INTRODUCTION

Bacterial pathogens live in the infection sites and use host as a growth medium. The nutritional environment of different infection sites remains largely unknown and might vary during the course of infection. Bacteria have evolved a set of regulatory pathways to adapt to the dynamic nutritional environment in nature (Nudler & Mironov, 2004; Wang & Levin, 2009). Thus it is no surprise that nutritional cues have a huge impact on bacterial physiology, including virulence (Palmer et al., 2005), motility (Klausen et al., 2003), quorum sensing (Shrout et al., 2006) and biofilm formation (Banin et al., 2005).

Pseudomonas aeruginosa is a ubiquitous environmental bacterium with a large genome (6.3 Mb) and incredible nutritional versatility (Stover et al., 2000). P. aeruginosa causes a wide range of opportunistic infections and secretes a series of virulence factors such as elastase, rhamnolipids and pyocyanin (Lyczak et al., 2000). P. aeruginosa is a model organism to study quorum sensing (QS) and encodes three sets of QS systems, las, rhl and pqs (Pearson et al., 1997; Pesci et al., 1999). QS systems employ small diffusible signal molecules to coordinate gene expression and group behaviours such as swarming and biofilm formation (Shrout et al., 2006; Yang et al., 2009).

Recent studies showed that nutritional cues, especially carbon sources, affect P. aeruginosa QS signalling (Palmer et al., 2007; Wagner et al., 2003). However, the underlying mechanisms of the regulatory effects by nutritional cues are unclear. The catabolite repression control protein (Crc) was previously shown to affect pyocyanin production (O’Toole et al., 2000), which is normally regulated by the Pseudomonas quinolone signal (pqS) QS system (Gallagher et al., 2002). Here, we investigated how Crc affects the P. aeruginosa pqs QS system in growth medium supplied with different carbon sources.

METHODS

Bacterial strains and growth conditions. P. aeruginosa PA14 was used as the wild-type strain in this study. The Crc-defective PA14Δcrc mutant (O’Toole et al., 2000) and its complementary PA14Δcdpcrc+ mutant (O’Toole et al., 2000) were kindly provided by Dr G. A. O’Toole (Dartmouth Medical School, Hanover, USA). The Escherichia coli DH5a strain was used for standard DNA manipulations. Luria–Bertani (LB) medium was used to cultivate E. coli strains. Batch cultivation of P. aeruginosa was carried out at 37 °C in AB minimal medium (Yang et al., 2007) supplemented with 5 g glucose l−1 (ABTG) or 5 g Casamino acids l−1 (ABTC). For plasmid maintenance in E. coli the medium was supplemented with 100 μg ampicillin ml−1, 15 μg gentamicin ml−1, 50 μg streptomycin ml−1, or 8 μg chloramphenicol ml−1. For marker selection in P. aeruginosa, 60 μg gentamycin ml−1 and 100 μg streptomycin ml−1 were used, as appropriate.

Pyocyanin assay. Pyocyanin quantification was carried out as previously described (Essar et al., 1990). Briefly, P. aeruginosa strains were cultivated at 37 °C in LB medium with shaking. After overnight
cultivation, 1 ml supernatant of each culture was collected after centrifugation and mixed with 0.6 ml chloroform. The chloroform phase of the mixture was then transferred into a new Eppendorf tube containing 200 μl 0.2 M HCl and mixed. The pyocyanin was extracted to the top HCl phase and quantified by measuring absorbance at 520 nm (OD_{520} nm).

**Quantification of eDNA from planktonic cultures.** Extracellular DNA (eDNA) from planktonic cultures was quantified as previously described (Das et al., 2013). Briefly, *P. aeruginosa* strains were cultivated at 37 °C in LB medium with shaking. After overnight cultivation, supernatant of each culture was collected after centrifugation and filtered using 0.22 μm Millipore filter units (Millipore). The concentration of eDNA in the supernatants was quantified by the fluorescent dye assay (dsDNA BR) from Qubit, using a Qubit 2.0 Fluorometer (Invitrogen).

