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「生物の多様性と新しい微生物学」

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■略歴

1956　東京大学農学部農芸化学科卒業
1958～60　米国パデュー大学、研究員(フルブライト交換留学生)
1963　東京大学大学院博士課程終了(農学博士)
1963　理化学研究所入所
1966～67　米国カリフォルニア大学デービス分校助教授
1974～93　理化学研究所主任研究員
1984～90　新技術開発事業団特殊環境微生物プロジェクト(スーパーバグプロジェクト)総括責任者
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■主要受賞歴

1979　科学技術庁長官賞研究功績者「アルカリ性発酵法によるβ－サイクロデキストリンの製法に関する研究」
1982　大河内賞「アルカリ性醱酵法による有用酵素の開発とこれを用いるシクロデキストリン製造技術の確立」
1987　紫綬褒章「好アルカリ性微生物によるサイクロデキストリン製造技術の開発」
1989　日本農芸化学会賞鈴木賞「好アルカリ性微生物とアルカリ酵素の研究」
1991　英国国際バイオテクノロジー協会ゴールドメダル「極限微生物についての研究」及びFellowship of International Institute of Biotechnology.

■主要著書

1983　「好アルカリ性微生物－その発見と応用－」掘越弘毅、渡部一穂(海鳴社)
1987　「転換するバイオテクノロジー－DNAの読解から生命現象の解明へ－」掘越弘毅、谷口維紹(三田出版会)
1991　「アルカリ環境下の微生物」(独VCH社英文)
1992　「酵素－科学と工学」掘越弘毅、虎谷哲夫、北爪智哉、青野力三(講談社サンエンティフィク)

■Personal History

1956　B.S.,Dept. of Agricultural Chemistry. University of Tokyo.
1958～60　Research Assistant at Purdue University, Indiana, U.S.A.
1963　Ph. D., Dept. of Agricultural Chemistry, University of Tokyo.
1963～70　Kenkyuin (equivalent to assistant professor) at Riken Institute.
1966～67　Invited Associated Professor at California University, Davis, Calif., U.S.A.
1974～93　Professor of the Dept. of Applied Microbiology at Riken Institute.
1984～90　Director of the Superbugs project of ERATO.
1988～93　Professor of the Tokyo Institute of Technology, Dept. of Bio-Engineering.
1990～　Director of the DEEP STAR project at the Japan Marine Science Research Center.
1993～　Professor of the Toyo University, Dept. of Applied Chemistry.

■Awards

1979　Award of the Ministry of Science and Technology "Industrial production of cyclodextrins".
1982　Award of the Ohkouchi Memorial "Alkalophilic Microorganisms and new fermentation techniques".
1987　The Purple Ribbon Medal from the Japanese Emperor "Industrial application of alkalophilic microorganisms".
1989　Award of the Agricultural Chemical Society of Japan "Discovery and studies of alkalophiles".
1991　Gold Medal Lecture of The International Institute of Biotechnology at The Royal Society, London "Superbugs and Biotechnology Innovation".

■Publications

1983　"Alkalophilic Microorganisms" (In Japanese) Kaimeisha.
1987　"Innovation in Biotechnology" (In Japanese) Mita-Shuppan.
1991　"Microorganisms in Alkaline Environments" VCH Publisher
1992　"Enzymes for engineers" (In Japanese) Kodansha Scientific.

Microbial diversity and a new microbiology

Lecture at the Conferring Ceremony on the 17th of November 1993, in Tokyo

Professor Koki Horikoshi

The Winner of the Honda Prize 1993
Director of the DEEP STAR project at the Japan Marine Science Research Center
Professor of the Toyo University, Dept. of Applied Chemistry

Your Excellencies, President of The Honda Foundation, Mrs. Honda, distinguished guests, ladies and gentlemen, it is indeed a great honor for me to be given The Honda Prize and to deliver a lecture on biodiversity and biotechnology innovation, with particular reference to the isolation of extremophiles and their industrial applications.

In autumn 1990, I had the chance to see one of the largest exhibition of Claude Monet's most famous series, at the Royal Academy of Arts in London. As Kenneth Clark explains in his famous book "Civilization," Monet attempted a kind of color symbolism to express the changing effects of light. For example he painted a series of cathedral facades in different lights--pink, blue and yellow--which seem to me too far from my own experience. The colors of these objects depend on the physical environment, such as sunlight, snow, the time of the day, the season, etc. Under different conditions, one object may show quite different properties. Who can be sure what is the absolute property ? The microbial world may have the same uncertainty and diversity.

