Investigations into the transcriptome of the toxigenic marine dinoflagellate *Alexandrium minutum*

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Contents

1 Summary

Dinoflagellates of the *A. minutum* species complex are widely distributed bloom-forming, photosynthetic protists typically producing potent neurotoxins which cause paralytic shellfish poisoning (PSP). Human intoxications usually occur via the ingestion of filter-feeding bivalves, but intoxications after consumption of predatory crabs have also been reported. The same group of toxins is produced by certain freshwater cyanobacteria, in which the gene cluster coding for the PSP toxin-producing enzymes has recently been identified. However, the identification of corresponding genes in dinoflagellates is pending, which might be due to their unusual and little understood genomes.

The aim of this thesis is to contribute to understanding gene expression and regulation in dinoflagellates by investigating the transcriptome-level gene expression in *A. minutum*. The gene expression experiments were based on an expressed sequence tag (EST) library, which was generated from pooled samples originating from different treatments to include the highest possible diversity of cDNA sequences. A set of microarray probes based on this library was used for different gene expression experiments, such as the comparison of toxic and non-toxic strains. Three stains were compared at two different times of the light phase, yielding a group of 192 genes differentially expressed between toxic and non-toxic strains at both timepoints.

Another experiment was based on the induction of a fivefold increase in toxin levels by the presence of a copepod grazer. In combination with the data obtained from the strain comparison experiment, this resulted in the identification of two sequences potentially correlated to the ability to produce the toxins.

Other treatments were designed to represent extreme values of ecologically significant parameters, such as different salinities or nutrient limitation. While the transcriptomic differences between cultures acclimatised to different salinities remained moderate and indicated a high prevalence of differences in post-transcriptional processes, the differences among growth phases and between nutrient-replete and nutrient-limited batch cultures were considerable. By using combinations of comparisons, several genes consistently up-regulated at the transition between exponential growth and stationary phase were identified, as well as 87 genes consistently associated with N- or P-limitation.

In the course of this thesis, I identified several *A. minutum* genes apparently associated with intracellular toxins, as well as patterns of gene expression indicative of culture growth status and of nutrient limitation. These data substantially add to the emerging field of dinoflagellate transcriptomics. Additionally, they provide a starting point for the potential development of new monitoring tools testing the physiological status or toxin-producing potential of *A. minutum* populations.

2 Zusammenfassung

Dinoflagellaten aus dem Artkomplex *A. minutum* sind weit verbreitete einzellige Phytoplanktonorgansimen, die häufig Massenvorkommen, sogenannte „Algenblüten“, bilden. Die meisten Stämme und Populationen produzieren starke Nervengifte, die paralytische Muschelvergiftungen (paralytic shellfish poisoning, PSP) verursachen können. Meistens werden diese Vergiftungen durch den Verzehr von Muscheln hervorgerufen, die sich zum Teil von giftbildenden Dinoflagellaten ernährt haben. Auch Vergiftungen nach dem Verzehr von räuberischen Strandkrabben sind bekannt. Die gleiche Toxingruppe wird auch von im Süßwasser lebenden Cyanobakterien gebildet, in denen vor Kurzem das Gencluster mit den Enzymen des PSP-Toxin-Biosynthesewegs ermittelt wurde. Die Identifizierung der entsprechenden Gene in Dinoflagellaten steht allerdings noch aus, was wahrscheinlich mit deren ungewöhnlichen Genomen zusammenhängt.

Ziel dieser Arbeit ist es, durch Untersuchung der Genexpression von *A. minutum* auf Transkriptomebene zum Verständnis der Genexpression und ihrer Regulation in Dinoflagellaten beizutragen. Grundlage der Genexpressions-Experimente war eine EST(expressed sequence tag)-Bank, zu deren Herstellung unter verschiedenen Bedingungen gewachsene Proben kombiniert wurden, um eine möglichst diverse Population von cDNA- Sequenzen zu erhalten. Anhand dieser Bank wurde ein Katalog von Microarray-Sonden erstellt, der dann für verschiedene Genexpressionsexperimente verwendet wurde. Eins dieser Experimente war ein Vergleich zwischen zwei toxischen und einem nicht toxischen Stamm zu zwei unterschiedlichen Zeitpunkten innerhalb der Lichtphase. Anhand dieses Versuchs wurde eine Gruppe von 192 Genen identifiziert, deren Expression zu beiden Zeitpunkten signifikant verschieden zwischen den zwei toxischen und dem nicht-toxischen Stamm war.

Bei einem anderen Experiment wurde die durch die Anwesenheit eines Copepoden hervorgerufene Induktion von fünffach erhöhten Toxinwerten innerhalb der *Alexandrium*-Zellen verwendet. In Verbindung mit den Daten aus dem Vergleich zwischen den verschiedenen Stämmen wurden dabei zwei Sequenzen identifiziert, die mit der Toxinproduktion in Verbindung zu stehen scheinen.

In weiteren Versuchen wurden extreme Werte ökologisch relevanter Parameter wie Salinität oder Nährstoffmangel verwendet. Während die Unterschiede im Transkriptom von an unterschiedliche Salinitäten akklimatisierten Kulturen nicht sehr ausgeprägt waren, aber auf Unterschiede in posttranskriptionellen Prozessen schließen ließen, wiesen Kulturen in unterschiedlichen Wachstumsphasen oder mit unterschiedlicher Nährstoffversorgung beachtliche Unterschiede auf. Durch Kombination paarweiser Vergleiche zwischen verschiedenen Wachstumsbedingungen konnten einige Gene identifiziert werden, deren erhöhte Expression mit dem Übergang von exponentieller zu stationärer Wachstumsphase assoziiert waren. Außerdem wurden 87 Gene identifiziert, deren Expression anscheinend spezifisch auf N- oder P-Mangel reagierte.

In dieser Arbeit identifiziere ich sowohl mehrere *A. minutum*-Gene, die mit dem intrazellulären Toxingehalt in Zusammenhang zu stehen scheinen, als auch charakteristische Genexpressionsmuster für bestimmte Phasen im Kulturzyklus und für Nährstofflimitation. Diese Informationen sind ein wesentlicher Beitrag zu dem aufkommenden Forschungsfeld der Transkriptomforschung an Dinoflagellaten. Außerdem sind sie ein Ansatzpunkt für die Entwicklung neuer Systeme zur Überwachung von Planktonpopulationen, die den physiologischen Zustand oder die Fähigkeit zur Toxinproduktion in *A. minutum*-Vorkommen testen könnten.

3 Introduction

3.1 Dinoflagellates – an unusual group of eukaryotes

Dinoflagellates are a highly diverse group of protists that can be found in most aquatic habitats worldwide. Exhibiting a wide diversity of lifestyles, they are often major components of both phytoplankton and micrograzer communities. Other species are benthic photo-, mixo- or heterotrophs, and some are symbionts or parasites of different uni- or multicellular organisms.

Photosynthetic dinoflagellates are among the most important phytoplankton groups in marine ecosystems. While exact data on their global biomass production are not available, they occur from the Arctic and Antarctic marine ice brine channels (Montresor *et al.*, 2003; Thomson *et al.*, 2006) to the tropics (Parab *et al.*, 2006). In some instances, dinoflagellates can regularly (Reid *et al.*, 1990) or even permanently (Landry & Kirchman, 2002) dominate the eukaryotic phytoplankton in the marine environment. Although less dominant in freshwater systems, they often contribute significantly to lake phytoplankton Many species are not purely autotrophic but will act as facultative mixotrophs when suitable prey organisms are available (Jeong *et al.*, 2005b). The “zooxanthellae” of various organisms, such as corals, marine flatworms (Lopes & Silveira, 1994), certain foraminifera (Lombard *et al.*, 2009) or radiolarians (Gast & Caron, 1996) are photosynthetic dinoflagellate symbionts.

Similar to the photosynthetic species, heterotrophic dinoflagellates contribute significantly to the heterotrophic plankton worldwide, and can even dominate marine micrograzer communities (Tillmann & Hesse, 1998; Levinsen & Nielsen, 2002; Yang *et al.*, 2004; Vargas & Martínez, 2009). Following an estimate by Dodge (1983), the proportion of heterotrophic dinoflagellates is usually reported to be about 50%. Their

feeding modes can vary considerably (reviewed in Tillmann, 2004). The prey items of the various groups range from bacteria over diatoms and phototrophic or other heterotrophic dinoflagellate species to small metazoa, such as copepod eggs or nauplii. Some other heterotrophic species are parasites of copepods (Elbrächter, 1988), fish (Landsberg *et al.*, 1994) or other organisms.

3.1.1 The dinoflagellate genome

The most unusual feature of dinoflagellates is the peculiar organisation of their genome, which is arguably one of the most bizarre eukaryotic machineries known. Both chromosome numbers and DNA content of core dinoflagellate ("dinokaryote") nuclei are unusually high; depending on the species, haploid dinoflagellate cells can contain 200 or more chromosomes (Shyam & Sarma, 1978). A survey of genome sizes of free-living species revealed DNA contents between 3.6 and 225 pg DNA per cell, which corresponds to roughly 1 to 75 times the haploid human value (LaJeunesse *et al.*, 2005). Most of this DNA is permanently condensed into a liquid crystalline state (Livolant & Bouligand, 1978), which is stabilised by high concentrations of bivalent cations (Levi-Setti *et al.*, 2008). This arrangement lacks histones and consequently nucleosomes, but the nuclei contain low abundances of basic histone-like proteins that were acquired by horizontal gene transfer from proteobacteria (Moreno Díaz de la Espina *et al.*, 2005; Chan *et al.*, 2006). The DNA itself contains high amounts of modified bases. In addition to the methylation of cytosine, which is common in other eukaryotes as well, between 12 and 68% of the dinoflagellate thymine is replaced by the unusual base hydroxymethyluracil.

While dinoflagellates contain the largest known protist transcriptomes - *A. tamarense* was estimated to have about 40,000 transcribed genes, and evidence for extensive genome duplication coupled with chromosome reorganisation has been reported (Zhang

et al., 2009; Moustafa *et al.*, 2010) - their gene content is considerably lower than would be expected based on genome scale. Their genome sizes appear uncoupled from gene numbers (Moustafa *et al.*, 2010).

The combination of these features has been interpreted as indicative of high amounts of non-coding structural DNA. This is supported by the restriction of transcriptional activity to DNA filaments extending from the main body of the chromosome into the nucleoplasm (Sigge, 1984), as well as by the results of the first available whole genome sequence survey of a dinoflagellate (McEwan *et al.*, 2008). About 90% of the examined *Heterocapsa triquetra* sequences were found to be apparently random, non-repetitive DNA (McEwan *et al.*, 2008).

Many dinokaryote genes are organised into large gene families with evidence for expression correlation among family members (Moustafa *et al.*, 2010), and highly expressed genes tend to be organised in tandem repeats (Bachvaroff & Place, 2008). Uniquely, many genes show signs of being descended from processed mRNAs re-integrated into the genome, apparently having been cycled through an mRNA intermediate once or several times during recent evolutionary history (Slamovits & Keeling, 2008).

For most or even all transcribed genes, mRNA maturation involves a trans-splicing process in which a common spliced leader (SL) sequence is added to the 5'-end of mature mRNAs (Lidie & Van Dolah, 2007; Zhang & Lin, 2009). Unlike most other core dinoflagellate peculiarities, SL trans-splicing has also been shown in earlier-diverging lineages such as the syndineans (Bachvaroff *et al.*, 2009) and perkinsids (Zhang *et al.*, 2007; Joseph *et al.*, 2010) (see below).

The unusual genomic setups, as well as some open questions regarding gene family structure and the regulation of gene expression, complicate the interpretation of genomic studies on dinoflagellates, but also reinforce the interest in such projects.

3.1.2 Evolutionary origin

Phylogenetically and systematically, dinoflagellates are one of the three main groups comprising the alveolates, the other two being the predominantly parasitic apicomplexa and the usually heterotrophic ciliates.

The earliest diverging group of the dinoflagellate lineage are the perkinsids (Gómez *et al.*, 2009; Hoppenrath & Leander, 2009), intracellular parasites of various marine (Moreira & López-García, 2003; Stelter *et al.*, 2007) and freshwater organisms (Lepère *et al.*, 2008). The perkinsids are sister to a group of three crown clades of unresolved branching order: the "Marine Alveolate Group I", the "Marine Alveolate Group II" also known as syndinean dinoflagellates and the core dinoflagellates which in this context are called "dinokaryotes" (Guillou *et al.*, 2008; Hoppenrath & Leander, 2009; Skovgaard *et al.*, 2009) (Fig.1). Both the syndineans and the Marine Alveolate Group I encompass relatively few known species, all of which appear to be parasites, and a vast diversity of organisms known only from environmental sequence surveys from a wide range of marine environments (Groisillier *et al.*, 2006; Dolven *et al.*, 2007; Guillou *et al.*, 2008; Skovgaard *et al.*, 2009).

While the physiology and indeed anatomy of most Group I and Group II alveolates remains elusive, the intracellular lifestyle of the perkinsids is reminiscent of that of derived apicomplexan parasites, and like apicomplexans they use an apical complex to access the cytoplasm of the host cell (reviewed in Leander & Keeling, 2003). By contrast the first-diverging apicomplexan clade, the colpodellids, are small free-living predatory flagellates, which use their apical complex in the myzocytosis of prey cells

(Leander *et al.*, 2003) (Fig 1). This feeding mode, which is also known from dinoflagellates (Calado *et al.*, 1998; Vogelbein *et al.*, 2002) and consists of the ingestion of prey cytoplasm through a perforation in the periplast, is thought to be ancestral within the clade containing apicomplexans, perkinsids and dinoflagellates (Leander & Keeling, 2003).

The available evidence suggests that this ancestor additionally contained a plastid that gave rise to both the dinoflagellate peridinin plastids and its highly reduced apicomplexan counterpart, the apicoplast. While dinoflagellates contain many heterotrophic lineages, both the early-diverging non-photosynthetic dinoflagellate *Crypthecodinium cohnii* (Sanchez-Puerta *et al.*, 2007) and the perkinsid *Perkinsus marinus* (Stelter *et al.*, 2007) were shown to contain plastid-derived genes and probably even cryptic plastids. The recently discovered sister taxon of apicomplexa, the Chromerida with the only known species *Chromera velia*, is photosynthetic and contains a plastid which is morphologically similar to those of peridinin-containing dinoflagellates (Moore *et al.*, 2008). Like the plastid of an even earlier branching photosynthetic alveolate which is only known by its strain designation CCMP3155, the *Chromera* plastid contains several morphological and genomic features retained in either apicomplexan or dinoflagellate plastids. The plastid genomes of the two basal lineages combine the genes found in the either apicomplexan or dinoflagellate plastid genomes (Janouškovec *et al.*, 2010).

The origin of this joint plastid remains under debate. The chromalveolate hypothesis (Cavalier-Smith, 2003) states that cryptomonads, haptophytes, stramenopiles and alveolates can be traced back to one chromalveolate ancestor which acquired a rhodopyhte as secondary endosymbiont. All non-photosynthetic members in these groups, such as the ciliates, are postulated to have subsequently lost their plastids.

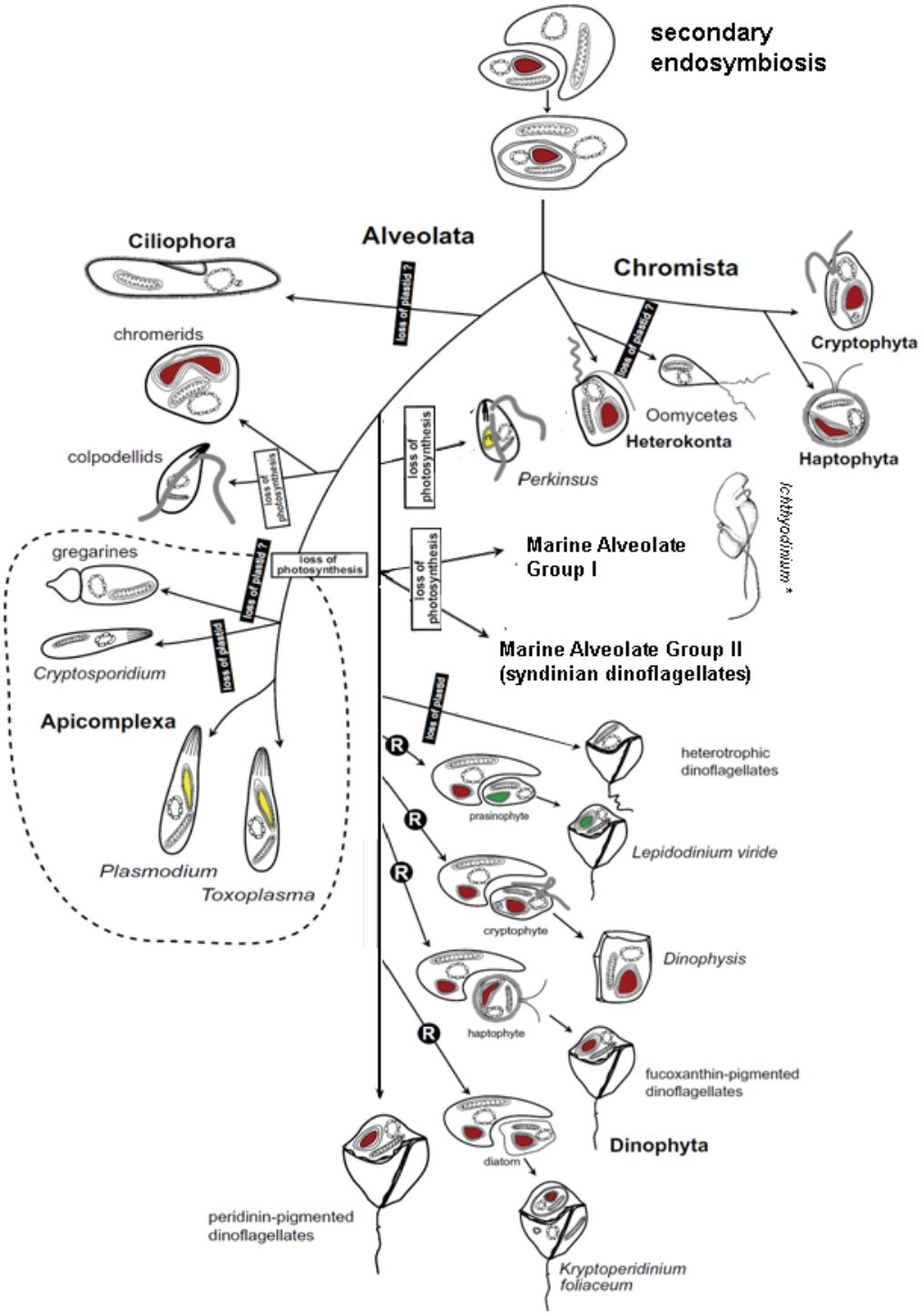


Fig. 1: Evolutionary history of Apicomplexa. Modified after Oborník *et al.* (2009). **Ichthyodinium chabelardi*, semi-schematic drawing of motile cell, Skovgaard *et al.* (2009).

While some phylogenies provided moderate support for a sister group relationship of the dinoflagellate peridinin plastids with haptophyte chloroplasts, apparently suggesting serial endosymbiosis (Bachvaroff *et al.*, 2005; Bodył *et al.*, 2009), whole-plastid genome phylogenies of *Chromera* and CCMP3155 supported a close relationship to the plastids of heterokonts. The picture is further complicated by supertree phylogenies

indicating that chromalveolates might not be monophyletic, as haptophytes and cryptomonads might be more closely related to plants than to alveolates and heterokonts, while the primarily non-photosynthetic Rhizaria cluster close to stramenopiles (Burki *et al.*, 2008; Lane & Archibald, 2008; Reeb *et al.*, 2009).

From a paleontological point of view, the oldest evidence of chemicals usually regarded as biomarkers for dinoflagellates was found to be associated with early Cambrian acritarchs (~520 million years ago). As acritarchs is a combined group for organic-walled microfossils that cannot be definitely allocated to known organisms, the presence of an early dinoflagellate ancestor in this group seems plausible. However, small amounts of the biomarkers in question have also been found in extant diatoms and prymnesiophytes (Volkman *et al.*, 1990; Volkman *et al.*, 1993), so their occurrence in fossil organisms not related to dinoflagellates cannot be ruled out.

The oldest known cysts that can be unambiguously allocated to dinoflagellates occur in mid-Triassic deposits at about 240 million years before present (Fensome *et al.*, 1996; John *et al.*, 2003), at the end of a 130 million year “phytoplankton blackout” lasting from the Devonian/Carboniferous boundary until the late Triassic. This gap in the phytoplankton record was probably caused by processes linked to the assembly of all continents into the single land mass Pangaea. The extensive loss of shelf seas, differences in global nutrient flows and other oceanographic and climatic effects are

likely to have caused a substantial reduction in phytoplankton primary production leading to the extinction of most of the older acritarch lineages (Riegel, 2008).

After the break-up of Pangaea and the return of shelf seas, the Mesozoic was characterised by the radiation of dinoflagellates, coccolithophorids and diatoms. The ongoing success of these "red lineage" groups has been linked to the different trace element requirements of red and green plastids. The "red lineage" is supposed to be slightly better adapted to the low availability of Fe and Mn in highly oxidised oceans, which may have helped them to attain dominance during the Mesozoic phytoplankton radiation (Quigg *et al.*, 2003; Falkowski *et al.*, 2004).

Tracing the development of sea levels and shelf sea areas, the diversity of cyst-producing dinoflagellates rapidly increased during the Upper Triassic (Sluijs *et al.*, 2005; Palliani & Buratti, 2006), reached maxima in the Jurassic (MacRae *et al.*, 1996), the Cretaceous and the early Eocene, after which cyst diversity started a long-term decline to the present values. This might be attributed to the relatively high wind speeds, thermohaline circulation and increased upper- ocean turbulence in the Quaternary, which lead to the present predominance of diatoms in oceanic systems (MacRae *et al.*, 1996; Sluijs *et al.*, 2005). However, dinoflagellates still remain a major part of planktonic systems, and regularly outcompete diatoms under low turbulence, stratified water conditions or under silicate limitation.

3.2 Phytoplankton Blooms

Many planktonic organisms can form mass occurrences in the water column. When the cell densities reach values considerably higher than their general background distribution, they are called blooms (see Smayda, 1997). Blooms can be almost monospecific, others are formed by a combination of species (Garcés *et al.*, 2005; Popovich *et al.*, 2008) . Many prominent blooms can be traced back to high nutrient

loads (Hallegraeff, 1993; Hwang & Lu, 2000; Yentsch *et al.*, 2008), but they can occur whenever a species is able to outgrow its competitors while partially reducing grazer pressure (Irigoien *et al.*, 2005). Some dinoflagellates bloom in unexpected places, such as below the Baltic winter ice cover (Spilling, 2007). Others are more benthic than planktonic, like the palytoxin-producing *Ostreopsis ovata* (Mangialajo *et al.*, 2008).

As the base of the marine food chains many phytoplankton blooms are beneficial for ocean productivity and hence benefit the fishing and shellfish industries (Hallegraeff, 1993). Other blooms however, most often of dinoflagellates, can significantly disrupt ecosystems (Wear & Gardner, 2001), harm commercially important species (Tang & Gobler, 2009) or produce substances toxic to humans (Van Dolah, 2000). Coastal eutrophication may in some cases lead to an increase in such harmful bloom events. Under nitrate or phosphate limited conditions, many diatom species can outcompete dinoflagellates due to generally faster nutrient uptake capabilities and subsequently higher growth rates. When the concentrations of these macronutrients are increased, diatom growth is likely to be limited by silicate, increasing the likelihood of dinoflagellate dominance (Smayda, 1990; Smayda, 1997). While part of the apparent global increase of harmful algal blooms (HABs) might be due to increased scientific awareness and increased commercial activities in coastal areas, eutrophication, climatic effects and the introduction of new harmful species by ship ballast water or import of shellfish stocks for aquaculture are likely to alter the current patterns of bloom occurrence (Hallegraeff, 1993; Scholin *et al.*, 1995; Nagai *et al.*, 2007; Leung & Dudgeon, 2008).

3.3 The genus Alexandrium

The gonyaulacoid dinoflagellate genus *Alexandrium* is reported to contain 28 species (Gómez, 2005), 11 of which are recognised as harmful, according to the IOC

Taxonomic Reference List of Toxic Algae (Moestrup, 2004). However, the determination of biologically meaningful species' boundaries is still in progress, and some of the listed species are probably conspecific or contain groups of several cryptic species (see below). *Alexandrium* species are marine, occur predominantly in coastal habitats and contain chloroplasts. They can apparently all survive as purely photosynthetic organisms in culture, but at least some members are known to be facultatively mixotrophic (e.g. Jeong *et al.*, 2005b).

According to molecular clock calculations by John *et al.* (2003), the genus *Alexandrium* probably originated around 77 million years ago, in the late Cretaceous, with confidence limits extending up to 119 million years ago (mid-Cretaceous). Each of these dates would coincide with one of the three all-time peaks in dinoflagellate cyst diversity, two of which are reported for mid- and late Cretaceous assemblages (Sluijs *et al.*, 2005).

The *A. tamarense* species complex, the most well-studied group within the genus, most likely originated during the early Neogene (23 million years ago, with confidence limits until the late Palaeogene, 45 million years ago). John *et al.* (2003) suggest that its radiation might have be determined by plate movements, oceanographic changes and climatic fluctuations, leading to apparent geographic clades in small- and large-subunit rDNA-based phylogenies (Scholin *et al.*, 1995; John *et al.*, 2003). Group affiliation was highly correlated with toxicity; all tested members of three of the groups were nontoxic, whereas every tested isolate of the remaining two groups was toxic (John *et al.*, 2003; Lilly *et al.*, 2007).

3.3.1 The *Alexandrium minutum* species complex

A similar pattern was discovered in *A. minutum*, the type species of *Alexandrium*. A review of the morphological and phylogenetic diversity among several closely related species revealed that *A. lusitanicum* and *A. angustitabulatum* could not be distinguished

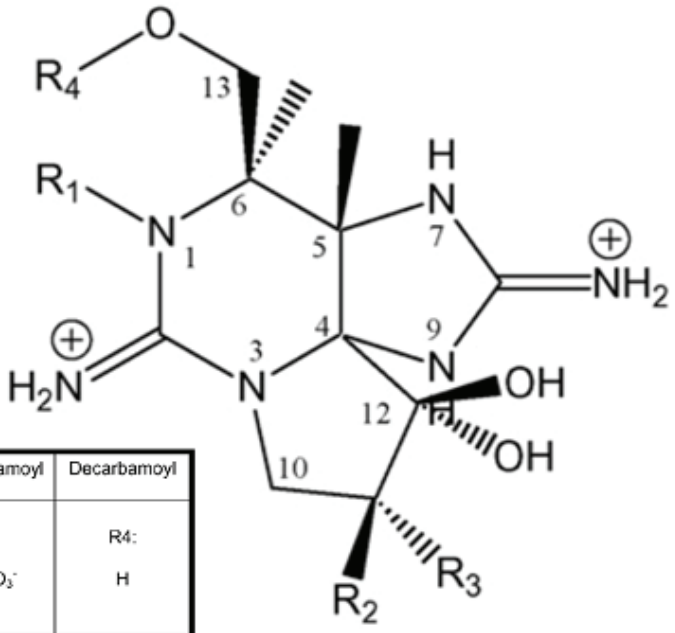
from *A. minutum*, whereas *A. tamutum*, *A. insuetum* and *A. andersonii* were shown to be morphologically and phylogenetically distinct (Montresor *et al.*, 2004; Lilly *et al.*, 2005). The *A. minutum* species complex was shown to consist of two major clades: a "Pacific clade" containing strains from New Zealand, Taiwan, Malaysia and Vietnam (Lilly *et al.*, 2005; Lim *et al.*, 2007), and a "Global clade" consisting of strains from Europe, South Africa and Australia. Within each clade, strains clustered according to origin, although the diversity within the Global clade could only be revealed by microsatellite analysis (McCauley *et al.*, 2009).

The ecological niche of *A. minutum* was extensively investigated along the Catalan coast (Bravo *et al.*, 2008). In this region, *A. minutum* cyst beds are present in all sampled harbours and at many beaches. The most important factor in determining the capacity for blooms was the available nitrogen source: *A. minutum* was dominant at high NO_3-levels, whereas additional high NH_4-levels favoured the co-occurring "*A. catenella*" populations.

3.4 Paralytic Shellfish Poisoning (PSP) toxins

Of the 11 toxic *Alexandrium* species listed by the Moestrup *et al.* (2004), 8 are reported to be associated with PSP toxin production. These tetrahydropurine neurotoxins form a group of more than 20 known analogues, the most intensively studied of which are probably saxitoxin and the gonyautoxins (GTX) (reviewed in Krock *et al.*, 2007). They act by selectively inhibiting sodium channels, which leads to the potentially severe human intoxication syndrome called PSP (Kao & Walker, 1982). Typical symptoms are paraesthesia, followed by distinct muscular weakness, loss of motor coordination, and paralysis. In severe cases or without adequate treatment, victims may die from respiratory failure (Lehane, 2000; García *et al.*, 2004). Most intoxications result from consumption of filter-feeding bivalves, which tend to accumulate the toxins after

feeding on PSP producers. However, toxins can also be propagated through the food chain, and intoxication through the ingestion of predatory invertebrates such as crabs is possible. Due to widespread resistance of marine invertebrates against PSP toxins, in both cases the resulting concentrations can be extremely high (Llewellyn *et al.*, 2002; Anderson *et al.*, 2005). Apart from the detrimental effects on humans, PSP-induced deaths of marine animals such as whales or seabirds are also occasionally reported (Geraci *et al.*, 1989; Trainer & Baden, 1999; Shumway *et al.*, 2003; Doucette *et al.*, 2006).



			Carbamoyl	N-Sulfocarbamoyl	Decarbamoyl
R1	R2	R3	R4: $CONH_2$	R4: $CONHSO_3^-$	R4: H
H	H	H	STX	B1	dcSTX
OH	H	H	NEO	B2	dcNEO
OH	H	OSO_3^-	GTX1	C3	dcGTX1
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 2: Naturally occurring PSP toxins, including carbamoyl, N-sulfocarbamoyl and decarbamoyl derivatives. Saxitoxin = STX; neosaxitoxin = NEO; gonyautoxins 1,2,3,4 = GTX 1,2,3,4; B1 (=GTX5); B2 (=GTX6); dc = decarbamoyl toxins. Modified after Krock *et al*. (2007).

In order to prevent PSP outbreaks, extensive monitoring programmes are indispensable. While these programmes ensure consumer safety, monitoring alone can do little to mitigate the economic effects of harmful algae occurrences. The necessary closure of shellfish harvesting areas whenever exposure exceeds safe limits, which is often followed by depression of consumer demand in the affected regions as well as with negative impacts on tourism industries, regularly leads to considerable economic losses (Lehane, 2000). HAB forecasting projects aim to decrease these impacts (e.g. NOAA Harmful Algal Bloom Forecast System, *http://tidesandcurrents.noaa.gov/hab/*; CSCOR: *http://www.cop.noaa.gov/news/fs/ne_hab_2009.html*) by providing seasonal and short-term forecasts based on data from oceanographic measurements, weather forecasts, cyst beds and monitoring results.

3.4.1 Toxin variability in *A. minutum*

Toxin content in *A. minutum* can vary substantially, and both toxic and non-toxic strains co-exist in both the global and the Pacific clade (Lilly *et al.*, 2005; McCauley *et al.*, 2009). Along the Irish coast, toxic and non-toxic *A. minutum* populations are reported to occur in different regions (Touzet *et al.*, 2007), but other populations contain otherwise indistinguishable toxic and non-toxic strains at the same time (McCauley *et al.*, 2009).

While the toxin profile of most *A. minutum* strains is dominated by gonyautoxins, even strains with identical LSU rDNA sequences can contain markedly different profiles (Hansen *et al.*, 2003). Unlike strains from other locations, those of New Zealand isolates were reported to contain substantial amounts of saxitoxin and neosaxitoxin (Chang *et al.*, 1997; MacKenzie & Berkett, 1997). A toxic Taiwanese strain and several other strains from the same region had very typical *A. minutum* toxin profiles dominated by GTX4 and GTX1 (Hwang & Lu, 2000; Chou *et al.*, 2004).

3.4.2 Biosynthesis of PSP toxins

Apart from *Alexandrium* species, PSP toxins are also produced by the dinoflagellates *Gymnodinium catenatum* and *Pyrodinium bahamense* var. *compressum*, as well as by various freshwater cyanobacteria (Moustafa *et al.*, 2009; Stucken *et al.*, 2010). This unusual distribution lead to the hypothesis that PSP toxin production in dinoflagellates might be due to symbiotic bacteria (e.g. Gallacher *et al.*, 1997; reviewed in Töbe, 2003). However, several studies demonstrated that removal of bacteria from *Alexandrium* cultures did not eliminate toxin production (Lu *et al.*, 2000; Hold *et al.*, 2001; Uribe & Espejo, 2003). The situation is complicated by bacterial production of non-PSP compounds which can mimic PSP toxins in HPLC analysis (Baker *et al.*, 2003; Prol *et al.*, 2009).

PSP toxin profiles are usually strain specific but stable within one strain (Cembella & John, 2006). Profile inheritance follows a Mendelian segregation pattern (Ishida *et al.*, 1998) indicative of the involvement of nuclear-coded enzymes. Several such interconverting enzymes have been characterised on the protein level. Based on crude cell extracts, Oshima (1995) characterised an *A. tamarense* oxidase transforming GTX 3 to GTX 4 and a *G. catenatum* N-sulfotransferase forming C2 from GTX3. The same sulfotransferase activity was observed in another toxic and a non-toxic *G. catenatum* isolate. In a later study, a similar enzyme was found in a crude enzyme extract of *A. tamarense* (Wang *et al.*, 2007). Sako *et al.* (2001) purified and characterised another PSP toxin sulfotransferase from *G. catenatum*. An O-sulfotransferase specifically transferring a sulfonyl group to O-22 of 11-hydroxy-STX to produce GTX 3 was purified and characterized by Yoshida *et al.* (2002). Based on the very specific activity of this enzyme, which specifically transfers a sulfate residue to a carbamoyl group present only in some saxitoxin analogues, the authors suggested that STX might be the

only toxin directly synthesized. Then all other PSP toxins might be derived by a fixed set of interconversions (Fig. 3).

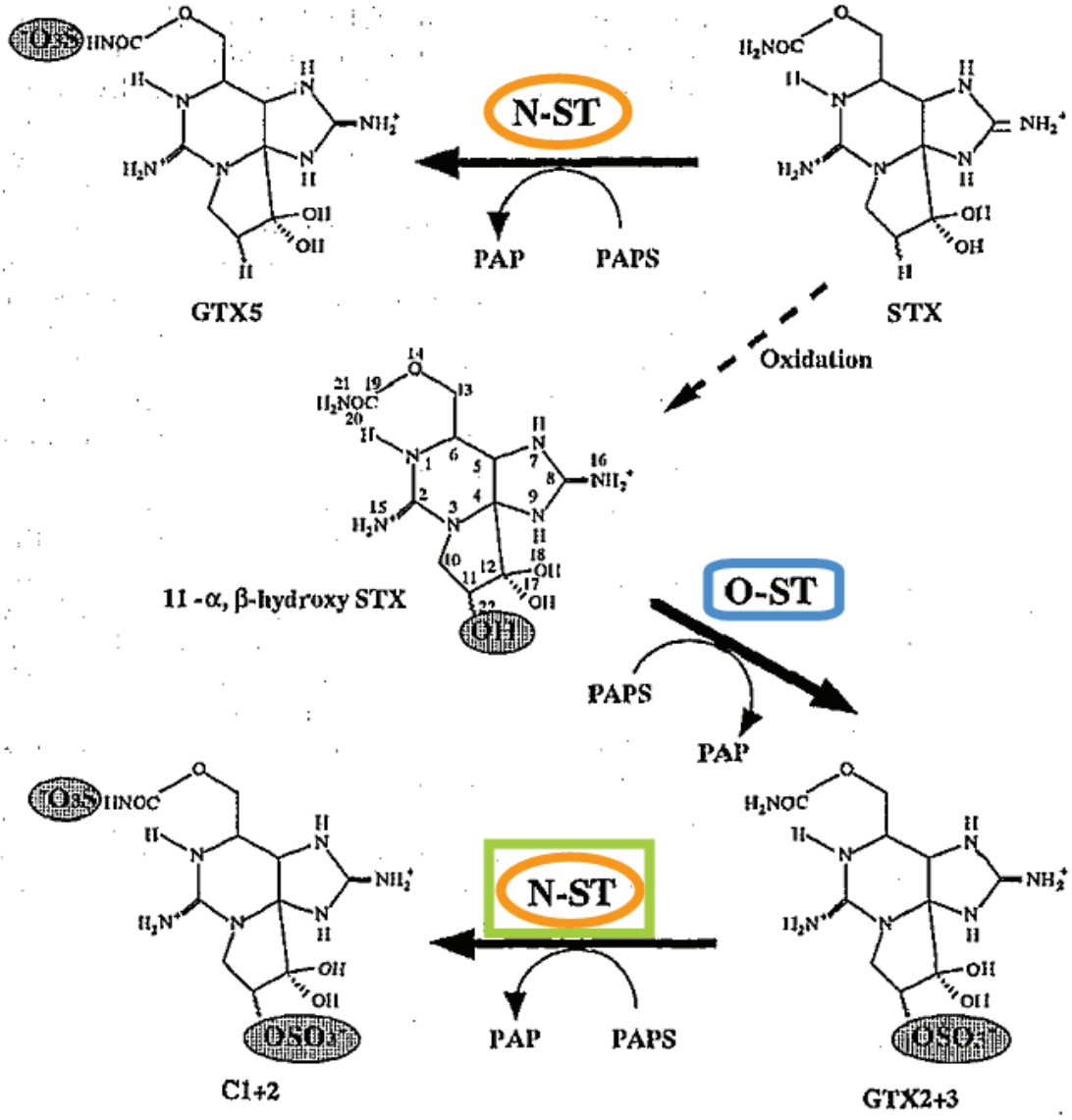


Fig. 3: Transformation of PSP toxins after Yoshida *et al.* (2002). Enzymes characterized by Sako *et al.* (2001) (orange ovals), Yoshida *et al.* (2002) (blue box) and (Wang *et al.*, 2007) (green rectangle).

While the data mentioned above combine to a comprehensive hypothesis of the biochemical processes determining the dinoflagellate PSP toxin profiles, the PSP toxin synthesis pathway is largely assumed to be identical to the cyanobacterial one, which is usually explained by horizontal gene transfer (Shimizu, 1996; Kellmann *et al.*, 2008a). The cyanobacterial pathway and the corresponding gene cluster have recently been identified in *Cylindrospermopsis raciborskii* T3 (Kellmann & Neilan, 2007; Kellmann *et al.*, 2008b). This cluster contains >35 kb of sequence coding for 26 proteins; PSP toxin synthesis is reported to be initiated with arginine, S-adenosylmethionine, and acetate by a new type of polyketide synthase. Similar clusters were found in other PSP toxin-producing cyanobacterial species (Mihali *et al.*, 2009), and further studies suggested that at least parts of this cluster have been frequently laterally transferred between different cyanobacterial genera (Kellmann *et al.*, 2008a). Conclusive evidence of the homology of this gene cluster to dinoflagellate sequences is pending.

3.5 Allelochemical properties of Alexandrium

In addition to the rather well characterised toxins, *Alexandrium* species produce less well understood extracellular compounds which cause negative effects against other protists and which are only now in the process of being chemically characterised (Ma *et al.*, 2009). These allelochemicals apparently act by attacking the outer cell membrane of target species, leading to cell deformation and eventually lysis (Tillmann & John, 2002). Susceptible organisms include potential grazers, like the predatory ciliate *Rimostrombidium caudatum* and various heterotrophic dinoflagellates, as well as autotrophic competitors (see Tillmann *et al.*, 2008). Similar allelochemicals have been found in other dinoflagellate species, such as *Peridinium aciculiferum* or *Fragilidium subglobosum* (Rengefors & Legrand, 2007; Tillmann *et al.*, 2008). In the case of the karlotoxin produced by *Karlodinium veneficum*, the substances apparently targeted at

protistan grazers can also exhibit cyto- and ichthytoxic properties and are thus officially recognised as toxins (Adolf *et al.*, 2007).

Independently of both species-specific and strain-specific PSP toxin content, allelochemical activity in *Alexandrium* is found in all species tested but can differ substantially between strains even within one *A. minutum* population. As activity between strains of different species varied widely among donor/target combinations, different *Alexandrium* species might produce different compositions of allelochemically active compounds (Tillmann & John, 2002; Tillmann *et al.*, 2008; Tillmann *et al.*, 2009).

While allelochemical interactions are usually discussed in the light of population-scale processes such as competition and bloom formation (reviewed in Legrand *et al.*, 2003; Granéli & Hansen, 2006), a recent simulation study suggests that their primary and possibly evolutionarily favoured functions might be predator-prey or host-parasite interactions mediated by a "chemical envelope" of allelochemical substances around each individual algal cell (Jonsson *et al.*, 2009). Independent of cell density, these envelopes might act as grazer or parasite deterrent, lyse prey cells or cause leakage of host cells during "casual parasitism". In this model, any bloom-promoting function of allelopathic interactions would be a side effect which can only set in after bloom formation of the producing species is well under way (Jonsson *et al.*, 2009). This might explain the low incidence of non-lytic clones within an *A. tamarense* population identified by Tillmann *et al.* (2009). The same authors also found a high intra-strain variability in lytic potency, indicating a low evolutionary pressure on the quantitative aspect of lytic activity apparently resulting from an intermittantly reduced or inconsistent evolutionary pressure for high allelochemical activity within the "chemical cloud" of local *A. tamarense* aggregations.

Once cell densities in a developing *Alexandrium* bloom are high enough to produce a "chemical cloud", allelochemical interactions can effectively change the plankton community. When natural plankton samples were exposed to cell-free filtrates of an *A. tamarense* culture at bloom concentration (corresponding to 34,000 cells l^{-1}), overall growth rate was considerably reduced. Especially ciliates, nanoflagellates and the autotrophic dinoflagellate *Scrippsiella trochoidea* were inhibited, whereas the cell numbers of small dinoflagellates were positively affected and bacterial numbers increased considerably (Fistarol *et al.*, 2004). This might imply a direct or indirect enhancement of putative prey organisms for the facultative mixotroph *Alexandrium* (Jeong *et al.*, 2005b) in addition to the repression of grazers and competitors. Even if these effects in dense *Alexandrium* populations are unlikely to convert direct evolutionary advantages to specific clones due to the probably rapid dissipation of related cells (Jonsson *et al.*, 2009), they are still a major ecological factor promoting the formation of even denser blooms as well as their maintenance.

3.6 Outline of the thesis

The aim of this thesis was to obtain transcriptome-level information on processes associated with PSP toxin production or with bloom-relevant processes such as growth, allelochemical activity, nutrient limitation, or stress, for the dinoflagellate *Alexandrium minutum*.

3.6.1 Rationale

A. minutum is a typical toxin-producing harmful algal bloom species (see Chang *et al.*, 1997; Hwang & Tsai, 1999; Bravo *et al.*, 2008). Although it does not achieve the high toxin cell quotas of the *A. tamarense* species group, the availability of toxic and non-toxic strains from the same population, which are similar enough to contain identical LSU and SSU rDNA sequences, recommend this species as a model for the study of

PSP toxin production. Due to its fast growth rate of up to one division per day, and to the availability of numerous published studies on its physiological reaction to a wide range of growth conditions, it also is a convenient model to investigate other bloom-related processes.

While many studies have investigated physiological phenomena associated with PSP toxin content and toxin production in different *Alexandrium* species (Flynn *et al.*, 1994; Taroncher-Oldenburg *et al.*, 1999; Hwang & Lu, 2000), only fragmentary data exist on the associated biochemical pathway in dinoflagellates. A hypothetical pathway based on labelled precursor studies carried out mainly in cyanobacteria (Shimizu *et al.*, 1984; Shimizu, 1996) was shown to be largely accurate for prokaryotic PSP toxin producers (Kellmann & Neilan, 2007; Kellmann *et al.*, 2008a), but the corresponding genes and enzymes in dinoflagellates remain elusive. Only a pathway for the conversion of saxitoxin into the other PSP toxins has been identified in dinoflagellates to date (Sako *et al.*, 2001; Yoshida *et al.*, 2002; Wang *et al.*, 2007).

Both the elucidation of the dinoflagellate PSP toxin pathway and the development of screening methods to assess the physiological status of natural populations of harmful species are bound to be greatly facilitated by the availability of sizeable molecular datasets for the species in question. The current method of choice for the production of such datasets is large-scale sequencing of the genome or the transcriptome of the study organism, which enables both the detection of conserved genes of known function and the discovery of new genes which can than be characterised based on sequence properties and expression analysis.

3.6.2 Characterisation of the *A. minutum* transcriptome

In non-model organisms without available genome sequences, projects aimed at the investigating of gene expression are usually based on a transcriptomic databases (e.g.

Okamoto & Hastings, 2003; Lidie *et al.*, 2005; Van Dolah *et al.*, 2007). Genome sequencing in dinoflagellates has so far proven extremely challenging, which is presumably due to their enormous size and unusual structure. While some sequencing initiatives are under way, no sequenced genome is available to date. However, given the large amounts of presumably "gene-empty" structural DNA (McEwan *et al.*, 2008) and a high prevalence of pseudogenes (Bachvaroff & Place, 2008), even a closed genome might be less useful for the investigation of actively transcribed genes than a carefully composed transcriptomic library.

Manuscript 1 describes the construction and characterisation of an *A. minutum* EST library, the first genomic database for its species. Similar libraries are published for other *Alexandrium* species (Hackett *et al.*, 2005; Jaeckisch *et al.*, 2008; Uribe *et al.*, 2008; Toulza *et al.*, 2010), but none of these are based on a comparable number of physiological conditions. As the investigation of gene expression is greatly facilitated by the availability of mRNA sequence data, the highly inclusive EST library introduced in Manuscript 1 is a very important resource for the study of transcriptomic responses of *Alexandrium* to different physiological and environmental conditions. These data were used to develop a microarray probe set for further gene expression analyses in *A. minutum*, the first sets of which are detailed below.

3.6.3 Search for sequences associated with PSP toxicity

The ability to synthesize the tetrahydropurine neurotoxins which cause paralytic shellfish poisoning is not unique to dinoflagellates, but also found in freshwater cyanobacteria (e.g. Stucken *et al.*, 2010). This led to the hypothesis that the dinoflagellate ability to produce these toxins might be a result of inter-kingdom lateral gene transfer (Shimizu, 1996; Kellmann *et al.*, 2008a). Given the high prevalence of mixotrophy in dinoflagellates (Jeong *et al.*, 2005b) in combination with their high

capacity to include new sequences such as organellar genes (Bachvaroff *et al.*, 2004; Nash *et al.*, 2007) or reverse-transcribed processed mRNAs (Slamovits & Keeling, 2008) into their genomes, the implied independent incorporation events into the genomes of several non-related dinoflagellate clades are not inconceivable. Following the recent elucidation of the PSP toxin gene cluster in the cyanobacterium *Cylindrospermopsis raciborskii* (Kellmann *et al.*, 2008b), the identification of dinoflagellate homologues based on sequence similarity should be trivial.

In the second part of Manuscript 1, these sequence similarity-dependent methods are combined with a microarray-based screening approach aimed to identify reproducible gene expression differences between toxin-producing and non-toxin-producing strains. The latter approach primarily measures differences in gene-specific mRNA abundances between pairs of strains, without discriminating between toxicity-related differences and toxin-unrelated variability. A combination of several such comparisons resulted in a manageable list of candidate genes that can be described as potentially associated with PSP toxin-related processes.

Several physiological or ecological functions of PSP toxins have been suggested, among others nitrogen storage (reviewed in Cembella, 1998), activity as pheromones (Wyatt & Jenkinson, 1997), and influences on the dinoflagellate-associated bacteria in the phycosphere (Jasti *et al.*, 2005). The hypothesis that PSP toxicity in dinoflagellates is linked to deterring effects on copepod grazers (Turner & Tester, 1997) was supported by an increase in *A. minutum* PSP toxin production in response to waterborne signals from certain copepod predators, which was correlated to an increased resistance of *A. minutum* to grazing by this copepod (Selander *et al.*, 2006). Bergkvist *et al.* (2008) found this effect to be proportional to the intrinsic toxicity of the different *A. minutum*

strains. Depending on the strain and the copepod tested, they report responses of up to 20 times higher toxin concentrations.

This induction is a highly convenient model to investigate the *A. minutum* genes associated with toxicity levels. A comparison of induced versus non-induced cultures will yield relative expression levels correlated with different levels of PSP toxicity within the same strain. The presence of predator cues is likely to elicit less severe physiological responses, and consequently a much more narrowly targeted gene expression signal, than other treatments known to influence cellular toxin content, such as nutrient limitation or suboptimal growth conditions. Manuscript 2 describes such a comparison between induced and non-induced cultures and relates the identified differences in gene expression to the differences between toxic and non-toxic strains found in Manuscript 1. Significantly, two of the sequences identified as higher expressed in toxic than in non-toxic *A. minutum* in Manuscript 1 were also found to be up-regulated in association with the induction of higher toxin content.

3.6.4 Transcriptome-level processes associated with growth, nutrient status and stress response

A. minutum is a eurythermal and euryhaline species which can bloom at a wide variety of temperatures and salinities (Vila *et al.*, 2005; Bravo *et al.*, 2008) and can achieve high growth rates at a wide range of light conditions (Hwang & Lu, 2000). The outbreak of *A. minutum* blooms is reported to be mainly determined by nitrate availability, irrespective of temperature or time of the year. The densest blooms usually occur at a combination of very high nitrate levels and elevated phosphate values (Bravo *et al.*, 2008), implying that under natural conditions *A. minutum* growth is mostly nutrient-limited. As nutrient input is often associated with freshwater runoff, elevated nutrient levels tend to coincide with changes in salinity. Optimal bloom salinities at the

Mediterranean coast were reported to be around 34-36 on the practical salinity scale (Bravo *et al.*, 2008).

