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Original Article

CarO promotes adhesion and colonization of acinetobacter baumannii through inhibiting NF-κB pathways

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Abstract: Acinetobacter baumannii has emerged as a major nosocomial pathogen. Cell adhesion and nasal colonization are the main forms of acinetobacter baumannii pathogenicity. However, mechanisms underlying acinetobacter baumannii adhesion and colonization remain poorly understood. The present study found a crucial role of carbapenem-associated outer membrane protein (CarO) in promoting cell adhesion of acinetobacter baumannii ATCC19606 *in vitro* and nasal colonization in mice. Furthermore, the present study showed that CarO could inhibit the activation of NF-κB signaling pathways and reduce expression of NF-κB target genes *TNF-α*, *IL-6,* and *IL-8* in WI38 and HNEpC cells. Consequently, CarO may enhance acinetobacter baumannii cell adhesion and nasal colonization ability mainly through inhibiting host cell inflammatory immunity response. In conclusion, CarO is a crucial promoter of acinetobacter baumannii cell adhesion and nasal colonization. Therefore, the present study provides novel insight into CarO's function in acinetobacter baumannii, suggesting that anti-CarO antibodies may provide novel insight into the development of novel treatments against acinetobacter baumannii.

Keywords: Acinetobacter baumannii, adhesion, colonization, CarO, NF-κB

Introduction

Acinetobacter baumannii is an important opportunistic pathogen associated with nosocomial infections, such as bacteremia, pneumonia, meningitis, urinary tract infections, and wound infections [1, 2]. Although acinetobacter baumannii has been regarded as a low-virulence pathogen, recent studies have demonstrated that this pathogen is more virulent than expected [3]. Acinetobacter baumannii can induce host cell death via many forms of pathogenicity, such as biofilm formation, adherence, and invasion of host cells [4-8]. Therefore, acinetobacter baumannii has gradually gained importance as a human pathogen in the hospital environment [9-11]. However, mechanisms underlying acinetobacter baumannii adhesion and colonization remain obscure. Discovery of these mechanisms may advance the development of more efficacious treatment.

Multidrug-resistant (MDR) strains have been increasing, posing a serious threat to human health. MDR strains are resistant to imipenem but sensitive to carbapenems [12]. Carbapenem-associated outer membrane protein (CarO) is the most characterized porin in acinetobacter baumannii. MDR strains could disrupt the CarO gene by various insertion elements [13, 14]. CarO plays an important role in carbapenem-resistant acinetobacter baumannii strains, mainly through participating in carbapenem influx [15-17]. However, the physiological functions of CarO remain poorly understood, especially its roles in acinetobacter baumannii adhesion and colonization.

The present study investigated the roles of CarO in the adhension and colonization of acinetobacter baumannii. This study reports a crucial role of CarO in promoting cell adhesion of acinetobacter baumannii ATCC19606 *in vitro*

and nasal colonization *in vivo*. Furthermore, the present study shows that CarO could inhibit the activation of NF-κB signaling pathways and reduce expression of NF-κB target genes TNF-α, IL-6, and IL-8 in WI38 and HNEpC cells. Results suggest that antibodies against CarO may provide insight into the development of novel treatments against acinetobacter baumannii.

Materials and methods

CarO genetic modification model preparation

CarO genomic deleted acinetobacter baumannii ATCC19606 strain was generated by Crispr/Cas9 recombination system. Plasmids used in this study (pCas9, pKD46, and pBAD) were purchased from YRgene (China). The sgRNA1-3 were designed in www.crispr.mit.edu to induce CarO dysfunction. Primers were as follows: sgRNA1-F: ATAAGCATGAGCACCGGTCATGG, sgRNA1-R: CCATGACCGGTGCTCATGCTTAT; sgRNA2-F: ATAAGCATGAGCACCGGTCATGG, sgRNA2-R: CCATGACCGGTGCTCATGCTTAT; sgRNA3-F: GCTACTTTCGTTGGTAACGATGG, sgRNA3-R: CCATCGTTACCAACGAAAGTAGC. Briefly, gRNAs were cloned onto pKD46 and co-electroporated with pCas9 into ATCC19606, respectively. Clones having the feature of CarO knockout (hereafter termed ATCC19606-ΔCarO) were selected on agar plates containing chloramphenicol (34 μg/mL) and ampicillin (100 μg/mL), identified by sequencing. Rescued CarO acinetobacter baumannii were established based on the CarO knockout strain via transfection with pBAD-CarO (ATCC19606-ΔCarO-CarO) and screening by kanamycin (100 μg/mL).

