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MtaR, a Regulator of Methionine Transport, Is Critical for Survival of Group B Streptococcus In Vivo

Daniel Shelver,† Lakshmi Rajagopal, Theresa O. Harris, and Craig E. Rubens*

*Department of Pediatrics, Division of Infectious Disease, Childrens' Hospital
and Regional Medical Center and University of Washington, Seattle, Washington 91805*

The group B streptococcus (GBS) is an important human pathogen that infects newborns as well as adults. GBS also provides a model system for studying adaptation to different host environments due to its ability to survive in a variety of sites within the host. In this study, we have characterized a transcription factor, MtaR, that is essential for the ability of GBS to survive in vivo. An isogenic strain bearing a kanamycin insertion in *mtaR* was attenuated for survival in a neonatal-rat model of sepsis. The *mtaR* mutant grew poorly in human plasma, suggesting that its utilization of plasma-derived nutrients was inefficient. When an excess of exogenous methionine (200 μg/ml) was provided to the *mtaR* mutant, its growth rate in plasma was restored to that of the wild-type strain. The *mtaR* mutant grew poorly in chemically defined medium (CDM) prepared with methionine at a concentration similar to that of plasma (4 μg/ml) but was able to grow normally in CDM prepared with a high concentration of methionine (400 μg/ml). Both the wild-type strain and the *mtaR* mutant were incapable of growth in CDM lacking methionine, indicating that GBS cannot synthesize methionine de novo. When the abilities of the strains to incorporate radiolabeled methionine were compared, the *mtaR* mutant incorporated fivefold less methionine than the wild-type strain during a 10-min period. Collectively, the results from this study suggest that the ability to regulate expression of a methionine transport system is critical for GBS survival in vivo.

The potential to identify new antibiotic targets has been a major driving force for the study of bacterial pathogens at the molecular level. The majority of research on bacterial pathogens has focused on uncovering the virulence mechanisms of bacteria, which are most commonly defined as bacterial activities that result in damage to the host or that allow the evasion of immune mechanisms. However, from the perspective of developing new antibiotics, many virulence factors do not provide effective targets because they are not essential for bacterial survival and growth. Historically, broad-spectrum antibiotics target essential bacterial metabolic processes, such as transcription and translation. It has been estimated that the antibiotics in current use target only 15 of ~300 essential genes in bacterial genomes (8). A broader understanding of the basic metabolisms of bacterial pathogens will facilitate the development of new antibiotics.

The group B streptococcus (*Streptococcus agalactiae*) is a leading cause of invasive infections in human neonates. GBS is a normal member of the human vaginal flora, and ~30% of adult females are colonized with GBS. GBS can be transmitted to the newborn during the childbirth process when the neonate aspirates GBS-containing amniotic fluid or vaginal contents. GBS infections of the neonate can manifest as pneumonia,

sepsis, and meningitis. GBS infections in adults also occur and are especially common among the immunocompromised.

The GBS infection pathway dictates that the bacterium must grow in a wide variety of host environments. GBS is considered to be primarily an extracellular pathogen, though it appears to have the ability to persist to some degree within host cells, including macrophages (3). During the infection process, growth of GBS occurs in a variety of body sites, including the maternal vagina, amniotic fluid, and the neonatal lung, as well as the bloodstream. Growth of GBS to high densities has been reported in amniotic fluid, as well as in the lungs of neonates (16). However, it is not well understood how GBS adapts to the different host environments it encounters.

Much remains to be learned about how basic bacterial metabolic pathways function when the bacterium is within the host. Nutrient concentrations in vivo vary dramatically from those used to culture bacteria in vitro, and pathogenic bacteria will inevitably face nutrient limitations in various host compartments. The metabolic characteristics of GBS in vivo, however, have not been thoroughly explored. While some pathogens have the ability to synthesize all amino acids and other nutrients required for growth, GBS is auxotrophic for multiple amino acids and must rely on transport of many nutrients from the host environment during in vivo growth. This is apparent from inspection of the GBS genome (6), which reveals the absence of most biosynthetic pathways for amino acids, as well as the presence of a large number of genes encoding components of ABC transporters. Collectively, these observations indicate that GBS obtains most of its nutrients from the host; this suggests that the GBS metabolism is most likely finely tuned to the availability of nutrients within its host, which would be essential for its ability to cause disease.

* Corresponding author. Mailing address: Department of Pediatrics, Division of Infectious Disease, Children's Hospital and Regional Medical Center, Mailstop 8G1, 4800 Sand Point Way NE, Seattle, WA 98105. Phone: (206) 987-2767. Fax: (206) 987-3890. E-mail: craig.rubens@seattlechildrens.org.
† Present address: Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, LA 71130-3932.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
S. agalactiae		
COH1	Type III GBS isolate from neonate with septicemia; highly virulent; wild-type reference strain	14
DS101	*mtaR::kan* derivative of COH1; Kan^r	This work
HY105	*cpsB::kan* derivative of COH1; acapsular; Kan^r	30
DS105	Derivative of DS101 (*mtaR::kan*) bearing pDS8 (*mtaR$^+$*); Kan^r Erm^r	This work
DS106	Derivative of DS101 bearing pTRKL2; Erm^r	This work
E. coli		
MC1061	Host strain for propagation of Erm^r plasmids	28
XL1-Blue	Host strain for general cloning	Stratagene
Plasmids		
pVE6007	*E. coli*-GBS shuttle vector; temperature-sensitive replication; pWV origin of replication; Cm^r	12
pACYC177	Medium-copy-number plasmid utilized for cloning	New England Biolabs (Beverly, Mass.)
pCIV2	Source of Ω Km-2 fragment used for mutagenesis	17
pHY304	Derivative of pVE6007; temperature sensitive; Erm^r	2
pTRKL2	*E. coli*-GBS shuttle vector; low-copy-number plasmid; pAMβ1 origin of replication; Erm^r	18
pDS6	pHY304 bearing *mtaR::kan* allele; used to construct DS101; Kan^r Erm^r	This study
pDS8	pTRKL2 bearing wild-type *mtaR* gene; used for complementation of DS101; Erm^r	This study

