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Original Article

Sodium azide induced mutaion of Actinomycetes II: Biochemical and Genetic Characterization

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ABSTRACT

Sodium azide was employed to generate mutants of an actinomycete isolate (Snp1e) from Sagarmatha National Park of Mount Everest region to establish biochemical and genetic changes among them. Nine mutants were selected from Starch Casein Agar plates containing 50, 75 and 100 ppm sodium azide based on observed morphological differences of individual colonies and purified subsequently. Individual isolates were tested for carbon assimilation tests, Nitrate reduction, Catalase production and hydrolysis tests. Five decamer primers were used to establish genomic polymorphisms among the isolates by Randomly Amplified Polymorphic DNA – PCR method. Most of the mutants grew faster than the wild type isolate in Starch-Casein broth. Many of the mutants were able to hydrolyze urea and Tween-20 and produce Nitrate reductase. All mutants were able to show one or more of either GOF mutation or LOF mutation or both. The primers allowed scoring of total 173 bands of which 53 (30.64%) were polymorphic. Primer P1 demonstrated DNA polymorphisms above 50% among the isolates and large numbers of bands were observed with primers P3 and P5. Ample biochemical and genetic variations were observed among mutants derived from an actinomycete using Sodium azide.

Key words: Actinomycetes, biochemical and genetic polymorphisms, Mt. Everest, mutation, sodium azide

INTRODUCTION

Actinomycetes are diverse groups of soil inhabiting microbes. They are aerobic Gram-positive bacteria that form branches, usually nonfragmented hyphae and asexual spores. They comprise 63 genera and are relatively well documented because of their value in discovery of various types of antibiotics. *Streptomyces* spp., a major group of Actinomycetes, are most populous and diverse soil bacteria producing various industrially and medically important secondary metabolites, such as enzymes (proteases, amylase, cellulase, lignase, chitinase etc.), chromogens and antibiotics [1]. Hence, investigation of actnomycetes from different ecological niches may yield novel isolates having more useful properties [2]. Bhattarai *et al*. [3] were first to observe sodium azide induced mutation of actinomycetes. Enhanced antibacterial property in a sodium azide induced mutant of *Streptomyces* strain - a Gain-of-Function (GOF) mutation - was reported by Bhattarai *et al*. [3] and Tiwari *et al*. [4]. The present study is the continuity of our previous study in Research Laboratory for Biotechnology and biochemistry (RLABB) on mutagenic effects of sodium azide on actinmycetes from Mt. Everest region. Genetic instability is very common among actinomycetes and display several distinct phenotype. Sodium azide is a mild mutagen and causes mutation of the genome non-specifically. The objective of the present work was to study genetic and biochemical polymorphisms among the sodium azide induced mutants of actinomycetes (SIMA).

MATERIALS AND METHODS

***Bacterial strain*:** An actinomycete isolate (Snp1e), previously isolated as pure culture from Sagarmatha National Park of Mt. Everest region, was obtained from RLAB and revived in Starch Casein agar (10g starch, 0.3g Casein, 2g KNO_3, 2g K_2HPO_4, 0.05g $MgSO_4$, 0.02g $CaCO_3$, 0.01g $FeSO_4$, 20g Agar, 1L distilled water, pH 7.2) by incubation the plate at 28ºC for five days.

***Generation of* mutants:** The isolate was streaked on the Starch-Casein agar plates containing 50, 75 and 100 ppm (sublethal concentrations) of sodium azide and incubated at 28ºC for 5 - 6 days. The

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colonies having morphology different from the wild type were considered as mutants. Nine distinct mutants (three from each concentration) were selected and subcultured into SCB and incubated at 28ºC in shaker water bath (150 rpm). After 5 days 0.1ml broth was spreaded on SCA plate and incubated as above for further analysis.

***Morphological characterization*:** Using a magnifying glass, colony morphology was noted with respect to color, aerial mycelium, size and nature of the colony, reverse side color and pigmentation.

***Biochemical characterization*:** Individual isolates were tested for carbon assimilation tests (viz., Arabinose, Glucose, Galactose, Xylose, Fructose, Maltose, Sucrose, Mannitol, Salicin and Citrate), Nitrate reduction, Catalase production and hydrolysis tests (viz., Urea, tween-20, Casein and starch) [1,2].

