Chemically Defined Medium for Growth and Sporulation of Clostridium perfringens

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A chemically defined medium was developed that could support sporulation and growth of Clostridium perfringens strains ATCC 12916 and H9. This medium consisted of a modification of the basal medium of Boyd et al. plus 0.1% sodium thioglycolate and 0.5% monosodium glutamate. Five other strains grew, but did not sporulate, in this medium. With the addition of more vitamins into the medium, two more strains grew but did not sporulate. The effects of glucose, monosodium glutamate, ammonium glutamate, and sodium thioglycolate on growth and sporulation of C. perfringens ATCC 12916 in the defined medium was investigated.

Clostridium perfringens causes about 40 to 45% of all reported food-borne poisoning cases in the United States (2). Detailed reviews of this organism were made by Bryan (4), Despaul (6), Dische and Elek (7), Hauschild and Thatcher (13), Hobbs (14), McClung (17), and others. The ubiquitous nature of C. perfringens and the ability to form heat-resistant spores complicate the effective control of this organism in food products. Recently, Duncan et al. (9) reported that sporulation and enterotoxin production by C. perfringens are directly related.

The nutritional requirements for growth and sporulation of C. perfringens in complex and semicomplex media were studied by Angelotti et al. (1), Duncan and Strong (8), Ellner (10), Gibbs and Hirsch (12), and Kim et al. (15). These media contained a combination of peptides, yeast extracts, Trypticase, Casein, Acids, starch, sodium thioglycolate, thiamine, and mineral salts.

Chemically defined media, developed for the growth of C. perfringens, were reported by Boyd et al. (2), Fuchs and Bonde (11), and Riha and Solberg (20). These authors, however, did not observe spores in the media that they developed. In 1967, an attempt to induce sporulation of 30 type A C. perfringens strains in a synthetic medium developed for Staphylococcus aureus was made by Meisel-Mikolajczyk and Osowiecki (18) without success. A chemically defined medium that can support growth of nine C. perfringens strains and sporulation of two of the strains is described in the present report.

MATERIALS AND METHODS

Culture and inoculum. Clostridium perfringens ATCC 12916 and ATCC 12917 were obtained from the American Type Culture Collection, Rockville, Md. Nine other C. perfringens strains, ATCC 3624 (no. 26), ATCC 10543, 215b, F42, FD1, H4, H9, H13, and HR1 were obtained through the courtesy of H.W. Walker of the Department of Food Technology, Iowa State University, Ames, Iowa. The purity and characteristics of these cultures were determined by Gram stain morphology, motility test, growth on sulfite-polymyxin sulfadiazine agar (BBL), litmus milk reactions, gelatin liquefaction, nitrate reduction, and the indole test. Stock cultures were made by growing the cells in 10% cooked meat medium (Difco) which was refrigerated (5 C) for storage of the culture. Frequent transfers were made to ensure viability.

Inocula were prepared by growing C. perfringens overnight at 37 C in Brewer thioglycolate medium (Difco). Active cultures were subsequently obtained by two serial transfers at intervals of 4 to 5 hr by using 10% inoculum for each transfer; incubation was at 37 C. Before inoculation into test media, active cultures (ca. 1 x 10⁸ cells/ml) were washed 3x in sterile phosphate buffer (0.1 M, pH 7.4). The cells were then resuspended and diluted to approximately 1 x 10⁷ cells/ml in sterile, distilled water before 1 ml was added to 9 ml of sterile chemically defined media. Thus, the final inoculum concentration was approximately 1 x 10⁴ cells/ml.

Growth and sporulation. C. perfringens ATCC 12916 was used throughout the study as the test organism unless stated otherwise. Tubes containing complex or chemically defined media with inoculum were incubated at 37 C in a Brewer anaerobic jar (BBL). Anaerobiosis was achieved by the Gas-Pak system (BBL) of Brewer and Allgeier (3) as well as by the reducing agents present in the media. Growth in terms of turbidity was estimated by spectrophotome-
try (600 nm; Spectronic-20, Bausch & Lomb, Inc., Rochester, N.Y.). Vegetative cells and spores were observed and enumerated in a Petroff-Hauser chamber under phase-contrast microscopy. The ratio of the number of spores per field in microscopy to the total number of cells plus spores was used to compute the percentage of sporulation in the test media.

