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ORIGINAL ARTICLE

In silico prediction and qPCR validation of novel sRNAs in *Propionibacterium acnes* KPA171202

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Abstract *Propionibacterium acnes* is an anaerobic, Gram-positive, opportunistic pathogen known to be involved in a wide variety of diseases ranging from mild acne to prostate cancer. Bacterial small non-coding RNAs are novel regulators of gene expression and are known to be involved in, virulence, pathogenesis, stress tolerance and adaptation to environmental changes in bacteria. The present study was undertaken keeping in view the lack of predicted sRNAs of *P. acnes* KPA171202 in databases. This report represents the first attempt to identify sRNAs in *P. acnes* KPA171202. A total of eight potential candidate sRNAs were predicted using SIPHT, one was found to have a Rfam homolog and seven were novel. Out of these seven predicted sRNAs, five were validated by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing. The expression of these sRNAs was quantified in different growth phases by qPCR (quantitative PCR). They were found to be expressed in both exponential and stationary stages of growth but with maximum expression in stationary phase which points to a regulatory role for them. Further investigation of their targets and regulatory functions is in progress.

1. Introduction

Propionibacterium acnes is a Gram-positive, non-spore forming, micro-aerophilic, pleomorphic rod shaped opportunistic pathogen with an optimal growing temperature of 37 °C. The bacterium has been found to be involved in a wide array of diseases ranging from acne [25] to prostate inflammation leading to prostate cancer [1,7]. Other diseases in which the bacterium was isolated from the site of inflammation include, rheumatoid arthritis, endophthalmitis, shunt-associated central nervous system infections, endocarditis, sarcoidosis, osteomyelitis, allergic alveolitis, pulmonary angitis, acne inversa and SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome [5]. Colonization by *P. acnes* in the pilosebaceous follicle is a key factor for inflammatory reaction in acne vulgaris [25]. Acne can manifest as a mild comedonal form to chronic inflammatory cystic acne on the face, chest, and back. The antibiotic therapy involves the use of erythromycin, clindamycin and tetracycline for weeks and months resulting in evolution of resistant strains. It thus becomes necessary to look for alternative therapeutics which do not lend themselves to development of resistance. The sequencing of *P. acnes* KPA171202 genome

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by Bruggemann and co-workers [6], leads to the annotation of genes involved in pathogenesis and virulence [4]. It has also brought up an opportunity to identify small regulatory RNAs among the *P. acnes* genome sequences.

Small RNAs (sRNAs) are small regulatory RNAs, occurring in prokaryotes; in addition to the already known messenger (mRNA), transfer (tRNA) and ribosomal (rRNA). Their sizes range from 30 to 600 nts approximately in length and are usually not translated into proteins. These are encoded by intergenic regions (IGRs) of bacterial chromosomes and are transcribed from their own promoters. Their transcription most often terminates at a strong Rho-independent terminator. The sRNAs might be transcribed in *cis* i.e. encoded on the strand opposite to the gene they regulate, or in *trans* i.e. away from the target genes. The *trans* acting-sRNAs act by partial or imperfect base pairing with the target transcripts while the *cis* acting-sRNAs have a region of perfect complementarity with the target transcript. In some cases, the sRNAs are expressed under highly specific growth conditions [10]. MicF was the first *trans*-encoded antisense RNA found in the *Escherichia coli* genome [22,23]. It showed partial and imperfect sequence complementarity to its target ompF (encoding outer membrane protein F) mRNA near the start codon, leading to strong translation inhibition. Classically, sRNAs were defined as short non-coding transcripts that, together with the RNA chaperone protein, Hfq, act in *trans* to control the translation or stability of target mRNAs. This definition was broadened by Liu and Camilli [16], as many coding, *cis*-acting and Hfq-independent sRNAs were also recognized. RNAIII of *Staphylococcus aureus* is a *cis*-acting regulatory RNA that also encodes a virulence factor δ-hemolysin [3]. SymR of *E. coli* is an example of Hfq-independent, *cis*-acting sRNA that represses the translation of symE which encodes for a toxic protein [13].

