Culture of *Clostridium pasteurianum* in Defined Medium and Growth as a Function of Sulfate Concentration

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*Clostridium pasteurianum* strain W-5 was selected as an anaerobe which may be grown from large inocula in defined media with sulfate as its primary sulfur source. Since it is important to keep inocula small in minimizing transfer of sulfur sources, culture conditions were optimized. The medium devised decreased lag period and generation time when compared with other media, but growth could not be induced consistently with $6 \times 10^4$ cells per ml or less. Addition of trace elements, chelating agents, reducing agents, metabolites, and spent medium from various stages of growth did not stimulate growth from small inocula. Generation time was 85 min on inoculation with $10^4$ or more cells per ml taken from young stocks, but the lag period decreased somewhat with larger inocula. On the other hand, generation time and lag period increased with age of the inoculum. The total yield of cells increased when buffer capacity was increased. Growth of *C. pasteurianum* W-5 was dependent upon sulfate at relatively low sulfate concentrations, and the organism is thus suitable for study of sulfur metabolism. No evidence of a maintenance requirement for sulfate was detected.

In surveying sulfur metabolites in bacteria, an anaerobic species was desired which could be grown in a simple, defined medium with an inorganic sulfur source. *Clostridium pasteurianum* was selected since strain W-5 is widely used in biochemical studies and several defined media have been described for culture of this species (2–4, 7–9, 11). All of these media are based on salts, sucrose as energy source, biotin and $p$-aminobenzoic acid as cofactors with inorganic sulfate as the sulfur source; buffering is provided by phosphate or by supplements of CaCO$_3$ (2, 3, 8). We report here the relationship of growth of *C. pasteurianum* W-5 to sulfate concentration in a defined medium modified from that of Sargeant et al. (9).

**MATERIALS AND METHODS**

*C. pasteurianum* strain W-5 (no. 9486) was obtained from the National Collection of Industrial Bacteria. Reagents of analytical grade (Aristar) were used to prepare media in glass-distilled water. After investigating several media and methods of preparation, e.g., Carnahan and Castle (2), Carnahan et al. (3), and Lovenberg et al. (7), modifications of that of Sargeant et al. (9) were finally chosen. The best experimental medium (DM 11) was prepared from stock solutions (Table 1) which were sterilized separately to decrease reaction between components: solutions A, B, and E by autoclaving, D by filtration, and C by allowing to stand. To avoid precipitation of hydrous iron oxide, stock solutions were combined as follows. Solution A was swirled and 0.1 ml of solution C was added. Then, solution E (1 ml) was added to make the sulfate concentration 0.6 mM, unless otherwise stated. Finally, all of solution B and 0.4 ml of solution D were added. Solutions C and E could be stored indefinitely, but solution D was prepared fresh every 2 weeks.

Medium DM 11 and variations were not suitable for preservation of stock cultures because cold storage for a week led to erratic growth, but subculturing daily (Carnahan et al. [3]) or every 2 to 3 days was satisfactory. Stock cultures were maintained on the potato medium of Jensen and Spencer (6).

Experimental cultures were grown in 75 ml of DM 11 in 125-ml Erlenmeyer flasks. Flasks had cotton plugs (wrapped in cheese cloth) each carrying a plugged Pasteur pipette for passage of gas to mix the cell suspension and displace O$_2$. Either N$_2$ or 95% N$_2$-5% CO$_2$ was bubbled through each culture system to sweep out O$_2$. Media were inoculated and incubated at 30°C, and cultures were sampled with sterile pipettes. Cultures were always resparged to displace any O$_2$ admitted during inoculation and sampling. Rapidly growing cultures of *C. pasteurianum* produced enough gas to make continuous sparging unnecessary for cultures in well plugged flasks or tubes.

Growth was measured by turbidity (absorbance at
The medium of Carnahan et al. (3) has an initial pH of 6.6 to 6.9, and several other media (7, 9) provide even higher initial pH values at 7.5, presumably to counteract the reported rapid pH decline during growth. Sargeant et al. (9) stated that the pH should not fall below 5.6, and, although continuous adjustment of pH was recommended (4, 9) for large scale cultures, it is impractical in work of the present type. Media containing powdered CaCO₃ (2, 8) effectively control pH but are unsuitable for turbidity or dry weight measurements and microscopic counts.

