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The Adc/Lmb System Mediates Zinc Acquisition in *Streptococcus agalactiae* and Contributes to Bacterial Growth and Survival

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ABSTRACT

The Lmb protein of *Streptococcus agalactiae* is described as an adhesin that binds laminin, a component of the human extracellular matrix. In this study, we revealed a new role for this protein in zinc uptake. We also identified two Lmb homologs, AdcA and AdcAII, redundant binding proteins that combine with the AdcCB translocon to form a zinc-ABC transporter. Expression of this transporter is controlled by the zinc concentration in the medium through the zinc-dependent regulator AdcR. Triple deletion of *lmb*, *adcA*, and *adcAII*, or that of the *adcCB* genes, impaired growth and cell separation in a zinc-restricted environment. Moreover, we found that this Adc zinc-ABC transporter promotes *S. agalactiae* growth and survival in some human biological fluids, suggesting that it contributes to the infection process. These results indicated that zinc has biologically vital functions in *S. agalactiae* and that, under the conditions tested, the Adc/Lmb transporter constitutes the main zinc acquisition system of the bacterium.

IMPORTANCE

A zinc transporter, composed of three redundant binding proteins (Lmb, AdcA, and AdcAII), was characterized in *Streptococcus agalactiae*. This system was shown to be essential for bacterial growth and morphology in zinc-restricted environments, including human biological fluids.

Streptococcus agalactiae (group B streptococcus [GBS]) is a Gram-positive commensal bacterium of the human gastrointestinal and uro-genital tracts. GBS carriage is mostly asymptomatic in healthy adults, and this bacterium is detected in the vagina of approximately 30% of pregnant women. Maternal carriage is the main source of transmission to neonates, in which *S. agalactiae* can cause invasive infections (pneumonia, septicemia, and meningitis), with an overall mortality rate of approximately 10% (1, 2). GBS is also an emergent pathogen among the elderly and in adults with underlying diseases (1, 3).

The ability of GBS to colonize different niches and cause infection is multifactorial, and many virulence-associated proteins have been identified (4, 5). Binding of GBS adhesins to components of the extracellular matrix constitutes a crucial first step in the process of infection (6–9). Among them, the Lmb protein has been identified as a GBS receptor for laminin, a glycosylated multidomain protein found in all human tissues (10). The gene encoding Lmb is located on a transposon with the *scpB* and *sht* genes, which encode a C5a peptidase and a histidine triad protein, respectively (11, 12). The *lmb* promoter region is a hot spot for the integration of two mobile genetic elements. One of them is associated with increased expression of *lmb*, resulting in increased binding of strains harboring the transposon to laminin (13). It has also been shown that Lmb may promote bacterial invasion in human brain microvascular endothelial cell lines (14).

Lmb is clustered by sequence homology as a metal-binding receptor. Indeed, Lmb has strong homology with the zinc-binding proteins AdcA and Lbp of other streptococcal species (15, 16). The crystal structure of Lmb has also been resolved and revealed the presence of a bound zinc in a metal-binding crevice (17, 18). Until now, studies have only been focused on its function as an adhesin,

despite evidence suggesting that Lmb may be involved in zinc uptake.

Zinc (Zn^{2+}) is a trace element that is essential for most living cells. It is a cofactor for a number of essential prokaryotic enzymes and transcriptional regulators (19). In the human body, the zinc concentration varies from 1.5 μM in cerebrospinal fluid to over 100 μM in lung tissue (20, 21). Pathogenic bacteria must adapt zinc transport mechanisms to accommodate these differences to both avoid toxicity and meet their requirements for this metal. An important determinant of resistance against elevated levels of Zn in *Streptococcus pneumoniae* is the CzcD-SczA system, which is conserved among *Streptococcus* species (22). In contrast, during starvation zinc acquisition in streptococci is mostly performed by an ATP-binding cassette (ABC) transporter. It is composed of one or several metal-binding proteins (AdcA, Lbp, or Lmb), an integral membrane component (AdcB), and an ATPase (AdcC) (21). The streptococcal AdcR repressor, a MarR family regulatory protein, is involved in the regulation of zinc uptake genes (23). In the

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presence of adequate intracellular zinc concentrations, AdcR binds to its target genes and inhibits their expression. AdcR repression is relieved during zinc starvation, allowing bacteria to efficiently adapt the expression of zinc acquisition systems to their needs for this metal (24–27).

In several pathogenic streptococci, deletion of one or several components of the Adc zinc-ABC transporter results in lower growth under zinc-restricted conditions as well as decreased virulence, adhesion, and biofilm formation, underlining the importance of zinc metabolism during colonization and infection (16, 21, 28–30). Concerning *S. agalactiae*, the role of zinc is still poorly documented, and no zinc transporter has yet been characterized.

In this study, we show that Lmb, together with the AdcA, AdcAII-binding proteins, and the AdcCB translocon, compose a zinc transporter. The expression and the role of this transporter in the bacterium's physiology were examined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The reference wild-type (WT) GBS strain used in this study was strain A909, a sequence type 7 (ST7), clonal complex 7 (CC7), serotype IA clinical isolate from a human case of septicemia. The *Escherichia coli* and *S. agalactiae* strains used in this study are listed in Table S1 in the supplemental material. *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (catalog number 1005317; MP, Solon, OH, USA) at 37°C with agitation (220 rpm) or on LB agar plates (1.5% agar). *S. agalactiae* strains were routinely grown in Todd-Hewitt (TH) broth (catalog number T1438; Sigma-Aldrich, St. Louis, MO) at 37°C without agitation and on TH agar or brain heart infusion agar (BHI agar; catalog number EAB140102; AES, Bruz, France). When necessary, *E. coli* and *S. agalactiae* strains were grown with erythromycin (150 µg/ml for *E. coli* and 10 µg/ml for *S. agalactiae*).

Growth of bacteria in chemically defined medium. *S. agalactiae* strains were cultured in a liquid chemically defined medium (CDM; 8.3 g/liter Dulbecco's modified Eagle medium base [catalog number D5030; Sigma-Aldrich], 1× BME vitamins, 74 µM adenine, 89.2 µM uracil, 65.7 µM xanthine, 66.2 µM guanine, 1123.5 µM D,L-alanine, 757 µM L-asparagine, 1,127 µM L-aspartic acid, 684.5 µM L-glutamine, 1,019.5 µM L-glutamic acid, 868.6 µM L-proline, 734.9 µM L-tryptophan, 4,125.4 µM L-cysteine, 12 µM lipoic acid, 1 µM pyruvate; pH 7.4). All CDM components were from Sigma-Aldrich.

To obtain zinc-restricted CDM, 500 µM EDTA (catalog number E5134; Sigma-Aldrich, St. Louis, MO), a chelating agent, was added to remove residual traces of metal ions. Addition of EDTA had no discernible effect on *S. agalactiae* growth compared to growth in CDM without EDTA or growth in CDM with a mix of metals (17.4 µM ZnSO₄, 10.5 µM CoCl₂, 0.4 µM CuSO₄, 147.9 µM MnSO₄).

Bacterial growth experiments were performed in 96-well microtiter plates (Greiner Bio-One; Cellstar) (300-µl culture volume). A bacterial overnight culture grown in TH was used to inoculate a zinc-restricted CDM culture (optical density at 600 nm [OD₆₀₀], 0.005) grown for 8 h that was then used to inoculate wells (OD₆₀₀, 0.005) containing zinc-restricted CDM with various ZnSO₄ concentrations (from 0 to 100 µM). Before each inoculation, cells were pelleted and washed in Milli-Q water. Plates were incubated for 18 h at 37°C in an Eon thermo-regulated spectrophotometer plate reader (BioTek Instruments). The OD₆₀₀ was measured every hour after double orbital shaking of the plate for 5 s.

Chromosomal and plasmidic DNA purification. Chromosomal DNA of *S. agalactiae* cultured overnight without agitation in TH (37°C) was purified as previously described (31). *E. coli* plasmids were purified with a NucleoSpin plasmid kit (Macherey-Nagel) according to the manufacturer's instructions. The DNA concentration was measured with a NanoDrop Lite spectrophotometer (Thermo Scientific). The ratio of absorbance at 260 nm and 280 nm was used to assess purity.

DNA sequencing. PCR products purified with the NucleoSEQ kit (Macherey-Nagel) were sequenced on both strands by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the ABI Prism 310 genetic analyzer.

PCR. Oligonucleotides (Eurogentec and Sigma-Aldrich) used in this study are listed in Table 1. Analytical PCR used standard OneTaq polymerase (New England BioLabs [NEB]) and PCR for cloning or sequencing was carried out with Q5 high-fidelity DNA polymerase (NEB). The resulting PCR fragments were purified with a NucleoSpin gel and PCR cleanup kit (Macherey-Nagel), according to the manufacturer's instructions.

RNA extraction. Total RNA was extracted from mid-exponential-phase cells (OD₆₀₀ of 0.5) growing in CDM. The bacteria were lysed mechanically with glass beads in a FastPrep-24 instrument, and total RNAs were extracted with a phenol/TRIzol-based purification method previously described (32). The concentration and purity of RNA were assessed with a NanoDrop Lite spectrophotometer (Thermo Scientific) and analysis of the A₂₆₀/A₂₈₀ ratio. A DNase (Turbo DNA-free DNase; Ambion) treatment of the purified RNAs was then realized. Control PCR mixtures, with 50 µg of purified RNAs, were performed to check for DNA contamination. (Primer pairs are detailed in Table 1.)

