

## Selective antimicrobial profile of a newly isolated thermostable *Brevibacillus borstelensis*

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### ABSTRACT

A thermophilic bacterium *Brevibacillus borstelensis* strain RH 102 was isolated from the municipal garbage dumping site through plate exposure method. The cell free supernatants (CFS) of this strain were screened for antimicrobial activity by agar diffusion assays against gram positive, gram negative and fungal pathogens. Methanolic extracts of CFS showed antimicrobial activity against the gram positive bacteria. The 16S rRNA gene sequence of this strain showed 99% nucleotide sequence homology with that of *Brevibacillus borstelensis* R-16402. The antimicrobial activity of the methanolic extract of CFS of RH 102 is abolished by protease. The protease sensitivity of the antimicrobial(s) produced by this *Brevibacillus* strain indicates it to be peptidyl in nature. Present study reports the production of antimicrobial compounds for the first time by *Brevibacillus borstelensis*. Selective spectrum activity of this metabolite(s) can have potential importance against emerging resistant gram positive pathogens.

**Key words:** *Brevibacillus borstelensis*, antibacterial activity, thermophile, 16S rRNA gene sequencing.

### INTRODUCTION

Recent years have seen a large number of pathogenic and clinically important bacteria becoming resistant to antibiotics in common use, presenting a significant threat to human health (Nathan, 2004, Projan, 2003 and Wenzel, 2004). Increasing population of immunocompromised in the developing world has also led to many nosocomial infections with lesser known common infections becoming pathogenic. In order to combat these infections, new antibiotics need to be developed to which bacteria are less likely to become resistant (Walsh, 2003). Current approach is to focus on the identification of antimicrobials with narrow specificities restricted to a single genus or species rather than the broad-spectrum approaches of the past (Mincey and Parkulo, 2001). Although broad spectrum antibiotics seem to have an edge over

narrow spectrum antibiotics in terms of economic incentives and faster treatment, research into the latter category would be more practical in terms of clinical practice (Walsh, 2003).

In the present work, we describe the isolation of a thermophilic *Brevibacillus* sp. strain RH102 from the garbage dumping site of the Municipal Corporation of Delhi, India. The site was chosen for isolation of microorganisms as the bacterial diversity of such sites is not well described and it is expected that different novel and biodegrading species may inhabit these sites that are able to produce antimicrobial compounds against competing flora, including spoilage and pathogenic bacteria. This study represents the first demonstration that *Brevibacillus* sp. produces antimicrobial compound(s).

## MATERIAL AND METHODS

### Isolation and identification of bacteria

The plates containing tryptic soy agar (TSA) and nutrient agar (NA) were exposed to municipal garbage dumping site for 30 min. These plates were kept at 55 °C for 24-48 h for colony appearance. Four prominent strains were selected and were further screened for their antimicrobial activities. The strain RH 102, growing on TSA, showed highest antimicrobial activity and was selected for further studies.

### 16S rRNA gene sequencing

Genomic DNA of strain RH102 was isolated by the method of Takagi et al. (1993). Amplification of 16S rRNA gene was performed as described by Weisburg et al. (1991) using universal primers fD1 (5' AGTTTGATCCTGGCTCA 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3'). In a 100µl reaction volume, 20 pmol of each primer was used for 20ng genomic DNA template. Amplification was performed on an automated thermocycler (MJ Research, USA) using 1U Taq polymerase (NEB), and the recommended buffer system. Amplification profile is as follows: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1min, 72°C for 1 min and a final extension for 7 min. Amplified DNA was purified using QIAGEN PCR purification kit (QIAGEN, Germany) and was sequenced at The Centre for Genomic Application (TCGA), New Delhi, India. Similarity searches of the sequence obtained were performed using BLAST algorithm of NCBI (Altschul et al., 1990).

