

## UV absorbing bacteria in coral mucus and their response to simulated temperature elevations

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### Abstract

Reef building corals encompass various strategies to defend against harmful ultraviolet (UV) radiation. Coral mucus contains UV absorbing compounds and has rich prokaryotic diversity associated with it. In this study, we isolated and characterized the UV absorbing bacteria from the mucus of the corals *Porites lutea* and *Acropora hyacinthus* during the pre-summer and summer seasons. A total of 17 UV absorbing bacteria were isolated and sequenced. The UV absorbing bacteria showed UV absorption at wavelengths ranging from  $\lambda_{\max} = 333$  nm to  $\lambda_{\min} = 208$ nm. Analysis of the DNA sequences revealed that the majority of the UV absorbing bacteria belonged to the family *Firmicutes* and the remaining belonged to the family *Proteobacteria* (class *Gammaproteobacteria*). Comparison of the sequences with the curated database yielded four distinct bacterial groups belonging to the genus *Bacillus*, *Staphylococcus*, *Salinicoccus* and *Vibrio*. The absorption peaks for the UV absorbing bacteria shifted to the UV-A range (320nm-400nm) when they were incubated at higher temperatures. Deciphering the complex relationship between corals and their associated bacteria will help us to understand their adaptive strategies to various stresses.

## **Introduction**

Coral reefs are unparalleled in terms of both prokaryotic and eukaryotic diversity. Bacteria associated with corals occupy different niches including the surface mucus layer (Ritchie and Smith 2004), tissues (Ceh et al. 2011) and skeleton (Sweet et al. 2011a). The immense diversity of bacteria associated with different species of corals was reported (Rohwer et al. 2001, 2002; Koren and Rosenberg 2006; Bourne and Munn 2005). It was observed that coral associated bacteria were species specific regardless of their geographical location (Rohwer et al. 2001; Bourne and Munn 2005). The rich species diversity of microbes on the surface of corals can be attributed to the presence of a surface mucus layer, with the composition of mucus being species specific for each coral (Meikle et al. 1988). The mucus has the ability to trap particulate organic matter from sea water and serves as an energy rich substrate for the heterotrophic activity of bacteria (Wild et al. 2004). The mucus also aids the corals in coping up with excessive sediment stress (Hubbard and Pocock 1972) and protects them from fouling (Ducklow and Mitchell 1979).

The nutrient rich coral mucus supports the growth of both beneficial and pathogenic bacteria, yet little is known about the microbial population associated with corals and their pathogenicity. The microbial communities associated with corals exhibit antimicrobial properties and potentially protect corals from pathogens by preventing their colonization via competition for space on the reef and deterring their growth by releasing antimicrobial compounds (Nissimov et al. 2008; Orland and Kushmaro 2009; Rypien et al. 2010; Orland et al. 2012). Raina et al. (2009) emphasized the role of microbial communities in the biogeochemical cycling of sulfur and stated that dimethylsulfoniopropionate (DMSP) and dimethylsulfide (DMS) are potential nutrient sources for coral associated bacteria. Shashar et al. (1994) reported the presence of nitrogen fixing bacteria in the coral skeleton playing an important role in the nitrogen recycling in the reef ecosystem. Coral probiotic hypothesis suggested that the coral holobiont could shift as an adaptive strategy of corals to the changing environmental conditions (Reshef et al. 2006). Recent research on coral associated bacteria suggested two contradictory scenarios following a stress event. The first is about irreversible replacement of the surface microbial biota with potential pathogens that makes the coral deprived of antimicrobial properties associated with the original microbial community. This shuffling causes disease in corals (Jones et al. 2010). The second, proposed by Garren et al. (2009) suggests that the microbial biota replaced with pathogens due to a stress is reversible in due course of time irrespective of the subsidence

of the stress and mere presence of pathogens do not cause disease in corals. This was supported by experimental evidence by Sweet et al. (2011b), in which an adjusted coral microbial community was restored back to its original composition after a time period.

