
Effect of polyoxyethylene sorbate compounds (Tweens) on colonial morphology, growth, and ultrastructure of *Mycobacterium paratuberculosis*

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Polyoxyethylene sorbate (Tween) compounds were tested to compare their growth stimulation effects on *Mycobacterium paratuberculosis*. Three low passage and three high passage clinical isolates and ATCC strain 19698 were used. Tween 20, 40, 60, and 80 were tested at concentrations of 0, 0.001, 0.01, 1.0, and 3.0% (w/v) in radiometric broth culture media and in Middlebrook 7H9 agar plates. Scanning and transmission electron microscopy were used to examine cell wall appearance and ultrastructure, respectively. In broth culture, 0.1% (w/v) Tween 60 most dramatically enhanced growth of *M. paratuberculosis* ATCC strain 19698. The effects of Tween 40 and 80 on growth took a bimodal form, enhancing growth at concentration ranges of 0-0.01% and 0.1-1.0% (w/v) but suppressing growth at concentrations of 0.01-0.1% (w/v). Two of three high passage clinical isolates grew optimally in the presence of 1.0% (w/v) Tween 80, while the remaining high passage isolate and all three low passage isolates grew best in media containing 0.1% (w/v) Tween 80. Colonial morphology of all strains grown on Middlebrook 7H9 agar without Tween 80 was irregular and granular whereas colonies on plate media containing > 0.01% (w/v) Tween 80 were entire, smooth, and domed. Scanning electron microscopy also revealed a transition from rough to smooth cell walls with increasing Tween 80 concentration. Transmission electron microscopy showed the presence of low electron dense intracellular vacuoles in Tween 80 grown *M. paratuberculosis* cells. Thus, Tweens altered colonial morphology, the cell wall surface, and ultrastructure of *M. paratuberculosis* and stimulated its growth *in vitro* in a concentration-dependent, and often bimodal, fashion. These data suggest that Tween may also influence the chemical or antigenic structure of *M. paratuberculosis*.

Key words: *Mycobacterium paratuberculosis*; colonial morphology; ultrastructure; growth rate; Tween; polyoxyethylene sorbate.

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Polyoxyethylene sorbate (Tween) compounds belong to a class of nonionic surface-active detergents normally used to lyse cell membranes by

solubilizing the membranous lipids and proteins (11). In addition, Tweens have been shown to enhance the growth of several species of mycobacteria (4, 5, 14-16). The mechanism of mycobacterial growth enhancement by Tween is poorly understood although several theories exist. The most common theory is that mycobacteria hydrolyze Tween 80 releasing oleic acid which serves as a substrate for growth (4, 5, 14-16).

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Oleic acid, the major hydrolysis product of Tween 80, has been shown to cause accumulation of low electron dense intracellular vacuoles in *Mycobacterium tuberculosis* (13). Transmission electron microscopy of Tween 80 grown *Mycobacterium avium* revealed similar intracellular vacuoles (10). In the present study we report the effects of Tweens, two additional nonionic surfactants, and oleic acid on the growth of *Mycobacterium paratuberculosis*, including a range of Tween concentrations not previously investigated. In addition, we describe Tween 80-induced changes in *M. paratuberculosis* colonial morphology and ultrastructure.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates of *M. paratuberculosis* UWIS B015, B025, B045, B047, B061, and B073 originated from feces of infected cattle and were isolated using modified Middlebrook 7H12B media (Johnston Laboratories, Towson, MD) by methods previously described (9). They were cultured for seedlot production in Middlebrook 7H9 broth (DIFCO Laboratories, Detroit, MI) supplemented with 10% OADC (oleate, albumin, dextrose, and catalase) enrichment (DIFCO Laboratories, Detroit, MI) and 1 µg/ml mycobactin J (Allied Laboratories, Inc.). *Mycobacterium paratuberculosis* ATCC strain 19698, and clinical isolates UWIS B025, B045, and B073 had been subcultured four or more times *in vitro*. Low passage isolates, B015, B047, and B061 were those subcultured less than three times. Cells were harvested after 8 weeks' incubation into 5 ml Middlebrook 7H9 broth using a sterile swab, dispersed with a motor-driven overhead stirrer and glass-teflon homogenizer (Wheaton Instruments, Millville, NJ), divided into aliquots in sterile freezer vials (Corning Glass Works, Corning, NY) and stored at -70 °C with 10% glycerol (Sigma, St. Louis, MO) added as a cryoprotectant. Viable counts on Middlebrook 7H9 agar plates were performed on all isolates before freezing to quantitate CFU/ml.

