Identification of novel small RNAs in *Burkholderia cenocepaica* KC-01 expressed under iron limitation and oxidative stress conditions

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**Abstract**

Small RNA (sRNA)-mediated regulation of gene expression is a major tool to understand bacterial responses to environmental changes. In particular, pathogenic bacteria employ sRNAs to adapt to the host environment and establish infection. Members of the *Burkholderia cepacia* complex, normally present in soil microbiota, cause nosocomial lung infection especially in hospitalized cystic fibrosis patients. We sequenced the draft genome of *Burkholderia cenocepaica* KC-01, isolated from the coastal saline soil, and identified several potential sRNAs *in silico*. Expression of seven small RNAs (Bc_KC_sr1–7) was subsequently confirmed. Two sRNAs (Bc_KC_sr1 and Bc_KC_sr2) were upregulated in response to iron depletion by 2,2'-bipyridyl and another two (Bc_KC_sr3 and Bc_KC_sr4) responded to the presence of 60 µM H2O2 in the culture media. Bc_KC_sr5, 6 and 7 remained unchanged under these conditions. Expression of Bc_KC_sr2, 3 and 4 also altered with a change in temperature and incubation time. A search in the Rfam and BSRD databases identified Bc_KC_sr4 as candidate in *B. pseudomallei* D286 and assigned Bc_KC_sr5 and 6 as tmRNA and 6S RNA, respectively. The novel sRNAs were conserved in *Burkholderiaceae* but did not have any homologue in other genera. Bc_KC_sr1 and 4 were transcribed independently while the rest were part of the 3’ UTR of their upstream genes. TargetRNA2 predicted that these sRNAs could target a host of cellular messages with very high stringency. Intriguingly, regions surrounding the translation initiation site for several enzymes involved in Fe–S cluster and siderophore biosynthesis, ROS homeostasis, porins, transcription and translation regulators, were among the suggested putative binding sites for these sRNAs.

**INTRODUCTION**

It is now abundantly clear that a major pathway of post-transcriptional gene regulation involves interaction between ‘small’ non-coding RNAs and their target protein-coding RNAs. These small RNAs (sRNAs) are 50–500 nucleotides long [1] and their activity can result in processing of the target RNA or regulation of its translation [2–9]. In most bacteria, trans sRNAs require Hfq, an RNA chaperone to help them interact with their targets [10]. Hfq-mediated sRNA–target RNA interaction occurs through the Hfq-mediated modification of the RNA secondary structure which facilitates the base-pairing interaction between sRNA with its target RNA [11]. Additionally, the sRNAs can mimic the secondary structure of nucleic acids at which certain regulatory proteins bind. 6S sRNAs inhibit transcription by soaking up the available RNA polymerases by mimicking the open promoter structure [12–14]. CsrB/C sRNAs display multiple CsrA binding sites thereby occluding CsrA from its natural sites of action [15]. tmRNAs rescue stalled ribosomes by providing both tRNA- and mRNA-like functions [16]. Reports are abound that document the pivotal role of sRNAs in the metabolic management of bacterial systems under both normal and stress conditions, including iron-limited environment [17], anaerobic conditions [18, 19], oxidative stress [20, 21], cell envelope stress [22, 23], high glucose-6-phosphate concentrations [24, 25] and glucose starvation [26–28].

Iron acts as a cofactor for a variety of enzymes involved in Krebs’ cycle, electron transport and oxidative stress [29]. Ferric iron found in aerobic environments is insoluble [30] and the concentration of available ferrous iron is extremely limited due to the presence of multiple iron-binding
proteins and enzymes [31–33]. In *E. coli*, the *ryhB* sRNA plays a central role in regulating the expression of transcripts of iron storage proteins, TCA cycle enzymes, succinate dehydrogenase and superoxide dismutase [34]. Several members of the Enterobacteriaceae also share *ryhB* homologues [35]. Iron-responsive sRNAs are also found in several bacterial species including *prrF1* and *prrF2* in *Pseudomonas aeruginosa* [36], *arrF* in *Azotobacter vinelandii*, [37] and *NrrF* of *Neisseria meningitidis* [38–40]. However, intracellular iron level and factors involved in its homeostasis would also influence the oxidative stress response in bacteria because of the Fenton chemistry for the generation of intracellular hydroxyl radicals and hydroperoxyl radicals. During infection also, pathogens are inundated with superoxide ions and peroxide synthesized by phagosomal membranes of macrophages. Bacteria belonging to *Burkholderia cepacia* complex (Bcc) survive intracellularly in monocytes and macrophages and endure an oxygen-dependent host defence mechanism [41]. Documented examples of oxidative stress management through sRNA in *E. coli* include *ryba* [42], *oxyS* [21] and the sRNAs already implicated in iron metabolism, *ryhB-1* and its paralogue *ryhB-2* [43]. In the purple bacterium *Rhodobacter sphaeroides*, four homologous sRNAs (CcTsR1-4) putatively increased the availability of reduced GSH, a major scavenger of ROS [44].

