Subinhibitory concentrations of the cationic antimicrobial peptide colistin induce the pseudomonas quinolone signal in Pseudomonas aeruginosa

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Colistin is an important cationic antimicrobial peptide (CAMP) in the fight against Pseudomonas aeruginosa infection in cystic fibrosis (CF) lungs. The effects of subinhibitory concentrations of colistin on gene expression in P. aeruginosa were investigated by transcriptome and functional genomic approaches. Analysis revealed altered expression of 30 genes representing a variety of pathways associated with virulence and bacterial colonization in chronic infection. These included response to osmotic stress, motility, and biofilm formation, as well as genes associated with LPS modification and quorum sensing (QS). Most striking was the upregulation of Pseudomonas quinolone signal (PQS) biosynthesis genes, including pqsH, pqsB and pqsE, and the phenazine biosynthesis operon. Induction of this central component of the QS network following exposure to subinhibitory concentrations of colistin may represent a switch to a more robust population, with increased fitness in the competitive environment of the CF lung.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen, and is associated with both chronic and acute infections, including sepsis and wound and pulmonary infections (Bodey et al., 1983; Govan & Deretic, 1996). It is a major cause of pulmonary damage, especially in patients suffering from cystic fibrosis (CF), and its emergence as a nosocomial pathogen is a growing concern. Infections with P. aeruginosa are particularly difficult to cure through antimicrobial therapy because of the bacterium’s intrinsically impermeable outer membrane and active efflux of toxic agents from the cytoplasm (Hancock, 1997a; Nikaido, 1989).

A major challenge has arisen regarding the treatment of infections caused by P. aeruginosa and other multi-drug resistant (MDR) Gram-negative bacteria (Bonomo & Szabo, 2006; Rahal, 2006). This has led to renewed interest in an ‘old class of antibiotics’ known as the polymyxins, first discovered in the 1950s in the soil bacterium Bacillus polymyxa (Ainsworth et al., 1947). Despite their effectiveness, intravenous formulations of colistin (polymyxin E) and polymyxin B were gradually abandoned in most parts of the world in the early 1980s because of the reported high incidence of nephrotoxicity (Beringer, 2001; Brown et al., 1970). Recently, a number of studies have confirmed the safety of colistin and provided increased support for treatment of acute pulmonary infections caused by P. aeruginosa as well as infections caused by other multi-resistant Gram-negative bacteria (Garnacho-Montero et al., 2003; Hanberger et al., 2001; Markou et al., 2003).

Despite the fact that polymyxins have been used clinically for nearly 60 years, there is a dearth of information regarding their pharmacokinetics and pharmacodynamics. This creates an underlying risk of sublethal treatment and the potential development of resistance. Indeed, the first reports of colistin-tolerant clinical isolates have already been published (Denton et al., 2002). P. aeruginosa has developed ‘adaptive tolerance’ to colistin, mediated through the PhoPQ and PmrAB two-component systems that control aminoarabinose modification of the LPS (McPhee et al., 2003). Pseudomonas strains isolated from patients treated with colistin have aminoarabinose-modi-
fied lipid A, providing evidence that *P. aeruginosa* can adapt to the presence of colistin in the airways of the CF lung (Denton et al., 2002; Frederiksen et al., 1999). While microbes have been subjected to antimicrobial agents throughout evolution, the clinical use of antibiotics is a relatively new phenomenon. The antibiotic concentrations that bacteria have encountered in nature are generally lower than the concentrations used to treat bacterial infections. Recent studies have shown that non-lethal concentrations can act as stimuli, in a dose-dependent manner, to elicit specific bacterial responses (Brazas & Hancock, 2005; Davies et al., 2006; Linares et al., 2006; Oh et al., 2000; Skindersoe et al., 2008; Tomasinsig et al., 2004). This modulation of transcription can lead to subtle changes in cell physiology with specific consequences for the collective behaviour of the bacterial population (Davies et al., 2006).