Ultraviolet (UV) radiation is one of the most dominant stressor to a variety of marine organisms, affecting their health and function of the marine ecosystems (Hader et al. 2011). UV radiation acts synergistically with elevated sea water temperature to cause coral bleaching (Lesser and Farrell 2004). UV-A radiation influences the productivity of marine ecosystems by affecting interactions between organisms at different trophic levels (Ochs and Eddy 1998). Most organisms synthesize UV absorbing compounds along with other UV protective mechanisms like photo reactivation and other repair mechanisms (Hader et al. 2007; Rastogi and Sinha 2011; Bhatia et al. 2011). Certain reef building corals possess UV absorbing compounds such as mycosporine like amino acids (MAA) that include mycosporine, palythine and palythanol which protect them from harmful UV rays (Dunlap and Chalker 1986). MAA was originally isolated from cyanobacteria and also synthesized by certain phytoplankton groups and macro algae (Hader et al. 2011). In addition to MAA, the calcium carbonate skeleton of corals also absorbs harmful UV radiation and emits it as yellow fluorescence, significantly reducing the tissue damage (Reef et al. 2009). However, the concentration of UV absorbing compounds decreases during a sharp rise in temperature, which in turn increases the risk of the harmful effects of UV radiation to both corals and zooxanthellae (Lesser et al. 1990). Corals get external protection from harmful UV rays by the presence of chromophoric dissolved organic matter (CDOM) in overlaying waters which absorbs harmful UV radiation, however, bleaching of the CDOM exposes corals to the harmful UV rays (Anderson et al. 2001).

In this study, UV absorbing bacteria associated with mucus of the massive coral *Porites lutea* and the table coral *Acropora hyacinthus* were isolated, identified and characterized phylogenetically. Their *in-vitro* UV absorbing property under elevated temperature conditions was also investigated.

## **Materials and methods**

### **Description of the study site**

Mucus was collected from the corals in Palk Bay, located on the south east coast of India (Fig. 1). Palk Bay is a shallow basin with an average depth of 9 meters and the reef here occurs at a depth of

1-4 meters. The reef itself is narrow and runs parallel to the land lying between longitudes 79° 17' 40" E and 79° 8' E and latitude 9° 17' N in an east-west direction. It is composed largely of massive boulder corals belonging to the genus *Porites*, *Favia*, *Favites*, *Platygyra*, *Symphillia* intermittent with sandy patches. In addition, few colonies of *Acropora sp.* can be found distributed in the reef crest.

### **Sample collection and isolation of bacteria**

Mucus samples were collected from *Porites lutea* and *Acropora hyacinthus* during pre-summer (March) and summer seasons (May) in 2010 using sterile syringes at a depth of 3 meters and 4 meters respectively. The syringes were immediately transported in ice to the laboratory under aseptic condition within 2 hours of collection.

The mucus samples were serially diluted (10 fold dilution) using filter sterilized and autoclaved seawater, plated on ZoBell marine agar plates and incubated at 28°C for 48 hours. After incubation, the microbial population of each plate was enumerated by counting the number of individual colonies grown using a colony counter and the number of colonies was expressed as cfu/ml of mucus. Colonies with unique morphology were further purified by quadrant streaking onto fresh nutrient agar plates prepared using aged seawater.

### **Screening of UV absorbing bacteria**

Bacterial isolates with unique morphology were inoculated individually into test tubes containing 50ml nutrient broth prepared in aged sea water. The broth was incubated for 24 hours at room temperature in an orbital shaker. After incubation, the cultures were scanned in the UV range (200nm-400nm) with a double beam UV-Visible spectrophotometer (Shimadzu UV-VIS 2550, Japan) using a 5cm path quartz cuvette against sterile nutrient broth as a reference. The isolates that showed an absorbance peak between 200nm-400nm were selected for further studies. Each isolate was given a unique code based on the time of collection (pre-summer and summer), coral species and the number of the isolate for the ease of presenting the data. For example : PSPL1 meant Presummer *P. lutea* isolate 1, PSAH1 -Presummer *A. hyacinthus* isolate 1, SPL1- Summer *P. lutea* isolate 1, SPH1- Summer *A. hyacinthus* isolate 1.

## **Effect of temperature on UV absorption**

The UV absorbing bacterial isolates were inoculated in the nutrient broth prepared in the aged seawater and incubated at a gradient of temperatures (28°C, 30°C, 32°C and 34° C) for a period of 24 hours. After incubation, the cultures were scanned between 200nm-400nm wavelength as described previously.

