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SHORT COMMUNICATION

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Cloning and characterization of the gene encoding an esterase from *Spirulina platensis*

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Abstract The gene encoding a 23 kDA serine esterase from the cyanobacterium *Spirulina platensis* has been identified, cloned, characterized and expressed in *Escherichia coli*. The primary structure of the esterase deduced from the DNA sequence displayed 32% sequence identity with the carboxylesterase (esterase II) encoded by *estB* of *Pseudomonas fluorescens*; the highest degree of homology is found in a stretch of 11 identical or highly conserved amino acid residues corresponding to the GXSXG consensus motif found in the catalytic site of many serine proteases, lipases and esterases.

Key words Microalgae · Cyanobacteria · Esterase
Gene cloning · DNA sequence

Spirulina sp. is a cyanobacterium used as a source of protein in human and animal nutrition and is also a promising solar energy converter in the fermentative conversion of biomass to fuel gas (Ciferri 1983; Ciferri and Tiboni 1985; Olguin 1986; Richmond 1986). The recent utilization of *Spirulina* in environmental decontamination (Mitchell and Richmond 1988; Gantar et al. 1991; Cañizares et al. 1993) has further broadened the spectrum of the possible biotechnological applications of this organism. Ultimately, the main factor limiting progress in this field is the scant knowledge of the genetics of *Spirulina*, since the absence of a known recombination system has so far prevented classical genetic analysis as well as the application of recombinant DNA technology to this organism. Until these problems have been experimentally solved, a useful way to proceed in the study of *S. platensis* is to characterize the largest possible number of its genes encoding functions which are either essential for the cell or potentially useful for biotechnological processes and eventually to align them on a physical map of its chromosome. Along these lines, we have recently started a program of shotgun cloning of DNA fragments of the *S. platensis* chromosome followed by the identification and characterization of genes based on: (a) their complementation of specific *Escherichia coli* mutations; (b) their sequence homology with already characterized genes from other prokaryotes; and (c) their capacity to express functions which are easily selectable or detectable. An example of the last type of approach is presented in this report. After shotgun cloning *S. platensis* DNA fragments in an expression vector, we screened for *E. coli* transformants positive for the production of esterase. This enzymatic activity was chosen because lipases and esterases are widespread in prokaryotes and several rare and unusual esters have been found in cyanobacteria (Richmond 1986). *S. platensis*, in particular, is characterized by the presence of abundant unsaturated fatty acids with two to three double bonds, such as the essential linoleic acid and γ-linolenic acid, esterified to glycerol derivatives (Richmond 1986).

Total chromosomal DNA extracted from *S. platensis* C1 (an axenic strain kindly provided by Dr. O. Tiboni, Pavia) was digested with *Bgl*II and 3-5 kb fragments isolated by electroelution were ligated into pPLc2833 (Remaut et al. 1981) linearized with *Bam*HI and dephosphorylated with alkaline phosphatase. Following transformation into *E. coli* HB101 carrying pcI857, recombinant clones were screened on agar plates containing L-broth and tributyrin (1,2,3-tributyrylglycerol; Kugimiya et al. 1986) with and without induction of the lambda promoter. A single colony forming a clear halo on a non-induced plate was isolated, restreaked and grown in liquid medium. The recombinant plasmid isolated from these cells revealed the presence of a 4.2 kb DNA insert with the restriction map shown in Fig. 1. When the esterase activity of total extracts from these tribu-

