Staphylococcus aureus ftnA 3′-Untranslated Region Modulates Ferritin Production Facilitating Growth Under Iron Starvation Conditions

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Iron acquisition and modulation of its intracellular concentration are critical for the development of all living organisms. So far, several proteins have been described to be involved in iron homeostasis. Among them, ferritins act as the major iron storage proteins, sequestering internalized iron and modulating its concentration inside bacterial cells. We previously described that the deletion of the 3′-untranslated region (3′UTR) of the ftnA gene, which codes for ferritin in Staphylococcus aureus, increased the ftnA mRNA and ferritin levels. Here, we show that the ferritin levels are affected by RNase III and PNPase, which target the ftnA 3′UTR. Rifampicin mRNA stability experiments revealed that the half-life of the ftnA mRNA is affected by both RNase III and the ftnA 3′UTR. A transcriptional fusion of the ftnA 3′UTR to the gfp reporter gene decreased green fluorescent protein (GFP) expression, indicating that the ftnA 3′UTR could work as an independent module. Additionally, a chromosomal deletion of the ftnA 3′UTR impaired S. aureus growth under conditions of iron starvation. Overall, this work highlights the biological relevance of the ftnA 3′UTR for iron homeostasis in S. aureus.

Keywords: Staphylococcus aureus, 3′UTRs, post-transcriptional regulation, RNase III, PNPase, mRNA decay, ferritin, iron homeostasis

INTRODUCTION

Iron is an essential micronutrient for several biological processes in living cells such as oxygen transport, methanogenesis, the tricarboxylic cycle (TCA), gene regulation, and DNA biosynthesis (Andrews et al., 2003; Price and Boyd, 2020). When infecting the host, pathogenic bacteria like Staphylococcus aureus need to overcome the restricted availability of free iron, a process known as nutritional immunity (Hood and Skaar, 2012; Marchetti et al., 2020). Staphylococcus aureus, one of the most relevant nosocomial bacteria worldwide (Tong et al., 2015), has developed diverse strategies to obtain iron by secreting siderophores and hemophores that chelate iron and heme molecules, respectively, as well as expressing iron and heme uptake systems (Wandersman and Delepelaire, 2004; Haley and Skaar, 2012).
Although iron is essential for life, at high cytosolic concentrations, it can be toxic for the bacterium. This is due to the formation of reactive oxygen species (ROS) that cause oxidative stress and damage DNA as well as other essential molecules (Meneghini, 1997; Price and Boyd, 2020). For this reason, bacteria dedicate a significant amount of resources to regulating intracellular iron concentration. In S. aureus, the ferric uptake regulator (Fur) and peroxide stress transcriptional regulator (PerR) are the two main transcriptional regulators that modulate the excess of iron and oxidative stress. When high levels of iron are sensed inside the cell, Fur inhibits the transcription of several genes involved in iron and heme uptake and promotes the expression of efflux pumps (Horsburgh et al., 2001; Friedman et al., 2006; Troxell and Hassan, 2013). Alternatively, the iron surplus is dealt with by the storage ferritin protein, FtnA, whose levels are increased under iron-rich conditions. However, when iron is scarce, ftnA expression is transcriptionally repressed by PerR (Morrissey et al., 2004; Zühlke et al., 2016).

3′-untranslated regions (3′UTRs) have recently emerged as impactful post-transcriptional regulatory elements that regulate the levels of the mRNAs in which they are encoded through different mechanisms (Miyakoshi et al., 2015; Zhao et al., 2018; Menendez-Gil and Toledo-Arana, 2021). For example, the 3′UTR of the icar mRNA modulates the production of IcaR, the main repressor of PIA-PNAG exopolysaccharide biosynthesis in S. aureus. This 3′UTR contains a UCCCC motif that binds to the Shine-Dalgarno (SD) sequence at the 5′UTR of the same mRNA and inhibits ribosome binding and translation. This interaction results in the formation of a double-stranded RNA (dsRNA) substrate for endoribonuclease III (RNase III) to process (de Los Mozos et al., 2013). The 3′UTR of the Bacillus subtilis hbs mRNA follows a similar pattern, interacting with its own 5′UTR, but in this case to prevent RNase Y cleavage (Braun et al., 2017). Another way in which 3′UTRs may influence RNA stability and protein expression is by carrying AU-rich motifs that are processed by RNase Y cleavage (Braun et al., 2017). The 3′UTR of the icar mRNA can influence RNA stability and protein expression by carrying AU-rich motifs that are processed by RNase Y cleavage (Braun et al., 2017). The 3′UTR of the icar mRNA can influence RNA stability and protein expression by carrying AU-rich motifs that are processed by RNase Y cleavage (Braun et al., 2017).

In a previous study, we unveiled that the S. aureus 3′UTR modulates ferritin production (Menendez-Gil et al., 2020). Here, we show that the ftnA 3′UTR could act as an independent regulatory module that is targeted by RNase III and PNPase to decrease ferritin expression. Deletion of the ftnA 3′UTR impaired bacterial growth under iron starvation conditions. In this scenario, the ftnA 3′UTR-mediated regulation would play an essential role in achieving a tightly regulated iron homeostasis in S. aureus.

