MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Deliverable D5.6

Assessment of biotic/abiotic factors on expression of bioactive biosynthetic genes

Organisation name of lead contractor: PharmaMar – partner 19

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Dissemination Level	
PU Public	
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	**X**

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v.1		

The following document is related to this deliverable:

1.

Please attach deliverable documents and any additional material if needed.

Summary

Objective:

- Identification of biotic and abiotic growth factors involved in the expression of biotechnology interesting biosynthetic genes in microorganisms, particularly in PKS gene clusters from deep-sea isolated actinobacteria [Partner 19] or PKS Type III and chalcone synthase in *Synechococcus* sp. WH7803 [Partner 13]

Rationale: WP5 focuses on identifying and investigating cell to cell communication and interaction pathways in natural marine microbial communities. This WP will also develop the identification of culture factors for improving the production of interesting compounds/metabolites by activation of biosynthetic genes in cultivable microorganisms.

Results:

Partner(s) involved in Deliverable production:

Partner(s) involved in Deliverable production:

Partner Name	Partner Number	Participation
UCC	12	y
UW	13	Y
PHM	19	y

Effect of biotic/abiotic factors for improving culture of deep-sea actinobacteria as a way to induce silent genes related to cytotoxic activity (PharmaMar, partner 19)

This Deliverable updated the results up to July 2015.

For partial results, see Deliverables D.3.3.- Improved conditions for novel groups of marine bacteria, D.3.10.- Optimized and standardized culture of known bioactive organisms and D.5.4.- Assessment of improved culture efficiency by using a living marine Microbiota.

♠ Rational

Nonribosomal peptides (NRPs) and polyketides (PKs) are two of the largest families of bioactive microbial metabolites, accounting for most of the antibiotic, antifungal, anticancer, and immunosuppressant compounds that have been characterized from cultured bacteria to date. Although NRPS and PKS biosynthesis is responsible for producing all biomedically relevant natural products, it leads to many of the metabolites used in the clinic including penicillin, vancomycin, rapamycin, erythromycin, rifamycin, and many others.

Many of these bioinformatically newly discovered secondary metabolism gene clusters are silent under standard laboratory conditions. Interesting examples can be seen using antiSMASH software, a tool PharmaMar is continuously applying to decide which marine strains must be re-cultivated for the expression of new metabolites.

The traditional method of a single culture restricts the metabolic pathways of microorganisms and as a result many metabolites cannot be formed. Recently, it has attracted much attention to use various techniques to activate those metabolic pathways restricted by the traditional method to get metabolic products with rich variety of structures. "One strain many compounds" (OSMAC) is a simple and effective approach for activating metabolic pathways and has been successfully applied. The common strategies of the OSMAC approach are altering cultivation parameters, co-cultivation, addition of enzyme inhibitors, etc. Here, we have been exploring the first option combined with genomics scanning.

PharmaMar has developed a battery of different production media, based on reformulations using Response Surfaces Optimization methodology.

This deliverable is based on the results obtained for the culture of more than 1,600 different deep-sea actinobacteria isolated as part of the MaCuMBa project using several stressing conditions highlighting the effect of high concentration of mannitol, dextrins, marine salts and diverse nitrogen sources on the antitumor activity of deep-sea actinobacteria. In addition to, and as a compass for, current and future target phylum isolation, we have been exploring the Genbank DDBB in NCBI for selecting the most promising groups of bacteria harboring PKS.

The results of active genera and potential groups harboring PKS are clearly coincident.

