Improved culture medium and kinetic studies of sterol reduction by *Eubacterium coprostanoligenes*

by

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INTRODUCTION

Physical and Chemical Methods of Cholesterol Reduction

Consumer demand for foods which are lower in cholesterol has stimulated research in the development of physical, chemical and biological technologies to decrease the amounts of cholesterol and triacylglycerols in foods (Arima et al, 1969; Bjorkhem et al., 1973; Dehal et al., 1991; Johnson and Somkuti, 1990; Marshek et al., 1972; Mazschner and Fine, 1989). Physical methods, such as steam-stripping have shown promise in that over 90% of cholesterol from butter oil and lard was removed without imparting off flavors to the products tested (Sperber, 1989). Supercritical extraction of cholesterol with carbon dioxide has also been successful to decrease the level of cholesterol in butterfat by 90% or more (Sperber, 1989; Randolph et al., 1988; Froning et al., 1990). Although both of the above mentioned methods show promise, neither has come into widespread use due to the problems of cost-efficiency and limited food types that can be processed. Other methods of cholesterol reduction in foods currently being investigated include the addition of high-molecular-weight hydroxypropylmethylcellulose to diets of hypercholesteremic patients and the enhanced degradation of cholesterol in the presence of cyclodextrins (Dressman et al., 1993; Jadoun and Bar, 1993).

Biological methods to reduce levels of cholesterol have also been investigated. These methods are advantageous in that they are less harsh on the products being treated, form fewer byproducts, are easier to control, and are considered more "natural" by consumers. Bacteria which degrade cholesterol have been studied for quite some time. Two types of bacterial cholesterol

degradation systems have been identified; a cholesterol oxidase system and a cholesterol reductase system (Stadtman et al., 1954; Smith and Brooks, 1976; Molnar et al., 1993; Noma and Nakayama, 1976; Brigidi et al. 1993; Snog-Kjaer et al., 1956; Bjorkhem et al., 1973; Rosenfeld and Gallagher, 1964; Wilkins and Hackman, 1974; Eyssen et al., 1973; Freier et al. 1994). Both methods of bacterial cholesterol reduction have shown promise in potential commercial applications.

Thesis Organization

This thesis consists of an introduction, literature review, one manuscript that will be submitted for publication, a summary and discussion, and acknowledgments. The literature cited for both the literature review and manuscript are listed in a single "literature cited" section. The master degree candidate, Ronald Guy Lulich, is the senior author and principal investigator for the manuscript.

LITERATURE REVIEW

Bacterial Oxidation of Cholesterol

Oxidation of cholesterol occurs in a number of species of bacteria (Turfitt, 1946; Noma and Nakayama, 1976; Smith and Brooks, 1976; Smith and Brooks, 1974; Ferreira et al., 1984; Smith et al., 1993). The cholesterol-oxidizing enzyme has been given several names including: cholesterol oxidase, cholesterol: O_2 oxidoreductase, and 3ß-hydroxysteroid oxidase based upon cofactor requirements and substrate specificities (Noma and Nakayama, 1976; Smith and Brooks, 1977; Turfitt, 1948; Uwajima et al., 1974; Molnar et al., 1993). Cholesterol oxidase catalyzes two different reactions. Cholesterol is oxidized to 5-cholesten-3-one, a transient intermediate, while at the same time, molecular oxygen is reduced to hydrogen peroxide. The stoichiometry of the reaction is 1:1 with one mole of hydrogen peroxide produced per mole of cholesterol oxidized (Stadtman et al., 1954; Uwajima et al., 1974; Richmond, 1973; Vrielink et al., 1991).

The next step involves an isomerization of the Δ^5- Δ^6 bond by a related Δ^5-3-ketosteroid isomerase resulting in the production of 4-cholesten-3-one as a final product (Noma and Nakayama, 1976; Smith and Brooks, 1976; Batzold et al., 1976; Kuliopulos et al., 1987). The formation of 4-cholesten-3-one is the first step in the catabolism of cholesterol in many bacteria (Turfitt, 1948; Stadtman et al., 1954; Smith and Brooks, 1976; Smith et al., 1993). It has been shown that the oxidation of the 3ß-hydroxyl group is slower than the rate of isomerization of the Δ^5-Δ^6 bond, thus making the oxidation the rate-limiting step (Uwajima et al., 1974; Brooks and Smith, 1975; Smith and Brooks, 1976).

Cholesterol oxidase exists as a single subunit enzyme with an M_r of 55,000Da (Uwajima et al., 1974; Vrielink et al., 1991). The enzyme is comprised of two binding domains, one an FAD-binding domain and the other a steroid binding region (Vrielink et al., 1991). Several hydrophobic loops in the steroid binding site are thought to provide a pathway of entry of the steroid into the active-site cavity (Vrielink et al., 1991). The active site region contains one charged glutamic acid residue which is thought to be involved as a proton acceptor in the isomerization step of cholesterol oxidation (Uwajima et al., 1974; Vrielink et al., 1991).

3ß-Hydroxysteroid oxidases have been isolated from other organisms such as *Streptoverticillium*, *Schizophyllum*, *Brevibacterium*, and *Nocardia* (Smith and Brooks, 1977; Fukuyama and Miyake, 1978; Inouye et al., 1982; Vrielink et al., 1991). The purified enzymes demonstrated similar characteristics placing them into the family of enzymes known as cholesterol: O_2 oxidoreductases (Molnar et al., 1993).

The cholesterol oxidases that have been characterized display a wide range of physical properties. The molecular weights of the purified products ranged from 31,000-32,500 Daltons (Da) for enzymes produced by *Brevibacterium* while enzymes from *Streptomyces* and *Corynebacterium* ranged from 56,000 to 61,000 Da. Spectral analysis of these proteins seems to exhibit a spectrum characteristic of a flavoenzyme (Kamei et al., 1978; Noma and Nakayama, 1976; Fukuyama and Miyake, 1978; Kenney et al., 1979; Inouye et al., 1982; Smith and Brooks, 1977; Vrielink et al., 1991; Molnar et al., 1993). The flavin moiety was identified as flavin adenine dinucleotide, and most cholesterol oxidases contain one mole of tightly-bound flavin adenine dinucleotide per mole of protein as a prosthetic group (Uwajima et al., 1974; Kamei et al., 1978; Kenney et al., 1979). However, in the

case of several species of *Nocardia*, no flavin adenine dinucleotide cofactor purified with active cholesterol oxidase (Richmond, 1973).

Cholesterol oxidase has high affinity not only for cholesterol, but other steroids with a *trans* A:B ring junction as well (Molnar et al., 1993; Vrielink et al., 1991). The probable enzymatic reaction consists of an oxidation step followed by, in the case of 5-ene steroids, by the isomerization of the C_5-C_6 double bond to the C_4-C_5 position (Smith and Brooks, 1976). The K_M for cholesterol oxidase isolated from various species ranged from 0.14-0.4 mM (Smith and Brooks, 1976; Tomioka et al., 1975; Fukuyama and Miyake, 1978; Inouye et al., 1982; Noma and Nakayama, 1976).

In the analysis of isolated cholesterol oxidases, cholesterol was usually the preferred substrate over many other 3-hydroxysteroids (Uwajima, 1974; Tomioka et al., 1975; Noma and Nakayama, 1976; Fukuyama and Miyake, 1978; Kamei et al., 1978; Inouye et al., 1982). Steroids lacking the 3ß-hydroxyl group generally were not oxidized (Smith and Brooks, 1976; Smith and Brooks, 1977; Kamei et al., 1978; Uwajima et al., 1974; Inouye et al., 1982). The lowest rates of oxidation occurred when ergosterol was used as a substrate which suggested that a C_4-C_5 conjugated bond is less easily oxidized (Smith and Brooks, 1974; Brooks and Smith, 1975). Epicholesterol and lanosterol did not serve as substrates (Smith and Brooks, 1976).

Various 4-methyl sterols and 4α- and 4ß-hydroxysterols tested were very slowly oxidized by the enzyme (Smith and Brooks, 1976). This may be associated with the elimination of the 3α– hydrogen atom during oxidation and the hindrance of a 4-methyl group during oxidation (Smith and Brooks, 1977). 5α-Cholestan-3ß-ol (cholestanol) appears to be readily oxidized at rates similar to cholesterol, but

when the 5ß-isomer (coprostanol) was added, no oxidation occurred (Brooks and Smith, 1975). Extremely low rates of oxidation were also observed for the 3α-hydroxy epimers of cholesterol and of 4-Cholesten-3ß-ol (Brooks and Smith, 1975).

Variations of the side-chain seem to also play an important role in the rates of sterol oxidation (Smith and Brooks, 1976). Sitosterol was readily oxidized; however, the introduction of a C_{22}-C_{23} or C_{24}-C_{24} double bond decreased the rate of oxidation (Smith and Brooks, 1974; Uwajima et al., 1974; Inouye et al., 1981). A study of the oxidation of various hydroxylated cholesterols (26-, 25-, 24-, 22-, and 20-hydroxycholesterol) yielded mixed results. An additon of the terminal substituent in 26-hydroxycholesterol significantly decreased the rate of oxidation of the 3ß-hydroxyl group, whereas a 24-hydroxyl group significantly stimulated the reaction (Brooks and Smith, 1975).

The length of the side-chain also seems to play an important role in the reaction rate of cholesterol oxidase (Richmond, 1973; Smith and Brooks, 1974). Kinetic studies were performed with 3ß-hydroxysteroids with hydroxylated side-chains ranging in size from 0-8 carbon atoms and a correlation of $V_{max.}$ against side-chain length was established. It was shown that the rate of oxidation ($V_{max.}$) of 5-en-3ß-hydroxysteroids increased as side-chain length increased to an optimum of 5 carbons with significant oxidation occurring with side-chains up to 8 carbons long (Smith and Brooks, 1976; Smith and Brooks, 1974; Richmond, 1973). The corresponding K_M values in this series indicated no difference in the degree of binding (Smith and Brooks, 1976).

The variations in V_{max} as related to side-chain length suggest that the longer side-chains of oxidizable compounds could serve to orient the bound sterol or steroid so that the 3ß-hydroxyl group and the catalytic site are compatibly aligned

for oxidation (Smith and Brooks, 1976; Smith and Brooks, 1974; Brooks and Smith, 1975). The length of the side chain is also important in the binding of steroids to the active site during the isomerization reaction (Smith and Brooks, 1977). Derivatives of 17-hydroxy and 17-ketosteroids as well as substituted oximes of dehydroepiandrosterone, which somewhat simulate the side-chains of 3ß-hydroxysteroids, served as very poor substrates (Smith and Brooks, 1976).

The gene for the oxidation of cholesterol, *choA*, was first isolated from *Streptomyces* (Murooka et al., 1986). The plasmid-containing DNA inserts coding for active cholesterol oxidation were transformed into wild-type strains of *S. griseus* and *S. lividans* which lacked cholesterol oxidase activity and these transformations yielded colonies with cholesterol oxidase activity (Murooka et al., 1986).

Transformed organisms produced both extracellular and intracellular cholesterol oxidase, and it has been shown that the *choA* gene directs the synthesis of both intracellular and extracellular cholesterol oxidase (Murooka et al., 1986). The strain *S. lividans*, containing the recombinant plasmid pIJ 702, produced a significant amount of cholesterol oxidase, of which up to 40% was secreted extracellularly (Murooka et al., 1986). The transformed cells produced cholesterol oxidase for up to 8 days and the amount of extracellular enzyme increased with culture time while production of intracellular enzyme plateaued at around 5 days (Molnar et al., 1991; Murooka et al., 1986). The continuous secretion of the enzyme by the cloned cells has provided a potential for commercial large-scale production of cholesterol oxidase.

