STUDIES ON MARINE MICROORGANISMS. III

TRANSPORT OF SPORES OF ACTINOMYCETE INTO SHALLOW SEA MUD AND THE EFFECT OF SALT AND TEMPERATURE ON THEIR SURVIVAL

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Actinomycete spores having various shapes and ornamentation were suspended in soil, and their removal by elution with water was examined. Spores of *Streptomyces* sp. strains SS-22, SS-28 and SS-254 isolated from shallow sea muds were rapidly removed. The precipitation of these spores in suspension with either distilled water or saline water was examined. Sodium chloride was found to accelerate the precipitation of most strains. The effect of temperature and salt concentration on actinomycete growth was examined and growth of most strains was found to be retarded by low temperature and high salt concentration. All strains survived under the test conditions, except *Streptomyces aureofaciens*. One strain of this species was unable to survive culturing in 3.5% NaCl, for 10 days at 15°C.

As described in a previous paper[1], many strains of actinomycete were isolated from shallow sea mud. Assuming that most originated from terrestrial soil, it was of interest to determine how they are transported from soil to sea water or mud. When such organisms reach sea environments, they would be exposed to water with different salt concentration and to temperatures different from those of terrestrial environments. To obtain information, the growth and survival of terrestrial and sea-mud isolates was examined under standardized conditions to determine whether they are able to grow and/or survive under benthic conditions.

Materials and Methods

Elution of spores from soil.

(1) Actinomycete strains tested: Strains studied are listed in Table 1. They were selected as follows: Strains SS-22 and SS-28 represent streptomycete isolated frequently from shallow sea mud. They were found to produce the antibiotic xanthomycin, a common secondary metabolite of marine microorganisms[1,4]. Strain SS-254 represents another type of streptomycete isolated frequently from shallow sea mud. Strains SS-1000 B and LS-147 were isolated from deep sea water and lake surface water, respectively. Four type strains of streptomycetes, each with a different kind of spore surface also were examined.

(2) Preparation of spores: Strains were cultured on slants of GG medium (glycerol 2.0%, glycine 0.25%, NaCl 0.1%, K_2HPO_4 (anhydrous) 0.1%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.01% and agar 1.7%; pH 7.4 before sterilization). After incubation for 1 week at 27°C, inocula (spores) from these cultures were spread on GG agar in Petri dishes (30 ml, 7.5×22.5 cm) and incubated for 10 days at 27°C. After incubation, the spores (including aerial mycelium) were harvested with a microspatula and suspended in distilled water. The materials were homogenized with a Potter glass homogenizer and the resultant spore and mycelial suspensions were centrifuged at 8,000 rpm for 10 minutes to give wet spore-mycelium

Table 1. Actinomycetes tested

Species and Strains	Source	Remarks
Streptomyces sp., SS-22	Shallow sea mud (15 m)*	Spore surface; smooth. Xanthomycin producer
Streptomyces sp., SS-28**	Shallow sea mud (20 m)	Spore surface; spiny. Xanthomycin-like antibiotic producer
Streptomyces sp., SS-254	Shallow sea mud (10 m)	Spore surface; smooth. Frequently isolated
Not identified, SS-1000 B***	Deep sea water (948 m)	Spore surface; smooth. 34°59′, N 155°01′W
Not identified, LS-147	Lake water (surface)	Spore surface; smooth. Lake Tama (Japan)
S. californicus, ISP 5058**	Land soil	Spore surface; smooth. Viomycin producer
S. bellus, ISP 5158	Land soil	Spore surface; spiny. Matamycin producer
S. griseoplanus, ISP 5009	Land soil	Spore surface; warty. Alazopeptin producer
S. prasinopilosus, ISP 5098	Land soil	Spore surface; hairy
Not identified, SS-17**	Shallow sea mud (5 m)	Pluramycin-like antibiotic producer
S. pluricolorescens, ISP 5019**	Land soil	Pluramycin producer
S. pseudogriseolus, ISP 5026**	Land soil	Xanthomycin producer
S. aureofaciens, ISP 5127**	Land soil	Low salt tolerance (4% or less)
S. echinatus, ISP 5013**	Land soil	Intermediate salt tolerance (7%)
S. rimosus, ISP 5260**	Land soil	High salt tolerance (10% or more)

* Depth (m).
** These strains were only used for the study on the effect of temperature and salt concentration on survival of actinomycetes.
*** Collected by Prof. N. TAGA of Tokyo University.

masses suitable for test. The number of viable units (spores and mycelial particles) was determined by a dilution plate count method on GG agar.