**2-Alkyl-4-quinolone production assay.** 2-Alkyl-4-quinolone quantification was performed using a biosensor assay as previously described, with modification (Fletcher et al., 2007). A PAO1 ΔpqsA mutant (Fletcher et al., 2007) containing the pqs signalling reporter vector pAC37 (Yang et al., 2007) was used as a biosensor for quantification of 2-alkyl-4-quinolones produced by various *P. aeruginosa* strains. The biosensor strain PAO1ΔpqsA/pAC37 was first grown in LB medium overnight, diluted 50-fold in fresh LB medium, and 100 μl of the diluted biosensor culture was added into each well of a 96-well microplate. The *P. aeruginosa* PA14, PA14Δcrc mutant and PA14Δcrc/pqr+ mutant were cultivated at 37 °C in LB, ABTG and ABTC medium, respectively, and 100 μl filtered culture supernatant of *P. aeruginosa* PA14, PA14Δcrc mutant and PA14Δcrc/pqr+ mutant were then added to microplate wells with the biosensor strain. As a positive control, 100 μl fresh LB medium containing PQS standard (Sigma-Aldrich) was added to each microplate well with biosensor strain. The biosensor cultures were then incubated at 37 °C. Relative fluorescence units (RFU) were monitored every 15 min after inoculation by using a Tecan Infinite 200 PRO microplate reader (Tecan Group). The maximum levels of RFU were recorded and compared for different cultures as an indication of 2-alkyl-4-quinolone synthesis.

**Autolysis assay.** *P. aeruginosa* strains were plated on LB agar plates and incubated at 37 °C for 5 days. Colony images were taken with a Zeiss Axiovert 200 light microscope (Carl Zeiss).

**Biofilm assay.** Green fluorescent protein (GFP)-tagged, cyan fluorescent protein (CFP)-tagged and yellow fluorescent protein (YFP)-tagged *P. aeruginosa* strains were constructed by insertion of mini-Tn7-cGFP-Strep, mini-Tn7-cCFP-Strep and mini-Tn7-cYFP-Strep, respectively, as previously described (Klausen et al., 2003). *P. aeruginosa* biofilms were cultivated at 37 °C in flow-chambers irrigated with ABTG or ABTC medium as previously reported (Sternberg & Tolker-Nielsen, 2006). Biofilm images were taken with a Zeiss LSM510 Confocal Laser Scanning Microscope, CLSM (Carl Zeiss). Vertical cross section images were generated using the IMARIS software package (Bitplane).

**Animal infection model.** Thirty 10-week-old healthy female BALB/cj mice were challenged by 10^9 c.f.u. ml⁻¹ of the *P. aeruginosa* PA14, PA14Δcrc mutant and PA14Δcrc/pqr+ mutant as described previously (Song et al., 2003). The concentrations of inocula were optimized in pilot studies. The anaesthetized mice were tracheostomized, and a 0.04 ml inoculum of bacteria was instilled in the lower left lung using a curved beaded-tipped needle. The incision was sutured. The bacterial burden at the beginning of infection was 4 × 10^6 c.f.u. lung⁻¹. The mice were maintained on standard mouse chow and water ad libitum for 1 week before challenge. All animal experiments were performed under authorization from the University Animal Experimentation Ethics Committees. All animals were sacrificed at 48 h after bacterial challenge, and lung samples were homogenized for bacteriology (n=10). The results were statistically assessed by Student’s t-test.

**RESULTS**

**Crc negatively regulates pqs signalling in *P. aeruginosa***

Previous studies showed that the catabolite repression control protein Crc regulates pyocyanin biosynthesis (Linares et al., 2010). The *P. aeruginosa* Crc defective PA14Δcrc mutant produced more pyocyanin compared to the wild-type PA14 and PA14Δcrc/pqr+ mutant (Fig. 1a). As pyocyanin biosynthesis is directly regulated by the pqs quorum-sensing system (Diggle et al., 2006), it is highly possible that Crc affects the pqs signalling. Biosensor assay indicated that PA14Δcrc mutant produces more 2-alkyl-4-quinolone signal molecules than the wild-type PA14 and PA14Δcrc/pqr+ mutant (Fig. 1b). Pqs signalling plays an important role for regulating release of extracellular DNA via autolysis (D’Argenio et al., 2002; Yang et al., 2007).

![Fig. 1. Pyocyanin production (a), 2-alkyl-4-quinolone synthesis (b) and eDNA release (c) of *P. aeruginosa* PA14 wild-type, PA14Δcrc and PA14Δcrc/pqr+ mutants in LB cultures. Means ± SD are from triplicate experiments. *P*<0.01.](image-url)
Previous study showed that the PQS overproducing mutant displayed autolysis phenotype on agar plates during long-term incubation (D’Argenio et al., 2002). We incubated the wild-type PA14, PA14Δcrc mutant and PA14Δcrc/pcrc+ mutant on LB agar plates at 37 °C for 5 days. Autolysis was detected in the colonies of PA14Δcrc mutant while not the wild-type PA14 and PA14Δcrc/pcrc+ mutant (data not shown). The high level of autolysis of the PA14Δcrc mutant might lead to release of a large amount of extracellular DNA (eDNA) than the cultures of wild-type PA14 and PA14Δcrc/pcrc+ mutant (Fig. 1c).

