Expression of the nuclear encoded OEE1 protein is required for oxygen evolution and stability of photosystem II particles in *Chlamydomonas reinhardtii*

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In *Chlamydomonas reinhardtii* the oxygen evolving enhancer protein 1 (OEE1), which is part of the oxygen evolving complex of photosystem II (PS II), is coded for by a single nuclear gene (*psbI*). The nuclear mutant FuD44 specifically lacks the OEE1 polypeptide and is completely deficient in photosynthetic oxygen evolution. In this mutant a 5 kb DNA insertion into the 5' region of the *psbI* gene results in the complete absence of OEE1 mRNA and protein. A revertant, FuD44-R2, which is capable of 30% of the photosynthetic oxygen evolution of wild-type cells, has lost 4 kb of the 5 kb DNA insert, and accumulates both OEE1 mRNA and protein, although at levels somewhat less than those of wild-type cells. Absence of the OEE1 protein in the FuD44 mutant does not affect the accumulation of other nuclear encoded PS II peripheral polypeptides. OEE1 absence does, however, result in a more rapid turnover of the chloroplast encoded PS II core polypeptides, thus resulting in a substantial deficiency of PS II core polypeptides in FuD44 cells. These PS II core proteins again accumulate in revertant FuD44-R2 cells.

Key words: *Chlamydomonas reinhardtii*/photosystem II particles/stability/oxygen evolution/OEE1 protein requirement

Introduction

Photosynthetic oxygen evolution requires the interaction of several different yet closely coupled biochemical reactions. The light capturing, charge separating capacities of photosystem II must work in close cooperation with an oxygen evolving complex capable of utilizing this oxidizing power to split water into its molecular species, oxygen and hydrogen. Electrons stripped from water during this reaction are funnelled back into photochemical reaction center II and then transported through the electron transport chain to photosystem I, eventually to be used for the reduction of NADP.

Photosystem II and the oxygen evolving complex are physically linked. Isolation of oxygen evolving particles yields a complex containing chlorophylls *a* and *b*, carotenoids, quinones, lipids and an array of at least eight polypeptides (Murata and Miyao, 1985). Five of these, known in *Chlamydomonas reinhardtii* as D1, D2, P5, P6 and C-b559, are chloroplast encoded (Rochaix, 1981) and localized within the PS II reaction center core (Inoue *et al.*, 1983; Erickson *et al.*, 1986). Associated with this core PS II particle are three extrinsic nuclear encoded polypeptides, oxygen evolving enhancer proteins (OEE 1, 2 and 3), which can

be removed from the particle by salt washing (Compiled in Inoue *et al.*, 1983). The removal of these water soluble extrinsic polypeptides from the PS II complex results in the loss of oxygen evolving activity, which can be partially restored by the re-addition of the extrinsic polypeptides to the PS II core (reviewed by Murata and Miyao, 1985; Critchley, 1985).

Here we report an *in vivo* analysis of polypeptides involved in photosynthetic oxygen evolution. Mutants of *Chlamydomonas reinhardtii*, a unicellular green alga utilizing the same photosynthetic scheme as most higher plants, were selected which displayed a high chlorophyll fluorescence compared to wild-type cells, which is indicative of the absence of active PS II centers. Several of these mutants were tested for photosynthetic oxygen evolution. One mutant, FuD44, which is completely lacking photosynthetic oxygen evolving activity, specifically lacks a 26 kd nuclear encoded PS II polypeptide (OEE1), known as the 33 kd polypeptide of spinach oxygen evolving particles (Kuwabara and Murata, 1979). Mutant FuD44 and a revertant of this mutant containing 30% of the oxygen evolving activity of wild-type cells, FuD44-R2, were characterized.

Results

Nuclear mutant FuD44 specifically lacks a 26 kd nuclear encoded polypeptide associated with PS II

