An investigation into the adsorption of cyanophages to their cyanobacterial hosts

BY

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Declaration

I hereby declare that the work described in this thesis was conducted by myself, under the supervision of Prof. N. H. Mann.

None of the information herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of reference.

Ying Jia

Summary

Cyanophages, viruses that infect cyanobacteria, are known to be abundant throughout the world's oceans. They are important because of the ecological significance of their hosts which are prominent primary producers. In the natural environment cyanobacteria undergo light-dark cycles, which might be expected to exert significant effects on the way in which cyanophages reproduce.

The results in this study show how light plays an important role in cyanophage adsorption to the host cell using a model system consisting of cyanophage S-PM2 and *Synechococcus* sp. WH7803. An initial investigation of the role of light on phage adsorption revealed a striking light-dependence. In the dark, the phage S-PM2 was virtually not capable of adsorbing to WH7803, but adsorption resumed as soon as the light was switched on. This light-dependent phage adsorption was not just limited to the phage S-PM2, four out of nine other cyanophages showed the same effect. The host photosynthetic activity and light/dark cycles were demonstrated not to influence phage adsorption. The presence of the photosynthetic reaction centre gene *psbA* in cyanophage genomes was not associated with the light-dependent phage adsorption. No photoreceptor was detected from the phage S-PM2 particle.

A phage-resistant mutant that S-PM2 can't adsorb to WH7803 was isolated. A putative multicopper oxidase was found to be absent from the outer membrane fraction of the mutant. This outer membrane fraction in the wild type showed a moderate phage neutralisation activity (up to ~ 30%). To test whether the putative multicopper oxidase was the S-PM2 receptor, a recombinant WH7803 strain was constructed by inactivating the putative multicopper oxidase gene. As S-PM2 can still adsorb to the knockout mutant as efficiently as to the wild type, it suggests that the multicopper oxidase is not the phage receptor and that loss of the putative multicopper oxidase is probably a pleiotropic consequence of the loss of the S-PM2 receptor or other components, such as lipopolysaccharide, that is needed for a successful S-PM2 adsorption.

Abbreviations

ASW	artificial sea water
bp	base pair (s)
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CFU	colony forming units
DCMU	dichlorophenyldimethyl urea
DGGE	denaturing gradient gel electropheresis
DMS	dimethylsulphide
DMSP	dimethylsulphoniopropionate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dCTP	deoxycytidine triphosphate
DOC	dissolved organic carbon
DOM	dissolved organic matter
DMS	dimethylsulfate
EDTA	diaminoethanetetraacetic acid
EFM	epifluorescence microscopy
FC	flow cytometry
FL	fluorescence
FSC	forward scatter
FVIC	the frequency of visibly infected cells
G+C	guanine + cytosine
g	gene
gp	gene product
h	hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kb	kilobase pairs
kDa	kilodalton
l	litre
LC-MS/MS	liquid chromatography/mass spectrometry/mass spectrometry
LD	light-dark
LPS	lipopolysaccharide
LTFs	long tail fibres

m	metres
MALDI-TOF	matrix assisted laser desorption/ionisation-time of flight
MC	marine cluster
mg	milligram
min	minute
ml	millilitres
mM	millimolar
MOI	multiplicity of infection
MOWSE	molecular weight search
MPN	most probable number
MW	molecular weight
ng	nanogram
nt	nucleotide(s)
OD	optical density
OMFs	out membrane fractions
ORF	open reading frame
PAM	pulse-amplitude-modulated
PBP	phycobiliprotein
PC	phycocyanin
PCC	Pasteur culture collection
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PFU	plaque forming units
PFGE	pulsed field gel electrophoresis
PI	isoelectric point
PMF	proton motive force
POM	particulate organic matter
PS	photosystem
PUB	phycourobilin
RCF	relative centrifugal force
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction

RS	Red Sea
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	second
sp.	species
SSC	side scatter
TEM	transmission electron microscope
T-RFLP	terminal restriction fragment length polymorphism
UV	ultra violet
WH	Woods Hole
XGal	5-bromo-4-chloro-3-indolyl-B-galactoside
YE	yeast extract
VLP	virus like particle
µg	microgram
µl	microlitre
µM	micromolar

Chapter 1 Introduction

1.1 Aims

The overall aim of this study was to further understand the interaction of marine cyanophage-host systems with respect to the first step of the infection process, namely adsorption. In particular, the role of light during the process of cyanophage adsorption to the host *Synechococcus* sp. WH7803 was to be established. Cyanophage-resistant mutants were to be isolated and purified. The cyanophage S-PM2 receptor(s) were to be investigated.

1.2 The marine environment

The oceans cover ~ 70% of the world's surface and accommodate a vast diversity of organisms that contribute to total primary production. Among them, strains of unicellular cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are prevalent throughout the oligotrophic regions of the oceans and contribute between 32% and 89% of primary production (Goericke and Welschmeyer, 1993; Li, 1995; Liu *et al.*, 1997; Veldhuis *et al.*, 1997). Marine viruses have been known as the most common biological entities in the marine environment with an abundance of ~ 10^7 viruses ml^{-1} (Bergh *et al.*, 1989; Proctor and Fuhrman, 1990; Suttle *et al.*, 1990). It is these marine viruses that infect the ecologically important cyanobacteria that are the subject of this thesis.

In the marine environment, sunlight acts as an important physical parameter that can affect the cyanobacteria-cyanophage interaction. First, sunlight drives the photosynthetic processes of cyanobacteria. Thus, the euphotic zone is created, which

is generally defined as the area between the sea surface and the depth where light has diminished to 1% of its surface value. The photosynthesis of the oceans occurs in the euphotic zone, which sometimes may be as deep as 150 meters in the open ocean (Kirk, 1994). Second, by heating the surface of the oceans, the sunlight helps to create a region called a thermocline, where temperature changes rapidly with depth. In the deep layer below the thermocline, where photosynthesis does not take place, both cyanobacterial numbers and primary production are generally low (Scavia and Laird, 1987; Simon and Tilzer, 1987).

In this study the effect of sunlight on the adsorption of marine viruses to the host cells is to be investigated by using a model system consisting of marine virus S-PM2 and cyanobacterium *Synechococcus* sp. WH7803.

1.3 Marine cyanobacteria

1.3.1 Introduction

Cyanobacteria are ancient forms of life on earth. They have the oldest known fossil record, more than 3.5 billion years old (Schopf and Packer, 1987; Awramik, 1992; Des Marais, 2000). All cyanobacteria are capable of photoautotrophic growth and are truly prokaryotic. Due to their oxygen-evolving capability, cyanobacteria are thought to have shaped the world's atmosphere (Schopf and Packer, 1987). They can flourish in seawater and freshwater (Mur *et al.*, 1999), in cold and hot springs (Mur *et al.*, 1999) and even in harsh environments where few or no other organisms can

exist (Mur *et al.*, 1999). The cyanobacteria make up a large component of marine plankton with a global distribution. It is estimated that 3.6×10^{28} prokaryotic cells are present in the upper 200 m layer of the world's oceans, and ~ 8% (2.9×10^{27}) of them are thought to be cyanobacteria (Whitman *et al.*, 1998).

Compared with their freshwater counterparts, marine cyanobacteria exhibit distinct physiological properties such as resistance to elevated salt levels and unique photosynthetic pigment compositions (MacColl, 1998). All these properties permit acclimation to different conditions in the marine environment.

1.3.2 Marine unicellular cyanobacteria, *Synechococcus* and *Prochlorococcus*

Synechococcus and *Prochlorococcus* are the two known genera of unicellular marine cyanobacteria. They dominate the prokaryotic component of the picophytoplankton (Veldhuis *et al.*, 2005), which are the phototrophic component of the microbial plankton community and were first discovered in the late 1970s (Johnson and Sieburth, 1979; Waterbury *et al.*, 1979).

Marine *Synechococcus* strains were discovered in 1979 with the aid of epifluorescence microscopy (Waterbury *et al.*, 1979). *Synechococcus* are unicellular rod to spherical shaped cells less than 3 μm in diameter (Figure 1.1) (Kana and Glibert, 1987; Waterbury and Rippka, 1989; Herdman *et al.*, 2001). As typical cyanobacteria, *Synechococcus* strains possess a large light-harvesting complex, the phycobilisome, which is associated with the thylakoid membrane (Glazer and Clark,

1986). Phycobilisomes (PBSs) are mainly comprised of pigmented phycobiliproteins (PBPs) as well as non-pigmented linker polypeptides and may amount to 50% of the soluble protein in many cyanobacteria (Grossman *et al.*, 1993). In marine *Synechococcus*, the dominant PBP is phycoerythrin (PE), which particularly absorb visible light in the green region of the spectrum (Ong and Glazer, 1991). *Synechococcus* was classified as subcluster 5.1, previously known as marine cluster A, members of which use phycoerythrin as their major light-harvesting pigment and distinguished from subcluster 5.2, previously known as marine cluster B, members of which have phycocyanin (PC) as their major light-harvesting pigment (Herdman *et al.*, 2001).

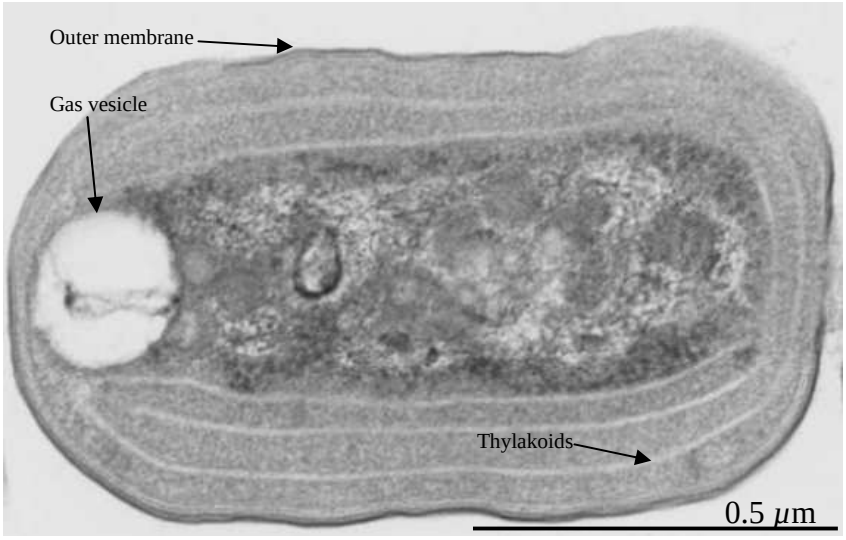


Figure 1.1 Electron micrograph of *Synechococcus* sp. WH7803.

Courtesy of Professor David Scanlan, Department of Biological Sciences, University of Warwick

Marine *Prochlorococcus*, a unicellular oxygenic photosynthetic prokaryote, was first identified by flow cytometry (Chisholm *et al.*, 1988) by virtue of their dim red fluorescence emitted by their unique light harvesting pigments — divinyl derivatives of chlorophyll *a* and *b* (Goericke and Repeta, 1992). Thus, *Prochlorococcus* is

unique amongst cyanobacteria in that it is able to fuel photosynthesis with the blue light that penetrates deepest down the water column in oceanic environments (Kirk, 1994).

The distribution of *Synechococcus* and *Prochlorococcus* in the world's oceans follow certain spatial patterns. *Prochlorococcus* is ubiquitous in the confined latitudinal bands between 40^0N and 40^0S in the oligotrophic regions of the oceans (Partensky *et al.*, 1999). It accounts for up to a third of the photosynthetic biomass in these vast areas. North of 40°N and south of 40°S it can still be found, but its concentrations decline very rapidly (Partensky *et al*., 1999). However, no *Prochlorococcus* is found in waters where the temperature is below 10°C (Partensky *et al.*, 1999). In contrast, *Synechococcus* has a broader distribution as it tolerates a wider range of temperatures and is found, albeit in low concentrations, in waters with temperatures as low as 2°C (Moore *et al.*, 1995). This group contributes up to 25% of the photosynthetic carbon fixation in oligotrophic oceans (Burkill *et al.*, 1993).

The vertical distributions of *Synechococcus* and *Prochlorococcus* exhibit a typical feature with *Synechococcus* in the upper layers and *Prochlorococcus* lower in the water column (Figure 1.2) (Ting *et al*., 2002). *Prochlorococcus* can be found dominating at depths of 100-200 m (Figure 1.2), and *Synechococcus* often dominates at, or near, the surface (~ 0-30 m) (Waterbury *et al.*, 1979; Grossman *et al.*, 1993; Worden *et al.*, 2000; Ting *et al.*, 2002). This *Synechococcus* dominance in the surface waters is explained by the finding that *Synechococcus* has a greater tolerance

to solar radiation, particular ultraviolet radiation, than *Prochlorococcus* (Sommaruga *et al.*, 2005).

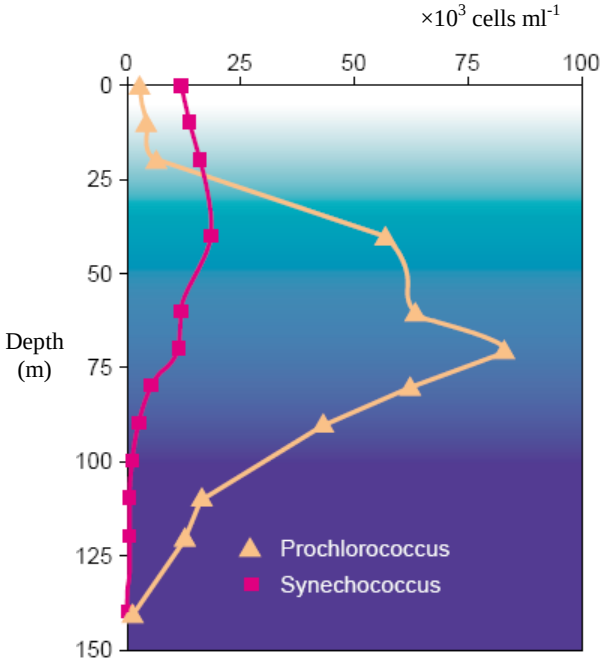


Figure 1.2 Distributions of *Synechococcus* (pink squares) and *Prochlorococcus* (orange triangles) through the water column at (32008N, 70002´W) on 10, June, 1996.

Flow cytometry was used for counting cells. The dominant wavelengths of light are indicated along the water column. Approximate depths of the open ocean water column are also shown in meters. From Ting *et al*., (2002)

In terms of average abundance, *Prochlorococcus* populations are generally about 1 order of magnitude higher than *Synechococcus* populations, excluding coastal waters (Partensky *et al*., 1999). However, since *Synechococcus* can adjust the relative proportions of its light-harvesting pigments through a process called chromatic adaptation (Palenik, 2001), it may dominate some oceanic regions and thus be greater in number than *Prochlorococcus* (Grossman, 2003).

1.3.3 Photosynthesis in cyanobacteria

Photosynthesis in cyanobacteria employs two photosystems; photosystem I (PSI) and photosystem II (PSII), which closely resemble those found in higher plants (Barry *et al.*, 1994; Golbeck, 1994). With their oxygenic photoautotrophic mode of nutrition, the cyanobacteria are distinct in the prokaryotic kingdom. They generally use water as an electron donor and produce oxygen as a by-product. Carbon dioxide is reduced to form carbohydrates via the Calvin cycle. In most cyanobacteria the photosynthetic machinery is embedded into thylakoid membranes.

The major difference in photosynthetic machinery between higher plants and cyanobacteria lies in the light-harvesting complexes. *Synechococcus* use PBSs to act as the antenna to harvest ambient light. PBSs are composed of chromophore-bearing PBPs and linker polypeptides and attached to the outer surface of the thylakoid membrane. PBSs diffuse rapidly between reaction centres along the thylakoid membranes in which both photosynthetic and respiratory electron flow occur, rather than the integral membrane chlorophyll-*a/b* binding proteins which capture light in plants. Therefore, the interaction between PBSs and reaction centres is transient (Mullineaux and Emlyn-Jones, 2005). It is these PBPs, together with chlorophyll *a* that give cyanobacteria their characteristic colouration; blue-green when PC is the major PBP and orange-red when PE predominates. Due to the different chromophore compositions in diverse cyanobacteria, a wavelength-specific light absorption is created. For example, open ocean *Synechococcus* WH8103 is nine times more effective at absorbing blue-green light (490 nm) than freshwater *Synechocystis* PCC

6701 because of the involvement of marine *Synechococcus*-specific chromophore, phycourobilin (PUB) (Ong and Glazer, 1991).

Different from the majority of cyanobacteria, including marine *Synechococcus*, *Prochlorococcus* possess a unique light-harvesting complex. It lacks organised phycobilisomes, although it still possesses PE-encoding genes (*cpeB* and *cpeA*) (Hess *et al.*, 1996; Penno *et al.*, 2000; Ting *et al.*, 2001). It contains divinyl derivatives of chlorophyll *a* (absorption maximum: ~ 443–450 nm) and *b* (absorption maximum: ~ 476–480 nm) as the major pigments (Goericke and Repeta, 1992; Morel *et al.*, 1993; Moore *et al.*, 1995). Thus, the *Prochlorococcus* light-harvesting antenna is better at absorbing blue wavelengths of light than green wavelengths, which explains the distribution pattern down the water column in Figure 1.2.

Once the energy adsorbed by PBSs is transferred to photosynthesis reaction centres, cyanobacteria and higher plants follow the same serial of reactions. By convention, the light reaction of photosynthesis in plants and cyanobacteria, is represented by the Z scheme; showing the pathway of electron transfer from water to $NADP^+$ (Figure 1.3). Briefly, PSII is the reaction centre which carries out the photolysis of water and excitation of chlorophyll P680. The excitation energy comes either from directly absorbed light or from light harvesting pigment. An excited electron is transferred from an excited $P680^+$ (which is reduced by electrons derived from photolysis of water molecules) to pheophytin (pheo), which will reduce a bound plastoquinone, named Q_A. Then Q_A reduces a second quinone (Q_B). From here, the electron flow follows the intersystem chain with decreasing negative potential to PS I associated with the generation of ATP. On excitation of PSI, electrons are consequently ejected

from P700, and the electron can therefore flow downhill to the ferredoxin and finally to NADP. In addition to this process, a cyclic electron flow can occur within this process when electrons flow from ferredoxin (FD) back into the intersystem electron transport pathway to generate ATP.

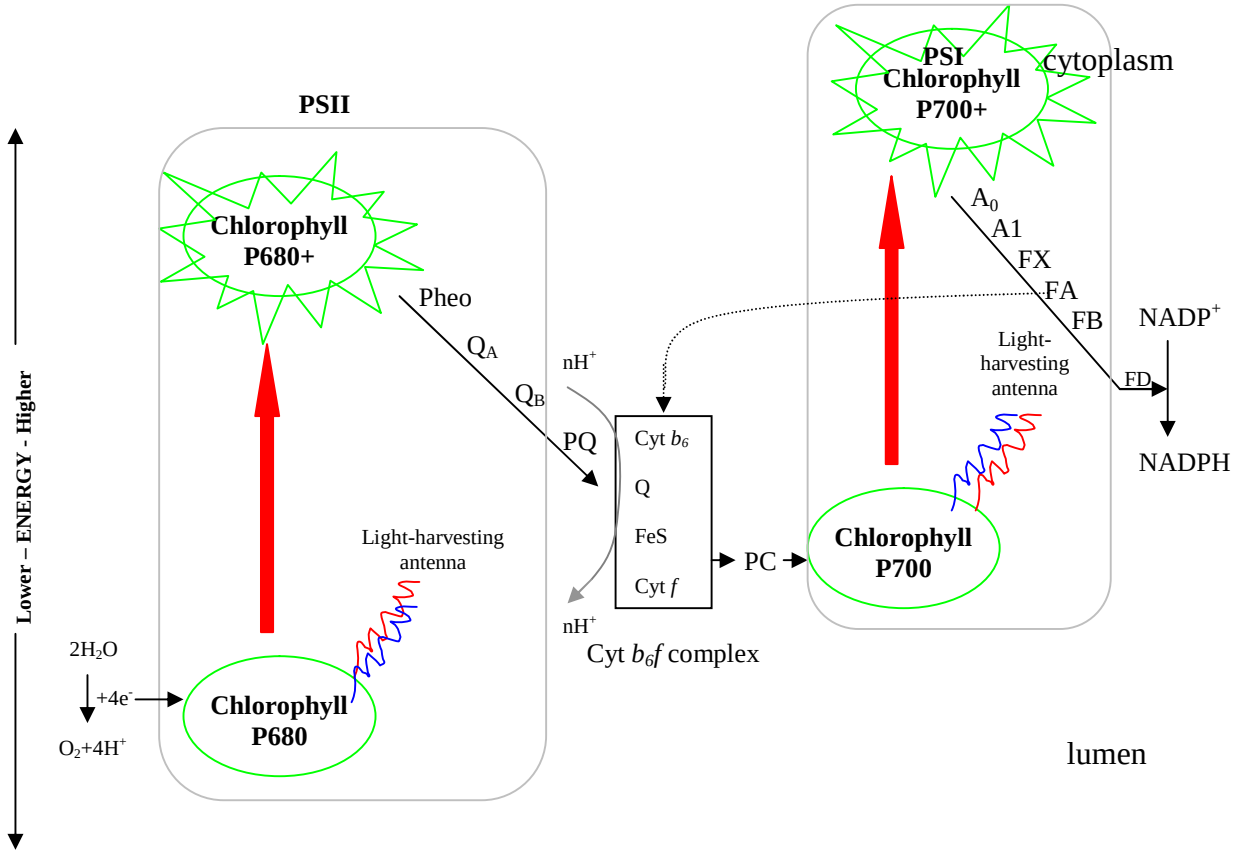


Figure 1.3 Diagram for the Z-scheme of oxygenic photosynthesis in cyanobacteria.

Most cyanobacteria, such as *Synechococcus*, use chromophore (phycobilin)-bearing PBSs to harvest light except for *Prochlorococcus* whose light-harvesting antenna contain divinyl derivatives of chlorophyll *a* and *b*. The light energy absorbed by the antenna is transferred into the reaction centres, PSII and PSI. In PSII, the absorption of light energy by the chlorophyll reaction centre P680 results in the transfer of an electron in an excited state from P680 to pheophytin (pheo), which reduces a tightly-bound and immovable plastoquinone molecule, Q_A. This in turn reduces a loosely bound plastoquinone molecule, Q_B, which detaches and becomes mobile and named PQ. The reduced Q_B diffuses through the hydrophobic core of the thylakoid membrane to a protein complex called Cyt b_6f complex (contains FeS, Cytochrome *f*, and two cytochrome b_6 molecules), which is reduced with the concomitant translocation of protons into the thylakoid lumen. The electrons are passed on to a mobile copper-protein (PC) which finally carries a single electron to PSI and reduces the oxidized

$P700^+$ to P700. This process results in the production of ATP. In PSI, the electron released from P700 (on A_0) is passed ultimately to $NADP^+$ via several other intermediates: A_1, a phylloquinone (vitamin K); F_X, F_A, and F_B which are bound iron-sulfur proteins; ferredoxin (FD), which is a somewhat mobile iron-sulfur protein molecule; and the enzyme ferredoxin-NADP reductase (FNR) and finally into $NADPH^+$ which is reduced to NADPH. Alternatively, the electron may return to the cyt b6f complex in a cyclic process that translocates additional protons into the thylakoid lumen. The electrons used for reducing excited $P680^+$ to P680 is from photolysis of water molecules showed on the left bottom of the diagram.

1.3.4 Ecological importance

The ecological importance of cyanobacteria was first determined by measuring the rate at which radioactively labelled carbon sources were incorporated into organic compound in the eastern tropical Pacific Ocean (Li *et al.*, 1983). It was found that up to 80% of primary productivity could be attributed to *Synechococcus* (Li *et al.*, 1983). More detailed estimation of the contributions of *Synechococcus* and *Prochlorococcus* to the primary production has been facilitated by the use of flow cytometry, which can distinguish *Synechococcus* and *Prochlorococcus* on the basis of their different photosynthetic pigments (Chisholm *et al.*, 1988; Chisholm *et al.*, 1992).

In the Atlantic, it was estimated that *Synechococcus* was responsible for 2% to 20% of primary productivity (Li, 1994), with *Prochlorococcus* contributing from 11% to 57% (Li, 1994). A study carried out in the East China Sea estimated that *Synechococcus* was responsible for up to 5% of the total primary production in March, by June this had increased to 63% (Chang *et al.*, 2003). In the Arabian Sea, it was found that on average *Synechococcus* was responsible for ~ 38% of the total gross primary production, whereas *Prochlorococcus* was responsible for ~ 15% of

primary production (Liu *et al.*, 1998). In contrast, *Prochlorococcus* was found to be a dominant primary producer in the tropical and subtropical oligotrophic regions (Liu *et al.*, 1999; Maranon *et al.*, 2003). Although these values vary from each other according to different geographical locations, it is apparent the contribution of both organisms is significant.

1.4 Marine viruses

1.4.1 Introduction

The first discovery of marine viruses can be traced back to the 1950's (Spencer, 1955). However, it was not for another 2 decades that the study of marine viruses became prominent. In 1979, Torrella and Morita reported a concentration of 10^4 marine viruses ml^{-1} by counting concentrated seawater samples passed through a 0.2 µm filter using transmission electron microscopy (TEM). Ten years later, a study using ultracentrifugation and TEM detected as high as 10^7 viral particles ml^{-1} present in seawater samples from the Atlantic and Chesapeake Bay (Bergh *et al.*, 1989). Following this discovery, viruses have been found to be abundant in a wide range of different regions in the marine environment, including the Antarctic (Kepner *et al.*, 1998; Madan *et al.*, 2005), Arctic (Steward *et al.*, 1996), the tropics and the oligotrophic open ocean (Boehme *et al.*, 1993; Cochlan *et al.*, 1993). In these studies, viruses are generally found in the range of ~ 10^4 to ~ 10^7 virus-like particles (VLPs) ml^{-1}. In general, surface waters contain higher concentrations of VLPs than deeper

waters, and the highest viral abundance is often accompanied by high numbers of bacterial hosts (Hara *et al.*, 1991; Boehme *et al.*, 1993; Jiang *et al.*, 2003).

In addition to variable abundance with respect to geographical locations, viral numbers also display a significant variability in temporal scales, such as seasonal variations. The seasonal change of viral abundance has been observed and depicted in several different marine environments including Chesapeake Bay, Tampa Bay, the Adriatic, Red Sea and Norwegian coastal waters (Jiang and Paul, 1994; Weinbauer *et al.*, 1995; Millard and Mann, 2006; Sandaa and Larsen, 2006). In the Tampa Bay estuary a strong seasonal pattern was found with the highest concentrations in the summer and lowest in the winter (Jiang and Paul, 1994). In the Adriatic Sea the highest virus numbers were observed in late summer or autumn, and the lowest numbers were in winter or early spring (Weinbauer *et al*., 1995). Total virus numbers are often closely correlated with their potential host abundance. For example, in the Norwegian coastal waters it was found that viral abundance was low in later summer (August) and mid winter (November) as were the number of bacteria (Sandaa and Larsen, 2006). Viral number dynamics also can be revealed by short-term studies. For example, in the Adriatic Sea oscillations of viral and bacterial numbers in the surface waters were detected during a 42-h cycle, where the major maxima in bacterial abundance were followed by viral peaks (Weinbauer *et al*., 1995).

1.4.2 Diversity of marine viruses

Marine viruses are extremely diverse. They can infect almost every type of marine organisms, including bacteria, planktonic microbes, algae, protozoa, molluscs,

crustaceans, reptiles, fish and mammals (Munn, 2006). The diversity of marine viruses that infect bacteria will be discussed in detail in the Section 1.4.5 using the best studied marine viruses that infect cyanobacteria (cyanophages) as an example. Apart from phages infecting marine cyanobacteria, significant advances have been made in understanding viruses infecting marine eukaryotic phytoplankton communities. The best described viruses belong to the family *Phycodnaviridae*, which are large icosahedral, and all contain double-stranded DNA genomes, ranging from 160 to 560 kb (180-560 kb) (Van Etten and Meints, 1999; Sandaa *et al.*, 2001; Van Etten *et al.*, 2002). Phycodnaviruses infecting the cocolithophorid *Emiliana huxleyi*, a well known bloom-forming marine coccolithophorid (Holligan *et al.*, 1993), have been shown to have major effect on the dynamics of *E. huxleyi* blooms (Wilson *et al.*, 2002; Schroeder *et al.*, 2003).

Viruses infecting the photosynthetic, eukaryotic marine picoflagellate *Micromonas pusilla* have also been shown to be genetically diverse, wide-spread (Cottrell and Suttle, 1991, 1995; Short and Suttle, 2002). In addition, viruses that infect the harmful boom-forming phytoplankton *Heterosigma akashiwo* were also investigated. These viruses were suggested to play an important role in regulating the demise of *H. akashiwo* red tide and were important factors in controlling the dynamics and diversity of *H. akshiwo* populations (Nagasaki *et al.*, 1994; Tarutani *et al.*, 2000). Although armed with the silica frustule, the bloom-forming diatom *Rhizosolenia setigera* was found to be subjected to infection by a single-stranded RNA (ssRNA) virus (Nagasaki *et al.*, 2004). A single-stranded DNA (ssDNA) virus was also detected to be able to infect diatom *Chaetoceros salsugineum* (Nagasaki *et al.*, 2005). In addition, a nuclear inclusion virus (CspNIV) which infects and lyses the diatom

Chaetoceros cf. *gracilis* was isolated and characterised from the Chesapeake Bay, USA (Bettarel *et al.*, 2005). Another group of phytoplankton, dinoflagellates, were also demonstrated to be susceptible to viral infection (Tarutani *et al.*, 2001; Tomaru *et al.*, 2004).

Other marine organisms, such as invertebrates, vertebrates and mammals are also susceptible to viral infections. For example, bivalve molluscs, *Crassostrea virginica*, were first reported to be infected by herpes-like virus almost 37 years ago (Farley *et al.*, 1972). Recently, a PCR-based method has been developed to detect a herpesvirus (Ostreid herpesvirus 1, OsHV1) that was identified from several species of bivalve molluscs (Batista *et al.*, 2005). One economically important virus, white spot syndrome virus (WSSV) that has an enveloped ds DNA, recently named as *Whispovirus* (Mayo, 2002) and sequenced (van Hulten *et al.*, 2001; Yang *et al.*, 2001). Viruses can also cause devastating effect in fish industry in terms of production and revenue, such as salmon anaemia virus (ISAV) (Kibenge *et al.*, 2004), viral haemorrhagic septicaemia virus (VHSV) (Skall *et al.*, 2005), lymphocystis virus (LV) (Borrego Garcia and Bergmann, 2005) and betanodavirus that can cause viral nervous necrosis disease in cultured marine fish (Maeno *et al.*, 2004). Viral infection is also a significant cause of mortality for marine mammals. For example, the phocine distemper virus (PDV) led to over 60% seal population losses in some areas of European 1988 and 2002 (Harkonen *et al.*, 2006).

Diversity of marine viruses is also studied at the molecular level. Based on the idea that separation of viral genomes by sizes should be able to provide a fingerprint of total virioplankton, pulse field gel electrophoresis (PFGE) has been successfully

applied in assessing the diversity of marine viruses from diverse environments (Steward *et al.*, 2000; Fuhrman *et al.*, 2002). The viral dynamic studies employing PFGE has shown that viral diversity changes with time, geographical location and depth of water column (Wommack *et al.*, 1999; Fuhrman *et al.*, 2002). The disadvantage of the use of PFGE alone in viral diversity studies is its inability of permitting definite conclusions on the presence of specific viral groups in aquatic environments. Recently, a culture-independent study of marine viral diversity has been performed by sequencing viral metagenomic libraries from two marine viral communities (Breitbart *et al.*, 2002). It was estimated that over 65% of the sequences were not significantly similar to previously reported sequences (Breitbart *et al.*, 2002). This discovery suggested that the majority of viral diversity was unknown. This may be due to the fact that most marine organisms are not culturable in the laboratory (Tramper *et al.*, 2003).

1.4.3 Ecological importance

1.4.3.1 The microbial loop

The concept of the microbial loop, as the process of reintroducing dissolved organic carbon (DOC) back into bacteria, has been established for over 20 years (Azam *et al.*, 1983). Following the discovery of abundant viral particles in the marine environment, the impact of viruses has been integrated into the marine microbial loop (Figure 1.4) (for reviews see Fuhrman, 1999; Wilhelm and Suttle, 1999; Weinbauer, 2004). Cyanophages, an important subset of marine viruses, play an integral part in the microbial loop (Figure 1.4), causing the lysis of phytoplanktonic

cyanobacteria and the release of dissolved organic matter (Fuhrman, 1999; Wilhelm and Suttle, 1999; Suttle, 2000).

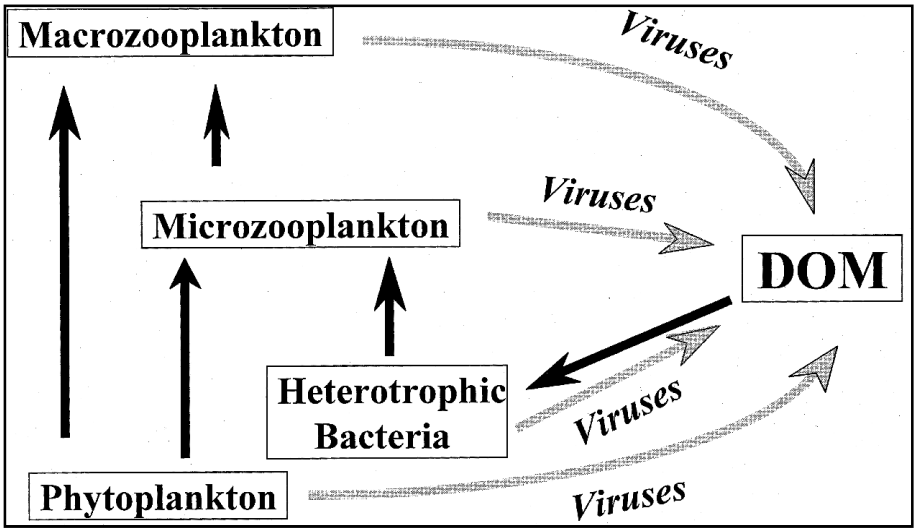


Figure 1.4 The microbial loop.

Phytoplankton are primary producers, which are subject to grazing by organisms at higher trophic levels. The function of viruses in this loop is to short-circuit the flow of carbon into the pool of dissolved organic matter (DOM). DOM then re-enters the loop via bacterial consumption. From Wilhelm and Suttle (1999)

As illustrated in Figure 1.4, the net effect of viral lysis is photosynthetic fixed carbon flowing away from higher trophic levels and being piped into the pool of dissolved organic matter (DOM). This pool is then gradually incorporated into the loop by heterophic bacteria (Middelboe *et al.*, 1996). It has been estimated that up to 47% of bacterial mortality may be due to viral lysis (Fuhrman and Noble, 1995). In terms of photosynthetic fixed carbon, up to 26% is recycled back into DOM by viral lysis (Wilhelm and Suttle, 1999).

1.4.3.2 Contribution to the global climate

Another potential impact that viruses have on a global scale is their potential involvement in the production of dimethyl sulphide (DMS), a critical component in cloud formation and climate regulation (Malin *et al.*, 1992). A study focusing on the DMS-generating marine alga, *Emiliania huxleyi*, has found that viral lysis could account for 25–100% of the demise of *E. huxleyi* during the decline of blooms in experimental mesocosms (Bratbak *et al.*, 1993). Another mesocosm study focusing on DMS-producing algae, *Micromonas* strains, also revealed that up to 34% of the populations could be lysed daily by viruses during the decline of blooms (Evans *et al*., 2003). As viral lysis of these organisms can facilitate the production of DMS (Hill *et al.*, 1998), marine viruses may thus have a role in the biological shaping of the global climate.

1.4.3.3 Impact on the community

Some theoretical and experimental analyses of the interaction between virus infection and community composition have demonstrated that viruses have a profound effect on the bacterial community composition in the marine environment (Thingstad and Lignell, 1997; Thingstad, 2000; Middelboe *et al.*, 2001; Fuhrman and Schwalbach, 2003b). Based on theoretical models for viral control of bacterial communities, the “kill the winner” hypothesis was proposed (Thingstad and Lignell, 1997; Thingstad, 2000). This hypothesis states that viruses would preferentially infect the most abundant hosts, thus other populations will be able to co-exist, otherwise, they would be out-competed. The experimental data also demonstrated that the presence of viruses reduced the dominance of specific organisms in a mixed

population, and thus are consistent with the “kill the winner” hypothesis (Fuhrman and Schwalbach, 2003a).

In addition, virus-mediated genetic exchange, occurring by a process known as transduction, was found in freshwater (Ripp *et al.*, 1994) and marine habitats (Dahlberg *et al.*, 1998; Jiang and Paul, 1998). Preliminary calculations indicated 10^{14} transduction events per year in Tampa Bay (Jiang and Paul, 1998). As viruses are generally specific to certain hosts, transduction would normally occur between strains of the same species. Nevertheless, with the discovery of broad-host-range phages, virus-mediated gene transfer could occur between genera (Sullivan *et al.*, 2003). A study has shown that phages infecting marine *Synechococcus* strains can package host DNA (Clokie *et al.*, 2003).

1.4.3.4 Virus-mediated mortality

Another important ecological consequence of viral infection is mortality of the host cells. Several different methods have been attempted to assess the viral mortality of cyanobacteria. The most direct method of detecting virus-infected cells is to use TEM to visualise mature phages inside hosts. This approach was first adopted in counting the number of visibly infected *Synechococcus* cells (Proctor and Fuhrman, 1990). It was found that up to 2.8% of cells contained mature phages, which was translated into 28% of *Synechococcus* mortality by a conversion factor of 10 to infer the frequency of infected cells from the frequency of visibly infected cells (FVIC).

In addition to the direct TEM method, a different approach using viral decay rate to infer the contribution of viruses to *Synechococcus* mortality gave an estimation of phage-mediated *Synechococcus* mortality between 0.2% and 15.0% for coastal and between 5% and 33% of mortality for open ocean systems (Suttle and Chan, 1994). Another indirect method using theoretical rates of phage absorption and host concentrations predicted the percentage of phage-induced *Synechococcus* mortality to be in the range of 0.005% to 3.2% (Waterbury and Valois, 1993a).

1.4.4 Bacteriophages

1.4.4.1 Classification

It has been suggested that viruses found in marine, freshwater and soil systems are dominated by phages (Weinbauer, 2004), which are viruses that infect bacteria. Traditionally, phages have been classified by morphological characteristics observed in the electron microscope. Currently, phages are classified into 1 order, 13 families, and 30 genera (Figure 1.5) (Ackermann, 2003), The tailed phages are classified into the order Caudovirales, which at present, contains three families: Myoviridae, Siphoviridae and Podoviridae (Ackermann, 2003). .

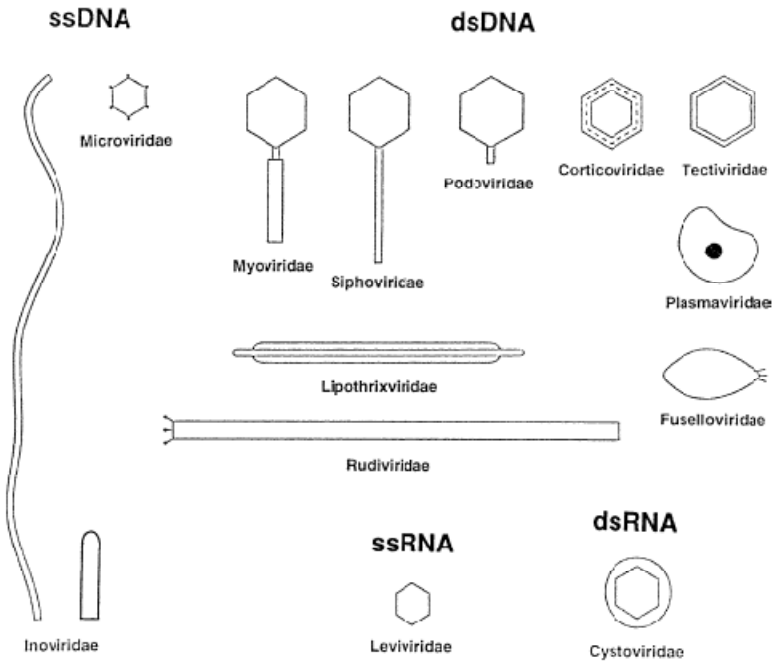


Figure 1.5 Schematic representations of the thirteen bacteriophage families categorised using transmission electron microscopy.

From Ackermann (2003)

The three families belonging to the tailed phages (Figure 1.6) all have dsDNA genomes. The myoviruses possess an icosahedral head with a long contractile tail, and compromise ~ 25% of phage (Ackermann, 2003). The most abundant siphoviruses also have an icosahedral head, but have a long non-contractile tail, and constitute ~ 61% of phages (Ackermann, 2003). The podoviruses again have an icosahedral head, but have a short non-contractile tail and constitute ~ 14% of all phages observed (Ackermann, 2003). Podovirus tails are sometimes so small, such as 3 nm in length, that they are not easily detected under an electron microscope (Ackermann, 1999). The three tailed families have been further subcategorised into different morphotypes based on head morphology, such as isometric, elongated and narrow in terms of head length to width ratio (Ackermann and Dubow, 1987).

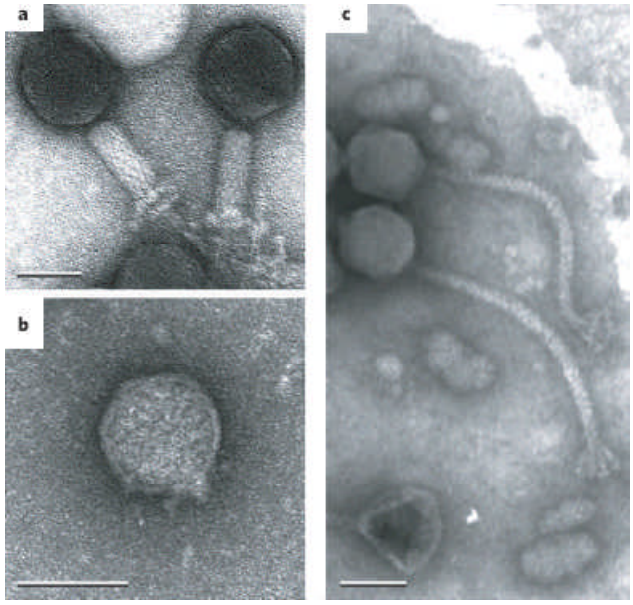


Figure 1.6 The three families of tailed phages as revealed by TEM.

a, Myoviruses. **b**, Podoviruses. **c**, Siphoviruses. Scale bar, 50 nm. From Suttle (2005)

As the traditional classification of phages is purely reliant on morphological characteristics, it has been questioned (Lawrence *et al.*, 2002). Accordingly, with the rapid accumulation of phage genomic data, a sequence-based taxonomic approach has been explored based on the overall similarity of 105 completely sequenced phage genomes (Rohwer and Edwards, 2002).

1.4.4.2 Host range

The large majority of tailed dsDNA phages carry thin tail fibres attached to a baseplate or to the tail shaft. The tail fibre proteins are the most important components involved in attachment to the host cell. For example, the adsorption specificity of T4 is determined by the distal tail fibers (encoded by gene 37), which bind to receptors on the bacterial surface (Oliver and Crowther, 1981; Wood *et al.*, 1994). Duplication of a small domain in the tail fibre adhesin was found to extend

the host range of bacteriophage T4 (Tetart *et al.*, 1996). Studies in T-even phages also showed that recombination between the tail fibre genes altered their adhesin specificity (Tetart *et al.*, 1998). DNA sequence analysis of a virulent double-stranded DNA bacteriophage, phage K1-5, revealed two open reading frames encoding two tail fibre proteins with different adhesin specificities, which allows them to infect both K5 and K1 *E. coli* strains (Scholl *et al.*, 2001).

Although bacteriophages are assumed to have a limited host range (Wommack and Colwell, 2000), a phage capable of infecting a broad range of host would conceivably have a better chance of surviving longer in the environment compared to one that has a narrow host range. In fact, a marine virus, vibriophage KVP40, has been shown to be able to infect eight *Vibrio* species and the closely related bacterium *Photobacterium leiognathi* (Matsuzaki *et al.*, 1992). Further study revealed that an outer membrane protein, OmpK, which functioned as the receptor for KVP40 determined host specificity (Inoue *et al.*, 1995).

1.4.5 Cyanophages

1.4.5.1 Introduction

Cyanophages are phages that specifically infect cyanobacteria. They were first isolated from freshwater over forty years ago (Safferman and Morris, 1962). The first marine cyanophage was isolated from the Black Sea in 1981 (Moisa *et al.*, 1981). No further research was carried out until Proctor and Fuhrman discovered that up to 5% of cyanobacteria contained phage particles (Proctor and Fuhrman, 1990).

Subsequently, novel cyanophages have been continually identified and characterised from different locations, such as Georgia estuarine rivers, Rhode Island's coastal waters, Gulf of Aqaba, the coastal waters of Texas, Woods Hole Harbor, the Sargasso Sea, the Gulf Stream, the English Channel, Chesapeake Bay, the Indian Ocean, and Norwegian coastal waters (Suttle and Chan, 1993a; Waterbury and Valois, 1993a; Wilson *et al.*, 1993a; Wilson, 1994a; Lu *et al.*, 2001; Zhong *et al.*, 2002; Marston and Sallee, 2003; Clokie *et al.*, 2006a; Millard and Mann, 2006). Most of these investigations of cyanophages mainly focused on the viruses infecting marine *Synechococcus*. However, phages infecting *Prochlorococcus* have also recently been isolated and characterised (Sullivan *et al.*, 2003).

1.4.5.2 Abundance and diversity

The cyanophage abundance in the world's oceans varies with geographical locations, with typical concentration ranging from 10^3 to10^5 viruses ml^{-1} (Suttle and Chan, 1993a; Waterbury and Valois, 1993a; Suttle and Chan, 1994; Lu *et al.*, 2001; Marston and Sallee, 2003), and sometimes with a concentration beyond 10^6 viruses ml^{-1} (Suttle and Chan, 1993a; Wilson *et al.*, 1993a; Suttle and Chan, 1994). In the case of phages infecting *Prochlorococcus*, the highest concentration detected so far has been 3×10^3 viruses ml^{-1} (Sullivan *et al*., 2003), which is within the range of phages infecting *Synechococcus*.

Cyanophages have been characterised on the basis of morphological analysis using TEM (Section 1.4.2), but molecular diversity of cyanophages based on DNA sequence analysis has increased rapidly. Analyses of restriction enzyme digestion of

cyanophage DNA have revealed different patterns (Wilson *et al.*, 1993a; Lu *et al.*, 2001), but Southern blotting analysis showed a limited degree of genetic conservation (Wilson *et al.*, 1993a). Further analysis of these conserved regions revealed that they contained a gene that was homologous to the *g20* region of the coliphage T4 (Fuller *et al.*, 1998). Gene *g20* of phage T4 encodes the portal vertex protein involved in capsid assembly. Moreover, this *g20* region from three *Synechococcus* myoviruses showed high variability as well as conserved regions. Based on these conserved regions, PCR primers specific for the *g20* region of cyanophages of the family *Myoviridae* were designed (Fuller *et al*., 1998). Denaturing gradient gel electrophoresis (DGGE) of the *g20* PCR products (Scanlan and Wilson, 1999) revealed high genetic diversity both in the surface water and throughout the water column.

Based on the forward PCR primer specific to *g20*, a new reverse primer was developed for amplifying a larger PCR product (Zhong *et al*., 2002) which has been used to reveal the enormous diversity of cyanophages. Phylogenetic analysis based on the sequenced PCR products revealed nine distinct phylogenetic groups with three groups constituting known phage isolates and six groups comprised of cloned environmental sequences. When terminal restriction fragment length polymorphism (T-RFLP) based on the *g20* gene was applied to the investigation of cyanophage diversity in Chesapeake Bay, it was found that both phage diversity and abundance changed over spatial and temporal scales (Wang and Chen, 2004). In another study of the oligotrophic Gulf of Aqaba, Red Sea, similar results showed cyanophage numbers and diversity varied over a temporal and a spatial scales (Muhling *et al.*,

2005; Millard and Mann, 2006) Moreover, it was also found that cyanophage could drive the diversity of their hosts (Muhling *et al.*, 2005).

1.4.5.3 Classification

All of the cyanophages isolated to date from the marine environment fall into the order Caudovirales (Mann, 2003). The majority of marine cyanophages characterised so far are myoviruses (Waterbury and Valois, 1993a; Wilson *et al.*, 1993a; Suttle and Chan, 1994; Lu *et al.*, 2001). Whereas, podoviruses dominate phages that infect *Prochlorococcus* (Sullivan *et al*., 2003). The naming of marine cyanophages was suggested as follows (Suttle, 2000): Cyanophage Xx-YYZaa where, Xx is the first letters of the genus (and sometimes only species names are adopted) of the host used for isolation (e.g. **S** for ***S****ynechococcus*), YY is the place of isolation (e,g. **RS** for **R**ed **S**ea), Z is the virus family (e.g. **M** for **M**yoviridae, **P** for **P**odoviridae and **S** for **S**iphoviridae), and aa is a sequential number (e.g. **3** and **4** in the names of S-RM**3**, S-RM**4**).

1.4.5.4 Host range

Considerable variation in the host ranges of phages infecting *Synechococcus* has been revealed (Waterbury and Valois, 1993a). Some phages were found to infect as many as 10 of the 13 subcluster 5.1 *Synechococcus* strains, whereas others would infect only the strain used for isolation. One phage was found to infect several subcluster 5.1 strains as well as one subcluster 5.2 strain (Suttle and Chan, 1993a; Wilson *et al.*, 1993a; Lu *et al.*, 2001). Although cyanophages have also been isolated

using subcluster 5.2 *Synechococcus* as host cells, it is reported to be easier to isolate phage on subcluster 5.1 strains than subcluster 5.2 strains (Lu *et al.*, 2001). It has also been found that phages infecting subcluster 5.1 *Synechococcus* strains had a broader host range (Lu *et al.*, 2001). Host range is clearly not constrained by the geographical locations where the phages and hosts were isolated. Phages that can infect subcluster 5.2 strains fall into all three tailed families, but all of the phages capable of infecting subcluster 5.1 strains are myoviruses (Mann, 2003). Thus, myoviruses tend to exhibit broader host ranges.

Recently, cyanophages capable of infecting both *Synechococcus* and *Prochlorococcus* have been identified (Sullivan *et al*., 2003). Furthermore, phages isolated on *Synechococcus* are more likely to cross infect with low-light-adapted *Prochlorococcus*, strains that are able to grow at extremely low irradiance (Moore and Chisholm, 1999), than high-light-adapted *Prochlorococcus*, strains that are able to grow maximally at higher light intensity (Moore and Chisholm, 1999), according to 16S rDNA phylogenetic analysis (Rocap *et al.*, 2002).

