Journal of General Microbiology (1970), **61**, 263–271
Printed in Great Britain

The Occurrence and Genetics of Some CO_2 Mutants in *Streptomyces coelicolor*

By A. VIVIAN* AND H. P. CHARLES
Microbiology Department, University of Reading, London Road, Reading, Berkshire, RG 1 5 AQ

(*Accepted for publication 29 January 1970*)

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 CO_2. Usually they have an alternative requirement for a specific growth factor such as arginine, purines or vitamins. Some of the CO_2 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 CO_2 in air stimulated the growth of some auxotrophic mutants of the fungus *Neurospora crassa* (Charles, 1962; Reissig & Nazario, 1962). Mutants stimulated by CO_2 were called CO_2 mutants (Charles & Broadbent, 1964). The purpose of the present study, and a related investigation on *Escherichia coli* (Charles & Roberts, 1968), was to see whether CO_2 mutants occurred in protokaryotic organisms. *Streptomyces coelicolor* was chosen because refined genetic analysis is possible (Hopwood, 1967). CO_2 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.

* Present address: John Innes Institute, Colney Lane, Norwich NOR 70 F.

Isolation of CO_2 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° 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 CO_2 mutants could be sought the CO_2 requirement of the wild-type strain, A3(2), had to be determined. Growth was slightly inhibited in desiccators containing potassium hydroxide to remove CO_2 from the gas phase (Charles, 1964); for this reason CO_2 mutants were isolated on the basis of growth stimulation by CO_2 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) CO_2. 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) CO_2. In this way CO_2-requiring mutants were distinguished from other auxotrophs. Cultures which grew better in the presence of CO_2 were isolated for further study. Many of the colonies that initially appeared to respond to CO_2 did not respond when tested again.

Table 1. *CO_2 mutants of Streptomyces coelicolor*

Mutation	Locus	Characteristics
ade-v9	—	Requirement for purines or CO_2
arg-v7	—	Requirement for arginine, partially satisfied by CO_2
cdx-v1	*cdxA*	Requirement for CO_2
nic-v1	*nicB*	Requirement for nicotinamide partially satisfied by CO_2
pdx-v1	*pdxA*	Requirement for pyridoxine, partially satisfied by CO_2

RESULTS

Classes of CO_2 mutants. Four CO_2 mutants came from platings on MM in air + 10 % (v/v) CO_2 and one (*ade-v9*) from CM in air (Table 1). Three of these mutants defined new loci (*cdx*A, *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) CO_2 stimulated the best growth of the CO_2 mutants. All the mutants responded moderately to CO_2; none grew as quickly

with CO_2 as did the parental strain without it. Mutant *cdxAv1* showed the strongest stimulation by CO_2 and was of special interest because no other substance has so far been found to replace CO_2 as a growth factor for it. The remaining four mutants grew well without supplementary CO_2 when supplied with other growth factors; growth stimulations by these other substances were generally better than by CO_2.

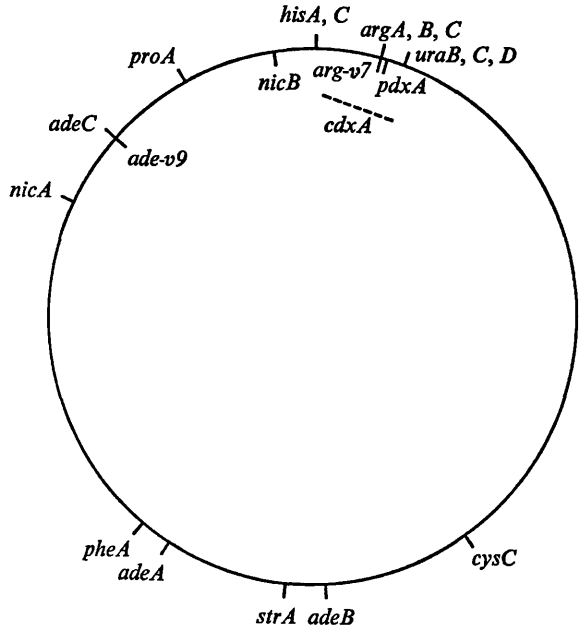

Fig. 1. *Streptomyces coelicolor*. Linkage map showing on the outside of the circle the location of previously known markers mentioned in text, and on the inside of the circle, the location of the CO_2 mutants. The marker designations are given by Hopwood (1967) with the exception of *adeC* which has a requirement for purines.