Binding of sRNA with its target may lead to translational activation or it may also lead to translational repression. These have been involved in the regulation of metabolism, growth processes, adaptation to stress, and pathogenesis of microorganisms [21]. Their regulatory nature also makes them attractive targets for developing nucleic acid based novel therapeutics. There are many approaches to identify and characterize sRNA molecules, their genes and targets in prokaryotes. These include genome-wide searches based on the bio-computational prediction of sRNA encoding genes. First evidence of sRNAs came in 2001 when three different groups [2,29,34] at the same time developed algorithms and identified 31 new sRNA in *E. coli*. Later on genomes of several organisms were explored for the presence of sRNA candidates. Some authors developed their own in silico approaches for sRNA prediction [33,36], while others used various web based tools singularly [28] or a combination of few tools [35]. Some researchers have used genome tilling microarrays for finding new sRNA transcripts and investigating sRNA expression. However, the microarray results need to be validated by northern blots or qPCR and analyzed further by RACE (rapid amplification of cDNA ends); for end-mapping to discriminate novel sRNA genes from leader sequences of genes. Microarrays were used for sRNA identification in *Caulobacter crescentus* by Landt and co-workers [15] and could verify only 27 out of 300 predicted sRNAs. Various other methods include cDNA library synthesis, next-generation sequencing, northern blotting, RT-PCR analysis of predicted genes and co-purification with proteins like hfq.

Present study systematically identified sRNAs in the *P. acnes* genome beginning with computational approach based on gene localization, intergenic sequence conservation, terminator and secondary structure prediction, followed by validation of predicted sRNAs in experimental approach using qPCR. The results indicate existence of seven and validation of five hitherto unreported sRNAs in *P. acnes* which might play a potential role in regulating gene expression and/or pathogenesis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. acnes (strain KPA171202/DSM 16379) was procured form DSMZ Germany (German Collection of Microorganisms and cell cultures). The culture was maintained in Brain Heart Infusion Broth at 37 °C for 48–72 h in anaerobic jar containing 5% CO_2 and on BHI plates supplemented with Vitamin K (10 μg/ml) and Haemin (5 μ/ml) at 37 °C. The growth rate of *P. acnes* in BHI media was monitored spectrophotometrically by observing OD at 600 nm after every 3 h till 78 h. Growth curve was plotted using optical density against time plot (Fig. 1).

2.2. In silico prediction of candidate sRNA genes

Genome sequences of *P. acnes* KPA171202 were downloaded from NCBI server for insilico prediction. Small RNA candidates of *P. acnes* were predicted using the web interface SIPHT (sRNA Identification Protocol using High-throughput Technologies) using default parameters [18]. SIPHT identifies sRNA encoding genes based on intergenic conservation and Rho-independent terminators. Each locus is annotated for many features like sequence conservation in other closely related species, promoter and transcription factor binding site, conserved secondary structure etc that provide information for its potential function. The performance and reliability of the tool was assessed and compared with other algorithms also [20].

2.3. Nomenclature of the sRNAs

Each candidate sRNAs detected by PCR and confirmed by sequencing are indicated by initial "s" (in lowercase), followed by genome identification as used in NCBI database (in uppercase); ending with the number of candidate as predicted by SIPHT. For example: 'sPPAK1' for sRNA of *Propionibacterium acnes* KPA171202 candidate 1.

2.4. Insilico validation of predicted sRNAs

The sequences were scanned for the presence of Shine-Dalgarno (SD) sequence, start codons, stop codons and rho independent terminators. ARNold was used for rho-independent transcription terminator prediction [26]. Secondary structure predictions were carried out using RNAfold program [12] with default parameters. The predicted sRNAs were scanned in Rfam database to check for novelty [11].

Table 1 Sequences of PCR primers used for amplifying sRNA genes.

Primer	Nucleotide sequence	PCR product length (bp)
sPPAK1	FWD-5′ ATCCTCGCGTTCTTACCACC 3′ REV-5′ TTGTTGGGGTCTGGATCTGC 3′	160
sPPAK2	FWD-5′ TGCGGCCCAGTCACCAC 3′ REV-5′ GCCACGAATGAGCGAGTCAG 3′	59
sPPAK4	FWD-5′ CTTACGGCGGGTTCCATC 3′ REV-5′ CAGTGGCTGCTGTGTGTGAC 3′	220
sPPAK5	FWD-5′ GGCAAAGCCCCAAGGCAC 3′ REV-5′ CCTAGCCGCCAAAAGGTG 3′	132
sPPAK7	FWD-5′ GCTGTGGGCCCGACGC 3′ REV-5′ CGAAAAAGCAGAGGCTCTG 3′	194
RecA	FWD-5′ GGCTCTCGAGATTGCTGACA 3′ REV-5′ GAATCACCCATCTCGCCCTC 3′	114

