Chapter 6
The Diversity of Deep-Sea Mussels and Their Bacterial Symbioses

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Contents

6.1 Introduction

Deep-sea chemosynthesis-based ecosystems are inhabited by diverse groups of metazoans. Although remote from the euphotic layer, and thus from photosynthetic primary producers, these ecosystems harbour high animal biomasses, orders of magnitude above biomasses usually reported in the deep-sea (Sibuet and Olu 1998; Van Dover 2000). The key to this high productivity is chemoautotrophy, a type of

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metabolism by which many prokaryotes fix inorganic carbon into organic molecules using the chemical energy resulting from the oxidation of reduced compounds present in their environment (Madigan et al. 2002). Indeed, hydrothermal vents and cold seeps are characterized by the occurrence of fluid emissions originating from the subsurface, which bring reduced compounds into mixing with bottom oxygenated seawater (reviewed in Von Damm 1995; Sibuet and Olu 1998; Van Dover 2000; Tunnicliffe et al. 2003). To summarize, hydrothermal vents occur mostly on oceanic ridges, where bottom seawater circulates into the newly formed crust. The high geothermic gradient linked with the presence of a magma chamber few kilometres below the ridge causes water to heat and to be enriched in reduced compounds (metals, sulphide…). Heated fluids, displaying lower densities, then reach back the seafloor along cracks and are emitted. Fluid interaction with cold seawater provokes the precipitation of dissolved metals and minerals in the form of complexes with sulphide, yielding typical (and often spectacular) chimneys. At cold seeps, which are mostly located along continental margins, fluids originate from the subsurface. The thermogenic or biogenic reduction of buried organic matter produces methane and other hydrocarbons which seep to the seafloor, and processes such as the anaerobic oxidation of methane coupled with sulphate-reduction can account for local enrichments in sulphide. Co-occurrence of effective electron donors in the form of reduced compounds such as sulphide or methane, and of good acceptors such as oxygen or nitrate, provides large amounts of energy to prokaryotes able to use them, and triggers significant primary production (Jannasch and Mottl 1985). Although free-living chemoautotrophs are abundant, the most remarkable feature of hydrothermal vents and cold seeps is the occurrence of symbiotic associations between chemoautotrophic bacteria and dominant invertebrates. Although no definition of ‘symbiosis’ is universally accepted, the term is used herein to describe a close association between a metazoan host and bacteria allowing the host to gain novel metabolic capabilities (Douglas 1994). Many metazoan taxa, such as siboglinid tubeworms, molluscs and arthropods indeed live in symbiosis with chemoautotrophic bacteria, mostly sulphur-oxidizers (this volume, Dubilier et al. 2008).

Among symbiotic metazoans found at deep-sea hydrothermal vents and cold seeps, members of a clade within the family Mytilidae are the group which has evolved interactions with the most diversified bacteria. Although related to coastal mytilids, they represent a highly specialized group with a restricted habitat range. Deep-sea mytilids occur worldwide at hydrothermal vents and cold seeps at depths from 200 to 3,600 m (Plate 11, Table 6.1). They are also reported from large sunken organic debris such as wood falls and carcasses of large vertebrates, types of habitats which present analogies with vents and seeps, in particular due to the presence of reduced compounds (Distel et al. 2000; Baco and Smith 2003; Pailleret et al. 2007). To date, no such mytilid was reported from the background deep-sea fauna. The most remarkable biological feature distinguishing deep-sea mussels from their coastal relatives is the presence of chemosynthetic bacterial symbionts associated with their gill epithelial cells. Understanding this symbiotic association

Table 6.1 List of mussel species from which molecular data regarding symbiosis are available. The name, when given, is displayed

Area	Name	HAB	Sites	Reported depth range (m)	Symbionts	Ref symbionts	Accession number symbionts	Ref host	Accession number host	I, E	δ^{13}C range (‰)	Ref isotopes
Atlantic	*B. azoricus*	H	Menez Gwen, Lucky Strike, Rainbow, Broken Spur	840–3,350	M, S	23	AM083950 & 74	24	AY649795	I	−21.3 to −36	7, 10, 11
	B. aff. *azoricus*	H	Lost City	800	M,S	22	DQ899960–900018					
	B. puteoserpentis	H	Broken Spur, Snake Pit, Logatchev, TAG	3,000–3,510	M, S	23	AM083950 & 74	24	AY649796	I	−21.4 to −37.3	5, 10 ′6, 11
	B. cf. *boomerang*	S	Regab, Barbados accretionary prism	3,150	M, S	18	AJ745717–8	26	DQ513451	I	−62.4 to −67.0	27
Gulf of Mexico	*B. heckerae*	S	Florida Escarpment, Blake Ridge	2,155–3,314	M, 2S, My	25	AM236325–8	24	AY649794	I	−56 to −93.8	4, 12, 21, 25
	B. brooksi	S	Alaminos Canyon, Atwater Canyon (Lower Louisiana Slope), Florida Escarpment	1,893–3,314	M, S	25	AM236330–1	24	AY649797	I	−44.4 to −67.3	4, 25
	I. macdonaldi	S	Louisiana Slope (Garden Banks)	650	S	31	EU326225	24	ay649804	?		

(continued)

Table 6.1 (continued)

Area	Name	HAB	Sites	Reported depth range (m)	Symbionts	Ref symbionts	Accession number symbionts	Ref host	Accession number host	I, E	$\delta^{13}C$ range (‰)	Ref isotopes
	B. childressi	S	Alaminos canyon (Lower Louisiana Slope), upper Louisiana Slope	540–2,226	M	3	AM236329	24	AY649800	I	−37.5 to −67.1	4
Eastern mediter ranean	*Idas* sp. Med	S	Central Province	1,150–3,000	M, 2S, My, G, CFB	28	AM402955–60	28	EF210072	?	−38.3	28
Eastern Pacific	*B.* sp. JDF	H	Juan de Fuca Ridge	2,200–2,500	S	19	DQ077893	19	DQ077892	?	−26.6	19
	B. thermophilus	H	East Pacific Rise 13°N to 22°S, 32°S, Galapagos Ridge	2,000–2,747	S	1	M99445	18	AF456285	I	−29.2 to −37.3	2, 16
	B. aff. *thermophilus*	H	East Pacific Rise (32°S)	2,331	S	31	DQ321717	14	AF456317	?		
Western Pacific	*B. japonicus*	H, S	Sagami Bay, Minami Ensei Knoll Iheya (Okinawa Through)	550–1,180	M	8	AB036711	15	AB101423	I		
	B. platifrons	H, S	Sagami Bay, Iheya Ridge and Izena Caldron (Okinawa Trough)	1,028–1,523	M	8	AB036710	15	AB101421	I	−67.5 to −68.1	9

B. septemdierum	H	Suiyo Seamount, Myojin Knoll (Izo Ogasawara Island Arc), Okinawa trough, Mariana back Arc	1,288–3,600	S	8	AB036709	15	AB101430	I	–37	7
B. brevior	H	Lau Basin, Mariana Through, north Fiji	1,750–3,289	S	31	DQ321714	24	AY649799	I	–30.8 to –35.8	5, 30
MOTU 16	W	Bohol sea	357–372	S	33	AM931532	33	EU350070	E		
A. longissima (BC 279)	W	Bohol sea	450–1,764	S	29	AM851094	33	EU350072	E		
B. sp. Manus	H	Manus Basin	1,629	S	31	EU326223	15	AB101431	?		
B. tangaroa	S	KERMadec Arc – Cape Turnagain – Cape Kidnappers	920–1,205	S	31	EU326222	17	AY608439	?		
G. gladius	H	KERM Rumble 3 and 5 seamount	216–755	S	31	EU326224	24	AY649802	?		
“B.” sp. NZ3	H	KERM – Maucauley Cone	200	S	31	DQ321718	17	AY608440	?		
Idas sp. C	W, B	Near Vanuatu	290–802	S	32	EU683305	32	EU702374	E		
Idas sp. D (= MOTU5, BC288 and 294)	W	Bohol Sea	762–1,764	S	29	AM503926, AM503922	32, 33	EU702359, EU350071	E		

