(II)-loaded rZitR, like untreated rZitR (Fig. 6B) that complexes Zn(II) (Fig. 5), is an active DNA-binding protein. This result showed that rZitR was able to compete with either EDTA or TPEN for Zn(II) binding and that its affinity for Zn(II) was higher than that of TPEN, i.e., more than 10^{15} M^{-1} (35). In the course of these experiments, a third shifted band appeared, possibly revealing a higher activity of rZitR (Fig. 6B). This suggested either that rZitR, when bound to both palindromes, was able to tetramerize (tetramerization was indeed observed during gel filtration and ESI-MS assays [see above]), thus bending DNA and affecting its migration, or, alternatively, that a third weak binding site might be present. Finally, the *in vitro* observation that, after inactivation by two chelator agents, Zn(II) is the only metal cation able to restore full rZitR activity, combined with our *in vivo* data, suggests that Zn(II) is the physiological corepressor of the ZitR protein.

rZitR operator sites. In our EMSA experiments (Fig. 6), the appearance of two new bands when rZitR was added to a

zitp-containing DNA was in agreement with the proposal that ZitR could bind the two TTAACYRGTTAA palindromes present in the *zitp* sequence (38). To test this hypothesis, the HpaI or BsrI enzyme was used to disrupt one or both palindromes (Fig. 1B) before EMSA. HpaI and AluI double digestion allowed a migration shift for only one of the two fragments (172 bp) obtained from the *zitp*-containing fragment of 277 bp (Fig. 7A). This indicated that, as expected, only one ZitR binding site was intact and that one palindrome was sufficient for rZitR binding. BsrI digestion impeded rZitR binding under conditions leading to a clear shift of the 277-bp fragment (compare the 6th and 3rd lanes from the left in Fig. 7B). These results demonstrate that the integrity of both palindromes is necessary for rZitR binding on *zitp* and confirm that they constitute the ZitR binding sites.

DISCUSSION

The physiological significance of *zit* regulation by extracellular zinc. The *L. lactis zit* promoter, *zitp*, was shown here to be fully repressed by extracellular Zn(II) in a wide concentration range, from high (including sublethal) to moderate (above 2 μM) concentrations, and to be induced at very low concentrations (below 10 nM), indicating that the repression threshold is low. It was indeed estimated to be around (slightly above) 100 nM (reference 28 and data not shown). At 2 μM extracellular Zn(II) (and above), the high cellular Zn content suggests that Zn(II) uptake could be achieved by another, unidentified system (Fig. 8A), as is the case in *E. coli* (4, 19) and *B. subtilis* (17) (for reviews, see references 5 and 33). Thus, rather than ensuring resistance to Zn(II) toxicity, *zit* repression could allow energy to be saved by preventing the synthesis of useless Zit proteins under Zn(II) depletion (Fig. 8A). The main physiological significance of *zit* regulation could be an emergency response to zinc scarcity and starvation, as zinc scavenging by the induced high-affinity ZitSQP transporter could allow cell survival under Zn(II) depletion (Fig. 8B).

The low regulation threshold appears to be physiological, as *L. lactis* can encounter zinc-poor media. Milk, a natural ecological niche for lactococci, has a soluble zinc content of only 0.3 nM (52). Also, during growth, extracellular Zn(II) could be progressively depleted, as supported by the decrease in the cellular Zn content observed here in a synthetic medium (28). In streptococcal pathogens, a μM range threshold for *adc* regulation could also be physiological. In *S. pneumoniae*, an *adc* mutant cannot grow on defined medium unless 0.8 μM ZnSO₄ is added (13). This suggests that *adc* genes for the Zn(II) uptake system are expressed only in less than 0.8 μM Zn(II), and their expression actually did not respond to Zn(II) addition in a rich medium initially containing 8 μM Zn(II) (24). Zinc exhaustion conditions can often be encountered in hosts, and the induction of Adc uptake systems could favor colonization. An increased risk of acute respiratory tract infections by *S. pneumoniae* is associated with zinc deficiency in children and can be reduced by zinc administration, and mice kept on a zinc-deficient diet show more extensive pneumococcal colonization in the nasal mucosa (46). Furthermore, as AdcR regulates genes encoding surface-exposed proteins involved in the invasion of Zn(II)-poor tissues (compared to blood), repression of these genes in response to elevated