2.5. Reverse transcriptase-polymerase chain reaction

Small RNA fraction was extracted from 36 h culture using RNAzol reagent (Sigma) according to the manufacturer's instructions incorporating minor modifications from small RNA enrichment method of Lu and co-workers [19]. Total small RNA fraction was first polyadenylated using Poly(A) polymerase (New England Biolabs, USA) and then reverse transcribed using oligo dT primer (Thermoscientific Fisher scientific) and Revertaid Reverse Tansranscriptase (Thermoscientific Fisher scientific) according to the manufacturer's protocol. The resulting cDNA was further used for gene specific polymerase chain reaction (PCR) using primers designed by PrimerBLAST as listed in Table 1 and Taq DNA polymerase (New England Biolabs, USA). Mastercycler® (Eppendorf, Hamburg, Germany) was used to perform gradient PCR, with an initial denaturation of 5 min at 95 °C, followed by 30 amplification cycles of 15 s at 95 °C, 10 s at 56–64 °C, and 15 s at 72 °C, and final extension was done for 5 min at 72 °C. The PCR products were visualized by silver staining 15% Polyacrylamide gel after electrophoresis for 2 h at 100 V along with pUC19/MspI digest DNA ruler (Merck). For sequencing, the same products were separated on 3% agarose gel electrophoresis and gel slices were excised for purification using Gel extraction Kit (Merck) according to the manufacturer's protocol. The samples were sent for sequencing to Xcelris Labs Ltd.

2.6. Quantitative real-time PCR

qPCR was performed to check the cellular abundance of the validated sRNA i.e. sPPAK1, sPPAK2, sPPAK4, sPPAK5 and sPPAK7, at different growth phases. *P. acnes* was grown for 36 (Exponential) and 72 (Stationary) hrs OD_{600} respectively. Cells were harvested and used for RNA isolation as described earlier. The polyadenylated and reverse transcribed cDNA aliquots of each sample in equal concentration were used in qPCR reaction mixture containing 5 µl of KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Cape Town, South Africa), 10 pM of each primer (forward and reverse) and final volume was made up to 10 µl with nuclease free water. *RecA* was used as internal control for normalization of gene expression. PCR was run on Mastercycler® (Eppendorf, Hamburg, Germany) with initial denaturation of 5 min at 95 °C and a subsequent run of 30 cycles each comprising 10 s at 95 °C, 10 s at 63 °C, and 15 s at 72 °C, and final extension was done for 5 min at 72 °C. The samples were run in triplicate. The $2^{-\Delta\Delta CT}$ method (relative quantitation) was used in which C_T value (threshold cycle) was normalized to endogenous reference gene *RecA* ($\Delta C_T = C_{T\,\text{target}} - C_{T\,\text{reference}}$) [17].

3. Results and discussion

3.1. In silico prediction of sRNAs in *P. acnes* KPA171202 genome

Potential sRNA candidates were predicted using SIPHT [18]. SIPHT has an automatic workflow based on the intergenic sequence conservation, presence of putative Rho-independent terminators and several other features including primary sequence conservation with previously annotated other regulatory RNAs. SIPHT has been widely used in sRNA prediction studies in other micro organisms like *Fransciella tularensis* [28], *Streptococcus mutans* [35], *Burkholderia pseudomalle* [14], *Mycobacterium smegmatis* [32], and *Brucella abortus* [8]. The complete genome of *P. acnes* KPA171202 was available at NCBI for sRNA candidate prediction in SIPHT web interface.

A total of eight sRNAs candidates were predicted in *P. acnes* KPA171202. Table 2 provides a detailed description of the predicted sRNAs including genomic position, size, GC percentage, Blast expect value, upstream and downstream genes. An additional file of results predicted by SIPHT explains this in detail [see Supplementary data]. However some changes have been made recently in Feb 2015 in the genome annotation of some genes of *P. acnes* KPA171202 by Bruggemann and coworkers (http://www.ncbi.nlm.nih.gov/nuccore/NC_006085.1) which have been taken into consideration during presentation of results.