(3) Soil column studies: Two g of garden soil or sandy soil were packed into a glass column (0.8×20 cm). The column was sterilized in an autoclave at 121°C for 30 minutes, 2 mg of wet spore mass was placed on top of the soil, and the column was washed with 100 ml of sterilized water. The number of viable units (spore and mycelial) in the eluate was determined by a dilution plate count method on GG agar and compared with the number of spores charged to the column.

Precipitation of spores in suspension with or without salt

(1) Actinomycete strains tested: Strains studied are listed in Table 1.

(2) Spore suspensions: Spore-mycelial masses were obtained in the same way as those for the previous experiment, suspended, and homogenized with a Potter glass homogenizer. Spores floating on the surface of suspension after centrifugation were discarded. One ml of homogenized spore was pipetted into a test tube (10×105 mm), and either distilled water, 0.67% NaCl solution (final conc. 0.5%), 4.0% NaCl solution (final conc. 3.0%), artificial sea

water (Jamarine*®), or aged sea water (natural sea water kept at room temperature) added in 3 ml amounts.

(3) Measurement of precipitation: Each spore suspension was adjusted with the appropriate diluent to a reading of approximately 100 using the Klett-Summerson Colorimeter Scale (Klett manufacturing Co., Inc.,). After 1, 3, 6, 12 and 24 hours at room temperature, 2 ml was gently pipetted from the surface of the suspension with a micropipette, and the light transmission of the removed suspension measured with the photoelectric colorimeter.

Effect of temperature and salt concentration on survival of actinomycetes

(1) Actinomycete strains tested: Strains studied are listed in Table 1.

(2) Cultural conditions: Each strain was shake-cultured in a flask (100 ml in a 500-ml SAKAGUCHI flask on a reciprocal shaker, 130 rpm, 7 cm amplitude) for 3 days at 27°C in medium I [glycerol 6.0%, soluble starch 1.0%, glucose 0.2%, Polypeptone® 0.5%, beef-extract 0.3%, K_2HPO_4 (anhydrous) 0.15%, KH_2PO_4 (anhydrous) 0.08%, $FeSO_4 \cdot 7H_2O$ 0.01%, dissolved in 1,000 ml potato extract (filtrate from 100 g sliced potato, boiled for 40 minutes), pH not adjusted]. One ml of the resultant culture was used for inoculating 100 ml of medium I in a 500 ml SAKAGUCHI flask, containing salt (NaCl) at 0% (control), 3.5%, 7.0%, or 14.0% concentration. The cultures were shaken for 5 days at 27°C or 10 days at 15°C and centrifuged at 3,000 rpm for 10 minutes. The precipitates were suspended in distilled water and centrifuged again at 10,000 rpm for 20 minutes. The packed cells obtained were weighed. If no growth occurred, the whole broth was aseptically centrifuged at 5,000 rpm for 10 minutes and the supernatant discarded. The precipitate was suspended in 2 ml of fresh medium I (no NaCl). This suspension was used in equal amounts to inoculate two flasks, each containing 100 ml of fresh medium I (no NaCl). One flask was shaken for 10 days at 15°C, the other for 5 days at 27°C, and observed for growth to determine whether the organism had survived.

Results and Discussion

Elution of Spores from Soil

Many kinds of actinomycete have been isolated from shallow sea muds and are believed to survive in spore form over long peridos of time. It is speculated that spores in soil are transported from one place to another by movement of rain water or underground water, or carried by animals or insects[2,3]. Ease of transport might depend on the types of organism.

Table 2. Removal of actinomycete spores from soil by elution with water

Strains tested	Spore type	Spores added to columns $\times 10^4$/ml	Garden soil		Sandy soil	
			Eluted spores $\times 10^4$/ml	Removal rate (%)	Eluted spores $\times 10^4$/ml	Removal rate (%)
Streptomyces sp., SS-22	Smooth	53*	24	45.3	34	64.2
Streptomyces sp., SS-28	Spiny	149	127	85.2	162	109.4
Streptomyces sp., SS-254	Smooth	940	610	65.0	760	80.9
SS-1000 B	Smooth	730	220	30.1	500	68.5
LS-147	Smooth	1,590	150	9.4	860	54.1
S. californicus, ISP 5508	Smooth	460	187	40.7	436	94.8
S. bellus, ISP 5185	Spiny	8	2	25.0	5	62.5
S. griseoplanus, ISP 5009	Warty	379	19	5.0	185	48.8
S. prasinopilosus, ISP 5098	Hairy	97	21	21.6	61	62.9

* Spore number determined by a dilution plate count method.

*® Jamarine Co., Osaka.