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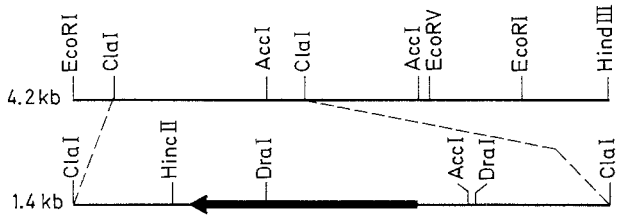
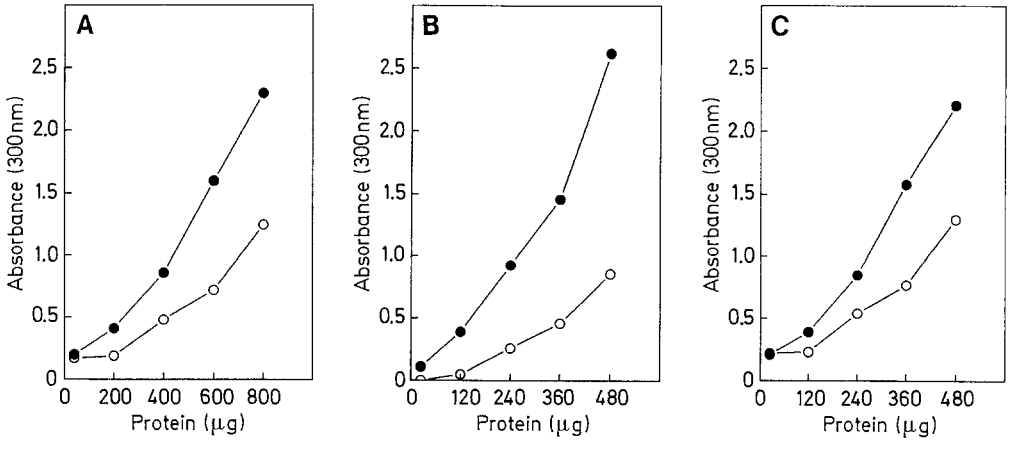


Fig. 1 Restriction map of the DNA fragment carrying the *Spirulina platensis* esterase gene. The upper row shows the relevant restriction sites present in the 4.2 kb *Eco*RI-*Hin*dIII fragment originally cloned in pPLc2388. The lower row presents the 1.4 kb *Cla*I fragment subcloned in pTZ18R. The *thick arrow* represents the gene coding for the esterase and points in the direction of transcription

tyrin-positive, insert-containing cells was measured, it was found to be substantially higher than that expressed by equivalent amounts of extracts prepared from control cells (Fig. 2A); the SDS-polyacrylamide (15%) electrophoretic analysis of the extracts of the cells carrying the 4.2 kb insert revealed that these extracts contained an additional band corresponding to a protein of approximately 23 kDa (not shown) not present in the control extracts prepared from the *E. coli* isogenic strain carrying the plasmid with no insert. Taken together, these results suggested that the 4.2 kb DNA fragment of *S. platensis* contains a gene encoding an esterase possibly corresponding to the 23 kDa protein; furthermore, since utilization of tributyrin, expression of the 23 kDa protein and high esterase activity were independent of the induction of the lambda P_L promoter, the results suggested that the *S. platensis* 4.2 kb insert also contains the promoter of the esterase gene or, at least, a promoter sequence recognized by the *E. coli* RNA polymerase. Upon subcloning different fragments of the 4.2 kb insert into pTZ18R and transformation into *E. coli* JM109, it was successively shown that the capacity to give rise to tributyrin-positive colonies, to produce the 23 kDa protein (not shown) and to express higher esterase activity than the controls is associated with the presence of the 3.5 kb *Eco*RI fragment (Fig. 2B) and the 1.4 kb *Cla*I fragment (Fig. 2C) derived from it (Fig. 1). These results indicated that the 23 kDa protein, the esterase activity and the promoter(s) necessary for their expression in *E. coli* are all encoded by the *Cla*I fragment; the small size of this fragment further suggested that the esterase activity is associated with the 23 kDa protein. The *Dra*I and *Dra*I-*Hin*cII fragments derived from the *Cla*I fragment (Fig. 1) were subcloned in pTZ18R and pTZ19R and subjected to DNA sequencing by the Sanger dideoxy chain-terminating method (Sanger et al. 1980); this allowed us to identify a 618 bp open reading frame (ORF) corresponding to a protein comprising 206 amino acids with M_r 23 kDa, beginning with an ATG initiation triplet and preceded by a potential 5'-AGGT-3' Shine-Dalgarno sequence (Fig. 3). Even though some sequences with potential homology to the *E. coli* Pribnow box (−10 element) are found upstream of the ORF, it is not clear at the moment where transcription begins. The primary structure of the protein deduced from the DNA sequence of the gene was identical, at least for the first 14 N-terminal amino acids, to that determined directly by automatic amino acid sequencing carried out on the electrophoretically resolved 23 kDa protein after blotting on Immobilon PVDF membranes (Millipore). Furthermore, the protein sequence displayed 32% identity with a carboxylesterase (esterase II) from *Pseudomonas fluorescens* (Hong et al. 1991). This is a carboxyl-esterase of 218 amino acids (23.8 kDa) encoded

