Distinctive Regulation of Carbapenem Susceptibility in *Pseudomonas aeruginosa* by Hfq

**Elisabeth Sonnleitner**, **Petra Pusic**, **Michael T. Wolfinger** and **Udo Bläsi**

1 Department of Microbiology, Immunobiology and Genetics, Max Perutz Labs, Vienna BioCenter (VBC), University of Vienna, Vienna, Austria, 2 Department of Theoretical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria, 3 Research Group Bioinformatics and Computational Biology, Faculty of Computer Science, University of Vienna, Vienna, Austria

Carbapenems are often the antibiotics of choice to combat life threatening infections caused by the opportunistic human pathogen *Pseudomonas aeruginosa*. The outer membrane porins OprD and OpdP serve as entry ports for carbapenems. Here, we report that the RNA chaperone Hfq governs post-transcriptional regulation of the *oprD* and *opdP* genes in a distinctive manner. Hfq together with the recently described small regulatory RNAs (sRNAs) ErsA and Sr0161 is shown to mediate translational repression of *oprD*, whereas *opdP* appears not to be regulated by sRNAs. At variance, our data indicate that *opdP* is translationally repressed by a regulatory complex consisting of Hfq and the catabolite repression protein Crc, an assembly known to be key to carbon catabolite repression in *P. aeruginosa*. The regulatory RNA CrcZ, which is up-regulated during growth of *P. aeruginosa* on less preferred carbon sources, is known to sequester Hfq, which relieves Hfq-mediated translational repression of genes. The differential carbapenem susceptibility during growth on different carbon sources can thus be understood in light of Hfq-dependent *oprD/opdP* regulation and of the antagonizing function of the CrcZ RNA on Hfq regulatory complexes.

**Keywords:** *Pseudomonas aeruginosa*, *opdP*, *oprD*, carbapenem resistance, riboregulation, catabolite repression

**INTRODUCTION**

The opportunistic human pathogen *Pseudomonas aeruginosa* can cause severe infections. It is particularly devastating for immunocompromised individuals and patients with cystic fibrosis, leading to high morbidity and mortality. As they are less prone to degradation by extended spectrum β-lactamases, carbapenems are frequently used to treat severe infections of Gram-negative bacteria including *P. aeruginosa* (Papp-Wallace et al., 2011; Fritzenwanker et al., 2018). Carbapenem-resistant *P. aeruginosa* strains are increasingly occurring (McDougall et al., 2013; Castanheira et al., 2014; Buehrle et al., 2017), which prompted the World Health Organization to rank carbapenem-resistant *P. aeruginosa* among the priority pathogens to investigate new drug treatments (Tacconelli et al., 2018). *P. aeruginosa* exhibits several resistance mechanisms toward carbapenems including the production of metallo-β-lactamases and carbapenemase (Sacha et al., 2008; Bassetti et al., 2018) as well as dedicated efflux systems (Chalhoub et al., 2016). Another notable feature of *P. aeruginosa*’s high intrinsic antibiotic resistance is the low outer membrane permeability (Hancock and Woodruff, 1988; Breidenstein et al., 2011). *P. aeruginosa* utilizes...
a variety of specialized outer membrane porins (Hancock and Brinkman, 2002; Eren et al., 2012). Two of them, OprD/OccD1 and OpdP/OccD3, show high sequence similarity (51%) and serve as entry ports for basic amino acids and small peptides as well as for certain carbapenems, e.g., imipenem and meropenem (Quinn et al., 1986; Trias et al., 1989; Tamber and Hancock, 2006; Papp-Wallace et al., 2011; Isabella et al., 2015). In fact, the first documented case of clinical resistance to carbapenems was found to be due to a loss of the monocistronic oprD gene (Quinn et al., 1986). The deletion of oprD resulted in a decreased susceptibility to carbapenems, but deletion of the opdP gene alone, residing in an operon together with genes encoding a dipeptide ABC transport system (Chevalier et al., 2017), did not cause a significant change. However, the deletion of both genes led to a remarkable increase in resistance when compared to the deletion of oprD alone (Isabella et al., 2015).

The RNA chaperone Hfq is a pleiotropic regulator and virulence factor in P. aeruginosa (Sonleitner et al., 2003, 2006, 2018). Hfq is involved in the control of mRNA translation through distinct mechanisms. In riboregulation, Hfq can act indirectly by facilitating base-pairing interactions of small regulatory RNAs (sRNAs) with cognate mRNA targets (Vogel and Luisi, 2011; Kavita et al., 2018). On the other hand, Hfq can directly repress translation, by binding to A-rich sequences at or in the vicinity of translation initiation sites (Sonleitner and Bläsi, 2014). Hfq has several distinct RNA binding sites. Crystalllographic and biophysical data showed that RNA recognition is mediated by distinct interactions with distal, proximal, and rim faces of the hexameric ring (Schumacher et al., 2002; Link et al., 2009; Sauer et al., 2012; Panja et al., 2013). Many sRNAs bind to the proximal side of Hfq via U-rich stretches (Schumacher et al., 2002; Mikulecky et al., 2004; Link et al., 2009) or through the poly-uridine tails derived from rho-independent terminators (Otaka et al., 2011; Sauer and Weichenrieder, 2011; Ishikawa et al., 2012). Internal U/A-rich regions in sRNAs as well as in mRNAs were found to interact with arginine patches on the lateral rim of the Hfq-hexamer (Sauer et al., 2012; Peng et al., 2014; Schu et al., 2015). The distal side recognizes A-rich regions, previously defined as ARN repeats, where A is an adenine, R is any purine nucleotide and N is any nucleotide, which are often present in mRNAs around the ribosome binding site (Link et al., 2009; Murina et al., 2013; Robinson et al., 2014; Sonleitner and Bläsi, 2014).

