Roles of the TonB1 and TonB2 proteins in haemin iron acquisition and virulence in *Riemerella anatipestifer*

Shuang Miao, Linlin Xing, Jingjing Qi, Hui Yu, Pan Jiang, Bingqing Sun, Junsheng Cui, Changcan Ou and Qinghai Hu

Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 518 Ziyue Road, Shanghai 200241, PR China

Two TonB systems in *Riemerella anatipestifer* were found and characterized as ExbB1–ExbD1–TonB1 and ExbB2–ExbD2–ExbD2–TonB2, but the significance of two sets of TonB complexes in *R. anatipestifer* is not clear. In this study, by deleting the tonB1 or tonB2 gene of *R. anatipestifer* strain CH3, we investigated the roles of the TonB1 and TonB2 proteins in iron acquisition and virulence. The results showed that strain CH3 could utilize haemin as the sole iron source in the presence of L-cysteine, but haemin iron acquisition was defective in the CH3 \( \Delta \) tonB1 mutant, and the deletion of either tonB1 or tonB2 significantly reduced adhesion to and invasion of Vero cells. Animal experiments indicated that the LD\(_{50}\) of the CH3 \( \Delta \) tonB1 and CH3 \( \Delta \) tonB2 mutants in ducklings was 224- and 87-fold, respectively, higher than that of the WT CH3 strain. Additional analysis indicated that blood bacterial loading of ducklings infected with CH3 \( \Delta \) tonB1 or CH3 \( \Delta \) tonB2 decreased significantly compared with that found for WT CH3-infected ducklings. Thus, our results indicated that the TonB1, but not TonB2 protein, is involved in haemin iron acquisition and that both TonB proteins are necessary for optimal bacterial virulence.

**INTRODUCTION**

Iron is an essential cofactor for several enzymes and metabolic pathways in microbes and their eukaryotic hosts (Huynh & Andrews, 2008), and a number of high-affinity iron transport systems have been identified in Gram-negative bacterial pathogens (Andrews et al., 2003; Braun & Hantke, 2011). The TonB–ExbB–ExbD complex is required for the energy-dependent, active transport of iron-bound substrates across the outer membrane of Gram-negative bacteria. The ExbB and ExbD proteins anchor TonB in the cytoplasmic membrane, whilst TonB, which serves as the energy-transducing protein, spans the periplasmic space as a dimer to interact with high-affinity outer membrane receptors (Krewulak & Vogel, 2011; Noinaj et al., 2010). Many Gram-negative bacteria harbour one or more sets of functional TonB systems, which specifically or preferentially drive the acquisition of various iron sources.

Abbreviation: DIP, 2,2'-dipyridyl.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the TonB1 and TonB2 systems in *Riemerella anatipestifer* strain CH3 are KM393215 and KM393216, respectively.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

In addition, TonB systems in Gram-negative bacteria are also involved in the uptake of vitamin B\(_{12}\), nickel, carbohydrates, phages and colicins (Blanvillain et al., 2007; Schauer et al., 2007). Furthermore, reflecting the importance of iron to the survival of bacterial pathogens within the host environment and as one of the components involved in the transduction of environmental signals, TonB protein has been shown to be essential for virulence in many bacterial pathogens (Beddek et al., 2004; Stork et al., 2004; Wang et al., 2008).

*Riemerella anatipestifer*, a member of the family Flavobacteriaceae, causes epizootic infectious disease in poultry and serious economic losses, especially to the duck industry, worldwide (Sandhu, 2008), and its iron acquisition systems are not well defined. In our previous reports, we found that 23 of 24 (95.83 %) virulent isolates and one of three avirulent *R. anatipestifer* isolates were positive for the sip gene, which encodes a siderophore-interacting protein that is involved in iron acquisition and is required for the virulence of the *R. anatipestifer* CH3 strain (Tu et al., 2014). In addition, the TonB-dependent receptor TbdR1 is involved in haemin iron acquisition and is necessary for optimal bacterial virulence (Lu et al., 2013). These previous results suggested that TonB-dependent pathways may play important roles in *R. anatipestifer* iron acquisition.
According to the full genome sequences of four *R. anatipestifer* strains in GenBank, this bacterium is predicted to contain two TonB–ExbB–ExbD systems (Mavromatis et al., 2011; Wang et al., 2014; Yuan et al., 2011). In this study, by deleting the *tonB1* or *tonB2* gene from *R. anatipestifer* strain CH3, the roles of the two TonB proteins in haemin iron acquisition and pathogenesis were investigated.