Growth measurement in radiometric broth media

Radiometric Middlebrook 7H12B is a broth medium contained in rubber septum-sealed vials. As bacteria grow, the ¹⁴C labeled palmitic acid present in the medium is metabolized releasing ¹⁴CO₂. The BACTEC 460 (Johnston Laboratories, Towson, MD), an automated ionization detector, measures the amount of ¹⁴CO₂ in the atmosphere above the broth and records it in growth index units. A growth index of 1 corresponds to 0.25 nCi ¹⁴CO₂. Cumulative growth indexes were obtained by summing the growth index measurements at each reading time (7).

Each study was carried out > 4 weeks with growth measurements made every 48 hours. However, growth

enhancement was evaluated only after substantial growth had occurred (> 10% total ¹⁴C labeled palmitic acid in each vial converted to ¹⁴CO₂, eg. a cumulative growth index of ≥ 1300); this was approximately day 20 for the ATCC strain and days 18-22 for the clinical isolates. This end point corresponded to the early to mid-exponential phase of growth (Fig. 1). Growth response was expressed as percent control, determined by dividing the cumulative growth index achieved in the supplemented broth by the cumulative growth index in a nonsupplemented broth. All treatments were tested in triplicate.

Colonial morphology studies

All isolates of *M. paratuberculosis* were diluted to concentrations of 10⁶ CFU/ml. Approximately 10³ CFU from each dilution were plated on Middlebrook 7H9 agar plates supplemented with 10% OADC and 1 µg/ml mycobactin J, and Tween 80 (Sigma, St. Louis, MO), Triton X-100, or Genapol 100 (CalBiochem, San Diego, CA) at the following concentrations: 0, 0.001, 0.01, 0.1, 1.0, and 3.0% (w/v). Plates were incubated at 36 °C under ambient air and checked weekly for growth.

Effect of Tweens on growth

A stock solution of Tween 80 was diluted and added to triplicate BACTEC vials containing Middlebrook 7H12B media supplemented with 1 µg/ml mycobactin J to obtain final concentrations of 0, 0.001, 0.01, 0.1, 1.0, or 3.0% (w/v). Approximately 10⁵ CFU of each isolate were inoculated and vials were incubated under ambient air at 36 °C. Identical experiments using Tween 20, 40, and 60 were performed but only using the ATCC strain.

Effect of nonionic surfactants on growth

Triton X-100 or Genapol 100 was added to BACTEC vials containing Middlebrook 7H12B media supplemented with 1 µg/ml mycobactin J to obtain final detergent concentrations of 0, 0.001, 0.01, 0.1, 1, and 3% (w/v). Approximately 10⁵ CFU of ATCC 19698 were added and the vials incubated under ambient air at 36 °C.

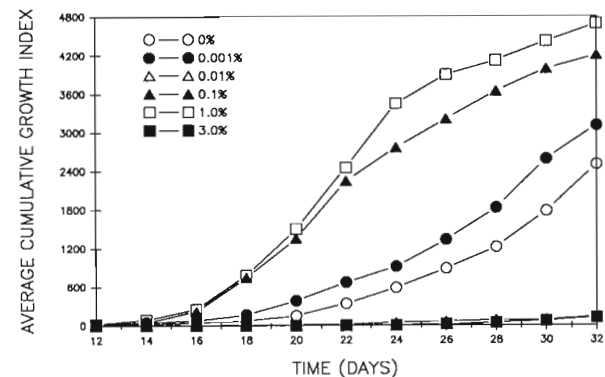


Fig. 1. Cumulative growth index curves of *M. paratuberculosis* ATCC strain 19698 grown in Middlebrook 7H12B media supplemented with Tween 80.