The focus of this study was to investigate the effects of subinhibitory concentrations of colistin, one of the last reserve agents available for MDR bacteria, on *P. aeruginosa*.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids used in this study are listed in Table 1. All cultures of *P. aeruginosa* PAO1 were routinely grown in Mueller–Hinton (MH) or Luria–Bertani (LB) media at 37 °C with shaking at 180 r.p.m. *Escherichia coli* strains were routinely grown in LB at 37 °C. Where appropriate, antibiotics were added to growth media at the following concentrations: 50 μg kanamycin ml⁻¹, 200 μg carbenicillin ml⁻¹, 60 μg tetracycline ml⁻¹ and 50 μg gentamicin ml⁻¹ for *P. aeruginosa*; and 40 μg X-Gal ml⁻¹, 25 μg gentamicin ml⁻¹ and 12.5 μg tetracycline ml⁻¹ for *E. coli*. *Colistin* (Sigma-Aldrich) was prepared in sterile distilled H₂O to a final volume of 20 μl. cDNA was synthesized according to the Affymetrix Expression Analysis Protocol guide. Transcripts corresponding to *Bacillus subtilis* genes *dap*, *thr*, *phe*, *lys* and *trp* were spiked into the cDNA synthesis reaction mixtures as controls to monitor cDNA synthesis, labelling, hybridization and staining efficiency. The cDNA was treated with 2 mg DNase-free RNase (Roche) at 37 °C for 1 h, purified using the QiAquick PCR Purification kit (Qiagen) and eluted in sterile distilled H₂O to a final volume of 20 μl. cDNA was synthesized according to the Affymetrix Expression Analysis Protocol guide.

**Determination of MIC.** The MIC of *P. aeruginosa* for colistin was determined using the broth microdilution technique, according to the National Committee of Laboratory Safety and Standards (NCLSS) guidelines. In brief, PAO1 was grown at 37 °C overnight in MH broth. The overnight culture was then diluted down to OD₆₀₀ 0.05 together with the appropriate final concentration of colistin and grown up in polypropylene 96-well plates. Colistin was added to the media at concentrations ranging from 0.15 to 2.4 μg ml⁻¹. Plates were incubated at 37 °C overnight in a growth curve machine (Bioscreen C). OD₆₀₀ readings were taken every hour.

**Microarray sample and preparation.** Three independent cultures of the *P. aeruginosa* strain PAO1 were exposed to 0.15 μg colistin ml⁻¹. This concentration was chosen as it has been clearly shown to have no effect on the growth of *P. aeruginosa* under the experimental conditions described (Fig. 1a). The untreated and treated samples were grown from OD₆₀₀ 0.05 to 0.8, and subsequently total RNA was extracted using the Ambion RiboPure Bacteria kit according to the manufacturer’s instructions. The RNA was treated with RQ1 RNase-free DNase (Promega) at 37 °C for 1 h and purified using the RNeasy Midi RNA Isolation kit (Qiagen). The RNA was precipitated with three volumes of ethanol and 1/10 volume of 3 M sodium acetate buffer (pH 5.2), and the RNA pellet was resuspended in sterile distilled H₂O to a final volume of 20 μl. cDNA was synthesized according to the GeneDirector and Gene Spring software to give a list of genes with altered expression greater than 1.5 and a P value ≤0.05.

**Semiquantitative RT-PCR.** Microarray data were validated by semiquantitative RT-PCR on RNA isolated from three independent experiments. Specific RT-PCR primers were designed for each gene

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>B. W. Holloway*</td>
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<td>pMP220 3552</td>
<td>PAO1 pmrH–lacZ transcriptional fusion; Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study</td>
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<td>Invitrogen</td>
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<td><strong>Plasmids</strong></td>
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<td>Promoterless lacZ vector, IncP; Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Spaink et al. (1987)</td>
</tr>
<tr>
<td>pCR2.1 TOPO</td>
<td>TOPO TA cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<sup>*</sup>Monash University, Clayton, Victoria, Australia.
promoter fusion analysis. The transcriptional fusion of the region upstream of PA3552 (including the transcriptional start site) was generated in pMP220, a plasmid containing a promoterless lacZ gene (Spaink et al., 1987). Specific primers were designed based on the PA01 genome sequence (Supplementary Table S1). The PCR product was amplified using High Fidelity Taq polymerase (Promega) and

<table>
<thead>
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<th>Gene</th>
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<th>Fold change 0.3μg ml⁻¹</th>
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</tr>
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</tr>
<tr>
<td>phzF</td>
<td>2</td>
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</table>

(b) Quantitative RT-PCR analysis of genes expressed in PA01 exposed to 0.15 μg colistin ml⁻¹ compared with untreated PA01. The values are the mean and SD of five independent replicates. (c) Semiquantitative RT-PCR analysis of genes expressed in PA01 exposed to 0.15 and 0.3 μg colistin ml⁻¹ compared with untreated PA01. The values are the mean and SD of three independent replicates.