Manuscripts 3 and 4 deal with the physiological and transcriptome-level responses of *A. minutum* to reduced and elevated salinities (Manuscript 3), in exponential or stationary growth phases and under nutrient starvation (Manuscript 4). In both studies physiological processes related to bloom development or maintenance, such as growth, allelochemical activity, PSP toxin contents and intracellular nutrient levels or acclimatisation to new salinity levels are combined with gene expression data comparing the respective treatments with exponentially growing control cultures.

In addition to contributing a new level of data to the understanding of *A. minutum* physiology, I examine the extent of differential mRNA abundances during these treatments, which adds new data to the ongoing discussion on the distinctive characteristics of dinoflagellate gene expression.

4 Manuscripts

4.1 List of Manuscripts

Manuscript 1: Comparative Gene Expression in toxic versus non-toxic Strains of the marine Dinoflagellate *Alexandrium minutum*. (I. Yang, U. John, S. Beszteri, G. Glöckner, B. Krock, Alexander Goesmann, A. Cembella). *BMC Genomics* 11(1): 248

Manuscript 2: Grazer induced toxin formation in dinoflagellates: A transcriptomic model study. (I. Yang, E. Selander, H. Pavia, U. John). *European Journal of Phycology*, accepted with minor revisions

Manuscript 3: Physiological and Gene Expression Responses to Salinity Stress in *Alexandrium minutum* (I. Yang, S. Beszteri, U. Tillmann, A. Cembella, U. John)

Submitted for the *Proceedings of the 13th International Conference on Harmful Algae*

Manuscript 4: Growth- and limitation-related gene expression in *Alexandrium minutum* (I. Yang, S. Beszteri, U. Tillmann, A. Cembella, U. John). To be submitted.

4.2 Declaration of contributions

Manuscript 1: The experiments were planned together with U. John, and the treatments included in the EST library were carried out together with S. Bestzeri. The in-house part of the bioinformatics analyses was carried out by the candidate, who also planned and carried out the strain comparison experiment and drafted the manuscript.

Manuscript 2: The experiment was planned together with E. Selander. The candidate carried out the genomic part of the experiment and the corresponding analysis and drafted substantial parts of the manuscript.

Manuscript 3: The experiment was planned together with U. John. The candidate was in charge of the physiological experiments, carried out the genomic part, analysed the data and drafted the manuscript.

Manuscript 4: The experiment was planned together with U. John and U. Tillmann. The candidate was in charge of the physiological experiments, carried out the genomic part, analysed the data and drafted the manuscript.

5 Manuscript 1

Comparative Gene Expression in toxic versus non-toxic Strains of the marine Dinoflagellate Alexandrium minutum

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Abstract

Background

The dinoflagellate Alexandrium minutum typically produces paralytic shellfish poisoning (PSP) toxins, which are known only from cyanobacteria and dinoflagellates. While a PSP toxin gene cluster has recently been characterized in cyanobacteria, the genetic background of PSP toxin production in dinoflagellates remains elusive.

Results

We constructed and analysed an expressed sequence tag (EST) library of *A. minutum*, which contained 15,703 read sequences yielding a total of 4,320 unique expressed clusters. Of these clusters, 72% combined the forward-and reverse reads of at least one bacterial clone. This sequence resource was then used to construct an oligonucleotide microarray. We analysed the expression of all clusters in three different strains. While the cyanobacterial PSP toxin genes were not found among the *A. minutum* sequences, 192 genes were differentially expressed between toxic and non-toxic strains.

Conclusions

Based on this study and on the lack of identified PSP synthesis genes in the two existent *Alexandrium tamarense* EST libraries, we propose that the PSP toxin genes in dinoflagellates might be more different from their cyanobacterial counterparts than would be expected in the case of a recent gene transfer. As a starting point to identify possible PSP toxin-associated genes in dinoflagellates without relying on *a priori* sequence information, the sequences only present in mRNA pools of the toxic strain can be seen as putative candidates involved in toxin synthesis and regulation, or acclimation to intracellular PSP toxins.

Background

Alexandrium minutum is a bloom-forming toxic dinoflagellate typically capable of producing paralytic shellfish poisoning (PSP) toxins. Occurring from northern Europe and the Mediterranean to Asia, Australia and New Zealand, *A. minutum* poses a widespread threat to seafood production and consumer health (for a review of biogeography and species boundaries see Lilly *et al.*, 2005). PSP is potentially fatal to both humans (García *et al.*, 2004) and marine fauna, particularly vertebrates (Geraci *et al.*, 1989; Trainer & Baden, 1999); human intoxications typically occur after ingestion of suspension-feeding bivalve molluscs.

PSP toxins are tetrahydropurine neurotoxins, which specifically bind to voltage-gated sodium channels of nerves and muscle cells. The most well known representative, saxitoxin, is probably also the parent compound (Sako *et al.*, 2001), of which more than 20 naturally occurring derivatives are known, including the gonyautoxins produced by *A. minutum* (reviewed in Krock *et al.*, 2007). Several physiological or ecological functions of PSP toxins have been considered (reviewed in Cembella, 1998), among others chemical defence (Cembella, 1998), nitrogen storage (Cembella, 1998), activity as pheromones (Wyatt & Jenkinson, 1997), and influences on the dinoflagellate-associated bacteria in the phycosphere (Jasti *et al.*, 2005). Several experimental studies link PSP toxicity in dinoflagellates to deterring effects on some species of copepod grazers (Turner & Tester, 1997; Selander *et al.*, 2006), which can lead to a redirection of grazing pressure onto non-toxic phytoplankton species competing for the same nutrient resources (Guisande *et al.*, 2002), but the interactions and deterrence responses are highly species-specific and not universal.

In addition to these effects, but independent of PSP toxin content, several *Alexandrium* species including *A. minutum* were shown to produce lytic substances which affect both competing algae and unicellular predators (Tillmann & John, 2002; Tillmann *et al.*, 2008). Both these factors potentially contribute to the formation and maintenance of *A. minutum* blooms.

Analytical and physical-chemical methods can resolve structures and provide basic biosynthetic pathways to phycotoxins, but provide little direct evidence on specific biosynthetic enzymes and regulatory functions (Cembella & John, 2006). Thus, a combined approach linked to genomics and proteomics is required to fully describe toxin biosynthesis and regulation. In the case of PSP toxins in dinoflagellates, however, this approach is rather challenging, as both the physiological regulation and evolutionary origin of PSP toxins remain enigmatic.

Apart from marine dinoflagellates, comprising several species of *Alexandrium* and a single species *Pyrodinium bahamense* of a closely related genus, plus *Gymnodinium catenatum*, which belongs to a very distantly related phylogenetic group (see Saldarriaga *et al.*, 2001; Levy *et al.*, 2007), the only confirmed PSP toxin producers are predominantly freshwater cyanobacteria, including members of the genera *Cylindrospermopsis*, *Anabaena*, *Lyngbya* and *Aphanizomenon.* This paraphyletic distribution was confirmed by several studies demonstrating that removal of bacteria from *Alexandrium* cultures did not eliminate toxin production (e.g. Lu *et al.*, 2000; Hold *et al.*, 2001; Uribe & Espejo, 2003). Along with the lack of conclusive evidence that toxigenesis could be definitively attributed to endosymbiotic bacteria within dinoflagellates, this supports the conclusion that the dinoflagellate genome is responsible for PSP toxin production. As the inheritance of toxin composition follows a Mendelian segregation pattern (Ishida *et al.*, 1998), at least the interconverting enzymes

are almost certainly encoded by nuclear genes. Since the early investigations into the biosynthetic pathway of PSP toxins (Shimizu *et al.*, 1984), the standing hypothesis has been that this pathway is the same in all PSP toxin-producing organisms, and that the corresponding genes should be homologous. In one of the most-cited papers on his model of the PSP toxin synthesis pathway, Shimizu (Shimizu, 1996) suggested that the unusual distribution of the ability to produce PSP toxins might be explained by a rare event of horizontal gene transfer from bacteria to dinoflagellates.

A cyanobacterial PSP toxin gene cluster (sxt) has recently been discovered in *Cylindrospermopsis raciborskii* T3 (Kellmann *et al.*, 2008b). This cluster contains >35 kb of sequence coding for 26 proteins; PSP toxin synthesis is reported to be initiated with arginine, S-adenosylmethionine and acetate by a new type of polyketide synthase. Similar clusters were found in other PSP toxin-producing cyanobacterial species (Mihali *et al.*, 2009; Stucken *et al.*, 2010), and further studies suggested that at least parts of this cluster have been frequently laterally transferred between different cyanobacterial genera (Kellmann *et al.*, 2008a). Nevertheless conclusive evidence of the homology of PSP toxin synthesis in dinoflagellates and prokaryotes is pending.

Attempts to find *Alexandrium* genes associated with PSP toxin production have remained inconclusive. While the physiological and circadian time-frame of PSP toxin production has been elucidated in *A. fundyense* (Taroncher-Oldenburg *et al.*, 1997), further experiments based on this information have failed to identify candidate genes directly linked to PSP toxins (Taroncher-Oldenburg & Anderson, 2000). Subtractive hybridization of cDNA was used to identify toxin-associated genetic differences between toxic and non-toxic subclones of one *Alexandrium tamarense* parental strain (Cho *et al.*, 2008). The differential gene fragments, however, did not seem to be directly related to toxins, as shown by polymorphism analysis of other subclones.

Genomic studies in dinoflagellates have long been hampered by their huge genomes, particularly of free-living species – up to 200 pg nuclear DNA cell^{-1} in *Prorocentrum micans*, which corresponds to roughly 75 times the DNA content of the human genome (LaJeunesse *et al.*, 2005) - and their unusual nuclear composition and organisation (reviewed by Moreno Díaz de la Espina *et al.*, 2005). Although most dinoflagellates are functionally haploid in the vegetative stage, the nucleus may contain up to 270 chromosomes (Moreno Díaz de la Espina *et al.*, 2005). The dinoflagellate nucleus typically lacks histones and nucleosomes and instead contains low abundances of basic histone-like proteins. The chromosomes are permanently condensed, and up to 70% of the nucleotides contain modified or rare bases. This unusual organisation was further illustrated in a low-redundancy sequence survey of the *Heterocapsa triquetra* genome (McEwan *et al.*, 2008); about 90% of the examined sequences were apparently random, non-repetitive DNA, whereas the highest number of recognizable sequences consisted of repeats, transposons or virus-specific protein sequences. These features pose major difficulties in directly studying the gene content of dinoflagellates, and while some sequencing initiatives are underway, no completely sequenced genome is yet available.

Most of the complexities associated with examining the dinoflagellate genomes can be circumvented by conducting genomic studies at the transcriptomic level (Rudd, 2003). In the last few years, a number of expressed sequence tag (EST) studies has been published (e.g. Tanikawa *et al.*, 2004; Hackett *et al.*, 2005; Lidie *et al.*, 2005; Patron *et al.*, 2005; Jaeckisch *et al.*, 2008; Uribe *et al.*, 2008), many of which led to further microarray-based studies of gene expression (e.g. Okamoto & Hastings, 2003a; Van Dolah *et al.*, 2007). In dinoflagellate species producing polyketide toxins of the spirolide and brevetoxin groups, search strategies based on EST- or cDNA-libraries were successful in identifying a range of polyketide synthases (PKS) (Jaeckisch *et al.*, 2008; Monroe & Van Dolah, 2008). These studies made use of the high degree of

sequence conservation in several PKS domains (reviewed in John *et al.*, 2008), but which of these genes are responsible for production of the corresponding toxins has not yet been established.

As well as being a member of the most well-studied dinoflagellate genus of PSP toxin-producers, for which a wealth of physiological and biosynthetic information on toxin production is available, *A. minutum* is an appropriate model for toxin production studies because of its simple toxin profile. Most *A. minutum* strains produce mainly or exclusively gonyautoxins and *ab initio* both toxic and non-toxic strains are available for comparative studies (Tillmann & John, 2002; Touzet *et al.*, 2007). We constructed a normalised EST library of *A. minutum* to search for PSP toxin-related genes in this dinoflagellate. To search for toxin-related or toxicity-influencing genes not readily apparent from EST library annotation, we used a microarray approach to compare the transcriptomes of toxic and non-toxic *A. minutum* clones and thus identified differences in gene expression potentially linked to toxin synthesis and/or regulation.

Methods

General culture conditions for Alexandrium minutum

Unless noted differently, *A. minutum* cultures (origin: Gulf of Trieste, Italy) were grown at 20°C in modified K-medium (Keller *et al.*, 1987) containing 440 µmol L^{-1} NO_3^-, 36 µmol L^{-1} NH_4^+, 25 µmol L^{-1} PO_4^{3-}, 10 nmol L^{-1} SeO_3^{2-}, 1000 µmol L^{-1} Trizma-Base (pH 8.3), K trace-metal solution and f/2 vitamin solution (Guillard & Ryther, 1962; Guillard, 1975). Illumination was provided from daylight fluorescent lamps at a photon flux density (PFD) of 200 µmol m^{-2} s^{-1} on a 16:8-hour light:dark photocycle. All experimental (but not stock) cultures were grown under antibiotic treatment (50 µg mL^{-1} ampicillin, 33 µg mL^{-1} gentamicin, 10 µg mL^{-1} ciprofloxacin, 1.13 µg mL^{-1}

chloramphenicol and 0.025 µg mL^{-1} streptomycin sulphate) using sterile handling techniques to minimize bacterial influence. To avoid any bias introduced by the antibiotics, treatment was stopped at inoculation of the cultures for toxin content and gene expression experiments, but aseptic handling techniques were maintained.

Culture and harvest of A. minutum for EST library construction

A. minutum clone AL3T was grown in 800 mL culture bottles for EST library construction. In order to include genes expressed under different physiological conditions, several alternative treatments were applied. Standard condition cultures were grown as detailed above. Two standard condition cultures were subjected to shock treatments (complete darkness or 5°C) for 24 h prior to harvesting. Cultures treated in high salinity (medium supplemented with 15 g L^{-1} NaCl) and low salinity (medium prepared with 1/3 aged seawater and 2/3 deionised water) were pre- conditioned in these media for ca. 40 cell cycles (about 40 days, over several sub-culturing transfers) before inoculation of the final cultures. To achieve N-limitation at a reasonably high cell concentration, NO_3^-, but not 36 µmol L^{-1} NH_4^+, was omitted from the culture medium. For P-limitation, cultures were grown in medium to which no phosphate was added. Light limitation of growth was attained under a PFD of 60 µmol m^{-2} s^{-1}, and for high and low temperature conditions, cultures were grown at 31.5°C and 13°C, respectively. The cultures were harvested at 7 time points through the photocycle to capture genes expressed at specific time points, and at different stages of the growth curve.

Cells were harvested by filtration upon an 8 µm pore-sized filters and rinsed with filter-sterilized seawater. Cells were immediately transferred into TriReagent (Sigma-Aldrich, Steinheim, Germany), or filters were quick-frozen in liquid nitrogen for later processing.

EST library construction and automated annotation

RNA was extracted using the Sigma TriReagent protocol, following cell lysis by 10 min incubation at 60°C in TriReagent, aided by repeated vortexing with glass beads included in the sample tube. The resulting RNA pellet was dissolved in 100 µL RNAse-free water (Qiagen, Hilden, Germany). RNA cleanup including on-column DNA digestion (27.3 u DNase per sample) followed the protocol supplied with the Qiagen RNeasy kit. RNA was eluted with 40 µL RNase-free water (Qiagen). When necessary, an additional cleanup and concentration step was applied using Qiagen MinElute or Microcon Ultracel YM-30 columns. RNA purity and quantity were determined with a NanoDrop spectrophotometer (PeqLab, Erlangen, Germany), and integrity and absence of DNA contamination was assessed with a Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Total RNA from harvested cells was pooled as equal amounts from each treatment for construction of a normalised cDNA library. The library was constructed and transformed into electrocompetent *Escherichia coli* cells by Vertis Biotechnologie AG (Freising-Weihenstephan, Germany).

Colonies were picked, and the DNA was extracted by magnetic beads on a robot platform (Qiagen, Hilden, Germany). Plasmid inserts were sequenced from both sides using Big Dye Chemistry (Applied Biosystems, Darmstadt, Germany) and separated on an ABI Prism 3700xl sequencing (Applied Biosystems) platform. Resulting high quality sequence reads (15,703) were clustered with a sequential assembly using decreasing identity thresholds (gap assembler) to avoid misassemblies due to polyA tails. Contigs were searched for potential alternative splicing by checking alignments of reads in clusters for the presence of gaps in at least one read compared to the consensus sequence of the cluster. Alternative splicing was assumed if the following criteria were

fulfilled: 1) the manually inspected difference between the transcripts was at least two bases, and 2) no other polymorphisms were present between the alternative transcripts, thereby indicating transcription from the same locus.

Contig sequences were loaded into SAMS (Sequence Analysis and Management System, Center for Biotechnology, Bielefeld University) for automated annotation based on BLAST comparisons against KEGG, KOG, SwissProt, InterPro and the Genbank nt and nr databases. Sequences were subject to open reading frame (ORF) prediction according to (Min *et al.*, 2005), and predicted ORFs were submitted to Pfam, TMHMM-2.0 and SignalP 3.0. To further assist manual annotation, all contigs were analyzed for phylogenetic association to SwissProt sequences using PhyloGena (Hanekamp *et al.*, 2007). Annotations of 192 sequences identified from the microarray results as differentially expressed in toxic and non-toxic strains were checked manually.

In-silico search for known sxt-related genes

The assembled *A. minutum* EST library was screened for contigs showing similarities to any of the publicly available (as of 13.1.2009) sxt-related cyanobacterial sequences in the NCBI protein database using translated protein – nucleotide (blastx) BLAST (Altschul *et al.*, 1997). Contigs and cyanobacterial sequences producing significant hits (e-value < 0.001) were subjected to a PhyloGena analysis in "top10select" mode to identify the most similar SwissProt sequences and to obtain the most likely protein translation of the EST contig sequence. BLAST-identified pairs of sxt proteins and translated *A. minutum* contigs were combined with the full-length versions of their respective SwissProt hits, and, in the cases of Amin_93i12r and Amin_73a05f, additionally with the 20 most similar nr sequences as identified by BLAST. These sequence sets were used for phylogenetic analysis following the method detailed in (John *et al.*, 2008) with modifications. Briefly, alignments were generated from kalign,

and alignment blocks out of the span of both the *A. minutum* and the sxt-related sequence were manually deleted with the CLC sequence viewer version 6.0.1 (Knudsen *et al.*). Phylogenetic trees were calculated with Phyml_3.0.1 (Guindon & Gascuel, 2003) using the subtree pruning and regrafting (SPR) method with a BioNJ starting tree, and the LG model of amino-acid substitution (Le & Gascuel, 2008) with gamma distribution parameters estimated from the data with four discretised substitution rate classes, the middle of which was estimated as the median. The same settings were used for a 100-replicate Bootstrap analysis.

Microarray design

Oligonucleotide probes covering both forward and reverse reading directions were designed in collaboration with CeBiTec, Bielefeld University. After a first test hybridisation of a microarray containing 22,264 oligos (2-14 per contig, mean 5.2), the probe set was reduced to the 8,609 best-performing oligos. Following a test for congruence with the contig ORF direction as determined by either manual annotation or a combination of PolyA-Tail identification and the direction of the best SwissProt hit, the best probe was chosen according to performance in triplicate hybridization with standard-strain (AL3T) RNA from 9 different physiological conditions. For some contigs without available manual annotation which could not be automatically assigned an ORF direction, two probes targeting both possible mRNA orientations were retained; these are marked as correct or false during manual annotation.

Toxin analysis

PSP toxins were extracted following a previously described method (Krock *et al.*, 2007). After centrifugation (3,000 x g, 4°C), pellets were suspended in 0.03 N acetic acid and homogenized in FastPrep tubes (Thermo Savant, Illkirch, France) containing 0.9 g of lysing matrix D by reciprocal shaking in a Bio101 FastPrep instrument (Thermo

Savant) at maximum speed (6.5 m s^{-1}) for 45 s. Cell debris was removed by centrifugation at 16,100 x g at 4°C for 15 min. The supernatant (400 µL) was filtered through a 0.45 mm pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) by centrifugation for 30 s at 800 x g.

Analytical Reagents

Water was deionised and purified (Milli-Q, Millipore, Eschborn, Germany) to 18 MΩ cm^{-1} quality or better. Formic acid (90%, p.a.), acetic acid (p.a.) and ammonium formate (p.a.) were purchased from Merck (Darmstadt, Germany), nitric acid (p.a.) and phosphoric acid (p.a.) were from AppliChem (Darmstadt, Germany), periodic acid, 1-heptanesulphonic acid, 1-octanesulphonic acid and diammonium hydrogenphosphate were from Sigma (Deisenhofen, Germany). The solvents, methanol, tetrahydrofurane (THF) and acetonitrile, were high performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany).

Standard solutions of PSP toxins (saxitoxin, STX; neosaxitoxin, NEO; decarbamoyl saxitoxin, dcSTX; gonyautoxins 1&4, GTX1/GTX4; gonyautoxins 2&3 GTX2/GTX3; decarbamoyl gonyautoxins 2&3, dcGTX2/dcGTX3; and B1) were purchased from the Certified Reference Material Programme of the Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada.

Analytical Methods

Liquid Chromatography with Fluorescence Detection (LC-FD)

The LC-FD analysis was carried out as previously described in detail (Krock *et al.*, 2007). Briefly, PSP toxins were separated by ion-pair chromatography on a ODS reverse-phase analytical column and detected fluorometrically after post-column derivatization. PSP toxins were resolved with two eluants. The first eluant for gonyautoxins and N-sulfocarbamoyl toxins consisted of 6 mM 1-octanesulphonic acid

and 6 mM 1-heptanesulfonic acid in 40 mM ammonium phosphate, adjusted to pH 7.0 with dilute phosphoric acid. Components of the saxitoxin group were separated by 0.75% THF and 13 mM 1-octanesulphonic acid in 50 mM phosphoric acid, adjusted to pH 6.9 with ammonium hydroxide, and 15% (v/v) acetonitrile and 1.5% THF. The flow rate was 1 mL min^{-1} with the following gradient: initial condition: 100% eluant A and isocratic elution until 15 min, gradient elution to 100% eluant B until 16 min, followed by isocratic elution with 100% eluant B until 35 min.

Post-column derivatization was performed with 10 mM of periodic acid in 550 mM ammonium hydroxide at a flow rate of 0.4 mL min^{-1} in a reaction coil set at 50°C. Subsequently, the eluate was continuously acidified with 0.75 N nitric acid at a flow rate of 0.4 mL min^{-1}. Toxins were detected by a dual monochromator fluorescence detector (λex 333 nm; λem 395 nm). PSP toxin concentrations were determined by external calibration.

Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

In order to confirm the LC-FD results using an independent method, mass spectral experiments were performed on an ABI-SCIEX-4000 Q Trap (Applied Biosystems, Darmstadt, Germany), triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent (Waldbronn, Germany) model 1100 LC. The LC equipment included a solvent reservoir, on-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A).

Toxin separation (5 µL of sample injected) for mass spectrometric analyses was performed by a hydrophilic interaction liquid ion-chromatography (HILIC) method (Diener *et al.*, 2007) with slight modifications. The analytical column (150 x 4.6 mm) was packed with 5 µm ZIC-HILIC, 200 Å, (SeQuant, Haltern, Germany) and

maintained at 35°C. A pre-column with the same packing material was also used. The flow rate was 0.7 mL min^{-1} and gradient elution was performed with two eluants. Eluant A was 2 mM formic acid and 5 mM ammonium formate in 20% water and 80% acetonitrile; eluant B was 10 mM formic acid and 10 mM ammonium formate in water. The gradient was as follows: 20 min column equilibration with 20% eluant B, linear gradient to 35% B until 5 min, then linear gradient to 40% B until 10 min, then linear gradient to 45% eluant B until 20 min, followed by isocratic elution with 45% eluant B until 24 min and finally return to initial 20% eluant B until 25 min.

Multiple reaction monitoring (MRM) experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion): *m/z* 412>332 and *m/z* 412>314 (for GTX1/GTX4 and C3/C4), *m/z* 396>316 and *m/z* 396>298 (for GTX2/GTX3, C1/C2 and B2), *m/z* 380>300 and *m/z* 380>282 (for B1), *m/z* 353>273 (for dcGTX2/dcGTX3), *m/z* 369>289 (for dcGTX1/dcGTX4), *m/z* 300>282 and *m/z* 300>204 (for STX), *m/z* 316>298 and *m/z* 316>196 (for NEO), *m/z* 257>196 and *m/z* 257>156 (for dcSTX) and *m/z* 273>255 (for dcNEO). Dwell times of 50–150 ms were used for each transition with the following source parameters: curtain gas: 30 psi, temperature: 650°C, ion-spray voltage: 5000 V, gas 1 and 2: 70 psi, interface heater: on, collision gas: high, declustering potential: 66 V, entrance potential 10 V, collision energy: 30 V, and collision cell exit potential: 12 V.

Determination of harvesting time for gene expression analysis

To determine the optimal point of the light-dark cycle at which putative toxin genes are most likely to be expressed, triplicate *A. minutum* AL3T cultures were grown in 5 L glass flasks with constant gentle aeration under controlled standard conditions, as previously described. Cultures were sampled for cell counts and toxin analysis at the onset of dark phase, at the onset of light phase, and at 2-h intervals until the beginning

of the next dark phase, with a sterile tube-sampling system (John *et al.*, 2000; Eschbach *et al.*, 2005). Duplicate samples for toxin analysis (125 – 200 mL of culture depending on harvesting time) were harvested by centrifugation for toxin analysis by LC-FD.

Inter-clonal comparisons of gene expression

Gene expression was compared between toxigenic (AL3T and AL9T) and naturally non-toxic (AL1T) *A. minutum* clonal strains originating from the same geographical population in the Gulf of Trieste, Italy (isolated by A. Beran, see Montresor *et al.*, 2004). Bacteria-reduced triplicate cultures were grown under the control conditions as stated above. Culture growth was monitored by daily manual cell counts of samples fixed in Lugol's iodine solution.

The toxin content of duplicate samples containing at least 2 x 10^6 AL3T and AL9T cells and non-duplicate samples containing at least 4 x 10^6 AL1T cells were analyzed by LC-FD. To confirm the toxin identification of specific PSP toxin analogues by LC-MS, samples of additional parallel cultures were combined into pellets containing 8.5 x 10^6 to 1.1 x 10^7 cells.

Samples for RNA extraction were taken during exponential growth phase, at 10-11 h (Sampling Time 1, ST1) and at 6-7 h (Sampling Time 2, ST2) after onset of light phase. Total RNA (500 ng sample^{-1}) was extracted as described herein, then amplified and labelled with a Low RNA Input Linear Amplification kit (Agilent, Waldbronn, Germany). The Agilent Low RNA Input Linear Amplification Kit protocol was followed for synthesis of Cy3- and Cy5-labelled cRNA and microarray hybridisation. Microarrays were scanned on an Agilent G2565AA scanner, and raw data were extracted with the Agilent Feature Extraction Software version 9.1.3.1 (FE). Array quality was monitored with the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07.

Pre-processed data were subjected to SAM (Significance Analysis of Microarrays, Tusher *et al.*, 2001) as implemented in MeV 4.0 (Saeed *et al.*, 2006), and SAM-based q-values (Storey, 2003) were calculated. Probes with a q-value of <1% were considered to indicate differential expression of the corresponding genes if the mean fold change of the sample triplicate was at least 1.5. To minimise the influence of physiological differences between the strains that were not related to the capacity for toxin production, only genes identified as significantly higher or significantly less expressed in both toxic strains at both sampling time-points were designated as “higher expressed” or “less expressed” in toxic strains.

Samples were clustered by Hierarchical Cluster Analysis (HCL) support trees as implemented in MeV 4.2. Trees calculated with different distance measures (Euclidean, Manhattan and Covariance values) and different distance metrics (average, complete and single linkage) were compared. Node confidence was tested by 1000 Bootstrap replicates.

To test reliability of the microarray, expression levels of six genes were evaluated by qPCR. Four of these genes had been identified as significantly higher expressed in the toxic strains, the other two as significantly higher expressed in the non-toxic strain. The qPCR was carried out as described (Krell *et al.*, 2007) with the lepidopteran genes MA and NSP as an artificial internal reference. Primers for qPCR were designed with PrimerExpress 3.0 (Applied Biosystems, Darmstadt, Germany) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Standard curves using cDNA plasmids corresponding to target sequence ESTs were plotted to test the primer pairs for consistent efficiency at different concentrations. These plasmids were amplified by M13-primed PCR; qPCR primers were tested on dilution series of plasmid PCR products spanning at least 8 orders of magnitude. The qPCR reaction was based on the

PowerSybrGreen PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, using a 7000 Real-Time PCR System (Applied Biosystems).

Prior to cDNA synthesis the samples were spiked with the artificial internal control RNAs MA (1 ng reaction^{-1}) and NSP (1 pg reaction^{-1}). Equal amounts of RNA from all strains were processed in parallel. All qPCR reactions based upon the same primer-set were run on the same plate, and reaction efficiencies were compared with MA and NSP-specific primers. To test for contamination with genomic DNA, negative controls consisted of full reactions in which cDNA was exchanged for RNA aliquots of all samples. Thresholds were determined manually for each primer set. Relative expression levels were recorded as the cycle threshold value (C_t).

PCR

To test for the presence of the cyanobacterial toxin gene cluster in *A. minutum*, amplification of the cyanobacterial genes from genomic DNA isolated from AL3T cultures grown under antibiotic treatment was attempted. The 14 primer pairs designed against the PSP toxin ORF in the *Raphidiopsis brookii* genome (K. Stucken et al., unpublished data; Additional file 1) were tested. A primer pair targeting the D1/D2 (Scholin *et al.*, 1995) region of the large ribosomal subunit was used as positive control, primers against the bacterial 16S ribosomal subunit controlled for bacterial contamination of the DNA.

Reactions were scaled to 30 µL and contained 20 ng genomic DNA, 0.2 mM forward and reverse primers, 0.1mM dNTPs, 0.6 units of U of HotMaster *Taq* DNA polymerase (Eppendorf) and HotMasterTaq buffer 1X (Eppendorf). Gradient PCR was employed to try each primer at 6 different annealing temperatures spanning a range of approximately 9°C. As the primers were of different melting temperatures, three different temperature

spans between 46 and 58°C were used. Cycling conditions were based on those optimal for these primers (Additional file 1): initial denaturation at 94°C for 5 min, 20 cycles with denaturation 94°C (20 s), annealing for 45 s, extension at 70°C (30 s), and a final extension step of 10 min at 70°C. PCR products were visualized after agarose gel electrophoresis.

Results

Characterisation of the EST Library

The cDNA fragments sequenced from both sides yielded 15,703 read sequences clustered into 4,320 contigs. Of these contigs, 3,112 (72%) combined the forward- and reverse reads of at least one bacterial clone, and thus can be considered full-length insert sequences, but might not represent the total length of the corresponding mRNA. Among contigs, 1,998 contained reads from more than one cDNA insert. Contig length varied between 100 and 2185 bp, with a mean of 791±240 bp (mean ± standard deviation). The mean GC content of contigs was 56.10 ± 4.67% (Table 1).

The examination of alignments of reads in the clusters for potential alternative splicing identified different splice variants in ca. 9% of the contigs containing reads from more than 1 cDNA insert, or ca. 3% of all genes examined.

Checking all contigs for evidence of transsplicing revealed that the complete dinoflagellate-specific spliced leader sequence (SL), DCCGTAGCCATTTTGGCTCAAG (D =U, A, or G) (Lidie & Van Dolah, 2007; Zhang *et al.*, 2007) was present in 204 contigs. Of these SLs, 37 (18%) started with A, 10 (5%) with G and 157 (77%) with U. In addition, we identified 35 contigs containing incomplete SL sequences. In 23 of these, the sequence was lacking 1-4 nucleotides at the 5'-end of the contig, while 23 SLs differed in 1-3 nucleotides within the SL

sequence. Of the SL-containing contigs, 193 contained an additional 5'polyA-region and thus are likely to contain the complete ORF (see Additional file 2).

Automated annotation with the SAMS platform yielded annotations for 1,200 contigs (28%), whereas 1,969 contigs (46%) did not produce any BLAST hit below tool cut-off. Another 1,151 contigs (27%) produced no hits that met the criteria for automated annotation. Automated classification to KOG categories based on the SAMS "BLAST 2x *vs*. KOG" output (cut-off e-value 10^{-4}) resulted in assignment of 1005 contigs (23%) to functions other than "General function prediction only" or "Function unknown" (Fig.1).

A BLAST survey of the *A. minutum* library against all available dinoflagellate EST libraries available in GenBank (as of 28.11.2007) plus a new *A. ostenfeldii* EST library (N. Jaeckisch, unpublished observations) detected similar sequences for 43% of the contigs at a moderately stringent cut-off value of $e = 1 \times 10^{-10}$ (Table 2).

In-silico search for sequences related to the cyanobacterial PSP toxin gene cluster

A BLAST search of the EST library against the cyanobacterial PSP toxin genes resulted in 14 hits at e-value <0.001. In 12 of 14 maximum likelihood phylogenies calculated from the BLAST-identified pairs of sxt proteins and translated *A. minutum* contigs, and their closest matches identified from the SwissProt database, the *A. minutum* and the cyanobacterial sequences clustered in different clades. Furthermore, these clades were separated by long branches, well-supported nodes with high bootstrap support, or both (see Additional file 3: Phyml-based likelihood trees with bootstrap support values). In the case of Amin_93i12r and Amin_73a05f, the phylogenies based on SwissProt hits were not that clear (Additional file 3 M.1 and N.1). This led to a further test for orthology of Amin_93i12r and Amin_73a05f with the cyanobacterial PSP toxin genes by including the results of a BLAST search against the nr database. In the case of

Amin_93i12r, the cyanobacterial gene in question was identified in this BLAST search as hit number 64; the BLAST result list of Amin_73a05f did not include its potential cyanobacterial counterpart within the best 100 hits. When we repeated calculation of the phylogenies for Amin_93i12r and Amin_73a05f with the best 20 BLAST *vs*. nr hits included in addition to the SwissProt database hits, the *Alexandrium* sequences did not cluster in the vicinity of the cyanobacterial PSP toxin genes (Additional file 3 M.2 and N.2).

Toxin production over the light-dark cycle

Under our standard growth conditions, the toxin-producing strain AL3T exhibited a growth rate (1.01 cell divisions day^{-1}; I. Yang, unpublished data) that approximated the total length of the light:dark period, which suggested that these cultures might have a naturally circadian-phased cell cycle. In the triplicate cultures examined, however, both cell number and culture toxin content increased over the entire light phase. The toxin content per cell stayed roughly the same during the whole day, while cell numbers doubled from 3,271 ± 296 to 7,167 ± 482 cells mL^{-1} within 24 h. The toxin content of the culture increased from 1.38 ± 0.16 ng μL^{-1} to 3.24 ± 0.21 ng μL^{-1} (Fig.2)

Strain-specific toxin content

A. minutum PSP toxin concentration as measured by LC-FD was strongly clone-dependent. No PSP toxins were found in AL1T, whereas the toxin profiles of AL3T and AL9T were virtually identical, although toxin cell quotas differed by an order of magnitude (Table 3). The toxin profiles as confirmed by LC-MS/MS (Fig. 3) in the extracted ion chromatograms clearly indicate the presence of the transitions *m/z* 396 > 298 for GTX3 and *m/z* 412 > 314 for GTX4. No signals were obtained from any other ion traces, indicating that no known PSP toxins other than GTX3 and GTX4 were present in the samples.

Gene expression

Gene expression differences between toxic and non-toxic isolates were identified by microarray experiments comparing the two toxic strains AL9T and AL3T to the non-toxic AL1T. With SAM at a gene-specific FDR cut-off of q=1% and a fold-change cut-off of 1.5, 145 contigs were identified as higher expressed in both toxic strains at both time points tested, whereas 47 genes were significantly higher expressed in the non-toxic strain (see Additional file 2 – List of differentially expressed genes). HCL support trees in which samples were clustered according to similarity of gene expression grouped the samples according to their clonal designations. Of the nine tested different combinations of clustering parameters, only the tree based on the Euclidean distance measure combined with complete linkage separated the midday and afternoon triplicates of the AL9T vs. AL1T microarrays. In all other analysis, samples did not cluster according to harvesting time.

Of the 145 genes identified as higher expressed in the toxic strains at both time points, 49 could be manually annotated based on BLAST-based automated annotation, domain comparison using Pfam and phylogenetic reconstruction with the PhyloGena program (Hanekamp *et al.*, 2007). Putative functions could be assigned to 31 of these genes (Table 4).

The group of 47 genes higher expressed in the non-toxic strain AL1T contained 8 genes for which a putative function could be identified (Table 4).

For 8 of the contigs higher expressed in the toxic strains, the signals of the non-toxic strain were identified as not “well above background” by the microarray data extraction software on at least 8 of the 12 arrays, and on at least one array for each strain-time-point combination. We consider these as either not expressed in the non-toxic strain AL1T or considerably different on the sequence level, which might imply functional

differences. This group contains two putative helicases and six hypothetical proteins for which no annotation could be found. For one of these, Amin_66c10r, we found a homologous sequence in the published *A. tamarense* library (Hackett *et al.*, 2005), but in none of the libraries of non-PSP toxin-producing dinoflagellates. Another five sequences examined did not produce significant BLAST hits against any other dinoflagellate library (see Additional file 2).

The analysis of relative mRNA levels of 6 genes re-examined by qPCR, as shown in Fig. 4, indicated that microarray and qPCR data agreed qualitatively, although most of the fold changes calculated from qPCR results were substantially higher than those identified by microarray.

Discussion

EST Library

The EST library presented here includes sequences from a variety of physiological states induced by different treatments and sampling time points. We aimed for a high diversity of transcripts ideally representing an overview over the complete transcriptome of this species, by combining samples from control, cold-shocked, darkness-shocked, high- and low-salinity stressed, nitrogen- and phosphorous-starved as well as heat- and cold-stressed *A. minutum* cultures and by normalization of the cDNA library. The 15,703 read sequences, which originated from 9,485 cDNA clones, clustered into only 4,320 contigs or putative genes. We estimated that our contigs account for approximately 80% of the potential genes within this particular cDNA library, based upon the rarefaction curve analysis (Additional file 4). Nevertheless, we recognize that not 80% of the potential gene candidates from the *A. minutum* genome are included in the library. Based on the experience of diverse genome projects, the total

number of gene candidates is probably two or three times higher. In any case, contig numbers for the *A. minutum* library are substantially lower than those found for EST libraries of the congeneric *A. tamarense* (6,723 unique sequences from 11,171 reads, normalised library) (Hackett *et al.*, 2005) and *A. catenella* (6,496 "unigenes" from 10,850 ESTs, library not normalised) (Uribe *et al.*, 2008). The latter two EST libraries were both obtained from cultures grown only under control conditions, and hence presumably do not reflect stress, cell cycle or different culture stage effects on gene expression. The relationship of unique genes within the EST library for *A. minutum* is reminiscent of that of the free-living marine dinoflagellate *Heterocapsa triquetra* (Patron *et al.*, 2005), for which the EST library was based on samples taken throughout the photocycle, but contained only 2,022 unique clusters out of 9,309 ESTs.

A BLAST-search of the *A. minutum* contigs against available dinoflagellate EST libraries found at least weakly similar sequences for about half of the contigs, which is mainly due to the availability of EST libraries for *A. tamarense* (Hackett *et al.*, 2005), *A. catenella* (Uribe *et al.*, 2008) and *A. ostenfeldii* (N. Jaeckisch et al, unpublished observations) (Table 2). The low novelty rate implied by these similarities, however, is not reflected in the annotation results; automated annotation was possible for 28% of the contigs. The attribution of these contigs among functional categories (depicted in Fig.1) is likely to reflect to a large extent the sequence conservation in the respective groups, and not necessarily the true distribution of gene functions. Studies examining the function of dinoflagellate genes are still rare, and as they are ecologically, physiologically and genetically very distinct from their better-studied relatives, the apicomplexa (Fast *et al.*, 2001), annotation of dinoflagellate genes remains notoriously difficult (see John *et al.*, 2004).

A recent study (Bachvaroff & Place, 2008) suggests that gene expression in dinoflagellates reflects genomic organisation. Most highly expressed genes seem to be in tandem arrays of slightly different gene copies, with few or no introns, short intergenic spacers, and are mostly spliced-leader trans-spliced (Lidie & Van Dolah, 2007; Zhang *et al.*, 2007). We found evidence for the existence of such gene families for 8 of 9 sequences tested; in addition to intra-specific differences among strains, there was considerable intra-strain variability on both the transcriptomic and genomic levels, with variation in the cDNA within a strain, comprising both synonymous and non-synonymous differences. This implies that microarray-measured differences in gene expression might be due to either differences in the frequencies of highly probe-complementary *vs*. less probe-complementary gene family members, or to the simultaneous up- or down-regulation of whole gene families.

In contrast to these multi-copy genes, moderately expressed genes appear to be single copy and exhibit a higher intron density; trans-splicing seems to occur less often (Bachvaroff & Place, 2008). Furthermore, we identified alternative splice variants in 9% of the genes containing reads from more than one cDNA, adding to the evidence that more conventional eukaryotic introns and splicing phenomena exist in dinoflagellates (Alverca *et al.*, 2006).

Based on our data and with reference to previous studies we cannot, however, exclude that in rare cases identical copies with different splicing patterns may exist. Yet since splicing accuracy and efficiency depends on the underlying sequence, this scenario is highly unlikely. In any case, to our knowledge, our work herein is the first report on the frequency of alternative splicing in dinoflagellates.

In-silico search for candidate saxitoxin-related genes

The known information on PSP toxin-related proteins in dinoflagellates is limited to enzymes involved in the interconversion of PSP toxins, which have been characterised on the protein level. An oxidase capable of transforming GTX 3 to GTX 4 has been found in crude extracts of *A. tamarense* cells (Oshima, 1995). In the dinoflagellate *Gymnodinium catenatum* the same research group also reported an N-sulfotransferase forming C2 from GTX3 in a reaction mix containing adenosine 3'-phosphate 5'-phosphosulfate. Nevertheless, this enzyme was present in both toxic and non-toxic isolates of this species. A similar enzyme was also found in a crude enzyme extract of *A. tamarense* (Wang *et al.*, 2007). A sulfotransferase that specifically transfers a sulfonyl residue to a carbamoyl group present in some saxitoxin analogues has been purified and characterised from PSP toxin-producing *G. catenatum* (Sako *et al.*, 2001). The authors suggested that STX might be the only toxin directly synthesized, and that all other PSP toxins are derived by a fixed set of interconversions. Consistent with this idea, an O-sulfotransferase specifically transferring a sulfonyl group to O-22 of 11-hydroxy-STX to produce GTX 3 has also been purified and characterized from *G. catenatum* (Yoshida *et al.*, 2002). If these interconversions are the same in all PSP-producing dinoflagellates, *A. minutum* should contain the characterized O-sulfotransferase (Yoshida *et al.*, 2002) and the oxidase transforming GTX 3 to GTX 4 (Oshima, 1995). Searching our *A minutum* EST library for corresponding candidate genes revealed several oxidases and one sulfotransferase of unknown specificity. The microarray data set shows two of the potential oxidases as higher expressed in the toxic strains - one hypothetical protein similar to iron/ascorbate family oxidoreductase was between 6.6 and 10.3 times higher expressed in the toxic strains AL3T and AL9T, and a putative steroid oxidoreductase superfamily member 2.9 to 4.5 times. Both oxidases had a very good BLAST match with the PSP toxin-producers *A. tamarense* and *A. catenella*,

respectively, but only weak or no similarities to other dinoflagellate sequences. The sulfotransferase had a potentially homologous match (BLAST e-value = 6 x 10^{-11}) in the *A. tamarense* EST library, but in none of the other dinoflagellate libraries. The microarray data did not identify it as differentially expressed between toxic and non-toxic strains. As sulfotransferases and oxidases are rather general enzyme types occurring in many other pathways including those of primary metabolism, it is not possible to determine at this point whether one of the enzymes found in *A. minutum* is related to PSP toxin modification.

The only available sequence information related to PSP toxin synthesis is a cyanobacterial gene cluster first identified in *C. raciborskii* (Kellmann *et al.*, 2008a). The standing hypothesis is that PSP toxin biosynthesis should follow the same pathways (Shimizu *et al.*, 1984) and be encoded by the same set of genes in dinoflagellates and cyanobacteria. This is usually explained by horizontal gene transfer (HGT) between the prokaryotic cyanobacteria and dinoflagellates (Shimizu, 1996; for other examples of inter-kingdom gene transfer see Lander *et al.*, 2001; Richards *et al.*, 2006). Although the gene cluster coding for the enzymes of the PSP synthesis pathway are known, corresponding genes in dinoflagellates have not been identified to date.

Searching our *A. minutum* EST library for contigs homologous to the cyanobacterial PSP toxin genes identified several sequences of moderate similarity. However, when we calculated phylogenies from these sequence pairs and their most similar matches as identified by BLAST against SwissProt or, in the case of *Amin_93i12r* and *Amin_73a05f* against SwissProt and nr, the *A. minutum* sequence and the *C. raciborskii* toxin gene appeared to be very distant relatives separated by many more closely related sequences and often by long branches in the Phyml-based likelihood trees. This indicates that if a suite of genes similar to the *C. raciborskii* PSP toxin gene cluster is

present in the *A. minutum* transcriptome, none of its sequences were recovered during EST library preparation.

A gradient PCR test using DNA from *A. minutum* AL3T cultures grown under antibiotic treatment and primers designed against the PSP toxin cluster from cyanobacteria recovered no PCR products except the positive control (I. Yang, unpublished data). However, negative results in a PCR experiment cannot prove non-existence of a sequence, and other primers or different PCR conditions might lead to a different outcome. In any case, no evidence of the cyanobacterial PSP toxin gene cluster in dinoflagellates has been published, in spite of the fact that the PSP-producing *Alexandrium* species are among the best-studied dinoflagellates (e.g. Hackett *et al.*, 2005; Jaeckisch *et al.*, 2008; Uribe *et al.*, 2008). Together with the data presented here, this suggests the hypothesis that the PSP toxin genes in dinoflagellates are rather more different from their cyanobacterial counterparts than would be expected in the case of a recent gene transfer. Whether or not this HGT event has occurred as hypothesized, or if the capacity for PSP toxin production arose independently in dinoflagellates, or if the bacterial genes were horizontally transferred to dinoflagellates but then changed during evolutionarily relevant time-spans to a point beyond recognition with current methods, can only be determined when the first dinoflagellate PSP toxin genes are identified.

Physiology-based approach

Although it has been suggested (Monroe & Van Dolah, 2008) that many genes in dinoflagellates are post-transcriptionally regulated, examination of mRNA pools regularly succeed in finding genes differentially expressed between different physiological treatments or at different times of the light-dark cycle (Taroncher-Oldenburg & Anderson, 2000; Okamoto & Hastings, 2003b; Okamoto & Hastings, 2003a; Van Dolah *et al.*, 2007). The ability to produce PSP toxins is genetically fixed

within strains, and as it is probably genetically determined, evidence should be traceable to the mRNA pool, whether transcriptionally or post-transcriptionally regulated. This interpretation serves to justify our attempt to identify possible candidate genes for involvement in PSP toxin production by comparing mRNA pools of toxic and non-toxic strains.

Certain experimental efforts to understand PSP toxin production in *Alexandrium* species have focussed on identifying critical time-points during the cell cycle. For example, in light-deprivation synchronized cultures of *Alexandrium fundyense*, PSP toxin production was shown to be coupled to an 8-10 h period in the G1 phase of the cell cycle (Taroncher-Oldenburg *et al.*, 1997). Several dinoflagellate species are reported to exhibit naturally phased cell cycles in both non-synchronized cultures and in field populations (van Dolah *et al.*, 1995; Leighfield & Van Dolah, 2001; Van Dolah *et al.*, 2008). As the toxic *A. minutum* strain AL3T is capable of a growth rate of one cell division per day under optimal conditions, we suspected that even experimentally non-manipulated cultures might exhibit a circadian-phased (perhaps even synchronised) cell cycle similar to that found for the dinoflagellate *Amphidinium operculatum* (Leighfield *et al.*, 2002). However, both cell numbers and intracellular toxin per culture volume increased continuously throughout the light phase (see Fig. 2) in non-synchronised AL3T cultures. A similarly broad time frame for cell division in a dinoflagellate is only known for a slow-growing culture of *Gyrodinium uncatenatum* (Cetta & Anderson, 1990) The apparent lack of phased cell division in non-synchronised *A. minutum* AL3T cultures might be an artefact of growth under conditions optimised for high growth rate and therefore may not reflect growth under natural conditions or in experimentally synchronised cultures. However, the failure of most gene expression – based HCL support trees to resolve the difference between mid-day and afternoon triplicates of the AL3T – AL1T and AL9T – AL1T- comparisons indicate that a similar lack of phased

physiological conditions might occur in other *A. minutum* strains grown under the same conditions, although we did not examine the timing of cell division and toxin production in the other strains in this study. Both laboratory cultures and field population of different *Alexandrium* species are known to be primarily in G1 throughout most of the light period or daylight time (Taroncher-Oldenburg *et al.*, 1997; Garcés *et al.*, 1998; Figueroa *et al.*, 2007). Thus, combining data from two different time-points during light phase should capture differences in the mRNA pool of toxic and non-toxic strains during PSP toxin production while excluding strain-specific differences in circadian responses not related to toxin production.

The microarray approach can identify differences in mRNA abundances of known sequences irrespective of the assigned function, to compare the gene expression of the different strains. This allows screening for gene expression patterns associated with different genetic traits coupled to physiological responses such as toxin production, and can be used to correlate non-annotatable sequences with biological connotation (Pir *et al.*, 2006). As the two-colour microarray setup used in this study directly measures the ratio of sequence abundances in the two samples hybridized, the detection of these differences is largely independent of overall expression levels.

