Minimal Growth Requirements for *Clostridium perfringens* and Isolation of Auxotrophic Mutants

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Received for publication 26 August 1974

The minimal growth requirements for two strains of *Clostridium perfringens* were defined, and both synthetic and semisynthetic plating media were developed. Plate counts of the wild-type strains on both of these minimal media were equivalent to those on complex media. A number of auxotrophic mutants of each strain were isolated, and their phenotypes were defined.

*Clostridium perfringens* appeared to be a good choice for use in studies of possible genetic exchange between cells of a strict anaerobe. It can be cloned on agar plates with relative ease and is not as sensitive to short-term exposure to oxygen as are many clostridia. A number of chemically defined media have been reported to support good growth of this species (1, 4, 8, 10). Also, a number of both virulent and temperate phages have been described for this species (5, 6, 9).

In preparation for genetic studies, it was necessary to obtain mutants for various specific markers. No auxotrophic mutants of a *Clostridium* have been described previously. Examination of the composition of the published synthetic media for *C. perfringens* (1, 4, 8) indicated that it might be possible to develop a more simplified minimal medium for use in isolating auxotrophic mutants. The present paper describes both synthetic and semisynthetic plating media which give excellent recovery of *C. perfringens* cells. Also, methods for isolation and descriptions of a number of auxotrophic mutants are reported.

**MATERIALS AND METHODS**

**Cultures and cultural methods.** Two strains of *C. perfringens* were used, BP6K-N<sub>4</sub> and PX7. The former is a highly toxigenic variant isolated from strain BP6K (7). Strain PX7 was isolated from strain NCTC 8798 (food poisoning strain, Hobb's serotype 9). This culture was cured of a prophage Y carried by the parent cells (M. Sebald, unpublished data). For the purposes of this paper, we will refer to strains BP6K-N<sub>4</sub> and PX7 as wild-type strains.

The complex media used for routine plating was thioglycolate medium (Difco) without glucose or indicator, which was supplemented with 1% glucose, bromocresol purple (40 mg/liter), and 1.5% agar (thio-BCP medium). Broth cultures were in a medium containing 30 g of Trypticase (BBL), 20 g of yeast extract, 5 g of glucose, and 1 g of sodium thioglycolate per liter of water, pH 7.4 (TGY broth). All dilutions of cultures and cell suspensions were in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.3, supplemented with 10<sup>-5</sup> M CaCl<sub>2</sub>, 10<sup>-3</sup> M MgSO<sub>4</sub>, and 1% glucose (TCMG buffer). The buffer was boiled for 15 min before use.

All cultures were incubated at 37 C in anaerobic jars (Baird and Taitlock) containing a hydrogen atmosphere and a palladium catalyst. Plates were incubated for 24 h when complex media were used and 48 or 72 h when Casamino Acids (Difco) or synthetic media were used.

**Nutritional studies.** The basal medium of Riha and Solberg (8), supplemented with 1.5% agar, enabled growth initiation when low inoculum levels were used. Batches of this medium (8) were prepared, omitting nutrients which previous studies (1, 4) had reported to be non-essential for this organism. The following groups were omitted from individual lots of media separately: group I, glycine, serine, lysine, proline, and hydroxyproline; group II, nicotinic acid and thiamine; and group III, adenine and uracil. When a group was found essential for growth, its components were omitted separately.

Plates poured with the individual lots of media were streaked with cells from early stationary phase cultures in TGY broth. The cells were washed and resuspended in TCGM buffer before use. All streaks showing positive growth after 2 or 3 days of incubation were restreaked on another plate containing the same medium.

The requirements for each of the other nutrients in the medium of Riha and Solberg (8) were determined by omitting each of them separately. Liquid media were used in these experiments. An inoculum of ~2 × 10<sup>4</sup> washed cells per 10 ml of broth was used; two serial transfers were made before quantitative growth measurements were made. The final optical density of cultures was taken after 24 h of incubation with a Meunier photolorimeter equipped with a 470-nm filter.

