# A novel proterhodopsin bearing *Flavobacterium* species from an Antarctic saline lake

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**Abstract**

Numerous *Flavobacterium* species have been officially described from Antarctic samples, and with recent investigations more will likely be described [1]. All Antarctic *Flavobacterium* species show some level of cold adaptation and have enhanced osmotolerance relative to other members of *Flavobacterium* (Fig. 1). The overall pattern of the distribution of cold-adapted species within the *Flavobacterium* phylogenetic radiation suggests the emergence of cold adaptation has occurred multiple times within different ecosystems, however most species cluster together suggesting ecophysiological specialisation amongst related *Flavobacterium* species. Within the largest of these cold-adapted clades (Fig. 2) resides one so far undescribed strain, designated ACAM 123, most closely related to the species *F. degerlachei* and *F. frigoris*. ACAM 123 likely represents a novel species, based on accumulated data [2]. ACAM 123 is both salt-requiring and psychrophilic (tolerates up to 6% NaCl, optimal temperature for growth ~10°C). ACAM 123 forms slimy orange colonies and contains carotenoids but not flexirubin pigments. The strain is not motile (or only weakly so at best) and is also non-fermentative, proteolytic, moderately saccharolytic and able to perform assimilatory denitrification, via reduction of nitrate to ammonia.

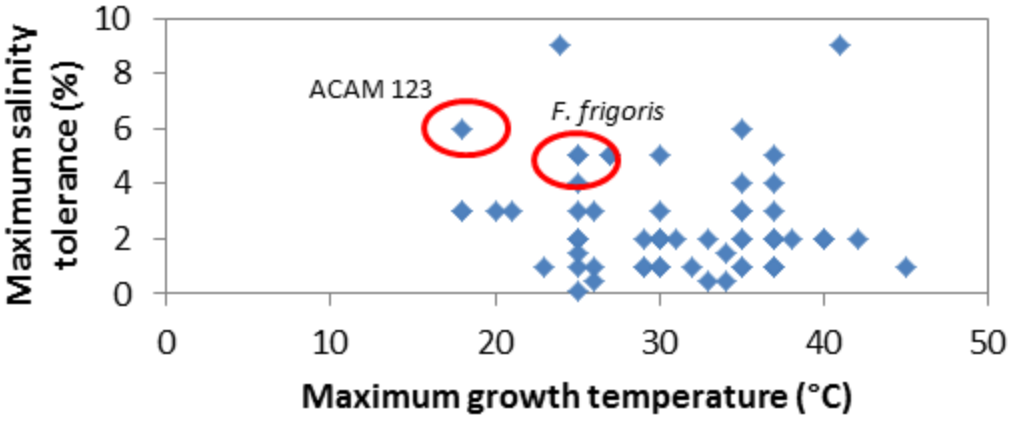


Figure 1. Distribution of maximum growth temperature and salinity tolerance of described *Flavobacterium* species. Strains isolated from Antarctica are circled.

ACAM 123 was isolated from algae collected from Burton Lake, a meromictic lagoon located within the Vestfold Hills ice free zone of eastern Antarctica (68°S, 78°E) during a much earlier pioneering effort to uncover novel Antarctic microbial diversity [3]. Of the isolates obtained from Burton lake ice, water and algal biomass samples, 27% were *Flavobacterium*-like with 57% being psychrophilic. Burton Lake has a salinity similar to seawater (37 to 43 psu) and the bottom 16 m (of 26 m total depth) is anoxic due to salinity stratification.