## **Genomic DNA extraction and 16S rRNA sequencing**

The isolates that showed UV absorption were subjected to identification using 16S rRNA sequencing. Genomic DNA was extracted from the cultures grown overnight following a modified phenol-chloroform method and the quality of the product was checked in 0.8% agarose gel. Gene amplification was done using the forward primer 27F (5'AGAGTTTGATCCTGG CTCAG 3'; Weisberg et al. 1991), and reverse primer 1492 R (5'GGTTACCTTACGACTT 3'; Reysenbach et al. 1992). PCR was done in a thermocycler (Applied Biosystems, USA) using the following cyclic program : initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were purified using Poly ethylene glycol precipitation as described by Liss (1980). The purified PCR products were further extended using the 27 F and 1492 R primers in a thermocycler using the following program: Initial denaturation at 96°C for 1 minute followed by 30 cycles of denaturation at 96°C for 10 seconds; annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes and final extension at 60°C for 1 minute. The final extension products were cleaned using the protocol described by Jaysankar and Ramaiah (2007). Sequencing was done using an ABI sequencer (ABI PRISOM model no. 3700). Forward and reverse sequences obtained were aligned using Sequencher V4.10.1. The sequences were then submitted in the GenBank. The accession numbers are GQ 281097 - –GQ 281107(Pre summer isolates) and KC431013 – KC431018 (summer isolates).

## **Phylogenetic characterization**

Aligned sequences were compared with the curated database of type strains of prokaryotes present in Eztaxon server (<http://www.eztaxon.org>; Chun et al. 2007) to determine approximate phylogenetic affiliations. 16S rRNA gene sequences were compiled and manually aligned using the ARB software package ([http:// www.arb-home.de](http://www.arb-home.de)) (Ludwig et al. 1998). Phylogenetic tree calculated

with 16S rRNA gene sequences for all target sequences and their close relatives using the neighbor-joining (Saitou and Nei 1987) method in ARB. The neighbor joining (NJ) tree topology was then evaluated by bootstrap analyses (Felsenstein 1985) based on 1000 re-samplings. The final phylogenetic tree was constructed using the MEGA5 (Tamura et al. 2011).

## Results

### Isolation and enumeration of bacteria

The culturable bacterial counts from the mucus sample of *P. lutea* and *A. hyacinthus* were  $4.1 \times 10^7$  cfu ml<sup>-1</sup> and  $1.2 \times 10^7$  cfu ml<sup>-1</sup> respectively for the pre-summer season. For the summer season, the culturable bacterial counts from the mucus sample of *P. lutea* and *A. hyacinthus* were  $7 \times 10^7$  cfu ml<sup>-1</sup> and  $2.13 \times 10^7$  cfu ml<sup>-1</sup> respectively. A total of 12 isolates from *P. lutea* and 31 isolates from *A. hyacinthus* were obtained during the pre-summer season. Subsequently, a total of 13 isolates from *P. lutea* and 20 isolates from *A. hyacinthus* were obtained during the summer season.

### UV absorbing bacteria

Of the total 43 isolates obtained from *P. lutea* and *A. hyacinthus* during pre-summer season, 6 isolates from *P. lutea* and 5 from *A. hyacinthus* exhibited UV absorbing property ( $\lambda_{\max} = 333$  nm; and  $\lambda_{\min} = 208$  nm respectively). Only 6 isolates out of a total of 33 isolates obtained during summer season exhibited UV absorbing property at a range of  $\lambda_{\max} = 282$  nm to  $\lambda_{\min} = 208$  nm (3 each *P. lutea* and *A. hyacinthus*).

### Phylogenetic analysis

The nearest Phylogenetic neighbors of the UV absorbing isolates were identified following BLAST analysis of 16S rRNA gene sequence. The DNA sequences exhibited <97% identity to the nearest Eztaxon entry. 16S rRNA sequence analysis of the UV absorbing isolates revealed the presence of two major bacterial groups. Majority of the UV absorbing isolates were of *Firmicutes* (class *Bacilli*) and the rest were of Phylum Proteobacteria (class *Gammaproteobacteria*) in both *P. lutea* and *A. hyacinthus*. Of the 11 UV absorbing isolates obtained during the pre-summer, 8 isolates PSPL 1, PSPL 3, PSPL 4, PSPL 5, PSPL 6, PSAH 1, PSAH 2 and PSAH 3 were clustered within the *Firmicutes* group (Fig. 2) comprising the genus *Bacillus* and the rest belonged to the *Gammaproteobacteria* comprising