In this study, we provide evidence that methionine availability can limit the growth of GBS in plasma. We demonstrate that a regulatory protein that controls methionine transport is essential for the ability of GBS to survive within a neonatal-rat host. Mutation of a gene encoding a LysR-type transcriptional regulatory protein, *mtaR*, resulted in a decrease in GBS growth rates in human plasma, and this phenotype was linked to a diminished ability to grow in defined media containing low levels of methionine. A methionine transport deficiency is implicated as one mechanism to explain the poor survival of the mutant in vivo. The findings of our study implicate methionine availability as an important determinant for the survival of GBS in vivo. Since MtaR homologues are found in the genomes of a number of other pathogenic bacteria, MtaR may also play an important role in the survival of other pathogens in vivo.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, Calif.) was used as a host for pACYC-derived and pTRKL2-derived (18) plasmids (Table 1). *E. coli* strain MC1061 was used as a host for pHY304-derived plasmids. COH1 is a virulent, highly encapsulated GBS isolate from a newborn with septicemia. Plasmid pHY304 (2), which was used for the construction of GBS mutants, is a derivative of pVE6007 (12) and replicates in a temperature-sensitive manner in *E. coli* and GBS. Plasmid pTRKL2 (18), which was used for complementation analysis, replicates with a p15A origin of replication in *E. coli* and replicates with a pAMβ1 origin of replication in GBS.

Bacterial growth and antibiotics. *E. coli* was cultured in Luria-Bertani medium. For routine culture, GBS strains were grown in Todd-Hewitt Broth (THB). For growth of GBS, antibiotics were added when necessary at the following concentrations: kanamycin, 500 μg/ml, and erythromycin, 5 μg/ml. The C-48 chemically defined medium (CDM) of Willett and Morse (29) was utilized for cultivation of GBS with defined nutrients. The concentrations of nutrients utilized in the present study were identical to those reported previously, except that the methionine concentrations were varied as specified in Results. To evaluate growth in CDM, GBS was initially grown to log phase (optical density at 600 nm[OD$_{600}$], 0.3) in THB. The cells were harvested by centrifugation, washed twice in an equivalent volume of phosphate-buffered saline (PBS) (130 mM

NaCl, 3 mM KCl, 1.5 mM KH$_2$PO$_4$, 4.3 mM Na$_2$HPO$_4$, pH 7.4), and diluted 1 to 50 in CDM. Growth was monitored spectrophotometrically at a wavelength of 600 nm. For growth experiments with human plasma, plasma was obtained from healthy human volunteers after consent. Prior to growth in plasma, GBS was grown in THB to log phase (OD$_{600}$, 0.3), collected by centrifugation, and washed twice in an equal volume of PBS. The resulting suspension was diluted to ~2 × 10^4 CFU/ml in PBS, and 75 μl was used to inoculate 750 μl of human plasma (~1,000-CFU/ml initial concentration of GBS) in a 2-ml microcentrifuge tube. The tubes were then incubated in hybridization tubes with slow rolling at 37°C. At designated intervals, growth was quantified by plating to Todd-Hewitt agar. For experiments in which plasma was supplemented with CDM components, the CDM components were used at the concentrations specified previously (29).

DNA and RNA techniques. Standard methodologies were utilized for cloning, PCR amplification, and Northern and Southern analyses (23). RNA was isolated by the method of Yim and Rubens (31) with minor modifications. Northern hybridization was performed with antisense RNA probes, utilizing the digoxigenin nonradioactive labeling and detection system (Roche, Indianapolis, Ind.).

Construction of a GBS *mtaR* mutant. The *mtaR* and flanking regions (including an IS*861* element and a *cpsA* region) were amplified from COH1 genomic DNA using Expand High Fidelity polymerase (Roche). PCR primers that incorporate *Xho*I and *Bam*HI sites for cloning were designed to amplify the *mtaR* region. The resulting PCR product was digested with *Xho*I and *Bam*HI and was ligated to similarly digested pACYC177, generating the plasmid pDS4. The region flanking *mtaR* was sequenced to insure that no mutations were introduced during PCR amplification. The kanamycin resistance cassette from pCIV2 was amplified using primers that incorporate *Pvu*II sites for cloning and was digested with *Pvu*II. Plasmid pDS4 was then digested with *Bsa*BI and ligated to the *Pvu*II-cut kanamycin resistance fragment, inserting the cassette into the *mtaR* gene. The plasmid insert, bearing the *mtaR::kan* allele, was liberated with *Pst*I and *Xho*I and ligated to similarly cut pHY304, generating plasmid pDS6, which was transformed directly into GBS strain COH1 (4).

