

## Analysing photosynthetic complexes in uncharacterized species or mixed microalgal communities using global antibodies

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Photoautotrophs share core pathways for primary productivity and elemental cycling. These paths are generally mediated by abundant protein complexes which are conserved across wide taxonomic ranges, and which quantitatively dominate the proteomes of photoautotrophs. Quantification of key protein pools is a powerful approach to measure resource allocations and maximal catalytic capacities for biological functions, for comparing species or communities, or for tracking change over time within communities. Protein quantification can be more definitive than transcript analyses for functional studies, since changes in transcript levels are often only weakly coupled to pool sizes of functional protein. For field samples protein detections are more generally applicable than enzyme assays which require individually specialized extractions and

assays, which are prone to interference by environmental contaminants. We are using bioinformatic analyses to design a series of peptide sequence tags that are conserved in all family members for a well-characterized subunit from each of the major catalytic complexes mediating photosynthesis and nitrogen metabolism. We use these peptide sequence tags to elicit production of 'global antibodies', intended to recognize all members of the target protein family with equal efficiency regardless of the species of origin. Uniform target detection is particularly important for tracking levels of key proteins in mixed communities, or for quantitative comparisons across plant species. In parallel we are creating quantification standards for these key protein pools for functional and (eco)physiological studies.

### Introduction

#### Photoautotrophic functions are catalysed by conserved protein complexes

Natural communities of phytoplankton, aquatic epiphytes and other communities of photoautotrophs can include representatives of numerous species of cyanobacteria, diatoms, green algae and other groups. These taxa have diverse physiological and ecological properties.

Nevertheless, these photoautotrophs, along with higher plants, share many core biochemical functions that support primary productivity and elemental cycling (reviewed in Falkowski and Raven 1997). These core, widely shared functions include light capture, conversion of excitation energy to photochemically generated electrons, electron transport, ATP synthesis, carbon fixation, and nitrogen assimilation. These main processes of photosynthetic metabolism are catalysed by abundant

protein complexes which quantitatively dominate the proteomes of cyanobacteria, algae and many photosynthetic tissues in plants. The compositions, catalytic and biochemical properties of these key protein complexes have generally been well characterized using controlled experiments on fairly wide ranges of taxa, usually grown under near ideal culture conditions (reviews in Aro et al. 1993, Douglas 1994, Frasch 1994, Gantt 1994, Golbeck 1994, Kallas 1994, Tabita 1994).

#### Rationale for quantitating proteins in natural samples of photoautotrophs and communities

Natural populations of photoautotrophs live in mixed communities that continually chase changing and non-ideal environmental conditions by changing allocations to pools of these key protein complexes, often within constraints imposed by resource limitations

*Abbreviations* – HMM, Hidden Markov Model; Ig, immunoglobulin; PSII, photosystem II.

(e.g. LaRoche et al. 1995, 1996, Ottander et al. 1995, Scanlan et al. 1997, MacKenzie et al. 2001). Furthermore, although the compositions and catalytic properties of the core complexes of photosynthetic metabolism are highly conserved, the allocation patterns, stoichiometric ratios among the complexes and even their ecophysiological roles can vary widely between different taxa or different growth conditions (e.g. LaRoche et al. 1995, 1996, Fujita 1997, Bhaya et al. 2000). Therefore, a means to quantify key protein pools can be a powerful element of ecophysiological studies to assess the allocation patterns and gross capacities for key metabolic transformations at the level of mixed photoautotroph communities or in uncharacterized species (Orellana and Perry 1992, 1995, Vrieling and Anderson 1996, Lin et al. 1998), to track acclimatory changes in these capacities (LaRoche et al. 1995, 1996, Scanlan et al. 1997), and to compare allocations across taxa or communities (Staley 2002).

### **Shared characteristics allow standard quantification of members of a protein family**

Studies of natural photoautotroph communities often require large sample sets comprising mixed and varying species compositions. Various community types require different sampling procedures, which must nonetheless be sufficiently simple and rapid for field applications. Samples can contain a wide range of contaminants or extraneous material. They are often limited in amount and may be unique or impractical to regenerate. Therefore a method for parallel, rapid and standard detection of a suite of key proteins from each sample preparation would enable protein quantification to be applied to ecophysiological studies.

