The Expression of the fim Operon Is Crucial for the Survival of Streptococcus parasanguinis FW213 within Macrophages but Not Acid Tolerance

Yi-Ywan M. Chen1,2*, Hui-Ru Shieh1, Ya-Ching Chang2
1 Department of Microbiology and Immunology, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan, 2 Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan

Abstract
The acquisition of transition metal ions is essential for the viability and in some cases the expression of virulence genes in bacteria. The fimCBA operon of Streptococcus parasanguinis FW213 encodes a Mn2+/Fe2+-specific ATP-binding cassette transporter. FimA, a lipoprotein in the system, is essential for the development of endocarditis, presumably by binding to fibrin monolayers on the damaged heart tissue. Recent sequence analysis revealed that Spaf_0344 was designated fimR. By using various fim promoter (p_fim) derivatives fused with a promoterless chloramphenicol acetyltransferase gene, the functions of the cis-elements of p_fim were analyzed in the wild-type and fimR-deficient hosts. The result indicated that FimR represses the expression of p_fim and the palindromic sequences 5’ to fimC are involved in repression of p_fim. A direct interaction between FimR and the palindromic sequences was further confirmed by in vitro electrophoresis gel mobility shift assay and in vivo chromatin immunoprecipitation assay (ChIP)-quantitative real-time PCR (qPCR). The result of the ChIP-qPCR analysis also indicated that FimR is activated by Mn2+ and, to a lesser degree, Fe2+. Functional analysis indicated that the expression of FimA in S. parasanguinis was critical for wild-type levels of survival against oxidative stress and within phagocytes, but not for acid tolerance. Taken together, in addition to acting as an adhesin (FimA), the expression of the fim operon is critical for the pathogenic capacity of S. parasanguinis.

Introduction
Streptococcus parasanguinis is a primary colonizer of the tooth surface and an important member of the dental plaque [1,2]. Occasionally, S. parasanguinis and other viridans streptococci can enter the bloodstream, causing a transient bacteremia and infective endocarditis on native and prosthetic heart valves [3,4]. Although the significance of S. parasanguinis in the oral ecosystem and systemic infection is well established, thus far the only known virulence factor associated with endocarditis is FimA of the FimCBA Mn2+/Fe2+ ATP-binding cassette (ABC) transporter [5]. FimA, a member of the lipoprotein receptor antigen I (LraI) family, participates in both metal transportation [5] and adherence to fibrin [6]. Binding to the fibrin and platelets deposited on the damaged heart tissues is critical for vegetation formation; therefore, it is proposed that FimA mediates the development of endocarditis by binding to the fibrin monolayer [6]. A FimA-deficient S. parasanguinis is avirulent in an animal model [6]. Immunization with the purified FimA protein prior to infection with S. parasanguinis FW213 also reduces the frequency and severity of infection in the rat model [7], further confirming the impact of FimA in disease development.

Genes encoding FimCBA transporter along with tpx, encoding a thiol peroxidase, are arranged as an operon in S. parasanguinis FW213 (fimCBA-tpx) [8]. The expression of the promoter located 5’ to fimC (p_fim), which transcribes fimCBA and tpx [5,8], is inhibited by 10 μM Mn2+, but neither Fe2+ nor Mg2+ influences the expression [5]. An additional constitutive promoter, p_psa, is located in the intergenic region between fimA and tpx [8], thus the expression of tpx is initiated from both p_fim and p_psa [8,9].

The fimCBA-tpx operon arrangement of S. parasanguinis is similar to the psa operon of Streptococcus pneumoniae, the ssa operon of Streptococcus sanguinis, and the slo operon of Streptococcus gordonii [10–12]. A homologous operon, sloABCR, is present in Streptococcus mutans [13]. However, instead of a tpx, the last gene of the slo operon encodes a metalloregulatory protein for the Slo system, whereas the loci encoding the specific regulators of the Psa, Ssa and Sca systems are not located in the flanking region of the structural genes [14,15]. In addition, FimA, along with PsaA of the Psa system, SsaB of the Ssa system, ScaA of the Sca system, and SloC of the Slo system all play a major role in the virulence capacity of the microbes [10,12,15–21].

The expression of psa, ssa and slo operons is subject to the regulation of PsaR, ScaR and SloR of the Diphtheria toxin repressor (DtxR) family, respectively, in the presence of excess amounts of cognate metal ions [14,22,23]. The consensus binding sequence of DtxR and its homologues have been determined as the

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* E-mail: mchen@mail.cgu.edu.tw
key cis-regulatory element in several systems [15,24]. The binding sequences of PsaR, ScaR and SloR all contain a palindromic sequence rich in A/T, albeit the overall lengths of the proposed operators vary among the three. Specifically, the predicted operators for PsaR [22] and SloR [25] contain one palindromic of an 8-nucleotide (nt) inverted repeat spaced by 6 nt (AAAT-TAACCTTGAATTA1TTT), whereas the proposed operator of ScaR contains an additional imperfect inverted repeat of 9 nt (TGTTAAGGTATATTA1TA), with a total length of 46 nt [14]. Although the second inverted repeat was also observed in psa and slo promoters with a distance to the first palindromic similar to that in sea operon, the function of the second palindromic in the binding of PsaR and ScaR is unknown. Both PsaR and ScaR are activated by Mn2+ and additional metal ions, such as Cr3+, but not Zn2+, and it is suggested that an excess amount of Zn2+ could ensure an optimal uptake of Mn2+ by inactivation of PsaR and ScaR [26,27]. On the other hand, SloR is a bifunctional regulator that exerts both positive and negative regulation when Mn2+ is available. SloR is a repressor if the SloR recognition element (SRE) is located within 50 bp of the transcription initiation site of the target gene. When the SREs are located further upstream, SloR acts as an activator [25]. Moreover, like many other metalloregulatory proteins, both PsaR and SloR regulate other genes in addition to the cognate metal uptake system [22,25], confirming the critical role of the intracellular metal homeostasis in the physiology and pathogenesis.

