Non-coding small (micro) RNAs of *Pseudomonas aeruginosa* isolated from clinical isolates from adult patients with cystic fibrosis

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ABSTRACT

MicroRNAs are a class of small non-coding RNAs widely reported in eukaryotic multicellular organisms. In this study, a number of small non-coding micro (mi)RNA species in clinical isolates of prokaryote *Pseudomonas aeruginosa* were obtained from the sputum of adult patients with cystic fibrosis (CF) utilising a DynaExpress miRNA cloning kit, and five miRNAs of 16–47 nucleotides that were smaller than those encountered or described (80–100 nucleotides) previously in bacterial systems were described. This report presents data on these unknown cellular miRNAs cloned from *P. aeruginosa* isolates from CF patients. Adapting a computational miRNA prediction model that takes advantage of the highly conserved known miRNA hair pin stems regions, the results revealed that the fold structure of the microRNAs had a high homology to the recently reported human bacterial infection response (BiR)-related microRNA, mi-146, associated with the Toll-like receptor (TLR) family, which is the primary evolutionarily conserved sensors of pathogen-associated molecular patterns (PAMPs), and known to trigger host inflammatory and immune responses.

KEY WORDS: Cell regulation.
MicroRNAs.
Therapeutics.
Virulence.

Introduction

Pseudomonas aeruginosa is a clinically significant bacterial pathogen responsible for increased morbidity and mortality in patients with cystic fibrosis (CF). Small non-coding micro (mi)RNA species, generally 80–100 nucleotides (nt) or less in prokaryotes, are involved in many cellular processes.[1,2] For example, iron homeostasis in *P. aeruginosa* is regulated by tandem duplicates of regulatory small RNAs (sRNAs).[3] These miRNAs act by base pairing with target mRNAs, imposing translational and stability changes culminating in the control and regulation of target mRNAs, which is crucial for bacterial stress responses and virulence to changing host cell environments (e.g., human airway epithelial cells). To date, there has been little description of smaller-sized non-coding miRNAs (<50 nt) and their regulatory function in *P. aeruginosa*. The aim of this study is to isolate small-sized miRNAs from organisms freshly isolated from the sputum of CF patients. This is a first report of preliminary characterisation of new miRNAs obtained from clinical isolates of *P. aeruginosa* in CF patients.

Materials and methods

Isolation and cloning of small RNAs

P. aeruginosa clinical isolates (n=6) were cultured from expectorated sputum obtained from chronically infected adult CF patients attending the Adult Regional CF Unit at Belfast City Hospital. Micro RNA (miRNA) was initially extracted using the Invitrogen PureLink miRNA isolation kit (K1570.01). The resultant miRNA was quantified using a Nanodrop ND 1000 spectrophotometer (Labtech International) and then purified by an Ambion FlashPage (small RNA size) fractionator. A DynaExpress miRNA cloning kit (Biodynamics, Tokyo, Japan) was used for the isolation of small RNAs including miRNA, siRNA or other small RNA (16–18 nt) from purified, fractionated RNA. Briefly, 500 ng purified fractionated RNA was exposed to a) dephosphorylation, b) ligation of 3′-linker and purification, c) phosphorylation, d) 5′-linker ligation and purification, e) reverse transcription and amplification, and f) cloning into a plasmid vector (Figs. 1A–C).

Isolation of cloning-ready miRNAs

A sample (100–500 µg) of purified, fractionated miRNA described elsewhere was subjected to dephosphorylation by alkaline phosphatase in a 1.5 mL nuclease-free tube together with 10 µL RNA, 4 µL 10x phosphatase buffer, 39 µL nuclease-free water and 1 µL BAP (0.6 µg/µL), and incubated at 37°C for 1 h. This was followed by standard phenol/chloroform extraction and ethanol precipitation. The dephosphorylated RNA (supernatant) was subjected to

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ligation using 18 µL nuclease-free water, 1 µL MI-3′-oligonucleotide DNA linker (5′pCTG TAA CTC GGG TCA ATddc), 4 µL T4RNA ligase (x10) buffer, 16 µL PEG (45%) and 1 µL T4 RNA ligase, and incubated at 37˚C for 1 h. This was followed by standard phenol/chloroform extraction and ethanol precipitation.[4]