Reverse transcription and quantitative reverse transcriptase PCR. For reverse transcription and quantitative reverse transcriptase PCR (qRT-PCR), the RNAs were reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Primers were selected with Primer3web software (<http://bioinfo.ut.ee/primer3/>) in order to design 100- to 200-bp amplicons (Table 1 lists the primer sequences). qRT-PCRs were performed in a 20-µl reaction volume containing 40 ng of cDNA, 0.5 µl of gene-specific primers (10 µM), and 1× LightCycler 480 SYBR green I mix (Roche). PCR amplification, detection, and analysis were performed with the LightCycler 480 PCR detection system and LightCycler 480 software (Roche). PCR conditions included an initial denaturation step at 95°C for 5 min, followed by a 40-cycle amplification (95°C for 10 s, 60°C for 20 s, and 72°C for 20 s). The specificity of the amplified product and the absence of primer dimer formation were verified by generating a melting curve (65°C to 98°C, continuous increase). The crossing point (C_p) was defined for each sample. The expression levels of the tested genes were normalized using the *gyrB* (primers OLM021 and OLM022) or *recA* (primers OLM321 and OLM322) genes ("reference genes" in the first equation below) of *S. agalactiae* as internal standards whose transcript levels did not vary under our experimental conditions (see Table 1 for the primer sequences). The fold change in the transcript level was calculated using the following equations: $\Delta C_p = C_{p(\text{target gene})} - C_{p(\text{reference gene})}$; $\Delta\Delta C_p = \Delta C_{p(\text{reference condition})} - \Delta C_{p(\text{test condition})}$; ratio = $2^{-\Delta\Delta C_p}$. Each assay was performed in duplicate and repeated with at least three independent RNA samples.

5'-RACE PCR. *S. agalactiae* strain A909 was grown in zinc-restricted CDM at 37°C without agitation until the mid-exponential phase of growth (OD₆₀₀, 0.5). Total RNAs of growing cells were extracted as described above, and the 5' end of the *lmb-sht* operon mRNA was determined using a 5'/3' rapid amplification of cDNA ends (RACE) PCR kit (second generation; Roche Applied Science). Two *lmb* antisense specific primers (OAH068 and OAH069) were designed (Table 1) to produce cDNAs. Control RT-PCRs, omitting reverse transcriptase, were performed to check for DNA contamination of the RNA preparation with appropriate primers (Table 1).

Construction of *lmb*, *adcA*, *adcAII*, *adcCB*, and *adcR* deletion mutants. *S. agalactiae* A909Δ*lmb* is a nonpolar mutant of strain A909 with a deletion of the entire coding sequence of *lmb* (*sak*₁₃₁₉) that was achieved via allelic exchange. Upstream and downstream flanking regions of *lmb* were amplified by PCR with primer pairs OAH024/OAH025 and OAH026/OAH027, respectively. A recombination cassette, consisting of a fusion between these two regions, was obtained by using splicing-by-overlap extension PCR with primers OAH024 and OAH027 (Table 1). To

TABLE 1 Oligonucleotides used in this study

Primer use and name	Sequence ^a
Deletion of <i>adcR</i>	
OAH003	TTAAT CGAATT CGGAATCTTCAAATGGACTATG
OAH004	ATGATTACCCTTCTAGTTCTAAAACTGTCATATA TACCTC
OAH005	TGACAGTTTTAGAACTAGAAGGGTAATCATGCGA TAT
OAH006	ACATT GGGAT CCCCGCCATGGTAAAGATTGGTTCC
Deletion of <i>adcCB</i>	
OAH074	ATGACAG AATT CGAACAAAAATTAGACCATTAG TGAG
OAH015	TGCGAAGCAAGCCTACGCATGATTACCCTTCTAA TTCTCT
OAH016	AAGGGTAATCATGCGTAGGCTTGCTTCGCAAACG TTAA
OAH075	ATACTT GGAT CCCAGATACAAATAATGTAGCTC CCC
Deletion of <i>lmb</i>	
OAH024	AGGCT GGAATT CTGGAAGGCGCTACTGTTCC
OAH025	CTCCTTTACTTCAACCCTTTTTCATAGTACCTC CTCAATT
OAH026	TACTATGAAAAAGGGTTGAAGTAAAGGAGATT ATTAGTGAAG
OAH027	GGCTT GGGAT CCAGCTAGCTCACTTGGAGAC
Deletion of <i>adcAII</i>	
OAH040	AAAAT CGAATT CCAACGTGTTAATCAAGCAAGTG
OAH062	TAAG GTACCT CCGTATCCTTTTCATTAAACCTCC
OAH063	TTAG GTACCT AGGTAGTTATATAAAGAAAGGACG
OAH043	TACTA AGGAT CCATCTCCCATGTCATCAATGAC
Deletion of <i>adcA</i>	
OAH086	AATCGT GGTAC CGCAGCTCTAGCAGATCCACAC
OAH076	ATA AAAGCTT GAAATTTCTTCTCATTTTTTCTCC
OAH077	ATGA AGCTT CATTAATATTAAAGATGATAT CGG
OAH087	CATCC AGGAT CCCGTCCAGTTGTTTTCTTAGA TAC
Complementation of <i>lmb</i>	
OAH115	TAAAT AGATCT GGAGGTAATGAAAAA
OAH102	TAATA CTGCAGT TACTTCAACTGTTGATAGAGC
Complementation of <i>adcAII</i>	
OAH119	AAAATA AGATCT GGAGGTTAATGAAAAGG
OAH104	TTATAT CTGCAGT ATTGATTTAACGATTG
Complementation of <i>adcA</i>	
OAH120	GTAAAA AGATCT GGAGGAAAAATGAGAAAG
OAH121	ATCTTT CTAGAT TAATGAGACATAAGGTC
Transcriptional fusion	
OAH122	TAGTTA GAATC CTTTCTTCTTGGGATTAGTAGC
OAH009	TT CGAT CCCCTCTCAATTATAATTAAACAGT TATTAAC

TABLE 1 (Continued)

Primer use and name	Sequence ^a
OAH034	GGTCT CGCCAGTTAATTTTACTCCTTTATCAA TGC
OAH035	GGTCT CGCTGGCCCATAACTGGTTAAATTATAA TTGAGG
OAH022	GGTCT CGCCCCCAGTTATTAACCAAGTTAA
OAH023	GGTCT CGGGGGATTATAATTGAGGAGGTACTATG
5'-RACE PCR	
OAH068	GCACCTGATTGGATCATCCTCAC
OAH069	GCTGGTTACAACGACATGCCTTG
qRT-PCR	
OAH053 <i>lmb</i>	TAGTAATGATAGCAGGGTGTGATAAGTC
OAH054 <i>lmb</i>	AGATACTTCTTTTGTTCATCGCATACATT
OAH047 <i>adcA</i>	TTGGGATTACAAAGCTAAATCTAAAAA
OAH048 <i>adcA</i>	ATCTTGATTGATTCTACGTCAGTCTTG
OAH051 <i>adcAII</i>	GCATAAAATCATAGGAAAGCATATCAA
OAH052 <i>adcAII</i>	GCTAAGTATGAGAATGCTGTATGTGAAG
OLM321 <i>recA</i>	CTGGTGGTCGTGCTTTGAAA
OLM322 <i>recA</i>	TATGCTCACCAGTCCCCTTG
OLM021 <i>gyrB</i>	GCTCACATCAGAAGCTTACTTTTAACTC
OLM022 <i>gyrB</i>	TTTAATCTCACTTCTACTTTGACACC

^a Added restriction site sequences are shown in bold.

carry out chromosomal gene inactivation, appropriate PCR fragments (overlap extension) were cloned into the EcoRI/BamHI restriction sites of the thermo-sensitive shuttle plasmid pG+host1 (see Table S1 in the supplemental material).

The same cloning strategy was applied to obtain the Δ *adcA* (*sak_0685*), the Δ *adcAII* (*sak_1898*), the Δ *adcCB* (*sak_0218* and *_0219*), and the Δ *adcR* (*sak_0217*) mutant strains, except that fusion between upstream and downstream regions of *adcAII* was obtained using the PstI restriction site. Upstream and downstream regions of *adcA* were amplified by PCR with primer pairs OAH086/OAH076 and OAH077/OAH087, respectively. Upstream and downstream regions of *adcAII* were amplified by PCR with primer pairs OAH040/OAH062 and OAH063/OAH043, respectively. Upstream and downstream regions of *adcR* were amplified by PCR with primer pairs OAH003/OAH004 and OAH005/OAH006, respectively. Upstream and downstream regions of *adcCB* were amplified by PCR with primer pairs OAH074/OAH015 and OAH16/OAH075, respectively.

The recombinant plasmids were electroporated in *E. coli* cells for amplification and then purified and electroporated in strain A909 (see Table S1 in the supplemental material). Allelic exchange was performed as previously described (33). Deletions of genes were confirmed by PCR and sequencing.

