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Derivation of ciliate from a dinoflagellate-like ancestor

Hiroshi ENDOH (Department of Biology, Faculty of Science, Kanazawa University)

When compared with simple flagellates, extant ciliates are so complicated in constitution that their evolutionary pathway has been unknown. Expanding molecular data appear to have recently led to a general agreement that ciliates are closely related with dinoflagellates and apicomplexans, the three phyla being grouped in Alveolata. On the other hand, parasitic opalinids are generally grouped in heterokonts, but a β-tubulin gene phylogeny brought opalinids within alveolates (A. Nishi, in this meeting). Based on this information, I propose a possible evolutionary pathway of ciliates from a multicellular dinoflagellate-like ancestor such as a genus *Polykrikos,* from which oplainids might have also originated. In this scenario, the common ancestor of ciliates and opalinids might have attained multinuclear and multiciliary state by multicellularization and a subsequent reunicellularization. Then the ancestor would have started parasitism, and all opalinids remain parasitic even now. After an ancestral ciliate diverged from opalinids, ciliates might have elaborated DNA elimination, resulting in spatial differentiation of germline and soma within a single cell. This compaction of the somatic genome might be a reflection of an adaptation to parasitism, as frequently seen in parasitic organisms. Ciliates could have recovered a free-living life shortly after the adaptation, followed by cytostome formation and development of polyploid macronucleus accompanied with DNA amplification. Formation of the macronucleus might have resulted in the loss of mitotic ability because of a large number of fragmented chromosomes, as seen in the primitive ciliates, karyorelictids, until ciliates invented amitotic division later.

Are opalinids alveolates?

Akane NISHI (Department of Biology, Faculty of Science, Kanazawa University)

Opalinids are endosymbionts that inhabit the large intestine of anuran amphibians. Opalinids possess many nuclei and are uniformly covered with numerous cilia, so that they look like ciliates. Opalinids share some characteristics with both flagellates and ciliates, so opalinids have been placed in various systematic positions since their discovery. At present researchers in the field seem to agree that opalinids are classified into the Class Opalinea in heterokonts, based on the presence of a ciliary transitional helix which is characteristic of heterokonts. However, opalinids do not share another synapomorphy of heterokonts, flagellar hair (mastigonemes). Here I propose a new phylogenetic position of opalinids based on β-tubulin gene phylogeny. Because of the difficulty of pure preparation of opalinid cells, molecular biology of opalinids has not been reported at all so far. In order to avoid contamination, DNA was extracted after repeated washing of 10 opalinid cells by micropipette. Thereafter the 18S rRNA gene and β-tubulin gene of opalinids were amplified. Partial 18S rDNA sequences commonly contained an opalinid-specific insertion in the 3' portion of the coding region, indicating no contamination by other protists. Phylogenetic analysis of the β-tubulin gene placed opalinids within alveolates, consisting of ciliates, apicomplexans and dinoflagellates, notin heterokonts. In fact, opalinids share more characteristics, e.g. multinucleate, many cilia, double stranded ciliary necklace, with alveolates than heterokonts. Considering these results, opalinids should be given an independent phylum in Alveolata, unlike the previous view.

Infection of symbiont-free *Paramecium bursaria* with yeasts

Toshinobu SUZAKI[1], Gen OMURA[1] and Hans-Dieter GÖRTZ[2]
([1]Dept. Biol., Fac. Sci., Kobe Univ.; [2]Biol. Inst., Univ. Stuttgart)

Chlorella-free *Paramecium bursaria* is known to be infected with various species of free-living microorganisms including yeasts and bacteria. In an attempt to understand the mechanism of establishment of the symbiosis, infection with three different species of yeasts was examined. When *Saccharomyces cerevisiae* was mixed with *Chlorella*-free *P. bursaria* in sterile conditions, ingested yeasts were retained in the cytoplasm of *P. bursaria* for more than one week. The yeasts did not associate with the cortical layer of cytoplasm, but kept moving around in the streaming cytoplasm. The yeasts were

readily ejected from the cytoproct when *P. bursaria* was fed with food organisms (*Chlorogonium elongatum* or *Micrococcus luteus*) or *Chlorella* that had been cultured outside *P. bursaria*. When other species of yeasts (*Rhodotorula rubra* or *Yarrowia lipolytica*) were introduced, the ingested yeasts stayed permanently in the cortical cytoplasm among the trichocysts and multiplied in the cytoplasm. With these species, yeast retention is not disturbed by food uptake, but they disappear when the paramecia are re-infected with *Chlorella*. When *P. bursaria* was fed with *Chlorella* or yeasts, trichocysts became detached from the cell cortex, and finally disappeared allowing the ingested symbionts to approach the cell cortex. At the same time, many small vesicles of about 0.5 μm in diameter were observed instead. These observations suggest that close association of yeast cells with the inner surface of the cell cortex may be important for establishing stable interaction with the host paramecia.

Preliminary studies on the estimation of biomass of soil ciliates by the MPN method

Tadao TAKAHASHI[1], Yurika AKASAKI[1], Chifumi NODA[1], Sadako HASHIGUCHI[1], Hiromi FUKUDA[1], Saori MOMOSAKI[1], Kuniyo WATANABE[1] and Tomoyoshi HASHIMOTO[2]
([1]Biol. Lab., Nishikyushu Univ., [2]Natl. Agr. Res. Cent. Kyushu Okinawa Reg.)

In order to understand the role of ciliates in the soil ecosystem, it is very important to determine their fauna and biomass. However, the methodology for the estimation of their biomass has not yet been sufficiently established. The purpose of this study is to examine whether the MPN (Most Probable Number) method, which is frequently used in the estimation of the biomass of soil bacteria and fungi, is applicable to soil ciliates. In this work, soil samples were collected from a farm, which had been supplied livestock slurry for a long period, in the Kyushu Okinawa agriculture research center at Miyazaki. The biomass of the soil ciliates was estimated using two MPN sub-methods. In sub-method 1, a 10-fold dilution series of soil samples was transferred to a 96-well microplate, 0.1 ml sample per well. The plates were maintained at 23°C and monitored regarding the appearance of ciliates in each well for about one month. In sub-method 2, a 10-fold dilution series of soil samples was transferred to 3-well-depression slides with 0.7 ml sample each. These were also maintained and monitored regarding the appearance of ciliates for about 2 weeks. Furthermore, we transferred the ciliates which were found in the depression slides to 1% methylcellulose and identified their genus. We obtained significantly different estimations of the biomass for the same soil samples when using different sub-methods. The cause of these differences should be investigated in the future.

Molecular cloning and characterization of farnesyltransferase of *Entamoeba histolytica*

Masahiro KUMAGAI[1], Asao MAKIOKA[1], Tsutomu TAKEUCHI[2] and Tomoyoshi NOZAKI[3,4] ([1]Dept. Trop. Med., Jikei Univ. Sch. Med., [2]Dept. Trop. Med. Parasitol., Keio Univ. Sch. Med., [3]Dept. Parasitol., Natl. Inst. Infect. Dis., [4]PRESTO, JST)

Farnesylation, one of the post-translational lipid modifications of proteins such as Ras, is catalyzed by farnesyltransferase (FT). Since farnesyaltion is essential for Ras to function, FT has been attracted attention as a target of cancer chemotherapy. We studied FT of *Entamoeba histolytica* biochemically to assess its biological importance and possibility as a target of chemotherapy. cDNA of α and β subunit (FTα, FTβ) of the enzyme were PCR amplified using primers designed on homology search of *E. histolytica* genome databases at NCBI. Those encoded 298 and 375 amino acid open reading frames, respectively, with 24-36% positional identity with those of human, *Saccharomyces cerevisiae* and *Trypanosoma brucei.* The coding regions of both subunits, and ribosome binding sites between them, were cloned in tandem in pQE31, and were expressed in *Escherichia coli*. Thirty-eight and 43 kD proteins, corresponding to FTα and FTβ were produced. The recombinant proteins farnesylated human recombinant H-Ras(-CVLS), and showed little activity on mutant Ras(-CVLL), when assayed by incorporation of [^{3}H]farnesyl pyrophosphate. Among 4 recombinant *E. histolytica* Ras homologues (-CIMF, -CELL, -CSVM, -CVVA), only Ras homologue-CVVA was the substrate. This suggests a difference in substrate specificity between human and *E. histolytica* FT, which may be exploitable for chemotherapy.

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Inactivation of *Giardia lamblia* cysts by ultraviolet irradiation

Shinji IZUMIYAMA, Kenji YAGITA, Tokiko ASAKURA and Takuro ENDO
(Dept. Parasitol., National Institute of Infectious Disease, Japan.)