### Extraction of active compounds

Cell free supernatants (CFS) of strain RH 102 grown at 55°C for 24 h was lyophilized and extracted separately with petroleum ether, ethyl acetate, chloroform and methanol. About one-third volume of each organic solvent was used for extraction of lyophilized powder obtained from 200 ml CFS. Extracts were evaporated to dryness under vacuum at 40°C using rotavapor (BÜCHI Labortechnik AG, Switzerland) and dissolved in milliQ water, phosphate buffered saline (PBS) and dimethylsulfoxide (DMSO) to screen for antimicrobial activities. All solvents were purchased from Sisco Research Laboratories, India.

### Antimicrobial assay

Following test organisms were used to screen antimicrobial compound(s) obtained from strain RH102. *Escherichia coli* 739, *Pseudomonas aeruginosa*, *Bacillus subtilis* NCIM 2063, *Micrococcus flavus*, *Staphylococcus aureus*, *Dietzia* sp. K44 and *Candida albicans*. All the bacterial test organisms were maintained on nutrient agar at 30°C; *Candida albicans* was maintained on Sabouraud dextrose agar (SDA). All media were purchased from BD Biosciences, India.

Antimicrobial activity of CFS and the solvent extracts were carried out by well agar assay as described by Gillespie et al. (2002) with slight modification. The CFS and solvent extracts were applied as 50 µl drops in the well and incubated at 30 °C. Inhibition zones were measured after 48 h in case of *Dietzia* sp. K44 and with other organisms after 24h. Dimethylsulfoxide was used as negative control while tetracycline (30 µg) and vancomycin (30 µg) were used as positive controls for gram positive bacteria. Ampicillin (10µg), and amphotericin B discs (100 U, HiMedia Laboratories, India) were used as positive control against gram negative bacteria and fungi respectively. All the assays were carried out in duplicates and the antimicrobial activity was expressed as mean of the diameter of zones of inhibition (mm) produced by the extracts. Inhibition was reported as diameter of the clear zone around the well in mm and was scored using ProtoCOL System, Synoptics Limited, United Kingdom.

### Thin layer chromatography (TLC)

The TLC of the methanolic extracts was carried out on silica gel cards with fluorescent indicator 254nm (Fluka, USA) and developed using solvent system containing n-butanol: acetic acid: H<sub>2</sub>O (4: 1: 1 v/v). Air-dried chromatograms were examined under both short- and long wave UV light (Metrex UV cabinet, Metrex Scientific Instruments Pvt. Ltd., India) and stained in an iodine chamber and/or by ninhydrin spray.

### Effect of proteolytic enzymes on the antimicrobial activity of the compound

Sensitivity of the methanolic extract to proteinase K (Sigma, USA) and trypsin (Sigma,

USA) was tested according to Collado *et al.* (2005). Intense digestions were carried out following the suppliers' protocols. Equal volumes of autoclaved digestion buffers containing same quantity of enzymes were used as negative controls. The antibacterial activity was determined against *M. flavus* and *Dietzia* sp. K44 before and after the protease treatments.

#### Phylogenetic tree

The 16S rRNA sequences of *Brevibacillus* sp. reported till the date were used for construction of phylogenetic tree (Table 1) (Shida *et al.*, 1996, Logan *et al.*, 2002, Goto *et al.*, 2004, Allan *et al.*, 2005). The sequences of 16S rRNA were aligned using CLUSTAL W software (Thompson *et al.*, 1994). Evolutionary distance matrices were constructed

