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Preliminary Study of Heterotrophic Bacteria Isolated from two Deep Sea Hydrothermal Vent Invertebrates: *Alvinella pompejana* (Polychaete) and *Bathymodiolus thermophilus* (Bivalve)

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Abstract

The existence of rich animal communities in the close vicinities of deep sea hydrothermal vents, has been explained by the abundance of bacterial trophic resources. These bacteria are found involved both in classical filter feeding via the digestive tract and in endo and ecto-symbiotic associations with invertebrates. In both cases, the autotrophic metabolism of these bacteria has been demonstrated. However, because of the high density of the animal vent communities, the occurrence of heterotrophic bacteria involved in degradative processes should also be expected. During the "Biocyarise" cruise in 1984, on the 13° N site of the East Pacific Rise, individuals of *Alvinella pompejana* and *Bathymodiolus thermophilus* were obtained using the submersible "Cyana". Seventy-three bacterial strains were isolated from cultures on media for heterotrophic and mixotrophic bacteria. This collection of strains is marked by the lack of fermentative metabolism, even for the strains isolated from the gut of invertebrates. Only a few strains showed proteolytic or amylolytic activities, but many were lipolytic. Most of the isolated strains produced hydrogen sulfide from cysteine. The bacteria isolated from the epidermis of *Alvinella* showed the highest index of carbohydrate and amino acid utilization as the sole carbon source. Fifty percent of the strains were able to grow at 50°C, which is a little above the estimated temperature of the worm's habitat. Further studies are now in progress to complete the description of these bacteria and improve our knowledge of the possible role of heterotrophic microorganisms in the hydrothermal vent ecosystem.

Keywords: hydrothermal vents, invertebrates, heterotrophic bacteria

1. Introduction

The rich animal communities living in the vicinity of deep sea hydrothermal vents on the East Pacific Rise constitute one of the main discoveries of the last years in the field of biological oceanography (Lonsdale, 1977). Among the hypotheses proposed to explain the existence of such communities, contrasting with the scarce fauna of usual deep sea ecosystems, is the trophic role of a local abundance of bacterial biomass which could serve the most probable food source (Lonsdale, 1977). The first estimations of bacterial abundance by Corliss et al. (1979) ranged from 10^8 to 10^9 cells per mi, corresponding to 1 g of bacterial organic carbon per litre of seawater. Further estimations (Jannasch and Wirsen, 1979) were lower, ranging from 5×10^5 to 10^6 cells per ml or 5 to 1 mg of organic carbon per litre. The activity measurements conducted by Tuttle et al. (1983) revealed the prevalence of the chemosynthetic activity of sulfur oxidizing bacteria. However, bacterial production appeared to be insufficient to support a biomass up to 50 kg wet weight per m^2 (Fustec et al., 1987). Moreover, the Pogonophoran *Riftia pachyptila* which represents a large proportion of the biomass, has no digestive tract (Jones, 1981), so being unable to use the suspended bacterial biomass, even if it constituted an adequate food supply. Further investigations, based on transmission and scanning electron microscopy (Cavanaugh et al., 1981; Gaill et al., 1987, Le Pennec and Prieur, 1984), $^{13}C/^{12}C$ ratios (Williams et al., 1981), led to the demonstration of the primordial trophic role of various bacterial communities, more or less closely associated with the invertebrate tissues or teguments. Moreover, several enzymes of the Calvin cycle were analysed in some invertebrate tissues (Felbeck, 1981), and indicated the autotrophic metabolism of the associated bacterial communities. However, although autotrophic bacteria obviously constitute the first level of the hydrothermal food chain, the occurrence of heterotrophic microorganisms could also be possible, taking into account the high invertebrate biomass, and subsequent biological processes like excretion, even death of animal communities. Bacterial heterotrophic activities were measured in seawater surrounding the vents (Tuttle et al., 1983), so demonstrating the occurrence of heterotrophic bacteria. Such bacteria associated with invertebrates, could provide detoxification benefits or trace nutrients to their hosts. The present study took place in 1984 during the "Biocyarise" cruise, on the 13° N site (Desbruyères et al., 1982) of the East Pacific Rise, and was conducted in part to look for heterotrophic bacteria associated with two invertebrates: the bivalve *Bathymodiolus thermophilus* and the polychaete *Alvinella pompejana.*