To determine the role of nuclear encoded PS II polypeptides in photosynthetic oxygen evolution we isolated nuclear mutants deficient in oxygen evolving activities. One such mutant FuD44, and a revertant of this mutant containing 30% of the oxygen evolving activity of wild-type cells, FuD44-R2, were characterized. Water soluble proteins were isolated from wild-type, FuD44, and FuD44-R2 cells, and separated on 7.5–15% polyacrylamide gels containing 0.1% SDS. Following electrophoresis the gels were either stained with Coomassie Blue (Figure 1A) or transferred to CNBr activated paper (Figure 1B). The protein blots were hybridized with antisera raised against the three extrinsic PS II polypeptides, OEE1, OEE2 and OEE3, which have been implicated in oxygen evolution. As shown in Figure 1B only one of these polypeptides, OEE1, is lacking in mutant FuD44. This 26 kd polypeptide (OEE1) is again present in revertant FuD44-R2 cells, although at only 50% of the wild-type levels (Figure 1B, wt X 1/2). It is interesting to note that while the growth rate of wild-type and FuD44-R2 cells is about equal on acetate containing media, the growth rate of FuD44-R2 on minimal media, therefore relying exclusively on photosynthesis for growth, is only about one half that of wild-type cells. The two other peripheral PS II polypeptides, OEE2 and OEE3, are present in each of the different strains (Figure 1B), although the level of OEE2 is somewhat reduced in both FuD44 and FuD44-R2 cells.

FuD44 specifically lacks OEE1 mRNAs

To determine if the absence of OEE1 protein in FuD44 cells was due to a mutation affecting expression of the gene encoding it (*psbI*), and not due to a pleiotropic effect of a mutation at another site, we cloned cDNAs encoding the three extrinsic polypeptides

Fig. 1. Polyacrylamide gel electrophoresis of water soluble proteins isolated from wild-type (wt), mutant FuD44, and revertant FuD44-R2 cells. Following electrophoresis the gels were either stained with Coomassie Blue (A), or electrophoretically transferred to CNBr activated paper (B). The location of the OEE1 protein is marked (→) on the stained gel. The protein blots were hybridized with antisera specific to three extrinsic polypeptides associated with PS II; OEE1, OEE2 and OEE3. A sample lane containing 50% of the protein loaded into the wt lane (wt X 1/2) is shown for comparison of OEE1 protein levels in the FuD44-R2 sample. Protein-antibody complexes were visualized by autoradiography after labeling with 125 I-labeled *Staph-A* protein.

Fig. 2. Northern analysis of mRNA isolated from wild-type (wt), FuD44 and FuD44-R2 cells. Total RNA was separated on denaturing formaldehyde gels and electroblotted to nylon membranes. The filters were hybridized with nick translated cloned cDNAs encoding the PS II peripheral proteins OEE1, OEE2 and OEE3.

associated with oxygen evolving particles, and measured the accumulation of their corresponding mRNAs. A *C. reinhardtii* cDNA library was constructed in a λ gt11 expression vector and the expressed fusion proteins were identified by hybridization with antisera specific for the three extrinsic polypeptides (Mayfield *et al.*, 1986). Several plaques were identified using OEE1 antisera, and individual phage were isolated. One of the recombinant phage contained a 1.6 kb cDNA insert which was subcloned into the *Eco*RI site of plasmid pUC19 to form plasmid

pPII-12.4. To confirm that pPII-12.4 encoded the OEE1 polypeptide the cDNA insert was sequenced. Comparison of the deduced amino acid sequence of pPII-12.4 with the N-terminal amino acid sequence determined from isolated mature OEE1 protein confirmed that pPII-12.4 encodes the OEE1 polypeptide (sequence to be reported elsewhere).

Total RNA was separated on denaturing agarose gels, blotted to nylon membranes and stained with methylene blue to verify equal loading and even transfer of the RNA (Khandjian, 1986). The membranes were hybridized with nick translated cDNA inserts from plasmid pPII-12.4. As shown in Figure 2 this cDNA hybridized to a 1.6 kb mRNA in wild-type cells which is completely missing from mutant FuD44, but is again present in the revertant FuD44-R2. From dilutions of wild-type mRNA we estimate that the revertant contains only about 25% of the OEE1 mRNA of wild-type cells (data not shown). Hybridization of identical filters with cDNA encoding OEE2 (Mayfield *et al.*, 1986) and OEE3 (cloning and sequence to be reported elsewhere) show that both of these mRNAs are present in equal quantities in wild-type, FuD44 and FuD44-R2 cells (Figure 2).