(continued)

Table 6.1 (continued)

Area	Name	HAB	Sites	Reported depth range (m)	Symbionts	Ref symbionts	Accession number symbionts	Ref host	Accession number host	I, E	δ¹³C range (‰)	Ref isotopes
	BC1007	W	Bohol Sea, Maribojoc island	387–453	S	29	AM503923			E		
	Adipicola crypta	W, B	Philippines, Vanuatu, Japan (off Kagoshima)	440	S	32	EU683302; EU683308	32	EU702317	I		
Indian ocean	*B. marisindicus* (= *B.* aff. *brevior*)	H	Central Indian Ridge (Kairei and Edmond)	2,450–3,300	S	20	DQ077891	20, Smith unp.	AY275543	I	−20 to −30.8	20

HABitats: H: hydrothermal; S: cold seeps; W: wood falls; B: bone falls. Symbionts: M: methanotroph; S: thiotroph; My: Methylotroph; G: unknown Gammaproteobacterium; CFB: Bacteroidetes. Accession numbers are given for bacterial 16 rRNA symbionts and COI hosts. I, E: Intra- or Extracellular localization of bacteria

1 Distel et al. 1988; 2 Fisher et al. 1988; 3 Distel and Cavanaugh 1994; 4 Nelson and Fisher 1995; 5 Dubilier et al. 1998; 6 Robinson et al. 1998b; 7 Trask and Van Dover 1999; 8 Fujiwara et al. 2000; 9 Barry et al. 2002; 10 Colaco et al. 2002; 11 Fiala-Médioni et al. 2002; 12 Van Dover et al. 2003; 13 Won et al. 2003b; 14 Won et al. 2003a; 15 Miyazaki et al. 2004; 16 Raulfs et al. 2004; 17 Smith et al. 2004; 18 Duperron et al. 2005; 19 McKiness et al. 2005; 20 McKiness and Cavanaugh 2005; 21 Salerno et al. 2005; 22 DeChaine et al. 2006; 23 Duperron et al. 2006; 24 Jones et al. 2006; 25 Duperron et al. 2007; 26 Olu-LeRoy et al. 2007a; 27 Olu-LeRoy et al. 2007b; 28 Duperron et al. 2008b; 29 Duperron et al. 2008a; 30 Henry et al. 2008; 31 Won et al. 2008; 32 Lorion et al. 2009; 33 Duperron et al. 2009

has stimulated the interest of many investigators since its discovery in the early 1980s. Many species harbour sulphur-oxidizing symbionts comparable to those described from other bivalves families (Lucinidae, Thyasiridae, Solemyidae and Vesicomyidae), but some species harbour methane-oxidizing symbionts, a very rare feature among bivalves. Multiple symbioses involving two to six distinct bacterial types are another originality of these deep-sea mussels, and appear as a good way to deal with highly variable environments they are experiencing (Fisher et al. 1993; Distel et al. 1995).

Although no symbiont has yet been isolated in pure culture, the diversity, role, physiology, ecology and evolution of mussel symbioses have been documented. The development of tools applied to deep-sea biology, such as molecular techniques or experimental devices, and the input from other disciplines such as deep-sea imagery or in situ chemistry has allowed a better understanding of the ecology of chemosynthesis-based ecosystems. The present contribution gives a general overview of the diversity of mussels associated with deep-sea chemosynthesis-based ecosystems and their bacterial symbionts, with an emphasis on recent advances in the understanding of interactions between hosts, bacteria, and their environment. Finally, some new questions and directions for future research are presented.

6.2 Mussel Distribution and Habitats

Mussels have been collected over the years from many deep-sea chemosynthesis-based environments spread over the world including hydrothermal vents, cold seeps and organic falls such as whale bone or wood deposits (Plate 11). Mussels are mostly epibenthic and live attached by byssal threads to hard substrates, sometimes forming tight aggregates (Fig. 6.1, Plate 12). Notable exceptions are *Bathymodiolus boomerang* from the Barbados accretionary prism and *Gigantidas horikoshi*, which live two-thirds buried into the sediment (Hashimoto et al. 1995; von Cosel and Olu 1998; Desbruyères et al. 2006). The mussels' reliance on chemosynthetic symbionts located in their gills implies that reduced compounds are needed in the surrounding fluid, as mussels do not have access to the anoxic sediment. This does not normally occur in the deep-sea despite the anoxy of the sediment because fluxes are not sufficient to emit reduced compounds into the bottom seawater. On the contrary, fluids emitted at cold seeps and hydrothermal vents contain huge amounts of reduced compounds such as sulphide and methane (Van Dover 2000; Le Bris et al. 2006b; Sarrazin et al. 2006). Similar compounds, for example sulphide and methane, are also produced during the microbially-mediated decay of large debris of organic matter such as whale or wood falls (Leschine 1995; Baco and Smith 2003; Goffredi et al. 2008; Treude et al. 2009).

Around vents, mussels usually occur on chimneys or in the vicinity of cracks where diffuse flow occurs (Fig. 6.1b) (Johnson et al. 1994; Fisher 1995; Cuvelier et al. 2009; Le Bris and Duperron in press). At seeps mussels usually live attached