A scAR homologue (Spaf_0344), approximately 2 kb 3′ to the fim operon, was identified previously by chromosomal walking (ACR24649). The recent transcriptomic analysis of S. parasanguinis FW213 further confirmed the expression of Spaf_0344 [28]. In this study, we investigated the regulatory function of Spaf_0344 on fim operon expression, and the impact of the regulation on the pathogenic capacity of S. parasanguinis FW213. Our results indicated that in addition to acting as an adhesin (FimA), the expression of the fim operon in S. parasanguinis is critical for the optimal capacity against oxidative stress and wild-type levels of survival within phagocytes.

**Results**

**Identification of fimR**

Sequence analysis of the 3′ flanking region of fim operon revealed two open reading frames (ORFs), Spaf_0345 and Spaf_0344, in opposite orientations (Figure 1). Both ORFs began within 50 bp of the transcription initiation site of the target gene. The relative location and transcription direction of each ORF are shown. Spaf_0345 and Spaf_0344 are indicated as 0345 and fimR, respectively. The limits of the sequence present in Figure 2A are indicated by two vertical arrows. The position of the erm in strain ΔfimR is indicated by an inverted triangle above the gene. The putative terminators for Spaf_0345 and fimR are indicated. The sizes of Spaf_0345 and fimR in nt, predicted molecular weight in kDa and pl of the gene products are shown. doi:10.1371/journal.pone.0066163.g001

Figure 1. Schematic diagram of the fim operon and its flanking regions of S. parasanguinis FW213. The relative location and transcription direction of each ORF are shown. Spaf_0345 and Spaf_0344 are indicated as 0345 and fimR, respectively. The limits of the sequence present in Figure 2A are indicated by two vertical arrows. The position of the erm in strain ΔfimR is indicated by an inverted triangle above the gene. The putative terminators for Spaf_0345 and fimR are indicated. The sizes of Spaf_0345 and fimR in nt, predicted molecular weight in kDa and pl of the gene products are shown.

FimR Negatively Regulated the Expression of p fim

Sequence analysis reveals two inverted repeats in the intergenic region of pepO and fimC (Figure 2A), the potential targets of the DtxR-family proteins [14]. To analyze the impact of FimR on fim operon expression and its possible binding region, a series of p fim-chloramphenicol (Cm) acetyltransferase gene (cat) fusion derivatives with different lengths of the 5′ flanking region were established in the wild-type and fimR-deficient (ΔfimR) S. parasanguinis as detailed in the materials and methods. Of note, all fusions were tagged with a spectinomycin (Sp) resistance gene (spe) [30] at the 5′ end of the fusion. The spe cassette contains a strong terminator and is in the same transcription direction as the fusion, thus preventing any possible read through effect from the 5′ flanking region. Since regulatory proteins of DtxR family are generally activated by multiple metal ions, the promoter activity in all strains was determined in cells grown in the complex medium, Todd-Hewitt (TH) broth. A basal and unregulated Cm acetyltransferase (CAT) expression was observed in strains with the p fim(33 b)-cat fusion (Figure 2B). With all other fusion constructs, a lower level of CAT activity was detected in the wild-type background than that in ΔfimR (P<0.01, Student’s t test), indicating that FimR represses the expression of p fim. A comparable expression level was detected in strains harboring p fim(445 b)-cat, p fim(239 b)-cat and p fim(151 b)-cat fusions, indicating that all cis-elements are located within the 151-base region. As p fim(445 b)-cat fusion contains the longest 5′ flanking region of p fim, this fusion was used as the full-length p fim control in the following analysis. Elevated CAT activities were detected in both the wild-type FW213 and ΔfimR harboring the p fim(109 b)-cat fusion, suggesting that the sequence between −151 and −109 contains a negative regulatory element. Further shortening the length of p fim by 30 bases (p fim(59 b)-cat) reduced the CAT expression, suggesting that the sequence from −109 to −59 is essential for optimal expression. Trans-complementation of fimR on pDL276 (pHR6) in...
strain ΔfimR harboring p_fim(445 b)-cat restored wild-type p_fim expression (Figure S1). Taken together, p_fim is negatively regulated by FimR. The different expression levels in ΔfimR harboring various fusions also suggest the presence of additional regulators.

The Expression of p_fim was Modulated by both Mn^{2+} and Fe^{2+}

Previous studies by Oetjen et al. demonstrated that fimCBA encodes an uptake system for manganese and iron [5]. However, the expression of the fim operon is repressed only by Mn^{2+} but not Fe^{3+}. To investigate whether Fe^{2+} is involved in the FimR-mediated regulation, the CAT activity in the wild-type FW213 and ΔfimR in the presence of various amounts of Mn^{2+} and Fe^{2+} was determined (Figure 3). To precisely control the content of the metal ions, cells were cultivated in the chemically-defined medium FMC supplemented with various amounts of Mn^{2+} and Fe^{2+} as detailed in the materials and methods. As expected, an up regulation of p_fim expression was consistently observed in ΔfimR under all conditions used, confirming the negative effect of FimR on p_fim expression. With 50 μM of Mn^{2+} and/or Fe^{2+}, the p_fim activity in ΔfimR was approximately twofold higher than that in the wild-type strain, and comparable expression levels were detected among the three conditions (Figure 3, lanes II to IV), indicating that FimR is active in the presence of Mn^{2+} or Fe^{2+}. However, when cells were grown under limited Mn^{2+} (0.01 μM) and Fe^{2+} (0.1 μM), a further up regulation was observed in both the wild-type and ΔfimR hosts (Figure 3, lane I), suggesting that additional regulation modulated by the amounts of Mn^{2+} and Fe^{2+} also participates in the regulation. To confirm that the observed differential expression in response to Mn^{2+} and Fe^{2+} was driven by the promoter but not the nature of CAT, we also monitored the activity of p_fap1, whose expression is insensitive to Mn^{2+} and/or Fe^{2+} contents, under various metal conditions by using a S. parasanguinis p_fap1-cat fusion strain. A comparable CAT activity was observed in this strain under all four metal conditions (Figure S2), confirming the regulation of p_fim in response to metal conditions.