In the second stage, the 3′-end of the linker was blocked to prevent circularisation of the RNA. The third stage involved 3′-linker-ligated product purification via polyacrylamide gel electrophoresis with a guide size marker. Usually, miRNAs ligated with 3′ linkers are RNA/DNA chimaera nucleotides and tend to migrate between 36–46 mer bands of the marker on a denaturing polyacrylamide gel (10%) run in 1x TBE buffer. The 36-46 mer gel region was carefully excised with a scalpel blade and transferred to a 1.5 mL tube. Using RNA extraction buffers, each band was eluted, centrifuged and the supernatant subjected to standard chloroform extraction.[4] The precipitated pellet was dried thoroughly. The resultant 3′ linker-ligated miRNA precipitate was phosphorylated using 34 µL nuclease-free water, 4 µL 10x PNK buffer, 1 µL each of 100 mmol/L ATP and T4 PNK (10 µg/µL), and the reaction mixture was held at 37˚C for 1 h prior to phenol/chloroform extraction and ethanol precipitation.[4]

In the following stage, the 5′ linker was ligated and purified. This was initially achieved via the 5′ end of the 3′-linker-ligated miRNA being phosphorylated in 18 µL nuclease-free water, 1 µL ribonucleotide MI-5′ linker (5′- AUC GUC UCG GGA UGA AA), 4 µL T4 RNA ligase (x10) buffer, 16 µL PEG (45%) and 1 µL T4 RNA ligase, and incubated at 37˚C for 1 h. The 5′ end of the ligated product was subjected to standard phenol/chloroform extraction and ethanol precipitation.[4] This new 'two-linker' ligated miRNA was purified via polyacrylamide gel electrophoresis (PAGE) as described elsewhere.

The miRNAs ligated with 3′ linkers and 5′ linkers migrate 'between' the 53–63 mer bands of the marker on a denaturing polyacrylamide gel, although they are not generally seen on staining. As described elsewhere, the putative 5′ linker-ligated miRNA bands were excised and eluted using standard RNA gel band extraction procedures,[4] the chloroform extracted supernatant removed and the precipitated pellet was dried.

Cloning protocol for putative miRNAs

The miRNAs obtained above were subjected to reverse transcription (RT) using 2 µL product, 1 µL 3′ RT primer DNA (5′ ATTGACCCGAGTTACAG), and heated to 70˚C for 3 min, cooled to room temperature for 2 min, then held on ice. The RT mixture (4 µL x5 RT buffer, 2 µL 10 mmol/L dNTPs, 1 µL reverse transcriptase [100 µg/µL]) was added and the tubes incubated at 37˚C for 10 min, 42˚C for 1 h, then 85˚C for 5 min. The resulting cDNAs in the RT (20 µL) were subjected to polymerase chain reaction (PCR) amplification using 30.5 µL nuclease-free water, 10 µL amplification (x10) buffer, 2 µL 10 mmol/L dNTPs, 1 µL 5′ primer DNA (5′ ATC GTC TCG GGA TGA AA), 3′ RT primer DNA (5′ ATT GAC CCG AGT TAC AG), *Thermus aquaticus* (*Taq*) polymerase (5 µg/µL) and the final 50 µL reaction mixture was exposed to the following conditions in a thermocycler: initial heating at 94˚C for 1 min, 25–30 cycles at 94˚C for 1 min, 50˚C for 1 min, 72˚C for 1 min, and a final extension at 72˚C for 10 min. After cycling, the amplified PCR products were separated by 3% agarose gel electrophoresis in TAE buffer.