3.2. Insilico validation

Sequences of the predicted sRNAs were analyzed using different tools. The secondary structure predictions were carried out using RNAfold program. Secondary structures based on the lowest minimum folding energy are shown in Fig. 2, all sRNAs were highly structured containing several stem loops similar to

Table 2 List of sRNAs predicted and their features. SIPHT (sRNA Identification Protocol using High-throughput Technologies) predicted eight sRNA candidates.

sRNA Name	Start/end position	sRNA length	GC content (%)	Up GENE name	Down GENE name	BLAST expect
sPPAK_1	951917–952181	264	57.2	Transposase	Excinuclease ABC subunit A	2.20E−07
sPPAK_2	2174934–2175009	75	61.3	Hypothetical protein	GTP-binding protein TypeA	1.70E−06
sPPAK_3	1372951–1372982	31	70.9	Sensory histidine kinase	RNA polymerase sigma factor RpoE	9.80E−06
sPPAK_4	1583666–1583898	232	61.2	ATP-dependent DNA helicase RecG	Ribonuclease	2.00E−05
sPPAK_5	1719301–1719433	132	56.8	HNH endonuclease	Hypothetical protein	1.00E−05
sPPAK_6	1937987–1938035	48	81.25	Molecular chaperone GroES	Hypothetical protein	1.00E−05
sPPAK_7	2515653–2515851	198	65.6	Glycerol-3-phosphate dehydrogenase	Hypothetical protein	0.0016
SRP_bact	252392–252480	88	67.04	RNA-binding protein S4	Hypothetical protein	9.70E−06

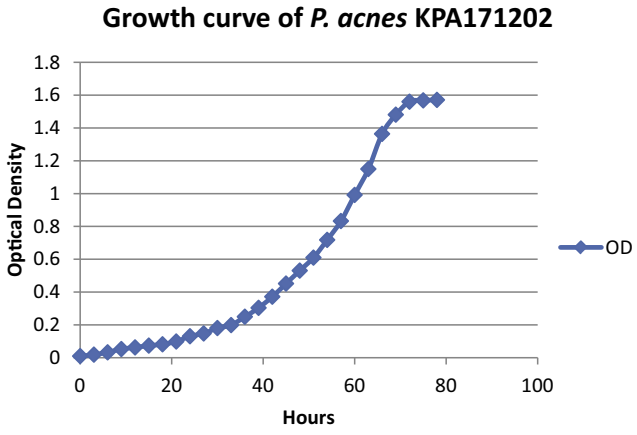

Figure 1 Growth curve of *Propionibacterium acnes* KPA171202.

sRNAs validated in other bacteria [14]. Genomic location and the orientations of sRNA, upstream and downstream genes were also analyzed (Fig. 2). The sequence of sRNA genes was analyzed for terminator prediction. Rho-independent terminators were predicted at the 3′ end using ARNold (Fig. 3). Although, the study aimed to explore sRNAs in the intergenic regions, one of the sRNA: sPPAK7 partially overlaps with the protein coding gene. Recent annotation of the genome of *P. acnes* KPA171202 shows that sPPAK4 completely lies within the 50S ribosomal protein L28 encoding region. However, sPPAK4 sequence on translation using ExPasy translate tool, did not yield any protein. Sequences of the predicted sRNAs have start codons which are immediately followed by stop codons. This indicates that these sRNAs are non-coding in nature.

The candidate sRNAs were scanned against Rfam families (version 10.1) to check the novelty of sRNAs. Out of eight predicted sRNAs one showed homology in Rfam database with a signal recognition particle and Rfam gave no significant hits for other seven candidates indicating that these sRNAs are novel bacterial sRNAs. Further experimental validation was carried out for five sRNAs viz. sPPAK1, sPPAK2, sPPAK4, sPPAK5 and sPPAK7.

The genomic location of the sRNA sheds some light to investigate the functions and targets of the sRNAs. The flanking genes could be possible targets of these sRNAs. In the present study, the upstream flanking genes of sPPAK4, sPPAK5 and sPPAK7 were found to be ATP-dependent DNA helicase RecG, HNH endonuclease and Glycerol-3-phosphate dehydrogenase respectively. All these genes show high sequence complementarity and point to the *cis* regulatory activity of respective sRNAs. Further investigations to prove the same are underway.

