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MtsR, an Iron-Dependent Regulator in *Streptococcus iniae*

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Abstract

Streptococcus iniae (*S. iniae*) is a major pathogen that is capable of resulting severe economic loss to cultured fish. Steadily iron availability from micro-environment is an important virulence factor for pathogens, and *S. iniae* encodes the iron-transporter MtsABC to accomplish heme utilization, but very little was known about the mechanisms involved in regulating and maintaining iron balance in *S. iniae*. In this study, the role of a putative iron-dependent transcriptional regulator MtsR was investigated, and the results showed that MtsR regulated the expression of iron-transport mtsABC to control iron homeostasis in *S. iniae*.

Keywords

Streptococcus iniae, Iron-Dependent Regulator, Iron Transporter, MtsR, MtsABC

1. Introduction

Iron plays a significant role in many biological processes [1] [2] [3], and is an essential nutrient that *Streptococcus iniae* (*S. iniae*) needs to survive. Despite its abundance in the natural environment, iron has low solubility in physiological condition which made iron capture is an important act in bacteria. Our previous study indicated that the iron-transporter *mtsABC* of *S. iniae* HD-1 was involved in hemeutilization [4] [5], but very little was known about the mechanisms involved in regulating and maintaining iron balance in *S. iniae*. The iron acquisition processes in bacteria are tightly regulated, and the homeostasis of iron was

typically controlled by iron-dependent transcription regulators belonging to the DtxR or the Fur family [6]. These two families regulated the production of iron-transport systems, and in pathogens, they often controlled the expression of virulence factors as well.

S. iniae is one of the most important fish pathogens that causes serious infections in kinds of fish [7] [8] [9] [10], and has been reported to cause opportunistic infection in humans [11]. In present study, BLAST-mediated sequences similarity search of *S. iniae* genome sequences resulted in the identification of gene *mtsR* that shared amino acid sequence homologies with iron-dependent transcription regulators of other Streptococcal pathogens. Therefore, the role of iron-dependent transcriptional regulator MtsR in *S. iniae* has been characterized, and the results demonstrated that MtsR regulated the expression of iron-transporter *mtsABC* in response to iron availability intracellular. This might provide information about the role of MtsR in iron homeostasis in *S. iniae* HD-1.

2. Materials and Methods

2.1. Iron Is Essential for *S. iniae*

To detect the iron requirement of *S. iniae*, HD-1 cells were cultured. HD-1 was isolated from *Plectorhynchus cinctus* in China, and had been described previously and characterized thoroughly by Zhou *et al.* [12]. HD-1 cells were grown in complete medium, brain heart infusion (BHI) at 28˚C, iron-restricted medium was prepared by adding 0.1285 g nitrilotriacetic acid trisodium salt (NTA), 3.6 g brain heart infusion in 100 ml dd H_2O, and supplementing it with 0.0043 g $MnCl_2$, 0.0038 g $ZnCl_2$, 0.0031 g $CaCl_2$, and 0.0033 g $MgCl_2$ [13].

2.2. Cloning and Sequence Analysis of *mtsR*

Genomic DNA was extracted from the *S. iniae* HD-1 using the Wizard genomic DNA purification kit, and the products were quantified by measuring the absorbance at 260 nm. PCR was carried out with 1 μg of genomic DNA using the primers 5'-*TTTTGTGACATATAGTTGGCGGGCA*-3' and 5'-*ATGACGCCTAACAAAGAAGATT*-3' as described by Zou [4], and the PCR products were sequenced at Invitrogen corporation to confirm their specificity. Briefly, the cycling conditions used for *mtsR* ORF were as follows: 1 cycle of 94˚C for 2 min, 35 cycles of 94˚C for 45 sec, 35 cycles of 61˚C for 45 sec, 35 cycles of 72˚C for 1 min, 1 cycle of 72˚C for 7 min. Nucleotide and deduced amino acid homology analysis of MtsR were carried out by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Conserved Domains of *mtsR* were detected by NCBI CD Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.3. qPCR Analysis

