Occurrence of a specific dual symbiosis in the excretory organ of geographically distant Nautiloids populations

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Summary

Nautilus is one of the most intriguing of all sea creatures, sharing morphological similarities with the extinct forms of coiled cephalopods that evolved since the Cambrian (542–488 mya). Further, bacterial symbioses found in their excretory organ are of particular interest as they provide a great opportunity to investigate the influence of host–microbe interactions upon the origin and evolution of an innovative nitrogen excretory system. To establish the potential of Nautilus excretory organ as a new symbiotic system, it is, however, necessary to assess the specificity of this symbiosis and whether it is consistent within the different species of present-day Nautiloids.

By addressing the phylogeny and distribution of bacterial symbionts in three Nautilus populations separated by more than 6000 km (N. pompilius from Philippines and Vanuatu, and N. macromphalus from New Caledonia), this study confirms the specificity of this dual symbiosis involving the presence of betaproteobacteria and spirochaete symbionts on a very wide geographical area. Overall, this work sheds further light on Nautiloids excretory organ as an innovative system of interaction between bacteria and cephalopods.

Introduction

Nautilus is one of the most famous of all sea creatures, inspiring many artists and giving its name to the wondrous craft imagined by Jules Verne in his novel. However, it is probably in the eyes of the naturalists that Nautilus has the most important place, being the last representative of the subclass of Nautiloidea, and the only extant cephalopod among hundreds of extinct, coiled cephalopod genera that evolved since the Cambrian (542–488 mya) (Ward, 1987; Kröger et al., 2011). Despite their morphological similarities with the extinct forms of Ammonoids, present-day Nautiloids are not ‘living fossils’ but rather highly specialized animals occupying a specific niche in the coral reef ecosystems of tropical and subtropical Indo-Pacific regions. Indeed, Nautiloids live on the external slope of coral reefs, where they undertake complex vertical migrations (depth ranging from 100 to 700 m) in order to avoid contact with predators, but also to explore and scavenge for crustaceans decapods rich in protein (Ward et al., 1984; Norman, 2000; Dunstan et al., 2011). After ingestion of their prey, the three steps of digestion (digestion, absorption and excretion) (Westermann et al., 2002) lead to the production of ammonia as main end-product of protein catabolism (Boucaud-Camou and Boucher-Rodoni, 1983).

Nautiloids excretory system features highly specialized organs, unique among cephalopods, referred to as pericardial appendages (Fig. 1A and B). These organs are responsible for most of the excretory processes including filtration (i.e. filtration of small molecules contained in the blood), reabsorption (i.e. active reincorporation of compounds from the pericardial coelom to the internal part of the organ) and secretion of ammonia rich fluid (Schipp et al., 1985; Mangold et al., 1989). Each pericardial appendage consists of numerous finger-like villi that collect the blood from capacious sinuses and produce the excretory fluid rich in ammonia [up to 200 ppm, Fig. 1B (Schipp and Martin, 1987)] which is firstly secreted in the pericardial coelom and then excreted into the mantle cavity (Mangold et al., 1989). This physiological innovation has a significant impact on Nautilus metabolism and more particularly on the management of nitrogen waste, the amount of ammonia released to the surrounding sea.
water by *Nautilus* being three to four times lower than by any other extant cephalopods (Boucher-Rodoni and Mangold, 1994).

The bacterial symbioses discovered in *Nautilus* pericardial appendages about two decades ago (Schipp *et al*., 1990) provide a great opportunity to shed light on the influence of host–microbes interactions on the origin and evolution of such an innovative excretory system (Fig. 1; Pernice *et al*., 2007b). However, before establishing *Nautilus* pericardial appendages as a new symbiotic system, it is necessary to assess the specificity of this symbiosis and whether it is a general rule in present-day Nautiloids. Currently, information about this symbiosis remains scarce due to the lack of biological material and the inability to cultivate the bacterial symbionts artificially. The present study identifies the specificity of this symbiosis by combining data concerning the phylogeny and the distribution of bacterial symbionts in three *Nautilus* populations separated by more than 6000 km (*N. pompilius* from Philippines and Vanuatu, and *N. macromphalus* from New Caledonia, Fig. 2). Some aspects concerning the evolution of this symbiosis and its potential implication in the ecophysiology of Nautilus excretory organ are further discussed.