The putative miRNA PCR products were digested with

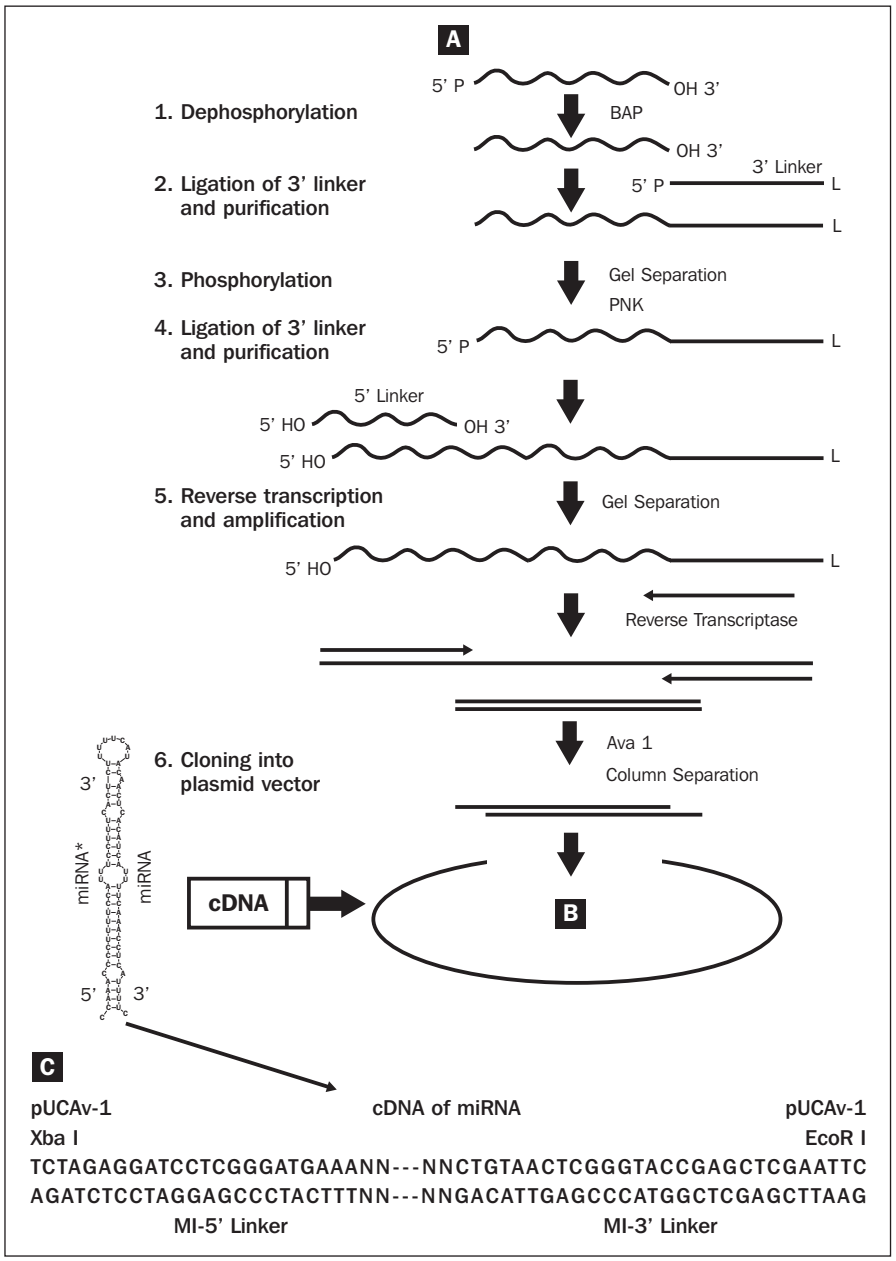

Fig. 1. Scheme depicting small RNA cloning steps (www.biodynamics.co.jp) and a model cloned micro RNA with its characteristic stem-loop RNA fold structures.

Ava I (50 µL RT-PCR product [cDNA of microRNA], 5 µL Ava I buffer [x10] and 1 µL Ava I [10 µg/µL]) and the reaction mixture was incubated overnight at 37°C. To the Ava I-digested products, taken in a spin column, 170 µL binding buffer (isopropanol) was added, centrifuged and the filtrate in the lower collection tube discarded. The miRNA product was purified in excess volumes of wash buffer (absolute ethanol). The spin column was attached to a DNA-elute (1.5 mL) tube and 30 µL elution buffer was added and allowed to stand for 1–2 min at room temperature, and the centrifuged.

The Ava I-digested, purified DNA fragments containing the miRNA sequences were cloned into a T-Vector (pUCAv-1 sequences 1–2686 bp; available on NCBI database) to derive 'directionally cloned' cDNAs (Fig. 1B) of the miRNAs isolated. The reaction mixture contained 5 µL Ava-digested PCR (DNA) product, 1 µL pUCAv-1 (linearised vector), 6 µL ligation buffer (x2) and 1 µL ligase mixture, and was incubated at 16°C for 30 min. The ligation mixture was stored at –20°C until transformation into *Escherichia coli* using high-efficiency competent cells grown on LB medium containing 50–100 µg/mL ampicillin.