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Based on the PA01 genome sequence, GenBank accession number NC002516 (Supplementary Table S1), RT-PCRs were performed using 50 ng template cDNA μl⁻¹, PCR Master Mix (Promega), and 0.5 μM each primer for 20–25 cycles. The constitutively expressed housekeeping gene proC (Savli et al., 2003) was used as a reference to ensure harmonization of samples in all replicates.

![Graph](image)

Fig. 1. (a) Growth curve of PA01 in Mueller–Hinton broth over 24 h. *P. aeruginosa* PA01 was exposed to increasing concentrations of colistin (0.15–2.4 μg ml⁻¹) to determine the MIC. Growth was analysed using Bioscreen C and was measured spectrophotometrically every hour at 600 nm. (b) Quantitative RT-PCR analysis of genes expressed in PA01 exposed to 0.15 μg colistin ml⁻¹ compared with untreated PA01. The values are the mean and SD of five independent replicates. (c) Semiquantitative RT-PCR analysis of genes expressed in PA01 exposed to 0.15 and 0.3 μg colistin ml⁻¹ compared with untreated PA01. The values are the mean and SD of three independent replicates.
subsequently cloned into pCR2.1 TOPO according to the manufacturer’s recommendations (Invitrogen). The PCR product was subcloned as an Asp718 and XbaI fragment into KpnI–XbaI sites of pMP220, transformed into DH5α competent E. coli cells, and subsequently electroprotopated into P. aeruginosa strain PAO1.

The untreated and treated (0.3 μg colistin ml⁻¹) samples were grown from OD₆₀₀ 0.05 to 0.4 in 40 ml MH. β-Galactosidase activity was measured as described by Miller (1972). All assays were carried out in triplicate and the mean and SE were calculated.

Pseudomonas quinolone signal (PQS) extraction and analysis. Cultures of P. aeruginosa were grown in the presence of 0.3 μg colistin ml⁻¹ in MH broth for 20 h. PQS was extracted and analysed by TLC using the protocol described by Fletcher et al. (2007).

Motility analysis. Swimming, swarming and twitching ability was analysed on LB 0.3 % (w/v) agar, Eiken 0.5 % (w/v) agar (Eiken Chemical Tokyo) and LB 1 % (w/v) agar plates, respectively. Colistin was added at the following concentrations: 0.15, 0.3 and 0.6 μg ml⁻¹. The plates were inoculated with bacteria grown overnight on LB agar using a sterile toothpick. The plates were assessed qualitatively by examining the turbid zone formed by bacterial cells migrating from the inoculation point. Unlike PA14, PAO1 does not typically form large tendrils on swarm agar plates, and this allows measurement of the diameter of the swarm front. The twitching plates were analysed by staining with 0.1 % (w/v) crystal violet.

Effect of supernatant from P. aeruginosa on the growth of Lactobacillus spp. The virulence assay to detect the antimicrobial effects of supernatant from P. aeruginosa on Lactobacillus spp. was adapted from that of Zaborina et al. (2007). In brief, P. aeruginosa PA01 was grown overnight in MH broth. This was diluted down to OD₆₀₀ 0.05 and colistin was subsequently added at a final concentration of 0.3 and 0.6 μg colistin ml⁻¹. After 24 h growth, the cells were pelleted by centrifugation and the supernatant was filtered through a 0.20 μm pore-size filter and maintained on ice. The Lactobacillus strains were grown overnight in DeMan, Rogosa and Sharpe (MRS) broth (Oxoid) and diluted down to OD₆₀₀ 0.01. Cultures (100 μl) were placed in 100-well plates, followed by 100 μl supernatant from P. aeruginosa. Control samples contained 100 μl MH and 0.6 μg colistin ml⁻¹. Plates with Lactobacillus spp. were incubated at 37 °C in the BioScreen C plate reader, and growth was monitored by measuring OD₆₀₀.

