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Deletion of the gene encoding the Photosystem II 33 kDa protein from *Synechocystis* sp. PCC 6803 does not inactivate water-splitting but increases vulnerability to photoinhibition

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Photosynthetic oxygen evolution is mediated by a Mn cluster at a site on the lumenal side of Photosystem II (PS II). The *psb*O gene product, the so-called extrinsic 33 kDa protein appears closely associated with this site, although its precise role remains unclear. We have used targeted mutagenesis to investigate whether the *psb*O protein is essential for PS II activity in the cyanobacterium *Synechocystis* sp. PCC 6803. The *psb*O gene was isolated and the locus was shown to be present as a single copy in the genome by low-stringency Southern hybridisations. Northern analysis detected a major *psb*O monocistronic transcript of 0.95–1.0 kb. Insertion and deletion *psb*O$^-$ mutant strains (named IC1 and IC2, respectively) were engineered and verified biochemically. The lesions in *psb*O have been confirmed by readdition of the *psb*O gene into both mutants using in situ complementation. This technique further indicated that the mutants may not be phenotypically identical. However, both mutants can grow photoautotrophically and exhibit appreciable rates of oxygen evolution. We therefore confirm independently the recently published results of Burnap and Sherman (Burnap, R.L. and Sherman, L.A. (1991) Biochemistry 30, 440–446). We also show that the mutated organism is highly susceptible to photoinhibition. This observation indicates that although the *psb*O protein is not absolutely required for water-splitting in *Synechocystis* sp. PCC 6803 it does play an important role in protecting against donor side photoinhibition.

Introduction

In all organisms performing oxygenic photosynthesis, Photosystem II (PS II) functions as a light-catalysed water-plastoquinone oxidoreductase, with molecular oxygen being produced as a side product. PS II is a multi-subunit pigment-protein enzyme which spans the thylakoid membrane [1].

The site of photochemistry, the PS II reaction centre, is now well characterised. The D1 and D2 polypeptides (*psb*A and *psb*D gene products respectively) bind the photooxidising chlorophyll species P680 and the primary electron acceptor pheophytin, form the plastoquinone-binding sites Q_A, Q_B and contain the tyrosine residues that produce the electron paramagnetic resonance (EPR) signals Z and D when oxidised [1,2]. This understanding of the PS II reaction centre has been greatly aided by comparison with the reaction centre crystal structure of purple photosynthetic bacteria determined by X-ray crystallography [3].

In contrast, the site of water-oxidation, situated on the lumenal side of the thylakoid membrane, is relatively poorly understood. A tetrameric manganese cluster plays a central catalytic role coupling the single-electron photochemistry of primary charge separation to the four electron process of water oxidation by cycling through a series of oxidation states S_0 to S_4 [4,5].

In plant and algal chloroplasts three extrinsic polypeptides situated on the lumenal side of PS II, with apparent molecular masses on SDS-PAGE of 33 kDa (the *psb*O gene product often called MSP, the

Abbreviations: PS II, Photosystem II; P680, primary electron donor to PS II; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance.

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'manganese-stabilising protein'), 23 kDa (*psb*P gene product) and 17 kDa (*psb*Q gene product) have also been closely implicated in oxygen evolution [6]. The *psb*O gene was formerly called *wox*A or *psb*1. A large volume of experimental evidence, including the fact that they are not present in cyanobacteria [7–9], has assigned non-catalytic roles to the 23 kDa and 17 kDa polypeptides. It seems that these polypeptides function to sequest or regulate Ca^{2+} and Cl^- levels in the vicinity of the water-oxidation site [6,10].

On the other hand, the 33 kDa protein is found in all oxygenic organisms including cyanobacteria and is firmly attached to the intrinsic PS II core in close proximity to the Mn cluster, visible through electron microscopy as a protrusion into the lumen [11,12]. Much experimental data concerning *psb*O protein function has come from in vitro systems. Most suggest the 33 kDa protein is not a major site of Mn coordination and that it can be functionally replaced, at least partially, by high, non-physiological Cl^- and Ca^{2+} levels [13]. The *psb*O protein can be washed from the intrinsic PS II core without concomitant Mn release [14]. Moreover Virgin et al. [15] have correlated Mn release to the degradation of the D1 polypeptide.

Despite the above, the simplest PS II preparations that evolve oxygen under near physiological Ca^{2+} and Cl^- concentrations retain the 33 kDa protein along with the chlorophyll-binding proteins CP-47 (*psb*B gene product) and CP-43 (*psb*C gene product), D1, D2, the α- and β-subunits of cytochrome *b*-559 (*psb*E and *psb*F gene products) [16,17] and probably one or more additional low molecular weight polypeptides, e.g., the *psb*I gene product [18,19]. A smaller particle, formed by chemical cross-linking consists predominantly of D1, D2 and the 33 kDa protein. This apparently retains Mn and is capable of catalysing H_2O_2 oxidation, but not water-splitting [20].

To date there have been far fewer in vivo studies of the *psb*O protein function. Groups studying *Synechocystis* sp. PCC 6803 and *Chlamydomonas reinhardtii* mutants lacking functional *psb*O genes have both concluded that the presence of the *psb*O polypeptide is essential for oxygen evolution in vivo [9,21]. However, recently Burnap and Sherman [22] and also Philbrick and Zilinskas (personal communication) have constructed mutants of *Synechocystis* sp. PCC 6803 in which the entire *psb*O gene had been deleted. Surprisingly these workers found that a *psb*O-less mutant still retains the ability to evolve oxygen and grow photoautotrophically. We have also chosen the cyanobacterium *Synechocystis* sp. PCC 6803 as a convenient organism in which to target mutagenesis of PS II genes [23,24] including *psb*O [25] so that the function of specific PS II components can be addressed in vivo. In this paper we define the molecular genetics of the *psb*O locus and confirm the conclusion that the 33 kDa protein is not essential for in vivo PS II water-splitting activity in *Synechocystis* 6803. We also report that the deletion mutant is highly sensitive to photoinhibition as compared with the wild-type.

Materials and Methods

All chemicals used were of reagent grade. Restriction enzymes and T4 DNA ligase were purchased from Northumbria Biologicals. For probe labelling, sequencing grade Klenow and deoxynucleoside triphosphates used were from Boehringer-Mannheim. DNAase-free RNAase and lysozyme were supplied by Sigma. All were used according to the manufacturers instructions. [α-^{32}P]dCTP (3000 Ci mmol^{-1}) was obtained from New England Nuclear, DuPont (U.K.). The PS II herbicides, atrazine and DCMU (3-[3,4,-dichlorophenyl]-1,1-dimethylurea), were obtained from British Greyhound, Birkenhead, U.K.

