Characterization and application of a novel cholesterol-reducing anaerobe, Eubacterium coprostanoligenes ATCC 51222

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Characterization and application of a novel cholesterol-reducing anaerobe, 

_Eubacterium coprostanoligenes_ ATCC 51222

by

Ling Li

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation is presented in an alternative format. Following the General Introduction, a Literature Review on topics related to the dissertation research is presented. Six papers, including two published ones and four manuscripts, follow the Literature Review. Each paper is complete in itself with an abstract, introduction, materials and methods, results, discussion, and references cited. A General Summary follows the sixth paper and presents in brief the major finding and conclusions of the dissertation research. References cited in the General Introduction and Literature Review are listed following the General Summary.

Background and Significance

Cholesterol, as an essential constituent of biological membranes and an obligatory precursor in the synthesis of bile salts and steroid hormones, plays an important role in mammalian system. The problem is that, however, there is often too much cholesterol in the blood and other organs. For many years, it has been recognized that excess serum cholesterol is a major risk factor for atherosclerosis that results in coronary heart disease (Gotto et al. 1990), which remains a leading cause of human mortality in the United States and other developed countries (Thom et al. 1992; American Heart Association, 1994). Therefore, numerous
cholesterol-lowering agents have been discovered and synthesized to treat hypercholesterolemic individuals (Endo 1992). The side effects of those drugs, however, have caused concerns about their clinical use (Erkelens et al. 1988). Much effort also has been made in the development of chemical, physical, and biological technologies to decrease cholesterol contents of foods because dietary cholesterol intake affects serum cholesterol directly in many people (Fraser 1994).

Biological methods (whole cell or enzymatic) are often the preferred method of food treatment because they are usually less harsh, form fewer by-products, are easier to control, and are considered "natural" by the consumer. Much of the research in decreasing cholesterol content of foods by biological methods has focused on the cholesterol oxidase that has been well characterized (Vrielink et al. 1991). Unfortunately, attempts to use cholesterol-oxidizing bacteria to decrease the cholesterol content of foods has produced few positive results (Aihara et al. 1988; Johnson and Somkuti 1990). A novel method of using cholesterol reductase or cholesterol-reducing bacteria has been proposed to decrease cholesterol content of foods and to lower serum cholesterol concentration in humans (Dehal et al. 1991). The enzyme, cholesterol reductase, catalyzes the reduction of cholesterol to coprostanol. Coprostanol, unlike cholesterol, is absorbed poorly from the human intestine (Bhattacharyya 1986). Therefore, foods treated with cholesterol reductase or cholesterol reductase-producing bacteria should have little or no cholesterol available for absorption when the foods are consumed. Furthermore, introduction of cholesterol reductase or cholesterol reductase-producing bacteria into the small
intestine is hypothesized to decrease absorption of both the endogenous and exogenous cholesterol because of in situ conversion to coprostanol.

Cholesterol-reducing bacteria have been isolated from the feces of rats (Eyssen et al. 1973), humans (Sadzikowsky et al. 1977), and baboons (Brinkley et al. 1982). These previously isolated bacteria, however, had growth characteristics that made common laboratory manipulations difficult. Growth requirements included the need for plasmalogen, which is found in high concentrations in mammalian brain tissue, and the need for complete anaerobiosis. Further, those bacteria did not form colonies on solid plating media. Attempts to detect cholesterol reductase activity in extracts of those bacterial cells were unsuccessful (Björkhem et al. 1973).

Recently, a unique cholesterol-reducing bacterium, designated Eubacterium coprostanoligenes ATCC 51222, that is much easier to grow in the laboratory was isolated (Freier et al. 1994). E. coprostanoligenes is a Gram-positive, anaerobic, nonfermentative bacterium that forms colonies on lecithin-containing solid media. It converts up to 90% of cholesterol to coprostanol in cholesterol-containing media, but it does not require plasmalogen or cholesterol for growth. Thus, E. coprostanoligenes seems more amenable than previously studied cholesterol-reducing bacteria for applications in the food and pharmaceutical industries. The objectives of the present study are as follows: (1) optimization of conditions for growth and coprostanol production of E. coprostanoligenes, (2) development of assays to analyze the cholesterol-reducing activity of the bacterium, (3)
characterization of the new cholesterol-reducing bacterium, and (4) investigation of possibilities to use the bacterium for decreasing plasma cholesterol concentrations in animal models. The results from this study are necessary for the future use of *E. coprostanoligenes* as a probiotic.
Absorption of Cholesterol and Other Related Sterols

Cholesterol absorption has been a subject of great interest because of its close link with plasma cholesterol concentration. It has been found in many cases that the fractional absorption of cholesterol is related positively with plasma cholesterol concentrations and apolipoprotein E phenotype (Kesäniemi et al. 1987; Kesäniemi and Miettinen 1987). The relationship between dietary cholesterol and plasma cholesterol was highlighted by the case of an 88-year-old man who ate 25 eggs per day and maintained a normal plasma cholesterol, which was explained partly by absorbing only 18% of his dietary cholesterol (Kern 1991) whereas usually 34-57% of dietary cholesterol is absorbed from the human intestine (Grundy and Mok 1977). Studies on cholesterol feeding in hyper- and hypo-responding nonhuman primates also have indicated that cholesterol absorption and plasma cholesterol are related positively (Eggen 1976; Parks et al. 1977; St. Clair et al. 1981).

Sterols structurally related to cholesterol are absorbed differently. Some plant sterols inhibit the absorption of cholesterol (Mattson et al. 1977; Heinemann et al. 1991) and have demonstrated hypocholesterolemic properties (Lees et al. 1977). Cholesterol is metabolized by intestinal bacteria into several derivatives, predominantly coprostanol, a saturated analog of cholesterol. The absorbability of coprostanol is of concern because a potential technology can be developed to
decrease cholesterol concentrations in foods and in human blood if coprostanol is not absorbed (Dehal et al. 1991).

Sources of cholesterol

Cholesterol available for absorption by the digestive tract includes cholesterol derived from the diet and from endogenous sources, predominantly bile and sloughed cells from the intestinal wall. A typical Western diet contains about 335 mg cholesterol/d (Beitz and Knight, 1994). Bile contains about 400 mg cholesterol/100 ml (Nakayama 1969). At an average flow of 250 to 1100 ml/d, this adds 1 to 4 g of cholesterol each day to the adult human digestive tract (Whyte et al. 1973). Grundy and Metzger (1972) estimated that biliary outputs of cholesterol range from 800 to 1200 mg/d in normal adults. The amount of cholesterol released from sloughed cells varies greatly, depending on the wear and tear on the mucosa (Sabine 1977). Therefore, the cholesterol present within the intestinal lumen during any one day is typically more than two-thirds from endogenous sources and less than one-third from dietary sources.

Sites of absorption

The main sites for cholesterol absorption are the duodenum and jejunum in humans (Borgström 1960). In rats, after administration of a test dose of radiolabeled cholesterol, the most radioactivity is accumulated in the proximal half of the small intestine (Sylvén and Nordström 1970). However, there is evidence
that the entire length of the small intestine has the capacity to absorb sterol from the lumen (Feldman and Henderson 1969). Cholesterol absorption occurs through the intestinal mucosal cells that cover the surface of the intestinal villi. The intestinal villus displays functional differences between the cells at the villus tip and those in the crypts. Sterol uptake is greatest in mucosal cells near the tip of the villus (Sylvén and Nordström 1970).

**Forms of cholesterol**

Biliary cholesterol is entirely unesterified, but a portion of dietary cholesterol is esterified with fatty acids. Only free cholesterol, however, seems to be absorbed from intestinal lumen. Any cholesterol ester entering the intestine is hydrolyzed rapidly by pancreatic cholesterol esterase (Treadwell and Vahouny 1968). Synthetic cholesterol esters, resistant to hydrolysis by pancreatic cholesterol esterase, are either poorly absorbed or not absorbed at all (Vahouny and Treadwell 1964). Cholic acid or its taurine or glycine conjugate is an absolute requirement for enzymatic activity of rat pancreatic cholesterol esterase (Hyun et al. 1971; Jacobson et al. 1990). Before absorption, cholesterol must be solubilized by micelles containing conjugated bile acids and hydrolytic products of triacylglycerols and lecithin--fatty acids, monoacylglycerols, and lysolecithin (Hofmann and Borgström 1964). Cholesterol is only sparingly soluble in bile salt solutions (Carey and Small 1970). The addition of phospholipid or monoacylglycerol to bile salt solutions markedly increases the solubility of cholesterol.
Uptake of cholesterol

To date, it is not known how cholesterol within the intestinal lumen moves from the lumen into the intestinal mucosal cells. Hofmann and Borgström (1962) suggested that the absorption of lipids, including cholesterol, occurs from micelles. However, both in vivo and in vitro studies indicate that micelles containing cholesterol are not taken up by the intestinal mucosa as intact aggregates (Simmonds et al. 1967; Hofmann and Yeoh 1971). Westergaard and Dietschy (1976) proposed that micelles promote cholesterol absorption by facilitating transport across the unstirred layer of water adjacent to the surface of the luminal cell. This transport occurs by simple diffusion. Movement of cholesterol through the unstirred layer, rather than penetration of the microvillus membrane, seems to be the rate-limiting step for its absorption. The micelle as a whole does not penetrate the cell membrane. Passage of cholesterol through the structural lipid of the membrane occurs by monomolecular passive diffusion. Several investigators, however, have looked for a protein mediator of cholesterol absorption. The discovery of such a protein would offer the unique opportunity to therapeutically interfere with cholesterol absorption by inhibition of the protein involved. An intrinsic membrane protein may indeed be involved in the absorption of cholesterol from mixed micelles by brush-border membrane vesicles (Thurnhofer et al. 1991). Further purification and characterization of the responsible protein(s) will confirm this new finding.
Events inside mucosal cells

Once inside the mucosal cells, the absorbed cholesterol is mixed with that synthesized within the intestinal cells. Even though cholesterol is absorbed in the unesterified form, cholesterol secreted into intestinal lymph following a cholesterol-rich meal is about 70-80% esterified (Treadwell and Vahouny 1968). It has been suggested that the cholesterol esterifying activity of the mucosa may be an important regulator of cholesterol absorption, because reesterification of absorbed free cholesterol within the mucosal cell would enhance the diffusion gradient for free cholesterol into the cell. Two enzymes are responsible for the esterification of cholesterol within intestinal mucosal cells: cholesterol esterase (CE, EC 3.1.1.13) and acyl coenzyme A:cholesterol acyltransferase (ACAT, EC 2.3.1.26).

Cholesterol esterase activity requires millimolar concentrations of conjugated bile salts (Swell et al. 1950). The role of CE in cholesterol absorption has not been consistent. Murthy et al. (1961) indicated that a CE-like activity increased in rat intestinal mucosa with cholesterol feeding. Other investigators also showed that pancreatic juice or functioning CE activity within the mucosa was required for normal cholesterol absorption (Borja et al. 1964; Hyun et al. 1969). In contrast, some other studies showed that CE status has no effect on the proportion of exogenous cholesterol recovered as cholesteryl ester in lymph (Watt and Simmonds 1981). By using Caco-2 cells, Huang and Hui (1990) found that there was no effect of CE on the association of radiolabeled cholesterol with these cells.

Acyl CoA:cholesterol acyltransferase is another enzyme that has been
implicated in intestinal mucosal cell esterification reactions. Unlike CE, ACAT is inhibited by bile salts (Norum et al. 1979) and is dependent on the CoA activation of fatty acids prior to esterification (Haugen and Norum 1976). Intestinal ACAT activity increases with cholesterol feeding and decreases with cholestyramine administration (Field and Salome 1982). Specific ACAT inhibitors usually decrease cholesterol absorption (Krause et al. 1992). The effectiveness of ACAT inhibitors in decreasing intestinal cholesterol absorption suggests a primary role for ACAT in the regulation of cholesterol absorption; the role of CE seems less established. Recently, Chang et al. (1993) successfully cloned and expressed human ACAT cDNA in mutant Chinese hamster ovary cells, which will allow more direct investigation about the role of ACAT in cholesterol absorption and other aspects of cholesterol metabolism.

Transfer of cholesterol to lymph

During cholesterol absorption, there is not a large accumulation of esterified cholesterol in the mucosal cells (Murthy et al. 1963), which indicates rapid disposition of the cholesterol esters as they are synthesized. This transfer of cholesterol esters and free cholesterol from mucosal cells into lymph is accompanied by the simultaneous transfer of protein, triacylglycerols, and phospholipids as stabilized fat particles, chylomicrons and very-low-density lipoproteins (VLDL) (Treadwell and Vahouny 1968).
Differential cholesterol absorption

It is assumed generally that the digestive machinery does not distinguish between cholesterol derived from these three sources—diet, bile, and desquamated cells. There has been evidence, however, showing the differential absorption of cholesterol from dietary and biliary sources. Biliary cholesterol actually may be absorbed with greater efficiency than is dietary cholesterol.

Intestinal perfusion studies have shown that efficiency of absorption of endogenous cholesterol is frequently in the range of 60-80% (Grundy and Mok 1977). Absorption of dietary cholesterol is reported usually to be lower (Grundy and Ahrens 1969). Results from other studies using rats and chickens also support the concept of differential absorption of endogenous and exogenous sources of cholesterol (Dulery and Reisser 1982; Sklan et al. 1977). Hofmann and Borgström (1962 and 1964) explained that the difference might be caused by the physical states of endogenous and exogenous cholesterol. Biliary cholesterol enters the intestine in micellar solution. Ingested cholesterol mostly is dissolved in dietary triacylglycerols. To be absorbed, dietary cholesterol must be transformed into a micellar state. This extra step may not be 100% efficient; if it is not, absorption of exogenous cholesterol would be less than that of endogenous cholesterol.

The studies of Samuel and McNamara (1983) also strongly suggest that micellarized sources of cholesterol are absorbed preferentially. They found that when exogenous cholesterol was administered in liquid formula, its absorption was significantly lower than that of endogenous cholesterol. When exogenous
cholesterol was dissolved and administered in monooleoylglycerol, however, its absorption was higher than that of endogenous cholesterol. These results indicate that the initial rate of cholesterol absorption is dependent on the physicochemical state of the cholesterol source. Data from studies of Samuel and McNamara (1983) also suggested that exogenous and endogenous cholesterol approach equilibrium as they travel down the absorptive gut.

In a recent review on cholesterol absorption, Wilson and Rudel (1994) stated that even though the efficiency of absorption of micellar cholesterol in bile is potentially greater, cholesterol from endogenous and dietary sources usually is mixed completely in the intestine. As a result, they concluded that the percentage absorption of cholesterol from both sources is equal. Therefore, maneuvers designed to decrease cholesterol absorption as a mechanism to lower plasma cholesterol concentrations must consider both endogenous and exogenous cholesterol as the target.

**Specificity of sterol absorption**

Sterol absorption in the intestine seems very selective. Structurally related sterols that differ from cholesterol only in the structure of the side chain at carbon-24 or in the degree of saturation of the sterol nucleus are absorbed less efficiently than cholesterol. Absorption of plant sterols, such as β-sitosterol, is normally poor and equal to only a few milligrams daily (Salen et al. 1970). Sitosterol differs from cholesterol only by the addition of an ethyl group on the side chain. Campesterol,
which has an additional methyl group on the side-chain, also is absorbed poorly. Cholestanol (5α-cholestan-3β-ol), a saturated analog of cholesterol, is absorbed only half as efficiently as cholesterol (Treadwell and Vahouny 1968). Coprostanol (5β-cholestan-3β-ol), another saturated analog of cholesterol, is a nonabsorbable sterol.

The mechanism for this differential absorption is not fully understood yet. The most likely differences lie in rates of esterification of the noncholesterol sterols within enterocytes before incorporation into chylomicrons. Plant sterols can act as substrates for esterification *in vitro*, but all are esterified to a lesser extent than is cholesterol (Swell et al. 1954; Bhattacharyya 1979; Field and Mathur, 1983).

Coprostanol and cholestanol differ only in the A/B ring configuration. Coprostanol is the A/B ring isomer of cholesterol, whereas cholestanol has an A/B ring configuration more similar to that of cholesterol. Bhattacharyya (1986) studied the differences in uptake and esterification of coprostanol and cholestanol by rat small intestine *in vivo* and *in vitro* with an attempt to explain the different absorbabilities of these two saturated cholesterol analogs. The results showed that coprostanol is nonabsorbable because its uptake by the intestinal mucosa and its esterification within the mucosal cells are extremely limited. In contrast, the uptake and esterification of cholestanol are not limited. Several possible reasons have been proposed for the lower uptake of coprostanol than that of cholestanol by the intestinal mucosa, although the two sterols differ so little from each other in structure. First, there may be differences in the solubility of the two sterols in
mixed micelles that could limit the availability of the sterol for uptake by the mucosal cells. No difference, however, was observed in concentrations of the two sterols per volume of mixed micellar layer isolated from the intestinal contents of sterol-fed animals (Bhattacharyya 1986). Second, the two sterols could form mixed micelles of different structures that could limit the partitioning of the sterols out of the mixed micelles into monomeric form, which, in turn, could limit the diffusion of the sterol and thus limit the uptake of the sterol by the mucosal cells. *In vitro* measurement of the rate of diffusion of the two sterols from mixed micellar solution individually across an artificial interface of phosphate buffer showed that the rate of diffusion of coprostanol was significantly lower than that of cholestanol (Bhattacharyya 1986). Third, the structural difference because of the cis- rather than trans-fusion of the ring A/B junction in coprostanol molecule leading to a difference in the 3-hydroxyl group from the axial to the equatorial position might have affected the incorporation into the phospholipid bilayer of the mucosal cell membrane, thereby affecting uptake. Therefore, it was concluded that not only the carbon numbers of sterols but the whole structure of sterol molecules seems to be an important determinant for its uptake and esterification, and probably absorption, in the small intestine (Bhattacharyya 1986).