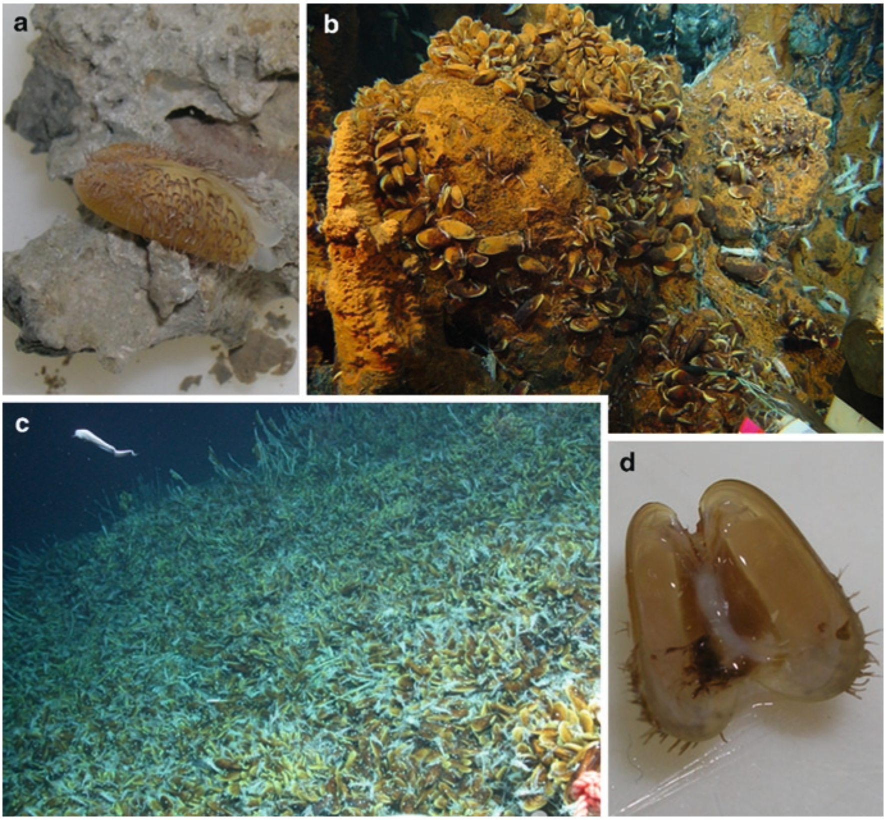

Fig. 6.1 Morphology and habitats of bathymodiolin mussels. (**a**) *Idas* sp. Med attached to a carbonate crust recovered from cold seeps in the eastern Mediterranean (MEDECO cruise, 2007, photo by O. Gros). Note the two whitish siphons in the posterior part. Shell length 1 cm. (**b**) *Bathymodiolus azoricus* specimens attached to a fluid-emitting edifice at the Rainbow hydrothermal vent site (Mid Atlantic Ridge, 2,277 m depth). Notice the iron deposits on chimneys and shells, and the presence of *Rimicaris exoculata* shrimps on the right of the image. Image © Ifremer, MOMARDREAM cruise, 2008 (chief scientist: J. Dyment). (**c**) Dense bed of the mussel *Bathymodiolus* sp. at a cold seep site situated in a depression on top of a diapir structure in the lower Congo Basin (2,700 m depth). The area displayed gas bubble sources. Other metazoans include tubeworms (*Lamellibrachia* sp., in the back), shrimps, and limpets (small white dots visible on mussel shells). Image © Marum, University of Bremen, GUINECO M76/3a cruise, 2008 (chief scientist: A. Boetius). (**d**) *Idas* sp. Med, opened valves. Note the white foot, brownish visceral mass below and gills in the left and right valves. A colour version of this figure can be found in Appendix (Plate 12)

to authigenic carbonates, sometimes covering large areas (Fig. 6.1c), or other hard substrates such as tubes of vestimentiferans (Olu-LeRoy et al. 2007b). Living at the oxic-anoxic interface can be risky. In a documented example, specimens of *B. childressi* form a ring, a few meters wide, surrounding a 190 m^2 methane-rich anoxic hypersaline brine pool in the northern Gulf of Mexico (GoM). However, some mussels located in the inner rim were dead after being entirely submerged by an elevation of the brine level (MacDonald et al. 1990). Similarly, small specimens of *B. azoricus* reach densities above 10,000 individuals m^{-2} on the walls of active venting chimneys at Lucky Strike despite the risk of exposure to deadly hot fluids (von Cosel et al. 1999). Specimens of *B. brevior* were shown to survive in acidic waters with pH values as low as 5.36 at the Eifuku volcano (Mariana Arc), although with shell thickness and daily growth rates only half those measured in mussels occurring in neutral waters (Tunnicliffe et al. 2009). Aside of such occasional extremes, mussels have to tolerate only a relatively moderate temperature range. *B. brevior* has an upper temperature limit of 35°C and tolerates 2 h exposure at 19°C (Henry et al. 2008). Heat shock experiments for 2 h at 25°C and 30°C were shown to result in global depression in gene expression in *B. azoricus*, suggesting that mussels are not adapted to 'hot' temperatures (Boutet et al. 2009). At vents, mussels are indeed usually out-competed at the hottest habitats by specialists such as the gastropod *Ifremeria nautilei* at Lau Basin, the annelid polychaete *Alvinella pompejana* at sites of the East Pacific Rise (EPR), or the shrimp *Rimicaris exoculata* at sites of the Mid Atlantic Ridge (MAR). They rather occupy more peripheral areas with more diffuse venting and temperatures slightly above those of ambient seawater, up to 15°C (Sarradin et al. 1999). Although in situ measurement of physico-chemical parameters is difficult, and some compounds such as methane can not be monitored directly, several studies have described local conditions around mussels. An important point is that physico-chemical characteristics are highly variable in both space and time, particularly at vents, resulting in varying sharp gradients to which the organisms must adapt (Johnson et al. 1994; Le Bris et al. 2006b). Mussels thrive in environments with moderate concentrations of reduced compounds (compared to end-member fluids), in the range of micromolars for sulphide and methane. For example, sulphide concentrations between 0.5 and 62 μM were reported in the vicinity of mussel beds at the Lucky Strike vent site (<0.5 to 26 μM at Menez Gwen), with larger mussels found at sulphide-rich locations (Sarradin et al. 1999), and up to 325 μM sulphide was recorded at Rose Garden (Galapagos Rift) (Johnson et al. 1986, 1994; Fisher et al. 1988). Methane concentrations up to 33.7 μM were reported from mussel beds at the Régab cold seep (Duperron et al. 2005; Olu-LeRoy et al. 2007b). The presence of additional chemical compounds can influence the habitat. For example, end member fluids at the Rainbow vent site display low sulphide concentrations, and the abundance of dissolved iron (which forms deposits on shells, see Fig. 6.1b) further limits the bioavailability of sulphide for mussels (Le Bris et al. 2006a). At vents, toxic heavy metals can also be abundant, even in diffuse flow, and mussels use a variety of systems to protect themselves (Geret et al. 1998; Rousse et al. 1998). Mussels 'engineer' their vent habitat by redirecting fluid flow and dispersing it throughout the mussel bed (Johnson et al. 1994). They also display a limited mobility, and can move a bit to optimize access to their substrates (Childress 1988; Johnson et al. 1994). Compared to vent and seep habitats, precise characteristics of organic falls have rarely been documented, although some data indicate enrichment in sulphide in the sediment close to whale bones, and the occurrence of methane (Smith et al. 1998; Fujiwara et al. 2007; Treude et al. 2009). Recent results confirm the direct emission of sulphide from wood deployed in a shallow mangrove habitat (Laurent et al. 2009). In summary, observations and results indicate that mussels require habitats where oxygen is available as well as electron donors and energy sources for their bacterial symbionts.

6.3 The Diversity of Mussels

6.3.1 General Description

Around 37 mussel species reported from chemosynthesis-based ecosystems have been assigned a name (22 large mussels within the genera *Bathymodiolus* and *Gigantidas*, and around 16 small species within the genera *Idas* and relatives including *Adipicola*, *Benthomodiolus*, or *Tamu*) (Dell 1987; von Cosel 2002; von Cosel and Marshall 2003; von Cosel and Janssen 2008; Pelorce and Poutiers 2009). Many more possible 'species' have been identified based on molecular studies (Smith et al. 2004; McKiness and Cavanaugh 2005; McKiness et al. 2005; Jones et al. 2006; Olu-LeRoy et al. 2007a; Samadi et al. 2007; Lorion et al. 2009). They display several common features. Shell is modioliform and usually brownish (Fig. 6.1). Adult shell length varies from ~10 to 360 mm, larger species (>40 mm) being restricted to hydrothermal vents and cold seeps. The mantle delimiting the pallial cavity displays different degrees of fusion. In some species (such as *Idas* sp. from the eastern Mediterranean, see Fig. 6.1a), siphons comparable to those of infaunal bivalve species have been described on living specimens despite their epibenthic lifestyle (von Cosel 2002; Okutani et al. 2003; Fujiwara et al. 2007). Compared to their shallow, non-symbiotic relatives, mussels display reduced labial palps and guts, the latter varying among species from curved or S-shaped to straight (Page et al. 1991; von Cosel 2002). Gills are typically enlarged, occupying for example 77% of shell length in *B. boomerang*, and very often dark coloured, a common trend in symbiotic bivalves (Fisher 1990; von Cosel and Olu 1998). The gills are filibranch. They display inner and outer demibranchs composed of ascending and descending lamellae, with few-to-no inter-lamellar junctions, and ciliary junctions between consecutive filaments (von Cosel et al. 1999). Gill epithelium is constituted of one layer of cells, of which some harbour symbionts (see below and Fig. 6.2) while others are almost symbiont-free and harbour cilia (intercalary cells) (Fiala-Médioni 1984). Gills retains the ability to filter feed at rates comparable to shallow mussels, as shown in *B. azoricus* and *"B." childressi* (the use of quotation marks is explained in Section 6.3.2), and the digestive tract is functional (Page et al. 1991; Pile and Young 1999). To colonize scattered habitats efficiently, mussels have developed specific strategies.

