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MUTANT STRAINS OF *RHODOPSEUDOMONAS SPHEROIDES* WHICH FORM PHOTOSYNTHETIC PIGMENTS AEROBICALLY IN THE DARK

GROWTH CHARACTERISTICS AND ENZYMIC ACTIVITIES

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SUMMARY

Mutant strains of *Rhodopseudomonas spheroides* have been isolated which contain 5–50 times more bacteriochlorophyll and carotenoids than the wild type when grown under highly aerobic conditions in the dark. Their pigment content is similar to the wild type when grown in the light. One of the mutants (TA-R) grew more slowly than its parent strain under aerobic conditions but formed pigments at about 60% of the rate observed under photosynthetic conditions. The other mutants grew at rates similar to the wild type under all conditions. Synthesis of bacteriochlorophyll by suspensions of the mutants began without delay upon transfer from conditions of high to low aeration. In contrast to the wild type, magnesium protoporphyrin–*S*-adenosylmethionine methyltransferase (EC 2.1.1.11) activity in particulate preparations from the mutants was not repressed by growth under aerobic conditions in the light or dark. Ribulose diphosphate carboxylase (EC 4.1.1.39) activity was repressed by O_2 in the mutants as in the wild type. Other enzyme activities were compared in mutant TA-R and its parent strain grown under the same conditions. NADH oxidase activity in particles from aerobically grown TA-R was about one third that found in the parent strain. However, the respiration rates of the intact cells did not differ. Light inhibited the respiration of aerobically grown TA-R, indicating that the bacteriochlorophyll formed under these conditions had photochemical activity. It is concluded that the insensitivity of the mutants to O_2 repression is due to defects in the regulatory system which controls formation of the enzymes concerned in pigment synthesis.

INTRODUCTION

R. spheroides and other members of the Athiorhodaceae are able to grow anaerobically in the light or aerobically in the dark but under the latter conditions synthesis of the chlorophyll and carotenoids pigments is suppressed provided that the O_2 concentration is sufficiently high[1,2]. In *R. spheroides* it is likely that repression of the biosynthetic enzymes contributes to the effect of O_2 on bacteriochlorophyll formation[3]. Some of the enzymes involved in early steps of bacteriochlorophyll synthesis up to the

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

stage of protoporphyrin are repressed by high aeration. However, information about enzymes of the magnesium branch of the pathway is lacking. The only step which has been detected *in vitro* is magnesium protoporphyrin–*S*-adenosylmethionine methyltransferase (EC 2.1.1.11) and this has been found by GIBSON *et al.*[4] to be repressed in cells grown with high aeration. O_2 also appears to inhibit the activity of enzymes of bacteriochlorophyll synthesis. Thus, pigment synthesis by cells growing anaerobically in the light and therefore rich in biosynthetic enzymes, is immediately halted upon introduction of O_2 (ref. 2). Observations with a mutant strain of *R. spheroides* requiring δ-aminolaevulate for growth suggest that the enzyme system for the introduction of magnesium into the protoporphyrin nucleus is particularly sensitive to inhibition by O_2 (ref. 5). Production of magnesium protoporphyrin by suspensions of this mutant ceased immediately upon introduction of O_2 into an anaerobic–light system, whereas formation of iron protoporphyrin continued.

In the hope of learning something of the mechanisms of these various effects of O_2 on pigment synthesis by *R. spheroides* we have sought mutant strains which are able to form pigments under highly aerobic conditions. The isolation and some of the properties of such mutants is the subject of the present work.

MATERIALS AND METHODS

Organisms and growth conditions

The wild-type strain of *R. spheroides* (NCIB 8253) and its maintenance has been described previously[6]. Strain 6-10 was derived from the wild type by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine[6]; it required methionine for growth but behaved like wild type in other respects. The pigment mutants were derived as follows. Mutant L-57R was a spontaneous revertant of strain L-75, which forms neither bacteriochlorophyll nor carotenoids[7]. Mutant DW-R was obtained by mutagenesis of the wild type. Mutant TA-R was derived from strain 6-10 by selection under conditions favoring organisms whose photosynthetic pigment system was not repressed by growth under high aeration. Beginning with an aerobic–dark culture, about $4 \cdot 10^8$ cells were transferred to 10 ml yeast extract–malate–glutamate medium[8] and incubated anaerobically in the light for 8 h. A sample of this culture was then transferred to 10 ml of fresh medium and incubated for 16 h under aerobic–dark conditions. After repetition of such subculturing through ten cycles the aerobic culture was pigmented and grew without delay upon transfer to anaerobic–light conditions. When plated on yeast extract–malate–glutamate agar the colonies were highly pigmented and mutant TA-R was isolated from one of these.

