ADAPTATION TO DEEP-SEA HYDROTHERMAL VENTS: SOME MOLECULAR AND DEVELOPMENTAL ASPECTS

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ABSTRACT

Alvinella pompejana is a polychaetous annelid inhabiting the surface of deep sea hydrothermal chimneys along the ridge of the east part of the Pacific ocean. The main characteristic of this emblematic species is its habitat, which is very aggressive considering its temperature. The exceptional thermotolerance of this species (up to 80°C) has been the subject of much controversy. This review is focused on the thermal adaptation of this worm regarding molecular data relative to its extracellular matrix and life history traits.

INTRODUCTION

Alvinella pompejana, the so-called Pompeii worm [15], is one of the emblematic animals living in the extreme environment of deep-sea hydrothermal vents. This tubicolous animal is found exclusively in association with high temperature venting, at the surface of hydrothermal chimneys on the East Pacific Rise (Figure 1). The strong gradient from the chimney wall to the surrounding seawater, depicted in Desbruyères *et al.* [16], has been more finely assessed with high resolution surveys (review in [44]). While the 2°C background seawater temperature is generally recorded less than ten centimeters above tube openings, temperatures largely above 100°C are measured in contact with the mineral substrate directly beneath the tubes (as in Figure 1b). The reported temperature maxima for *Alvinella* colonies on different chimneys range from 125°C in [45] to 175°C in [18]. In contrast to these temperature extremes, moderately warm conditions were reported at tube openings, ranging from 6°C to 45°C on average [45]. *Alvinella pompejana* are living inside tubes they secrete

at the smoker surface [27]. These exoskeletons allow the *Alvinella* juveniles to colonize the mineral substrate of the smokers.

In the eighties, deep-sea biologists have come up with spectacular observations which strongly suggested that this species could withstand unusually high

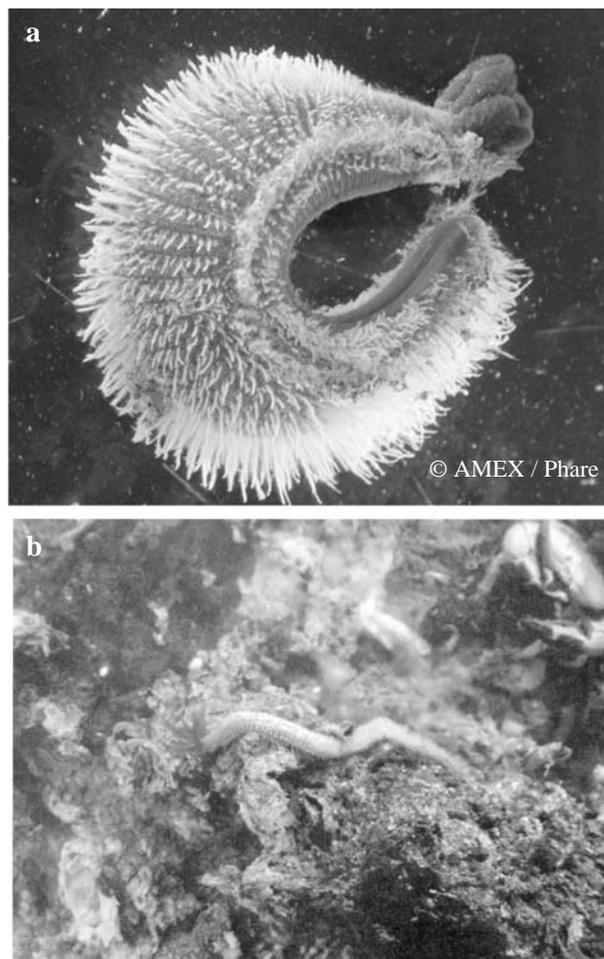**Fig. 1.** **(a)** *Alvinella pompejana* **out of its tube after recovery. The animal is about 10 cm long; (b) Alvinella pompejana crawling on bare mineral surface at vent sites.**

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temperatures, (up to more than 100°C [10]), and which triggered an on-going debate on its upper thermal limits [12]. Temperature inside *A. pompejana* tubes was initially determined by [7]. An average temperature of 68 ±6°C monitored inside a tube over 2 hours, with spikes as high as 81°C was reported. The relevance of this value was strongly debated and several artifacts were suggested, including disturbance of the animal behavior or piercing the tube when inserting the probe [12]. Since that date, Cary and co-workers have confirmed the ability to reproduce hour-long temperature monitoring inside tubes [18].

Not only the extreme temperatures encountered in some tubes, but also the variable temperature over time support the idea that the medium is not necessarily in thermal equilibrium with the worm body. The 2 to 4 hours long temperature records presented in [7] and [18] exhibit frequent temperature changes of 10 to 20°C over a few minutes and frequent sharp spikes of up to 40°C in amplitude. The modulation of mean temperature over more than two hours in one of the hottest tubes was shown to range between 60°C and 100°C [18]. Furthermore, the large external temperature gradient along a tube length of c.a. 15 cm suggests a substantial longitudinal gradient inside the tube supporting the idea that the pompeii worms are also the most eurythermal animals ever known in the oceans.

Besides *in situ* measurements conducted on and within colonies, *in vivo* experiments using pressure aquaria confirmed the exceptional thermotolerance of representatives of the alvinellid family. A northern Pacific relative of *A. pompejana*, *Paralvinella sulfincola*, was very recently shown to be tolerant to temperature of 50 to 55°C [35], the highest ever found for a marine metazoan. Even more surprising, this worm was shown to prefer temperature in the range 40°C to 50°C.

Several reviews devoted to *A. pompejana* have been published in the past 20 years, assessing current knowledge in its ecology [16], biology [27] or providing a general overview of the various ecological, physiological and biochemical studies related to this organism [17]. New tools have been used in the last decade to precise the adaptation strategies of *A. pompejana* to the extreme environmental conditions of its habitat and these recent data have been reviewed by [44]. Part of this paper will use data which were discussed in previous reviews including the most recent one [44], but will focus on the thermal adaptation of *A. pompejana* through the description of its extra-cellular matrix characteristics and life history traits.

After a brief review of the thermal behavior of the animal, the thermotolerance of *Alvinella pompejana* will be considered in the light of the findings concerning the properties of its exoskeleton, the tube, and of its

collagen molecules. Extra-cellular biopolymers are supposed to protect the animals from the harsh surroundings and would give indication about the upper temperature limit the animal may support. In this respect comparison with extra-cellular matrix (ECM) from the giant tube worm *Riftia pachyptila* will allow us to precise what is specific to the alvinellid family from what is more widely found in annelids. We will start with ECM covering the animal body, i.e. the tubes and/ or mucus, and further deal with the main components of the ECM, which are the collagen molecules. We will see that data obtained on the biopolymers are in favor of a worm body of less than 50°C, even though this animal can withstand up to 65°C. How such a high thermostability may be reached by structural proteins like collagens? Part of the review will be dedicated to the thermal adaptation at the molecular level.