**MATERIALS AND METHODS**

**Strains, Plasmids, Oligonucleotides, and Growth Conditions**

Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Tables 1–3, respectively. *Staphylococcus aureus* strains were grown in Tryptic Soy Broth (Pronadisa) supplemented with 0.25% glucose (TSBg) or, when indicated, in modified chemically defined medium (Toledo-Arana et al., 2005). To prepare *S. aureus* and *E. coli* competent cells, B2 (casein hydrolysate, 10 g L⁻¹; yeast extract, 25 g L⁻¹; NaCl, 25 g L⁻¹; K₂HPO₄, 1 g L⁻¹; glucose, and 5 g L⁻¹; pH 7.5), and SuperBroth (tryptone, 30 g L⁻¹; yeast extract, 20 g L⁻¹; and MOPS, 10 g L⁻¹; pH 7) media were used, respectively. For selective growth, media were supplemented with the appropriate antibiotics at the following concentrations: ampicillin (Amp), 100 μg ml⁻¹ for *E. coli* transformants; erythromycin (Erm), 1.5 μg ml⁻¹ or 10 μg ml⁻¹ for *S. aureus* cells harboring the pMAD or pCN plasmids, respectively.

**Plasmid Construction**

Most of the plasmids used in this study were engineered as previously described (Caballero et al., 2018; Menendez-Gil et al., 2020). pMAD plasmids used for chromosomal deletions were constructed by amplifying flanking sequences (AB and CD) of the target regions using primers A/B and C/D (Table 3). PCR fragments were digested and ligated into pMAD through a double-fragment ligation process using BamHI, EcoRI, and KpnI or NheI (Tables 2, 3).

The green fluorescent protein (GFP) reporter plasmids were constructed using the *Listeria monocytogenes* PAD-CGF plasmid as a template (Balestrino et al., 2010). To build pGFP, the hly 5′UTR and GFP sequences were amplified with primers Sal-GFP-fw and Bcll-TT-BamHI-GFP-rvs (Table 3) and the resulting PCR fragment was cloned into the pEW plasmid. The 3′UTR of *ftnA* was amplified using primers BamHI-EcoRI-ftn and SalI-GFP-rvs as a template and primers SalI-GFP-fnt and KpnI-D3UTR-term-fnt (Table 3) and inserted downstream of the *gfp* gene using restriction sites BamHI and SmaI. The pGFP-Δ3′UTR₅₇ was constructed using the pGFP-Δ3′UTR₅₇ as a template and primers Sall-GFP-fw and KpnI-D3UTR-term-fnt (Table 3). The amplification product was ligated into the pGFP plasmid using Sall and KpnI.

The plasmids expressing 5′XFtnAA 3′UTR₁₉₋₅₆ and 5′XFtnAA 3′UTR₁₉₋₅₆ and 5′XFtnAA 3′UTR₁₉₋₅₆ and 5′XFtnAA 3′UTR₁₉₋₅₆ were constructed using the p5′XFtnAA plasmid backbone. For the 5′XFtnAA 3′UTR₁₉₋₅₆ plasmid, an overlapping PCR was performed using oligonucleotide pairs +1-fnt and 3′UTR-fnt-19-56-fw and 3′UTR-fnt-19-56-rv and term-fnt. Analogously, oligonucleotides +1-fnt and 3′UTR-fnt-term-1/2 were used for the 5′XFtnAA 3′UTR₁₉₋₅₆ plasmid (Table 3). The resulting amplicons were then inserted into pEW using BamHI and KpnI (Table 2).
TABLE 1 | Strains used in this study.

