

Formation and cell-medium partitioning of autoinhibitory free fatty acids and cyclodextrin's effect in the cultivation of *Bordetella pertussis*

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Abstract

Palmitic, palmitoleic and stearic acids were found in the extracted cellular lipids of virulent *Bordetella pertussis* as unesterified acids in confirmation of earlier taxonomic analyses. The same free fatty acids (FFAs) were found in the spent culture supernatant in concentrations higher than in the uninoculated medium, indicating that they are released into the extracellular medium. These long-chain fatty acids are known to inhibit the growth of *B. pertussis* at concentrations as low as 1 ppm. Measurement of palmitate cell-medium partitioning demonstrated a strong tendency of FFAs for cellular adsorption. Inhibition kinetics indicated that the cell-bound FFA was responsible for inhibition and that the specific cellular FFA concentrations actually found during growth were similar to those determined to be inhibitory. Autoinhibition by these endogenous FFAs provides an explanation of the low maximum cell concentrations currently attainable in liquid media. Addition of soluble dimethyl- β -cyclodextrin (Me β CD) to FFA-inhibited cultures resulted in a rapid reversal of the inhibition. A corresponding shift in the distribution of FFAs from the cells to the extracellular medium demonstrated that Me β CD sequesters FFAs. Although Me β CD did not increase final cell concentrations and even had an adverse effect on growth at concentrations above 1 g l⁻¹, it did (at 1 g l⁻¹) extend the initial period of high growth rate leading to shorter cultivation times.

Keywords: *Bordetella pertussis*; Growth; Formation of long-chain fatty acids; Growth inhibition; Cell-medium partitioning; Cyclodextrin

1. Introduction

Bordetella pertussis, the human pathogen causing pertussis (whooping cough), has often been described as a fastidious organism. The difficulty encountered in in vitro cultivation initially impeded the

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progress of large-scale vaccine production as well as clinical diagnostics (Ley and Mueller, 1946). It was formerly believed that the whole-blood and potato extract of the first isolation medium (BG medium) of Bordet and Gengou (1906) were required to satisfy the bacterium's complex nutritional requirements. However, the minimal nutrient requirement of *B. pertussis* has since been shown to be quite simple (Jebb and Tomlinson, 1957; Stainer and Scholte, 1971). Successful growth in the BG medium can be attributed to the presence of substances which neutralized the effects of various growth inhibitors including peptone, sulfur, sulfides, manganese, iron, peroxides, and fatty acids (Proom, 1955; Rowatt, 1957; Cameron and Dunning, 1970; Cameron et al., 1985; Pollock, 1947, 1949).

Of these inhibitors, fatty acids may be the most critical. Growth of *B. pertussis* is inhibited at long-chain fatty acid concentrations as low as 1 ppm (Field and Parker, 1979) and free fatty acids appear to be formed by *B. pertussis* (Pollock, 1947) and thus could be autoinhibitory. A significant fraction of unesterified free fatty acids (FFAs) have been identified in the lipids of *B. pertussis* (Thiele and Schwinn, 1973; Kawai and Moribayashi, 1982). Albumin and starch, critical components in the original isolation medium, have an affinity for lipids. The starch was demonstrated by Ungar et al. (1950) to act by adsorbing a growth inhibitor. These adsorbents have been replaced by other compounds with similar properties such as charcoal (Holt, 1962), anion exchange resins (Sutherland and Wilkinson, 1961), polyvinyl acetate and polyvinyl alcohol, methyl cellulose (Greenspan, 1985; Nikolajewski et al., 1990), and cyclodextrins (Imaizumi et al., 1983a, b). Linear carbohydrate chains of starch are known to bind fatty acids by helical encapsulation (Schoch and Williams, 1944). Similarly, cyclodextrins bind fatty acids by forming inclusion complexes (Szejtli, 1982).

Low concentrations of fatty acids have been shown to uncouple oxidative phosphorylation in mitochondrial preparations (Weinbach and Garbus, 1969) and may inhibit bacteria by a similar mechanism (Sheu et al., 1975). However, the degree of inhibition should also depend on the partitioning of the fatty acids between the cell membrane and the bulk medium (Levin and Freese, 1978). Freese et al. (1979) correlated the octanol/water partition coefficient of vari-

ous lipophilic acids with their inhibition of *Bacillus subtilis* and HeLa cells, suggesting that the more hydrophobic the fatty acid the more effective it is as an inhibitor.

If the endogenously formed FFAs of *B. pertussis* are freely diffusible, then their inhibitory activity would be equivalent to FFAs added or present as medium contaminants. An accumulation during growth could limit growth rate and maximum cell concentrations in bioreactors. In this study, we quantify growth inhibition by the fatty acids that are also produced by *B. pertussis* and the degree of binding with heptakis (2,6-*O*-dimethyl) β -cyclodextrin (Me β CD). We also demonstrate that the behavior of these inhibitors can be described by an equilibrium partitioning model and that FFAs act as autoinhibitors to limit the in vitro growth of *B. pertussis*.

1.1. Quantifying partitioning, cyclodextrin binding, and growth inhibition

The equilibrium distribution of a lipophilic inhibitor in a culture can be expressed by a partition coefficient (PC) defined as:

$$PC = I_c / I_e \quad (1)$$

where I_c is the specific cellular concentration of the inhibitor associated with the cell and I_e is the concentration of the inhibitor in the extracellular medium. In the presence of an inhibitor-binding ligand such as Me β CD, some of the inhibitor will be bound with the ligand. At equilibrium, the concentration of the inhibitor–ligand complex (IL), the concentration of the free ligand (L), and I_e , can be related by a binding, or complex 'formation', constant.

$$K_f = IL / (I_e)(L) \quad (2)$$

Eq. 2 assumes a 1:1 host/guest complexation ratio; however, up to three cyclodextrin rings have been found to complex with a single long-chain fatty acid of 16 to 18 carbons (Szejtli, 1982). Thus, the K_f defined here is an overall or apparent binding constant.