ECM data were obtained on adult tissues. However, we still do not know to which extent the larvae may face high temperature, since early steps of development of *Alvinella* have been shown to occur in rather cold environment, i.e. less than 20°C [57]. So, a specific description of the life history traits of the pompeii worms and sister species will help us in the understanding of the life cycle of this unusual thermophilic metazoan. We will first review current knowledge on the reproduction mode of the alvinellids which exhibit specific reproduction strategies [56, 59] and the development process of the animal [57, 60].

1. Thermal tolerance of alvinellids and related species

New types of high-pressure aquaria [35, 58, 64] have allowed to examine in vivo the thermal limits of several hydrothermal vents species inhabiting chimney walls [35, 46, 61, 62, 65, 66]. Although very promising high-pressure experiments have demonstrated the capacity to maintain *A. pompejana* alive up to 24 hours after recovery [64], its limited survival after collection did not allowed, to date, *in vivo* experimentation on this species.

In an effort to characterize the thermal tolerance of various species living in close proximity to high temperature emissions, Shillito *et al.* [65] considered the hesionid worm, *Hesiolyra bergi*, that was observed crawling at the surface of *A. pompejana* colonies and often entering their tube for a few second to several minutes. These authors have demonstrated an escape behavior at 35°C and a lethal limit at 41°C for this congener, which indicated that high thermal tolerance is not a prerequisite to live among *A. pompejana* colonies. The large thermal heterogeneity characterizing these colonies over space and time, however, precludes considering this mobile species as a biological 'thermometer', as it was suggested by [65]. As reviewed in

[44], it is now well established that the surface of the colony is exposed to temperatures ranging from a few degrees to 45°C, and that the highest temperatures in this range are restricted to the vicinity of localized fluid outflows. Another example with the alvinellids supports the idea that non-thermotolerant species can in fact inhabit high temperature chimney wall. *Paralvinella sulfincola* and *Paralvinella palmiformis*, the two alvinellids species of the Juan de Fuca Ridge, were shown to have very different temperature tolerances while sharing the same habitat [35]. While *P. sulfincola* preference to temperature in range 40-50°C was established *in vivo*, the later consistently avoided temperatures above 35°C.

P. sulfincola is the only animal that is now firmly identified to prefer chronic exposure to temperature as high as 50-56°C. Although lower than for some terrestrial animals (55 to 65°C; see examples in [10, 35]), the temperature preference and tolerance of this species stand at the upper limited of the accepted range for metazoans. While most hydrothermal vent animal species were indeed observed to live at 'room temperature' (c.a. about 20°C) (see review in [77]), some species, such as *P. sulfincola*, reveal outstanding temperature preference in *in vivo* experimental settings offering them the possibility to chose their thermal environment. In this regard, *in vivo* experimentation should now allow us to investigate thermal behavior of vent organisms. The association of *Alvinella pompejana* to extremely hot substrates, comparable in this respect to the environment of *P. sulfincola* [41], supports the idea that this species would have a similarly high thermotolerance. If the limits of its thermal preference and tolerance remain to be empirically defined, its exceptional thermal adaptation is now firmly assessed from molecular markers.

2. Alvinellid extracellular matrix

Alvinella extra-cellular matrices are composed of two different tissues: the tube, which is the exoskeleton of the animal, allowing the worm to settle on the chimney wall, and the collagen which is the main molecular component of the tissues covering the worm body. Other alvinellid species such as *Paralvinella grasslei* dot not have solid exoskeleton and secrete soft mucus, which allow their adhesion to organic or mineral substrate [27].

(1) Alvinellid tubes

(a) Composition and structure

The material which *A. pompejana* uses to build its tube originates from an anterior-ventral glandular shield [79]. The bulk of the material is formed from homogeneous granules secreted by the deep main cells of the shield, while an accessory component is provided by mucocytes secreting sulfated glycosaminoglycans. The granules are proteinaceous but also contain minerals (e. g., phosphorus, calcium, iron, and lesser amounts of magnesium and zinc, see next section). Although the organic material of the tube is mainly protein, about 7% of the tube is hexose sugar, presumably as poly- or oligosaccharide material bound to the protein.

The tube is a concentrically multilayered, fibrous structure in which the superimposed layers of parallel fibrils vary in direction from one sheet to the next. Neither the number nor the thickness of the layers is constant and varies between different parts of the same tube. Old and mineralized tubes tend to have thinner walls than those that have been secreted more recently [26]. The inner surface of alvinellid tubes is covered by filamentous bacteria, which, as the tube is being laid down, become trapped under consecutive layers of material. Each layer of tube is composed of sub-layers and individual layers are separated by entrapped bacteria. Within the layers of secreted protein, a novel distribution of fibrils is displayed, reminiscent of the arrangement of the polymeric units in a cholesteric liquid crystal. Observation of the *Alvinella* behaviour during the Phare cruise, allowed us to infer the role of the worm motion in this twist variation [44]. Mucous secretion, and bacteria associated with that secretion, may provide the initial structure-dictating constraint and is later modified by the fibrous structure itself as it is laid down or by pulses of additional mucus production and bacterial growth. Such biopolymeric organisation is thought to provide a specific thermal resistant to the worm [27].

(b) Mineral content

The mineral content of the *A. pompejana* tubes, as reflected by the ash content, is high (29%). In addition to this involatile inorganic content, there is also between 12 and 25% of elemental free sulphur, the amount depending upon the age and the area where the tube comes from [26]. The mineral seems to be present as a mixture of sulfides, phosphates, and carbonates.

Minerals show specific patterns of association with tubes. Zinc-iron sulphide nanocrystals grouped in submicrometer-sized clusters were described between proteinaceous tube layers [84]. These minerals show a specific zinc-iron signature, and have a conserved size contrary to mineral precipitations found on the outside of the tubes. The nanometer size of individual minerals within tubes and their specific constant composition suggested the biological origin of these crystals, most

probably induced by bacteria associated to the tube [84]. Mineral particles were also seen as useful markers for evaluating the chemical characteristics of the microenvironment [85]. Gradients in mineral crystals size and composition were described and hypothesized to reflect gradients in chemical characteristics between the inside and the outside of the tubes, as well as decimetre-scale gradients between tubes located more or less deep in the thickness of the *alvinella* colony. From these results, the authors hypothesized that the tube acts as an efficient barrier to the external environment [85].

(c) Associated bacteria and sulphur

The dense covering of filamentous bacteria on the internal face of the tube is not uniform. Some region may be free of bacteria, perhaps reflecting differences in secretory activity of the epidermis of the worm. The bacteria can generate iron oxides from pyrite within the tube and can become embedded in amorphous silica. Crystals of pure sulphur have been observed by us on the inside surfaces of the tubes in association with filamentous bacteria, and free amorphous sulphur is present in mucus of *Paralvinella* which has been laid down for some time and which is in the process of mineralizing [27].