**METHODS**

**Bacterial cultures.** *R. anatipestifer* strain CH3 and its derivatives were cultured at 37 °C in tryptic soy broth (TSB) or agar (TSA) (BD) in a 5% CO2 atmosphere. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani broth or agar. For selective growth of bacterial strains, antibiotics were added at the following concentrations, unless otherwise stated: ampicillin, 100 μg ml−1; kanamycin, 50 μg ml−1; spectinomycin, 80 μg ml−1; chloramphenicol, 34 μg ml−1; erythromycin, 1 μg ml−1.

**DNA manipulation and analysis.** Genomic DNA was extracted using a QIAaamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. Plasmid DNA was extracted using a TIANprep Mini Plasmid kit (Tiangen Biotech). PCRs were performed using a high-fidelity PCR system (Roche Diagnostics). All the sequences of the primers used are listed in Table S1 (available in the online Supplementary Material). PCR products were cloned into a pGEM-T Easy vector (Promega). Automated DNA sequencing was performed at Invitrogen. DNA analysis was completed using DNASTAR 7.01 software (DNASTAR) and BLAST network services.

**Construction of deletion mutants CH3ΔtonB1 and CH3ΔtonB2.** The *tonB1* or *tonB2* genes of *R. anatipestifer* strain CH3 were deleted by allelic exchange in which a recombinant suicide vector was used to replace a 577 bp fragment of the 861 bp *tonB1* ORF for a 529 bp fragment of the 846 bp *tonB2* ORF fragment with a 1119 bp spectinomycin resistance cassette, as described previously (Lu et al., 2013; Tu et al., 2014) (Fig. S1). Upstream and downstream regions (700–1200 bp) flanking each gene were amplified with specific primers (Table S1). The 1119 bp spectinomycin resistance cassette was PCR-amplified from plasmid pFW5 using the primers Spc-F1 and Spc-F2 (Hu et al., 2011). The PCR-derived fragments were ligated into the suicide vector pDS132 (Philippe et al., 2004). *E. coli* S17-1pir was transformed with the pDS132 derivatives and conjugated with the *R. anatipestifer* strain CH3, and the transconjugants were selected on TSA agar containing kanamycin and spectinomycin. Mutants were confirmed by PCR amplification of the 16S rRNA, *spec*, *tonB1* or *tonB2* genes using specific pri-mers (Table S1). The mutant strains with the phenotype 16S rRNA "Spec" *tonB1* and 16S rRNA "Spec" *tonB2* were designated CH3Δ *tonB1* and CH3Δ *tonB2* respectively.

**Construction of the complemented strains.** For complementation analysis, recombinant plasmids were generated by cloning the intact *tonB1* or *tonB2* ORF into the *E. coli–Riemerella anatipestifer* shuttle vector pRES (Hu et al., 2013). The *tonB1* or *tonB2* ORF was ligated into plasmid pRES to generate pRES- *tonB1* and pRES- *tonB2*, respectively. The expression of *tonB1* or *tonB2* in the complementing plasmids was under the control of the putative promoter pSr. Then, plasmid pRES- *tonB1* or pRES- *tonB2* was introduced into the corresponding mutant by conjugation, as described previously (Hu et al., 2011), to generate the complemented strains CH3Δ *tonB1* (pRES- *tonB1*) for the mutant CH3Δ *tonB1* and CH3Δ *tonB2* (pRES- *tonB2*) for the mutant CH3Δ *tonB2*.

**Iron utilization assays.** The CH3 and derivative strains were tested for iron-limited growth in TSB containing the iron chelator 2,2′-dipyridyl (DIP; Sigma-Aldrich) at 0, 50, 100, 150, 200 and 250 μM. All bacterial strains were grown at 37 °C with shaking and growth curves were constructed as described previously (Hu et al., 2002). Iron utilization assays were performed in liquid culture as described elsewhere (Hagan & Mobley, 2009), with slight modifications.