The present paper deals with the sensitivity of *G. lamblia* cysts to ultraviolet irradiation as a possible measure for the inactivation of enteric pathogens transmitted via the drinking water. *G. lamblia* cyst suspensions to be irradiated were placed in open 6cm plastic Petri dishes. The depth of the suspensions was 4.7 mm. The ultraviolet source was a 5 W low-pressure mercury lamp, which gave an intensity of 0.05 mW/cm^2 at a distance of 39 cm for the *G. lamblia* exposures. The cyst suspensions were exposed at the constant ultraviolet intensity mentioned above with varying exposure time so as to give exposure doses for up to 1.2 mJ/cm^2 at varying water temperatures ranging from 4 to 30°C. The cysts viability was tested by cultivation in TYI-S-33 culture medium. Briefly, the irradiated cysts were treated with excystation medium, and were concentrated into inocula - 0.1 mL each, containing between 10^0 and 10^4 cysts with decimal dilutions — which were transferred into screw-capped glass vials filled with the culture medium to minimize gas phase. The cultures were observed microscopically for signs of *Giardia* growth for up to 2 weeks, and titers were calculated as most probable numbers (MPN). A 2-log$_{10}$ reduction in activity, namely 99% inactivation, was obtained at 1 mJ/cm^2 (= mW · s/cm^2) regardless of water temperatures examined. The results obtained in the present experiment demonstrate that ultraviolet irradiation is a plausible method for the inactivation of *G. lamblia* cysts.

First isolation of pathogenic *Naegleria australiensis* in Japan

Tokiko ASAKURA, Kenji YAGITA, Shinji IZUMIYAMA, Rieko SHIMOGAWARA and Takuro ENDO (Dept. Parasitol., National Institute of Infectious Disease, Japan.)

Free-living amoeboflagellates of the genus *Naegleria* are found worldwide in varieties of waters. In this genus, there have been three species living in thermal waters reported to be pathogenic to human and animals (*N. fowleri*) or to have potential to kill experimental animals (*N. australiensis*, *N. italica*). The objective of this study is to demonstrate a possible occurrence of pathogenic *Naegleria* in man-made thermal waters including whirlpool baths commonly used in Japan. Water samples were collected mainly from whirlpool baths, and cultured at 42°C for up to 7 days on nonnutrient agar plates coated with heat inactivated *Escherichia coli*. Amoebae isolated from the culture with the morphology of Vahlkampfiidae were cloned and analyzed by means of PCR/RFLP for the ITS region according to the method of Pelandakis and Pernin (2002) with slight modifications. The 400 bp PCR product can be obtained from amoebae in the genus *Naegleria*, of which RFLP for *Mse* I gives a major fragment of 330 bp for *N. australiensis* and two fragments of 287 bp and 100 bp for *N. fowleri*. Among 26 *Naegleria* isolates so far examined, 11 isolates showed the PCR/RFLP pattern of *Naegleria* including *N. australiensis*. Further investigation by the direct sequencing of the ITS region revealed that there were two types of *N. australiensis*; one had ITS sequence identical to the authentic strain of *N. australiensis* (PP-397) while the other one had an insertion of 2 thymine residues at +322 in the sequence. These two strains were also confirmed to have identical isozyme patterns for both acid phosphatase and propionyl esterase to those of PP-397. This is the first report for the isolation of *N. australiensis* in Japan. The isolates are presently processed to test for their ability to kill mice after intranasal instillation.

Comparative study of protein profiles on pathogenic and nonpathogenic *Naegleria* species by 2D-PAGE (2)

Mako OMURA, Kenji YAGITA, Shinji IZUMIYAMA, Rieko SHIMOGAWARA and Takuro ENDO
(Dept Parasitol., NIID, Japan)

Naegleria fowleri, a causative agent of primary amoebic meningoencephalitis (PAM), is known to be morphologically indistinguishable from non-pathogenic *N. lovaniensis*. Their protein profiles by isoelectric focusing (IEF) separation were reported to be also quite the same. Recent technological progress of protein analyses makes it possible to compare proteomes of the genus *Naegleria* by analyzing the spots into amino acid level after being separated by 2D-PAGE. An average gel of *N. fowleri* which ideally represented protein spot patterns common in the species was obtained from the 5

strains of this species, such as 4 authentic strains (Nf66, KUL, LEE, 76/14/S3) and one Japanese isolate (KURUME), and detected 228 protein spots common in the species. Similarly, an average gel of *N. lovaniensis* was obtained from 2 strains (Aq/9/1/45D, TS), and detected 246 spots. The pairwise comparison of average gels using computer-assisted spot matching showed that these two species exhibited marked diversity, with percentage of matching being 38.6% (88 common spots). Among 140 spots specific for *N. fowleri*, 16 were selected and analyzed the N-terminal amino acid sequences, and revealed that a 17 kDa protein (pI ~ 5.2) was identified as Mp2CL5 membrane protein specific for *N. fowleri*. Four additional protein spots could be demonstrated to be highly related to other known proteins, namely HSP-70 and three enzymes of carbohydrate metabolism.

Inhibition of mitochondrial respiration by climacostol

Yoshinori MUTO[1], Yumiko TANABE[2], Kiyoshi KAWAI[2] and Hideo IIO[3]
([1]Dept. Basic Health Sci. and Fundamental Nurs., Gifu Univ. School of Med., [2]Fac. Wellness, Chukyo Women's Univ., [3]Dept. Material Sci. Graduate School Sci., Osaka City Univ.)

Climacostol, found by Miyake et al., is a resorcinol derivative that exists in ciliate *Climacostomum virens*. Exposure to purified climacostol results in lethal damage to the predatory ciliate *Dileptus margaritifer* and several other ciliates. To elucidate the mechanism of climacostol action, we investigated the effects of this compound on the swimming behavior of *Tetrahymena* and respiration of rat liver mitochondria. When added to living *Tetrahymena* cells, climacostol markedly increased the turning frequency that is accompanied by a decrease in swimming velocity and subsequently followed by cell death. Observation by fluorescence microscopy showed morphological changes in mitochondria stained with Mitotracker, indicating that mitochondria may be a target site for climacostol. Furthermore, climacostol inhibited NAD-linked respiration in rat liver mitochondria, but had no apparent effect on succinate-linked respiration in submitochondrial particles. This finding indicates that climacostol specifically inhibits respiratory chain complex I in mitochondria. Taken together, these results suggest that the inhibition of mitochondrial respiration may be a cytotoxic mechanism of climacostol's defenses against predatory protozoa.

Purification of bacteria-derived protein that neutralizes toxin in WGP used for the growth of *Paramecium*

Naomi MIZOBUCHI[1], Kumio YOKOIGAWA[2, 1], Terue HARUMOTO[3, 1], Hiromi FUJISAWA[1, 3] and Yoshiomi TAKAGI[3, 1]
([1]Div. Human Envir. Sci., Grad. Sch. Human Cult., Nara Women's Univ.; [2]Dept. Food Sci. Nutr., Fac. Human Life Envir., Nara Women's Univ.;[3]Dept. Biol., Fac. Sci., Nara Women's Univ.)

Paramecium cells are usually cultured in bacterized plant infusion; wheat grass powder (WGP) infusion inoculated with *Klebsiella pneumoniae*. However, WGP infusion without bacteria is toxic to paramecia, and bacteria derived substances detoxifies the killing substance. Here, the detoxifying substance, which was found proteinaceous, was purified to homogeneity from *K. pneumoniae*. The protein had an apparent molecular mass of about 200 kDa by gel filtration and 92 kDa by SDS-gel electrophoresis. Although the amino acid sequence of amino terminal region did not show sequence identity with any reported proteins, amino acid sequences of internal regions of the protein were almost identical with catalase HPII from *Escherichia coli*. When the WGP infusion (3 ml) was treated at 25°C for 1 h with 0.8 unit of commercially available catalase from bovine liver, the toxicity of the infusion against paramecia was completely abolished. The initial concentration of hydrogen peroxide in the WGP infusion was about 30 μM and completely decomposed by the catalase treatment. Therefore, the toxic substance in the WGP infusion and the detoxifying substance from *K. pneumoniae* are considered hydrogen peroxide and catalase, respectively.

Analysis of mechanisms of cortical pattern formation in the ciliate *Paramecium* using a microsurgical technique

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Norikazu MIYOSHI and Tadao TAKAHASHI (Bio. Lab., Nishikyushu Univ.)

It is well known that the cell surface of the ciliate *Paramecium* consists of cortical units which are arranged in a certain regular pattern. It was previously considered that the organizing center of the cortical structures might exist in the center of the ventral equatorial region. In contrast, Takahashi et al. (1998) reported that in *P. trichium* the special cortical units, which are located in the most posterior position of 1C-2BB (two basal bodies with one cilium) units region, might contribute continuously to cortical reproduction; that is, they may be the stem units. The purpose of this study was to examine this hypothesis using a microsurgical technique. We transected and removed the posterior 1/4 region of the cell, which probably included the hypothetical stem unit region, by using a fine glass needle, and then traced their regeneration processes during the three cell generations. We found that the missing part of the cell was gradually regenerated and completely recovered until the completion of the third cell division after the operation. These observations suggest that the formation of the whole cell surface structures is accomplished according to the positional information which is assumed to exist along the longitudinal cell axis, although the stem units may continuously supply new cortical units.

Breeding analysis of the mutant with long autogamy-immaturity in *Paramecium tetraurelia*

Rie KOMORI[1], Terue HARUMOTO[2, 1], Hiromi FUJISAWA[1, 2] and Yoshiomi TAKAGI[2, 1]
([1]Div. Human Envir. Sci., Grad. Sch. Human Cult., Nara Women's Univ., [2]Dept. Biol. Sci., Fac. Sci., Nara Women's Univ.)

