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Growth and Cholesterol Oxidation by *Mycobacterium* Species in Tween 80 Medium

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***Mycobacterium* strain DP was isolated from marine coastal sediment and tested for its ability to oxidize cholesterol in Tween 80-cholesterol (2.59 mM) medium. Strain DP degraded cholesterol to 4-cholesten-3-one (cholestenone), 4-androsten-3,17-dione (AD), 1,4-androstadien-3,17-dione (ADD), testosterone, and 1-dehydrotestosterone (DHT). Cholesterol disappeared in about 4 days. Cholestenone, AD, testosterone, and DHT accumulations were transient with peak concentrations of 300, 600, 30 to 40, and 21 μM. ADD production peaked after 6 days with a concentration of 1,100 μM. Peak ADD concentrations and production rates compared well with those reported for strain NRRL B3683 on cyclodextrin medium. Tween 80 medium was superior to finely dispersed cholesterol particles for both strains. In comparison, NRRL B3683 (patented for its ability to accumulate AD and ADD) on Tween 80 medium transiently accumulated more AD (~1,000 μM) than did strain DP, but ADD accumulations (200 μM) were significantly lower than those for strain DP. Strain DP could be adapted to grow on ADD, which was initially inhibitory at 3.25 mM. ADD-adapted strain DP cultures produced approximately four times as much DHT from ADD than unadapted cultures did from cholesterol, showing that additional manipulation might enhance testosterone production. We believe that ADD toxicity might account for the low ADD accumulations by NRRL B3683 in Tween 80 medium.**

Cholesterol decomposition is widespread among microorganisms (1, 3, 5). Cholesterol is usually completely oxidized to carbon dioxide and water (13, 14). Sterol oxidation inhibitors were used to delineate cholesterol oxidation pathways through accumulation of intermediates (14–17). Mutation and selection procedures yielded mutants that accumulated 4-androsten-3,17-dione (AD) and/or 1,4-androstadien-3,17-dione (ADD) (13, 22). AD and ADD are useful precursors for manufacturing pharmaceutical steroids that can be generated from cheap sterols (cholesterol and sitosterol) (7, 8) and numerous patents were issued for AD- and ADD-accumulating strains (6, 10–12).

Of 1,589 strains of bacteria, yeasts, and molds tested, only 46 strains consumed 50% or more of a finely dispersed suspension (1 g/liter) of cholesterol particles within 7 days (1). However, surveys of particulate sterol transformation rates might more reflect rates of cholesterol-solubilizing emulsifier production than rates of cholesterol metabolism.

Oxidation rates can be improved by adding surface active or other sterol-solublizing agents to the bacterial growth media. Organic solvent phases tended to inhibit sterol side chain oxidations and reduce AD and ADD yields (8); they were therefore not suitable as sterol solubilizers.

More recently, a mutant *Mycobacterium* strain (NRRL B-3683) (13) produced greatly improved AD and ADD accumulations from cholesterol or sitosterol when the sterol was complexed with cyclodextrins compared with when the sterol was provided as a fine particle suspension (7). Sterols solubilized by emulsifying agents would provide a better standard for comparison than particle suspensions, because sterol transformations rates should reflect metabolism rather than sterol solubilization. The cyclodextrin effects were attributed to improved sterol solubility and inhibition of side oxidations leading to premature sterol ring cleavage (7). Elimination of product inhibitions by complex formation was equally possible (18, 23).

We report experiments measuring rapid cholesterol oxidation by *Mycobacterium* strain NRRL B-3683 and strain DP (a *Mycobacterium* isolated from marine sediment) in media containing cholesterol emulsions in Tween 80 in order to compare intermediate accumulations with accumulations reported for cyclodextrins.

