A suicide vector for allelic recombination invoking the gene for glutamate 1-semialdehyde aminotransferase in the cyanobacterium Synechococcus PCC 7942

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A suicide vector for allelic recombination involving the gene for glutamate 1-semialdehyde aminotransferase in the cyanobacterium *Synechococcus* PCC 7942

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Abstract Gabaculine (2,3-dihydro 3-amino benzoic acid) is a potent inhibitor of tetrapyrrole biosynthesis in organisms that use the C_5 pathway for the synthesis of δ-aminolaevulinic acid. Glutamate semialdehyde aminotransferase (GSA-AT), the enzyme catalysing the formation of this key precursor of tetrapyrroles, is normally inhibited by concentrations of gabaculine in the order of 5 μM. However, in *Synechococcus* 6301 strain GR6, a cyanobacterium that is resistant to 100 μM gabaculine, this enzyme has undergone two changes in structure: a deletion of three amino acids from positions 5 to 7 and the substitution of isoleucine for methionine at position 248. To establish the effect in vivo of these specific changes in the gene for GSA-AT (*hemL*), a suicide vector (pHS7) containing an antibiotic cassette was constructed to achieve the replacement, by homologous recombination, of the wild-type *hemL* gene in the chromosome by a modified form of the gene. Recombinant strains of *Synechococcus* 7942 obtained using pHS7-*hemL*^{GR6} were indistinguishable from *Synechococcus* 6301 GR6 in terms of the resistance of growth and of chlorophyll accumulation to high concentrations of gabaculine, while a wild-type recombinant produced using pHS7-*hemL*^{WT} had retained its sensitivity. Southern hybridisation using gene probes for *hemL*, *amp*^{r} and *cm*^{r} confirmed that chromosomal integration of the plasmids had occurred in both WT and GR6 recombinants. Growth and chlorophyll accumulation in equivalent strains with the *hemL* gene containing either the deletion or the transition characteristic of *Synechococcus* 6301 GR6 were inhibited by 10 μM gabaculine. Consequently, resistance in vivo to high concentrations of this compound is dependent on both the changes in gene/enzyme structure. This investigation has established the effectiveness of the suicide vector pHS7 for studying the effect in vivo of specific changes in the *hemL* gene. It has also demonstrated that replacement of the wild-type gene by that from *Synechococcus* 6301 GR6 is sufficient to confer resistance in vivo to high concentrations of gabaculine.

Key words δ-Aminolaevulinic acid · Glutamate 1-semi-aldehyde aminotransferase · Suicide vector · Gabaculine resistance · Allelic recombination

Introduction

δ-Aminolaevulinic acid (ALA) is the first intermediate committed to the biosynthesis of tetrapyrroles. In higher plants (Wang et al. 1981), eukaryotic algae (Wang et al. 1984; Weinstein et al. 1987; Houghton et al. 1989), cyanobacteria (O'Neill et al. 1988; Bull et al. 1989a) and several other prokaryotes (Friedmann et al. 1987; Avissar et al. 1989; Li et al. 1989; O'Neill et al. 1989; Hansson et al. 1991), it is made from glutamyl-tRNA^{Glu} by the C_5 pathway. The final step in this sequence is catalysed by glutamate 1-semialdehyde (GSA) aminotransferase (Kannangara and Gough 1978; Wang et al. 1981) and involves the rearrangement of keto and amino functions in the substrate.

Various observations indicate that this enzyme contains vitamin B_6 as an essential cofactor (Bull et al. 1989a, 1990; Grimm et al. 1991b; Nair et al. 1991). Studies of the mechanism of the reaction catalysed by the enzyme from the cyanobacterium *Synechococcus* 6301 (Smith et al. 1991) and from pea plants (Pugh et al. 1992) suggest that bound cofactor cycles between the pyridoxamine phosphate (PMP) and pyridoxal phosphate (PLP) forms during the conversion of GSA to ALA via an intermediate equivalent to diaminovaleric acid (DAVA). All GSA aminotransferases investigated are very sensitive to 3-amino 2,3-dihydrobenzoic acid (gabaculine) (Kahn and Kannangara 1987; Hoober et al.

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1988; Grimm et al. 1991b; Reible and Beale 1991). This is thought to interact with enzyme in which the cofactor is in the PLP form to produce a stable inhibitor-cofactor derivative as has been reported for γ-aminobutyric acid aminotransferase (Rando 1977). The sensitivity of GSA aminotransferase to gabaculine accounts for the effect of this compound at concentrations greater than 20 μM on growing cultures of *Synechococcus* 6301: a rapid, complete inhibition of chlorophyll accumulation and the eventual inhibition of growth (Hoult et al. 1986).

