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Isolation and Abundant Production of Anti-HIV Small Single-Stranded RNA by *Bacillus subtilis* MTCC5480 from Fermented Soybean

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Abstract: *The increasing demand for antiviral RNA therapy is driven by the rise of new viral infections. However, the exploration of small non-coding regulatory RNAs (sRNA) from microbial sources for antiviral activity remains largely untapped. This study focuses on evaluating the anti-HIV potential of sRNA produced by 12 distinct microbial species isolated from naturally fermented foods in North-East India. The sRNA was selectively extracted from microbial cultures, its single-stranded nature confirmed through immunoblotting, and the cDNA library deep-sequenced using the Illumina platform. Employing conventional algorithms, we predicted potential targets of the sRNA sequences within the 3'-UTR region of HIV-1. Notably, a 34-base sRNA fragment with the sequence 3'-UUGGUACACGAGAUGGUUCGACUCGAUGAAGGGC-5', abundantly produced (constituting 9.17% of the total sRNA fraction) by Bacillus subtilis MTCC5480, demonstrated significantly higher base complementarity values compared to previously analyzed miRNAs against HIV-1. The isolated sRNA fraction was separated, and its anti-HIV activity against human peripheral blood mononuclear cells (PBMC) infected with the JRCSF strain of HIV-1 virus was validated, with an EC50 value ranging from 0.2 to 0.3 μM. The small sRNA produced by B. subtilis MTCC5480 holds promise for further exploration as a potential antiviral therapeutic agent.*

Keywords: *sRNA, Bacillus subtilis, HIV-1.*

1. Introduction

The primary source of anti-HIV activity in natural products is commonly attributed to secondary metabolites of plant and microbial origin. Numerous studies have highlighted alterations in the expression profile of host cellular small non-coding regulatory microRNAs (miRNA) during HIV-1 infection. These studies have identified an upregulation of miR-122, miR-370, miR-373, miR-297, and miR-223, along with the suppression of miR-18, miR-19a, miR-20a, miR-19b, miR-921, miR-29, and miR-155.

Computational target prediction, followed by experimental validation, has implicated specific miRNAs, including miR-28, miR-125, miR-150, miR-223, and miR-382, in inhibiting viral replication primarily by targeting the 3'-untranslated region (3′ UTR) of human immunodeficiency virus-1 (HIV-1) RNA. These small RNAs exert their effects by repressing the translation of target genes through base-pairing with complementary sequences in the 3′ UTR of the targeted transcripts.

Among the miRNAs validated for their anti-HIV activity, miR-29a and miR-326 have demonstrated particularly robust repression of HIV-1 replication, exceeding 40%. The introduction of small-stranded RNA (sRNA) into the cell has been shown to specifically degrade mRNA containing the same sequence. Subsequent efforts have focused on the development of synthetic and engineered miRNAs, as well as multiplexing multiple miRNAs, to enhance anti-HIV activity. However, despite these advancements, miRNAs from natural sources have yet to be thoroughly explored for their anti-HIV potential.

Fermented foods are well known for several health-promoting and therapeutic properties. Emerging evidence shows anti-inflammatory properties of the small single-

stranded RNA (sRNA) produced by food fermenting bacteria. As part of our research program, we are

characterizing the sRNAs produced by different food fermenting bacteria isolated from traditional fermented foods of India, in-silico prediction of its biological function from its sequences and further validation in the cell line models. During this screening, we identified a small sRNA produced by Bacillus subtilis has high anti-HIV potential. This sRNA frac- tion occupied N9% relative abundance of the total sRNA produced by B. subtilis and showed a much higher base complementarity values than the miRNA analysed against HIV-1 in the earlier studies. Here, we separated, sequenced and validated the anti-HIV activity of the sRNA fraction from B. subtilis in human peripheral blood mononu- clear cells (PBMC) infected with JRCSF strain of HIV-1 virus.

2. Materials and methods General experimental procedure

The RNA fraction from the bacterial cell lysate was extracted by using a temperature-controlled benchtop centrifuge (5810R, Eppendorf). The RNA fraction was separated and purified by agarose gel electrophoresis system (Sub Cell GT, Biorad) and polyacrylamide gel electrophoresis system (Protean-II xi, Biorad). The GelRed stained RNA bands were visualised by Gel documentation system (Gel DocXR, Biorad). The RNA quantity was measured by Qubit fluorometer (Invitrogen) and Nanodrop Spectrophotometer (Thermo). The quality of the RNA fraction was assessed by Bioanalyser (2100, Agilent). The cDNA library from the small RNA fraction was prepared by PCR thermal cycler (C100 Touch, Biorad). The cDNA library was sequenced in the Illumina platform with 1 × 50 base chemistry (HiSeq 2000). The se- quence data were processed by Tyrone workstation (Intel Xeon Proces- sor E5). The in-silico target prediction of the sRNA sequences using miRanda, PITA , and TargetScan algorithms was performed by PARAM supercomputing facility, Centre for Development of Advanced Computing (C-DAC, Pune, India).