It is only three hundred years ago, Antony van Leewenhoek first observed microorganisms through his microscope. In the middle of the 19th century, Louis Pasteur conducted one of the most important experiments in the field of microbiology, as a result of which he was able to refute the theory of spontaneous generation. As you know, Sir Alexander Fleming made his famous serendipitous discovery of the first antibiotic, Penicillin in 1928 that has lengthened our average life-span. The industrial production of Penicillin has resulted in the development of basic microbiology, such as physiology and genetics, as well as industrial microbiology. And in 1977, only one decade ago, the first DNA sequence of the virus SV40 was determined by Maxam and Gilbert. I would like to say that the decoding of the three and a half billion year history of life has only just begun.

EXTREME ENVIRONMENTS

Not too many years ago, almost all biologists believed that life could survive only within a very narrow range of temperature, pressure, acidity, alkalinity, salinity and so on, in so called moderate environments. So when microbiologists looked around for interesting bacteria and other life-forms, they attempted to isolate microorganisms only from moderate environments.

Nature, however, contains many extreme environments (Table 1), such as acidic or hot springs, saline lakes, deserts, alkaline lakes and

Table 1. Extreme Environments

Temperature	:	60℃< 10℃>
pH	:	pH9< pH3>
Salt concentration	:	>15% (NaCl)
Others	:	pressure, organic solvents, radiation

the ocean bed. All of these environments would seem to be too harsh for life to survive.

However, in recent times, many organisms have been found in such extreme environments. Moreover, some of them connot survive in so called "moderate" environments. For example, thermophilic bacteria, high temperature-loving bacteria, grow in environments with extremely high temperatures, but will not grow at 20 to 40℃. Some alkali-loving bacteria cannot grow in a nutrient broth at pH 7.0, but flourish at pH 10.5. If a moderate environment for conventional organisms such as *Escherichia coli* that is very popular in the field of genetic engineering were superimposed on that for thermophilic organisms, for example, the "moderate" environment would seem very cold for thermophiles. Thus, the idea of an extreme environment is relative, not absolute. Clearly we have been too anthropocentric in our thinking. We should therefore extend our consideration to other environments in order to isolate and cultivate new microorganisms.

DISCOVERY OF ALKALIPHILES

In 1968, I was visiting Florence, in Italy. I was looking at the Renaissance buildings, which were so very different from Japanese architecture. About 500 years ago, no Japanese of Muromachi period could have imagined this Renaissance culture. Then suddenly a voice whispered in my ear, "There might be a whole new world of microorganisms in different unexplored cultures." "Could there be an entirely unknown domain of microorganisms at alkaline pH ?" The acidic environment was being studied, probably because most food is acidic. However, hardly any work had been done in the alkaline region.

Science, just as much as the arts, relies upon a sense of romance and intuition. Upon my return to Japan, I prepared an alkaline medium, placed a small amount of soil in it, and incubated it overnight at 37℃. To my surprise, various microorganisms flourished in all 30 test tubes. Here was a new alkaline world which was utterly different from the neutral world discovered by Pasteur. I named these microorganisms which thrive in alkaline environments "alkaliphiles," and coducted systematic microbial physiological studies of them. At the same time I focused my interest on the enzymes produced by these alkaliphilic microorganisms as well as on their molecular genetics. The results showed that these microorganisms, which are completely different from any previously reported, were widely distributed on the globe and they produced new products. This was my first encounter with a kind of superbugs, and subsequent to my work with alkaliphiles.

SUPERBUGS PROJECT

A five-year project, the Superbugs Project at the ERATO program in Japan was launched in 1984 to search for superbugs that grow in the extreme environments, and to try to use their unique properties to establish a "New Biotechnology". I was put in cahrge of the project which was initially called the "Project for studying microorganisms in extreme environments." This is something of a mouthful, so I renamed it the "Superbugs Project." This five year research project resulted in many significant scientific discoveries.
Let me show you some of the results.

1) Triangular bacteria

Key event in the superbug saga occurred in 1985, when we found a thin, triangular bacterium living in high concentrations of salt, for example in saltern soil in Japan. No previously known bacteria were triangular, usually they are spheres, rods or their derivatives.