**Table 1: NCBI sequences used in this study**

Strain	Nucleotide sequence Accession no.	Reference
<i>Brevibacillus agri</i>	D78454	Shida <i>et al.</i> 1996
<i>Brevibacillus borstelensis</i>	D78456	Shida <i>et al.</i> 1996
<i>Brevibacillus borstelensis RH 102</i>	DQ144420	Present study
<i>Brevibacillus brevis</i>	D 78457	Shida <i>et al.</i> 1996
<i>Brevibacillus centrosporus</i>	D78458	Shida <i>et al.</i> 1996
<i>Brevibacillus choshiensis</i>	D78459	Shida <i>et al.</i> 1996
<i>Brevibacillus formosus</i>	D78460	Shida <i>et al.</i> 1996
<i>Brevibacillus invocatus</i>	AF378232	Logan <i>et al.</i> 2002
<i>Brevibacillus laterosporus</i>	D78461	Shida <i>et al.</i> 1996
<i>Brevibacillus levickii</i>	AJ715378	Allan <i>et al.</i> 2005
<i>Brevibacillus limnophilus</i>	AB112717	Goto <i>et al.</i> 2004
<i>Brevibacillus parabrevis</i>	D78463	Shida <i>et al.</i> 1996
<i>revibacillus reuszeri</i>	D78464	Shida <i>et al.</i> 1996
<i>Brevibacillus thermoruber</i>	DZ26921	Shida <i>et al.</i> 1996

using the algorithm of Jukes & Cantor and evolutionary trees for the datasets were inferred from the neighbor joining method using MEGA version 3 (Kumar *et al.*, 2004). The stability of relationships was assessed by performing bootstrap analysis of neighbor joining data based on 1,000 replications.

#### Nucleotide sequence accession number

The 16S rDNA sequence obtained in this study was deposited in the GenBank nucleotide sequence database under accession number **DQ144420**.

## RESULTS

#### Isolation and characterization of bacteria

The isolated strains were capable of growing on tryptic soy medium but only one bacterium was found to produce antimicrobial compounds after 24 h of its growth. This thermophilic bacterium was gram positive, having optimum growth temperature and pH of 55°C and 8.0, respectively. It showed positive reactions of urease, catalase, amylase, gelatinase but did not produce lipase, and was designated as RH102 (Table 2).

**Table 2: Characteristics of *Brevibacillus borstelensis* RH102**

Properties	Results
Gram Staining	Positive
Morphology	Rods
Colony colour	Yellowish
Glossy	-
Optimum temperature	55°C
Optimum pH	8
Catalase Reaction	Positive
Urease Reaction	Positive
Hydrolysis of starch	Positive
Hydrolysis of tributyrin	-
Hydrolysis of gelatine	Positive

Sequence analysis of the 16S rRNA gene was carried out for identification of the organism. BLAST search for the partial 935 nt 16S rDNA sequence revealed 99% sequence homology to *Brevibacillus borstelensis* R-16402.

**Agar well diffusion assay**

The CFS as well as extracts obtained with petroleum ether and ethyl acetate showed no antimicrobial activity against the test organisms. Chloroform extract showed very low activity (6-8mm) against *M. flavus*, while methanol extract was found to have good activity against the gram positive bacteria, *M. flavus*, *S. aureus* and *Dietzia* sp. K44 Table 3.

**Table 3: Well agar diffusion assay**

Target organisms	Inhibition zone diameter (mm)*				
	A	B	C	D	E
<i>E. coli</i> 739	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-
<i>M. flavus</i>	-	-	-	†	24.5
<i>B. subtilis</i> NCIM 2063	-	-	-	-	-
<i>S. aureus</i>	-	-	-	-	14.69
<i>Dietzia</i> sp. K44	-	-	-	-	16.5
<i>C. albicans</i>	-	-	-	-	-

A: Cell free supernatant, B: Petroleum ether extract dissolved in DMSO, C: ethyl acetate extract dissolved in DMSO, D: CHCl<sub>3</sub> extract dissolved in DMSO, E: Methanolic extract dissolved in DMSO