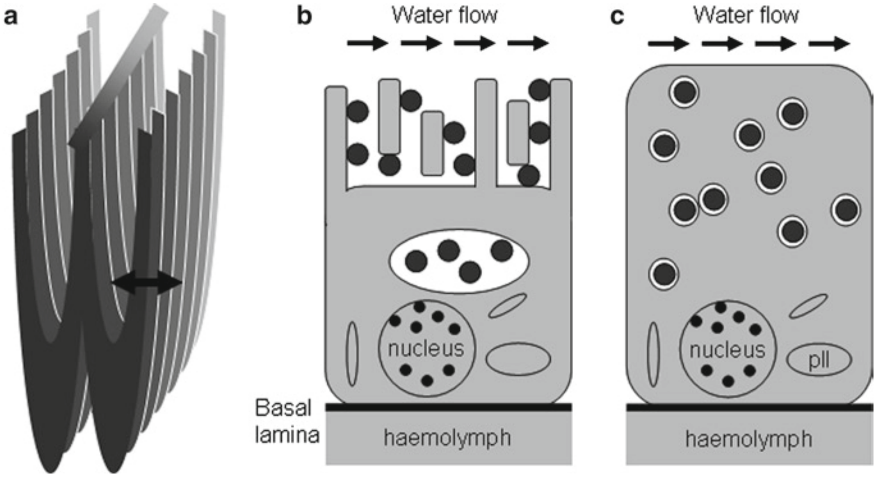

Fig. 6.2 (**a**) Frontal view of a single mussel gill. Arrow indicates how transverse sections through filaments (used for electron and fluorescence microscopy) are obtained. (**b–c**) Detail of two mussel gill epithelial cells as seen in transverse sections. Notice the basal lamina underlying the gill epithelium and separating cells from the hemolymph, and the nucleus and phagolysosome-like bodies (pll) in the basal part of cells. Arrows indicate the direction of water flow. Sulphur-oxidizing bacteria are displayed in dark grey. (**b**) Extracellular bacteria located between microvilli, or engulfed in a large vacuole, as typically observed in some small bathymodiolines from organic falls; (**c**) Intracellular bacteria inside of host cells within vacuoles, as observed in most large species from hydrothermal vents and cold seeps

Bathymodiolinae are dioecious or successive hermaphrodites, but simultaneous hermaphrodites rarely occurred in the species investigated (Le Pennec and Beninger 2000). For example *Idas washingtonia* displayed evidence of protandric hermaphroditism (Tyler et al. 2009). Males represented 74% of individuals investigated and included the smallest mussels. Female and hermaphrodites represented 10% and 14% of specimens, respectively. Although not well-documented, the development of mussels appears to involve small and numerous gametes and eggs (Tyler et al. 2007, 2009). The large size of the larval shell indicative of a long larval life, planktotrophic larvae probably have large dispersal abilities (Lutz et al. 1980, 1984; Comtet et al. 2000; Arellano and Young 2009, reviewed in Le Pennec and Beninger 2000 and Tyler and Young 2003). Mussel lifespan is hard to estimate compared to that of many coastal bivalves, because no comparable typical cycles (seasonal, lunar, circadian, tidal) are proven to be linked with shell increments. Rough estimates of an age around 18 years for a 14 cm-long specimen of *B. brevior* were suggested, and *B. thermophilus* specimens may get older than 25 years (Schöne and Giere 2005). This is not an unusual lifespan among mytilids, as *Modiolus modiolus*, a non symbiotic mussel, was reported to live 50 years (von Cosel and Olu 1998).

6.3.2 *Taxonomy and Classification*

The taxonomy of mussels from chemosynthesis-based ecosystems is under discussion. Not many distinctive features allow comparisons among species, and some could be attributed to allometric growth (von Cosel and Olu 1998). Most large species found at hydrothermal vents and cold seeps are classified within the genus *Bathymodiolus* of which the type species is *B. thermophilus* from the EPR (Kenk and Wilson 1985). However, detailed morphological investigations indicate that *Bathymodiolus* can be subdivided in three groups: the *"B." childressi*, the *B. thermophilus*, and the *B. aduloides* group (von Cosel 2002; von Cosel and Janssen 2008). A second genus of large mussels is *Gigantidas*, morphologically distinct from *Bathymodiolus* and described from vents around New Zealand (von Cosel and Marshall 2003). Although known since the end of the nineteenth century from trawls (example in Jeffreys 1876), smaller species (shell lengths <3 cm, Fig. 6.1a) have been less investigated and morphological descriptions are sometimes ancient and rarely backed by molecular data. Members of the genera *Idas* and *Adipicola* (classified within the sub-family Modiolinae), or the more recently erected genus *Tamu*, have been reported from cold seeps as well as organic falls from various locations. Except for their smaller size, their morphology resembles that of large *Bathymodiolus*. Soft parts have however not been studied in great detail.

Molecular phylogenies based on host nuclear and mitochondrial genes support the monophyly of symbiont-associated mussels within the family Mytilidae. The subfamily name 'Bathymodiolinae' has been proposed. The monophyly of *Bathymodiolus* is not supported. Analyses of COI, ND4 and 28S rRNA-encoding genes distinguish three *Bathymodiolus* clades, not contradictory to the groups suggested by von Cosel (Miyazaki et al. 2004; Jones et al. 2006; Samadi et al. 2007; Lorion et al. 2009). The three groups emerge from a multifurcation which includes smaller mussels classified as *Idas*, *Tamu* or *Adipicola*. To distinguish between 'true' *Bathymodiolus* from the *B. thermophilus* group and others, Gustafson suggested the use of quotation marks for representatives of other groups until the taxonomy is fully clarified (Gustafson et al. 1998). Molecular studies also point to the existence of cryptic species (Moraga et al. 1994; Won et al. 2003a; Smith et al. 2004; Olu-LeRoy et al. 2007a). A re-assessment of the nomenclature is thus needed which should combine morphological as well as molecular data, as used for example by Maas and co-workers to distinguish between mid-Atlantic ridge (MAR) vent species (Maas et al. 1999).

6.4 The Diversity of Bacterial Symbionts

Bacteria have been shown to occur in all Bathymodiolinae species investigated to date. As for other chemosynthetic symbioses, no symbiont has been isolated in pure culture despite attempts (Nelson et al. 1995). Bacteria are associated with gill epithelial cells located in the lateral zone of gill filaments (Fig. 6.2). This allows bacteria to gain access to compounds present in circulating fluid, including energy and carbon sources. Comparable gill symbioses, though involving only sulphur-oxidizers (thiotrophs), have also been reported from the bivalve families Thyasiridae, Lucinidae, Solemyidae and Vesicomyidae (Fisher 1990; Stewart et al. 2005; Stewart and Cavanaugh 2006; Taylor and Glover 2006).

