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Characterization of Culturable Thermophilic Actinobacteria from Livingston Island, Antarctica

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Abstract

Thermophilic microorganisms in Antarctica are poorly investigated. This paper reports partial characterization and biosynthetic abilities of 26 thermophilic actinobacteria isolated from soil and penguin excrements samples from Livingston Island, Antarctica. About 15% of the tested Antarctic actinobacteria were able to tolerate up to 4% NaCl in the growth medium. We found that all strains are sensitive to eight, and 77% to ten of the 12 tested antibiotics suggesting relatively low anthropogenic impact in this Antarctic region. The Antarctic actinobacteria were tested for hydrolytic enzymes activity, antibiotic and hemolytic activity. It was found that all strains were able to hydrolyze starch, 81% - tributirin, and 65% - casein. All Antarctic actinobacteria demonstrated hemolytic activity, and about 27% - antimicrobial activity against some common bacterial pathogens. The results obtained revealed promising strains producers of industrially important thermostable enzymes and antibiotic compounds.

Keywords: Thermophilic actinobacteria, antarctica, antibiotic susceptibility, antimicrobial activity, hemolytic activity, hydrolytic enzymes activity.

Introduction

Actinomycetes are one of the most widely distributed groups of Gram positive, mainly aerobic, filamentous bacteria. They are known for their metabolic versatility enabling them to survive even under extreme environmental conditions. Many actinomycetes are ecologically important and are used for production of antibiotics and enzymes[1-3]. Microorganisms isolated from extreme environments have been found to be promising producers of enzymes and metabolites with novel properties[4-6]. In recent years, researchers have shown great interest in thermophilic actinomycetes because of their economical potential in useful biological processes such as biodegradation and waste treatment[7-9], and in production of antibiotics and enzymes[4, 10-12]. Thermophilic microorganisms are especially suitable for industrial application due to their rapid growth rate and reduced risk of contamination[13]. Actinomycetes have been isolated from a number of Antarctic environments[14,15]. In Antarctica, the presence of thermophilic microorganisms including actinomycetes has been reported[16-18], which possibly due to several geothermal regions in Antarctica. Only few reports have shown the presence of thermophilic bacteria on this continent including volcanic Deception Island[16-22]. Previously, different ecological types of microorganisms including thermophilic actinomycetes were isolated in the Institute of Microbiology from different samples collected in the region of Livingston Island, and a culture collection was created[23,24]. In the present research, 26 thermophilic actinomycetes from this collection were characterized and their biosynthetic abilities investigated aiming to detect promising producers of thermostable industrially relevant enzymes and antimicrobial compounds.

Material and Methods

Collection of samples: Samples of soil and penguin's excrements were collected near the Bulgarian scientific station "St. Kliment Ohridski" in Livingston Island (62°36′S 60°30′W), South Shetland Islands, Antarctica, during several Bulgarian Antarctic Research Expeditions since 1998 year. The samples were shipped to the laboratory under ice and stored frozen at –20°C in sterile containers, until processing.

Isolation of thermophilic actinomycetes: Thermophilic actinomycetes used in this study were isolated previously in the Institute of Microbiology from soil (14 isolates) and penguin excrements (12 isolates) samples collected at Livingston Island, using peptone-corn steep extract agar (PCA) medium at 55°C, as described previously[23,24]. All isolated colonies were obtained in pure form by a series of regular subculture carried out on PCA. The identity of isolated cultures was confirmed using conventional methods of the morphological and cultural characteristics of each organism. The strains were stored at +4°C in PCA tubes and transferred after every three months onto fresh medium.

The effect of temperature and NaCl: The Antarctic actinomycetes were tested for their growth in PC liquid medium at different temperatures. After inoculation, the strains were cultivated for 24 h under shaking at temperatures 30, 40, 45, 50,

55, 60, 65 and 70°C. Growth of the strains was measured by monitoring the turbidity at 570 nm. Salt tolerance of the strains was tested on PCA medium containing 3, 4, 5 and 6% (w/v) NaCl. After inoculation, the tubes were incubated at 50°C for 3 days.

