A salient method to prevent Nosocomial infection causing pathogens: Bacillus from Mesophilic soil

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Abstract:

The focus of this study was the in vitro antimicrobial activities of Bacillus, bacteria commonly found in the soil and not a very well known for producing antibiotics. Bacillus isolated from mesophillic soil of rajasthan were evaluated for their inhibitory activities on three microorganisms which posses many antibiotic resistances potential are *E. coli, P. aeruginosa and S. aureus*. Three isolates exhibited antibiotic activity against at least one of the test organisms were characterized by conventional as well as advanced methods. Results indicated that one isolates was highly active against E. coli strains. Another isolate showed antibiotic activity against S. aureus strains including methicillin resistant *Staphylocoocus aureus* (MRSA) and is having biological activity against P. aeruginosa. Another isolates was highly active with an inhibition zone more than 16 mm in diameter. Compounds responsible for biological actiivity are formed by these isolates were confirmed by contact TLC microbial autobiography method and check by drug library of compounds. The bacterial isolates having antimicrobial activity were sent to **Vision Ecologica Pvt Ltd, 8D Raisoni Industrial Park, Village Maan, Rajiv Gandhi IT Park, Phase II, Pune** with ID code for identification based on 16S rRNA and was processed, and the report for the same was provided confirming the isolates were of bacillus. Identification report was generated using EZ-Taxon Database and the confidence in identification is limited by both the availability and the extent of homology shown by the ~700 bp sequence of our sample with its closest neighbor in the database. For details and sequence fasta, please see the results and discussion part.

Keywords: Antimicrobial, infection, Nsocomial, Antibiotic, Multidrug resistant.

Introduction

In 21st century major global healthcare problem is bacteria which are resistant to many day to day use antibiotics and they are responsible to cause severe infections **(1)**. Staphylococcus aureus, for instance, a virulent pathogen that is responsible for a wide range of infections including hospital acquired infections and bacteremia has developed resistance to most classes of antibiotics **(2**). Split up in proteins, namely proteolytic activity is seen mostly in the members of Bacillus genus found in the soil. Protease enzymes not only have essential industrial uses, but also the proteases of these microorganisms take part in an important function in the nitrogen cycle, which contribute to the productiveness of the soil. Most of the nitrogen source is stored as biomass protein and decomposes gradually to low molecular mass amino acids by the activity of soil protease. Soil protease is thought to be mainly supplied by soil microorganisms **(3-6).** While many antibiotics are known to exist, efforts to discover new antibiotics still continue. Antibiotics have been recognized as the only means of effective microbial growth control **[7],** after the discovery of penicillin and other antimicrobial agents by Alexander Fleming in 1928 **[8].** Since that time to date, there has been continues search for more effective antibiotics that can stand the emerging menace of drug resistance among microorganisms world wide **[9, 10, 11]** Therefore, many species such as Streptomyces, Bacillus and Penicillium have been studied continuously for their ability to produce antibiotics **(12)**. In addition, due to the fact that Bacillus species have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective in studies conducted to date, these microorganisms are preferable for commercial production. Currently, the target is to produce antibiotics such as polymyxin and bacitracin from Bacillus **(13, 14).**

It was reported that members of the species Bacillus generally produced polypeptide type bacteriocines, and that these antibiotics generally affect gram (+) bacteria **(10, 15)**. It was also reported that since most Bacillus species populate the same ecosystems as Streptomyces and other antibiotic producers, they might have acquired resistance to antibiotics produced under natural conditions **(16).** Studies conducted to date have shown that the resistance in many bacteria against antibiotics, bacteriocine production fertility and many specific biochemical functions were controlled by plasmid DNAs **(17).**.

