Microbes need their vitamins too: The role of vitamin B12 in microbial light-sensing

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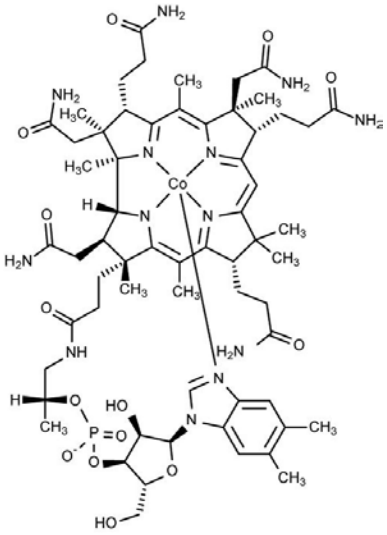
Microbial Diversity Course 2017



Research Rationale

Microorganisms must be able to mitigate the harmful effects of light in order to be successful. Photooxidative stress produces reactive oxygen species that greatly damage living cells (Ziegelhoffer & Donohue, 2009), requiring that microorganisms are able to sense light and orchestrate the expression of 'microbial sunscreens', such as carotenoids (Armstrong, 1997) and mycosporine-like amino acids (MAAs). Microorganisms use photoreceptor proteins with light-absorbing molecules (chromophores) to detect light in the visible and UV wavelengths (Purcell & Crosson, 2008; van der Horst et al., 2007). A class of photoreceptors utilising vitamin B12 (cobalamin) as a chromophore to sense light and mediate the transcription of carotenoids has been characterised in *Thermus themophilus* and *Myxococcus xanthus* (Burchard & Dworkin, 1966; Ortiz-Guerrero et al., 2011; Elias-Arnaz et al., 2008). In these microorganisms the production of CarH, a transcriptional repressor of carotenoid synthesis, is dependent upon the presence of adenosylcobalmin (Perez-Marin et al) for oligomerisation (Fig.1). The microbial utilization of cobalamin as a chromophore in CarH like proteins has been identified in various bacterial genomes spanning across several taxological groups (Ortiz-Guerrero et al., 2011). The presence of CarH-enconding genes in many microorganisms suggests that microbial photo-sensing may play a key role in the biogeochemical cycling of vitamin B12, which is often a limiting nutrient in the environment. However, CarH expression, and it's dependence on B12 availability, has not been characterised in natural environments exposed to high levels of sunlight. This study aims to characterize CarH expression both

the environment and pure culture, with emphasis on the role of vitamin B12 in these processes. To this end, the following research questions will be addressed:

1. Does the transcription of carotenoid genes repressed by CarH , *carA*, (Fig.1) increase in response to incremental increases in sunlight exposure duration in a pure culture of *Myxococcus xanthus*? Does carotenoid transcription depend on vitamin B12 availability?
2. Are CarH encoding genes present in environments exposed to high levels of sunlight?