In inter-strain comparisons using microarrays, mRNA differences between the strains might result in differences in hybridisation efficiency which from the array data alone cannot be distinguished from expression level differences. However, comparison of microarray results (see Additional file 2:_List of differentially expressed genes) with qPCR data (Fig. 4) indicated that in this respect, our microarray setup is more likely to pick up slightly divergent sequences than the gene expression “gold standard” qPCR.

According to microarray data we identified 145 genes as higher expressed in both toxic strains at both time-points examined. Among the eight of these that were not

significantly expressed in the non-toxic strain were two putative helicase sequences and six hypothetical proteins for which no annotation could be found. We consider these eight genes as candidates for genes associated with the biosynthesis or regulation of PSP toxins, or for adaptive responses to intracellular PSP toxins. For one of the non-annotatable candidates we obtained a significant BLAST hit (E-value $6x10^{-14}$) against ESTs of the PSP toxin-producing species *A. tamarense*, but not against any of the non-PSP-toxin-producing dinoflagellates tested. As we do not know the proportion of the respective transcriptomes represented in either EST library, we rate the match but not the lack of matches as significant. Therefore we consider these eight genes candidates for genes associated with the biosynthesis or regulation of PSP toxicity, or for acclimatisation to intracellular PSP toxins.

The putative functions of 31 of the genes higher expressed in the toxic strains, and of 8 of the 47 genes higher expressed in the non-toxic strain AL1T, were identified by manual annotation. A number of these were involved in post-transcriptional processes, which in dinoflagellates seems to be an important level of gene regulation (Bachvaroff & Place, 2008).

Most of the gene expression differences mentioned herein are probably due to variations in growth rate, overall metabolic rate, or other physiological differences between individual strains. Nevertheless, the genes that were not significantly expressed in the non-toxic AL1T match the expression pattern expected for genes involved in toxin biosynthesis, regulation or sequestration. We therefore propose a particular focus on these sequences as gene targets to be further comparatively studied in other dinoflagellates and species complexes for which toxigenic and non-toxigenic strains are available. All authors read and approved the final manuscript.

Conclusions

An in-silico approach to identify genes related to the recently published cyanobacterial PSP toxin gene cluster in our new EST library of the PSP toxin-producing dinoflagellate *A. minutum* did not yield evidence for cyanobacteria-like sxt genes in our species. A semi-extensive PCR approach involving *A. minutum* DNA and primers designed for the cyanobacterial genes also failed to detect congruent sequences related to PSP toxin biosynthesis. Although EST libraries such as the one presented here represent only a subsample of the transcriptome, the lack of evidence for all of these genes in our library, as well as in other published EST libraries of PSP toxin-producing *Alexandrium* species, suggests that the PSP toxin genes in dinoflagellates are more different from their cyanobacterial counterparts than would be expected in the case of a recent gene transfer. This does not rule out a cyanobacterial ancestry for the dinoflagellate PSP toxin genes, because the possibility of rapid change of newly obtained genes in the dinoflagellate nucleus cannot be excluded.

Microarray-based comparisons of toxic and non-toxic strains of *A. minutum* indicated that many genes were higher expressed in the toxic than in the non-toxic strain. Among contigs candidates for PSP toxin-related genes are the several genes that could not be annotated due to a lack of known similar sequences but were expressed only in the toxic strains. Further testing of these candidates, by expression analysis with different toxic and non-toxic strains and by physiological manipulations that affect the biosynthetic rate and cell content of particular toxin analogues is clearly warranted.

While transformation of dinoflagellates has rarely been achieved (ten Lohuis & Miller, 1998), several studies have demonstrated successful expression of dinoflagellate genes in *E. coli* (Bae & Hastings, 1994; Suzuki-Ogoh *et al.*, 2008) or yeast (Lippmeier *et al.*, 2002; Ho *et al.*, 2007).To obtain conclusive evidence for the function of PSP toxin

genes it might be necessary to use heterologous expression of the identified candidates in a suitable expression system, followed by purification of the enzymes and assessment of their activity on known PSP toxin precursors.

Authors' contributions

UJ and AC conceived of the study, participated in its design and helped to draft the manuscript; UJ also participated in the day-to-day management of this study and in the design of the microarray. GG sequenced the cDNA library, constructed the EST contigs and provided part of the bioinformatic analysis. BK conceived of the LC-MS/MS measurement protocol, carried out these measurements and provided the LC-FD protocols. AG provided the SAMS part of the automated annotation. IY and SB determined the growth conditions, grew and harvested the input cultures for the EST library. SB conceived of the RNA extraction protocol. IY carried out the remainder of the molecular genetics studies, participated in the bioinformatics analysis, carried out the manual annotation and drafted the manuscript. All authors read and approved the final paper.

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Figures

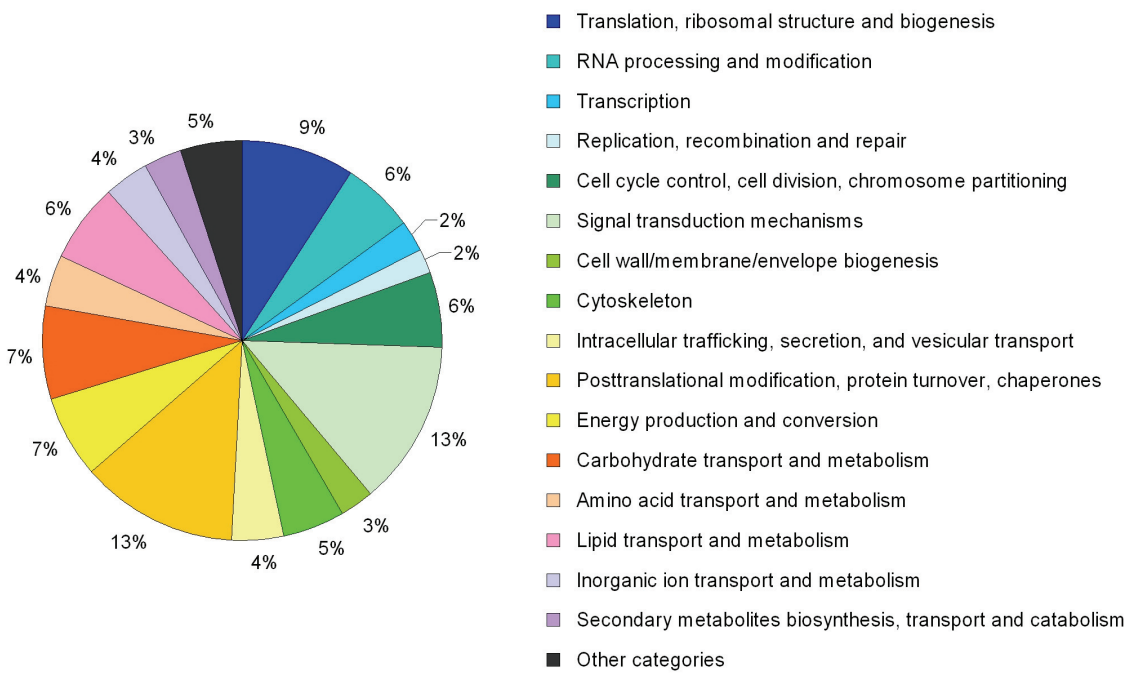


Figure 1 KOG-based functional classification of 1005 contigs as obtained by BLAST-identified similarities

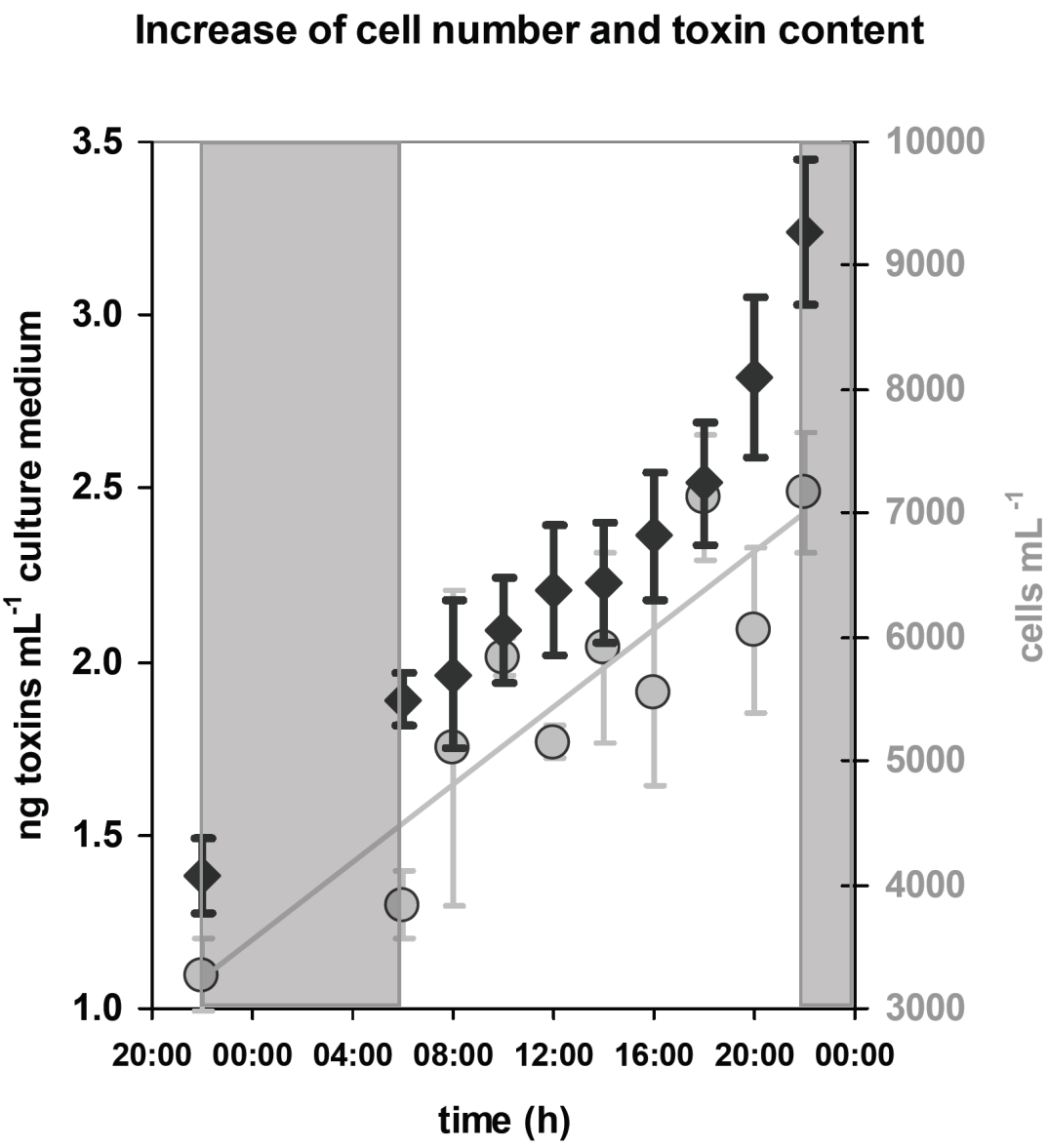


Figure 2 Increase of cell number and culture toxin content in strain AL3T over 24 hours. Grey boxes =dark phase, circles = cell numbers, diamonds = total PSP toxins mL^{-1}.

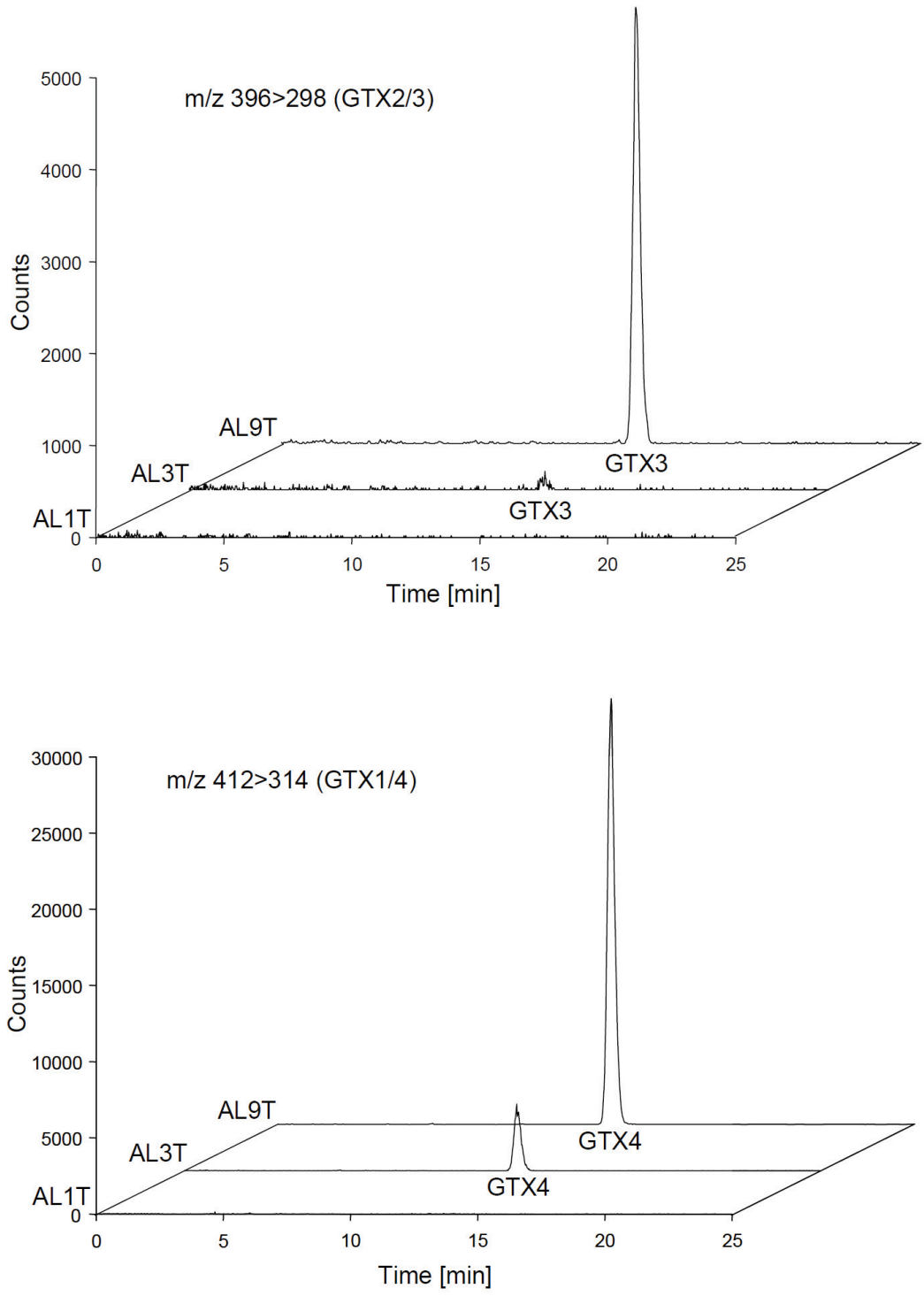


Figure 3 Extracted ion traces of LC-MS/MS chromatograms of the *A. minutum* strains AL1T, AL3T and AL9T. Top: transition 396 > 288 for GTX2/3 (retention time GTX3: 14.1 min); bottom: transition 412 > 314 for GTX1/4 (retention time GTX4 13.3 min)

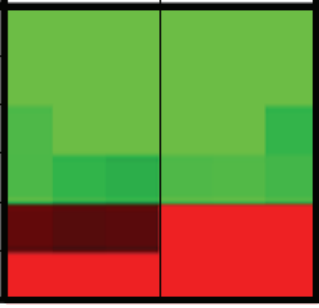
	Heatmap microarray data		Relative Abundance according to Microarray		Abundance according to qPCR		
Arrays / strains Genes	AL3T /AL1T (tox / n-tox)	AL9T/AL1T (tox / n-tox)	AL3T /AL1T (tox / n-tox)	AL9T/AL1T (tox / n-tox)	AL3T (tox)	AL9T (tox)	AL1T (n-tox)
Amin_81i24r			++	++	++	+	N.D.
Amin_56a03r			++	++	+	+	N.D.
Amin_89d06r			+	+	+	+	N.D.
Amin_52d01f			+	+	++	+	N.D.
Amin_88h09r2			(-)	-	+	-	++
Amin_44h03r			-	-	+	+	++

Figure 4 Comparison of expression ratios based on microarray- and qPCR- data.

Array data: more than 15x higher expressed in toxic strains, ++; more than 5x, +; more than 5x higher expressed in non-toxic strain, -; 2-5x higher expressed in non-toxic strain, (-); raw data see supplementary table 1. qPCR: Ct < 25, ++; Ct<27, +; Ct>27, -; N.D., not detected at Ct < 35.

Tables

Table 1 Base composition of the *A. minutum* EST library

GC content	no. of contigs	fraction of contigs
<40%	75	1.7%
40-50%	243	5.6%
50-60%	3454	80.0%
≥ 60%	548	12.7%
22%	minimum	
66%	maximum	

Table 2 Result of BLAST search of *A. minutum* contigs against all dinoflagellate ESTs available in GenBank (as of 28.11.2007).

E-value cut-off	all available dinoflagellates	*A. tamarense*	*A. catenella*	*A. ostenfeldii*
10^{-5}	2209 (51.1%)	1487 (34.4%)	940 (21.8%)	1543 (35.7%)
10^{-10}	1864 (43.1%)	1203 (27.8%)	715 (16.6%)	1335 (30.9%)
10^{-30}	1123 (26.0%)	703 (16.3%)	360 (8.3%)	867 (20.1%)

Table 3 Strain-specific toxin content as measured by LC-FD (in fmol $cell^{-1}$ ± standard deviation, n=3)

strain	Aggregate toxins	GTX 1/4	GTX 2/3	STX
AL3T	1.11 ± 0.06	1.09 ± 0.06	0.02 ± 0.003	traces
AL9T	10.33 ± 1.95	10.02 ± 1.94	0.29 ± 0.01	0.02 ± 0.001
AL1T	0	0	0	0

Table 4 Putative functions of differentially expressed genes

Contig name	Gene product	Putative function	Log mean fold changes		
			AL3T / AL1T	AL9T / AL1T	Tox / Nontox
Higher expressed in toxic strains					
Amin_34h03r	putative small nuclear ribonucleoprotein polypeptide E	gene expression	1.7	2.4	2.0
Amin_36k19f	translation elongation factor-like protein	gene expression	24.4	25.8	25.1
Amin_44h09f	glycyl-tRNA synthetase	gene expression	4.3	4.6	4.5
Amin_56k21r	probable translation initiation factor E4	gene expression	22.6	28.5	25.4
Amin_65d02r	putative Ubiquitin-like domain-containing CTD phosphatase 1	gene expression	3.1	2.3	2.7
Amin_75e04r	putative helicase	gene expression	24.3	26.1	25.2
Amin_48i15f	putative helicase	gene expression	26.9	44.8	34.7
Amin_56g23f	putative Alpha-L-fucosidase 1	protein glycosylation	1.8	5.5	3.2
Amin_95c08r	sialyltransferase	protein glycosylation	20.3	23.5	21.9
Amin_87p16r	casein family protein kinase	signal transduction	9.7	13.0	11.2
Amin_98a08r	endonuclease / exonuclease / phosphatase family protein	intracellular signalling	3.6	4.8	4.2
Amin_63e03r	hypothetical protein similar to Sentrin-specific protease 8	cell cycle control	3.7	3.2	3.4
Amin_68d07f	rrm family protein similar to mei2	cell cycle control	2.9	2.5	2.7
Amin_63c06r	RNA-binding protein similar to mei2	cell cycle control	1.9	2.6	2.2
Amin_53e04r	chlorophyll a-c binding protein	chloroplast	5.5	4.9	5.2
Amin_81i24r	translation elongation factor P	organellar translation	37.4	45.2	41.1
Amin_97a05r	putative mitochondrial import receptor subunit tom40	mitochondrial	4.6	5.8	5.1
Amin_70g03r	ferrochelatase	mitochondrial	3.0	2.8	2.9
Amin_40e21r	putative hydrolase	hydrolytic enzyme	5.5	3.1	4.1
Amin_82n24r	phospholipase/carboxylesterase family protein	hydrolytic enzyme	7.2	8.8	8.0
Amin_46a02r	Abhydrolase domain-	hydrolytic	5.6	4.0	4.7

	containing protein	enzyme			
Amin_95b07r	putative sulfatase, similar to Ats family arylsulfatases	hydrolytic enzyme	3.2	4.2	3.7
Amin_79g11r	hypothetical protein similar to various hydrolytic enzymes	hydrolytic enzyme	2.1	3.4	2.7
Amin_62h07r	hypothetical protein similar to glutathione S-transferase	detoxification	4.7	7.0	5.8
Amin_12c01r	aminotransferase, class I or II	Amino-transferase	2.1	3.0	2.5
Amin_93i12r	hypothetical protein similar to branched-chain-amino-acid aminotransferase	Amino-transferase	2.5	4.2	3.3
Amin_46c04r	hypothetical protein similar to Iron/ascorbate family oxidoreductases	Oxido-reductase	7.9	8.8	8.3
Amin_83a03f2	galactose-1-phosphate uridylyltransferase	sugar metabolism	5.2	8.6	6.7
Amin_44a04r	putative steroid oxidoreductase superfamily member	secondary metabolites	3.2	4.0	3.6
Amin_53f01f	putative lipolytic enzyme, G-D-S-L family	lipid metabolism	3.5	2.3	2.8
Amin_17d04f	putative CorA-like Mg2+ transporter protein	metal ion transport	3.5	3.7	3.6
Amin_59d02r	GFA family protein	putative carbon-sulphur lyase	20.4	22.8	21.6
Higher expressed in non-toxic strain AL1T					
Amin_30a05r	cyclin-dependent kinases regulatory subunit 1	signal transduction	-3.9	-2.9	-3.3
Amin_74g08f	dynein heavy chain family protein	intracellular transport	-5.5	-4.3	-4.9
Amin_44h03r	fibronectin type III domain-containing protein	putatively involved in signalling	-9.2	-11.3	-10.2
Amin_09b02f	glutaredoxin family protein	DNA nucleotide synthesis	-4.7	-3.2	-3.9
Amin_89h11r	hypothetical protein containing a putative 'Cold-shock' DNA-binding domain	stress response	-2.4	-3.2	-2.8
Amin_101h04r	inorganic H+ pyrophosphatase, 3'-region.	proton pump	-3.0	-1.9	-2.4
Amin_16h04r	peptide chain release factor 1	translation	-8.5	-7.5	-8.0

Amin_42k04f	hypothetical protein similar to taurine catabolism dioxygenase TauD	putative dioxygenase	-2.7	-29.7	-9.0

Additional files

Additional file 1: Primer tables and respective PCR conditions (see appendix)

All primers used and the respective PCR protocols of the study are listed in this file.

Additional file 2: List of differentially expressed genes (see file)

Table of contigs identified as differentially expressed between both toxic strains and the non-toxic strain at two time-points during light phase. Included are contig IDs, microarray-based gene expression data, characterisation of the contigs, manual annotation and results of various database searches.

Additional file 3: Phyml-based likelihood trees with bootstrap support values (see appendix)

14 phylogenies of *A. minutum* EST contigs that produced significant ($e<10^{-4}$) BLAST hits with cyanobacterial sxt-related genes. Phylogenies were calculated including the *A. minutum* contig sequence, the corresponding cyanobacterial gene, and their closest SwissProt matches as identified by PhylogGena (Top10Select-mode). Phylogenies M.2 and N.2 additionally include the best 20 hits produced by blasting the *Alexandrium* sequences against the NCBI non-redundant protein sequence database (nr).

Additional file 4: Rarefaction curve (see appendix)

Generated ESTs and assembled cluster as contigs were analysed using http://www.biology.ualberta.ca/jbrzusto/rarefact.php#Calculator.

6 Manuscript 2

Grazer induced toxin formation in dinoflagellates: A transcriptomic model study

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Abstract

The cosmopolitan marine dinoflagellate *Alexandrium minutum* is known to produce paralytic shellfish poisoning (PSP)-causing toxins. As has recently been shown, this toxicity can be induced to higher levels by the presence of certain copepod species. Inducible defences are known from a variety of marine organisms, but the associated transcriptomic changes have only been investigated in model angiosperms. Here, we use a microarray approach to investigate the changes in gene expression during the copepod-provoked induction of higher toxicity in *A. minutum*. We found a very limited set of 14 genes (0.35% of the tested sequences) to be affected by copepod presence; gene expression changed by a factor of 1.9 to 11.4 when compared to control samples. We compared the differentially expressed genes after one day - before the onset of augmented toxicity – and after 3 days, when cellular toxin content in the induced samples was 5 times higher than in control cultures. Comparison to previously found differences between toxic and non-toxic strains revealed two sequences apparently associated with PSP toxin content. Our study is the first investigation into the transcriptomal effects associated with grazer-induced induction of elevated toxicity in a protist. This provides a novel approach to study the ecology of predator-prey interactions, as well as a highly targeted method to investigate the genes associated with toxin production and regulation in toxic dinoflagellates.

Introduction

The potent sodium channel blocking neurotoxins collectively known as paralytic shellfish toxins (PSTs), including the well-known saxitoxin, are produced by at least eleven different species in the marine dinoflagellate genus *Alexandrium* (Hansen *et al.*, 2003), the dinoflagellates *Gymnodinium catenatum* and *Pyrodinium bahamense* as well as by some fresh water cyanophyceans (e.g. *Aphanizomenon flos-aqua*).

A grazer-deterring function of dinoflagellate toxins has long been suggested, but as the specific interaction strongly depends on both the toxin composition of the prey and on the grazer tested (for a review see Turner & Tester, 1997), this effect has remained controversial (Cembella, 2003). Recent results hint to a complex interplay between dinoflagellate toxicity and grazer adaptation. Colin and Dam (2002) investigated PSP toxin susceptibility in copepods from different populations and found a detrimental effect of PSP toxin-producing, but not of non-toxic, *Alexandrium* strains on the ingestion and egg production rates of copepods from populations not historically exposed to toxic *Alexandrium* blooms. Copepods from historically exposed populations were less affected and able to acclimate to the exposure within a few days. Investigating the ingestion rate of *Alexandrium* cells added to natural plankton samples, Teegarden *et al*. (2008) showed 3 of 4 tested copepod species to preferentially ingest non-toxic *Alexandrium*, but to avoid PSP-producing species. Teegarden *et al*. (2008) suggest the magnitude of this response is dependent on both the *Alexandrium* cellular toxicity and concentration relative to non-toxic prey items. Further support for a grazer-deterring function of PSP toxins was provided by Selander et al (2006) who found that PST-producing dinoflagellates increase their cellular toxin content in response to waterborne cues from zooplankton grazers. Naturally occurring densities of copepods may provoke a more than twenty-five fold increase in toxicity (Bergkvist *et al.*, 2008) in *Alexandrium*

minutum, which has also been shown to correlate with increased resistance to copepod grazing (Selander *et al.*, 2006). Besides its ecological implications for planktonic interactions and the formation of toxic algal blooms, this induction of toxin production allows for a new type of experimental design in which genetically identical algal cultures can be induced to different levels of toxin content. In combination with the recent development in genomic resources for *Alexandrium* spp. (Mansucript 1) it also offers a new approach to identify elicitor-sensitive genes and in particular the genes involved in toxin formation.

The cyanobacterial PST gene cluster (*sxt* cluster) recently identified in *Cylindrospermopsis raciborskii* (Kellmann *et al.*, 2008b) revealed a complex phyolgenetic pattern suggesting that its assembly involved both native genes and multiple horizontal gene transfers (HGTs) from different prokaryotic sources (Moustafa *et al.*, 2009). While several recent publications deal with the identity and evolution of the *sxt* genes in cyanobacteria (e.g. Kellmann *et al.*, 2008a; Mihali *et al.*, 2009; Moustafa *et al.*, 2009; Stucken *et al.*, 2010) the known similarities to dinoflagellate genes remain extremely sparse. So far, the only published similarities in dinoflagellates refer to *sxtA*, a cyanobacterial putative polyketide synthase. Two different parts of this saxitoxin cluster gene were found to be somewhat similar to two dinoflagellate EST, one from an *A. catenella* library and one from *A. tamarense* (Moustafa *et al.*, 2009). At the time of the study by Moustafa *et al.* (2009), several large-scale sequence databases for PST-producing *Alexandrium* species were available (Hackett *et al.*, 2005; Erdner & Anderson, 2006; Uribe *et al.*, 2008), so the sparseness of identified similarities might be due to a considerable change in the *sxt* genes after their HGT to dinoflagellates. During the long evolutionary timescale the *sxt* genes might have changed beyond recognition. In a recent experiment (Mansucript 1), we used an *in silico* approach to identify homologs of the *sxt* gene cluster in a normalised *A. minutum* cDNA library sequenced to

approx. 80% saturation. Although we found several sequences with similarities to the different *sxt* genes, phylogenetic analysis demonstrated all potential homologs to be closer related to sequences of organisms not known to produce PSTs. While these data are not completely conclusive, they suggest that the dinoflagellate PSP toxin genes might be too different from their cyanobacterial counterparts to be easily recognizable, and that similarity-independent ways of identifying these genes in dinoflagellates might be more promising.

Attempts to identify these genes from dinoflagellate gene expression patterns can be based either on comparisons between strains of different toxicity, or on physiological conditions affecting PSP toxin accumulation (Plumley, 1997; Cembella & John, 2006). The main challenge in both these approaches is the inherent difficulty in discerning the differences leading to the different toxin levels from those due to unrelated physiological effects. The predator-induced differences in PSP toxin accumulation provide a system in which samples of the same strain with substantially different toxin quotas can be compared without resorting to severe P-starvation to increase toxin cell quotas or to other harsh conditions known to lead to a reduction in PSP toxin content (Flynn *et al.*, 1994; Hwang & Lu, 2000; Touzet *et al.*, 2007).

The goal of this study was to identify *A. minutum* genes regulated by copepod presence and associated with the copepod-stimulated induction of higher toxin cell quotas. In order to do so, we employed EST-based oligonucleotide microarrays to compare the gene expression in copepod-challenged and untreated cultures. As the first gene expression analysis of grazer-induced toxin formation in phytoplankton primary producers, this study demonstrates the capability of this method to identify elicitor responsive genes in phytoplankton-grazer interactions.

Materials and Methods

Induction experiment

We used *A. minutum* strain GUMACC #83 (synonymous with CCMP 113 Al and Al 1V), which originates from the Ria de Vigo in NW Spain (Franco *et al.*, 1994), well within the distribution range of *C. typicus* (Beaugrand *et al.*, 2007). A culture of this strain was diluted with K/10 medium (Keller *et al.*, 1987) to an initial concentration of 8800 cells ml-1 and divided between 12 flasks. Each flask received 500 mL culture and half of them were supplemented with four adult female copepods (*Centropages typicus*). The flasks were incubated in a temperature and light controlled room (18ºC, 16:8 h light:dark cycles, ~150 µmol m-2 s-1 fluorescent light). Half of the replicates (three controls and three copepod containing bottles) were harvested after one day, the other half received a small volume of K medium (50 ml) on day two to avoid nutrient limitation and was harvested on day three. Day three cell counts were corrected for the additional dilution. At each sampling occasion a well mixed sample of 60 mL was extracted from each replicate and analysed with triplicate counts on a particle counter (Elzone 180XY). Based on the particle counts, duplicate samples containing at least 2*106 cells (200 mL at the Day 1 sampling occasion and 150 mL at the Day 3 sampling occasion) were gently prefiltered through a 67 µm nylon plankton mesh to eliminate copepods and copepod eggs, before vacuum filtration onto 47 mm nucleopore filters (8 µm). Each sample was rinsed twice with approximately 50 mL autoclaved filtered seawater, the filter was vacuum dried and immediately transferred to 50 mL centrifuge tubes with forceps and plunged into liquid nitrogen. The samples were stored in a -80ºC freezer until RNA extraction. A known volume of the remaining culture was suction filtered onto 25 mm glass-fibre filters, lyophilized and stored frozen until toxin analysis.

5 mL nutrient samples were taken from the remaining volume filtered through a 0.45 µm syringe filter and stored frozen until analysis.

Toxin and nutrient analysis

PST samples from the bioassays were extracted with 1 mL 0.05 M acetic acid (aq) through three consecutive freeze-thaw cycles. The extract was filtered (Whatman GF/A) and frozen in HPLC glass vials until analysis with high performance liquid chromatography with fluorescence detection [HPLC-FD (Asp, 2004 #104)]. HPLC analyses were carried out on a Hitachi-7000 system equipped with a Genesis C8 column, (Vymac, 4 µm, 150*3 mm). Gonyautoxin (1-4) standards were from NRC-CNRC, Halifax, Canada. The *A. minutum* strain used here is only known to produce GTX 1/4 and GTX 2/3 (Franco *et al.*, 1994; Selander *et al.*, 2006), which can be completely separated by an isocratic elution with 50 mM ammonium-phosphate buffer (pH 7.1) and 2 mM sodiumheptanesulfonate at 0.4 mL min-1. After the separation, toxins were oxidized with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0, 0.2 mL min-1) in a PEEK capillary (10 m, 80°C). The oxidation was terminated with 0.5 M acetic acid (0.2 mL min-1) before fluorescent detection at λex = 330 nm, λ_{em} = 390 nm.

Differences in cell specific total toxicity were analysed with a two factor ANOVA, with the fixed orthogonal factors time (two levels; one and three days) and treatment (two levels; copepod present or absent). The Student-Newman-Keul post hoc procedure was used to evaluate significant differences between groups. Toxin profiles were compared between treatments by comparing the ratio between the epimers GTX 1/4 and GTX 2/3 on Day 1 and Day 3 using a two-tailed t-test with α set to 0.025 after Bonferroni correction for multiple comparisons.

Nutrient concentrations (nitrate plus nitrite, phosphate) were determined using standard methods (Strickland & Parsons, 1972) to verify that the cultures were not nutrient limited during experiments.

Microarray analysis

RNA extraction followed a modified TriReagent protocol (Sigma-Aldrich, Steinheim, Germany) with additional cleaning steps. Cells were briefly lysed by 10 min incubation at 60°C in TriReagent, aided by repeated vortexing with glass beads included in the sample tube. After addition of 200 µL chloroform per mL TriReagent, samples were centrifuged for 15 min at 12000 g and 4°C. The aqueous phase was mixed with an equal volume of isopropanol and incubated at -20°C for at least 10 minutes. An RNA pellet was obtained by centrifugation at 12 000 g for 10 min at 4°C. It was washed by addition of 75% ethanol, followed by another centrifugation step. After removal of the ethanol, the pellet was allowed to dry until hyaline and then dissolved in 100 µL RNAse-free water (Qiagen, Hilden, Germany). RNA cleanup and DNA digestion followed the protocol supplied with the Qiagen RNeasy kit: RNA samples were mixed with 350 µL binding buffer RLT (Qiagen) containing 1% β-mercaptoehtanol. After mixing with 250 µL ethanol, samples were applied to an RNeasy column (Qiagen) containing a silica membrane. Columns were washed by 1 min incubation with 700 µL RW1 and centrifugation, before 10 µL DNase I mixed with 70 µL Buffer RDD (both Qiagen) were applied for 15 min. To interrupt DNase digestion, columns were washed with 700 µL RW1. Samples were incubated for 1 min in Buffer RPE (Qiagen), centrifuged, and washed again with the same buffer. After 2 minutes centrifugation and another 1 min high-speed centrifugation in a new collection tube, RNA was eluded with 40 µL RNase-free water (Qiagen). To increase final RNA concentration, the flow-through was applied to the membrane a second time. Where necessary, an additional cleanup and

concentration step using Microcon Ultracel YM-30 columns (Millipore, Schwalbach, Germany) was applied. RNA purity and quantity were determined using a NanoDrop (PeqLab, Erlangen, Germany), RNA integrity was assessed using a Bioanalyzer (Agilent Technologies, Böblingen, Germany). Total RNA (250 ng/sample) was amplified and labelled using an Agilent low-input linear amplification kit. Microarrays were scanned on a Agilent G2565AA scanner, and raw data was extracted with the Agilent Feature Extraction Software version 9.1.3.1 (FE). Array quality was monitored using the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07.

Pre-processed data were subject to SAM (Significance Analysis of Microarrays (Tusher *et al.*, 2001)) as implemented in MeV 4.0 (Saeed *et al.*, 2003), and SAM-based q-values (Storey, 2003) were calculated. Probes with a q-value of <1% were considered to indicate differential expression of the corresponding genes if the mean fold change of the sample triplicate was at least 1.5.

Results

Cell-specific toxin content was not significantly different after one day of incubation ($p=0.32$ according to Student–Newman–Keul's multiple comparison procedure (SNK)). After three days however, the toxin content per cell was approximately five times higher in the copepod-exposed cultures compared to controls (SNK $p<0.001$, ANOVA $F_{1,8}=460$, $p<0.001$; Fig. 1). The toxin profiles were dominated by gonyautoxin (GTX) 1 and 4 (97.8±1.0% of total toxin content) with small amounts of GTX 2/3 present in all samples (2.2±1.0%). The relative contribution of GTX 1/4 to GTX 2/3 was not different between treatments on the first sampling occasion ($t_{df=4}=0.96$; $p=0.39$), but on the second sampling occasion, GTX 2/3 were slightly more common in controls (3.7±0.4%) compared to grazed treatments (1.9±0.1%; mean ±SD $t_{df=4}=-11.7$; $p<0.001$).

Growth rates were similar between treatments over the course of the experiment (tdf=4=0.28; p=0.79) and averaged 0.29 d-1. The density of Alexandrium cells approximately doubled over the experimental time, and the highest increase in cell numbers was observed over the first 24 hours (Fig. 2). Day 3 cell numbers differed by less than 4% between copepod-exposed and control cultures, indicating that only a small part of the Alexandrium cells was consumed. This is in agreement with theoretical considerations based on published C. typicus ingestion rates (Bonnet & Carlotti, 2001) indicating that four adult females should not consume more than 47000 cells, or 1.07% of the initial cell number, per day.

Nutrient concentration was still high at the end of the experiment (105±6.8 μM nitrate and 1,9±0.22 μM phosphate mean +-SD) indicating that the cultures were not nutrient limited.

Microarray data identified 14 genes as being significantly higher expressed in the copepod-exposed cultures (Table 1); no gene was identified as higher expressed in the control. 7 of these genes were significantly up-regulated only on Day 1, 3 only on Day 3, and 4 genes were identified as higher expressed in the copepod-exposed cultures at both sampling occasions.. The highest measured difference was the 11.4-fold higher expression of the sequence Amin_06b12r on Day 3. The same gene was also the one with the highest relative expression on Day 1, when it showed 9.9 fold greater expression in the treatment than in the control.

Two of the differentially expressed genes could be annotated (Table 1): Amin_08 10f, which was 3.8 fold higher expressed in the copepod-conatining cultures on Day 1, was identified as a peptidylprolyl isomerase based on sequence similarity searches against various nucleotide and protein sequence databases.Amin_56a03r, which showed 10 fold greater expression in copepod-challenged cultures on Day 3, contained a 139 amino

acid domain recognised by Pfam (http://pfam.sanger.ac.uk/search) as indicative of a NAD:arginine ADP-ribosyltransferase (ART); this was verified by BLAST hits at E-values below our threshold for BLAST-based annotation (e-value>10-4).

The other differentially expressed genes could only be annotated as "hypothetical proteins" due to a lack of similar sequences in the searched databases. For some of these sequences, prediction of the protein coding open reading frame (orf) remained uncertain as well, so signal peptides and transmembrane regions could not be predicted with confidence. BLAST searches against other dinoflagellate EST libraries revealed similar sequences (e-value < 10-5) for five of the differentially expressed genes (supplementary table).

Discussion

Inducible defences have been found in widely different organisms from prokaryotes (Patterson & Bolis, 1997; Matz *et al.*, 2004) to diatoms (Lürling & Van Donk, 2000), ciliates (e.g. Hammill *et al.*, 2009) and various green, red and brown macroalgae (Toth & Pavia, 2007), but investigations into the transcriptomal changes associated with induced chemical defences have largely been restricted to angiosperm model organisms (Arimura *et al.*, 2004; Reymond *et al.*, 2004; De Vos *et al.*, 2005). To our knowledge, this is the first study to link gene expression in a planktonic organism to its reaction to grazer presence.

Grazer induced elevation of PST formation in *Alexandrium* is mediated by distant chemoreception without the involvement of mechanical damage (Selander *et al.*, 2006) and thus is a specific defensive response not confounded by wounding reactions. Consequently, measured differences in gene expression have a higher probability of being involved in the induction of higher toxin cell quotas. Of the 14 genes found to be

significantly up-regulated in copepod-exposed cultures, two have been identified in a previous study as considerably higher expressed in toxin-producing strains of *A. minutum*, as compared to a non toxin-producing strain (genes Amin_89d06r and Amin_56a03r, expressed 9.88 and 51.01 folds higher in toxic strains, respectively (Mansucript 1). As both studies identified only a small proportion of the tested genes as differentially expressed – 0.35% in this study and 4.82% in the strain comparison – this pattern suggests that these two sequences are good candidates for future studies of the regulation of PSP toxin content at the gene level.

The number of significantly differentially expressed genes in this study was lower than those identified between other physiological treatments in *A. minutum*, such as exponentially growing cultures under different salinities (Mansucript 2). While a slight strain-specific effect of the microarray probes cannot be ruled out, this further indicates that the differences in gene expression observed in this experiment are likely to be directly associated with either copepod recognition or induction of higher toxin content. Typically for genomic studies in dinoflagellates (John *et al.*, 2004; Jaeckisch *et al.*, 2008), most of the sequences identified as differentially expressed could not be annotated due to a profound lack of well-characterised similar sequences in all available databases.

Of the two annotatable genes, Amin_08c10f, which was up-regulated in the copepod-containing cultures on Day 1, was identifiable as a peptidylprolyl isomerase (PPIase). This protein family catalyzes the cis-trans isomerization of proline imidic peptide bonds and thus accelerates protein folding (e.g. Pfam: *pfam.sanger.ac.uk//family/Rotamase*, uniprot: *www.uniprot.org/keywords/?query=name:%22Rotamase%22*). In some cases, PPI-mediated peptidy- prolyl cis/trans isomerisation has been reported to act as a molecular switch mechanism between different confirmations of protein native states, which differ in ligand recognition or activity (Andreotti, 2003).

The sequence Amin_56a03r that which showed 10fold greater expression in copepod-challenged high-toxin-containing cultures on Day 3, and which has also been associated with toxic strains (Mansucript 1), was identified as an NAD:arginine ADP-ribosyltransferase (ART). The ART domain catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD^+) to an arginine residue of an acceptor protein. This mono-ADP-ribosylation is a phylogenetically ancient mechanism of reversible posttrantlational modification found both in prokaryotes and eukaryotes (reviewed in Hassa *et al.*, 2006). It was originally identified as the pathogenic mechanism of several bacterial toxins, including cholera toxin, and is generally implicated in changing the activity of target proteins. The most well-studied eukaryotic ART family members are a suite of mammalian extracellular free or membrane-bound enzymes acting on extracellular proteins (e.g. Okazaki *et al.*, 1996), but arginine-specigic mono-ADP-ribosylation is also known from cytoplasmic and nuclear targets including histones. ADP-ribosylation of different proteins has been implicated in the control of proliferation, apoptose and differentiation of specific mammalian cell types (Hassa *et al.*, 2006).

The low proportion (0.35%) of 14 up-regulated genes out of 3983 sequences tested is reminiscent of the initial response of *Arabidopsis* towards insect attack. Reymond *et al.* (2004) extrapolated the differentially expressed genes in *Arabidopsis* after 3-5 h of attack to 1.3% of the total transcriptome. Similar to our experiment, their study identified almost exclusively up-regulated transcripts. In contrast to this, the transcriptome of *Nicotiana attenuata* contained both up- and down-regulated genes after 24 h of attack by either mirids or hornworms (Voelckel & Baldwin, 2004). Although the 3 to 5 h response identified by Reymond *et al*. (2004) was almost identical between a specialist and a generalist insect, the 24 h *Nicotiana* responses against the mirids and the hornworms included more genes differentially expressed between attacking species than

commonly regulated genes. This difference vanished within 5 days, leading to the conclusion that the plant's first reaction is a rapid recognition response, which declines while the defense is mounted. A similar mechanism seems to exist in the response of *A. minutum* to *C. typicus*: While more genes are significantly induced after 1 day of exposure to the grazer, the physiological response of a considerably higher intracellular toxin content was measured after 3 days. This is consistent with the hypothesis of a short recognition and acclimation period, followed by a physiological switch to higher toxin accumulation.

It should be noted that *Alexandrium* spp produce several other bioactive compounds besides PST, e.g. spirolides (Cembella *et al.*, 1999), and the hereto unidentified lytic compounds associated with the allelophathic effects in *Alexandrium* spp. (Tillmann & John, 2002; Tillmann *et al.*, 2008; Ma *et al.*, 2009), and it is likely that there are additional bioactive secondary metabolites that we are not yet aware of and consequently will not detect when targeting only known substances. The microarray procedure on the other hand provides a non-biased tool to detect regulation of secondary or primary metabolism as long as this regulation is mediated through a change in transcriptional activity.

Conclusions

Overall, the results suggest that grazer-induced toxin formation may be a good starting point for investigations of genes involved in PST formation. Toxin levels can be manipulated with trace amounts of copepod cues, without the confounding effects of wounding (Selander *et al.*, 2006; Selander, 2007). The results show that corresponding change in transcription is modest, suggesting that toxin induction is not accompanied by a massive amount of confounding factors, as is the case when toxicity is manipulated

with e.g. phosphate limitation (Barreiro *et al.*, 2006) or the use of different species (Teegarden, 1999). A comparison of the gene expression response associated with grazer-induced elevated toxin formation with expression differences between toxin-producing and non-toxin-producing strains revealed two sequences with expression patterns suggesting involvement in the regulation of toxin content. The NAD:arginine ADP-ribosyltransferase domain-containing sequence Amin_56a03r, which has been identified as having the highest relative expression of toxic vs non-toxic cultures, was also the gene with the highest relative expression in the high-toxicity-induced cultures on Day 3. We consider this sequence as a particularly strong candidate for involvement in the regulation of cellular toxicity.

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Figures

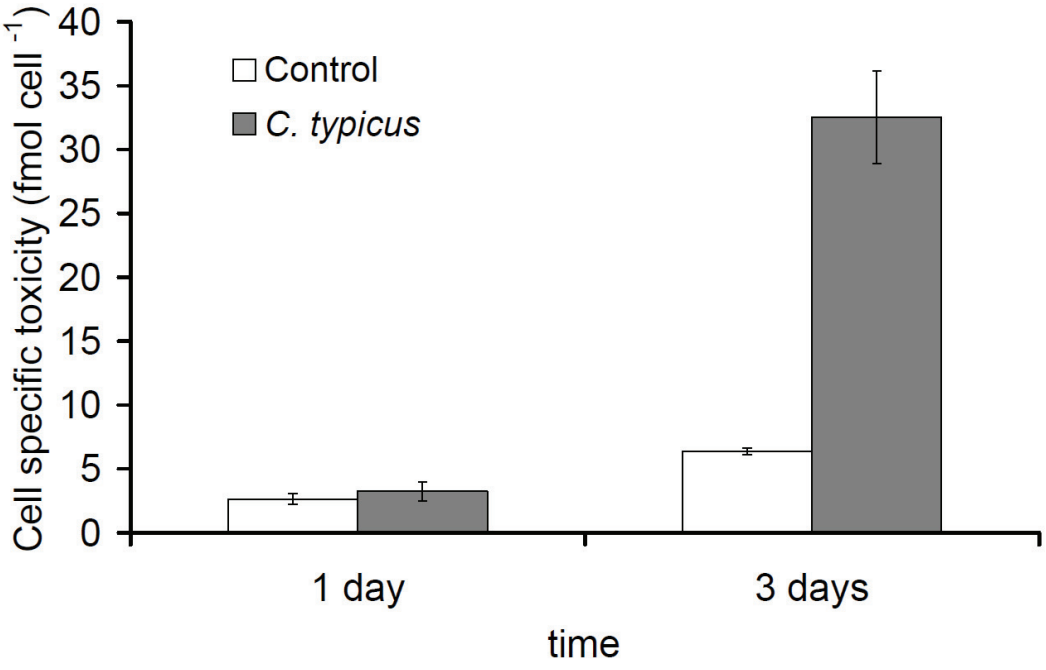

Figure 1: Cell specific toxin content in *Alexandrium minutum* cultures exposed to copepod grazers (grey bars) and controls without copepods added (white bars), after one and three days of incubation. Bars show mean values of three replicates, error bars denote standard deviation of mean.

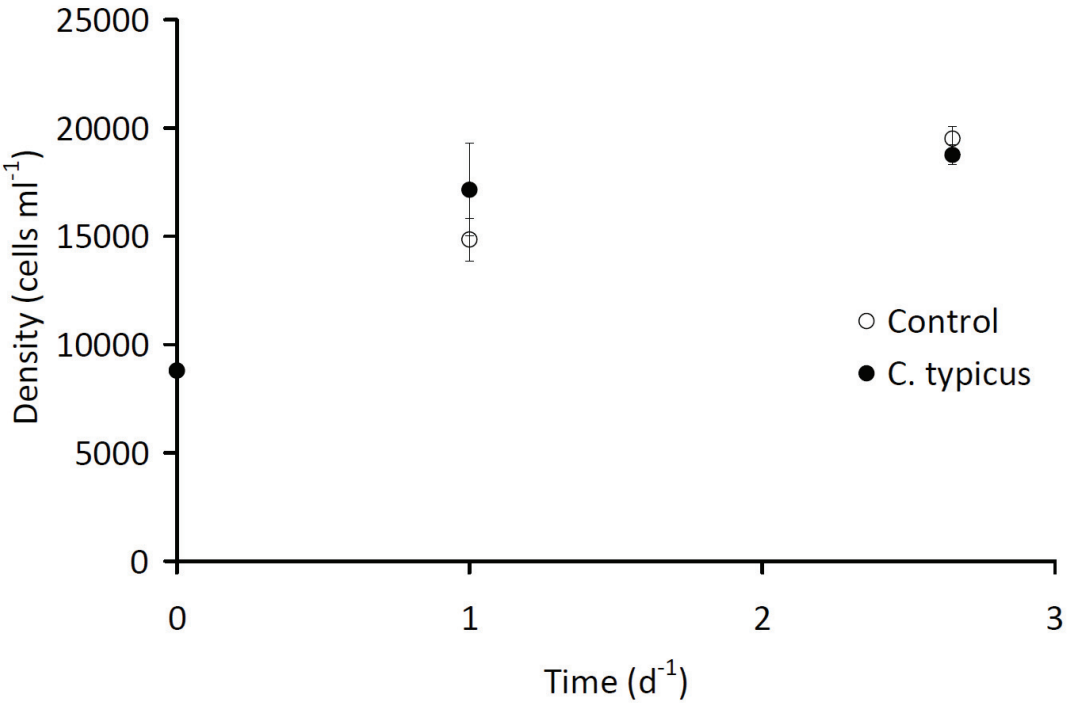

Figure 2: Density of Alexandrium minutum cells over the experimental period. Start concentrations represents a single sample whereas all other symbols represents the mean value of three replicates. Error bars denote standard deviation of mean.

