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Encystment and excystment of kinetoplastid *Azumiobodo hoyamushi*, causal agent of soft tunic syndrome in ascidian aquaculture

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ABSTRACT: Soft tunic syndrome in the edible ascidian *Halocynthia roretzi* is caused by the kinetoplastid flagellate *Azumiobodo hoyamushi*, which was found to assume a fusiform cell form with 2 flagella in axenic, pure culture. When the flagellate form was incubated in sterilized artificial seawater (pH 8.4), some of the cells became cyst-like and adhered to the bottom of the culture plate. The cyst-like forms were spherical or cuboidal, and each had 2 flagella encapsulated in its cytoplasm. Encystment was also induced in culture medium alkalified to the pH of seawater (8.4) but not in unmodified (pH 7.2) or acidified media (pH 6.4). More than 95% of the cyst-like cells converted to the flagellate form within 1 d following transfer to seawater containing ascidian tunic extracts from host ascidians. The cyst-like cells were able to survive in seawater with no added nutrients for up to 2 wk at 20°C and for a few months at 5 to 15°C. The survival period in seawater depended on temperature: some cyst-like cells survived 3 mo at 10°C, and ca. 95% of these converted to flagellate forms in seawater containing tunic extracts. Thus, *A. hoyamushi* is able to persist under adverse conditions in a cyst-like form able to adhere to organic and inorganic substrata for protracted periods of time.

KEY WORDS: Kinetoplastid flagellate · *Halocynthia roretzi* · Soft tunic syndrome · Tunic extract · Temporary cyst · *In vitro* culture · pH

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INTRODUCTION

The flagellate protozoan *Azumiobodo hoyamushi* (Kinetoplastea: Neobodonida) is the causal agent of soft tunic syndrome in the edible ascidian *Halocynthia roretzi* (Kumagai et al. 2010, 2011, Hirose et al. 2012, Kim et al. 2014). This protozoan is found exclusively in the ascidian tunic, which softens and ruptures when infected, thereby killing the host. This infestation has caused mass mortalities of *H. roretzi* in Korean and Japanese aquaculture facilities (Jung et al. 2001), with diseased ascidians also occurring in natural populations (Kumagai et al. 2013).

In pure culture, the flagellate is susceptible to high (>20°C) and low (<5°C) temperatures (Kim et al. 2014), which is consistent with the absence of soft tunic syndrome during summer at our study site (Miyagi, Japan) when the water temperature is 20°C or higher (Kumagai et al. 2010). The mechanism by which the parasite survives summer conditions is

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presently unknown, but as temperatures decrease, soft tunic syndrome reappears, indicating that the flagellates have a persistent phase in their life history. Resistant cysts may play a role in persistence through summer. Indeed, a cyst-like form of *A. hoyamushi* has been observed in a diseased tunic (Jang et al. 2012).

In long-term cultures of *A. hoyamushi,* we often found spherical cells lacking flagella that resembled the cysts reported by Jang et al. (2012). We also observed reversion of some cyst-like forms to the flagellate form when tunic extract was supplied instead of culture medium. In the present study, we examined the morphological characteristics of the cyst-like forms and the conditions for encystment and excystment. Our findings may explain the infection process and the seasonal occurrence of soft tunic syndrome.

MATERIALS AND METHODS

Azumiobodo hoyamushi culture conditions

The strain of *A. hoyamushi* used in this study was isolated from a diseased ascidian reared near Samenoura, Miyagi Prefecture, Japan (Kumagai et al. 2011). The flagellate was cultured in maintenance medium (10% [w/v] MEM, 2.5% [v/v] fetal bovine serum, 5 mM HEPES [pH 7.2], 2 mM L-glutamine, 2 mM sodium bicarbonate, and 1% [v/v] penicillin–streptomycin in mixed solution [Nacalai Tesque] prepared in artificial seawater) at 15°C and subcultured by 10-fold dilution into fresh maintenance medium at ca. 1 wk intervals. We used 112th to 176th passage flagellates in the following experiments.

Microscopy

Flagellated cells of *A. hoyamushi* were incubated in sterilized artificial seawater (SASW) to induce encystment (see next section) on plastic coverslips. Six days after incubation in SASW, we fixed the cyst-like cells adhering to the coverslips in a 2.5% glutaraldehyde, 0.45 M sucrose, and 0.1 M sodium cacodylate solution (pH 7.4). To induce flagellate formation, some of the coverslips were incubated in tunic extract for 15 min before fixation (see 'Induction of excystment with tunic extract'). Prior to the post-fixation procedure, we observed some of the specimens under a light microscope equipped with differential interference contrast optics. The specimens on the coverslips were then rinsed in a 0.1 M cacodylate and 0.45 M sucrose solution and post-fixed for 1.5 h in a 1% osmium tetroxide and 0.1 M cacodylate solution.

