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Studies on the distribution of alkalophilic and alkali-tolerant soil fungi II: Fungal flora in two limestone caves in Japan

Koji Nagai[1], Kenichi Suzuki[1] and Gen Okada[2]

[1] Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 1–1–8, Azusawa, Itabashi-ku, Tokyo 174–8511, Japan

[2] Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), 2–1, Hirosawa, Wako-shi, Saitama 351–0198, Japan

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In a series of studies on the distribution of alkalophilic and alkali-tolerant fungi, soil fungi were isolated from five alkaline calcareous soil samples in two closely located limestone caves (stalactite caves) in Japan using slightly acidic and alkaline media. Some common soil fungi that can grow in alkaline conditions were obtained in high frequencies. The growth response to pH of the isolates revealed that approximately one third (30.8%) of the isolates had the optimum pH in the alkaline range. All isolates of four *Acremonium* species and two *Chrysosporium* species grew well in alkaline conditions, of which *Acremonium* sp. and *Chrysosporium* sp. were pronounced alkalophiles. These fungi were thought to be indigenous species in this alkaline environment. The fungal flora in the Japanese alkaline soils was considerably different from the flora reported in alkaline environments in other countries.

Key Words——alkalophilic; distribution; limestone cave; soil fungi.

In a series of studies on the distribution of alkalophilic and alkali-tolerant fungi, the effect of isolation media has been evaluated by Nagai et al. (1995) using the materials from Indonesia and Japan. In this paper, we report on the fungal flora in an alkaline environment in Japan.

Nagai et al. (1995) isolated soil fungi from acidic soils from Japan and alkaline soils from Indonesia using slightly acidic and alkaline media to study the distribution of alkalophilic and alkali-tolerant fungi. Different species were obtained on each isolation medium from both soils. Some fungi, especially *Acremonium* species, were alkalophiles or alkali-tolerants that could grow at pH 10. Most of them were isolated from alkaline soils, but a few species also existed in acidic soils. As those species were not isolated on acidic media, the use of an alkaline medium (ACMA; Nagai et al., 1995) facilitated the isolation of alkalophilic soil fungi.

Although alkaline soils have been examined for their fungal flora in other countries (Warcup, 1951; Stenton, 1953; Nicholls, 1956; Mukerji, 1965; Pugh and Dickinson, 1965; Rai et al., 1971; Nagai et al., 1995), the distribution of fungi in the alkaline environments in Japan has not been reported as far as we know. Using slightly acidic and alkaline media, we isolated here the fungi from Japanese alkaline soils in a soil type different from that in Indonesia (Nagai et al., 1995): i.e., the soils in two limestone caves (stalactite caves) in Japan. For each isolate, the growth response to pH of the medium was measured to evaluate the alkalophilic or alkali-tolerant nature.

Materials and Methods

Soil samples Five alkaline calcareous soils (pulverized limestone) having high free calcium carbonate content (pH 7.7 to 8.8 in H_2O) were randomly collected in two limestone caves, Takikandou and Hakurendou, in Iwate Pref., Japan on 3 June 1993.

Isolation of fungi Fungi were isolated on cornmeal agar (CMA) and alkaline cornmeal agar (ACMA) by the dilution plate method (Nagai et al., 1995). Dilution plates were prepared from 1 g (fresh weight) of each soil sample, at dilutions of 10, 10^2, 10^3 in test-tubes with sterile physiological saline solution, on CMA (pH 6.0; Nissui, Tokyo) and ACMA (pH ca. 9.7), both containing 100 mg/L chloramphenicol.

ACMA was prepared with solution A (17 g of CMA powder, 900 ml of distilled water) and solution B (3 g of Na_2CO_3, 3 g of $NaH_2PO_4 \cdot 2H_2O$, 100 ml of distilled water). After sterilization, 900 ml of solution A and 100 ml of solution B were mixed. The final pH of the mixture was about 9.7.