3.3. Experimental validation of predicted sRNAs

Out of seven predicted sRNAs, experimentally five sRNAs were tested and validated. Expression of the chosen sRNA candidates was confirmed by RT-PCR. RNA was polyadenylated and reverse transcribed to produce cDNA which was amplified using primers designed specific for sPPAK1, sPPAK2, sPPAK4, sPPAK5 and sPPAK7. The PCR products visualized were 160, 59, 220, 132 and 194-bp bands confirming the presence of sPPAK1, sPPAK2, sPPAK4, sPPAK5 and sPPAK7 respectively among the sRNAs isolated from *P. acnes* KPA171202. The same samples were electrophoresed on 3% agarose gel and PCR products of exact predicted size were obtained. Bands of sPPAK1, sPPAK4, sPPAK5 and sPPAK7 were excised from the gel and were purified for sequencing. The sequencing results were consistent with the predictions and the sequence of sPPAK1, sPPAK4, sPPAK5 and sPPAK7 was submitted to NCBI database with accession numbers [Genbank: KF722796, KP881479, KP881480 and KP218043] respectively.

3.4. Expression analysis by qPCR under different growth phases

It is well established that sRNA gene expression is growth phase and stress related. The cellular abundance of each validated sRNA was checked at different growth phases to monitor their biosynthesis. The transcript level of all the sRNAs, identified in this study was found to be most abundant in stationary growth phase (Fig. 4). A number of sRNAs of gram positive pathogens are growth phase dependent.