Microbial cultures and growth conditions

A total of 12 microbial cultures isolated from different traditional fermented foods of Northeast India, identified by 16S rRNA sequence similarity analysis were used for this study. The details of the source of isolation, strain and NCBI sequence accession details are listed below. Tetragenococcus halophilus KL13 (KJ699143) and Lactobacillus pobuzihii LP1 (PRJNA591754) were isolated from a fermented fish prod- uct “Ngari” . Lactobacillus acetotolerans LA1 (PRJNA591747), Lacto- bacillus brevis H3S2L4 (KT757206), and Lactobacillus spicheri H1S2A5 (KT757211) were isolated from a fermented bamboo shoot “Hikhu”. Weissella cibaria SD2S4L4 (KJ095644), Leuconostoc citreum SD3S4L4 (KJ095642), Lactococcus lactis subsp. lactis SD1S6L3 (KJ095654), Lacto- bacillus plantarum SD1S6L2 (KJ095651) and Pediococcus pentosaceus SD2S6L8 (KJ095667) were isolated from a fermented bamboo shoot “Soidon” . Bacillus subtilis MTCC5480 (GQ268319) (deposited in Mi- crobial Type Culture Collection and Gene Bank, Chandigarh, India) was isolated from a fermented soybean “Hawaijar” and Saccharomyces cerevisiae PH3Y10 (JF427814) was isolated from a rice wine starter “Hamai” . The bacterial strains L. plantarum, L. brevis, W. cibaria,L. citreum and L. pobuzihii were grown in de Man Rogosa and Sharpe (MRS) broth (Merck), P. pentosaceus, and
L. spicheri were grown in MRS broth supplemented with 1% (w/v) maltose and 1% (w/v) fructose,T. halophilus in MRS broth with 7% (w/v) NaCl (pH 7.2), L. lactis subsp. lactis in M17 broth (Himedia), L. acetotolerans in Briggs liver broth medium (pH 5.0) , and B. subtilis was grown in LB broth (Himedia). The lactic acid bacteria were incubated at 30 °C under an O2-depleted and CO2-enriched atmosphere in anaerobic jars using anaerobic gas packs (Anaerocult® C, Merck) for 60–96 h. The S. cerevisiae was incubated aer- obically at 28 °C for 48–60 h, and the B. subtilis was incubated aerobi- cally at 37 °C for 24 h under 200 rpm shaking condition.

Extraction of sRNA

The total RNA from the bacterial cell pellet was extracted by a hotphenol method as described by Jahn et al. with some modifications. Further, the sRNA fraction was separated from the total RNA by treating with RNase-A (Sigma-Aldrich) in 10 mM Tris-Cl (pH 8.0) with 0.3 M NaCl and RQ1 RNase-free Dnase (Promega). The reaction was stopped by adding EDTA after 20 min incubation at 37 °C. The sRNA fraction was precipitated overnight at −20° by adding 2.5 vol- ume of ice-cold absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and then centrifuged at 16,000 ×g for 30 min at 4 °C. The pre- cipitated RNA fraction was washed twice with 70% (v/v) ethanol, dried in laminar airflow for 10 min and dissolved in DEPC-treated deionized water. The sRNA fraction recovered was quantified by RNA Qubit assay (Invitrogen, USA). The purified sRNA fraction was run on 2.5% (w/v) agarose gel in 0.5× TBE buffer (pH 8.2) at room temperature to confirm the presence and the intactness of sRNA, further separated by 20% Urea-poly acrylamide gel electrophoresis (Urea-PAGE), purified by gel elution and extracted by gel-breaker tubes (IST Engineering). The extracted sRNA fraction was stored at −80

°C until required.

RNA immunoblotting

RNA immunoblotting was performed with sRNA-specific monoclonal antibody J2 (mAb J2, English Scientific Consulting Kft., Hungary) as described by Lybecker et al. Electrophoresis on an 8% native polyacrylamide gel was then transferred to a nylon membrane (Sigma) and the RNA was cross-linked by UV irradiation for 3 min. The membrane was first incubated overnight with the primary antibody (J2 monoclonal sRNA antibody), then washed and incubated with the secondary anti-mouse IgG-alkaline phosphatase (AP) antibody (Sigma).

