Understanding the interaction between Anabaena sp. and a heterocyst-specific epibiont

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Abstract

Anabaena sp. and an associated heterocyst-specific epibiontic bacterium were isolated from School Street Marsh in Woods Hole, MA in 1997 during the Microbial Diversity course. A two-member culture of the cyanobacterium and the epibiont have been maintained at WHOI by John Waterbury. Anabaena species with similar heterocyst-specific epibionts have also been isolated from other locations (7, 8, 9). Successful culture of the epibiont separately from the School Street Marsh cyanobacterium was achieved on two occasions (1, 10), but the isolates were not preserved. The epibiont was identified to be an α-proteobacterium within the Rhizobiaceae group (10).

The close and specific association between the cyanobacterium and the epibiont strongly suggests there might be a physiological benefit or benefits to the cyanobacterium, the epibiont, or to both partners. This study was designed to understand the potential benefits of the interaction for the epibiontic bacterium.

Several approaches were used to isolate the epibiont in pure culture, unsuccessfully. This report describes the rationale for undertaking the project and the approaches used to isolate the epibiont, in the hopes that this will be useful information for the future isolation of an axenic epibiont culture.

Rationale

Cyanobacterial associations with heterotrophic bacteria from environmental isolates have been described before and have been implicated in increasing the growth of the cyanobacterium (5, 6, 7). Benefit to the cyanobacterium may be derived from a range of associations, including the presence of heterotrophic bacteria in the culture medium, attraction of such bacteria to cyanobacterial secretions (either along the whole filament, or secretions stemming from the heterocyst in filamentous bacteria), or the attachment of the bacterium to the cyanobacterium outer surface (5, 6, 7, 9).

The attachment of bacteria to the outer surface, and the specific attachment to heterocysts to the exclusion of vegetative cells, is very interesting as the closeness and specificity of the interaction indicates that it may have been selected for over time, with a benefit to one or both partners. The specificity of epibiont attachment is not under question, as the epibiont has not been observed to bind to vegetative cells. Importantly, the epibiontic bacterium was shown to associate de novo to heterocysts that are very likely to be non-metabolizing (10). The issue regarding this two-species interaction is whether the specificity is based on metabolic benefits in addition to the specific attachment. The epibiont side of the interaction, while more likely to involve benefits than the cyanobacterial side (i.e., a parasitic interaction is more likely for the epibiont than for the cyanobacterium), has not been investigated and was the focus of this proposal.

A specific association of epibiont to heterocyst based on metabolism is not hard to envision, as heterocyst cells are physiologically distinct from vegetative cells. Heterocysts are the site of nitrogen fixation in filamentous cyanobacteria that harbor them (3). Moreover, they are thought to have evolved as a result of selection for a specialized low-oxygen environment for nitrogen fixation, an extremely oxygen-sensitive process. One of these specializations is a thick cell wall, with a specific lipopolysaccharide (LPS) on the surface of the cell, which can serve as a site for attachment and as a source of nutrients for the epibiont (3). The Anabaena LPS contains glucose, galactose and mannose (12).

Nitrogen fixation in the heterocyst provides other possible nutrients for the epibiont. First, ammonia is generated, which through a series of intermediates (e.g., glutamine) is converted to phycocyanin. Phycocyanin is a polypeptide made up of repeating units of arginine and aspartate, which serves for nitrogen storage and transport. At the ends of the heterocyst, the degradation products of phycocyanin, arginine and aspartate, are found. Second, nitrogenase produces as a co-product molecular hydrogen, which is an attractive electron donor for many bacteria, and could be used by the epibiont. Importantly, there is some preliminary evidence that hydrogen may play a role in the Rhizobium-legume symbiosis. Many such symbioses harbor Rhizobium strains that are defective in uptake hydrogenase (HUP), an enzyme that oxidizes hydrogen with energy conservation, and hydrogen is found to leak out of the nodules (2, 11). We currently don't know whether the School Street Marsh Anabaena sp. has HUP, and if it does, how much of the molecular hydrogen is metabolized by this enzyme.

Based on the above considerations, the following two major experimental approaches were designed to address the potential benefits for the epibiont of the association with the cyanobacterium:

1. Determine growth requirements of epibiont
Currently, the epibiont has only been grown in complex rich media (MP media, see recipe below). One approach is to test different sources of carbon and nitrogen, in the presence or absence of hydrogen gas, and measure biomass accumulation. A second approach is to do chemotaxis assays to analyze epibiont chemoattraction to different sources of carbon and nitrogen, and to hydrogen gas.

2. Quantitative electron microscopic studies of de novo association and dissociation between epibiont and Anabaena sp.
For the association experiments, the two axenic cultures will be mixed in different media, and the following parameters measured over time using electron microscopy: a) the number of heterocysts harboring epibionts, b) the number of epibionts attached to each heterocyst, c) the number of epibionts not attached to the Anabaena sp., and d) whether any vegetative cells have epibionts attached to them. For the disassociation experiments, a growing two-membered culture will be monitored over time for the association between epibionts and newly formed heterocysts in various media. The prediction is that if the epibiont attaches to obtain hydrogen and/or carbon sources from the cyanobacterium, it will not attach when these are available in the environment.