**Results and discussion**

**Nautiloid symbiont diversity and phylogeny**

Comparison of bacterial 16S rRNA gene sequence analysis of a total of 525 clones indicated the predominance of two bacterial phylotypes (betaproteobacteria and spirochaetes) in the pericardial appendages of the three
Nautilus populations analysed. In situ hybridization using specific probes confirmed that both the betaproteobacteria and the spirochaete symbionts were present and closely associated to Nautilus tissue in the three different populations. Another bacterial phylotype belonging to the gammaproteobacteria (Vibrionacae) was detected in N. pompilius population from Philippines by amplification of 16S rRNA gene (Pernice et al., 2007a) but in situ hybridization analyses using a Gammaproteobacteria-specific probe [Gam42A (Manz et al., 1992)] failed to confirm a close association of this bacterial phylotype with Nautilus tissue. This lack of corroborative evidence calls into question the symbiotic status of these bacteria (Table 1). Indeed, the excretory organs of Nautiloids are connected to the external environment through the pallial cavity and, therefore, the detection of Vibrionales by polymerase chain reaction (PCR) amplification of 16S rRNA gene is likely to result from an environmental contamination as previously suggested (Pernice et al., 2007a).

Comparison of the 16S rRNA-gene sequences obtained for betaproteobacteria and spirochaete symbionts indicates that (i) the sequence variation within each phylotype was remarkably low (below 0.5% based on 1411 bp of 16S rRNA gene sequence) and (ii) neither the betaproteobacteria nor the spirochaete symbionts were closely related to any others symbionts associated with other hosts species or any free-living bacteria (≤ 90% of similarity based on 1411 bp of 16S rRNA gene sequence), supporting the specificity of this dual symbiosis on a very wide geographical area (Nautilus collection sites being distant from more than 6000 km, Fig. 2). In addition, phylogenetic analysis of 16S rRNA gene reinforces this hypothesis by clustering the two phylotypes within two Nautilus-specific bacterial groups (Fig. 3). Based on the analysis of 16S rRNA gene, the closest relatives of the betaproteobacterial symbionts are members of a clade of free-living ammonia-oxidizing bacteria from the family Nitrosomonads including Nitrososphaera multiformis and Nitrosospira briensis (Teske et al., 1994). The spirochaete symbionts belong to a monophyletic group that includes the free-living spirochaetes Spirocheta bajas-californiensis (Magot et al., 1997) and Spirocheta smaragdinae (Fercek and Stolz, 1985) and the symbionts of gutless marine oligochaetes.

Sequencing and analysis of the gene coding for the 16S rRNA of Nautilus bacterial symbionts provides new information that may help elucidating the evolutionary history of Nautiloids and their symbioses. In respect to the evolution of Nautiloids, the strong genetic similarity observed between N. macromphalus and N. pompilius bacterial symbionts supports the hypothesis proposed by Wray and colleagues (1995) that New Caledonia-endemic species N. macromphalus, may in fact represent a geographic variant within a divergent, widespread N. pompilius species. However, it is now recognized that the gene coding for the 16S rRNA does not have sufficient resolution to define the phylogenetic relationships of closely related bacterial strains (Kowalchuk and Stephen, 2001). Future co-phylogeny studies should, therefore, use molecular markers with greater phylogenetic resolution. In this respect, the gene coding for the glyceraldehyde phosphate dehydrogenase (gapA) has been recently used to investigate the phyleogeography of the mediterranean sepiolids squid-Vibrio symbioses (Zamborsky and Nishiguchi, 2011) and could, therefore, represent a promising candidate.