Strains and culture conditions

The *Synechocystis* sp. PCC 6803 strain used throughout these experiments was the glucose-tolerant strain *Synechocystis* 6803-G [26], a kind gift from Dr. J.G.K. Williams (DuPont, Delaware, U.S.A.). This strain is referred to throughout as *Synechocystis* 6803 or the wild-type. It was routinely grown in BG-11 medium supplemented with 5 mM glucose at 30–33°C. Plate medium was supplemented as described by Pakrasi et al. [27]. Liquid cultures were grown with continuous orbital shaking; large cultures in sterile conical flasks, small 4 ml cultures in 30 ml disposable universal bottles (Sterilin). *psb*O$^-$ strains were routinely grown in medium supplemented with 5 mM glucose, 100 μg ml^{-1} kanamycin and 20 μM atrazine to maintain the PS II lesion. Continuous illumination was provided by a mixture of white and Gro-light fluorescent light. Liquid cultures were illuminated at approx. 30 μE m^{-2} s^{-1}, BG-11 plates at approx. 50–60 μE m^{-2} s^{-1}. The pUC19 recombinant plasmid pTK1 was a kind gift from Dr. T. Kuwabara and contains the entire *Anacystis nidulans* R2 *psb*O gene within a 1.2 kb *Xba*I-*Hin*dIII fragment (see Ref. 28).

DNA manipulation, library screening and Southern hybridisation

General DNA manipulations and library screening were all performed essentially as described by Sambrook et al. [29]. The *Synechocystis* 6803 library screened was also a kind gift from Dr. J.G.K. Williams. It was made commercially by Stratagene (U.S.A.) who ligated partial *Sau*3A chromosomal fragments into the *Bam*HI site of λEMBL3. λ clones containing *psb*O were isolated by screening with the 1.2 kb *Xba*I-*Hin*dIII pTK1 insert using overnight hybridisation at 55°C in 5 × SSC, 5 × Denhardt's, 0.5% SDS, 100 μg ml^{-1}

denatured sonicated calf thymus DNA, with subsequent filter washing to 1 × SSC, 0.1% SDS at 55°C (20 × SSC is 3.0 M NaCl, 0.26 M trisodium citrate; 50 × Denhardt's is as described in Sambrook et al. [29]). Maxiprep cyanobacterial chromosomal DNA was isolated according to Williams [26]. For Southern analysis, restricted DNA was fractionated on 0.8% agarose (BRL) gels and transferred to Schleicher and Schuel BA 85 nitrocellulose. Following ethidium bromide visualisation and before denaturation and neutralisation, gels were soaked for 10 min in 0.25 M HCl to facilitate the transfer of high molecular weight DNA fragments. [α-^{32}P]-radiolabelling of double-stranded DNA probes to high activity was achieved essentially as described by Feinburg and Vogelstein [30]. In the *Synechocystis* 6803 *psb*O copy-number determination the lowest stringency conditions used were hybridisation for 24 h at 55°C in 10 ml 5 × SSPE, 5 × Denhardt's, 0.2% SDS 1 mM ATP, 100 μg ml^{-1} calf thymus DNA with subsequent filter washing in 4 × SSPE, 0.2% SDS at 50°C followed by prolonged washing at 25°C in 2 × SSPE, 0.2% SDS. (20 × SSPE is 3.6 M NaCl, 0.2 M sodium dihydrogen orthophosphate, 2 mM EDTA). Fig. 3 Southern conditions consisted of hybridisation overnight at 65°C in 10 ml 5 × SSC, 5 × Denhardt's, 0.5% SDS, 100 μg ml^{-1} denatured sonicated calf thymus DNA, with filter washing to 0.1 × SSC, 0.1% SDS at 65°C. Chromosomal minipreps of *Synechocystis* 6803 mutant DNAs were performed from 4 ml cultures using the amalgamation of two published methods. A scaled-down version of the protocol of Williams [26] was followed through the NaI and lysozyme treatment steps. Then the procedure of Bustos et al. [31] was adopted from the proteinase K step onwards. Single-stranded template for sequencing using the Sequenase sequencing kit (USB, supplied by Cambridge BioScience) was prepared via directional subcloning of DNA into M13 vectors mp18 and mp19 [32].

Northern analysis

Total *Synechocystis* 6803 RNA was prepared from exponential growth phase (A_{730nm} = 0.5) and late exponential/stationary growth phase (A_{730nm} = 2) cells according to Mohamed and Jansson [33]. It was separated on 1.2% formaldehyde denaturing agarose gels and transferred to Hybond-N membranes (Amersham) according to the manufacturer's instructions. Prehybridisation, hybridisation and filter washing were performed as in Ref. 33. Boehringer-Mannheim RNA molecular-weight marker III was used for transcript sizing. High activity single-stranded DNA probes were prepared using the method of Burke [34] modified by Dr. J. Paterson (personal communication). The complementary radiolabelled strand was synthesised from single-stranded M13 clone mSMO1 (see Fig. 1A) using Klenow fragment and the universal primer, in the presence of dTTP, dGTP, dATP and [α-^{32}P]dCTP. The reaction products were then digested with *Hin*cII and electrophoresed on a 1.5% low-gelling-temperature agarose gel (FMC BioProducts) using 30 mM NaOH and 10 mM EDTA buffer. The *psb*O-specific single-stranded probe was located by autoradiography, excised, melted and added directly to the hybridisation.

Transformation of Synechocystis 6803 and isolation of psbO$^-$ mutants

Transformation of *Synechocystis* 6803 was carried out essentially as described by Williams [26]. Exponential-phase cells were spun down and resuspended to $1 \cdot 10^9$ cells ml^{-1} concentration in BG-11. 0.3–1.0 μg of unrestricted plasmid was added to 200 μl of cells in a sterile disposable 7 ml Bijoux bottle (Sterilin) and incubated in the growth room for 4–6 h with occasional shaking. The transformation mixture was then spread onto 50 mm diameter Sartorius SM 113 (0.45 μm pore size) nitrocellulose filters overlaid on 60 mm diameter BG-11 plates supplemented with 5 mM glucose to permit the growth of potentially non-photoautotrophic mutants. After approx. 20 h incubation the filters were transferred to fresh BG-11 plates containing 5 mM glucose, 5 μg ml^{-1} kanamycin and 10–20 μM atrazine. Incubation for about a week produced visible colonies. Several rounds of re-streaking from single colonies onto BG-11 plates containing 5 mM glucose, 100 μg ml^{-1} kanamycin and 20 μM atrazine then ensued to segregate mutants for the *psb*O mutation. For both strains IC1 and IC2 a number of independent transformants were segregated, though Southern analysis ultimately showed them to be indistinguishable.

Immunoblotting

Membranes were isolated from 800 ml cultures of cells in late exponential growth phase. The cells were broken by passage through a pre-chilled French pressure cell at 20000 p.s.i., according to Gounaris et al. [35]. The protein content of each cyanobacterial preparation was determined using the Pierce BCA assay system (Rockford, IL). Immunoblotting was performed as described previously by Nixon et al. [36] using antibodies to the extrinsic 33 kDa protein from pea [37].