Within the N-terminal region of cholesterol oxidase, a high degree of sequence homology to alcohol dehydrogenase, *p*-hydroxybenzoate hydroxylase,

and glutathione reductase was found (Ishizaki et al., 1989). In this region of homology, the conserved sequences Gly-X-Gly-X-Gly was found characteristic of the flavin adenine dinucleotide binding region of flavoproteins and dehydrogenases (Ishizaki et al., 1989).

Another open reading frame 70 base pairs upstream from the *choA* gene was discovered in the search for the promoter region of cholesterol oxidase and termed the *choP* gene (Horii et al., 1990). The *choP* gene encodes a protein product of 381 amino acids with a molecular weight of 41,668 Da (Horii et al., 1990). The nucleotide sequence of the *choP* gene has a high sequence homology to cytochrome P-450 genes from humans and cytochrome P-450 genes from *Pseudomonas* species (Horii et al., 1990). A region of homology with cytochrome P-450s was identified in the *choP* gene and may represent a binding site for heme-iron (Horii et al., 1990). This was confirmed by CO difference spectral analysis of an extract of *S. lividans* cells transformed with a *choP* gene-containing plasmid in which a unique peak characteristic of cytochrome P-450 was detected (Horii et al., 1990). This peak was identical to that found in the parental strains of *Streptomyces* with already-existing cholesterol oxidases (Horii et al., 1990).

The new open reading frame is located 70 base pairs upstream from the *choA* gene (Horii et al., 1990). Data from deletion analysis, nucleotide sequences, primer extension mappings, and Northern blot analysis suggest that the *choA* gene is transcribed from the promoter region of the *choP* gene (Horii et al., 1990). It has been postulated that the *choP* and *choA* genes are transcribed both polycistronically and from an operon (Horii et al., 1990). This operon has been designated the *cho* operon (Horii et al., 1990).

Another cholesterol oxidase gene, *choB*, was cloned from *Brevibacterium*. The nucleotide sequence of *Brevibacterium* was comprised of a single open reading frame encoding the 552 amino acid protein preceded by a 45-amino acid signal sequence (Ohta et al., 1991; Fujishiro et al., 1990). *ChoB* showed a 64% homology with the structural region within the *choA* gene from *Streptomyces,* but was dissimilar in the 5' and 3' flanking regions (Ohta et al., 1991; Fujishiro et al., 1990). *ChoB* also has the FAD-binding consensus sequence also found in the *choA* gene, but *choB* lacks any operon or regions of homology with cytochrome P-450's as in the *choP* gene (Ohta et al., 1991). The signal sequences for each of the two genes varied greatly, but a 58% amino acid sequence homology within the structural genes does place the derivation of the two genes to common ancestry (Ohta et al., 1991; Horii et al., 1990).

Cholesterol oxidase has been used in widespread applications, notably its use in the determination of total serum cholesterol (Richmond, 1973; Allain et al., 1974; Smith and Brooks, 1977). Assay methods for cholesterol oxidase activity include quantitation of 4-cholesten-3-one production, evolution of hydrogen peroxide coupled to horseradish peroxidase, as well as numerous fluorescence, chromatographic and spectral assays (Richmond, 1973; Allain et al., 1974; Smith and Brooks, 1976; Uwajima et al., 1973; Inouye et al., 1982).

Cholesterol oxidase enzymes are also being used to lower cholesterol in milk and dairy products (Smith et al., 1991). However, several problems have developed with the use of cholesterol oxidase in milk to lower dietary cholesterol. One problem is the production of hydrogen peroxide which might result in the generation of toxic compounds or undesirable flavor components (Smith et al., 1991). 4-Cholesten-3-one, the principal product from the oxidation of cholesterol,

is believed to be toxic because a 5% solution in dimethyl sulfoxide caused nuclear aberrations in mouse tissue when instilled intrarectally into mice (Suzuki et al., 1986).

Cholesterol oxidase is also being used in the degradation of egg yolk cholesterol in an attempt to produce eggs with lower levels of dietary cholesterol (Christodoulou et al., 1994; Johnson and Somkuti, 1990; Aihara et al., 1988). The cholesterol levels in the eggs were reduced by as much as 50% and the oxidation occurred over a wide range of temperatures (Christodoulou et al., 1994). Requirements for lower concentrations of enzyme and the ability of cholesterol oxidase to degrade egg yolk cholesterol at 4°C make this a promising and practical method of dietary cholesterol reduction (Christodoulou et al., 1994).

Cholesterol oxidase genes have also been cloned into several Gram-positive bacteria including *Streptococcus thermophilus* , *Lactobacillus casei* , *L. reuteri*, several species of *Bacillus*, and the Gram-negative *Escherichia coli* (Brigidi et al., 1993). Cloning cholesterol oxidase into these commercially important dairy and intestinal organisms has provided a potential for safe and effective delivery of cholesterol oxidase into food systems for processing (Brigidi et al., 1993). The cholesterol oxidase-transformed intestinal bacteria could find potential use as a probiotic in the treatment of hypercholesteremia in humans (Brigidi et al., 1993). Unfortunately, the Gram-positive transformants, even though stable, did not show cholesterol oxidase activity (Bridigi et al., 1993).

Bacterial Reduction of Steroids and Sterols

Interest in bacteria which chemically reduce sterols such as cholesterol, steroids, and bile acids evolved in response to a need for the understanding of the processes involved in the regulation of levels of these sterols and steroids in humans. Recently, sterol-reducing bacteria have been again studied in light of current research showing the many potential ill-effects of high levels of neutral steroids and sterols as related to arteriosclerosis. Isolation and purification of these enzymes that convert substrates to commercially important products could be of great interest to drug and pharmaceutical companies. Since there has been evidence that some of the endproducts of the oxidation of cholesterol could be toxic and that there are problems associated with expression of the gene for cholesterol oxidase in GRAS (Generally Regarded As Safe) hosts for the use in food systems, the study of sterol-reducing bacteria has re-emerged (Suzuki et al., 1986; Bridigi et al., 1993).

Many species of sterol, steroid, and bile acid-reducing bacteria have been isolated and characterized, including *Eubacterium*, *Clostridium*, *Lactobacillus, Mycobacterium*, *Bacillus, and Serratia* (Samuelson, 1960; Arima et al., 1969; Marshcek et al., 1972; Essyen et al., 1973; Essyen et al., 1974; Sadzikowski et al., 1977; Mott and Brinkley, 1979; Mott et al., 1980; White et al., 1980; Brinkley et al., 1980; Brinkley et al., 1982; Macdonald et al., 1982; Gilliland et al., 1985; Stanton, 1987; Stanton and Cornell, 1987; Freier et al., 1994).

Genus *Eubacterium*

Although, many sterol, steroid, and bile acid-reducing bacteria have been isolated and characterized, much of the more recent work has centered around isolates identified as the genus *Eubacterium* (Mott et al., 1980; Macdonald et al., 1982; White et al., 1980; Feighner and Hylemon, 1980; Bokkenheuser et al., 1980; Masuda and Oda, 1983; Glass and Burley, 1985; Watkins and Glass, 1991; Freier et al., 1994).

In context of this thesis, it should be noted that the genus *Eubacterium* should not be confused with the kingdom classification of the eubacteria, or "true" bacteria. Members of the genus *Eubacterium* are Gram-positive, non-sporing, chemoorganotrophic, obligately anaerobic rods (Moore and Moore, 1984). Species of *Eubacterium* produce mixtures of organic acids such as butyric, acetic, and formic, while growing at an optimal temperature of 37°C with a pH near neutrality (Moore and Moore, 1984; Brinkley et al., 1982; Freier et al., 1994). Eubacteria have been isolated from cavities of man and other animals, plant and animal products, infections of soft tissue, and soil (Mott et al., 1980; Brinkley et al., 1982; Sadzikowski et al., 1977; Freier et al., 1994; Moore and Moore, 1984; George and Falkler, 1992). Enumeration of the fecal material of baboons have shown counts of *Eubacterium* as high as 3.3×10^{10} per gram of dry fecal matter, which comprised around 16.7% of the total number of viable organisms recovered (Brinkley and Mott, 1978). Biochemically, members of the genus *Eubacterium* vary a great deal from overall morphology to growth substrates and culturing conditions (Moore and Moore, 1984; Brinkley et al., 1982; Mott et al., 1980).

Further interests in metabolic activities of species of *Eubacterium* were pursued when methylotrophic/acidogenic strains of *Eubacterium limosum* were isolated and characterized (Pacaud et al., 1986; Pacaud et al., 1985; Loubiere et al., 1986; Genthner and Bryant, 1982). *E. limosum* cometabolizes methanol, along with carbon monoxide (CO) or carbon dioxide (CO_2) into acetic and butyric acids (Pacaud et al., 1985). Media were developed with initial levels of these acids to favorably produce one acid or the other in commercial acid production applications (Pacaud et al., 1986).

These one-carbon substrates appear to enter a common metabolic pathway where they are incorporated into the acids produced by this fermentation. The common intermediate of the entry of methanol and CO_2 is the formation of acetyl-coenzyme A (CoA) (Pacaud et al., 1985). In the proposed model for methyltrophic growth by these strains of *E. limosum*, methanol is preferentially incorporated into the methyl group of acetyl CoA while the CO_2 is the precursor to the carboxyl group of the acids produced (Pacaud et al., 1985). The acetyl CoA produced enters into one of three following pathways: acetic acid production, butyric acid production, or biomass generation (Loubiere et al., 1986; Pacaud et al., 1985). The reactions yield reducing equivalents of nicotinamide adenine dinucleotide ($NADH_2$) which appear to be balanced by the regeneration of NAD^+ by the production of butyric acid (Pacaud et al., 1985).

The formation of acetyl CoA from the condensation of equimolar amounts of methanol and CO_2 has been proposed in a general assimilation model (Kerby et al., 1983; Zeikus, 1983). Although the CO_2 is provided in the growth medium, the model proposes another source of CO_2 via methanol dissimilation which is thought to involve tetrahydrofolate (THF) intermediates (Pacaud et al., 1985). This results

not only in the production of NAD(P)H_2 reducing equivalents, but also generation of additional ATP from the terminal steps of organic acid production via acetate and butyrate kinases (Zeikus, 1983). The excess $NADH_2$ is thought to be recycled by two reverse-action dehydrogenases involved in the butyric acid biosynthetic pathway (Pacaud et al., 1986).

The amount of CO_2 present in the methylotrophic medium is the determining factor in the growth and nature of the organic acids produced (Loubiere et al., 1986; Pacaud et al., 1985; Pacaud et al., 1986). The production of the organic acid overflow metabolites can be altered by adjusting the initial growth substrate concentrations of acetic and butyric acid (Pacaud et al., 1985). The inclusion of acetate (50 mM) into the initial growth medium results in a homobutyric fermentation at improved rates of growth, and the added acetic acid is not metabolized during this fermentation (Pacaud et al., 1986).

Strains of *E. limosum* have also been grown with carbon monoxide (CO) as the sole carbon and energy source (Genthner and Bryant, 1982). Growth on CO or CO_2 supplemented with H_2 resulted in normal growth of *E. limosum* at a pH optimum of 7.0-7.2 with a generation time of 7 hours (Genthner and Bryant, 1982). Bacterial growth was uninhibited in cultures with up to 50% CO and the CO was preferentially utilized before H_2 utilization is significant (Genthner and Bryant, 1982).