Table

Table 1 Up-regulated genes. Sequences identified as significantly differentially expressed between copepod-exposed and control cultures. No sequence significantly higher expressed in the control cultures was detected. Fold changes as geometric mean of biological triplicates.

Contig name	Fold change exposed / control (Day 1)	Fold change exposed / control (Day 3)	Gene product
Amin_06b12r	9.92	11.40	hypothetical protein
Amin_08b02r	5.36	9.26	hypothetical protein
Amin_07b11f	2.88	4.61	hypothetical protein
Amin_88h09r2	5.11	2.81	hypothetical protein
Amin_26k02r	5.10		hypothetical protein
Amin_78b09r	4.81		hypothetical protein
Amin_06h01f	4.45		hypothetical protein
Amin_08c10f	3.80		peptidylprolyl isomerase
Amin_86k10r	2.39		hypothetical protein
Amin_79e01f	2.18		hypothetical protein
Amin_89d06r	1.91		hypothetical protein
Amin_88f04r2		3.54	hypothetical protein
Amin_61f03r		3.99	hypothetical protein
Amin_56a03r		9.77	hypothetical protein

Supplementary Table

Additional file: information on the genes up-regulated at Day 1 or Day 3 of copepod-induced PSP toxin production (see appendix)

Log_2 values of fold change exposed / control (mean of triplicates), standard deviation of log_2 values, fold change exposed / control (geometric mean of triplicates), annotation or lack of annotation confirmed manually. Geometric mean toxic strains / non-toxic strains (Manuskript 1). With value of best BLAST hit against other dinoflagellate EST libraries, NCBI Transcriptome Shotgun Assembly (TSA) database accession numbers and miscellaneous sequence characteristics.

7 Manuscript 3

Physiological and Gene Expression Responses to Salinity Stress in Alexandrium minutum

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Abstract

We investigated the effect of high- and low-salinity stress on the PSP-toxin-producing dinoflagellate *Alexandrium minutum*. The photosynthetic efficiency parameter F_v/F_m was affected throughout exponential growth but was most reduced in low salinity. Chloroplast-related gene expression changed most in high salinity, whereas chloroplast efficiency was less affected, suggesting that these gene expression changes are linked to chloroplast acclimation. In exponential growth, 8.8% of genes tested were differentially expressed under salinity stress, but the amplitude of regulation was low (max 3.5 fold). Salt stress acclimation seems to be regulated mainly post-transcriptionally, as 27% of the functionally annotated differentially expressed genes were involved in mRNA modification or translation-related processes. Consistent with other dinoflagellates, the high abundance of post-transcriptional and translational sequences identified in this microarray experiment suggests that this might be a general feature of dinoflagellate gene expression.

Introduction

Alexandrium minutum is a bloom-forming toxic dinoflagellate occurring from northern Europe and the Mediterranean to Asia, Australia and New Zealand. European strains typically produce gonyautoxins (GTX 1,2,3,4) associated with paralytic shellfish poisoning (PSP). This species preferably blooms in confined coastal habitats, such as harbours, estuaries or lagoons, where blooms are often initiated by input of nutrient-rich freshwater (Bravo *et al.*, 2008). Recorded salinity preferences range from s = 15 (for strains isolated from brackish water, see Hwang & Lu, 2000) to s = 34-38 (Bravo *et al.*, 2008).

This study is part of the EU project Expressed Sequence Tags of Toxic ALgae (ESTTAL; http://genome.imb-jena.de/ESTTAL/cgi-bin/Index.pl), in which we aim to examine the physiological and genetic basis for toxin production and bloom formation. Here we focus on the physiology and gene expression of *A. minutum* at the strain-specific limits of growth tolerance at extreme salinities.

Methods

Following bacteria-reduction by gauze filtration and antibiotic treatment (Manuscript 1), triplicate cultures of *A. minutum* strain AL3T (origin: Gulf of Trieste, Italy) were grown at three different salinities. Control cultures were grown in modified K-medium based on sterile-filtered aged seawater (salinity s = 32.8 ± 0.2,; all salinities according to the practical salinity scale). High salinity medium was supplemented with 15 g l^{-1} NaCl to a final salinity of 49.7 ± 0.1; low-salinity medium (s = 11.9 ± 0.2) was prepared with ⅓ aged seawater and ⅔ deionised water. Cultures were kept at 20°C at a photon flux density of 200 µmol m^{-2} s^{-1} on a 16:8-h light:dark cycle. Cultures were monitored daily

by pH measurements, microscopic cell counts and measurements of potential quantum efficiency (F_v/F_m) of Photosystem II by Pulse-Amplitude-Modulated (PAM) fluorometry. Specific growth rates were calculated: $\mu=(\ln(N_{t2})-\ln(N_{t1}))(t_2-t_1)^{-1}$ with N=cells ml^{-1} and t=sampling time. Significance of physiological data was tested according to Student's t-test at $p<0.05$. Cells were harvested in mid- and late exponential growth and stationary phase.

PSP toxins were analyzed by liquid chromatography with fluorescence detection according to Krock *et al.* (2007). Allelochemical activity (as EC_{50}) was determined by co-incubation of *A. minutum* with the cryptophyte *Rhodomonas salina* (Tillmann *et al.*, 2008).

Samples for gene expression analysis were taken during exponential growth, *ca.* 12 h after beginning of the light phase. RNA was extracted using the Sigma TriReagent protocol, followed by a modified Qiagen RNeasy cleanup (as in Manuscript 1). Total RNA (500 ng $sample^{-1}$) was amplified and labelled using an Agilent low-input linear amplification kit and hybridized to microarrays. Microarrays were scanned (Agilent G2565AA scanner), and raw data were extracted and normalized by Agilent Feature Extraction Software version 9.1.3.1. Output data were subject to SAM (Significance Analysis of Microarrays; Tusher *et al.*, 2001) as implemented in MeV. Probes with a SAM-based q-value of <1% and a minimum fold change of 1.5 were considered to indicate differential expression of the corresponding genes.

Results and Discussion

Physiological Responses

After a highly variable lag-phase, specific growth rates in exponential phase and cell numbers at beginning stationary phase were not significantly different among

treatments. We therefore consider that from the start of exponential phase the cultures were salt-stress acclimated.

The pH increased continuously during exponential phase, and during stationary phase was in the range found limiting for different dinoflagellate species (Siu *et al.*, 1997; Hansen *et al.*, 2007). The values at the beginning of stationary phase and on the two consecutive days differed significantly (p=0.05) among salinities: The three-day mean in control medium was pH 9.07 ± 0.04, in low salinity pH 8.95 ± 0.04 and in high salinity 9.27 ± 0.05.

Dark-adapted values of F_V/F_m showed major differences among treatments. Whereas control values increased during exponential growth from 0.61 ± 0.04 to 0.71 ± 0.01, high salinity values stayed at 0.57 ± 0.08 until late exponential phase, when they increased to 0.66 ± 0.06. Low salinity exponential phase Fv/Fm values (0.55 ± 0.06) were below the range of control culture stationary phase values (0.58 ± 0.04). These low values are interpreted as an indication of damage to Photosystem II, suggesting differences in chloroplast efficiency at different salinities.

Intracellular PSP toxin content was significantly lower in all salinity-stressed cultures than in control cultures, and significantly lower in high salinity than in low salinity (Fig.1a). Our results correspond to those of Hwang and Lu (2000), whose *A. minutum* strain T1, which originated from an aquaculture pond in Taiwan, grew best at s = 15, showed reduced toxicity in s = 7.5 and reached even lower toxicity values at s = 25 and s = 30.

Irrespective of salinity, allelochemical activity against *R. salina* was higher in stationary phase than in mid-exponential phase, suggesting an accumulation of allelochemical compounds in the culture. Whereas the high salinity and control cultures showed overlapping confidence intervals of their EC_{50}-values, the low salinity cultures were

considerably less allelochemically active both in late exponential and in stationary phase (Fig.1b). This reinforces the finding by Tillmann & John (2002) that allelochemical activity in *Alexandrium* is not linked to intracellular PSP toxin content.

Here we showed that even within a strain, conditions that moderately affect PSP toxin content can drastically reduce allelochemical activity, whereas others leading to a more pronounced reduction in PSP toxin content do not significantly alter allelochemical potency.

Gene expression

The microarray analysis identified 320 genes as significantly differentially expressed in salinity-stressed when compared to control cultures, accounting for 8.8% of microarray probes. Of these, 23% (74 genes) could be functionally annotated (Fig. 2, table available on request), which is in the range expected for a dinoflagellate (John *et al.*, 2004; Lidie *et al.*, 2005; Jaeckisch *et al.*, 2008). The average fold change of the genes identified as differentially expressed was 1.8, with a maximum value of 3.5.

In cultures under acute stress, general stress response genes should be expressed irrespective of the actual treatment. However, we observed no genes regulated in the same direction under both salinity-stress treatments. Instead, 12 genes were differentially expressed in both treatments but with opposite regulation; 8 of those were up-regulated in high- and down-regulated in low salinity, 4 others conversely. This response and the overall low gene expression differences may reflect the fact that acclimation was largely complete after lag phase.

Of the *A. minutum* genes differentially expressed in the salinity-stress treatments, 242 were up-or down-regulated in high salinity, and 90 changed their expression in low salinity. One of the most crucial steps of acclimation to salt stress is the re-establishment of ion homeostasis (Krell *et al.*, 2008). Some of the ion transporters are

likely still needed even after acclimation is achieved. We detected four differentially expressed sequences putatively involved in ion transport in our salt-stress acclimated samples, two of which were ion transporters up-regulated in high salinity.

Twenty of the functionally annotatable genes (27%) were involved in post-transcriptional or translational processes. Most were up-regulated in high salinity (7 genes) or down-regulated in low salinity (8 genes). Among these were genes involved in pre-mRNA processing, ribosome biogenesis and translation (table available on request). In high salinity, we also found three differentially expressed genes for protein modification. Overall these data suggest an increase in mRNA processing, translation and protein modification in high salinity and a decrease in low salinity. The high proportion of sequences involved in post-transcriptional and translational processes among the differentially abundant mRNAs suggests that an important part of gene regulation in *A. minutum* occurs during these stages. This is in accordance with the finding that 33% of diurnally controlled identifiable genes in *Karenia brevis* were involved in posttransciptional processing/turnover or protein processing (Van Dolah *et al.*, 2007).

Genes implicated in chloroplast metabolism were mainly affected by high salinity. We identified 2 down-regulated cytochrome b6 genes, whereas 8 other essential chloroplast-related genes were up-regulated. These included 4 light-harvesting complex proteins, a photosystem reaction centre and 2 different ATP synthase subunits. The only putative chloroplast gene differentially expressed in low salinity was a PPR repeat protein, which was down-regulated. This conspicuous reaction of chloroplast-associated genes to high salinity is similar to the results showing restructuring of the light harvesting complexes in the diatom *Fragilariopsis cylindrus* (Krell *et al.*, 2008) in high-salinity shocked cultures. In contrast to the situation in high salinity, where major changes in

chloroplast-associated gene expression are combined with a moderate reduction in Fv/Fm values, the major drop in chloroplast efficiency seen in low salinity is not directly reflected in the expression of known chloroplast genes. However, the down-regulation of genes associated with RNA processing and translation in low salinity hints to an overall reduction in metabolic activity.

Protein-kinases probably mediate most of the differences among *A. minutum* cultures grown in different salinities, either via signal transduction or by direct phosphorylation of target enzymes. A total of 9 sequences identified as differentially expressed were annotated as proteins involved in signal transduction pathways. These included 8 protein kinases of different specificities. A putative calcium-dependent protein kinase was up-regulated in high salinity and down-regulated in low salinity and thus might be a candidate for the regulation of salinity-specific responses.

From this dataset we cannot draw any direct conclusion on which genes might be involved in toxicity or allelochemical activity. The cyanobacterial PSP toxin gene cluster found by Kellmann *et al.* (2008) has no identifiable homologues in our EST database, nor in the published databases of other PSP-producing *Alexandrium* species (Hackett *et al.*, 2005; Uribe *et al.*, 2008), which might be due either to necessarily incomplete coverage of the dinoflagellate transcriptomes or to the huge phylogenetic distance between cyanobacteria and dinoflagellates. However, the data presented in this paper can be used in comparisons with other physiological treatments to find genes that are expressed in correlation with the production of PSP toxins or allelochemical substances.

Acknowledgements

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Figures

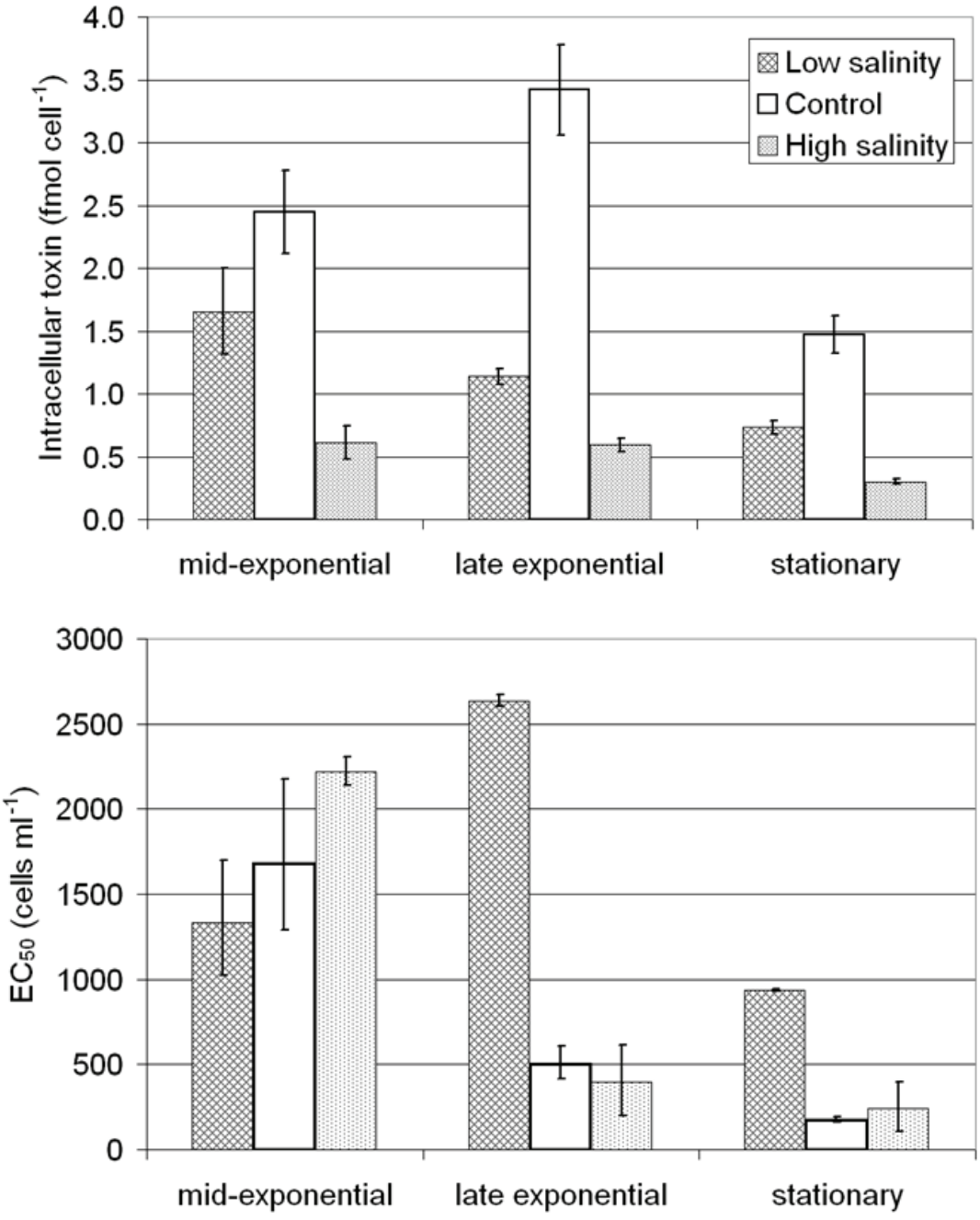

Figure 1a Intracellular toxin content. Mean values of triplicate cultures. Bars = SD (n=3). **1b** Allelochemical activity against *R. salina*. Bars = 95% confidence intervals, low salinity, s = 11.9 ± 0.2; control, s = 32.8 ± 0.2; high salinity, s = 49.7 ± 0.1

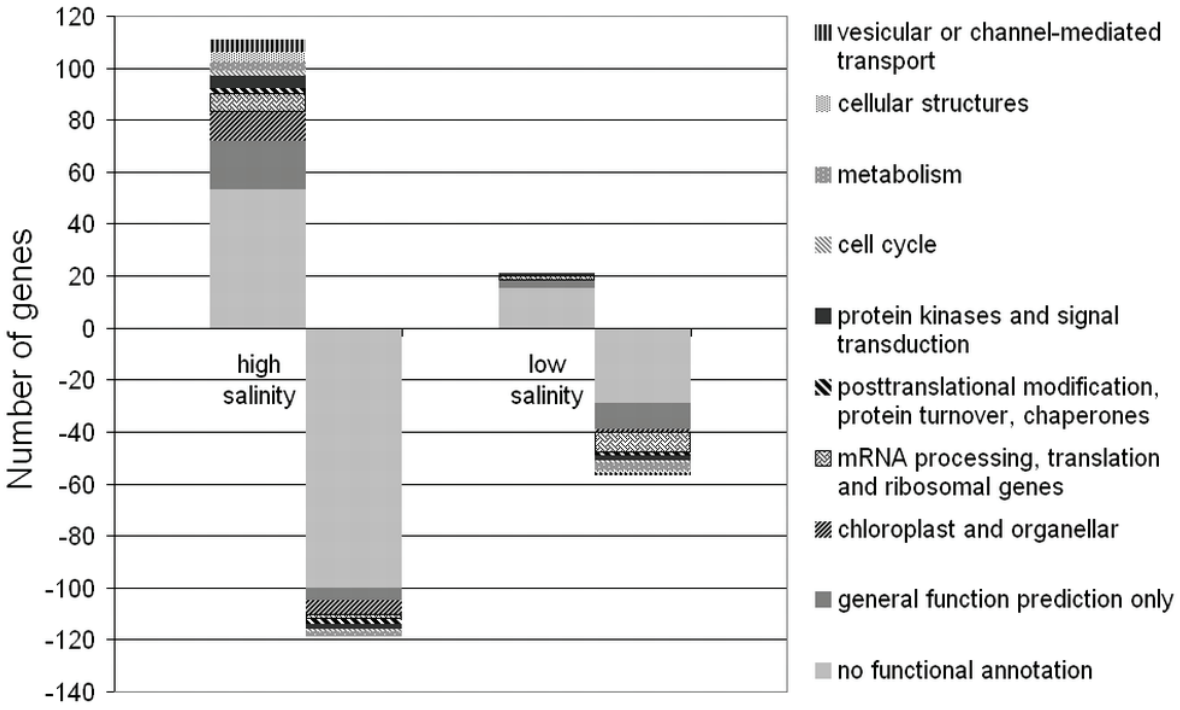

Figure 2 Functional categories of genes identified as differentially expressed between salinities. Positive range = up-regulated, negative range = down-regulated.

8 Manuscript 4

Growth- and nutrient- dependent gene expression in Alexandrium minutum

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Abstract

The toxigenic marine dinoflagellate *Alexandrium minutum* forms toxic blooms causing paralytic shellfish poisoning (PSP), primarily in coastal waters, throughout the world. We examined effects on physiology and gene expression patterns associated with growth and nutrient starvation in a toxic strain of *A. minutum*. Bloom-relevant factors, including growth rate, intracellular toxin content, allelochemical activity and nutrient status were investigated in *A. minutum* cultures grown under different environmental regimes. Allelochemical potency of *A. minutum* increased with culture age but was independent of nutrient status.

The phenotypic data were integrated and compared with gene expression in cell samples taken at selected points along the growth curve. We observed 489 genes consistently differentially expressed between exponentially growing and growth-limited cultures. The expression pattern of stationary-phase cultures was characterised by conspicuous down-regulation of translation-associated genes, up-regulation of sequences involved in intracellular signalling and some indications of increased activity of selfish genetic elements such as transposons. Treatment-specific patterns included five genes regulated in parallel in all nutrient-limited cultures. The conspicuous decrease in photosynthetic performance identified in N-starved cultures was paralleled by down-regulation of chloroplast-associated genes.

The particular gene expression patterns we identified as specifically linked with exponential growth, cessation of growth or nutrient limitation may be suitable biomarkers for indicating the beginning of growth limitation in field- or mesocosm studies.

Introduction

Dinoflagellates are ubiquitous protists and key components of marine and freshwater food webs worldwide. In many marine systems, chloroplast-containing dinoflagellates are among the most important biomass producers (Yallop, 2001; Anderson *et al.*, 2008; Thompson *et al.*, 2008). Many dinoflagellate species can form dense blooms. To a much larger extent than those of other planktonic groups, dinoflagellate blooms often pose serious health and ecosystem threats through the production of noxious, toxic or other ecosystem-disruptive substances.

Alexandrium minutum is a widely distributed toxic dinoflagellate that tends to form toxic blooms associated with paralytic shellfish poisoning (PSP) in warm temperate coastal regions worldwide, from the Mediterranean (Bravo *et al.*, 2008) to subtropical Asia (Hwang & Lu, 2000), and New Zealand (Chang *et al.*, 1997). *Alexandrium minutum* can grow under a relatively wide range of temperatures and salinities, and under low-turbulent conditions bloom development seems to be largely dependent on nutrient availability (Vila *et al.*, 2005; Bravo *et al.*, 2008). This implies that under non-bloom conditions *A. minutum* growth has a tendency to be nutrient-limited, therefore data on the physiological and gene expression differences between exponentially growing and nutrient-limited cultures are of high ecological significance. As the different growth stages in laboratory batch cultures correspond to profound physiological differences that develop over a time-scale of several cell cycles (John & Flynn, 2000), this species is also a convenient model to examine transcriptional regulation associated with acclimation of a dinoflagellate over physiologically relevant time-scales.

Dinoflagellates are often considered to exhibit poor nutrient uptake efficiency and relatively slow growth rates when compared with other phytoplankton, such as diatoms (Smayda, 1997), but this may be compensated by the typical dinoflagellate traits of circadian nutrient-retrieval migrations, high prevalence of mixotrophy, and production of allelochemicals and toxins targeted against interspecific competitors and predators (Smayda, 1997; Cembella, 2003). Some of these substances are active against other protists (Tillmann & John, 2002), while others have harmful or toxic effects on other organisms, including humans.

In order to better understand mechanisms of population dynamics and bloom formation in dinoflagellates, more knowledge of the intrinsic regulation of growth, nutrient uptake and starvation responses, as well as the biosynthesis and regulation of toxins and allelochemical substances, is required. A combination of chemical characterisation, physiological experimentation and gene expression comparisons under a variety of environmental regimes seems most promising (Cembella & John, 2006). The chemistry of dinoflagellate toxins is well known, and apart from a few newly discovered species (e.g., Tillmann *et al.*, 2009), physiological responses related to growth and toxin production in toxin-producing dinoflagellates is often well studied (Flynn *et al.*, 1994; Chang & McClean, 1997; Yamamoto & Tarutani, 1999; Hwang & Lu, 2000; Leong *et al.*, 2004; Touzet *et al.*, 2007).

Genomic studies on dinoflagellates, however, are complicated by profound doubts as to what extent methods and concepts developed in other model organisms, including protists, are applicable to dinoflagellates (Moreno Díaz de la Espina *et al.*, 2005; Bachvaroff & Place, 2008; Monroe & Van Dolah, 2008). Dinoflagellates arguably contain the most unusual eukaryotic genetic machinery known. Their huge genomes (LaJeunesse *et al.*, 2005) comprise both major proportions of apparently random, non-

repetitive DNA with very little recognizable gene content (McEwan *et al.*, 2008) and unusually high numbers of transcribed genes (Moustafa *et al.*, 2010). For example, *Alexandrium tamarense*, with about three times the nuclear DNA content of *A. minutum*, was shown to contain about 40,000 transcribed genes occurring in complex families (LaJeunesse *et al.*, 2005). Dinoflagellate chromosomes are permanently condensed into a liquid crystal state (Livolant & Bouligand, 1978; Moreno Díaz de la Espina *et al.*, 2005), and transcription as well as most of the coding sequences seem to be restricted to DNA filaments protruding into the nucleoplasm (Anderson *et al.*, 1992). Partly owing to these genomic peculiarities, fundamental aspects about the regulation of dinoflagellate gene expression are currently under debate.

Contradictory evidence exists regarding the extent of gene regulation on the transcriptomic level. Both regulation of mRNA abundances (Taroncher-Oldenburg & Anderson, 2000; Okamoto & Hastings, 2003; Hosoi-Tanabe et al., 2005; Toulza et al., 2010) and a high prevalence of translational regulation (Rossini et al., 2003; Lidie, 2007; Lapointe & Morse, 2008) have been reported in dinoflagellates. The discovery of spliced-leader trans-splicing (Lidie & Van Dolah, 2007; Zhang et al., 2007; Slamovits & Keeling, 2008) and of single-domain transcripts apparently derived from multi-domain genes led to the suggestion of trypanosome-like mechanisms of spliced-leader-associated constitutive translational gene regulation in dinoflagellates (Monroe & Van Dolah, 2008). In analogy to trypanosomes, highly expressed dinoflagellate genes were proposed to be constitutively transcribed and regulated during mRNA processing in a mechanism involving trans-splicing (Bachvaroff & Place, 2008). This model would predict transcriptional regulation to be restricted to low-copy genes mostly lacking spliced leader sequences. However, this has been challenged by the discovery of spliced leader sequences in the 5'-regions of the genes postulated to lack them (Zhang & Lin, 2009).

We investigated growth-related processes in batch cultures of A. minutum in exponential versus stationary growth phase and under nutrient starvation, to gain a deeper understanding of the physiological and transcriptomic processes associated with bloom formation and development. In addition to determining the phenotypic effect on toxin content and allelochemical activity, we compared the transcriptional response of exponentially growing and growth-limited batch cultures under different growth regimes along the growth curve. At three characteristic points of the culture cycle, we determined allelochemical activity, intracellular toxin content and intracellular and extracellular nutrient status. By means of DNA microarrays, we compared gene expression differences among cultures in exponential growth (control), at the transition to stationary phase, and several days after onset of stationary phase. Cross-comparison of the resulting patterns of differential gene expression enabled us to propose characteristic expression patterns associated with specific physiological phenomena.

Methods

Strain and culture conditions

Alexandrium minutum strain AL3T (origin: Gulf of Trieste, Italy) was grown at 20°C at a photon flux density of 200 µmol m^{-2} s^{-1} on a 16:8 h light:dark cycle. Stock cultures were kept in modified K-medium consisting of aged seawater (salinity ca. 32 practical salinity units) enriched with 440 µmol L^{-1} NO_3^-, 36 µmol L^{-1} NH_4^+, 25 µmol L^{-1} PO_4^{3-}, 10 nmol L^{-1} SeO_3^{2-}, 1000 µmol L^{-1} Trizma-Base (pH 8.3), K trace metal solution and f/2 vitamin solution (Keller *et al.*, 1987). Preparatory cultures were filtered over a 10 µm gauze, washed with sterile-filtered seawater to reduce bacterial load and grown under antibiotic treatment (50 µg mL^{-1} ampicillin, 33 µg mL^{-1} gentamicin, 10 µg mL^{-1} ciprofloxacin, 1.13 µg mL^{-1} chloramphenicol and 0.025 µg mL^{-1} streptomycin sulfate)

for 13 days, during which they were kept in exponential growth phase by repeated sub-culturing. Only starter cultures in which no bacteria could be detected by Acridine orange staining (Hobbie *et al.*, 1977) followed by fluorescence microscopy were used to inoculate the experimental treatments.

Experimental cultures were grown in 5 L Duran bottles (Schott AG, Mainz, Germany) under constant gentle aeration and sampled with a sterile tube-vacuum system as described in Eschbach *et al.* (2005). Control cultures were grown in complete K-medium as defined above; for P- or N-limited cultures, the phosphate source or the nitrate and ammonium sources were omitted, respectively, from the medium.

Sampling and daily measurements

Cultures were monitored daily by pH measurements, microscopic cell counts and measurements of potential quantum efficiency (F_v/F_m) of Photosystem II. The F_v/F_m values were determined by Pulse-Amplitude-Modulated (PAM) fluorometry using a Xenon-PAM-Fluorometer (WALZ GmbH, Effeltrich, Germany) after 15 ±5 min of dark incubation, following the method detailed by Mock and Hoch (2005). Specific growth rates were calculated: $\mu = (\ln(N_{t2})-\ln(N_{t1}))(t_2-t_1)^{-1}$ with N = cells mL^{-1} and t = sampling day. Stationary phase was defined as the growth phase where $\mu < 0.1$ d^{-1}. Samples for nutrient measurements, allelochemical assays, PSP toxin measurements, and RNA extraction were taken on Days 4 and 5 for all cultures and two to three days after each treatment triplicate had entered stationary growth phase. On the last treatment-specific sampling date, aliquots of each culture were transferred into 50 mL Erlenmeyer flasks to serve as run-on cultures for further monitoring of cell growth. In order to confirm nutrient limitation, two aliquots per nutrient-limited culture were taken, one of which was supplemented with the missing nutrient.

Nutrient analysis

Filtered medium samples for dissolved nutrient analysis were preserved by adding 3 µL 3.5% (w/w) $HgCl_2$ per mL sample and stored at 4 °C until analysis. Dissolved nutrients were analysed by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany). For total dissolved phosphorus and nitrogen, the analysis was preceded by digestion with peroxodisulphate in an autoclave.

Samples for particulate nutrient analysis were filtered on pre-combusted glass fibre GF/F filters (Whatmann, Omnilab, Bremen, Germany) and stored at -20°C. Filters for C/N-measurements were dried at 60°C and encapsulated into chloroform-washed tin containers. Samples were analysed on an NA 1500 C/N Analyzer (Carlo Erba Instrumentazione, Milan, Italy). Particulate phosphate was measured photometrically by continuous-flow analysis with photometric detection (AA3 Systems, Seal GmbH, Norderstedt, Germany) after digestion with peroxide and sulphuric acid (Kattner & Brockmann, 1980). Mean C/N values were calculated from the C/N measurements for individual filters; C/P and N/P values were determined from the average of all possible pairs of measurements for each culture at a given sampling point.

Toxin analysis

PSP toxins were extracted and prepared for analysis following the method described in Krock *et al.* (2007) (see Manuscript 1). Briefly, cells were harvested by centrifugation (3,000 x g, 4 °C). Pellets were suspended in 0.03 N acetic acid and homogenized in FastPrep tubes containing 0.9 g of lysing matrix D with a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France) at maximum speed (6.5) for 45 s. Cell debris was removed by centrifugation at 16,100 x g at 4 °C for 15 min. The supernatant was filtered through a 0.45 µm pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) by centrifugation for 30 s at 800 x g. PSP toxins were separated by ion-pair

liquid chromatography and detected fluorometrically after post-column derivatization (LC-FD) as described in Krock *et al*. (2007).

Determination of allelochemical activity

Allelochemical activity was determined by co-incubation of *A. minutum* cells with intact cultured cells of the cryptophyte *Rhodomonas salina* (Tillmann *et al.*, 2008). Briefly, different concentrations of *A. minutum* cells (in biological triplicates) were incubated with *R. salina* cells for 24 h in 20 ml glass vials in darkness. Incubations were stopped by addition of Lugol's iodine solution and numbers of intact *R. salina* cells were counted using an inverted microscope (Zeiss, Jena, Germany) at 200 - 400X magnification The *Alexandrium* cell concentrations yielding a 50% decline in intact *R. salina* cells (EC_{50}) were estimated by fitting the following equation to the cell count data using the non-linear fit procedure of Statistica (Statsoft, Germany):

$$N_{final} = N_{control} / (1 + (x / \log EC_{50})^{h})$$

with N_{final} = *R. salina* cell concentration after incubation with *A. minutum*, $N_{control}$ = *R. salina* cell concentration after incubation without *A. minutum*, x = log-transformed cell concentration of *A. minutum*, and the fit parameters log EC_{50} and h. Results are expressed as EC_{50} including 95% confidence intervals. Because of the high number of data points required to fit the equation, the data from all three replicate cultures were combined to calculate one EC_{50} value per treatment and time-point.

RNA extraction and microarray experiments

RNA extraction and microarray hybridisation were carried out as described in Manuscript 1. Cells were harvested by filtration upon an 8 µm pore-sized filter (TETP04700, Millipore Schwalbach, Germany) and rinsed with filter-sterilized seawater. Filters were quick-frozen in liquid nitrogen and later thawed by rinsing with

heated (60°C) TriReagent (Sigma-Aldrich, Steinheim, Germany). RNA was extracted according to the TriReagent protocol, following cell lysis by 10 min incubation at 60 °C in TriReagent, aided by repeated vortex mixing with glass beads included in the sample tube. Briefly, after addition of 200 mL chloroform per mL TriReagent, samples were centrifuged for 15 min at 12,000 x g at 4 °C. The aqueous phase was mixed with an equal volume of isopropanol and incubated at -20 °C for at least 10 minutes. An RNA pellet was obtained by centrifugation at 12,000 x g for 10 min at 4°C. The pellet was washed by addition of 75% ethanol, followed by another centrifugation step. After removal of the ethanol, the pellet was dried until hyaline and then dissolved in 100 mL RNAse-free water (Qiagen, Hilden, Germany). RNA cleanup and DNA digestion followed the protocol supplied with the Qiagen RNeasy kit: RNA samples were mixed with 350 mL binding buffer RLT containing 1% β-mercaptoethanol. After mixing with 250 mL ethanol, samples were applied to an RNeasy column (Qiagen) containing a silica membrane. Columns were washed by 1 min incubation with 700 mL RW1 followed by centrifugation before 10 mL DNase I mixed with 70 mL Buffer RDD (both Qiagen) were applied for 15 min. To interrupt DNase digestion, columns were washed with 700 mL RW1. Samples were incubated for 1 min in Buffer RPE (Qiagen), centrifuged, and washed again with the same buffer. After 2 min centrifugation and another 1 min high-speed centrifugation in a new collection tube, RNA was eluted with 40 mL RNase-free water (Qiagen). To increase final RNA concentration, the flow-through was applied to the membrane a second time. When necessary, an additional cleanup and concentration step using Qiagen MinElute or Microcon Ultracel YM-30 columns was applied. RNA purity and quantity were determined with a NanoDrop ND-1000 Spectrophotometer V3.1.0 (PeqLab, Erlangen, Germany), and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Total RNA (500 ng sample^{-1}) was amplified and labelled using an low-input linear amplification kit (Agilent, Waldbronn, Germany), following the Agilent protocol for synthesis of Cy3- and Cy5-labelled cRNA and microarray hybridisation. Agilent custom-made microarrays were based on the oligonucleotide probe set previously developed (Manuscript 1). Day 5 and stationary-phase samples from all treatments were hybridised against control-treatment Day 4 samples. Microarrays were scanned on an Agilent G2565AA scanner, and raw data was extracted with the Agilent Feature Extraction Software version 9.1.3.1 (FE). Array quality was monitored using the Agilent QC Tool (v1.0) with the metric set GE2_QCMT_Feb07.

Pre-processed data were analyzed using SAM (Significance Analysis of Microarrays Tusher *et al.*, 2001) as implemented in MeV 4.0 (Saeed *et al.*, 2006), and SAM-based q-values (Storey, 2003) were calculated. The SAM one-class option served to compare each treatment to the control treatment Day 4 samples hybridised on the same arrays. Probes with a q-value of <1% were considered to indicate differential expression of the corresponding genes if the mean fold change of the sample triplicate was at least 1.5. As all treatments had been hybridised against the same control samples, two-class SAM was applied to directly compare between treatments on Day 5 respectively in stationary phase. After identification of probes recognised as differentially expressed in several comparisons, the corresponding contig sequences in the *A. minutum* EST library were manually annotated.

Statistical analysis

Except for allelochemical activity (see above), physiological values are reported as the mean of biological triplicates with the associated standard deviation. Where not otherwise stated, significance of physiological data was tested according to the Student's t-test at $p<0.05$ (t-test). Normality was assessed by the Shapiro-Wilk-test as

implemented in R and variances were compared by Fisher's F-test. Significance of physiological data for which Fisher's F-test indicated unequal variances was tested according to the Welch's t-test for unequal variances at $p<0.05$ (Welch test). Microarray-based expression values are given as the geometric mean of three microarray measurements based on biological triplicates.

Results

Growth and physiological parameters

The nutrient-limited and control cultures displayed similar growth patterns during the early growth stages (Fig.1a). None of the cultures exhibited a pronounced lag phase, and mean exponential-phase growth rates were not significantly different between samples and among treatments (two-tailed *t*-test, $p=0.1$; Fig. 1b). Cell concentrations increased exponentially until Day 4, after which growth rate began to decrease under all treatments. Stationary phase was reached at Day 6 in the N-limited culture, Day 7 in the P-limited culture and Day 8 in the control treatment (see Table 1 for stationary-phase cell counts, pH and nutrient ratios). Follow-up cultures after the stationary-phase harvesting point confirmed the specific nutrient limitation: aliquots of both the P- and the N-restricted treatments resumed growth after addition of the limiting nutrient, but those without added nutrients remained in stationary phase (Fig. 1c). Follow-up cultures of the control treatment, presumably containing sufficient extracellular and/or intracellular residual N- and P-nutrients, resumed growth as well. The pH values increased with increasing cell concentration; the highest values were reached in the control treatment just before stationary phase (Figs. 1b and 2).

Throughout the experiment, the nutrient-limited culture media contained greatly reduced amounts of the limiting nutrient (Fig. 3a), which was reflected in the

intracellular nutrient levels and ratios (Fig. 3b, c). The stationary-phase control cultures had depleted the dissolved phosphate to levels similar to the exponentially growing P-limited culture by the time of the last harvesting, but intracellular P levels and P/C ratios were not significant different from those of N-limited cultures (Fig. 3c and Table 1).

The potential quantum efficiency of Photosystem II (PSII), measured as Fv/Fm, increased with increasing cell concentrations in exponentially growing control and P-limited culture (Fig. 4) and slowly decreased during stationary phase. In N-limited cultures, Fv/Fm did not change significantly during exponential phase (one-way ANOVA Fv/Fm versus time) but decreased rapidly during stationary phase.

The changes in intracellular PSP toxin content along the growth curve were strongly treatment-dependent (Fig. 5a; significance tested according to Student's t-test at $p<0.05$). At the first two sampling points, toxin content per cell was not significantly different between nutrient-replete control and P-limitation conditions. Stationary-phase cellular toxin concentrations significantly declined in control cultures. In P-limited cultures, toxin per cell was significantly elevated both with respect to values from control or N-limited cultures and to values at the second sampling point from all treatments. Intracellular toxin content in the N-limited culture was significantly lower than in the other treatments at all sampling time points but did not change significantly over the culture cycle (t-test repectively Welch test at $p<0.05$).

Allelochemical activity against Rhodomonas salina followed the same trend in all cultures. Whereas the P-limited culture was much less allelochemically active at the first two sampling points, as indicated by much higher EC50 values, allelochemical activity increased with culture age in all three treatments (Fig. 5b).

Gene expression

Out of 4,298 *A. minutum* sequences represented in the database, 1,781 (41%) were identified as differentially expressed between exponential-phase control samples and at least one of the treatments at the second or third sampling time-point (Table 2 a,b). Among these genes, 1,025 were differentially expressed between one or both of the nutrient-limited treatments and the exponentially growing control.

The set of 1,565 genes up-regulated in the stationary phase compared to exponentially growing control cultures contained two carbonic anhydrase (CA) sequences. A dinoflagellate-type extracellular delta-CA, Amin_85n03r, was expressed 1.65-fold higher in the stationary phase control. This sequence was also 2.09-fold up-regulated in the stationary-phase N-limited cultures when compared to exponentially growing controls. The second sequence Amin_77b06f is an intracellular CA similar to a sequence known in the pennate diatom *Phaeodactylum tricornutum*. This gene was 2.12- fold up-regulated in the stationary-phase control relative to the exponentially growing control. For both Amin_85n03r and Amin_77b06f, expression differences between all other tested pairs of growth conditions remained non-significant.

In searching for consistent patterns of up- and down-regulation among datasets, we identified 554 sequences repeatedly associated with one of the tested physiological regimes (Table 3). The expression of 489 genes was linked to the difference between exponential growth and all tested growth-limiting conditions for A. minutum cultures. Table 4 depicts a selection of these genes for which a function was assignable.

In stationary-phase control cultures 8 genes were down-regulated relative to exponential-phase controls and relative to both nutrient-limited treatments (Table 5); these were identified as characteristic for the stationary phase in control cultures.

In both the comparisons with exponential- and stationary-phase cultures (Tables 3 and 6), 87 genes were associated with N- or P-limitation. Among these genes, 5 were regulated in parallel under all nutrient-limited regimes (Table 3, supplementary Table 1), but none of them could be annotated to function.

Analysis of the frequency of complete spliced leader (SL) sequences in the set of differentially expressed genes in comparison with the whole underlying EST library revealed no apparent pattern. Examination of the associated EST contigs showed that 4.4% of the genes differentially expressed between one of the treatments and the exponential-phase control samples contained a complete SL sequence. The same was true for 5.8% of the sequences that could be linked to physiological conditions. Both percentage values are similar to the 4.7% SL-containing sequences in the whole library.

Discussion

Nutrient limitation

For both nutrient-limited and control cultures, nutrient quotas and ratios were in the range of values previously published for *A. minutum* in laboratory experiments (Flynn *et al.*, 1994; Maguer *et al.*, 2007), and also agreed with those reported for other *Alexandrium* species (John & Flynn, 2000; Juhl, 2005). Molar nutrient ratios (C:N, C:P, N:P) in the control cultures were at the lower range of the reported values for nutrient-replete cultures of various marine microalgal species (Geider & La Roche, 2002). Control culture values were also lower (Fig. 3c) than the canonical Redfield ratios (Redfield, 1958) of mol C:N:P = 106:16:1 considered to represent balanced growth conditions in natural populations. Similar to other *Alexandrium* species, *A. minutum* is known to be a specialist for intracellular storage of P (Labry *et al.*, 2008), and to a lesser extent of N (Flynn *et al.*, 1996; Maguer *et al.*, 2007), during nutrient pulses. The

nutrients stored during the periods of "luxury consumption" can later be mobilised for growth when extracellular nutrients are depleted. Laboratory cultures are typically grown on "excess" inorganic N and P often at high external N:P ratios. Our *A. minutum* control cultures were inoculated into K-medium at 476 µmol L^{-1} total inorganic N and 25 µmol L^{-1} PO_4^{3-}, apparently triggering significant intracellular nutrient storage and correspondingly reduced nutrient ratios.

As expected, in the nutrient-limited cultures, the intracellular amount of the limiting nutrient decreased both in absolute and in relative terms (Fig. 3b,c), indicating that acclimation to these conditions involves major changes in intracellular biochemistry. The N:P ratio of 44.6+/-11.8 reached in the P-limited cultures were somewhat lower than those typically attained by a variety of marine microalgae under P limitation (Geider & La Roche, 2002). However, given the high DNA content of about 29.9 pg DNA per cell in A. minutum (Figueroa et al., 2010) and the approximate elemental composition of DNA (Geider & La Roche, 2002), the genomic DNA alone should account for about 56% of the intracellular P in stationary phase P-limited cultures (0.16+/-0.03 pmol cell-1).

Stationary phase in control cultures

Even with abundant aeration, high pH in batch cultures and natural populations of microalgae is both a cause and indicator of insufficient biologically available dissolved C to sustain further growth (Berman-Frank *et al.*, 1994; Lundholm *et al.*, 2005).Growth in the stationary-phase control cultures, where N- and P-nutrients were replete, was apparently limited by availability of dissolved CO_2, as indicated by the high pH in the culture vessels (Fig. 2) and the resumption of growth after transfer to the small-volume follow-on culture. Limitation by low dissolved inorganic carbon is a phenomenon often encountered in stationary-phase batch cultures, but it has also been reported from

dinoflagellate blooms in freshwater (Berman-Frank *et al.*, 1994) and brackish water (Hansen, 2002). In addition to its effect on carbon availability, high pH in itself has been shown to be a possible limiting factor for dinoflagellate growth (Hansen *et al.*, 2007), an effect supposedly mediated by a partial inhibition of cellular proton pumps leading to increased intracellular pH which can disrupt pH-sensitive enzymatic processes and lead to cell leakage (Søderberg & Hansen, 2007). Carbon or pH limitation of dinoflagellate growth is unlikely to occur in marine ecosystems, due to higher seawater buffer capacity (see Wolf-Gladrow & Riebesell, 1997). However, *A. minutum* can occur and also bloom in brackish coastal waters (Hwang & Lu, 2000; Piumsomboon *et al.*, 2001) and therefore responses to high pH and low CO_2 availability are potentially ecologically relevant.

Photosynthetic performance

The potential quantum efficiency of Photosystem (PS)II, measured as F_v/F_m , is a sensitive indicator of photosynthetic performance and as such is often used as a general stress indicator for photosynthetic cells (Nedbal *et al.*, 2000; Kim *et al.*, 2006; Hsu, 2007; Tillmann *et al.*, 2008). The F_v/F_m increase in exponentially growing control- and P-limited cultures was associated with increasing cell concentrations (Fig. 3), and apparently results from acclimation to decreasing light availability in increasingly dense cultures. Similar effects are known from the green macroalga *Cladophora* sp., where increased F_v/F_m values are reported for light-limited environmental samples (Hiriart-Baer *et al.*, 2008), and from cultured isolates (clades A, B and F) of the symbiotic dinoflagellate S*ymbiodinium*, in which acclimatisation to high light was associated with reduced F_v/F_m values (Robison & Warner, 2006).

The decrease in F_v/F_m measured in all stationary cultures may correspond to a higher proportion of damaged PSII centres due to impaired repair mechanisms (Lippemeier *et*

al., 2001; Takahashi & Murata, 2008) or to down-regulation of photosynthesis-associated processes in non-growing cells. The lower demand for carbon compounds, ATP and redox equivalents (see Geider *et al.*, 1993) in combination with the reduced availability of dissolved CO_2 at higher pH values in older cultures, can lead to a diversion of photosynthetic electrons to oxygen, resulting in the production of reactive oxygen species (ROS) (Vardi *et al.*, 1999). Limitation of photosynthetic CO_2 fixation can decreases the consumption of the reducing agent NADPH, potentially leading to depletion of its reduced form $NADP^+$, the major acceptor of electrons for Photosystem I. This increases the transfer of electrons from PSI to molecular oxygen. The generated ROS impede the repair of photodamaged PSII by inhibiting the synthesis of new D1 protein at the elongation step of translation, leading to photoinhibition (reviewed by Takahashi & Murata, 2008).

Factors influencing toxin content

Similar to other studies on various PSP toxin-producing *Alexandrium* species (John & Flynn, 2000; Lippemeier *et al.*, 2003; Leong *et al.*, 2004), intracellular PSP toxin content in our cultures was closely linked to nutritional status. This sensitivity to nutrient limitation has been attributed to variation in intracellular concentrations of arginine (John & Flynn, 2000), which is a biosynthetic precursor of PSP toxin biosynthesis in cyanobacteria and likely also in dinoflagellates (Shimizu, 1982; Kellmann & Neilan, 2007). Anderson *et al.* (1990) found that under most growth conditions, in laboratory cultures of *A. fundyense*, cellular concentrations of free arginine were low when toxin content peaked but increased rapidly as toxin content declined. This relationship also held for P-limited cultures. While intracellular toxin quota cannot be interpreted as a direct measure for the rate of toxin production (Cembella, 1998), P-limitation in *Alexandrium* tends to lead to an increase in toxin

productivity not only on a cellular but also on a culture volume basis (Lippemeier *et al.*, 2003). Anderson *et al.* (1990) explained this observation by the existence of a saxitoxin biosynthetic pathway that continues to operate even after the cessation of cell division, and which then depletes cellular arginine pools with greatly reduced competition from other pathways. Lippemeier *et al.* (2003) attributed the elevated toxin synthesis during P limitation to a potential arrest of cell cycle in G1, which is the cell cycle stage in which toxin production occurs (Taroncher-Oldenburg *et al.*, 1997). This arrest in G1 would imply a continuous expression of G1-specific genes, which should include at least some of the genes coding for PSP toxin biosynthetic enzymes.