| Strains | Relevant characteristic(s) | BGR ID* | Source or reference |
|---------|-----------------------------|---------|---------------------|
| Staphylococcus aureus | | | |
| 15981 | MSSA (methicillin sensitive Staphylococcus aureus) clinical isolate from an otitis infection; biofilm positive; PIA-PNAG-dependent biofilm matrix. | 8 | Valle et al., 2003 |
| 15981 ΔtnA | 15981 carrying a chromosomal deletion of tna gene. | 933 | Menendez-Gil et al., 2020 |
| ΔtnA p3xFtnA | 15981 ΔtnA carrying the p3xFtnA plasmid. | 1831 | Menendez-Gil et al., 2020 |
| ΔtnA p3xFtnAA3'UTR | 15981 ΔtnA carrying the p3xFtnAA3'UTR plasmid. | 1832 | Menendez-Gil et al., 2020 |
| 15981 p3xFtnA | 15981 carrying the p3xFtnA plasmid. | 793 | This study |
| 15981 p3xFtnAA3'UTR | 15981 carrying the p3xFtnAA3'UTR plasmid. | 794 | This study |
| 15981 p3xFtnAA3'UTR19-56 | 15981 carrying the p3xFtnAA3'UTR19-56 plasmid. | 1657 | This study |
| 15981 p3xFtnAA3'UTR37-56 | 15981 carrying the p3xFtnAA3'UTR37-56 plasmid. | 2807 | This study |
| 15981 p3xFtnAA3'UTR55-56 | 15981 carrying the p3xFtnAA3'UTR55-56 plasmid. | 1644 | This study |
| 15981 pGFP-3'UTR | 15981 carrying the pGFP-3'UTR plasmid. | 1809 | This study |
| 15981 Δmc | 15981 with a deletion of the mc gene. | 1760 | This study |
| Δmc p3xFtnA | 15981 Δmc carrying the p3xFtnA plasmid. | 1771 | This study |
| Δmc p3xFtnAA3'UTR | 15981 Δmc carrying the p3xFtnAA3'UTR plasmid. | 1772 | This study |
| Δmc pGFP-3'UTR55-56 | 15981 Δmc carrying the pGFP-3'UTR55-56 plasmid. | 1774 | This study |
| 15981 ΔpnpA | 15981 with a deletion of the pnpA gene. | 242 | Lasa et al., 2011 |
| ΔpnpA p3xFtnA | 15981 ΔpnpA carrying the p3xFtnA plasmid. | 1628 | This study |
| ΔpnpA p3xFtnAA3'UTR | 15981 ΔpnpA carrying the p3xFtnAA3'UTR plasmid. | 1629 | This study |
| ΔpnpA pGFP-3'UTR55-56 | 15981 ΔpnpA carrying the pGFP-3'UTR55-56 plasmid. | 1646 | This study |
| 15981 Δmr | 15981 with a deletion of the mr gene. | 243 | Lasa et al., 2011 |
| Δmr p3xFtnA | 15981 Δmr carrying the p3xFtnA plasmid. | 1630 | This study |
| Δmr p3xFtnAA3'UTR | 15981 Δmr carrying the p3xFtnAA3'UTR plasmid. | 1631 | This study |
| 15981 Δnr | 15981 with a deletion of the mrn gene. | 1762 | This study |
| Δnr p3xFtnA | 15981 Δnr carrying the p3xFtnA plasmid. | 1777 | This study |
| Δnr p3xFtnAA3'UTR | 15981 Δnr carrying the p3xFtnAA3'UTR plasmid. | 1778 | This study |
| 15981 Δny | 15981 with a deletion of the my gene. | 1761 | This study |
| Δny p3xFtnA | 15981 Δny carrying the p3xFtnA plasmid. | 1783 | This study |
| Δny p3xFtnAA3'UTR | 15981 Δny carrying the p3xFtnAA3'UTR plasmid. | 1784 | This study |
| 15981 ΔnjA | 15981 with a deletion of the mya gene. | 1768 | This study |
| ΔnjA p3xFtnA | 15981 ΔnjA carrying the p3xFtnA plasmid. | 1797 | This study |
| ΔnjA p3xFtnAA3'UTR | 15981 ΔnjA carrying the p3xFtnAA3'UTR plasmid. | 1798 | This study |
| 15981 ΔtnA3'UTR | 15981 carrying a deletion of the tna 3'UTR. | 931 | This study |

*Identification number of the strains stored at the Laboratory of Bacterial Gene Regulation, XAB-CSIC.

TABLE 2 | Plasmids used in this study.

| Plasmids | Relevant characteristic(s) | Source and/or reference |
|----------|-----------------------------|-------------------------|
| pEW | A derivative pCN40 plasmid including the transcriptional terminator region of the pCN47 plasmid downstream of the multiple cloning site. | Menendez-Gil et al., 2020 |
| pAD-CGFP | Listeria monocytogenes plasmid carrying the GFP gene with the 5'UTR from hly gene under the control of the Phyper promoter. | Balestino et al., 2010 |
| pMAD | Escherichia coli-Staphylococcus aureus shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria. The vector contains the bgaB gene encoding a β-galactosidase under the control of a constitutive promoter as reporter of plasmid presence. AmpR, EmR. | Arnaud et al., 2004 |
| pMAD-Δ3'UTR55-56 | pMAD plasmid containing the allele for deletion of the 3'UTR of tna A gene. | This study |
| pMAD-Δmc | pMAD plasmid containing the allele for deletion of mc gene. | This study |
| pMAD-Δmrn | pMAD plasmid containing the allele for deletion of mrn gene. | This study |
| pMAD-ΔnjA | pMAD plasmid containing the allele for deletion of mya gene. | This study |
| p3xFtnA | pEW plasmid expressing the 3xFLAG-tagged tnaA mRNA. | Menendez-Gil et al., 2020 |
| p3xFtnAA3'UTR | pEW plasmid expressing the 3xFLAG-tagged tnaA mRNA lacking the 3'UTR while preserving the transcriptional terminator. | Menendez-Gil et al., 2020 |
| p3xFtnAA3'UTR19-56 | pEW plasmid expressing the 3xFLAG-tagged tnaA mRNA lacking a region of the 3'UTR including nt 19-56 after the stop codon. | This study |
| p3xFtnAA3'UTR37-56 | pEW plasmid expressing the 3xFLAG-tagged tnaA mRNA lacking a region of the 3'UTR including nt 37-56 after the stop codon. | This study |
| pGFP | pCN40 plasmid expressing GFP with the 5'UTR from hly of L. monocytogenes. | This study |
| pGFP-3'UTR55-56 | pEW plasmid expressing a chimeric mRNA including the gfp gene fused to the 3'UTR of tnaA. | This study |
| pGFP-Δ3'UTR55-56 | pEW plasmid expressing a chimeric mRNA including the gfp gene fused to the transcriptional terminator of the tnaA mRNA. | This study |
TABLE 3 | Primers used in this study.