Although much of the recent attention has centered around cholesterol-reducing strains of *Eubacterium*, strains have been isolated which degrade and utilize a number of other commercially important sterols, steroids, and bile acids (Glass and Burley, 1985; Glass et al., 1991; Watkins and Glass, 1991; White et al., 1980; White et al., 1981; Masuda et al., 1984; Bokkenhauser et al., 1980; Feighner

et al., 1979; Coleman et al., 1987; Feighner and Hylemon, 1980). Some of the steroid, sterols, and bile acids which are reduced include: cholesterol, progesterone, 16-dehydroprogesterone, corticosterone, deoxycortisol, cholic acid, and chenodeoxycholic acid.

Progesterone derivatives have been chemically reduced by strains of *Eubacterium* (Glass and Burley, 1985; Watkins and Glass, 1991). Cell suspensions of *Eubacterium* species strain 144 have been shown to quickly reduce 16-dehydroprogesterone to 17-isoprogesterone via a 16-dehydroprogesterone reductase (16-DHPR) along with the provision of hemin or exogenous electron donors such as H_2 or pyruvic acid (Glass and Burley, 1985). This strain also possessed additional steroid conversion activities, including the reduction of Δ^4-Δ^5 double bond and the 3-keto group on the A ring of these steroids (Glass and Burley, 1985). These activities were not observed in the absence of electron donors and hemin, suggesting that a cytochrome-containing electron transport system may be involved in the flow of electrons from pyruvic acid or H_2 to these enzymes which is consistent with strains of *E. lentum* when grown in hemin (Sperry and Wilkins, 1976; Glass and Burley, 1985).

Several types of cytochromes have been identified in cell extracts of *Eubacterium lentum* (Sperry and Wilkins, 1976). *E. lentum* was shown to contain cytochromes *a, b*, and *c* along with an unidentified carbon monoxide binding pigment (Sperry and Wilkins, 1976). These cytochromes functioned only under anaerobic conditions and were rapidly reduced by dithionite, but not by ascorbate or reduced glutathione (Sperry and Wilkins, 1976). This was the first obligate anaerobe to be isolated which contained these three cytochromes (Sperry and Wilkins, 1976).

The enzyme which catalyzes the reduction of the Δ^4-Δ^5 double bond in the A ring of 17α-progesterone was termed a 5α-steroid reductase (Glass et al., 1991). The 5α-reductase also required electron donors such as pyruvate, dithionite, and H_2, resulting in the generation of reduced methyl viologen, which was essential for the reductase activity (Glass et al., 1991). NADH or NADPH, with or without flavin nucleotides did not serve as electron donors to the 5α-reductase (Glass et al., 1991). The similarity in the electron donor requirements for 5α-reductase and 16-DHPR suggests a common electron transport mechanism which links pyruvic acid and H_2 oxidation to steroid double bond reduction in *Eubacterium* strain 144 (Glass et al., 1991). No activity was found when 5ß-steroids were assayed suggesting that no 5ß- to 5α-steroid isomerase was present (Glass et al., 1991).

The 3-keto group of steroids was reduced by a 3α-hydroxysteroid dehydrogenase (3α-HSDH) in *Eubacterium* species strain 144 (Glass and Burley, 1985). This $NADP^+$-linked enzyme converts the Δ^4-Δ^5 reduced steroid via the 5α-steroid reductase to a pregnanolone derivative commonly isolated from rat fecal material (Glass et al., 1991). The 3α-HSDH and 5α-steroid reductase activities were shown to be performed by separate enzymes and that 3α-HSDH activity was dependent upon the addition of oxidized NAD^+ or $NADP^+$ (Glass et al., 1991).

The 5α-reductase activity was localized to the membrane fraction and the enzyme was found to be solubilized by Triton X-100 (Glass et al., 1991). It has also been suggested that the genes for 16-DHPR and 5α-steroid reductase are differentially regulated (Glass et al., 1991). It was shown that 16-dehydropregnanalone induced synthesis of 16-DHPR, but not 5α-reductase whereas, progesterone-induced cells contained 5α-reductase, but not 16-DHPR activity (Glass et al., 1991).

Intestinal microflora have long been known to be involved in the conversion of many sterols and steroids within humans and animals. As a result, these bacteria play a significant role in the regulation of concentrations of particular steroids and sterols in the intestine and colon (Feighner et al., 1979). A strain of *E. lentum* has been shown to readily convert the substrates 11-deoxycorticosterone or corticosterone to progesterone via a 21-hydroxylase (Feighner et al., 1979; Feighner and Hylemon, 1980). Kinetics utilizing corticosterone yielded an apparent K_M of 7.35 μM and a V_{max} of 15.4 nmol (Feighner and Hylemon, 1980). The dehydroxylase activity was optimized when reduced pyridine nucleotides and an oxidized flavin coenzyme (FMN or FAD oxidoreductase) were added to reaction mixtures (Feighner et al., 1979). The resulting NAD(P)H: flavin oxidoreductase activity was spectrophotometrically measured (Feighner et al., 1979).

Some strains of *E. lentum* which undergo 21-dehydroxylation of steroid hormones also have been shown to perform a 3α-hydroxysteroid dehydrogenase activity as described in other strains of *Eubacterium* (Feighner et al., 1979; Glass et al., 1991). The physiological and metabolic significance of the dehydroxylation of steroids by these intestinal anaerobes is unknown. However, these non-assimilating steroid transformations might serve to determine routes of excretion of neutral steroid hormones (Feighner and Hylemon, 1980). The steroids themselves might serve as terminal electron acceptors since they have been shown not to stimulate growth (Feighner et al., 1979; Feighner and Hylemon, 1980).

The most well-known and characterized enzymatic activity of strains of *Eubacterium* is the bile-acid 7α-dehydroxylase, or 7α-hydroxysteroid dehydrogenase (White et al., 1980; White et al., 1981; Macdonald et al., 1982; Masuda et al., 1983; Masuda and Oda, 1983; Masuda et al., 1984; Coleman et al.,

1987; Coleman et al., 1987; Coleman et al., 1988; Franklund et al., 1990). Members of *Eubacterium*, as well as many other intestinal microorganisms modify taurine and glycine derivatives of primary bile acids via this enzyme (Franklund, et al., 1990).

Purification of the constitutively expressed 7α-hydroxysteroid dehydrogenase (7α-HSDH) resulted in the isolation of a protein with a subunit molecular mass of 27 kDa (Franklund et al., 1990). Gel filtration utilizing Sepharose CL-6B yielded a native molecular mass of about 124 kDa, suggesting that the enzyme exists as a tetramer of four identical subunits (Franklund et al., 1990). As in the cholesterol oxidase system, 7α-HSDH activity was inhibited by sulfhydrl inhibitors, while the addition of metal chelators had no effect upon catalytic activity (Franklund et al., 1990; Uwajima et al., 1973). The enzyme also demonstrated a great range of substrate specificity with the ability of the enzyme to utilize a number of bile acids and bile acid analogs (Falklund et al., 1990).

It has been shown that 7α-HSDH follows Michaelis-Menten kinetic models (White et al., 1980; White et al., 1981; Franklund et al., 1990). Substrate saturation kinetics plots showed that most bile acid substrates demonstrated K_M values between 4 and 20 μM, while the V_{max} range was from 601-674 μmol/min·mg protein (White et al., 1981; Franklund et al., 1990). The kinetic plots along with substrate inhibition products demonstrated an ordered, sequential direction of bile acid oxidation and reduction with NAD(P)H binding first (Franklund et al., 1990). The N-terminal amino acid sequence of purified enzyme revealed homology to several short, non-zinc alcohol/polyol dehydrogenases, and a possible bile acid binding domain (Franklund et al., 1990).

Genes for 7α-HSDH were cloned into *Escherichia coli* (Coleman et al., 1987). Transformants containing the 2.2-kb fragment encoding the 27 kDa structural gene product for 7α-HSDH showed activity with the addition of cholic acid (Coleman et al., 1987). However, it appears that cholic acid does not play a role in the regulation of expression of the catalytic peptide in *Escherichia coli* (Coleman et al., 1987). The synthesized peptide from transformed cells showed cross reactivity with antibodies which were raised from the purified 7α-HSDH from *Eubacterium* (Coleman et al., 1987).

The expressed protein was shown to have sequence homology to a number of other bacterial alcohol-polyol dehydrogenases which contain pyridine nucleotide binding domains (Coleman et al., 1988). This gene represents the first cloned gene from a species of *Eubacterium* (Coleman et al., 1987).

Cholesterol-Reducing *Eubacterium* Species

Interest in bacteria which chemically reduce cholesterol evolved in response to a need for the understanding of the processes involved in the regulation of levels of dietary cholesterol in humans. Recently, cholesterol-reducing bacteria have been again studied in the light of current research of the cholesterol oxidase system. Since there has been evidence that some of the endproducts of the oxidation of cholesterol could be toxic and problems associated with expression of the gene for cholesterol oxidase in GRAS hosts for use in food systems, the study of cholesterol-reducing bacteria has re-emerged (Suzuki et al., 1986; Dehal et al., 1991; Bridigi et al., 1993).

The isolation of bacteria that chemically reduce cholesterol has been of interest due to research that indicated that many fecal neutral sterols are derived from dietary origins rather than biliary or intestinal wall sources (Wilson and Reinke, 1968). It has been found that during digestive activities within the intestine, cholesterol is converted to two bacterial conversion products, coprostanol and coprostanone (McNamara et al., 1981). Cholesterol, coprostanol, and coprostanone together account for over 95% of the neutral steroid content of feces with the remainder being primarily cholestanol. It has been shown that up to 50% of the fecal sterols, depending upon diet, are present in the form of coprostanol (Heftman, 1970).

The isolation of bacteria that chemically reduce cholesterol has focused upon those bacteria which convert cholesterol to coprostanol. Coprostanol, the 5ß-hydrogenated product of the reduction of cholesterol by these bacteria, has been shown to be poorly absorbed within the human gastrointestinal tract (Bhattacharyya, 1986). Until the advent of improved isolation techniques for these bacteria, a body of earlier work was done with cecal contents and fecal material from animals and humans where rates of conversion of cholesterol to coprostanol were detected and quantitated (Dam, 1934; Rosenfeld et al., 1954; Snog-Kjaer et al., 1956; Rosenfeld and Gallagher, 1964; Bjorkhem and Gustafsson, 1971; Bjorkhem et al., 1973; Wilkins and Hackman, 1974; Ashes et al., 1978).

Several species of bacteria that chemically reduce cholesterol have been isolated and characterized, including species of *Eubacterium*, *Clostridium*, *Lactobacillus, Mycobacterium*, *Bacillus, and Serratia* (Arima et al., 1969; Marshcek et al., 1972; Essyen et al., 1973; Essyen et al., 1974; Sadzikowski et al., 1977; Mott and Brinkley, 1979; Mott et al., 1980; Brinkley et al., 1980; Brinkley et al., 1982;

Gilliland, et al., 1985; Stanton, 1987; Stanton and Cornell, 1987; Freier et al., 1994). Although, many bacteria that chemically reduce cholesterol have been isolated and characterized, much of the more recent work has centered around isolates identified as the genus *Eubacterium* (Mott et al., 1980; Brinkley et al., 1982, Dehal et al., 1991; Freier, 1991; Lulich et al., 1994; Freier et al., 1994; Lulich et al., 1995).