Oxygen evolution measurements

Wild-type and mutant strains were grown at 31° C in liquid culture supplemented with 5 mM glucose and also 25 μg ml^{-1} kanamycin for mutants. Exponential-phase cells (A_{730nm} of 0.65–0.8) were harvested by centrifugation for 10 min at 4500 × g at 15°C. The chlorophyll *a* content was determined [38] following methanol extraction of the chlorophyll from cells. O_2 evolution was measured at 31°C using a Hansatech DW2 O_2 electrode unit (Hansatech, King's Lynn, Norfolk, U.K.). Illumination was provided by a Flexilux

HL150 lamp to an intensity of 3000 μE m^{-2} s^{-1}. The light intensity was measured inside the O_2 electrode reaction chamber using a fibre optic probe with the Skye quantum measuring system SKP200 (Skye Instruments, Llandrindod Wells, Powys, U.K.).

In situ genetic complementation

The procedure described in Ref. 39 was modified to establish a rapid screening system where many plates could be screened simultaneously if required. A 4 ml culture of mutant strain cells was spun down, washed twice in 5 ml BG-11 and after the third spin, resuspended in 1–2 ml BG-11. The A_{730nm} was measured and the cells then diluted to a concentration of $1.33 \cdot 10^8$ cells ml^{-1}. An A_{730nm} of 0.25 corresponds to a concentration of $1 \cdot 10^8$ cells ml^{-1} [26]. 150 μl ($2 \cdot 10^7$) cells were mixed briefly with 1.5 ml 0.8% BG-11 agar maintained at 42°C and overlaid onto a 60 mm diameter BG-11 plate. After the top agar had solidified transforming plasmids were spotted onto the agar surface adjacent to positions marked on the base of the plate. The spots were allowed to evaporate by leaving the plate lids off for 15 min. The lids were then sealed on with Parafilm (American National Can) and removed from the sterile hood.

Results

Isolation and characterisation of the Synechocystis 6803 psbO gene

The *Synechocystis* 6803 *psb*O gene was isolated from a λEMBL3 genomic library as detailed in Materials and Methods (a preliminary report has already been published in Ref. 25). Three positive phage clones were further characterised by restriction-mapping and Southern blotting (data not shown). Two of these possessed a 5.5 kb *Hin*dIII-*Hin*dIII fragment and the third, a 4.1 kb *Hin*dIII-*Bam*HI fragment adjacent to the λEMBL3 boundary, which hybridised to the *Anacystis nidulans* R2 *psb*O probe. Both these fragments, which are shown in Fig. 1A, were subcloned into the plasmid pUC18 [40]. The 5.5 kb fragment generated pSMO1 and the 4.1 kb fragment pSMO2.

Restriction-mapping of pSMO1 and pSMO2 together with partial sequencing of the region established that the *psb*O gene already described by Philbrick and Zilinskas [9] had been independently isolated and thus is not a second *psb*O locus in *Synechocystis* 6803. Fig. 1A extends the restriction data already reported and also shows the extent of the DNA sequencing performed. Only the *Hin*cII-*Bam*HI fragment was sequenced in both orientations and this sequence is identical to that already published. The 5.5 kb *Hin*dIII insert of pSMO1 corresponds to the signal detected in the low stringency Southerns.

Copy-number determination of the Synechocystis 6803 psbO gene

A series of low stringency Southern blots were performed using both the *A. nidulans* R2 and *Synechocystis* 6803 *psb*O genes as probes. The stringencies were lowered progressively to accomodate a range of possible mismatches between probe and target sequence and also a reduction in the kinetics of hybridisation (see Ref. 41 for discussion of the relevant parameters). These experiments were performed to discount the possibility that the mutant phenotype observed later upon inactivation of the *psb*O gene was not due to a second functional copy of the *psb*O gene in the *Synechocystis* 6803 chromosome. Results, even at the very low stringency conditions described in the methods, confirmed previous reports [9,22,28] that *Synechocystis* 6803 and *A. nidulans* have only one *psb*O gene copy per chromosome. Furthermore, the *Anabaena* sp. PCC 7120 *psb*O gene is also single-copy [42] so that it appears to be a trend in cyanobacteria that *psb*O exists as a single copy unlike the genes *psb*A, *psb*D and *psb*G [26,43].

Investigation of psbO transcription in Synechocystis 6803

Prior to mutagenesis it is important to establish the pattern of gene expression from the target locus. We therefore sought to establish at two stages of growth the nature of transcripts derived from *psb*O and the flanking DNA. It can be seen from Fig. 2 that a short autoradiogram exposure after hybridising with either probe (panels II, V) clearly identifies the same major transcript. This was sized as 0.95–1.0 kb long by comparing the mobility to the RNA size markers run on the same gels. Burnap and Sherman [22] also detected this transcript, although they sized it at 1.2 kb.

The *Synechocystis* 6803 *psb*O coding region is 822 bp long so this transcript is just large enough to cover the complete *psb*O gene. A much fainter 0.7 kb signal is also detected in exponential-phase RNA but not late exponential-phase RNA, using both probes. As the Southern analysis indicates there is only one *psb*O locus, it is likely that this 0.7 kb signal is a specific degradation product of the 1.0 kb *psb*O message and does not arise from a separate locus.

Over-exposure of the autoradiograms (panels III and VI) shows two things. Most importantly, no obvious transcript larger than 1.0 kb is observed. The signals above the major 1.0 kb transcript which are particularly visible in the exponential-phase RNA preparation result from probe hybridisation to trace DNA contamination and to ribosomal RNA. Therefore, the *psb*O transcript size implies that *psb*O is very likely not co-transcribed with any other gene in *Synechocystis* 6803 in either of the growth phases investigated. Secondly, upon over-exposure two additional signals of approximately 0.5 kb and 0.35 kb are de-

tected in late exponential-phase RNA using the longer probe (panel VI) but not the internal *psb*O probe (panel III). The significance of these signals is not known at present, but it is possible that they originate from sequences flanking the *psb*O gene. It should be noted that the sequence of approx. 0.4 kb downstream of *psb*O that was included in the double-stranded probe and which was removed with *psb*O in the deletion mutant (see below) has not been sequenced. This region could possibly be functional.

The intensity of the 0.95–1.0 kb signal is greater from the late exponential-phase RNA than the exponential-phase RNA using either probe. As approximately equal amounts of RNA were loaded this may be significant or it may reflect that the exponential-phase RNA preparation is slightly degraded.