\* Mean of inhibition zone diameters including cup diameter of 6mm

† Inhibition zone was not greater than 8mm

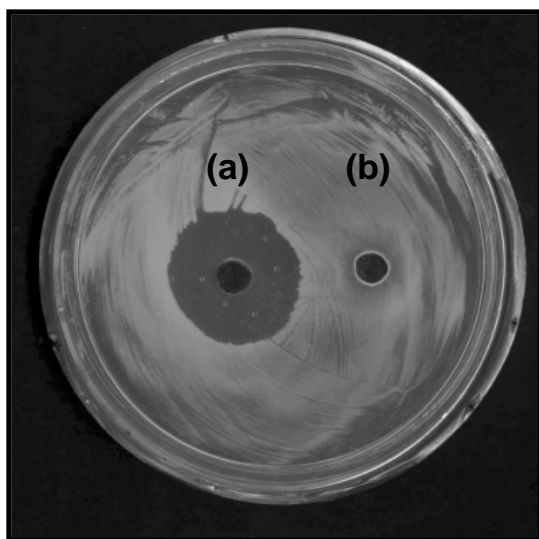
As the methanol extract was found to be mostly soluble in water, antimicrobial activity was ascertained with aqueous solution (Fig. 1). However, both PBS and DMSO solutions also showed equally potent inhibitory activity (data not shown). Antimicrobial activity of the methanol extract and its solubility in aqueous solvent suggest the polar nature of the active compound(s).

Tetracycline and vancomycin showed inhibition zone diameters ranging from 19 to 28 mm.

Ampicillin showed 20 mm diameter inhibition zone. Inhibition zone of amphotericin B was 14 mm against *C. albicans*. DMSO showed no inhibitory effect (results not shown).

**Characterization of antimicrobial compound**

The TLC profile of the methanolic extracts showed presence of components with a wide range of retention factor ( $R_f$ ) values that were visible under UV light and also when exposed to iodine vapours. These spots with the  $R_f$  values being 0.575, 0.497,



**Fig. 1: Antibacterial activity of methanolic extract against *Micrococcus flavus*.**  
(a) Inhibition zone (b) Control

0.26 and 0.0958 respectively, were also found to take ninhydrin stain. Yellow coloured spots revealed on exposure to iodine vapours and violet coloured spots revealed on spraying with ninhydrin corresponded to spots visible under UV light.

#### **Effect of proteases on antimicrobial compound(s) from *Brevibacillus* sp. RH102**

The inhibitory activities of methanolic extract on treatment with proteases are compiled in Table 4. Methanolic extract treated with trypsin was found to show a reduction in the activity against gram-positive bacteria. The zone of inhibition zone was found to reduce to almost half after treatment with trypsin.

Treatment of methanolic extract with proteinase K led to an increase in the zone of inhibition against gram-positive bacteria. To see the effect of proteinase K alone on the micro organisms, it was used as control and was found to be inhibitory.

**Table 4: Effect of proteolytic enzymes on the activity of antimicrobial compound from *Brevibacillus borstelensis* RH102**

Test	Mean of diameters of inhibition zone (in mm) against	
	<i>M. flavus</i>	<i>Dietzia</i> sp. K44
Methanolic extract	24.43	17.29
Proteinase K + methanolic extract	29.72	22.63
Proteinase K + water	22.10	16.04
Trypsin + methanolic extract	13.51	9.71
Trypsin + water	0.00	0.00

When the zone of inhibition of proteinase K was deducted from that of methanolic extract treated with proteinase K, there was a significant decrease in the activity against the test organisms, *M. flavus* and *Dietzia* sp. K44 (7.62 and 6.59 respectively) indicating that the active compound(s) responsible for antimicrobial activity was being degraded by proteinase-K.

#### **Phylogenetic analysis based on 16S rRNA gene**

To further identify the proximity of RH 102 with other *Brevibacillus* sp., representatives of all

isolates belonging to the genus *Brevibacillus* on the basis of 16S rRNA gene sequences were taken into consideration for constructing phylogenetic tree (Fig. 2). Analysis of the strain RH 102 reiterates its close relationship to *Brevibacillus borstelensis* and *Brevibacillus thermoruber*, the other two thermophilic bacteria and strains belonging to the same genus.