A GRIL-seq approach identified two base-pairing small regulatory sRNAs, Sr0161, and EsrA, as negative translational regulators of oprD (Zhang et al., 2017). EsrA is transcriptionally controlled by the envelope stress response regulator AlgU/T (Ferrara et al., 2015), and its expression is further up-regulated after a shift from high to low oxygen supply, and upon entry into stationary phase (Ferrara et al., 2015; Zhang et al., 2017). Sr0161 did not show any phase dependent expression in full broth (Zhang et al., 2017). The study by Zhang et al. (2017) also suggested an interaction between Sr0161 and opdP mRNA. RT-qPCR showed that the opdP levels were decreased after overexpression of the sRNA and modestly increased in a sr0161 deletion mutant (Zhang et al., 2017). However, in contrast to Hfq-Sr0161/EsrA-mediated negative translational regulation of oprD, it remains unknown whether Sr0161 directly regulates translation of opdP (Zhang et al., 2017).

In P. aeruginosa, carbon catabolite repression (CCR) operates at the post-transcriptional level. In the presence of preferred C-sources (e.g., succinate), the RNA chaperone Hfq binds to the translation initiation region (TIR) of mRNAs encoding proteins important for the uptake and utilization of less preferred C-sources, and represses their translation (Sonleitner and Bläsi, 2014). Upon binding, the Hfq/RNA complex forms a “platform” to which the catabolite repression protein Crc can bind, which results in stabilization of the repressive complex (Sonleitner et al., 2018; Pei et al., 2019). When the preferred C-source is exhausted, the levels of the Hfq-titrating RNA CrcZ increase, which in turn leads to relieve of Hfq/Crc-mediated CCR (Sonleitner and Bläsi, 2014). Previous studies provided some hints that oprD is regulated by CCR (Ochs et al., 1999; Linares et al., 2010). However, recent omics studies are somewhat inconsistent. A ChIP-seq approach identified opdP mRNA among the mRNAs that were co-immunoprecipitated with antibodies directed against Hfq as well as against Crc (Kambara et al., 2018). Nevertheless, transcriptomic and proteomic analyses designed to reveal the Crc regulon revealed only opdP as a candidate for Crc-mediated post-transcriptional regulation but not oprD (Corona et al., 2018).

Taken these studies together with the observation that a PAO1 hfq⁻/-crc⁻ deletion mutant was more susceptible to carbapenems when compared with the parental strain (Ducret et al., 2016; Pusic et al., 2018), it appeared safe to assume that Hfq is involved in regulation of both, oprD and opdP. In this study, we addressed the question whether post-transcriptional regulation of oprD and opdP occurs through riboregulation and/or through CCR, i.e., through Hfq/Crc repressive complexes. To dissect the regulatory mechanism(s) for oprD and opdP, we used a genetic approach employing translational reporter constructs in conjunction with different P. aeruginosa O1 mutant strains. These studies verify that oprD is regulated by Hfq-mediated riboregulation and strongly indicate that opdP is directly translationally repressed by Hfq/Crc.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Strains and plasmids used in this study are listed in Supplementary Table S1. If not indicated otherwise, the cultures were grown at 37°C in either Lysogeny-broth (LB) (Sambrook et al., 1989) or basal-salt medium (BSM) supplemented with the indicated carbon sources (Sonleitner et al., 2009). When required, the following concentrations of antibiotics were used: 15 µg/ml gentamicin, 25 µg/ml tetracycline, and 100 µg/ml ampicillin for Escherichia coli; 50 µg/ml gentamicin, 100 µg/ml of tetracycline, and 250 µg/ml carbenicillin for P. aeruginosa.

Construction of PAO1 Deletion Strains

The deletion of the first 82 nucleotides (nt) of ersA (coordinates 6183580-6183661 of the PAO1 genome; Winsor et al., 2016)
and the deletion of the sr0161 3’ end including additional 24 nt downstream of sr0161 (coordinates 184302–184482 of the PAO1 genome; Winsor et al., 2016) was created by homologous recombination, respectively (Zhang et al., 2017). Briefly, plasmids pEXG2-sr0161 (for generation of PAO1Δsr0161 and PAO1ΔhfqΔsr0161) and pEXG2-ersA (for generation of PAO1ΔersAΔsr0161 and PAO1ΔhfqΔersAΔsr0161) were mobilized into the strains PAO1, PAO1Δhfq, PAO1Δsr0161, and PAO1ΔhfqΔsr0161 with the aid of E. coli strain S17-1, and then chromosomally integrated through selection for gentamicin. Excision of the vector by a second crossover event was achieved by selection of sucrose insensitive cells as the pEXG2 vector encodes the Bacillus subtilis sacB gene, whose gene product – levan sucrase – renders Gram-negative bacteria sensitive to sucrose (Rietsch et al., 2005; Hmelo et al., 2015).

Construction of Plasmid pME6015P_{tac}

For construction of a cloning vector suited for the generation of translational lacZ fusions under the control of the P_{tac} promoter, a 1,522-base pair (bp) fragment encoding the LacI\(^9\) repressor gene and the P_{tac} promoter together with the operator sites located downstream of the transcriptional start site were amplified by PCR using the oligonucleotide pairs A123 (5′-AAC TTC GCC GTC ACT GCG GCA C-3′) and M85 (5′-TTT TTT GAT GTA CCA ATT GTT ATC CGC TCA CAA TTC C-3′) and plasmid pME6032 as template. The PCR fragment was cleaved with EcoRI and BamHI, and then ligated into the corresponding sites of plasmid pME6015.

Construction of Plasmids pME6015P_{tac}oprod::lacZ and pME6015P_{tac}opdP::lacZ

To construct translational gene fusions between opd\(^P\) and opd\(^P\), respectively, and lac\(^Z\) under transcriptional control of the P_{tac} promoter, a 71-bp fragment of the TIR of the P_{opd\(^P\)} (coordinates 1045365–1045294 of the PAO1 genome; Winsor et al., 2016) and a 118-bp fragment of the TIR of opd\(^P\) (coordinates 5038800–5038918 of the PAO1 genome; Winsor et al., 2016) were amplified by PCR using the oligonucleotide pairs A123 (5′-AGC TGG ATC GAC AAG AAC TAG CCG TCA C-3′)/H112 (5′-ACG TCT GCA GCC TCT ACT TCA TCA CCT TCA TTG-3′) for opd\(^P\) and Q144 (5′-ACG TGG ATC TCT GCC GCG CCG TCT TCG-3′)/Q148 (5′-AGC TCT GCA GCC TCT GG TGG CCG AAC-3′) for opd\(^P\), respectively, and chromosomal DNA of PAO1 as template. The PCR fragments were cleaved with BamHI and PstI, and then ligated into the corresponding sites of plasmid pME6015P_{tac}. Despite the presence of LacI\(^9\), the P_{tac} promoter was leaky in the pME6015P_{tac} derivatives (not shown). Thus, IPTG was not added in the experiments described below.