Cartridge mutagenesis of the Synechocystis 6803 psbO gene

Two mutant *Synechocystis* 6803 strains with disrupted *psb*O loci were engineered. An insertion mutant strain, named IC1 was constructed. In this strain the gene encoding aminoglycoside 3′-phosphotransferase that confers resistance to the antibiotic

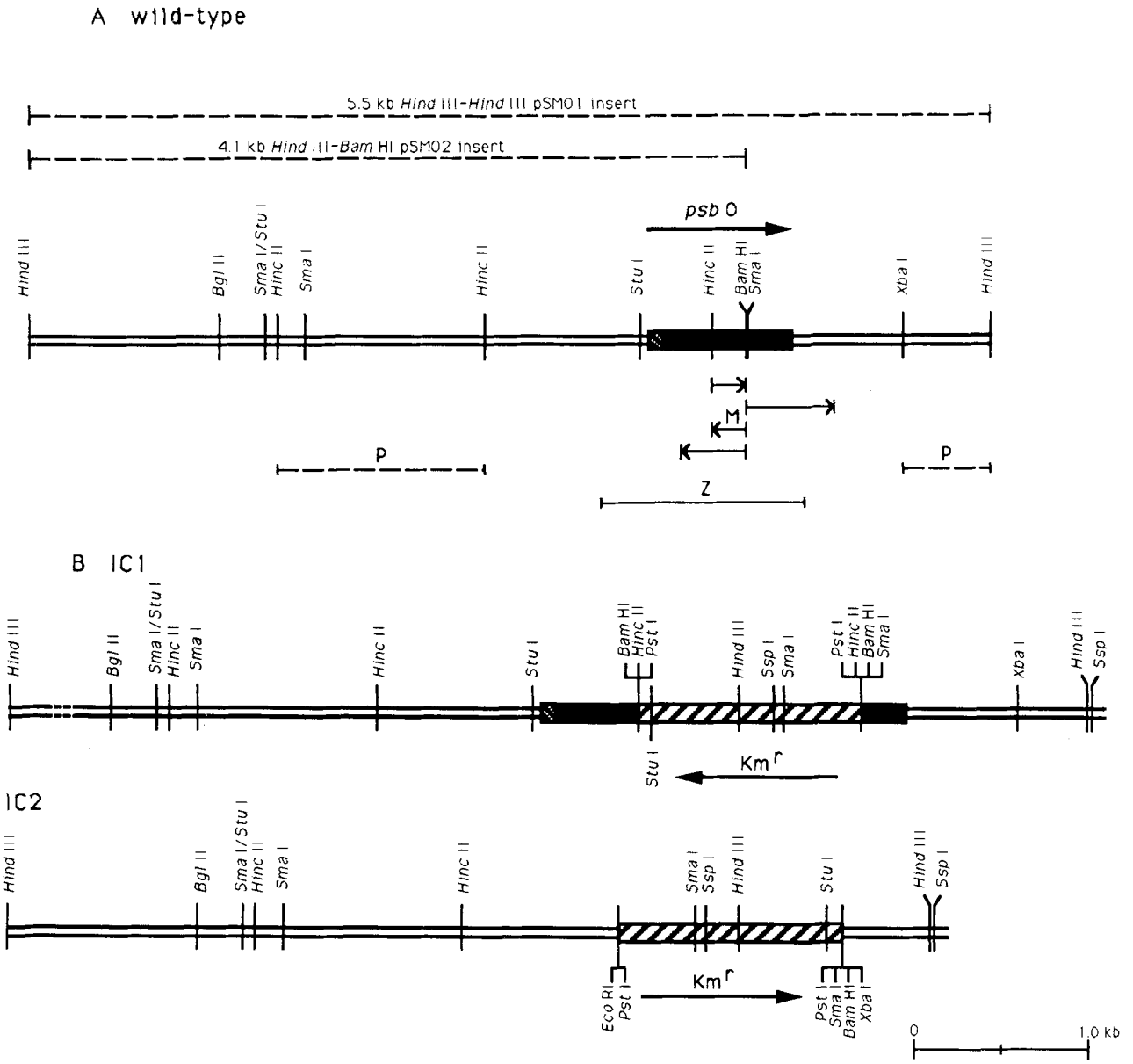

Fig. 1. Restriction maps of the *Synechocystis* 6803 *psb*O locus from wild-type and mutant strains IC1, IC2. (A) Wild-type locus. This shows the location of the *psb*O pre-sequence (hatched box) and mature sequence (solid box). Also illustrated are the inserts of the clones pSMO1 and pSMO2 and the DNA used as probe in Fig. 3 (dotted lines denoted P). The region of sequencing performed by Philbrick and Zilinskas [9] is indicated (Z). The fine arrows show the extent of DNA sequencing we have performed and the orientation of the sequencing clone mSMO1 (labelled M) used in the Northern analysis for the generation of a single-stranded *psb*O probe. (B) Mutant strains IC1 and IC2. The direction of transcription of the Km^r gene is arrowed in each strain.

kanamycin (hereafter referred to as Km^r) was inserted into the *psb*O *Bam*HI site. Plasmid pSMO1 was partially digested with *Bam*HI, electrophoresed and the linearised form purified from the excised gel slab. This was ligated with the 1.3 kb *Bam*HI Km^r fragment from plasmid pUC4K (variant described in Ref. 44) and transformed into *Escherichia coli*. Recombinant clones were screened by restriction-mapping and plasmid pSMO4, where the Km^r cartridge interrupts the *psb*O gene, was selected. The orientation of the Km^r cartridge was also elucidated in this analysis. Plasmid pSMO4 was transformed into *Synechocystis* 6803 to ultimately generate mutant strain IC1.

At the same time, a deletion strain named IC2 was made where the 1.5 kb *Stu*I-*Xba*I fragment containing all of *psb*O was replaced by the Km^r cartridge in the *Synechocystis* 6803 chromosome. Briefly, the 5.5 kb insert from pSMO1 was subcloned into the plasmid vector pUC9 to generate pSSO1. The desired orientation was selected by restriction-mapping recombinant clones. Concurrently the *Pst*I Km^r fragment of pUC4K was subcloned into Bluescript SK + (Stratagene, U.S.A.) to form plasmid pSSKm1 and the cassette orientation was also verified by restriction-mapping. Next, pSSO1 was partially digested by *Stu*I, then totally digested by *Xba*I and the longest restriction fragment purified. This fragment was ligated to the *Eco*RV-*Xba*I Km^r fragment of pSSKm1 to generate pSSO3. Construct pSSO3 was transformed into *Synechocystis* 6803 to generate strain IC2 after segregation of the homozygous mutant.

The rationale for generating these particular mutants was two-fold. Firstly, IC1 was made to correspond as closely as possible to the original insertion mutant constructed by Philbrick and Zilinskas [9]. The only possible differences between the insertion strains are the source of the Km^r cartridge and its orientation in the chromosome. Secondly, since IC1 still contains all the wild-type *psb*O gene, albeit in an interrupted manner, IC2 was also made. As the wild-type *psb*O gene has been completely removed IC2 not only lacks an inherent ability to revert to a *psb*O$^+$ strain but also this strain may be more useful for future site-directed mutagenesis experiments.