SCANNING ELECTRON MICROSCOPY

1. Cyanobacterial filaments were placed onto a glass slide with appropriate medium, and the clumps were teased apart gently and placed onto a 0.2 μm filter.
2. Samples were fixed for 4 hours in 2% glutaraldehyde, 1.5% formaldehyde solution.
3. Samples were dehydrated with 2,2-dimethoxypropane (only one was sufficient) and kept in absolute ethanol overnight.
4. The next day, the samples were dried using a critical-point drier, mounted on an SEM stub and sputter-coated, and stored in a dessicator.
5. SEM was performed on a JEOL JSM-840 scanning electron microscope.

Figures 1-4 show some of the photomicrographs obtained. Figure 1 shows a cyanobacterial clump. Figures 2-4 are close-ups of single filaments with heterocysts and attached epibionts. Note the range in length of the filaments from ~1 to ~8 μm.

APPROACHES FOR THE ISOLATION OF AN EPIBIONT CULTURE

Media
Both liquid and solid media was used for the purifications. The first medium described below is for culture of cyanobacteria; the other media are heterotrophic media used for the isolation of the epibiont. All recipes are for 1 L of medium. All media used had 25% seawater, which is the same salinity as the media used for the two-member culture. All solid media were with 1% agar.

BOX and BNAX media
These are the media that John Waterbury routinely uses in his lab for culture of cyanobacteria. Since I was using modular media (different combinations), I made stock solutions and would mix them as desired, which is a different approach from how this media is typically prepared. There is no need to adjust pH, as the seawater acts as a very efficient buffer.

	BOX (ml)	BNAX (ml)	Cyano trace metals (g/L)	
ddH$_2$0	750	750	ZnS0$_4$x7H$_2$0	0.222
Filtered seawater	250	250	MnCl$_2$x4H$_2$0	1.4
20 mM Na$_2$C0$_3$	0.1	0.1	Co(N0$_3$)$_2$x6H$_2$0	0.025
20 mM K$_2$HP0$_4$	0.1	0.1	Na$_2$MoO$_4$x2H$_2$0	0.39
200 mM Na$_2$N0$_3$	--	1.0	Citric Acid hydrate	6.25
200 mM NH$_4$Cl	--	0.1	Ferric Ammonium	
Cyano trace metals	0.5	0.5	Citrate (brown)	6.0
0.1 mM EDTA (disodium)	0.5	0.5		

Oligotrophic CYPS medium
Many bacteria grow optimally under limiting amounts of nutrients. I also thought that this media could provide a way to promote the growth of the epibiont and discourage the growth of the contaminating bacteria (see below, "Major problem: culture contamination"). The CYPS medium is commonly used by Jeanne Pointdexter for the growth of oligotrophic marine organisms.

Casamino acids	0.05%	Peptone	0.05%
Yeast extract	0.05%	Seawater	25%

Marine Purity medium

This is the medium that was used successfully for the purification of the epibiont (10). Please note that this medium does not necessarily have the optimal concentrations and combinations of nutrients for epibiont growth, as indicated by the slow growth of the epibiont under these conditions (10, John Waterbury, personal communication).

	MP medium (g)	
NaCl	20	Make separately NaCl/AC broth, $MgSO_4$, and $CaCl_2$ solutions
AC broth*	17	with dH_2O, autoclave, cool down to $<65^0C$, and mix.
$MgSO_4x7H_2O$	8	
$CaCl_2x2H_2O$	0.6	

*The manual directions for AC broth (Difco) is 34 g/L. Brad Stevenson (10) used 17 g/L. I used both 17g/L and 8.5 g/L.

Other media combinations

Other combinations of oligotrophic media were also used to better define the growth requirements of the epibiontic bacterium and to provide (as above) a selection against the contaminating bacteria. The major principle was to use a base medium and provide different carbon sources:

Base medium	Carbon sources (additional)
BOX medium components	All at 0.05%
0.05% peptone	Mannose, malate, glucose, galactose, cellobiose, acetate, succinate

Combinations
Base medium + one carbon source

Other culture parameters

Cyanobacterial cultures were grown in a 30^0C incubator with a 12 h light/12 h dark cycle, with no shaking. Previous studies clearly indicate that the epibiont is an aerobic organism (4, 10). Thus, to provide sufficient oxygenation for the isolation of the epibiont, liquid cultures were grown on the lab bench with shaking at 100 rpm. Cultivation on solid media was in the dark, in a drawer in the lab room.

Major problem: culture contamination

The two-member culture of Anabaena sp. and epibiont was found to harbor other bacteria, which were also heterotrophic organisms that grow in the presence of oxygen. Such community associations between different bacteria in culture are not unusual and have the potential to improve the growth of the bacteria.

Two factors exacerbated the problem. First, the contaminating organisms are very fast growers. Second, some (and potentially most/all) of the bacteria were found to be closely associated with the dense cyanobacterial clumps that are typical of the Anabaena sp. when it is grown without shaking.