About 600 samples were collected from various areas of high salt concentration in nature and small amounts of soil were suspended in a medium containing 20% NaCl. After 6 day's cultivation in the liquid medium at 37℃ with continuous shaking, the cell shape was examined using a phase contrast microscope. An isolate from a Japanese saltern soil of Ishikawa Prefecture exhibited triangular cells in the liquid medium. The bacterium is motile with a flagellum in the liquid medium containing high concentrations of salts.

The method of cell division of TR-1 attracted our scientific curiosity because of its characteristic shape. The cell division was recorded with a time lapse cinephotomicroscope equipped

with a 16 mm movie camera. The cell plate is formed between the apex of a triangle and the middle of the opposite side, or between the middle of any two sides. The average time for one cell cycle was 3.7 hours.

The strain TR-1 is an extremely salt-loving, halophilic archaebacterium. From taxonomical studies, we proposed TR-1 as a new species called *Haloarcula japonica*. We are currently interested in the salt stable enzymes of this isolate. (Fig. 1)

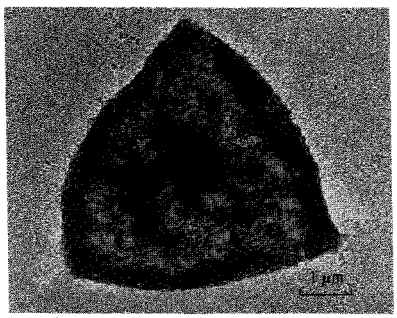

Fig 1 Haloarcula Japonica

2) Solvent tolerant life-form

Bacteria that thrive at high temperatures and in extremely saline or alkaline conditions are strange enough. But a recent discovery has exceeded all our conceptions of what constitutes a moderate environment. This novel life-form can thrive in a 50% solution of toluene, an organic solvent used in the manufacture of explosives. Normal organisms perish in even a 0.1% solution of this chemical. The newly discovered bug also tolerates high concentrations of xylene, styrene and other organic solvents that are toxic to most forms of life.

Surprisingly, the bug was found in normal soil. Researchers had looked in oil wells and tar pits, as well as factory discharges and refining sites, but they had not been able to find a toluene resistant bug. The media used for the isolation of the bacteria contained 30% toluene. A small amount of soil was suspended in the media in test tubes and the cultures incubated at 37℃ for one week in a test-tube shaker.

From 800 soil samples collected from all over the world, one strain, IH-2000 which grew well in the toluene medium, was isolated from a soil sample taken in Kumamoto, Kyushu in Japan. From taxonomical studies, the isolate IH-2000 (Fig. 2) was identified as closely resembling *Pseudomonas putida* except its toluene resistance. Unlike the *P. putida* strain, however

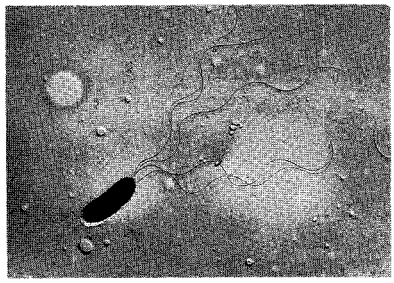

Fig 2 Strain IH2000: solvent-tolerant microorganism

IH-2000 is unable to utilize toluene as a nutrient. This is an electron micrograph of IH-2000 The doubling time for growth was one hour in the presence of toluene. And the strain IH-2000 has broad tolerance to other solvents, as I mentioned before. However, benzene, fluorobenzene lower alcohols, ethers and ketoses exhibit strong toxicity.

To investigate the genetics of solvent tolerance of strain IH-2000, we isolated solvent-sensitive mutants. Using these mutants as hosts. we have identified a fragment of DNA that appears to be responsible for the toluene tolerance. Although the nucleotide sequence analysis of the DNA fragment is still in progress, this is the first finding of a gene or genes for toluene tolerance.

Toluene resistance is more than a laboratory curiosity. It has potential uses in industry. For instance, certain fermentation processes such as the bioconversion of steroid hormones require huge amounts of water and extremely large fermentation vessels because of the low solubility of some of the compounds involved. But if we use an organic solvent in reactor vessels, we can readily dissolve water-insoluble compounds to effect efficient conversion. Recently, we have isolated many organic solvent tolerant microorganisms from deep-sea. Some of them can degrade crude oil in sea water as you can see in Fig.3. Therefore, these microorganisms have potential application to bioremidiation of oil-pollution.