MATERIALS AND METHODS

Bacterial strains. We isolated *Mycobacterium* strain DP from a mineral enrichment medium (BM [2]) containing 1 g of a sonicated suspension of cholesterol per liter and inoculated with marine sediment (from Point Reyes, Calif.). Cultures on nutrient agar (BBL Microbiology Systems) were sent to the National Veterinary Services Laboratories, Ames, Iowa, for identification. *Rhodococcus erythropolis* ATCC 4277 and *Mycobacterium* sp. NRRL B-3683 (ATCC 29472, U.S. patent 3,684,657) were purchased from the American Type Culture Collection, Rockville, Md.

Chemicals. Cholesterol, cholestenone, AD, ADD, testosterone, 1-dehydrotestosterone, and cholesterol oxidase (EC 1.1.3.6) were purchased from Sigma Chemical Company, St. Louis, Mo. *o*-Phthalaldehyde was from ICN (Cleveland, Ohio).

Culture media. Our cultures were maintained on our modification of Arima medium (1), consisting of the following (in grams per liter): NH_4Cl, 1.0; K_2HPO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.001; yeast extract, 5.0; steroid-Tween 80, 20.0 ml; and H_2O, 980 ml. Steroid-Tween 80 was prepared by adding 5 g of steroid to 100 ml of Tween 80 and heating until the steroid dissolved. Steroid-Tween 80 mixture was added to the medium, and the pH was adjusted to 7.0 to 7.2. The medium was autoclaved at 121°C for 15 min. For solid media, agar was added to a 1.5% (wt/vol) final concentration before autoclaving. Modified

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Arima medium required swirling at intervals during cooling after autoclaving to maintain clarity. Unless specified otherwise, stock cultures were maintained on modified Arima medium with 2.59 mM cholesterol, and modified Arima medium was used for all of our experiments. In this paper, the terms Arima medium and modified Arima medium are used interchangeably.

Growth. Growth measurements were in triplicate in 100-ml cultures incubated in 250-ml Erlenmeyer flasks. We estimated growth from increases in A_{540}. We also monitored viable counts by plating serial 10-fold dilutions of cultures (diluent 20 mM potassium phosphate, pH 7.0) onto Arima agar containing 1 g of cholesterol per liter and 2% (vol/vol) of Tween 80. Colonies were counted after the plates were incubated for 1 week at 30°C. Unless stated otherwise, cultures were incubated at 30°C with shaking (150 to 200 rpm) for 16 days. Growth rate constants (mean [μ], ln(2)/doubling time) were computed from a regression analysis of $\ln(A_{540})$ versus time.

Growth temperature profiles on 100-ml cultures in Arima medium (2.59 mM cholesterol) and Arima medium without cholesterol were determined. Incubation was at 15, 20, 25, 30, 35, and 40°C in a shaking water bath. Cultures were inoculated with a 1% (vol) inoculum of strain DP in Arima–2.59 mM cholesterol medium, and A_{540}s were monitored on 1-ml samples (30-min to 1-h intervals).

Time courses of growth and cholesterol oxidation by strains DP and NRRL B-3683 were determined by inoculating 100-ml cultures in Arima medium and incubating with shaking at 30°C. Triplicate cultures were sacrificed at intervals of 48 h over at least 16 days, A_{540}s were measured, viable counts were determined, and *o*-phthalaldehyde assays for cholesterol concentration were performed on 5 ml of culture. The remaining 95 ml of culture was extracted with ethyl acetate and assayed by high-pressure liquid chromatography (HPLC). The time course of AD oxidation by strain DP was performed identically to the cholesterol time course experiments, except that 3.49 mM AD was substituted for the cholesterol in the Arima medium.

Assays. All assays were in triplicate. Cholesterol was assayed colorimetrically with 1-ml saponified samples of whole cultures, and the saponification, extraction methods, and *o*-phthalaldehyde assay were previously described (21). The *o*-phthalaldehyde assay measured only the first step in cholesterol oxidation, its conversion to cholestenone.