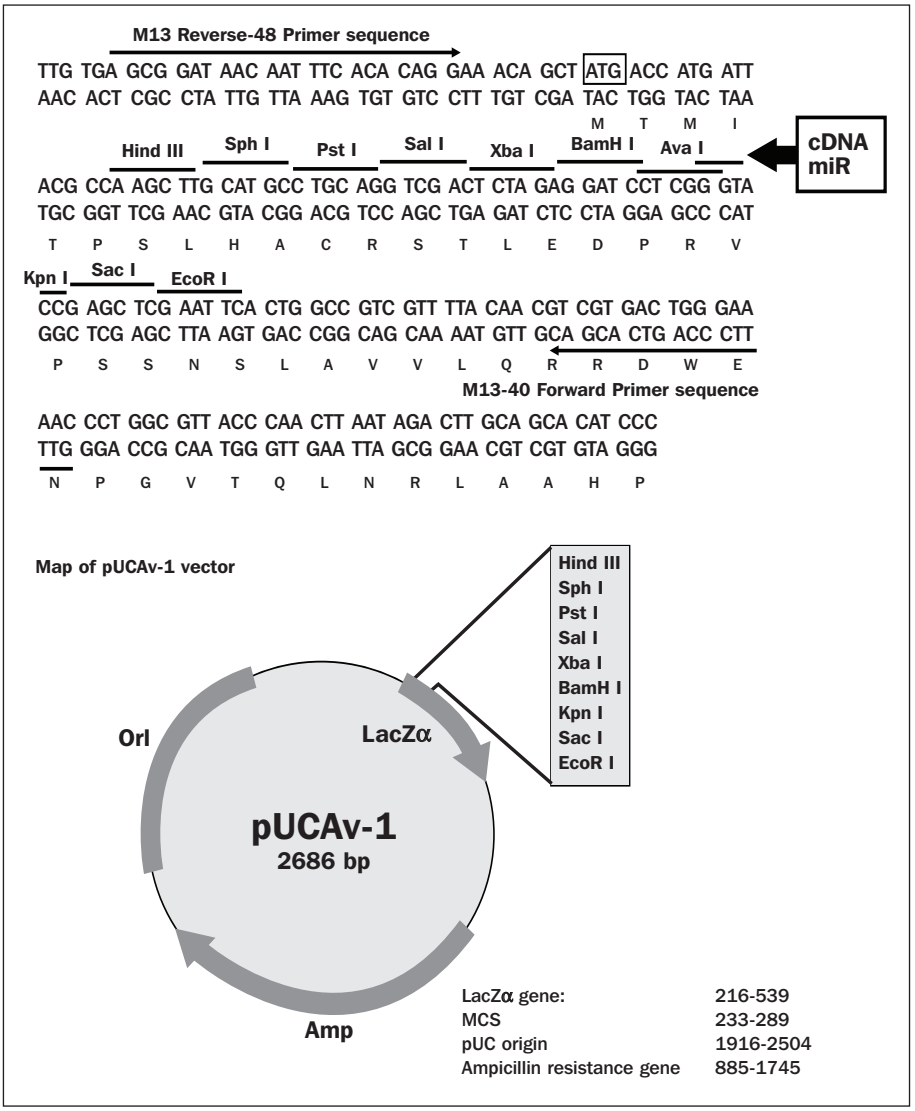

Fig. 2. Sequence around the cloning site in the pUCAv-1 vector plasmid construct (adapted from Biodynamics, MiRNA Cloning Kit AV, Tokyo, Japan).

Cloned miRNA sequence analysis

In the plasmid constructs, the cDNAs of the miRNAs were flanked by Mi-5 linker and the XbaI site of pUCAv-1, while MI-3 linker was located on the EcoRI site of pUCAv-1 (Fig. 1C). The sequences chosen (Fig. 2) were 1916–2504 of pUCAv-1 (1–2686 bases), and the marker sequences were ampicillin resistance genes (885–1745 bp) and the *lacZ*α gene (216–539 bp). Sequencing was performed on miRNA plasmid inserts using the M13 reverse 48 primer sequence (A GCG GAT AAC AAT TTC ACA CAG GA) and M13-40 forward primer sequence (GCA GCA CTG ACC CTT TTG). Discrete PCR products were recovered from agarose gels using a Qiaquick PCR nucleotide removal and gel extraction kit (Qiagen) and cloned by ligation into the pUCAv1 plasmid vector and transformed into *E. coli* DH5α competent cells. Positive colonies were cultured overnight in 50 mL LB broth containing 125 µL ampicillin (50 mg/mL) and plasmid DNA was extracted using the Wizard Plus Miniprep DNA purification kit (Promega).

The plasmid insert was sequenced using the dye terminator cycle sequencing kit (PE Applied Biosystems) using the following cycle parameters: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, repeated for 25 cycles. Reactions were purified with 95% ethanol and 3 mol/L sodium acetate before being washed in 70% ethanol and vacuum-dried for 20 min. The samples were resuspended in 3 µL loading buffer (5:1 formamide:50 mmol/L EDTA [pH 8] with 2 mg/mL blue dextran). The samples were then denatured for 2 min at 90°C before being placed on ice prior to sequencing using ABI Big Dye Terminator v.3.1 (ABI, Warrington, UK) and an ABI 3100 DNA analyser (sequencer rig). Sequences obtained were analysed using ABI SEQ collection v.2 software and edited using sequence analyser v.5.1.1. software

Computational prediction analyses

The miRNAs isolated were subjected to Hierarchical Hidden Markov Model (HHMM) analysis, described previously.[5] For this preliminary study, the training of initial datasets was based on at least 300 random hairpins, secondary structures and their minimum fold energy values. The structural details were elucidated using the Vienna RNAfold programme,[6] and sequence comparisons were based either on RNA database (BLASTn) searches or from microRNA registry (miRBase) compilations.[7,8]