Antibiotic susceptibility assay: Susceptibility of the Antarctic actinomycetes to 13 antibiotics was tested using the conventional disk diffusion assay. The following antibiotics were tested: erythromycin, gentamicin, amikacin, tobramycin, lincomycin, tetracycline, chloramphenicol, vancomycin, novobiocin, ciprofloxacin, ampicillin and cefazolin. Disks impregnated with known amounts of antibiotics (BUL BIO, NCIPD, Ltd, Sofia, Bulgaria, table 1) were placed on the surface of the inoculated PCA plates. The plates were then incubated at 45°C for 24 h and the diameter of the inhibition zones was measured (including the diameter of the disks).

Antimicrobial and hemolytic activity assay: The Antarctic actinomycete strains were screened for antibiotic activity using conventional well diffusion assay as described previously[25]. Aliquots of cell suspensions (100 µl) were added into wells (6 mm in diameter) cut in PCA plates previously seeded (1% v/v) with the indicator cultures: *Bacillus subtilis* ATCC 6633, *Micrococcus luteus*, *Pseudomonas aeruginosa* NBIMCC 1390 and *Xanthomonas oryzae*. After incubation for 24 h at 45°C, the diameter of the growth inhibition zones was measured. For extracellular hemolytic activity testing, each actinomycete culture was streaked onto blood agar plates. After 24 h incubation at 45°C, the zones of hemolysis of erythrocytes were measured.

Hydrolytic enzymes activity assay: The production of extracellular hydrolytic enzymes by the Antarctic actinomycetes was determined by the agar well diffusion assay using specific substrates. After inoculation of each isolate on agar, the plates were incubated at 45°C for 24 h. Zones of clearing around the colonies on respective specific media were used as an indication of the presence of the tested enzyme activity. The production of extracellular protease was determined using 30% (v/v) skim-milk (caseinase activity). The α-amylase activity was determined after growth of the strains on 1% (w/v) soluble starch and detected as a clear halo surrounding colonies after adding iodine reagent[26]. Lipolytic activity was detected by tributirin degradation on PCA medium supplemented with 1% (v/v) tributirin.

Table-1
Growth temperature and antibiotics resistance profiles of thermophilic actinobacteria from Livingston Island, Antarctica