(d) Specificity of the *Alvinella* tube composition

Unlike vestimentiferan tubes [25], *Alvinella* tubes do not contain chitin. There is no indication from X-ray diffraction studies of an ordered secondary structure of proteins, although the amino acid composition, with its high glycine, alanine, and serine levels, is typical of the type of beta-pleated fibrous proteins such as silk fibroins [27]. Polychaete worms exhibit a peculiar versatility in regard to the composition of their tube materials and, therefore, the amino acid composition of the alvinellid tube is probably of little use in establishing comparison with other polychaetes. However one characteristic has to be retained: it is its highly hydrophobic nature.

(e) Chemical and physical stability

The material of the *Alvinella* tube has considerable chemical stability. While it is not unusual for invertebrate structural materials to be very chemically stable, usually as a consequence of extensive cross-linking, most will swell eventually disrupt at room temperature in strongly acidic or alkaline solutions or else in chaotropic agents such as anhydrous formic and haloacetic acids or lithium thiocyanate. The *Alvinella* tube shows little response to these or to disulfide bond-breaking agents, although a cycle of concentrated hydrochloric acid and potassium hydroxide treatments will cause delamination, swelling, and a little solubilization [26]. Thermal stability is great also, with little swelling or shrinkage taking place over the 0 to 100°C temperature range. This too probably reflects a high degree of cross-linking.

(2) Alvinellid Mucus

Tubes are not secreted by the genus *Paralvinella*, but extensive mucus production helps to serve a similar protective purpose. This material eventually can form permanent structures as mineralization takes place. Freshly secreted mucus is transparent with no visible deposits in it but then becomes yellowed due to elemental sulphur at levels as high as 80% of the dry weight (5). Sulphur found in mucus is probably a product of bacterial metabolism since mucus is extensively colonized by sulfide-oxidizing bacteria.

(a) Amino acid composition

The amino acid composition of the freshly secreted mucin of *P. grasslei* is quite different from that of the tube of *Alvinella*, discounting the view that the latter might simply represent a cross-linked from of mucin secretion (5). Instead the aspartic and glutamic acid contents are high, the glycine content lower, and the alanine and serine contents appreciably less. However, as the deposited mucus ages and mineralizes, the proteins degrade and leave a core of very hydrophobic proteins, which is richer in glycine, alanine, and valine.

(b) Mineral composition

The secretion of mucins may fulfil many roles, among which protection against a spectrum of environmental affronts, feeding, and detoxification. Thiolic metal-binding proteins are detectable in the mucins, and iron, zinc, copper, and uranium have all been detected, with concentration of uranium ranging from less than 0.45 to 3.0 μg Kg^{-1}. These concentrations exceed that of seawater itself. Uranium enrichment in worm tubes is not confined to animals living on the white smokers, but has also been found in tubes from the black smokers and the Galapagos hydrothermal mounds.

(3) Alvinellid collagens

The collagen molecules belong to a family of extra-cellular proteins, which are characterized by a

triple helical domain. This domain is formed by association of 3 similar peptides, called α chains, which are composed of a succession of Gly-X-Y amino acid triplets. Usually the Y position is occupied by a proline amino-acid that is often hydroxylated. The collagen is synthetized inside the cell, and secreted outside the cell in the extra-cellular compartment. The intracellular collagen is composed of a central triple helical domain, which is conserved during the whole life time of the molecule. The carboxy- and amino-propeptides (C-pro and N-pro respectively), ending the triple helical central part, are removed when the molecule is secreted in the extra-cellular matrix.

The collagen characteristics may provide a relevant set of information relative to the characteristics of the surroundings. This molecule is one of the most well known extra-cellular proteins of the animal kingdom and is a relevant marker of thermal adaptation [25]. This molecule has been well characterized in *Alvinella pompejana* [29, 30, 32, 33] and vent species [48] and the origin of the thermostability of the *Alvinella* collagen has been determined by [68].

(a) Interstitial and cuticular collagens

Vent annelid species possess two abundant collagen types, which differ in composition, size, domain structure [33] and immunological properties [30]. Whereas the interstitial collagen is similar in morphology to the fibrillar collagen of vertebrates, the cuticular collagen is rather unusual. The length of the triple helix is of 280 to 300 nm for all interstitial collagens of the alvinellid studied. A similar length is reported for the interstitial collagen of another vent endemic annelid, *Riftia pachyptila*. In contrast, the cuticle collagens of annelids, with lengths of up to 2.5 μm in the alvinellid species, are the longest collagenous protein known [25]. They possess a terminal globular domain and no comparable counterpart has so far been identified in other invertebrates or in vertebrates. The cuticular collagen of *R. pachyptila* is not so long as its length is about 1.5 μm. It has been shown that the cuticular collagen of tube worms from various chemosynthetic environments has a similar length and that such a characteristic is phylogenetic.

Except for the thermostability of the molecule, cuticular collagens from coastal and vent species shared similar structural characteristics. This is also true for the interstitial collagen of annelids from various habitats. These characteristics were apparently conserved in various annelid families including a substantial and not very variable level of 4-hydroxyprolinee in the Y position of the Gly-X-Y sequence triplets.

(b) C-propeptide

The C-propeptides (C-pro) from the annelids collagens share overall feature of the mammalian fibrillar collagen C-pro. They all present a potential cleavage site, similar in type I, II and III collagen chains, leaving in the collagen molecule a telopeptide of about 30 amino acid long, which is consistent with the length found in other fibrillar collagen chains [68]. Specific residues are also conserved such as the cyclic ones (F, Y,W), the charged ones (D,N,E,Q,R,K) and proline residues as well as the glycosylation site (NXT/S).

The main difference between annelids and mammalian C-pro is related to the number of cystein residues. So far C-pro contained 7 or 8 cysteins depending on the type of association, hetero- or homo-trimer, of the α chains. The reduced number of cysteins (6) observed in the *Arenicola* collagen [67] is common to all worms collagen. The missing cysteins (2 and 3 in the α1(I) chain) are thought to form intermolecular bonds but have also been shown of minor importance in realizing triple helix [5]. The alvinellid and Riftia C-pro are a natural example of the Bulleid observations [5] that the molecular mechanism for chain recognition does not solely rely on the number of cystein residues, but also on the divergent regions of the C-propeptides.

(c) Thermal stability

Alvinella has the most thermostable protein ever known [32] and [1] have shown that pressure is not involved in such a characteristic. The temperature at which the collagen molecule is denatured (Tm) is 46°C for the cuticular collagen covering the animal epidermis and 45°C for the interstitial one which is found in the worm tissue [32]. Among the fibrillar collagens of 40 other vertebrates and invertebrates, the *A. pompejana* collagen is positioned at the upper limit for melting temperature, only before that of thermostable synthetic collagens (review in [44]).