We have previously isolated a strain of *Synechococcus* 6301 (Bull et al. 1989b) that will accumulate chlorophyll and grow in the presence of gabaculine at concentrations greater than 100 μM. The gene for GSA aminotransferase (*hemL*) has been cloned from this strain (Grimm et al. 1991b) and its sequence compared with that from the wild-type cyanobacterium (Grimm et al. 1991a). The *hemL* gene from the resistant strain contains two alterations: a base transition at position 743, resulting in the replacement of methionine 248 by isoleucine, and a deletion of nine nucleotides from positions 12 to 20 that eliminates the amino acid sequence Ser-Pro-Phe from positions 5 to 7. As preliminary, in vitro studies by other workers (Smith and Grimm 1992) have suggested that the amino acid substitution alone confers significant resistance on the enzyme, the role, if any, of the accompanying deletion remains uncertain. That it has a significant effect is implied by our observation that other isolates of *Synechococcus* 6301 with high tolerance to gabaculine possess not only the internal base transition but also a deletion of 9 or 12 nucleotides close to the 5′ end of the gene (unpublished results).

To investigate the effect of these and other changes in gene/enzyme structure in vivo, a suicide vector was constructed to achieve the replacement of the GSA aminotransferase gene (*hemL*) in the chromosome of a cyanobacterium with engineered variants by homologous recombination. The strain selected for this investigation, *Synechococcus* 7942, is closely related to *Synechococcus* 6301 (Golden et al. 1989) except that it is readily transformable (Porter 1985). The strategy used in the present investigation depended on a suicide vector containing an antibiotic cassette, a *hemL* insertion site, and upstream and downstream sequences that flank the *hemL* gene in *Synechococcus* 7942.

Materials and methods

Microorganisms and plasmids

Synechococcus PCC 6301 was obtained originally from M. M. Allen (Biological Sciences, Wellesley College, Middletown, Mass., USA) and strain 7942 from S. Shestakov (Department of Genetics, Moscow State University, Moscow, Russia). Both strains were grown photoautotrophically in medium BG-11 as described previously (Hoult et al. 1986). When required, medium was solidified using 1.5% (w/v) agar; inhibitor and antibiotics were sterilised by filtration and added to sterile medium. *Escherichia coli* DH5α [*supE44ΔlacU169* (*ϕ* 80*lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was obtained from Bethesda Research Laboratories. The plasmids pBR322 and pACYC184 were obtained from New England Biolabs and pBluescript SK⁻ from Stratagene.

Molecular biology techniques

Protocols for DNA manipulation were taken from Sambrook et al. (1989) or were those recommended by the manufacturers. Digests of genomic DNA (10 μg) were fractionated by agarose gel electrophoresis, and transferred to charged nylon Zeta-Probe GT membranes (Bio-rad). They were then probed by Southern hybridisation with nick-translated [α-^{32}P]dCTP-labelled DNA produced from the coding region of the *hemL* gene, cloned as described by Grimm et al. (1991a), to locate fragments containing the *hemL* gene. Libraries of size-fractionated cyanobacterial genomic DNA were constructed in λZAPII with in vitro packaging using Gigapack II and transfection using the standard protocol (Stratagene). After amplification overnight, plaques were screened by hybridisation with the *hemL* gene probe. Gene probes used to locate specific genes in digests of genomic DNA from recombinant strains by Southern hybridisation were prepared in a similar manner. These included probes for the ampicillin resistance gene (*amp^r*) obtained in a *Pst*I-*Cla*I fragment from pBR322, for the chloramphenicol resistance gene (*cm^r*) obtained in an *Ava*I-*Eco*RI fragment from pACYC184 and for the *hemL* gene obtained in a *Not*I fragment from pHS7-*hemL*^{WT}.

RNA for Northern hybridisation was extracted from organisms obtained from 25 ml of a cyanobacterial culture in log phase. Pelleted cells were lysed at 65° C by suspension in 0.5 ml of 200 mM sodium acetate, pH 5.2, 10 mM EDTA, 1% (w/v) SDS. Phenol that had been pre-equilibrated with 50 mM sodium acetate buffer, pH 5.6 (0.5 ml) was added to the cell lysate at the same temperature. After mixing and incubating for 5 min at 65° C, the sample was mixed with 0.5 ml of chloroform/iso-amyl alcohol (24/l, v/v) and the incubation continued for 10 min. Samples were cooled to room temperature and centrifuged for 2 min to separate the phases. The aqueous layer was re-extracted with acid phenol followed by chloroform/iso-amyl alcohol. RNA in the aqueous layer was precipitated overnight at 4° C after adding 0.2 vol. 12 M LiCl. RNA was recovered by centrifugation, washed two times with 90% ethanol, air dried and taken up in 50 μl of diethyl pyrocarbonate (DEPC)-treated water. RNA was size-fractionated by electrophoresis in 0.6 M formaldehyde denaturing 0.6% (w/v) agarose gels and transferred to Zeta-Probe GT nylon membrane (Bio-Rad). RNA on these membranes was hybridised with the appropriate labelled probe at 48° C in buffer containing 50% (w/v) formamide.