FimR Binds to p_fim Directly

As the predicted FimR binding site overlaps with −35 and −10 elements of p_fim, the reporter assay described above does not allow us to analyze directly the impact of this region in p_fim expression. Thus, electrophoretic mobility shift assay (EMSA) was used to determine if FimR directly interacts with p_fim. A biotin-labeled DNA fragment containing both inverted repeats 5′ to the +1 of p_fim (Figure 2A) was incubated with increasing amounts of purified histidine-tagged FimR (His-FimR) in the presence Mn^{2+}. Two probe-FimR complexes were evident with 40 μM His-FimR, and the complexes with the slower mobility become clear in the

Figure 2. The regulation of FimR on p_fim expression. (A) The nt sequence of the 5′ flanking region of fimC. The pepO and fimC are transcribed from the opposite DNA strands, thus the sequence of pepO presented here is the noncoding strand, and the sequence of fimC is the coding strand. The transcription initiation sites (+1) of fimC and pepO are shown by a solid triangle above the sequence, and two open triangles below the sequence, respectively. The putative −10 and −35 sequences of p_fim are shaded. The potential Per box is underlined. The inverted repeat sequences are shown by horizontal arrows above the sequence. The sequence of the probe used in EMSA is boxed. The limits of the deletion derivatives are indicated by the numbers. (B) The CAT activities in wild-type FW213 and ΔfimR harboring various p_fim-cat fusions. All strains were grown in TH. Values shown are means and standard deviations of three independent experiments. All experiments were done in triplicate reactions and negative controls were reactions carried out in the absence of Cm. doi:10.1371/journal.pone.0066163.g002
reaction with 80 μM His-FimR (Figure 4). The shift pattern remained the same in the presence of additional unlabelled tcrB fragment, indicating that FimR binds specifically to the p_tcrB probe. This result also suggests the presence of two FimR binding sites on the probe.

To confirm the in vivo binding of FimR to p_tcrB and to determine the impact of Mn^{2+} and Fe^{2+} on the binding activity of FimR, chromatin immunoprecipitation (ChIP) assay-quantitative real time PCR (qPCR) with anti-FimR antibody was employed as detailed in the materials and methods. The strongest binding of FimR to p_tcrB was detected in cells grown in the presence of 50 μM MnCl₂ and 50 μM FeSO₄ (Figure 5, lane IV), whereas minimal amounts of MnCl₂ (0.01 μM) and FeSO₄ (0.1 μM) led to the weakest binding (Figure 5, lane I). Although both Fe^{2+} and Mn^{2+} at 50 μM can activate FimR, an 1.8-fold increase in the relative quantity was observed when 50 μM Mn^{2+} was provided in the culture medium compared to medium containing 50 μM Fe^{2+} (Figure 5, lanes II and III), indicating that Mn^{2+} is more effective than Fe^{2+} for FimR activation. Taken together, in the presence of excess amounts of Mn^{2+} or Fe^{2+}, FimR repressed the expression of p_tcrB by directly binding to the target sequence.

**Figure 3. Effect of Mn^{2+} and Fe^{2+} on p_fim expression.** Wild-type FW213 and ΔfimR harboring p_tcrB(P44s b)-cat were grown in FMC containing 0.01 μM MnCl₂ and 0.1 μM FeSO₄ (I), 0.01 μM MnCl₂ and 50 μM FeSO₄ (II), 50 μM MnCl₂ and 0.1 μM FeSO₄ (III), 50 μM MnCl₂ and 50 μM FeSO₄ (IV). All cultures were supplemented with 1 mM MgSO₄ and 1 mM CaCl₂. Values are means and standard deviations of three independent experiments. Significant differences between samples were determined by two-way ANOVA using SPSS Statistic 17.0. The ρ values between the wild-type strain and ΔfimR under all four conditions are less than 0.01. P values between condition I and II are indicated in the figure. *, P<0.05; **, P<0.01. doi:10.1371/journal.pone.0066163.g003

**Figure 4. EMSA demonstrating the interaction between FimR and p_tcrB.** Lanes 1 to 4 are reactions containing 0, 20, 40, and 80 μM His-FimR, respectively; lane 5 is reaction containing 80 μM His-FimR and unlabeled tcrB. The positions of the FimR-probe complexes are indicated by triangles. doi:10.1371/journal.pone.0066163.g004

**Figure 5. ChIP-qPCR demonstrating the relative quantity of p_fim bound by FimR.** Cells were grown under 0.01 μM MnCl₂ and 0.1 μM FeSO₄ (I), 0.01 μM MnCl₂ and 50 μM FeSO₄ (II), 50 μM MnCl₂ and 0.1 μM FeSO₄ (III), and 50 μM MnCl₂ and 50 μM FeSO₄ (IV). The ΔCq of the sample from III was used as the reference. Significant differences between samples were determined using one-way ANOVA. A significant difference (P<0.05) was detected between all pairs of comparison. doi:10.1371/journal.pone.0066163.g005

FimA is Required for *S. parasanguinis* Defense against Oxidative Stress

As intracellular metal homeostasis is linked closely to the oxidative stress response, the possible role of FimA and FimR regulation in avoiding oxidative challenge was examined. Generally, regulatory proteins of DtxR family modulate not only metal homeostasis but also the expression of other genes, thus a fimA and fimR double mutant strain (VT930_AfimR) was also included in the following studies to differentiate the impact of fimA and other genes regulated by FimR. The growth of all strains in the presence of paraquat, a redox-cycling compound that can cause oxidative stress by generating superoxide radical in the cytoplasm, was monitored. It was noticed that inactivation of fimA (VT930) [31] or both fimA and fimR (VT930_AfimR) enhanced the growth in TH broth, whereas fimR-deficiency alone (ΔfimR) led to a longer doubling time than the wild-type strain (Figure 6A). The estimated doubling time for the wild-type FW213, VT930, ΔfimR and VT930_AfimR in TH is 80, 50, 105 and 50 min, respectively. In the presence of 2 mM paraquat, a reduced growth rate was detected in both the wild-type FW213 and AfimR. The lag phase in ΔfimR was slightly shorter than that in the wild-type strain in the presence of 2 mM paraquat (Figure 6B), and the difference between these two strains was more pronounced under 4 mM paraquat (Figure 6C). On the other hand, the growth of VT930 and VT930_AfimR was severely hampered in the presence of paraquat. Thus, a functional FimCBA transport system is essential for optimal oxidative stress responses in *S. parasanguinis*. As VT930 and VT930_AfimR bear a similar capacity against paraquat challenge, it is concluded that the expression of fimA plays a key role in this process.