Nonabsorbability of coprostanol has led to development of a novel technology to decrease cholesterol contents of foods and in the blood of hypercholesterolemic individuals (Dehal et al. 1991).
Microbial Reduction of Cholesterol to Coprostanol

Historical perspective

Search for cholesterol-reducing microorganisms dates back to the nineteenth century when it was recognized that cholesterol of exogenous or endogenous origin was eliminated with the feces mainly in the form of its saturated derivative, coprostanol (Bondzynski and Humnicki 1896-97). An important statement was made by Schoenheimer in the 11 December 1931 issue of SCIENCE that even the slightest structural changes in sterols influence their absorbability in the digestive tract (Schoenheimer 1931). He showed that cholesterol was absorbed easily, but coprostanol, known as koprosterol or coprosterol then, was absorbed much more difficultly. Soon after the discovery of this compound, it was suggested that coprostanol is formed by bacterial hydrogenation (reduction) of cholesterol in the intestine because coprostanol was not found in the tissue but always in large amount in the feces. However, early investigators were unable to reproduce cholesterol reduction to coprostanol by bacteria in vitro (Bondzynski and Humnicki 1896-97; Schoenheimer et al. 1930; Beumer and Bischoff 1930). On the other hand, it was demonstrated that putrefaction of feces increased the degree of saturation of fecal sterols, which provided evidence for coprostanol production by bacterial action (Dam 1934).

Later, a number of investigators succeeded in converting cholesterol into coprostanol with human feces in vitro (Rosenfeld et al. 1954; Snog-Kjaer et al. 1956; Coleman and Baumann 1957). It was also found that coprostanol was absent
in the feces of germ-free animals (Danielson and Gustafsson 1959; Evrard et al. 1964; Gustafsson et al. 1966; Kellogg and Wostmann 1969). In conventional animals, coprostanol formation was abolished by the administration of antibacterial agents (Rosenheim and Webster 1943; Wainfan et al. 1952; Coleman and Baumann 1957; Kellogg et al. 1969.). Certain anaerobic bacteria from human feces were found responsible for cholesterol reduction (Snog-Kjaer et al. 1956). Schoenheimer hoped in 1931 that a cholesterol-reducing bacterium would be cultivated in the near future. It was not until 42 years later, however, when a pure culture of a cholesterol-reducing microorganism, *Eubacterium* ATCC 21408, was isolated (Eyssen et al. 1973). Since then, extensive research has been conducted to isolate more cholesterol-reducing microorganisms, to study their physiological and biochemical characteristics, and to investigated their potential applications in food and pharmaceutical industries.

**Cholesterol-reducing bacteria**

To date, a total of 13 cholesterol-reducing bacteria have been isolated and characterized (Eyssen et al. 1973; Sadzikowski et al. 1977; Mott and Brinkley. 1979; Brinkley et al. 1982; Freier et al. 1994). All of these bacteria have been classified as strains of *Eubacterium* species.

The first cholesterol-reducing bacterium, *Eubacterium* ATCC 21408, was isolated from rat cecal contents with use of a brain-thioglycollate medium under anaerobic conditions (Eyssen et al. 1973). *E. ATCC 21408* was described as a
small, strictly anaerobic, and gram positive in very young cultures but became gram negative in cultures more than 3 d old. In young cultures, many diploforms were observed.

*E. ATCC 21408* failed to grow on many common culture media such as fluid thioglycollate medium, brain-heart infusion broth, trypticase soy broth, cooked meat medium, and the like. Growth on these media was not improved by addition of blood, serum, or yeast extract. Growth occurred, however, when those media were supplemented with 1-2 mg/ml of cholesterol in lecithin-containing media. Cholesterol could be replaced by $\Delta^5$-3β-hydroxy plant sterols such as β-sitosterol, campesterol, and stigmasterol or certain steroid hormones such as testosterone. These media, however, supported growth only for a limited number of transfers. After approximately 10 transfers, these media no longer supported growth of this bacterium. Long-term propagation could only be carried out on media containing brain powder. It was concluded that the bacterium had an absolute requirement for cholesterol or a related unsaturated sterol and that growth stimulating activity of various $\Delta^5$-sterols was limited to those compounds that could be hydrogenated by *E. ATCC 21408* (Eyssen et al. 1973).

Growth of *E. ATCC 21408* was very sparse on media containing 0.125 or 0.25 mg cholesterol/ml, and all strains were lost on further subculture. With media containing cholesterol in amounts between 0.25-2.0 mg/ml, growth was directly proportional to the amount of sterol present. At least 1 mg sterol/ml was required for maintenance of the cultures, and maximal growth was not obtained unless the
medium contained 1.5-2 mg cholesterol/ml. The large amount of steroid required for maximal growth indicated that cholesterol was not acting as a true growth factor. It was assumed logically that the 5,6-double bond of cholesterol, plant sterols, and steroid hormones was acting as a hydrogen acceptor in the metabolism of the microorganism (Eyssen et al. 1973).

*E.* ATCC 21408 grew optimally at 37 to 40°C and did not produce spores. Tests for production of indole, acetyl-methyl-carbinol, H$_2$S, or catalase were negative, and nitrate was not reduced to nitrite. The microorganism was not motile, and it produced small amounts of acid and gas from glucose, lactose, and melibiose. No acid or gas was produced after growth in media supplemented with maltose, D-fructose, raffinose, mannose, trehalose, melezitose, xylose, or rhamnose. The bacterium was classified into the genus *Eubacterium* because the general characteristics of this microorganism were compatible with those of the genus. The special growth requirements and the ability to produce coprostanol from cholesterol, however, differentiated this strain from any species listed in the genus *Eubacterium* in Bergy's manual at the time.

In addition to cholesterol, *E.* ATCC 21408 reduced the 5,6-double bond of campesterol, β-sitosterol, stigmasterol, 5-androsten-3β-ol-17-one, and 5-pregn-3,20β-diol, exclusively yielding the corresponding 5β-saturated derivatives. Similarly, the 4,5-double bond of allocholesterol was reduced to coprostanol. Neither the 7,8-double bond in 7-dehydrocholesterol nor in lathosterol nor the 22,23-double bond in the side chain of stigmasterol were reduced. In the absence
of a 3-hydroxy function (e.g. in cholest-5-ene), when the 3-hydroxyl function was in 3α-position (e.g. in epicholesterol) or was substituted (e.g. in 3β-chloro-cholest-5-ene and in cholesteryl esters), no saturation of the double bond was observed. The bacterium also reduced 4-cholesten-3-one and coprostanone to coprostanol (Eyssen et al. 1973).

Biohydrogenating activity of *E. ATCC 21408* in vivo was investigated to determine its effect on serum cholesterol concentration and fecal sterol output (Eyssen and Parmentier 1974). It had been shown that conventional rats excreted 50 to 100% more neutral sterols through the feces than did germ-free rats (Evrard et al., 1964; Kellogg and Wostmann 1969) and that cholesterol was more efficiently absorbed from the intestine of germ-free rats (Wostmann 1973). These findings led to an assumption that intestinal bacteria might interfere with the absorption of cholesterol and promote the fecal output of neutral sterols. To investigate whether transformation of cholesterol into the poorly absorbed coprostanol might explain these phenomena, gnotobiotic rats were associated with *E. ATCC 21408*. Because of its strictly anaerobic character, *E. ATCC 21408* could not be established as a monoassociate in the intestine of germ-free rats where the prevailing oxidation-reduction potential was slightly positive. To obtain a more negative oxidation-reduction potential, the rats were exposed first to a *Clostridium* species. Gnotobiotic rats associated with *E. ATCC 21408* plus *Clostridium* Cl-8 converted cholesterol into coprostanol with an efficiency of approximately 70%. However, the extensive conversion of cholesterol into coprostanol did not result in an
increase in fecal C-27 sterol output.

In another experiment, conventional and germ-free rats were fed a 1% cholesterol diet. After 2 wk of cholesterol feeding, conventional rats had 11% and 28% less cholesterol in serum and liver, respectively, than did germ-free rats. The cholesterol concentrations in serum and liver of rats associated with C. Cl-8 plus E. ATCC 21408, however, were not lower than those of germ-free rats. It was concluded that conversion of cholesterol into poorly absorbed coprostanol was not the cause of the higher output of fecal C-27 sterols or the less efficient absorption of cholesterol in conventional rats (Eyssen and Parmentier 1974). It was explained by the observation that coprostanol formation predominantly occurred in the cecum and the large intestine, sites from which no cholesterol absorption takes place (Kellogg 1973). Other activities of the microflora were thought to be responsible for these phenomena. The microflora might contribute to fecal excretion of sterols by promoting sloughing of mucosal cells (Kellogg and Wostmann, 1969). On the other hand, the microflora also could interfere with absorption and excretion of cholesterol via an action on the bile acids. It was shown that the bile acid pools in the small intestine were three times larger in germ-free rats than in conventional rats and this might well explain the improved absorption of cholesterol in germ-free animals (Kellogg and Wostman 1969).

The role of the cecum in maintaining the \( \Delta^5 \)-steroid-reducing activity was investigated because E. ATCC 21408 was found exclusively in the cecum and the large intestine on microscopic examination of the intestinal contents of gnotobiotic
rats associated with *C. Cl*-8 plus *E. ATCC* 21408 (Eyssen and Parmentier 1974). In the study, 10 conventional rats were cecectomized. An equal number of animals were kept as unoperated controls, and a further 10 rats were sham-operated. Formation of coprostanol and β-reduction of 5,6-double bonds in β-sitosterol and campesterol were abolished completely within 2 d after cecectomy. Similar results were obtained after cecectomy of gnotobiotic rats associated with *E. ATCC* 21408 and *C. Cl*-8 (Eyssen et al. 1972). It was concluded that *E. ATCC* 21408 was unable to grow in the intestine of cecectomized rats. One possible explanation was that in cecectomized animals normal peristalsis of the colon resulted in a rapid elimination of slowly growing indigenous microorganisms such as *E. ATCC* 21408. The *Eubacterium* might require a site where stasis of intestinal contents occurs. This hypothesis was compatible with the observation that coprostanol formation could be restored in cecectomized rats by surgically producing a self-filling blind loop on the small intestine. It was pointed out that the observation could also explain why, in spite of the absence of a cecal sac similar to that of rodents, coprostanol formation takes place in the human colon where the intestinal contents are retained for 18 to 24 h. Crowther et al. (1973), however, determined that in humans the intestinal transit time was not crucial in maintaining cholesterol-reducing bacteria. When healthy volunteers were fed a fat-free liquid diet, the percentage of neutral sterols excreted in the feces as coprostanol decreased. Fecal transit time in these volunteers was actually longer than that in controls.

Wilkins and Hackman (1974) categorized people into two distinct patterns in
terms of cholesterol conversion to coprostanol: low conversion and high conversion. In a group of 31 volunteers, fecal samples from 8 people contained less than 30% of total sterols as coprostanol, whereas the fecal samples from the other 23 volunteers contained greater than 60% of total sterols as coprostanol. Because all the volunteers were consuming similar diets and the patterns of sterol excretion were stable with time, it was thought that the differences were related to the bacterial flora of an individual rather than to difference in the colonic environment (Wilkins and Hackman 1974).

Several investigators observed that feeding of diets containing large amounts of incompletely absorbed carbohydrates resulted in suppression of coprostanol formation (Wells and Cooper 1958; Kellogg and Wostmann 1966; Subbiah et al. 1974). The effect of lactose on Δ⁵-steroid-reducing activity of E. ATCC 21408 was investigated (Eyssen et al. 1974). A group of 10 germ-free rats was associated with C. Cl-8 plus E. ATCC 21408. A second group was monoassociated with C. Cl-8, whereas a third group was kept germ-free. After 3 wk on the starch diet, five rats out of each group were given a diet containing 40% of lactose in the place of cornstarch. In rats associated with E. ATCC 21408, biohydrogenation of cholesterol was more than 80% inhibited 5 d after starting the lactose diet. Biohydrogenation of β-sitosterol followed a similar curve. At the same time, the number of E. ATCC 21408 decreased significantly in lactose-fed rats. The pH of fecal contents decreased from a value of 6.8 in starch-fed rats to as low as pH 6.0 in lactose-fed rats. The oxidation-reduction potential in the intestine was not
affected by the type of carbohydrate. When the rats were fed the lactose-containing diet for more than 3 wk, however, production of coprostanol again increased to become stabilized after 5 to 6 wk at values 75-80% of those observed in starch-fed animals; the pH and the numbers of E. ATCC 21408 also increased to the values of those in starch-fed rats. Similar results were observed in starch- or lactose-fed conventional rats. The results suggested that the acid pH resulting from fermentation of unabsorbed lactose by the microflora was responsible for inhibition of E. ATCC 21408.

Addition of glucose or lactose to pure cultures of E. ATCC 21408 in vitro did not inhibit the cholesterol-reducing activity. In these cultures, the pH dropped slowly from pH 7.3 before incubation to pH 6.5 after a 72-h incubation. When grown in a basal brain-thioglycollate medium without added carbohydrates, production of coprostanol by E. ATCC 21408 was not inhibited by cocultivation with lactose-fermenting bacteria such as C. Cl-8, Escherichia coli, or Streptococcus faecalis. When glucose or lactose were added to these mixed cultures, however, the pH dropped to values below pH 6 within 24 h. Under these conditions, E. ATCC 21408 failed to grow adequately and coprostanol was not produced. The inhibitory effect of the carbohydrate in mixed cultures, however, could be overcome by maintaining the pH of the culture medium above 6.5 throughout the incubation period, e.g., by addition of appropriate amounts of KOH every 6 h or by addition of CaCO₃ to the medium before incubation. It was concluded that the adverse effect of lactose diets on coprostanol formation in gnotobiotic and conventional rats was
an indirect effect mediated by the acids resulting from fermentation of unabsorbed lactose (Eyssen et al. 1974).

Isolation of a cholesterol-reducing bacterium from human feces was first reported by Sadzikowski et al. (1977). The microorganism was isolated and cultivated in an anaerobic medium containing homogenized pork brains (naturally high in cholesterol). The human isolate was similar to E. ATCC 21408 from the cecum of rat (Eyssen et al. 1973). Both of them are small, gram-positive, anaerobic diplobacilli that require cholesterol for growth. Both organisms could use cholesteryl palmitate for limited growth, and the double bond of the cholesterol in the ester form was reduced, even though Eyssen et al. (1973) reported that E. ATCC 21408 did not reduce the double bond of cholesterol when cholesterol was present as an ester. The human isolate was not able to grow on a solid medium. The inability to form colonies necessitated laborious and unconventional isolation procedures—serial dilution procedures. It was noted that fecal samples with low initial coprostanol concentrations produced low amounts of coprostanol in vitro, and that the converse was true for samples with high concentrations.

In an attempt to explain differences in coprostanol excretion between high and low converters observed by Wilkins and Hackman (1974), the human isolate and E. ATCC 21408 were incubated with several strains of bacteria to check for inhibition because cholesterol-reducing organisms from low converters seemed to be inhibited by certain members of the fecal flora. E. ATCC 21408 was inhibited significantly by the mixed flora isolated from a low converter. One organism,
identified as *Fusobacterium russi*, caused over 40% inhibition of coprostanol formation by *E. ATCC 21408*. The human isolate was inhibited only slightly by *F. russi*. It was concluded that differences in the amount of coprostanol in the feces of individuals may be because of differing susceptibilities of the cholesterol-reducing organisms to certain antagonistic bacteria of the fecal flora. It has been indicated that the cholesterol-reducing organisms seem uniquely adapted to a specialized niche in the environment of the colon. Most other intestinal bacteria are not capable of attacking the abundant cholesterol of feces; the cholesterol-reducing organisms, however, seems to have evolved a means of using cholesterol as a terminal electron acceptor (Eyssen et al. 1973). This cholesterol reduction could be advantageous because it might allow adenosine 5'-triphosphate (ATP) to be generated via a compound that is not used by the rest of the fecal flora (Sadzikowski et al. 1977). Besides the human isolate, Sadzikowski et al. (1977) also isolated a strain from rat cecal contents.