**Complex media.** Four complex media were also used for growth and sporulation of *C. perfringens* ATCC 21916 to provide data that could be compared with that obtained in the chemically defined media. The test organism was grown in the media devised by Angelotti et al. (1), Duncan and Strong (8), Ellner (10), and Kim et al. (15). After 20 hr of incubation at 37 C, the percentage of sporulation was determined in each of the complex media.

**Basal medium.** The basal medium of Boyd et al. (2) was used as the starting medium for this study. L-amino acids were substituted for the D-amino acids at the same concentrations, and DL-amino acids were replaced by L-amino acids at levels half of those specified by Boyd et al. (2). Procedures for the preparation of media, other than the changes noted above, were identical to those described by Boyd et al. (2).

**Nutritional requirements for sporulation.** L-Arginine (0.25%, 0.5%), L-alanine (0.5%), L-glutamic acid (0.5%), and thiamine (0.12%), reported by others (16, 19; B. C. Wooley and R. E. Collier, Bacteriol. Proc., p. 16, 1966; J. F. Charba and H. M. Nakata, Bacteriol. Proc., p. 16, 1966) to stimulate sporulation of *Bacillus* spp. and *Clostridium* spp., were added to the modified basal medium of Boyd et al. (2) at the concentrations indicated. In some experiments several compounds, such as sodium thioglycolate (0.1%), monosodium glutamate (0.5%), and ammonium glutamate (0.5%), were added in different combinations to test for combined effects of these compounds on sporulation. Effects of varying concentrations of monosodium glutamate (0 to 110 mg/10 ml of medium) and glucose (0 to 300 mg/10 ml of medium) on sporulation were also tested. All the components (except FeSO₄ and glucose) of the medium were mixed together and sterilized. FeSO₄ and glucose were filter-sterilized and heat-sterilized, respectively, and later were aseptically added to the medium. The pH of the medium was adjusted to 7.4 prior to sterilization. After the addition of components and washed inoculum the test media were incubated anaerobically at 37 C. Measurement of growth and observation of sporulation were made at appropriate intervals of incubation up to 22 hr. For growth studies, 45 ml of chemically defined medium was placed in a 100-ml flask and inoculated with 5 ml of washed inoculum. Samples were aseptically removed for measurements of turbidity, pH, total cell count, and spore counts at suitable intervals up to 26 hr. Anaerobiosis was maintained throughout the experiment by using Gas-Pak (BBL) after each sampling.

**Growth and sporulation of other *C. perfringens* strains.** After the chemically defined medium was formulated for *C. perfringens* ATCC 12916, 10 other strains were tested for growth and sporulation in the medium. In a separate experiment, additional vitamins (0.0001% of p-aminobenzoic acid and 0.0001% each of nicotinic acid, thiamine, and folic acid) were supplied to the defined medium before testing growth and sporulation of all 11 strains.

**Stability of the medium.** Tubes of the sterilized, defined medium were stored at refrigerated temperature (1 C) and freezing temperature (−18 C) for a period up to 1 month before *C. perfringens* ATCC 12916 was inoculated into these tubes to test for growth and sporulation.

**RESULTS AND DISCUSSION**

A comparative study on sporulation of *C. perfringens* ATCC 12916 in the four complex media showed that sporulation was best (74%) in Ellner medium (10). Although substantially less than 74% in other complex media, the percentage sporulation in Duncan and Strong medium (8), Kim et al. medium (15), and Angelotti et al. medium (1) was 18.7, 13.0 and 8.6, respectively. Heat resistance of spores obtained from these complex media was not tested.

After repeated testing of various compounds, individually or in combinations (data not shown), a chemically defined medium was developed that supported growth and consistent sporulation (1.6 × 10⁸ spores/ml, 2 × 10⁸ cells/ml, 8.0%) for *C. perfringens* ATCC 12916. The medium consisted of the modified basal medium of Boyd et al. (2) with added sodium thioglycolate (0.1%) and monosodium glutamate (0.5%). Subsequent experiments on growth and sporulation were then performed by using the developed defined medium.