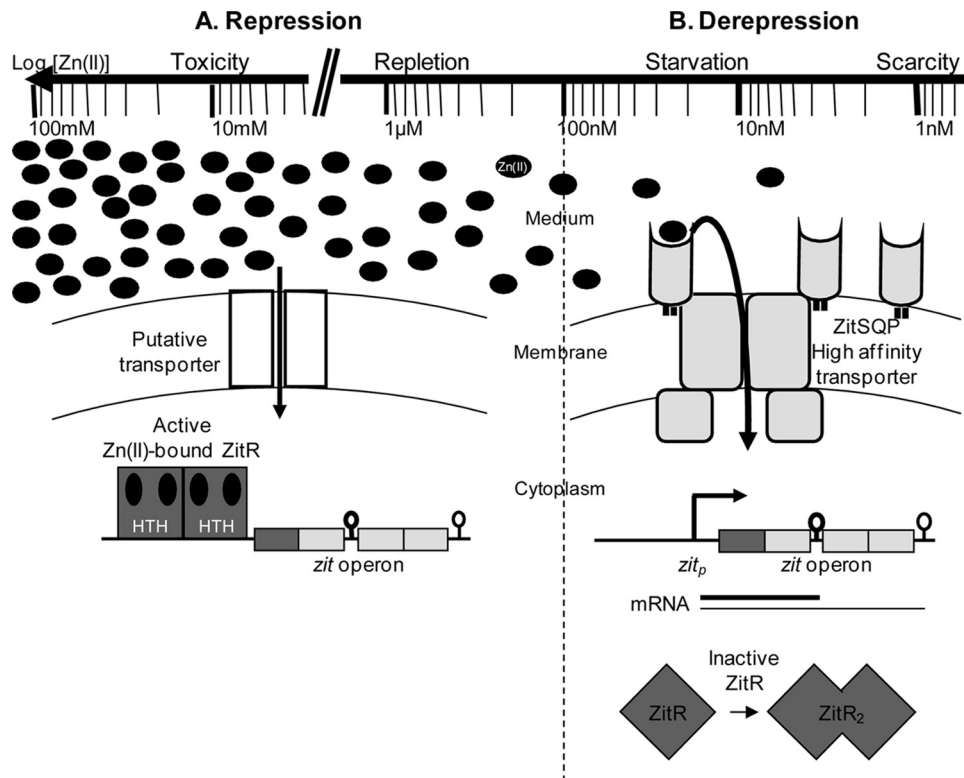


FIG. 8. Model for *zit* regulation by Zn(II). Extracellular concentrations of soluble Zn(II) are shown at the top on a logarithmic scale (large horizontal arrow), and the estimated regulation threshold (100 nM) is indicated by a dashed vertical line. (A) Repression. In a wide concentration range above the threshold, from repletion to profusion and finally toxicity, repression is achieved by active holo-ZitR proteins able to bind the *zitp* promoter (active Holo-ZitR is shown as a Zn(II)₄-ZitR₂ complex, but other forms could also be active). As *zit* repression by Holo-ZitR is effective under repletion conditions, it could be a way for the cells to save energy. If another Zn(II) uptake system existed in *L. lactis*, as is the case in *E. coli* and *B. subtilis* (see Discussion), under toxic conditions, *zit* repression would not be sufficient to ensure resistance to Zn(II). (B) Derepression. Under Zn(II) deprivation, inactive ZitR forms that have lost their allosteric ligands appear. Under prolonged or extreme Zn(II) deprivation, or because of neosynthesis, apo-ZitR appears (as, in particular, the unbound ZitR₂ dimer, although other forms could be inactive). Inactive ZitR forms could ensure continual sensing of intracellular Zn(II) and fast repression in response to a sudden Zn(II) increase. However, the main significance of *zit* regulation could be to allow efficient adaptation to zinc scarcity: *zit* induction under extreme Zn(II) deprivation could be an emergency response to allow zinc scavenging by the high-affinity Zit transporter and to ensure cell survival.