Generation of *lmb*, *adcA*, and *adcAII* complementation constructs.

To complement the Δ *lmb* mutant strain, the entire coding sequence of *lmb* was amplified by PCR with primers OAH115 and OAH102. The PCR fragment was cloned into the PstI/BglII restriction sites of the pTCV-P_{Tet} plasmid, a derivative of the shuttle vector pTCV and carrying a constitutively expressed Gram-positive promoter sequence (34) (see Table S1 in the supplemental material). In the same way, the Δ *adcA* mutant strain was complemented using primers OAH120 and OAH121 and cloned with the XbaI/BglII restriction sites. The Δ *adcAII* mutant strain was complemented using primers OAH119 and OAH104 and cloned with the BglII/PstI restriction sites.

The recombinant pTCV-P_{Tet} plasmids were electroporated in *E. coli* cells for amplification and purified, and then gene sequences were confirmed before electroporation in the A909 strain.

Construction of *lacZ* transcriptional fusions. Plasmid pTCV-*lac*, which carries a promoterless *lacZ* gene (35), was used to construct transcriptional *lacZ* reporter fusions (see Table S1 in the supplemental material). The promoter region of *lmb* was amplified by PCR using primers OAH122 and OAH009, and the PCR fragment was cloned into the EcoRI/BamHI restriction sites of the plasmid pTCV-*lac* (Table 1).

The sequence of the conserved 10-bp palindromic motif potentially constituting the AdcR-binding site (AdcR box) was obtained from the RegPrecise database maintained by the Lawrence Berkeley National Laboratory (<http://regprecise.lbl.gov/>). Site-tagged mutagenesis was performed to obtain the *adcR box*₁* and *adcR box*₂* coding sequences, in which 3 nucleotides for *adcR box*₁* and 4 nucleotides *adcR box*₂* were replaced so as to destroy the palindromic operator sequence without disturbing RNA polymerase fixation. For each construction, two DNA fragments were generated by PCR using primer pairs OAH122/OAH034 and OAH035/OAH009 for the *adcR box*₁* mutation or OAH122/OAH022 and OAH023/OAH009 for the *adcR box*₂* mutation. The point mutations were obtained using oligonucleotides containing 3 or 4 mismatches (OAH035 and OAH023) and also carrying BsaI restriction sites; the type IIS restriction endonuclease, which cleaves after its restriction site, generated DNA fragments with tetranucleotide cohesive ends (Table 1). Following digestion with BsaI (2 h at 50°C), the two fragments were purified with a NucleoSpin PCR cleanup kit (Macherey-Nagel) and ligated using the sticky-end instant ligase (NEB), seamlessly fusing the fragments together without adding any nucleotides. The resulting fragments were reamplified by PCR using the external oligonucleotides OAH122 and OAH009 (Table 1) and cloned in the pTCV-*lac* plasmid.

Electroporation. Electrocompetent *E. coli* and *S. agalactiae* cells were produced as previously described (36, 37). Both bacterial species were then transformed by electroporation using the Micropulser (Bio-Rad) and the Ec2 conditions (2.5 kV) with 1 to 2 µg of appropriate plasmids, respectively. Transformants were selected on LB agar (*E. coli*) or on 5% horse blood tryptic soy (TSH) agar plates (bioMérieux) (*S. agalactiae*), with the appropriate antibiotics.

β-Galactosidase assays. For β-galactosidase assays, bacteria were grown in CDM and harvested (10-ml samples) during the mid-exponential phase of growth (OD₆₀₀, 0.5). Cells were resuspended in 500 µl of Z buffer (38) and lysed mechanically with glass beads in a FastPrep-24 instrument, and cell debris were eliminated by centrifugation (5 min; 8,000 × g). Supernatant was incubated with 0.5 U/ml DNase I (NEB) at 37°C for 30 min and used for assays. Assays were performed as previously described, and β-galactosidase specific activities were expressed in arbitrary units per milligram of protein (38). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). All experiments were carried out in triplicate.

Cell size and quantification of chain length. Bacteria were grown in CDM containing 1 or 10 µM zinc. At mid-exponential phase, bacteria were deposited onto glass slides and examined under bright-field conditions at ×1,000 magnification with the Nikon Eclipse 80i optical microscope. Image acquisition and processing were performed using the NIS-Elements D software. Cell sizes (in micrometers squared) of 100 bacteria from three different pictures of three independent experiments were measured. To determine the bacteria chain length, 100 chains of three different pictures of three independent experiments were counted. Chain length values were calculated as percentages of all counted chains.

Competition assays in human biological fluids. To easily discriminate the wild-type and mutant strains in mixed cultures, we used the pTCV-*lac* vector, which carries a promoterless *lacZ* gene, or the pTCV-*lac*-P_{Cyl} vector, with a *lacZ* gene under the control of the strong and constitutively active promoter P_{Cyl} (34) (see Table S1 in the supplemental material). When plated on TH agar containing erythromycin (10 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 60 µg/ml), cells carrying the pTCV-*lac*-P_{Cyl} vector appeared as blue colonies, while cells carrying pTCV-*lac* remained white.

Each strain was transformed with pTCV-*lac* and pTCV-*lac*-P_{Cyl} plas-

mids. Mixed cultures were inoculated alternately with a combination of the wild-type strain carrying a pTCV-*lac* and a mutant strain carrying pTCV-*lac*-P_{Cyl} or a combination of the wild-type strain carrying pTCV-*lac*-P_{Cyl} and a mutant strain carrying pTCV-*lac*, to ensure that plasmids had no deleterious effect on bacterial growth. All strains were grown during 8 h in TH with erythromycin at 10 µg/ml and then in zinc-restricted CDM with erythromycin at 10 µg/ml overnight, and mixed cultures were inoculated with approximately 5 × 10⁵ CFU/ml of each strain in human biological fluids supplemented with erythromycin at 10 µg/ml. Bacterial growth was monitored during 48 h, and wild-type and mutant strains were discriminated by plating culture dilutions on TH agar containing erythromycin (10 µg/ml) and X-Gal (60 µg/ml). For each competition experiment between the WT and a mutant strain, both combinations (WT pTCV-*lac*/ΔpTCV-*lac*-P_{Cyl} or WT pTCV-*lac*-P_{Cyl}/ΔpTCV-*lac*) were tested. Each experiment was repeated at least three times.

Human plasma was obtained from healthy donors of the Etablissement Français du Sang (EFS Centre Atlantique, France). It was decontaminated by heating at 56°C during 30 min. Amniotic fluid and cerebrospinal fluid (CSF) were collected in the Bretonneau University Hospital (Tours, France) from, respectively, 2 and 3 donors who were not taking medications that would influence the analysis, such as antimicrobial agents.

Macrophage survival assays. For macrophage survival assays, bacteria were grown in zinc-restricted CDM overnight. Cultures were washed in PBS and adjusted to the desired inoculum in RPMI 1640 medium (Gibco), and CFU counts were verified by plating serial dilutions on TH plates. Macrophage RAW 264.7 cells, grown to confluence in RPMI–10% fetal calf serum (Gibco), were counted and incubated with bacteria (multiplicity of infection [MOI], ~10) in RPMI 1640 at 37°C with 5% CO₂ for 1 h to allow bacterial phagocytosis. Cells were then incubated in RPMI–10% fetal calf serum–gentamicin (500 µg/ml)–streptomycin (100 µg/ml)–penicillin (100 U/ml) in order to kill extracellular bacteria during 2 h, which represented the time zero (T₀) of the assay. At the indicated times (6 and 24 h after T₀), infected macrophages were washed once with RPMI and then lysed by incubation in 1 ml of ice-cold Milli-Q water for 30 min. CFU counts were determined by plating serial dilutions on TH plates. Assays were performed in duplicate and were repeated three times.

Statistical analyses. Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using the unpaired Student *t* test. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Two homologs of the Lmb protein are present in *Streptococcus agalactiae*. *lmb* is located on a 16-kb composite transposon containing the *scpB*, *lmb*, and *sht* genes (11, 12). We performed BLASTN searches on available genomic DNA sequences of *S. agalactiae* (*n* = 271) and identified the presence of the *lmb*-containing transposon in 96.8% of the 187 human isolates and only 26.7% of the 84 animal isolates (mainly fish and cattle), in agreement with previous observations for 30 human and 38 bovine strains (11).