1.4.5.5 Temperate cyanophages

Compared with the information about the lytic phages infecting *Synechococcus*, only limited work has been carried out on temperate marine *Synechococcus* phages. The presence of temperate phages has been indicated as an increase in viral counts and a decrease in *Synechococcus* counts when *Synechococcus* populations were induced by mitomycin C (Ortmann *et al.*, 2002).The lysogenised host has been shown to account for 0.6% of *Synechococcus* populations. Another report demonstrated the occurrence

of lysogenic *Synechococcus* in natural populations and revealed that prophage induction was inversely correlated with *Synechococcus* abundance and primary productivity during an annual cycle (McDaniel *et al.*, 2002). Studies on lysogeny in *E. coli* have revealed that lysogenic phages can improve the fitness of the host in certain environment; they may alter the phenotype of the host and can confer immunity to the host (Wommack and Colwell, 2000). Whether or not the temperate phages of marine *Synechococcus* have similar implications for their hosts has yet to be determined.

1.5 Virus receptor

Viruses are intracellular parasites that commonly bind to the cell surface and inject their genetic material and other needed components through the host cell barriers. This first step in virus-host interactions, the recognition of the host cell, represents one of the critical factors determining a successful life cycle. Potentially, any host surface components can serve as virus receptors that were specifically bound to viral receptor-binding proteins (Heller, 1992; Haywood, 1994). For example, influenza C virus uses *N*-acetyl-9-*O*-acetylneuraminic acid as the receptor (Herrler *et al.*, 1985). Bovine coronavirus (BCV), a virus that can cause severe diarrhoea in newborn calves, also use this sialic acid as the primary receptor (Schultze and Herrler, 1992). Apart from carbohydrates, proteins are also served as viral receptors, such as a 46-kDa membrane glycoprotein that is referred to as CAR (coxsackie adenovirus receptor) is the primary receptor to which most of the human adenoviruses bind (Bergelson *et al.*, 1997; Tomko *et al.*, 1997); LamB, an outer membrane protein of *E.*

coli involved in maltose and maltodextrin transport, acts as the receptor protein for phage lambda (Boulain *et al.*, 1986; Heller, 1992).

Experimental evidence also showed that some viruses are using multiple receptors, such as human immunodeficiency virus (HIV) recognise both the CD4 glycoprotein (Sattentau and Weiss, 1988) and galactosylceramide (Fantini *et al.*, 1993). One of the best studied bacterial viruses, T-even-type phage T4, also bind to two different receptors on the bacterial cell surface. The first, reversible step in the adsorption is the binding of the long tail fibers to lipopolysaccharide (LPS) (Wilson *et al.*, 1970) in the outer membrane of *E. coli* B or the outer membrane proteins, OmpC of *E. coli* K-12 (Morona *et al.*, 1985). In phage T2, the long tail fibre binds to the out membrane protein OmpF (Hantke, 1978). Once the long tail fibres binding to the outer membrane receptors, the short tail fibres are released from their stored and folded position to irreversibly bind to either the *E. coli* LPS core region including heptose (Riede, 1987) or lipid A including keto-desoxy-octonate (Heller *et al.*, 1983). In this second step, all T-even phages bind to the LPS but not to the outer membrane proteins (Mosig and Eiserling, 2006).

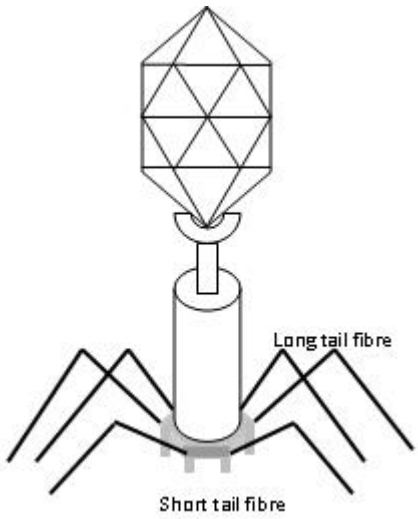


Figure 1.7 Schematic representations of the bacteriophage T4.

The long and short tail fibres are indicated.

The two phage receptor proteins stated above, OmpC, OmpF and LamB from *E. coli*, are bacterial pore-forming outer membrane proteins that were named porins in 1976 (Nakae, 1976). Porins are large water-filled channels in lipid bilayer membranes (Nakae, 1976). In most Gram negative bacteria, porins are unusually monomers or trimers in the outer membrane with a molecular mass between 30 and 40 kDa (Benz and Bauer, 1988). They are highly expressed proteins and can reach about 10^4-10^6 copies per cell (Koebnik *et al.*, 2000). Most of them, including the three porins stated above, show slightly cation selectivity (Benz *et al.*, 1985). Some of them, such as PhoE and NmpC of *E. coli* and protein P of *P. aeruginosa*, show anion selectivity (Benz *et al.*, 1985). None of them show particular substrate specificity as maltoporin LamB from *E. coil*, which are maltooligosaccharid-specific (Benz *et al.*, 1986). Given the porin's special location and high level of expression, it is not surprising that some of them were first identified as phage receptor before their physiological function was determined. For example, LamB was firstly identified as a receptor for phage lambda, hence, reflected by its name, LamB (Szmelcman and Hofnung, 1975).

In cyanobacteria, such as *Synechococcus* sp. PCC 6301 and *Anabaena variabilis*, porins are bigger and composed of monomers of about 50 to 70 kDa (Benz and Bohme, 1985; Hansel and Tadros, 1998). However, whether cyanobacterial outer membrane proteins can also act as phage receptors remains to be established.

1.6 Environmental and physiological effects on phage-host interactions

1.6.1 Introduction

Due to the fact that phages propagate by hijacking the host cells, the production and distribution of phages are subject to the productivity and density of the host bacterial populations. As one would expect, anything that affect the host will have an effect on the phage-host interactions. Thus, environmental factors, such as temperature, pH, cations and light, and host physiological state can potentially have profound effects on every stage of the cyanophage life cycle from adsorption to cell lysis.

The study of marine phage-host interactions is essential in order to understand the role that phages play in the marine environment. Phages can interact with the bacterial host cells in at least three distinct ways, resulting in lytic, lysogenic, and pseudolysogenic relationships (Figure 1.8) (Weinbauer, 2004). The life cycle of phages starts with attachment and ends with lysis of the host cells. During a lysogenic cycle, the phage genome may incorporate into the genome of the host. The

phage genome (prophage) remains as part of the genome of the host until the lytic cycle is induced (Fuhrman, 1999; Weinbauer, 2004). In contrast to a lysogenic phage, during pseudolysogenic cycle, phage DNA will act as a free episome and remain dormant within the host for some time before environmental conditions favour the lytic or lysogenic life cycles (Weinbauer, 2004)

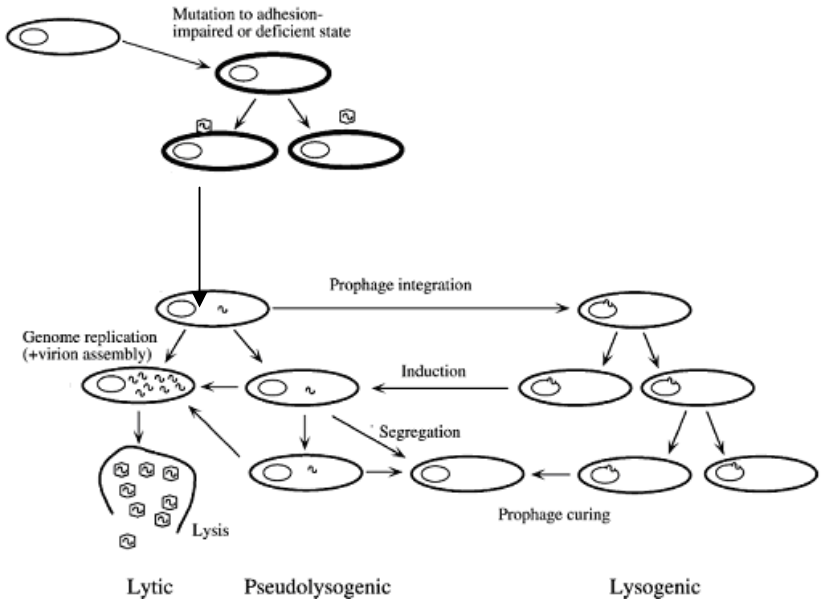


Figure 1.8 Different types of phage life cycles.

Lytic cycle starts with phage adsorption and ends with the release of phage progenies. During lysogenic cycle, phage genomes integrate into bacterial genomes and stay with the host bacteria until the lytic cycle is induced. Alternatively, phage genomes may neither replicate nor integrate into the host genome. They remain as intact and inactive for some time until environmental conditions favour the lytic or lysogenic life cycles. Adapted from Weinbauer, (2004)

1.6.2 Effect of temperature, pH and cations

Temperature is a key environmental parameter and phages in general have been found to retain their infectivity over a wide range temperature conditions from sea ice to hot springs (Weinbauer, 2004). Studies on cyanophages revealed that they can

be stored at 4ºC for several months without significant loss of infectivity (Safferman and Morris, 1964; Safferman *et al.,* 1969). With respect to high temperatures, cyanophages display varied tolerance; some are stable at 40ºC (Safferman and Morris, 1964; Safferman *et al.,* 1969) for one hour or more, others can't form plaques at above 35ºC (Padan *et al.*, 1971).

Data on pH tolerance is only available in freshwater cyanophages, which show a broad range of pH from 5 to 11 (Safferman and Morris, 1964; Safferman *et al.*, 1969). This observation coincides with the fact that some cyanobacteria show maximum growth at pH values above 8, some even at pH 11 (Padan and Shilo, 1973). By comparison, many bacteriophages lose their infectivity beyond pH 8 or 9 (Ackermann and Dubow, 1987).

The availability and concentration of cations, such as Mg^{2+}, also can affect cyanophage infectivity and adsorption. For instance, Mg^{2+} is crucial for stabilisation of freshwater cyanophages LPP-1 and SM-2 in deionised water. In contrast, cyanophage SM-1 is stable in distilled water and Mg^{2+} does not enhance its infectivity (Safferman *et al*., 1969).

1.6.3 Effect of light

In laboratory studies on phage-host systems infection is commonly carried out under optimised light supply for host growth. However in natural environments cyanobacterial hosts are subject to light-dark cycles. Thus, it is important to know how daily alternation of light and darkness will affect the phage-host interactions.

Previous studies have found that light can strongly influence the attachment of cyanophage AS-1 (Safferman *et al.*, 1972) to the cyanobacterium *Synechococcus* sp. strain PCC6301 (formerly *Anacystis nidulans*) (Cseke and Farkas, 1979b), because only 40% of the phage were adsorbed to the cells in the dark, compared to 70% adsorption in the light. However, a 10-fold increase in Na^+ concentration in the medium counteracted the effect of darkness and restored the adsorption rate of AS-1 to the level of light adsorption in the original medium. This phenomenon has been explained as due to light-induced charge neutralisation at the cell surface or by light-induced changes in the ionic composition adjacent to the cell surfaces (Suttle, 2000). So far there has been no report about the effect of light on marine cyanophage adsorption and so little work to identify the specific receptors on the host cell which cyanophage can recognise and bind to. Clearly, the effect of light on cyanophage adsorption is an area that needs more study.

In addition to adsorption, cyanophage replication is also sensitive to light conditions. Studies on freshwater cyanophage LPP-1 infection of cyanobacterium *Plectonema boryanum* have revealed that the dark incubation of phage-infected cells not only reduced the burst size to 10-15% of that observed in the light, but also delayed the appearance of free phage particles (Sherman and Haselkor, 1971). Despite keeping the infected cells in the dark for various times, phage replication would resume normally but with a larger burst size (Sherman and Haselkor, 1971). Similar results were also observed when cyanophage AS-1 infected *Synechococcus elongatus* were kept in light for 3 h before they were transferred into darkness for 10 hours (Kao *et al.*, 2005). In contrast, studies of freshwater cyanophage N-1 infection of the

filamentous cyanobacterium *Nostoc muscorum* and AS-1 infection of *Synechococcus* sp. strain PCC6301 have found that light was crucial for phage release from the hosts, because incubation of phage-infected cells in the dark significantly reduced burst sizes (Adolph and Haskelkorn, 1972; Allen and Hutchison, 1976). These data clearly demonstrate that at least for some cyanophages, the host photosynthetic activity is required for phage replication.

Apart from the dependence of phage replication on photosynthesis, phage infection can have very different impacts on the host's photosynthetic activity. In the case of cyanophage infection of unicellular cyanobacteria, photosynthetic activity was typically kept unchanged prior to lysis of the cells (MacKenzie and Haselkorn, 1972; Sherman, 1976; Suttle and Chan, 1993a; Clokie *et al.*, 2006c), despite the fact that AS-1 infection of cyanobacterium *Anacystis nidulans* resulted in a gradual inhibition of photosynthetic electron transport of PSII, whereas the activity of PSI was not altered during phage infection (Teklemariam *et al.*, 1990).

1.6.4 Effect of host physiology

Phage-host interactions can also be affected by the physiological status of the host. There is clear evidence from studies on *E. coli* phage T4 that increased growth rate of the host caused an increase in phage T4 adsorption rate and burst size, but a decrease in the latent and eclipse period (Hadas *et al.*, 1997). There has been very little work done to investigate the effect of host physiology on cyanophage-host interactions. The only report is in the cyanophage S-PM2 and *Synechococcus* sp. WH7803 system, for which adsorption kinetics were unchanged when the cells were

subjected to phosphate-starved conditions, but the latent period was longer and the burst size was reduced to 20% of that with phosphate-containing medium (Wilson *et al.*, 1996). Further research found that 100% of the phosphate-replete cells lysed, compared to only 9% of the phosphate-depleted cells. This finding indicated that although the majority of phosphate-depleted cells were infected with cyanophage S-PM2 (unchanged adsorption kinetics); most phages remained dormant inside the host cells, only a small proportion entered the lytic life cycle. This phage-host relationship has been termed pseudolysogeny and has been discovered in other phage-host systems (Ripp and Miller, 1997).

1.6.5 Cyanophage genomics

Cyanophage strain S-PM2 was first isolated from the coastal waters off Plymouth, UK (Wilson *et al*., 1996). It is a lytic myovirus with an icosahedral head and long contractile tail that infects several strains of the abundant and ecologically important marine *Synechococcus* strains (Wilson *et al*., 1996). It resembles coliphage T4 that infects *E. coli* in morphology (Hambly *et al.*, 2001), and was thus classified as a cyanomyovirus. *Synechococcus* strain WH7803 is commonly used as its host and this system has been used in studying phage-host interactions in this study. Recently, the fully sequenced S-PM2 genome was published (Mann *et al.*, 2005) (Figure 1.9). It shows that S-PM2 has a genome size of ~ 196 kb with 237 probable protein-coding sequences and 25 tRNA genes. There are at least 40 ORFs that are homologues of phage T4, 19 ORFs encoding proteins that could be associated with the cell envelope, and 20 ORFs encoding homologues of the cyanobacterial host. Most strikingly, S-PM2 encodes homologues of the *Synechococcus* PSII reaction

centre proteins D1 and D2 (Mann *et al.*, 2003; Mann *et al.*, 2005), which suggests that S-PM2 may have a role in the photosynthesis of the host cells for the sake of the providing energy for phage propagation (Mann *et al*., 2003).

The concept of interactions between phage infection and the host photosynthetic performance was not a new topic. It was first reported three decades ago that phage infection was dependent on the host photosynthesis although the extent of the dependence varied (Adolph and Haskelkorn, 1972; MacKenzie and Haselkorn, 1972). This concept was reinforced by following studies using different phage-host systems (Sherman, 1976; Lindell *et al.*, 2005; Clokie *et al.*, 2006c; Shan *et al.*, 2008). Recently, phage-encoded photosynthesis genes of cyanobacterial origin, such as *psbA* encoding photosystem II core reaction centre protein D1 and *hli* (high-light inducible) genes, are expressed during infection (Lindell *et al.*, 2005; Clokie *et al.*, 2006b; Lindell *et al.*, 2007). Some degree of light dependence for adsorption is also reported for the freshwater cyanophage AS-1 (Cseke and Farkas, 1979a; Kao *et al.*, 2005). It is thought that the expression of virus-encoded photosynthesis genes in infected cells allows the infected cell to maintain its photosynthetic activity so that there is enough energy for viral replication and assembly.

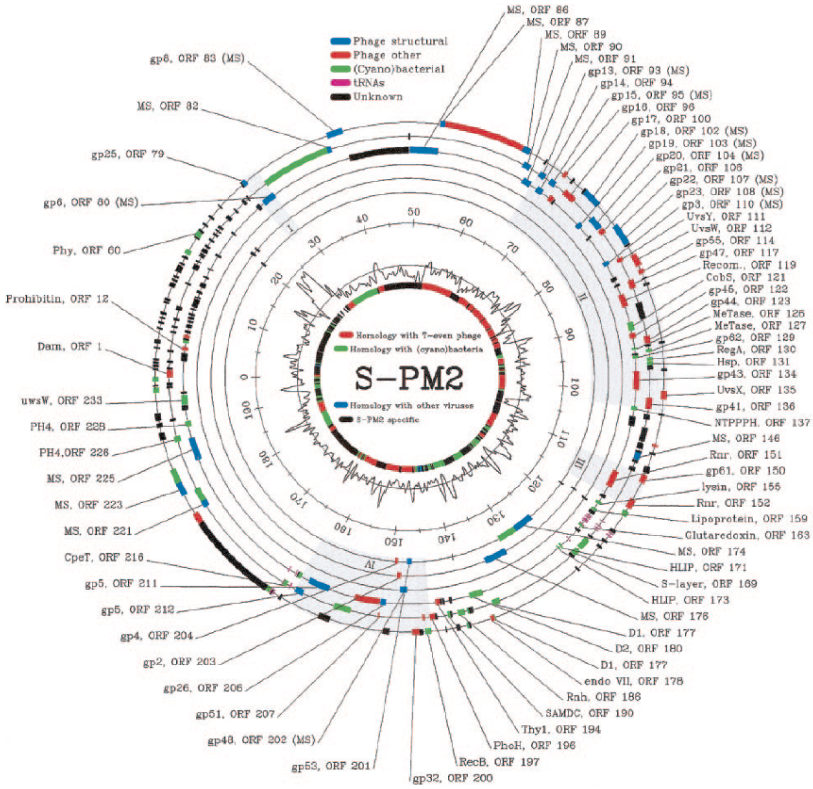


Figure 1.9 Diagram showing the circularly permuted genome of phage S-PM2.

The circles from outside to inside indicate the six reading frames, the scale bar in kilobases, G + C content and homology with other organisms. Labels show ORF numbers and gene designations named after their corresponding homologues in T4 or the host cyanobacteria. MS in parentheses indicates the presence of S-PM2 proteins that have been demonstrated by mass spectrometry. Shaded regions indicate the four genetic modules that encode T4-like genes. From Mann *et al.*, (2005)

In addition to the S-PM2 genome, six other cyanophage (Syn9, Syn5, P60, P-SS7, P-SSM2 and P-SSM4) genomes have been sequenced and annotated to date (Table 1.1), among which four phages (S-PM2, Syn9, P-SSM2 and P-SSM4) are T4-like and the others are T7-like. Four of them, S-PM2, P60, Syn5 and Syn9, were isolated on marine *Synechococcus* strains (Waterbury and Valois, 1993a; Wilson *et al.*, 1993a; Lu *et al.*, 2001), while the other three are *Prochlorococcus* cyanophages (Sullivan *et al.*, 2005).

Table 1.1 General features of the sequenced cyanophages

Name	Family	Original host	Size (bp)	ORFs	Presence of *psbA*	Presence of *psbD*	Reference
P60	*Podoviridae*	*Synechococcus*	47,872	80	N	N	(Chen and Lu, 2002)
S-PM2	*Myoviridae*	*Synechococcus*	196,280	239	Y	Y	(Mann *et al.*, 2005)
P-SSP7	*Podoviridae*	*Prochlorococcus*	44,970	54	Y	N	(Sullivan *et al.*, 2005)
P-SSM2	*Myoviridae*	*Prochlorococcus*	252,401	327	Y	N	(Sullivan *et al.*, 2005)
P-SSM4	*Myoviridae*	*Prochlorococcus*	178,249	198	Y	Y	(Sullivan *et al.*, 2005)
Syn5	*Podoviridae*	*Synechococcus*	46,214	61	N	N	(Pope *et al.*, 2007)
Syn9	*Myoviridae*	*Synechococcus*	177,300	226	Y	Y	(Weigele *et al.*, 2007)

'Y' indicates that the gene is present. 'N' indicates that the gene is absent

With the exception of P60 and Syn5, the other sequenced cyanophages contain the gene *psbA*, encoding for the photosynthesis reaction centre protein D1, and three of them also contain the gene *psbD* encoding for another core photosynthetic protein D2 (Table 1.1). Studies on the presence of the core photosynthetic genes in marine cyanophages have established that the occurrence of the genes is widespread among marine cyanophages (Millard *et al.*, 2004; Sullivan *et al.*, 2006).

1.7 Hypothesis and outline

Marine cyanophage S-PM2 needs light to complete its adsorption to *Synechococcus* sp. WH7803. This light-dependent phage adsorption is not just limited to S-PM2. Different light wavelengths and light-related processes, such as cyanobacterial circadian clock and photosynthetic activity, may have a role in this light-dependent phage adsorption. To gain a better understanding of cyanophage adsorption process, S-PM2-resistant *Synechococcus* sp. WH7803 is to be screened from lab collections. With the availability of the phage-resistant mutant, more effort can be put into characterisation of the mutant and investigation of S-PM2 receptor material, which

may be a light-sensitive molecule(s), such as protein(s), located on the outer membrane of WH7803. Experiments including SDS-PAGE can be carried out to compare the protein profile of wild-type and phage-resistant strains. The proteins present in the wild-type but not in the mutant (they may represent S-PM2 receptors) are to be analysed using mass spectrometry. To confirm their identity as S-PM2 receptors, gene knock-out mutants are to be constructed.

Chapter 2 Materials and Methods

2.1 Strains and Plasmids

2.1.1 Cyanobacterial and cyanophage strains

Table 2.1 *Synechococcus* strains used in this study

Strain	Area of isolation	Reference
WH7803	North Atlantic	(Waterbury *et al.*, 1986)
WH8018	Woods Hole, USA	(Waterbury *et al.*, 1986)
WH8109	Woods Hole, USA	(Waterbury *et al.*, 1986)
Dim	Gulf of Aqaba, Red Sea Blanes Bay,	(Fuller *et al.*, 2003)
BL36	Blanes Bay, Mediterranean Sea	Laure Guillou, unpublished
BL161	Blanes Bay, Mediterranean Sea	Laure Guillou, unpublished
BL164	Blanes Bay, Mediterranean Sea	Laure Guillou, unpublished
RS9916	Gulf of Aqaba, Red Sea	(Fuller *et al.*, 2003)

The *Synechococcus* strains used in this study have been maintained in artificial sea water (ASW) medium at the University of Warwick for a number of years.

Cyanophage S-PM2 was the main phage strain used throughout this study. It was originally isolated from English Channel (Wilson *et al.*, 1993a). S-PM2 was maintained by infecting *Synechococcus* sp. strain WH7803 and phage stocks (Section 2.8) were kept at 4ºC in the dark for up to one month. The first 9 phages (from S-PM2 to S-BM3) listed in Table 2.2 were also used for challenging WH7803 to isolate cyanophage-resistant mutants. In addition, other cyanophage strains involved in the phage adsorption experiment were from the laboratory collection and are listed in Table 2.2.

Table 2.2 Cyanophages used in this study

Phage strain	Family	Host (s)	Area of isolation	Reference
S-PM2	*Myoviridae*	WH7803, WH8012, WH8018	Plymouth, UK	(Wilson *et al.*, 1993a)
S-PWM1	*Myoviridae*	WH7803	Gulf of Mexico	(Suttle and Chan, 1993a)
S-PWM3	*Myoviridae*	WH7803, SYN48, SNC2, SNC1*	Gulf of Mexico	(Suttle and Chan, 1993a)
S-BP3	*Podoviridae*	WH7803	Bermuda	(Fuller *et al.*, 1998)
S-BnM1	*Myoviridae*	WH7803	Bergen, Norway	(Wilson, 1994a)
S-MM1	*Myoviridae*	WH7803	Miami, Florida	(Wilson, 1994a)
S-MM4	*Myoviridae*	WH7803	Miami, Florida	(Wilson, 1994a)
S-MM5	*Myoviridae*	WH7803	Miami, Florida	(Wilson, 1994a)
S-BM3	*Myoviridae*	WH7803, WH8103	Sargasso Sea	(Fuller *et al.*, 1998)
S-RSM1	*Myoviridae*	WH7803, WH8103	Red Sea	(Wilson, 1994b)
S-RSM2	*Myoviridae*	WH7803, WH8012	Red Sea	(Wilson, 1994b)
S-RSM3	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM5	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM6	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM7	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM8	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM9	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM11	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM12	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM13	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM14	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM15	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM18	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM19	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM22	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM23	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM25	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM26	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM30	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)
S-RSM34	*Myoviridae*	WH7803	Red Sea	(Millard and Mann, 2006)

*** SYN48 and SNC2 are phycoerythrin-containing strains of marine *Synechococcus*, and SNC1 is a phycocyanin-containing strain of marine *Synechococcus***

2.1.2 *Escherichia coli* strains

Table 2.3 *E. coli* strains

Strain	Genotype	Reference
TOPO 10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)Φ80lacZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
MC1061	*ara*D139, Δ (*ara*, *leu*)7697, Δ*lac*X74, *gal*U$^-$, *gal*K$^-$, *hsr*$^-$, *hsm*$^+$, *str*A	(Casadaban and Cohen, 1980)
DH5α	*supE44 Δ lacU169 f80 lacZΔM15 hsdR17 recA1 'endA1 gyrA96 thi-1 relA1*	(Hanahan, 1983)

2.1.3 Plasmids

Table 2.4 Properties of plasmids

Plasmid	Relevant Characteristic (s)	Reference
pCR2.1-TOPO	Ap^R, Kan^R	Invitrogen
pMUT100	Kan^R, Tet^R	(Brahamsha, 1996)
pRL24	Tc^r Ap^r; conjugal plasmid; derivative of RK2	(Meyer *et al.*, 1977)
pRL528	Cm^r; helper plasmid; carries *mob*	(Elhai and Wolk, 1988)
pCR-XL-TOPO	Kan^R, Zeocin^R	Invitrogen
pYJ01	0.37 kb *Eco*RI insert cloned from *Synechococcus* sp. WH7803 ORF0948	This study
pYJ02	0.4 kb *Eco*RI insert cloned from *Synechococcus* sp. WH7803 ORF0948	This study

2.2 Chemicals

All chemicals were of analytical grade from Sigma Chemicals, unless otherwise stated. Restriction enzymes and DNA modifying enzymes were supplied by Helena Biosciences, unless other wise stated. Bacto-agar was supplied by Difco Laboratories Ltd.

2.3 Equipment and kits

Gel tanks for DNA gel electrophoresis were supplied by Pharmacia, Bucks. QIAquick Gel Extraction kit and QIAquick PCR Purification Kit from Qiagen Ltd, UK were used to purify PCR product. A Mini prep kit (Qiagen Ltd, UK) was used to extract plasmid from *E. coli*. PCR was performed using a Biometra thermal cycler. Visualisation of PCR products was performed using Geneflash from SYNGENE, USA. A French press from the American Instrument Company, USA was used to disrupt cells. Two systems were used to set up protein gels to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). One system was the vertical slab gel system, 21 cm long and 1 mm thick from C.B.S. Scientific Company, Del Mar, CA. The second system was the Mini-PROTEAN 3 system (BIO-RAD, USA). A Becton Dickinson FACScan was used to count cyanophages. Protein concentration was measured using the DC Protein Assay kit (Bio-Rad Laboratories; Richmond, CA) using bovine serum albumin (Sigma) as standard.

A bench top Heraeus Biofuge Pico was used for centrifuging small volumes (< 2 ml) of material with a maximal relative centrifugal force (RCF) of 15 000 *g*. For volumes

ranging from 10 to 350 ml, an Avanti® J-25 centrifuge (Beckman-coulter) was used. Two different rotors were used with the centrifuge; JA25.50 for volumes up to 30 ml, JLA-10.500 for volumes up to 350 ml. For ultracentrifugation, an Optima™ L-80XP Ultracentrifuge (Beckman-coulter) with a SW40Ti rotor was used. A Backman TLA-100 with a TLA-100.3 rotor was used for membrane preparation.

2.4 Medium

Water used throughout this study was obtained from a Milli-Q plus 185 water purification system, Millipore Gloucester, UK.

2.4.1 Purification of agar for plating *Synechococcus* strains

In order to remove the impurities in Bacto agar for the successful growth of *Synechococcus* strains on plates, Bacto agar used throughout this study was subject to a process of purification according to a previous report (Waterbury and Willey, 1988). Briefly, 250 g Bacto agar was washed by mixing with 5 l of water through constant stirring in a large beaker for 30 min. Then, a Buchner funnel with 2 sheets of 3MM Whatman paper was used to collect the agar by filtration. This procedure was repeated at least 3 times until the filtrate was visibly clear. Then, ethanol (5 l) and acetone (5 l) were sequentially used for washing the agar. The resultant agar was air dried in a fume hood. The purified agar was stored in a plastic airtight container.

2.4.2 Media for growth of cyanobacteria

Both artificial seawater (ASW) and medium SN were used throughout this study for the growth of *Synechococcus* strains.

Table 2.5 ASW medium (Wyman and Carr, 1988)

Compounds	g l^{-1}
NaCl	25
$MgCl_2.6H_20$	2
KCl	0.5
$NaNO_3$	0.75
$MgSO_4.7H_20$	3.5
$CaCl_2 \cdot H_20$	0.5
Tris base	1.1
$K_2HPO_4 \cdot 3H_20$	0.03
ASW trace metals solution	1 ml

Table 2.6 ASW trace metals solution (Wyman and Carr, 1988)

Compounds	g l^{-1}
H_3BO_3	2.86
$MnCl_2.4H_20$	1.81
$ZnSO_4.7H_20$	0.222
$Na_2Mo0_4.2H_20$	0.39
$CuSO_4.5H_20$	0.008
$Co(N0_3)_2.6H_20$	0.0494
$FeCl_3.6H_20$	3.0
EDTA	0.5

HCl was used to adjust the pH of the medium to 8.0. ASW was routinely made from stocks prepared by the University media preparation service. Solid ASW agar plates (1%) were made by the following procedures: ASW (double concentrated) was

autoclaved separately from the agar solution (2%, w/v) before mixing together and pouring the plates.

Table 2.7 SN medium salt stocks (100×) (Waterbury and Willey, 1988)

Compounds	g l^{-1} (for preparation of liquid SN medium)	g l^{-1} (for preparation of solid SN medium)
EDTA	0.56	0.56
$NaNO_3$	76.5	21.2
NH_4Cl	-	0.531
K_2HPO_4	1.57	0.390
$Na_2CO_3.H_2O$	1.06	1.06

Table 2.8 SN trace metals solutions and Va Vitamin mix (Waterbury and Willey, 1988)

SN trace metal solutions compounds	g l^{-1}
$MnCl_2.4H_2O$	1.4
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.390
$Co(NO_3).6H_2O$	0.025
Ferric ammonium citrate ($C_6H_8O_7.nFe.nH_3N$)	6.0
Citric acid hydrate ($C_6H_{10}O_8$)	6.250

Va Vitamin mix Compounds	mg l^{-1}
Thiamin-HCl	200
Biotin	1
Vitamin B12	1
Folic Acid	2
PABA	10
Nicotinic acid	100
Calcium pantothenate	200
Pyridoxine-HCl	100

For preparation of liquid SN medium, 200 ml of water was added to 750 ml of

seawater. After autoclaving at 121ºC for 15 min, 10 ml of sterile salt stock was added, followed by the addition of 0.5 ml of filter-sterilised Va Vitamin mix and 1 ml of trace metals solution.

For the preparation of solid SN (0.3%) used in pour plating, 3 g of agar in 200 ml of water was autoclaved separately from 750 ml of seawater before being combined. Then 10 ml of sterile salt stock, 0.5 ml of Va Vitamin mix (filter-sterilised) and 1ml of trace metals solution (filter-sterilised) were added. Finally sodium sulphite (filter-sterilised) was added to a final concentration of 2 mM in a total volume of 40 ml.

For the preparation of solid SN (1.0%) used in solid agar plates, 10 g of agar in 200 ml of water was autoclaved separately from 750 ml of seawater before being combined. Then 10 ml of sterile salt stock, 0.5 ml of Va Vitamin mix (filter-sterilised) and 1 ml of trace metals solution (filter-sterilised) were added aseptically. Finally sodium sulphite (filter-sterilised) was added to a final concentration of 2 mM in a total volume of 40 ml.

2.4.3 Media for growth of *E. coli*

2.4.3.1 Luria Bertani (LB) Medium

Table 2.9 LB medium

Compounds	g l^{-1}
Bacto tryptone	10
Bacto yeast extract	5
NaCl	5
pH adjusted to 7.5 with 10 M NaOH	-

2.4.3.2 SOC Medium

Table 2.10 SOC medium

Compounds	g l^{-1}
Bacto tryptone	20
Bacto yeast extract	5
NaCl	0.5
KCl 1 M	2.5 ml
Glucose * 1 M	20 ml

*Filter-sterilised glucose was added after the media had been autoclaved; SOC media was used in transforming *E. coli*.

2.4.4 Contamination test medium for *Synechococcus* strains

To test for heterotrophic contaminants, samples from *Synechococcus* cultures were plated on a modified ASW medium enriched with 2% (w/v) glucose, 0.15% (w/v) yeast extract and 1.5% (w/v) agar. Double concentrations of the enriched ASW and agar were autoclaved separately followed by the addition of filter-sterilised glucose (1 M) and mixing before pouring the plates. The plates were incubated in the dark at 25ºC for 14 days and checked regularly for the appearance of colonies.

2.5 Phage titration

Phage titration was based on a previous reported protocol with minor modifications (Wilson *et al.*, 1996). Briefly, 10 fold dilutions of cyanophage was performed using ASW, and samples were left to adsorb to 100 fold concentrated exponentially growing *Synechococcus* sp. WH7803 (OD_{750} of 0.35-0.40) for 1.5 h at 25ºC with

gentle occasional shaking. These phage-cell suspensions were then mixed with 3 ml molten ASW agar (0.3%) and poured onto solid ASW agar (1%) plates before being kept on the bench at room temperature for at least 2 h. Incubation of these plates was carried out at 25ºC with illumination at 15-25 μE m^{-2} s^{-1}. The agar used for this procedure had been previously cleaned as described in Section 2.4.1. Plaques (normally appeared within 7 days) were counted manually using a colony counter. Control plates received no cyanophage.

2.6 Culture conditions

Synechococcus stock strains were cultured in 50 ml, 100 ml and 250 ml volumes in conical flasks under constant illumination; 2 –25 μE m^{-2} s^{-1}, at 25ºC in ASW without agitation in a Gallenkamp cooled incubator. Large volumes of *Synechococcus* were cultured in 1 and 5 l glass vessels to which 0.5 g l^{-1} $NaHCO_3$ was added. These vessels were provided with aeration by filtered air and continuous stirring under the same condition as stated above. Sub-culturing was performed every month by a 10^{-1} dilution of stock into fresh medium. Stocks were also tested for contamination by the streaking of culture onto contamination test medium (Section 2.4.3). When the growth curve was monitored, *Synechococcus* cultured in 1 l glass vessel was used. The cell density was recorded using the absorbance at 750 nm (OD_{750}) (Wilson *et al.*, 1996).

2.7 Spot test

A spot test was used to detect the susceptibility of host cells to phage infection. Briefly, 10 µl of serially diluted phage stocks (from 10^{-1} to 10^{-7}) were spotted onto a lawn of host cells growing on solid ASW agar (1%) plates, and kept under illumination at 10-25 µE m^{-2} s^{-1}, at 25ºC until plaques appeared.

2.8 Preparation of phage stocks

Phage lysate was prepared by infecting an exponentially growing 1 l culture of *Synechococcus* sp. WH7803 (OD_{750} between 3.5 and 4) with cyanophage at a MOI (the ratio of viruses to hosts) of 1. When lysis was complete in 2-5 days (culture became clear), centrifugation at 6 000 *g* was used to remove cell debris. The titre of the resulting S-PM2 lysate was in the range of 1.5×10^8 - 7.2×10^8 pfu ml^{-1}. This was used as phage stock and kept at 4ºC for up to six months before a new phage stock was prepared.

2.9 Cyanophage concentration and purification

2.9.1 Extraction from agar plates

For every 5 plates the sloppy top agar was scraped off and resuspended in 5 ml of ASW containing 0.5 ml of chloroform in 50 ml Oakridge centrifuge tubes. To each

plate that has been scraped, 3 ml of ASW was added and left for 1 h, before combining them with the resuspended sloppy agar. This suspension was shaken vigorously, left to stand for 1 h to allow phage particles to diffuse into the medium and was then centrifuged at 8000 *g* for 10 min. DNase I and RNase I were added to the resulting supernatant to a final concentration of 1 μg ml^{-1} and lysozyme to a final concentration of 5 mg ml^{-1}. This mixture was left for 2 h at room temperature. Then NaCl was added to a final concentration of 0.5 M, mixed and left on ice for 1 h, before being centrifuged at 8000 *g* for 10 min. The supernatant was transferred to a clean Oakridge tube followed by polyethylene glycol (PEG) 8000 addition to a final concentration of 10% (w/v). After gentle mixing, this was kept overnight at 4ºC. The crude phage lysates were centrifuged at 8000 *g* for 10 min to pellet the precipitated phage, and washed once with 4 ml of ice-cold 10% PEG/0.5 M NaCl. The final resulting pellet was resuspended in 2 ml of ASW. Finally, an equal volume of chloroform was mixed with this phage suspension by gentle inversion of the tube followed by centrifugation at 8000 *g* for 5 min. The resulting top aqueous phase was collected and subject to further phage DNA purification steps.

2.9.2 Extraction from liquid culture

Cyanophages were also extracted from phage-lysed cultures. Lysozme, DNase I, RNase I and NaCl were added to phage lysate as described in Section 2.9.1 before removing cell debris by centrifugation at 4 000 *g* for 20 min. PEG 8000 was then added to the supernatant at a final concentration of 10% (w/v), until all of the PEG was dissolved. The mixture was left over night at 4ºC followed by centrifugation at 8 000 *g* for 10 min. The resulting pellet was resuspended in 5 ml of ASW with the

addition of an equal volume of chloroform and mixing by gentle inversion of the tube followed by centrifugation at 8000 *g* for 5 min. The resulting top aqueous phase was collected and subject to further phage DNA purification steps.

2.9.3 Caesium chloride purification of cyanophage

Phages were further purified using caesium chloride gradients as previously described (Sambrook and Russell, 2001). Phage suspensions from Section 2.9.1 or Section 2.9.2 were layered on the top of 7 ml of 0.75 g ml^{-1} caesium chloride (made up in ASW) held in Ultra-Clear™ centrifuge tubes (14 × 95 mm) (Beckman). These were centrifuged at 4°C in an Optima™ L-80XP Ultracentrifuge with a SW40Ti rotor at 86 000 *g* for 24 h. Concentrated cyanophages were revealed as a milky blue band, which was removed and dialyzed against ASW at 4°C for 3 days with daily changes of ASW. The dialysis membrane was Spectra/Por® 6 dialysis membrane (Spectrum Laboratories, Inc.) with a molecular weight Cut Off (MWCO) of 12-14 KDa. The titre of the CsCl-purified S-PM2 was in the range of 1.7×10^{10} - 6.4×10^{10} pfu ml^{-1}. The CsCl-purified S-PM2 was stored at 4°C in the dark.

2.10 Flow cytometry

Use of flow cytometry for direct counting of cyanophage S-PM2 was attempted using a FACScan flow cytometre equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter setup. Methods were developed and applied to phage counting according to previous work (Marie *et al.*, 1999; Brussaard, 2004).

CsCl-purified S-PM2 (10 times diluted in ASW), S-PM2 lysate, ASW (control), and *Synechococcus* sp. WH7803 culture that have been filtered through 0.2 µm were fixed with glutaraldehyde at a final concentration of 0.5% (v/v) for 30 min at 4°C followed by freezing in liquid nitrogen and storage at -80°C. When thawed, samples were diluted in TE buffer (10 mM Tris, 1mM EDTA, pH8) and incubated with three different dyes, SYBR Green I, YOYO-1 and SYBR Gold (Molecular Probes) at a final concentration of 5×10^{-5} dilutions of commercial stock, respectively and incubated at 80ºC for 10 min in the dark with Triton X-100 added to a final concentration of 0.1% (v/v).

10 µl of stained phage sample were added to 990 µl TE buffer in a 5 ml polystyrene round-bottom tube (BD Falcon) immediately prior to analysis. For the non-stained phage sample, 100 µl of phage sample were added to 900 µl TE buffer. For calibration, 10 µl Fluoresbrite® YG (Polysciences) calibration beads (optional; 2×10^6 beads ml^{-1}) were added to 990 µl TE buffer. Samples were analyzed by a FACScan flow cytometre for 1 min at a low flow rate. The flow rate was checked before and after running using the method recommended in the manual. The average flow rate (normally between 30 and 40 µl ml^{-1}) was used for calculation (Campbell, 2001). The discriminator was set to green fluorescence, which is proportional to the nucleic acids–SYBR-I complex, and the detection threshold was progressively decreased until viruses could be detected (see below), but care was taken to avoid detecting more than 1 000 events per s, a threshold above which coincidence occurs, resulting in the underestimation of particle abundance. Relative green fluorescence (FL1), total counts, and relative side scatter (SSC) were recorded on logarithmic

scales and analyzed with the CellQuest Software (provided by BD Biosciences) that discriminates cell populations by using combinations of all recorded parameters.

2.11 Influence of light on cyanophage adsorption

To determine the kinetics of adsorption under light and dark conditions, S-PM2 was added to two identical cultures of *Synechococcus* sp. WH7803 (OD_{750} of 0.35-0.40) at a MOI of 0.02. The 'dark culture' were wrapped in aluminium foil and incubated with the 'light culture' at 25ºC at 15 μE m^{-2} s^{-1}. At intervals, 2 ml samples were withdrawn and centrifuged for 15 min at 15 000 *g* at 4ºC. The cell-free supernatant was titred as described in Section 2.5. The results were expressed as percentages of the initial phage titre. In addition, adsorption of another eight cyanophages to WH7803 under light and dark conditions, including S-BnM1, S-BP3, S-MM1, S-MM4, S-MM5, S-PWM1, S-PWM3 and S-BM3, was also investigated (Table 2.1).

To determine the influence of light wavelength on phage adsorption, the same phage-cell suspensions as indicated above were exposed to blue (460 nm-490 nm), green (500-570 nm), yellow (580-590 nm) and red light (785-620 nm) with the same intensity of 15 μE m^{-2} s^{-1} as the white light (460-620 nm). Different light wavelength were supplied from the Schott KL 1500 Electronic Light Source (Schott-Fostec, LLC, Auburn, NY) at 25ºC. At intervals, 2 ml samples were withdrawn and centrifuged for 15 min at 15 000 *g* at 4ºC. The cell-free supernatant was titred as described in Section 2.5. The results were expressed as percentages of the initial phage titre.

2.12 Influence of DCMU and CCCP on cyanophage adsorption

Photosynthetic inhibitor DCMU (or uncoupler CCCP) was first dissolved in 50 ml ethanol to a final concentration of 0.2 mM to make stock solutions. Working solutions (10^{-4} M) were then prepared by dilution with water. *Synechococcus* sp. WH7803 cultures were pre-treated with DCMU or CCCP (both at 10 µM) for 1 h before the adsorption test. The cyanophage S-PM2 adsorption test was carried out as for the 'light culture' described in Section 2.11 with a non-treated control. The cell-free supernatant was titred during a 3-h period. To investigate if S-PM2 can still propagate in DCMU/CCCP-treated WH7803, S-PM2 titre was determined at 19 and 24 h post-infection according to Section 2.5. The results were expressed as percentages of the initial phage titre.

2.13 Cyanophage adsorption to entrained *Synechococcus* sp. WH7803

As *Synechococcus* sp. WH7803 has been demonstrated to be easily entrained by light-dark (LD) cycle (Sweeney and Borgese, 1989). 1 litre culture of *Synechococcus* sp. WH7803 (OD_{750}=0.042) was incubated under a continuously modulated 12 h – 12 h LD cycle at 22ºC for at least 7-10 days. When the culture reached OD_{750} =0.5 (this represents 3.6 generations), sampling began over a 24-h period (Figure 2.1). Specifically, aliquots of cells (20 ml) were collected at 6 time points, which were 15 min post-light (named as early-light), 6 hrs post-light (named as middle-light), 15 min before switching to dark (named as late-light), 15 min post-darkness (named as early-darkness), 6 hrs post-darkness (named as middle-darkness), and 15 min before

switching to light (named as late-darkness). These six samples were subject to the light/dark adsorption test as described in Section 2.11.

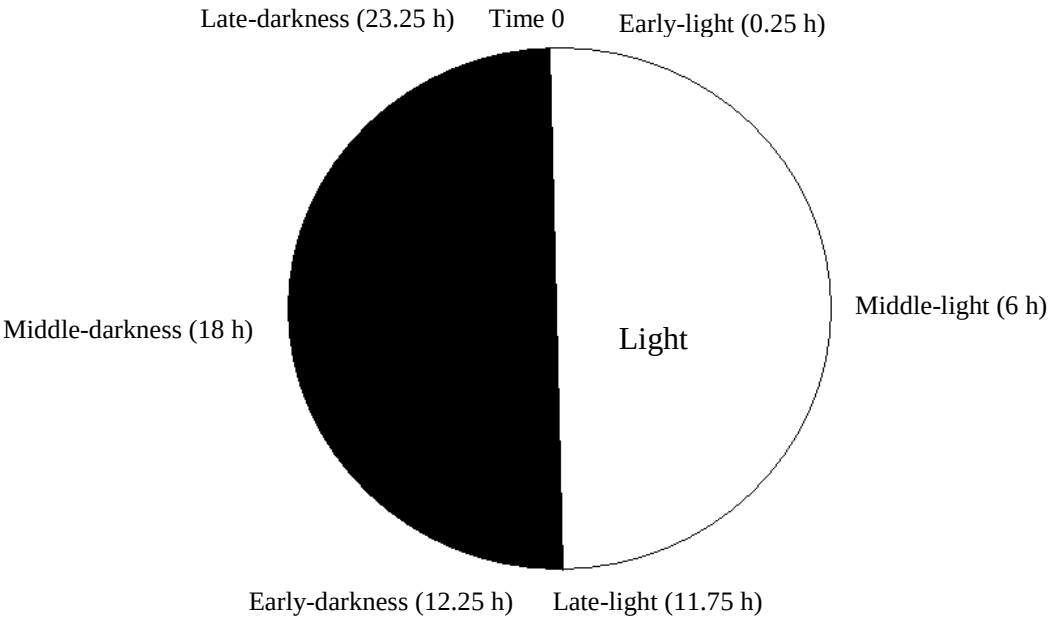


Figure 2.1 Diagram showing the sampling times during a 12 h – 12 h LD cycle.

2.14 Pour plating of single colonies of *Synechococcus* strains

The plating technique was based on previous work (Brahamsha, 1996) with modifications. *Synechococcus* strains were serially diluted in SN (or ASW) medium and 0.1 ml of each dilution was added to a Petri dish followed by the addition of 35 ml of molten SN (or ASW) agar (0.3%). Gently shaking was needed to spread the agar evenly. All plates were left on the bench for 2 h before incubating at 25ºC at a light intensity of 5 μE m^{-2} s^{-1} for 2 days, after which a higher light intensity of 25 μE m^{-2} s^{-1} was used to illuminate them for up to 8 weeks or until the appearance of single colonies.

2.15 Isolation of cyanophage-resistant mutants

Samples of exponentially growing *Synechococcus* sp. WH7803 were infected with S-PM2, S-BP3, S-PWM3, S-MM5, S-MM1, S-MM4, S-BM3, S-PWM1, and S-BnM1, respectively, at a MOI of 1, and kept at 25ºC at 15 μE m^{-2} s^{-1}. Following prolonged incubation (3 months), putative phage-resistant mutants were detected as renewed growth and were purified by the pour plating method as described in Section 2.14. Single colonies were grown in 2 ml of ASW medium under constant illumination of 10 μE m^{-2} s^{-1} at 25ºC for 4 weeks or until the OD_{750} was above 0.3. Then they were scaled up in 25 ml of ASW medium as stock cultures under the same condition (Section 2.6).

In order to prove that these phage resistant mutants are genuinely mutants of *Synechococcus* sp. WH7803, three pairs of primers targeting 16S rRNA, *mpeBA* and *cpeT* genes (Table 2.11) were used to perform PCRs over the genomic DNA extracted from putative phage-resistant mutants. *mpeBA* and *cpeT* genes are characteristic marine *Synechococcus* genes (Cobley *et al.*, 2002), whose products function in photosynthesis. The PCR products were subsequently sequenced (provided by the University of Warwick sequencing service) using primers listed in Table 2.11 and the sequences were compared with the sequenced genome of WH7803 using ClustalX (1.81).

Table 2.11 Primers used in sequencing phage-resistant mutants

Primers	**5'-3'**
27F-OXY1313R	GGACGGGTGAGTAACGCG
OXY107F-1522R	CTTCAYGYAGGCGAGTTGCAGC

16S rRNA359F	GGGGAATYTTCCGCAATGGG
16S rRNA778R	ACATACTCCACCGCTTGTGC
7803mpeBAF-704	TCATCATGCGCTACGTCTCC
7803mpeBAR-1330C	CGCCAACGACCAAGGAGTAG
7803cpeTF39	AACGCTAGCCGGCCACTACA
7803cpeTR612C	CCAACGCAGCCAGTGCTCAT

2.16 Isolation of the outer membrane fraction (OMF)

Two different protocols were attempted to isolate the OMF of *Synechococcus* sp. WH7803.

1. Isolation of the OMF was performed at 4ºC to maintain the native structure of proteins. 100 ml of exponential growing WH7803 (OD_{750} of 0.35) was harvested by centrifugation at 4 000 *g* for 20 min. Cell pellets were resuspended in 3 ml of 10 mM HEPES containing 10 mM $MgCl_2$ buffer (pH 7.4), and broken by passing six times through a French pressure cell at 1.01×10^5 kPa. After removal of unbroken cells and cell debris by centrifugation at 8 000 *g* for 20 min, the supernatant was subjected to centrifugation at 103 320 *g* for 30 min at 4ºC with the rotor TLA-100.3. The resulting pellet was resuspended in 200 µl of the same buffer with the addition of Triton X-100 to a final concentration of 2% (v/v) followed by centrifugation at 103 320 *g* for 30 min at 4ºC. The resulting pellet was dissolved in 100 µl of 10 mM HEPES containing 10 mM $MgCl_2$ buffer (pH 7.4) and stored at 4ºC. 10 µl of the sample was used for SDS-PAGE.