6.4.1 Sulphur-Oxidizing Bacteria

Of the 27 mussel 'species' for which molecular information about symbiosis is available, 24 harbour bacteria related to chemoautotrophic sulphur-oxidizing (or thiotrophic) Gammaproteobacteria, based on comparative analysis of their16S rRNA-encoding gene (Table 6.1). Initially discovered in the EPR vent species *B. thermophilus*, sulphur oxidizing symbionts are small (0.5 µm diameter on electron micrographs) Gram negative bacteria (Rau and Hedges 1979; Felbeck et al. 1981; Cavanaugh 1983; Fiala-Médioni 1984). They fix carbon autotrophically using the Calvin Benson Bassham (CBB) cycle as demonstrated by the presence of the functional form I of Ribulose 1,5 bisphosphate carboxylase/oxygenase (RubisCO) and its encoding gene in several species (Fisher et al. 1988; Robinson et al. 1998a; Pimenov et al. 2002; Spiridonova et al. 2006). Measurements of enzyme activities and gene sequencing also demonstrated the presence of adenosine phosphosulfate (APS) reductase and ATP sulfurylase, two enzymes involved in sulphur metabolism (Nelson and Fisher 1995). Thiosulphate and sulphide stimulate the fixation of inorganic carbon based on incubation experiments of animal tissue or living specimens of *B. thermophilus* and *B. azoricus* (Belkin et al. 1986; Nelson et al. 1995; Riou et al. 2008). The use of alternate energy sources, such as hydrogen, has been suspected but has not been proven to date. Contrary to what is observed for example in lucinid clams, symbionts do not deposit sulphur granules (Dando et al. 1986).

In most species, thiotrophic symbionts occur within vacuoles containing one to several bacteria each that are located inside of host gill epithelial cells named 'bacteriocytes' (Fig. 6.2) (Fiala-Médioni 1984; Dubilier et al. 1998). Recently however, thiotrophs were shown to occur extracellularly between microvilli of epithelial cells in several small species such as MOTU 16, *A. longissima*, *Idas* sp. C and *Idas* sp. D from organic falls of the western Pacific (Fig. 6.2) (Gros and Gaill 2007; Gros et al. 2007; Duperron et al. 2008a; Lorion et al. 2009). *Idas simpsoni*, *I. washingtoniana* and *Adipicola* sp. JDF (for Juan de Fuca ridge) also display extracellular bacteria as evident from microscopic investigation (Southward 2008). In many species, symbiont localization was not investigated, or ambiguous such as in *Bathymodiolus* sp. from the Juan de Fuca ridge (McKiness et al. 2005; Duperron et al. 2008b; Won et al. 2008). Either intra- or extracellular, symbiont localization appears to be consistent and conserved within a given host species (Lorion et al. 2009).

All sulphur-oxidizing symbionts of mussels cluster within a clade of Gammaproteobacteria that also includes sequences from a few free-living uncultivated bacteria, and a clade of maternally-inherited endosymbionts associated with vesicomyid clams (Distel et al. 1988; Peek et al. 1998; Duperron et al. 2008a). Grouping free-living bacteria as well as extracellular, intracellular, and maternally-inherited (with reduced genome) symbionts, this clade is a good target group to study the processes leading from free-living to strictly endosymbiotic bacteria. One 16S rRNA symbiont phylotype (a phylotype is a version of the sequence) is usually associated specifically with a single mussel species, although exceptions occur. The two MAR vent species *B. azoricus* and *B. puteoserpentis* for example share a single 16S rRNA symbiont phylotype (Duperron et al. 2006), and the co-occurrence of two distinct thiotroph-related 16S rRNA phylotypes representing two distinct symbiont strains was reported in *B. heckerae* and in *Idas* sp. Med (Duperron et al. 2007, 2008b).

6.4.2 Methane-Oxidizing Bacteria

To date, at least ten species from cold seeps and hydrothermal vents are reported to harbour bacteria related to Type I methane-oxidizing (or methanotrophic) Gammaproteobacteria, of which three have only methanotrophs, five also have sulphur-oxidizers, and two – *Bathymodiolus heckerae* and *Idas* sp. Med – have four and six symbiont types in total, respectively (Table 6.1). Methanotrophic symbiosis is rare among metazoans, and was suggested only for some sponges (Vacelet et al. 1996), a few siboglinid annelids (Schmaljohann et al. 1990), the gastropod *Ifremeria nautilei* (Borowski et al. 2002), the thyasirid bivalve *Conchocele bisecta* (Kamenev et al. 2001), and epibionts of the shrimp *Rimicaris exoculata* (Zbinden et al. 2008). Methanotrophic symbionts were initially discovered in mussels from the Gulf of Mexico cold seeps. *B. childressi* grows using methane as a carbon and energy source (Childress et al. 1986; Cary et al. 1988). *Bathymodiolus heckerae* harbours large bacteria with a diameter of 1.5 µm and stacked internal membranes typical of free-living Type I methanotrophs. This species also displays unexpectedly negative $\delta^{13}C$ tissue values, very close to those reported for local methane (Cavanaugh et al. 1987). Methanol dehydrogenase activity, the second enzyme of the aerobic oxidation of methane, was shown (Fisher et al. 1987; Robinson et al. 1998b; Pimenov et al. 2002). The presence of methane mono-oxygenase, the diagnostic enzyme for aerobic methane oxidation, was confirmed based on gene sequence analysis as well as in situ hybdridization of its mRNA. However, its activity is difficult to measure because of its unstability (Pernthaler and Amann 2004; Spiridonova et al. 2006; Duperron et al. 2007). Besides methane utilization, RubisCO appears to be present in methane-oxidizing symbionts of a *Bathymodiolus* from Okinawa Through, indicating the possible presence of a functional CBB cycle (Elsaied et al. 2006). When present, methanotrophs are always intracellular, located within vacuoles inside bacteriocytes.

Symbiotic methanotrophs form a clade within the Gammaproteobacteria which is the sister-group of free-living Type I methanotrophs (Distel and Cavanaugh 1994; Duperron et al. 2005). To date, very few free-living bacteria cluster within this clade questioning the existence of active free-living forms of symbionts. Similar to thiotrophs, one 16S rRNA symbiont phylotype is usually associated with a single mussel species. Exceptions again are *B. azoricus* and *B. puteoserpentis* that share a single methanotroph-related phylotype (Duperron et al. 2006).

6.4.3 Additional Symbionts and Multiple Symbiosis

Besides the presence of methanotrophic symbionts, the main originality of bathymodiolines is the occurrence of multiple symbioses. 'Dual symbiosis' involving sulphur- and methane-oxidizers co-occurring within a single bacteriocyte was first suspected in *B. heckerae* (Cavanaugh et al. 1987), hypothesized in *B. brooksi* from the Gulf of Mexico based on microscopy (Fisher et al. 1993), and demonstrated formally using 16S rRNA gene sequencing and fluorescence in situ hybridization (FISH) in the vent species *B. puteoserpentis* (Distel et al. 1995). Dual symbiosis has subsequently been documented in *B. azoricus*, *B.* aff. *azoricus* from Lost City, *B. boomerang* from the Barbados accretionary prism, *B.* cf. *boomerang* from Régab (Plate 13a–b) and re-investigated in *B. brooksi* (Olu et al. 1996; Fiala-Médioni et al. 2002; Duperron et al. 2005, 2007; DeChaine et al. 2006).

Recently, thanks to the sequencing of larger bacterial 16S rRNA clone libraries from gill tissues, new types of bacterial symbionts have been identified in the gills of two cold seep species, *B. heckerae* and *Idas* sp. Med, and confirmed using FISH (Duperron et al. 2007, 2008b). Both mussel species harbour one methanotroph- and two distinct thiotroph-related 16S rRNA symbiont phylotypes, labelled M, S1 and S2 in Table 6.1, respectively (Plate 13d). Both mussels also harbour phylotypes related to free-living methylotrophs of the genus *Methylophaga* (Plate 13c). Although the metabolism of these symbionts is unknown, it can be hypothesized that they are actually methylotrophs, i.e. bacteria utilizing mono carbon compounds such as methanol, but not methane. Use of methane is restricted to the subgroup of methanotrophs (Anthony 1982). Finally, *Idas* sp. has two additional symbionts compared with *B. heckerae*. One is a Gammaproteobacterium unrelated to any cultivated bacterium, making it impossible to propose any metabolic scheme. The other is a member of the Bacteroidetes group, related to members of the genus *Tenacibaculum* and cellulose-degrading bacteria such as *Polaribacter dokdonensis*. Its role is unknown, and could be linked either to the degradation of organic compounds or pathogenicity. Intra- or extracellular localization of these recently discovered symbionts has not yet been ascertained.