2. Materials and Methods

All the samples were taken in March 1984, on the East Pacific Rise (12°59'N, 103°56'W), at a depth of 2600 m, using the inhabited submersible "Cyana" from its mother ship N.O. "Nadir" (Ifremer, France). Invertebrate samples were collected by the arm of the submersible, brought to the surface by a free diving and operating shuttle, and prepared for analysis, as soon as they arrived aboard the mother ship. Samples were rinsed in sterile 0.2μm filtered sea water and processed by dissection. In the case of *Alvinella pompejana*, worm tube fragments, worm epidermis, and a whole animal were sampled. For *Bathymodiolus thermophilus*, the digestive gland, the gill filaments, and the mantle, were separated, strongly rinsed five times in sterile 0.2μm filtered seawater, and ground in similar water with an "ultra turrax" grinder. Ground material was diluted and dilutions were plated on petri dishes containing one of 3 media: 2216E (ME) medium (Oppenheimer and Zobell, 1952); MF medium (2216E with double concentrations of organic matter) or inoculated in a liquid medium (MM) for mixotrophic sulfur oxidizing bacteria (Güde et al., 1981). The cultures were incubated for 1 week under atmospheric pressure, at temperatures of 8°C and 30°C for the samples of *Bathymodiolus* and *Alvinella*, respectively. After isolation and purification, the strains were described by the following features: Gram, morphology, sporulation, motility, growth at 5°C, 37°C, 41°C, 45°C, 50°C, fermentation metabolism, catalase, gelatinase, lipase, amylase, nitrate reduction, production of hydrogen sulfide from cysteine, utilization as sole carbon source of 8 carbohydrates and 8 amino acids.

3. Results

Origins of the isolates

The number of isolates per origin and medium are given in Table 1. Only 11 strains were isolated from *Bathymodiolus* samples, all from the MF medium. Sixty-two strains were purified from the *Alvinella* samples on the 3 media used. The most important group of strains came from the worm epidermis sample cultivated on ME medium.

Description of the isolates

The biochemical and ecological features of the isolates are given in Table 2, the nutritional features, in Table 3.

Table 1. Numbers of isolates per culture medium and origin

origins	*A. pompejana*			*B. thermophilus*		
	Tube	Epidermis	Whole worm	Gills	Mantle	D. gland
Media						
MB	5	8	7	0	0	0
ME	6	20	6	0	0	0
MF	6	0	4	3	4	4
Total	17	28	17	3	4	4

Table 2. Biochemical and ecological features of the isolates. The results are expressed in percentages of positive responses to the tests

Origins	*A. pompejana*			*B. thermophilus*
	Tube	Epidermis	Whole worm	Gills + Mantle + D. gland
Features				
Rods	100.0	100.0	100.0	100.0
Motility	64.6	78.5	100.0	45.4
Gram	0	0	0	0
Fermentation	0	0	11.7	0
Oxydase	70.5	75.0	88.2	36.3
Catalase	88.2	100.0	82.3	72.7
NO_3^- NO_2	58.8	64.2	82.3	9.0
H_2S	82.3	100.0	64.7	54.5
Gelatinase	0.5	0	0	0
Lipase	52.9	78.5	70.5	63.6
Amylase	0.5	17.8	0.5	27.2
5°C	76.4	57.1	58.8	90.9
41°C	100.0	100.0	100.0	73.0
45°C	82.3	92.8	100.0	63.6
50°C	0	35.7	0.5	9.0

Strains isolated from Bathymodiolus thermophilus

Eleven strains were isolated from *Bathymodiolus*, 3 from the gills, 4 from the mantle, and 4 from the digestive mass. Because of this small amount of isolates, the results are presented together. All the strains were isolated from the MF medium. They are Gram negative rods, 45.8% being motile. No strain showed a fermentative metabolism, and only 1 is able to reduce nitrates to nitrites. No strain is proteolytic, 27.2% are amylolytic and 63.6%

Table 3. Nutritional features and Average Utilization Index (AUI) of the isolates. The results are expressed as percentages of positive responses to the tests