2. 400 ml exponentially growing WH7803 cells were harvested by centrifugation at 4 000 *g* for 20 min. Cells were incubated in 40 ml of 10 mM HEPES (pH 7.2) containing 10 mM EDTA for 30 min at 37ºC. The cells were removed by centrifugation at 4 000 *g* for 20 min, the supernatant was then centrifuged at 4ºC at 160 000 *g* for 1 h in an Optima™ L-80XP Ultracentrifuge (Beckman-coulter) with a SW40Ti rotor. The yellow layer of the resulting pellet was rinsed into 200 µl of 10 mM HEPES (pH 7.2) followed by centrifugation at 13 000 rpm for 20 min. The resulted supernatant was the OMF and stored at 4ºC.

Protein concentration in the OMFs was measured using the DC Protein Assay kit (Bio-Rad Laboratories; Richmond, CA) using bovine serum albumin as standard.

2.17 SDS-PAGE

50 ml of exponentially growthing (OD_{750} of 0.35-0.40) *Synechococcus* sp. WH7803 and S-PM2-resistant mutants were collected by centrifugation, washed twice with 10 mM HEPES pH 7.4, and resuspended in 500 µl of the same buffer. Then, 100 µl of sample was mixed with 50 µl of Blue Loading Buffer Pack (New England Biolabs). The sample was heated at 100ºC for 5 min before loading onto a stacking gel (5%) over a separating gel (12%). SDS-PAGE was performed as previously described (Laemmli, 1970) in a vertical slab gel system (C.B.S. Scientific Company, Del Mar, CA) 21 cm long and 1 mm thick with SeeBlue® Plus2 Pre-stained Standard (Invitrogen) as molecular weight markers. The gel was run at 300 V for 8 h at 4ºC and stained overnight with Coomassie Blue with continuous shaking. Destaining was

performed using 45% methanol and 10% acetic acid for 3 h or until individual bands were easily visualised. Images were collected using a scanner (Fujitsu) or using a Geneflash system (SYNGENE).

Alternatively, the Mini-PROTEAN 3 system (BIO-RAD) was used to analyse the outer membrane fractions (OMFs) of *Synechococcus* sp. WH7803, the protein concentration was measured using the DC Protein Assay kit. 90 µg of the sample was loaded onto the gels that were run at 4ºC, 200 V for 1 h and stained with Coomassie Blue with continuous shaking for more than 3 h, then destained for 1 h or until individual bands were easily visualised. SilverQuest^{TM} Silver Staining Kit (Invitrogen) was used for silver staining. The manufacturer's protocol was followed. When the C.B.S. vertical slab gel system was used to analyse the OMFs, 600 µg of the sample was loaded onto the gels. The same procedure was followed as stated above.

2.18 Protein identification and prediction

Protein samples from SDS-PAGE were sent to an outside source for protein identification. **Proteomics and sequence analysis tools** from ExPASy Proteomics Server (http://expasy.org/) were used to translate nucleotide sequences to protein sequences. To determine the subcellular localisation of the target protein the PSORTb program (http://www.psort.org/psortb/) was used.

2.19 Phage neutralisation

10 μl of S-PM2 phage stock at a titre of 1.5×10^8 pfu ml^{-1} was mixed with 10, 20 and 30 μl of the OMF prepared using the method 3, respectively. The mixtures were incubated at 25ºC at 15 μE m^{-2} s^{-1} for 1 h. Immediately after incubation, the mixtures were titred according to Section 2.5. The control mixture was similarly prepared using 30 μl of 10 mM HEPES (pH 7.2). Phage neutralisation experiments were performed in triplicate as two biological replicates. The neutralisation rate (Rn) was expressed as a percentage and calculated as follows:

Rn = (the initial amount of free phages – the amount of free phages after the addition of the OMF)/the initial amount of free phages × 100%

2.20 Molecular techniques

Routine cloning techniques such as restriction enzyme digestion (Fermentas), DNA ligation (New England Biolabs) and TA cloning (Invitrogen) followed the manufacturer's instructions. Routine sequencing service was provided by the University of Warwick sequencing service. When necessary, individual sequencing results were assembled using SEQMAN (DNASTAR, Inc. Madison, Wis.). Sequence comparisons were performed using ClustalX (1.81).

Table 2.12 Buffers and reagents

DNA Gel Loading Buffer (10x)	0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene cyanol, 15% (w/v) Ficoll

Buffer R	10mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg ml^{-1} BSA
Tango™ Buffer	33mM Tris-acetate (pH7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg ml^{-1} BSA
TBE Buffer (10x)	0.89 M Tris-HCl, 0.89 M boric acid, 0.02 M EDTA pH 8
TE Buffer	10 mM Tris-HCl pH 8, 1 mM EDTA
T4 DNA Ligation Buffer (10x)	400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25ºC
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)	20 mg ml^{-1} dimethylformamide
Elution Buffer (EB)	10 mM Tris-HCl, pH8.5

2.20.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was used routinely throughout this study. An optimisation procedure for individual PCR was carried out using 25 µl reaction systems. Typically, the following basic conditions with varied final magnesium concentrations and annealing temperatures were adopted in PCR optimisation.

Table 2.13 PCR optimisation system

2.5 µl	2.5 mM dNTP`s
2.5 µl	10× Buffer
1.0 µl	10 µM Forward primer
1.0 µl	10 µM Reverse primer
A* µl	25 mM $MgCl_2$
0.5 µl	10 ng μl^{-1} template DNA
0.1 µl	5U μl^{-1} Taq Polymerase
X µl	Sterile H_20 to make final volume of 25 µl

Table 2.14 Thermal cyler conditions

2 min	94ºC	1 ×
45 s	94ºC	
45 s	B*ºC	
1 min	72ºC	34×
10 min	72ºC	1×

A* 4 different volumes of $MgCl_2$ were used in PCR optimization system; 1, 2, 3, and 4 µl corresponding to final concentrations of 1 mM, 2 mM and 3 mM and 4 mM. B* 3 different annealing temperatures were used 50ºC, 55ºC and 60ºC.

2.20.2 Primer design

Primers were analysed and selected using Primer Designer (Science Educational Software), the criteria for selecting primers included; a length of 20 bp, a mol G+C content of 50-60%, melting temperature in the range 55-80ºC, no theoretical hairpins or self-priming. In the case of no ideal primers being found (not all criteria were able to be met), the primer set closest to the ideal conditions was selected.

2.20.3 Purification of DNA fragments

A QIAquick PCR purification kit from Qiagen was used to purify DNA fragments ranging in size from 100 bp to 10 kb from a single PCR reaction according to the manufacturer's instructions. Alternatively, a QIAquick Gel Extraction Kit was used to extract DNA fragments ranging in size from 70 bp to 10 kb from the agarose gel derived from PCR reactions and enzymatic reactions according to the manufacturer's instructions.

2.20.4 DNA agarose gel electrophoresis

1.0% (w/v) agarose gels were used unless otherwise stated; gels were run in 0.5 × TBE buffer at 100 volts. Ethidium bromide was added to gels to give a final concentration of 5 $\mu g\,ml^{-1}$.

2.20.5 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* by the use of the QIAprep® Miniprep kit according to the manufacturer's instructions (Qiagen).

2.20.6 Cyanophage DNA extraction

An equal volume of phenol was added to concentrated and purified phage sample obtained in Section 2.9.3, mixed well and left to stand for 2 min before being centrifuged at 15 000 *g* for 5 min. The aqueous layer was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), mixed well and left to stand for 2 min before being centrifuged at 15 000 *g* for 5 min. The aqueous layer was extracted with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), mixed well, left for 2 min and centrifuged at 15 000 *g* for 5 min. The resulting aqueous layer was mixed well with 0.4 volumes of 7.5 M ammonium acetate and 2 volumes of isopropanol, and left on ice for 1 h before being centrifuged at 15 000 *g* for 20 min at 4°C. After removing the supernatant and briefly air-drying, the resulting DNA pellet was washed once with 100 µl of 70% (v/v) ethanol. Finally, the DNA pellet was

recovered by a final centrifugation of 10 min at 15 000 *g*. The dried pellet was dissolved in 50 µl of Elution Buffer (EB, 10mM Tris-HCl, pH 8.5). A NanoDrop® ND-1000 spectrophotometer was used to quantify the phage DNA samples.

2.20.7 RNA extraction from *Synechococcus* sp. WH7803

50 ml samples were collected from exponentially growing *Synechococcus* sp. WH7803 cultures. Cells were recovered by centrifugation at 4 000 *g* for 20 min and were snap frozen in liquid nitrogen and stored at -20°C until RNA extraction.

After thawing and removing excess medium from the cell pellets, they were resuspended in 1.5 ml of TRIZOL reagent (Invitrogen) and incubated for 10 min at room temperature. $CHCl_3$ was then added to a final concentration of 0.2% (v/v) and the samples were shaken vigorously for 30 s before being incubated for a further 10 min at room temperature. After centrifugation at 15 000 *g* for 10 min, the upper (aqueous) phase was precipitated by the addition of 0.5 volumes of isopropanol and left on ice for 12 min. The RNA pellet was recovered after centrifugation at 15 000 *g* for 10 min. The pellet was washed with 1 ml of 75% (v/v) ethanol, and after a final centrifugation at 15 000 *g* for 10 min the supernatant was removed and the pellet air-dried for a maximum of 15 min. The RNA was dissolved in 90 µl of RNase-free water and 10 µl of 10 × DNase buffer (Ambion). Eight units of DNase I (Ambion) were added to the samples which were incubated at 37°C for 20 min. The RNA was purified using an RNeasy Mini Kit (Qiagen) with the addition of an on-column DNase digestion with the RNase-free DNase Set (Qiagen), according to the manufacturer's instructions. In order to eliminate contaminating phage and host genomic DNA the

procedure of DNase I digestion was repeated followed by the RNeasy Mini Kit purification according to the manufacturer's instructions. The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified using a NanoDrop® ND-1000 spectrophotometer.

2.20.8 cDNA synthesis

cDNA synthesis was carried out using Taqman reverse transcription reagents (Applied Biosystems). Each 100 µl reaction volume contained 800 ng of total RNA to which random hexamers were added to a final concentration of 5.0 µM, and 500 µM of each nucleotide triphosphate and 6.0 mM $MgCl_2$ were added. A total of 312.5 units of MultiScribe reverse transcriptase, 0.4 units of RNase inhibitor and 5.0 µl of 10× MultiScribe buffer were also added and amplification of cDNA was carried out for 10 min at 25°C, 60 min at 42°C and 5 min at 95°C in a Biometra thermal cycler.

2.20.9 DNA extraction from *Synechococcus* sp. WH7803

20-30 ml of cells in the late exponential phase of growth (OD_{750}>0.5) were harvested by centrifugation at 4 000 *g* for 20 min. The cell pellets were washed once with 2.0 ml of sterile TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) and resuspended in a lysis buffer containing 567 µl of TE, 30 µl of 10% (w/v) SDS solution and 3 µl of proteinase K (20 mg/ml). This solution was mixed thoroughly by gentle inversion prior to incubation at 60ºC for 4 h. 100 µl of 5M NaCl and 80 µl of 10% CTAB/NaCl solution (prepared by adding 10 g CTAB to 100 ml of 0.7 M NaCl while agitating on

a heated stirrer) were the added to the solution and incubated at 65ºC for a further 10 min. The mixture was allowed to cool for 30 s before adding one volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and mixed by gentle inversion. After centrifugation (15 000 *g* for 10 min), the supernatant (aqueous phase containing DNA) was transferred to a sterile eppendorf tube followed by extraction with one volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifugation at 15 000 *g* for 10 min. 0.6 volume of isopropanol was then added to the resulting supernatant. After mixing by gentle inversion, DNA was precipitated at 15 000 *g* for 10 min at 4ºC. The resulting pellet was then washed in progressive ethanol concentrations; 50%, 75% and 100%. Finally, the DNA pellet was air-dried at room temperature for a maximum 10 min prior to dissolving in 80 µl of sterile water or EB buffer.

2.20.10 Construction of gene knockout mutants of *Synechococcus* sp. WH7803

2.20.10.1 Suicide plasmind pYJ01 and pYJ02 construction

The broad host range suicide plasmid pMUT100 (Brahamsha, 1996) was used to inactivate ORF0948 (coding for a putative multicopper oxidase) from *Synechococcus* sp. WH7803. To do this, PCR primers (table 2.15) with *EcoR*I enzyme digestion sites were designed to amplify two DNA fragments, ORF0948-500 (407 bp in length) and ORF0948-1000 (370 bp in length), within ORF0948. The gel-purified PCR products were then introduced into the multiple cloning sites in the plasmid pMUT100 using *EcoR*I.

The first step in this process was to clone the PCR products into the commercially available plasmid pCR2.1 using a TA cloning kit (Invitrogen) according to the manufacturer's instructions. Then, the PCR products of the ORF0948 fragments were cut out of the recombinant pCR2.1 using the following enzyme digestion system: 2 µl *EcoR*I (Fermentas), 4 µl 10× buffer *EcoR*I, and 1.5-2 µg recombinant pCR2.1™ DNA. This system was kept at 37ºC for 1 h. Meanwhile, 1-2 µg of the plasmid pMUT100 was also digested at 37ºC for 1 h using a system consisting of 3 µl *EcoR*I, and 4 µl 10× buffer *EcoR*I. After enzyme digestions, the plasmid pMUT100 was treated with calf intestine alkaline phosphatase (New England Biolabs) as previously described (Sambrook and Russell, 2001); the ORF0948 fragments were gel-purified.
These fragments were ligated with T4 DNA Ligase according to the manufacturer's instructions.

The resulting ligation reaction mixtures were used to transform competent DH5α cells (Laboratory's own collection) as previously described (Sambrook and Russell, 2001), using kanamycin (50µl/ml) as a selection. The nature of the putative recombinant plasmids was confirmed by amplifying two ORF0948 fragments using the primer sets shown in Table 2.15. In addition, sequencing (provided by the University of Warwick sequencing service) was performed using the primers (pMUTsequencing5516: TGCCACCTGACGTCTAAGAA). Thus plasmid pYJ01 (plasmid pMUT100 harbouring the DNA fragment amplified with PCR primers ORF0948-500) and pYJ02 (plasmid pMUT100 harbouring the DNA fragment amplified with PCR primers ORF0948-1000) were constructed.

Table 2.15 PCR primers for the ORF0948

Names	Forward 5'-3'	Reverse 5'-3'
ORF0948-500	ACTGTCCGCAGACTTGCTAA	GGCAGCGTAAGAATGACATC
ORF0948-1000	GAACGCAGTTGGAGTTGTTG	GCCATAGGTGCTGTTGCTAA

2.20.10.2 Conjugation

The recombinant plasmids (pYJ01 and pYJ02) were electroporated into *E. coli* MC1061 (containing plasmids pRK24 and pRL528) strains using Gene pulser II system (BioRad Laboratories, Richmond, CA) according to a published method (Brahamsha, 1996). LB agar plates supplemented with ampicillin (100 µg/ml), chloramphenicol (10 µg/ml) and kanamycin (50 µg/ml) were used to screen for successfully transformed *E. coli* MC1061 strains by incubating overnight at 37ºC. The resulting *E. coli* MC1061 single colonies were grown in LB medium supplemented with ampicilin (100 µg/ml), chloramphenicol (10 µg/ml) and kanamycin (50 µg/ml) overnight at 37ºC with vigorous aeration. 1 ml of culture was centrifuged, washed 3 times in 1 ml of fresh LB medium, and resuspended in 500 µl of SN (or ASW) containing 10% (vol/vol) LB broth. Cells from 50 ml of exponentially growing WH7803 culture (OD_{750} between 3.5 and 4.0) was harvested and resuspended in 150 µl of SN (or ASW). 10-50 µl of *E. coli* MC1061 was mixed with 50 µl of cyanobacterial suspension to obtain serial conjugation ratios and spotted immediately onto a week-old SN (or ASW) plate containing 0.3% (w/v) agar. These plates were kept on the bench at room temperature for approximately 2 h and were then incubated at a light intensity of 10 µE m^{-2} s^{-1} at 25ºC.

After a 2-day incubation, the inoculated areas were cut out of the agar with a sterile stainless steel spatula and placed in 2 to 10 ml of SN (or ASW). Then, the phage T4 (Laboratory's own collection) was added and the suspension was left overnight at room temperature to allow T4 to kill the remaining *E. coli* and the *Synechococcus* cells to diffuse into SN (or ASW) liquid. After serial dilutions, pour plating was performed according to Section 2.14 using 0.3% SN (or ASW) agar (wt/vol) containing 25 μg/ml kanamycin. Within 3 months, single colonies were transferred from the agar plates into 2 ml of SN (or ASW) liquid medium containing kanamycin (15 mg/ml), and cultured at a light intensity of 10-25 μE m^{-2} s^{-1} at 25ºC.

Chapter 3 Investigation of the effect of light and dark on cyanophage adsorption to the host *Synechococcus* sp. WH7803

3.1 Introduction

3.1.1 Enumeration of marine viruses

A variety of methods have been developed for estimating total viral abundance in marine samples. Among these techniques, plaque assay (PA) measures infectivity of phages by counting plaques appeared on solid medium; the most probable number (MPN) is a method of getting quantitative estimation of phage titres from serial diluted phage suspensions in liquid culture (Borsheim, 1993; Waterbury and Valois, 1993a). They have the advantage of examining only viruses that are infective for a specific host. However, this is also a major limitation, because the host strain used in plaque assays and MPN methods will by no means have the same susceptibility to infection by all viruses present in a natural sample (Suttle and Chan, 1993a; Waterbury and Valois, 1993a; Wilson *et al.*, 1993a). Additionally, the use of culture-based methods to examine viral abundance is biased as it only detects viruses that can infect hosts that can be cultured and it is estimated that < 1% of organisms present in the oceans can be cultured (Rodriguez-Valera, 2004).

The most direct method of detecting marine viruses is Transmission Electron Microscope (TEM). TEM uses a beam of electrons instead of light. It was the use of TEM that allowed the first discovery of high viral abundance in the marine environment (Bergh *et al.*, 1989; Proctor and Fuhrman, 1990). However, the major disadvantages for TEM lie in its inability to differentiate viral particles that will infect different organisms and the time consuming process.

Alternatively, direct counting can be done by using epifluorescence microscopy (EFM), in conjugation with nucleic acid-specific stains such as Yo-Pro and SYBR Green (Hennes and Suttle, 1995; Noble and Fuhrman, 1998). EFM uses light from the objective lens to excite the stains in a sample, rather than through the sample towards the objective lens. The advantage of viral counts by EFM compared with the use of TEM is that it is less time consuming and more cost effective. However, counting using DNA stains gives much higher concentrations than using TEM methods (Hennes and Suttle, 1995; Noble and Fuhrman, 1998), which has been attributed to the staining of particles other than viruses. Yo-Pro and SYBR Green-based viral counting methods only allow total viral numbers to be obtained and cannot distinguish individual viral populations.

3.1.2 Flow cytometric detection of marine viruses

Flow cytometry (FC) can be defined as a technique for analysing the light scatter and fluorescence emitted from individual particles and cells as they pass single-file through an intensely focused light source (i.e. a laser) (Campbell, 2001). As illustrated in Figure 3.1, the sample is injected into the sheath where the particles are hydrodynamically focused in a laminar flow and aligned in single file before intercepting a light source. As the cells or particles of interest intercept the light source they scatter light and fluoresce. The scattered and fluoresced light is sensed and converted to electrical signals by electronic detectors. Thus, in contrast to spectrophotometry where fluorescence is measured for a bulk volume of sample, flow cytometry measures fluorescence per cell or particle.

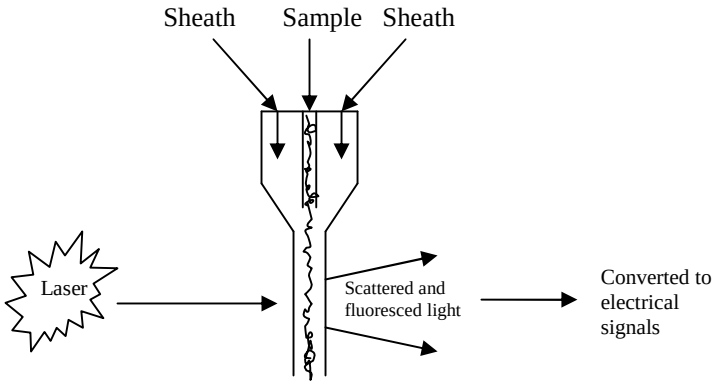


Figure 3.1 A simplified illustration of flow cytometry.

The sample is injected into the sheath. Hydordynamic focusing will insure cells are in single file to flow through the sensing path under the illumination of a laser light. The signals resulting from light scatter and fluorescence is collected and analysed (Campbell, 2001).

Several parameters can be collected through flow cytometric analysis using a Becton-Dickinson FACSort. These include forward scatter (FSC) and side scatter (SSC), which index size and internal complexity or granularity, respectively, three typical fluorescence parameters including red (FL3), orange (FL2) and green (FL1) wavelengths, as defined by the optical filters.

The idea of counting virus particles using FC was previously suggested to be impossible because of their small size (Porter *et al.*, 1997). FC has been used to enumerate and discriminate the different picophytoplanktonic populations, including marine *Synechococcus* (Olson *et al.*, 1985). The discovery of *Prochlorococcus* was made possible only because of the introduction of flow cytometric analysis into picoplankton research (Chisholm *et al.*, 1988). In conjunction with the nucleic acid staining dyes, such as SYBR Green that fluoresce at 488 nm, FC has been found to be a promising technique in enumerating marine viruses in natural samples (Marie *et al.*,

1999; Brussaard *et al.*, 2000). Apart from high correlation to counts obtained by TEM and EFM, FC also showed a potential capability to distinguish two and sometimes three distinct virus populations (Marie *et al.*, 1999). Similarly, two populations of VLPs were identified by FC in an attempt to assess the contribution of viral lysis to the mortality of a *Micromonas* spp. population (Evans *et al.*, 2003).

Although the application of FC alone seems to have made counting total virus numbers less time consuming (Brussaard *et al.*, 2000), EFM and TEM can still provide complementary information, such as viral counts by FC were generally higher than those obtained by TEM and EFM (Marie *et al.*, 1999). Indeed, different virus counting methods can result in different estimation of viral numbers (Marie *et al.*, 1999). Moreover EFM and FC generally give higher viral estimates than those estimated by TEM (Weinbauer and Suttle, 1997; Marie *et al.*, 1999). In addition, the use of different fluorochromes can lead to different estimates for the same sample (Bettarel *et al.*, 2000). Thus, comprehensive optimisation procedures are proposed to minimise the errors associated with counting with a FC (Brussaard, 2004).

In addition to culture-based and direct counting methods, viral abundance has been estimated by reverse transcriptase PCR (RT-PCR) (Rose *et al.*, 1997). Compared to the traditional plaque assay for viral detection, the RT-PCR method has been shown to be useful in detecting large amounts of non-cultivatable viruses. However, problems like existence of RT-PCR inhibitors and the inability of detection of low levels of coliphage in samples were present (Rose *et al*., 1997).

3.1.2 Phage adsorption

The first key step in the phage-host interaction is adsorption. This is a diffusion-limited process that relies on protein(s) (adhesin) on the phage recognising and binding to a molecule on the bacterial cell surface (receptor). Generally, this process does not require energy. After the phage has attached to the host cell, energy is required for the entry of the phage nucleic acid into the bacterial cytoplasm, leaving the phage capsid outside of the host cell. In the natural environment, light-dark cycles can potentially affect the first step of cyanophage-host (cyanobacteria) interactions because light induces a completely distinct host physiological status, which may induce changes in the properties of the cell surface. Thus, it is important to know how the first step of cyanophage-host interactions might be affected by the shift from light to dark. This chapter deals with the effect of light on cyanophage adsorption.

Limited research has been performed with regard to this aspect of cyanophage biology. Cyanophage AS-1 adsorption to its host was found to be significantly dependent on light, though this dependence could be reduced by increasing the concentration of Na^+ ions (Cseke and Farkas, 1979b). Lipopolysacharride (LPS) and proteins have been shown to be involved in the process of cyanomyovirus AS-1 adsorption, based on the finding that purified polysaccharide can partially inactivate AS-1 and protease treatment of proteins decreased the ability to inactivate AS-1 (Samimi and Drews, 1978). A recent study on short-term variations in the abundance of natural cyanophage populations of the Indian Ocean revealed that the highest number of free phages was after midnight (0100 h) (Clokie *et al.*, 2006a). A reasonable interpretation

is that this represents a time when phages reduce/stop adsorption to the host cells, and phages adsorbed during the light period are being released.

Many of the previous studies on the relationship between photosynthesis and cyanophage infection were carried out using chemical inhibitors such as photosynthetic inhibitor, DCMU, and photosynthetic uncoupler, CCCP. DCMU inhibits photosynthetic electron transport at the acceptor side of photosystem II (PSII) by binding to the D1 protein of PSII competing with quinone for binding to the Q_B site and thereby inhibiting electron transport from the primary quinone acceptor Q_A to the secondary quinone acceptor Q_B. CCCP is an uncoupler that abolishes both photosynthetic and respiratory ATP production by breaking down the proton motive force (PMF).

In the case of the lytic cyanophage AS-1 infection of the unicellular cyanobacterium *Synechococcus* sp. strain PCC 6301 (formerly *Anacystis nidulans*) (Allen and Hutchison, 1976), DCMU treatment reduced the phage yield by 73%, for which a reasonable explanation was that cyclic photophosphorylation via PSI could support a reduced level of phage production. The uncoupler CCCP, not surprisingly, completely abolished phage production. Compared to the limited degree of phage AS-1 replication in the presence of DCMU, cyanophage SM-1 replication following infection of *Synechococcus* strain NRC-1was completely abolished by DCMU treatment at a concentration of 10^{-6} M (MacKenzie and Haselkorn, 1972). Thus, replication of SM-1 was completely dependent on a fully functional photosynthetic apparatus and could not be even partially sustained by cyclic phosphorylation.

3.2 Aims

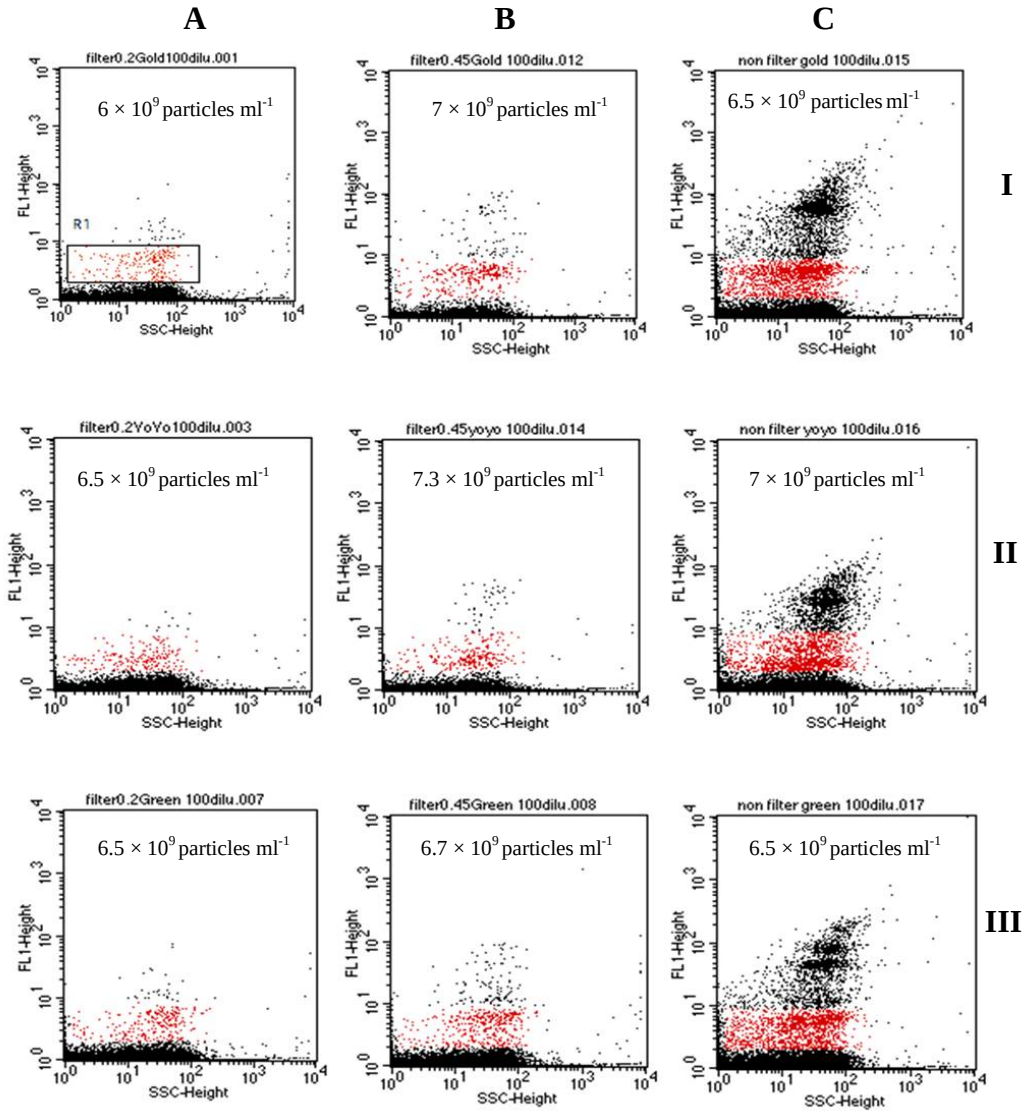
To determine the role of light in the process of cyanophage adsorption to *Synechococcus* sp. WH7803

3.3 Results

3.3.1 Flow cytometric analysis of the phage lysate

In order to clearly define the phage S-PM2 population, CsCl-purifed S-PM2 with a titre of 2.5×10^{10} puf ml^{-1} were stained using three different DNA-specific fluorescent dyes, SYBR Green I, YOYO-1 and SYBR Gold. The results are shown in Figure 3.2. The horizontal and vertical axes represent relative side scatter and relative green fluorescence, respectively. Row I, II and III are S-PM2 stained with SYBR Gold, YOYO-1 and SYBR Green I, respectively. Column A and B are S-PM2 samples subjected to filtration with 0.2 μm and 0.45 μm syringe filters (Sartorium Minisart) before staining. Column C is S-PM2 sample without filtration. As seen in Figure 3.2, filtered S-PM2 samples showed a cluster above the background noise. This region was gated and their numbers were calculated and shown inside each plot. However, non-filtered S-PM2 samples showed at least another population representing particles larger than S-PM2. These particles can be removed using 0.2 or 0.45 μm filters (Figure 3.2). These 'impurities' can't be bacteria because the S-PM2 has been purified using CsCl ultracentrifugation. They may be S-PM2 aggregates. The titres based on different combinations of dyes and filtrations were very similar and in the range of 6

$\times 10^9$ to 7.3×10^9 particles ml^{-1}. No particles were detected from the control experiment in which ASW was diluted with TE buffer pH8 and stained using SYBR Green I (Figure 3.2D). However, when the same gated region was applied to non-stained S-PM2 samples (Figure 3.2E), a titre of 6×10^8 particles ml^{-1} was detected.



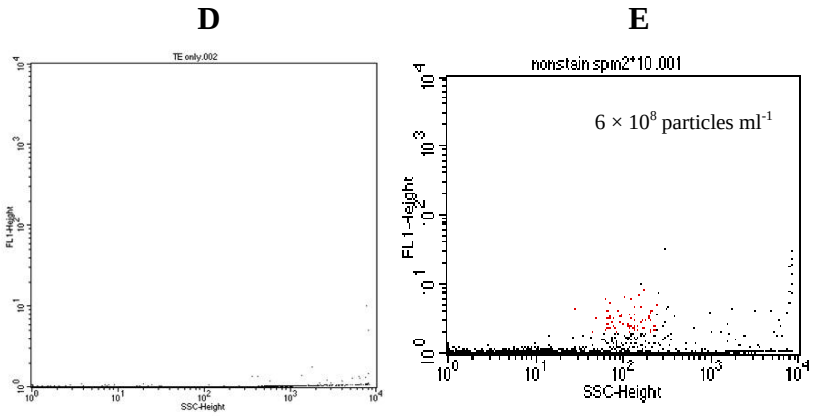


Figure 3.2 Cytograms (4 decades log scale) of green fluorescence (Y Axis) versus side scatter (X Axis) for CsCl-purified phage S-PM2 (AI to CIII) and the controls (D and E).

(**AI to CIII**), CsCl-purified S-PM2 samples were subjected to filtration using 0.2 and 0.45 μm pore size syringe filter, fixed with glutaraldehyde at a final concentration of 0.1% (v/v), frozen in liquid nitrogen, diluted in TE buffer pH8, stained for 10 min at 80ºC with three different dyes, SYBR Green I, SYBR Gold and YOYO-1 at final dilution of 5 $\times 10^{-5}$ dilutions of commercial stock, respectively. (**D**), ASW (control) samples were subjected to filtration using 0.2 and 0.45 μm pore size syringe filter, fixed with glutaraldehyde at a final concentration of 0.1% (v/v), frozen in liquid nitrogen, diluted in TE buffer pH8, stained for 10 min at 80ºC with SYBR Green I at final dilution of 5 $\times 10^{-5}$ dilutions of commercial stock. (**E**), CsCl-purified S-PM2 samples were subjected to filtration using 0.2 μm pore size syringe filter, fixed with glutaraldehyde at a final concentration of 0.1% (v/v), frozen in liquid nitrogen, diluted in TE buffer pH8. The gated region for analysis was marked red.

When a fresh S-PM2 lysate (which was passed through a 0.2 μm filter) was used for flow cytometric analysis S-PM2 population was detected in the same gated region after staining with SYBR Green I, although this S-PM2 signature was not as discrete as CsCl-purified ones (Figure 3.3A). The count was 7.4×10^7 particles ml^{-1}. The count based on a stained blank (Figure 3.3B) was 1.3×10^7 particles ml^{-1}. This is probably due to non-specific staining of cellular debris and auto fluorescence of the water soluble photosynthetic pigments of *Synechococcus* sp. WH7803, such phycoerythrins (Ong *et al.*, 1984). There were also some particles above the S-PM2 population (indicated by a blue circle) (Figure 3.3A). These were probable due to aggregations of phage particles.

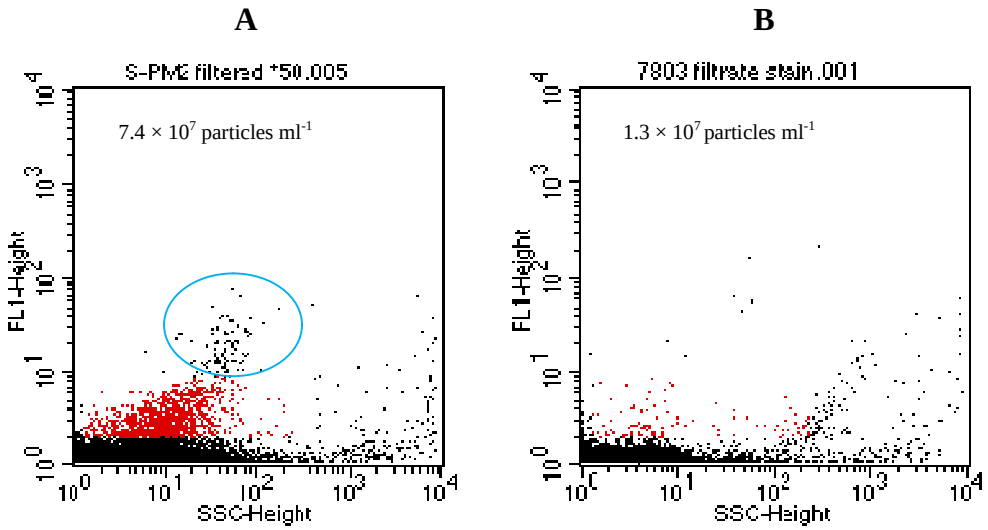


Figure 3.3 Cytograms (4 decades log scale) of green fluorescence (Y Axis) versus side scatter (X Axis) for cell-free crude phage lysate.

(**A**), The S-PM2 lysate after filtration with 0.2 µm syringe filter was fixed with glutaraldehyde at a final concentration of 0.1% (v/v), frozen in liquid nitrogen, diluted in TE buffer pH8, stained for 10 min at 80ºC with SYBR Green I at a final dilution of 5×10^{-5} dilutions of commercial stock. (B), A stained blank was WH7803 culture (filtered through 0.2 µm) stained with SYBR Green I. The gated region for analysis was marked red. Particles inside blue line may represent aggregated phage particles.

In order to establish that this staining protocol for flow cytometric analysis of phages can function well, the same methodology was applied to a *Streptomycete* phage Φc31 (Alexander and McCoy, 1956) in crude phage lysate (Courtesy of Dr. Leonides A Calvo-Bado). As seen in Figure 3.4A, a tight phage population was detected. No phage populations were detected from a stained blank (Figure 3.4B).

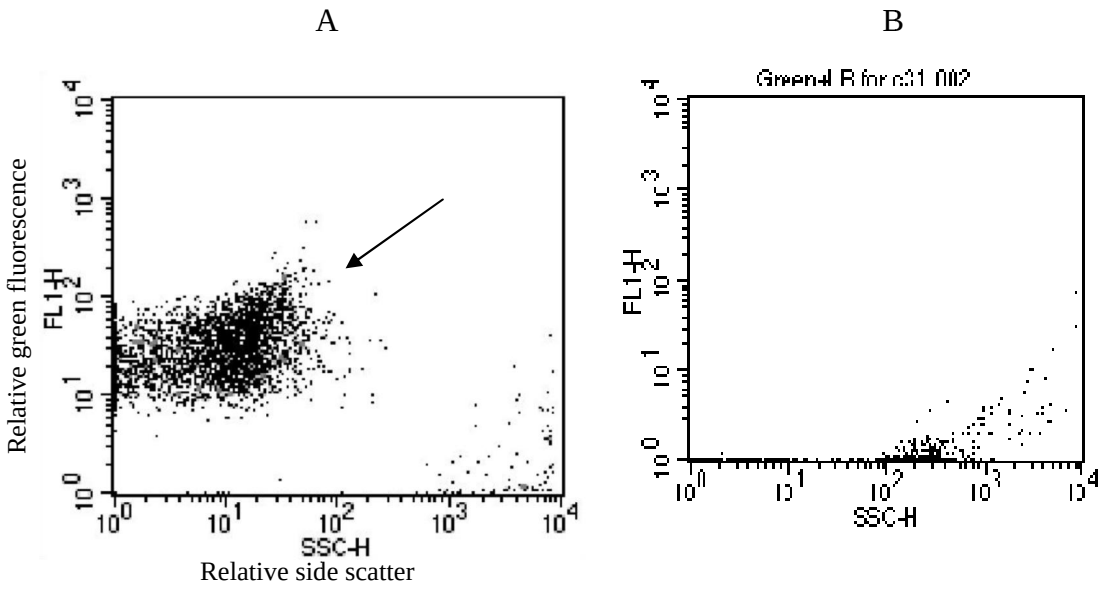


Figure 3.4 Cytograms (4 decades log scale) of green fluorescence (Y Axis) versus side scatter (X Axis) for cell-free crude phage lysate.

(**A**), The *Streptomycete* phage Φc31 lysate after filtration with 0.2 μm syringe filter was fixed with glutaraldehyde at a final concentration of 0.1% (v/v), frozen in liquid nitrogen, diluted in TE buffer pH8, stained for 10 min at 80ºC with SYBR Green I at a final dilution of 5 $\times 10^{-5}$ dilutions of commercial stock. (B), A stained blank was *Streptomycete* culture (filtered through 0.2 μm) stained with SYBR Green I. The phage population was detected as a discrete cluster (indicated by an arrow).

3.3.2 The role of light in the cyanophage S-PM2 adsorption to *Synechococcus* sp. WH7803

Using a model system consisting of the marine cyanomyovirus S-PM2 and *Synechococcus* sp. WH7803, the adsorption of cyanophage S-PM2 to the host WH7803 was found to be rapid in the light; nearly 90% of the phages were adsorbed to the cells within 45 min (Figure 3.5). There was hardly any further adsorption for a further 15 min, and a plateau was reached (Figure 3.5). The abrupt increase of the number of unattached phages at around 6 h post-infection is due to the release of

progeny phages from the infected host cells (Figure 3.5). Compared to the rapid adsorption in light, the adsorption in dark was considerably slower; about 15% of the phages were adsorbed within 3 h (Figure 3.5). However, the adsorption resumed almost instantaneously upon illumination 3 h post-infection in the dark (Figure 3.5).

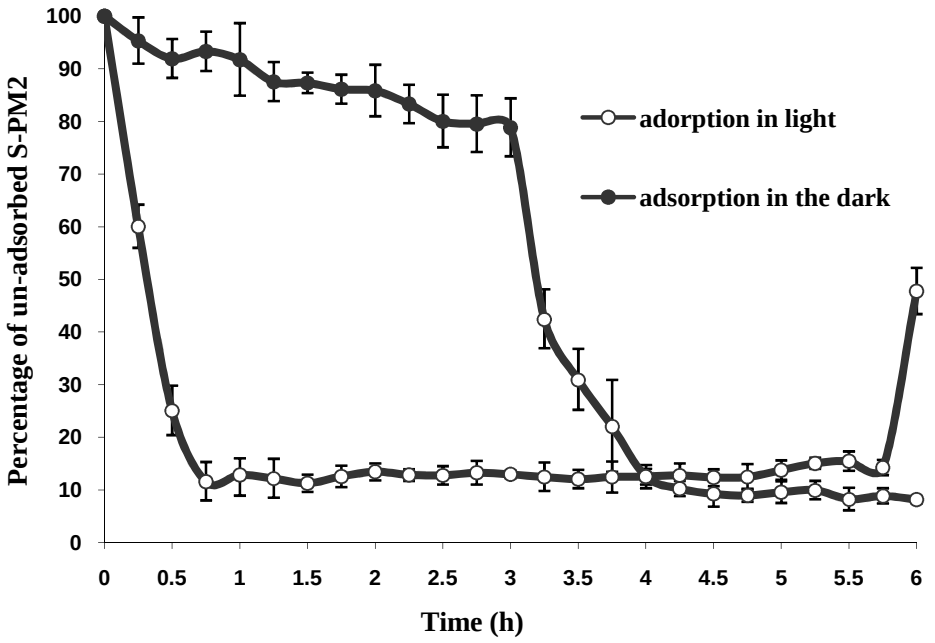


Figure 3.5 Effect of light on the adsorption of cyanophage S-PM2 to *Synechococcus* sp. WH7803 cells.

Synechococcus cells (OD_{750}= 0.35) in the logarithmic growth phase were incubated with phage at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed every 15 min for 6 h in the cell-free supernatant remaining after centrifugation. Empty circles (○) represent phage adsorption in the light and solid circles (●) represent phage adsorption in the dark. Data are the mean of three biological replicates with standard deviation (SD) values.

3.3.3 Investigation of the role of light in the adsorption of 8 other cyanophages to *Synechococcus* sp. WH7803

In order to get an idea of whether this light-dependent phage adsorption is a universal phenomenon in the process of cyanophage infection or unique to the cyanophage S-PM2, a simplified version of this light/dark adsorption experiment was applied to 8 other cyanophages by examining the number of un-adsorbed phages 45 min post-infection. All these cyanophages can infect the host strain *Synechococcus* sp. WH7803 and form plaques. Figure 3.6 shows the proportions of un-adsorbed phages either in the light or dark. On the basis of their adsorption patterns, three different groups can be identified. Four of the phages (S-PWM3, S-BP3, S-BnM1 and S-PM2) show light-dependent adsorption (~ 90% adsorption in the light, ~ 10% adsorption in the dark 45 min post-infection). One cyanophage, S-MM5, shows a decreased adsorption rate that was light/dark-independent (~ 50% adsorption in light/dark 45 min post-infection). The other four cyanophages (S-MM1, S-MM4, S-BM3 and S-PWM1) display a light-independent adsorption pattern with a markedly decreased adsorption rate (~ 10% adsorption in light/dark 45 min post-infection). These data show that light plays an important role in the cyanophage adsorption and suggest that at least some of the cyanophage in the natural environment may display the light-dependent adsorption.

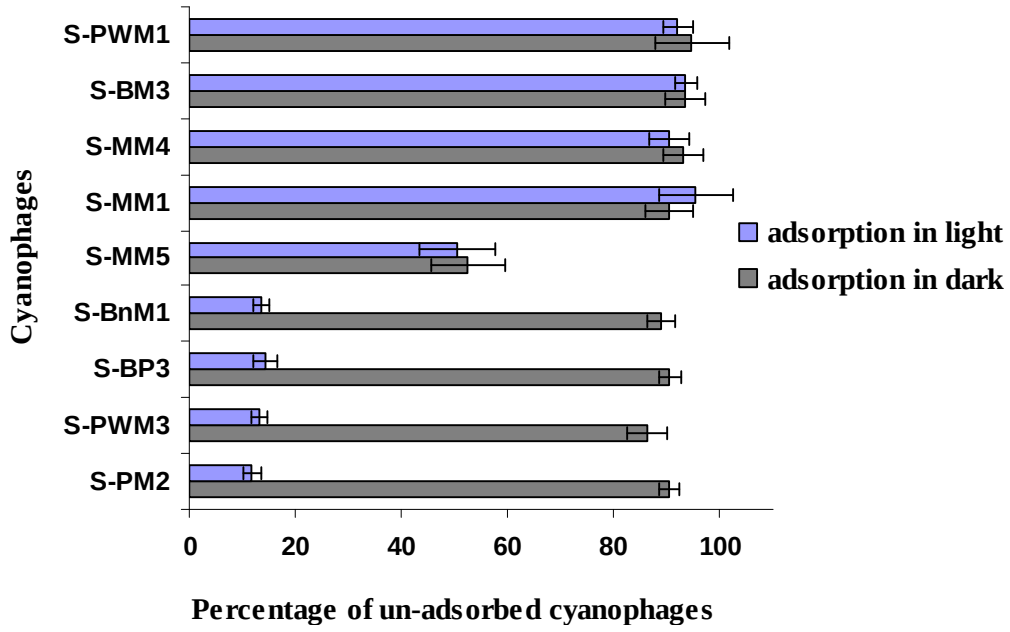


Figure 3.6 Adsorption of different cyanophages to *Synechococcus* sp. WH7803 cells under illumination and in darkness.

Synechococcus cells (OD_{750}= 0.35) in the logarithmic growth phase were incubated with different phage strains at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1} and in the dark respectively. The free phage titer was assayed 45 min post-infection in the cell-free supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

3.3.4 Light wavelength-associated cyanophage adsorption

As light is a type of electromagnetic radiation that exists at a wide range of wavelengths, cyanophage adsorption under different wavelengths was studied. As shown in Figure 3.7, no marked differences in the phage adsorption patterns were detected by exposure to blue (460 nm-490 nm), green (500-570 nm) or yellow (580-590 nm) light compared to the adsorption kinetics in white light (460-620 nm). However, cyanophage adsorption in red light showed a much lower rate. Interestingly, in *Synechococcus* sp. WH7803, red light (785-620 nm) is the only wavelength that can not be harvested by the photosynthetic light-harvesting system to fuel

photosynthesis owing to the constitution of the light-harvesting pigments, which have absorption maxima lie between 490 and 570 nm (Ong and Glazer, 1991; Swanson *et al.*, 1991)). The red light's inability to drive S-PM2 adsorption suggests that a functional photosynthesis of the host may be required for cyanophage adsorption.

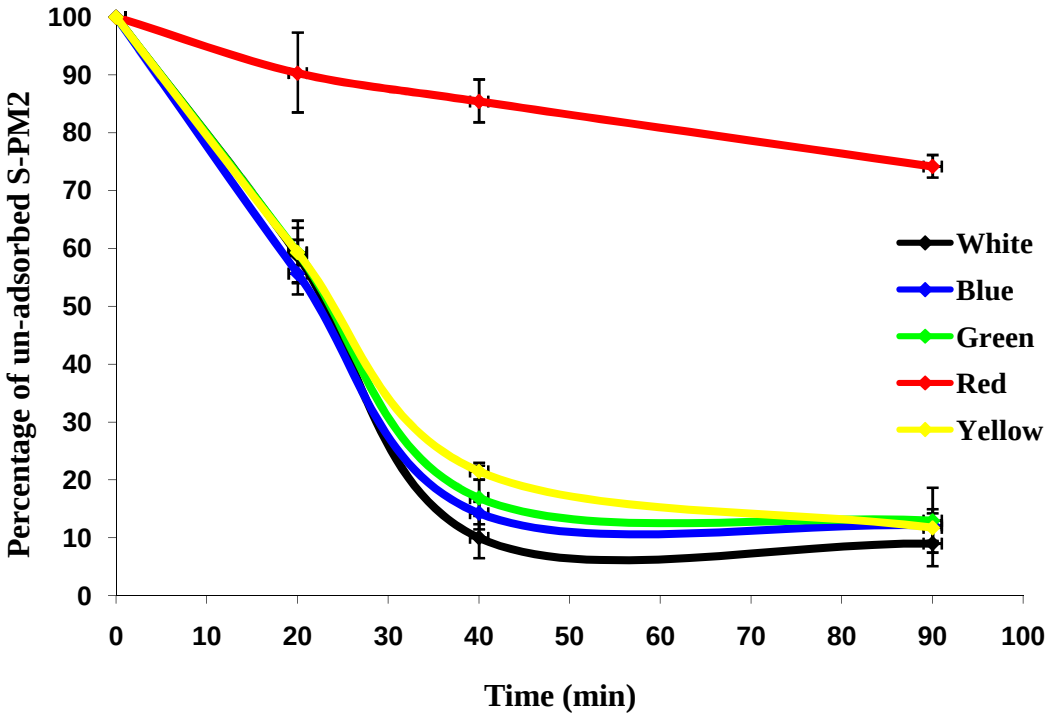


Figure 3.7 Effect of wavelengths on the adsorption of cyanophage S-PM2 to *Synechococcus* sp. WH7803 cells.

Synechococcus cells (OD_{750}= 0.35) in the logarithmic growth phase were incubated with phage at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1} and were subjected to white (460-620 nm), blue (460-490 nm), green (500-570 nm), red (785-620 nm), or yellow light (580-590 nm). Aliquots were withdrawn at 20, 40 and 90 min post-infection and the free phage titer was determined in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

3.3.5 S-PM2 can still adsorb to DCMU/CCCP-treated *Synechococcus* sp. WH7803

In order to investigate whether photosynthesis of the host has a role in S-PM2 adsorption to *Synechococcus* sp. WH7803, photosynthesis inhibitors, DCMU, and uncoupler, CCCP, were used to treat cells prior to adsorption. At time intervals, free phage was titered. Two control reactions were included in this experiment. Control 1 was using non-treated cells. Control 2 was the same as control 1 except for the inclusion of ethanol at a concentration of 0.5% (v/v) due to the fact that ethanol was used to prepare the DCMU and CCCP solutions. As seen in Figure 3.8, no alternations were detected when comparing S-PM2 adsorption kinetics under different conditions and a plateau was reached after 1 h post-infection. This demonstrates that DCMU and CCCP treatment of the host cell did not influence S-PM2 adsorption kinetics. When the whole experiment was repeated in the dark, no adsorption was observed (Figure 3.9).