Effect of oleic acid on growth

A 10 mg/ml stock solution of oleic acid (Sigma, St. Louis, MO) was diluted and added to BACTEC vials containing Middlebrook 7H12B media supplemented with 1 µg/ml mycobactin J to obtain final oleic acid concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0% (w/v). Approximately 10⁵ CFU of ATCC 19698 were added and the vials incubated under ambient air at 36 °C.

Scanning electron microscopy

Suspensions of each *M. paratuberculosis* isolate grown in nonradiometric Middlebrook 7H9 broth supplemented with 10% OADC, 1 µg/ml mycobactin J and Tween 80 at concentrations of 0, 0.01, and 1.0% (w/v) were adjusted to an absorbance of 0.05 to 0.08 at 650 nm. 1.0 ml was filtered through a 0.8 µm polycarbonate filter membrane (Nucleopore, Pleasanton, CA), fixed for 1 hour in buffered 2.5% glutaraldehyde, postfixed for 15 minutes in buffered 1% OsO₄, dehydrated through an alcohol series, and dried by the critical point procedure using the Samdri (Tousimas Corporation, Rockville, MD). Duplicate samples were freeze dried to ensure observations were not artifacts of cell shrinkage. Samples were dried at an average temperature of -60 °C for 48 hours and allowed to warm to room temperature for 24 hours. After coating, the samples were viewed on a Hitachi S-900 scanning electron microscope.

Transmission electron microscopy

Cells for transmission electron microscopy were pelleted, rinsed three times with sterile distilled water, fixed with Karnovsky's fixative, postfixed with 1% OsO₄, dehydrated by alcohol series and embedded in EPON 812. Blocks were sectioned using a diamond knife, stained with 1% uranyl acetate, and viewed with a Philips 410 transmission electron microscope.

RESULTS

Colonial morphology of *M. paratuberculosis* on Middlebrook 7H9 agar plates was dependent on the Tween 80 concentration in the media. At concentrations of 0 and 0.001% (w/v), colonies of both ATCC and clinical isolates were rough and irregular (Fig. 2A). Colonies on Middlebrook media containing 0.01% (w/v) had a fried egg morphology (Fig. 2B), while those grown on media containing 0.1 to 1.0% (w/v) Tween 80 were entire, smooth, and domed (Fig. 2C). Two of the low passage clinical isolates failed to grow in the presence of 1.0% Tween 80. Addition of Tween 40 and Tween 60 to Middlebrook 7H9 agar plates caused similar rough to smooth colony transition patterns. Colonial morphology on plates supplemented with Tween 20, Triton X-100, or Genapol