FuD44 contains a 5 kb DNA insert in the 5' region of the single OEE1 gene

To characterize the mutation resulting in the specific absence of OEE1 mRNA and protein we examined the chromosomal region containing the *psbI* gene by Southern analysis. DNA was isolated from wild-type, FuD44 and FuD44-R2 cells, digested with restriction endonucleases, separated on agarose gels, and blotted to nitrocellulose membranes. The DNA blots were then hybridized with nick translated cloned OEE1 cDNA insert. As shown in Figure 3B the cDNA hybridized to a single fragment in both the *Eco*RI and *Hind*III digests in each of the different DNA samples. However, in mutant FuD44 the DNA fragment containing the *psbI* gene is approximately 5 kb larger than the fragment in wild-type cells. In the revertant FuD44-R2 the fragment containing *psbI* is approximately 4 kb smaller than the frag-

Fig. 3. Southern analysis of wild-type, FuD44 and FuD44-R2 genomic DNA. DNA was digested with restriction endonuclease as indicated on the figure, separated by agarose gel electrophoresis, and blotted to nitrocellulose. For each digestion indicated the sample lanes are left to right; wild type, FuD44 and FuD44-R2. (A) The blot was hybridized with a nick translated 750 bp *HindIII*–*HindIII* cloned wild-type genomic fragment located at the 5' end of the *psbI* gene (marked on genomic map C). (B) After removal of this probe the filter was hybridized with a cDNA encoding OEE1, this cDNA lies completely 3' to the *PstI* site within the coding region. (C) Genomic map of the chromosomal fragment containing the *psbI* gene. Restriction sites for *PstI* (P), *HindIII* (H), *SalI* (S) and *AvaI* (A) are shown, as are the 5' and 3' ends of the coding region (■), the site of the DNA insert (□), the location of the genomic fragment used as the 5' probe (▨), and the 5' limit of the cDNA probe (⋈).

ment in FuD44, but still about 1 kb larger than the wild-type fragment (Figure 3A and B).

Genomic clones containing the *psbI* gene were isolated from a EMBL3–*C. reinhardtii* genomic library, and mapped using common restriction endonucleases. A restriction map of the chromosomal segment containing the wild-type *psbI* gene is presented in Figure 3C. Wild-type, FuD44 and FuD44-R2 DNA was restricted with endonucleases *PstI*, *SalI* and *HindIII*, all of which cut the *psbI* gene into two or more fragments. The DNAs were separated on agarose gels, blotted to nitrocellulose filters and hybridized with a 750 bp *HindIII*–*HindIII* cloned wild-type genomic fragment, which is located at the extreme 5' end of the single *psbI* gene (crossed line in Figure 3C). As shown in Figure 3A this probe hybridized to a single fragment in the *PstI* and *HindIII* digests and to two fragments in the *SalI* digest. Both the *PstI* and the 1.8 kb *SalI* fragments are larger than their wild-type counterpart in FuD44 and FuD44-R2, while the 750 bp *HindIII* fragment and the 3.5 kb *SalI* fragment are the same in all three DNA samples. This probe was removed from the filters and the blot was rehybridized with a cDNA probe which lies 3' to the *PstI* site as indicated in Figure 3C. As shown in Figure 3B the *PstI* fragments of each sample are the same size, while the 3' *HindIII* fragments of FuD44 and FuD44-R2 are approx-

imately 5 kb and 1 kb larger than their wild-type counterpart. This analysis identified the site of insertion of foreign DNA into the *psbI* gene to a 220 bp *HindIII*–*PstI* fragment that is located at the 5' end of the *psbI* gene (see map Figure 3C). Sequencing of the 220 bp wild-type *PstI*–*HindIII* genomic fragment showed it to contain 60 nucleotides of an exon, and 160 nucleotides of an intron, located at the 5' end of the *psbI* gene (data not shown). The exact location of the 5 kb insert within this 220 bp fragment in FuD44 has not yet been determined.

The absence of OEE1 results in the reduction of core PS II polypeptides, but not in a deficiency of their mRNAs

To assess what effect the absence of OEE1 had on PS II core polypeptides, membrane proteins were isolated from wild-type, FuD44 and FuD44-R2 cells, and separated on 7.5–15% polyacrylamide gels. Following electrophoresis the gels were either stained with Coomassie Blue (Figure 4A) or transferred to CNBr activated paper (Figure 4B). The protein blots were then hybridized with antisera specific to the PS II core polypeptides D1, D2, P5 and P6, and to the PS II antenna chlorophyll binding protein, LHC II. As shown in Figure 5B all of the PS II core polypeptides accumulate in FuD44 cells, but only to approximately 10–15% of wild-type levels. These PS II polypeptides accumulate to a much greater extent in the revertant FuD44-R2 cells, but are still reduced to ~50% of the wild-type levels. The LHC II polypeptides, like most of the membrane proteins as visualized by the stained protein gels, accumulate to similar levels in all three strains (Figure 4B).

