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Microbial degradation of 7-ketocholesterol

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Abstract 7-Ketocholesterol (7KC) is an oxidized derivative of cholesterol suspected to be involved in the pathogenesis of atherosclerosis and possibly Alzheimer's disease. While some oxysterols are important biological mediators, 7KC is generally cytotoxic and interferes with cellular homeostasis. Despite recent interest in preventing the accumulation of 7KC in a variety of matrices to avoid adverse biological effects, its microbial degradation has not been previously addressed in the peer-reviewed literature. Thus, the rate and extent of biodegradation of this oxysterol was investigated to bridge this gap. A wide variety of bacteria isolated from soil or activated sludge, including *Proteobacterium* Y-134, *Sphingomonas* sp. JEM-1, *Nocardia nova*, *Rhodococcus* sp. RHA1, and *Pseduomonas aeruginosa*, utilized 7KC as a sole carbon and energy source, resulting in its mineralization. *Nocardia nova*, which is known to produce biosurfactants, was the fastest degrader. This study supports the notion that microbial catabolic enzymes could be exploited to control 7KC levels in potential biotechnological applications for agricultural, environmental, or medical use.

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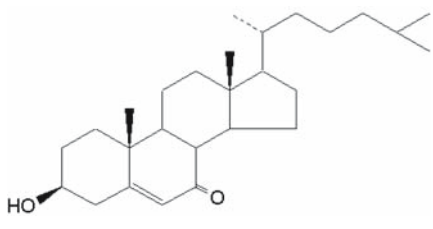
Introduction

Oxysterols are oxidized derivatives of cholesterol that are widely distributed in nature and often exert potent and diverse biological effects. Although these compounds normally make up only a minute fraction of total sterol present when found naturally, usually 10^{-6}–10^{-3} in most cases (Bjorkhem 2002), oxysterols display high biological activity with synergistic effects observed even at low concentrations (Larsson et al. 2006).

Many of the most commonly encountered oxysterols are those with either a keto or hydroxyl group at the C-7 position of the steroid nucleus. Amongst these compounds, 7-ketocholesterol (7KC) (Fig. 1) is one of the most predominant and widely studied, principally due to its suspected involvement in various human pathological conditions such as atherosclerosis (Hughes et al. 1994) and Alzheimer's disease (Nelson and Alkon 2005). There is considerable evidence suggesting that 7KC promotes these conditions through various cytotoxic modes of action, including induction of apoptosis (Nishio et al. 1996). In addition, 7KC has been found to increase Na+/K+ ATPase activity in fibroblasts (Sevanian et al. 1995),



Name:	5-Cholesten-3β-ol-7-one
7KCFormula:	$C_{27}H_{44}O_2$
Synonyms:	7-ketocholesterol
	3β-Hydroxy-5-cholesten-7-one
	7-oxocholesterol
MW:	400.64
Water Solubility:	~10^{-8}M
Melting Point:	170 - 172ºC

Fig. 1 Structure and properties of 7KC

decrease Ca^{2+} uptake in human erythrocytes (Neyses et al. 1985), stimulate monocyte differentiation (Hayden et al. 2002), inhibit proliferation and stimulate differentiation in lens epithelial cells (Girao et al. 2003), and promote oxidative stress through reactive oxygen intermediate generation in murine macrophages (Dushkin et al. 1998). Many oxysterols, including 7KC, have also been shown to inhibit HMG CoA-reductase (Kandutsch and Chen 1973), the first enzyme in the mevalonate pathway which controls a multitude of cellular processes such as cholesterol and sterol biosynthesis, proliferation, apoptosis, and possibly inflammation.

In addition to human pathogenicity, some plant-derived sterols (phytosterols) are endocrine disruptors that can adversely affect environmental health. For example, β-sitosterol (a phytosterol commonly present in pine pulp and paper mill effluents (Conner et al. 1976)) has been linked to the masculinization of resident fish populations (Tremblay and Van Der Kraak 1999), possibly through its microbial conversion to androstenedione (Jenkins et al. 2003). Furthermore, structural analogues of 7KC, such as 7-ketositosterol and 7β-hydroxysitosterol which are derived from β-sitosterol, have been found to be cytotoxic to a number of cell lines (Ryan et al. 2005) and accumulate in the bodies of animals (Tomoyori et al. 2004).

