Chemically Defined Medium for the Growth of Clostridium perfringens

WILLIAM E. RIHA, JR., AND MYRON SOLBERG
Department of Food Science, Rutgers The State University, New Brunswick, New Jersey 08903

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A defined medium which supports growth of Clostridium perfringens at low inoculum levels was developed. Generation time for strain 8797 was 1.5 times greater than previously reported for growth in purged fluid thioglycolate medium.

In preparation for a study of Clostridium perfringens metabolism under challenge in a model cured-meat system, it was necessary to develop a defined medium capable of supporting growth at a near optimal rate from a very small inoculum. Chemically defined media for the growth of C. perfringens strains have been described by Boyd et al. (1), Fuchs and Bonde (2), and Murata et al. (3). All of these media required large inoculum levels and, in some cases, special techniques to obtain anaerobiosis if regular growth support was to be achieved. This note describes a chemically defined medium capable of supporting rapid growth of several strains of C. perfringens from low inoculum levels at pH 6.3.

Inocula of seven strains of C. perfringens were obtained by the technique described by Boyd et al. (1), Fuchs and Bonde (2), and Murata et al. (3) but, in some cases, special techniques to obtain anaerobiosis if regular growth support was to be achieved. This note describes a chemically defined medium capable of supporting rapid growth of several strains of C. perfringens from low inoculum levels at pH 6.3.

Inocula of seven strains of C. perfringens were taken from cultures grown twice for 8 hr at 43 C in fluid thioglycollate medium (FTM; Difco) and then serially diluted in either 0.1% peptone water or in distilled water. Pour plate counts were made on SPS agar (Difco). Plates were incubated at 43 C in Anaero-Jars (Case Laboratories, Inc., Chicago, Ill.) in a nitrogen atmosphere.

Table 1 shows each component of the new medium and its final concentration. To prepare the medium, the ascorbic acid and all amino acids except cystine were weighed into a large flask. Distilled water was added, and the amino acids were dissolved by bringing the solution to a boil. The cystine was dissolved in a few milliliters of 0.1 N HCl and added to the cooled amino acid solution. Stock solutions of the vitamins and nucleic acids were prepared as suggested by Boyd et al. (1) and stored at 5 C. Samples from stock solutions were added to the cooled amino acid solution as required. Salts were weighed and added directly to the solution. Glucose could be added directly to the medium when it was to be filter sterilized, but, when the medium was to be autoclaved (15 min, 121 C), the glucose was made up to a 50% (w/w) stock solution, sterilized at 121 C for 15 min, and added aseptically to the medium. The medium was adjusted to pH 6.3 before sterilization.

Preliminary studies which measured growth spectrophotometrically showed that autoclaved medium and filter-sterilized medium were equivalent. The addition of glucose before autoclaving did not yield as much growth. The addition of fructose, as reported by Murata et al. (3), also yielded lower levels of growth. Filter sterilization eliminated precipitate of magnesium, manganese, iron, and zinc phosphate salts which occurred during autoclaving. A reduction of the concentration of these salts by 0.1 reduced the precipitate appreciably but increased the minimum inoculum level required to produce outgrowth for all strains by a factor of 10^6.

To determine the lowest inoculum which would produce visible growth, 10.0 ml of the autoclaved medium was inoculated from serial dilution of the seven 8-hr cultures in FTM. Dilutions of 10^6 to 10^8 were made in 9.0 ml of sterile distilled water or 0.1% peptone water blanks. An inoculum of 0.1 ml was then delivered from each dilution to 10 ml of the new medium (R & S medium) and Boyd's medium. All tubes were incubated for 24 hr at 43 C. The number of cells delivered from each dilution was determined by pour plate counts made from the culture. Tests conducted with the seven strains with both distilled water and 0.1% peptone water as diluent show that, in some cases, the peptone water protects the organism during dilution so that initial viable counts are slightly higher. The use of peptone water dilution blanks did not change the inoculum level necessary to produce visible outgrowth in either R & S medium or Boyd's medium.