FimA Enhances the Intracellular Survival of *S. parasanguinis* within Macrophages

Macrophages are critical for defending microbial infection, thus the impact of FimA and FimR regulation in the survival of *S. parasanguinis* within macrophages was analyzed. Of note, inactivation of fimA does not inhibit the uptake of the bacteria by granulocytes [6]. The intracellular survival rate of VT930 and VT930_AfimR within THP1 was less than 30% of that of wild-type FW213, whereas the survival rate of ΔfimR was twofold greater than that of wild-type FW213 (Figure 7A). A similar survival pattern between wild-type FW213, VT930, ΔfimR and VT930_AfimR was detected in RAW264.7 macrophages.
As the expression of *fim* operon was negatively regulated by FimR, and VT930 and VT930_ΔfimR exhibited a comparable survival rate in both macrophages used, these results indicated that the expression of FimA is critical for wild-type levels of survival within macrophages.

The Expression of the *fim* Operon is not Required for the Acid Tolerance of *S. parasanguinis*

As oxidative stress responses are known to overlap with acid tolerance [32–35], the possible function of FimA and FimR in acid tolerance was determined by an acid killing assay. When the survival rates at pH 3 was examined, a time-dependent decline in survival rate was observed with all strains tested. Interestingly, the viability of ΔfimR was lower than that of the wild-type FW213, whereas inactivation of *fimA* or both *fimA* and *fimR* enhanced the survival at pH 3 (Figure 8). These results indicated that, opposite to the oxidative stress responses, *S. parasanguinis* was more sensitive to acidic challenges when the *fim* operon was highly expressed.

**Discussion**

This study set to investigate the regulation and expression of the FimCBA transport system on the pathogenic capacity of *S. parasanguinis* FW213. We found that the expression of the *fim* operon is regulated by FimR and additional trans-acting element(s), and the expression of the *fim* operon is critical for the oxidative stress responses and survival of *S. parasanguinis* against phagocytic killing. Our results also indicated that the expression of *p_fim* is sensitive to both Mn²⁺ and Fe²⁺. Such regulation will ensure an adequate uptake of Mn²⁺ and Fe³⁺ for growth and avoid potential toxicity caused by excess amounts of intracellular Fe²⁺. As homologues of FimR are known global regulators, it is likely that *S. parasanguinis* possesses a FimR regulon. However, all phenotypes of ΔfimR observed in this study result from up regulation of the *fim* operon, indicating that the intracellular homeostasis of Mn²⁺ and Fe³⁺ is critical for the described phenotypes.

That metal uptake systems are regulated by multiple regulators is not unique to the *S. parasanguinis* FimCBA system. For instance, the expression of the *mtsABC* of *Streptococcus pyogenes*, encoding an ABC transporter for Mn²⁺ (mainly) and Fe³⁺, is regulated by both MtsR and PerR [36]. Both MtsA and FimA belong to the LraI
family [37], and MsxR, a member of the DtxR family proteins, represses the expression of mtsABC in response to Mn^{2+}. PerR, a parologue of Fur, generally acts as a metal-dependent and oxidative-responsive repressor. However, PerR positively regulates the expression of mtsABC unresponsive to Mn^{2+}, Fe^{3+}, and Zn^{2+} in S. pyogenes [36,30]. Sequencing analysis of the 5′ flanking region of S. parasanguinis fimC revealed a putative Per box located at -71 to -57 of p_{fim} (Figure 2A). This motif differs from the consensus sequence (TTANATNNATTNA) derived from Bacillus subtilis and S. pyogenes [39] by 3 bases (Figure 2A). Furthermore, the BlastX search result found that Spaf_616 encodes a Fur family transcriptional regulator that shares 83% identity with the PerR of S. pyogenes MGAS6180 (AAX71273). As a positive effect on expression was detected between -109 to -59 of p_{fim}, it is possible that the expression of the fim operon in S. parasanguinis is positively regulated by Spaf_616. Unfortunately, multiple tries failed to generate a Spaf_616 mutant, thus the possible involvement of Spaf_616 in fim operon expression remains unknown.

It is peculiar that the highest p_{fim} activity was detected in strain p_{fim}(109 b)-cat, whereas both extending and reducing the promoter by 42 b (p_{fim}(151 b)-cat) and 50 b (p_{fim}(59 b)-cat), respectively, reduced the promoter activity (Figure 2C). Further sequence analysis revealed a putative catabolite responsive element (cre) (TGTAACAGTACCAT), the binding sequence of the catabolic control protein A (CcpA), located at -146 to -133 of p_{fim}. This motif is only 2 bases different from the proposed cre of S. pyogenes (TGWAANSBHTWHHH) [40]. Interestingly, inactivation of ccpA led to a higher CAT activity in FW213 harboring p_{fim}(151 b)-cat. However, the increase in CAT activity was also detected in strains p_{fim}(109 b)-cat and p_{fim}(59 b)-cat (data not shown), indicating that the predicted cre is not involved in the regulation and CcpA modulates p_{fim} expression indirectly. As the FimCBA transport system also transports iron, presumably the CcpA-mediated repression of p_{fim} could provide an additional control of the intracellular iron and subsequently reduce the oxidative damage resulting from the Fenton reaction. The link between metabolism and oxidative stress response via the regulation of CcpA has been reported in Lactococcus lactis [41]. CcpA activates the expression of FhuR, the repressor for the haem uptake system FhuBGD, and thus prevents oxidative damage caused by excess amounts of intracellular iron at the onset of exponential growth in L. lactis [41]. Of note, no potential cre was detected in the 5′ flanking region of fimR, thus, the function of CcpA on p_{fim} remains unclear.