The requirements for purine and pyrimidine bases and for vitamins were also determined in a Casamino

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Acids medium (semisynthetic). The composition of this medium was the same as that of Riha and Solberg (8), except that all of the amino acids were replaced with 1.5% vitamin-free Casamino Acids (Difco) and 0.025% tryptophane. The glucose was filter sterilized and added to the autoclaved medium. The individual bases and vitamins were omitted from different lots of the medium, and plates were poured and streaked as outlined in the previous paragraphs.

Isolation and characterization of mutants. Tubes of TGY broth were inoculated with a 1% inoculum of an overnight culture in the same broth. When the cultures had attained mid-log phase, N-methyl-N'-nitrosoguanidine was added to give a final concentration of 50, 100, or 200 μg/ml. After 30 min of incubation at 37°C, the cells were centrifuged from 10 ml of the broth, washed in 10 ml of TCMG buffer, and resuspended in 20 ml of TGY broth. These cultures were incubated overnight. A few mutants (experiments I and II, Table 3) were isolated by plating the cultures at this point; however, most mutants were isolated after enrichment with penicillin as follows. Subsamples of the overnight cultures in TGY were centrifuged; the cells were washed with 5 volumes of TCMG buffer and resuspended in 5 volumes of either the synthetic or semisynthetic medium. After 4 h of incubation to permit initiation of growth, 100 U of penicillin per ml was added, and incubation was continued for 8 to 10 additional h. After centrifugation, the cells were washed in TCMG buffer, resuspended in the buffer at one-fifth the volume of the penicillin-treated culture, and then plated on thio-bromocresol purple agar. The plates were incubated 48 h and then replicated on incomplete media. Cultures from the synthetic penicillin media were replicated on both minimal and complete synthetic agar, whereas those from the semisynthetic penicillin media were replicated on both minimal and complete semisynthetic agar. The synthetic medium plates were incubated for 3 days, and the semisynthetic medium plates were incubated for 2 days.

All the colonies which grew on the complete but not on the minimal media were restreaked on both complete and minimal media of the same type. After streaking and reisolating the auxotrophic mutants, their identity was confirmed by microscopic examination, by the aspect of colonies on blood agar, and by their sensitivity to phages of the laboratory collection. The growth requirements of the mutants were confirmed by plating 2× concentrated overnight broth cultures on the appropriate minimal medium and plating a 10⁻⁷ dilution on the minimal medium supplemented with the required nutrient(s). In some instances, growth rates of the mutants were studied in supplemented and unsupplemented minimal media.

For each marker, only one mutant from a single mutation experiment was saved to insure that each had a different origin. All mutants were lyophilized for preservation, and they are available from our collection.

Lac⁻ mutants were isolated using N-methyl-N'-nitrosoguanidine treatment and a penicillin selection. The procedures used were the same as those described except that the penicillin treatment was in the Casamino Acids medium described above, but with the glucose replaced with lactose. After penicillin treatment, cells were plated on Thio-BCP agar containing 1% lactose instead of glucose. Colonies which failed to show an acid reaction in the medium were reisolated twice on thio-BCP agar.

RESULTS

Nutritional requirements. Omission experiments using the synthetic and semisynthetic agar media demonstrated that neither strain of C. perfringens required nor was greatly stimulated by L-lysine, L-proline, hydroxyproline, adenine, uracil, nicotinic acid, p-aminobenzoic acid, or thiamine. Variable growth of the two strains was observed when either glycine or serine was omitted, but no growth occurred when both were absent. Apparently these cultures have some ability to interconvert these two amino acids.

Using liquid cultures in the synthetic medium with individual nutrients omitted, it was found that 11 amino acids and two vitamins were essential for growth: arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane, tyrosine, valine, pantethenic acid, and pyridoxamine. A number of other nutrients were either required or were greatly stimulatory to one or the other of the two strains (Table 1).