Initial studies on biological cholesterol reduction were performed *in vitro* with human feces or colon contents (Dam, 1934). The cholesterol added to the fecal material was shown to be hydrogenated into coprostanol; however, the organism(s) responsible were not isolated (Rosenfeld et al., 1943). The rate of hydrogenation occurred at rates as high as 80% *in vitro*, and it was shown that the common intestinal microflora were not responsible for the hydrogenation of cholesterol to coprostanol (Snog-Kjaer et al., 1956).

The enzyme responsible for the reduction of cholesterol to coprostanol has been named a "cholesterol reductase" (Dehal et al., 1991; Freier et al., 1994). The proposed mechanism involves the reduction of the Δ^5-Δ^6 bond of ring B resulting in the A/B ring structure having a *cis* configuration (Bjorkhem and Gustafsson, 1971; Margalith, 1986)(Figure 1). As a result of the configuration change, the C-3 hydroxyl group is in an equatorial position (Bjorkhem and Gustafsson, 1971). The combination of an equatorial hydroxyl group, coupled with the *cis* ring configuration is thought to be the main reason for the poor uptake of coprostanol by animals (Bhattacharyya, 1986). The 5α-hydrogenated derivative cholestanol, as well as cholesterol, have *trans* ring junctions with the hydroxyl group in the axial position resulting in better uptake by the intestine (Bhattacharyya, 1986).

Two mechanisms for the bacterial biotransformation of cholesterol to coprostanol by have been proposed (Figure 1). One is the direct reduction of the Δ^5-Δ^6 double bond of cholesterol to yield coprostanol (Rosenfeld et al., 1954; Rosenfeld and Gallagher, 1964). The other mechanism involves the indirect reduction of cholesterol via three intermediates: 5-cholesten-3-one, 4-cholesten-3-one, and coprostan-3-one (Bjorkhem and Gustafsson, 1971; Bjorkhem et al., 1973; Ren, 1991). The intermediates 4-cholesten-3-one and coprostan-3-one have been identified, but 5-cholesten-3-one has yet to be identified in the indirect reduction of cholesterol (Bjorkhem, et al., 1973; Ren, 1991). It is thought that the possible isomerization of the Δ^5-Δ^6 double bond to the Δ^4-Δ^5 position is the rate-limiting step in the indirect reduction of cholesterol to coprostanol (Bjorkhem et al., 1973). Due to the high rate of this isomerization, 5-cholesten-3-one may only briefly exist as a transient intermediate, thus providing an explanation for the inability to detect it by current methods (Bjorkhem et al., 1973; Ren, 1991).

The exact mechanism of the bacterial reduction of cholesterol to coprostanol by these strains of *Eubacterium* is largely unknown. Since early work was performed with rat cecal contents, a possibility might be that both mechanisms of cholesterol reduction are occurring simultaneously due to the complex mixture of the bacteria used. The current mechanism supports the possibility of both mechanisms occurring simultaneously in the bacterial conversion of cholesterol to coprostanol (Bjorkhem et al., 1973; Ren, 1991). Detergent extracts of cecal contents used in radio-labeled studies of chemical cholesterol reduction were unsucessful due to the inability to recover fractions containing cholesterol reduction activity (Bjorhem et al., 1973).

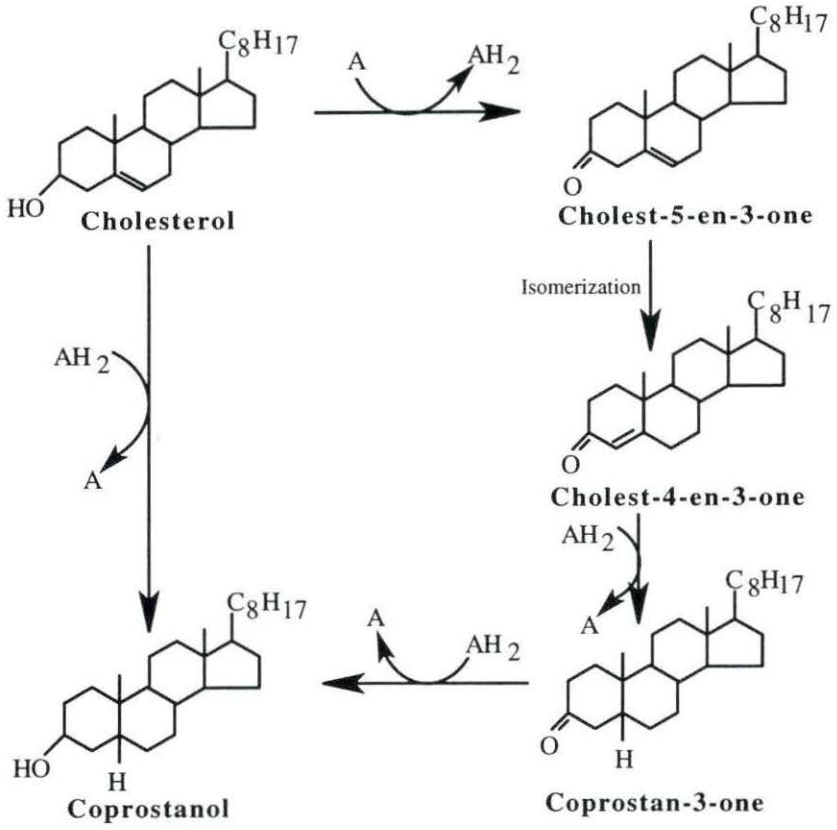

Figure 1. Proposed reaction mechanisms of cholesterol reduction by *E. coprostanoligenes* (Bjorkhem et al., 1973)

Purification of cholesterol reductase from fecal material was attempted by ion-exchange and gel filtration chromatography (Bjorkhem et al., 1973). Enzyme activity which was NADH-dependent, was observed in crude soluble fractions, but was quickly lost upon further purification.

The use of ion-exchange chromatography was unsuccessful due to the apparent lability of the enzyme as indicated by an almost 70% loss of activity in crude supernate samples stored at 4°C for up to 48 hours (Bjorkhem et al., 1973).

There have been many strains of the genus *Eubacterium* isolated which chemically reduce cholesterol to coprostanol (Ashes et al., 1978; Brinkley et al., 1982; Mott et al., 1980; Sadzikowski et al., 1977; Freier et al., 1994). Many of the strains of *Eubacterium* isolated required either homogenized brain or lipid extracts of brain for growth and long-term maintenance (Brinkley et al., 1980; Mott and Brinkley, 1979). Many of the strains isolated also required large amounts of cholesterol for growth and a great many of the isolates grew very poorly, or not at all, on solid growth medium (Mott and Brinkley, 1979; Brinkley et al., 1980; Freier, 1991; Lulich et al., 1994). Isolates which did plate on solid media yielded colonies which were extremely small (0.05-0.5mm) in diameter, and plating efficiencies were extremely low (Brinkley et al., 1980; Sadzikowski et al., 1977; Mott et al., 1979; Essyen et al., 1973; Lulich et al., 1994). Direct assays of cholesterol reductase activity were very difficult due to the heterogeneity of growth media; however, *in vitro* studies showed that up to 90% of the cholesterol present in the growth medium was reduced to coprostanol (Brinkley et al., 1980; Freier et al., 1994). The high apparent rates of cholesterol reduction by these bacteria that chemically reduce cholesterol showed promise of a new and potentially safer way to reduce levels of dietary cholesterol in humans.

As previously mentioned, many of the cholesterol-reducing isolates of *Eubacterium* were difficult to culture and when culturable, difficult to work with due to the high amount of lipids and cholesterol present in the growth medium (Lulich, et al., 1994). As a result, cholesterol-reducing strains with better growth characteristics were sought which would facilitate better biochemical studies of the cholesterol reductase. An isolate from a hog sewage lagoon, named *Eubacterium coprostanoligenes*, showed several unique properties making this isolate more amenable to biochemical and genetic studies of cholesterol reduction by the cholesterol reductase (Freier, 1991; Freier et al., 1994).

E. coprostanoligenes is unique, compared to other cholesterol-reducing eubacteria in that it does not require brain, lipid extracts of brain, or plasmenylethanolamine for growth and long-term maintenance (Freier, 1991; Freier et al., 1994). However, *E. coprostanoligenes* does require lecithin for growth (Freier, 1991). The significance of lecithin as a growth requirement is currently unknown (Freier et al., 1994). Another unique aspect is that, as opposed to previously isolated strains of eubacteria that chemically reduce cholesterol , *E. coprostanoligenes* grew in the absence of large amounts of cholesterol or other related sterols (Freier et al., 1994; Lulich et al., 1994).

E. coprostanoligenes provided an isolate that was easier to work with and quickly reduced cholesterol to coprostanol. In actively growing cultures of *E. coprostanoligenes*, the concentration of cholesterol in the growth medium was lowered from 2.0 mg/ml to less than 0.003 mg/ml over a three-day incubation period, which was significantly lower than previously isolated bacteria that chemically reduced cholesterol (Lulich et al., 1994). This strain has shown promise in that it can theoretically decrease the concentration of cholesterol to levels 10-to

100-fold lower than those found in meat and dairy products (Bechtel, 1986; Price and Schweigert, 1987).

E. coprostanoligenes was shown to metabolize pyruvic acid, and this metabolism has been postulated to be related to the chemical cholesterol reduction where pyruvic acid serves as either an electron donor or cofactor in the reduction of cholesterol to coprostanol (Freier et al., 1994). This postulation is supported by other work which showed that pyruvic acid acts as an electron donor for the 16-dehydroprogesterone reductase in cell extracts of *Eubacterium* sp. strain 144 (Glass and Burley, 1985; Watkins and Glass, 1991; Freier et al., 1994).

Since coprostanol is not absorbed by the human gastrointestinal tract, there exists potential use for cholesterol reductase in the reduction of dietary cholesterol. New technologies for lowering levels of cholesterol utilizing cholesterol reductase are being developed (Dehal et al., 1991). Cholesterol reductase also shows promise in that the potential rates of reduction could result in significantly lower levels of cholesterol in foods and hypercholesteremic patients. More promise exists in that no toxic endproducts are produced in the reduction of cholesterol and further characterization and purification of cholesterol reductase shows great commercial promise.

IMPROVED CULTURE MEDIUM AND KINETIC STUDIES OF STEROL REDUCTION BY *Eubacterium coprostanoligenes*

A paper to be submitted for publication to

Anaerobe

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Abstract

Eubacterium coprostanoligenes is a Gram-positive, obligately anaerobic bacterium isolated from a swine lagoon on the basis of chemical cholesterol reduction. Biochemical studies on the cholesterol reductase system in *E. coprostanoligenes* have been hampered by poor growth yields and the production of a capsule in the isolation medium. To overcome these problems, the standard culture medium was modified to increase cell yields and reduce the production of extracellular capsular polymers. First, the cholesterol reductase system was determined to be constitutive, and elimination of cholesterol and lactose from the growth medium drastically reduced capsular polymer formation. Second, increased levels of lecithin in the growth medium reduced the level of capsular formation further and also increased the cell yield four-fold.

E. coprostanoligenes was also grown in a modified, lecithin-free methylotrophic medium. Growth rates were comparable to those observed in the original isolation medium; however, growth of *E. coprostanoligenes* in this

methylotrophic medium occurred in the absence of lecithin. Cells grown in the new culture medium also showed higher rates of chemical cholesterol reduction.

