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Isolation and Characterization of *Azospirillum* Strains Isolated from Different Agroclimatic Zones of Virudhunagar District, Tamil Nadu

KEYWORDS: *Azospirillum*, identification, biology, sources

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ABSTRACT The present study was carried out to explore the Azospirillum strains isolated from different agroclimatic zones of Virudhunagar District, Tamil Nadu. The isolated strains were identified and studied their biology. The result revealed that the population of Azospirillum was higher in soil collected from Sattur and followed by Rajapalayam. Among ten selectes strains, eight were identified as Azospirillum lipoferum and remaining identified as Azospirillum brasilense. In vitro studies such as tolerance level to pH, temperature and antibiotics were studied to know about the biology of selected strains. Azospirillum strains preferred temperature ranging from 15°C to 35°C. The optimum pH for the growth of Azospirillum ranged from 6.0 – 7.5. All the Azospirillum strains showed tolerance up to 300 ppm for penicillin, 200 ppm for chloramphenicol and 200 ppm for streptomycin. Further, the utilization various chemical sources like carbon, nitrogen, amino acids and vitamins varied among the strains.

INTRODUCTION

Azospirillum is a rhizosphere bacterium colonizing the roots of crop plants making use of root exudates and fixes substantial amount of atmospheric nitrogen. At least 15 *Azospirillum* species have been described, but in terms of physiology and genetics the most studied ones are *A. lipoferum* and *A. brasilense* described by Tarrand *et al*. (1978). The third species *A. amazonense* (Magalhaes *et al*. 1983) was isolated forage grasses planted in Amazonian region. The other species of are *A. halopraeferans* (Reinhold *et al*. 1987), *A. irakense* (Khammas *et al*. 1989), *A. largimobile* (Sly and Stackebrandt 1999), *A. dobereinerae* (Eckert *et al*. 2001, *A. oryzae* (Xie and Yokota 2005), *A. melinis* (Peng *et al*. 2006), *A. canadense* (Mehnaz *et al*. 2007a), *A. zeae* (Mehnaz *et al*. 2007b), *A. rugosum* (Young *et al*. 2008), *A. palatum* (Zhou *et al*. 2013), *A. picis* (Lin *et al*. 2009) and *A. thiophilum* (Lavrinenko *et al*. 2010).

Azospirillum is microaerophilic, gram negative and spiral shape bacterium. It is asymbiotic nitrogen fixers able to fix the atmospheric nitrogen make it available to plants. It is beneficial to plants by mechanisms related to enhancement of plant growth, increases the mineral uptake, increases the dry matter, improve the water absorption and improve the yield.

Azospirillum grown in N-free medium behaves as microaerophilic and fixes nitrogen and when supplemented with nitrogen it grows as an aerobe (Day and Dobereiner, 1976). In culture tubes of semisolid medium with a suitable carbon and energy source, *Azospirillum* develops a growth pellicle just below the surface and fix the nitrogen only under microaerophilic condition, because its nitrogenase is poorly protected from oxygen (Okon *et al.,* 1977; Nelson and Knowles, 1978).

Azospirillum prefers acidic pH for their growth and activity. The optimum pH for the growth of *A. amazonense, A. lipoferum* and *A. brasilense* strains isolated from a variety of habitats found to be 5.7 - 6.5, 5.7 - 6.8 and 6.0 - 7.3 respectively (Baldani *et al.,* 1986). The optimum temperature for growth of *Azospirillum* was found to be between 32 and 40°C (Day, 1977). The growth response of *Azospirillum* strains indicated that D-fructose, D-mannitol, sorbitol, sucrose, tyrosine and tryptophan were poor carbon sources, while α-keto glutarate, L-alanine, L-glutamate, lactate, pyruvate and succinate were good carbon sources (Rai and Gaur, 1982; Del Gallo *et al.,* 1984). *Azospirillum* spp. differed in their utilization of amino acids. *A. lipoferum* and A. *brasilense* readily utilized many amino acids as the sole source of carbon and nitrogen (Hartmann, 1988). Dobereiner and Baldani (1979) found diversity among *Azospirillum* strains with respect to their resistance to various antibiotics. *Azospirillum amazonense* strains were resistant to penicillin but relatively tolerant to chloramphenicol and erythromycin (Magalhaes *et al.,* 1983).