Duplicate cultures of *S. iniae* HD-1 were harvested at early logarithmic phase

($OD_{600\ nm}$ = 0.35), mid-logarithmic phase ($OD_{600\ nm}$ = 0.65), and final-logarithmic phase ($OD_{600\ nm}$ = 0.80) in BHI orin iron-restricted medium. The total RNA was extracted and was reverse transcribed to cDNA. Real-time fluorescence PCR (qPCR) analysis was performed with a LightCycler480 system. The primers of *mtsABC*, *mtsR* and *gyrA* used in qPCRas listed in **Table 1** [4], and the cycling protocol were an initial denaturation at 94˚C for 2 min followed by 40 cycles of 94˚C for 15 s, 56˚C for 50 s, and 79˚C for 10 s. Finally, a melting curve was performed to ensure that there was no contamination. qPCR of the genes of interest, *mtsA*, *mtsB*, *mtsC*, and *mtsR* and a normalizer gene, *gyrA*, were performed in triplicate for each sample, and included a no-template control to rule out contamination and primer-dimer formation. Gene *gyrA* was chosen as a normalizing gene because its expression in other streptococci is stable under different test conditions [14]. The expression fold change of gene was calculated base on the comparison with the normalized *gyrA*. All statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., USA).

3. Results

3.1. Iron Supports *S. iniae* HD-1 Growth

To detect the importance of iron for *S. iniae*, the growth curves of HD-1 in BHI and iron-restricted medium were characterized. The results showed that the growth of *S. iniae* HD-1 was inhibited by adding NTA to BHI medium supplemented with additional cations, and this inhibition was the result of iron limitation. In iron-restricted medium, the HD-1 cells needed 9.5 h to reach the stationary phase, which was 3 h more than that of in BHI medium, and the growth level was dropped 10% (**Figure 1**). The gram stain was used to observe the morphological features of *S. iniae* HD-1 cells at the logarithmic phase, and the results showed that the cells in iron-restricted medium had long chain length (**Figure 2(b)**) than that of in BHI medium (**Figure 2(a)**), which indicated that

Table 1. Primer pairs used in qPCR analysis of *mtsABC* and *mtsR*.

Gene*	*Sequence* (5'-3')	*Amplicon size* (bp)	Annealing temperature (˚C)
mtsA F	AGCCGTTGCCAGAAGATGTTG	99 bp	56˚C
mtsA R	AGTTTCGTAAACCAGGCTTGCC	99 bp	56˚C
mtsB F	GTGGTACGGTAACTATTGGTGAAC	76 bp	56˚C
mtsB R	CATACTTCGTTGCTCCACATAGG	76 bp	56˚C
mtsC F	ATATTCTGGCGGTTCAAGATAGTG	123 bp	56˚C
mtsC R	TTAGCAAGCACTGGGTCAAATG	123 bp	56˚C
mtsR F	TTGGTCTTTTCTTGGAATGCTAC	179 bp	56˚C
mtsR R	AACACCGCTTGATTGAACTCTTT	179 bp	56˚C
gyrA F	AGTTCACCGTCGTATTCTTTATGGTATGA	150 bp	56˚C
gyrA R	CCATACGAACCATGGCTTCATAAATA	150 bp	56˚C

*Gene correspond to *mtsABC* gene designations in *S. iniae* HD-1. F, forward, R, reverse.

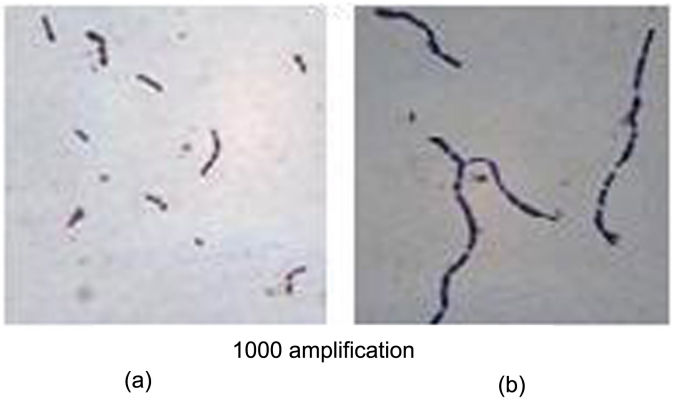

Figure 1. Iron influences on the growth of *S. iniae* HD-1. (BHI: Positive control; 5 mM NTA: Add 5 mM NTA to BHI medium).