Results

Generic comparison of microRNAs

Using the Biodynamics MiRNA kit and the procedures represented in Figures 1A–C, 27 presumptive miRNAs were obtained from clinical *P. aeruginosa* isolates. Five of the putative miRNAs obtained from the clinical isolates which yielded small non-coding RNAs (16–47 nt) were chosen and the corresponding cDNAs (Fig. 1C) were cloned into plasmid constructs (Fig. 2). As *P. aeroginosa* is principally an environmental (e.g., soil/plant) bacterium, the study aimed to compare these presumptive small RNAs to those from other important soil bacteria. The results (Fig. 3) showed that *Rhizobium* spp. (Fig. 3, Lane 1) and *Agrobacterium tumefaciens* (Fig. 3, Lane 4) did not yield miRNAs in this size range. However, *P. aeruginosa* plant pathogenic strain PA14 (Lane 2) and CF type strain PA01 (Lane 3) and the human clinical isolates (Lanes 5, 6 and 7) contained miRNAs of similar size. The miRNAs (>70 bp) common to *Pseudomonas* bacteria also occurred in the CF type strain PA01. The smaller-sized miRNA sequences of the clinical isolates (Fig. 3, Lanes 5, 6 and 7) when compared to those of the pseudomonad database (sRNAPredict www.tufts.edu/sackler/waldorlab/) but were not recognised. However, such sequences may have to be reviewed against new genomic data arising from the unfinished regions of the genomes of host or bacterial cells. In light of these results, the putative miRNA sequences obtained from the clinical isolates (Table 1) were subjected to an m-fold program (www.bioinfo.rpi.edu/applications/mfold), which showed that they generated a statutory stem-loop structure conformation to the surrounding genomic sequences (e.g., miRNA precursors), and their integrity was validated by Northern blot analysis with size-fractionated miRNAs as probes against the corresponding total RNA of the organism/sample.

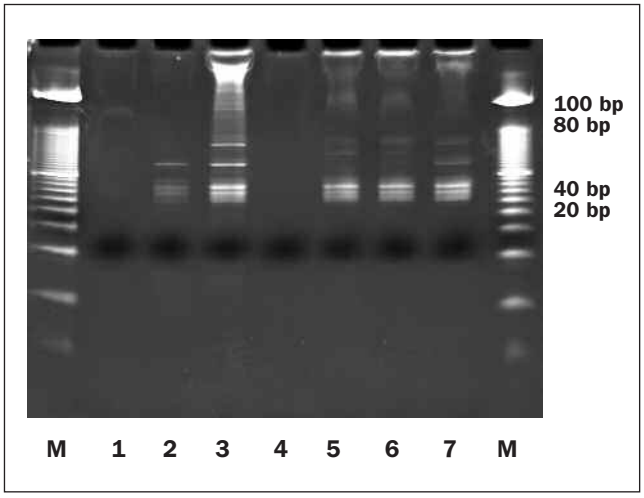

Fig. 3. Isolation of microRNA (miRNAs) in *Pseudomonas* spp. (40–80 bp) from agriculture environment, plant pathogen (PS) or soil-related (SPS) bacteria and in a collection of clinical isolates of *Pseudomonas aeruginosa* (cfPA) from adult cystic fibrosis patients chronically infected with *P. aeruginosa*. PCR amplification of cloned sequences using DynaExpress miRNA Cloning Kit AV (BioDynamics Laboratory, Japan). Lane M: 10-bp Invitrogen Trackit ladder (Invitrogen, Paisley, Scotland); Lane 1: *Rhizobium* spp. (plant root infecting bacteria); Lane 2: *P. aeruginosa* (PS-phytopathogen); Lane 3: *P. aeruginosa* (PAO1 type strain); Lane 4: *Agrobacterium* sp. (root-gall soil bacteria); Lane 5: *P. aeruginosa* cfPA (clinical isolate 3188.2); Lane 6: *P. aeruginosa* cfPA (clinical isolate 3188.3); Lane 7: *P. aeruginosa* cfPA (clinical isolate 3188.4). In order to rule out any degraded/derivative small fragments of non-target cellular RNA species (e.g., mRNA, tRNA or rRNA) and to confirm that the cloned sequences were microRNAs, the above sequences were subjected to a series of computational approaches.