Plasmids were isolated routinely by the alkaline lysis method. Specific DNA fragments produced by restriction endonuclease digestion were isolated after agarose gel electrophoresis using Sephaglas (Pharmacia). The sequence of the *Not*I oligonucleotide inserted in the *Eco*RI site of pBR322 was 5′AAGCGGCCGCTT3′ and those of the *Not*I primers for amplification of *hemL* from the wild-type (WT) and GR6 strains of *Synechococcus* 6301 by the polymerase chain reaction (PCR) were 5′ATAAGAATGCGGC CGCTAGCGGTTGTGTTGCAACAGTCCC3′ (NotS1, which included an upstream segment identical to nucleotides 620 to 644 of the published sequence) and 5′ATAAGAATGCGGCCGCAATT CCCAGCAGAATCAAGCCGAT3′ (NotX1, with a downstream segment complementary to nucleotides 2148 to 2168 of the published sequence) (Grimm et al. 1991a). These were obtained from Genosys. The products of amplification were treated with *Not*I prior to ligation into *Not*I-cleaved pHS7. Plasmid constructs containing one or other of the two changes characteristic of *hemL*^{GR6} were obtained by specific endonuclease digestion of pHS7-*hemL*^{WT} and *hemL*^{GR6}, separation of the fragments and ligation of these in appropriate combinations to form two appropriate hybrid vectors: pHS7 *hemL*^{NTD} containing only the 5′deletion and pHS7 *hemL*^{MTI} containing only the base transition. The identity of these was confirmed by sequencing the relevant segment of the gene by the dideoxy chain termination method.

Transformation of *Synechococcus* 7942 was based on standard procedures (Golden and Sherman 1984). Organisms in the log

phase of photoautotrophic growth were resuspended to a density of 3×10^8 cells/ml in medium BG-11; 25 ml of this suspension was centrifuged for 5 min at 4066 *g* and 22° C, the cells washed and finally resuspended in 900 µl of BG-11. The cells were incubated with 15 µg of plasmid DNA, incubated in the dark at 30° C for 16 h, and spread on plates of BG-11 agar. After incubation in the light for 24 h at 30° C, 0.5 ml of antibiotic solution (ampicillin, 30 µg/ml or chloramphenicol, 750 µg/ml) was added under the agar and the plates incubated in the light until distinct colonies were visible. Recombinants were selected on the basis of resistance to ampicillin and chloramphenicol and grown routinely in BG-11 medium containing ampicillin and chloramphenicol at final concentrations of 0.5 µg/ml and 5 µg/ml, respectively.

Other methods

The effect of gabaculine on the photoautotrophic growth of cyanobacteria was monitored in terms of change in culture density and of chlorophyll accumulation over 30 h. Organisms were grown as described previously (Allen and Smith 1969) and culture density was determined turbidimetrically using a Klett-Summerson colorimeter fitted with a glass filter transmitting light of wavelength greater than 730 nm (Melles Griot). Chlorophyll a was extracted from samples of cell material using acetone and estimated spectrophotometrically as described in Lichtenthaler and Welburn (1983).

Results

Location and expression of the *hemL* gene in *Synechococcus* 7942

The *hemL* gene was located in fragments of 8 or 2 kb from genomic DNA of *Synechococcus* 7942 digested respectively with either *Xba*I or *Eco*RI by Southern hybridisation with a nick-translated probe encoding the gene for the aminotransferase from *Synechococcus* 6301. A sub-library of size-fractionated fragments was prepared using λZAPII and screened to identify *hemL* clones using the same gene probe. Restriction analysis of a cloned *Xba*I fragment containing *hemL* (Fig. 1A) showed it to be very similar though not identical to the corresponding fragment isolated from *Synechococcus* 6301 previously reported to be 7.6 kb (Grimm et al. 1991a). The fragments from both strains contained specific *Eco*RI sites on either side of *hemL*, as expected from the isolation of the gene in a smaller *Eco*RI fragment cloned from *Synechococcus* 7942 in the present investigation that was equivalent to the full-length gene fragment used to sequence the *hemL* gene in *Synechococcus* 6301 (Grimm et al. 1991a).

In species of *Bacillus* (Hansson et al. 1991), the *hemL* gene is in an operon together with several others that encode enzymes involved in the early stages of tetrapyrrole biosynthesis. The possibility that the *hemL* gene in cyanobacteria is in a similar grouping of functionally related genes was investigated by Northern blot analysis of total RNA from both strains of cyanobacteria to determine the size of *hemL* mRNA. The gene probe hybridised with RNA from *Synechococcus* 7942 of approximate size 1.9 kb, indicating that the RNA containing the transcript of the *hemL* gene is monocistronic (Fig. 1B). This is consistent with the observation that

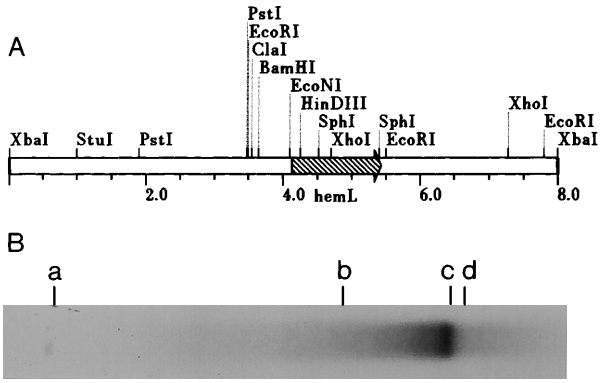

Fig. 1A, B Location of the *hemL* gene in *Synechococcus* 7942. **A** Restriction map of the approximately 8 kb *Xba*I fragment containing *hemL*. The *shaded region* shows the location of the *hemL* gene. **B** Northern blot of RNA. Total RNA was size-fractionated by agarose gel electrophoresis in the presence of formaldehyde, blotted onto a nylon membrane and hybridised with the *hemL* probe. *a* Sample well, *b* 3.6 kb standard, *c hemL* transcript, *d* 1.8 kb standard. The standards were the large and small rRNAs from *Escherichia coli*

the *Xba*I fragment containing the *hemL* gene does not hybridise with a probe for *hemB*, the gene for ALA dehydratase, the enzyme that catalyses the next step in tetrapyrrole biosynthesis (unpublished results). In view of this it seemed likely that the insertion of antibiotic resistance genes on either side of *hemL* would not disrupt its expression in *Synechococcus* 7942.