Four plates were prepared for each dilution series, and all were incubated at 24°C for 1–3 wk. The growing fungi were observed under the light microscope, and representative strains were isolated. All isolates are maintained in Yamanouchi Pharmaceutical Co., Ltd. (YBLF).

Identification of fungi Identification was carried out by using the literature described in Nagai et al. (1995).

Growth rates of the isolates at various pH In each species, one strain was selected at random as a representa-

tive. The strains were inoculated onto malt extract agar (MA: 10 g of malt extract powder (Difco), 2.5 g of peptone, 20 g of agar, 1 L of distilled water) of which the initial pH was adjusted after autoclaving to 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with buffers. The media were prepared by mixing 900 ml of the above MA formula with 100 ml of one of the buffer solutions listed in Table 1. Growth patterns of the species were obtained by measuring twice the colony diameters of the representative strains after incubation for 1–2 wk.

Classification of the isolates based on the growth patterns The species were grouped into alkalophobic, alkali-tolerant and alkalophilic fungi according to the definitions of Nagai et al. (1995): strains which could not grow at pH 10 are regarded as alkalophobic, those which grew at pH 10 as alkali-tolerant, and those which could grow at pH up to 10, but not at pH 5–6, as alkalophilic.

Results

Fungal species isolated from alkaline soils in the limestone caves Table 2 shows the species or infraspecific names with citation of the authors and unidentified taxa of fungi isolated from the alkaline soils in the limestone caves. Fifty-two species were recorded in total on CMA and ACMA. Thirty-three species were recorded on CMA and 26 species on ACMA. Only 7 species were common to both media. Twenty-six and 19 species were recorded only on CMA and ACMA, respectively. The species of *Mortierella*, *Mucor*, *Penicillium* and *Trichoderma* were isolated only on CMA. The species common to both media were *Acremonium furcatum*, *A. murorum*, *Beauveria bassiana*, *Fusarium solani*, *Gliocladium cibotii*, *G. roseum*, and *Volutella ciliata*. The strains of *Acremonium*, *Doratomyces*, *Fusarium*, and *Plectosporium* were more frequently isolated on ACMA than on CMA. In *Acremonium*, 2 species and all 4 species were recorded on CMA and ACMA, respectively. In *Fusarium*, 1 species was found on CMA and all 4 species on ACMA. *Alternaria* sp. 2, *Arthrinium* sp., *Chrysosporium* spp., *Metarhizium anisopliae*, *Trichobotrys effusa*, *Verticillium tenerum*, *Verticillium* sp. 1, *Wardomyces* sp., the anamorph of *Nectria ellisii*, and an unidentified hyphomycete were isolated only on ACMA.

Growth patterns of the isolates For 52 species, the optimum growth pH was determined based on the colony diameter measurement at 24°C (Table 3). Two species showed the best growth at pH 10, 6 species at pH 9, 8 species at pH 8, 12 species at pH 7, 13 species at pH 6, and 11 species at pH 5. Sixteen species (30.8%) had the optimum pH in the alkaline range, 12 species (23.1%) in the neutral range, and 24 species (46.2%) in the acidic range. Thirty-two species (61.5%) were able to grow at pH 10.

Sixteen species (30.8%) isolated on CMA were alkalophobic: *Chaetomium* sp., *Cladosporium* sp. 1, *Cladosporium* sp. 2, *Cylindrocarpon* sp. 2, *Epicoccum nigrum*, *Geomyces pannorum* var. *pannorum*, *Mortierella* sp., *Mucor* sp., *Paecilomyces* sp. 2, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 4, *Pestalotiopsis* sp., *Trichoderma hamatum*, *T. harzianum*, and *Trichoderma* sp. The species that grew better at pH 10 than at pH 5 were *Acremonium furcatum*, *A. strictum*, *Acremonium* sp., *Chrysosporium* sp., *Stachybotrys cylindrospora*, and *Verticillium tenerum* (data not shown). As *Acremonium* sp. (YBLF 810) and *Chrysosporium* sp. (YBLF 831) grew very well at pH 10 but not at all at pH 5, these 2 species were designated as alkalophilic fungi. Their growth patterns are shown in Fig. 1. The remaining 30 species grew under alkaline conditions and might be alkali-tolerant species.

