The Occurrence and Genetics of Some CO₂ Mutants in *Streptomyces coelicolor*

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SUMMARY

We have isolated auxotrophic mutants of *Streptomyces coelicolor* which can grow on a minimal medium without growth factors when the gas phase is supplemented with CO₂. Usually they have an alternative requirement for a specific growth factor such as arginine, purines or vitamins. Some of the CO₂ mutants resemble those already known in *Neurospora crassa* and *Escherichia coli* but others present novel phenotypes.

INTRODUCTION

Carbon dioxide is present in ordinary air at a concentration of at least 0.03 % (v/v). It is essential for the growth, or initiation of growth, of bacteria and other micro-organisms (Valley & Rettger, 1927; Rockwell & Highberger, 1927). Recently it was shown that high concentrations of CO₂ in air stimulated the growth of some auxotrophic mutants of the fungus *Neurospora crassa* (Charles, 1962; Reissig & Nazario, 1962). Mutants stimulated by CO₂ were called CO₂ mutants (Charles & Broadbent, 1964). The purpose of the present study, and a related investigation on *Escherichia coli* (Charles & Roberts, 1968), was to see whether CO₂ mutants occurred in protokaryotic organisms. *Streptomyces coelicolor* was chosen because refined genetic analysis is possible (Hopwood, 1967). CO₂ mutants were obtained in this investigation; these are discussed in relation to mutants obtained in other micro-organisms.

METHODS

*Media.* Media used were the minimal (MM) and complete (CM) media of Hopwood (1967). Glucose was autoclaved separately as a 50 % (w/v) solution and added to each medium just before pouring into Petri dishes.

*Culture methods.* Incubation was at 30°, unless stated otherwise. When it was necessary to incubate the cultures in a gas phase other than air, 5 l. vacuum desiccators were used according to the procedure described by Broadbent & Charles (1965).

*Strains.* The strain of *Streptomyces coelicolor* in which mutants were induced and the multiply-marked mutant stocks used in linkage analysis were generously provided by Professor D. A. Hopwood. The terminology and mutant designations, and the procedures for making and analysing crosses, are those of Hopwood (1967). Mutants isolated during this study are identified by a v as a prefix to the isolation number.

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Isolation of CO₂ mutants. Mutants were induced by ultraviolet (u.v.) irradiation or by N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Spore suspensions were made by suspending spores, from the surface of 5 day slope cultures on CM, in liquid MM and filtering through non-absorbent cotton wool to remove large mycelial fragments. For u.v. mutagenesis the spore suspension was irradiated with a Camag universal u.v. lamp, to a survival of about 0.5%. For treatment with NTG the spore suspension was centrifuged and the spores suspended in 5 ml. liquid MM enriched with 2.5% (v/v) liquid CM; NTG was added (4 mg./ml.) and the suspension incubated at 37°C for 45 min. Liquid CM (5 ml.) was then added and incubation continued for 90 min.; this treatment killed about 99% of the spores. At the time of this investigation no enrichment procedure for the isolation of auxotrophic mutants of Streptomyces was known, nor were the recently discovered optimal conditions for mutagenesis by NTG (Delić, Hopwood & Friend, 1970).

Before CO₂ mutants could be sought the CO₂ requirement of the wild-type strain, A3(2), had to be determined. Growth was slightly inhibited in desiccators containing potassium hydroxide to remove CO₂ from the gas phase (Charles, 1964); for this reason CO₂ mutants were isolated on the basis of growth stimulation by CO₂ at concentrations greater than that in ordinary air.

Following mutagenic treatment, suitable dilutions of the spore suspensions were plated on either CM and incubated in air, or MM and incubated in air enriched with 10% (v/v) CO₂. After 5 days the colonies from each Petri dish were replicated to MM with velvet pads and the replicas incubated in air. Those colonies that failed to grow on the replicas were picked from the original plates and purified by streaking on CM. They were then each replicated to two plates of MM, one of which was incubated in air and the other in air+10% (v/v) CO₂. In this way CO₂-requiring mutants were distinguished from other auxotrophs. Cultures which grew better in the presence of CO₂ were isolated for further study. Many of the colonies that initially appeared to respond to CO₂ did not respond when tested again.