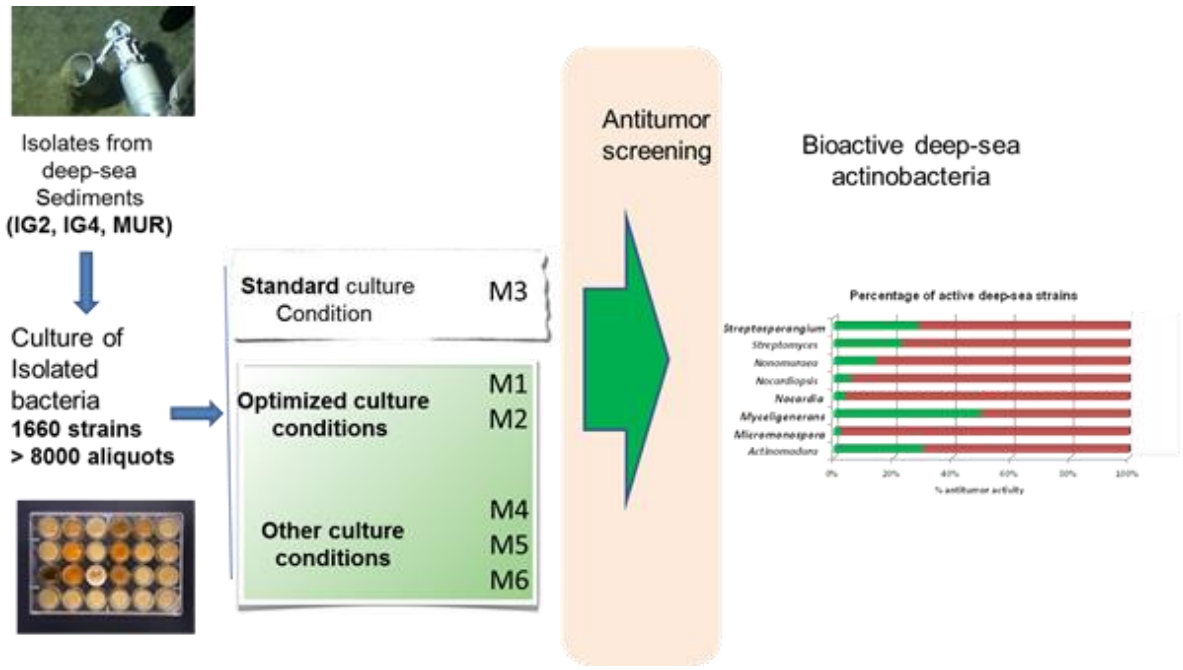

Figure 1.1.- *(From D3.10) Summary of PharmaMar experiments aimed at optimizing and standardizing culture conditions of known bioactive organism cultured grown under standard (M3) and stress conditions (M1, M2, M4, M5 and M6)*

♠ Materials and Methods

Strains

More than 1,600 deep-sea bacteria (mainly actinobacteria) have been cultured under standard and optimized conditions and the whole broth have been tested for bioactivity under antitumor screening platform. 15% of the strains have shown activity in at least one condition.

Fermentation conditions

As described in a previous deliverable (see D.3.10) we have used a combination of parameters such as different inoculum development for activation of initial biomass, 12 well microtiter format (3ml) and 24 well microtiter Format (1ml), different media and different timing course of fermentation (95h, 120h, 144h, 168h, up to 2 weeks) to define the best conditions for culturing. After fermentation, the plates are lyophilized and extracted with organic solvent to get crude extracts for screening.

Production media

With respect to fermentation conditions, our standard culture conditions were based on the production medium, coded as **M3** in other deliverables, (see D3.3, D5.4 and D5.5), comprising balanced quantities of dextrin, dextrose, mannitol, soyflour, artificial sea water and micro/oligoelements. Using Plackett & Burmann method and Surface Response

Optimization algorithms, other optimized media were designed as 2-7 **(M1**) applying an high mannitol concentration (50g/L) and 2-36 **(M2)** stressing medium harboring dextrin (68g/L). Additional media have been used as FA-1 **(M4),** composed by dried yeast and partially hydrolysed starch (Glucidex©, >40g/L), **M5** mainly contained glycerol and corn steep power, nitrates and high concentration of marine salts. This M5 has been used an excellent isolation medium. Finally, **M6** was a highly complex medium using all M1-M5 ingredients.

Screening

Each extract will be screened to determinate the *in vitro* GI50 activity (50% growth inhibition) against 3 human tumor cell lines, related to lung (A549) , colon (HT-29) and breast (MDA-MB231) cancer.

♠ Active (anti-tumor) biodiversity

Based on the results of 1660 strains, belonging to 47 genera and cultured in several production media, only 8 genera have shown activity in at least one culture condition.

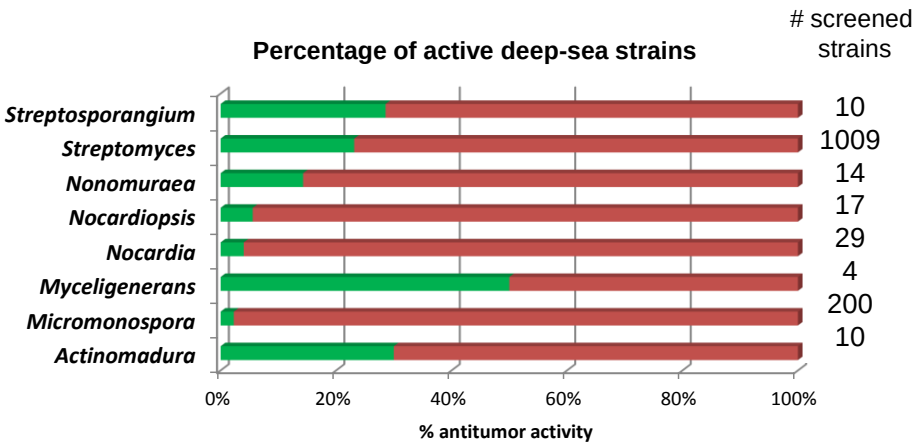

Figure 1.2.- *(From D3.3) Antitumor deep-sea actinobacteria.*

Those active genera belonged to filamentous actinobacteria, with large genome size and pre-selected as groups harboring interesting gene clusters such as PKS.

These results confirm the genomic mining exploration of PKS that PharmaMar is developing (both in MicroB3 and MaCuMBA) as compass for selection of interesting bacterial groups for drug discovery.