The following procedure was then utilized to derive a strain bearing a replacement of the wild-type *mtaR* allele with the *mtaR::kan* allele described above. The GBS strain carrying pDS6 was cultured at 30°C overnight in THB with selection for kanamycin. The culture was then washed in THB without antibiotics, diluted 1/20 in THB without antibiotics, and incubated at 37°C until the culture was saturated. This dilution-and-outgrowth procedure was repeated for a total of five cycles. After the last outgrowth, dilutions of the culture were plated onto kanamycin-containing THB agar. GBS colonies from the kanamycin plates were then screened for sensitivity to erythromycin. A Km^r Em^s clone was designated DS101 and was selected for further study. The presence of the *mtaR::kan* allele on the chromosome of DS101 was verified by Southern blotting.

Construction of GBS strains used for complementation studies on *mtaR*. The *mtaR* gene and surrounding region, including the 5′ end of the adjacent *cpsA* gene and the 3′ end of the IS*861* element, were amplified from GBS strain COH1 using Expand High Fidelity DNA polymerase. The resulting amplification product was purified, digested with *Xmn*I and *Bgl*II, and ligated to pTRKL2 (18) digested with *Eco*RV and *Bam*HI, generating the plasmid pDS8. Plasmid pDS8 was introduced into strain DS101 by electroporation, generating GBS strain DS105.

Competition and LD$_{50}$ experiments in a neonatal-rat model. The ability of GBS to survive within a neonatal rat was measured by a competition assay and 50% lethal-dose (LD$_{50}$) analysis. For in vivo competition assays, wild-type and *mtaR* mutant GBS were cultured in THB to an OD$_{600}$ of 0.3, harvested by centrifugation, washed with an equal volume of PBS, and resuspended in PBS. It was determined that for both the wild-type and mutant strains, equivalent numbers of wild-type and mutant CFU were present at an OD$_{600}$ of 0.3. The wild-type and *mtaR* mutant strains were mixed in equivalent numbers and diluted in PBS to a density of ~2.1 × 10^5 CFU/ml, and 100 μl was injected into the peritoneal cavities of newborn (24- to 48-h-old) rat pups. The following day, the spleens were harvested and homogenized in PBS, and dilutions of the homogenates were plated for CFU. The mutant was distinguished from the wild-type strain by its Kan^r phenotype. The survival of the mutant in relation to the survival of the wild type was expressed as a competitive index, defined as follows: [(CFU of mutant recovered from spleen)/(CFU of wild type recovered from spleen)]/[(CFU of mutant input)/(CFU of wild-type input)]. For LD$_{50}$ analysis, wild-type (COH1), mutant (DS101), and *mtaR*-complemented strains were grown to an OD$_{600}$ of 0.3 in THB. Tenfold dilutions of the test strains were prepared and used to inoculate five or six rat pups per dilution for each strain tested.

Measurement of amino acid transport. The transport of amino acids was evaluated using a procedure based on that of Kadner (10). GBS was grown in CDM supplemented with methionine at 400 μg/ml to ensure equivalent growth of wild-type and *mtaR* mutant GBS. Cultures of GBS were grown at 37°C and harvested at an OD$_{600}$ of 0.30. Ten milliliters of each culture was harvested by centrifugation, and the cells were washed three times in CDM without methionine and leucine and supplemented with chloramphenicol at 50 μg/ml. The resulting suspension was adjusted to an OD$_{600}$ of 0.36 in the same medium. The culture was preincubated at either 4 or 37°C. When the transport of methionine was measured, an equivalent volume of CDM containing 10 μCi of L-[*methyl*-³H]methionine (Amersham-Pharmacia, Piscataway, N.J.)/ml, 2 μM total L-methionine, and 6.6 μM unlabeled leucine was added to the cell suspension. For measuring the transport of leucine, an equivalent volume of CDM was added to the cells to achieve a final concentration of 2 μCi of L-[U-¹⁴C]leucine (Amersham-Pharmacia)/ml, 6.6 μM total L-leucine, and 2 μM total L-methionine. After incubation at either 4 or 37°C for 10 min, 600 μl of the cell suspension was filtered in triplicate onto AcetatePlus membranes (Osmonics, Minnetonka, Minn.). The membrane filters were washed with 10 ml of PBS and dried. Radioactivity was then determined by scintillation counting after the addition of 10 ml of BCS-NA scintillation fluid (Amersham-Pharmacia).

RESULTS

MtaR encodes a LysR-type transcriptional regulator that is conserved in pathogenic streptococci. The sequence analysis of the GBS *mtaR* gene was originally reported by Koskiniemi and coworkers (11), who named the gene *cpsY* (see below) based upon its physical linkage to the capsular polysaccharide biosynthesis cluster. The sequence of *mtaR* predicts that it encodes a transcriptional regulator from the LysR family (24). MtaR shows the typical characteristics of LysR-type transcriptional regulators, including a putative DNA-binding domain at its N terminus and an effector-binding region at its C terminus (24). Koskiniemi et al. suggested that MtaR may regulate the adjacent polysaccharide synthesis locus (*cps*) (11). However, no experimental evidence was provided to support this hypothesis.