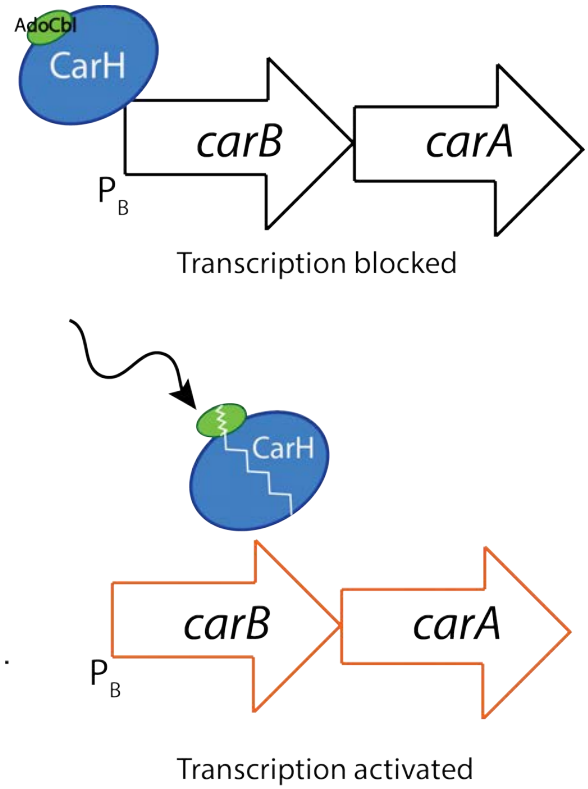


Figure 1. CarH as a transcriptional regulator. A) In the dark, the N-terminus of the CarH tetramer binds to DNA, preventing the carotenoid synthesis through transcription of *carB* and *carA* operons. B) Upon exposure to blue, green or UV light, the Co-C bond of AdoCbl is cleaved, and the tetramer dissembles – causing CarH to detach from DNA and allowing transcription to ensue.

Methods & Materials

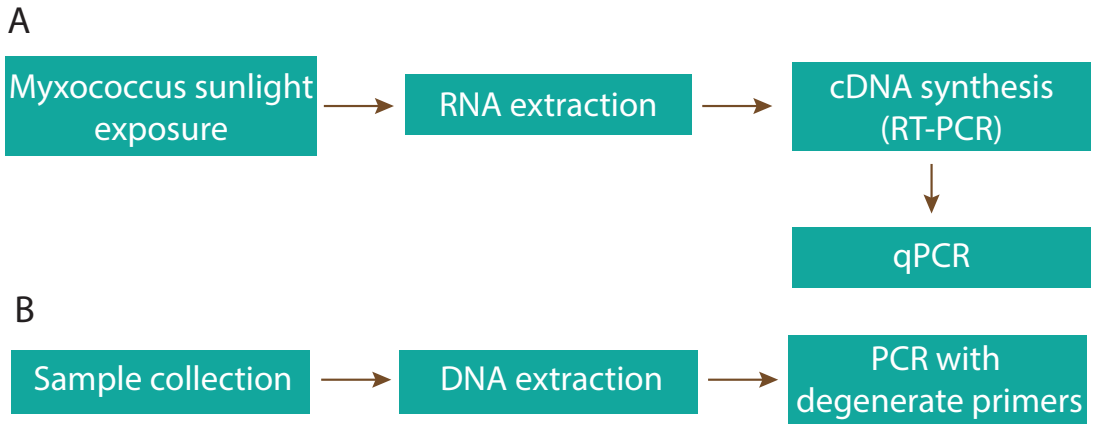


Figure 2. Schematic of experimental protocols used to address research question 1 (A) and 2 (B).

Bacterial strains and growth conditions. *Myxococcus xanthus* strain DK101, herein referred to as *M.xanthus* (Chen et al., 1991) was grown on 1% Casitone-Tris (CTT) medium supplemented with and without 1µM vitamin B12 (Ortiz-Guerrero et al., 2011). Cultures were allowed to grow for 48 hours at 30°C in dark conditions prior to any sun exposure.

RNA extraction following sun exposure. Following 48 hours incubation, pure culture plates of *M.xanthus* were exposed to direct sunlight for either 0,5,15,30 or 60 minutes duration (Table 2). All experiments were kept in the same temperature conditions throughout the experiment duration of 1 hour (28°C). RNA of colonies scraped from plates was immediately extracted using a Maxwell® Instrument with a 16 LEV simplyRNA Purification kit. RNA was quantified using Thermo NanoDrop™ One^{c} microvolume UV-Vis Spectrophotometer.

***carA* primer design.** Specific primers designed to amplify the *carA* gene (Table 1) of *M.xanthus* were designed from sequence data downloaded from NCBI using Primer3Plus (Untergasser et al., 2012).

