NITROGEN ASSIMILATION DEFECTS IN A MUTANT OF RHODOPSEUDOMONAS CAPSULATA BLOCKED IN α-KETOGLUTARATE GENERATION

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1. Introduction

_Rhodopseudomonas capsulata_ lacks glutamate dehydrogenase and normally utilizes the glutamine synthetase (GS)/glutamate synthase (GOGAT) sequence of reactions as the primary pathway for the photosynthetic assimilation of both N\textsubscript{2} and ammonia [1]. Recent work of Brown and Herbert [2,3] indicates that the sequence noted is also a major biosynthetic process in various other photosynthetic bacteria during growth on low concentrations of exogenous ammonia. As part of a research program on mutants of _R. capsulata_ defective in N assimilation, we have examined the properties of a strain unable to grow photoheterotrophically with either N\textsubscript{2} or NH\textsubscript{3} as N source and malate as the C source. This communication summarizes experiments on the mutant, strain WE29, which show that its phenotype is due to a genetic lesion resulting in inability to produce α-ketoglutarate, a necessary substrate for the glutamate synthase reaction.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

The properties of wild type _R. capsulata_ strain B10 have been described by Weaver et al. [4]. Mutant WE29 is a derivative of B10, isolated after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis [5] and penicillin selection by standard procedures; the strain was isolated by P. Weaver and B. Errede during a screening for mutants unable to grow on minimal malate + NH\textsubscript{3} medium without added L-glutamate or L-glutamine. Strain T10 is a spontaneous revertant of WE29 capable of growing in the minimal medium noted; strain T100 has the same phenotype as T10 and is a genetic recombinant of WE29 isolated as indicated in the text (see section 3.4).

The minimal medium used, designated as RCVB, contained 30 mM DL-malate, 7.5 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.01 M KH\textsubscript{2}PO\textsubscript{4}, 1 mg thiamine hydrochloride per liter, 15 μg biotin per liter, and additional inorganic salts as specified by Ormerod et al. [6]. Strains B10, T10, and T100 were routinely subcultured in RCVB medium, whereas mutant WE29 was maintained in RCVB modified by addition of 10 mM L-glutamate. For experiments in which N\textsubscript{2} was the N source for growth, (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was omitted from the RCVB medium and the cultures were continuously gassed with 1% CO\textsubscript{2} in N\textsubscript{2}.

All cultures were grown photosynthetically (at 30°–33°C, unless noted otherwise) under anaerobic conditions, using saturating illumination provided by Lumilene incandescent lamps.

2.2. Preparation of cell-free extracts and enzyme assays

For preparation of extracts, cells were grown in RCVB modified by omission of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and addition of 10 mM L-glutamate, and harvested in the late exponential phase. The cells were washed with 50 mM Tris—HCl buffer (pH 7.5) and disrupted in the same buffer by passage through a French pressure cell (operated at 18 000 lb/in\textsuperscript{2}). Extracts were centrifuged at 140 000 X g for 90 min to remove debris, and the clear supernatant fluids retained for assay of citrate synthase (EC 4.1.3.7) and isocitrate dehy-
drogenase (EC 1.1.1.42) activities as described by Reeves et al. [7].

3. Results and Discussion

3.1. Growth of R. capsulata wild type and mutant WE29 with ammonia and N₂

Typical wild type isolates of R. capsulata, such as strain BIO, can use either ammonium salts or N₂ as sole N sources for photosynthetic growth with malate as the C source [4]. Growth kinetics of strain BIO on NH₄⁺ + malate are illustrated in Fig. 1, which also shows results obtained with mutant WE29. The latter is unable to use NH₄⁺ as the N source under the conditions noted and, in addition, cannot grow on N₂ + malate. Strain WE29, however, grows readily when the minimal NH₄⁺ + malate medium is supplemented with either L-glutamate or α-ketoglutarate (Fig. 1). This observation indicates that strain WE29 is unable to synthesize glutamate from NH₄⁺ + malate, presumably due to a metabolic block in generation of α-ketoglutarate, a direct precursor of glutamate. In separate experiments it was found that WE29 can grow photosynthetically with N₂ as the sole N source in a minimal malate + α-ketoglutarate medium (the doubling time, however, is ca. 2.5 × that of the wild type parent under the same conditions).

3.2. The enzymatic defect in mutant WE29

The growth tests described implied that WE29 is not genetically defective in respect to ability to synthesize GS and GOGAT, and this was substantiated by enzyme assays using extracts of cells grown on malate + glutamate (specific activities of GS and GOGAT were comparable to those observed in extracts of wild type cells grown on malate + NH₄⁺ (see ref. 1)). Since WE29 can grow on NH₄⁺ or N₂ when α-ketoglutarate is added to the minimal malate medium, it is apparent that this strain has at least one metabolic block in the citric acid cycle segment: malate → oxaloacetate ←α-ketoglutarate → isocitrate → α-ketoglutarate + CO₂. The results of Table 1 show that extracts of WE29 cells grown in a malate + glutamate medium show virtually no citrate synthase (CS) or isocitrate dehydrogenase (ICD) activities; the absence of either activity obviously could account for the inability of WE29 to grow on malate + NH₄⁺ or N₂. Control experiments in which extracts of BIO and WE29 were mixed showed that extracts of the mutant do not contain an inhibitor of either CS or ICD activity. Aconitate hydratase activities were found to be similar in all strains.