Co-immunoprecipitation of mRNAs Bound to Hfq

A total of 40 ml of PAO1 culture was grown in BSM complex medium (Sonnleitner et al., 2018) and harvested at an OD_{600} of 1.5. The cells were first washed in lysis buffer (20 mM Tris pH 8.0, 150 mM KCl, 1 mM MgCl_2, 1 mM DTT, and 0.05% Triton X-100) and then snap frozen in liquid nitrogen. The cells were lysed by sonication (six times for 10 sec on ice) in 800 µl lysis buffer in the presence of 200 U RibonLock\(^\text{TM}\) RNase inhibitor (Fermentas). Cell debris were removed by centrifugation and anti-Hfq antibodies (Pineda) were added to 60 µl supernatant and incubated for 2 h at 4°C on a rotating wheel. Then, 5 µl Dynabeads\(^\text{TM}\) Protein G beads (Novex) were added, and the incubation was continued for 1 h. The beads were washed three times with lysis buffer and finally collected in 200 µl of lysis buffer without Triton X-100. Then, 100 µl phenol was added and the beads were shaken at 900 rpm for 30 min at room temperature. The RNA was purified by phenol-chloroform extraction, which was followed by ethanol precipitation. Libraries were constructed using NEBNext\(^\text{TM}\) Ultra\(^\text{TM}\) Directional RNA Library Prep Kit from Illumina. A total of 100 bp single end sequence reads were generated using the Illumina HiSeq 2000 platform at the Vienna BioCenter Core Facility\(^1\). Sequencing adapter removal was performed with cutadapt (Martin, 2011). Mapping of opd\(^P\) and opd\(^P\) RNA against the PAO1 reference genome (NCBI accession number NC_002516.2) was performed with Segemehl (Hoffmann et al., 2009) with default parameters. The mapped sequencing data were prepared for visualization using the ViennaNGS tool box, and visualized with the UCSC Genome Browser (Wolfinger et al., 2015). The raw sequencing data were deposited in the European nucleotide archive (ENA) as a study under the accession number PRJEB37368.

Microscale Thermophoresis

For in vitro transcription of the 100 nt long opd\(^P\) RNA fragment [nt −61 to nt +39 with regard to the A (+1) of the AUG start codon] and the 150 nt long opd\(^P\) RNA fragment [nt −111 to nt +39, with regard to the G (+1) of the GUG start codon], the AmpliScribe T7-Flash Transcription Kit (Epicentre Biotechnologies) was used according to the manufacturer’s instructions. First, PCR fragments were generated with the primer pairs Y133 (5′-TCT AGA CGG TAT CCC TAG CCG TCA C-3′)/H112 (5′-ACG TCT GCC TCC TCA TCA ACT TCA TTG-3′) for opd\(^P\), and Q144 (5′-ACG TGG ATC TCT GCC GCG CCG TCT TCG-3′)/Q148 (5′-AGC TCT GCA GCC TCT GG TGG CCG AAC-3′) for opd\(^P\), respectively, and chromosomal DNA of PAO1 as template. The PCR fragments were cleaved with BamHI and PstI, and then ligated into the corresponding sites of plasmid pME6015P_{tac}. Despite the presence of LacI\(^9\), the P_{tac} promoter was leaky in the pME6015P_{tac} derivatives (not shown). Thus, IPTG was not added in the experiments described below.

---

\(^1\)https://www.viennabiocenter.org/facilities/
Facility NT.115 Green/Red instrument at the Vienna BioCenter Core Facility. The MST measurements were performed in duplicate. The data for MST analysis were recorded at 25°C using the red LED (excitation: 625 nm and emission: 680 nm); MST Power 40%, LED Power 50%. Data analysis was performed with NTAffinityAnalysis v2.0.2 for thermophoresis and T-jump analysis 0 and 5 s after the pulse. For determination of the $K_T$-values the Hill-fit model within the NTAffinityAnalysis software was used.

**Agar Disk Diffusion Assay**

PAO1 and mutants thereof were grown in BSM supplemented with either 40 mM succinate or mannitol to an OD$_{600}$ of 1.8–2.0. Then, 200 µl of cultures were plated on agar plates containing the respective media and filter disks loaded with 10 µg imipenem were applied on top (Oxoid). The plates were incubated at 37°C for 24 h.

The diameter of the growth inhibition zones was measured in mm.

**β-Galactosidase Assays**

The β-galactosidase activities were determined as described (Miller, 1972). The cells were permeabilized with 5% toluene. Unless indicated otherwise, the β-galactosidase units in the different experiments were derived from at least two independent experiments, and are shown as mean. The error bars in the different figures represent standard deviations. Except for the data in Figure 3, statistical analyses were performed in Excel with a two-tailed Student’s t-test of two sample arrays with unequal variance, ns (non-significant); $P > 0.05$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Due to the multiple comparison, the results were statistically analyzed by an ANOVA test with post hoc multiple comparison. In short, Levene’s test was used to test for equality (homogeneity) of variances between the tested groups. Statistical significance was determined by one-way ANOVA with the Tukey’s post hoc test when more than two groups with normal distribution were compared (Figure 3A). When more than two groups with unequal variance were compared, the Brown-Forsythe and Welch’s ANOVA test with the Dunnett’s T3 post hoc test was used (Figures 3B–D), ns (non-significant); $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant results.

**Western-Blot Analyses**

Equal amounts of total proteins were separated on a 12% SDS-polyacrylamide gel, and then electro-blotted onto a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and probed with rabbit anti-Hfq, rabbit anti-Crc or rabbit anti-E. coli-S1 antibodies (Sonleitner and Bläsi, 2014). Immunodetection of ribosomal protein S1 served as a loading control. The antibody-antigen complexes were visualized with horseradish peroxidase (HRP) conjugated anti-rabbit antibodies (Cell Signaling Technology) using the SuperSignalTM West Pico PLUS chemiluminescent substrate kit (Thermo Scientific). The signals were detected with ChemiDocTM Touch Imaging System (BioRad) and analyzed with ImageLab 5.2.1 (BioRad).