Minimal media. Based on these data, two minimal media were devised for mutational studies. The minimal synthetic medium (SI) (Table 2) was devised for the isolation of mutants auxotrophic for lysine and proline. This medium is essentially the same as the R & S liquid medium of Riha and Solberg (8), except that lysine, proline, hydroxyproline and riboflavin are omitted. The level of L-arginine was reduced by 50% of that in the Riha and Solberg medium, since growth experiments indicated that levels as low as 0.1 mg/ml would support good growth of our two strains. It was recently shown that D,L-amino acids used at a 2× concentration could be substituted for all L-amino acids in the SI medium; only D,L-arginine was not checked, since it was not available in the laboratory. In addition, it was necessary to add sodium thioglycolate to the plating medium to obtain high recoveries of cells. Routinely, the thioglycolate was added at the optimal level (0.1%) just prior to pouring the plates, and the plates were inverted with their lids off and dried for 45 min at 37°C and then spread. Drying overnight after pouring the plates resulted in low recoveries of cells.

A minimal Casamino Acids medium (CA-I) was used for isolation of mutants auxotrophic
This medium and autoclaved However, was the adenine volume colonies. The wild-type to those obtained medium: tions with the SI medium. 

Although the SI medium was incubated at 105 C for 20 min. A 50% (wt/vol) solution of glucose was autoclaved separately, and the appropriate volume was added to give a 1% concentration. To prepare complete Casamino Acids medium (Ca-C), the indicated volumes of sterile solutions of the following were added to 100 ml of medium: 0.4 ml of nicotinic acid (0.25 mg/ml), 0.4 ml of thiamine HCl (0.25 mg/ml), 1 ml of adenine sulfate (1.74 mg/ml) in 0.2 M HCl, and 1 ml of uracil (1 mg/ml) in 0.2 M HCl. It was unnecessary to add sodium thioglycollate to this medium. Plate counts of cultures of both wild-type strains on CA-I were comparable to those obtained on the complex medium (Thio-BCP).

The growth curves for both strains in TGY, CA-I, and SI media are reported in Fig. 1. No lag was observed when media were inoculated with mid-exponential phase cells from the same medium. Although non-essential for growth in SI liquid medium, 0.1% sodium thioglycollate was used for growth curves in that medium, an irregular lag being observed when it was omitted. The biphasic aspect of the curves obtained with the strain PX7 was due to spontaneous induction of the phage X, which had not been cured of the strain (Sebald, unpublished data).

**Auxotrophic mutants.** A variety of auxotrophic mutants of each of the two strains of *C. perfringens* was isolated after mutagenic treatment and penicillin selection. No spontaneous auxotroph mutants were found in nonmutagenized cultures. As outlined previously, the penicillin selection is a non-essential step, but it makes the isolation much easier. The mutants which we have preserved are listed in Tables 3 and 4 along with their phenotype, origin, and

### Table 1. Effects on growth of various nutrients with which variable growth responses were observed

| Nutrient omitted | % maximal OD |
|------------------|--------------|
|                  | BP6K-N | PX7 |
| None             | 100     | 100 |
| L-Alanine        | 71      | 47  |
| DL-Aspartic acid | 9       | 0   |
| L-Cystine        | 78      | 40  |
| Glycine          | 28      | 0   |
| L-serine         | 27      | 37  |
| Biotin           | 20      | 77  |

* Evaluated from final optical density (OD) at 470 nm after two serial transfers in a synthetic (SI) liquid medium with the single omissions indicated. Cultures were incubated for 24 h.

