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Heptakis(2,6-*O*-Dimethyl)β-Cyclodextrin: a Novel Growth Stimulant for *Bordetella pertussis* Phase I

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The effect of cyclodextrins on the growth of *Bordetella pertussis* Tohama phase I in synthetic medium was evaluated. The addition of cyclodextrins, especially heptakis(2,6-*O*-dimethyl)β-cyclodextrin (MeβCD), to a complete synthetic medium such as Stainer-Scholte medium gave the same number of individual colonies and growth rates as those on Bordet-Gengou medium. Furthermore, with the addition of MeβCD, growth inhibition by fatty acids such as oleic or palmitic acid was overcome and normal cell growth was observed. This modified Stainer-Scholte medium, designated as cyclodextrin solid medium (CSM), supported excellent growth of 20 lyophilized clinical isolates. Serotypes of the organisms after 10 passages on this CSM plate were not changed. These results suggest that MeβCD is a significant growth stimulant and CSM is one of the most suitable synthetic media for culture of *B. pertussis* phase I.

Bordetella pertussis phase I is a finicky, fastidious, slow-growing bacterium that is difficult to isolate on laboratory media. Fresh isolates were thought to have complex nutritional requirements, but *B. pertussis* phase I is now thought to have rather simple nutritional needs. However, they are sensitive to some growth inhibitors such as peptone, sulfur, peroxide, manganese, and fatty acids (12–15). The effects of these inhibitors can be partially overcome by the addition of blood, albumin, charcoal, soluble starch, or anion-exchange resins to the medium, which serves to inactivate or to sequester one or more inhibitors (2, 11, 16, 20).

However, solid synthetic medium does not support the growth of isolates unless they are seeded in very large numbers, and there is a need for an improved medium to allow growth from small inocula. The medium described by Cohen and Wheeler (3) to which charcoal and agar have been added is widely used as a solid medium for the growth of *B. pertussis* (6). The presence of charcoal in this medium is essential, but it is difficult to achieve uniform distribution and growth. Parker (11) described a solid medium which supported the good growth of *B. pertussis* phase I in which Dowex-1 resin and agarose were added in place of agar. We evaluated this medium but failed to obtain good growth, and the discrepancy was probably due to differences in the sources of agarose.

We attempted to find a compound which would support the growth of *B. pertussis* phase I well and ideally would have a low molecular weight, dissolve uniformly, and have no toxicity. Therefore, we investigated the effect of various types of cyclodextrins (CDs) on the cell growth of *B. pertussis* phase I compared with soluble starch since they are known to have the ability to form inclusion complexes and hence may be able to remove inhibitors. In this paper, we describe the effect of CDs especially heptakis(2,6-*O*-dimethyl)β-cyclodextrin (MeβCD), on the growth of *B. pertussis* phase I.

MATERIALS AND METHODS

Bacterial strain. *B. pertussis* Tohama phase I and other clinical isolates with the characteristics of virulent strains were used in our lyophilized stock cultures.

Basal medium. Stainer-Scholte (SS) liquid medium (17) was used. For clinical isolation, we used modified SS medium, designated as cyclodextrin solid medium (CSM). CSM consisted of basic medium and supplement. The basic medium contained the following: 10.7 g of sodium glutamate (mono), 0.24 g of L-proline, 2.5 g of NaCl, 0.5 g of KH_2PO_4, 0.2 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 6.1 g of Tris, 0.5 g of Casamino Acids (certified; Difco Laboratories, Detroit, Mich.), 1.0 g of MeβCD, and 15.0 g of Bacto-Agar (Difco) per liter of distilled water. It was adjusted to pH 7.4 and autoclaved at 121°C for 15 min. The supplement preparation for CSM contained 40 mg of L-cysteine monohydrochloride, 10 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of ascorbic acid, 4 mg of niacin, and 150 mg of reduced glutathione per 10 ml of distilled water. It was sterilized by filtration, and 10 ml was added to each liter of basic medium. Bordet-Gengou (BG) medium was used for comparison.

Inocula and culture conditions. Lyophilized stock

cultures of all strains used were suspended in 1% Casamino Acids solution and cultured on plates of BG medium for 72 h at 35°C. The harvested cells from these plates were subcultured on BG medium and incubated for an additional 24 h. Cells were harvested in unsupplemented SS medium and adjusted to a cell concentration of about 2×10^{11} cells per ml. One milliliter of seed suspension was inoculated into a culture medium consisting of 200 ml of SS medium in a 500-ml shaker flask. Incubation was carried out on a reciprocal shaker, using 35-mm strokes and 125 oscillations per min at 35°C.