**RESULTS**

**Hfq Is Involved in Translational Regulation of oprD and opdP**

Recent studies revealed that *P. aeruginosa* hfq deletion mutants showed an increased susceptibility to imipenem (Ducret et al., 2016; Pusic et al., 2018), which coincided with higher oprD and opdP transcript levels in the hfq mutant strain when compared with the parental strain during growth in different media (Supplementary Figure S1 and Supplementary Table S2) (Ducret et al., 2016; Pusic et al., 2018). To verify the impact of Hfq on oprD and opdP translation, oprD::lacZ and opdP::lacZ translational gene fusions were constructed and the β-galactosidase activities conferred by the respective fusion proteins were determined in strains PAO1 and PAO1Δhfq harboring plasmids pME6015P$_{lac}$oprD::lacZ and pME6015P$_{lac}$opdP::lacZ, respectively. Transcription of either chimeric gene was driven by the P$_{lac}$ promoter to control for potential transcriptional effects of Hfq (Ducret et al., 2016). The strains were grown in LB medium to an OD$_{600}$ of 2.0, as the sRNAs ErsA and Sr0161 are transcribed under these conditions (Zhang et al., 2017). As reported by Zhang et al. (2017), our RNAseq analysis confirmed growth independent quantities of Sr0161 RNA in LB medium and increased levels of ErsA RNA in stationary phase when compared to logarithmically growing cells (Supplementary Figure S2). Translation of both reporter fusions was repressed in the presence of Hfq. However, the effect of Hfq on oprD::lacZ translation was less pronounced (Figure 1A: 1.5-fold difference between PAO1 and PAO1Δhfq harboring pME1615P$_{lac}$oprD::lacZ) than on opdP::lacZ translation (Figure 1B: 10-fold difference between PAO1 and PAO1Δhfq harboring pME1615P$_{lac}$opdP::lacZ). The differential translation rates observed for oprD::lacZ and opdP::lacZ in the wild-type strain and the corresponding hfq deletion strains coincided with a 2–3-fold and ≥10-fold higher transcript abundance of oprD and opdP, respectively, in PAO1 and PAO1Δhfq during cultivation in different media (Supplementary Table S2). As anticipated, the complementation of hfq by ectopic expression of a plasmid borne hfq$_{B32}$ allele in strain PAO1Δhfq resulted again in repression of the oprD::lacZ and opdP::lacZ reporter genes (Figures 1C,D), respectively.

As the mRNA sequences of the TIRs of oprD and opdP genes in the clinical isolate PA14 showed only minor differences to PAO1 (Winsor et al., 2016), the same experiments were performed with the clinical isolate PA14 and the corresponding PA14Δhfq mutant harboring plasmids pME1615P$_{lac}$oprD::lacZ and pME1615P$_{lac}$opdP::lacZ, respectively, after growth in synthetic cystic fibrosis medium (SCFM) containing 100 µM FeSO$_4$ (Palmer et al., 2007; Táta et al., 2016). The outcome of these experiments was comparable with the results obtained for PAO1 after growth in LB medium (Supplementary Figures S3A–D).

**Binding of Hfq to the Translation Initiation Regions of oprD and opdP**

To test whether Hfq binds to the TIRs of oprD and opdP, a co-immunoprecipitation assay with Hfq specific antibodies was
FIGURE 1 | Continued
performed to identify protein bound RNA fragments. Subsequent RNA-seq revealed a distinct sub-sequence upstream of the oprD start codon (Figure 2A). This region contains several ARN motifs, which have been shown to bind to the distal side of Hfq (Link et al., 2009). The sub-sequence also comprises the EsrA and Sr0161 interaction sites (Figure 2A) (Zhang et al., 2017).

The co-immunoprecipitated RNA sub-sequence including the start codon of oprD comprised as well several ARN-triplets and overlaps with the predicted Sr0161 binding-site (Figure 2B) (Zhang et al., 2017).

The binding affinity of Hfq for the respective co-immunoprecipitated oprD and oprD sub-sequences was determined by MST. Hfq exerted a high affinity for the oprD−61→39 sub-sequence ($K_d = 19.7 \pm 2.8$ nM) and a somewhat lower affinity for the oprD−111→39 sub-sequence ($K_d = 78.1 \pm 1.97$ nM) (Figure 2C).

At this junction it seems worth noting that binding of Hfq to the TIRs of either oprD or oprD is anticipated regardless of whether Hfq/sRNA-mediated regulation is the underlying mechanism of translational repression, or whether this occurs through Hfq/Crc repressive complexes.

The sRNA Sr0161 Does Not Impact Translation of oprD

Next, we re-examined whether sRNA Sr0161, which was identified by GRIL-seq and suggested to base-pair with the oprD TIR (Zhang et al., 2017) can translationally regulate oprD identified by GRIL-seq and suggested to base-pair with the TIRs of either oprD (Zhang et al., 2017) can translationally regulate oprD identified by GRIL-seq and suggested to base-pair with the TIRs of either oprD (Zhang et al., 2017). The binding affinity of Hfq for the respective co-immunoprecipitated oprD and oprD sub-sequences was determined by MST. Hfq exerted a high affinity for the oprD−61→39 sub-sequence ($K_d = 19.7 \pm 2.8$ nM) and a somewhat lower affinity for the oprD−111→39 sub-sequence ($K_d = 78.1 \pm 1.97$ nM) (Figure 2C).

At this junction it seems worth noting that binding of Hfq to the TIRs of either oprD or oprD is anticipated regardless of whether Hfq/sRNA-mediated regulation is the underlying mechanism of translational repression, or whether this occurs through Hfq/Crc repressive complexes.

