Cloning and Oxygen-regulated Expression of the Bacteriochlorophyll Biosynthesis Genes \textit{bch} E, B, A and C of \textit{Rhodobacter sphaeroides}

By C. NEIL HUNTER* AND SHIRLEY A. COOMBER

Department of Pure and Applied Biology, Imperial College of Science and Technology, London SW7 2BB, UK

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Four mutants of the photosynthetic bacterium \textit{Rhodobacter sphaeroides} were isolated which were incapable of photosynthetic growth due to inability to synthesize bacteriochlorophyll. A \textit{Rb. sphaeroides} gene bank was constructed in the mobilizable vector pSUP202 and was transferred into these mutants using the helper plasmid pRK2073. Three clones that produced photosynthetic transconjugants from one or more of the \textit{bch} mutants were isolated and characterized. These clones were used as probes to estimate levels of specific transcripts in cells undergoing a 100-fold increase in bacteriochlorophyll content. The maximum level of transcripts was observed at an early stage of photosynthetic membrane synthesis when only 7\% of the eventual level of pigment had been synthesized.

\section*{INTRODUCTION}

The purple bacterium \textit{Rhodobacter sphaeroides} provides a useful model system in which to study the role of bacteriochlorophyll synthesis in photosynthetic membrane assembly, due to its ability to grow aerobically in the dark as well as photosynthetically. This facultative property has permitted the isolation of stable non-photosynthetic mutants that are unable to synthesize bacteriochlorophyll; such mutants excrete biosynthetic intermediates into the growth medium. Several workers have proposed biosynthetic pathways based on these intermediates (Lascelles, 1966; Richards \& Lascelles, 1969; Pudek \& Richards, 1975; Jones, 1978; Rebeiz \& Lascelles, 1982).

Further progress depends upon the development of genetic tools for this organism. In this regard, cosmid mobilizing techniques have already been used to investigate genes for the carotenoid biosynthetic pathway in \textit{Rb. sphaeroides} (Pemberton \& Harding, 1986). Marrs and coworkers have used chromosome and plasmid mobilizing techniques to map and isolate a 45 kb gene cluster in \textit{Rb. capsulatus} which contains genes for bacteriochlorophyll and carotenoid biosynthesis, and reaction centre and light-harvesting polypeptides (Yen \& Marrs 1976; Marrs, 1981; Taylor \textit{et al.}, 1983). A highly efficient mobilization system which employs plasmid pRK2073 (Hunter \& Turner, 1988) has been used here to transfer a bank of \textit{Rb. sphaeroides} genes into mutants unable to synthesize bacteriochlorophyll. In this way clones carrying genes designated \textit{bch} E, B, A and C according to the scheme outlined by Biel \& Marrs (1983) have been isolated. In view of the widespread use of \textit{Rb. sphaeroides} as a model for bacteriochlorophyll biosynthesis, the work described here seeks to provide a molecular genetic analysis of the pathway.

It has been known for some time that bacteriochlorophyll synthesis and more generally photosynthetic membrane assembly are repressed by oxygen (Cohen-Bazire \textit{et al.}, 1957). Under conditions of high aeration, it is possible to repress cellular bacteriochlorophyll to less than 1\% of maximum. At the onset of low aeration, this repression is lifted and over a period of approximately 20 h the cell elaborates a system of pigmented membranes which houses the photosynthetic apparatus (Niederman \textit{et al.}, 1976). In the early stages of membrane assembly,
there is a rapid rise in the level of transcripts specific for reaction centre and light-harvesting apoproteins of *Rb. sphaeroides* (Hunter *et al.*, 1987). In *Rb. capsulatus* the transcription of several *bch* genes is influenced by oxygen (Biel & Marrs, 1983), but there has hitherto been no information on the influence of oxygen on *bch* genes in *Rb. sphaeroides*. In this paper we describe the use of the cloned probes for *bch E, B, A* and *C* to determine how the levels of specific transcripts are affected by oxygen during derepression of photosynthetic membrane assembly.

**METHODS**

**Growth of cultures.** *Escherichia coli* strains were grown in Luria media as described by Maniatis *et al.* (1982). *Rb. sphaeroides* strains were grown in M22 medium (Sistrom, 1977) supplemented with sodium succinate (4-6 g l⁻¹), sodium glutamate (0.27 g l⁻¹) and aspartic acid (0.04 g l⁻¹). This is designated M22+. Where indicated, 0.1% Casamino acids were used to supplement this medium.

**Induction of bacteriochlorophyll synthesis in suspensions of *Rb. sphaeroides* under conditions of low aeration.** This was performed as described by Niederman *et al.* (1976). DNA (50 μg) was partially digested by *TaqI* and size-fractionated on a NaCl gradient (1.5-5 M NaCl in 10 mM-Tris pH 7.5, 1 mM-EDTA). Fragments in the range of 10-14 kb were ligated into the ClaI site of pSUP202 which had been treated with calf intestinal alkaline phosphatase (BCL), and the mixture was transformed into *E. coli* DH5. The gene bank consisted of 2500 clones of average insert size 1 kb (f = 0.999; Maniatis *et al.*, 1982). The library was subdivided into 40 sublibraries each representing approximately 50 clones, which were harvested and frozen in 25% (v/v) glycerol in LB medium at −80 °C.

