Staphylococcus aureus pigmentation is not controlled by Hfq

Wenfeng Liu, Pierre Boudry, Chantal Bohn and Philippe Bouloc*

Abstract

Objective: The golden color of Staphylococcus aureus is due to the synthesis of carotenoid pigments. In Gram-negative bacteria, Hfq is a global posttranscriptional regulator, but its function in S. aureus remains obscure. The absence of Hfq in S. aureus was reported to correlate with production of carotenoid pigment leading to the conclusion that Hfq was a negative regulator of the yellow color. However, we reported the construction of hfq mutants in several S. aureus strains and never noticed any color change; we therefore revisited the question of Hfq implication in S. aureus pigmentation.

Results: The absence or accumulation of Hfq does not affect S. aureus pigmentation.

Keywords: Staphylococcus aureus, Hfq, Pigmentation, Staphyloxanthin, Regulation

Introduction

Staphylococcus aureus is a major pathogen responsible for numerous diseases from minor skin infection to septicemia, affecting humans and other animals. Its name “aureus” comes from the golden color of strains that express carotenoid pigments [1]. These pigments contribute to oxidative stress and neutrophil resistance, and virulence [2]. The carotenoid biosynthetic operon (crt-MNOPQ) leading to the synthesis of staphyloxanthin is regulated by σB [3, 4], an alternative σ factor that also controls a large number of general stress genes. σB activity depends on RsbU, its positive regulator [5, 6]. Numerous strains, including the S. aureus model NCTC8325, have rsbU mutations that prevent σB activity and crt operon expression, such that colonies are white. In addition, mutations in 37 genes were shown to result in the loss of a yellow pigmentation [5, 7].

Hfq is an RNA chaperone needed for activity of numerous regulatory RNAs in Gram-negative bacteria [8]. However, its role in Gram-positive bacteria, with the exception of Clostridium difficile [9], remains enigmatic [10]. Hfq functionality from different species is often tested by interspecies complementation tests. However, expression of hfq genes from Gram-positive bacteria S. aureus and Bacillus subtilis in Salmonella could not compensate the absence of endogenous hfq, indicating a functional difference between Gram positive and negative Hfq [11, 12].

We previously compared phenotypes of S. aureus hfq mutants with their isogenic parental strains and observed no detectable difference associated with the absence of Hfq in the tested conditions [13]. However, our results were partly challenged by a publication reporting that carotenoid pigment production was increased in hfq-negative strains [14]. Here we use nine different S. aureus strains to show that Hfq absence or overexpression has no effect on pigment expression.

Main text

Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. Allelic replacements of hfq+ by Δhfq::cat were either performed by φ11-phage

*Correspondence: philippe.bouloc@cnrs.fr
Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198 Gif-sur-Yvette, France
mediated transduction using RN4220 hfq::cat as a donor strain or by homologous recombination using pMADΔhfq::cat [13, 15]. The Δhfq::cat deletion in SAPHB5 was verified by Southern blot and subsequent Δhfq::cat transductants were verified by PCR as described [13].

Engineered plasmids were constructed as described [16]. Conditional hfq expression was obtained by cloning hfq under the xyl/tetO promoter in pRMC2 [17] and pRMC2FLAG (Table 1). pRMC2Hfq allowing hfq conditional expression was obtained as follows: pRMC2 and PCR-amplified hfq (using primers 39/49 on HG003