In preparation for scanning electron microscopy, the specimens on the coverslips were rinsed in distilled water, dehydrated through a graded ethanol series, immersed in *t*-butanol, and then freeze-dried. The dried specimens were sputter-coated with gold–palladium and examined with a JEOL JSM-6060LV scanning electron microscope at 15 kV.

In preparation for transmission electron microscopy (TEM), the specimens on the coverslips were dehydrated through a graded ethanol series and embedded in epoxy resin. We stained thin sections with lead citrate and uranyl acetate and examined them with a JEOL JEM-1011 transmission electron microscope at 80 kV.

Induction of encystment in seawater

Following subculture in maintenance medium at 15°C for 10 d, the flagellates were transferred to SASW or maintenance medium (as a control). We immediately centrifuged 2 ml of flagellate suspension at 500 × *g* for 1 min at 10°C. We discarded 1.8 ml of supernatant and resuspended the pellet in 1.8 ml of SASW. We repeated this series of steps, then added 2 ml of SASW. The numbers of flagellates were determined using a Thoma hemocytometer. Finally, we incubated all flagellates (2.4×10^5 to 1.98×10^6 cells in 4 ml of suspension) in the wells of a 6-well plate (Iwaki Microplate; Asahi Glass). After incubation for 2 d at 15°C, we discarded the SASW and washed the wells 5 times with SASW to remove swimming flagellates. In the control, we replaced SASW with the maintenance medium. The numbers of the cyst-like cells adhering to the bottoms of the wells were counted in 10 viewing fields (3.49 mm^2 in sum) using an inverted microscope to estimate the number of cyst-like cells in each well (962 mm^2 substratum per well). We calculated the encystment index (after incubation) as the number of cyst-like cells per 100 flagellated cells before the incubation. The experiments were replicated 8-fold.

Induction of encystment in acidified or alkalified medium

Following subculture in intact maintenance medium (pH 7.2) at 15°C for 4 d, we incubated 4 ml of the flag-

ellate suspension (3.65×10^6 to 1.57×10^7 cells in 4 ml of suspension) in each of 3 wells of a 6-well plate. Subsequently, we (1) acidified the maintenance medium with 0.01 N HCl to pH 6.4, (2) alkalified it with 4% NaOH to pH 8.4, or (3) left the pH unchanged. After incubation for 1 d at 15°C, we calculated the encystment index using the procedure detailed in the previous subsection. The experiments were replicated 10-fold.

Induction of excystment with tunic extract

Tunic segments (18 g in total) from healthy ascidians were cut into small pieces (ca. 5 × 5 × 3 mm) and incubated in 90 ml of SASW for 1 d at 15°C. The tunic extract was obtained as a filtrate following passage through a 0.45 µm filter (Millex-HA; Millipore). We induced encystment of *A. hoyamushi* by incubating flagellated cells in SASW for 3 d, as described above in 'Induction of encystment in seawater'. After the induction procedure, individual wells of the well plates contained 7.88×10^4 to 7.89×10^5 cyst-like cells. We subsequently discarded the incubation medium (SASW) and added 4 ml of tunic extract to each well. Fifteen minutes later, we examined the cells in several of the wells for a few minutes (using an inverted microscope at a magnification of 300×) to check for damage or exfoliation. Following incubation for 1 d at 15°C, we checked cell condition microscopically in all of the wells and then discarded the incubation medium (tunic extract) and washed the wells with SASW 5 times to remove swimming flagellates. We counted the number of cysts in 10 viewing fields and calculated the number of excysting cells as the difference between the number of cyst-like cells before and after incubation in the tunic extract. The excystment rate (%) was obtained by dividing the excysting cell numbers by the numbers of cyst-like cells before incubation. We used SASW instead of tunic extract in the controls. Comparisons were made between paired control and experimental wells. Experiments were replicated 10-fold.

Longevity and temperature tolerance of cyst-like cells

Flagellated cells were incubated in SASW to induce encystment (1.4×10^6 cells in a 25 cm^2 tissue culture flask). Culture flasks were maintained at 15°C. After 3 d of incubation, we poured out the SASW, thereby removing any remaining flagellates, and added SASW to the flasks, which were then incubated at 5, 10, 15, 20, and 25°C. After incubations of 7, 14, 30, 60, 90, and 120 d duration, we counted the numbers of cyst-like cells in 10 viewing fields to estimate the numbers per flask. We subsequently replaced the incubation medium (SASW) with tunic extract. Following incubation for 1 d at each of the temperatures, we again counted the number of cyst-like cells in 10 viewing fields. The number of excysting cells in each flask was calculated as the difference between the numbers of cyst-like cells before and after the induction of excystment with tunic extract. The excystment rate (%) was obtained by dividing the numbers of excysting cells by the numbers of cyst-like cells before incubation.