While microbial degradation of cholesterol has been extensively studied (Soehngen 1913; Tak 1942; Van der Geize et al. 2007), and considerable research has been performed on the biological effects and fate of a number of oxysterols within mammalian systems, to date there is very limited research on the biotransformation of most oxysterols, and no peer-reviewed publications regarding the microbial degradation of 7KC. Knowledge of how these compounds are transformed and degraded by bacteria could prove beneficial for a number of reasons. For example, most oxysterols with C-7 bound oxygen groups have deleterious effects, and the identification and use of microbial enzymes that degrade 7KC has been proposed as a possible therapeutic approach for the treatment of certain age-related diseases (de Grey et al. 2005). Furthermore, dietary intake of oxysterols, the largest percent of which was 7KC, has been reported to cause significant accumulation within the plasma of humans (Linseisen and Wolfram 1998). Thus, information concerning the microbial degradation of 7KC and other oxysterols could also be used to help remove some of these substances from food. Finally, the endocrine disrupting potential of some oxysterols also motivates research on their biodegradation to mitigate their potential ecological and agricultural impact.

In this paper, we report the isolation of various bacteria capable of using 7KC as a sole carbon and energy source. Thus, this study serves as a stepping stone to understand the microbial degradation of oxysterols for potential applications in wastewater treatment, food technology, and medical bioremediation.

Materials and methods

Media and substrate

7KC was obtained from Sigma (97.1% pure as determined by gas chromatography). A modified Hunter's Mineral Base medium (MSB) consisting of a dilution of three stock solutions was used in all experiments. The medium consists of 40 ml of solution A (141.2 g Na_2HPO_4 and 136 g KH_2PO_4 per liter, pH 7.25), 20 ml of solution B (10 g nitrilotriacetic acid, 14.45 g $MgSO_4$, 3.33 g $CaCl_2{\cdot}2H_2O$, 9.25 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 99 mg $FeSO_4{\cdot}7H_2O$, and 50 ml Metals 44 per liter) and 5 ml of solution C (200 g

$(NH_4)_2SO_4$ per liter). Metals 44 is composed of 2.50 g EDTA, 10.95 g $ZnSO_4 \cdot 7H_2O$, 1.54 g $MnSO_4 \cdot 7H_2O$, 5.00 g $FeSO_4 \cdot 7H_2O$, 392 mg $CuSO_4 \cdot 5H_2O$, 248 mg $Co(NO_3)_2 \cdot 6H_2O$, and 177 mg $Na_2B_4O_7 \cdot 10H_2O$ per liter (final pH 7).

Enrichment and isolation of bacteria

Activated sludge samples were acquired from the 69th Street Wastewater Treatment Plant and the Shell Westhollow Technology Center, both in Houston, TX. Soil samples were collected from numerous sites within Houston. Both the activated sludge and soil were exposed to 7KC within 2 h of sampling. Enrichment cultures (100 ml) were created by transferring 1 ml of activated sludge (10^{-3} dilution with sterile water) or 1 ml of soil seed (suspended 10 g 100 ml^{-1} into 100 ml of MSB amended with 7KC (1 g l^{-1} or 0.0025 M) as the sole carbon source. Because 7KC is relatively insoluble (Fig. 1), the medium was sonicated prior to inoculation for 30 min in a bath sonicator to homogenize the solution and decrease 7KC particle size. 7KC initially floats on top of the medium until saturated and subsequently settles to the bottom of the flask. Sonication notably increases the turbidity of the solution and breaks up larger aggregates. Each culture was incubated at 30°C and 150 rpm in a gyratory shaking incubator until growth could be determined through visual inspection, and positive cultures were aliquoted (100 µl) onto MSB agar plates with 5.0 mg of 7KC in methanol evaporated to dryness on the surface. Plates were then incubated for 72 h at 30°C and all colonies were picked and grown in batch cultures as above. One-ml samples of that the pure cultures that grew on 7KC were then added to 1 ml of sterile 40% glycerol and stored at −70°C until needed.

Carbon dioxide measurements

Heterotrophic activity was measured per CO_2 accumulation and respiration rates using a MicroOxymax respirometer (Columbus Instruments, Columbus, OH). Batches were prepared as above, though the volume used was 50 ml and the temperature was 25°C. Each batch was inoculated with 5 µl of cells that had been grown overnight in LB media, centrifuged at 10,000*g* for 10 min and washed in MSB minimal media a total of three times. Cells were resuspended to an OD_{600} of 1.0 prior to inoculation.