The possibility that R. capsulata may be able to generate α-ketoglutarate from metabolic sequences other than the citric acid cycle segment indicated was considered since in certain Pseudomonas species, α-ketoglutarate can be produced from metabolic conversions of L-arabinose [8] or D-glucaric acid [9]. These compounds, however, did not support growth of WE29 when added to the minimal malate + NH₄⁺ medium. Since glutamate addition can circumvent the
TABLE 1

Enzyme activities in extracts of R. capsulata wild type strain B10, mutant WE29, and derivatives of WE29 capable of growing in minimal malate + NH₄⁺ medium a

<table>
<thead>
<tr>
<th>Strain</th>
<th>CS specific activity (µmoles CoASH/min · mg protein)</th>
<th>ICD specific activity (µmoles NADPH/min · mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>WE29</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T10</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td>T100</td>
<td>0.26</td>
<td>0.22</td>
</tr>
</tbody>
</table>

a CS activities were determined by measuring (net) substrate-dependent change in absorbance at 412 nm due to reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by CoASH. Reaction mixtures contained (in 1 ml total volume): Tris-HCl buffer (pH 8.1), 200 mM; DTNB, 0.2 mM; enzyme extract (0.140–0.325 mg protein); acetyl coenzyme A, 0.1 mM; and oxaloacetate, 0.5 mM; reaction was started by addition of oxaloacetate. For ICD assay, the reaction mixtures (1 ml) contained: sodium D,L-isocitrate, 0.5 mM; NADP⁺, 0.5 mM; MnCl₂, 2.0 mM; Tris—HCl buffer (pH 7.5), 20 mM; and enzyme extract (0.033–0.160 mg protein); reaction was initiated by addition of D,L-isocitrate and rate of increase of absorbance at 340 nm was measured.

3.3. Properties of revertants of WE29

Spontaneous revertants of WE29 capable of growing on NH₄⁺ + malate were observed at frequencies expected for a single site mutation (ca. 10⁻⁷ to 10⁻⁸). The growth rate of one such revertant, T10, was approximately that of the parental B10 strain, and this was also true for growth on N₂ + malate. From Table 1 it can be seen that CS and ICD activities are present in extracts of T10 at the wild type level. These observations reinforce the conclusion that the WE29 phenotype is due to loss of ability to synthesize α-ketoglutarate.

3.4. Properties of genetic recombinants of WE29

Genetic recombinants of R. capsulata can be obtained by treatment of cells with "gene transfer agent", a phage-like vector produced by many strains of this organism [10]. Cells of WE29 were incubated with a "gene transfer agent" preparation derived from wild type strain J1 [10] using the procedures described by Wall et al. [5], and recombinants capable of growth in minimal NH₄⁺ + malate medium were isolated. As expected, extracts of one recombinant (strain T100) showed CS and ICD activities at the wild type levels (Table 1).

3.5. Other observations and remarks

Strain WE29 readily gives rise to mutants which grow more rapidly on complex media (containing Bactopeptone and yeast extract), especially when cultured aerobically in darkness. These derivatives (like the parental strain) still require a source of α-ketoglutarate for growth; CS and ICD activities could not be detected in extracts of one such isolate (strain T200). Attempts to obtain spontaneous revertants or genetic recombinants of strain T200 capable of growth without an added source of α-ketoglutarate have thus far given negative results. It is of interest that secondary mutants with faster growth rates appear spontaneously in Escherichia coli strains lacking ICD activity [11]; it seems that accumulation of an inhibitory compound is prevented by a second lesion that eliminates CS activity in such mutants. This explanation, however, does not readily account for faster growing secondary mutants of R. capsulata WE29 since this strain lacks both CS and ICD activities initially.

The frequencies with which prototrophic recombinants and spontaneous revertants (of WE29) are observed are consistent with the conclusion that a single site mutation is responsible for the WE29 phenotype. Thus, a single mutational event apparently results in the loss of two enzyme activities. There are several alternative possible explanations of this type of effect (for example, the mutation may inactivate a regulatory element essential for expression of both enzyme activities or may be a polar mutation in an operon containing the structural genes for both enzymes). Analysis of the WE29 phenotype has provided data substantiating the significance of the GS/GOGAT sequence in inorganic N assimilation by R. capsulata, and further study of the mutants described will hopefully give new insights into metabolic regulation by citric acid cycle intermediates.
Acknowledgments

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References