MATERIAL AND METHODS

ISOLATION AND ENUMERATION OF AZOSPIRILLUM

Soil and feeder root samples were collected from different agroclimatic zones of the growing areas (Rajapalayam, Srivilliputtur, Sivakasi, Virudhunagar and Sattur) in Virudhunagar district, Tamil Nadu. For the isolation of *Azospirillum*, the rhizosphere soil samples were serially diluted up to 10^6 dilutions. Enumeration of *Azospirillum* in soil samples were carried out by Most Probable Number (MPN) method (Cochran, 1950) and expressed as number of *Azospirillum* per gram dry weight of soil samples.

CHARACTERIZATION OF AZOSPIRILLUM STRAINS

In vitro studies such as tolerance level to pH, temperature and antibiotics were studied to know about the biology of selected strains of *Azospirillum.* In all these cases, the growth was graded as no growth (-), poor growth (+), medium growth (++), good growth (+++) and very good growth (++++).

Antibiotic resistance

The antibiotic resistance was tested on yeast extract glucose agar (Allen, 1953) incorporated with various antibiotics like penicillin (50 – 300 ppm), chloramphenicol (50 -300 ppm) and streptomycin (50 - 300 ppm). The antibiotic solution was prepared, filter sterilized and added to the medium before plating. The plates were streaked with selected strains of *Azospirillum* and incubated at 35±2°C and observed the growth.

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Utilization of carbon, nitrogen, amino acid and vitamin sources

The utilization of different carbon, nitrogen, amino acids and vitamins sources by *Azospirillum* strains was studied. Different sources were individually incorporated into the medium at 1% level. The *Azospirillum* cultures were inoculated at the rate of 1.0 ml and incubated at room temperature. The growth was observed by turbidity of the broth read at 560 nm.

IDENTIFICATION OF BACTERIAL STRAIN

The selected bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology (Krieg and Dobereiner, 1984).

RESULTS AND DISCUSSION

ISOLATION OF AZOSPIRILLUM

Attempts were made to isolate the native strains of *Azospirillum* from the soil samples collected from different areas in Virudhunagar district. Totally 10 (R1, R 2, SP 1, SP 2, SK 1, SK 2, V 1, V 2, S 1and S 2) *Azospirillum* strains were isolated and brought to pure culture (Table1). All ten isolates showed white dense and undulating fine pellicle. The pellicle formation in the nitrogen free semi solid malic acid medium is characteristic of *Azospirillum*. Semisolid malate medium has been recommended to isolation and detection of nitrogen fixing spirilla in soil and rhizosphere. Azospirilla were isolated from a wide variety of plants including many grasses and cereals from all over the world, in tropical, temperate and cold climates, from desert plants, from water-flooded rice paddies and from salt affected soils (Haahtela *et al.,* 1981; Reinhold *et al.,* 1987).

POPULATION DYNAMICS OF AZOSPIRILLUM STRAINS

In addition to isolation, the population level of *Azospirillum* was enumerated. The results showed that the population level of *Azospirillum* was higher in the soil collected from Sattur followed by Rajapalayam and least in Srivilliputtur (Table 1). The associations of the *Azospirillum* spp were initially reported to be restricted to the graminaceae (Dobereiner and Day, 1976); seasonal variation (Bezbaruah, 1999); host plant (Hu *et al.,* 2006) and soil type (Yadav and Singh, 1991).

