# Carotenoid expression in nonphotosynthetic bacteria isolated from Sippewissett marsh soil.

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## Abstract

Carotenoids comprise a major class of pigment molecules with a broad natural distribution from bacteria to higher plants. In this project I tried to examine carotenoid production in six nonphotosynthetic bacteria isolated from Sippewissett marsh soil. Pigment extraction and spectrophotometer analysis showed that these isolates contain, carotenoid pigments. The pigments of five of these strains were expressed only under aerobic conditions; one of them expressed its pigmentation both aerobically and anaerobically. Phylogenetic analysis of the different strains clustered them into three phylogenetic groups: Two of the strains fell into the gamma proteobacteria group both closely related to *Vibrio gazoganes*, two were found to belong to the *Flexibacter-Bacteroides-Cytophaga* phylum (one closely related to *Flectobacillus* sp. and the other to *Polaribacter glomeratus*), and the last sequenced isolate belonged to the gram-positive bacteria the *Bacillus* genera. I also attempted to generate a cartenoid biosynthesis mutant with trasposon mutagenesis, using the strains closely related to *Vibrio gazoganes* and the *Flectobacillus* sp. closely related strain. A few suspected transconjugants were found in the later strain, however only two showed a suspected mutation in carotenoid biosynthesis.

## Introduction

Carotenoids represent one of the most widely distributed and structurally diverse classes of natural pigments. They are important in photosynthesis, nutrition, and protection against photooxidative damage. Carotenoids can be found in photosynthetic organisms ranging from bacteria to higher plants. May nonphotosynthetic organisms including certain bacteria, fungi and red yeasts synthesize carotenoids. In mammals, certain dietary carotenoids such as β-carotene serve as the precursors for vitamin A, retinoic acid and retinal, and may offer protection against some types of cancer. It has been found that colored carotenoids protect against the damaging effect of excited state triplet photosesitizers, singlet oxygen and radicals by the virtue of their polyene chromophore. In photosynthetic organisms carotenoids are not only serving physical quenchers of excited state triplet chlorophyll, bacterial chlorophyll and oxygen radicals but also serve as the accessory light harvesting pigment (Gottschalk 1986).

Carotenoids are composed of isoprene subunits and are often termed isoprenoids. The yellow and red pigments of bacteria are composed of isoprene units. The branched isoprenoidsare that are commonly found in carotenoids of pigmented bacteria are synthesized from acetyl CoA and mevalonic acid (Schmidt 1978). The biosynthesis of carotenoids are catalyzed by a sequential array of enzymes and proceeded via a series of colored intermediates. Previous research has shown that some mutations in genes coding for the biosynthesis of carotenoids in a number of photosynthetic bacteria, such as the purple non-sulfur photosynthetic bacteria *Rhodobacter* (Armstrong et al 1990) result in novel color phenotypes. Thanks to this, many mutants can be identified by their pigmentation and genes responsible for these mutations can be easily cloned and identified (Page and Sockett 1999). Mutagenesis using transposon elements has been

widely used in *Rhodobacter* has generated, carotenoid biosynthetic mutants that are available for study (Armstrong et al 1990).

Most of the work done thus far was focused on carotenoids produced by photosynthetic bacteria. In this study, I have tried to characterize nonphtosynthetic carotenoid producing bacteria isolated from Sippewissett marsh soil. The characterization was done by means of their morphology, pigment expression, phylogenetic analysis, growth and pigment extraction under different growth conditions. I also tried to generate carotenoid biosynthesis mutants with transposon mutagenesis.

## Material and Methods

**Strain isolation.** Soil samples from Sippewissett marsh were homogenized by hand, suspended in seawater, diluted and inoculated on Marine agar (see below) plates. The plates were incubated at room temperature both aerobically and anaerobically for 1-2 days. Pigmented colonies were selected and subcultured for purification.

**Media and culture conditions**. *E.coli* strains were either grown in LB broth or LB agar (1.5% Difco agar). The marine strains were grown using Marine broth (Difco) or Marine Agar (Difco). Antibiotics were added to the LB medium and to the Marine medium in the following concentrations: ampicillin 50, 100, 150 ug/ml; naladixic acid 50, 100 ug/ml; chloramphenicol 25, 50, 100 ug/ml; kanamycine 30, 50, 100 ug/ml; tetracycline 10, 20 ug/ml; rifampicine 25, 50 ug/ml. the *E. coli* strains were incubated at 37°C, and liquid cultures were aerated by shaking at ~200 rpm. The anaerobic marine strains, which were inoculated on solid medium, were incubated at R.T. in the anaerobic chamber. Cultures

inoculated in liquid media were incubated in anaerobic tubes at 30°C. The aerobic marine strains were incubated at 30°C, and liquid cultures were aerated by shaking at ~200 rpm.