RESULTS

Transcriptome analysis of P. aeruginosa PAO1 exposed to a subinhibitory concentration of colistin

There is growing evidence that subinhibitory concentrations of antibiotics can modulate gene expression in bacteria, leading to adaptive responses. To understand the implications of P. aeruginosa exposure to subinhibitory concentrations of colistin, global changes in the pattern of gene expression were assessed by microarray analysis. The experimental approach for the microarray study was designed to encapsulate the direct and indirect effects of exposure to subinhibitory concentrations of colistin, some of which might be expected to be subtle. A Student’s paired t test was performed on the RMA-normalized data, using a 1.5-fold cut-off to ensure detection of these subtle effects, which could then be further validated. Approximately 0.5 % (30 out of the 5500 probe sets present on the array) of genes showed significantly altered expression in the colistin-treated sample. Of these, 13 were upregulated and 17 were downregulated (Table 2 and Supplementary Table S2). Data analysis revealed upregulation of genes involved in quorum sensing (QS), LPS modification and biofilm formation, while genes involved in motility and osmorelance were downregulated (Table 2 and Supplementary Table S2). The low fold-change is indicative of the subtle but biologically significant effect that exposure to subinhibitory concentrations of colistin has on P. aeruginosa cellular physiology. The unexpected prevalence of PQS-associated genes led us to investigate the hypothesis that subinhibitory concentrations of colistin induce the PQS signalling molecule and its regulon in P. aeruginosa.

An expected feature of the microarray data was the upregulation of genes known to be involved in tolerance to polymyxins (Supplementary Table S2). The pnmrHFIJKLM-ugd (PA3552–PA3559) operon has been shown to be induced in response to subinhibitory concentrations of colistin (McPhee et al., 2003), and a sixfold induction of a pnmrH–lacZ fusion was observed 3 h post-inoculation (Fig. 2a). Quantitative real-time RT-PCR (qRT-PCR) was carried out on selected targets and alteration of these transcripts in response to 0.15 μg colistin ml⁻¹ was confirmed (Fig. 1b). Results obtained from the qRT-PCR were in agreement with the findings from the microarray data, further indicating that it was a biologically relevant dataset. To determine whether an increase in colistin concentration would increase the magnitude of the transcriptional response, we carried out semiquantitative RT-PCR on cells treated with 0.3 μg colistin ml⁻¹ (Fig. 1c). Although this approach is less sensitive than qRT-PCR, there was a clear increase in expression of target genes at 0.3 μg colistin ml⁻¹ (Fig. 1c), and this concentration was chosen for analysis of colistin-responsive genes identified in the microarray analysis.

Colistin exposure triggers induction of 4-hydroxy-2-heptyquinoline (HHQ) and PQS

An unexpected yet striking feature of the transcriptome analysis was the upregulation of genes involved in synthesis of 2-alkyl-4-quinolones (AHQs), including HHQ and PQS (Table 2 and Supplementary Table S2). PQS is one of three QS molecules found in P. aeruginosa along with C₄-HSL and C₁₂-HSL, and its expression is usually induced during the onset of the stationary phase of growth. Genes involved in synthesis of AHQs (pqsB) and conversion of HHQ to PQS (pqsH) were upregulated in response to subinhibitory concentrations of colistin (Supplementary Table S2). Furthermore, pqsE, which plays a role in mediating the effects of the PQS signal molecule (Bredenbruch et al., 2006), was also upregulated in response to colistin (Table 2). Upregulation of pqsB and pqsE was confirmed by RT-PCR (Fig. 1b, c). As the pqsABCDE operon is known to be
controlled by the pqsA promoter, a P_{pqsA–lacZ} fusion was used to monitor gene expression. Exposure to subinhibitory concentrations of colistin led to fivefold upregulation of the pqsABCDE operon after 3 h (Fig. 2b). Expression of the pqsABCDE operon remained high relative to untreated cells until entry to stationary phase, at which point the operon was induced in the absence of colistin (Fig. 2b). Increasing the concentration of colistin led to a further increase in the level of pqsABCDE induction (data not shown), indicating that the response is dose-dependent. Induction of PQS biosynthetic gene expression in P. aeruginosa cells in response to subinhibitory concentrations of colistin might be expected to lead to increased production of PQS. Therefore, PQS was extracted from the supernatant of P. aeruginosa cultures that had been treated with subinhibitory concentrations of colistin. Samples were analysed by TLC and increased production of PQS was observed in the colistin-treated samples (Fig. 3).

