NUMBER 6

INSTITUTE FOR FERMENTATION
OSAKA

RESEARCH COMMUNICATIONS

(ANNUAL REPORT 1971-1972)

1973

財団法人 発酵研究所

RESEARCH COMMUNICATIONS

No. 6

(*Annual Report 1971-1972*)

1973

INSTITUTE FOR FERMENTATION, OSAKA

4–54, Juso-Nishinocho, Higashiyodogawa-ku,
Osaka 532, Japan

CONTENTS

Confirmation System for the ISP Strains Preserved in Japan.
.... Takezi HASEGAWA and Yoshiro OKAMI 1

A Method for Preservation of Bacteria and Bacteriophages.
by Drying *in vacuo*. .... Teiji IIJIMA and Takeshi SAKANE 4

Successive Fungal Flora on Sterilized Leaves in the Litter of Forest. II.
.... Keisuke TUBAKI and Tatsuo YOKOYAMA 18

Successive Fungal Flora on Sterilized Leaves in the Litter of Forest. III.
.... Keisuke TUBAKI and Tatsuo YOKOYAMA 27

Experiments on the Protection of Gnotobiotic Silkworm Larvae from Bacterial Diseases by Oral Antibiotics.
.... Reijiro KODAMA and Yugoro NAKASUJI 50

Inhibition of Development of Viral Diseases in Gnotobiotic Silkworm Larvae by Nalidixic Acid. .... Reijiro KODAMA and Yugoro NAKASUJI 59

Bacteria Isolated from Silkworm and Their Pathogenicity in Gnotobiotic Silkworm Larvae.
.... Yugoro NAKASUJI, Akira KOBAYASHI and Reijiro KODAMA 68

Descriptive Catalogue of I.F.O. Culture Collection.
Fungal Collection. 83
Bacterial Collection. 95

Abstracts, 1971–1972. 99

Presentation of Papers at Scientific Meetings. 104

Annual Report, 1971–1972. 105

Contents of No. 1–5. 106

IFO Res. Comm. 6,
1–3, 1973 (*January*)

CONFIRMATION SYSTEM FOR THE ISP STRAINS PRESERVED IN JAPAN

Takezi HASEGAWA and Yoshiro OKAMI*

Cooperative taxonomic evaluation studies of *Streptomyces* (*Actinomyces*) and *Streptoverticillium* species were undertaken by the Sub-committee on Taxonomy of Actinomycetes which was constituted by IAMS in 1958. Dr. David Gottlieb, University of Illinois, Chairman of the Subcommittee, organized these studies because of the importance of these organisms as antibiotics producers and because of the weakness of the criteria for these particular taxa. The International Streptomyces Project (ISP), recently shortened from the International Cooperative Project for Description and Deposition of Type Cultures of *Streptomyces*, was started in 1964 staffed by specialists from 18 nations with Dr. Elwood B. Shirling of Ohio Wesleyan University as Director of the Project.[1]

The basic philosophy of the program was that description of any species must be reproducible by competent investigators anywhere in the world, and that all type cultures of streptomycete species should be deposited in collections so as to be freely available to all investigators.

A manual[2] of experimental methods was compiled from previous investigations to standardize interpretation of characteristics and descriptions. Media were prepared by the Difco Laboratories under standard procedures for the entire project. More than four hundred cultures of streptomycete species were collected and coded at the project office at Ohio Wesleyan University. An unidentified subculture was sent to three investigators in different countries. Only after the subcultures had been re-described were the species names distributed by the project office. When all three descriptions agreed or were generally consistent there was no problem. If the results for any character from all three collaborators differed, that character was considered unsuitable for species description. If agreement was found only by two of the three investigators, differences were usually settled by submitting the culture to a fourth investigator.

The reexamined descriptions were published in the International Journal of Systematic Bacteriology,[3,4,5,6] and the described organisms were deposited in the Centraalbureau voor Schimmelcultures, Baarn, the American Type Culture Collection, Rockville, U.S.S.R. Research Institute for Antibiotics, Moscow, and the Institute for Fermentation, Osaka.

The Institute for Fermentation, Osaka (IFO) has been the main center in Japan responsible for the Project since 1969 in compliance with the request from the Society

* Institute of Microbial Chemistry, Kamiosaki, Shinagawaku, Tokyo

for Actinomycetes, Japan, that had cooperated in the execution of the Project. In 1970, the Society organized a Japanese committee for confirmation of the ISP cultures kept in Japan.

The current members of the committee are:

Dr. Tadashi Arai, Institute of Food Microbiology, Chiba University, Chiba
Dr. Tamotu Furumai, Microbiological Chemistry Research Laboratory, Tanabe Seiyaku Co. Ltd., Tokyo
Dr. Masa Hamada, Institute of Microbial Chemistry, Tokyo
Dr. Akihiro Matsumae, The Kitasato Institute, Tokyo
Dr. Yuzuru Mikami, Institute of Food Microbiology, Chiba University, Chiba
Dr. Koiti Nakazawa, Institute for Fermentation, Osaka
Dr. Taro Niida, Central Research Laboratories, Meiji Seika Kaisha, Ltd.
Dr. Yoshio Okami, Institute of Microbial Chemistry, Tokyo (chairman)
Dr. Masanori Okanishi, National Institute of Health, Tokyo
Dr. Akio Seino, Research Laboratories, Kaken Chemical Co. Ltd., Tokyo
Dr. Takashi Shomura, Central Research Laboratories, Meiji Seika Kaisha Ltd., Tokyo
Dr. Akira Shimazu, Institute of Applied Microbiology, University of Tokyo, Tokyo

The program of the committee is:
1. Cultures maintained in IFO are checked at about four year intervals with their description as published by the Project.
2. If a culture differs from its Project description it is suspended from free distribution. If already distributed, the receivers would be so advised.

The aims of the committee are not only to prevent ISP strains in IFO from causing any taxonomic confusion, but also to confirm the characteristics as keys for identification and to contribute to the establishment of a reliable systematics in this taxon. The first examination by the committee was carried out in February, 1971. Several criteria, i.e., color of aerial hyphae, reverse color of the colony, diffusion of water-soluble pigments and microscopic morphology were tested with 300 strains sent by the Project office to IFO and maintained there as lyophilized stocks for one to two years.[7] One culture was discrepant in comparison with ISP description and was replaced with another culture of the same strain received again from the Project office. Two other cultures showed questionable discrepancy and are under further investigation.

Recently, another 115 cultures[6] were sent to IFO from the Project office and they are under study by the above Japanese committee.

References

1) Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int'l. J. System. Bact. 16(3) 313–340.
2) Gottlieb, D. and E.B. Shirling, 1967. Cooperative description of type cultures of *Streptomyces*. I, The International Streptomyces Project. *ibid.* 17(4) 315–322.
3) Shirling E.B. and D. Gottlieb, 1968. Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. *ibid.* 18(2) 69–189.
4) Shirling E.B. and D. Gottlieb, 1968. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. *ibid.* 18(4) 279–392.
5) Shirling, E.B. and D. Gottlieb, 1969. Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. *ibid.* 19(4) 391–512.
6) Shirling, E.B. and D. Gottlieb, 1972. Cooperative description of type cultures of *Streptomyces*. V. *ibid. in press.*
7) IFO List of Cultures, 1972.

IFO Res. Comm. 6,
4–17, 1973 (*January*)

METHOD FOR PRESERVATION OF BACTERIA AND BACTERIOPHAGES BY DRYING *IN VACUO*

Teiji IIJIMA and Takeshi SAKANE

Preservation of microorganisms in the living state involves risk of loss of certain strains, cultural variation or contamination even under careful control. These risks are partly overcome by a method of preservation in dried state which can be achieved by drying suspensions of microorganisms either from frozen state or directly from liquid state.

In genetical studies, a number of mutant strains have been isolated and they should be preserved in several years without loss of their viability and without changing their genetic characteristics. For preservation of these mutants a similar method is also available. However, the possibility of cross-contamination in drying procedures among ampoules of different mutant strains, must be minimized because these mutant strains sometimes have a similar phenotype.

We have been attempting to establish a simple and efficient method to preserve a number of mutant strains of *Escherichia coli* and a fairly satisfactory method was established by a modification of the method of Annear (1958, 1962).

Some of the results in the present paper were published earlier (Iijima and Sakane 1970 1971).

Methods

Preparation of dried specimens

(1) Round bottomed and straight necked tubes (110 mm — 8 mm) are plugged with cotton wool and sterilized in a oven.

(2) 0.1 ml (or 0.2 ml) aliquot of bacterial suspension are poured into the sterile tubes.

(3) The cotton wool plugs are trimmed at the top of the tubes and are pushed half way into the tube by a sterilized glass rod.

(4) The tubes are constricted about 1.5 cm above the plug in a gas flame.

(5) Tubes are attached to the horizontal manifold of a drying apparatus (Japan Vacuum Engineering Co. Ltd.). The manifold has sixty side arms which are connected to Vertis stoppers.

(6) When the valve to the manifold is opened, the water of the bacterial suspension is evaporated *in vacuo*.

(7) Temperature of the suspension decreased to below 10 C, but for impediment of the cotton wool plug in the tubes, it did not fall below the freezing point.

(8) The suspension superficially appeared to be dried after about one hour but drying was continued for further one hour after this stage has been reached.

(9) The tubes are sealed at the constricted portion by gas flame *in vacuo*.

(10) The ampoules are tested for their maintenance of vacuum by a High Frequency Tester (Edward High Vacuum Ltd. Model T2).

(11) The ampoules are stored at 5 C.

(12) For the accerelated storage test suggested by Greiff (1967, 1968) a part of the ampoules are stored at 37 C and checked their viability for 24 months.

Bacterial strains: *Escherichia coli* K12 mutant strain F682 (W4573 nonlysogenic) was used throughout the experiments to determine the optimal condition for drying *in vacuo*. The mutant strain has the following characteristics; lactose-, galactose-, arabinose-, maltose-, mannitol- and xylose-nonfermenting, streptomycin resistant, F^- strain. To test the availability of the method, a number of bacterial strains from the culture collection of the Institute for Fermentation, Osaka (IFO) were used.

Preparation of bacterial suspension: A bacterial strain was cultured overnight in nutrient broth. The cells are harvested by centrifugation and resuspended in a phosphate buffer containing sodium glutamate. The composition of the suspending medium is 1/10 M potassium phosphate buffer (pH 7.0) containing 3% sodium glutamate. The cells in the stationary phase gave a satisfactory result which maintained high viability during drying procedure and preservation. The cells in the logarithmic growing phase were rather sensitive to drying but there was no difference in sensitivity to drying between the cells harvested after 16 hr and 24 hr incubation.

Tris-buffer or borate buffer gave unsatisfactory results, even at pH 7.0.

Rehydration: After opening an ampoule, 0.5 ml of broth is added to the ampoule and the content is mixed carefully and made a homogeneous suspension. After appropriate dilution the surviving cells are estimated by plating on nutrient agar plates by the soft-agar double layer method.

Measurement of residual moisture of dried samples: As the amount of dried sample in a tube is extremly small, so five ampoules which were prepared at the same time, are combined and measured together.

(1) Each ampoule are applied a file mark around the tube and washed with aceton to remove the dirts.

(2) The tubes are put into a drying apparatus shown in Fig. 1 and dried for 1 hr at room temperature *in vacuo*.

(3) In a small chamber dried with P_2O_5, these ampoules are opened and checked their combined weight (W_1).

(4) After drying for 3 hr at 70 C *in vacuo* the residual weight (W_2) are checked.

(5) The ampoules are washed with water and dried in 120 C for 1 hr and dried further in the drying apparatus *in vacuo*.

(6) Weight of the dried empty ampoules is measured (W_3).

(7) The residual moisture is calculated from the following formula.

$$\frac{W_1-W_2}{W_1-W_3}\times 100$$

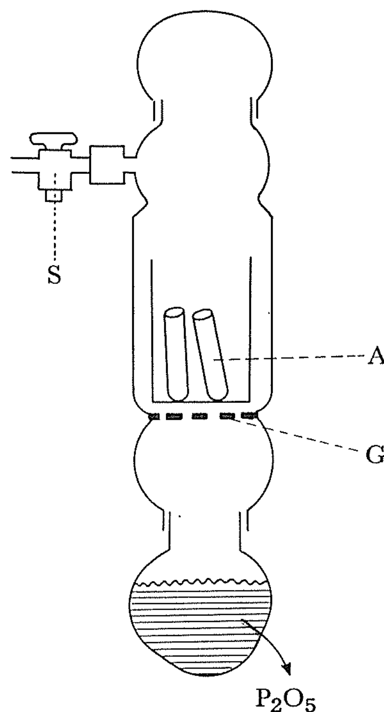


Fig. 1. Drying apparatus for measuring residual moisture

A : Ampoules containing dried organism
G : Glid
S : Stopper connected to a vacuum pump

Results and Discussion

The dried specimens were prepared by the method mentioned above and were stored at three different temperatures, 5, 25 and 37 C respectively. The stored ampoules were drawn at intervals; and checked their viability by the soft agar double layer method. The result is shown in Fig 2.

We can estimate the life-span of the dried specimens to be about 10 years at 25 C. The result shows that the method is efficient and practical for the preservation of the strain. In the method mentioned above, it is necessary to resuspend bacterial cells in the suspending medium after centrifugation. When a number of different strains are

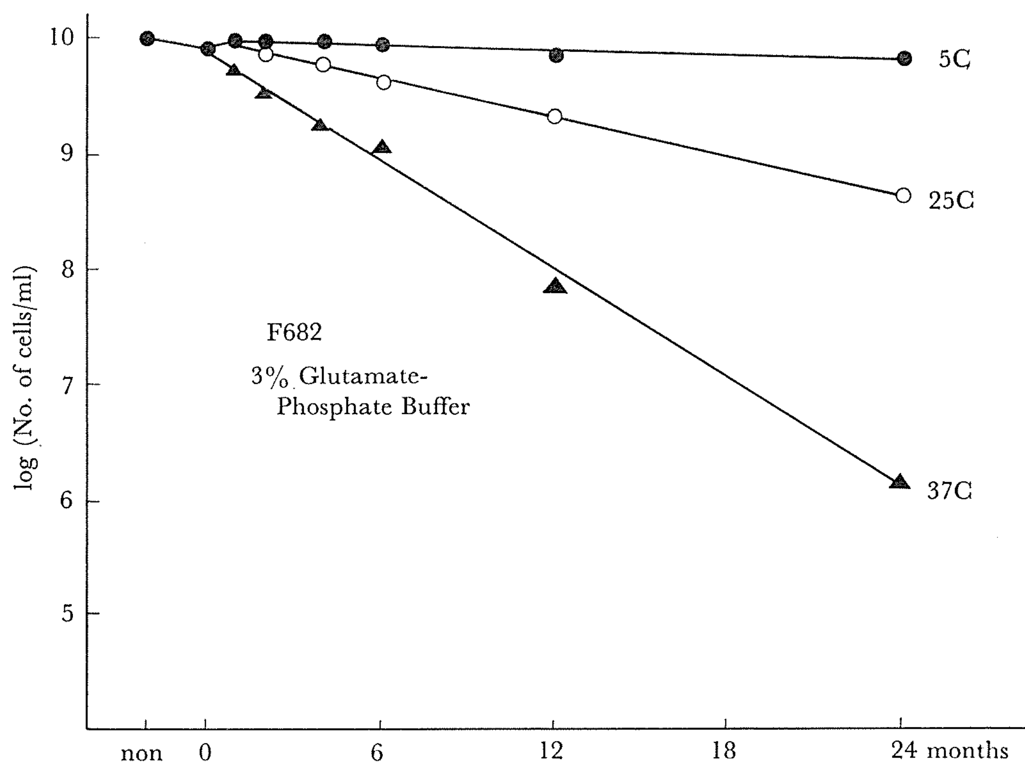


Fig. 2. Surviving fraction after storage at various temperatures
Ampoules contained a dried specimen were stored at various temperatures; 5, 25 and 37C. Ampoules were opened and surviving cells were counted after rehydration.

intended to be dried *in vacuo* simultaneously, the process of centrifugation will be somewhat troublesome. As shown in Table 1, the direct drying of the culture gave an unsatisfactory result. However when fresh broth was added to the culture to be dried, the survivor of cells increased to the level of 10%. Therefore the following modification was adopted when the centrifugation process was omitted.

An overnight culture was diluted five-fold with fresh broth then phosphate buffer containing sodium glutamate is added to the culture at a final concentration of 1/10 M potassium phosphate and 3% sodium glutamate. The suspension was used for drying according to the method mentioned above. Furthermore, when cells from agar slant were suspended directly in the suspending medium and used for drying, they gave a satisfactory result.

The drying apparatus in this laboratory has sixty sidearms, so sixty ampoules can be dried at the same time. Since one cycle of the drying procedures will take about two to three hours, one can make about 200 ampoules in a day.

The genetic markers such as nutritional requirement, sugar fermentation ability, lysogeny and resistance to drugs were stably maintained after preservation.

Table 1. The effects of the addition of fresh broth, sodium glutamate or phosphate buffer on the survival of drying samples.

Ratio[A]	Addition of		Titer of the initial	Stored at 37C			
				O day		30 days	
	glutamate[B]	phosphate[C] buffer	log N	log N	survivor %	log N	survivor %
1 : 0	—	—	9.5	8.7	13	8.5	9
	+	—	9.5	8.7	15	8.1	3
	+	+	9.5	9.1	37	8.3	5
1 : 1	—	—	9.3	8.5	14	8.3	10
	+	—	9.3	8.5	15	8.2	8
	+	+	9.3	8.9	40	8.2	8
1 : 3	—	—	9.0	8.0	10	8.0	10
	+	—	9.0	8.4	26	8.2	17
	+	+	9.0	8.6	40	8.3	19
1 : 9	—	—	8.5	7.1	3	7.2	4
	+	—	8.5	8.1	37	8.0	29
	+	+	8.5	8.1	34	7.9	22

A) Ratio of overnight culture : fresh broth added
B) Final concentration of glutamate in the mixture is 3%
C) Final concentration of phosphate buffer is 1/10 M.

Overnight culture of a strain was diluted by fresh broth containing glutamate or/and phosphate buffer. The suspension was dispensed in tubes and dried *in vacuo*. Samples were stored at 37°C and checked their survival fraction by plating.

Measurement of the drying condition.

When bacterial samples were dried in tubes without cotton wool plug, the temperatures of the samples fell below the freezing point and true sublimation was achieved during the whole process. However, when samples were dispensed in tubes with a cotton wool plug, frozen by dryice-alcohol and dried *in vacuo*, thawing of the sample occured at some stage of the drying, because of impediment of the cotton wool plug, hence true sublimation was not achieved. This fact suggests that the presence of the cotton wool plug would change the condition in the drying process, although the plug have been used to prevent cross-contamination. We intended to measure the temperature and the vacuum in the tubes in the both cases; that is, in the presence and the absence of the plug. An apparatus for this purpose is shown in Fig 3.

We can estimate the temperature and the vacuum inside of the cotton wool plug by a thermometer (T_1) connected to a thermocouple (t), and a vacuum gauge (V_1). Similarly the temperature of a sample without a cotton wool plug was indicated by thermometer T_2. Vacuum gauge V_2 will show the value outside of the cotton wool plug as well as the value in a tube without a cotton wool plug. Figure 4 shows a change of the vacuum and of the temperature measured by this method.

When a tube with a cotton wool plug (designated as type E specimen) was connected to a vacuum pump, temperature of the sample rapidly decreased from the room

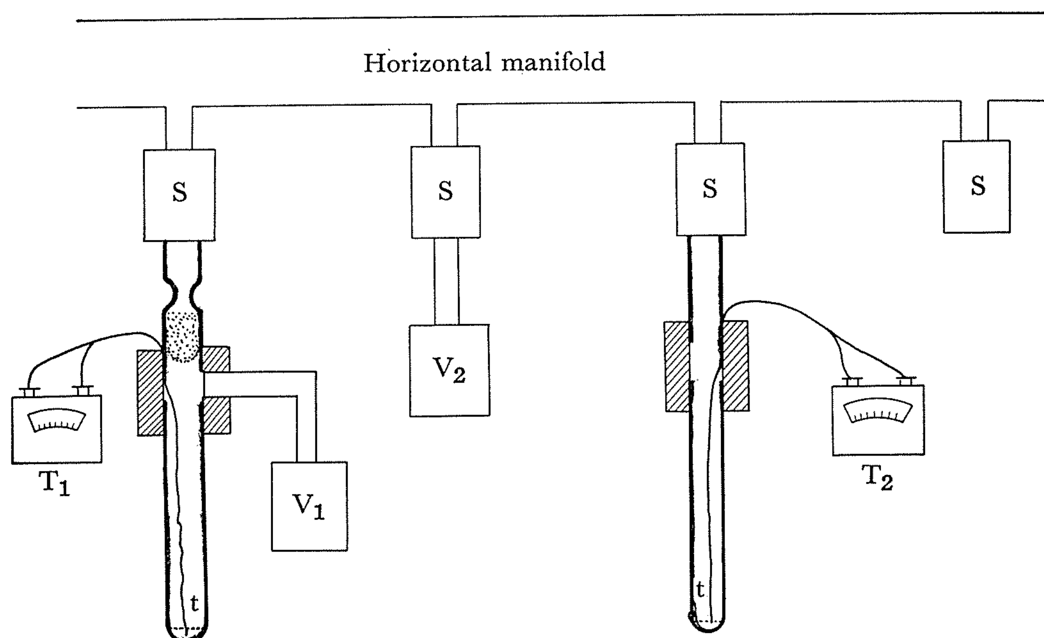


Fig. 3. Apparatus to measure the temperature of the sample and vacuum inside and outside of the cotton wool plug

T_1 : Thermometer to measure the temperature of sample which is dried inside of the cotton wool
T_2 : Thermometer to measure the temperature of sample which is dried without cotton wool plug
V_1 : Vacuun gauge to measure the vacuum inside of the cotton wool
V_2 : Vacuum gauge to measure the vacuum outside of the cotton wool
t : Thermocouple

temperature to 5 to 2C and maintained these level for 20 min (Fig 4 A). The water was evaporated under this condition. When the sample superficially appeared to be dried the temperature of the sample gradually rose and levelled at the room temperature after 60 min (curve T). Vacuum inside of the cotton wool plug fell to 10^{-1} mm Hg and remained constant for about 30 min, until the evaporation of water is completed (curve V). The vacuum outside of the tube is 1 order higher than that of inside. If a sample is dried without a plug, the change of vacuum and temperature is more drastic and proceeds along the curve of V and T in Fig 4B. However, if the vacuum and the temperature were controlled by manipulation of the valve connected to the vacuum pump, we would realized a similar condition as in Fig 4A even in the absence a of cotton wool plug. Figure 4C shows the change of the temperature and of the vacuum under the manipulation of the valve, and also shows that these changes are the same as those obtained in the presence of a cotton wool plug (Fig 4A). The samples prepared by the manipulation of the valve were designated as type F specimen. The specimen E and F were prepared in a similar condition as indicated in Fig 4A and Fig 4C except the presence of cotton wool plug. However the survival curve obtained from these two samples when they are stored at 37C, was different from each other as shown in Fig 5.

The result shown in Fig 5 indicated that the both specimens E and F, had the same

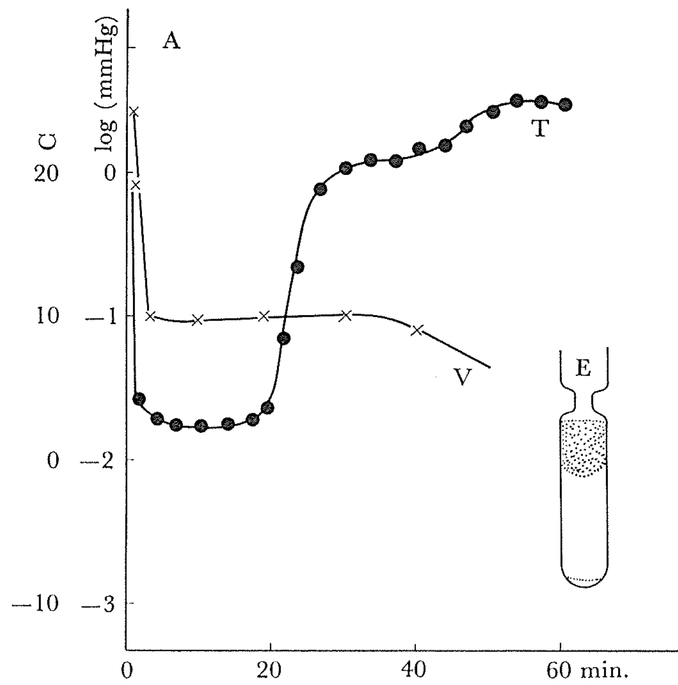
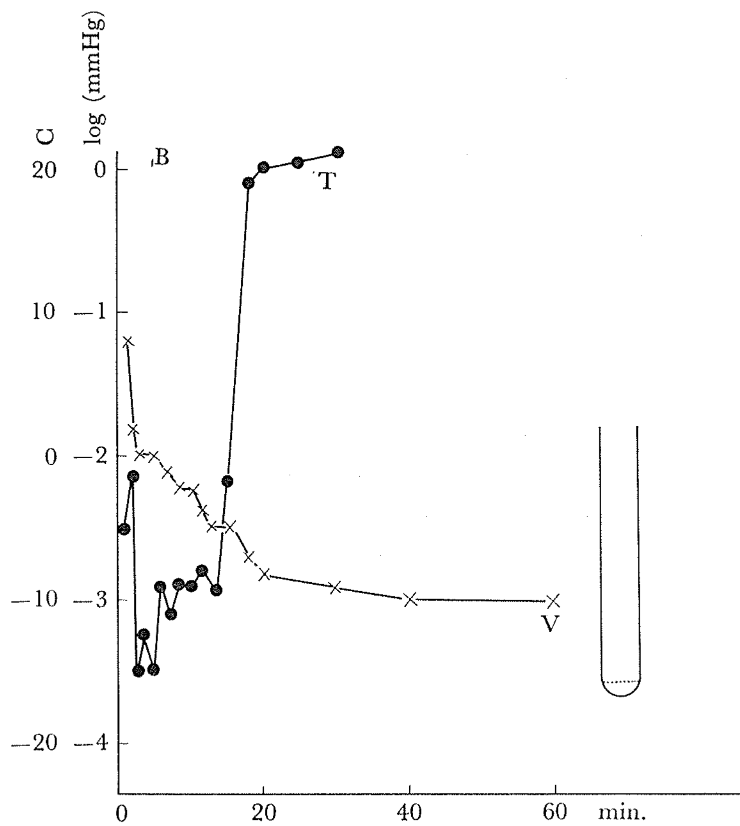
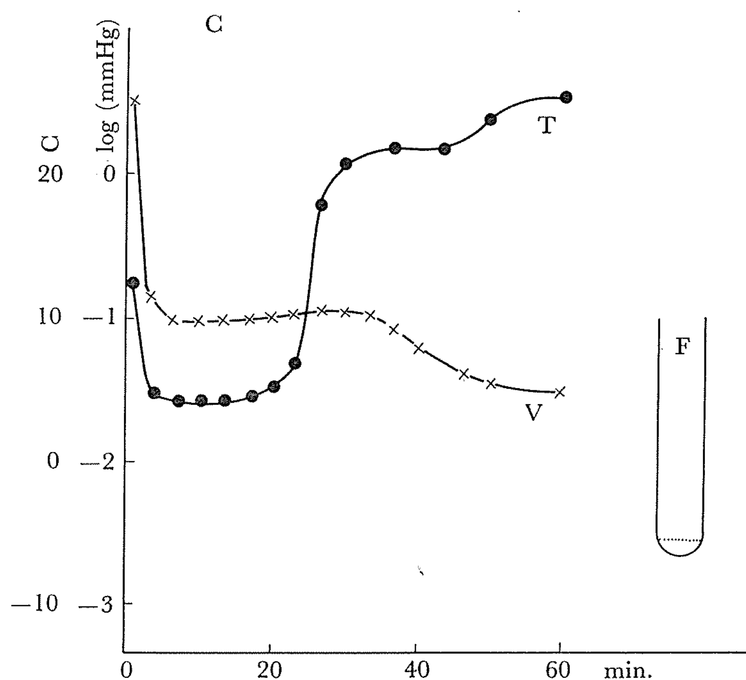


Fig. 4. Change of temperature and of vacuum druing the drying *in vacuo*

4A: Drying with a cotton wool plug



4B: Drying without a cotton wool plug



4C: Drying with manipulation of valve
T : Temperature of sample
V : Vacuum in a tube

amount of survivor before and after the drying because of a similar condition during the drying process. However after the preservation for 1.5 month, the survival percent in the specimens E was about a hundred-fold higher than that in the specimen F. This may indicate that the presence of a cotton wool plug in the tube gave a critical effect on the stability of the specimens. The role of a cotton wool plug was replaced by absorbent cotton, filter paper or some kinds of desiccants, but not by glass wool. This fact suggest that the cotton wool plug would act as a desiccant and decrease the residual moisture in the sample after the sealing.

Measuring the residual moisture of dried sample.

To elucidate whether the residual moisture would give a critical effect on a life-span of the specimen the residual moisture in various specimens were measured by the method described above. Specimens were prepared by various procedures such as type E, F and freeze-drying and were sealed at various stage during the drying procedures. The results are shown in Fig. 6 and Fig. 7.

The residual moisture in a freeze-drying specimen decreased slowly and fell to 25% in 1 hr, to 10% in 3 hr and to 8% in 5 hr after the commencement of drying. In contrast to this, the residual moisture of specimen with cotton wool plug (E) reached to 10% level in 30 min and remained in the constant value of 10% during drying procedure. (Fg 6). In the case of drying with valve-controlling (F) the change of residual moisture proceeded sa shown in Fig 7.

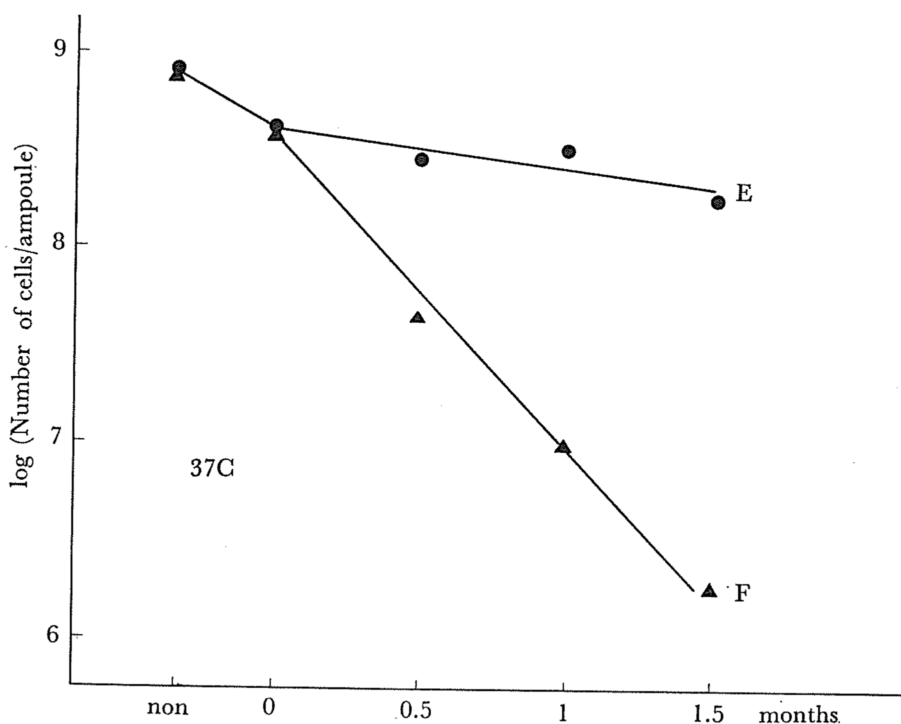


Fig. 5. Surviving fraction of two types of specimens after storage at 37C
E: Surviving fractions in a type E specimen
F: Surviving fractions in a type F specimen
Two kinds of specimens had been stored at 37C and the number of survivors were counted at intervals by plating on nutrient plates

From these results it is concluded that in freeze-drying method, residual moisture decreased slowly, so it is necessary to continue more than 5 hr to diminish the residual moisture below 10%, whereas in the cotton-plugged specimen (E), the decrease of residual moisture is somewhat rapid. In this case the residual moisture reached to 10% level within 1 hr but did not change by further drying.