The sRNA Sr0161 Does Not Impact Translation of oprD

Next, we re-examined whether sRNA Sr0161, which was identified by GRIL-seq and suggested to base-pair with the oprD TIR (Zhang et al., 2017) can translationally regulate oprD identified by GRIL-seq and suggested to base-pair with the TIRs of either oprD (Zhang et al., 2017). The binding affinity of Hfq for the respective co-immunoprecipitated oprD and oprD sub-sequences was determined by MST. Hfq exerted a high affinity for the oprD−61→39 sub-sequence ($K_d = 19.7 \pm 2.8$ nM) and a somewhat lower affinity for the oprD−111→39 sub-sequence ($K_d = 78.1 \pm 1.97$ nM) (Figure 2C).

At this junction it seems worth noting that binding of Hfq to the TIRs of either oprD or oprD is anticipated regardless of whether Hfq/sRNA-mediated regulation is the underlying mechanism of translational repression, or whether this occurs through Hfq/Crc repressive complexes.

The sRNA Sr0161 Does Not Impact Translation of oprD

Next, we re-examined whether sRNA Sr0161, which was identified by GRIL-seq and suggested to base-pair with the oprD TIR (Zhang et al., 2017) can translationally regulate oprD identified by GRIL-seq and suggested to base-pair with the TIRs of either oprD (Zhang et al., 2017). The binding affinity of Hfq for the respective co-immunoprecipitated oprD and oprD sub-sequences was determined by MST. Hfq exerted a high affinity for the oprD−61→39 sub-sequence ($K_d = 19.7 \pm 2.8$ nM) and a somewhat lower affinity for the oprD−111→39 sub-sequence ($K_d = 78.1 \pm 1.97$ nM) (Figure 2C).

At this junction it seems worth noting that binding of Hfq to the TIRs of either oprD or oprD is anticipated regardless of whether Hfq/sRNA-mediated regulation is the underlying mechanism of translational repression, or whether this occurs through Hfq/Crc repressive complexes.
FIGURE 2 | Co-immunoprecipitation of *oprD* and *opdP* mRNA fragments with Hfq. Hfq-bound RNAs were isolated from lysates of the PAO1 culture after co-immunoprecipitation with Hfq-specific antibodies as described in section “Materials and Methods.” The identity of Hfq-bound RNAs in the supernatant was revealed by RNA-seq. (A,B) The read coverage visualized in the Genome Browser is shown for *oprD* (A) and *opdP* (B), respectively. The sequences of the co-immunoprecipitated mRNA fragments corresponding to the TIR of *oprD* (A) and *opdP* (B), respectively, are shown below. The numbers refer to the PAO1 genome coordinates (http://www.pseudomonas.com) (Winsor et al., 2016). The start codons of *oprD* and *opdP* are underlined. Putative Hfq binding sites are indicated in red. The interacting sequences for EsrA and Sr0161 in the *oprD* TIR (A) (Zhang et al., 2017) are indicated by gray and green bars, respectively. The putative interacting sequence for Sr0161 in the *opdP* TIR (B) is indicated by a green bar (B) (Zhang et al., 2017). (C) Microscale thermophoresis reveals the *Kd* of Hfq for *oprD*−61−39 RNA (yellow circles) and *opdP*−111−39 RNA (green triangles) with 19.7 ± 2.8 nM and 78.1 ± 1.97 nM, respectively. Increasing amounts of the non-labeled *in vitro* transcribed *oprD*−61−39 and *opdP*−111−39 fragments were added to 56 nM fluorescently labeled Hfq protein. The dissociation constant (*Kd*) of *oprD*−61−39 and *opdP*−111−39 was determined as described in section “Materials and Methods,” and was expressed as mean EC50 ± EC50 confidence interval of two independent experiments. Thermophoresis/T-jump analysis is shown. LED power of 50% and MST power of 40% were used.

the *oprD*:lacZ reporter gene was unaffected by Crc (Figure 4B). In line, ectopic expression of *crc*-flag resulted in repression of *opdP*:lacZ in strain PAO1Δcrc(pME6015P<sub>lac</sub>opdP::lacZ) (Figure 4C), whereas ectopic expression of *crc*-flag did not significantly change *oprD*:lacZ translation in strain PAO1Δcrc(pME6015P<sub>lac</sub>oprD::lacZ) (Figure 4D). These data strongly suggested that Crc contributes to Hfq-mediated direct translational regulation of *opdP*, i.e., that its translation is controlled by a Hfq/Crc repressive complex. As translational regulation of *oprD* was rather independent of Crc, these studies further support the notion that translational regulation of *oprD* is only subject to Hfq/EsrA/Sr0161-mediated riboregulation.

**High CrcZ Levels Increase *oprD* and *opdP* Translation**

Transcription of the Hfq titrating RNA CrcZ is known to be induced by less preferred carbon sources such as mannitol.
Sonnleitner et al. Carbapenem Susceptibility Regulation by Hfq

**FIGURE 3** | The sRNAs Sr0161 does not regulate opdP translation. (A) The strains PAO1 (blue bar), PAO1Δsr0161 (green bar), and PAO1ΔhfpΔsr0161 (pink bar) harboring plasmid pME6015P_tac::opdP::lacZ were grown in LB medium. Samples were withdrawn at an OD_{600} of 2.0. The bars represent the β-galactosidase values conferred by the opdP::lacZ translational fusion gene encoded by plasmid pME6015P_tac::opdP::lacZ. (B) The strains PAO1 (pKH6) (blue bar), PAO1(pKH6::sr0161) (gray bar), PAO1Δsr0161(pKH6) (green bar), and PAO1Δsr0161(pKH6::sr0161) (striped green bar) harboring plasmid pME6015P_tac::opdP::lacZ were grown in LB medium supplemented with 0.2% arabinose to induce sRNA gene expression from plasmid pKH6::sr0161. Samples were withdrawn at an OD_{600} of 2.0. The bars represent the β-galactosidase values conferred by the opdP::lacZ translational fusion gene encoded by plasmid pME6015P_tac::opdP::lacZ. (C) The strains PAO1 (blue bar), PAO1ΔersA::sr0161 (green bar) and PAO1ΔhfpΔersA::sr0161 (pink bar) harboring plasmid pME6015P_tac::oprD::lacZ were grown in LB medium. Samples were withdrawn at an OD_{600} of 2.0. The bars represent the β-galactosidase values conferred by the oprD::lacZ translational fusion gene encoded by plasmid pME6015P_tac::oprD::lacZ. (D) Ectopic expression of ersA and sr0161 in strains PAO1ΔersA::sr0161 (green bars) and PAO1ΔhfpΔersA::sr0161 (pink bars) harboring plasmid pME6015P_tac::oprD::lacZ. The β-galactosidase values conferred by the oprD::lacZ translational fusion gene in either strain in the absence of ersA::sr0161 expression (solid bars) and in the presence of ersA (dotted bars) or sr0161 (striped bars) are indicated by bars. Statistical analyses were performed with AGOVA test with post hoc multiple comparison as described in section “Materials and Methods.” ns (non-significant); *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
FIGURE 4 | Continued