We have attempted to isolate mutants with modified life cycle features in order to anatomize time-measuring process in *Paramecium*. We reported the stock d4-RK with unusually long autogamy-immaturity: 0% autogamy at the age of 27 fissions when 97.3% autogamy on average was observed in the wild-type stock 51. Autogamy in d4-RK began to be induced at about age 50 in some clones and scarcely even after age 200 in other clones. This phenotype was expressed only at 25°C, and at 32°C there occurred frequent autogamy, indicative of a conditional mutant. Breeding analysis indicated that this mutant phenotype is attributable to a single recessive mutation. We expected that this mutation might also extend the clonal life span, because the correlation between the length of sexual maturation and the life span was reported in mammals and ciliates. However, d4-RK was shorter in the clonal life span and slower in fission rate than the wild-type. This result, however, does not rule out the possibility that some mutations other than the mutation in question caused by mutagen-treatment may be responsible for these phenotypes. To examine this possibility, progeny clones after repeating back-crosses should be studied about their clonal life span.

Purification and molecular identification of mating-type substances in *Paramecium caudatum*

Kozue SUZUKI and Mihoko TAKAHASHI (Institute of Biological Sciences, Univ. of Tsukuba)

In the mating reaction of *Paramecium caudatum*, specific cell-cell recognition between complementary mating types takes place due to the interaction between mating-type substances. Although previous studies have shown that the mating-type substances are proteins, none has succeeded in the direct identification of the molecules. To identify the molecules, two analyses were performed. After mild treatment with trypsin, ciliary supernatant was subjected to 2D-PAGE, native-PAGE and SDS-PAGE. Alternatively, it was subjected to a Mono Q column, ammonium sulfate fractionation and a Phenyl Superose column. Mating-type substances were monitored with a mating-type specific monoclonal antibody. The two analyses identified only a 95 kDa protein in common. The 95 kDa protein appeared at mating-reactive periods in culture age. These results suggest that the 95 kDa protein is a mating-type substance.

A destiny of maternal macronuclei after conjugation in *Paramecium caudatum*

Naomi KIMURA (Dept. of Biology, Kanazawa Univ.)

In *Paramecium caudatum*, a maternal macronucleus which is fragmented into 40 pieces during conjugation does not degenerate at once, but persists until the 8th cell cycle after conjugation. It is known that during the period their fragments maintain their regeneration competence and are simply distributed to daughter cells without dividing. Their subsequent fate has been still uncertain. Here I demonstrate that the initiation of the maternal macronuclear degeneration occurs at the 5th cell cycle after conjugation. When the fragments were measured, the size continued to increase from the 1st to 4th cell cycle, and then shifted to rapidly decrease, while a new macronucleus gradually grew and reached the maximum in size at the 4th cell cycle. In addition, small pycnotic fragments were observed at the 6th or later stage, suggesting DNA degradation of the maternal macronucleus. In order to detect the degradation, total DNAs were isolated from conjugants and exconjugants in various stages. Southern blot analysis revealed that a slightly sized-down DNA appeared around at 5th cell cycle. These observations lead me to the following conclusion: The moment the new macronucleus is completed at the 4th cell cycle, the maternal macronuclear fragment might be destined to degenerate. Until then, the maternal fragments would assist the function of the imperfect new macronucleus.

Analysis of genes expressed prior to nuclear differentiation of *Paramecium caudatum*

Akiko TAKASE[1], Masahiko SHIBAZAKI[2], Hideaki KIKUCHI[2] and Kazuyuki MIKAMI[1]
([1] EEC Miyagi Univ. Edu., [2] IDAC Tohoku Univ.)

Paramecium caudatum is a unicellular animal having a large somatic macronucleus and a small germinal micronucleus. The macronuclei and micronuclei differentiate from division products of the micronucleus after conjugation. In order to understand the nuclear differentiation mechanism, we investigated genes that were expressed at the time of macro- and micronuclear differentiation by using differential mRNA display. In this study, we obtained fragments of about 30 genes that were expressed specifically at the stage of nuclear differentiation. A BLAST search with the BLASTN program revealed that some of these fragments had homology with the mitochondrial large ribosomal RNA (lrRNA) gene (*P. tetraurelia*), the alpha-tubulin gene (*P. caudatum*), or the 14-3-3-protein gene (*Tetrahymena pyriformis*). So far, the remaining fragments have not been found to be homologous with already known genes. The mitochondrial lrRNA brings to mind a determining factor in germ cells in *Drosophila melanogaster*. However, we did not obtain any evidence to show that the same system as in *Drosophila* is present in protozoa. We then investigated whether the transcriptional activity of mitochondria has any effect on nuclear differentiation. When an antibiotic, rifampicin (Wako) (1 mM, 20–30 *pl* per cell), was injected into cells, no macronuclear anlagen developed. This suggests that the transcriptional activity of mitochondria has some relationship with macronuclear differentiation.

Analysis of genes expressed at gametogenesis and fertilization in *Paramecium caudatum*

Ikuno TAGA[1], Mayuko KUMASAKA[2], Hiroaki YAMAMOTO[2] and Kazuyuki MIKAMI[1]
([1]EEC, Miyagi Univ. Edu., [2]Biol. Inst., Grad. Sch., Tohoku Univ.)

The germ micronucleus undergoes meiosis and produces four haploid nuclei when conjugation is induced in *Paramecium caudatum*. Three of the nuclei disappear but one of them survives. This remaining nucleus divides once and produces gametes (migratory nucleus and stationary nucleus). The degeneration of meiotic products seems to be a type of apoptosis, because they became pycnotic and produced oligonucleosomal-length fragments during degeneration. We consider this nuclear apoptosis according to Mpoke *et al* (1997). It is known that expression of some key genes for degeneration versus survival of meiotic products occurs just after meiosis I. In this study, we cloned some specific genes that were expressed at this stage by the method of subtractive hybridization. We sequenced 100 clones but, so far, these clones do not contain genes that participate in the cascade of caspase in apoptosis. We also investigated whether the process of nuclear apoptosis was caspase-dependent. To probe the possibility, conjugating pairs were isolated into solutions of a caspase inhibitor, Z-VAD-FMK (0.2 - 2.0 mM, LD_{50} = 0.75 mM), just after meiosis I. Moreover, the solutions (0.2, 2 or 20 mM, 20–30 *pl* per cell) were injected into conjugational pairs just after meiosis I or meiosis II. However, neither of these

treatments had any effect on nuclear degeneration. These results suggest that nuclear apoptosis of haploid nuclei does not depend on caspase, though we cannot prove it.

Stable expression of GFP-gene in the last cells of the life history in *Paramecium*

Yasuhiro TAKENAKA[1] and Nobuyuki HAGA[2]
([1]National Institute of Agrobiological Science, [2]Dept. of Biotech. Ishinomaki Senshu Univ.)

We have examined the pattern of gene expression in the last phase of clonal aging in *Paramecium* using the GFP-gene as a reporter. Many cells unable to undergo cell division because of aging were collected from subclonal cultures derived from a single cell that was injected with the GFP-gene. Although the morphological characteristics of the last cells of the life history were slim and clean in most cases, with occasional abnormal shape and incomplete cell division, all cells examined showed strong green fluorescence in cytoplasm and nuclei. This indicates the presence of a stable protein synthesis pathway from the GFP-gene in the last phase of clonal aging. On the other hand, the pattern of longevity in the subclones showed two subgroups; one with a relatively short life span and the other long. Both groups had a normal distribution pattern with similar standard deviations. This result suggests that random events are involved in the causes of longevity in clonal aging. It is important to analyze DNA sequences in the region where the GFP-gene is located in the gemone. An understanding of stable GFP-gene expression in the last phase of clonal aging may provide the way for extending the life span in *Paramecium*.

Stability of genomic DNA during clonal aging in *Paramecium caudatum*

Satoshi OHTA and Nobuyuki HAGA (Dept. of Biotech. Ishinomaki Senshu Univ.)

During clonal aging, the cells of *Paramecium* show remarkable changes in cellular functions, such as decline of cell growth ratio, decrease in mating activity, increase in progeny mortality and aberration in cell morphology. In *P. tetraurelia*, dramatic changes in chromosomal DNA size according to clonal aging were also reported. To analyze the stability of chromosomal DNA size distribution during clonal aging in *P. caudatum*, we have established a method for the separation of intact chromosomal DNA by a modified method of CHEF (clumped homogeneous electric fields electrophoresis). The DNA banding pattern examined in cells of three different ages, 100, 300 and 600 fissions after conjugation, showed five major DNA bands (1100, 900, 97, 48 and 25 Kbp) in all ages of cell lines. These results indicate that all chromosomal DNA is stably maintained during clonal aging in *P. caudatum*. The cells at a 600 fission-age, however, showed a significant reduction in cell division ratio in comparison with the other two cell lines. Therefore, it is important to analyze local changes in chromosomal DNA to detect the genetic causes of this reduction in cell division ratio.

Tolerance and adaptation to of low temperature in *Paramecium*

Hajime SASAKI and Nobuyuki HAGA (Dept. of Biotech., Ishinomaki Senshu Univ.)