In 1979, Mott and Brinkley isolated a cholesterol-reducing bacterium from baboon feces by techniques similar to those reported by previous investigators (Mott and Brinkley, 1979). The isolate closely resembled *E. ATCC 21408* (Eyssen et al. 1973) and was designated *Eubacterium* 403. To determine the growth factors for cholesterol-reducing *Eubacterium*, they extracted and isolated the lipid fractions of calf brain. The brain lipid fractions and pure lipid standards were tested in lecithin-cholesterol medium for their ability to support growth of *E. 403*. Lecithin-cholesterol-medium is a brain-free medium that alone did not support growth of
the cholesterol-reducing bacterium. The results showed that both plasmenylethanolamine and 2-lyso-plasmenylethanolamine supported growth of *E. 403* in lecithin-cholesterol-medium. These plasmalogens or plasmenyl lipids are a class of glycerophospholipids characterized by an alkenyl ether linkage in the 1 position. No growth occurred in lecithin-cholesterol-medium supplemented with acid-hydrolyzed Folch extract (Folch et al. 1957), as indicated by lack of coprostanol formation and absence of cells by Gram stain. Addition of acid-hydrolyzed Folch extract to lecithin-cholesterol-medium-plus-plasmenylethanolamine, however, enhanced coprostanol production, indicating that other brain lipids stimulated this activity. Acetic acid hydrolysis destroyed the growth-supporting activity of plasmenylethanolamine. Plasmenylethanolamine obtained from a commercial source also supported growth in lecithin-cholesterol-medium; no other lipid standard, however, tested was active. Therefore, it was concluded that an intact alkenyl ether linkage is a growth requirement of the cholesterol-reducing *Eubacterium in vitro*. *E. 403* actually catabolized the alkenyl ether linkage of plasmenylethanolamine during coprostanol formation (Mott and Brinkley, 1979).

Later, Mott et al. (1980) further characterized cholesterol-reducing bacteria, including *E. ATCC 21408* and *E. 403*, by conducting conventional biochemical tests and by testing different sterols and glycerolipids as potential growth factors. In media containing cholesterol and plasmenylethanolamine, the tests for nitrate reduction, indole production, and gelatin and starch hydrolyses were negative, and
no acid was produced from any of 22 different carbohydrates. The cholesterol-reducing bacteria had β-glycosidase activity because esculin was hydrolyzed to esculentin. It also was found that four other alkenyl ether residue-containing lipids also supported growth of \( E. \ 403 \) in lecithin-cholesterol-medium, in addition to plasmenylethanolamine and 2-lyso-plasmenylethanolamine. Lysoplasmanylcholine, a 1-alkyl glycerophospholipid that does not contain an alkenyl residue, did not support growth. Of six steroids tested, cholesterol, 4-cholesten-3-one, 4-cholesten-3β-ol (allocholesterol), and 5-androsten-3β-ol-17-one supported growth of \( E. \ 403 \). All four steroids were reduced to the 3β-ol, 5β-H products. The \( \Delta^5 \) steroids 5-cholesten-3α-ol (epicholesterol) and 22,23-bisnor-5-cholenic acid-3β-ol were not reduced and did not support growth of the bacterium. At a high concentration (2.0 mg/ml) of 4-cholesten-3-one in the medium, coprostanone was the major product after incubation, but at a low concentration (0.2 mg/ml), 4-cholesteren-3-one was converted principally to coprostanol. The authors noted that members of the genus \( \text{Eubacterium} \) generally produced energy for growth by fermenting sugars to short-chain acids. Like \( \text{Eubacterium lentum} \), however, the cholesterol-reducing organisms did not produce acids from carbohydrate-containing media. Unlike \( E. \ lentum \), cholesterol-reducing strains of \( \text{Eubacterium} \) did not derive energy from the arginine dihydrolase pathway (Mott et al. 1980). Therefore, it was concluded that the cholesterol-reducing bacteria resembled \( E. \ lentum \), but, because they lacked the arginine dihydrolase pathway and had a unique requirement for certain sterols and alkenyl ether lipids, they could not be classified in any existing species within the
Isolation and characterization of the cholesterol-reducing strains of Eubacterium has been difficult because these organisms fail to grow on solid media. Brinkley et al. (1980) developed a cholesterol-brain agar medium that sustained the growth and colony formation of these bacteria under anaerobic conditions. Cholesterol was thought to be a limiting factor because it would not readily diffuse in agar. Therefore, 5% cholesterol was added to the lecithin-cholesterol medium containing plasm-enylethanolamine (0.5 mg/ml of medium) and 1% agarose. Colonies of E. ATCC 21408 developed within 6 d on this medium when incubated under anaerobic conditions. Then, cholesterol concentrations of 1 to 10% in a brain agar medium was tested and 5% was found to be the optimal concentration. Deletion of amino acids or yeast extract from cholesterol-brain agar resulted in poorer growth, suggesting that free amino acids and vitamins are required for optimal growth of the cholesterol-reducing eubacteria. Trypan blue was added to improve the contrast between the small white colonies and the medium. This dye was not toxic and was not readily decolorized (reduced) by an anaerobic environment or by the cholesterol-reducing eubacteria. Many other anaerobes reduced the dye to a colorless form that aided in differentiation of the cholesterol-reducing eubacteria from contaminants.

Two colony types on cholesterol-brain agar were observed with both E. ATCC 21408 and E. 403. The colonies were composed of small, gram-positive bacilli and fibers; the fibers were seen protruding from both colony types when
observed microscopically. The unusual colony morphology and the presence of protruding fibers helped to differentiate these organisms from other bacteria. *E.
ATCC 21408 and 403 were subcultured repeatedly on cholesterol-brain agar by picking isolated colonies and streaking for isolation. No loss of viability was observed. Isolated colonies were transferred into standard brain medium and were confirmed as cholesterol-reducing organisms by the presence of coprostanol as a major product of growth (Brinkley et al. 1980).

Brinkley et al. (1982) isolated nine new strains of cholesterol-reducing bacteria from baboons by plating dilutions of feces and intestinal contents directly to cholesterol-brain agar. All strains had similar colony and cell morphology and hydrolyzed the β-glucosides esculin and amygdalin. Unlike the previously reported cholesterol-reducing bacteria, all of the nine new strains did not require cholesterol and an alkenyl ether lipid for growth. All strains reduced cholesterol to coprostanol in lecithin-cholesterol medium containing plasmenylethanolamine. Only two strains, 103 and 104, reduced cholesterol in the absence of plasmenylethanolamine. These two strain also produced succinate as an end product. Carbohydrate fermentation was variable; some strains produced weak acids (pH 5.5 to 6.0) from only a few different carbohydrates, whereas other strains produced strong acid reactions (pH \(\leq 5.5\)) from a wide variety of carbohydrates. All nine strains metabolized pyruvate and produced acetate and acetoin (3-hydroxy-2-butanone). Succinate production by strains 103 and 104 was increased in the presence of pyruvate. Carbon dioxide production also was enhanced by pyruvate.
Hydrogen was not produced. All isolates were negative for indole production, nitrate reduction, urease production, and gelatin and starch hydrolysis. No products of the arginine dihydrolase pathway were detected, and lactate and threonine were not fermented.

It was stated that cholesterol-reducing organisms were a much more diverse group of bacteria than previously described (Brinkley et al. 1982). The diversity seemed to be related to the metabolism of substrates for energy production. The most probable mechanism for ATP production was thought to be the dehydrogenation of pyruvate to acetyl-CoA. The role of cholesterol in growth of cholesterol-reducing bacteria again was questioned. Eyssen et al. (1973) suggested that cholesterol is the terminal electron acceptor, and all of the isolates previously reported required cholesterol for growth (Eyssen et al. 1973; Sadzikowski et al. 1977; Mott and Brinkley, 1979). Because all of the nine isolates grew in the absence of cholesterol, an alternate electron acceptor must have been used by these strains when cholesterol was not available. The role of alkenyl ether lipids in cholesterol reduction also was questioned. An alkenyl ether lipid (e.g., plasmenylethanolamine) was required for the growth of E. ATCC 21408 and 403. These nine strains grew without cholesterol and plasmenylethanolamine, and strain 103 and 104 reduced cholesterol in the absence of plasmenylethanolamine. It was indicated that most of their isolates belonged to the genus Eubacterium (Brinkley et al. 1982). Strains 103 and 104 did not fit the description for the genus Eubacterium because they produced significant amounts of succinate, a characteristic of
Actinomyces. Because these strains did not produce lactate or show any branching or other irregular cell morphology, however, they would be atypical of the genus Actinomyces. Because strains 103 and 104 shared so many characteristics with other isolates, it was suggested that all of these bacteria should remain in a single taxon (Brinkley et al. 1982).

Recently, a novel cholesterol-reducing bacterium was isolated from a swine sewage lagoon (Freier et al. 1994). The new isolate has been designated as Eubacterium coprostanoligenes and deposited in the American Type Culture Collection as strain ATCC 51222. E. coprostanoligenes shares many properties with previously isolated cholesterol-reducing bacteria. The new isolate is a small, anaerobic, gram-positive coccobacillus. The bacterial cells are 0.5 to 0.7 μm in diameter and 0.7 to 1.0 μm in length and occur singly and in pairs. The cells are nonmotile and non-spore forming. Surface colonies on anaerobic lecithin agar plates are small, white, and circular with a powdery texture. Growth and coprostanol production are optimal at pH 7.0 to 7.5 and at 35°C to 39°C. The bacterium was described as an aerotolerant anaerobic chemooorganotroph. It reduces cholesterol to coprostanol but does not require cholesterol or plasmalogen for growth. Phosphatidylcholine is metabolized and is required for growth. Nitrate is not reduced, and indole is not produced. The bacterium does not hydrolyze starch and gelatin, but hydrolyzes esculin and indoxylacetate and produces β-glucosidase. It ferments amygdalin, lactose, and salicin and produces much acid, but it only weakly ferments L-arabinose, cellobiose, fructose, glucose, mannose, and
melibiose. The bacterial culture produces moderate amounts of H₂ and small amounts of CO₂. It produces acetic, formic, and succinic acids, but not alcohols.

The genomic DNA of *E. coprostanoligenes* has a G + C content of 41 mol% (Freier et al. 1994). In contrast to the previous isolates, *E. coprostanoligenes* is much easier to grow in the laboratory. It forms colonies on lecithin-containing solid media and survives exposure to air for at least 48 h. The bacterium converts up to 90% of the cholesterol to coprostanol in cholesterol-containing medium. The cholesterol-reducing activity is possessed constitutively by the bacterium. Washed bacterial cells from cholesterol-free media efficiently reduced cholesterol to coprostanol under either anaerobic or aerobic conditions (Li et al. 1995). The cholesterol-reducing activity in bacterial cells decreased as a function of storage time at 22°C, 4°C, and -20°C. Hydrogen, pyruvate, and reducing agents increased the activity. Optimal cholesterol reduction occurred at pH 7.5 in a sodium phosphate buffer. *E. coprostanoligenes* seems more amenable than previously studied cholesterol-reducing bacteria for applications in food and pharmaceutical industries.

**Mechanisms of cholesterol reduction**

Long before a pure cholesterol-reducing bacterium was isolated, two hypotheses had been proposed for the mechanism of cholesterol reduction reaction: (1) a direct stereospecific reduction of the double bond (Bondzynski and Humnicki 1896; Schoenheimer et al. 1930) and (2) a multistep conversion from cholesterol
involving the intermediates 4-cholestene-3-one and coprostanone (Rosenheim and Starling 1933; Schoenheimer et al. 1935; Rosenheim and Webster 1943). Since then, the debate on whether cholesterol is reduced biologically to coprostanol through a direct or an indirect pathway has been continuing.

Radioactive cholesterol was used usually to study the mechanism of cholesterol reduction. With [3-\(^2\text{H}, 4-\(^{14}\text{C}\)]-cholesterol, Rosenfeld et al. (1954) did \textit{in vivo} experiments by feeding the dual-labeled cholesterol to human subjects and \textit{in vitro} experiments by incubating the radioactive cholesterol with human fecal homogenates. They found that coprostanol isolated in both \textit{in vivo} and \textit{in vitro} studies contained deuterium and \(^{14}\text{C}\) and that the dilutions of deuterium and \(^{14}\text{C}\) are essentially of the same order. The retention of the isotopic label at C-3 provided evidence that the hydroxyl group at C-3 was not involved in the process of cholesterol reduction to coprostanol. They argued that, if coprostanol arose from cholesterol principally by the multistep pathway, the deuterium atom at C-3 would be completely lost and the coprostanol would have contained only \(^{14}\text{C}\) but no deuterium. Therefore, it was suggested that coprostanol is produced from cholesterol principally by a process not involving the hydroxyl group at C-3, but by direct saturation of the 5, 6 double bond of cholesterol. Later, however, Rosenfeld et al. (1956) found that coprostanol obtained from the biochemical reduction of 3\(\alpha\)-\(^2\text{H}\)-cholesterol contained an appreciable amount of isotope in ring B. It was concluded that some of the cholesterol molecules must have served as deuterium donors for other cholesterol molecules that were converted ultimately to
coprostanol. Then, by using 3α-3H-cholesterol, Rosenfeld and Gallagher (1964) again observed that cholesterol was reduced to coprostanol by fecal suspensions primarily with retention of tritium at C-3. However, they also found incorporation of isotope into other stable positions in coprostanol. It was suggested that cholesterol could be reduced by more than one bacterial species via different biochemical pathways.

Björkhem and Gustafsson (1971) questioned the conclusion of Rosenfeld and Gallagher that the main pathway for the formation of coprostanol is direct reduction of cholesterol. They argued that the label in the C-3 position of cholesterol could be transferred to a cofactor in an oxidative step and that the labeled cofactor could transfer its label to the steroid in a reduction step. They investigated the importance of the two pathways for cholesterol reduction to coprostanol by cecal contents from rats. The reaction was carried out in deuterated water and with [4β-3H, 4-14C]- and [3α-3H, 4-14C]cholesterol as substrates. It was found that conversion of [3α-3H, 4-14C]cholesterol into coprostanol occurred with loss of 50% of the tritium. The tritium retained in coprostanol was located in the C-3α position. Part of the retained tritium might have been introduced in a reductive step following an initial oxidative step. This hypothesis was supported by the finding that coprostanol formed from [4-14C]cholesterol in the presence of [3α-3H]β-sitosterol contained significant amounts of tritium in the C-3α position.

After conversion of part of [3α-3H, 4-14C]cholesterol into coprostanol,
unchanged cholesterol had a higher $^{3}\text{H}/^{14}\text{C}$ ratio than did the cholesterol added to the incubation mixture, indicating that oxidation of the 3β-hydroxyl group is at least partly rate-limiting in the over-all conversion of cholesterol into coprostanol (Björkhem and Gustafsson 1971). After cholesterol was incubated with cecal contents in the presence of deuterated water, the isolated coprostanol had a deuterium content of about 1.4 atoms at C-2, C-3, and C-4, about 0.5 atoms at C-5, and about 0.1 atoms at C-6, indicating extensive oxido-reduction at C-3 during or after conversion of cholesterol into coprostanol.

Coprostanol isolated after incubation of $[4\beta-^{3}\text{H}, 4-^{14}\text{C}]$cholesterol retained 60% of the tritium. Most of this tritium had been transferred to the C-6 position, showing that the conversion of cholesterol into coprostanol involves isomerization of the $\Delta^5$ double bond to $\Delta^4$ double bond. They also showed that incubation of $\Delta^4-[4-^{14}\text{C}]$cholesten-3-one with cecal contents resulted in efficient formation of coprostanol. Therefore, the authors concluded that the conversion of cholesterol into coprostanol proceeds to at least 50% by means of the intermediate formation of 4-cholesten-3-one (Björkhem and Gustafsson 1971). It was pointed out, however, that the reaction had been studied in a system consisting of a complex mixture of microorganisms. Thus, it was possible that different microorganisms might use different mechanisms to reduce cholesterol to coprostanol and that the contribution of the two mechanisms might be expected to vary.