Approaches to handle culture contamination
After washing using one or a combination of the below approaches, the cultures were either streaked onto plates or plated after serial limiting dilution down to 10^{-7} or 10^{-8}. For liquid incubations a small volume (typically 5 µl, and up to 100 µl) was inoculated into 5 ml of media.

Single filament wash
This is a standard approach used for the purification of filamentous cyanobacteria. A single cyanobacterial filament is washed in small volumes (~200 µl) in wells on a plate with 9 wells by transferring from well to well using a micropipette. All materials were kindly provided by John Waterbury (WHOI, Woods Hole).

This technique requires some skill in order to avoid losing the filament within the wash volume or breaking the filament. Initially, I would wash several single filaments at a time to prevent these problems, but it is preferable to handle a single filament only to reduce carry-over of the contaminating bacteria.

Large volume wash
This approach was reported for isolation of epibiont cultures in the literature (5). Cyanobacterial filaments (e.g., a small clump) would be washed 3 times in 50 ml of 25% seawater.

Filtering
Cyanobacterial filaments (after one large volume wash as above) were filtered through a 2 µm filter and washed with 150 ml of 25% seawater. Attached cyanobacterial filaments were resuspended into 25 ml of 25% seawater. Only cultures that had been stirred (see below) were used.

Stirring of cyanobacterial cultures
Cyanobacterial cultures are typically grown without any agitation. A culture was incubated with a magnetic stir bar and stirred at a moderate setting of 4. As a result, larger clumps were found to disassociate into smaller clumps. All clumps appeared much looser. Microscopically, many epibiontic bacteria were found disassociated within the matrix of the clumps.

Blending of cyanobacterial cultures
Cyanobacterial filaments (after one large volume wash as above) were blended for 20 minutes in a household blender in a volume of 20 ml of 25% seawater. Only cultures that had been stirred (as above) were used. Microscopically, no disassociation of the epibionts could be detected, though this was analysis was not quantitative and the large media volume may have prevented detecting dissociated epibionts.

Approaches for epibiont enrichment
A heterocyst-enrichment procedure (10) was used to increase the proportion of epibionts relative to the contaminating bacteria. Separation of the epibionts from the filaments was attempted with 2 mM EDTA for 5, 10, 15, and 20' and immediate plating onto solid media. This did not result in epibiont-containing colonies.

Isolation approaches – summary and discussion

The single filament wash is a standard technique that is routinely used to isolate single filaments and to purify filaments from other bacteria. It requires some time, as the filament is inoculated onto a plate, within a few days single hormogonia are isolated and put into liquid media. Within one or two weeks (due to the slow growth of cyanobacteria), a pure culture can be obtained. A variation of this technique was to incubate the purified two-membered filaments with purified Anabaena filaments that do not have attached epibionts, and wait for colonization of heterocysts by the epibiont. Upon skilful application of this technique and when enough time is available, these approaches will yield a purified culture containing only Anabaena sp. filaments with attached epibionts. This is the approach that should be given highest priority.

The other approaches are less likely to be successful. Even a combination approach of consecutive wash-stir-wash-filter-blend procedure, followed by either a) limiting dilution of supernatant and plating on solid media or inoculating liquid media or b) streaking of filaments, yielded a large number of colonies of the contaminating organisms. Tom Schmidt has suggested an alternative approach, the separation of contaminating bacteria from the two-membered filaments using Percoll gradients. However, this approach is not very likely to be successful in the presence of contaminating bacteria within the clumps of filaments, even after intensive washing, unless the media gives strong preference for epibiontic growth.

Another suggestion by Tom Schmidt is to use a high-throughput screen of colonies, such as an epibiont-specific PCR screen, rather than a microscopic screen based on cell morphology under 400 and 1000 magnification. This method should undoubtedly be used to screen in the future, as it allows for fast and, importantly, comprehensive screening of all colonies. Another important argument for the use of a PCR screen is that culture conditions may affect the appearance of the epibiont, as has been shown for other organism.

It is important to note that the lack of such a PCR screen in this work is unlikely to be the reason for the failure to identify the epibiont. First, previous isolations showed that the epibiont grows slowly in MP media, one of the major isolation media used in this work. The contaminating bacteria formed very large colonies within 1 or 2 days of inoculation. Second, a large number of colonies were screened using microscopy, and the predominating colonies of very distinct morphologies (with little square patches on top, yellow, pink), were always found to harbor the same morphological types (size and shape). Third, no colonies were ever found to harbor epibiont-looking organisms, even in mixture with other bacteria. Fourth, the appearance of the epibiont in MP media (albeit liquid) from previous isolations remained the same to the attached bacterium (10).

The previous successful cultivations of the epibiont involved only growth in liquid cultures, and streaking onto solid media, even after isolation of the epibiont in liquid culture, was either not attempted or not remembered. Therefore, there is no indication that the epibiont can grow well or at all on solid medium. Therefore, liquid media techniques should be preferntially used for isolation of an epibiont culture.

There is no doubt that a pure culture of the epibiontic bacterium that is specifically associated with heterocysts of the Anabaena sp. from School Street Marsh can be obtained. Many exciting studies of the nature of the relationship between these two closely associated organisms are to come!

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