Reverse-Transcriptase qPCR

Promega GoTaq® 2-step Reverse Transcriptase Real-Time quantitative PCR (RT-qPCR) System was used to quantify transcription of *carA* in cultures exposed to varying durations of sunlight.

Standard calibration curve. Standards for qPCR were generated by extracting DNA (see following protocol) from non-exposed colonies. DNA was quantified using Thermo NanoDrop™ One^{c} microvolume UV-Vis Spectrophotometer following purification and copy number was calculated using EndMemo online calculator (2017).

cDNA synthesis. cDNA synthesis using GoScript™ Reverse Transcriptase was performed using the following protocol: 25°C for 5 minutes, 42°C for 60 minutes and finally 70°C for 15 minutes.

Real-Time PCR. Real-Time PCR of cDNA (quantities of 1ng and 10ng) was performed with GoTaq® qPCR Master Mix and *carA* specific primers on QuantStudio 5 Real-Time PCR system. qPCR conditions were as follows: 95°C for 2 mins, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

16s rRNA PCR amplification. Total cDNA and RNA from *M.xanthus* was amplified using 16S rRNA specific primers. The following PCR protocol was used: 95°C for 2 minutes, followed by 30 cycles consisting of 95°C for 30s, 55°C for 30 s, 72°C for 90s and finally 72°C for 15 minutes.

DNA extraction from sediment. Unconsolidated surface sediment was collected from Great Sippewissett Salt Marsh, Falmouth, MA (41.58,-70.644). Sediment to 1cm depth was collected in an area exposed to fluctuating tides and light levels. Sediment was predominantly mud and silt sized (<0.2µM). Genomic DNA was extracted from samples using QIAamp PowerFecal DNA Kit, followed by quantification using a Promega Quantus™ flourometer.

Protein modeling of CarH-like proteins. Degenerate primers were designed to amplify CarH-like protein encoding genes from environmental samples, with emphasis on proteins with tertiary structures that are able to accommodate vitamin B12 as a chromophore. NCBI Protein BLAST search results with a percentage identity match to *M.xanthus* exceeding 40% were initially selected for 3D structure analysis. *M.xanthus* was used rather than *Thermus thermophilis (*herein referred to as *T.thermophilis)* to increase likelihood of capturing mesophilic CarH like proteins, however tertiary protein modeling was performed using *T.thermophilis* as this is a resolved protein structure (Jost et al., 2015). The 3D structure of selected proteins were modeled using MPI bioinformatics toolkit (Alva et al., 2016) using FASTA sequences downloaded from UniProt. *T.thermophilis* CarH was used for alignment, to check whether proteins could likely house a cobalamin molecule (Fig.3). The top 12 proteins (Table 1) with the lowest root mean square distances (RMSD) of atomic positions (<0.4) when aligned to *T.thermophilis* were selected to be used in primer design.

Degenerate Primer Design. Sequences were aligned using MUltiple Sequence Comparison by Log- Expectation (MUSCLE) in JalView software (Waterhouse et al., 2009). Primers designed are shown in Table 1.

PCR amplification and gel electrophoresis. Degenerate primer products were amplified using thermal gradient (45-65°C) PCR with the following conditions: 95°C for 2

minutes, followed by 30 cycles of 45-65°C for 30s, 72°C for 90 s and finally 72°C for 10 minutes.

	Myxococcus *carA* primers	**Degenerate primers**	**Organism**	**RMSD**
	5' – 3' sequence	5' – 3' sequence	*Myxococcus xanthus*	0.235
1	TGCTGGATGACGCACAGAC	ATGGCNGARNGNMCNAC	*Myxococcus fulvus*	0.312
	CCAGCTCTTCCGATGACTCC	ATHNGNGCNTGGGAR	*Myxococcus hansupus*	0.387
2	CCATCGCCCAGGTGAAGA	TGYTTYCCNGARGAR	*Myxococcus stipitatus*	0.399
	CGAGCGAGAAAATGGCCTTC	YTCYTCNGGRAARCA	*Stigmatella erecta*	0.322
3	CTGGAAGGGGACGAGGTGA	GCNGCNCCNCCNACCCA	*Hyalangium minutum*	0.231
	GCGAGAAAATGGCCTTCAGG		*Cystobacter fuscus*	0.255
4	ACGCACAGACCGTCATGG		*Cystobacter ferrugineus*	0.218
	CCACCAGCTCTTCCGATGAC		*Archangium viola*	0.231
			Archangium sp.Cb.G35	0.389
			Archangium gephyra	0.249
			Corallococcus coralloide	0.355