Prior to inoculation, strains were washed with PBS containing 250 μM DIP and then ~10^7 c.f.u. inoculated into TSB containing 200 μM DIP supplemented with FeCl3 (200 μM) or haemin (1–100 μM; Sigma-Aldrich) as the sole iron source and incubated at 37 °C with shaking. The OD_{600} was determined as indicated.

**Bacterial adherence and invasion assays.** To determine the influence of *tonB1* or *tonB2* deletion on the adherence and invasion capacity of the *R. anatipestifer* strain CH3 to African green monkey kidney epithelial cells (Vero cells; ATCC CCL-81), a quantitative adherence assay was performed using the CH3Δ *tonB1* and CH3Δ *tonB2* mutants, the complemented strains, and the WT CH3 strain, as described previously (Hu et al., 2011). All assays were performed in duplicate and repeated on at least 3 separate days.

**Virulence assays.** Cherry Valley ducklings (1 day old) were obtained from Zhuhuang Duck Farm (Shanghai, PR China) and housed in cages under controlled temperatures ranging from 28 to 30 °C and a 12 h light/dark cycle, with free access to food and water. Animal experiments in this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee set by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Veterinary Research Institute (Permit 13-10).

To determine whether inactivation of the *tonB1* or *tonB2* gene influenced *R. anatipestifer* virulence, the LD₅₀ values of the CH3Δ *tonB1*, CH3Δ *tonB2*, CH3Δ *tonB1* (pRES- *tonB1*), CH3Δ *tonB2* (pRES- *tonB2*) and WT CH3 strains were measured as described previously (Hu et al., 2011). Briefly, for each strain, Cherry Valley ducklings (8 days old) were divided randomly into five groups (10 ducklings per group) and injected intramuscularly with 10^7, 10^8, 10^9 or 10^10 c.f.u. each bacterial strain. Ducks that became moribund were killed humanely and counted as dead. Dead ducks were subjected to *R. anatipestifer* identification. Ducks were monitored daily for clinical symptoms and death rate until 10 days post-infection and LD₅₀ values were calculated using the Reed–Muench method (Reed & Muench, 1938).

Bacterial loads in the blood from CH3Δ *tonB1*-, CH3Δ *tonB2*-, CH3Δ *tonB1* (pRES- *tonB1*), CH3Δ *tonB2* (pRES- *tonB2*)- and WT CH3-infected ducklings were compared. Cherry Valley ducklings (8 days old) were injected intramuscularly with 10^7 c.f.u. each bacterial strain, and blood samples were collected at 12 and 24 h post-inoculation (six ducklings per group per time point), and plated on TSA agar plates for counting.

**Statistical analysis.** The statistical significance of the data were determined by one-way ANOVA using Prism 5 (GraphPad). P < 0.05 was considered statistically significant.

**RESULTS**

**Genetic analysis of the TonB1 and TonB2 systems of *R. anatipestifer* strain CH3**

In this study, we cloned the genes encoding two putative TonB systems in *R. anatipestifer* strain CH3 according to
the whole-genome sequence of strain DSM15868 (GenBank accession number CP002346). The TonB1 system in *R. anatipestifer* CH3 consisted of three ORFs (ExbB1–ExbD1–TonB1) and the TonB2 system included four closely linked ORFs (ExbB2–ExbD2–ExbD2′–TonB2; Fig. S2). The two TonB systems and their flanking genes in strain CH3 were the same as those found in strains DSM15868, RA-GD, CH-1 and CH-2. Moreover, both the TonB1 and TonB2 genes of *R. anatipestifer* were located downstream of the exbB and exbD genes. There are two alleles that were found in both tonB1 and tonB2 ORFs, due to eight and two amino acid replacements, respectively. In both tonB1 and tonB2 genes, allele 1 was shared by strains CH3 and CH-1, and allele 2 was found in strains DSM15868, CH-2 and RA-GD. TonB1 and TonB2 proteins from *R. anatipestifer* CH3 shared 15.8% amino acid sequence identity. In addition, although *R. anatipestifer* strain HXb2 does not carry the *sip* gene (Tu et al., 2014), it does carry the TonB1 and TonB2 systems.