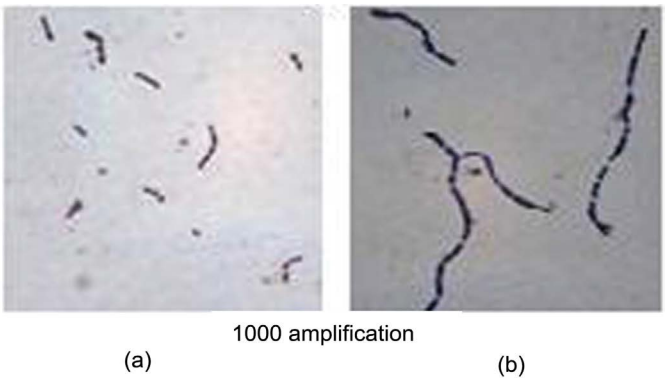

Figure 2. Morphological features of *S. iniae* HD-1 in BHI and iron-restricted medium. (Gram stain and microscopic examine of *S. iniae* HD-1. A: HD-1 in BHI medium at 5 h; B: HD-1 in iron-restricted medium at 5 h).

the reproduction of *S. iniae* HD-1 cells had been affected without the support of iron. These results indicated that iron was the essential nutrient that *S. iniae* needed to survive.

3.2. Cloning and Sequence Analysis of *mtsR*

Iron acted as a regulatory factor having influence on proteins production, but the mechanisms processes in *S. iniae* for iron homeostasis have not been characterized. Screening of *S. iniae* genome sequences resulted in the identification of gene *mtsR* (GeneBank No: JN177478) that shared amino acid sequence homologies with DtxR family which were the metal-dependent transcription regulators. The closest homologs for MtsR are the iron-dependent repressors from *Streptococcus pyogenes* MGAS9429 (**Table 2**, http://blast.ncbi.nlm.nih.gov/Blast.cgi). *mtsR* has 624 bp which located at 5' proximal of the iron-transporter *mtsABC*, and was transcribed in the opposite direction (**Figure 3(a)**). The localization of *mtsR*, and its similarity to other iron-dependent transcriptional regulators suggested that MtsR may function as the *mtsABC* repressor, which can regulate

Table 2. The proteins to which MtsR have close identity and similarity.

Function	Organism	% Identity/ similarity	Length of aa compared
copyIron-dependent repressor	*Streptococcus pyogenes MGAS*9429	155/181	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes SSI*-1	155/181	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes MGAS*315	155/181	213
Iron-dependent repressor	*Streptococcus pyogenes MGAS*6180	155/181	213
Iron-dependent repressor	*Streptococcus dysgalactiae subsp. equisimilis GGS*_124	155/180	213
Iron-dependent repressor	*Streptococcus pyogenes MGAS*5005	155/181	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes NZ*131	155/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes MGAS*8232	154/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes str. Manfredo*	154/180	213
Iron-dependent repressor	*Streptococcus pyogenes MGAS*10394	154/180	213
Iron-dependent repressor	*Streptococcus pyogenes MGAS*10270	154/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus pyogenes M*1	154/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus equi subsp. Zooepidemicus*	151/180	213
Transcriptional regulator of metal ABC transporter	*Streptococcus equi subsp. zooepidemicus MGCS*10565	151/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus equi subsp. equi* 4047	150/180	213
Putative metal-dependent transcriptional regulator	*Streptococcus uberis* 0140*J*	144/180	213
Iron-dependent repressor	*Streptococcus pyogenes MGAS*2096	87/105	131
Iron-dependent repressor	*Streptococcus agalactiae A*909	129/169	213
Hypothetical protein	*Streptococcus agalactiae NEM*316	129/169	213
Iron-dependent transcriptional repressor	*Streptococcus agalactiae* 2603 *V/R*	129/169	213
Putative iron-dependent transcriptional regulator	*Streptococcus gallolyticus UCN*34	129/164	214
Putative metal-dependent transcriptional regulator	*Streptococcus suis P*1/7	123/164	214

the transcription of *mtsABC* in response to iron availability intracellular.