Small RNA deep sequencing

Purified sRNA fractions were used to prepare cDNA libraries using the TruSeq Small RNA Library Preparation Kit (Illumina #RS200-0012) according to the manufacturer's instructions. Briefly, 3′ and 5′ RNA adapters were sequentially ligated into a 200 ng sRNA fraction, reverse transcribed, and subsequently amplified using 15 cycles of polymerase chain reaction (PCR) with a thermal cycler. PCR products were analyzed on a 6% polyacrylamide gel for size selection. cDNA libraries were purified using a QIAquick gel extraction kit (Qiagen, New Delhi, India) according to the manufacturer's instructions. The quantity of purified libraries was assessed using the Qubit dsDNA HS assay kit in a Qubit 2.0 fluorescence instrument (Invitrogen), and the quality of the libraries was examined using the High Sensitivity DNA kit from 2100 Bioanalyzer (Agilent). The cDNA library was sequenced on an Illumina platform with 1 × 50 bp chemistry (Xcelris Genomics, Ahmedabad, India) and the relative abundance of each unique representative sequence was further calculated.

n-silico prediction

Potential targets of sRNA sequences in the Nef-3′ -LTR region, which serves as the 3′ -UTR of all HIV-1 transcripts (NCBI accession number: NC_001802), were determined by the three most commonly used and timely updated target prediction algorithms. and it; miRanda, PITA and Target Scan on the Linux platform. To compare the anti-HIV potential, we used 2656 human mature miRNA sequences obtained from the miRBase database. An sRNA sequence with high base-pairing potential to the 3′ UTR region of the HIV-1 genome (En-energy value b −35 kcal/mol, N score 185, and seed match 8 mer) werepredicted. Clustering and sequence matching of 42 sRNA sequences with ~11% relative abundance from B. subtilis MTCC5480 with a high HIV prediction score were further subjected to multiple sequence alignment (ClustalW) and clustering based on the Maximum Likelihood and Kimura 2-parameter method in MEGA X shows similar sequences sRNA, also predicted the secondary structure of the sRNA.

Cytotoxicity test

Human PBMCs collected from a healthy volunteer were used for sRNA cytotoxicity using the MTT assay (HiMedia). PBMCs were isolated from whole blood using Ficoll–Hypaque (HiMedia) density gradient separation. Briefly, PBMCs were plated at 2 x 10 5 cells/well in a 96-well plate in 150 µl/well of serum-free RPMI 1640 medium. Cells were then transfected with various amounts of sRNA (10 nM, 50 nM, 100 nM, 250 nM, 500 nM and 1000 nM) using Lipofectamine 2000 (Invitrogen) with 50 µl of RNA-lipid complex. The negative control was treated with water and the experiment was repeated in triplicate. One hour later, cells were spun down for 30 min at 7 x g using a centrifuge (5810 R, Eppendorf), then 100 µl of 3 x complete medium (RPMI1640, 15% FBS and Pen-Strep) was added and the cells were cultured at 37 °C in a humid atmosphere containing 5% CO2. At 24 h and 48 h post-transfection, cell viability was then assayed by adding 10 µl of 5 mg/ml 3-[4,5-dimethylthiazol-yl]-2,5-diphenyltetrazolium bromide (MTT) followed by incubation for 4 h at 37° C. Then, 100 µL of solubilization solution was added to each well and the optical density was recorded using a Multiskan EX plate reader (Thermo Fisher Scientific) at 570 nm. Cell viability was calculated as the percentage of cell survival compared to the untreated control by sampling in triplicate.

Anti-HIV test

PBMC stimulated with phytohemagglutinin (PHA, 2.5 µg/ml) and interleukin-2 (IL-2, 20 units/ml; 2 µg/ml polybrene) were challenged with HIV-1 strain JRCSF from the NIH AIDS Repository. HIV infection was performed by mixing JRCSF stock virus with stimulated PBMC culture at 37°C in a humidified atmosphere containing 5% CO 2 . On the fourth day of infection, cells were washed twice with phosphate-buffered saline (PBS) and infected cells were plated at 8 x 10 4 cells/well in a 24-well culture plate in serum-free RPMI 1640 medium. . Transfection of purified sRNA at different concentration (10–250 nM) was performed in PBMCs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 1 h of transfection, cells were spinoculated at 7 × g for 30 min using a centrifuge (5810 R, Eppendorf, Germany), then 200 µl of 2 × IL-2 GM (RPMI1640, 15% FBS, Pen-Strep and polybrene was added). At 24 h and 48 h post-transfection, the supernatant was collected for virus production. Cells transfected with Lipofectamine and RNA alone were treated in the same manner and used as a control