Kinetic studies indicate that the rate limiting step in whole cell, chemical cholesterol reduction was substrate accessibility. Cholesterol availability was increased using lecithin/cholesterol micelles, which resulted in a 20-fold increase in the rate of cholesterol reduction. The rate of cholesterol reduction in the new assay system showed an apparent V_{max} of 2.9 μmol/min·mg cell protein and an apparent K_M of 111 μM. From this whole-cell assay, a cell-free assay was developed using ascorbate as a reductant.

The cholesterol reductase activity demonstrated a broad substrate specificity. Cholesterol, 4-Cholesten-3-one, coprostanone, corticosterone, 17-Hydroxyprogesterone, anhydroxyprogesterone, and progesterone demonstrated comparable $K_{M\ app}$ and $V_{max\ app}$values of reduction. Mixed substrate studies with cholesterol, 4-Cholesten-3-one, coprostan-3-one, and progesterone indicate a competitive type of inhibition. Coprostan-3-one or 4-cholesten-3-one, proposed intermediates in a bacterial multiple step reduction of cholesterol showed a competitive type of inhibtion.

Introduction

Consumer demand for reduced-cholesterol foods has stimulated research in the development of physical, chemical, and biological technologies to decrease the amounts of cholesterol and triacylglycerols in foods (Arima et al., 1969; Bjorkhem et al., 1973; Dehal et al., 1991; Johnson and Somkuti, 1990; Marschek et al., 1972; Mazschner and Fine, 1989). Of the three, biological methods are often preferred,

since they are often less harsh, form fewer by-products, and are considered "natural" by the consumer. Much of the research in decreasing cholesterol content of foods biologically has focused on the cholesterol oxidase system. The microorganisms catalyzing this reaction often completely oxidize cholesterol to carbon dioxide and water (Turfitt, 1946; Noma and Nakayama, 1976; Smith and Brooks, 1976; Ferreira and Tracey, 1984; Smith et al., 1993). The enzyme responsible for the initial oxidation, cholesterol oxidoreductase, catalyzes the oxidation of cholesterol to 4-cholesten-3-one via the intermediate 5-cholesten-3-one (Noma and Nakayama, 1976; Molnar et al., 1993; Uwajima et al., 1974; Smith and Brooks, 1977; Turfitt, 1948; Stadtman et al., 1954; Richmond, 1973).

Identification of bacteria that chemically convert cholesterol to coprostanol has provided a second enzyme system to decrease cholesterol content in foods. Coprostanol, the hydrogenated product of this chemical reduction, is poorly absorbed by humans as well as in several test animals (Dam, 1934; Ashes et al., 1978; Bjorkhem and Gustafasson, 1971; Rosenfeld et al., 1954; Rosenfeld et al., 1963; Rosenfeld and Gallagher, 1964; Snog-Kjaer et al., 1956; Bhattacharyya, 1986). Several strains of intestinal bacteria have been shown to catalyze this reduction, the most common being different species of the genus *Eubacterium.* Members of the genus *Eubacterium* are Gram-positive, chemoorganotrophic, nonspore forming, obligately anaerobic rods (Ashes et al., 1978; Brinkley et al., 1982; Eyssen et al., 1973; Mott and Brinkley, 1979; Sadzikowski et al., 1977; Freier et al., 1994).

Two mechanisms of bacterial reduction of cholesterol to coprostanol have been proposed (Fig. 1). One is the direct reduction of the $\Delta^{5,6}$-double bond of cholesterol to coprostanol (Bjorkhem et al., 1973; Bjorkhem and Gustafasson,

1971; Rosenfeld and Gallagher, 1964). The other proposed mechanism involves a multi-step reduction. Cholesterol is first oxidized to 5-cholesten-3-one, followed by an isomerization to 4-cholesten-3-one, a reduction to coprostan-3-one, followed by a second reduction to coprostanol (Bjorkhem et al., 1973; Bjorkhem and Gustafasson, 1971; Ren, 1991).

The physiological function of chemical cholesterol reduction has not been identified (Mott and Brinkley, 1979; Sadzikowski et al., 1977; Freier et al., 1994). The poor growth characteristics of *Eubacterium* sp. have hampered studies on this enzyme system. Most strains of cholesterol-reducing *Eubacterium* required either homogenized brain tissue, lipid extracts of brain, or plasmenylethanolamine for long-term maintenance (Mott and Brinkley, 1979). Many also required cholesterol or a related sterol for growth (Mott and Brinkley, 1979; Sadzikowski et al., 1977). An isolate from a hog lagoon, *Eubacterium coprostanoligenes*, showed several unique properties that made the isolate more amenable to biochemical and genetic studies (Freier, 1991). First, this strain did not require plasmalogen or mammalian brain tissue extracts, making the medium less viscous than previous cholesterol-reducing *Eubacterium* media. Second, in actively growing cultures, *E. coprostanoligenes* showed higher affinity for cholesterol and higher reduction rates than the other cholesterol-reducing strains tested. The high rates and high affinities of the cholesterol reductase system in *E. coprostanoligenes* makes this bacterium an ideal test system to examine the potential use of cholesterol-reducing bacteria in food production processes.

Although an improvement over other cholesterol-reducing bacteria, the growth characteristics of *E. coprostanoligenes* were still too poor to attempt biochemical or genetic studies. In addition to long generation times (approximately

3.5 hours) and low yields (150 - 200 μg cell protein per ml culture medium), *E. coprostanoligenes* produces a large capsule when grown on either isolation medium (Lulich et al., 1994). This capsular material has made whole cell and cell-free assays very difficult because the lipids in the medium are concentrated in the capsular material. The high lipid content associated with the capsular material has made harvesting cells difficult, and the membrane fraction associates with the capsular fraction upon cell lysis, making membrane isolations difficult.

This paper describes two new growth media that significantly improve yields and decrease concentration of extracellular polysaccharides. These media have made physiological and biochemical studies on the cholesterol reductase system in *E. coprostanoligenes* possible. Cells from these media were used to examine the specificity and kinetics of cholesterol reduction.

Materials and Methods

Strains and growth conditions

Eubacterium coprostanoligenes (ATCC #51222) was cultured by modified serum bottle "Hungate" techniques (Freier et al., 1994; Miller and Wolin, 1974). The media used in these experiments were prepared as follows: Basal cholesterol (BC) medium was prepared as previously described (Freier, 1991). The medium was reduced by boiling and was dispensed anaerobically in 90-ml aliquots into 125-ml Hypo-vials (Pierce Chemical, Rockford, IL.) which were subsequently sealed with butyl rubber stoppers and autoclaved. The final pH upon cooling was 7.2. Inoculations were performed in an anaerobe chamber utilizing a 10% inoculum, and cultures were incubated at 37°C for 5-7 days.

Eubacterium (EM) medium was prepared as above with the following changes: no cholesterol, 0.075% lecithin, no lactose, and 0.1% pyruvic acid. Inoculations were performed in the same manner as described above. For batches above 3 liters, transfers were performed anaerobically with Masterflex pumps and oxygen-impermeable Tygon tubing lines run from each carboy with vents adapted to the anaerobic chamber (Cole-Parmer, Inc. Chicago) (Norton Industries) (Lulich et al., 1994).

Methylotrophic medium (MSM) for the growth of *E. coprostanoligenes* on methanol and CO_2/CO was prepared as previously described with the following modifications (0.5 L formulation) (Pacaud et al., 1986; Pacaud et al., 1986; Loubiere et al., 1986; Sharak Genthner and Bryant, 1982; Lidstrom, 1988): Solution A: 5 ml Whittenbury's Trace Elements (Whittenbury et al., 1970), 0.25 g NH_4Cl, 4 mM sodium thioglycolate, 0.005 g $NaMoO_4$, 4 µM resazurin, 5 µM $CuSO_4$, 0.25 g casitone, 0.25 g yeast extract, and 0.7 mM $CaCl_2$ (all anhydrous). Part A was reduced by boiling, cooled under oxygen-free N_2, and transferred to an anaerobic chamber where 80-ml aliquots were dispensed into 125-ml hypovials, sealed, and sterilized.

To 80-ml aliquots of Solution A, the following were aseptically added under anaerobic conditions: 1.25 ml 1 M sodium acetate, 7.5 ml 1 M methanol, 1 ml 0.5 M Na/KPO_4 buffer (pH 7.2), and 25 µl Staley's vitamin solution. Upon inoculation, vial atmospheres were sparged with approximately 5 psi CO or CO_2 prior to incubation. Cultures were incubated at 37°C for 3-5 days and transferred five consecutive times within the test medium prior to assaying for cell yield and cholesterol reductase activity.

Isolation of cells, soluble and membrane fractions

Cells were harvested by centrifugation 10,000 x *g* for 20 minutes at 4°C and washed in degassed 10 mM sodium/potassium phosphate, 4 mM sodium thioglycolate buffer, pH 7.2 (reducing buffer) and immediately placed into Hypo-vials under anaerobic conditions . Vials containing whole cells were incubated at 37°C when used in whole cell assays or put on ice in preparation for lysis. Cells were lysed by passage through a French pressure cell (Aminco Instruments, Inc.) three times at 18,000 psi (piston diameter = 1 inch); anaerobic conditions were maintained by flushing with oxygen-free nitrogen when the cell was opened . A canula with nitrogen was also utilized to stream nitrogen into the collection vial during lysis. The mixture was then centrifuged at 10,000 x *g* for 20 minutes at 4°C to remove unlysed cells and debris. The supernate (cell-free fraction) was placed in Hypovials and stored under nitrogen until utilized for assays or fractionation studies. In cell fractionation studies the supernate was centrifuged at 150,000 x *g* for 90 minutes at 4°C. The pellet from this preparation was used as the membrane fraction, and the supernate was used as the soluble fraction.

Quantitation of extracellular polysaccharides

E. coprostanoligenes was grown to log phase. 1.5-ml aliquots from each culture were dispensed in microfuge tubes and sonicated for 20 seconds at 26 W with a Braun-sonic 15 sonicator (R Braun, Melsungen, AG). Sonicated cells were then centrifuged at 13,200 x *g* for 20 minutes at 4°C The supernates (1 ml) were assayed for total extracellular polysaccharides by the phenol/sulfuric reaction utilizing glucose as a standard (Hanson and Phillips, 1981; Powell et al., 1982). *Escherichia coli* strain K-12 was used as a control.

Preparation of cholesterol/lecithin mixtures

Blended cholesterol /lecithin was prepared by suspending a 1:2 molar ratio of cholesterol/soy lecithin (lecithin-97% phosphatidyl choline; Calbiochem, La Jolla, CA.) in assay buffer. The mixture was homogenized in a Waring blender for 15 minutes under a stream of nitrogen. The homogenate was placed into vials and put under anaerobic conditions.

Cholesterol/lecithin mixed micelles were prepared at described by Luk *et al.* (1993) using a 1:2 molar ratio of cholesterol:lecithin and maintaining anaerobic conditions as much as possible. Micelles were added to reaction mixtures to give a final cholesterol concentration of 0.5 mM (0.2 mg/ml) as determined by gas chromatography.

Protein determination

Protein concentrations were determined by Folin phenol method as described by Lowry *et al.* (1951)using bovine serum albumin as the standard.

Sterol and steroid reduction quantitation assay

Sterol and steroid reduction assays were performed in sealed Hypovials at 37°C under anaerobic conditions. All sterols and steroids used as experimental substrates were obtained from Steraloids (Wilton, NH). Experimental substrates were prepared in lecithin to form micelles as previously described (Luk et al., 1993). The reaction mixtures contained per 0.5 ml washed cells: 2.0-2.5 mg protein; 0.005 mM, 0.1 mM, 0.05 mM, 0.075 mM, 0.10 mM, or 0.5 mM sterol or steroid ; pyruvic acid, 10 mM; $CaCl_2$, 50 mM. Assay buffer (10 mM

sodium/potassium phosphate buffer, pH 7.2) was added to bring the total volume to 0.5 ml, and the vials were pre-incubated at 37°C for 1 hr prior to assay.