Cell adhesion assay

Human lung embryonic lung fibroblasts WI38 and nasal epithelial cells HNEpC (Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences) were cultured in DMEM/F12 containing 20% (v/v) fetal bovine serum and 100 U/mL penicillin G/streptomycin sulfate at 37°C in a 5% CO_2 incubator. WI38 and HNEpC cells were seeded at a concentration of 5×10^5 cells/well in 12-well plates. Prior to co-culturing with acinetobacter baumannii strains, the cells were washed twice with PBS. The cells were then co-cultured with ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO strains with a 1:10 ratio at 37°C for 2 hours.

To analyze the number of adherent bacteria, WI38 and HNEpC cells were washed three times with PBS to remove dissociative bacteria thoroughly. They were harvested after lysing by adding 1 mL RIPA buffer. Bacterial counts were confirmed by the growth of serial dilutions of the bacterial suspension on LB agar in terms of CFU after 24 hours of incubation at 37°C.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

After cells were co-cultured with different acinetobacter baumannii strains for 6 hours, followed by sterile PBS washing three times, total RNA was extracted by TRIzol Reagent (Invitrogen), according to manufacturer instructions. Before performing reverse transcription, RNA was treated with 5U DNase I (Beyotime) on ice for 10 minutes to remove the bacteria genomic DNA and purified by isopropanol and 3 M sodium acetate. It was then washed by 75% ice ethanol. Reverse transcription was then performed using the QuantiTect Reverse Transcription Kit (Qiagen). RT-PCR reactions were done using the Taq PCR MasterMix (Tiangen), in triplicate with the following conditions: 95°C/2 minutes, 30 cycles of 95°C/15 seconds, 55°C/15 seconds, 72°C/1 minute, and 72°C/10 minutes. Next, 5 μl of each PCR product was run with 1% agarose gel and imaged and semi-quantitative analyzed by Tanon 2500 imaging system. Sequences of primer pairs used were: CarO-F: AATCTGAATATGTTGACACAACTGCA, CarO-R: AAGCGAATTGGTTAGCAACACTTG; 16S ribosomal RNA (IX87_RS17290)-F:, 16S ribosomal RNA (IX87_RS17290)-R:; TNF-α-F: AGACCAAGGTCAACCTCCT, TNF-α-R: AAAGTAGACCTGCCCAGAC; IL-6-F: GAAAGCAGCAAAGAGGCACTG, IL-6-R: GCAAGTCTCCTCATTGAATC; IL-8-F: CATACTCCAAACCTTTCCACC, IL-8-R: CAACCCTCTGCACCCAGTTTTC; β-actin-F: TCCCTGGAGAAGAGCTACG, β-actin-R: GTAGTTTCGTGGATGCCACA. Relative mRNA levels were normalized by β-actin.

Western blot analysis

After adding RIPA buffer to harvest cells, total protein concentrations of the cells transfected with different acinetobacter baumannii strains were determined using the bicinchoninic acid (BCA) assay. Aliquots of protein resolved by SDS-PAGE were immunoblotted with NF-κB pathway (phosphorylation of IκB, p65) associated

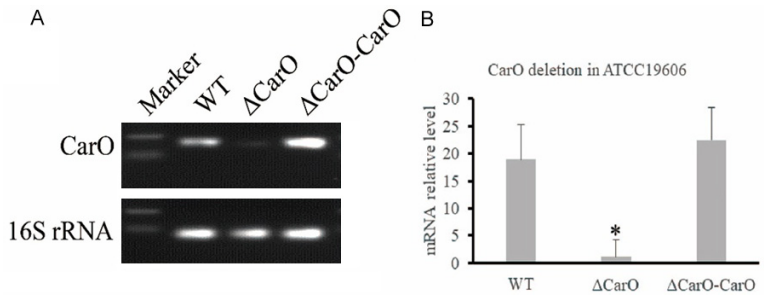

Figure 1. Identification of CarO genetic editing in ATCC19606. The transcriptional level of CarO in ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO strains using RT-PCR (A) and semi-quantitative analysis normalized by β-actin (B). "*" represents *p*-values less than 0.05.

antibodies at 4°C overnight. They were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. Images of Western blotting were acquired using Image Lab (Bio-Rad).