By material balance, the total global concentrations of inhibitor (I_t) and ligand (L_t) are:

$$I_t = I_e + I_c X + IL \quad (3)$$

$$L_t = L + IL \quad (4)$$

where X is the cell concentration. PC is determined by measuring the fraction of inhibitor associated with the cells (f_c) in the absence of cyclodextrin. K_f is then determined by repeating measurements of f_c in the presence of cyclodextrin at a known concentration. f_c is:

$$f_c = I_c X / I_t \quad (5)$$

Combining Eq. 5 with Eqs. 1 and 3 where $IL = 0$,

$$PC = (1/X)(f_c/(1-f_c)) \quad (6)$$

The quantity $f_c/(1-f_c)$ is commonly referred to as the partition ratio (PR). A plot of X vs. PR should yield a straight line of slope PC . K_f is calculated by combining Eqs. 1, 2, 3, 4, and 5 where PC , X , L_t , I_t , and a new f_c are known.

$$K_f = \frac{(1-f_c)f_c^{-1}PCX - 1}{(L_t - I_t) + f_c I_t (1 + (PCX)^{-1})} \quad (7)$$

Growth inhibition is normally quantified by the reduction in growth rate observed for a known total dose of inhibitor (I_t) added to an actively growing culture. For a cell-bound inhibitor, it is more descriptive to express the inhibitor concentration in terms of the specific cellular concentration (I_c). In the absence of cyclodextrin ($IL = 0$), I_c can be calculated from Eqs. 1 and 3:

$$I_c = I_t / (X + PC^{-1}) \quad (8)$$

Inhibitor potency is often described by the inhibitor concentration required to inhibit growth by 50% (IC_{50}). In this article, we use $I_t C_{50}$ to distinguish it from $I_c C_{50}$ which describes the I_c that inhibits by 50%. $I_c C_{50}$ is a more general parameter since it is independent of cell concentration.

2. Materials and methods

2.1. Media

A modified version of the medium of Imaizumi et al. (1983b) was used in this study containing per l: 6.10 g tris(hydroxymethyl)aminomethane as buffer, 3 g casamino acids (Difco Certified), 17 g monosodium glutamate, 0.24 g proline, 2.5 g NaCl,

0.5 g KH_2PO_4 , 0.2 g KCl, 0.1 g $MgCl_2 \cdot 6H_2O$, and 0.02 g $CaCl_2$ (Licari et al., 1991). pH was adjusted to 7.6 with 5 N HCl. Growth factors were added separately (10 ml l^{-1}) at time of inoculation as a concentrated solution consisting of per l: 0.4 g nicotinic acid, 0.1 g $FeSO_4 \cdot 7H_2O$, 4.0 g L-cysteine hydrochloride monohydrate, 15 g glutathione, and 40.0 g ascorbic acid (pH 7.2). Me β CD, when included, was at a concentration of 1 g l^{-1} or as otherwise noted.

2.2. Bacteria

Bordetella pertussis strain SK101 (18323C1::Kan^r) is resistant to antigenic modulation as induced by sulfate and niacin and to kanamycin (Knapp and Mekalanos, 1988). The strain has similar growth behavior and pertussis toxin yields as its wild-type parent. The work-up of frozen seed cultures for later inoculation of experimental cultures has been described elsewhere (Frohlich et al., 1995).

2.3. Bioreactor studies

Batch cultivations were carried out in a New Brunswick 2-l 'Bioflow' unit with a 1 to 1.3 l working volume ($36.5 \pm 0.2^\circ\text{C}$, pH controlled at 7.2 ± 0.05 using 4 N HCl, air flow at 0.8 standard l min^{-1} , agitation rate $\geq 500 \text{ rpm}$ to keep the dissolved oxygen concentration above 50% of saturation). Sigma Antifoam C was used (Frohlich et al., 1995). The bioreactor was inoculated with an actively growing preculture grown at 36°C for 24 h in a 250-ml spinner flask (Bellco Biotechnology).

2.4. Cell concentration measurements

Cell growth was monitored by optical density (OD) on a Beckman (DU 50) spectrophotometer at 530 nanometers (OD_{530}). Correlation between OD and dry cell weight concentration was determined as described previously (Frohlich, 1993; Frohlich et al., 1995). One (1.0) OD_{530} unit corresponded to about 0.34 g dry cell weight per liter (g l^{-1} DCW) or roughly 2.2×10^9 cells per ml. This correlation was determined to be linear over at least 10 OD_{530} . Cell clumping was not observed in media with or without cyclodextrin as long as vigorous agitation was used.

2.5. Growth inhibition studies

To determine the effect of fatty acids on growth rate, initial-rate experiments were performed using 100 ml cultures in 250-ml spinner flasks ($36 \pm 0.5^\circ\text{C}$) inoculated from a preculture grown 18–24 h. The fatty acid inhibitors were added after inoculation as a sterile concentrate in 95% ethanol. An equivalent amount of ethanol added to the control cultures did not significantly affect growth. Care was taken to acid clean the spinner flasks to minimize the inhibiting effects of contaminating fatty acids and soaps.