We performed BLASTP searches on the *S. agalactiae* A909 genome and identified two putative homologs of Lmb (SAK_1319). We named them AdcA (SAK_0685) and AdcAII (SAK_1898), based on sequence and functional homology with *S. pneumoniae* proteins. The Lmb and AdcAII proteins are very similar, sharing 58% identity. The *lmb* and *adcAII* genes exhibit the same transcriptional organization, as they are each cotranscribed with a gene encoding the histidine triad proteins Sht (SAK_1318) and ShtII (SAK_1897), respectively (Fig. 1). The AdcA protein shares only 34% identity with Lmb, and its corresponding gene is monocistronic (Fig. 1). In contrast to *lmb*, which is more specific to human isolates, the *adcA* and *adcAII* genes are highly conserved

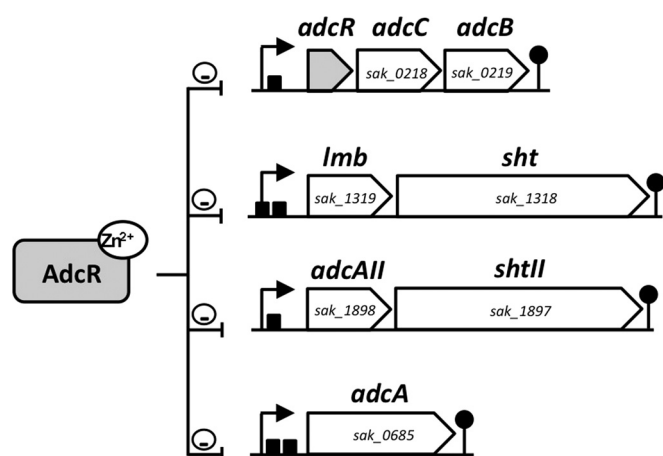


FIG 1 Transcriptional organization of the *lmb*, *adcA*, *adcAII*, and *adcRCB* genes and a model of Zn-dependent regulation by AdcR in *Streptococcus agalactiae*. In the presence of Zn^{2+} , AdcR can bind to the AdcR-binding motifs (TTAACNNGTTAA) located in the promoter regions of the *lmb-sht*, *adcAII-shtII*, and *adcRCB* operons and the *adcA* gene, causing repression of their expression. On the left of each sequence diagram is the promoter; the symbol on the right is the transcriptional terminator; black boxes are AdcR-binding boxes; the small circle with the dash inside it, prior to the sequence itself, indicates repression.

(at least 98% identity) in all of the 271 available *S. agalactiae* genomes.

The AdcA/Lmb family proteins are conserved among most streptococci and share a high degree of homology with zinc-binding proteins, in particular the AdcA and AdcAII proteins of *S. pneumoniae* (Table 2), whose role in zinc transport has been extensively studied (16, 39, 40). Following sequence alignment, we observed that the residues forming the Zn^{2+} ion-containing binding site of Lmb (His66, His142, His206, and Glu281) are conserved in the AdcA and AdcAII proteins (18) (Fig. 2). Analysis of the structure-based alignment between the proteins also revealed that AdcA possesses a histidine-rich loop and an extended C-terminal region exhibiting homology with the *Escherichia coli* ZinT protein and containing three conserved histidine residues that could potentially form a supplementary Zn-binding site (Fig. 2). The histidine-rich loop and ZinT domain of the *S. pneumoniae* AdcA homolog have been suggested to aid in recruiting Zn^{2+} (40). The structural differences between *S. agalactiae* AdcA and Lmb/AdcAII proteins are similar to those observed between *S. pneumoniae* AdcA and AdcAII proteins and correspond to distinct zinc acquisition mechanisms (40, 41).

Regulation of the *lmb-sht*, *adcA*, and *adcAII-shtII* gene operons is Zn^{2+} dependent. Zinc-binding proteins in other bacteria are upregulated under zinc limitation (26). We therefore examined *lmb-sht* expression in the presence of various concentrations of extracellular zinc, using a transcriptional *lacZ* fusion approach. We grew the *S. agalactiae* A909 strain in a metal-restricted CDM containing no zinc in its formulation and named here zinc-restricted CDM. This medium was supplemented with up to 100 μM Zn^{2+} . *S. agalactiae* A909 grew equally under all conditions (see Fig. S1 in the supplemental material). We measured *lmb-sht* promoter activity during mid-exponential growth, as there were no significant growth phase-dependent differences under all tested conditions (data not shown). In contrast, β -galactosidase

activity was significantly and gradually repressed in the presence of 0.5 to 100 μM Zn^{2+} ($P < 0.01$) (Fig. 3A); *lmb-sht* promoter activity was totally repressed in the presence of 10 μM added Zn^{2+} (approximately 20-fold less activity than in zinc-restricted CDM). It was also fully repressed in rich medium (Todd-Hewitt or brain heart infusion), in which the Zn^{2+} concentration is approximately 20 μM (data not shown). The repression of *lmb* expression by zinc appeared to be specific to this metal, as supplementation with various concentrations of Mn^{2+} , Cu^{2+} , Ni^{2+} , Fe^{2+} , or Co^{2+} did not change *lmb-sht* promoter activity (Fig. 3A).

Using qRT-PCR, we confirmed the strong repression of *lmb* in the presence of 10 μM extracellular Zn^{2+} , as well as that of the *adcA* and *adcAII* genes, suggesting that these genes are coregulated (Fig. 3B). The *lmb* gene also appeared to be the most highly expressed gene relative to *adcA* and *adcAII* when bacteria were grown in zinc-restricted CDM, but the difference was significant only with *adcA* (about 4-fold more highly expressed; $P < 0.05$) (Fig. 3B).

Zn^{2+} repression of the *lmb-sht*, *adcA*, and *adcAII-shtII* gene operons is mediated by AdcR. We have shown that the expression of the *lmb*, *adcA*, and *adcAII* genes is zinc dependent. Zn^{2+} -dependent regulation is mediated in *S. pneumoniae* and *S. pyogenes* by the AdcR regulator (26, 27, 42). *S. pneumoniae* AdcR has a unique effector in zinc, and its crystal structure revealed the presence of two metal-binding sites (43). A putative AdcR protein (SAK_0217; 53% identity with its pneumococcal homolog) containing the conserved metal-binding residues, is also present in *S. agalactiae* (see Fig. S2 in the supplemental material). The AdcR-encoding gene is present in all *S. agalactiae* sequenced genomes within an *adcRCB* operon (Fig. 1). We obtained a mutant of the *adcR* gene (Δ adcR) in *S. agalactiae* A909 and measured the effect of this mutation on *lmb-sht* promoter activity in a β -galactosidase assay. In the absence of AdcR, *lmb-sht* promoter activity was no longer repressed by zinc (Fig. 4A). The same results were confirmed for *lmb* and observed for the *adcA* and *adcAII* genes by qRT-PCR (Fig. 4B).

Our results indicate that the expression of the three putative zinc-binding proteins is controlled by AdcR. We thus analyzed their promoter regions to identify AdcR operator sites (TTAACN NGTTAA) (23, 42). One conserved 10-bp palindromic motif was present in the promoter region of the *adcAII-shtII* and *adcRCB* operons, and two overlapping copies were also present upstream from the *lmb-sht* and *adcA* genes (Fig. 1). We performed RACE-PCR to identify the transcription start site of the *lmb-sht* operon. We found it to be preceded by appropriately spaced -10 and -35 promoter regions, which are included in the first putative AdcR-binding motif (AdcR box) (Fig. 5). We introduced point mutations, designed to destruct the palindromic operator sequence

TABLE 2 Identity between the Lmb, AdcA, and AdcAII proteins of *Streptococcus agalactiae* and the AdcA and AdcAII proteins of *Streptococcus pneumoniae*

<i>S. agalactiae</i> protein	% identity with <i>S. pneumoniae</i> protein	
	AdcA	AdcAII
Lmb	35	67
AdcA	62	37
AdcAII	37	61