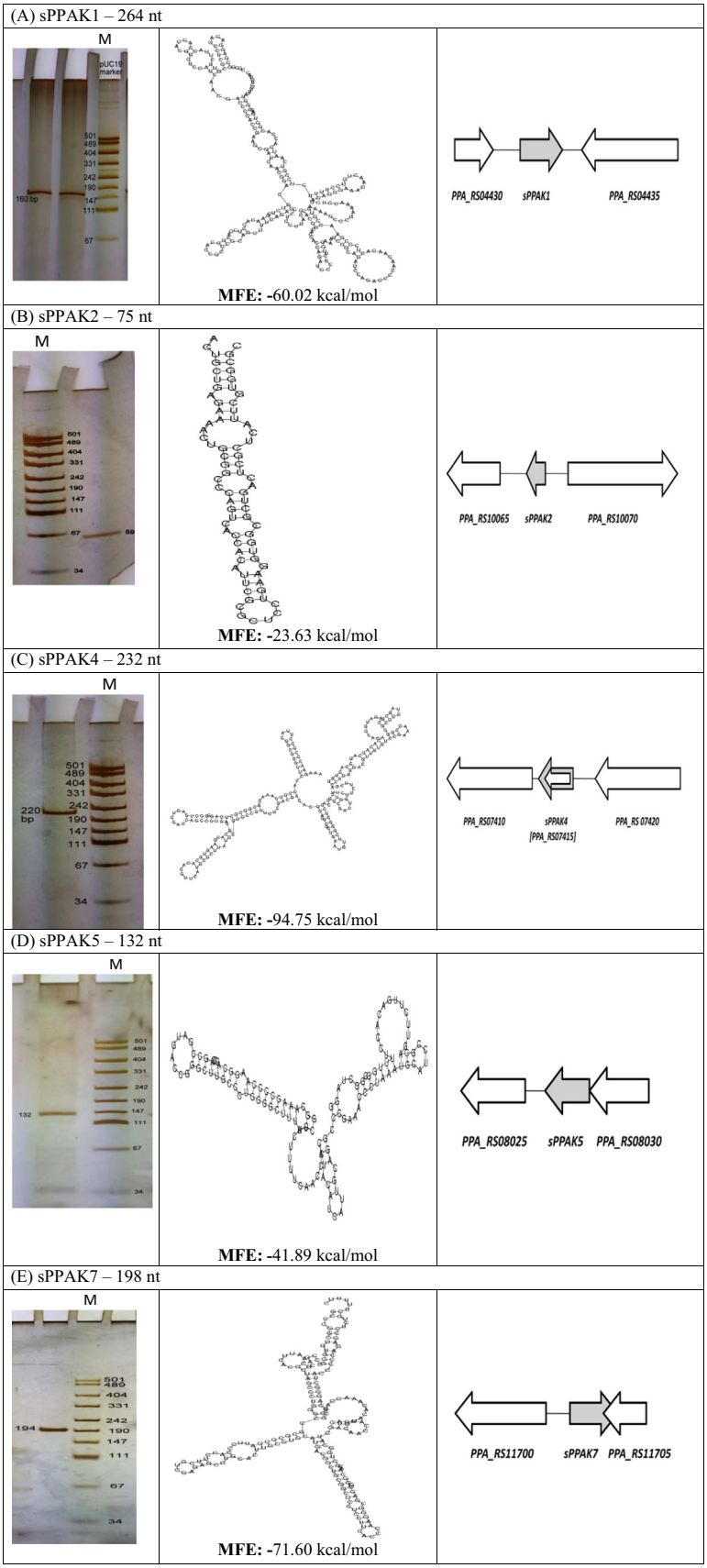

Figure 2 Experimental detection, secondary structure and genomic orientation of five selected sRNAs of *Propionibacterium acnes* KPA171202. (a) sPPAK1, (b) sPPAK2, (c) sPPAK4, (d) sPPAK5, (e) sPPAK7. The left column shows RT-PCR results of PCR amplicons run on 15% PAGE with pUC19/Msp I digest as marker (Lane marked as 'M'). The middle column displays secondary structures predictions performed using RNAfold with lowest minimum folding energy. The right column shows genomic orientation of the validated sRNAs. The gray arrow represents sRNA and white arrows are upstream and downstream genes; the direction of arrows shows orientation.

sPPAK1- 264 nt

+1 *5′-start*

GCCTGGCCGCGGGCGGTCTGCAAGCTTGGCCGGGTCCTGACCAAG**CGCGGCGAGGACATCCTCGCG**
TTCTTACCACCACGGCTCCAGCAACGACCCCACCGAAGCGATCAACGGACGTCTCGAACACCTCCAC
GGCACCGCCCGCAGCTTTCAGACCCTCCTTACCCACGGCACCACCAGATCCCTCTTGGAAATCGGCA
GATCCAGACCCCAACAACACTCCCGAACCGTGAAAAGCCCGAAAACCTCTAGAGGGAAATAACTGT
CCTTTTCTCCGTCAATCACCACGGTGACGGGTATTTTCTTTACCCTAAAGCAGC

3′-end +264

sPPAK2- 75 nt

+1 *5′-start*

TGCAAACACACCACAGTGAAGTGTAGGTCAGGGCAGGGTT**GCGCCACGAATGAGCGAGTCAGCGCC**
ACCTTCAGGAGCGCGAATGTGGTGACTGGGCCGCAGTTTTCTCAGCAGTCGTGTGGGTTCC

3′-end +75

sPPAK4- 232 nt

+1 *5′-start*

GCGTCGGGTGCATCTTGCACCCGGCCCTGAACAGGAGATCTC**CCAGTGGCTGCTGTGTGTGACATCT**
GCGCTAAGCGCCCCGGTTTCGGACACAACGTCCCCTGGTCGAAGAAGAAGACCAATCGCCGGTGGA
GCCCGAACATTCAGCGCGTCCGTGTCGTCGAGAACGGCACGCATAAGCGCATGAACGTGTGCACTT
CGTGCCTCAAGGCTGGTCGAGTCAGCCGCTGAGCGCCGCGTTTGACGATGGAACCCGCCGTAAGGC
GGGTTCTTTGTCGTTATGGGTCGAT

3′-end +232

sPPAK5- 132 nt

+1 *5′-start*

GCCCGGCTGGCGTCGGCGCTGTATGGGCAGGCCGCGTGAA**CCTAGCCGCCAAAAGGTGTCAAGAAC**
TGCGGATGCATTTAGGGTTTCGGGCCTGCAATGATGTAGGTGGTTGAAAAGTAGGAAAAGCCCCAG
GGCAAGCCCGGTCATCGGCTGGGTGCCTTGGGGCTTTGCCGTGTCCGGGAAAGGGG

3′-end +132

sPPAK7- 198 nt

+1 *5′-start*

CTGCAATCCTGAGCATCTTCGTACCTCCGAAGATGGGTTGAG**GCCCGGCTGTGGGCCCGACGCAATT**
CACGCTAGCCCTGGGCCGCGCGCGATTCCCACCTTCGTCGAGAGCCTGCACTTGCGTGCATGACGGC
TGCGGCTCCTCCTTCACCCAAGGGCGAGCTCCGCAACGCTGGCATCGACAACCAGGTCAGTACACAA
ACCACCGCGCAGGGCTACCCCCAGAGCCTCTGCTTTTTCGGCTCCGGATGCGAC

3′-end +198

Figure 3 Sequence analysis of validated sRNAs. The sequence shown in bold letters is sRNA sequence and unbold letters show upstream and downstream sequences. The region highlighted in green and yellow shows start and stop codons respectively. The region highlighted with gray color depicts putative rho independent terminator. 5′ and 3′ start and ending sites respectively are as predicted by SIPHT.