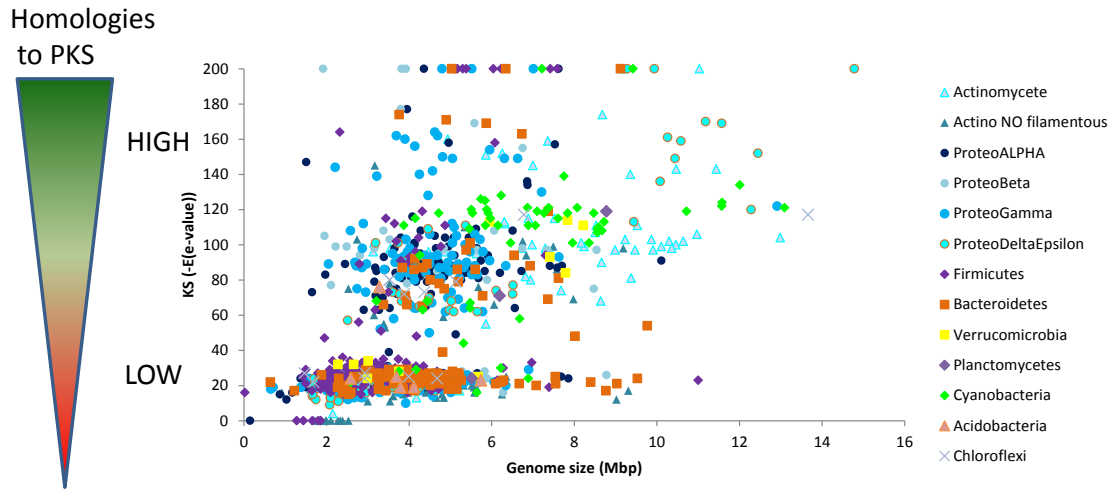

Figure 1.3.- Genome mining for PKS using >4.000 bacterial genomes in NCBI. The relationship between genome size and *e-value* for *ketosynthase*. Each point represents the best match per genus.

Except *Myceligenerans*, no sequenced species has been registered in NCBI yet, the rest of the active strains belonging to pre-selected genera due to their large genome size and high homology to PKS.

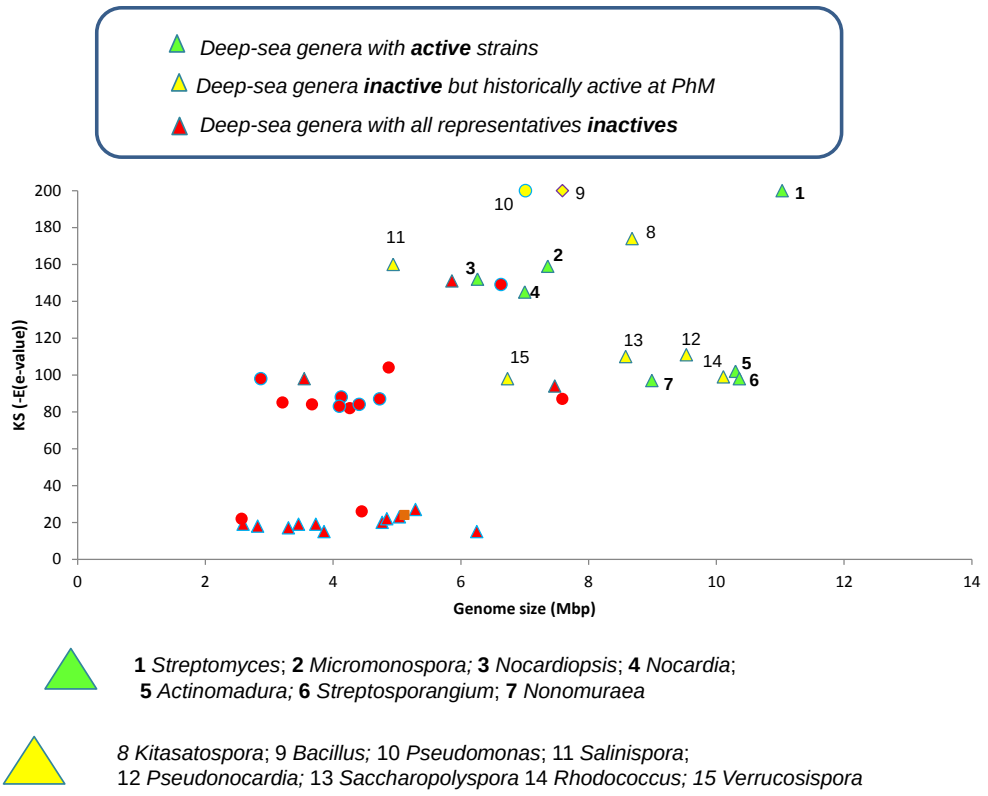

Figure 1.4.- *MaCuMBA isolates. Genome mining for PKS of relationship with antitumor activity*

Some of the inactive genera isolated during the MaCuMBA project have been active in other PharmaMar's isolations as the case of 8 to 15 genera.