Cell concentration. The optical density at 650 nm of a cell suspension was compared with that of the National Institute of Health (Japan) opacity standard, and the cell concentration was expressed in international opacity units. One international opacity unit is defined as being equivalent to 10^9 organisms.

Chemicals. Fatty acids in the free acid form were obtained from Nakarai Chemical Co., Tokyo, Japan, and were the highest grade available. All were dissolved in 99% ethanol and used without sterilization. Soluble starch was obtained from Difco Laboratories and the grade was certified.

CDs. αCD (cyclohexaamylose), βCD (cycloheptaamylose), γCD (cyclooctaamylose), and their hexakis, heptakis, and octakis (2,6-*O*-dimethyl) derivatives were used. Reagent grades of α- and γCD were supplied by N. Nakamura of Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan. Their purity had been determined to be not <99% by thin-layer chromatography and high-pressure liquid chromatography. βCD was prepared in our laboratory by a standard method described briefly as follows. Crude supernatant containing 50 Tilden-Hudson units (19) of cyclodextrin glycosyltransferase (EC 3.2.1.19), prepared by the cultivation method of Lane and Pirt (9), using *Bacillus macerans* IFO 3490, was mixed with 50% (wt/vol; final concentration) of dextrin (lot NSD-210, Nihon Shiryo Co., Ltd.). The enzymatic reaction was carried out for 2 days at 37°C with dropwise addition of trichloroethylene (10% [vol/vol] final concentration) as the complexing agent. By repeated steam distillation, trichloroethylene was removed; then, repeated crystallization gave the purified βCD. The purity was determined to be >98% by thin-layer chromatography and gel filtration, using Sephadex G-15.

Each 2,6-*O*-dimethylated derivative of cyclodextrin was prepared basically by the method of Boger et al. (1). Their purity as 2,6-*O*-dimethylated derivatives was determined to be >90% by thin-layer chromatography (toluene-ethanol [4:1]; no. 5715, E. Merck AG, Darmstadt, West Germany) and nuclear magnetic resonance. The residual portions consisted of partially methylated derivatives.

Factor sera. Factor sera were prepared by the method of Eldering et al. (4). These sera were used for the determination of antigenic factors of *B. pertussis* cells.

RESULTS

Effect of CDs and soluble starch on solid medium. The effect of CDs and soluble starch on SS agar medium (SSA) was studied. A 100-μl portion of a cell suspension (10^8/ml) was spread on SSA, and then various concentrations of CDs or soluble starch were dropped onto the SSA. Plates were incubated for 4 days at 35°C and the cell growth was evaluated. Good growth was observed in the places where MeβCD (>125 μg per droplet) was dropped (Fig. 1). The heaviest growth was obtained with 500 μg of MeβCD per droplet. Soluble starch, αCD, βCD, and γCD were not effective, at least under these experimental conditions.

Table 1 shows the effect of CDs and soluble starch when the plates were seeded with various concentrations of cells. Various cell concentrations (10^7, 10^8, 10^9, and 10^{10} cells per plate) of *B. pertussis* were inoculated onto SSA plates followed by the addition of paper disks (8-mm diameter, thin; Toyo Seisakusho Co., Ltd., Tokyo, Japan) which had been dipped into a solution containing 20 mg of the various CDs or soluble starch per ml. The plates were incubated for 4 days at 35°C. Cell growth was observed around the paper disks, which were dipped into αCD, βCD, MeαCD, and MeβCD (Table 1). MeβCD was the most effective. The efficacy sequence was as follows: MeβCD > MeαCD > αCD > βCD. Soluble starch, γCD, and MeγCD were not effective even at high inoculations. MeβCD was found to be the most effective for supporting the growth of *B. pertussis* (Fig. 1; Table 1), and further studies were carried out to compare cell growth on SSA containing MeβCD with that on BG medium.

Table 2 shows the cell growth on SSA containing various CDs or soluble starch and on BG medium. Plates containing 500 μg of CDs or soluble starch per ml were inoculated with various levels of organisms from 10^1 to 10^8 cells per plate, using a special 5-μl inoculating apparatus

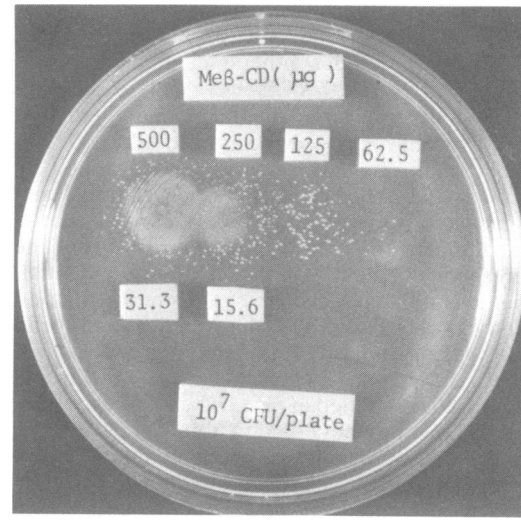

FIG. 1. Effect of MeβCD on the growth of *B. pertussis* Tohama phase I on SSA medium.