Stock cultures of mutant and parent strains were maintained as stab cultures in yeast extract–malate–glutamate agar, which were incubated in the light for 24 h. They were stored at 4° and subcultured at six weekly intervals.

Cultures for experimental purposes were grown in malate–glutamate medium[8] supplemented with 0.4 mM L-methionine in the case of strains 6-10 and TA-R. Unless stated otherwise the standard conditions of incubation were as follows. Aerobic cultures were grown in the dark in flasks containing 20% of their nominal capacity of medium and shaken on a gyrorotary shaker at 200 rev./min. Such conditions suppressed pigment synthesis in the wild type to barely detectable levels. Cultures grown anaerobically in the light were in flat-sided bottles filled to the neck

and illuminated with incandescent light at an intensity of 700–800 ft candles. In some experiments cultures were grown in the light with aeration; this was achieved by sparging the illuminating cultures with a stream of air. All cultures were grown at a temperature of 34°.

Growth was measured with a Klett colorimeter using the red filter. A reading of 120 Klett units was equivalent to 0.43 mg dry weight of cells per ml.

Cell suspension experiments

Tetrapyrrole synthesis by cell suspensions incubated with low aeration was determined as described previously[11]. The cells were grown under high aeration and resuspended to a density of 2 mg dry weight per ml in malate–glutamate medium with glycine and succinate. ^{59}Fe was added for measurement of haem synthesis. The suspensions were incubated with low aeration.

Preparation of extracts for enzyme assays

Harvested cells were washed by centrifugation in 40 mM Tris buffer (pH 7.5) and suspended in this buffer to a density of 20 mg dry weight per ml. Extracts were prepared by the French press (American Instrument Co., Silver Springs, Md.) and were centrifuged for 10 min at 12000 × *g*. The supernatant, designated as crude extract, was centrifuged at 100000 × *g* for 90 min and the supernatant ("soluble fraction") removed. The particulate fraction was rinsed with 40 mM Tris buffer (pH 7.5) and suspended in the same buffer containing 30% (v/v)glycerol to a protein concentration of about 10 mg/ml. All operations were at 0–4°.

Analytical methods and enzyme assays

Bacteriochlorophyll and carotenoids were determined by extraction of the cells with acetone–methanol (7:2, by vol.)[2]. The concentration of bacteriochlorophyll was calculated using the ε_{mM} value of 76 at 770 nm (ref. 9). The mixture of carotenoids formed by this organism, particularly when grown in the presence of O_2, exhibited a broad maximum at 484 nm; the concentration was determined from the absorbance at 484 nm, using the ε_{mM} value of 128 calculated from the data of COHEN-BAZIRE *et al.*[2]. Protein was determined by the Folin method[10] with bovine serum albumin as standard. Magnesium protoporphyrin (or its methyl ester) formed by cell suspensions was estimated by its absorbance at 420 nm (ε_{mM} = 308)[12]. Haem synthesis was determined using ^{59}Fe as described by LASCELLES AND HATCH[11].

Magnesium protoporphyrin–*S*-adenosylmethionine methyltransferase activity was assayed in the particulate fraction of cell-free extracts by a method based on that of GIBSON *et al.*[4]. The complete system contained in 1 ml final volume: Tris buffer (pH 8.5), 50 μmoles; Tween 80, 0.2% (v/v); magnesium protoporphyrin, 0.06 μmole; *S*-adenosyl-L-[*Me*-^{14}C]-methionine, 0.1 μC, 0.06 μmole; particles, 0.5–1.5 mg protein. Incubation was for 1 h at 37° and the reaction was terminated by the addition of 4 ml ethyl acetate–glacial acetic acid (3:1, by vol.). This mixture was centrifuged and the supernatant transferred to a separatory funnel. The pellet was re-extracted with 3 ml ethyl acetate–acetic acid and the supernatant combined with the first extract. The extract was washed twice with saturated sodium acetate (6 ml) and twice with water (3 ml). Porphyrins were extracted from the ethyl acetate with 10% (w/v) HCl (6 ml followed by 1 ml). The acid extract was collected in a measuring

cylinder and was rinsed twice with 4 ml of ethyl acetate. Ethyl acetate (4 ml) was then added and the porphyrins were driven into the solvent layer by careful addition of solid $NaHCO_3$. Radioactivity was determined by planchet counting using a Nuclear Chicago planchet counter, Model 1042; the concentration of protoporphyrin and methyl ester was determined spectrometrically at 408 nm after dilution in 5% (w/v) HCl[13].