pH in the tunic

We collected healthy *Halocynthia roretzi* from an aquaculture farm site where the disease has never been recorded. To infect them with the disease, we injected 2 ml of flagellate suspension (1.7×10^6 cell ml^{-1}) through 26 gauge needles under the tunic matrix in the lateral sector of the body. The injected ascidians were reared at a density of ca. 1.0 ind. l^{-1} in aquaria containing aerated seawater (ca. 19°C). After 14 d, 14 of 51 individuals showed clinical symptoms of soft tunic syndrome. We selected diseased specimens (n = 10) with largely or entirely soft tunics (grade 3 infection; see Kitamura et al. 2010 for grade criteria) for pH measurements. Controls comprised intact, healthy individuals (n = 10). We measured pH values in the lateral sector of the tunic matrix using a pH meter equipped with a Spear electrode (EUtech Instruments). The electrode was inserted directly into the tunic matrices of the healthy specimens, but this procedure was impossible for diseased individuals because their tunics were too thin for complete immersion of the electrode. We therefore wrapped the electrodes in the tunic matrices of diseased animals.

RESULTS

Morphologies of cyst-like and flagellate forms

Azumiobodo hoyamushi cells maintained in the maintenance medium were usually fusiform with 2 subapical flagella in anterior and posterior locations (Fig. 1A,D). Within 6 d of incubation in SASW, many

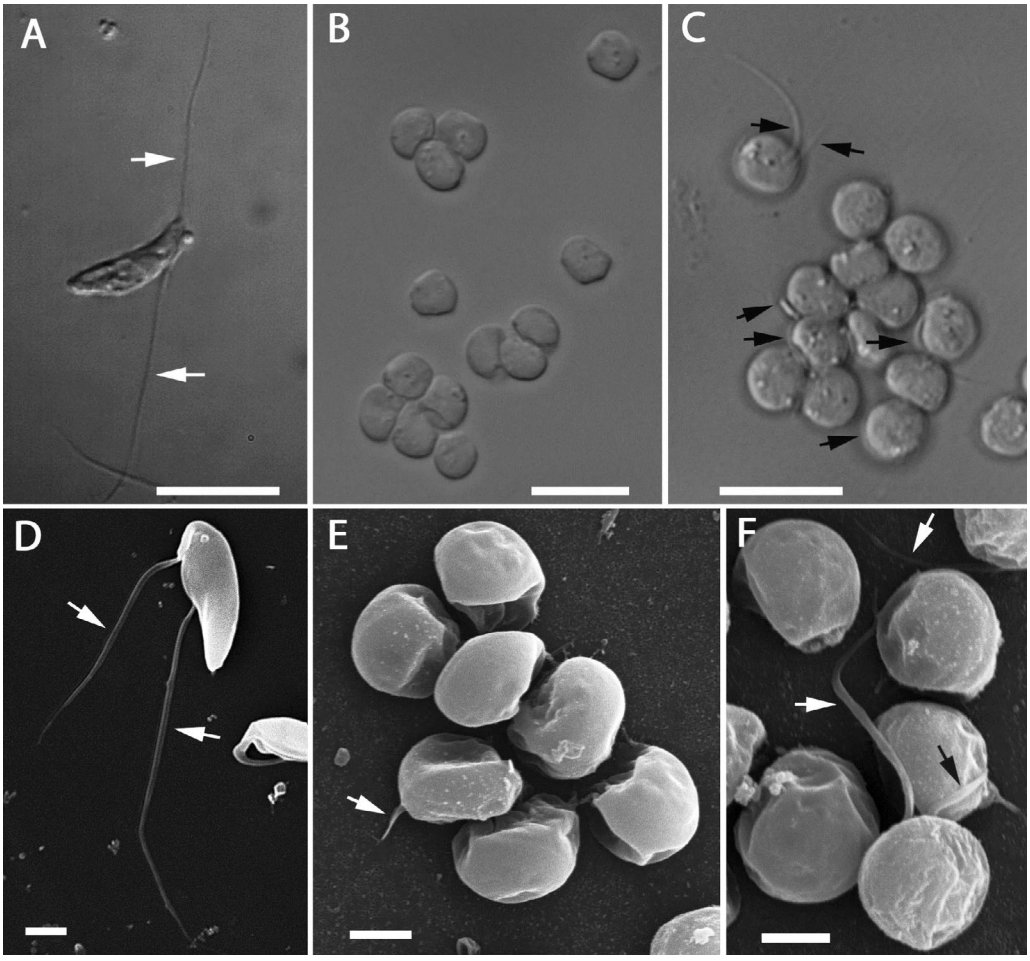

Fig. 1. *Azumiobodo hoyamushi.* (A–C) Light and (D–F) scanning electron micrographs. (A,D) Fusiform, flagellated cells in maintenance culture medium; (B,E) cyst-like cells incubated in sterilized artificial seawater for 6 d; (C,F) flagellate formation from a cyst-like cell after induction with tunic extract for 15 min. Arrows indicate flagella. Scale bars = (A–C) 10 µm, (D–F) 2 µm

A. hoyamushi cells became immobile, ovoid, and devoid of flagella (Fig. 1B). We refer to these as cyst-like cells, which often formed aggregates of cuboidal cells (Fig. 1E). We sometimes found flagella encapsulated in the cytoplasm of cyst-like cells (Fig. 1E). Within 15 min of incubation in the tunic extract, 1 or 2 flagella had emerged from some of the cyst-like cells (Fig. 1C,F), which were round to ovoid in shape and ca. 5 µm on the long axis.