**Bacterial strains, plasmids and transposons.** The *E. coli* bacterial strains used in this project are listed in table 2. The marine bacterial strains used in this study are presented in tables 1 and 2.

**Phenotypic characterization.** The cells morphology and motility was examined microscopically (zeiss MC 80). Catalase activity was tested using 3% $H_2O_2$. Growth rate and antibiotic resistance (ampicillin; naladixic acid; chloramphenicol; kanamycine; tetracycline; rifampicine) was checked only in two strains.

**Phylogenetic analysis.** DNA from a single colony was extracted using the PrepMan™ kit (Applied Biosystems). The 16S-rDNA gene was amplified with a PCR with 8f and 1492r 16S rDNA primers. The PCR product was sequenced using a Didioxy Fluorescent Terminator Chemistry on an ABI 377 automated DNA sequencer.
BLAST was used to compare the sequencse obtained to previously sequenced 16S-rDNA genes available in the GenBank nucleotide library (U.S. National Institutes of Health Internet site). These Sequences were then aligned to their closest related sequences in the arb database. Phylogenetic relationships were reconstructed using the neighbor joining method, and the outgroup sequence was *E. coli*.

**Pigment extraction and characterization.** Total pigments were extracted from exponentially grown cells. 5 ml of the cells were pelleted by centrifugation and extracted two times with acetone and methanol 7:2 mixture. Extracts were centrifuged each time to

pellet the cellular debris. The extract absorbance was analyzed using a spectrophotometer (Carry 50) and then applied to a thin layer plate. The plate was developed in the dark using petroleum ether and acetone (9:1), the bands were marked and extracted from the silica gel using acetone and methanol 7:2 and analyzed by the spectrophotometer.

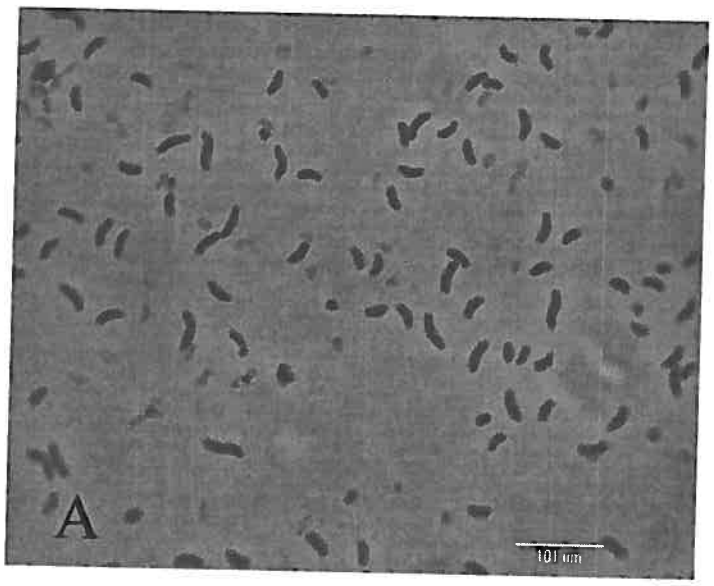
**Conjugation.** Mating of the bacterial strains was done on a sterile filter, which was placed on a solid medium plate. The mating was preformed by mixing equal volumes of early logarithmic phase cultures ($O.D._{600}$ is 0.3-0.2) of the donor and the recipient strains. The mating mix was incubated for 12-18hr at 37°C and 30°C for the E. coli and the marine recipient strains respectively. The filters were then resuspended in fresh medium and plated on appropriate selective media (Cooper et al 1997).

**Table 2**: bacteria strains and plasmids.

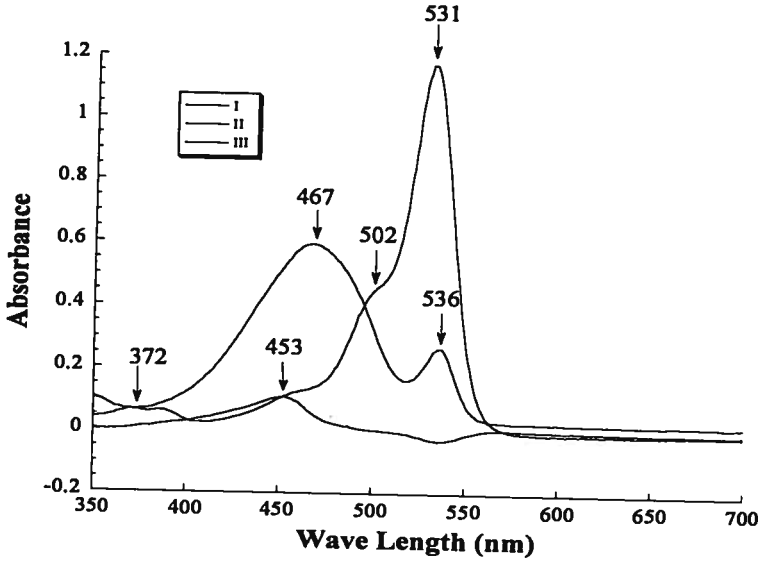
| **Strain** | **Genotype** | **Relevant phenotype** | **Reference** |
|---|---|---|---|
| Bacteria strains | | | |
| *E. coli* | | | |
| BW19857 | S17-1, RP4 uid::pir(R6K)$^+$ | | Metcalf et al 1994 |
| EM24NR | pir(R6K)$^-$ | $Rif^R$, $Nal^R$ | |
| UPA | wild type | red, $Tc^R$ | |
| XF2 | wild type | pink, $Kn^R$ | |
| plasmids | | | |
| pEP4351 | $Tc^R$, $Em^R$, $Cm^R$ (R751::Tn4351) | | Cooper et al 1997 |