Table 1 Staphylococcus aureus strains, plasmids and primer used for this study

| Strain name | Key features | Reference or construction |
|-------------|--------------|--------------------------|
| RN4220 | Transformable by DNA from E. coli | [25] |
| SAPhB5 | RN4220 Δhfq::cat | [13] |
| NCTC8325 | Clinical isolate | [26] |
| SAPhB224 | NCTC8325 Δhfq::cat | NCTC8325 + φ11(SAPHB5) |
| NCTC8325-4 | NCTC8325 Δφ11 Δφ12 Δφ13 | [27] |
| SAPhB197 | NCTC8325-4 Δhfq::cat | NCTC8325-4 + φ11(SAPHB5) |
| RN6390 | NCTC 8325-4 φ6390 | [28] |
| SAPhB22 | RN6390 Δhfq::cat | [13] |
| HG001 | NCTC8325 rsbU repaired | [22] |
| SAPhB199 | HG001 Δhfq::cat | HG001 + φ11(SAPHB5) |
| HG002 | NCTC8325 tcaR repaired | [22] |
| SAPhB201 | HG002 Δhfq::cat | HG002 + φ11(SAPHB5) |
| HG003 | NCTC8325 rsbU and tcaR repaired | [22] |
| SAPhB203 | HG003 Δhfq::cat | HG003 + φ11(SAPHB5) |
| COL | Methicillin resistant clinical isolate | [29] |
| SAPhB16 | COL Δhfq::cat | [13] |
| Newman | Clinical isolate | [30] |
| SAPhB17 | Newman Δhfq::cat | [13] |
| SAPhB142 | RN4220 pRMC2 | RN4220 + pRMC2 |
| SAPhB248 | RN4220 pRMC2Hfq | RN4220 + pRMC2Hfq |
| SAPhB251 | RN4220 pRMC2HfqFLAG | RN4220 + pRMC2HfqFLAG |
| SAPhB233 | HG003 pRMC2 | HG003 + pRMC2 |
| SAPhB249 | HG003 pRMC2Hfq | HG003 + pRMC2Hfq |
| SAPhB257 | HG003 pRMC2HfqFLAG | HG003 + pRMC2HfqFLAG |

| Plasmid name | Key features | Reference/construction |
|--------------|--------------|------------------------|
| pRMC2 | Anhydrotetracycline (aTc) inducible promoter P_xyl/tetO | [17] |
| pRMC2Hfq | hfq inducible expression | See “Methods” |
| pRMC2FLAG | pRMC2 derivative for translational gene fusions with 3xflag coding sequence | See “Methods” |
| pRMC2HfqFLAG | hfq:3xflag inducible expression | See “Methods” |

| Primer name | Sequence | Purpose |
|-------------|----------|---------|
| 39 | GGGGTACCATGATTGCAACCGAAAAC | hfq amplification (with a KpnI site) |
| 49 | GGGGAATTCATATCTATTCACCTTCAGTAGATGC | hfq amplification (with an EcoRI site) |
| 856 | GGTACGTTAAGACATGCTAG | pRMC2 amplification |
| 918 | GCCTATTTCAATCTATTCATCAATGATAGAG | pRMC2 and pRMC2FLAG amplifications |
| 858 | TCAGATCTGTAAACGCTGAGCTGAACTGGTTGAGGAA | 3xflag amplification |
| 919 | GATAGAGTTAACATTTAAAGCCGAGCTCGAATCTAAAGACCA | 3xflag amplification |
| 865 | GCTTACAGGACAGACCGG | pRMC2FLAG amplification |
| 939 | GATAGAGTTATCTAAAGGCTGAAAGGATCGACAGATGA | hfq amplification for cloning in pRMC2FLAG |
| 940 | CCGTCTAGGCCTGTGTTTGTAGTCTTTCACTTCATGATGCTGG | hfq amplification for cloning in pRMC2FLAG |
DNA) were KpnI-EcoRI digested and ligated together. pRM2FLAG was engineered for conditional expression of 3xFLAG-tagged proteins as followed: pRM2 and pSUB11 [18] were PCR-amplified using primers 856/918 and 858/919, respectively. The two resulting products, i.e. pRM2 and 3xflag sequence coding, were assembled using the Gibson method [19]. pRM2HfqFLAG, allowing conditional expression of Hfq::3xFLAG, was obtained as follows: pRM2FLAG and hfg HG003 were PCR-amplified using primers 918/865 and 939/940, respectively. The two resulting products were assembled using the Gibson method.