Bacteria suspected to be deleterious have also been identified in mussel gill tissues. "Candidatus *Endonucleobacter bathymodioli*", a Gammaproteobacterium related to sequences from a bacterium causing mass mortalities in Pacific razor clams, has recently been identified within nuclei of gill intercalary cells in several species of *Bathymodiolus* from various geographical origins (Zielinski et al. 2009). This filament-shaped bacterium apparently establishes itself within host cells nuclei, multiplies and destroys the content of the nucleus. It then causes the nucleus to break open, releasing offspring bacteria to the environment, similar to the way that is used by some viruses. "Ca. *E. bathymodioli*" is apparently closely related to several bacterial species based on sequences recovered from marine animal samples, including two which also have been hypothesized to originate from endonuclear bacteria (Kerk et al. 1992). This led Zielinski et al. (2009) to propose that endonuclear bacteria could be widespread among marine invertebrates.

Clearly, recent discoveries of new bacterial partners and of the occurrence of up to six symbionts within a single mytilid host indicate that the diversity of bacteria associated with mussel tissues has been underestimated. Cheaper high-throughput sequencing technologies should allow in the future to gain a more exhaustive and systematic overview of bacteria associated with bathymodioline mussels.

6.5 The Symbiotic System: How Does It Work?

Mussels and their sometimes diverse associated bacteria form complex 'symbiotic systems' in which each partner interacts with others and with the environment. Although the nature of these interactions is rarely well-understood, the following chapter aims at summarizing current knowledge concerning aspects of these interactions.

6.5.1 Interactions Between Mussels and Their Bacterial Symbionts

How bacteria are acquired by host tissue has not been fully resolved. Although early developmental stages of dual symbiotic species display sulphur- and methane-oxidizing bacteria (Salerno et al. 2005), these were not observed from gonad tissue or eggs, suggesting an early environmental acquisition. The extracellular localization of thiotrophs in several small mussel species, and the observations of open vacuoles engulfing sulphide-oxidizing bacteria in the most apical part of bacteriocytes in many species with intracellular symbionts support this hypothesis (Dubilier et al. 1998; Duperron et al. 2008a; Southward 2008; Lorion et al. 2009). Detailed genetic investigations have shown the co-occurrence of several 16S rRNA or ITS (Internal Transcribed Spacer, a more variable marker gene allowing to distinguish between closely related bacterial strains) phylotypes of sulphide-oxidizers in the gills of mussels from the Mid-Atlantic Ridge, Gulf of Mexico, eastern Mediterranean and western Pacific (Won et al. 2003b; Duperron et al. 2007, 2008b; Lorion et al. 2009). Because some of these strains were usually found in mussel species occurring nearby, these observations are interpreted as further support for environmental acquisition. The transmission of methanotrophs and other types of symbionts has not been studied in detail. To date only one 16S rRNA methanotroph-related phylotype was reported from a given mussel species. Genomic investigations will help documenting whether bacteria can live outside of their hosts, and how hosts and bacteria recognize each other and establish the association.

Contrary to tubeworms such as *Riftia pachyptila*, or clams such as *Calyptogena* spp., no specific H_2S transporter has been identified in mussels. In tubeworms, bacteria are located inside a special organ, the trophosome, remote from the gill where H_2S is taken up. In *Calyptogena* (vesicomyid) clams the foot mines for sulphide in the sediment or emitted waters whereas bacteria sit in the gills (Childress et al. 1993; Scott and Fisher 1995; Flores et al. 2005). Both are highly-integrated symbioses in which reduced sulphur needs to be transported to the symbionts. In mussels, symbionts occurring in (or on) gill epithelial cells are very close to the circulating fluid that contains both their electron donors, including sulphide, and acceptors (Fig. 6.2). Transporters are therefore not needed.

The nutritional role of symbionts has been well-documented. Stable isotope analyses are a classical tool used to trace carbon sources in ecology (Lajhta and Michener 1994). Tissue carbon isotope signatures can be compared with signatures of carbon sources to estimate their quantitative importance in the nutrition of animals. In hydrothermal vents and cold seeps, the role of sulphur- or methane-oxidizing symbionts in mussel nutrition can be, to a certain extent, evaluated using this approach (Fisher 1995). Along with direct incubations of gill material or whole living mussels in the presence of radio-labelled inorganic carbon and methane, these methods have demonstrated the transfer of bacterially-acquired carbon to the host tissue from both sulphur- and methane-oxidizing symbionts (Table 6.1) (Childress et al. 1986; Fisher et al. 1987; Fisher and Childress 1992; Kochevar et al. 1992; Jahnke et al. 1995; Nelson et al. 1995; Riou et al. 2008). Estimates for *B. heckerae* mussels from Blake Ridge show 60% of their organic carbon from methane- and 40% from sulphur-oxidizers based on stable isotopes investigation (Van Dover et al. 2003). How carbon transfer is achieved is however still debated. Early work has shown the digestion of bacteria within phagolysosome-like bodies located in the basal part of bacteriocytes (Fig. 6.2) (Fisher et al. 1987; Fiala-Médioni et al. 1994). No such phagolysosome-like bodies were convincingly observed in extracellular sulphur-oxidizers, questioning their role as carbon providers (Duperron et al. 2008a). Other ways of transfer are known from other symbioses, for example the release of organic compounds by photosynthetic symbionts in corals. Another possibility was suggested following the observation of bacteria-filled bacteriocytes, detached from the gill, in the food groove. Mussels could possibly perform a type of autophagy. Direct evidence is lacking, but these alternate pathways can not be ruled out and could play a role. Besides their role in carbon nutrition, thiotrophs were also suggested to detoxify sulphide, which is toxic for host cytochrome oxidase, protecting mussels against occasional bursts of sulphide-rich fluids (Powell and Somero 1986; Dubilier et al. 1998; Duperron et al. 2005). Methanotrophs were suggested to contribute nitrogen uptake (Lee and Childress 1994; Lee et al. 1999; Dattagupta et al. 2004).

6.5.2 Interactions Among Bacteria

Dividing stages of sulphur- and methane-oxidizers have been observed in many mussel species, confirming that symbionts benefit from their cellular environment (examples in Fiala-Médioni et al. 1986; Duperron et al. 2005). In the limited volume available for growth within bacteriocytes, competition is likely. In multiple symbioses, direct competition between symbionts with distinct metabolisms is possibly prevented by their use of distinct carbon and energy sources. Relative densities appear to depend on environmental parameters that favour either metabolism (see below). Competition between two symbionts with the same presumed metabolism was suggested based on the observation of mutual exclusion between the two thiotrophs S1 and S2 from *B. heckerae*. They very rarely co-occur within a single bacteriocyte, but instead tend to occupy distinct areas of the gill filaments (Plate 13d) (Duperron et al. 2007). A similar co-occurrence of two thiotrophs has been reported in *Idas* sp. Preliminary observations indicate that one phylotype occurs mostly in the periphery of filaments while the other is spread over the whole lateral zone, but mutual exclusion has not been observed (Duperron et al. 2008b; Halary et al. personal observation). In both cases, it is not clear if the presence of two thiotrophs leads to true functional redundancy, or whether they display slight differences in their metabolisms.