Currently, immunological methods are the most practical generalized approach to detect and quantify a specific protein in a complex mixture (Orellana and Perry 1992, 1995, LaRoche et al. 1995, 1996, Vrieling and Anderson 1996, Scanlan et al. 1997, Lin et al. 1998, Schade et al. 2000). Antibodies are usually elicited using either purified or over-expressed protein from a particular species on a peptide selected to match the sequence of a region of a particular protein. Such antibodies show variable cross-reactivity to related proteins from other species depending on their antigenic similarity to the initial target molecule (e.g. Orellana and Perry 1992, 1995, LaRoche et al. 1995, 1996). This is problematic because differences in immunological signals between species, or in sample series from a mixed community can result from: (a) changes in the level of the targets; (b) differences in the mix of target molecules, either from species distinctions or from changes in the population composition of the community, or a combination of (a) and (b) (Orellana and Perry 1992, 1995, LaRoche et al. 1995, 1996).

It is not practical to develop individual antibodies to detect each protein of interest specifically from each species in most natural communities. The species in a community may be poorly characterized at the molecular

level, unknown, or even unculturable (Staley 2002). We thus need a system to evenly and universally recognize all members of a defined protein family or subfamily based on shared characteristics.

We have addressed this need with a method for quantitating the members of a defined target protein family in an uncharacterized or mixed sample by identifying 'peptide sequence tags' that are present in all members of the target protein family, and are exclusive to the target protein family. We use the peptide sequence tag to elicit production of antibodies designed for even, pooled recognition of all members of the target protein family, thus allowing quantifications by comparison to known standards.

## **Methodology and applications**

### **Identifying peptide sequence tags diagnostic for conserved protein families**

Most of the abundant protein complexes mediating photosynthetic functions contain subunits that belong to highly conserved families (e.g. Aro et al. 1993). In most cases representatives of these subunit families have been sequenced from a wide range of taxa (e.g. Badger et al. 2002, SwissProt public database, <http://www.expasy.ch>, NCBI database, <http://www.ncbi.nlm.nih.gov>). Sequence database searches for the target protein families show a range of representatives from different taxonomic groups, sharing key conserved regions and core biochemical functions. Therefore, inspecting alignments of protein sequences (Corpet 1988) from known members of a subunit family allows us to identify conserved peptide sequence tags that can then be tested for exclusivity to the target protein family using BLAST (Altschul et al. 2001, Schaffer et al. 2001) searches against all known protein sequences. We have designed a series of such peptide sequence tags (Campbell 2002) diagnostic for subunits from most of the major protein complexes of photosynthetic and nitrogen metabolism, and assessed them for their potential antigenicity and lack of known or putative post-translational modifications.

A target protein family is selected on the basis of prior knowledge that it mediates a function of interest, or because it represents a quantitatively significant investment of resources by the organisms of communities of interest. For example, all oxygenic photosynthetic organisms allocate significant resources to synthesize large pools of the RbcL protein, the conserved large subunit of the hyperabundant Rubisco enzyme responsible for carbon fixation (Tabita 1994). Different photoautotrophs contain somewhat different versions of the RbcL protein, which nevertheless share conserved regions (<http://ncbi.nlm.nih.gov>) and similar core properties.

To date our efforts have been directed to conserved subunits of the major complexes of photosynthetic and nitrogen metabolism including GlnA (glutamine synthetase), NirB (nitrite reductase), PsaA (photosystem I),

PsbA (photosystem II), NifH (nitrogenase) and RbcL (Rubisco). In principle the approach can be applied to any conserved protein family. We scan public protein sequence databases (<http://www.expasy.ch>; <http://www.ncbi.nlm.nih.gov>) for published sequences of proteins catalysing the target function, using either keyword or enzyme descriptor searches of the sequence annotation, or by submitting a representative query sequence to a BLAST (Altschul et al. 2001, Schaffer et al. 2001) search of known sequences. We seek representatives of the protein family from a wide range of organisms, but in particular, we seek coverage of taxa present in the intended study environments or communities, or from their nearest available phylogenetic relatives.

Based on the retrieved sequences, annotations and published references we analyse whether the biological function of interest is performed by members of one protein family, or if instead similar functions, for instance carbon-concentrating mechanisms in microalgae (Badger et al. 2002), are catalysed by more than one protein sequence family, or by highly divergent members of one superfamily.