for the assay.HIV-1 production was monitored by quantifying the amount of p24 antigen produced in the culture supernatant using the HIV-1 p24 Antigen ELISA kit (Xpress- Bioscience Life, USA) according to the manufacturer's instructions. For the pre-infection assay (first RNA transfection, then viral infection), PHA- and IL-2-stimulated PBMCs were plated at 2 x 10 5 cells/well in a 96-well plate. Cells were transfected with sRNA (250 nM) using Lipofectamine and left for 24 h at 37°C in a humidified atmosphere containing 5% CO 2 .

Azidothymidine (AZT) at a concentration of 60 µM was used as a positive control. At 24 h post- transfection, the cells were washed twice with phosphate-buffered saline (PBS) and infected with the JRCSF strain of HIV-1. After 5 h of virus exposure, cells were washed twice with PBS and fresh medium was added. On the fourth day of incubation, the supernatant was collected for quantification of p24 antigen level. Percent inhibition was calculated as the percentage of the p24 antigen level in the treatment versus the control level, and the effective concentration EC50 was calculated as the concentration of the fraction of sRNA required to reduce the p24 level to 50% based on three independent experiments.

Statistical analysis

The arithmetic mean and standard error of the mean was calculated using MS Office Excel 2007 package. The statistical significance of differ- ent values was estimated using two-tailed Students t-test (p-values) for the comparison of values.

3. Results and discussion

Highly diverse small non-coding sRNA sequences

For this study, the sRNA fraction extracted from 12 different microbial cultures (isolated from naturally fermented foods in India) was analyzed. We selectively extracted a small fraction of sRNA from the total RNA of the microbial cell by treatment with RNase-A at a concentration of

0.3 M NaCl. This selective extraction resulted in 9–43% of the sRNA fraction from total RNA, corresponding to 19–157 µg of sRNA fraction from 10–15 mL of microbial culture. We first confirmed the double-stranded nature of the RNA fraction using an sRNA-specific J2 monoclonal anti-sRNA antibody (mAb J2) by immunoblotting. Urea-PAGE used for separation showed a small size (b60 bp) fraction of sRNA. A cDNA library prepared from this small sRNA fraction was deeply sequenced using the Illumina platform, resulting in thousands of extremely diverse unique species-specific sRNA sequences 18–51 bp in length. Previous studies predicted the genome-wide distribution of small non-coding RNAs in bacteria by in-silico analysis and found that nearly 30% of these sRNAs are viral RNA-like contigs of unexplained origin. Functional annotation of these sRNA sequences by the MG-RAST server did not show protein- coding features, indicating the non-coding nature of the extracted sRNA fraction.

Abundance of sRNAs with anti-HIV potential in B. subtilis MTCC5480

We used a conventional target prediction approach using three miRNA target prediction algorithms, miRanda PITA and TargetScan, to predict potential targets of sRNA sequences obtained from 12 different food-fermenting microbes in the 3'-UTR region of HIV-1 under the most stringent criteria (energy value b −35 kcal /mol, N score 185 and seed match 8 mer). The 3′ -UTR region was chosen in accordance with the notion that, in most cases, sRNA binds to the 3′ -UTR region of a transcript to regulate gene expression at the post-transcriptional level [9]. Among the 12 different microbial species analyzed, we found that sRNA sequences (42 out of 5144 sRNA sequences)

Giant. 1. Shown here is a small fraction of sRNA extracted from Bacillus subtilis and separated in denaturing Urea-PAGE (20% acrylamide). M: Low range 10–300 bp ladder DNA (SM 1211, Thermo Fisher Scientific) Lanes 1–6: sRNA fraction extracted from B. subtilis strains MTCC5480, MTCC1747, AKB13, MCC2516 and MCC2511: Lanes . The sRNA fraction extracted from Saccharomyces cerevisiae strains MTCC180, H20Y5 and PH3Y10.