As DCMU/CCCP causes damage to the host photosynthetic energy production system, it is reasonable to infer that DCMU/CCCP treatment will abolished S-PM2 propagation although they can still adsorb to *Synechococcus* sp. WH7803. As S-PM2 has a latent period of 9 h for infecting a culture of WH7803 (Wilson *et al.*, 1996), samples were taken at 19 and 24 h post-infection to count un-adsorbed S-PM2 using plaque assay. No plaques were detected that meant no phage progenies were produced. In contrast, the positive control reaction (control 1) produced a typical one-step growth curve of S-PM2 on *Synechococcus* sp. WH7803, which agreed with a previous report (Wilson *et al.*, 1996).

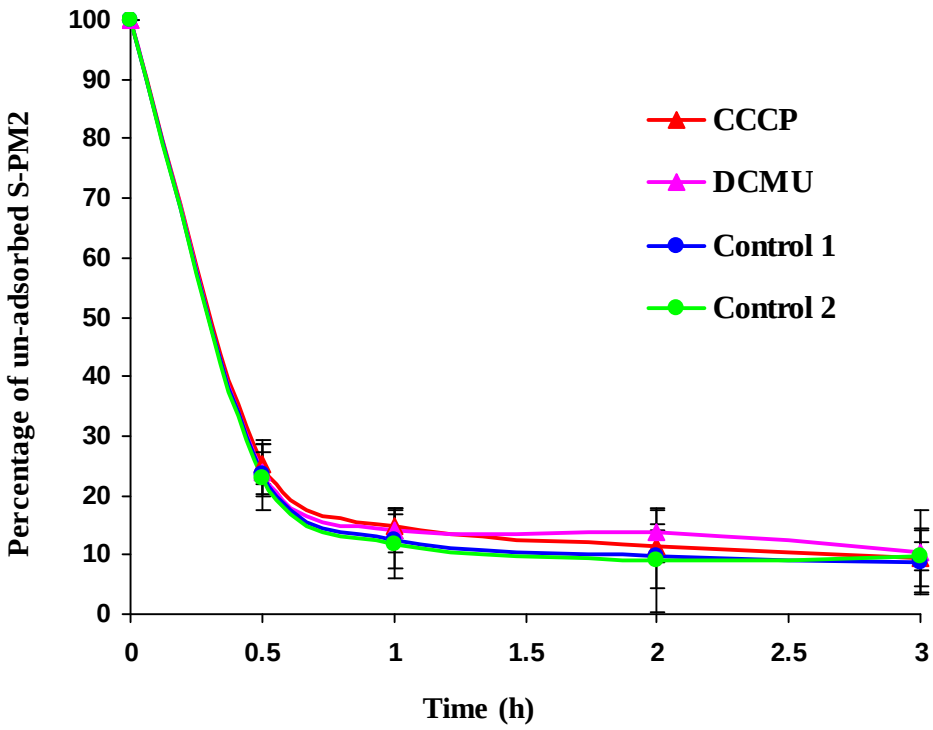


Figure 3.8 Cyanophage S-PM2 adsorption to DCMU (10^{-5}M) / CCCP (10^{-5}M) treated *Synechococcus* sp. WH7803 cells in constant light.

In the presence of DCMU/CCCP, *Synechococcus* cells in the logarithmic growth phase ($OD_{750} \geq 0.35$) were incubated with phage at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed at 0.5, 1, 2, and 3 h in the supernatant remaining after centrifugation. Control 1 was S-PM2 adsorption to non-treated WH7803 cells. Control 2 was S-PM2 adsorption to non-treated WH7803 cells in the presence of ethanol (0.5% v/v) due to the use of ethanol in preparing the DCMU and CCCP solutions. Data are the mean of three biological replicates with SD values.

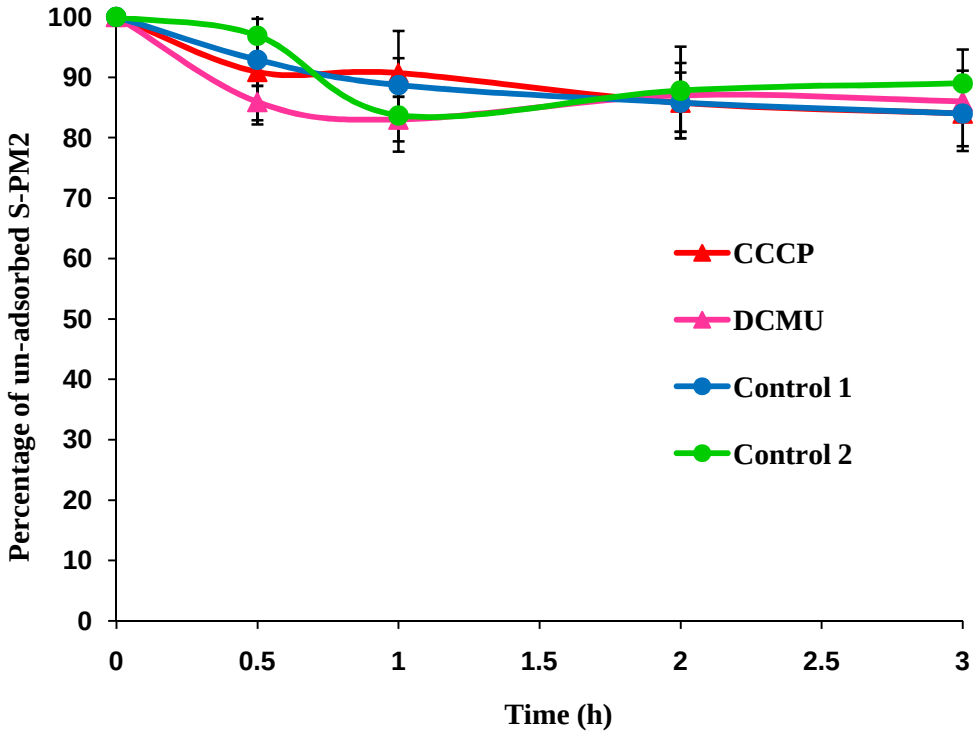


Figure 3.9 Cyanophage S-PM2 adsorption to DCMU (10^{-5}M) / CCCP (10^{-5}M) treated *Synechococcus* sp. WH7803 cells in the dark.

In the presence of DCMU/CCCP, *Synechococcus* cells in the logarithmic growth phase ($OD_{750} \geq 0.35$) were incubated with phage at a MOI of 0.02 in the dark. The free phage titer was assayed at 0.5, 1, 2, and 3 h in the supernatant remaining after centrifugation. Control 1 was S-PM2 adsorption to non-treated WH7803 cells. Control 2 was S-PM2 adsorption to non-treated WH7803 cells in the presence of ethanol (0.5% v/v) due to the use of ethanol in preparing the DCMU and CCCP solutions. Data are the mean of three biological replicates with SD values.

3.3.6 Cyanophage adsorption to *Synechococcus* host strains grown under an artificial 24-h light-dark (LD) cycle

Light/dark cycles are environmental stimuli that synchronize the circadian clock and it has been demonstrated that cyanobacteria possess an endogenous 24-hour circadian clock, which regulates cell division, nitrogen fixation, photosynthesis, amino acid uptake, carbohydrate synthesis and respiration (Sweeney and Borgese, 1989; Lorne *et*

al., 2000; Golden, 2003). *Synechococcus* sp. WH7803 has been demonstrated to be readily entrained to an artificial 24 hour LD cycle (Sweeney and Borgese, 1989). How this circadian clock in WH7803 influences diel phage adsorption has never been studied. In order to address the question of light-dependent phage adsorption, phage adsorption to entrained *Synechococcus* strains was investigated.

As shown in Figure 3.10, S-PM2 adsorption to cells derived from 6 different time points (Figure 2.1) over a 12 h – 12 h LD cycle followed the same pattern: ~ 90% adsorption in the light and ~ 10% adsorption in the dark. These data clearly show that no matter what stage the cells were withdrawn from phage adsorption followed the same simple rule of light-dependent adsorption. This indicates that the circadian rhythm of the host cells did not have a role in S-PM2 adsorption.

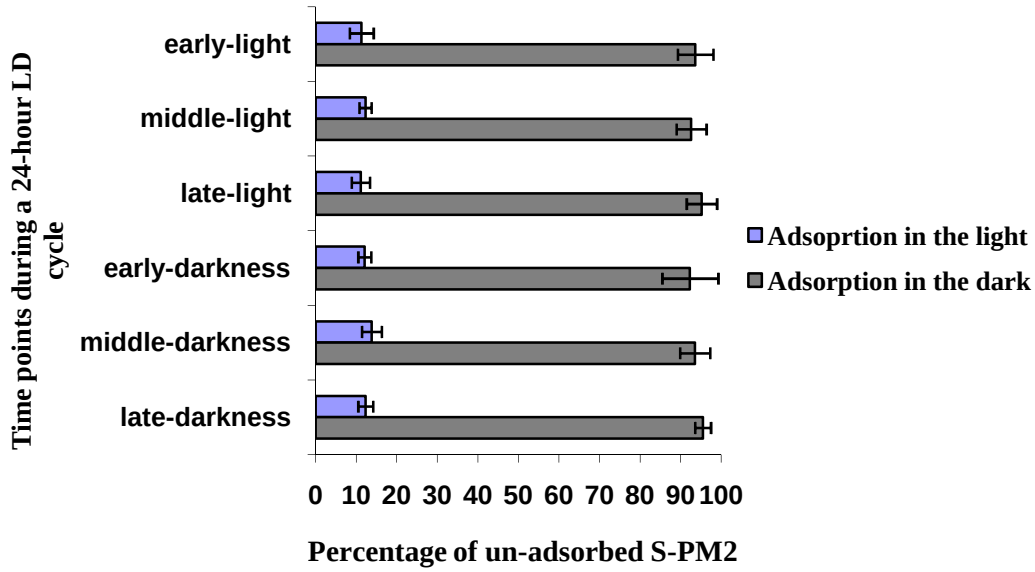


Figure 3.10 Adsorption of cyanophage S-PM2 to *Synechococcus* sp. WH7803 cells under a modulated LD cycle.

Synechococcus cells (OD_{750}= 0.35) grown under 12:12 h LD cycle were incubated with S-PM2 at a MOI of 0.02 in light (15 μE m^{-2} s^{-1}) and dark at 25ºC, respectively. The free phage titer was assayed 45

min post-infection in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

3.3.7 Investigation of the role of the *psbA* gene in the light-dependent cyanophage adsorption to *Synechococcus* sp. WH7803

As many, but not all marine cyanophages, have been found to contain the gene *psbA*, coding for the photosynthetic reaction centre protein D1 (Millard *et al.*, 2004; Sullivan *et al.*, 2006), is it possible that the presence of the gene *psbA* in the cyanophage genomes determines light-dependent phage adsorption. To answer this question, a set of degenerate PCR primers targeting the *psbA* gene was designed based on known *psbA* gene sequences which include 23 cyanobacterial *psbA* gene sequences and 15 cyanophage *psbA* gene sequences (courtesy of Dr. Andy Millard, this laboratory). The detailed sequence information can be found in Appendix 1. The resulting degenerate primers were: F-primer, 5'-CTTCTATCCNATYTGGGAAG-3'; R-primer, 5'-TNAGGTTGAANGCCATNGTR-3' (R=A+G ,Y=C+T, N=A+C+G+T). PCRs were carried out to amplify the *psbA* gene from the 9 cyanophage stains as described above in a total volume of 50 µl, containing 0.25 mM dNTPs, 3 mM $MgCl_2$, 0.4 µM primers, 10 ng of phage DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C.

As seen in Figure 3.11, PCR products were obtained from all the cyanophages. However, a PCR product was also generated from *Synechococcus* sp. WH7803

(Figure 3.11), which indicated that these degenerate primers were able to amplify the *Synechococcus psbA*. In order to eliminate false postive cyanophage *psbA* PCR products generated from contained host genomic DNA contamination, cyanophages were CsCl-purified before DNA extraction. The putative cyanophage DNAs were then tested by PCR targeting the 16S rRNA gene of *Synechococcus* sp. WH7803 using the following primers: 27F-OXY1313R and OXY107F-1522R (Fuller *et al.*, 2003). PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers, 10 ng of DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. No 16S rRNA PCR products were obtained based on the cyanophage DNA (Figure 3.12), which ensured the absence of host genomic DNA.

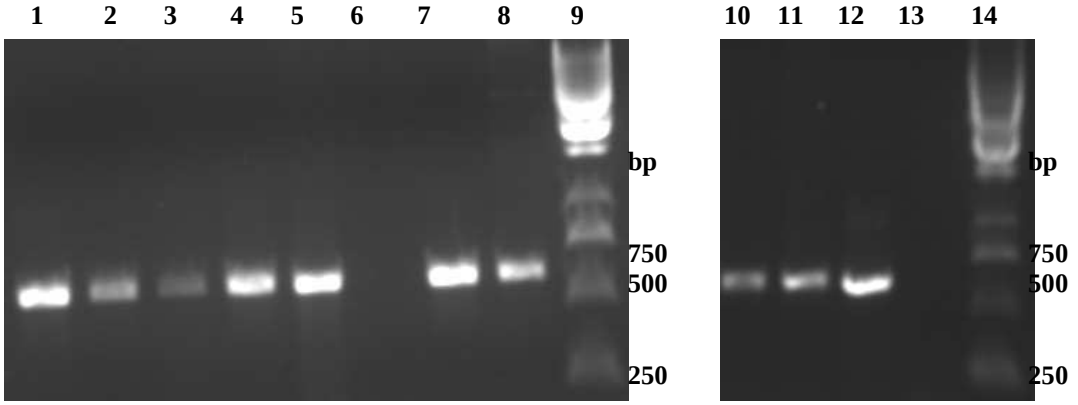


Figure 3.11 Gel images of PCR products of the *psbA* gene generated by a set of degenerate primer.

DNA extracted from 9 cyanophages infecting *Synechococcus* sp. WH7803 were used as templates to perform PCRs. S-PWM3 (Lane 1), S-BP3 (Lane 2), S-BnM1 (Lane 3), S-PM2 (Lane 4), S-MM5 (Lane 5), negative control (Lane 6), S-MM1 (Lane 7), WH7803 (Lane 8), GeneRuler™ 1kb DNA ladder in bp from Fermentas (Lane 9), S-MM4 (Lane 10), S-BM3 (Lane 11), S-PWM1 (Lane 12), negative control (Lane 13), GeneRuler™ 1kb DNA ladder in bp from Fermentas (Lane 14).

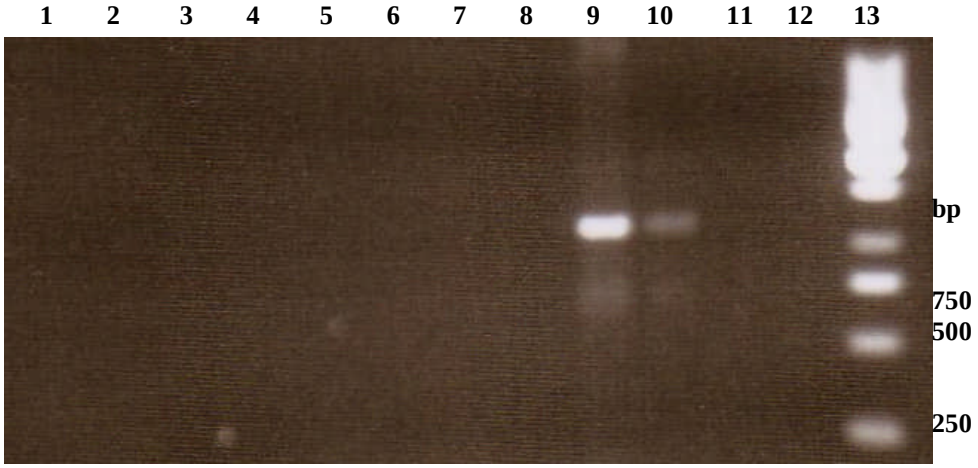


Figure 3.12 Gel image of PCR products generated by PCR primers targeting the 16S rRNA gene of *Synechococcus* sp. WH7803.

DNA extracted from 9 cyanophages infecting *Synechococcus* sp. WH7803 were used as templates to perform PCRs. S-PWM3 (Lane 1), S-BP3 (Lane 2), S-BnM1 (Lane 3), S-PM2 (Lane 4), S-MM5 (Lane 5), a negative control (Lane 6), S-MM1 (Lane 7), S-MM4 (Lane 8), 50 ng of WH7803 DNA (Lane 9), 1 ng of WH7803 DNA (Lane 10), S-BM3 (Lane 11), S-PWM1 (Lane 12), GeneRuler™ 1kb DNA ladder in bp from Fermentas (Lane 13).

The nucleotide sequences of the 9 cyanophage *psbA* products were very similar to each other and to the cyanobacterial *psbA* gene sequence from their host strain, *Synechococcus* sp. WH7803 (Figure 3.13). This demonstrated that all the 9 cyanophages carried the *psbA* gene. Therefore, light-dependent cyanophage adsorption has nothing to do with the presence or absence of the *psbA* gene in cyanophage genome. These data also indicated that the presence of the *psbA* gene in cyanophage genomes was prevalent and this gene may function for cyanophage replicating in photoautotrophic hosts (Clokie *et al.*, 2006c).

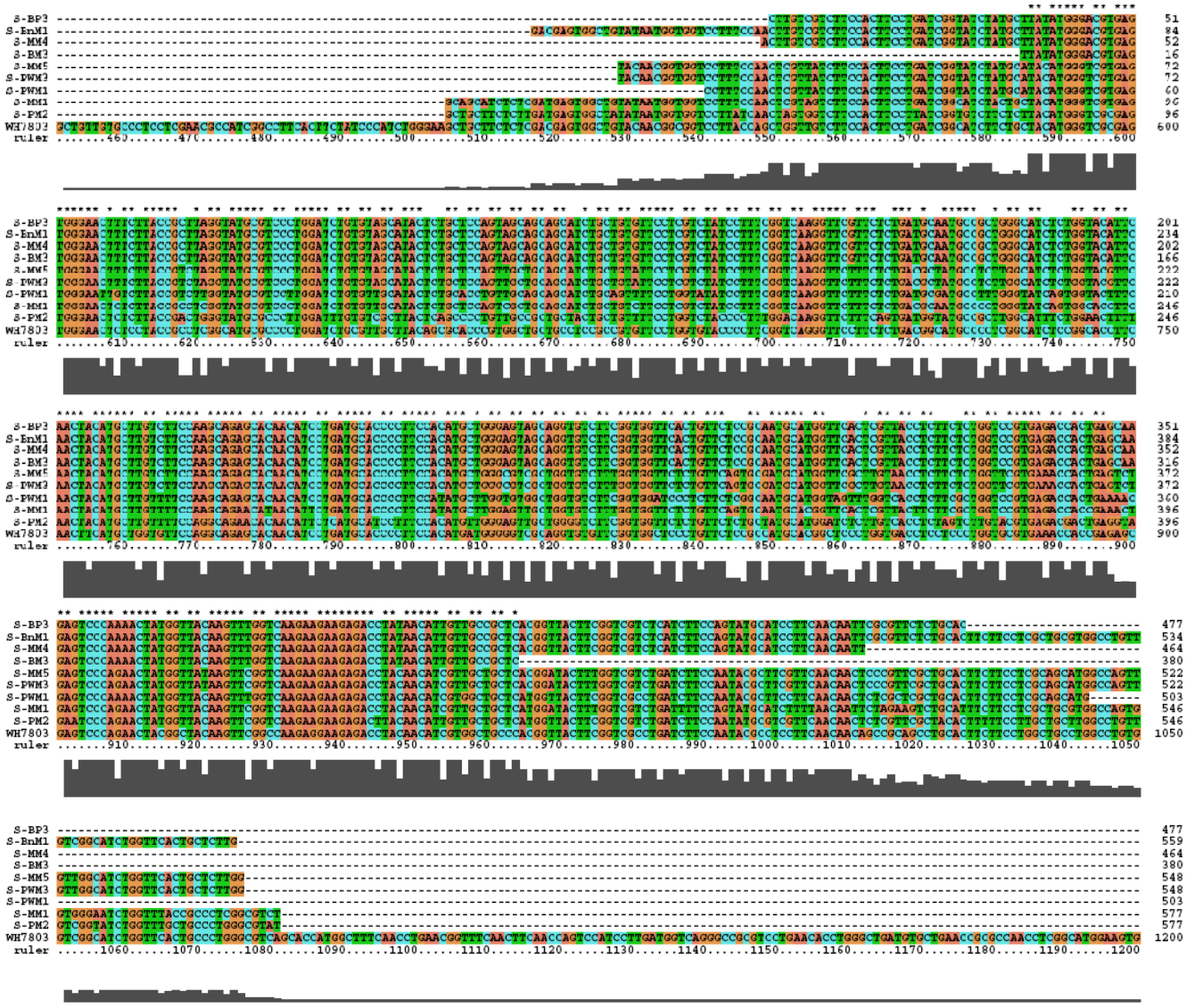


Figure 3.13 Sequence alignment of the *psbA* PCR product from 9 cyanophage strains with the cyanobacterial *psbA* gene sequence from their host strain, *Synechococcus* sp. WH7803.

A degenerate PCR primer was used to amplify the *psbA* gene from 9 cyanophage strains (S-MM5, S-PWM3, S-PWM1, S-BP3, S-BnM1, S-MM4, S-BM3, S-MM1 and S-PM2). The sequences were aligned using ClustalX.

3.3.8 Absorption properties of S-PM2 particles

In order to establish whether S-PM2 itself carries a photoreceptor to detect ambient light, the absorption properties of CsCl-purified S-PM2 particles were determined

using a NanoDrop spectrophotometer (Figure 3.14). No absorption peaks were detected within the range of visible wavelentgh (400 nm to 750 nm), which indicated the absence of a chromophore. Attention was then focused on the host to find out more about the cyanophage S-PM2 receptor.

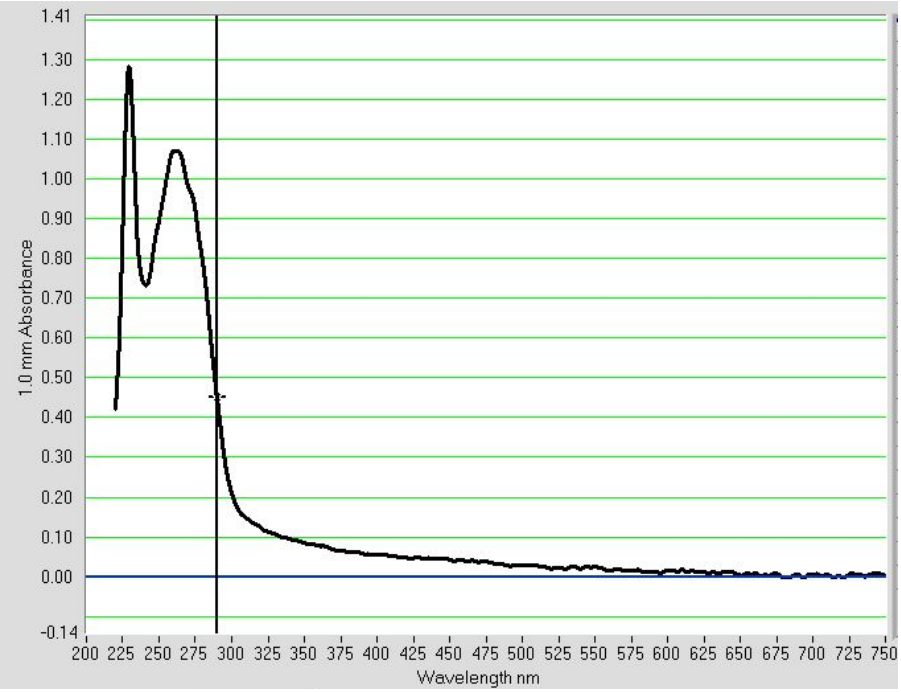


Figure 3.14 Absorption spectrum of CsCl purified Cyanophage S-PM2.

1 µl of CsCl purified S-PM2 at a concentration of 3×10^8 pfu ml^{-1} was analysed using a NanoDrop spectrophotometer. ASW was used as a blank.

3.3.9 Preliminary investigation of cyanophage-cyanobacterium recognition

Many molecules located on the cell surface can potentially function as phage receptors. In order to gain a better understanding of the nature of cyanophage receptor molecules, 8 different cyanobacterial hosts were challenged with 9 different

cyanophages using a spot test (Section 2.7). The diversity and complexity of cyanophage-cyanobacterium recognition can be seen in Table 3.1. The '+' was used to signify the detection of plaques, which indicates successful lytic phage infection. The '-' was used to signify no plaque formation. As seen in Table 3.1, specific phages can infect multiple hosts, this is presumably due to the presence of the same phage receptor(s) on the different cell surfaces. Specific hosts show different susceptibilities to different phages (except WH7803-original isolation host). This table can serve as a guide to design experiments to examine the adsorption patterns of different cyanophage-host systems in the light and dark. For example, light-dependent adsorption (~ 90% adsorption in light, ~ 10% adsorption in dark 45 min post-infection) was also discovered in two other cyanophage-host systems: S-MM5 and Dim (Fuller *et al.*, 2003), S-MM4 and Dim. These data support the notion that the light-dependent cyanophage adsorption is not just limited to S-PM2 and *Synechococcus* sp. WH7803.

Table 3.1 Cyanophage-host systems

	S-PM2	S-PWM3	S-BP3	S-BnM1	S-MM5	S-MM1	S-MM4	S-BM3	S-PWM1
WH7803	+	+	+	+	+	+	+	+	+
WH8109	+	-	+	-	-	-	-	-	-
Dim	+	+	+	-	+	+	+	+	+
BL161	+	-	+	+	-	+	+	+	+
BL164	+	-	+	+	-	+	+	+	-
RS9916	-	+	-	-	-	-	-	-	+
WH8018	-	-	+	-	-	+	+	+	-
BL36	-	+	-	-	-	-	-	-	-

+: positive infection -: no infection

In order to establish whether S-PM2 light-dependent adsorption was specific to WH7803, another S-PM2 host, marine *Synechococcus* strain BL161 (Laure Guillou, unpublished), was used to perform adsorption test in the light and dark by counting

free phages at the beginning and 45 min post-infection following the same protocol as for *Synechococcus* sp. WH7803. It was found that ~ 60% of the phages adsorbed to BL161 in the light and ~ 40% of the phages adsorbed to BL161 in the dark (Figure 3.15). This pattern was quite distinct from the S-PM2 adsorption to WH7803 (90% adsorption in the light and 10% adsorption in the dark). This suggests that the nature of the host cell has a big impact on the S-PM2 light-dependent adsorption. Therefore, it is crucial to examine the cell surface of the host cells and define the component to which cyanophage S-PM2 attaches.

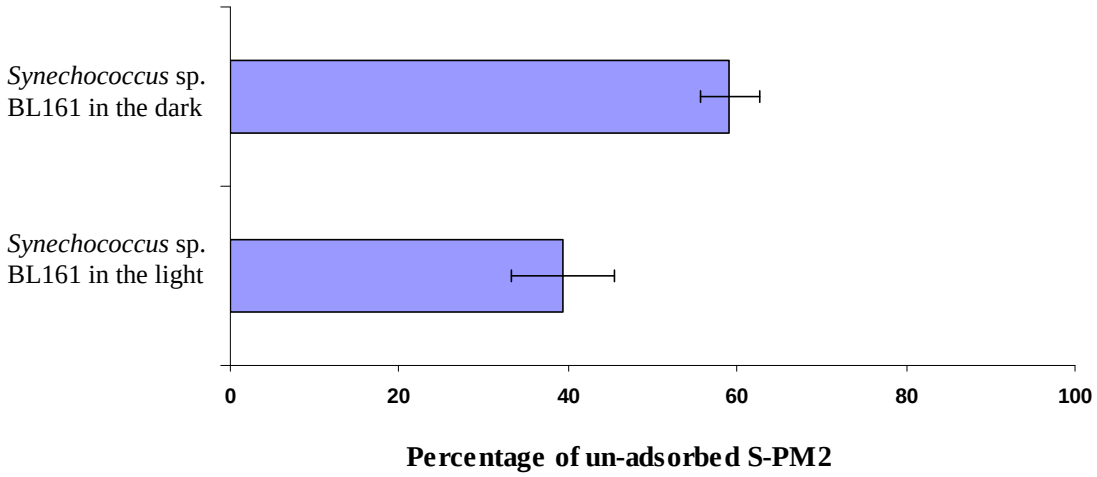


Figure 3.15 Adsorption of S-PM2 to *Synechococcus* sp. BL161 in the light and dark.

BL161 cells in the logarithmic growth phase (OD_{750}= 0.35) were incubated with S-PM2 at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed 45 min post infection in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

3.4 Discussion

Using flow cytometry for counting phage S-PM2 is because the sheer volume of the plaque assays used in counting phage. For example, when the experiment of the effect of light on the adsorption of cyanophage S-PM2 to *Synechococcus* sp. WH7803 cells

was carried out (Figure 3.5), phage samples were collected from 25 time points and each time point needed 9 plates to do plaque assays, which means there was a total of 450 plates (225 for phage adsorption in the light and 225 for phage adsorption in the dark). Although plaque assays give reliable phage counts, it is time-consuming. In order to do large scale phage adsorption experiments, namely assaying adsorption of other phages to *Synechococcus* sp. WH7803 in the light and dark, counting phage using plaque assays obviously becomes a tedious task. A quick and reliable way of counting is needed.

Although the nucleic acid staining dye, SYBR Green, has been used in flow cytometry to count marine viruses from crude cell lysate (Marie *et al.*, 1999; Brussaard *et al.*, 2000; Brussaard, 2004), it was not the case for marine phage S-PM2 infection of *Synechococcus* sp. WH7803. Even with CsCl-purified S-PM2 phage particles, 2-3 individual populations were observed (Figure 3.2, Column C). When the CsCl-purified S-PM2 was subject to an extra procedure of filtration, populations representing particles bigger than S-PM2 were removed (Figure 3.2, Columns A and B). These data indicates that cyanophage S-PM2 tend to form aggregates, which may explain the observation that count of CsCl-purified S-PM2 based on FC (6×10^9 to 7.3×10^9 particles ml^{-1}) were almost 10 time lower than plaque assay (2.5×10^{10} puf ml^{-1}). When fresh S-PM2 lysates were used to perform FC, the phage count (7.4×10^7 particles ml^{-1}) was almost 10 times lower than plaque assay (5.8×10^8 puf ml^{-1}). It is not an unusual phenomenon for lytic phages to form aggregates. For example, a lytic phage, named 0305Φ8-36 infecting *Bacillus thuringiensis* has been reported to be able to form aggregates during plaque formation (Serwer *et al.*, 2007a; Serwer *et al.*, 2007c; Serwer *et al.*, 2007b). To overcome the problem of aggregation, S-PM2 may need to

be diluted to dissociate phage aggregates before counting using FC. Apart from phage aggregation, the autofluorescence of the phycoerythrins in WH7803 (Ong *et al.*, 1984) can also be a problem based on the following observations: (1) CsCl-purified S-PM2 without staining with SYBR Green I showed particles in the gated region (6×10^8 particles ml^{-1}, Figure 3.2E). (2) The stained blank also showed particles in the gated region (1.3×10^7 particles ml^{-1}, Figure 3.3B). These counts may be due to the autofluorescence of photosynthetic pigments of WH7803. To make FC work for S-PM2, phycoerythrins would need to be removed. As the same protocol functioned on a *Stratomycete* phage Φc31, as seen in Figure 3.4, the problem with S-PM2 was not a technical fault. Therefore, due to aggregation and background signals, phage counting in following study resumed to plaque assay.

Light is one of the most important environmental factors that effect cyanobacterial growth. In the natural environment cyanobacteria are subject to light/dark cycles and it is important to know how phage infection will be affected by the shift from light to dark. It has been previously reported that the light can considerably affect the adsorption of the freshwater cyanophage AS-1 to *Synechococcus* PCC 7942 (previously named *Anacystis nidulans*) (Cseke and Farkas, 1979b); 40% of the phage was adsorbed to the cells in the dark, whereas 80% of the phage was adsorbed to the cell in the light. Compared to the 10% of phage adsorption in dark and 90% of phage adsorption in light, S-PM2 is far more sensitive to light in terms of the adsorption step. Apart from light availability, higher light intensity has been indicated to be more effective in cyanophage AS-1 adsorption to *Synechococccus elongates* (Kao *et al.*, 2005).

As DCMU/CCCP-treatment of *Synechococcus* sp. WH7803 cells did not prevent phage S-PM2 from adsorbing, this suggests that the adsorption of S-PM2 to WH7803 does not need the ATP generated through the process of photophosphorylation. However, DCMU/CCCP did abolish the replication of S-PM2 in *Synechococcus* sp. WH7803, which agrees with previous observation that the use of DCMU or CCCP completely abolished cyanophage SM-1 replication in *Synechococcus* strain NRC-1 (MacKenzie and Haselkorn, 1972). This leads to two implications, either the process of S-PM2 adsorption does not need energy or other unknown energy sources in the hosts are required for S-PM2 adsorption.

LD cycles can induce different physiological statuses and synchronise the circadian clock of the photoautotrophic host cell, which may influence phage adsorption. Furthermore, *Synechococcus* sp. WH7803 has been reported to be easily entrained under LD cycles (Sweeney and Borgese, 1989; Jacquet *et al.*, 2001). Consequently, phage adsorption to LD-entrained WH7803 cells was investigated. Data showed that LD cycles did not bring any effect to S-PM2 adsorption kinetics, which agrees with a previous observation that circadian rhythm of *Synechococccus elongatus* had little effect on the cyanophage AS-1 diel infection (Kao *et al.*, 2005). However, to unequivocally establish the role of circadian clock of WH7803 in S-PM2 adsorption, the entrainment of the WH7803 circadian clock needs to be confirmed (Ishiura *et al.*, 1998) as the strain has been cultured in lab under constant light for many years.

One possible scenario for the light-dependent adsorption is that light may introduce some ‘critical changes’ (which could not be initiated in the dark) in either the cell receptor or the phage itself so that successful adsorption could occur. If light could

trigger some change in the phage itself, S-PM2 must contain a photoreceptor to detect ambient light. In higher plants, the photoreceptor is commonly phytochrome, a protein with bilin chromophores that is sensitive to light in the red and far-red region of the visible spectrum (Quail *et al.*, 1995). However, red light (785-620 nm) did not bring about phage adsorption to a similar level as blue (460 nm-490 nm), green (500-570 nm) and yellow (580-590 nm) light did (Figure 3.7). As a matter of fact red light (785-620 nm) had a very similar effect as the darkness to S-PM2 adsorption (Figure 3.5 and 3.6). This implies that no phytochrome-like molecule exists in S-PM2. In addition, absorption spectrometry of S-PM2 also confirmed the absence of light-detection molecules in S-PM2. Furthermore, no chromophore-coding gene was discovered in the S-PM2 genome (Mann *et al.*, 2005).

As S-PM2 showed decreased adsorption rate to *Synechococcus* strain BL161 compared to that of WH7803 in the light but no adsorption to both of them in the dark, this indicates that light-triggered S-PM2 receptor(s) on the cell surfaces of BL161 and WH7803 may be different molecule(s). If the same molecule(s) were used as S-PM2 receptor(s), the abundance of that receptor molecule(s) may be higher on the cell surfaces of WH7803 than BL161 in the light.

It has been reported that the gene *wac* of phage T4 encodes a protein whisker, which can interact with the long tail fibers (LTFs) and hold the LTFs in a retracted conformation that prevents the phage from adsorbing (Letarov *et al.*, 2005). Moreover, the sensitivity of the wac to ambient conditions, such as pH and temperature, enables T4 to adapt itself to its environment (Letarov *et al.*, 2005). Phage S-PM2 may use a similar mechanism as phage T4 to maintain the LTFs of phage S-PM2 in an extended

format that enables the phage to adsorb. Elimination of light may force the LTFs to fold and form a compressed format that inhibits contact between phage adhesin and cell receptor, thus blocked adsorption.

The possibility that the expression of the phage S-PM2 receptor-coding gene was under the control of light was ruled out by the observation that when the phage-host suspension was exposed to light adsorption resumed almost immediately (Figure 3.5). This indicates that the receptor molecule must already be on the cell surface, which leads to the possibility that the receptor molecule could undergo a conformational change in the light to favour phage recognition. Therefore, the research now focuses on the host *Synechococcus* sp. WH7803, trying to identify the molecule that S-PM2 binds to.

Chapter 4 Isolation of cyanophage-resistant mutants

4.1 Introduction

Mutant *E. coli* strains that are resistant to phage infection have been reported for all the T-series phages (Lenski, 1988b). For lytic phages, resistance is often the result of loss or modification of the receptor molecule on the cell surface to which the phage binds. These mutations often simultaneously reduce the cell's competitiveness because the receptor molecules often have a functional role in bacterial physiology (Lenski, 1988a). Despite the trade-off between resistance and competitiveness, sensitive and resistant *E. coli* strains have been shown to stably co-exist in laboratory communities (Lenski, 1988b).

The occurrence of phage-resistant cyanobacteria has been suggested to be widespread in the marine environment (Waterbury and Valois, 1993a; Suttle and Chan, 1994; McDaniel *et al.*, 2006). Indeed, Waterbury and Valois (1993) and McDaniel *et al.* (2006) reported that some *Synechococcus* isolates from the Sargasso Sea, Woods Hole Harbor, the Gulf Stream and the Gulf of Mexico were resistant to cyanophage isolates obtained from the same location. Suttle and Chan (1994) suggested that the phage-resistant *Synechococcus* strains were more likely to occur in the mesotrophic rather than the oligotrophic regions of the ocean.

There are three potential ways for *Synechococcus* strains to become phage-resistant in the natural environment (Mann, 2003). Firstly, a number of strains within the *Synechococcus* population are naturally resistant to certain phages due to their genotype. This has been shown to be the case in laboratory studies. Some *Synechococcus* strains showed susceptibly to as many as 11 phage isolates, whereas

others were only infected by 1 or 2 phages (Waterbury and Valois, 1993a). Secondly, *Synechococcus* strains can mutate into phage-resistant forms under the selection pressure for the strains to be resistant, though these mutants pay a physiological cost (Lennon *et al.*, 2007). Thirdly, normally sensitive cells may become resistant to phage infection under certain physiological conditions or in particular phases of the cell cycle.

Phage receptor studies in *E. coli* and related species were facilitated by the availability of phage-resistant mutants (Heller, 1992). However, little work has been carried out on identifying cyanophage-resistant cyanobacteria. Recently, a number of phage-resistant *Synechococcus* mutants have been isolated and characterised (Stoddard *et al.*, 2007). Phage adsorption tests also suggested that resistance was likely due to changes in phage receptors (Stoddard *et al.*, 2007).

Limited work has been carried out to identify cyanophage receptors. One early report suggested that the lipopolysaccharide (LPS) may be involved in binding of the cyanophage AS-1 to the unicellular cyanobacterium *Anacystis nidulans* KM based on the fact that the LPS layer was able to inactivate the cyanophage AS-1 (Katz *et al.*, 1977; Samimi and Drews, 1978). In this chapter, spontaneous cyanophage-resistant mutants from *Synechococcus* sp. WH7803 were isolated and their susceptibilities to cyanophage infection were tested.

4.2 Aims

To isolate cyanophage-resistant mutants derived from *Synechococcus* sp. WH7803.

4.3 Results

4.3.1 Isolation and confirmation of cyanophage-resistant mutants

As part of an effort to understand light-dependent S-PM2 adsorption, 9 different cyanophages (see Section 2.11) were used to infect *Synechococcus* sp. WH7803 at a MOI of 1. During prolonged incubations (3 months), re-growth was detected from 3 out of 9 phage-infected WH7803 cell cultures, including phages S-PM2, S-BP3 and S-BnM1. No re-growth was observed for the other six cyanophage-host incubations. These 3 putative phage-resistant strains were purified using the pour plate method (Section 2.14) to get single colonies, which were then subject to culture in liquid medium. The resistant strains were named by adding 'R' (for 'resistant') and the name of the cyanophage to the end of the host strain WH7803. Consequently, the S-PM2-resistant strain was named 'WH7803RS-PM2'; the S-BP3-resistant strain was named 'WH7803RS-BP3'; the S-BnM1-resistant strain was named 'WH7803RS-BnM1'.

To confirm that these 3 putative phage-resistant mutants, derived from single colonies, were still resistant to phage infection, cultures (OD_{750}=0.3) were challenged with their corresponding phages at an MOI of 1 and were kept under illumination (Section 2.6) for 10 days. Wild-type WH7803 cultures (with or without phage addition) were grown as controls. All the putative mutants showed obvious growth (OD_{750} ≥0.75), but the phage-infected wild-type cell cultures died. This indicated that the phage-

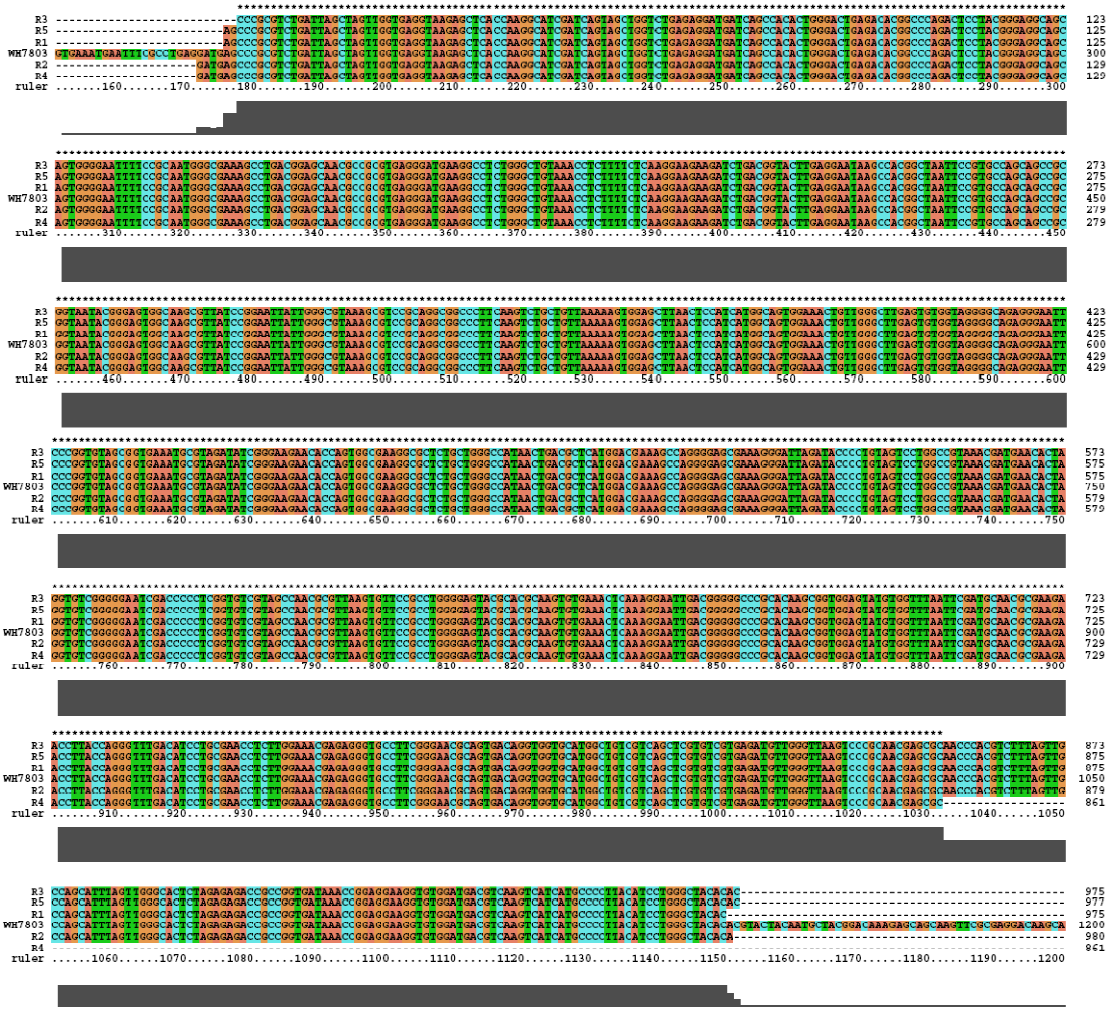
resistance was not just a result of a temporary phenomenon and but was probably due to a permanent modification in their phage receptors.

In order to rule out the possibility of contamination and to further confirm that these 3 putative phage-resistant mutants were genuinely *Synechococcus* sp. WH7803 derivatives, all 3 phage-resistant mutants derived from single colonies was examined by PCR coupled with DNA sequencing using primers targeting three *Synechococcus* sp. WH7803 genes; the 16S rRNA gene and two photosynthesis-related genes, *mpeBA* and *cpeT* (Table 2.11). The PCR product of the 16S rRNA gene was amplified using the following primers: 27F-OXY1313R and OXY107F-1522R (Fuller *et al.*, 2003). PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers, 10 ng of DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. The PCR product of the *mpeBA* operon was amplified using the following primers: 7803mpeBAF-704 and 7803mpeBAR-1330C. The PCR product of the *cpeT* gene was amplified using the following primers: 7803cpeTF39 and 7803cpeTR612C. Both PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 1 mM $MgCl_2$ (for *mpeBA*) or 4 mM $MgCl_2$ (for *cpeT*), 0.4 µM primers, 10 ng of DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Both amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. Sequencing reactions were carried out using the same primers as for the PCRs, except that two more primers, 16S rRNA359F and 16S

rRNA778R, were involved in 16S rRNA sequencing in order to sequence the full length of 16S rRNA gene.

When the sequence results were aligned with the corresponding genes of wild-type *Synechococcus* sp. WH7803, 100% identity was revealed (Figure 4.1-4.3). This demonstrated that the 3 phage-resistant mutants were derived from *Synechococcus* sp. WH7803.

A



B

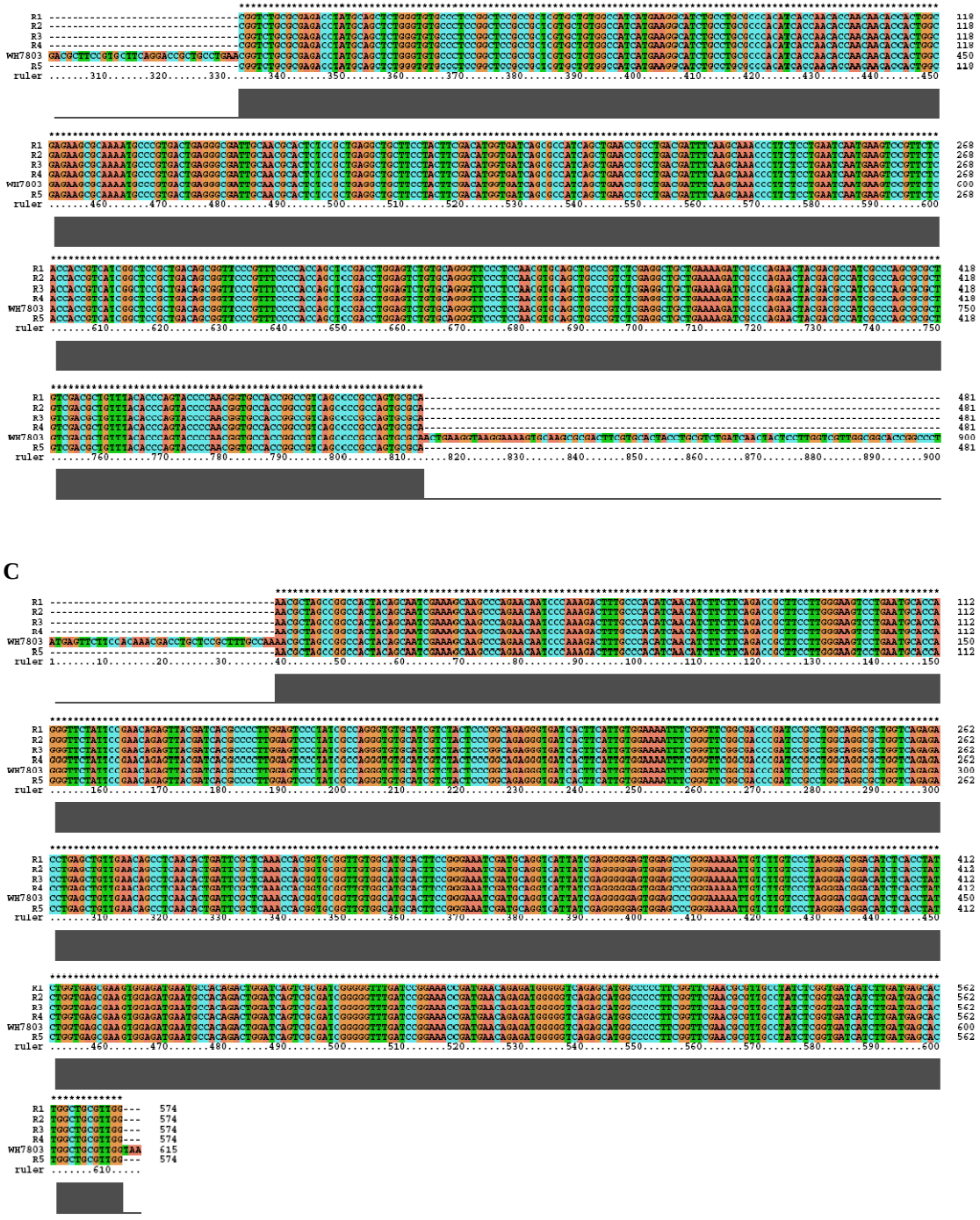
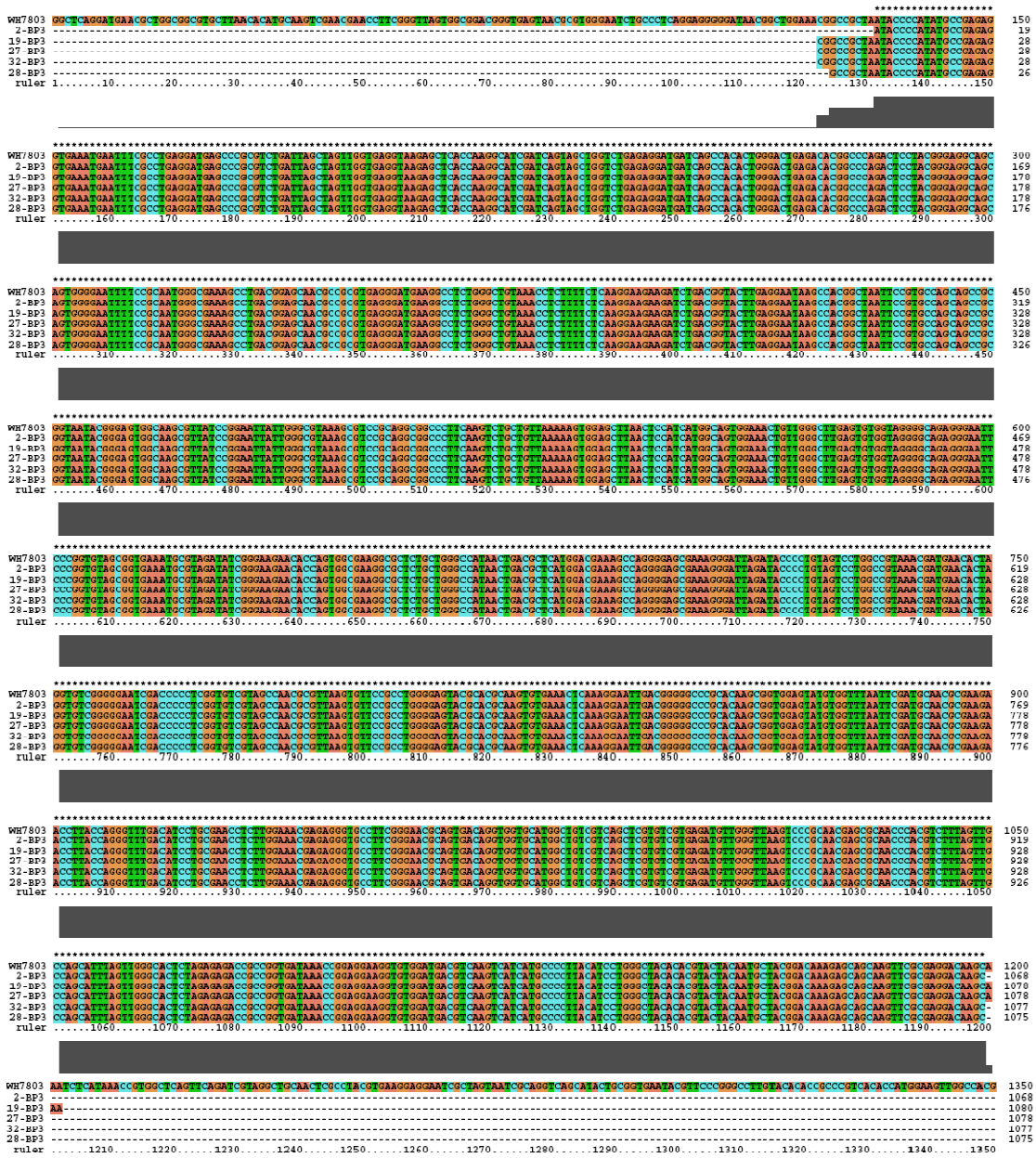


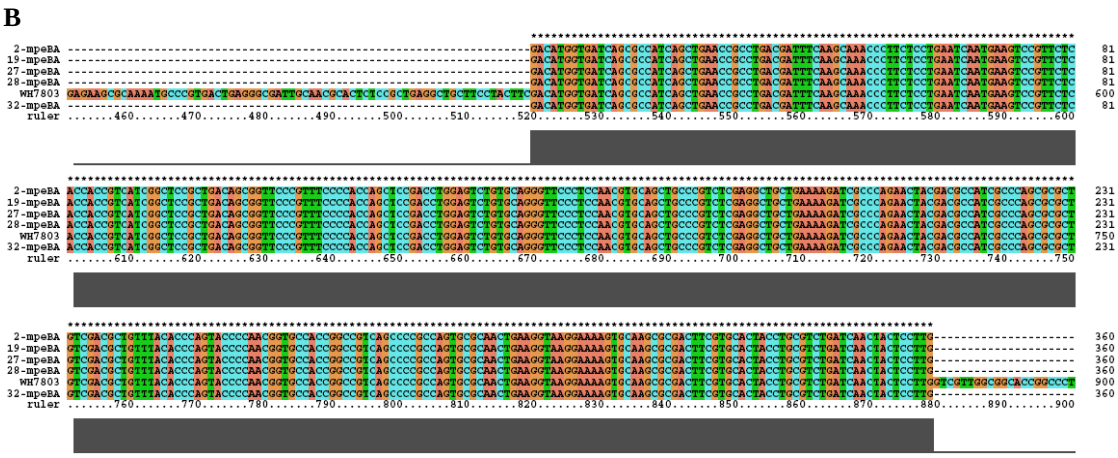
Figure 4.1 Sequence alignments of the genes in the S-BnM1-resistant strain and *Synechococcus* sp. WH7803 (WH7803).