Our TEM observations showed that kinetoplastids always had 2 flagella emerging from the apical poles of the fusiform cells (Fig. 2A). Both flagella had previously been completely enclosed in the cytoplasm of cyst-like cells (Fig. 2B,C). In the image captured in Fig. 2D, 1 flagellum is emergent and the other remains coiled within the cytoplasm of an ovoid cell. Kinetoplasts occurred in both fusiform and cyst-like cells (Fig. 2).

Formation of cyst-like forms

The encystment index after a 2 d incubation in SASW (11.0 ± 4.93, mean ± SD, n = 8) was significantly higher than the index in maintenance medium (0.56 ± 0.49, n = 8, $p > 0.01$, Wilcoxon matched-pairs signed-rank test; Fig. 3). Many of the *A. hoyamushi* cells remained fusiform and flagellated, even in SASW. Cells were motile in both SASW and the maintenance medium after the 2 d incubations.

Encystment was also induced in alkalified medium at the pH of seawater (8.4); the encystment index in this treatment was significantly higher than indices at pH 7.2 (unmodified maintenance medium) or pH 6.4 (acidified medium) ($p > 0.001$ and $p > 0.05$, respectively, Dunn's multiple comparison test; n = 10). However, encystment indices were not signifi-

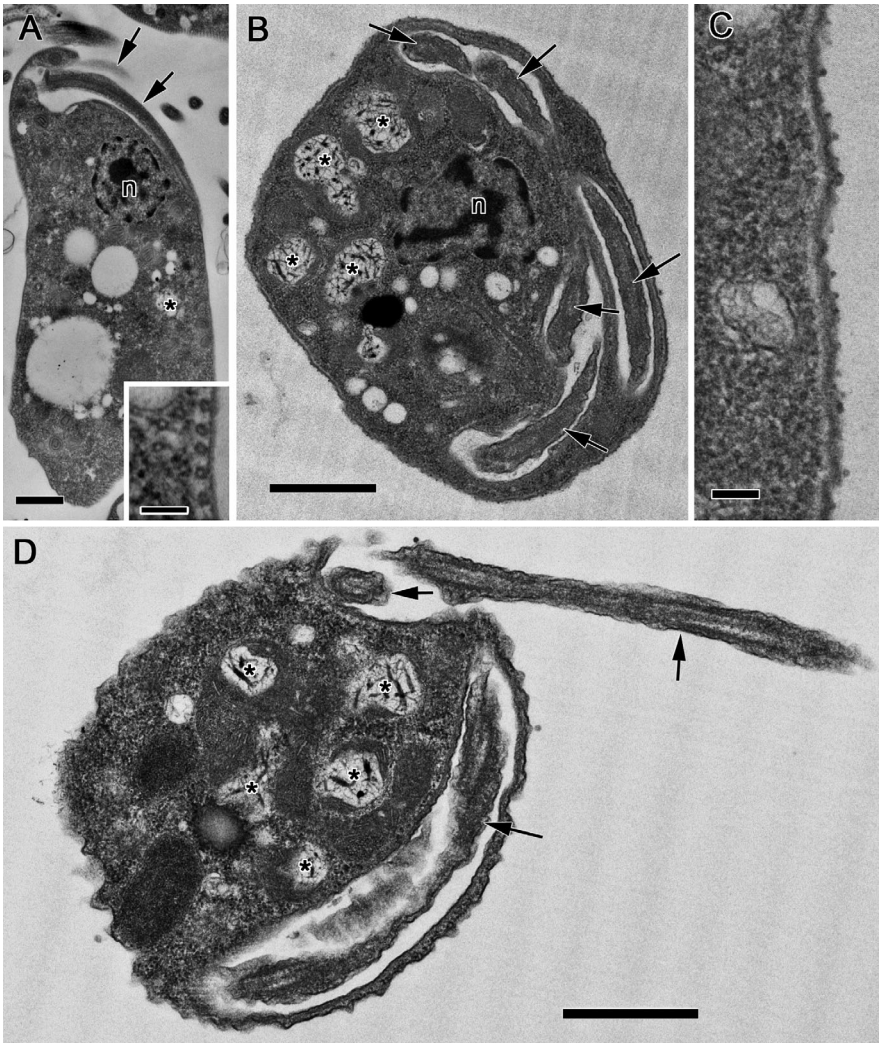

Fig. 2. Transmission electron micrographs of *Azumiobodo hoyamushi*. (A) Fusiform, flagellated cells in maintenance culture medium and enlargement of the cortical part of the cell (inset); (B) cyst-like cells incubated in sterilized artificial seawater for 6 d; (C) enlargement of the cortical part of the cell in (B); (D) flagellate formation from a cyst-like cell. Arrows indicate flagella; asterisks indicate kinetoplasts; n indicates nucleus. Scale bars = (A,B,D) 1 µm, (inset of A,C) 0.1 µm