***DNA isolation*:** Actinomycetes were grown to the late exponential phase in SCB at 28ºC for two days and DNA was isolated by Phenol: Chloroform method [5]. One milliliter of the broth in a microfuge tube (MFT) was centrifuged at 5000 rpm, washed twice with SSC buffer (3 M NaCl, 0.3 M trisodium citrate, pH 7.0) and re-suspended in lysis solution (50 mM Glucose, 25 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.6). After 5-6 cycles of boiling and chilling, the suspension was incubated at 37ºC with lysozyme (50 µl of 10 mg/ml) for an hour. Proteinase K (5 µl of 4 mg/ml) was added and incubated for 30min at 37ºC followed by addition of 2 µl of RNAse (5 mg/ml) and allowed to stand for 10min at 37ºC. Then, equal volume of Phenol: Chloroform: Iso-amylalcohol (25:24:1 v/v) was added, mixed by gentle inversions, centrifuged at 8000 rpm for 10 min at 4ºC, and upper aqueous layer was transferred into new MFT. DNA was precipitated by adding equal volume of isopropanol and placed at -20ºC for overnight and subsequently centrifuged with 15000 rpm for 10min at 4ºC. The DNA was washed with 70% ethanol and, then, pellet was dissolved in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA concentration and purity were assayed spectro-photometrically by reading absorbance at 260 nm and 280 nm.

***RAPD-Primers*:** Five decamer RAPD primers from BIONEER Company (P1: AAGAGCCCGT, G+C%=60; P2: GTTTCCGCCC, G+C%= 70; P3: AACGCGCAAC, G+C%=60; P4: GCGATCCCCA, G+C%=70; P5: CTGGATGCGA, G+C%=60) were used to amplify DNA by RAPD-PCR method.

***PCR condition*:** Separate 25 µl reaction volume containing PCR buffer (10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, pH 8.3), 100 µM dNTPs, 40 pmol primer, 1 U of Taq DNA polymerase, 1% DMSO and 25ng of genomic DNA. Amplification was done with initial denaturation (95ºC for 10 min); four cycles of denaturation (95ºC for 1 min), annealing (35ºC for 3 min) and extension (72ºC for 5 min); 35 cycles of denaturation (95ºC for 1 min), annealing (36ºC for 2 min) and extension (72ºC for 3 min); and final extension (72ºC for 10 min). PCR reactions included negative control lacking DNA.

Agarose gel electrophoresis: Amplified products (15 µl of mixture of 5 volumes of PCR products and 1 volume of 6X Loading dye containing 6 ml of 50% Glycerol, 1 ml of 2% bromophenol blue and 3 ml DW) were electrophoresed in 0.5 µg/ml ethidium bromide containing 1% agarose gel electrophoresis in 1X TAE buffer (50X TAE: 24.2g Tris, 5.71 ml acetic acid, 11.1 ml of 0.5 M Na_2EDTA, 100 ml distilled water, pH 8.0) for 90 min at 60V. PCR marker (USB Corporation) was also loaded in a well per gel. The gels were visualized by UV Trans-illuminator and photographs were captured.

RESULTS AND DISCUSSION

Morphological characterization: Mutants were selected based on morphological differences. They were, further, differed on color of their substrate mycelia, aerial spore mass and growth patterns in SC broth (Table 1). Possibly, azide hits many target sites in bacterial chromosome and, collectively, the phenotypic changes as colonial morphology changes in the mutants.

Growth patterns: Most of the mutants grew faster in SC broth than the wild type isolate. Though the amounts of bacteria were not calculated before inoculation into the broth, marked increase in cell mass might indicate enhanced physiological sum-up capability of mutants compared to the wilt type isolate.

Types of mutations in carbon metabolism: Mutations were characterized for various carbon sources (Table 2). Altogether four mutants having negative mutation (LOF) in loci of utilization of sucrose and citrate were observed. Among them, two were found to have double mutations in utilization of both substrates. All mutants had one or more positive mutation/s (GOF) in loci of fructose, mannitol, arabinose and salicin utilization. Contrary to the previous finding [6], the mutants were found to gain (or, possibly, regain) fructose assimilation ability. Five mutants had all four GOF mutations (single; Fru, Man, Ara and Suc), two had double mutations (single; Fru and Suc). Among them, four had both GOF and LOF mutations.