| Oligonucleotide name | Sequence |
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**Synthesis of riboprobes**

- NB-probe-3xF-ftn-fw
- T7prom-NB-3xF-ftn-rvs
- NB-probe-ftn-fw
- T7-NB-probe-ftn-rvs

**Construction of chromosomic mutant strains**

- D3UTR_ftn_A (BamHI)
- D3UTR_ftn_B (NheI)
- Dftn-C (NheI)
- Dftn-D (EcoRI)
- Dftn-E
- Dftn-F
- Drnc-A (BamHI)
- Drnc-B (KpnI)
- Drnc-C (KpnI)
- Drnc-D (EcoRI)
- Drnc-E-n
- Drnc-F-n
- Dmrnc-A (BamHI)
- Dmrnc-B (KpnI)
- Dmrnc-C (KpnI)
- Dmrnc-D (EcoRI)
- Dmrnc-E
- Dmrnc-F
- Drny-A (BamHI)
- Drny_B (KpnI)
- Drny-C (KpnI)
- Drny-D (EcoRI)
- Drny-E
- Drny-F
- DrnjA-A (BamHI)
- DrnjA-B (KpnI)
- DrnjA-C (KpnI)
- DrnjA-D (EcoRI)
- DrnjA-E
- DrnjA-F
- pMAD-1
- pMAD-2

**Construction of plasmids expressing ftmA mRNAs**

- +1-ftn (BamHI)
- 3’UTR-ftn-19-56-fw
- 3’UTR-ftn-19-56-rv
- 3UTR-ftn-term-1/2 (KpnI)

**Construction of plasmids expressing GFP**

- Sall-GFP-fw
- Bcui-TT-BamHI-GFP-rvs
- BamHI-EcoRI-3UTR-ftn-fw
- Smal-3UTR-ftn-rvs
- Term ftp (KpnI)
- KpnI-3UTR-term-ftn

*Restriction enzymes sites and T7 promoter sequence are indicated in italic and bold, respectively.

**Rifampicin mRNA Stability Assay and Northern Blotting**

Precultures were grown in 5 ml of TSBg supplemented with Erm (TSBg+Erm) and incubated overnight (ON) at 37°C and 200 rpm. Bacterial concentrations of the precultures were estimated by measuring their optical density (OD_{600}). Erlermeyer flasks containing fresh TSBg+Erm were inoculated with precultures to reach starting OD_{600} of 0.02. For rifampicin mRNA stability assays, test cultures were grown at 37°C and 200 rpm until an OD_{600} of 0.5 (exponential phase). Six aliquots of 20 ml of the culture were transferred to 50 ml Falcon tubes containing 300 μg ml\(^{-1}\) of rifampicin and incubated at 37°C for 0, 2, 4, 8, 15, and 30 min. Then, 5 ml of stop solution (95% ethanol and 5% phenol) were added to the samples and centrifuged for 2 min at 4,400 g. Pellets were frozen in liquid nitrogen and stored at −80°C. RNA extraction and Northern blot analysis were performed as described in Toledo-Arana et al. (2009) and Menendez-Gil et al. (2020). Radiolabeled riboprobes were synthesized from a PCR carrying the T7 promoter.
(Table 3) using the MAXIscript T7 transcription kit (Ambion) and [α32P]-UTP, following the manufacturer’s recommendations. These riboprobes were designed to target the ftnA or the 3′UTR mRNAs. The mRNA levels were quantified by densitometry of Northern blot autoradiographies using ImageJ.1 Each of the mRNA levels was normalized to the levels of the sample at 0 min of rifampicin incubation.

**Chromosomal Mutagenesis**

The mutants generated in this study (Table 1) were obtained as previously described (Valle et al., 2003) by a two-step homologous recombination that exchanges a specific chromosomic region by the mutant allele present in the pMAD plasmid (Arnaud et al., 2004). The marker-less mutants were verified by PCR using oligonucleotides E and F (Table 3) and Sanger sequencing.