cantly different between pH 7.2 and 6.4 (Fig. 4). Many *A. hoyamushi* cells were fusiform, flagellated, and motile in all pH treatments. Flagellated cells had closely similar morphologies across treatments.

The pH in the tunic matrix was 6.73 ± 0.14 (mean ± SD) in apparently healthy individuals and 6.75 ± 0.17 in diseased individuals. These means are not significantly different ($p > 0.01$, Student's *t*-test).

Induction of excystment

Approximately 15 min after the initiation of incubation with tunic extract, cyst-like cells began moving while still attached to the plastic substratum. Thereafter, the movement became active, and the ovoid cells gradually assumed a fusiform shape. Within a few minutes, 2 flagella emerged, and the cells began swimming (see video Supplement at www.int-res.com/articles/suppl/d115p253_supp/). Damaged cells and cell exfoliation were rarely observed under the microscope after 1 d of incubation. The excystment rates in SASW with and without the tunic extract were 96.9 ± 0.51% (mean ± SD, n = 10) and 7.08 ± 1.66% (n = 10), respectively. Rates were significantly different between treatments with and without tunic extract ($p < 0.005$; Wilcoxon matched-pairs signed-rank test; Fig. 5).

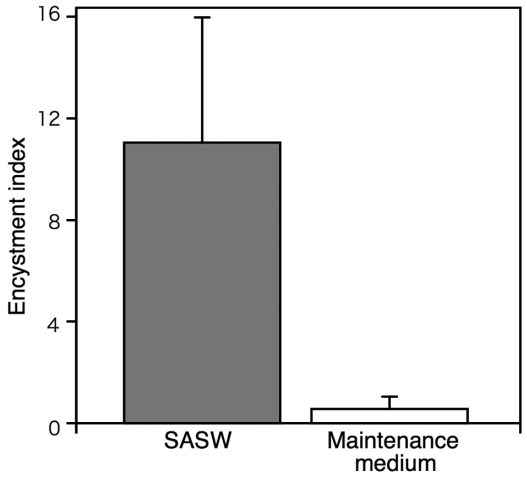

Fig. 3. Encystment indices for *Azumiobodo hoyamushi* incubated in sterilized artificial seawater (SASW) and maintenance medium. The encystment index (after incubation) was calculated as the number of cyst-like cells per 100 flagellated cells before incubation

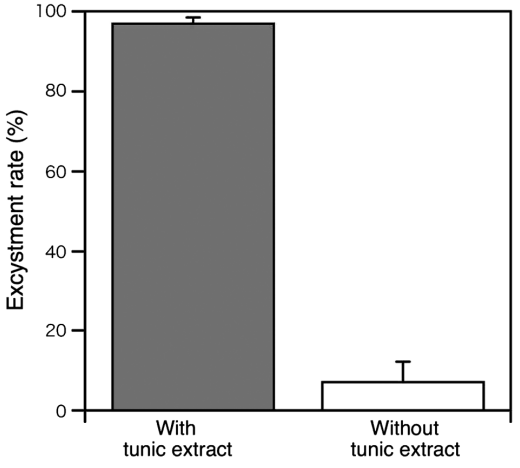

Fig. 5. Excystment rates of the cyst-like form of *Azumiobodo hoyamushi* incubated in sterilized artificial seawater with or without tunic extract. The excystment rate (%) was obtained by dividing the excysting cell numbers by the numbers of cyst-like cells before incubation

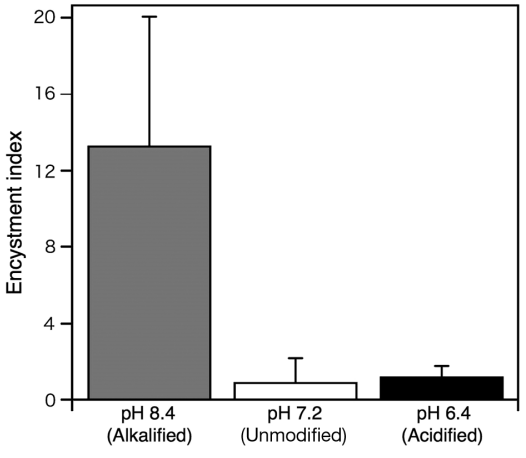

Fig. 4. Encystment indices of *Azumiobodo hoyamushi* at different pH values in maintenance media: pH 8.4 (alkalified medium), pH 7.2 (unmodified medium), pH 6.4 (acidified medium). The encystment index (after incubation) was calculated as the number of cyst-like cells per 100 flagellated cells before incubation