Isolation of a pure cholesterol-reducing microorganism, *Eubacterium* ATCC 21408, provided more specific information about the mechanism of cholesterol
reduction to coprostanol (Eyssen et al. 1973). Parmentier and Eyssen (1974) studied the mechanism of biohydrogenation of cholesterol to coprostanol by incubating *E. ATCC 21408* with \([3\alpha^{-3}\text{H}, 4^{-14}\text{C}]\text{cholesterol or } [4\beta^{-3}\text{H}, 4^{-14}\text{C}]\text{cholesterol in a brain-thioglycollate medium under anaerobic conditions.}

Conversion of \([3\alpha^{-3}\text{H}, 4^{-14}\text{C}]\text{cholesterol to coprostanol by } E. ATCC 21408\) resulted in 65% loss of tritium. Part of the tritium, however, might have been lost after but not during reduction of cholesterol to coprostanol because incubation of preformed \([3\alpha^{-3}\text{H}, 4^{-14}\text{C}]\text{coprostanol with } E. ATCC 21408\) had caused loss of 40% of the tritium at the C-3 position of coprostanol. Thus, it was stated that loss of tritium during conversion of \([3\alpha^{-3}\text{H}]\text{cholesterol to coprostanol could not warrant the conclusion that reduction of the 5,6-double bond involved the formation of 3-oxo intermediates.}

To investigate whether biohydrogenation of cholesterol involved the formation of a 4,5-double bond, *E. ATCC 21408* was incubated with \([4\beta^{-3}\text{H}, 4^{-14}\text{C}]\text{cholesterol (Parmentier and Eyssen 1974). The isolated coprostanol retained 81% of the tritium originally present in cholesterol. It was found, however, that the label was not in the C-3 position but more than 90% of the tritium present in coprostanol was in the C-6 position. Therefore, it was concluded that more than 70% of 4\beta^{-3}\text{H} of cholesterol was transferred by an intramolecular shift to the C-6 position of coprostanol during the process of biohydrogenation, indicating that the reaction involved isomerization of the 5,6-double bond to a 4,5-double bond. The data supported the hypothesis that the major pathway for biohydrogenation of
cholesterol by \textit{E.} ATCC 21408 involves the intermediate formation of 4-cholesten-3-one followed by reduction of the latter to coprostanol (Parmentier and Eyssen 1974; Eyssen and Parmentier 1974).

Recently, Ren et al. (1995) studied the mechanism of cholesterol reduction to coprostanol by using \textit{Eubacterium coprostanoligenes} ATCC 51222. The bacterium was incubated with [4-\textsuperscript{2}H, 4-\textsuperscript{14}C]cholesterol as a mixture of \(\alpha\) and \(\beta\) isomers. After 5 d of incubation, 90\% of cholesterol was converted to coprostanol. No intermediates were found in the culture. The isolated coprostanol retained 97\% of the tritium originally present in cholesterol. The majority of the label (64\%), however, was located in the C-6 position, and only 36\% of tritium remained in the C-4 position. It was shown that no solvent exchange occurred during proton transfer form C-4 to C-6 by the bacterium. Transferring of tritium from C-4 to the C-6 position indicated isomerization of a 5,6-double bond to a 4,5-double bond during the conversion of cholesterol to coprostanol. The data suggested that \textit{E. coprostanoligenes} reduces cholesterol through an indirect pathway with the formation of 4-cholesten-3-one and coprostanone.

In resting cell assays where washed cells of \textit{E. coprostanoligenes} were incubated with micellar cholesterol in phosphate buffer at 37\(^\circ\)C, both 4-cholesten-3-one and coprostanone were produced in addition to coprostanol (Li et al. 1995b). Furthermore, 5-cholesten-3-one, 4-cholesten-3-one, and coprostanone were converted efficiently to coprostanol by \textit{E. coprostanoligenes}. Therefore, it was concluded that the major pathway for reduction of cholesterol to coprostanol by \textit{E.}
coprostanoligenes involves the intermediate formation of 4-cholesten-3-one and coprostanone followed by reduction of the latter to coprostanol (Ren et al. 1995).

Potential applications

Microbial reduction of cholesterol results in coprostanol that is absorbed poorly from the gastrointestinal system (Schoenheimer 1931; Bhattacharyya 1986). The nonabsorbability of coprostanol has led to the development of potential technology to lower cholesterol concentration in the blood of hypercholesterolemic individuals and to decrease cholesterol contents of foods (Dehal et al. 1990). By introducing cholesterol-reducing bacterium into the digestive tract, it is possible that little or no endogenous (e.g., biliary) and dietary cholesterol would be absorbed so that the cholesterol concentration in blood might be decreased. Treating foods with the bacteria may produce low-cholesterol or cholesterol-free foods so that consumers would have little or no concerns about the cholesterol in animal-derived foods.

Early investigators have assumed that microbial conversion of cholesterol to coprostanol could affect sterol balance of the host (Iritani and Wells 1966). It was found that conventional animals excreted 50 to 100% more neutral sterols through feces than did germ-free animals (Evrard et al. 1964; Kellogg and Wostmann 1969; Eyssen et al. 1974). It was also observed that cholesterol was absorbed more efficiently from the intestine of germ-free animals and that germ-free animals had 2 to 3 times greater liver cholesterol content than similar conventional animals when
moderate amounts of cholesterol were fed with the diet (Wostmann 1973). These findings indicate that intestinal bacteria interfere with the absorption of cholesterol and promote the fecal output of neutral sterols.

Gnotobiotic rats associated with *E. ATCC 21408*, however, failed to promote the fecal output of neutral sterols (Eyssen and Parmentier 1974). The phenomenon was explained by the observation that coprostanol formation predominantly occurred in the cecum and the large intestine, an anatomical site from which no cholesterol absorption takes place.

Recently, with the new cholesterol-reducing bacterium, *E. coprostanoligenes ATCC 51222*, Li et al. (1995a) investigated its effect on plasma cholesterol concentration in dietary-induced hypercholesterolemic rabbits. Experimental rabbits received 4 ml of *E. coprostanoligenes* suspension (*ca* 10⁸ cells/ml) daily *per os* for 10 d; control rabbits received the same dose of boiled bacterial suspension. It was found that plasma cholesterol concentration of experimental rabbits was 26% lower (*P* < 0.001) than that of controls after bacterial treatment. The hypocholesterolemic effect of *E. coprostanoligenes* continued for at least 34 d after the last bacterial feeding. The sustained effect was explained by colonization of the bacterium in the digestive tracts. Also, coprostanol-to-cholesterol ratios in contents of digestive tracts (especially small intestines) of experimental rabbits were significantly greater than those of controls, which suggested that the colonized bacteria were reducing cholesterol to coprostanol actively in the intestine. When the intestinal contents were inoculated into the growth medium, *E.*
coprostanoligenes and its cholesterol-reducing activity were recovered from the experimental group but not from the control group. It was concluded that oral administration of E. coprostanoligenes caused a significant hypocholesterolemic effect in rabbits and that this effect can be explained by the conversion of cholesterol to coprostanol in the intestine. This study was different from the study of Eyssen and Parmentier (1974) because, after oral administration of E. coprostanoligenes to rabbits, the bacteria were found to colonize and reduce cholesterol in the jejunum and ileum, which are major sites for cholesterol absorption. Similar hypocholesterolemic responses were observed by using milk as a carrier to deliver E. coprostanoligenes to the digestive tract of dietary-induced hypercholesterolemic rabbits (Madden 1995). Absence of an effect on serum cholesterol concentration and fecal sterol output with E. ATCC 21408 in gnotobiotic rats might result from the failure of colonizing the small intestine.

Treatment of foods with cholesterol-reducing bacteria has provided another potential way to decrease cholesterol contents in foods (Dehal et al. 1991). It was observed that cholesterol reductase from plant sources converted some cholesterol in homogenized milk, homogenized cream, ground beef and pork, and fresh and dried egg yolks to coprostanol. However, the amount of conversion was small. With E. coprostanoligenes, preliminary data have shown that cholesterol in homogenized milk can be reduced significantly (≈ 10%) by incubation with the bacterium (unpublished data). Sonication of the milk made cholesterol therein more accessible to the bacterium. Incorporation of E. coprostanoligenes in yogurt
starter cultures resulted in lower cholesterol yogurt (unpublished data).

Inoculation of *E. coprostanoligenes* into ground pork and mutton led to about 5% cholesterol reduction after 15 h of incubation (Madden 1995). Therefore, a microbial cholesterol-reducing system seems promising for future applications in food-processing industries.

**Cholesterol Oxidase and Cholesterol-Oxidizing Microorganisms**

Microbial cholesterol oxidases (EC 1.1.3.6) catalyze the transformation of cholesterol to 4-cholesten-3-one with concomitant reduction of molecular oxygen to hydrogen peroxide. This transformation is the initial step in the microbial catabolism of cholesterol. Cholesterol oxidase has been used widely for determination of serum cholesterol concentration in clinical specimens by coupling with peroxidase (Richmond 1973; Allain et al. 1974; Lolekha and Jantaveesirirat 1992). The enzyme also has been used for steroid identification (Smith and Brooks 1974) and as tools in cell biology for cholesterol quantification and for the subcellular localization of unesterified cholesterol (Heider and Boyett 1978; Lange and Ramos 1983; Patzer et al. 1978; Thurnhofer et al. 1986). Interest in cholesterol oxidases and cholesterol-oxidizing microorganisms is also partly because of their potential use in decreasing cholesterol content of foods, in particular fermented dairy products (Smith et al. 1991). Cholesterol degradation at the intestinal level by bacterial cultures can be used as probiotics.
Sources of cholesterol oxidase

Since the report by Stadtman et al. in 1954 on crude enzyme preparations from *Mycobacterium* sp. (Stadtman et al. 1954), cholesterol oxidases have been described in a number of microorganisms, including *Arthrobacter* (Arima et al. 1969), *Norcardia* sp. (Cheetham et al. 1980), *Brevibacterium* sp. (Uwajima et al. 1973), *Streptomyces* sp. (Tomioka et al. 1976), *Corynebacterium* sp. (Shirokane et al. 1977), *Actinomyces* sp. (Petrova et al. 1979), *Streptoverticillium* sp. (Inouye et al. 1982), *Schizophyllum* sp. (Fukuyama and Miyake 1979), *Rhodococcus* sp. (Watanabe et al. 1986), and *Pseudomonas* sp. (Lee et al. 1989). Some of these microorganisms produce only membrane-bound cholesterol oxidase such as *Nocardia* (Cheetham et al. 1980), some produce only extracellular enzymes such as *Brevibacterium*, *Streptomyces*, and *Streptoverticillium* (Uwajima et al. 1973; Tomioka et al. 1976; Inouye et al. 1982; Fukuyama and Miyake 1979), and others produce both a secreted and a membrane-bound form such as some *Rhodococcus* strains (Aihara et al. 1986).

Purification of cholesterol oxidase

Cholesterol oxidase was first purified and crystallized from *Brevibacterium sterolicum* ATCC 21387 (Uwajima et al. 1973). *B. sterolicum* is a soil bacterium. It can grow on cholesterol as the sole source of carbon and energy and produces an extracellular form of cholesterol oxidase. Therefore, the enzyme was purified from the culture filtrate by a procedure involving ammonium sulfate fractionation,
DEAE-cellulose and hydroxyapatite column chromatographies, and Sephadex G-75 gel filtration. Crystals of the enzyme were obtained from solutions of the purified preparation by the addition of ammonium sulfate. The crystals appeared as fine rods, with a bright yellow color. The crystalline cholesterol oxidase showed an absorption spectrum characteristic of a flavoprotein. Addition of sodium dithionite to the enzyme under anaerobic conditions produced a "bleaching" of the absorption peaks. Thus, these observations suggest that flavin is a prosthetic group of cholesterol oxidase and is responsible for its catalytic activity (Uwajima et al. 1973). This finding has been confirmed by later studies with cholesterol oxidase (Uwajima et al. 1974; Kamei et al. 1978; Kenney et al. 1979). It was found that most cholesterol oxidases contain one mole of tightly bound flavin adenine dinucleotide (FAD) per mole of protein as a prosthetic group. However, some of them, for example the enzyme from Nocardia erythropolis, lack this cofactor (Richmond 1973). The crystal structure of the enzyme from B. sterolicum was determined at 1.8 Å resolution (Vrielink et al. 1991).

Richmond (1973) characterized, extracted, and purified a membrane-bound cholesterol oxidase from Nocardia sp. Disintegrating Nocardia sp. cells with mechanical techniques did not release much enzyme from the cell membrane. Incubation with a detergent, Triton X-100, effectively solubilized the membrane-bound enzyme. Then, the enzyme was purified by a procedure involving ammonium sulfate precipitation and ion-exchange chromatography. Cholesterol oxidase from Nocardia sp. is very stable at 4°C and is also highly heat stable, which
allows it to be used analytically at temperatures as high as 50°C. It is active in aqueous ethanolic solutions containing as much as 30% of ethanol and in buffers ranging from pH 4.0 to pH 9.0. The enzyme is specific for 3β-sterols and requires a double bond in the Δ^5 or Δ^4 positions. Shortening the cholesterol side chain markedly diminishes the affinity of the enzyme for these substrates. Cheetham et al. (1980) further developed the extraction method to obtain high yield of cholesterol oxidase from *Nocardia rhodochrous* by treatment either with a detergent, Triton X-100, or with trypsin. It was found that the enzyme extracted with detergent, after removal of the detergent, could be reabsorbed by *Nocardia*, whereas enzyme extracted by using trypsin was water-soluble and could not be reabsorbed by cells. The results indicate that cholesterol oxidase from *Nocardia* is an intrinsic membrane-bound protein possessing a hydrophobic anchor region that can be removed by trypsin (Cheetham et al. 1980).

Lately, Rhee et al. (1991) described the synthesis of a novel cholesterol affinity adsorbent and a very simple purification procedure that gives a high yield of homogeneous cholesterol oxidase from culture broth of a *Pseudomonas* sp.

*Rhodococcus equi* ATCC 33706 possesses a membrane-bound and a secreted form of cholesterol oxidase. Johnson and Somkuti (1991) isolated both forms of the enzyme from the microorganism. They tested several detergents (*n*-octyl glucoside, Tween-20, Tween-60, Tween-80, and Brij-35) in phosphate buffer for efficacy to release protein from membrane fragments and prevent aggregation of the secreted enzyme. It was found that 0.5% Brij-35 was most efficient for
releasing membrane-bound protein. n-Octyl glucoside decreased the activity of the membrane-bound enzyme and failed to release protein from the fragments. Octyl glucoside at a 2.0% concentration, however, prevented aggregation of the secreted enzyme. The membrane-bound enzyme was purified 38-fold by Bio-Gel A-1.5 m chromatography, whereas isolation from a Sephacryl S-200 column resulted in a 58-fold purification of the extracellular enzyme. Cholesterol oxidase activity for both enzyme preparations was optimal at approximately 40°C; however, the pH optimum (8.0) for the membrane-bound enzyme was much broader for the secreted enzyme (pH 6.0-8.0).

Recently, Kreit et al. (1994) reported the production and extraction of membrane-bound cholesterol oxidase from Rhodococcus sp. cells and proposed a topology model at the cytoplasmic membrane level for the cell-bound cholesterol oxidases of the noncardioform bacteria. Enzyme solubilization was achieved by cell treatment with Triton X-100 or other nonionic detergents that have low critical micelle concentration. They suggested that the enzyme active domain is located at the outer surface of the membrane and substrate catalysis in vivo occurs externally to the cytoplasm. This topology was indicated by the following: (a) The rapid release of Δ⁴-3-keto derivatives by cell action on cholesterol or phytosterols seemed to show an easy and direct contact between the enzyme system and the substrate (b) The enzyme activity could be measured by using whole cells in a colormetric assay, indicating that substrate should not intersect the cytoplasmic membrane for catalysis (c) The enzyme could be extracted from whole cells with nonionic
detergents without cell disintegration, indicating an amphiphilic nature of the enzyme. It was concluded that this topology model might be a general one for the microbial enzyme (Kreit et al. 1994).

**Substrate specificity of cholesterol oxidase**

The substrate specificity of cholesterol oxidases has been studied with the enzyme from different microbial species (Richmond 1973; Smith and Brooks 1975; Smith and Brooks 1977; Kamei et al. 1978; Inouye et al. 1982; Uwajima et al. 1974). The results of these studies indicated that the 3-hydroxy group in the β-position of the sterol molecule is an absolute substrate requirement to allow enzymatic oxidation. Variations in the C(17)-linked side chain of the sterol affects oxidation rates. Alterations in the ring structure of the sterol are oxidized slower when compared with cholesterol.

Cholesterol oxidase from *Nocardia* sp. catalyzed oxidation of 3β-hydroxy-5-cholene-24-oic acid, 5-pregnen-3β-ol, and androst-5-ene-3β-ol but with a much slower rate than cholesterol, whereas 5α-cholestan-3β-ol or 5β-cholestan-3β-ol (coprostanol) was not oxidized by the enzyme (Richmond 1973).