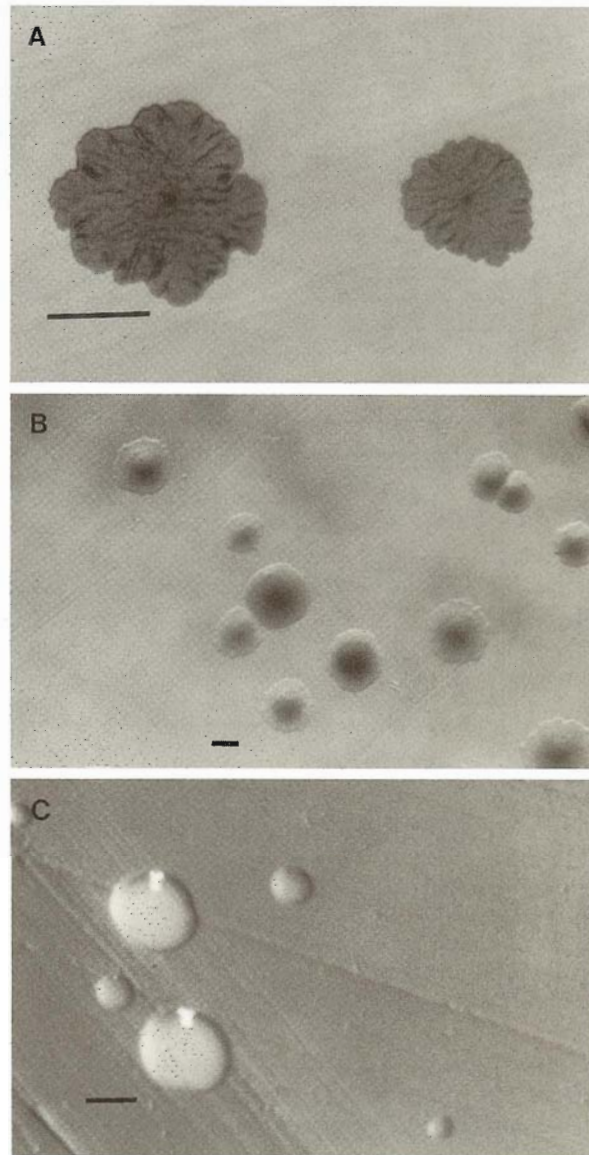


Fig. 2. Colonial morphology of *M. paratuberculosis* clinical isolate UWIS B025 grown on Middlebrook 7H9 agar supplemented with Tween 80.

- A. 0% (w/v) Tween 80. Bar marker = 1 mm.
 B. 0.01% (w/v) Tween 80. Bar marker = 1 mm.
 C. 1.0% (w/v) Tween 80. Bar marker = 1 mm.

100 appeared to be less rough and irregular. As concentrations of these detergents were increased, however, no growth occurred at concentrations > 0.01% (w/v) and colonial morphology never became completely smooth.

All isolates had bimodal growth enhancement patterns when grown in media with increasing concentrations of Tween 80 (Fig. 3). The ATCC

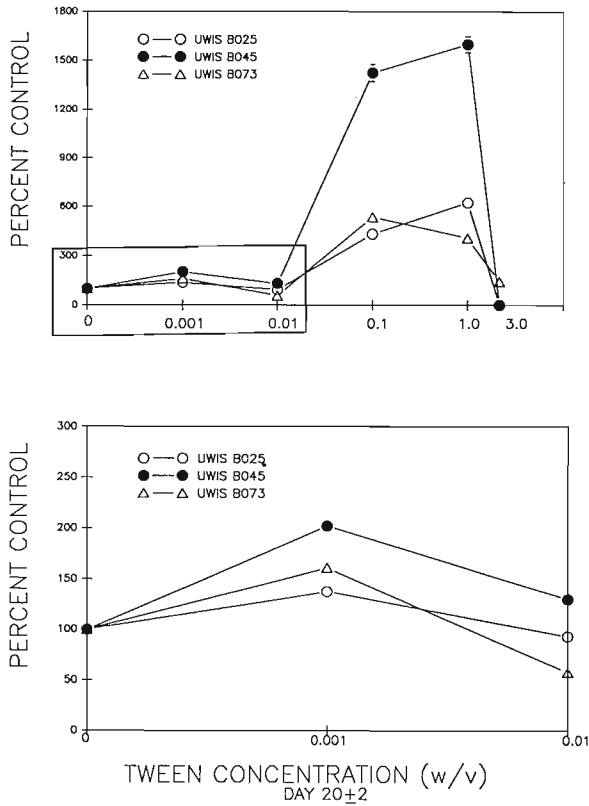


Fig. 3. Effect of Tween 80 in radiometric Middlebrook 7H12B media on growth of *M. paratuberculosis* high passage clinical isolates, UWIS B025, B045, and B073. A. Full graph.

B. Enlargement of Fig. 3A to show effects of low Tween concentrations.

strain, and 2 of the 3 high passage clinical isolates grew optimally in the presence of 1.0% (w/v) Tween 80. Peak growth for all 3 low passage clinical isolates and the remaining high passage clinical isolate was at 0.1% (w/v). Growth of the ATCC strain in Tween 40-containing media also followed the bimodal pattern observed with Tween 80 although the second mode of growth enhancement was not as dramatic. Growth enhancement of *M. paratuberculosis* ATCC strain 19698 by Tween 20 and 60 was not bimodal (Fig. 4).