2.6. Free fatty acid analysis

The free fatty acids (FFAs) in the cellular lipids as well as the cell-free culture supernatant were analyzed. Bacterial suspensions of known volume and optical density were centrifuged at $6000 \times g$ for 1 h to recover the cells. The culture supernatant was ultracentrifuged at 37°C and at $100\,000 \times g$ for 2 h to remove any remaining cell debris.

For analysis of FFA in the cellular lipids, the cells were first sonicated and total lipids extracted by the method of Bligh and Dyer (1959) using chloroform and methanol. Solvents used were of a grade pure enough for trace organic analysis. The organic phase was concentrated to approx. 1 ml in a rotary evaporator and transferred to a pre-weighed vial. Total lipid weights were determined by drying the extract in a stream of nitrogen. Separation of the neutral lipid fraction (FFAs) from the more polar phospholipids was accomplished using a procedure similar to that described by Walstad et al. (1974). After initial identification, however, the preparative thin layer chromatography (TLC) was replaced with solid phase extraction (SPE) for routine analyses. Concentrated extract was loaded onto a minicolumn (Supelco LC SPE tube) preconditioned with heptane and the neutral fraction eluted using 5 ml of a petroleum ether/diethyl ether/acetic acid (70:30:1 by volume) mixture. Eluent was evaporated to dryness three times with heptane to azeotropically remove the acetic acid before exposing the sample to diazomethane vapors. The methyl esters formed were injected into a gas chromatograph (Hewlett Packard, HP 5890) equipped with a polar column (Supelco, SP1000) and either a mass spectrophotometer (HP

5988A) for initial identification or a flame ionization detector for routine quantification. Very little oleic acid is contained in the lipids of *B. pertussis* (Thiele and Schwinn, 1973) and was used as the internal standard.

For quantitative analysis of the extracellular medium, a known volume (approx. 20 ml) of the culture supernatant (ultracentrifuged) was acidified to pH 2.0 after the addition of the internal standard. This supernatant was then extracted with one volume of diethyl ether and then twice more with one half volume. The remainder of the procedure is the same as that used for the cellular lipids. TLC or SPE was used here to separate FFAs from other coextractants like hydrophobic amino acids and denatured proteins before identification by GC/MS.

2.7. Cell - medium partitioning of palmitic acid

Radiolabeled palmitic acid [$1\text{-}^{14}\text{C}$] (New England Nuclear, Boston, MA) was used to determine partition coefficients between cells and medium using a method similar to Sallee (1978). Stock solutions in 95% ethanol (undenatured) were prepared of palmitic acid [$1\text{-}^{14}\text{C}$] at 0.36 mM ($20 \mu\text{Ci ml}^{-1}$), and of unlabeled palmitic acid (Sigma Chemical Co.) at 20 mM. These solutions were mixed together before adding to cell suspensions to give varying concentrations. Fatty acid concentrations in the test cultures ranged from 0.1 to $10 \mu\text{g ml}^{-1}$ but always contained $0.1 \mu\text{g}$ palmitic acid [$1\text{-}^{14}\text{C}$] per ml giving $0.023 \mu\text{Ci ml}^{-1}$ equivalent to 50 000 counts per min per ml (cpm ml^{-1}). Cells were prepared for addition from a spinner culture in a cyclodextrin-free medium by centrifuging at $5100 \times g$, resuspending the cells in equal volume of fresh medium, centrifuging again, and resuspending them in a few milliliters of medium. Concentrated cells were suspended in fresh culture media at 37°C in 50- or 125-ml Erlenmeyer flasks siliconized with Sigmacote (Sigma Chemical Co.). After 15 min from the addition of the fatty acid solution, 1-ml samples were withdrawn, placed in 1.5-ml polypropylene centrifuge tubes, and spun down at $10\,000 \times g$ for 10 min at $37 \pm 2^\circ\text{C}$. Supernatant (0.8 ml) was removed and counted with 15 ml scintillation fluor (UltimaGold, Packard). Remaining supernatant was counted along with the cell pellet. The pellet was recovered by cutting off the tip of the

centrifuge tube with a razor blade and solubilized in the vial with 1 ml of solubilizer ('Solvable', New England Nuclear) before adding 15 ml scintillation fluor.

Partition coefficients were calculated from Eq. 6 where $f_c = 1 - [s1/v1/(s1 + p)]$; $s1$ is the cpm in first supernatant aliquot, $v1$ is the volume of the first aliquot (normally 0.8 ml), and p is the cpm in the recovered pellet plus remaining supernatant. This assumes that the pellet has negligible volume. Correction for interstitial media in pellet was not necessary due to the high degree of cell binding of FFA in comparison to the media.

2.8. Fatty acid-cyclodextrin binding

Binding constants for palmitic acid–Me β CD complex were estimated from partition data in cyclodextrin-containing media. The procedure used is parallel to the one described above. Calculation of the binding constant is described in the Introduction.

3. Results

3.1. Free fatty acid analysis

Both the cells and the spent extra-cellular medium, recovered from bioreactor cultures, were analyzed for free fatty acids. Table 1 summarizes the quantitative analysis of FFAs in cultures grown in media with (at 1 g l⁻¹) or without dimethyl- β -cyclodextrin (Me β CD). FFAs in the cellular lipids were found to be predominantly palmitic, palmitoleic, and stearic acids. Their identity was verified by the parent peak molecular weight of their respective mass spectra. The composition of this neutral lipid fraction is generally in agreement with the results of Thiele and Schwinn (1973) who found free palmitic, palmitoleic, and stearic acids to comprise 60, 23, and 7% of total FFAs, respectively; other minor fatty acids made up the remaining portion.