To determine if the reduction in PS II core proteins was a consequence of reduced mRNA levels we measured the accumulation of chloroplastic mRNAs encoding the PS II core proteins. Equal amounts of RNA from wild-type, FuD44 and FuD44-R2 cells were separated on denaturing gels and electroblotted to nylon membranes. The filters were then hybridized with nick translated cloned chloroplastic genomic fragments encoding the PS II core polypeptides D1, D2, P5 and P6 (Rochaix, 1981). As shown in Figure 5 all of the mRNAs encoding these PS II core polypeptides accumulate in FuD44 and revertant FuD44-R2 cells to levels similar to those of wild-type cells.

Deficiency of core PS II polypeptides in FuD44 is due to instability of the core complex

To determine if the deficiency of PS II core proteins in FuD44 cells was due to an increased instability of the complex or due to a reduction in synthesis of the core polypeptides, wild-type, FuD44 and FuD44-R2 cells were pulse-labeled with [14 C]acetate. As shown in Figure 6A the pattern of proteins from cells labeled for 10 min with [14 C]acetate is identical in both wild-type and FuD44 cells. Because this pattern of labeled proteins is complex we also labeled cells in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis. As shown in Figure 6C labeling of membrane proteins in this way allows for the easy visualization of the PS II core proteins. The upper panel in Figure 6C is an autoradiograph of proteins labeled for 45 min in the presence of 8 μ g/ml cycloheximide prior to separation on polyacrylamide gels containing 8 M urea, and shows that the D1 and D2 proteins are synthesized in approximately equal amounts in all three strains. The lower panel in Figure 6C is an autoradiograph of the same labeled proteins separated on non-urea containing gels, which allows for the separation of polypeptides P4 and P5, and shows that both polypeptide P5 and P6 are synthesized in all three strains.

Cells labeled without the addition of cycloheximide were chased for 90 min in the presence of cold acetate following the 10 min

Fig. 4. Polyacrylamide gel electrophoresis of membrane proteins isolated from wild-type (wt), FuD44 and FuD44-R2 cells. Following electrophoresis the gels were either stained with Coomassie Blue (A), or transferred to CNBr activated paper (B). The protein blots were hybridized with antisera specific to the PS II core proteins D1, D2, P5 and P6, and to the PS II antenna chlorophyll binding protein LHC II. The proteins were visualized by autoradiography after labelling of the antigen-antibody complexes with ^{125}I -labeled *Staph-A* protein.

Fig. 5. Northern analysis of mRNA isolated from wild-type (wt), FuD44 and FuD44-R2 cells. Total RNA was separated on denaturing formaldehyde gels and electroblotted to nylon membranes. The filters were hybridized with nick translated cloned chloroplastic genomic fragments encoding the PS II core proteins D1, D2, P5 and P6.

pulse. As shown in Figure 6B the patterns of labeled proteins in wild-type and FuD44 cells show some differences. This difference is most easily observed in the D1 and P5 proteins which are clearly underrepresented in the FuD44 cells after the 90 min chase (Figure 6B). Polypeptides P6 and D2 co-migrate with other labeled proteins on these gels and are thus not as easily observed in the autoradiograph (Figure 6B). The loss of P5 and D1 protein, and we assume P6 and D2 as well, in FuD44 cells must therefore be due to a more rapid turnover of the protein in FuD44 cells compared to wild-type cells.

Fig. 6. Autoradiograph of *in vivo* labeled membrane proteins from wild-type (wt), FuD44 and FuD44-R2 cells. (A) Cells labeled for 10 min with [^{14}C]acetate, and (B) chased for 90 min in the presence of non-radioactive acetate. The site of migration of polypeptides P5, P6, D1 and D2 is marked. (C) Cells labeled for 40 min in the presence of cycloheximide. Upper panel shows the proteins separated on polyacrylamide gels containing 8 M urea, and the lower panel shows a section of the autoradiograph with similar samples separated on non-urea gels which allows for the separation of polypeptides P4 and P5.