Construction of the suicide vector pHS7

An insertion cassette was constructed containing the Tn*3* ampicillin resistance gene (*amp*^{r}) from pBR322 and the Tn*9* chloramphenicol resistance gene (*cm*^{r}) from pACYC184, separated on one side by a synthetic *Not*I site to accommodate the *hemL* gene and on the other by DNA from *Synechococcus* 7942 immediately upstream and downstream relative to the *hemL* gene. A 12 bp DNA sequence containing a *Not*I site was ligated into the end-filled *Eco*RI site of pBR322 to give pBR_{NOT}. This plasmid was cut with *Ava*I, end-repaired and then cut with *Cla*I to eliminate the gene for tetracycline resistance. The other plasmid, pACYC184, was cut with *Eco*NI, end repaired and then cut with *Cla*I to remove part of the tetracycline resistance gene. The larger restriction product was recovered from the digest of each plasmid and these were ligated. Transformants of *E. coli* DH5α were selected on the basis of resistance to ampicillin and chloramphenicol. One of these yielded plasmid pBA (6.6 kb), which contained the *Not*I site, the two antibiotic resistance genes *amp*^{r} and *cm*^{r}, and two origins of replication: a ColE1 origin from pBR322 and a p15A origin from pACYC184. The latter was removed by digesting pBA with *Nhe*I and *Xba*I and ligating the compatible cohesive ends to yield pΔBA (5.8 kb), containing the antibiotic cassette with the *Not*I site for the insertion of the *hem*L gene. In the remaining stages of vector construction, segments of DNA flanking the

hemL gene in *Synechococcus* 7942 were inserted into the vector. A small segment of upstream DNA (approximately 0.5 kb) was obtained from the 2 kb *Eco*RI fragment containing *hemL* (Fig. 1A). This fragment was self-ligated, cut with *Eco*NI, end-repaired and blunt-ligated into pΔBA cut with *Ava*I. The product was end-repaired and ligated to give pΔBAE (approximately 7.8 kb). The *hemL* gene from *Synechococcus* 7942, the *rop* gene from pBR322 and the remaining segment of the tetracycline resistance gene originating from pACYC184 were deleted from pΔBAE by digestion with *Nde*I, end-repaired and digested with *Bam*HI. The larger fragment contained the antibiotic resistance cassette, the *Not*I site, the ColE1 *ori* and the small segment of DNA that was immediately upstream of the *hemL* gene in *Synechococcus* 7942. Additional cyanobacterial DNA upstream and downstream relative to the *hemL* gene was obtained from the 8 kb *Xba*I fragment containing the *hemL* gene from *Synechococcus* 7942 (Fig. 1A). This fragment was self-ligated, cut with *Bam*HI and ligated into the *Bam*HI site of pBluescript SK$^-$. The *hemL* gene and all of the vector DNA were deleted from this construct by partial digestion with *Eco*RI to cleave at the site immediately downstream of *hemL*. The appropriate full-length product of digestion was end-repaired and cut with *Bam*HI to give a fragment containing upstream (approximately 3.6 kb) and downstream (approximately 2.5 kb) cyanobacterial DNA. Ligation of this with the large fragment obtained from pΔBAE by digestion with *Nde*I and *Bam*HI gave pHS7 (approximately 10.7 kb) in which the two antibiotic resistance genes, *amp*^{r} and *cm*^{r}, are separated on one side by segments of *Synechococcus* 7942 DNA upstream and downstream relative to *hemL* and on the other side by the *Not*I cloning site (Fig. 2). DNA containing the required form of the *hemL* gene, wild type or inhibitor tolerant (GR6), was prepared from the appropriate strain of *Synechococcus* 6301 by PCR using two primers containing *Not*I sites near their 5′ ends: these primers were homologous to DNA just upstream (NotS1) and downstream (NotX1) of *hemL* in *Synechococcus* 6301. After cleavage with *Not*I the PCR product was ligated into the *Not*I site of pHS7. Transformants of *E. coli* containing pHS7-*hemL*^{WT} and pHS7-*hemL*^{GR6} constructs were selected on the basis of resistance to ampicillin and chloramphenicol.