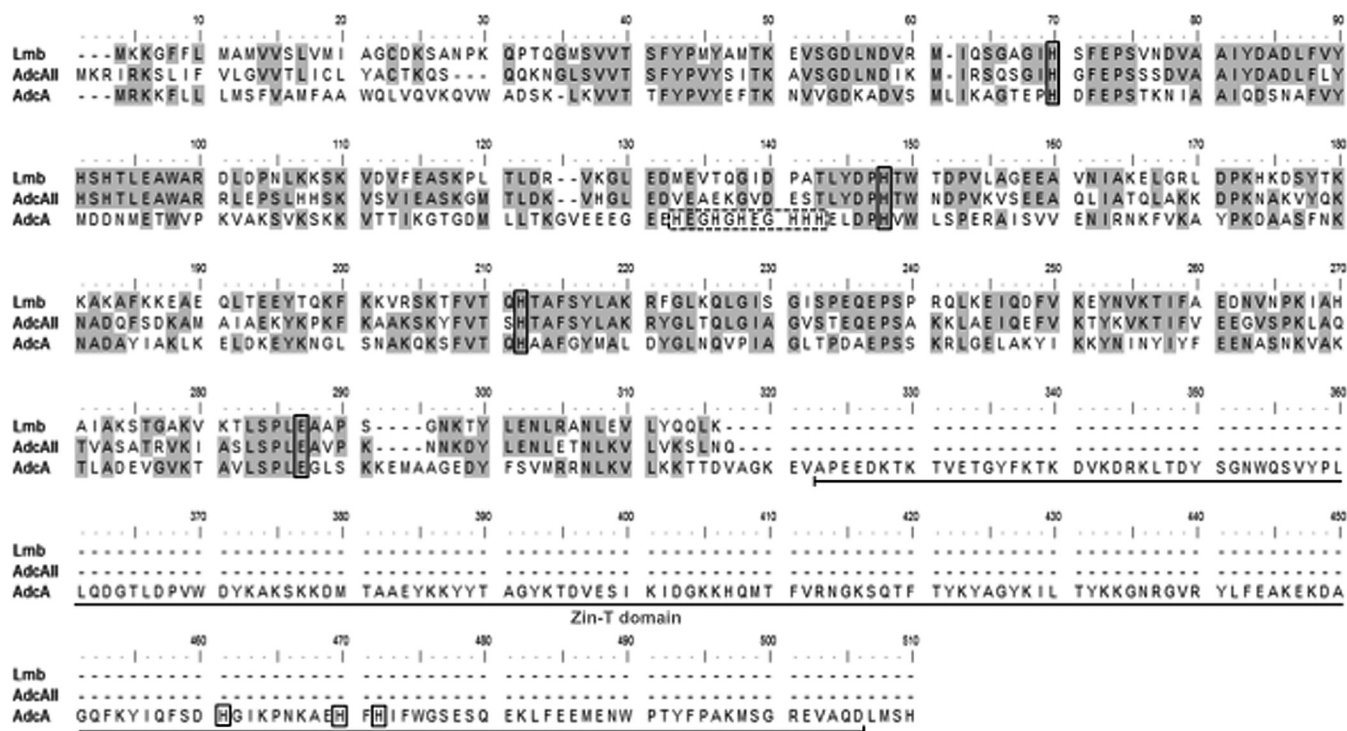


FIG 2 Amino acid sequence alignment of Lmb, AdcA, and AdcAII proteins of *Streptococcus agalactiae*. Identical residues are highlighted in gray. Potential zinc-binding residues are boxed in black. The His-rich loop of AdcA, which has been suggested to aid in recruiting Zn²⁺, is boxed by the black dotted lines. The Zin-T domain of AdcA, which may contain a supplementary zinc-binding site, is underlined. Sequence alignment was performed using the BioEdit program.

without disturbing RNA polymerase fixation, by PCR-mediated site-tagged mutagenesis, into each or both putative AdcR boxes. Mutation of either AdcR box led to weaker repression of *lmb-sht* promoter activity by zinc, indicating that both boxes are necessary for optimal AdcR fixation (Fig. 5). When both boxes were mutated, *lmb-sht* promoter activity was completely derepressed in the presence of zinc, as observed in the Δ adcR strain (Fig. 5). Altogether, these results strongly suggest that Zn²⁺-dependent repression of the *lmb-sht* promoter is mediated by the AdcR regulator, which binds to the TTAACNNGTTAA target sequence in the presence of zinc.

We used this sequence to identify potential AdcR-regulated genes in *S. agalactiae* by using the search pattern function of the SagaList software (<http://genolist.pasteur.fr/SagaList>). In addition to genes encoding the Adc/Lmb system, we found a perfect AdcR box in the promoter region (400 bp upstream from the start codon) of 7 supplementary genes or operons (Table 3), in particular, upstream of the *adh* gene (*sak_0087*), encoding a potential zinc-containing alcohol dehydrogenase, which has already been identified to be a direct binding target of AdcR in *S. pneumoniae* (26, 42).

Lmb, AdcA, and AdcAII are involved in zinc acquisition. We constructed mutant strains containing single and combined deletions of each gene encoding a putative zinc-binding protein in *S. agalactiae* A909 to evaluate the relative contributions of Lmb, AdcA, and AdcAII in zinc transport. Bacteria in which the *lmb*, *adcA*, and *adcAII* genes were fully expressed were grown in zinc-restricted CDM (Fig. 3B). The growth of all single and double mutant strains was similar to that of the wild-type strain (see Fig. S3 in the supplemental material). In contrast, we observed almost

no growth for the triple mutant of the binding proteins (Δ *lmb* Δ *adcA* Δ *adcAII*) (Fig. 6A). We cloned the *lmb*, *adcA*, and *adcAII* genes downstream of the constitutive promoter P_{Tet} (34) to generate complementing vectors (see Table S1 in the supplemental material). We introduced each construction in the Δ *lmb* Δ *adcA* Δ *adcAII* triple mutant strain, and complemented bacteria were grown in zinc-restricted CDM. Recombinant plasmids expressing any of the three proteins fully restored the growth of the Δ *lmb* Δ *adcA* Δ *adcAII* triple mutant, suggesting that the three proteins are redundant as zinc suppliers (see Fig. S4 in the supplemental material).

We then added increasing concentrations of Zn²⁺, from 0.5 to 100 μ M, to the medium. We observed no difference in growth for the wild-type strain (see Fig. S1 in the supplemental material), but the growth of the Δ *lmb* Δ *adcA* Δ *adcAII* mutant strain was gradually restored by Zn²⁺ addition, reaching a maximum restored growth rate from a concentration of 10 μ M (Fig. 6A). The addition of 10 or 100 μ M Mn²⁺, Cu²⁺, Ni²⁺, Fe²⁺, Mg²⁺, or Co²⁺ had no impact on growth (see Fig. S5 in the supplemental material), suggesting that the detrimental effect observed in the triple mutant is specifically due to zinc deprivation.

Lmb, AdcA, and AdcAII use the same AdcCB translocon. Lmb, AdcA, and AdcAII belong to the metal binding receptor class of proteins that classically associate with an AdcCB translocon, consisting of a permease, called AdcB (SAK_0219), and an ATPase, AdcC (SAK_0218), to form an ABC transporter. In all sequenced *S. agalactiae* strains, genes encoding a putative AdcCB translocon are present within an *adcRCB* operon which lacks a substrate-binding protein-encoding gene (Fig. 1). We constructed the mutant strain of the translocon Δ *adcCB* to test whether Lmb,

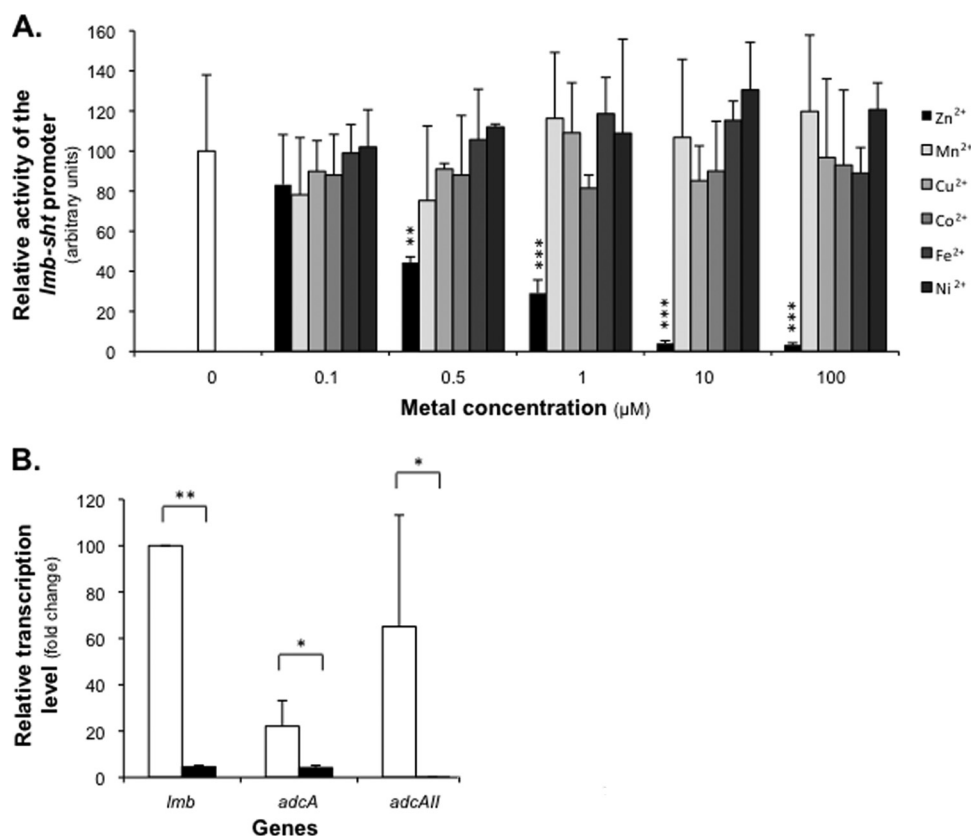


FIG 3 Regulation of the *Streptococcus agalactiae* *lmb*, *adcA*, and *adcAII* genes is Zn dependent. (A) *lmb-sht* operon promoter activity was measured in Zn-restricted CDM supplemented with various amounts of added metals (0 to 100 μM). A909 cells were grown until the mid-exponential phase of growth (OD_{600} , 0.5), and β -galactosidase assays were performed as described in Materials and Methods. The reference value (100%) is the *lmb-sht* promoter activity of cells grown in zinc-restricted CDM (0 μM Zn^{2+} added; white bar), and activities were calculated based on this reference. The values shown are mean results \pm standard deviations. The asterisks indicate *P* values obtained using unpaired Student's *t* test, comparing promoter activity of wild-type (WT) cells grown in zinc-restricted CDM and cells grown in CDM with the various added Zn^{2+} concentrations. **, *P* < 0.01; ***, *P* < 0.001. (B) *lmb*, *adcA*, and *adcAII* expression levels were measured in zinc-restricted CDM containing 0 (white bars) or 10 μM added Zn^{2+} (black bars). qRT-PCR was performed on RNA extracts of *S. agalactiae* A909 grown until the mid-exponential phase. The amount of transcripts of each gene was normalized against *recA* transcript levels. The reference value (100%) is the level of *lmb* transcripts after growth in zinc-restricted CDM (0 μM added Zn^{2+}). Gene expression is presented as the fold change. Results are presented as the means \pm standard deviations of three independent experiments. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare gene expression of cells grown in zinc-restricted CDM versus cells grown with 10 μM added Zn^{2+} . *, *P* < 0.05; **, *P* < 0.01.

