APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1979, p. 237-240
0099-2240/79/08-0237/04$02.00/0

Vol. 38, No. 2

Effect of Carbon Dioxide on Growth of *Pseudomonas fluorescens*

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Received for publication 17 May 1979

In minimal medium at 30°C, growth of *Pseudomonas fluorescens* was stimulated when the pressure (p) of CO_2 in solution was 100 mm of Hg, but at higher concentrations the growth rate declined linearly with increasing pCO_2. All concentrations of CO_2 were inhibitory for growth in complex medium, and at 30°C the maximum degree of inhibition was attained when pCO_2 was 250 mm of Hg. The degree of inhibition at a constant pCO_2 in solution increased with decreasing temperature. The degree of inhibition was directly proportional to temperature for growth in complex medium, but not in minimal medium. The inhibition of cell respiration by CO_2 was the same whether cells had been grown in air or in the presence of CO_2, indicating that adaptive enzyme synthesis does not occur in response to CO_2.

Carbon dioxide can both stimulate and inhibit growth of microorganisms (12). Stimulation of growth occurs because some anabolic reactions involve CO_2 fixation and, in the absence of an external source of the gas, CO_2 concentration in the cell can be rate limiting for these reactions, with resultant decreased growth rates (7). The basis of CO_2 inhibition has not been clearly established. Some reported inhibitory effects may have resulted from reduction of media pH, but a direct effect of CO_2 undoubtedly occurs (2).

The inhibitory effects of CO_2 have been exploited in the extension of storage life of fresh fruit and meat (9, 11). The increasing use of vacuum packages for preservation of fresh meat has stimulated renewed interest in the effect of CO_2 on development of spoilage floras at chill temperatures (−2 to 5°C). Although most workers observed significant extensions of storage life in atmospheres containing 10 to 20% CO_2, the detailed results are somewhat conflicting (1, 4, 8, 10). These differences may be the result of differences in the composition of the spoilage floras, but they could also arise because of poor definition of the conditions to which the bacteria were exposed. Bacteria respond to the concentration of CO_2 in solution, which varies with the pH and temperature of the medium. There have been no attempts to determine the actual concentrations of CO_2 to which spoilage bacteria are exposed in practical situations. Moreover, there are few detailed quantitative data available on inhibition by CO_2 of individual bacterial species. We have therefore examined the effect of CO_2 on growth of *Pseudomonas fluorescens*, a major food spoilage organism, cultured under controlled conditions.

MATERIALS AND METHODS

Culture conditions. *P. fluorescens* PDD 3513 was grown on minimal medium containing (grams/liter) $Na_2HPO_4 \cdot 12H_2O$, 12.9; KH_2PO_4, 3.1; $(NH_4)_2SO_4$, 0.2; glucose, 2.0; nitrilotriacetic acid, 0.1; $MgSO_4 \cdot 7H_2O$, 0.04; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.02; $CaCl_2$, 0.01; or on the same medium supplemented with (grams/liter) Casamino Acids, 0.5; and yeast extracts, 0.5.

The inoculum was taken from a continuous culture growing under glucose limitation at the maximum dilution rate obtainable with the conditions of temperature, pH, and medium composition which were to be used in the experiment. Growth rates were determined from the increase in optical density at 550 nm (OD_{550}) of cultures growing in a magnetically stirred vessel of a 500-ml working volume, fitted with Radiometer (Copenhagen) CO_2 and pH electrodes, and a New Brunswick (New Brunswick Scientific Co., New Brunswick, N.J.) oxygen electrode for continuous monitoring of pH, the pressure (p) of CO_2, and pO_2 in the liquid phase.

Mixtures of O_2, N_2, and CO_2 were prepared by adjusting the flow rates of these gases to a 5-liter vessel, where they were mixed before passing to the culture vessel at 200 ml/min. The oxygen concentration of the gas was maintained at 10% (vol/vol) throughout the experiments. The medium was equilibrated with the gas mixture, and the pH was adjusted to the required value by addition of 2 N NaOH before inoculating with sufficient cell suspension to give an initial OD_{550} of about 0.1.

Oxygen consumption. The respiration of washed cell suspensions was measured in an oxygen electrode cell with a volume of 1.9 ml, using a Beckman oxygen

analyzer (model 777) connected to a chart recorder (Hitachi QD 25).

The cell was maintained at 30°C and filled with 10 mM phosphate buffer or with CO_2-bicarbonate buffer in equilibrium with a gas phase of 80% CO_2–20% O_2, which was diluted with phosphate buffer when filling the cell, to give the required CO_2 concentrations. Both buffers were at pH 7.0. Cell suspensions (0.05 ml) and substrate solutions (0.1 M, 0.1 ml) were injected into the sealed cell as required.