Oleic acid stimulated the growth of *M. paratuberculosis* optimally at 0.2% (w/v). Enhancement of growth by Triton X-100 and Genapol 100 did not mimic the bimodal effect of Tween 80. Growth enhancement was limited to concentrations $0\% < X < 0.01\%$ and resembled the effect of Tween 20 on *M. paratuberculosis* growth (Fig. 5).

Scanning electron microscopic examination of

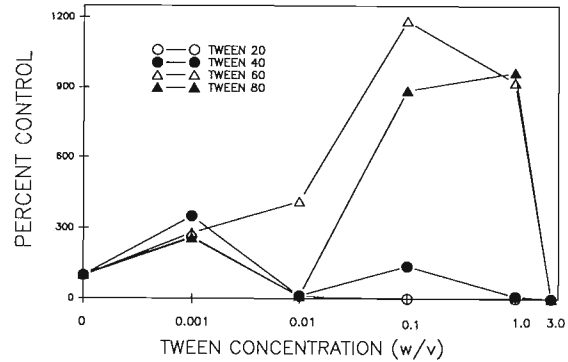


Fig. 4. Effect of Tween 20, 40, 60, and 80 on growth of *M. paratuberculosis* ATCC strain 19698 in radiometric Middlebrook 7H12B media supplemented with 1 $\mu\text{g/ml}$ mycobactin J.

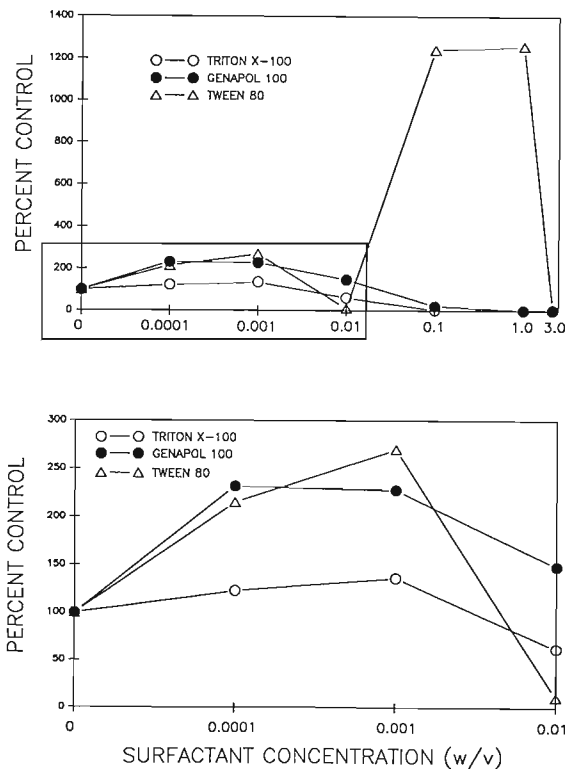


Fig. 5. Effect of the nonionic surfactants, Genapol 100, Triton X-100, and Tween 80 on growth of *M. paratuberculosis* ATCC strain 19698 in radiometric Middlebrook 7H12B media supplemented with 1 $\mu\text{g/ml}$ mycobactin J.

A. Full graph.

B. Enlargement of Fig. 5A to show effects of low detergent concentrations.

ATCC and clinical strains grown in the presence of Tween 80 revealed an effect of Tween 80 on cell wall appearance. Cell walls appeared less rough at