Figure 1 shows typical growth and sporulation curves for *C. perfringens* ATCC 12916 in the defined medium. According to total cell count, log phase began at 3 hr and ended at 8 hr.
However, according to the absorbance curve, log growth began at 7 hr and ended at 11 hr. At 14 hr of incubation a count of $2 \times 10^8$ cells/ml was obtained from an initial count of approximately $1 \times 10^6$ cells/ml. Effervescence was observed in culture flasks at the end of log-phase growth. Nonsporulating cells at this time became granulated in appearance under a phase-contrast microscope. Autolysis of some cells was observed also. Forespores first appeared at 10 to 12 hr, when the vegetative cells reached late log phase. Mature, refractile spores were observed by 13 hr ($6 \times 10^7$ spores/ml, $2 \times 10^8$ cells/ml). Most spores remained in sporangia rather than as free spores at 15 hr. The position of the spores was located from centrally to terminally (Fig. 2). The maximum number of spores and cells was obtained at 17 hr ($2 \times 10^8$ spores/ml, $2.5 \times 10^9$ cells/ml). Free spores started to be released from the sporangia by 18 hr. At 26 hr, both the number of spores and vegetative cells declined ($4.9 \times 10^6$ spores/ml, $6.2 \times 10^8$ cells/ml); however, the percentage of spores remained about 8%. The pH of the synthetic medium (7.4) remained essentially unchanged in the first 6 hr of growth. During onset of log-phase growth, pH started to decrease gradually. After 13 hr of growth, the pH reached a low of 6.3 and did not change appreciably thereafter.

When 10 other C. perfringens strains (ATCC 3624 [no. 26], ATCC 10543, ATCC 12917, 215b, F42, FD1, H4, H9, H13, and HR1) were investigated for their abilities to grow and sporulate in the defined medium, six strains grew (ATCC 3624 [no. 26], ATCC 10543, ATCC 12917, F42, H9 and HR1) and one strain (H9) sporulated ($2.3 \times 10^6$ spores/ml, $2.5 \times 10^8$ cells/ml) in the defined medium. The fact that four strains of C. perfringens (215b, FD1, H4, and H13) failed to grow in the defined medium probably indicated that these strains may require extra vitamins or other factors. After incorporation of four extra vitamins (p-aminobenzoic acid, nicotinic acid, thiamine, and folic acid), two more strains (215b and FD1) were able to grow in the defined medium. However, no stimulatory effect on sporulation by these added vitamins was observed for all strains. C. perfringens ATCC 12916 and H9 remained the only two strains that sporulated in the chemically defined medium.

Table 1 shows the effect of adding sodium thioglycolate, monosodium glutamate, and ammonium glutamate into the defined medium individually and in combinations. The presence of either sodium thioglycolate (0.1%) or monosodium glutamate (0.5%) alone in the basal medium provided growth but not sporulation. The simultaneous presence of the above two compounds in the basal medium resulted in sporulation. Addition of ammonium glutamate (0.5%) alone into the basal medium resulted in stimulation of sporulation. However, growth

Fig. 2. Spores (ca. 14 hr) of Clostridium perfringens ATCC 12916 in the chemically defined medium among dividing cells. About 8% of the cells sporulated in this defined medium. x1,400.
sodiumthioglycolate When both medium containing both monosodium glutamate and sodium thioglycolate (OD, 0.34). When both ammonium glutamate (0.5%) and sodium thioglycolate (0.1%) were added to the basal medium, growth (OD, 0.12) and sporulation of the test organism occurred.

The optimal concentration of monosodium glutamate to be used in the chemically defined medium for C. perfringens ATCC 12916 was found to be 50 mg/10 ml of medium (Table 2). Other concentrations of monosodium glutamate resulted in less growth and sporulation. Growth but no sporulation occurred in the absence of monosodium glutamate in the defined medium. A similar study on the effect of glucose showed that the best growth and largest number of spores were obtained when 100 mg of glucose per 10 ml of medium was used (Table 3). Other concentrations of glucose in the medium resulted in less growth and sporulation. In the absence of glucose no growth was recorded, although monosodium glutamate (0.5%) was present in the medium as carbon source.

The above data showed that glutamate has an important role in sporulation of C. perfringens ATCC 12916 in the defined medium. Glutamate was required for sporulation but was not essential for growth when glucose was present as the carbon source. The central role of glutamate in stimulating sporulation of aerobic Bacillus spp. was reported by Buono et al. (5), Charba and Nakata (Bacteriol. Proc., 16, 1966), and Singh (21).

The presence of sodium thioglycolate in the defined medium probably provided appropriate oxidation-reduction potential conducive to good sporulation. The stimulatory effect of sodium thioglycolate on sporulation was discussed by Lund (16) and Kim et al. (15).