for vitamins and purine and pyrimidine bases. This medium contained the same levels of salts A and B, ascorbic acid, biotin, calcium D-pantothenate, pyridoxamine, and agar as the SI. However, all of the amino acids were replaced with 15 g of Casamino Acids (vitamin free; Difco) and 25 mg of tryptophane per liter. In addition, 40 mg of bromoresol purple was added per liter, which made it easy to visualize colonies. The pH was adjusted to pH 6.8, and the medium was autoclaved at 105 C for 20 min. A 50% (wt/vol) solution of glucose was autoclaved separately, and the appropriate volume was added to give a 1% concentration. To prepare complete Casamino Acids medium (Ca-C), the indicated volumes of sterile solutions of the following were added to 100 ml of medium: 0.4 ml of nicotinic acid (0.25 mg/ml), 0.4 ml of thiamine HCl (0.25 mg/ml), 1 ml of adenine sulfate (1.74 mg/ml) in 0.2 M HCl, and 1 ml of uracil (1 mg/ml) in 0.2 M HCl. It was unnecessary to add sodium thioglycollate to this medium. Plate counts of cultures of both wild-type strains on CA-I were comparable to those obtained on the complex medium (Thio-BCP).

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**Auxotrophic mutants.** A variety of auxotrophic mutants of each of the two strains of *C. perfringens* was isolated after mutagenic treat-

### Table 2. Solutions used for preparation of minimal synthetic media

| Stock solutions | Composition | ml of stock solutions/liter |
|-----------------|-------------|---------------------------|
| Salts A         | Per liter: Na2HPO4, 57 g; K2HPO4, 14 g | 50 |
| Salts B         | Per 800 ml: MgSO4·7H2O, 4 g; MnSO4·4H2O, 2 g; ZnSO4·7H2O, 2 g; FeSO4·7H2O, 2 g; Acids with 1 ml of 1 N HCl. | 20 |
| Amino acids and bases Solution I | Per liter: L-alanine, 2.5 g; L-arginine, 25 g; glycine, 5 g; DL-histidine, 5 g; DL-isoleucine, 5 g; L-leucine, 5 g; DL-methionine, 3.5 g; L-phenylalanine, 2.5 g; L-serine, 3.75 g; DL-threonine, 5 g; DL-valine, 3.75 g. Dissolve in water. | 100 |
| Solution II     | Per liter: DL-aspartic acid, 10 g; L-glutamic acid, 10 g. Dissolve in about 500 ml of water by adding 5 N NaOH slowly. Make to volume. | 100 |
| Solution III    | Per liter: L-tyrosine, 2.5 g; L-tryptophane, 2.5 g. Dissolve in about 800 ml of water by adding 5 N NaOH slowly. Make to volume. | 100 |
| Solution IV     | Per 100 ml: L-cystine, 4 g; adenine sulfate, 0.174 g; uracil, 0.1 g. Suspend cystine in about 10 ml of water and dissolve by adding 5 N HCl slowly. Make to volume, add bases, and dissolve by warming. | 10 |
| Vitamins        | Biotin, 2 mg/100 ml; Calcium D-pantothenate, 2 mg/ml; Pyridoxamine, 0.5 mg/ml; Nicotinic acid, 1 mg/ml, and thiamine, 1 mg/ml | 0.25 0.5 1.0 1.0 |

* To prepare the minimal medium (SI medium) combine the stock solutions, add 0.25 g of L-ascorbic acid per liter, and adjust to pH 7.0 with 5 N NaOH. Add 15 g of agar per liter, dissolve, and autoclave for 20 min at 105 C. Sterile glucose solution (2 ml) (50%, wt/vol) is added per 100 ml of the sterilized medium, and 0.4 ml of a fresh filter-sterilized solution of sodium thioglycollate (25%, wt/vol) is added per 100 ml just prior to using the medium. To prepare the complete synthetic medium, 1 ml of a solution of L-proline (25 mg/ml) and 1 ml of a solution of L-lysine (50 mg/ml) are added per 100 ml of SI medium.
**Fig. 1.** Growth of *C. perfringens* BP6K-Ns (A) and PX7 (B) in TGY broth (●), CA-I (×), and SI (O) media.