Bcc is a group of closely related Gram-negative Betaproteobacteria, consisting of 18 species [45]. Bcc has drawn serious scientific attention since their isolation as the causative agent of nosocomial infection in the respiratory tracts of patients with cystic fibrosis [46, 47]. Treatment of these patients is difficult as Bcc are intrinsically resistant to the majority of commonly used antibiotics [48, 49]. Several sRNA genes have already been identified in *B. cenocepacia*, one of the predominant *Burkholderia* species infecting humans. An *in silico* approach identified 213 putative non-coding sRNA genes in the *B. cenocepacia* J2315 genome of which the expression of four was confirmed by microarray hybridization [50]. Another study showed transcriptomic overexpression of 19 intergenic regions of J2315 upon chlorhexidine treatment, but none of them had a significant match to the sequences in the RFAM database [51]. An identification strategy based on co-purification with Hfq, identified 24 sRNAs in J2315, of which three were exclusive to Bcc and the rest shared homology with other organisms [52]. Recently, a genome-wide transcription start site profiling of J2315 identified 15 non-coding sRNAs highly expressed in biofilms [53]. Unfortunately, even this limited number of identified sRNAs in *B. cenocepacia* has not been experimentally addressed to their possible targets.

In the present study, we explored *B. cenocepacia* KC-01, a natural isolate from the coastal saline soil from the Sunderbans in India, for its display of sRNAs specifically expressed under iron limitation and in the presence of peroxide. We have sequenced the genome of *B. cenocepacia* KC-01 (accession number NZ_AWOP00000000.1) and searched for non-coding sRNA through _sRNA_ identification protocol using high-throughput technology (SIPHT) [54]. Experimental validation was carried out in different conditions including iron depletion, oxidative stress and variations in growth temperature. We have experimentally verified seven sRNAs of which four were novel. 6S RNA, tmRNA and a previously identified sRNA (candidate738_SIPHT) were the other sRNAs identified in our analysis. Two distinct sets of sRNAs were upregulated under iron depletion and oxidative stress. These sRNAs were predicted to bind to regions surrounding translation start sites of several important enzymes involved in iron metabolism, oxidative stress management and translation.

**METHODS**

**Growth conditions**

Generation time of KC-01 cells grown in LB broth at 37 °C under constant shaking at 250 r.p.m. was 80 min. For iron-depletion experiments, a 300 µM concentration of 2,2'-bipyridyl [55] was chosen from a range of 100–500 µM at 37 °C since growth was inhibited beyond 300 µM. 50 ml of LB broth was inoculated with 0.1 % of overnight culture of KC-01 and shaken at 250 r.p.m. at 37 °C till the OD₆₀₀ reached 0.2. Then, 2,2'-bipyridyl was added and shaking was continued for a further 160 min (two generation times) with aliquots taken out at regular intervals. For induction of oxidative stress, a final concentration of 60 µM H₂O₂ [21] was added to exponentially growing KC-01 cells at 37 °C. Altogether, 20 ml of LB broth was inoculated with 0.1 % of overnight culture and shaken at 250 r.p.m. at 37 °C till the OD₆₀₀ reached 0.5. The culture was harvested after 5 and 15 min of H₂O₂ addition. For studies on the expression pattern of sRNAs at different temperatures, 20 ml of LB broth was inoculated with 0.1 % of overnight culture and shaken at 250 r.p.m. at 37°, 50° and 25°C. Aliquots of the bacterial culture were harvested after the fifth, sixth, seventh, eighth and ninth hour.

**Target prediction of small RNAs**

For the prediction of mRNA targets of novel sRNAs of *B. cenocepacia* KC-01, online algorithms like IntaRNA [56] and TargetRNA2 [57] were used with their default parameters.

**Total RNA isolation**

Bacterial cultures were centrifuged at 5000 g for 5 min at 4°C. RNA was isolated using Trizol reagent. Cells were lysed by pipetting several times and were centrifuged at 12000 g for 10 min at 4°C. Chloroform was next added to the supernatant, shaken vigorously for 15 s and incubated for 15 min at room temperature. Following centrifugation for 15 min at 12000 g for 15 min at 4°C, RNA was precipitated by 100 % isopropanol at room temperature. Pellet was air dried and resuspended in RNase-free water. Trizol, chloroform and isopropanol was added at a ratio of 5 : 1 : 2.5.
Northern blot
In total, 30 µg of DNase-free RNA were run on 8 % denaturing urea polyacrylamide gel using 0.5X TBE buffer. Gel was transferred on a Brightstar Plus nylon membrane at a constant current of 5.5 mA cm⁻² for 1 h and UV cross-linked at 120 mJ cm⁻². Uniformly labelled RNA probes were synthesized in vitro using [α-³²P]UTP with either T7 or SP6 RNA polymerase on the M13 PCR product which was performed using pGEM-Teasy plasmids with respective inserts as the template. The size of the RNA probes for Bc_KC_sr1, 2, 3, 4, 5, 6 and 7 was 295, 315, 282, 319, 391, 306 and 339 nucleotides, respectively.

5’ rapid amplification of cDNA ends (RACE)
Overall, 10 µg RNA was reverse transcribed using a gene-specific primer, Protoscript RT, 10x DTT, Ribolock and 10 mM dNTPs were taken in 20 µl reaction and incubated at 42 °C for 90 min followed by heat inactivation at 65 °C for 20 min. Gene-specific primer sequences are given in Table S1 (available in the online version of this article). Terminal transferase with CoCl₂ as the cofactor and 2.5 mM ATP was used to add poly dA at the 3’ end of cDNA in the reaction. The reaction mix was incubated at 37 °C for 1 h. PCR reaction was carried out using the gene-specific nested primer and the oligo(dT) anchor primer.

RT- PCR and RT-qPCR
Reverse transcription was carried out on 1 µg of DNase-treated RNA at 42 °C for 90 min with Protoscript II (NEB) and RNase inhibitor (NEB) in a reaction mixture of 20 µl. Then, 2 µl of this cDNA was used for PCR reaction. Primers are listed in Table S1.