Actinomycete code	Source type	Optimum growth temperature (°C)	Antibiotic											
			Cp	G	Am	Tb	Nb	L	T	A	C	V	E	Cfz
			Inhibition zone (mm in diameter)											
3A	excrements	40-55	R (-)	S(21)	S(26)	I(20)	R (-)	R(7)	S(45)	S(30)	S(30)	S(25)	S(25)	S(37)
6A	excrements	40	I(18)	S(21)	S(35)	S(23)	R (-)	S(22)	S(55)	I(20)	S(27)	S(45)	S(25)	S(45)
9A	excrements	45	R(10)	I(20)	S(45)	S(28)	S(21)	R(12)	S(40)	S(26)	S(27)	S(30)	S(27)	S(50)
10A	soil	45-55	R(10)	S(26)	S(38)	S(28)	R (-)	I(16)	S(40)	S(40)	S(30)	S(30)	S(28)	S(40)
11A	soil	50	R(10)	S(30)	S(39)	S(28)	S(21)	I(18)	S(50)	S(40)	S(25)	S(30)	S(35)	S(40)
12A	soil	40-55	R (7)	I(18)	S(39)	S(28)	R(7)	I(18)	S(47)	S(40)	S(30)	S(30)	S(35)	S(50)
13A	soil	40	R (7)	S(26)	S(36)	S(30)	R (-)	S(21)	S(40)	S(40)	S(32)	S(30)	S(35)	S(45)
14A	soil	45-55	R(10)	S(30)	S(36)	S(22)	R (-)	I(20)	S(38)	S(40)	S(37)	S(38)	S(37)	S(38)
15A	excrements	40-55	R(12)	S(44)	S(45)	S(25)	R (-)	I(18)	S(40)	S(45)	S(45)	S(45)	S(45)	S(45)
16A	excrements	45-60	R(10)	S(30)	S(36)	I(20)	R (-)	I(16)	S(40)	S(40)	S(40)	S(39)	S(39)	S(41)
17A	excrements	55	R (-)	S(30)	S(41)	S(25)	R (-)	S(35)	S(40)	S(35)	S(30)	S(23)	S(35)	S(42)
18A	excrements	40-50	R(10)	S(26)	S(40)	S(42)	R (-)	S(28)	S(50)	S(37)	S(30)	S(27)	S(30)	S(50)
20A	excrements	40-55	R (-)	S(29)	S(35)	S(22)	R (-)	I(16)	S(35)	S(30)	S(40)	S(22)	S(25)	S(40)
22A	excrements	40	R (-)	S(22)	S(35)	S(22)	R (-)	I(18)	S(43)	S(31)	S(26)	S(22)	S(26)	S(45)
23A	excrements	50	I(16)	S(38)	S(40)	S(35)	R(10)	S(38)	S(50)	S(30)	S(35)	S(26)	S(25)	S(45)
24A	excrements	40	R (-)	S(23)	S(32)	S(21)	R (-)	S(39)	S(40)	S(38)	S(25)	I(20)	I(20)	S(45)
25A	excrements	40	R(8)	S(38)	S(40)	S(25)	R (-)	S(25)	S(50)	S(40)	S(35)	S(26)	S(32)	S(50)
26A	soil	40	R (-)	S(30)	S(40)	R (-)	R (-)	S(28)	S(45)	S(40)	S(32)	S(30)	S(45)	S(50)
29A	soil	45-55	R (-)	S(39)	S(50)	S(50)	R(9)	R(10)	S(50)	S(35)	S(50)	S(50)	S(30)	S(50)
31A	soil	40-45	R(8)	S(30)	S(28)	S(32)	R(10)	R (-)	S(52)	S(36)	S(35)	S(26)	S(40)	S(50)
33A	soil	40-50	R(8)	S(27)	S(32)	R(8)	R(9)	R(10)	S(45)	S(45)	S(45)	S(43)	S(45)	S(45)
34A	soil	40-45	R (-)	S(30)	S(46)	S(24)	R (-)	I(18)	S(45)	S(34)	S(29)	S(34)	S(27)	S(43)
35A	soil	40-55	R(10)	S(25)	S(40)	I(20)	R(8)	S(30)	S(40)	S(39)	S(40)	S(39)	S(45)	S(45)
36A	soil	40-60	R(10)	S(32)	S(58)	S(25)	R (-)	I(20)	S(45)	S(40)	S(40)	S(30)	S(30)	S(45)
37A	soil	40	R(11)	S(26)	S(36)	S(22)	R (-)	R(11)	S(40)	S(32)	S(32)	S(25)	S(30)	S(50)
39A	soil	45	R(10)	S(30)	S(31)	S(21)	R (-)	S(25)	S(50)	S(50)	S(50)	S(50)	S(40)	S(55)

Degree of susceptibility: S, sensitive (≥ 21 mm); I, intermediate (16-20 mm); R, resistant (≤ 15 mm). Cp, ciprofloxacin (5 µg); G, gentamicin (10 µg); Am, amikacin (30 µg); Tb, tobramycin (10 µg); Nb, novobiocin (5µg); L, lincomycin (15 µg); T, tetracycline (30 µg); A, ampicillin (10 µg); C, chloramphenicol (30 µg); V, vancomycin (30 mg); E, erythromycin (15 µg); Cfz, cefazolin (30 µg)

Results and Discussion

Growth temperature range and halotolerance: We found that all Antarctic actinomycetes are thermophilic in that they grew at 55°C[27]. The optimum temperature for growth of most strains ranges between 40 to 55°C (table 1).

The ash layers originating from the volcanic activity on the neighboring Deception Island are typical of the glaciology of Livingston Island. Thermal springs, hot soils and fumarolic emissions in Deception Island are evidence of its continuing activity, which provides excellent conditions for thermophilic bacteria[21,22]. Therefore, it is likely that thermophilic microorganisms are transported by ash particles from Deception Island to Livingston Island.

We found that all tested actinomycete strains tolerate up to 3% NaCl in the growth medium, and four strains (6A, 9A, 10A and 22A) tolerate up to 4% NaCl. It could suggest that halotolerance of microbial isolates is due to permanent impact of high salinity sea water during formation of ground microcenoses in maritime Antarctica.