AdcA, and AdcAII all use the AdcCB proteins to import zinc. The ΔadcCB mutant was unable to grow in zinc-restricted CDM, but its growth was gradually restored by the addition of Zn^{2+} (Fig. 6B). The growth of the Δlmb ΔadcA ΔadcAII triple mutant and that of the ΔadcCB mutant were similar for all tested Zn^{2+} concentrations. This was also the case for the mutant strain of the entire putative transporter ΔadcCB Δlmb ΔadcA ΔadcAII (data not shown). Altogether, these results strongly suggest that the three binding proteins use the same translocon to transport zinc.

The absence of the Adc/Lmb zinc transporter affects cell morphology. In bacteria, several proteins involved in cell division, cell wall synthesis, and remodeling, including peptidoglycan deacetylases and metalloproteases, use zinc as a cofactor (21, 44). We examined whether the absence of the Lmb/AdcA/AdcAII zinc-binding proteins or the AdcCB translocon affected cell morphology and/or chain formation. We cultured *S. agalactiae* strains in zinc-restricted CDM with 1 or 10 μM added zinc, examined them by optical microscopy, and calculated the size of individual bacteria and the number of cells per chain for each strain. We observed no significant difference between the individual cell size for any

strain (data not shown). However, under low zinc concentrations (1 μM Zn^{2+}), the Δlmb ΔadcA ΔadcAII and ΔadcCB mutant strains formed aggregates (clumps) or were mostly in the form of single cells, whereas the wild-type strain formed chains of mostly 2 to 4 cells per chain (Fig. 7). In the presence of 10 μM added zinc in the medium, the Δlmb ΔadcA ΔadcAII and ΔadcCB strains regained the capacity to form chains with a size distribution comparable to that of the wild-type strain, indicating that the observed abnormal cell morphology was due to zinc deficiency (Fig. 7).

The Adc/Lmb zinc transporter does not promote bacterial survival in murine macrophages. Phagocytic cells sequester iron to improve the clearance of pathogens, including bacteria (45). Other transition metals, specifically manganese and zinc, are also actively sequestered by the host during infection in order to hinder bacterial growth (46), and a $\text{Mn}^{2+}/\text{Fe}^{2+}$ transporter has been shown to play a role in GBS intracellular survival in macrophages (47). We compared the survival of the *S. agalactiae* wild-type strain and its isogenic Δlmb ΔadcA ΔadcAII mutant strain in the widely used murine macrophage cell line RAW 264.7. This phago-

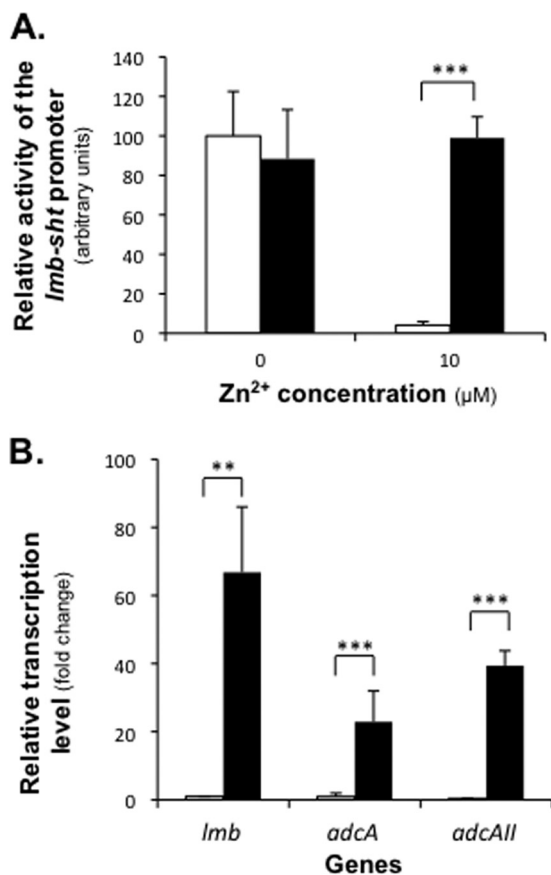


FIG 4 AdcR is required for Zn-dependent repression of the *lmb*, *adcA*, and *adcAII* genes. (A) WT A909 (white bars) or its isogenic Δ *adcR* mutant (black bars) were grown either in zinc-restricted CDM or in medium supplemented with 10 μ M Zn²⁺ until reaching the mid-exponential phase (OD₆₀₀, 0.5). β -Galactosidase assays were performed as described in Materials and Methods. The reference value (100) is the *lmb-shf* promoter activity of WT cells grown in zinc-restricted CDM, and the results are presented as the fold change compared to this reference. The values shown are the means \pm standard deviations of three independent assays. (B) WT A909 (white bars) or its isogenic Δ *adcR* mutant (black bars) strains were grown in zinc-restricted CDM supplemented with 10 μ M Zn²⁺ until reaching the mid-exponential phase (OD₆₀₀, 0.5). The amount of transcript of each gene was normalized against *recA* transcript levels. The reference value (1) is the level of *lmb* transcript of the wild-type strain. Gene expression is presented as the fold change. Results are presented as means \pm standard deviations of three independent experiments. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare promoter activity (A) or gene expression (B) of the WT strain and that of its isogenic Δ *adcR* mutant. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

cytic cell line efficiently killed *S. agalactiae* with both strains, displaying similar survival kinetics (Fig. 8). This result indicates that the Adc/Lmb zinc transporter has no significant role in bacterial survival in macrophages.

The Adc/Lmb zinc transporter supports growth and survival of *Streptococcus agalactiae* in two human biological fluids. During infection, *S. agalactiae* has to cross or colonize several human anatomic sites that have variable zinc concentrations: approximately 1.5 μ M in the amniotic fluid, more than 14 μ M in plasma, and 2.3 μ M in CSF (21, 48, 49). We thus examined the effect of the Adc/Lmb transporter on the ability of the bacteria to develop and survive in these human biological fluids. We performed a competition assay between the Δ *lmb* Δ *adcA* Δ *adcAII* or Δ *adcCB* mutant

strains and their wild-type parent by inoculating human biological fluids (amniotic fluid, plasma, or CSF) with equal amounts and monitoring the proportion of each strain. Both mutant strains were clearly outcompeted by the wild-type strain in CSF and amniotic fluid. Indeed, the decrease of the mutant strains began during the growth phase and was accentuated during the persistence stage, until they constituted less than 10% of the global *S. agalactiae* population after 48 h (Fig. 9). In contrast, the wild-type/mutant strain ratio remained relatively unchanged after growth in plasma, suggesting that the Adc/Lmb zinc transporter is dispensable in this biological fluid (Fig. 9).

The *lmb* gene appears to be specific to human isolates. We thus performed a competition assay between the wild-type and simple Δ *lmb* mutant strain in human cerebrospinal and amniotic fluids. There was no significant difference in their growth (see Fig. S6 in the supplemental material), indicating that the Lmb and AdcA/AdcAII proteins were also redundant in these human biological fluids.

DISCUSSION

In this study, we identified the Lmb/AdcA/AdcAII lipoproteins and the AdcCB translocon as components of a zinc transporter in *Streptococcus agalactiae*. We showed that expression of the *lmb*, *adcA*, and *adcAII* genes is controlled by the zinc concentration of the medium through the zinc-dependent regulator AdcR. Bacteria were unable to grow in zinc-restricted chemically defined medium in the absence of the Adc/Lmb transporter. To our knowledge, this is the first time that this metal has been shown to be essential for optimal growth of *S. agalactiae*. Zinc is a cofactor for a number of prokaryotic enzymes. In bacteria, there are several important enzymes that are zinc-containing proteins, including alcohol dehydrogenase, peptidoglycan deacetylase, metalloprotease, and ribosomal proteins (21, 44). Homologs of these proteins are present in *S. agalactiae* and are also likely to require zinc for their proper activity. Thus, a severe zinc restriction could impair the structure and function of essential proteins, explaining the growth delay observed for the Adc/Lmb zinc transporter mutant strains and the abnormal cell division. Indeed, the Δ *lmb* Δ *adcA* Δ *adcAII* and Δ *adcCB* mutant strains only formed aggregates or very short chains under zinc-restricted conditions. The origin of this defect is still unknown and may be related to mismetallation or undermetallation of a key enzyme(s) involved in cell division. For example, the L,D-carboxypeptidase DacB of *Streptococcus pneumoniae*, which is essential for peptidoglycan turnover and crucial to preserve cell shape, contains a mononuclear Zn²⁺ catalytic center (50). A homolog of this enzyme is present in *S. agalactiae*, and an alteration of its activity may explain part of the division phenotype and of the observed growth deficiency.