Immunofluorescence assay

WI38 and HNEpC cells (1×10^3) were passaged and grown in 6-well dishes containing sterilized cover glass for 24 hours. After co-culturing with acinetobacter baumannii, cells were washed by PBS gently and fixed with 1% paraformaldehyde at room temperature (RT) for 10 minutes, then permeabilized with 0.1% Triton X-100. They were then blocked with 2% horse serum/PBS for 30 minutes at RT and subsequently incubated with phosphorylated p65 antibody at 4°C overnight. After washing, cells were further incubated with appropriate Alexa Fluor secondary antibody at 1:10000 dilutions for 30 minutes at RT. Cells were washed and mounted in mounting media with DAPI (Vector Laboratories, Burlingame, CA). Cells were observed on an Olympus BX-51 microscope. Images were acquired and analyzed using Image J software.

Nasal colonization model

Male BALB/c mice were used for the nasal colonization model. They were raised in specific pathogen-free conditions (6-8 weeks old, Shanghai Institute of Materia Medical, Chinese Academy of Sciences). Mice were housed at six per cage with a 12-hour light/dark schedule at 25 ± 1°C. They were fed an autoclaved chow diet and water. Animal care and experimental protocols were approved by the Animal Ethics Committee of Xiamen University. Mice were randomly divided into indicated groups before the injection and double-blinded evaluations were performed. Acinetobacter baumannii strains (ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO) were grown to the mid-exponential growth phase, washed, and resuspended in sterile PBS at $1 * 10^{10}$ CFUs per mL. Mice were anesthetized with isoflurane and pipetted 10 μl inoculum containing $1 * 10^8$ CFUs slowly into the nares without touching the nose (six mice per group). Three days after inoculation, the mice were sacrificed. Levels of acinetobacter baumannii in the nasal carriage were evaluated. The nasal region was wiped externally with 70% ethanol and nasal tissue was homogenized in 0.5 mL TSB. The total number of acinetobacter baumannii CFUs per nose was assessed by plating 100 μl diluted nasal suspensions on TSB agar containing ampicillin.

Statistical analysis

Statistical analysis of the data was performed using Prism 7 software (GraphPad Software). Comparisons between groups were carried out by two-tailed Student's *t*-tests. Data are presented as mean ± SD, unless otherwise stated. *P*-values less than 0.05 are considered statistically significant.

Results

CarO enhances the cell adhesion ability of acinetobacter baumannii

To assess the functional involvement of CarO in ATCC19606 cell adhesion *in vitro*, this study constructed an isogenic gene deletion mutant in CarO in ATCC19606, verifying the most effective CarO silencing by gRNA2 (Supplementary Figure 1) and rescue of CarO expression in the CarO-gRNA2 strain. This study then validated CarO expression by RT-PCR (**Figure 1A**, **1B**). The above strains were co-cultured with WI38 and HNEpC cells. The attachment of Acinetobacter baumannii was then measured. The number of attached and internalized CarO mutant strain (ATCC19606-ΔCarO) was markedly