Although there was no observable change in pqsR expression in response to colistin, induction of the PQS biosynthetic genes by colistin requires a functionally intact PqsR (data not shown). PqsR (also called MvfR) is a LysR-type regulator that binds directly to the pqsA and phnA promoters and positively influences expression of pqsABCDE and phnAB (Cao et al., 2001; Wade et al., 2005; Xiao et al., 2006). Expression of pqsR is positively influenced by LasR and negatively influenced by RhlR, creating a regulatory loop between the three QS circuits (Wade et al., 2005). Promoter fusion analysis revealed that the colistin-mediated PQS gene expression was not a direct result of upregulation of the LasIR and RhlIR QS systems, which were unchanged in the presence of subinhibitory concentrations of colistin (data not shown).

### Physiological consequences of colistin-induced AHQs

To date, P. aeruginosa and Burkholderia spp. are the only organisms in which AHQs have been identified (Dubern & Diggle, 2008), and the antimicrobial properties of these compounds towards Gram-positive bacteria have been reported (Hays et al., 1945; Wells et al., 1952). However, the role of PQS in this is uncertain as a ΔpqsE mutant had the same killing effect as PA14 wild-type (Déziel et al., 2004). As induction of AHQs may increase antimicrobial activity against Gram-positive bacteria, supernatants from colistin-treated cultures were tested for inhibition of Lactobacillus rhamnosus GG growth. While colistin alone did not affect growth, P. aeruginosa culture supernatants had a slight inhibitory effect on the growth of L. rhamnosus GG. Addition of a subinhibitory concentration of colistin to P. aeruginosa cultures resulted in supernatant that had a marked inhibitory effect on the growth of L. rhamnosus GG. Addition of a subinhibitory concentration of colistin to P. aeruginosa cultures resulted in supernatant that had a marked inhibitory effect on the growth of L. rhamnosus GG. Although there was no significant increase in growth inhibition in the presence of culture supernatants treated with 0.3 μg colistin ml\(^{-1}\), a pronounced inhibitory effect was observed upon addition of supernatant from cells treated with 0.6 μg colistin ml\(^{-1}\). Increased inhibition was not observed upon addition of supernatant from colistin-treated pqsR (Fig. 4b) or pqsA (data not shown) mutant cells. This would suggest that the increased antimicrobial activity is a direct result of increased AHQ.

### Table 2. Microarray analysis of P. aeruginosa PAO1 exposed to a subinhibitory concentration of colistin

Shown are genes with a twofold or greater altered expression (P ≤ 0.05) in the presence of 0.15 μg colistin ml\(^{-1}\). Positive values represent genes that are upregulated and negative values represent genes that are downregulated in the presence of colistin.

<table>
<thead>
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<th>Gene number</th>
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<th>Description</th>
<th>Fold change</th>
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<tr>
<td>PA1000</td>
<td>pqsE</td>
<td>Quinolone signal response</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>phzF*</td>
<td>Phenazine biosynthesis protein PhzF</td>
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<td>psIC</td>
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<td></td>
<td>Probable acyl carrier protein</td>
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<td>Rhamnosyltransferase chain B</td>
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<td>−2.2</td>
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<td>PA2821</td>
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<td>Probable glutathione S-transferase</td>
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<td>PA5372</td>
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*The Affymetrix P. aeruginosa microarray contains probe sets for only one copy of each phzA–G gene.
Subinhibitory concentrations of colistin induce PQS

production in response to subinhibitory concentrations of colistin.