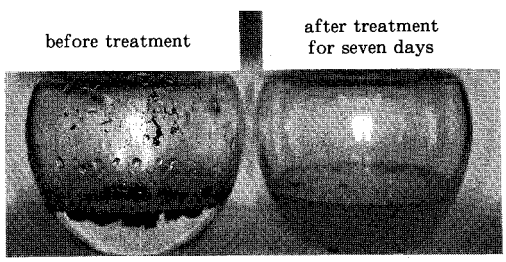

Fig 3 Degradation of oil-Spill in sea water by microorganisms

ALKALIPHILES.

As I mentioned, I discovered a whole new world of alkaliphilic microorganisms growing at around pH 10, and isolated several thousand strains from soil samples. One of the most important factors in the isolation of alkaliphiles from nature is pH control of the isolation media. On the laboratory scale, the media commonly contain 1 % sodium carbonate or sodium bicarbonate, which is usually sufficient to maintain the pH above 10 (Table 2).

Table 2. Basal Media for Alkalophilic Microorganisms

Ingredients	Horikoshi-I (g/l)	Horikoshi-II (g/l)
Glucose	10	–
Soluble starch	–	10
Polypeptone	5	5
Yeast extract	5	5
KH_2PO_4	1	1
$Mg_2SO_4 \cdot 7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20

1) Genetic applicatoins of alkaliphiles.

Basic genetic engineering has also benefit from superbugs technology. It may, for example, boost the use of *Escherichia coli*, the most common bacterium for genetic engineering. Segments of genetic material, DNA, can be added to the chromosome that cause the bacterium to produce specific molecules to order. A major problem is that *E. coli* has three biomembranes on its cell surface. Usually 500-1000 species of proteins remain trapped inside the cells. This makes the isolation of tailor-made proteins from genetically engineered *E. coli* very difficult.

To overcome the problem, we found a gene in a special alkaliphilic superbug that made the outer cell membrane permeable. When the fragment was introduced into *E. coli*, the improved *E, coli* possessed a changed cell surface structure that allowed specific proteins to be easily secreted, making them easier to collect and to purify. We have managed to produce several enzymes, human growth hormones and human immunoglobulin by using our improved *E. coli*.

Table 3. Enzymes Produced by alkaliphiles

	optimin pH	Stability pH	MW. $\times 10^4$
alkaline protease			
No.221	11.5-12.0	4-11	3
No.8-1	10.5-11.0	6-9	3
No.D-6	10.5-11	4-12	2-3
alkaline amylase			
No.A-40-2	10.5	7-9	7
No.H167	10-11	6-11	6.0
			7.3
			8.0
No.17-1	4.5,10	6-10	5-6
No.38-2	4.5	6-10	8.8
	7.0	6-9	8.5
	8-9	–	8.5
No.313	8	6-8	6.4
alkaline pectinase			
No.P-4-N	10.0	5-9	6-7
alkaline pullulanase			
No.202-1	9.0	6-10	9.2
alkaline cellulase			
No.N-4	6-11	5-11	4-8
No.212	6-8	5-11	5
No.1139	9	5-11	9.2
alkaline alginase			
No.M-2	9.0	8-10	4
alkaline catalase			
No.Ku-1	10.0	7-9	12.58
alkaline RNase			
No.243	9.0	6-10	1.2
alkaline DNase			
No.M-29	9.0	6-10	4
restriction enzyme			
No.170	7.5	–	–

	optimin pH	Stability pH	MW. $\times 10^4$
β-1,3-glucanase			
No.K-12-5	5.5-9	6-8	4
No.221	8.5	5-9	3.6
xylanase			
No.C-59-2	5.5-9	5-9	3.58
No.212	5.7	4-9	2.3
	6.8		3.7
	7.8		1.45
No.C-125	6-7	5-11	1.6
	6-10	4-12	4.3
α-galactosidase			
No.31-2	7.7	7.5-8.0	–
β-galactosidase			
No.31-2	6.5	5.5-9	18.5
penicilinase			
No.170	6-7	7-10	2-3
maltose dehydrogenase			
No.93-1	10.2	6-10	3.9
	9.8	7-8	4.8
glucose dehysrogenase			
No.93-1	9.8	6-8	5.1
uricase			
No.H-3	9	10	10
polyamine oxidase			
No.PO-1	4.0	3-6	6.4×2
	6.0		
β-mannnanase			
No.AM001	9.0	8-9	5.85
	9.0	8-9	5.95
	8.5	8-9	4.20
β-mannosidase			
No.AM001	6.0	6-5	9.4