The TBLASTN analysis showed that the predicted amino acid sequence of MtsR was highly conserved (**Table 2**), and the NCBI CD Search predicted that MtsR has three conserved domains: WHTH_GntR, Fe_dep_repr_C, and FeoA (**Figure 3(c)**). WHTH_GntR, and Fe_dep_repr_Cdomains are highly conserved in MtsR, which found in other DtxR homologues thatare responsive to iron, manganese, or both (**Figure 1(b)**). FeoA domain was found at the C-terminus of a variety of metal-dependent transcriptional regulators, which in most cases likely to be either iron or manganese [15]. Based on these observations, we concluded that *mtsR* is a member of the metal-dependent transcriptional regulators.

3.3. Determination of MtsR as the Iron-Dependent Transcriptional Regulator

Total RNA was isolated from HD-1 cells in BHI and in iron-restricted medium, and qPCR analysis was performed to determine whether MtsR regulated the expression of iron-transporter *mtsABC* at the transcriptional level. The housekeeping gene *gyrA* was used as an internal control in qPCR, and similar levels of

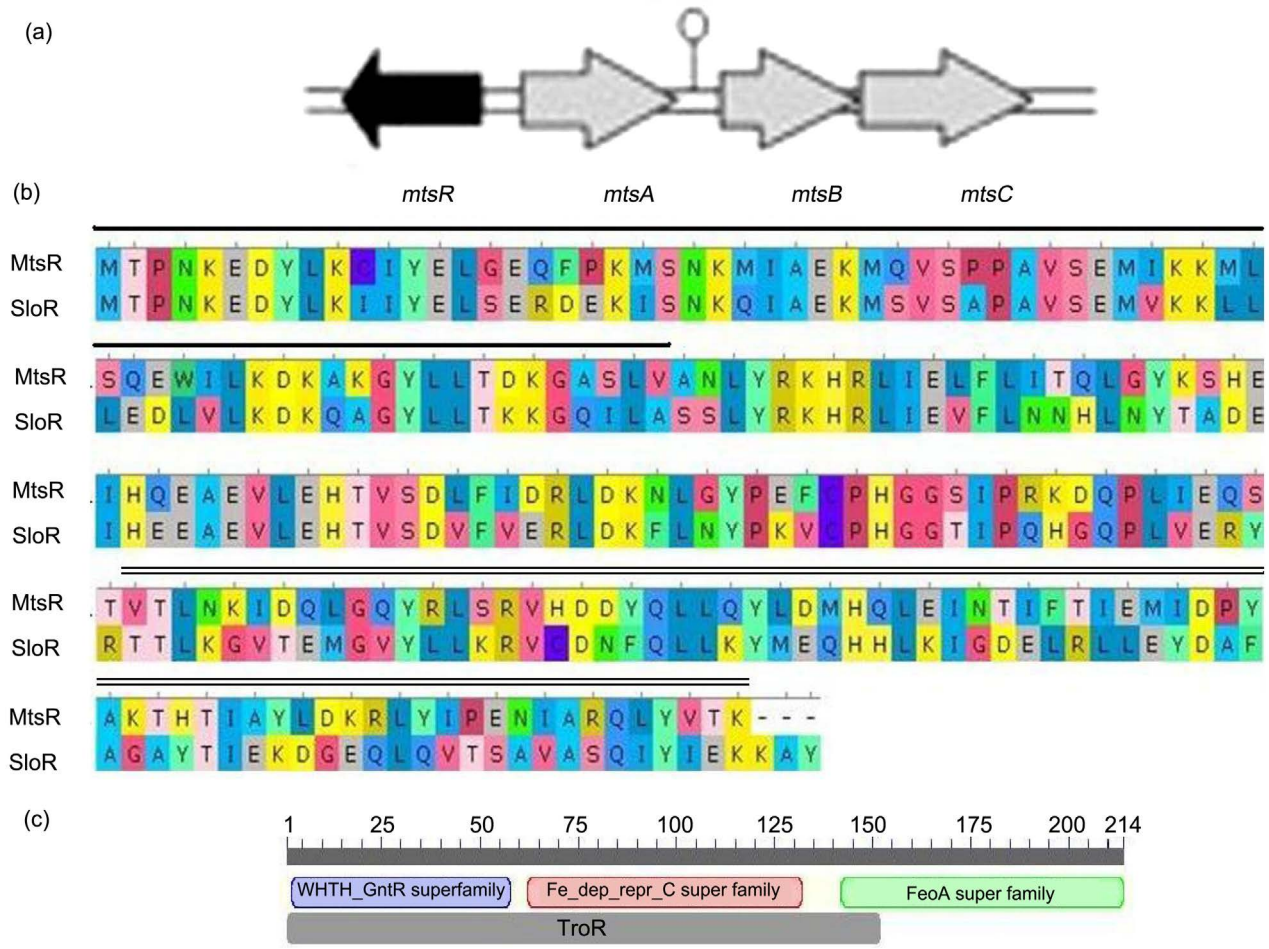

Figure 3. The streptococcal iron transport repressor (MtsR).