Identification and analysis of miRNAs

Putative miRNA sequences are shown in Table 1. Small non-coding RNAs and their unique hairpin structures play a major part in biological functions among various taxonomic groups within vertebrates, invertebrates, plants and in microbes. The secondary structures generated by HHMM analysis using RNAfold software are depicted in Figure 4. The output of this HHMM model examined against the BLASTn mammalian library exhibited the lowest matches (<70%) and the highest match (>80%) with miRNA hairpins in a wide variety of mammals. Database analysis of RNAfold when restricted to searches for human BiR miRNAs most relevant to the present study yielded a high degree (~94.5%) of homology to the secondary folds of a recently discovered human NF-κB-dependent miRNA, miR-146, an inhibitor targeted to signalling proteins of innate immune responses usually associated with conserved components of microbial infection. Also obtained were RNA stem-loop structures generated (Fig. 4) via RNAfold (www.bioinfo.rpi.edu/applications/mfold) as examples to demonstrate the variation in miRNAs with increasing size.

Discussion

Isolation, cloning and sequencing of miRNAs

Recent advances in molecular cell biology involving RNAs include the description of versatile small RNAs that regulate gene expression. Of these, the coding RNAs are the essential 'connecting links' for gene expression steps, while non-coding RNA subspecies (e.g., ribosomal and transfer RNA) possess 'key' structural, catalytic and information decoding motifs that dictate their roles in protein synthesis. Non-coding small RNAs characterised thus far act as post-transcriptional regulators and in bacteria they are normally 80–100 bp.[1,3,13]

Based on the miRNA extraction methodologies and cloning strategies followed in this study and the comparison to strategies adopted for bacterial small RNAs,[9] the detection of small (micro) RNAs reported here (Fig. 3) can be regarded as novel insofar as CF-related *P. aeroginosa* clinical isolates are concerned. Although there are a number of approaches currently available for miRNA isolation and identification, the current strategy was based on a breakthrough in large-scale miRNA identification.[10,11]

Endogenous short interfering (si) RNAs and the miRNAs have a central biogenesis and can in theory perform interchangeable biochemical functions for gene expression. However, these two classes of small RNAs have distinctly different cellular origin, evolutionary conservation and the types of gene they target for regulation,[12] and thus their variation is revealed in their structural characteristics.

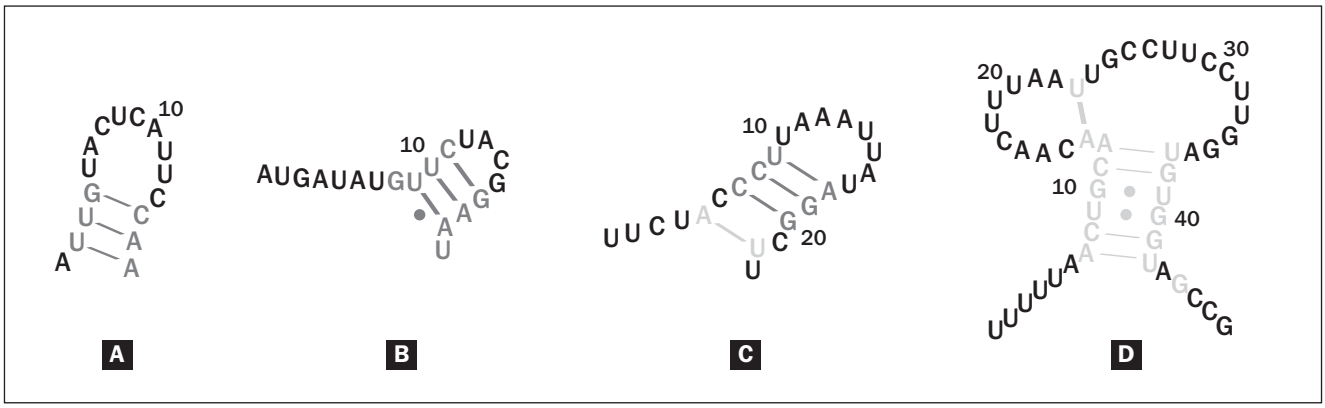

Fig. 4. Examples of RNA stem-loop structures generated via RNAfold (www.bioinfo.rpi.edu/applications/mfold) for putative miRNAs obtained from clinical *P. aeroginosa*. A: cfPA3188.2 (size 16); B: cfPA3188.3 (size 19); C: cfPA3188.4 size 23; and, D: cfPA3188.5 (size 47). The predictive model depicts the increasing variations that can be brought about in structural specificity in stem-loop folding as demonstrated between miRNAs A, B and C with as little as 4nts and a total change in the folding modes with higher species of miRNAs (D) bringing about the potential for variation in their gene regulatory, tissue/site-specific expression roles.