Recently, Slotte (1992) suggested that the physico-chemical state of the substrate might affect how efficiently the enzyme can bind its substrate. Therefore, he studied substrate specificity of cholesterol oxidase from *Streptomyces cinnamomeus* with cholesterol present in monomolecular monolayers at the air/water interface so that the orientation of the substrate molecules relative to the
enzyme could be controlled. Of the cholesterol analogs with structural alterations in the A- or B-ring that were examined, it was observed that removal of the Δ^5 double bond, to yield 5α-cholestan-3β-ol, had no effect on the rate of oxidation. When the Δ^5 double bond in cholesterol was instead at the Δ^4 position, the oxidation rate became 3-fold slower. A similar 3-fold decrease in the average oxidation rate was observed when the Δ^5 double bond in cholesterol was instead at the Δ^7 position (to yield 5α-cholest-7-en-3β-ol). 5,7-Cholestadien-3β-ol, which had an additional double bond at Δ^7, was oxidized 5-fold slower than was cholesterol, whereas 3β-hydroxy-5-cholesten-7-one and 5β-cholestan-3β-ol (coprostanol) were not substrates for cholesterol oxidase. With C(17) side chain analogs of cholesterol, it was observed that the complete lack of the C(17) side chain (5α-androsten-3β-ol), or the insertion of a double bond at Δ^{24} (desmosterol), or even an ethyl group at C(24) (24b-ethyl-5,22-cholestadien-3β-ol) had no appreciable effects on sterol oxidation rate, implying that the enzyme did not recognize the side chain in oriented sterol monolayers.

The results were explained by three-dimensional, energy-minimized structures of the sterols with molecular modeling (Slotte 1992). It was shown that 5α-cholestan-3β-ol is structurally very similar to cholesterol (as is the oxidation rate), whereas the A-ring of 5β-cholestan-3β-ol (coprostanol) is markedly reoriented away from the plane of the sterol ring system. This A-ring distortion, together with the resulting reorientation of the 3β-hydroxy group, makes this sterol essentially a non-substrate for cholesterol oxidase. The 3-D structure of 3β-
hydroxy-5-cholest-7-one is very similar to the structure of cholesterol, except for the keto-group extruding at C(7). This 7-keto derivative of cholesterol is not a substrate of cholesterol oxidase (Slotte 1992), suggesting that obtruding functional groups at positions in the B-ring will interfere with successful binding of the sterol molecule to the catalytic site of the enzyme. Sterol analogs that have obtruding methyl groups at position 4 in the A-ring, such as lanosterol, are also non-substrate molecules for cholesterol oxidase (Wortberg 1975).

**Mechanism of cholesterol oxidation**

The catalytic mechanism of cholesterol oxidase also has been studied (Smith and Brooks 1977; Kass and Sampson 1995). Cholesterol oxidase is a bifunctional enzyme. It catalyzes the oxidation of cholesterol to the temporary intermediate 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide (Stadtman et al. 1954; Uwajima et al. 1974) and the isomerization of the $\Delta^5$-bond (Smith and Brooks 1977) via a mechanism analogous to that of the $\Delta^5$-3-ketosteroid isomerase from *Pseudomonas testosterini* (Batzold et al. 1976; Kuliopulos et al. 1987).

5-Cholesten-3-one, however, is usually not accumulated in the oxidation of cholesterol to 4-cholesten-3-one. A faster rate of isomerization than of oxidation explains this phenomenon. The trapping of [4-$^{14}$C]5-cholesten-3-one in a pool of unlabeled ketone after the incubation of [4-$^{14}$C]cholesterol with *N. erythropolis* cholesterol oxidase demonstrates that the sequence of oxidation followed by
isomerization does occur (Smith and Brooks 1977). Incubation of [4α-2H]-and [4β-2H]-cholesterol with cholesterol oxidase showed that the 4β-hydrogen atom can be transferred to the 6β-position. Incubations of cholesterol, 5-cholesten-3-one, and 4-cholesten-3-one with the enzyme in 2H2O, however, led to some incorporation of 2H into the 4-cholesten-3-one products, mostly at position 6β, suggesting that reverse isomerization can take place. It was found that both the isomerase and the oxidase activities of cholesterol oxidase were inhibited by the acetylenic 3-oxo seco-steroids (5,10-seco-19-nor-5-cholestone-3,10-dione), indicating either that there are two active sites on one protein or that there are two enzymes that are closely associated (Smith and Brooks 1977).

Cholesterol oxidase from Brevibacterium sterolicum, however, was found to be a monomer (55 kD) with a single active site for both oxidation and isomerization and requires one FAD per active site (Uwajima et al. 1974; Li et al. 1993). The three-dimensional structure of the B. sterolicum has been published (Li et al. 1993; Vrielink et al. 1991). Two structures have been solved, one in the absence of substrate and one with dehydroepiandrosterone, a moderately active substrate, in the active site. The enzyme has an active site sequestered from solvent by two loops that act as "lids". In addition, there is one charged residue in the active site, glutamate-361. This residue is positioned directly over the α-face of the bound sterol. This observation led to the proposal that glutamate-361 is the base responsible for isomerization of the double bond into conjugation with the ketone of the intermediate (Li et al. 1993). Furthermore, it is presumed that the
isomerization reaction proceeds in a stepwise manner via an enol(ate) intermediate. The mechanism entails deprotonation of the axial 4-hydrogen by glutamate-361 and reprotonation by the conjugated acid at the axial position of carbon-6. This proposed mechanism is analogous to those determined for the cholesterol oxidase from N. erythropolis (Smith and Brooks 1977) as well as ketoisomerases (Schwab and Henderson 1990). The degree of sequence similarity between the Nocardia and the Brevibacterium oxidases is unknown. They seem to have different physical properties. The Nocardia oxidase is a cytosolic protein, and there is no evidence for a flavin cofactor in the Nocardia oxidase (Smith and Brooks 1977). Furthermore, they have different substrate specificities (Smith and Brooks 1977).

Recently, Kass and Sampson (1995) determined whether the isomerization reaction of B. sterolicum cholesterol oxidase is stereospecific, i.e., if the proposed mechanism is correct, by incubating $[4\alpha^-H]$- and $[4\beta^-H]$-cholesterol with cholesterol oxidase and analyzing the product composition. Additionally, they measured the degree of direct proton transfer between carbon-4 and carbon-6 to determine if the isomerization was catalyzed by one or two bases. Their results showed that $[4\alpha^-H]$-cholesterol was converted completely to 4-cholesten-3-one when it was incubated with the enzyme and that 84% of the deuterium label remained in the product as determined by mass spectrometry. $^1$H-NMR spectral analysis of the product revealed that no deuterium label was transferred to carbon-6. All of the deuterium label remained at carbon-4. When $[4\beta^-H]$-cholesterol was incubated with the enzyme, it also was converted completely to 4-cholesten-3-one.
It was found that 50% of the deuterium label remained in the product and that all of the deuterium label remained at carbon-4. Cholesterol was incubated in deuterated phosphate buffer with the enzyme that had not been preequilibrated with D_2O and was converted completely to 4-cholesten-3-one. None of the product contained deuterium. When cholesterol was incubated in the same deuterated phosphate buffer with the enzyme that had been preequilibrated with D_2O, 13% of the product contained deuterium and the label was in the 6β position.

Therefore, it was concluded that the isomerization reaction catalyzed by *B. stercolicum* cholesterol oxidase proceeded via a stereospecific proton transfer from the 4β-carbon to the 6β-carbon to form 4-cholesten-3-one (Kass and Sampson 1995). Fifty percent of the 4β deuterium label was transferred directly to the 6β-carbon; 50% of the label was lost to solvent. The 4α-deuterium label remained on carbon-4. These results imply that there is one active site base that is positioned over the β-face and is responsible for isomerization. Based on crystallographic evidence (Li et al. 1993; Vrielink et al. 1990), glutamate-361 is most likely the general base. The observation of direct transfer of deuterium label indicates that the active site base must be quite mobile to accomplish both deprotonation and reprotonation (Kass and Sampson 1995).

Very surprisingly, Molnár et al. (1993) found that bacterial cholesterol oxidases are able to act as flavoprotein-linked ketosteroid monooxygenases and catalyze the hydroxylation of cholesterol to 4-cholesten-6-ol-3-one. The new metabolite of cholesterol originally was found in reaction mixtures containing
cholesterol or 4-cholesten-3-one as a substrate and extra- or intracellular protein extracts from recombinant *Streptomyces lividans* and *Escherichia coli* strains carrying cloned DNA fragments of *Streptomyces* sp. SA-COO, the producer of *Streptomyces* cholesterol oxidase. Commercially purified cholesterol oxidase of a *Streptomyces* sp., as well as of *Brevibacterium sterolicum* and a *Pseudomonas* sp., and a highly purified recombinant *Streptomyces* cholesterol oxidase also were able to catalyze the 6-hydroxylation reaction. Cholesterol oxidases of different bacterial origins, however, catalyze 6-hydroxylation at different rates. With the same amounts of enzyme, the ratio of 4-cholesten-3-one and 4-cholesten-6-ol-3-one produced in the reactions showed wide variation, with the *Pseudomonas* enzyme producing mainly the 6-hydroxylated derivative while the *Brevibacterium* enzyme formed only trace amounts of this steroid. Hydrogen peroxide accumulating in the reaction mixtures as a consequence of the 3β-hydroxysteroid oxidase activity of the enzyme was shown to have no role in the formation of the 6-hydroxylated derivative. The authors proposed a possible scheme for a branched reaction pathway for the concurrent formation of 4-cholesten-3-one and 4-cholesten-6-ol-3-one by cholesterol oxidase. At this point, cholesterol oxidase therefore can be considered as a trifunctional enzyme (Molnár et al. 1993).

**Cloning of cholesterol oxidase genes**

The study of cholesterol oxidase has progressed to the molecular level. Genes encoding cholesterol oxidase (*cho*) have been cloned from *Streptomyces* sp.
strain SA-COO (choA) (Murooka et al. 1986), *Brevibacterium sterolicum* ATCC 21387 (choB) (Fujishiro et al. 1990), and *Streptomyces* sp. strain A19249 (choM) (Corbin et al. 1994). The expression of these genes in heterologous bacterial hosts has been observed (Brigidi et al. 1993; Corbin et al. 1994; Ohta et al. 1992; Solaiman and Somkuti 1991; Somkuti et al. 1991; Somkuti et al 1992).

Murooka et al. (1986) cloned the cholesterol oxidase gene (choA) from *Streptomyces* sp. strain SA-COO into *S. lividans* with a multicopy plasmid, pIJ702, which resulted in an several-fold increase in cholesterol oxidase production. Deletion analysis of the recombinant plasmid, pCO-1, showed that the entire coding sequence of the choA gene was located within a 2.4 kb segment of the chromosomal DNA, which allows for production of about a 91,000 dalton protein. The results also showed that the choA gene on the plasmid could direct the synthesis of both extracellular and intracellular cholesterol oxidase (Murooka et al. 1986).

Ishizaki et al. (1989) determined the nucleotide sequence of a 2.1 kb fragment containing the choA gene, which codes secreted cholesterol oxidase. The data show that a single open reading frame encodes a mature cholesterol oxidase of 504 amino acids with a calculated $M_r$ of 54,913. The leader peptides extend over 42 amino acids and have the characteristics of a signal sequence, including basic amino acids near the amino terminus and a hydrophobic core near the signal cleavage site. Analysis of the total amino acid composition and amino acid sequencing of the first 21 amino acids from the N terminus of the purified
extracellular enzyme agree with data deduced from nucleotide sequencing data (Ishizaki et al. 1989).

Horii et al. (1990) determined the nucleotide sequence of the promoter region of choA and found an open reading frame (choP) located between a potential promoter sequence and the structure gene for ChoA protein. Deletion analysis showed that the promoter region for choP is essential for expression of the choA gene. Northern (RNA) blot analysis of the transcript revealed a 2.9 kb transcript that is identical in size to the total sequence of the choP and choA genes. These results suggest that the two genes, choP and choA, are transcribed polycistronically under the control of the promoter that is upstream from the structural gene for choP. The choP gene encodes a protein of 381 amino acids with a calculated $M_r$ of 41,688. The nucleotide sequence of the choP gene has a high degree of similarity to the sequence of the genes for cytochrome P-450s from humans and Pseudomonas species. A region of homology with the cytochrome P-450s from different microorganisms was identified in the choP protein and may represent a region associated with a binding site for heme iron. It was speculated that the gene product of choP might be involved in the metabolism of cholesterol (Horii et al. 1990). The natural substrate for the choP protein or the inducer for the cho operon, however, has not been found. Molnár et al. (1991) deleted a 1.2 kb fragment adjoining upstream of the choP-choA operon and subcloned the operon into a multi-copy shuttle vector composed of pIJ702 and pUC19 in Streptomyces lividans, which resulted in the overproduction of Streptomyces
cholesterol oxidase extracellularly about 70-fold more than that of the original producer, *Streptomyces* sp. SA-COO. The amount of overproduction of cholesterol oxidase was found to be dependent on the copy numbers of the plasmids as well as the presence of the sequences derived from pUC19.

Fujishiro et al. (1990) cloned another cholesterol oxidase gene (*choB*) from *Brevibacterium sterolicum* ATCC 21387. The gene was selected from a pUC19-based gene bank in *E. coli* MM294 by colony hybridization by using a synthetic DNA probe. The gene was shown to encode a protein having the same amino acid sequence as that determined from amino acid sequence analysis. The expression of *choB* in *E. coli* was not observed probably because of transcriptional failure (Fujishiro et al. 1990).

Ohta et al. (1991) sequenced the *choB* gene and found that the nucleotide sequence contained an open reading frame with a G + C content of 64.9 mol% that would encode a protein of 552 amino acids. N-terminal amino acid sequence analysis of the extracellular enzyme of *B. sterolicum* confirmed that the mature enzyme consisted of 507 amino acids with a predicted *M*ₘ of 54,902 and was preceded by a 45-amino acid signal sequence. Sequences of *choA* and *choB* were compared also by Ohta et al. (1991). The results show that the nucleotide sequence and deduced amino acid sequence of these two genes have identities of 64% and 58%, respectively, indicating that the genes derived from a common ancestor. There are at least six highly conserved regions between the two enzymes that may play structurally or functionally important roles such as substrate binding
Recently, an insecticidally active cholesterol oxidase gene (choM) has been cloned and sequenced from *Streptomyces* sp. strain A19249 (Corbin et al. 1994). The primary translation product was predicted to be a 547 amino acid protein whose first 43 amino acids constitute a secretory signal peptide.

**Heterologous expression of cholesterol oxidase genes**

Ideas of developing a biotechnological approach to decrease the cholesterol content of milk have lead to attempts to engineer starter culture bacteria (lactococci, lactobacilli, and streptococci) genetically, to express the phenotypic traits that are related to cholesterol metabolism, and to use these cultures in the production of fermented dairy foods (yogurt and cheeses).

Somkuti et al. (1991) transferred and expressed *Streptomyces* cholesterol oxidase gene in *Streptococcus thermophilus*, a thermophilic bacterium used primarily for lactic acid synthesis in dairy fermentation. The gene transfer involved construction of the recombinant plasmid pNCO937 from the *Streptomyces* sp. SA-COO cho gene and the bifunctional shuttle vector pNZ19. The transformants were confirmed by Southern hybridization experiments. The biosynthesis of a functional cholesterol oxidase in *S. thermophilus* transformants was confirmed also by thin-layer chromatographic analysis of reaction products following incubation of sonically disrupted cells with cholesterol. Unlike *S. lividans* (Murooka et al. 1986), however, *S. thermophilus* did not secrete cholesterol oxidase into the culture
medium. The data provided evidence that the promoter sequence of the
*Streptomyces cho* gene was recognized in *S. thermophilus*. Instability of the
insertion, however, was obvious, as indicated by the loss of cholesterol oxidase
activity from transformants after repeated transfers. Agarose gels clearly showed
that mutational events involved primarily the 2.4 kb component of pNCO937 that
contained the *cho* gene.

Later, Somkuti et al. (1992) also electrotransformed three strains of
*Lactobacillus casei* with pNCO937. Transformation frequency was generally low
and strain dependent. *L. casei* transformants, however, stably maintained
pNCO937 with no indication of deletion mutational events. Transformants
produced active cholesterol oxidase, although the enzyme that is extracellular in
*Streptomyces* sp. was detectable only in sonicated cells of *L. casei*.

Brigidi et al. (1993) studied the cloning and the expression of the *cho* from
*Streptomyces* sp. into some species of *Bacillus*, in the intestinal species *Lactobacillus
reuteri* and in *E. coli*. The cloning of the *cho* gene in those bacteria was successful.
Only the transformants of *E. coli*, however, showed a significant intracellular
enzyme activity. The heterologous gene was stably maintained in Gram-positive
transformants but no enzyme activity was detected, indicating that these hosts could
not use the *Streptomyces* promoter. It was concluded that strong promoters known
to function efficiently in Gram-positive bacteria and new cloning strategies have to
be used to express the *cho* gene in intestinal and dairy lactobacilli (Brigidi et al.
1993).
Recently, Corbin et al. (1994) expressed an insecticidally active cholesterol oxidase gene (choM) in E. coli and in plant protoplasts. Expression of the gene with the signal sequence in E. coli resulted in production of a protein that had enzymatic and insecticidal properties. Expression of the gene with or without the signal sequence in tobacco protoplasts resulted in production of an enzymatically active cholesterol oxidase. This study was the first description of expression of cholesterol oxidase in a plant cell expression system. Expression of the choM gene in stably transformed plants should provide significant insight into the utility of this gene as a novel insecticidal agent for transgenic plant technology.