**Table 3. Auxotrophic mutants of *C. perfringens* BP6K-Ns**

| Mutant no. | Phenotype* | Expt no.* | No. of colonies after 2x concn* | No. of colonies after 10⁻¹ dilution |
|------------|------------|-----------|-------------------------------|------------------------------------|
|            |            | Medium    | No.                           | Supplemented minimal†               | Thio-BCP                           |
| 350-5      | Ade⁻       | I         | CA-I                          | 0                                  | 121                                 | 36                                  |
| 350-17S    | Ade⁻       | II        | CA-I                          | 0                                  | 67                                  | 5                                   |
| 352-14     | Ade⁻       | III       | CA-I                          | 0                                  | 38                                  | 48                                  |
| 350-22S    | Ade⁻       | IV        | CA-I                          | 0                                  | 118                                 | 46                                  |
| B-5        | Ade⁻       | V         | CA-I                          | 0                                  | 100                                 | 300                                 |
| 1-12       | Ade⁻       | VII       | CA-I                          | 0                                  | 200                                 | 50                                  |
| 352-20S    | Ura⁻       | III       | CA-I                          | 0                                  | 66                                  | 53                                  |
| 350-3S     | Ade⁻ Ura⁺  | I         | CA-I                          | 0                                  | 30                                  | 8                                   |
|            |            | CA-C,-ade | 0                             |                                    | -                                   | -                                   |
|            |            | CA-C,-ura | 300 (tiny)                    |                                    | -                                   | -                                   |
| 352-17     | Ade⁻ Ura⁺  | III       | CA-I                          | 0                                  | 100                                 | 85                                  |
|            |            | CA-C,-ade | 0                             |                                    | -                                   | -                                   |
|            |            | CA-C,-ura | 64 (tiny)                     |                                    | -                                   | -                                   |
| 352-2      | Nic⁻       | I         | CA-I                          | 0                                  | 10                                  | 10                                  |
| 366-A4     | Nic⁻       | VI        | CA-I                          | 0                                  | 300                                 | 300                                 |
| 350-21S    | Ura⁺ Nic⁺  | IV        | CA-I                          | 0                                  | 36                                  | 22                                  |
|            |            | CA-C,-ura | 125                           |                                    | -                                   | -                                   |
|            |            | CA-C,-nic | 178                           |                                    | -                                   | -                                   |
| 2⁻         | Pro⁻       | V         | SI                            | 0                                  | 66                                  | 23                                  |
| 1          | Pro⁻       | VIII      | SI                            | 0                                  | 35                                  | 5                                   |
| 6⁻         | Lys⁻       | VI        | SI                            | 0                                  | 500 (tiny)                          | 115                                 | 124                                 |
| 7⁻         | Lys⁻       | VII       | SI                            | 0                                  | 58                                  | 46                                  |
| 41         | Lys⁻       | VII       | SI                            | 0                                  | 206                                 | 164                                 |
| 2⁻-2       | Pro⁻ Lys⁻  | IX        | SI                            | 0                                  | 100                                 | 20                                  |
|            |            | SC,-pro   | 0                             |                                    | -                                   | -                                   |
|            |            | SC,-lys   | Tiny                          |                                    | -                                   | -                                   |

* Ade, Adenine; Ura, uracil; Nic, nicotinic acid; Pro, proline; and Lys, lysine.
* N-methyl-N'-nitrosoguanidine (100 μg/ml) was used in all experiments except for experiments II (50 μg/ml) and IV (200 μg/ml).
* Approximately 10⁶ cells were plated.
* CA-I was the minimal medium used for adenine, uracil, and nicotinic acid mutants, and SI was used for lysine and proline auxotrophs. The media were supplemented with the nutrient(s) indicated for the phenotype of the mutant plated.
* Not tested.
growth responses in minimal and supplemented media. Most of these mutants are very stable and have an absolute requirement for the nutrient(s) for which they are auxotrophic. The plate counts on minimal media supplemented with the appropriate nutrient(s) were very similar to the counts on complex media. Some of the cultures which are auxotrophic for more than one nutrient appear to be leaky in one or both mutations; these are designated ± in Tables 3 and 4.