A

\[ \text{opdP} \]

\( \beta \)-galactosidase activity (Miller Units)

\[ \text{pME6015P}_{\text{tac-opdP::lacZ}} \]

PAO1  PAO1Δcrc

B

\[ \text{oprD} \]

\( \beta \)-galactosidase activity (Miller Units)

\[ \text{pME6015P}_{\text{tac-oprD::lacZ}} \]

PAO1  PAO1Δcrc

C

\[ \text{opdP} \]

\( \beta \)-galactosidase activity (Miller Units)

\[ \text{PAO1Δcrc} \]

\[ \text{pME6015P}_{\text{tac-opdP::lacZ}} \]

\[ \text{pME4510} \]

\[ \text{pME4510Crc\_Flag} \]

D

\[ \text{oprD} \]

\( \beta \)-galactosidase activity (Miller Units)

\[ \text{PAO1Δcrc} \]

\[ \text{pME6015P}_{\text{tac-oprD::lacZ}} \]

\[ \text{pME4510} \]

\[ \text{pME4510Crc\_Flag} \]
(Sonleitner et al., 2009). Therefore, we next tested whether the susceptibility toward imipenem is increased in an oprD deletion strain during growth in the presence of mannitol (high levels of CrcZ; Sonleitner et al., 2009) when compared to the presence of succinate (low levels of CrcZ; Sonleitner et al., 2009), i.e., whether translational repression of oprD by Hfq/Crc is relieved in the presence of CrcZ and in the absence of CCR. The disk diffusion assay revealed extended growth inhibition zones for the oprD mutant strain during growth in mannitol (Table 1), and therefore increased susceptibility to the tested antibiotic, consistent with the notion that oprD translation is controlled by Hfq/Crc. The same experiment conducted with an oprD deletion strain revealed a comparable result, indicating that CrcZ also interferes with Hfq/EsrA/Sr0161-mediated riboregulation of oprD. In support, ectopic overexpression of crcZ from plasmid pMMBcrcZ in strains PAO1(pME6015P_tac_oprD::lacZ) (Supplementary Figure S4A) and PAO1(pME6015P_tac_opdP::lacZ) (Supplementary Figure S4B) confirmed that high levels of CrcZ increase both oprD and opdP translation. Again, these findings are in line with a reduced minimal inhibitory concentration (MIC) of imipenem upon ectopic overexpression of crcZ in PAO1 (Supplementary Figure S4C).

**DISCUSSION**

The outer membrane porins OprD and OpdP are required for the uptake of carbapenems (Tamber and Hancock, 2006). Here, we have verified and shown that Hfq is involved in negative translational riboregulation of oprD by the sRNAs EsrA and Sr0161. Moreover, we have provided evidence for Hfq/Crc-mediated regulation of opdP. Hence, the increased susceptibility of *P. aeruginosa* hfq deletion mutants toward imipenem (Pusic et al., 2018) can be rationalized at the molecular level by these studies. However, we cannot exclude that hitherto unknown Hfq-mediated regulatory circuits additionally impact oprD and opdP regulation.

Zhang et al. (2017) observed no specific amplicons of chimeras formed by Sr0161 and oprD mRNA in a Δhfq strain. In addition, they showed that the oprD mRNA levels are increased in a Δhfq strain. These experiments indicated that Sr0161-dependent negative regulation of oprD is Hfq dependent (Zhang et al., 2017). Using the triple mutant strain PAO1ΔhfqΔesrAΔsr0161(pME6015P_tac_oprD::lacZ), we have shown directly that translational repression of oprD::lacZ upon ectopic expression of either esrA or sr0161 depends on Hfq (Figure 3D). Ectopic expression of Crc in strain PAO1Δcrc(pME6015P_tac_oprD::lacZ) insignificantly increased translation of oprD::lacZ (Figure 4D) rather than decreased it. This observation is consistent with our previous results in that over-expression of crc can interfere with Hfq-mediated riboregulation (Sonleitner et al., 2018), and would argue against negative regulation of oprD translation by a repressive Hfq/Crc complex. In this context, it is also worth noting that the absence of Hfq in strain PAO1ΔhfqΔesrAΔsr0161(pME6015P_tac_oprD::lacZ) (Figure 3C) did not result in increased de-repression of oprD::lacZ translation when compared with strain PAO1ΔesrAΔsr0161(pME6015P_tac_oprD::lacZ) (Figure 3C), again indicating that direct translational repression of oprD by Hfq/Crc does not occur in addition to Hfq/EsrA/Sr0161-mediated riboregulation (Figure 5A). This hypothesis also agrees with other studies (Corona et al., 2018), which indicated that oprD is not post-transcriptionally regulated by Crc.

Zhang et al. (2017) reported that deletion and over-expression of sr0161 resulted in increased and decreased opdP mRNA levels, respectively, which was indicative for Sr0161-dependent regulation of opdP. However, our experiments

**TABLE 1** | Sensitivity toward imipenem during growth on different carbon-sources.

| Antibiotic | Strains | PAO1 Media | PAO1 BSM + 40 mM succinate | PAO1 ΔoprD BSM + 40 mM succinate | PAO1 ΔopdP BSM + 40 mM succinate | PAO1 ΔoprD BSM + 40 mM mannitol | PAO1 ΔopdP BSM + 40 mM mannitol | PAO1 ΔoprD BSM + 40 mM mannitol | PAO1 ΔopdP BSM + 40 mM mannitol |
|------------|---------|------------|----------------------------|-----------------------------------|-----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Imipenem   | 10 µg   | 22.25 ± 1.26 | 25.5 ± 1.29 | 18.5 ± 0.58 | 20.5 ± 0.58 | 27.75 ± 2.06 |