Ribulose diphosphate carboxylase (EC 4.1.1.39) was assayed in crude cell-free extracts. The complete system contained in a final volume of 0.5 ml: Tris buffer (pH 7.9), 95 μmoles; EDTA, 0.025 μmole; $MgCl_2$, 2.7 μmoles; GSH, 9 μmoles; ribulose diphosphate, sodium salt, 0.3 μmole; $Na_2{}^{14}CO_3$, 15 μmoles, 1.5 μC; extract, 0.05–0.5 mg protein. Incubation was for 20 min at 30° and the reaction was stopped by addition of 0.1 ml of 6 M HCl. The tubes were heated at 100° for 2 min to remove excess $^{14}CO_2$. Samples (0.1 ml) were added to vials containing 20 ml counting fluid (Nuclear Chicago "Spectrafluor–PPO–POPOP" diluted 42 ml to 1 l with toluene) and counted in a Beckman liquid scintillation system counter. Activity is expressed as μmoles CO_2 fixed per h after subtraction of control values without ribulose diphosphate.

δ-Aminolaevulate synthetase was assayed in the supernatant fraction by the method of BURNHAM AND LASCELLES[14].

Succinate dehydrogenase (EC 1.3.99.1) was assayed in the particles by following the rate of reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm in the presence of phenazine methosulphate[15]. The cuvettes contained in 1 ml: potassium phosphate buffer (pH 7.5), 100 μmoles; KCN, 2 μmoles; DCIP, 0.1 μmole; phenazine methosulphate, 0.32 μmole; particles, 0.1–0.4 mg protein. The reaction was started by addition of 10 μmoles sodium succinate.

Dihydroorotate dehydrogenase (EC 1.3.3.1) was assayed in the particles as described for succinate dehydrogenase, except that dihydroorotic acid (1 μmole) was used as substrate, and the protein concentration was increased 10-fold.

NADH oxidase activity was assayed in particles by following the decline in absorbance at 340 nm. Experimental cuvettes contained in a 1-ml volume: potassium phosphate buffer (pH 7.5), 50 μmoles; NADH, 0.1 μmole, particles, 0.5–1 mg protein. The reference cuvette lacked NADH.

All spectrophotometric assays were at room temperature (25°) using a Cary spectrophotometer, Model 14, equipped with a 0–0.1 absorbance slide wire.

Respiratory activity was determined by the Warburg technique. Vessels contained in a 2-ml volume: potassium phosphate buffer (pH 7.5), 100 μmoles; washed cells, 2.5–5 mg dry weight; substrate, 10 μmoles (added from the side arm). The centre well contained NaOH to absorb CO_2. Incubation was in air at 27° and activity is expressed as μl O_2 consumed per h per mg dry weight cells.

Special chemicals

Magnesium protoporphyrin was prepared from protoporphyrin dimethylester (Calbiochem, Los Angeles, Calif.) by the method of BAUM *et al.*[16]; conversion to the potassium salt was by the method of GRANICK[12]. Stock solutions (1–2 mM) were dissolved in 40 mM Tris buffer (pH 7.5) containing 2% Tween 80 and their concentration determined spectrophotometrically at 590 nm, using the ε_{mM} value of 174 (refs. 12,17). *S*-Adenosyl-L-[*Me*-^{14}C]methionine was obtained from ICN (Irvine, Calif.)

and the unlabeled compound was from Sigma Chemical Co. (St. Louis, Mo.); stock solutions were stored frozen at pH 3–4. Ribulose diphosphate, dibarium salt, was from Sigma Chemical Co. and was converted to the sodium form by treatment with Dowex-50.

RESULTS

Effect of growth conditions on pigment synthesis by mutant and parental strains

Aerobic slant cultures of mutant strains L75-R. DW-R, and TA-R differed from the parental strains in being intensely pigmented. Isolated colonies showed red pigmentation throughout whereas colonies of the parental strains were pigmented only at the centre where dense growth resulted in a low concentration of O_2.

The concentration of bacteriochlorophyll and carotenoids was determined in cells of mutant and parent organisms harvested during the exponential phase of growth under various conditions (Table I). When grown under high aeration the wild type and strain 6-10 contained only traces of these pigments. In contrast, the mutants had readily detectable levels of bacteriochlorophyll and carotenoids. In cells grown anaerobically in the light the pigment concentration in mutant and parental strains was similar. The mutants responded to changes in light intensity in the manner observed with normal strains of photosynthetic bacteria[2]. The pigment concentration was decreased by raising the light intensity (Table I). In cultures growing under low

TABLE I

EFFECT OF GROWTH CONDITIONS ON PIGMENT CONTENT OF WILD TYPE AND MUTANT STRAINS OF *R. spheroides*

Bacteriochlorophyll and carotenoids were determined in cells harvested during the logarithmic phase of growth at a cell density of about 0.4 mg dry weight per ml; malate-glutamate medium was used supplemented with 0.4 mM L-methionine in the case of strains 6-10 and TA-R. Cultures grown in the light were illuminated at an intensity of 460 ft candles (low) or 1300 ft candles (high).