Regarding the origin and evolution of the Nautilus-bacteria association, the important genetic distance between Nautilus symbionts and any bacterial strains referenced in the databases reflects the specificity of this symbiosis and may suggest a potential host–symbiont coevolution. Indeed, phylogenetic analyses of 16S rRNA sequences presented in this study show that all the different Nautilus populations analysed so far share symbiont phylotypes evolutionarily very close (Fig. 3). Further,
according to the most reliable rates of base substitution in the 16S rRNA gene for prokaryotes (1% per 50 MA; Ochman and Wilson, 1987; Moran et al., 1993; Droge et al., 2006), the large genetic distance (≥ 10%) observed in comparative 16S rRNA gene analysis would suggest (i) that the last common ancestors of Nautilus symbionts must have existed earlier than 500 million years ago (515–630 mya) and (ii) that the diversification of these symbionts may have begun with their acquisition by the common ancestor of extant Nautiloids, probably before its divergence from Coleoids (i.e. squids, cuttlefish and octopods) at the Silurian/Devonian boundary (416 ± 60 mya) (Kröger et al., 2011). However, the rates of molecular evolution can vary considerably among bacterial lineages (Ochman et al., 1999) and the use of such a fixed rate of base substitution is likely to result in biased estimates (Kuo and Ochman, 2009). It is reasonable to suggest that this association occurred long ago, but in order to better understand when this symbiosis evolved, further work should focus on different species of Nautiloids that have never been analysed concerning their potential symbiotic populations, including N. belauensis, N. stenomphalus, and N. repertus, but also and most importantly, Allonautilus scrobiculatus which is the only species of the genus Allonautilus (Wray et al., 1995; Ward and Saunders, 1997). In this respect, the study of Nautiloids populations living off New Guinea, the only place in the world where N. pompilius is sympatric with A. scrobiculatus, would be of the greatest interest as it could allow comparing bacterial symbionts in two divergent lineages of Nautiloids living within the same environment.

Distribution of bacterial symbionts in relation to the ecophysiology of the symbiotic organ

Imaging of the bacterial symbionts in Nautilus pericardial appendages by using in situ hybridization (Text S1) revealed a remarkably stable spatial distribution in the different Nautilus bacterial populations, with two levels of structuration within the host tissue (Fig. 4). A first level of

Fig. 3. The phylogenetic relationships of the spirochaete and betaproteobacterial symbionts of N. pompilius (from Philippines and Vanuatu) and N. macromphalus (from New Caledonia) inferred from 16S rRNA gene analysis using maximum likelihood (ML) (954 sites analysed). Numbers at each branch point are the bootstrap values for percentages of 1000 replicate trees calculated by MP (upper) and ML (lower) methods. Only values > 60% are shown. Trichodesmium thiebautii (cyanobacteria, AF013027) is included as an out-group.

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organization was observed by using a probe recognizing most bacteria [Eub388 (Aman et al., 1990)] in longitudinal section of the excretory organs and concerned the predominance of bacterial symbionts in the baso-median region of the villi and their complete absence in the apical region (Fig. 4A–C). A second level of spatial distribution, specific to each group of symbionts, was revealed by using the spirochaete symbiont-specific probe NauSpiro255 and the Betaproteobacterium-specific probe NauBet66 (Pernice et al., 2007b) and indicated the predominance of betaproteobacteria symbionts in the cavities formed by baso-medial invaginations of the villi while the spirochaete symbionts were mainly present in the peripheral areas (Fig. 4D–I). In accordance with the functional organization revealed by the ultrastructure of the pericardial villi (Schipp et al., 1985), this spatial distribution suggests that the bacterial population may interact specifically with the host tissue for two main reasons. First, the bacterial symbionts are concentrated and attached to the outer epithelium in the baso-medial region of the pericardial villi which is highly active in the ultrafiltration and the reabsorption processes while the apical part of the villi is devoid of any symbionts (Schipp and Martin, 1987). Second, the ultrastructure of the outer epithelium and its polar organization in the symbiotic region of pericardial villi are characteristic of an energy-requiring, transport active tissue with a high density of mitochondria and ionic pumps (Martin, 1983; Pernice et al., 2007c). The precise distribution of the bacterial symbionts in this transport active region of the excretory tissue is likely to reflect metabolic interactions with their host tissue based on a three-step process: (i) the secretion of the excretory fluid by the host tissue; (ii) the degradation and assimilation of compounds present in the excretory fluid by the bacterial symbionts; and (iii) the reabsorption of compounds derived from bacterial assimilation by the host tissue (Fig. 5). The ecophysiology of this symbiotic system seems to be primarily governed by pH and ammonia