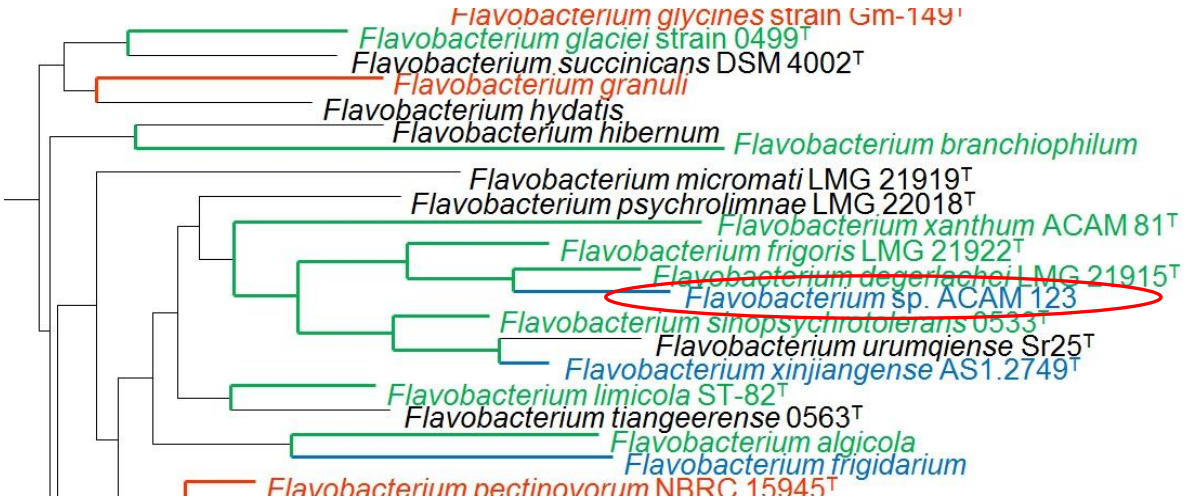


Figure 2. 16S rRNA gene sequence based phylogenetic tree (distances based on the maximum likelihood algorithm with distances clustered using neighbour-joining) showing ACAM 123 in relation to other *Flavobacterium* species. The label and branch colour indicates the inferred temperature relation of the species (red- mesophilic, green- psychrotolerant, blue- psychrophilic i.e. no growth >25°C)

The average annual temperature is very similar to the coastal seawater (-1.7°C) due to the lake having a narrow inlet to Krok Fjord. The inlet recharges the lake with seawater during summer when the inlet is ice free. The nutrient recharge could be the reason why Burton Lake has an unusually high *Bacteroidetes* diversity, which is yet to be studied in any depth [4]. ACAM 123 likely also exists in the upper water layer as well as in coastal sea-ice. The related species *F. derglachei* and *F. frigoris* also occur in similar marine salinity ecosystems, including sea-ice.

Interestingly, ACAM 123 possesses a proteorhodopsin system. Proteorhodopsin (PR) represents a paradigm in marine microbiology overturning the old concept that only algae and specific groups of bacteria capable of synthesizing chlorophyll and its analogues are able to harness light energy in the sea [5]. Proteorhodopsin provides chemoheterotrophic bacteria the means to energise their membranes and helps enhance the chemiosmotic gradient required to generate ATP as well as empower electron transport [6]. This is possible because proteorhodopsin acts as a transmembrane proton pump. Light is harvested by PR via a retylidine cofactor, derived from carotenoid via a carotenoid monooyxgenase coded by the *bhl* gene, which is adjacent to the PR gene. PR has key amino acids that maximize absorption of light energy within certain regions of the spectrum. PR can be tuned to green or blue light and this has been linked with econiche preference within pelagic zones. PR is distributed widely in marine bacteria especially in ultraoligotrophic clades. In particular alphaproteobacterial SAR clades (e.g. *Candidatus* "Pelagibacter ubique"), which mainly have green type PR, and uncultured oligotrophic gammaproteobacteria, which mainly possess blue type PR. PR also occurs in marine group II, an uncultured clade within *Euryarchaeota* that dwells in seawater. PR also occurs in members of the phylum *Bacteroidetes*, including strictly marine (e.g. *Polaribacter*, *Psychroflexus*, *Dokdonia*) and non-marine species (e.g. *Runella*, *Spirosoma*). PR analogues have also been discovered in terrestrial freshwater habitats (i.e. actinorhodopsin). Distantly related, much more studied rhodopsins are the halorhodopsins of extremely halophilic Archaea. PR is still comparatively rare amongst