Identification of Azospirillum

The *Azospirillum* strains were identified up to species level based on biochemical tests following Bergey's Manual of Determinative Bacteriology. The isolated strains were found to be identical with *Azospirillum* sp. forming typical white, dense and undulating fine pellicle in nitrogen free semi-solid medium and Gram negative and spiral movement. Among ten isolates, the strains, R2, SP1, SP2, SK1, SK2, V2, S1 and S2 were identified as *Azospirillum lipoferum.* The rest of the isolates, R1 and V7 were identified as *Azospirillum brasilense* (Table 2). On the basis of colony morphology, gram staining, and carbon/nitrogen utilization pattern the isolated strains from maize (*Zea mays* L.) were identified as members of genus *Azospirillum* (Ilyas *et al.*, 2012). *Azospirillum* strains from paddy field rhizosphere soil of Thanjavur district were identified by morphological, physiological and biochemical characters.All the 10 *Azospirillum* strains were identified based on morphological character such as shape , temperature, pH and biochemical character such as IMViC, catalase, citrate,starch hydrolysis and urease test were studied (Usha and Kanimozhi, 2011).

CHARACTERIZATION OF AZOSPIRILLUM STRAINS

Temperature Tolerance

The result clearly indicated that all the *Azospirillum* strains preferred temperature ranging from 15°C to 35°C above and below which the growth was retarded (Table 3). The optimum temperature for growth of *Azospirillum* was reported to be between 32 to 40°C (Day, 1977) but none of the *Azospirillum* isolates tested showed an optimum temperature of N_2-dependent growth at 41°C as *A. halopraeferens* does, but some isolates showed still significant acetylene reduction activity at 41°C (Reinhold *et al.,* 1987).

pH Tolerance

Tolerance to pH varied within the selected strains of *Azospirillum*. These variations are mainly due to the nature of strains and the nature soil conditions. The optimum pH for the growth of *Azospirillum* ranged from 6.0 – 7.5 (Table 4). The pH ranges for the optimum growth of *A. amazonense, A. lipoferum* and *A. brasilense* strains isolated from variety of habitats were found to be 5.7 - 6.5, 5.7 - 6.8 and 6.0 - 7.3 respectively (Baldani *et al.,* 1986).

Antibiotic resistance

All the *Azospirillum* strains showed tolerance up to 300 ppm for penicillin, 200 ppm for chloramphenicol and 200 ppm for streptomycin (Table 5). Within the same species of microorganism, variation in the resistance to antibiotics was observed between strains isolated from comparable habitats or even from the same habitat (Balandreau, 1986). Merline Sheela (1991) studied the resistance of *Azospirillum* isolates to various antibiotics viz, streptomycin (150 µg/ml) rifampicin (3 µg/ml) chloramphenicol (150 µg/ml) tetracycline (5µg/ml) kanamycin (20 µg/ml) ampicillin (100µg/ml) erythromycin (20 µg/ml) and neomycin (10 µg/ml). Magalhaes *et al.,* (1983) observed that all the *Azospirillum amazonense* strains were resistant to penicillin but relatively tolerant to chloramphenicol and erythromycin. Govindarajan and Prushothaman (1984) reported that out 80 isolates of *Azospirillum* only one isolate was found to be resistance to 50 µg ml^{-1} of streptomycin, 15 per cent of isolates were resistant to 20µg / ml and 7 per cent isolates were 1.0µg / ml.

Utilization of carbon sources

Azospirillum strains utilized different types of chemicals compounds as carbon source. The utilization of different types of carbons sources varied from strain to strain. Most of the *Azospirillum* strains were preferred well glucose, fructose as carbon source. Sucrose and lactose were moderately utilized by all *Azospirillum* strains. Further, starch and cellulose were poorly utilized by all strains (Table 6). There was a marked difference between the *Azospirillum* species in the pattern of carbohydrate utilization. *A.lipoferum* grew on D-glucose (Goebel and Krieg, 1984; Loh *et al.,* 1984). *Azospirillum brasilense* preferably grew well in succinate, glycerol, D-fructose, D-gluonate, D-galectose, and L-arabinose but not on D-glucose and *Azospirillum lipoferum* grow well on succinatte, glucose and mannose (Konde, 1984).