As for *Acremonium*, eight isolates were classified into four species. These *Acremonium* species all grew very well under alkaline conditions and the optimum growth was observed on the alkaline side (above pH 8).

Discussion

Though we collected five soil samples in two closely located limestone caves to isolate fungal strains in this study, we did not compare the five samples or the two sites with each other, mainly because the environments in those two limestone caves were thought to be very similar. Rather, we examined the fungal flora of the

Table 1. Composition of buffer solution for the pH-adjustment of malt extract agar.

Final pH	Composition of buffer solution (mmol/100 ml)			
	Na_2CO_3	$NaHCO_3$	Na_2HPO_4	$NaH_2PO_4 \cdot 2H_2O$
11.0	50.0[a]			
10.0	27.5	22.5		
9.0	3.0	47.0		
8.0			49.0	1.0
7.0			27.5	22.5
6.0			5.0	45.0
5.0				50.0[b]

a) pH adjusted to 12.7 with 1 N NaOH.

b) pH adjusted to 3.8 with 1 N HCl.

Table 2. Frequency of occurrence of fungi in five alkaline soil samples.

Species/Taxon	Frequency (Max. 5)	
	CMA	ACMA
Acremonium furcatum	1	1
A. murorum	3	1
A. strictum		1
Acremonium sp.		1
Alternaria sp. 1	1	
Alternaria sp. 2		2
Arthrinium sp.		1
Aspergillus sp.	1	
Beauveria bassiana	1	2
Chaetomium sp.	2	
Chrysosporium pseudomerdarium		1
Chrysosporium sp.		1
Cladosporium sp. 1	1	
Cladosporium sp. 2	2	
Cylindrocarpon sp. 1	1	
Cylindrocarpon sp. 2	1	
Doratomyces nanus		2
D. stemonitis		1
Epicoccum nigrum	1	
Fusarium lateritium		1
F. oxysporum		1
F. solani	1	3
F. subglutinans		1
Geomyces pannorum var. *pannorum*	1	
Gliocladium cibotii	2	2
G. roseum	2	3
Mariannaea elegans	1	
Metarhizium anisopliae		1
Mortierella sp.	2	
Mucor sp.	4	
Paecilomyces sp. 1	1	
Paecilomyces sp. 2	1	
Penicillium sp. 1	1	
Penicillium sp. 2	1	
Penicillium sp. 3	2	
Penicillium sp. 4	1	
Pestalotiopsis sp.	1	
Plectosporium tabacinum		3
Stachybotrys chartarum	1	
S. cylindrospora	1	
Trichobotrys effusa		1
Trichoderma hamatum	1	
T. harzianum	2	
Trichoderma sp.	1	
Verticillium tenerum		1
Verticillium sp. 1		1
Verticillium sp. 2	1	
Verticillium sp. 3	1	
Volutella ciliata	1	1
Wardomyces sp.		1
anamorph of *Nectria ellisii*		1
unidentified hyphomycete		1

Table 3. Optimum pH of MA for growth of isolates.