The level of thermal stability of *Alvinella pompejana* cuticular interstitial collagen is significantly higher than that of other vent annelids. In comparison, *Riftia pachyptila* molecular collagen stability only reaches 29°C and the collagen of *Paralvinella grasslei* has a denaturation temperature of only 35°C [48].

(d) Thermal stability process

The origin of the collagen stability is not well understood but it is obvious that the rate of proline hydroxylation is an important factor. In collagen-like peptides that form triple helices, the substitution of hydroxyproline (Hyp) for proline in the Y position of

the repeating Gly-X-Y triplets provides additional sites for hydrogen bonding of water molecules in crystals of the peptides (review in [68]). In fact the water bridges would only contribute in part to stability. Different explanations have been proposed, involving the entropy state of the chain and an electron withdrawing inductive effect of the hydroxyl group. No one knows today the exact correlation between the thermal stability score and the different processes involved in the molecular stability.

In *Alvinella*, as almost all the collagen proline residues in the Y position of the Gly-X-Y triplets have been shown to be fully hydroxylated [32], the collagen stability process would be the same than what is known in vertebrate and human fibrillar collagen. Sicot *et al.* [68] have demonstrated that there is a clear correlation between the thermal stability and the proline content in Y position. This would indicate that in living organisms, proline in the Y position of the GXY triplet would be a decisive factor involved in the collagen thermal stability.

The relative percentage of proline in the Y position of the triplets is 3 times higher in *Alvinella* than in *Riftia*, which has the lowest relative percentage. The frequency of the double-P triplets (GPP) relies only on the frequency of the overall proline content among the various aminoacids present at the second and third position of the triplets. Hence, an increase in proline content would automatically lead to an increase in GPP triplets. Since GPP triplets are among the most stabilizing triplets, an increase in proline content would result in an increase in the thermal stability of the triple helix.

Bachinger and Davis [2] have proposed the concept of sequence specific relative stability of the collagen triple helix to quantify the molecule stability. Considering this point, *Alvinella* has also the highest score of the fibrillar collagens analyzed so far, with the highest stabilizing triplets and the highest GPP frequency. Moreover, all the stabilizing factors known today are amplified in the *Alvinella* collagen including the percentage of stabilizing triplets, the proline content and the frequency of hydroxyproline in the Y position of the Gly-X-Y triplets.

(e) Diversity of stability process

Results obtained on the second type of fibrillar collagen, the cuticular collagen, observed in the alvinellid and sioglinid worms [30, 48], indicate that the same type of protein family would exhibit two different strategies of thermal stability. One which had a great success in the course of evolution is that found in the alvinellid interstitial collagen, relying on the proline hydroxylation of the residues in the Y position of the GXY triplets; and one which seems to be original

up to now, which relies on the glycosylation of the threonine in the same position of the triplet [48]. The latter is found in the cuticular collagen of *R. pachyptila*. Both these examples underline the importance of post-translational processes in the molecular stability. No one knows today if these differences are phylogenetically related or collagen type specific and additional data are needed on the cuticular collagen sequence to answer this question.

Another potential stability process is the number and distribution of Gly-X-Y triplets in α chains. In theory, there are more than 400 possible Gly-X-Y triplets, but analysis of sequences from fibrillar and non fibrillar collagens shows that only a limited set of triplets are found in significant numbers and many are never observed (review in [68]). The distribution of the triplets through the chain length of these collagens has not been yet precisely analysed. It would be of interest to determine the frequency and distribution of the triplets through the chain length of the available collagen molecules.

(f) Thermostability and collagen evolution

The alvinellid collagen sequences obtained by [68] have confirmed the monophyly of the annelid interstitial collagens [67]. However, depending on the considered collagen domain, the triple helix or the C-pro, the resulting phylogenetic trees are different. If the C-pro is solely considered in the phylogenetic analysis, the obtained tree is in accordance with the species classification published in [67]. *A. pompejana* and coastal annelid *A. marina* collagen domains are grouped whereas *R. pachyptila* has a distinct location. In contrast, the *R. pachyptila* collagen groups with that of *A. marina* when the triple helical domain is analysed. This result indicates that different selective constraints have been applied on the 2 domains during evolution (Table 1). This has been hypothesized to be related to the temperature the worms inhabit, *A. marina* and *R. pachyptila* living in a colder habitat than the thermophilic worm *Alvinella*.

The collagen is a modular protein where the triple helical domain has a longest life time than the C-propeptide, which is removed once the collagen is secreted outside the cell. The triple helix and the C-Pro domains would have evolved independently, the selective pressure affecting more the triple helical domain which is exposed to high temperature in *A. pompejana* ECM. The collagen evolution being not neutral, the conclusion of Sicot *et al.* [68] leads to the hypothesis that the collagen triple helix part would have evolved in different direction according to the living temperature of the animal, the evolution of the C-propeptide remaining constant.

Table 1. **Evolutionary distances between annelids' fibrillar collagens (from [68]).** **Evolutionary distances between** *Alvinella pompejana*, *Riftia pachyptila* **and** *Arenicola marina* **collagen chains were computed.** **Distances computed from the helical domain are always greater than distances computed from the C-propeptide.** **This means that the selective constraints on the two domains of the same molecule are different.** **Indeed, the helix only is maintained in the extracellular matrix, while the role of the C-propeptide is to catalyze the formation of the triple helix.** **It can be assumed that the selective constraints exerted on the C-pro would not differ from one species to the other, while the helix might be more prone to environmental influences.** **Hence, the C-propeptide domain was taken as an internal control measuring the part of evolutionary drift after speciation in the evolution of the molecules.** **The rate of evolution of the helical domain is about twice the rate of evolution of the C-propeptide along the distance from** *Riftia* **to** *Arenicola* **and along the distance from** *Riftia* **to** *Alvinella*. **This means that changes in the helical domains after the divergence of** *Alvinella* **and** *Arenicola* **from their common ancestor are more numerous than expected.** **This was taken as circumstantial evidence for an adaptive effect.**

| | Total sites | Variable sites | Distance between | | |
			Alvinella /Riftia	*Arenicola/Riftia*	*Arenicola/Alvinella*
Helix	354	237	98.21	98.88	77.62
C-pro	246	114	54.33	49.82	31.43
Relative rate Helix/C-pro			1.81	1.98	2.47

(g) Molecular thermostability and animal thermotolerance

Since the thermal stability of the collagen molecule is 45°C, it can be assume that synthesis of the collagen would be stopped at a higher temperature, and the *Alvinella pompejana* maximum body temperature would be less than 45°C. However, the collagen is not found in tissues in a molecular form, but in a supramolecular state. Fibrillar structure is the physiological state of the collagen in metazoan tissues. Once synthesized, the collagen molecules assemble in fibrils and such a polymeric organization has a thermal stability which can exceed by 20°C that of collagen molecules [28]. *A. pompejana* fibrillar collagen assemblage may thus resist up to 65°C, which is consistent with experimental data (review in [44]). If molecular data let us think that above 45°C, the collagen cannot be synthesized by the epidermal cells, the worm could still sustain such a high temperature without any damage in its collagen assemblage. This means that thermal fluctuations between 40 and 60°C could be easily supported by this animal. This would also support the idea of a thermal gradient along the animal body length [6], which is consistent with the observation that the cuticle of the oldest worms disappears on their posterior part [31]. Additional data on the *Alvinella pompejana* prolylhydroxylase [42] indicate that the worm is not only facing the highest temperature ever known for marine invertebrates but would have a metabolic machinery adapted for working in low oxygen environments (review in [44]).