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environment, ammonia being the main end-product of Nautilus excretion (Martin, 1983); and (iii) transformation of ammonia could have an important ecological role for Nautilus by detoxifying its tissue and/or by providing the nitrogen gas filling its chambered shell (containing over 90% of nitrogen as gas), responsible for its neutral buoyancy (Denton, 1974; Boucher-Rodoni and Mangold, 1994).

Preliminary results concerning the potential implication of bacterial symbionts in the transformation of ammonia are contrasted. Indeed, a molecular approach using PCR amplification has failed to detect the presence of genes coding for enzymes related to nitrogen metabolism such as amoA (Purkhold et al., 2000), nirK (Braker et al., 1998) and nosZ (Scala and Kerkhof, 1998) in the DNA extracted from Nautilus pericardial appendages (Pernice et al., 2007b). A second approach, using isotopic incubation of the symbiotic organ in seawater enriched in 15N-ammonia and 14N-nitrate (Text S1) has highlighted a series of interesting metabolic responses including rapid production of nitrates (in less than 6 h) followed by nitrates assimilation (from 12 to 18 h) and small but significant accumulation of 15N-labelled nitrogenous gas (Fig. S1). Such metabolic responses could indicate a combination of nitrification (i.e. oxidation of ammonia to nitrate) and further denitrification (oxidation of nitrite to dinitrogen gas) suggesting that the two bacterial symbionts may share a mutualistic relationship with each other in an endosymbiotic nitrogen cycle, in addition to their symbiotic relationship with Nautilus host. However, the rate of 15N-labelled nitrogenous gas production observed (c. 0.3 nM/h) was remarkably low in comparison to the rate of nitrate assimilation (c. 8 µM/h), which calls into question this hypothesis of nitrification combined to denitrification. An alternative hypothesis concerning the metabolic pathways of the symbionts is that the symbiotic bacteria could allow Nautilus to better conserve nitrogen by degrading proteins present in the coelomic fluid into amino acids. Such metabolic activity could ultimately facilitate the reabsorption process in baso-median region of Nautilus pericardial appendages (Pernice et al., 2007b). Further investigations are clearly needed to understand how the betaproteobacteria and spirochaete symbionts establish their own ecological niche within this micro-ecosystem.

Bacterial symbioses involved in the recycling of digestive or waste products have been described in a number of metazoans during the past decades and their activities are now widely recognized as essential for the functioning of all ecosystems including the human body (Turnbaugh et al., 2006; Douglas, 2009; Wagner, 2009). In Nautilus, the identity of the compounds involved in the host–symbiont interaction remains unclear. Most of the preliminary work has been focused on the potential role of bacterial symbions in nitrogen metabolism as (i) Nautilus betaproteobacteria symbionts are phylogenetically affiliated to Nitrosomonadaceae, an ammonia-oxidizing lineage; (ii) the symbiotic bacteria live in an ammonia-rich...
References


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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Metabolic activity of Nautilus bacterial symbionts in seawater enriched in labelled nitrogen compounds (ammonia, 15NH4+; nitrate, 14NO3–).

**Text S1.** Supplementary information for Material and Methods.

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