RT-qPCR experiments were employed to analyse the expression levels of sRNAs against 5S rRNA as the reference gene. Only those sRNAs that generated a slope of less than 0.1 in ΔCₜ versus the log(RNA concentration in ng) plot was used for quantitation by the 2⁻¹ΔΔCₜ method (ΔCₜ=ΔC but between sRNA and 5S rRNA) [58]. The sRNA expression level in KC-01 grown at 37 °C for 5 h was used as a calibrator. The reactions were performed in triplicate with three biological replicates. qPCR reaction was carried out with cDNA from 10 ng of the total cellular RNA in each well. The primers used for amplification are specified in Table S1. The fluorescence of SYBR Green (Dynamo ColorFlash SYBR Green qPCR Kit, Thermo) was captured in the ABI StepOne instrument and analysed.

Determination of the transcription pattern of sRNAs
Overall, 2 µg of total RNA was reverse transcribed using Protoscript II (NEB) as described above. Next, 1 µl of reverse transcription mix was used for each PCR. Either a forward or reverse primer was positioned in the neighbouring gene with the cognate primer on the candidate sRNA to detect transcripts harbouring sRNA in the 3’ or 5’ UTR of the adjacent genes. In every set, transcription of the sRNA and its neighbouring genes were ascertained by individual RT-PCR experiments with appropriate controls. No DNA (negative) and genomic DNA (positive) controls were run for every PCR.

RESULTS
Identification of candidate sRNAs in B. cenocepacia KC-01
Analysis of the draft genome sequence of B. cenocepacia KC-01 predicted a total of 5306 coding regions (2748 genes transcribed from the positive strand and 2558 from the negative strand), of which 4070 (77 %) could be functionally annotated. The genome-coding density in KC-01 is 85 % with an average gene length of 896 bp. The annotated genome has 108 genes responsible for metabolism of aromatic compounds, including 72 genes for metabolism of central aromatic intermediates. A total of 141 genes are present for the stress response, including 30 for osmotic stress, 79 for oxidative stress and 18 for detoxification. A MLSA tree generated from 16 housekeeping genes depicting the phylogenetic relationship of B. cenocepacia KC-01 to other closely related strains is presented in Fig. S1.

To find the sRNAs in B. cenocepacia KC-01, we initiated our search by first compiling a library of predicted sRNAs in all the published genomes of Burkholderia using SIPHT. The algorithm generated output files for 7144 predicted sRNAs and from them, we initially selected 279 sRNA candidates. This selection was based on several criteria, viz., high BLAST score of intergenic conservation, low BLAST E-value, concordance between different terminator predictions, etc. These candidate sRNAs were then searched for in the KC-01 draft genome distributed in 283 contigs (NCBI accession: NZ_AWOP00000000.1). Our search generated 109 sRNA candidates within the KC-01 contigs that demonstrated >70 % sequence identity with the predicted sRNAs. Since these 109 candidates contained representations of the same sequence in different contigs of the draft genome, we sorted them into 30 distinct candidate sRNA sequences. We further pruned this list of the sRNAs shorter than 60 nucleotides, and finally proceeded with 17 candidates to investigate their expression under iron-depletion and oxidative stress conditions. Therefore, our experimental approach ensured that only the sRNAs expressed under these conditions were selected for further analysis.

Validation of expression of sRNAs under iron depletion
Exponentially growing KC-01 cells were depleted of ferrous iron by 300 µM of 2,2’-bipyridyl for up to 160 min (two generation times). Initial screening by non-quantitative RT-PCR analysis indicated expression of nine out of the 17 sRNAs upon iron depletion. Northern blot analyses demonstrated significant upregulation of two sRNAs (Bc_KC_sr1, 83 nt and Bc_KC_sr2, 129 nt) compared to when iron was abundant in the medium or in cells treated with EtOH, the solvent for the chelator (Fig. 1). The expression levels of the two sRNAs reached their maximum within 120 min. The
Northern blot for Bc_KC_sr2 also indicated that a longer RNA of 139 nt crossreacted with the probe. However, five other sRNAs (Bc_KC_sr3, 244 nt; Bc_KC_sr4, 162 nt; Bc_KC_sr5, 370 nt; Bc_KC_sr6, 175 nt; and Bc_KC_sr7, 167 nt) were also expressed but their levels remained similar to iron-abundant growth conditions.

Expression of sRNAs under oxidative stress

To induce oxidative stress in the KC-01 cells, 60 µM H₂O₂ was added to exponentially growing culture and incubated for up to 15 min. We observed that both Bc_KC_sr1 and Bc_KC_sr2 showed only a moderate increase while Bc_KC_sr3 showed a more pronounced expression upon H₂O₂ treatment (Fig. 2). Nonetheless, the most dramatic increase was observed in Bc_KC_sr4 expression, as shown in Fig. 2(d). We also noted that Northern blot revealed multiple RNA fragments being recognized simultaneously by the probes against both Bc_KC_sr3 and Bc_KC_sr4. While Bc_KC_sr3 was the longest amongst the RNAs recognized by the probe, both longer and shorter RNA fragments hybridized to the Bc_KC_sr4 specific probe. Therefore, it remains possible that both the sRNAs are subject to degradation or themselves could be the product of degradation of other RNAs. Similar to iron-depletion experiments, Bc_KC_sr5, Bc_KC_sr6 and Bc_KC_sr7 did not show any alteration in their expression when H₂O₂ was introduced in the media.