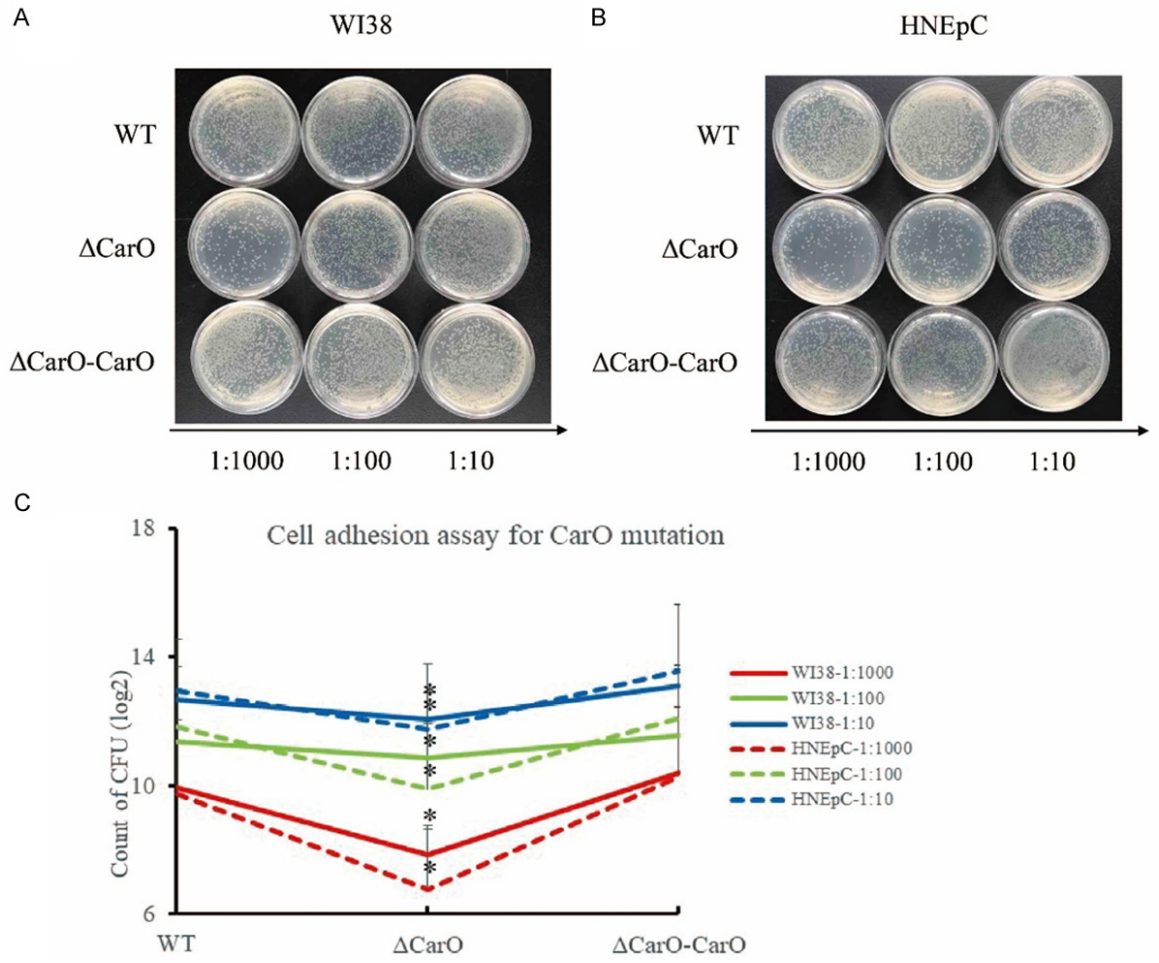

Figure 2. Cell adhesion assay for CarO knockout or compensation strains *in vitro*. The CFU of the adhesive or internal acinetobacter baumannii strains (ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO) from the WI38 (A) and HNEpC (B) cells diluted as 1:1000, 1:100, and 1:10, respectively, for three duplications and statistical analysis (C). "*" represents *p*-values less than 0.05.

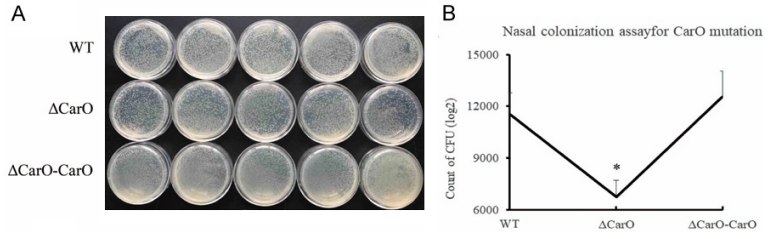

Figure 3. Nasal colonization assay for CarO knockout or compensation strains *in vivo*. The CFU of the nasal colonization of acinetobacter baumannii strains (ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO) from six repeated mouse nasal region diluted as 1:1000 (A) and statistical analysis (B). "*" represents *p*-values less than 0.05.

decreased, compared with the wild type ATCC19606 strains (ATCC19606-WT) and complement strains (ATCC19606-ΔCarO-CarO) (**Figure 2A-C**). Results indicate that CarO promotes the cell adhesion ability of acinetobacter baumannii *in vitro*.