Allelochemical activity

Allelochemical activity against *Rhodomonas salina*, unlike PSP toxin content, did not respond in a notably treatment-specific pattern (Fig. 4b). In contrast to the situation in the haptophyte *Prymnesium parvum*, in which lytic activity is considerably induced by both N- and P-starvation (Granéli & Johansson, 2003), the pattern observed in *A. minutum* is more reminiscent of an accumulation with culture age that might be secondarily influenced by nutrient availability (Fig. 5b). An induction by growth limitation acting non-discriminately under P-, N- and pH- or C-limitation cannot be ruled out. In order to further illuminate the factors influencing synthesis and accumulation of the lytic compounds, highly targeted datasets combining a high number of measurements of allelochemical activity with finely graded variations of the physiological parameters seem most promising. The elucidation of the chemical composition and structure of those compounds is currently under way (Ma *et al.*, 2009).

Gene expression in A. minutum

Dinoflagellates are evolutionarily distant from most model organisms, even among apicomplexa. Functional annotation of their genes is therefore often difficult (John *et*

al., 2004). With the *A. minutum* EST library herein analysed, BLAST-based automated annotation as a starting point for manual annotation was available for only 28% of the sequences (Manuscript 1). In any case, physiological responses of the toxigenic dinoflagellate *A. minutum*, including growth kinetics and nutrient-dependent limitation, PSP toxin biosynthesis and cell content, and allelochemical activity, are reflected in comparisons of gene expression among cultures grown under different environmental regimes. While this restricted availability of genomic information precluded pathway reconstruction, we nevertheless observed patterns characteristic for the processes involved in acclimation to the different treatments.

Growth stage dependent gene expression across all treatments

The high number of genes consistently up- or down-regulated in all stationary-phase cultures when compared to the exponentially growing control (Table 2b) mirrors the fundamental physiological differences between the different culture growth stages. Gene expression differed considerably more among growth stages than between different treatments at the same growth stage (Tables 2a, 2b, 3), demonstrating an essential dissimilarity between actively growing and non-growing cells. An equivalent effect is documented in yeast, where the stages of fermentation, which correspond to the different growth stages in batch cultures, can affect the overall gene expression to a considerably greater degree than the fermentation medium or even the strain examined (Rossouw & Bauer, 2009).

Not unexpectedly, the largest functional group among the genes higher expressed in exponential phase were genes involved in translational processes (Table 4). This is in agreement with the high number of sequences involved in protein synthesis and translational regulation identified in a recent study in *A. catenella*, which involved a gene expression library of exponential-phase *A. catenella* subtracted with a stationary-

phase sample (Toulza *et al.*, 2010). These authors considered the expression of the ribosomal component 5.8S rRNA as reflecting the whole-cell metabolic activity. Following this suggestion, the reduced expression of 14 ribosome-connected sequences is an indication of reduced translational and metabolic activity associated with stationary phase. While a high incidence of post-transcriptional regulation in dinoflagellates has frequently been reported (Rossini *et al.*, 2003; Lidie, 2007; Lapointe & Morse, 2008), we found only one translation elongation factor-like protein and two splicing-related proteins to be down-regulated across all treatments.

In all stationary-phase treatments, gene expression patterns revealed evidence of down-regulation of photosystem components. Reduction of the number of photosystem reaction centres, otherwise known as a photoacclimation strategy (e.g. Ragni *et al.*, 2010), is apparently linked to the reduced F_v/F_m values in stationary-phase cultures. This is likely to be either a response to photoinhibition due to ROS production linked to reduced photosynthetic CO_2 fixation, or an acclimatisation response to avoid such damage. The main causes for the stationary-phase reduction in carbon fixation differ among treatments. In control and N-limited cultures, this effect may be directly caused by limiting CO_2 concentrations (see above) and reduced amounts of the CO_2-fixing enzyme (Huang *et al.*, 2004), respectively. The reason for the reduced carbon fixation in P-limited cultures is less well understood, but appears to be related to a decrease in photosynthetic electron transport capacity, which decreases the ATP content and hence CO_2 fixation, while increasing the likelihood of damage from excess absorbed excitation energy (Moseley *et al.*, 2006; Lin *et al.*, 2009b). Both photosynthetic performance and the related gene expression are most affected in the N-limitation treatment.

The most prevalent functional group among the genes higher expressed in all of the stationary-phase cultures were sequences involved in intracellular signalling, which might be due to an increase in stress-related effects. Like the differential expression of translation – and photosynthesis-related gene expression, the importance of intracellular signalling-related sequences has also been noted in *A. catenella* (Toulza *et al.*, 2010).

As the group of growth-state indicative genes identified here is correlated with the difference between exponentially growing cells and cells limited by different environmental factors, it provides an interesting starting point to identify marker genes or marker gene expression profiles to identify the level of growth limitation in mesocosm experiments or in potential *A. minutum* bloom populations.

Gene expression linked to stationary phase in control cultures

While a high number of genes were differentially expressed between exponentially growing controls and the same cultures in stationary phase, significant expression differences between the stationary-phase samples from different treatments were rare (Table 2b). Of the 8 genes identified as specifically down-regulated in the stationary-phase control cultures as compared to both the exponential-phase control and the nutrient-limited cultures, three sequences were identified as ribosomal proteins, indicating a further down-regulation of the translational machinery when compared to the nutrient-limited cultures (Table 5).

Growth in these cultures was apparently limited by availability of dissolved organic carbon (see above). The typical dinoflagellate response to a reduced concentration of dissolved CO_2 is an increase in amount and per-cell-activity of the enzymes involved in the dehydration of HCO_3, the extracellular and intracellular carbonic anhydrases (CAs) (Berman-Frank *et al.*, 1994; Ratti *et al.*, 2007; Lapointe *et al.*, 2008). We identified an extracellular δ-CA similar to that of the bloom-forming marine dinoflagellate

Lingulodinium polyedrum (Lapointe *et al.*, 2008; see Toulza *et al.*, 2010), as well as an intracellular CA, which were both significantly higher expressed in the stationary-phase control culture than in the exponentially growing control (supplementary Table 2).

Nutrient limitation-related gene expression patterns

Metabolic pathways and related gene expression conceivably affected by nutrient supply include mechanisms of active transport, nutrient assimilation and sequestration, as well as biosynthetic pathways leading to core components (proteins, nucleic acids, lipids) and secondary metabolites. Indeed, this is reflected in the gene expression data for *A. minutum*, as 23% of the genes tested were differentially expressed between at least one of the nutrient-limiting treatments and exponentially growing control cultures. Most of these changes were associated with the difference between exponentially growing and stationary-phase cultures irrespective of the limiting factor, but 87 genes specifically responding to N- or P-starvation were identified (Tables 3, 6).

The most conspicuous group among these nutrient-limitation responsive sequences were the chloroplast-associated genes differentially regulated under N-limitation. For example, 8 mRNAs encoding cytochromes and photosystem proteins were on average 9.3 times less expressed under N-limitation than in the exponentially growing control, and those for two chlorophyll a-c-binding proteins and a ribulose-bisphosphate carboxylase (RubBisCO) were up-regulated by an average factor of 5.4 (Table 6). This is consistent with the marked N-dependency of these two core components of the photosynthetic apparatus. The key carbon fixation enzyme RubBisCO catalyzes the rate-limiting step of light-independent photosynthetic reaction and is generally the most abundant protein in photosynthetic organisms. Levels of RubBisCO can be severely compromised under N-limitation (Sims *et al.*, 1998; Huang *et al.*, 2004), which can limit overall photosynthesis (Parry *et al.*, 2008). The reduced CO_2 fixation can lead to

substantially increased ROS production (see above) and cause enhanced photoinhibion (Takahashi & Murata, 2008). An up-regulation of RubBisCO on the mRNA level might partially compensate this effect. Synthesis of the chlorophyll tetrapyrrole skeleton is dependent on the availability of amino acid precursors (reviewed in Nogaj *et al.*, 2005), and lower chlorophyll quotas in N-limited cells are common (Verhoeven *et al.*, 1997; Sciandra *et al.*, 2000; de Groot *et al.*, 2003). This can be associated with reduced amounts of light harvesting complexes, while other components of the light harvesting machinery shift abundance (Peltier & Schmidt, 1991). The observed adjustments of the mRNA levels of chlorophyll-binding proteins in connection with reduced mRNA amounts of other photosystem proteins and different cytochromes is consistent with these phenomena. They are reflected in the decreased potential quantum efficiency of Photosystem 2 (PSII) (Fig. 4) in the nitrogen-limited cultures. Similar strong effects of N-starvation on photosynthetic performance are known from a variety of phytoplankton species (Flynn *et al.*, 1994; Lippemeier *et al.*, 2001; Juhl, 2005).

Transition to stationary phase

The 5 genes up-regulated at the transition to stationary phase in all treatments (Table 3) are associated with the onset of limitation or with a slowing of growth. As such, they constitute putative candidates as markers for the onset of growth limitation and potentially bloom breakdown.

In *Alexandrium tamarense*, nutrient limitation can lead to bloom breakdown by induction of resting cyst formation, which tends to remove considerable fractions of the vegetative population (Anderson & Lindquist, 1985; Ichimi *et al.*, 2001; Yamamoto & Seike, 2003). While N- and P-limitation can promote cyst production in *A. minutum* under laboratory conditions (Blanco, 1995; Figueroa *et al.*, 2007), *A. minutum* bloom decline cannot usually be attributed to encystment (Garcés *et al.*, 2004), as cyst

production in bloom populations apparently is a constant process, involving a continual small percentage of cells throughout most of the bloom duration (Garcés *et al.*, 2004; Pitcher *et al.*, 2007).

Nevertheless, in addition to the obvious implications of a marker for restricted growth, physiological state might influence the susceptibility of *A. minutum* to parasites and pathogens (Llaveria *et al.*, 2010). This would indicate an increased likelihood of bloom termination by otherwise tolerated (but see Chambouvet *et al.*, 2008) levels of infection (Figueroa *et al.*, 2008). Furthermore, a decrease in growth rate can lead to significant reductions of the dinoflagellate population by microzooplankton grazing (Calbet *et al.*, 2003), which might otherwise have little effect on an established bloom (Van Lenning *et al.*, 2007; Estrada *et al.*, 2010).

The genes associated with the transition to stationary phase included a serine/threonine-protein phosphatase, potentially a regulator of enzyme activity, and an ABC transporter probably involved in active transport across membranes. The transporter sequence was the only one of the transition phase-associated sequences that was not identified as associated with stationary phase as well. This reinforces the potential utility of these genes as markers for early stages of limitation.

Toxin-related gene expression

Our gene expression results contribute little to the elucidation of the regulation of PSP toxin content. While more than half of the genes differentially expressed between the highly toxin-containing P-limited cultures and the low-toxin N-limited cultures could be annotated, ,all inferred functions suggested involvement in the regulation of core metabolic responses (Table 7). Nevertheless, the differential expression pattern of two of the non-annotatable sequences was consistent with an involvement in the regulation of PSP toxin levels: Amin_21a03r was up-regulated under both beginning and severe P-

limitation and down-regulated under severe N-limitation when compared to any other condition, and Amin_78e07r was higher expressed under P-limited than in the N-limited conditions, less expressed under N-limited than under stationary phase control conditions, and not significantly differentially expressed any other comparison (Table 7). Additionally, earlier BLAST comparisons had identified a sequence similar to Amin_78e07r in the PSP toxin-producing related species *A. tamarense*, but not in any other dinoflagellate, including several other PSP toxin producing and non-PSP-toxin-producing *Alexandrium* species (Manuscript 1). However, as these BLAST comparisons were based on non-exhaustive EST databases, and absence of a sequence in these databases is not conclusive evidence of absence in the respective organism. Nevertheless, as these two sequences are genes of unknown function with an expression pattern suggestive of involvement in PSP toxin regulation, they warrant further examination in more targeted experiments.

Gene expression – general trends

The data presented here show substantial changes in the *A. minutum* transcriptome associated with medium-term differences in growth and nutrient status, adding to a complicated pattern of transcriptomic and translation-level regulation of gene expression in dinoflagellates:

We identified 41% of all tested sequences to be differentially expressed between any of the growth-limited treatments and the exponential-phase control samples (Table 2 a,b). This is a somewhat higher change rate than reported from the related species *A. tamarense* in a recent MPSS study (Moustafa *et al.*, 2010): In *A. tamarense*, 73% of transcripts remained uniformly abundant irrespective of N-or P-limitation or the presence or absence of bacteria. Whether this difference is due to true differences in the

prevalence of transcriptome-level gene regulation, or due to the different experimental setups, remains to be tested.

In different dinoflagellate species, regulation of the mRNA pool seems to be mainly found in association with longer-term physiological differences, such as adaptation to different light regimes on a timescale of days to weeks (Roman *et al.*, 1988; ten Lohuis & Miller, 1998),or during different stages of the sexual life-cycle (Hosoi-Tanabe *et al.*, 2005). While part of the gene regulation associated with different stages of the cell cycle is associated with differences in transcript abundance (Taroncher-Oldenburg & Anderson, 2000), several studies confirmed translational regulation of marker genes over the circadian cycle (Mittag *et al.*, 1994; Rossini *et al.*, 2003; Lapointe & Morse, 2008). A microarray investigation revealed that about 10% of the *K. brevis* transcriptome changes over the duration of the light-dark cycle. Genes involved in posttranscriptional processing of RNA and protein turnover were unusually highly represented among these sequences. On the other hand, cell cycle genes did not seem to be transcriptionally regulated (Van Dolah *et al.*, 2007). In the same species, acute peroxide stress lead to conspicuous changes in protein levels which in most cases were not associated with corresponding changes in transcript levels (Lidie, 2007). Instead, short-term shock response, which was monitored during the first 240 min after shock treatment, seemed to be associated with changing representations of stress-related mRNAs in the actively translated pool. In a similar stress study on a slightly longer timescale, about 4% of *P. lunula* genes were differentially expressed on the transcriptome level 6h after beginning redox-stress treatments (Okamoto & Hastings, 2003). The transcriptomic response affected several groups of conventional stress-response genes, including several kinases, proteins involved in protein ubiquination and degradation, and transcription factors.

These differences might be strongly dependent on the timescale investigated: a strong translational component of the short-time response to shock treatments is also known from other organisms such as yeast (Melamed *et al.*, 2008), in which the inhibition of protein synthesis caused by salt shock is reversed over a timescale of 5h. In human cell cultures under hypoxia, rapid translational responses are mediated by the translation factor elF2, resulting in a drop in the average number of ribosomes per translated transcript. This effect reaches a maximum at 1–2 h. The response to prolonged anoxia (~16 h) is associated with disruption of mRNA cap-binding complex, resulting in a reduction in fraction of mRNA found within polysomes. While the overall result is an inhibition of global mRNA translation, specific mRNAs are preferentially translated or even translationally induced (Koritzinsky *et al.*, 2006). Another factor differentially influencing the translation of mRNAs is the activity of microRNAs, single-stranded RNA molecules that form part of a ribonucleoprotein complex which selectively mediates the degradation, destabilisation or translational inhibition of target mRNAs (reviewed in Winter *et al.*, 2009). MicroRNAs are well studied in animals and plants, but have also been identified in various protists (Lin *et al.*, 2009a, and references therein).

In general, translational gene expression is a phenomenon well known from model organisms and is probably especially prominent during rapid stress response. Nevertheless, available data suggest a particularly strong component of post-transcriptional regulation in dinoflagellate gene expression, not only during shock response but also associated with other short-term physiological changes such as differences over the light-dark cycle. Even in long-term adaptation genes involved in the regulation of translation and protein degradation tend to be among the most highly regulated functional groups, but this is not associated with a systematic lack of regulation on the transcriptomic level.

In analogy to the situation in trypanosomes, the phenomenon of mRNA transsplicing in dinoflagellates has been suggested to be associated with post-transcriptional control of gene expression (Bachvaroff & Place, 2008; Monroe & Van Dolah, 2008). Our data on the percentages of differentially expressed *A. mintum* sequences which contain the typical transsplicing-associated spliced leader sequence suggests that spliced-leader transsplicing in dinoflagellates is not associated with a lack of regulation at the transcriptional level. Whereas 4.7% of all the sequences in our normalised EST library contained a spliced leader sequence, the same was true for 4.4% of the genes differentially expressed between one of the treatments and the exponential-phase control samples, and for 5.8% of those showing the same trend of differential expression in several comparisons. As many of these ESTs are incomplete and 3' end-biased representations of the corresponding mRNAs, SL sequences are very probably hugely underrepresented. The mentioned percentages should therefore not be taken as indicative of the prevalence of SLs in *A. minutum* mRNAs. While the equal representation of spliced leader sequences among the differentially expressed genes does not imply any specific function of this sequence, the data presented here are in agreement with those of Zhang and Lin (2009), who found spliced-leaders associated with all sequences examined. This indicates that even if SL trans-splicing is involved in the post-transcriptional regulation of gene expression, e.g. in mRNA stabilisation or in the recruitment into the actively translated mRNA pool, it is probably not restricted to constitutively transcribed genes.

Conclusions

The dataset presented here constitutes a comprehensive series of measurements related to bloom-relevant factors such as toxin content, allelochemical activity, nutrient limitation and growth. While the growth stages of batch cultures are a rather crude

model for the stages of bloom development, they nevertheless permit a comparison between actively proliferating and non-growing cells. Using extensive cross-comparisons of the differential gene expression responses, we identified narrow gene expression patterns linked with specific physiological factors such as exponential growth, cessation of growth or nutrient limitation.

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Figures

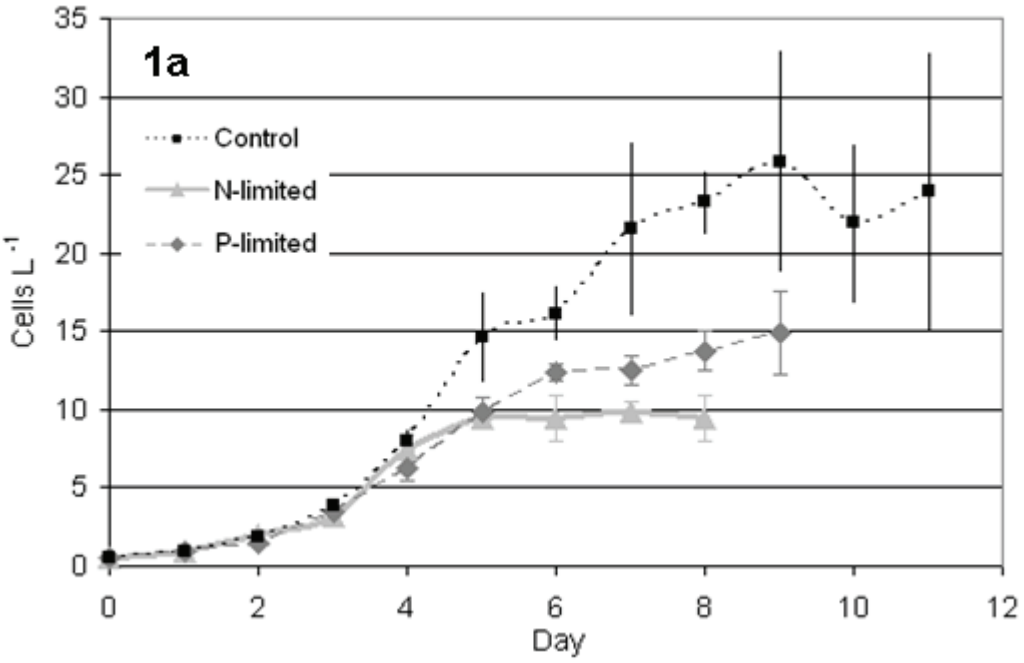
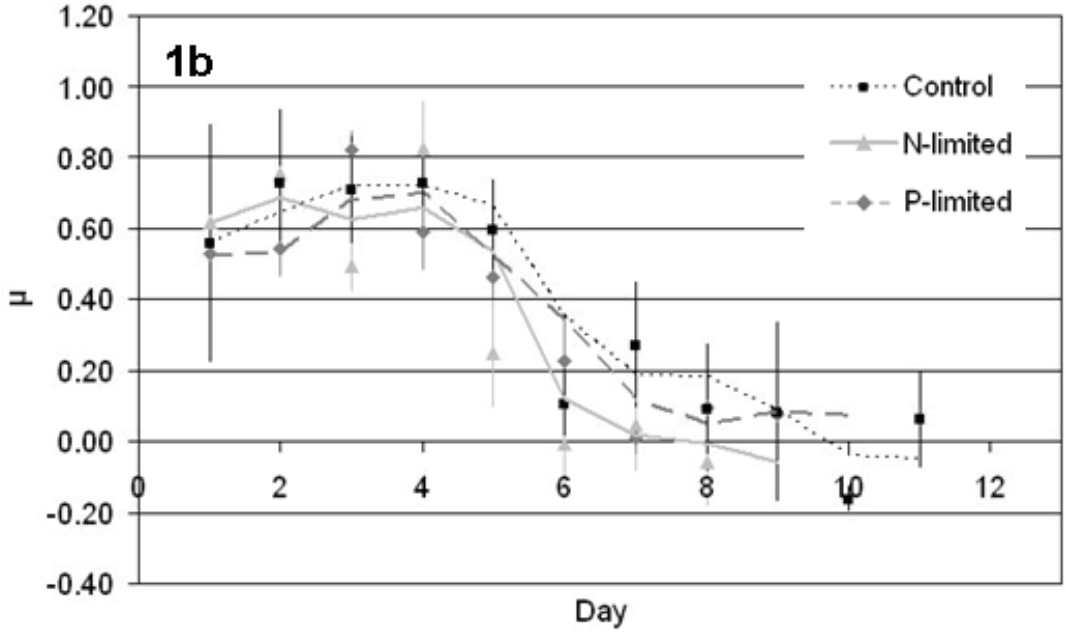
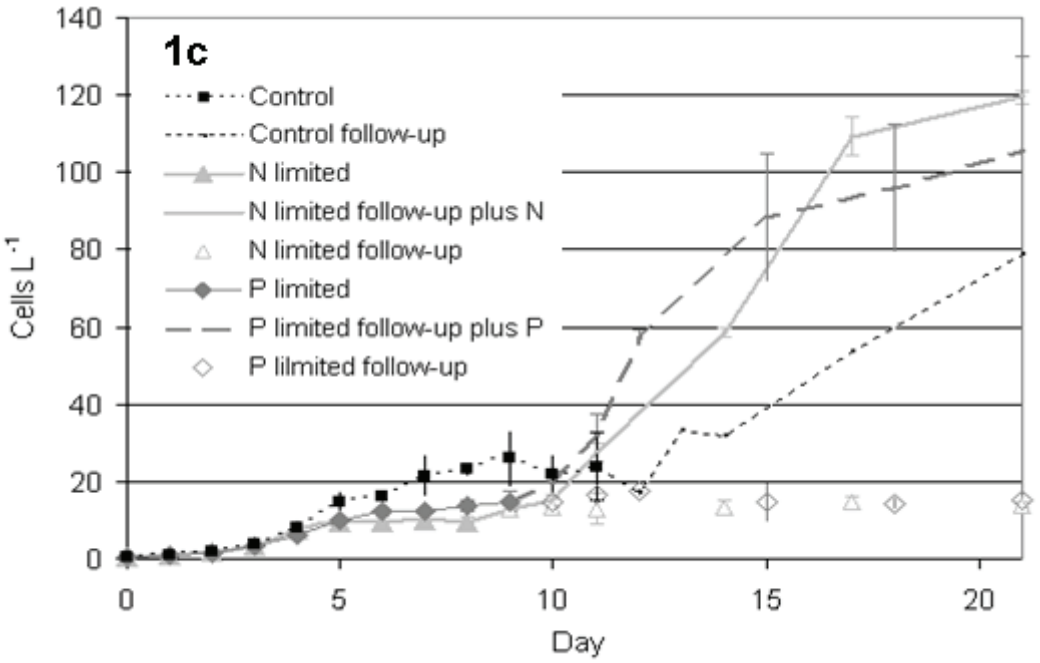


Figure 1 Growth kinetics of *Alexandrium minutum* AL3T in batch culture experiments: **a)** cell concentrations versus day after inoculation.



b) growth rate (μ) versus day after inoculation (μ=ln(cell count (day2))-ln(cell count(day1)), lines: 2-day moving average.



c) cell concentrations versus day after inoculation including nutrient-spiked (solid lines) and non-spiked (open symbols) run-on cultures. All values are mean ± standard deviation of biological triplicates.

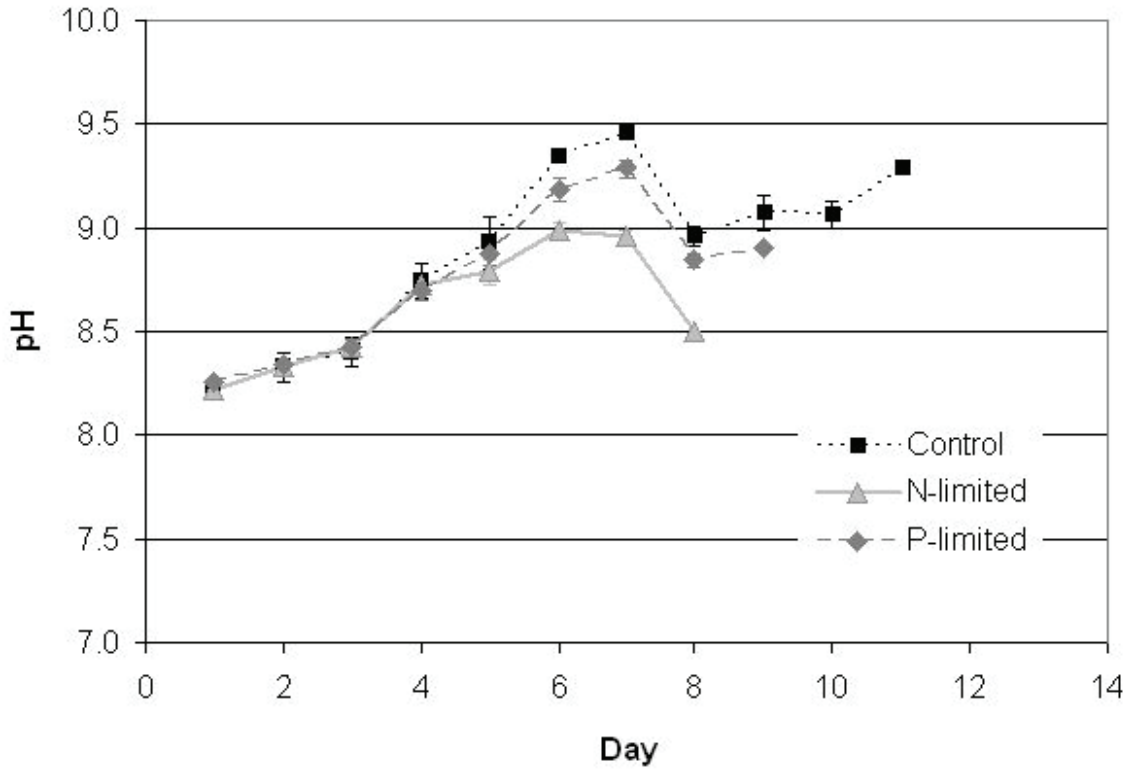


Figure 2 pH of the culture medium for various treatments given as mean ± standard deviation of biological triplicates. The last data point corresponds to the treatment-specific stationary-phase harvesting date.

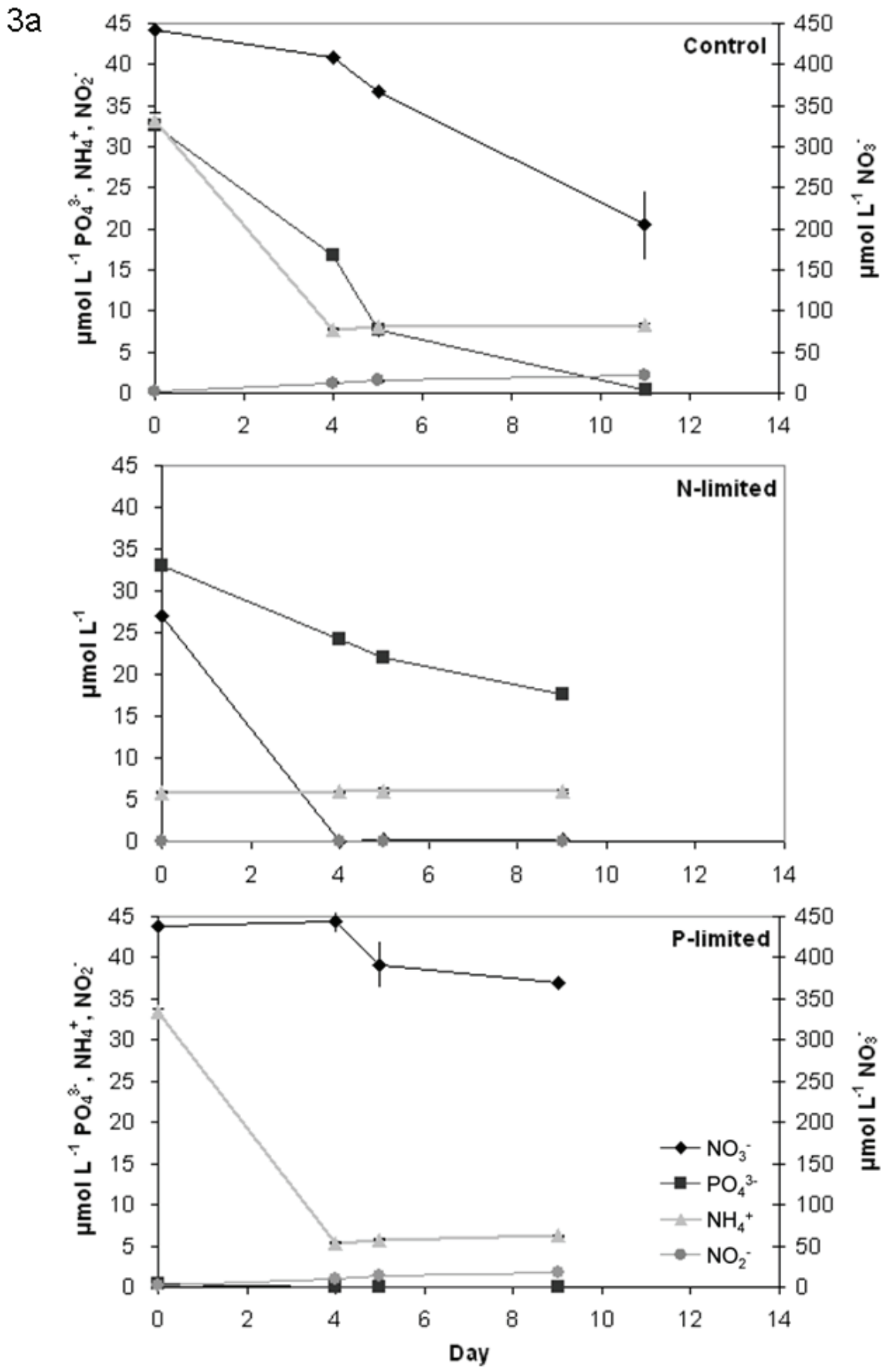
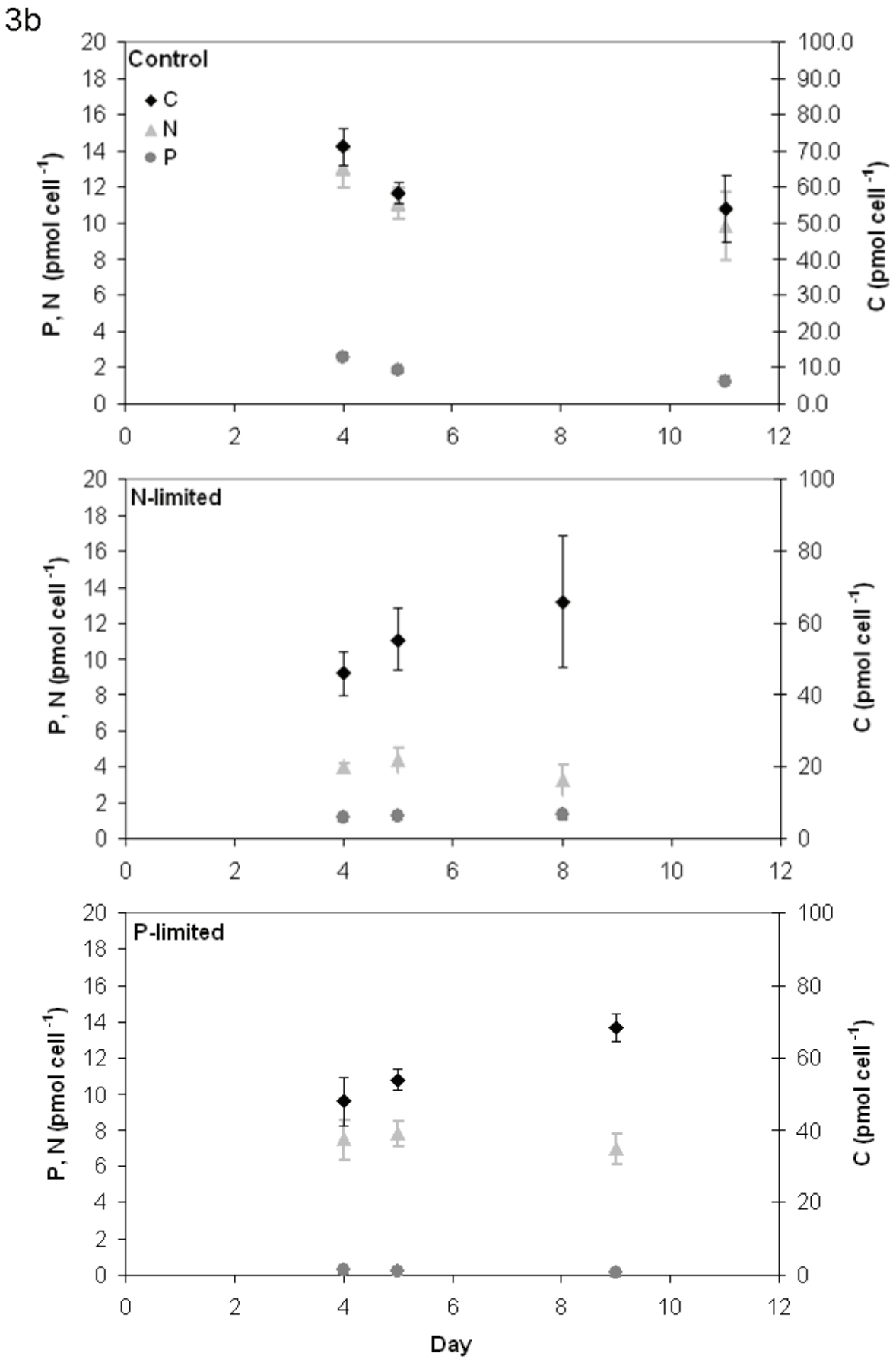
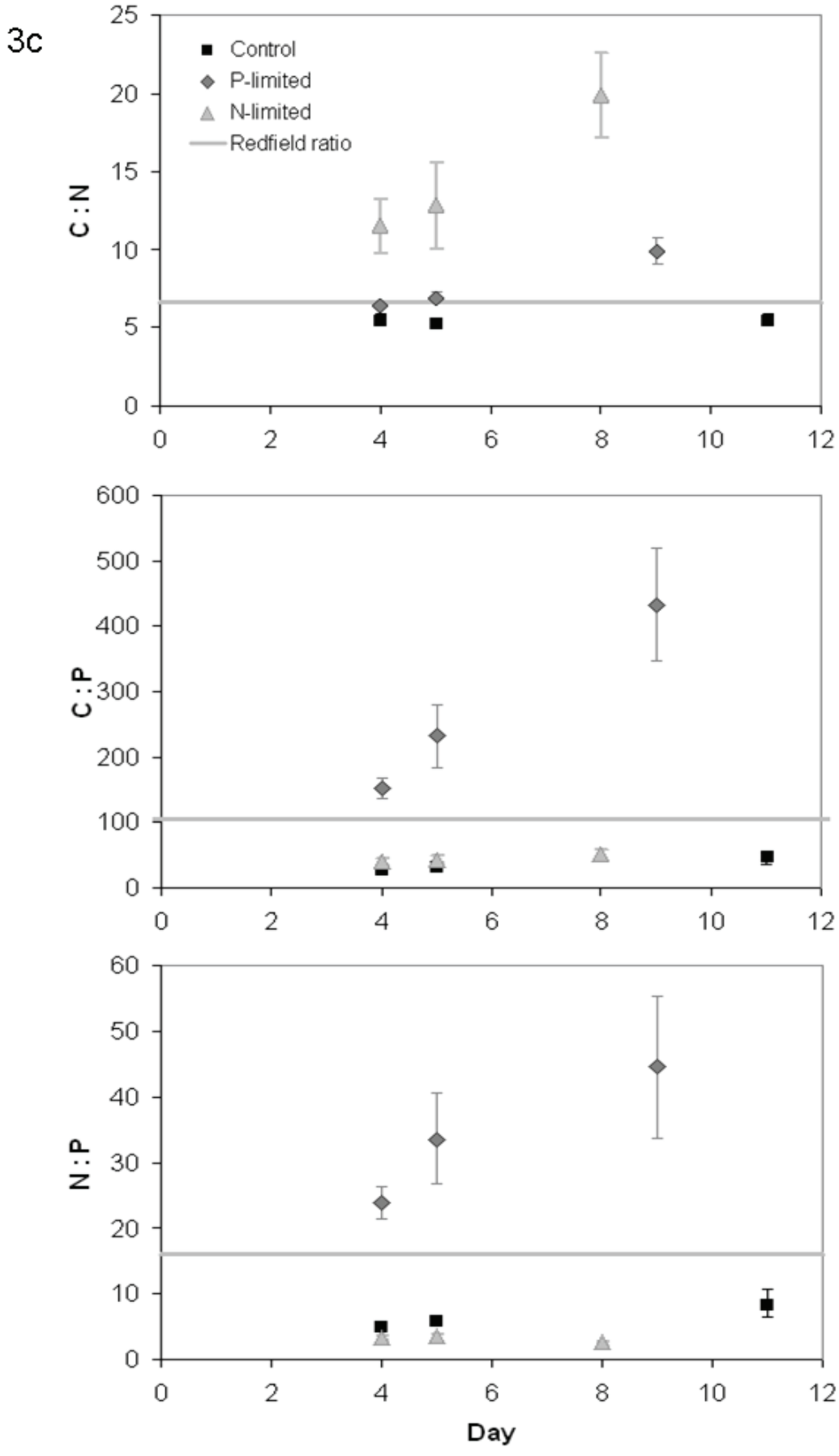


Figure 3 Nutrient status of cultures. **a)** Dissolved nutrients in culture medium.



b) Intracellular nutrients.



c) Element ratios. Average of biological triplicates, with standard deviation.

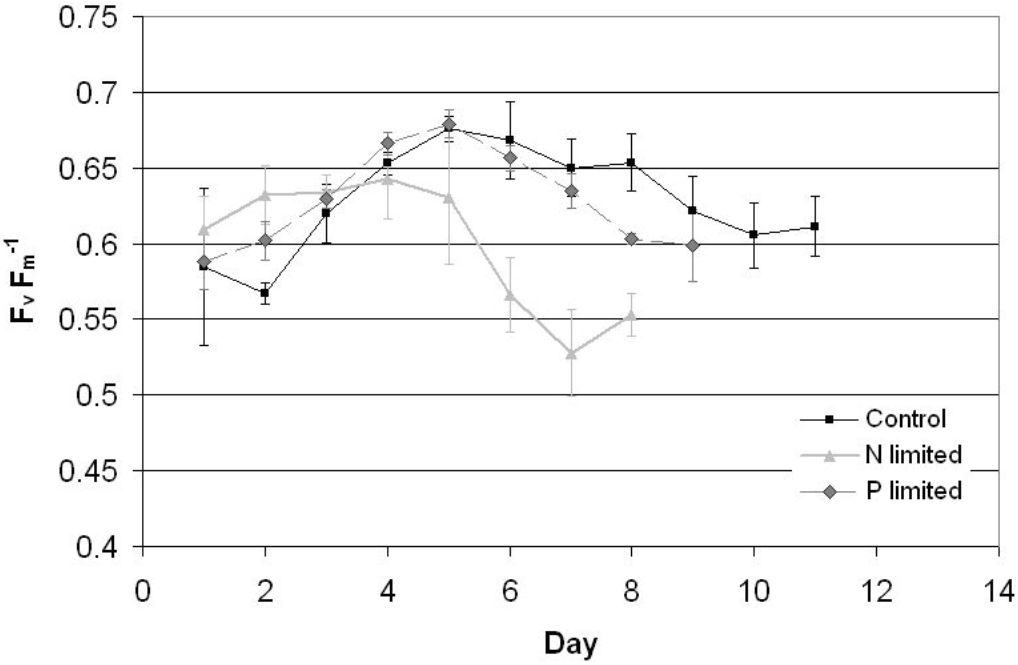


Figure 4 Potential quantum efficiency of PSII, measured as dark-adapted F_v/F_m. Mean of triplicates, with standard deviation.

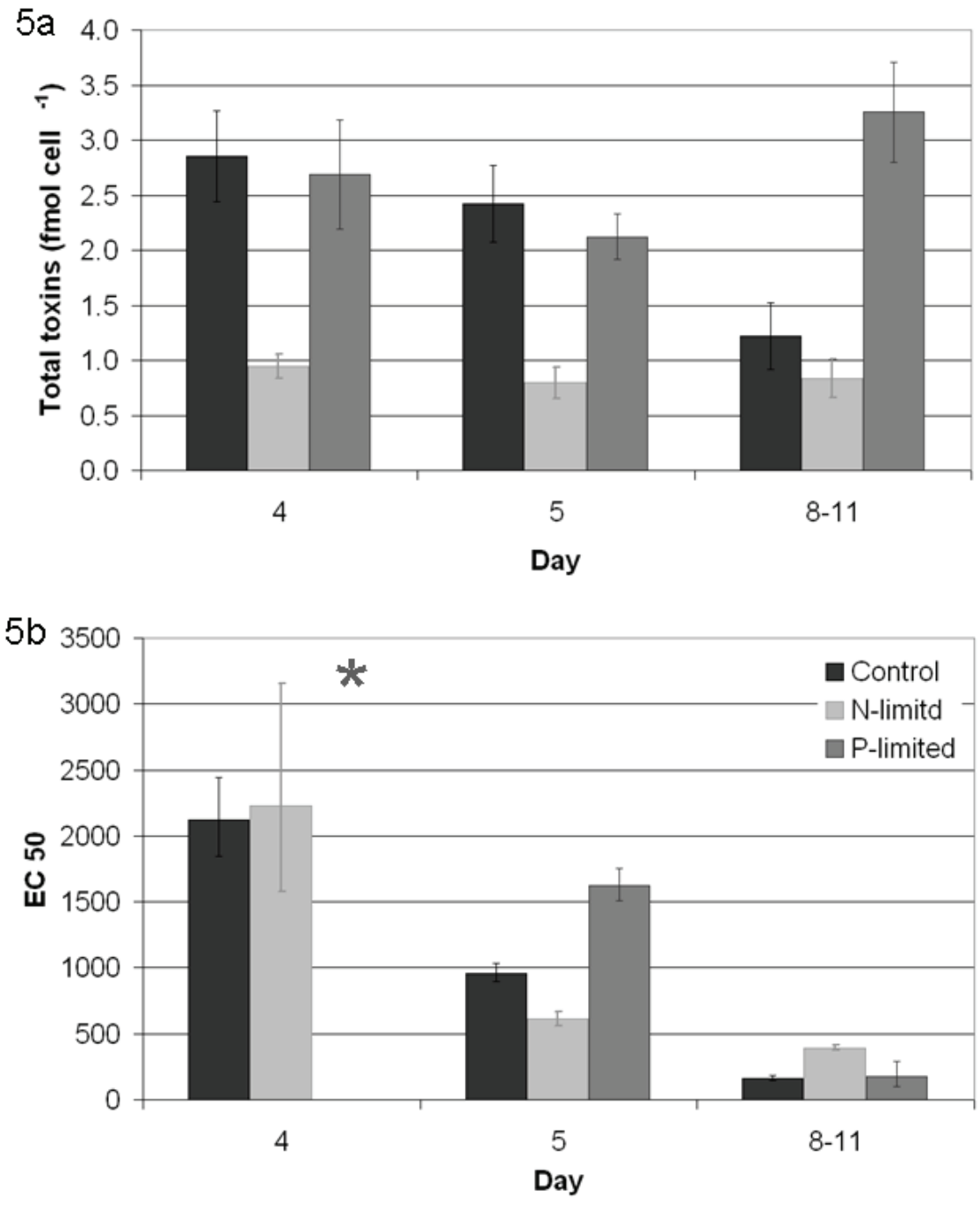


Figure 5 Toxicity and allelochemical activity. a) Intracellular toxins (fmol cell^{-1}) as measured by liquid chromatography with fluorescence detection (LC-FD). Mean of biological triplicates, with standard deviation. b) Allelochemical activity against *Rhodomomas baltica*, calculated as half-effective concentration (EC_{50}) of *A. minutum* cells, with 95% confidence intervals. * at limit of measured range, between 3500 and 7200 – no fit to model possible.

Tables

Table 1 Values for physiological variables in stationary phase, defined as when $\mu < 0.1$ d^{-1}. All values are mean ± standard deviation of biological triplicates, with pH measurements and cell counts averaged over the stationary phase, and cellular nutrient ratios determined at the last harvesting point at the end of the experiment. (1)significantly different ($p \leq 0.05$) from all other values in this column as determined by Student's *t*-test on ln-transformed data ($p \leq 0.05$). (2)significantly different from all other values in this column according to Welch's *t*-test (unequal variances t-test, $p \leq 0.05$).

Treatment	Cells L^{-1}	pH	C:N	N:P	C:P
Control	23.7 ± 5.6 (1)	9.10 ± 0.13	5.5 ± 0.4 (2)	8.5 ± 2.2	45.9 ± 10.5
P-limited	13.7 ± 1.8 (1)	9.01 ± 0.21	9.9 ± 0.8 (2)	44.6 ± 10.8 (2)	432.4 ± 86.6 (2)
N-limited	9.6 ± 1.1 (1)	8.82 ± 0.24	19.9 ± 2.7 (2)	2.6 ± 0.4	50.4 ± 6.8

Table 2a Numbers of genes differentially expressed between treatments at transition to stationary phase and when compared to exponentially growing control cultures.

		transition to stationary phase		
		Control culture	**N-limited**	**P-limited**
transition to stationary phase	N-limited	430	-	13
	P-limited	6	13	-
exponential phase	Control culture	6	126	21

Table 2b Numbers of genes differentially expressed between treatments in stationary phase and when compared to exponentially growing control cultures.

		stationary phase		
		Control culture	**N-limited**	**P-limited**
stationary phase	N-limited	143	-	19
	P-limited	77	19	-
exponential phase	Control culture	1565	778	764

Table 3 Numbers of differentially expressed genes (SAM-based q-value <1% and fold-change ≥ 1.5) showing the same trend in several comparisons. Stationary phase: Significantly up- or down-regulated in all stationary-phase samples in relation to the control culture in exponential phase; N- / P-limitation: differentially expressed and showing the same trend between stationary-phase N- or P- limited cultures and both exponentially growing and stationary-phase control samples; nutrient limitation: identified for N- and P-limitation and same trend in both; stationary phase – control: differentially expressed between stationary and exponentially growing control and stationary phase control and both stationary-phase N- and P-limited cultures; transition to stationary phase: differentially expressed and showing the same trend in all comparisons between Day 5 samples and the control cultures in exponential phase.