the genus *Vibrio* (PSPL 2, PSAH 4 and PSAH 5). In contrast, all the UV absorbing bacterial isolates obtained during summer were clustered within *Firmicutes* group comprising three different genus namely *Bacillus* (SPL 2, SPL 3, SAH 1 and SAH 2), *Staphylococcus* (SPL 1) and *Salinicoccus* (SAH 3). To compare the levels of novelty with other bacterial communities, we assembled 16S rDNA libraries from previously published data for coral-associated bacteria. The Phylogenetic tree constructed to determine their affiliations (Fig 2) formed four groups. Group A represented *Bacillus* species, the most abundant bacterial species associated with the coral mucus examined in this study. Group B and C represented *Staphylococcus* and *Salinicoccus* respectively. The isolates related to *Bacillus kochii* (GQ281101; GQ281098; KC431016), *Bacillus gibsonii* (GQ281102) and *Bacillus oceanisediminis* (GQ281103; GQ281100) were not previously isolated from corals and this is the first report of the isolation of these species from corals. Group D represents *Vibrio* species, the second most abundant bacterial species associated with corals in the present study. The *Vibrio* sp. (GQ281099; GQ281105; GQ281106), reported in this study was 99% similar to *Vibrio owensii* (JX127215), which is reported to cause *Montipora* white syndrome in the Hawaiian reef coral *Montipora capitata* (Ushijima et al. 2012). Groups A, B and D were aligned with representatives of coral associated bacteria found from previous studies, whereas group C, was not, as there were no previous reports of these bacterial sp. associated with corals.

### **Effect of temperature on UV absorbance**

For UV absorbing isolates incubated at 28°C, peaks of absorption wavelength were below 300nm (ranging between 208nm to 282nm), with the exception of four isolates (PSPL 2, PSAH 4, SAH 1 and SAH 3) which showed absorption peaks above 300nm. Incubating all the isolates at higher temperatures triggered additional absorption peaks in the UV-A range ( $\lambda_{\max} = 367$  nm; and  $\lambda_{\min} = 317$ nm). The absorption wavelengths for the UV absorbing bacterial isolates during the pre-summer and summer season incubated at different temperature are presented in Figures 3 and 4 respectively.

### **Discussion**

The UV absorbing bacterial community isolated from the mucus of *P. lutea* and *A. hyacinthus* during the pre-summer and summer season was dominated by *Bacillus* and *Vibrio* species. When comparing the distribution of the UV absorbing bacterial population, *A. hyacinthus* showed a greater

microbial diversity than *P. lutea* in both the pre-summer and summer seasons. However the abundance of UV absorbing bacteria was more or less equal between the two species.

Shallow water corals receive higher amounts of UV radiation than those found at greater depths especially during summer seasons and they showed to possess MAAs to help protect them from UV rays. Shick et al. (1996) reported that the coral surface mucus layer offers protection against UV rays, as it consists of MAAs secreted by the algal symbionts. In addition, we reported the presence of UV absorbing bacteria in the coral mucus, which has the potential to play an important role in protecting the corals from UV radiation.

The number of UV absorbing bacteria isolated was significantly low relative to the other heterotrophic bacteria in coral mucus during the summer season for both *A. hyacinthus* and *P. lutea* as compared to the pre-summer season. This could potentially be attributed to the rise in sea water temperatures. When the corals were under thermal stress, the population of algal symbionts was significantly reduced which subsequently affected mucus composition and secretion (Brown and Bythell 2005). Also, Ritchie and Smith (1995) stated that the chemical nature and quantity of mucus could change when the coral was stressed. The bacterial communities outcompete one over the other during the incidence of high temperature and bleaching (Ritchie et al. 1994). In our study, the change in the composition of mucus (especially during an increase in sea water temperature), would have favored other heterotrophic bacteria to outcompete the UV absorbing bacteria associated with the coral mucus. Currently, nothing is known about the UV absorbing properties and numerical abundance of UV absorbing bacteria within the un-cultured mucus associated bacterial population and this should be the focus of the next area of research on this topic. Rising incubation temperatures for the UV isolates from 28°C to 34°C shifted the wave length of UV absorption to a higher range. This is of particular importance, as Gleason and Wellington (1993) state that UV-A radiation at the wavelength band of 280nm-400nm is one of the major contributing factors in coral bleaching. Prevalence of these UV absorbing bacteria during an episode of brief warming period could be an indication of the adaptive mechanism in the corals to shuffle and proliferate a particular group of bacteria that can absorb these lethal UV radiations.