Bacillus species are gram-positive aerobic or facultative anaerobic, sporulating rod shaped bacteria that are widely spread in nature **[18, 19]**, being implicated in food poisoning **[20, 21]**. Bacillus species exhibit a wide range of physiologic abilities that allow the organism to flourish in every environment and compete favorably with other organisms within the environment, due to its ability to form spores produce metabolites that are heat stable, cold, radiation, and desiccation disinfect ants and have antagonistic effect on other microorganisms **[7].** Resistance

to antibiotics has resulted in morbidity and mortality from treatment failures and increased health care costs **(22).**

Microbial communities are in close contact with soil micro-environments, and thus easily liable to undergo alterations if there are changes in soil chemical properties **(23)**. Thus, the type of area of soil collection and its geographic as well as physiological conditions are likely to affect the microbial parameters such as respiratory capacities, microbial biomass and extracellular enzymatic activities (**24, 25**). Nutrient availability often plays a critical role in regulating the microbial community structure and function **(26, 27, 28)**. Bacteria are easy to isolate culture and maintain, also allow human techniques to improvise their strains. Microbes are omni-present and exist in a competitive environment. Bacillus species being the predominant soil bacteria, because of their ability to produce resistant endospore and imperative antibiotics like bacitracin etc., are always found inhibiting the growth of the other organisms.

The greatest limitation while studying the soil micro-flora is the inability to culture them in *in-vitro* conditions. Almost 99% of the actual soil microbial population can't be grown under lab conditions and we are only able to grow 1% of the microbial species (**29**). Among the immense pool of secondary metabolites obtained from microorganisms, antibiotics are the ones most exploited at the commercial level. Surprisingly, most of these antibiotic producing bacteria and other species have been isolated from soil. Many potential fungal strains are used for synthesis of antibiotic drugs on large scale. The ability of these microbes to synthesize such compounds is due to presence of antibiotic biosynthesis genes (**30**). In the prsent study, we describe the isolation Bacillus strains from mesophillic soil of Rajasthan, India region having antimicrobial activities gram positive and gram negative hospital acquired pathogens. Identification of these isolates was done by conventional and molecular methods, as well as the optimal conditions for antimicrobials formation.

Materials and Methods

Sample Collection

Soil samples were collected in sterile air tight plastic bags by sterile showel from four locations of Jaipur, Rajasthan i.e. slum area of jawahar circle, SMS hospital, Durgapura and Mansarovar. The Samples collected were named as S-I, S-II, S-III and S-IV respectively. Selection of site was also based on various factors, temperature of the particular area, population load around the area, presence of any organic or inorganic additive/contaminant to the soil of the particular region, ease of repetition of sample collection, ease of accessibility of the area. Soil was collected from 4 cm deep each of these locations. It was then brought to the laboratories and stored at 4^{o}C till further processing.

Isolation of microorganisms

Suspension of all the four soil samples were prepared using physiological and followed by serial dilution method. Spread plate technique on nutrient agar plates was used for the isolation of microorganisms. Serial tenfold dilution of mesophilic soil samples were spread on sterile nutrient agar and incubated at 36^{o}C for 24 hrs. Total 29 isolates were obtained out of which only six were morphologically dominant which were selected for further study.

Identification

Out of the six isolates, all the four isolates were gram positive, confirmed by gram staining. All the isolates were assigned a specific number according to sampling location i.e. S-I (Jawahar Circle) A, B, C, D, S-II (SMS) A, B, C, D, S-III (Durgapura) A, B, C, D, S-IV (Mansarovar) A, B, C, D. All the isolates were identified by carrying out several biochemical tests and microscopic morphological studies and assesment by Bergy's manual.

Isolates were checked for the presence of enzyme like catalase, urease, oxidase and hydrolase qualitatively by standard method.

Optimization of cultural conditions

Optimum temperature, pH, NaCl concentration required for optimum growth was checked for all isolates by standard method. Different temperatures, pH, NaCl concentrations were used during optimization.

Fermentation

The colonies screened from the dilutions of soil sample were inoculated in Luria broth. Shaker treatment for 2 hrs daily at 700 rpm was provided for 30 days at different temperature ranges. Regular testing of metabolites (primary/secondary) was done after 7 days, 15 days, 21 days, and 27 days.

Isolation of Mixture containing Bioactive compounds

After 27 days of incubation, each culture was centrifuged at 8000 rpm for 10 minutes and supernatant was collected separately. Metabolites were extracted using three solvents i.e. benzene, ethyl acetate and chloroform. Supernatant was mixed in 2:1 ratio with each of the three solvents, shaken and allowed to mix properly. The mixture was left undisturbed to allow the separation of the solvent having the dissolved metabolites from the culture. The solvent was

then decanted from the culture and allowed to vaporize at 40-50°C in the oven. The method used for separating bioactive compounds from extracellular secondary metabolites was Liquid-liquid extraction.