Nevertheless the surviving fraction of type E specimen was high when they had been stored at 37 C. As mentioned above, the cotton wool plug was supposed to act as a desiccant, so the residual moisture in the specimen would be changed during storage. This suggestive action of the cotton wool plug may be revealed by the measuring the change of residual moisture during storage.

Figure 8 shows that the residual moisture decreased gradually and reached below 10% after four weeks of preservation at 5 C. This means that the residual moisture in the specimen reaches to a suitable value for preserving specimen in drying state, even if its residual moisture was somewhat higher than the optimal condition immediately after the drying. This might be a reason why the clear difference was observed between type E and type F specimen.

In summary an efficient and practicable method was established to preserve the bacterial strain. Drying conditions were examined by measuring temperature, vacuum

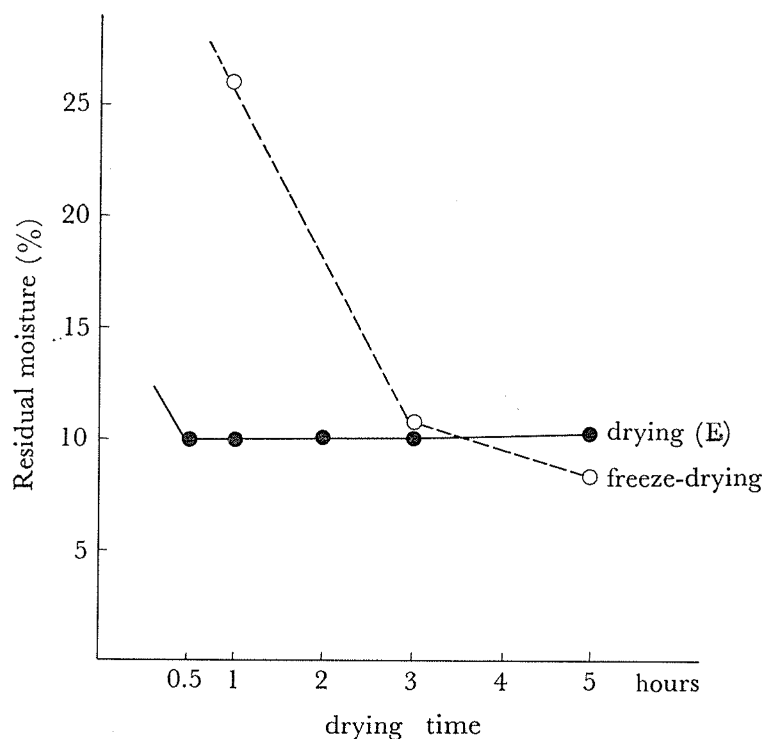


Fig. 6. Residual moisture in freeze-drying specimen and type E specimen
The specimens prepared by freeze-drying or drying *in vacuo*. During the drying procedures, ampoules were sealed and their residual moisture were measured by the method described in the text

and residual moisture. From these measurement, it was found that a cotton wool plug acted as a buffer for the drying condition, and in the presence of a plug the change of atomospheric condition inside the plug was efficiently controlled against the change of external condition. In contrast when a plug was absent, the change of external condition must directly affect on the sample to be dried. As indicated in Fig. 8 the residual moisture of a cotton wool is 1% (or less) and the volume of a cotton wool plug is much larger than that of the specimen, thus the plug acts as a moderate desiccant during storage.

The method has another point of advantage, the temperature of sample during the drying is 5 to 2 C, hence the evaporation of water is rapid and the time to the completion is shorter than that of lyophilization.

Application for other bacterial strains

These data were obtained mainly from the experiments with a mutant strains of *Escherichia coli*, but if composition of the suspending medium or condition of preculture were modified, a similar method may apply to other bacterial strains and bacteriophages as well.

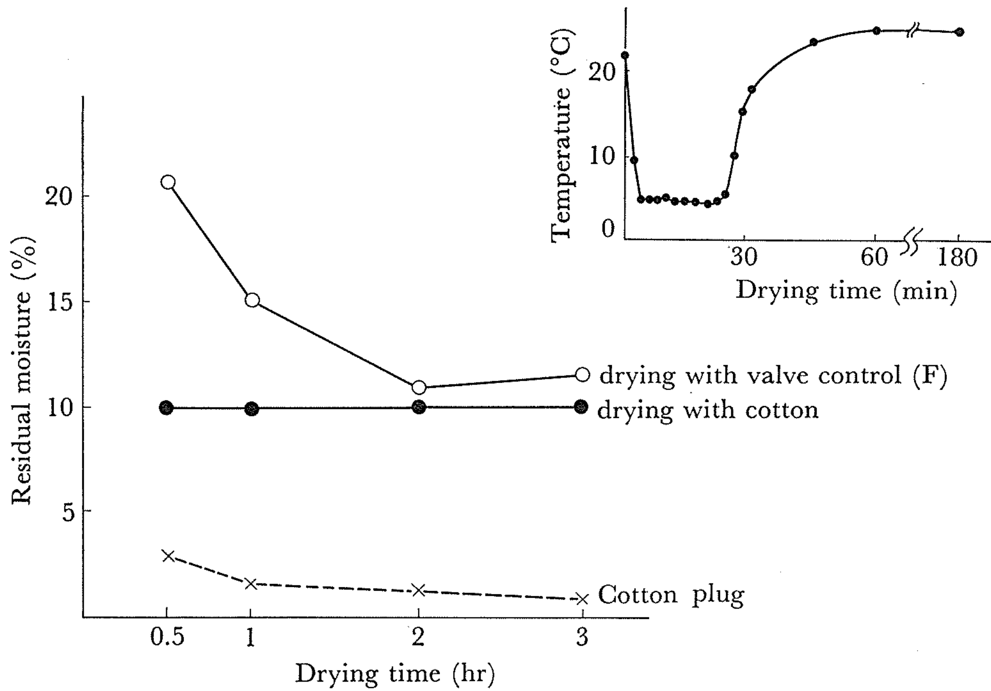


Fig. 7. Residual moisture of type E and type F specimen

Two types of specimens were prepared by the method described in the text. Ampoules were sealed at various stages in drying procedures, and their residual moisture were measured. Temperature during the drying of the specimens is shown in the figure.

●—●: Type E specimens ○—○: Type F specimen, ×…×: Residual moisture of a cotton wool plug

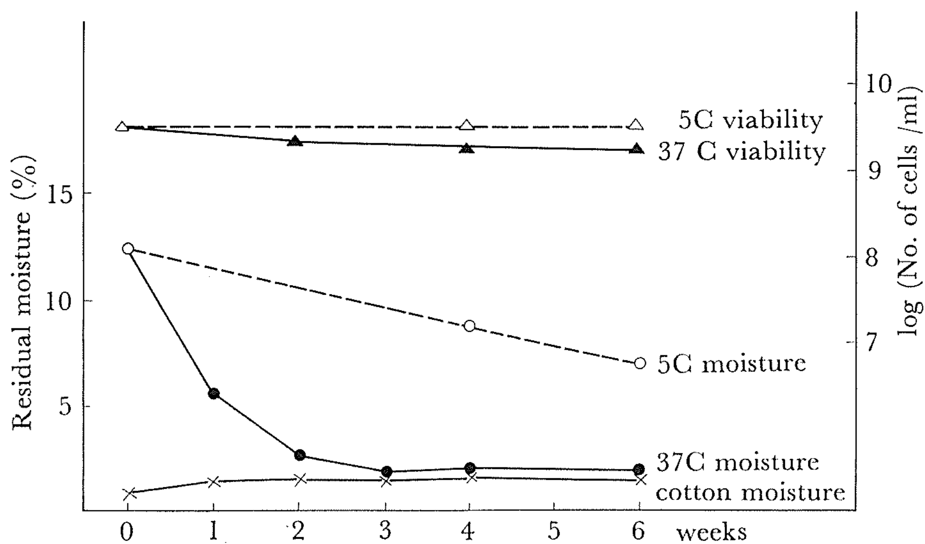


Fig. 8. Viability and residual moisture during storage

The specimens were stored at 37C or 5C. During the storage, the specimens are drawn and measured their viability and residual moisture.

Table 2. Viability of various bacteria after drying *in vacuo*.

Species	IFO No.	Control log N	fraction of survivor after drying (%)				
			stored at 37°C				stored at 5°C
			months				months
			0	1	3	6	6
Ps. aeruginosa	3756	10.4	42	19	15	8	52
X. oryzae	3825	9.9	5.9	6	3	0.5	77
Vib. anguillarum	12710	9.7	0.4	0.01	10^{-4}	10^{-4}	0.6
Chr. violaceum	3740	10.0	12	3	0.3	10^{-3}	12
Achr. xerosis	12606	9.3	50	42	38	17	50
Alc. faecalis	3160	10.2	4	1	1	0.3	4
Esch. coli	3310	10.1	49	9	8	3	42
Aer. aerogenes	3320	10.1	85	74	55	42	75
Erw. herbicola	12686	10.1	92	62	55	30	92
Ser. marcescens	3046	10.3	89	24	6	0.5	74
Mic. lysodeikticus	3333	9.4	100	111	96	93	89
St. aureus	3061	9.3	81	57	52	39	76
Cor. poinsettiae	2161	10.0	23	50	30	23	39
Arth. sp.	12724	9.5	2	5	0.08	0.01	16
Str. faecalis	12580	8.9	65	19	15	11	54
Lact. casei	3425	8.5	57	23	5	2	46
Bac. cereus	3001	8.7	38	14	12	3	29
Str. griseus ATCC	11984	9.2	100		71		65

After 16 hr incubation, cells were harvested by centrifugation and resuspended in the suspending medium. Number of viable cell per ml (N) in the suspension was counted by plating before drying (shown in log N) and after the drying (shown in the Table in % of the initial). Samples were stored at 37 C and 5 C respectivery. After 1, 3 and 6 months, samples were rehydrated and number of survivors are counted by plating. Table shows the surviving fraction in % of the initial.

Table 2 shows some of results obtained by various strains of bacteria in the culture collection. The survival percentages of some strains were decreased rapidly in storing at 37 C. For example in cases of *Vibrio anguillarum* (IFO 12710), *Chromobacterium violaceum* (IFO 3740) and *Serratia marcescens* (IFO 3046), the survival percentage after 6 months at 37 C were very small, but when these specimens were stored in 5 C, they were fairly stable as indicated in the Table 2. The small survival percentage, immediately after the drying, may be improved by changing a suspending medium and condition of preculture.

Application to phage lysates

The same method was applied to phage lysates of *E. coli*. The lysates were

Table 3. Viability of bacteriophages by drying *in vacuo*.

Bacteriophages	log (PFU/ml) before drying	log (PFU/ml) after drying	survivor 0 hr %
T1	9.3	9.1	74
T2	9.3	8.1	7
T3	9.2	9.2	88
T4	10.3	9.4	10
T5	10.7	10.0	21
T6	10.4	8.0	0.4
T7	10.4	10.0	4.2
T4 r164	9.3	8.0	6
T4 r184	9.7	8.5	7
T4 r196	9.0	7.8	6
T4 r271	9.6	8.7	10
f1	10.3	10.3	111
f2	10.4	10.4	100
Ms 2	10.3	10.0	58
M 12	10.2	10.2	100
ZD	9.4	9.4	100
Qβ	10.4	10.4	100
lambda	10.0	9.5	31
lambda-vir	10.8	10.5	57
ϕ170	9.8	8.9	13
ϕ170 vir	9.9	9.1	17
ϕ80	10.8	10.1	17
ϕX 174	10.4	10.4	100
Actinophage ATCC 11984–B	8.4	8.1	61

PFU: Plaque forming unit

Phage lysates were diluted five-fold with fresh broth. Phosphate buffer containing glutamate was added to the lysate at a final concentration of 1/10 M potassium phosphate and 3% of sodium glutamate. The lysate was dispensed in tubes and dried *in vacuo* according the method described in the text.

prepared by agar layer method or lysis of liquid culture (Adams 1959). The lysate were centrifuged at 6000 rpm to remove the bacterial debris. After the addition of a phosphate buffer containing sodium glutamate and broth, the lysates are dispensed into the tubes and dried in vacuo. The survival percent was shown in Table 3. When these specimens were stored at 5 C they were fairly stable.

T even phages are more sensitive than T odd phages. Clark (1962) mentioned that T even phages were more sensitive than T odd phage in freeze-drying method.

Summary

A modified method for preservation of bacterial strains and bacteriophages was described. The method comprised to dry a bacterial suspension *in vacuo* without freezing. Cotton wool plug is used to prevent cross-contamination and the important role of the plug in drying and during storage were discussed.

Literatures Cited

Adams, M.H. Bacterilphages. Interscience, New York 1959.
Annear, D.I. Australia. J exp. Biol. Med. Sci. **36:** 1–4 1958.
Annear, D.I. Australia. J. exp. Biol. Med. Sci. **40:** 1–8 1962.
Clark, W.A. Appl. Microbiol. **10**: 466–471 1962.
Greiff, D. and W.A. Rightsel Cryobiol. **3:** 432–444 1967.
Greiff, D. and W.A. Rightsel Appl. Microbiol **16:** 835–840.
Iijima, T. and T. Sakane J. Japan. Soc. Research Freez. and Dry. **16:** 87 1970.
Iijima, T. and T. Sakane J. Japan. Soc. Research Freez. and Dry. **17:** 16 1971.

IFO Res. Comm. 6,
18–26, 1973 (*January*)

SUCCESSIVE FUNGAL FLORA ON STERILIZED LEAVES IN THE LITTER OF FORESTS. II.

Keisuke TUBAKI and Tatsuo YOKOYAMA

Following the previous paper (1971) concerning with the succession of fungi invading the embedded sterilized leaves in the litter of forests, the present paper is a continuation and abbreviation trying to get subsequent data on the succession of microfungi which develop on a fallen leaf. In the previous paper, fifty nine genera of fungi were encountered and discussions were given on succession, autoecology, effect of variation in a host leaf and effect of leaf-condition which were the results of the examination carried out in the vicinity of Ootsu, Shiga Pref. from October, 1969 through March, 1970. As noted previously, the investigation has been extended further

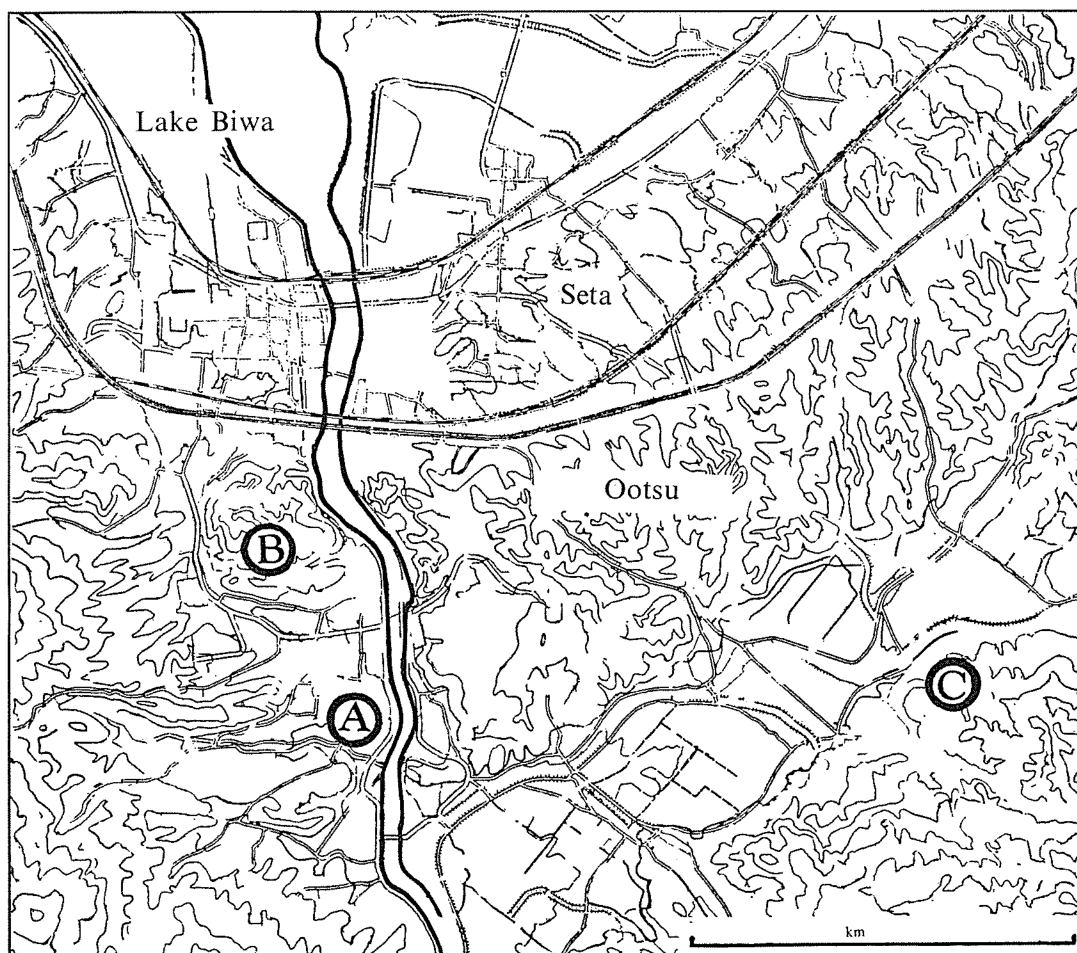


Fig. 1. Details of stations (A, B & C) in Ootsu area.

to study fungal flora in different seasons under the same procedure in the same localities, the Stations of A, B and C (Fig. 1).

Materials and Method

The selected plants are the same as those given in the previous paper, *Castanopsis cuspidata* and *Quercus phillyraeoides*. Both trees are dominant in the temperate and also in the warm temperate foiests in Japan. Green and newly detached leaves of both plants were collected and completely dried. Then ten leaves were autoclaved and sandwiched between a sterile square-net (10×15 cm). As immediately as possible, nine traps each were embedded in the litter of three stations at the end of April, 1970 and each trap was taken to the laboratory once a month during the period of May, 1970 through January, 1971. All fungi developed on the leaves were isolated and identified in much the same manner as described in the previous paper.

Results and Discussion

A list of genera observed is given in Table 1. Those genera printed in boldface are additional ones developed on the embedded leaves to the list of the previous paper (see Table 1, 1971). Those which developed only the naturally decayed leaves were included indicating by *. Seasonal occurrence of fungi on the sterilized embedded leaves during the period of May through February in these three stations is shown in Table 2 (A, B and C).

What is obvious for the reader in comparing both results obtained during the winter and the summer is the general tendency of the occurrence in some fungi. Fourteen members whose occurrence has a tendency to incliminate towards winter or summer are summarized in Table 3. Those of Mucorales which were regarded as one of the first invadors in the winter as described in the previous paper were much reduced in their development in the summer and became no longer a member of the primary colonizing groups on the leaves in the present study.

As shown in the table, *Ceratocystis* was common on the leaves of the very early decay producing numerous perithecia, and it continued to rise in the winter, while it was recovered rarely in the summer and was decreasing over a long period of time.

A similar decreasing pattern of occurrence was observed also in *Toxotrichum*. In the winter, the cleistothecia of this fungus, *T. cancellatum*, were in abundance even on the leaves of an early decay and were encountered on seven occasions in all stations during the latter half of the period, January through March. On the contrary, in the summer, only a few cleistothecia of the same fungus were found in Stations B and C only in July and September, respectively. Both records during the summer were obtained only on the newly detached leaves of *Castanopsis cuspidata* in the same way as that shown previously.

Table 1. List of fungi developed on the embedded leaves in the three communities examined.

Zygomycotina		
	Mucor	*Mortierella*
Ascomycotina		
	Ceratocystis	*Myxotrichum**
	*Leptosphaeria**	***Nectria***
	*Mollisia**	
Basidiomycotina		
	Lepista	***Rhizoctonia***
	Marasmius	
Deuteromycotina		
	*Actinopelte**	*Monacrosporium*
	Arthrinium	***Neottiosporella***
	Beltrania	*Oidiodendron*
	Botrytis	*Paecilomyces*
	Calcarisporium	*Penicillium*
	Candelabrum	*Pestalotia*
	Catenularia	*Phialocephala*
	Cephalosporium	*Polyscytalum*
	Chalara	*Psudobotrytis*
	Cladosporium	*Ramularia*
	*Clonostachys**	*Rhinocladiella*
	Codinaea	***Scolecobasidium***
	Crinula	*Scolecosporium*
	Cryptophiale	*Sporothrix**
	*Cylindrocladium**	*Stilbum*
	*Ellisiopsis**	*Subulispora*
	*Gliocladium**	***Sympodiella***
	Helicosporium	*Trichoderma*
	Idriella	*Verticillium*
	Illosporium	
Myxomycotina		
	Stemonitis	

Basidiomycetous fungi are significant in the occurrence in the present study. In the winter, none of them was found on the embedded leaves so far. However, in the present study, white mycelium of a basidiomycete spread widley covering the entire surface of all kinds of leaves of five-month decay, September, in the Station A. Then the mycelium gradually changed to pale purple in color and the fruitbodies of *Lepista subnuda* Hongo developed on the leaves. In the Station C, *Marasmius* sp. developed with the fruitbodies on the leaves of seven-month decay, November, which were markedly decomposed.

Beltrania was completely absent on the embedded leaves in the winter though

Table 2. Seasonal occurrence of fungi on the sterilized leaves in three stations (A, B & C). ○...growth is apparent but less intensive, not appearing on entire surface. ●...growth in abundance. 1 & 2 sterilized green and dead leaves of *Castanopsis*. 3 sterilized leaves of *Quercus*.

Station A

genus	May			June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella								○																			
Mucor								○	○						○				○								
Ceratocystis				○	○	○	○	○	○																		
BASIDIOMYCETES														○	○	●	●	●	●	●	●	●	●	●	●	●	●
Beltrania						○			○																		
Calcarisporium	○			○			○	○	○			○															
Cephalosporium				○	○	○																					
Chalara						○					○		○	○	○			○									○
Cladosporium												○											○				
Crinula										○		○															
Codinaea				○	○	○	○	○	○			○															
Idriella		○				○																					
Illosporium				○	○	○	○																				
Oidiodendron					○					○		○															
Paecilomyces						○																					
Penicillium	○	○	●	○	○	○			○											○	○						
Polyscytalum					○																						
Pseudobotrytis															○												
Scolecobasidium																											○
Stilbum				○																							
Subulispora	○	○		○			○		○			○															
Sympodiella					○	○	○	○	○																		
Trichoderma	○	○		○		○	○	○	○		○	○	○	○	○				○	○							
MYXOMYCETES													○														

Station B

genus	May			June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella								○	○	○		○					○										
Ceratocystis		○		○		○	○	○								○											
Toxotrichum														○													
DISCOMYCETES										○			○											○			
Beltrania	○		○				○			○			○					○									
Botrytis																						○		○			
Calcarisporium										○		○															
Cephalosporium						○	○																○			○	
Chalara	○	○	○										○							○	●				○	○	
Cladosporium								○											○			○					
Codinaea				○			○	○	○	○	○	○	○			○			○		○	○		●	●	○	●
Crinula																○			○								
Cryptophiale										○			○		○	○	○		○	○				○	●	●	○
Fisidium																											○
Helicosporium								○																			
Idriella	○	○	○	○	○		○		○	○	○	○				○								○	○	○	
Illosporium				○						○											●			○	○		○
Monacrosporium						○										○			○								
Oidiodendron					○			○				○			○			○			○	○	○	○			
Penicillium	○○	○	○	○	○	○	○		○			○	○	○	○	○		○	○	○	●	○	○	○	○	○	○
Pestalotia																						○					
Scolecobasidium																									○		
Subulispora	○	○	○																								
Sympodiella								○	○	○	○	○	○	○	○	○	○	○	○	○	●				●	●	●
Trichoderma		○	○	○	○	○	○	○	○		○				○	○	○			○	○	○	●				

Station C

	May			June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella	○													○				○					○	○			
Mucor							○																				
Ceratocystis				○				○																			
Nectria		○																									
Toxotrichum								○																			
BASIDIOMYCETES																			●							○	
Beltrania		○							○			○				○		○			○			○			○
Botrytis	○	○	○																								
Calcarisporium													○														
Candelabrum										○		○	○	○		○	○	○					○			●	○
Catenularia				○	○			●	○	○																	
Cephalosporium										○																	
Chalara									○	○	○	○	○	○	○	○	○	○	○	○	○				○	○	○
Cladosporium		○	○						○	○	○	○		○			○	○			○	○		○			
Codinaea		○						○		○						○	○	○				○			○	●	○
Cryptophiale											○		○	○	○	○	○	○				○					
Fusarium		○			○																						
Idriella	○	○	○	○			○	○		○		○															
Monacrosporium							○					○						○			○						
Neottiosporella							○																				
Oidiodendron																○				○			○			○	
Paecilomyces																				○			○	○			○
Penicillium	○	○	○	○		○	○	○	○						○					○				○	○		○
Pestalotia	○																										
Phialophora																							○				
Rhinocladiella																				○							
Scolecobasidium																										○	○
Subulispora	○			○			○	○	○		○														○	○	
Sympodiella					○	○	○	○	○	○	○	○	○	○	○		○	○		○	○		○	○	○	○	●
Trichoderma	○	○			○	○	○	○	○		○	○		○	○				○	○			○	○			
Verticillium			○							○	○		○	○													

Table 3. List of fungi which seasonal occurrence incliminates towards winter or summer.

Organisms	Occurrence on the leaves	
	winter Oct.-March (1971)	summer– May-January (1971–1972)
Mucorales	early period of decay, then decrease.	less common
Ceratocystis	common throughout	2–3 months
Toxotrichum	very early and continues	only in 2nd and 5th month
Basidiomycetes	——	after 5–7 months
Beltrania	——	common throughout
Codinaea	early and continues	2–3 months & in 9th month
Chalara	early	common throughout
Cryptophiale	——	after 4 months
Idriella	common throughout	1–4 months
Oidiodendron (yellow)	very early, then decreases	——
" (gray)	after 3 months	2–4 months
Penicillium	common throughout	early and decreases
Ramularia	common throughout	——
Subulispora	early and decreases	1–4 months
Sympodiella	common throughout	2–4 months & in 9th month

it was common on the naturally decaying leaves in the same place. In the summer, it developed commonly throughout the whole process of decay even of one-month.

A similar result was obtained in *Cryptophiale* which was absent in the winter. It developed first on the leaves of four-month decay in the summer and continued to develop over a long period of time.

Chalara was the most frequently occurring fungus on the leaves of two-month decay in the summer and afterwards.

Idriella developed even on the leaves of one-month decay and was not uncommon during the latter decay of the leaves in the winter. In the summer, it developed mostly on those leaves of one- through four-month decay.

Oidiodendron consisted of the two groups in the winter, yellowish *O. citrinum* and blue-grayish conidial state of *Toxotrichum cancellatum*, and the former developed at first on the leaves of very early decay and tend to decrease in number over a long period of time, while the latter group appeared in a latter half of the decay. In the summer and afterwards, *Oidiodendron*-species was less common and has no tendency of the occurrence. However, it is significant that only blue-grayish species developed. Yellowish *Oidiodendron* was completely absent in the present case.

Penicillium was an extremely common colonizer on the leaves in the winter and was very common especially on those of one- to two-month decay. Many pinkish

sclerotia of *P. thomii* were significant in number throughout the study carried out in the winter. In the present case, the above tendency was nearly similar, but the sclerotia were less common and developed only on the one-month decayed leaves. *P. implicatum* which was very common in the winter was infrequent in the summer.

Ramularia appeared in the winter especially on the sterilized green leaves in all stations throughout the period of decay. However, in the summer, it was absent though the fungus was common on the naturally decaying leaves around the stations.

Subulispora, consisting of two species, *S. procurvata* and *S. rectilineata*, was common in the winter in its luxriant growth throughout the study, especially on the leaves of one- to four-month decay and was considered to be a first invador. In the summer, this fungus was less common and the occurrence was restricted to the period of one- through four-month.

In addition to these fungi which showed the difference in the occurrence, *Codinaea* and *Sympodiella* are also peculiar in another point. Both genera are the dominant primary fungi and developed on the leaves of rather early decay and continued to develop in the latter half of the period in the winter. In the summer and afterwards, on the contrary, both developed less commonly on the leaves of more than two-month decay and then appeared in abundance again at the last process of the decay during the period of December through February. The reason is uncertain at present.

Other genera, not mentioned above, were more or less similar in the tendency of the occurrence as compared with the results obtained during the period of both the winter and summer.

To summarise our interpretation of the results, we can give the following points.

1. In summer in which tempertaure and humidity are higher because of the rainy season's passing, the decomposition of the embedded leaves is prompted naturally more than that in winter. Therefore, those decomposers which take up to six months to colonize leaves in winter because of less activities of the first and proceeding invadors, are now able to come up early on the leaves during the warmer period of summer and autumn. *Candelabrum*, a hyphomycete, is an example.

2. For the same reason, succession of fungi upon the leaves in summer and autumn may be more promoted than that in winter, so many fungi which appeared evenly under the whole process of decay during the cold season may have a tendency to show the peak of the growth during the early process of decay in summer and then the growth reduces rapidly. This tendency agrees well with the peak in numbers shown by *Idriella*, *Oidiodendron*, *Penicillium* and *Subulispora*. In other wards, the life cycles of those first invadors are shortened in summer and metabolism of them is faster than those in winter and the primary seres consisting of these genera may be soon converted into the secondary ones.

3. Those genera which mainly comprise tropical, subtropical or warm temperate species are unable to colonize on the leaves during the winter season. However, they can grow on the leaves during the period of summer through autumn as the temperature rises together with the humidity. In this case, the temperature may act as a main

factor controlling the occurrence. *Beltrania* and *Cryptophiale* are such examples.

Conversely some fungi prefer a rather lower temperature for the growth and the rate of the growth is somewhat reduced in the winter season. *Ceratocystis* and *Chalara* are such examples. Members of the Mucorales and the yellowish *Oidiodendron*, *O. citrinum*, may be included in this group. This point is related with a problem in the optimum temperature for the growth.

4. Some fungi can grow in the face of competition from other fungi. After a certain period during the succession of fungi, mycelium of a peculiar fungus develop to overgrow the leaves and eventually occupied the whole surface of the leaves on which other fungi are suppressed to colonize and can not but disappear. *Lepista subnuda* is an example which covered the whole surface within five months competing with other fungi and makes the microflora more simple.

Summary

Following the previous paper which was carried out during winter, an additional study was made on the successive colonization of fungi on the embedded sterilized leaves of *Castanopsis cuspidata* and *Quercus phillyraeoides* in the naturally developing litter of the forests during the period of summer through autumn. Embedded leaves were taken to the laboratory once a month during the period of May, 1970 to January, 1971, and the fungi developed on the leaves were isolated and identified. Fifty genera were encountered and discussions are made on autoecology, succession and difference in microflora between winter and summer.

All things considered, the temperature plays the most important role on the succession of fungi. A higher temperature changes the approximate order of frequency of occurrence of first invadors on the leaves and also makes the climax of each sere shorter as compared with that in a lower temperature. The optimum temperature for the growth of each fungus itself may control its time on stage which makes the flora different depending on seasons.

We are grateful to Dr. T. Hongo, Shiga University, for his helps in setting up the stations in the Ootsu area.

We are grateful to Dr. T. Hasegawa, the Director, for his encouragement and also to Mr. T. Ito for isolation and identification of many fungi. Most of the penicillia were identified by Mr. I. Asano to whom we owe many thanks. We were also favored to have the assistance of Miss. M. Mashimo.

References

Tubaki, K. & T. Yokoyama. 1971. Successive fungal flora on sterilized leaves in the litter of forests. I. Res. Comm. Inst. Ferm. **5**: 24–42.

IFO Res. Comm. 6,
27–49, 1973 (*January*)

SUCCESSIVE FUNGAL FLORA ON STERILIZED LEAVES IN THE LITTER OF FOREST. III.