Antibiotic susceptibility: The Antarctic actinobacteria were tested for antibiotic resistance as a measure of human impact in this Antarctic region. All strains were found to be sensitive to most of the 12 tested antibiotics (table 1). All cultures showed high sensitivity to cefazolin, tetracycline, amikacin and chloramphenicol (inhibition zone range 37-55 mm, 38-55 mm, 26-58 mm and 25-50 mm, respectively). 96% of the strains were sensitive to ampicillin, vancomycin and erythromycin (inhibition zone range 26-50, 22-50 and 25-45 mm, respectively), 92% were sensitive to gentamicin (inhibition zone range 21-44 mm), and about 80% were sensitive to tobramycin (inhibition zone range 21-50 mm). About 92% of the cultured actinomycete strains were highly resistant only to ciprofloxacin and novobiocin, 23% - to lincomycin, and less than 8% - to tobramycin.

The presence of antibiotic-resistant strains is widespread in the environment irrespective of the human use of antibiotics and may arise in response to various impacts. In some cases, antibiotic resistance can due to the presence of plasmids able to be horizontally transferred from one bacterium to another, even occasionally between phylogenetically distant bacteria, which greatly contributed to the widespread dissemination of antibiotic resistance genes in the environment[28-30]. Transfer of resistance to antimicrobial agents could be an essential mechanism of bacteria to adapt and survive in extreme environments[31]. Analysis and isolation of bacterial strains from areas with limited human activity could be a good indicator of the occurrence of anthropogenic impact on industry-free and clean natural areas, such as the Antarctic region[32]. Siebert et al.[33] have found that some of the bacteria isolated from Antarctic sand stone in McMurdo valley were resistant to one or more antibiotics. Multiple antibiotics strains of environmental bacteria were also found in the ice core from the Greenland[34], in the Arctic permafrost subsoil in Siberia[35], and in King George Island, Antarctica[36]. We found that the majority of the tested Antarctic actinobacteria are sensitive to most antibiotics used, which suggest relatively low anthropogenic impact in this Antarctic region.

Antimicrobial activity: The Antarctic actinobacteria were screened for antimicrobial activity against some common bacterial pathogens. The results showed relatively low potential of the tested strains to produce at the used conditions antimicrobial compounds (table 2).

Only one strain inhibited the growth of two indicator cultures, and six strains showed antimicrobial activity against one of the target cultures. Strains 14A, 34A and 36A showed significant inhibition zones (in the range 26-30 mm) against *Bacillus subtilis*. Three strains (14A, 16A and 36A) exhibited antimicrobial activity against *X. oryzae*, and two strains (10A and 17A) inhibited the growth of *P. aeruginosa*. Biosynthesis of antimicrobial compounds is a strategy of many bacteria to survive in the environment and compete with other microorganisms for resources[37]. A large number of actinomycetes have been isolated and screened in the past decades, accounting for 70-80% of relevant secondary metabolites available commercially[2,38]. The resistance problem demands discovery of new antimicrobial agents effective against resistant pathogenic bacteria and fungi. Isolation of microorganisms from unusual environments such as sediments from deep sea water, hyper saline areas, hot sulfur springs, glacier regions, are expected to be potential producers of novel secondary metabolites[39]. Actinomycetes that produce antibiotics have also been isolated from Antarctica[40,41]. The secondary metabolism of thermophilic microorganisms is poorly understood, and thermophilic actinobacteria from Antarctica are poorly explored for antagonistic activity[42-44]. In the present study, we detected thermoactinomycete strains promising as potential producers of antimicrobial compounds: strains 13A, 34A and 36A showing antibiotic activity against *B. subtilis*; 10A and 17A exhibiting antibiotic activity against *P. aeruginosa*. Strains 14A, 16A and 36A showing antibiotic activity against phytopathogenic strain *X. oryzae* are prospective for application in the agriculture for plant protection.

Hemolytic activity: All tested thermophilic actinobacteria exhibited relatively high hemolytic activity on blood agar plates (table 2). Of them, six strains showed broad hemolytic zone in the range 30-35 mm, 18 strains - in the range 20-28 mm, and two strains - in the range 16-18 mm. Hemolysis followed on blood agar plates has been widely used to screen microorganisms for production of surface-active compounds[45]. Due to their amphiphilic character, surfactants often induce hemolysis at a given concentration[46]. Surface-active compounds of microbial origin are of biotechnological importance due to their potential application in different industries[47]. The search for different types of surface-active compounds is important since their use in

industry requires different physicochemical properties. To the best of our knowledge, there are no reports on surfactants production by thermophilic actinobacteria from Antarctica. Six Antarctic thermophilic actinobacteria, 10A, 13A, 14A, 16A, 34A and 36A, showing hemolytic activity together with antimicrobial activity can be suggested as new potential surfactant-producing strains.