The intracellular manganese and zinc homeostasis are co-regulated by PsaR and AdcR in S. pneumoniae [26]. AdcR is the repressor of the AdcCBA Zn^{2+} ABC transporter that represses the expression of adcCBA in the presence of excess amounts of Zn^{2+} [42]. Excess amounts of intracellular Zn^{2+} resulted from adcR-deficiency can compete with Mn^{2+} in binding to PsaR, albeit at a lower efficiency, and subsequently derepress p_{fim} operon [26]. An adcRCBaI homologue is present in the genome of S. parasanguinis FW213. However, in contrast to the regulation in S. pneumoniae, inactivation of adcR with a non-polar erm lowered p_{fim} expression in S. parasanguinis, regardless the amount of Zn^{2+} in the growth medium (data not shown), indicating that AdcR positively regulates p_{fim} expression. As we did not observe any potential AdcR binding sequence in the 5′ region of fimC, nor did we detect any interaction between AdcR and p_{fim} DNA fragment in EMSA (data not shown), it is more likely that AdcR binds to a yet-to-be-identified protein and regulates p_{fim} indirectly.

The generation of reactive oxygen species and reactive nitrogen species by activated immune cells is essential for animal and plant innate immune defenses against invading pathogens. It has also been suggested that phagocytes control the replication of invading bacteria within phagosomes partially via the activity of natural resistance-associated macrophage protein (Nramp1), which catalyzes the efflux of divalent cations in a H^{+}-dependent manner [43]. Mn^{2+} is an important cofactor for several bacterial enzymes, including the Mn^{2+}-dependent superoxide dismutase (MnSOD), and enzymes participating in carbon metabolism and stringent response [44], therefore an elevated Mn^{2+} uptake capacity, as seen in ΔfimR, will enhance the survival of S. parasanguinis within phagocytes. Although we could not rule out the possibility that additional genes/operons regulated by FimR may also contribute to the survival of S. parasanguinis within phagocytes, the impact of FimA in this process is very clear.

Studies by Bruno-Bárcena revealed that activation of MsxSOD can enhance the resistance of Streptococcus thermophilus against acid stress by reducing the frequency of the intracellular iron-mediated oxidative stress [35]. Such regulation also suggests that a low intracellular iron concentration coincides with optimal acid tolerance. As the content of iron in brain-heart-infusion-based medium is approximately 100-fold higher than that of manganese [26], it is possible that inactivation of fimR could lead to an increased intracellular concentration of iron over manganese via the transport of the FimCBA system, and subsequently enhanced Fenton reaction and reduced survival at pH 3. On the other hand, inactivation of fimA would result in a minimal amount of intracellular Mn^{2+}/Fe^{2+} and enhanced acid tolerance.

**Conclusions**

In conclusion, this study demonstrated that the expression of the fim operon in S. parasanguinis provides protection against phagocytic killing. Over expression of this system disrupts the acid survival in S. parasanguinis, presumably via an enhanced intracellular Fenton reaction. The complexity of the p_{fim} regulation suggests that an optimal expression of the fim operon is critical for the survival of S. parasanguinis.

**Materials and Methods**

**Bacteria Strains, Plasmids, Culture Media and Growth Conditions**

*S. parasanguinis* FW213 [45] and its derivatives were cultivated routinely in TH broth at 37°C in a 10% CO_{2} atmosphere. Where indicated, spectinomycin (Sp) at 500 μg ml^{-1}, erythromycin (Em) at 5 μg ml^{-1}, or kanamycin (Km) at 200 μg ml^{-1} were included in the media for maintaining recombinant *S. parasanguinis* strains. To analyze the effects of metal ions on growth, the chemically defined medium FMC [46] was used with modifications. Where indicated, the FMC was treated with Chelex-100 (Sigma, United States) at 55°C for 24 h to remove all divalent metal ions. The essential metal ions were then replenished by the addition of 0.01 or 50 μM MnCl_{2}, 0.1 or 50 μM FeSO_{4}, 1 mM MgSO_{4}, and 1 mM CaCl_{2}. Recombinant *E. coli* strains were routinely cultured in LB broth containing ampicillin (Ap) at 100 μg ml^{-1}, Km at 50 μg ml^{-1}, Em at 200 μg ml^{-1}, or Cm at 25 μg ml^{-1} as needed. The bacterial strains and plasmids used in this study are listed in Table 1.

**General Genetic Techniques**

Genomic DNA and total cellular RNA were isolated from *S. parasanguinis* as previously described [47,48]. Plasmid DNA was isolated from recombinant streptococcal strains by the method of Anderson and McKay [49]. Plasmid DNA was introduced into *S. parasanguinis* and its derivatives via electroporation as previously described [48]. PCR was carried out by using Vent® (NEB, United States) or Blunt Taq® DNA polymerase (TOYOBO, Japan). All primers used in this study are listed in Table 2.
Construction of the Recombination \textit{S. parasanguinis} \textit{p}^{\textit{fim-}}\textit{cat} Fusion Strain and its Deletion Derivatives