Steroids other than cholesterol were assayed by HPLC of ethyl acetate extracts of whole cultures. Cultures (95 ml) were extracted in a separatory funnel by shaking three times with 95 ml of ethyl acetate and combining the extracts. The combined ethyl acetate extract (12 ml of a 285-ml total volume) was evaporated to dryness under nitrogen. The remaining ethyl acetate extract was saved for possible nuclear magnetic resonance and mass spectrometer analysis. Losses from evaporation and handling during extraction were accounted for in computing the results. Molar extraction efficiencies based on cholesterol, cholestenone, AD, ADD, testosterone, or 1-dehydrotestosterone standards were typically 40%. The residue from the combined extract was dissolved in 4 ml of HPLC solvent mixture (75:25, hexane-isopropanol) and then injected (100 μl) into an isocratic ISCO (Lincoln, Nebr.) HPLC system containing a silica column (4.6 by 250 mm; 5-μm packing, at a constant temperature of 25°C). Hexane-isopropanol (75:25 [vol/vol]) was used for the mobile phase. Peaks were detected by their UV A_{240}s. A diode array detector (Applied Biosystems, Foster City, Calif.) was sometimes connected in tandem to aid in peak identification by providing complete UV absorption spectra. When necessary to ensure identification, HPLC runs were repeated with samples spiked with standards (cholesterol, cholestenone, AD, ADD, testosterone, and 1-dehydrotestosterone). Peak areas were compared with the standards for quantitation.

Cholesterol oxidase was assayed by the colorimetric procedure of Smith and Brooks (20) on concentrated culture supernatant fractions after centrifugation (10,000 × *g* for 30 min). Culture supernatant fractions from 1 liter of strain DP cultures were concentrated by membrane ultrafiltration with a model 8400 Amicon ultrafiltration cell (Amicon Div., W. R. Grace and Co., Danvers, Mass.) and membrane filters (Pharmacia, Inc., Piscataway, N.J.; 10,000-Da exclusion limit).

Identification of sterol intermediates. Cholesterol, cholestenone, AD, ADD, testosterone, and 1-dehydrotestosterone were identified in culture extracts by their comigration with standards in HPLC mobile phases of various compositions. Identifications were confirmed on the basis of UV absorption spectra, mass spectrometry, and proton nuclear magnetic resonance data from pooled, purified peak fractions.

RESULTS

Strain DP identification. Our cholesterol-oxidizing bacterium grew in complex media such as nutrient broth or Arima medium. Pinpoint colonies appeared in complex agar media in 48 h; smooth, orange-yellow colonies with entire edges developed in Arima agar after incubating for 5 days at 30°C. Our isolate was identified as a rapidly growing *Mycobacterium* species on the basis of its strong acid-fast reaction, lack of motility, and positive reaction in the 3-day arylsulfatase test (19). Tests performed at the National Veterinary Laboratories showed that the isolate produced mycolic acids, was catalase and urease positive, and grew in nutrient broth containing 6% NaCl. Its metabolism was aerobic. Acid was not produced from D-glucose, lactose, D-mannitol, D-xylose, or sucrose. Negative results were reported for starch, gelatin, tyrosine, casein, adenine, and DNA hydrolysis; oxidase reaction; nitrate reduction; and growth on sodium acetate or acetamide.

Our isolate hydrolyzed Tween 80 and did not grow in Simmons citrate medium. Tween 80 hydrolysis suggested that fatty acids from Tween 80 could be used as an energy source during growth in our cholesterol medium.

Temperature profile. Strain DP grew optimally at 30 to 35°C in Arima medium containing 2% (vol/vol) Tween 80 with or without 2.59 mM cholesterol. Growth rate constants (μ) on Arima medium without cholesterol were as follows: 0.045, 15°C; 0.069, 20°C; 0.118, 25°C; 0.149, 30°C; 0.159, 35°C; and 0, 40°C. On Arima medium (containing 2.59 mM cholesterol), growth rate constants were as follows: 0.046, 15°C; 0.076, 20°C; 0.106, 25°C; 0.147, 30°C; 0.131, 35°C; and 0, 40°C. Standard deviations were less than 0.006.