Bacteria without the Adc/Lmb transporter grew similarly to the wild-type strain when 10 μ M zinc was added to the medium, suggesting the presence of one or several secondary zinc transporters with lower affinity. A good candidate may be the MtsA manganese transporter, whose encoding gene is present in the *S. agalactiae* genome and which has been shown to bind zinc with low affinity in *S. pneumoniae* (51, 52).

We showed that the growth and survival of the Δ *lmb* Δ *adcA* Δ *adcAII* and Δ *adcCB* mutant strains in human cerebrospinal and amniotic fluids were clearly impaired. These biological fluids represent potential infection sites. In contrast, we observed no significant role of the zinc transporter in human plasma. The variable

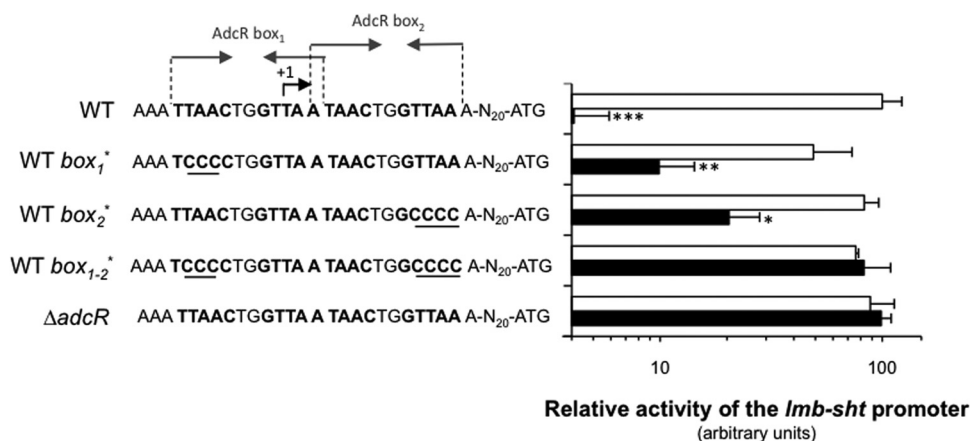


FIG 5 Both putative AdcR boxes within the *lmb-sht* operon promoter are required for full Zn-dependent promoter repression. Transcriptional *lacZ* fusions with the *lmb-sht* promoter region carrying point mutations that destroyed an inverted repeat of the first putative AdcR-box (*box*₁^{*}), or point mutations that destroyed an inverted repeat of the second putative AdcR-box (*box*₂^{*}), or combined mutations (*box*₁₋₂^{*}), were constructed. *lacZ* fusions containing the native *lmb-sht* promoter region and its derivatives were introduced into the WT strain. The native *lmb-sht* promoter region was also introduced in a Δ*adcR* mutant strain. β-Galactosidase assays were performed as described in Materials and Methods. The relative activity of the promoters was measured in zinc-restricted CDM containing 0 (white bars) or 10 μM added Zn²⁺ (black bars). The reference value (100) is the *lmb-sht* promoter activity of WT cells grown in zinc-restricted CDM, and the results are presented as the fold change against this reference. The values shown are the means ± standard deviations of three independent assays. The positions are numbered with respect to the start codon, and the transcription initiation site (with small right arrow) is labeled +1. Less relevant nucleotides are replaced with an N. Nucleotides in bold represent the two putative AdcR-binding sites, and underlined nucleotides indicate the introduced point mutations. The asterisks indicate P values obtained using an unpaired Student *t* test to compare promoter activity of the WT strain grown in zinc-restricted CDM and the other tested strains and conditions. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

importance of the Adc/Lmb transporter, depending on the human biological fluid, could be easily correlated with the zinc content. Indeed, the zinc concentration in plasma varies between 12 and 16 μM, which is much higher than that usually observed in amniotic and cerebrospinal fluids (approximately 1.5 and 2.3 μM, respectively). Thus, the Adc/Lmb zinc transporter may be dispensable in plasma because zinc concentrations are high enough to allow import by secondary transporters. Moreover, recent studies pointed out that vertebrates can sequester zinc upon bacterial infection, in particular by the action of the human calprotectin released from neutrophils (46). This strategy has been shown to efficiently inhibit microbial growth inside abscessed tissues and contributes to host defense by rendering bacterial pathogens more sensitive to host immune effectors (53, 54). Thus, *in vivo*, available zinc concentrations in a defined host compartment could vary during infection. Under these conditions, the presence of a high-affinity zinc uptake system such as the Adc/Lmb transporter of *S. agalac-*

tiae could be essential for the bacteria, and this opens interesting perspectives for future studies.

Our results support the concept that the Adc/Lmb zinc transporter is composed of three binding proteins, Lmb, AdcA, and AdcAII, that all use the same AdcCB translocon. This multiplicity of substrate-binding proteins is common among ABC transporters. In the three pathogenic streptococci, *S. pneumoniae*, *S. pyogenes*, and *S. gordonii*, the Adc system is composed of one AdcCB translocon and two zinc-binding proteins, the genes of which are found either within an *adcRCBA* operon or independently of the *adc* cluster (21). In *S. pneumoniae*, either AdcA or AdcAII is sufficient for zinc acquisition during growth *in vitro* and for systemic virulence *in vivo*, but both are necessary for optimal nasopharynx colonization (40). A recent study, again in *S. pneumoniae*, showed that the absence of AdcAII but not AdcA negatively affected early colonization of the nasopharynx (55). These results suggest that during infection, *S. pneumoniae* encounters environments with

TABLE 3 Identification of potential AdcR regulon members based on *in silico* *Streptococcus agalactiae* A909 genome scanning

Position(s) ^a	Gene ID and/or name	Function(s)	Pattern sequence ^b
-126	<i>sak_0087; adh</i>	Alcohol dehydrogenase	TTAAAGATTAAACCAGTTAAGAATA
-28	<i>sak_0217; adcR</i>	Repressor, zinc metabolism	TATAATTTACTGGTTAACAAAA
-96	<i>sak_0252</i>	Binding protein, peptide/nickel ABC transporter	TCTAATTAACCAGTTAAGTAAT
-28 and -39	<i>sak_0685; adcA</i>	Binding protein, zinc ABC transporter	AAATATTAACCGGTAAATAACGGGTTAAATAAA
-50	<i>sak_0693</i>	Similar to surface proteins (LPXTG motif)	AAAACCTTAACCGGTTAATTATT
-42	<i>sak_1023</i>	Similar to histidine triad protein, putative internalin	TATAATTAAGTAACTAACTAAA
-32 and -43	<i>sak_1319; lmb</i>	Binding protein, zinc ABC transporter	TAAAATTAAGTGGTTAATAACTGGTTAAATTAT
-52	<i>sak_1468</i>	Similar to flavoprotein, involved in K ⁺ transport	TATTGTTAACTGGTTAAGTATT
-39	<i>sak_1469</i>	Similar to ammonium transporter	AATACTTAACCAGTTAACAATA
-340	<i>sak_1542</i>	Binding protein, peptide/nickel ABC transporter	TATACTTAAGTGGTTAAGTATA
-38	<i>sak_1898; adcAII</i>	Binding protein, zinc ABC transporter	AAATGTTAACTGGTTAAGTATT

^a Nucleotide numbering is from the start codon of the gene to the end of the putative AdcR binding site.

^b Nucleotides in bold indicate the putative AdcR binding site.

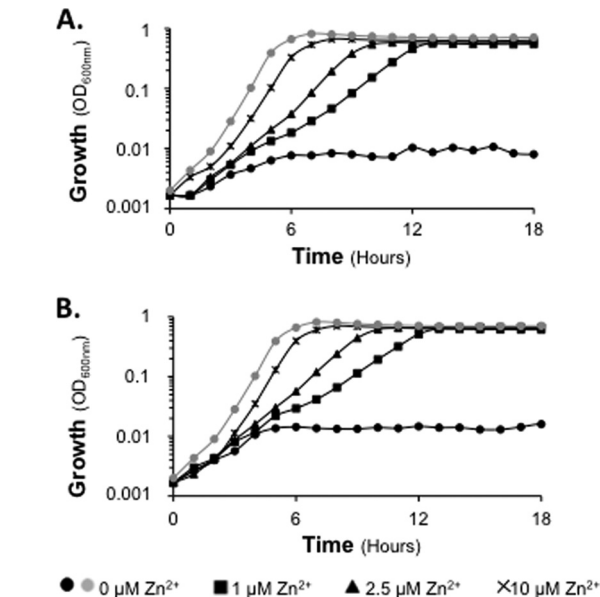


FIG 6 The Lmb, AdcA, and AdcAII proteins are involved in zinc acquisition in association with the AdcCB translocon. A909 WT (gray) and $\Delta lmb \Delta adcA \Delta adcAII$ mutant (black) strains (A) or WT (gray) and $\Delta adcCB$ (black) mutant strains (B) were grown in zinc-restricted CDM with various amounts of added Zn^{2+} (from 0 to 10 μM). The growth was monitored by assessing the OD₆₀₀ at 1-h intervals during 18 h. Data are representative mean OD₆₀₀ measurements from three independent experiments.

various zinc concentrations and that, in specific niches and conditions, the bacteria benefit from having two distinct zinc-binding proteins. The particularity of the *S. agalactiae* Adc/Lmb transporter is the presence of an additional binding protein, Lmb, which is almost specific to human isolates. In *S. agalactiae*, Lmb,

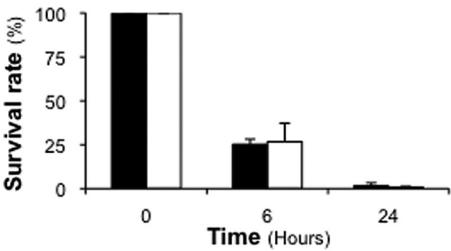


FIG 8 Intracellular survival of *Streptococcus agalactiae* in RAW 264.7 macrophages. RAW 264.7 cells were infected with A909 WT (black bars) or $\Delta lmb \Delta adcA \Delta adcAII$ (white bars) strains at an MOI of 10, and phagocytosis was allowed to proceed for 1 h. Antibiotics were then added, and the cells were incubated for a period of 2 h to kill the extracellular bacteria. This initial antibiotic treatment, which represents time zero of the experiment, was extended for different times up to 24 h, and the cells were lysed to quantify the intracellular survival rates of the bacteria. Results are presented as the means \pm standard deviations of three independent experiments performed in triplicate for each strain.