**Conjugation, and plasmid isolation and analysis.** The techniques were as described in the accompanying paper (Hunter & Turner, 1988).

**Construction of the *Rb. sphaeroides* library in pSUP202.** *Rb. sphaeroides* genomic DNA was prepared using the method described by Hunter & Turner (1988). DNA (50 μg) was partially digested by *TaqI* and size-fractionated on a NaCl gradient (1.5-5 M NaCl in 10 mM-Tris pH 7.5, 1 mM-EDTA). Fragments in the range of 10-14 kb were ligated into the *ClaI* site of pSUP202 which had been treated with calf intestinal alkaline phosphatase (BCL), and the mixture was transformed into *E. coli* DH5. The gene bank consisted of 2500 clones of average insert size 11 kb (f = 0.999; Maniatis *et al.*, 1982). The library was subdivided into 40 sublibraries each representing approximately 50 clones, which were harvested and frozen in 25% (v/v) glycerol in LB medium at −80 °C.

**Conjugation, and plasmid isolation and analysis.** The techniques were as described in the accompanying paper (Hunter & Turner, 1988).

**Induction of bacteriochlorophyll synthesis in suspensions of *Rb. sphaeroides* under conditions of low aeration.** This was performed as described by Niederman *et al.* (1976).

**Preparation and analysis of RNA.** Total RNA was prepared from cells of *Rb. sphaeroides* harvested at various times following pigment induction, using the method described by Hunter & Turner (1988). Northern blots were made from formaldehyde denaturing gels as described by Maniatis *et al.* (1982). RNA dot and DNA hybridization analyses were performed as described by Thomas (1983). Autoradiographs of blots were scanned on a Shimadzu CS-930 dual-wavelength TLC scanner.
RESULTS AND DISCUSSION

Characterization of bacteriochlorophyll mutants

A number of strains carrying lesions in bacteriochlorophyll biosynthesis were obtained following chemical or transposon Tn5 mutagenesis of the wild-type. These are listed in Table 1. Initially, mutants were isolated on the basis of an inability to grow photosynthetically (Psg⁻). Psg⁻ mutants were grown under oxygen-limited heterotrophic conditions which promote the excretion of biosynthetic intermediates of bacteriochlorophyll into the growth medium; absorption spectra are shown in Fig. 1. Following extraction of cell pellets by acetone/methanol and diethyl ether a variety of absorption and fluorescence emission spectra were obtained and the results (Table 2) were compared with similar work on bacteriochlorophyll-less mutants of *Rb. sphaeroides* (Lascelles, 1966; Richards & Lascelles, 1969). We conclude that N6 excretes magnesium protoporphyrin monomethyl ester, N5 excretes magnesium divinyl phaeoporphyrin a₅ monomethyl ester, N22 excretes 2-desvinyl-2-hydroxyethyl chlorophyllide a, and T127 excretes 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide a. However, further work is needed in order to see if a range of spectrally similar pigments are present within mutant N5 for example, since our methods do not provide a means to resolve monovinyl or divinyl derivatives of protochlorophyllide. Further analysis by HPLC indicates that mono- and divinyl species may be excreted by mutant N5 (B. White, W. T. Griffiths, S. A. Coomer & C. N. Hunter, unpublished results). The scheme of Pudek & Richards (1975) predicts that disruption of the
Table 2. Major absorption and fluorescence emission peaks of acetone/methanol and diethyl ether extracts of whole cells of bch mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Solvent</th>
<th>Excitation wavelength (nm)</th>
<th>Solvent</th>
<th>Possible bacteriochlorophyll intermediate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetone/methanol</td>
<td>423</td>
<td>Diethyl ether</td>
<td>590</td>
</tr>
<tr>
<td>N6</td>
<td>584</td>
<td>588</td>
<td></td>
<td>592</td>
</tr>
<tr>
<td>N5</td>
<td>624</td>
<td>622</td>
<td></td>
<td>629</td>
</tr>
<tr>
<td>N22</td>
<td>656</td>
<td>656</td>
<td></td>
<td>668</td>
</tr>
<tr>
<td>T127</td>
<td>704</td>
<td>708</td>
<td></td>
<td>718</td>
</tr>
</tbody>
</table>

Possible bacteriochlorophyll intermediate

- Mg protoporphyrin monomethyl ester
- Mg divinyl phaeoporphyrin a₃ monomethyl ester
- 2-desvinyl-2-hydroxyethyl-bacteriochlorophyllide a
- 2-desacetyl-2-hydroxyethyl-bacteriochlorophyllide a

Gene encoding reduction of protochlorophyllide to chlorophyllide may yield such a mixture of intermediates. For the present, the mutants have been assigned to the linear pathway proposed by Jones (1978). The genes are allocated to the stages of this pathway according to Jones and to the scheme of Biel & Marrs (1983) although they provide no designation for the gene encoding protochlorophyllide reduction which appears in Fig. 2 as bchL. Another area of uncertainty in bacteriochlorophyll and chlorophyll synthesis includes the number of steps and genes involved in the cyclization reactions that lead to the formation of the E ring of mono- or divinyl magnesium protoporphyrin monomethyl ester.