Apart from these seven sRNAs, two additional sRNAs were detected in non-quantitative RT-PCR experiments (Fig. S2). However they were undetectable in Northern blot analyses. Since the absence of Northern blot data precludes an estimate of their actual length and also indicates a low level of expression, we decided against any further analysis of these two sRNAs. For the seven sRNAs, we checked whether transcription was strand-specific or both strands were being transcribed. Northern blotting could not detect any hybridization signal with probes targeting the opposite strand of the expressed sRNA sequences.
Expression profiles of novel sRNAs under different temperatures

We checked the temporal expression pattern of five sRNAs (Bc_KC_sr1, 2, 3, 4 and 7) under different temperatures (Fig. 3). Since Bc_KC_sr5 and Bc_KC_sr6 were homologous to already well-characterized sRNAs (see the section on the homology search of candidate sRNAs), we concentrated only on these five sRNAs for subsequent characterizations. From an overnight culture of cells, KC-01 cells were subcultured at three different temperatures at 37°C, 30°C and 25°C for up to 9 h. However, the quantitation of Bc_KC_sr1 and Bc_KC_sr7 by RT-qPCR was not possible since these sRNAs displayed a slope greater than 0.1 in the ΔCt versus log (RNA concentration) plot. Non-quantitative RT-PCR reactions detected the expression of BC_KC_sr1 and Bc_KC_sr7 at all temperatures (Fig. 3a). However, we noted the absence of Bc_KC_sr7 at 5 h at 25°C. In Fig. 3b, we depicted RT-qPCR quantitation of the other three sRNAs. Bc_KC_sr2 expression level showed a marked increase (3.5-fold) at 9 h at 30°C. Its expression, however, was low at 25°C. The Bc_KC_sr3 level was slightly upregulated with decreasing temperature and was maximum (2.5-fold) at 30°C at 5 h. However, prolonged incubation diminished its expression at all temperatures. Bc_KC_sr4 expression was similar at all temperatures at 5 h but similar to Bc_KC_sr3 decreased with increasing incubation time across the studied temperature range.

Determination of the 5’ end of expressed sRNAs by 5' RACE

For Bc_KC_sr3 and Bc_KC_sr7, our 5’ RACE attempts were unsuccessful and the 3’ ends of these sRNA genes were taken as the last T in the T-stretch following a hairpin in the candidate locus and the 5’ end was surmised as per length obtained from Northern blot. The 5’ ends of the other five

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Fig. 2. Northern blot analysis for expression of sRNAs under oxidative stress. Bc_KC_sr1 (a) and Bc_KC_sr2 (b) showed only a moderate increase after the fifth and fifteenth minute of treatment with 60 µM H2O2 compared to that of the control (no H2O2 added). H2O2 was added when KC-01 cells were at an OD600 value of 0.5 at 37°C. Similar to Fig. 1(b), the probe against Bc_KC_sr2 recognized an additional longer RNA. Bc_KC_sr3 (c) increased with H2O2 exposure. However, increase in Bc_KC_sr4 level (d) was most significant and reached a maximum within 5 min of H2O2 exposure. Bc_KC_sr5 (e), Bc_KC_sr6 (f) and Bc_KC_sr7 (g) levels remained unaltered. RNA loading was normalized by levels of 5S rRNA. Arrows indicate the position of the sRNA. In (g), the impression of Bc_KC_sr5 remained in the blot even after repeated stripping. One blot was repeatedly probed for all sRNAs except for (d), which came from a separate blot.
sRNAs have been successfully determined by 5' RACE and their 3' ends were deduced from its length calculated from the Northern blot and the presence of stem-loop forming sequences followed by stretch of uridines. The sequences of the sRNAs are shown in Table 1.

**Homology search of candidate sRNAs**

When we searched for possible structural homologues of the identified sRNAs (Table S2), the RFam database [59] revealed Bc_KC_sr4 is homologous to Bp_1_candidate_738_SIPHT in the RF02422 family, a sRNA previously identified in *B. pseudomallei* D286 [60]. Bc_KC_sr5 was identified as the transfer messenger RNA (tmRNA) of the RF00023 family and a high stringency search in the GLASSgo (http://rna.informatik.uni-freiburg.de) platform to identify homologous sRNAs in proteobacteria indicated that Bc_KC_sr5 (tmRNA) has homologous sRNAs in *Ralstonia solanacearum*, *R. mannitolilytica* and *R.eutropha*. Bc_KC_sr6 was identified as 6S RNA in the Bacterial Small RNA Regulatory Database (BSRD) [61] and its homologues were also found in different bacteria. We could not find any homology for the remaining four sRNAs in RFam, BSRD and GLASSgo platforms and therefore concluded that these four sRNAs are unique to *Burkholderia* species and possibly regulate bacterial metabolic response under specific environmental challenges.

**Location of the sRNAs in the *B. cenocepacia* KC-01 genome and their transcription patterns**

We next compared the predictions from SIPHT with the experimentally verified data for these sRNAs (Table S3). The sRNA sequences and their neighbours were conserved among *B. cenocepacia* strains MC0-3, HI2424, AU1054, ST32, H111, J2315, DWS37E-2 and DDS22E-1. An independent transcription unit at a minimum distance of 70 nt from both the neighbouring 5' and 3' ORFs was set as a criteria for 'intergenic' sRNAs. Such selection criteria put Bc_KC_sr1, 4 and 6 in the intergenic category, Bc_KC_sr2 in the 3' UTR and Bc_KC_sr7 in the 5' UTR categories. Bc_KC_sr5, the putative tmRNA, is antisense to the 3' UTR of phosphoglycolate phosphatase. The neighbourhood of Bc_KC_sr3 needs separate attention. The 5' end of this sRNA is within the gene-encoding tRNAVal and its 3' end overlaps with the 5' end of a hypothetical protein. Accordingly, we designated this as part of mRNA transcribed from the entire genomic location. We hypothesize that Bc_KC_sr3 is generated from processing of a longer mRNA transcript.