Bacteria were grown in BHI medium (BD Difco, ref: 237500) at 37 °C under vigorous agitation. BHI solid media were obtained by the addition of Bacto Agar 15 g l	extsuperscript{−1} (BD Difco, ref: 214010). For strains containing pRM2 and derivatives, chloramphenicol (Sigma-Aldrich, ref: C0378) 5 µg ml	extsuperscript{−1} was added to media. Expression from pRM2 and derivatives was achieved by anhydrotetracycline (aTc, Chemodex, ref: CDX-A0197-M500) 250 ng ml	extsuperscript{−1} addition to growth media.

**Protein extraction, Western blotting and staphyloxanthin spectral measurement**

Overnight cultures were diluted 1000 times in fresh medium. After 3 h, aTc was added. 10 min and 30 min later, cells were harvested by centrifugation (16,000g for 2 min), resuspended in 400 µl Tris HCl buffer (50 mM, pH 6.8) and lysed using a FastPrep (3 cycles of 45 s at 6.5 m s	extsuperscript{−1}). Cell debris was removed by centrifugation (16,000g for 10 min). Protein concentration was determined by Bradford assays [20]. For each sample, 3 µg of protein extract was separated on a polyacrylamide gel (Blot	extsuperscript{TM} 4–12% Bis–Tris Plus, Invitrogen, ref: NW04122BOX). After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (iBlot	extsuperscript{TM} Flex Western System (ref: SLF2000S) was used according to supplier’s instructions. Membranes were probed with the primary polyclonal ANTI-FLAG antibody produced in rabbit (Sigma, ref: F-7425) at a 1/15,000 dilution. A rabbit secondary antibody conjugated to horseradish peroxidase (Advansta, ref: R-05072-500) was used at a 1/25,000 dilution. Bluminescent signal was detected with the WesternBright	extsuperscript{™} ECL-spray (Advansta, ref: K-12040-D50) using a digital camera (ImageQuant	extsuperscript{™} 350, GE Healthcare).

The *S. aureus* pigments were extracted as described [21]. In brief, strains were grown in BHI under vigorous agitation for 24 h. Cells were harvested by centrifugation, the pellet was rinsed twice with sterile water and pigments were extracted by methanol. Absorbance between 330 and 550 nm was measured on a microplate reader (CLARIOstar BMG LABTECH).

**Results**

**The absence of Hfq does not alter *S. aureus* pigmentation**

In 2010, Liu et al. reported that “deletion of *hfq* gene in *S. aureus* 8325-4 can increase the surface carotenoid pigments” [14]. Their work was performed using an allele called Δ*hfq*-8325 in which the *hfq* coding sequence was replaced by a kanamycin cassette. The *hfq* chromosomal deletion was constructed in strain RN4220 and then transduced into NCTC8325-4, RN6390, COL and ATCC25923 by phage φ11. We constructed a similar *hfq* deletion in RN4220, except that the *hfq* coding sequence was replaced by a chloramphenicol resistant gene (Δ*hfq*-cat); this allele was transduced into RN6390, COL and Newman by φ11-phage mediated transduction [13]. Note that RN4220, RN6390 and COL strains were used for both studies. As we did not notice a change of color when the Δ*hfq*-cat allele was introduced into these strains, this information was not reported [13]. In view of the previous report, we focused this work on the possibility that Hfq could affect *S. aureus* pigment expression.

NCTC8325 isolated in 1960 from a sepsis patient is the progenitor of numerous strains including NCTC8325-4 (cured of three prophages) which itself gave RN6390 and RN4220 [22]. As these descendants were mutagenized, they carry several mutations that may affect their phenotypes. NCTC8325 has a deletion of 11 bp in *rsbU* and a point mutation in *tcaR*. The derivatives HG001 (*rsbU* restored), HG002 (*tcaR* restored), HG003 (*rsbU* and *tcaR* restored) were constructed to perform physiological studies in a non-mutagenized background [22]. All these NCTC8325 derived strains, except HG001 and HG003 (which have a functional σB factor), give rise to white colonies (Fig. 1). In addition to those reported [13], we constructed Δ*hfq*-cat derivatives in NCTC8325, NCTC8325-4, HG001, HG002 and HG003 (Table 1). In contrast to results reported in Liu et al., deletion of the *hfq* gene in all tested strain backgrounds had no effect on pigmentation (Fig. 1a). Note that COL, Newman are not NCTC8325 derivatives.