Isolation and characterization of pSUP202 recombinant clones carrying bch genes

Following transfer of the *Rb. sphaeroides* gene library to the mutants N5, N6, N22 and T127, newly photosynthetic recombinant clones were isolated. Direct recovery of the *bch* genes from the recombinants was not attempted because of the instability of pSUP202 in *Rb. sphaeroides* (Hunter, 1988). Instead the clones were isolated by repeated subdivision and transfer of the gene library. Each of the 40 sublibraries (see Methods) was transferred to mutants N5, N6 and N22 using the helper plasmid pRK2073, followed by selection for photosynthetic growth. On average, three sublibraries out of 40 gave 20–30 photosynthetic transconjugants. These transconjugants have fully restored levels of photosynthetic apparatus, are stable in aerobic culture and no detectable pigment is excreted into the growth medium. Clones which produced photosynthetic growth were analysed by restriction mapping (Figure 3). These plasmids, which were named pSCN6-1, pSCN5-1 and pSCN22-1, have inserts of 11.3, 12.1 and 10.5 kb respectively, and complement mutants N6, N5 and N22 respectively. No overlaps in complementation were noted between the three plasmids and the three mutants, although pSN22-1 also restores mutant T127 to photosynthetic growth. Moreover, pSCN5-1 and pSCN6-1 overlap and share 6 kb in common. None of these clones overlap with pJW1, which contains a 12.2 kb BamHI fragment carrying *puf* genes A, B, L and M encoding subunits of the LH1 and reaction centre complexes. Pemberton & Harding (1986) have already shown that several carotenoid (*crt*) genes map closely together in *Rb. sphaeroides*. Work is currently being undertaken to link pSCN6-1, pSCN5-1 and pSCN22-1 with the *puf* and *puh* genes of the photosynthetic cluster as found in *Rb. capsulatus* (Taylor et al., 1983).

Influence of oxygen on mRNA levels for *bch* E, B, A and C

Cohen-Bazire *et al.* (1957) first showed that oxygen represses bacteriochlorophyll biosynthesis in purple non-sulphur bacteria. A number of groups have shown that oxygen represses the levels of mRNA for LH2, LH1 and reaction centre complexes, in both *Rb. capsulatus* and *Rb. sphaeroides* (Clark *et al.*, 1984; Zhu & Kaplan, 1985; Klug *et al.*, 1985; Zhu & Hearst, 1986; Zhu *et al.*, 1986; Hunter *et al.*, 1987). The transcription of several genes for bacteriochlorophyll
biosynthesis has been shown to be regulated by oxygen in *Rb. capsulatus* (Biel & Marrs, 1983). Also, the levels of mRNA for the *bch* genes of *Rb. capsulatus* are affected by light and oxygen (Clark *et al.*, 1984; Zhu & Hearst, 1986; Zhu *et al.*, 1986).

Total mRNA was prepared from *Rb. sphaeroides* cultures at various stages of pigmentation after the oxygen concentration was lowered; over the time course the level of cellular bacteriochlorophyll increased 100-fold. A series of dot blots were probed with the plasmids pSCN6-1, pSCN5-1 and pSCN22-1 (Fig. 4). The results show that the maximum level of transcripts is achieved 1 h after the lowering of oxygen concentration and represents a two- to threefold increase. The timing of this increase can be compared with a recent study on the induction of mRNA for reaction centre and light-harvesting polypeptides (Hunter *et al.*, 1987): the maximum levels of transcripts for reaction centre and LH1 complexes were achieved 2 h after the start of induction, and for LH2, which binds much of the bacteriochlorophyll, a gradual increase was observed over the 6 h of the experiment. Thus, it can be concluded that the rise in mRNA for the bacteriochlorophyll biosynthesis pathway within 1 h is a primary event in photosynthetic membrane assembly. It is interesting that the products of this pathway are needed to stabilize the polypeptide components of photosynthetic complexes in *Rb. capsulatus* (Dierstein, 1983).

Northern blots probed with these plasmids did not reveal bands representing *bch* transcripts with sufficient clarity for densitometry. We attribute this to the low abundance of these transcripts, in comparison with those encoding structural proteins. Nevertheless, the sizes of these transcripts could be measured. pSCN5-1 encoded five transcripts of 1-4, 1-0, 0-8, 0-73 and
0.43 kb, pSCN6-1 encoded four transcripts of 1.95, 1.45, 1.07 and 0.84 kb, and pSCN22-1 encoded four transcripts of 1.4, 1.26, 1.06 and 0.8 kb. The levels of all these transcripts increased two- to threefold when oxygen concentration was lowered. These studies show that oxygen tension affects the amount of transcripts encoded by clones carrying bch genes although they do not differentiate between increase in transcription or decrease in degradation of these mRNA species. Work is currently underway to position the bch genes and transcripts on the clones obtained so far, and to clone genes for the remainder of the pathway.

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REFERENCES


**Rb. sphaeroides** bacteriochlorophyll genes