**Table 1. CO₂ mutants of Streptomyces coelicolor**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Locus</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>ade-v9</td>
<td></td>
<td>Requirement for purines or CO₂</td>
</tr>
<tr>
<td>arg-v7</td>
<td></td>
<td>Requirement for arginine, partially</td>
</tr>
<tr>
<td></td>
<td></td>
<td>satisfied by CO₂</td>
</tr>
<tr>
<td>cdx-v1</td>
<td>cdxA</td>
<td>Requirement for CO₂</td>
</tr>
<tr>
<td>nic-v1</td>
<td>nicB</td>
<td>Requirement for nicotinamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>partially satisfied by CO₂</td>
</tr>
<tr>
<td>pdx-v1</td>
<td>pdxA</td>
<td>Requirement for pyridoxine,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>partially satisfied by CO₂</td>
</tr>
</tbody>
</table>

**RESULTS**

Classes of CO₂ mutants. Four CO₂ mutants came from platings on MM in air+10% (v/v) CO₂ and one (ade-v9) from CM in air (Table 1). Three of these mutants defined new loci (cdxA, nicB, pdxA); their positions and those of mutants that have not yet been assigned to a locus are shown in Fig. 1.

It was found that a gas phase of air+10% (v/v) CO₂ stimulated the best growth of the CO₂ mutants. All the mutants responded moderately to CO₂; none grew as quickly
Streptomyces CO₂ mutants

with CO₂ as did the parental strain without it. Mutant cdxAv~ showed the strongest stimulation by CO₂ and was of special interest because no other substance has so far been found to replace CO₂ as a growth factor for it. The remaining four mutants grew well without supplementary CO₂ when supplied with other growth factors; growth stimulations by these other substances were generally better than by CO₂.

Adenine/CO₂ mutant ade-vg. This mutant grew well when supplied with adenine or supplementary CO₂, and to a lesser extent with guanine, hypoxanthine or xanthine. The mutation was mapped by the method of haploid recombinant selection (Hopwood, 1967) as follows. The mutant, ade-vg, was crossed with strain 876 carrying a series of well-separated markers, and spores from the mixed culture were plated on a medium selecting distant markers, hisCg+ and strA1. Fig. 2 shows the marker arrangements in the parental strains and gives the results. The allele ratios for non-selected markers amongst the 142 selected recombinants indicated a position for ade-vg either between proA and pheA (as shown) or between argA and cysC. The former location was indicated by a consideration of the frequencies of individual segregant classes: with this location only one recombinant (ade, pro), required more than the minimum number of one crossover in each arc between the selected markers, whereas with the alternative location 16 recombinants (the pro phe and phe classes) required complex crossover patterns.

A sample of 27 adenine segregants all responded well to air + 10 % (v/v) CO₂ in the absence of adenine, suggesting that the adenine/CO₂ phenotype resulted from a single mutation.

Fig. 1. Streptomyces coelicolor. Linkage map showing on the outside of the circle the location of previously known markers mentioned in text, and on the inside of the circle, the location of the CO₂ mutants. The marker designations are given by Hopwood (1967) with the exception of adeC which has a requirement for purines.
An adenine locus had not been defined in the region of ade-v9, but another adenine mutant isolated during this study, ade-vr, not a CO₂ mutant, also mapped in this region. Recently a new locus, adeC, has been defined between cysA and proA (E. J. Friend, personal communication) and it seems probable that ade-v9 is either at this locus or near it.