Host-bacterial responses

The data on sRNAs warrant a brief overview of these RNA elements in the wider context of plant, animal and microbial systems.[20] Over the past few years, many small non-coding RNAs have been identified in plant-microbe interactions (e.g., root gall, root nodule and root rot) and have been implicated as crucial mRNA expression and regulatory elements in bacterial stress responses and in bacterial virulence.[13] The host-bacterial responses vary in the 'recognition' of the type of host cell invaded by the (environmental) bacterial infection; however, the miRNA species could be different (Fig. 3, Lanes 1–7). For example, miRNAs of the same size as pathogenic *Pseudomonas* were not present in either *Agrobacterium tumefaciens* or *Rhizobium* spp., although these bacteria could have other forms of small RNAs. In plant host-bacterial relationships, miRNAs are known to have near-perfect complementaries, enabling convincing target predictions of miRNA pairings.[12] In light of such miRNA patterns and the ability of *P. aeruginosa* to infect multiple hosts, this suggests the potential use of plants as hosts for *in vitro* model systems to study human pathogenesis and host responses. A review[20] of cross-species infections previously highlighted the prospect of using plant cells for genetic dissections on 'host responses' to virulent pathogens. Data presented here (Fig. 3) show that non-coding RNAs found in clinical *P. aeruginosa* isolates can be regarded as unique to CF hosts, although structural or size variants can also exist in other *P. aeruginosa* depending on the host and their responses to the invading bacteria.

Computational analysis

The functions of most regulatory ~22 nt RNAs discovered so far are not readily perceived from sequence data alone. Taking into account the nuances of sRNA searches[8] using computational tools, a group in the Tufts School of Medicine[14] recently developed an improved bioinformatics tool (sRNAPredict2) which facilitated identification of *P. aeruginosa* sRNAs. An initial search (data not shown) to match small RNAs reported in the present study using this bioinformatics tool failed to reveal the genomic annotations for these novel miRNA sequences in the intergeneric regions of the pathogenic pseudomonads.

Recently, microRNAs have been discovered across the genome, with over 100 sRNAs isolated and identified in *Escherichia coli* using integrative bioinformatic approaches searching for sRNA-associated encoded regions including promoters and Rho-independent terminators or intergeneric sequence conservation.[15] Fewer examples have been identified in other bacterial species mentioned as they are based solely on primary sequence homology with known *E. coli* sRNAs. However, these small non-coding RNA are now recognised as the major regulatory network of genes in animals, plants and microbes.

The most challenging problems in identifying and understanding miRNA are in uncovering specific 'structural features' of its RNA stem loop. While deciphering the structure is critical, it is not always easy to achieve entirely by complex experimental protocols[16] involving cloning, deep sequencing size fractionated cDNA libraries, with restrictions applicable to single cell type or conditions. This study utilised the directional cloning of miRNAs because success had been achieved using this strategy in previous studies.[9–11] The HHMM approach is useful to ascertain miRNA roles in cellular functions under pathogenic influences in CF lung epithelial tissues.

The computational prediction (HHMM) model (Table 1) indicates that these show high levels of sensitivity and specificity in their folding structures. Apart from being structurally orientated to more than ~70% of the known miRNAs of mammalian descriptions, the miRNAs reported here have a high (~95%) related structural specificity to human bacterial infection (immune) response microRNAs,[17] based on RNAfold searches.

Stem loop predictive structures versus functional significance

The structural specificity of small RNAs is related to biogenesis and for base pairing with their target messenger RNA (mRNA).[18] These small RNAs form duplexes at the 5′ prime end of target mRNA thus influencing the stability and/or altering the access of mRNAs to the translational machinery. A single sRNA can regulate many genes and has telling effects on cell physiology. Some miRNA structure/function information is available for certain fungi,

to a limited extent for bacteria and more recently in Epstein Barr virus.[19]

Quite frequently, such sRNA-miRNA interactive molecular modulations are stabilised by Hfq, an RNA chaperone protein conserved in a number of bacterial pathogens.[1] For example, deletion of Hfq in *Vibrio cholerae, Salmonella typhimurium, Brucella abortus, Yersinia enterocolitica* and *P. aeruginosa* attenuates virulence, and deletion of four functionally redundant sRNAs nullifies the virulence factor in some of these bacteria, suggesting that sRNA modulation is a well-conserved mechanism in bacterial virulence.[13]