♠ Effect of biotic factors on antitumor activity

As mentioned in the *material & methods*, several stressing production media were used to increase the rate of active strains, using an excess (>40g/L) of alternative carbon sources. This means a stressing situation where the microorganism develops different routes to metabolize the excess of mannitol or polysaccharides having an environmental induction of same biosynthetic routes.

The next table shows the antitumor activity per production media in expeditions of IG2 and MUR. No activity was reported in the IG4 expedition.

IG2 + MUR expeditions			Standard	Optimized from M3		Orther stressing conditions		
Genus	Total strains	Actives	M3 Control	M1 Mannitol 52g/L	M2 Dextrin 68g/L	M4 Glucidex >40g/L	M5	M6 Complex
Actinomadura	10	3	AA	AA	II	II	II	II
Micromonospora	197	4	AI	II	IA	II	II	AA
Myceligenerans	4	2	A	I	A	I	I	I
Nocardia	9	1	A	A	I	I	I	I
Nocardiopsis	17	1	I	A	A	I	I	I
Nonomuraea	14	2	AA	AI	AI	AA	II	II
Streptomyces	1005	231	AA	AA	AA	AA	AA	AA
Streptosporangium	2	2	II	AI	II	II	II	AI
Unidentified	13	6	AA	AA	II	AI	AI	AI
Other strains	389	0	I	I	I	I	I	I

Table 1.1.- *Antitumor activity of deep-sea actinobacteria using different production media.* ***AA:*** *Antitumor activity in the two expeditions (IG2+MUR).* ***AI*** *Active in IG2 but inactive in MUR.* ***II****: Inactive in both expeditions.*

♠ Conclusions

In conclusion, improved culture conditions (as a measure of antitumor activity) depends on the taxonomy. There is not a "super" production medium for all deep-sea actinomycetes, although the composition of M3 (dextrin (10g/L), dextrose (2g/L), mannitol (20g/L) and 50% artificial sea water) has demonstrated the best formulation for the majority of the active genera.

- ***Actinomadura*****:** Good production under high concentration of mannitol, but excess of dextrins can be inhibitory of the production of secondary metabolism.

- ***Micromonospora*:** The best results has been obtained using M2 (dextrin) and M6, a high complex media without any nutrient upper than 20 g/L.
- ***Myceligenarans*.** Clearly production on dextrin with mannitol as inhibitor.
- ***Nocardia**:* No high concentration of nutrients triggers clearly the cytotoxic activity.
- ***Nocardiopsis**,* clearly the biosynthesis of secondary metabolites is related with stressing concentration of mannitol of destrins.
- ***Nonomuraea*:** Best results using stressing concentrations.
- ***Streptomyces:*** The activity depends on the quality of the strains (or the type of specie) more than genus. Good results in all cases in general.
- ***Streptosporangium***, the excess of mannitol and high complex media have been the best scenario for productions.

This result confirms the relationship between genera and identification of secondary gene clusters as PKS (polyketide synthases).

Assessment of biotic/abiotic factors on expression of bioactive biosynthetic genes in *Synechococcus*. (The University of Warwick, partner 13)

Using the AntiSMASH software, the *Synechococcus* sp. WH7803 genome was screened for gene clusters responsible for production of secondary metabolites. Only one type III polyketide synthase (T3PKS) gene cluster was found (Table 2.1).

Cluster	Type	From	To
Cluster 1	Bacteriocin	60425	71303
Cluster 2	Bacteriocin	359679	370002
Cluster 3	Bacteriocin	497385	507975
Cluster 4	Bacteriocin	551259	562567
Cluster 5	Bacteriocin	710512	740547
Cluster 6	Bacteriocin	873198	883473
Cluster 7	T3PKS	902671	943765
Cluster 8	Bacteriocin	993365	1003733
Cluster 9	Bacteriocin	1199409	1210296
Cluster 10	Bacteriocin-terpene	1451011	1472589
Cluster 11	Bacteriocin	1501047	1511295
Cluster 12	Terpene	2081620	2102561

Table 2.1 *Types and locations of secondary metabolite gene clusters in* Synechococcus *sp. WH7803.*

The T3PKS gene cluster is located on a genomic island and is present in a number of *Synechococcus* spp. and *Prochlorococcus* spp. (Figure 2.1). Unfortunately, several of the genes in the cluster are hypothetical with no specific motifs which could help predict their functions (Table 2.2).