3. Reproduction and development in Alvinellid species

Hydrothermal vents are highly unstable ecosystems both on a spatial and on a temporal scale. For this reason, the question of the maintain of species and of population distribution has immediately been raised and is still not answered. Genetic studies carried out on most vent taxa evidenced exchanges between populations over distances greater than the average intervals that occur between vent sites. Fairly wide dispersal capabilities were thus inferred for most species inhabiting vent sites.

Many vent species are sessile and cannot survive as adults outside of vent sites. Dispersal was thus mainly attributed to the larval phase. However, this phase of the life cycle of vent organisms remains unknown for most species. In Alvinellids, reproductive strategies have now been investigated in several species, and early developmental stages were obtained in *Alvinella pompejana* [57].

A. pompejana has been described as a pioneer on newly formed chimneys [17]. Colonization experiments have further confirmed the settlement of the first individuals within a few days on colonization devices deployed over a smoker wall, following the formation of filamentous microbial mats [71]. The formation of several centimeter-thick assemblages of tubes (cf Figure 2) within two months, partly encrusted in mineral precipitates, underlines a fast colonization process in response to rapid production of newly available substrates [85]. Among the processes that can govern the formation of these new settlements, we [59] have con-
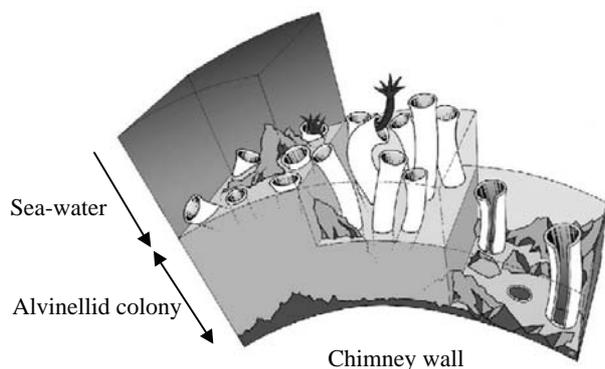
Fig. 2-3 D diagram of the *Alvinella* colony. In the upper sea-water layer, temperature and pH vary according to local fluid outflows. Within the thickness of the colony, tubes are surrounded by a matrix of mineral and organic deposits. Non-diluted acidic hydrothermal fluids circulate through this matrix. The inside of tubes is mainly sea-water entering tubes through their opening, heated by thermal conduction. From [45].

sidered two alternative possibilities: the recruitment of larvae or the migration of post-larvae stages. Almost nothing is known on the life cycle and dispersal strategies of the major vent species. To date, embryos of only two vent species (*Riftia pachyptila* and *A. pompejana*) have been studied for temperature and pressure tolerance [49, 57]. This is mainly limited by the fact that catching larvae directly *in situ* remains highly challenging. As an alternative to sampling, *in vitro* fertilization methods combined to *in vivo* experiments revealed to be a very pertinent approach to obtain essential information on the ability of early stages to deal with the extreme environment of adult colonies [57].

(1) Reproductive strategy

Because vent systems are unstable, vent species were expected to exhibit efficient reproductive strategies allowing them to survive unpredictable environmental changes. That means an early sexual maturity, and the quick production of a large number of offsprings and an efficient fertilization mode. If environmental constraints may have influenced the evolution of reproductive strategies on one hand, on the other hand, in several vent taxa, reproductive strategies were found to be similar to those of non-hydrothermal relatives, suggesting that phylogenetic constraints may be stronger than environmental constraints in some cases [20, 76, 78]. Then, although polychaete species exhibit a great plasticity in their reproductive strategies [34, 81], most hypotheses originally emitted concerning reproduction in the Alvinellidae were based on known reproductive characteristics from species of the Terebellida group to which the Alvinellidae family belongs.

(a) Reproductive modes in Terebellida

The closest families to the Alvinellidae are Ampharetidae, Terebellidae and Trichobranchidae [22, 63]. Different reproductive modes are found in these families : continuous or discontinuous reproduction, species that reproduce once in their life time (monotelic) or several times (polytelic), free spawners or species incubating the embryos inside their tube or even inside the female body. Some species such as the Terebellidae *Eupolymnia nebulosa* Montagu, may even exhibit 2 types of spawning, with free spawning in Atlantic populations, and spawning in a gelatinous cocoon in Mediterranean populations [47]. Some characteristics appear to be relatively conserved. Oocyte size often lay between 100 and 300 μm in diameter. Development is for most species direct (i.e. without a free larval phase) or lecithotrophic (i.e. free larval phase that do not feed in the plankton but exclusively on oocyte reserves), but rarely planktotrophic (free living phase that feeds in the plankton).

(b) Morphological characteristics

All Alvinellid species are gonochoric and exhibit sexual dimorphism (Table 2). In all species examined, males display a pair of modified peribucal tentacles, which are lacking in females [17, 40, 82, 86]. Males of *Paralvinella grasslei* have a pair of small blind cavities on the peristomium [82], and in two *Paralvinella* species, females have papillae at the base of the gills which are absent in males [82, 86]. Finally, genital pore dimorphism was also observed in several species [56, 86].

The organisation of the reproductive apparatus is similar in all species analyzed so far [17, 39, 51, 56, 82, 86]. Females have a single pair of oviducts that extends trough the anterior part of the coelomic cavity. The oviducts are connected to a pair of spermathecea located in the dorsal part of the most anterior setigerous segment. The spermathecae communicate with the exterior by a short common canal, located medially at the base of the most posterior gills. In males, the reproductive apparatus is similar with one pair of spermiducts connected to seminal vesicles, which open to a unique genital pore also located at the base of the posterior gills. The presence of only one pair of gonoducts was described in the Ampharetidae *Ampharete grubei* [21]. This is a rather unusual feature in Terebellida where several pairs of gonoducts are found for the majority of the species [69, 70]. The Alvinellidae is, to date, the only taxon in the order Terebellida known to possess spermathecae.

Table 2. Reproductive characteristics of alvinellid species.