flavobacteria and its presence appears to have emerged in specific lineages. The idea that PR has been horizontally transferred has been raised but based on accumulated data many species appear to have acquired PR long ago. Essentially PR is similar to catabolic metabolism genes in terms of evolutionary volatility. Deletion of the PR gene in a strain of *Vibrio harveyi* demonstrated that PR provides enhanced survival during carbon starvation [7]. Enhanced growth yield at low carbon levels while under illuminated conditions (relative to dark conditions) though initially demonstrated has yet to be shown in most PR bearing strains.

PR in ACAM 123 was uncovered during a PCR-based screen of Antarctic isolates. The PR gene discerned is of the "green" type as is the case for all flavobacteria PR found to date suggesting the strain prefers surface waters rather than less illuminated deeper waters. PR was detected in a range of Antarctic flavobacteria isolates, mainly from saline lake systems as well strains related to *Glaciecola* spp. and *Colwellia* (both members of the class *Gammaproteobacteria* and possessing blue type PR) as well as a strain identified as a member of genus *Erythrobacter.* This latter strain is a true photoheterotroph prodigy possessing not only bacteriochlorophyll but also two proteorhodospin genes, one of which is green type while the other is the blue type. In regards to *Flavobacterium* only the species *F. frigoris*, a sea-ice species and BAL38, an unspeciated strain (closely related to *F. cheniae*) isolated from the Baltic Sea, possess PR based on their respective genome sequences.

Our data suggests that PR is widespread but sporadic in its appearance amongst Antarctic bacterial strains. This could be linked to their econiche specialisation and requirements. Functionality of PR is still being discerned and it is possible that PR function may vary considerably, however knowledge of ecological preferences and lifestyles of Antarctic bacteria is still poorly defined. We would surmise ACAM 123 like other Antarctic *Flavobacterium* species prefer not only cold marine habitats (lakes, pelagic zone, sea-ice) but also depend on primary production formed nutrients within these locations. PR could aid survival either when resources are scarce or as a means to gain energy non-selectively when light is available. In that sense PR-bearers most likely concentrate and successfully compete in areas that have photosynthetically available radiation (PAR). PAR penetration can be highly attenuated by ice cover and water depth thus it is likely PR-bearing bacteria concentrate in the upper layers of waterbodies. In Burton Lake PAR is 0.01% of the full solar insolation but PAR can increase 40-fold in summer when the ice cover thins and thaws.

We obtained the genome of ACAM 123 via 454 pyrosequencing in order to reveal traits related to its response to light as well understand its ecological adaptations. The genome of ACAM 123 is 3.95 megabases in size with a mol% G+C of 33.5 based on 248 contigs of >500 bp (total 88.9 million bp) and a coverage of 22-fold. After annotation using the RAST server and GLIMMER v. 3.02, 3583 protein coding genes (excluding pseudogenes and misannotated short annotations) and 62 rRNA/tRNA coding regions were defined. The genome has been deposited in GenBank under accession number AJX01000000.1. The genome of ACAM 123 is similar in size to that of sister species *F. frigoris* PS1, which is being studied for its anti-freeze proteins (Raymond and Kim, unpublished). Between these two strains 2282 ORFs were in common between the strains (Fig. 3) while the pangenome extended to 4836 ORFs. Within the core genome, most ORFs have relatively strong synteny. The spectrum of carbon source utilisation patterns and growth requirements are similar. Taking into account other ecophysiological properties allow both taxa to occupy essentially the same econiches and despite substantial differences in their genomes.