Fig. 1

# Strain UPA



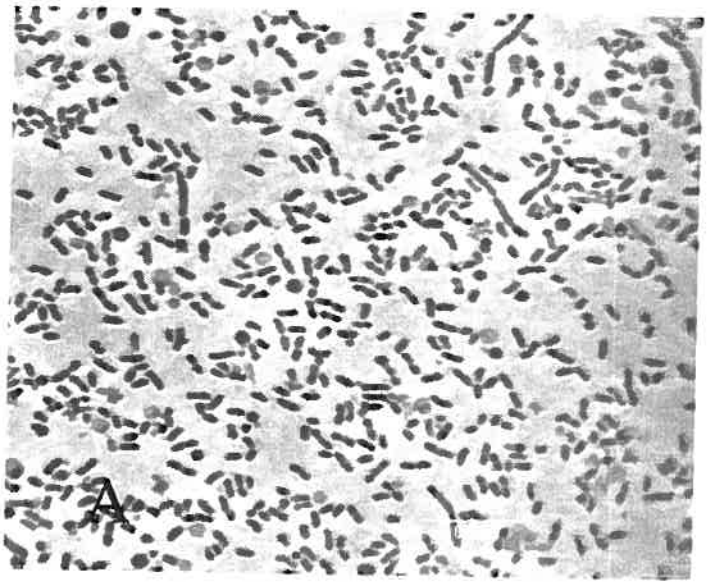

Specrum of strain UPA extraction



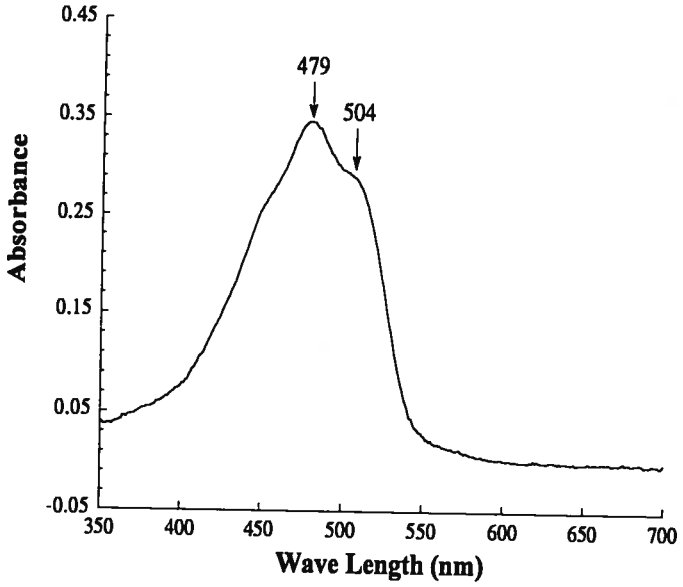

B

Fig 2.