HPLC analysis

High performance liquid chromatography (HPLC) was used to verify 7KC degradation by the strains isolated. Batches (25 ml) were prepared as above and incubated at 30°C and 150 rpm. Five 100 µl samples were taken from each batch using large bore pipette tips and pooled for extraction. Three replicate batches were used for each strain tested. 7KC was then extracted from the pooled samples twice, using 3 ml of 3:2 hexane/isopropanol, and 1.0 ml of the organic layer was filtered through a 0.22 µl Whatman syringe filter and capped inside an analytical vial. This filtrate was analyzed by reverse phase HPLC (0.72 ml min^{-1}, 85:10:5 methanol/water/acetonitrile) using a Waters 2695 Separation Module with a Waters 996 photodiode array (235 nm) and a Waters NovaPak C_{18} column (3.9 by 150 mm). Chromatographic data was analyzed with the Empower 2 software suite (Waters, Milford, MA).

Growth on 7KC in the presence of a surfactant

To overcome grow-rate limitation by the rate of dissolution of 7KC, growth experiments were also conducted in the presence of Tween 80, a surfactant that is widely used to enhance the solubility of hydrophobic compounds. *Pseudomonas aeruginosa* was grown in 100 ml batches containing 2 mM 7KC in MSB (30°C, 150 rpm). The 7KC was first dissolved in 7 ml of methanol, filter sterilized, and the methanol was evaporated off, allowing a fine crystalline layer to form along the bottom of the flasks to which 0.5-ml Tween 80 and 99.5-ml MSB were added. Controls were prepared in the same manner without the addition of 7KC. Optical density (600 nm) was measured at 1 h time intervals until log phase was reached. Thereafter measurements were taken at 30 min intervals. Samples with OD measurements over 0.4 were diluted and re-measured until readings were under 0.4. The final OD in these cases was determined by multiplying by the dilution factor.

DNA extraction and amplification

DNA extractions were performed using a MoBio UltraClean Microbial DNA Isolation Kit according to the manufacturer's protocol. Amplification of 16S rDNA was performed by polymerase chain reaction (PCR) using extracted DNA with a Taq PCR Master

Mix Kit (Qiagen) and DGGE primers (DGGE-F: 5′-ATGGCTGTCGTCAGCT-3′ and DGGE-R: 5′-CG CCCGCCGCGCCCCGCGCCCGGCCCGCCGCCC CCGCCCCACGG GCGG TGTGTAC-3′) on a T-Gradient thermocycler (Biometra). Amplification mixtures had a final volume of 100 µl and contained 0.5 µM of each primer, 200 µM dNTPs, 1X PCR buffer, and 2.5 units of Taq polymerase. The temperature cycle for the PCR was 1 min of denaturation at 94°C, 1 min of annealing, and 3 min of primer extension at 72°C. The annealing temperature was initially set at 53°C and reduced 1°C each cycle until it reached 43°C. This was followed by 20 additional cycles at 43°C and a final primer extension for 10 min. Amplification yielded a final product of approximately 350 bp as determined by sequencing.

Phylogenetic analysis

Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rDNA gene sequence was used to ensure strain purity and identify bacteria for which DNA had been extracted. Analysis was performed on a Bio-Rad DCode^{TM} system (35 volts, 14 h) and the gel (using denaturing gradients from 30 to 80%) stained in ethidium bromide before visualization under UV. Samples which displayed one or a small number of bands had those excised, the DNA reamplified, and were sequenced (LoneStar Labs, Houston, TX). The sequences were matched against the NCBI GenBank Database (http://ncbi.nlm.nih.gov/Blast) and the Sequence Match facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP) for phylogenetic identification of the bacteria.

Results and discussion

Isolation and identification of 7KC-degraders

Numerous bacterial species were isolated from soil and activated sludge by virtue of their ability to grow on 7KC as sole carbon and energy source. Two of five soil sample enrichment cultures, and all of the activated sludge samples tested utilized 7KC. After incubating aliquots of the cultures on MSB agar plates with 7KC as the sole carbon source, numerous colony-forming units (CFUs) could be observed with distinct morphology and color. Each of these CFUs was then transferred into batch culture as described above, and those that retained the ability to grow were analyzed for strain purity using DGGE. Based on DGGE analysis, several cultures were found to contain more than one strain of bacteria, but no polymorphisms were found in pure cultures.