Adenine/CO_2 mutant ade-v9. This mutant grew well when supplied with adenine or supplementary CO_2, 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-v9*, 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, *hisC9*$^+$ 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-v9* 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) CO_2 in the absence of adenine, suggesting that the adenine/CO_2 phenotype resulted from a single mutation.

An adenine locus had not been defined in the region of *ade-v9*, but another adenine mutant isolated during this study, *ade-v1*, not a CO_2 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.

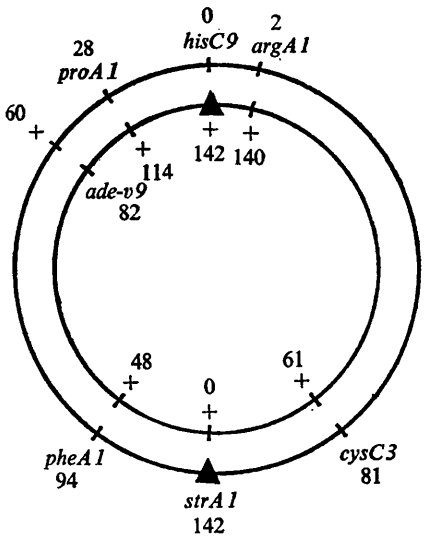

Fig. 2

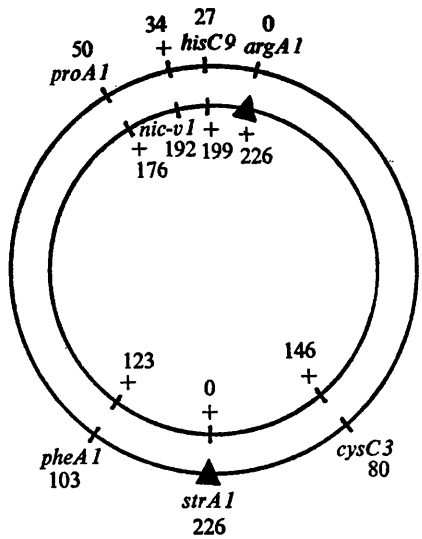

Fig. 3

Fig. 2. Location of *S. coelicolor* mutant *ade-v9*. Results of a cross between CO_2 mutant *ade-v9* (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.

Segregant phenotype	Number	Segregant phenotype	Number
arg, pro, phe, cys	2	*ade, phe, cys*	20
pro, phe, cys	19	*ade, phe*	14
pro, phe	6	*ade, cys*	17
phe, cys	23	*ade*	30
phe	10	*ade, pro*	1
		Total	142

Fig. 3. Location of *S. coelicolor* mutant *nic-v1*. Results of a cross between CO_2 mutant *nic-v1* (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.

Segregant phenotype	Number	Segregant phenotype	Number
nic	97	*nic, pro, phe, cys*	10
nic, cys	26	*pro, phe*	7
nic, phe, cys	31	*pro, phe, his*	14
nic, phe	22	*pro,phe, his, cys*	13
nic, pro, phe	6	Total	226

Arginine/CO_2 mutant arg-v7. This mutant was slightly leaky, in that some growth occurred on MM without supplementary CO_2 or arginine. Arginine allowed good growth and CO_2 gave a weaker stimulation. Ornithine and citrulline were not stimulatory. Arginine/CO_2 mutants are known in *Neurospora crassa* (Charles, 1962) and

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 CO_2 inhibited by pyrimidines, and not having its response to arginine further enhanced by pyrimidines. In *N. crassa* and *E. coli* the CO_2 stimulation is explained in terms of a defect in carbamyl phosphate (CAP) synthesis which is overcome, directly or indirectly, by CO_2. 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 *hisA1*$^+$ 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 CO_2 response.

Nicotinamide/CO_2 mutant nic-v1. This mutant responded moderately to CO_2 on MM, and nicotinamide caused growth stimulation which was not further increased by CO_2. Some other *nic* mutants (*nicA1*, *nicA3*, *nic-v2*, *nic-v3*, *nic-v4*) were tested for stimulation by CO_2 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/CO_2 mutant pdx-v1. This mutant showed a weak to moderate response to supplementary CO_2; 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*.

CO_2 mutant cdx-v1. This mutant did not grow on MM except when the air was supplemented with 10% (v/v) CO_2. Slow growth occurred on CM without supplementary CO_2, 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 CO_2 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 CO_2, and many of the resulting colonies were very small. These defects of growth on MM + yeast extract, as compared with CO_2, may perhaps be attributed to delayed germination.