Inactivation of *mtaR*. To test whether MtaR influenced expression of the GBS capsular polysaccharide, we disrupted the wild-type copy of *mtaR* with a kanamycin resistance cassette. The *mtaR::kan* mutation was constructed as described in Ma-
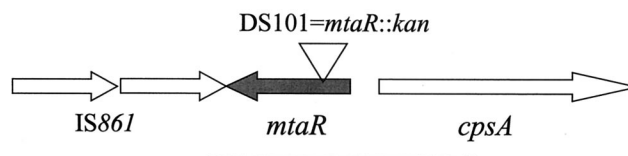

FIG. 1. The *mtaR* region of GBS. The open arrows depict the coding regions of the genes described in this study and indicate the direction of transcription. The *cps* gene cluster, which directs synthesis of the extracellular capsular polysaccharide of GBS, is transcribed divergently from *mtaR*. The IS*861* element (22) is adjacent to *mtaR* in strain COH1 (14). The open triangle depicts the location of the kanamycin omega cassette in GBS strain DS101 (*mtaR::kan*). The solid bar represents the DNA fragment bearing *mtaR* that was used for complementation of DS101 (*mtaR* mutant).

terials and Methods and subcloned to a temperature-sensitive *E. coli*-GBS shuttle plasmid (pHY304 [2]), generating pDS6. Subsequently, pDS6 was electroporated into GBS strain COH1, which is a heavily encapsulated, highly virulent type III clinical isolate. The plasmid was cured, and a strain bearing an allelic replacement of *mtaR* was isolated, as detailed in Materials and Methods. Southern blots of genomic DNA (not shown) confirmed the presence of the desired mutation on the chromosome of a representative isolate, designated DS101.

Cell surface-associated polysaccharides from COH1 and DS101 were quantified by competitive enzyme-linked immunosorbent assay. No significant difference was observed between the two strains (data not shown). As an independent test to determine if transcription of the *cps* region was altered in DS101, we evaluated *cps* transcript accumulation with Northern blots. No difference in *cps* transcript accumulation was observed when total RNAs extracted from log-phase COH1 and DS101 were compared (data not shown). Thus, under the in vitro conditions tested, we found no evidence that MtaR controls transcription of the adjacent *cps* operon.

The *mtaR* gene shows strong identity with two uncharacterized open reading frames from other important human pathogens. The organization of the *mtaR* region is presented in Fig. 1. When we searched the available databases for homologues of MtaR by using BLAST (1), we found homology to open reading frames from two other pathogenic streptococci. MtaR showed 78% identity and 86% similarity to the product of an open reading frame (Spy0898) from the genome of an M1 strain of *Streptococcus pyogenes*. MtaR also showed 75% identity and 84% similarity to the predicted product of an open reading frame (SP0676) from the genome of a virulent isolate of *Streptococcus pneumoniae*, TIGR4 (26). *S. pneumoniae* also produces a polysaccharide capsule, but the *S. pneumoniae mtaR* homologue is not physically linked to its capsule-biosynthetic locus (26). MtaR shows lower levels of similarity to a large number of other members of the LysR family (23).

MtaR is required for GBS survival in vivo in a neonatal rat. We considered the possibility that, since *mtaR* does not regulate expression of the GBS capsular polysaccharide, it may instead regulate a distinct cellular process that is important for the virulence of the organism. A number of transcription factors from the LysR family regulate virulence-related genes in diverse species of bacteria (24). To test if MtaR contributes to GBS virulence, we compared the ability of DS101 (*mtaR* mu-

tant) to survive in an animal model in relation to its isogenic parent strain, COH1. Neonatal rats were infected by intraperitoneal injection with a mixture of the wild type and mutant in a ratio of 1:1. The next day, spleens were harvested from infected animals and the surviving bacteria were enumerated. In three separate experiments, the *mtaR* mutant survived much more poorly than the wild-type strain (competitive-index values, 0.0000136, 0.00042, and 0.00000019; see Materials and Methods for a definition of competitive index). We also conducted LD_{50} analysis using the neonatal-rat sepsis model. The wild-type (COH1) LD_{50} was 1.0×10^3, while the *mtaR* mutant (DS101) displayed an LD_{50} of 1.1×10^6, a decrease in virulence of ~3 log units. These results suggest that MtaR is essential for survival within the host.

MtaR is necessary for wild-type growth in human plasma. In the sepsis model that was utilized in our experiments, bacteria access the bloodstream shortly after intraperitoneal injection. Thus, we hypothesized that poor survival in the animal may reflect a defect in the ability of the mutant to grow in blood. To determine if the attenuated survival of DS101 was due to poor growth in blood, we tested its ability to grow in human plasma in vitro. The use of plasma, as opposed to whole blood, allowed the evaluation of bacterial growth in the absence of the bactericidal effect of blood-derived immune cells. When COH1 (wild type) and DS101 (*mtaR* mutant) were compared, the growth rate of DS101 was substantially less (~2.5-fold) than that of the wild type (Fig. 2A). Control experiments in which the growth rates of the strains in THB (data not shown) and in the CDM of Willett and Morse (29) were compared revealed no difference in growth rates (see below). We also tested another mutant bearing the kanamycin resistance element, HY105 (*cpsB*::*kan*), to verify that the presence of the kanamycin cassette was not the cause of the slow growth of the strain; no growth defect was present for the strain in plasma (data not shown). Together, these results suggested that mutation of *mtaR* resulted in a methionine-related metabolic defect that is observable specifically in plasma.