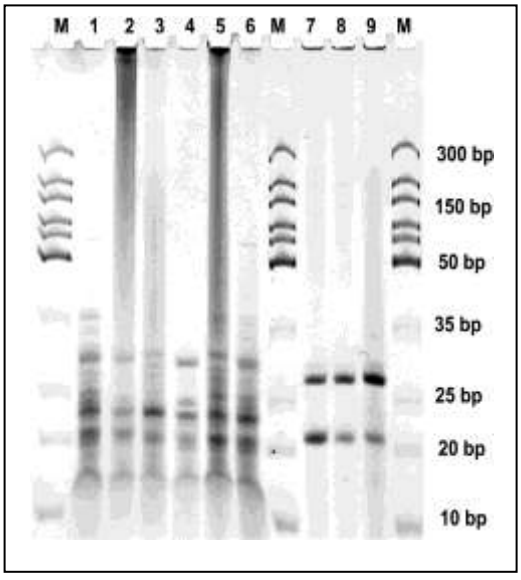

Fig. 1. The small sRNA fraction extracted from Bacillus subtilis and separated in a denaturing Urea-PAGE (20% acrylamide) is shown here. M: Low range DNA ladder of 10– 300 bp in size (SM 1211, Thermo Fisher Scientific) Lanes 1–6: sRNA fraction extracted from B. subtilis strains MTCC5480, MTCC1747, AKB13, MCC2516, and MCC2511, Lanes 7–9: sRNA fraction extracted from Saccharomyces cerevisiae strains MTCC180, H20Y5, and PH3Y10 from Bacillus subtilis MTCC5480 predicted to have high base-pairing ability in the HIV-1 3'-UTR region according to strict criteria. Base complementarity values and targets of 42 sRNA sequences from B. For comparison, we also used human mature miRNAs (2656 sequences) obtained from the miRBase database and not even a single miRNA hit in the HIV-1- 3′ UTR region under the stringent conditions used for in-silico prediction. The predicted anti-HIV potential of sRNA sequences from B. subtilis MTCC5480 was much higher than miRNAs (both human and synthetic) analyzed against HIV-1 by previous studies, moreover, the most abundant sRNA sequence (Bst_UNIQ000001 , 9.17% relative abundance) present in the fraction The B. subtilis MTCC5480 sRNA had the highest anti-HIV potential in contrast to other studied species that had trace concentrations of sRNA sequences with anti-HIV potential.

Two major sRNA sequence clusters with anti-HIV potential

In further analysis, the predicted 42 sRNA sequences from B. subtilis with high anti-HIV potential were compared and clustered by the Maximum Likelihood method. This resulted in two major clusters (Cluster-I and Cluster-II) of overlapping sRNA sequences of 34–37 bases (14 sequences) and 51 bases (18 sequences). These sRNA sequences with a slight difference in base complementarity values had their target site in the most conserved region 8656–8709 of the HIV- 1 3′ -UTR region. When we compared these two major sRNA clusters, we noticed a similar seed region for sRNA sequences from both clusters at position 8698–8708 of the HIV-1 3'-UTR region. The seed region sequence from positions 2 to 8 at the 5' end of the miRNA is critical for RNA/target interaction. Our analysis showed a much stronger seed region at position 2–10 at the 5' end of the sRNA sequences from the above two major sRNA clusters. In-silico analysis identified the 34-base Bst_UNIQ000001 as the most abundant (9.17% relative frequency) sRNA produced by B. subtilis MTCC5480 with high base-pairing ability at the 3'-UTR region of HIV-1 as a potential candidate. for validation of anti-HIV activity. We experimentally tested an ∼34- base sRNA fraction purified by Urea-PAGE gel extraction from B. subtilis MTCC5480, hereafter named “BS1” for anti-HIV activity

sRNAs and cytotoxicity

Next, we investigated the cytotoxicity of BS1 on human PBMCs. PBMCs were transfected with 10 to 1000 nM concentration of BS1 and cell viability was assessed 48 hours after transfection by MTT assay. Although we did not observe toxicity of sRNA alone up to a concentration of 1000 nM, sRNA together with the transfecting agent Lipofectamine (18 µl) reduced PBMC viability to 60% (Supplementary Figure S2). Since Lipophetamine is a cationic lipid, it is toxic at higher concentrations. In addition, the RNA-lipofectamine complex showed less toxicity to PBMCs than lipofectamine alone. The RNA-lipofectamine complex normally reduces the charge ratio between the cationic lipid and the RNA, thereby reducing toxicity. From this result, it is clear that the cytotoxicity observed in our study is due to the higher concentration of Lipofectamine and not to the higher concentration of the sRNA fraction from Bacillus subtilis. At a safer level, we set the upper concentration limit for the transfection experiment as 250 nM of the sRNA fraction and 4.5 µL of Lipofectamine.