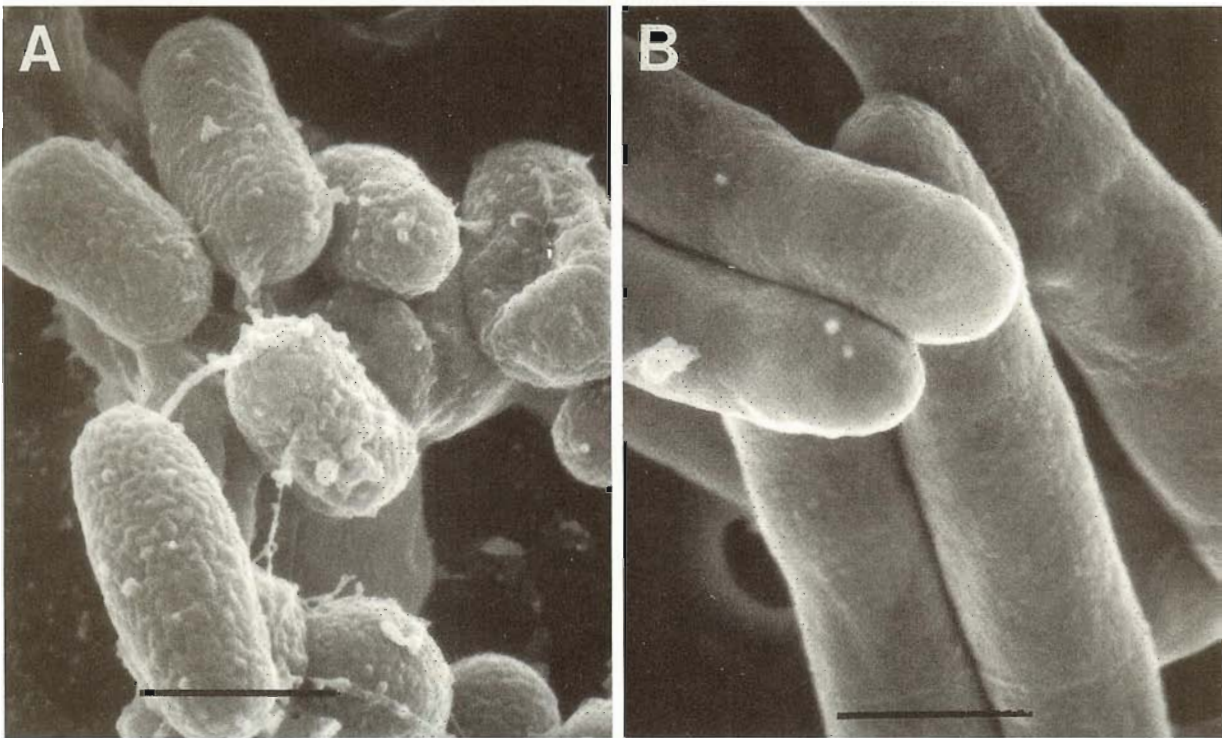


Fig. 6. Scanning electron micrograph of *M. paratuberculosis* clinical isolate UWIS B025 grown in Middlebrook 7H9 broth supplemented with Tween 80. Bar markers = 500 nm.

- A. 0% (w/v) Tween 80.
- B. 1.0% (w/v) Tween 80.