Keisuke TUBAKI and Tatsuo YOKOYAMA

On the previous reports (Tubaki & Yokoyama, 1971; and in this journal), attemps were made to obtain a new interpretation about the successive colonization by fungi on the embedded sterilized leaves of *Castanopsis cuspidata* and *Quercus phillyraeoides* in the naturally developing litter of the forests in the three stations (A, B and C) in vicinity of Ootsu, Shiga Pref. during the period of October, 1969 to March, 1970, and of May, 1970 to February, 1971. Fifty eight and fifty genera of fungi were encountered in the first and the second papers respectively, and discussions were given on the succession and autoecology of these fungi.

As discussed in the first paper (1971), we have investigated the fungal flora under the same procedure in the two other additional subtropical stations which were set up on Tanegashima and Yakushima Islands, Kagoshima Pref. The investigation was carried out to know a difference of the successive colonization of the leaf litter fungi growing on the embedded sterilized leaves in the central and the southern parts of Japan. This paper presents a problem on such difference in the fungal succession in both localities.

Materials and Method

The plant stands examined are in five naturally developing forests in Kagoshima Pref. as shown below (Fig. 1 & 2).

Forests of Onoaida on Yakushima Is. (D & E) with the station's latitude of ca. 30°20′N with the annual temperature average of ca. 21°C and the forest of the Station D consists of *Quercus phillyraeoides* mixed with *Cinnamomum japonicum* Sieb., *Daphniphyllum teijsmanni* Zo., *Litsea japonica* Juss, etc.; the forest of the Station E mainly consists of *Castanopsis cuspidata*, *Ficus wightiana* Wall., and *Quercus glauca* var. *lacera* Mtsum. mixed with *Mallotus japonicus* Muell, bamboo, etc.

Forests of Noma on Tanegashima Is. (F, G & H) with the station's latitude of ca 30°30′N with the annual temperature average of ca. 19°C and the forest of the Station F consists mainly of *Castanopsis cuspidata*, *Camellia* sp., *Ficus erecta* Thunb., *Quercus myrsinaefolia* Blum., *Viburnum japonicum* Thunb. etc; that of the Station G consists mainly of *Castanopsis cuspidata* mixed with *Camellia* sp., *Machilus thunbergii* Sieb. & Zucc., *Meliosma rigida* Sieb. & Zucc., *Tarenna gyokushinkwa* Ohwi, etc.; that of the Station H consists mainly of *Castanopsis cuspidata*.

The experimental procedure is the same as that descrived in the previous paper

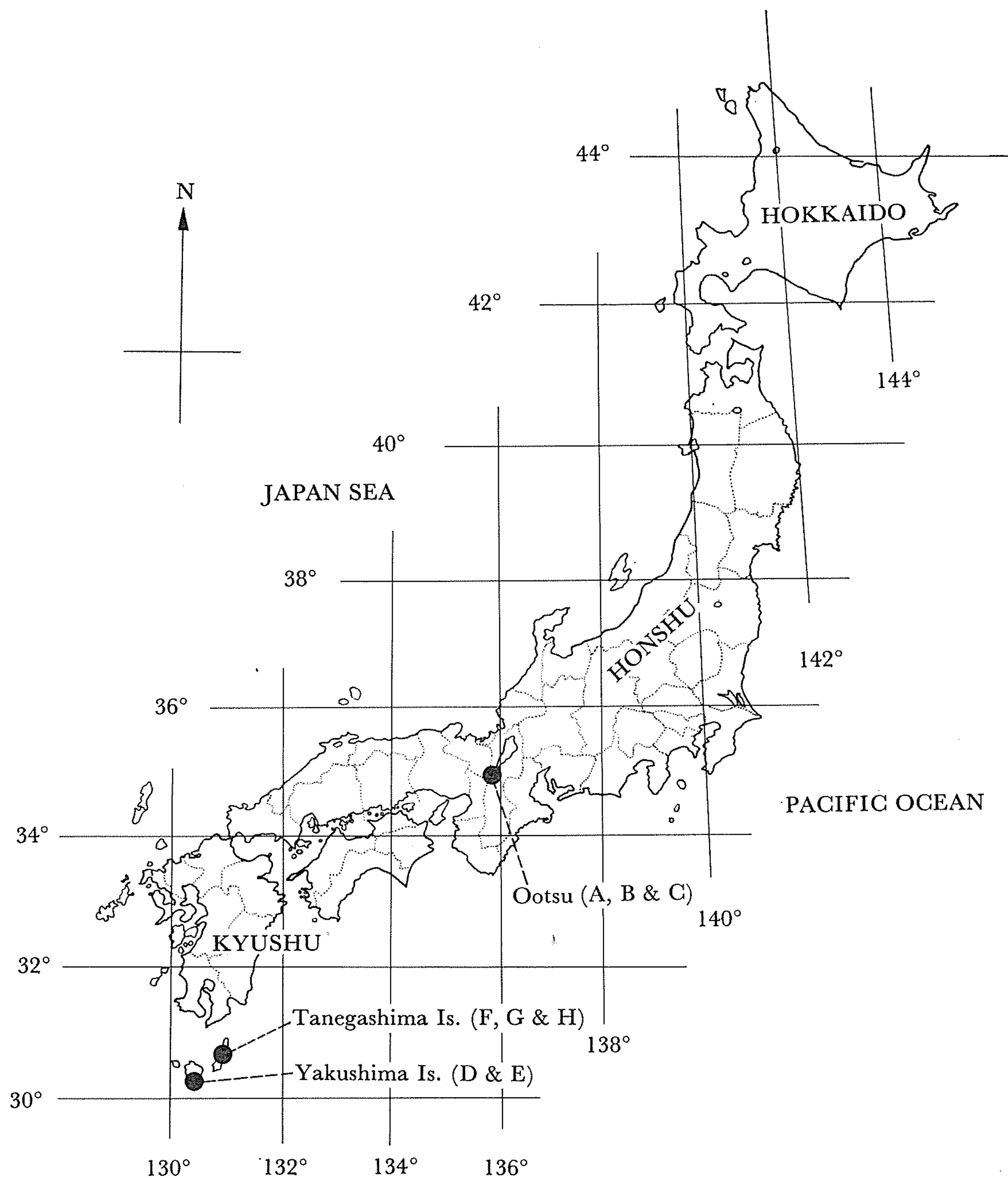


Fig. 1. Locations of stations. Stations where sterilized leaves were embedded are shown as solid circles.

using two species of sterilized leaves, *Castanopsis cuspidata* and *Quercus phillyraeoides*. Six leaf-traps each was embedded in each one of the Stations of D-H at the beginning of summer and winter season respectively. Experiments were carried out twice from June through November, 1970 and from December, 1970 through May, 1971. Each trap was taken once a month to the laboratory during the periods and every fungus

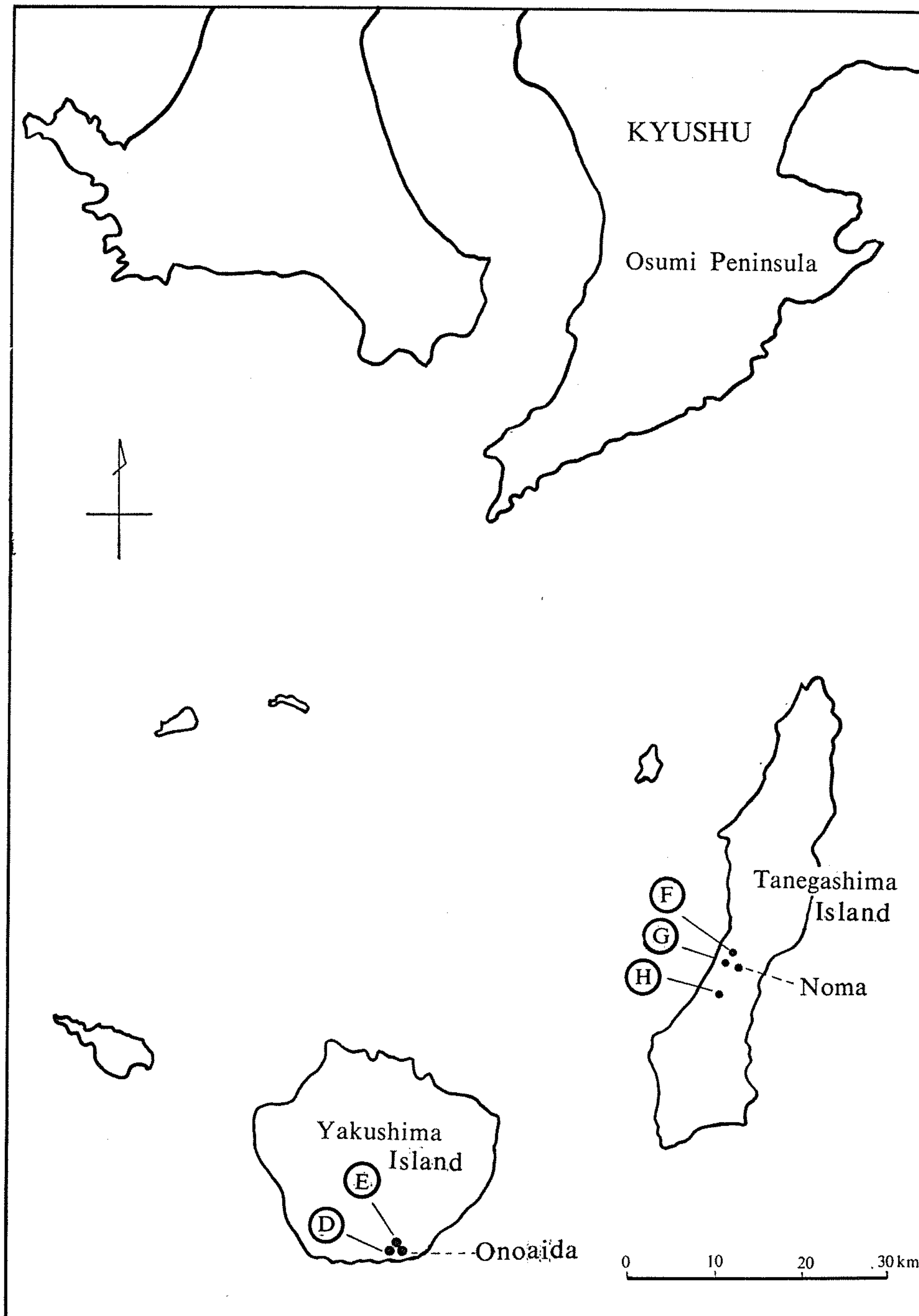


Fig. 2. Details of stations (D–H) on Yakushima and Tanegashima Is.

developed on the leaves was isolated and identified. Unfortunately those of the samples of the last two months in the Stations D and E were not in hand at last, so the latter term data in these two stations were made from December, 1970 through March, 1971.

Results and Considerations

1. Difference in microflora.

Genera of fungi observed are listed in Table 1 excluding unidentified members, and the station in which each genus was found is indicated by D-H. Those which developed only on the naturally decayed leaves were also included in the list indicating by * and the difference in the distribution of the genera will be described in detail otherwise. Those genera printed in boldface were not found previously on the embedded leaves in the Stations A-C.

2. Seasonal occurrence of fungi found in the Stations D-H.

List of the genera found on the leaves together with their frequency of seasonal occurrence is shown in Table 2 (D-H).

Because the decomposition of the leaves progressed rapidly on these stations on both southern islands, the leaves embedded were decomposed markedly even after three months (Pl. 3) and, after four months, the leaves were skeletonized with only veins and petioles left making somewhat difficult to compare the fungus flora developed on the leaves themselves. This rapid decomposition of the leaves in contrast to that in Ootsu area (Pl. 2) probably took place by the higher temperature, higher humidity and heavy rain fall on these islands. In addition, active behavior of insects and other small invertebrates on the leaf surface should not be overlooked.

Among these genera listed, fifteen genera are significant in their occurrence developing more dominantly during the period of the summer season, June through September. On the contrary, seven genera are found mostly during the period of the winter season, December through March. These genera of both types are arranged in Table 3. From the table, it is agreeable that the fungi indicated by * developed dominantly in the summer season as known from the references on the world distribution of them. They are known to distribute mainly in the warmer area, while there has been no marked tendency in other five genera on the distribution. Perhaps high temperature stimulated their growth on the leaves. On the contrary, among the fungi dominant in the winter season, only *Oidiodendron* and *Ceratocystis* are known to be distributed in the cooler area and developed in abundance on the leaves embedded in the previous study carried out in the Ootsu area, central Japan. The other group consists of both subtropical and high temperate fungi. It is uncertain why *Penicillium* developed so dominantly in the winter season. It may be a problem of succession as discussed below.

There is no seasonal difference in the occurrence of the following genera: *Chalara*, *Paecilomyces* and *Trichoderma*.

3. Autoecology of each fungus with a discussion on the sucession.

We extended our investigation of the autoecology of the genera and the species found on the embedded leaves, some of which were mentioned already in our previous

Table 1. List of genera of fungi on the embedded leaves in the five communities examined.

Genus	Communities	Genus	Communities
Zygomycotina			
Gongronella	F	*Mucor*	D E F G
Mortierella	D E F G H		
Ascomycotina			
Calonectria	D E F G	*Nectria*	E F H
Ceratocystis	E G	*Toxotrichum*	D E G
Leptosphaeria	D F		
Deuteromycotina			
*Acrothecium**	E	*Menisporopsis*	D E F G
Beltrania	D E F G	*Monacrosporium*	D E F G H
Blastophorum	F	*Mirandina*	H
Candelabrum	D G	*Neottiosporella*	D E F G H
Catenularia	D E G	*Oidiodendron*	D E F G H
Cephalosporium	D G H	*Paecilomyces*	D E F G H
Chalara	D E F G H	*Penicillium*	D E F G H
Chaetopsina	D E F G H	*Pestalotia*	D E F G H
*Chloridium**	F	*Phialocephala**	H
Circinotrichum	D E	*Phoma**	E
Cladosporium	D E F G H	*Phyllosticta**	E
Clonostachys	D G H	*Polyscytalum*	D E F H
Codinaea	D E F G H	*Pseudobotrytis*	H
Crinula	E F	*Ramularia**	D F
Cryptophiale	D G H	*Rhinocladiella**	D
Cylindrocladium	D E F G H	*Scolecobasidium*	D E F G H
Dactylaria	D F	*Scolecosporium*	G
Diheterospora	D E F	*Selenophoma*	G
*Discosia**	D E	*Selenosporella*	F G
Ellisiopsis	D E F G H	*Septonema*	G
Endophragmia	D	*Solosympodiella*	E G H
Fusarium	E	*Sporothrix*	G H
Fusidium	D E H	*Stilbum*	E
Gliocladium	D E H	*Subulispora*	D E G H
Gonatobotrys	E F	*Sympodiella*	D E
Gonytrichum	D F G	*Thysanophora*	E
Gyrothrix	D E F G H	*Trichoderma*	D E F G H
Helicosporium	D G	*Verticillium*	D E F G H
Idriella	D E F G	*Zanclospora*	E F
Mahabatella	F	*Zygosporium*	F
Basidiomycotina			
Marasmius	G		
Mycena	E		
Rhizoctonia	D E F		
Myxomycota	D		

Table 2. Seasonal occurrence of fungi on the sterilized leaves in five stations (D–H) during the period of June through November and of December through May (March in the stations D and E).

Station D

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella	○																				○		○						○	
Mucor		○	○	○																										
Calonectria			○																			○	○							
Leptosphaeria			○										○	○		○	○													
Toxotrichum																														○
BASIDIOMYCETES			○					○								○	○													
Rhizoctonia								○																						
Beltrania	○		○			○	○								○				○		○			○			○			
Candelabrum													○	○	○	○										○				
Catenularia																	○				○			○	○	○	○			
Cephalosporium			○							○														○						
Chaetopsina				○		○									○													○		
Chalara					○								○	○	○				○		○			○	○	○	○	○	○	○
Circinotrichum																														○
Ciadosporium										○	○	○			○															
Clonostachys																								○						
Codinaea	○				○		○	○	○		○		○				○	○								○			○	○
Cryptophiale						○								○											●		○	○		
Cylindrocladium	○	○					○	○			○											●			○		○	○		○
Dactylaria																						○								
Diheterospora											○																			
Ellisiopsis							●		○			○												○			○			
Endophragmia								○					○	○																
Fusidium			○																											

Station D (continued)

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Gliocladium		○																												
Gonytrichum		○		○	●																						○			
Gyrothrix																									○		○			
Helicosporium	○					○							○																	
Idriella										○								○		○		○	○	○	○	○	○	○	○	○
Monacrosporium				●		○	○	○	○					○																
Menisporopsis										○										○				○						
Neottiosporella	○	○	○	○	○	○	○	○	○			○		○		○			○				○							
Oidiodendron																							○	○	○	○	○			○
Paecilomyces	○		○	○		○			○									○					○					○		○
Penicillium		○		○		○						○				○			○	○	○	○		○		○	○			○
Pestalotia	○		○																											
Polyscytalum	○		○			○	○		○																○					
Scolecobasidium																									○	○	○	○		
Selenophoma	○	○	○			○										○														
Subulispora			○	○	○																					○				
Sympodiella		○		○	○													○												
Trichoderma	○	○	○	○	○	○	○	○	○	○	○	○	○		○			○	○	○		○	○		○	○	○	○	○	
Verticillium														○							○									○
Volutina										○									○	○			○							
Wiesneriomyces	○												○					○			○	○								○
MYXOMYCETES														○																

Station E

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella																														○
Mucor																		○												
Calonectria	○																													
Ceratocystis																										○				
Nectria																			○		○	○	○	○			○			
Toxotrichum																														○
BASIDIOMYCETES								○	○								○													
Rhizoctonia									○																					
Beltrania	○		○				○	○	○				○	○					○			○								○
Catenularia										○								○												
Chaetopsina					○						○											○					○	○		○
Chalara	○		○	○		○							○					○					○		○	○	○	○	○	○
Circinotrichum																												○		
Cladosporium																					○	○			○	○		○		○
Codinaea	○		○		○	○											○						○		○			○	○	○
Crinula			○			○																								
Cylindrocladium	○		○	○			●												●		○	○		●			○			○
Diheterospora																													○	
Ellisiopsis									○	○																	○			
Fusarium																													○	
Fusidium																				○										
Gliocladium																														○
Gonatobotrys		○																												
Gyrothrix																						○			○					

Station E (continued)

genus	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Idriella	○						○			○									○									○		
Monacrosporium	○				○				○																					
Menisporopsis																													○	○
Neottiosporella	○			○				○					○				○	○						○	○	○	○			
Oidiodendron																									○					○
Paecilomyces								○	○																○			○		
Penicillium																			○		○	○	○					○		○
Pestalotia		○																	○		○	○								
Polyscytalum				○					○																○					
Scolecobasidium		○			○	○	○		○	○																		○		
Solosympodiella				○																							○			
Stilbum																				○										
Subulispora	○	○		○			○																	○						
Sympodiella				○	○	○		○	○								○											○		
Thysanophora										○																				
Trichoderma	○	○	○		○		○	○	○	○			○	○		○	○	○	○	○	○	○	○	○	○	○	○	○	○	
Verticillium																														○
Volutina															○					○	○									○
Wiesneriomyces	○			○		○	○		○										○		○	○	○	○		○	○	○		○
Zanclospora																											○			

Station F

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March			April			May		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Gongronella												○																								
Mortierella																													○	○						
Mucor																										○	○									
Calonectria	○																																			
Leptosphaeria				○	○																						○									
Nectria																								○		○										
BASIDIOMYCETES																													○							
Beltrania	○		○	●		○	○		●	○		○	○		○							○		○						○						
Blastophorum														○																						
Chaetopsina	○	○	○					○		○		○			○																					
Chalara				○			○						○		○									○		○	○		○							
Cladosporium		○				○									○										○						○		○			○
Codinaea								○	○			○																				○				
Crinula			○																																	
Cylindrocladium	○	○	○			○	○									○				○				○	○		○	○		○				○		○
Dactylaria																													○			○				
Diheterospora												○																	○							
Ellisiopsis										○																	○									○
Gonytrichum		○			●		○	○		○	○			○															○							
Gonatobotrys		○																																		
Gyrothrix																			○																	

Station F (continued)

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March			April			May		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Idriella																												○				○				
Mahabatella						○																														
Monacrosporium	○						○		○	○		○	○		○							○							○							○
Menisporopsis	○	○	●						○		○	●					○	●		○	○					○	○	○	○			○				
Neottiosporella	○	○	○	●	●		○	○	○	○	○	○	○				○	○							○										○	
Oidiodendron																			○	○			○													
Paecilomyces																								○							○			○		
Penicillium																			○	○	○	○	○	○	○	○	○	○								
Pestalotia		○								○									○	○	○	○	○	○	○											
Polyscytalum	○		○			○										○									○									○		
Scolecobasidium		○	○		○					○																										
Selenosporella																						○														
Trichoderma	○	○	○	○	○			○	○	○	○	○			○				○	○	○		○	○	○	○	○		○					○	○	○
Volutina																																○				
Wiesneriomyces	○		○																				○	○			○									
Zanclospora									○																											
Zygosporium																																				○

Station G

genus	June			July			Aug.			Sept.			Oct.			Nov.		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella																		
Mucor		○														○		○
Calonectria																		
Ceratocystis																		
Chaetomium					○													
Toxotrichum																		
BASIDIOMYCETES									○									
Beltrania	○	○	○	○	○	○	○			○					○			
Candelabrum																		
Catenularia																○	○	
Cephalosporium																		
Chaetopsina																		
Chalara			○						○				○		○	○		
Cladosporium																		
Clonostachys		○					○											
Codinaea			○	○		○	○	○			○	○	○	○				○
Cryptophiale			○															
Cylindrocladium																		
Ellisiopsis																○		
Gonytrichum																		
Gyrothrix																		

genus	Dec.			Jan.			Feb.			March			April			May		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella												○						
Mucor																		
Calonectria			○															
Ceratocystis	○																	
Chaetomium																		
Toxotrichum																		○
BASIDIOMYCETES																		
Beltrania																		
Candelabrum																	○	
Catenularia																		
Cephalosporium													○					
Chaetopsina	○						○									○		
Chalara							○	○	○	○	○	○	○		○			
Cladosporium												○						
Clonostachys																		
Codinaea			○	○	○		○		○	○		○	○	○	○	○	○	○
Cryptophiale																		
Cylindrocladium	○																	
Ellisiopsis						○			○									
Gonytrichum																	○	
Gyrothrix						○												

Station G (continued)

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March			April			May		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Helicosporium				○			○																													
Idriella	○	○		○				○												○		○	○	○	○	○	○	○		○	○		○			
Monacrosporium								○	○			○		○	○									○												
Menisporopsis										○										○	○			○			○			○						
Neottiosporella	○		○	●	●															○					○											
Oidiodendron																							○					○	○		○	○	○	○	○	○
Paecilomyces			○		○																	○		○							○				○	
Penicillium	○		○									○							○	○	○	○		○	○		○	○		○						
Pestalotia																			○	○	○															
Scolecobasidium																			○	○																
Scolecosporium	○	○																																		
Selenosporella													○										○													
Septonema																																		○		
Solosympodiella																							○													
Sporothrix			○																																	
Subulispora	○	○	○	●	○	○	○	○		○		○	○					○					○					○	○	○	○			○		
Trichoderma	○	○	○	○	○	○	○			○		○	○						○	○	○		○	○	○	○	○		○		○	○		○		
Verticillium																													○							
Volutina			○																																	
Wiesneriomyses																							○						○							
MYXOMYCETES														○	○																					

Station H

	June			July			Aug.			Sept.			Oct.			Nov.			Dec.			Jan.			Feb.			March			April			May		
genus	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mortierella																													○	○						
Nectria																					○															
Cephalosporium		○																																		
Chaetopsina																									○											
Chalara			○			○		○					○						○	○	○				○		○	○	○	○	○		○		○	
Cladosporium																					○	○														○
Clonostachys			○																																	
Codinaea		○	○				○		○				○	○	○								○	○	○		○	○	○	○						
Cryptophiale													○																							
Cylindrocladium	○	○	○																				○													
Ellisiopsis							○		○												○						○									
Fusidium		○																					○		○											
Gliocladium	○																																			
Gyrothrix																															○					
Monacrosporium													○								○	○											○	○		○
Mirandina																													○							
Neottiosporella		○					○							○		○	○	○								○							○	○	○	○
Oidiodendron																							○					○		○						
Paecilomyces						○	○			○	○	○							○		○						○									
Penicillium			○	○					○											○	○		○	○			○	○		○						
Pestalotia																			○	○		○	○	○		○	○									
Phialocephala																					○															
Polyscytalum																											○									
Pseudobotrytis																																	○			
Scolecobasidium																								○												
Solosympodiella		○																		○	○															
Sporothrix			○								○																									
Subulispora			○						○																○											
Trichoderma	○	○	○	○	○	○	○			○	○	○							○	○	○	○	○	○	○	○	○	○	○	○			○			○
Verticillium														○																						
Volutina		○																						○		○	○									
Wiesneriomyces	○	○	○			○	○						○	○						○				○	○	○	○	○	○	○						

Table 3. Genera of fungi characteristic in their seasonal occurrence. Only dominant ones were listed.

Dominant in summer	Dominant in winter
Nectria	*Ceratocystis*
Mucor	*Leptosphaeria*
*Beltrania**	*Catenularia*
*Chaetopsina**	*Gyrothrix*
*Codinaea**	*Oidiodendron*
*Cylindrocladium**	*Penicillium*
*Ellisiopsis**	*Pestalotia*
Helicosporium	
Menisporopsis	
*Monacrosporium**	
*Neottiosporella**	
*Polyscytalum**	
Selenophoma	
*Subulispora**	
*Sympodiella**	

paper (1971). In the present paper, we discuss on the autoecology of those fungi found on both islands comparing with the data found in the Station A-C. The reader may refer to the previous data given in 1971.

Zygomycotina:

Mucorales is less common in the present investigation than that found in the Stations A–C. Especially species of *Mortierella isabellina*-series was apparently less common and found only during the winter on the leaves in contrast to the previous data in which these fungi were much frequent during the process of an early decay of the leaves though they tended to decrease in number in the summer together with other mucoraceous members. It may be speculated that they were encountered on the leaves during the process of very early decay, one month or shorter. *Mucor* was restricted to the leaves of an early decay as shown in the previous data.

Ascomycotina:

Calonectria is not uncommon on the leaves of an early decay, one month decay during the summer and two months during the winter. Most species of *Calonectria* belong to *C. kyotensis* Terashita.

Ceratocystis was found only during the winter on the leaves of two months or three months decay and the species are the same as that of the previous paper giving *Sporothrix*- and *Calcarisporium*-conidial states.

Toxotrichum was found only in the Stations D and E on Yakushima Is. on the leaves of four months decay in the winter. All isolates belong to *T. cancellatum* (Ph.) Orr & Kuehn.

Basidiomycotina:

It is significant that although only a few basidiomycetes developed on the embedded leaves of the six months treatment in Ootsu area, in the present study, basidiomycetes are not uncommon on the embedded leaves. *Marasmius* and *Mycena* developed even on the one month decayed leaves in the Station D and were not uncommon on the two or three months decayed leaves throughout the study in the summer season. *Rhizoctonia* sp., probably be *R. repens* Bernard, developed twice on the three and four months decayed leaves in the summer with mycelium provided with clamp-connections. *Rhizoctonia*-state of *Thanatephorus orchidiola* Wark. & Talb. developed also on the leaves of three months decay.

Deuteromycotina:

Beltrania is a more or less fast growing fungus and was fairly common during the summer throughout the study although this was not observed on the embedded leaves from the Ootsu area in the previous study even after a six months treatment. Most species belong to *B. querna* Harkness or *B. rhombica* Penzig.

Candelabrum is unique in the characterisitc shape of the conidia and was found on the leaves of five to six months decay in the summer and three months decay in the winter. Species belongs to *C. spinulosum* v. Bev.

Catenularia was not quite predominant on the leaves of an early decay.

Chaetopsina, not found previously on the embedded leaves, was not uncommon on the leaves of an early decay in the summer and is rapid in the growth spreading on the leaf-surface. The species mostly belongs to *C. fulva* Ramb.

Codinaea was very common in the present study and was one of the predominant fungi on the leaves of one month decay in the summer, developed after two months in the winter. *C. parva* Hugh. & Kendr. is predominant.

Cryptophiale is special in terms of its peculiar shape of the conidial apparatus and was found on the leaves of an early decay in the summer and of two or three months decay in the winter. *C. udagawae* Pir. was predominant.

Cylindrocladium was very common on the leaves of an early decay, growing rapidly on the surface of the leaves even after one month. Most species belong to *C. ilicicola* (H.) Boed. & Reit. and *C. scoparium* Morgan.

Diheterospora was found only on the leaves of four months decay in the summer and all isolates belong to *D. catenulata* Kamyschko.

Ellisiopsis developed only after three months in the summer, but was found even after one month in the winter. Most species belong to *E. gallesiae* Bat. & Nascim.

Fusarium was very rare as known from the previous paper and was found only once on the leaves of four months decay in the Station D. As discussed previously (1971), this fungus will be rare in the natural, not-cultivated, soil.

Gonytrichum mostly developed on the leaves of an early decay and was predominant on those of two to three months decay. All isolates belong to *G. macrocladum* (Sacc.) Hughes.

Gyrothrix tends to develop during the winter season rather, probably according to the slow-growing. *G. microsperma* (Hohn.) Pir. is representative.

Idriella-species was fairly common throughout the study being analogous to the previous data.

Monacrosporium was frequent in its occurrence on the leaves of an early decay but rather rare in those of the winter season. *M. ellipsosporum* (Grove) Cooke & Dickinson is predominant.

Neottiosporella developed in abundance on the leaves of an early decay and was one of the most common fungi in the present study. Most isolates belong to *N. radiata* Morris.

Oidiodendron was found only on the leaves of the winter season although it was a common leaf-fungus of an early decay in the winter in the Ootsu area.

Penicillium, known as ubiquitous and omnivorous, was very common on all leaves in all stations in the previous study in the Ootsu area, and *P. thomii* was dominant. On the contrary to the previous data, in the present study, *Penicillium*-species was more or less predominant on the leaves of very early decay in the winter, and the sclerotia of *P. thomii* Maire were hardly seen on the leaves, which were characteiistic in the previous study. *P. herquei* B. & S. was most dominant and *P. citreoviride* Biourge, *P. citrinum* Biourge, *P. corylophylum* Dierckx, *P. crustosum* Thom, *P. fellutanum* B., *P. funiculosum* Thom, *P. implicatum* B., *P. janthinellum* B., *P. nigricans* B. ex T., *P. puberulum* Bain., *P. roqueforti* Thom, *P. terlikowski* Zal. and *P. velutinum*

Pestalotia has been found mostly on the sterilized green leaves during the decay of the process in the winter season. This result, preferring the green leaves, is the same as described previously.

Polyscytalum was found characteristically on the leaves of an early decay in the summer and, in the winter, it was found on the leaves of three months decay.

Selenophoma was also significant because of the development only on the leaves of an early decay in the summer. The species is probably *S. donacis* (Pass.) Spr. & John.

Subulispora developed in a very early stage of a decay process the same as observed in the previous study. However, it seems to be restricted rather on the leaves of one- to three months decay differing from the previous data in which the fungus continues to colonize throughout the whole process. *S. procurvata* Tub. was dominant, but *S. rectilineata* Tub. was also observed.

Trichoderma was observed to colonize throughout the whole process like those in the previous study.

4. Comparison between the successive colonization of fungi on the leaves in the central parts of Japan and that in the southern part of Japan.

Comparing the present data with those shown in the previous studies, what is evident is that there is a significant difference in the order of frequency of occurrence of some fungi in addition to the difference of the flora in two localities. Following

four genera are good examples to show such difference of the order and also to refer to the succession of fungi.

Cladosporium is known as one of the commonest primary saprophytes on the decaying leaves and, in the previous study carried out in the Ootsu area, this fungus developed throughout the whole process of the decay. In the present study, however, the occurrence of it reduced markedly in contrast to that obtained in the previous study and was found mostly during the winter season on the southern islands.

Codinaea was common on the leaves of more than two months decay in the previous study, while in the present case, it developed in abundance even on those of one month decay and reduced in its frequency in the winter.

Conversely, in the present case, *Penicillium* was less common on the leaves of an early decay in the summer and was in abundance during the whole process of the decay in the winter. This fungus, *Penicillium*, was very common on those of the whole process of the decay in Ootsu.