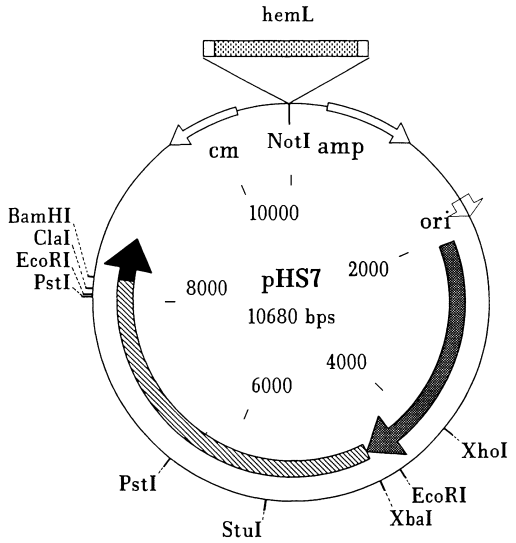

Fig. 2 Map of the suicide vector pHS7 constructed for allelic recombination in *Synechococcus* 7942 involving *hemL*. The *partial inner circle* identifies the segments of DNA from *Synechococcus* 7942 that are upstream (◀ and ▨) and downstream (◀) of *hemL*. The smaller segment of upstream DNA was obtained from the *hemL Eco*RI fragment and the larger upstream and also the downstream segment were obtained from the *hemL Xba*I fragment (Fig. 1A). The DNA segment containing the *hemL* gene (▩) was inserted at the *Not*I site

Use of the pHS7 construct for *hemL* gene replacement in Synechococcus 7942

Organisms from log phase, photoautrophic cultures of *Synechococcus* 7942 were transformed with suicide vector containing either the 6301 WT or the 6301 GR6 *hemL* gene and recombinants recovered that were resistant to ampicillin and chloramphenicol at concentrations that inhibited the growth of *Synechococcus* 6301 and 7942. One wild-type and five GR6 recombinants were selected for comparison with wild-type *Synechococcus* 6301 and 7942, and *Synechococcus* 6301 GR6 as control strains for effect of gabaculine on growth and on chlorophyll accumulation in cultures.

The effect of gabaculine (80 μM) on the wild-type recombinant was essentially the same as for both wild-type strains of cyanobacteria: compared with inhibitor-free controls, the growth rate in the presence of gabaculine over 30 h was much lower (Fig. 3A). In contrast, all of the GR6 transformants grew in the presence of this concentration of gabaculine and the increase in culture density was comparable to that in the inhibitor-free controls (Fig. 3B).

As the inhibitory effect of gabaculine on the growth of sensitive strains develops with time and is only complete after exposure for more than 30 h, a better indicator of the effect of inhibitor is the amount of chlorophyll accumulated during this period. Following the addition of inhibitor (80 μM), there was little if any increase in the amount of chlorophyll in cultures of wild-type strains of *Synechococcus* 6301 and 7942 or in the wild-type recombinant (Fig. 4). In contrast, pigment accumulation in the GR6 recombinants in the presence of 80 μM gabaculine was 75–105% of that in inhibitor-free cultures. The GR6 recombinants were indistinguishable in this respect from *Synechococcus* 6301 GR6. Similar growth experiments established that chlorophyll accumulation and growth of one of the GR6 recombinants was unaffected by gabaculine at 200 μM (data not shown). This demonstrated that the effect of gabaculine on recombinants was determined by the form of the *hemL* gene used to replace that in the chromosome of the wild-type cyanobacterium.

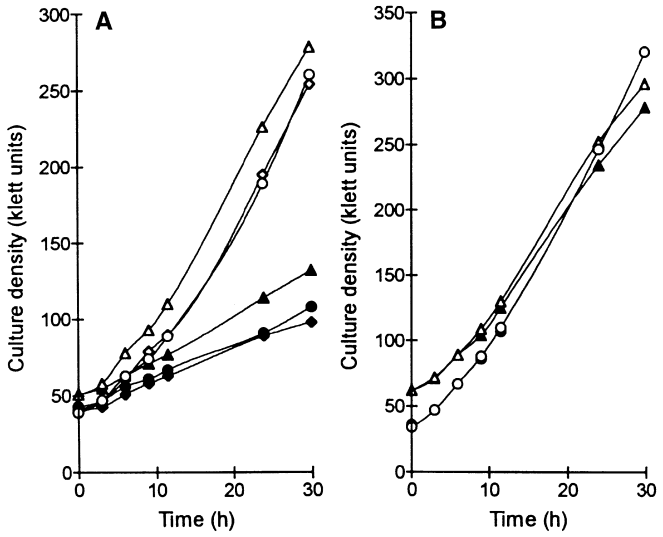

Fig. 3A, B Effect of gabaculine on the growth of *Synechococcus* 6301 and 7942 wild-type (WT) strains and of *Synechococcus* 7942 recombinants. **A** *Synechococcus* 6301 WT (○, ●) and 7942 WT (Δ, ▲) and 7942 WT recombinant (◊, ◆) and **B** *Synechococcus* 6301 GR6 (Δ, ▲) and 7942 GR6 recombinant 3 (○, ●) were grown photoautotrophically and the culture density monitored turbidimetrically. The other four GR6 recombinants gave essentially the same growth curves as GR6 recombinant 3. *Open symbols* represent gabaculine-free cultures and *full symbols* cultures to which inhibitor was added at time zero at a final concentration of 80 μM