Besides competition, syntrophic relationships have been suggested between methanotrophs and methylotroph-related bacteria in *B. heckerae* (Duperron et al. 2007). Methylotrophs were suggested to use methanol, a C1 intermediate compound in the methane oxidation pathway used by methanotrophs. This hypothesis was based on the close proximity between the two symbiont types observed in hybridized sections (Plate 13c). Syntrophy among symbionts was previously hypothesized between the sulphur-oxidizing and sulphate-reducing bacteria associated with gutless oligochetes (Dubilier et al. 2005; Woyke et al. 2006).

The nature of inter-bacteria interactions is very hard to study. Furthermore, the host likely controls what occurs within bacteriocytes, maybe not only through digestion. In vesicomyid clams for example, recent investigation of the genome content of the thiotrophic symbiont *Vesicomyosocius okutanii* indicated the absence of *ftsZ*, an important gene involved in bacterial division. Authors suggested a close control of bacterial division directly by the host (Kuwahara et al. 2007).

6.5.3 Interaction Between the Symbiotic System and Its Environment

Generally, even mussels associated with symbionts can filter-feed. Filter feeding can significantly contribute to mussel nutrition, as suggested by stable isotope-based evidence from *"B." childressi* (Page et al. 1991; Pile and Young 1999). Recently, modelling approaches have indicated a shift from reliance on photosynthesis-derived carbon towards total reliance on bacterial-derived carbon during the growth of *B. azoricus* (Martins et al. 2008). However, transplant experiments in which *B. thermophilus* specimens were transferred from their vent habitat to bare rock or sediment devoid of fluid emissions have shown that mussels were quickly losing their symbionts and dying (Raulfs et al. 2004). This fits with the observation that specimens from a waning vent site were in bad condition, displaying low condition indexes and thin bacteriocytes with almost no symbionts. Altogether, this makes clear that organic matter available in the deep-sea is not sufficient to sustain symbiotic mussels without the help of their symbionts (Raulfs et al. 2004).

Deep-sea mussels can not survive in the absence of reduced compounds. However, detailed studies of the interaction between mussel symbioses and environmental parameters are still in their infancy due to our lack of precise knowledge about the physico-chemical environment of mussels and to the difficulties of experimentation on deep-sea animals. Deep-sea environments, in particular vent habitats, are highly variable in space and time even at the scale of centimeters. These steep gradients are caused by multiple distinct sources of fluids, biological activity, convective processes and fluid circulation, currents, and so on (see section 6.2). Determination of local physico-chemical parameters in the deep-sea requires the use of adapted in situ sensors, if they exist, and necessitates time-series measurements that are expensive to perform. Despite these limits, transplant experiments in which specimens of *"B." childressi* were transferred from a low-methane to a high-methane site (and vice versa) have clearly demonstrated increased mussel growth caused by increased abundance of methane, confirming the direct link between environmental parameters and mussels health (Nix et al. 1995; Bergquist et al. 2004). Experimentation is another difficulty. Certain mussel species from shallower sites, such as *B. azoricus* and *"B". childressi*, can be kept alive in aquaria at ambient pressure (Kadar et al. 2005; Riou et al. 2008). For deeper-dwelling species, pressurized aquaria in which specific compounds can be injected and monitored, such as the IPOCAMP™ device, are becoming available (Shillito et al. 2001). However, without detailed knowledge of habitat characteristics, it is not possible to properly simulate a realistic deep-sea vent or seep environment in an experimental setting. Even though observed trends can usually be extrapolated to the 'real' environment, exact physiological rates must therefore be taken with caution (Cary et al. 1988; Kochevar et al. 1992).

Mussels from different sites can display markedly different carbon stable isotope signatures that indicate different relative contributions of sulphur- and methane-oxidizers, even within a single species. (Trask and Van Dover 1999). This was shown for mussels from the Mid-Atlantic ridge associated with both sulphur- and methane-oxidizing symbionts, where isotope signatures were correlated with sulphide concentration in the end-member fluid (Colaco et al. 2002). To test whether these differences in the nutritional role of symbionts arise from differences in bacterial abundances, accurate quantification methods are required. However quantifying bacterial densities is not straightforward. Using bacteria-specific indicators such as lipids or quinones, densities of 1.7×10^{10} to 2×10^{11} bacteria per gram of gill tissue were estimated (Powell and Somero 1986; Yamamoto et al. 2002). Methanotrophic symbionts of *"B." childressi* were estimated at 5.6×10^{9} bacteria per gram tissue (Kochevar et al. 1992). Quantifying variations of symbiont densities is also difficult. Several methods have been employed, including direct counts on TEM pictures (benefiting from the distinct morphologies of sulphur- and methane-oxidizers), quantification of 16S rRNA, and quantification of volumes occupied by distinct symbionts in bacteriocyte sections (Fiala-Médioni 1984; Trask and Van Dover 1999; Fiala-Médioni et al. 2002; Duperron et al. 2007; Halary et al. 2008; Riou et al. 2008). Using such methods on mussels collected from several MAR hydrothermal vent sites, the authors demonstrated that symbiont populations were flexible. Relative abundances of methane- and sulphur-oxidizers were correlated with the composition of the end-member vent fluids, with the former becoming predominant at methane-rich sites and vice versa. Symbiont densities in *B. azoricus* specimens that were experimentally exposed to either sulphide or methane responded within hours to changes in their environment, with an increased abundance of sulphur- or methane-oxidizers, respectively (Kadar et al. 2005; Halary et al. 2008; Riou et al. 2008). Dynamic multiple symbioses thus appear to allow mussels to quickly optimize their access to available resources in variable environments, and cope with more diverse types of habitats, as suggested shortly after their discovery (Distel et al. 1995). Recently, we suggested that the specific bacterial densities observed in specimens of a single mussel species found on wood or whale falls are a consequence of sulphide availability (Lorion et al. 2009). Tools are becoming available to study the interaction between mussels, symbiont populations, and the environment in detail. However, more interdisciplinary studies coupling biological, chemical and modelling approaches are required (Martins et al. 2008).

6.6 The Symbiotic System: How Did It Evolve?

6.6.1 The Evolution of Mussels

Mussels occurring at chemosynthesis-based ecosystems and organic falls group in a single clade within the Mytilidae. The question of how the group evolved, what its common ancestor looked like, what the environment it lived in was or whether symbiotic bacteria were present, is hard to answer. Bathymodiolines are known from fossil seeps since the late Eocene, but the occurrence of symbionts is not possible to establish in the fossil record (Kiel and Goedert 2006). Molecular studies suggest an age between 23.7 and 74.3 MY for the origin of the group (Little and Vrijenhoek 2003; Jones et al. 2006). Early work suggested the evolution of vent mussel species from seep ancestors (Craddock et al. 1995). The later addition of small species from organic falls in phylogenetic reconstructions lead Distel et al. to propose the hypothesis that vent and seep mussels derived from ancestors associated with wood and whale fall ecosystems (Distel et al. 2000). However, results from recent bathymodioline phylogenies draw a more complex picture. Some species associated with organic falls such as *Benthomodiolus lignicola* or *Idas* sp. Sal 3 appear to be basal to seep and vent species (Samadi et al. 2007) while at least one vent species, "*Bathymodiolus*" NZ3, possibly a relict from an early vent colonization event, is also quite basal (Jones et al. 2006). Apart from these deep-branching species, the bathymodioline tree today is a large multifurcation which includes at least three vent/seep clades (named the *B. thermophilus*, *"B." childressi* and *"B." aduloides* clades) as well as numerous small species from whale and wood falls (Iwasaki et al. 2006; Jones et al. 2006; Lorion et al. 2009). Authors identified a recurrent trend from shallow (<1,000 m) to deep habitats in the evolution of the three groups of *Bathymodiolus*-like mussels. This would advocate multiple events of seep and vent colonization from a pool of small species associated with organic falls (Fig. 6.3, Plate 12). Habitat shifts during the evolution of mussels are plausible. Indeed, although most species today are specialists of a single type of environment, several ubiquist species were recently identified using reliable genetic approaches. *B. platifrons* and *B. japonicus* occur both at seeps and vents around Japan, and *Adipicola crypta* and *Idas* sp. C were both associated with wood- and whale-falls in the western Pacific (Miyazaki et al. 2004; Lorion et al. 2009).