Adaptation to low temperature seems to be an essential strategy for wild animals and plants to survive in the winter season. Little is known about the lifestyle of ciliates in the winter season. To understand the wintering of *Paramecium* at low temperature, we have examined cell growth ratio under low temperature conditions using 8 cell lines of *P. caudatum*. We found that KNZ2 showed remarkable tolerance to low temperature compared with the other 7 cell lines. The cells of KNZ2 underwent several cell divisions at 5°C during the 20 days of single cell isolation-line culture. The progeny of selfing conjugation of KNZ2 showed 4 different types of growth pattern under low temperature conditions: 1) stable growth, 2) unstable growth with a mixture of live and dead cells in the daughter cell population, 3) living, without cell division, and 4) dying, without cell division. The segregation ratio of these 4 types was approximately 1:1:2:1. These results suggest that several genes may be involved in growth regulation under low temperature conditions. We think that it is useful to assume the existence of "wintering genes" in a working hypothesis of tolerance and adaptation to low temperature.

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Studies on 12 kDa FK506-binding protein (TFKBP12) in *Tetrahymena thermophila*

Ryoko OSAWA, Yasuo NAKASHIMA, Hironori UENO and Osamu NUMATA

The 12-kDa FK506-binding protein (FKBP12) is well conserved from yeast to human, and is known to bind with the immunosupressant FK506 [(2)]. FKBP12 is also known to bind with a calcium channel called type-1 ryanodine receptor [(1,3,4,5)]. This FKBP12 also exists in *Tetrahymena thermophila*. The present study was undertaken to investigate the functions of FKBP12 in *Tetrahymena thermophila* (TFKBP12). The TFKBP12s ORF size was 330 bp, the domain for PPIase was conserved, and the TFKBP12 gene was a single gene. TFKBP12 was localized around basal bodies and studies with immuno-EM showed that TFKBP12 was localized on the epiplasmic layer near basal bodies. From the results of our studies and the fact that other FKBP12s work as regulators of calcium, TFKBP12 may be working as part of a calcium regulating system near cilia. References: [1]Brillantes, A. B., Ondrias, K., Scott, A., Kobrinsky, E., Moschelle, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks A. R (1994) Cell 77, 513-523. [2]Hamilton, G. S. and Steiner, J. P. (1998) J. Med. Chem. 41, 5779-5143. [3]Jayaraman, T., Brillantes, A-M. B., Timerman, A. P., Erdjument-Bromage, H., Fleicher, S., Tempst. P., and Marks, A. R (1992) J. Biol. Chem. 267, 9474-9477. [4]Show W., Agdasi B., Hamilton S. L., Matzuk M. M. (1998) Nature 39 489-492. [5]Schiene-Fischer C.,Yu C. (2001) FEBS letters 495 1-6.

Analysis of TCBP-25 and tubulin in the conjugation mutants, *bcd2*, *cnj7* and *cnj10*, during sexual reproduction

Tomomi NAKAGAWA[1], Eric S. COLE[2], Kenta FUJIU[1] and Osamu NUMATA[1]
([1]University of Tsukuba, [2] St. Olaf College.)

The *Tetrahymena* Ca^{2+}-binding protein of 25 kDa ,TCBP-25, is a calmodulin family protein containing four calcium-binding domains of the EF-hand type. TCBP-25 is localized around both the migratory and stationary gametic pronuclei and in the conjugation junction at the pronuclear exchange stage during sexual reproduction (Hanyu et al., 1995). To investigate the function of TCBP-25 during conjugation, we have analyzed the localization of TCBP-25 in the conjugation mutants *bcd2* (Cole, 1991), *cnj7* and *cnj10* (Cole and Soelter, 1997). Our data indicate that TCBP-25 is not involved in the distinction between the pronuclei and the degenerative nuclei, and there is no relation between the localization of TCBP-25 and pronuclear fusion, and also that TCBP-25 plays some role in migratory pronuclear exchange. We further analyzed the *cnj10* mutant, which is unable to exchange pronuclei, by investigating the localization of α-tubulin. We observed astral localization of α-tubulin around migratory pronuclei only in wild type pairs. Our data suggest that the localization of TCBP-25 around pronuclei could be related to astral localization of α-tubulin. We propose that TCBP-25 and tubulin play some role in migratory pronuclear exchange.

Genetic analysis of a morphological mutant in *Paramecium tetraurelia*.

Megumi KANNO and Isoji MIWA (Dept. Biol., Fac. Sci., Ibaraki Univ.)

We isolated four trichocyst-nondischarge mutants of *Paramecium tetraurelia* by treatment with N-methyl-N'-nitro-N-nitrosoguanidine. Furthermore, we found a slow-growing clone among the mutants. The cells of this clone showed a normal shape like wild type cells in mass culture, but they became bigger and more spherical when they were cultured in isolated conditions. To analyze its genetic characteristics, F1 progeny cells were obtained after crossing with the wild type. F1 cells were then allocated to two experiments. In one, autogamy was induced, and in the other, they were backcrossed with parent cells of the mutant. In autogamy progeny, the ratio of spherical cells to normally shaped cells was different in every experiment. On the other hand, the ratio in the progeny obtained from backcrossing was close to 1:1. Therefore, the shape-altering phenotype of the mutant seems to be controlled by the gene.

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Effects of symbiotic *Chlorella* on the expression of circadian rhythm in *Paramecium bursaria*

Satoshi OHMORI and Isoji MIWA (Dept. Biol., Fac. Sci., Ibaraki Univ.)

Paramecium bursaria shows many kinds of circadian rhythm, including a mating reactivity rhythm and a photoaccumulation rhythm. Cells of *P. bursaria* normally contain several hundred cells of the green alga *Chlorella* established in the cytoplasm as endosymbionts. *Chlorella*-free white cells can be obtained from natural green cells by rapid growth in constant darkness (DD). Symbiotic *Chlorella* were isolated easily from their host cells and re-infected into *Chlorella*-free white cells. It is known that the phase of photoaccumulation rhythms in re-infected cells was shifted in constant light (LL) depending on the phase of infecting *Chlorella* which had been kept under a light and dark cycle. Moreover, symbiotic *Chlorella* play a similarly important role in the expression of mating reactivity rhythms. The phase shift of photoaccumulation and mating reactivity rhythms in LL occurred 3 days after the white cells were infected with *Chlorella* entrained by the opposite phase of the LD cycle. This time, we infected *Chlorella* cultured under the temperature cycle (CW12:12) into white cells, and measured the mating reactivity rhythm. The phase of the mating reactivity rhythm of recipient cells was shifted by infecting *Chlorella* after 4 days in LL. But a phase shift did not occur in DD.

Predator-prey interaction between 6 species of ciliates and the turbellarian *Stenostomum sphagnetorum*

Akio MIYAKE, Sara TAMBURRINI, Federico BUONANNO, Piero SALTALAMACHIA (Dept. Mol. Cell. Animal Biol., Univ. Camerino, Italy)

The haptorian ciliate *Dileptus margaritifer* attacked with the toxicysts-bearing proboscis the catenulid turbellarian *Stenostomum sphagnetorum*. The *Stenostomum* released a mass of cloudy material from the attacked site and swam away, while the *Dileptus* ate the released material. If the attack was repeated, the *Stenostomum* was gradually destroyed. On the other hand, *Stenostomum* preyed on *Dileptus* by engulfing it. *Dileptus* required a numerical superiority over *Stenostomum* to prey on it. Lysozyme, which induces a massive discharge of extrusomes in various ciliates, induced (at 500 μg/ml) a rapid and massive secretion from all over the surface of *Stenosstomum*. The secreted material formed a capsule around the treated *Stenostomum*, just as in the "capsule shedding" in ciliates. Such an induction of massive secretion by lysozyme (125 μg/ml, 10 min) did not affect the ability of *Stenostomum* to reproduce, but it significantly reduced the capacity of *Stenostomum* to defend itself against *Dileptus*, suggesting that the secretion is effective for defense against predators just as the discharge of defensive extrusomes in ciliates. *Stenostomum* took into the pharynx *Blepharisma japonicum*, *Climacostomum virens*, *Loxodes striatus*, *Paramecium tetraurelia* and *Spirostomum teres*, but *Loxodes* and *Spirostomum* were regurgitated, apparently unharmed. The phenomenon was similar to the regurgitation of engulfed *Paramecium* by *Climacostomum* (Sugibayashi & Harumoto, 2000).

Ca^{2+}-dependent contraction of isolated nuclei in the heliozoon *Actinophrys sol*.

Mikihiko ARIKAWA and Toshinobu SUZAKI (Dept. Biol., Fac. Sci., Kobe Univ.)

In this study, isolated nuclei of the heliozoon *Actinophrys sol* were found to show Ca^{2+}-dependent contraction. The isolated nuclei showed 50 - 80% contraction despite variation in their size. Nuclear contraction was not induced by addition of Mg^{2+}, and was inhibited by neither colchicine nor cytochalasin B. The nuclear contraction was repeatedly induced by alternate addition of Ca^{2+} and EGTA. The degree of nuclear contraction and Ca^{2+} concentration showed a sigmoidal relationship, with the threshold level of Ca^{2+} in the order of 10^{-7} M. The nuclear contractility remained even after treatment with 2 M NaCl, suggesting a possible involvement of nucleoskeletal components in the nuclear contraction. Electron microscopy showed that thin filaments which were spread in the nucleus as a meshwork structure became aggregated into thicker filaments when nuclear contraction occurred. These observations suggest that Ca^{2+}-dependent transformation of the thin filaments may be responsible for the nuclear contraction. Similar nuclear contractility was found to exist in nuclei

of other protozoon species and even in HeLa cells, and the possibility is presented that eukaryotic cells in general possess a Ca^{2+}-dependent nuclear contractility.