Fig. 2 A–C Esterase activity of cell extracts derived from *Escherichia coli* harboring the expression vectors carrying the *S. platensis* esterase gene. *E. coli* cells grown to saturation in L broth were pelleted by centrifugation and ruptured by sonication. The total amount of protein present in each extract was determined (Bradford 1976) and equivalent amounts of cell extracts, normalized for their protein content, were used for the determination of esterase activity, essentially as described by Iwai et al. (1983). Each reaction mixture (1 ml) contained 0.9% NaCl, 5 mM methyl-5-acetylsalicylate and the indicated amounts of cell extracts. After 20 min incubation at 37° C, the methyl salicylate produced was determined from its absorbance at 300 nm. **A** *E. coli* HB101 transformed with pPLc1833 with (●) or without (○) the 4.2 *Eco*RI-*Hin*dIII insert. **B** *E. coli* JM109 transformed with pTZ18R with (●) or without (○) the 3.5 kb *Eco*RI fragment and **C** the 1.4 kb *Cla*I fragment



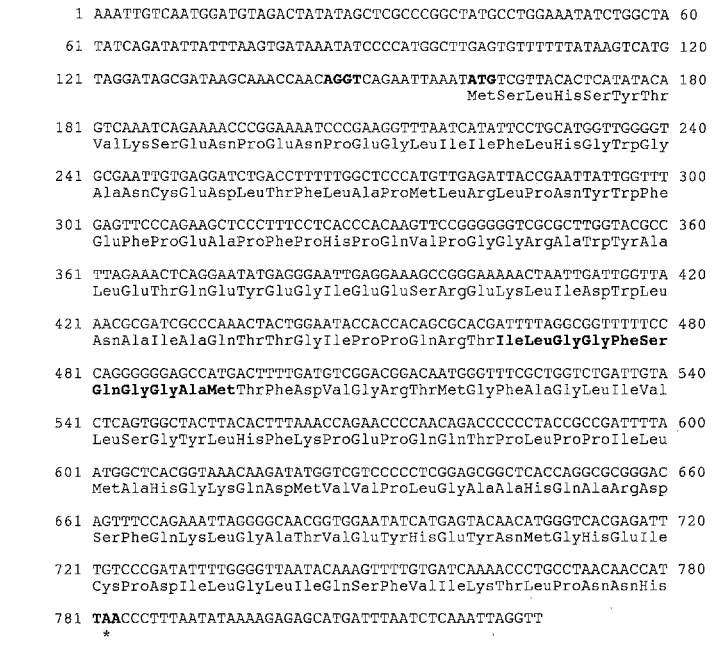


Fig. 3 DNA and deduced protein sequences of the *S. platensis* esterase gene. Two DNA fragments (*Dra*I and *Dra*I-*Hin*cII) were subcloned in pTZ18R and 19R and sequenced on both strands by the dideoxy chain-terminating method (Sanger et al. 1980). The presumed Shine-Dalgarno sequence, the initiation and the termination triplets are indicated by *bold letters*. The serine-containing peptide indicated in *bold letters* represents the presumed active site of the esterase. The N-terminal Ile and the C-terminal Met are conservative replacements, all other residues are identical to those found in *Pseudomonas fluorescens* carboxylesterase II

by the *estB* gene, which preferentially hydrolyzes phenylacetate and methyl and *p*-nitrophenyl esters of short fatty acids. The highest degree of homology between *S. platensis* and *P. fluorescens* esterases is found around the middle of the molecule and includes 11 identical or highly conserved amino acid residues (Fig. 3) including the GFSQG sequence which corresponds to the GXSXG consensus motif found in the catalytic site of many serine proteases, lipases and esterases (Brenner 1988; Oakeshott et al. 1987; Mickel et al. 1989). This finding strongly suggests also that the *S. platensis* esterase is a serine enzyme; further work is necessary to prove this point and to define more precisely the substrate specificity of the *S. platensis* esterase reported here.

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