Inhibition of HIV-1 virus production in the post-infection phase

We next checked whether the transfected sRNA fraction of BS1 would affect HIV-1 production in PBMCs infected with the JRCSF strain of HIV-1 (obtained from the NIH AIDS Reagent Program) and assessed whether BS1 suppresses HIV-1 dose-dependently. - in a dependent manner. 72 hours after HIV-1 infection, we confirmed the infection by measuring the p24 antigen level in the PBMC supernatant. At this post-infection stage, we tested suppression of HIV-1 production by BS1. Infected PBMCs were transfected with different concentrations of BS1 (10–250 nM). At 24 hours post-transfection, p24 antigen level measured from cell supernatants showed a reduction in HIV-1 virus production in BS1-transfected cells compared to the untreated control. Our result showed a 43% reduction in HIV-1 virus production by the sRNA fraction BS1 at the 250 nM concentration in comparison to the control. Similar to our results, miR-29 at a concentration of 200 nM was shown to suppress almost 40% of HIV-1 viral particle production in a luciferase assay model. Our results also showed a dose-dependent suppression of HIV-1 virus production by BS1 sRNA fractions. expressed here as a decrease in p24 antigen level. The LA sRNA fraction from L. acetotolerans LA1, which showed a low hit to the 3′ -UTR region of HIV-1 during in-silico analysis, was also used here as a control for comparison. At a concentration of

250 nM, the LA sRNA fraction showed no significant reduction in virus production. SRNA can only target bare cytoplasmic genomic RNA of HIV-1 by direct RNA-RNA interaction during the retroviral life cycle. It represses translation of the target gene through complementary sequence pairing in the 3′ -UTR of transcripts, leading to sequence-specific cleavage. Because the HIV-1 Nef-3′ LTR region serves as the 3′ UTR of all HIV-1 transcripts, sRNA binding to the Nef-3′ LTR region can inhibit the translation of almost all HIV-1 encoded proteins. It includes the Tat and Rev proteins, which are regulators of viral gene transcription and viral RNA translocation.

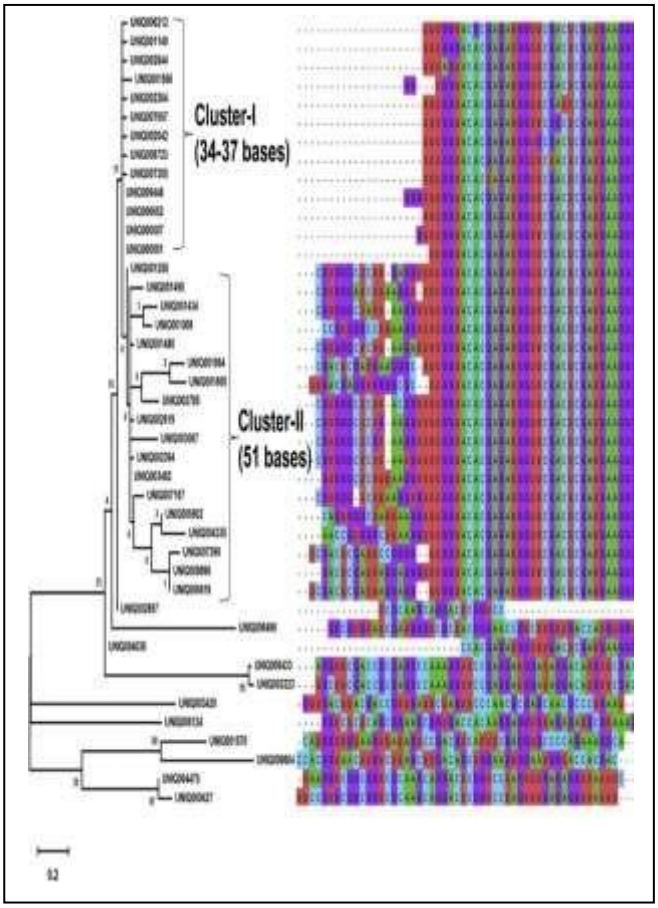

Fig. 2. Sequence similarity matching and clustering of the sRNA sequences derived from Bacillus subtilis MTCC5480 with a high base-pairing ability on 3′ -UTR region of the HIV-1 genome RNA. The sequence clustering based on Maximum Likelihood method and Kimura 2-parameter and similarity matching show two major clusters (Cluster-I and Cluster-II) with a highly conserved region. The sRNA sequences with a high base-pairing potential on 3′ UTR region of HIV-1 genome RNA with the threshold energy value b−35 kcal/mol, score N185 and seed match of 8 mer by target prediction algorithms were used for the analysis