Utilization of nitrogen sources

It was observed that *Azospirillum* isolates poorly utilized nitrogenous compounds such ammonium sulphate, ammonium nitrate, ammonium chloride, potassium nitrate and sodium nitrate (Table 7). It was observed that *Azospirillum* isolates fairly utilized both inorganic and organic nitrogenous compounds (Konde, 1984). Ammonia compounds like potassium nitrate, ammonium nitrate, ammonium sulphate, ammonium chloride, sodium nitrate were found to support fair growth of all *Azospirillum* isolates (Postgate, 1978). *A. brasilense* was preferentially utilized organic acids such as malate, succinate, lactate and pyruvate. It was observed that *Azospirillum* isolates fairly utilized both inorganic and organic nitrogenous compounds as a source of nitrogen (Del - Gallo *et al.,* 1984).

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Utilization of amino acids

All the amino acids were found to be supported the growth of *Azospirillum* strains. The utilization of amino acids was differed from strains to strains. Amino acids like lysine, and valine were preferred more compared to other amino acids (Table 8). Amino acids such as alanine, leucine, lysine, isoleucine, valine supported good growth of both *A. brasilense* and *A. lipoferum* (Konde, 1984). *Azospirillum* spp. differed in the utilization of amino acids. *A. lipoferum* and *A. brasilense* were readily used many amino acids as the sole sources of carbon, nitrogen and energy. Glutamate was a good nitrogen source for *A. lipoferum* and *A. amazonense*, but a poor nitrogen source for *A. brasilense* and *A. halopraeferens* (Hartmann, 1988).

Utilization of vitamins

All vitamins were found to support growth of *Azospirillum* strains. The utilization of different types of vitamins varied from strain to strain. Myoinositol was utilized more by all *Azospirillum* strains and nicotinic acid, thiamine, pyridoxine and ascorbic acid were moderately utilized by all *Azospirillum* strains (Table 9). For the large-scale production of *Azospirillum* inoculants, Okon's medium was used having macro and micronutrients with malate as carbon source and yeast extract as source for vitamins, serves as appropriate medium. Vitamins improved the growth and population of *Azospirillum* in different types of growth medium. The microaerophilic growth of *Azospirillum* was strongly stimulated by thiamine. The positive effect was differed species to species (Rozycki *et al.,* 1992).

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Table 1: Population level of Azospirillum in the soil samples collected from Virudhunagar district

S. No.	Soil sample	Population level (x 10 /g soil dry wt.)	Azospirillum Isolates
1.	Rajapalayam	4.6	R1 & R 2
2.	Srivilliputtur	0.8	SP 1 & SP 2
3.	Sivakasi	1.9	SK 1 & SK 2
4.	Virudhunagar	3.9	V 1 & V 2
5.	Sattur	6.2	S 1 & S 2

Table 2: Identification steps of Azospirillum

Tests	**Azospirillum *Strains***									
	R1	**R2**	**SP1**	**SP2**	**SK1**	**SK2**	**V1**	**V2**	**S1**	**S2**
1.Grams staining	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2.Biotin requirement	-	+	+	+	+	+	-	+	+	+
3.Acidification in peptone based glucose medium	-	+	+	+	+	+	-	+	+	+
4. Shape	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid	Vibrioid
5. Cell wider in Niyrogen free semi-solid medium	-	+	+	+	+	+	-	+	+	+
6.Motility	+	+	+	+	+	+	+	+	+	+
7.Root associate N-fixers	+	+	+	+	+	+	+	+	+	+
8. Colonies in BMS agar	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink	Pink
9. PHB	+	+	+	+	+	+	+	+	+	+
10. Oxidase	+	+	+	+	+	+	+	+	+	+
11. Catalase	+	+	+	+	+	+	+	+	+	+
12.Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+
13.Indole production	-	-	-	-	-	-	-	-	-	-
14. VP	-	-	-	-	-	-	-	-	-	-
15.NO_3 dependent Anaerobic growth	+	+	+	+	+	+	+	+	+	+
16.Nitrate Reduction	+	+	+	+	+	+	+	+	+	+
17.Acid production from Glucose	-	+	+	+	+	+	-	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Ribose	-	+	+	+	+	+	-	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-
18.Carbon source	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Glucose	-	+	+	+	+	+	-	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-
Arabinose	-	+	+	+	+	+	-	+	+	+
Mannitol	-	+	+	+	+	+	-	+	+	+
Ribose	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-