Growth and cholesterol oxidation. The low solubility of cholesterol in aqueous environments limits cholesterol metabolism by bacteria, so it is a common practice to add an emulsifier to solubilize sterols. Strain DP grew well on medium containing Tween 80 as an emulsifier, but Tween 20 inhibited growth. Growth rates on Arima medium without sterol were not changed by omitting Tween 80.

Tween 80 medium accelerated cholesterol (2.6 mM) degradation rates relative to those in media containing sonicated cholesterol suspensions (2.6 mM); complete degradation occurred in 2 to 4 days for a 1% (vol) inoculum at 30°C rather than the 6 to 7 days required for sonicated suspensions.

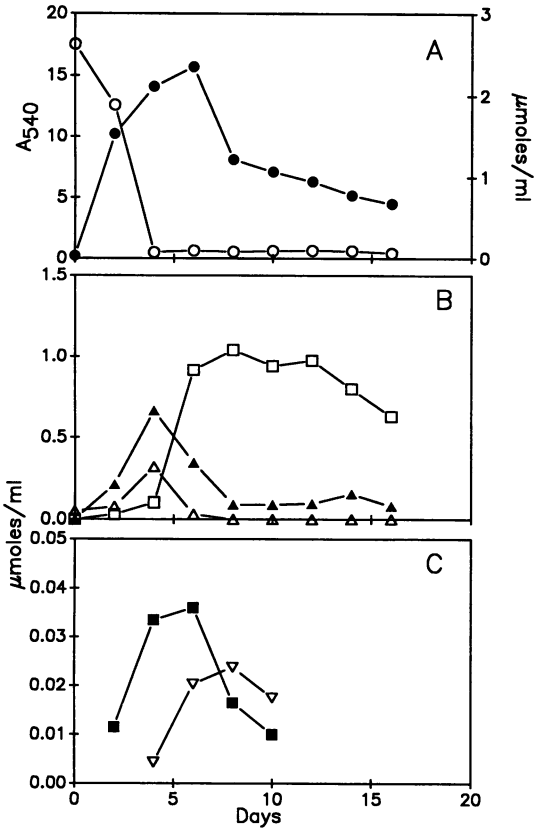

FIG. 1. Strain DP growth and cholesterol oxidation. Strain DP cultures were inoculated (1% [vol/vol]) into 100 ml of Arima medium (2.59 mM cholesterol) and incubated for 16 days at 30°C. The results are mean values of triplicate cultures. (A) A_{540} (●) and cholesterol (○); (B) cholestenone (△), AD (▲), and ADD (□); (C) testosterone (■) and 1-dehydrotestosterone (▽).