To pre-incubated vials, 0.5 ml of cells or cell-fractions were anaerobically injected into the sealed vials. The vials were incubated at 37°C for 1 hour. Following incubation, the reaction mixtures were extracted with methanol/chloroform/water as described by Bligh *et al.* (Bligh and Dyer, 1959). Extracted organic phases were dried down under nitrogen to completeness. The dried extracts were derivatized with a 1:1 ratio of N-O-*bis* (trimethylsilyl) Trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA in 1% TMCS) (Pierce, Rockford, IL) and acetonitrile.

Derivatized mixtures were sealed and heated at 65°C for 1 hr prior to injection. Detection of steroids and sterols was determined by gas chromatography with an Autosystems gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a flame ionization detector. Analysis column was a 1/8", 0.91m glass column packed with SP-2250 on 100/120 Supelcoport (Supelco, Inc. Bellafonte, PA). Running conditions were as follows: Oven, 250°C; injector, 290°C; detector, 325°C; carrier gas flow rate, 20 ml/min.

Cell-free assay

Potential reductants were screened in the following concentrations: pyruvate, 10 mM; succinate, 10 mM; reduced nicotinamide adenine dinucleotide (NADH), 2 mM; lumiflavin + EDTA, 20 μM; dithionite, 2 mM; ascorbate, 2 mM; sodium thioglycolate, 5 mM. Some of the reductants were screened in tandem with the following redox dyes: methyl viologen, 5 mM; benzyl viologen, 5 mM. Sodium ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylene diamine hydrochloride (TMPD) was

prepared as outlined by Ferguson-Miller *et al.* (1976). Ascorbate, methyl and benzyl viologen, reduced NADH, and ascorbate/TMPD were prepared fresh in amber vials. Lumiflavin was prepared as described by DiSpirito *et al.* (1987) and photoreduced in reaction vials with a 120 watt flood lamp with a water filter between reaction vials and light source for 10-20 minutes prior to assaying. All redox dyes along with ascorbate, reduced NADH, and dithionite were prepared fresh and added just before initiation of reactions.

Vials were pre-prepared with a total volume of 1 ml. To the vials, the following were added under anaerobic conditions: cholesterol/lecithin micelles, 0.5 mM; lecithin stock solution (as described above), 40 µl; $CaCl_2$, 5 mM; and assay buffer (pH 7.2) added to bring the total volume to 0.5 ml. Reductants and/or redox dyes were added right before assay and vials were pre-incubated at 37°C for 50-60 minutes before the addition of crude lysate. When specified, H_2 gas was added to atmospheres of vials to a total concentration of 50% along with reduced nitrogen. All other vials contained 100% nitrogen. The reductants, redox dyes, and cell lysate were added to reaction vials with Hamilton gas-tight syringes (Hamilton, Reno, NV.) which were purged with N_2 before drawing.

Steroid/sterol kinetics

Kinetic studies were carried out with 6-7 day washed whole-cell cultures of *E. coprostanoligenes*. All sterols and steroids were obtained form Steraloids (Wilton, NH) and checked for purity prior to use. From preliminary compatibility studies, the following steroids and sterols were chosen: cholesterol, 4-Cholesten-3-one, coprostan-3-one, corticosterone, and progesterone. Reaction vials were

prepared as previously described for the other whole cell assays, and all assays were performed under anaerobic conditions.

Kinetic studies of chosen sterols and steroids were carried out utilizing micellar solutions of steroid/sterol with lecithin. These micellar solutions were prepared as previously described for whole cell and cell-free studies in this paper. Reactions were incubated for 1 hour and upon completion, were extracted, derivatized, and assayed for reduction activity as already described in this paper.

Results

Culture medium

All of the components in the growth media described by Freier *et al.* (1994) were determined to be in excess except lecithin. Optimal growth of *E. coprostanoligenes* was obtained with the removal of lactose and cholesterol and increasing the lecithin concentration to 2.0 mg/ml (Table 1). Levels of lecithin in excess of 2.2 mg/ml appeared to be toxic to *E. coprostanoligenes*. There was no difference in total cell yields in EM medium with cholesterol versus EM without cholesterol (Table 1). The lecithin-optimized EM medium resulted in almost a 4-fold increase in cell yields versus the original isolation medium (BC) (Table 1).

Growth of *E. coprostanoligenes* in defined minimal salts medium

The growth of *E. coprostanoligenes* in lecithin-free minimal salts medium was successful (Table 1). Highest yields were obtained when *E. coprostanoligenes* was grown in MSM with CO added as the main atmospheric gas (Table 1). Previous characterization of *E. coprostanoligenes* stated an absolute requirement for lecithin in the growth medium. Cells harvested from MSM

Table 1. Growth yields and cholesterol reductase activity of *E. coprostanoligenes* as grown in modified EM, BC, and MM media.

Medium	Yield (μg protein/ml	Cholesterol Reductase Activity (pmol coprostanol/ min·mg protein	Rate of Intermediate Formation (pmol/min·mg protein)	
			4-Cholesten-3-one	Coprostan-3-one
BC	390	409	ND*	ND
BC-No Cholesterol	407	421	ND	ND
EM	975	1007	235	ND
EM-No Cholesterol	943	996	262	ND
EM-2.0 mg/ml Lecithin	1474	1566	405	128
EM-2.0 mg/ml Lecithin-No Cholesterol	1456	1534	422	117
MSM w/o Acetate+ Lecithin+CO_2	884	355	ND	ND
MSM w/o Acetate+CO_2	985	385	ND	ND
MSM+Lecithin +CO_2	867	381	ND	ND
MSM w/o Acetate+CO	1031	610	105	ND
MSM+CO_2	922	385	ND	ND
MSM+CO	1152	669	116	ND
MSM+Lecithin +CO	1169	672	123	ND

*ND = Not Detected

demonstrated cholesterol reductase activity. However, growth was similar with or without lecithin.

When *E. coprostanoligenes* was grown in MSM medium with CO, cell yields were comparable to previously developed isolation media were observed (Table 1). However, cholesterol reductase activity was somewhat lower than had been observed from cells grown in EM medium (Table 1). The cholesterol reductase activity of cells grown in MSM was highest under CO. The presence of lecithin in the growth medium did not significantly affect the rate of cholesterol reductase activity. When *E. coprostanoligenes* was grown in MM with CO_2 in the presence or absence of lecithin, rates of cholesterol reductase activity were almost 40%-45% lower than with washed whole cells grown in the same medium under a CO atmosphere (Table 1).

EM, BC, and MSM media were prepared in agar form as well. There was no improvement in colony size or plating efficiency when *E. coprostanoligenes* was grown on EM, BC, or MSM agar plates. Colonies were about 0.5 mm in diameter, taking 5-7 days to appear, which was consistent in previous plating attempts of *E. coprostanoligenes* (Freier, 1991; Freier et al., 1994, Lulich and DiSpirito, 1994).

Reduction of extracellular polysaccharides

In addition to lowering cell yields, lactose and cholesterol were associated with the production of extracellular polysaccharides by *E. coprostanoligenes.* Removal of these components in the growth medium decreased the concentration of extracellular polysaccharides by 68 to 79%. The concentration of extracellular polysaccharides was minimized with growth in EM medium with 2.0 mg/ml lecithin and without lactose (Table 2).

Table 2. Levels of extracellular polysaccharides produced by *Eubacterium coprostanoligenes* as grown in selected test media

Sample	Total extracellular polysaccharide index (μg/ml)	Cell yield (μg/ml of cells)
BC	26.6	407
BC+1.5% Pyruvate	33.4	551
BC+1.5% Pyruvate & 1.5% Lactose	40.7	499
EM + Cholesterol and Lactose	22.7	883
EM-No Lactose	4.3	879
EM-No Lactose-No Cholesterol	7.2	886
EMO*	15.5	1070
EMO+Phosphate Buffer-No Lactose*	4.5	1081
EMO-No Lactose	6.2	1062
Escherichia coli K-12	1.8	1916

*EMO = Optimized EM medium with 2.0 mg/ml lecithin

Whole cell kinetics of cholesterol reduction

Initial kinetic studies of cholesterol reduction revealed a problem with substrate accessibility. Optimal whole cell activity required cholesterol/lecithin micelles (Fig. 2). Michaelis-Menten plots of whole cell assays utilizing cholesterol/lecithin micelles over blended cholesterol/lecithin showed a four-fold increase of activity. Blended cholesterol/lecithin showed a maximal rate of cholesterol reductase activity of 0.7 nmol/min·mg cell protein at a substrate concentration of 2 mM (Fig. 2). However, the kinetic data obtained with the use of blended cholesterol-lecithin as substrate did fit into conventional kinetic models. Using cholesterol/lecithin micelles, whole-cell cholesterol reductase activity showed an apparent K_M of 111 μM and an apparent V_{max} of 2.9 nmol cholesterol reduced/ min·mg cell protein (Fig. 2).

The proposed intermediates of the multiple-step reduction of cholesterol to coprostanol, 4-cholesten-3-one and coprostan-3-one, were identified (Table 1). However, cholesten-3-one and coprostan-3-one were only detected in whole cells showing high cholesterol reductase activity (Table 1).

Cell-free/fractionation studies

Highest cell-free rates of cholesterol reductase activity were obtained with crude lysates using NADH or ascorbate as a reductant (Table 3). The cholesterol reductase activity was observed in membrane fractions (Table 4). The loss of activity between the cell-free fraction and membrane fraction could not be recovered by addition of the soluble fraction. The highest cholesterol reduction activity in washed membrane fractions was observed when ascorbate and methyl viologen were used as the reductant/redox dye combination (Table 4).

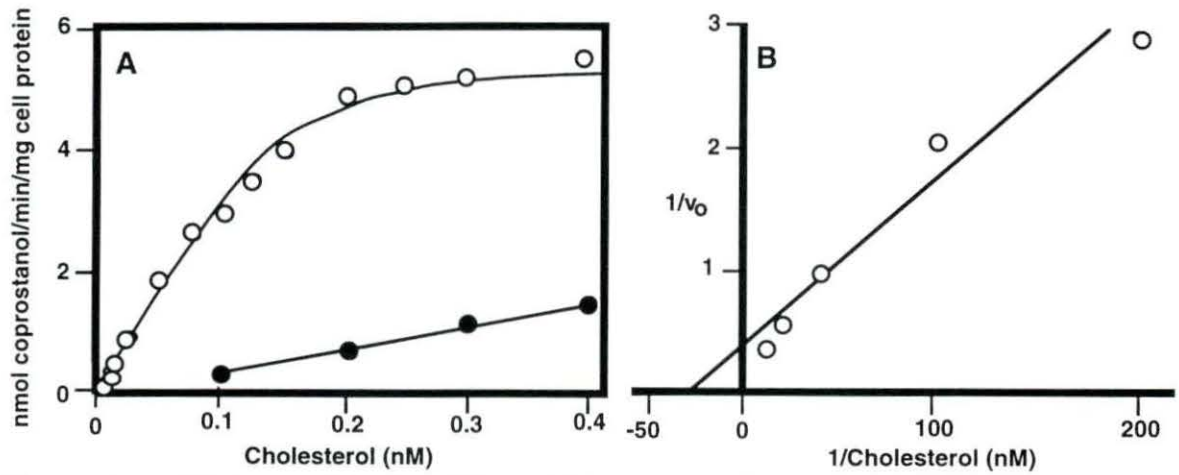

Figure 2. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of cholesterol reduction by *E. coprostanoligenes*; assayed using blended cholesterol (●) and cholesterol-lecithin micelles (❍).