Strain XF2



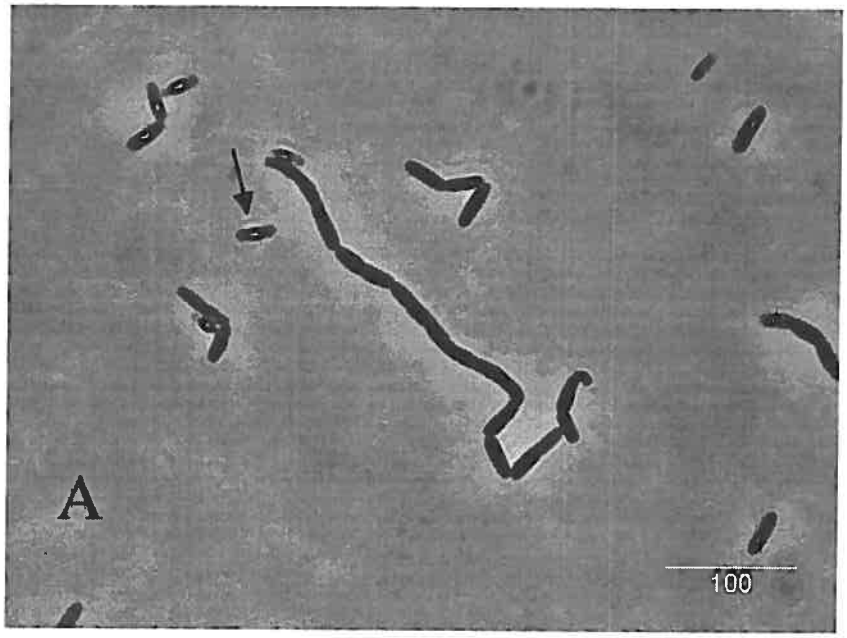

Spectrom of starin XF2 extraction.




B

Fig 3.

# Strain HF4

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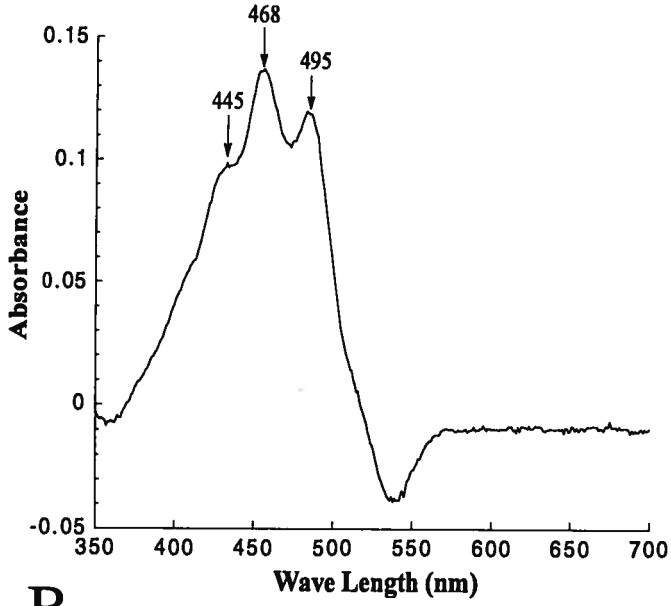
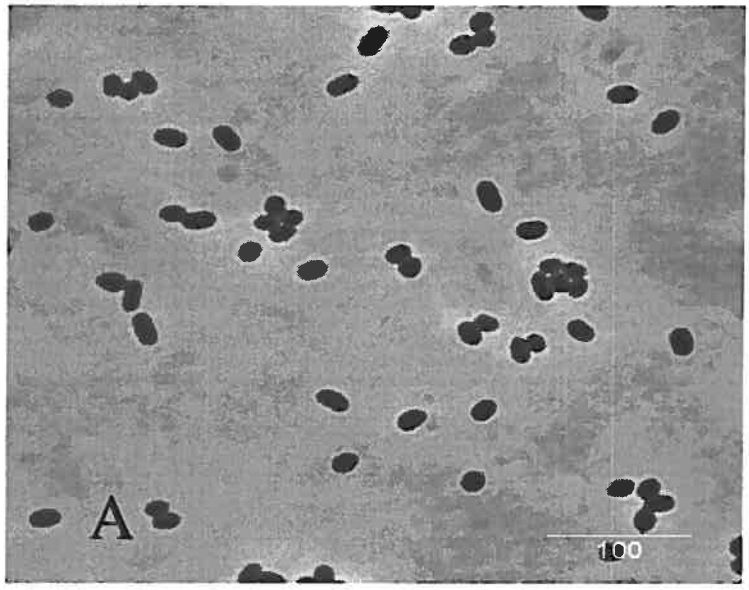



B

Fig 4.