Contraction of contractile vacuole isolated from *Amoeba proteus*

Eri NISHIHARA, Gohki TSURU, Teruo SHIMMEN, and Seiji SONOBE
(Dep. Life Sci., Grad. Sch. Sci., Himeji Inst. Tech.)

The contractile vacuole (CV) of *Amoeba proteus* is an osmoregulatory organelle and its mechanism of contraction is poorly understood. CV is not fixed at the cell surface and moves in the cytoplasm, contracting about every 3 minutes. Prusch, R. D. (1970) reported that isolated CV of *A.proteus* shrank in the presence of 5 mM ATP. We re-examined Prusch's experiment with slight modifications. When a cell was squashed between slide glass and cover glass, CV was released into the medium. Contraction of isolated CV was induced by ATP, but not by GTP, ITP, ADP, AMP-PNP and ATPγS. To examine a possibility that contraction was caused by breakdown of CV membrane due to mechanical pressure, isolated CV was burst mechanically. In this case CV was broken without contraction. Since the presence of actin and myosin around CV has been reported in *Acanthamoeba*, cell fragments containing CV were obtained by centrifugation of cells in Percoll. The surface of CV in the cell fragments was strongly stained with rhodamine-palloidin, suggesting that actin filaments are associated with CV and involved in contraction.

Effect of proteasome inhibitors on the growth and differentiation of *Entamoeba*

Asao MAKIOKA[1], Masahiro KUMAGAI[1], Hiroshi OHTOMO[1], Seiki KOBAYASHI[2]
and Tsutomu TAKEUCHI[2]
([1]Dept. Trop. Med., Jikei Univ. Sch. Med., [2]Dept. Trop. Med. Parasitol., Keio Univ. Sch. Med.)

Protein degradation by ubiquitin-proteasome pathway plays an essential role for a wide variety of cellular processes in eukaryotic cells. Studies with selective proteasome inhibitors such as lactacystin have demonstrated possible contribution of protein degradation by this pathway in cellular processes. Therefore, we examined whether proteasome inhibitors affect growth and encystation as well as excystation of *Entamoeba*. Three proteasome inhibitors, lactacystin, *clasto*-lactacystin β-lactone, and MG-132 inhibited *E. histolytica* growth, with lactacystin being most potent. *E. invadens* was more resistant to these drugs than *E. histolytica*. Encystation of *E. invadens* was also inhibited by these inhibitors and was more sensitive to the drugs than the growth, with the β-lactone being most potent. The inhibitory effect of lactacystin and the β-lactone on encystation was slightly or little abrogated by the removal of the drug, respectively. Multinucleation occurred in *E. histolytica* trophozoites treated with these drugs, being most marked by lactacystin. Electron microscopy revealed that treatment of *E. histolytica* trophozoites with lactacystin led to an increase in the number of cells with many glycogen granules in the cytoplasm. These inhibitors had no or little effect on the excystation and metacystic development of *E. invadens*. These results show that proteasome function plays an important role for *Entamoeba* growth and encystation, but has no obvious effect on the excystation and metacystic development.

Killing mechanism of *Leishmania amazonensis* by eosinophils.

Yoshiya WATANABE and Masato FURUYA (Kochi Medical School, Institute for Lab. Animals)

Leishmaniasis is an important endemic currently affecting 12 million people in 88 countries on 4 continents. *Leishania* are intracellular obligate protozoan parasites that infect host macrophages and cause a number of diseases with a wide spectrum of clinical features such as cutaneous, visceral and mucocutaneous leishmaniasis. It is reported that the tissue eosinophilia is remarkable in the chronic phase of murine cutaneous leishmaniasis, but a role of eosinophils in this disease is unknown. To determine the possible role of eosinophils in *Leishmania* infection, both interleukin-5 transgenic mice (IL-5 Tg) with a constant eosinophilia condition and back ground mice (non-Tg) were infected with *Leishmania*

amazonensis (*L.amazonensis*). IL-5 Tg mice demonstrated greater resistance than non-Tg mice to *L.amazonensis* infection. Furthermore, the killing activity of eosinophils to *L.amazonensis* was induced by IL-4 and IFN-γ in vitro, and this activity was specifically inhibited by catalase. It is an accepted theory that protection of mice against *Leishmania* infection depends on the ability to generate macrophage-activating Th1 responses resulting in production of IFN-γ. However, we show that eosinophils which induced by Th2 cytokine of IL-5 were effective in controlling *L. amazonensis* infection and found that hydrogen peroxide produced by eosinophils in response to either IFN-γ or IL-4 effectively killed this protozoan parasites.

Does the soil ciliate eat the plantpathogenic fungi?

S. SHIMANO[1], K. YAMAHATSU[1] and T. TAKAHASHI[2]
([1] Nat. Agri. Res. Cent. Tohoku, [2] Nishikyusyuu Univ.)

Colpoda cucullus and *Platophrya spumacola* were isolated by culture with phytopathogenic fungi, *Fusarium oxysporum* f. sp. *conglutinans,* from soil of a field and a hydroponic culture, respectively. When *F. o. cong.* was given to the ciliates as food, the highest cell density (cells/ml) of *C. cucullus* (mean ± S.D.) was 1938 ± 226, 3280 ± 113, 1205 ± 186 and 245 ± 25 at 30°C, 25°C, 20°C and 15°C, respectively. On the other hand, the highest cell density of *P. spumacola* reached 128 ± 36, 3133 ± 324, 4426 ± 746 and 1150 ± 105 at 30°C, 25°C, 20°C and 15°C. These results suggest that the adaptation temperature for *C. cucullus* is higher than that for *P. spumacola*. When *C. cucullus* were given the transformant of phytopathogenic fungus, *F. o. cong.* as food, fungal fragments expressing GFP were taken in the food vacuole of *C. cucullus*. These results suggest that *C. cucullus* feed on the transformant of phytopathogenic fungus, *F. o. cong.* We conclude that the use of the mycophagous ciliate, *C. cucullus*, and a newly produced transformant would be hopeful to clarify how the mycophagous ciliates control phytopathogenic fungi in the field.

Survey for the water quality of the Shirakawa River using ciliate community

Noriko GOTO[1] and Tadao MATSUSAKA[2]
([1]Syst. Nat. Env., Grad. Sch. Sci. Technol., [2]Dept. Env. Sci., Fac. Sci., Kumamoto Univ.)

Many investigators have described that freshwater ciliate community structure reflects the water quality, stressing its effectiveness of biological evaluation of watercourses. We clearly demonstrated that the water quality is well correlated with the total numbers of ciliates and with the percentage based on the numbers classified by their feeding preferences (Nagata, N. *et al*. (2002) Jpn. J. Protozool. 35, 48). Since our and most of other reports were performed on the wide range of pollution levels, the present study examines if the ciliate community structure could be a bioindicator even among the stations of similar pollution levels in a single river. Samplings were done at monthly intervals from January to October 2002 at 8 stations from the upper to lower streams of the Shirakawa River, Kumamoto Pref. Ciliate species were categorized by their feeding preferences; bacteriovore, omnivore, and carnivore. Pollution was determined by bacterial counts and by several physico-chemical parameters (temperature, pH, DO, COD, total nitrogen, NO_3^--N, NH_4^+-N, total phosphorus, PO_4^--P and alkyl benzene sulphonate). Total numbers of ciliates reflected well positively with the pollution levels. Although clear correlations between pollution and species numbers, or the percentages based on the numbers categorized as above were not found, the percentage of bacteriovorous species correlated well positively with the pollution and the percentage of omnivorous species well correlated negatively.

Multivaliate analysis on symbiotic flagellate community of termites

Osamu KITADE (Fac. Sci., Ibaraki Univ.)

Most of the termite families and a xylophagous cockroach genus *Cryptocercus* possess symbiotic flagellate community in their hindguts. The symbiotic flagellates belong to orders Trichononadida, Hypermastigida and Oxymonadida and most of them have mutualistic relationship with their hosts through cellulose degradation. Usually, the symbiont composition is host-species specific and is expected to reflect host phylogeny. In this study, similarity of the symbiotic

flagellate community was analyzed to describe its property and evaluate the effect of host phylogeny. The flagellate composition data were extracted from a checklist by Yamin (1979) and some descriptive papers, and through field investigation. A total of 81 flagellate genera have been reported from 38 host genera. Results of cluster analysis based on the similarity of symbiont generic composition (similarity index: 1-r, clustering: UPGMA) indicated that the host genera in Rhinotermitidae and Kalotermitidae formed almost exclusive clusters. Serriterimitidae was included in the cluster of Rhinotermitidae. *Cryptocercus* and Mastotermitidae had characteristic symbiont composition. Results of Hayashi's quantification type III was almost the same as that of the cluster analysis, which corresponded to the tentative phylogeny of hosts inferred from mitochondrial gene sequences.

Bioactivity of pigment of ciliate *Blepharisma japonicum* – mitochondria toxicity –

Masayo TERAZIMA[1], Terue HARUMOTO[2], Yumiko TANABE[3] and Kiyoshi KAWAI[3]
([1]Tokai Women's Junior College, [2]Dep. Biol., Fac. Sci., Nara Women's Univ., [3] Fac. Wellness, Chukyo Women's Univ.)