It is not known whether the UV absorbing property exhibited by the bacterial isolates from our study was due to the synthesis of UV absorbing compounds like MAAs. MAAs (Mycosporin like



Amino Acids) is a collective term given to a group of amino acids synthesized as secondary metabolites in the shikimic acid pathway involved in the synthesis of aromatic amino acids that protect aquatic organisms against harmful solar radiations. MAAs consists of cyclohexenone or cyclohexinimine chromophore conjugated with the nitrogen substituent of an aminoacid (Sinha et al. 1998). More than 20 different MAAs has been reported in various organisms each having their own absorption maxima due to the difference in attached side groups and nitrogen substituents (Singh et al. 2008). Dunlap and Shick (1998) reviewed the presence of MAAs in reef associated organisms that either synthesize their own MAAs or acquired them from their algal symbionts. Biosynthesis of MAAs via the shikimic acid pathway (Favre-Bonvin et al. 1987) was reported to be absent in the animal tissues (Bentley 1990) suggesting that zooxanthellae might be the source. Contrary to this, there are also reports of zooxanthellae not being the source of MAAs (Banaszak and Trench 1995; Ishikura et al. 1997). However, in the majority of the studies the same type of MAAs was isolated from both the zooxanthellae and host animal tissue indicating that they share this compound (Dunlap and Shick 1998).

In general, heterotrophic bacteria lack UV protective pigments and compounds (Karentz 1994), but Arai et al. (1992) isolated and characterized UV absorbing compounds from a marine bacterium *Micrococcus*. In the study presented here, bacteria isolated from coral mucus and cultured independently under laboratory conditions do exhibit UV absorbing properties, indicating the synthesis of UV absorbing compounds by the bacteria itself. When these UV absorbing bacterial isolates were incubated at temperatures higher than 28°C, additional absorption peaks appeared in the UV-A range (315nm - 400nm). This may be due to the synthesis of additional UV absorbing compounds as previously described in Cyanobacteria (Sinha et al. 2001) and in the marine macro algae *Chondrus crispus* (Karsten et al. 1998). The synthesis of UV-A absorbing compounds by these bacteria in response to elevated temperatures suggests that a rise in temperature could indirectly aid in diluting the effects of UV-A radiation on coral hosts.

In conclusion, this study suggests that the UV absorbing bacteria associated with the coral mucus might play an important role in protecting the corals during incidences of increased UV radiation. Unravelling the complex relationship between corals and their associated bacteria will help us in understanding the adaptive strategies corals and their associates use against various environmental stresses. Moreover, in this study, only culturable bacteria were shown to absorb UV radiation. Exploring

the uncultured bacteria associated with the coral mucus will show the actual magnitude of the UV protection rendered by these coral associated bacteria.

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## Figure legends

**Figure 1:** Sampling sites in Palk Bay, southeast coast of India. (ArcGIS v 9)

**Figure2:** Neighbor Joining (NJ) phylogenetic tree for 16S rRNA gene sequences showing the relationship between the coral associated bacterial strains (GENBANK sequences, in dark blue), UV absorbing bacterial isolates from this study [*Acropora hyacinthus* (green), *Porites lutea* (purple) and reference strains (black)]. The sequence of *Aquifex pyrophilus* (M83548) was used as an outgroup. Bootstrap values >50 are indicated at corresponding nodes. Sequence accession numbers are in parentheses.

**Figure 3:** Absorption wavelength of UV absorbing bacteria isolated during Presummer season in response to increasing temperature. With increase in temperature, additional absorption wavelength was observed in the UV A range.

**Figure 4:** Absorption wavelength of UV absorbing bacteria isolated during summer season in response to increasing temperature.