Zn(II) could allow their expression to be limited in the early stages of infection, when they are needed (7, 38). Finally, ZitR/AdcR regulators seem to play an important role in adaptation to the environment, including the host for pathogenic species.

Unbalanced expression of *zit* genes. As the *zit* operon is weakly transcribed compared to *zitRS* genes, probably because of the stem-loop structure downstream of *zitS*, there could be an excess of ZitS lipoprotein compared to the ZitPQ proteins (Fig. 8B). Such a transcriptional regulation involving an internal stem-loop structure was first described for the *mtsABC* metal uptake operon in *Streptococcus pyogenes* (21). It was proposed that large amounts of the binding lipoprotein MtsA at the cell surface could serve as a reservoir of metal cations while awaiting contact with the less available MtsBC proteins embedded in the membrane (21). Another gene involved in Zn(II) binding, the *E. coli znuA* gene, which is adjacent to but divergent from the *znuBC* operon, is 2-fold more upregulated than the *znuB* and *znuC* genes under Zn(II) depletion conditions (18). Interestingly, the *adcA* genes in *Streptococcus mutants* and *S. pyogenes* are orphan genes (29, 38). It is conceivable that the lack of selective pressure to maintain all the genes

involved in Zn(II) ABC uptake in an operon might be related to the need for higher expression of the gene encoding the binding protein to improve transport.

The *L. lactis* ZitR protein is a zinc-responsive repressor. The HTH domain of the *L. lactis* ZitR protein was found to be required for *zitp* repression. In the case of the *E. coli* Zur repressor, the *zurΔ46-91* allele was identified from a genomic library cloned on a plasmid by its ability to derepress a reporter fusion to *znuA* in an otherwise *zur*⁺ strain (39, 40). Purified ZurΔ46-91 protein failed to bind the *znu* operator site, thus defining amino acids 46 to 91 as part of the Zur DNA-binding domain (39). When mixed, WT and mutant proteins could form heterodimers, highly suggesting that the formation of inactive heterodimers was responsible for reporter derepression *in vivo* (39). Similarly, in the case of ZitR, reporter derepression in the presence of both plasmid-borne *zitR*_{ΔHTH} and chromosomal WT *zitR* alleles, and the fact that rZitR protein was purified as dimeric forms endowed with DNA-binding activity, supports the model that truncated ZitR_{ΔHTH} protein could titrate WT ZitR protein into inactive heterodimers. Furthermore, in the WT active homodimer, both C-terminal HTH domains should be necessary for stable DNA binding. The fact

that full induction was observed only at high Zn(II) concentrations suggests both that heterodimer stability could be improved at high Zn(II) concentrations and that at moderate Zn(II) levels, some WT homodimers could lead to residual repression.

Here, ZitR protein was one of the first members of the AdcR metalloregressor group to be characterized *in vitro*: purified rZitR protein was found to be a dimer able to bind up to four Zn(II) cations. Only two AdcR proteins were previously purified (1, 3, 44), and the *S. pneumoniae* AdcR protein has only recently been characterized as a dimer bound to three to five Zn(II) cations (44). An even number of bound cations is generally observed for dimeric forms of metalloregulators, in agreement with symmetric binding of both monomers (30, 44). In the case of the rZitR protein, Zn(II) was shown to be required to restore full DNA-binding activity after protein inactivation by two chelator agents, suggesting that Zn(II) might exert an allosteric effect on DNA-binding activity. Although the activity of each individual dimeric form was not assessed here, we found that purified rZitR protein was active, meaning that among its three dimeric forms that complex two to four Zn(II), at least one is active [in Fig. 8A, active ZitR is shown as species E or Zn(II)₄-ZitR₂]. In the future, it would be interesting to test whether the ZitR protein, like the *E. coli* Zur protein, could bind two structural and two regulatory Zn(II) per dimer (37). If a similar model also applies to the ZitR protein, the dimeric forms that bind up to two Zn(II) per dimer [up to species C or Zn(II)₂-ZitR₂] could be inactive. In particular, ZitR proteins newly synthesized under Zn(II) deprivation should rapidly dimerize into an inactive apo-ZitR form (species A or ZitR₂) that could ensure continual sensing of intracellular Zn(II) (Fig. 8B) and fast repression resetting once Zn(II) again became available (Fig. 8A). Our data, in particular the low regulation threshold, the correlation between the intracellular Zn content and the *zitp* repression level, and the high affinity of rZitR for Zn(II), suggest that ZitR, like Zur (35), could be a sensitive sensor of intracellular Zn(II) to efficiently respond to Zn(II) variations in the environment.