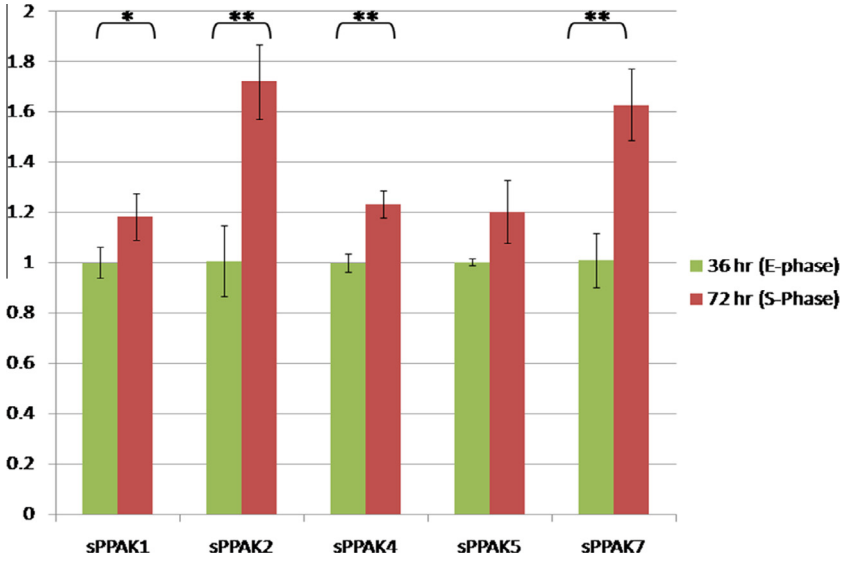

Figure 4 qPCR detection the transcript levels of validated sRNAs under different growth phases. Statistical significance ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$) was obtained using Anova test.

In *S. aureus*, 11 novel growth phase dependent sRNAs were validated, many of them accumulated in the late-exponential phase of growth and responded to acid stress [9]. Shioya and co-workers [30] experimentally characterized 11 sRNAs in *Enterococcus faecalis* V583 out of which, six sRNAs were specifically expressed at exponential phase, two sRNAs were observed in stationary phase, and three were detected during both phases. Expression of twenty-four sRNA genes of *Streptococcus pyogenes M49*, were also regulated in a growth phase and/or medium dependent fashion [27]. In *Clostridium difficile* expression of six sRNAs was growth phase dependent out of which three sRNAs (RCd4, RCd5 and SQ1002) were induced at the onset of stationary phase, whereas the expression of three others (RCd2, RCd6 and SQ1498) was high during exponential phase and decreased at the onset of stationary phase. It was proposed that some of these growth phase regulated sRNAs are involved in the control of virulence determinants and associated factors which are also growth phase dependent [31]. In the case of the present study, the five validated sRNAs appear during both exponential and stationary phases of growth but these are found to be maximally expressed during stationary growth phase. These results provide a starting point toward understanding of complex sRNA-based regulatory network. Further transcriptome based investigations will help to understand the molecular mechanism and biological pathways regulated by these sRNAs.

4. Conclusions

This report presents the first study of small non-coding RNAs reported so far on *P. acnes* KPA171202. Eight sRNAs were predicted by SIPHT in *P. acnes*, out of which one showed homology in Rfam database with a signal recognition particle. Seven sRNAs thus were selected as putative regulatory molecules. The experimental validation was carried out successfully by RT-PCR for five sRNAs. These sRNAs were further analyzed and found to be maximally expressed under stationary growth phases. Their growth phase dependence point to their regulatory nature, with a probable role in pathogenesis, host pathogen interaction, environmental stress coping mechanisms and other physiological pathways. Further studies to gather such information are being carried out. The unique nature of sRNAs can be exploited for the development of novel diagnostic tools and therapeutic interventions such as antisense PNAs (peptide nucleic acids) as anti-bacterial drugs [24].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jgeb.2016.03.002.

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