Inactivation of *mtaR* results in a requirement for increased exogenous methionine. One hypothesis to explain the poor growth of DS101 in plasma is that the mutant strain experiences a nutritional deficiency in plasma. As noted above, the mutant strain grew comparably to the wild-type strain in the CDM of Willett and Morse (29). CDM contains 20 amino acids at levels that are higher than those required for growth of GBS. The normal growth of the *mtaR* mutant in CDM suggested that some component(s) of the CDM was deficient in plasma. We thus screened the individual components of the CDM for a compound that, when added to plasma, could stimulate growth of the mutant. We found that methionine, when added to plasma at the concentration present in CDM (400 μg/ml), increased the growth rate of the mutant to a rate equivalent to that of the wild-type strain (Fig. 2A). As a control, we tested if methionine specifically stimulated the growth of the mutant strain or if instead it stimulated GBS growth in general under the conditions present in plasma. We thus cultured the wild-type strain with and without the addition of methionine at 400 μg/ml. The addition of methionine did not change the growth rate of the wild-type strain.

The stimulation of the growth of the *mtaR* mutant by methionine supplementation strongly suggested that the *mtaR*
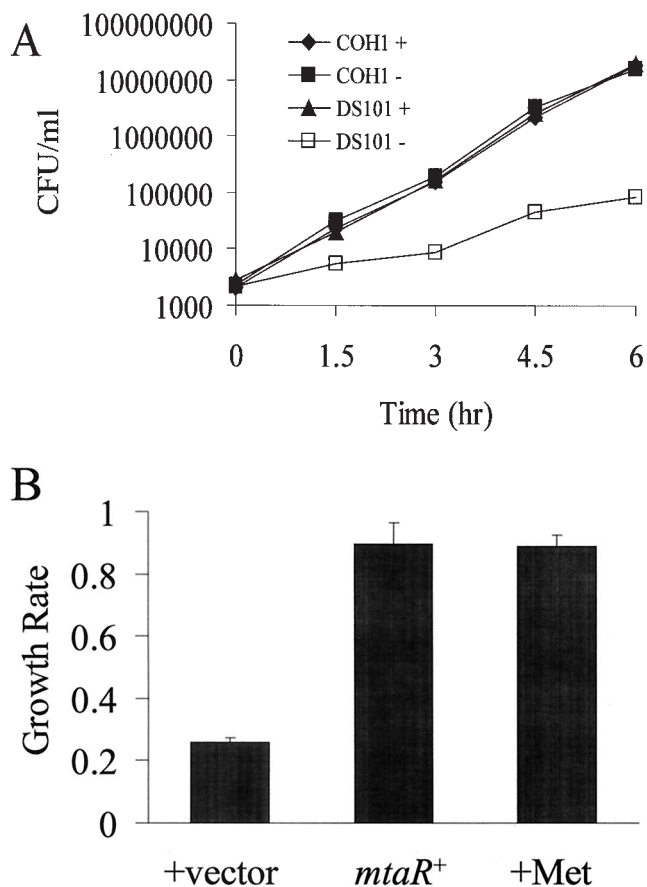

FIG. 2. Growth of GBS in plasma. (A) Growth of wild-type (COH1) and MtaR⁻ (DS101) GBS in plasma with and without methionine. GBS was grown in THB to an OD_{600} of 0.3, washed twice in PBS, and resuspended to a final OD_{600} of 0.3. After a dilution of 1/1,000 with PBS, 75 μl of the suspension was used to seed 675 μl of plasma obtained from a healthy adult human volunteer, achieving a bacterial density of ~1,000 CFU/ml. The plasma was either supplemented with methionine at 200 mg/ml (+) or not supplemented (−). The plasma containing the bacteria was then incubated at 37°C with end-over-end rotation, and dilutions were plated for CFU at the indicated time points. The experiment was conducted twice with similar results; one replicate is shown. (B) Complementation of growth defect of MtaR⁻ GBS. The growth rates in plasma of GBS strain DS101 (MtaR⁻) and DS105 (DS101 bearing a plasmid that contains a wild-type *mtaR* gene) were compared. As a positive control, DS101 was incubated in the presence of methionine (400 μg/ml). Growth experiments were conducted similarly to the experiment described in the legend to panel A, except the average growth rate over a 3-h period was calculated. Shown are the growth rates of DS101 with the vector portion of the complementing plasmid alone (+vector) (strain DS106), DS101 bearing a vector with the wild-type *mtaR* gene (*mtaR⁺*) (strain DS105), and DS101 grown in plasma supplemented with methionine (+Met). The error bars represent the standard deviations of three growth experiments.