Synechocystis 6803 possesses approx. 12 chromosomal DNA copies per individual cell [45] so that after several rounds of re-streaking from single colonies it was necessary to check whether the segregation of

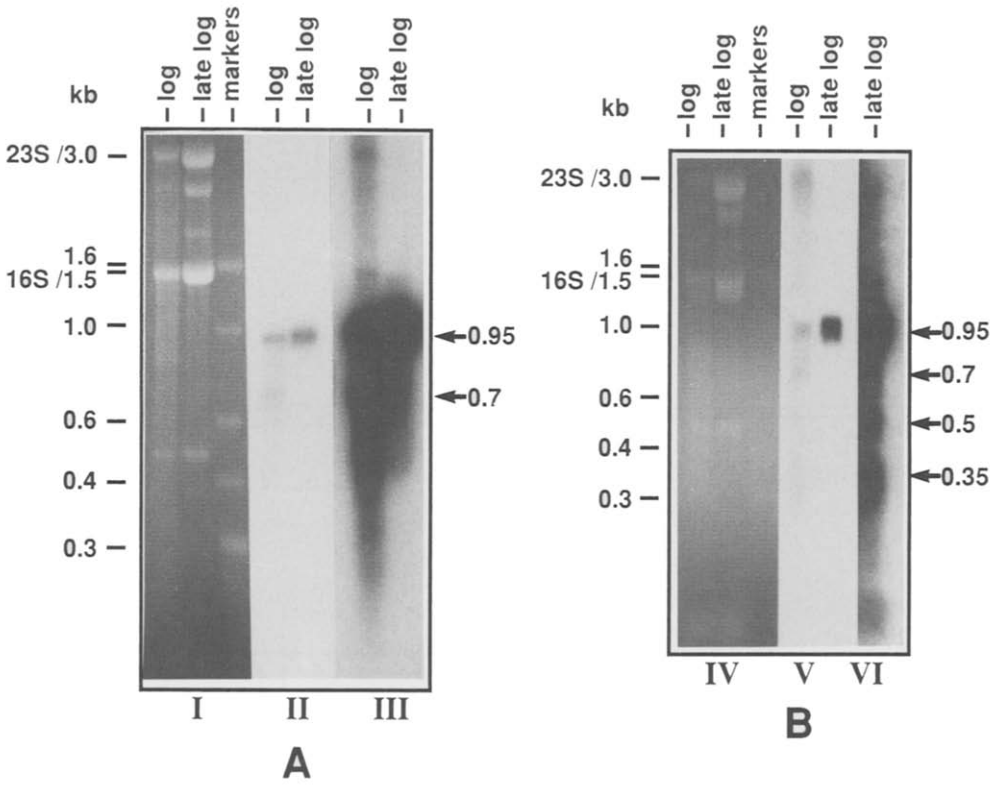

Fig. 2. Northern analysis of transcripts from the *Synechocystis* 6803 *psb*O locus present in exponential-phase and late-exponential phase RNA. Two different *psb*O probes were hybridised to Northern blots of total *Synechocystis* 6803 wild-type RNA isolated from exponential-phase (abbreviated to log phase) and late exponential-phase (late log) cultures. Probe A was a 198 bp single-stranded internal *psb*O probe generated from clone mSMO1 (see Fig. 1A). Probe B was formed by radiolabelling both strands of the purified 1.5 kb *Stu*I-*Xba*I restriction fragment containing all of *psb*O, plus flanking DNA. Approx. 10 μg total RNA per lane was loaded. Panels I, IV show the stained agarose gels prior to transfer. Panels II, V show the resulting autoradiograms following exposure at $-80\,^{\circ}$C for 60 h and 11 h respectively. Panels III, VI show over-exposure of the same filters.

homozygous mutants had been achieved. Genomic DNA was isolated from each mutant strain and used for Southern analysis to confirm all the wild-type *psb*O copies had been substituted. Fig. 3 shows quite clearly that the wild-type *psb*O restriction fragments, for example, the 5.5 kb *Hin*dIII fragment, are no longer present in either mutant strain. Instead the mutant restriction profiles verify the mutant *psb*O loci restriction maps shown in Fig. 1B. A hybridising 0.2 kb fragment must have run off the end of the gel in each *Sma*I digest in Fig. 3.

These results are clear confirmation that each mutant contains no functional *psb*O gene copies. There is no question that copies of the wild-type *psb*O gene remain in the mutants but at too low a level to be detected by Southern analysis. Experience we have gained constructing meridiploids during the course of other *Synechocystis* 6803 mutagenesis work confirms that if disruption of an essential gene is attempted then, in order to retain the gene function, at least one wild-type gene copy per cell is maintained and its resulting Southern signal intensity is at least one twelfth

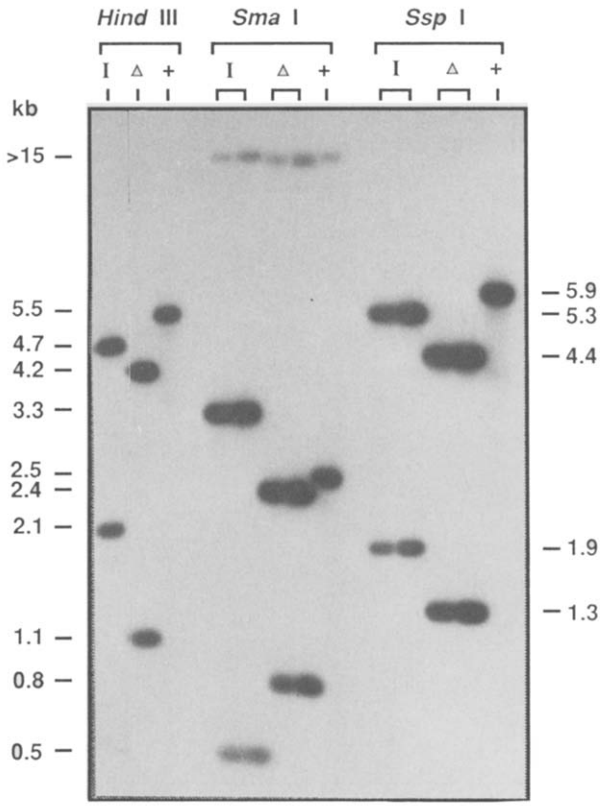

Fig. 3. Verification of *psb*O$^-$ strain genotypes by Southern analysis. Wild-type, strains IC1 (I) and IC2 (Δ) chromosomal DNAs were digested with *Hin*dIII, *Sma*I or *Ssp*I, electrophoresed and transferred to nitrocellulose. The filter was hybridised at high stringency with a DNA probe consisting of a 1:1:1 molar mixture of the *psb*O *Hin*cII-*Hin*cII and *Xba*I-*Hin*dIII fragments shown in Fig. 1A together with the pUC4K Km^r insert. An autoradiogram exposed for 2 days at −80°C with intensifying screens is shown.