Polyscytalum was not infrequent in Ootsu on the leaves of four months decay, while in the present study, it developed only on a very early decay of the leaves.

Subulispora developed in the present case on the leaves of a very early decay and its occurrence reduced markedly after three to four months and was very rare during the winter although it was found previously colonizing throughout the process.

Many other examples can be found in addition to these fungi in the occurrence on the leaves, and the difference of the occurrence between these fungi in two localities must have their own reasons.

At first we thought that the difference might be floral discrepancies present between the central and the southern localities mentioned above. However, this thought was found not necessarily correct as the result of a comparative invetigation on the occurrence of fungi. We believe that the speed and process of an early decay of the leaves influenced markedly the succession of the fungi. As described above, even the leaves of three months decay were decomposed heavily in the present southern localities under high temperature, high humidity and heavy rain fall. Accordingly one month decay of the leaves in the present localities, especially during the summer, may correspond roughly to that of more than three months or more in the central Japan where the climate is rather moderate. As one of the proofs of such consideration, a comparison of the relative degree of the leaf-decomposition is shown by the photographs (Pl. 0,000).

We have discussed previously on the difference of the status in the colonization of fungi on the leaves between two main seasons of the winter and the summer appeared in the Ootsu area, central Japan, and the temperature was taken as the most significant factor to bring about the difference. While, in the case of the comparison of the data taken in both localities of central and southern Japan, such seasonal differences either in fungus development or in physical condition of the leaf itself have appeared in form of the promotions. Differences in the frequency and the sequence of the development of fungi are stressed.

Of course, it is apparent that there are much more problem to be studied before explaining the difference of the succession of fungi. Here we only submit the data up to the present.

Summary

Successive colonization by fungi on the embedded sterilized leaves in the naturally developing litter of the forests has been examined in five stations set up on Yakushima and Tanegashima Islands, southern parts of Japan, during the priod of December, 1970 through May, 1971. Seventy one genera were encountered and discussions are given on autoecology, seasonal difference in the flora and also on the difference of the successive colonization of fungi between two distant parts, central and southern localities of Japan, Ootsu area and two islands in Kagoshima Pref.

The authors are grateful to Mr. M. Taki, the Head of the Tanegashima Branch of the National Institute of Hygienic Sciences, and also to Mr. M. Hidaka, Kamiyama Primary School on Yakushima Is., for their considerable helps in providing the leaf-samples. The authors also wish to thank to Dr. T. Hasegawa, the Director of the Institute for Fermentation, for his encouragement and to Mr. T. Ito for isolation and identification of fungi. Most of the penicillia were identified by Mr. I. Asano to whom we owe many thanks. The authors were favored to have the assistance of Miss. M. Mashimo.

Reference

Tubaki, K. and T. Yokoyama. 1971. Successive fungal flora on sterilized leaves in the litter of forests. I. Res. Comm. Inst. Ferm., **5:** 24–42.

————. 1972. ————. II. in this journal, p.

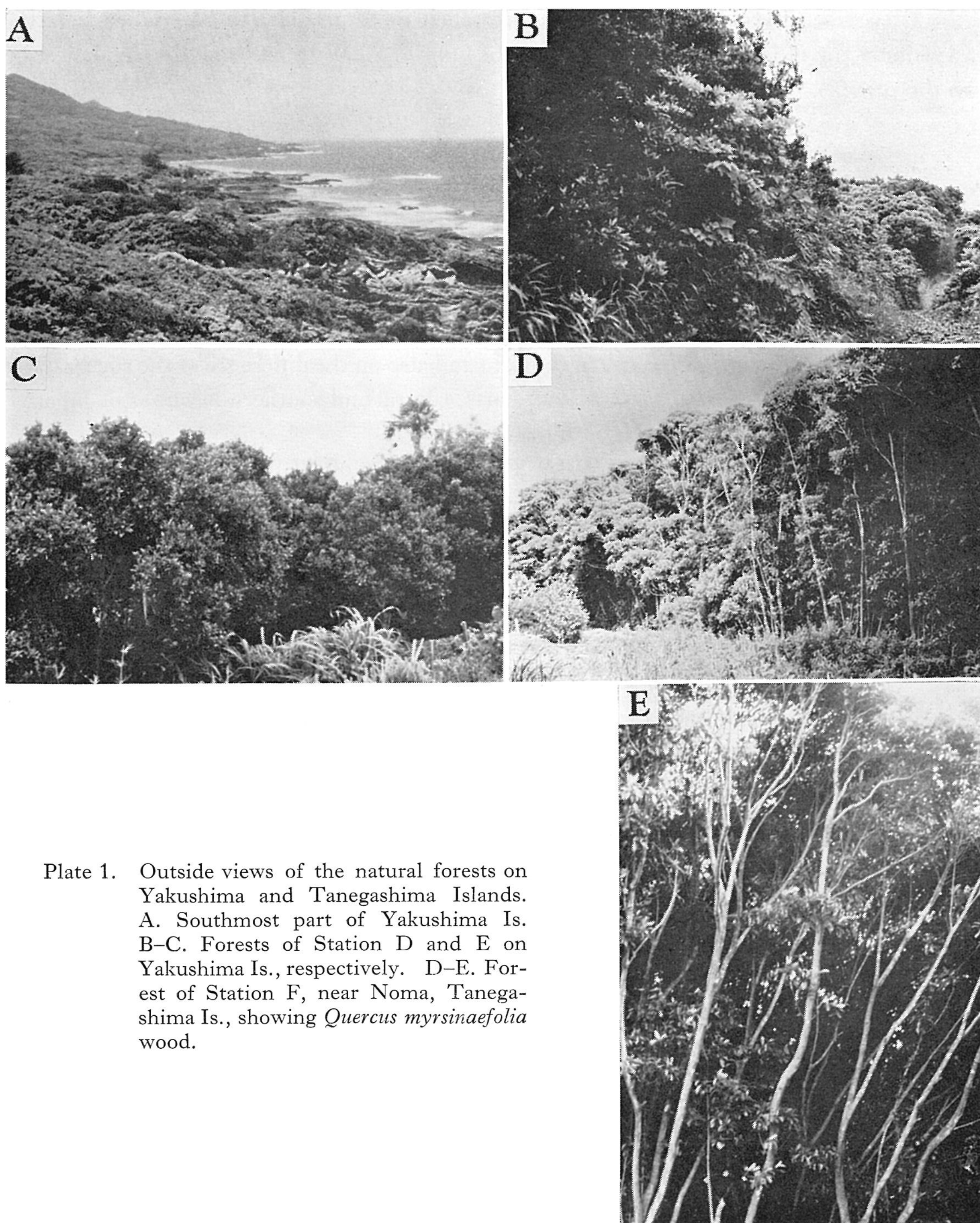


Plate 1. Outside views of the natural forests on Yakushima and Tanegashima Islands. A. Southmost part of Yakushima Is. B–C. Forests of Station D and E on Yakushima Is., respectively. D–E. Forest of Station F, near Noma, Tanegashima Is., showing *Quercus myrsinaefolia* wood.

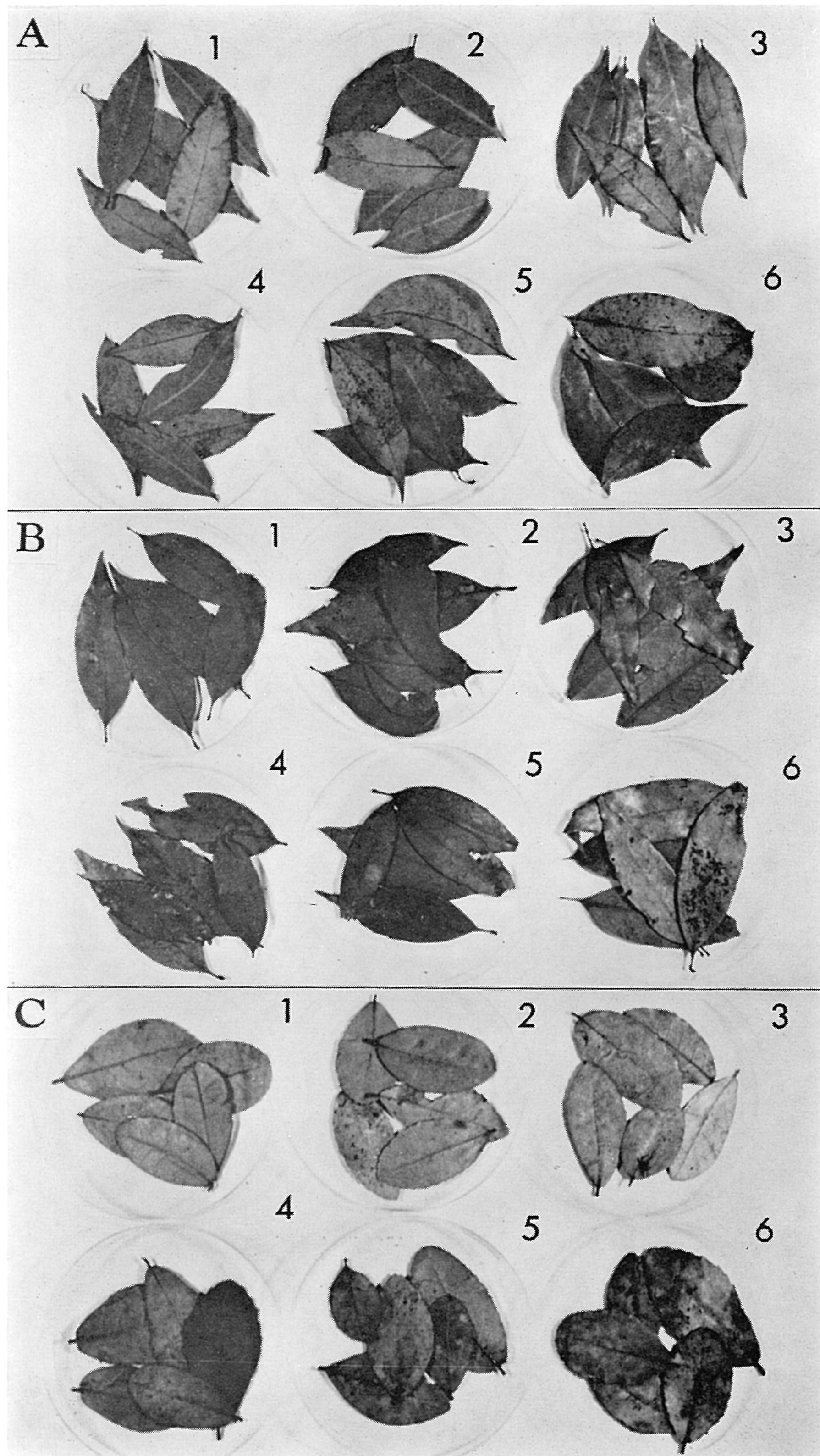


Plate 2. Comparison of the relative degree of the leaf-decomposition in Station A, Ootsu area, during six months (1–6) from May through October. A. Green leaves of *Castanopsis cuspidata*, B. Newly detached leaves of *Castanopsis cuspidata*, C. Newly detached leaves of *Quercus phillyraeoides*.

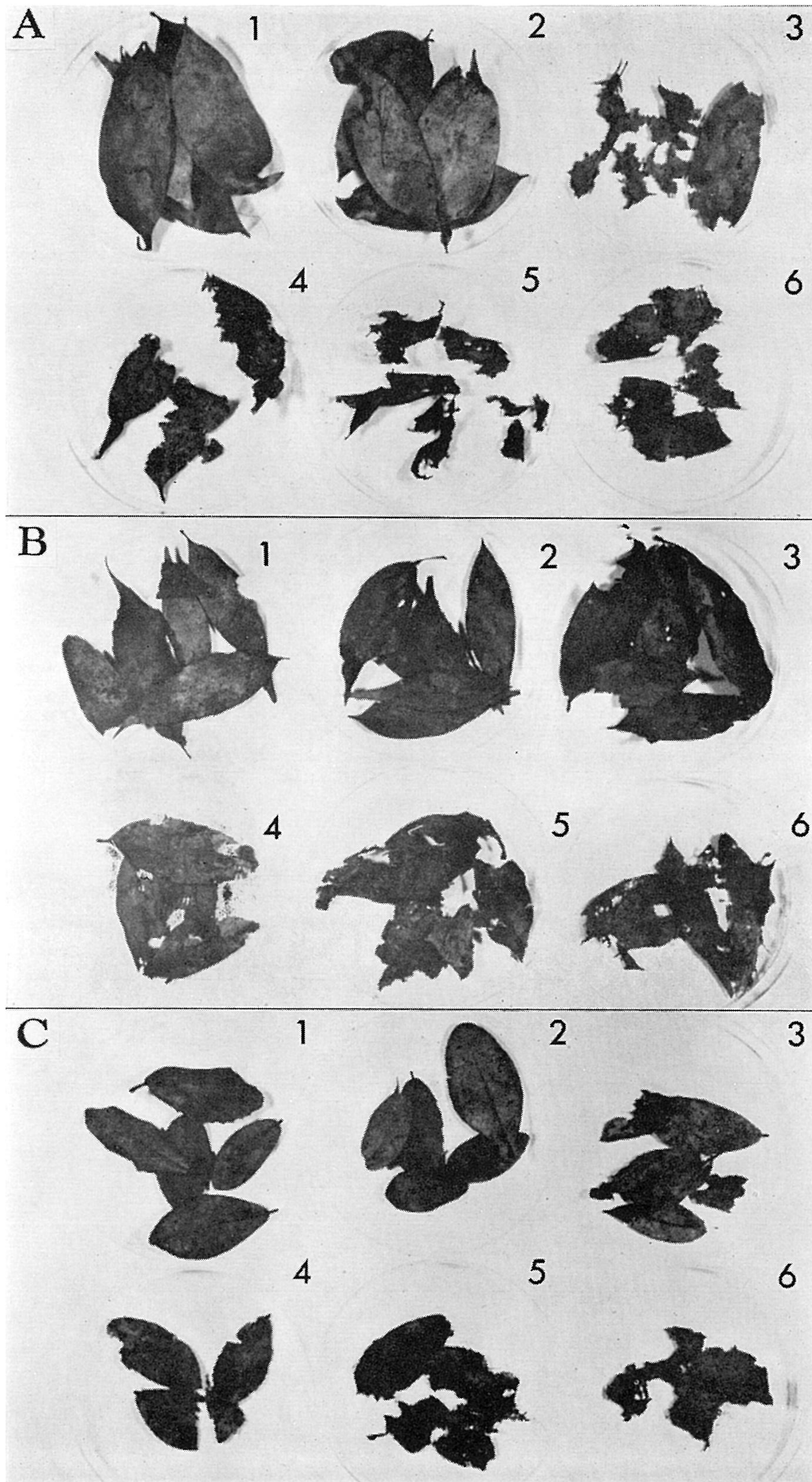


Plate 3. Comparison of the relative degree of the leaf-decomposition in Station D, Yakushima Is., during six months from June through November. A–C. Plants are same with those in Pl. 2. Note rapid decomposition even after three months in contrast to that in Pl. 2.

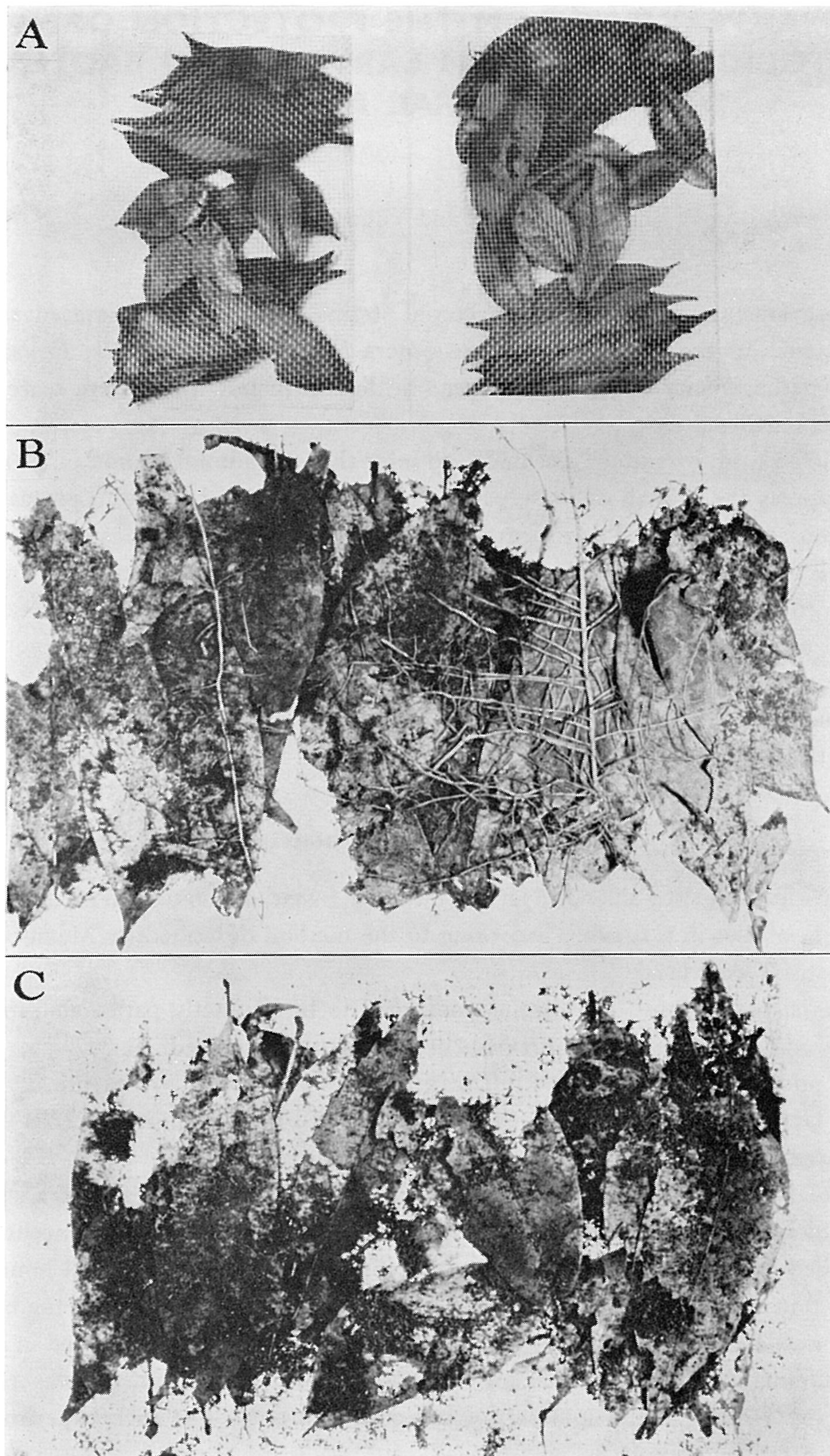


Plate 4. A. Salan-nets, each containing sterilized leaves. B–C. Marked decomposition of the leaves of *Cast. cuspidata* after six months in Station D, Yakushima Is., and Station F, Tanegashima Is., respectively.

IFO Res. Comm. **6**,
50–58, 1937 (*January*)

EXPERIMENTS ON THE PROTECTION OF GNOTOBIOTIC SILKWORM LARVAE FROM BACTERIAL DISEASES BY ORAL ANTIBIOTICS

Reijiro KODAMA and Yugoro NAKASUJI

As previously reported,[3] some bacterial strains isolated from diseased or dead silkworm, such as those belonging to the genera *Streptococcus*, *Serratia*, *Proteus*, etc., had great pathogenicity for silkworm larvae in the 5th instar, which were reared aseptically on an artificial diet.

In such a case, it is quite reasonable to infer that the administration of antibiotics, which suppress the growth of these pathogenic bacteria, may be able to protect larvae from bacterial diseases. There are only a few studies[1-2] on the susceptibility of bacterial isolates from diseased silkworm to antibiotics. Recently, a study was conducted as to whether gnotobiotic silkworm larvae could be protected from diseases to be caused by the oral inoculation of pathogenic bacteria, if an artificial diet containing the effective antibiotics was supplied.

In this paper, the results obtained from the protection experiments and the behavior of antibiotics added to an artificial diet are presented.

Materials and Methods

Silkworm. Healthy silkworm larvae in the 5th instar, which were reared aseptically on an artificial diet in test tubes according to the method described by Matsubara and his associates,[5] were used.

Bacterial pathogens. The strains confirmed to be distinctly pathogenic for aseptically reared silkworm larvae in previous experiments[3] were used.

Gut juice of silkworm larvae. The gut contents vomited by aseptically reared silkworm larvae with an electric shock were centrifuged for 30 minutes at 12,000 r.p.m. The supernatant was preserved in refrigerator until use.

Inoculation with bacteria and administration of antibiotics. Each 5g of a sterilized diet, to which each antibiotic to be tested was added aseptically at desired concentrations, was supplied to each larva immediately after ecdycis. After feeding for 24 hours, each larva was transferred in a tube containing diet infected with each strain of the bacteria, and was inoculated by feeding for 4 hours. Then, each larva was transferred in another tube containing freshly prepared, antibiotic-added diet. The transfer was repeated every 20 to 24 hours, until spinning of coccoon occurred. For each experiment, 10 larvae were used.

Measurement of antibacterial activity of antibiotics in test tube. Media A and B were used for measurement of antibacterial activity against streptococci and gram-

negative bacteria respectively.

Medium A consisted of yeast extract, 5 g; peptone, 5 g; glucose, 20 g; K_2HPO_4, 1 g; Salts B[6)], 10 ml; distilled water, 1000 ml; pH 9.0.

Medium B consisted of meat extract, 10 g; peptone, 10 g; NaCl, 10 g; distilled water, 1000 ml; pH 7.0.

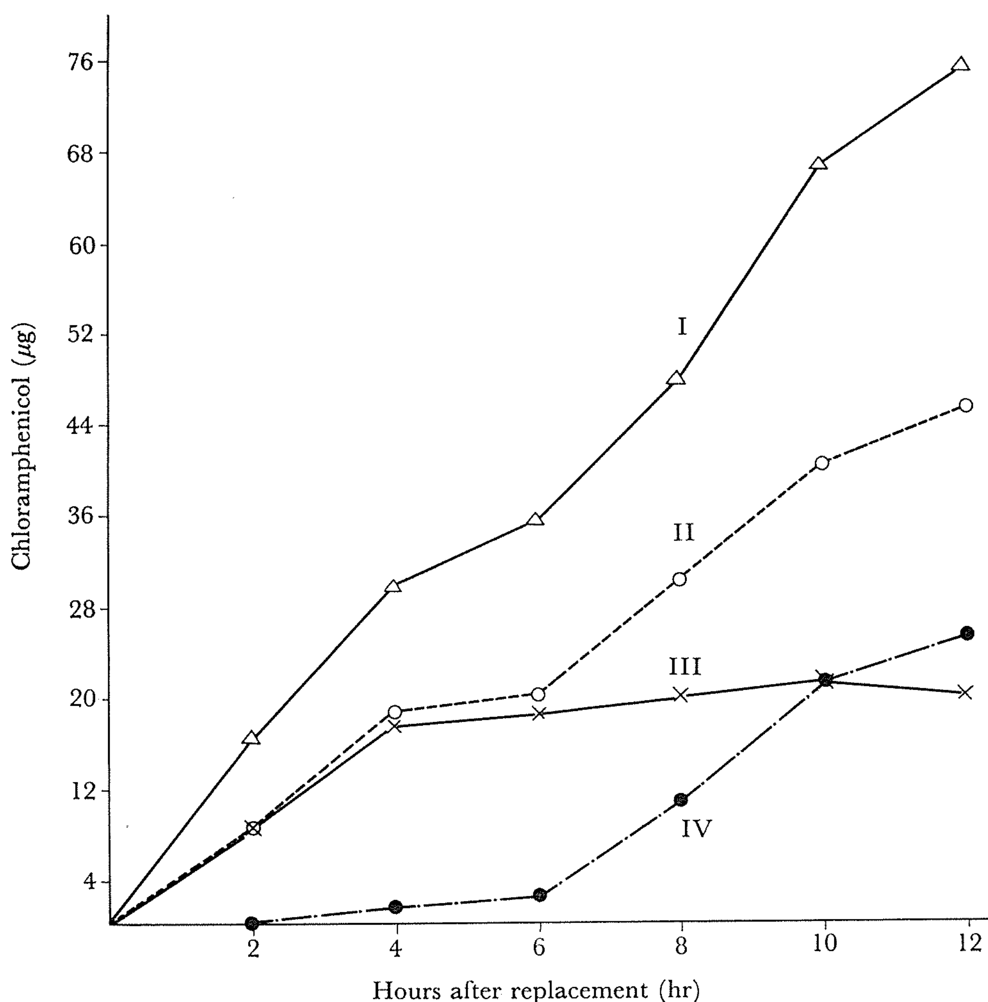


Fig. 1. Amount of chloramphenicol recovered from gut contents and feces.

I (△—△) Amount of chloramphenicol added to diet (values calculated from weight of diet ingested, average of 5 larvae).

II (○···○) III+IV

III (×—×) Amount of chloramphenicol recovered from gut contents (average of 5 larvae).

IV (●···●) Amount of chloramphenicol recovered from feces (average of 5 larvae).

Stability test of antibiotics in the gut juice. To 4 ml of the gut juice was added a 0.1% solution of each antibiotic. The mixture was incubated at 25°, and then changes in antibacterial activity were checked after 1, 24, 48 and 72 hours by the cylinder-plate method employing *Bacillus subtilis* P.C.I. 219 as the test organism.

Determination of chloramphenicol in diet, gut contents and feces. Determination of chloramphenicol in these materials was carried out by the method employing *Aeromonas hydrophila* IFO 3920 as the test organism, because this method was perferable in sensitivity to chloramphenicol to that employing *Bac. subtilis* P.C.I. 219.

(1) Diet. KH_2PO_4-NaOH buffer solution (pH 6.0) was added to 1 g of a chloramphenicol-containing diet. After crushing the diet with a glass rod, the suspension was filled up to 3 ml with distilled water, and then centrifuged out for 30 minutes at 3,000 r.p.m. The supernatant was tested for analysis.

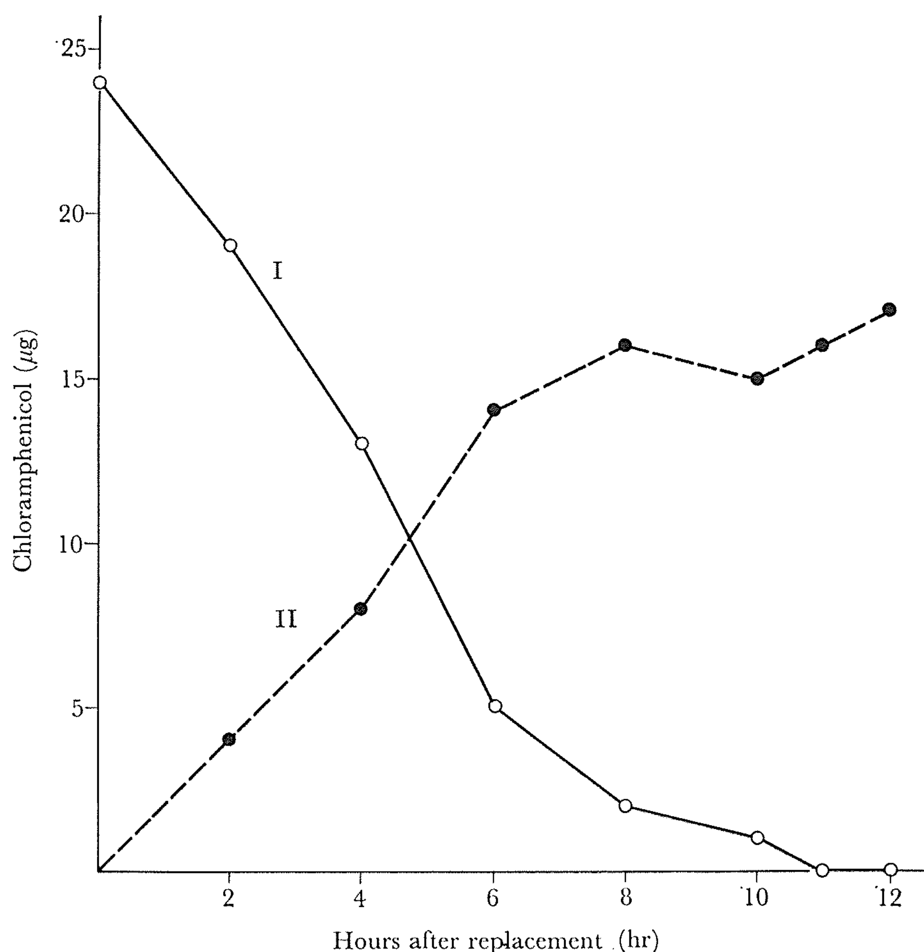


Fig. 2. Vicissitudes of chloramphenicol in gut contents and feces.
I (○—○) Amount of chloramphenicol recovered from gut contents (average of 5 larvae).
II (●···●) Amount of chloramphenicol recovered from feces (average of 5 larvae).

(2) Gut contents. Buffer solution was added to the alimentary canal removed after incision of the abdominal region. Subsequent treatment was the same as above.

(3) Feces. Buffer solution was added to the feces, which were excreted in each given period as indicated in Figs. 1 and 2. Subsequent treatment was the same as above.

Results

I. Suppression of growth of bacteria in test tubes

The results are summarized in Table 1. Macrolide antibiotics and enduracidin are highly effective against the growth of the streptococci whether they are primary invaders (*Streptococcus faecalis-Streptococcus faecium* intermediate) or secondary invaders (*Streptococcus faecalis* var. *liquefaciens*), while the gram-negative bacteria, considered practically as secondary invaders, were suppressed effectively by the members of streptomycin- and neomycin-group.

Table 1. Suppression of growth of bacterial pathogens in test tubes

Bacterial pathogen	Number of strains tested	Antibiotics effective against growth of bacteria
Streptococcus faecalis-Streptococcus faecium intermediate	3	Macrolide antibiotics (oleandomycin, triacetyloleandomycin, leucomycin, tylosin, carbomycin, erythromycin, pikromycin, spiramycin, tertiomycin, and others), enduracidin, CA*
Streptococcus faecalis var. *liquefaciens*	4	CA, Macrolide antibiotics
Achromobacter superficialis	1	CA, DSM, NM
Aerobacter cloacae	1	CA, DSM, KM, NM, ZM
Aeromonas hydrophila	1	DSM, NM
Flavobacterium aquatile	1	DSM
Proteus inconstans	2	DSM, KM, NM, ZM
Proteus mirabilis	1	DSM, NM
Proteus morganii	2	DSM*, NM*
Proteus vulgaris	2	CA, DSM*, NM, ZM
Pseudomonas aeruginosa	1	DSM
Serratia indica	1	CA, DSM, NM
Serratia marcescens	5	DSM*, KM*, NM, ZM
Serratia piscatorum	3	KM*, NM, ZM

In above table, CA, DSM, KM, NM and ZM designate chloramphenicol, dihydrostreptomycin, kanamycin, neomycin B and zygomycin, respectively.
* Antibacterial activity varied with the strains tested.