We verified these predictions by non-quantitative RT-PCR experiments (Fig. 4). For determination of 3' UTR, we designed the forward primer to bind to the upstream ORF and the reverse primer to the candidate sRNA gene. Conversely, forward primers spanning the candidate sRNA and reverse primers annealing to downstream ORF were used for RT-PCR verification of being in the 5' UTR of the downstream gene mRNA transcript.

Concordant to their assignment as intergenic sRNAs, Bc_KC_sr1 and sr4 were found to be unique transcription units. The other three small RNAs tested were synthesized as the UTR of either its upstream or downstream or both flanking ORFs. However, we did not ascertain the absence of any independent promoter activity in their upstream sequences. Bc_KC_sr2 was transcribed as the 3' UTR of its upstream ORF, AphC/TSA family protein/peroxidase. Given the very close proximity of this sRNA to the upstream ORF (21 nt) and absence from the downstream gene transcript, its assignment to the 3' UTR appears valid. Northern blot analysis of its expression during both iron depletion and H2O2 assault also depicts the presence of a slightly larger (139 nt) fragment alongside Bc_KC_sr2 (see Figs 1b,2b). The presence of such a doublet could indicate processing from a larger transcript. It is also interesting to note its presence in the 3' UTR of a peroxidase. Since Bc_KC_sr2 is also upregulated in the presence of H2O2, we are tempted to speculate its involvement in oxidative stress mitigation. Bc_KC_sr3, on the other hand, is part of a large transcript that spans both its upstream and downstream ORFs. As already discussed, the 5' end of Bc_KC_sr3 is within the tRNAVal gene and a hypothetical protein overlaps with its 3' end. For Bc_KC_sr7, RT-PCR signal was detected with both its upstream and downstream neighbours. A strong RT-PCR signal for its existence in the 3' UTR of the upstream gene was observed notwithstanding a 94 nt

![Fig. 3](image-url)

*Fig. 3.* Expression profile of sRNA candidates in different temperatures in the absence of iron limitation or oxidative stress. From an overnight culture, *B. cenocepacia* KC-01 cells were diluted in LB medium and were grown at specified temperatures. Aliquots were taken out at indicated times and total RNA was isolated and non-quantitative RT-PCR or RT-qPCR experiments were carried out. (a) Non-quantitative RT-PCR experiments for Bc_KC_sr1 and Bc_KC_sr7 reveal their expression at all temperatures. Bc_KC_sr7 was not detected after 5 h incubation at 25 °C. 55 rRNA was used as the loading control in the RT reaction. (b) RT-qPCR experiments demonstrate that expression levels of Bc_KC_sr2, 3 and 4 were dependent on incubation time and temperature. The 2-ΔΔCt method was followed with expression at 37°C for 5 h taken as the calibrator and 5S rRNA as the housekeeping gene. Bc_KC_sr2 showed a strong stimulation of expression at 9 h at 30°C. For all sRNAs, expression levels generally diminished with longer periods of incubation.
separation between them. However, even though it is located within 41 nt of its downstream ORF, the RT-PCR signal for its presence in the 5′ UTR is weak. We hypothesize that a small fraction of the elongating RNA polymerases escape the Bc_KC_sr7 intrinsic terminator and moves on to the downstream gene. A long transcript similar to Bc_KC_sr3 is not detected in this case. To summarize, Bc_KC_sr1 and Bc_KC_sr4 are independent transcription units while Bc_KC_sr2, 3 and 7 are nestled in the 3′ UTR of the upstream genes, even though their transcription from individual promoters is not explicitly ruled out. A cartoon depicting the sRNAs with direction of transcription of their surrounding ORFs is shown in Fig. 5(a). We used RNAfold web server to predict the secondary structures of these novel sRNAs (Fig. 5b). All sRNAs displayed structures with extensive intramolecular base-pairing characteristics of small regulatory RNAs.

**Predicted targets of novel sRNAs**

Since transcription of these loci took place from one strand of DNA only, as evidenced by the absence of any signal from the antisense strand in Northern blots, the sRNAs generated from these regions cannot act in cis to regulate expression of their neighbouring genes. We performed a computational target prediction for possible mRNA targets with IntaRNA and TargetRNA2 for Bc_KC_sr1, 2, 3, 4 and 7 using the default parameters in these algorithms. This computational search was carried out considering all the annotated ORFs in the B. cenocepacia MC0-3, J2315, HI2424 and AU 1054; B. thailandensis MSMB121 and E264; B. mallei NCTC 10247, ATCC 23344, SAVP1, NCTC 10229; B. pseudomallei 1106a, MSHR346, MSHR305, 668, BPC006, 1710b, K96243, 1026b; B. xenovorans LB400; B. ambifaria AMMD, MC40-6; B. multivorans ATCC 17616; B. phenoliruptrix BR3459a; B. vietnamiensis G4 and B. cepacia GG4. TargetRNA2 predicted a large number of possible targets for these sRNAs. We listed here only the targets that were predicted to interact around their translation initiation sites (Table 2) and if the interaction was suggested in a minimum of two B. cenocepacia strains. Moreover, a high stringency of $P$-values was set to reduce the chance of false-positives in the predicted targets.