A 16S rRNA gene, **B** *mpeBA* gene, **C** *cpeT* gene. Five individual phage-resistant single colonies (indicated as R1-5) were chosen for DNA sequencing.

A



B



C

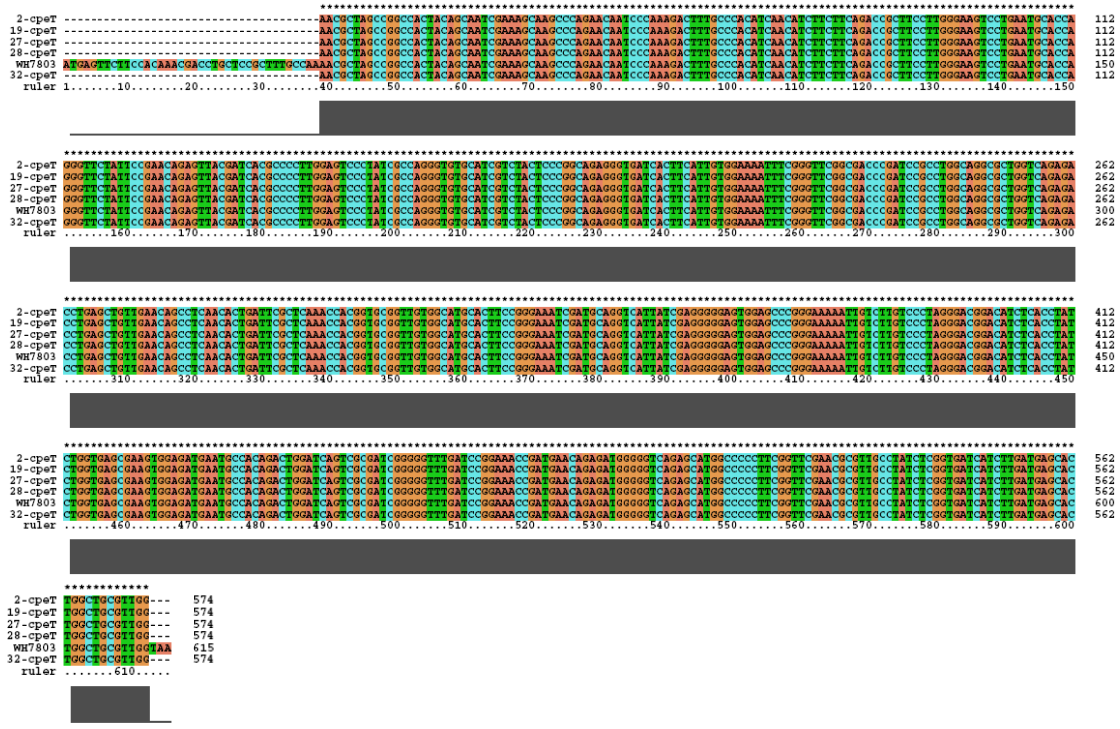
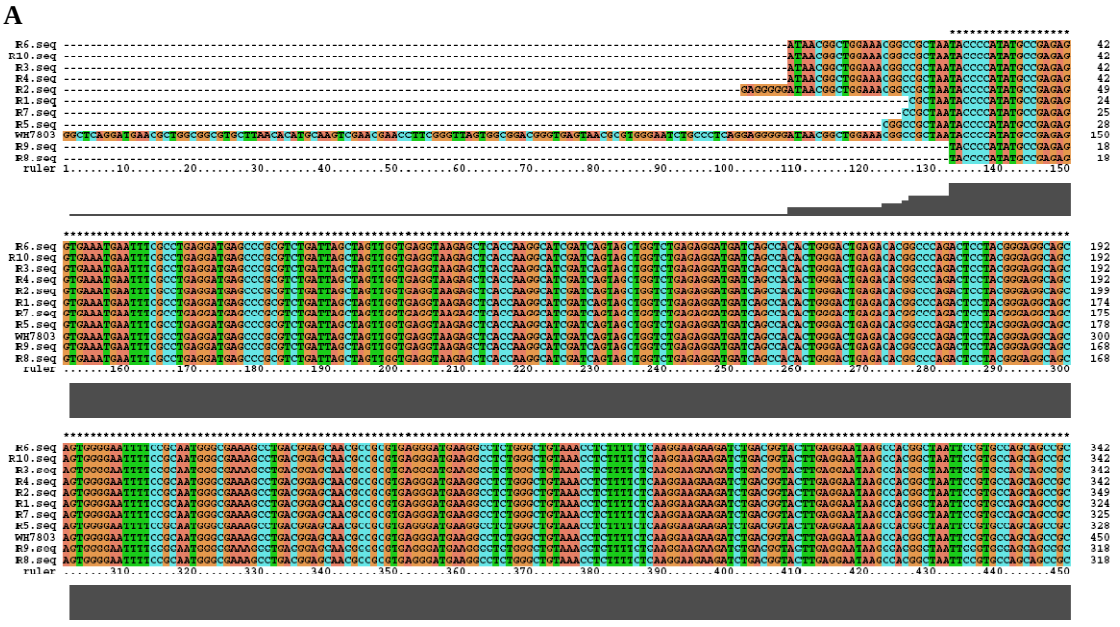
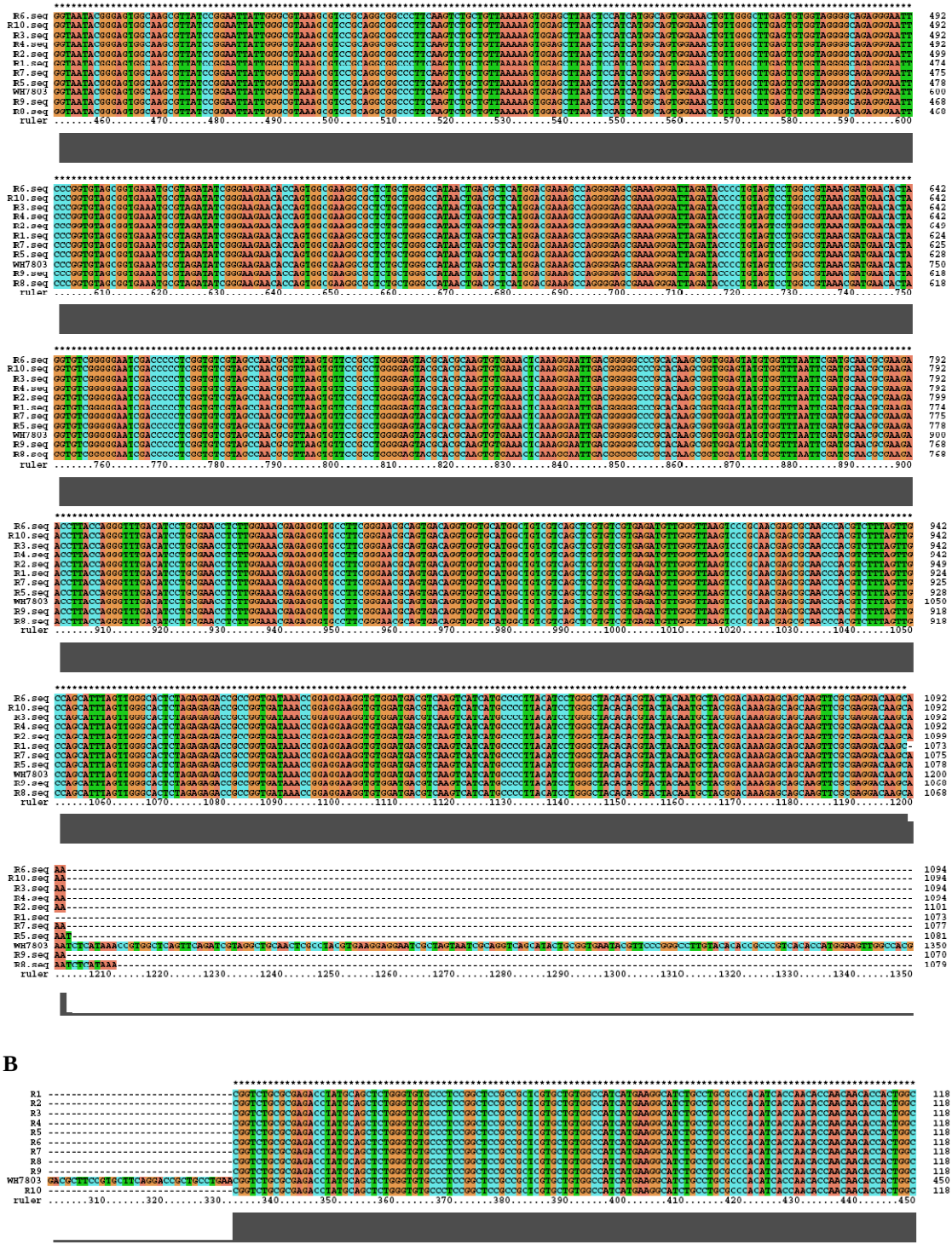


Figure 4.2 Sequence alignments of the genes in the S-BP3-resistant strain and *Synechococcus* sp. WH7803 (indicated WH7803).

A 16S rRNA gene, **B** *mpeBA* gene, **C** *cpeT* gene. Five individual phage-resistant single colonies (indicated as 2, 19, 27, 28 and 32) were chosen for DNA sequencing.

A





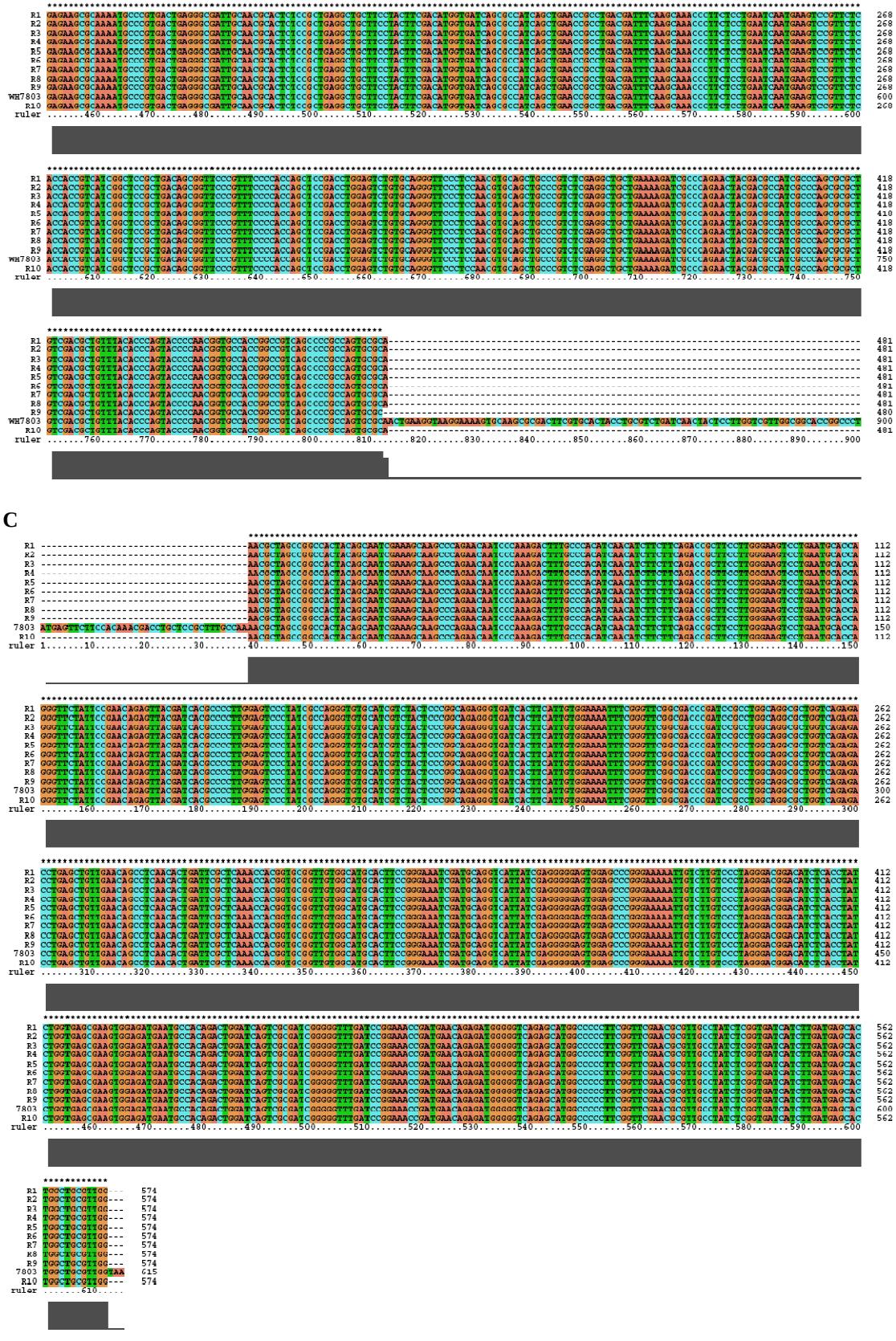


Figure 4.3 Sequence alignments of the genes in the S-PM2-resistant strain and *Synechococcus* sp. WH7803 (indicated as 7803/WH7803).

A 16S rRNA gene, **B** *mpeBA* gene, **C** *cpeT* gene. 10 individual phage-resistant single colonies (indicated as R1-10) were chosen for DNA sequencing.

Since time constraints did not allowed me to perform a comprehensive analysis of all three cyanophage-resistant strains, only WH7803RS-PM2 was chosen for detailed analysis.

4.3.2 Examination of the binding capacity of S-PM2 to WH7803RS-PM2

The binding capacity of WH7803RS-PM2 to S-PM2 was examined by performing the adsorption experiment described in Chapter 3 using WH7803RS-PM2 as the host. It was found that virtually no phage adsorbed to the mutant within 45 min post infection in light compared to ~ 90% adsorption to WH7803 (Figure 4.4). This lack of adsorption suggests that resistance to S-PM2 occurred through mutations of the S-PM2 receptor molecules to which S-PM2 binds.

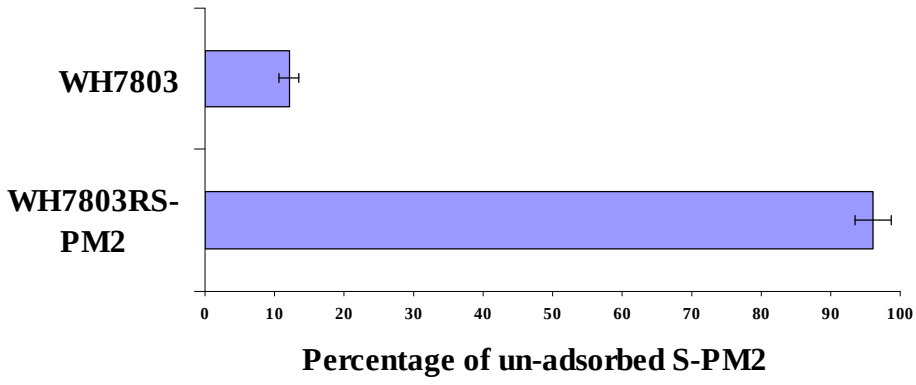


Figure 4.4 Adsorption of S-PM2 to WH7803RS-PM2 and WH7803 in the light.

WH7803RS-PM2 and WH7803 in the logarithmic growth phase (OD_{750}= 0.35) were incubated with S-PM2 at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed 45 min post infection

in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

4.3.3 Examination of the growth curve of WH7803RS-PM2 cells

Although no obvious visible phenotypic alterations were observed in the mutant and wild-type cells, the growth rates were examined in order to determine any effect exerted by phage-resistance. No marked difference was observed in the growth curves of the wild-type and mutant (Figure 4.5). This indicated that loss of the S-PM2 receptor molecules on the cell surface did not significantly impair the cell's physiological status under these particular growth conditions.

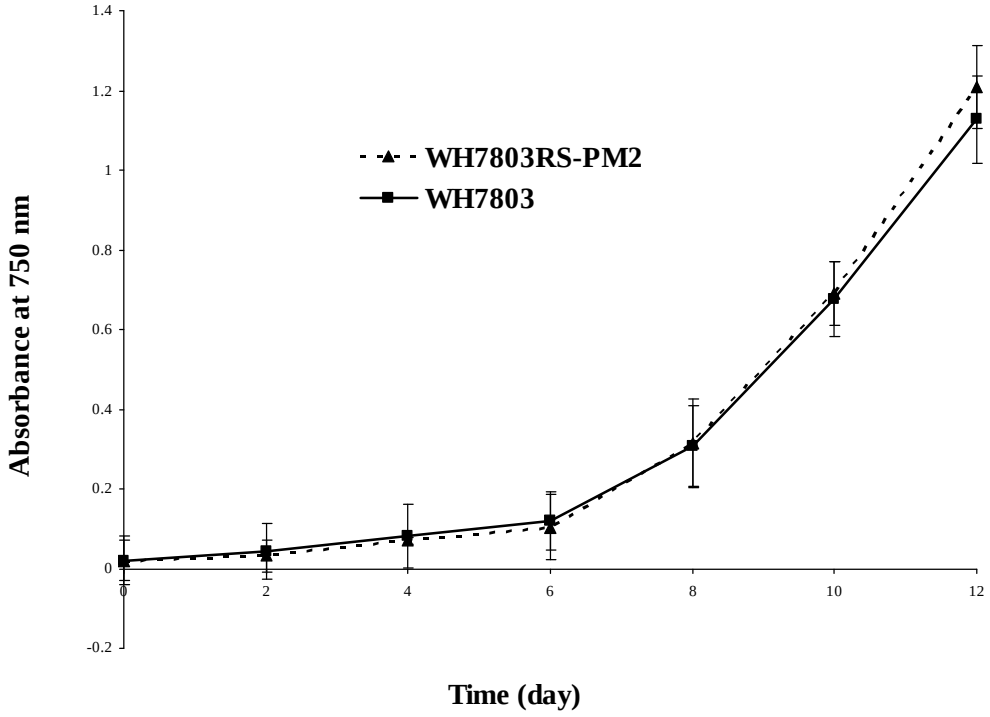


Figure 4.5 Growth curves of *Synechococcus* sp. WH7803 and WH7803RS-PM2 in ASW medium.

Cells were cultured in 1 l glass vessel with aeration and stirring. Cell growth was monitored by absorbance at 750 nm. The points are the mean of three biological replicates. The error bars represent SD (n=3).

4.3.4 Examination of the binding capacity of other cyanophages to WH7803RS-PM2

In order to gain further insight into the specificity of the S-PM2 receptors, the susceptibility of WH7803RS-PM2 to 30 cyanophages (listed in Table 2.2) was investigated by the use of a spot test (see Section 2.7). The wild-type *Synechococcus* sp. WH7803 strain was used as a positive control because it is susceptible to all of the 30 cyanophages and always yields plaques. Freshly prepared stocks of the 30 phages (Section 2.8), which had a similar titration of ~10^8 pfu ml^{-1}, were serially10-fold diluted and each dilution was used in the spot test. As shown in Table 4.1, WH7803RS-PM2 was still infected by 13 cyanophages with a similar susceptibility to the control cells based on the fact that plaques were formed on both strains at dilutions down to 10^{-7}. This indicates that these 13 cyanophages probably do not bind to the same cell receptor molecule as S-PM2. Apart from these 13 cyanophages that form clear plaques on WH7803RS-PM2 at dilutions down to 10^{-7}, one particular cyanophage, S-MM5 was able to form plaques on WH7803RS-PM2 at dilutions down to 10^{-4} and form plaques on the control cells, WH7803 at dilutions down to 10^{-7} (Figure 4.6). This decreased resistance to cyanophage S-MM5 indicates that WH7803RS-PM2 is only partially resistant to S-MM5 infection. This may result from an alteration in a secondary phage receptor. In addition, WH7803RS-PM2 was resistant to the other 16 cyanophages with no plaques being formed at any dilution. Thus, they probably use the same cell receptor molecules as S-PM2.

4.3.5 Examination of the binding capacity of 9 cyanophages to WH7803RS-BP3 and WH7803RS-BnM1

The same methodology as described above was applied to WH7803RS-BP3 and WH7803RS-BnM1 to test their susceptibility to 9 cyanophages. Both were still resistant to S-BP3, S-BnM1, S-PWM1 and S-BM3 and sensitive to the others (Table 4.2). This indicated that the two phages, S-BP3 and S-BnM1, probably shared the same phage receptor(s).

Table 4.1 Binding capacity of 30 cyanophages to *Synechococcus* sp. WH7803RS-PM2

Phage strain	*Synechococcus* sp.WH7803RS-PM2	*Synechococcus* sp.WH7803
S-PM2	—	‡
S-PWM1	‡	‡
S-PWM3	‡	‡
S-BP3	‡	‡
S-BnM1	‡	‡
S-MM1	‡	‡
S-MM4	‡	‡
S-MM5	†	‡
S-BM3	—	‡
S-RSM1	—	‡
S-RSM2	—	‡
S-RSM3	—	‡
S-RSM5	—	‡
S-RSM6	—	‡
S-RSM7	—	‡
S-RSM8	‡	‡
S-RSM9	‡	‡
S-RSM11	—	‡
S-RSM12	—	‡
S-RSM13	—	‡
S-RSM14	—	‡
S-RSM15	‡	‡
S-RSM18	‡	‡
S-RSM19	—	‡

S-RSM22	—	‡
S-RSM23	—	‡
S-RSM25	‡	‡
S-RSM26	‡	‡
S-RSM30	—	‡
S-RSM34	‡	‡

‡: sensitive, with similar susceptibility to the control cells

†: sensitive, with decreased susceptibility compared to the control cells

—: resistant

Table 4.2 Binding capacity of 9 cyanophages to WH7803RS-BP3 and WH7803RS-BnM1

Phage strain	*Synechococcus* sp.WH7803RS-BP3	*Synechococcus* sp.WH7803RS-BnM1	*Synechococcus* sp.WH7803
S-PM2	‡	‡	‡
S-PWM1	—	—	‡
S-PWM3	‡	‡	‡
S-BP3	—	—	‡
S-BnM1	—	—	‡
S-MM1	‡	‡	‡
S-MM4	‡	‡	‡
S-MM5	‡	‡	‡
S-BM3	—	—	‡

‡: sensitive, with similar susceptibility to the control cells

—: resistant

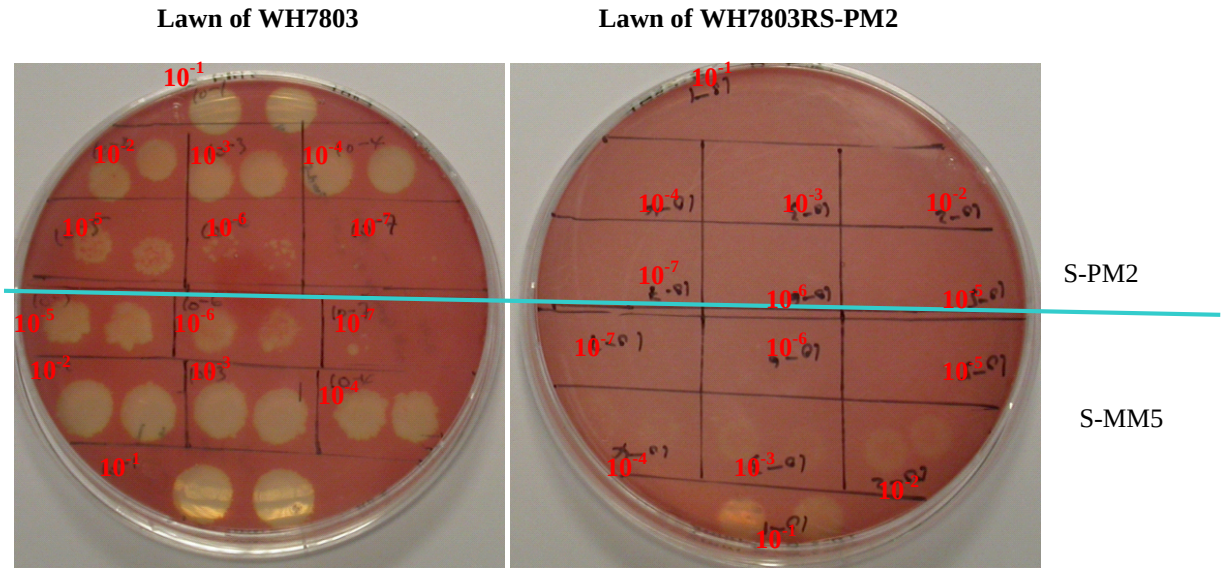


Figure 4.6 Spot tests of the infectivity of cyanophage S-MM5 on WH7803RS-PM2 and WH7803.

Serial diluted (10^{-1} to 10^{-7}) S-PM2 and S-MM5 phage stocks with a titer of ~ 10^{8} pfu ml^{-1} were spotted on lawns of WH7803 (left) and WH7803RS-PM2 (right) cells. S-PM2 was spotted on the upper half of the plate, while S-MM5 was spotted the bottom half.

4.4 Discussion

The successful isolation of 3 cyanophage-resistant mutants, WH7803RS-PM2, WH7803RS-BP3 and WH7803RS-BnM1, out of 9 phage-infected *Synechococcus* sp. WH7803 cell suspensions demonstrates that spontaneous phage-resistant strains do exist among wild-type strains, these phage-resistant strains will show the advantage when challenged with phage. As resistance can be passed from single colonies to several transfers in liquid cultures, this suggests the resistance is due to heritable genetic changes not physiological cost. Furthermore, mutation to resistance to one phage does lead to resistance to other phages based on the fact that the S-PM2-resistant mutant also showed resistance to other 15 cyanophages. According to the theory that phage-resistant marine *Synechococcus* strains are accompanied by a fitness

cost (Lennon *et al.*, 2007), one would expect that mutation to resistance to 16 cyanophages would impose a large trade-off in the fitness of WH7803RS-PM2. However, no obvious fitness cost has been detected from the S-PM2-resistant mutant because it showed a very similar growth rate as the wild-type strain. Armed with this resistance to multi-phage infections and the ability to maintain the cell's physiological status, this S-PM2-resistant *Synechococcus* strain certainly has a competitive advantage over sensitive strains, which has been generally assumed (Bohannan and Lenski, 2000). Although the multi-phage-resistant strains were discovered in the laboratory environment, it remains to be investigated whether or not this multi-phage-resistant and physiologically intact mutant exists in the natural environment. The possibility that the observed phage resistance is due to lysogeny was ruled out by the fact that these cyanophages are strictly lytic.

The fact that the S-PM2-resistant *Synechococcus* strain is not able to bind to S-PM2 implies mutation(s) in the phage receptor(s). A recent report also showed that cyanophage-resistant marine *Synechococcus* strains may gain their resistance by changing the host receptor sites (Stoddard *et al.*, 2007). The mutations conferring resistance to S-PM2 also caused resistance to other 15 cyanophages. This cross-resistance to other phages suggests that different cyanophages may share the same receptor(s). In this study, two cyanophages, S-BP3 and S-BnM1, probably use the same molecule(s) as the receptor according to the evidence that WH7803RS-BP3 and WH7803S-BnM1 strains showed the same susceptibility pattern to 9 cyanophages.

The fact that the mutant strain WH7803RS-PM2 was still susceptible to 16 out of 30 cyanophage indicated that mutation to resistance to one phage does not necessarily

lead to resistance to other phages, which agrees with previous observations (Bohannan and Lenski, 2000). This discovery also confirms that different phages use different receptor(s) and also implies that *Synechococcus* strains in the natural environment may be composed of many populations that probably differ in their phage receptors, such as *Synechococcus* strain WH7803 may be composed of one population of S-PM2-resistant strains, one population of wild-type strains and one population of S-PM2-sensitive but S-BP3 and S-BnM1-resistant strains.

Apart from the possible universality and specificity in the phage receptor, a possible partial resistance was indicated by the fact that WH7803RS-PM2 strain (compared to the wild-type *Synechococcus* sp. WH7803) showed a decreased susceptibility to phage S-MM5 infection (Figure 4.2). A similar result has also been discovered in coliphage-resistant strains (Lenski, 1984). Mutants of *E. coli* B that exhibited complete resistance to bacteriophage T4 were shown to be partially resistant to bacteriophage T2, and this was due to the involvement of two receptors in the adsorption of T2 to *E. coli* B cells (Lenski, 1984). Thus, it is possible that two receptor molecules on the cell surface are required for a successful S-MM5 adsorption to *Synechococcus* sp. WH7803. As numerous studies in the past have provided considerable evidence that some viruses recognise more than one receptor (Haywood, 1994), it is possible S-PM2 binds to two receptors on the surface of WH7803. Therefore, any change to one of the receptor molecules may lead to resistance to S-PM2 infection. Another possibility leading to resistance is changes on the cell surface morphology (could be due to missing of outer membrane proteins), which makes the phage receptor(s) inaccessible for phage binding. A detailed study focusing on the nature of the S-PM2 receptor will be described in Chapter 5.

Chapter 5 Characterisation of a phage S-PM2-resistant mutant derived from *Synechococcus* sp. WH7803

5.1 Introduction

5.1.1 SDS-PAGE

SDS-PAGE is a standard technique used to separate proteins according to their primary structure or size (Shapiro *et al.*, 1967). By mixing protein samples with SDS, an anionic detergent, any secondary, tertiary or quaternary structure will be denatured. As a result, all proteins are reduced to the same linear shape with negative charge due to the attachment of the SDS anions. The proteins are then separated on a polyacrylamide gel, which is formed from the polymerization of two compounds, acrylamide and a cross-linking agent, bisacrylamide (Bis). The relative size of the pores formed within the gel is determined by two factors, the total amount of acrylamide and the amount of cross-linker. The pore size increases when the total amount of acrylamide increases. With respect to cross- linking, 5% gives the smallest pore size. Higher or lower than 5% will increases the pore size (Shapiro *et al.*, 1967; Weber and Osborn, 1969). Therefore, different pore-sized gels can be obtained by adjusting the concentration of polyacrylamide and Bis for the purpose of separating a mixture of proteins.

In practice, a multiple gel system comprising of a stacking gel over a separating gel is used. These gels are made from different concentrations of acrylamide using different buffer system. For examples, the stacking gel is usually a large pore-sized polyacrylamide gel that is prepared with Tris/HCl buffer pH 6.8. As a result, SDS-coated proteins are concentrated to a thin starting zone. The resolving gel is a small

pore polyacrylamide gel that is typically made using a pH 8.8 Tris/HCl buffer (Laemmli, 1970). It is the resolving gel that has the power to separate proteins according to their size.

A major drawback to the one-dimensional SDS-PAGE is that it can not distinguish two proteins made of different amino acids but with the same molecular weight. This problem was resolved more than 20 years ago by introducing another dimension of immobilised pH gradient (Gorg *et al.*, 1998), so called two-dimensional SDS-PAGE. The 1st dimension uses isoelectric focusing (IEF) that separates proteins on the basis of their isoelectric points (p*I*s) (Dossi *et al.*, 1983). In the 2nd dimension, proteins are separated by their size.

5.1.2 Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

Mass spectrometry has been successfully used for identification and analysis of molecules for more than half a century (Burlingame *et al.*, 1992). However, it has limitation when analysing large molecules due to their low volatility and thermal instability (Burlingame *et al.*, 1992). To obviate these problems, one techniques that has significant impact on large molecule identification is MALDI-TOF MS, which allows for determination of large biomolecules greater than 200 KDa (Hillenkamp *et al.*, 1991; Beavis *et al.*, 1992). MALDI-TOF MS was introduced by Karas *et al*. and Tanaka *et al*. in 1987-1988 for UV-laser desorption/ionisation of large polypeptides and proteins about 10 kDa (Karas *et al.*, 1987; Karas and Hillenkamp, 1988; Tanaka *et al.*, 1988). The MALDI technique is based on an ultraviolet absorbing matrix,

which is normally an organic aromatic weak acid that can facilitate desorption and ionisation of compounds in samples (Karas *et al.*, 1987; Karas and Hillenkamp, 1988). The principle underlying MALDI-TOF MS is illustrated in Figure 5.1. Typically, the sample/matrix is placed in vacuum and subjected to multiple laser shots. The resulted ions are then accelerated by an applied high voltage, separated in a field-free flight tube. The smaller ions arrive at the detector in a shorter amount of time than the more massive ions. These ions are detected as an electrical signal and recorded as a function of time to yield a TOF mass spectrum. MALDI-TOF MS offers promise for the fast and unequivocal identification of relatively pure material. For example, identification of a protein can be accomplished by breaking it into specific peptide fragments using amino acid specific proteolytic enzymes such as endoprotease trypsin. The enzymatic digestion results a unique 'mass-map' or profile, which is used to search against databases to allow unambiguous identification.

5.1.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

LC or high performance liquid chromatography (HPLC) is a form of column chromatography to separate and identify compounds. LC system comprises a column packed with stationary phase, a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. MS/MS is a technique that involves two mass spectrometers. Typically, the first MS functions in selecting a single mass. The second MS is used to separate the mass-selected fragment ions (Wysocki *et al.*, 2005). By coupling an LC system, LC-MS/MS becomes a

powerful technique in proteomics, such as peptide sequencing and protein identification (Delahunty and Yates, 2005).

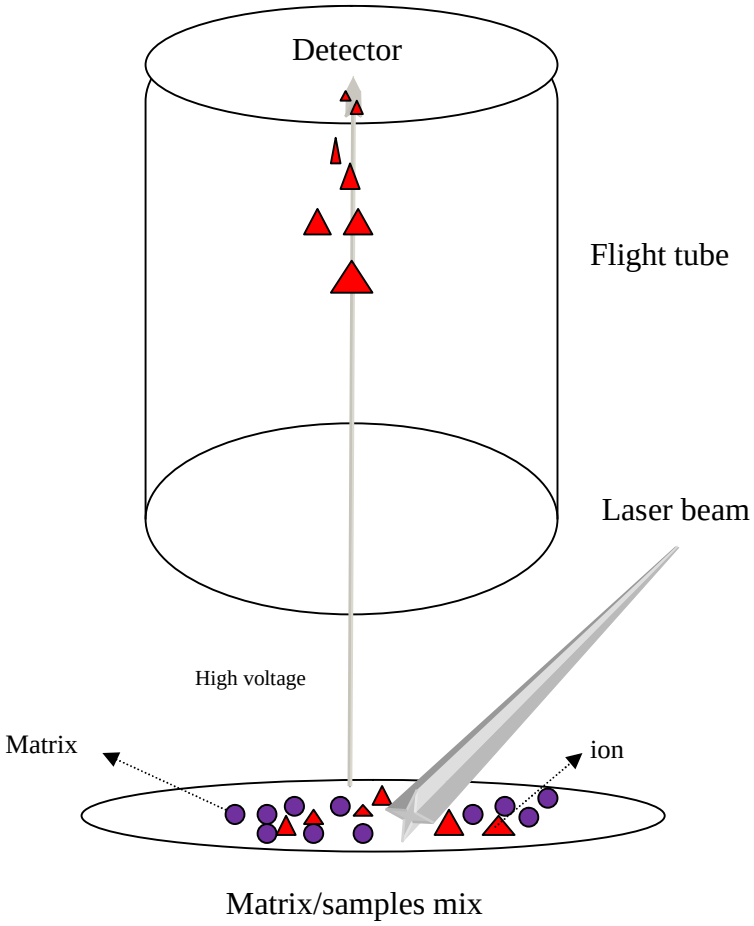


Figure 5.1 Cartoon to show the principles of MALDI-TOF MS.

The matrix/sample mix is subjected to laser shot. The ions are separated in a flight tube where small ions reach the detector first.

5.1.4 Cyanobacterial cell surface

The cell surface is critical in determining the susceptibility of a bacterium to bacteriophage infection as phage adsorption is dependent on the presence of specific attachment sites, receptors, on the cell surface. The cyanobacterial cell surface

displays a combination of Gram positive and Gram negative characteristics. It has a considerably thicker peptidoglycan layer than that of most Gram negative bacteria (Golecki, 1977; Hoiczyk and Baumeister, 1995) (Figure 5.2). Frequently, cyanobacterial outer membranes (OMs) are covered by external surface layers such as the S-layer, which is protein surface layer formed by a single protein (for a review see Sara and Sleytr, 2000). As part of the Gram negative cell envelope structure, S-layers may function as a protective coat (Koval, 1997) or molecular sieves providing sharp cutoff levels (Sara and Sleytr, 1987). They also act as adhesion sites for cell-associated exoenzymes (Egelseer *et al.*, 1995) and may contribute to virulence when they are part of the cell envelope of pathogens (Doig *et al.*, 1992).

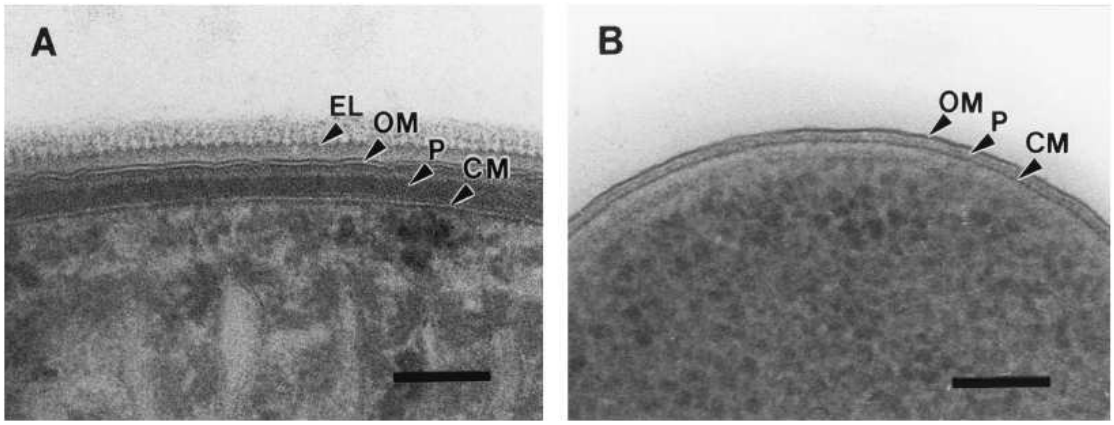


Figure 5.2 Electron micrograph images showing the cell envelope of the cyanobacterium *Phormidium uncinatum* (A) and *E. coli* (B).

The cyanobacterial cell wall possesses a much thicker peptidoglycan layer and a serrated external layer (e.g. an S-layer) outside the outer membrane. CM, cytoplasmic membrane; EL, serrated external layer; OM, outer membrane; P, peptidoglycan layer. Bars, 100 nm. From Hoiczyk and Hansel (2000)

5.1.5 Cyanophage receptors

Limited work has been carried out to identify the cell receptors recognised by cyanophages. One early report based on the model system consisting of cyanophage AS-1 and *Anacystis nidulans* (*Synechococcus* sp. PCC 7942) suggested that both the LPS and protein fractions may be involved in the binding process based on the fact that AS-1 was inactivated by LPS and protein fractions extracted from the cell surface (Katz *et al.*, 1977; Samimi and Drews, 1978). Disruption in *Anabaena* sp. strain PCC 7120 of the genes thought to encode undecaprenyl-phosphate galactosephosphotransferase (*rfbP*) and mannosyl transferase (*rfbZ*) led to resistance of the obligately lytic phage A-1 and the temperate phage A-4 (Xu *et al.*, 1997). Both these enzymes are involved in the synthesis of the O-antigen component of LPS and electrophoretic analysis showed that the interruption of the *rfbP* and *rfbZ* genes led to a change in, or loss of, the characteristic pattern length of the LPS (Xu *et al.*, 1997). The O-antigen is comprised of serially repeated, strain-specific oligosaccharide units. Thus this study suggests that LPS may play an important role in cyanophage adsorption and variation in the nature of the O-antigen component may determine cyanophage host range.

5.2 Aims

To characterise the phage S-PM2-resistant mutant derived from *Synechococcus* sp. WH7803 and localise the cell receptor(s) in *Synechococcus* strain WH7803 to which cyanophage S-PM2 binds.

5.3 Results

5.3.1 SDS-PAGE of whole-cell proteins of WH7803 and WH7803RS-PM2

In order to identify the potential host components that can act as S-PM2 receptor(s), whole-cell SDS-PAGE was used to characterise the protein profiles of *Synechococcus* sp. WH7803 and WH7803RS-PM2 cells. As shown in Figure 5.3, a single protein band with a size of ~180 kDa is only present in *Synechococcus* sp. WH7803 (The poor quality of the picture is due to the resolution limit in printing and an electronic version of Figure 5.3 can be found in the CD accompanied this thesis). Apart from this protein band no other difference can be revealed by comparing the protein profile based on the one dimensional PAGE (identical results were obtained from three biological replicates). The identity of this protein was established.

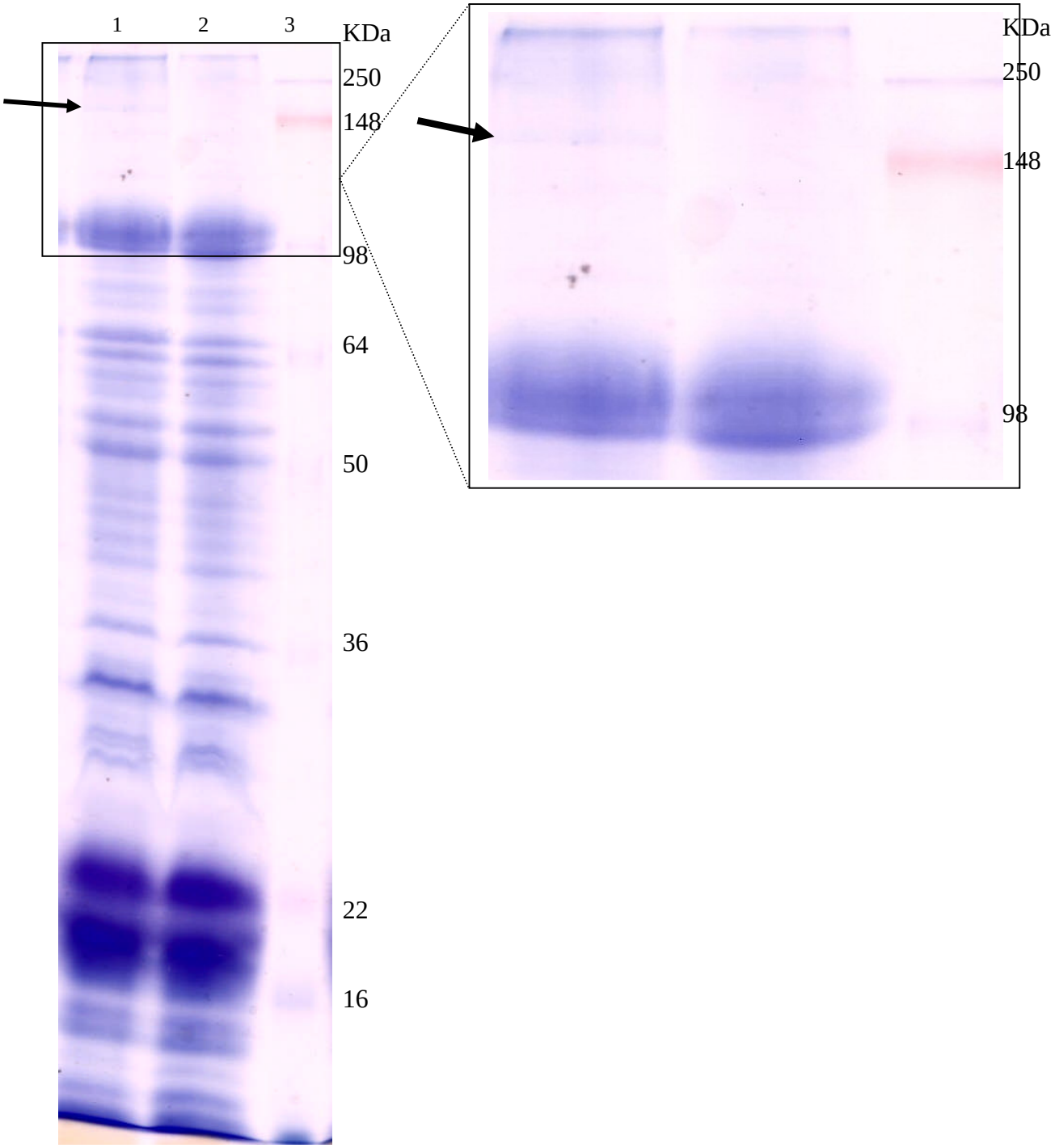


Figure 5.3 SDS-PAGE profiles of whole-cell protein extracts from *Synechococcus* sp. WH7803 and WH7803RS-PM2 cells.

Lane 1 *Synechococcus* sp. WH7803, Lane 2 *Synechococcus* sp. WH7803RS-PM2, Lane 3 SeeBlue® Plus2 Pre-Stained protein standards (Invitrogen) that were used to estimate protein molecular weight. The missing band from the mutant is indicated by an arrow (a better view can be achieved by referring to the electronic version of this picture in the CD).

5.3.2 Protein identification by MALDI-TOF and LC-MS/MS

The protein band present only in *Synechococcus* sp. WH7803 was excised from the gel and analysed by MALDI-TOF MS to obtain a 'mass fingerprint' and the remainder peptides separated using a nano-scale HPLC system, followed by online MS/MS (this part was performed and analysed by Samuel J.H. Clokie).

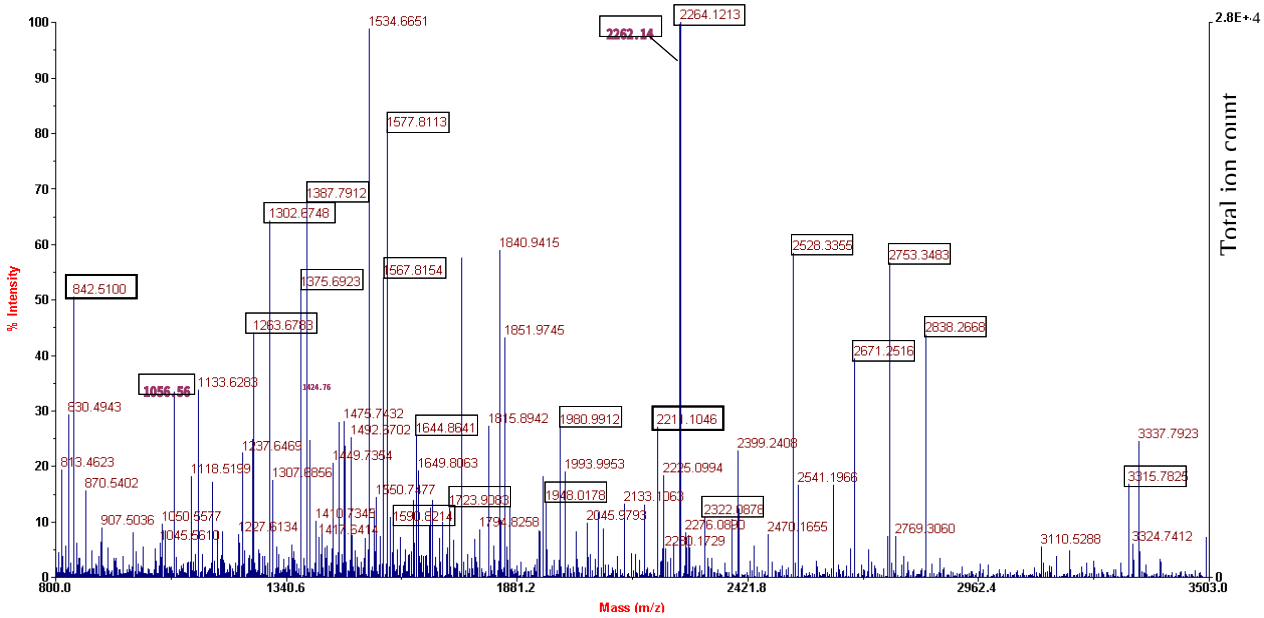
MALDI-TOF analysis (Figure 5.4B) yielded 118 peptides of sufficient quality (signal to noise ratio) to search against the *Synechococcus* sp. WH7803 genome (Genbank accession NC009481) using the Protein Prospector program (UCSF, CA). The results clearly showed that the protein is the putative multicopper oxidase (MCO) (encoded by the ORF0948 in *Synechococcus* sp. WH7803) with a MOWSE score significantly (~10^6) above the runner-up hit (Figure 5.4A and 5.4C). As a control no significant hits were recorded when the mass list was searched against the entire NCBI non-redundant database using the MASCOT (www.matrixscience.com) web-based search program or the Protein Prospector program. A nano-scale HPLC also confirmed the MALDI-TOF identification as the putative MCO (Figure 5.4D, 5.4E and 5.4F). The experiment was repeated three times and the mass spectral analysis performed on three separate experiments.