Taking this into account, the elution rate of spores of various streptomycetes was examined. The results are given in Table 2. In garden soil, spores of the actinomycete tested showed significantly different elution rates. It is of interest that the spores of actinomycete isolated from shallow sea mud, especially strain SS-28, showed high elution rates. In contrast, the elution rates of spores of strain LS-147 and strain ISP 5009 *Streptomyces griseoplanus* BACKUS, TRESNER and CAMPHBELL were notably low. In sandy soil, all actinomycete showed higher elution rates than they did in garden soil. These results suggest that elution rates might represent a selective factor affecting the transport of actinomycete from terrestrial sites to the sea. Before these studies, it was thought that the nature of spore-surface might affect the elution rate[3], but as shown in Table 2, strains SS-28 and ISP 5185 *Streptomyces bellus* MARGALITH and BERETTA (spiny spore surface), and strains SS-254 and LS-147 (smooth spore surface) showed no clear difference in their elution rates.

Precipitation of Spores in Suspension with or without Salt

As indicated above, most actinomycete spore are easily removed from soil by water, and some might be able to reach a sea environment. When spores of actinomycete reach the sea, they encounter an entirely different aqueous milieu (sea water). After mixing with sea water, it is speculated that some actinomycete spores might float and others might be precipitated and fall to the sea bottom. The precipitation of the spores, as affected by salt concentration, was examined to obtain information on this point. As shown in Table 3 most spores in distilled water were precipitated after 12 hours, but it is significant that precipitation of spores of strains SS-22, SS-28, SS-254, SS-1000 B and ISP 5185 (*S. bellus*) were remarkably accelerated by NaCl. Precipitation of spores of strain ISP 5058 [*Streptomyces californicus* (WAKSMAN and CURTIS) WAKSMAN and HENRICI] and strain ISP 5098 (*Streptomyces prasinopilosus* ETTLINGER, CORBAZ and HÜTTER) were not accelerated by NaCl, although precipitation of the former was accelerated by artificial sea water and by aged sea water and that of the latter only by aged sea water. Fig. 1 shows the time course of precipitation of various spores suspended in aged sea water. The results suggested that most spores of actinomycete reaching the sea might be precipitated in

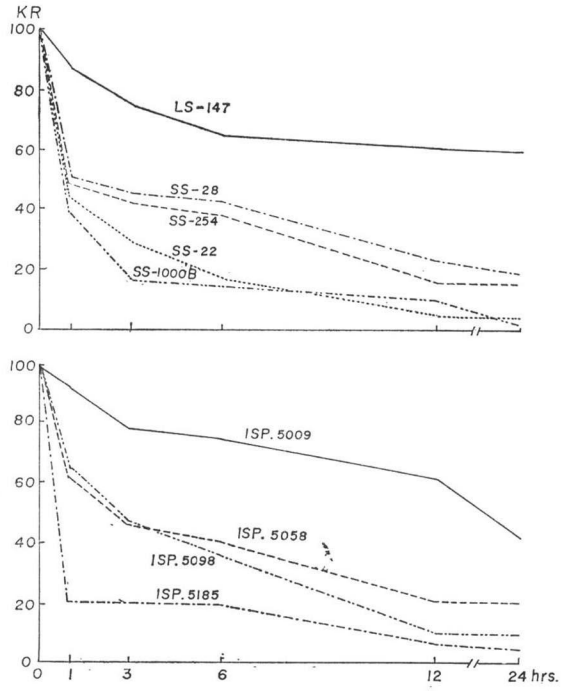
Table 3. Precipitation of actinomycete spores in suspension with or without salts

Strains tested	Spore type	Initial spore suspended	Precipitation in				
			Dist. water	0.5% NaCl dist. water	3.0% NaCl dist. water	Artificial sea water	Aged sea water
Streptomyces sp., SS-22	Smooth	104*	42**	6**	6**	4**	4**
Streptomyces sp., SS-28	Spiny	105	43	20	21	20	19
Streptomyces sp., SS-254	Smooth	99	50	28	24	21	15
SS-1000 B	Smooth	96	30	12	10	12	13
LS-147	Smooth	104	62	60	60	60	60
S. californicus, ISP 5058	Smooth	99	43	42	43	18	20
S. bellus, ISP 5185	Spiny	108	40	30	12	12	6
S. griseoplanus, ISP 5009	Warty	100	61	65	60	65	61
S. prasinopilosus, ISP 5098	Hairy	102	32	36	34	34	9

* Reading of light transmission (by Klett-Summerson Colorimeter Scale) of spore suspension.
** Reading of light transmission (by Klett-Summerson Colorimeter Scale) after 12 hours at room temperature. Reduced number means increased transparency corresponding to reduced turbidity.

a short time. In contrast, strain LS-147 and strain ISP 5009 (*S. griseoplanus*) showed very low rates of precipitation. In this study, no relation between the nature of spore surfaces and rate of precipitation was observed.