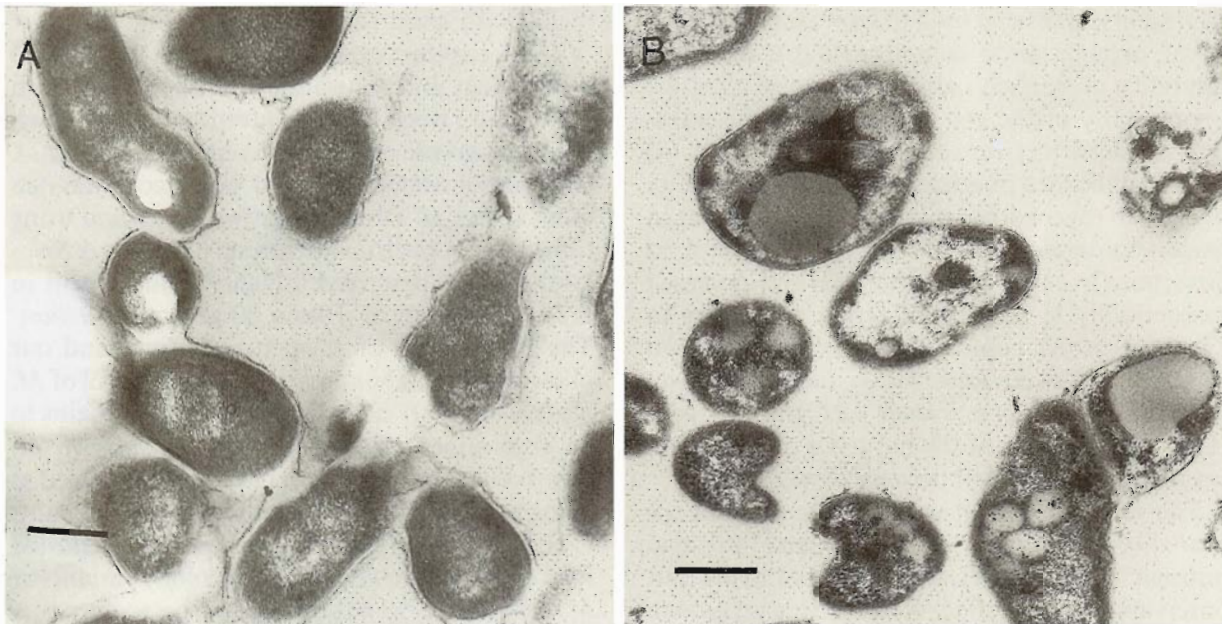


Fig. 7. Transmission electron micrograph of *M. paratuberculosis* clinical isolate UWIS B025 grown in Middlebrook 7H9 broth supplemented with Tween 80. Bar markers = 0.5 μm .

- A. 0% (w/v) Tween 80.
- B. 1.0% (w/v) Tween 80.

higher concentrations of Tween 80 (Fig. 6) although the effect was subtle in the ATCC strain. Also notable was an increase in size of the bacteria as Tween 80 concentration was increased. The cell wall appearance of freeze-dried samples did not differ from those of critical-point-dried samples.

Transmission electron microscopy revealed the presence of low electron dense intracellular vacuoles in *M. paratuberculosis* cells grown in the presence of Tween 80. The number of vacuoles increased as the Tween 80 concentration increased but they were absent in cells grown on media without Tween 80 (Fig. 7). In addition, cells grown in the absence of Tween appeared to have a more irregular surface than those grown in Tween 80-containing media.

DISCUSSION

Each mode in the Tween 40 and 80 bimodal growth enhancement phenomenon may be due to a different mechanism of action. Fig. 5 illustrates how growth enhancement caused by low concentrations of Tween 80 (0.0001 to 0.001% w/v), was imitated by Triton X-100 and Genapol 100, non-ionic surfactants, at the same concentrations. Thus growth enhancement by low Tween concentrations was probably due to a detergent effect. Detergents have been used to increase the solubility of mycobacterial components (6). Increased solubility of surface components may alter the permeability of the cell wall and facilitate transport of substrates into the cell. *Cutler et al.* (4) proposed the idea of altered cell wall permeability to explain mycobacterial growth enhancement in Tween-containing media. *Naik et al.* (10) showed distortions in outer cell integuments and increased susceptibility to subsurface acting antibiotics in Tween 80 grown clinical isolates of the *M. avium* complex. Investigations which measure movement of labeled compounds into the cell may clarify the question of the influence of Tween on mycobacterial cell wall permeability.

The second mode of growth enhancement appeared to be a combined detergent and fatty acid nutrient effect. It was difficult to compare our observations to the effect of fatty acids on growth of other mycobacterial species described in the literature. Fatty acid concentrations and the mycobacterial species tested previously vary greatly, and wide species and strain differences have been

reported (2, 3, 8, 13). In the present study, oleic acid was shown to stimulate growth of *M. paratuberculosis*. Optimal growth occurred at 0.2% (w/v) and was less at higher concentrations, however, growth in oleic acid-supplemented media was always well above the oleic acid free control (% control = 112.9) at 0.8% (w/v).