mutant had a defect in some aspect of methionine metabolism. However, the metabolism of methionine by GBS has not been well characterized, so we performed growth studies in the CDM of Willett and Morse (29). As mentioned above, both strains grow normally in CDM with 400 μg of methionine/ml (Fig. 3A). When methionine was completely eliminated from CDM, neither the wild-type nor the mutant strain grew over
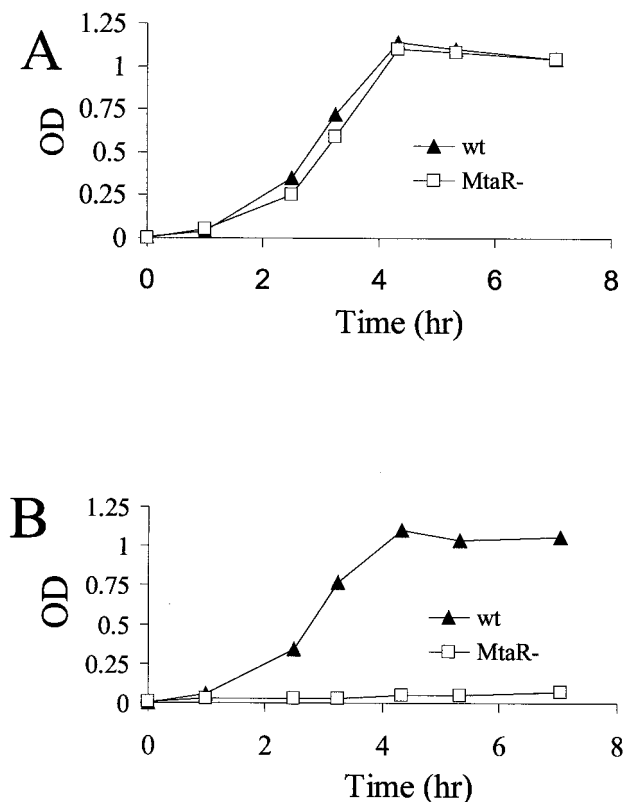
FIG. 3. Growth of GBS in chemically defined medium (CDM). GBS was grown in THB to log phase (OD$_{600}$, 0.3), washed twice in PBS, and resuspended to a final OD$_{600}$ of 0.3. The resulting suspension was diluted 1/100 and was used to inoculate CDM (29). The cultures were incubated statically at 37°C. Growth over time was monitored spectrophotometrically by measuring the OD$_{600}$. Shown are the growth rates of GBS strains in CDM with methionine at 400 (A) and 4 (B) μg/ml. The experiment was conducted three times with similar results, and one replicate is shown. wt, wild type.

the time frame of the experiment (data not shown). Thus, COH1 was incapable of de novo synthesis of methionine, at least when cultured in CDM.

The concentration of free methionine present in adult human plasma is ~4 μg/ml, which is ~50-fold lower than the concentration of methionine in CDM (29). The *mtaR* mutant was next evaluated for its ability to grow at low methionine concentrations. In CDM prepared with methionine at 4 μg/ml, the wild-type strain grew well, but the mutant strain did not grow over the time frame of the experiment (Fig. 3B). Collectively, the results of the CDM growth experiments indicate that GBS must scavenge methionine from an exogenous source and that the *mtaR* mutation renders the mutant unable to utilize exogenous methionine at physiological concentrations.

We next verified by complementation analysis that the virulence and growth defects that were observed were attributable to the *mtaR* mutation. A low-copy-number plasmid (pDS8 [see Materials and Methods]) bearing the wild-type *mtaR* allele was introduced into DS101. The introduction of pDS8 (*mtaR*$^+$) to DS101 decreased the LD$_{50}$ of the resulting strain to 1.5 × 10^4, a decrease of ~72-fold (compared to the *mtaR* mutant). We are not sure why the introduction of pDS8 did not

restore the virulence of DS101 to the level of COH1. We have observed that pTRKL2-based plasmids are stable in GBS during animal passage and do not alter the virulence of plasmid-bearing strains. However, it is possible that non-wild-type levels of MtaR are expressed from pDS8 due to its episomal nature and that this decreases the virulence of DS105 relative to COH1.

The complemented strain (DS105) was also tested for the ability to grow in plasma. The introduction of *mtaR* into DS101 restored the ability of the mutant to grow normally in plasma, to a rate that was comparable to that of DS101 grown in plasma supplemented with methionine (Fig. 2B). Thus, the *mtaR*::*kan* mutation was responsible for the attenuated virulence and the growth defect in plasma displayed by DS101.

MtaR is necessary for efficient methionine transport. The growth defect of the *mtaR* mutant in medium with low levels of methionine could be readily explained if DS101 were defective in its ability to transport methionine. To test this hypothesis, we compared the abilities of DS101 and COH1 to incorporate [³H]methionine. DS101 and COH1 were grown in CDM supplemented with high levels of methionine (400 μg/ml) to ensure that both strains grew equivalently. After growth to mid-log phase (OD$_{600}$, 0.3), the cells were washed in CDM lacking methionine and leucine and supplemented with chloramphenicol (50 μg/ml) to stop protein synthesis, as described in Materials and Methods. After being washed, the cells were incubated in CDM at 37°C with [³H]methionine or [¹⁴C]leucine. After 10 min, the cells were filtered and washed with PBS, and the radioactivity in the filters was determined by scintillation counting. Figure 4 depicts the amount of labeled amino acid incorporated by the cells. When incubated at a temperature of 37°C, the wild-type strain incorporated approximately fivefold more [³H]methionine than the *mtaR* mutant over a 10-min period. When an identical experiment was performed at 4°C, there was an ~47-fold decrease in [³H]methionine incorporation by the wild-type strain. This suggested that the incorporation of methionine by GBS was an enzymatic process, which typically shows temperature dependence. As a control, [¹⁴C]leucine incorporation was measured under conditions identical to those used to measure [³H]methionine transport. Wild-type and *mtaR* mutant GBS incorporated similar amounts of [¹⁴C]leucine (Fig. 4B); more leucine was incorporated at 37 than at 4°C. The results of the amino acid incorporation experiments together indicated that the *mtaR* mutation confers upon GBS a defect in its ability to efficiently transport methionine.