A

Rank	Score	% coverage	MW/PI	Protein
1	1.87e+^{010}	30/118 (25%)	187712.2/4/21	Putative MCO
2	1.45e+^{004}	12/118 (10%)	67890.9/7/41	Porin, P stress induced
3	3.26e+^{004}	7/118 (5%)	19058.8/5.84	CpeB C-phycoerythrin class I beta chain
4	785	6/118 (5%)	17825.1/5.84	MpeA C-phycoerythrin class II alpha chain
5	245	9/118 (7%)	48480/7/74	CtpA Carboxyl-terminal processing protease

B

Mass Spectrum of 180 kDa band



C

>putative MCO, MW: 187712.2 / PI:4.21

MATKLIMNPLDFLASGWLNR**SLLGTTDQPYQFKIAADTYIDLLRLDTQGR**DLDFGFESLSEQTWLSEFQQANASSLD
LEKLR**TDRWLQTEGVSLGGRRATPISADNWRPIARDSSGELLSLEEVTLTGNSALARFSGGVEAVYSVGGSGLLA**
NAIPEGPQVVVEGTVRRLANYNNGIAIYEADPLNGAVDGLSPGDPGYLQAALRDAKESGLVFSAFQLPENGSIGSINLN
LSPDKNYGFLLLVDGDESNLFSSYSSANPDNAVQVVSFTTSDGSLALGFEDQLVTGESDQDFNDVILTLPITTSTDILSEV
NYR**IGSLFPTLEDGSPNLILSSGLELR**TIDGESR**VVSSLQSGTLDTPMRISSDPVRIPGIDR**FLRDWFIPQLTDTANSQS
LFNAYKK**GVLSKWTTHARNR**SMEDLLKIGYVGPGNDQSATNDDYWNAVGVVGSDLYK**AASDSSNYKPAELWDAY**
DGSTTVFSRFDTDALLQRLEALTDPEANPNPDLWYPSMLYTFGVPGEGTSYPAPVLMMQPGDGMNLNFTNDIKVDGL
NEEQNQAASLVSNSTYGNAAGDGLGALNAVNYHLHGSHTNPGGFGDNVVARYTTGQQWTTEIDLPADHGQGSYWY
HPHYHPSVNQMVYGGMSGPFQVGDPLSKIPLFKDIPR**NWAVLK**TMDVGIDAETGK**LRLDGFDNLGGVVNR**MTMVT
VNGEFQPTAEAGEGGWQAITLSNQTNQA**FHNISLIHTDSDGNRTTLPLYLYGEDGHQYPQIR**AATDGIFGASGASNQ
LPTGYTQAVDLLSLPPAK**RVDVLVYLPEGK**TEMASTYSFEQDGVDYTINNAGSYPELTEINTGFGSKTGAGPLALGNV
EGGQALPTTAELDAVIAQANAGIDVQQILPTTSQADYDPLQVPSVDLFAQDADGSDLWDPIRQQFNWTK**GTLVGPASE**
YDAATVELLKHYSMMNDGATYEPYTSLPVGKPGVDNWLGYNNPFLINDHVFPYGNMTIAQLGTIEEWVNRNWSINSP
SK**YIGHPFHIHINDYQVK**DSDTELQNKR**NLEDTTSLNSSGYEFYDPAAKEVVSLEPQRGEFHSIPEAQDPEKISLATF**
GANDQTIRMLYQDYLGTYVFHCHILPHEDAGMMQIVTVVENTDSSWLVEAQGFTQNESGVR**LYQAQTFDSVQLQAL**
PDSGQTWTRAQAGNLGADFVQDIALAAGGGGEAGIIELFDGAALRGETLRTSR**LTPYADSSLAPWVFIEDFSGDGQR**
DLVTAGFDQVQSDVVNLKDLEIKAFLPGEAPGSWDEQFNFDPFDDISLMAPHSVMPRMGLSADQVSVAMADMNLDN
FQDVAIAYAVEGGVRLVVIDGAALSLMFQTGEMEGGFFADSNVLADAVFLDSGLSDLSQLVLTSGFNSYAQSALENLV
LTTQSSAGSQQFTLQLQAGHFIATNLPDSSESGGHGGHGGHGGAGLSPDERITNLRNNSLPLFLVDELQLANGTEAVTPT
ISAGLGHGGTLLDGHAVIAQGNEVNGNASNSDILINTTQQLVIPLDGLNLINADDLTGIVDTTSSSTFTAEQVQQRYQLTS

MTYLAYTGKLLWPSALASQAASILGTGEQASALVTNLLSSPAYAGEIEALYGGPLADQSVNDIVEIAYSTLYKRSATASE
LQSWQDQVSAGLDQTLLPQAILQSTQEADRFRVALLSDITQWTALQWGTTAEVSGSYGQGLVGDEQVSNQLDALASSL
GSYASFEDAQQGFDLFTTEALQELIGTPVSKSGFF

D

Rank	# matched peptides*	% coverage by aa no.	MW/PI	Protein
1	11 (25)	17.2%	187712.2/4/21	Putative MCO
2	2 (12)	17.5%	67890.9/7/41	Porin, P stress induced
3	1 (9)	36.8%	22361/	Uncharacterised conserved secreted protein

E

#	GBU	TIC	File	z	dM	MH+	xC	dCn	Sp	RSp	Ions	Sf	Ref	()	Sequence	Prob
A gi|5958 248 31|Σ:8.3e8|4%|avg:2.7e7 {29,1,1,1,1,4}																
294		5.3e7	1788-1797	2	0.6	1947.4	6.02	0.68	3186	1	26/34	--	gi|5958		(R)DLVTAGFDQVQSDVVNLK	
263		2.5e8	1521-1554	2	0.8	1933.2	5.38	0.75	1959	1	25/36	--	gi|5958		(K)GTLVGPASEYDAATVELLK	
232		8.6e7	1326-1329	2	0.6	1375.1	4.51	0.61	1607	1	21/24	--	gi|5958		(R)LDGFDNLGGVVNR	
216		4.0e7	1257-1302	2	0.7	1577.1	4.43	0.69	957	1	18/28	--	gi|5958		(K)IASLATFGANDQTIR	
334		2.3e7	2220-2262	2	0.6	2670.7	4.35	0.74	678	1	22/46	--	gi|5958		(R)LTPYADSSLAPWVFIEDFSGDGQR	
244		7.9e6	1404-1407	2	0.6	2263.6	3.52	0.69	1366	1	21/36	--	gi|5958		(R)TTLPLYLYGEDGHQYPQIR	
222		1.9e7	1284	2	1.3	1395.4	3.30	0.55	1372	1	19/22	--	gi|5958		(R)SLLGTDQPYQFK	
256		1.7e6	1482	2	-0.2	2264.3	3.25	0.67	706	1	15/36	--	gi|5958		(R)TTLPLYLYGEDGHQYPQIR	
260		1.8e7	1509	2	0.6	2837.7	3.12	0.60	471	1	18/50	--	gi|5958		(K)AASDSSNYKPAELWDAYDGSTTVFS	
170		2.2e7	0960	2	0.3	1583.5	3.12	0.46	681	1	16/26	--	gi|5958		(R)GEFHSIPEAQDPEK	
314		1.0e6	2028	2	0.8	2527.6	2.73	0.65	857	1	22/46	--	gi|5958		(R)IGSLFPTLEDGSPNLILSSGLELR	
190		3.5e7	1101-1224	2	1.0	1566.8	2.44	0.43	257	1	12/26	--	gi|5958		(R)ATPISADNWRPIAR	
312		1.3e6	2004	2	0.7	2545.7	2.35	0.60	522	1	15/44	--	gi|5958		(R)DLVTAGFDQVQSDVVNLKDLEIK	
258		4.7e7	1497-1500	1	0.4	1263.3	2.32	0.38	256	1	12/20	--	gi|5958		(K)IAADTYIDLLR	
175		2.5e7	0984	2	0.3	1056.3	2.23	0.50	420	2	11/16	--	gi|5958		(K)EVVSLEPQR	
259		2.1e7	1503	1	-1.7	1265.4	2.13	0.43	251	1	11/20	--	gi|5958		(K)IAADTYIDLLR	
174		1.4e7	0975-0981	1	0.2	1056.4	1.82	0.39	273	1	11/16	--	gi|5958		(K)EVVSLEPQR	
180		1.8e6	1032	1	-0.7	1033.2	1.80	0.30	300	1	11/16	--	gi|5958		(R)NWSINSPSK	
179		2.1e7	1020-1029	1	0.7	822.7	1.68	0.46	74	1	8/10	--	gi|5958		(R)QFNWTK	
249		4.2e6	1434	1	0.6	1231.1	1.54	0.38	493	2	10/20	--	gi|5958		(R)VDVLVYLPEGK	
214		2.6e7	1245-1248	1	0.3	1078.3	1.51	0.55	329	1	12/16	--	gi|5958		(R)FDTDALLQR	
188		3.3e7	1086-1089	1	0.3	730.1	1.51	0.41	447	1	8/10	--	gi|5958		(R)NWAVLK	
167		1.5e7	0924-0927	1	0.2	670.2	1.45	0.17	135	2	7/10	--	gi|5958		(R)IPGIDR	
231		1.8e7	1323	1	0.4	1375.3	1.41	0.49	108	1	9/24	--	gi|5958		(R)LDGFDNLGGVVNR	
186		3.6e7	1071-1134	1	0.5	616.9	1.39	0.32	162	2	6/8	--	gi|5958		(K)IPLFK	
204		3.5e6	1185	1	1.1	834.3	1.38	0.37	396	1	8/12	--	gi|5958		(R)SMEDLLK	
168		2.4e6	0930	1	0.2	851.2	1.22	0.23	88	2	7/12	--	gi|5958		(R)SM*EDLLK	
93		3.0e6	0495-0693	1	-0.2	503.6	1.08	0.05	260	2	6/8	--	gi|5958		(K)GVLSK	
145		2.1e6	0810	1	0.3	773.1	0.96	0.06	78	9	5/12	--	gi|5958		(R)ISSDPVR	
59		3.9e5	0291-0330	1	-0.1	503.4	0.64	----	39	26	3/8	--	gi|5958		(K)GVLSK	
264		1.8e5	1527-1569	1	-1.0	690.3	0.60	----	30	26	2/10	--	gi|5958		(R)LDTQGR	

F

>putative MCO, MW: 187712.2 / PI:4.21

MATKLIMNPLDFLASGWLNR**SLLGTTDQPYQFKIAADTYIDLLRLDTQGR**DLDFGFESLSEQTWLSEFQQANASSLD
LEKLRTDRWLQTEGVSLGGRR**ATPISADNWRPIAR**DSSGELLSLEEVTLTGNSALARFSGGVEAVYSVGGSGLLANAIP
EGPQVVVEGTVRRLANYNNGIAIYEADPLNGAVDGLSPGDPGYLQAALRDAKESGLVFSAFQLPENGSIGSINLNLSPD
KNYGFLLLVDGDESNLFSSYSSANPDNAVQVVSFTTSDGSLALGFEDQLVTGESDQDFNDVILTLPITTSTDILSEVNYR**I
GSLFPTLEDGSPNLILSSGLELR**TIDGESRVVSSLQSGTLDTPMR**ISSDPVRIPGIDR**FLRDWFIPQLTDTANSQSLFNAY
KK**GVLSK**WTTHARNR**SMEDLLK**IGYVGPGNDQSATNDDYWNAVGVVGSDLYK**AASDSSNYKPAELWDAYDGSTT
VFSRFDTDALLQR**LEALTDPEANPNPDLWYPSMLYTFGVPGEGTSYPAPVLMMQPGDGMNLNFTNDIKVDGLNEEQN
QAASLVSNSTYGNAAGDGLGALNAVNYHLHGSHTNPGGFGDNVVARYTTGQQWTTEIDLPADHGQGSYWYHPHYHP
SVNQMVYGGMSGPFQVGDPLSK**IPLFK**DIPR**NWAVLK**TMDVGIDAETGKLR**LDGFDNLGGVVNR**MTMVTVNGEFQ
PTAEAGEGGWQAITLSNQTNQAFHNISLIHTDSDGNR**TTLPLYLYGEDGHQYPQIR**AATDGIFGASGASNQLPTGYTQ
AVDLLSLPPAKRVDVLVYLPEGKTEMASTYSFEQDGVDYTINNAGSYPELTEINTGFGSKTGAGPLALGNVEGGQALPT
TAELDAVIAQANAGIDVQQILPTTSQADYDPLQVPSVDLFAQDADGSDLWDPIRQ**QFNWTKGTLVGPASEYDAATVE
LLK**HYSMMNDGATYEPYTSLPVGKPGVDNWLGYNNPFLINDHVFPYGNMTIAQLGTIEEWVNR**NWSINSPSK**YIGHP

FHIHINDYQVKDSDTELQNKRNLEDTTSLNSSGYEFYDPAA**KEVVSLEPQRGEFHSIPEAQDPEKISLATFGANDQTI**
RMLYQDYLGTYVFHCHILPHEDAGMMQIVTVVENTDSSWLVEAQGFTQNESGVRLYQAQTFDSVQLQALPDSGQTW
TRAQAGNLGADFVQDIALAAGGGGEAGIIELFDGAALRGETLRTSR**LTPYADSSLAPWVFIEDFSGDGQRDLVTAGF**
DQVQSDVVNLKDLEIKAFLPGEAPGSWDEQFNFDPFDDISLMAPHSVMPRMGLSADQVSVAMADMNLDNFQDVAIA
YAVEGGVRLVVIDGAALSLMFQTGEMEGGFFADSNVLADAVFLDSGLSDLSQLVLTSGFNSYAQSALENLVLTTQSSA
GSQQFTLQLQAGHFIATNLPDSSESGGHGGHGGHGGAGLSPDERITNLRNNSLPLFLVDELQLANGTEAVTPTISAGLGH
GGTLLDGHAVIAQGNEVNGNASNSDILINTTQQLVIPLDGLNLINADDLTGIVDTTSSSTFTAEQVQQRYQLTSMTYLAY
TGKLLWPSALASQAASILGTGEQASALVTNLLSSPAYAGEIEALYGGPLADQSVNDIVEIAYSTLYKRSATASELQSWQD
QVSAGLDQTLLPQAILQSTQEADRFRVALLSDITQWTALQWGTTAEVSGSYGQGLVGDEQVSNQLDALASSLGSYASF
EDAQQGFDLFTT**EALQE**LIGTPVSKSGFF

Figure 5.4 The protein band that was absent in the WH7803RS-PM2 cells migrating to approximately 180 kDa corresponds to a putative MCO, confirmed by MALDI-TOF mass spectrometry and peptide mass fingerprinting.

A The table shows top scoring hits from the 118 masses submitted to the *Synechococcus* sp. WH7803 genome in house, indicating MOWSE (Pappin *et al.*, 1993) scores and the sequence coverage. The table shows the putative MCO as the clear winner, with a lead of over 10^6 to the next match. **B** the mono-isotopic spectrum shows the masses of peptides recovered from the tryptic digest of the 180 kDa band found in *Synechococcus* sp. WH7803. Peptide masses corresponding to the theoretical digest of MCO are shown – not all matching masses are displayed in this image. Heavily lined boxes indicate trypsin masses (842.51 and 2211.10) used for internal calibration of the spectra. Altogether 30 matching peptides were recovered, corresponding to 25% of the MCO protein. The distribution of the matched peptides within the protein sequence is shown in **C** with matching peptides shown in bold. Although there are no matches in the C-terminal 500 amino acids of this protein, there is only one possible tryptic peptide (due to the low abundance of Arginine and Lysine residues) within this region that is in the range of MALDI-TOF analysis. The mass tolerance used to search the database was set at 50 ppm. **D** shows the most significant hit as the MCO from data derived from LC-MS/MS analysis of the 180 kDa band. **E** shows a summary of data for each peptide with good cross-correlation scores corresponding to the MCO. **F** shows in bold, the location of the peptides in the protein sequence. As control, both sets of data were searched against the NCBI nr database and no significant hits were reported.

5.3.3 Sequencing the putative MCO gene in *Synechococcus* sp. WH7803RS-PM2 strains

One possibility that could lead to the absence of the putative MCO protein in the S-PM2-resistant strain was a mutation in the gene sequence. To test this hypothesis, the gene in the mutant strain was sequenced and compared with its counterpart in the wild

type strain. To do this, a 5256-bp PCR product covering the region of the putative MCO gene was amplified from *Synechococcus* sp. WH7803 and WH7803RS-PM2 using the 'Expand Long Template PCR System' (Roche) with the following primers: ORF0948F 7, 5'-ATGGTTAATGGCGACGAAGC-3'; ORF0948R+13, 5'-TGGCGTTCAGCAATCAGAAG-3'. PCRs were carried out in a total volume of 50 µl, containing 0.35 mM dNTPs, 0.35 µM primers, 100 ng of DNA, 0.75 µl of Expand Long Template Enzyme mix, and 5 µl of Expand Long Template buffer 1. Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 20 sec, 65°C for 30 sec, 68°C for 4 min + 20 sec/cycle, with a final extension of 7 min at 68°C.

When different amounts of input template DNA (200, 10 and 5 ng) were used, single PCR products with the expected size of 5256-bp were revealed (Figure 5.5A). A well-defined PCR product was generated from 100 ng of input DNA template (Figure 5.5B), which was then purified and used as the DNA template in sequencing. Based on the known sequence of ORF0948 in *Synechococcus* sp. WH7803, 10 sequencing primers were designed (Table 5.1). The primer, 0948R3638C (bolded in Table 5.1), was used to sequence the gap between the primers 0948F2720 and 0948F3267.

In addition, PCR primers (upstream0948F848, 5'-AGCATCGCCAACCAGCTCAC-3'; upstream0948R+422, 5'-ACCGCCTCAACACCACCAGA-3') targeting a 1270-bp region (including an 848-bp upstream region with respect to the transcription start point of the ORF0948) were also designed to examine if there were any alternations in the promoter regions of ORF0948 in wild-type and mutant strains. PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM

primers, 10 ng of DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 65°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. The resulting PCR products (Figure 5.6) were sequenced using the primers of upstream0948F848 and upstream0948R+422.

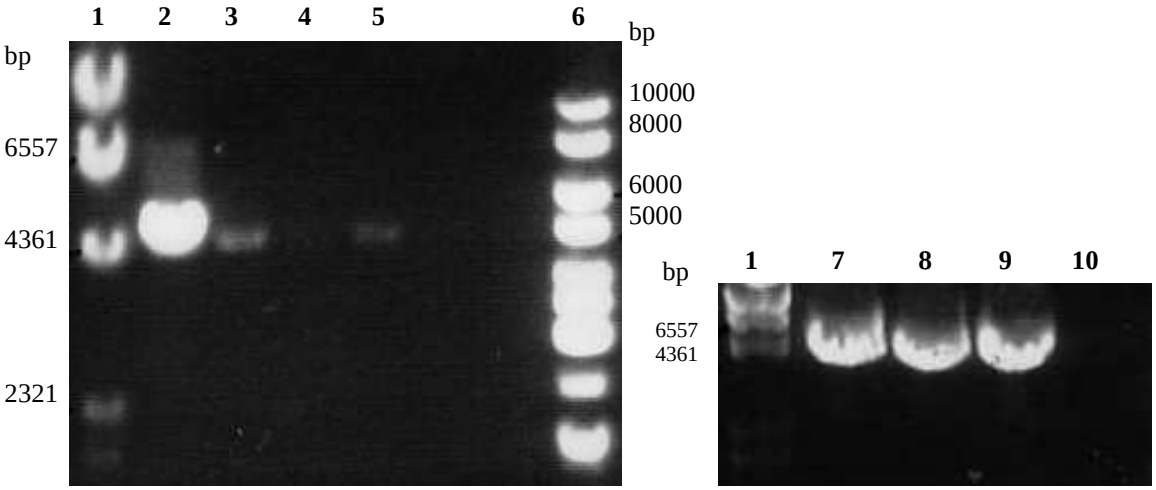


Figure 5.5 Gel image of PCR products generated by the PCR primer targeting the putative MCO gene.

DNAs from *Synechococcus* sp. WH7803 and WH7803RS-PM2 were used as templates to perform PCRs. Lane 1 DNA ladder of λDNA *Hind*III digest in bp, Lane 2 200 ng of WH7803RS-PM2 DNA as template, Lane 3 10 ng of WH7803RS-PM2 DNA as template, Lane 4 a negative control, Lane 5 5 ng of WH7803RS-PM2 DNA as template, Lane 6 GeneRuler™ 1kb DNA ladder from Fermentas in bp, Lane 7 100 ng of WH7803 DNA as template, Lane 8 and 9 100 ng of WH7803RS-PM2 DNA as template, Lane 10 a negative control.

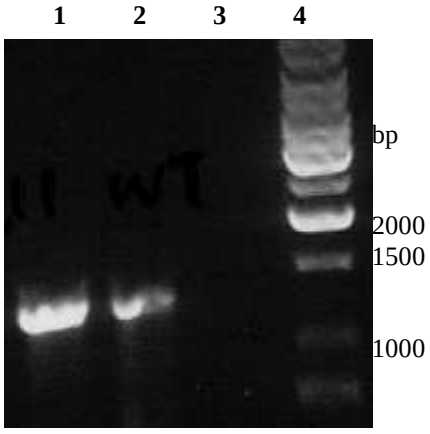


Figure 5.6 Gel image of PCR products generated by the PCR primer targeting the upstream region of the putative MCO gene.

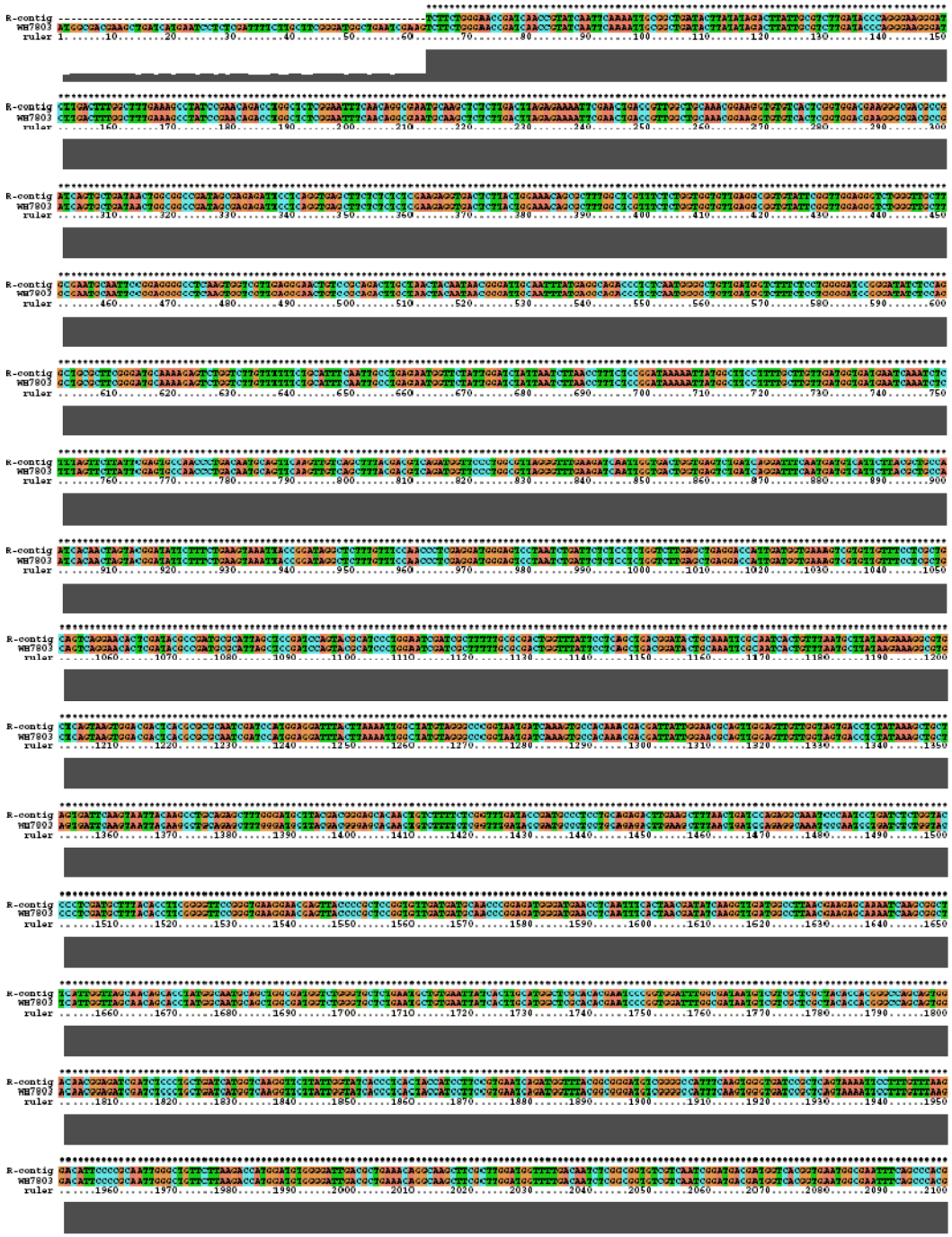
DNAs from *Synechococcus* sp. WH7803 and WH7803RS-PM2 were used as templates to perform PCRs. Lane 1 WH7803RS-PM2 DNA as template, Lane 2 WH7803 DNA as template, Lane 3 a negative control, Lane 4 GeneRuler™ 1kb DNA ladder in bp from Fermentas.

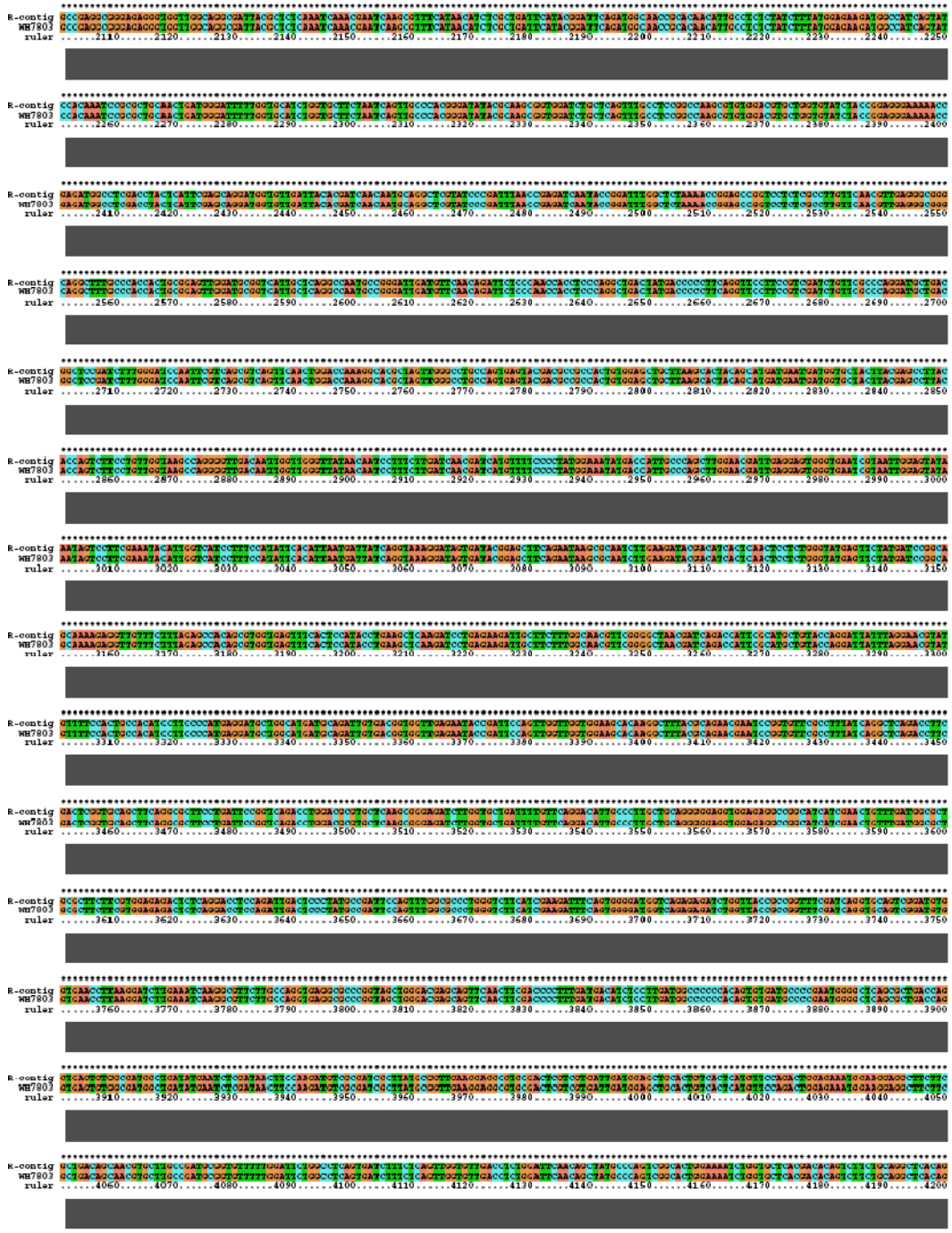
Table 5.1 Primers used for sequencing the ORF0948 in *Synechococcus* sp. WH7803RS-PM2

Primers	**5'-3'**
0948F498	CCGCAGACTTGCTAACTACA
0948F1078	CGCATTAGCTCCGATCCAGT
0948F1644	AGCGGCTTCATTGGTTAGCA
0948F2190	TTCAGATGGCAACCGCACAA
0948F2720	CAATTCGTCAGCGTCAGTTC
0948F3267	TCGCATGCTGTACCAGGATT
0948F3622	CTCAGGACCTCCAGATTGAC
0948F4000	GCTGCACTGTCACTCATGTT
0948F4850	TTGCCTATTCCACGCTCTAC
0948R3638C	AATCTGGAGGTCCTGAGAGT
0948R5111C	GCATCCAACTGATTGGAGAC

When the sequencing results of the ORF0948 of *Synechococcus* sp. WH7803RS-PM2 were assembled and aligned with that of *Synechococcus* sp. WH7803, a 100% identity was revealed (Figure 5.7A). This demonstrated that the putative MCO gene sequences were the same in S-PM2-resistant and wild-type strains. In addition, the 848-bp upstream regions of the ORF0948 also showed 100% identity (Figure 5.7B). These date demonstrated that there were no mutations in the putative MCO.

A





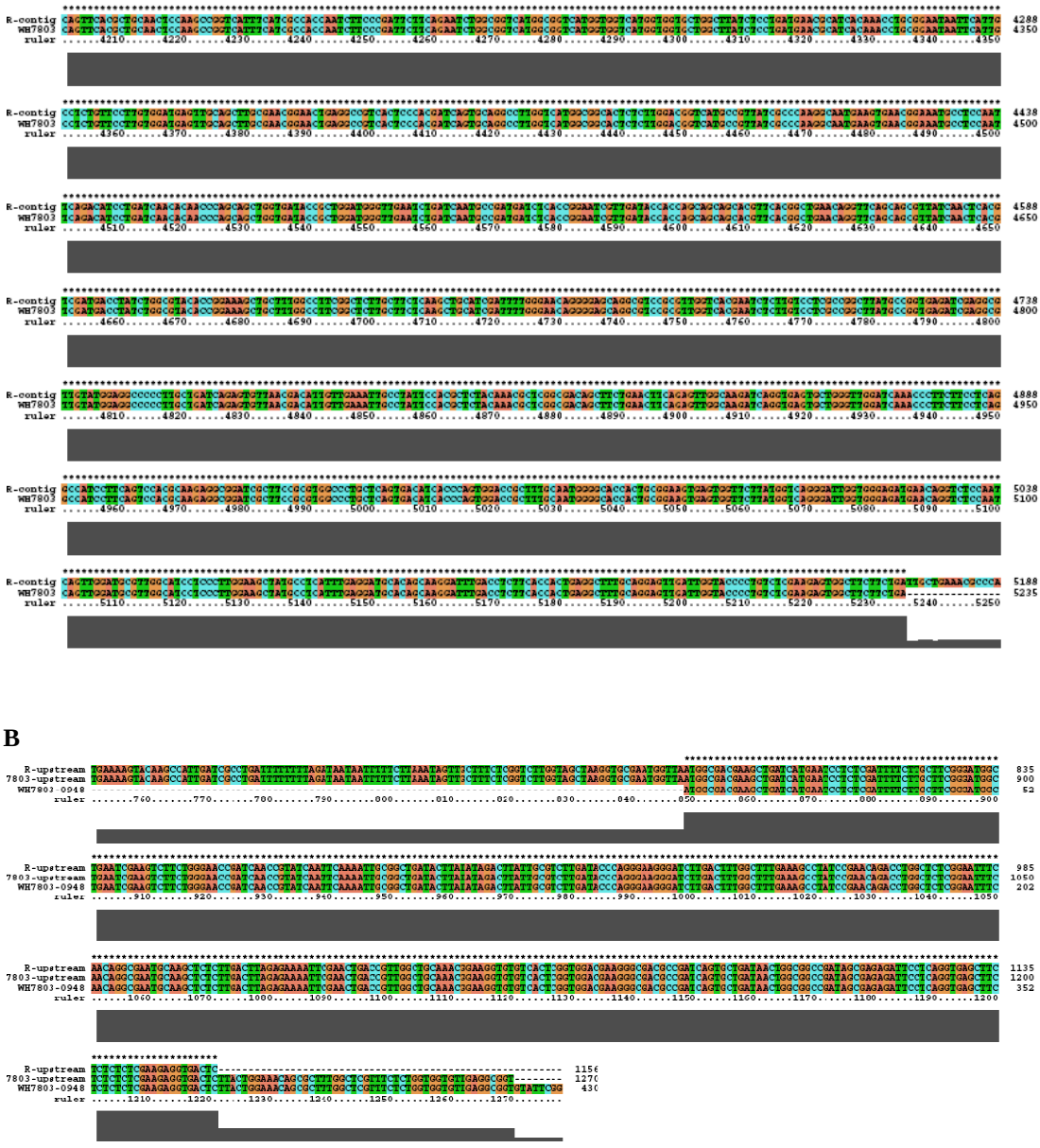


Figure 5.7 Sequence alignments of the putative MCO gene and its 848-bp upstream region in S-PM2-resistant strain and *Synechococcus* sp. WH7803.

A The putative MCO gene. **B** The 848-bp upstream region. A 5256-bp PCR product covering the putative MCO-coding region were amplified and subjected to DNA sequencing. A 1270-bp region including a 848-bp upstream segment with respect to the transcription start point of the ORF0948 was amplified and were subjected to DNA sequencing.

5.3.4 Reverse transcriptase (RT)-PCR analysis of the putative MCO gene expression in *Synechococcus* sp. WH7803 and WH7803RS-PM2 strains

Another possibility that could lead to the absence of the putative MCO protein in the S-PM2-resistant strain was a mutation affecting transcription. To test this hypothesis, RT-PCR was used to determine the expression of the MCO-coding gene (ORF0948) in *Synechococcus* sp. WH7803 and WH7803RS-PM2 cells. A 407 bp PCR product was amplified from regions within ORF0948 by using the following primers: KO407F493, 5'-ACTGTCCGCAGACTTGCTAA-3'; and KO407R899C, 5'-GGCAGCGTAAGAATGACATC-3'. Another 307 bp PCR product was amplified from other regions within ORF 0948 by using the following primers: KO307F1308, 5'-GAACGCAGTTGGAGTTGTTG-3'; and KO307R1677C, 5'-GCCATAGGTGCTGTTGCTAA-3'. Both PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 4 mM $MgCl_2$, 0.4 µM primers, 10 ng of cDNA (or DNA for positive control), 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C.

Furthermore, a 249 bp PCR product representing the structural gene of *mpeBA* coding for the major light-harvesting protein was amplified to demonstrate the quality of the cDNA. The following primers were used mpeBAF492, 5'-ACTCTCCGCTGAGGCTGCTT-3'; and mpeBAR740C, 5'-GCGATGGCGTCGTAGTTCTG-3'. The PCR was carried out in a total volume of 50

µl, containing 0.25 mM dNTPs, 1 mM $MgCl_2$, 0.4 µM primers, 10 ng of cDNA (or DNA for positive control), 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C.

As shown in Figure 5.8, no PCR products were obtained when using RNA as templates, which rules out the possibility of genomic DNA contamination. As expected, a PCR product for *mpeBA* was obtained using cDNA derived from WH7803 and WH7803RS-PM2 cells. The two sets of PCR primers targeting two different regions of the putative MCO gene generated products on cDNA derived from WH7803 and WH7803RS-PM2, which demonstrated that this gene was still transcribed in the WH7803RS-PM2 cells.

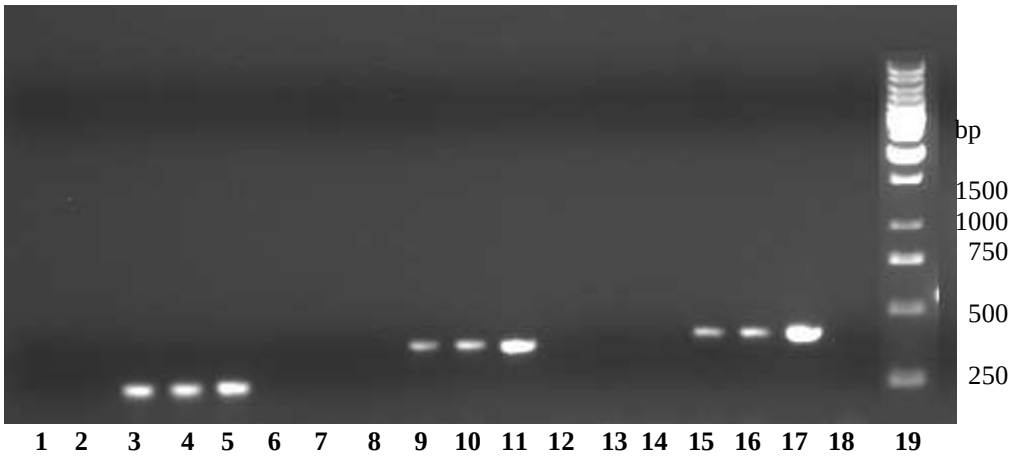


Figure 5.8 Gel image of PCR products using the primer pair to amplify a region within ORF 0948 (coding for muticopper oxidase) against cDNA from wild-type and S-PM2-resistant cells.

Lanes are numbered from left to right. PCR reactions with different primers and templates are as follows: Lane 1 *mpeBA* primers + RNA extracted from WH7803RS-PM2 cells, Lanes 2 *mpeBA* primers + RNA extracted from WH7803 cells, Lane 3 *mpeBA* primers + WH7803RS-PM2 cDNA, Lane 4 *mpeBA* primers + WH7803 cDNA, Lane 5 *mpeBA* primers + WH7803 DNA, Lane 6 a negative

control (*mpeBA* primers + water), Lane 7 ORF 0948 (370) primers + RNA extracted from WH7803RS-PM2 cells, Lanes 8 ORF 0948 (370) primers + RNA extracted from WH7803 cells, Lane 9 ORF 0948 (370) primers + WH7803RS-PM2 cDNA, Lane 10 ORF 0948 (370) primers + WH7803 cDNA, Lane 11 ORF 0948 (370) primers + WH7803 DNA, Lane 12 a negative control (ORF 0948 (370) primers + water), Lane 13 ORF 0948 (407) primers + RNA extracted from WH7803RS-PM2 cells, Lanes 14 ORF 0948 (407) primers + RNA extracted from WH7803, Lane 15 ORF 0948 (407) primers + WH7803RS-PM2 cDNA, Lane 16 ORF 0948 (407) primers + WH7803 cDNA, Lane 17 ORF 0948 (407) primers + WH7803 DNA, Lane 18 a negative control (ORF 0948 (407) + water), Lane 19 GeneRuler™ 1kb DNA ladder in bp from Fermentas.

5.3.5 Isolation and characterisation of the outer membrane fractions (OMFs) of *Synechococcus* sp. WH7803 and WH7803RS-PM2

In order to establish the role this MCO protein may play in S-PM2 adsorption, its subcellular localisation was investigated.

5.3.5.1 Bioinformatic prediction

The translated amino acid sequence (Figure 5.9A) from the DNA sequence of the ORF0948 of *Synechococcus* sp. WH7803 genome was subjected to localisation prediction using PSORTb version 2.0.4. The most likely predictions were outer membrane or extracellular (Figure 5.9B).

A

>SynWH7803_0948 Putative MCO

MATKLIMNPLDFLASGWLNRSLLGTDQPYQFKIAADTYIDLLRLDTQGRDLDFGFESLSEQTWLSEFQQANASSLDLEK
IRTDRWLQTEGVSLGGRRATPISADNWRPIARDSSGELLSLEEVTLTGNSALARFSGGVEAVYSVGGSGLLANAIPEGPQ
VVVEGTVRRLANYNNGIAIYEADPLNGAVDGLSPGDPGYLQAALRDAKESGLVFSAFQLPENGSIGSINLNLSPDKNYG
FLLLVDGDESNLFSSYSSANPDNAVQVVSFTTSDGSLALGFEDQLVTGESDQDFNDVILTLPITTSTDILSEVNYRIGSLFP
TLEDGSPNLILSSGLELRTIDGESRVVSSLQSGTLDTPMRISSDPVRIPGIDRFLRDWFIPQLTDTANSQSLFNAYKKGVLS
KWTTHARNRSMEDLLKIGYVGPGNDQSATNDDYWNAVGVVGSDLYKAASDSSNYKPAELWDAYDGSTTVFSRFDTD
ALLQRLEALTDPEANPNPDLWYPSMLYTFGVPGEGTSYPAPVLMMQPGDGMNLNFTNDIKVDGLNEEQNQAASLVSN
STYGNAAGDGLGALNAVNYHLHGSHTNPGGFGDNVVARYTTGQQWTTEIDLPADHGQGSYWYHPHYHPSVNQMVY
GGMSGPFQVGDPLSKIPLFKDIPRNWAVLKTMDVGIDAETGKLRLDGFDNLGGVVNRMTMVTVNGEFQPTAEAGEGG
WQAITLSNQTNQAFHNISLIHTDSDGNRTTLPLYLYGEDGHQYPQIRAATDGIFGASGASNQLPTGYTQAVDLLSLPPAK
RVDVLVYLPEGKTEMASTYSFEQDGVDYTINNAGSYPDLTEINTGFGSKTGAGPLALFNVEGGQALPTTAELDAVIAQA

NAGIDVQQILPTTSQADYDPLQVPSVDLFAQDADGSDLWDPIRQRQFNWTKGTLVGPASEYDAATVELLKHYSMMND
GATYEPYTSLPVGKPGVDNWLGYNNPFLINDHVFPYGNMTIAQLGTIEEWVNRNWSINSPSKYIGHPFHIHINDYQVKD
SDTELQNKRNLEDTTSLNSSGYEFYDPAAKEVVSLEPQRGEFHSIPEAQDPEKIASLATFGANDQTIRMLYQDYLGTYVF
HCHILPHEDAGMMQIVTVVENTDSSWLVEAQGFTQNESGVRLYQAQTFDSVQLQALPDSGQTWTRAQAGDLGADFV
QDIALAAGGGGEAGIIELFDGAALLRGETLRTSRLTPYADSSLAPWVFIEDFSGDGQRDLVTAGFDQVQSDVVNLKDLEI
KAFLPGEAPGSWDEQFNFDPFDDISLMAPHSVMPRMGLSADQVSVAMADMNLDNFQDVAIAYAVEGGVRLVVIDGA
ALSLMFQTGEMEGGFFADSNVLADAVFLDSGLSDLSQLVLTSGFNSYAQSALENLVLTTQSSAGSQQFTLQLQAGHFIA
TNLPDSSESGGHGGHGGHGGAGLSPDERITNLRNNSLPLFLVDELQLANGTEAVTPTISAGLGHGGTLLDGHAVIAQGN
EVNGNASNSDILINTTQQLVIPLDGLNLINADDLTGIVDTTSSSTFTAEQVQQRYQLTSMTYLAYTGKLLWPSALASQAA
SILGTGEQASALVTNLLSSPAYAGEIEALYGGPLADQSVNDIVEIAYSTLYKRSATASELQSWQDQVSAGLDQTLLPQAI
LQSTQEADRFRVALLSDITQWTALQWGTTAEVSGSYGQGLVGDEQVSNQLDALASSLGSYASFEDAQQGFDLFTTEAL
QELIGTPVSKSGFF

B

```
SeqID: SynWH7803_0948 Putative MCO
  Analysis Report:
    CMSVM-            Unknown                        [No details]
    CytoSVM-          Unknown                        [No details]
    ECSVM-            Extracellular                  [No details]
    HMMTOP-           Unknown              [No internal helices found]
    Motif-            Unknown                        [No motifs found]
    OMPMotif-         Unknown                        [No motifs found]
    OMSVM-            OuterMembrane                  [No details]
    PPSVM-            Unknown                        [No details]
    Profile-          Unknown          [No matches to profiles found]
    SCL-BLAST-        Unknown           [No matches against database]
    SCL-BLASTe-       Unknown           [No matches against database]
    Signal-           Unknown            [No signal peptide detected]
  Localization Scores:
    Cytoplasmic           0.00
    CytoplasmicMembrane   0.00
    Periplasmic           0.02
    OuterMembrane         3.73
    Extracellular         6.26
  Final Prediction:
    Unknown (This protein may have multiple localization sites.)
```

Figure 5.9 Prediction of the subcellular localisation of the putative MCO of *Synechococcus* sp. WH7803 by the use of PSORTb version 2.0.4.

A The predicted amino acid sequence of the putative MCO. **B** The result predicted using PSORTb version 2.0.4.

5.3.5.2 SDS-PAGE analysis of the outer membrane fractions (OMFs)

To experimentally establish the localisation of the putative muticopper oxidase protein on the *Synechococcus* cell surface, two outer membrane isolation methods were used. Method 1 is based on differential centrifugation (Resch and Gibson, 1983). Method 2 is based on the fact that the outer membrane is not covalently bound to the

peptidoglycan network, but bound primarily by physical forces, i.e. ionic and/or hydrophobic. As a result, treatments such as incubation with a chelating agent like EDTA cause release of the OMF, a complex of LPS, protein, and phospholipid into the surrounding medium (Lindberg, 1973). The detergent Trition X-100 was used in the method 1 to remove cytoplasmic membrane (Schnaitm, 1971).

90 μg of the OMFs from *Synechococcus* sp. WH7803 and WH7803RS-PM2 obtained using the method 1 showed a profile with three predominant bands at approximate 98, 64 and 50 kDa (indicated by solid arrows) (Figure 5.10). No protein bands corresponding to the MCO (~180 kDa) were identified, which may be due to limitation of the isolation method. One faint protein band with an apparent molecular mass lower than 16 kDa (indicated by a dashed arrow) seemed to be only present in the OMFs of mutant cells (Figure 5.10) (identical results were obtained from three biological replicates). This difference was confirmed using the silver staining of the OMFs obtained using method 2 (lower part of lane 3, Figure 5.13). However this protein band with an apparent molecular mass lower than 16 kDa was found both in the mutant and wild-type cells using whole-cell SDS-PAGE analysis (Figure 5.3), it was probably due to the presence of other similar sized proteins in the whole cell.

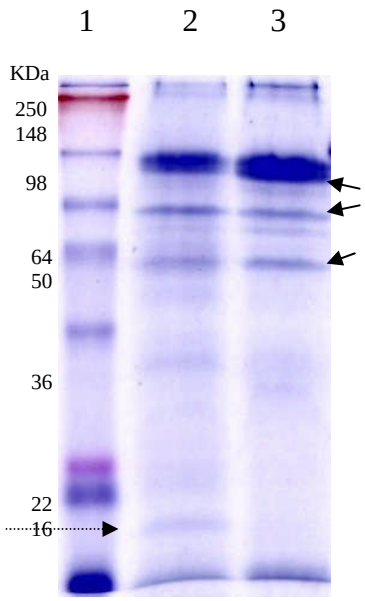


Figure 5.10 SDS-PAGE profiles of OMFs obtained by method 1 from *Synechococcus* sp. WH7803 and WH7803RS-PM2 cells.

Lane 1 SeeBlue® Plus2 Pre-Stained protein standard (Invitrogen) that was used to estimate protein molecular weight, Lane 2 *Synechococcus* sp. WH7803RS-PM2, Lane 3 *Synechococcus* sp. WH7803.

Since SDS-PAGE analysis of the OMFs obtained using method 1 did not reveal the MCO band, method 2 was tried. The resulting pellet obtained after centrifuging the cell-free fraction of EDTA-treated WH7803 had a green base covered with a yellow layer (Figure 5.11). Since the OMF contains no chlorophyll and phycobilins, the yellow layer should be the OMF and the green pellet should be a membrane fraction containing chlorophyll. A similar finding was observed previously (Resch and Gibson, 1983).

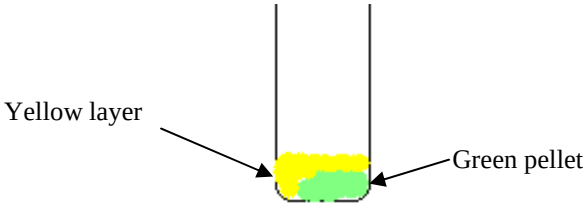


Figure 5.11 Diagram to indicate the pellets obtained after centrifuging the supernatant of EDTA-treated WH7803.