As PQS is maximally produced at the onset of the stationary phase of growth, it may act as a secondary regulatory signal for a subset of QS-controlled genes (Déziel et al., 2004). Therefore, induction of PQS in the early exponential phase might be expected to affect the cellular physiology of *P. aeruginosa*. Indeed, an interesting feature of PQS is that, unlike the acyl homoserine lactone (AHL) signal molecules, addition of exogenous PQS can overcome density-dependent gene expression in *P. aeruginosa* (Diggle et al., 2003). Recent work by Häussler and colleagues has indicated that PQS predisposes *P. aeruginosa* to exogenous stress (Häussler & Becker, 2008). To study the effects of colistin-induced PQS on *P. aeruginosa* fitness, cells were challenged with both subinhibitory concentrations of colistin and purified PQS. An increased lag-time relative to addition of either colistin or PQS alone was observed (Fig. 5a). Under the same conditions, no effect was observed in the *pqsR* mutant (Fig. 5b). Increasing the concentration of exogenous PQS in the presence of colistin led to an increased lag-time in the *pqsR* mutant. However, growth was also inhibited in the presence of PQS alone at these higher concentrations (data not shown), suggesting that a threshold had been reached. It would therefore appear that colistin-induced PQS, once accumulated above a threshold level, has a negative effect on the cellular physiology of *P. aeruginosa*.

As well as playing a role in signalling and virulence, PQS is also a critical component in the formation of biofilms in *P. aeruginosa*. Expressed in cells that form the stalk of maturing biofilms, PQS contributes to the formation of the mushroom structure and secretion of extracellular DNA (Yang et al., 2007). Transcriptome analysis revealed regulation of several genes involved in biofilm formation and maturation in response to subinhibitory concentrations of colistin (Fig. 1c, Table 2 and Supplementary Table S2). Phentotypic assays revealed that both swarming (44% inhibition) and swimming motility (31% inhibition) were affected; however, no effect on twitching motility was observed (Supplementary Table S3). To examine the possibility that colistin exposure influences biofilm formation in *P. aeruginosa*, crystal violet assays were carried out on static cultures in the presence of subinhibitory concentrations of colistin. No difference in attachment or
pellicle formation was observed. Flow-cell analysis also revealed no difference in biomass or ability to form mushroom structures in the presence of the antimicrobial (data not shown). This may be a reflection of the distinct environments and expression profiles that exist in planktonic cultures and mature biofilms (Waite et al., 2005, 2006; Whiteley et al., 2001). It must also be considered that other genes, which are not responsive to subinhibitory concentrations of colistin under the conditions tested, are required for induction of the biofilm mode of growth.

**Exposure to subinhibitory concentrations of colistin results in increased pyocyanin production in* P. aeruginosa*

PQS regulates the production of several virulence factors in *P. aeruginosa*, including elastase, rhamnolipids and phenazines (Déziel et al., 2004). Enhanced production of phenazines in response to subinhibitory concentrations of other antibiotics has already been reported (Mitova et al., 2008; Shen et al., 2008). Exposure to subinhibitory concentrations of colistin resulted in upregulation of the phenazine biosynthesis operon (Table 2). qRT-PCR confirmed increased expression of *phzF* and this was found to coincide with an increase in pyocyanin production, particularly evident in the earlier stages of growth (Fig. 6). Induction of the *phzA–G* operon by colistin provides evidence that exposure to subinhibitory concentrations influences *P. aeruginosa* pathogenicity, a finding that has implications for the use of this antibiotic as a monotherapeutic option.

**DISCUSSION**

Colistin is considered one of the last reserve agents for treating Gram-negative MDR bacterial infections. To develop an effective response to the emergence of colistin resistance, it is imperative that targets of the antibiotic are identified and characterized. While the mechanism of polymyxin-mediated killing has been studied by several groups, it is becoming increasingly clear that suboptimal doses of antibiotics modulate markedly different changes in bacterial gene expression (Brazas & Hancock, 2005; Gerber et al., 2008; Labro et al., 1992; Skindersoe et al., 2008). The
Fig. 5. (a) Growth kinetics of *P. aeruginosa* PAO1 in the presence of colistin (0.6 μg ml\(^{-1}\)) and PQS (1 μl ml\(^{-1}\)). In the presence of both colistin and PQS there is an inhibiting effect on growth. (b) Growth kinetics of *P. aeruginosa* pqsR mutant cells under the same conditions. There was no effect on growth when PQS was present in the media while there was a slight effect on growth in the presence of colistin. This experiment was repeated three times with independent cultures and the outcome of one representative experiment is shown.