Strain	*Growth conditions*	*Pigment (nmoles/mg dry wt. cells)*	
		Bacteriochlorophyll	*Carotenoids*
Wild type	Aerobic–dark	<0.1	<0.1
	Anaerobic–light, low	3.9	3.8
	Anaerobic–light, high	1.9	2.1
6-10	Aerobic–dark	<0.1	<0.1
	Anaerobic–light, low	5.2	3.8
L-57R	Aerobic–dark	0.75	0.71
	Anaerobic–light, low	3.3	3.1
	Anaerobic–light, high	2.0	2.3
	Aerobic–light, low	<0.1	0.34
DW-R	Aerobic–dark	0.21	0.59
	Anaerobic–light, low	4.9	3.8
TA-R	Aerobic–dark	2.9	3.4
	Anaerobic–light, low	4.6	3.9
	Anaerobic–light, high	2.0	3.0
	Aerobic–light, low	<0.1	1.3

light the pigment concentration of the cells rose progressively as the cultures became more dense, reaching values of 8–10 nmoles/mg dry weight of cells; the values shown in Table I are for cells harvested during the mid-exponential phase.

The effect of growth conditions was examined in more detail with mutant strain TA-R which formed considerably more pigment than the other strains under aerobic conditions. When cultures of this strain were transferred from anaerobic–light conditions to aerobic–dark conditions bacteriochlorophyll synthesis continued at a differential rate which was about 60% of that observed anaerobically in the light (Fig. 1a). In contrast, a similar shift of cultures of the parent strain, 6–10, resulted in complete cessation of pigment synthesis (Fig. 1b). Mutant strain TA-R behaved similarly to strain 6-10 when shifted from anaerobic conditions to aerobic conditions in the light, in that bacteriochlorophyll synthesis was completely prevented (Table I, Figs. 1a and 1b). Thus, pigment synthesis by TA-R was relatively insensitive to repression by O_2 in the dark but was completely prevented by aeration in the light.

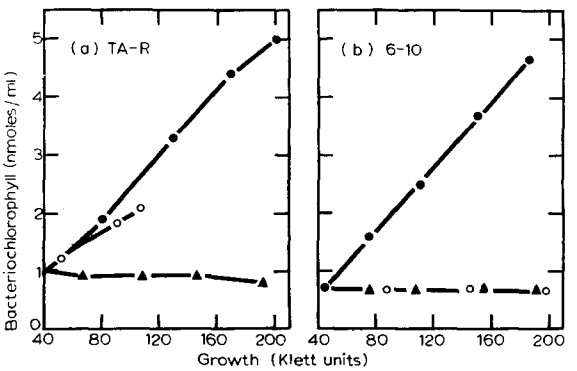

Fig. 1. Differential rate of bacteriochlorphyll synthesis by cultures of strains TA-R and 6-10. Cultures were grown in malate–glutamate medium with 0.4 mM L-methionine under the conditions: anaerobic–light (●); aerobic–dark (○); aerobic–light (▲).

Growth rates

Mutants and parental strains grew at similar rates when incubated anaerobically in the light in malate–glutamate medium, supplemented with methionine in the case of strains 6-10 and TA-R. Under these conditions, the doubling time was 2.5–3 h. With the exception of strain TA-R, similar generation times were observed under aerobic conditions in the dark. The aerobic growth rate of strain TA-R was considerably slower than that of its parent, 6-10; the doubling times was about 5 h in malate–glutamate medium with methionine (Table II). The slow growth rate of strain TA-R under aerobic conditions in the dark was not due to inhibition of growth by O_2, since both mutant and parent strains grew at the usual rate under aerobic conditions in the light (Table II). Also, supplementation of the medium with yeast extract restored the aerobic–dark rate of TA-R to that observed with strain 6-10 (Table II).

It was possible that the high rate of pigment synthesis observed with strain TA-R under aerobic–dark conditions was an indirect consequence of the slow growth rate. Therefore, bacteriochlorophyll levels were compared in cultures of the mutant and parent organism grown aerobically in the dark on a variety of media which promoted

TABLE II

GROWTH RATE AND BACTERIOCHLOROPHYLL CONTENT OF STRAINS 6-10 AND TA-R UNDER VARIOUS CONDITIONS

Growth rates were determined under the standard conditions as described in the text and the bacteriochlorophyll content was estimated in cells harvested in the exponential phase.