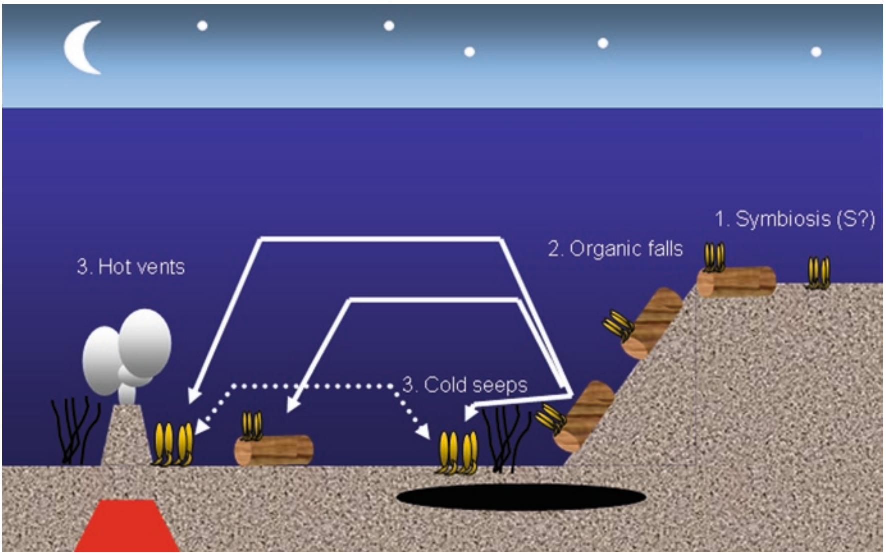

Fig. 6.3 The 'stepping stone' hypothesis. Ancestors of bathymodiolines were shallow species (on the right), which might have acquired the ability to associate with bacteria (1), most likely sulphur oxidizers. Mussels able to derive nutrition from both filter-feeding and bacterial chemoautotrophs colonized organic habitats such as wood and whale falls occurring at various depths (2). From such habitats, mussels then colonized hydrothermal vents at ridges and cold seeps on margins (3), giving rise to large *Bathymodiolus*-like species. This event probably occurred several times during the evolution of the group. Shifts between vent and seep probably occur, as evidenced by the existence of species colonizing both habitats (dotted arrow). A color version of this figure can be found in Appendix (Plate 12)

6.6.2 The Evolution of Mussel Symbiosis

Bacterial symbionts do not co-speciate with their mussel hosts (Won et al. 2008). However, very closely related mussel species, for example *B. azoricus* and *B. puteoserpentis* from the MAR, *B. boomerang* and *B.* aff. *boomerang* from the Gulf of Mexico (GoM) and Gulf of Guinea (GoG), or *Idas* sp. C and *Idas* sp. D from the Vanuatu and Philippine waters, harbour very closely related bacteria (Duperron et al. 2006, 2007; Lorion et al. 2009). Overall, symbiont phylogenies are not well resolved and sometimes ambiguous due to the low variation levels observed in 16S rRNA gene sequences (Duperron et al. 2005, 2008b; DeChaine et al. 2006; Won et al. 2008). Clearly, the understanding of symbiont biogeography and evolution would greatly benefit from the use of multi-marker approaches besides 16S rRNA. Reconstructions indicate that the occurrence of thiotrophic symbionts in the latest common ancestor of bathymodiolines is likely (Won et al. 2008; Duperron et al. 2009). Symbiont localization in the ancestor is however not clear. Thiotrophs are consistently intra- or extracellular depending on the species and mostly intracellular in seep and vent mussels and often extracellular in small *Idas*-like species from organic falls. However, symbiont localization is not documented or ambiguous for the deepest-branching species such as *Bathymodiolus* from the Juan de Fuca Ridge, *Benthomodiolus lignicola* or *"Bathymodiolus"* NZ3, thus not allowing a prediction (McKiness et al. 2005; Lorion et al. 2009). Whatever the localization, the acquisition of a flexible sulphur-oxidizing symbiosis in the common ancestor of bathymodiolines must have constituted an advantage for the mussels. Such an adaptation opened new potential ecological niches, and could explain the subsequent specialization of the group towards reduced ecosystems. It probably triggered a rapid diversification of the group which translated into the large multifurcation observed in host phylogenetic trees. Based on our current knowledge, methanotrophic symbiosis seems to appear several times in host trees, and could be more opportunistic from the animal's point of view. It apparently occurred only at methane-rich sites. However, host – symbiont specificity appears to be high as evidenced by the consistent occurrence of two distinct methanotrophs in the gills of *B. brooksi* and *B. childressi*, two mussels which co-occur within the same mussel beds at Alaminos Canyon (Fujiwara et al. 2000; Duperron et al. 2007). Other symbioses involving methylotrophs, Bacteroidetes or other Gammaproteobacteria have been discovered recently and it is too early to propose any hypothesis. Nevertheless, it is intriguing that dual and multiple symbioses are to date reported only from the Atlantic area, including MAR, the GoM, GoG and Mediterranean (Table 6.1). Noteworthy, at least three mussel species from this area, two *Bathymodiolus* and one *Idas*, display amphi-Atlantic distributions (Olu-LeRoy et al. 2007a; Duperron et al. 2008b; Génio et al. 2008). No mussel with multiple symbionts is reported to date from the Pacific, despite 16 mussel 'species' investigated in the area. Clearly, although hypotheses can be proposed based on current knowledge, much information is needed to clarify our understanding of symbiosis evolution in mytilids.

6.7 Conclusion

Mytilids associated with deep-sea chemosynthesis-based ecosystems provide fascinating examples of symbiosis. How the diversity of symbionts is accommodated within a single bacteriocyte in some species is not understood, but the worldwide occurrence of mytilids at seeps, vents and organic falls and the diversity of their associated bacteria illustrate the success of this group. The ability to associate with metabolically diverse bacteria and to adapt symbiont densities to environmental parameters is probably a key to this success, allowing mytilids to cope with diverse and variable habitats.

Many questions remain. Our inability to cultivate symbionts, and difficulties of both in situ and ex situ experimentation on living mussels are major issues hampering research on these ‘symbiotic systems’. The existence of free-living forms of symbionts remains to be proven, and how associations establish is not known yet. The metabolic potential and diversity of symbionts are probably underestimated. Genomic approaches should provide indications, yield testable hypotheses, and allow comparisons between mytilid symbioses and other well-documented associations for which genomic information is available, be they chemosynthetic (*Riftia*, *Calyptogena*…) or not (insects).

Finally, geographical trends observed and evolutionary hypotheses proposed to date result from the study of a limited number of species from regions where oceanographic research has focused over the last decades (GoM, GoG, MAR…), leaving many blank spots on the map (Indian Ocean, southern Atlantic, Antarctic…). There is no doubt that new species discovered in newly explored areas will in the future considerably change our views, and yield a more complete picture of the biogeography and evolution of symbiosis in deep-sea mytilids.

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