Table 1. *carA* specific primers used in qPCR and degenerate primers used for environmental samples. Organism CarH sequences used in degenerate primer design and root mean square distances of atomic position values (RSMD) of modeled CarH tertiary structures aligned to *T.thermus* CarH.

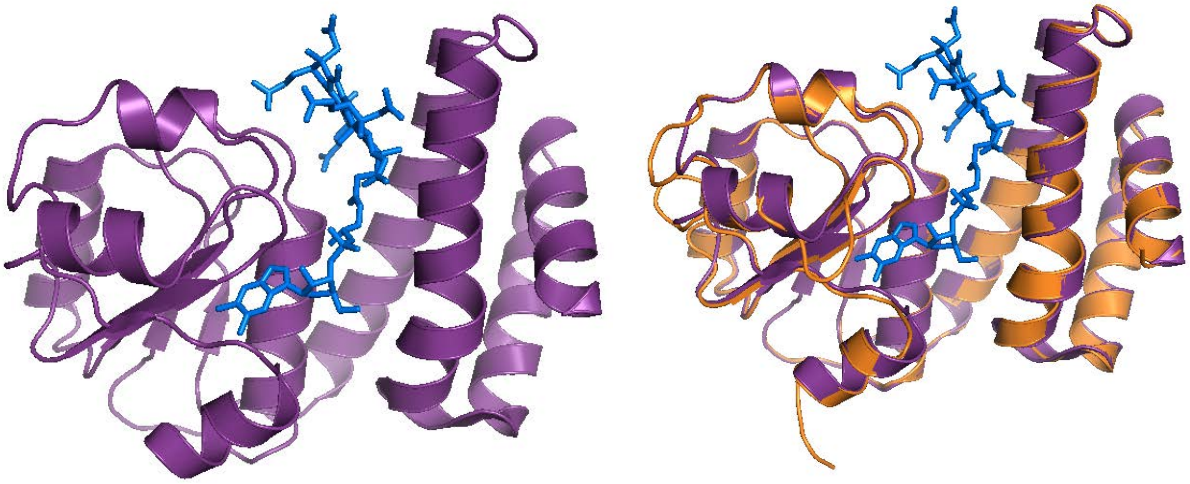


Figure 3. A) Resolved tertiary structure of CarH of *Thermus thermophilis* showing cobalamin in blue (Jost et al., 2015). B) MPI Bioinformatics toolkit modeled *M.xanthus* CarH (orange) aligned with known *T.thermophilis* CarH structure (purple).

Results & Discussion

Sunlight exposure experiments

Only one primer set was successful in amplifying the *carA* gene from a pure culture of *Myxococcus xanthus* (Fig.4A & B), so was therefore chosen as the primer set for the qPCR reaction. Following exposure to light, colonies did not display any noticeable colour change from yellow to white (Fig.5), indicative of enhanced carotenoid production, as have been reported in other studies (Ortiz-Guerrero et al., 2011).