Table 3. Cell-free cholesterol reductase assays utilizing cell-free lysates.

Reductant	Redox Dye	Cholesterol Reductase Actitvity (pmol/min/mg protein)
Pyruvate*	-	102
NADH	-	0
NADH	Benzyl Viologen	0
NADH	Methyl Viologen	576
NADH plus dithionite	-	0
Succinate	-	48
Succinate	Benzyl Viologen	0
Succinate	Methyl Viologen	90
Lumiflavin	EDTA	0
Lumiflavin	EDTA plus Benzyl Viologen	50
Lumiflavin	EDTA plus Methyl Viologen	170
Ascorbate	-	0
Ascorbate	Benzyl Viologen	350
Ascorbate	Methyl Viologen	230
Ascorbate	TMPD	90
H_2	Benzyl Viologen	88
H_2	Methyl Viologen	63
Sodium Thioglycolate	-	0
Sodium Thioglycolate	Benzyl Viologen	0
Sodium Thioglycolate	Methyl Viologen	230

* = No stimulation of cholesterol reductase activity was observed with the addition of methyl or benzyl viologen.

Table 4. Reductant/redox dye screenings of cholesterol reduction by membrane fractions of *E. coprostanoligenes.*

Reductant	Redox Dye	Cholesterol Reductase Activity (pmol/min • mg protein)
Sodium Thioglycolate		119
Sodium Thioglycolate+Pyruvate		124
Sodium Thioglycolate +H_2		149
Pyruvate		120
Pyruvate + H_2		166
Ascorbate		245
Dithionite	Benzyl Viologen	56
Dithionite	Methyl Viologen	79
NADH + H^+	-	0
NADH + H^+	Benzyl Viologen	145
NADH + H^+	Methyl Viologen	158

Steroid/sterol kinetics

The cholesterol reductase activity from *E. coprostanoligenes* demonstrated a broad specificity for sterol/steroid derivatives other than cholesterol (Table 5). The rate of reduction for both coprostan-3-one and 4-Cholesten-3-one were similar to the rate of cholesterol reductase activity (Table 5). 4-Cholesten-3-one has been identified as a stable intermediate in a multiple step mechanism of the chemical reduction of cholesterol to coprostanol by Bjorkhem *et al.* (Bjorkhem et al., 1973). However, Bjorkhem's studies were conducted with mixed cultures.

The rates of corticosterone, progesterone, 17-hydroxyprogesterone, and anhydroxyprogesterone reduction activity were somewhat lower than those observed with cholesterol derivatives. There was no reduction of 17-hydroxypregnenalone or ß-sitosterol by whole cells of *E. coprostanoligenes* (Table 5).

The sterol/steroid reductase activity of *E. coprostanoligenes* showed high affinity for the steroids and sterols assayed (Table 5). Cholesterol appears to be the preferred substrate (Table 5). The affinity for cholesterol and rates of cholesterol reductase activity are significantly higher than previously reported bacterial reduction rates (Mott and Brinkley, 1979; Freier et al., 1994). 4-Cholesten-3-one and coprostan-3-one, proposed intermediates in a multiple-step reduction of cholesterol, were both converted to coprostanol at rates comparable to that of cholesterol (Table 5).

Corticosterone and progesterone were also chemically reduced by washed whole cells of *E. coprostanoligenes* (Table 5). Although the $V_{max\ app.}$ values obtained were somewhat lower than that of the cholesterol derivatives, the enzyme showed higher substrate affinities.

Table 5. Steroid/sterol reduction by *E. coprostanoligenes*.

Steroid/Sterol	Steroid/Sterol Reductase Activity		
	Rate*	K_M+	V_{Max}†
Cholesterol	-	2.9	111
4-Cholesten-3-one	-	2.5	63
Coprostan-3-one	-	2.5	81
Corticosterone	-	0.7	24
Progesterone	-	0.5	28
17-Hydroxyprogesterone	800	ND	ND
Anhydroxyprogesterone	615	ND	ND
ß-Sitosterol	0	ND	ND
17-Hydroxy Pregnenalone	0	ND	ND

ND = Not Determined
* = pmol/min·mg protein
+ = µmol as calculated
† = nmol/min·mg protein

Mixed substrate studies of sterol/steroid reduction by *E. coprostanoligenes* indicated that 4-cholesten-3-one and coprostan-3-one were intermediates in the reduction of cholesterol. When cholesterol and either 4-cholesten-3-one, coprostan-3-one or progesterone were added to reaction mixtures, a competitive type of inhibition was also observed (Figs. 3 - 5).

Discussion

E. coprostanoligenes, unlike previously isolated strains of cholesterol-reducing *Eubacterium*, does not require homogenized brain tissue or lipid extracts of brain for growth. Results of these studies illustrate that lecithin is also not required for growth. The growth of *E. coprostanoligenes* in a methanol-acetate based medium (i.e. MSM) yielded comparable growth and cholesterol reductase activity rates with cells cultured in the absence of lecithin. The minimal salts medium developed could serve as an alternative and more cost-effective medium. The acidogenic properties of *E. coprostanoligenes* when grown under methylotrophic conditions are very similar to acidogenic strains of *Eubacterium limosum* (Pacaud et al., 1986; Pacaud et al., 1986; Loubiere et al., 1986; Sharak Genthner and Bryant, 1982).

The lower rates of cholesterol reductase activity observed when *E. coprostanoligenes* was grown in MSM could be due to several reasons. The generation of energy and biomass by *E. coprostanoligenes* when grown in MSM may cause a shift in the reductant, from cholesterol to butyryl-CoA resulting in production of butyric acid (Pacaud et al., 1986). Final culture pH might also play a role in the decreased cholesterol reduction rates as seen when lactose was used in the initial isolation media (Freier et al., 1994; Freier, 1991). *E. coprostanoligenes*

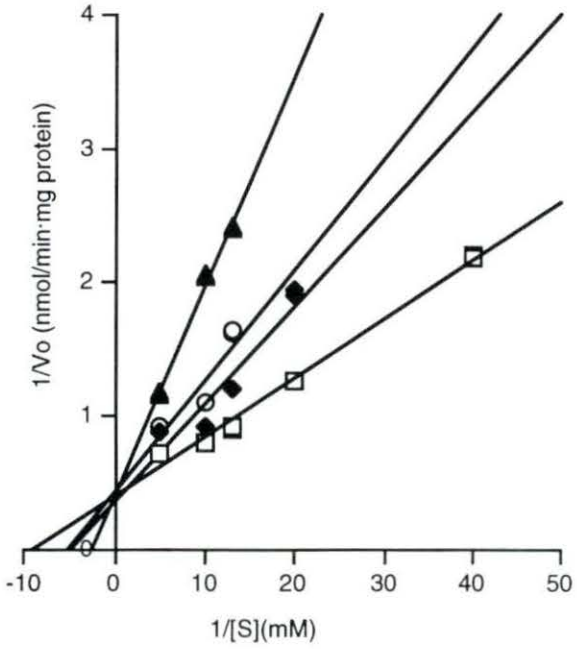

Figure 3. Lineweaver-Burk plots of cholesterol reduction by *E. coprostanoligenes* with increasing levels of 4-cholesten-3-one (❑-no addition; ◆-+ .01 mM 4-cholesten-3-one; ○-+ .02 mM 4-cholesten-3-one; ▲-+ .03 mM 4-cholesten-3-one).

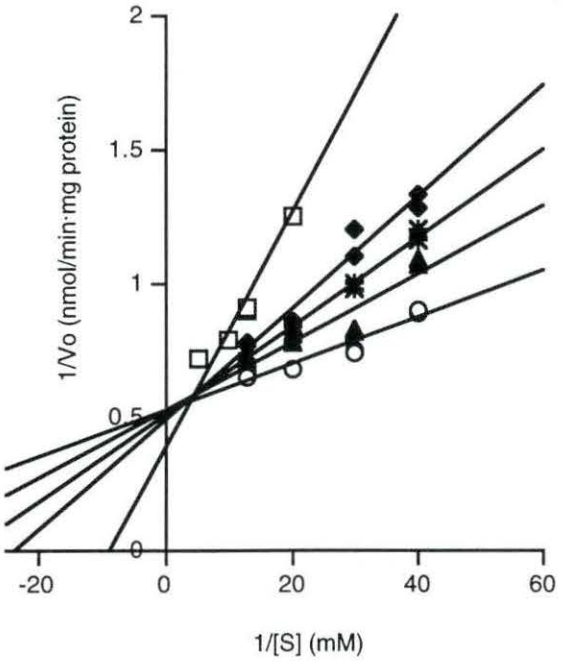

Figure 4. Lineweaver-Burk plots of cholesterol reduction by *E. coprostanoligenes* with increasing levels of coprostan-3-one (❑-no addition; ◆-+ .04 mM coprostan-3-one; ✳-+ .05 mM coprostan-3-one; ▲-+ .06 mM coprostan-3-one; ❍-+ 0.1 mM coprostan-3-one).

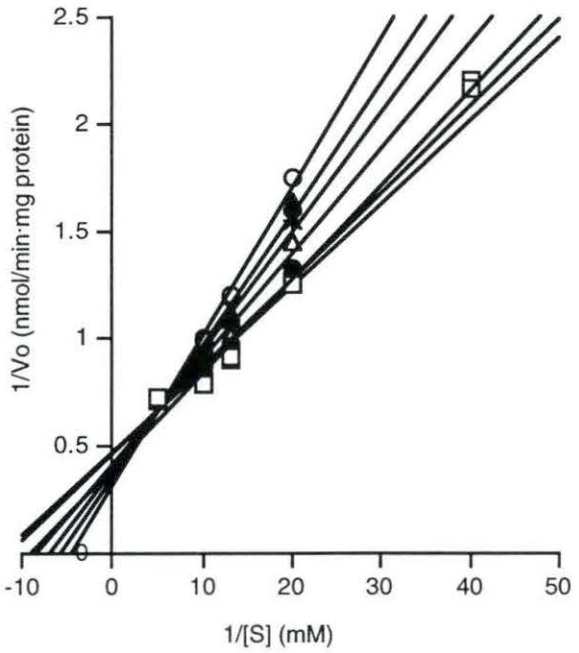

Figure 5. Lineweaver-Burk plots of cholesterol reduction by *E. coprostanoligenes* with increasing levels of progesterone (❑-no addition; ◆-+ .01 mM progesterone; ●-+ .025 mM progesterone; Δ-+ .05 progesterone; ✳-+ .075 mM progesterone; ▲-+ 0.1 mM progesterone; ❍-+ 0.2 mM progesterone).

is the second species of *Eubacterium* shown to be a facultative methylotroph. As a result, further research into other members of the genus *Eubacterium* may provide a variety of commercially applicable bacteria for large-scale acid production.

The lecithin requirement in BC and EM media is still in question. The substitution of soy lecithin containing 97% phosphatidyl choline in place of less pure soy lecithin used in the EM medium resulted in a sharp decline in cell yields and lower levels of cholesterol reductase activity (Lulich and DiSpirito, 1994). Substitution of egg-yolk lecithin for soy lecithin confirmed that the growth requirement is not phosphatidyl choline but some other component in the crude lecithin mixture that is necessary for growth in EM-type media (Lulich and DiSpirito, 1994). Attempts to isolate and optimize essential components were unsuccessful.