AdcA, and AdcAII are redundant for zinc acquisition in chemically defined medium, which is consistent with what was observed in *S. pneumoniae* (40). In human biological fluids, bacterial growth was not affected by the loss of the Lmb protein alone, whereas the $\Delta lmb \Delta adcA \Delta adcAII$ mutant strain was clearly out-competed by the wild-type strain in cerebrospinal and amniotic fluids. Thus, the three *S. agalactiae* zinc-binding proteins were redundant under the conditions we tested. However, we cannot exclude that, in specific environments or when in competition with other bacteria, Lmb, AdcA, and AdcAII could be complementary, and possessing three copies of these zinc-binding proteins could give an advantage to *S. agalactiae*. *In vivo* experiments would be useful to answer these questions.

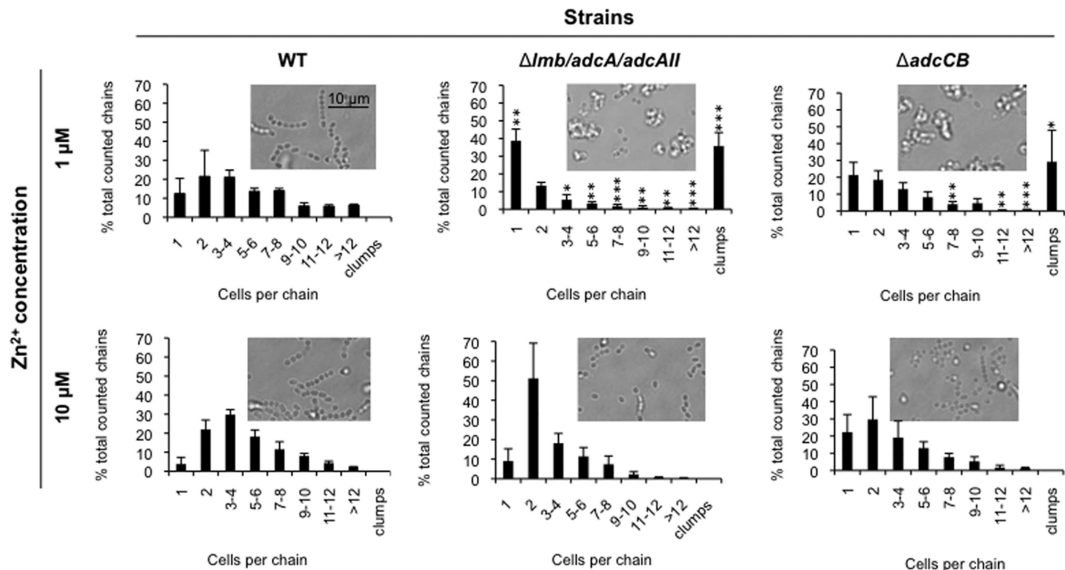


FIG 7 The Adc/Lmb zinc transporter affects cell morphology. Bacterial chain length of the A909 wild-type, $\Delta lmb \Delta adcA \Delta adcAII$, and $\Delta adcCB$ mutant strains was observed after growth in zinc-restricted CDM with 1 μM (top) or 10 μM (bottom) added zinc. Visualization of chain length was performed at a magnification of $\times 1,000$. Cells were collected during the mid-exponential phase. Images were captured from three separate experiments, and at least 100 chains were counted from each set, for a total of 300 or more chains counted for each strain. Chain length values were distributed between arbitrarily set numerical categories and calculated as percentages of all counted chains. Results are presented as the means \pm standard deviations for three independent counts. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare chain-length counts of the WT strain and the $\Delta lmb \Delta adcA \Delta adcAII$ and $\Delta adcCB$ mutant strains. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

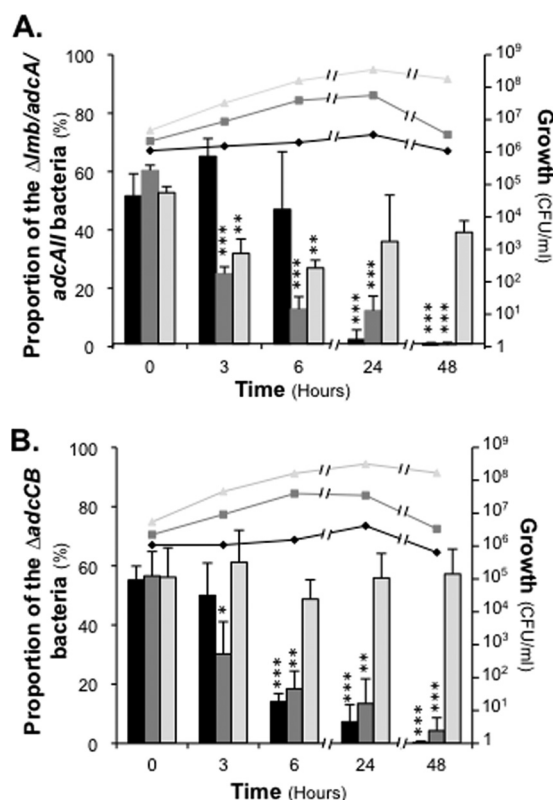


FIG 9 The Adc/Lmb zinc transporter is beneficial for *Streptococcus agalactiae* survival in two human biological fluids. A909 WT and $\Delta lmb \Delta adcA \Delta adcAII$ mutant strains (A) or WT and $\Delta adcCB$ mutant strains (B) were inoculated in equivalent numbers into human cerebrospinal fluid (diamonds and black bars), amniotic fluid (squares and dark gray bars), or plasma (triangles and light gray bars). Cocultures were incubated at 37°C for 48 h without agitation, the CFU were counted, and the growth curves were traced. The proportion of each strain was monitored by plating diluted cultures on TH agar containing erythromycin (10 μ g/ml) and X-Gal (60 μ g/ml) (see Materials and Methods). Results are presented as the means \pm standard deviations for three independent cocultures. The asterisks indicate *P* values obtained using an unpaired Student *t* test to compare the proportion of the strain at T0 and its proportion at the indicated times. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Another explanation for the multiplicity of these proteins may be that they have a secondary function beyond their involvement in zinc transport. Indeed, Lmb is a laminin-binding adhesin (10, 18). We expected to observe a difference between the binding of wild-type and $\Delta lmb \Delta adcA \Delta adcAII$ mutant strains to laminin because of the strong homology between Lmb and AdcAII (73% similarity) and because we determined conditions (zinc-restricted CDM) in which these proteins were optimally expressed. We performed binding assays with immobilized human laminin, fibrinogen, or collagen and did not observe any significant difference between the wild-type strain, Δlmb , or $\Delta lmb \Delta adcA \Delta adcAII$ mutant strains after bacterial growth in zinc-restricted CDM or TH broth (data not shown). There was also no effect on bacterial adhesion to A549 human alveolar basal epithelial cells (data not shown). These results do not agree with previous observations (10, 18). An explanation may be that we did not use the same *S. agalactiae* genetic background that was used in previous studies and that, despite strong conservation of the Lmb sequence, the ability to bind laminin may be strain dependent. It may be infor-

mative to test purified recombinant Lmb, AdcA, and AdcAII proteins for their binding ability, as previously done for the Lmb protein of *S. agalactiae* or Lbp of *S. pyogenes* (15, 18, 56). However, a recent study on Lmb homologs of *S. pneumoniae* also failed to show any binding to laminin (55).

Both the *S. agalactiae* *lmb* and *adcAII* genes are encoded within an operon containing the *sht* and *shtII* genes. These genes encode polyhistidine triad proteins, and *S. agalactiae* Sht has been shown to promote complement degradation by binding to factor H (12). In *S. pneumoniae*, the Sht homologs, called pht proteins, aid in zinc delivery to the ABC transporter substrate-binding protein AdcAII (40, 41) and play a role in pneumococcal adhesion to the respiratory epithelium (57). The involvement of *S. agalactiae* Sht proteins in zinc transport, their interaction with the Adc/Lmb system components, and their putative redundancy are now under study and should provide new insights to better understand mechanisms of zinc acquisition in *S. agalactiae*.

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