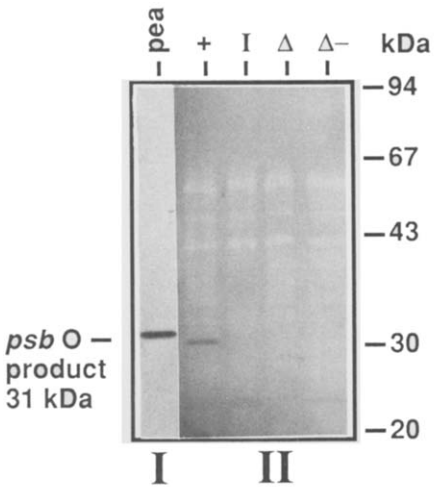

Fig. 4. Immuoblotting of wild-type and mutant thylakoid membranes with an antibody raised against the pea extrinsic 33 kDa polypeptide. Thylakoid membranes from pea (panel I, <1 μg protein loaded) and *Synechocystis* 6803 wild-type and *psb*O$^-$ mutant strains (panel II) were challenged with antibodies against the extrinsic 33 kDa protein from pea, following SDS-PAGE and transfer to a nitrocellulose membrane. Two antibody serum dilutions were employed: 1:3000, panel I; 1:500, panel II. The cyanobacterial preparations were made from wild-type (+), IC1 (I) and IC2 (Δ) cells grown with a supplement of glucose and IC2 (Δ −) without glucose. 40 μg protein were loaded for each *Synechocystis* 6803 lane.

that of the mutant signal. This level of wild-type signal would be readily visible in Fig. 3.

Analysis of mutant phenotypes

It was found that both IC1 and IC2 could grow photoautotrophically on BG-11 plates or in culture, albeit with doubling times significantly longer than the wild-type. Therefore, PS II seems to be at least partly functional in both these mutants. To check that photoautotrophic growth was really being monitored, identical BG-11 plates, supplemented with either 20 μM atrazine or 10 μM DCMU were prepared. Neither wild-type *Synechocystis* 6803 nor either of the mutants grew on these plates, indicating that the media contained no ingredients capable of sustaining photoheterotrophic growth where PS II activity is not required.

Immunoblotting

Immunoblotting of wild-type and mutant membranes was performed at high antibody concentration. Nonetheless, Fig. 4 shows that although the pea antibody cross-reacted well with the wild-type membrane preparation, no *psb*O protein is visible in any of the mutant samples. The result from IC2 grown in BG-11 without glucose supplement is particularly important as it demonstrates that even under photoautotrophic conditions where PS II is absolutely required, no *psb*O protein can be detected. Under the electrophoresis conditions employed here the *Synechocystis* 6803 *psb*O

TABLE I

Oxygen evolution rates dependent on CO_2 fixation for Synechocystis 6803 wild-type and psbO$^-$ mutant cells

Cells were harvested and resuspended in fresh BG-11 containing 10 mM $NaHCO_3$. Oxygen evolution rates, expressed in μmol O_2 mg (Chlorophyll *a*)$^{-1}$ h^{-1}, reflect electron transport from H_2O to fixation of CO_2. The mutant rates as a percentage of the rates from wild-type cells are given in parentheses.

Strains	Rate of O_2 evolution
Wild-type	272.2
IC1	204.1 (75)
IC2	180.5 (66.3)

gene product migrated slightly further than its pea counterpart.

Oxygen evolution measurements

Table I shows that both *psb*O$^-$ mutant strains evolve oxygen at similar appreciable rates, although both are reduced relative to the control rate. It is not possible to draw any quantitative conclusions from this data since values for O_2 evolution vary by up to 30% in different experiments. Similar variations in oxygen evolution data have been reported in Ref. 46. However, we have discovered that the rate of oxygen evolution from the deletion mutant decreases more rapidly than from wild-type following preillumination in high light conditions well above saturating light intensities (Fig. 5). The reduction in the rate of photosynthetic electron transport after illumination is termed photoinhibition. A similar sensitivity was found with the insertion mutant (data not shown).

In situ genetic complementation of the psbO$^-$ mutant strains

To genetically verify the mutant strains created the wild-type *psb*O gene was reintroduced to both IC1 and IC2. The clear difference in photoautotrophic growth rates between wild-type *Synechocystis* 6803 and the *psb*O$^-$ mutants was used as a convenient visual assay in the 'in situ dot transformation' procedure developed by Dzelzkalns and Bogorad [39]. Suitable controls were also performed to demonstrate that the results below are due to introduction of specific DNA sequences (data not shown).

The results of complementing both IC1 and IC2 are shown in Fig. 6A–C. Plasmid pSMO1, housing the 5.5 kb *Hin*dIII insert containing all of *psb*O plus flanking DNA regions (see Fig. 1A), was consistently able to complement either *psb*O$^-$ strain growing photoautotrophically. This complementation was manifested by an obvious increase in colony size in the region of each plate where pSMO1 had been spotted. Figs. 6A and B show that this increase was just apparent after 7 days for strain IC1. Fig. 6B illustrates that one of the most interesting features of the in situ complementation technique is the production of a sharp interface, which