Blepharismin markedly suppressed the oxygen uptake of a protozoan *Tetrahymena thermophilia*, suggesting the impairing effect on the respiratory system of the *Tetrahymena* mitochondria. The effects of blepharismin on the mitochondrial reactions have been therefore investigated by means of isolated rat liver mitochondria and submitochondrial particles (SMP), which are the reverted membrane vesicles of the inner membranes, to gain insight into the molecular mechanism of the toxicity. Because the isolation procedure of rat liver mitochondria is now well established and the mechanism of respiratory system is elucidated in detail. Blepharismin exhibited a weak uncoupling and inhibitory effects on the NAD-linked respiration oxidizing L-glutamate and exerted the uncoupling effect on succinate-linked respiration. NADH oxidase of SMP was not interfered by blepharismin, indicating that blepharismin inhibits the NAD-linked respiration by disturbing ion translocation systems of mitochondrial inner membrane. Blepharismin induced a swift swelling of mitochondria suspended in the isotonic solutions of alkali metal chlorides, indicating the induction of the ion permeability transition of the inner membrane. These results suggest that blepharismin may interfere mitochondrial reactions in *Tetrahymena* by the similar mode of reactions as those to rat liver mitochondria, though the detailed mechanism is not available at present.

A novel citrate synthase gene in *Tetrahymena thermophila*: Evidence for lateral gene transfer

Atsushi MUKAI (Dept. Biol., Fac. Sci., Kanazawa Univ.)

Citrate synthase in *Tetrahymena thermophila* is known to catalyze the initial reaction of TCA cycle in mitochondria, while forms 14-nm filament in cytosol. In this study, a new citrate synthase gene homolog (CSH) was identified. Characterization of the CSH revealed the following characteristics: 1) The cloned fragment contains 3' region of ORF and 3' UTR; 2) ORF is interrupted by a 52-bp intron; 3) TAA is used for glutamine, indicating ciliate-specific codon usage; and 4) The CSH is constitutively expressed in vegetative phase and conjugation. Based on the molecular phylogeny of citrate synthase genes, I provide evidence for lateral gene transfer of *Tetrahymena* CSH. Surprisingly, *Tetrahymena* CSH was found to be closely related to citrate synthase genes of green sulfur bacteria and somewhat to alpha-proteobacteria, but not related to mitochondria-type genes. The clade including *Tetrahymena* CSH additionally contains a few sequences from cellular slime mold and two plants, suggesting that CSH may be used in glyoxylate cycle. In addition, Southern blot analysis showed the absence of the corresponding gene in other relatives of ciliates such as *Blepharisma japonicum, Colpoda inflata* and *Paramecium tetraurelia.* These results suggest that *Tetrahymena* CSH might have directly derived from green sulfur bacteria by lateral gene transfer after *Tetrahymena* diverged from other ciliate lineage.

Molecular phylogenetic analysis of syngens of *Paramecium caudatum* based on cytosolic hsp70 sequences

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Manabu HORI, Izumi TOMIKAWA and Masahiro FUJISHIMA
(Biol. Inst., Fac. of Sci., Yamaguchi Univ.)

Morphological species *P. caudatum* comprise several syngens that can be distinguished by their mating reaction. However, attempts to classify them by isozyme analyses and RFLP analyses have not succeeded yet. The aim of the present study was to classify syngens of *P. caudatum* using the cytosolic hsp70 gene as a molecular phylogenetic marker. Previously, we demonstrated that a phylogenetic tree of the genus *Paramecium* can be reliably constructed based on the cytosolic hsp70 gene. In the present study, sequences of 428 bases of the cytosolic hsp70 molecule from 32 strains of 6 syngens in *P. caudatum* were isolated by the polymerase chain reaction. A *Paramecium* phylogenetic tree was constructed for each set of sequences using the MOLPHY program package on the basis of maximum likelihood. The resultant tree had two clusters of *P. caudatum,* implying that *P. caudatum* has two cytosolic hsp70 genes. Within either cluster, however, the relationships between the syngens were not clear.

Antibody to a protein specific for the infectious form of *Holospora obtusa* of the ciliate *Paramecium caudatum*.

Hideo DOHRA[1] and Hidemi SAKURAI[2]
([1]Inst. for Genet. Res & Biotech., [2]Grad. School of Sci. & Engin., Shizuoka Univ.)

We detected a 63-kDa protein (MW 63 kDa, pI 8.9) specific for the infectious form of *H. obtusa*, a macronuclear endosymbiont of the ciliate *P. caudatum* by two-dimensional polyacrylamide gel electrophoresis using the immobilized pH gradient system. We developed anti-63-kDa protein antiserum by injecting the purified protein into a mouse. Immunoblotting with the antiserum showed that the 63-kDa protein is present only in the infectious form of *H. obtusa*, but not in the reproductive form. Indirect immuno-fluorescent microscopy with anti-63-kDa protein antiserum showed that the protein localized in the recognition tip of the infectious form of *H. obtusa*, suggesting that the protein was involved in the infection process of *H. obtusa*. Cloning and sequencing of the gene encoding for the 63-kDa protein showed that the gene encoded a protein composed of 523 amino acids, and the polypeptide is preceded by a putative signal peptide of 14 amino acids, which was involved in the transport of the 63-kDa protein from cytoplasm to periplasm. The function of the 63-kDa protein during the infection process of *H. obtusa* is not clear yet, because the 63-kDa protein did not show any domains and amino acid similarity with any known proteins.

Chemical modification of histidine residues in spasmoneme

Jie FANG[1], Bei ZHANG[2] and Hiroshi ASAI[1] ([1]Waseda University, [2]Nankai University, China)

Glycerinated stalk of *Vorticella* can repeat contraction/extension cycle many times by the addition/removal of Ca^{2+}. In order to identify the essential amino acid residues for spasmoneme contraction, glycerinated spasmoneme in the stalk was modified with diethl-pyrocarbonate (DEPC). We observed that the inhibition of spasmoneme contractility degree was dependent on DEPC concentration and modification time at 0°C and pH6.0. Hydroxylamine treatment the modified spasmoneme can partially restore the contractility degree. Ca^{2+} provides almost complete protection against inhibition and modification. Those suggested histidine residue was critical for spasmoneme contraction.

Axopodial contraction evoked by extracellular Ca^{2+} and external stimuli, and ultrastructural observation in the heliozoon *Raphidiophrys contractilis*

S. M. Mostafa Kamal KHAN, and Toshinobu SUZAKI (Dept. Biol., Fac. Sci., Kobe Univ., Kobe)

In this study, we investigated axopodial contraction of the centrohelid heliozoon *Raphidiophrys contractilis* induced by external stimuli. To induce axopodial contraction, extracellular Ca^{2+} was found to be required. In the absence of Ca^{2+}, axopodial contraction could not be induced at all. The threshold level of extracellular Ca^{2+} was between 10^{-6} and 10^{-7} M.

The axopodial contraction took place within one frame of a video recording and just after application of a mechanical shock, which indicates that the velocity of the contraction was more than 3.0 μm/s. Re-elongation of axopodia was observed immediately after the contraction, and its velocity was about 0.7 μm/s. For electron microscopic observation, an improved fixative using ruthenium red and Taxol was developed and applied to the heliozoons, which prevented artificial retraction of the axopodia during fixation and resulted in better preservation of the cytoskeleton. A bundle of hexagonally-arranged microtubules was observed in an axopodium, but no other filamentous structures were detected at all. This observation suggests that the contractile machinery in the axopodia of *R. contractilis* may be different from that in the actinophryid heliozoons in which Ca^{2+}-dependent contractile structures (contractile tubules) are employed for contraction.

Structural analysis of outer-arm dynein heads from *Tetrahymena* cilia.

Hideki ISHIDA[1] and Etsuko MASUYAMA[2] ([1]Dept. Biol. Sci., Fac. Life and Environmental Sci., Shimane Univ., [2]Dept. Health Sci., Hiroshima Pref. Women's Univ.)

Axonemal dyneins are force-generating ATPases that produce movement of eukaryotic cilia and flagella. The outer-arm dynein from *Tetrahymena* cilia is a large particle (Mr 2,000 kD) and contains three heavy chains (Mr > 500 kD; α, β, and γ), each forming a large globular head and a short tail domain. Due to their large size and complex compositions, it has been difficult to investigate molecular interactions between dynein and tubulin. We tried to prepare small and simple dynein fragments by limited proteolysis of outer-arm dynein. Dynein digested with thermolysin yielded two stable fragments (Mr 400 kD) which were derived from β and γ heavy chains. Transmission electron microscopic analysis revealed that negatively stained fragments were ellipsoidal or spherical in shape with approximate sizes of 10 - 20 nm. This observation implies that the fragment might correspond to the head domain of a dynein molecule. Furthermore, conformational changes of the fragments were examined by using acrylodan, a cystein-specific fluorescence probe. Binding of bovine brain tubulin to acrylodan-labeled fragments induced a significant blue shift (from 506 nm to 457 nm). Moreover, with the addition of 1 mM ATP, fluorescence intensity increased 1.5 times in quantum yield coupled with stimulation of ATPase activity. These results indicate that dynein heads cause conformational changes by interaction with tubulin and ATP, since acrylodan is known to show a blue-shifted emission spectrum on exposure to a hydrophobic environment.