Strain	*Growth conditions*	*Medium**	*Generation time (h)*	*Bacteriochlorophyll (nmoles/mg dry wt. cells)*
6-10	Aerobic–dark	M–G–met	2.5	<0.1
	Aerobic–dark	G–met	5	<0.1
	Anaerobic–light	M–G–met	2.75	5.5
	Aerobic–light	M–G–met	2.75	<0.1
TA-R	Aerobic–dark	M–G–met	5	3.3
	Aerobic–dark	G–met	6	7.8
	Aerobic–dark	M–G–met + 0.1% yeast extract	2.25	1.5
	Anaerobic–light	M–G–met	3	4.6
	Aerobic–light	M–G–met	3	<0.1

* M–G–met, malate–glutamate medium + 0.4 mM L-methionine; G–met, as M–G–met but without malate and containing 40 mM L-glutamate.

different growth rates (Table II). In all cases, pigment synthesis was repressed in strain 6-10 but not in the mutant. In particlar, both mutant and parent strain grew at a similar slow rate on glutamate as sole carbon source, yet pigment synthesis by the latter was still completely repressed.

Tetrapyrrole synthesis by cell suspensions

Cell suspensions of wild-type *R. spheroides*, grown with high aeration, form bacteriochlorophyll and haem when incubated with limited aeration in malate–glutamate medium supplemented with glycine and succinate[8,11]. There is a lag in the production of bacteriochlorophyll under these conditions, possibly reflecting the derepression of enzymes required for pigment synthesis.

The behavior of mutant L-57R was examined under these conditions. In

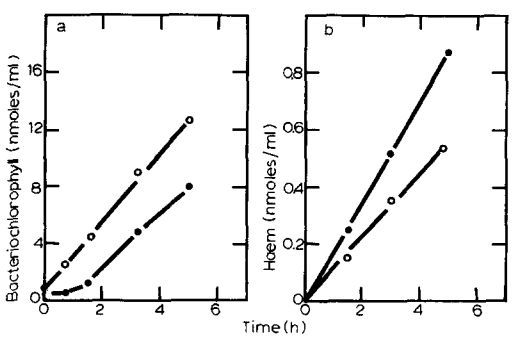

Fig. 2. Bacteriochlorophyll and haem synthesis by suspensions of wild type (●) and mutant L-57R (○). The cells were incubated under low aeration with glycine and succinate and with ^{59}Fe for measurement of haem synthesis as described previously[11].

contrast to the wild type bacteriochlorophyll by the mutant began without delay (Fig. 2a). The addition of δ-aminolaevulate to the suspensions resulted in the accumulation of magnesium protoporphyrin by both strains (Fig. 3). In this respect mutant L-57R was again more active than the wild type during the initial stages of incubation. These observations suggested that the enzymes of the magnesium pathway are present in the mutant at the time of transfer from high to low aeration.

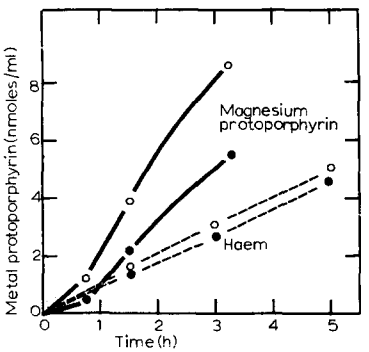

Fig. 3. Magnesium protoporphyrin and haem synthesis by suspensions of wild type (●) and mutant L-57R (○). Experimental conditions were as in Fig. 2 with the addition of 0.5 mM δ-aminolaevulinate and 0.1% (w/v) Tween 80 for measurement of magnesium protoporphyrin (—) and of 1 mM δ-aminolaevulinate and ^{59}Fe for measurement of haem (– – –).

Haem synthesis, measured by incorporation of ^{59}Fe, was also compared in mutant L-57R and the wild type. With glycine and succinate as primary substrates, the mutant was less active than the wild type (Fig. 2b). This difference might be due to enhanced diversion of the common intermediate protoporphyrin to the magnesium pathway in the mutant. Addition of δ-aminolaevulate to suspensions of both strains increased haem synthesis by 8–10-fold (Fig. 3). Apparently, the rate-limiting step in haem synthesis by both strains is the δ-aminolaevulinate synthetase.

Magnesium protoporphyrin–S-adenosylmethionine methyltransferase activity

More direct evidence for the presence of enzymes of the magnesium pathway was sought by assay of the activity of the methyltransferase in particles from mutant and parental strains grown under various conditions. In the parental strains grown with high aeration, the enzyme activity was only about one-tenth that found in preparations from cells grown anaerobically in the light (Table III). In contrast, the levels of methyltransferase in the mutants grown aerobically in the dark or in the light were as high or even higher than in cells grown anaerobically in the light (Table III).