The FFAs found in the extracellular medium were primarily palmitic and palmitoleic in roughly equal

Table 1
Analysis of total lipid and free fatty acid content of *B. pertussis* cultivated in liquid media with and without dimethyl- β -cyclodextrin^a

Growth medium:	Medium without Me β CD ^b	Medium with 0.1% Me β CD ^b	Uninoculated medium
Total lipid content			
% of dry cell weight	8.2 ± 2.8 (n = 3)	13.5 ± 1.1 (n = 4)	–
Free fatty acid content of cellular lipids			
% of dry cell weight	0.53 ± 0.13 (n = 3)	0.45 ± 0.19 (n = 4)	–
% of total lipid	7.24 ± 3.58 (n = 3)	3.62 ± 1.5 (n = 4)	–
Composition			
% palmitic (16:0)	61.3 ± 5.5 (n = 5)	64.3 ± 1.2 (n = 4)	–
% palmitoleic (16:1)	15.3 ± 4.4 (n = 5)	18.8 ± 4.1 (n = 4)	–
% stearic (18:0)	23.4 ± 6.8 (n = 5)	16.9 ± 2.8 (n = 4)	–
% others (14,15,17:0)	< 5	< 5	–
Free fatty acid concentration in culture supernatant			
Total concentration (μ g ml ⁻¹)	3.0 ± 1.8 (n = 3) ^c	8.6 ± 3.4 (n = 3) ^d	0.6 ± 0.3 (n = 2)
Composition:			
% palmitic (16:0)	42.5 ± 8.4 (n = 4)	42.9 ± 4.1 (n = 5)	40 ± 5 (n = 2)
% palmitoleic (16:1)	29.8 ± 10.1 (n = 4)	45.4 ± 9.2 (n = 5)	0
% stearic (18:0)	27.7 ± 15.3 (n = 4)	11.8 ± 6.1 (n = 5)	60 ± 5 (n = 2)

^a Given as mean ± standard deviation and number of determinations (n).

^b Post log-phase samples.

^c Concentrations ranged from 0.7 μ g ml⁻¹ at start to 4.7 μ g ml⁻¹ at end of batch growth.

^d Concentrations ranged from 2.8 μ g ml⁻¹ at start to 12.3 μ g ml⁻¹ at end of batch growth.

concentrations. Stearic acid was also detected, particularly in samples with low total FFA concentrations, and may be a medium contaminant in addition to being released by the cells. Only a small amount of FFAs, mostly stearic acid, were present in the uninoculated medium ($0.6 \mu\text{g ml}^{-1}$) as opposed to the culture supernatants. As expected, the 1 g l^{-1} Me β CD spent medium supernatant contained more FFA than the supernatant of the medium without Me β CD, supporting the idea that cyclodextrins can enhance the aqueous solubility of long-chain fatty acids.

3.2. Growth inhibition by palmitic and palmitoleic acids

Because palmitic and palmitoleic acid were present in the culture supernatant at the highest concentrations and are both highly inhibitory (Field and Parker, 1979), we focused our analysis on these two fatty acids. Representative growth curves are shown in Fig. 1 in which cultures were injected with vary-

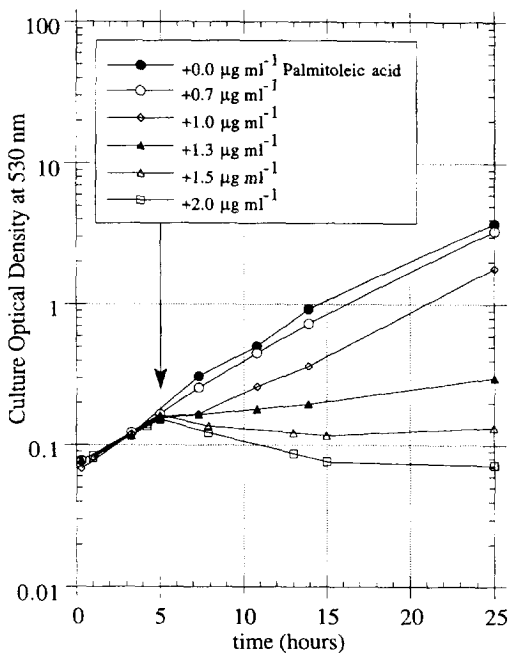


Fig. 1. Spinner flask growth with varying final concentrations of palmitoleic acid. The fatty acid (in ethanol) was added when the cell concentration reached $0.15 OD_{530}$ (0.051 g l^{-1} DCW).