Fig. 1. Precipitation of actinomycete spores in aged sea water



Effect of Temperature and Salt concentration on Survival of Actinomycete

As described above, actinomycete spores can be transported through the soil by water and some can be precipitated to the bottom of the sea in a short time. It is believed that environmental conditions in shallow sea are very different from terrestrial ones. Therefore, the effects of temperature and salt concentration on survival of actinomycete were selected as parameters for study. As shown in Table 4, the growth of most strains was retarded by low temperature and high salt concentration. Although the conditions employed do not directly reflect those actually present in marine environment (experiments were conducted with shaking, an unlikely condition at sea bottom), all strains failed to grow in the presence of 14% NaCl. One strain ISP 5260, *Streptomyces rimosus* (SOBIN, FINLAY and KANE) WAKSMAN in WAKSMAN and LECHEVALIER 1953, was able to grow at 7% NaCl concentration. In contrast, strains SS-28, ISP 5026 (*Streptomyces pseudogriseolus* OKAMI and

Table 4. Effect of temperature and NaCl concentration on growth of actinomycetes under shaking conditions

Strains tested	0%		3.5%		7.0%		14.0%	
	15°C	27°C	15°C	27°C	15°C	27°C	15°C	27°C
Streptomyces sp., SS-17	2,492**	2,878	924	1,404	—***	563	—	—
S. pluricolorescens, ISP 5019	2,131	3,447	1,773	2,757	—	1,160	—	—
Streptomyces sp., SS-28	1,522	664	—	1,715	—	—	—	—
S. pseudogriseolus, ISP 5026	2,420	1,604	—	2,035	—	—	—	—
SS-1000 B	2,321	2,035	1,437	1,375	—	2,483	—	—
LS-147	1,777	2,962	250	1,652	—	—	—	—
S. californicus, ISP 5058	5,169	2,843	—	424	—	—	—	—
S. aureofaciens, ISP 5127	1,797	1,485	—	—	—	—	—	—
S. echinatus, ISP 5013	1,607	5,029	—	930	—	—	—	—
S. rimosus, ISP 5260	4,280	5,360	3,589	5,986	462	1,268	—	—

* Incubation on reciprocal shaker (130 rpm, 7-cm amplitude) for 10 days at 15°C and 5 days at 27°C in basal medium I.

** Wet weight mg/100 ml after centrifugation at 8,000 rpm for 20 minutes.

*** — = No growth.

UMEZAWA), ISP 5058 (*S. californicus*), ISP 5127 (*Streptomyces aureofaciens* DUGGAR) and ISP 5013 (*Streptomyces echinatus* CORBAZ, ETTLINGER, GÄUMAN *et al.*) gave no growth when they were shaken at 15°C in medium I containing 3.5% NaCl. Half of the actinomycete tested grew at 15°C in medium I containing 3.5% NaCl. These results suggest that some actinomycete which reach the sea may possibly grow if proper nutrients and oxygen are available. The other half

Table 5. Recovery of actinomycete which failed to grow in salt medium at 15°C

Strains tested	Culture		Recovered growth* (15°C, 10 days) mg/100 ml***	Recovered growth** (27°C, 5 days) mg/100 ml***
	Salt con. (%)	Growth (15°C, 10 days)		
Streptomyces sp., SS-17	3.5	+		
	7.0	−***	2,242	686
	14.0	−	1,813	1,071
S. pluricolorescens ISP 5019	3.5	+		
	7.0	−	3,076	2,073
	14.0	−	−	2,308
Streptomyces sp., SS-28	3.5	−	−	574
	7.0	−	−	761
	14.0	−	−	−
S. pseudogriseolus ISP 5026	3.5	−	−	1,371
	7.0	−	−	1,602
	14.0	−	−	1,736
SS-1000 B	3.5	+		
	7.0	−	706	1,777
	14.0	−	−	1,534
LS-147	3.5	+		
	7.0	−	−	1,808
	14.0	−	−	−
S. californicus ISP 5058	3.5	−	1,813	1,977
	7.0	−	−	4,722
	14.0	−	−	4,209
S. aureofaciens ISP 5127	3.5	−	−	−
	7.0	−	−	−
	14.0	−	−	−
S. echinatus ISP 5013	3.5	−	999	3,752
	7.0	−	−	2,874
	14.0	−	−	1,952
S. rimosus ISP 5260	3.5	+		
	7.0	+		
	14.0	−	3,186	3,890

* The broth (showing no growth, when incubated at 15°C, 10 days) was centrifuged and the precipitate was transferred to fresh medium without salt and incubated for 10 days at 15°C.