**Protein Extraction and Western Blotting**

Bacteria were grown as described above. At OD₆₅₀ 0.5, 30 ml samples were taken for protein extraction as described by Menendez-Gil et al. (2020). Western blotting was performed as previously described (Caballero et al., 2018). The 3xFLAG tagged protein samples were incubated with mouse monoclonal anti-FLAG M2-Peroxidase (HRP) antibodies (Sigma) diluted 1:1,000, whereas the GFP samples were incubated with mouse monoclonal anti-GFP antibodies 1:5,000 (Living Colors, Clontech). Membranes were developed using the SuperSignal West Pico Chemiluminiscent Substrate kit (Thermo Scientific). Mean intensities of developed protein bands were quantified by densitometry of Western blot images using ImageJ and plotted as arbitrary units (A.U.). Statistical significances were calculated by running a paired t-test in GraphPad Prism; asterisks (*) indicate p-values lower than 0.05 (p<0.05) while ns indicate not significant differences.

**RESULTS**

**Deletion of the ftnA 3’UTR Increases the Half-Life of Its mRNA**

In a previous study, we showed that deletion of the ftnA 3’UTR increased both the ftnA mRNA and ferritin protein levels in *S. aureus* (Menendez-Gil et al., 2020). To evaluate whether the ftnA 3’UTR deletion affected ftnA mRNA stability, we performed rifampicin mRNA stability assays and half-life determinations. To that end, we transformed the *S. aureus* 15981 ΔftnA strain with the p3XXftnA and p3XXFtnAΔ3’UTR plasmids, which expressed the WT and Δ3’UTR ftnA mRNAs under the control of the P₆₅₀ constitutive promoter, respectively. This allowed us to exclusively monitor the plasmidic ftnA gene using a strand-specific riboprobe. The resulting strains were grown until exponential phase and their total RNAs extracted at different time points after rifampicin addition. Northern blots revealed that the half-life of the ftnA mRNA was higher in the p3XXFtnAΔ3’UTR strain (2.5 min) when compared to the p3XXFtnA strain (0.8 min; Figure 1). Note that since the decrease in concentration of the two mRNAs was not exponential for all

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1http://rsbweb.nih.gov/ij/
time point, we used only the first three time points (0–4 min) for the WT mRNA and the first four time points (0–8 min) for the Δ3’UTRftnA deletion mutant to calculate their half-life (Figure 1B). These results indicated that the ftnA 3’UTR may be targeted by unknown RNases to modulate FtnA expression.

**RNase III and PNPase Target and Process the 3’UTR of the ftnA mRNA**

In order to identify the RNases that could potentially target the ftnA 3’UTR, we transformed the most relevant non-essential RNase mutants (Bonnin and Bouloc, 2015) of the *S. aureus* 15981 WT strain with the p3xFtnA and p3xFtnAΔ3’UTR plasmids, which expressed the 3xFLAG-tagged ferritin protein (3xFtnA) from the WT and Δ3’UTR ftnA mRNAs, respectively. The selected RNase mutants included Δrnc (RNase III, a dsRNA endonuclease; Lasa et al., 2011; Liolliou et al., 2012), Δmrnc (mini-RNase III, a double-stranded RNA endonuclease paralog to RNase III, which was initially identified in *B. subtilis*; Olmedo and Guzmán, 2008, with an uncharacterized ortholog in *S. aureus*), ΔpnpA (PNPase, 3′-5′ exonuclease; Anderson and Dunman, 2009), Δrrr (RNase R, 3′-5′ exonuclease; Oussenko et al., 2002), Δrny (RNase Y, a single-stranded RNA endonuclease; Marincola et al., 2012), and ΔrnyA (RNase J1, a bifunctional RNase with endonuclease and 5′ to 3′ exonuclease activities; Linder et al., 2014). We then determined their 3xFtnA protein levels by Western blot using anti-FLAG antibodies (Figure 2A). As expected, the *S. aureus* 15981 WT strain that carried the p3xFtnAΔ3’UTR plasmid expressed higher 3xFtnA protein levels than the strain expressing the whole ftnA mRNA. Such protein increase was also obtained when ferritin was expressed from the p3xFtnAΔ3’UTR plasmid in the Δrrr, Δmrnc, and Δrny mutant strains. Since 3xFtnA could not be detected in the ΔrnyA strain, we performed the same experiment but loading a higher amount of total protein (Figure 2B). The ΔrnyA mutant strains also showed an increase in ferritin expression when the 3’UTR was deleted (Figures 2A,B). These results suggested that RNase R, mini-RNase III, RNase Y, and RNase J1 were not involved in the 3’UTR-mediated processing of the ftnA mRNA. In contrast, the Western blots revealed that the Δrrr mutant strains carrying the p3xFtnA and p3xFtnAΔ3’UTR plasmids expressed similar levels of the 3xFtnA protein regardless of the 3’UTR deletion from the 3xFLAG-ftnA mRNA. Similar results were obtained in the ΔpnpA mutant strains (Figure 2A). This suggests that RNase III and PNPase could be targeting the ftnA 3’UTR to process the ftnA mRNA.

mRNA decay is often initiated by endoribonucleases, including RNase Y and RNase III (Durand et al., 2015). To confirm the implication of RNase III in the ftnA mRNA decay, we evaluated the half-life of the WT and Δ3’UTRftnA mRNAs in *S. aureus* 15981 WT and its isogenic Δrrr mutant strain. The rifampicin mRNA stability assays revealed that the ftnA mRNA half-life increased from 1.3 min in the WT strain to 7.0 min in the Δrrr mutant (Figures 2C,D). This was higher than the half-life observed for the Δ3’UTRftnA mRNA mutant, indicating that the RNase III might target the ftnA mRNA through additional mechanisms. Note that the half-life of the ftnA mRNA and its Δ3’UTR mutant were similar when expressed from the Δrrr mutant (7.0 vs. 5.8 min, respectively; Figures 2C,D). Taken together, these results indicate that RNase III promotes ftnA mRNA decay in a process in which the ftnA 3’UTR plays a critical role.