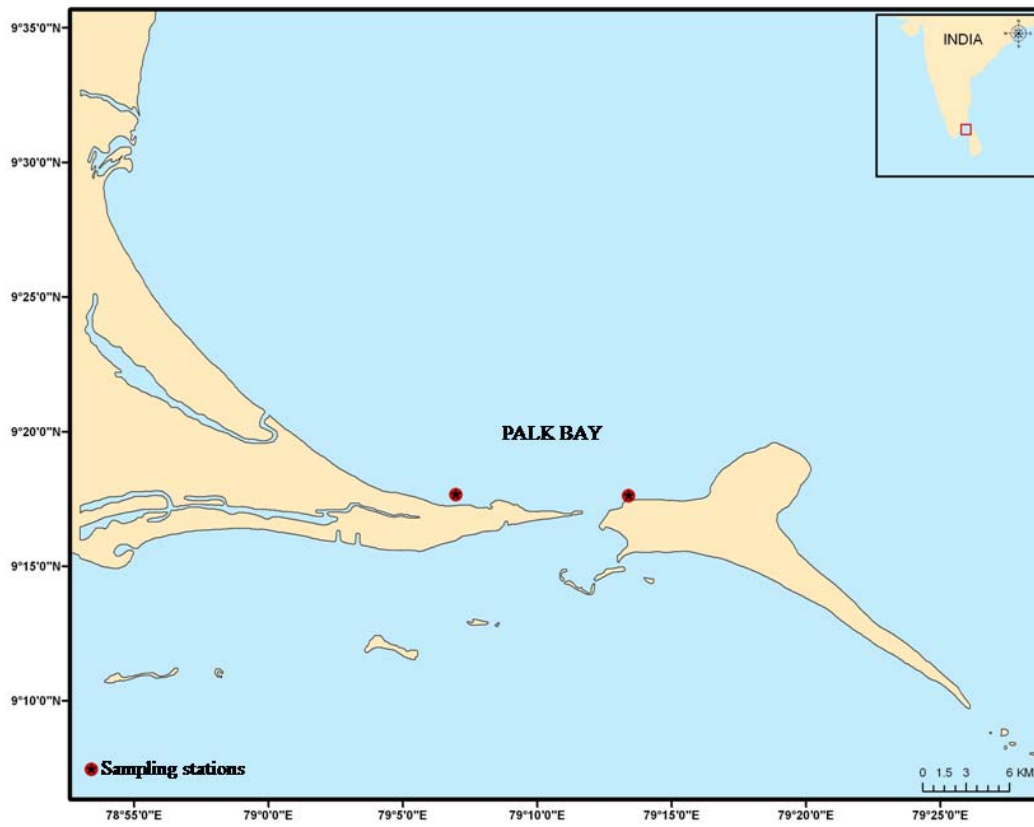


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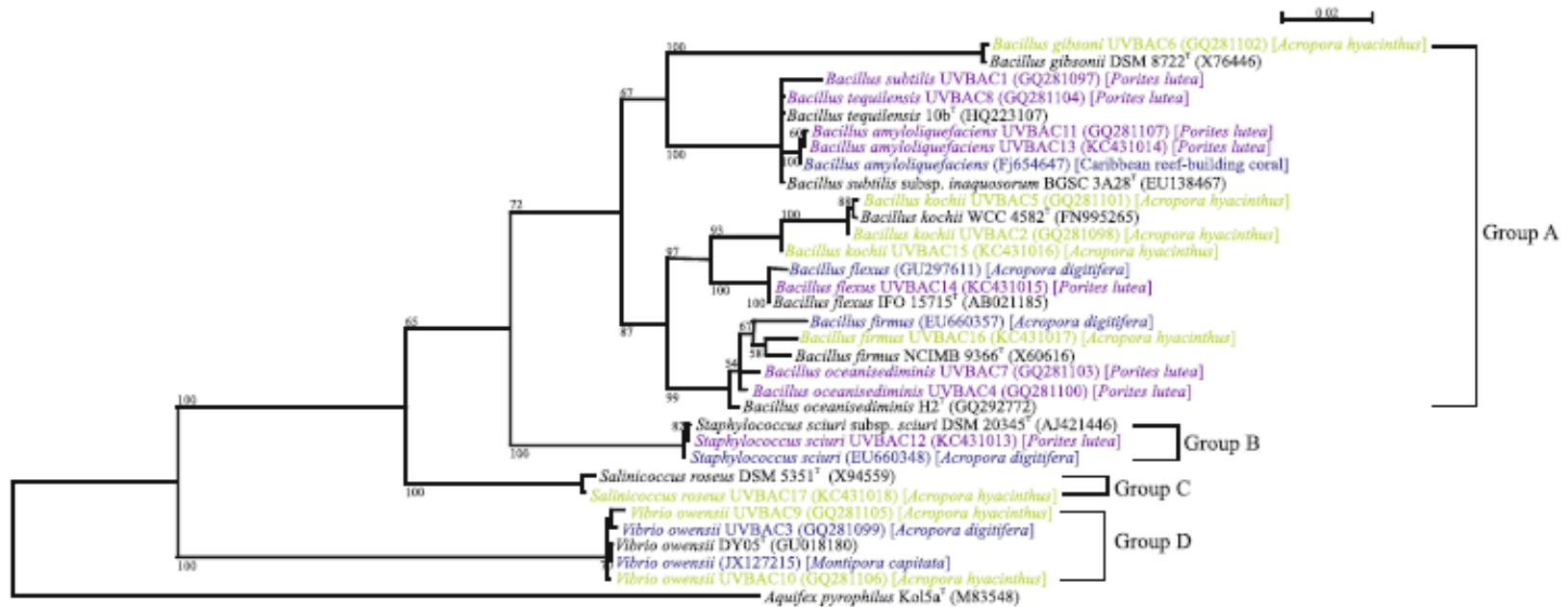