DISCUSSION

In this study, we provide evidence that a putative regulatory gene, *mtaR*, is required for the growth of GBS in human plasma and CDM in the presence of physiological methionine levels (4 μg/ml). Since GBS is auxotrophic for methionine, and since mutation of *mtaR* confers an inability to efficiently transport methionine, we conclude that MtaR is essential for methionine scavenging.

It was surprising that the *mtaR* mutant was not able to scavenge methionine through the degradation of plasma proteins. In order to utilize extracellular proteins as sources of amino acids, gram-positive bacteria, such as *Lactococcus lactis*,
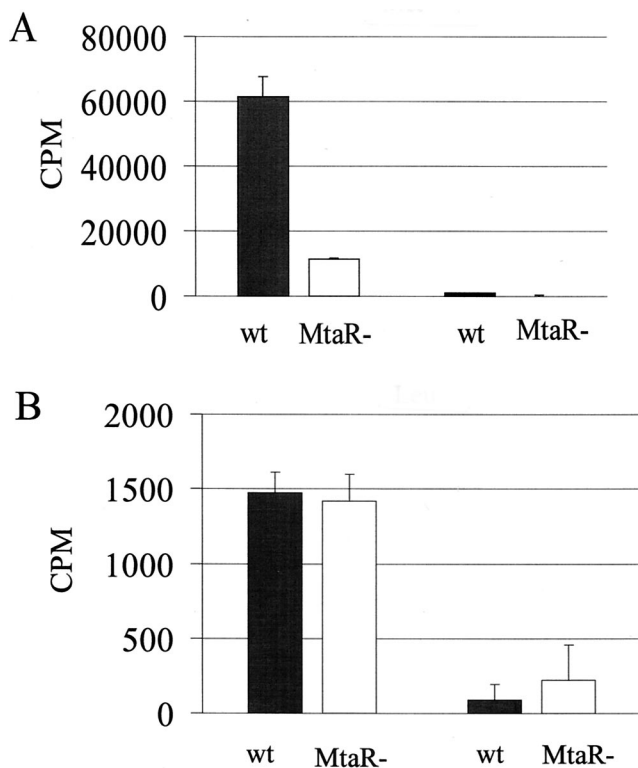
FIG. 4. Methionine incorporation by GBS. Wild-type (wt) and *mtaR* mutant (MtaR−) GBS were grown to log phase in CDM supplemented with methionine at 400 µg/ml. After being washed in PBS, the GBS cells were suspended in CDM at equivalent cell densities. After the cell suspension was prewarmed, [³H]methionine (1 µM total methionine) or [¹⁴C]leucine (3.3 µM total leucine) was added to initiate the assay. After incubation for 10 min at 4 or 37°C, 600 µl of the cell suspension was filtered, in triplicate, to AcetatePlus membranes (Osmonics), and the filters were washed with 10 ml of PBS by using a vacuum manifold (Millipore, Bedford, Mass.). The filter membranes were dried, and the radioactivity on the filters was determined by scintillation counting. The background radioactivity was determined by performing the experiment with CDM in place of the cell suspension and was subtracted from the results. The radioactivity (CPM) incorporated for COH1 (solid bars) and DS101 (open bars) is depicted. The error bars represent the standard deviations of triplicate samples. The two left-hand bars of each panel represent the results obtained when the incubation was performed at 37°C; the two right-hand bars represent the results obtained at 4°C. (A) [³H]methionine incorporation; (B) [¹⁴C]leucine incorporation.

hydrolyze proteins via an extracellular protease, transport the resulting peptides via peptide permeases, and degrade the peptides to amino acids with multiple peptidases. Inspection of the genome of another GBS serotype III strain, NEM316, reveals the presence of two putative extracellular proteases, four exported peptidases, three oligopeptide transporters, and 21 genes encoding intracellular peptidases. This suggests that GBS has the capacity to utilize extracellular proteins as nutritional sources. Several possibilities could explain the inability of the GBS *mtaR* mutant to grow at wild-type rates when utilizing plasma proteins as sources of methionine. One possibility is that utilization of plasma proteins is not as efficient as utilization of free amino acids and thus may not support rapid growth. Alternatively, GBS may lack an oligopeptidase with the proper specificity to release methionine from plasma-de-

rived oligopeptides. Finally, in addition to regulating the transport of free methionine, MtaR may be required for the expression of proteases, transport systems for oligopeptides, oligopeptidases, or some other component required for utilization of proteins as a source of methionine.

The ability of bacterial pathogens to adapt to in vivo environments, to acquire nutrients, and to grow in vivo is of fundamental importance to the disease process. In order to survive in vivo, pathogens must be able to adapt and utilize nutrients that are present. Despite the essential role of nutrition in the persistence of pathogens, surprisingly little research has been devoted to understanding what nutritional limitations pathogenic bacteria face in vivo and how the bacteria adapt to these limitations. Notable exceptions are the mechanisms of iron acquisition and its regulation, which have been well studied in a number of pathogenic bacteria. Animals have evolved sophisticated mechanisms to sequester iron in order to restrict the growth of pathogenic bacteria, and bacteria have countered by evolving strategies to acquire iron from their hosts. As for other nutrients, tryptophan availability may restrict the growth of pathogenic bacteria in eukaryotic cells. It has been suggested that macrophages can limit intracellular growth of bacteria such as *Toxoplasma gondii* (20) and *Legionella pneumophila* (5) by catabolizing tryptophan. Auxotrophic mutants of *Listeria monocytogenes* have been examined for virulence; a threonine auxotroph displayed an LD_{50} that was 1 log_{10} unit higher in a mouse model of infection. A number of in vivo expression technology and signature-tagged mutagenesis studies have implicated nutrition-related genes as important in virulence. Taken together, these observations suggest that nutrients can be expected to limit bacterial growth in the host environment, and pathogenic bacteria must have evolved mechanisms to counter these limitations. However, the nature of these nutritional limitations needs to be studied in more detail.