Fig. 6. Quantification of pyocyanin. White bars, untreated; black bars, treated with 0.3 μg colistin ml\(^{-1}\). Results are the mean and SE of triplicate measurements.
lack of definitive data regarding the pharmacokinetics and pharmacodynamics of colistin necessitates elucidating the subinhibitory colistin signature as a matter of priority.

Antibiotics are increasingly being thought of as hormetic compounds; in other words, they have contrasting effects at low and high concentrations. Exposure to subinhibitory concentrations is not necessarily detrimental to susceptible bacteria and may in some cases result in phenotypes that are advantageous (Linares et al., 2006). Biofilm formation, cytotoxicity, motility, expression of virulence determinants and QS may be induced or repressed by bacteria in response to subinhibitory concentrations of different classes of antimicrobials (Brazas & Hancock, 2005; Linares et al., 2006; Skindersoe et al., 2008). The transcripional changes induced tend to be both species- and antibiotic-specific. For instance, while tobramycin and colistin may inhibit biofilm formation in *Klebsiella pneumoniae*, tobramycin increases biofilm formation in *P. aeruginosa* and *E. coli* (Hoffman et al., 2005; Hostacka & Ciznar, 2008; Linares et al., 2006). Tetracycline increases the cytotoxicity of *P. aeruginosa*, while ciprofloxacin induces the R2/F2 pyocins in *P. aeruginosa*, causing susceptibility to fluoroquinolones (Brazas & Hancock, 2005; Linares et al., 2006). Plate assays reveal increased expression of *lasIR* in *E. coli* in the presence of both colistin and polymyxin B, while expression is unchanged in liquid cultures (Goh et al., 2002). In contrast, several recent studies have reported downregulation of the *rhlIR* and *lasIR N*-acylhomoserine lactone signalling systems in *P. aeruginosa* in response to sublethal concentrations of LL-37, azithromycin, ceftazidime and ciprofloxacin (Nalca et al., 2006; Overhage et al., 2008; Skindersoe et al., 2008). Therefore, the emerging model is one where subinhibitory concentrations of antibiotics elicit a niche-specific response in bacteria, which may contribute to adaptation within a natural ecosystem. Revealing the induction of the PQS virulence regulon in response to subinhibitory concentrations of colistin is an important contribution to this emerging field.

The transcriptional response to subinhibitory concentrations of antibiotics can often involve genes that appear to be unrelated to the previously defined targets of the antibiotic. Polymyxins are known to displace the calcium and magnesium bridges that stabilize the LPS (Evans et al., 1999; Hancock, 1997b; Hermsen et al., 2003; Tam et al., 2005). Both *pmrHFIJKLMNOP* and PQS are induced in the exponential growth phase when *P. aeruginosa* is grown in magnesium-limiting media (Guina et al., 2003). Therefore, it is possible that the displacement of \( \text{Mg}^{2+} \) following colistin binding is responsible, at least in part, for the observed transcriptional changes. Furthermore, ~60% of PQS appears to be cell wall-associated (Diggle et al., 2007; Lépine et al., 2003), and this may also be a critical factor in the response. A recent study has shown that inactivation of *bptS* (PA1396), a sensor kinase, results in upregulation of *PA4773*, a predicted polyamine biosynthesis gene, and increased tolerance to colistin and polymyxin B (Ryan et al., 2008). Understanding how subinhibitory concentrations of antimicrobials elicit changes in bacterial gene expression will require a comprehensive analysis of the nature of the interaction and identification of all sensory and regulatory components involved.