amplification confirmed that the RNA quantities used as the templates in all qPCR reactions were equal. When use the RNA isolated from the HD-1 grown in BHI medium as templates, the expression of *mtsA*, *mtsB*, and *mtsC* were significantly up-regulated and reached the peak at the early logarithmic, de-regulated at the mid-logarithmic, and back to housekeeping gene level at the final-logarithmic phase ($p < 0.05$, **Figure 4**). This representation owed to the high-efficiency of procaryotic cells. The cells needed iron for growth, they stored iron through up-regulate iron-transporter, and once they captured enough iron, the expression of iron-transporter was up-regulated [16]. The expression tendency of the *mtsABC* in iron-restricted medium was consistent with that of in BHI medium, but the expression levels of *mtsA*, *mtsB*, and *mtsC* were showed 1.83, 13.3, and 2.11 times higher than that of in BHI medium, respectively (**Figure 5**). In contrast, both in **Figure 4** and **Figure 5** the results showed that the high transcription of the *mtsABC* is observed when the *mtsR* remain inactivate, suggesting that MtsR up-regulate *mtsABC* expression was very likely to lead to an increase in iron uptake by *S. iniae*. Meanwhile, activation of MtsR resulted in de-regulation of the *mtsABC* transcription, which demonstrated that *mtsR*