Typically, visual evaluation of growth occurred between five and ten days for any culture surviving transfer from the plates to enrichment culture. 16S rDNA gene analysis based on percent similarity from NCBI BLAST search revealed *γ-Proteobacterium* Y-134 (99%), *Sphingomonas* sp. JEM-1 (98%), *Nocardia nova* (99%), and *Pseduomonas aeruginosa* (97%). Another mixed culture which we have been unsuccessful at plating on 7KC and MSB agar, but which grows well in enrichment culture, could not be reliably identified and were most closely associated with *Xanthomonas* sp. (85%) and uncultured *α-Proteobacterium* sp. (79%). This mixed culture was isolated from an activated sludge sample as were the *γ-Proteobacterium* Y-134 and *Pseudomonas aeruginosa*. *Sphingomonas* sp. JEM-1 and *Nocardia nova* were both isolated from soil samples. In addition to those isolated, *Rhodococcus* sp. RHA1 (provided by William Mohn, University of British Columbia) was also tested for growth on 7KC. All of these isolates are either from the Probacterium or Actinobacteria phyla.

Evidence of degradation

The results of the HPLC analysis for 7KC degradation are presented in Fig. 2. The most rapid degradation was observed with *Nocardia nova*, a known producer of biosurfactants (Margaritis et al. 1979), which removed the 7KC below detection levels within 20 days. It is likely that the degradation rate was dissolution limited, as is the case with many hydrophobic compounds that experience faster degradation in the presence of biosurfactants (Oberbremer et al. 1990; West and Harwell 1992). All other cultures still contained measurable amounts of 7KC at 25 days. UV detection from 190 to 400 nm revealed no measurable accumulation of metabolites within that range. The absorption maximum for 7KC is 233 nm, allowing accurate determination of 7KC concentration. However, other oxysterols are generally not as strong UV absorbers, so the extracellular presence of degradation intermediates cannot be ruled out.

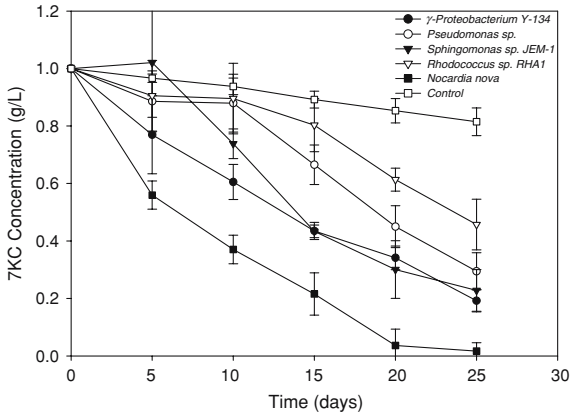


Fig. 2 HPLC analysis of 7KC degradation by *γ-Proteobacterium* Y-134, *Sphingomonas* sp. JEM-1, *Nocardia nova*, and *Rhodococcus* sp. RHA1. This experiment was conducted at 30°C, pH 7. Three replicate batch reactors were sampled for each bacteria and the control

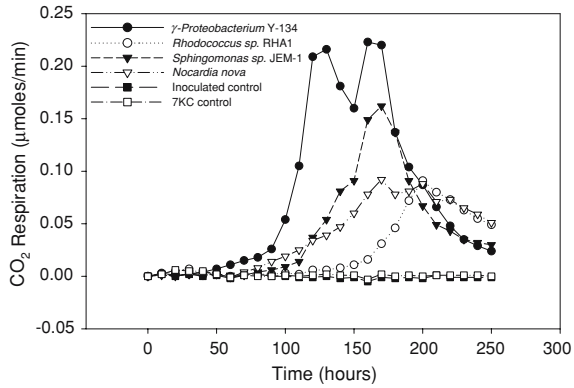


Fig. 4 CO_2 evolution rate (μmoles/min) in batch reactors (single replicates) inoculated with *γ-Proteobacterium* Y-134, *Rhodococcus* sp. RHA1, *Sphingomonas* sp. JEM-1, or *Nocardia nova* in mineral salts media with 0.1% 7KC as the sole carbon source. Controls contained no 7KC. This experiment was conducted at 25°C, pH 7

CO_2 accumulation and respiration rates were determined through respirometry as illustrated in Figs. 3 and 4, respectively. For the *γ-Proteobacterium* Y-134, *Nocardia nova*, and the *Sphingomonas* sp. JEM-1, noticeable respiration did not begin to occur until approximately 100 h after inoculation, and peaked at just past 200 h for all three. *Rhodococcus* sp. RHA1, however, did not begin noticeable respiration until 150 h time, and peaked at approximately 250 h. The percent mineralization over a 300 h period was 43% for *γ-Proteobacterium* sp., 24% for *Sphingomonas* sp., 21% for *Nocardia nova*, and 14% for *Rhodococcus* sp. RHA1. The purity of the 7KC was 97.1%, allowing a maximum of approximately 100 μmoles of CO_2 generated from other potential co-substrates. *Rhodococcus* sp. RHA1 gave the lowest amount of accumulated CO_2, which was 460 μmoles. *γ-Proteobacterium* sp. released the highest amount of CO_2 at 1,442 μmoles.

