Brief Note

Lactonase SsoPox modulates CRISPR-Cas expression in gram-negative proteobacteria using AHL-based quorum sensing systems

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ABSTRACT

Quorum sensing (QS) is a molecular communication system that bacteria use to harmonize the regulation of genes in a cell density-dependent manner. In proteobacteria, QS is involved, among others, in virulence, biofilm formation or CRISPR-Cas gene regulation. Here, we report for the first time the effect of a QQ enzyme to alter the regulation of CRISPR-Cas systems in model and clinical strains of Pseudomonas aeruginosa, as well as in the marine bacterium Chromobacterium violaceum CV12472. The expression of CRISPR-Cas genes decreased in most cases suggesting that enzymatic disruption of QS is promising for modulating phage-bacteria interactions.

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1. Introduction

For billions of years, bacteria have developed sophisticated systems to adapt to their environment and hostile surroundings [1,2]. Among these systems, CRISPR-Cas (clustered regularly interspaced short palindromic repeat and CRISPR associated proteins) [3,4] and quorum sensing (QS) [5] have been selected and are found in many bacteria for cooperating and resisting to predators such as bacteriophages. On the one hand, CRISPR-Cas is an adaptive-immune system used by bacteria to cannibalize exogenous DNA that contributes to resisting invaders including viruses [6]. On the other hand, QS is a communication system based on the secretion and perception of small signal molecules that bacteria use to coordinate the regulation of genes in a cell density-dependent manner [7]. In proteobacteria, QS mainly relies on the secretion and perception of N-acyl homoserine lactones (AHL) and recent studies have pointed out interconnections with CRISPR-Cas systems [8,9]. It has been demonstrated that two proteobacteria with AHL-based QS: Pseudomonas aeruginosa PA14 and Serratia sp. ATCC39006 use QS to activate cas gene expression in order to target exogenous DNA at high cell density and promote CRISPR adaptation. QS deficient mutants of Serratia and P. aeruginosa were further found to be less adaptable to invaders. Both activity and acquisition of resistance were shown to be tunable through the administration of pro- and antiquorum-sensing molecules. Since bacteriophages represent the main predators of bacteria in nature and that phage therapy is constantly gaining interests for medical purposes, disruption of QS, a strategy referred to as quorum quenching (QQ), is of prime interest to simultaneously disorganize bacteria, decrease virulence, reduce biofilm formation and increase susceptibility to bacteriophages [10].

In this report, we investigate for the first time the potential of a QQ enzyme to modulate the regulation of the CRISPR-Cas system. To this end, we used the robust lactonase SsoPox-W263I, an enzyme widely studied for its capacity to degrade QS involved acyl-homoserine lactones (AHL) including the N-(3-Oxododecanoyl)-L-homoserine lactone involved in P. aeruginosa communication [11–13]. This enzyme has already been shown to reduce virulence and biofilm formation in model strains of P. aeruginosa PA01 and PA14, as well as in 51 clinical isolates of diabetic foot ulcers [14,15]. Here, the potential of SsoPox-W263I to deregulate the CRISPR-Cas system in P. aeruginosa was investigated. Strain PA14 was first targeted, as the link between QS and CRISPR-Cas system was previously established [8]. Moreover, PA14 is a model strain that may not be representative of all P. aeruginosa strains, including clinical isolates, and a recent study underlined the strong diversity of CRISPR-
Cas distribution in antibiotics-resistant strains [16]. To address this point, 13 clinical strains of P. aeruginosa isolated from diabetic foot ulcers [14] were targeted to determine whether QQ would impact their CRISPR-Cas system. In complement to P. aeruginosa strains, we also considered bacteria from aquatic environment. The oceans are a huge reservoir of bacteriophages [17] and marine bacteria are promising candidates to decipher the regulation of phage resistance mechanisms. Therefore, we targeted the marine model bacteria Chromobacterium violaceum CV12472 that possesses AHL-based QS and CRISPR-Cas, and the potential interaction between both systems was studied using SsoPox-W263I.