Origins	*A. pompejana*			*B. thermophilus*
	Tube	Epidermis	Whole worm	Gills + Mantle + D. gland
Features				
Saccharose	47.0	57.1	58.8	54.5
Arabinose	29.4	64.2	23.5	27.2
Glucose	47.0	71.4	47.0	9.0
Maltose	47.0	71.4	52.9	36.3
Galactose	41.1	64.2	29.4	36.3
Mannose	52.9	75.0	41.1	63.6
Mannitol	52.9	71.4	64.7	63.6
Sorbitol	47.0	64.2	70.5	54.5
AUI	0.45	0.67	0.48	0.43
Proline	70.5	64.2	94.1	72.7
Glycine	0	0	5.8	0
Leucine	41.1	78.5	76.4	72.7
Arginine	47.0	67.8	47.0	27.2
Serine	0	0	0	0
Asparagine	47.0	60.7	47.0	36.3
Aspartate	41.1	50.0	58.8	36.3
Glutamate	5.8	14.2	11.7	18.1
AUI	0.31	0.41	0.41	0.32

lipolytic. Six of the 11 isolates (54.5%) produce hydrogen sulfide from cysteine. Concerning the nutritional features, the average utilization indexes (AUI) of organic compounds are 0.43 and 0.32 for the carbohydrates and amino acids, respectively. Proline, leucine, mannose and mannitol are the most utilised compounds. These strains are adapted to low temperatures and 90.9% grow at 5°C. However, 63.6% can still grow at 45°C and 1 strain isolated from the mantle is able to grow at 50°C.

Strains from the epidermis of Alvinella pompejana

Twenty-eight strains were isolated from the epidermis, on the media MB and ME. All the strains are Gram negative rods and 78.5% are motile. No strain has a fermentative metabolism. Only 1 strain is proteolytic, 17.8% are amylolytic, but 78.5% have a lipase. The nitrates are reduced to nitrites by 64.2% of the strains, and all of them produce hydrogen sulfide from cysteine. The AUI are 0.67 and 0.41 for the carbohydrates and the amino and leucine are the most utilised compounds. Only 57.1% of the strains grow at 5°C, but 92.8% of the strains grow at 45°C, and 37.5% at 50°C. The most thermophilic strains were isolated from ME medium. All of them produce hydrogen sulfide from cysteine. Their AUI are 0.91 and 0.52 for the carbohydrates and amino acids, respectively.

Strains from a grinding of Alvinella pompejana

Seventeen strains were isolated from a grinding of a small, whole *A. pompejana*, on the 3 media used. All the strains are Gram negative motile rods. Two of them have a fermentative metabolism. No strain is proteolytic, 1 is amylolytic, but 70.5% have a lipase. The AUI are rather similar, 0.48 and 0.41 for the carbohydrates and the amino acids, respectively. Proline, leucine, mannitol and sorbitol are the most utilised compounds. All the strains can grow at 45°, 58.8% at 5°C, and only 1 at 50°C.

4. Discussion and Conclusion

The bacteriological studies dealing with the deep sea hydrothermal vents of the East Pacific rise first demonstrated the role of autotrophic microorganisms. However, Jannasch and Wirsen (1979) reported the culture of some heterotrophic bacteria, and Ruby et al. (1983) found heterotrophic activity in seawater. During the "Biocyarise" cruise, some counts of heterotrophic bacteria in water samples indicated that these microorganisms could reach a concentration of up to a tenth of the total counts obtained by epifluorescence microscopy (Prieur, unpublished data).

For the 2 invertebrate species studied during this cruise, the results indicated the occurrence of heterotrophic microflora associated with the invertebrates. The question of contaminants must be considered because all the samples are washed in the water column (2600 m), when they are lifted to the surface. If some contamination occurred, it is likely to be negligeable for the following reasons. One individual of the polychaete *Paralvinella grasslei* was ground and analysed at the time, with the same methods as the *Alvinella* and *Bathymodiolus* samples. Most of the cultures were negative, and in fact this species has no obvious associated microflora (Desbruyères and Laubier, 1982). Moreover, the strains isolated from the different parts of *Alvinella* and *Bathymodiolus* are different, but the contamination sources are identical. Their growth temperatures also reflect their biotops and not contaminating seawater. The numerical taxonomy of isolates, which is in progress at the present time could confirm this point.

It is possible that the temperature incubation of the enrichment cultures (8°C for *Bathymodiolus*, 30°C for *Alvinella*) could play a role in the selection of the microflora. However, 90.9% of the isolates from *Bathymodiolus* grow at 5°C, 100% at 30 and 37°C, and the percentages of cultivable strains decreased for the highest temperatures. On the contrary, only 57.1% of the bacteria isolated from the epidermis of *Alvinella* can grow at 5°C, whereas 35.7% grow at 50°C. The isolates appeared to be well related to the estimated temperature of their biotops (Desbruyères et al., 1982). However, these results are a little different from those of Ralijaona and Bianchi (1982), who reported that the gut bacteria of a cold deep sea holothurian grew better at 44°C than at 4°C.