# Strain HF6

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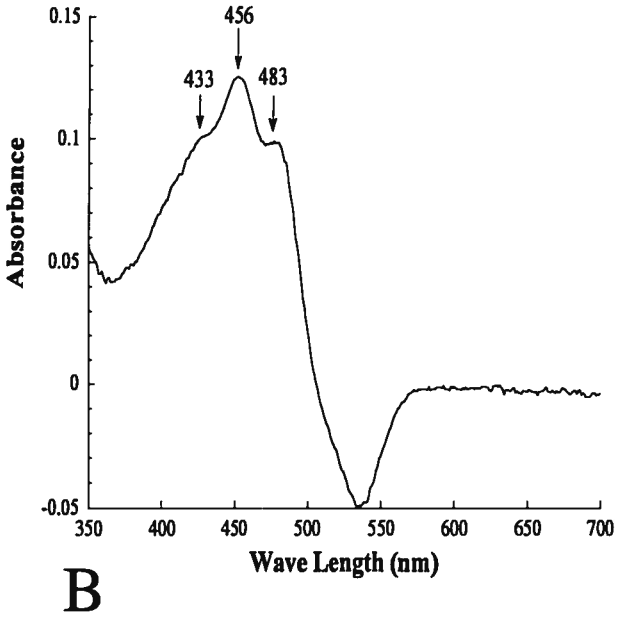
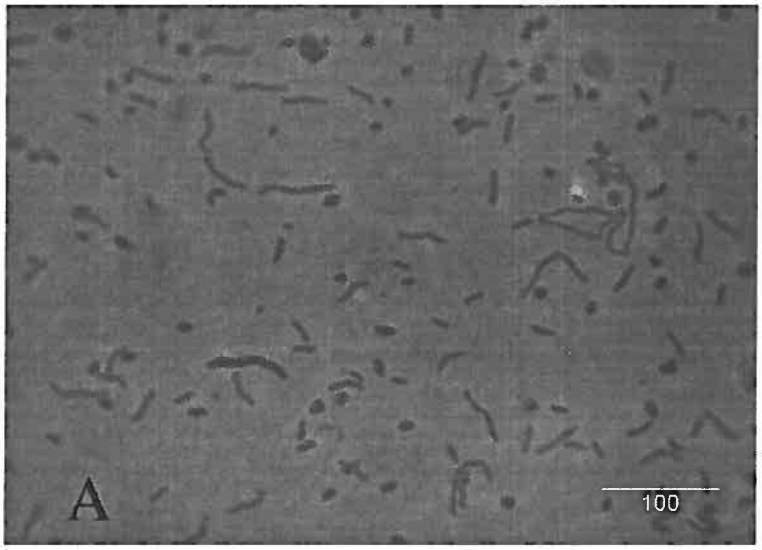



B

Fig 5.

# Strain UP4

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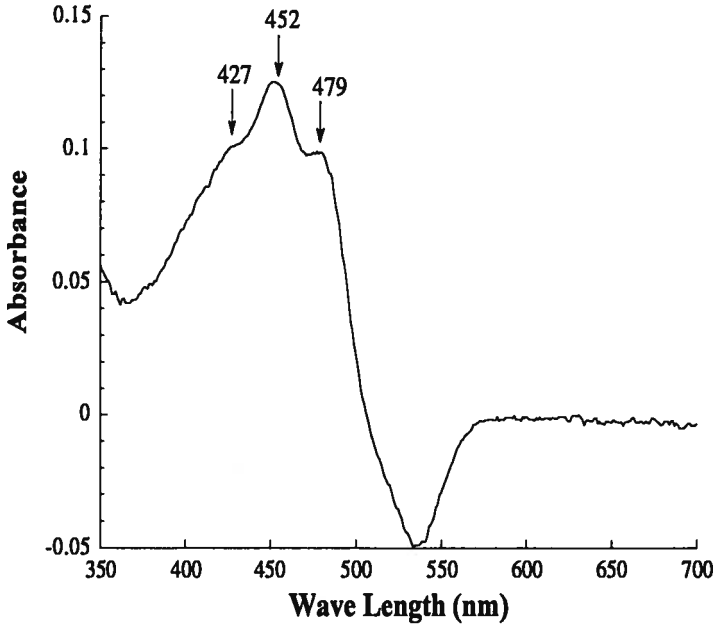


B

**Table 1**: charechterizations of strains isolated from the Sippewissett marsh soil.

| Strain | 16S rDNA identification (closest neighbor) | Sequence similarity | Phenotype | Pigment production (nm) | Facultative anaerobes | Anaerobic pigment production | Catalase activity | Reference |
|---|---|---|---|---|---|---|---|---|
| UPA | *Vibrio gazeganes* | 98% | red | 531 | + | - | + | Ruimy et al 1994 |
| C6 | *Vibrio gazeganes* | 97% | red | 532 | + | + | + | Ruimy et al 1994 |
| XF2 | *Flectobacillus* sp. | 93% | pink | 479 | + | - | + | Bowman et al 1997 |
| HF4 | *Bacillus* oleronius | 96% | pink | 468 | - | | + | Kuhnigk et al 1995 |
| HF6 | | | orange | 456 | + | - | + | |
| UP4 | *Polaribacter glomeratus* | 90% | yellow | 452 | - | | - | Gosink et al 1998 |