Discussion

Several groups have isolated oxygen evolving particles which contain the PS II reaction center core proteins and three extrinsic nuclear encoded proteins (reviewed by Critchley, 1985; Murata and Miyao, 1985). There is, however, some debate as to which of the extrinsic proteins are really necessary for photosynthetic O₂ evolution. Several groups have reported that *in vitro* the PS II core and OEE1 comprise the minimal particle capable of oxygen evolution (Ono and Inoue, 1984; Ikeuchi *et al.*, 1985; Preston and Critchley, 1985; Ghanotakis *et al.*, 1984), others have reported that the PS II core and OEE2 are sufficient for O₂ evolution *in vitro* (Moller and Hoj, 1983; Henry *et al.*, 1982), while still others (Tang and Satoh, 1985) have concluded that O₂ evolution is associated with the PS II core alone.

Recently we have shown that *in vitro* OEE2 is required for high levels of O₂ evolution, but that in its complete absence there is still some O₂ evolution (5% of wild-type levels, Mayfield *et al.*, 1986). Here we show that *in vivo* OEE1 is absolutely required for any photosynthetic oxygen evolution, and that cells deficient for OEE1, unlike those deficient for OEE2, are incapable of photoautotrophic growth, thus OEE1 is absolutely required for photosynthesis.

Two interesting observations can be made from the Western analysis of PS II polypeptides. The first is that the absence of OEE1 does not result in the loss of either OEE2 or OEE3. Thus OEE1 is not required for the stability of the other OEE polypeptides. We have not, however, attempted to localize OEE2 or OEE3 within the chloroplast and thus we can not say whether these polypeptides remain anchored to the membrane in the absence of OEE1, only that they accumulate to near wild-type levels and are the correct, mature, size. The second observation is that the absence of OEE1 causes a substantial reduction of core PS II polypeptides and active PS II centers, but not a reduction in other polypeptides closely associated with PS II, like the LHC II polypeptides. The *in vivo* labelling experiments clearly show that the deficiency of the PS II core proteins in FuD44 cells is not due to a decrease in synthesis of these proteins, but rather due to a more rapid turnover of the newly synthesized polypeptides in the absence of OEE1 protein. This suggests that OEE1, or perhaps a portion of it, is required for the stable formation of the PS II core particle, perhaps even having some structural role within the complex. An alternative explanation could be that the loss of OEE1 exposes the PS II core or one of its proteins in such a way that the particle becomes susceptible to proteolytic degradation. Recently we have shown that another O₂ evolving deficient mutant of *C. reinhardtii*, which is missing the OEE2 polypeptide (Mayfield *et al.*, 1986), accumulates almost wild-type levels of all of the core PS II proteins, thus the instability of the PS II core proteins in FuD44 cells must be due to the absence of OEE1 and not due to some pleiotropic effect of the loss of O₂ evolving activities.

The mutation which results in the absence of OEE1 mRNA in FuD44 cells is a large, 5 kb, DNA insertion into the 5 prime region of the single *psbI* gene. Revertants of the original mutant, which are again capable of oxygen evolution, have lost 4 kb of this DNA insert. These revertants, although still harboring a 1 kb DNA insert in the *psbI* gene, again accumulate OEE1 mRNA. We assume from this that the DNA insert must be in the non-coding region of the 220 bp genomic fragment which contains the insert, as the RNA is still correctly spliced to yield functional mature OEE1 mRNA of the correct size. Analysis of 12 revertants of FuD44 (data not shown) shows that with one

exception all of them have identical patterns on Southern analysis, indicating that the reversion of this mutant is probably not a random event. It is also interesting to note that the revertant, although able to accumulate both OEE1 mRNA and protein, does not accumulate either to wild-type levels. This data suggest that this DNA insert may be a transposable element, but proof of this must await the cloning and analysis of the mutant OEE1 locus and its revertants.