Types of mutations for enzymatic tests: Among nine mutants, two had double GOF mutations (Urea, Nit), two had single GOF mutation (Nit), and other three had both GOF (single, Urea) and LOF (single, Tween 20) mutations (Table 3).

Table 1 Morphological Characterization of Snp1e and corresponding mutants

Strains	**Colony color**	**Configuration**	**Elevation**	**Margins**	**Growth in SC broth**	**Growth amount**
Snp1e	LYG	RRM	Umbonate	Smooth	Flocculent	++
Snp1e.50.i	LYG	RRM	Umbonate	Smooth	Granular	+
Snp1e.50.ii	LYG	RRM	Umbonate	Smooth	Granular	+++
Snp1e.50.iiii	LYG	RRM	Umbonate	Smooth	Turbid	+
Snp1e.75.i	LYG	RRM	Umbonate	Smooth	Granular	++
Snp1e.75.ii	LYG	RRM	Umbonate	Smooth	Turbid	++
Snp1e.75.iii	LYG	RRM	Umbonate	Smooth	Granular	+++
Snp1e.100.i	LG	C	Raised	Smooth	Turbid	++
Snp1e.100.ii	LG	RRM	Umbonate	Smooth	Flocculent	+++
Snp1e.100.iii	LG	C	Raised	Smooth	Flocculent	+++

Light yellowish green, LYG; Light gray, LG; Round with raised margin, RRM; Concentric, C

Table 2 Carbohydrate assimilation test of Snp1e and corresponding mutants

Strains	Gluc	Fru	Gal	Mal	Man	Xyl	Suc	Ara	Sal	Lac	Cit	Remarks
Snp1e	++	-	++	++	-	+	+	-	-	-	+	Wild type isolate
Snp1e.50.i	++	++	++	++	-	+	+	-	-	-	+	GOF (Fru)
Snp1e.50.ii	++	++	++	++	-	+	++	-	-	-	+	GOF (Fru)
Snp1e.50.iii	++	++	++	++	++	++	++	+	++	-	+	GOF (Fru+Man+Ara+Sal)
Snp1e.75.i	++	++	++	++	++	++	-	+	++	-	-	GOF (Fru+Man+Ara+Sal), LOF (Suc+Cit)
Snp1e.75.ii	++	++	++	++	++	++	+	+	++	-	+	GOF (Fru+Man+Ara+Sal)
Snp1e.75.iii	++	++	++	++	++	++	++	+	++	-	-	GOF (Fru+Man+Ara+Sal), LOF (Cit)
Snp1e.100.i	++	++	++	++	-	+	-	-	+	-	+	GOF (Fru+Sal), LOF (Suc)
Snp1e.100.ii	++	++	++	++	-	+	+	-	+	-	+	GOF (Fru+Sal)
Snp1e.100.iii	++	++	++	++	++	+	-	+	++	-	-	GOF (Fru+Man+Ara+Sal), LOF (Suc+Cit)

Glu=Glucose, Fru=Fructose, Gal=Galactose, Mal=Maltose, Man=Mannitol, Xyl=Xylose, Suc=Sucrose, Ara=Arabinose, Sal=Salicin, Lac=Lactose, Cit=Citrate utilization

Table 3 Enzymatic test of Snp1e and corresponding mutants

	Hydrolysis				Catalase	Nitrate reduction (Nitrate reductase)	Remarks
Strains	Casein	Starch	Urea	Tween-20			
Snp1e	+	+	-	+	+	-	Wild type isolate
Snp1e.50.i	+	+	-	+	+	+	GOF (Nit)
Snp1e.50.ii	+	+	-	+	+	+	GOF (Nit)
Snp1e.50.iii	+	+	-	+	+	-	
Snp1e.75.i	+	+	+	+	+	+	GOF (Urea+Nit)
Snp1e.75.ii	+	+	+	+	+	+	GOF (Urea+Nit)
Snp1e.75.iii	+	+	-	+	+	-	
Snp1e.100.i	+	+	+	-	+	-	GOF (Urea), LOF (Tween)
Snp1e.100.ii	+	+	+	-	+	-	GOF (Urea), LOF (Tween)
Snp1e.100.iii	+	+	+	-	+	-	GOF (Urea), LOF (Tween)