The putative conserved domains of the TonB1 and TonB2 proteins from *R. anatipestifer* strain CH3 were analysed with the National Center for Biotechnology Information Conserved Domain Database tools. The TonB1 and TonB2 proteins were shown to contain a TonB domain (COG0810) at aa 14–163 (TonB1) and 47–275 (TonB2), respectively, whilst TonB2 contained a TonB_C superfamily (pfam03544) domain at aa 209–279. In *E. coli*, the TonB_C domain could interact with the N-terminal TonB box of the outer membrane transporter that binds the Fe^{3+}–siderophore complex (Pawelek et al., 2006). This indicated that the TonB2 protein may be involved in siderophore utilization in *R. anatipestifer*.

**Phenotypic characteristics of the tonB deletion mutants**

The mutants CH3ΔtonB1 and CH3ΔtonB2 were phenotypically characterized and compared with the WT CH3 strain. The colony morphologies of the TonB mutants on TSA agar were similar to that of the WT CH3 strain (data not shown).

The growth curves of the WT CH3 strain, the CH3ΔtonB1 mutant and its complement strain CH3ΔtonB1(pRES-TonB1), and the CH3ΔtonB2 mutant and its complement strain CH3ΔtonB2(pRES-TonB2) were determined in TSB medium at 37°C with shaking. The results showed that the growth of the mutant CH3ΔtonB2 was significantly slower than that of CH3 parent strain (P<0.05; Fig. 1a) and the complemented plasmid pRES-TonB2 could restore the growth ability of the mutant CH3ΔtonB2 (P>0.05).

**Both TonB proteins play a critical role in iron acquisition**

To determine whether the TonB1 and TonB2 systems play a role in iron acquisition in *R. anatipestifer* strain CH3, the growth of the mutants CH3ΔtonB1 and CH3ΔtonB2, and the complemented strains CH3ΔtonB1(pRES-TonB1) and CH3ΔtonB2(pRES-TonB2) in TSB supplemented with different concentrations of DIP was measured. As shown in Fig. 1b, the growth of both CH3ΔtonB1 and CH3ΔtonB2 was significantly slower compared with that of the WT CH3 strain when grown in the presence of 50 μM DIP (P<0.05) and the growth defect of the mutant CH3ΔtonB2 was more severe than that of CH3ΔtonB1. In addition, the growth of CH3ΔtonB2 was almost non-existent in TSB supplemented with 100 μM DIP and so was that of CH3ΔtonB1 in TSB with 200 μM DIP. Furthermore, although the WT CH3 strain, the CH3ΔtonB1 and CH3ΔtonB2 mutants, and the complemented strains CH3ΔtonB1(pRES-TonB1) and CH3ΔtonB2(pRES-TonB2) did not grow in TSB containing 200 μM DIP, the addition of 200 μM FeCl₃ to the cultures could restore the growth of the WT CH3 strain and the complemented strains (Fig. 1c). These results suggested that both TonB1 and TonB2 were involved in iron acquisition by *R. anatipestifer* strain CH3, and that the tonB2 mutant was more impaired in its ability to utilize iron. Moreover, the growth defect of the CH3ΔtonB1 and CH3ΔtonB2 mutants may have been due to their decreased capacity for iron acquisition.

**TonB1, but not TonB2, plays an important role in haemin utilization**

As shown in Fig. 2, the growth of strain CH3 was unaffected in TSB supplemented with 0–10 μM haemin, but decreased in TSB containing 20 μM haemin and was non-existent in TSB containing 40 μM haemin. Additionally, in TSB containing 250 μM DIP, the growth of strain CH3 was almost non-existent in TSB supplemented with 0–40 μM haemin (Fig. 2a). It was suggested that *R. anatipestifer* cannot utilize haem as the sole iron source under these conditions. To test whether L-cysteine plays a role in haemin utilization in *R. anatipestifer*, the growth of CH3 in TSB supplemented with 250 μM DIP, 10 μM haemin and 0–6 mM cysteine was measured. The results showed that the growth defect of CH3 in iron-depleted TSB could be rescued by haemin in the presence of 1–6 mM L-cysteine (Fig. 2b). Growth of CH3 in TSB containing 4 mM cysteine, 250 μM DIP and 0.5–20 μM haemin was almost the same as that in TSB medium, whilst it was defective in the presence of 50 μM haemin (Fig. 2c). This suggested that *R. anatipestifer* CH3 could utilize haemin as the sole available iron source in the presence of L-cysteine.