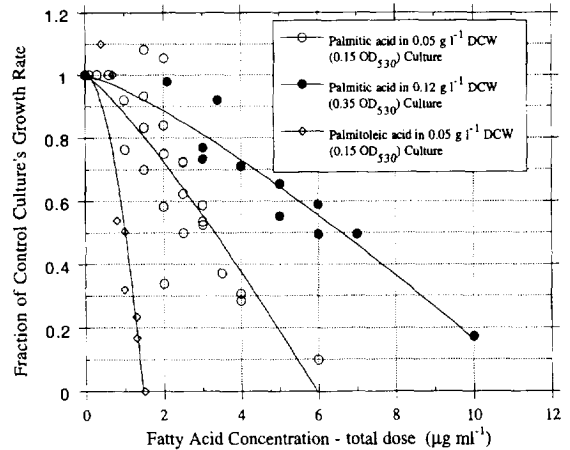


Fig. 2. Growth inhibition of *B. pertussis* by palmitoleic acid and by palmitic acid at two cell concentrations. Growth rates were determined for the first 10 h following inhibitor addition.

ing amounts of palmitoleic acid or an ethanol blank (control) at a specified cell concentration. Inhibition of growth occurs almost immediately and total inhibition is observed at palmitoleic acid concentrations greater than $1.3 \mu\text{g ml}^{-1}$ for the cell concentration in this case ($0.15 OD_{530}$ or 0.051 g l^{-1} DCW). Fig. 2 summarizes the effect of both palmitic and palmitoleic acids on growth rate. The $I_{tC_{50}}$ for palmitoleic acid is approx. $1 \mu\text{g ml}^{-1}$ at the test cell concentration of 0.051 g l^{-1} DCW ($0.15 OD_{530}$). The $I_{tC_{50}}$ for palmitic acid is about $3.3 \mu\text{g ml}^{-1}$ and $6.6 \mu\text{g ml}^{-1}$ when tested at cell concentrations of 0.051 g l^{-1} DCW ($0.15 OD_{530}$) and 0.12 g l^{-1} DCW ($0.35 OD_{530}$), respectively. This cell concentration-dependent response indicates that it is the cell-bound FFAs that inhibit growth. Interestingly, FFA concentrations found in the supernatant of the 1 g l^{-1} Me β CD as well as no-CD culture media (Table 1) are in excess of the amount determined above to halt growth. However, the final cell concentrations in the bioreactor were about 3.0 g l^{-1} DCW (e.g., Fig. 5).

3.3. Cell-medium partitioning of palmitic acid

Partitioning of palmitic acid between medium and cells was examined. Both PC and PR are expected to be independent of the inhibitor concentration. Initial experiments were carried out to verify that neither the extracellular liquid phase nor the cell

Table 2
Test for phase saturation by palmitic acid in cell-medium partitioning measurements

Free fatty acid concentration (total dose; $\mu\text{g ml}^{-1}$)	Cell-bound fraction ^a (f_c)	Partition ratio ^a ($f_c/(1-f_c)$)
0.3	0.77 ± 0.01	3.34 ± 0.25
1.0	0.74 ± 0.04	2.85 ± 1.00
3.0	0.75 ± 0.03	3.00 ± 0.75

^a Mean \pm standard deviation of three determinations.

phase is saturated with FFA at the normal test concentration of $3 \mu\text{g ml}^{-1}$ palmitic acid. Table 2 indicates that this is indeed so since a concentration 10-fold less gave the same *PR*. Also, to demonstrate that these values were determined at equilibrium, the *PR* was measured at 5, 10, and 15 min after the addition of radio-labeled palmitic acid to a no-CD medium. Equilibrium was established within 10 min (Fig. 3, initial time course).

f_c was determined at several cell concentrations without Me β CD. *PR*, calculated from f_c , was plotted against X in Fig. 4. *PC* is estimated to be $15000 \pm 2000 \text{ l kg}^{-1} \text{ DCW}^{-1}$ from the slope of the line (Eq. 6). Assuming that cellular lipid comprises about 10% of dry cell mass, this value corresponds to about 150000 l per kg total lipid. This high partition coefficient demonstrates the relative hydro-

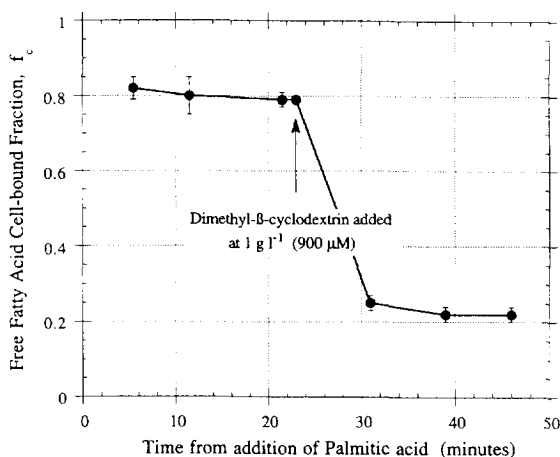


Fig. 3. Redistribution of palmitic acid between cells and medium upon addition of dimethyl- β -cyclodextrin at 37°C. The cells were initially re-suspended in fresh medium without the cyclodextrin. Total palmitic acid concentration was $3 \mu\text{g ml}^{-1}$.

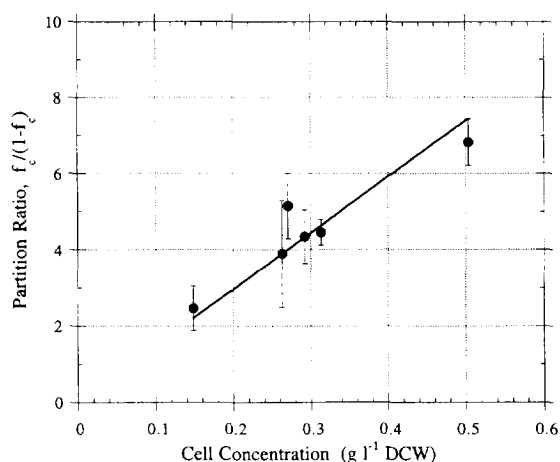


Fig. 4. Cell-medium partition ratio of palmitic acid as a function of cell concentration. Total concentration of palmitic acid was $3.0 \mu\text{g ml}^{-1}$ for all points. The line shown is a least-squares fit of data through the origin where the slope equals the partition coefficient.

phobicity of the membranes and the strong tendency of FFAs to associate with the membranes rather than remain in solution.