Species	Sexual dimorphism	Fecundity (average)	Oocyte maximum diameter	Spermatozoa	Fertilization	Reproductive synchrony	Development	References
Alvinella pompejana	• Pair of modified buccal tentacles in � • Morphological difference in ♂ / ♀ genital pore	80000	200	• No acrosome • No midpiece • Small size (4 μm) • Conical shape • Short flagellum	• Sperm transfer to spermathecae • External fertilization	No	• Lecitotrophic or direct • Temperature sensitive early development • Negative egg buoyancy	[17, 39, 40, 56, 57, 60]
Alvinella caudata	• Pair of modified buccal tentacles in � • Morphological difference in ♂ / ♀ genital pore	?	?	• No acrosome • No midpiece • Small size (4.5 μm) • Conical shape • Short flagellum	• Sperm transfer to spermathecae	?	• Lecitotrophic or direct	[39] (autors personal obs.)
Paralvinella grasslei	• Pair of modified buccal tentacles in � • Pair of blind cavities on peristonium in � • Papillae on the stem of posterior gills in ♀	3900	275	• No acrosome • No midpiece • Oval "head" (10 μm) • No flagellum • Long caudal process.	• Sperm transfer to spermathecae	Yes, at the scale of a single vent	• Lecitotrophic or direct (*in situ* observation of erpochaete larva)	[39, 82, 83]
Paralvinella pandorae pandorae	• Pair of modified buccal tentacles in �	4500	215	• Elongated "head" (19 μm) • Atypical midpiece • Long flagellum inserted poteriorly but directed anteriorly	• Sperm transfer to spermatheca • Spermatozoa attached to the spermathecal walls	No	• Lecitotrophic or direct	[39, 51, 83]
Paralvinella pandorae irlandei	?	?	?	• Elongated "head" (19 μm) • Atypical midpiece • Long flagellum inserted poteriorly but directed anteriorly	• Sperm transfer to spermatheca • Spermatozoa attached to the spermathecal walls	?	• Lecitotrophic or direct (*in situ* observation of erpochaete	[15, 39]
Paralvinella palmiformis	• Pair of modified buccal tentacles in � • Morphological difference in ♂ / ♀ genital pore • Papillae on the stem of posterior gills in ♀	18000	260	• No acrosome • No midpiece • Oval "head" (10 μm) • No flagellum • Short process.	• Sperm transfer to spermatheca	Yes, over several vents for colonies at a similar stage.	• Lecitotrophic or direct	[13, 14, 39, 51, 86]
Paralvinella sulfincola	?	?	250			No	• Lecitotrophic or direct	[13]
Paralvinella dela	• Pair of modified buccal tentacles in ☭males	?	?	?	?	?	?	[86]

(c) Gametogenesis

Early steps of gametes development occur in the coelomic cavity. To date, the gonades, where the reproductive cells start their differenciation, have not been identified in any Alvinellid species. In *P. grasslei*, glands located on both sides of the medio ventral nervous chain from setigerous segment 5 (S5) to S35-40

were first described as gonades [82]. However, later on, very similar glands were observed in *P. palmiformis* and *P. dela*, but interpreted as mucus glands [86].

The earliest stages of spermatozoa development were described in the coelomic cavity of *P. palmiformis* and *P. pandorae pandorae* [51]. The spermatogonia divide into spematocytes rosettes. Spermatocytes then mature into groups of spermatocytes with flagellae called sperm morulae. Spermatozoa are further stored into the spermiducts.

Ultrastructural investigations on spermatozoa revealed modified structures in all studied species, being flagellated (*P. pandorae*) or not (*A. pompejana, A. caudata, P. grasslei, P. palmiformis*), having no acrosome and an atypical or absent midpiece (Table 2). Other special features were also described and thought to be involved in sperm storage into the female spermathecae and/or fertilization process. In *A. pompejana* and *A. caudata* spermatozoa exhibit a flat vesicular surface that could be involved in adhesiveness in the spermathecae. Spermatozoa of *P. palmiformis* also possess a vesicular surface that could produce some adhesive material favouring clustering and storage in the spermathecae. In *P. p. irlandei* and *P. p. pandorae*, spermatozoa are implanted in the spermathecal walls after the sperm transfer [39].

During vitellogenesis oocytes float freely in the coelomic cavity [17, 51, 56, 82]. Oocytes have a flattened discoid shape, and in *P. grasslei* and *A. pomejana* they exhibit a micropyle , which was related to the absence of acrosome in spermatozoa [17, 82]. Maximal diameter in coelomic ocytes vary between Alvinellid species from 200 to 275 (m [13, 14, 17, 51, 56, 82].

In *A. pompejana*, ultrastructural analyses showed that coelomic oocytes do not rely on any type of helper cells for nutrition, but may use autosynthetic mechanisms for yolk production [56]. Combination of extraovarian vitellogenesis and autosynthesis of yolk suggest a rather slow oogenesis process, which is contradictory with the original hypothesis of fast egg production. After completing yolk synthesis, grown oocytes enter the oviduts through funels opening into the coelomic cavity. At this stage, a selection process based on oocytes size or membrane characteristics would occur (Figure 3) [56]. Such selection mechanism of ripe gametes by gonoducts was already suggested in other Terebellida species [50, 69]. Thus, the organisation of the genital tract seems to allow the storage of a distinct pool of ripe oocyte. This pool could then be spawn at any time, possibly in response to specific environmental cues (Figure 3). Such mechanism would give spawning processes enough flexibility to face chaotic environmental conditions, despite a slow oocyte production.
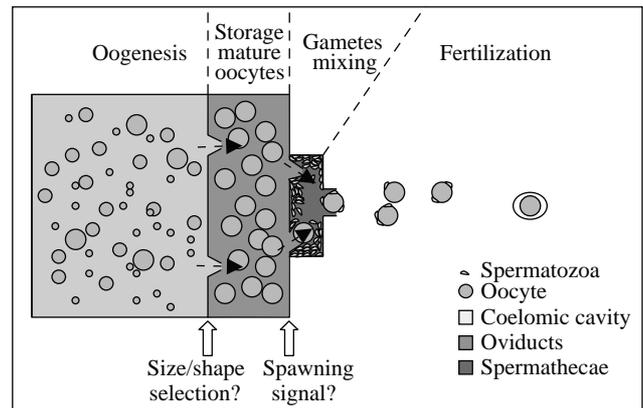

Fig. 3. **Diagram of the gametogenesis, fertilization and spawning mechanisms in *Alvinella pompejana*.**

(d) Fecundity

Fecundity is highly variable in Alvinellids. Average values range from 3900 oocytes per female in *P. grasslei*, up to 80 000 in *A. pompejana* [8, 17, 59]. Such high fecundity values can not, however, be compared with fecundities of other Terebellida species, since studies in these species report the number of eggs per spawning event, whereas estimates for Alvinellid species were calculated from the total amount of coelomic oocytes. Spawning in Alvinellids will most probably involve only mature oocytes stored in the oviducts. In *A. pompejana*, full oviducts may contain about 3000 oocytes, which would be the size of a single spawning event [60].