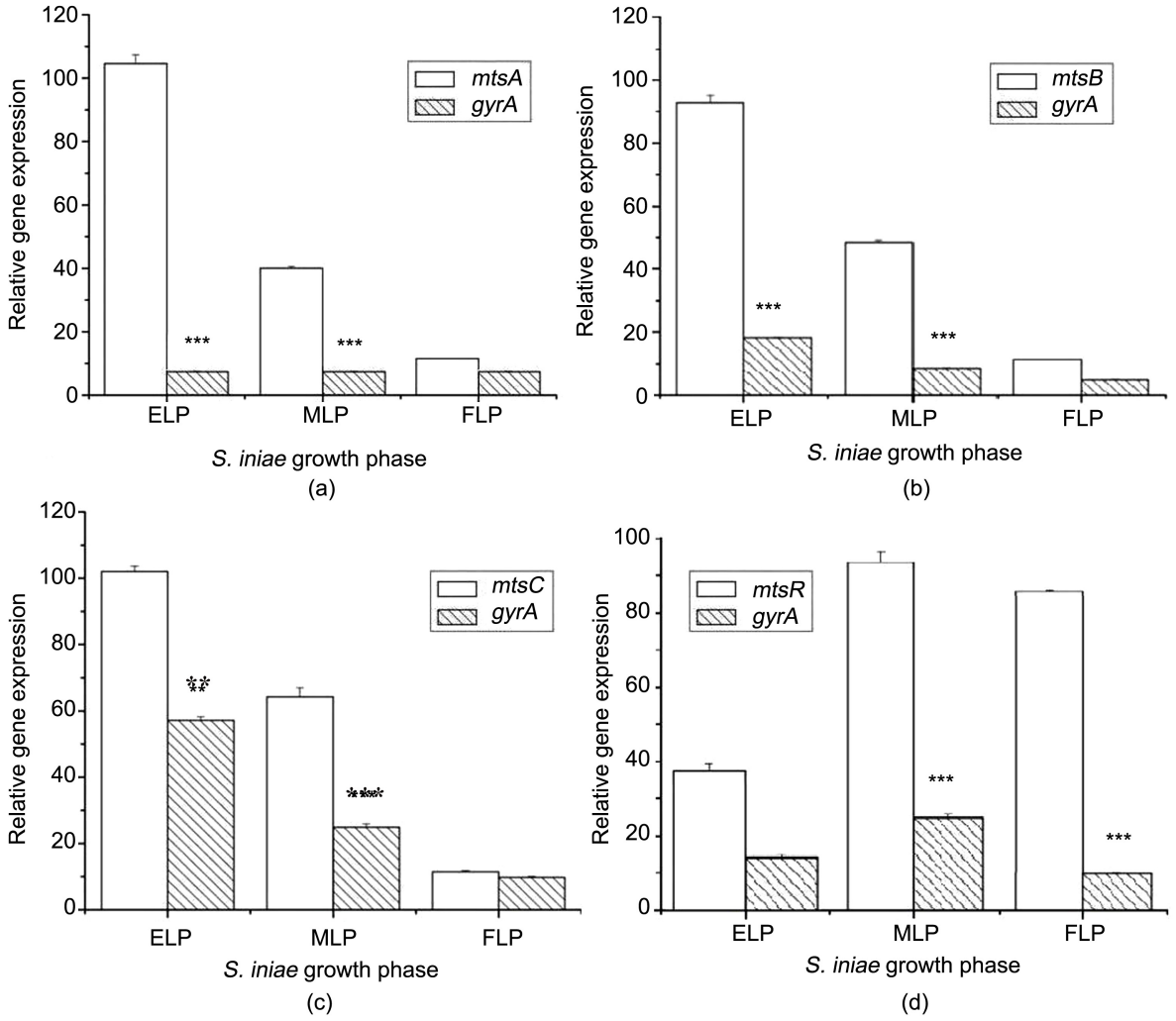

Figure 4. The relative gene expression levels of *mtsABC* and *gyrA* in BHI medium.

up-regulated the expression of *mtsABC* in response to iron availability intracellular to control the iron homeostasis in *S. iniae* HD-1.

4. Discussion

Iron is an important nutrient for various pathogens, which can often use low environmental iron levels as a signal for the induction of virulence genes [2]. Bacteria face the problem that in acquiring sufficient iron from their surroundings is particularly acute for pathogens. For bacterial pathogen, scavenging iron from the environment is less effortless than synthesizing it *de novo*. Our study showed that *S. iniae* HD-1 in the iron-restricted medium caused by addition of NTA in the final concentration 5 mM postpones the log phase of bacterial growth 3 h. When the morphological features were examined by the Gram stain, HD-1 cells showed a long chain appearance in iron-restricted medium than that of in BHI medium. These results indicated that iron was required for the growth of *S. iniae* HD-1.