A study of the effect of initial pH on culture (Fig. 1) revealed that growth rate was significantly decreased by an initial pH of 5.5 and much depressed at pH 7.2, even though this latter value was lower than that recommended by some workers (9). Both lag period and generation time were affected by pH 7.2; growth was optimal over the pH range of 5.8 to 6.5.

pH also influenced the final yield of cells with the maximum for an initial pH of 6.2 to 6.5 in 0.1 M phosphate buffer (Fig. 2). The total yield could be increased further by adjustment of buffering capacity. For example, 0.2 M phosphate produced 10% more cells, and addition of solid CaCO₃ to 0.1 M phosphate increased the

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**TABLE 1. Stock solutions for defined medium DM 11 used to culture C. pasteurianum**

| Solution | Ingredients                                                                 | ml combined |
|----------|-----------------------------------------------------------------------------|-------------|
| A        | Sucrose (20 g)                                                              | 800         |
|          | MgCl₂·6H₂O (200 mg)                                                         |             |
|          | Na₂MoO₄·2H₂O (10 mg)                                                        |             |
|          | NH₄Cl (660 mg)                                                              |             |
|          | H₂O (to 800 ml)                                                             |             |
| B        | KH₂PO₄ (10.9 g)                                                             | 200         |
|          | NH₄HPO₄ (2.84 g)                                                            |             |
|          | H₂O (to 200 ml)                                                             |             |
| C        | FeCl₃·6H₂O (1 g)                                                            | 0.1         |
|          | Absolute ethanol (20 ml)                                                    |             |
| D        | Biotin (1 mg)                                                                | 0.4         |
|          | p-Aminobenzoic acid (1 mg)                                                  |             |
|          | H₂O (20 ml)                                                                 |             |
| E        | (NH₄)₂SO₄ (8 g)                                                             | 1.0         |
|          | H₂O (to 100 ml)                                                             |             |

650 nm) determinations in 1-cm cuvettes with a Unicam SP 600 spectrophotometer. When necessary, samples were diluted to give readings in the range of 0.1 to 0.6. Calibration (dry weight and cell count) was linear to at least 4.0 absorbance at 650 nm, corresponding to 1.8 mg/ml and 1.2 x 10⁶ cells per ml. Cells were counted in a Petroff-Hauser chamber. For dry weights, aqueous washed suspensions were dried in vacuo for 24 h over P₂O₅. Cultures from acceleration, exponential, stationary, or declining growth phases fit the same calibration, suggesting that there is little change in cell size with growth phase. Generation times were calculated from the linear portions of semilogarithmic growth plots.

Original stocks were preserved as lyophilized stab or Petri plate cultures on clostridium agar or on agar plates of DM 11 in an anaerobic jar under hydrogen.

**RESULTS AND DISCUSSION**

As preliminary experiments revealed that not all factors important to culture of *C. pasteurianum* in simple defined medium had been identified, study of culture conditions was essential before utilization of sulfate could be investigated. Major problems included choice of initial pH and the level and nature of the inoculum culture. Large inocula (5 to 10%) used by most workers (H. C. Winter, personal communication) interfered with study of sulfate metabolism by decreasing the specific activities of labeled compounds. Unfortunately, *C. pasteurianum* failed to grow regularly in previously described media (2, 3, 7, 9) with 1% inocula. Therefore, the effect of pH was reexamined first.

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**Fig. 1. Effect of initial pH on growth of C. pasteurianum W-5. Medium DM 11 was modified by separate addition of Na₂HPO₄ and KH₂PO₄, solutions to produce 75-ml cultures of different initial pH. A 2% inoculum was employed after sparging with 95% N₂, 5% CO₂. Suspensions were incubated at 30°C with sparging continued. Samples were withdrawn aseptically for turbidity and pH measurement. For cultures in the most favorable pH range, the average was 127 min per generation.**
yield about 30% at two different initial pH values. The shortest generation time (127 min) in this type of experiment occurred during exponential growth in 0.1 M phosphate in the pH range 5.5 to 5.6. Contrary to a report (9) that this organism does not grow below pH 5.2, continuing growth was repeatedly observed until the pH declined to 4 or a little below.

It became evident during these studies that the nature of the inoculum played a major role in culture of *C. pasteurianum*. Figure 3 illustrates this influence in terms of the age of cultures used as inocula. A culture in DM 11 just approaching stationary phase was maintained under 95% N₂, 5% CO₂. Seven 4-ml samples were removed at intervals and used to inoculate 75-ml cultures of DM 11 which were incubated under 95% N₂, 5% CO₂ and subsequently used as inocula for the final cultures illustrated in Fig. 3. Of these seven, sample A was transferred from the still growing stock culture at a time designated zero and ultimately provided the oldest inoculum used in the last stage of the experiment; sample B was taken after 2 h, C after 4 h, D at 6.5 h, E at 9 h, and F and G after 13 h to form cultures subsequently providing the youngest inocula. Incubation was continued for 12 h after initiation of the youngest cultures (F and G). All had reached stationary growth phase with turbidity readings of 4.5 to 5.0. The two 12-h inocula had just grown to this level, whereas the others had been affected progressively more extensively by their exposure to low pH. One experimental growth culture was inoculated from each of these inoculum cultures (1.6 ml into 75 ml of DM 11) for the growth curves of Fig. 3. Both generation time and duration of lag period increased with age of the inoculum. CaCO₃, added with young inoculum G, increased generation time without much effect on the lag period, although it increased the total yield of cells. Generation times depended upon the age of the inoculum and were increased by addition of CaCO₃. A generation time of 84 min for the 12-h-old inoculum equaled the best achieved in the present work on *C. pasteurianum*. A 24-h subculture increased the lag period to 3 days.