(e) Fertilization

In several Alvinellid species, spermatozoa were observed inside the spermathecae, and a transfer of sperm from males to females during a probable pseudocopulation process was suggested [17, 39, 82]. In *situ* observations show that Alvinellids frequently leave their tubes to enter tubes of other individuals [9, 24] and sperm transfer may occur during these events. In *P. pandorae irlandei* and *P. grasslei*, pairs individuals were observed in mucus cocoons at the base of the tubes of vestimentiferen tubeworms, suggesting that an appariement of sexes might take place in Alvinellids during reproduction [38, 83]. Sperm transfer to the spermathecae is interpreted as a way to avoid gamete losses and increase fertilization efficiency in the highly dynamic environment [83].

However, sperm transfer to the spermathecae does not necessarily mean that fertilization is internal as suggested before. In *A. pompejana*, fertilized eggs were never found in the spermathecae, although it was filled

with spermatozoa, and the oviducts of the same female were packed with ripe oocytes. This suggests that oocytes are not incubated in the spermathecae, but rather go through the spermathecae during spawning, where spermatozoa could possibly attach to their surface through their vesicular surface, and fertilization would take place outside after spawning [56] (Figure 3). Such strategy has now also been suggested in Siboglinid species [36].

(f) Synchrony in reproductive processes

In a number of marine invertebrates from coastal environments, gametogenesis processes are controlled by endogenous rhythms of hormones production, which are under the influence of external factors such as temperature, photoperiod or moon phases [3, 4, 43, 53, 54, 80]. In the abyssal environment, where food abundance is limited, and environmental variations almost non-existent, the initial hypothesis was that of a continuous reproduction [55]. A number of abyssal species nevertheless exhibit clearly synchronised reproduction with a periodic maturation of reproductive tissues, followed by spawning events [73]. Such periodicity was explained by the arrival of seasonal phytoplankton blooms on the oceanic seafloor [72]. At hydrothermal vents, photoperiod cannot be detected, and organic matter input from the surface is negligible compared to the high local production rates. The prevailing hypothesis was therefore that of a continuous reproduction for vent species.

Most vent species display continuous reproduction [74, 75]. Alvinellids, however, seem to have evolved diverse strategies. In the alvinellid family, asynchronous reproduction was suggested in *Paralvinella sulfincola* [13] and *P. pandorae* [51]. In contrast, *P. grasslei* [68] and *P. palmiformis* [14] were suggested to reproduce synchronously at vent scale, responding to periodic variation linked to tidal regime in environmental factors such as temperature [11, 37]. In *P. palmiformis*, however, spatial variation in reproductive patterns was found at vent scale, which may reflect the successional mosaic of the vent community, with immature individuals in earlier successional stages [14]. In *A. pompejana*, the dynamic disturbance/colonisation process similarly results in a mosaic of patches harbouring individuals at different reproductive stages [60]. New surfaces are colonised within a few days by juveniles and non-reproductive individuals (Figure 4). In such patches all individual are non-reproductive, similarly to what was observed in early successional stages in *P. palmiformis* [14]. In older colonies, reproductive females were found, but females of a same patch did not show synchronism in reproductive stages. On
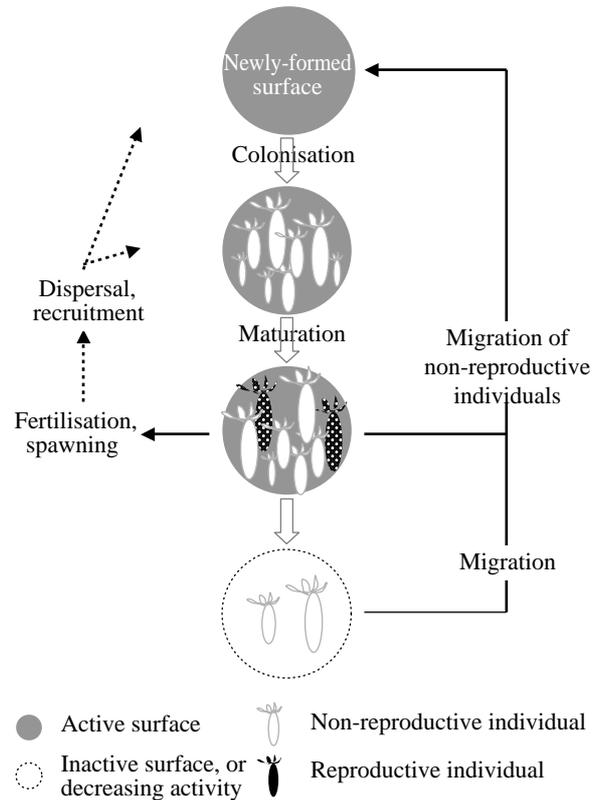

Fig. 4. Reproduction and colonization patterns in *Alvinella pompejana*, at th escale of local patches on a vent chimney. From [59].

the contrary, the diversity of reproductive stages suggested that spawning episodes would occur repeatedly and would concern only a fraction of the adult population [59].

If physico-chemical gradients may be steep on the interface between the sea-water and the chimney wall, *Alvinella* colonies modify fluid circulations by building tubes [45] (Figure 2). Then well-established colonies form an isolating layer that may greatly reduce temperature gradients [45]. Since *A. pompejana* females are only found in such colonies, this might reflect their preference for milder environment during reproduction.

(2) Development

Developmental characteristics such as the length of the cells cycles during embryonic cleavages, the developmental mode, the embryos buoyancy or physiological tolerance to physical environmental parameters have a strong influence on dispersal capabilities of larvae. However, deciphering these characteristics require first to be able to obtain the embryos. In the abyssal environment, collecting larvae has been challenging, and in the cases where larvae were indeed collected, they could not be easily identified.

(a) Development mode of Alvinellids

Alvinellid embryos were never identified *in situ* so far, and development was suggested to be direct or lecithotophic (i.e. with no or limited dispersal capabilities) because of hypotheses based on the oocyte size [51, 83] and observation of larval stages in *in situ* collection which were similar to those of ampharetid polychaetes [16, 83].

Embryos of *A. pompejana* were obtained using *in vitro* fertilization methods [57], which were also used to study embryos of the Siboglinid tubeworm *Riftia pachyptila* [49]. Since *A. pompejana* naturally experience pressure of 250 atmospheres (250 atm. ≈ 2500 m. depth) at vent sites, specific pressure equipment including a microscopy imaging system were designed for developmental studies. In *A. pompejana*, first divisions are asymmetrical, the mechanism of this pattern being the formation of polar lobe [60]. Early embryos have a typical spiral development as found in most polychaete [19] (Figure 5).