Concordant with our observation that Bc_KC_sr1 was upregulated upon iron depletion, its targets included cysteine desulfurase and isochorismatase. Cysteine desulfurase is required for Fe-S cluster synthesis [62] and isochorismatase-like hydrolase is crucial for siderophore biosynthesis [63]. Its other putative targets included the 2Fe-2S iron-sulfur cluster proteins, ABC transporter-like proteins, which are already established players maintaining iron homeostasis. Porins, the transmembrane channels, also seem to be targeted by Bc_KC_sr1 in all surveyed B. cenocepacia strains. Bc_KC_sr2, on the other hand, did not reveal many mRNAs where translation start sites could be targeted by the sRNA. The MerR family of transcriptional regulators, like SoxR identified in E. coli, regulates oxidative stress response and responds to perturbation of [2Fe-2S] clusters.

**Table 1.** Sequences of the small RNAs identified in B. cenocepacia KC-01

<table>
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<tr>
<th>Name of the sRNA</th>
<th>Sequence (5′-3′)</th>
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<td>Bc_KC_sr1 (83 nt)</td>
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<td>Bc_KC_sr2 (129 nt)</td>
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*For Bc_KC_sr3 and Bc_KC_sr7, 5′ RACE experiments were unsuccessful.*

Ghosh et al., *Microbiology* 2017;163:1924–1936
Bc_KC_sr2 is suggested to target this family of important transcriptional regulators and dihydropicolinate synthetase, a key enzyme in lysine biosynthesis. Bc_KC_sr3, which was induced upon introduction of H$_2$O$_2$, is capable of association with trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase (Proline utilization A, PutA). This correlation is particularly interesting since PutA is implicated in promoting cell death by increasing the ROS levels in various kingdoms [65]. The sRNA can also target bifunctional 5, 10-methylene tetrahydrofolate dehydrogenase/cyclohydrolase, an enzyme crucial for synthesis of 10-formyltetrahydrofolate which is a key metabolite required for purine biosynthesis and formylation of initiator-tRNA$_{Met}$. Isocitrate dehydrogenase, the enzyme that controls the flux between TCA and glyoxylate cycles, is also suggested to be a target of this sRNA. The presence of LysR-type transcriptional regulators and ABC transporter-like proteins as potential targets of Bc_KC_sr3 probably indicates a global response that can be elicited by this sRNA during peroxide accumulation. In the case of Bc_KC_sr4, polyprenyl synthetase and an inner membrane component of protein transport systems were primarily suggested as putative targets. Since polyprenyl synthetase synthesizes isoprenoids leading to among others formation of isopentenyladenosine-tRNA$_{Met}$ derivatives [66], stimulation of Bc_KC_sr4 will probably affect translation efficiency.

Bc_KC_sr7, the sRNA which was constitutively expressed in B. cenocepacia KC-01, possibly targets the GTPase, ObgE. ObgE acts as the ribosomal anti-association factor and its association with 50S ribosomal subunit is enhanced by ppGpp [67, 68]. It could be postulated that Bc_KC_sr7 is involved in regulating ribosome assembly during translation. The other putative targets for Bc_KC_sr7 were enzymes involved in amino acid metabolism.