The morphology of different isolates was very homogeneous, and composed of rods. The spheric, curved or filamentous forms reported previously (Gaill et al., 1987) have not been observed in the cultures. Two explanations are possible: these bacteria were not able to grow in the proposed media or the culture conditions were not convenient for the exhibition of the particular morphologies because of the lack of substrate (many bacteria were observed attached), or the lack of hydrostatic pressure.

The number of pure strains was very different, according to invertebrate species. These results must be considered with the data of microscopic observations. In the case of *B. thermophilus*, the bacteria have been observed in the digestive tract and in the gill filaments (Le Pennec and Prieur, 1984; Le Pennec and Hily, 1984). There is no observation of bacteria on the mantle, and the isolates have probably originated from the mantle cavity. Only few strains were isolated from the digestive gland, and this could indicate that ingested bacteria are digested at this level of the digestive tract. If the gill associated bacteria are strict autotrophs, it is possible that they are not cultivable on the media used. But these bacteria also could be in a weak state and so unable to grow on a culture medium. Le Pennec (1987) reported that some of the gill bacteria were lysed.

The isolates from *A. pompejana* were more numerous, and this can be explained by the abundance of bacteria observed on the animal (Gaill et al., 1987); the inner wall of the worm tube is covered by bacteria and the epidermis is colonized by various bacterial types.

The biochemical features of the isolates are difficult to appreciate because of the very few number of papers dealing with the microflora of deep sea invertebrates. However, some features are particularly interesting. First of all, the number of fermentative strains is very low: only 2 for seventy-three isolates. Nevertheless, some parts of digestive tracts were analysed, which generally contain fermentative bacteria (Prieur, 1981; Ralijaona and Bianchi, 1982). But this result does not exclude the possibility of anaerobic metabolism, because of the high percentage of strains that reduce nitrates to nitrites. It must also be noted, that the cultures were done under atmospheric pressure, which is not the normal condition of these deep sea bacteria.

The other interesting feature is the high percentage of bacteria producing hydrogen sulfide from cysteine. Although it is not possible to assume that this metabolism really exists "in situ", it is possible to consider that a part of the energy source (hydrogen sulfide) of some autotrophic sulfur oxidizing could be produced by the heterotrophic communities.

The possible symbiotic role of the heterotrophic bacteria associated with the hydrothermal invertebrates can be estimated on the basis of previous works. In a review of deep sea microbial energetic, Morita (1979) suggested that deep sea bacteria could provide the deep sea invertebrates with essential nutrients, like methionine, which are not synthesized by the animals. The production by the heterotrophic communities of trace compounds like vitamins could also be hypothesized.

However, the most probable role of these bacteria should be detoxification. The hydrothermal fluids are particularly enriched with heavy metals (Corliss et al., 1979) and the soft tissues of hydrothermal vent invertebrates have been found contaminated (Gaill et al., 1984; Roesijadi and Crecelius, 1984). Moreover, in several cases, the highest metal concentrations were found in tissues containing endocellular bacteria (Chassard-Bouchaud et al., 1986). The hypothesis of a role of bacteria in the concentration of metals and detoxification of animals have been proposed (Chassard-Bouchaud et al., 1986), some bacteria being able to synthesize metallothioneins, which have been detected in the tissues of the polychaete *Alvinella pompejana* (Cosson-Mannevy et al., 1986). Such a role could also be suggested for the epibiotic microflora of the worm: Johnson et al. (1981) demonstrated that the concentration of chromium by the crab *Helice crassa* was due to periphytic bacteria colonizing the crab carapace and gills.

The preliminary results about the heterotrophic bacteria associated with deep sea hydrothermal vent invertebrates which are reported here are not adequate to improve the understanding of the interactions between bacteria and invertebrates. However, the occurrence of heterotrophic bacteria within these associations, particularly in the case of of *Alvinella pompejana* has been pointed out. Some laboratory tests which are now in progress should provide more information on the detoxification role of heterotrophic bacteria, and improve the understanding of the hydrothermal vent ecosystems.

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