The \textit{p}_{fim} fragment, containing the 445 bp 5’ to the transcription initiation site (+1) of \textit{p}_{fim}, and the region from +1 to the translation start codon of \textit{fimC} [\textit{p}_{fim}(445 b)], was amplified from \textit{S. parasanguinis} by PCR using primer pair pC0p/AS320SacI and \textit{fimC}/ASBamHI. A SacI and a BamHI recognition site were incorporated in the two primers, respectively, to facilitate the cloning. The promoter region was subsequently ligated to the 5’ end of a promoterless \textit{cat} gene from \textit{Staphylococcus aureus} pC194 [50]. The \textit{p}_{fim}(445 b)-\textit{cat} fusion was confirmed by sequencing analysis, and a 3’ cassette was subsequently cloned into the 5’ end of the correct fusion. To facilitate the integration into FW213 chromosome, an internal fragment of \textit{tcrB}, encoding a copper-(or silver)-translocating P-type ATPase, was amplified by PCR with primers \textit{tcrB}/S and \textit{tcrB}/AS, and subsequently cloned into \textit{pGEM3Zf}(+) vector. The \textit{spe}/\textit{p}_{fim}-\textit{cat} fusion was then cloned into the EcoRV site in \textit{tcrB} to generate \textit{pHR3}. To generate \textit{p}_{fim} deletion derivatives, an inverse PCR approach was employed by using \textit{pHR3} as the template. Briefly, five sense primers, pDS-1, 2, 3, 4, and 5, starting at 239, 151, 109, 59 and 33 bases 5’ to the +1 of \textit{p}_{fim}, were paired with an antisense primer, spe/AS, and used in inverse PCRs. The PCR products were subsequently self-ligated and established in \textit{E. coli}. The identity of each clone was confirmed by sequencing analysis. Plasmid \textit{pHR3} and the 5 derivatives were introduced into \textit{S. parasanguinis}, and the correct double-crossover recombination event at the \textit{tcrB} locus was verified by colony PCR using a \textit{tcrB}-specific primer pair. The resulting recombinant strains were designated \textit{p}_{fim}(239 b)-\textit{cat}, \textit{p}_{fim}(151 b)-\textit{cat}, \textit{p}_{fim}(109 b)-\textit{cat}, \textit{p}_{fim}(59 b)-\textit{cat} and \textit{p}_{fim}(33 b)-\textit{cat}, respectively.

Construction of the \textit{fimR}-deficient Strain, the \textit{fimR}-\textit{fimA} Double Deficient Mutant and Complementation of \textit{fimR}-deficiency

A 2.9-kbp amplicon containing \textit{fimR} and its flanking region was generated by PCR using the primer pair \textit{fimR}/S8023Spel and \textit{fimR}/AS10915SacI. The PCR product was subsequently cloned into the \textit{XbaI} and \textit{SacI} sites of \textit{pSU21} [51]. The 19\textsuperscript{th} to 203\textsuperscript{rd} nt 3’ to the ATG start codon of \textit{fimR} was deleted by inverse PCR with primers \textit{fimR}/AS9576SmaI and \textit{fimR}/S9761SmaI and replaced by an Em resistance gene (\textit{erm}). The resulting plasmid was introduced into VT930, the wild-type \textit{p}_{fim}(445 b)-\textit{cat} strain and its derivatives to inactivate \textit{fimR} by allelic exchange. The correct inactivation was confirmed by colony PCR using a \textit{fimR}-specific primer pair, and the resulting recombinant strains were designated VT930\textit{ΔfimR}, \textit{Δ}_{fimR}(445 b)-\textit{cat}, \textit{Δ}_{fimR}(151 b)-\textit{cat}, \textit{Δ}_{fimR}(109 b)-\textit{cat}, \textit{Δ}_{fimR}(59 b)-\textit{cat} and \textit{Δ}_{fimR}(33 b)-\textit{cat}, respectively. To generate a \textit{fimR} complementation strain, a DNA fragment containing the intact \textit{fimR}, its 5’

\begin{table}[h]
\centering
\caption{Bacterial strains and plasmids used in this study.}
\begin{tabular}{|l|l|l|l|}
\hline
Strain or Plasmid & Relevant phenotypes & Description & Source \\
\hline
\textit{S. parasanguinis} & & & \\
FW213 & Wild-type strain & & [45] \\
\textit{p}_{fim}(33 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(33 b)-\textit{cat} at tcrB & This study \\
\textit{p}_{fim}(59 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(59 b)-\textit{cat} at tcrB & This study \\
\textit{p}_{fim}(109 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(109 b)-\textit{cat} at tcrB & This study \\
\textit{p}_{fim}(151 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(151 b)-\textit{cat} at tcrB & This study \\
\textit{p}_{fim}(239 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(239 b)-\textit{cat} at tcrB & This study \\
\textit{p}_{fim}(445 b)-\textit{cat} & Sp{'} & FW213 harboring \textit{p}_{fim}(445 b)-\textit{cat} at tcrB & This study \\
VT930 & Km{'} & FW213 \textit{fimC}\textsuperscript{aphA3} & [31] \\
VT930\textit{ΔfimR} & Km{'} & VT930 containing a deletion in \textit{fimR} & This study \\
\textit{Δ}_{fimR}(33 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(33 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}(59 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(59 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}(109 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(109 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}(151 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(151 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}(239 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(239 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}(445 b)-\textit{cat} & Sp{’}, Em{’}, FimA{’} & \textit{fimR}-deletion mutant harboring \textit{p}_{fim}(445 b)-\textit{cat} at tcrB & This study \\
\textit{Δ}_{fimR}/\textit{PHR6} & Sp{’}, Em{’}, Km{’} & Strain \textit{Δ}_{fimR}(445 b)-\textit{cat} harboring \textit{PHR6} & This study \\
\hline
\textit{Plasmids} & & & \\
pDL276 & Km{’} & \textit{Streptococcus-E. coli} shuttle vector & [52] \\
pGEM3Zf(+) & Ap{’} & \textit{General E. coli} cloning vector & Promega \\
\textit{pHR3} & Ap{’}, Sp{’} & \textit{pGEM3Zf}(+) / \textit{tcrB-Spe-P}_{fim}(445 b)-\textit{cat} & \textit{pGEM3Zf}(+) vector & Promega \\
\textit{PHR6} & Km{’} & \textit{pDL276/fimR} & This study \\
pQE30 & Ap{’} & Expression vector of His-tagged proteins & Qiagen \\
pQE30/\textit{fimR} & Ap{’} & \textit{pQE30} harboring the coding sequence of \textit{fimR} & This study \\
pSU21 & Cm{’} & \textit{pACYC184-based E. coli} cloning vector & [51] \\
\hline
\end{tabular}
\footnote{Resistance; \textit{Δ} deficiency.}
\end{table}
Table 2. Primers used in this study.