Regulation of Crc to pqs signalling in P. aeruginosa is nutrient dependent

Crc was suggested to repress carbohydrate metabolism in the presence of TCA intermediates (Wolff et al., 1991). Thus, we hypothesized that the regulation of Crc on pqs signalling in P. aeruginosa is nutrient dependent. To test this hypothesis, we measured the production of 2-alkyl-4-quinolone signal molecules by P. aeruginosa wild-type PA14, PA14Δcrc mutant and PA14Δcrc/pcrc+ mutant cultivated in ABTG medium and ABTC medium, respectively. The PA14 produces more 2-alkyl-4-quinolone signal molecules than the PA14Δcrc mutant in ABTG medium (Fig. 2). In contrast, the PA14Δcrc mutant produces more 2-alkyl-4-quinolone signal molecules than the PA14 in ABTC medium (Fig. 2). The PA14Δcrc/pcrc+ mutant produces a similar level of 2-alkyl-4-quinolone signal molecules to the PA14 (Fig. 2).

Crc affects biofilm formation of P. aeruginosa

We have previously shown that Crc affected early stage biofilm formation of the P. aeruginosa strain in ABTG medium (Zhang et al., 2012). The deficiency of early stage biofilm formation by the PA14Δcrc mutant in ABTG medium might be due to the deficiency of pili and flagellum-mediated motility (O’Toole et al., 2000). Since we showed that the PA14Δcrc mutant has a higher level of pqs signalling in ABTC medium compared to the wild-type PA14, we hypothesized that cultivation in ABTC medium might rescue the early stage biofilm deficiency of the PA14Δcrc mutant. Unlike in ABTG medium (Zhang et al., 2012), early stage biofilm formation was not impaired in the PA14Δcrc mutant when it was cultivated in ABTC medium (Fig. 3). To measure the fitness of PA14Δcrc mutant over its parent wild-type PA14 strain, we tagged the PA14 strain and PA14Δcrc mutant with CFP and YFP, respectively. The PA14 CFP and PA14Δcrc YFP overnight cultures were mixed at 1:1 ratio before inoculation into flow cell biofilm systems. After 24 h cultivation, CLSM observation revealed that PA14Δcrc mutant was inefficient at biofilm formation and only weakly associated with the PA14 wild-type in ABTG medium (Fig. 4a). In contrast, the PA14Δcrc mutant was efficiently associated with the PA14 wild-type and formed substantial amount of biofilms in ABTC medium (Fig. 4b).

Crc affects colonization of P. aeruginosa in a mouse lung infection model

To evaluate the impact of Crc on host–pathogen interactions, we measured the capability of P. aeruginosa wild-type PA14 strain, PA14Δcrc mutant and PA14Δcrc/pcrc+ mutant against host immune clearance in a mouse model of lung infection. Bacterial loads in the lungs of mice were 4 × 10^6 c.f.u. lung^-1 in the beginning, and reached median values of approximately 10^7 (PA14Δcrc/pcrc+ mutant), 10^4 (PA14) and 10^6 (PA14Δcrc mutant) c.f.u. lung^-1, respectively, at the 48 h time point of infected mice. Significantly higher bacterial loads in the lungs (P<0.05) were found in PA14Δcrc mutant compared to the wild-type PA14 strain and PA14Δcrc/pcrc+ mutant (Fig. 5).

DISCUSSION

The dynamic nutritional environment of the host might have a huge impact on the virulence and social behaviour of bacterial pathogens. Investigating the regulation mechanisms of nutrition on bacterial physiology might facilitate better understanding of bacterial adaptation during chronic infections. The catabolite repression control systems exist in a huge impact on the virulence and social behaviour of bacterial pathogens. Investigating the regulation mechanisms of nutrition on bacterial physiology might facilitate better understanding of bacterial adaptation during chronic infections. The catabolite repression control systems exist in a wide range of micro-organisms and allow efficient utilization of available nutrients in the natural environment, where nutrition source is very limited (Görke & Stulke, 2008). The catabolite repression control systems regulate a large set of virulence traits in pathogens (Almengor et al., 2007; Gilbreth et al., 2004; Linares et al., 2010; O’Toole et al., 2000).