TABLE 1. Effects of CDs or a soluble starch disk on cell growth on SSA medium[a]

Compound	Cells per plate			
	10^{10}	10^9	10^8	10^7
αCD	++[b]	+	+	−
βCD	+	−	−	−
γCD	−	−	−	−
MeαCD	+++	++	+	+
MeβCD	+++	++	++	++
MeγCD	−	−	−	−
Soluble starch	−	−	−	−

[a] Inocula were spread on the plate, and then paper disks (8-mm diameter) were dipped into 2.0% CD or a soluble starch solution. The cell growth around the paper disk was estimated after 4 days of incubation.

[b] Growth is expressed as: +++, full growth; ++, 10^2 to 10^3 colonies; +, <100 colonies; −, no growth.

(Microplanter, model MIT-P; Sakuma Seisakusho, Tokyo, Japan), and then were incubated for 4 days at 35°C. SSA containing MeβCD (500 μg/ml) showed cell growth similar to that on BG medium (Table 2). αCD, βCD, and γCD were less effective than MeβCD, and soluble starch was not effective.

It is known that the growth rate of *B. pertussis* is accelerated by the addition of Casamino Acids to the SS medium. Additive or potential effects of Casamino Acids and MeβCD on colony formation were estimated. One hundred cells per plate were spread on SSA containing MeβCD and Casamino Acids (Difco, certified); the plates were incubated for 3 days at 35°C, and then the number of colonies per plate was counted. Results are shown in Table 3. The number of colonies on SSA containing MeβCD (500 μg/ml to 1 mg/ml) was the same as that on BG medium even if Casamino Acids were omitted, but the colonies growing on Casamino Acids-free medium were smaller than those growing on Casamino Acids-supplemented medium. SSA containing 1 mg of MeβCD and 500 μg of Casamino Acids per ml, designated as CSM, successfully supported the growth of *B. pertussis*. The total number of CFU and the size of the colonies on BG medium and CSM were comparable (Fig. 2).

Effect of MeβCD on growth inhibition mediated by fatty acids. *B. pertussis* phase I organisms are susceptible to a number of inhibitors present in both solid and liquid media, including peptone, sulfur, manganese, peroxide, and fatty acids (11). Recently, Field and Parker (5) reported the inhibitory effect of fatty acids on the growth of *B. pertussis* in defined medium. Therefore, we studied the effect of MeβCD on growth inhibition mediated by fatty acids. Incubation was carried out in a 500-ml shaker flask containing 200 ml of SS medium seeded with 10^9 cells of *B. pertussis* per ml. Figure 3A illustrates the effect of MeβCD on growth inhibition mediated by palmitic acid ($C_{16:0}$). As reported by Field and Parker (5), palmitic acid inhibited growth even at a concentration of 10 μM, but the addition of MeβCD (0.5 mg/ml) at the start of the culture reversed that inhibition. This was also the case with the unsaturated fatty acid oleic acid ($C_{18:1}$), which showed stronger growth inhibition than palmitic acid (Fig. 3B).

Effect of addition time on reversal of MeβCD of fatty acid inhibition. To determine whether the inhibitory effects of fatty acids are reversible, MeβCD was added to the culture broth at various cultivation periods. Results are shown in Fig. 4A. Reversal of palmitic acid inhibition could be observed when MeβCD was added up to 3 h after the start of culturing. However, with oleic acid, the addition of MeβCD 1 h after the start of culturing failed to support growth (Fig. 4B).

Growth of clinical isolates on CSM. Twenty lyophilized stock cultures of recent clinical isolates were tested. Lyophilized stock of the clinical isolates was suspended in 1% Casamino Acids solution, and the suspension was spread on CSM plates. The plates were incubated for 72 h at 35°C. The harvested cells from these plates

TABLE 2. Cell growth from various inocula on SSA medium containing CDs or soluble starch

Compound (500 μg/ml)	Inoculum size (cells)[a]							
	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
None	+++[b]	−	−	−	−	−	−	−
αCD	+++	+++	++	−	−	−	−	−
βCD	+++	++	+	−	−	−	−	−
γCD	+++	++	+	−	−	−	−	−
MeβCD	+++	+++	+++	+++	+++	++	+	+
Soluble starch	+++	+	−	−	−	−	−	−
BG medium	+++	+++	+++	+++	+++	++	+	+

[a] 5-μl inoculum.