The difference between mutant and parental strains with respect to regulation of the methyltransferase was clearly shown in growing cultures shifted from anaerobic–light to aerobic conditions. The results of such an experiment with wild type and mutant L-57R are illustrated in Fig. 4, by plotting the specific activity of the enzyme against growth. In the wild type formation of the enzyme was repressed upon transfer of the cultures to aerobic conditions. The decline in specific activity was proportional to the increase in culture density, suggesting that the enzyme initially present was

TABLE III

MAGNESIUM PROTOPORPHYRIN–*S*-ADENOSYLMETHIONINE METHYLTRANSFERASE ACTIVITY IN PARENTAL AND MUTANT STRAINS

The enzyme activities was assayed in particles prepared from cells grown under various conditions as described in MATERIALS AND METHODS. The activity is expressed as nmoles of methyl ester formed per h per mg protein.

Strain	*Methyltransferase activity in particles from cells grown*		
	Anaerobic–light	*Aerobic–dark*	*Aerobic–light*
Wild-type	9.5	1.4	1.2
L-57R	8.7	12.6	10.0
DW-R	19.4	20.4	Not tested
6-10	9.9	1.3	Not tested
TA-R	9.8	20.2	24.0

diluted out, rather than inactivated, as the cells continued to grow in the presence of O_2. Cultures of L-57R continued to form the enzyme after transfer to aerobic conditions and the differential rate of synthesis was similar to that in cultures growing anaerobically in the light.

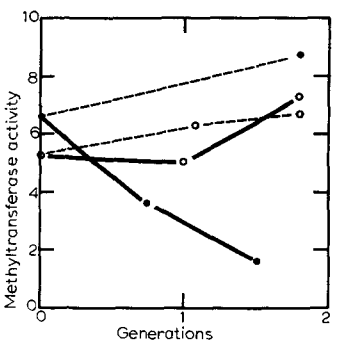

Fig. 4. Methyltransferase activity in growing cultures of wild type (●) and mutant L-57R (○). Cultures of the organisms growing in malate–glutamate medium under anaerobic–light conditions were diluted in fresh medium and shifted to aerobic–dark conditions (—). Control cultures were incubated anaerobically in the light (– – –). Samples were removed at intervals and the enzyme activity determined in the particles. The activity is expressed as nmoles methyl ester formed per h per mg protein.

Other enzyme activities

In *R. spheroides* and related bacteria the levels of many enzyme activities differ in cells grown anaerobically in the light or aerobically in the dark[3]. Some of these were assayed in preparations from strains 6-10 and TA-R grown under the two conditions (Table IV). Of the activities associated with the particulate fraction the only significant difference between the two strains was in the NADH oxidase in cells grown aerobically. In strain 6-10 this activity was about 3 times higher than in strain TA-R. Particles from both strains grown anaerobically in the light had similar activity, which was considerably less than that found in preparations from aerobically grown cells. A similar difference in NADH oxidase activity in cells grown aerobically or photosynthetically has been observed in other Athiorhodaceae[18,19].

TABLE IV

ENZYMIC ACTIVITIES IN PREPARATIONS FROM STRAINS 6-10 AND TA-R

Enzyme activities were assayed with preparations from cells harvested during the logarithmic phase of growth as described in MATERIALS AND METHODS. The values represent at least three different preparations. Units of enzyme are defined as nmoles of substrate oxidized per min for NADH oxidase, succinate dehydrogenase and dihydroorotate dehydrogenase, and as nmoles of δ-aminolaevulic acid formed or CO_2 fixed per h in the case of the synthetase and ribulose diphosphate carboxylase, respectively.

Enzyme system	*Cell fractions assayed*	*Enzyme activity (units/mg protein) in cells grown*			
		Aerobic–dark		*Anaerobic–light*	
		6-10	*TA-R*	*6-10*	*TA-R*
NADH oxidase	Particles	58	21	9	7
Succinate dehydrogenase	Particles	358	352	112	119
Dihydroorotate dehydrogenase	Particles	19	21	5	6
δ-Aminolaevulinate synthetase	Soluble	71	157	191	199
Ribulose diphosphate carboxylase	Crude extract	21	126	1374	1335

Succinate and dihydroorotate dehydrogenase in particles from strains 6-10 and TA-R grown under comparable conditions did not differ significantly; in each case these activities were lower in preparations from cells grown anaerobically in the light.

In wild type *R. spheroides* δ-aminolaevulate synthetase is repressed by growth under high aeration to a level which is 20–30% that in cells grown photosynthetically[8]. In mutant TA-R this enzyme activity was found to be high under both conditions of growth whereas the usual repression by O_2 was observed in the parent strain 6-10 (Table IV). In contrast to mutant TA-R, the activity of the synthetase in strain L-57R was repressed by O_2; the levels of enzyme were similar in this strain to those found in the wild type grown under the same conditions.