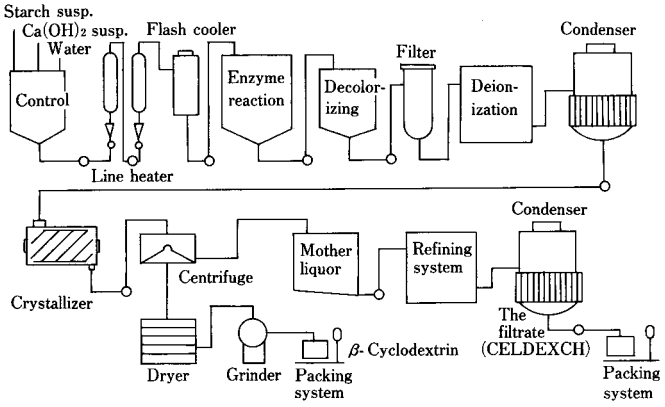

Fig 4 Flow chart of production of β—cyclodextrin

I should now like to turn my attention to enzymes produced by alkaliphiles and their industrial applications.

Studies of alkaliphiles have led to the discovery of many types of enzymes which exhibit unique properties in many respects. In our laboratories, about 35 new kinds of enzymes have been isolated and purified (Table 3). Some of them have been produced in industrial scale plants.

2) Cyclodextrin production

I would particularly like to highlight Cyclodextrin-forming enzymes and the industrial production of Cyclodextrin (CD), since the first industrial application of enzymes produced by alkaliphilic bacteria was the production of cyclodextrin from starch.

CD is a derivative of starch and a polymer of seven glucose units. This slide shows the molecular structure of cyclodextrin. This doughnut-shaped molecule has a hydrophobic cavity. It can be used to make so-called molecular capsules. Volatile compounds are trapped by CD and become non-volatile, liquids change to powder and unstable compounds become stable.

Mass production of this unique compound on an industrial scale has been attempted several times in the past. However, there were serious problems in the production processes. Firstly, the CD-forming enzyme were not thermostable enough for industrial use. Secondly, the yield of CD from starch was not high, usually 20 to 30 %. Thirdly, toxic organic solvents such as trichloroethylene, bromobenzene, toluene etc were used to precipitate CD because of the low conversion rate. The use of such harmful organic solvents is now strictly prohibited in various fields. Thus, the development of large-volume systems was quite limited.

We solved the problems by isolating several CD-forming enzymes from alkaliphilic bacteria. One of them overcame all these drawbacks and allowed mass-production of crystalline CD at low cost without the use any organic solvents.

The simple method we developed was able to reduced the cost of CD from 50,000 yen to 2,000 yen per kilogram (Fig.4). This success has paved the way for its use in a large variety of foodstuffs, chemicals and pharmaceuticals (Table 4).

This table summarizes some of the industrial applications of CD.

3) A new industrial application of alkaline cellulase

Another superbug investigated by our group possessed very strong cellulose degrading enzymes that could function in high alkalinity. No cellulase with an alkaline optimum pH for activity, pH 10 or higher, had been reported at that time.

About 15 years ago, we found that a newly isolated bacterium produced cellulases which could hydrolyze only modified cellulose in alkaline conditions. This was the first finding of alkaline cellulase in the world (Fig.5)

Cotton absorbs sweat, grease and other stains very well, but it is very difficult for conventional laundry detergents to remove stains from cotton fabrics at lower temperatures. Stains on cotton fibers are largely trapped in amorphous hydrated cellulose. It was shown that in the presence of alkaline cellulases, a part of this hydrated cellulose was modified and soil was easily removed by a detergent. Our alkaline cellulase were mixed with laundry detergents and the change in the washing effect was analyzed. One of our alkaline cellulases