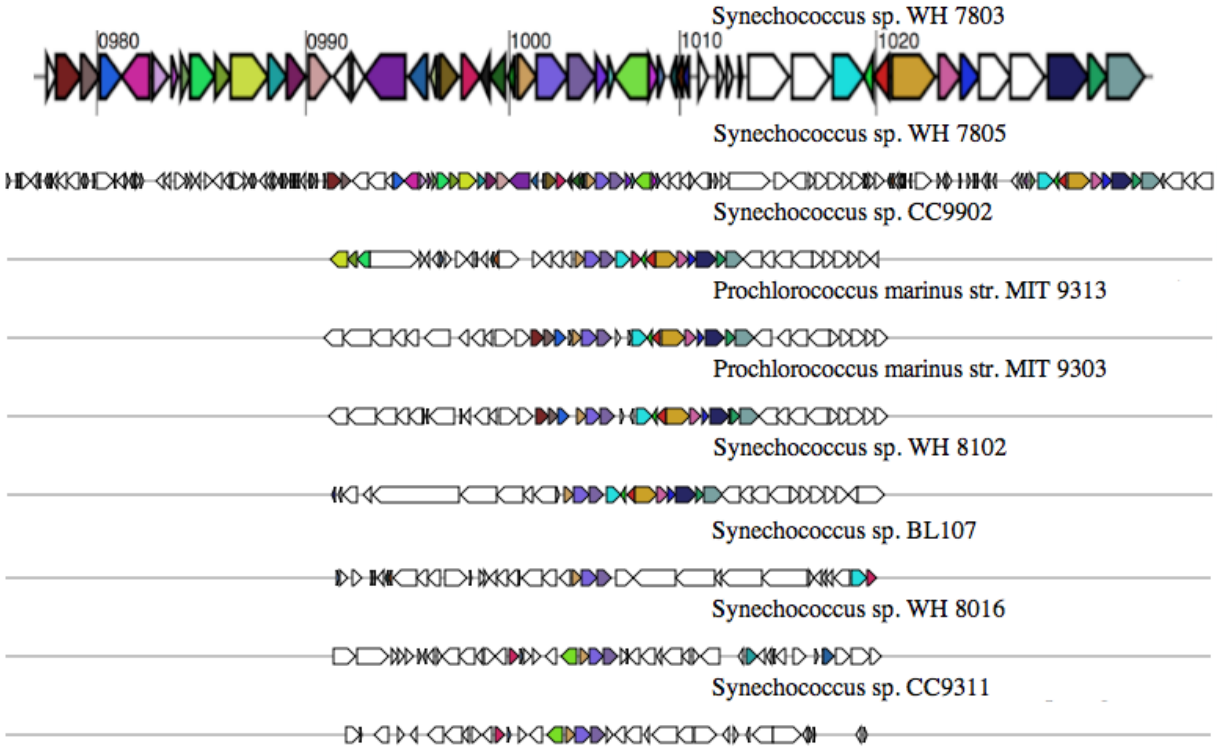

Figure 2.1 *Type III polyketide synthase clusters in* Synechococcus *spp. and* Prochlorococcus *spp. with published genomes.*

Gene	Annotation	Possible functions based on blastp and motifs found
synWH7803_0977	hypothetical protein	hypothetical protein
synWH7803_0978	ABC-type Mn2+ transport system periplasmic protein	manganese/zinc ion ABC transporter, substrate binding protein, metal transporter
synWH7803_0979	ABC-type Mn2+ transport system, ATPase component	manganese/zinc ion transporter, iron-chelate-transporting ATPase, ABC transporter
synWH7803_0980	ABC-type Mn2+ transport system permease	iron ABC transporter permease, membrane protein
synWH7803_0981	DMT family permease	DMT (drug/metabolite transporter) permease, membrane protein
synWH7803_0982	hypothetical protein	hypothetical protein
synWH7803_0983	hypothetical protein	hypothetical protein
synWH7803_0984	cytochrome C6	cytochrome C biogenesis protein CcsB, cytochrome C553 (soluble cytochrome f), cytochromo C6
synWH7803_0985	permease	permease
synWH7803_0986	hypothetical protein	hypothetical protein, membrane protein
synWH7803_0987	hypothetical protein	hypothetical protein
synWH7803_0988	Mn2+/Zn2+ ABC transporter ATPase	ABC transporter ATP-binding protein, phosphonate-transporting ATPase, ABC-type Mn/Zn transport system ATPase component