Once we have determined that the target function maps to a defined protein family, we align (Corpet 1988) all the available full-length sequences retrieved from the target protein family. If multiple sequences are available from closely related taxa, we may eliminate near-relatives to avoid biasing the alignment towards a well-represented cluster of closely related taxa. This can be the case when many sequences of proteins in the family have been determined for taxonomic studies, as for instance the RbcL or PsbA protein families (<http://www.ncbi.nlm.nih.gov>). We generally use only full-length sequences where practical, to avoid biasing the alignment process by partial sequences (Lesk 2002), and to avoid incorrect interpretations of conservation in sequence regions not covered by partial sequences. Once we have identified conserved peptide sequences we submit them to BLAST (Altschul et al. 2001) to determine if the selected peptide sequence is exclusive to the target protein family and if the selected peptide sequence shows absolute conservation in all members of the protein family, or in a useful subset of the protein family.

Our efforts have been directed towards peptide sequence tags that are conserved in protein families over the widest possible range of taxa, at least within the photoautotrophs. In principle peptide sequence tags can also be selected for specificity to a protein subfamily from a particular taxonomic range. In practice, however, regions that vary among different subfamilies within a protein family often also show variation within protein subfamilies. Therefore it can be difficult to select peptide sequence tags absolutely conserved within a subfamily and absent from all sister subfamilies. Understanding the level of conservation and specificity of the peptide tags is crucial in order for results to be meaningful. It is difficult to design peptide sequence tags with confidence of their range of conservation, and hence their range of reliable taxonomic application, unless there is a good range of

sequences available for the target protein family, spanning the taxonomic range of interest. Alignment of hundreds of sequences from higher plants will not necessarily lead to a peptide sequence tag conserved in the homologous protein in dinoflagellates. Therefore when possible we include protein family representatives from all known oxygenic photoautotroph groups when seeking peptide sequence tags globally conserved for subunits of complexes of photosynthetic metabolism.

In one example, we were able to design a peptide sequence tag specific to form I RbcL proteins only (Fig. 1), typical of the L8S8 enzyme complexes common to most oxygenic photoautotrophs (Tabita 1994), and absent from the form II RbcL proteins typical of the L2 enzyme complexes common to many bacteria and also to dinoflagellates (Morse et al. 1995, Whitney et al. 1995). We also designed an alternate peptide sequence tag that is conserved in all RbcL proteins, both form I and form II.

In addition to the assessments of taxonomic conservation, we predict whether the putative peptide sequence tag is maintained unadulterated in the mature proteins. We avoid peptide sequences known or predicted to be susceptible to post-translational modifications such as glycosylation, which could interfere with detection of the mature members of the protein family. In many cases conserved sequence motifs are characteristic of general functions such as nucleotide binding or hydrolytic activities which occur in a range of functionally different protein families. Paralogues, members of the same protein superfamily diverged to catalyse distinct functions, can often share conserved regions. Therefore, we use motif databases such as the Protein families database of alignments and HMMs (Pfam; <http://www.sanger.ac.uk/cgi-bin/Pfam/>), sequence annotations and the relevant literature to help determine if peptide sequences are characteristic of such generic functions, or whether they are specific to a usefully defined protein family. Finally, the putative peptide sequence tags are assessed for empirically predicted ease of synthesis, stability and antigenicity.

In summary the chosen peptide sequence tags are conserved in all published members of the defined target protein family or subfamily, do not align significantly with members of other known protein families, and have acceptable predicted antigenic and synthesis properties.

### Global antibodies generated using peptide sequence tags

We are currently using peptide sequence tags to elicit the production of a series of global antibodies (Fig. 1) raised in chickens, which secrete stable immunoglobulin (Ig)-Y antibodies into their egg yolks (Schade et al. 2000). The global antibodies can be applied to detect subunits of major protein complexes in a range of species, including uncharacterized species, with confidence that the detection efficiency of the antibody is standard for all denatured members of the target protein family, regardless of the species of origin. Furthermore a quantity standard