To find out the protein composition of the pellet obtained by the second method, 600 μg of the yellow layer and green pellet were analysed using SDS-PAGE alongside whole-cell protein extracts. As seen in Figure 5.12, the whole-cell protein profiles of *Synechococcus* sp. WH7803 and EDTA-treated WH7803 appear to be the same, which indicated that EDTA treatment was mild enough to preserve most of the cell structures. A well-defined protein band was revealed in the yellow fraction (highlighted by a yellow rectangle), but almost invisible in the green material. This band was analysed by MALDI-TOF and demonstrated to be the putative MCO (this was performed and analysed by Samuel J.H. Clokie using the same methodology as described in Section 5.3.3). From now on the yellow fraction will be referred to as the OMF. This band can also be visualised as faint bands in the whole cells (due to the limitation of resolution in printing, they can't be seen in the printed format, but can be see on the electronic version of Figure 5.12 stored on the CD accompanying this thesis). Additionally, the OMF and the green material displayed marked difference. A band of approximately 98 kDa (highlighted by a black rectangle) that was observed using method 1 was almost indistinguishable from the background in the OMF, but prominent in the green material as well as in the whole cells. The 50 kDa band (highlighted by a blue rectangle) that was observed using method 1 was also revealed in the OMF obtained by method 2 (Figure 5.12). In addition, the 64 kDa band (highlighted by a red rectangle) that was observed using the methods 1 was only present in the green material. Both the OMF and the green material were devoid of phycobiliproteins, which were the most noticeable bands in the whole-cell extracts. Identical results were obtained from three biological replicates.

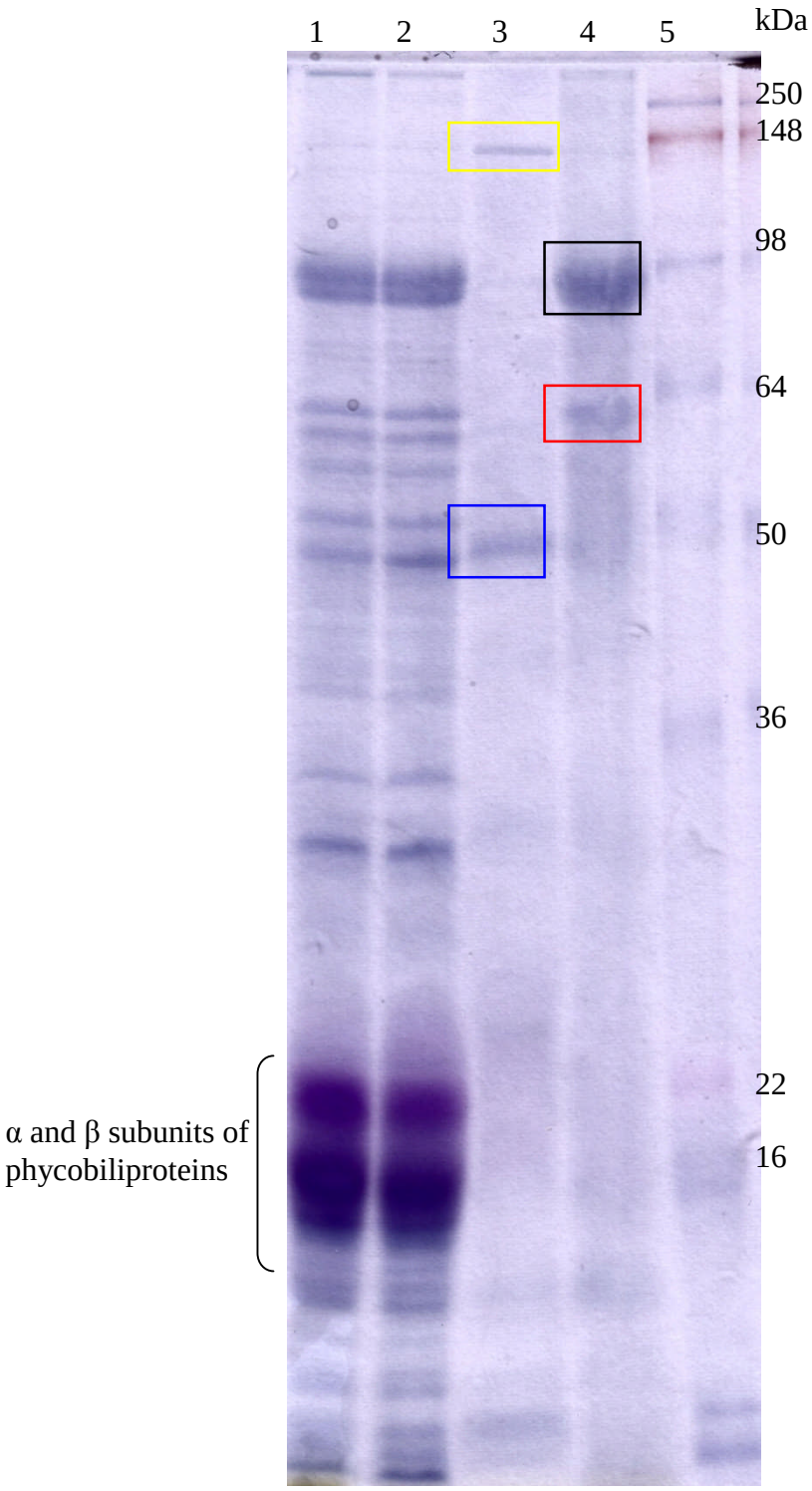


Figure 5.12 SDS-PAGE profiles of OMFs obtained by the use of method 2.

Lane 1 wild-type WH7803 cells, Lane 2 EDTA-treated WH7803 cells, Lane 3 the yellow pellet obtained from the supernatant of EDTA-treated WH7803 cells, Lane 4 the green pellet obtained from the supernatant of EDTA-treated WH7803 cells, Lane 5 the SeeBlue® Plus2 Pre-Stained protein standard (Invitrogen) in kDa. α and β subunits of phycobiliproteins were labelled. The band highlighted by a yellow rectangle was the putative multicopper oxidase. The band highlighted by a black rectangle was the protein band that was only revealed using method 1 to prepare the OMF. The band highlighted by a red rectangle was only present in the OMF prepared using method 1 and the green material

prepared using method 2. The band highlighted by a blue rectangle was revealed using both method 1 and 2.

5.3.5.3 Comparison of the OMFs of *Synechococcus* sp. WH7803 and WH7803RS-PM2 cells

As the putative MCO protein has been demonstrated to be present in the OMF of *Synechococcus* sp. WH7803 and absent in WH7803RS-PM2 cells, it is logical to predict that this protein should be absent in the OMF of WH7803RS-PM2. To unambiguously confirm this prediction and gain a better understanding of the composition of the OMFs from the wild-type and phage-resistant cells, both Coomassie staining and silver staining techniques were used to visualise 60 μg of the OMFs after the protein electrophoresis as the silver staining is about 10-100 times more sensitive than the Coomassie staining (Switzer *et al.*, 1979).

As seen in Figure 5.13, more protein bands are revealed using silver staining (lane 2) than Coomassie staining (lane 1). The putative MCO protein band (confirmed by MALDI-TOF) was faint in Coomassie staining but very distinct in silver staining (please refer to the electronic version of Figure 5.12 on the CD accompanying this thesis to see the putative MCO protein band in Coomassie staining). No corresponding protein band was revealed in the OMF of WH7803RS-PM2 (lane 3) using silver staining (identical results were obtained from three biological replicates). This confirmed that the putative MCO was absent from the OMF in the S-PM2-resistant mutant, which indicated that this protein could play an important role in the adsorption of S-PM2 to *Synechococcus* sp. WH7803. In addition, silver staining also revealed several differences in the protein band profiles of the two OMFs, such as the

three extra bands (indicated by red arrows) that seemed to be present in the S-PM2-resistant mutant, but missing from the wild-type strain (lane 3) (identical results were obtained from three biological replicates). However, the presence of two protein bands (indicated by blue arrows) that seemed only to be present in the S-PM2-resistant mutant was not a repeatable observation.

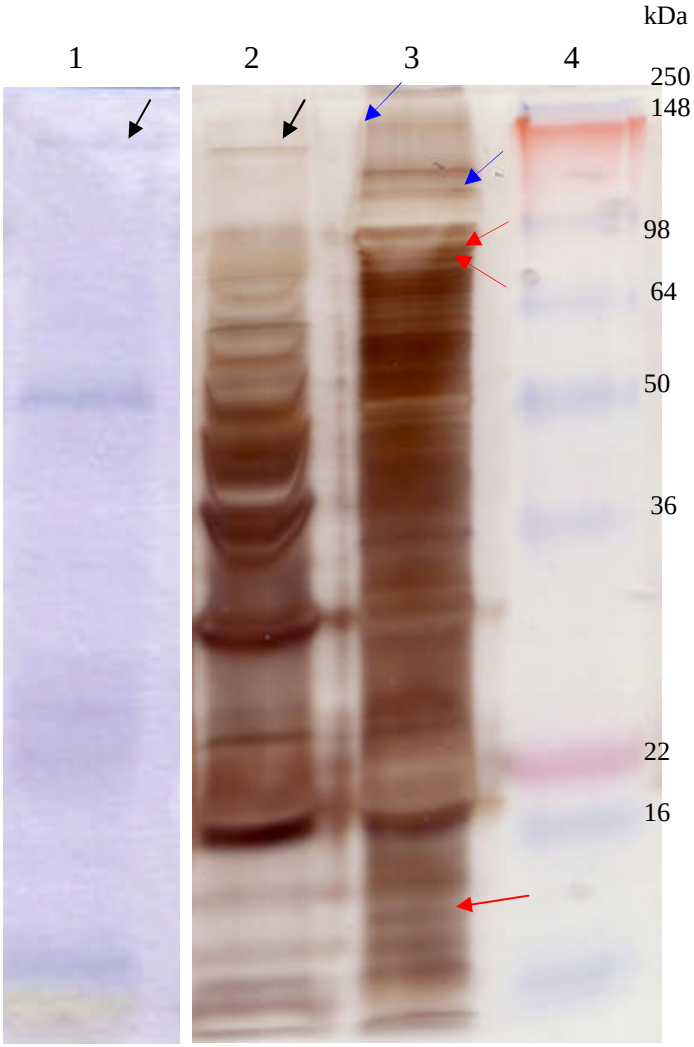


Figure 5.13 SDS-PAGE profiles of the OMFs obtained by method 2 from *Synechococcus* sp. WH7803 and WH7803RS-PM2.

SilverQuestTM Silver Staining Kit (Invitrogen) was used to stain the protein gel. Lane 1 the OMF from *Synechococcus* sp. WH7803 cells after Coomassie staining, Lane 2 the OMF from *Synechococcus* sp. WH7803 cells after silver staining, Lane 3 the OMF from WH7803RS-PM2 cells, Lane 4 the SeeBlue® Plus2 Pre-Stained protein standard (Invitrogen) in kDa. The protein band indicated by black arrows represented the putative MCO protein. Three protein bands indicated by red arrows were only revealed in the resistant mutant. Two protein bands indicated by blue arrows were not a repeatable observation.

5.3.6 Examination of the binding capacity of S-PM2 to EDTA-treated *Synechococcus* sp. WH7803

To test if the EDTA treatment of *Synechococcus* sp. WH7803 can remove the phage S-PM2 receptor material, an adsorption experiment as described in the Chapter 3 was performed using EDTA-treated *Synechococcus* sp. WH7803 as the host. It was found that ~ 70% of phage adsorbed to the EDTA-treated cells within 45 min post infection compared to ~ 90% adsorption to the non-treated cells (Figure 5.14). This reduced adsorption suggested that the material removed by EDTA treatment of *Synechococcus* sp. WH7803 contained the S-PM2 receptor materials.

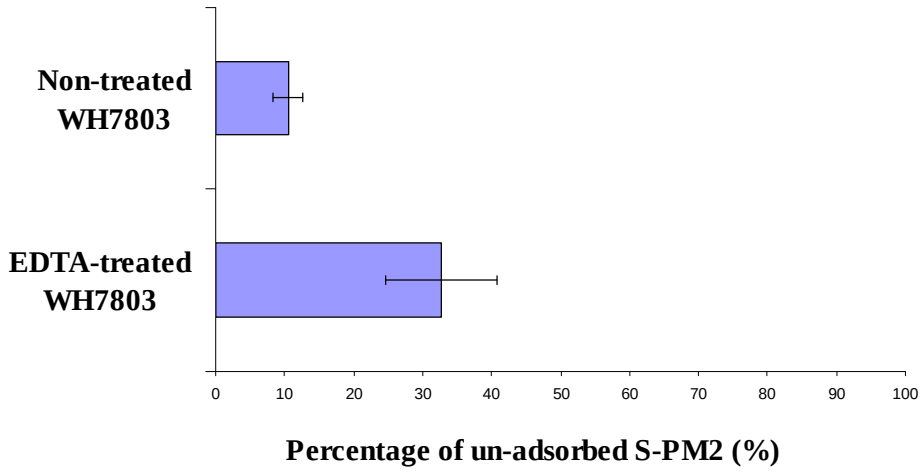


Figure 5.14 Adsorption of S-PM2 to EDTA-treated *Synechococcus* sp. WH7803.

Synechococcus sp. WH7803 cells in the logarithmic growth phase (OD_{750}= 0.35) were firstly treated using 10 mM HEPES (pH 7.2) containing 10 mM EDTA followed by ASW washing for 3 times and incubated with S-PM2 at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed 45 min post infection in the cell-free supernatant remaining after centrifugation. Data are the mean of 3 biological replicates with SD values.

5.3.7 Examination of the binding capacity of S-PM2 to the OMF of *Synechococcus* sp. WH7803

To demonstrate that the OMF obtained by EDTA treatment contains phage S-PM2 receptor material, a phage neutralisation experiment was performed. It was found that 30 µl of the OMF (contains 1.5 mg protein) neutralised ~ 30% of 10 µl of S-PM2 stock at a titre of 1.5×10^8 pfu ml^{-1} (Figure 5.15). A similar neutralisation effect was also observed with the addition of 20 µl of the OMF (containing 1 mg protein) (Figure 5.15). 10 µl of the OMF (containing 0.5 mg protein) neutralised ~ 18% of S-PM2 stock (Figure 5.15). Without the addition of the OMF, the control reaction with the addition of 30 µl of 10 mM HEPES (pH 7.2) showed a negligible neutralisation effect (~ 3%) (Figure 5.15). This suggests that the OMF contains S-PM2 receptor material that could be the putative MCO.

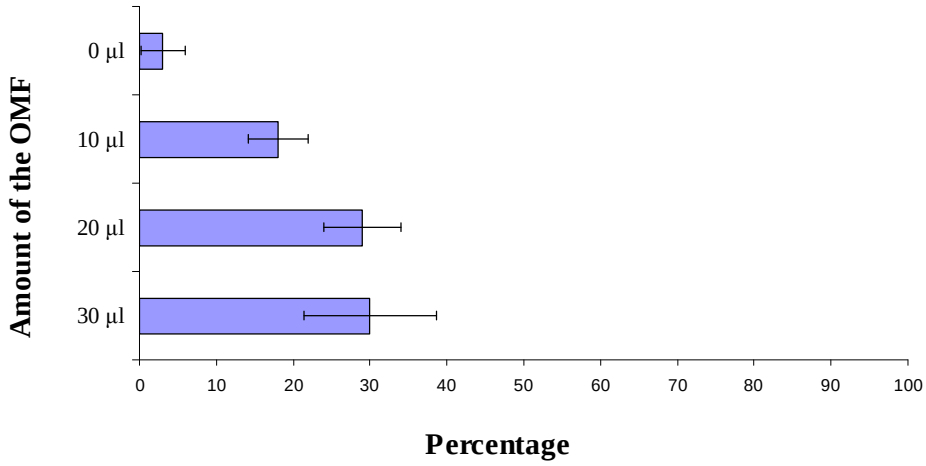


Figure 5.15 Phage neutralisation of the OMF of *Synechococcus* sp. WH7803 obtained by method 3.

10 µl of S-PM2 phage stock at a titre of 1.5×10^8 pfu/ml was mixed with varying amount of the OMFs and incubated at 25ºC at 15 µE m^{-2} s^{-1} for 1 h. The mixtures were then subject to phage titration. Data are the mean of 3 biological replicates with SD values.

5.4 Discussion

Based on the 1D-PAGE coupled with MS analyses of *Synechococcus* sp. WH7803 and WH7803RS-PM2, the putative MCO was found to be absent from WH7803RS-PM2 cells. To demonstrate the possibility of the putative MCO involving in S-PM2 adsorption, both bioinformatic and biochemical analyses were used to localise the putative MCO protein from *Synechococcus* sp. WH7803. Although two different methods, differential centrifugation and EDTA treatment, were tried, only the latter method revealed the presence of the putative multicoppper oxidase in the OMF obtained from *Synechococcus* sp. WH7803. The protein band representing the putative MCO showed reduced apparent molecular weight in the OMF (Figure 5.12) compared to that in the whole-cell (Figure 5.3). This is probably due to the different preparation method used. The use of EDTA in preparing the OMF may remove some metal ions from the MCO that otherwise would bind to the MCO in the whole-cell protein preparations. Consequently, the MCO from the OMF showed reduced size on a SDS-PAGE gel. As expected, the OMF obtained from WH7803RS-PM2 cells showed the absence of the putative MCO. Sequencing and RT-PCR analyses demonstrated that the putative MCO gene in WH7803RS-PM2 cells remained complete at the nucleotide level and was actively transcribed into mRNA. This led to two possibilities, that either the mutant cell may have lose the ability to translate this particular mRNA into protein or more likely the putative MCO could not be properly anchored into the specific site on the cell surface, which may be due to a structural change on the cell membrane, and thus was secreted into the surrounding environment based on the fact that the putative MCO can only be detected in the OMF from the wild-type strain, but not from the S-PM2-resistant mutant.

MCOs (MCOs) are a diverse family of proteins that utilise the unique redox feature of copper ion as cofactors in the oxidation of a wide variety of substrates (Messerschmidt, 1997). The best known MCOs are laccase, ascorbate oxidase and ceruloplasmin (Nakamura and Go, 2005). Recently, novel MCOs that function in the transport systems of metal ions were discovered. These include hephaestin, a copper-dependent ferroxidase (Nittis and Gitlin, 2004), Fet3 protein for Fe(II) transfer (Askwith *et al.*, 1994), CueO (Copper efflux oxidase), an enzyme involved in converting Cu(I) to the less toxic Cu(II) (Outten *et al.*, 2000), and CumA for Mn(II) oxidation (Francis and Tebo, 2001). They have been suggested to be involved in Mn^{2+} oxidation in several different bacterial strains, including a marine Gram-negative bacterium (Brouwers *et al.*, 2000; Francis *et al.*, 2001). However, there is little research about the function of this protein in the marine *Synechococcus* strains. A putative MCO has been discovered bioinformatically from the genome of *Synechococcus* CC9311 (Palenik *et al.*, 2006). Alongside the putative MCO discovered from *Synechococcus* sp. WH7803, the cyanobacterial MCO may function in oxidation of organic compounds or detoxifying high levels of reduced copper as both *Synechococcus* sp. WH7803 (Waterbury *et al.*, 1986) and CC9311 (Toledo and Palenik, 1997) were isolated from costal environment where copper is an important component, both naturally occurring and as a pollutant.

The discovery that this putative MCO was absent from the S-PM2-resistant mutant derived from *Synechococcus* sp. WH7803 leads to speculation that if this protein functions as a phage receptor. it may be present as two forms, active and non-active, which are activated by light. To prove this speculation, future work including the

purification and characterisation of the putative MCO of *Synechococcus* sp. WH7803 is needed. Alternatively, if the process of S-PM2 adsorption needs energy, it may be derived from Mn^{2+} oxidation, which has been demonstrated to be a energy source for a marine bacterial strain SSW22 (Ehrlich and Salerno, 1990). If this speculation is right, it explains why phage adsorbed to the host occurs even when energy production was abolished by DCMU and CCCP. The lack of adsorption in the dark and wavelength-associated adsorption could be due to the possibility that the visible spectrum of light is need to induce Mn^{2+} oxidation in *Synechococcus* sp. WH7803. To prove this speculation, future work including demonstration of the presence of energy production via Mn^{2+} oxidation in the strain WH7803 and the energy requirement for phage S-PM2 adsorption is needed.

As a decreased rate of S-PM2 adsorption to the EDTA-treated WH7803 cells was observed (Figure 5.13), a phage neutralisation experiment using S-PM2 and the OMF obtained using the method 2 was performed in order to demonstrate that the S-PM2 receptor materials were present in the OMF of *Synechococcus* sp. WH7803. A moderate neutralisation effect (up to ~ 30% neutralisation) was achieved using 1 mg of the OMF. No further neutralisation was observed even when the amount of the OMFs was increased to 1.5 mg (Figure 5.14). The failure in achieving a near 100% neutralisation could be due to the involvement of other molecules (such as lipopolysaccharide that will be discussed in Chapter 6) not present in this OMF for S-PM2 adsorption. Alternatively, the integrity (or the partial integrity) of the outer membrane may be required for S-PM2 adsorption.

Since the use of one dimensional PAGE in comparing the protein profiles of the wild type and mutant strains, questions rise about its limited resolving power compared to two dimensional PAGE (Klose, 1975; Ofarrell, 1975; Ofarrell *et al.*, 1977). Although one dimensional PAGE revealed only one proteins band absent in the mutant, it did not necessarily mean there are no other differences between the two strains. In addition, two dimensional PAGE has been used in analysis of the *E. coli* outer membrane (Molloy *et al.*, 2000). Therefore, to better understand the protein differences between the wild type and mutant, two dimensional PAGE would be a better choice.

Chapter 6 Construction of a putative MCO gene knockout mutant of *Synechococcus* sp. WH7803

6.1 Introduction

Generally, there are three methods that can be employed in gene transfer in cyanobacteria; conjugation, electroporation and natural transformation (Koksharova and Wolk, 2002). Bacterial conjugation is a process of transmitting genetic material from a bacterium to another cell through cell-to-cell contact. Although it is time-consuming, conjugation has proven to be the most general means for gene transfer in cyanobacteria, mediated by the broad-host-range, incompatibility-group P (IncP) plasmid RP4 and its close relatives, e.g., RK2 (Elhai and Wolk, 1988; Koksharova and Wolk, 2002). Those conjugal plasmids have been successfully used to mobilise DNA from *Escherichia coli* to a wide variety of cyanobacteria including oceanic cyanobacteria of the genus *Synechococcus* (Brahamsha, 1996). Two criteria must be satisfied for DNA/plasmid to be mobilised by a conjugal plasmid. First, it must contain an "origin-of-transfer" (*oriT*), a stretch of DNA that is also called the *bom* site (for basis of mobilisation). Second, it must carry or be provided with a *mob* gene whose product is a DNA-nicking protein that specifically recognises the *bom* site (Elhai and Wolk, 1988). One plasmid that can fulfil the criteria stated above is the incompatibility-group Q (IncQ) plasmid RSF1010, a high-copy-number, broad-host-range plasmid known to be efficiently transmitted in the presence of IncP plasmids (Bagdasarian *et al.*, 1981). Electroporation appears to be a general technique for the introduction of exogenous DNA into cyanobacterial cells (with varied efficiencies), although it has a potential for causing mutagenesis (Thiel and Poo, 1989; Koksharova and Wolk, 2002). A few cyanobacteria, such as *Synechococcus* and *Synechocystis* strains, are naturally transformable by exogenous DNA (Lorenz and Wackernagel,

1994). It has been reported that *Synechocystis* sp. strain PCC6803 can take up virtually any sequence of DNA (Grigorieva and Shestakov, 1982).

The conjugation system used in this study is based upon a previous report (Brahamsha, 1996), in which *Synechococcus* cells were mated with an *E. coli* conjugal donor strains MC1061 containing three plasmids, pRK24, pRL528 and the recombinant plasmid pMUT100 (or original pRL153 in the control experiment). The broad-host-range conjugal plasmid pRK24 has been shown to be able to mediate DNA transfer to a wide range of bacteria including myxobacteria, thiobacilli, and cyanobacteria (Elhai and Wolk, 1988). The second plasmid pRL528 is a helper plasmid that provides the Mob protein facilitating mobilisation (Elhai and Wolk, 1988). The third plasmid pMUT100 is a kanamycin-resistant suicide vector that is a derivative of pBR322 (Brahamsha, 1996). In order to find out the function of the putative MCO in the process of S-PM2 adsorption, this suicide vector was used to inactivate ORF0948 that encodes the putative MCO of *Synechococcus* sp. WH7803. A recombinant pMUT100 harbouring an internal portion of ORF0948 was generated and introduced into WH7803. Integration of the recombinant vector into the WH7803 genome by homologous recombination thus impaired the function of ORF0948. Subsequent examination of the adsorption properties of phage S-PM2 to the knockout mutant cells would answer the question of whether the MCO plays a role in S-PM2 adsorption.

6.2 Aim

To inactivate the ORF0948 of *Synechococcus* sp. WH7803 that encodes a putative MCO.

6.3 Results

6.3.1 Construction of suicide plasmids pYJ01 and pYJ02

Two PCR products of 407 bp and 370 bp in length within ORF0948 were amplified using the following primer sets: ORF0948-500 and ORF0948-1000 (Table 2.15). PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.1 µM primers, 10 ng of DNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C.

The two PCR products were firstly cloned into the commercially available plasmid pCR2.1 (Invitrogen). Then, the PCR products of the ORF0948 fragments were cut out of the recombinant pCR2.1 using *EcoR*I enzyme digestion and were ligated with plasmid pMUT100 that was also digested with *EcoR*I. The recombinant plasmids, pYJ01 (accommodating the 370-bp fragment) and pYJ02 (accommodating the 407-bp fragment), were firstly confirmed by PCR to amplify the two ORF0948-derived fragments (Figure 6.1A and 6.1B). Then, *EcoR*I enzyme digestion of the putative plasmids, pYJ01 and pYJ02, gave the expected patterns with two bands, the insertions (370 and 407 bp in length) and the linearised pMUT100 (Figrue 6.1C and 6.1D). Finally, the putative recombinant plasmids were sequenced using the following primer: pMUTsequencing5516: TGCCACCTGACGTCTAAGAA. When the

sequenced results were aligned with the genome of *Synechococcus* sp. WH7803, a 100% identity was revealed. This demonstrated that the putative recombinant plasmids, pYJ01 and pYJ02, were truly recombinant pMUT100 carrying two ORF0948-derived fragments.

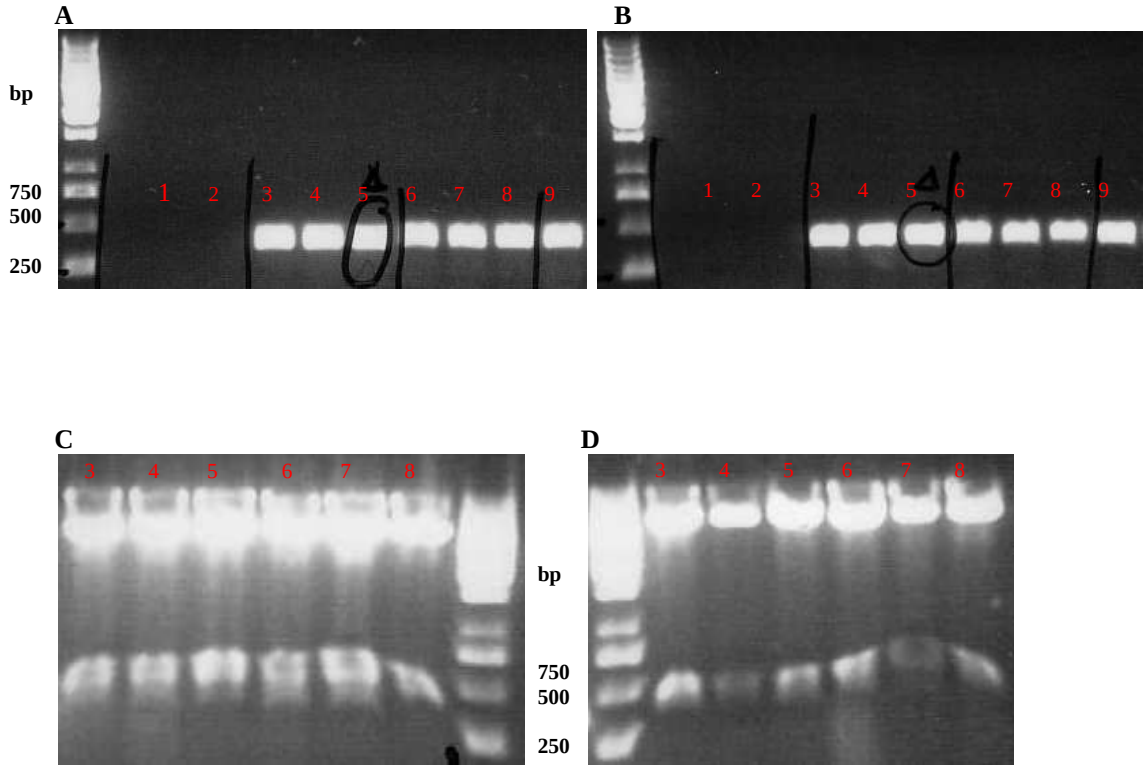


Figure 6.1 Gel images of PCR products and *EcoR*I enzyme digestions.

The putative recombinant plasmids (pYJ01 and pYJ02) derived from 6 individual DH5α single colonies grown on kanamycin (50µl/ml) were subject to PCR and *EcoR*I digestion. Lane 1 a negative control, Lane 2 PCR against plasmid pMUT100, Lane 3 to 8 the plasmid DNA derived from 6 individual DH5α single colonies, Lane 9 the DNA derived from *Synechococcus* sp. WH7803. **A** 370-bp PCR products amplified using the primer set ORF0948-1000, **B** 407-bp PCR products amplified using the primer set ORF0948-500, **C** *EcoR*I digestion of plasmid pYJ01, **D** *EcoR*I digestion of plasmid pYJ02. GeneRuler™ 1kb DNA (in bp) ladder from Fermentas was used.

6.3.2 Conjugation

Two different media SN and ASW were used for growing *Synechococcus* sp. WH7803 throughout the conjugation experiments. As no differences were detected, only the data from SN medium is presented here. The efficiency of conjugation was $(2.5 \pm 0.73) \times 10^{-4}$. Efficiency is expressed as the number of transconjugants per recipient cell and calculated as described by Brahamsha (1996). Values are the averages of three independent experiments (± the standard deviation). Single colonies of putative transconjugants, which displayed a kanamycin-resistant phenotype, were inoculated into liquid medium for further analyses.

After obtaining kanamycin-resistant single colonies it was important to demonstrate that this resistance was due to the presence of the recombinant plasmid, pYJ01 or pYJ02, and that no viable *E. coli* was present in the culture. Firstly, portions of the SN liquid cultures of the putative transconjugants were spotted on LB solid agar plates and contamination test medium (Section 2.4.4) to ensure that they did not contain any *E. coli* contamination. Both media failed to detect even a single live cell of *E. coli*. This demonstrated that no viable *E. coli* cells were present in kanamycin-resistant single colonies. Secondly, one set of PCR primers was used to amplify an 872-bp region covering the kanamycin-resistance gene in the plasmid pMUT100 using the following primers: KanF, 5'-ATGGCAAGATCCTGGTATCG-3'; and KanR, 5'-GGAACCGGAGCTGAATGAAG-3'. The PCRs were carried out directly on 1 µl each of transconjugant and wild-type WH7803 cells (OD_{750}=0.35) in a total volume of 50 µl, containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.4 µM primers, 1 units of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification

conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C.

It was found that only the transconjugant culture yielded PCR products, while the wild-type culture yielded no such PCR products (Figure 6.2). These tests confirmed that the kanamycin-resistance was due to the presence of the recombinant plasmids, pYJ01 and pYJ02, in *Synechococcus* sp. WH7803. Consequently, the knockout strains accommodating the plasmid pYJ01 or pYJ02 were named 'WH7803/pYJ01' or 'WH7803/pYJ02'.

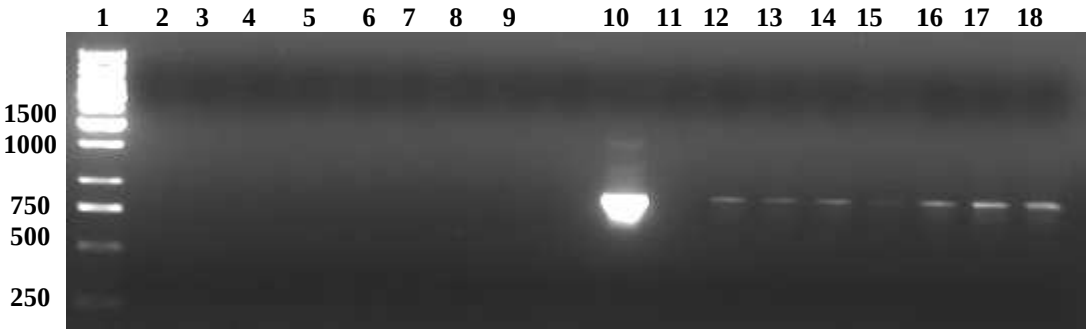


Figure 6.2 Gel image of PCR product of kanamycin gene fragment.

Lanes are numbered from left to right. Lane1 GeneRuler™ 1kb DNA ladder from Fermentas (bp), Lane 2-8 PCR reactions using cell cultures derived from 7 single colonies of *Synechococcus* sp. WH7803 as template, Lane 9 a negative control, Lane 10 PCR reaction using 100 ng of pMUT100 DNA as template, Lane 11 a negative control, Lanes 12-15 PCRs using WH7803/pYJ01 cultures derived from 4 kanamycin-resistant single colonies, Lanes 16-18 PCRs using WH7803/pYJ02 cultures derived from 3 kanamycin-resistant single colonies.

6.3.3 RT-PCR analysis of the expression of the putative MCO gene

To determine whether the putative MCO gene is inactivated by conjugal transfer of the pMUT100-derived plasmids pYJ01 and pYJ02, two PCR primer sets, ORF0948-

500 and ORF0948-1000 (Table 2.15), were used to amplify the cDNA derived from the WH7803/pYJ01, WH7803/pYJ02 and WH7803 cultures. PCRs were carried out in a total volume of 50 µl, containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.1 µM primers, 10 ng of cDNA, 1 unit of *Taq* polymerase (Fermentas), and 5 µl 10× *Taq* buffer (Fermentas). Amplification conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. As seen in Figure 6.3, no PCR products were obtained using cDNA derived from WH7803/pYJ01 and WH7803/pYJ02 cultures. These confirmed that the putative MCO gene in *Synechococcus* sp. WH7803 has been successfully inactivated.

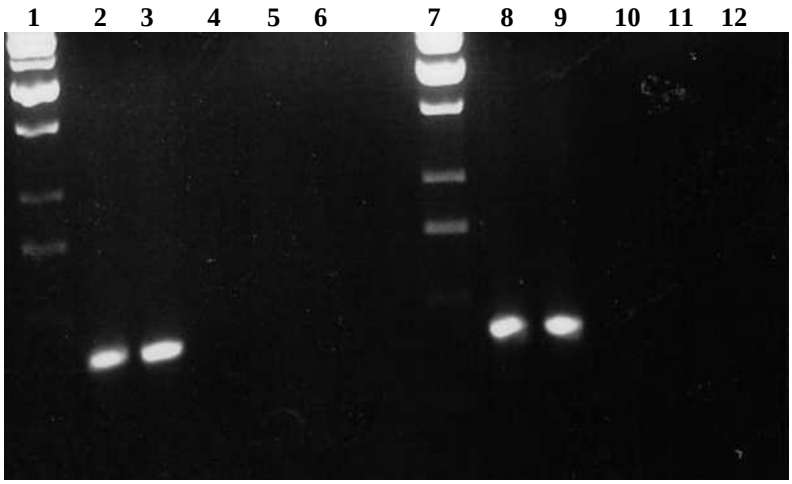


Figure 6.3 Gel image of PCR products of 370 and 407-bp within the putative MCO gene.

Lanes are numbered from left to right. Lane 1 GeneRuler™ 1kb DNA ladder from Fermentas (bp), Lane 2 370-bp PCR products amplified using WH7803 DNA as a template, Lane 3 370-bp PCR products amplified using pYJ01 DNA as a template, Lanes 4 PCR using the primer set of ORF0948-500 and WH7803/pYJ01 cDNA, Lane 5, PCR using the primer set of ORF0948-500 and WH7803/pYJ02 cDNA, Lane 6 a negative control, Lane 7 GeneRuler™ 1kb DNA ladder from Fermentas (bp), Lane 8 407-bp PCR products amplified using WH7803 DNA as a template, Lane 9 407-bp PCR products amplified using pYJ02 DNA as a template, Lanes 10 PCR using the primer set of ORF0948-1000 and WH7803/pYJ01 cDNA, Lane 11, PCR using the primer set of ORF0948-1000 and WH7803/pYJ02 cDNA, Lane 12 a negative control.

6.3.4 SDS-PAGE of whole-cell proteins of WH7803/pYJ01 and WH7803/pYJ02

To determine whether the knockout mutants lacked the putative MCO, whole-cell SDS-PAGE analysis was used to characterise the protein profiles of WH7803/pYJ01 and WH7803/pYJ02 cultures. As shown in Figure 6.4, the putative MCO band (indicated by an arrow) is only present in *Synechococcus* sp. WH7803 but not in WH7803/pYJ01 and WH7803pYJ02 (Please refer to the CD accompanied this thesis for an electronic version of Figure 6.4). This demonstrates that the knockout strains had lost the ability to produce the putative MCO.

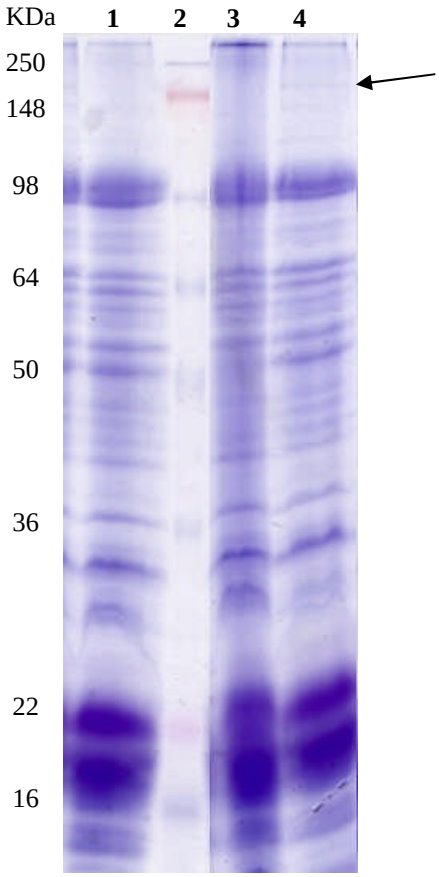


Figure 6.4 SDS-PAGE profiles of whole-cell protein extracts from WH7803, WH7803/pYJ01 and WH7803/pYJ02.

Lane 1 WH7803/pYJ01, Lane 2 SeeBlue® Plus2 Pre-Stained protein standard (Invitrogen) in KDa, Lane 3 WH7803/pYJ02, Lane 4 *Synechococcus* sp. WH7803. The putative MCO band in WH7803 strain is indicated by an arrow.

6.3.5 Examination of the growth curve of WH7803/pYJ01 and WH7803/pYJ02

Although no obvious visible phenotypic alternations were observed in the knockout mutants, their growth rates were compared with the wild-type strain. As seen in Figure 6.5, no marked difference was observed in the growth curves. This indicated that loss of the putative MCO did not do much harm to the cell's physiological status under these particular growth conditions.

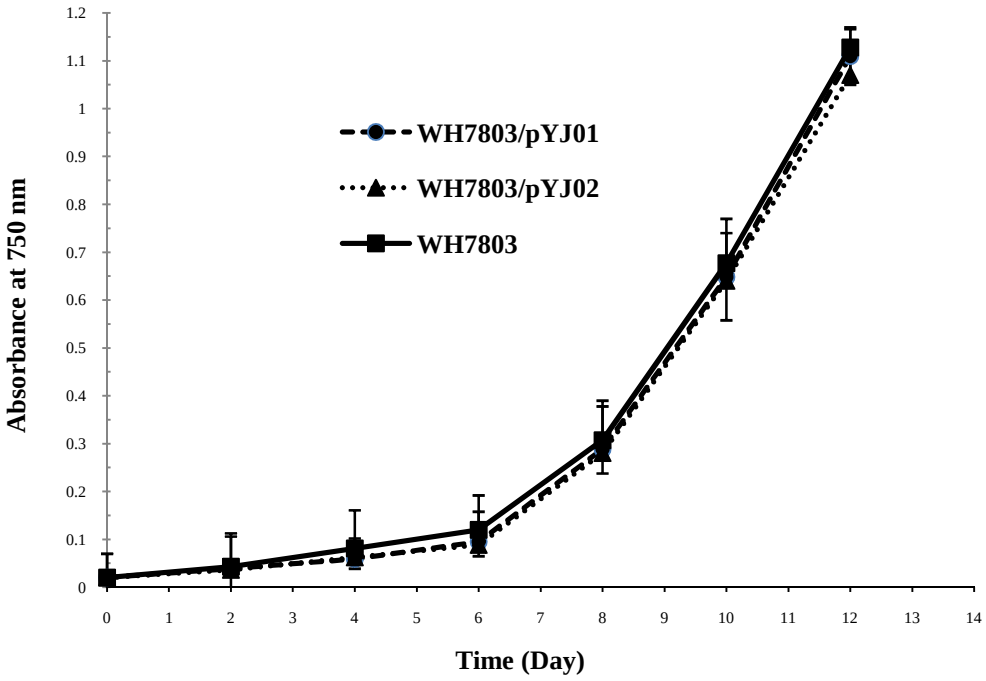


Figure 6.5 Growth curves of *Synechococcus* sp. WH7803 and WH7803/pYJ01 and WH7803/pYJ02 in ASW medium.

Cells were cultured in 1 l glass vessel with aeration and stirring. Cell growth was monitored by absorbance at 750 nm. The points are the mean of three biological replicates. The error bars represent SD (n=3).

6.3.6 Examination of the binding capacity of S-PM2 to WH7803/pYJ01 and WH7803/pYJ02

To determine whether or not the phage S-PM2 is still able to adsorb to the putative MCO gene knockout mutant in the light and dark, the adsorption kinetics were investigated using the same method as described in Section 3.3.1. WH7803 and the S-PM2-resistant mutant strains were used as controls. As seen in Figure 6.6, ~ 85% phage adsorbed to both knockout mutants, WH7803/pYJ01 and WH7803/pYJ02 within 45 min post infection in the light. Compared to ~ 90% adsorption to WH7803 and no adsorption to the phage S-PM2-resistant mutant, the adsorption rate suggest that the inactivation of the putative MCO does not eliminate the phage S-PM2 receptor. S-PM2 showed no adsorption to the resistant mutants in the dark (Figure 6.7). This suggests that the loss of the putative MCO in the S-PM2-resistant mutant may be a pleiotropic effect of the loss of the phage S-PM2 receptors, or it may be part of the phage S-PM2 receptors, and other components are required for a successful adsorption.

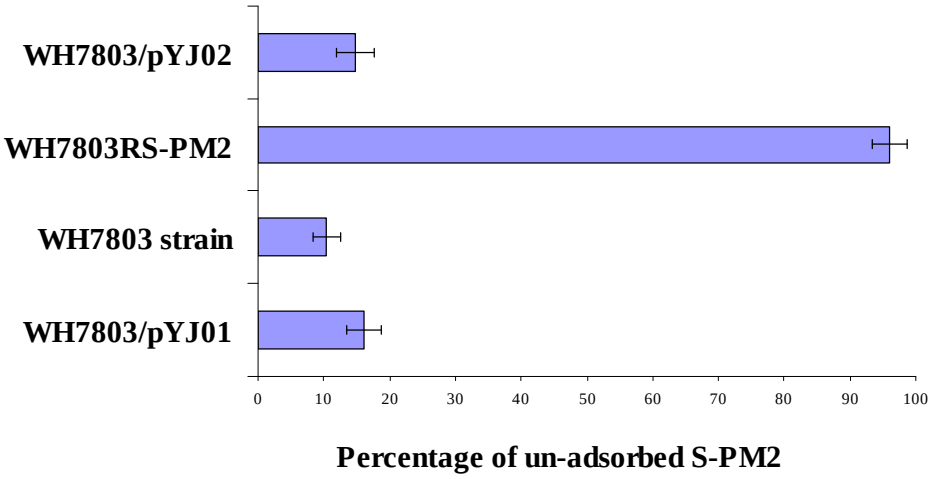


Figure 6.6 Adsorption of S-PM2 to WH7803RS-PM2, WH7803 and the knockout WH7803 mutants in the light.

WH7803RS-PM2, WH7803, WH7803/pJY01 and WH7803/pYJ02 in the logarithmic growth phase (OD_{750}= 0.35) were incubated with S-PM2 at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed 45 min post infection in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

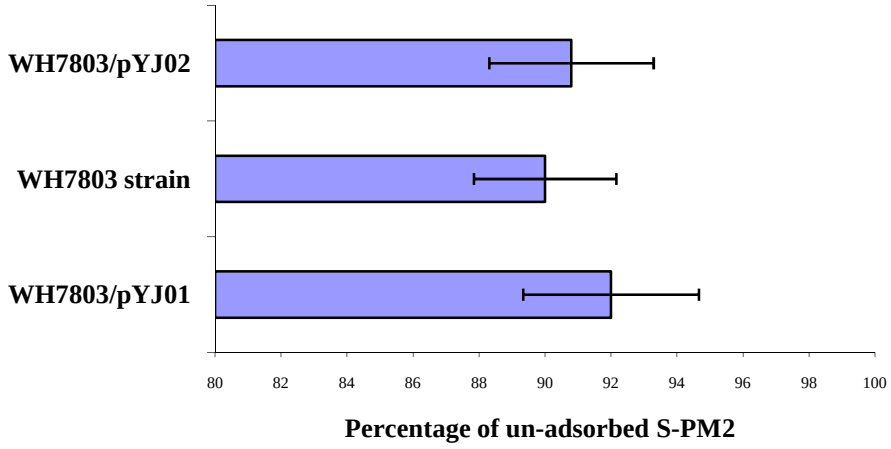


Figure 6.7 Adsorption of S-PM2 to WH7803 and the knockout WH7803 mutants in the dark.

WH7803, WH7803/pJY01 and WH7803/pYJ02 in the logarithmic growth phase (OD_{750}= 0.35) were incubated with S-PM2 at a MOI of 0.02 at 25ºC at 15 μE m^{-2} s^{-1}. The free phage titer was assayed 45 min post infection in the supernatant remaining after centrifugation. Data are the mean of three biological replicates with SD values.

6.4 Discussion

The idea of inactivating the putative MCO gene stems from two experimental observations: firstly, this particular protein is absent in the S-PM2-resistant mutant; secondly, the OMF of *Synechococcus* sp. WH7803 containing this protein has a neutralisation effect on the phage S-PM2. If the putative MCO was the S-PM2 receptor, the knockout strain of WH7803 would lose its susceptibility to phage infection. To test this hypothesis, a suicide plasmid pMUT100, which had been previously constructed for the purpose of conjugal transfer of *Synechococcus* sp. WH7803 (Brahamsha, 1996), was used to inactivate the putative MCO gene in *Synechococcus* sp. WH7803. A conjugation frequency of $(2.5 \pm 0.73) \times 10^{-4}$ was observed, which was comparable to the previous reported data (Brahamsha, 1996).

Compared with the wild-type strain, the knock-out WH7803 mutants can still grow well in ASW, almost the same growth rate as the wild-type. It could be due to the loss of MCO is not 'life-threatening' for WH7803, and its function could be compensated by other proteins. A recent report also demonstrated that a knock-out strain of *Shewanella oneidensis* MR-1 showed the same growth rate under anaerobic conditions, but a decreased growth rate when cultured aerobically compared to the wild-type strain (Gao *et al.*, 2006). In addition, it has been reported that the growth rate of *Escherichia coli* did not change after the *cyaY* gene was knocked out (Li *et al.*, 1999).

As S-PM2 still can adsorb to the knockout mutant as it does to the wild type, it shows that the loss of the putative MCO can not block S-PM2 adsorption. Based on the fact

that the putative MCO-containing OMF showed an S-PM2-neutrilisation effect (Chapter 5), other components in the OMF may be S-PM2 receptors. Previous studies on the phage T4 receptors have revealed that both lipopolysaccharide (LPS) and outer membrane proteins, such as OmpC, were required for a successful adsorption to *E. coli* K-12 (Yu and Mizushima, 1982). Furthermore, the LPS of the unicellular cyanobacterium *Anacystis nidulans* KM has been indicated to be involved in binding of the cyanophage AS-1 based on the fact that the LPS fraction was found to be able to inactivate the cyanophage AS-1 (Samimi and Drews, 1978). Therefore, it is possible that LPS is required for the adsorption of the phage S-PM2 to *Synechococcus* sp. WH7803. Further experiments would be to investigate the LPS fractions from S-PM2-resistant mutant and the wild type.

Chapter 7 Conclusions

Viruses in general and bacteriophages in particular, have been shown to be ubiquitous in the marine environment and are thought to play an important role in marine food webs and influence global carbon cycling (Fuhrman, 1999; Wilhelm and Suttle, 1999). Cyanophages capable of infecting marine unicellular cyanobacteria were first characterised in 1993 (Suttle and Chan, 1993b; Waterbury and Valois, 1993b; Wilson *et al.*, 1993b). In contrast to phage infection of heterotrophic hosts, novel features of cyanophage–host interactions have been revealed during the course of this study. Cyanophage replication depends on the host photosynthetic performance, although the extent of the dependence varies (Adolph and Haskelkorn, 1972; MacKenzie and Haselkorn, 1972; Sherman, 1976; Lindell *et al.*, 2005). Phage-encoded photosynthesis genes of cyanobacterial origin, such as the *psbA* encoding photosystem II core reaction centre protein D1 and *hli* (high-light inducible) genes, are expressed during infection (Lindell *et al.*, 2005; Clokie *et al.*, 2006c; Lindell *et al.*, 2007). These discoveries indicate that light may play an important role in cyanophage adsorption. However, regarding the first key step in the cyanophage–host interaction, the adsorption of the phage to the host cell through highly specific binding to receptors on the cell surface, there is little information as to the nature of the receptors for *Synechococcus* phages and little work has been carried out to investigate the influence of environmental factors on cyanophage adsorption.

The broad aim of this study was to further understand the interaction of marine cyanophage-host systems based on a model system consisting of cyanophage S-PM2 and the marine cyanobacterium *Synechococcus* sp. WH7803. *Synechococcus* strains are marine unicellular cyanobacteria and are inevitably subject to light-dark cycles in the natural environment. The particular aim of this study was to investigate the role of

the light during the process of cyanophage adsorption to the host *Synechococcus* sp. WH7803. Another aim of this study was to isolate and purify cyanophage-resistant mutants and to investigate the S-PM2 receptor(s).

The results from this study show for the first time that marine phage adsorption was light-dependent. Compared to 40% adsorption of a freshwater cyanophage AS-1 to *Synechococcus* PCC 7942 (previously named *Anacystis nidulans*) in the dark, (Cseke and Farkas, 1979b), elimination of light almost completely abolished phage S-PM2 adsorption to *Synechococcus* sp. WH7803. Once the light was switched on, S-PM2 adsorption resumed immediately. Moreover, this light-dependent phage adsorption was not just limited to S-PM2. Four out of nine other cyanophages also showed the same effect. This suggests that at least some cyanophages in the marine environment can only infect cyanobacteria in the presence of daylight. This light-dependent cyanophage adsorption explains why the abundance of cyanophages from the Indian Ocean showed a maximum at 0100 h over a 24 h period (Clokie *et al.*, 2006a).