Intraciliary calcium imaging during ciliary reversal in *Didinium nasutum*.

Yoshiaki IWADATE (Fac. Integr. Arts Sci., The Univ. Tokushima)

Internal Ca^{2+} level controls the pattern of ciliary and flagellar beating in eukaryotes. In ciliate, many authors have suggested that the reversal of the direction of ciliary beating (ciliary reversal) is induced by a rise in intra-ciliary Ca^{2+} level. However, this putative increase in intra-ciliary Ca^{2+} level have not as yet been recorded during ciliary reversal. We injected the fluorescent Ca^{2+} indicator Calcium Green into a ciliate *Didinium nasutum* and observed the intra-ciliary Ca^{2+} level during spontaneous ciliary reversal. *D. nasutum* showed a rise in Ca^{2+} level all over the cilium accompanied by the ciliary reversal. We injected caged Ca^{2+} into *D. nasutum* and applied ultraviolet light to the middle region of the cilia. The cilia bent at the UV applied region. These results strongly suggest that not only Ca^{2+} influx region but also Ca^{2+} binding sites and bending devices for ciliary reversal is distributed all over the cilium.

Behavioral and membrane potential responses of *Paramecium caudatum* to external Na^+

Kazunori OAMI and Mihoko TAKAHASHI (Inst. Biol. Sci. Univ. Tsukuba)

To understand the mechanisms underlying the sensation of the external inorganic ions in *Paramecium caudatum*, we examined behavioral and membrane potential responses of the cells to external Na^+. Wild-type cells immersed in the Na^+-containing solution (32 mM or more) exhibited initial continuous backward swimming followed by repeated transient backward swimming. A wild-type impaled by a microelectrode showed initial action potential followed by sustained

membrane depolarization in response to an application of the Na^+-containing test solution. Depolarizing afterpotential took place subsequently after termination of the application. CNR mutant defective in voltage-gated Ca^{2+} channels showed only sustained membrane depolarization but not the action potential and the depolarizing afterpotential to the application. Both wild-type and CNR exhibited transient depolarization overlapping the sustained depolarization when the application was prolonged. It is concluded that *Paramecium* shows two kinds of behavioral responses to Na^+. The initial backward swimming is controlled by the mechanism similar to that underlying the K^+-induced backward swimming while the repeated backward swimming is controlled by the transient depolarization.

Activity change of sphingosine kinase and phospholipase D in synchronized cell division process of *Tetrahymena* cell

Osamu UENO[1,2], Shulin WANG[3], Masahiro KOHDA[4], Kenji OOGUCHI[2], Yoshinori NOZAWA[2] and Yoshiko BANNO[4]
([1]FAIS [2]Gifu Intl. Inst. Biotech. [3]Univ Pennsylvania, [4]Dept. of Biochemistry, Gifu Univ. Sch. of Med.)

Sphingosine kinase (SPHK) and phospholipase D (PLD) have attracted increasing attention as signal transduction factors associated with cell survival, growth, and death. SPHK and PLD activities were examined during the synchronous cell division induced by cyclic heat treatments in *Tetrahymena* cells. SPHK and PLD activity transiently increased at 30 to 45 min after the end of heat treatment (EHT) prior to initiation of the synchronous cell division (75 min after EHT). Synchronous cell division was delayed and decreased by these enzyme inhibitors. Moreover, PLD activity changes were inhibited by SPHK and SPHK activity changes were inhibited by PLD inhibitor. These results suggest that these activity changes were associated with the cell cycle and that the up-regulated SPHK and PLD activity would be required for the initiation of the oncoming synchronous cell division in *Tetrahymena* cells.

Morphological changes of germ-nucleus in conjugation of *Paramecium caudatum*: Continuous observation on living cells

Kenichi IWATA, Yuta SHOJI and Kazuyuki MIKAMI (EEC, Miyagi Univ. of Education)

It is known that the germ-nucleus (micronucleus) of *Paramecium caudatum* changes its form from ovoid to crescent-shaped. To study the specific morphological change of micronuclei, the micronucleus in a living cell was observed under a phase contrast microscope and recorded on videotape continuously. We employed Cell-tak™ (BD Biosciences) to stick cells on a glass slide. Prior to observation, cells were treated in DAPI solution (1 μg/ml). After observation under phase contrast, the micronucleus stained with DAPI was irradiated with UV and recorded on videotape for some seconds. The area of DNA stained with DAPI was compared with the nuclear area that was observed under the phase microscope. Speed of protoplasmic streaming was also determined on the monitor screen by measuring migration distance of a small granule (0.5 μm). The speed of protoplasmic streaming tended to be faster than that of vegetative cells after 2 hrs of conjugation. The micronucleus moved around the macronucleus in accordance with protoplasmic streaming, and changed its form from ovoid to a long form, and eventually to a crescent. This means that the micronucleus is elongated by development of the intranuclear cytoskeleton. At 6 hrs after conjugation, the shape of the micronucleus became a typical crescent under phase contrast. However the area of DNA became thinner and a DNA-free area appeared around the DNA area. The function of the DNA-free area in the micronucleus remains to be resolved.

Changes in trichocysts during re-infection of white *Paramecium bursaria* by *Chlorella*

Gen OMURA and Toshinobu SUZAKI (Dept. Biol., Fac. Sci., Kobe Univ.)

Trichocysts in *Paramecium bursaria* are regularly arranged in the cell cortex. The density of trichocysts in *Chlor-*

ella-bearing "green" *P.bursaria* was less than that in *Chlorella*-free "white" *P. bursaria.* This indicates that, during re-infection of white *P. bursaria* with *Chlorella*, trichocysts become diminished in the cell cortex. Light microscopy showed that many granules appeared in the cytoplasm of white *P. bursaria* that were re-infected with *Chlorella* for 6 hours. In parallel with the appearance of the granules, the number of trichocysts decreased. The granules were not derived from food vacuoles, because the granules remained unchanged even after the paramecia were washed extensively and continuously with fresh culture medium for 15 hours. Trichocyst discharge was not observed at all during the entire process of re-infection with *Chlorella*. Electron microscopy showed that the granules consist of electron dense material without apparent covering membranes. The granules were observed particularly in the vicinity of trichocysts, with frequent attachment to trichocysts. Some of the granules were observed to be fused with trichocysts. In the paramecia re-infected with *Chlorella* and successively washed for 15 hours, many granules were observed but almost all trichocysts had disappeared. These observations suggest that trichocysts were transformed into smaller electron-dense granules during the process of *Chlorella* infection.

Bacteria-free culture of a colorless euglenoid flagellate *Peranema trichophorum* and establishment of a method for flagellar isolation

Yasutaka SUETOMO[1], Akira SAITO[1,2] and Toshinobu SUZAKI[1]
([1]Dept. Biol., Fac. Sci., Kobe Univ., [2]Dept. Histol. Cell Biol., Grad. Sch. Biomed. Sci., Hiroshima Univ.)

We have recently shown that the anterior flagellum and its mastigonemes play an important role in gliding movement in a colorless euglenoid flagellate *Peranema trichophorum*. For further biochemical analysis of the molecular mechanism of flagellar surface motility, we have developed an improved monoxenic sterile culture method for *P. trichophorum* and a method for flagellar isolation. By adjusting initial cell densities of *P. trichophorum* and co-existing food flagellates, *Chlorogonium elongatum*, to 800 and 30,000 cells/ml, respectively, *P. trichophorum* reached its maximum cell density of 10,000 cells/ml in only one week. A brief cold-shock procedure allowed an efficient isolation of flagella. Light and electron microscopic observations showed that the isolated flagella retained their structure with abundant mastigonemes.

A possible involvement of mitochondria in apoptotic nuclear death of conjugating *Tetrahymena thermophila*

Takashi KOBAYASHI (Dept. Biol., Kanazawa Univ.)

In the ciliated protozoan *Tetrahymena thermophila*, old parental macronucleus is degenerated *via* apoptosis-like process during conjugation. This process controlled by a set of specific genes is called Programmed Nuclear Death (PND). Previously caspase-like activity was detected in macronuclear degradation stage during PND. Here we demonstrate mitochondrial association in PND. It is well known that various apoptosis-related molecules are released from mitochondria in apoptosis of metazoans, resulting from the loss of mitochondrial membrane potential ($\Delta\Psi m$). Using the dye (DePshipher) to detect change ($\Delta\Psi m$) of mitochondrial potential, conjugants and exconjugants were stained. The dye unexpectedly localized in the degenerating parental macronucleus as well as mitochondria. Other nuclei, macronuclear anlagen and micronuclei, were not stained. The degenerating macronucleus in this stage is known to be surrounded by autophagosome. The localization of the dye in the autophagosome containing the degenerating macronucleus did not coincide with that of acidic organelles detected by Acridine Orange staining, suggesting that it is not the response to low pH. In addition, other mitochondria staining method (MitoTracker) also supported mitochondrial localization in the autophagosome. These results suggest that part of mitochondria are taken in autophagosome together with the degenerating macronucleus. The mitochondria in the autophagosome would lose their membrane potential and then degrade. In this mechanism, it can be rationalized why the release of apoptosis inducing factors and the subsequent caspase activation are restricted in autophagosome.

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Genetic structure of the genus *Frontonia* (Peniculida, Nassophorea)

Yuuji TSUKII (Lab. Biol., Sci. Res. Cent., Hosei Univ.)