Longevity and temperature tolerance of the cyst-like form

The number of cyst-like cells gradually decreased through the incubation period in SASW (Fig. 6A). These cells survived for 14 d at 20°C, 60 d at 5 and 15°C, and 90 d at 10°C, while none survived for 7 d at 25°C. Among the surviving cyst-like cells in each treatment, >60% transformed to the flagellate form following induction with tunic extract (Fig. 6B). Among the temperatures tested, 10°C was the most favorable for survival in SASW: ca. 95% of cyst-like cells were capable of excystment after incubation for 90 d in SASW.

DISCUSSION

Azumiobodo hoyamushi usually has a biflagellate fusiform morphology in both *in vitro* culture and diseased tunics (e.g. Kumagai et al. 2011, Hirose et al. 2012), although some cells are occasionally spherical and lack flagella (Jang et al. 2012). Our electron microscopic observations showed that nonmotile cyst-like cells retained 2 flagella coiled in the cytoplasm. Cytoplasmic contents were microscopically similar between flagellate and cyst-like cells. In the bodonid kinetoplastids *Parabodo caudatus* and *Dimastigella trypaniformis*, cyst formation begins with the rounding up of flagellated cells and proceeds with the progressive formation of cyst walls and alterations of cytoplasmic contents (Brooker & Ogden 1972, Breunig et al. 1993). The cyst-like cells of *A. hoyamushi* were similar to cells in the precystic period of encystment in these 2 species, but we found no alteration in cytoplasmic contents in the species we studied. However, we cannot rule out the possibility that *A. hoyamushi* is able to form resting cysts enveloped in thick cyst walls under other circumstances (the culture conditions that we tested may have been unsuitable for the cyst maturation process reported for other kinetoplastids).

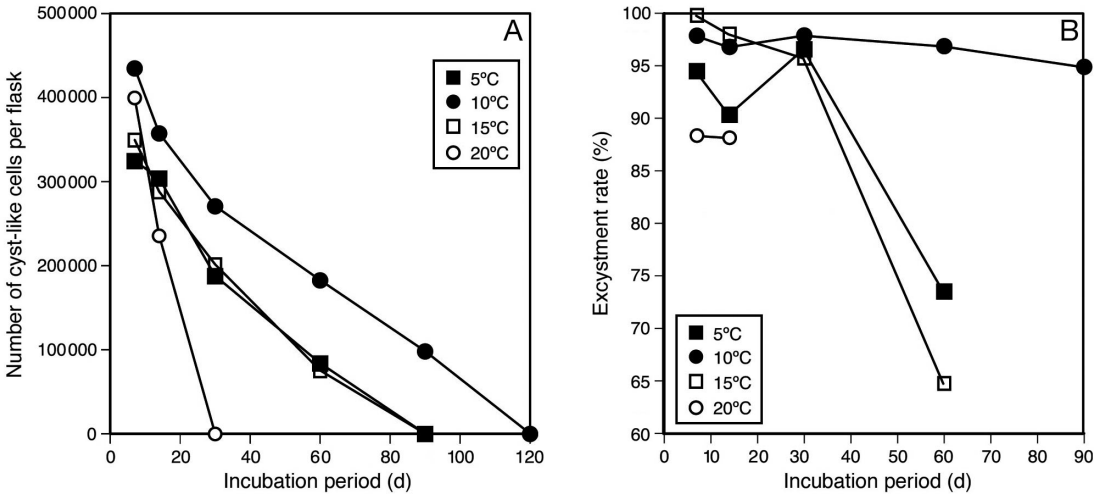

Fig. 6. Longevities of cyst-like cells incubated in sterilized artificial seawater at different temperatures. (A) Numbers of cyst-like cells after incubations of 7 to 120 d duration; (B) excystment rates (%) of cyst-like cells following induction with tunic extract after incubations of 7 to 90 d duration

In trypanosomatid kinetoplastids, several species are known to produce cysts such as straphangers that attach to the flagella of mother cells (e.g. Maslov et al. 2010, 2013 and references therein); these cysts are resting cysts and persist under adverse conditions for a long period. In the trypanosomatid cysts, the cell components are condensed, and electron-dense layers are formed on both sides of the cell membrane (Tieszen et al. 1985, Reduth & Schaub 1988). Therefore, straphangers of trypanosomatids are not similar in structure to the cyst-like cells in *A. hoyamushi.* In *Leptomonas* species (Trypanosomatidae) from fleas, ovoid cells were found in a rosette of the flagellates, and they were assigned as cyst-like forms that have uncondensed cell components, a short free flagellum, and no apparent cyst wall (Molyneux & Croft 1980). These morphological features are similar to those in the cyst-like cells of *A. hoyamushi*. The cyst-like forms of the *Leptomonas* species were supposed to be contained in the feces of fleas and ingested by larval fleas, supposing the capacity to survive in the feces outside the host, whereas excystment was not described for the cyst-like cells in *Leptomonas* species.