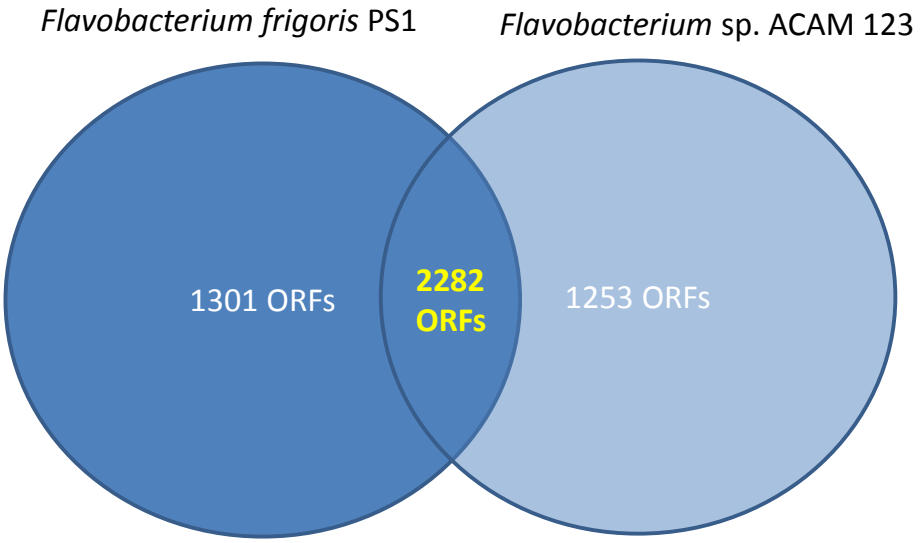


Figure 3. Overlap in numbers of protein coding genes between strain ACAM 123 and *F. frigoris* PS1.

The PR and *bhl* genes of ACAM 123 and PS1 are located adjacent to the *dnaK* (molecular chaperone), *corA* (manganese/cobalt uptake protein), *pta* (phosphotransacetylase) and *ackA* (acetate kinase) genes (Fig. 4). The similarity of the surrounding ORFs and their conserved nature strongly suggests PR is ancestral and relatively ancient in this *Flavobacterium* lineage. Further surveys of other related *Flavobacterium* species will likely yield additional PR-bearing strains. ACAM 123 and PS1 contain 5 to 7 putative bacteriophytochrome signal transduction systems further suggesting light is influential on its growth. These two-component histidine kinase/response regulator systems have PAS and/or GAF domains linked to responses to light in photosynthetic and other signals in non-photosynthetic bacteria [8], however the role of these sensors in the context of ACAM 123 is rather speculative and obviously requires much further investigation. Both ACAM 123 and PS1 also possess two cryptochrome homologs, putative RNA-binding blue light regulatory proteins [9]. These proteins are universal in plants and animals (i.e. CRY1, CRY2) and are involved in governing circadian rhythms. Such proteins are rare in bacteria and are often annotated as DNA repair photolyases, which are DNA binding proteins. Whether cryptochromes have regulatory roles in light-oriented responses remains to be determined.

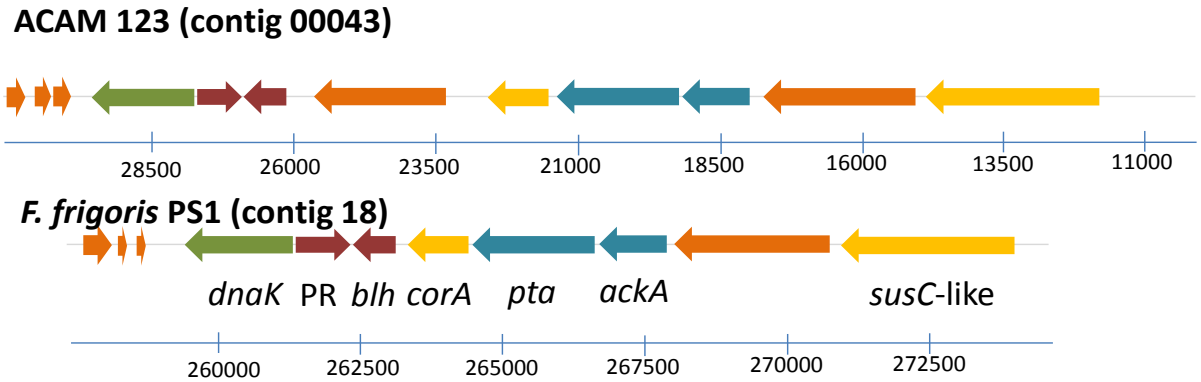


Figure 4. Proteorhodopsin genetic neighbourhood of ACAM 123 and *F. frigoris* PS1 showing the conserved nature of this genomic region. ACAM 123 has an 89 kDa putative metalloprotease located between the *blh* and *corA* homologs that is absent in PS1. Contig numbers indicate the loci of the genetic regions shown amongst the draft sequence data.