3.4. Inhibitory cellular FFA concentration

The specific cellular concentration, $I_c C_{50}$, corresponding to the observed $I_t C_{50}$ can be calculated from Eq. 8. For palmitic acid, the $I_c C_{50}$ calculated from the $I_t C_{50}$ s reported above for the two different cell concentrations ($X = 0.051$ and $0.120 \text{ g l}^{-1} \text{ DCW}$) are 2.8 and 3.8% of DCW, respectively. As expected, these two sets of experiments yielded the same $I_c C_{50}$ within the error of these measurements. Adsorption of FFA to glass was observed which effectively reduced I_t in these experiments, indicating that *B. pertussis* is even more sensitive to palmitic acid than the calculated $I_c C_{50}$ suggests.

For palmitoleic acid, using the partition coefficient determined for palmitic acid, the $I_c C_{50}$ calculated from the $I_t C_{50}$ observed in Fig. 2 is approx. 0.85% of DCW. Since the mono-unsaturated palmitoleic acid is less lipophilic than its saturated C16 counterpart (Sallee, 1978), the $I_c C_{50}$ is expected to be even lower. From this comparison, palmitoleic is significantly more inhibitory than palmitic acid. This is in agreement with previous work which indicates

that unsaturated fatty acids are more potent antimicrobial agents (Weinbach and Garbus, 1969; Cherrington et al., 1991).

3.5. Palmitic acid-cyclodextrin binding

It is likely that Me β CD reverses growth inhibition by sequestering fatty acids; however, the binding between FFA and cyclodextrin has not been examined quantitatively. To simulate the actual removal of fatty acids from cells by cyclodextrin, Me β CD was added to a cell suspension initially equilibrated with a dose of fatty acids as was done to measure the cell-medium partition coefficient. The short time required to reach a new distribution (Fig. 3) implies rapid desorption and binding. From the new f_c established after the addition of Me β CD, K_f can be calculated from Eq. 7. This calculation was done for several experiments ($n = 7$) at various cell concentrations. An average K_f was determined to be $20\,400 \pm 4000\text{ M}^{-1}$. These results indicate that equilibrium partitioning is an acceptable model to de-

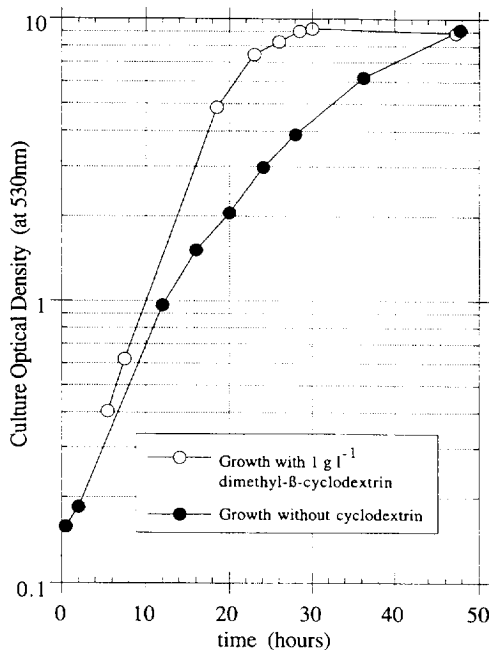


Fig. 5. Comparison of batch growth in the bioreactor using media with and without dimethyl- β -cyclodextrin. The same lot of base medium was used and pH was controlled at 7.2 using hydrochloric acid in both experiments.

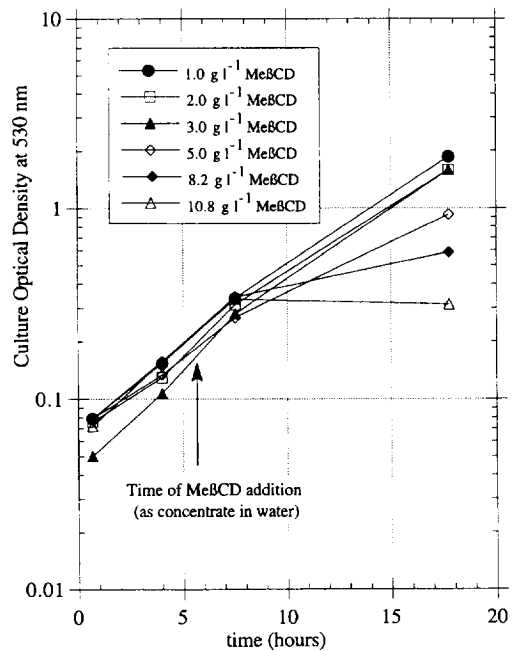


Fig. 6. Spinner flask growth with increased concentrations of dimethyl- β -cyclodextrin (Me β CD) in the medium.

scribe the distribution of FFAs in a growing culture. Also, the actual distribution of FFAs in the 1 g l^{-1} Me β CD medium approximates that calculated using the measured PC and K_f .