Genetic polymorphisms: Morphological and biochemical variations among mutants were also redefined by molecular method, RAPD – PCR. Five decamer primers (P1 – P5) with G+C% of 60 and 70 were used to establish genomic polymorphisms among the isolates. Linear regression line equations were established for each agarose gel electrophoresis set with each primer for all isolates. The primers allowed scoring of total 173 bands of which 53 (30.64%) were polymorphic. Primer P1 demonstrated DNA polymorphisms above 50% among the isolates. Large numbers of bands were observed with primers P3 and P5 while fewer bands were observed with primer P2. RAPD-PCR typing method may be sufficiently sensitive to access genomic DNA polymorphism, such as multiple or even single nucleotide insertions and deletion or substitution [7]; however, optimization of the reagents in the reaction mixture such as concentration of primer, magnesium chloride, template DNA, Taq polymerase, DMSO, is necessary to obtain amplification product generated by RAPD [8]. No DNA contamination is extremely important in order to obtain clear and discriminatory pattern, since primer can amplify very small amount of DNA from any organism [9]. Growing actinomycetes for not more than 48hrs is valuable to obtain high quality chromosomal DNA by preventing avoiding stationary growth phase of organism in which organism starts to form extensive mycelial growth and sporulation that may make the organism be refractory to common chemicals for DNA extraction and purification.

Table 4: Polymorphic band sizes of Snp1e and corresponding mutants with RAPD-Primers (P1 – P5)

Organisms	**Band sizes* (bp) with respective primer**				
	P1	**P2**	**P3**	**P4**	**P5**
Snp1e	2103, 1851, 1304, 533	1060, 918, 555, 481	1295, 994, 843, 691, 567, 496, 356	1880, 1249, 779, 571	1822, 1587, 1205, 825, 605, 492, 350
Snp1e.50.i	456	-	-	-	3507, 1822, 1587, 1383
Snp1e.50.ii	1647, 1206, 1466, 756, 422	795	1632, 1173, 994, 843, 356	1075, 860, 715, 492	3507, 1822, 1587, 1383
Snp1e.50.iii	2744, 2103, 474	1268, 795	2057, 1632, 1212, 843, 625, 567, 356	770, 715	1822, 1587, 1336, 205, 1087, 883, 719, 584
Snp1e.75.i	1116, 756, 474, 439	795	2126, 1579, 1212, 843, 625, 356	1075, 860, 741, 457	3507, 1760, 1587, 1383, 1205, 980, 825
Snp1e.75.ii	1032	795	1579, 789, 356	1075, 830, 715, 457	3507, 1822, 1587, 1383, 1205, 980
Snp1e.75.iii	2431, 1160, 883, 673	4918, 795, 348	994, 789	440	1822, 1587, 1383, 980, 695
Snp1e.100.i	2103, 1206, 850, 673, 533	1464, 1060, 795, 640	1990, 1528, 1212, 900	1249, 893, 440, 394, 292	1087, 825, 695
Snp1e.100.ii	2528, 2103, 1713	1060, 640	1990, 1528, 1212, 900, 420	1249, 893, 492, 408	565
Snp1e.100.iii	2628, 2002, 1647, 405	795, 640	1940, 1579, 496	530	1291, 1205, 1050
No. of total bands (TB)	34	20	42	29	48
No. of polymorphic bands (PB)	18	7	7	10	11
% of polymorphisms**	52.94	35.0	16.67	34.48	22.92

*Band sizes were calculated using semi log graph prepared with log of molecular weights of marker bands in Y-axis (y) and distance travelled by each band in centimeter in X-axis (x) for each primer (viz., P1: y = -0.7785x +

10.56, R^2 = 0.9885; P2: y = -0.7181x + 10.197, R^2 = 0.994; P3: y = -0.6611x + 10.538, R^2 = 0.9881; P4: y = -0.7447x + 10.332, R^2 = 0.9932; P5: y = -0.6892x + 10.368, R^2 = 0.9872).