**The ftnA 3’UTR Works as an Independent Functional Module**

In order to investigate whether the ftnA 3’UTR had functional capacities on its own, we fused the ftnA 3’UTR downstream of the gfp gene, which encodes the GFP, thus, generating the pGFP-3’UTRmut plasmid. As a control, we constructed a plasmid that included the transcriptional terminator (TT) of the ftnA mRNA downstream of the gfp gene (pGFP-Δ3’UTRmut; Figure 3A). Then, we transformed the *S. aureus* 15981 WT strain with these plasmids and determined the GFP levels by Western blot analysis. The results revealed that the pGFP-3’UTRmut plasmid expressed lower GFP levels when compared to the pGFP-Δ3’UTRmut plasmid (Figure 3B). This confirmed that the ftnA 3’UTR alone worked as an independent module able to reduce the expression of a heterologous gene like gfp.

To analyze whether such GFP expression reduction was mediated by RNase III and PNPase, we introduced the pGFP-3’UTRmut plasmid into the Δrrr and ΔpnpA mutant strains. Western blot results showed that the Δrrr and ΔpnpA mutants expressed similar GFP levels to the ones produced by the strain carrying the pGFP-Δ3’UTRmut plasmid, indicating that the 3’UTR would still be targeted and processed by RNase III and PNPase regardless of the CDS (Figure 3C).

**The ftnA 3’UTR Is Highly Conserved in *Staphylococcus aureus* and *Staphylococcus argenteus***

Since RNase III is a double-stranded endoribonuclease, we looked for putative double-stranded RNA regions within the ftnA 3’UTR secondary structure. We used the RNAstructure version 6.2 software to predict the ftnA mRNA conformation. We could not find any evident secondary structures susceptible to RNase III within the ftnA 3’UTR, nor a hypothetical 5’UTR-3’UTR interaction, as previously described for the icaR mRNA (de Los Mozos et al., 2013). A plausible alternative would be for the ftnA 3’UTR to be targeted by a trans-acting small RNA that, upon interaction, generated a double-stranded substrate for RNase III to process. This idea would require the putative paring region to be conserved among *S. aureus* strains and close relatives. Previous multiple sequence alignment analyses revealed that 8,193 out of the 10,000 *S. argenteus* differences (Menendez-Gil et al., 2020). Although, such a high degree of conservation suggested an important role for the ftnA 3’UTR, it prevented us from identifying a putative functional region. Previous analyses also showed that among other

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2http://rna.urmc.rochester.edu/RNAstructure.html
FIGURE 2 | Role of RNases in ftnA 3’UTR-mediated regulation. Western blots showing: (A) 3xFtnA levels from different Staphylococcus aureus 15981 RNase mutants harboring the p3xFtnA and p3xFtnAΔ3’UTR constructs. (B) 3xFtnA levels in the Δmja mutant increasing the protein load used in (A). Western blots were developed using peroxidase-conjugated anti-FLAG antibodies. Coomassie (Coom.) stained gel portions are shown as loading controls. Western blot images show representative results from at least three independent replicates. The mean intensity of the bands was estimated by densitometry of blot images using ImageJ (A.U., arbitrary units). Statistical significances were determined through paired t-tests in GraphPad Prim; *p < 0.05; and ns, not significant. (C) Rifampicin half-life assays of 3xFLAG ftnA WT and Δ3’UTR mRNAs expressed from a constitutive promoter in the S. aureus 15981 WT strain and its isogenic Δmja mutant. Strains were grown in TSBg at 37°C until exponential phase when 300 μg/ml of rifampicin was added. Samples were taken at the indicated time points (min). rRNAs stained with Midori Green are shown as loading control. Representative images of the experiment, which was repeated twice, are shown. (D) The mRNA levels were quantified by densitometry of Northern blot images in Image J (http://rsbweb.nih.gov/ij/) and normalized using time 0 as a reference. The mean of the mRNA levels was plotted in function of time. Error bars represent the SD from two independent replicates.
**Deletion of the *ftnA* 3′UTR Impairs *Staphylococcus aureus* Growth During Iron Starvation**

To evaluate the biological relevance of the *ftnA* 3′UTR-mediated control of ferritin production, we constructed a chromosomal *ftnAΔ3′UTR* mutant in the *S. aureus* 15981 genetic background. First, to control that the chromosomal mutant behaved as the plasmidic one, we performed Northern blot analyses to monitor the *ftnA* mRNA levels. Total RNAs were extracted from the WT and Δ3′UTR mutant strains grown until exponential phase in a rich medium (TSBg). Northern blot results showed that the chromosomal *ftnAΔ3′UTR* mutant expressed higher *ftnA* mRNA levels than the WT strain as it occurred with the plasmidic mutant (Figure 5A).