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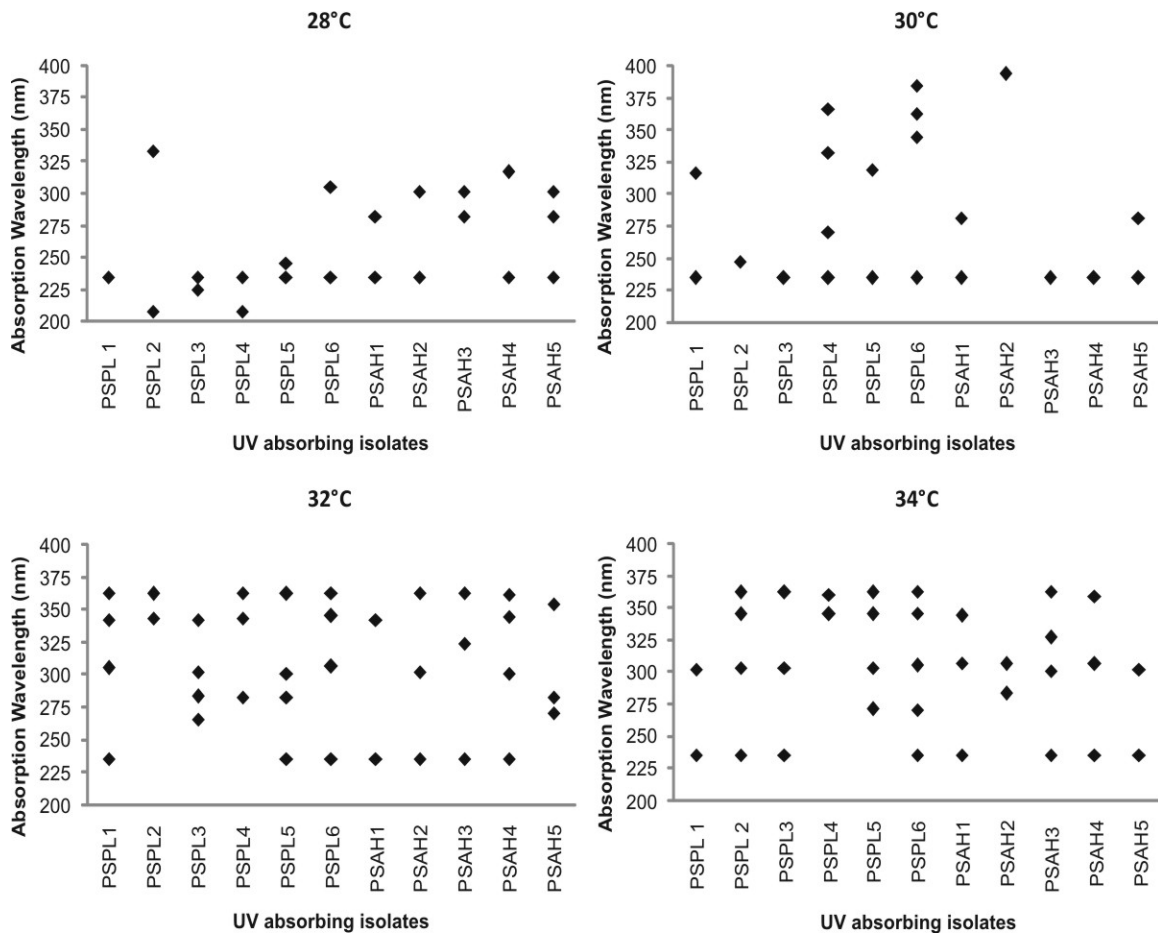