Because mutant *cdx-v1* required CO_2 for uniform colony initiation and growth it was necessary, in mapping experiments, to incubate the cultures in a gas-phase containing 10 % (v/v) CO_2; so far as could be determined this did not adversely affect the growth of any of the segregants. A cross (Fig. 5) located *cdx-v1* close to *argA*, and

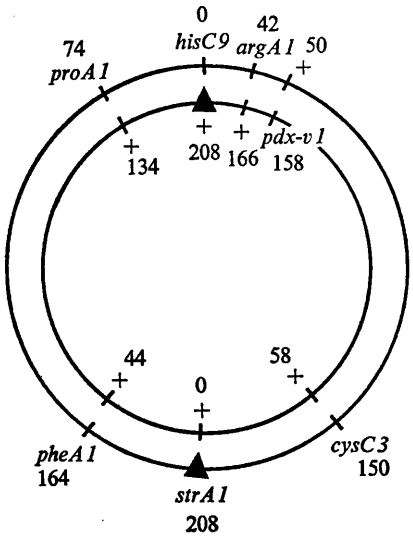

Fig. 4

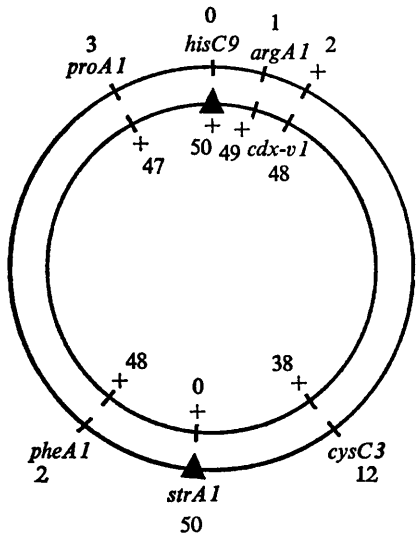

Fig. 5

Fig. 4. Location of *S. coelicolor* mutant *pdx-v1*. Results of a cross between CO_2 mutant *pdx-v1* (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.

Segregant phenotype	Number	Segregant phenotype	Number
pdx, phe, pro, cys	40	*pdx, pro, phe*	10
pdx, phe, cys	49	*arg, pro, cys*	2
pdx, phe	23	*arg, cys*	5
pdx, cys	13	*phe*	2
pdx	22	+	1
arg, phe, pro, cys	21	*arg, pdx, pro, phe, cys*	1
arg, phe, cys	13	*cys*	1
phe, cys	5	Total	208

Fig. 5. Location of *S. coelicolor* mutant *cdx-v1*. Results of a cross between CO_2 mutant *cdx-v1* (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.

Segregant phenotype	Number	Segregant phenotype	Number
cys, cdx	10	*pro, phe, cys, cdx*	1
cdx	34	*arg*	1
pro, cdx	2	*cys*	1
phe, cdx	1	Total	50

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 CO_2 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 CO_2 response in *cdx-v1* is considered in the Discussion.

DISCUSSION

CO_2 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 CO_2 mutants are probably of general occurrence in micro-organisms.

So far the CO_2 mutants that are most easily understood are those defective in the incorporation of CO_2 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/CO_2 mutant of *Streptomyces coelicolor* may be of this type. Many other CO_2 mutants (Broadbent, 1965; Roberts, 1968; Vivian, 1968; H. P. Charles, unpublished results) offer no obvious explanations for their response to CO_2. The vitamin/CO_2 mutants of *S. coelicolor* are examples: no CO_2 incorporation reaction has been shown to be involved in the biosynthesis of either nicotinamide or pyridoxine. Possibly CO_2 causes growth without being incorporated into the final product of the defective biosynthetic sequence, for example by a regulatory function. CO_2 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, CO_2 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 CO_2.

Some answers to the more fundamental aspects of CO_2 metabolism, particularly the dependence of heterotrophic organisms upon CO_2 for growth (Valley & Rettger, 1927; Rockwell & Highberger, 1927) might come from a study of some of the CO_2 mutants poorly understood at present. The work of Lwoff & Monod (1947) and Ajl & Werkman (1949) indicated that the CO_2 requirement of heterotrophs was partly due to the incorporation of CO_2 in biosynthetic reactions, although CO_2 could not be entirely replaced as a growth factor under all conditions of inoculum size. If wild-type *Streptomyces coelicolor* requires small quantities of CO_2 in essential reactions for which no other growth factor will substitute, the CO_2 requirement of *cdx-v1* might result from an enzyme involved in the utilization of CO_2 having a decreased affinity for CO_2, or because some defect of permeability hinders the uptake of CO_2. This is an interesting possibility because CO_2, 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-v1* may have a defect in the germination process causing an increased requirement for CO_2. The problem of germination of spores of *S. coelicolor*

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 CO_2 for initiation of growth (Gladstone, Fildes & Richardson, 1935) presumably because the 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 CO_2 from the yeast extract to initiate growth.