Gene	Annotation	Possible functions based on blastp and motifs found
synWH7803_0989	Mn2+/Zn2+ ABC transporter permease	manganese ABC transporter, zinc ABC transporter, ABC-type transporter, integral membrane subunit
synWH7803_0990	Mn2+/Zn2+ ABC transporter periplasmic protein	ABC transporter ATP-binding protein, manganese/zinc ion ABC transporter
synWH7803_0991	hypothetical protein	hypothetical protein with transmembrane domains
synWH7803_0992	hypothetical protein	uncharacterized conserved secreted protein
synWH7803_0993	porin-like protein	porin
synWH7803_0994	hydrogenase accessory membrane protein	hydantoin utilization protein, HupE-UreJ family cobalt transporter
synWH7803_0995	hypothetical protein	hypothetical protein
synWH7803_0996	hypothetical protein	dehydrogenase, uncharacterized conserved secreted protein, N-acetylmuramoyl-L-alanine amidase
synWH7803_0997	16S ribosomal RNA methyltransferase RsmE	ribosomal RNA small subunit methyltransferase E
synWH7803_0998	redox protein	redox protein, regulator of disulfide bond formation
synWH7803_0999	hypothetical protein	steroid 5-alpha reductase
synWH7803_1000	hypothetical protein	hypothetical protein, membrane protein
synWH7803_1001	hypothetical protein	nucleotide-binding protein, methyltransferase type 12
synWH7803_1002	dehydrogenase	dehydrogenase, NAD binding site, flavin dependent oxidoreductase
synWH7803_1003	chalcone synthase	chalcone synthase, naringenin chalcone synthase
synWH7803_1004	hypothetical protein	alpha-ketoglutarate decarboxylase, translation initiation factor 2
synWH7803_1005	hypothetical protein	uncharacterized conserved membrane protein
synWH7803_1006	cytochrome P450	cytochrome P450
synWH7803_1007	hypothetical protein	hypothetical protein
synWH7803_1008	hypothetical protein	hypothetical protein, ATP-binding protein
synWH7803_1009	hypothetical protein	fumarate reductase
synWH7803_1010	hypothetical protein	gibberellin regulated protein
synWH7803_1011	hypothetical protein	uncharacterized conserved membrane protein, TetR family transcriptional regulator in *Streptomyces iakyrus* (identity 34%)
synWH7803_1012	hypothetical protein	hypothetical protein
synWH7803_1013	hypothetical protein	hypothetical protein, transketolase, membrane protein
synWH7803_1014	hypothetical protein	hypothetical protein, membrane protein, malate dehydrogenase
synWH7803_1015	hypothetical protein	hypothetical protein, 3-hydroxyacyl-CoA dehydrogenase
synWH7803_1016	hypothetical protein	conserved exported protein of unknown function
synWH7803_1017	hypothetical protein	conserved exported hypothetical

Gene	Annotation	Possible functions based on blastp and motifs found
		protein
synWH7803_1018	hypothetical protein	conserved hypothetical protein
synWH7803_1019	hypothetical protein	conserved hypothetical protein
synWH7803_1020	signal peptidase I	peptidase A26, thylakoidal processing peptidase, serine peptidase, leader peptidase I, signal peptidase
synWH7803_1021	menaquinone biosynthesis protein MenD	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase
synWH7803_1022	naphthoate synthase	dihydroxynaphthoic acid synthetase, naphthoate synthase
synWH7803_1023	hypothetical protein	ErfK/YbiS/YcfS/YnhG family protein, protein erfK/srfK precursor, transpeptidase, conserved hypothetical protein
synWH7803_1024	phosphate ABC transporter substrate-binding protein	protein sphX, phosphate ABC transporter substrate-binding protein
synWH7803_1025	hypothetical protein	hypothetical protein with transmembrane domains
synWH7803_1026	glycogen synthase	glycogen synthase, ADP glucose-glucosyltransferase
synWH7803_1027	hypothetical protein	hypothetical protein, flagelliform silk protein, peptidase M48 Ste24p, cell division protein ZipA
synWH7803_1028	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D- alanine ligase	UDP-N-acetylmuramyl peptide synthase, cytoplasmic peptidoglycan synthetase

Table 2.2 *Genes present in the T3PKS cluster in* Synechococcus *sp. WH7803, their annotations and possible functions based on motifs found within gene sequences and BLASTp searches for proteins with high identities.*

Based on the data, the synWH7803_1003 gene was chosen for inactivation by insertional mutagenesis. Chalcone synthase is the first committed enzyme in flavonoid biosynthesis in higher plants. Flavonoids can have a range of functions including recruitment of nitrogen-fixing bacteria, chemical messengers and inhibitors against pathogens. However, there is a dearth of information about the role of chalcone synthase in bacteria.

Moreover, an exometabolomic analysis showed that *Synechococcus* sp. WH7803 produces a small molecule in high abundance only when the culture is axenic (see also deliverable 5.3). The exometabolome of the chalcone synthase mutant does not appear to have a peak for the molecule, suggesting that the gene is involved in the production of this compound (Figure 2.2).