Stationary phase		**N-limitation**		**P-limitation**		**nutrient limitation**		**stationary phase - control**		**transition to stationary phase**	
down	up	down	up	down	up	down	up	down	up	down	up
197	292	26	35	3	19	2	3	8	0	5	0

Table 4 Selection of genes identified as differentially expressed in all stationary-phase regimes when compared to control in exponential growth

Contig name		Stationary vs exponential control		Gene product	Function
Canonical name	Control	P-limited	N-limited		
Amin_09g12r	-2.91	-2.33	-3.65	ABC (ATP-binding-cassette) transporter protein	transport
Amin_15e01r	-2.98	-2.62	-2.78	calcium-activated potassium channel	transport
Amin_51h24f	-3.74	-2.66	-3.31	putative sugar transporter family protein	transport
Amin_07f04r	-2.38	-2.76	-2.50	calcium/calmodulin-dependent protein kinase	intracellular signalling
Amin_08d07f	-9.27	-5.28	-6.98	putative intracellular signaling protein	intracellular signalling
Amin_42g08r	-3.74	-4.11	-3.93	putative mitochondrial protein, PRR repeat-containing	organellar RNA-binding protein (putative)
Amin_12a02r	-4.38	-2.79	-2.12	60S ribosomal protein L10a.	organellar translation
Amin_34c10r	-1.99	-2.42	-6.07	Photosystem I P700 chlorophyll a apoprotein A1	chloroplast
Amin_26m06r	-1.82	-2.05	-6.43	Photosystem II D2 protein	chloroplast
Amin_06c10r	-3.82	-4.22	-4.59	malate dehydrogenase	Citric acid cycle
Amin_57n13r	-5.78	-8.05	-23.75	cytochrome b	mitochondrial
Amin_38k24f	-1.75	-2.11	-8.58	cytochrome c oxidase polypeptide I	mitochondrial
Amin_52g07r	-1.69	-2.42	-3.87	DNA-3-methyladenine glycosylase	DNA repair
Amin_26g08f2	-2.17	-2.04	-2.29	pre-mRNA-processing-splicing factor 8, C-terminal domain	Splicing
Amin_34h03r	-1.88	-2.15	-2.33	putative small nuclear ribonucleoprotein polypeptide E	Splicing
Amin_33f12f	-6.92	-9.07	-9.81	18S rRNA	translation
Amin_44f03f	-3.72	-2.41	-2.01	40S ribosomal protein S11.	translation
Amin_06b02r	-3.02	-2.94	-2.71	40S ribosomal protein S15	translation
Amin_83a11r	-3.81	-2.35	-2.18	40S ribosomal protein S16.	translation
Amin_03c10r	-3.34	-2.50	-2.15	40S ribosomal protein S4	translation
Amin_57f03r	-3.99	-3.10	-2.56	40S ribosomal protein S7	translation
Amin_90e06f	-10.52	-7.02	-6.06	40S ribosomal protein S7.	translation
Amin_48e09r	-1.94	-2.70	-3.48	50S ribosomal protein L14, mitochondrial or chloroplast	translation
Amin_14a02r	-5.53	-7.67	-6.25	60S ribosomal protein L12.	translation

Amin_24d11f2	-4.33	-3.65	-2.89	60S ribosomal protein L6	translation
Amin_13g03r	-4.49	-3.24	-2.21	60S ribosomal protein L6.	translation
Amin_78c12f	-4.21	-2.76	-2.80	60S ribosomal protein L7a	translation
Amin_26m14r	-8.93	-15.02	-16.98	ribosomal operon external transcribed spacer	translation
Amin_75d07r	-4.67	-3.08	-2.64	ribosomal protein S13	translation
Amin_36k19f	-4.41	-3.29	-3.43	translation elongation factor-like protein	translation
Amin_07g02r	-3.07	-2.21	-2.52	tRNA (guanine-N1-)-methyltransferase	translation
Amin_95c08r	-4.34	-2.26	-3.27	sialyltransferase involved in protein glycosylation	protein glycosylation
Amin_95c05r	-1.59	-2.15	-2.34	probable E3 ubiquitin-protein ligase, HECT domain-containing	protein degradation
Amin_11c08r	-2.76	-3.36	-3.70	aspartyl proteinase family protein	protein degradation
Amin_24h08f2	1.53	1.75	1.77	ABC-transporter protein	transport
Amin_13d06r	3.76	3.59	3.27	ABC-transporter family protein	transport
Amin_32d04f	2.18	2.18	2.27	ion channel similar to voltage-gated cation channels	transport
Amin_93m06f	1.61	2.40	1.91	TPT transporter family protein	transport
Amin_68f12f	2.97	2.66	2.66	inorganic H+ pyrophosphatase, vacuolar-type	intracellular pH regulation
Amin_08d12r	2.19	3.48	4.19	vacuolar ATP synthase subunit B	intracellular pH regulation
Amin_03h02f	2.18	2.33	3.96	14-3-3 protein	intracellular signalling
Amin_84i17r	3.13	2.40	5.91	3'5'-cyclic nucleotide phosphodiesterase family member	intracellular signalling
Amin_56m09r	2.12	2.00	2.19	calmodulin-like protein	intracellular signalling
Amin_49d23r	2.25	2.00	1.86	cGMP-dependent protein kinase	intracellular signalling
Amin_52f11f	1.60	2.05	2.04	dual specificity phosphatase	intracellular signalling
Amin_98e10f	2.06	2.53	2.47	predicted Traf-like protein	intracellular signalling
Amin_01h11r	3.94	3.05	6.62	protein kinase similar to shaggy-related protein kinases	intracellular signalling
Amin_88g07r2	3.54	2.85	3.53	protein kinase, putatively calcium-dependent	intracellular signalling
Amin_07c10r	6.15	5.15	6.04	Ras small GTPase, Rab type, probably involved in vesicle trafficking	intracellular signalling
Amin_06f11f	1.59	2.42	2.65	serine/threonine-protein kinase	intracellular signalling
Amin_09a03r	3.11	2.70	2.76	serine/threonine-protein phosphatase	intracellular signalling
Amin_68g05r	3.86	5.22	6.06	pentatricopeptide (PPR) repeat-containing protein	organellar RNA-binding protein (putative)
Amin_84m07f	4.21	2.42	4.20	pentatricopeptide (PPR) repeat-containing protein	organellar RNA-binding protein (putative)
Amin_34g06f	2.61	4.45	4.10	putative PPR repeat protein	organellar RNA-binding protein

					(putative)
Amin_64f10f	2.36	3.33	6.73	caroteno-chlorophyll a-c-binding protein	chloroplast
Amin_78d01f	6.17	4.41	4.83	light-harvesting chlorophyll a-c binding protein	chloroplast
Amin_41p17r	2.87	2.87	5.50	light-harvesting chlorophyll a-c binding protein	chloroplast
Amin_06b05r	1.80	2.00	2.63	phosphofructokinase family protein	chloroplast
Amin_61f12r	2.23	3.02	2.55	fumarate hydratase, putative	mitochondeial
Amin_07a03r	2.28	1.92	1.98	Arp2/3 complex, subunit 2 (p34-Arc)	cytoskeleton
Amin_21f05r	2.05	2.21	3.03	actin	cytoskeleton
Amin_09e07f	2.69	2.95	2.66	C-terminal motor kinesin	cytoskeleton
Amin_74g08f	3.59	5.16	4.85	dynein heavy chain family protein	cytoskeleton
Amin_07f07r	1.76	1.96	4.48	LisH domain-containing protein	cytoskeleton
Amin_55d08r	2.05	2.16	2.51	putative myosin, N-terminal WD 40-repeats	cytoskeleton
Amin_06h09r	2.54	2.36	2.19	ribonuclease HII	putatively reverse transcription
Amin_58c02r	2.81	3.45	3.74	putative reverse transcriptase	reverse transcriptase
Amin_09f06f	1.71	1.96	2.08	amine oxidase	amine metabolism
Amin_33f09r	2.59	2.97	3.45	putative D-3-phosphoglycerate dehydrogenase	amino acid biosynthesis
Amin_77b01r	3.53	2.93	3.16	putative sterol 3-beta-glucosyltransferase , partial sequence	sterol modification
Amin_60e01f	2.02	4.96	3.30	glycoside hydrolase family 28 family member	glycoside hydrolase
Amin_47c09f	3.42	2.87	2.67	adenylosuccinate lyase	nucleotide metabolism
Amin_83d02r2	2.87	2.25	2.15	guanine deaminase	nucleotide metabolism
Amin_46e08r	4.05	4.23	2.56	putative Uridine-binding protein	nucleotide metabolism
Amin_08h02r	1.79	2.11	2.16	DNA-directed RNA polymerases I, II, and III subunit RPABC3	transcription
Amin_03c05r	2.17	2.47	3.81	ribosomal operon external transcribed spacer	translation
Amin_44h09f	2.46	2.97	3.13	glycyl-tRNA synthetase	translation - tRNA-related
Amin_69g10f	1.74	2.73	2.00	phenylalanine-tRNA synthetase	translation - tRNA-related
Amin_74a06r	1.82	2.09	2.19	tRNA-dihydrouridine synthase 3-like	translation - tRNA-related
Amin_08c10f	2.74	2.72	3.19	peptidylprolyl isomerase	protein folding
Amin_46g06r	2.42	2.22	2.35	diphthine synthase	protein modification
Amin_07g04f	2.07	1.94	2.47	aspartic protease	protein degradation
Amin_07g03r	2.21	2.36	2.44	proteasome subunit alpha	protein degradation
Amin_42o14f	1.51	2.35	1.90	ubiquitin-specific protease, putative	protein degradation

Table 5 Genes in stationary phase control cultures which were down-regulated relative to both exponential-phase and nutrient-limited cultures.

Contig name	Control stationary phase vs Control exponential	Stationary vs stationary		Gene product
		N-limited / Control	P-limited / Control	
Amin_17h12f	-6.50	16.60	7.04	hypothetical protein similar to subtilase family peptidases
Amin_09f12f	-5.93	5.47	2.58	putative aminopeptidase
Amin_07e10f	-5.13	5.97	8.56	hypothetical protein similar to alcohol dehydrogenase
Amin_12a02r	-4.38	2.07	1.57	60S ribosomal protein L10a
Amin_64e04r	-4.04	2.44	1.89	60S ribosomal protein L22
Amin_61a05r	-2.95	1.77	1.99	60S ribosomal protein L21
Amin_07h11f	-4.52	3.10	2.48	hypothetical protein
Amin_54e08r	-2.67	2.38	1.85	hypothetical protein

Table 6 Selection of genes identified as differentially expressed in stationary-phase nutrient-limited cultures when compared to control cultures in both exponential and stationary growth phase

Contig name	N-limitation	P-limitation	Stationary vs exponential control			Stationary vs stationary			Gene product
			Control	P-limited	N-limited	N-limited / Control	P-limited / Control	P-limited / N-limited	
Amin_57f07r		down		-3.31			-3.21		sodium-glucose cotransporter
Amin_60e01f		up	2.02	4.96	3.30		2.51		glycoside hydrolase family 28 family member
Amin_69g10f		up	1.74	2.73	2.00		1.57		phenylalanine-tRNA synthetase
Amin_37h11f		up		2.08	2.67		1.54		putative NALP-related protein similar to NOD3
Amin_53g06f	down				-1.85	-1.81			aldo-keto reductase family protein
Amin_57n13r	down		-5.78	-8.05	-23.75	-4.22		2.96	cytochrome b
Amin_14a08f	down				-3.14	-2.39		3.02	cytochrome b6.
Amin_04f11f	down		-2.35		-10.25	-4.76		5.85	cytochrome b6-f complex subunit 4
Amin_38k24f	down		-1.75	-2.11	-8.58	-4.34			cytochrome c oxidase polypeptide I
Amin_34c10r	down		-1.99	-2.42	-6.07	-3.06			Photosystem I P700 chlorophyll a apoprotein A1
Amin_89e05r	down			-2.26	-6.28	-3.48		2.97	Photosystem I P700 chlorophyll a apoprotein A1
Amin_26m06r	down		-1.82	-2.05	-6.43	-3.50		3.05	Photosystem II D2 protein
Amin_46g07r	down			-2.43	-9.71	-5.69			photosystem Q(B) protein
Amin_83b01r	down			-2.23	-4.26	-3.47			putative esterase
Amin_64f10f	up		2.36	3.33	6.73	2.62			caroteno-chlorophyll a-c-binding protein
Amin_41p17r	up		2.87	2.87	5.50	1.74			light-harvesting chlorophyll a-c binding protein
Amin_98b11f	up		1.90		3.92	1.93		-2.94	ribulose-bisphosphate carboxylase form II
Amin_11a02f	up		2.35	3.62	6.69	2.94			hypothetical protein similar to metal-dependent hydrolase ElsH
Amin_18f03f	up				2.50	2.39			hypothetical protein similar to sodium channel proteins
Amin_100c02r	up			2.03	2.70	2.18			major intrinsic protein, putative aquaporin

Amin_71d02r	up	1.72		2.88	1.68	-2.77	-4.57	pre-mRNA-processing factor
Amin_36g07r	up	1.51		2.61	1.70			skp1 family protein
Amin_84i17r	up	3.13	2.40	5.91	1.84		-2.48	3'5'-cyclic nucleotide phosphodiesterase family member
Amin_95f08r	up			1.96	1.58			ADP-ribosylation factor-like protein 3
Amin_71f07r	up			1.92	1.98			vWF domain-containing protein

Table 7 Complete list of genes differentially expressed between P-limited and N-limited cultures

Contig name	Stationary vs exponential control			Stationary vs stationary			Gene product
	Control	P-limited	N-limited	N-limited / Control	P-limited / Control	P-limited / N-limited	
Amin_71d02r	1.72		2.88	1.68	-2.77	-4.57	pre-mRNA-processing factor
Amin_84i17r	3.13	2.40	5.91	1.84		-2.48	3'5'-cyclic nucleotide phosphodiesterase family member
Amin_97h03r	1.64		2.73			-2.39	high-affinity nitrate transporter, putative
Amin_95g04f	-2.76		-3.36		2.73	3.39	ubiquitin conjugation factor E4 B
Amin_98b11f	1.90		3.92	1.93		-2.94	ribulose-bisphosphate carboxylase form II
Amin_57n13r	-5.78	-8.05	-23.75	-4.22		2.96	cytochrome b
Amin_89e05r		-2.26	-6.28	-3.48		2.97	Photosystem I P700 chlorophyll a apoprotein A1
Amin_14a08f			-3.14	-2.39		3.02	cytochrome b6
Amin_26m06r	-1.82	-2.05	-6.43	-3.50		3.05	Photosystem II D2 protein
Amin_04f11f	-2.35		-10.25	-4.76		5.85	cytochrome b6-f complex subunit 4
Amin_09e10r						-4.46	hypothetical protein
Amin_60e06r	-1.88	-2.28		2.13		-2.60	hypothetical protein
Amin_78e07r				-1.72		2.16	hypothetical protein
Amin_93o20f	-4.47	-6.92	-18.07	-4.17		2.68	hypothetical protein
Amin_22d03r			-2.19			2.79	hypothetical protein
Amin_42m14r	2.69	6.78	2.46		2.51	2.82	hypothetical protein
Amin_66a07f	-2.13	-2.25	-6.69	-3.33		2.99	hypothetical protein
Amin_25j23r			-5.59	-3.87		3.32	hypothetical protein
Amin_21a03r		3.37	-2.06	-2.72	2.58	7.08	hypothetical protein

Additional files

Supplementary Table 1: Complete list of differentially expressed genes showing the same trend in several comparisons (see file)

Empty expression data cells = no significant expression difference (cutoffs: SAM-based q-value <1% and fold-change ≥ 1.5). Includes differential expression pattern across all comparisons, expression patterns found in other studies, as well as results of manual and automated annotation.

Supplementary Table 2: Carbonic anhydrases differentially expressed between stationary phase and exponential phase in control cultures (see file)

Empty expression data cells = no significant expression difference (cutoffs: SAM-based q-value <1% and fold-change ≥ 1.5). Includes differential expression pattern across all comparisons as well as results of manual and automated annotation.

9 Synthesis

9.1 Characterisation of the A. minutum transcriptome

The dinoflagellate genome is highly unusual and little understood (Hackett *et al.*, 2004; Moreno Díaz de la Espina *et al.*, 2005; McEwan *et al.*, 2008), and the same is true for their gene structure and regulation (Bachvaroff & Place, 2008; Monroe & Van Dolah, 2008; Zhang & Lin, 2009). Dinoflagellates contain excessively large gene families (Machabée *et al.*, 1994; Sharples *et al.*, 1996) with high intra-family sequence diversity (Reichman *et al.*, 2003) and a frequent occurrence of pseudogenes (Bachvaroff & Place, 2008). While no information on their gene content exists, a combination of large genome sizes (LaJeunesse *et al.*, 2005) with an unknown but apparently dominant share of structural DNA (Sigge, 1984; Levi-Setti *et al.*, 2008) results in an exceedingly low density of genes within the genomic sequences (McEwan *et al.*, 2008). In order to circumvent these complications, most projects aimed at the large-scale discovery and characterisation of dinoflagellate nuclear genes are based on EST libraries (Bachvaroff *et al.*, 2004; Tanikawa *et al.*, 2004; Hackett *et al.*, 2005; Uribe *et al.*, 2008), which allow an overview over the genes represented in the transcriptome at the moment of sampling.

In order to use this approach to detect the highest possible number of genes in *A. minutum*, the EST library introduced in Manuscript 1 was based on pooled RNA samples from 10 different physiological treatments and 7 different timepoints over the light – dark-cycle, and the library was normalised to boost the relative abundance of less highly expressed mRNAs. Partial sequencing of 9585 cDNA library inserts (mostly sequenced from both sides, yielding 15 703 read sequences) resulted in detection of 4320 contigs, or putative genes. Of these, 28% could be automatically annotated, which is in the usual range for dinoflagellate EST libraries (John *et al.*, 2004; Jaeckisch *et al.*,

2008). The two largest functional categories among the genes annotated to KOG categories were signal transduction mechanisms and the functional complex of posttranslational modification, protein turnover and chaperones. This might be an indication of the importance of these processes in dinoflagellates, but is probably also dependent on the extent of sequence conservation of the corresponding genes across large evolutionary distances.

Within this library, 9% of all contigs containing reads from more than one cDNA insert contained evidence for alternative splice variants. Alternative splicing is a mechanism creating multiple mRNA transcripts from a single gene, which can lead to the production of different functional proteins coded by the same sequence or to non-functional mRNAs that are thus post-transcriptionally repressed (reviewed in Barbazuk *et al.*, 2008). About 80% or the human genes and 20% of both rice and *Arabidopsis* genes are known to exist in different splice variants, but their discovery is greatly dependent on EST coverage of the genome (Barbazuk *et al.*, 2008). While the existence of the typical eukaryotic splicing machinery has been shown before (Alverca *et al.*, 2006), this is the first report of the frequency of alternative splicing. The variants add to the transcriptomic variation originating from the existence of the non-identical dinoflagellate gene families (Reichman *et al.*, 2003).

A better-known dinoflagellate splicing phenomenon, the dinoflagellate-specific spliced leader (Lidie & Van Dolah, 2007; Zhang *et al.*, 2007), was recovered in about 5% of this library. As the cDNA library is enriched in mRNA 3'-ends due to technical reasons, this is no indication of its absence in the other mRNAs, but rather an indication of complete 5'-ends in the corresponding ESTs. The percentages of SL-containing sequences among the differentially expressed genes identified in manuscript 4 were in the same range as those for the whole library. 4.3% for genes differentially expressed

between any growth-limiting treatment and exponentially growing control and 5.8% for the much smaller set identified as associated with a specific treatment. This equal representation of SL sequences among the different groups of genes suggest that it is probably not restricted to a special class of constitutively transcribed multi-copy genes as has recently been suggested (Bachvaroff & Place, 2008); it is, however, in agreement with the suggestion that SL sequences can be found with all dinoflagellate mRNAs (Zhang & Lin, 2009). This neither implies nor rules out an involvement of this sequence in the post-transcriptional regulation of gene expression, e.g. in mRNA stabilisation or in the recruitment into the actively translated mRNA pool.

- The EST library described in Manuscript 1 was composed of RNA from *A. minutum* cultures subject to a variety of treatments and harvested at different circadian times. This should ensure a high versatility of the microarray developed based on this data to detect relevant mRNA level differences in a wide variety setups.
- The library contained indications of a rather high frequency of alternative splicing; this is the first such report in a dinoflagellate.
- Manuscript 1 reports the proportion of identifiable dinoflagellate-specific SL sequences in the EST library. In Manuscript 4, examination of the gene expression patterns under different physiological conditions for involvement of the SL-containing sequences reveals a very similar representation to that in the complete library, suggesting that the regulation patterns for trans-spliced mRNAs are probably not very different from those acting on the majority of sequences.

9.2 Search for sequences associated with PSP toxicity

An identification of dinoflagellate genes correlated with the ability to synthesise PSP toxins would allow for the development of tools for the rapid and culture-independent discrimination of PSP-producing and non-PSP-producing *Alexandrium* populations, ideally independently of the exact identification of the species complex clade (John *et al.*, 2003) or the *A. minutum* population of origin (Touzet *et al.*, 2006).

The PSP toxin gene cluster of cyanobacteria has been identified (Kellmann *et al.*, 2008b), and its distribution and evolution among prokaryotes is now being investigated (Kellmann *et al.*, 2008a; Mihali *et al.*, 2009; Moustafa *et al.*, 2009). The second part of Manuscript 1 describes a search for a dinoflagellate counterpart of this gene cluster. As an attempt to identify genes similar to the cyanobacterial ones lead to no result both in the *A. minutum* library and in other EST libraries of PSP toxin-producing *Alexandrium* species, an approach independent of available sequence information was chosen. As *A. minutum* contains closely related toxic and non-toxic clones (Montresor *et al.*, 2004), the method of choice was a gene expression comparison between three such strains. Two independent trials yielded 145 genes consistently higher expressed in the toxic strains, as well as eight genes not significantly expressed in the non-toxic strain. These 145 genes, and especially the 8 genes specific for the toxic strains, can be considered tentative candidates for PSP toxin-associated genes.

Switching to a very different system involving the induction of higher intracellular levels of PSP toxins, Manuscript 2 describes the response of *A. minutum* towards a copepod grazer. The chemical cues excreted by copepods have been shown to induce toxin production in *A. minutum* and to lead to up to 20x augmented intracellular toxin levels, which in turn lead to an increased resistance against grazing by these copepods. The magnitude of this response depends on both the copepod species used and the

susceptibility of the *A. minutum* strain (Selander *et al.*, 2006; Bergkvist *et al.*, 2008; Selander *et al.*, 2008). Using this system, a very limited set of 14 genes was shown to be affected by copepod presence, while intracellular toxin content increased to levels 5 times higher than in the control. Among the genes up-regulated at the first day was one of the genes that had been identified as about 10x less expressed in the non-toxic *A. minutum* strain in Manuscript 1. At the third day, another sequence that had been identified as 51x higher expressed in toxic strains was among the three differentially expressed genes. This second sequence was not detected in a qPCR assay comparing the toxic and non-toxic strains in manuscript 1 (see manuscript 1, Fig.4), and attempts to amplify this sequence from cDNA and genomic DNA were only successful in the two toxic strains (data not shown). This combination of transcriptomic comparisons between strains of different physiological capabilities and between induced and non-induced cultures of the same strain are a promising approach for further investigations into the genetics of *A. minutum* toxin production.

- While no homologues of the cyanobacterial gene cluster could be found in the *A. minutum* EST library, a microarray comparison of the genes expressed in toxic and non-toxic strains of *A. minutum* lead to the identification of candidates potentially correlated to the ability to produce toxins.
- Two of these candidates were also up-regulated in *A. minutum* cultures during the induction of higher toxin cell quotas by predator cues.

9.3 Transcriptome-level processes associated with growth, nutrient status and stress response

Manuscripts 3 and 4 use the microarray probe set to investigate the transcriptome-level gene expression in *A. minutum* over an array of different treatments designed to study bloom-relevant conditions. As *A. minutum* is a coastal species that tends to bloom in relatively enclosed water bodies after nutrient input, which is often associated with freshwater runoff (Bravo *et al.*, 2008), acclimation to different salinities should be a highly relevant feature of *A. minutum* biology. Manuscript 3 examines the physiological and gene expression responses under high and low salinity conditions. After a variable lag-phase, growth characteristics in the two salinity treatments paralleled those of control cultures, indicating successful acclimation. However, both high and low salinity cultures showed signs of photoinhibition, suggesting differences in chloroplast efficiency at these salinities. This was corroborated by the differential expression of several chloroplast or non-assigned organellar genes, especially in high salinity, where photoinhibition was less severe.

Overall, 8.8% of the sequences tested were identified as differentially expressed at the salinities tested. However, they displayed a very moderate amplitude with a maximum fold change of 3.5 between control and treatment. No genes were regulated in parallel between both treatments, but 12 were regulated in opposite directions. This implied an overall lack of transcriptomic stress response at this stage of acclimation. 27% of the functionally annotated differentially expressed genes were involved in mRNA modification or translation-related processes, which might hint at a high prevalence of post-transcriptional regulation under high and low salinity conditions.

The set of physiological conditions examined in Manuscript 4 was targeted at the examination of growth- and limitation-associated processes. In the course of this study,

41% of all sequences represented on the microarray were identified as differentially expressed between some limiting condition and exponentially growing control cultures.

Among the identified patterns of regulation were 5 genes up-regulated in each treatment at the transition between exponential growth phase and stationary phase. These might be interesting candidates to test for suitability as biomarkers for the beginning of limitation in potential field- or mesocosm studies.

Stationary phase was mainly characterised by a major down-regulation of translational apparatus, as well as an up-regulation of genes involved in intracellular signalling. 87 genes were identified as associated with N-or P-limitation; 3 of these were up- and 2 were down- regulated in both nutrient limited treatments. This is another set of potential gene expression markers which might be useful for assessing the level of nutrient limitation in a population.

In combination with the available literature on transcriptional and translational gene regulation in dinoflagellates and model eukaryotes, the data presented in manuscripts 3 and 4 suggest that dinoflagellate gene expression is modulated at both the transcriptomic and the translational level and that the relative importance of these different mechanisms might be highly treatment specific.

- While limited sets of mRNAs are differentially expressed between *A. minutum* cultures acclimatised to different salinities, other physiological factors such as nutrient limitation or culture growth phase lead to substantial alterations of the transcriptome.
- Manuscript 4 identifies 5 genes consistently up-regulated at the transition between exponential growth and stationary phase, which might be useful indicators for the onset of limitation.
- 87 genes were consistently associated with either N- or P-limitation and might thus be useful to identify the nutrient status of an *A. minutum* population.

9.4 Conclusion

This thesis contains the most comprehensive study of the interplay between physiological factors and gene expression in the genus *Alexandrium* to date. The *A. minutum* transcriptome was characterised on the basis of an EST library, which was designed to include the highest possible diversity of cDNA sequences. Automated annotation of the of contigs produced from this library revealed a high percentage of the functionally annotatable genes to be involved in either signal transduction or the functional category combining posttranslational modification, protein turnover and chaperones. The library allowed for the first report on the frequency of alternative splicing in dinoflagellates. An analysis of another splicing phenomenon, the dinoflagellate-specific spliced leader transsplicing, contributed new data to the current discussion on the regulation of dinoflagellate gene expression. A comparison of the prevalence of spliced leader sequences in the whole library with that in different groups of differentially expressed genes revealed no significant difference, supporting the

suggestion that SL transsplicing is a general feature of dinoflagellate mRNA processing and not restricted to certain groups of genes with distinct expression patterns.

The PSP toxin genes known from cyanobacteria could not be found in *A. minutum*, nor in EST libraries of other PSP toxin-producing dinoflagellates. This lead to the suggestion that the corresponding dinoflagellate genes might be too different from their cyanobacterial counterparts to be identified based on sequence similarity. A comparison between toxic and non-toxic *A. minutum* strains revealed a number of tentative candidates for PSP toxin-associated genes. While some of the genes differentially expressed between toxic and non-toxic strains were apparently involved in gene expression-related processes or in cell cycle control, others could only be recognised as members of certain functional groups. In spite of annotations as “hydrolytic enzyme” or “Amino-transferase”, these genes could not be assigned to biochemical reactions or pathways. The function of the majority of the genes differentially expressed between toxic and non-toxic strains could not be determined due to a lack of similarity to known sequences. Two of the sequences identified as associated with toxin-producing strains were also involved in the very specific gene expression response to a copepod grazer, which was associated with a considerable increase in PSP toxin content. These two sequences can be considered very strong candidates for genes involved in PSP toxin production or regulation.

An examination of the *A. minutum* gene expression response to a variety of physiological stress factors revealed very different magnitudes of transcriptome-level changes associated with the different stimuli. Acclimatisation to extreme values of high and low salinity was associated with the differential expression of a small number of genes, and amplitudes of gene expression change were moderate. While no genes were regulated in parallel, some were regulated in opposite directions in high- and low-

salinity cultures. A set of gene expression comparisons between different culture growth stages and between nutrient-replete and nutrient-limited batch cultures revealed nearly half of all sequences tested to be differentially expressed between some limiting condition an the exponentially growing control. A subsequent series of cross-comparisons between the different responses identified narrow patterns associated with specific physiological conditions such as cessation of growth or nutrient limitation, identifying potential biomarkers for several physiological factors related to bloom development.

10 Future Perspectives

In the course of this thesis, I identified a number of genes associated with specific physiological conditions. These genes are promising markers for the occurrence of the respective conditions in laboratory experiments or mesocosms. If they are to be established as strain-independent markers for the physiological state of *A. minutum*, their validity in different strains should be tested. These comparisons might yield universally valid cut-offs for gene expression under certain physiological conditions, possibly regarding the ratios of several specific up- and down-regulated genes in the same sample, or in relation to reference genes. This would reduce the need for measuring expression of these marker genes in relation to control samples from the same strain, enabling examination of samples containing unknown proportions of several strains, such as after several days of growth in a mixed culture or in a natural population. Such universal cut-offs would allow the development of new tools for the prediction of Alexandrium bloom probability based on the physiological status of a low-density population.

The gene expression patterns associated with the induction of higher toxin cell quotas in *A. minutum* allow not only for the unusually specific study of physiological and transcriptomal changes associated with the built-up and maintenance of higher toxin levels, but also for an investigation of the sensory response of *Alexandrium* cells to copepod presence. Further studies on the physiology and gene expression response of *A. tamarense* challenged with copepod cues are currently under way (Wohlrab *et al*, in prep).

The most interesting group among the genes identified as potentially involved in toxicity are certainly the two genes for which both higher expression in toxin-producing strains and as up-regulation under copepod-induced higher toxin content was shown.

The other 145 sequences identified as higher expressed in toxin-producing strains and especially the eight genes identified as specific for toxin producers also clearly warrant further investigation. These sequences could be further tested for their distribution and expression among additional toxin-producing and non-toxin-producing strains, ideally taking into account the newest available data on genetic similarity within *A. minutum* (e.g. McCauley *et al.*, 2009) to combine the few available non-toxin producers with the closest-related available toxin-producers and to include a range of distantly related toxin-producing strains.

If toxin-related gene expression patterns prove universal within *A. minutum*, their distribution in other PSP toxin-producing *Alexandrium* species such as *A. tamarense* and PSP toxin-producing and spirolide-producing *A. ostenfeldii*, as well as in the other dinoflagellate PSP toxin producers, may be of interest. Comparing the expression of dinoflagellate genes robustly associated with toxicity but not directly involved in the biosynthesis pathway with gene expression patterns in PSP toxin-producing cyanobacteria might yield common patterns of regulation or of interaction with other biochemical pathways.

EST libraries like the one the transcriptomic data of this thesis are based on typically contain only a subset of the investigated transcriptome, hence the *A. minutum* library is unlikely to contain the full set of genes involved in the PSP toxin synthesis pathway. Nevertheless, given the conspicuous lack of evidence for close homologs of the cyanobacterial gene cluster in dinoflagellates to date, concentrating on de novo detection and characterisation of toxin production-related genes in Alexandrium appears more promising then searching for sequences related to the cyanobacterial genes. Depending on the similarity of the cyanobacterial and dinoflagellate pathways and the level of homology between the corresponding genes, this might or might not apply after

the first dinoflagellate PSP toxin synthesis genes have been confirmed and characterised.

The sequences for which the pattern of higher expression in toxic *A. minutum* strains persist should be further examined for their whole-transcript sequence, inter-and intra-strain variability between homologous mRNAs, genomic context of the corresponding genes, genomic copy number and organisation of their gene families. If this characterisation leads to a hypothesis on their biochemical function or a specific regulatory role in the toxin-synthesis pathway, further characterisation could be facilitated by targeted production of the proteins using heterologous expression of the corresponding gene. Such an expression of dinoflagellate proteins in both *Escherichia coli* and yeast has been successfully used in the characterisation of *Lingulodinium polyedrum* (as "*Gonyaulax polyedra*") luciferase and cyclin enzymes, respectively (Bae & Hastings, 1994; Bertomeu & Morse, 2004). This method tends to greatly simplify isolation of protein in question for direct verification of its function.

If knowledge of the whole mRNA sequences of reconfirmed candidate genes does not lead to a testable hypothesis on their function, further investigations might only be possible after establishing procedures for targeted gene inactivation in dinoflagellates. One such method that is routinely used for the ciliate *Paramecium tetraurelia*, another alveolate with highly peculiar genome arrangements and extremely high gene copy numbers in the somatic nucleus, is gene silencing. In *P. tetraurelia*, both transformation with high-copy untranslatable but transcribable transgenes or feeding with bacteria producing double-stranded RNAs leads to post-transcriptional silencing of homologous mRNAs (Ruiz *et al.*, 1998; Galvani & Sperling, 2002; Lepère *et al.*, 2009). This effect has been shown to operate on subfamilies with up to about 25% sequence difference on the nucleotide level, but not between subfamilies (Ruiz *et al.*, 1998), and so should be

applicable to the extensive gene families of dinoflagellates. As *A. minutum* has been shown to ingest small prey cells when grown under low light (Jeong *et al.*, 2005a), gene silencing by feeding might be applicable if ingestion of prey can be maximised. Transformation of dinoflagellate cells has been demonstrated (ten Lohuis & Miller, 1998) but has not yet been adapted for thick-walled species such as *A. minutum*.

Gene expression studies such as the ones used in this thesis link expressed genes to physiological processes. If the studied organism is well understood and the approximate function of many expressed genes is known, then gene expression patterns can be used to examine the response of various biochemical pathways and their regulation (e.g. Rossouw & Bauer, 2009). In less well-studied organisms such as *A. minutum*, in which few genes can be readily annotated based on similar sequences from better-studied organisms, patterns of gene expression can reveal association with physiological parameters. This can help to more precisely define the physiological function of genes annotated to broad functional categories, and can be a first step towards functional characterisation of genes which are newly discovered and have not yet been annotated. Identified gene expression signatures for key physiological processes such as growth or nutrient limitation can be used to assess the physiological status of a population, e.g. when investigating the metatranscriptome of a bloom or potentially in new phytoplankton monitoring applications.

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Appendix

I. Manuscript 1, additional file 1: Primer tables and respective PCR conditions

Primers for cyanobacterial sxt gene cluster

gene	Amplicon size (nt)	primer sequence 5'-3'	tm
sxtG	675	sxtGF GATTTACCAATCGCCAAGCA	53.6
		sxtGR GTAACCATTCTATGCCCAAACC	53.9
sxtU	690	sxtUF ATTGGATGGAAAAGTGGCGATTA	54.9
		sxtUR TGATCGGGTTGTGTCACTGCATA	58.5
sxtF	762	norM8F ATCTGATATTAGAAGCACGAGCA	53.7
		norM8R CGTTAAATAGTGCCGTTTCCG	54.3
sxt15	601	sxt15F GGTACTATGGCGCAGTTACGA	56.7
		sxt15R TAGCAGTTTCGGAGGATTTC	52.2
sxt14	481	sxt14F TTTTGACTCAGCAGGTAATG	50.3
		sxt14R GAATTGGTTTGGAAGGATTT	48.7
sxtX	645	sxtXF GAGAAGATAAATGTGCTATT	44.8
		sxtXR GAGCCGTTGTAACCGTA	51.7
sxtA	680	sxtA1F TAATCGTTATACTCACGGCTTTG	52.7
(ACP domain)		sxtA1R AAGCATCTCTTTGGAATACGG	52.7
sxtA	740	sxtA3F CAAGGTCGCAAGGTGCTAA	55.4
(Aminotransferase domain)		sxtA3R CCTCCTGCTTACAAGGCAAA	55.3
sxtH	660	sxtHF AAACCAGGAAGTATCACCACGGC	59.4
		sxtHR GCATCAAATCCGCAGTCCG	56.9
sxtI	714	sxtIaF CTACCACGATAGTGCTGCCG	57.8
		sxtIaR CCCCACCGTGTAGTTGAAGT	57
sxtT	662	sxtTF GCACGTAGTCGCCAATGTAGAAG	57.8
		sxtTR GGGGATTCTGAAACGCAGTATTG	56.1
sxtM	770	norM16F ATGATGGGCTTACTTGGTACTCA	55.7
		norM16R CCCGATTCCAAGAGGTATCACT	56.1
sxtN	347	sxtNF TACCCGTGCTTTTGAGAACTTAG	54.8
		sxtNR CCTGTCTTTCTCTCCGCTATA	53.3
sxtO	446	sxtOF AATTAGAGCAGACTTTGTTC	47.8
		sxtOR TGTTTTCCACGTATTTG	44.4

Gradient a: 6 temperature steps, approximately 48 – 58 °C;
Gradient b: 6 temperature steps, approximately 48.2 – 58.5 °C

30 µl reactions:

- 20 ng genomic DNA
- 0.2 µmol l^{-1} forward and reverse primers
- 0.2 mmol l^{-1} dNTPs

- HotMasterTaq buffer 1X (Eppendorf)
- 1.5 units of HotMaster *Taq* DNA polymerase (Eppendorf)

sxtO: 5 temperature steps, approximately 46 - 53.5 °C; 2 DNA concentrations at 46°C: 33 ng DNA, 100 ng DNA

Cycling conditions were based on those optimal for these primers (K.Stucken, pers. comm.).: 94°C for 5 min, 20 cycles with denaturation 94°C (20 s), annealing for 45 s, extension at 70°C (30 s), and a final extension step of 10 min at 70°C. PCR products were visualized using agarose gel electrophoresis.

M13-primer PCR for amplification of cDNA plasmid inserts to use in calibration curves:

50 µl reactions:
- 0.5 µl plasmid stock
- 0.2 µmole l^{-1} forward and reverse primers
- 0.2 mmole l^{-1} dNTPs
- HotMasterTaq buffer 1X (Eppendorf)
- 2.5 units of HotMaster *Taq* DNA polymerase (Eppendorf)

Cycling conditions:

Cycling conditions: 94°C for 2 min, 24 cycles with denaturation 94°C (1 min), annealing for 1 min, extension at 72°C (2 min), and a final extension step of 10 min at 72°C. PCR products were visualized using agarose gel electrophoresis.

qPCR

primers: 5'-3'

Amin_44h03_406F	ACAAGGTCAGAATGCGGAAGA
Amin_44h03_505r	GCAGCAACAGAGCCCATGT
Amin_52d01_503F	GAGGAGAGCTGCGACCACAT
Amin_52d01_602r	CAAGTTGGGAGTCACATTTCCA
Amin_56a03_417F	CAGCACGAACGCACCTTGTA
Amin_56a03_516r	TTGACGTGGATTGCCTGAGA
Amin_81i24_193F	ATGCGGAGCCTGTTGAGATC

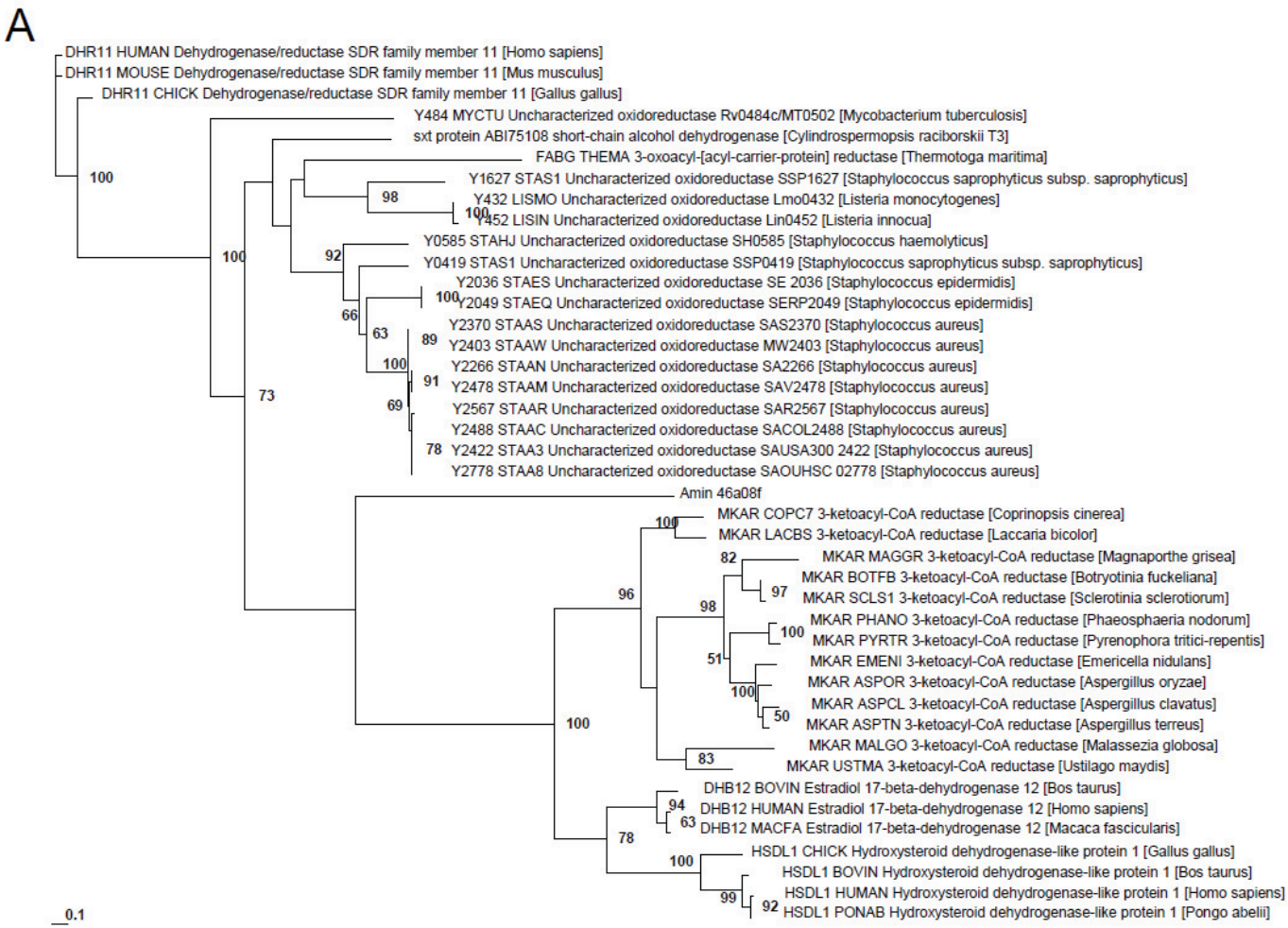
Amin_81i24_292r	GTAGCTCCAGTGCGCAAGTG
Amin_88h09_564F	ACAGTGAGCCAGCGAGTGAA
Amin_88h09_663r	TCCATGCTCTGCCAATCTTG
Amin_89d06_358F	CGAACCCGAACTGGAAGGT
Amin_89d06_457r	TCATCGCCTACCCGGTACAC

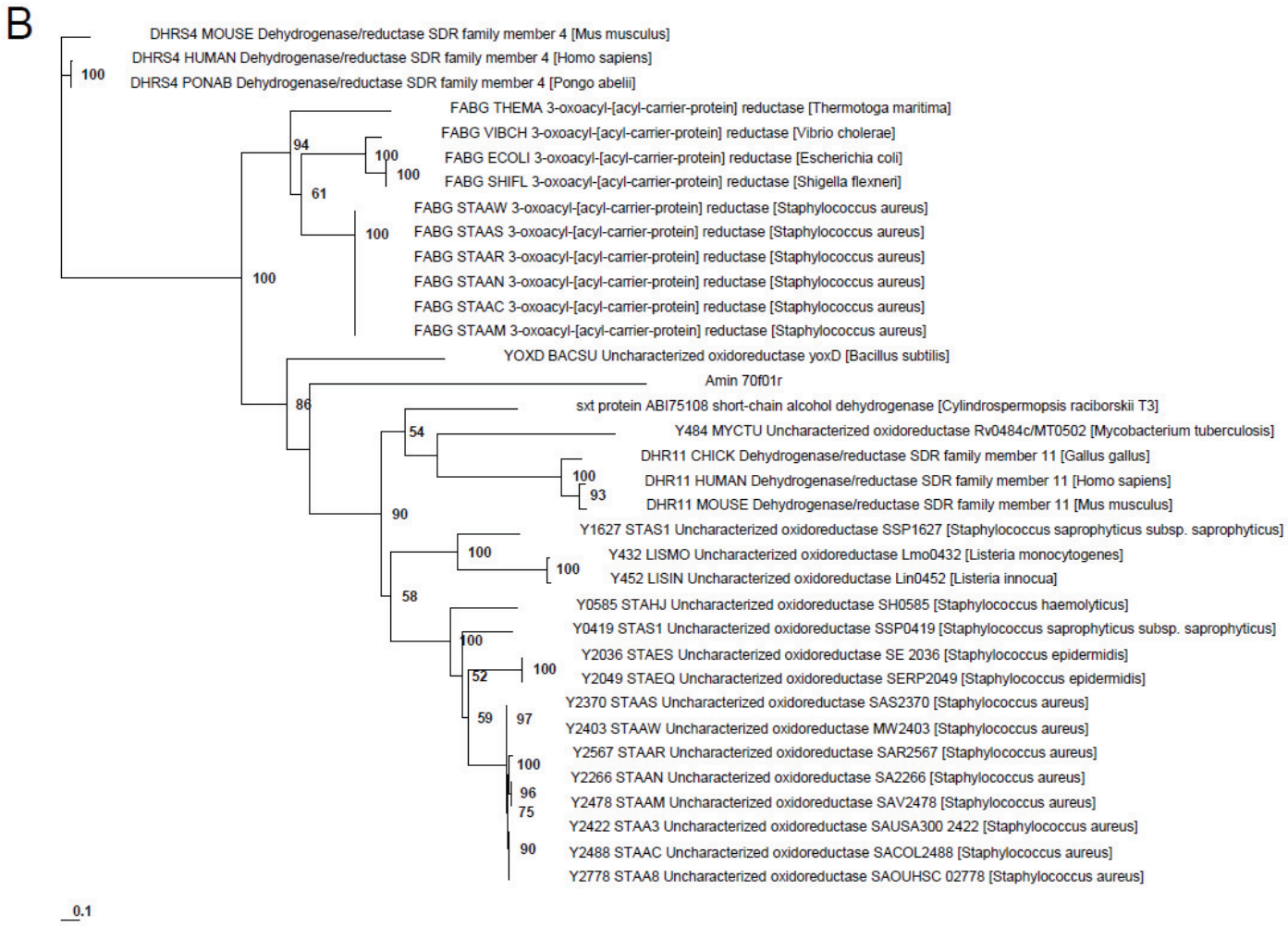
cDNA synthesis

cDNA was synthesised using SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instruction. Each reaction contained 500 ng total RNA supplemented with the artificial control RNAs (1 ng reaction^{-1}) and NSP (1 pg reaction^{-1}) and 50 pmol oligo dT primer.

II. Manuscript 1, additional file 3: Phyml-based likelihood trees with bootstrap support values

14 phylogenies of *A. minutum* EST contigs that produced significant ($e<10^{-4}$) BLAST hits with cyanobacterial sxt-related genes. Phylogenies were calculated including the *A. minutum* contig sequence, the corresponding cyanobacterial gene, and their closest SwissProt matches as identified by PhylogGena (Top10Select-mode). Phylogenies M.2 and N.2 additionally include the best 20 hits produced by blasting the *Alexandrium* sequences against the NCBI non-redundant protein sequence database (nr).