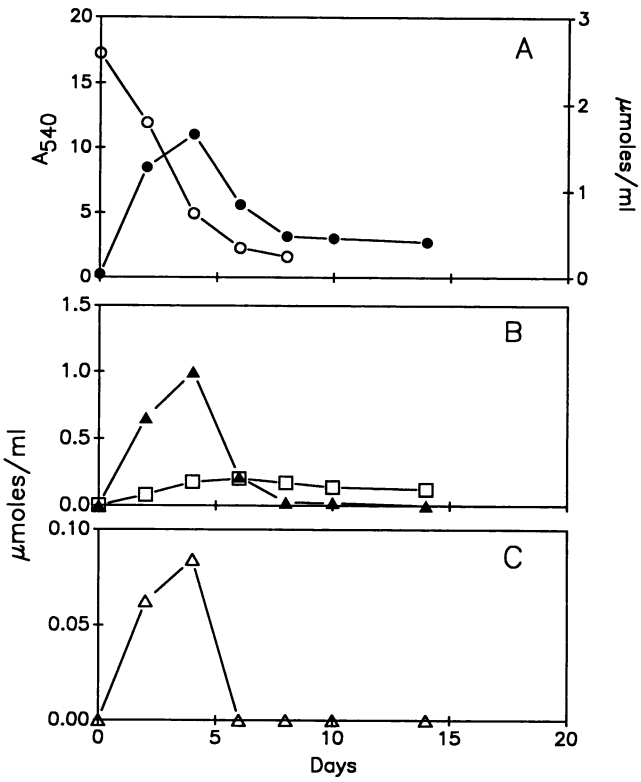

FIG. 2. Strain NRRL B-3683 growth and cholesterol oxidation. Strain NRRL B-3683 was inoculated into 100 ml of Arima medium (2.59 mM cholesterol) and incubated for 16 days at 30°C. The results are mean values of triplicate cultures. (A) A_{540} (●) and cholesterol (○); (B) AD (▲) and ADD (□); (C) testosterone (△).

Tween 80 also increased medium clarity to the point where cell growth could be monitored in the presence of cholesterol by absorbance measurements. Tween 80, other medium components, and cholesterol oxidation products did not interfere in the *o*-phthalaldehyde assay.

At 30°C, A_{540} on Arima medium with 2.59 mM cholesterol peaked 6 days after cultures were inoculated (Fig. 1A) and then declined rapidly. Viable counts (not shown) indicated rapid loss of culture viability in step with decreasing absorbances. Cholesterol concentrations (Fig. 1A, measured by *o*-phthalaldehyde assay) declined rapidly after culture inoculation; cholesterol consumption was essentially complete within 4 days.

HPLC analysis of whole culture extracts showed that cholestenone and AD (Fig. 1B) accumulated during the first 4 days after inoculation and then declined to low levels. AD concentrations did not rise above 700 μM. We detected ADD (Fig. 1B) on the fourth day after inoculation. ADD concentrations reached a nearly maximum level at 6 days and then fell slowly after attaining maximal values (1.2 mM) at 8 days.

Small amounts of testosterone (35 to 40 μM) and 1-dehydrotestosterone (20 to 25 μM) accumulated transiently (Fig. 1C); testosterone accumulated first, reaching a peak 4 to 6 days after inoculation, and then nearly disappeared within 10 days. 1-Dehydrotestosterone appeared at about the same time as ADD, peaked at 8 days, and then began slowly declining at 10 days after inoculation.

Cholesterol oxidase. We wanted to know if strain DP produced a soluble cholesterol oxidase for carrying out the first step in cholesterol oxidation. Chipley et al. (4) reported that culture supernatant fractions of *Mycobacterium* ATCC 19652 degraded significant quantities of cholesterol within 24 h.

Cholesterol degradation in strain DP culture supernatant fractions and cholesterol oxidase activity in 100-fold concentrates of strain DP culture supernatant fractions were not observed. Control concentrates of *R. erythropolis* culture supernatant fluids exhibited easily detectable cholesterol oxidase activity. (*R. erythropolis* is known to produce a soluble cholesterol oxidase.) We concluded that the initial step in cholesterol oxidation by strain DP was mediated by a cell-associated enzyme system.

Strain NRRL B-3683 on Arima medium. We tested *Mycobacterium* NRRL B-3683 on Arima medium with 2.59 mM cholesterol (Fig. 2) to compare growth and cholesterol metabolism with strain DP and to serve as a basis for comparing performance on Tween 80 medium to reported performance on cyclodextrin medium (7). NRRL B-3683 absorbances (including cell death and lysis, as indicated by decreasing absorbance and viable counts) and cholesterol disappearance (Fig. 2A) resembled those obtained for strain DP, suggesting growth rates similar to those of strain DP.