3.6. Batch growth with and without Me β CD

The effect of dimethyl- β -cyclodextrin (Me β CD) on the growth of *Bordetella pertussis* in an air-sparged bioreactor is demonstrated by the two batch growth curves superimposed in Fig. 5. The initial growth over the first 10 to 15 h is nearly exponential with a specific growth rate of $0.17\text{--}0.20\text{ h}^{-1}$ in the cyclodextrin-supplemented (1 g l^{-1} Me β CD) medium and approx. 0.16 h^{-1} in the unsupplemented (no-CD) medium. Whereas the initial growth rates are only marginally different, there is a marked reduction in growth rate later in the growth cycle in the no-CD medium as compared to the 1 g l^{-1} Me β CD medium. Final cell concentrations, however, were not significantly different. The no-CD cultivation was terminated at the last point shown because of severe foaming even though growth did not appear to have stopped completely. These foam-

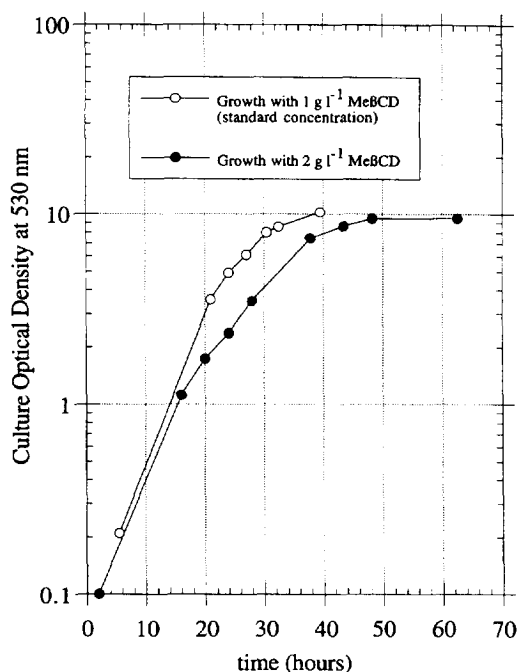


Fig. 7. Comparison of batch growth in the bioreactor using media containing 1 g l^{-1} (standard concentration) and 2 g l^{-1} dimethyl- β -cyclodextrin. The same lot of base medium was used and pH was controlled at 7.2 using hydrochloric acid in both experiments.

ing problems were not experienced with media containing Me β CD.

Higher Me β CD concentrations did not further increase final cell concentrations. Fig. 6 shows the effect on growth of increasing concentrations of Me β CD above 1 g l^{-1} on spinner cultures. From this experiment it is apparent that Me β CD concentrations above 1 g l^{-1} are detrimental to growth. This was confirmed in the bioreactor (Fig. 7) where Me β CD at 2 g l^{-1} decreased the extent of growth compared to Me β CD at 1 g l^{-1} . Initial growth rates, however, were the same.

4. Discussion

4.1. *B. pertussis* and growth inhibition by FFAs

In support of earlier studies, palmitic, palmitoleic, and stearic acids were found in the lipids of *B. pertussis* in a free, unesterified, form. This neutral lipid composition reflects the relatively simple esteri-

fied lipid composition of *B. pertussis* from which the FFAs are most likely derived. Both Thiele and Schwinn (1973) and Kawai and Moribayashi (1982) found 90% of the esterified lipids to be palmitate and palmitoleate in approximately equal proportions predominantly in the form of phosphatidyl-ethanolamine. These long-chain FFAs are known inhibitors of *B. pertussis*. Total FFA contents were about 0.5% of DCW and 5% of total lipid (Table 1). These cellular concentrations are somewhat below those reported by others (Kawai and Moribayashi, 1982; Thiele and Schwinn, 1973), but are comparable to those determined to be inhibitory (palmitoleic $I_c C_{50} \leq 0.85\%$ DCW and palmitic $I_c C_{50} \leq 3.8\%$ DCW) in this study.

Palmitic, palmitoleic, and stearic acids were also found in the extracellular medium during growth. FFA concentrations in the spent culture supernatant, which had not been previously measured, were significantly higher than those found in the uninoculated medium indicating that they are released by the cells. These extracellular concentrations are as high as those found previously (Field and Parker, 1979) to inhibit growth. Thus, this study supports the hypothesis that the growth of *Bordetella pertussis* in bioreactor culture is limited by the autoinhibitory effects of long-chain fatty acids.

The FFA autoinhibition hypothesis can explain much of the puzzling in vitro growth behavior of *B. pertussis* including: (a) the low maximum cell concentrations achieved with conventional media; (b) the distinct deceleration of growth in batch culture before the onset of stationary growth (Rowatt, 1957; Van Hemert, 1974) and; (c) the apparent washout (reduction in steady state cell concentrations) in continuous culture at dilution rates well below those corresponding to the maximum growth rates in dilute cultures (Van Hemert, 1974; Novotny and Cownley, 1978; Frohlich, 1993). Maximum cell concentrations of virulent *B. pertussis* are limited to about 3.5 g l^{-1} DCW in conventional media (Frohlich, 1993). In a recent study (Frohlich et al., 1995), no oxygen or nutrient limitations or inhibition by major growth products could be identified. Although salt concentration can affect the maximum cell concentration, no direct effect of salt on growth rate is observed when tested at low cell concentration. However, the formation and accumulation of FFAs does explain

the gradually declining growth rate observed in batch cultivation which becomes more pronounced towards the end of the growth period.

Although endogenously formed FFAs cause the larger problem in bioreactor culture, this analysis can also shed light on some of the earlier problems associated with exogenous FFAs present as contaminants in both solid and liquid media. Often, at least a 5–10% inoculum was necessary to initiate growth. By cell-medium partitioning, the larger the inoculum, the less FFA adsorbed per cell. In early work, liquid medium preparations, most likely of lesser purity than newer media, often required pretreatment with lipid-adsorbing albumin, starch or charcoal to achieve satisfactory growth from small inocula. Similarly, we have found that considerably smaller inoculum sizes can be used in media containing cyclodextrin (data not shown).