II. Protection of gnotobiotic silkworm larvae from bacterial diseases

The relationship between the amount of antibiotics added to diet and the outbreak of bacterial diseases was investigated, employing some of the strains listed in Table 1, which were all shown to be distinctly pathogenic for aseptically reared silkworm larvae

Table 2. Protective effect of antibiotics on development of bacterial disease

Bacterial pathogen	Viable cells added (per gram of diet)	Antibiotic	Amount of antibiotic added to diet (μg/g)	Lethality (%)	Amount of antibiotic required for suppressing growth of bacteria in test tube (μg/g)
Streptococcus faecalis-Streptocooccus faecium intermediate E–5	2.3×10^3	Spiramycin	0	100	0.1–0.5
			1	80	
			10	10	
			100	0	
	2.3×10^3	Tylosin	0	100	0.5–1.0
			1	80	
			10	20	
			100	0	
	9.6×10^3	Leucomycin	0	100	0.5–1.0
			1	90	
			10	0	
			100	0	
Streptococcus faecalis var. *liquefaciens* A–1	6.2×10^3	Spiramycin	0	30	0.1–0.5
			1	10	
			10	0	
			100	0	
	6.2×10^3	Tylosin	0	30	0.5–1.0
			1	30	
			10	0	
			100	0	
	1.7×10^3	Leucomycin	0	20	0.5–1.0
			1	10	
			10	0	
			100	0	
Proteus inconstans A–142	1.1×10^6	Neomycin B	0	20	1.0–2.5
			1	10	
			10	0	
			100	0	
Proteus vulgaris I–215	1.1×10^6	Neomycin B	0	40	1.0–2.5
			1	10	
			10	20	
			100	0	
Pseudomonas aeruginosa IFO 3898	2.0×10^6	Dihydro-streto-mycin	0	30	5.0–10.0
			1	30	
			10	0	
			100	0	
Serratia indica IFO 3759	1.8×10^6	Neomycin B	0	40	2.5–5.0
			1	10	
			10	10	
			100	0	
Serratia marcescens A–21 (non-chromogenic strain)	1.2×10^6	Neomycin B	0	30	2.5–5.0
			1	20	
			10	10	
			100	0	
Serratia piscatorum E–15	1.6×10^6	Neomycin B	0	50	2.5–5.0
			1	40	
			10	30	
			100	0	

in previous experiments.[3] As seen from Table 2, lethality dropped with increasing amounts of antibiotics added to diet, and survival rate of pupae reached 100% by addition of 10 to 100 μg of antibiotics per gram of diet. These results indicate that the oral administration of antibiotics, which effectively suppress the growth of pathogenic bacteria in test tubes, makes it possible to protect the larvae from diseases caused by orally inoculated bacteria. However, there was a marked difference between the amount of antibiotics in test tubes required for suppressing the growth of bacteria and the amount in diet necessary for protecting the larvae from bacterial diseases.

III. Stability of antibiotics in the gut juice

The pH value of the gut juice derived from aseptically reared silkworm larvae lies at 10.0 or higher. An investigation was made on the stability of antibiotics under such an alkaline condition as the gut juice, when antibiotics were taken into the gut together with diet.

Neomycin, dihydrostreptomycin, spiramycin and chloramphenicol were used as the test antibiotics. The results are summarized in Tables 3 and 4. No loss of antibacterial activity was observed after 72 hours in distilled water. However, the activity of dihydrostreptomycin as well as spiramycin declined slightly in buffer solution (pH 10.86), and the decline of activity, especially that of spiramycin, was greater in the gut juice of the same pH value as the buffer solution. Among the macrolide antibiotics tested, triacetyloleandomycin was most stable, while the others were more or less unstable.

Table 3. Stability of antibiotics in gut juice. I

Antibiotic	Substrate	Decline in percentage of antibacterial activity (%)			
		Duration of contact with substrate at 25° (hr)			
		1	24	48	72
	DW	0	0	0	0
Neomycin B	BS	0	0	0	0
	GJ	2	0	4	2
	DW	0	0	0	0
Dihydrostreptomycin	BS	0	0	4	8
	GJ	0	0	0	16
	DW	0	0	0	0
Spiramycin	BS	0	0	8	20
	GJ	6	8	52	60
	DW	0	0	0	0
Chloramphenicol	BS	0	0	0	0
	GJ	0	0	0	0

DW: distilled water, BS: [0.02M Na_2CO_3+0.02M $NaHCO_3$] buffer solution (pH 10.86), GJ: gut juice (pH 10.86).

Table 4. Stability of antibiotics in the gut juice. II

Macrolide antibiotic	Substrate	Decline in percentage of antibacterial activity (%) Duration of contact with substrate at 25° (hr) 1	24	48	72
	DW	0	0	0	0
Erythromycin	BS	0	24	24	28
	GJ	0	36	32	44
	DW	0	0	0	0
Leucomycin	BS	0	8	8	12
	GJ	0	4	12	20
	DW	0	0	0	0
Spiramycin	BS	0	0	8	20
	GJ	6	8	52	60
	DW	0	0	0	0
Tertiomycin	BS	0	41	50	53
	GJ	8	64	68	80
	DW	0	0	0	0
Triacetyloleandomycin	BS	0	0	0	0
	GJ	0	0	0	4
	DW	0	0	0	0
Tylosin	BS	0	0	12	20
	GJ	0	0	20	36

DW: distilled water, BS: [0.02 M Na_2CO_3+0.02 M $NaHCO_3$] buffer solution (pH 10.86), GJ: gut juice (pH 10.86).

IV. Behavior of antibiotics added to diet

Investigations were carried out on the behavior of antibiotics, when added to diet and then ingested by the silkworm larvae. In these experiments, chloramphenicol was used as the test antibiotic because of the stability on contact with the gut juice as described above.

(1) Recovery of chloramphenicol from diet, gut contents and feces

As seen from Table 5, only about 60% of the added chloramphenicol was recovered from the diet, which was left standing for 5 and 20 hours after the addition; consequently about 40% was lost. It was later established that the loss was caused by adsorption of the antibiotic on diet.

When a chloramphenicol-supplemented diet was supplied to the larvae for 5 and 20 hours, the antibiotic was recovered from both the gut contents and the feces in slightly less amounts than those calculated from the weight of the diet ingested.

(2) Vicissitudes of chloramphenicol in the gut

When a chloramphenicol-free diet was replaced by a chloramphenicol-supplemented diet, the amount of the antibiotic in the gut contents reached a maximum about 4 hours after the larvae began to ingest the antibiotic-supplemented diet. At about

Table 5. Recovery of chloramphenicol from diet, gut contents and feces

Duration of supplying with chloramphenicol-supplemented diet (hr)	Chloramphenicol (μg/g)		Quantity of chloramphenicol-supplemented diet ingested (g/larva)	Quantity of Chloramphenicol ingested (μg/larva)	
	Added to diet (A)	Recovered from diet (B)		Values calculated from (A) (C)	Values calculated from (B) (D)
5	200	120	0.24	48	29
20	200	117	1.07	214	125

Chloramphenicol recovered (μg/larva)			Rate of recovery of chloramphenicol (%)	
Gut contents (E)	Feces (F)	Total ((E)+(F))	Values calculated from (C)	Values calculated from (D)
20	4	24	50	83
23	92	115	54	92

An average of 5 larvae.

the same time, the amount of the antibiotic excreted in the feces began to increase (Fig. 1).

On the contrary, when a chloramphenicol-supplemented diet, which was continued for about 16 hours, was replaced by a chloramphenicol-free diet, the amount of the antibiotic in the gut contents continued to decrease, while the amount in the feces gradually increased. At 10 to 11 hours after the replacement, the antibiotic could not be detected in the gut contents, and only 63 to 67% of the initial amount of the antibiotic could be recovered from the feces (Fig. 2). The reason for this was not further investigated.

Discussion

In our previous paper,[4] it was concluded that the streptococci produce an intestinal disease in aseptically reared silkworm larvae as primary invaders, and also play an inductive role for secondary invaders such as the gram-negative bacteria. From such a pathogenic mechanism it may be assumed that the primary invaders, which are active principally in the gut and not in the haemocoele, play a most important role in the outbreak of bacterial diseases of larvae. The vicissitudes of antibiotics, when added to an artificial diet and then taken into the gut, are of considerably important to protect the larvae from diseases. It has been observed that supplemented antibiotics are adsorbed on the diet and inactivated by the gut juice. One of the causes of the inactivation is probably alkalinity of the gut juice. Howevei, other factor or factors responsible for the inactivation may also be present in the gut juice.

In an attempt to protect gnotobiotic silkworm larvae from bacterial diseases by

supplying a diet containing antibiotics, the kind as well as the amount of antibiotics to be used should be selected in due consideration of the adsorption, the inactivation and the vicissitudes of the antibiotics.

Summary

Experiments were carried out to protect gnotobiotic silkworm larvae from bacterial diseases by the oral administration (feeding) of antibiotics. The behavior of antibiotics, when added to an artificial diet and then taken in the gut of larvae, was also investigated. The following results were obtained.

(1) The oral administration of antibiotics, which effectively suppress the growth of pathogenic bacteria in test tubes, made it possible to protect the larvae from diseases caused by orally inocultated bacteria. However, there was a marked difference between the amount of antibiotics required for suppressing the growth of the bacteria in test tubes and the amount in diet required for protecting the larvae from diseases.

(2) Some of the antibiotics tested were inactivated by contact with the gut juice of aseptically reared silkworm larvae. Chloramphenicol and triacetyloleandomycin were stable, while tertiomycin and spiramycin were relatively unstable.

(3) Using chloramphenicol as the test antibiotic,

a) When the antibiotic was added to diet, it was considerably adsorbed on the diet.

b) At about 4 hours after the larvae began to ingest the antibiotic-supplemented diet, the amount of the antibiotic in the gut contents reached a maximum, and at about the same time the amount of the antibiotic excreted in the feces began to increase.

c) On the contrary, when the antibiotic-supplemented diet was replaced by an antibiotic-free diet, the antibiotic could not be detected in the gut contents at 10 to 11 hours after the replacement.

References

1) Afrikian, E.G. 1960. Causal agents of bacterial diseases of the silkworm and the use of antibiotics in their control. J. Insect Path. **2**: 299–304.
2) Kawakita, T., T. Watanabe, T. Ishisaka and K. Hayashi. 1966. On the susceptibility to antibacterial agents in various bacteria isolated from the silkworm larvae infected with flacherie and unidentified bacteria taken from mulberry leaves. J. Sericult. Sci. Japan **35**: 248–252 (in Japanese).
3) Kodama, R. and Y. Nakasuji. 1969. Pathogenicity of bacteria for silkworm larvae reared aseptically on an artificial diet. Ann. Rep. Inst. Fermentation, Osaka No. (1967–1968), p. 1–11.
4) Kodama, R. and Y. Nakasuji. 1971. Further studies on the pathogenic mechanism of bacterial diseases in gnotobiotic silkworm larvae. Res. Commun. No. 5 (Ann. Rep. 1969–1970), p. 1–9.
5) Matsubara, F., M. Kato, K. Hayashiya, R. Kodama and Y. Hamamura. 1967. Aseptic rearing of silkworm with prepared food. J. Sericult. Sci. Japan **36**: 39–45 (in Japanese).
6) Snell, E.E. and L.D. Wright. 1941. A microbiological method for the determination of nicotinic acid. J. Biol. Chem. **139**: 675–678.

IFO Res. Comm. 6,
59–67, 1937 (*January*)

INHIBITION OF DEVELOPMENT OF VIRAL DISEASES IN GNOTOBIOTIC SILKWORM LARVAE BY NALIDIXIC ACID

Reijiro KODAMA and Yugoro NAKASUJI

A review of the literature reveals several investigations[1,7,11,14,15] on the protection of silkworm larvae from viral diseases by the use of chemical agents. It is needless to say that these studies have been carried out using silkworm larvae fed only with mulberry leaves, which usually contain various kinds of pathogens in the period used for the test.

Recently, it was reported by Ayuzawa et al.[2] and Ebihara and Iioka[5] that a synergistic effect in the pathogenicity of viruses and that of bacteria was found in silkworm larvae fed with mulberry leaves or with an artificial diet. The results of these studies offer a useful suggestion for the investigation of the pathogenic mechanism of silkworm diseases and the protection from the diseases. Under the present conditions, however, it may be quite difficult to expect satisfactory protection, unless some means of inhibiting both the activity of viruses and that of bacteria are devised.

In the preceding paper[9] it was substantiated that the outbreak of bacterial diseases in gnotobiotic silkworm larvae was suppressed by supplying an artificial diet containing antibiotics, which effectively suppress the growth of pathogenic bacteria in test tubes. As the next step in our studies, chemical agents inhibiting viral activity in gnotobiotic silkworm larvae were investigated. During the study it was found that the development of viral diseases of the larvae infected orally either with the infections flacherie virus (FV) or with the nuclear polyhedrosis virus (NPV) was conspicuously inhibited by the oral administration of nalidixic acid (NA).

In this paper the inhibitory effect of NA and some related results are presented.

Materials and Methods

Silkworm. Silkworm larvae reared aseptically on an artificial diet according to the method by Matsubara et al.[10] were used.

Virus. The infectious flacherie virus, the cytoplasmic polyhedrosis virus (CPV) and the nuclear polyhedrosis virus were used as the test virus. FV and CPV were supplied by Dr. C. Ayuzawa (The Sericultural Experiment Station, Tokyo), and NPV by Dr. F. Matsubara (Kyoto University of Industrial Arts and Textile Fibers, Kyoto).

Inoculation with virus. The suspension of each virus was prepared as follows:

(1) FV 10% Emulsion of silkworm larvae suffering from infectious flacherie was centrifuged at 10,000 r.p.m. for 10 min. The supernatant was filtered through a

membrane fillter (0.22μ) after appropriate dilution with distilled water. The filtrate was used for the inoculation, and the concentration of the viral suspension was expressed as 10^{-1}.

(2) CPV After the polyhedra were suspended in 0.5% aqueous Na_2CO_3 at the rate of 10^8/ml, the suspension was filtered through a membrane filter (0.22μ). The filtrate was used for the inoculation.

(3) NPV The preparation of the inoculum was carried out according to the same method as for CPV.

The suspension of each virus or its dilution was added to a sterilized diet in proportion of 0.04 ml/g, and then supplied to aseptically reared silkworm larvae on the 2nd day in the 5th instar for 24 hours.

Inoculation with bacteria. *Streptococcus faecalis-Streptococcus faecium* intermediate G-27 was used as the test organism. The inoculation with the strain was conducted as described in the preceding paper[8] using larvae on the 2nd day in the 5th instar.

Results

I. Inhibition of development of infectious flacherie and nuclear polyhedrosis by the administration of NA

The development of viral diseases due to orally inoculated FV or NPV was inhibited by the oral administration of NA, while that of cytoplasmic polyhedrosis was not inhibited (Tables 1 and 2).

Table 1. Inhibition of development of infectious flacherie by NA

FV*	Amount of NA added to diet** (μg/g)	Number of larvae tested	Number of dead larvae				Lethality (%)
			3rd instar	4th instar	5th instar	cocoon	
Inoculated	0	10	0	0	8	1	90
	1	10	0	0	3	1	40
	10	10	0	0	1	1	20
	100	10	0	0	0	0	0
	500	10	0	0	0	0	0
	1000	10	0	0	0	0	0
Not inoculated	0	10	0	0	0	0	0
	1	10	0	0	0	0	0
	10	10	0	0	0	0	0
	100	10	0	0	0	0	0
	500	10	0	0	0	0	0
	1000	10	0	0	0	0	0

* Viral suspension diluted to 10^{-4} was used as the inoculum.

** The supply of NA-containing diet continued throughout the 3rd to the 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with virus.

Table 2. Inhibition of development of infectious flacherie and nuclear polyhedrosis by NA

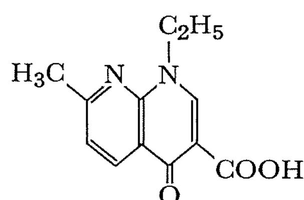
Viral suspension added to diet		Amount of NA added to diet* (μg/g)	Number of larvae tested	Number of dead larvae				Lethality (%)
Virus	Inoculum			3rd instar	4th instar	5th instar	cocoon	
FV	Suspension diluted to 10^{-4}	0	10	0	0	4	3	70
		10	10	0	0	1	3	40
		100	10	0	0	0	0	0
NPV	Suspension containing 10^8 polyhedra per ml	0	10	0	0	3	3	60
		10	10	0	0	1	2	30
		100	10	0	0	0	0	0
CPV	Suspension containing 10^8 polyhedra per ml	0	10	0	0	2	3	50
		10	10	0	0	1	3	40
		100	10	0	0	1	4	50

* The supply of NA-containing diet continued throughout the 3rd to the 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with each virus.

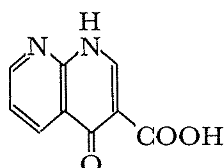
Table 3. Inhibitory effect of NA and its derivatives on development of infectious flacherie

FV*	Derivative**	Amount added to diet (μg/g)	Number of larvae tested	Number of dead larvae				Lethality (%)
				3rd instar	4th instar	5th instar	cocoon	
Inoculated	Not added		10	0	0	3	2	50
	NA	100	10	0	0	0	0	0
	I	100	10	0	0	2	1	30
	II	100	10	0	0	5	1	60
Not inoculated	Not added		10	0	0	0	0	0

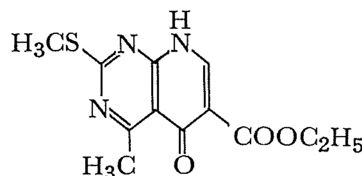
* Viral suspension diluted to 10^{-4} was used as inoculum.
** The supply of NA-or its derivative-containing diet continued throughout the 3rd to 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with virus.



NA



I



II

Two NA-related compounds, I and II, exhibited little or no effect (Table 3).

II. Relation between concentration of FV and amount of NA

The results from an experiment carried out on the relationship between the concentration of FV inoculum and the amount of NA required for inhibiting the development of the viral disease, are summarized in Table 4. When the concentration of the viral suspension was lower than 10^{-4}, inhibition by NA was conclusive, and less NA was required for the inhibition with decreasing concentrations of the viral suspension. However, when the concentration was higher than 10^{-3}, inhibition by NA was not obtained.

Table 4. Relation between viral concentration of FV suspension and amount of NA

Viral concentration of FV suspension	Amount of NA added to diet* ($\mu g/g$)	Number of larvae tested	Number of dead larvae				Lethality (%)
			3rd instar	4th instar	5th instar	cocoon	
10^{-1}	0	10	0	0	10		100
	50	10	0	0	10		100
	100	10	0	0	10		100
	250	10	0	0	10		100
	500	10	0	0	10		100
10^{-2}	0	10	0	0	10		100
	50	10	0	0	9	0	90
	100	10	0	0	9	0	90
	250	10	0	0	4	4	80
	500	10	0	0	8	2	100
10^{-3}	0	10	0	0	9	0	90
	50	10	0	0	5	3	80
	100	10	0	0	6	1	70
	250	10	0	0	4	3	70
	500	10	0	0	4	0	40
10^{-4}	0	10	0	0	6	1	70
	10	10	0	0	3	1	40
	25	10	0	0	2	0	20
	50	10	0	0	0	1	10
	100	10	0	0	0	0	0
10^{-5}	0	10	0	0	2	2	40
	5	10	0	0	0	2	20
	10	10	0	0	0	1	10
	25	10	0	0	1	0	10
	50	10	0	0	0	0	0
Not inoculated	0	10	0	0	0	0	0

* The supply of NA-containing diet continued throughout the 3rd to the 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with virus.

Table 5. Inhibition of development of disease under pathogenic synergism between FV and *S. faecalis-S. faecium* intermediate G–27

Pathogen		Amount of drug added to diet (μg/g)		Number of larvae tested	Number of dead larvae				Lethality
FV*	*S. faecalis-S. faecium* intermediate G–27**	NA†	Leucomycin††		3rd instar	4th instar	5th instar	cocoon	(%)
+	−	0	0	10	0	0	1	0	10
−	+	0	0	10	0	0	2	0	20
+	+	0	0	10	0	0	6	2	80
+	+	100	0	10	0	0	5	0	50
+	+	0	10	10	0	0	1	1	20
+	+	100	10	10	0	0	0	0	0
−	−	0	0	10	0	0	0	0	0

* Viral suspension diluted to 10^{-4} was used as inoculum. +, inoculated; −, not inoculated.
** 3.2×10^2 viable cells per gram were added to diet.
† The supply of NA-containing diet continued throughout the 3rd to the 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 5th instar) necessary for inoculation with virus and a period of 4 hours (on the 2nd day in the 5th instar) necessory for inoculation with bacteria.
†† The supply of leucomycin-containing diet continued throughout the 5th instar except for a period of 4 hours (on the 2nd day in the same instar) necessary for inoculation with bacteria.

Table 6. Relation between inhibitory effect of NA and duration of administration

FV*	NA added at						Number of larvae tested	Number of dead larvae					Lethality
	2nd instar	3rd instar: On the 1st day	3rd instar: On the 2nd day	3rd instar: On andafter the 3rd day	4th instar	5th instar		2nd instar	3rd instar	4th instar	5th instar	cocoon	(%)
Inoculated	−	−	(FV inoculated)	−	−	−	10	0	0	0	6	0	60
	+	+		−	−	−	10	0	0	0	3	2	50
	+	+		+	−	−	10	0	0	0	1	0	10
	+	+		+	+	−	10	0	0	0	0	0	0
	−	+		+	−	−	10	0	0	0	3	1	40
	−	+		+	+	−	10	0	0	0	1	0	10
	−	+		+	+	+	10	0	0	0	0	0	0
	−	−		+	−	−	10	0	0	0	5	1	60
	−	−		+	+	−	10	0	0	0	2	1	30
	−	−		+	+	+	10	0	0	0	3	0	30
Not inoculated	+	+	+	+	+	+	10	0	0	0	0	0	0
	−	−	−	−	−	−	10	0	0	0	0	0	0

In above table, + and − indicate addition and non-addition of 100μg NA per gram of diet respectively.
* Viral suspension diluted to 10^{-4} was used as inoculum.

Table 7. Inhibitory effect of fructosazine on development of nuclear polyhedrosis and infectious flacherie

Viral suspension added to diet		Amount of fructosazine added to diet* (mg/g)	Number of larvae tested	Number of dead larvae				Lethality (%)
Virus	Inoculum			3rd instar	4th instar	5th instar	cocoon	
NPV	Suspension containing 10^6 polyhedra per ml	0	10	0	0	3	0	30
		2	10	0	0	1	0	10
		5	10	0	0	1	0	10
		10	10	0	0	1	0	10
	Suspension containing 10^8 polyhedra per ml	0	10	0	2	4	0	60
		2	10	0	0	3	1	40
		5	10	0	0	3	0	30
		10	10	0	0	4	0	40
FV	Suspension diluted to 10^{-6}	0	10	0	0	3	0	30
		2	10	0	0	1	0	10
		5	10	0	0	0	2	20
		10	10	0	0	1	0	10
	Suspension diluted to 10^{-4}	0	10	0	0	7	0	70
		2	10	0	0	4	1	50
		5	10	0	0	4	1	50
		10	10	0	0	3	0	30
Not added		0	10	0	0	0	0	0

* The supply of fructosazine-containing diet continued throughout the 3rd to the 5th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with virus.

Table 8. Amount of NA required for inhibition of development of infectious flacherie in the presence of fructosazine

FV*	Amount of drug added to diet**		Number of larvae tested	Number of dead larvae				Lethality (%)
	Fructosazine (mg/g)	NA (μg/g)		3rd instar	4th instar	5th instar	cocoon	
Inoculated	0	0	10	0	0	5	2	70
	0	1	10	0	0	4	3	70
	0	10	10	0	0	0	3	30
	0	25	10	0	0	0	1	10
	0	50	10	0	0	0	1	10
	0	75	10	0	0	0	0	0
	0	100	10	0	0	0	0	0
	2	0	10	0	0	1	3	40
	2	1	10	0	0	0	4	40
	2	10	10	0	0	0	2	20
	2	25	10	0	0	0	0	0
	2	50	10	0	0	0	0	0
	2	75	10	0	0	0	0	0
	2	100	10	0	0	0	0	0
Not inoculated	0	0	10	0	0	0	0	0

* Viral suspension diluted to 10^{-4} was used as inoculum.
** The supply of drug- or drugs containing diet continued throughout the 3rd to the 4th instar except for a period of 24 hours (extending over the 2nd to the 3rd day in the 3rd instar) necessary for inoculation with virus.

III. Inhibition of development of diseases under a condition of pathogenic synergism between bacteria and virus

As seen from Table 5, a synergistic effect on lethality was observed between FV and *S. faecalis-S. faecium* intermediate G–27. The synergistic effect disappeared practically by the oral administration of leucomycin, one of the macrolide antibiotics, which suppress the growth of the streptococci. However, lethality was not lowered so markedly by the administration of NA alone. Only by the combined administration of leucomycin and NA, was the development of the disease under synergism inhibited satisfactorily.

IV. Period of administration of NA

An experiment was carried out on the period in the larval stage at which NA should be administered in order to obtain satisfactory inhibition, when FV was inoculated orally to larvae on the 2nd day in the 5th instar. Satisfactory inhibition was not obtained by the administration of NA at a period either before or after the inoculation of the virus, but the inhibition was apparent when this compound was administered both before and after inoculation (Table 6).

The inhibitory effect of NA was greater with increasing duration of administration, and a satisfactiry effect was observed when the supply of NA continued from the 2nd to the 4th or the 3rd to the 5th instar (Table 6).

V. Amount of NA required for the inhibition in the presence of fructosazine

According to the communication from Dr. K. Hayashiya[7] (Kyoto University of Industrial Arts and Textile Fibers, Kyoto), silkworm larvae fed with an artificial diet containing fructosazine were moderately resistant to infection with NPV. We also confirmed that the compound had the same effect not only in larvae infected with NPV but also in those infected with FV (Table 7), and that the amount of NA required for inhibiting the development of infectious flacherie decreased by the addition of fructosazine to the diet (Table 8).

Discussion

There have been never found the descriptions on an antiviral activity of NA, an antibacterial substance active principally against gram-negative bacteria[4]. During the course of our research on chemical agents protecting silkworm larvae from viral diseases, it was found that the oral administration of NA inhibited the development of viral diseases in gnotobiotic silkworm larvae infected either with NPV or with FV, but not CPV. The primary action of NA in *E. coli* is believed to be an inhibition of the synthesis of DNA.[6] From this point of view it is convincible that the administration of NA made it possible to protect gnotobiotic silkworm larvae from nuclear polyhedrosis, but not from cytoplasmic polyhedrosis, because the causal agents of the two viral diseases are substantiated to be DNA virus and RNA virus respectively. A question arises,

however, with respect to infectious flacherie in reference to the communication from Sudo and Kawase[12] (Faculty of Agriculture, Nagoya University, Nagoya) that the causal agent is probably a RNA virus.

Recently, several workers, including Temin and Mizutani[13] and Baltimore,[3] indicated the presence of an enzyme, RNA-dependent DNA polymerase, in some of the RNA tumour viruses, suggesting that synthesis of DNA may be involved in the process of synthesis of RNA in these viruses. If RNA-dependent DNA polymerase as well as DNA-dependent DNA polymerase are involved in the proliferation of FV, it may be comprehensible that the development of disease caused by the virus was inhibited by the administration of NA. These questions are to be solved in future.

The fact that the synergistic effect observed between the pathogenicity of FV and that of *S. faecalis-S. faecium* intermediate G–27 practically disappeared by the administration of the antibiotic alone, while lethality was not lowered so markedly by the administration of NA alone, is of much interest from the standpoint of the pathogenic mechanism of the synergism.

Summary

Chemical agents protecting gnotobiotic silkworm larvae from viral disease were investigated. Following results were obtained.

(1) The development of viral diseases caused by oral inoculation of infectious flacherie virus (FV) or nuclear polyhedrosis virus (NPV) was inhibited by the oral administration of nalidixic acid (NA), but that of the cytoplasmic polyhedrosis (CPV) was not inhibited.

(2) Using FV as the test pathogen,

a) More NA was required for inhibiting the development of disease with increasing concentrations of the viral suspension added to the diet. When very high concentrations of the suspension were used as the inoculum, the inhibition by NA was not achieved.

b) The development of disease under pathogenic synergism between FV and *S. faecalis-S. faecium* intermediate G–27, a strain of pathogenic streptococci, was inhibited by the combined administration of leucomycin and NA. In this experiment, the lethality was considerably lowered by the oral administration of the antibiotic alone, but was not greatly affected by the administration of NA alone.

c) Among NA and its related compounds, though small in number, only NA exhibited satisfactory inhibitory effect on the development of disease.

d) Satisfactory inhibition by NA was produced when this compound was administered both before and after the inoculation of the virus, and the inhibitory effect was greater with increasing duration of administration.

e) The amount of NA required for inhibiting the development of disease decreased by the addition of fructosazine to the diet.

References

1) Aruga, H., N. Yoshitake and M. Owada. 1963. Factors affecting the infection *per os* in the cytoplasmic polyhedrosis of the silkworm, *Bombyx mori* L. J. Sericult. Sci. Japan **32:** 41–50 (in Japanese).
2) Ayuzawa, C., Y. Furuta, R. Kodama and Y. Nakasuji. 1968. On the synergism between the viruses and the bacteria in the development of flacherie of the silkworm, *Bombyx mori* L. J. Sericult. Sci. Japan **37:** 395–402 (in Japanese).
3) Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature **226:** 1209–1211.
4) Deitz, W. H., J. H. Bailey and E. J. Froelich. 1963. *In vitro* antibacterial properties of nalidixic acid, a new drug active against gram-negative organisms. Antimicrobial agents and chemotherapy 1963 p. 583–587.
5) Ebihara T. and K. Iioka. Unpublished.
6) Goss, W. A., W. H. Deitz and T. M. Cook. 1965. Mechanism of action of nalidixic acid on *E. coli*. J. Bacteriol. **89:** 1068–1074.
7) Hayashiya, K., F. Matsubara, J. Nishida and H. Kushida. Personal communication.
8) Kodama, R. and Y. Nakasuji. 1969. Pathogenicity of bacteria for silkworm larvae reared aseptically on an artificial diet. Ann. Rep. Inst. Fermentation, Osaka No. 4 (1967–1968), p. 1–11.
9) Kodama, R. and Y. Nakasuji. 1970. Experiments to protect gnotobiotic silkworm larvae from bacterial diseases by oral administration of antibiotics. Res. Commun. (Ann. Rep. Inst. Fermentation, Osaka) No. 6 (1971–1972), p. x–y.
10) Matsubara, F., M. Kato, K. Hayashiya, R. Kodama and Y. Hamamura. 1967. Aseptic rearing of silkworm with prepared food. J. Sericult. Sci. Japan **36:** 39–45 (in Japanese).
11) Miyajima, S. and S. Kawase. 1965. The inhibitory effect of 5-fluorouracil on the outbreak of infectious flacherie in the silkworm, *Bombyx mori*. J. Sericult. Sci. Japan **34:** 359–365 (in Japanese).
12) Sudo, C. and S. Kawase. Personsl communication.
13) Temin, H.M. and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature **226:** 1211–1213.
14) Ueda, K., Y. Okimoto, H. Sakai and K. Arima. 1955. Antibiotic against silkworm jaundice virus, grasseriomycin, produced by Streptomyces species. J. Antibiotics, Ser. A **8:** 91–15.
15) Utsumi, S. and H. Chikushi. 1963. Studies on the inhibition of polyhedrosis in the silkworm, *Bombyx mori* L. (1) The effect of taurine and cystine on the incidence of polyhedrosis. J. Sericult. Sci. Japan **32:** 232–239 (in Japanese).

IFO Res. Comm. 6,
68–82, 1973 (*January*)

BACTERIA ISOLATED FROM SILKWORM AND THEIR PATHOGENICITY IN GNOTOBIOTIC SILKWORM LARVAE

Yugoro NAKASUJI, Akira KOBAYASHI and Reijiro KODAMA

Up to the present, many taxonomical and pathological studies have been made on bacteria isolated from silkworm larvae. In most of these studies, the examination of pathogenicity was carried out utilizing larvae fed with mulberry leaves. However, such larvae are not always suitable for the investigation of the pathogenicity of a special kind of pathogen. Recently, Kodama and Nakasuji[5–7] proposed that the use of gnotobiotic silkworm larvae affords an advantage for studies on bacterial pathogenicity.

In this paper, the results obtained in studies on the pathogenicity of bacterial isolates for aseptically reared silkworm larvae are presented.

Materials and Methods

I) Bacteria

The isolation of bacteria from silkworm larvae was made by the method previously reported.[6] Several cultures from Institute for Fermentation, Osaka, were also used in this study.

II) Silkworm

Healthy silkworm larvae in the 5th instar, reared aseptically on an artificial diet according to the method of Matsubara et al.,[11] were used.

III) Bacteriological tests

These were carried out by the methods described in the MANUAL FOR THE IDENTIFICATION OF MEDICAL BACTERIA[3] and IDENTIFICATION METHODS FOR MICROBIOLOGISTS.[4]

VI) Examination of pathogenicity

Inoculation with bacteria by feeding and by injection was conducted as described previously.[7]

Results

I) Taxonomical studies

Of 61 strains of bacteria isolated from diseased silkworm in several parts of Japan, 22 were classified as the families *Lactobacillaceae*, 14 as *Micrococcaceae*, 19 as *Enterobacteriaceae*, 2 as *Achromobacteraceae* and 4 as *Pseudomonadaceae*, as shown in Table 1.