Why do *Streptococcaceae* use ZitR/AdcR metalloregulators rather than Zur proteins to control zinc uptake? The *L. lactis* zinc-responsive ZitR repressor is a model of ZitR/AdcR metalloregulators that are specific to the *Streptococcaceae* group (10, 30, 38). Remarkably, in *S. pneumoniae*, Zn(II) efflux by the cation diffusion facilitator CzcD is not controlled by the CzcA regulator (ArsR-SmtB family), as in *B. subtilis* (31), but by SczA, the first described metalloregulator of the TetR family (23). It is quite interesting from an evolutionary point of view that *Streptococcaceae* seem to have peculiar metalloregulators to control all Zn(II) homeostasis aspects. The streptococcal specificity for ZitR/AdcR proteins is particularly intriguing, as they appear to be the functional analogs of Zur regulators, fulfilling the same physiological function and sharing the same biochemical properties. *In vivo*, both Zur (25, 39, 40) and the ZitR/AdcR proteins allow zinc acquisition when cells are starved for zinc. *In vitro*, for both *E. coli* Zur (37, 39) and *L. lactis* ZitR proteins, DNA-binding activity requires Zn(II) bound in up to two cations per monomer and the HTH domain, whereas Zn(II) is not required for protein dimerization.

Despite their similarities, there is a major difference between Zur and the ZitR/AdcR proteins: whereas Zur has four

conserved cysteine residues, including two probably involved in Zn(II) binding, by homology to Fur proteins (Fig. 2B) (37, 39), the ZitR/AdcR proteins have a single one (30) (Fig. 2A), indicating that most, if not all, Zn(II)-binding sites [as the cysteine residue in *S. pneumoniae* AdcR protein was shown not to be required for Zn(II) binding (44)] involve only acidic or histidine residues. ZitR/AdcR proteins exemplify cysteine exclusion in the proteomes of aerobic *Firmicutes* (11) and, in particular, of lactococci and streptococci (see the supplemental material in reference 11; R. Daniels, personal communication). The difference in cysteine contents between Zur and the ZitR/AdcR proteins could be crucial for their folding and activity. *In vitro*, the *E. coli* Zur protein, which contains nine cysteine residues in total, is highly sensitive to oxidation that abolishes both Zn(II) and DNA binding, and the latter can be restored only by DTT treatment (39). On the other hand, rZitR DNA-binding activity was found to be insensitive to DTT (data not shown). *In vivo*, protein sensitivity to oxidation could be a problem for bacteria that encounter highly oxidative environments and/or whose cytoplasm is not reducing enough to deal with incorrect disulfide bonds, like *Streptococcaceae*. Pathogenic streptococci colonize and infect oxygen-exposed tissues, like the epidermis and the respiratory tract, and cysteine exclusion was proposed to provide such bacteria with a selective advantage (11). In *L. lactis*, most strains are not able to synthesize glutathione, one of the two major systems for disulfide bond reduction (27). Whether *Streptococcaceae* could need ZitR/AdcR instead of Zur as a Zn(II)-responsive repressor more resistant to oxidation and better suited either to a highly oxidative environment or to a poorly reducing cytoplasm deserves further investigation.

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