To know genetic relationships within and between species of *Frontonia*, RAPD (Random Amplified Polymorphic DNA) profiles of 25 stocks belonging to 5 different species (*F. leucas*, *F. vernalis*, *F. marina*, *F. acuminata*, *F. depressa*) were examined. Totally 257 RAPD bands were detected using 20 primers. RAPD profiles of all species except *F. leucas* were highly variable (up to 95% of dissimilarity). On the contrary, those of all 7 stocks of *F. leucas* were quite similar (up to 25% of dissimilarity or nearly identical when only major bands were compared). This is peculiar to this species when compared to other *Frontonia* species as well as other ciliate species, e.g. *Paramecium caudatum* (Tsukii, 1996) or other protists, e.g. *Chilomonas paramecium* (Tsukii, 2001), suggesting that *F. leucas* is very young in the evolutionary history of *Frontonia* or some unknown mechanism keeps this species homogenous. Two stocks of *F. vernalis* were found clearly separated from those of *F. leucas*. Thus, it is highly possible that *F. vernalis* is not a variety of *F. leucas* as described by Kahl (1933) but a valid species. Furthermore, 11 stocks of *F. marina* were subdivided into two groups based on their cell shapes and these two subgroups were also separated on the RAPD tree.

Bioassay of acrylamide cytotoxicity using the ciliate *Paramecium bursaria*

Toshiyuki TAKAHASHI[1], Masanobu YOSHII[2], Tomonori KAWANO[1], Toshikazu KOSAKA[1] and Hiroshi HOSOYA[1] ([1]Department of Biological Science, Graduate School of Science, Hiroshima University, [2]Time Associates Inc.)

The Swedish National Food Administration has announced that acrylamide (AA), a known carcinogen, has been found in elevated levels in starch-containing foods cooked at high temperatures (Press Release, WHO/32, 26 April, 2002). Thus, elucidation of the mechanism of AA cytotoxicity to animal cells and a search for an inhibitor of AA cytotoxicity are urgently required. We have established a bioassay system using P. bursaria for assessing the effect of environmental indicator substances. In this study, various amounts of AA were added to the culture medium of *P. bursaria* to investigate AA cytotoxicity. At high concentrations (≥ 0.15%, w/v), a lethal effect of AA was observed on *P. bursaria*. At low concentrations (0.0015-0.05%, w/v), an inhibitory effect on proliferation of P. bursaria and a decrease in the number of endosymbiotic algae in *P. bursaria* were observed. Interestingly, in the presence of 0.005-0.015% AA, algae-free paramecia were observed in the culture medium. These results indicate that the endosymbiotic algae in *P. bursaria* are more sensitive to AA than other organisms such as brown trout and Crustacea. Taken together, these data indicate that *P. bursaria* as well as its endosymbiotic algae are very useful indicators for assessing the effect of AA and AA cytotoxicity on animal cells.

Infection of endosymbiotic bacterium *Holospora obtuse* depresses surface antigen gene expression of the host *Paramecium caudatum*

Yoshimitsu NAKAMURA, Manabu HORI and Masahiro FUJISHIMA (Biol. Inst., Fac. Sci., Yamaguchi Univ.)

Holospora obtusa is an endonuclear symbiotic bacterium of the ciliate *Paramecium caudatum*. In order to identify host genes of which expressions are altered by infection of *H. obtusa*, mRNAs expressed in bacteria-bearing cells (symbiotic cells) and non-bearing cells (aposymbiotic cells) were compared by differential display analyses. Twelve aposymbiotic cell-specific and three symbiotic cell-specific cDNAs were detected. The putative amino acid sequence of an aposymbiotic-specific cDNA bore a 64.9% similarity to a surface antigen of *P. primaurelia* stock 168G. Northern blot analysis with DIG-labelled cDNA as a probe showed that the surface antigen G homologous gene expressed only in the aposymbiotic cell whereas not in the symbiotic cell. It was found that the cell surface antigen-like protein with more than 200 kDa in molecular mass was extracted from the aposymbiotic cell by salt/alcohol extraction method, whereas not from the symbiotic cell. Indirect immunofluorescence microscopy with a monoclonal antibody specific for the extracted

surface antigen-like protein also showed that FITC-fluorescence appeared only on the cell surface of the aposymbiotic cell, but not on the symbiotic cell. These results strongly suggest that infection of *H. obtusa* depresses surface antigen gene expression of the host *Paramecium caudatum*.

Paramecium caudatum acquires a heat-shock resistant nature by infection of endonuclear symbiotic bacterium *Holospora obtusa*

Miki KAWAI, Ryo YAMAMOTO and Masahiro FUJISHIMA
(Biol. Inst., Fac. Sci., Yamaguchi Univ.)

Holospora obtusa is a macronucleus-specific bacterium of the ciliate *Paramecium caudatum*. Aposymbiotic cells, reproductive form of *H. obtusa*-bearing and predominantly infectious form of *H. obtusa* -bearing cells were cultivated at 25°C, and then these cells were transferred to 4, 10, 25, 35, and 40°C . During 2-3 min after the temperature-shifts, the cells were photographed for 2 sec exposures and their mean swimming velocities were measured from lengths of their swimming traces. Although the aposymbiotic cell almost ceased their swimming activity at both 4 and 40°C, the reproductive form-bearing cell showed active swimming velocities even under these temperatures. Predominantly infectious form-bearing cell also showed similar swimming velocities with the reproductive form-bearing cell though the effect was lower than that of the reproductive form-bearing cell. These results show that the host cell can acquire a heat-shock resistant nature if the cell was infected by *H. obtusa* in the macronucleus. This is the first evidence to show that *Holospora* contributes to the host survival at unsuitable temperatures.

Tetrahymena multifunctional proteins II: regulation of multiple functions of peptide elongation factor 1α

Kunie ANDO, Fumihide BUNAI, Hironori UENO and Osamu NUMATA
(Inst. Biol. Sci., Univ. Tsukuba)

Elongation factor-1α (EF-1α) is known to be a multifunctional protein. On ribosomes it functions as a peptide elongation factor, while in the cytoskeleton it functions as an actin-binding protein. In *Tetrahymena*, EF-1α has been reported to localize to the division furrow during cytokinesis and has the ability to bind to filamentous actin (F-actin) and to form F-actin bundles. With Ca^{2+}/calmodulin (CaM), EF-1α cannot bundle F-actin even though it still binds to F-actin. Using gel filtration column chromatography, we examined the native molecular weight of purified EF-1α in the presence or absence of Ca^{2+}/CaM, and revealed that EF-1α itself exists as a monomer, a dimer or a tetramer. Furthermore, EF-1α dimer seems to be the most stable under conditions where it bundles F-actin *in vitro*. When Ca^{2+}/CaM was added to EF-1α, EF-1α monomer increased, while the dimer decreased, suggesting that Ca^{2+}/CaM changes EF-1α dimer into monomer and inhibits F-actin bundle formation. On the other hand, EF-1α monomer functions as a peptide elongation factor on ribosomes. Therefore, these results support the idea that the multiple functions of EF-1α are regulated by oligomer formation.

Analysis of negative-gravi-responses in Triton-extracted models of *Paramecium caudatum*

Toyoaki KINOSHITA (Dept. of Physics, School of Science and Engineering, Waseda Univ.)

I analysed gravitactic behavior of *Paramecium caudatum* and Triton-extracted-models of *Paramecium* in media whose specific gravities had been increased from 1.00 to 1.04 and 1.08 by addition of Percoll. *Paramecium* in the solution with a specific gravitiy of 1.08 showed positive gravitaxis, and in a specific gravity of 1.04 showed no gravitaxis. However, Triton-extracted-models in the solution with a specific gravity of 1.08 showed negative gravitaxis. This could be interpreted to mean that Triton-extracted-models show negative gravitaxis but rise due to buoyancy. Then, I recorded and analysed the sedimentation rate (S), the gravity-independent rate of propulsion (P), and the inclination angle of the cell (θ; 0°=horizontal) of Triton-extracted-models. The upward speed of Triton-extracted-models is given by $\Delta Y = S + P \sin\theta$.

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Transduction of Ca^{2+}-signaling and its energy in 3 nm-diameter biofilaments

Hiroshi ASAI and Takeshi ITABASHI (Graduate School of Science and Engineering, Waseda Univ.)

The spasmoneme composed of a bundle of 2-4 nm-diameter filaments in a stalk of protozoan ciliate *Vorticellidae* contracts in the presence of micrmolar amounts of free calcium ions (Ca^{2+}) *in vitro* and *in vivo*. The contraction can take place many times by changing the ambient Ca^{2+} in the medium without involving in a direct hydrolysis of ATP or any other organic substrate. Thus, the binding of Ca^{2+} to a spasmoneme protein is thought to be the immediate source of contraction energy. We have determined cDNA sequences encoding the two kinds of Ca^{2+}-binding proteins (spasmin 1 and spasmin 2) in spasmoneme of *Zoothamnium arbuscula* strain Kawagoe. Furthermore, it was demonstrated that a thermally flexible protein in 2-4 nm-diameter filament is a probable candidate for elastically, in another word entropically, contractile one (200 kDa or its monomeric 50 kDa protein). Transduction of Ca^{2+}-signalling and its energy in the 3 nm-diameter bofilaments was discussed.