NRRL B-3683 produced different levels of AD, ADD, and testosterone (Fig. 2B) than did strain DP. AD accumulated

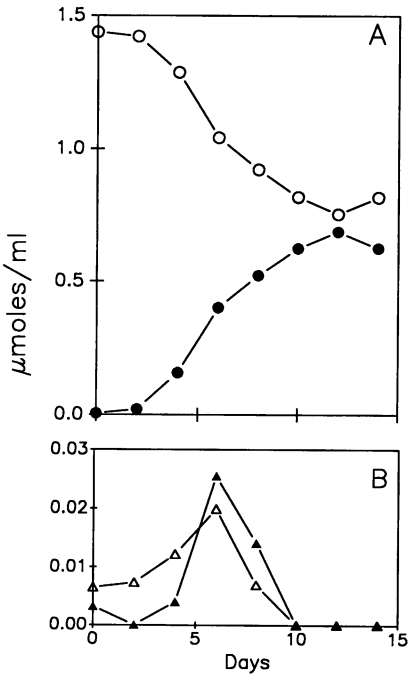

FIG. 3. Oxidation of AD. Strain DP (1% [vol] inoculum) was inoculated into Arima medium (100 ml) containing 3.49 mM AD and incubated for 14 days at 30°C. (A) AD (○) and ADD (●); (B) testosterone (△) and 1-dehydrotestosterone (▲).

to high (1 mM) concentrations and then rapidly disappeared. However, maximum ADD concentrations (at 6 days) did not exceed 300 μM, suggesting that AD was preferentially transformed to a (unidentified) substance other than ADD. Small amounts of testosterone (80 μM [Fig. 2C]) and 1-dehydrotestosterone (not shown) accumulated transiently. Previous reports showed that NRRL B-3683 produced low 1-dehydrotestosterone concentrations (13), but testosterone formation was not reported.

Other steroid transformations. Strain DP cultures inoculated into Arima medium with 3.49 mM AD transformed AD to ADD (Fig. 3A) as expected, confirming that AD was the source of ADD. AD oxidation to ADD appeared nearly quantitative but not complete and ceased when ADD concentrations reached 600 μM. Growth rates on cholesterol, AD, and ADD (Table 1) were faster on cholesterol than on 3.49 mM AD or 3.52 mM ADD, suggesting that ADD was toxic to strain DP.

Testosterone and 1-dehydrotestosterone were produced transiently from AD (Fig. 3B). Testosterone concentrations peaked at about the same time (6 days) as 1-dehydrotestosterone concentrations. Peak testosterone and 1-dehydrotestosterone concentrations (20 and 26 μM) were not higher than in cholesterol medium.

Cultures also grew on medium with 3.47 mM testosterone as a substrate. After 6 days on Arima-testosterone medium (30°C, 1% [vol/vol] inoculum), testosterone concentrations dropped to 0.41 mM, AD and ADD accumulated to 1.89 mM and 0.39 mM, respectively, and a trace of 1-dehydrotestosterone was present.

ADD toxicity. ADD reportedly inhibited growth and cholesterol oxidation by *Mycobacterium* strain NRRL-B3683 (9). ADD toxicity might contribute to cell death and lysis and lowered cholesterol transformation rates and efficiencies, even though it was a desirable end product. Growth rates were measured for strain DP on Arima medium with Tween 80 and various concentrations of ADD (Table 2) and no other sterol substrates.

Strain DP cultures grew at initial rates comparable to those on cholesterol medium when we added ADD to 176 to 352 μM (Table 1), but growth rates were slower than at 0 mM ADD. For reasons not clear to us, growth at 0 mM ADD slowed earlier in this experiment than in other experiments, resulting in lower A_{540} values at 29.5 h than we obtained for 176 or 352 μM ADD. ADD concentrations above 352 μM inhibited growth rates, indicating that ADD was somewhat toxic for strain DP.

Strain DP cultures reduced ADD to AD, testosterone, and 1-dehydrotestosterone (Table 3), indicating that AD oxidation to ADD was reversible. HPLC analysis of ethyl acetate extracts showed that nearly equimolar AD and 1-dehydrotestosterone concentrations were produced from 1.8 mM ADD after cultures were incubated for 56 h. The final 1-dehydrotestosterone concentrations were approximately

TABLE 1. Comparison of growth rates on steroid substrates[a]

Substrate	μ (h^{-1})	SD	Doubling time (h)
None	0.157	0.009	4.4
Cholesterol	0.144	0.029	4.8
AD	0.125	0.005	5.5
ADD	0.069	0.003	10.0

[a] Cultures (1% [vol]) were inoculated into 100 ml of Arima medium containing 2% (vol/vol) Tween 80 and 1 g of the indicated substrate per liter. The cultures were shaken (200 rpm) at 30°C, and growth was monitored by measuring A_{540}. The growth rate constants are mean values of triplicate determinations.