4.2. Action of Me β CD in *B. pertussis* cultivation

Of all the adsorbents previously tried with *B. pertussis*, cyclodextrin seems to be the best in terms of growth, foam control, and antigen production (Imaizumi et al., 1983a, b; Irons and Gorringe, 1988; Frohlich, 1993). Since Me β CD was shown to have a high binding affinity for FFAs and to rapidly reverse FFA-induced inhibition, the extension of the initial period of high growth rate is easily explained in terms of binding of endogenously formed FFAs. This effect on the later stages of batch growth has not been previously noted most likely because prior growth studies used shake flasks (rather than bioreactors) where other factors can limit growth. Others have noted an initial lag phase which was reduced by Me β CD, an effect probably due to FFA contaminants.

Another reason shake flasks were not used in this study is because bacterial aggregation was observed in these devices. Menozzi et al. (1994) have recently shown Me β CD to abolish autoagglutination of *B. pertussis*. Bacterial aggregation can cause an apparent difference in growth curves in media with and without Me β CD since cell clumps can influence light scattering determination of cell concentrations. We employed spinner flasks or bioreactors under conditions of vigorous agitation where bacterial aggregation was not observed in either medium.

Our inability to attain higher concentrations of cells by using more cyclodextrin suggests that Me β CD might also have an adverse effect on growth. Thus, the use of cyclodextrin in the medium could involve a trade off between binding inhibitory fatty acids and binding necessary nutrients. Imaizumi et al. (1983b) observed an interaction between glutathione and cyclodextrin. Alternatively, a small amount of FFA may be beneficial to growth which may be removed if too much Me β CD is used. However, the Me β CD had little effect on initial growth rates. If FFAs were required, this early growth period would be most affected by cyclodextrin since the unbound FFA would be at its lowest concentration.

4.3. Physiology of *B. pertussis* and its similarity to *Neisseria gonorrhoeae*

The formation of autoinhibitory FFAs is not unique to *B. pertussis*. *Neisseria gonorrhoeae*, also a Gram-negative pathogen that colonizes human epithelial tissue, has been shown to produce long-chain FFAs (Walstad et al., 1974). Like *B. pertussis*, growth of *N. gonorrhoeae* is also inhibited by them (Miller et al., 1977). Most Gram-positive bacteria exhibit sensitivity to long-chain fatty acids, while Gram negatives do not (Kodicek, 1949; Nieman, 1954). Sheu and Freese (1973) speculated that the outer membrane of Gram-negative bacteria protects them from the action of fatty acids. The Gram-negative pathogens *B. pertussis* and *N. gonorrhoeae*, however, are sensitive to free fatty acids indicating perhaps a fundamental difference in their outer-membranes. Quite possibly this difference lies in the structure of their lipopolysaccharides (LPS). *B. pertussis* is known to possess a lipooligosaccharide (a LPS with a shorter carbohydrate chain; LeDur et al., 1980; Pepler, 1984).

The presence of unesterified fatty acids in cellular lipids is uncommon in bacteria. Since the FFAs are inhibitory to the bacteria, their formation seems counterproductive. An interesting possibility is that the release of FFAs has a function in pathogenesis. They might function alone or in conjunction with other virulence factors. For instance, palmitic acid was shown to augment the hemolytic activity of *Staphylococcus aureus* (Kapral, 1976). Recent stud-

ies showing that the short-chain fatty acids of anaerobic bacteria can inhibit phagocytosis by human neutrophils and lung phagocytes (Eftimiadi et al., 1990), suggest that the free long-chain fatty acids of *B. pertussis* might also play a role in avoiding bacterial clearing by immune cells. Alternatively, FFAs might play a role in or be a by-product from the release of other virulence factors. Both *N. gonorrhoeae* and *B. pertussis* release a peptidoglycan-derived cytotoxin (Goldman, 1988).

5. List of symbols

DCW,	dry cell weight
f_c ,	fraction of fatty acid inhibitor (radio-labeled) associated with cell pellet in culture sample
FFA,	free fatty acid
I_c ,	specific cellular inhibitor concentration ($\mu\text{g ml}^{-1}$; μM)
IL ,	inhibitor-associated ligand (cyclodextrin) concentration (μM)
I_t ,	total inhibitor concentration in culture ($\mu\text{g ml}^{-1}$; μM)
$I_c C_{50}$,	specific cellular inhibitor concentration that inhibits growth by 50% ($\mu\text{g g}^{-1}$ DCW $^{-1}$; % of DCW)
$I_t C_{50}$,	total inhibitor concentration that inhibits growth by 50% ($\mu\text{g ml}^{-1}$; μM)
K_f ,	equilibrium formation constant of inhibitor–ligand (cyclodextrin) complex (kg l^{-1})
L ,	free ligand concentration in culture (μM)
L_t ,	total ligand concentration in culture (μM)
$Me\beta\text{CD}$, OD_{530} ,	heptakis (2,6- <i>O</i> -dimethyl) β -cyclodextrin optical density (absorbance) at 530 nm wavelength
PC ,	equilibrium partition coefficient of inhibitor between cells and extracellular medium (1 kg^{-1} DCW $^{-1}$)
PR ,	partition ratio of inhibitor between cells and extracellular medium (dimensionless)
X ,	cell concentration (g l^{-1} DCW; OD_{530})

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