Suppression of HIV-1 virus production by preinfection treatment

Furthermore, we analyzed the anti-HIV activity of the sRNA fraction BS1 by treatment in the pre-infection stage (transfection of PBMCs first with the sRNA fraction followed by HIV-1 infection). After 24 h of transfection with 250 nM of the sRNA fraction, PBMCs were infected with the JRCSF strain of HIV-1. 72 hours after infection, we measured the level of p24 production from the cell supernatant of infected PBMCs. We observed that the BS1 sRNA fraction at a concentration of 250 nM suppressed HIV-1 production more strongly in the pre- infection treatment (66% reduction in virus production than in the post-infection treatment. An effective concentration of the BS1 sRNA fraction required a 50% reduction in HIV-1 virus production in the range of 0, 2–0.3 μM Our observation suggests that the BS1 sRNA fraction from B. subtilis can effectively inhibit HIV-1 infection at an early stage before integration rather than after integration Similar to post-infection treatment, pre-infection treatment with L. acetotolerans sRNA fraction showed no significant effect on HIV-1 virus production These results confirmed the in-silico prediction of sRNA from B.

subtilis to exhibit high anti-HIV potential, possibly through further pairing directly with HIV-1 genomic RNA and subsequent inhibition of downstream replication processes Little interference in bare cytoplasm ic HIV-1 genomic RNA at the initiation point of the viral cycle before reverse transcription can lead to a drastic reduction in viral infection.SRNA complementary to the 3'LTR region of HIV-1 genomic RNA can inter fer with the activation process that is required to initiate reverse transcription. Such type of inhibition of reverse transcription affects the integration of viral RNA with human genomic DNA. This approach of stopping at the entry level of the retroviral cycle will provide a drastic reduction in subsequent replication processes. Our study also noted a much higher reduction in viral load by pre-infection treatment with BS1 fraction sRNA than by post-infection treatment. Further studies with the chemically synthesized sRNA fragment and screening for various assays such as nef expression, monocyte chemotactic protein (MCP)-2 release, the ability of macrophages/monocyte-derived dendritic cells (MDDCS) to infect or reduce dendritic In addition to p24 expression will determine the mode of action of anti

-HIV activity specific cell intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) expression. Several RNA-based antiviral therapies have been studied for the treatment of HIV using antisense approaches. However,

sRNA provides a more reliable method of viral gene inactivation than antisense RNA inactivation.