2. Material and methods

2.1. Stains and culture condition

Experiments were performed with C. violaceum ATCC 12472, P. aeruginosa PA14 and P. aeruginosa clinical isolates from samples held by the Department of Microbiology of the Nîmes University Hospital. Clinical strains have been isolated from diabetic patients with a new suspected episode of diabetic foot infection for a period of one year (2014). This study was approved by the local ethics committee (South Mediterranean III) and was carried out in accordance with the Declaration of Helsinki as revised in 2008. After a preculture of 6 h in LB, the cultures were diluted by a factor 1000 and P. aeruginosa strains and C. violaceum CV12472 were grown in MOPS glutamate medium [18] and LB medium respectively for 24 h at 37 °C with shaking at 650 rpm. The enzyme SsoPox was added at 0.5 mg ml⁻¹ in the culture only.

2.2. Enzyme production

SsoPox variant W263I was produced in the E. coli BL21(DE3)-pGro7/GroEL strain (TakRa) in ZYP medium, as previously described [14]. The purification was performed on a size-exclusion chromatography column (HiLoad 16/600 Superdex™ 75 pg, GE Healthcare; AKTA Avant). The purity of the protein was checked by 10% SDS-PAGE separation and protein concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.3. QS-regulated factors measurements in P. aeruginosa

Protease activity in cell-free culture supernatants was measured using azocasein (30 mg ml⁻¹) as substrate in a Phosphate-buffered saline (PBS) solution at pH 7.0 [19]. Reaction was performed for 1 h at 37 °C and stopped with 20% (w/v) trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at OD690 nm using a microplate reader (Synergy HT, BioTek).

Crystal violet was further resolubilized with ethanol 96%. The so-lution was transferred to a 96 well plate. The crystal violet was measured at OD595 nm using a plate reader (Synergy HT, BioTek).

2.4. Violacein measurement in C. violaceum

The amount of violacein was estimated by measuring the absorbance of cell-free supernatants at OD575 nm using a plate reader (Synergy HT, BioTek).

2.5. Biofilm quantification

Biofilm was quantified using crystal violet (Sigma) staining as previously described [22]. Briefly, planktonic cells (non-attached) were first removed by washing the wells with PBS. Plates were dried at 37 °C and the biofilm was colored with crystal violet 0.5%. Crystal violet was removed and each well was rinsed with PBS. Crystal violet was further resolubilized with ethanol 96%. The solution was transferred to a 96 well plate. The final concentration of crystal violet was measured at OD595 nm using a plate reader (Synergy HT, BioTek).

2.6. Identification of CRISPR-Cas genes in clinical isolates of P. aeruginosa

Genomic DNA was extracted with QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer’s instructions. cas1, csy3, csy1, csy2, csy3, csy4 genes were amplified by PCR using DNA, Taq polymerase, (RED Taq® ReadyMix™ PCR Reaction Mix, Sigma–Aldrich) and 10 μM of forward and reverse primers specifically conceived for each gene (Supplementary Table 1). The PCR method consists of a first denaturation step of 5 min at 94 °C, followed by 29 cycles of [1 min at 94 °C, 1 min at 55 °C, 30 s at 72 °C] for amplification, followed by a final elongation step for 7 min at 72 °C. Amplification products were visualized on agarose gel (1.5%).

2.7. Expression of CRISPR-Cas genes

RNA was extracted and purified using mini kit RNA PureLink® (ThermoFisher) according to the manufacturer’s recommendations and treated with TURBO DNA-free™ kit (ThermoFisher) to remove genomic DNA. Process quality was checked by migration on agarose gel 1.5%, and nucleic acid quantity was measured by spectrophotometer NanoDrop 2000 (Thermo Scientific) at OD260nm. CDNA was synthetized using Reverse transcription reagents TaqMan® kit (ThermoFisher) according to manufacturer’s recommendations. Real time quantitative PCR was realized using LuminoCt® SYBR® Green qPCR ReadyMix™, CFX thermocycler (Bio–Rad) and a gene specific pair of primers. Amplification steps are the same as for PCR. Fluorescence was measured at the end of each cycle and melting curves were analyzed using the CFX Manager™ software (Bio–Rad). As for gene expression studies, each gene was normalized according to the expression of housekeeping gene 55 RNA.

2.8. Statistical analysis

For virulence factors and biofilm, statistical analyses were performed on raw optical density data of 3 biological replicates. A Student’s t test was performed to determine the p-values between control and enzymatic treatment. For the CRISPR-Cas gene expression, statistical analyses were performed on 2 ΔΔCt values. Standard deviations were determined using 2 biological and 2 technical replicate experiments. The p-values were calculated according to Student’s t-test.