Table 1. Strains isolated from silkworm larvae during 1962–1965 and their classification

Family	Genus	Species	Strain
Lactobacillaceae	*Streptococcus*	*S. faecalis-S. faecium* intermediate	
		group I	A–12*, A–33, A–217, I–111, I–221, K–106, MY–222
		group II	MY–121, MY–122, MY–221, G–51, ME–111, I–122
		S. lactis (Lister) Löhnis	MT–6, MT–7
		S. faecalis var. *liquefaciens* (Sternberg, emend. Orla-Jensen) Mattick	K–2, K–4, K–6, A–1, K–23, I–313, A–39
Micrococcaceae	*Micrococcus*	*M. freudenreichii* Guillebeau	A–43
		M. flavus Trevisan	I–1204
		M. candidus Cohn	MY–111, MY–112, MY–212, ME–11
		M. caseolyticus Evans	A–73, I–1109, I–2107, A–97, A–174
	Staphylococcus	*S. epidermidis* (Winslow et Winslow) Evans	MY–113, MY–114, MY–211
Enterobacteriaceae	*Proteus*	*P. vulgaris* Hauser	I–215
		P. morganii (Winslow et al.) Rauss	MT–3
		P. inconstans (Ornstein) Shaw et Clarke	A–142, A–223
	Aerobacter	*A. aerogenes* (Kruse) Beijerinck	A–122, A–14, A–121
		A. cloacae (Jordon) Bergey et al.	A_2–4, A–115, A–187, MT–1, MT–2, ME–12
	Serratia	*S. piscatorum* (Lehmann et Neumann) Breed	A–16, A–65, A–1214, A–1527
		S. marcescens Bizio (nonchromogenic)	A–21, A–190
Achromobacteraceae	*Alcaligenes*	*A. bookeri* (Ford) Bergey et al.	A–123
	Achromobacter	*A. superficialis* (Jordan) Bergey et al.	I–1102
Pseudomonadaceae	*Pseudomonas*	*P. ovalis* Chester	I–2106, I–1201, K–101, A–210

* The alphabetical markings indicate the names of places in which silkworm larvae were collected; prefectures of Kyoto (A), Gifu (G), Ibaragi (I), Saitama (K), Mie (ME), Nagano (MT) and Miyazaki (MY).

A) *Lactobacillaceae* (22 strains)

All strains were facultative anaerobes, gram-positive, non-sporeforming and non-motile. Growth on the surface of agar slants was slight to moderate and the optimum temperature was 30–37C. They were usually coccal or ovoid form and occurred singly, in pairs, or sometimes in short chains. They tolerated 40% bile, survived 60C for 30 minutes, did not produce indole, and were MR test-positive. They required calcium pantothenate, nicotinic acid, biotin and B_6-group vitamin essentially for growth. Thiamine, ascorbic acid, cyanocobalamin and p-aminobenzoic acid were not required. All fermented starch, dextrin, maltose, cellobiose, trehalose, glucose, fructose, mannose, galactose, salicin, sorbitol, mannitol, glycerol and esculin, and none fermented sorbose, arabinose, dulcitol and adonitol. They varied with respect to the fermentability of inulin, raffinose, melezitose, sucrose, lactose, melibiose, rhamnose, xylose, ribose, α-methylglucoside and inositol.

Over 83% of the glucose fermented was converted to lactic acid. Among the 22 strains tested, 16 strains produced dextro lactic acid and 6 strains (*S. faecalis* var. *liquefaciens*) produced inactive lactic acid from glucose. The other variable characteristics are shown in Table 2.

These strains were classified as the genus *Streptococcus*. Of 22 strains, 13 were identified as *S. faecalis-S. faecium* intermediate, 2 as *S. lactis* and 7 as *S. faecalis* var. *liquefaciens*. At least two groups were observed among *S. faecalis-S. faecium* intermediate strains. These groups differ from one another in the following properties: reduction of nitrate at pH 9.65, tyrosine decarboxylation, utilization of arginine as energy source, coagulation of milk, and fermentability of rhamnose and α-methylglucoside.

B) *Micrococcaceae* (14 strains)

The strains grew on nutrient agar slant, were gram-positive cocci, non-sporeforming, catalase-positive and VP test-negative. They produced ammonia from peptone, did not produce indole, did not utilize urea as sole nitrogen source for growth, and did not hydrolyze urea and starch. They did not produce acid from raffinose and inositol. They varied with respect to the production of acid from dextrin, maltose, sucrose, lactose, cellobiose, glucose, galactose, rhamnose, arabinose, xylose, salicin, α-methylglucoside, mannitol and glycerol. The other variable characteristics are shown in Table 3.

Of 14 strains, 11 were classified as the genus *Micrococcus* and 3 as the genus *Staphylococcus*.

(1) *Micrococcus*; Of 11 strains, 1 was identified as *M. freudenreichii*, 1 as *M. flavus*, 4 as *M. candidus* and 5 as *M. caseolyticus* in Bergey's Manual.[1]

(2) *Staphylococcus*; Three strains were identified as *S. epidermidis* in Bergey's Manual.

Table 2. Characteristics of the strains belonging to *Streptococcus*

species / characteristics	*S. faecalis-S. faecium* intermediate group I (7 strains)	*S. faecalis-S. faecium* intermediate group II (6 strains)	*S. lactis* (2 strains)	*S. faecalis* var. *liquefacience* (7 strains)
Gelatin liquefaction	−	−	−	+
VP test	+	2/6*±, 4/6+	+	+
Reduction of nitrate				
pH 6.75	−	−	−	−
pH 9.65***	−	5/6+, 1/6−	−	−
Reduction of tetrazolium	6/7+, 1/7±	+	+	+
Production of				
catalase	−	−	−	1/7±, 6/7−
ammonia from pepton	+	1/6+, 5/6±	+	+
Hydrolysis of sodium hippurate	−	−	−	+
Litmus milk				
reduction of litmus (8hrs.)	+	+	+	+
acid production	+	+	+	+
coagulation	−	5/6+, 1/6−	−	+
peptonization	−	−	−	+
Growth				
at 10C	+	+	+	+
at 45C	−	1/6+, 5/6−	−	+
in 6.5% Nacl broth	6/7+, 1/7−	+	−	+
in pH 9.6 broth	+	+	−	+
in 0.1% methylene blue milk	+	+	+	+
in 0.3% methylene blue milk	1/7+, 6/7−	3/6+, 3/6−	−	+
0.04% potassium tellurite tolerance	4/7+, 2/7±, 1/7−	+	1/2+, 1/2−	+
Tyrosine decarboxylation	+	−	−	+
Energy utilization				
pyruvate	−	−	−	+
arginine	+	−	1/2+, 1/2−	+
citrate	−	−	−	1/7+, 6/7−
serine	−	−	−	+
gluconate	−	4/6+, 2/6−	+	+
malate	−	4/6+, 2/6−	−	+
Vitamin requirement				
riboflavin	N	N	E	6/7E, 1/7N
folic acid	E	E	N	N
Haemolysis on cows blood agar	2/7 nonhaemolytic 5/7 gamma haemolytic (indifferent)	nonhaemolytic	nonhaemolytic	nonhaemolytic
Reaction with group D serum	+	5/6+, 1/6±	−	5/7+, 2/7±
Maximum pH for the growth	10.49–11.15**	10.40–11.50	9.20–9.45	10.00–10.71

+; positive, ±; weak positive, −; negative
E; essentially required, N; not required.
*; Number of cultures which gave each reaction/number of cultures tested.
**; Grew in pH 10.49 broth, but not in pH 11.15 broth.
***; pH values adjusted aseptically to 9.65 just before inoculation.

Table 3. Characteristics of the strains belonging to *Micrococcus* and *Staphylococcus*

species / haracteristics	*M. freudenreichii* (1 strain)	*M. caseolyticus* (5 strains)	*M. candidus* (4 strains)	*M. flavus* (1 strain)	*S. epidermidis* (3 strains)
Size (μ)	0.8–1.0 ×0.8–1.0	0.6–1.0 ×0.6–1.0	0.5–1.0 ×0.5–1.0	0.6–1.0 ×0.6–1.0	0.5–1.0 ×0.5–1.0
Motility	−	−	−	+	−
(position of flagella)				(polar)	
O–F test	oxidation	oxidation	no action	no action	fermentztion
Gelatin liquefaction	+	+	−	+	+
MR test	−	−	−	−	+
Utilization of					
sodium citrate	+	+	−	−	−
$NH_4H_2PO_4$	+	+	−	−	−
Production of					
cytochrome oxidase	−	−	−	−	1/3*+, 2/3±
phosphatase	+	+	−	±	+
hydrogen sulfide	−	−	−	+	−
Hydrolysis of					
esculin	−	−	−	+	+
sodium hippurate	−	−	−	−	NT
Reduction of nitrate	−	−	−	−	+
Limus milk					
reduction of litmus	+	+	3/4+, 1/4−	+	+
acid production	+	+	2/4+, 2/4−	+	+
coagulation	−	+	−	+	+
peptonization	−	+	−	+	+
Haemolysis on cows blood agar	β	β	no change	no change	no change
Novobiocin resistance (γ/ml)	>80	>80	2/4 2.5–5.0 1/4 30–40 1/4 >80	5–10	40–50
Growth					
at 10C	+	+	+	+	+
at 45C	−	−	−	−	1/3+, 2/3−
in broth containing					
4% NaCl	+	+	+	+	+
6% NaCl	+	+	3/4+, 1/4−	+	+
8% NaCl	−	−	3/4+, 1/4−	−	+
10% NaCl	−	−	1/4+, 3/4−	−	+
Optimum temperature (C)	30–37	25–37	25–40	25–30	37–45
Maximum pH for the growth	9.43–9.80**	9.43–9.80	9.43–10.45	9.43–9.80	9.80–10.45

+; positive, ±; weak positive, −; negative, NT; not tested.
*; Number of cultures which gave each reaction/number of cultures tested.
**; Grew in pH 9.43 broth, but not in pH 9.80 broth.

C) *Enterobacteriaceae* (19 strains)

All strains were gram-negative rods, catalase-positive, cytochrome oxidase- and protopectinase-negative, and reduced nitrate to nitrite. They metabolized glucose fermentatively in Hugh and Leifson medium. The other variable characteristics are shown in Table 4.

Of 19 strains, 4 were classified as the genus *Proteus*, 9 as the genus *Aerobacter* and 6 as the genus *Serratia*.

(1) *Proteus*; All strains decarboxylated leucine and valine, grew in KCN broth, and reduced trimethylamine oxide. They fermented glucose, fructose, galactose and glycerol, and did not ferment dextrin, raffinose, lactose, arabinose, dulcitol and sorbitol. They varied with respect to the fermentability of maltose, sucrose, mannose, xylose, salicin, mannitiol and inositol.

Of 4 strains, 1 was identified as *P. vulgaris*, 1 as *P. morganii* and 2 as *P. inconstans* in Bergey's Manual.

(2) *Aerobacter*; All strains were uncapsulated, produced ammonia from peptone, utilized uric acid, and did not decompose alginic acid. They fermented dextrin, raffinose, maltose, sucrose, lactose, cellobiose, trehalose, glucose, fructose, mannose, galactose, rhamnose, arabinose, xylose, mannitol and glycerol. They varied with respect to the fermentability of starch, inulin, sorbose, salicin, α-methylglucoside, dulcitol, sorbitol and esculin.

Of 9 strains, 3 were identified as *A. aerogenes* and 6 as *A. cloacae* in Bergey's Manual.

(3) *Serratia*; All strains fermented dextrin, maltose, sucrose, trehalose, glucose, fructose, mannose, galactose, salicin, inositol, mannitol, sorbitol and glycerol. They did not ferment starch, inulin, raffinose, lactose, sorbose, rhamnose, arabinose, xylose, α-methylglucoside and dulcitol.

Of 6 strains, 2 were identified as *S. marcescens* (nonchromogenic) and 4 as *S. piscatorum* in Bergey's Manual.

D) *Achromobacteraceae* (2 strains) and *Pseudomonadaceae* (4 strains)

All strains were gram-negative rods, non-sporeforming, non-haemolytic on bovine blood agar, and did not grow at 40C. They produced catalase, did not produce pigments and indole, and did not decompose alginic acid. Both MR test and VP test were negative. No acid and no gas were produced from lactose, sucrose, maltose, cellobiose, raffinose, starch, dextrin, inulin, mannitol, sorbitol, dulcitol and α-methylglucoside. They varied with respect to the production of acid from glucose, fructose, mannose, galactose, rhamnose, arabinose, xylose, salicin, inositol and glycerol. The other variable characteristics are shown in Table 5.

Of 6 strains, 1 was identified as *Alcaligenes bookeri*, 1 as *Achromobacter superficialis* and 4 as *Pseudomonas ovalis* in Bergey's Manual.

Table 4. Characteristics of the strains belonging to *Aerobacter*, *Proteus* and *Serratia*

characteristics \ species	*A. aerogenes* (3 strains)	*A. cloacae* (6 strains)	*P. vulgaris* (1 strain)	*P. morganii* (1 strain)	*P. inconstans* (2 strains)	*S. marcescens* (nonchromogenic) (2 strains)	*S. piscatorum* (4 strains)
Pleomorphic in young culture	−	−	+	+	+	−	−
Swarming	−	−	+	−	−	−	−
Motility	−	+	+	+	+	+	+
(position of flagella)		(peritrichous)	(peritrichous)	(peritrichous)	(peritrichous)	(leteral)	(leteral)
Gelatin liquefaction	1/3*+, 2/3−	+	+	−	−	+	+
MR test	−	−	+	+	+	−	1/4±, 3/4−
VP test	+	+	−	−	−	+	+
Utilization of sodium citrate	+	+	−	−	+	+	+
Production of pigment	−	−	−	−	−	−	rose (water soluble)
urease	−	−	+	+	−	+	+
hydrogen sulfide	1/3+, 2/3−	−	+	+	+	−	1/4+, 3/4−
indol	2/3+, 1/3−	−	+	+	+	−	−
Litmus milk							
reduction of litmus	+	5/6+, 1/6±	−	−	−	+	+
acid production	+	+	+	−	+	+	+
coagulation	+	+	+	−	−	+	+
peptonization	−	+	+	−	−	+	+
Deamination of phenylalanine	−	−	+	+	+	−	−
Hydrolysis of sodium hippurate	2/3+, 1/3±	1/6±, 5/6−	NT	NT	NT	NT	NT
Vitamin requirement							
nicotinic acid	NT	NT	E	E	NE	NT	NT
pantothenic acid	NT	NT	NE	E	NE	NT	NT
Otpimum temperature (C)	30–37	30–37	25–30	25–37	25–37	28	28
Maximum pH for the growth	9.11–9.45**	8.84–9.45	8.52–8.81	8.81–9.24	8.81–9.24	9.26–9.40	9.14–9.40

+; positive, ±; weak positive, −; negative, NT; not tested.
E; essentially required, NE; not required.
*; Number of cultures which gave each reaction/ number of cultures tested.
**; Grew in pH 9.11 broth, but not in pH 9.45 broth.

Table 5. Characteristics of the strains belonging to *Alcaligenes*, *Achromobacter* and *Pseudomonas*

characteristics \ species	*Alcaligenes bookeri* (1 strain)	*Achromobacter superficialis* (1 strain)	*Pseudomonas ovalis* (4 strains)
Size (μ)	0.3–0.6×0.5–1.0	0.6–0.9×0.6–0.9	0.6–0.8×0.7–1.0
Motility	+	+	+
O–F test	no action	no action	oxidation
Gelatin liquefaction	+	—	—
Reduction of nitrate	+	—	+
Utilization of sodium citrate	±	—	1/4* +, 2/4±, 1/4—
Production of			
cytochrome oxidase	±	—	+
urease	—	—	+
hydrogen sulfide	+	—	—
Litmus milk			
reduction of litmus	—	—	—
acid production	—	+	—
alkali production	+	—	+
coagulation	+	+	—
peptonization	+	—	—
Growth			
on Holding's inorganic nitrogen medium	—	—	2/4+, 1/4±, 1/4—
in broth containing			
4% NaCl	+	+	+
6% NaCl	+	+	+
8% NaCl	—	+	—
Decomposition of arginine	—	+	+
Optimum temperature (C)	25–37	25–30	30–37
Maximum pH for the growth	9.30–10.00**	9.30–10.00	9.06–10.00

+; positive, ±; weak positive, —; negative.
*; Number of cultures which gave each reaction/number of cultures tested.
**; Grew in pH 9.30 broth, but not in pH 10.00 broth.

II) Pathological studies

The examination of pathogenicity of the above strains and ten strains maintained in the IFO Culture Collection for aseptically reared silkworm larvae were carried out.

A) *Lactobacillaceae* infections

The results of the examination of pathogenicity are summarised in Table 6. As is evident from this table, the strains used were divided into pathogenic and non-pathogenic groups and furthermore, there were two patterns—an intestinal disease and a septicemic disease—in the pathogenic strains according to the pathogenic effects.

The strains producing an intestinal disease exhibited pathogenicity only by feeding. The symptoms of this disease were the same as described previously,[6,7] i.e., the larvae suffering from the intestinal disease lost appetite, became sluggish in movement and showed diarrhoea. On the other hand, the strains producing a septicemic disease exhibited little or no pathogenicity by feeding, but exhibited marked pathogenicity by injection. The larvae injected with the strains producing a septicemic disease became paralyzed during 2 to 4 days after injection. Soon after death, the body became dark brown or nearly black in color, becoming soft, but the body could be removed without disrupting the body wall.

A trial was made to find a correlation between the bacteriological characters of the strains and their pathogenicity. A conclusion can not be drawn due to the insufficient number of strains used, but it is noteworthy that the strains producing an intestinal disease had an alkali-resistant property, and were unable to liquefy gelatin, and the strains producing a septicemic disease were able to liquefy gelatin and the maximum pH value for growth was relatively lower than that of the strains producing an intestinal disease.

B) *Micrococcaceae* infections

Among 24 strains tested, 1 strain of *M. flavus* and 2 strains of *M. candidus* caused septicemia in 3 days after injection. After death, the body became dark brown in color and softened a little. The other strains exhibited no pathogenicity either by feeding or by injection within the limits of this experiment (Table 7).

C) *Enterobacteriaceae*, *Achromobacteraceae* and *Pseudomonadaceae* infections

The results of the examination of pathogenicity are summarised in Table 8. As is evident from this table, *S. piscatorum*, *S. marcescens* (nonchromogenic), *P. vulgaris*, *P. morganii* and *P. inconstans* exhibited pathogenicity both by feeding and by injection, especially showing 100% lethality by injection. The larvae infected with these strains fell in a dead within 12 hrs after injection, and after the death, the body changed rapidly to a brown or black color. It became very soft and could not be removed without disrupting the body wall.

One strain of *Aerobacter aerogenes* and 5 strains of *Aerobacter cloacae* exhibited pathogenic effects only by feeding. In this case, infected larvae became sluggish in movement. The dead bodies softened slightly. *Achromobacter superficialis* exhibited pathogenic effects only by injection. The infected larvae became paralyzed in 3 days after injection, and the dead larvae became somewhat soft. *Pseudomonas ovalis* and *Alcaligenes bookeri* had little or no pathogenic effect.

Table 6. Pathogenicity of the strains belonging to *Streptococcus*

Strains tested			Infection by feeding*														Infection by injection**		
	characteristics			Number of dead silkworms															
				on indicated day after inoculation															
species	gelatin liquefaction	maximum pH values for the growth	Viable cells added to one gm of a diet	1	2	3	4	5	6	7	8	9	10	11	in cocoon	lethality	Viable cells injected per larvae	Lethality	Pattern of pathogenic effect
S. faecalis -*S. faecium* intermediate			$\times 10^3$													%	$\times 10^5$	%	
group I																			
A-12			0.2	1	2			1	3	1	1		1			100	6.7	0	
A-33			0.3				1	5		2		2				100	4.2	0	
A-217			0.2							1			2			30	4.4	0	
I-111		10.49	0.2		2				1	2	1	1	1	2		100	4.6	0	
I-221	−	~	0.2						1	2		3	1	3		100	6.1	0	intestinal disease
K-106		11.15	0.2		1		2		1		2	1	1	2		100	5.7	0	
MY-222			0.2						2							20	1.5	0	
group II																			
MY-121			0.2						1	3	1	1	2			80	7.0	0	
MY-122			0.2						1	1	1	2				50	2.9	0	
MY-221			0.2							2	3					50	4.7	0	
ME-111			0.2						1	1	5	1				80	3.8	0	
G-51			0.1								1					10	5.7	0	
I-112			0.2										2	1		30	5.0	0	
S. faecalis var. *liquefaciens*																	$\times 10^3$		
K-2		10.00	9.4													0	1.5	100	
K-23	+	~	8.0									1				10	0.7	100	septicemic disease
A-1		10.71	1.5												1	10	1.5	80	
A-39			3.6							1					1	20	0.9	80	
I-313			3.8						1							10	1.7	100	
K-4			2.1												1	10	1.7	80	
K-6			9.8												2	20	1.9	80	
S. lactis		9.20															$\times 10^5$		
MT-6	−	~	0.1													0	3.3	0	
MT-7		9.45	0.2													0	2.0	0	
control			0.85% saline***													0	0.85% saline***	0	
																	no injection	0	

*; Larvae were infected with bacteria on the 2nd or 3rd day of the 5th instar.
**; Larvae were infected with bacteria on the 4th day of the 5th instar.
Lethality was calculated on the basis of the number of dead silkworms within 4 days after injection.
***; The bacterial suspension was replaced by the same amount of a sterile 0.85% saline.

Table 7. Pathogenicity of the strains belonging to *Micrococcus* and *Staphylococcus*

Strains tested	Infection by feeding*		Infection by injection**	
	viable cells added to one gm of a diet	Lethality	viable cells injected per larva	Lethality
Micrococcus	$\times 10^6$	%	$\times 10^3$	%
M. aurantiacus IFO 12422	3.3	0	5.1	0
M. candidus MY–111	2.1	0	2.3	0
MY–112	1.2	0	4.0	80
MY–212	1.6	0	4.1	100
ME–11	1.4	0	2.0	0
M. caseolyticus A–73	2.3	0	3.3	0
A–97	1.1	0	3.3	0
A–174	1.7	0	4.3	0
I–1109	2.1	0	5.0	0
I–2107	2.1	0	8.1	0
M. flavus I–1204	1.0	0	3.0	60
IFO 3242	2.8	0	4.4	0
M. freudenreichii A–43	2.6	0	5.0	0
M. luteus IFO 3763	2.4	0	4.4	0
M. roseus IFO 3764	3.1	0	3.9	0
M. rubens IFO 3768	4.2	0	5.3	0
M. varians IFO 3765	3.8	0	4.5	0
Staphylococcus				
S. afermentans IFO 3333	1.2	0	3.9	0
S. aureus IFO 3061	3.1	0	5.3	0
IFO 3183	2.3	0	6.3	0
S. epidermidis MY–113	1.1	0	2.8	0
MY–114	2.4	0	1.6	0
MY–211	1.3	0	1.4	0
IFO 12993	5.1	0	4.8	0
Control	0.85% saline***	0	0.85% saline***	0
			no injection	0

*; Larvae were infected with bacteria on the 2nd or 3rd day of the 5th instar.
**; Larvae were infected with bacteria on the 4th day of the 5th instar. Lethality was calculated on the basis of the number of dead silkworms within 3 days after injection.
***; The bacterial suspension was replaced by the same amount of a sterile 0.85% saline.

Table 8. Pathogenicity of the strains belonging to *Serratia*, *Proteus*, *Aerobacter*, *Alcaligenes*, *Achromobacter* and *Pseudomonas*

Strains tested	Viable cells added to one gm of a diet	Infection by feeding*											Infection by injection**	
		Number of dead silkworms										Lethality	Vaible cells injected per larva	Lethality
		on indicated day after inoculation									in cocoon			
		2	3	4	5	6	7	8	9	10				
Serratia	$\times 10^5$											%	$\times 10^3$	%
S. piscatorum A–16	3.8										2	20	3.2	100
A–65	4.6			1	1						2	40	4.0	100
A–1214	6.0	1	1									20	3.0	100
A–1527	4.6		2								3	50	2.9	100
S. marcescens (nonchromogenic) A–21	4.4					2	1				1	40	3.2	100
A–190	3.6								1	1	2	40	4.3	100
Proteus														
P. vulgaris I–215	5.4	1		1	1		2	2			3	100	2.1	100
P. morganii MT–3	5.6										3	30	4.0	100
P. inconstans A–142	6.4										2	20	2.2	100
A–223	6.8										1	10	3.7	100
Aerobacter														
A. aerogenes A–14	4.8											0	3.2	0
A–122	4.4				1						1	20	1.6	0
A–121	2.6											0	2.0	0
A. cloacae A_2–4	3.6										1	10	2.7	0
A–115	3.2										1	10	2.2	0
A–187	3.4										2	20	3.5	0
MT–1	3.0											0	2.7	0
MT–2	4.2										2	20	2.0	0
ME–12	4.6			1	1	2					1	50	1.9	0
Alcaligenes														
A. bookeri A–123	2.2											0	2.6	0
Achromobacter														
A. superficialis I–1102	4.0											0	2.8	60
Pseudomonas														
P. ovalis A–210	4.2											0	1.5	0
K–101	3.0											0	1.8	0
I–1201	2.4										1	10	1.7	0
I–2106	2.8										1	10	4.3	0
Control	0.85% saline***											0	0.85% saline***	0
													no injection	0

*; Larvae were infected with bacteria on the 2nd or 3rd day of the 5th instar.

**; Larvae were infected with bacteria on the 4th day of 5th instar.
Lethality was calculated on the basis of the number of dead silkworms within 3 days after injection.

***; The bacterial suspension was replaced by the same amount of a sterile 0.85% saline.

Discussion

The results given in this paper emphasize that there were two patterns—an intestinal disease and a septicemic disease—in the pathogenic effects exhibited by the pathogenic streptococci, and that, within the limits of this experiment, there was a clear relationship between the physiological properties on the liquefaction of gelatin and each of these pathogenic patterns. From these facts, it is suggested that among the physiological properties of strains belonging to the genus *Streptococcus*, both the ability to liquefy gelatin and the alkali-resistant character[6,7] are the representatives from the point of pathological view.

The streptococci causing an intestinal disease are included into a group of primary invader, as described previously.[8,9] On the other hand, the streptococci causing a septicemic disease are included into a group of secondary invader, but it is regarded that when increased amounts of viable cells are inoculated by feeding, they may play a role of primary invader and then cause septicemia. It was pointed out that strains belonging not only to *Serratia* but also to *Proteus*, *Pseudomonas aeruginosa*,[7] *Aeromonas hydrophila*[7] and *Flavobacterium aquatile*[7] caused a lethal septicemia in silkworm larvae reared aseptically on an artificial diet. It is regarded that these gram-negative bacteria causing a septicemic disease play a role of secondary invader. Most strains belonging to *Aerobacter*, *Alcaligenes*, *Achromobacter*, *Pseudomonas*, *Micrococcus* and *Stahylococcus* had little or no pathogenic effect either by feeding or by injection. It is regarded that these non-pathogenic strains and these pathogenic strains of doubtful status do not play any important role in bacterial disease of the silkworm.

It was found that the streptococci causing an intestinal disease were not a member of any of the four pathogenic bacterial groups classified by Bucher,[2] because they do not require the specialized condition for growth, are non-sporeforming bacteria, and have little or no ability to multiply in the haemocoele.[9]

Among the bacteria tested, the strains producing a septicemic disease are included in a group of potential pathogens described by Bucher,[2] because they do not require the specialized conditions for growth, produce septicemia by injection of doses of less than 10^4 cells, and usually do not cause disease by feeding in small doses of bacterial cells. In the case of the pathogenicity for silkworm larvae reared aseptically on an artificial diet, however, some strains belonging to the genera *Serratia* and *Proteus* exhibited a considerable pathogenic effect either by feeding or by injection, and therefore, it is reasonable that they are included in a group of facultative pathogens described by Bucher.[2]

Within the limits of the test on the pathogenicity of non-sporulating bacteria for silkworm larvae reared on an artificial diet, pathogenic bacteria can be divided into the following two groups:

1) Bacterial group causing intestinal disease; This group includes bacterial strains that multiply in the gut from small inocula and cause an intestinal disease of chronic character, but do not multiply significantly in the haemocoele and do not cause

a septicemic disease. They play an inductive role for the strains of various species of gram-negative and gram-positive bacteria functioning as the secondary invaders or potential pathogens.[7-9]

2) Bacterial group causing septicemic disease; This group includes bacterial strains that multiply in the haemocoele and cause a septicemic disease. They do not kill larvae when small numbers of viable cells are inoculated by feeding, but exhibits a pathogenic effect when increased amounts of viable cells are inoculated by feeding.

According to Takizawa and Iizuka,[12] some species of *Staphylococcus* are members of bacterial flora of the gut. They, however, did not mention about the ability of the bacteria to grow in the gut. From the results in the present paper, it is regarded that staphylococci do not grow in the gut, because they do not cause a disease by feeding, and the maximum pH values for their growth were considerably lower than that of the gut. It is a subject for a future study to determine whether staphylococci are a member of the bacterial flora of the gut.

Langston and Williams[10] showed that two strains of streptococci isolated from silage were able to reduce nitrate, and that optimal reduction of nitrate occurred at pH 8.0 and the pH range varied from 6.8 to 9.2. The optimum pH for the nitrate-reducing streptococci isolated from silkworm larvae was between 9 to 9.7 and the pH range varied from 8 to 10. These results proved that the reduction of nitrate by streptococci is related to pH values of the medium, and emphasize that the test should be carried out not only at pH 7.0 but also at pH 8.0–9.7.

Summary

Taxonomical and pathological studies were carried out on bacteria isolated from silkworm larvae. The results obtained were;

I) Sixty-one strains isolated from diseased larvae were identified as the following species taxonomically; thirteen as *Streptococcus faecalis-Streptococcus faecium* intermediate (classified into two groups), seven as *Streptococcus faecalis* var. *liquefaciens* (Sternberg, emend. Orla-Jansen) Mattick, two as *Streptococcus lactis* (Lister) Löhnis, one as *Micrococcus freudenreichii* Guillebeau, one as *Micrococcus flavus* Trevisan, four as *Micrococcus candidus* Cohn, five as *Micrococcus caseolyticus* Evans, three as *Staphylococcus epidermidis* (Winslow et Winslow) Evans, one as *Proteus vulgaris* Hauser, one as *Proteus morganii* (Winslow et al.) Rauss, two as *Proteus inconstans* (Ornstein) Shaw et Clarke, three as *Aerobacter aerogenes* (Kruse) Beijerinck, six as *Aerobacter cloacae* (Jordon) Bergey et al., two as *Serratia marcescens* Bizio (nonchromogenic), four as *Serratia piscatorum* (Lehmann et Neumann) Breed, one as *Alcaligenes bookeri* (Ford) Bergey et al., one as *Achromobacter superficialis* (Jordan) Bergey et al. and four as *Pseudomonas ovalis* Chester.

II) The pathogenicity of each strain for healthy silkworm larvae in the 5th instar, which were reared aseptically on an artificial diet, was tested by feeding and by injection.

A) It was found that there were two patterns—an intestinal disease and a septicemic disease—in the pathogenic effect exhibited by pathogenic streptococci.

B) *Serratia marcescens* (nonchromogenic), *Serratia piscatorum*, *Proteus vulgaris*, *Proteus morganii* and *Proteus inconstans* caused a lethal septicemia both by feeding and by injection.

C) One strain of *Achromobacter superficialis*, two of four of *Micrococcus candidus* and one of two of *Micrococcus flavus* caused septicemia only by injection. The other strains of *Micrococcus* exhibited no pathogenic effects.

D) Most strains of *Staphylococcus*, *Aerobacter* and *Pseudomonas* had little or no pathogenic effect either by feeding or by injection, within the limits of the investigation.

III) A correlation between the bacteriological characters of the pathogenic streptococci and their pathogenicity was discussed.