RNA extraction following sunlight exposure of cultures was successful, with RNA yields between 4-6ng/µl (Table 2). RT-qPCR indicates a high level of amplification following 30 cycles (Fig.6), shown by a rapid increase in fluorescence signal (ΔRn). However, this likely represents amplification of non-specific genetic material, rather than of the *carA* gene which was the focus of this study. It is not immediately clear whether lack of *carA* specific amplification is due a low signal of *carA* transcription, as a result of low UV exposure, or whether a component of RT-qPCR failed. Unfortunately, the instrument failed to run the standards which would help clarify this. To determine whether the standards had successfully amplified, the qPCR product of standards was visualized on a 1% agarose gel (Fig.4C), indicating that amplification of *carA* was successful through Real-Time PCR. To determine whether lack of sample amplification was due to cDNA failure, 16s rRNA PCR amplification of the initial RNA and cDNA was performed. No amplification of genetic material was seen (Fig.4D), indicating that cDNA synthesis from extracted RNA was unsuccessful.

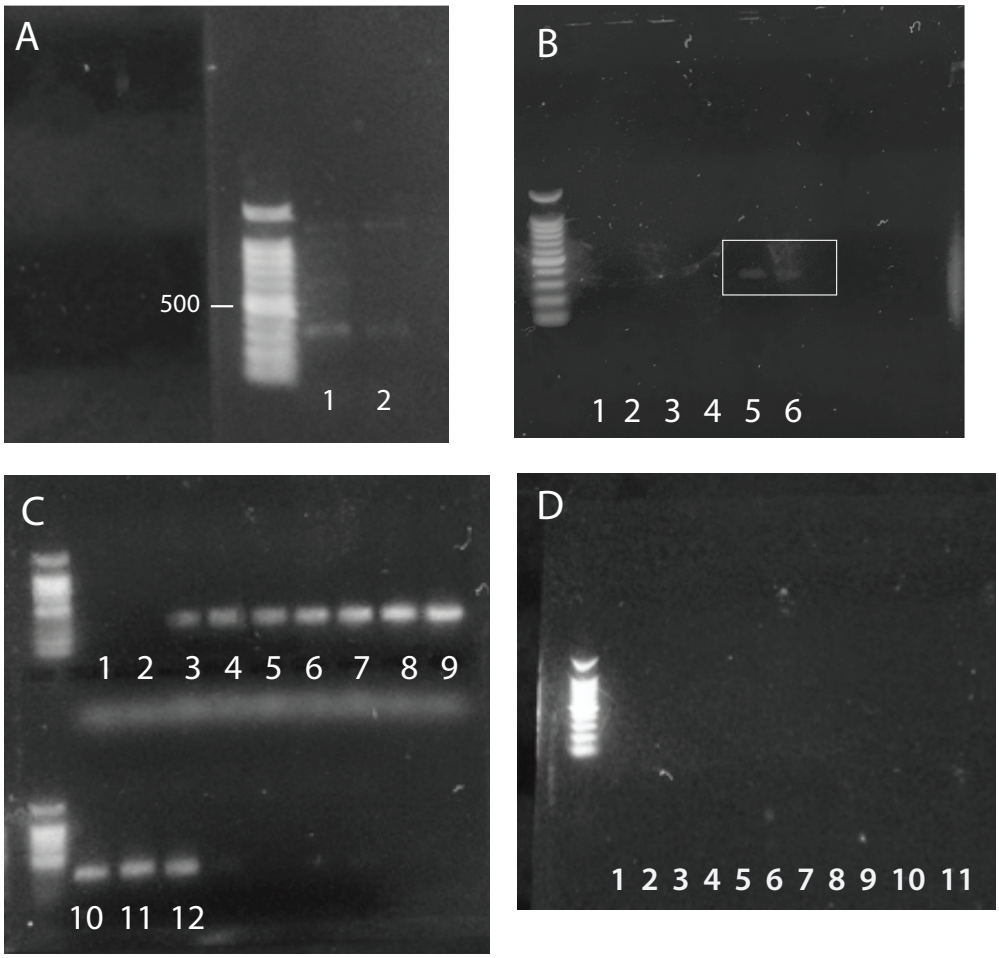


Figure 4. A) 1% agarose gel visualization of PCR primer set 3 (Table 1) amplified product of *M.xanthus*, in duplicate (shown alongside a 100bp ladder) B) Lanes 1 & 2 show primer set 1, lanes 2 & 4 show primer set 2, lanes 5 & show primer set 3 (100bp ladder). C) qPCR standards showing 10-part dilution series of *carA* from original DNA extraction (lane 12) which contains 4.59 x 10^{10}copies of *carA*. Ladder shown is 100bp. D) PCR product using CarH degenerate primers showing unsuccessful amplification.