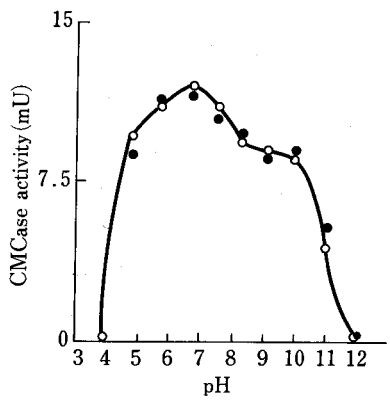

Fig 5 Effect of pH on unpurified CMCase activity

Table 4 Industrial Applications of Cyclodextrins

Functions	Guests	End Products
Foods		
(1)Emulsification	Oils and fats	Margarine, Cake, Whipping cream, French dressing
(2)Stabilization	Flavors, Spices, Colors and pigments	Horse radish paste, Mustard paste Cakes and cookles, Pickled vegetables, Dried vegetables
(3)Masking of taste and odor		Juices, Soy milk, Bone powder, Boiled rice
(4)Improvement of quality		Hard candy, Cheese, Soy sause, Canned citrus fruits and juices
(5)Reduce volatility	Ethanol	Food preservatives
(6)Otheres		Breath mints
Cosmetlcs and toiletries		
(1)Emulsification	Oils and fats	Face creams, Face lotion, Tooth pastes
(2)Stabilization	Flavors and fragrances	Bath refresher crystals
Agrochemicals		
(1)Stabilization	Pyrolnitrin	Fungicide
	Pyrethroids	Insecticide
(2)Reduce volatility	Organic Phosphates (DDVP)	Insecticide
	Thiocarbamic acid	Herbicide
(3)Reduce toxicity	2-Amino 4-methyl-phosphynobutyric acid	Fungicide

Functions	Guests and end products
Pharmaceuticals	
(1)Improve solubility	Prostaglandins, Steroids, Cardiac glycosides, Non-steroidal antl-inflammatory agents, Barbiturates, Phenytoin, Sulfonamides, Sulfonylureas, Benzodiazepines
(2)Chemical stabilization	
(A)Hydrolysis	Prostacyclin, Cardiac glycosides, Aspirin, Atropine, Procaine
(B)Oxidation	Aldehydes, Epinephrine, Phenothiazines
(C)Photolysis	Phenothiazines, Ubiquinones, Vitamins
(D)Dehydration	Prostaglandin E_1, ONO-802
(3)Improve bioavailability	Aspirin, Phenytoin, Digoxine, Acetohexamide, Barbiturates, Non-steroidal antiinflammatories
(4)Powdering	ONO-802, Clofibrate, Benzaldehyde, Nitroglycerin, Vitamin K_1, K_2, Methylsalicylate
(5)Reduce volatility	Iodine, Naphthalene, *d*-Camphor, *l*-Menthol, Methylcinnamate
(6)Improve taste, smell	Prostaglandins, Alkylparabens
(7)Reduce irritation to stomach	Nonsteroidal antiinflammatory agents
(8)Reduce hemolysis	Phenothiazines, Flufenamic acid, Benzylalcohol, Antibiotics

showed the best results. The alkaline cellulase does not attack cotton, but releases dirt attached to amorphous cellulose fibers. Fabrics are mostly crystalline cellulose, and this kind of cellulose is never degraded by our alkaline cellulases. Several laundry detergents containing the alkaline cellulase are now commercially available. In Japan, such laundry detergents enjoy approximately 60% of the market. To the best of my knowledge, this is the first large scale industrial application of cellulase, I am convinced that no laundry detergent contaning alkaline cellulase would have been developed, i we had not found and not reported the production of alkaline cellulase by alkaliphilic bacteria.

CONCLUSION

In closing my talk, I would like to say that a new kind of microbiology has been developed very rapidly. This new microbiology is not restricted by the conventional anthropocentric view of microbiology, but a microbiology based

on studying microorganisms in their optimal conditions for life. We must study them as they are. Soil contains 10 million to one billion counts of microorganisms per gram, if you count them under the microscope. How many of these microorganisms can we isolate and grow? The answer is very disappointing. Recovery is only about 1 to 10% even by skillful microbiologists. We have not discovered how to grow all of them, although these microorganisms thrive in nature. This is proof enough that our knowledge is still insufficient. We know even less about extreme environments, but a great variety of these undiscovered microorganisms are distributed on the Earth, indicating the boundlessness of the information they have to offer.