Although not evident in Tables 3 and 4, we observed spontaneous revertants in all of these mutant cultures at a frequency of $10^{-8}$ to $10^{-9}$. Therefore, they are probably point mutations. All of the auxotrophs were obtained by a single mutagenic treatment except for the 2'-2', Pro-Lys$^-$ culture from strain BP6K-N$_5$. This double auxotroph was isolated after NTG treatment of

strains 2'-2', Pro$. No spontaneous revertants have been observed with this mutant.

Lac$^-$ mutants. Lac$^-$ mutants were obtained from the strain BP6K-N$_5$. A small number of them were stable, with a spontaneous reversion rate of $10^{-8}$, but many were unstable.

**DISCUSSION**

For the first time, defined media have been developed which will yield plate counts of *C. perfringens* equivalent to those obtained in complex media. To our knowledge, the mutants selected during this investigation are the first auxotrophic mutants obtained with anaerobic bacteria. This makes it possible to pursue genetic studies of this organism under defined conditions. Genetic recombination could help in further investigations on phage restriction and modification (2) and on sporulation (3) of *C.
perfringens. We have conducted a number of experiments in attempts to demonstrate transduction in this organism and have also attempted transformation and conjugation, without success. However, new phages are now being isolated for further efforts in this area.

It is quite possible that with at least some strains auxotrophic mutants for alanine, serine, cystine, and biotin might be isolated. These nutrients were not absolutely essential but were stimulatory for the two strains used in this investigation, so no attempts were made to use them as markers. In this study, the most frequent mutant phenotypes isolated were Ade- and Nic-. Ura- and Thia- were less frequent, and most of the Thia- mutants appeared leaky and were discarded. Of the nutrients studied, p-aminobenzoic acid was the only one for which we failed to obtain auxotrophs.

ACKNOWLEDGMENTS
This investigation was supported by INSERM grant CRL 73.4.046.3 and by a grant from La Fondation pour la Recherche Médicale Française.

LITERATURE CITED
1. Boyd, M. J., M. A. Logan, and A. A. Tytell. 1948. The growth requirements of Clostridium perfringens (welchii) BP6K. J. Biol. Chem. 174:1013-1025.
2. Dreyfus-Fourcade, M., M. Sebald, and M. Zavadova. 1972. Restriction et modification du phage par Clostridium perfringens NCTC 8798 et ses mutants. Ann. Inst. Pasteur. (Paris) 122:1117-1127.
3. Duncan, C. L., D. H. Strong, and M. Sebald. 1972. Sporulation and enterotoxin production by mutants of Clostridium perfringens. J. Bacteriol. 119:578-591.
4. Fuchs, A.-R. and G. J. Bonde. 1957. The nutritional requirements of Clostridium perfringens. J. Gen. Microbiol. 16:317-329.
5. Mahony, D. E., and K. B. Easterbrook. 1970. Intracellular development of a bacteriophage of Clostridium perfringens. Can. J. Microbiol. 16:963-986.
6. Mahony, D. E., and G. G. Kalz. 1968. A temperate bacteriophage of Clostridium perfringens. Can. J. Microbiol. 14:1085-1093.
7. Murata, R., T. Yamada, and S. Kameyama. 1956. Preparation of the reproducible peptone medium for the production of the toxin of high potency. Jap. J. Med. Sci. Biol. 9:81-91.
8. Riha, W. E., Jr., and M. Solberg. 1971. Chemically defined medium for the growth of Clostridium perfringens. Appl. Microbiol. 22:738-739.
9. Smith, H. W. 1959. The bacteriophages of Clostridium perfringens. J. Gen. Microbiol. 21:622-630.
10. Ting, M. N., and D. Y. C. Fung. 1972. Chemically defined medium for growth and sporulation of Clostridium perfringens. Appl. Microbiol. 24:755-759.