3. Results

The most common intact CRISPR-Cas system present in P. aeruginosa strains, including PA14, is type I–F [16]. In order to identify the presence of this CRISPR-Cas system in clinical strains, primers targeting genes involved in adaptive exogenous DNA integration (cas1) or genes involved in bacterial response to previously encountered invaders (csy3, csy1, csy2, csy3 and csy4) were designed using PA14 genome (Supplementary Table 1). Interestingly CRISPR-Cas genes were effectively detected in only 6 out of
the 13 strains that were assayed, suggesting that strong genetic variations may be found in clinical environments. These 6 strains (A11, B10, C5, C11, D10 and F3) along with PA14 were further used for this study. C. violaceum CV12472 also possesses a type I–F CRISPR-Cas system and specific primers were designed according to the deposited genome (Supplementary Table 1). To evaluate the potential of SsoPox-W263I as QQ enzyme in these strains several QS-associated factors were measured with and without enzymatic treatment. Pyocyanin, proteases, elastases and biofilm formation were studied in P. aeruginosa strains together with violacein and biofilm formation in C. violaceum. Each factor was found to be strongly affected by SsoPox-W263I, confirming efficient QQ effect in all strains (Fig. 1A). SsoPox-W263I drastically decreased the level of secreted factors (pyocyanin, protease and elastase) in all Pseudomonas strains, while the biofilm was only reduced for PA14 and C5. No change in biofilm was observed for F3 conversely to A11, B10, C11 and D10, for which a significant increase was measured. We also demonstrated that QQ by SsoPox-W263I effectively reduced the production of violacein and biofilm, by 50% in C. violaceum 12472.

To determine whether QQ may impact the regulation of CRISPR-Cas genes, the expression level of cas1, cas3 and csy1-4 were measured with and without enzymatic treatment (Fig. 1B, Supplementary Table 2). Of note, csy1 expression was not detected for A11, B10 and C11 in these conditions. As expected, the expression of cas and csy genes was totally abolished in PA14 [8]. Although SsoPox-W263I triggered all-or-nothing shift in PA14, the effects in clinical isolates were more variable and underlined the influence of the genetic context on the regulation of CRISPR-Cas by QS. The expression of each detected gene was significantly reduced in B10 and C11 with an average decrease of 5.9 and 9 times respectively. A significant reduction was also observed in C5 albeit with a lower range. No variation in cas1 and cas3 expression levels in the presence of enzyme was noticed for A11 nor D10 but csy1–4 expression levels were significantly reduced. Conversely, both cas and csy genes were increased by a 1.7-fold on average in F3. For C. violaceum CV12472 significant reduction in cas3 and csy2–4 were measured in presence of SsoPox-W263I, confirming that SsoPox-W263I effect on CRISPR-Cas genes is not limited to P. aeruginosa.

4. Discussion

In this report we investigated the impact of a QQ enzyme on CRISPR-Cas system expression in P. aeruginosa clinical isolates as well as in C. violaceum, a bacterium which inhabits completely different environmental niches than P. aeruginosa. To this end we used the lactonase SsoPox-W263I to degrade QS signaling molecules. The disruption of QS was checked by measuring several QS-regulated factors prior to CRISPR-Cas expression studies. Although cas and csy genes expression levels were completely abolished in the model strain PA14 with SsoPox-W263I, no comparable effect was achieved with the clinical isolates. Albeit the expression of cas and csy genes were significantly decreased in most clinical isolates, one strain out of seven (F3) showed an inverse effect with a significant increase in the expression of the CRISPR-Cas system. The differences between clinical isolates might be explained by different genomic organizations of the CRISPR-Cas genes in these strains as compared to P. aeruginosa PA14. Nevertheless, although the range of expression modulation was variable between strains, all were affected in the regulation of CRISPR-Cas genes with stronger or weaker effects upon addition of the QQ lactonase indicating that independently from the strain QS contributes to the regulation of CRISPR-Cas expression. Although the combination of QQ and bacteriophages would be a promising therapeutic perspective, further investigations are required to understand how bacterial diversity may affect the efficacy of combined treatment on bacterial virulence and their ability to modulate CRISPR-Cas mediated phage resistance.

Conflict of interest statement

EC has a patent WO2014167140 A1 licensed to Gene&GreenTK. LP, DD, BR, and EC report personal fees from Gene&GreenTK during the conduct of the study. All the other 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2019.06.004.

References