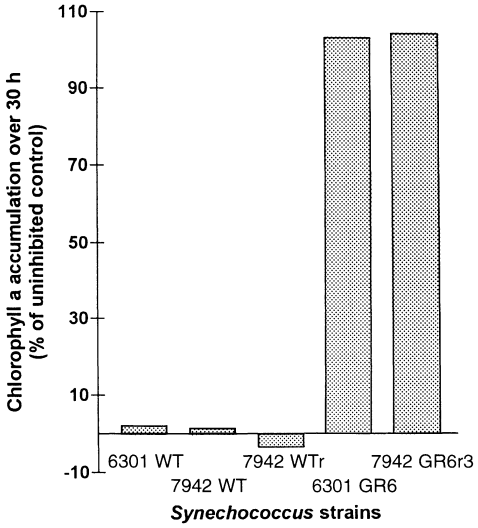

Fig. 4 Effect of gabaculine on the accumulation of chlorophyll a by *Synechococcus* 6301 and 7942, and by 7942 recombinants. Chlorophyll a accumulation over 30 h in the photoautotrophic cultures in the presence of gabaculine (80 μM) is expressed as a percentage of the accumulation in inhibitor-free cultures of the same strain. The amounts of chlorophyll a that accumulated in the inhibitor-free cultures of various *Synechococcus* strains over 30 h were (μg/ml of culture): 15.5 (6301 WT), 12.7 (7942 WT), 15.0 (7942 WT recombinant), 19.9 (6301 GR6) and 12.3 (7942 GR6 recombinant 3). The other GR6 recombinants gave essentially the same results

Confirmation of genomic recombination mediated by pHS7-*hemL* constructs by Southern hybridisation

Genomic DNA from the wild-type strain of *Synechococcus* 7942 and two antibiotic-resistant recombinants, obtained by transformation of this cyanobacterium with the suicide vector containing the *hemL* gene from either wild-type or GR6 strains of *Synechococcus* 6301, was digested with *Xba*I and *Not*I. After separation of the fragments in the digests by agarose gel electrophoresis and transfer to a charged nylon membrane, the blots were screened by Southern hybridisation (Fig. 5) using three different gene probes: the ampicillin resistance gene (*amp*^{r}) from pBR322, the chloramphenicol resistance gene (*cm*^{r}) from pACYC184 and the *hemL* gene from pHS7-*hemL*^{WT}. The probes for the two antibiotic resistance genes did not hybridise with DNA from the natural, wild-type strain of *Synechococcus* 7942, while they gave clear signals with DNA from both recombinants (Fig.5A, B); the *amp*^{r} and *cm*^{r} probes hybridised with fragments of 4.5 and 5.8 kb, respectively, which are in close agreement with the sizes predicted for these fragments (4.6 and 6.1 kb). The *hemL* probe gave the expected band at 8.0 kb with DNA from the wild-type cyanobacterium (Fig. 5C, lane 3). With DNA from both of the *Synechococcus* 7942 recombinants, the *hemL* gene probe hybridised with fragments of two different sizes (Fig. 5C, lanes 1, 2). The major band seen at 1.6 kb for both recombinants was close in size to that of the *hemL* segment (1.4 kb) inserted into the *Not*I site in the vector cassette. The minor bands at 4.5 and 5.8 kb were of the same size as those that hybridised with the probes for the antibiotic resistance genes. There are two alternative explanations for these additional bands. Small differences were found in the order of restriction endonuclease sites on either side of the *hemL* gene in the two *Synechococcus* strains. A consequence of this could be

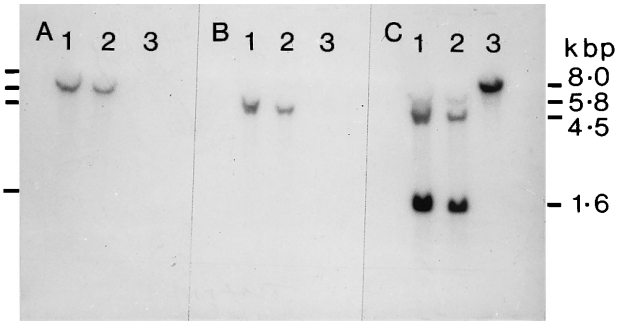

Fig. 5 Southern blots of genomic DNA from *Synechococcus* 7942 WT and from WT and GR6 recombinants. Genomic DNA was digested with *Not*I and *Xba*I and hybridised with probes for: **A** *cm*^{r}, **B** *amp*^{r} and **C** *hemL* genes. The three lanes in each group are for digests of genomic DNA from: 1 *Synechococcus* 7942 WT recombinant; 2 *Synechococcus* 7942 GR6 recombinant and 3 *Synechococcus* 7942 WT. The sizes in kilobases of the DNA fragments that hybridised with the various probes were calculated from the position in the original agarose gel of standard fragments obtained from λDNA by digestion with *Hind*III

that sequences in strain 6301 that are in the segment between the *Not*I sites introduced during *hemL* amplification are outside this segment in strain 7942. Consequently, the *hemL* gene probe would hybridise with the fragments of genomic DNA containing the amp^r and cm^r genes as well as *hemL*. However, the possibility that these additional bands were due to hybridisation with probe derived from template containing traces of undigested plasmid cannot be excluded.