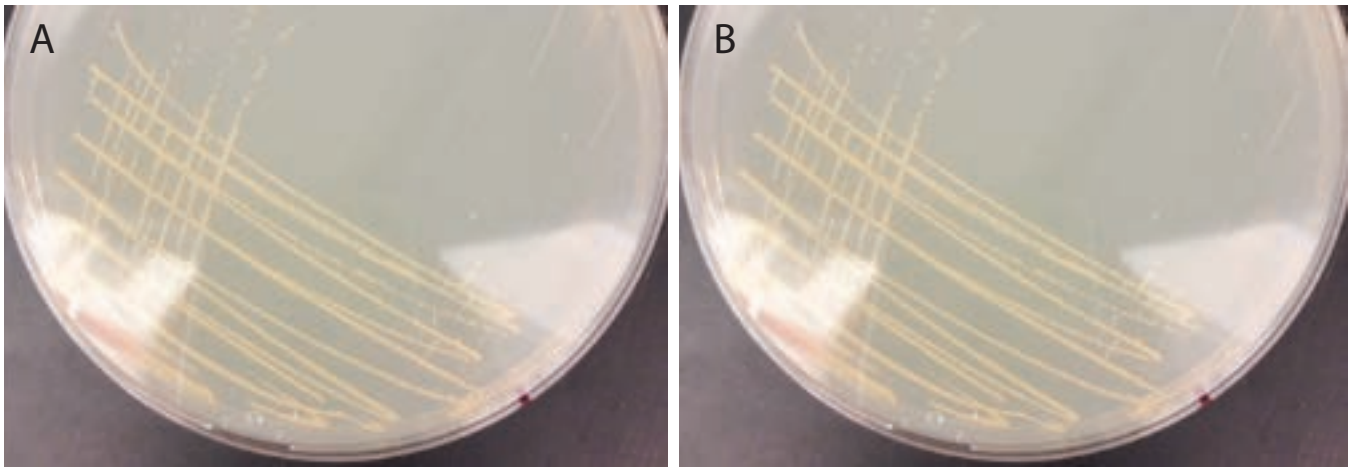


Figure 5. Streaked plates of *M.xanthus* prior to sun exposure (A) and following sun exposure (B).

Sample	RNA (ng/µl)	Sample	RNA (ng/µl)
B12- 0 mins	4.9	No B12- 0 mins	4.9
B12 – 5 mins	4.5	No B12 – 5 mins	4.4
B12 – 15 mins	5.0	No B12 – 15 mins	3.8
B12 – 30 mins	6.9	No B12 – 30 mins	4.8
B12 – 1 hour	4.3	No B12 – 1 hour	4.0

Table 2. Sunlight exposure experimental times and vitamin B12 conditions. B12 supplemented experiments have 1µM vitamin B12.