Antimicrobial Analysis

The dried form of compounds collected was again mixed in 2-3 ml of respective solvents. Sterilized circular discs were cut and soaked in solvents containing bioactive compounds. Lawn of five common nosocomial infection causing pathogens i.e. *Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis, Pseudomonas aerogninosa* and *E. coli* was prepared on nutrient agar. The antimicrobial activity was then tested against these pathogens using disc diffusion method. Plates were incubated at 38°C for 48 hrs.

Contact TLC bio-autographic detection

The procedure in bio-autographic methods is similar to the one used in agar diffusion method. Bio-autography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity. Contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact. Ten µl (10 mg/ml) of each extract was loaded onto TLC plates in a narrow band and eluted using the three different mobile solvent systems (specific). The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. Fresh cultures of pathogenic bacteria i.e. *E. coli* and *Pseudomonas aeruginosa* were spread over agar plates. Developed TLC plates were cut like stripes of 0.8 cm width in a way that the compounds separated should not disrupt. Now, gently merge the TLC plates perpendicularly in the agar plates containing lawn of pathogenic cultures (as shown in figures). Kept the plates for 24 – 36 hours and observe the zone of inhibition

Test pathogens

Pure cultures of five test pathogens were obtained from Department of Microbiology, JECRC University, Jaipur and SMS Hospital. These included *Pseudomonas aeruginosa* MTCC 7093, *Staphylococcus aureus* MTCC 7443, *Escherichia coli* MTCC 40, *Klebsiella pneumoniae* MTCC 530, *and Bacillus subtillis* MTCC 121. Microbial isolates were tested against these five bacteria using disc diffusion, well diffusion and perpendicular cross streak methods.

Separation and Characterization of bioactive compounds by TLC

Centrifugation

Isolates kept for fermentation were centrifuged at 8000 rpm for 10 min. Supernatants were treated with LLE I, LLE II and LLE III i.e. benzene, ethyl acetate and chloroform respectively.

Thin Layer Chromatography (TLC)

Glass plates of 18 cm ×18 cm were used to perform TLC, so that approximately 4 samples could be run together. Slurry was made with silica gel and water. Mixing and shaking of silica gel in water should be proper for homogenous and adhesive mixture. Thin layer on glass plates were formed and kept for 3-4 hrs on the plain surface for drying and later in the oven at 70°C. The temperature of the oven was raised to 110 ^{0}C for 1 hour for activation of the plates. Taken the plates out and allowed to cool. Mobile phase (solvent) prepared and poured in TLC glass chamber in which plates spotted with extract were placed. The plates were placed in the chamber till it run or develop upto ¾ of the TLC plate and then again kept in oven for drying. Plates were sprayed with specific reagents and spots were observed. The developed plate was baked at 110 ^{0}C for half hour and observed under UV light chamber and the displacement of development (mobile phase) and extract were measured and recorded.

Soil Sampling Location	Soil Sample I.D.	Total No. of bacterial isolates	Isolate I.D.
JC slum area	S – I	7	A,B,C,D,E,F,G

Results

Table 1: Isolation of microorganisms

SMS hospital	S – II	8	A,B,C,D,E,F,G,H
Durgapura	S – III	7	A,B,C,D,E,F,G
Mansarover	S – IV	7	A,B,C,D,E,F,G

Table 2: Isolation of extract containing bioactive compounds

Extracts	**Benzene**	**Ethyl Acetate**	**Chloroform**
Sample	**Extract/100mL**	**Extract/100mL**	**Extract/100mL**
S-1 E	0.07	0.06	0.02
S-II A	0.19	0.04	0.05
S-IV B	0.16	0.15	0.16

Table 3: TLC R_f value and color of spot

Isolates	**R_f Value**			**Colour of spot**		
	Benzene	E.A.	Chloroform	**Benzene**	**E.A.**	**Chloroform**
S-1 E	-	0.3	-	-	Dark brown	-
S-II A	0.4	0.13	-	N.D.	Dark orange	-
S-IV B	-	-	0.09	-		Grayish