As no previous work has been carried out to understand the light-dependent adsorption, the investigation was started using different light wavelength to examine the phage adsorption rate. It was found that S-PM2 exhibited a considerably decreased adsorption rate under illumination at red wavelengths compared to blue, green, and yellow light. This discovery suggested a possible link between phage adsorption and the host photosynthetic energy production for the reason that red light can't be effectively utilised by *Synechococcus* sp. WH7803 for fuelling photosynthesis (Ong and Glazer, 1991). In order to establish whether photosynthetic energy production is needed in the process of S-PM2 adsorption, chemical inhibitors of photosynthesis and

the proton motive force, DCMU and CCCP, were used to treat the host before adsorption. No differences were detected when comparing the S-PM2 adsorption to 'treated' and 'non-treated' hosts. This finding rules out the possibility that ATP generated by photophosphorylation is needed for phage S-PM2 adsorption.

As *Synechococcus* sp. WH7803 has been demonstrated to establish a circadian rhythm in response to light/dark (LD) cycles (Sweeney and Borgese, 1989), it was important to establish whether this circadian rhythm could vinfluence the light-dependent adsorption. It was found that no matter what stage the cells were at in the 12 h – 12 h LD cycle, phage adsorption was still light-dependent. This showed that the circadian rhythm of the host cells did not have a role in cyanophage adsorption.

Recent studies have shown that the *psbA* gene is present in cyanophage genomes (Mann *et al.*, 2003; Millard *et al.*, 2004; Sullivan *et al.*, 2006). The importance of the D1 protein has been well-explained (Melis, 1999). As many, but not all marine cyanophages, have been found to contain the *psbA* gene (Millard *et al.*, 2004; Sullivan *et al.*, 2006). This led me to investigate the possible role of the phage-encoded *psbA* gene in the light-dependent adsorption. Nine cyanophages that displayed different adsorption patterns to *Synechococcus* sp. WH7803 in the light and dark were sequenced for the presence of the *psbA* gene. All of them were discovered to contain the *psbA* gene. This indicated that the genomic occurrence of the phage-encoded D1 protein was not associated with the light-dependent cyanophage adsorption.

Since phage adsorption can be rapidly restored when exposed to light, a photoreceptor may be responsible. Therefore, the absorption property of S-PM2 particles was

examined in order to establish whether S-PM2 itself carries a photoreceptor to detect ambient light. No absorption peaks were detected within the range of visible wavelength (400 nm to 750 nm). This indicated that S-PM2 did not carry detectable chromophore-containing molecule(s) responsible for detecting ambient light, which led to focus on the host.

To gain a better understanding of whether this light-dependent S-PM2 adsorption to WH7803 is common in the natural environment, more cyanophage-host pairs were tested. Interestingly, the cyanophage S-MM5 only displayed light-dependent adsorption to *Synechococcus* strain Dim, but not to WH7803. Darkness was demonstrated to be less efficient in preventing S-PM2 from adsorption to another S-PM2 host BL161 than to WH7803. This suggested that the nature of the cell surface could play a major role in the light-dependent cyanophage adsorption. Since information about *Synechococus* phage receptors remains unknown, isolation and characterisation of phage-resistant mutants was carried out.

An S-PM2-resistant mutant of *Synechococcus* sp. WH7803 was isolated and shown not to bind the S-PM2, which suggested a mutation in the S-PM2 receptor. This mutant was also resistant to 16 out of 30 cyanophages. No obvious fitness cost has been detected from the S-PM2-resistant mutant because it showed a very similar growth rate as the wild-type strain. Consequently, this S-PM2-resistant *Synechococcus* strain would have a competitive advantage over sensitive strains.

Since the restoration of phage adsorption was immediate when the phage-host suspension was shifted from the dark to the light, this indicated that a protein conformational change may be induced by the light to favour phage recognition. The protein profiles of *Synechococcus* sp. WH7803 and the S-PM2-resistant mutant were analysed. A putative multicopper oxidase was found to be absent in the S-PM2-resistant mutant and was demonstrated to be present in the outer membrane fraction (OMF) of *Synechococcus* sp. WH7803, but not in the OMF of the mutant. The OMF of the wild type showed a neutralisation effect on S-PM2. This demonstrated that the OMF contained molecules that had S-PM2 receptor activity.

Due to the absence of the multicopper oxidase in the OMF of the mutant, this made the protein a good candidate to be tested as the S-PM2 receptor. The corresponding gene in WH7803 was knocked out. However, S-PM2 was still able to adsorb to the knockout mutants at a similar rate as to the wild type. This indicated that the putative multicopper oxidase was not the S-PM2 receptor and suggested that the loss of this protein in the S-PM2-resistant mutant may be a pleiotropic effect of the loss of the phage S-PM2 receptor. As the putative multicopper oxidase gene in the mutant remained complete at the nucleotide level and was transcribed into mRNA, this led to the possibility that the putative multicopper oxidase could not be properly anchored into the specific site on the cell surface, which may be due to a structural change in the cell envelope and thus was secreted into the surrounding environment.

Since the OMF of the wild type showed a neutralisation effect on S-PM2, components in the OMF should be responsible for this effect. As 1-D SDS-PAGE has limited

resolution, further experiment using 2-D SDS-PAGE analysis over the OMFs would be a better choice.

Based on the results presented in this thesis, a hypothesis (Figure 7.1) was formulated. For a successful S-PM2 adsorption, two receptors, MCO and a second receptor (probably LPS) are needed. S-PM2's light-dependent adsorption and adsorption only at a certain wavelength range suggested at least one receptor, most likely MCO, is light sensitive. In the presence of the light, MCO is activated which leads to MCO conformation change. Only this conformation will be recognised and binded by S-PM2. After the successful initial binding, the irreversible binding to the second receptor follows. The adsorption process doesn't need energy derived from the host photosynthesis because S-PM2 can still adsorb to DCMU/CCCP-treated WH7803 in the light.

But surprisingly, S-PM2 can still infect the MCO-knockout mutants and adsorption to these mutants only happened in the light, but not in the dark. It can be explained that other surface ion-transporters may have a similar function role as MCO thus they compensated MCO to act as the first receptor to make the initial binding happen. The growth rate of these knockout mutants was similar to the wild type, which supports the compensation theory on cell fitness point of view. If the second phage receptor was also knocked out, S-PM2 would not be able to absorb to WH7803. The OMF prepared using EDTA treatment of WH7803 only showed a moderate (30%) neutralisation effect on S-PM2. This is due to the absence of the second phage receptor (LPS) in the OMF.

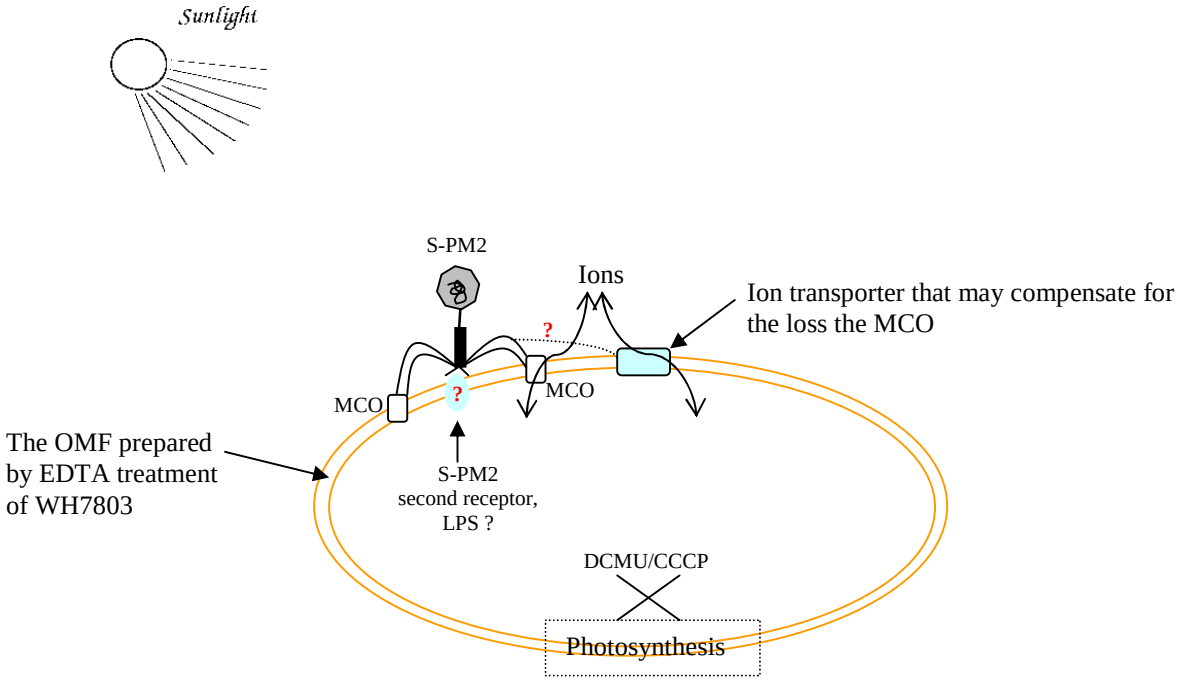


Figure 7.1 Diagram illustrating the possible role of two S-PM2 receptors involved in S-PM2 adsorption to *Synechococcus* sp. WH7803.

S-PM2 adsorption involved two steps and two sets of receptors. Firstly, MCOs (▢) and other similar ion transporters (▭) changed conformation in the presence of light and become recognisable to S-PM2 to initiate the adsorption. After the initial first step, the irreversible adsorption to the second receptor ( ), such as LPS in the cell wall followed. As S-PM2 adsorption doesn't need energy derived from the host photosynthesis, S-PM2 can still adsorb to DCMU/CCCP-treated WH7803. But adsorption did not only depend on light, but also need certain wavelength range. EDTA treatment of WH7803 only stripped off OMF (including MCO) but no cell wall fraction, which explain why the OMF only showed a 30% neutralisation effect on S-PM2. If the second S-PM2 receptor was added in the OMF, a 100% neutralisation of S-PM2 was expected.

The discoveries made in this study provide with some clues about the phage-host relationships in the marine environment. S-PM2 illustrates the critical role of the daylight in some phage life cycles. S-PM2 displays intriguing characteristics that are not found in phages infecting heterotrophic bacteria. The study also suggests that some cyanophages in the natural environment can only infect cyanobacteria in the daytime and that multi-phage-resistant stains may occur in the natural environment.

Considering that there is so little information about cyanophage receptors, this study provides the basis for the eventual identification of a cyanophage receptor.

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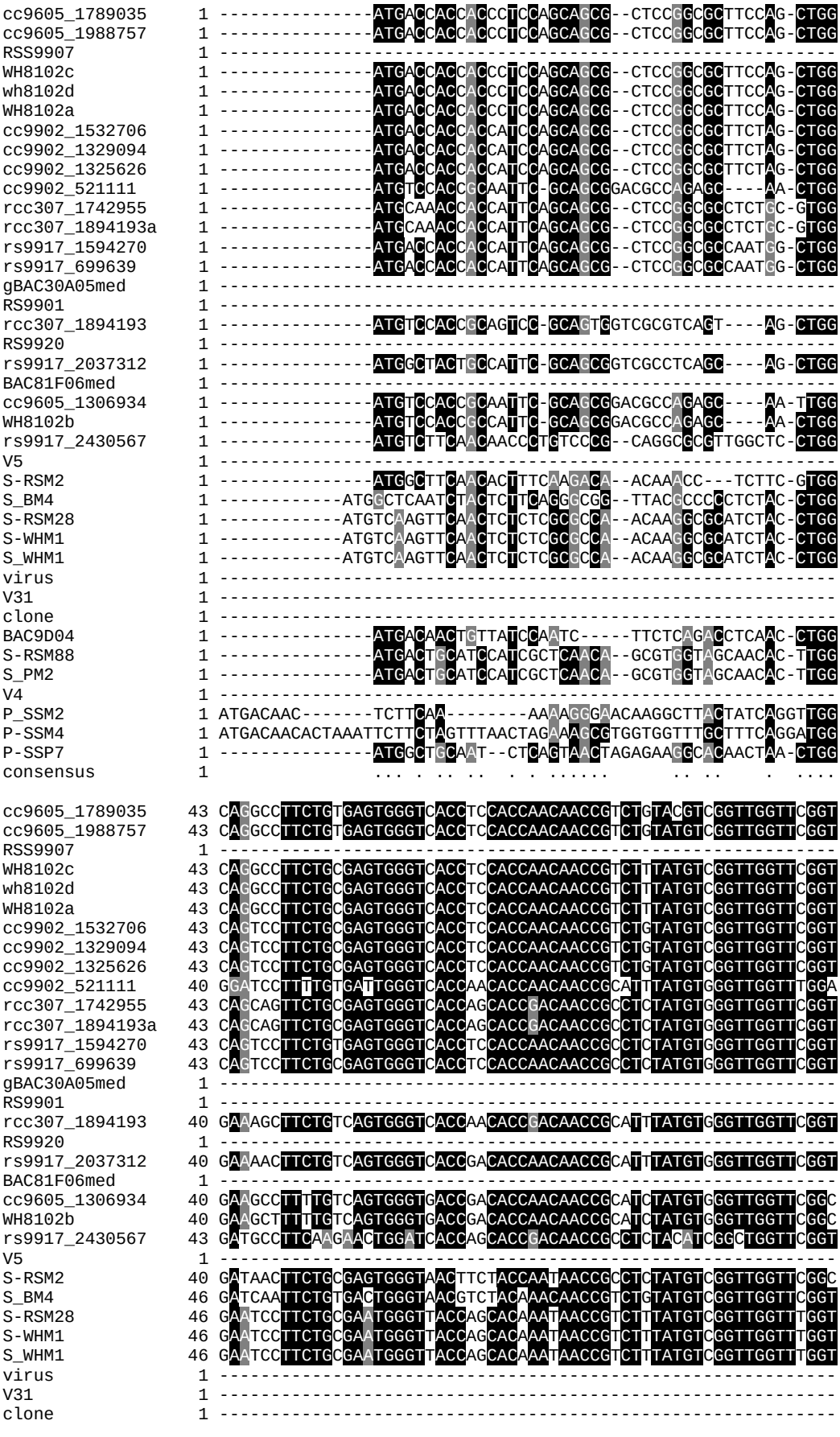
Appendix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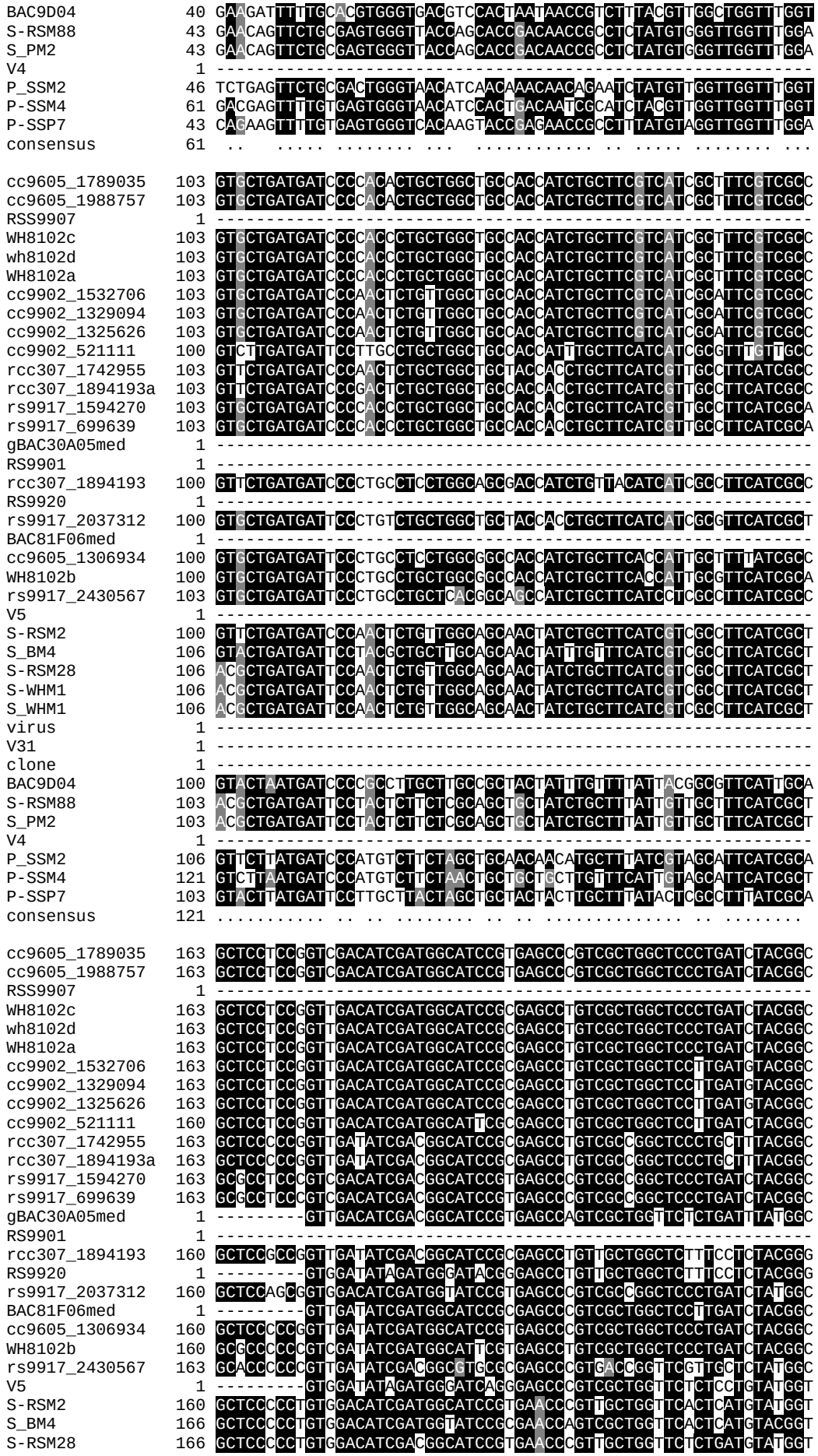
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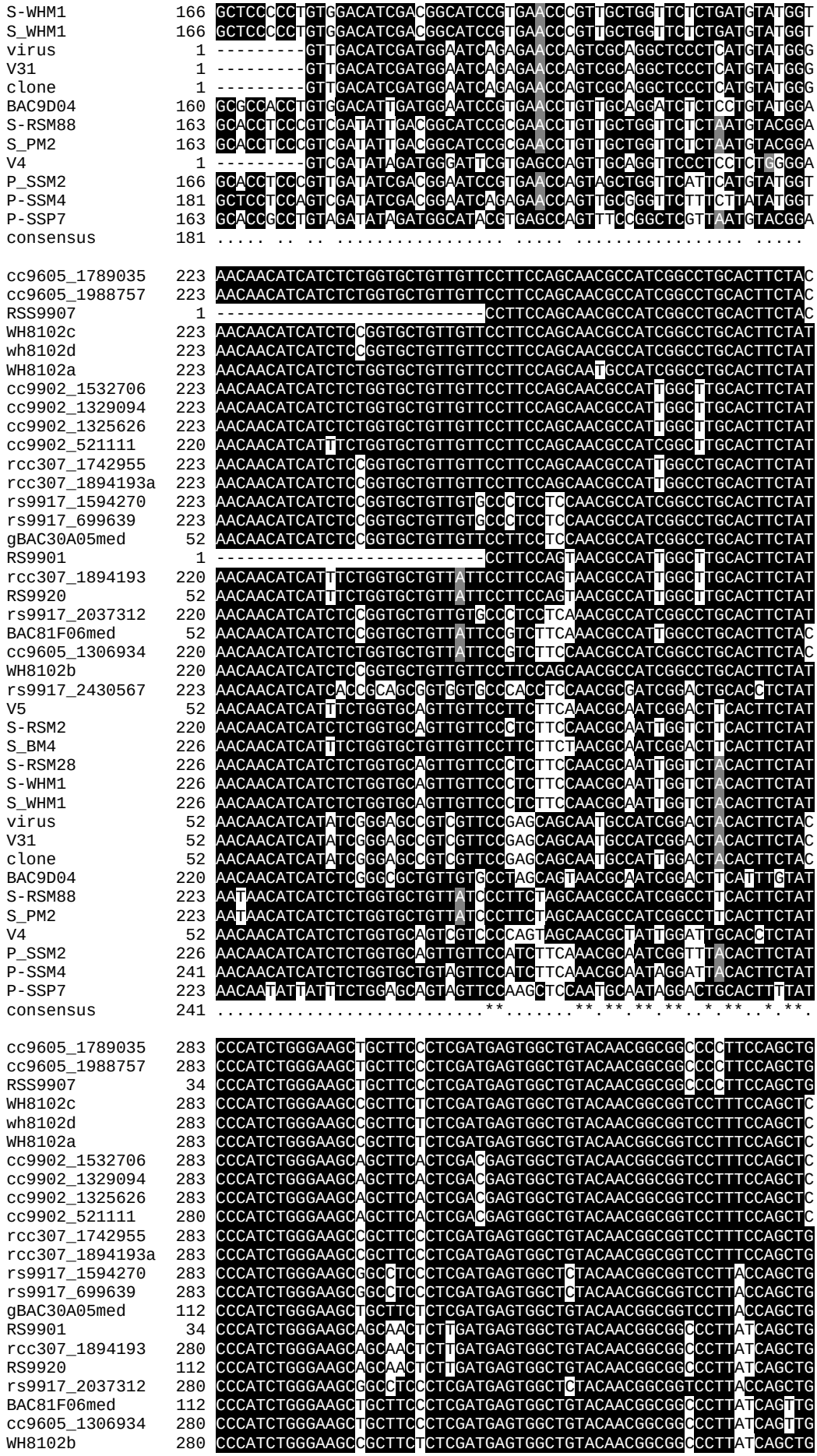
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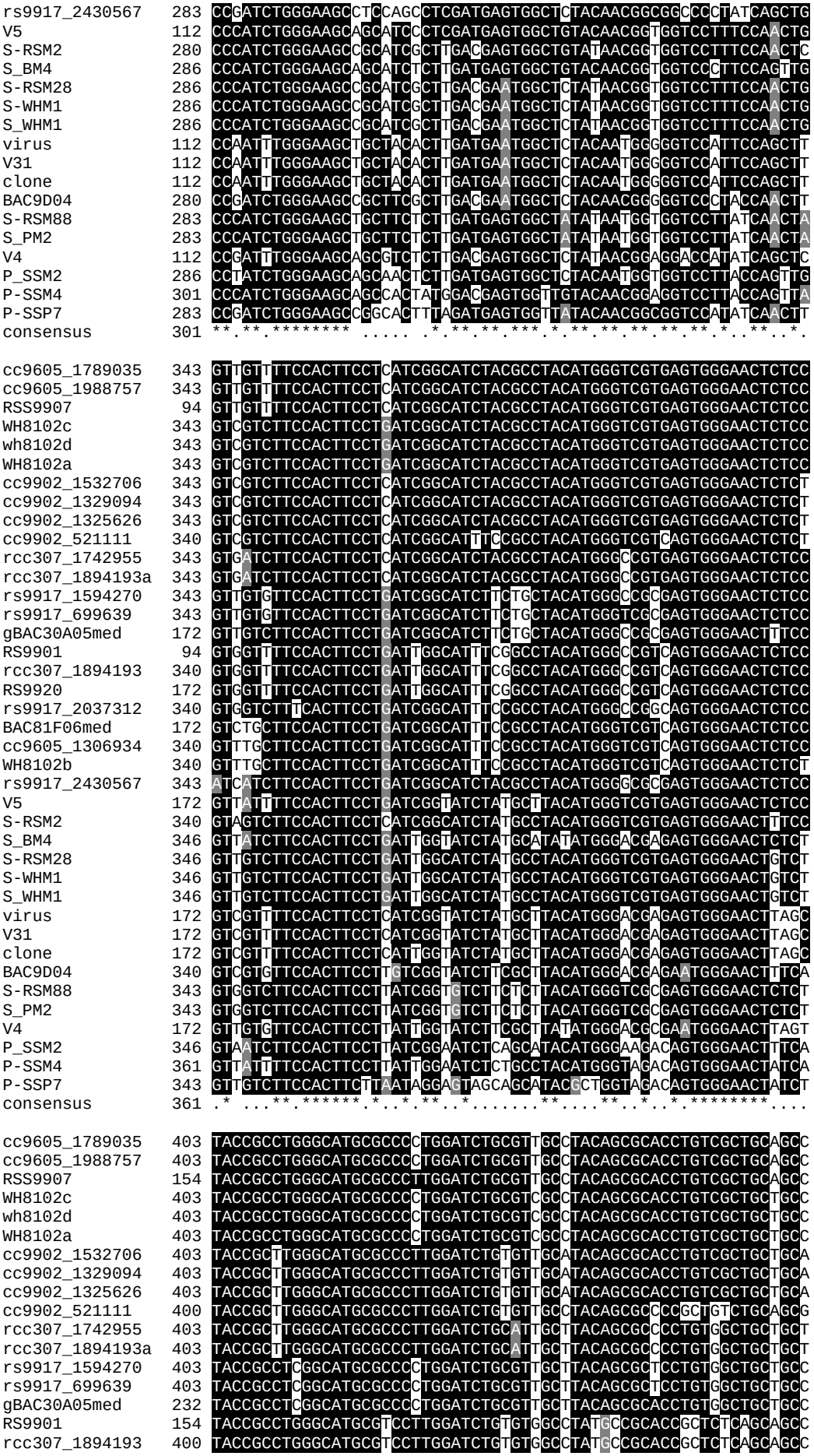
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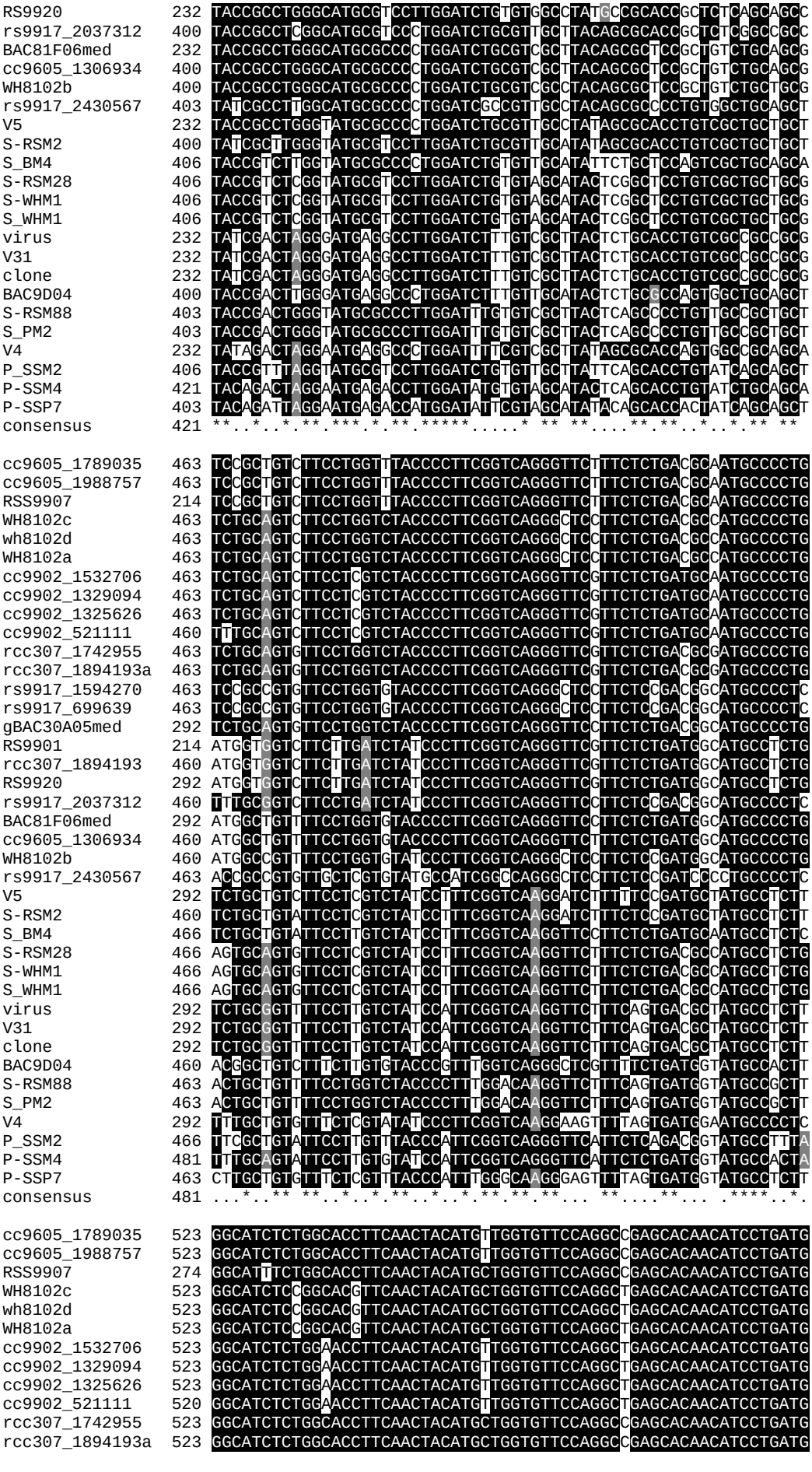
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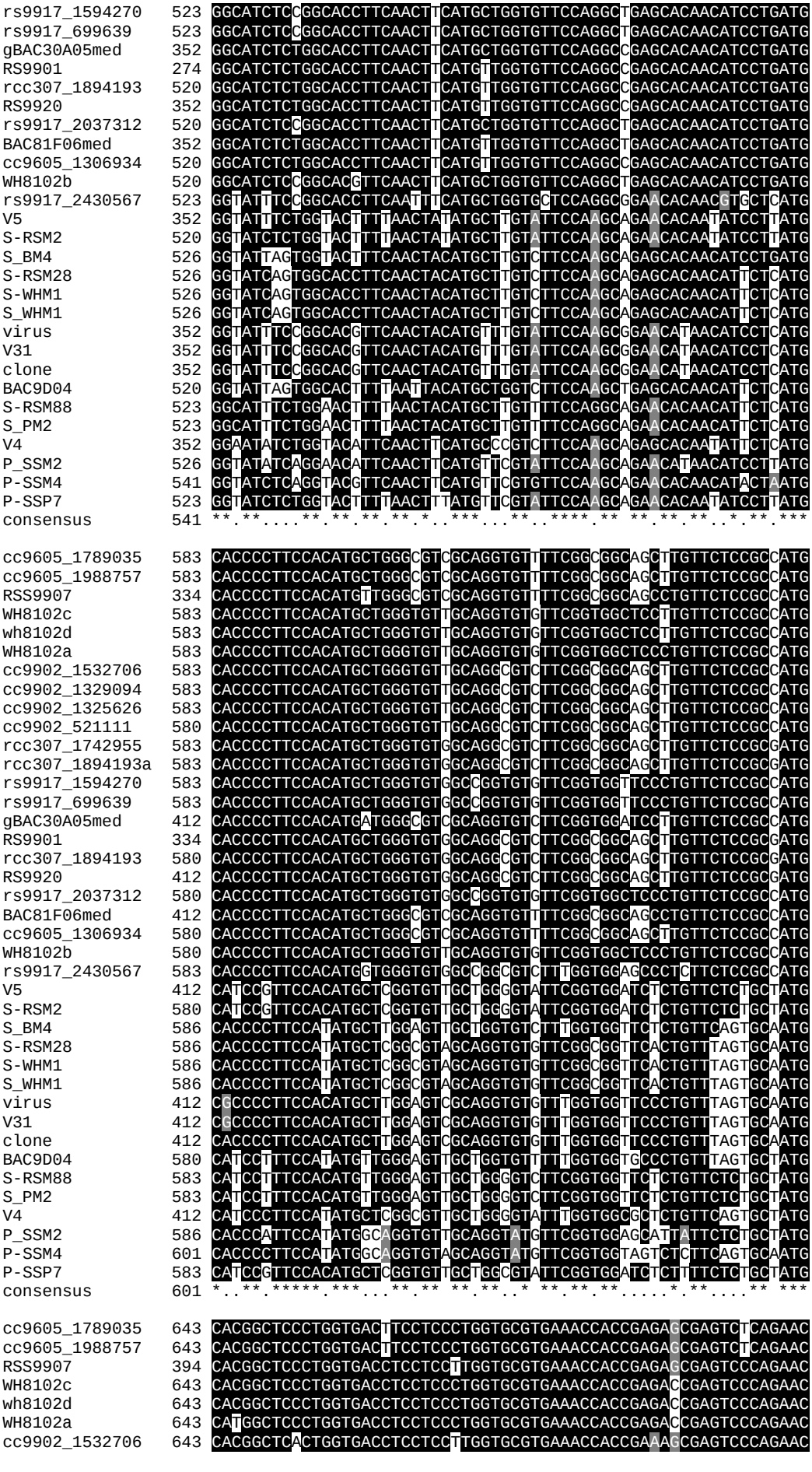
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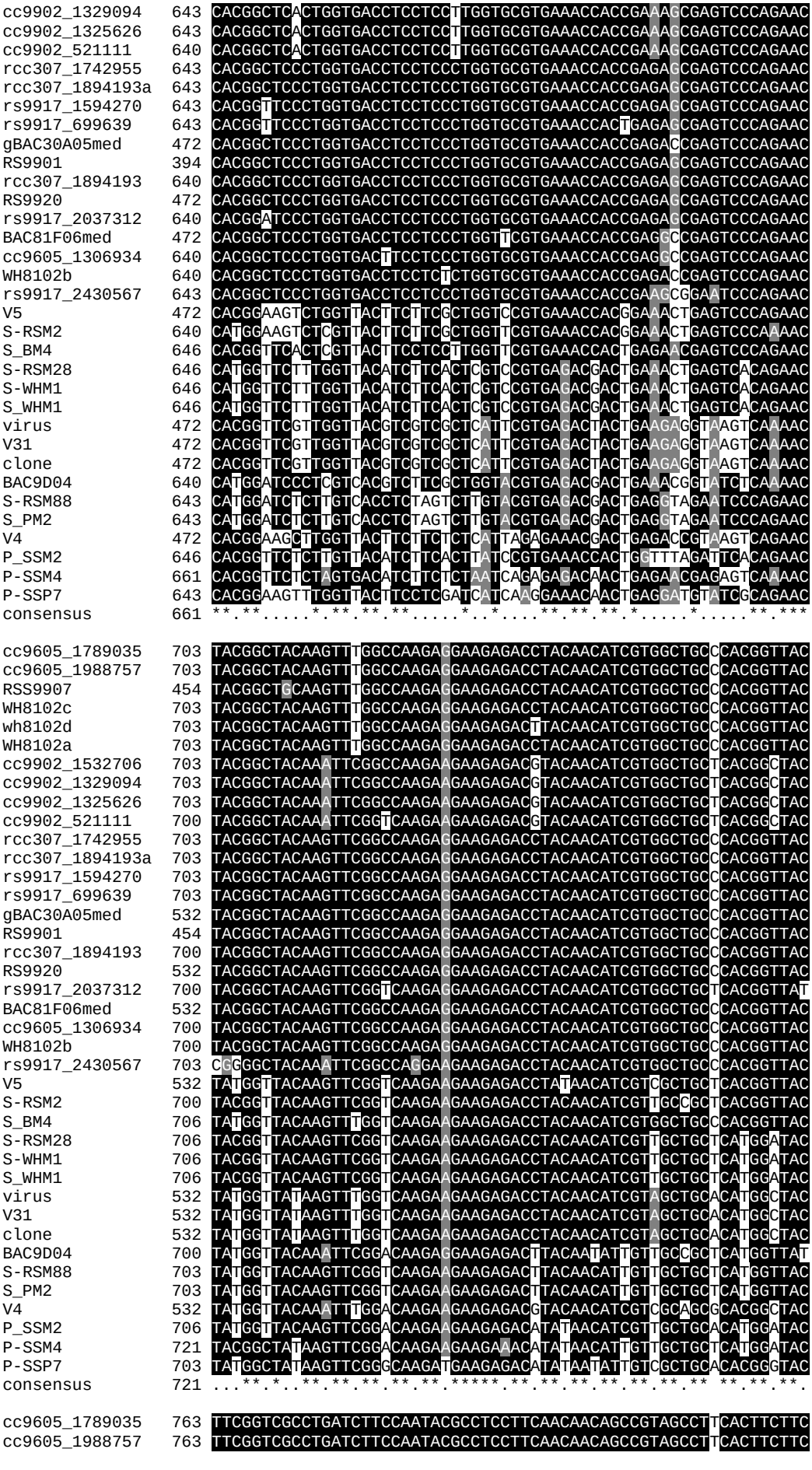
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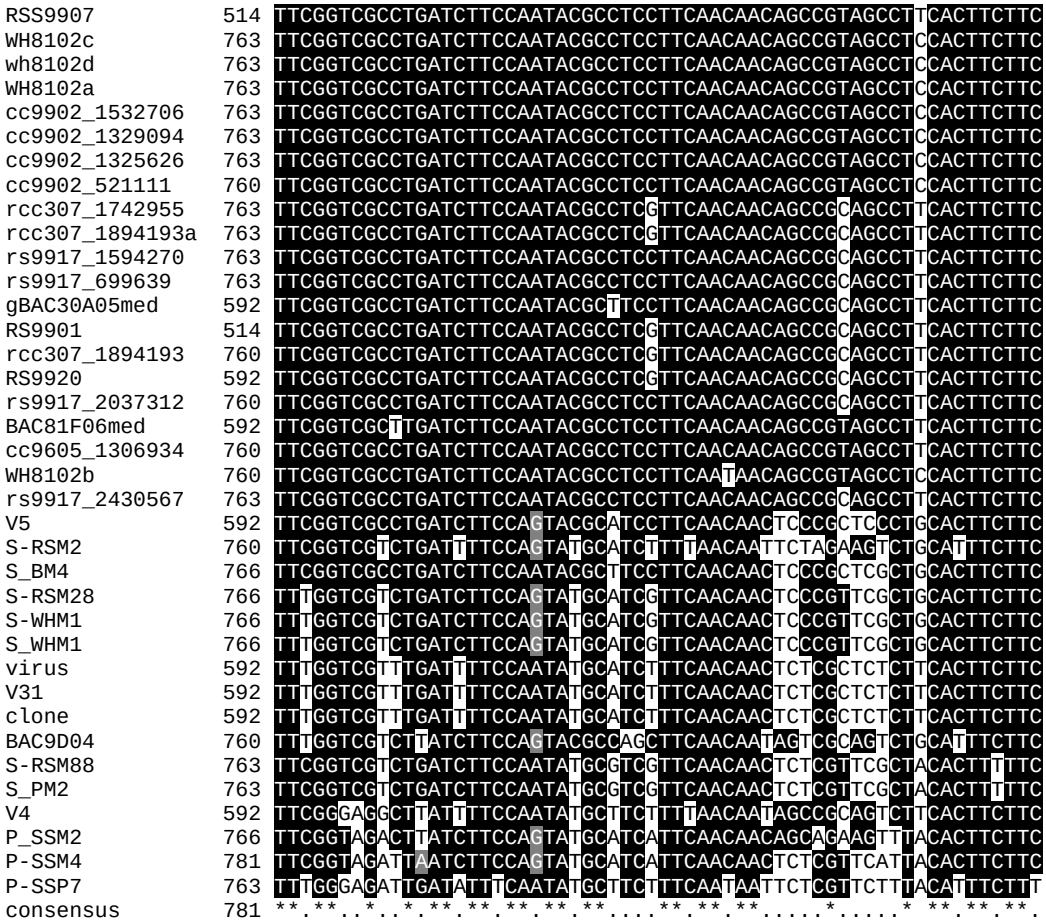
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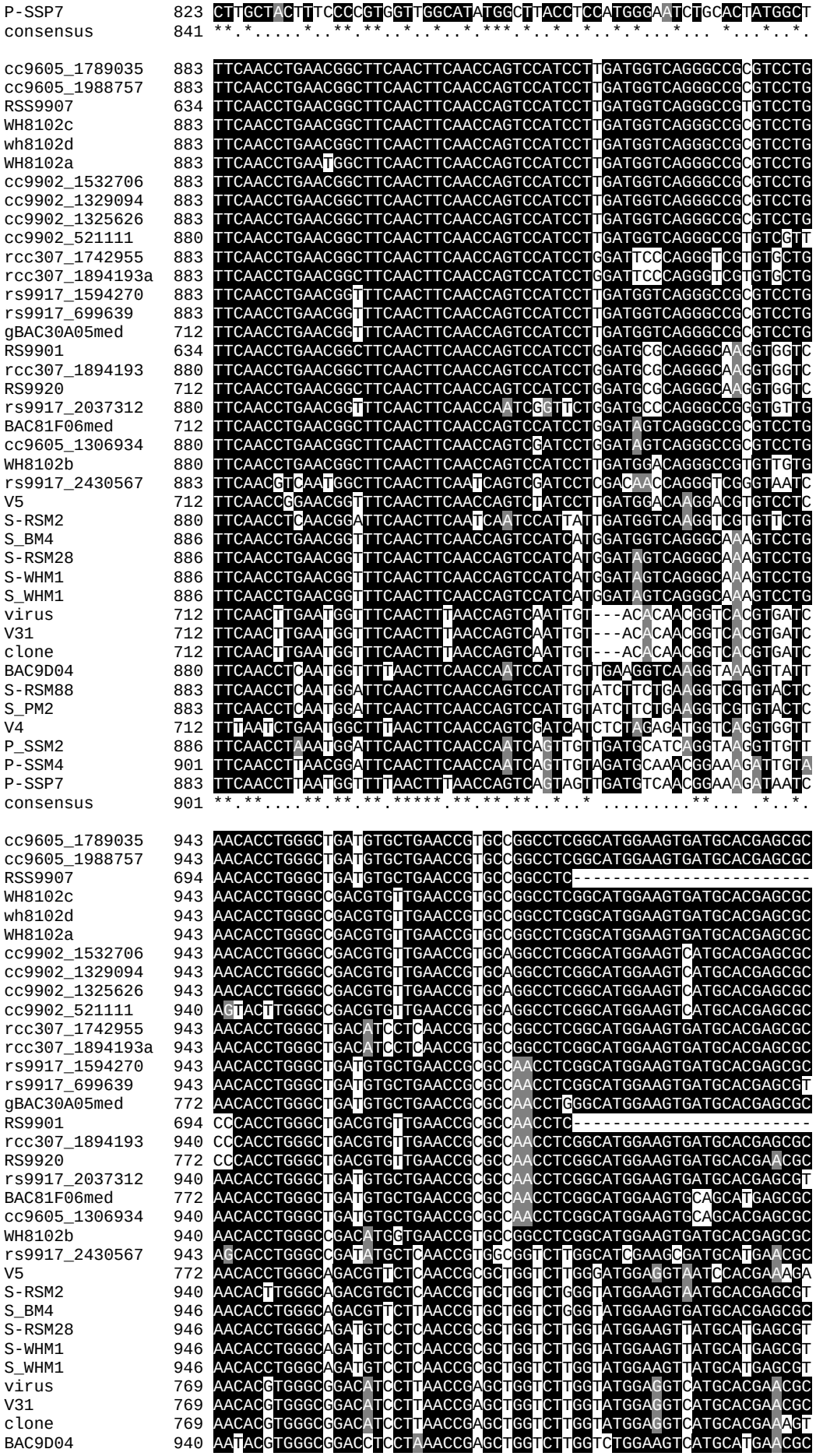
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V5 772
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S-RSM88          943 AACACCTGGGCAGATGTGCTGAATCGTGCTGGTCTGGGTATGGAGGTGATGCACGAGCGT
S_PM2            943 AACACCTGGGCAGATGTGCTGAATCGTGCTGGTCTGGGTATGGAGGTGATGCACGAGCGT
V4               772 AACACTTGGGCAGACATCCTAAACCGAGCAAACCTTGGTATGGAAGTGATGCACGAAAGT
P_SSM2           946 CCTACTTGGGGTGATGTTCTTAACAGAGCAAACCTTGGTATGGAAGTTATGCACGAAAGA
P-SSM4           961 CCAACATGGGGAGATGTTCTTAACAGAGCAAACCTTGGAATGGAAGTTATGCATGAAAGA
P-SSP7           943 CCAACATGGGCTGATGTATTAAATAGAGCTAACCTCGGATTTGAAGTTATGCACGAGCGC
consensus        961 ...**.****. **..*..*.**..* *. ...**.......................

cc9605_1789035  1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
cc9605_1988757  1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
RSS9907              ------------------------------------------------------------
WH8102c         1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
wh8102d         1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
WH8102a         1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
cc9902_1532706  1003 AACGCTCACAACTTCCCCCTCGACCTGGCAGCTGCTGAGTCCACACCTGTGGCACTGCAA
cc9902_1329094  1003 AACGCTCACAACTTCCCCCTCGACCTGGCAGCTGCTGAGTCCACACCTGTGGCACTGCAA
cc9902_1325626  1003 AACGCTCACAACTTCCCCCTCGACCTGGCAGCTGCTGAGTCCACACCTGTGGCACTGCAA
cc9902_521111   1000 AACGCTCACAACTTCCCCCTCGACCTGGCAGCTGCTGAGTCCACACCTGTGGCACTGCAA
rcc307_1742955  1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCCGAGAGCGCCCCTGTGGCTCTCCAG
rcc307_1894193a 1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCCGAGAGCGCCCCTGTGGCTCTCCAG
rs9917_1594270  1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACCCCCGTGGCTCTGCAG
rs9917_699639   1003 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACCCCCGTGGCTCTGCAG
gBAC30A05med     832 AACGCTCACAACTTCCCCC-------------------------------------------
RS9901               ------------------------------------------------------------
rcc307_1894193  1000 AACGCTCACAACTTCCCCCTCGACCTGGCAACGACTGAGAGCGCCCCTGTGGCTCTCCAG
RS9920           832 AACGCACACAACTTCCCA--------------------------------------------
rs9917_2037312  1000 AACGCTCACAACTTCCCCCTCGACCTGGCTGCGGCTGAGTCCACCCCCGTGGCTCTGCAG
BAC81F06med      832 AACGCTCACAACTTCCCC--------------------------------------------
cc9605_1306934  1000 AACGCTCACAACTTCCCCCTCGACCTGGCTGCTGCTGAGTCCACTCCTGTGGCTCTGCAG
WH8102b         1000 AACGCTCACAACTTCCCCCTCGACCTGGCAACTGTTGAGTCCACTCCTGTGGCTCTGCAG
rs9917_2430567  1003 AACGTGCATAACTTCCCGCTCGATCTGGCAGCGGTCGACAGTCAACCCGTGGCGCTCACA
V5               832 AATGCGCACAACTTTCCA--------------------------------------------
S-RSM2          1000 AATGCTCACAACTTCCCACTCGACCTCGCTGCTGCTGAGTCCACCCCTGTGGCACTCACC
S_BM4           1006 AACGCTCACAACTTCCCTCTCGACCTTGCTGCTGCTGAGTCTACCCCTGTAGCACTCACC
S-RSM28         1006 AACGCACACAACTTCCCTCTCGACCTCGCTGCTGCTGAGTCCACACCTGTGGCACTCACC
S-WHM1          1006 AACGCACACAACTTCCCTCTCGACCTCGCTGCTGCTGAGTCCACACCTGTGGCACTCACC
S_WHM1          1006 AACGCACACAACTTCCCTCTCGACCTCGCTGCTGCTGAGTCCACACCTGTGGCACTCACC
virus            829 AACGCACACAACTTTCCCA--------------------------------------------
V31              829 AACGCACACAACTTTCCCA--------------------------------------------
clone            829 AATGCCCACAACTTCCCA--------------------------------------------
BAC9D04         1000 AACGCCCACAACTTCCCGCTTGACTTGGCTGCTGCCGAAACAACTTCTGTTGCTTTGGTT
S-RSM88         1003 AATGCACACAACTTCCCTCTTGACCTTGCAGCAGCAGAAGCAACACCAGTTGCCTTGACT
S_PM2           1003 AATGCACACAACTTCCCTCTTGACCTTGCAGCAGCAGAAGCAACACCAGTTGCCTTGACT
V4               832 AACGCGCACAGCTTCCCA--------------------------------------------
P_SSM2          1006 AATGCACACAACTTCCCACTTGACTTAGCATCTGCTAACGAGACAGAAGTTGCTCTTGTT
P-SSM4          1021 AATGCACACAACTTCCCACTAGACCTAGCATCTGCTGAGTCTACAACAGTTGCATTAACT
P-SSP7          1003 AATGCTCATAACTTCCCACTTGATTTAGCTTCGGCTGAGTCAACAAACATTGCACTAACA
consensus       1021 ..................  ...........  ..............  ........  ..

cc9605_1789035  1063 GCTCCTGCCATCGGTTGA
cc9605_1988757  1063 GCTCCTGCCATCGGTTGA
RSS9907              ------------------
WH8102c         1063 GTTCCTGCCATCGGTTGA
wh8102d         1063 GCTCCTGCCATCGGTTGA
WH8102a         1063 GCTCCTGCCATCGGTTGA
cc9902_1532706  1063 GCACCTGCAATCGGTTGA
cc9902_1329094  1063 GCACCTGCAATCGGTTGA
cc9902_1325626  1063 GCACCTGCAATCGGTTGA
cc9902_521111   1060 GCACCTGCAATCGGTTGA
rcc307_1742955  1063 GCTCCTGCCATCGGTTGA
rcc307_1894193a 1063 GCTCCTGCCATCGGTTGA
rs9917_1594270  1063 GCTCCCGCCATCGGTTGA
rs9917_699639   1063 GCTCCCGCCATCGGTTGA
gBAC30A05med         ------------------
RS9901               ------------------
rcc307_1894193  1060 GCTCCTGCCGTCGGCTGA
RS9920               ------------------
rs9917_2037312  1060 GCTCCCGCCATCGGTTGA
BAC81F06med          ------------------
cc9605_1306934  1060 GCTCCTGCCATCGGTTGA
WH8102b         1060 GCTCCTGCCATCGGTTGA
rs9917_2430567  1063 GCACCTGCAATCGGTTAG
V5                   ------------------
S-RSM2          1060 GCACCTAGCATCGGTTGA
S_BM4           1066 GCACCTGCAATCGGTTGA
S-RSM28         1066 GCTCCTGCGATCGGTTGA
S-WHM1          1066 GCTCCTGCGATCGGTTGA
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S_WHM1 1066 GCTCCTGCGATCGGTTGA
virus ----------
V31 ----------
clone ----------
BAC9D04 1060 GCTCCAACGATTGGCTAA
S-RSM88 1063 GCACCCGCAATCGGTTGA
S_PM2 1063 GCACCCGCAATCGGTTGA
V4 ----------
P_SSM2 1066 GCTCCTTCTATAGGTTAA
P-SSM4 1081 GCACCTTCAATCGGTTAA
P-SSP7 1063 GCTCCAGAAATTGGTTGA
consensus 1081 .. ..