Then, we aimed at comparing the capacities of the *S. aureus* 15981 WT and the chromosomal *ftnAΔ3′UTR* mutant strains to grow under iron starvation conditions. We incubated microplates containing minimal medium lacking iron (MM\textsuperscript{wo/Fe}) at 37°C and measured bacterial growth by registering the optical density every 30 min. However, no growth differences were observed between the WT and Δ3′UTR mutant in the MM\textsuperscript{wo/Fe} (Figure 5B). Since iron traces could be still present in this medium, iron starvation might be difficult to achieve under laboratory conditions without the use of chelating agents (Pf and Helmann, 2017). Therefore, the MM\textsuperscript{wo/Fe} was complemented with increasing concentrations of 2,2′-dipyridyl (DIP), a strong iron chelator. Figure 5B shows that the addition of the DIP chelator at a concentration of 100 μM significantly affected the growth of the *ftnAΔ3′UTR* mutant, while adding 500 μM of DIP completely impaired it. Altogether, these data portrayed the *ftnA* 3′UTR as an essential module to control the *ftnA* mRNA expression and maintain proper iron levels for *S. aureus* growth under iron starvation conditions.

**DISCUSSION**

The right amount of iron concentration inside the cells is essential for bacterial growth since it is utilized as a cofactor for a wide variety of enzymes. However, intracellular iron excess can lead to oxidative stress and, ultimately, cell damage (Andrews et al., 2003; Hood and Skaar, 2012). For this purpose, the existence of regulating agents such as the ferritin, which removes free intracellular iron, is paramount for protecting cells from its potential toxic effects (Zühlke et al., 2016). As a consequence, the levels of ferritin must also be tightly regulated and in accordance with iron availability (Morrissey et al., 2004). In this study, we showed that ferritin expression is controlled at the post-transcriptional level by the *ftnA* 3′UTR, which is mainly targeted by RNase III and PNPase (Figure 2). The *ftnA* 3′UTR seems to work as an independent cis-regulatory module since its fusion to the heterologous *gfp* reporter gene few nucleotides are not enough to reproduce the effect generated by the Δ3′UTR mutant (Figure 4C). At the same time, it indicated that both conserved regions contribute to RNase III action to modulate ferritin expression.

**Staphylococcus** species carrying the *ftnA* gene, the *ftnA* 3′UTR conservation only applied to *S. argenteus* (Menendez-Gil et al., 2020). Further nucleotide comparison analysis between the *S. aureus* and *S. argenteus* *ftnA* 3′UTRs revealed two conserved regions comprised between nucleotides 18–56 (conserved region I) and 68–89 (conserved region II), respectively, besides the putative TT (Figure 4A). To determine whether one of these regions could be involved in the *ftnA* mRNA processing, we constructed two plasmids expressing the 3xFLAG tagged *ftnA* mRNA carrying either a deletion between nucleotides 19 and 56 (p\textsuperscript{3xFtnAΔ19-56}), or 57 and 93 (p\textsuperscript{3xFtnAΔ3′UTR57-93}), which selectively eliminated conserved regions I and II, respectively (Figure 4B). We used such plasmids to transform the *S. aureus* 15981 WT strain and evaluated their 3xFtnA protein expression. Western blot analyses revealed that both mutations produced similar 3xFtnA protein levels when compared to the full-length *ftnA* mRNA, suggesting that deletions of a few nucleotides are not enough to reproduce the effect generated by the Δ3′UTR mutant (Figure 4C). At the same time, it indicated that both conserved regions contribute to RNase III action to modulate ferritin expression.