[b] See footnote *b*, Table 1.

TABLE 3. Colony formation on SSA medium containing MeβCD and Casamino Acids[a]

Casamino Acids (μg/ml)	No. of colonies per plate at given MeβCD concn (μg/ml)					
	0	50	100	250	500	1,000
0	0	0	0	0	116	89
500	0	0	1	64	123	109
1,000	0	0	0	0	98	112
2,500	0	0	0	0	99	121
5,000	0	0	0	0	102	137
10,000	0	0	0	0	0	74

[a] About 100 cells per plate were spread on SSA medium containing MeβCD or Casamino Acids or both; the plates were incubated for 3 days at 35°C, and then the number of colonies per plate was counted. BG plates (N = 5) were used as controls, and the mean number of colonies per plate was 104 ± 16.

were suspended in saline; cell concentrations were adjusted to 5×10^2 cells per ml; and 0.2 ml of this suspension was spread on CSM and BG medium. The plates were incubated for 4 days at 35°C. The cell growth of clinical isolates was observed. All clinical isolates showed full cell growth, and the total number of CFU and the size of the colonies on BG medium and CSM were comparable.

Antigenic factor of cells cultured on the CSM plate. It is well known that *B. pertussis* phase I changes to an avirulent strain and loses its antigenic factor after several passages on defined medium. The *B. pertussis* Tohama phase I (serotypes 1, 2, and 3) and 10 recent clinical isolates (serotypes 1 and 3) were passaged 10 times on CSM plates. The cells were harvested each time in phosphate-buffered saline and stored in ice water. After 10 passages, the antigenic factor of all harvested cells was determined. There was no detectable antigenic change in any of the harvested cells.

DISCUSSION

It has not yet been possible to grow *B. pertussis* on synthetic media such as SS medium with small inocula. We show in Table 2, inocula of $<10^7$ cells were insufficient for growth. On the other hand, the addition of CDs, especially MeβCD, to a complete synthetic medium gave the same number of colonies and growth rate as BG medium. This finding prompted us to investigate the factors required for growth.

To determine which element in SS medium is responsible or obligatory for cell growth, we evaluated the component on which MeβCD confers a benefit(s). The effect of MeβCD might be brought about by (i) neutralizing or relieving the growth inhibitor, (ii) enhancing or stimulating the uptake of a component(s) essential for growth, and (iii) stabilizing such an essential component(s). In the case of (i), fatty acids such as oleic or palmitic acid were highly inhibitory to cell growth, but by the addition of MeβCD at the start of incubation, the inhibition was overcome and normal cell growth was observed. Machida et al. (10) reported studies on the stimulatory effect of MeβCD on fatty acid synthesis in *Mycobacterium smegmatis*, and Kawaguchi and Bloch (8) elucidated that MeβCD reversed the inhibition of yeast glucose-6-phosphate dehydrogenase by palmitoyl-coenzyme A. Both phenomena were found to be caused by complex formation between MeβCD and hydrophobic acyl residue (chain) in the palmitoyl-coenzyme A molecule. Similar interaction between fatty acids and MeβCD might also occur in our experiments because strengthening the hydropho-

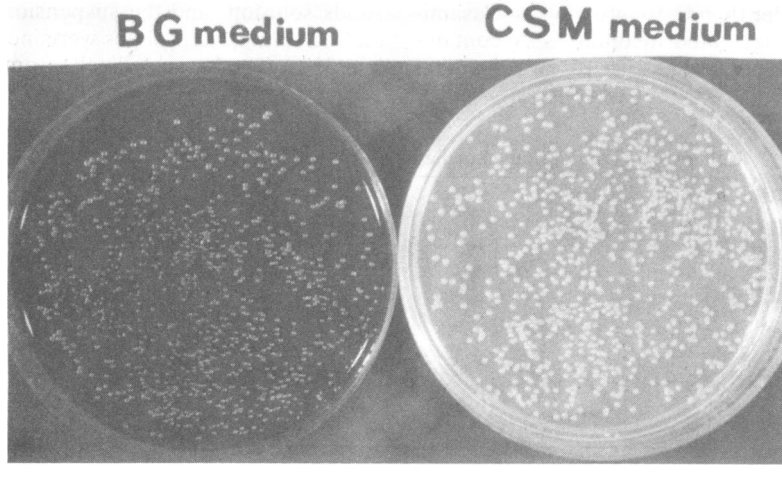

FIG. 2. Comparison of colony formation of *B. pertussis* on CSM and BG medium.