Table 4: Test pathogens

Test pathogens	**MTCC code**	**Pathogen I.D.**
Pseudomonas aeruginosa	MTCC 7093	P1
Staphylococcus aureus	MTCC 7443	P2
E. coli	MTCC 40	P3
Klebsiella pneumonia	MTCC 530	P4
Bacillus subtilis	MTCC 121	P5

Table 5: Antimicrobial analysis

Identification of Isolates shown antibiotic activity		**Zone of inhibition(cm)**				
		Test Pathogens				
		P1	**P2**	**P3**	**P4**	**P5**
Soil samples	**S – I E**	1.1	0	0	0	0
	S – II A	0	0	0.5	0	0
	S – IV B	0	.6	0	0	0

Table 6 showing results of Biochemical tests of screened isolates

Sample Location		**Catalase Test**	**Of Basal Test**	**SIM Test**	**MacConkey Test**	**Starch Hydrolysis**	**Motility**	**Mannitol Test**	**Urease Test**	Tentative Microbe
	A	-	+	Red	-	+	-	+	+	Actinomycetes
	B	-	+	Black	-	+	-	-	+	Actinomycetes

S – I	C	-	-	Red	+	-	-	-	-	Enterobacter
	D	+	-		+	+	+	-	-	Vibrio
	E	+	-	Black	-	-	-	-	-	Bacilli
	F	-	+		-	-	-	+	-	Enterobacter
	G	+	-	Black	+	+	+	+	+	Actinomycetes
S – II	A	-	+	Red	-	-	-	+	-	Bacilli
	B	-	+	Black	+	-	-	+	-	Enterobacter
	C	+	-	Red	+	-	+	+	-	Vibrio
	D	-	+	Red	-	-	+	-	-	
	E	-	-	Black	+	-	-	-	+	Enterococci
	F	+	+	Black	-	-	-	-	+	Actinomycetes
	G	+	-	Red		+	+	-	+	Bacilli
	H	-	+	Red	+	+	-	+	-	
S – III	A	-	-	Red	+	+	-	+	-	Enterobacter
	B	-	-	Black	-	+	-	-	+	Streptococci
	C	+	-	Red	-	+	+	-	-	Enterobacter
	D	+	-		+	-	-	-	-	Enterobacter
	E	+	-	Red	+	+	+	-	-	Vibrio
	F	-	+	Black	+	+	-	-	-	Enterobacter
	G	+	+	Black	+	+	+	-	-	
S – IV	A	-	+	Red	+	-	+	-	-	Bacilli
	B	-	-	Black	-	+	+	-	-	Bacilli
	C	+	+	Red	-	+	-	+	-	Enterobacter
	D	+	-		+	+	-	-	+	Enterobacter
	E	+	+		+	-	-	-	+	Stephylococci
	F	-	-		-	-	-	-	+	Stephylococci
	G	-	+	Black	+	+	-	-	-	Actinomycetes
	H	-	+	Red	-	+	-	+	+	pseudomonas

Table 7: showing morphological characteristics of screened isolates

Sample Location		Shape	Colour	Opacity	Elevation	Surface	Consistency/Texture	Gram + ve/-ve
S – I	A	Filamentous	white	opaque	Negligible	rough	viscid	positive
	B	Filamentous	white	Translucent	Negligible	Smooth	Butyrous	positive
	C	Rods	Buff	Opaque	curve	rugose	Brittle	negative
	D	Rods curve	Buff	Opaque	curve	Glistening	Brittle	negative
	E	Rods	White	Translucent	Negligible	Smooth	Butyrous	positive
	F	Rods	Buff/orange	Translucent	curve	Rough	Brittle	negative
	G	Filamentous	Buff	Translucent	Negligible	Rough	Viscid	positive
	A	Rods	Buff/yellow	Opaque	curve	Smooth	Butyrous	positive