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Table 3: Temperature tolerance of *Azospirillum* strains

S. No.	*Azospirillum* Strains	Temperature				
		5°C	15°C	28°C	35°C	50°C
1.	R1	+	++	+++	+++	-
2.	R2	+	++	+++	+++	-
3.	SP1	+	++	+++	+++	-
4.	SP1	+	++	+++	+++	-
5.	SK1	+	++	+++	+++	-
6.	SK2	+	++	+++	+++	-
7.	V1	+	++	+++	+++	-
8.	V2	+	++	+++	+++	-
9.	S1	+	++	+++	+++	-
10.	S2	+	++	+++	++	-

- no growth; +poor growth; ++medium growth; +++good growth

Table 4: pH tolerance of *Azospirillum* strains

S. No.	*Azospirillum* Strains	pH				
		5.0	6.0	7.0	7.5	8.0
1.	R1	+	++	+++	+++	+
2.	R2	+	++	+++	+++	+
3.	SP1	+	++	+++	+++	-
4.	SP1	+	++	+++	++	-
5.	SK1	+	++	+++	+++	+
6.	SK2	+	++	+++	++	-
7.	V1	+	++	+++	+++	-
8.	V2	+	++	+++	++	-
9.	S1	+	++	+++	+++	-
10.	S2	+	++	+++	++	+

Table 5: Antibiotic resistance of selected *Azospirillum* strains

Azospirillum strains	Chloramphenicol (ppm)			Streptomycin (ppm)				Penicillin (ppm)				
	100	200	400	100	200	300	400	100	200	300	400	500
R1	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
R2	+++	+++	-	+++	+++	+	-	+	+++	+++	+	-
SP1	+++	+++	-	+++	+++	+	-	+	+++	+++	+	-
SP1	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
SK1	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
SK2	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
V1	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
V2	+++	+++	-	+++	+++	+	-	+	+++	+++	+	-
S1	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-
S2	+++	+++	-	+++	+++	+	-	++	+++	+++	+	-

Table 6: Utilization of carbon sources by *Azospirillum* strains

S. No.	*Azospirillum* strains	Carbon Source					
		Glucose	Fructose	Sucrose	Lactose	Starch	Cellulose
1.	R1	++ (0.837)	+++ (1.454)	++ (1.019)	++ (0.898)	++ (0.825)	++ (0.815)
2.	R2	++ (0.845)	+++ (1.467)	++ (1.027)	++ (0.904)	++ (0.827)	++ (0.819)
3.	SP1	++ (0.915)	+++ (1.537)	++ (0.799)	++ (0.735)	++ (0.742)	++ (0.625)
4.	SP1	++ (0.930)	+++ (1.543)	++ (0.811)	++ (0.763)	++ (0.745)	++ (0.628)
5.	SK1	++ (1.017)	+++ (1.507)	+++ (1.005)	++ (0.827)	++ (0.650)	++ (0.534)
6.	SK2	++ (1.020)	+++ (1.512)	++ (1.106)	++ (0.834)	++ (0.657)	++ (0.539)
7.	V1	+++ (1.119)	+++ (1.437)	++ (0.854)	+++ (1.137)	++ (0.913)	+ (0.457)
8.	V2	+++ (1.126)	+++ (1.455)	++ (0.865)	+++ (1.141)	++ (0.917)	+ (0.461)
9.	S1	+++ (1.355)	+++ (1.521)	+++ (1.521)	+++ (1.457)	++ (0.628)	+ (0.369)
10.	S2	+++ (1.373)	+++ (1.551)	+++ (1.551)	+++ (1.463)	++ (0.632)	+ (0.372)

Values in parentheses indicate OD value

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Table 7: Utilization of nitrogen sources by *Azospirillum* strains