It is not clear if the decreased virulence of DS101 is due only to a defect in methionine transport. The nearly 3-log-unit decrease in virulence is striking, and while it appears that MtaR regulates methionine transport, it may directly regulate other virulence traits of GBS, and this may account for such a prominent attenuation in virulence. Another possibility is that slow growth of the *mtaR* mutant due to a methionine transport defect predisposes the mutant to clearance by the immune system.

The survival of a GBS in the sepsis model is primarily influenced by two factors: first, the growth rate of the GBS, and second, the ability of the GBS to resist clearance by the immune system. Interestingly, recent studies have shown that the two traits are linked; the ability of GBS to resist immune clearance is directly related to its growth rate. In vitro, growth rates of GBS affect the expression of several known GBS virulence factors. Paoletti and coworkers have shown that in vitro, fast-growing GBS resists opsonophagocytic killing more readily than does slow-growing GBS (19). Rapid growth rates stimulate high-level expression of the antiphagocytic polysaccharide capsule (19); this is one potential mechanism to explain the resistance of fast-growing GBS to immune clearance. A number of other potential GBS virulence factors appear to be regulated in response to the growth rate (21), and the invasiveness of GBS strains for epithelial cells is also regulated

by the growth rate (13). Taken together, these studies suggest that after entry into the bloodstream, GBS must grow rapidly or face clearance by the immune system. Thus, it is also possible that the slow growth of the *mtaR* mutant leads to decreased expression of virulence factors, predisposing it to clearance by the immune system. Further studies are needed to determine the impact of MtaR on the expression of other virulence factors.

It is noteworthy that the genomes of two other important human pathogens, *S. pyogenes* and *S. pneumoniae*, contain highly similar MtaR homologues. As obligate human pathogens, *S. pyogenes* and *S. pneumoniae* also rely on the transport of nutrients from the host, and like GBS, the other two streptococcal species are auxotrophic for multiple amino acids. We hypothesize that MtaR may also play a role in the regulation of nutrient uptake in these species in vivo.

What is the methionine transport system that is regulated by MtaR? The data presented in this study strongly indicate that MtaR is required for high-level expression of a methionine uptake system. Since *mtaR* encodes a protein that is similar to transcriptional regulatory proteins, the data presented in this study can be explained if MtaR normally effects activation of a gene encoding a methionine transport system. An alternative explanation is that loss of *mtaR* results in expression of a protein that blocks methionine transport. Members of the LysR family of transcriptional regulators, such as MtaR, activate or repress gene expression by binding directly to DNA sites and either activating or repressing transcription. The gene encoding the postulated methionine transport system controlled by MtaR, however, is unknown. One could hypothesize that MtaR regulates a gene located 5′ to IS*861*. However, when the genomes of the type III (6) and type V (27) GBS isolates are inspected, there are no open reading frames that encode products similar to amino acid transporters in the immediate vicinity of the IS element. Additionally, there has been no functional characterization of methionine transporters in gram-positive bacteria, so our ability to speculate on the nature of the gene encoding the transporter is limited. The methionine transport systems of *E. coli* (10) and *Salmonella enterica* serovar Typhimurium (7) are, to our knowledge, the only prokaryotic methionine transporters that have been described functionally. *E. coli* synthesizes two systems, a high-affinity methionine transport system from the ABC transporter family (MetD) (15) and a low-affinity system encoded by an unknown gene (9). To our knowledge, no homologues of methionine transporters for gram-positive bacteria have been characterized in the literature. We found no close homologues of the *metD* system of *E. coli* and *S. enterica* serovar Typhimurium in the genome of type III GBS or in the genomes of other gram-positive bacteria that have been released to date. This may indicate that gram-positive bacteria utilize different methionine transport systems than gram-negative bacteria.

What is the signal that is recognized by MtaR? MtaR shows similarity to the LysR family of transcriptional regulatory proteins, which incorporate signal sensing, DNA binding, and transcriptional regulatory activities in a single protein. Proteins from this family are typically cytoplasmic and recognize intracellular signals, in contrast to members of the two-component family of transcriptional regulatory proteins (5), which frequently recognize extracellular signals. Thus, we expect MtaR

to respond to an intracellular molecule. Inspection of the MtaR sequence reveals that the signal-sensing domain of the LysR family, which is located at the C terminus, is present in MtaR. The putative signal-sensing domain of MtaR, however, does not show strong similarity to the corresponding region of other LysR-type regulators; this may indicate that MtaR senses a signal distinct from those sensed by the other characterized LysR family members.

In summary, we have identified a regulatory protein from GBS that is distributed among pathogens, including *S. pyogenes* and *S. pneumoniae*. The finding that close homologues of MtaR exist in other important human pathogens suggests that MtaR may play a role in the pathogeneses of other gram-positive bacteria. In GBS, MtaR is essential for in vivo survival in a sepsis model and is also essential for normal growth in plasma and normal methionine transport. Taken together, our results suggest that methionine availability in blood may limit the growth of GBS and potentially other pathogens.

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