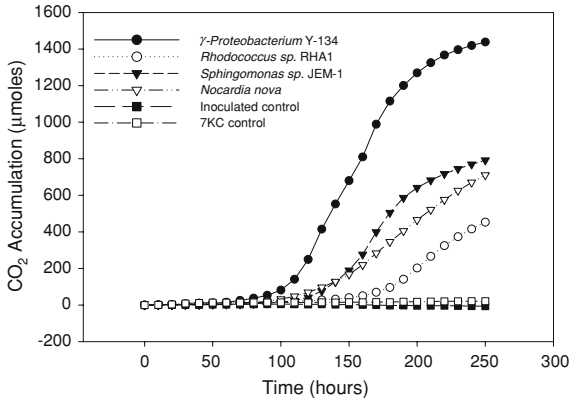


Fig. 3 CO_2 accumulation (μmoles) in batch reactors (single replicates) by *γ-Proteobacterium* Y-134, *Rhodococcus* sp. RHA1, *Sphingomonas* sp. JEM-1, and *Nocardia nova* in mineral salts media with 0.1% 7KC as the sole carbon source. Controls contained no 7KC. This experiment was conducted at 25°C, pH 7

An interesting observation is that *Nocardia nova*, the fastest degrader, exhibited a lower extent of mineralization than two of the other cultures. Whether this reflects incomplete degradation of 7KC by *N. nova* or differences in carbon utilization pathways remains to be determined. One plausible explanation is that production of biosurfactants by *N. nova* consumes organic carbon that would otherwise be oxidized to CO_2 by catabolic pathways.

Growth on 7KC in the presence of a surfactant

Due to the hydrophobicity of 7KC, which results in polydispersed colloidal particles with a tendency to precipitate, it is difficult to measure optical density unless the solution is properly suspended and diluted. In order to achieve a fine suspension, 7KC was first dissolved in methanol and filter-sterilized and evaporated to form a crystalline layer before the addition of Tween 80 (0.5%) and MSB. This surfactant enhanced bacterial growth on 7KC. As shown in Fig. 5, exponential growth was achieved within hours

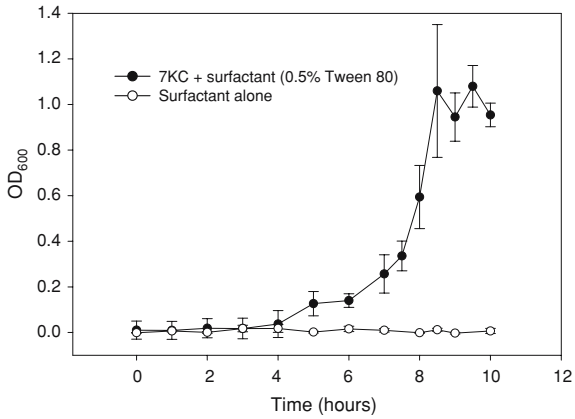


Fig. 5 Growth of *Pseudomonas* sp. in MSB media and 0.5% Tween 80 withand without 7KC. Optical density was determined at 600 nm. This experiment was conducted at 30°C, pH 7

when 7KC was present while no growth occurred without 7KC. This proves that 7KC rather than the surfactant or residual methanol served as sole carbon and energy source to support growth. In contrast, the HPLC (Fig. 2) and respirometry data (Fig. 4) suggest that, in the absence of surfactant, overcoming the lag phase took much longer, normally at least five days. These results support the notion that 7KC biodegradation might be dissolution limited, and that compounds that enhance its effective solubility and bioavailability are likely to enhance degradation kinetics.

Conclusion

Several bacteria from the Proteobacteria or Actinobacteria phyla were isolated from soil and activated sludge from wastewater treatment plants, based on their ability to exploit the biodegradation of 7KC as a metabolic niche. Carbon dioxide evolution in incubations with 7KC (but not in unamended controls) indicated extensive mineralization. Growth on 7KC as sole carbon source was also demonstrated, and noted to proceed faster in the presence of a surfactant; this suggests that 7KC dissolution might be a rate-limiting step. Overall, these results support the notion that oxysterol levels might be controlled in different matrices by biodegradation processes, and motivate further investigation into the specific pathways involved in microbial 7KC degradation, with the goal of identifying novel enzymes capable of transforming oxysterols for potential environmental, industrial, pharmaceutical, and medical applications.

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