Optimum pH	Species/Taxon	Isolated on CMA	Isolated on ACMA	Strain No.
10	***Acremonium* sp.**[a]		○	YBLF 810
	***Chrysosporium* sp.**		○	YBLF 831
9	***Acremonium murorum***	○	○	YBLF 802
	A. strictum		○	YBLF 808
	Chrysosporium pseudomerdarium		○	YBLF 830
	Stachybotrys cylindrospora	○		YBLF 982
	***Verticillium* sp. 1**		○	YBLF 801
	unidentified hyphomycete		○	YBLF 829
8	***Acremonium furcatum***	○	○	YBLF 834
	Beauveria bassiana	○	○	YBLF 817
	Dratomyces stemonitis		○	YBLF 839
	Fusarium lateritium		○	YBLF 826
	Plectosporium tabacinum		○	YBLF 824
	Verticillium tenerum		○	YBLF 812
	***Wardomyces* sp.**		○	YBLF 805
	anamorph of *Nectria ellisii*		○	YBLF 820
7	*Arthrinium* sp.	○		YBLF 827
	Doratomyces nanus		○	YBLF 804
	Fusarium solani	○	○	YBLF 823
	F. subglutinans		○	YBLF 837
	Gliocladium cibotii	○	○	YBLF 809
	Metarhizium anisopliae		○	YBLF 832
	Penicillium sp. 3	○		YBLF 988
	Stachybotrys chartarum	○		YBLF 972
	Trichobotrys effusa		○	YBLF 828
	***Verticillium* sp. 2**	○		YBLF 842
	Verticillium sp. 3	○		YBLF 1006
	Volutella ciliata	○	○	YBLF 816
6	***Aspergillus* sp.**	○		YBLF 990
	Chaetomium sp.	○		YBLF 993
	Cladosporium sp. 1	○		YBLF 968
	Cladosporium sp. 2	○		YBLF 995
	***Cylindrocarpon* sp. 1**	○		YBLF 967
	Cylindrocarpon sp. 2	○		YBLF 983
	Fusarium oxysporum		○	YBLF 836
	Gliocladium roseum	○	○	YBLF 822
	Mariannaea elegans	○		YBLF 966
	Mortierella sp.	○		YBLF 976
	Mucor sp.	○		YBLF 969
	***Paecilomyces* sp. 1**	○		YBLF 984
	Penicillium sp. 4	○		YBLF 1005
<5	***Alternaria* sp. 1**	○		YBLF 971
	***Alternaria* sp. 2**		○	YBLF 807
	Epicoccum nigrum	○		YBLF 975
	Geomyces pannorum var. *pannorum*	○		YBLF 962
	Paecilomyces sp. 2	○		YBLF 1002
	Penicillium sp. 1	○		YBLF 964
	Penicillium sp. 2	○		YBLF 970
	Pestalotiopsis sp.	○		YBLF 973
	Trichoderma hamatum	○		YBLF 965
	T. harzianum	○		YBLF 987
	Trichoderma sp.	○		YBLF 960

a) Species that can grow at pH 10 are indicated in bold.

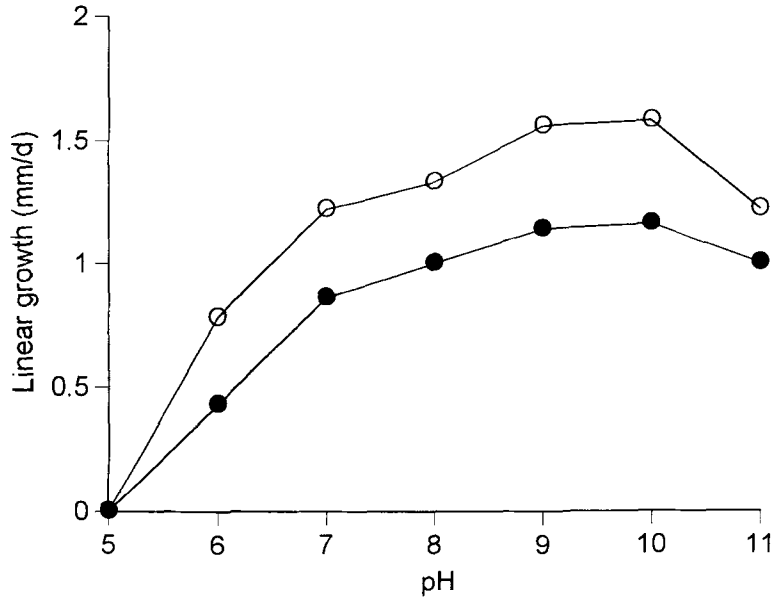

Fig. 1. Growth patterns of alkalophilic hyphomycetes on MA.
○: *Acremonium* sp. (YBLF 810). ●: *Chrysosporium* sp. (YBLF 831).

limestone caves dealing collectively with the five soil samples.