RESULTS

Addition of CO_2 to cultures growing at 30°C in either minimal or complex medium caused an immediate reduction in growth rate. This initial inhibition increased with increasing CO_2 concentration, and in minimal medium a lag phase was induced at the highest CO_2 concentration used (450 mm of Hg). Over a period, which lengthened with increasing CO_2 concentration, growth rates rose to constant values lower than the growth rates in air, except for cells growing in minimal medium, which attained a higher growth rate with low CO_2 concentrations (<100 mm of Hg) than they did in air. Low concentrations of CO_2 produced a large decrease of the steady growth rate of cultures in complex medium (Fig. 1). With higher CO_2 concentrations the growth rates in minimal medium decreased linearly with increasing CO_2 concentration, but in complex medium maximum inhibition was attained with a CO_2 concentration of 250 mm of Hg (Fig. 2).

The inhibitory effect of CO_2 became more pronounced with decreasing temperature. In complex medium the degree of inhibition increased linearly with decreasing temperature, but in minimal medium the effect of temperature was greatest in the middle of the growth temperature range (Fig. 3). At high CO_2 concentrations and low temperature (300 mm of Hg; 3°C), no growth was observed in minimal medium during 72 h of incubation, although growth readily occurred in complex medium.

In both media, the degrees of inhibition at 30°C were unaffected by changes in pH between 6.0 and 7.5 when a constant pCO_2 in solution (150 mm of Hg) was maintained.

Inhibition of respiration by CO_2. Endogenous respiration was not inhibited by the presence of CO_2, but was stimulated at low CO_2 concentrations. With glucose or glucose and yeast extract as substrates, the inhibitions of respiration with increasing CO_2 concentration

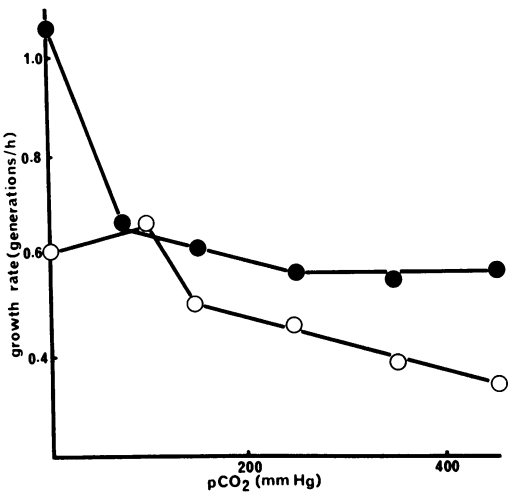

FIG. 2. *Effect of CO_2 concentration in solution on the rate of growth of P. fluorescens at 30°C in simple (○) or complex (●) medium.*

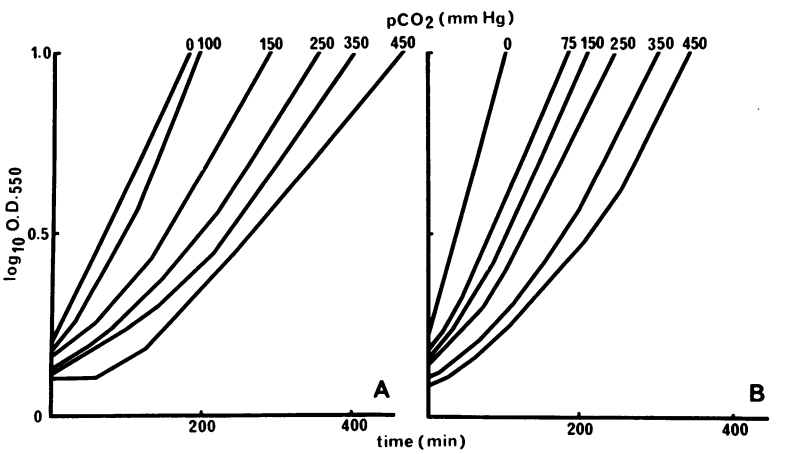

FIG. 1. *Growth of P. fluorescens at 30°C in (A) simple or (B) complex medium in air and with various concentrations of CO_2 in solution.*

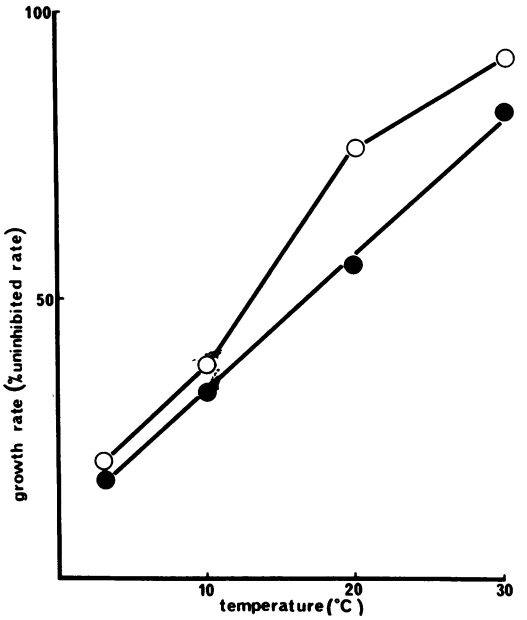

FIG. 3. *Effect of temperature on the growth rate of P. fluorescens growing in simple* (○) *or complex* (●) *medium with pCO_2 in solution at 150 mm of Hg.*