Ribulose diphosphate carboxylase is formed by the wild type only when grown anaerobically in the light and it is repressed by O_2 (ref. 20). The same pattern of repression of this enzyme occurred in mutant TA-R. Only low levels of the carboxylase were found in aerobically grown cells whereas the activity in cells grown anaerobically in the light was similar to that in strain 6-10. Similar observations were made with all the other mutants.

Respiration of intact cells

The relatively low NADH oxidase activity found in aerobically grown cells of TA-R suggested that a defect in its respiratory chain might account for its slow growth rate under aerobic conditions. However, the respiratory activity of intact cells of the mutant did not differ significantly from that of the parent strain. With malate as substrate the RQ values for cells grown aerobically were 36 and 32 for strains 6-10 and TA-R, respectively. In both cases, respiration was inhibited completely by 2 mM KCN.

Athiorhodaceae grown anaerobically in the light exhibit O_2 uptake which is inhibited by light. The effect (at least in *Rhodospirillum rubrum*) is attributable to interaction between the photosynthetic and the respiratory electron transport chains,

which share identical or closely related components[18,21]. The effect of light on the respiration of mutant TA-R was examined to determine whether the pigments formed under aerobic conditions had photochemical activity. The respiration of cells grown aerobically in the dark was severely inhibited by light (Fig. 5). As expected, light had no effect on the respiration of the parent strain 6-10 when grown aerobically and which was consequently devoid of photosynthetic pigments (Fig. 5).

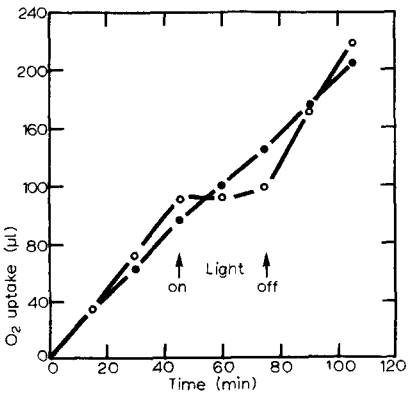

Fig. 5. The effect of light on respiration of aerobically grown 6-10 (●) and TA-R (○). The washed cells were incubated aerobically as described in MATERIALS AND METHODS. The Warburg vessels were illuminated with light at an intensity of about 800 ft candles as indicated by arrows. The dry weight of cells per vessel was, respectively, 3.6 and 4.4 mg for strains 6.10 and TA-R.

DISCUSSION

Bacteriochlorophyll synthesis is prevented by O_2 in two distinct ways. The activity of one or more of the biosynthetic enzymes is inhibited; the insertion of magnesium into the protoporphyrin nucleus appears to be a particularly sensitive step. O_2 also acts by repression of formation of the methyltransferase[4]. It is likely that other enzymes of the magnesium pathway were similarly repressed but this has yet to be established experimentally. The mutants described in this work all formed considerably more bacteriochlorophyll than the parental strains when grown with high aeration in the dark. However, the activity of the biosynthetic enzymes was still affected by O_2 because in all cases bacteriochlorophyll synthesis was curtailed upon shifting cultures from anaerobic–light conditions to highly aerobic conditions. In this respect strain TA-R showed the least response to O_2. The mutants did not accumulate magnesium protoporphyrin in such a shift, even upon addition of δ-aminolaevulate. This suggests that the enzyme responsible for insertion of magnesium into the protoporphyrin nucleus is still inhibited by O_2; the possibility that it is less sensitive than that in the wild type can only be established by direct enzymic analysis.

The most significant difference between mutant and parental strains was in the activity of the methyltransferase. In all the mutants the level of this enzyme in cells grown in the presence of O_2 was at least as high as that in cells grown anaerobically in the light. Therefore, it appears that the mutants are defective in the normal mechanisms which regulate formation of the methyltransferase. Probably, other

enzymes of the magnesium pathway are insensitive to repression by O_2 in the mutants; when grown under highly aerobic conditions, suspensions synthesized bacteriochlorophyll and magnesium protoporphyrin without a lag under conditions of low aeration (Figs. 2,3). The conclusion from the present observations is that failure of O_2 to repress enzyme formation may account for the ability of the mutants to form bacteriochlorophyll under aerobic–dark conditions. Carotenoid synthesis is also less susceptible to suppression by O_2 in these mutants but there is a complete lack of information about the enzymes concerned in the formation of these pigments.