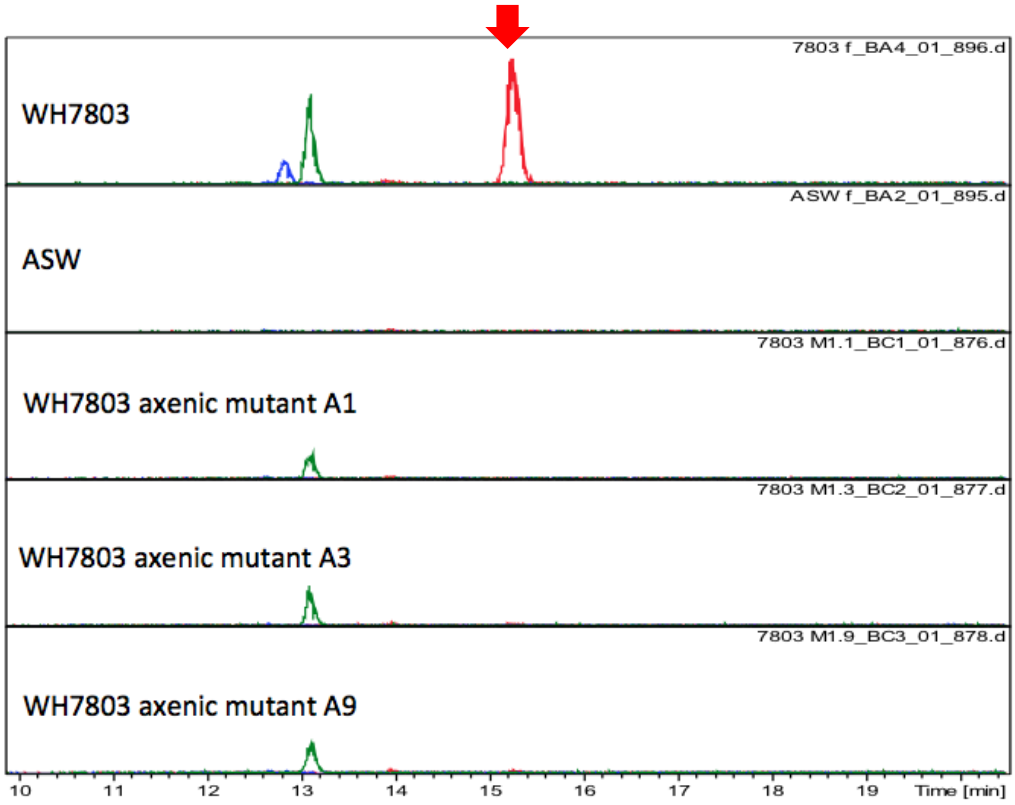

Figure 2.2 *No peak for the molecule of interest is visible in* Synechococcus *sp. WH7803 chalcone synthase mutants (WH7803 axenic mutants A1-9) compared to axenic wild type control (WH7803).*

RNA was extracted from axenic *Synechococcus* sp. WH7803 cultures in exponential and stationary phase of growth to generate cDNA. qPCR primers for the chalcone synthase (synWH7803_1003) and phosphoenol pyruvate carboxylase (synWH7803_454) genes were designed and optimized (PCR efficiencies of 99.136% and 101.397% respectively; single amplicon). When normalized to the housekeeping gene (phosphoenol pyruvate carboxylase) expression level following the Livak method, expression of chalcone synthase in stationary phase of growth was higher 1.26-fold compared to the exponential phase of growth. Such a small change does not suggest differential expression of the gene during growth.

In terms of the production of the molecule of interest, there was no significant difference between different nutrient conditions other than that caused by different cell densities (Figures 2.3 and 2.4; see also deliverable 5.3). This suggests that the molecule is produced irrespective of nutrient conditions.

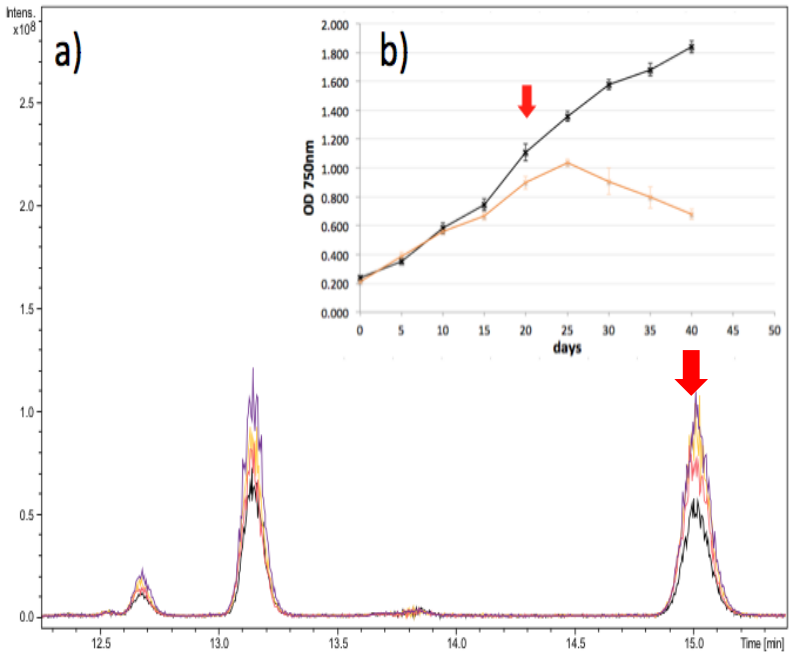

Figure 2.3 *Production of the molecule (a) in* Synechococcus *sp. WH7803 cultures grown in sulphur-limited artificial seawater (yellow, red and violet chromatograms) compared to cultures grown in standard artificial seawater (black chromatogram) on day 20 at similar (b) optical density at 750 nm. Although treatment cultures display signs of limitation, such as poor growth, metabolite production is not affected and peaks in late exponential and stationary phase of growth*.