The infection site is an environment rich in nutrition such as amino acids and sugars (Rohmer et al., 2011). Adaptive evolutions were observed during chronic infections by pathogens and these mutations changed their metabolism

![Fig. 2. 2-Alkyl-4-quinolone synthesis of P. aeruginosa PA14 wild-type, PA14Δcrc and PA14Δcrc/pcrc+ mutants in ABTG and ABTC minimal medium, respectively. Means ± SD are from triplicate experiments. *P<0.01.](http://mic.sgmjournals.org)
and pathogenesis profiles (McAdam et al., 2011; Yang et al., 2011a, c). A large number of these mutations are involved in adaptation to nutrient conditions. For example, mutation in the lasR gene of cystic fibrosis (CF) P. aeruginosa isolates can reduce oxygen consumption and enhance nitrate utilization (Hoffman et al., 2010). The P. aeruginosa lasR mutant displays an autolysis phenotype on agar plates and increases resistance to two antibiotics used frequently in CF treatment, tobramycin and ciprofloxacin (Hoffman et al., 2010).

Another study showed that adaptive mutations of carbon catabolism regulatory genes were selected in CF isolates (Silo-Suh et al., 2005). In that study, Silo-Suh and colleagues showed the expression of zwf, encoding glucose-6-phosphate dehydrogenase (G6PDH) was refractory to catabolite repression in CF isolates while under stringent control of catabolite repression in environmental wild-type strains (Silo-Suh et al., 2005). zwf is essential for alginate biosynthesis and thus the deregulated zwf expression might guarantee a high level of alginate synthesis by P. aeruginosa in the dynamic CF environment. Alginate serves as an important P. aeruginosa biofilm matrix material and protects P. aeruginosa cells against the host immune responses (Song et al., 2003).

Our studies suggest other reasons for altered regulation of carbon catabolism for CF isolates of P. aeruginosa. We have previously shown that P. aeruginosa Crc mutant had a higher level of metabolic activity compared to its wild-type strain even in late stationary phase and biofilm cultures (Zhang et al., 2012). The high level of metabolic activity of catabolite-repression-deregulated mutants is more resistant to the treatment of antimicrobial peptides such as colistin.

![Fig. 3. GFP-tagged P. aeruginosa PA14 wild-type, PA14Δcrc and PA14Δcrc/pcrc+ mutants in biofilms after 1 day of cultivation in ABTC medium. The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. Bars, 20 μm.](image)

![Fig. 4. CFP-tagged P. aeruginosa PA14 wild-type and YFP-tagged PA14Δcrc mutant in biofilm co-cultures after 1 day of cultivation in ABTG (a) and ABTC (b) media. The central pictures show horizontal CLSM optical sections, and the flanking pictures show vertical CLSM optical sections. Bars, 20 μm.](image)
suggests that deregulation of the catabolite repression by et al. formation and impair the host immune response (Jensen release of bacterial DNA to the infection sites (Yang aeruginosa (Sonnleitner pqs downregulated the expression of the is supposed to bind to Crc and antagonize its effect, contains several conserved catabolite activity motifs and accord with our study, another recent study showed that level of crc mutant had an autolysis phenotype and a higher PA14 wild-type, PA14crc and PA14Δcrc/pcr+ mutants in a mouse model of lung infection. The bacterial counts in lungs at 48 h were expressed as c.f.u. lung⁻¹. The mean bacterial counts are shown as the dark line in the middle of box. *P<0.01.

( Zhang et al., 2012). In the present study, we showed that the Crc mutant had an autolysis phenotype and a higher level of pqs signalling than the wild-type PA14 strain. In accord with our study, another recent study showed that mutation in a small RNA (sRNA) termed CrcZ, which contains several conserved catabolite activity motifs and is supposed to bind to Crc and antagonize its effect, downregulated the expression of the pqs genes in P. aeruginosa (Sonntleitner et al., 2012). Autolysis will lead to release of bacterial DNA to the infection sites (Yang et al., 2007), which was shown to enhance bacterial biofilm formation and impair the host immune response (Jensen et al., 2010; Yang et al., 2011b). In summary, our study suggests that deregulation of the catabolite repression by P. aeruginosa might enhance its fitness during CF infections.

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