Table 2 presents a summary of the results obtained with Boyd's medium and R & S medium. The range of minimum inoculum levels (the smallest number of cells delivered in 0.1 ml to
TABLE 1. Composition of chemically defined medium for the growth of Clostridium perfringens (R & S medium)

| Component       | Final concn. (mg/ml) | Component       | Final concn. (mg/ml) |
|-----------------|----------------------|-----------------|----------------------|
| DL-Alanine      | 0.5                  | DL-Serine       | 0.75                 |
| L-Arginine      | (HCl or free base)   | DL-Threonine    | 0.5                  |
|                 | 5.0                  | L-Tryptophan    | 0.25                 |
| L-Aspartic acid | 0.5                  | L-Tyrosine      | 0.25                 |
| L-Cystine       | 0.4                  | DL-Valine       | 0.375                |
| Glycine         | 0.5                  | Uracil          | 0.010                |
| L-Glutamic acid | 1.0                  | Adenine         |                      |
| L-Histidine     | 0.25                 | Ascorbic acid   | 0.25                 |
| L-Hydroxyproline | 0.20                | Riboflavin      | 0.0005               |
| DL-Isoleucine   | 0.5                  | Ca-D-pantothenate | 0.001              |
| L-Lysine        | 0.5                  | Pyridoxamine    | 0.0005               |
| L-Leucine       | 0.5                  | Biotin          | 0.000005             |
| DL-Methionine   |                      | Nicotinic acid  | 0.001                |
| L-Proline       | 0.35                 | Thiamine        | 0.001                |
| DL-Phenylalanine| 0.25                 | NaHPO₄          | 2.55                 |
|                 | 0.5                  | KH₂PO₄          | 0.70                 |
|                 |                      | MgSO₄₂H₂O       | 0.10                 |
|                 |                      | MnCl₂·4H₂O      | 0.05                 |
|                 |                      | ZnSO₄₂H₂O       | 0.05                 |
|                 |                      | FeSO₄·7H₂O      | 0.05                 |
|                 |                      | Glucose         | 12.50                |

Each tube containing 10 ml of medium) required to elicit visible growth during several experiments is presented. In all cases but two, R & S medium supported growth at lower inoculum levels. R & S medium also demonstrated a smaller range of minimum inoculum levels needed to produce growth.

From Table 2 it is evident that an inoculum level as low as 10 cells/ml would assure outgrowth for four of the strains in R & S medium and none of the seven strains in Boyd's medium. There is a reasonable probability that 10 cells/ml would result in outgrowth of all seven strains in R & S medium, since every strain demonstrated this ability at concentrations below 6 cells/ml in at least two out of every three experiments.

To demonstrate the efficiency of R & S medium with respect to growth rate, low inoculum levels of strain 8797 were studied. The medium was filter-sterilized, and 200 ml was placed in 300-ml screw-top Erlenmeyer flasks fitted with small (16 by 25 mm) septated side arms through which a sample could be removed by syringe. Quantities of cells (10² to 10⁶) were added to each culture flask and incubated at 43 C. The duration of the adjustment phase was approximately 8 hr with an inoculum of 10 cells/ml and 5 hr with an inoculum of 200 cells/ml. These durations were somewhat greater than the 1.0-hr adjustment phase of 8797 in FTM at 43 C as reported by Parekh and Solberg (4), whose inoculum levels were approximately 10⁶ cells/ml. The slopes of the exponential growth phase for high and low inoculum levels were determined by regression analysis. When Student's t test was used, the slopes were not significantly different. The generation time for the high inoculum level was 20.8 min; for the low inoculum experiments, it was 21.2 min. These generation times are somewhat greater than the 13.8-min generation time reported by Parekh and Solberg (4) for strain 8797 in CO₂ or N₂-flushed FTM.

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