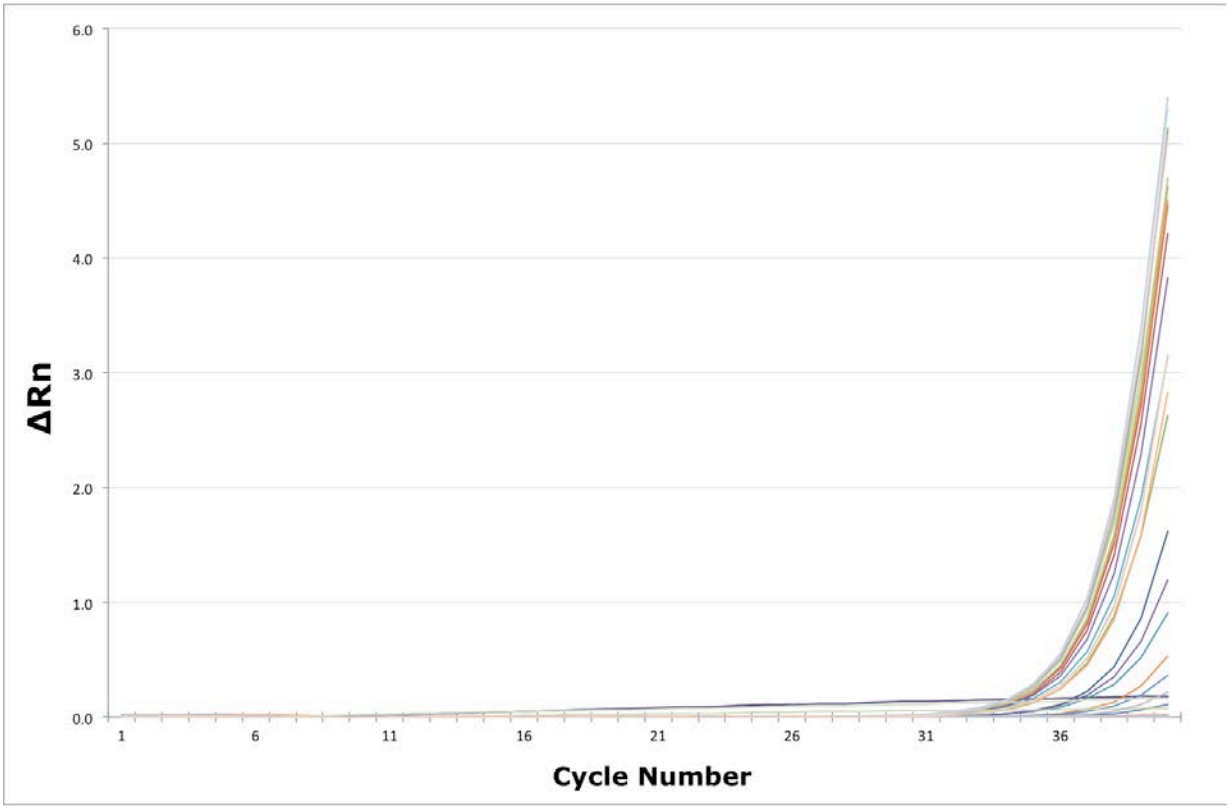


Figure 6. qPCR amplification plot showing late amplification of non-specific genetic material.

CarH in environmental samples

Degenerate primers were designed in order to amplify genes of encoding for CarH-type proteins from a natural environmental sample, using tertiary protein modeling to select proteins that could likely accommodate vitamin B12 as a chromophore. Genomic DNA extraction yielded 112ng/µl of DNA from Sippewissett Salt Marsh sediments. However, the primer failed to amplify genes of interest. This does not indicate lack of CarH in the natural environment, but rather unsuccessful annealing, as the positive control failed to show amplification of the CarH encoding gene. The amino acid sequence of CarH is highly rich in serine, arginine and leucine, resulting in difficulty in designing specific primers as these are very degenerate amino acids.

Concluding remarks

This project aimed to explore whether CarH like proteins are pervasive in the environment and to characterise the role of vitamin B12 and sunlight in the expression of the *carA* operon which is modulated by CarH. The lack of specificity in primer design and the failure to synthesise cDNA preclude answering the questions set out by this project. Future work should focus on a more sophisticated primer design approach, employing more sequences to ensure that a robust, specific primer was designed. Given the wish to amplify a long RNA sequence, it would be prudent to also increase the RNA incubation time with retrotranscriptase to ensure that cDNA sythnesis is successful.

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