The defined medium was found to retain its effectiveness to support growth and sporulation after 90 days of storage at refrigerated (1 C) and freezing (−18 C) temperatures. However, the extent of growth and sporulation of the test

| Table 1. Effect of sodium thioglycolate, monosodium glutamate, and ammonium glutamate on growth and sporulation of Clostridium perfringens ATCC 12916 |
|---------------------------------|-----------------|-----------------|-----------------|
| Medium                          | Spores/ml       | Cells/ml        | Absorbance (600 nm) |
| BM                             | 0               | 8.0 x 10⁴       | 0.12             |
| BM + 0.1% STG                  | 0               | 8.9 x 10⁴       | 0.20             |
| BM + 0.5% MSG                  | 0               | 8.5 x 10⁴       | 0.17             |
| BM + 0.5% AG                   | 4.8 x 10⁷       | 5.1 x 10⁹       | 0.10             |
| BM + STG + MSG                 | 1.6 x 10⁶       | 2.0 x 10⁸       | 0.34             |
| BM + STG + AG                  | 1.6 x 10⁶       | 8.2 x 10⁸       | 0.12             |

Optical density determination, cell count, and spore count were made after 20 hr of anaerobic incubation at 37 C. BM, Basal medium; STG, sodium thioglycolate; MSG, monosodium glutamate; AG, ammonium glutamate.

| Table 2. Growth and sporulation of Clostridium perfringens ATCC 12916 in different monosodium glutamate concentrations |
|---------------------------------|-----------------|-----------------|-----------------|
| Mono-sodium glutamate          | Spores/ml       | Cells/ml        | Absorbance (600 nm) |
| 0                              | 0               | 7.7 x 10⁴       | 0.19             |
| 10                             | 4 x 10⁴         | 1.0 x 10⁶       | 0.20             |
| 30                             | 1.6 x 10⁷       | 1.2 x 10⁸       | 0.22             |
| 50                             | 1.6 x 10⁴       | 2.0 x 10⁶       | 0.34             |
| 70                             | 3.9 x 10⁷       | 1.3 x 10⁷       | 0.23             |
| 90                             | 3.8 x 10⁷       | 1.5 x 10⁷       | 0.19             |
| 110                            | 1.8 x 10⁷       | 7.1 x 10⁴       | 0.18             |

Optical density determination, cell count, and spore count were made after 20 hr of anaerobic incubation at 37 C.

| Table 3. Growth and sporulation of Clostridium perfringens ATCC 12916 in different glucose concentrations |
|---------------------------------|-----------------|-----------------|-----------------|
| Glucose (mg/ml)                 | Spores/ml       | Cells/ml        | Absorbance (600 nm) |
| 0                              | 0               | <1.0 x 10⁴      | 0.20             |
| 25                             | 2.2 x 10⁵       | 1.1 x 10⁶       | 0.20             |
| 50                             | 3.8 x 10⁵       | 1.2 x 10⁶       | 0.20             |
| 75                             | 1.1 x 10⁵       | 1.9 x 10⁶       | 0.33             |
| 100                            | 1.6 x 10⁵       | 2.0 x 10⁶       | 0.34             |
| 150                            | 4.8 x 10⁵       | 1.2 x 10⁶       | 0.25             |
| 200                            | 5.7 x 10⁵       | 7.1 x 10⁶       | 0.18             |
| 300                            | 5.5 x 10⁵       | 1.1 x 10⁶       | 0.21             |

Optical density determination, cell count, and spore count were made after 20 hr of anaerobic incubation at 37 C.

| Table 4. Stability of the chemically defined medium |
|---------------------------------|-----------------|-----------------|-----------------|
| Temperature of storage          | Time of storage (days) | Spores/ml       | Cells/ml        | Absorbance (600 nm) |
| Refrigerated at 1 C             | 18              | 1.0 x 10⁴       | 1.2 x 10⁶       | 0.28             |
| Frozen at −18 C                 | 18              | 3.7 x 10⁷       | 8.3 x 10⁷       | 0.19             |

Optical density determination, cell count, and spore count were made after 20 hr of anaerobic incubation at 37 C.
organism in the stored, defined medium was less than that obtained from freshly prepared medium (Table 4).

In conclusion, a chemically defined medium was developed that can support growth of nine strains of \textit{C. perfringens} and sporulation of two strains. Concentrations of several compounds optimal for sporulation of \textit{C. perfringens} ATCC 12916 in the medium were evaluated. Stability of the medium under cold storage was tested. Heat resistance of spores of \textit{C. perfringens} ATCC 12916 and H9 obtained from the defined medium is currently under investigation (M. N. Ting, J. Funk, and D. Y. C. Fung, Abst. Annu. Meet. Amer. Soc. Microbiol., p. 5, 1972).

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