Materials and methods

Isolation of mutant FuD44, and cell culture conditions

Wild-type cells 137c were treated with 5-fluorodeoxyuridine and metronidazole as described (Bennoun *et al.*, 1978; Schmidt *et al.*, 1977). Mutant FuD44 was isolated as a high chlorophyll fluorescent colony which was back crossed to wild-type cells and the mutation shown to be inherited in a Mendelian (nuclear) fashion. Complementation analysis in young zygotes (Bennoun *et al.*, 1980) with mutant BF25, a nuclear mutant which affects the expression of the OEE2 polypeptide (Mayfield *et al.*, 1986), showed that the two mutations are in different genes (data not shown). Revertants of mutant FuD44 were selected by plating the mutant on agar plates containing minimal media in the light, thus selecting for photosynthetic growth. Revertants were observed at a frequency of $\sim 1 \times 10^{-6}$ to 10^{-7} cells, and 12 colonies, capable of growth on minimal media, were isolated. With one exception all of these revertants showed similar patterns on Southern analysis so only one revertant, FuD44-R2, was used for further characterization. Oxygen evolution was measured on whole cells with a Clark-type oxygen electrode (Rank Brothers, England).

Wild-type *C. reinhardtii* strain 137c, nuclear mutant FuD44 and revertant FuD44-R2 were grown in liquid Tris-acetate-phosphate media pH 7.0 (Gorman and Levine, 1965) under dim fluorescent lighting to a density of $1-2 \times 10^6$ cells/ml. The cells were harvested by centrifugation at 8000 g for 10 min resuspended in 1/20 volume fresh media and pelleted again at 8000 g for 10 min.

Protein isolation, polyacrylamide gel electrophoresis, electroblotting and antibody hybridization

Protein isolation and sample preparation were described previously (Mayfield *et al.*, 1986). Electrophoresis of proteins on urea containing gels was described in Piccioni *et al.* (1981). Polyacrylamide gel electrophoresis of non-urea containing gels, protein blotting, antibody hybridization, and autoradiography were as described by Mayfield and Taylor (1984). Following autoradiography the filters (CNBr activated paper) were stripped of the primary antibodies by washing the filters for 30 min in 7.5 M guanidinium hydrochloride, 5% β -mercaptoethanol and then for 15 min in 0.2 M Glycine-HCl pH 2.8. The filters were then washed for 30 min in hybridization buffer before being rehybridized with a new antisera. As long as the filters were never allowed to completely dry they could be reused several times without apparent loss of signal.

Isolation of cDNA and genomic clones, and RNA and DNA isolation, electrophoresis and blotting

Isolation of cDNA clones was described by Mayfield *et al.* (1986), but basically involved identification of OEE1 protein expressed in a λ gt11 expression vector (Young and Davis, 1985) using the same rabbit polyclonal antisera used for the Western blots. Genomic clones were then isolated from a *C. reinhardtii* EMBL3 genomic library (Goldschmidt-Clermont, 1986) by the method of Benton and Davis (1977) using nick translated OEE1 cDNA inserts from the λ gt11 clones.

RNA was isolated with guanidinium hydrochloride as described (Nelson *et al.*, 1984). Total RNA, 10 μ g per sample lane, was separated on denaturing formaldehyde agarose gels and then electroblotted to nylon membrane (genescreen) in 25 mM phosphate pH 6.5. Following electroblotting the RNA was fixed to the filters by u.v. irradiation, and then stained with methylene blue to verify even loading and transfer of RNA (Khandjian, 1986). The filters were then prehybridized and hybridized as described (Johnson *et al.*, 1984).

Isolation of *C. reinhardtii* DNA, digestion with restriction endonucleases, electrophoresis and blotting to nitrocellulose membranes was as previously described (Rochaix, 1978).

Pulse labeling of proteins with [¹⁴C]acetate

Cells were grown in complete media to a cell density of $1-2 \times 10^6$ cells/ml, then transferred to media lacking acetate for 1 h prior to labelling. The cycloheximide treated cells were made 8 μ g/ μ l final concentration 10 min prior to the addition of label, and were labeled for 45 min with 2 μ Ci/ml [¹⁴C]acetate (56 μ Ci/ μ M). 50 μ Ci of [¹⁴C]acetate was added to 40 ml of cells which had not been treated with cycloheximide and allowed to incorporate for 10 min. The media was then made 50 mM acetate by the addition of 2 M non-radioactive acetate, and the cells quickly harvested by centrifugation for 2 min at 10 000 g. Half

of the cells were quick frozen in dry ice ethanol and the other half returned to complete media containing non-radioactive acetate and allowed to grow for an additional 90 min, then harvested and frozen as above. Protein samples were prepared and electrophoresed exactly as the samples used for the stained gels except that following destaining the gels were soaked for 20 min in Enlighting (New England Nuclear), dried onto filter paper under vacuum, and then exposed to film at -70°C .

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