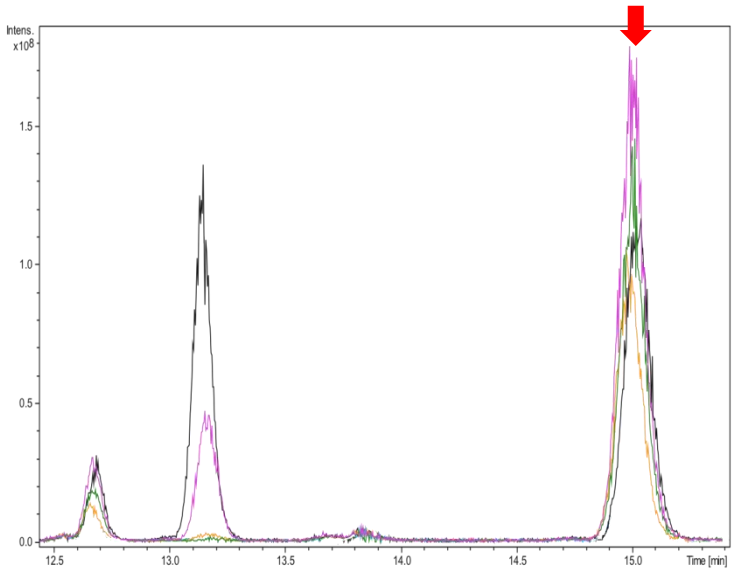

Figure 2.4 *Production of the metabolite in* Synechococcus *sp. WH7803 cultures grown in iron (pink), phosphorus (green) and sulphur (pink) limited artificial seawater compared to cultures grown in standard artificial seawater (black chromatogram) at a similar optical density, when limited cultured reached stationary phase.*

Genomics analysis for the transcriptional awakening of silent biosynthetic gene clusters. (UCC, partner 12)

Many silent biosynthetic gene clusters carry transcriptional regulators which in most cases control the production of the key secondary metabolites. Previously, UCC have performed a phylogenomics approach to one of the most abundant classes of transcriptional regulator, the LysR-type transcriptional regulator (LTTR) family (Reen et al., 2013). This revealed an abundance of LTTRs encoded in the genomes of organisms from which the greatest frequency of natural product isolation has been achieved, namely Actinobacteria, Firmicutes, and Proteobacteria. This is highly significant in light of the fact that these regulators are signal responsive, in many cases to small molecular ligands or biotic/abiotic factors. We consider the possibility that they may therefore be an important link between the addition of growth compounds and cluster activation. Therefore, a more detailed sequence based phylogenomic study is currently being undertaken to further elucidate the role of these signal responsive regulators in awakening silent biosynthetic gene clusters.

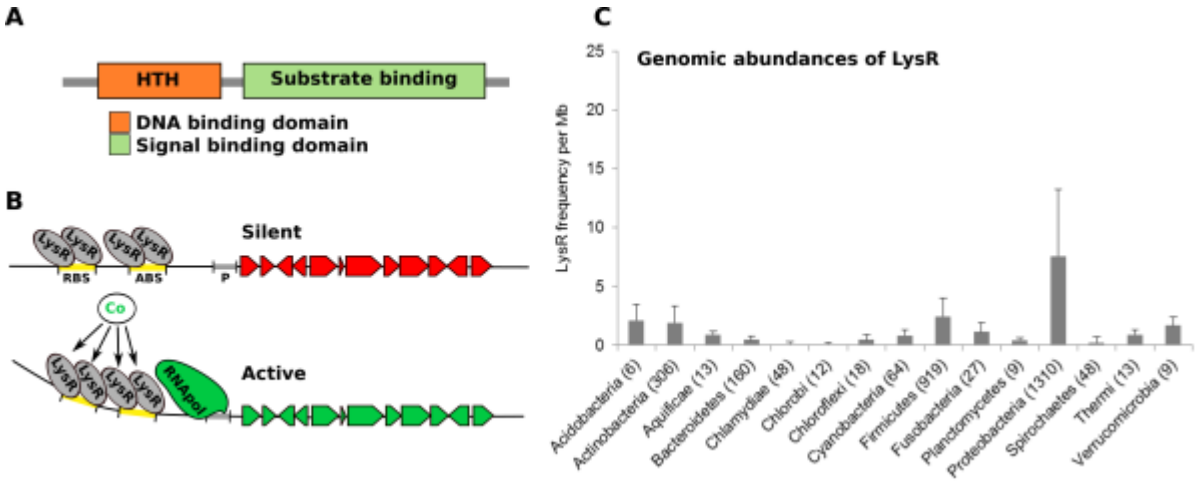

Figure 3. *LysR regulation and cryptic clusters. (A) LysR transcriptional regulators are comprised of two domains, a DNA binding HTH domain typically found at the N-terminal, and a co-inducer or signal binding domain. (B) As with several other families of transcriptional regulators, LTTRs are signal responsive, present either in an inactive or sometimes repressive state on the promoters of biosynthetic genes in the absence of an environmental cue. Upon addition of the appropriate signal or co-inducer, conformational changes occur at the promoter eliciting activation of the target gene(s). (C) The frequency of genes encoding LTTR proteins is significantly higher among the genomes of families known to include well characterized bioactive producing organisms. The specificity with which these proteins regulate gene expression and their inactivity in the absence of an activating signal make them likely candidates for control of cryptic biosynthetic gene expression.*