**% of polymorphism = PB X 100 / TB

Total polymorphism score (%) = (18+7+7+10+11) X 100 / (34+20+42+29+48) = 5300 / 173 = 30.64

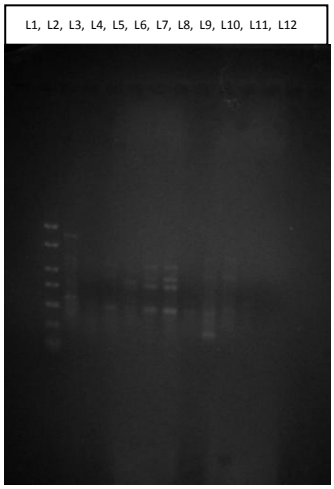

Fig. 1a DNA polymorphisms of SIMA using P1 RAPD primer

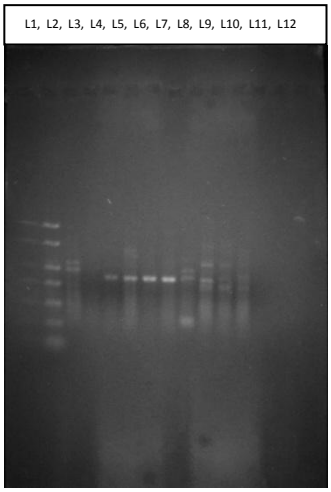

Fig. 1b DNA polymorphisms of SIMA using P2 RAPD primer

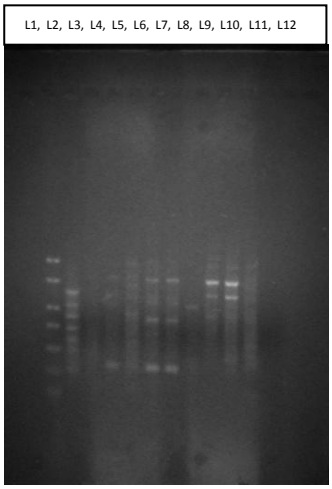

Fig. 1c DNA polymorphisms of SIMA using P3 RAPD primer

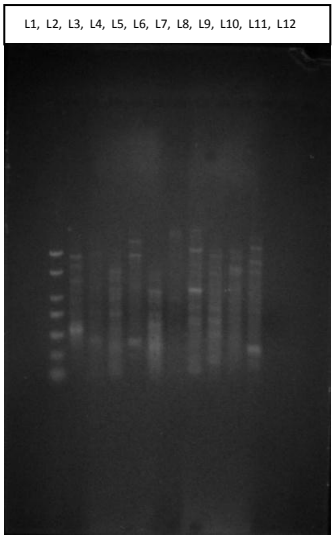

Fig. 1d DNA polymorphisms of SIMA using P4 RAPD primer

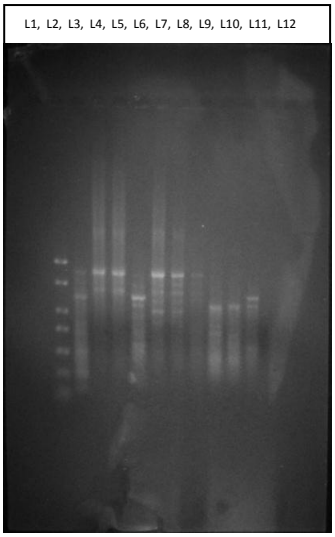

Fig. 1e DNA polymorphisms of SIMA using P5 RAPD primer

Lane definitions:

L1: PCR Marker (USB corporation),
L2: S1e,
L3: S1e.50.i,
L4: S1e.50.ii,
L5: S1e.50.iii,
L6: S1e.75.i,
L7: S1e.75.ii,
L8: S1e.75.iii,
L9: S1e.100.i,
L10: S1e.100.ii,
L11: S1e.100.iii,
L12: Negative control

Fig. 1 Polymorphic RAPD-PCR bands of SIMA using five primers (P1-P5)

CONCLUSION

For last eight years, RLABB has been studying diversity of actinomycetes in soil samples of Mount Everest region under CNR, Italy supported program. RLABB scientists have the honor of discovering the mutagenic effect of sodium azide on actinomycetes [3,4]; results presented here further confirm this discovery. This work has been supported by International Foundation for Science (IFS) Grant – 2008 in order to expand the limited study to encompass the wide spectrum of actinomycetes from Mount Everest region. The present work extensively and conclusively established previous preliminary findings for genetic differences in wild and mutant strains by RAPD – PCR. These mutations are not epigenetic, because mutants can be propagated extensively. We have found that some mutants have gained antimicrobial activity which we are studying now.

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