CarO promotes the nasal colonization ability of acinetobacter baumannii ATCC19606

The human nose is the primary reservoir of acinetobacter baumannii [18]. Thus, the effects of CarO on the nasal colonization ability of acinetobacter baumannii *in vivo* were further assessed. ATCC19606-WT, ATCC19606-ΔCarO, and ATCC19606-ΔCarO-CarO strains were intranasally inoculated into mice, respectively (1 × 10^8 CFU/mouse, n = 6 mice/

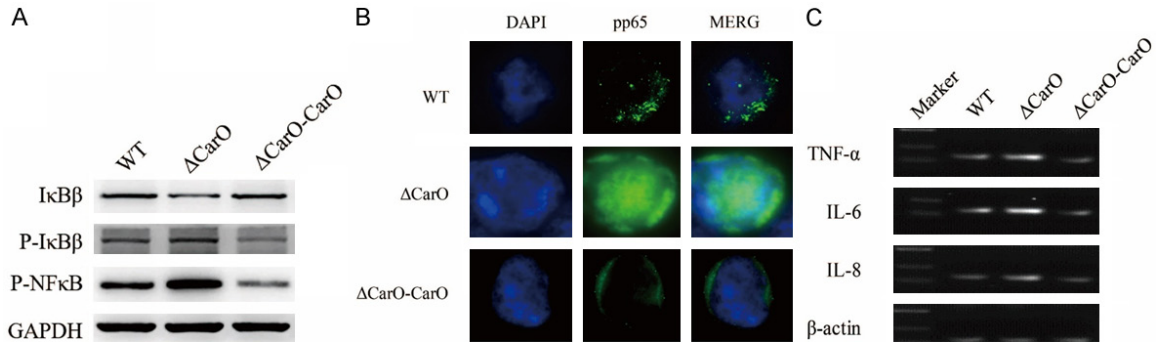

Figure 4. Relationship between CarO and NF-κB signaling pathways. The phosphorylation and expression levels of IκB and p65 in the HNEpC cells with CarO knockout or compensation acinetobacter baumannii strains using Western blot (A). The localization of phosphorylated p65 in HNEpC cells with CarO knockout or compensation acinetobacter baumannii strains using IF assay (B). The transcriptional level of TNF-α, IL-6, and IL-8 measured RT-PCR in HNEpC cells with CarO knockout or compensation acinetobacter baumannii strains (C).

group). After three days, mice were euthanized and the nasal bacterial burden was determined. Consistent with results of cell experiments, colonization of the nasal cavity in CarO deletion mutant strain was obviously less than the wild type and CarO-rescued strains (**Figure 3A**, **3B**). Taken together, results indicate that CarO promotes the nasal colonization ability of acinetobacter baumannii in a mice model.

CarO reduces activation of NF-κB signaling pathways in HNEpC cells

It has been established that LPS or other antigens from bacteria can induce the activation of nuclear factor κB (NF-κB) pathways for immune response in host cells [19, 20]. To explore the underlying pathological mechanisms of CarO's promotion of acinetobacter baumannii cell adhesion and nasal colonization ability, this study investigated the relationship between CarO and NF-κB pathways in HNEpC cells. It was observed that rescued CarO could effectively suppress the phosphorylation of IκBβ and maintain higher levels of IκBβ than ATCC19606-WT and ΔCarO (**Figure 4A**). This study further observed remarkable phosphorylation and nuclear localization of p65 in HNEpC cells treated with CarO deletion (**Figure 4B**), both of which are commonly considered as indicators of NF-κB signaling activation. Furthermore, the transcriptional activity of TNF-α, IL-6, and IL-8 was upregulated in HNEpC cells with ATCC19606-ΔCarO, compared to the wild type and complement CarO strains (**Figure 4C**). Taken together, results indicate that CarO could restrain the activation of NF-κB signaling pathways in human cells.