Spectral profiles highlighting *S. aureus* carotenoid production were determined as described [21] for three strains and their *hfq* derivatives after growth for 24 h in BHI. HG003 and HG003 Δ*hfq*-cat gave equivalent profiles with three pics characteristic of carotenoid production. In contrast, NCTC8325-4 and RN1 had spectra characteristic of no or very little carotenoid production. As expected from our visual observation (Fig. 1a), the spectra of Δ*hfq*- derivatives did not differ from those of their respective parental strains (Fig. 1b).
Hfq overexpression does not alter S. aureus pigmentation

In the above-described strains, hfq is possibly poorly expressed, in which case hfq deletions would not lead to detectable phenotypes. We therefore tested the effects of an inducible Hfq expression system on pigment production. If the absence of Hfq leads to yellow colonies as proposed [14], the presence of Hfq could lower pigment production and lead to white colonies. To address this point, hfq was cloned under the control of the Pxyl/tetO promoter in multi-copy plasmid pRMC2 [17] leading to pRMC2Hfq. hfq expression in strains harboring pRMC2Hfq was induced upon aTc addition to media. To confirm that Pxyl/tetO was effectively driving hfq expression, a pRMC2Hfq derivative was engineered harboring a 3xflag sequence inserted in frame at the end of the hfq open reading frame. The resulting plasmid, pRM2HfqFLAG is a proxy for expression from pRMC2Hfq. HG003 was transformed with pRMC2, pRMC2Hfq and pRMC2HfqFLAG. The protein Hfq::3xFLAG was detected upon aTc induction by western blotting using FLAG antibodies (Fig. 2a). We inferred from this result that addition of aTc to strains harboring pRMC2Hfq lead to Hfq synthesis. The RN4220 white and HG003 yellow colors were not affected by the presence of either pRMC2, pRMC2Hfq or pRMC2HfqFLAG and remained identical upon aTc addition to growth medium (Fig. 2b).

Conclusion

Our results show that neither the absence, nor the accumulation of Hfq affects pigmentation of S. aureus: Hfq does not appear to regulate staphyloxanthin synthesis. Our conclusions are supported by Tarrant PhD dissertation showing an NCTC8325 hfq mutant that remained unpigmented [23]. Of note, Pseudomonas aeruginosa reportedly induces pigment production of a non-pigmented phenotypic variant of S. aureus, however, this effect was independent of hfq transcription [24]. In addition, color variation in USA300 strain was screened in a genome-wide transposon mutant

Fig. 1 Absence of Hfq does not affect S. aureus pigmentation. The indicated strains were grown overnight in BHI and then a streaked on BHI agar or b assayed for spectral profiles as described [21]
library, and the hfq inactivation was not reported to affect S. aureus pigmenta
tion [7].

While the hfq gene is absent in some Firmicutes (e.g. Lacto-
tobacillales), it is conserved in all S. aureus, suggesting that it plays a crucial function, however not related to pigment expression. The quest to find the Staphylococcal Hfq function remains open.

Limitations
Our conclusion is in contradiction with Liu et al. results concerning the effect of Hfq on S. aureus pigmenta
tion [14]. We cannot rule out that our observation is limited to specific S. aureus strains. However, we used an NCTC8325-4 hfq derivative similar the one used in the previous study. Furthermore, the present results are strengthened by the construction of hfq mutants in numerous S. aureus backgrounds. The discrepancy between our and Liu et al. 2010 [14] results, is a possible inadvertent selection of mutants with altered color patterns (as shown in [23]) in the former study.

Abbreviations
PCR: Polymerase chain reaction; BHI: Brain heart infusion; aTc: Anhydrotetracycline.

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Authors’ contributions
PhD designed the experiments and wrote the manuscript. WL, PiB and CB performed the experiments, analyzed data and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article. Strains and plasmids are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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