Cysts in dinoflagellates are either resting or temporary. The resting cyst has a thick wall and cytoplasmic contents that are reorganized and condensed for dormancy. In contrast, temporary cysts have thin walls and unmodified cytoplasmic contents. Temporary cysts are able to endure short-term or sudden stress but are less resistant to decay than resting cysts (Chapman et al. 1982). Temporary cysts are formed to survive allelochemicals, prolonged darkness, nutrient stress, and other environmental stress (Fistarol et al. 2004, Olli 2004, Rintala et al. 2007). The cyst-like cells of *A. hoyamushi* appear to be functionally equivalent to the temporary cysts of dinoflagellates, and for this reason, we refer to cyst-like cells as temporary cysts in this report.

Encystment was induced by the incubation of flagellates in seawater and in alkalified maintenance medium at the pH of seawater (8.4); neither unmodified (pH 7.2) nor acidified (pH 6.4) media induced encystment. Therefore, in addition to nutrient deficiency, the pH of seawater is one of the potential factors for inducing formation of temporary cysts in *A. hoyamushi*, which is unsurprising because raising or lowering a medium pH rapidly induces the formation of cysts, spores, gametes, and zygotes in diverse protists (see Agrawal 2012). The pH values in the tunics of both intact and diseased ascidian individuals were ca. 6.7. This value is a rough estimate because pH can be slightly lowered by the lysate of blood cells during measurements. The pH values in ascidian tunics and blood plasma are generally more acidic than the pH of seawater; the pH of blood plasma in *Pyura stolonifera* is 7.3 to 7.4 (Warr et al. 1977), and indicator dyes show that the pH in the vascular lumen of *Botrylloides simodensis* is lower than that of seawater (Hirose et al. 1995). Moreover, pH values of the media used for *in vitro* culture of ascidian cell lines are in the range of 6.8 to 7.0 (Rinkevich & Rabinowitz 1993, Kawamura & Fujiwara 1995). The tunic pH of *Halocynthia roretzi* was significantly lower than the pH of seawater; pH values in this range did not induce encystment in *A. hoyamushi*. This finding is consistent with previous observations of flagellate forms of *A. hoyamushi* in the tunics of diseased *H.*

roretzi individuals (Kumagai et al. 2010, 2011). The tunic extract we formulated was a remarkable excystment promoter; temporary cysts transformed to flagellate forms immediately after exposure to soluble factor(s) derived from the tunic. The temporary cysts of *A. hoyamushi* rapidly converted to proliferative, motile flagellates. Cyst cells began moving ca. 15 min after induction with tunic extracts and transformed to swimming flagellates a few minutes later. We assume that transformation is accelerated by the presence of coiled flagella in the unmodified cytoplasm of temporary cysts.

Temporary cysts were able to survive in seawater with no nutrient sources for a few months at temperatures of 5 to 15°C. Longevity was temperature-dependent. Some cysts transformed to the flagellate form (following induction with tunic extract) after a 90 d incubation in seawater. However, cysts survived for 14 d or less at 20°C, and none survived 7 d at 25°C. Thus, temporary cysts of *A. hoyamushi* were susceptible to heat stress. According to Kim et al. (2014), *A. hoyamushi* proliferates *in vitro* within a temperature range of 5 to 20°C. Therefore, heat and cold stress tolerances do not differ between temporary cysts and flagellated cells, which have anatomically similar cytoplasmic contents. This structural similarity may be related to the cytophysiological similarities in stress tolerance between the 2 phases and the remarkable transformation abilities of cyst-like cells. Nevertheless, a large proportion of temporary cysts were able to excyst *in vitro* after being held for protracted time periods in seawater (Fig. 6B). Thus, *A. hoyamushi* is able to persist *ex hospite* as a temporary cyst form for long periods. Longevity under these conditions was temperature-dependent, but any surviving cysts retained the ability to rapidly transform into infectious flagellates.