A new 15-year research program, called International DEEP STAR (Deep-sea Environment Exploration Program, Science and Technolgy for Advanced Research) was launched in October, 1990. I have been placed in charge of this program and would like to expand the sources of microorganisms for study from the surface of the globe to the deep sea. We have the use of two submarines, and we can dive to 6,500 meters by using the Shinkai 6500 of the Japan Marine Science and Technology Center and collect samples from deepest parts of the oceans (Fig.6)

Many microorganisms in the deep sea are true superbugs, because they may experience the extreme conditions, of low temperatures, high temperatures, high pressure (Fig.7), or in high concentrations of inorganic compounds. Imagine: They have never experienced solar energy, and they have to eat foodstuffs not derived from sunlight. Some of them have an utterly defferent metabolic pathway from conventional life-forms and metabolize inorganic sulfur or iron as their energy source. It is distinctly possible that very ancient life-forms may be in hibernation in the worlds largest refrigerator. Genetic engineering, protein engineering and the so called modern biology of microbes isolated from the deep sea will give us new information on the origins of life and its evolution.

Finding new life-forms will definitely develop basic science and new biotechnologies. Basic science is the one common language of all human beings. We have just started to communicate with nature by using basic science. Science is just as a sheet of white paper. If Monet placed his colors on this paper, the paper would become a painting. If Beethoven wrote on the paper, the paper would become music, In conclusion, I am convinced that microbiologists will have the opportunity to create a new biotechnology, if they know how to ask the microorganisms.

Thank you for your attention.

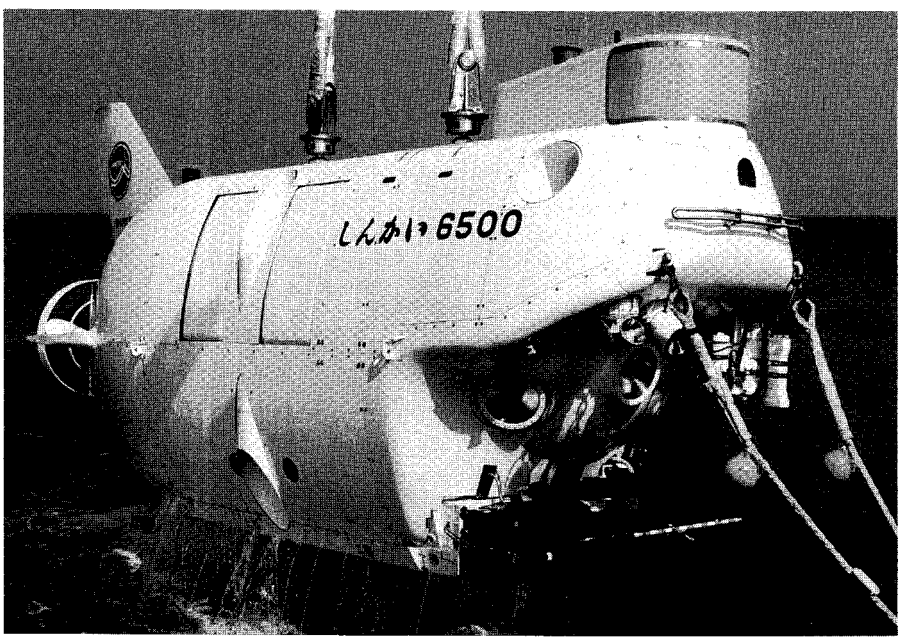

Fig 6 Shinkai 6500

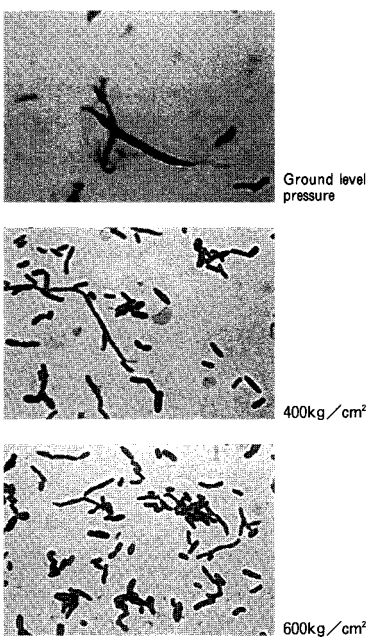

Fig 7 DB6906 morphology cultured under the various pressure