**DISCUSSION**

Here we report the discovery of several small RNAs expressed in B. cenocepacia KC-01 under different physiological challenges. Their differential expression pattern in iron depletion, oxidative stress, variation in growth temperature as evidenced from our study, may be indicative of their regulatory roles in the survival mechanism of bacteria. We also demonstrated here that these sRNAs might be expressed differently under different stress conditions.
Fig. 5. Genomic neighbourhood of the novel sRNAs and their predicted secondary structures. (a) The cartoon represents the novel sRNAs and their neighbouring genes in *B. cenocepacia* KC-01. The sRNAs are represented by grey regions and the direction of transcription indicated by arrows. The depicted genes are not drawn to scale. (b) RNAfold predicted secondary structures of indicated sRNAs are displayed with their minimum free energies.
<table>
<thead>
<tr>
<th>sRNA ID</th>
<th>Description of the target gene</th>
<th>Location (when known)</th>
<th>Presence of targets in B. cenocepacia strain</th>
<th>Locus (name of the target gene if known); putative binding position in target mRNA</th>
<th>Presence of targets in B. cenocepacia strain</th>
<th>Locus (name of the target gene if known); putative binding position in target mRNA</th>
<th>Presence of targets in B. cenocepacia strain</th>
<th>Locus (name of the target gene if known); putative binding position in target mRNA</th>
<th>Presence of targets in B. cenocepacia strain</th>
<th>Locus (name of the target gene if known); putative binding position in target mRNA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bc_KC_sr1</td>
<td>Ammonium transporter</td>
<td>–</td>
<td>Bcen2424_2865; –22 to –8</td>
<td>Bcen2424_2865; –22 to –8</td>
<td>Bcen2424_2865; –22 to –8</td>
<td>0.001</td>
<td></td>
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<tr>
<td></td>
<td>Cysteine desulphurase</td>
<td>BCAL2198 (iscS); –14</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Isocitrate dehydrogenase</td>
<td>–</td>
<td>Bcen2424_4527; –15 to –5</td>
<td>Bcen2424_4527; –15 to –5</td>
<td>Bcen2424_4527; –15 to –5</td>
<td>0.002</td>
<td></td>
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<td></td>
<td>Rieske (2Fe-2S) domain-containing protein</td>
<td>–</td>
<td>Bcen2424_4406; –18 to –5</td>
<td>Bcen2424_4406; –18 to –5</td>
<td>Bcen2424_4406; –18 to –5</td>
<td>0.002</td>
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<tr>
<td></td>
<td>Nitrate/sulfonate/bicarbonate ABC transporter periplasmic ligand-binding protein</td>
<td>–</td>
<td>Bcen2424_3532; –23 to –9</td>
<td>Bcen2424_3532; –23 to –9</td>
<td>Bcen2424_3532; –23 to –9</td>
<td>0.002</td>
<td></td>
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<tr>
<td></td>
<td>ABC transporter-like protein</td>
<td>–</td>
<td>Bcen2424_0909; –15 to +4</td>
<td>Bcen2424_0909; –15 to +4</td>
<td>Bcen2424_0909; –15 to +4</td>
<td>0.004</td>
<td></td>
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<td>Porin</td>
<td>1. BCAM1974; –11 to +1</td>
<td>Bcen2424_4776; –11 to +1</td>
<td>Bcen2424_4776; –11 to +1</td>
<td>Bcen2424_4776; –11 to +1</td>
<td>0.005</td>
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<td></td>
<td>2. BCAM2187; –19 to –2</td>
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<td>3. BCAM2723; –11 to +10</td>
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<tr>
<td>Bc_KC_sr2</td>
<td>MerR family transcriptional regulator</td>
<td>–</td>
<td>Bcen2424_3445; +9 to +20</td>
<td>Bcen2424_3445; +9 to +20</td>
<td>Bcen2424_3445; +9 to +20</td>
<td>0.002</td>
<td></td>
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<tr>
<td></td>
<td>Dihydropicolinate synthetase</td>
<td>–</td>
<td>Bcen2424_5804; –72 to –63</td>
<td>Bcen2424_5804; –72 to –63</td>
<td>Bcen2424_5804; –72 to –63</td>
<td>0.002</td>
<td></td>
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<tr>
<td>Bc_KC_sr3</td>
<td>2Functional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase</td>
<td>BCAL2121 (folD); –12 to +8</td>
<td>Bcen2424_2140; –12 to +8</td>
<td>Bcen2424_2140; –12 to +8</td>
<td>Bcen2424_2140; –12 to +8</td>
<td>0</td>
<td></td>
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<td></td>
<td>Heat-inducible transcriptional regulator</td>
<td>BCAL3275 (hrcA); –9</td>
<td>Bcen2424_0745 (hrcA); –9</td>
<td>Bcen2424_0745 (hrcA); –9</td>
<td>Bcen2424_0745 (hrcA); –9</td>
<td>0.001</td>
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<tr>
<td></td>
<td>ABC transporter-like protein</td>
<td>BCAL2456; –8 to +6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Isocitrate dehydrogenase</td>
<td>BCAL2735; +4 to +15</td>
<td>Bcen2424_2523; –6 to +6</td>
<td>Bcen2424_2523; –6 to +6</td>
<td>Bcen2424_2523; –6 to +6</td>
<td>0.001</td>
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<tr>
<td></td>
<td>Trifunctional transcriptional regulator/proline dehydrogenase/pyrrole-5-carboxylate dehydrogenase</td>
<td>BCAL0042 (putA); –12 to +9</td>
<td>Bcen2424_0113 (putA); –12 to +9</td>
<td>Bcen2424_0113 (putA); –12 to +9</td>
<td>Bcen2424_0113 (putA); –12 to +9</td>
<td>0.001</td>
<td></td>
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<tr>
<td></td>
<td>LyS-R family transcriptional regulator</td>
<td>–</td>
<td>1. Bcen2424_0667; +13 to +20</td>
<td>1. Bcen2424_0667; +13 to +20</td>
<td>1. Bcen2424_0667; +13 to +20</td>
<td>0.005</td>
<td></td>
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<td></td>
<td></td>
<td>2. Bcen2424_4768; +1 to +15</td>
<td>2. Bcen2424_4768; +1 to +15</td>
<td>2. Bcen2424_4768; +1 to +15</td>
<td>2. Bcen2424_4768; +1 to +15</td>
<td>0.005</td>
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<tr>
<td>Bc_KC_sr4</td>
<td>Polypropenyl synthetase</td>
<td>–</td>
<td>Bcen2424_3878; –9 to +5</td>
<td>Bcen2424_3878; –9 to +5</td>
<td>Bcen2424_3878; –9 to +5</td>
<td>0.002</td>
<td></td>
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<td></td>
<td>Binding-protein-dependent transport systems inner membrane component</td>
<td>–</td>
<td>Bcen2424_4593; +12 to +20</td>
<td>Bcen2424_4593; +12 to +20</td>
<td>Bcen2424_4593; +12 to +20</td>
<td>0.003</td>
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<tr>
<td>Bc_KC_sr7</td>
<td>GTPase-ObgF</td>
<td>BCAL3440 (obgF); –17 to –1</td>
<td>Bcen2424_0583 (obgF); –17 to –1</td>
<td>Bcen2424_0583 (obgF); –17 to –1</td>
<td>Bcen2424_0583 (obgF); –17 to –1</td>
<td>0</td>
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<td></td>
<td>Putative l-isoaspartate O-methyltransferase</td>
<td>BCAL1894; +2 to +20</td>
<td>Bcen2424_1822; +2 to +20</td>
<td>Bcen2424_1822; +2 to +20</td>
<td>Bcen2424_1822; +2 to +20</td>
<td>0</td>
<td></td>
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<td></td>
<td>Sensor signal transduction histidine kinase</td>
<td>–</td>
<td>Bcen2424_5810; –13 to +4</td>
<td>Bcen2424_5810; –13 to +4</td>
<td>Bcen2424_5810; –13 to +4</td>
<td>0.003</td>
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<tr>
<td></td>
<td>FAD-dependent oxidoreductase</td>
<td>–</td>
<td>Bcen2424_5828; –12 to +8</td>
<td>Bcen2424_5828; –12 to +8</td>
<td>Bcen2424_5828; –12 to +8</td>
<td>0.005</td>
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</tbody>
</table>
Bc\_KC\_sr3 and Bc\_KC\_sr4 were upregulated on oxidative stress but did not follow a similar temperature-dependent expression pattern. While Bc\_KC\_sr4 demonstrated similar expression under different temperatures, Bc\_KC\_sr3 displayed robust expression at 30°C. The identified novel sRNAs thus possibly perform specific regulatory roles under different stress conditions. Moreover, since they do not share homology with the known sRNAs identified in different bacteria, iron and oxidative stress response in Burkholderiaceae could possibly occur through an unknown set of transcripts.