Based on the present findings, we propose a qualitative life cycle schema for *A. hoyamushi* during the outbreak phase in an aquaculture facility (Fig. 7). (1) Flagellates invade the tunic of *H. roretzi.* Although the main body tunic is entirely covered by a cuticle sufficiently dense to repel agents of infection, the tunic of the inner siphon walls is often damaged, and the damaged areas are potential entry sites for the flagellates (Hirose et al. 2014). (2) *A. hoyamushi* cells in the tunic have a flagellate form, which proliferates and softens the tunic. The tunic pH remains constant in this phase (ca. 6.7); pH values are similar in intact and diseased individuals. (3) The tunic disintegrates and tears, releasing some of the flagellates, which are able to swim for short periods in the seawater; they readily find new hosts because the ascidians are crowded in aquaculture facilities. Seawater (ca. pH 8.4) induces encystment of some of the flagellates in the water column. In our *in vitro* experiment, ca. 10% of flagellates formed temporary cysts within a few days. (4) Some of the temporary cysts drift and disperse in the water, some attach to fixed substrata (e.g. the aquaculture facility and resident sessile organisms), and others sink to the seafloor (ca. 20 to 30 m below the water surface in the vicinity of facilities in Miyagi Prefecture). (5) When the host ascidians filter drifting temporary cysts out of the seawater, unknown factors leaking from the tunic induce rapid excystment. The cysts transform to the flagellate form, likely in tissues surrounding the damaged tunic lining the inner walls of the siphons; the newly formed flagellates infest the host tunic.

At our study site (Miyagi, Japan), incidence of the disease falls rapidly toward the end of summer when the surface water temperature is ca. 20°C or higher

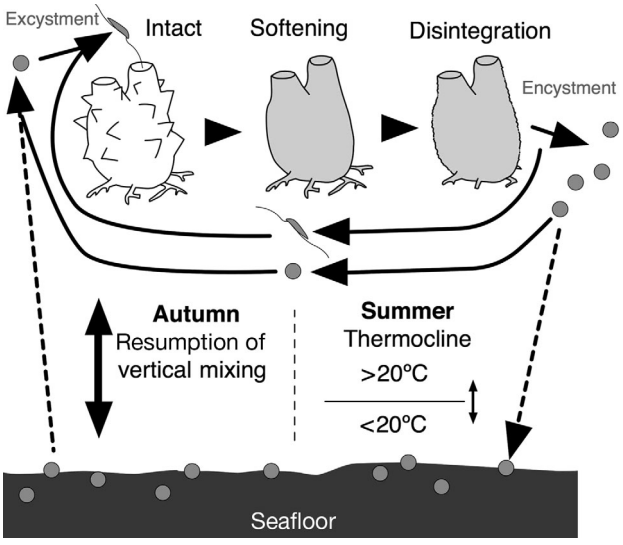

Fig. 7. Infection cycle of *Azumiobodo hoyamushi* in the outbreak (solid lines) and resting (dashed lines) phases. Flagellates enter the tunic of inner walls of the siphons (upper left) and proliferate as flagellate forms in the superficial layers of the host animals. Flagellates are released from the disintegrating tunics. Some of the released flagellates infect the tunics of new hosts. Those that do not are induced by seawater (ca. pH 8.4) to form temporary cysts that drift and disperse in the water column. When host ascidians filter out drifting temporary cysts from seawater, they rapidly transform to flagellate forms that infect the host tunic. Some cysts sink to the seafloor (where the temperature is always lower than that in the shallower aquaculture facilities) and are able to persist for extended periods in the cool waters beneath the thermocline. Vertical mixing of the previously thermally stratified water column in the fall–winter period brings the temporary cysts into surface layers, where they are able to infect intact host ascidians

(Kumagai et al. 2010). At this time of the year, the host ascidians are often dormant, their seawater intake rates are greatly diminished, and the rates of infection decline (Kumagai et al. 2010). Many of the *A. hoyamushi* cells are inactive or morbid in aquaculture facilities (with depths <15 m) at this temperature. However, temporary cysts are able to persist on the seafloor at depths of ca. 30 m or more, where the temperature is lower than that in the shallower aquaculture facilities (Kumagai et al. 2010), due to thermal stratification of the unmixed water column (Kaneda et al. 2002). During the fall to winter period, temperature gradients disappear in the upper 1 to 20 m of the water column; this indicates the resumption of vertical mixing (Kumagai et al. 2010), which may transport temporary cysts from the seafloor to the surface where they are filtered out by ascidians, initiating a new cycle of infection (Fig. 7). This schema provides a plausible explanation for the seasonal fluctuation in the soft tunic syndrome in our aquaculture facilities.

We do not know precisely why soft tunic syndrome appeared suddenly in Japan, but the epizootiology of the syndrome strongly suggests a relationship between disease outbreak and the introduction of Korean spat (Kumagai et al. 2010). However, contradictory data come from infection tests and ultrastructural observations showing that juvenile ascidians are not susceptible to *A. hoyamushi* (Hirose et al. 2014). In this report, we showed that *A. hoyamushi* is able to form adherent temporary cysts that are able to persist for several months in seawater. Therefore, these cysts may originally have been transferred to Japan adhering to substrata to which the Korean ascidian spat were attached.

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