To further explore the roles of the TonB1 and TonB2 proteins in haemin transport in *R. anatipestifer*, the WT CH3 strain, the CH3ΔtonB1 and CH3ΔtonB2 mutants, and the complemented strains CH3ΔtonB1(pRES-TonB1) and CH3ΔtonB2(pRES-TonB2) were grown in iron-depleted TSB supplemented with 10 μM haemin as the sole available iron source in the presence of 4 mM L-cysteine. The results showed that the WT CH3 strain, CH3ΔtonB2 and the complemented strain CH3ΔtonB2(pRES-TonB2) grew normally under these conditions, whilst CH3ΔtonB1 and the complemented strain CH3ΔtonB1(pRES-TonB1) displayed...
a severe growth defect (Fig. 2d); however, the recombinant shuttle plasmid pRES-TonB1 could not restore the growth defect of mutant CH3\textit{tonB1}. The results indicated that \textit{TonB1}, but not \textit{TonB2}, of \textit{R. anatipestifer} strain CH3 was involved in haemin iron transport.

To investigate whether the deletion of \textit{tonB1} or \textit{tonB2} played a role in adhesion to a biotic surface, we assessed the capacity of the WT CH3 strain, the CH3\textit{tonB1} and CH3\textit{tonB2} mutants, and the complemented strains CH3\textit{tonB1}(pRES-TonB1) and CH3\textit{tonB2}(pRES-TonB2) to adhere to Vero cells. The adhesion and invasion capacities of the CH3\textit{tonB1} and CH3\textit{tonB2} mutants to Vero cells were significantly decreased compared with those of the WT CH3 strain (\(P<0.01\); Fig. 3), and complementation of the mutants with plasmids pRES-TonB1 or pRES-TonB2 significantly restored their adhesion and invasion capacities (\(P<0.01\)). These results suggested that the deletion of \textit{tonB1} or \textit{tonB2} influenced the interaction between \textit{R. anatipestifer} and host cells.

**TonB1 and TonB2 systems are required for the virulence of \textit{R. anatipestifer}**

The ability to acquire iron under low-iron conditions is required for the virulence of a variety of bacterial pathogens (Tai \textit{et al.}, 1993). To determine the effect of the \textit{tonB1} or \textit{tonB2} deletions on the virulence of the \textit{R. anatipestifer} strain CH3, groups of Cherry Valley ducklings (8 days old) were inoculated with the WT CH3 strain, the \textit{tonB} mutants or the complemented strains. All of the strains were able to invade and infect deeper tissues, and to cause systemic infection and death. At 10 days post-infection, the calculated LD\textsubscript{50} values of the WT, CH3\textit{tonB1}, CH3\textit{tonB1}(pRES-TonB1), CH3\textit{tonB2} and CH3\textit{tonB2}(pRES-TonB2) strains were \(2.29 \times 10^7\), \(5.13 \times 10^9\), \(4.9 \times 10^9\), \(2.0 \times 10^9\) and \(2.14 \times 10^8\) c.f.u., respectively. For the CH3\textit{tonB1} and CH3\textit{tonB2} mutants, the \(\sim 224\)- and \(\sim 87\)-fold increases, respectively, in the LD\textsubscript{50} values compared with that of the WT CH3 strain indicated that
disruption of either tonB1 or tonB2 resulted in the attenuation of *R. anatipestifer* virulence. Meanwhile, the LD$_{50}$ values of the complemented strains CH3$\Delta$tonB1(pRES-TonB1) and CH3$\Delta$tonB2(pRES-TonB2) were similar to that of the CH3 strain.

In addition, to investigate the effect of the deletion of tonB1 or tonB2 during systemic *R. anatipestifer* infections *in vivo*, bacteria were recovered from the blood of infected ducklings at 12 and 24 h post-inoculation and bacterial colonies were determined by plate counting. As shown in Fig. 4, the bacterial loads in the blood of ducklings infected with the CH3$\Delta$tonB1 or CH3$\Delta$tonB2 mutants decreased significantly in comparison with those from ducklings infected with the WT CH3 strain ($P<0.01$). Taken together, these results provided evidence that both TonB1 and TonB2 were involved in *R. anatipestifer* pathogenesis.