S – II	B	Irregular	Orange	Opaque	curve	Smooth	Butyrous	negative
	C	Rods	White	Translucent	Negligible	Rough	Brittle	negative
	D	curve	Buff/yellow	Translucent	Negligible	Rough	Mucoid	negative
	E	Irregular		Translucent	Negligible	Rough	Brittle	positive
	F	Round	Buff	Translucent	Negligible	Smooth	Butyrous	positive
	G	Filamentous	white	Translucent	Negligible	Glistening	Mucoid	positive
	H	Rods	White	Translucent	Negligible	Rough	Viscid	negative
S – III	A	Irregular	Buff	Opaque	curve	Rough	Brittle	negative
	B	Rods	White	Opaque	curve	Smooth	Brittle	positive
	C	Round	White	Opaque	curve	Smooth	Butyrous	negative
	D	Rods	Buff	Opaque	curve	Smooth	Butyrous	negative
	E	Rods	yellow	Opaque	Negligible	Smooth	Butyrous	negative
	F	curve	yellow	Opaque	Negligible	Smooth	Butyrous	negative
S – IV	A	Rods	Buff	Opaque	curve	Smooth	Butyrous	negative
	B	Rods	White	Opaque	curve	Rough	Viscid	positive
	C	Rods	Buff	Translucent	Negligible	Smooth	Brittle	positive
	D	Rods	Buff	Translucent	Negligible	Smooth	Viscid	negative
	E	Rods	Buff	Translucent	Negligible	Rough	Brittle	negative
	F	Round	White	Opaque	Negligible	Smooth	Butyrous	positive
	G	Round	Orange	Opaque	curve	Smooth	Butyrous	positive
	H	Irregular	White	Translucent	Negligible	Rough	Brittle	positive
		Rods	Green	Translucent	Negligible	Smooth	Butyrous	negative

Table 8 Summary of the closest neighbour(s) for sample S-I E from Ez Taxon:

Sl. No.	Name	Strain	Accession	Pairwise Similarity (%)	Diff/Total nt
1	*Bacillus licheniformis*	ATCC 14580(T)	AE017333	99.53	3/638
2	*Bacillus sonorensis*	NBRC 101234(T)	AYTN01000016	99.37	4/638
3	*Bacillus aerius*	24K(T)	AJ831843	99.21	5/636
4	*Bacillus dabaoshanensis*	GSS04(T)	KJ818278	99.06	6/638
5	*Bacillus*	LMG 18435(T)	AJ250318	98.74	8/636

shackletonii

Table 9 Summary of the closest neighbour(s) for sample S IIA from Ez Taxon:

Sl.No.	Name	Strain	Referance	Accession No	PairwiseSimilarity (%)
1	*Bacillus Cereus*	ATCC 14579(T)	Frankland and Frankland1887	AE016877	99.27
2	*Bacillus anthracis*	ATCC 14578(T)	Cohn 1872	AB190217	99.16
3	*Bacillus anthracis*	Ames	Cohn 1872	AE016879	99.12
4	*Bacillus thuringiensis*	ATCC 10792(T)	Berliner 1915	ACNF01000156	98.98
5	*Bacillus toyonensis*	BCT-7112(T)	Jiménez et al. 2014	CP006863	98.98

Table 10 Summary of the closest neighbour(s) for sample S-IV B from Ez Taxon

Sl. n.	Name	Strain	Reference	Accession no.	Pairwise similarity%
1	*Bacillus subtilis* subsp. inaquosorum	KCTC 13429(T)	Rooney et al. 2009	AMXN01000021	99.68
2	Bacillus tequilensis	KCTC 13622(T)	Gatson et al. 2006	AYTO01000043	99.68
3	*Bacillus subtilis* subsp.subtilis	NCIB 3610(T)	(Ehrenberg 1835)	ABQL01000001	99.59
4	*Bacillus subtilis* subsp. spizizenii	NCIB 3610(T)	Nakamura et al. 1999	CP002905	99.51
5	*Brevibacteriumha lotolerans*	DSM 8802(T)	Delaporte and Sasson 1967	AM747812	99.51

Isolation, selection and biochemical testing was done for primary screening of microorganisms. Main objective is to isolate a microorganism which produces compound which can be useful in manufacturing drug against nosocomial infection causing pathogens. After plating methods, microbes were selected on the basis of their growth pattern and morphological features. 29 colonies were targeted as different among all the colonies. These 29 colonies were further sub-cultured to isolate as pure and for further testing. First objective was to find out the microbes having biological activity against some pathogens as well as nosocomial infection causing pathogens. 29 isolates were tested through different activity test methods against 5 test pathogens i.e. E.coli, *Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia and Staphylococcus aureus*. This narrowed down the search for extraction of compound of main interest. Out of 29 colonies, 3 showed biological activity against above mentioned 5 test pathogens.