References

1) Breed, R.S., E.G.C. Murray, N.R. Smith, and ninety-four contributors 1957: Bergey's Manual of Determinative Bacteriology, 7th Ed. (Baltimore).
2) Bucher, G.E. 1960: Potential bacterial pathogens of insects and their characteristics. J. Insect Pathol. **2**: 172–195.
3) Cowan, S.T. and K.J. Steel 1965: Manual for the identification of medical bacteria. Cambridge university press (London).
4) Gibbs, B.M. and F.A. Skinner 1966: Idendification methods for microbiologists. Academic press (London).
5) Kodama, R. and Y. Nakasuji 1967: Application of gnotobiotic silkworm larvae to studies on pathogenicity of bacteria for the insects. Advance in germfree research and gnotobiology p. 417–423. edited by M. Miyakawa and T.D. Luckey, CRC Press (Ohio).
6) Kodama, R. and Y. Nakasuji 1968: Bacteria isolated from silkworm larvae. I. The pathogenic effects of two isolates on aseptically reared silkworm larvae. J. Sericult. Sci. Japan, **37**: 477–482. (Japanese with English abstract).
7) Kodama, R. and Y. Nakasuji 1969: Pathogenicity of bacteria for silkworm larvae reared aseptically on an artificial diet. Annual Report of the Institute for Fermentation, Osaka, No. 4, p. 1–11.
8) Kodama, R. and Y. Nakasuji 1969: Bacteria isolated from silkworm larvae. IV. A study on the pathogenic mechanism of bacterial diseases in aseptically reared silkworm larvae. J. Sericult. Sci. Japan, **38**: 406–412. (Japanese with English abstract).
9) Kodama, R. and Y. Nakasuji 1971: Further studies on the pathogenic mechanism of bacterial disease in gnotobiotic silkworm larvae. Institute for Fermentation, Osaka, Research Communications No. 5, p. 1–9.
10) Langston, C.W. and P.P. Williams 1962: Reduction of nitrate by streptococci. J. Bact. **84**: 603.
11) Matsubara, F., M. Kato, K. Hayashiya, R. Kodama and Y. Hamamura 1967: Aseptic rearing of silkworm with prepared food. J. Sericult. Sci. Japan **36**: 39–45. (Japanese with English abstract).
12) Takizawa, Y. and T. Iizuka 1968: The aerobic bacterial flora in the gut of larvae of the silkworm, *Bombyx mori* L. (1) The relation between media and the numbers of living cells. J. Sericult, Sci. Japan, **37**: 295–305. (Japanese with English abstract).

IFO Res. Comm. **6**,
83–94, 1973 (*January*)

DESCRIPTIVE CATALOGUE OF I.F.O. CULTURE COLLECTION

FUNGUS COLLECTION III.

Following to the previous papers in this series (1969, 1971), the present eleven interesting organisms have been added to our collection, all of which have been not yet or insufficiently recorded from Japan. Descriptions of them are given with explanatory notes. Unless otherwise stated, the dried materials were deposited in the Herbarium of the Institute for Fermentation. New taxa will be described in other mycological periodicals.

(K. Tubaki)

13. **Backusella circina** Ellis & Hesseltine (Pl. 1 A-C) Mucorales
Mycologia **61**: 863 (1969).

Growth on malt agar rapid filling a petri dish in 4–5 days at 25°, pale gray to olive gray; reverse light gray. Sporangiophores circinate when young, erect in mature, hyaline, at first unbranched, later sporangiolar branches arise, 4–12μ wide, tapering to 4–5μ just below sporangia. Sporangia globose, 60–80μ in diam., smokey gray, with deliquescent and glistening wall; columellae short oval, 11–24$\times$11–23μ, smooth walled, hyaline with granular contents, collar indistinct. Sporangiospores subglobose, short oval or irregular in shape, 7–12(—14)$\times$6–10μ, smooth walled. Conidia borne singly on circinate branches, globose, 13–15μ in diam., thick walled, echinulate markedly. Sporangioles also present, arise in same manner as conidia, containing several sporangiospores, up to 30μ in diam.

Hab.: Isolated from the embedded sterilized leaves of *Castanopsis cuspidata* Schottky in the litter of forest, Ishiyama-Hiratsu Machi, Shiga Pref., Nov. 11, 1969 (A–1–1).

Strain preserved: IFO–9231 Isol. K. Tubaki
Det. J.J. Ellis

The present fungus was isolated from the decaying leaves as described (in this journal, 5, 26, 1971). Because the morphological characteristics of it agrees closely in many respects with the description given by Ellis & Hesseltine (1969) at that time, the strain was sent to Dr. C. W. Hesseltine, Northern Utilization Research and Development Division, Peoria, for the identification. Then according to the personal communication from Dr. J. J. Ellis, zygospores developed when the present isolate was contrasted with *B. circina* NRRL 3293(—). Therefore, the present isolate has a (+) mating reaction.

14. **Brachysporiella gayana** Batista (Pl. 1 D) Hyphomycetes

Bolm. Secr. Agric. Ind. Com. Ext. Pernambuco, **19:** 109 (1952); Ellis, Mycol. Pap. **72:** 15 (1959).

Conidiophore on natural substrate arise singly, thick walled, stout, erect, straight or slightly flexuous, simple or bearing short branches near apex, dark reddish brown, paler above, 140–230μ long, 8–10μ wide at base, 3–4μ at apex. Conidia develop terminally as blown-out ends of conidiophore and at tip of each short branch, obovoid or pyriform, truncate at base, pale to dark brown, thick and smooth walled, usually 3-septate, apical cell larger and basal cell often collapse, 24–28(—30)$\times$11–14μ, usually containing a large circular guttule; conidiophore or short branch proliferate through scar after first conidia has fallen and forms another conidia; conidiogenous short branches flask-shaped, 9–11$\times$4–6μ, remain attached to conidiophore in a chain or may adhere to fallen conidia.

Growth on malt agar moderate, restrict, convex, velutinous, dark olive to dark olive gray; reverse almost black.

Hab.: On rotten wood, Daimonji, Kyoto, Sept. 24, 1972.

Strain preserved: IFO–9632 Coll. T. Ito
Det. K. Tubaki

The present fungus is characteristic in development of the conidia on short branches of the conidiophores. In 1958, Subramanian erected a new genus of hyphomycete, *Edmundmassaria*, based on *E. pulchra* Sub. (J. Ind. Bot. Soc. **37**, 401, 1958) and in 1964, Rao & Rao added a second species of the genus, *E. bulbosa* Rao & Rao (Mycopath. Mycol. Appl. **22**, 242, 1964). Because the description and the drawing of *E. pulchra* given by Subramanian are very close to those of *Brachysporiella*-species, the genus *Edmundmassaria* probably is congeneric with the present genus.

15. **Brachysporium nigrum** (Link) Hughes (Pl. 1 E-F) Hyphomycetes

Hughes, in Can. J. Bot., **36:** 742 (1958).

Conidiophores on natural substrate erect from vegetative hyphae, straight or often flexuous, dark brown except for apical cells, up to 220μ long, commonly swollen at base to 8–11μ, 7–8μ wide just above the basal swelling, tapering to 3–4.5μ at hyaline apical cell which bears thorn remains of hyaline pedicels after conidia liberate. Conidia 3–septate, elliptical, 20–23$\times$10–11μ, two middle cells brown, end cells more or less equal, hyaline or very pale colored.

Growth on malt agar very reduced, slow, restrict, pale cream to brown; reverse same colored.

Hab.: On rotten wood (*Quercus* sp. ?), Mt. Katsuragi (Yamato-Katsuragi), Gosho, Nara Pref., June 11, 1972 (198–1).

Strain preserved: IFO–9588 Coll. K. Tubaki
Det. K. Tubaki

Although this species is very common in Europe and Canada, no record has been appoered in Japan. Conidia of the present fungus are somewhat longer than that given in the literature (17–20×9–10μ) and, in this respect, it approaches to *B. dingleyae* Hughes (N.Z.J. Bot. **3**: 27, 1964). Because, however, the end cells of ellipsoid conidia in this collection are more or less equal that is a charactristic in *B. nigrum*, the present identification was made.

16. **Diheterospora catenulata** Kamyschko (Pl. 2 A-B) Hyphomycetes
Bot. Matr. **15**: 140 (1962); Barron & Onions, Cand. J. Bot. **44**: 861 (1966).

Growth on malt agar spreading, consists of a convex, dense, floccose mycelium, at first white becoming pale yellow; reverse hyaline to pale yellow.

Vegetative hyphae slender, 1. 5–2μ in diam., hyaline. Conidiophores slender, septate, simple to branched in a verticillate manner, 12 to 15μ or more in length; terminal branches (phialides) slender, swollen at base, tapering gradually to a long narrow apex, 8–12 (—15)×1.5–2.0μ, hyaline. Conidia, of the phialospore-type, subglobose, borne in a long beaded chain, smooth, 2–2.5(—3.0)×1.5–1.7(—2.0)μ, hyaline. Aleuriospores large, hyaline to pale colored, muriform, indented at the septa, thick walled, globose, ovoid or irregular in shape, 20–40μ in diam., borne terminally on short branches; branches 12–22×2–3μ, broading towards the attachment up to 5μ, narrower below to 2μ.

Hab.: On the rotten leaves, *Quercus phillyraeoides* A. Gray, Noma, Tanegashima Is., Kagoshima Pref., Sept., 1969 (F–3–4–7).

Strain preserved: IFO–9373 Isol. K. Tubaki
Det. K. Tubaki

This fungus is not uncommon on decaying leaves in the southern part of Japan. Although the diameter of conidia of the present isolate is smaller than that in the description given by Barron & Onions (as 3–4×2–2.5μ), other characteristics agree closely with the description. Because *D. chlamydosporia* (G.) Barron & Onions, a type for the genus, was previously found in Japan by Hirayama & Kobayashi (Ronso, Matsuzaka Woman's College, **6**, 60, 1969) on the wool fabric, two described species of *Diheterospora* have been found in Japan now.

17. **Listeromyces insignis** Penzig & Saccardo (Pl. 3 A-F) Hyphomycetes
Malpighia **15**: 259 (1901); Goos, Mycologia **63**: 213 (1971).

Stromata on natural substrate scattered, solitary or in a few groups, clavate, simple or branched, at first dingy-white to gray, becoming dark brown to blackish brown, finally black, granulate, covered with large, black, phragmosporous conidia on whole surface; inner texture pseudoparenchymatous, composed of hyaline, thin-walled, compactly-packed irregular cells; sporogenous surface hymenia-like, composed of hyaline, clavate to fusiform conidiogenous cells with round and brownish apex, lining to outer side of stroma. Conidia broadly fusiform to elliptical, rounded at distal end,

somewhat narrowed towards proximal end, rough walled, yellowish brown to fuscous with olivaceous tint, 6–11 (mostly 7–9)–septate, septal plate thickened at center but with distinct central pore, 40–58 × 22–27μ (mostly 48–52 × 23–25μ). Microconidia globose, hyaline, smooth, 1–1.5μ in diam., produced from phialide born on growing end of dichotomous branch of sporodochial hyphal structure, spermatia-like.

Growth on malt agar rapid, floccose, at first white, then becoming pinkish-buff, pale purple gray to vinaceous gray; reverse concolor. Sporulation occurs in abundance under the culture.

Hab.: On the bark of dead trunk of *Cinnamomum japonicum* Sieb., Mt. Myoken, Katano, Osaka Pref., Dec. 2. 1968 (4312–2–3).

Strain preserved: IFO–9104 Coll. T. Yokoyama
Det. T. Yokoyama

This fungus was once collected by Penzig in Java in 1897, and was subsequently reported by Penzig & Saccardo in 1901 as a new species of the monotypic genus, *L. insignis*. Goos reported as the second described record of the occurrence in 1970 from Hawaii. At the almost same time this fungus was also collected in Japan. Morphologically the present fungus agrees well with the original description. As already pointed out by Goos, Hawaiian isolate produces somewhat larger conidia, however, the septation of the conidia of both Hawaiian and Japanese isolates is mostly nine in number. Conidia of the Japanese isolate developed either on the natural substrate or on the culture media are quite similar in the morphological properties. Japanese isolate produces conidia in a great abundance on the agar slants, especially of malt extract, potato sucrose and corn meal agar, even after several transfers. Attemps were made to produce possible perfect state of the present fungus under mixed culture with the Hawaiian isolate which was kindly sent by Dr. Goos, but was never observed so far.

18. **Menisporopsis novae-zelandiae** Hughes & Kendrick
(Pl. 2 C) Hyphomycetae

N. Z. J. Bot. **6:** 369 (1968).

Fructification on natural substrate consists of single seta and a group of unbranched conidiophores, provided with a subiculum at base. Setae developed from vegetative hyphae or directly from subiculum, stout, septate, 320–380μ long, 6–9μ wide at base, tapering to 4μ at apical cells, dark brown to almost black and paler apex. Conidiophores arise at base of seta, closely bound into a synnematum, unbranched, parallel, diverging slightly at conidia-bearing apex, 80–110(—140)μ long, 1.5–2.0μ wide, pale brown, bearing terminal phialide at each end. Phialides cylindrical, straight or slightly curved, 14–16 × 3–3.5μ, narrowed toward apex provided with funnel-shaped collarette. Phialospores develop successively from phialide and grouped in a slimy colorless droplet at apex of cluster of conidiophore, long and slightly asymmetrical, 1–septate, pointed at each end, 16–20 × 2.0–2.7μ, hyaline, with a gently curved setulae (4–6μ long) at each end.

Growth on malt agar moderate, spreading, pale olive to dark olive; reverse concolor.

Hab.: On decayed leaves of deciduous tree, Mt. Amagi, Tanegashima Is., collected by K. Yokoyama, Nov. 23, 1968 (Mi–27–1).

Strain preserved: IFO–9179 Isol. K. Tubaki
Det. K. Tubaki

The present species is characteristic in the genus as a single species having 1–septate conidia. This species was originally found in New Zealand and the present collection was the second found.

Under culture on malt agar, conidiophores are sinuous and variable in the length, developed solitarily and branched rarely; in this case, the microscopical features are very close to *Codinaea*; setae also developed not providing with the conidiophores.

19. **Menisporopsis theobromae** Hughes (Pl. 2 D) Hyphomycetes
Mycol. Pap. **48:** 59 (1952).

Setae on natural substrate erect, tough, 250–300μ long, 7–8μ wide at base, 6–7μ at middle, tapering gradually to 4μ at apex, dark brown. Conidiophores closely packed into a synnema which slightly expands at apex, unbranched, parallel, septate, 100–110μ long, 1.5–2.5μ wide, bearing terminal phialides, pale brown. Phialides cylindrical, straight or slightly curved, $14–17 \times 2–3.5\mu$, provided with apical funnel-shaped collarette, light brown. Phialospores unseptate, $14–20 \times 2–2.7\mu$, hyaline, with a gently curved setulae (4–8μ).

Growth on malt agar moderate, restrict, flat, velutinous, dark olive gray; reverse dark olive to almost black.

Hab. On decaying leaves of *Quercus phillyraeoides*, A. Gray, Onoaida, Yakushima Is., Kagoshima Pref., March, 1970 (F–3–10–13)

Strain preserved: IFO–9590 Isol. K. Tubaki
Det. K. Tubaki

This fungus is fairly common on the fallen leaves in the southern part of Japan. Rarely, especially in the old fructification, upper half of the phialospore becomes empty separated by a median septum from the other half. In this cae, a difference of the present species from *M. novae-zelandiae* becomes unclear.

20. **Phaeoisaria clavulata** (Grove) Mason & Hughes
(Pl. 2 E) Hyphomycetes
Mason & Hughes, Mycol. Pap. **56:** 42 (1953).

Synnemata on natural substrate erect, composed of closely packed, septate conidiophores, up to 390μ long, sometimes very short to 70μ, 24–40μ wide at base, 10–12μ at middle, crowned with a dry solid conidial head, dark brown. Conidiophores straight or flexuous, 2μ in diam., branched towards apex, pale to mid brown; branches geniculate, developed along entire or upper length of a synnema, hyaline. Conidia

develop successively on each slender denticle of geniculate branches, dry, broadly elliptical, 5–6 × 2.0–2.5μ, hyaline.

Growth on malt agar rapid, spreading, flat, velutinous, olive gray to dark olive; reveise dark olive to almost black. Synnema-production is very reduced and the conidia develop on the solitarily developed conidiogenous hyphae; globose or elliptical olive-colored aleuriospores are produced in abundance directly on hyphae.

Hab.: On rotten wood of *Machilus thunbergii* Sieb. & Zucc., Isen, Tokunoshima Is., Kagoshima Pref., April 13, 1971 (187–28).

Strain preserved: IFO–9591 Coll. K. Tubaki
Det. K. Tubaki

This fungus is fairly common in Japan.

21. **Rhopalomyces strangulatus** Tbaxter (Pl. 4 A-D) Mucorales
Bot. Gaz. **16:** 14 (1891).

Conidiophores simple, upright, slender, aseptate, hyaline, supported by rhizoidal hyphae at base, 3–5 mm high, 50–60μ wide at base, 60–80(—88)μ wide at middle, thick walled (wall 3–4μ in diam.), sharply narrowed into a slender neck which is 30–40μ long and 10–12μ wide, and develop to a vesicle. Vesicles globose, ca. 280–450μ in diam., easily collapsing, wall smooth with evenly scattered spicules over entire surface; each spicule bearing a single conidium, short cylindrical, 3–4 × 1.5μ. Conidia oblong-elliptical, 30–44 × 8–10μ, smooth walled, pale olive brown.

Hab.: On decaying animal material with old bones, Shugakuin, Kyoto, Sept. 11, 1968 (Sagara–1341).

Specimen pieserved: IFO. H–11636

Coll. N. Sagara, Kyoto Univ.
Det. K. Tubaki

Conidiophores and conidia in the present collection are a little bit different from the description given by Thaxter (conidiophores are 40–50μ wide and vesicles are 250μ large by Thaxter). Other characteristics, however, agree well with the original description. As pointed out by Drechsler (Bull. Toriey Bot. Club. **82**, 473, 1955), Thaxter didn't mention the color of conidia which is pale olive brown in this collection. This can abandon the Berlese's suspicion (Bull. Soc. Mycol. Fr. **8**, 94, 1892) that the conidia of this species were hyaline. This species is significant in the genus in the conidiophore with a narrow neck below the vesicle. It is interesting that the present habitat is the same as that described by Thaxter, "old bones and other decaying animal matter". The present finding of this remarkable fungus is probably the second one from nature so far since Thaxter described the species.

Germination of the conidia was obtained on the nutrient agar medium which was contaminated by *E. coli* and reautoclaved.

After the present manuscript was submitted in this journal, a similar fungus was

collected and sent by Mr. S. Murata from Toyohashi City, Aichi Pref, which fits closely to the present species in all respects. The fungus was growing on the decaying animal material (fish ?) with ammonical odor. The present species, therefore, seems to be distributed not uncommonly in Japan.

22. **Sporoschisma mirabile** Berk. & Br. (Pl. 2 F-G) Hyphomycetes

Berkeley, in Gdnr'a Chron. p. 540 (1847); Hughes, Mycol. Pap. **31:** 1 (1949).

Conidiophores on natural substrate solitary or scattered, thick walled, with a bulbous base leading to erect stalk, 160–300μ high, 14–16μ wide at base, dark brown; stalk enlarges gradually into a slightly swallen part, 16–18μ wide, and then it narrows into a cylindrical tube, 60–90μ long and 14–16μ wide, with very slightly paler apex; upper cylindrical tube often narrowed slightly at base into 11–14μ wide. Capitate setae often accompany conidiophores, 120–180μ long, 6–8μ wide at base, tapering to 6μ, with swallen club-shaped apex measuring 4–7μ in diam. Conidia develop endogenously in basipetal succession, cylindrical with truncate ends, thick walled, mostly 1–septate, 26–34 × 12–14μ, dark brown.

Growth on malt agar very reduced, restrict, consists of only pale brown sterile mycelium.

Hab.: On rotten drift wood, Lake Kugushi, Fukui Pref., July 2, 1971 (189–1d–1).

Strain preserved: IFO–9593 Coll. K. Tubaki
Det. K. Tubaki

The present fungus differs from the description given by Berkeley & Broome, Hughes or Ellis in septation of the conidia and length of the setae. As described by them, the conidia are almost always 3–septate, whereas those of the present collection are mostly 1–septate. In addition, setae of the present collection are much shorter although seta is generally variable in length. As pointed out by Hughes, however, abnormal conidia may present consisting of 2–, 3– or 5–cells. Because of such general variation of the conidia and the solitary or scattered stalks in the present material, the present collection must be an unmatured stage of this species.

23. **Torula caligans** (Batista & Upadhyay) M. B. Ellis (Pl. 2 H) Hyphomycetes

Ellis, in Dem. Hyph. CMI, Kew, p. 337 (1971).

Growth on malt agar rapid, spreading, flat, velutinous, pale olive to olive gray; reverse dark colored.

Hyphae slender, septate, minutely verrucose, 2.0–2.5μ wide. Conidiophores (sporogenous cells)short, sub-globose, with or without basal stalks, often lobbed, 6–16μ long, 4–5μ in diam. at widest part, minutely verrucose, hyaline. Conidia develop

at first as sub-hyaline, thick walled primordia from a pore on conidiophore, then elongate and septate basipetally; broadly fusiform, consist of four cells, dark brown, apical cells smaller and paler, intermediate cells larger and darker, thick walled, constricted markedly at septa, with verrucose or warty walls, 16–20μ long, 7–9μ wide at broadest part; develop in a single or branched chain; apical smaller cell may act as a separating cell and becomes empty. On germination, germ-tubes develop from one or both ends of conidia.

Hab.: Isolated from a bottum mad, Lake Sengari, Hyogo Pref., collected by Y. Matsuda, Dec., 1971 (XXII-4-1).

Strain preserved: IFO-9594 Isol. T. Ito
Det. T. Ito

The present collection is a second of the genus in Japan and this isolate differs clearly from *T. herbarum*, a very common species. Sporulation is most pronounced on corn meal agar.

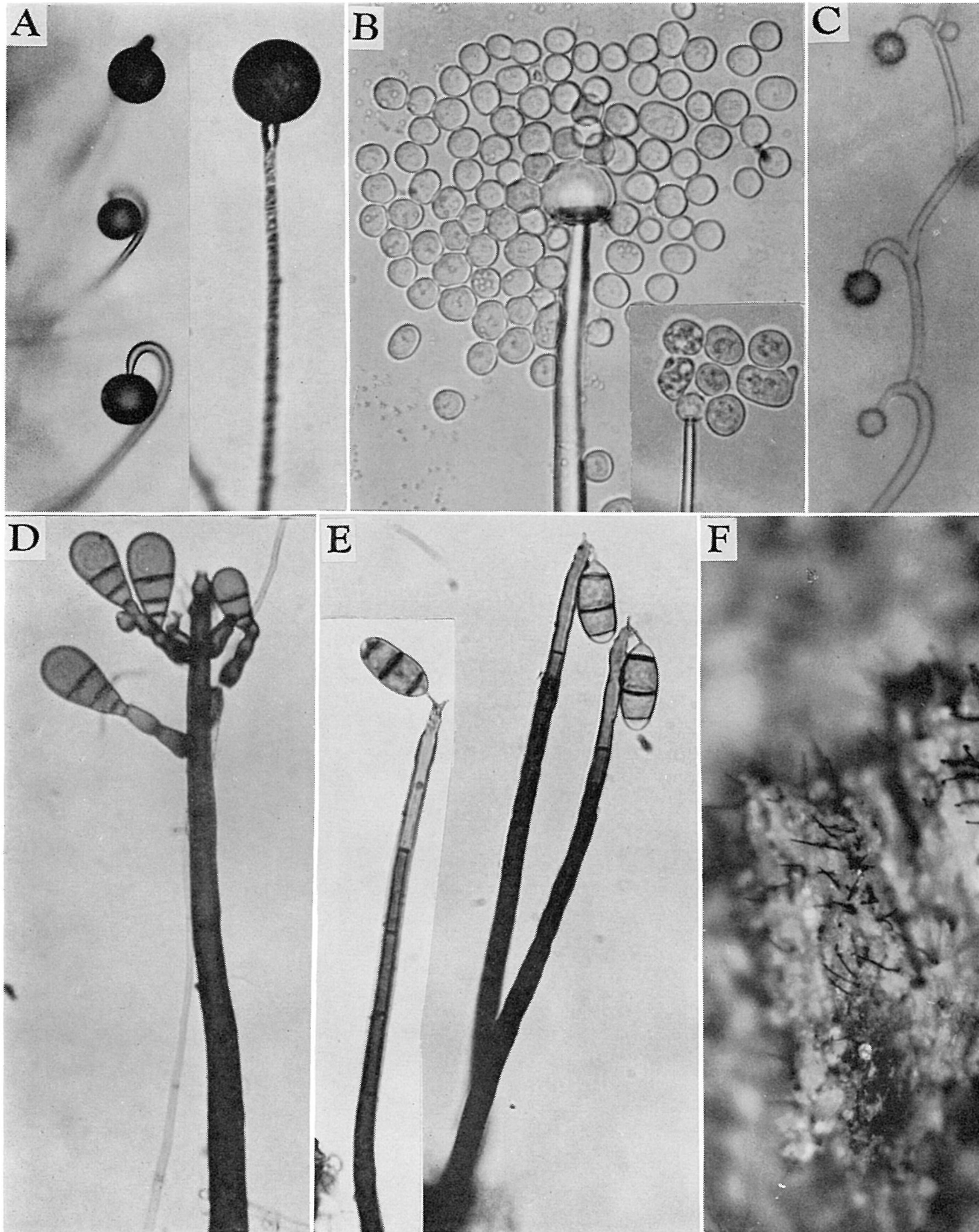


Plate 1. A-C. *Backusella circina* A. Sporangia (×100), B. Columellae of sporangia and sporangiole with spores (×400), C. Conidia on circinate branches (×250), D. *Brachysporiella gayana* (×400), E-F. *Brachysporium nigrum* E. Conidiophores and conidia (×400), F. Habit on wood.

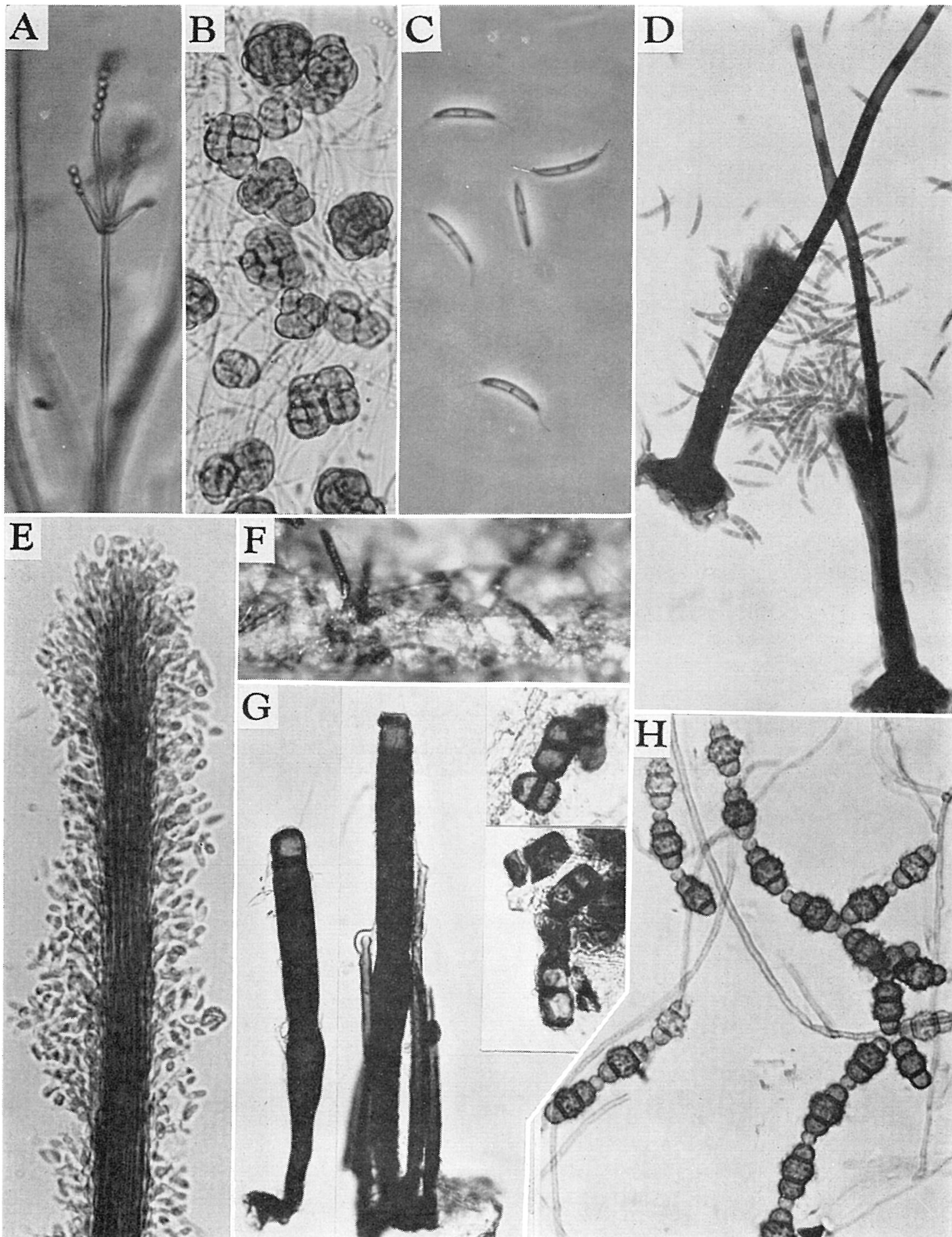


Plate 2. A-B. *Diheterospora catenulata* A. *Verticillium*-stage (×400), B. Aleuriospores (×250), C. Septate conidia of *Menisporopsis novae-zelandiae* (×400), D. *Menisporopsis theobromae* (×250), E. *Phaeoisaria clavulata* (×400), F-G. *Sporoschisma mirabile* F. Habit on wood (Ultropak, ×65), G. Conidiophores, setae and conidia (×250), H. *Torula caligans* (×250)

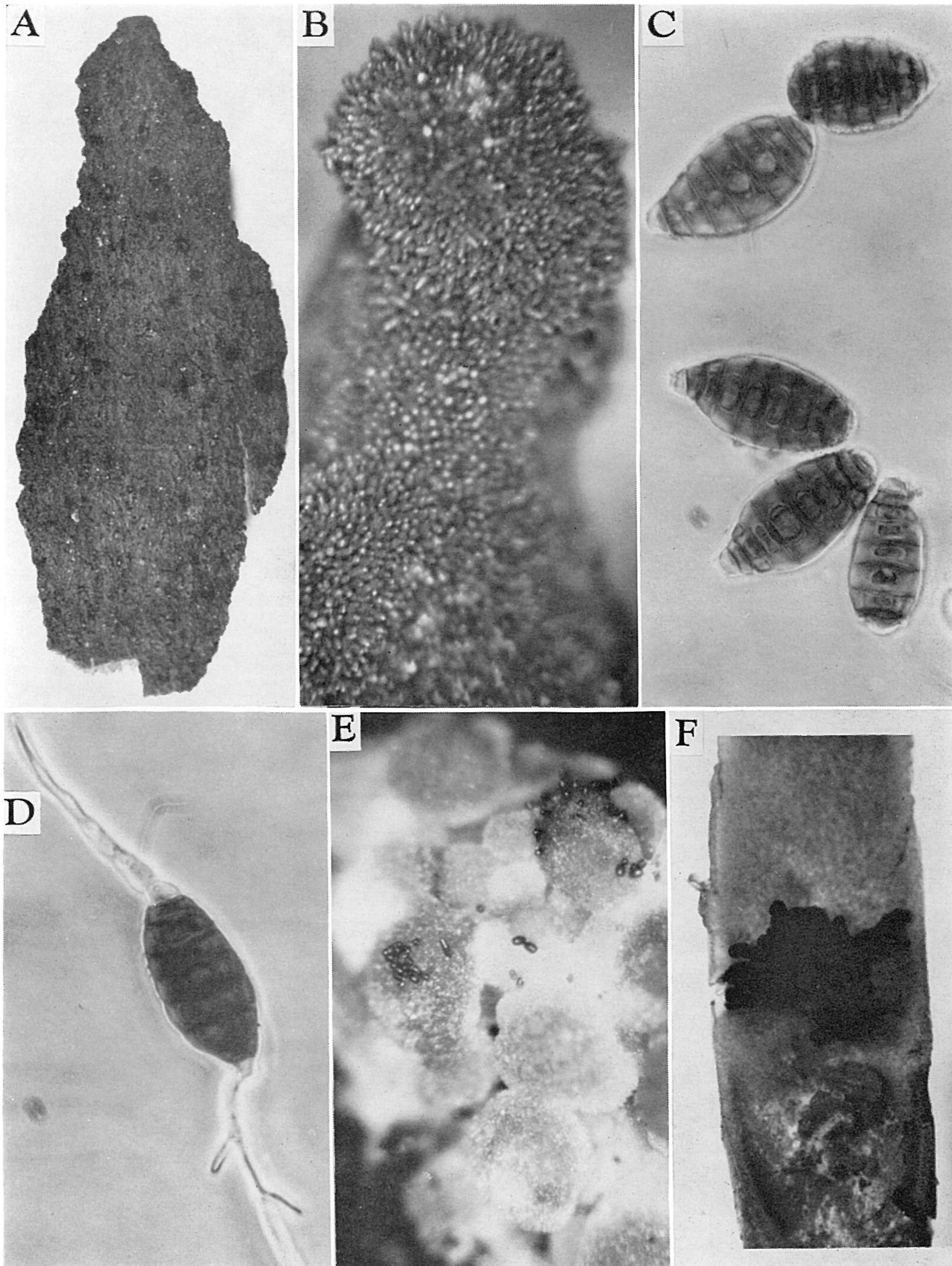


Plate 3. A-F. *Listeromyces insignis* A. Habit on bark, B. Stroma (Ultropak, ×38), C. Conidia (×400), D. Germination of conidium (×400), E. Stroma-like structure bearing microconidia in culture (Ultropak, ×38), F. Stroma developed on malt agar slant.