Potential applications

There have been attempts to use cholesterol oxidases or cholesterol-oxidizing bacteria to decrease the cholesterol content of foods. Aihara et al. (1988) investigated the degradation of egg yolk cholesterol with Rhodococcus equi No. 23, a strain isolated from butter. R. equi was incubated in a sterile egg yolk medium containing 0.3-1.7 mg cholesterol/ml. It was found that the cholesterol content decreased with increasing incubation time. With cholesterol less than 0.9 mg/ml in the medium, cholesterol was degraded completely after 3 d of incubation. With higher initial cholesterol concentration in the medium, however, the degradation of cholesterol was incomplete after 3 d. In addition to the residual cholesterol, small amounts of 4-cholesten-3-one could be detected but no other degradation products were detectable by TLC, indicating that the 4-cholesten-3-one formed was
converted rapidly into nonsteroid compounds with almost no accumulation of steroid intermediates in the culture medium.

Johnson and Somkuti (1990) tested strains of the genera *Rhodococcus*, *Norcardium*, *Brevibacterium*, and *Microbacterium* for their ability to degrade cholesterol. It was found that the rate of cholesterol degradation by rhodococci was generally higher than that shown by *Nocardia* strains. With *R. equi*, it was observed that the actively growing cultures metabolized cholesterol without accumulation of large amounts of steroid intermediates, which was in agreement with other reports (Aihara et al. 1986; Watanabe et al. 1986). If the rate of cellular metabolism was decreased as in the case of resting cells, however, certain intermediate degradation products such as 4-cholesten-3-one and 1,4-cholestadiene-3-one could be detected. When sonicated extracts of *R. equi* strains were tested for activity with free cholesterol or egg yolk as the substrate, all *R. equi* strains degraded free cholesterol at a higher rate. The rate of cholesterol degradation by cell-free extracts with egg yolk preparation was much lower. Some differences were seen among *Rhodococcus* strains in their ability to degrade egg yolk cholesterol even though rates of dissimilation of free cholesterol were similar. When the cholesterol in the cream preparation was used as the substrate, the degradation rate was even lower, indicating that cholesterol in cream preparations was even less accessible to the cholesterol dissimilating system of rhodococci than was cholesterol in egg yolk preparations. Therefore, it was concluded that the discovery of more efficient microbial enzymes and the development of procedures resulting in greater
substrate availability to enzymatic degradation are needed before further progress in reducing the cholesterol content of complexed food systems biologically can be made (Johnson and Somkuti 1990).

Xiansheng et al. (1990) reported the cholesterol degradation and formation of oxidation products by cholesterol oxidase in a buffer system and in commercially homogenized pasteurized milk at a range of temperatures. Only one byproduct, 4-cholesten-3-one, generated by cholesterol oxidase both in buffer and in milk was identified. In phosphate buffer, cholesterol (1 mg/ml) was oxidized to 4-cholesten-3-one completely in less than 24 h at 25°C or 37°C. At 6°C, the enzyme was still active, but the reaction rate was slower. Interestingly in milk, the highest cholesterol degradation was achieved at the lowest temperatures tested, up to 50% being degraded in 8 h at both 7°C and 3°C. The result was explained by possible attachment of cholesterol oxidase to the fat globules (therefore more accessible to cholesterol) in milk at a low temperature. This feature offers the potential to use cholesterol oxidase in the same way as lactase is sometimes commercially applied to milk. Enzymes aseptically added after the heat treatment could decrease the cholesterol more than 50% before the milk reaches the consumer.

Smith et al. (1991) further assessed reactivity of milk cholesterol with bacterial cholesterol oxidases from *Pseudomonas*, *Streptomyces*, and *Rhodococcus*. It was shown that *P. fluorescens* cholesterol oxidase oxidized cholesterol rapidly in homogenized/pasteurized milk or in sonicated raw milk but catalyzed cholesterol oxidation only slowly in intact raw milk. Pasteurization had little influence on
oxidation rates. Oxidases from *Streptomyces* and *Rhodococcus* species were significantly less active in milk than was the *Pseudomonas* enzyme. Cholesterol oxidation in homogenized milk depends on temperature and enzyme concentration. A maximum of 85% of the initial whole milk cholesterol (100-138 μg/ml) was oxidized in 96 h to a final cholesterol concentrations of 20-30 μg/ml. Sonicated skim milk cholesterol concentrations (initially 17-20 μg/ml) were decreased to 4-6 μg/ml. In addition to sonication, other procedures have been developed to make the water-insoluble substrate, cholesterol, more accessible to the water-soluble cholesterol oxidase or cholesterol-oxidizing bacteria. For example, cyclodextrins have been used for enhancing the bioavailability of organic substrates to biocatalysts.

Jadoun and Bar (1993) conducted cholesterol oxidation by resting *Rhodococcus erythropolis* cells and soluble cholesterol oxidase in a cyclodextrin medium. It was found that both the enzyme and microbial oxidation of cholesterol were enhanced by the dimethylated β-cyclodextrin. The microbial transformation, however, was subject to a larger enhancement effect than was the enzymatic one, which was interpreted to indicate a stronger affinity of dimethylated β-cyclodextrin-complexed substrate for the microbial cells.

In summary, studies have shown that it is possible to remove or decrease cholesterol in foods by enzymes or enzyme-producing microorganisms. Cholesterol oxidase (alone) or bacteria that produce it, however, might not be suitable for removing food cholesterol because the hydrogen peroxide formed might
generate toxic compounds or undesirable flavor components (Smith et al. 1991). Further, the safety of the oxidized cholesterol products needs to be assured.

**Cholesterol-Lowering Agents**

Increased serum cholesterol concentrations are a major risk factor for coronary heart diseases. There is substantial evidence that lowering total and LDL-cholesterol concentrations will decrease the incidence of coronary heart disease (Lipid Research Clinics Program 1988). To date, numerous cholesterol-lowering agents have been discovered and synthesized.

**3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors**

The HMG-CoA reductase inhibitors are the most commonly prescribed cholesterol-lowering drugs and are also the most recently introduced hypocholesterolemic agents. These inhibitors were initially discovered in Japan in 1976 (Endo et al. 1976). The first potent inhibitor was named mevastatin (formerly called ML-236B or compactin). Subsequently, many mevastatin analogs were developed. By 1990, lovastatin (formerly called mevinolin), simvastatin, and pravastatin had been approved and marketed in many countries (Grundy 1988; Hunninghake 1992). The potency of lovastatin and pravastatin seems to be similar, and simvastatin is about twice as potent (Hoffman et al. 1986). These drugs are very efficient in lowering total cholesterol and LDL cholesterol concentrations, particularly in people resistant to diet-induced decreases in plasma cholesterol.
concentrations. Beneficial effects from their administration in patients with coronary heart disease have been observed (Brown et al. 1990).

HMG-CoA reductase is the rate-limiting enzyme in the \textit{de novo} biosynthesis of cholesterol. The HMG-CoA reductase inhibitors are administered primarily to lower the plasma concentrations of total and LDL-cholesterol. These inhibitors share some molecular similarities with HMG-CoA, particularly the 3,5-dihydroxy heptanoic acid portion; other regions are also functionally essential (Endo 1992). These features lead to the result that the statins have a 10,000-fold greater affinity for HMG-CoA reductase than does the natural substrate, thus effectively blocking the conversion of HMG-CoA to mevalonic acid and thus also blocking cholesterol synthesis.

Theoretically, an HMG-CoA reductase inhibitor lowers plasma cholesterol by two mechanisms: (a) an inhibition of the hepatic formation of plasma lipoproteins and (b) enhanced lipoprotein catabolism in the liver (Endo, et al. 1979). Kovanen et al (1981) demonstrated in dogs that lowering of LDL-cholesterol occurs via these two mechanisms. Bilheimer et al (1983), however, showed that the principal action of lovastatin is to increase the number of LDL receptors and to enhance LDL receptor-mediated catabolism of lipoprotein rather than to inhibit synthesis of lipoproteins. This conclusion was drawn from the observation that these drugs are extremely effective in patients with heterozygous familial hypercholesterolemia (FH). Cholesterol concentrations fall by 25-30\% and 35-40\% with twice-daily doses of 20 mg and 40 mg lovastatin, respectively. In
receptor-negative patients with homozygous FH, however, no cholesterol-lowering has been observed during the treatment with lovastatin, even at very high doses (Uauy et al. 1988).

Rates of synthesis of LDL receptors are correlated inversely with the amount of cholesterol in cells (Goldstein and Brown 1977). The mechanism responsible for up-regulating LDL receptors in receptor-deficient patients starts with inhibition of HMG-CoA reductase. The consequent reduction in hepatic cholesterol production results in an increase in receptor numbers, which "pull" cholesterol out of blood, thereby lowering serum cholesterol concentration. Studies with experimental animals revealed that HMG-CoA reductase inhibitors increase messenger RNA for LDL receptors in the liver (Ma et al. 1986) and enhance the number of LDL receptors expressed on the surface of liver cells (Kovanen et al. 1981).

The HMG-CoA reductase inhibitors are considered generally as safe cholesterol-lowering drugs that decrease the death rate from heart attacks. Undesirable side effects, however, have been observed during clinical use of these drugs (Erkelens et al. 1988; Walkwe 1989). Some adverse clinical events include headache and gastrointestinal upset. Most common adverse laboratory events include elevation of serum transaminase activity and serum creatine kinase activity. Also, several studies have shown that HMG-CoA reductase inhibitors increase the risk of death from noncardiac mortality, including cancer and violent deaths, suggesting that over-all survival is not improved (Muldoon et al. 1990; Oliver 1992;
Ravnskov 1992; Rossouw, et al. 1990). Recently, a report from the Scandinavian Simvastatin Survival Study Group (1994), however, has indicated that long-term treatment with simvastatin is safe and improved survival in 4444 coronary heart disease patients.

**Bile acid sequestrants and cholesterol absorption inhibitors**

Bile acid sequestrants are recommended as first-line therapy for decreasing LDL if dietary therapy is unsuccessful in lowering LDL-cholesterol concentrations sufficiently (Ad Hoc Committee 1984). They have been used routinely for lowering of elevated concentrations of LDL-cholesterol for more than two decades (National Cholesterol Education Program 1988). Two bile acid sequestering anion-exchange resins, cholestyramine (a quaternary ammonium salt of a copolymer of styrene and divinylbenzene) and colestipol (a tertiary amine, copolymer of diethylenetriamine and epichlorohydrin), although quite different in chemical structure, provide parallel and alternative approaches to normalization of plasma total and LDL-cholesterol concentrations. The Lipid Research Clinics Coronary Primary Prevention Trials demonstrated that cholestyramine was effective in lowering LDL-cholesterol concentrations and subsequently decreasing the incidence of coronary heart disease (Lipid Research Clinics Program 1984a and 1984b).

Both cholestyramine and colestipol bind bile acids in exchange for chloride ions, an interaction that is irreversible and independent of temperature or pH. Cholestyramine, however, is more effective than colestipol in cholesterol-lowering
action. Because of their very large molecular size, resins are retained in the intestine where they sequester bile acids and thus promote their excretion in the feces. The augmented disposal of bile acid causes an increase in hepatic synthesis of bile acids by stimulating activity of the key liver enzyme cholesterol 7α-hydroxylase. Increasing bile acid synthesis requires cholesterol as the precursor, which results in the depletion of the cholesterol pool in liver. Therefore, on one hand, the number of LDL receptors on liver cells is increased in order to capture more circulating LDL-cholesterol (thus lowering the plasma LDL-cholesterol concentration). On the other hand, the synthesis of cellular cholesterol is increased by up-regulating the activity of the HMG-CoA reductase (Kim et al. 1977). The increase of cholesterol synthesis by bile acid sequestrants partially offsets the beneficial effect of increased steroid excretion (Kim et al. 1977). Thus, the mechanism of action has two perceived defects. One is that LDL lowering is dependent on the capacity of new receptor synthesis, meaning that FH homozygotes are refractory, but these patients are rare. The other is that LDL lowering is limited by the accompanying rise in cholesterol synthesis, but this disadvantage can be remedied by combination therapy with HMG-CoA reductase inhibitors (Contermans and Erkelens 1992). Even though cholestyramine is highly effective in the treatment of many patients with high plasma cholesterol concentrations, it unfortunately is not tolerated by all patients. Therefore, in spite of its proven usefulness, the bile acid sequestrant is not an ideal cholesterol-lowering agent (Endo 1994).
Neomycin is a cation aminoglycoside antibiotic that has a cholesterol-lowering effect. It acts by precipitating cholesterol within the intestinal lumen and thus inhibiting its absorption (Samuel 1979). Side effects, including nausea, diarrhea, and renal toxicity, limit long-term administration. Other agents acting to inhibit cholesterol absorption are not of outstanding efficacy—possibly because there is always the potential for a compensatory increase in cholesterogenesis—but the unabsorbed plant sterol β-sitosterol and the hydrogenated derivative, sitostanol, may be beneficial on low-dose administration (Heinemann et al. 1986).

Lifibrol (4-[4-[4-(1,1-dimethylethyl)phenyl]-2-hydroxybutoxy-benzoic acid) is a novel lipid-lowering agent. Lifibrol was initially studied in humans by Hasibeder et al. (1991). Lifibrol has primary effects on LDL-cholesterol, total cholesterol apo B, and Lp(a). The potency is similar to high doses of HMG-CoA reductase inhibitors or nicotinic acid and bile acid sequestrant combinations (Locker et al. 1995). The effects on serum triacylglycerols and HDL-cholesterol are also similar to the HMG-CoA reductase inhibitors. The mechanism of action of lifibrol is complex and seems to involve several modes of action, including inhibition of cholesterol synthesis (but lifibrol is not a HMG-CoA reductase inhibitor), inhibition of sterol absorption, and enhanced sterol excretion, or possibly enhanced LDL-receptor activity or bile acid negative feedback on HMG-CoA reductase. Lifibrol is well tolerated and could be useful in the therapy of primary hypercholesterolemia (Locker et al. 1995).

Ikeda et al. (1992) found that tea catechins, in particular their gallate esters,
effectively decrease cholesterol absorption by decreasing solubility of cholesterol in mixed micelles. This observation accounts for the hypocholesterolemic effect of tea catechins. Recently, Burnett (1994) discovered a very potent class of cholesterol absorption inhibitors represented by azetidinone. The effect of this compound on serum cholesterol concentrations has not been reported yet.

Fibric acid derivatives

The fibrates include clofibrate and its analogs, gemfibrozil, bezafibrate, and fenofibrate. They are useful for lowering serum triacylglycerols and increasing HDL concentrations. The principal action of clofibrate is on triacylglycerols, i.e. VLDL. The other analogs are more potent, particularly with respect to the elevation of HDL-cholesterol. Some (e.g., gemfibrozil) also decrease LDL-cholesterol concentrations in some patients (Fidge 1993). The mechanism of action of these fibric derivatives is still not fully understood. Their primary effect is a lowering of VLDL concentration. The concentration of other major lipoproteins, LDL or HDL, may also be altered, depending on which of the derivatives is used (Brewer 1992).

Clofibrate is the ethyl ester of p-chlorophenoxyisobutyric acid. The ester form is necessary for absorption from the intestine, but tissue and plasma esterases rapidly hydrolyze it to give the acid form, which is subsequently bound to albumin. Moreover, p-chlorophenoxyisobutyric acid increases the lipoprotein lipase activity that accelerates the hydrolysis of VLDL-triaclylglycerol and, consequently, the rate
of formation of LDL (Shepherd and Packard 1986). Other biochemical actions include decreases in plasma free fatty acid (FFA) concentrations, possibly by inhibition of adipocyte cyclic adenosine monophosphate (cAMP) generation, and a lowering of FFA output into the blood (Brewer 1992). In addition, the fibrates have been shown to inhibit acyl coenzyme A:cholesterol acyl transferase (ACAT) activity, thereby causing a decrease in the rate of cholesteryl ester formation and an associated rise in free cellular cholesterol concentration. This ACAT effect may explain both the increased biliary secretion of cholesterol and gallstone formation that sometimes accompany the use of these drugs. Also, fibrates inhibit HMG-CoA reductase activity (Bernt et al. 1978), a surprising result because fibrates clearly are not competitive inhibitors of the enzyme. The resultant inhibition of cholesterol synthesis and subsequent up-regulation of the LDL receptor pathway results in more cholesterol being removed from the plasma. This reasoning explains why some of the drugs lower both cholesterol and triacylglycerol concentrations.

Gemfibrozil is a member of the fibrate family that is tolerated better than clofibrate. It exerts additional influence on HDL metabolism by inducing synthesis of the nascent peptide (selectively increasing HDL₃) and by preserving apo E levels so that the uptake of VLDL remnants (apo E-receptor mediated) is augmented (Saku et al. 1985; Newton and Krause. 1986). Bezafibrate and fenofibrate are newer potent hypolipidemic agents that lower both triacylglycerol and cholesterol concentrations. They also decrease hepatic VLDL secretion, possibly by enhancing
β-oxidation of liver fatty acids, thus depleting the FFA pool available for triacylglycerol production (Fidge 1993). In addition to a decrease in VLDL-triacylglycerol concentration, these compounds also lead to a decrease in LDL-cholesterol concentration.