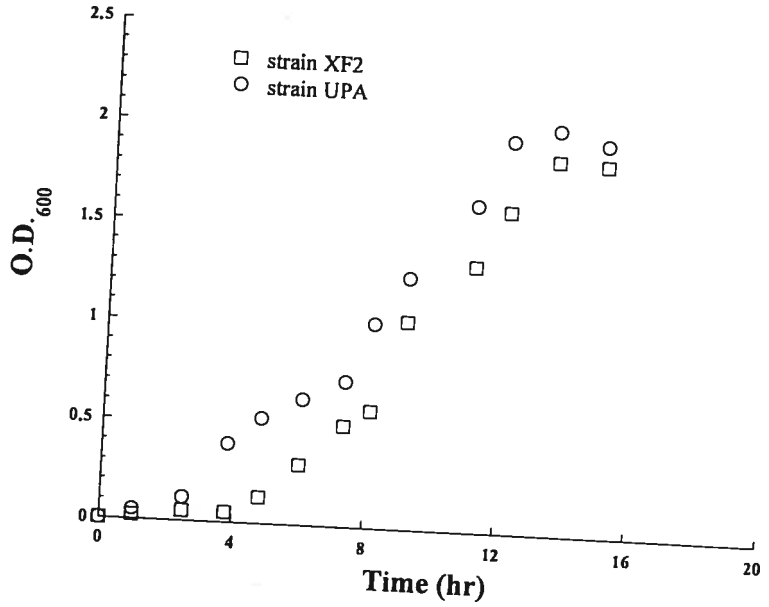


Fig 6. Growth curve of the strains UPA and XF2.

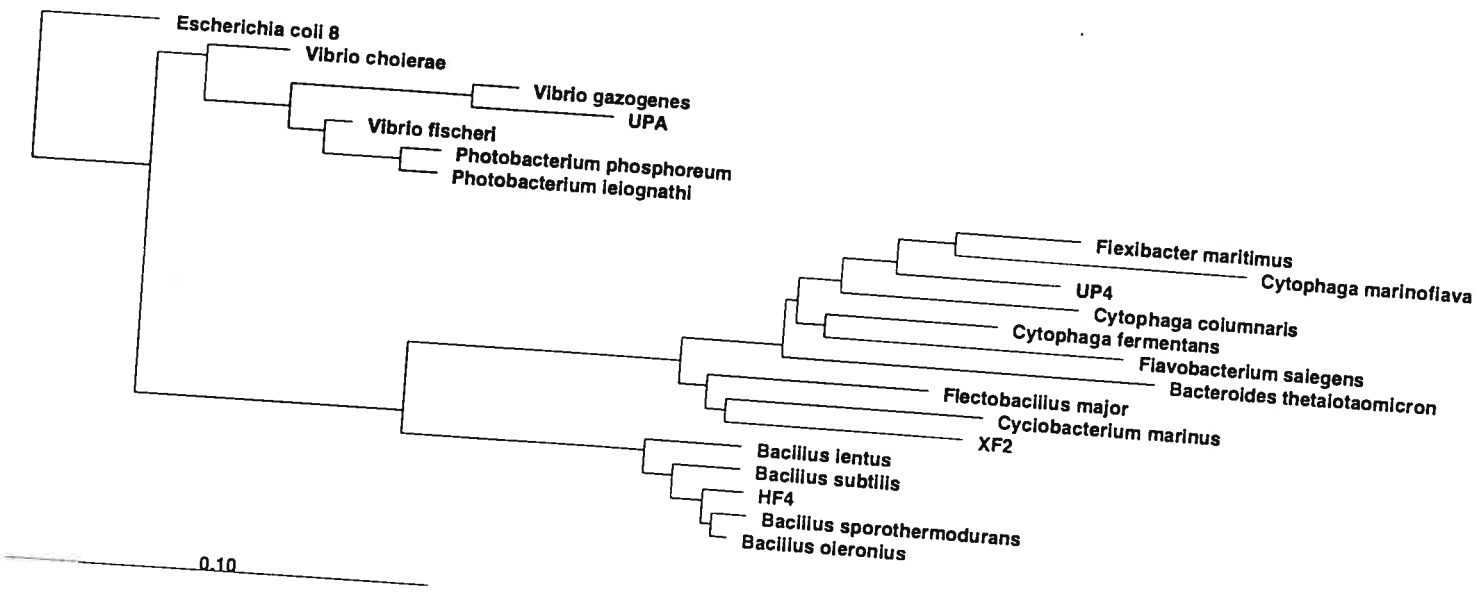


Fig 7. Phylogenetic tree derived from 16S rDNA sequence of the strains UPA, XF2. UP4, HF4. *E, coli* was used as the out group.

## Results and discussion

Close examination of the bacterial isolates indicated that some of the isolated aerobic colonies and only one of the anaerobic colonies were pigmented. Among these pigmented colonies, I focused on six isolates with red, yellow and orange colors. These colonies were analyzed under the microscope and most of them were found to have distinguishable morphotypes (fig 1-5A) with the expression of the aerobic strain UPA and the anaerobic strain C6 which had identical morphotypes. PCR amplification of these morphologically similar isolates showed that they are closely related. Under anaerobic conditions, strain UPA did grow however, it did not produce its characteristic pigment. When other aerobic bacteria were grown under anaerobic conditions, none of the isolates expressed their pigments (see table 1). When the plates were in addition taken out of the anaerobic chamber and transferred to aerobic condition pigmentation was observed within a few hours.