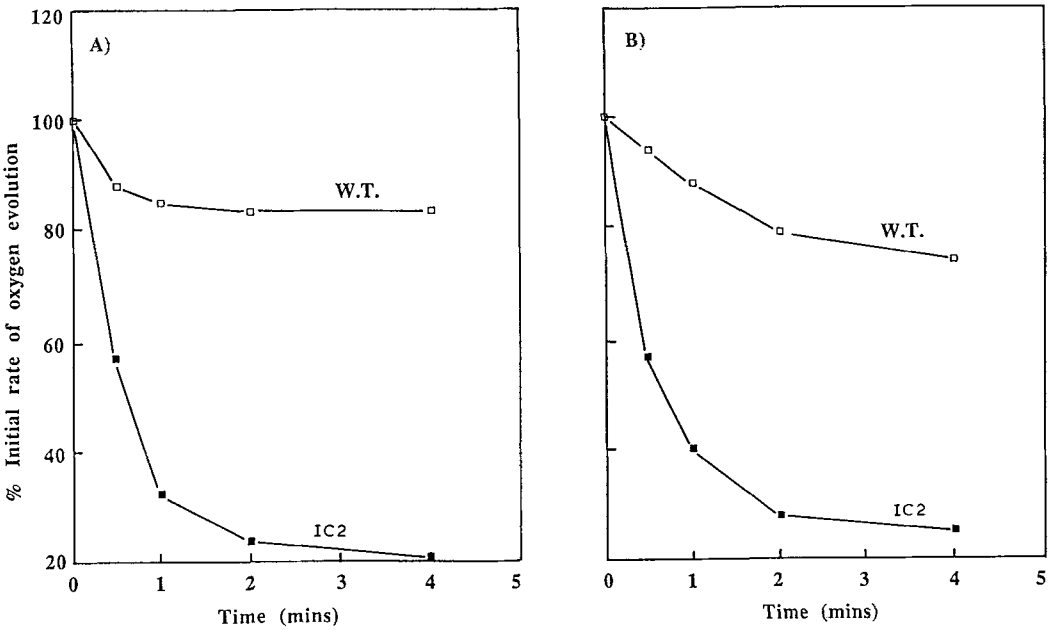

Fig. 5. Initial rates of oxygen evolution of wild-type (WT) and IC2 mutant of *Synechocystis* 6803 harvested in (A) exponential or (B) late exponential phase of growth and the effect of preillumination with strong light. Cells were harvested by centrifugation, diluted to 10 μg chlorophyll ml^{-1} and subjected to various periods of preillumination with white light having an intensity of approximately 8500 μE m^{-2} s^{-1}. The temperature for the preillumination and the subsequent oxygen evolution measurements was 31°C. 10 mM $NaHCO_3$ was added as an electron acceptor. In (A) the absolute rates for oxygen evolution for the non-treated wild-type and for the deletion mutant were 246 and 140 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1} respectively, and in (B) the respective rates were 273 and 129 μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1}.

we term the transformation interface (TI), between colonies with the mutant phenotype and those that have been complemented. This interface provides a direct comparison of the differing genetic backgrounds. Many of the colonies to the right of the TI arrow in Fig. 6B have received the *psb*O gene and are increased in size. Fig. 6B also shows that there is not a great difference in colony density on each side of the transformation interface. This means that there is probably little difference between the *psb*O$^-$ and *psb*O$^+$ strains in the initial viability of single cells at the onset of colony establishment.

As pUC plasmids are non-replicating in *Synechocystis* 6803 (J.G.K. Williams, personal communication), the wild-type *psb*O gene must be propagated by incorporation into the mutant chromosome. Recombination between the pSMO1 plasmid insert and the homologous sequences in the *Synechocystis* 6803 chromosome must occur. It is probable that the wild-type *psb*O sequence is added back at the site of the engineered *psb*O lesion through a double-crossover event between sequences common to pSMO1 and the mutant chromosome. In this case the Km^r marker should be simultaneously excised and it is for this reason that kanamycin was not added as a supplement to the BG-11 plates during these experiments.

IC1 and IC2 did not behave in an identical manner when complemented with pSMO1. Routinely, complementation was visible after a week when IC1 was spotted, whereas 2–3 weeks were needed when spotting a IC2 lawn. However, as Fig. 6C shows, after 3 weeks the appearance of both complemented mutant lawns was very similar.

On the other hand, Fig. 6A and C shows that the control plasmid pSMO2 (see Fig. 1A) failed to complement visibly in situ either IC1 or IC2. This was not due to the integrity of the pSMO2 DNA as judged by agarose gel electrophoresis and ethidium bromide staining. It was concluded that this plasmid was probably taken up by the mutant strains, but that the pSMO2 insert could not rescue the mutant phenotype. This demonstrates the mutant phenotypes are not caused by lesions upstream of the *psb*O gene.

The results presented above confirm that both mutants can grow photoautotrophically but with an increased rate when the *psb*O gene is replaced. They

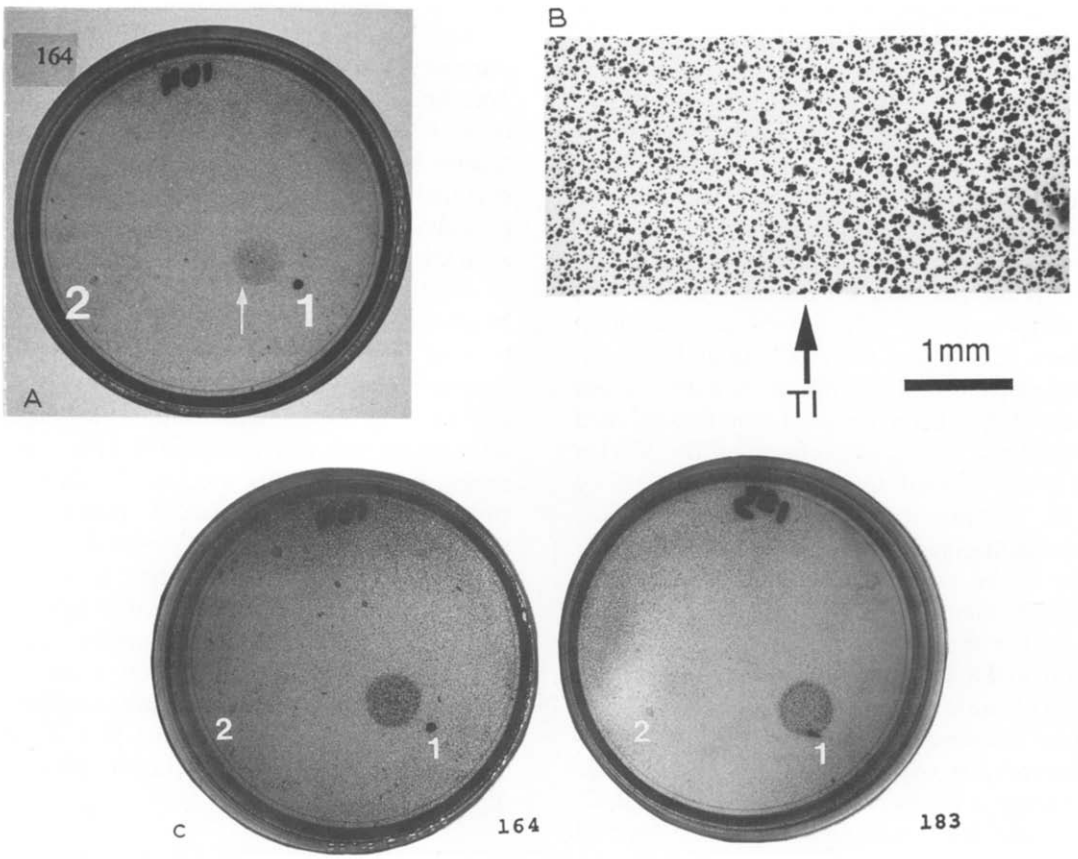

Fig. 6. in situ complementation of strains IC1 and IC2 with clones pSMO1 and pSMO2. 0.4–0.6 μg of unrestricted pSMO1 (spot 1) and pSMO2 (spot 2) plasmid DNAs were spotted in a 4–5 μl volume of TE buffer onto separate regions of a BG-11 plate overlaid by soft agar containing *psb*O$^-$ mutant cells (see Materials and Methods). (A) shows the appearance of the IC1 plate after 7 days growth. The transformation interface arrowed (arrow TI) is shown magnified in B. (C) shows the similar appearance of both the IC1 (plate 164) and IC2 (plate 183) transformations after three weeks growth.

also indicate that the primary phenotypes observed are not likely due to independent secondary mutations outside the *psb*O gene.