**DISCUSSION**

In Gram-negative bacteria, TonB, ExbB and ExbD form a cytoplasmic membrane complex required for high-affinity iron transport. In *E. coli* and most other Gram-negative bacteria, one single set of the TonB–ExbB–ExbD complex mediates the assimilation of multiple types of iron-loaded siderophores or ligands (Faraldo-Gómez & Sansom, 2003). However, *R. anatipestifer*, similar to some other bacteria, such as *Vibrio cholerae*, *Vibrio anguillarum*, *Actinobacillus pleuropneumoniae* and *Pseudomonas aeruginosa*, has been shown to harbour two or more sets of functional TonB...
systems. The significance of two sets of TonB complexes in *R. anatipestifer* is not clear. Generally, two distinct TonB systems may play different roles in iron acquisition. The two TonB systems in *V. cholerae* were shown to have unique, as well as common, functions. Both TonB systems functioned well in the transport of the siderophore vibriobactin, whilst the TonB2 system was required for utilization of enterobactin and for haem transport via HasR (Seliger *et al.*, 2001; Wyckoff *et al.*, 2007). In addition, although the TonB1 system specifically contributed to haemin and haemoglobin utilization in *Vibrio alginolyticus* MVP01, both TonB systems showed the ability to support the utilization of iron sources from ferrichrome and vibrioferrin, the endogenous siderophore of *V. alginolyticus* (Wang *et al.*, 2008). In this study, our results suggested that although the two TonB systems are involved in iron utilization by *R. anatipestifer* CH3, the TonB1, but not TonB2, system may play an important role in haemin iron utilization. It was noteworthy that *R. anatipestifer* CH3 utilized haemin as an iron source in the presence of L-cysteine. This may be due to the specific culture requirements of *R. anatipestifer*. Most *R. anatipestifer* strains grew better on TSA in a 5% CO2 incubator or in a candle jar than that on TSA without additional CO2, but also grew well with shaking in TSB without additional CO2. In addition, the fact that the tonB1 mutant was more attenuated in virulence than the tonB2 mutant may be related to a haemin iron acquisition defect in the mutant CH3ΔtonB1.

Whether the TonB1 or TonB2 systems are involved in the uptake of other substances, such as haemoglobin, holotransferrin, vitamin B12, nickel or carbohydrates, remains to be clarified. Some *R. anatipestifer* strains can secret siderophores (our unpublished data), but we have not purified siderophores from *R. anatipestifer* to test which TonB system contributes to the import of iron–siderophore complexes into the cytosol. In addition, we also did not determine why the tonB2 deletion in *R. anatipestifer* strain CH3 exhibited a greater growth defect than that of the tonB1 deletion. These questions remain to be clarified in future studies.

The control of iron homeostasis is central in the host–pathogen interplay and influences the course of an infectious disease in favour of either the host or the pathogenic invader (Naiz *et al.*, 2010). In our previous studies, we showed that the deletion of the *tbdR1* or *sip* genes, which were also involved in iron acquisition, reduced the pathogenicity to ducklings, biofilm formation, and adherence to and invasion of Vero cells (Lu *et al.*, 2013; Tu *et al.*, 2014). In this study, consistent with their roles in iron acquisition, both TonB1 and TonB2 were found to be involved in host infections. In fact, this is a common phenomenon. Many previous studies of Gram-negative bacteria suggested that iron may serve as the ‘critical determinant’ that decides the outcome of host–bacteria interactions (Banin *et al.*, 2005; Crosa, 1989; Weinberg, 1978) and that iron is an important environmental signal that controls the differential expression of a large number of genes, some of which encode important bacterial virulence factors (Rhodes *et al.*, 2007). In addition, the expression of adherence factors by several bacterial species is influenced by the environmental iron supply (Alves *et al.*, 2010). In a previous study, iron was shown to have a regulatory role in the adhesion of diphtheria bacilli to cells of the human respiratory tract (Hep-2 cells) and blood (erythrocytes), and low iron availability modulated the expression of *Corynebacterium*...

In conclusion, our results showed that l-cysteine plays an important role in haemin utilization in R. anatipestifer and that TonB1, but not TonB2, was involved in haemin iron acquisition in the presence of l-cysteine. Moreover, both the tonB1 and tonB2 mutants showed an impaired adherence capacity to Vero cells, as well as reduced virulence.

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