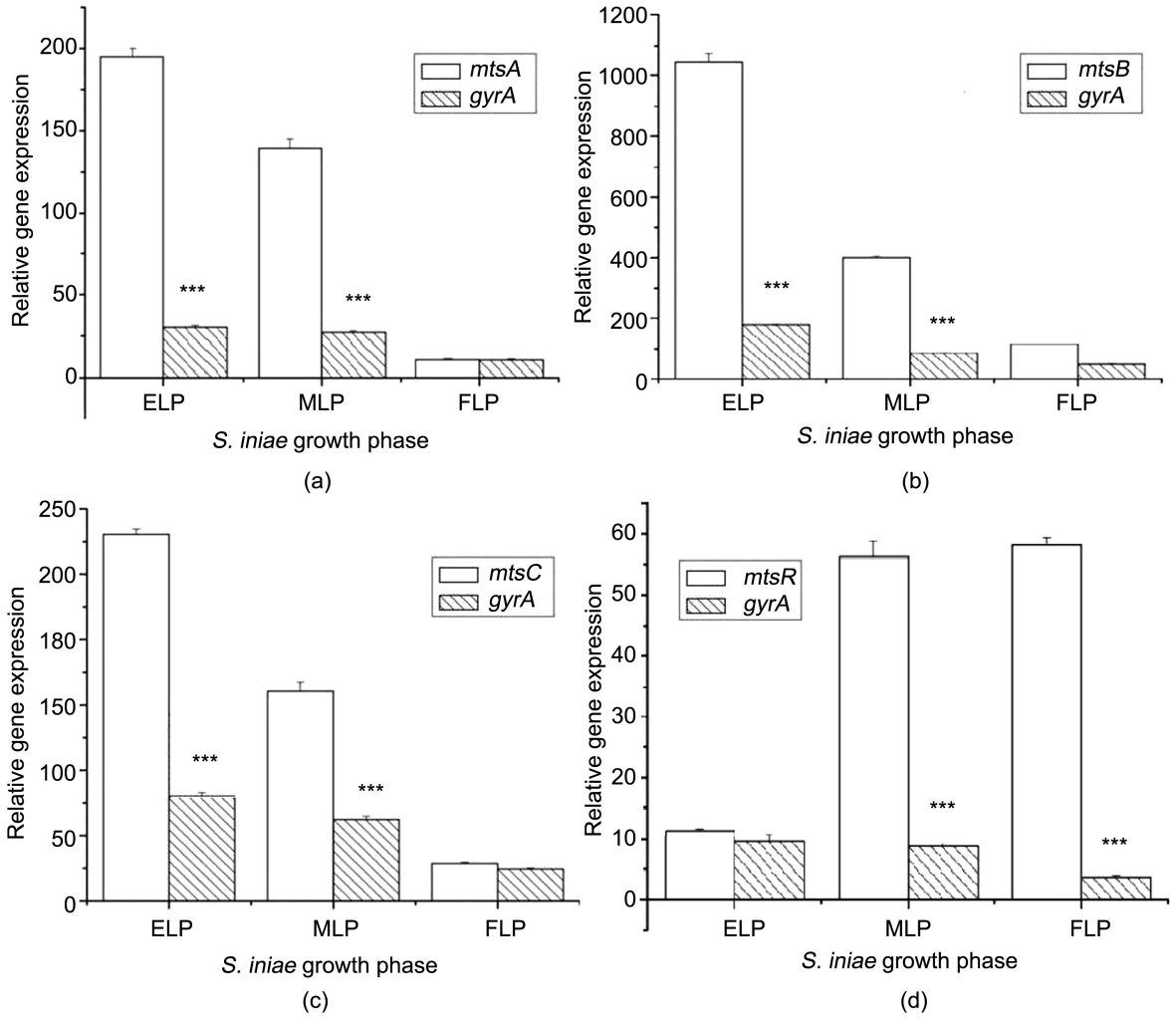

Figure 5. The relative gene expression levels of *mtsABC* and *gyrA* in BHI-NTA medium.

Iron withholding by the human host is a challenge for pathogen, as the bacterium requires iron for optimal growth. At the same time, maintaining iron homeostasis is important for the bacterial physiology as well. Therefore, like other bacterial pathogens, *S. iniae* needs to modify iron uptake in response to changes of iron availability in the environment. To address the conundrum of iron homeostasis in *S. iniae*, the gene of putative iron-dependent transcriptional regulators, *mtsR*, was cloned fromHD-1. MtsR may have both negative and positive roles in *mtsABC* expression, depending on the iron availability in the cells. Using qPCR analysis, we have demonstrated that high transcription of the *mtsABC* genes is observed when the *mtsR* maintained inactivate, suggesting that MtsR repressed *mtsABC* expression in cells growing in BHI medium. MtsR de-regulated the expression of *mtsABC* in cells grown in restricted-medium, and this de-regulation as a result of MtsR activation is very likely to lead to an increase in iron uptake by *S. iniae*. This has demonstrated that MtsR is a DtxR homologue with an important role in iron homeostasis.

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