Both PS1 and ACAM 123 possess two glycolate oxidase homologs (*glcDC*), which code proteins for the conversion of glycolate to glyoxylate. Glycolate has been shown recently to be an important source of carbon for aquatic bacteria, potentially structuring microbial communities during seasonal transitions [10]. Glycolate is an excretion product produced during photorespiration by photosynthetic organisms. Since regeneration of glycolate via phosphoglycolate is energetically inefficient glycolate is usually not further metabolised. Glycolate can instead be metabolised by bacteria to either L-serine or malate. Glycolate thus can be used for energy or form carbon units via gluconeogenesis and the tricarboxylic acid cycle, respectively. ACAM 123 can use glycolate as well as phosphoglycolate and downstream intermediates (glyoxylate, serine, glycerol) as sole sources of carbon and energy suggesting that it may use glycolate photosynthate in its natural habitat.

It is possible that ACAM 123 is an epiphyte, dwelling on the surface of marine and ice algae that occur in Antarctic lake and sea-ice environments since it was isolated from algal biomass. Based on the genome data ACAM 123 possesses putative antifreeze proteins homologous to those observed in *F. frigoris* PS1. These surface proteins can affect the crystallisation of ice in such a way to prevent ice penetrating cell membranes during freezing and/or may allow attachment to ice crystals [11]. ACAM 123 colonies have a slimy mucoid appearance and thus it likely secretes capsular polysaccharides and exopolysaccharide (EPS). Several loci were identified on the genome involved in the synthesis of EPS. The genome also revealed a number of putative adhesin genes. Taken together these capabilities would allow surface colonisation and biofilm formation, including on algal surfaces. EPS could also allow nutrient acquisition by acting as a ligand, and provide environmental modification of local ice structures. The latter aspect is connected to survival in sea-ice where EPS is suspected to act as a cryopreservant factor and improves mass transfer of nutrients through the ability to act as delivery vehicles for antifreeze proteins and extracellular enzymes [12].

Based on the genome contents most gliding genes and the associated Por secretion system genes are present in ACAM 123; based on what is known about the genetics of gliding motility [13]. ACAM 123, however does not possess the rapid gliding phenotype of *F. johnsoniae* and appears largely immobile. Closer analysis of the genome suggests homologs of *sprB*, *sprC* and *sprD* are absent thus suggesting the apparent epiphytic lifestyle of ACAM 123 does not require it to be actively motile. ACAM 123 possesses extensive foreign DNA defence systems including several restriction modification enzymes and multiple CRISPR loci, the latter representing phage immunity systems. Sea-ice and lake-ice are known to have very high phage particle populations due to their concentration within brines [14], thus it is logical that enhanced phage defence systems potentially allows persistence of populations within such locations. ACAM 123 also contains an operon that suggests it is able to synthesize a lantibiotic-type oligopeptide that may provide defence against other bacteria. The strain also possesses several glycosidases and proteases indicating it has a role in secondary production degradative processes. A schematic is shown indicating the role of different genome inferred functional aspects could allow for survival of ACAM 123 in its native Antarctic lake/ice habitat (Fig. 5).