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 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 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

AJL, S. J. & WERKMAN, C. H. (1949). Anaerobic replacement of carbon dioxide. *Proceedings of the Society for Experimental Biology and Medicine* **70**, 522.

BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1962). Induction of yeast-like development in *Mucor* by carbon dioxide. *Journal of Bacteriology* **84**, 829.

BROADBENT, J. A. (1965). *Carbon dioxide-requiring mutants in Neurospora crassa.* Ph.D. thesis, Reading University, England.

BROADBENT, J. A. & CHARLES, H. P. (1965). Some carbon dioxide-requiring mutants of *Neurospora crassa. Journal of General Microbiology* **39**, 63.

CHARLES, H. P. (1962). Response of Neurospora mutants to carbon dioxide. *Nature, London* **195**, 359.

CHARLES, H. P. (1964). Relationships between certain pyrimidine and arginine mutants of Neurospora, as revealed by their response to carbon dioxide. *Journal of General Microbiology* **34**, 131.

CHARLES, H. P. & BROADBENT, J. A. (1964). Carbon dioxide mutants: a large and interesting class of Neurospora mutants. *Nature, London* **201**, 1004.

CHARLES, H. P. & ROBERTS, G. A. (1968). Carbon dioxide as a growth factor for mutants of *Escherichia coli. Journal of General Microbiology* **51**, 211.

DELIĆ, V., HOPWOOD, D. A. & FRIEND, E. J (1970). Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in *Streptomyces coelicolor. Mutation Research* **9**, 167.

DE SERRES, F. J. (1966). Carbon dioxide stimulation of the *ad*-3 mutants of *Neurospora crassa. Mutation Research* **3**, 420.

GLADSTONE, G. P., FILDES, P. & RICHARDSON, G. M. (1935). Carbon dioxide as an essential factor in the growth of bacteria. *British Journal of Experimental Pathology* **16**, 335.

HOPWOOD, D. A. (1967). Genetic analysis and genome structure in *Streptomyces coelicolor. Bacteriological Reviews* **31**, 373.

HOPWOOD, D. A., WILDERMUTH, H. & PALMER, H. M. (1969). A morphogenetic system in *Streptomyces coelicolor. Heredity, London* **24**, 517.

LOOMIS, W. F. (1961). Feedback factors affecting sexual differentiation in *Hydra littoralis. The Biology of Hydra and of Some Other Coelenterates*, p. 337. Edited by H. M. Lenhoff & W. F. Loomis, Coral Gables, Florida, U.S.A.: University of Miami Press.

LWOFF, A. & MONOD, J. (1947). Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Annales de l'Institut Pasteur, Paris* **73**, 323.

REISSIG, J. L. & NAZARIO, M. (1962). Regulación genetica de la sintesis de arginina y uridina. *Sesiones Cientificas de Biologica*, p. 76. Cordoba, Argentina.

ROBERTS, G. A. (1968). *Studies on carbon dioxide stimulated and carbon dioxide inhibited mutants of Neurospora crassa and Escherichia coli.* Ph.D. thesis, Reading University, England.

ROCKWELL, G. E. & HIGHBERGER, J. H. (1927). The necessity of carbon dioxide for the growth of bacteria, yeasts and moulds. *Journal of Infectious Diseases* **40**, 438.

SERMONTI, G., MANCINELLI, A. & SPADA-SERMONTI, I. (1960). Heterogeneous clones ('heteroclones') in *Streptomyces coelicolor* A3(2). *Genetics, Princeton* **45**, 669.

SWANSON, H. D. & OGG, J. E. (1969). Carbon dioxide regulation of formate hydrogenlyase in *Escherichia coli. Biochemical and Biophysical Research Communications* **36**, 567.

VALLEY, G. & RETTGER, L. F. (1927). The influence of carbon dioxide on bacteria. *Journal of Bacteriology* **14**, 101.

VIVIAN, A. (1968). *Carbon dioxide-requiring mutants in Neurospora crassa and Streptomyces coelicolor.* Ph.D. thesis, Reading University, England.

WILDERMUTH, H. (1970). Development and organization of the aerial mycelium in *Streptomyces coelicolor. Journal of General Microbiology* **60**, 43.

WILDERMUTH, H. & HOPWOOD, D. A. (1970). Septation during sporulation in *Streptomyces coelicolor. Journal of General Microbiology* **60**, 51.