Several characteristic fungal species were reported from alkaline soils using dilution plate methods. Nicholls (1956) mentioned that *Penicillium nigricans*, *Mortierella alpina*, and *M. minutissima* were dominant in alkaline chalk soils in UK. Many studies showed that *Gliocladium roseum* was a common soil fungus particularly in neutral and alkaline soils (Tribe, 1957; Griffin, 1960; Pugh and Dickinson, 1965). Mukerji (1965) and Rai et al. (1971) investigated the fungal flora of alkaline soils in India (Usar), and they found *Aspergillus* to be the most dominant genus. From five alkaline soils in two limestone caves in Japan, we frequently isolated *Acremonium murorum*, *Fusarium solani*, *Gliocladium roseum*, *Mucor* sp., and *Plectosporium tabacinum* (Table 2). Apart from *F. solani*, these species were also isolated from weakly acidic soils in Japan (Nagai et al., 1995). They are characteristic species growing well over a wide pH range (Tables 2, 3), and are different from the Indonesian species reported by Nagai et al. (1995). These results probably reflect the nature of the dilution plate method, which has been used in most studies on the distribution of soil fungi. This method tends to result in isolation of fast-growing fungi (common soil fungi), many of which originate from resting stages (Gams, 1992). In this study, many alkali-tolerant common soil fungi are thought to have been present as resting structures in alkaline soils and to have started growing as a result of the isolation procedure.

Fungi are generally considered to grow well on acidic (below pH 7) substrata (Bååth et al., 1984). Twenty-four species (46.2%) isolated from the soils in the limestone caves in Japan preferred acidic conditions and 16 species (30.8%) were alkalophobic. In Japanese acidic soils, the isolation rates of acidophilic and alkalophobic fungi were 69.0% and 31.0%, respectively (Nagai et al., 1995). Fungi with acidic pH optima were less frequently isolated from alkaline soils, but several alkalophobic fungi including common soil fungi were isolated, perhaps as a result of the resting mechanisms mentioned above. Approximately one third (30.8%) of the isolates from these alkaline soils had the optimum pH in alkaline range. It is interesting that fungi with alkaline pH optima were more frequently isolated from alkaline soils than acidic soils in Japan: 30.8% of the isolates from alkaline soils (this study) and 13.8% of the isolates from acidic soils (Nagai et al., 1995). In these circumstances, it is probable that soil types and fungal physiological characteristics are closely related, and that soil fungi are selected by their substrata.

All isolates of four *Acremonium* species preferred alkaline conditions. The ability to grow under alkaline conditions is thought to be common to many *Acremonium* spp. *Acremonium* sp. (YBLF810) did not grow at all at pH 5 and was designated as the most representative alkalophilic species. Two isolates of *Chrysosporium* also grew well in alkaline conditions, and *Chrysosporium* sp. (YBLF831) was designated as an alkalophile. As far as we know, this is the first report on an alkalophilic *Chrysosporium* sp. Besides the species of these two genera, some species showed good growth in an alkaline range: *Stachybotrys cylindrospora*, *Verticillium* sp. 1, and unidentified hyphomycete grew best at pH 9; *Beauveria bassiana*, *Doratomyces stemonitis*, *Fusarium lateritium*, *Plectosporium tabacinum*, *V. tenerum*, *Wardomyces* sp., and the anamorph of *Nectria ellisii* grew best at pH 8. These species are thought to be indigenous fungi in the alkaline environment examined in this study.

The fungal species isolated from alkaline soils at two limestone caves in Japan were considerably different from the species reported in alkaline environments in other countries. The fungal flora in alkaline environments in Japan should be clarified further by analyzing many samples in the future. Another important task is to resolve morphologically and physiologically how fungal strains live in alkaline soils.

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