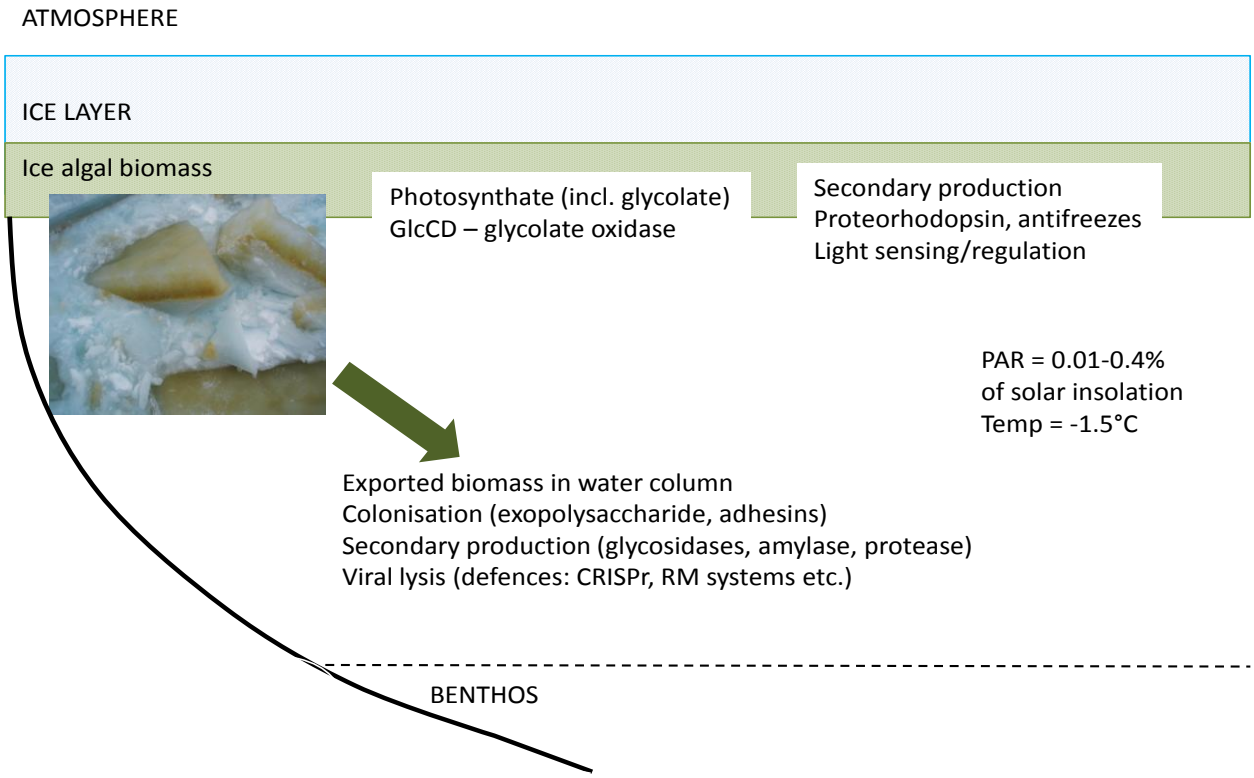


Figure 5. Schematic showing the environmental associations of ACAM 123 and its relevant physiological capacity, based on genome-derived functionalities. The PAR, insolation and temperature data is the annual average for Burton Lake surface waters. PAR increases 40-fold during ice cover melt in summer.

In conclusion, the results of the comparative genome analysis of ACAM 123 suggests *Flavobacterium* species have not only successfully adapted to cold ecosystems but species can be econiche specialists that bear little resemblance to each other in terms of ecological preferences. The presence of proteorhodopsin in Antarctic saline lake flavobacteria indicates light may aid in competition in these environments. The genome data of ACAM 123 also provides a means to begin to understand more mechanistically how Antarctic bacteria might survive under conditions of perpetual cold as well as providing preliminary understanding on how they function within the microbial loop and the larger aquatic food web. Further studies related to PR are clearly needed, including in-frame gene deletion in order to link light-harvesting functionality to cellular physiology and also to understand whether light can influence genetic regulation. Further studies are also needed to also compare different PR-bearing strains to determine if light harvesting energy-productive capacity is ecologically influenced or is a more generalised, non-specific trait.

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