Effect in vivo of the separation of the changes in the *hemL* gene in strain GR6

Gabaculine resistance in vitro has been attributed to the substitution of isoleucine for methionine at position 248 (Smith and Grimm 1992). Nevertheless, the deletion near the 5′ end of the gene also appears to be of importance because we have found it in independent isolates of *Synechococcus* 6301 with high resistance to gabaculine; in one of these (DC80), the deletion was exactly the same as in strain GR6 (positions 12–20) while in another (DC40) it was three nucleotides longer (positions) (9–20) unpublished results). To resolve the matter, the suicide vector was used to establish the inhibitor sensitivity of recombinant strains in which the *hemL* gene contained only one of the two changes present in strain GR6. The appropriate constructs were made by digesting pHS7-$hemL^{WT}$ and pHS7-$hemL^{GR6}$ with *Hin*dIII and *Xba*I. The digestion products were separated and ligated in the appropriate combinations to give pHS7-$hemL^{NTD}$ containing the nine nucleotide 5′ deletion and pHS7-$hemL^{MTI}$ containing the base transition at position 743. These were used to transform *Synechococcus* 7942 to give recombinants resistant to ampicillin and chloramphenicol. One recombinant of each type was characterised in terms of its resistance to gabaculine. Neither the NTD nor the MTI recombinant was resistant to 80 μM gabaculine as measured by increase in culture density (Fig. 6) or chlorophyll accumulation (Table 1) over 30 h. The minimum concentration of gabaculine that gave the decrease in growth rate observed at high concentrations of inhibitor was determined for NTD, MTI and WT recombinant strains: it was approx. 10 μM for the NTD and the MTI recombinants compared with approximately 2.5 μM for the *Synechococcus* 7942 WT recombinant. The minimum concentration that had the same effect on *Synechococcus* 6301 WT was approximately 20 μM (data not given). A comparison of the chlorophyll content of these cultures demonstrated that the minimum concentration of inhibitor that decreased pigment accumulation relative to

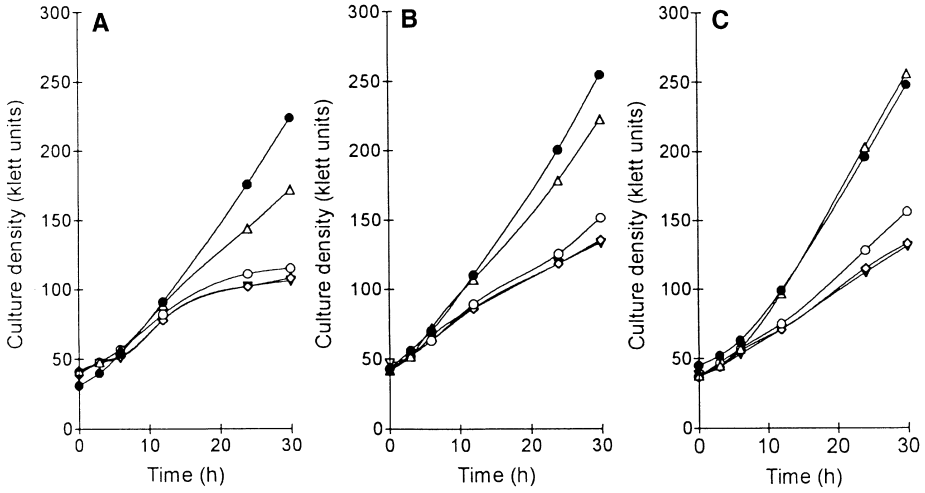

Fig. 6A–C Response to gabaculine of the growth of recombinant strains of *Synechococcus* 7492. Recombinant strains of *Synechococcus* 7942 were grown photoautotropically from time zero in the presence of various concentrations of gabaculine and the density of cultures monitored turbidimetrically: **A** the WT recombinant at final concentrations of 0 (●), 1.25 (Δ), 2.5 (○), 3.75 (◊) and 5.0 μM gabaculine (∇), and **B** the NTD (nine nucleotide deletion) recombinant, and **C** the MTI (base transition) recombinant, both at final concentrations of 0 (●), 5 (Δ), 10 (○), 15.0 (◊) and 20.0 μM gabaculine (∇)

Table 1 Response of chlorophyll a accumulation to gabaculine in recombinant strains of *Synechococcus* 7942. Chlorophyll a accumalation over 30 h in the photoautotrophic culures of recombinants described in Fig. 6 is expressed as a percentage of the accumulation in inhibitor-free cultures of the same strains. The amounts of chlorophyll a that accumulated in the inhibitor-free cultures of the recombinant strains of *Synechococcus* 7942 over 30 h were (μg/ml of culture): 11.0 for the wild-type recombinant (WTr), 9.2 for the MTI recombinant (MTIr) and 10.0 for the NTD recombinant (NTDr)

WTr		NTDr		MTIr	
gabaculine (μM)	chlorophyll a (% control)	gabaculine (μM)	chlorophyll a (% control)	gabaculine (μM)	chlorophyll a (% control)
1.25	40.1	5	109.5	5	102
2.50	1.7	10	51.3	10	108
3.75	1.3	15	13.5	15	82.9
5.00	1.3	20	5.2	20	26.5

that of inhibitor-free cultures by at least 50% was 15 to 20 μM, approximately 10 μM, and less than 1.25 μM for the MTI, NTD and WT recombinant strains respectively (Table 1). These observations indicated that the NTD and the MTI recombinants were both marginally more tolerant to gabaculine than the corresponding WT recombinant, but neither displayed resistance that was significant compared with that shown by the GR6 recombinants in which the *hemL* gene contained the 5′ deletion and the internal transition.