Functional significance of predictive structures

The presence and detection of novel miRNA species (Table 1) raises the possibility of significance as regulators of bacterial stress responses. Critical to any discussion on *Pseudomonas* infection, mechanisms such as biofilm formation,[21] quorum sensing[22] and associated *P. aeruginosa* strains, or the genetic elements governing infection of plant or animals by the bacterium, could be expected to differ to those of humans. In plants, stress-related factors (*Pseudomonas* bacterial lipopolysaccharides or flagellin lipopeptides) elicit pathogen-associated molecular patterns (PAMPs) by pattern recognition proteins (PRMs).[23] However, in insects and vertebrates, Toll-like receptors (TLRs) and lysine-rich repeat (LRR) signalling proteins induce an innate immune defence to modulate mRNA gene expression.[20] Using the predictive HHMM model, the present study showed that the miRNA sequences may vary, but they appear to have RNA stem loop structural (Fig. 1) similarities to those of a recently reported human (BiR)-related lipopolysaccharide-induced microRNA, mi-146, associated with the TLR family[17] – primary evolutionarily conserved sensors of PAMPs. Follow-on studies including Northern blot analysis should ascertain endotoxin response gene (*miR-146*) expression in CF cell lines. It is also known[28] that *P. aeruginosa* flagella activate airway epithelial cells through asialoGM1 and TLR2 as well TLR5 through induction of Ca^{2+} fluxes, Src, Ras and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (MAPK) and nuclear factor-κB activation, a pathway previously associated with asialoGM1-mediated stimuli; and the availability of exposed receptors on the apical surface of polarised airway epithelial cells is a major factor in the activation of signalling pathways by bacterial flagella.

Further investigation is required to uncover the nature and role of such miRNAs, which occur unusually in smaller sizes (16–47nt) than previously reported in bacterial systems (80–100 nt). For example, the change in RNA stem loop structural specificity is remarkable when miRNA size increases by as little as 4 nt (Fig. 2). Their significance as regulators of *P. aeruginosa* stress responses in CF, the mechanisms of biofilm and quorum sensing systems[24] that are critical in bacterial pathogenesis and host cell death[20] also requires fuller investigation.

Current update on non-coding RNAs

Intensive study of the changes to host cellular regulatory pathways in response to bacterial stress and/or pathogen response molecules is required. Non-coding RNAs of the type encountered in the present study are rare, and may well be an adaptive response to stressful environments such as the biofilm in CF lungs,[24] infective airway epithelial cells[25] and in other CF bacteria.[26] They could prove critical in the evolution of new therapeutic regimes for clinical application. Most recently, an electronic compilation of putative sRNA and non-coding RNA genes of *P. aeruginosa* PA01 has been reported.[27] However, further work is needed to assess the occurrence of unusually small non-coding miRNAs in CF-associated prokaryotes and their expression levels in relation to environmental variations, given that these regulatory miRNAs have been found in eukaryotes.

Given the considerable advances in the bioinformatics[29] of pathogenic *P. aeroginosa* genome database projects (www.pseudomonas.com) and the tantalising yet diverse correlations drawn between miRNAs modulating tumours[30] and their potential involvement in microbial pathogenesis, future therapeutic evaluations will prove critical. Emerging guidelines[8] will improve the understanding of computational predictions of targets, and help to focus the rules for target complementarity. These new initiatives in miRNAs will enhance the field of laboratory miRNomics to achieve the ultimate goal of clinical applications[18] such as rapid diagnostics and the development of new therapies for *Pseudomonas* infection, thus improving the quality of life in CF sufferers.

Table 1. Computational prediction results of putative miRNAs obtained from clinical *P. aeroginosa*, freshly isolated from the sputum of patients with cystic fibrosis.

Isolate	miRNA sequence	Size	Triplet (UUU) formation (%)	Minimum folding energy (mfe) kcal/mol	HHM MiR model (%) BiR	Sn (%)	Sp (%)	FPR (%)	FDR (%)	BLASTn Mam. miRNA (%)	(RNA fold) Human mi-146 TLR/NF (%)
cfPA3188.2	ATTGTACTCATTCCAA	16	94.5	–2.9	96.5	75	90.0	11.0	13.0	<77	91.0
cfPA3188.3	ATGATATGTTCTACGGAAT	19	94.0	–3.2	97.0	74	89.0	9.0	8.0	<71	92.0
cfPA3188.4	TTCTACCCTTAAATTATAGGCTT	23	94.5	–3.5	95.5	72	91.0	6.5	12.0	80	94.5
cfPA3188.5	TTTTTAACTGCAACAACTTTAAT TGCCTTCCTTGGATGTGGTAGCC	47	95.0	–3.8	96.5	74	94.0	5.0	10.0	66 75	92.0
cfPA3188.10	TTAAATTGTACTCATTCCAA	20	94.5	–3.5	97.0	76	95.0	11.0	9.0	70	94.0

HHMM: Hierarchical Hidden Markov Model; Bi R: Bacterial infection response (human relevance miRNA);
Sn: Sensitivity; Sp: Specificity; FPR: False-positive rate; FDR: False discovery rate; BLASTn: BLAST alignments;
Mam. miRNA: Mammalian microRNAs (e.g., miR1271, miR26).

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