**Antilipolytics:** nicotinic acid, acipimox, and acifran

Nicotinic acid, unrelated to its vitamin effect, when administered in large doses, has a more pronounced impact on circulating triacylglycerol than on total cholesterol concentration, but there is a differential effect on lipoproteins such that VLDL declines and HDL rises. Nicotinic acid acts by inhibiting lipolysis, which is catalyzed by hormone-sensitive lipase, in white adipose tissue, probably by inhibiting adenylate cyclase activity (Saggerson, 1986). The inhibition of lipolysis in adipose tissue results in lower FFA availability for liver triacylglycerol production. The clinical use of nicotinic acid has been hampered by prostaglandin-mediated flushing, experienced after initial doses, which may be related to the rapidity of the attainment of nicotinic acid peak concentration in the circulation (Fears 1987). Therefore, several analogs have been developed. Among them are acipimox and acifran.

Acipimox (5-methylpyrazine carboxylic acid-4-oxide) is more potent as a hypolipidemic agent (Sommariva et al. 1985). However, the half-life of acipimox in blood is shorter than that for nicotinic acid. Like nicotinic acid, acipimox produced a decrease in VLDL and an increase in HDL₂ with an enhanced activity of
lipoprotein lipase.

Acifran (AY-25712; 4,5-dihydro-5-methyl-4-oxo-5-phenyl-furan-2-carboxylic acid) is also more potent than is nicotinic acid in laboratory animals (Cayen 1985). The half-life of plasma acifran is about the same as nicotinic acid. Acifran has been shown to cause a small decrease in total and LDL-cholesterol with a small increase in HDL-cholesterol.

**Antioxidants including probucol**

Oxidized lipoproteins have been of a great interest because they are suspected to cause increased atherogenicity (Berry 1992). Oxidized LDL activates differentiation and migration of macrophages. The scavenger receptors on macrophages recognize oxidized LDL and allow for subsequent phagocytosis. When the macrophage becomes filled with oxidized LDL-cholesterol, it becomes the foam cell that is typically observed in early atherosclerotic lesions. Under the continuing influence of excessive oxidative stress, the foam cells necrose, and the toxic contents are spilled into the intimal space. This process stimulates an inflammatory response whereby the neutrophils and more macrophages are recruited to the site, with resultant neointimal and smooth muscle proliferation and eventual plaque formation (Steinberg et al. 1989). Most of the oxidation occurs when lipoproteins leave the plasma and enter extracellular environments that lack adequate concentrations of natural antioxidants (Fidge 1993).

Antioxidants are chemical compounds with sparsely populated outer electron
shells that readily accommodate the unpaired electron characteristic of an oxygen free radical and thus neutralize this highly reactive species. Antioxidants that may be relevant to preventing the development of atherosclerosis in human include vitamin E (α-tocopherol), β-carotene, vitamin C (ascorbic acid), flavonoids, and selenium. It has been shown that vitamin E is probably the most effective antioxidant relative to preventing atherogenesis. Uptake of large doses of dietary vitamin E is associated with a decreased risk of coronary heart disease (Rimm et al. 1993; O'Keefe et al. 1995). Vitamin E inhibits the oxidation of LDL-cholesterol both *in vitro* (Esterbauer et al. 1991) and *in vivo* (Riemersma et al. 1991).

Probucol (2,2-bis[3,5-di-t-butyl-4-hydroxyphenylmercapto]propene) is a cholesterol lowering drug that decreases both LDL- and HDL-cholesterol concentrations but has no effect on VLDL-cholesterol or on triacylglycerol metabolism (Fears 1987). The mechanism of action is different from that of other hypolipidemic agents. Probucol increases the fecal excretion of bile acids and total fat but not sterol (Beynen 1986). It inhibits both hepatic cholesterogenesis and apo B synthesis. Even though probucol decreases the "good" (HDL) cholesterol, it has maintained an anti-atherogenic effect in several animal studies and in human trials (Walldius 1992). The presence of probucol in LDL prevents oxidation and impedes macrophage degradation (Parthasarathy et al. 1986), so that a direct anti-atherosclerotic action may coexist with the quantitative effect on LDL.
Other potential cholesterol-lowering agents

**ACAT inhibitors**: ACAT, acyl-coenzyme A:cholesterol acyltransferase, plays a key role in the intracellular cholesterol esterification (Suckling and Stange 1985). Cholesterol esterification is an essential step in cholesterol absorption in the intestine, cholesterol secretion from the liver, and cholesterol accumulation and the foam cell formation in the vascular wall. Therefore, an ACAT inhibitor may exhibit both hypocholesterolemic and antiatherosclerotic effects by blocking cholesterol esterification. A number of ACAT inhibitors have been reported and are classified into two major chemical types, one being long-chain aliphatic carboxylic acid amide derivatives and the other being urea derivatives (Picard 1993). Recently, Tawada et al. (1994) reported the synthesis of novel ACAT inhibitors known as 3-quinolylurea derivatives. These compounds were found to inhibit ACAT activity effectively *in vitro* and cause hypocholesterolemic effects *in vivo*.

**Squalene Synthase Inhibitors**: Squalene synthase catalyzes the second committed step in cholesterol biosynthesis (Agnew 1985). In the reaction, two molecules of farnesyl pyrophosphate (FPP) are condensed in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH) and Mg$^{2+}$ to form squalene. Therefore, squalene synthase has been the second potential therapeutic target for controlling cholesterol biosynthesis besides HMG-CoA reductase. There have been many reports of different classes of compounds that are potent inhibitors of squalene synthase. Three fungal metabolites, the zaragozic acids (sometimes...
called squalestatins), have been show in vitro to be inhibitors of rat liver squalene synthase between nanomolar and picomolar concentrations as well as able to substantially decrease cholesterol biosynthesis in vivo (Hasumi et al. 1993; Bergstrom et al. 1993). A second class of "nanomolar" inhibitors are biophosphonates (Amin et al. 1992; Ciosek et al. 1993), and a third class, which are FPP analogs, are the isoprenoid (phosphinylmethyl)phosphonates (Biller and Forster 1990; Biller et al. 1991). LoGrasso et al. (1994) compared the inhibition of yeast, rat, and human squalene synthase by zaragozic acid C and two FPP analogs. It was found that zaragozic acid C was a "nanomolar" inhibitor of all three isoenzymes. The isoprenoid phosphonate was a "micromolar" inhibitor of all three isoenzymes. An ether-linked phosphonate, however, was found to be 15- and 42-fold more potent an inhibitor of the mammalian forms of squalene synthase than it was for yeast squalene synthase. The results suggest potential differences in active-site binding residues in those isozymes (LoGrasso et al. 1994). Syntheses of squalestatin S1 (also known as zaragozic acid A) and zaragozic acid C have been reported (Kelly and Roberts 1995). These syntheses are considerable achievements because natural compounds that are identified as having a desirable biological activity may have low activity in vivo, a poor therapeutic selectivity, or detrimental side effects that potentially preclude them from medical use. Many derivatives must be synthesized and their biological activity evaluated to identify one with the greatest potency and selectivity, and the least toxicity. Clinical use of squalene synthase inhibitors will not be possible until outstanding preclinical data are
available.

Cyclophane: A cholesterol "cage" has been built recently that ultimately might be used to filter out excess cholesterol from the blood stream (Bradley 1994). Cyclophane is a ring-shaped molecule that can be tailored to large sizes by using organic synthesis. Two molecules of cyclophane, linked with acetylenes, create a molecule with a cavity 0.9 by 1.3 nanometers wide and 1.1 nanometers deep--just the right size for a cholesterol molecule. It was reported that when cholesterol was added to a cyclophane-water solution, cholesterol was accepted 190 times more than in normal water. Testosterone, a steroid hormone with a similar chemical structure as cholesterol, was trapped much less often, indicating the cage was fairly selective. It was believed that cyclophanes eventually could be incorporated into the membrane of a dialysis machine that would "vacuum" cholesterol from the bloodstream of patients with severe hypercholesterolemia in a "blood washing" procedure. However, much more needs to be learned about the selectivity of the cage before it is ready for clinical use.

Cholesterol Vaccine: Although cholesterol is a common constituent of cells, antibodies directed specifically against cholesterol could be induced by immunization (Swartz et al. 1988). Protection against atherosclerosis with vaccines has been discussed (Travis 1993). Variations of this approach have been tested in animals (Bailey 1994). It was shown that when cholesterol-fed rabbits were immunized with synthetic antigens in which cholesterol-esters (as haptens) were covalently linked to various protein carriers, significant decreases in serum
cholesterol and up to 90% protection against atherosclerotic plaques were observed. Travis (1993), however, noted possible long-term adverse effects of the vaccination procedure. In experiments lasting up to 9 months, the hypocholesterolemic effects of immunization persisted, but the protective effects against atherosclerosis gradually declined, despite monthly booster shots. Nevertheless, it is believed that cholesterol vaccination could become a useful adjunct to diet or drug therapy if the procedure can be validated in a more appropriate model. Many technical obstacles, however, would need to be overcome before the scenario of a routine "cholesterol vaccination" could become a reality (Bailey 1994).

Technologies to Decrease Cholesterol Content of Foods

Cholesterol content of animal-derived foods is a concern of consumers because intake of cholesterol may result in an increase of plasma cholesterol concentration, which is a major risk factor for coronary heart disease. The primary therapeutic intervention for patients at high risk for development or progression of coronary heart disease is to prescribe a cholesterol-lowering diet (The Expert Panel 1993). Therefore, much effort has been made to develop physical, chemical, and biological methods to remove or decrease the cholesterol content of animal-derived foods.
Physical processes

**Supercritical CO₂ Extraction**: Supercritical CO₂ extractions are performed under high pressure above the critical temperature of the solvent (31°C for CO₂). The intense pressure densifies the CO₂ that then solubilizes a portion of the lipid components and removes them from the food matrix. This technology has been used to extract cholesterol and other lipids from spray-dried egg yolk powders (Froning et al. 1990; Rossi et al. 1990). Cholesterol and triacylglycerols were preferentially removed from egg powders, while retaining the phospholipids that contribute to the functionality of the product. Supercritical fluid extraction also can be applied to meats and meat byproducts. For example, Hardardottir and Kinsella (1988) extracted 97% of cholesterol and 78% of other lipids from trout muscle with this process.

King et al. (1989) studied techniques for performing analytical extraction of lipids from meat products. They found that it was difficult to obtain efficient extraction of lipids from intact muscle because of the fibrous nature of the muscle structure and the high moisture content, both of which serve as barriers to penetration by CO₂. Comminuting and dehydrating the muscle prior to extraction improved the efficiency of the extraction process. King et al (1993) recently, however, reported that supercritical fluid extraction could be used effectively to decrease the total lipid and cholesterol content of preformed meat products, without requiring comminution of the sample. Beef patties (raw, raw freeze-dried, cooked, and cooked freeze-dried) were used in the study. It was found that freeze-
drying of the patties prior to supercritical CO₂ extraction improved removal of total lipids and cholesterol. Freeze-drying enhanced cholesterol extraction, whereas precooking had limited effects (King et al. 1993). The process, however, is far more successful with dehydrated meat products because of the physical nature and low moisture contents of these products. Also, dehydrated meats are usually cooked products; so, protein denaturation during extraction is not a concern.

Wehling et al. (1992) investigated (1) several factors that affect the extraction of lipid compounds from dehydrated beef and (2) different extraction conditions that affect the composition and sensory properties of the products. The results demonstrated that lipids were more easily extracted from chunks than from powders, allowing for the successful removal at lower temperatures and pressures. Extracted samples were lighter in color than the controls. The residual lipids in the extracted sample also contained higher relative percentage of linoleic and linolenic acids than did the lipids in the control samples, suggesting differential solubilities of lipids on the basis of fatty acid compositions. Taste panel evaluation of control and extracted powders found no significant differences in beef flavor, the presence of off-flavors, or overall acceptability (Wehling et al. 1992)

*Vacuum Steam Distillation:* Substances can be fractionated selectively by using vacuum molecular distillation (Boudreau and Arul 1993). Cholesterol is a compound of low volatility but is more volatile than are the major triacylglycerols of milk fat. Superheated steam can be bubbled through the oil, and the oil can be heated directly to provide for the latent heat of vaporization of the distilling
compounds and to prevent the condensation of steam; thus, temperature and pressure can be varied independently. When the sum of the partial vapor pressures of water vapor and the distillates is equal to the total pressure, water vapor and the low volatile compounds such as cholesterol, free fatty acids, and aroma distill over. It has been reported that this process can be used to decrease cholesterol content by over 90% from butter oil or milk (Marschner and Fine 1989). The advantages of steam distillation include that the equipment is commercially available and less expensive to operate than that for supercritical fluid extraction. The dairy industry can offer consumers a series of functional new products such as low cholesterol milk, yogurt, ice cream, cheese, and butter. Cholesterol removal through the steam-stripping process, however, may cause the formation of toxic oxidation products of cholesterol, particularly 7-hydroxy and 25-hydroxy cholesterols and cholestanetriol (Boudreau and Arul 1993).

Complexation processes

*Cyclodextrins*: $\alpha$-, $\beta$-, and $\gamma$-cyclodextrins, enzymatically derived from starch, are cyclic molecules that contain six, seven, and eight glucose units, respectively. They have a hydrophobic central cavity and form inclusion complexes with a large variety of hydrophobic and amphiphilic molecules including cholesterol (Abadie et al. 1994). $\beta$-Cyclodextrin usually is selected for this application because its ring structure best fits the cholesterol molecules for selective encapsulation. The encapsulated cholesterol then can be separated from the cyclodextrin. $\beta$-
Cyclodextrin has been used to remove cholesterol from animal fats, including milk cholesterol and egg cholesterol (Michael Foods 1992). *Simply Eggs*, liquid whole eggs with 80% less cholesterol than shelled eggs, were available on the shelf in food markets for a period of time but now are not produced because of a lack of sufficient commercial interest. Although cyclodextrin is used as a processing aid and not as an ingredient, FDA approval is needed because treated foods usually have a residual amount of cyclodextrin. Cyclodextrins, however, are approved for food use in Japan and several European nations.

*Saponins*: Sidhu and Oakenful (1989) have developed a method that is based on complexation of cholesterol onto saponin or cyclodextrin chemically bound to an inert solid support. This method can remove up to 90% of the cholesterol from milk or cream. It was claimed that this technology does not affect the components of the fat globule membrane of milk apart from its cholesterol. Use of saponins for removing cholesterol from foods is the subject of a pending patent; therefore, the full details have not been revealed at this stage.

**Biological processes**

Biological processes to remove or decrease the cholesterol content of foods involve the use of enzymes or enzyme-producing microorganisms, which have been reviewed under the topics of Cholesterol-Reducing Bacteria and Cholesterol Oxidase and Cholesterol-Oxidizing Microorganisms previously.
OPTIMIZATION OF CONDITIONS FOR GROWTH AND COPROSTANOL PRODUCTION OF *EUBACTERIUM COPROSTANOLIGENES* ATCC 51222

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Abstract

Conditions for growth and coprostanol production of *Eubacterium coprostanoligenes* ATCC 51222 were optimized in this study. The bacterium did not require cholesterol for growth, but it retained cholesterol-reducing activity in the absence of cholesterol. Lecithin was required for growth, but excess lecithin in media did not improve bacterial growth. Pyruvate, lactose, and most reducing agents improved bacterial growth and coprostanol production. The optimal pH range was 7.0 to 8.0 for both bacterial growth and coprostanol production. Addition of a buffer system in media did not seem necessary for bacterial growth but increased the coprostanol production. Optimal incubation temperature was 37°C to 39°C. Most antibiotics inhibited bacterial growth with the exception of tetracycline at a final concentration of 50 μg/ml. Resistance to tetracycline was dose-dependent. Ethanol as the solvent for dissolving tetracycline did not show any effect with a final concentration up to 1.3%. The mechanism for the tetracycline
resistance is unknown. No plasmid was found in the bacterium. Bile salts showed some inhibitory effects on bacterial growth. Knowledge of these growth conditions and effectors is necessary for future characterization and applications of *E. coprostanoligenes*.

**Introduction**

There has been a great interest in cholesterol because of its relationship with cardiovascular diseases. Excess serum cholesterol is a major risk factor for coronary heart disease, which remains a leading cause for human death in many developed countries including the United States (Thom et al. 1992; American Heart Association, 1994). Many cholesterol-lowering agents have been discovered and synthesized to treat hypercholesterolemic individuals (Endo 1992). Potential side effects of those drugs, however, have caused concerns about their clinical use (Erkelens et al. 1988). Also, many efforts have been made to develop physical, chemical, and biological technologies to decrease cholesterol contents of foods because dietary cholesterol intake affects serum cholesterol directly in many people (Fraser 1994; Pyörälä 1987).