Phylogenetic analysis of the 16S rDNA has shown a wide diversity in the carotenoid-producing bacteria (see fig 7 for phylogenetic tree). Two of the isolates belong to the gamma proteobacteria UPA and C6 both closely related (97 and 98% respectively) to *Vibrio gzoganes*. These halophilic *Vibrio* specie that produces characteristic red colonies in marine agar (Farmer et al 1992). It is interesting to note that this bacterium was first isolated from the salt marshes of Massachusetts by a student of this course (Farmer et al 1992; Leadbetter personal communication). Under the microscope one can see that the bacteria form curve rods (Fig 1A) which is also consistent with the morphology of *Vibrio gzoganes*. The spectrum analysis of the acetone methanol pigment extraction showed a peak at 531nm (fig 1.B. curve I). For further characterization of the carotenoid, we applied the pigment extraction to a thin layer chromatography silica gel. After extracting

the bands we saw two peaks: one at 467 nm (fig 1.B. curve II) and the other at 536nm (fig 1.B. curve III). The data suggest that this bacterium produces two different carotenoids, however, this assumption needs to be tested experimentally.

Two other isolates belong to the *Flexibacter-Bacteroides-Cytophaga* phylum; XF2 is closely related to a marine isolate IC025 isolated from the Antarctic Sea ice (Bowman et al 1997). Similar to the IC025 isolate XF2 is halophilic, gliding and has a red pigment but unlike IC025 isolate XF2 shows a filamentous, rod morphology (fig 2A) and is facultative aerobic. The acetone methanol extraction peak of its carotenoid is at 479 nm (fig 2B). The strain UP4 is also closely related to the novel species *Polaribacter glomeratus*, (Gosink et al 1998) which was isolated from the Antarctic Sea ice. Like its closely related relative it can form short filaments (Fig 5A). The acetone methanol extraction peak of its carotenoid at 452 nm (fig 5B).

The last strain isolated was clearly a spore former (fig 3A) by its morphology and the 16S rDNA analysis showed it is most closely related to *Bacillus oleronius*, a bacillus strain isolated from a termite hindgut (Kuhnigk et al 1995). The acetone methanol extraction peak of its carotenoid is at 468 nm (fig 5B).

HF6 sequence did not succeed, and the undistinguished coccoid morphology (Fig 4A) of this strain did not allow e to determine its bacterial group, however acetone methanol extraction revealed the presence of a carotenoid peak of 456 nm (fig 5B).

Table 1 summarizes the characteristics of the different strains. Although the bacteria are diverse in morphology and in their phylogenetic links it can be noted that their acetone methanol extraction peak is quite similar whether their carotenoids have common

features in their biosynthetic pathway encoding genes or in their regulation awaits further research.

**Table 3**: the resistance of strain UPA & XF2 to different antibiotics and ITS concentrations.

| Antibiotics | Concentration (ug/ml) | Strain UPA | Strain XF2 |
|---|---|---|---|
| Chloramphenicol | 25 | - | - |
| | 50 | - | - |
| | 100 | - | - |
| Ampiciline | 50 | - | - |
| | 100 | - | - |
| | 150 | - | - |
| Kanamycin | 30 | + | + |
| | 50 | - | + |
| | 100 | - | + |
| Tetracycline | 10 | + | + |
| | 20 | + | + |
| Naladixic acid | 5 | - | - |
| | 10 | - | - |
| Rifanpicine | 25 | - | - |
| | 50 | - | - |

My attempt to generate a carotenoid biosynthesis mutant in either *Vibrio gazoganes* closely related strain UPA and/or the *Flectobacillus* sp. closely related strain XF2 was only mildly successful (table 4). As a control I used *E. coli* strain pEP4351 as donor and *E. coli* strain EM24NR as the acceptor. After a few false attempts I succeeded in generating a low number of transcojugants (table 4). In order to select for my isolates I had to find a selective marker for these bacteria. I tested these strains on number of different antibiotics at different concentration (table 3). I found that XF2 is resistant to kanamycin while UPA is resistant to tetracycline (though it showed resistance at the low concentration of the kanamycin). These data suggest that I do not have a selective marker

for UPA and the two attempts to conjugate those organisms yielded no results. The conjugation of *E. coli* strains pEP4351 and XF2 looked more promising after I learned that it belongs to the *Flexibacter-Bacteroides-Cytophaga* phylum to which successful transconjugation had been performed. For plating the donor and recipient cells after mating I used kanamycin 100ug/ml, tetracycline 20ug/ml with or without chloraphenicol at 50ug/ml. In order to achieve successful mating the growth rate of the XF2 and UPA was determined (fig 6). With that data I have made a few conjugation experiments using XF2 as the recipient strain. One of them yielded a few colonies, which were resistant both to kanamycin and tetracycline (table 4), No colonies resistant to kanamycin, tetracycline and chloramphenicol were detected. Whether these colonies are indeed the result of a successful transcojugation needs additional study.