The length of the lag phase and the generation time were dependent upon the size of inoculum used. With an inoculum of 0.5%, 6 x 10⁶ cells per ml, cultures did not grow consistently even though the subculture used was a young one. The lag period was substantially decreased by using moderate inocula, e.g., from 7 h in one group of experiments with inocula of 9 x 10⁸ cells per ml to 2 h with 17 x 10⁸ cells per
ml. Under the best conditions devised, the generation time increased markedly from a fairly constant minimal value of about 85 min as the inoculum size was decreased from about 10⁶ cells per ml (turbidity of 0.04, approximately 1% inoculation from a culture at absorbance of 5 at 650 nm to infinity with 10⁸ cells per ml.

The possibility that an unknown factor might initiate growth in still more dilute cultures of C. pasteurianum led to unsuccessful attempts to demonstrate growth-promoting activity. These attempts included: control of redox potential of the medium with thioglycolate, cysteine, or ascorbic acid; addition of biologically important metal ions (5); removal of oxygen by sparging with inert gases under a variety of conditions; incubation in anaerobic jars under an H₂ atmosphere from which the last traces of O₂ were removed with a hot platinum filament; addition of citrate, ethylenediaminetetraacetate, or nitriotriacetate to chelate any metals that might be toxic; addition of glutamate, aspartate, succinate, and cyclic adenylyl to initiate metabolic pathways and cell division; addition of used medium from which cells had been centrifuged at various stages of growth. Finally, because traces of toxic materials, e.g., detergent, adhering to glassware might inhibit growth, new glassware was cleaned with boiling H₂SO₄–HNO₃–H₂O (1:1:1, vol/vol/vol) and rinsed five times with water, which had been glass-distilled, passed through an Elgastat B102 ion exchanger (resistance in test circuit greater than 4 × 10⁶ ohms), then distilled from glass previously cleaned with the acid mixture and rinsed with deionized water. However, even with cleaned equipment and fresh medium DM 11 prepared from this redistilled water, there was no improvement in growth from small inocula. Since the earlier cultures of C. pasteurianum in defined media required 5 to 10% inocula for consistent growth with generation times of 90 to 120 min, present reductions to 1% inoculum and 85 min may be of value in biochemical work on this organism.

The culture conditions established herein were suitable for study of sulfur metabolism and, assuming that biotin (80 nM) was not a significant source of sulfur for C. pasteurianum in DM 11, growth was studied as a function of sulfate concentration. Starting first with a 5% inoculum, Fig. 4 reveals a significant effect of sulfate concentration on total growth but not on growth rate. The extensive growth observed without added sulfate presumably reflected carry-over in the inoculum grown originally in 0.6 mM sulfate. This inoculum could provide up to 0.03 mM, enough for considerable growth. When sulfate concentration was suboptimal, the population became stable. However, at the highest sulfate concentrations, attainment of maximal turbidity was followed by decline in turbidity and cell count.

For Fig. 5, sulfate accompanying the inoculum was limited by using only enough rapidly growing cells to provide initial populations of 1.5 × 10⁷ cells per ml. The maximal sulfate level thus transferred into the experimental systems was 12 μM. Not all of this sulfate was metabolized during growth of the inoculum because the final turbidity in absence of additional sulfate had increased more than the factor of two recorded (1) for growth of Escherichia coli on
internal reserves alone. As in Fig. 4, growth at the highest sulfate concentrations passed a maximum and declined by 25%. No threshold value of sulfate concentration, below which growth did not occur, could be determined. This result is similar to that reported (1) in the much more sensitive experiments with E. coli which did not reveal a maintenance requirement for sulfur. Modified experimental approaches, such as successful culture with much smaller inocula or use of washed cells as inocula, will be necessary before this analysis with C. pasteurianum can be made more sensitive. However, the present limited growth induced by small additions of sulfate will permit labeling at high specific radioactivity and isolation and identification of sulfur metabolites by methods used earlier (10).

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