bic properties in cavities of cyclodextrin molecules such as MeαCD and MeβCD gave better results than did the parent unmethylated compounds such as αCD and βCD. In the case of (ii), Yamane et al. (21) noted that CDs possessed the same functions as albumin, i.e., operating as carriers of fatty acids in tissue cultures. Albumin was also beneficial in the culture of the genus *Leptospira* (7). This result might be brought about by its enhancing or stimulating uptake of essential components such as fatty acids. The possible compensatory role of CDs for albumin in our case is now under investigation. In the case of (iii), CDs are known to be useful as stabilizers of various unstable compounds in the pharmaceutical field (18). Preliminary experiments were carried out to examine the effect of MeβCD on stabilization of the respective components in SS medium. Mutual interaction exclusively between MeβCD and glutathione was observed. Inspection of space-filling models showed that MeβCD could incorporate monomeric, reduced glutathione in its cavity. This phenomenon suggests the possibility that MeβCD acts as a stabilizer of glutathione, but another possibility, that MeβCD facilitates the uptake or incorporation of glutathione into *B. pertussis* cells, cannot be ruled out.

As defined solid medium, SSA containing MeβCD has various advantages for genetic and nutritional studies. Until now, many studies concerning requirements of amino acids and vitamins for *B. pertussis* growth have been carried out with charcoal. This insoluble additive has undesirable disadvantages such as nonspecific adsorption of amino acids and vitamins. On the other hand, SSA containing MeβCD permits uniformity and overcomes these disadvantages.

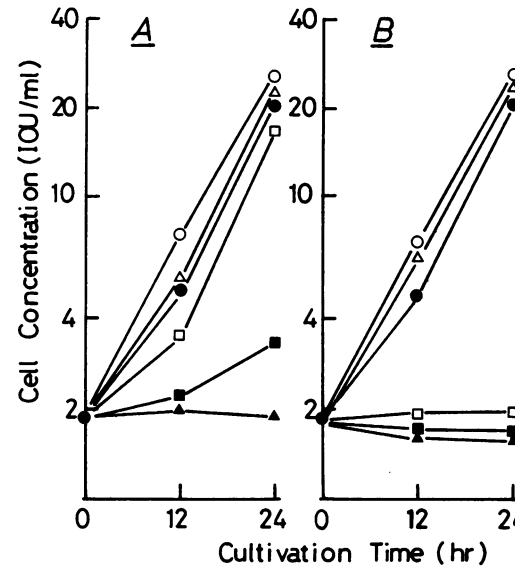

FIG. 4. Effect of addition time on reversal by MeβCD of fatty acid inhibition. (A) Palmitic acid; (B) oleic acid. Additional concentrations of MeβCD and fatty acid were 0.5 mg/ml and 10 μM, respectively. Symbols: (●) growth control (MeβCD and fatty acid not included); (○) only MeβCD added; (▲) only fatty acid added; (△) MeβCD added at start of culture; (□) MeβCD added 1 h after start of culture; (■) MeβCD added 3 h after start of culture.

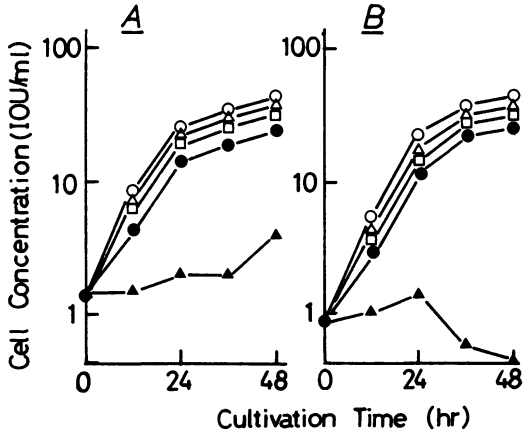

FIG. 3. Effect of MeβCD on growth inhibition mediated by fatty acids. (A) Palmitic acid; (B) oleic acid. Cell concentration is expressed as international opacity units (IOU) per milliliter. One IOU was defined as being equivalent to 10^9 cells. MeβCD and fatty acid were added to SS liquid medium at the start of culture. Symbols: (●) growth control (MeβCD and fatty acid not included); (○) only MeβCD (0.5 mg/ml) added; (▲) only fatty acid (10 μM) added; (△) fatty acid (10 μM) and MeβCD (0.5 mg/ml) added; (□) fatty acid (10 μM) and MeβCD (1.0 mg/ml) added.

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