*a Average of two independent experiments ± standard deviation.
FIGURE 5 | Model for distinctive translational regulation of oprD and opdP by Hfq/EsrA/Sr0161 and Hfq/Crc, respectively. (A) Under conditions of envelope stress, low oxygen and during stationary phase, EsrA is synthesized and represses translation of oprD in a Hfq-dependent manner if the levels of the Hfq-titrating CrcZ RNA are low as it is the case during CCR (+CCR) (Sonnleitner et al., 2009). The environmental cues leading to synthesis of Sr0161, the Hfq-dependent second negative regulator of oprD, are unknown. (B) Alleviation of CCR results in increased levels of the RNA CrcZ, which will titrate Hfq and result in increased translation of oprD. (C) When CCR is in place, translation of opdP is reduced by a repressive Hfq/Crc complex. (D) Alleviation of CCR results in CrcZ synthesis and thus in titration of Hfq and/or Hfq/Crc complexes, which in turn will relieve translational repression of oprD. Translational repression of oprD by Hfq/ErsA/Sr0161 and of opdP by Hfq/Crc, respectively, will lead to reduced influx of carbapenems and thus increased resistance toward these antibiotics. In opposite, titration of Hfq and or Hfq/Crc by the RNA CrcZ will lead to increased translation of both, oprD and opdP concomitantly with an increased influx of and susceptibility to carbapenems.

performed with the translational opdP::lacZ reporter gene did not reveal direct evidence for an involvement of the sRNA Sr0161 in opdP translation (Figures 3A,B). Hence, we can only speculate that the observations made by Zhang et al. (2017) result from indirect effects. It is also worth noting that the putative Hfq and Sr0161 binding sites appear to overlap (Figure 2B). Thus, Hfq would be assumed to compete with sRNA binding rather than support the interaction with opdP. Our results are rather consistent with a model wherein a repressive Hfq/Crc complex prevents opdP translation when CCR is in place (Figure 5C). In support, our studies showed that opdP::lacZ translation is de-repressed and repressed in the absence of Crc and upon ectopic expression of crc-flag, respectively.

Hfq is titrated by the regulatory RNA CrcZ, which abrogates its function in Hfq-Crc-mediated translational repression during CCR (Sonnleitner and Bläsi, 2014) as well as in Hfq/sRNA-mediated riboregulation (Sonnleitner and Bläsi, 2014; Sonnleitner et al., 2017). Previous studies have shown that the levels of CrcZ are comparatively low when the cells are cultured in the presence of succinate (CCR) when compared with cells cultured in the presence of mannitol (no CCR) (Sonnleitner et al., 2009; Valentini et al., 2014). The model shown in Figure 5 therefore specifies that in the presence of low levels of CrcZ (succinate; CCR), the translation of both, oprD and opdP, is negatively regulated by Hfq/EsrA/Sr0161-mediated riboregulation (Figure 5A) and by Hfq/Crc repressive complexes (Figure 5C), respectively. In opposite, increasing levels of CrcZ (mannitol; no CCR) are anticipated to result in titration of Hfq, and in translation of oprD and opdP (Figures 5B,D). Hence, the differential imipenem susceptibility (Table 1) during growth on succinate and mannitol can thus be rationalized in light of Hfq-dependent oprD/opdP regulation and of the antagonizing function of the RNA CrcZ on Hfq regulatory complexes.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

UB, ES, and PP conceived and designed the experiments. ES and PP performed the experiments. ES, PP, UB, and MW analyzed the data. ES and UB wrote the manuscript.

FUNDING

The work was supported by the Austrian Science Fund (www.fwf.ac.at/en) through project P28711-B22 (UB and ES) and the Special Research Program RNA-REG F43, subproject AF4311 (UB). PP was supported through the FWF funded doctoral program RNA-Biology W-1207.

ACKNOWLEDGMENTS

We are grateful to Dr. S. Lory and Dr. Y. F. Zhang, Harvard Medical School, and to Dr. A. A. Miller, AstraZeneca Infection Innovative Medicines, United States, for providing plasmids and strains as well as to Beatrice Krennmayr and Dorothea A. Heitzinger for excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01001/full#supplementary-material