Genetic analysis of *E. coprostanoligenes* have been hampered by poor plating efficiencies and small colony size (Freier, 1991). Many solid media combinations were tried in an effort to increase colony size and enhance plating efficiencies. Solid media variations of EM, BC, and MSM were tried with various liquid overlays consisting of either cholesterol, lecithin, or reductants. None of the combinations attempted resulted in any significant improvements in either colony size or overall plating efficiency (Lulich and DiSpirito, 1994). Different agars were also screened including incubation of plates under various atmospheric conditions which yielded no improvements as well (Lulich and DiSpirito, 1994).

Detection of 4-cholesten-3-one and coprostan-3-one in washed-cell assays confirmed the multiple-step reduction mechanism proposed by Bjorkhem *et al.* (1973). These intermediates were only observed in assays showing high cholesterol reduction rates. In cells showing lower cholesterol reduction rates, the

rate of reduction to 5-cholesten-3-one must be equal to or less than the rate of isomerization to 4-cholesten-3-one.

Lineweaver-Burk plots of mixed substrate studies of cholesterol and either 4-cholesten-3-one, coprostan-3-one or progesterone showed a competitive type of inhibition (Figures 2-4). However, the lines did not cross on the y-axes (Figures 2-4). This shift was probably the result of substrate accessibility. Although the use of micellar substrates greatly enhanced activities, there still could be problems with substrate accessibility in this assay system or other factors may have affected the formation of substrate enzyme complexes.

Cholesterol reductase activity is biochemically similar to a number of previously characterized related sterol and steroid degradation enzymes (Table 6). Like many bacterial cholesterol oxidases, the chemical reduction of cholesterol by *E. coprostanoligenes* involves the generation of 4-cholesten-3-one as a stable intermediate. Many of these bacteria will further metabolize 4-cholesten-3-one as a carbon and energy source via other enzymes with H_2O and CO_2 as end products (Smith and Brooks, 1976). However, coprostanol is not further metabolized by *E. coprostanoligenes*, thus raising questions of the metabolic role of cholesterol reductase (Lulich and DiSpirito, 1994). When *E. coprostanoligenes* was cultured in media containing cholesterol, there was no increase in growth yields (Table 2).

Cholesterol reductase activity also shows the broad substrate specificity observed with cholesterol oxidase (Smith and Brooks, 1976). Cholesterol oxidase will oxidize a number of sterols and steroids, but the rates are affected by side-chain length and distribution of hydroxyl groups within the ring system (Smith and Brooks, 1976). Cholesterol reductase activity is also influenced by side chain length and hydroxyl group positioning (Table 5). Rates of sterol reduction by *E.*

Table 6. Properties of sterol/steroid oxidzing and reducing enzymes characterized from various organisms.

Organism	Enzyme	Primary Substrate	V_{max}*	KM_{app}†	Ref.
Eubacterium coprostanoligenes	sterol reductase	cholesterol	2.9	111	This Study
Eubacterium sp. Strain 144	16-DHPR	16-dehydro-progesterone	N/A	150	[53][54]
Eubacterium sp. Strain 144	16α-dehydroxylase	16α-hydroxy-progesterone	N/A	250-520	[53][54]
Eubacterium sp. VPI 12708	7α-hydroxysteroid dehydrogenase	bile acids with 7-α hydroxyl group	601	4-20	[55]
Eubacterium sp. VPI 12708	"	cholic and cheno-deoxycholic acids	674	25	[56]
Streptomyces violascens	cholesterol oxidase	cholesterol,3ß-hydroxysteroids	78	450-670	[57]
Nocardia erythropolis	"	"	18.5	2.9-7	[9][10][18]
Schizophyllum commune	"	"	10.4	330	[58]
Brevibacterium sterolicum	"	"	11.3	2.9	[10][14]
Pseudomonas testosteroni	"	"	6.4	29	[10]

* = nmol/min·mg protein
†= µmol as calculated

coprostanoligenes were also similar to the bile-acid and sterol hydroxylases and reductases characterized in other strains of *Eubacterium* (Table 6).

Although cholesterol oxidase has been more extensively studied, attempts to use cholesterol oxidase in food systems to lower levels of cholesterol have not been successful (Aihara et al., 1988; Smith et al., 1991; Suzuki et al., 1986; Brigidi et al., 1993). One of the major problems with cholesterol oxidase is the generation of 4-cholesten-3-one as a stable by-product. 4-Cholesten-3-one has been shown to cause nuclear aberrations when administered to mice and could pose a concern if present at high levels in food products (Suzuki et al., 1986).

The use of cholesterol reductase has several distinct advantages over previously studied cholesterol degrading systems. One is that cholesterol is exclusively reduced to coprostanol. Coprostanol, a neutral sterol, has been shown to be poorly absorbed by the human body (Bhattacharyya, 1986). Coprostanol, thought to represent some 50% of the total fecal sterols, has not been shown to be toxic (Bhattacharyya, 1986). The use of cholesterol reductase to lower levels of cholesterol in foods serves as a novel, natural and possibly economical alternative to the previously developed methods.

SUMMARY AND DISCUSSION

This series of studies involved the biochemical characterization of a cholesterol-reducing enzyme from the obligate anaerobe *Eubacterium coprostanoligenes*. The work outlined in this thesis supports a previously proposed multiple-step model of the chemical reduction of cholesterol to coprostanol with the generation of several stable intermediates (Figure 1) (Bjorkhem et al., 1973).

The first series of studies involved the optimization of growth and cholesterol reductase activity. The original isolation media, although useful, made important biochemical studies difficult. The optimized growth medium developed not only increased cell yields by 62%, but also resulted in a 68-79% reduction in the amount of extracellular polysaccharides (Table 2). With the optimized medium, there was also an increase in cholesterol reductase activity making the development of working whole-cell and cell-free assays more feasible (Table 1).

The growth of *E. coprostanoligenes* on MSM media provided several advantages over modified EM and BC media. Lecithin is not required for growth, and comparble growth yields and cholesterol reduction rates were obtained. The minimal salts medium could serve as an alternate, more cost-effective medium. The lecithin requirement for EM and BC media is still in question. Obviously, some component of the less pure soy lecithin used is essential for growth of *E. coprostanoligenes* in these media. Attempts to isolate and optimize these essential components were unsuccessful.

With an optimized growth medium, a whole-cell and a cell-free cholesterol reductase activity assay were developed. Whole-cell and cell-free cholesterol

reductase activity assays did not appear to be dependent upon other coupled enzymes such as NADH dehydrogenases, or related enzymes (Tables 3, 4). Cholesterol reductase activity was localized to the membrane fraction and cell-free activities were somewhat lower than those obtained in whole-cell screens (Table 3, 4). The loss of activity could be due to sensitivity of cholesterol reductase to oxygen or the loss of necessary cofactor(s) during fractionation.

The increased levels of cholesterol reductase activity resulted in the detection and elucidation of several intermediates (Table 1). 4-Cholesten-3-one and coprostan-3-one, proposed intermediates in the multiple step mechanism of chemical cholesterol reduction, were detected (Figure 1, Table 1). 5-Cholesten-3-one, the third of the proposed intermediates in this mechanism was not detected during any assays performed. This could be due to the fact that in the proposed reduction model, the isomerization of 5-Cholesten-3-one to 4-Cholesten-3-one could happen quickly enough that detection within the established assay systems was not possible (Bjorkhem et al., 1973).

Cholesterol reductase activity showed high affinity for substrate with $V_{max\ app}$ and $K_{M\ app}$ values of 2.9 nmol cholesterol reduced/min·mg protein and 111μM, respectively (Table 5, Figure 2). These measurements were made possible by the use of cholesterol/lecithin mixed micelles as the principal substrate delivery system. Chemical reduction of cholesterol to coprostanol followed Michealis-Menten enzyme kinetic models when micellar solutions of substrates were used (Figure 2). Use of blended cholesterol/lecithin as a substrate not only resulted in lower whole-cell rates of cholesterol reduction, but the activity obtained did not follow many enzymatic models tested (Figure 2). The improved substrate delivery system has made further characterization of this novel enzyme system possible.

As shown in the cholesterol oxidase system, cholesterol reductase activity also has a broad substrate specificity. Cholesterol, along with 4-cholesten-3-one, and coprostan-3-one, were reduced to coprostanol by *E. coprostanoligenes*. Other substrates reduced by *E.coprostanoligenes* included corticosterone, progesterone, 17-hydroxyprogesterone, and anhydroxyprogesterone (Table 5). No activity was observed with ß-sitosterol or 17-hydroxypregnenalone (Table 5). The lack of activity with ß-sitosterol suggests that side-chain positioning of terminal substituents as well as overall length of the side chain, may affect substrate binding and catalysis, as was seen with cholesterol oxidase (Smith and Brooks, 1976).

Kinetic studies of chemcial cholesterol reduction with various substrates revealed a competitive type of inhibition (Figures 3, 4, 5). These results, coupled with reduction of the intermediates 4-cholesten-3-one and coprostan-3-one to coprostanol, support the multiple-step, chemical reduction of cholesterol to coprostanol. The competitive type of inhibition observed shows that the chemical reduction of cholesterol to coprostanol is most likely a one-enzyme system.

The accumulation of intermediates is probably due to the increased rate of growth of *E. coprostanoligenes* in optimized media. The intermediates likely accumulate until cholesterol, the preferred substrate, is consumed in active reaction mixtures. Once cholesterol is consumed, the conversion of the intermediates to coprostanol occurs. The artificial assay conditions probably alter the equilibrium state of enzyme and intermediates and have a profound effect upon the regulation of this system.

The rates of chemical cholesterol reduction are comparable to a number of steroid/sterol oxidation/reduction systems (Table 7). Use of cholesterol oxidase to reduce levels of cholesterol in food systems has shown little promise. One problem

of cholesterol oxidase is the endproducts that are generated in the oxidation of cholesterol. Hydrogen peroxide, a highly reactive endproduct, has been shown to impart off-flavors in test food systems while the other product, 4-cholesten-3-one, has been implicated in nuclear aberrations in mouse tissue (Smith et al., 1991; Suzuki et al., 1986).

Although 4-cholesten-3-one is produced during the reduction of cholesterol to coprostanol by cholesterol reductase, the intermediate is rapidly converted to coprostanol and is not present as a stable endproduct. Since over 98% of cholesterol within reaction mixtures is converted to coprostanol, questions concerning a metabolic role (if any) of cholesterol reductase have been raised. Coprostanol, the endproduct of cholesterol reduction, comprises some 50% of fecal sterols present in humans and has not demonstrated any toxic effects (Bhattacharyya, 1986).

The use of cholesterol reductase in the reduction of cholesterol in food systems shows great promise. Other, non-biological methods, although effective, have been disappointing due to high costs of equipment and complexity of processes. Other cholesterol-degrading enzymes have been employed with limited results. Cholesterol reductase represents a novel and natural cholesterol-lowering enzyme that is not only efficient, but does not generate any potential toxic end products.

For commercial use of cholesterol reductase, further studies involving isolation and stabilization of component(s) of cholesterol reductase will be necessary. Isolation of purification of protein(s) of cholesterol reductase will further provide insight and a potential commercial use of this relatively unknown system in the reduction of cholesterol.

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