Hydrolytic enzymes activity: The Antarctic actinomycetes were screened for proteolytic, lipolytic and amylolytic activity on solid media. All strains showed broad zones of hydrolysis of starch; lipase activity on tributirin was detected in 81% of the strains, and caseinase activity -in 65% of the strains (table 3).

Microbial extracellular enzymes play important role in degradation and utilization of a wide range of organic polymers[48]. Patterns of enzyme activities can be a useful tool for assessing organic matter turnover in ecosystems[49]. Proteases, α-amylases and lipases are among the most important enzymes of great significance in present-day biotechnology, and several thermostable enzymes from thermophilic actinomycetes have been reported[10,50]. Thermophilic actinomycetes isolated from Antarctica have also screened for hydrolytic enzymes production[51]. In the present study, we detected several promising thermophilic actinobacteria that could be applied for production of industrially important thermostable proteases, α-amylases and lipases.

Conclusion

The results obtained from this study revealed perspective Antarctic thermophilic actinobacteria, which can be promising producers of industrially important thermostable enzymes and bioactive metabolites. Estimated low antibiotic resistance in the tested group suggests relatively low anthropogenic impact in the region of Livingston Island.

Table-2
Antimicrobial and hemolytic activity of thermophylic actinobacteria from Livingston Island, Antarctica

Actinomycete code	Inhibition zone (mm in diameter)				Hemolytic activity (mm)
	Bacillus subtilis	*Xanthomonas oryzae*	*Pseudomonas aeruginosa*	*Micrococcus luteus*	
3A	-	-	-	-	25
6A	-	-	-	-	26
9A	-	-	-	-	25
10A	w	-	15	-	22
11A	-	-	-	-	30
12A	-	-	w	w	18
13A	30	-	-	-	26
14A	w	16	-	-	25
15A	-	-	w	-	25
16A	w	20	-	-	20
17A	w	-	17	-	16
18A	w	w	-	-	28
20A	-	-	w	-	35
22A	-	-	-	-	27
23A	-	-	-	-	20
24A	-	-	w	w	20
25A	-	-	-	-	25
26A	-	-	-	-	26
29A	-	-	-	-	29
31A	-	-	-	-	30
33A	-	-	-	-	25
34A	30	-	-	-	22
35A	-	-	-	-	24
36A	26	15	-	-	34
37A	-	-	-	-	30
39A	-	-	-	-	26

Symbols: -, no zone detected; w, weak zone ≤ 1 mm

Table-3
Hydrolytic enzymes activity of thermophylic actinobacteria from Livingston Island, Antarctica, tested on solid media (zones, mm in diameter)

Actinomycete code	Protease (skim-milk)	Amylase (starch)	Lipase (tributirin)
3A	+ +	+ + +	+ +
6A	+	+ + +	+ +
9A	+ +	+ + +	-
10A	-	+ + +	-
11A	+ + +	+ +	+ + +
12A	+ + +	+ + +	+
13A	-	+ + +	+ +
14A	+ +	+ + +	+ +
15A	+ +	+ + +	-
16A	+ +	+ + +	+
17A	-	+ + +	+
18A	-	+ +	+
20A	+ +	+ + +	+
22A	-	+ + +	+ + +
23A	+ +	+ + +	+ + +
24A	-	+ + +	+ +
25A	+ +	+ +	+ + +
26A	+ +	+ + +	+
29A	+ +	+ + +	+ +
31A	+ +	+ + +	+ +
33A	-	+ + +	+ +
34A	+ + +	+ + +	+ + +
35A	+ +	+ + +	-
36A	-	+ + +	+
37A	-	+ + +	+
39A	+ +	+ + +	-

Symbols: +, ≤ 15 mm; + +, 16-29 mm; + + +, ≥ 30 mm; -, no zone detected

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