**Table 4**: transcojugation assay of E. coli EM24NR and marine strains as recipients and E. coli bearing pEP4351as donor.

| **Donor strain** | **Recipient strain** | **# of transcojugants (CFU/ml)** |
|---|---|---|
| *E. coli* ($Tc^R$, $Em^R$, $Cm^R$) | *E. coli* ($Rif^R$, $Nal^R$) | 117.5 |
| *E. coli* pEP4351 ($Tc^R$, $Em^R$, $Cm^R$) | UPA ($Tc^R$) | - |
| *E. coli* pEP4351 ($Tc^R$, $Em^R$, $Cm^R$) | XF2 ($Kn^R$) | 105 |

## Conclusions

Carotenoids are distributed among many photosynthetic and nonphotosynthetic bacteria. In this project I showed that carotenoids are ubiquitous among phylogenetically diverse nonphototrophic bacteria. The carotenoids in the facultative aerobic bacteria are usually produced under aerobic growth conditions. It might be for a few reasons but mainly because these pigments require oxygen for their biosynthesis, another possible explanation is that the bacteria might use these pigments as antioxidants, but they are inhibited under anaerobic conditions. I favor the second explanation due to the fact that one of the bacteria did produced the carotenoids under anaerobic conditions while a closely related isolate did not do so. This suggests that these pigments be regulated by oxygen availability.

## Reference


Armstrong G.A., Schmidt A., Sandmann G. and Hearst J. E. Genetic and biochemical characterization of carotenoid biosynthesis mutants of *Rhodobacter capsulatus*.Biol Chem May 15;265(14):8329-38 (1990).

Bowman J. P., McCammon S.A., Brown M. V., Nichols D. S. and McMeekin TA Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl Environ Microbiol. 63(8):3068-78 (1997).

Cooper A. J., Kalinowski A.P., Shoemaker N.B. and Salyers A.A. Construction and characterization of a Bacteroides thetaiotaomicron recA mutant: transfer of Bacteroides integrated conjugative elements is RecA independent. J. Bacteriol. 179(20):6221-7 (1997)

Farmer J. J., and Hickman-Brenner F. W. the genera Vibrio and Photobacterium. in: The Prokaryots. Balows A., Truper H. G., Dowrkin M., Harder W. and Schleifer K. H. (eds) 2nd edition Springer-Verlag. pp. 2593-3011.

Gosink,J.J., Woese,C.R. and Staley,J.T. Polaribacter gen. nov., with three new species, P. irgensii sp. nov., P. franzmannii sp. nov. and P. filamentous sp. nov., gas vacuolate polar marine bacteria of the Cytophaga-Flavobacterium-Bacteroides group and reclassification of 'Flectobacillus glomeratus' as Polaribacter glomeratus comb. nov. Int. J. Syst. Bacteriol. 48, 223-235 (1998).

Gottschalk G. Bacteria metabolism. Springer-Verlag. pp. 303-304 (1986).

Kuhnigk,T., Borst,E.M., Breunig,A., Konig,H., Collins,M.D.Hutson,R.A. and Kampfer,P. Bacillus oleronius sp.nov., a member of the hindgut flora of the termite Reticulitermes santonensis (Feytaud). Can. J. Microbiol. 41 (8), 699-706 (1995).

Metcalf W.W,, Jiang W. and Wanner B. L Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. Gene 28;138(1-2):1-7 (1994)

PageM. D. and Socket R. E. Molecular genetic methods in Paracoccus and Rhodobacter with particular reference to the analysis of respiration and photosynthesis. In:Genetic methods for diverse prokaryotes. Smith M. C. M. and Sockett E. eds. pp. 434 (1999)

Ruimy R., Breittmayer V., Elbaze P., Lafay B., Boussemart O., Gauthier M. and Christen R. Phylogenetic analysis and assessment of the genera Vibrio, Photobacterium, Aeromonas, and Plesiomonas deduced from small-subunit rRNA sequences. Int J Syst Bacteriol. 44(3):416-26 (1994).

Schmidt K. Biosynthesis of carotenoids. In: The photosynthetic bacteria. Clayton R. K. and Sistrom W. R. (eds). Plenum press. pp. 729-732. (1978).