Discussion

Low stringency Southern hybridisation, immunoblotting and Northern transcript analyses confirm, to the limit of the sensitivity of each technique, that the *psb*O gene exists as a single copy in *Synechocystis* sp. PCC 6803 and is transcribed as a monocistronic message. *psb*O$^-$ insertion and deletion mutant strains, which have been verified both in vitro and in vivo, can still grow photoautotrophically and evolve oxygen at appreciable rates. Therefore, a main conclusion from these studies is that the *psb*O gene product is not absolutely required for the water-splitting reaction of Photosystem II in *Synechocystis* 6803. Perhaps of equal importance is the finding that when the *psb*O gene is deleted the photosynthetic activity of the mutant is readily inhibited by exposure to strong illumination as compared with the wild-type. We also show that an in situ complementation technique provides a convenient method for both rapidly confirming a mutant genotype and highlighting phenotypic changes following targeted mutagenesis in *Synechocystis* 6803.

Implications for psbO protein function

Our results are consistent with biochemical studies using isolated oxygen-evolving preparations which have also indicated that oxygen evolution can occur in the absence of the *psb*O protein. These studies showed that the *psb*O protein could be washed from the surface of the PS II complex without releasing the Mn [14] and that S-state turnover [47] and oxygen evolution occurred, albeit at a slower rate, as long as high concentrations of calcium and chloride ions were present [48]. However, it has been debated whether in vitro washing procedures can actually remove all the 33 kDa protein [49]. With regard to this point, the use of defined *psb*O$^-$ mutants reported here avoids the possibility of accumulating even trace amounts of the *psb*O protein in the membranes. Therefore, observations with *psb*O$^-$ mutants of *Synechocystis* 6803 support the view that it is not residual extrinsic 33 kDa protein bound to PS II particles which is responsible for the oxygen evolution activity in vitro.

The minimal biochemically-isolated PS II unit capable of photosynthetic oxygen evolution under near-physiological ionic conditions contains D1, D2, CP-47, CP-43, cytochrome *b*-559 subunits and the extrinsic 33 kDa protein [16,17]. The observation that the 33 kDa polypeptide is not essential for oxygen evolution is consistent with the proposal that it is the intrinsic PS II core which binds the Mn and contains the catalytic site for water-oxidation [50].

Existing in vivo data concerning the function of the *psb*O gene product, though much less plentiful, is seemingly more at odds with the *Synechocystis* 6803 result. In *Chlamydomonas reinhardtii* a mutation of the *psb*O gene abolishes PS II activity completely [21] whilst in *Euglena gracilis* the onset of PS II activity has been correlated specifically with the accumulation of the *psb*O gene product [51]. In *Synechocystis* 6803 the initial studies of Philbrick and Zilinskas [9] also indicated that the *psb*O gene product was essential for photoautotropic growth. Recently, however, this laboratory (J.B. Philbrick and B.A. Zilinskas, personal communication) has reappraised their initial finding and found, as we have, that the *psb*O gene product is not absolutely essential for water-splitting activity. Burnap and Sherman [22] have also arrived at the same conclusion for *Synechocystis* 6803. Therefore, the data available at present seems to indicate that the *psb*O protein is required for oxygen evolution in eukaryotic algae but not in *Synechocystis* 6803. This is a surprising conclusion since the primary sequences of this protein are relatively well conserved across species and in reconstitution experiments the spinach and *Synechococcus vulcanus psb*O proteins are, to some degree, interchangeable for restoring oxygen evolution [8]. However, other factors, be they protein, ionic or lipid may vary. For example the *psb*O protein cannot be removed from *Synechocystis* 6803 thylakoids using washing procedures that readily release the protein from plant thylakoids [52] and eukaryotic organisms have additional proteins associated with the *psb*O protein, namely the products of the *psb*P and *psb*Q genes. It is worth noting here that the 9 kDa polypeptide reported to be a constituent of the water-oxidation machinery of *Phormidium laminosum* [7,53] does not seem to be present in all cyanobacteria. It was not identified in *Synechococcus vulcanus* [54] and low stringency Southern blotting experiments similar to those described here, using the *P. laminosum* 9 kDa protein gene as probe, failed to detect a corresponding gene in *Synechocystis* 6803 (S.R. Mayes, J. Barber, T.P. Wallace and C.J. Howe, unpublished results).

In reconciling all the existing in vitro and in vivo data, together with that presented here, it is evident that the *psb*O gene product is not the major site of Mn binding and performs a regulatory rather than catalytic role in PS II. However, our results are not inconsistent with the widely-held view that the 33 kDa protein serves to stabilise the Mn cluster, particularly in the higher S-states [55].

Implications for the oxidising side of PS II and photoinhibition

Our findings that the *psb*O-less mutants have decreased oxygen evolving capacity and are more susceptible to photoinhibition than the wild-type suggests that

the removal of the *psb*O protein perturbs in some way the efficiency of water-splitting. There is increasing evidence that photoinhibition can occur when the rate of electron donation to the PS II reaction centre becomes limiting [56]. So that under strong illumination highly oxidising species, such as $P680^+$, can accumulate and bring about damage to the protein and pigment components of the reaction centre [57,58]. Therefore although the *psb*O protein is not an absolute requirement for the water-splitting reaction, it seems likely that its presence is required for efficient donation of electrons to $P680^+$ especially under conditions when the photochemical activity of the reaction centre is high. Burnap and Sherman [22] also suggested from their chlorophyll fluorescence studies that the absence of the *psb*O protein decreased the efficiency of electron donation to the reaction centre. Our results therefore indicate that the *psb*O protein has a protective role and is important for normal physiological growth where organisms are exposed to varying conditions.

A principal role for the *psb*O protein in PS II assembly seems unlikely in *Synechocystis* 6803 as PS II obviously still assembles in the *psb*O$^-$ strains. In general gross mutations in genes encoding the intrinsic PS II core components in *Synechocystis* 6803 have a far greater destabilising effect on PS II assembly (discussed in Ref. 59). For example in a *Synechocystis* 6803 *psb*A$^-$ mutant, the D2 and CP-47 polypeptides fail to accumulate appreciably even though the *psb*O protein binds to the membrane [52].

Finally, it is appropriate to point out that we observed a difference in the time taken for visible in situ complementation with the *psb*O gene between the two *psb*O$^-$ mutant strains. This could mean that the mutants, IC1 and IC2 are not phenotypically identical. Differences could conceivably arise either because IC1 still expresses the 5′ portion of the *psb*O gene or because additional functional DNA has been deleted along with the *psb*O gene in IC2. This latter possibility could not be ruled out from the transcription studies. A more detailed phenotypic study of both mutants is currently in progress.

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