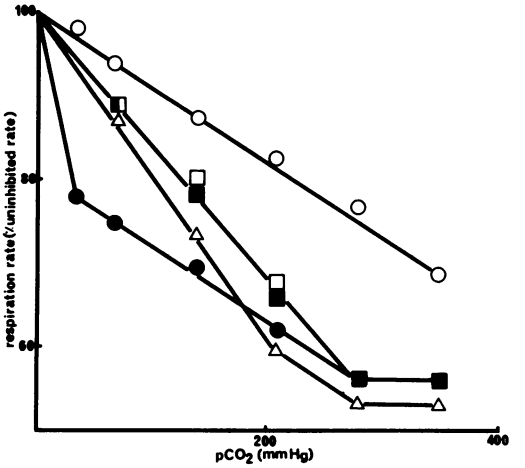

FIG. 4. *Effect of increasing concentrations of CO_2 on the respiration rates of P. fluorescens at 30°C oxidizing glucose* (○), *glucose and yeast extract* (●), *pyruvate* (△), *succinate* (□), *and malate* (■). *Uninhibited respiration rates were 138, 187, 78, 66, and 70 nmol of O_2/min per mg of cell (dry weight), respectively.*

were similar to the inhibition of growth rate observed in minimal and complex media, respectively. With other substrates, pyruvate, succinate, and malate, inhibition of respiration increased linearly with CO_2 concentration, but attained a maximum value when pCO_2 was about 250 mm Hg (Fig. 4). No difference in response was observed between cells grown in air or in an atmosphere containing 50% CO_2.

DISCUSSION

The only previous study of comparable detail on CO_2 inhibition of bacterial growth is that of King and Nagel (5). They grew *Pseudomonas aeruginosa* at a single suboptimum temperature (24°C) on minimal medium and concluded that inhibition varied linearly with CO_2 concentration. Although a similar relationship was observed with *P. fluorescens* growing at 30°C over a limited range of CO_2 concentrations, there were significant deviations. Notably, low CO_2 concentrations produced marked inhibition of growth in complex medium, but stimulated growth in minimal medium, and in complex medium a maximum degree of inhibition was attained at relatively low CO_2 concentrations. It is possible that a more detailed investigation of *P. aeruginosa* would reveal an equally complex response to CO_2. If other species respond similarly to CO_2 when growing on nutritionally complex substrates such as meat, this would explain why increasing the atmospheric content of CO_2 above 20% has little further inhibitory effect on spoilage flora growing at chill temperatures. However, it remains to be determined whether the response to CO_2 of the strain *P. fluorescens* used in this study can be regarded as typical for any group of spoilage bacteria.

Increase in the degree of CO_2 inhibition of mold growth with decreasing temperature has been reported to be due to the increased solubility of the gas at lower temperatures (3). It has been assumed that this observation is applicable to bacteria, but it is clear that with *P. fluorescens* there is a direct enhancement of CO_2 inhibition with decreasing temperature. The nonlinearity of the relationship between temperature and the degree of inhibition with minimal medium probably arise because of the competing inhibitory and stimulant effect of CO_2 during growth in this medium. Because the inhibitory effects of CO_2 can vary with temperature and medium composition, it is obviously not valid to compare results from different sources unless these factors are taken into account.

King and Nagel were unable to detect any change in enzyme levels between cells of *P. aeruginosa* grown in air and in the presence of CO_2 and concluded that adaptive enzyme synthesis in response to the presence of CO_2 does not occur (5). Our observation that the rates of respiration in air and in the presence of CO_2 were the same whether cells of *P. fluorescens* had been grown in the presence or absence of

CO_2 supports their conclusion. In attempting to define the mode of action of CO_2, King and Nagel could find no direct evidence of enzyme inhibition in vivo. However, on the basis of the linear increase in inhibition with increasing CO_2 concentrations of some enzymes in vitro, they concluded that CO_2 inhibits by a mass action effect on decarboxylating enzymes (6). This explanation seems plausible, although as yet it is based on inadequate circumstantial evidence. Our results indicate that a number of enzymes are affected by CO_2 in vivo as the pattern of inhibition varies with the nature of the substrate being metabolized. For a satisfactory explanation of the complex inhibitory effects of CO_2, further work will be necessary to identify the affected enzymes and the metabolic consequences of their inhibition by CO_2.

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