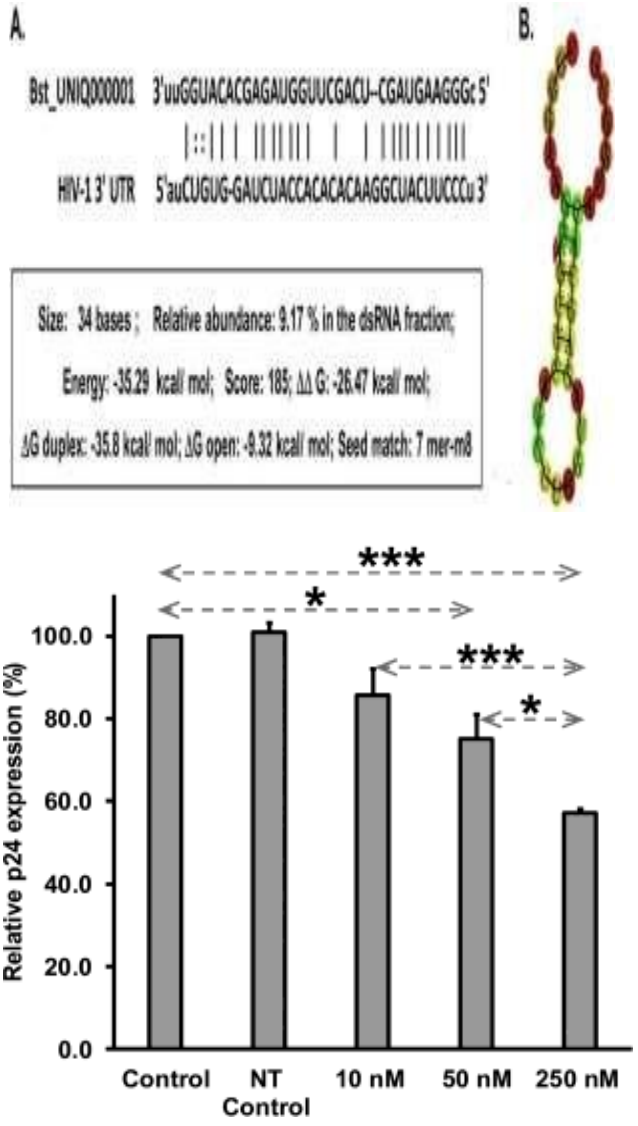

Fig. 4. Dose-dependent suppression of HIV production expressed here as p24 antigen level in HIV-1 infected PBMC culture transfected with sRNA fraction BS1 from Bacillus subtilis MTCC 5480. The y-axis represents the relative p24 values (as percentages) normalized to the control (PBMC treated with 4.5 µL of Lipofectamine). NT control represents the non- transfection control treated with 250 nM of the sRNA fraction BS1. Different concentrations of the sRNA fraction transfected on HIV infected PBMC was analysed after 24 h of treatment. The significant difference was calculated by Students t-test, two- tailed with equal variance and indicated as *p b0.05, ***p b0.001.

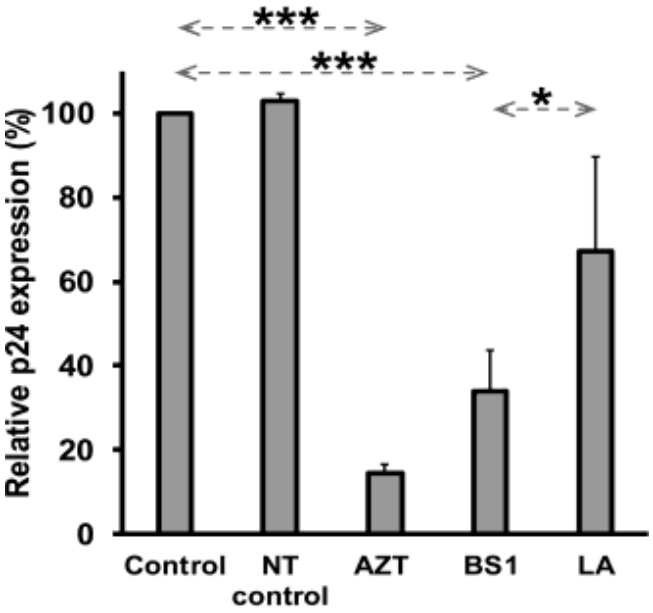

Fig. 5. The PBMC transfected at the pre-infection stage with the sRNA fraction BS1 from B. subtilis MTCC5480 reduced the HIV-1 infection. The y-axis represents the relative p24 antigen expression (as percentages) in HIV-1 infected PBMC, normalized to the control (PBMC treated with 4.5 µL of Lipofectamine). NT control represents the non- transfection control treated with 250 nM of sRNA fraction BS1. AZT: Azidothymidine, an antiretroviral drug at 60 µM concentration. LA: 250 nM of sRNA fraction from Lactobacillus acetotolerans LA1. The significant difference was calculated by Students t- test, two-tailed with equal variance and indicated as *p b0.05, ***p b 0.001.

4. Conclusions

This study represents the inaugural exploration of the anti-HIV potential inherent in a small sRNA generated by bacteria involved in food fermentation. A specific sRNA fragment, 3'-UUGGUACACGAGAUGGUUCGACUCGAUGAAGGG C-5', exhibited noteworthy anti-HIV capabilities and was produced abundantly (constituting 9.17% of the total sRNA fraction) by B. subtilis MTCC5480 bacteria, isolated from naturally fermented soybean food in India. In contrast to previously reported anti-HIV microRNAs, this sRNA fragment displayed heightened efficacy.

Following further purification of the sRNA fraction and subsequent validation experiments, the suppression of HIV-1 virus production was confirmed using a human PBMC model. Importantly, the effective inhibition of HIV-1 occurred at an early stage, before integration, rather than

post-integration transcription. The proposed mechanism of action suggests that the sRNA fraction from B. subtilis MTCC5480 may target HIV-1 genomic RNA at two distinct stages of the retroviral cycle, imparting dual effects. First, inhibition of reverse transcription at the early stage of infection (pre-nuclear import and integration), and second, suppression of translation at the post-integration stage.
Given the higher abundance of this sRNA fragment with potent anti-HIV activity produced by B. subtilis, it emerges as a promising candidate for further investigation into its mechanism of action and potential development as a novel anti-HIV therapeutic agent.

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