| Primer            | Sequence         |
|-------------------|------------------|
| fimC/AS BamH1     | GAATCATGAGATCGGCTCCTTTTACTATT |
| fimC/AS SspI      | GGAGCTCGGTGGTATAGTCTT |
| fimC_S/S4731      | GCTGTCTCAAGCCCATTCCTCCTGGACG |
| fimR/AS SspI      | GAAGATCTCAGAGCAAGAGGATCTTT |
| fimR/AS SspI 1    | GCCGGAGCTCTACTGGTTAAGGACCT |
| fimR/AS S803SpeI  | AGGAGCATGAGCTCTTCTATATCCTAC |
| fimR/AS S9221BamH1| CATGAGATCTCACAAGAGGTGGGC |
| fimR/AS S761Smal  | AACCCGGGTGCTCTGATCTCTAC |
| fimR/SsacI        | TATTTTATAATTAACTTGACTTAATTT |
| fimR/stopASPstI   | ATCAAGTCCATAAAACCAC |
| fimR/AS S9761SmaI | TATTTTATAATTAACTTGACTTAATTT |
| fimR/S9576Smal    | ATCAAGTCCATAAAACCAC |
| fimR/AS S10331PstI| CATCTTGTAAACGTACCATGATC |
| fimR/AS S10915SacI| ATCAAGTCCATAAAACCAC |
| fimR/AS S11015PstI| CATCTTGTAAACGTACCATGATC |
| fimR/AS S11115SacI| ATCAAGTCCATAAAACCAC |

*Inserted restriction recognition sites are underlined.

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flanking region of 340 bp, and its 3' flanking region of 120 bp was generated by PCR using primers fimR/S9221BamHI and fimR/AS10331PstI. The product was subsequently cloned into the E. coli-streptococcal shuttle vector, pDL276 [52]. The identity of the PCR fragment was confirmed by sequencing analysis, and the correct chimeric plasmid (pHR6) was introduced into E. coli generating strain fimCBA(445 by-cat) to generate strain ΔfimR/pHR6. The presence of pHR6 in the complementation strain was confirmed by plasmid isolation and restriction endonuclease analysis.

CAT Assay

Mid-log phase cultures (optical density at 600 nm [OD₆₀₀] = 0.6) grown in TH or FMC containing various amounts of metal ions were harvested, washed once with 10 mM Tris, pH 7.8, and resuspended in 2.5% of the original culture volume in the same buffer. Total protein lysates from concentrated cell suspensions were obtained as described previously [53]. The protein concentration was measured by Bio-Rad protein assay (United States) and bovine serum albumin (BSA) was served as the standard. CAT activities were determined by the method of Shaw [54], and the specific activities were calculated as nmole Cmin⁻¹ (mg total protein)⁻¹.

Purification of His-FimR and Preparation of Polyclonal Antiserum

The coding region of fimR was amplified from wild-type FW213 by PCR using primers fimR/MSSacI and fimR/stopASPstI. The amplicon was digested with SacI and PstI, and cloned into pQE30 (Qiagen, United States) at the compatible sites to generate pQE30/fimR. The identity of pQE30/fimR was confirmed by sequencing analysis. The induction and purification of His-FimR under native conditions was carried out according to the manufacturer’s instruction. Briefly, the E. coli strain harboring pQE30/fimR was grown to an OD₆₀₀ of 0.2 initially, to which IPTG was added to a final concentration of 1 mM and the culture was incubated at 37°C for an additional hour to induce the expression of His-FimR. At the end of the induction, cells were collected, washed and lysed by French Press. His-FimR was purified from the total cell lysate by using the nickel-affinity chromatography. The bound protein was eluted with 250 mM imidazole. The identity of the purified His-FimR was further confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF). For EMSA, the purified protein was dialyzed against 5 liter of buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA and 1% (v/v) glycerol, at 4°C for 16 h prior to use. To generate polyclonal anti-FimR antiserum in rabbits (Yao-Hong Biotecnology Inc., Taiwan), the isolated protein was first separated on 12% PAGE. The region containing His-FimR was excised and then used as an antigen. The specificity and titer of the antiserum were examined by Western blot analysis (Figure S3).

EMSA

Two 53-mer oligos (pfimbox/S and pfimbox/AS) containing the complement sequences of the 13th to 65th nt 5' to the ATG of fimCBA were annealed and end-labeled with biotin using Biotin 3' end DNA labeling kit (Pierce, United States). The binding reaction between the His-FimR and pEms probe was carried out in the presence of 0.1 mM MnCl₂, 5 mM MgCl₂, 50 mM KCl, 1 mM DTT, 5% (v/v) glycerol, 10 mM Tris (pH 7.5), 250 μg ml⁻¹ BSA and 50 μg ml⁻¹ poly(dI-dC). To each 20 μl binding reaction, 40 fmol labeled probe was used. Non-specific competition was carried out by including an internal fragment of terB (300 bp) without labeling in 10-fold excess in the reaction mixture. All reactions were incubated at room temperature for 20 min and then resolved on 6% native polyacrylamide gels. The DNA and protein complex was electro-transferred on to Nylon membranes, then resolved on 6% native polyacrylamide gels. The DNA and protein complexes were detected by chemiluminescent nucleic acid detection module kit (Pierce).