A
DHR11 HUMAN Dehydrogenase/reductase SDR family member 11 [Homo sapiens]
DHR11 MOUSE Dehydrogenase/reductase SDR family member 11 [Mus musculus]
DHR11 CHICK Dehydrogenase/reductase SDR family member 11 [Gallus gallus]
Y484 MYCTU Uncharacterized oxidoreductase Rv0484c/MT0502 [Mycobacterium tuberculosis]
sxt protein ABI75108 short-chain alcohol dehydrogenase [Cylindrospermopsis raciborskii T3]
FABG THEMA 3-oxoacyl-[acyl-carrier-protein] reductase [Thermotoga maritima]
Y1627 STAS1 Uncharacterized oxidoreductase SSP1627 [Staphylococcus saprophyticus subsp. saprophyticus]
Y432 LISMO Uncharacterized oxidoreductase Lmo0432 [Listeria monocytogenes]
Y452 LISIN Uncharacterized oxidoreductase Lin0452 [Listeria innocua]
Y0585 STAHJ Uncharacterized oxidoreductase SH0585 [Staphylococcus haemolyticus]
Y0419 STAS1 Uncharacterized oxidoreductase SSP0419 [Staphylococcus saprophyticus subsp. saprophyticus]
Y2036 STAES Uncharacterized oxidoreductase SE 2036 [Staphylococcus epidermidis]
Y2049 STAEQ Uncharacterized oxidoreductase SERP2049 [Staphylococcus epidermidis]
Y2370 STAAS Uncharacterized oxidoreductase SAS2370 [Staphylococcus aureus]
Y2403 STAAW Uncharacterized oxidoreductase MW2403 [Staphylococcus aureus]
Y2266 STAAN Uncharacterized oxidoreductase SA2266 [Staphylococcus aureus]
Y2478 STAAM Uncharacterized oxidoreductase SAV2478 [Staphylococcus aureus]
Y2567 STAAR Uncharacterized oxidoreductase SAR2567 [Staphylococcus aureus]
Y2488 STAAC Uncharacterized oxidoreductase SACOL2488 [Staphylococcus aureus]
Y2422 STAA3 Uncharacterized oxidoreductase SAUSA300 2422 [Staphylococcus aureus]
Y2778 STAA8 Uncharacterized oxidoreductase SAOUHSC 02778 [Staphylococcus aureus]
Amin 46a08f
MKAR COPC7 3-ketoacyl-CoA reductase [Coprinopsis cinerea]
MKAR LACBS 3-ketoacyl-CoA reductase [Laccaria bicolor]
MKAR MAGGR 3-ketoacyl-CoA reductase [Magnaporthe grisea]
MKAR BOTFB 3-ketoacyl-CoA reductase [Botryotinia fuckeliana]
MKAR SCLS1 3-ketoacyl-CoA reductase [Sclerotinia sclerotiorum]
MKAR PHANO 3-ketoacyl-CoA reductase [Phaeosphaeria nodorum]
MKAR PYRTR 3-ketoacyl-CoA reductase [Pyrenophora tritici-repentis]
MKAR EMENI 3-ketoacyl-CoA reductase [Emericella nidulans]
MKAR ASPOR 3-ketoacyl-CoA reductase [Aspergillus oryzae]
MKAR ASPCL 3-ketoacyl-CoA reductase [Aspergillus clavatus]
MKAR ASPTN 3-ketoacyl-CoA reductase [Aspergillus terreus]
MKAR MALGO 3-ketoacyl-CoA reductase [Malassezia globosa]
MKAR USTMA 3-ketoacyl-CoA reductase [Ustilago maydis]
DHB12 BOVIN Estradiol 17-beta-dehydrogenase 12 [Bos taurus]
DHB12 HUMAN Estradiol 17-beta-dehydrogenase 12 [Homo sapiens]
DHB12 MACFA Estradiol 17-beta-dehydrogenase 12 [Macaca fascicularis]
HSDL1 CHICK Hydroxysteroid dehydrogenase-like protein 1 [Gallus gallus]
HSDL1 BOVIN Hydroxysteroid dehydrogenase-like protein 1 [Bos taurus]
HSDL1 HUMAN Hydroxysteroid dehydrogenase-like protein 1 [Homo sapiens]
HSDL1 PONAB Hydroxysteroid dehydrogenase-like protein 1 [Pongo abelii]
0.1


B
DHRS4 MOUSE Dehydrogenase/reductase SDR family member 4 [Mus musculus]
DHRS4 HUMAN Dehydrogenase/reductase SDR family member 4 [Homo sapiens]
DHRS4 PONAB Dehydrogenase/reductase SDR family member 4 [Pongo abelii]
FABG THEMA 3-oxoacyl-[acyl-carrier-protein] reductase [Thermotoga maritima]
FABG VIBCH 3-oxoacyl-[acyl-carrier-protein] reductase [Vibrio cholerae]
FABG ECOLI 3-oxoacyl-[acyl-carrier-protein] reductase [Escherichia coli]
FABG SHIFL 3-oxoacyl-[acyl-carrier-protein] reductase [Shigella flexneri]
FABG STAAW 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
FABG STAAS 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
FABG STAAR 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
FABG STAAN 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
FABG STAAC 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
FABG STAAM 3-oxoacyl-[acyl-carrier-protein] reductase [Staphylococcus aureus]
YOXD BACSU Uncharacterized oxidoreductase yoxD [Bacillus subtilis]
Amin 70f01r
sxt protein ABI75108 short-chain alcohol dehydrogenase [Cylindrospermopsis raciborskii T3]
Y484 MYCTU Uncharacterized oxidoreductase Rv0484c/MT0502 [Mycobacterium tuberculosis]
DHR11 CHICK Dehydrogenase/reductase SDR family member 11 [Gallus gallus]
DHR11 HUMAN Dehydrogenase/reductase SDR family member 11 [Homo sapiens]
DHR11 MOUSE Dehydrogenase/reductase SDR family member 11 [Mus musculus]
Y1627 STAS1 Uncharacterized oxidoreductase SSP1627 [Staphylococcus saprophyticus subsp. saprophyticus]
Y432 LISMO Uncharacterized oxidoreductase Lmo0432 [Listeria monocytogenes]
Y452 LISIN Uncharacterized oxidoreductase Lin0452 [Listeria innocua]
Y0585 STAHJ Uncharacterized oxidoreductase SH0585 [Staphylococcus haemolyticus]
Y0419 STAS1 Uncharacterized oxidoreductase SSP0419 [Staphylococcus saprophyticus subsp. saprophyticus]
Y2036 STAES Uncharacterized oxidoreductase SE 2036 [Staphylococcus epidermidis]
Y2049 STAEQ Uncharacterized oxidoreductase SERP2049 [Staphylococcus epidermidis]
Y2370 STAAS Uncharacterized oxidoreductase SAS2370 [Staphylococcus aureus]
Y2403 STAAW Uncharacterized oxidoreductase MW2403 [Staphylococcus aureus]
Y2567 STAAR Uncharacterized oxidoreductase SAR2567 [Staphylococcus aureus]
Y2266 STAAN Uncharacterized oxidoreductase SA2266 [Staphylococcus aureus]
Y2478 STAAM Uncharacterized oxidoreductase SAV2478 [Staphylococcus aureus]
Y2422 STAA3 Uncharacterized oxidoreductase SAUSA300 2422 [Staphylococcus aureus]
Y2488 STAAC Uncharacterized oxidoreductase SACOL2488 [Staphylococcus aureus]
Y2778 STAA8 Uncharacterized oxidoreductase SAOUHSC 02778 [Staphylococcus aureus]
100
94
100
100
61
100
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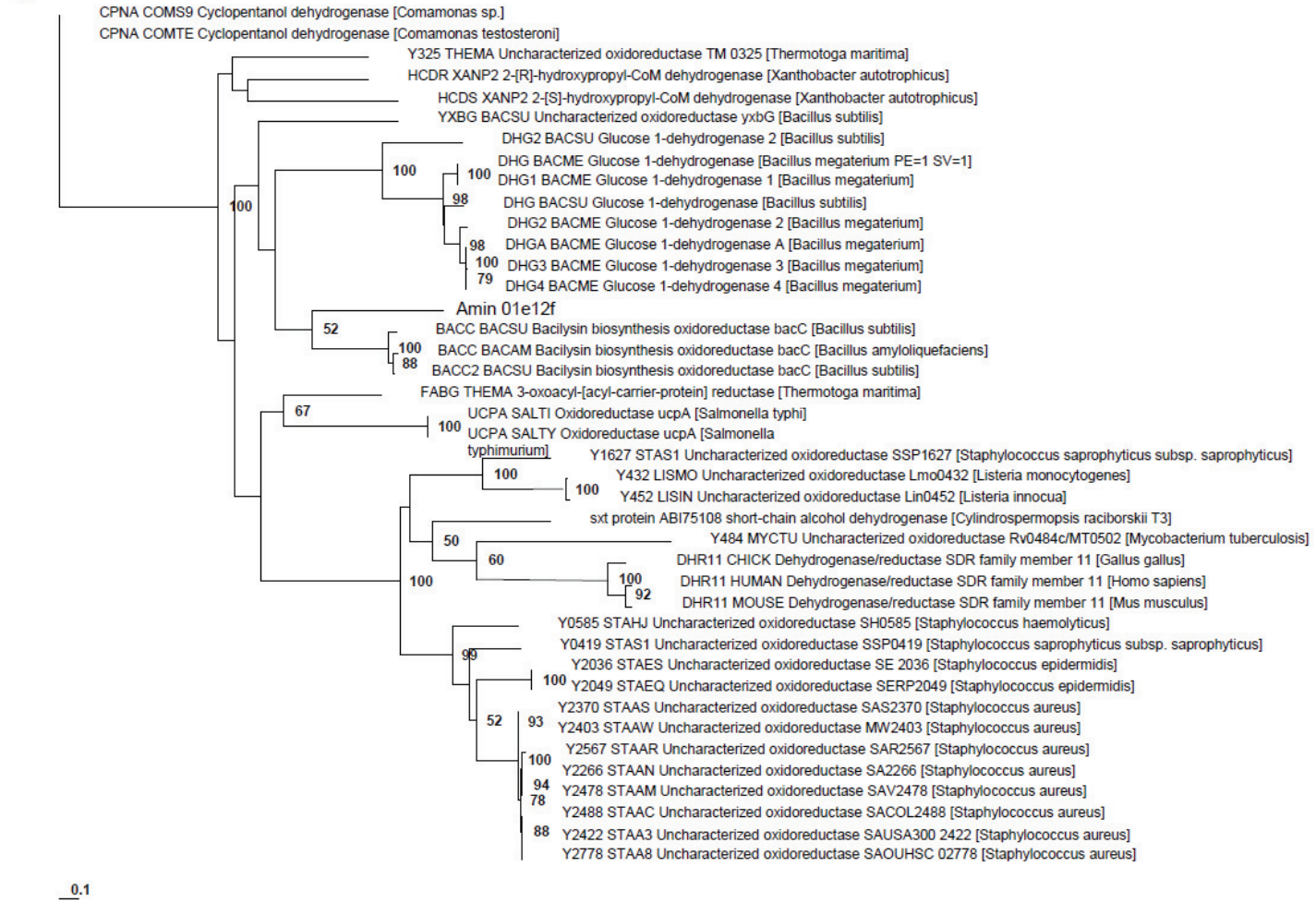
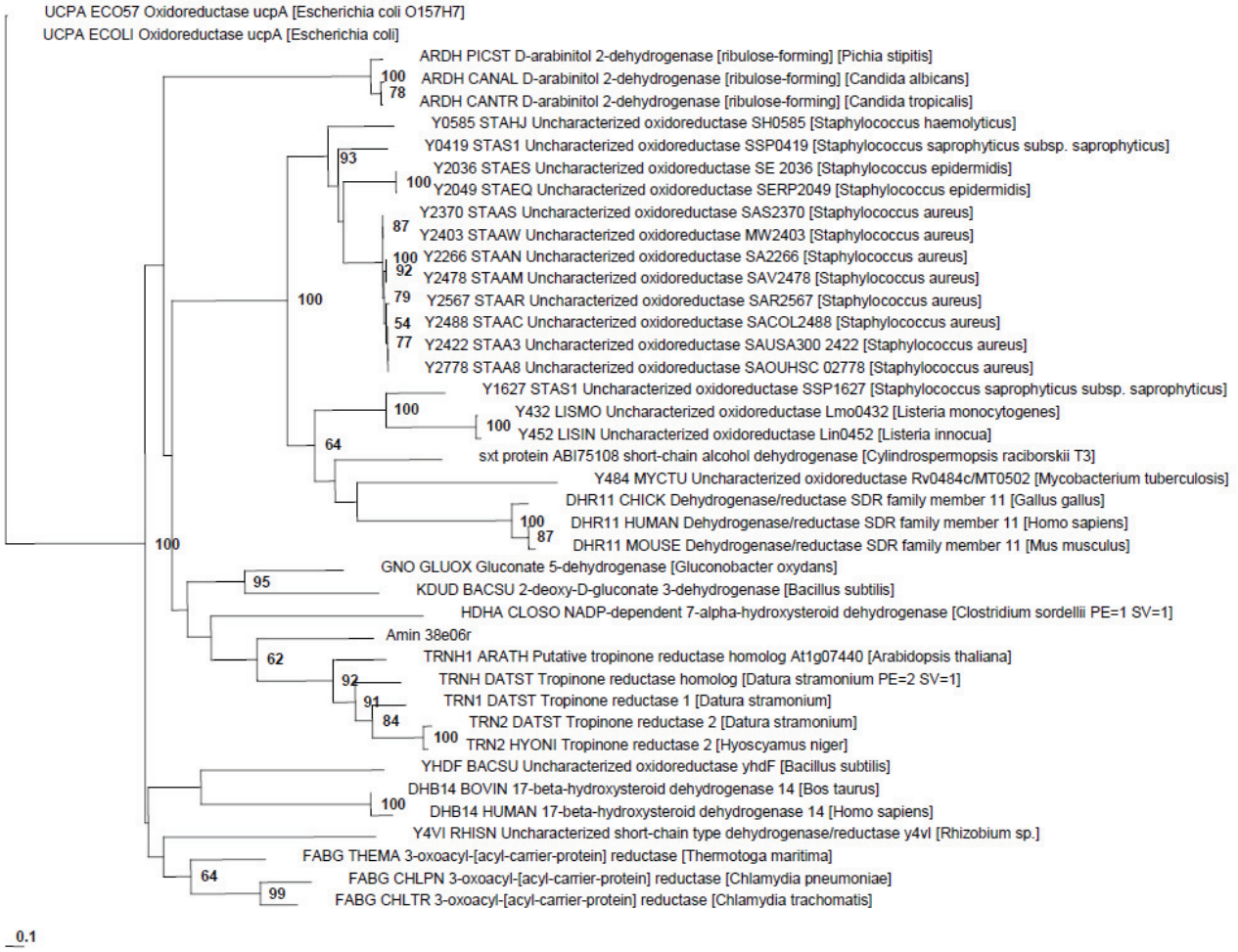

CPNA COMS9 Cyclopentanol dehydrogenase [Comamonas sp.]
CPNA COMTE Cyclopentanol dehydrogenase [Comamonas testosteroni]
Y325 THEMA Uncharacterized oxidoreductase TM 0325 [Thermotoga maritima]
HCDR XANP2 2-[R]-hydroxypropyl-CoM dehydrogenase [Xanthobacter autotrophicus]
HCDS XANP2 2-[S]-hydroxypropyl-CoM dehydrogenase [Xanthobacter autotrophicus]
YXBG BACSU Uncharacterized oxidoreductase yxbG [Bacillus subtilis]
DHG2 BACSU Glucose 1-dehydrogenase 2 [Bacillus subtilis]
DHG BACME Glucose 1-dehydrogenase [Bacillus megaterium PE=1 SV=1]
DHG1 BACME Glucose 1-dehydrogenase 1 [Bacillus megaterium]
DHG BACSU Glucose 1-dehydrogenase [Bacillus subtilis]
DHG2 BACME Glucose 1-dehydrogenase 2 [Bacillus megaterium]
DHGA BACME Glucose 1-dehydrogenase A [Bacillus megaterium]
DHG3 BACME Glucose 1-dehydrogenase 3 [Bacillus megaterium]
DHG4 BACME Glucose 1-dehydrogenase 4 [Bacillus megaterium]
Amin 01e12f
BACC BACSU Bacilysin biosynthesis oxidoreductase bacC [Bacillus subtilis]
BACC BACAM Bacilysin biosynthesis oxidoreductase bacC [Bacillus amyloliquefaciens]
BACC2 BACSU Bacilysin biosynthesis oxidoreductase bacC [Bacillus subtilis]
FABG THEMA 3-oxoacyl-[acyl-carrier-protein] reductase [Thermotoga maritima]
UCPA SALTI Oxidoreductase ucpA [Salmonella typhi]
UCPA SALTY Oxidoreductase ucpA [Salmonella typhimurium]
Y1627 STAS1 Uncharacterized oxidoreductase SSP1627 [Staphylococcus saprophyticus subsp. saprophyticus]
Y432 LISMO Uncharacterized oxidoreductase Lmo0432 [Listeria monocytogenes]
Y452 LISIN Uncharacterized oxidoreductase Lin0452 [Listeria innocua]
sxt protein ABI75108 short-chain alcohol dehydrogenase [Cylindrospermopsis raciborskii T3]
Y484 MYCTU Uncharacterized oxidoreductase Rv0484c/MT0502 [Mycobacterium tuberculosis]
DHR11 CHICK Dehydrogenase/reductase SDR family member 11 [Gallus gallus]
DHR11 HUMAN Dehydrogenase/reductase SDR family member 11 [Homo sapiens]
DHR11 MOUSE Dehydrogenase/reductase SDR family member 11 [Mus musculus]
Y0585 STAHJ Uncharacterized oxidoreductase SH0585 [Staphylococcus haemolyticus]
Y0419 STAS1 Uncharacterized oxidoreductase SSP0419 [Staphylococcus saprophyticus subsp. saprophyticus]
Y2036 STAES Uncharacterized oxidoreductase SE 2036 [Staphylococcus epidermidis]
Y2049 STAEQ Uncharacterized oxidoreductase SERP2049 [Staphylococcus epidermidis]
Y2370 STAAS Uncharacterized oxidoreductase SAS2370 [Staphylococcus aureus]
Y2403 STAAW Uncharacterized oxidoreductase MW2403 [Staphylococcus aureus]
Y2567 STAAR Uncharacterized oxidoreductase SAR2567 [Staphylococcus aureus]
Y2266 STAAN Uncharacterized oxidoreductase SA2266 [Staphylococcus aureus]
Y2478 STAAM Uncharacterized oxidoreductase SAV2478 [Staphylococcus aureus]
Y2488 STAAC Uncharacterized oxidoreductase SACOL2488 [Staphylococcus aureus]
Y2422 STAA3 Uncharacterized oxidoreductase SAUSA300 2422 [Staphylococcus aureus]
Y2778 STAA8 Uncharacterized oxidoreductase SAOUHSC 02778 [Staphylococcus aureus]
0.1

D


UCPA ECO57 Oxidoreductase ucpA [Escherichia coli O157H7]
UCPA ECOLI Oxidoreductase ucpA [Escherichia coli]
ARDH PICST D-arabinitol 2-dehydrogenase [ribulose-forming] [Pichia stipitis]
ARDH CANAL D-arabinitol 2-dehydrogenase [ribulose-forming] [Candida albicans]
ARDH CANTR D-arabinitol 2-dehydrogenase [ribulose-forming] [Candida tropicalis]
Y0585 STAHJ Uncharacterized oxidoreductase SH0585 [Staphylococcus haemolyticus]
Y0419 STAS1 Uncharacterized oxidoreductase SSP0419 [Staphylococcus saprophyticus subsp. saprophyticus]
Y2036 STAES Uncharacterized oxidoreductase SE 2036 [Staphylococcus epidermidis]
Y2049 STAEQ Uncharacterized oxidoreductase SERP2049 [Staphylococcus epidermidis]
Y2370 STAAS Uncharacterized oxidoreductase SAS2370 [Staphylococcus aureus]
Y2403 STAAW Uncharacterized oxidoreductase MW2403 [Staphylococcus aureus]
Y2266 STAAN Uncharacterized oxidoreductase SA2266 [Staphylococcus aureus]
Y2478 STAAM Uncharacterized oxidoreductase SAV2478 [Staphylococcus aureus]
Y2567 STAAR Uncharacterized oxidoreductase SAR2567 [Staphylococcus aureus]
Y2488 STAAC Uncharacterized oxidoreductase SACOL2488 [Staphylococcus aureus]
Y2422 STAA3 Uncharacterized oxidoreductase SAUSA300 2422 [Staphylococcus aureus]
Y2778 STAA8 Uncharacterized oxidoreductase SAOUHSC 02778 [Staphylococcus aureus]
Y1627 STAS1 Uncharacterized oxidoreductase SSP1627 [Staphylococcus saprophyticus subsp. saprophyticus]
Y432 LISMO Uncharacterized oxidoreductase Lmo0432 [Listeria monocytogenes]
Y452 LISIN Uncharacterized oxidoreductase Lin0452 [Listeria innocua]
sxt protein ABI75108 short-chain alcohol dehydrogenase [Cylindrospermopsis raciborskii T3]
Y484 MYCTU Uncharacterized oxidoreductase Rv0484c/MT0502 [Mycobacterium tuberculosis]
DHR11 CHICK Dehydrogenase/reductase SDR family member 11 [Gallus gallus]
DHR11 HUMAN Dehydrogenase/reductase SDR family member 11 [Homo sapiens]
DHR11 MOUSE Dehydrogenase/reductase SDR family member 11 [Mus musculus]
GNO GLUOX Gluconate 5-dehydrogenase [Gluconobacter oxydans]
KDUD BACSU 2-deoxy-D-gluconate 3-dehydrogenase [Bacillus subtilis]
HDHA CLOSO NADP-dependent 7-alpha-hydroxysteroid dehydrogenase [Clostridium sordellii PE=1 SV=1]
Amin 38e06r
TRNH1 ARATH Putative tropinone reductase homolog At1g07440 [Arabidopsis thaliana]
TRNH DATST Tropinone reductase homolog [Datura stramonium PE=2 SV=1]
TRN1 DATST Tropinone reductase 1 [Datura stramonium]
TRN2 DATST Tropinone reductase 2 [Datura stramonium]
TRN2 HYONI Tropinone reductase 2 [Hyoscyamus niger]
YHDF BACSU Uncharacterized oxidoreductase yhdF [Bacillus subtilis]
DHB14 BOVIN 17-beta-hydroxysteroid dehydrogenase 14 [Bos taurus]
DHB14 HUMAN 17-beta-hydroxysteroid dehydrogenase 14 [Homo sapiens]
Y4VI RHISN Uncharacterized short-chain type dehydrogenase/reductase y4vI [Rhizobium sp.]
FABG THEMA 3-oxoacyl-[acyl-carrier-protein] reductase [Thermotoga maritima]
FABG CHLPN 3-oxoacyl-[acyl-carrier-protein] reductase [Chlamydia pneumoniae]
FABG CHLTR 3-oxoacyl-[acyl-carrier-protein] reductase [Chlamydia trachomatis]
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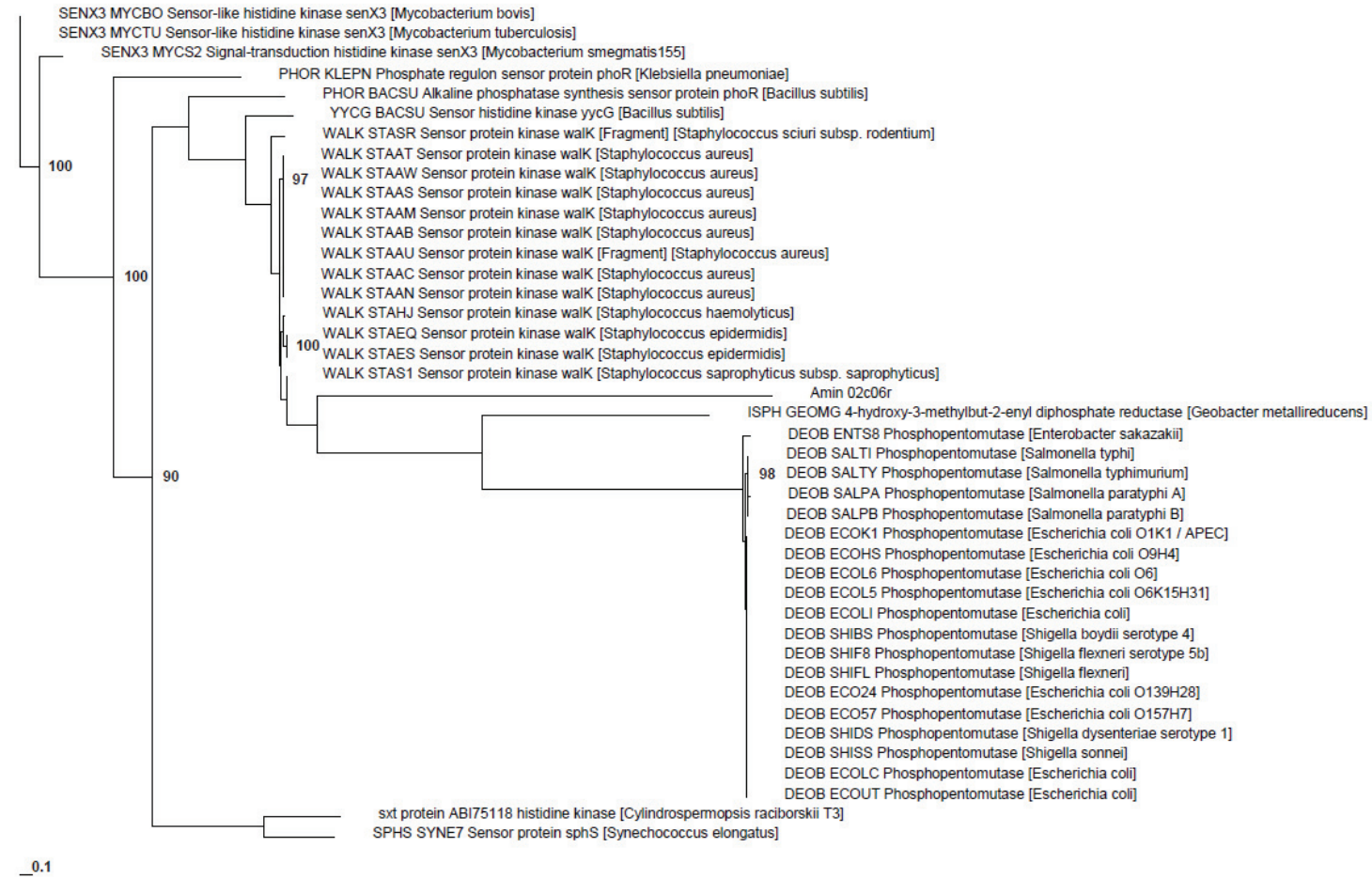
SENX3 MYCBO Sensor-like histidine kinase senX3 [Mycobacterium bovis]
SENX3 MYCTU Sensor-like histidine kinase senX3 [Mycobacterium tuberculosis]
SENX3 MYCS2 Signal-transduction histidine kinase senX3 [Mycobacterium smegmatis155]
PHOR KLEPN Phosphate regulon sensor protein phoR [Klebsiella pneumoniae]
PHOR BACSU Alkaline phosphatase synthesis sensor protein phoR [Bacillus subtilis]
YYCG BACSU Sensor histidine kinase yycG [Bacillus subtilis]
WALK STASR Sensor protein kinase walK [Fragment] [Staphylococcus sciuri subsp. rodentium]
WALK STAAT Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAW Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAS Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAM Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAB Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAU Sensor protein kinase walK [Fragment] [Staphylococcus aureus]
WALK STAAC Sensor protein kinase walK [Staphylococcus aureus]
WALK STAAN Sensor protein kinase walK [Staphylococcus aureus]
WALK STAHJ Sensor protein kinase walK [Staphylococcus haemolyticus]
WALK STAEQ Sensor protein kinase walK [Staphylococcus epidermidis]
WALK STAES Sensor protein kinase walK [Staphylococcus epidermidis]
WALK STAS1 Sensor protein kinase walK [Staphylococcus saprophyticus subsp. saprophyticus]
Amin 02c06r
ISPH GEOMG 4-hydroxy-3-methylbut-2-enyl diphosphate reductase [Geobacter metallireducens]
DEOB ENTS8 Phosphopentomutase [Enterobacter sakazakii]
DEOB SALTI Phosphopentomutase [Salmonella typhi]
DEOB SALTY Phosphopentomutase [Salmonella typhimurium]
DEOB SALPA Phosphopentomutase [Salmonella paratyphi A]
DEOB SALPB Phosphopentomutase [Salmonella paratyphi B]
DEOB ECOK1 Phosphopentomutase [Escherichia coli O1K1 / APEC]
DEOB ECOHS Phosphopentomutase [Escherichia coli O9H4]
DEOB ECOL6 Phosphopentomutase [Escherichia coli O6]
DEOB ECOL5 Phosphopentomutase [Escherichia coli O6K15H31]
DEOB ECOLI Phosphopentomutase [Escherichia coli]
DEOB SHIBS Phosphopentomutase [Shigella boydii serotype 4]
DEOB SHIF8 Phosphopentomutase [Shigella flexneri serotype 5b]
DEOB SHIFL Phosphopentomutase [Shigella flexneri]
DEOB ECO24 Phosphopentomutase [Escherichia coli O139H28]
DEOB ECO57 Phosphopentomutase [Escherichia coli O157H7]
DEOB SHIDS Phosphopentomutase [Shigella dysenteriae serotype 1]
DEOB SHISS Phosphopentomutase [Shigella sonnei]
DEOB ECOLC Phosphopentomutase [Escherichia coli]
DEOB ECOUT Phosphopentomutase [Escherichia coli]
sxt protein ABI75118 histidine kinase [Cylindrospermopsis raciborskii T3]
SPHS SYNE7 Sensor protein sphS [Synechococcus elongatus]
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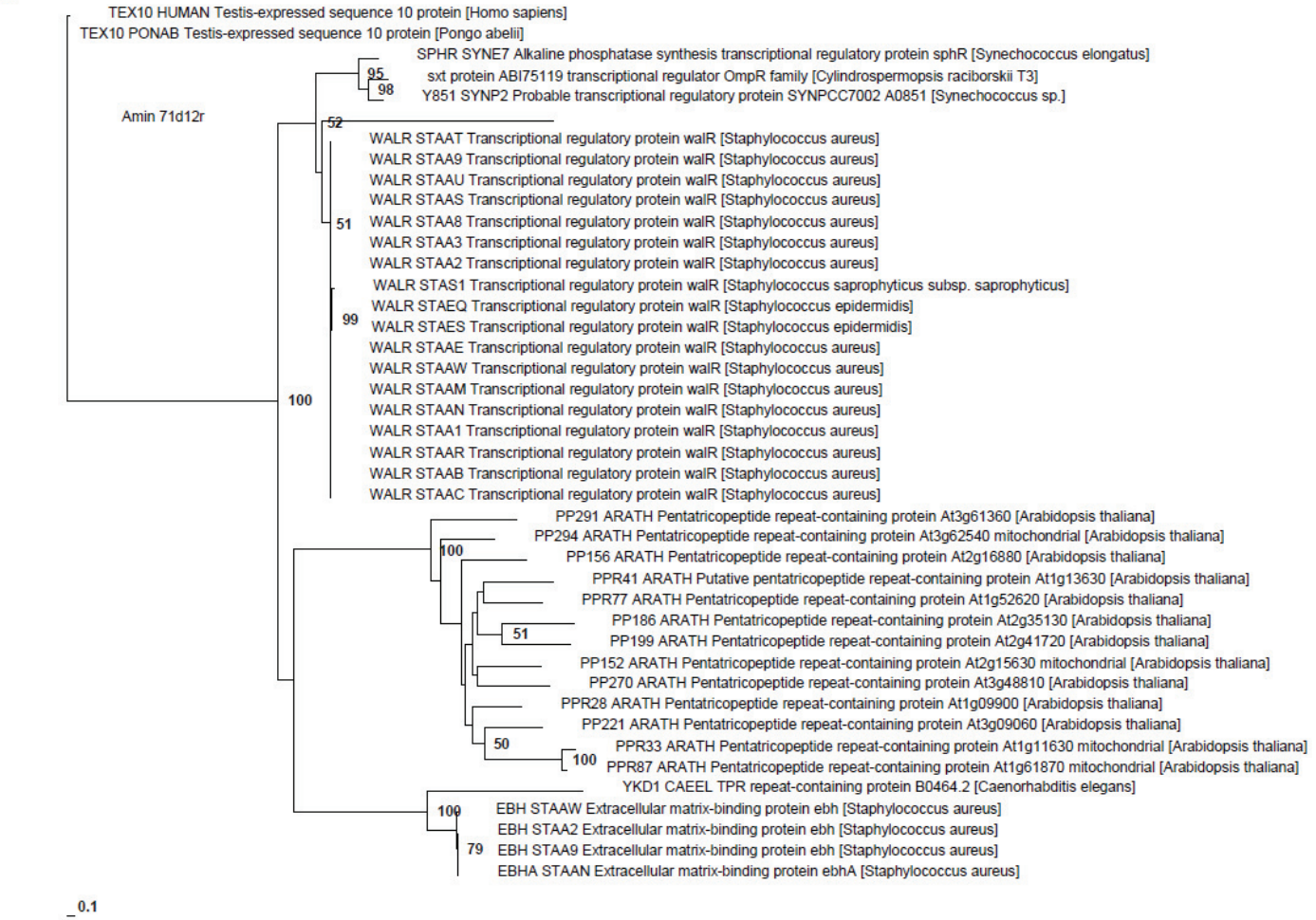
TEX10 HUMAN Testis-expressed sequence 10 protein [Homo sapiens]
TEX10 PONAB Testis-expressed sequence 10 protein [Pongo abelii]
SPHR SYNE7 Alkaline phosphatase synthesis transcriptional regulatory protein sphR [Synechococcus elongatus]
sxt protein ABI75119 transcriptional regulator OmpR family [Cylindrospermopsis raciborskii T3]
Y851 SYNP2 Probable transcriptional regulatory protein SYNPCC7002 A0851 [Synechococcus sp.]
Amin 71d12r
WALR STAAT Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA9 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAU Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAS Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA8 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA3 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA2 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAS1 Transcriptional regulatory protein walR [Staphylococcus saprophyticus subsp. saprophyticus]
WALR STAEQ Transcriptional regulatory protein walR [Staphylococcus epidermidis]
WALR STAES Transcriptional regulatory protein walR [Staphylococcus epidermidis]
WALR STAAE Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAW Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAM Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAN Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA1 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAR Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAB Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAC Transcriptional regulatory protein walR [Staphylococcus aureus]
PP291 ARATH Pentatricopeptide repeat-containing protein At3g61360 [Arabidopsis thaliana]
PP294 ARATH Pentatricopeptide repeat-containing protein At3g62540 mitochondrial [Arabidopsis thaliana]
PP156 ARATH Pentatricopeptide repeat-containing protein At2g16880 [Arabidopsis thaliana]
PPR41 ARATH Putative pentatricopeptide repeat-containing protein At1g13630 [Arabidopsis thaliana]
PPR77 ARATH Pentatricopeptide repeat-containing protein At1g52620 [Arabidopsis thaliana]
PP186 ARATH Pentatricopeptide repeat-containing protein At2g35130 [Arabidopsis thaliana]
PP199 ARATH Pentatricopeptide repeat-containing protein At2g41720 [Arabidopsis thaliana]
PP152 ARATH Pentatricopeptide repeat-containing protein At2g15630 mitochondrial [Arabidopsis thaliana]
PP270 ARATH Pentatricopeptide repeat-containing protein At3g48810 [Arabidopsis thaliana]
PPR28 ARATH Pentatricopeptide repeat-containing protein At1g09900 [Arabidopsis thaliana]
PP221 ARATH Pentatricopeptide repeat-containing protein At3g09060 [Arabidopsis thaliana]
PPR33 ARATH Pentatricopeptide repeat-containing protein At1g11630 mitochondrial [Arabidopsis thaliana]
PPR87 ARATH Pentatricopeptide repeat-containing protein At1g61870 mitochondrial [Arabidopsis thaliana]
YKD1 CAEEL TPR repeat-containing protein B0464.2 [Caenorhabditis elegans]
EBH STAAW Extracellular matrix-binding protein ebh [Staphylococcus aureus]
EBH STAA2 Extracellular matrix-binding protein ebh [Staphylococcus aureus]
EBH STAA9 Extracellular matrix-binding protein ebh [Staphylococcus aureus]
EBHA STAAN Extracellular matrix-binding protein ebhA [Staphylococcus aureus]
95
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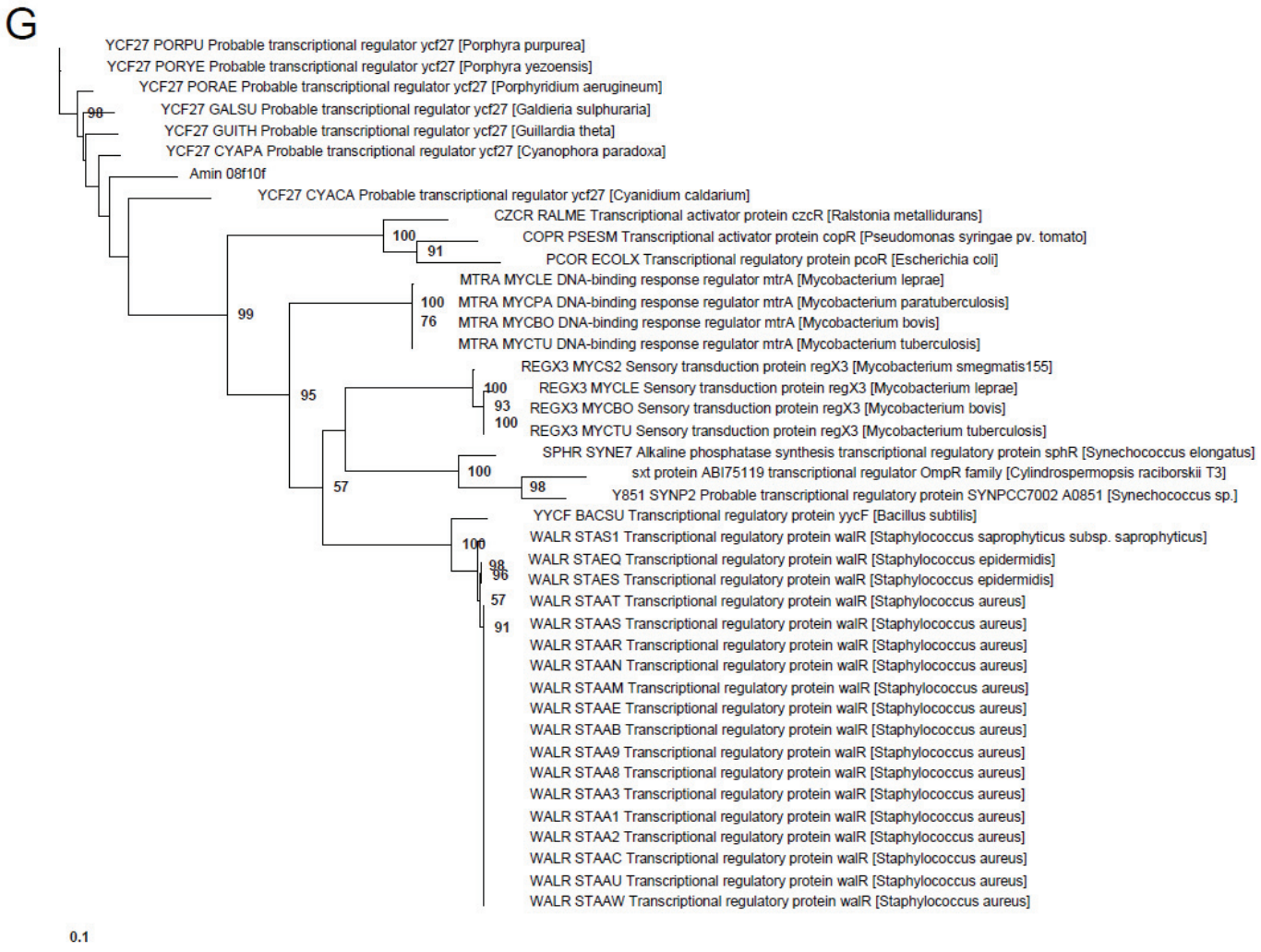
YCF27 PORPU Probable transcriptional regulator ycf27 [Porphyra purpurea]
YCF27 PORYE Probable transcriptional regulator ycf27 [Porphyra yezoensis]
YCF27 PORAE Probable transcriptional regulator ycf27 [Porphyridium aerugineum]
98
YCF27 GALSU Probable transcriptional regulator ycf27 [Galdieria sulphuraria]
YCF27 GUITH Probable transcriptional regulator ycf27 [Guillardia theta]
YCF27 CYAPA Probable transcriptional regulator ycf27 [Cyanophora paradoxa]
Amin 08f10f
YCF27 CYACA Probable transcriptional regulator ycf27 [Cyanidium caldarium]
CZCR RALME Transcriptional activator protein czcR [Ralstonia metallidurans]
100
COPR PSESM Transcriptional activator protein copR [Pseudomonas syringae pv. tomato]
91
PCOR ECOLX Transcriptional regulatory protein pcoR [Escherichia coli]
MTRA MYCLE DNA-binding response regulator mtrA [Mycobacterium leprae]
100
MTRA MYCPA DNA-binding response regulator mtrA [Mycobacterium paratuberculosis]
99
76
MTRA MYCBO DNA-binding response regulator mtrA [Mycobacterium bovis]
MTRA MYCTU DNA-binding response regulator mtrA [Mycobacterium tuberculosis]
REGX3 MYCS2 Sensory transduction protein regX3 [Mycobacterium smegmatis155]
100
REGX3 MYCLE Sensory transduction protein regX3 [Mycobacterium leprae]
95
93
REGX3 MYCBO Sensory transduction protein regX3 [Mycobacterium bovis]
100
REGX3 MYCTU Sensory transduction protein regX3 [Mycobacterium tuberculosis]
SPHR SYNE7 Alkaline phosphatase synthesis transcriptional regulatory protein sphR [Synechococcus elongatus]
100
sxt protein ABI75119 transcriptional regulator OmpR family [Cylindrospermopsis raciborskii T3]
57
98
Y851 SYNP2 Probable transcriptional regulatory protein SYNPCC7002 A0851 [Synechococcus sp.]
YYCF BACSU Transcriptional regulatory protein yycF [Bacillus subtilis]
100
WALR STAS1 Transcriptional regulatory protein walR [Staphylococcus saprophyticus subsp. saprophyticus]
98
WALR STAEQ Transcriptional regulatory protein walR [Staphylococcus epidermidis]
96
WALR STAES Transcriptional regulatory protein walR [Staphylococcus epidermidis]
57
WALR STAAT Transcriptional regulatory protein walR [Staphylococcus aureus]
91
WALR STAAS Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAR Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAN Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAM Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAE Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAB Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA9 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA8 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA3 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA1 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAA2 Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAC Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAU Transcriptional regulatory protein walR [Staphylococcus aureus]
WALR STAAW Transcriptional regulatory protein walR [Staphylococcus aureus]
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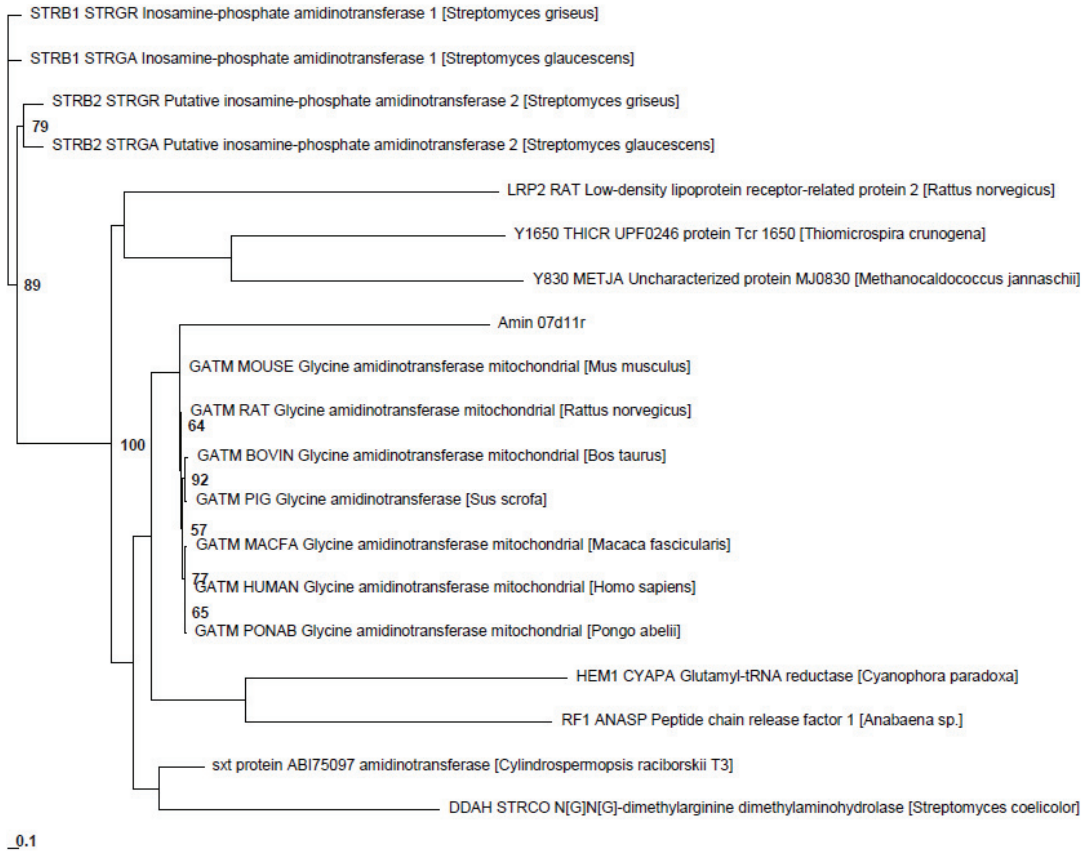
STRB1 STRGR Inosamine-phosphate amidinotransferase 1 [Streptomyces griseus]
STRB1 STRGA Inosamine-phosphate amidinotransferase 1 [Streptomyces glaucescens]
STRB2 STRGR Putative inosamine-phosphate amidinotransferase 2 [Streptomyces griseus]
79
STRB2 STRGA Putative inosamine-phosphate amidinotransferase 2 [Streptomyces glaucescens]
LRP2 RAT Low-density lipoprotein receptor-related protein 2 [Rattus norvegicus]
Y1650 THICR UPF0246 protein Tcr 1650 [Thiomicrospira crunogena]
Y830 METJA Uncharacterized protein MJ0830 [Methanocaldococcus jannaschii]
89
Amin 07d11r
GATM MOUSE Glycine amidinotransferase mitochondrial [Mus musculus]
GATM RAT Glycine amidinotransferase mitochondrial [Rattus norvegicus]
64
100
GATM BOVIN Glycine amidinotransferase mitochondrial [Bos taurus]
92
GATM PIG Glycine amidinotransferase [Sus scrofa]
57
GATM MACFA Glycine amidinotransferase mitochondrial [Macaca fascicularis]
77
GATM HUMAN Glycine amidinotransferase mitochondrial [Homo sapiens]
65
GATM PONAB Glycine amidinotransferase mitochondrial [Pongo abelii]
HEM1 CYAPA Glutamyl-tRNA reductase [Cyanophora paradoxa]
RF1 ANASP Peptide chain release factor 1 [Anabaena sp.]
sxt protein ABI75097 amidinotransferase [Cylindrospermopsis raciborskii T3]
DDAH STRCO N[G]N[G]-dimethylarginine dimethylaminohydrolase [Streptomyces coelicolor]
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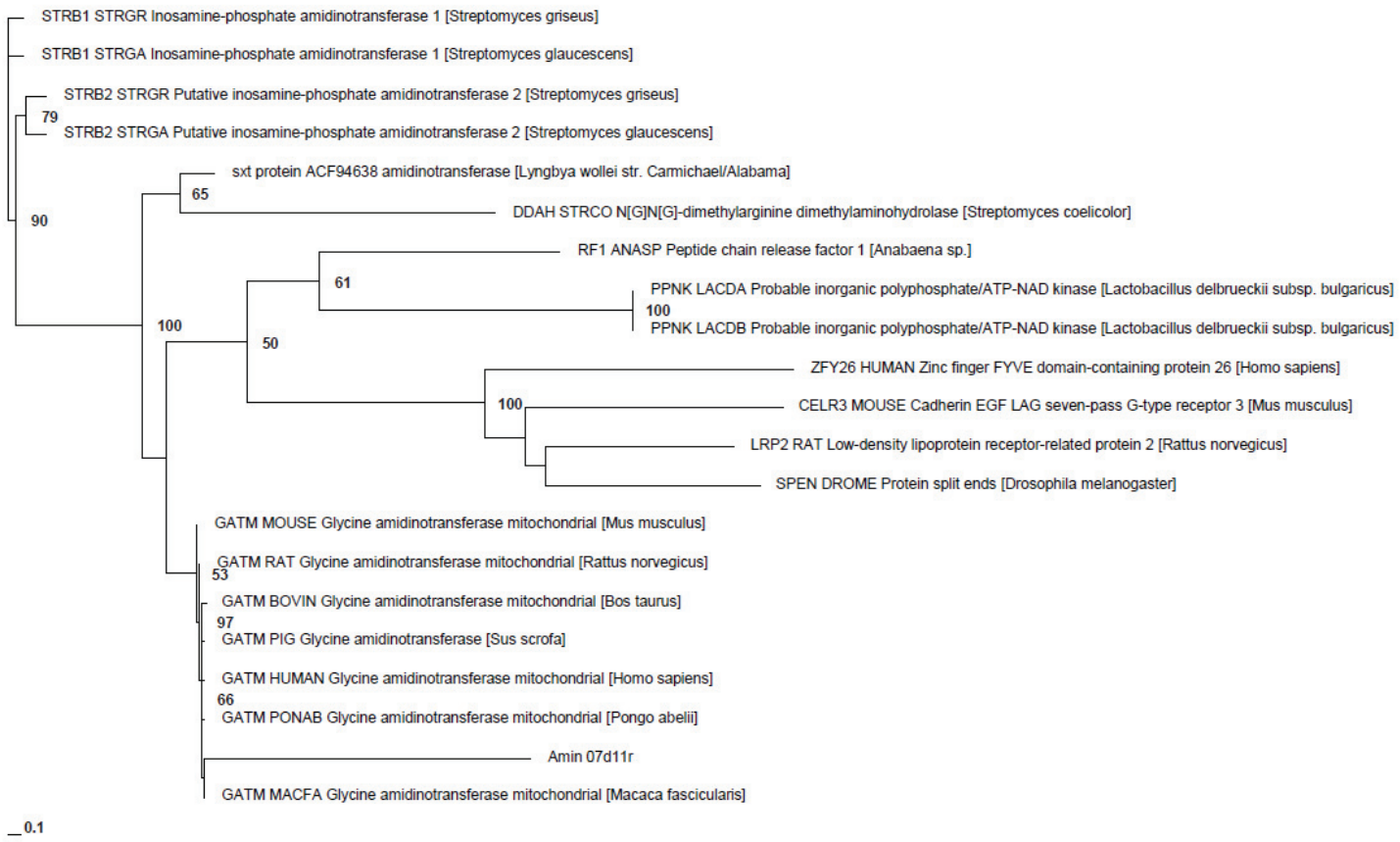
STRB1 STRGR Inosamine-phosphate amidinotransferase 1 [Streptomyces griseus]
STRB1 STRGA Inosamine-phosphate amidinotransferase 1 [Streptomyces glaucescens]
STRB2 STRGR Putative inosamine-phosphate amidinotransferase 2 [Streptomyces griseus]
79
STRB2 STRGA Putative inosamine-phosphate amidinotransferase 2 [Streptomyces glaucescens]
sxt protein ACF94638 amidinotransferase [Lyngbya wollei str. Carmichael/Alabama]
65
DDAH STRCO N[G]N[G]-dimethylarginine dimethylaminohydrolase [Streptomyces coelicolor]
90
RF1 ANASP Peptide chain release factor 1 [Anabaena sp.]
61
PPNK LACDA Probable inorganic polyphosphate/ATP-NAD kinase [Lactobacillus delbrueckii subsp. bulgaricus]
100
PPNK LACDB Probable inorganic polyphosphate/ATP-NAD kinase [Lactobacillus delbrueckii subsp. bulgaricus]
100
50
ZFY26 HUMAN Zinc finger FYVE domain-containing protein 26 [Homo sapiens]
100
CELR3 MOUSE Cadherin EGF LAG seven-pass G-type receptor 3 [Mus musculus]
LRP2 RAT Low-density lipoprotein receptor-related protein 2 [Rattus norvegicus]
SPEN DROME Protein split ends [Drosophila melanogaster]
GATM MOUSE Glycine amidinotransferase mitochondrial [Mus musculus]
GATM RAT Glycine amidinotransferase mitochondrial [Rattus norvegicus]
53
GATM BOVIN Glycine amidinotransferase mitochondrial [Bos taurus]
97
GATM PIG Glycine amidinotransferase [Sus scrofa]
GATM HUMAN Glycine amidinotransferase mitochondrial [Homo sapiens]
66
GATM PONAB Glycine amidinotransferase mitochondrial [Pongo abelii]
Amin 07d11r
GATM MACFA Glycine amidinotransferase mitochondrial [Macaca fascicularis]
0.1