** The same as above except incubation for 5 days at 27°C.

*** Wet weight (mg/100 ml after centrifuged at 8,000 rpm, for 20 min.).

**** − = No growth.

did not grow at 15°C in medium I containing 3.5% NaCl, but they were not killed because they grew in fresh medium without addition of NaCl at 15°C or 27°C. *Streptomyces aureofaciens* strain ISP 5127 was an exception and was found to be extremely sensitive to NaCl as reported elsewhere[5] (Tables 4, 5). Strain SS-28 failed to grow at 15°C in medium containing 3.5% NaCl and failed to grow even though transferred to fresh medium without NaCl. It did grow poorly at 27°C. With some exceptions, *e.g.*, strains SS-28 and ISP 5127 (*Streptomyces aureofaciens*), many actinomycete survive in media containing NaCl in concentrations as high as 7 or 14%, as revealed by their survival at 27°C in fresh medium without NaCl. In summary, many actinomycete can survive in a salty environment at low temperature (15°C) for considerable periods of time. Some may be able to grow in salty (about 3.5% NaCl) environments at low temperature (15°C) if proper nutrients and oxygen are supplied.

Discussion

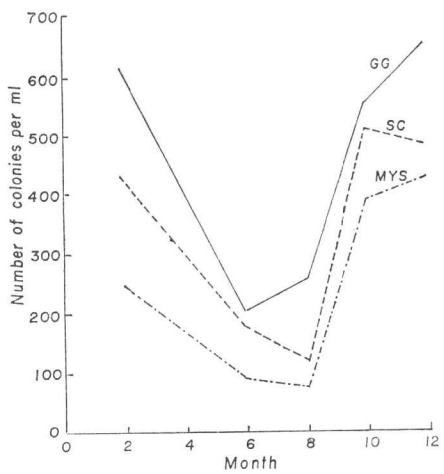
Actinomycete have been isolated from various terrestrial soils and are believed to exsist in spore form for much of the time[2,6,7]. Furthermore, S. T. WILLIAMS *et al.*[2,3,8,9,10,11] have reported that movement of detached spores was influenced by the fine structure of the spore surface, spore wettability and spore size. We were not able to ascertain the form (spores/vegetative mycelium) of actinomycete existed in marine environments, but spores of the test strains were used to facilitate quantitative work. Elution rates of the various strains of actinomycete were quite different and were not related to the types of spore surface. The discrepancy between WILLIAMS' data and ours may be due to differences in particle size of the soil or sand used for the studies. In WILLIAMS' experiments the effect of spore surfaces on movement was observed when fine and coarse sand was used but not when rough sand was used. In our case, we used garden soil consisting of both rough and fine particles, and no clear effect of spore surface was found. Nevertheless, significant differences between kinds of actinomycete were noted in terms of elution rates (removal) from soil or sand columns. These findings suggest that natural conditions in soil affect spore transport selectively and are independent of the nature of spore surface. Similarly, precipitation rates did not depend on the nature of spore surfaces, although again significant differences in precipitation rates were observed among different kinds of actinomycete. In summary, natural conditions during transport of actinomycete from terrestrial sites to the marine environment are complex, and they might lead to flora patterns in sea bottoms different from those in terrestrial soil. As shown, many mesophilic actinomycete can survive for considerable periods of time at the bottom of the sea, although certain strains sensitive to low temperature and salty condi-

Fig. 2. Seasonal change in numbers of actinomycete in Koajiro Inlet, Sagami Bay, Japan

GG medium: glycerol 2.0%, Glycine 0.25%, NaCl 0.1%, K_2HPO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.01%, agar 1.7% pH 7.4

SC medium: soluble starch 1.0%, Casein 0.1%, agar 1.7%, artificial mixed sea water 500 ml + dist. water 500 ml pH 7.4

MYS medium: maltose 1.0%, yeast extract 0.4%, agar 1.7% pH 7.2



tions may not. Because temperature at sea bottom in winter is lower and more stable than those in summer, preservation of actinomycete in shallow sea environments would be more favorable in winter than in summer. This was revealed in our record of colony counts of samples taken at inlets of Sagami bay (Fig 2). In conclusion, actinomycete in soil can be transported from terrestrial environments to the sea despite complex factors affecting their movement such as elution rate and sensitivity to temperature and salt concentration. Many actinomycete can survive for a period of time in the sea and some can grow or survive in shallow sea bottoms. Therefore, shallow sea sites represent an interesting source of special actinomycete which can survive and live under both terrestrial and marine environments.

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