Among the identified sRNAs that were homologous to already known ones, Bc\_KC\_sr4 is homologous to Bp\_1\_candidate\_738\_SIIPHT in the RF02422 family, a sRNA previously identified in B. pseudomallei D286. The sRNA harboured CRC-binding motif, CsrA/RsmA-binding motif and GNRA (N=any nucleotide, R=purine) tetraloop. Since both CRC (carbon repression control) proteins and CsrA/RsmA inhibit translation by preventing association of the ribosomes to the mRNA and/or affecting its stability, Bc\_KC\_sr4 is postulated to facilitate translation by sequestration of RNA-binding regulatory proteins. Association of sRNAs like CrpC and CrpZ to CRC protein [69] and CsrB/C sRNA families to CsrA proteins [70] are already very well established in other genera. However, homologues of CRC proteins or CsrA/RsmA are not yet characterized in Burkholderiaceae. The presence of GNRA tetraloop also equips this sRNA to attain its tertiary structure via long-range interactions between the tetraloop and a variety of its receptor sequences [71]. For Bc\_KC\_sr5, a homologue of tmRNA, we hypothesize that it performs a similar function in the rescue of ribosomes stalled on the mRNAs [72, 73]. These two sRNAs therefore act toward stimulation or rescue of translation. 6S RNA, the homologue of Bc\_KC\_sr6, mimics the DNA contained in a melted promoter and inhibits transcription by binding directly to the housekeeping holoenzyme form of RNA polymerase (i.e. σ\^70\_RNA polymerase in E. coli) [14]. Since the role of 6S RNA is to globally regulate transcription under conditions of nutrient limitation and important for modulating stress response, we assume that Bc\_KC\_sr6 possibly performs this function in KC-01.

For an intracellular pathogen like Burkholderia, where cytosolic levels of iron is limiting and superoxide level could be high, the novel RNAs described here represent a gamut of stress response elicited by iron depletion and peroxide assault. In Table 2, we highlighted the mRNAs that interacted with the sRNAs through the regions surrounding the translation initiation sites. Enzymes involved in the biosynthesis of Fe-S clusters, ammonium transporter, ABC transporters and porins were among the targets for Bc\_KC\_sr1. Bc\_KC\_sr2, on the other hand, could bind to the MerR family of transcriptional regulators. Targets for Bc\_KC\_sr3 included enzymes required for purine biosynthesis, ROS response and TCA cycle enzymes and Bc\_KC\_sr4 probably altered the isopentenylation profile of tRNA. Since intracellular iron plays a pivotal role in maintaining the intracellular redox equilibrium, it is expected that some of the stress response pathways between iron depletion and peroxide assault would overlap. Expectedly, ABC transporters and the LysR family of transcription regulators are suggested as targets of both sets of sRNAs. Bc\_KC\_sr7 presumably targets the pathways of ribosome assembly and amino acid metabolism.

Taken together, we present here evidence for the expression of four novel sRNAs in Burkholderiaceae under conditions of iron depletion and oxidative stress. In addition, expression of homologues of tmRNA and 6S RNA in Bcc have also been demonstrated. It is interesting to find the fact that KC-01 does not utilize the sRNAs present in Pseudomonas but employs an entirely novel set to mitigate oxidative stress or iron depletion. This underscores its ability to employ different strategies to suit its genetic wherewithal. Several important biological pathways are predicted targets of these novel sRNAs. Experimental validation of these targets will generate more insight about the survival strategies of Bcc bacteria inside macrophages and provide clues for therapeutic intervention.

Funding information
The work was supported by Department of Biotechnology (Government of India) grant no. BT/PR11424/BRB/10/680/2008 to S.G. (corresponding author) and S.R. (institutional code for the grant: GAP78). Instrumentation support was partly provided by the Department of Biotechnology-BUILDER program to University of Calcutta. S.G. and I.K. received research fellowship from UGC.

Acknowledgements
We thank the C-CAMP (www.ccamp.res.in/) next-generation genomics facility for help in obtaining the genome sequence. Authors also wish to thank Dr Alok Kumar Sil and members of his laboratory for their help in quantitative PCR experiments.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Edited by: N. Le Brun and F. Sargent

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