ChIP-qPCR

ChIP assay was performed by the method of Grainger et al. [55] with minor modifications. Briefly, the mid-exponential phase culture of S. parasanguinis FW213 in FMC supplemented with 0.01 or 50 μM MnCl₂, 0.1 or 50 μM FeSO₄, 1 mM MgSO₄, and 1 mM CaCl₂ was cross-linked with formaldehyde, washed, and then resuspended in 1/50 of the original culture volume in the lysis buffer [55]. The cell suspensions were subjected to mechanical disruption as described above, and the cellular DNA in the clear lysate was sonicated by sonication to generate DNA fragments with an average size of 0.5 to 1 kb. Prior to precipitation with the antiserum, the DNA suspension was incubated first with A/G agarose (Merck Millipore, United States), salmon sperm DNA and BSA at 4°C for 1 h. The insoluble complexes were removed by centrifugation and an aliquot of the supernatant was used in immuno precipitation reactions with the polyclonal anti-FimR antiserum. The negative control was carried out by using the pre-immunized rabbit serum, and the supernatant of this reaction was used as an input control. Immunoprecipitated samples were uncross-linked at 65°C for 12 h. DNA was then purified from the samples by phenol chloroform extraction and precipitation. 1/15 of the final product was then used in the qPCR analysis. The
qPCR was carried out using the Power SYBR® Green PCR Master Mix and 7500 Fast real-time PCR system (Applied Biosystem, United States). The data were analyzed by using 7500 software v2.0.5. Each PCR reaction contains 250 nM of primers fimC/AS9025 and fimC_5’/S4731. Thermal cycler conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each reaction was run in triplicates, and at least three samples were analyzed. Of note, a melting curve analysis was performed at the end of the amplification to ensure the amplification efficiency. The ΔCq of each sample was normalized with pre-immunized serum control. As Mn²⁺ is a known cofactor for FimR, the ΔCq derived from the sample grown in 50 μM MnCl₂ and 0.1 μM FeSO₄ was used as the reference. The relative quantity of each sample was calculated as the ΔCq of the sample compared to the reference using the formula 2^ΔΔCq.

The Effect of Paraquat on Growth

To examine the sensitivity of S. parasanguinis to oxidative stressors, cultures at OD₆₀₀ = 0.4 were diluted at 1:50 in TH medium containing various amounts of paraquat. The growth was monitored at OD₆₀₀ using a Bioscreen C growth monitor (Oy Growth Curves AB Ltd., Finland). Sterile mineral oil was added over the cell suspension to create a reduced oxygen environment, and the plate was shaken for 15 s prior to each reading. For each strain and condition, at least four samples were examined.

Acid Killing

Cultures at OD₆₀₀ = 0.4 were harvested, washed once with 0.1 M glycine buffer, pH 7, and then resuspended in 1/10 of the original culture volume in 0.1 M glycine buffer at pH 3. The viable counts of the bacterial suspension in pH 3 at 15, 30, and 45 min were determined by serial dilution and plating. The survival rate was expressed as a percentage of the viable count at each time point compared to the count prior to acid treatment. For each strain, at least three independent experiments were performed and all plating was done in triplicates.

Macrophage Survival Assays

Human monocytic cell line, THP-1, and mouse RAW264.7 macrophages (Bioresource Collection and Research Center, Taiwan) were maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum and 2 mM L-glutamine. THP-1 cells (2×10⁵ ml⁻¹) were activated by phorbol 12-myristate 13-acetate (PMA) at a final concentration of 1 μg ml⁻¹ for two days before use. Mouse RAW264.7 macrophages (3×10⁵ ml⁻¹) were allowed to adhere to plastic plates for 12 h prior to infection with bacteria. S. parasanguinis FW213 and its derivatives were grown to OD₆₀₀ = 0.4, washed once with PBS and resuspended in RPMI1640 or IMDM (without serum) at 2−8×10⁶ cells ml⁻¹. All infections were done at a MOI of 100 for 1 h. At the end of infection, non-internalized bacteria were removed by washing twice with PBS and the remaining extracellular bacteria were killed by adding penicillin-gentamicin at a final concentration of 100 units ml⁻¹ and 200 μg ml⁻¹, respectively, followed by incubation at 37°C for 1 h. The culture medium was removed and washed twice with PBS to remove the residual antibiotic. The cells were lysed in PBS containing 0.1% Triton X-100 for 10 min. Bacterial counts in the cell lysates were then determined by serial dilutions and plating. The survival rate was calculated as a percentage of the recovered bacterial counts compared to the number of bacteria used in each infection.

Supporting Information

Figure S1 The CAT activities in wild-type S. parasanguinis FW213, FimR, and the fimR complementation strain (FimR PhR6) harboring a single copy of p(fimCBA)_cat at the tcrB locus. All strains were grown in TH to OD₆₀₀ = 0.6. Values are means and standard deviations of three independent experiments.

Figure S2 The activity of p(fimCBA)_cat under various metal growth conditions. S. parasanguinis FW213 harboring a single copy of p(fimCBA)_cat at the tcrB locus was cultivated in FMC containing 0.01 μM MnCl₂ and 0.1 μM FeSO₄ (I), 0.01 μM MnCl₂ and 50 μM FeSO₄ (II), 50 μM MnCl₂ and 0.1 μM FeSO₄ (III), 50 μM MnCl₂ and 50 μM FeSO₄ (IV). All cultures were supplemented with 1 mM MgSO₄ and 1 mM CaCl₂. Values are means and standard deviations of three independent experiments.

Figure S3 Western analysis with the anti-FimR antiserum. 25 μg of total cellular proteins prepared from wild-type S. parasanguinis FW213 (I) and the fimR-deficient strain (II) were separated on 12% SDS-PAGE, transferred to a piece of membrane and probed with the polyclonal antibody against FimR. The primary antibody was used at a dilution of 1:20000 (A) and 1:10000 (B), respectively, and the secondary antibody, goat anti-rabbit IgG, was used at 1:10000. The molecular weight of FimR in kDa is indicated.

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Author Contributions

Conceived and designed the experiments: YMC HS. Performed the experiments: HS YC. Analyzed the data: YMC HS. Wrote the paper: YMC.

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