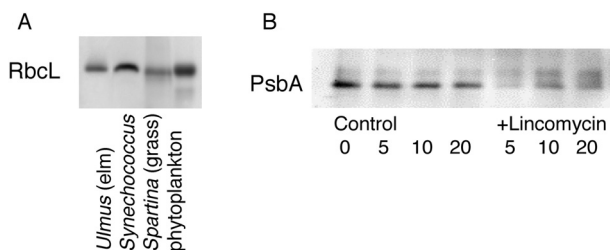


Fig. 1. Detection of proteins from field samples using immunoblotting with global antibodies. Total proteins were extracted using sonication and denaturing buffer, separated by denaturing SDS-PAGE, electrophoretically transferred to a membrane followed by chemiluminescent detection with a global antibody raised against a peptide sequence tag and protocols from <http://www.agrisera.se>. (A) Detection of RbcL large subunit of the Rubisco enzyme in a range of species. The RbcL global antibody was raised against a peptide sequence tag conserved in form I RbcL proteins, but not in the form II RbcL proteins found in dinoflagellates and some photosynthetic bacteria. (B) Community level PsbA (D1) turnover in a tropical phytoplankton community. This community off Ubatuba, Brazil has a rapid PSII repair cycle (Aro et al. 1993) to regenerate PsbA protein. When protein synthesis is inhibited with lincomycin the repair cycle is blocked and net PsbA protein is lost rapidly from the community, resulting in photoinhibition (Bouchard et al. 2002). The PsbA global antibody was raised against a peptide sequence tag conserved in all known PsbA proteins.

protein from one species or a synthetic quantity standard can be used for comparative quantification of members of the protein family across species. A set of antibodies and standards increases the utility of the system by allowing comprehensive detection of key molecules from a sample set from a population or community. In contrast, applying conventional antibodies to mixed communities such as phytoplankton communities (Orellana and Perry 1992, 1995, LaRoche et al. 1995, 1996) is more difficult because variable interactions between the antibodies and the proteins from different species cause variable detection efficiencies among members of the protein families.

The subunit stoichiometries of the major complexes of photosynthetic metabolism are well conserved, so quantifying one subunit allows us to infer the quantities of the other subunits in each complex, and thus the total cellular or community allocation of protein resources to a target complex. Measuring 5–10 such key subunits, for example using immunoblotting (Fig. 1), or spectroscopy for pigment proteins (Paerl 1997), allows us to determine the quantitative allocation of most (>90%) of the pooled proteome of a cyanobacterial or microalgal community, along with the molar ratios between complexes and capacities for key ecosystem transformations.

Quantitating protein pools gives different information from detecting their cognate transcripts or genes. For many ecophysiological and functional studies protein quantification has some advantages in comparison to transcript or gene analyses. Detection of a gene in a population or community demonstrates that the genetic capacity for a process is present, and is useful for assessing molecular diversity through sequencing or other techniques. Detecting changes in transcripts can

demonstrate when or under what conditions a population or community activates the potential to synthesize the cognate protein, and can therefore give valuable information on factors that influence gene expression. There are, however, strong post-transcriptional and post-translational regulations on the levels of protein subunits of the abundant protein complexes that mediate photosynthetic metabolism, so that the accumulated protein pool sizes are often only weakly coupled to changes in transcripts, and vice versa (Gygi et al. 1999, Fulda et al. 2000, Simpson and Dorow 2001, MacDonald et al. 2003).

For studies of resource allocation and cost-benefit analyses, proteins account for much larger fractions of cellular resources than do the relatively small and transient pools of transcripts. Measuring accumulated protein pool sizes allows estimation of maximal activities for reactions or processes mediated by the target protein complex. Furthermore, proteins are generally relatively stable in comparison with transcripts that can degrade rapidly and differentially during sampling, resulting in biases in quantifications of transcripts from field samples. The polymerase chain reaction for genes or reverse transcriptase-polymerase chain reaction for transcripts are the most feasible means of quantitating gene or transcript pools from natural samples (e.g. MacKenzie et al. 2001). These methods are highly sensitive and can be quantitative, but require two conserved, appropriately spaced nucleotide primer sequences. In contrast, one conserved region in a protein suffices as a peptide antibody target. Since protein amino acid sequences are in general more highly conserved than the cognate nucleotide sequences, the task of designing conserved targets for antibody binding to proteins is often more feasible than designing primer binding sites for transcript or gene detection.