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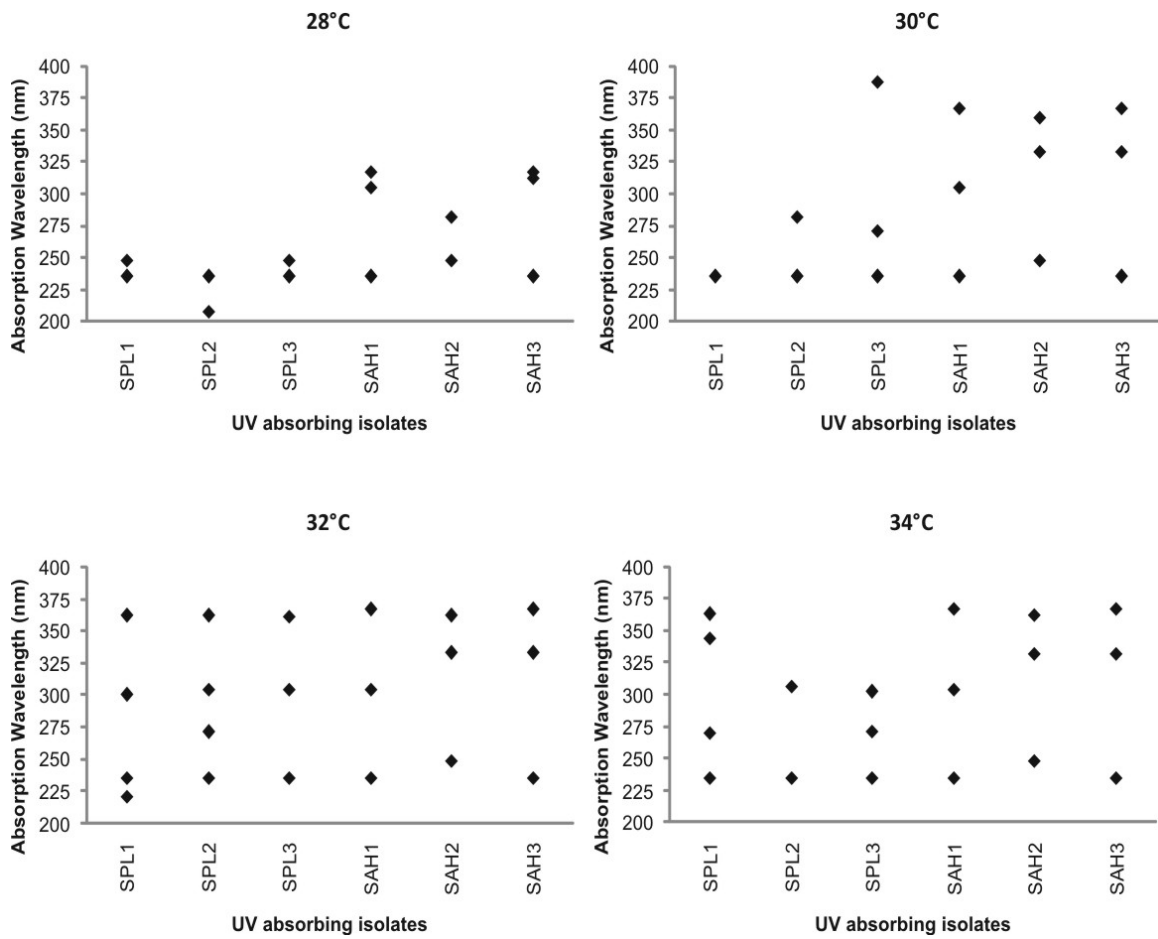


Figure 4: Absorption wavelength of UV absorbing bacteria isolated during summer season in response to increasing temperature