TABLE 2. Initial growth rates on ADD[a]

ADD (mM)	μ (h^{-1})	SD	Growth rate inhibition (%)	A_{540} increase[b]
0	0.170	0.006	0	0.238
0.176	0.152	0.018	10.6	0.470
0.352	0.151	0.008	11.2	0.478
1.76	0.127	0.001	25.3	0.195
3.52	0.080	0.004	52.9	0.057

[a] Arima medium contained 2% (vol/vol) Tween 80 and the indicated concentrations of ADD. The media were inoculated with strain DP (1% [vol/vol]), incubated at 28°C with shaking, and A_{540}s were monitored. μ, mean value from triplicate cultures.
[b] After 29.5 h of incubation (initial A_{540}, 0.020).

TABLE 3. Metabolism of ADD[a]

Initial ADD (mM)	Final product (mM)			
	ADD	AD	T	DT
0.208	0.075	0.059	0.016	0.030
0.386	0.158	0.092	0.030	0.060
1.844	1.154	0.187	ND[b]	0.174
3.703	3.452	0.085	ND	0.172

[a] The cultures from the experiments in Table 2 were extracted with ethyl acetate at the end of the growth, and the products were analyzed by HPLC. T, testosterone; DT, 1-dehydrotestosterone.
[b] ND, not determined.

fourfold higher than concentrations produced from cholesterol or AD.

DISCUSSION

Mycobacterium strains DP and NRRL B-3683 grew rapidly in cholesterol-Tween 80 medium and accumulated AD and ADD. ADD accumulated from strain DP without applying mutation and selection procedures or adding metabolic inhibitors.

Strain DP resembled strain NRRL B-3683 in rapid growth and cholesterol disappearance on Tween 80 medium, but strain DP accumulated more ADD than did strain NRRL B-3683. Strain DP produced ADD concentrations on Tween 80 medium that were comparable to concentrations reported for NRRL B-3683 on cyclodextrin medium (7).

The relatively poor performance of Tween 80 versus cyclodextrin for AD and ADD production by NRRL B-3683 might result from ADD inhibition in Tween 80 medium. Although Hesselink et al. (7, 8) reported that cyclodextrins improved AD and ADD yields by inhibiting side oxidations of sterol ring structures, it is also possible that cyclodextrins improved yields by sequestering ADD. End product inhibitions were reported in other steroid-metabolizing bacteria (23) and growth experiments with *Saccharomyces cerevisiae* (18) suggested that cyclodextrins might overcome an inhibitor. ADD was reported toxic to strain NRRL B-3683 (9) and inhibited strain DP growth in our experiments. We suspect that ADD yields in Tween 80 medium could be improved by overcoming ADD toxicity.

The low testosterone and 1-dehydrotestosterone concentrations produced on cholesterol medium in our experiments were not commercially useful, but the experiments show that *Mycobacterium* species can directly reduce AD and ADD to testosterone and 1-dehydrotestosterone. The testosterone (1-dehydrotestosterone) concentrations reached were similar to those reported elsewhere (13). The Δ-1,4 dehydrogenating enzyme of strain DP that oxidized AD to ADD apparently had low activity with testosterone, as suggested by the low 1-dehydrotestosterone concentrations but comparatively high AD and ADD concentrations reached in Arima-testosterone medium. Strain DP produced fourfold-higher 1-dehydrotestosterone concentrations from ADD after the strain was adapted to grow on ADD than it produced from AD or cholesterol before adaptation, suggesting that additional strain or culture manipulation with strain DP might result in commercially valuable 1-dehydrotestosterone concentrations.

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