Tween 60 (0.1% w/v) most dramatically enhanced growth of the ATCC 19698 strain of *M. paratuberculosis*. Tween 40 enhanced growth at concentrations of 0.01% or 0.1% (w/v). Tween 20 enhanced growth only at concentrations less than 0.01%. Since Tween 20, 40, 60, and 80 differ only in fatty acid chain length, differences in their growth enhancement patterns for *M. paratuberculosis* presumably resulted from this. Since most studies report only the effects of Tween 80 on mycobacteria, most of our work focused on this compound and experiments on the effects of Twens 20, 40, and 60 on clinical isolates were not performed.

Slight differences were observed between low and high passage clinical isolates of *M. paratuberculosis*. Two of three high passage isolates grew optimally in media containing 1.0% (w/v) Tween 80, while all low passage isolates and one of three high passage isolates grew best at 0.1% (w/v). This observation may indicate an adaptation of *M. paratuberculosis* to growth in Tween-containing media with serial passage *in vitro*.

The transition from rough to smooth colonial morphology and cell wall surface appearance with increasing Tween 80 concentration to our knowledge has not previously been reported. *Naik et al.* (10) demonstrated damaged polysaccharide-rich outer layers of Tween 80 grown *M. avium* using transmission electron microscopy. *Stinson & Solorovsky* (15) described a higher lipid content in cell wall extracts of Tween 80 grown *M. avium*. The relationship of these two findings and our observations on the apparent Tween control of *M. paratuberculosis* colonial morphology remains to be determined. Rough colony variants of *M. tuberculosis* and *M. avium* have been described as being more virulent than smooth colony variants (12), however, no such studies have been reported for *M. paratuberculosis*. Rough colony variants of *M. avium* have been shown to lack polar glycopeptidolipids found in smooth colony variants (1). Studies are underway to examine the glycopeptidolipids in rough and smooth colony forms of *M. paratuberculosis*.

The accumulation of low electron dense intracellular vacuoles has been reported with *M. avium* and *M. tuberculosis* by investigators using either Tween 80 (10) or oleic acid (13) as a growth substrate. Presumably, these vacuoles contain oleic acid or some metabolite thereof. Also notable in the transmission electron micrographs was the irregular surface of cells grown in the absence of Tween 80. This observation is consistent with the appearance of cells in scanning electron micrographs.

Interpretation of growth enhancement of mycobacteria by Tween using the BACTEC system is potentially complicated by the hydrolysis of Tween 40 and 80 to palmitic acid and oleic acid, respectively. These hydrolysis products might compete with the ^{14}C labeled palmitic acid substrate in the radiometric medium. The result, however, would then be lower growth index measurements, giving a false impression of decreased growth and thus could not explain the increased growth (as defined by $^{14}\text{CO}_2$ production) measured in our experiments. The objective of our study was not to define the mechanism of growth enhancement of *M. paratuberculosis* by Tween, but rather to describe the bimodal nature of the growth response and its relationship to colonial and cellular morphology. Performing viable cell counts on solid media may clarify the interpretation of this data regarding the mechanism of Tween action, however, the time, cost and technical difficulty of accurately quantitating this organism that clumps and requires 6 to 12 weeks to form visible colonies would be significant. In a limited study to examine oleate competition with radiolabeled palmitate (unpublished) we found that free oleic acid only competed with ^{14}C palmitate at very high molar ratios, ie. > 1:10, and no significant effect was observed at lower ratios. In studies done by Lambrecht (PhD Dissertation, University of Wisconsin, 1987) using egg yolk as a growth supplement for clinical isolates of *M. paratuberculosis*, BACTEC data correlated well with plate count data ($R^2 = 99.1\%$) suggesting minimal interference by that medium supplement containing fatty acids.

In summary, Tweens altered colonial morphology, cell wall surface appearance, ultrastructure and growth rate of *M. paratuberculosis in vitro*. The ability of Tween to alter other physical, chemical or biological properties, including antigen expression and virulence remains in question.

However, investigations on antigen expression or other *M. paratuberculosis* characteristics should take heed of the potentially profound influence of *in vitro* cultivation conditions.

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