Discussion

Acinetobacter baumannii has emerged as a major nosocomial pathogen [9, 21]. Although several genes associated with acinetobacter baumannii adhesion and colonization have been identified recently [6], mechanisms governing the pathogenicity and adhesion and colonization of acinetobacter baumannii still remain elusive. Therefore, the present study focused on the roles of CarO in acinetobacter baumannii adhesion and colonization ability. This study demonstrated that loss of CarO may suppress the cell adhesion ability of acinetobacter baumannii ATCC19606 *in vitro* and nasal colonization *in vivo*, while compensation of CarO compromised these effects. This study verified CarO as one of the driving factors for acinetobacter baumannii invasion towards the eukaryocyte. Results suggest that small molecular medicine or antibodies developed against CarO may provide novel insight into acinetobacter baumannii infection prevention.

Acinetobacter baumannii infection in human lung epithelial cells can trigger the upregulation of proinflammatory cytokines [22], which play a vital role in bacterial clearance [23]. NF-κB is an important and well-studied transcription factor for regulating the immune response to viral or bacterial infections [24-26], mostly presenting the induction of proinflammatory genes such as TNF-α, IL-6, and IL-8 [27, 28]. Present observations bridged the regulatory network between CarO and NF-κB signaling pathways in HNEpC cells, indicating that CarO silencing can enhance activation of NF-κB signaling pathways. Moreover, the presence of upregulated

transcriptional levels of TNF-α, IL-6, and IL-8 implies the consequences of activated NF-κB.

In conclusion, present results determined that CarO protein of acinetobacter baumannii can enhance cell adhesion and nasal colonization ability mainly through inhibiting NF-κB signaling pathways for host cell inflammatory immunity response. The precise mechanisms underlying CarO inhibition of NF-κB signaling pathways remain an interesting issue for future research. The present study suggests that anti-CarO medicine or antibodies may provide novel insight into clinical therapy for bacterial infection prevention.

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Disclosure of conflict of interest

None.

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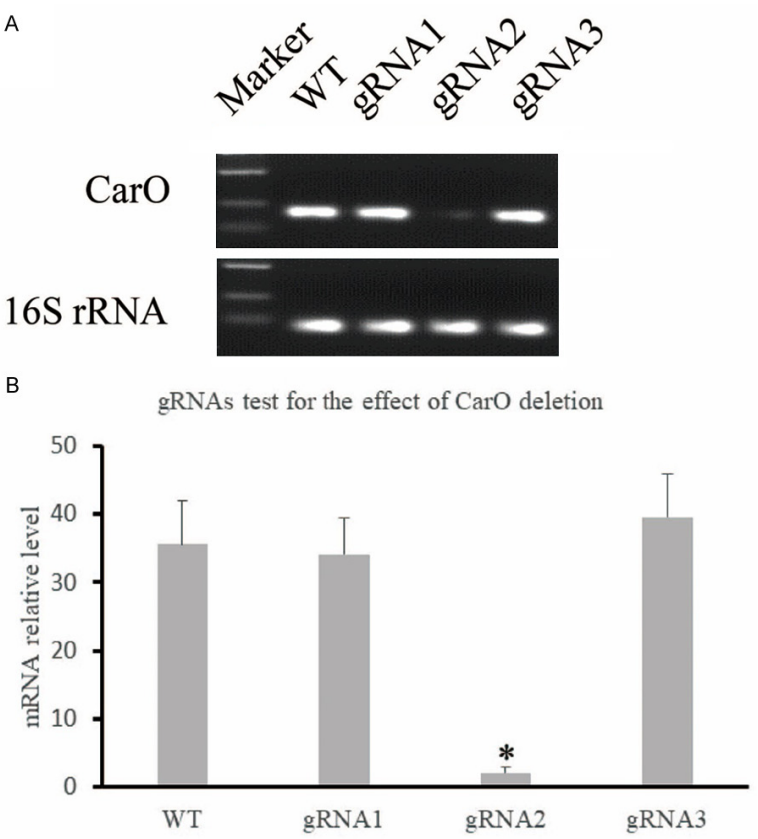

Supplementary Figure 1. gRNAs test for the effects of CarO deletion. The transcriptional level of CarO in ATCC19606 treated with different CarO gRNAs using RT-PCR (A) and semi-quantitative analysis normalized by β-actin (B). ATCC19606 gRNA2 termed as ATCC19606-ΔCarO is used for the next experiments in this study. "*" represents *p*-values less than 0.05.