REFERENCES

Bassetti, M., Vena, A., Croxatto, A., Righi, E., and Guery, B. (2018). How to manage Pseudomonas aeruginosa infections. Drugs Context 7:212527. doi: 10.7573/dic.212527
Breidenstein, E. B., de la Fuente-Nunez, C., and Hancock, R. E. (2011). Pseudomonas aeruginosa: all roads lead to resistance. Trends Microbiol. 19, 419–426. doi: 10.1016/j.tim.2011.04.005
Buchel, D. J., Shields, R. K., Clarke, L. G., Potoski, B. A., Clancy, C. J., and Nguyen, M. H. (2017). Carbapenem-resistant Pseudomonas aeruginosa bacteremia: risk factors for mortality and microbiologic treatment failure. Antimicrob. Agents Chemother. 61:1216. doi: 10.1128/AAC.01243-1216
Castanheira, M., Deshpande, L. M., Costello, A., Davies, T. A., and Jones, R. N. (2014). Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible Pseudomonas aeruginosa collected during 2009-11 in 14 European and Mediterranean countries. J. Antimicrob. Chemother. 69, 1804–1814. doi: 10.1093/jac/dku048
Chaloub, H., Saenz, Y., Rodriguez-Villalobos, H., Denis, O., Kahl, B. C., Tulkens, P. M., et al. (2016). High-level resistance to meropenem in clinical isolates of Pseudomonas aeruginosa in the absence of carbapenemases: role of active efflux and porin alterations. Int. J. Antimicrob. Agents 48, 740–743. doi: 10.1016/j.ijantimicag.2016.09.012
Chevalier, S., Bouffartigues, E., Bodilis, J., Maillo, O., Lesouhaitier, O., Feuillole, M. G. J., et al. (2017). Structure, function and regulation of Pseudomonas aeruginosa porins. FEMS Microbiol. Rev. 41, 698–722. doi: 10.1093/femsre/fix020
Corona, F., Reales-Calderon, J. A., Gil, C., and Martinez, J. L. (2018). The development of a new parameter for tracking post-transcriptional regulation allows the detailed map of the Pseudomonas aeruginosa Crc regulon. Sci. Rep. 8:16793. doi: 10.1038/s41598-018-34741-34749
Ducret, V., Gonzalez, M. R., Scrignari, T., and Perron, K. (2016). OprD repression upon metal treatment requires the RNA chaperone Hfq in Pseudomonas aeruginosa. Genes 7:82. doi: 10.3390ogenes7080082
Eren, E., Vijayaraghavan, J., Liu, J., Cheneke, B. R., Touw, D. S., Lepore, B. W., et al. (2012). Substrate specificity within a family of outer membrane carbohydrate channels. PLoS Biol. 10:e1001242. doi: 10.1371/journal.pbio.1001242
Ferrara, S., Carfoni, S., Fulco, R., Falcone, M., Macchi, R., and Bertoni, G. (2015). Post-transcriptional regulation of the virulence-associated enzyme AlgC by the signal(22) -dependent small RNA EraS of Pseudomonas aeruginosa. Environ. Microbiol. 17, 199–214. doi: 10.1111/1462-2920.12590
Fritzenwanker, M., Imirzalioglu, C., Herold, S., Wagenlehner, F. M., Zimmer, K. P., and Chakraborty, T. (2018). Treatment options for carbapenem-resistant gram-negative infections. Dtsch. Arztebl. Int. 115, 345–352. doi: 10.3238/arztebl.2018.0345
Hancock, R. E., and Brinkman, F. S. (2002). Function of pseudomonas porins in uptake and efflux. Annu. Rev. Microbiol. 56, 17–38. doi: 10.1146/annurev.micro.56.012302.160310
Hancock, R. E., and Woodruff, W. A. (1988). Roles of porin and beta-lactamase in beta-lactam resistance of Pseudomonas aeruginosa. Rev. Infect. Dis. 10, 770–775. doi: 10.1093/clinids/10.4.770
Hmelo, L. R., Borlee, B. R., Almblad, H., Love, M. E., Randall, T. E., Tseng, B. S., et al. (2015). Precision-engineering the Pseudomonas aeruginosa genome with two-step allelic exchange. Nat. Protoc. 10, 1820–1841. doi: 10.1038/nprot.2015.115
Hoffmann, S., Otto, C., Kurtz, S., Sharma, C. M., Khaitovich, P., Vogel, J., et al. (2009). Fast mapping of short sequences with mismatches, insertions and deletions using index structures. PLoS Comput. Biol. 5:e1000502. doi: 10.1371/journal.pcbi.1000502
Isabella, V. M., Campbell, A. J., Manchester, J., Sylvester, M., Nayar, A. S., Ferguson, K. E., et al. (2015). Toward the rational design of carbapenem uptake in Pseudomonas aeruginosa. Chem. Biol. 22, 535–547. doi: 10.1016/j.chembiol.2015.03.018
Ishikawa, H., Otaka, H., Maki, K., Morita, T., and Aiba, H. (2012). The functional Hfq-binding module of bacterial sRNAs consists of a double or single hairpin preceded by a U-rich sequence and followed by a 3’ poly(U) tail. RNA 18, 1062–1074. doi: 10.1261/rna.031575.111
Kambara, T. K., Ramsey, K. M., and Dove, S. L. (2018). Pervasive targeting of nascent transcripts by Hfq. Cell Rep. 23, 1543–1552. doi: 10.1016/j.celrep.2018.03.134
Kavita, K., de Mets, F., and Gottesman, S. (2018). New aspects of RNA-based regulation by Hfq and its partner sRNAs. Curr. Opin. Microbiol. 42, 53–61. doi: 10.1016/j.mib.2017.10.014
Linares, J. F., Moreno, R., Fajardo, A., Martinez-Solano, L., Escalante, R., Rojo, F., et al. (2010). The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in Pseudomonas aeruginosa. Environ. Microbiol. 12, 3196–3212. doi: 10.1111/j.1462-2920.2010.02292.x
Link, T. M., Valentin-Hansen, P., and Brennan, R. G. (2009). Structure of Escherichia coli Hfq bound to polyriboadenylate RNA. Proc. Natl. Acad. Sci. U.S.A. 106, 19292–19297. doi: 10.1073/pnas.0908744106
Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17:10. doi: 10.14806/ej.17.1.200
McDougall, D. A., Morton, A. P., and Playford, E. G. (2013). Association of ertapenem and antipseudomonal carbapenem usage and carbapenem resistance in Pseudomonas aeruginosa among 12 hospitals in Queensland. Austr. J. Antimicrob. Chemother. 68, 457–460. doi: 10.1093/jac/dks385
Sacha, P., Wieczorek, P., Hauschild, T., Zorawski, M., Olszanska, D., and Schumacher, M. A., Pearson, R. F., Moller, T., Valentin-Hansen, P., and Brennan, D. J., Zhang, A., Gottesman, S., and Storz, G. (2015). Alternative Hfq-
Sauer, E., and Weichenrieder, O. (2011). Structural basis for RNA 3'-end
Sauer, E., Schmidt, S., and Weichenrieder, O. (2012). Small RNA binding to the
Molecular Cloning: A
Robinson, K. E., Orans, J., Kovach, A. R., Link, T. M., and Brennan, R. G.
Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonomo, R. A. (2011).
Ochs, M. M., Lu, C. D., Hancock, R. E., and Abdelal, A. T. (1999). Amino acid-
Murina, V., Lekontseva, N., and Nikulin, A. (2013). Hfq binds ribonucleotides in
Panja, S., Schu, D. J., and Woodson, S. A. (2013). Conserved arginines on the
Pseudomonas aeruginosa
Palmer, K. L., Aye, L. M., and Whiteley, M. (2007). Nutritional cues control
Frontiers in Microbiology | www.frontiersin.org
Rietsch, A., Vallet-Gely, I., Dove, S. L., and Mekalanos, J. J. (2005). ExsE, a secreted
Sonnleitner et al. Carbapenem Susceptibility Regulation by Hfq
10.1093/emboj/cdf322
Copyright © 2020 Sonnleitner, Pusic, Wolflinger and Bläsi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.