S. No.	*Azospirillum* strains	Nitrogen Source				
		Potssium Nitrate	Ammonium Nitrate	Ammonium Sulphate	Ammonium Chloride	Sodium nitrate
1.	R1	+ (0.244)	+ (0.163)	+ (0.424)	+ (0.064)	+ (0.336)
2.	R2	+ (0.264)	+ (0.150)	+ (0.316)	+ (0.065)	+ (0.320)
3.	SP1	+ (0.156)	+ (0.114)	+ (0.386)	+ (0.077)	+ (0.292)
4.	SP1	+ (0.140)	+ (0.113)	+ (0.382)	+ (0.073)	+ (0.293)
5.	SK1	+ (0.105)	+ (0.136)	+ (0.344)	+ (0.128)	+ (0.333)
6.	SK2	+ (0.134)	+ (0.110)	+ (0.388)	+ (0.107)	+ (0.271)
7.	V1	+ (0.152)	+ (0.125)	+ (0.297)	+ (0.088)	+ (0.272)
8.	V2	+ (0.131)	+ (0.137)	+ (0.330)	+ (0.086)	+ (0.278)
9.	S1	+ (0.129)	+ (0.131)	+ (0.463)	+ (0.118)	+ (0.411)
10.	S2	+ (0.115)	+ (0.120)	+ (0.390)	+ (0.105)	+ (0.502)

Table 8: Utilization of amino acid sources by Azospirillum strains

S.No.	Azospirillum strains	Amino acid Source				
		Alanine	Leucine	Lysine	Isoleucine	Valine
1.	R1	++ (0.875)	++ (0.889)	++ (0.997)	++ (0.895)	++ (0.987)
2.	R2	++ (0.920)	++ (0.913)	++ (1.005)	++ (0.901)	++ (0.910)
3.	SP1	++ (0.945)	++ (0.935)	++ (1.027)	++ (0.913)	++ (1.012)
4.	SP1	++ (0.960)	++ (0.945)	++ (1.035)	++ (0.920)	++ (1.020)
5.	SK1	++ (1.023)	++ (1.003)	+++ (1.103)	++ (1.011)	+++ (1.119)
6.	SK2	++ (1.035)	++ (1.011)	+++ (1.111)	++ (1.015)	+++ (1.123)
7.	V1	+++ (1.123)	+++ (1.145)	+++ (1.231)	+++ (1.113)	+++ (1.221)
8.	V2	+++ (1.325)	+++ (1.157)	+++ (1.237)	+++ (1.120)	+++ (1.227)
9.	S1	+++ (1.404)	+++ (1.231)	+++ (1.301)	+++ (1.210)	+++ (1.321)
10.	S2	+++ (1.415)	+++ (1.245)	+++ (1.311)	+++ (1.216)	+++ (1.327)

Table 9: Utilization of vitamin sources by Azospirillum strains

S.No.	*Azospirillum* strains	Vitamin Source				
		Nicotinic Acid	Thiamine	Pyridoxine	Myoinositol	Ascorbic Acid
1.	R1	++ (0.957)	++ (0.857)	++ (0.981)	++ (0.988)	++ (0.889)
2.	R2	++ (0.983)	++ (0.878)	++ (0.985)	++ (0.995)	++ (0.945)
3.	SP1	++ (0.921)	++ (0.901)	++ (0.997)	++ (1.003)	++ (0.987)
4.	SP1	++ (0.928)	++ (0.911)	++ (1.012)	++ (1.015)	++ (1.023)
5.	SK1	++ (1.035)	++ (0.989)	++ (1.023)	+++ (1.126)	++ (1.037)
6.	SK2	++ (1.047)	++ (0.990)	++ (1.030)	+++ (1.132)	++ (1.128)
7.	V1	+++ (1.128)	++ (1.045)	+++ (1.115)	+++ (1.228)	++ (1.195)
8.	V2	+++ (1.132)	++ (1.053)	+++ (1.128)	+++ (1.238)	++ (1.237)
9.	S1	+++ (1.285)	+++ (1.117)	+++ (1.234)	+++ (1.303)	++ (1.345)
10.	S2	+++ (1.293)	+++ (1.223)	+++ (1.245)	+++ (1.312)	+++ (1.357)

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