The mechanism by which O_2 exerts its effect on the synthesis of the biosynthetic enzymes may involve a system consisting of products of regulatory genes acting in conjunction with effectors, whose intracellular concentration is influenced by O_2. Such an effector could be a component of the electron transport chain which must attain a certain critical ratio of reduced to oxidized form before enzyme synthesis is permitted. Alternatively, the concentration or ratio of adenine nucleotides might be critical. Either type of effector could be influenced by the concentration of O_2 in the environment. Of the mutants investigated, strain TA-R seems to be the most likely to be abnormal with respect to production of the hypothetical effector. This conclusion is based on its slow aerobic growth rate and its exceptionally high pigment content under these conditions. The observation that particles from aerobically grown cells had low NADH oxidase activity in comparison with that found in the parental strain is compatible with the involvement of the electron transfer chain in the repression mechanism. The absence of a genetic system for experimental analysis together with the lack of information at the enzymic level precludes further speculation about the mechanism of O_2 repression of pigment synthesis in *R. spheroides*.

Ribulose diphosphate carboxylase was repressed by O_2 to the same extent in mutant and parental strains. Apparently, formation of the carboxylase is controlled independently of the enzymes for pigment synthesis. This is consistent with observations of bacteriochlorophyll and of the carboxylase by wild-type *R. spheroides* when grown in the dark with low aeration. Such conditions promote bacteriochlorophyll synthesis but little carboxylase is formed until the cells are shifted to anaerobic–light conditions[20,22]. δ-Aminolaevulate synthetase is repressed by O_2 in wild-type *R. spheroides*, whereas this enzyme activity was found to be high in mutant strain TA-R grown under high aeration. However, the synthetase in strain L-57R exhibited the same response as the wild type to aerobic conditions. Therefore, no conclusion can be made about the possible relationship between control of this enzyme and that of enzymes of the magnesium pathway. Extracts of *R. spheroides* contain inhibitors of the synthetase one of which may be a protein of low molecular weight[23,24]. The degree of inhibition varies with the growth conditions and may also be ameliorated by appropriate pretreatment of extracts before assay. In view of these complexities, it is premature to interpret the relevance of the high activity found in strain TA-R to the problem of O_2 repression of pigment synthesis.

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REFERENCES

1 C. B. VAN NIEL, *Bacteriol. Rev.*, 8 (1944) 1.
2 G. COHEN-BAZIRE, W. R. SISTROM AND R. Y. STANIER, *J. Cellular Comp. Physiol.*, 49 (1957) 25.
3 J. LASCELLES, *Advan. Microbiol. Physiol.*, 2 (1968) 1.
4 K. D. GIBSON, A. NEUBERGER AND G. H. TAIT, *Biochem. J.*, 88 (1963) 325.
5 J. LASCELLES AND T. ALTSHULER, *J. Bacteriol.*, 98 (1969) 721.
6 J. LASCELLES, *Biochem. J.*, 100 (1966) 175.
7 J. LASCELLES, *Biochem. J.*, 100 (1966) 184.
8 J. LASCELLES, *Biochem. J.*, 72 (1959) 508.
9 R. K. CLAYTON, *Photochem. Photobiol.*, 5 (1966) 669.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 J. LASCELLES AND T. HATCH, *J. Bacteriol.*, 98 (1969) 712.
12 S. GRANICK, *J. Biol. Chem.*, 175 (1948) 333.
13 J. E. FALK, *Porphyrins and Metalloporphyrins*, B.B.A. Library, Vol. 2, Elsevier, Amsterdam, 1964.
14 B. F. BURNHAM AND J. LASCELLES, *Biochem. J.*, 87 (1963) 462.
15 O. ARRIGONI AND T. P. SINGER, *Nature*, 193 (1962) 1256.
16 S. J. BAUM, B. F. BURNHAM AND R. A. PLANE, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1439.
17 R. COOPER, *Biochem. J.*, 89 (1963) 100.
18 A. THORE, D. L. KEISTER AND A. SAN PIETRO, *Arch. Mikrobiol.*, 67 (1969) 378.
19 J.-H. KLEMME AND H. G. SCHLEGEL, *Arch. Mikrobiol.*, 68 (1969) 326.
20 J. LASCELLES, *J. Gen. Microbiol.*, 23 (1960) 499.
21 T. HORIO AND M. D. KAMEN, *Biochemistry*, 1 (1962) 1141.
22 M. HIGUCHI AND G. KIKUCHI, *Plant Cell Physiol.*, 10 (1969) 149.
23 S. TUBOI, H. J. KIM AND G. KIKUCHI, *Arch. Biochem. Biophys.*, 130 (1969) 92.
24 J. MARRIOTT, A. NEUBERGER AND G. H. TAIT, *Biochem. J.*, 111 (1969) 385.