Arginine/CO₂ mutant arg-v7. This mutant was slightly leaky, in that some growth occurred on MM without supplementary CO₂ or arginine. Arginine allowed good growth and CO₂ gave a weaker stimulation. Ornithine and citrulline were not stimulatory. Arginine/CO₂ mutants are known in Neurospora crassa (Charles, 1962) and


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Escherichia coli (Charles & Roberts, 1968). The Streptomyces coelicolor mutant differed from the mutants in N. crassa and E. coli in not responding to citrulline, not having its response to CO₂ inhibited by pyrimidines, and not having its response to arginine further enhanced by pyrimidines. In N. crassa and E. coli the CO₂ stimulation is explained in terms of a defect in carbamyl phosphate (CAP) synthesis which is overcome, directly or indirectly, by CO₂. This explanation requires that the mutants should respond to citrulline. It is probable that the S. coelicolor mutant arg-v7 has the same defect: failure to respond to citrulline is expected because arginine mutants of S. coelicolor that grow on ornithine respond very poorly to citrulline (Hopwood, 1967), suggesting a possible impermeability to citrulline. CAP is also required for pyrimidine synthesis, and the failure of mutant arg-v7 to be inhibited or stimulated by pyrimidines suggests differences in the regulatory effect of pyrimidines on CAP utilization in S. coelicolor.

A preliminary cross indicated a location for arg-v7 rather close to hisA1, either clockwise or anticlockwise. Three previously recorded loci, argA, B, C (Hopwood, 1967), lie clockwise of hisA, and an attempt was made to see if arg-v7 was closely linked with this cluster of arg loci. A strain carrying the arg-v7 mutation was crossed with multiply-marked strains carrying either argB2 or argC4, and spores were plated in parallel on media selecting for recombination between arg-v7+ and argB2+ or argC4+ on the one hand, and between the distant markers hisA+ and pheA+ on the other. Colony counts for the first selection were some 7 to 40 times lower than for the second, indicating close linkage between arg-v7 and the argB, C cluster. In both crosses arg-v7 was quite leaky in air, so that background growth on the Petri dish made it impossible to decide whether heteroclones (Sermonti, Mancinelli & Spada-Sermonti, 1960), which would have indicated complementation between the arg mutations (Hopwood, 1967), were present on the medium selecting for recombination between pairs of arg loci. Consequently it was not possible to decide whether arg-v7 was an allele of either argB or argC. Mutant arg-v7 resembles mutants at argB in responding to arginine but not to ornithine or citrulline; mutants at argB have not been tested for a CO₂ response.

Nicotinamide|CO₂ mutant nic-v1. This mutant responded moderately to CO₂ on MM, and nicotinamide caused growth stimulation which was not further increased by CO₂. Some other nic mutants (nicA1, nicA3, nic-v2, nic-v3, nic-v4) were tested for stimulation by CO₂ on MM but did not show it. The nic-v1 mutant was crossed with strain 876 and argA1+, strA1 recombinants were selected (Fig. 3). The results indicated a location for the nic-v1 mutation between proA and hisC, that is in a clearly different region from the previously known nicA locus; nic-v1 therefore defined a new locus, nicB.

Pyridoxine|CO₂ mutant pdx-v1. This mutant showed a weak to moderate response to supplementary CO₂; pyridoxal, pyridoxine or pyridoxamine caused better growth stimulations. This was the first pyridoxine mutant of Streptomyces coelicolor to be studied. The results of a cross between strain 876 and pdx-v1 (Fig. 4) indicated a location for the pdx-v1 mutation clockwise of argA at a locus designated pdxA.

CO₂ mutant cdx-v1. This mutant did not grow on MM except when the air was supplemented with 10% (v/v) CO₂. Slow growth occurred on CM without supplementary CO₂, and yeast extract was implicated as a source of an alternative growth factor; auxanographic experiments did not reveal its identity. Yeast extract was a
less effective growth factor than CO₂ in two ways: it caused only a small proportion
(about 10%) of the plating units in a suspension to give rise to colonies, as compared
with CO₂, and many of the resulting colonies were very small. These defects of growth
on MM + yeast extract, as compared with CO₂, may perhaps be attributed to delayed
germination.