For secondary screening, these 3 isolates were processed through fermentation process with regular shaker treatment for a specific duration and different incubation period and temperature. Centrifugation followed by extraction with solvents LLE – I, LLE – II and LLE – III respectively. Activity confirmation was performed by Contact TLC microbial bioautography method from the extracts of culture containing compounds this time, against five most common nosocomial infection causing pathogens. This test is important test for confirming which isolate is actually forming secondary metabolites or compounds and under what conditions it is responsible for activity against the two test pathogens.

Above strategies and tests confirmed that the isolates S – I A, S – II A and S – IV B are having the compounds which need to be targeted. For detailed information about the compounds they were processed through TLC, contact microbial. Applications and medical significance of every compound was analyzed. Isolates which shown biological

activities against Nosocomial infections pathogens were tentatively identified on the basis of biochemical tests performed and their features sutdy under microscopic analysis. After primary and secodary screening these isolates were for 16s RNA sequencing for the detailed identification and genome sequencing.

CONCLUSION

From the experiment it can be concluded that all the compounds detected in Sample S – I A, S-II A and S-IV B are responsible for bioactivity against nosocomial infection causing pathogens. Similarly, above mentioned activity tests clearly shows the number of biological active isolates. Contact TLC microbial autobiography method is used to confirm the biological activity against the pathogens among the all isoates.Tentative identification of active isoltes was done by biochemical tests and microscopic studies. Identified active isolates were sent to **Vision Ecologica Pvt Ltd, 8D Raisoni Industrial Park, Village Maan, Rajiv Gandhi IT Park, Phase II, Pune** with ID code for identification based on 16S rRNA and was processed, and the report for the same was provided confirming the isolates were of bacillus. Identification report was generated using EZ-Taxon Database and the confidence in identification is limited by both the availability and the extent of homology shown by the ~700 bp sequence of our sample with its closest neighbor in the database. The whole experiment confirms that the bacillus strain isolated from mesophilic soil of rajasthan (sample area mentioned above) has the potential to inhibit the growth of Nosococomial infection causing pathogens.

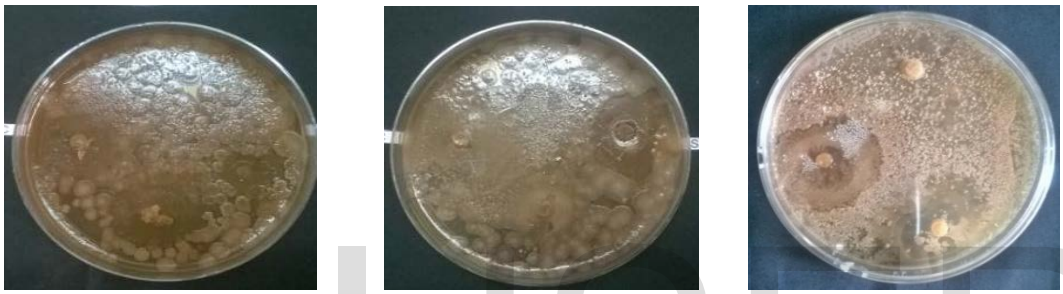

Figure 1: Clear zones showing antimicrobial activity against lawn of test pathogens.

Figure2: contact TLC microbial autobiography results.

Squence 1.
S-I E
Reference:

Kim et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with Phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 62: 716–721.

Sequence (in FASTA format):

>D1_08_15_003
CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
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CAAACGCAT

Squence 2
S IIA
Reference:
Kim et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database withphylotypes that represent uncultured species. Int J SystEvolMicrobiol. 62: 716–721.
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>D1_09_15_020
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AAAACCGTTCTCAGTTCGGATTGTAGCGTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATG
CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT

Squence 3.
S-IV B
Reference:
Kim et al. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database withphylotypes that represent uncultured species. Int J SystEvolMicrobiol. 62: 716–721.
Sequence (in FASTA format):
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TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CGTGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCC
CCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCGTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGA
AACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC

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Conflict of Interest

There is no conflict of interest

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