**FIGURE 3** | The *ftnA* 3′UTR can act as an independent module. (A) Schematic representation of the green fluorescent protein (GFP) constructs generated. P\textsubscript{const} constitutive promoter; TT transcriptional terminator. (B) Western blot showing the GFP levels of the *Staphylococcus aureus* 15981 WT strain carrying either pGFP-3′UTR\textsuperscript{wt} or pGFP-Δ3′UTR\textsuperscript{wt}. (C) Western blot showing the GFP levels of the 15981 Δmc and ΔpnpA strains carrying pGFP-3′UTR\textsuperscript{wt}. Western blots were developed using monoclonal anti-GFP antibodies and peroxidase-conjugated goat anti-mouse immunoglobulin G and M antibodies. Coomassie (Coom.) stained gel portions are shown as loading controls. Western blot images show the representative results from at least three independent replicates. Mean intensity bands were quantified by densitometry of blot images in ImageJ (A.U., arbitrary units). Statistical significances were determined by running paired *t*-tests using the GraphPad Prim software; *p* < 0.05, and ns, not significant.
also decreased GFP expression with the participation of RNase III and PNPase (Figure 3). PNPase is a 3′-5′ exoribonuclease whose activity is inhibited by the presence of strong RNA secondary structures (Spickler and Mackie, 2000; Dar and Sorek, 2018; Ingle et al., 2021). The 3′ end of the ftnA mRNA contains a putative intrinsic Rho-independent terminator that should avoid PNPase processing. Therefore, one would expect the action of RNase III to trigger ftnA mRNA processing, which would provide an mRNA carrying now a 3′ end accessible for PNPase cleavage. This is in agreement with the canonical mechanism of RNA degradation found in the majority of Gram-positive bacteria, which it is initiated by either RNase Y or RNase III and followed by the action of 3′-5′ exoribonucleases such as PNPase and RNase R (Broglia et al., 2001; Dar and Sorek, 2018; Ingle et al., 2021).
Our data indicated that RNase III would process the ftnA mRNA, at least in part, through its 3'UTR (Figure 3). Note that the rifampicin mRNA stability assays also showed that RNase III affects the ftnA mRNA independently of the 3'UTR (Figure 2). This mechanism would require further investigations.

RNase III cleaves dsRNAs. However, no internal double-stranded RNA structures were predicted in the 3'UTR that could provide a dsRNA substrate for RNase III as previously described (de los Mozos et al., 2013). This suggested that either a cis-antisense RNA or a trans-acting sRNA may be required to create such RNA substrate for RNase III to cleave (Lasa et al., 2011; Lioliou et al., 2012). Although, we predicted some putative interactions between the ftnA 3'UTR and previously identified sRNAs in S. aureus (Geissmann et al., 2009; Bohn et al., 2010; Carrol et al., 2016), we failed to validate such interactions in vivo (data not shown). Whether other sRNAs and/or asRNAs interact with the ftnA 3'UTR remains to be explored. Moreover, knowing that global regulatory RNA chaperones have been already shown to bind 3'UTRs (Holmqvist et al., 2016, 2018; Potts et al., 2017) and iron-sensing proteins like aconitase bind their own mRNA (Benjamin and Masse, 2014), it would be interesting to evaluate whether RNA-binding proteins or even the ferritin itself could interact with the ftnA mRNA through the 3'UTR to control ferritin expression.

It is noteworthy that the whole ftnA 3'UTR was highly conserved in S. aureus and S. argenteus. This suggested that the ftnA 3'UTR sequence may be relevant for both species, which preserve throughout evolution a similar post-transcriptional control of ferritin production. It is also interesting that the corresponding 3'UTR sequences from other Staphylococcus species were completely different (both in length and sequence) despite the ftnA CDS being conserved (Figure 6). We previously showed that constructs of chimeric mRNAs including the S. aureus ftnA CDS and the ftnA 3'UTRs from Staphylococcus simiae, Staphylococcus epidermidis, and Staphylococcus capitis were unable to decrease FtnA expression (Menendez-Gil et al., 2020), indicating the presence of species-specific 3'UTR-mediated regulatory mechanisms. How the ftnA 3'UTRs from different Staphylococcus species participate in the modulation of ferritin production remains to be investigated.

These putative regulatory differences are not restricted to the post-transcriptional level. It was also shown that the transcriptional regulation of ferritin expression in response to metals in S. epidermidis was significantly different from S. aureus (Morrissey et al., 2004), suggesting that members of the Staphylococcus genus have developed different strategies to regulate iron homeostasis in species-specific manners.

In addition to the ftnA 3'UTR, we recently found 3'UTR sequence variability in several staphylococcal genes, indicating that this phenomenon may be widespread among bacteria. Aside from iron homeostasis, long 3'UTRs with evolutionary variability (Menendez-Gil et al., 2020) also affect relevant biological processes such as metabolism (Maeda and Wachi, 2012), biofilm formation (de los Mozos et al., 2013; Zhu et al., 2016), and hemolysin production (Menendez-Gil et al., 2020). We proposed that these regions may be prone to changes that reflect in bacterial diversity in a similar way as it occurred for eukaryotes, promoting the diversification of species (Menendez-Gil and Toledo-Arana, 2021).

Another relevant observation in this study was the impaired growth of S. aureus under iron starvation conditions upon chromosomal deletion of the ftnA 3'UTR (Figure 5). Considering that deletion of the ftnA 3'UTR increased ferritin concentration (Figure 2; Menendez-Gil et al., 2020), it could be speculated that higher ferritin levels would sequester the scarce iron available inside the cells. As a result, the essential functions carried out by enzymes requiring iron as a cofactor would be affected, leading to bacterial growth arrest.
In summary, our study highlights the relevance of 3'UTRs to fine-tune the expression of genes involved in relevant processes such as iron homeostasis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PM-G and AT-A conceived and designed the experiments. PM-G and AC-M performed the experiments. PM-G, AC-M, CC, and AT-A analyzed the data and contributed to the interpretation of results. PM-G, CC, and AT-A wrote the manuscript. All authors contributed to the article and approved the submitted version.

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