Because mutant cdx-vI required CO₂ for uniform colony initiation and growth it
was necessary, in mapping experiments, to incubate the cultures in a gas-phase con-
taining 10% (v/v) CO₂; so far as could be determined this did not adversely affect the
growth of any of the segregants. A cross (Fig. 5) located cdx-vI close to argA, and

![Diagram](image)

**Fig. 4. Location of S. coelicolor mutant pdx-vI.** Results of a cross between CO₂ mutant
dpx-vI (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele
frequencies in a sample of recombinants containing the selected markers indicated by
triangles.

<table>
<thead>
<tr>
<th>Segregant phenotype</th>
<th>Number</th>
<th>Segregant phenotype</th>
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</thead>
<tbody>
<tr>
<td>pdx, phe, pro, cys</td>
<td>40</td>
<td>pdx, pro, phe</td>
<td>10</td>
</tr>
<tr>
<td>pdx, phe, cys</td>
<td>49</td>
<td>arg, pro, cys</td>
<td>2</td>
</tr>
<tr>
<td>pdx, phe</td>
<td>23</td>
<td>arg, cys</td>
<td>5</td>
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<tr>
<td>pdx, cys</td>
<td>13</td>
<td>phe</td>
<td>2</td>
</tr>
<tr>
<td>pdx</td>
<td>22</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>arg, phe, pro, cys</td>
<td>21</td>
<td>arg, pdx, pro, phe, cys</td>
<td>1</td>
</tr>
<tr>
<td>arg, phe, cys</td>
<td>13</td>
<td>cys</td>
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</tr>
<tr>
<td>phe, cys</td>
<td>5</td>
<td>Total</td>
<td>208</td>
</tr>
</tbody>
</table>

**Fig. 5. Location of S. coelicolor mutant cdx-vI.** Results of a cross between CO₂ mutant
cdx-vI (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele
frequencies in a sample of recombinants containing the selected markers indicated by
triangles.

<table>
<thead>
<tr>
<th>Segregant phenotype</th>
<th>Number</th>
<th>Segregant phenotype</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>cys, cdx</td>
<td>10</td>
<td>pro, phe, cys, cdx</td>
<td>1</td>
</tr>
<tr>
<td>cdx</td>
<td>34</td>
<td>arg</td>
<td>1</td>
</tr>
<tr>
<td>pro, cdx</td>
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<td>cys</td>
<td>1</td>
</tr>
<tr>
<td>phe, cdx</td>
<td>1</td>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>
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further analysis suggested a location clockwise of argA, that is in the region of uraB,C,D. The mutant is provisionally accepted as representative of a new locus, designated cdxA. Mutations at uraD result in a requirement for arginine + uracil (Hopwood, 1967) probably due to defective CAP synthesis. In *Escherichia coli* some mutations at the equivalent locus cause a CO₂ requirement (Charles & Roberts, 1968; M. Mergeay, personal communication) and this should also be true of uraD in *Streptomyces coelicolor*, in which case there may be a functional relationship between cdxA and uraD. A possible alternative explanation of the CO₂ response in *cdx-vt* is considered in the Discussion.

DISCUSSION

CO₂ mutants have previously been investigated in the mould *Neurospora crassa* (Broadbent & Charles, 1965; Broadbent, 1965; de Serres, 1966), and in the eubacterium *Escherichia coli* (Charles & Roberts, 1968). The present study has shown that they can also be isolated in *Streptomyces coelicolor*, a protokaryote not closely related to *E. coli*, suggesting that CO₂ mutants are probably of general occurrence in microorganisms.