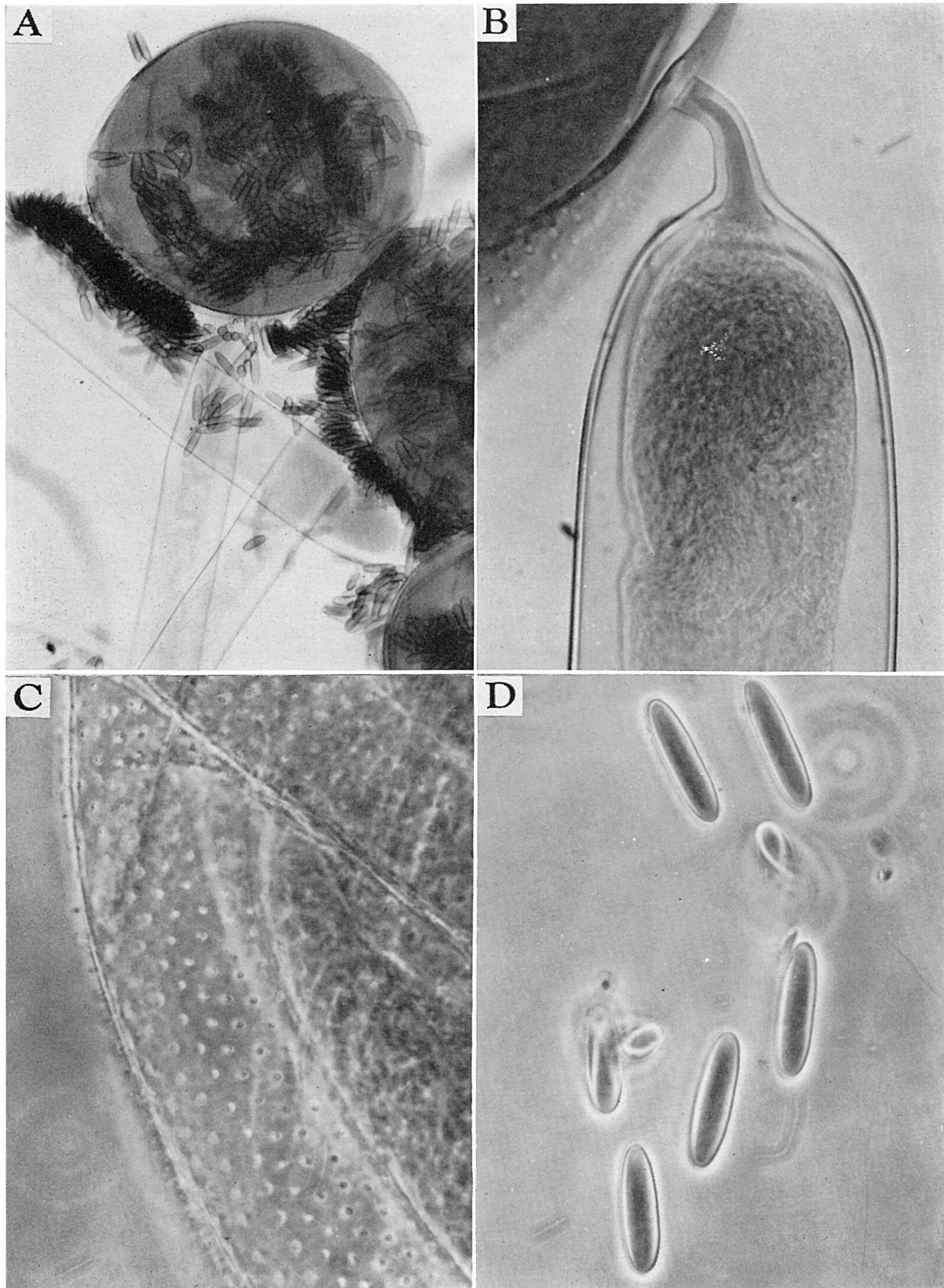


Plate 4. A-D. *Rhopalomyces strangulatus* A. Conidiophores, vesicles and conidia (×100), B. Narrow neck of conidiophore strongly contact with a vesicle (×400), C. Surface of vesicle covered by numerous spicules (×400), D. Conidia (×400).

IFO Res. Comm. 6,
95–98, 1973 (*January*)

BACTERIAL COLLECTION

Some of bacterial strains which were newly isolated at the Institute and obtained from other organizations were identified as known species. However, in most cases, their descriptions were not published yet or insufficiently described in published papers. The object of this catalogue is to provide these strains with valid descriptions. Each description is arranged below in alphabetical order of the scientific name of the strain.

1. Escherichia coli (Migula, 1895) Castellani and Chalmers, 1919.
IFO 13168

Obtained from Y. Takagi, Medical School, Osaka University (Ruth F. Hill, strain B) determined by T. Sakane.

Vegetative cells: rod, 0.6–0.8 × 1.0–2.0μ, varying coccoid to long rod, occurring singly or in pair, motile by peritrichous flagella, gram negative.

Colonies on agar medium: circular, undulate, gray, smooth surface.
Growth in liquid medium: heavy turbid, no pellicle.
NaCl tolerance: growth at 3% NaCl, slight growth at 7% NaCl.
Litmus milk culture: acid, coagulated.
Growth on potato: creamy to gray colony.
Utilization of citrate: negative.

VP test: slightly positive.

MR test: positive.

Reduction of nitrate to nitrite: positive.
Acid and gas produced from glucose, galactose, mannose, fructose, xylose, arabinose, lactose, trehalose, mannitol and sorbitol. Acid but no gas from maltose, starch (latently), and glycerol. Acid and gas not produced from sucrose and inositol.

Hugh & Leifson's OF test: acid and gas fermentatively produced.
Anaerobic gas production from nitrate: positive.
Anaerobic growth in glucose broth: positive with aerogenesis.
Aerobic growth is better than anaerobic.

Hydrolysis of starch: negative.
Hydrolysis of gelatin: negative.

Production of indole: positive.
Production of H_2S: positive in Christensen's medium.

Urease: negative.

Decarboxylation of

arginine; positive, lysine; positive, ornithine; positive.

Growth temperature: 15 to 45 C, optimum 30 to 36 C.
Specific characteristics: sensitive to T-series phages.

2. Bacillus circulans Jordan, 1890 emend. Ford, 1916.
IFO 13294

Isolated by Y. Sakamoto from air in the laboratory, April, 1971.
Determined by T. Sakane and K. Mikata.

Vegetative cells: rod, 0.4–0.6×2.0–2.8μ, with rounded end, mostly single, actively motile by peritrichous flagella, gram staining mostly negative but some cells stained.

Sporangia: definitely swollen.
Spores: oval to ellipsoidal, 0.7—1.5μ, central to terminal, thick and easily stainable wall.
Colony on agar medium: thin, spreading, translucent, wrinckled surface; there are motile micro-colonies rotating from point of inoculation on dry agar plate.

Growth in liquid medium: light to fair turbidity with fine sediment.
Litmus milk culture: weakly acid, the indicator reduced.
NaCl tolerance: scant growth in 7% NaCl.
Growth on potato: scanty, spreading.
Utilization of citrate: negative.
Utilization of malonate: negative.
VP test: negative.
MR test: weakly positive.
Reduction of nitrate to nitrite: positive.

Acid but no gas produced from arabinose (variable), xylose, glucose (weak), galactose, mannose, fructose, lactose, maltose, sucrose, trehalose, melibiose, raffinose, soluble starch, sorbitol (variable), glycerol (weak), and mannitol; acid and gas not produced from sorbose, rhamnose, and inositol.

Hugh & Leifson's OF test: fermentative acid formation.
Anaerobic gas production from nitrate: negative.
Anaerobic growth in glucose broth: positive without gas.
Formation of crystalline dextrin from starch: negative.
Hydrolysis of starch: positive; starch strongly enhances the growth.
Hydrolysis of gelatin: negative after 21 days.
Hydrolysis casein: negative.
Production of indole: negative.
Production of H_2S: positive.
Urease: negative.
Reduction of methylene blue: positive.
Hydrolysis of aesculin: positive.
Hydrolysis of tween 80: weakly positive.

Hydrolysis of tributyrin: negative.
Deamination of phenylalanine: negative.
Decomposition of tyrosine: negative.
Ketogenesis of glycerol: negative.
 ,, gluconate: negative.
Reduction of trimethylamine oxide: weakly positive.
Catalase: positive.
Oxidase: positive (Kovac's method).
β-Galactosidase: positive.
Phosphatase: negative in alkaline.
Requirement for growth: thiamine and pyrimidine base.
Growth temperature; 15–45 C, optimum 25–30 C.
Growth pH: 6–10, optimum 9.

Specific characteristics: micro-colony migration; on dry nutrient agar plate, three types of moving colonies were observed.

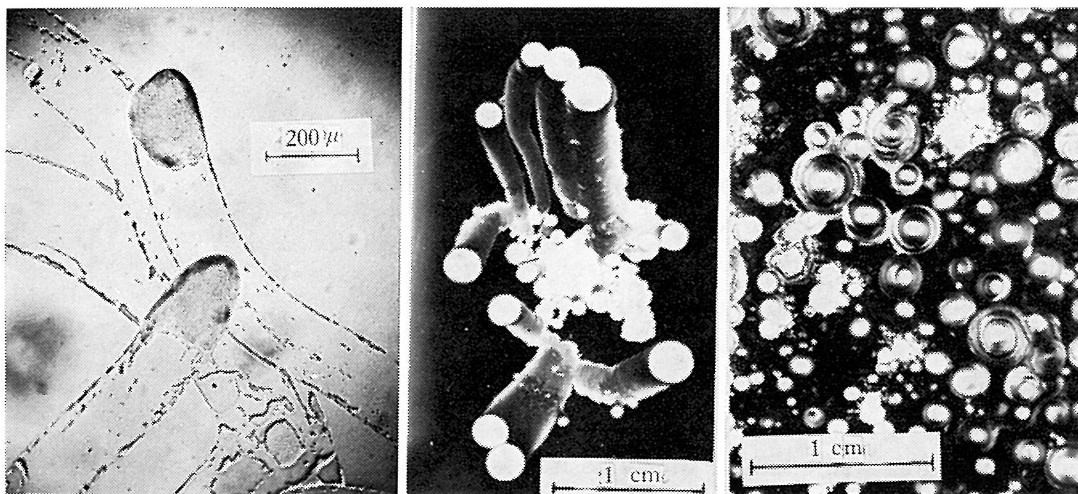


Fig. 1 Fig. 2 Fig. 3

The first was a young, bullet-shaped colony, about 100 to 150μ in length and width, moving at a rate of 100μ/min from the point of inoculation and leaving two parallel trails of cells in large arc- or circular-patterns (Fig. 1). The second type was a large, revolving disk-shaped colony, 1 to 3 mm in diameter, migrating at a rate of 3 to 5 mm/ day with rotation and leaving a trail of semicircular-pattern on the plate containing about 0.2% starch and 1% NaCl (Fig. 2). The third was a swirling colony, 3 mm or more in diameter, appearing after rapid and growth on a alkaline agar-plate (Fig. 3).

3. Bacillus subtilis Cohn, 1872 emend. Prazmowski, 1880.
IFO 13169

Isolated by S. Yamauchi from fermented soy beans (Natto). Sep., 1969. Determined by T. Sakane.

Vegetative cells: rod, 0.8–0.9 × 1.2–2.0μ, mostly in chains, shadow-forms, motility not clear, peritrichous flagella, gram positive.
Sporangia: not definitely swollen.
Spores: oval to cylindrical, central.
Colony on agar medium: irregular, rough, spreading, gray, dull.
Growth in liquid medium: heavy turbidity with pellicle.
Litmus milk culture: peptonized.
Growth on potato: abundant, spreading, pale brown.
NaCl tolerance: good growth in 7% NaCl.
Utilization of citrate: positive.
VP test: positive.
MR test: latently weak positive
Reduction of nitrate to nitrite: positive.
Acid but no gas produced from glucose, mannose, and sucrose, weakly produced from galactose, trehalose, sorbitol, and mannitol; no acid produced from xylose, lactose and starch.
Hugh & Leifson's OF test: fermentative acid formation.
Anaerobic gas production from nitrate: negative.
Anaerobic growth in glucose broth: scanty.
Hydrolysis of starch: positive.
Hydrolysis of gelatin: positive.
Hydrolysis of caseine: strongly positive.
Production of indole: negative.
Production of H_2S: positive.
Urease: negative.
Reduction of methylene blue: slowly positive.
Growth temperature: 15–55 C, optimum 40–45 C.
Remark: This strain has been preserved in TIME CAPSULE which was burried under the ground near Osaka Castle in Osaka city in 1970.

ABSTRACTS 1971-1972

Reijiro KODAMA, Yugoro NAKASUJI and Masaaki NISHIO

Bacteria Isolated from Silkworm Larvae

VIII. Experiments to Protect Aseptically Reared Silkworm Larvae from Bacterial Diseases by Oral Administration of Antibiotics

J. Sericult. Sci. Japan **39,** 425–428 (1970)

Experiments were carried out to protect larvae from bacterial diseases by oral administration (feeding) of antibiotics, employing healthy silkworm larvae in the 5th instar, which were reared aseptically on an artificial diet.

The oral administration of the antibiotics, which suppressed effectively the growth of the pathogenic bacteria used in the test tubes, made it possible to protect the larvae from diseases to be caused by the bacteria orally inoculated. However, there were marked differences between the amounts of antibiotics in the test tubes required for suppressing the growth of the bacteria and those in the diet required for protecting the larvae from bacterial diseases.

Reijiro KODAMA and Yugoro NAKASUJI

Bacteria Isolated from Silkworm Larvae

IX. The Behavior of Antibiotics Added to Artificial Diet

The behavior of antibiotics were investigated, when they were added to an artificial diet and then taken in the gut of larvae with the diet.

(1) Some of the antibiotics tested were inactivated by keeping them in contact with the digestive juice of larvae. Chloramphenicol and triacetyloleandomycin were stable, while tertiomycin and spiramycin were relatively unstable.

(2) Using chloramphenicol, the following results were obtained.

1) When the antibiotic was added to the diet, it was considerably adsorbed on the diet itself.

2) At the period of roughly 4 hours after the larvae began to ingest the antibiotic-containing diet, the amounts of the antibiotic in the gut contents reached maximum, and in the vicinity of the same period of time the amounts of the antibiotic excreted in the feces began to increase.

3) On the contrary, when the antibiotic-containing diet, which was continued to be given till that time, was replaced by the antibiotic-omitted diet, the antibiotic could not be detected from the gut contents at the period of 10 to 11 hours after the replacement.

Isao BANNO, Takezi HASEGAWA and Hiroshi IIZUKA*

A Taxonomic Investigation of Acid Producing Yeasts

J. Ferment. Technol. **49** (3) 165–179 (1971)

An examination for acid formation from glucose was carried out on the authentic strains of *Candida* and *Brettanomyces* and on unnamed NP yeasts which produced citric and isocitric acids from n-paraffins. Thirty-six strains of *Candida* species and 23 NP yeasts showed as much ability to form a considerable amount of acid from glucose in an agar medium as did 4 species and 1 variety of *Brettanomyces*. These yeasts produced citric and isocitric acids in a vigorously aerated culture, but ~~not~~ acetic acid and ethanol under a micro-aerobic condition. It was concluded that the strong formation of acid could not be used as a sole diagnostic key to distinguish the genus *Brettanomyces* from other genera of yeasts.

On a morphological survey of these yeasts the ogive shape of cell was confirmed to be a property that is exclusive with *Brettanomyces*.

As a result, of 50 NP yeasts, 30 were identified with *Candida lipolytica*, 16 with *C. tropicalis*, 2 with *C. parapsilosis* var. *intermedia*, 1 with *C. brumptii*, and 1 with *C.* spec.

* Institute of Applied Microbiology, University of Tokyo, Tokyo

Teiji IIJIMA and Kyo KAZIWARA*

Absence of Mutagenic Action of Thiamine Tetrahydrofurfuryl Disulfide Hydrochloride (TTFD-HCl) on *Escherichia coli*

Takeda Kenkyusho Ho **30:** 771–775 (1971)

The mutagenic action of TTFD-HCl was tested by using several tryptophan requiring mutants of *Escherichia coli* K12, *i.e.*, ochre mutant, GC→AT type, AT→GC type and frame-shift mutant. N-methyl-N′-nitro-N-nitrosoguanidine showed mutagenic action on these strains, but the mutagenic action of TTFD-HCl was not observed under experimental conditions.

Ko IMAI and Teiji IIJIMA

Inhibition of Bacterial Growth by Citrate 2

Inhibition of Glucose Permeation by Citrate in *Arthrobacter simplex* IFO 12069

J. Gen. Appl. Microbiol. **17:** 457–468 (1971)

The growth of *Arthrobacter simplex* IFO 12069 (ATCC 6946) was specifically inhibited by citrate but not by isocitrate or *cis*-aconitate in the glucose basal medium. For this phenomenon was given the reason that citrate bound to the cell surface and inhibited the glucose permeation. This conclusion was drawn from the following facts: (1) Glucose utilization by intact cells was inhibited by 0.3% or more of sodium citrate, but that by cell-free extract did still occur even when 1% of sodium citrate existed. (2) The glucose uptake by the hexokinaseless mutant cells was inhibited by citrate. (3) In citrate-treated cells whose intracellular citrate level was about 0.3%, glucose utilization was markedly repressed and it was restored by the addition of metal ions. (4) The intracellular citrate level in citrate-treated cells corresponded to the critical concentration of citrate to inhibit the growth in the glucose basal medium and to inhibit the glucose utilization by intact cells. (5) Citrate bound to citrate-treated cells was not removed by washing with distilled water or saline but easily excluded with metal ions.

---

* Biological Research Laboratories, Central Research Division, Takeda Chemical Industries Ltd., Osaka.

Teiji IIJIMA and Takeshi SAKANE

Preservation of *Escherichia coli* by drying *in vacuo*

J. Japan. Soc. Res. Freez. Dry. **16:** 87–94 (1970), **17:** 16–20 (1971)

A modified method for preservation of *Escherichia coli* and its bacteriophages was described. The method comprised to dry a bacterial suspension or phage lysate *in vacuo* without freezing. Temperature of the sample and vacuum in the ampoules during the drying procedures were measured. The cotton plug which has been used to prevent cross-contamination plays a role to bufferize the atomospheric condition in the tube and acts a descicant during the storage.

Yoshikazu EMOTO* and Keisuke TUBAKI

Gonytrichella, A New Genus of Hyphomycetes

Trans. Mycol. Soc. Jap. **11:** 95–97 (1970).

A new genus, *Gonytrichella*, based on *G. olivacea* was described.

Keisuke TUBAKI and Tatsuo YOKOYAMA

Notes on the Japanese Hyphomycetes, V

Trans. Mycol. Soc. Jap. **12:** 18–28 (1971).

New genera, *Stachybotryna* and *Subulispora*, are proposed based on *Stachybotryna columare* and *Subulispora procurvata* respectively. New species, *Dicranidion inaequalis* and *Sympodiella multiseptata* are described.

Keisuke TUBAKI and Tatsuo YOKOYAMA

Cultural Aspects of *Graphiola phoenicis*

Mycopath. Mycol. Appl. **43:** 49–60 (1971).

An experiment was carried out to study the cultural behavior of *Graphiola*

* Department of Conservation Science, Tokyo National Research Institute of Cultural Properties, Tokyo

phoenicis (Moug.) Poiteau because nothing had been known on the morphological and physiological characters on artificial media. The daughter cell sprouted out from the sporidium of *G. phoenicis* reproduced by budding so that a pinkish yeast-like colony resulted on the agar medium. Physiological characters of the isolate were investigated and a discussion on the possible relationships to the reddish yeast was given.

Isao BANNO and Takezi HASEGAWA

Acid formation as the criterion for classification of yeasts

Fermentation Technology Today (Proceedings of the IV International Fermentation Symposium 1972). Soc. Ferment. Technol. Japan, Osaka. 775–780 (1972)

It was found that strains of 55 species of 8 yeast genera besides *Brettanomyces* and *Dekkera* could form a remarkable amount of acids from glucose. From the result, it seems improbable that acid production is an useful criterion for the classification of yeast genera. The acid forming yeasts were divided into 3 groups according to the pattern of the kinds of acids produced. The first group was yeasts which exclusively produced acetic acid; the second produced acetic and citric acids; the third produced citric acid exclusively. There was a correlation of these acid producing patterns with the method of cell reproduction: yeasts of the first group reproduced their cells by fission or bud-fission while those of the second and third groups by budding.

The first group included the genera *Schizosaccharomyces*, *Hanseniaspora*, *Nadsonia*, *Wickerhamia*, *Kloeckera* and 3 species of *Torulopsis*. All budding yeasts belong to the second and third groups.

MISCELLANEOUS SCIENTIFIC PAPERS

1. Hiroshi IIZUKA and Takezi HASEGAWA 1970. Proceedings of the First International Conference on Culture Collections. 625 pp. University of Tokyo Press, Tokyo.
2. Classification and identification methods of industrial microorganisms: Takezi HASEGAWA 1971. Principles of the International Code of Botanical Nomenclature. Kagaku to Seibutsu 9 (8): 531–537. Keisuke TUBAKI 1971. Classification and identification method of mold. Kagaku to Seibutsu 9 (10): 664–670. [In Japanese]
3. Keisuke TUBAKI 1971. Several problems in classification of Deuteromycetes. Jap. J. Med. Mycol. 12: 18–28. [In Japanese]
4. Isao BANNO 1971. Numerical taxonomy for bacteria. Technol. Biol. 2: 350–352, 423–425, 507–508, 582–586, 670–672. [In Japanese]
5. Keisuke TUBAKI 1971. Classification for fungi. Technol. Biol. 2: 745–748, 837–840, 925–928. [In Japanese]

PRESENTATION OF PAPERS AT SCIENTIFIC MEETINGS, 1971-1972

Author(s)	Title	Scientific Meeting
Y. YOKOYAMA	Infection of lettuce seeds by *Myrothecium roridum.*	Meeting of the Kansai Plant Protection Society in Nagoya (February, 1971)
T. YOKOYAMA & K. TUBAKI	On Japanese species of the genus *Arthrinium.*	Phytopathological Society of Japan. Meeting in Sendai (April, 1971)
R. KODAMA & Y. NAKASUJI	Inhibition of development of viral Diseases in gnotobiotic silkworm by nalidixic acid.	Society of Sericultural Sciences of Japan. Meeting in Tokyo (April, 1971)
K. IMAI & T. IIJIMA	Inhibition of bacterial growth by citrate. III.	Agricultural Chemical Society of Japan Meeting in Tokyo (April, 1971)
K. TUBAKI, T. ITO & Y. MATSUDA*	On marine fungi V. Polluted water	Mycological Society of Japan. Meeting in Tokyo (May, 1971)
T. YOKOYAMA	Successive fungal flora of forest leaf litter.	The same as above
Y. SAKAMOTO & T. IIJIMA	High mutability and *uvrB* gene deletion. I.	Genetics Society of Japan. Meeting in Fukuoka (October, 1971)
I. BANNO & T. HASEGAWA	Acid formation as the criterion for classification in Yeasts.	IV International Fermentation Symposium in Kyoto (March, 1972)
K. IMAI & T. IIJIMA	Transport of the tricarboxylic acids in *Salmonella typhimurium.* I.	Agricultural Chemical Society of Japan. Meeting in Sendai (April, 1972)
K. TUBAKI	Conidial states of Basidiomycetes.	Mycological Society of Japan. Meeting in Hiroshima (May, 1972)
T. YOKOYAMA	On the conidial state of *Pseudomassaria corni.*	The same as above
Y. SAKAMOTO & T. IIJIMA	High mutability and *uvrB* gene deletion II.	Genetics Society of Japan. Meeting in Okayama (October, 1972)
T. IIJIMA & T. SAKANE	Preservation of *E. coli* and other bacteria by drying *in vacuo*	U.S.-Japan Seminar of Freezing and Freeze-Drying in Berkeley Springs, U.S.A. (October, 1972)

* Kobe Municipal Hygienic Laboratories, Kobe

ANNUAL REPORT, 1971-1972

The Group Training Course in Fermentation Technology in the fiscal year 1971 was given by the Japanese Government as a part of its technical cooperation scheme with the support of UNESCO. It was held from February 14th to March 17th 1972 at the Department of Fermentation Technology, Osaka University. (The Training Course was arranged by the Overseas Technical Cooperation Agency, the National Commission of UNESCO, the Osaka University and the Society of Fermentation Technology, Japan.) Dr. Hasegawa was invited as a lecturer on the microbial classification and he accomplished his duty through the assistance of Dr. Tubaki, and of Dr. Kazuo Komagata, Institute of Applied Microbiology, University of Tokyo.

The Fourth International Fermentation Symposium took place at the International Conference Hall in Kyoto during the week of March 19–25, 1972, and the non-medical part of the Second Specialized International Symposium on Yeasts was held in connection with the 4th IFS. (The medical part was held at the lecture hall of Japan Science Council's building in Tokyo, August 7–10, 1972.) Drs. Banno and Hasegawa contributed to this yeast symposium a paper concerning the significance of acid formation of yeasts in the criteria for the classification.

The U.S.-Japan Seminor on "The Preservation of Culture Collections by Freezing and Freeze-Drying" was held at Berkeley Springs, West Virginia, U.S.A. under the auspices of the U.S.-Japan Cooperative Science Program during the period of October 15–20, 1972. Dr. Iijima presented a paper of his studies on the preservation of bacteria by drying *in vacuo* at this meeting.

In May, 1972 Emeritus Professor Hirosuke Naganishi, Hiroshima University, offered to present the Institute with his fine library of microbiological literature and the Institute accepted this offer with sincere thanks. Prof. Naganishi is one of excellent mycologists in Japan. He established the famous CLMR Culture Collection at the Central Laboratory of the South Manchurian Railway Company in cooperation with Prof. Kendo Saito during the period of 1912–1930. Since 1946, he has often made important contributions to the IFO Culture Collection. He is still in green old age of eighty-two.

The fifth edition of the IFO List of Cultures was brought out in March 1972. It comprised 5451 strains of 601 genera together with 24 bacteriophages. With regard to activities of the Collection during two years of 1971 and 1972, 462 cultures of fungi and bacteria were obtained either from natural sources or through the courtesy of other collections and research organizations, and 7198 subcultures were distributed in Japan and abroad. The total number of cultures in the Collection reached 8214 at the end of 1972.

(T. Hasegawa)

BOARD OF TRUSTEES

Chobei TAKEDA, *Chairman*

Koji ANDO, Hideo KATAGIRI, Kozo Miki, Kin'ichiro SAKAGUCHI, Sueo TATSUOKA, Gyozo TERUI, Tadao TODA

Takezi HASEGAWA, *Director*

COUNCILORS

Shigeyasu AKAI, Kei ARIMA, Toshinobu ASAI, Tsunesaburo FUJINO, Motoyoshi HONGO, Hiroshi IIZUKA, Hideo KIKKAWA, Kakuo KITAHARA, Yosio KOBAYASI, Ryodi NAKAZAWA, Koichi OGATA, Yuji SASAKI, Rokuro TAKEDA

RESEARCH COMMUNICATIONS TABLE OF CONTENTS

No. 1, 1963

The nutrition of *Lactobacillus fructosus* and its application to microbiological determination of nicotinamide and fructose......R. KODAMA 11–24

Taxonomic study of Hyphomycetes......................................K. TUBAKI 55–60

Isolation of temperate phages from natural sources....................T. IIJIMA 55–60

Inactivation and induced mutation of *Rhodotorula glutinis* by irradiation. Part 1. With ultraviolet rays................................I. BANNO 61–66

Inactivation and induced mutation of *Rhodotorula glutinis* by irradiation. Part 2. With X-rays..I. BANNO 67–71

No. 2, 1965

A report on the taxonomy of red to orange *Rhodotorula*...........T. HASEGAWA 1–25

Microbiological activities of thiamine and its related compounds for nineteen strains of lactic acid bacteria......R. KODAMA and S. MARUOKA 27–32

Localization of ϕ 170 prophage on the chromosome of *Escherichia coli* K 12..T. IIJIMA 33–38

Contributions towards the fungus flora of Australia and New Zealand...K. TUBAKI 39–62

No. 3, 1967

Mycological studies of the Alaskan Arctic
........................Yosio KOBAYASI, Naohide HIRATSUKA, Richard P. KORF, Keisuke TUBAKI, Kiyowo AOSHIMA, MASAMI SONEDA and Junta SUGIYAMA 1–138
Japanese culture collections of microorganisms in the field of industry—their histories and actual state.................T. HASEGAWA 139–143

No. 4, 1969

Pathogenicity of bacteria for silkworm larvae reared aseptically on an artificial diet......................................R. KODAMA and Y. NAKASUJI 1–11
Studies on the Japanese marine fungi—lignicolous group. III. Algicolous group and a general consideration........................K. TUBAKI 12–41
Attachment site of bacteriophage ϕ 170 and other λ-related phages on the chromosome of *Escherichia coli* K 12
....T. IIJIMA and Y. SAKAMOTO 42–52
Occurrence of *Rhodosporidium toruloides* and *Rhodotorula* yeasts in forest..I. BANNO and K. MIKATA 53–59
Descriptive catalogue of IFO fungus collection...................................... 60–68

No. 5, 1971

Further studies on the pathogenic mechanism of Bacterial diseases in gnotobiotic silkworm larvae....................
.... R. KODAMA and Y. NAKASUJI 1– 9
Formation and segregation of double lysogens of λ-related phages... T. IIJIMA and Y. SAKAMOTO 10–19
A natural variant of *Streptomyces lavendulae* ....K. NAKAZAWA 20–23
Successive fungal flora on sterilized leaves in the litter of forests I. ....K. TUBAKI and T. YOKOYAMA 24–42
Cultural and taxonomical studies on the genus *Actinopelte*......
.... T. YOKOYAMA and K. TUBAKI 43–77
Descrptive catalogue of I.F.O. fungus collection II................................ 78–90
Subject Index (No. 1–No. 5) 103–110
Author Index (No. 1–No. 5) 111

発酵研究所研究報告　第６号

昭和48年１月15日　印刷
昭和48年１月25日　発行

定価 800 円

編集兼発行人　長　谷　川　武　治

発行所　財団法人　発　酵　研　究　所
大阪市東淀川区十三西之町４丁目54番地

印刷所　日 本 印 刷 出 版 株 式 会 社
大阪市福島区亀甲町２丁目62番地