Discussion

As the vector pHS7 lacks an origin that is recognised by the replication system in the cyanobacterium, its genes and any gene insert it contains cannot be maintained within the cell by autonomous replication. The insertion into the vector of segments of DNA flanking the *hemL* gene in *Synechococcus* 7942 was designed to achieve homologous recombination with the cyanobacterial genome, resulting in the displacement of the wild-type gene in the chromosome of the cyanobacterium by the two antibiotic resistance genes and the particular form of *hemL* placed between them at the *Not*I site. This was confirmed by Southern hybridisation of restriction digests of genomic DNA. With the exception of the two additional minor bands in the Southern blots of recombinant DNA that hybridised with the *hemL* gene probe, the results were very similar to those expected. The suicide vector should, therefore, be a suitable vehicle for replacing the *hemL* gene in the natural wild-type strain of *Synechococcus* 7942 with other forms of the gene. This was demonstrated by the successful isolation of several ampicillin/chloramphenicol-resistant recombinants with phenotypes determined by the form of the *hemL* involved: transformation with pHS7-*hemL*^{GR6} yielded several recombinants all of which were resistant to high concentrations of gabaculine while a recombinant obtained by transformation with pHS7-*hemL*^{WT} was sensitive to very low concentrations of the inhibitor.

Since it had been demonstrated that the pHS7 vector system achieves the replacement of the wild-type gene in the chromosome of *Synechococcus* 7942 with an altered version of *hemL,* this vector was used to investigate the effect, in vivo, of each of the two changes in the *hemL* gene in *Synechococcus* GR6 on the sensitivity of organisms to gabaculine when introduced separately into the gene. Neither change alone was sufficient to confer resistance to 80 μM gabaculine on recombinants. Clearly, both changes are necessary for the cyanobacterium to express the phenotype of resistance to high concentrations of inhibitor. These in vivo effects are wholly consistent with the properties of GSA aminotransferase revealed by specific cleavage of the product of expression of a *malE*/*hemL* fusion in *E. coli*. Enzyme containing one or other of the two changes present in the enzyme in the GR6 mutant of *Synechococcus* 6301 does not show significant resistance to gabaculine in vitro; both changes have to be present for the enzyme to exhibit resistance (unpublished data). The contribution that the replacement of Met^{248} by isoleucine makes to the resistance of the GSA aminotransferase in strain GR6 to high concentrations of gabaculine may be due to its likely proximity to the active site of the enzyme (Smith and Grimm 1992). An explanation for the effect of the N-terminal deletion on the interaction of the enzyme with inhibitor can be found in the roles of the N-terminal domain of the related enzyme, aspartate aminotransferase of porcine cytosol (Fukumoto et al. 1991). This domain not only interacts with the neighbouring subunit in this dimeric enzyme but is also involved in direct contacts with its substrate, aspartate, through an induced-fit conformational change. If the N-terminal domain of GSA aminotransferase has similar roles in enzyme stability and active site contacts, a change in structure such as the deletion in the GR6 enzyme is likely to have an effect on its interactions with substrate- and mechanism-based inhibitors such as gabaculine.

These data contrast with the preliminary observation of Smith and Grimm (1992) that the properties of a GSA aminotransferase in which isoleucine replaces methionine at position 248, produced using an *E. coli* expression system that adds four amino acids at the N-terminal end of the enzyme, indicate that the amino acid substitution confers resistance to inhibitor. The suicide vector pHS7 will be used, in conjunction with the gene-fusion expression system, to resolve this contradiction as part of a wider investigation of the effect, in vivo and in vitro, of specific changes in gene/enzyme structure. These will include changes achieved by site-directed mutagenesis as well as others that are the result of spontaneous mutations that confer resistance to inhibitors other than gabaculine.

Several observations made in vivo and in vitro have suggested, but have not proved, that GSA aminotransferase is the prime target site for gabaculine in the cyanobacterium and that the inhibition of tetrapyrrole biosynthesis leads ultimately to the inhibition of growth. These include the complete inhibition of chlorophyll accumulation in intact cells that have been exposed to gabaculine at concentrations greater than 20 μM (Hoult et al. 1986), and the sensitivity of GSA aminotransferase in wild-type *Synechococcus* 6301 to low concentrations of gabaculine compared with the lack of an effect on the activity of the enzyme from strain GR6 at inhibitor concentrations greater than 100 μM (Grimm et al. 1991b). However, the possibility cannot be excluded that the use of a continuous culture to obtain inhibitor-resistant strains (Bull et al. 1989b) might select changes in other genes as well as in the *hemL* gene. In the present investigation, the resistance of GR6 recombinants to gabaculine at the high concentrations tolerated by the original GR6 strain confirms that replacement of the wild-type aminotransferase with an inhibitor-resistant version of the enzyme is all that is necessary to confer resistance to high concentrations of inhibitor on the cyanobacterium.

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