#### **DISCUSSION**

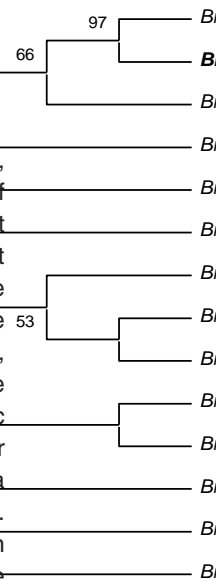
The antimicrobial activity of *Brevibacillus borstelensis* RH 102 was identified and the nature

**Fig. 2: Unrooted neighbor-joining phylogenetic tree constructed by using 16S rRNA gene sequences of species belonging to *Brevibacillus* genus. The names of bacterial strains and the DDBJ, EMBL and Gen Bank accession numbers are indicated in Table 1. The distance scale indicates 0.1% nucleotide substitutions. Numbers at nodes indicate percent bootstrap values above 50 (1000 replicates). Strain isolated in this study is indicated in bold letters.**

of its antimicrobial metabolite(s) was partially characterized. This is the first study to report the production of antimicrobial compounds from thermophilic *Brevibacillus borstelensis*. Although micro organisms producing antimicrobial compounds have been studied in the past, little has been studied about antimicrobial activity of thermophilic bacteria.

The TLC of the methanol extracts of strain RH 102 resolved it into four major spots that were visible when exposed to ninhydrin stain. Ninhydrin (1, 2, 3-triketo-hydrindene monohydrate) has been recognized since around the turn of the century as a reagent for detecting amino acids. It reacts with free amines to produce a deep blue or purple color known as Ruhemann's purple (Kaiser *et al.*, 1970). Production of purple spots on exposure to ninhydrin suggests the presence of  $-NH_2$  groups and hence the proteinaceous nature of the compound.

On treatment with proteolytic enzymes, there was a marked decrease in the activities of antimicrobial metabolite(s) against the test organisms. It is well known that trypsin acts against any peptide bonds in protein molecules next to lysine and arginine (Wilson, 1995). In case of proteinase K, the enzyme itself was inhibitory to the bacteria, as it is a versatile protease that cleaves peptide bonds at carboxylic sides of any aliphatic or aromatic amino acids. Thus proteinase K chops off the outer peptidoglycan layer of the gram positive bacteria resulting in an inhibition zone because of cell lysis. When the effect of proteinase K was subtracted from the overall effect of antimicrobial metabolite containing proteinase K, there was a reduction in the inhibition zone diameter of the antimicrobial compound(s). In case of both *M. flavus* and *Dietzia* K44, the decrease in the zone size indicates degradation of the active compound by proteases thereby confirming its proteinaceous nature.



The proteinaceous nature of the antimicrobial compound might be responsible for its selective activity against gram positive bacteria. Gram positive bacteria have a porous outer peptidoglycan layer, which does not impede movement of molecules less than 50 kDa. However, gram negative bacteria are surrounded by negatively charged lipopolysaccharides (LPS) in their outer membrane that function as efficient permeability barriers (Hogan and Kolter 2002).

The antimicrobial peptide(s) produced by thermophilic RH 102 against *M. flavus*, *S. aureus* and *Dietzia* sp. K44 can have potential applications with regard to its selective spectrum against gram positive pathogens. *Dietzia* sp. K44 bears close similarity to *Dietzia maris*, an actinomycetes that has been twice reported as nosocomial pathogen (Pidoux *et al.* 2001) and as an aetiological agent of reticulated papillomatosis (Natarajan *et al.* 2005).

Research on selective spectrum antibiotics would be more advantageous as they are effective against only a tapered class of pathogens. This approach becomes more rational with rapid advances in real time diagnosis, which would pave way for faster diagnosis of diseases in the near future. Availability of narrow spectrum antibiotics would prevent misuse of broad-spectrum antibiotics, thereby slowing the emergence of clinically significant resistant pathogens (Walsh 2003). This study summarizes evidence to the proteinaceous nature of antimicrobial substances produced by RH 102 and might be considered sufficient for further investigations aimed at purification and characterization of the active fractions.

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