(b) Thermal tolerance of the embryos

One of the objectives of these developmental studies were to determine physiological tolerance of embryos to physico-chemical parameters, which would give an idea of the favourable developmental conditions *in situ*, and could be used to deduce possible development area and dispersal capabilities. *A. pompejana* is a

Fig. 5. Embryos of *A. pompejana* obtained by *in vitro* fertilization and incubated under pressure. (a) fertilized egg; (b) 2-cell embryos exhibiting asymmetric division; (c) 4-cell embryo; (d) 96h embryo. Modified from [60].

thermotolerant species as an adult, and for embryos, two main hypotheses were first analyzed : (1) either embryos are able to develop in the abyssal sea-water with temperature typically around 2°C, (2) or they are also thermotolerant, and are able to develop on the vent chimney walls within adult colonies (>20°C). In the first hypothesis, dispersal could occur through transportation with marine currents over tens to hundreds of kilometres, allowing colonization of new distant sites. In the second hypothesis, embryos would develop without dispersal.

In vitro, embryos of *A. pompejana* exhibit low temperature tolerance, being unable to survive above 20°C. At optimal temperature (around 10°C), cleavage rates are very slow with approximately 1 division every 24h. In polychaete species living in the coastal environment, larvae may already be obtained after 24h [84]. In addition, the developmental process was shown to be arrested at 2°C, but a transient temperature increase could trigger development of arrested embryos [57].

(c) What is the in situ embryo behaviour?

The thermal tolerance window determined for early embryos of *A. pompejana* is therefore restricted to temperature lower than those encountered most of the time in adult colonies. This would suggest that embryos cannot develop there. However, recent studies evidenced that a great diversity of habitat with various hydrothermal influence [45]. Diffuse flow areas, with mild temperatures would be compatible with development of embryos of *A. pompejana*. Also, part of the embryos could be entrained with bottom currents where they would be exposed to very low temperature that would arrest their development. This kind of dormancy would then be stopped by temperature increase if the embryos happen to arrive close to a vent. This mechanism can potentially allow wide dispersal capabilities. However, the potential duration of this dormancy is still an open question.

To test these hypotheses, incubators containing embryos of A. pompejana were deployed in different habitats of a single edifice, along a gradient of hydrothermal influence [60]. Only 10% of the embryos incubated above an *Alvinella* colony survived after 5 days, among the surviving embryos, none of them had developed. On the contrary, 70% of the embryos incubated in milder area (*Riftia* clump 1 meter below the *Alvinella* colony, and on the bare mineral seafloor) (4 to 6°C), developed [60]. These results supported the idea that development outside of the colony is possible, while it would not be viable in the adult colony. Temperature measurement close to the incubation device in
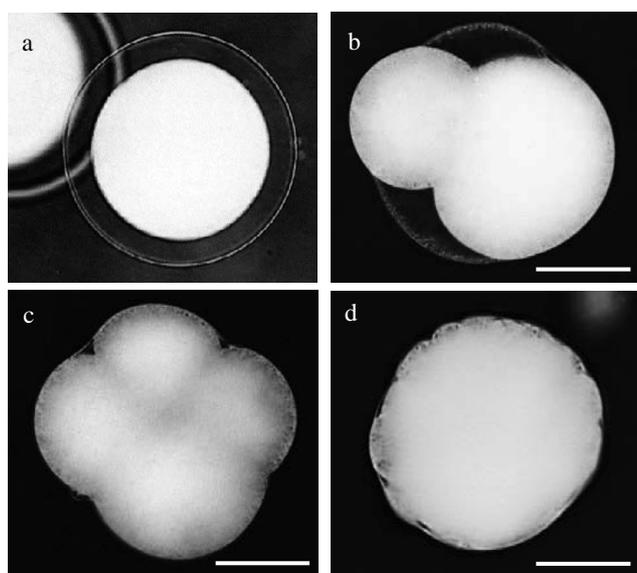
the *Alvinella* colony indicated an average of 13°C during the 5 days of the experiment, but frequent burst above 20°C were also recorded. In addition, sulphide levels were up to 10 fold higher in the *Alvinella* colony than in the 2 other habitats. These experiments, however, did not allow us to decipher which parameters predominantly affect embryos survival and development.

(d) Larvae dispersal

Both *in vitro* and *in situ* studies indicate that *A. pompejana* embryos could disperse through abyssal seawater and develop when they find conditions around 10°C. Development in the shallow part of the ocean can be excluded since embryos can not develop at atmospheric pressure [60]. Since pressure tolerance has not been precisely determined, the range of possible vertical movement is still matter of speculation, and embryos might be entrained by the hydrothermal plume far enough above the sea bottom to be further entrained by upper layers of currents with possibly different regimes than those running on the bottom [52]. Recent studies showed that low temperatures may be found at the surface of adult colonies because the tubes build by this species may isolate the surface of the colony from the hot chimney wall [45]. This could provide suitable habitat for embryos, and lead to reconsider the assumption that early development is excluded from the adult environment.

CONCLUSIONS

The ability of *Alvinella pompejana* to colonize high temperature substrates is far from being fully understood, but the exceptional properties of its extracellular biopolymers and the behavior of the worm can be now considered as major clues in the colonization process. *A. pompejana* could thus stand at the limits authorized for its biological machinery in a highly dynamic environment where temperature can readily reach lethal limits, but where temperature regulation by the animal itself would prevent exposure to deleterious thermal spikes. The animal has a specific cellular machinery which has been selected during the course of evolution. *Alvinella* collagen is the most thermostable protein ever known (Tm 46°C). This species exhibits enzymes able to synthesize these unique extra-cellular proteins in an anoxic environment. Sicot *et al.* [68] have demonstrated that the collagen stabilization process would be the same than that known in vertebrate and human fibrillar collagen. Moreover, all the stabilizing factors known today are amplified in the *Alvinella* collagen including the percentage of stabilizing triplets, proline content and the frequency of hydroxyproline in

the Y position of the Gly-X-Y triplets.

Such a thermostability results from an adaptation process to high temperature. This thermophilic metazoan worm occupies a very specific niche being a pioneer at the surface of the vent smoker. Once recruited at the surface of the smoker, the animal is able to secrete very specific biopolymers, allowing it to colonize new warm mineral surfaces and to optimize the interactions with the hydrothermal fluids. If we know now that this animal is thermophilic in its adult stage, the worm would prefer the cold abyssal sea-water in its early steps of development [57, 60]. This would be a good strategy to survive in the deep sea-water far away from the vents while dispersing. However, what we do not know yet, is the mechanism of the larval recruitment. It is possible that the larvae travel in between vent sites using the currents. However, how these larvae are able to find a new vent site is still mysterious. What are the signals indicating that a new smoker surface is available? How larvae find them and what are the signals indicating that it is time to settle on a new smoker surface? This is one aspect of the future of the research on this worm. Proteomic and genomic data will be available in the future and will bring new insights in the thermotolerance process involved in the biology of this unique deep-sea vent animal.

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