Applying global antibodies to studies of communities also has some advantages over more specific enzyme analyses (e.g. Burns and Dick 2002). One standard denaturing total extraction of proteins from field samples allows detection of multiple protein pools by multiple antibodies, in contrast to enzyme assays, which can be difficult to apply to contaminated samples and which require individual extraction and assay conditions. Quantitating key protein pools therefore extends and complements studies of genes (Wilmotte et al. 2002), transcripts (Pichard et al. 1997), pigments (Paerl 1997, Wilmotte et al. 2002) or enzyme activities in communities.

### Applications of global antibodies

Gel electrophoresis of protein extracts followed by immunoblotting is currently our method of choice for detecting members of protein families in environmental samples (Fig. 1; Bouchard et al. 2002, Schofield et al. 2003). Protein extraction, electrophoresis, immunoblotting and quantification are still labour intensive compared to high-throughput enzyme linked immunosorbent assays applied in clinical detections, but the electrophoresis separates the target

proteins from non-specific background and cross-reactions, allowing the investigator to quantify only those immunoreactions in the appropriate size range.

The major limiting step on high-throughput protein quantifications from natural samples is the current lack of generally applicable, high-efficiency and high-throughput methods for extraction of total proteins with consistent good yields from natural samples of photoautotrophic cells. In general the diverse and sometimes tough cell walls of photoautotrophs (e.g. Orellana and Perry 1995) and the presence of environmental contaminants preclude efficient enzymatic or chaotropic lysis of all community members. Our current method of choice is microprobe sonication at 4°C in the presence of denaturing buffers and protease inhibitors (<http://www.agrisera.se>). Although efficient, probe sonication is prone to differentially shearing some proteins (MacDonald et al. 2003). Furthermore probe sonication is slow and labour intensive, although in principle it could be automated.

We (Fig. 1; Bouchard et al. 2002, MacDonald et al. 2003, Schofield et al. 2003) and others are now applying these global antibodies to samples from controlled, uncharacterized or mixed natural communities of photoautotrophs, confident that the antibodies will react evenly with all members of the target protein families present. MacDonald et al. (2003) used global antibodies for a comparative study of the transcripts and proteins for phycocyanin, photosystem II (PSII) and Rubisco in controlled cultures of the model cyanobacteria *Synechococcus* sp. PCC 7942, exposed to moderate UVB. In this case the study organism and conditions were well characterized but the global antibodies provided a convenient and relatively inexpensive means to detect key protein complexes. *Synechococcus* sp. PCC 7942 contains multiple isoforms of the PsbA (D1) protein of PSII, but the global antibody allowed us to evenly detect the total pool of protein. Schofield et al. (2003) used global antibodies to track allocations of resources to key proteins in the green algal photobiont of the lichen *Lobaria pulmonaria*. There was no direct information available on the key protein complexes of photosynthetic and nitrogen metabolism in this species. Therefore the global antibodies were applied to quantify the proteins, based on the prediction that the highly conserved peptide sequence tags for each protein family are also present in the target proteins of the uncharacterized species *Lobaria pulmonaria*. Bouchard et al. (2002) (Fig. 1B) are applying global antibodies against PsbA (D1 protein) and RbcL (Rubisco large subunit protein) to compare the roles of the PSII repair cycle in UVB responses of mixed coastal phytoplankton communities at sites along a long latitudinal gradient. The various phytoplankton communities are comprised of a diverse range of taxa growing under very different ecophysiological conditions. The global antibody allows a standard basis of detection of total community allocations and turnover of pools of PsbA (D1), which is a major element in the PSII repair cycle involved in resistance to UVB and photoinhibition.

Our goals in these and other projects are analyses at the community level, or between taxa, of integrated allocations of resources and maximal capacities for the major processes of photosynthetic metabolism and acclimation, which both sustain ecosystem function and which demand heavy investments of nitrogen and other resources for synthesis.

## Future directions

We are working to produce additional global antibodies to cover subunits from the remaining complexes of photosynthetic metabolism including the ATP synthase, cyt b6f and the CP43 subunit of PSII, and preparing concentration standards for comparative quantification of proteins. We are exploring options for high-throughput quantifications using optimized protein extraction procedures, membrane-based immunological detections, capillary electrophoresis of antibody/antigen complexes or direct Q-TOF mass spectroscopic detection of the peptide sequence tags from mixtures.

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