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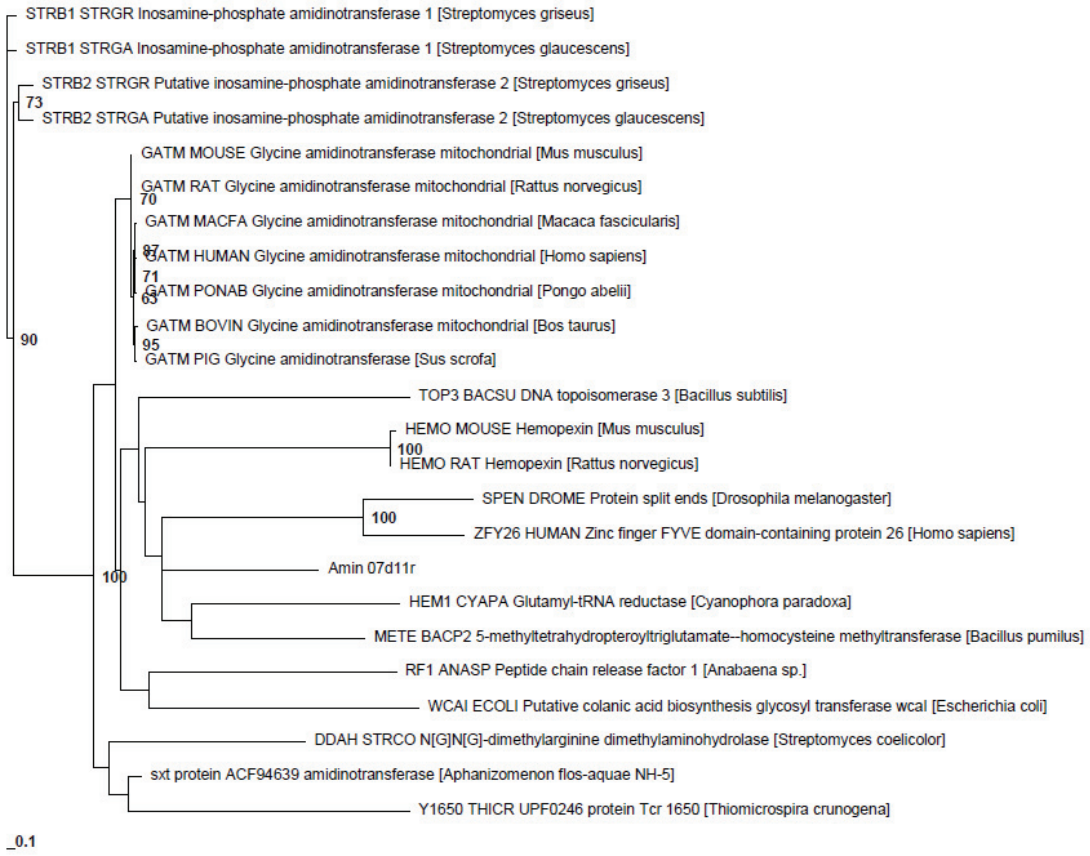
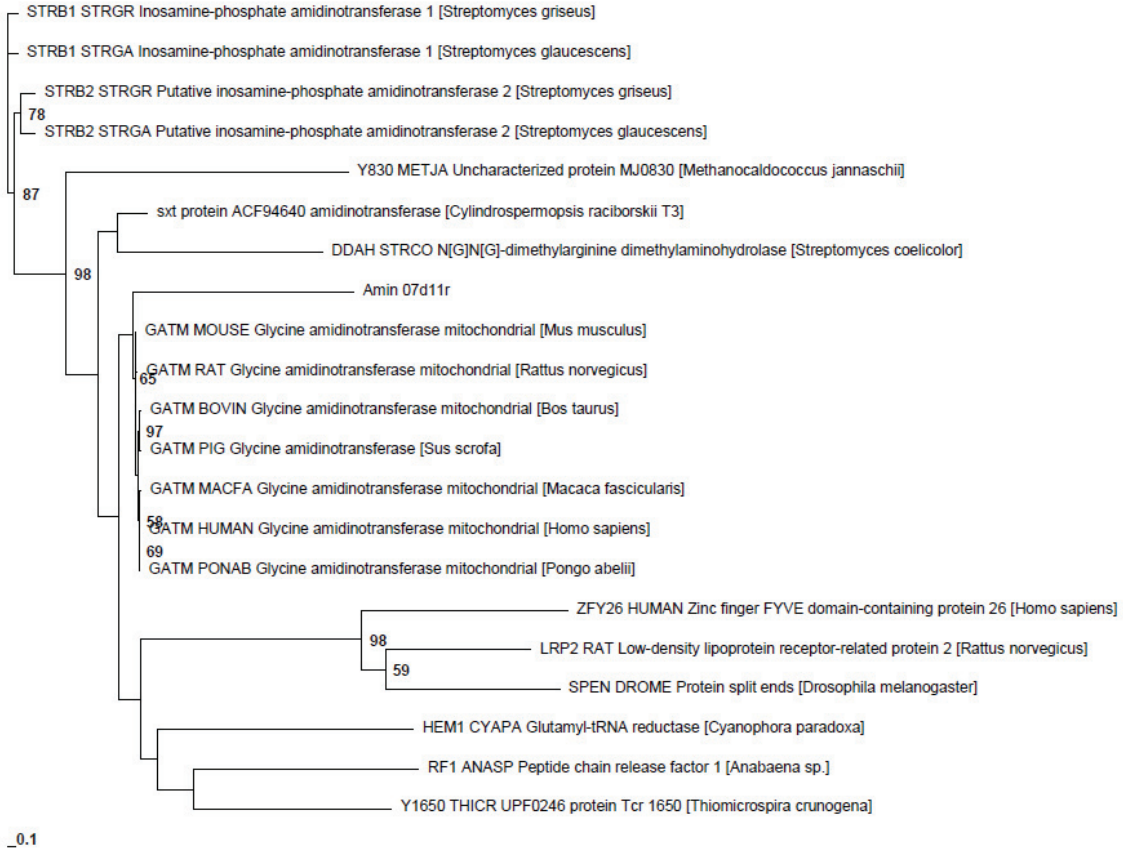
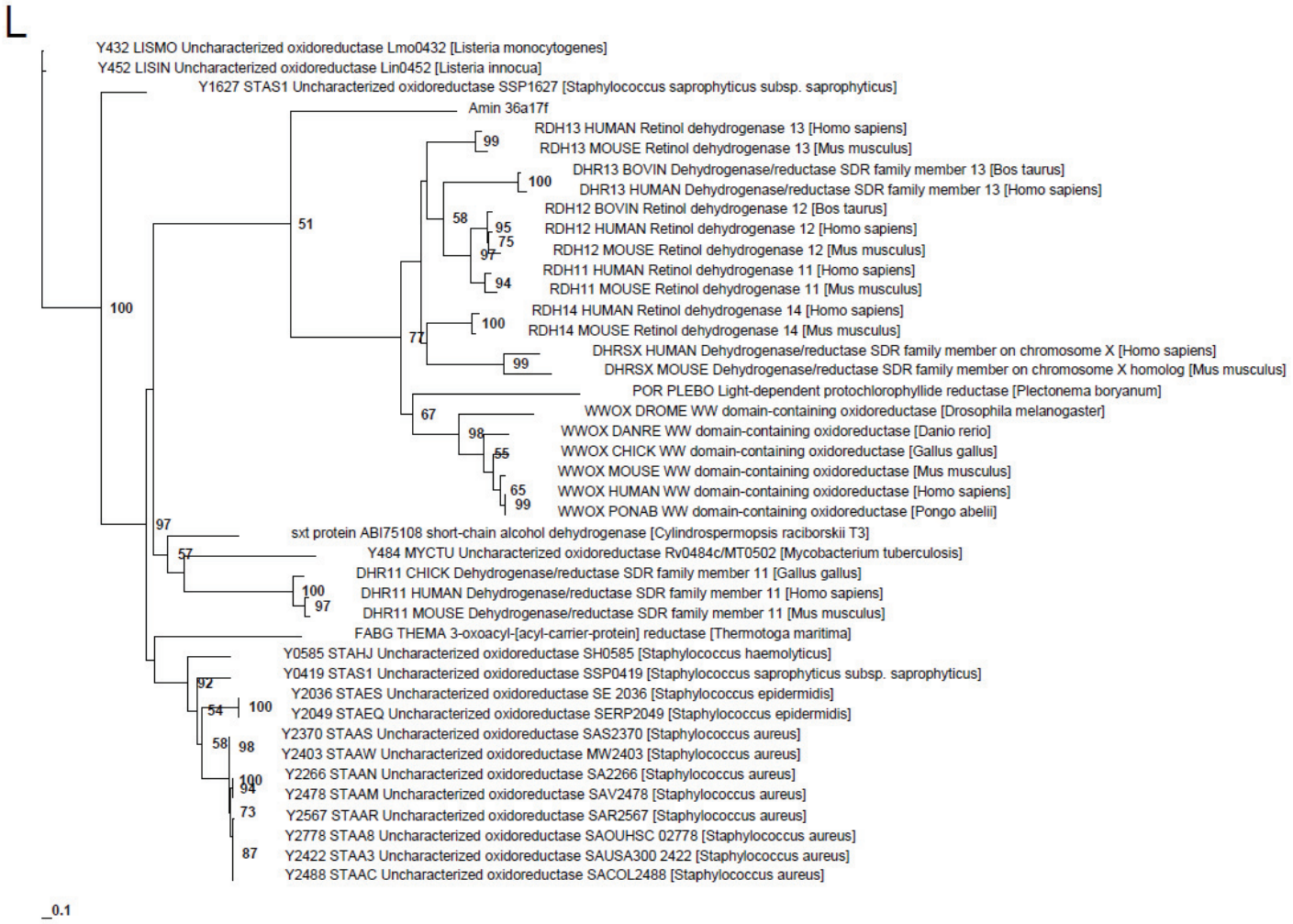

STRB1 STRGR Inosamine-phosphate amidinotransferase 1 [Streptomyces griseus]
STRB1 STRGA Inosamine-phosphate amidinotransferase 1 [Streptomyces glaucescens]
STRB2 STRGR Putative inosamine-phosphate amidinotransferase 2 [Streptomyces griseus]
73
STRB2 STRGA Putative inosamine-phosphate amidinotransferase 2 [Streptomyces glaucescens]
GATM MOUSE Glycine amidinotransferase mitochondrial [Mus musculus]
GATM RAT Glycine amidinotransferase mitochondrial [Rattus norvegicus]
70
GATM MACFA Glycine amidinotransferase mitochondrial [Macaca fascicularis]
97
GATM HUMAN Glycine amidinotransferase mitochondrial [Homo sapiens]
71
GATM PONAB Glycine amidinotransferase mitochondrial [Pongo abelii]
63
GATM BOVIN Glycine amidinotransferase mitochondrial [Bos taurus]
90
95
GATM PIG Glycine amidinotransferase [Sus scrofa]
TOP3 BACSU DNA topoisomerase 3 [Bacillus subtilis]
HEMO MOUSE Hemopexin [Mus musculus]
100
HEMO RAT Hemopexin [Rattus norvegicus]
SPEN DROME Protein split ends [Drosophila melanogaster]
100
ZFY26 HUMAN Zinc finger FYVE domain-containing protein 26 [Homo sapiens]
Amin 07d11r
100
HEM1 CYAPA Glutamyl-tRNA reductase [Cyanophora paradoxa]
METE BACP2 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase [Bacillus pumilus]
RF1 ANASP Peptide chain release factor 1 [Anabaena sp.]
WCAI ECOLI Putative colanic acid biosynthesis glycosyl transferase wcaI [Escherichia coli]
DDAH STRCO N[G]N[G]-dimethylarginine dimethylaminohydrolase [Streptomyces coelicolor]
sxt protein ACF94639 amidinotransferase [Aphanizomenon flos-aquae NH-5]
Y1650 THICR UPF0246 protein Tcr 1650 [Thiomicrospira crunogena]
_0.1

K


STRB1 STRGR Inosamine-phosphate amidinotransferase 1 [Streptomyces griseus]
STRB1 STRGA Inosamine-phosphate amidinotransferase 1 [Streptomyces glaucescens]
STRB2 STRGR Putative inosamine-phosphate amidinotransferase 2 [Streptomyces griseus]
78
STRB2 STRGA Putative inosamine-phosphate amidinotransferase 2 [Streptomyces glaucescens]
Y830 METJA Uncharacterized protein MJ0830 [Methanocaldococcus jannaschii]
87
sxt protein ACF94640 amidinotransferase [Cylindrospermopsis raciborskii T3]
DDAH STRCO N[G]N[G]-dimethylarginine dimethylaminohydrolase [Streptomyces coelicolor]
98
Amin 07d11r
GATM MOUSE Glycine amidinotransferase mitochondrial [Mus musculus]
GATM RAT Glycine amidinotransferase mitochondrial [Rattus norvegicus]
65
GATM BOVIN Glycine amidinotransferase mitochondrial [Bos taurus]
97
GATM PIG Glycine amidinotransferase [Sus scrofa]
GATM MACFA Glycine amidinotransferase mitochondrial [Macaca fascicularis]
58
GATM HUMAN Glycine amidinotransferase mitochondrial [Homo sapiens]
69
GATM PONAB Glycine amidinotransferase mitochondrial [Pongo abelii]
ZFY26 HUMAN Zinc finger FYVE domain-containing protein 26 [Homo sapiens]
98
LRP2 RAT Low-density lipoprotein receptor-related protein 2 [Rattus norvegicus]
59
SPEN DROME Protein split ends [Drosophila melanogaster]
HEM1 CYAPA Glutamyl-tRNA reductase [Cyanophora paradoxa]
RF1 ANASP Peptide chain release factor 1 [Anabaena sp.]
Y1650 THICR UPF0246 protein Tcr 1650 [Thiomicrospira crunogena]
_0.1


L
Y432 LISMO Uncharacterized oxidoreductase Lmo0432 [Listeria monocytogenes]
Y452 LISIN Uncharacterized oxidoreductase Lin0452 [Listeria innocua]
Y1627 STAS1 Uncharacterized oxidoreductase SSP1627 [Staphylococcus saprophyticus subsp. saprophyticus]
Amin 36a17f
RDH13 HUMAN Retinol dehydrogenase 13 [Homo sapiens]
RDH13 MOUSE Retinol dehydrogenase 13 [Mus musculus]
DHR13 BOVIN Dehydrogenase/reductase SDR family member 13 [Bos taurus]
DHR13 HUMAN Dehydrogenase/reductase SDR family member 13 [Homo sapiens]
RDH12 BOVIN Retinol dehydrogenase 12 [Bos taurus]
RDH12 HUMAN Retinol dehydrogenase 12 [Homo sapiens]
RDH12 MOUSE Retinol dehydrogenase 12 [Mus musculus]
RDH11 HUMAN Retinol dehydrogenase 11 [Homo sapiens]
RDH11 MOUSE Retinol dehydrogenase 11 [Mus musculus]
RDH14 HUMAN Retinol dehydrogenase 14 [Homo sapiens]
RDH14 MOUSE Retinol dehydrogenase 14 [Mus musculus]
DHRSX HUMAN Dehydrogenase/reductase SDR family member on chromosome X [Homo sapiens]
DHRSX MOUSE Dehydrogenase/reductase SDR family member on chromosome X homolog [Mus musculus]
POR PLEBO Light-dependent protochlorophyllide reductase [Plectonema boryanum]
WWOX DROME WW domain-containing oxidoreductase [Drosophila melanogaster]
WWOX DANRE WW domain-containing oxidoreductase [Danio rerio]
WWOX CHICK WW domain-containing oxidoreductase [Gallus gallus]
WWOX MOUSE WW domain-containing oxidoreductase [Mus musculus]
WWOX HUMAN WW domain-containing oxidoreductase [Homo sapiens]
WWOX PONAB WW domain-containing oxidoreductase [Pongo abelii]
sxt protein ABI75108 short-chain alcohol dehydrogenase [Cylindrospermopsis raciborskii T3]
Y484 MYCTU Uncharacterized oxidoreductase Rv0484c/MT0502 [Mycobacterium tuberculosis]
DHR11 CHICK Dehydrogenase/reductase SDR family member 11 [Gallus gallus]
DHR11 HUMAN Dehydrogenase/reductase SDR family member 11 [Homo sapiens]
DHR11 MOUSE Dehydrogenase/reductase SDR family member 11 [Mus musculus]
FABG THEMA 3-oxoacyl-[acyl-carrier-protein] reductase [Thermotoga maritima]
Y0585 STAHJ Uncharacterized oxidoreductase SH0585 [Staphylococcus haemolyticus]
Y0419 STAS1 Uncharacterized oxidoreductase SSP0419 [Staphylococcus saprophyticus subsp. saprophyticus]
Y2036 STAES Uncharacterized oxidoreductase SE 2036 [Staphylococcus epidermidis]
Y2049 STAEQ Uncharacterized oxidoreductase SERP2049 [Staphylococcus epidermidis]
Y2370 STAAS Uncharacterized oxidoreductase SAS2370 [Staphylococcus aureus]
Y2403 STAAW Uncharacterized oxidoreductase MW2403 [Staphylococcus aureus]
Y2266 STAAN Uncharacterized oxidoreductase SA2266 [Staphylococcus aureus]
Y2478 STAAM Uncharacterized oxidoreductase SAV2478 [Staphylococcus aureus]
Y2567 STAAR Uncharacterized oxidoreductase SAR2567 [Staphylococcus aureus]
Y2778 STAA8 Uncharacterized oxidoreductase SAOUHSC 02778 [Staphylococcus aureus]
Y2422 STAA3 Uncharacterized oxidoreductase SAUSA300 2422 [Staphylococcus aureus]
Y2488 STAAC Uncharacterized oxidoreductase SACOL2488 [Staphylococcus aureus]
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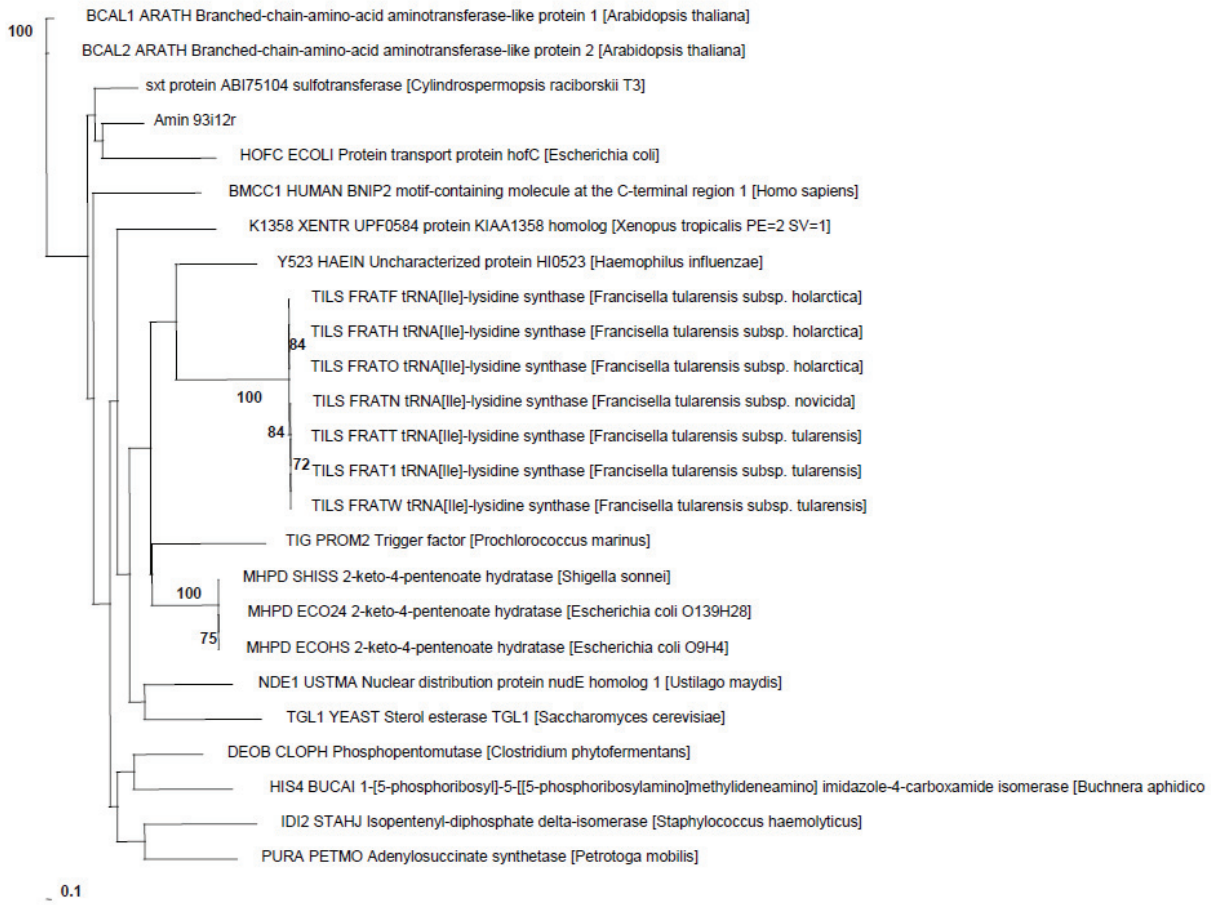
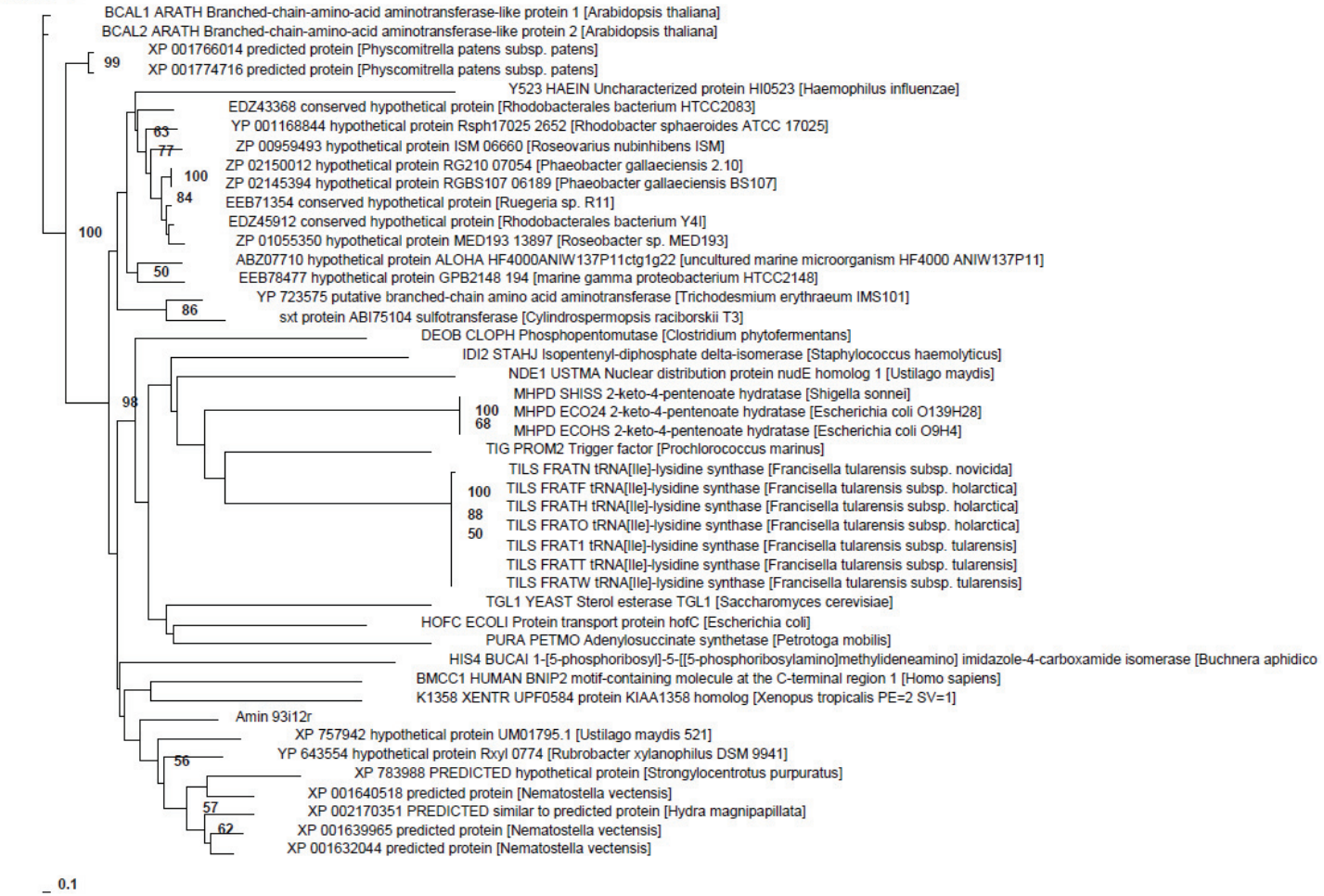
M.1


BCAL1 ARATH Branched-chain-amino-acid aminotransferase-like protein 1 [Arabidopsis thaliana]
BCAL2 ARATH Branched-chain-amino-acid aminotransferase-like protein 2 [Arabidopsis thaliana]
sxt protein ABI75104 sulfotransferase [Cylindrospermopsis raciborskii T3]
Amin 93i12r
HOFC ECOLI Protein transport protein hofC [Escherichia coli]
BMCC1 HUMAN BNIP2 motif-containing molecule at the C-terminal region 1 [Homo sapiens]
K1358 XENTR UPF0584 protein KIAA1358 homolog [Xenopus tropicalis PE=2 SV=1]
Y523 HAEIN Uncharacterized protein HI0523 [Haemophilus influenzae]
TILS FRATF tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRATH tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRATO tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRATN tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. novicida]
TILS FRATT tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TILS FRAT1 tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TILS FRATW tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TIG PROM2 Trigger factor [Prochlorococcus marinus]
MHPD SHISS 2-keto-4-pentenoate hydratase [Shigella sonnei]
MHPD ECO24 2-keto-4-pentenoate hydratase [Escherichia coli O139H28]
MHPD ECOHS 2-keto-4-pentenoate hydratase [Escherichia coli O9H4]
NDE1 USTMA Nuclear distribution protein nudE homolog 1 [Ustilago maydis]
TGL1 YEAST Sterol esterase TGL1 [Saccharomyces cerevisiae]
DEOB CLOPH Phosphopentomutase [Clostridium phytofermentans]
HIS4 BUCAI 1-[5-phosphoribosyl]-5-[[5-phosphoribosylamino]methylideneamino] imidazole-4-carboxamide isomerase [Buchnera aphidico
IDI2 STAHJ Isopentenyl-diphosphate delta-isomerase [Staphylococcus haemolyticus]
PURA PETMO Adenylosuccinate synthetase [Petrotoga mobilis]
100
84
100
84
72
100
75
0.1

M.2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BCAL2 ARATH Branched-chain-amino-acid aminotransferase-like protein 2 [Arabidopsis thaliana]
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XP 001774716 predicted protein [Physcomitrella patens subsp. patens]
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EDZ43368 conserved hypothetical protein [Rhodobacterales bacterium HTCC2083]
YP 001168844 hypothetical protein Rsph17025 2652 [Rhodobacter sphaeroides ATCC 17025]
ZP 00959493 hypothetical protein ISM 06660 [Roseovarius nubinhibens ISM]
ZP 02150012 hypothetical protein RG210 07054 [Phaeobacter gallaeciensis 2.10]
ZP 02145394 hypothetical protein RGBS107 06189 [Phaeobacter gallaeciensis BS107]
EEB71354 conserved hypothetical protein [Ruegeria sp. R11]
EDZ45912 conserved hypothetical protein [Rhodobacterales bacterium Y4I]
ZP 01055350 hypothetical protein MED193 13897 [Roseobacter sp. MED193]
ABZ07710 hypothetical protein ALOHA HF4000ANIW137P11ctg1g22 [uncultured marine microorganism HF4000 ANIW137P11]
EEB78477 hypothetical protein GPB2148 194 [marine gamma proteobacterium HTCC2148]
YP 723575 putative branched-chain amino acid aminotransferase [Trichodesmium erythraeum IMS101]
sxt protein ABI75104 sulfotransferase [Cylindrospermopsis raciborskii T3]
DEOB CLOPH Phosphopentomutase [Clostridium phytofermentans]
IDI2 STAHJ Isopentenyl-diphosphate delta-isomerase [Staphylococcus haemolyticus]
NDE1 USTMA Nuclear distribution protein nudE homolog 1 [Ustilago maydis]
MHPD SHISS 2-keto-4-pentenoate hydratase [Shigella sonnei]
MHPD ECO24 2-keto-4-pentenoate hydratase [Escherichia coli O139H28]
MHPD ECOHS 2-keto-4-pentenoate hydratase [Escherichia coli O9H4]
TIG PROM2 Trigger factor [Prochlorococcus marinus]
TILS FRATN tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. novicida]
TILS FRATF tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRATH tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRATO tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. holarctica]
TILS FRAT1 tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TILS FRATT tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TILS FRATW tRNA[Ile]-lysidine synthase [Francisella tularensis subsp. tularensis]
TGL1 YEAST Sterol esterase TGL1 [Saccharomyces cerevisiae]
HOFC ECOLI Protein transport protein hofC [Escherichia coli]
PURA PETMO Adenylosuccinate synthetase [Petrotoga mobilis]
HIS4 BUCAI 1-[5-phosphoribosyl]-5-[[5-phosphoribosylamino]methylideneamino] imidazole-4-carboxamide isomerase [Buchnera aphidico
BMCC1 HUMAN BNIP2 motif-containing molecule at the C-terminal region 1 [Homo sapiens]
K1358 XENTR UPF0584 protein KIAA1358 homolog [Xenopus tropicalis PE=2 SV=1]
Amin 93i12r
XP 757942 hypothetical protein UM01795.1 [Ustilago maydis 521]
YP 643554 hypothetical protein Rxyl 0774 [Rubrobacter xylanophilus DSM 9941]
XP 783988 PREDICTED hypothetical protein [Strongylocentrotus purpuratus]
XP 001640518 predicted protein [Nematostella vectensis]
XP 002170351 PREDICTED similar to predicted protein [Hydra magnipapillata]
XP 001639965 predicted protein [Nematostella vectensis]
XP 001632044 predicted protein [Nematostella vectensis]
99
100
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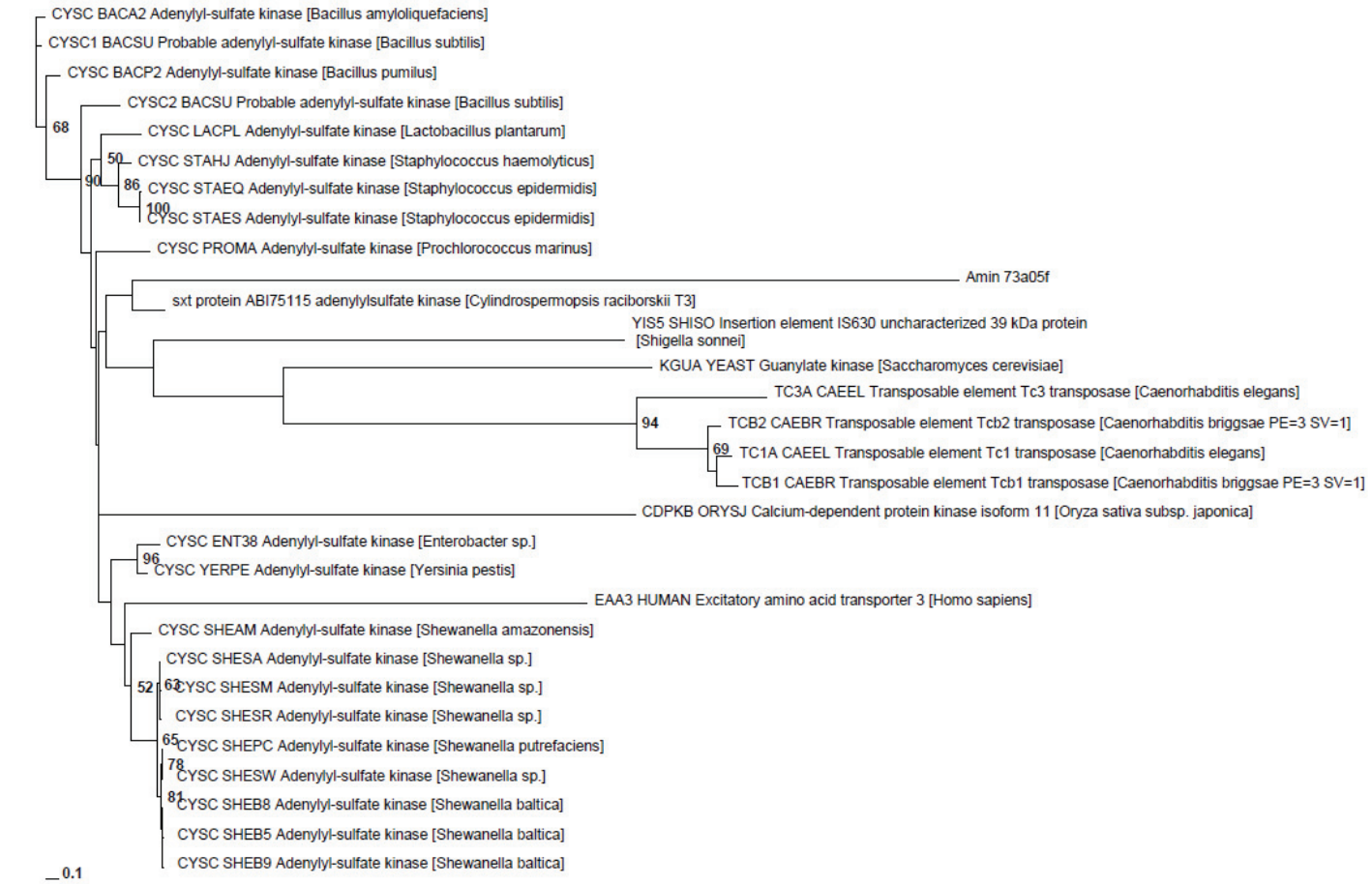
0.1

N.1


CYSC BACA2 Adenylyl-sulfate kinase [Bacillus amyloliquefaciens]
CYSC1 BACSU Probable adenylyl-sulfate kinase [Bacillus subtilis]
CYSC BACP2 Adenylyl-sulfate kinase [Bacillus pumilus]
CYSC2 BACSU Probable adenylyl-sulfate kinase [Bacillus subtilis]
CYSC LACPL Adenylyl-sulfate kinase [Lactobacillus plantarum]
CYSC STAHJ Adenylyl-sulfate kinase [Staphylococcus haemolyticus]
CYSC STAEQ Adenylyl-sulfate kinase [Staphylococcus epidermidis]
CYSC STAES Adenylyl-sulfate kinase [Staphylococcus epidermidis]
CYSC PROMA Adenylyl-sulfate kinase [Prochlorococcus marinus]
Amin 73a05f
sxt protein ABI75115 adenylylsulfate kinase [Cylindrospermopsis raciborskii T3]
YIS5 SHISO Insertion element IS630 uncharacterized 39 kDa protein [Shigella sonnei]
KGUA YEAST Guanylate kinase [Saccharomyces cerevisiae]
TC3A CAEEL Transposable element Tc3 transposase [Caenorhabditis elegans]
TCB2 CAEBR Transposable element Tcb2 transposase [Caenorhabditis briggsae PE=3 SV=1]
TC1A CAEEL Transposable element Tc1 transposase [Caenorhabditis elegans]
TCB1 CAEBR Transposable element Tcb1 transposase [Caenorhabditis briggsae PE=3 SV=1]
CDPKB ORYSJ Calcium-dependent protein kinase isoform 11 [Oryza sativa subsp. japonica]
CYSC ENT38 Adenylyl-sulfate kinase [Enterobacter sp.]
CYSC YERPE Adenylyl-sulfate kinase [Yersinia pestis]
EAA3 HUMAN Excitatory amino acid transporter 3 [Homo sapiens]
CYSC SHEAM Adenylyl-sulfate kinase [Shewanella amazonensis]
CYSC SHESA Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHESM Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHESR Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHEPC Adenylyl-sulfate kinase [Shewanella putrefaciens]
CYSC SHESW Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHEB8 Adenylyl-sulfate kinase [Shewanella baltica]
CYSC SHEB5 Adenylyl-sulfate kinase [Shewanella baltica]
CYSC SHEB9 Adenylyl-sulfate kinase [Shewanella baltica]
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90
86
100
94
69
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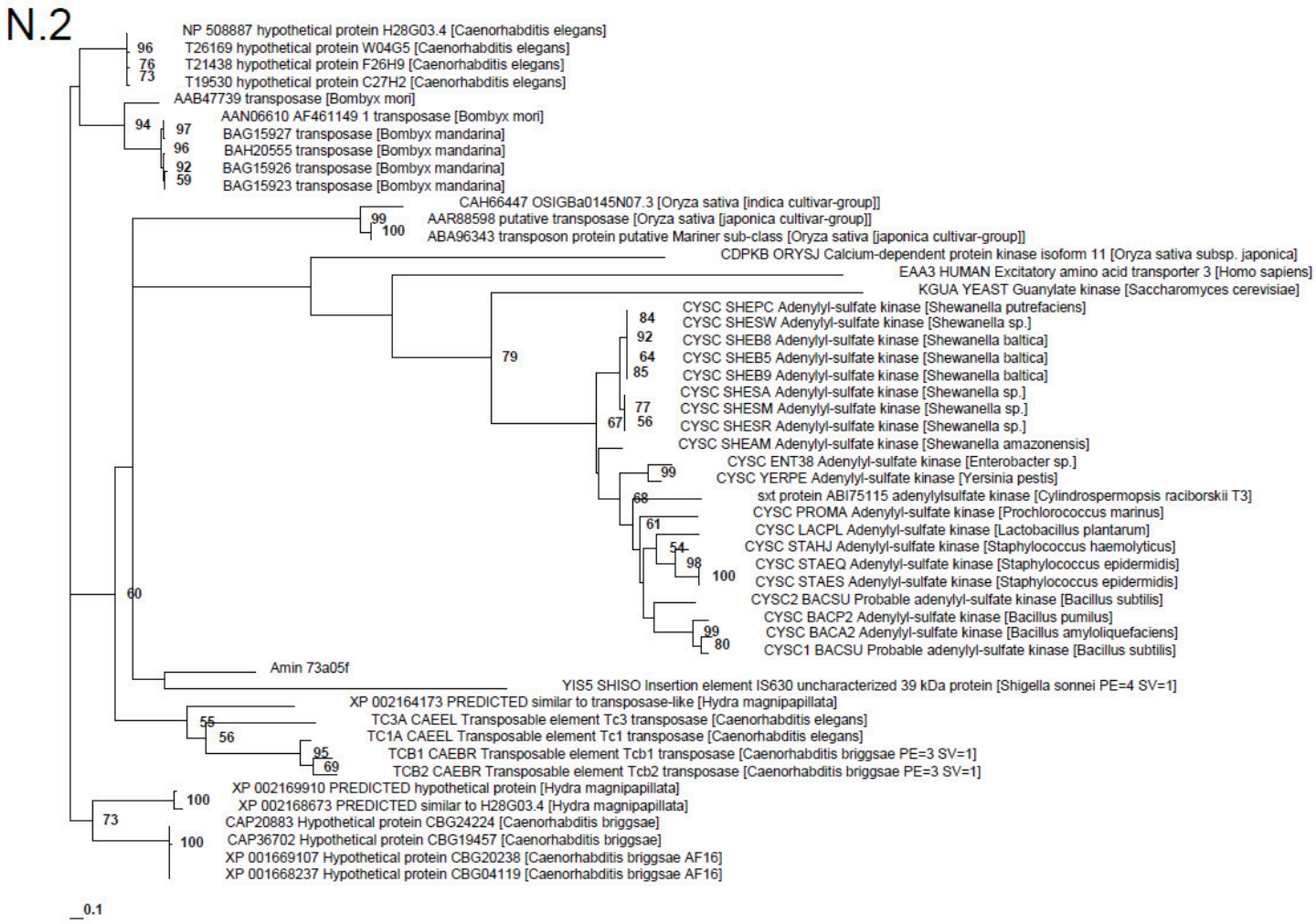
81
0.1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T26169 hypothetical protein W04G5 [Caenorhabditis elegans]
T21438 hypothetical protein F26H9 [Caenorhabditis elegans]
T19530 hypothetical protein C27H2 [Caenorhabditis elegans]
AAB47739 transposase [Bombyx mori]
AAN06610 AF461149 1 transposase [Bombyx mori]
BAG15927 transposase [Bombyx mandarina]
BAH20555 transposase [Bombyx mandarina]
BAG15926 transposase [Bombyx mandarina]
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CAH66447 OSIGBa0145N07.3 [Oryza sativa [indica cultivar-group]]
AAR88598 putative transposase [Oryza sativa [japonica cultivar-group]]
ABA96343 transposon protein putative Mariner sub-class [Oryza sativa [japonica cultivar-group]]
CDPKB ORYSJ Calcium-dependent protein kinase isoform 11 [Oryza sativa subsp. japonica]
EAA3 HUMAN Excitatory amino acid transporter 3 [Homo sapiens]
KGUA YEAST Guanylate kinase [Saccharomyces cerevisiae]
CYSC SHEPC Adenylyl-sulfate kinase [Shewanella putrefaciens]
CYSC SHESW Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHEB8 Adenylyl-sulfate kinase [Shewanella baltica]
CYSC SHEB5 Adenylyl-sulfate kinase [Shewanella baltica]
CYSC SHEB9 Adenylyl-sulfate kinase [Shewanella baltica]
CYSC SHESA Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHESM Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHESR Adenylyl-sulfate kinase [Shewanella sp.]
CYSC SHEAM Adenylyl-sulfate kinase [Shewanella amazonensis]
CYSC ENT38 Adenylyl-sulfate kinase [Enterobacter sp.]
CYSC YERPE Adenylyl-sulfate kinase [Yersinia pestis]
sxt protein ABI75115 adenylylsulfate kinase [Cylindrospermopsis raciborskii T3]
CYSC PROMA Adenylyl-sulfate kinase [Prochlorococcus marinus]
CYSC LACPL Adenylyl-sulfate kinase [Lactobacillus plantarum]
CYSC STAHJ Adenylyl-sulfate kinase [Staphylococcus haemolyticus]
CYSC STAEQ Adenylyl-sulfate kinase [Staphylococcus epidermidis]
CYSC STAES Adenylyl-sulfate kinase [Staphylococcus epidermidis]
CYSC2 BACSU Probable adenylyl-sulfate kinase [Bacillus subtilis]
CYSC BACP2 Adenylyl-sulfate kinase [Bacillus pumilus]
CYSC BACA2 Adenylyl-sulfate kinase [Bacillus amyloliquefaciens]
CYSC1 BACSU Probable adenylyl-sulfate kinase [Bacillus subtilis]
Amin 73a05f
YIS5 SHISO Insertion element IS630 uncharacterized 39 kDa protein [Shigella sonnei PE=4 SV=1]
XP 002164173 PREDICTED similar to transposase-like [Hydra magnipapillata]
TC3A CAEEL Transposable element Tc3 transposase [Caenorhabditis elegans]
TC1A CAEEL Transposable element Tc1 transposase [Caenorhabditis elegans]
TCB1 CAEBR Transposable element Tcb1 transposase [Caenorhabditis briggsae PE=3 SV=1]
TCB2 CAEBR Transposable element Tcb2 transposase [Caenorhabditis briggsae PE=3 SV=1]
XP 002169910 PREDICTED hypothetical protein [Hydra magnipapillata]
XP 002168673 PREDICTED similar to H28G03.4 [Hydra magnipapillata]
CAP20883 Hypothetical protein CBG24224 [Caenorhabditis briggsae]
CAP36702 Hypothetical protein CBG19457 [Caenorhabditis briggsae]
XP 001669107 Hypothetical protein CBG20238 [Caenorhabditis briggsae AF16]
XP 001668237 Hypothetical protein CBG04119 [Caenorhabditis briggsae AF16]
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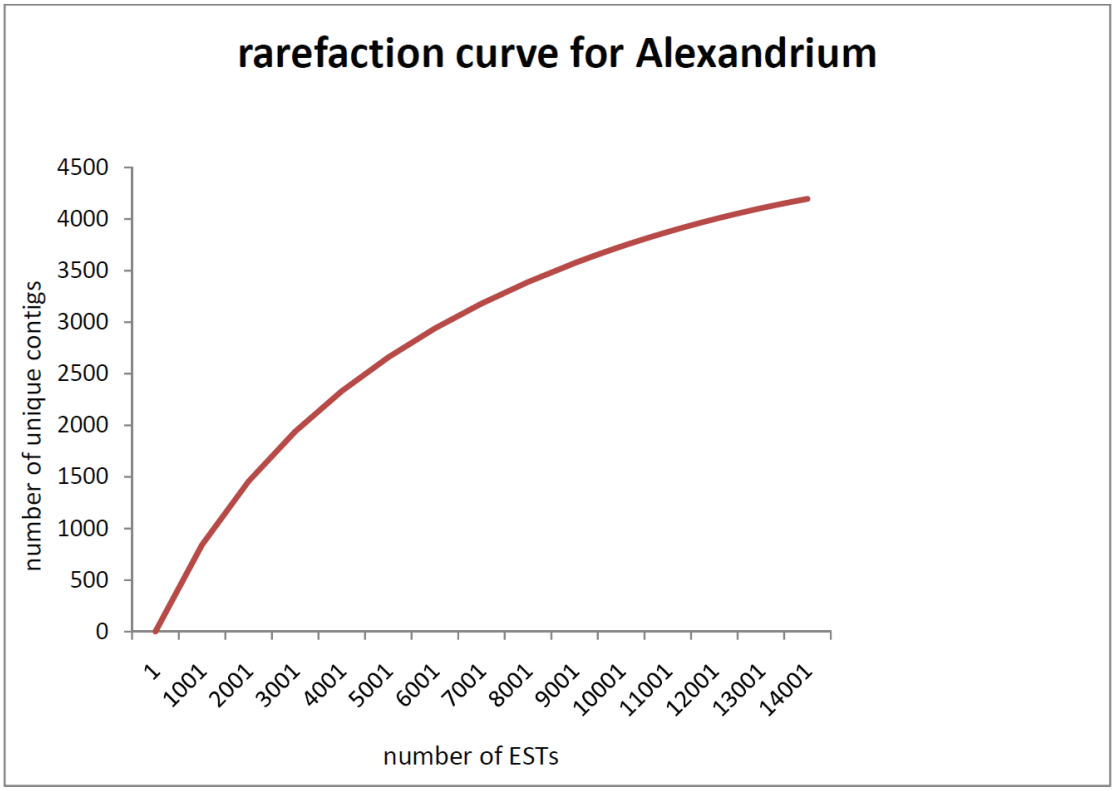
III. Manuscript 1, additional file 4: Rarefaction curve

Generated ESTs and assembled cluster as contigs were analysed using http://www.biology.ualberta.ca/jbrzusto/rarefact.php#Calculator.


rarefaction curve for Alexandrium
number of unique contigs
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
1001
2001
3001
4001
5001
6001
7001
8001
9001
10001
11001
12001
13001
14001
number of ESTs

IV. Manuscript 2, Supplementary table: information on the genes up-regulated at Day 1 or Day 3 of copepod-induced PSP toxin production

Log_2 values of fold change exposed / control (mean of triplicates), standard deviation of log_2 values, fold change exposed / control (geometric mean of triplicates), annotation or lack of annotation confirmed manually. Geometric mean toxic strains / non-toxic strains (Manuskript 1). With value of best BLAST hit against other dinoflagellate EST libraries, NCBI Transcriptome Shotgun Assembly (TSA) database accession numbers and miscellaneous sequence characteristics.

Contig name	TSA accession number	Log2 of fold change exposed / control		Standard deviation of log2 of fold change exposed / control		Geometric mean of fold change exposed / control		Gene product	Pfam search results	Geometric mean toxic strains / non-toxic strain	BLAST hits in other dinflagellate libraries			contig characteristics	
		Day 1	Day 3	Day 1	Day 3	Day 1	Day 3		evalue: pfam id		A. tamarense	A. ostenfeldii	other species	Length in bp	%GC content
Amin_06b12r	HP451726	3.31	3.51	0.13	0.21	9.92	11.40	hypothetical protein						827	49.82
Amin_08b02r	HP451727	2.42	3.21	0.37	0.31	5.36	9.26	hypothetical protein						678	54.42
Amin_07b11f	HP451728	1.53	2.20	0.12	0.23	2.88	4.61	hypothetical protein					4.00E-019	664	49.55
Amin_88h09r2	HP451729	2.35	1.49	0.54	0.18	5.11	2.81	hypothetical protein		-5.44				936	55.34
Amin_26k02r	HP451730	2.35		0.22		5.10		hypothetical protein				9.00E-016	6.00E-035	992	55.34
Amin_78b09r	HP451731	2.26		0.55		4.81		hypothetical protein		-2.19				722	57.20
Amin_06h01f	HP451732	2.15		0.46		4.45		hypothetical protein						818	55.62
Amin_08c10f	HP451733	1.93		0.44		3.80		peptidylprolyl isomerase	1.9E-19: Rotamase					791	47.79
Amin_86k10r	HP451734	1.26		0.23		2.39		hypothetical protein			6.00E-022	3.00E-021		961	55.36
Amin_79e01f	HP451735	1.12		0.18		2.18		hypothetical protein					5.00E-016	557	56.01
Amin_89d06r	HP451736	0.93		0.07		1.91		hypothetical protein		9.88				732	56.28
Amin_56a03r	HP451737		3.29		0.13		9.77	predicted NAD:arginine ADP-ribosyltransferase	2.1e-12: ART	51.01				962	52.81
Amin_61f03r	HP451738		2.00		0.20		3.99	hypothetical protein				4.00E-006		706	58.50
Amin_88f04r2	HP451739		1.82		0.17		3.54	hypothetical protein						258	55.81

Danksagung

Prof. Dr. Ulrich Bathmann und Prof. Dr. Dieter Wolf-Gladrow danke ich ganz herzlich für die Begutachtung meiner Arbeit. Prof. Dr. Dietmar Blohm danke ich, dass er die Rolle des dritten Prüfers übernimmt.

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Danke an Dr. Bánk Beszteri, Stefan Neuhaus, Stefan Pinkernell und Prof. Dr. Stephan Frickenhaus von der AG Bioinformatik für die vielen kleinen und großen Hilfen bei der Datenauswertung.

Danke an die aktuellen und ehemaligen Mitglieder der AG Cembella für die freundliche Arbeitsatmosphäre und die vielen gemeinsamen Mittagspausen:

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Danke an Steffi Gäbler, Maddalena Bayer, Linda Medlin und Jessica Kegel.

Special thanks to Dr. Thomas Richards.

Vielen Dank an meine Eltern, Stephi, und natürlich Chaoliang für Eure Unterstützung und Geduld.

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