So far the CO₂ mutants that are most easily understood are those defective in the incorporation of CO₂ into a biosynthetic sequence, for example that of adenine (Broadbent, 1965; de Serres, 1966) or arginine (Charles, 1962, 1964; Charles & Broadbent, 1964; Charles & Roberts, 1968). The adenine/CO₂ mutant of *Streptomyces coelicolor* may be of this type. Many other CO₂ mutants (Broadbent, 1965; Roberts, 1968; Vivian, 1968; H. P. Charles, unpublished results) offer no obvious explanations for their response to CO₂. The vitamin/CO₂ mutants of *S. coelicolor* are examples: no CO₂ incorporation reaction has been shown to be involved in the biosynthesis of either nicotinamide or pyridoxine. Possibly CO₂ causes growth without being incorporated into the final product of the defective biosynthetic sequence, for example by a regulatory function. CO₂ has recently been shown to have a regulatory function in an inducible anaerobic enzyme system in *Escherichia coli* by Swanson & Ogg (1969), who concluded that, under appropriate conditions, CO₂ could function either as a metabolic stimulator or as an inhibitor. Alternatively, growth might be possible because of a partial restoration of activity to a mutant enzyme by an effect of CO₂.

Some answers to the more fundamental aspects of CO₂ metabolism, particularly the dependence of heterotrophic organisms upon CO₂ for growth (Valley & Rettger, 1927; Rockwell & Highberger, 1927) might come from a study of some of the CO₂ mutants poorly understood at present. The work of Lwoff & Monod (1947) and Ajl & Werkman (1949) indicated that the CO₂ requirement of heterotrophs was partly due to the incorporation of CO₂ in biosynthetic reactions, although CO₂ could not be entirely replaced as a growth factor under all conditions of inoculum size. If wild-type *Streptomyces coelicolor* requires small quantities of CO₂ in essential reactions for which no other growth factor will substitute, the CO₂ requirement of *cdx-vt* might result from an enzyme involved in the utilization of CO₂ having a decreased affinity for CO₂, or because some defect of permeability hinders the uptake of CO₂. This is an interesting possibility because CO₂, usually in low concentration, is required for uniform germination of many micro-organisms, and this is probably true of *S. coelicolor* spores. The mutant *cdx-vt* may have a defect in the germination process causing an increased requirement for CO₂. The problem of germination of spores of *S. coelicolor*
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is complicated because a spore suspension contains not only separate spores, but also clumps of spores and mycelial fragments. Bacteria in clumps require less exogenously supplied \( \text{CO}_2 \) for initiation of growth (Gladstone, Fildes & Richardson, 1935) presumably because the \( \text{CO}_2 \) they themselves generate may reach a higher concentration within a clump. The colonies which arise on yeast-extract medium may arise from clumps which may generate a sufficiently high concentration of \( \text{CO}_2 \) from the yeast extract to initiate growth.

\( \text{CO}_2 \) has been found to have some effect on morphogenesis in various organisms, from moulds (Bartnicki-Garcia & Nickerson, 1962) to coelenterates (Loomis, 1961). Hopwood (1967) has pointed out the suitability of *Streptomyces coelicolor* for the study of morphogenesis in protokaryote systems. It could be worth future investigation to see whether \( \text{CO}_2 \) does have any significance as a morphogenetic agent in *S. coelicolor*. A start has been made towards unravelling the development of the aerial stages of growth in *S. coelicolor* (Wildermuth, 1970; Wildermuth & Hopwood, 1970) by the study of mutants defective in sporulation (Hopwood, Wildermuth & Palmer, 1969); it would be interesting to see if the defects in some of these mutants could be overcome by supplementary \( \text{CO}_2 \).

This investigation was supported by a grant from the Agricultural Research Council. We wish to thank Professor D. A. Hopwood for instructing one of us (A. V.) in the techniques of crossing in *Streptomyces coelicolor* and for his advice during the preparation of this manuscript. We thank Mr I. McMurray for technical assistance.

**REFERENCES**


Streptomyces CO₃ mutants