To address the question of how exposure to colistin may influence *P. aeruginosa* within a natural ecosystem, the consequences of PQS induction were assessed. Subinhibitory concentrations of colistin result in a PqsR-dependent increase in antimicrobial activity against *L. rhamnosus* GG. A recent report by Zaborina and colleagues has shown similar results using a subinhibitory concentration of the K-opoid dynorphin (Zaborina et al., 2007). Accumulation of PQS also has a significant effect on the cellular physiology of *P. aeruginosa*. Treatment of *P. aeruginosa* with both PQS and colistin resulted in a PqsR-dependent enhanced inhibitory effect on growth relative to either PQS or colistin alone. Häussler and colleagues propose that PQS mediates an endogenous stress that predisposes *P. aeruginosa* to subsequent external stresses (Häussler & Becker, 2008). They suggest that PQS moderates the *P. aeruginosa* population through its dual function as both pro- and anti-oxidant. The increased susceptibility may also be due to the interaction of PQS with the LPS layer of the cell, whereby it maintains the LPS in a well-ordered state (Mashburn-Warren et al., 2008). Alterations in membrane fluidity are known to contribute to protection against antimicrobial peptides (CAMPs), with fluid membranes being more resistant (Peschel, 2002). The contrasting roles of PQS highlight the need for a systems biology approach in studying the effect of subinhibitory concentrations of antibiotics on mixed microbial communities.

While an increase in PQS may enable *P. aeruginosa* to adapt in a multi-species ecosystem, it also has important implications for the use of colistin as a monotherapeutic strategy. PQS controls a battery of genes necessary for virulence and biofilm formation (Diggle et al., 2007). Its production results in upregulation of virulence factors such as phenazines and rhamnolipid, which are important for pathogenesis in several virulence models (Gallagher & Manoil, 2001; Mahajan-Miklos et al., 1999; Mavrodi et al., 2001; Pesci et al., 1999; Rahme et al., 2000). PQS is also known to promote biofilm formation and DNA release in *P. aeruginosa*, when iron is limiting (Yang et al., 2007). While our genetic and phenotypic data would suggest that subinhibitory concentrations of colistin influence biofilm formation in *P. aeruginosa*, no significant difference was observed in biomass using flow cells (data not shown). It is important to note that gene expression varies in *P. aeruginosa* planktonic cultures relative to the biofilm mode of growth (Waite et al., 2005, 2006; Whiteley et al., 2001). Furthermore, culture conditions may influence the patterns of gene expression in response to subinhibitory concentrations of colistin. Exposure to different nutrients and media constituents affects cellular physiology and may influence the response of *P. aeruginosa* to colistin. Indeed,
while this study provides clear evidence that exposure to subinhibitory concentrations of colistin induces PQS production in P. aeruginosa, the extent to which all metabolic enzymes for PQS synthesis are influenced remains to be elucidated.

Subinhibitory concentrations of tetracycline (1/16 MIC) have recently been found to positively influence expression of phzA1, phzA2, rhlA, lasB, exoS, exoY and rhlR in P. aeruginosa, whereas rifampicin (1/4 MIC) reduces expression of rhlA, lasB and phzA (Liang et al., 2008). A novel regulator of the PQS regulon has been identified in a YebC family protein, PA0964, which was found to regulate expression of the PQS regulon in response to subinhibitory concentrations of tetracycline. Surprisingly, in that study, polymyxin did not induce expression of phzA2 or rhlA, although neither the concentration of antibiotic used in these assays nor the specific polymyxin used was stated. As we have shown, induction of the PQS biosynthetic operon is dose-dependent, with increased activation as the concentration approaches sublethal levels. It is probable, therefore, that the concentration used in the study described by Liang and colleagues was simply too low to elicit activation of this system. Microarray analysis has also revealed that exposure to azithromycin (1/64 MIC) increases pqsA expression in P. aeruginosa (Nalca et al., 2006; Skinderso et al., 2008), suggesting that this critical component of the QS network is responsive to a wide class of compounds, perhaps reflecting an ability to command a rapid adaptive response in diverse ecological niches.

Conclusion

The results presented in this paper offer an important contribution to the field of subinhibitory antibiotics and have clear implications for the treatment of P. aeruginosa infections with colistin. Concerns have already been raised about colistin monotherapy, not least with the emergence of heteroresistance among Gram-negative pathogens (Hawley et al., 2008; Li et al., 2006). In the light of the role played by PQS in P. aeruginosa infection and its induction by subinhibitory concentrations of colistin, combinatorial strategies may have to be considered. An integrated approach is required to fully understand the effect of subinhibitory concentrations of colistin on P. aeruginosa in mixed microbial communities, including the CF lung. This knowledge is crucial for the successful development and application of colistin therapies in the future.

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REFERENCES


Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Camara, M. & Williams, P. (2003). The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol 50, 29–43.


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