Biological methods (whole cell or enzymatic) are often the preferred method of food treatment because they are usually less harsh, form fewer by-products, are easier to control, and are considered "natural" by consumer. Much of the research in decreasing cholesterol content of foods by biological methods has focused on the cholesterol oxidase that has been well characterized (Vrielink et al. 1991).
Unfortunately, attempts to use cholesterol-oxidizing bacteria to decrease the cholesterol content of foods has produced few positive results (Aihara et al. 1988, Johnson and Somkuti 1990). A novel method of using cholesterol reductase or cholesterol-reducing bacteria has been proposed to decrease cholesterol content of foods and to lower serum cholesterol concentrations in humans (Dehal et al. 1991). The enzyme, cholesterol reductase, catalyzes the reduction of cholesterol to coprostanol. Coprostanol, unlike cholesterol, is absorbed poorly from the human intestine (Bhattacharyya 1986). Therefore, foods treated with cholesterol reductase or cholesterol reductase-producing bacteria will have less or no cholesterol available for absorption when the foods are consumed. Introduction of cholesterol reductase or cholesterol reductase-producing bacteria into the small intestine is hypothesized to decrease absorption of both the endogenous and exogenous cholesterol.

Cholesterol-reducing bacteria have been isolated from feces of rats (Eyssen et al. 1973), humans (Sadzikowsky et al. 1977), and baboons (Brinkley et al. 1982). All these previously isolated bacteria, however, had characteristics that made common laboratory manipulations difficult. Recently, a unique cholesterol-reducing bacterium, *Eubacterium coprostanoligenes* ATCC 51222, was isolated and is much easier to culture in the laboratory (Freier et al. 1994). *E. coprostanoligenes* is a Gram-positive, anaerobic, nonfermentative bacterium that forms colonies on petri plates and converts up to 90% of cholesterol to coprostanol in its growth media but does not require cholesterol for growth. Thus, *E. coprostanoligenes* seems more
amenable than previously studied cholesterol-reducing bacteria for applications in the food and pharmaceutical industries. The present study was undertaken to optimize the growth conditions for culturing *E. coprostanoligenes* to achieve the maximal bacterial growth and coprostanol production. The information is necessary when proceeding toward further characterization and application of this cholesterol-reducing bacterium.

**Materials and Methods**

**Cultivation of bacteria**

A standard growth medium to culture *E. coprostanoligenes* contains the following components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g lactose, 5 g sodium pyruvate, 0.5 g sodium thioglycolate, 0.1 g lecithin (type IV-S, Sigma), 0.1 g CaCl₂·2 H₂O, and 1 mg resazurin. Cholesterol (ash-free, Sigma Chemical Co., St. Louis, MO) was added at a concentration of 0.02% in the medium if coprostanol production was of concern. The pH of the medium was adjusted to 7.5 with 3 N KOH. The Hungate technique was used for the anaerobic preparation of media and handling of the culture (Holdeman *et al.* 1977). Fresh medium was inoculated with 1% of a 2-d culture and incubated at 37°C.

**Protein determination**

Bacterial cultures were harvested by centrifugation at 10,000 x g for 10 min at 4°C, usually after incubation for 2-4 d at 37°C. The cell pellets then were
washed once and suspended in 25 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5). Total cellular protein was determined by the Bradford method with microtiter plates (Redinbaugh and Campbell 1985). A portion of the cell suspension was mixed with an equal volume of 2 N NaOH and incubated at 37°C overnight to solubilize the protein. Bovine serum albumin, treated in the same manner, was used as a standard. Total cellular protein concentration was used as an indicator of bacterial growth.

**Determination of coprostanol production**

After incubation at 37°C for 2-4 d, aliquots of bacterial cultures in cholesterol-containing media were taken through the lipid extraction procedure (Bligh and Dyer 1959). The amounts of coprostanol produced and residual cholesterol were determined by gas-liquid chromatography by the method of Oles et al. (1990). The lipid extract from the samples was evaporated to dryness under nitrogen. Sterols in the residue were etherified with 1,3-bis-(trimethylsilyl)trifluoroacetamide. A 5830A Hewlett Packard gas chromatograph was used. A 0.91 m x 4 mm internal diameter glass column, was packed with 3% SP-2250 on 100/120 Supelcoport (Supelco, Inc., PA). Operating conditions were: oven, 250-280°C; injector, 290°C; flame ionization detector, 290°C; nitrogen flow rate, 20 ml/min.
Results and Discussion

Effects of cholesterol

Cholesterol is not required for growth of *E. coprostanoligenes* (Freier et al. 1994). But, cholesterol was added to the growth medium because cholesterol-reducing activity of *E. coprostanoligenes* then could be determined in the same culture. The bacterium was cultured either in the presence or the absence of cholesterol, and the bacterial growth was determined (Figure 1). The results show that the growth rate and yield of *E. coprostanoligenes* were similar with or without cholesterol. Bacterial cells grown in cholesterol-free media retain cholesterol-reducing activity when incubated with micellar cholesterol substrate in resting cell assays (Li et al. 1995). Therefore, cholesterol reductase seems to be synthesized constitutively in *E. coprostanoligenes*. Most previously isolated cholesterol-reducing bacteria require cholesterol and plasmalogen for growth and cholesterol-reducing activity (Eyssen et al. 1973; Sadzikowski et al. 1977; Mott and Brinkley 1979). Cholesterol was suggested as a terminal electron acceptor in the metabolism of the microorganism (Eyssen et al. 1973). Because *E. coprostanoligenes* does not require cholesterol for growth, an alternate electron acceptor must be used when cholesterol is not available. Therefore, the role of cholesterol in the metabolism of this microorganism needs to be investigated further.
Figure 1. Effect of cholesterol on bacterial growth. Cell protein is a measure of growth. Chol-Free and Chol refer to growth media without and with cholesterol, respectively. Data are expressed as the mean of five samples ± standard error.

Effects of lecithin

Lecithin is required for the growth of *E. coprostanoligenes* (Freier et al. 1994). Effects of different concentrations of lecithin in the medium on bacterial growth was determined in this study (Figure 2). The bacterial growth rate was proportional to lecithin concentrations up to 0.05 mg/ml. The growth of *E. coprostanoligenes* was independent of lecithin concentrations over 0.05 mg/ml in the growth medium. *E. coprostanoligenes* has phospholipase activity and metabolizes lecithin, as indicated by the disappearance of lecithin and appearance of free fatty acids in the culture (Freier et al. 1994).
Figure 2. Effect of lecithin on bacterial growth. Cell protein is a measure of growth. Chol-Free and Chol refer to growth media without and with cholesterol, respectively. Each data point is an average of duplicate samples.

Mott and Brinkley (1979) observed that most previously isolated strains of cholesterol-reducing bacteria required plasmenylethanolamine for growth and cholesterol reduction to coprostanol. The plasmalogen was metabolized by those bacteria into unidentified end products. The first isolate of cholesterol-reducing bacterium, *Eubacterium* sp. strain ATCC 21408, was shown to have sphingomyelinase activity (Eyssen et al. 1973). None of the metabolites of sphingomyelin, however, supported growth of the bacterium. The ways in which phospholipase and sphingomyelinase activities are related to the bacterial growth and cholesterol reduction remain unknown. When glycerol or fatty acids were
added along with cholesterol to a growth medium, *E. coprostanoligenes* did not grow or reduce cholesterol, although growth occurred in the presence of phosphatidylcholine (Freier et al. 1994). Therefore, the enzymatic hydrolysis of phosphatidylcholine seems necessary for growth and cholesterol reduction. Calcium chloride was found to stimulate coprostanol formation by *E. coprostanoligenes*. The mechanism for the stimulation might be related to the fact that Ca$^{2+}$ is an activator of phospholipase C (Freier et al. 1994).

**Effects of pyruvate**

Pyruvate increases coprostanol production by *E. coprostanoligenes* in cholesterol-containing media (Freier et al. 1994). In resting cell assays, pyruvate was found also to stimulate cholesterol reductase activity of the bacterium (Li et al. 1995). The effect of different concentrations of pyruvate on the bacterial growth was determined in this study (Figure 3). The results show that cultures grew poorly without pyruvate in the medium. The yield of bacterial growth was proportional to the pyruvate concentrations up to 27 mM in the media. Greater concentrations of pyruvate did not improve the growth of *E. coprostanoligenes*. Freier et al. (1994) showed that pyruvate was metabolized by the bacterium because it disappeared from the growth medium and that, when pyruvate was added into the growth medium, significantly more acetic acid was produced. Pyruvate probably acts as an electron donor in cholesterol reduction. A similar observation was reported with 16-dehydroprogesterone reductase activity of *Eubacterium* sp. strain 144; pyruvate
Figure 3. Effect of pyruvate on bacterial growth. Cell protein is a measure of growth. Data are expressed as the mean of triplicate samples ± standard error.

functioned as a electron donor for 16-dehydroprogesterone reductase in both whole cells and cell-free extracts of the bacterium (Watkins and Glass 1991).

**Effects of lactose**

*E. coprostanoligenes* ferments lactose to produce much acid (Freier et al. 1994). Lactose was added to growth media to potentially increase the yield of bacterial growth. It was of concern, however, that the lower pH of the lactose-containing culture might affect the bacterial growth and coprostanol production. Therefore, media with or without lactose were prepared, inoculated, and cultured. The bacterial growth, coprostanol production, and the pH values of the cultures
were determined (Figure 4). The bacterial yield was significantly higher in cultures with lactose than without lactose. The highest bacterial yield was achieved when both lactose and pyruvate were in the growth medium (Figure 5). Coprostanol production was also much higher in cultures containing lactose. After 48 h of incubation, the pH was 6.38 ± 0.12 (mean ± SE, n=8) in cultures with lactose and 7.22 ± 0.01 (mean ± SE, n = 8) in cultures without lactose. Clearly, E. coprostanoligenes used energy produced from lactose fermentation for growth and cholesterol reduction. The lower pH of the culture can be used as a indicator for better bacterial growth, because, with the same medium, cultures with lower pH usually yield more bacteria than do the ones with higher pH. The effects of initial pH values in the growth medium on bacterial growth and coprostanol production will be shown and discussed later in this report.

Lactose and other carbohydrates were thought to inhibit coprostanol formation by cholesterol-reducing bacteria (Kellogg and Wostmann 1966; Subbiah et al. 1974; Wells and Cooper 1958) in vivo. With a pure culture of E. ATCC 21408, however, Eyssen et al. (1974) found that addition of lactose or glucose in the culture did not inhibit cholesterol-reducing activity of this bacterium and that the pH value dropped slowly from 7.3 before incubation to 6.5 after a 72-h incubation. But, when the cholesterol-reducing bacterium was cultured with lactose-fermenting bacteria such as Clostridium Cl-8, Escherichia coli, or Streptococcus faecalis, the pH of these mixed cultures dropped to values below 6 within 24 h, E. ATCC 21408 failed to grow adequately, and coprostanol
Figure 4. Effect of lactose on bacterial growth (cell protein) and coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Med+Lac and Med-Lac refer to media with and without lactose, respectively. Data are expressed as the mean of quadruple samples ± standard error.

Figure 5. Effect of lactose and pyruvate on bacterial growth. Cell protein is a measure of growth. Data are expressed as the mean of quadruple samples ± standard error.
production was inhibited. The inhibitory effect of the carbohydrate in mixed cultures could be overcome by maintaining the pH of the culture medium above 6.5 throughout the incubation period. In the present study, lactose not only did not show inhibitory effects but stimulated growth and coprostanol production of *E. coprostanoligenes*.

**Effects of reducing agents**

Reducing agents are particularly effective in stabilizing microbial enzymes that are derived from a reducing environment (Linn 1990). Some reducing agents such as dithiothreitol sometimes, however, cause inactivation of enzyme activities (Cayama and Apitz-Castro 1973; Gomez-Moreno and Palacian 1974; Trotta et al. 1974).

Sodium thioglycolate usually is added in the growth medium to culture *E. coprostanoligenes*. Effects of sodium thioglycolate and other reducing agents including β-mercaptoethanol, dithiothreitol, glutathione (reduced form), and L-cysteine on bacterial growth and coprostanol production were determined (Table 1). Sodium thioglycolate and β-mercaptoethanol stimulated the bacterial growth, dithiothreitol and glutathione had no effect, and L-cysteine inhibited bacterial growth. Coprostanol production was increased by sodium thioglycolate, β-mercaptoethanol, and glutathione but not by the other reducing agents. In resting cell assays, all the reducing agents except L-cysteine were stimulatory to cholesterol reductase activity of *E. coprostanoligenes* (Li et al. 1995).
Table 1. Effect of reducing agents on bacterial growth and coprostanol production.

<table>
<thead>
<tr>
<th>Reducing Agents</th>
<th>Cell Protein (μg/ml)(^a)</th>
<th>Coprostanol (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>331 ± 11</td>
<td>47.4 ± 1.3</td>
</tr>
<tr>
<td>Sodium thioglycolate</td>
<td>497 ± 38</td>
<td>56.6 ± 1.6</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>414 ± 14</td>
<td>50.1 ± 1.1</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>339 ± 16</td>
<td>47.2 ± 0.5</td>
</tr>
<tr>
<td>Glutathione (reduced form)</td>
<td>306 ± 13</td>
<td>52.3 ± 0.7</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>64 ± 9</td>
<td>39.0 ± 4.0</td>
</tr>
</tbody>
</table>

\(^a\) Mean of triplicate samples ± standard error. Cell protein is a measure of bacterial growth. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol.

Effects of initial pH values

Growth media with different initial pH values were prepared by addition of appropriate amounts of KOH or HCl. After incubation at 37°C for 2 d, the bacterial growth and coprostanol production were determined (Figure 6). Initial pH at 7.0 in the growth medium was the optimal pH for the bacterial growth. Growth was significant with the initial pH values between 6.5 and 8.5, whereas growth was sparse with the initial pH below 6.5 or above 8.5. Coprostanol production increased with the increase of initial pH values of growth media up to 7.0, remained stable between pH 7.0 and 8.5, and decreased when the initial pH value was above 8.5. When PIPES buffer (25 mM) was added into the growth medium at pH 7.5, bacterial growth was not improved but coprostanol production was increased significantly (Figure 7). In resting cell assays, the optimal pH for cholesterol reductase activity of *E. coprostanoligenes* was 7.5 with both PIPES buffer.
Figure 6. Effect of initial pH values of media on bacterial growth (cell protein) and coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Data are expressed as the mean of triplicate samples ± standard error.

Figure 7. Effect of PIPES buffer in media on bacterial growth (cell protein) and coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Med and Med+Buffer refer to growth media without and with PIPES buffer. Data are expressed as the mean of triplicate samples ± standard error.
and sodium phosphate buffer (Li et al. 1995). With different buffer systems, however, cholesterol reductase activity responded differently to the pH values.

Effects of incubation temperatures

Effects of different incubation temperatures on bacterial growth and coprostanol production were determined (Figure 8). The highest bacterial yield was achieved at 39°C. At 37°C, the bacterium growth was about 25% less. At 41°C, the bacterial yield was only half of that at 39°C. When incubation temperatures were above 41°C or below 35°C, no significant growth occurred. Similar results

![Figure 8](image)

Figure 8. Effect of incubation temperatures on bacterial growth (cell protein) and coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Data are expressed as the mean of triplicate samples ± standard error.
were observed with coprostanol production except that the optimal temperature for coprostanol production was 37°C instead of 39°C. Therefore, the temperature range for optimal bacterial growth and coprostanol production seems to be from 37°C to 39°C.

Effects of antibiotics

Antibiotics, including ampicillin, kanamycin, streptomycin, tetracycline, and chloramphenicol, were tested for their effects on the growth and coprostanol production of *E. coprostanoligenes* (Figure 9). All of these antibiotics except

![Figure 9. Effect of antibiotics on bacterial coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Amp = ampicillin, Kan = kanamycin, Str = streptomycin, Tet = tetracycline, and Chl = chloramphenicol. The final concentrations of antibiotics in media were 50 μg/ml. Incubation times are presented as 48 h and 96h. Data are expressed as the average of duplicate samples.](image-url)
tetracycline inhibited bacterial growth and coprostanol production at a final concentration of 50 μg/ml. After 48 h of incubation, cultures with tetracycline produced less coprostanol than did the controls; however, by 96 h, the cultures with tetracycline produced the same amount of coprostanol as did the controls.

This tetracycline resistance of *E. coprostanoligenes* was investigated further for dose and incubation time responses. To determine effects of ethanol, which was used as a solvent for tetracycline, the same concentrations of ethanol as in tetracycline cultures were added to the cultures without the antibiotics. Coprostanol productions in those cultures were determined (Figure 10). After 24 h of incubation, coprostanol production in cultures was not affected by ethanol.

**Figure 10.** Effect of ethanol and tetracycline on bacterial coprostanol production. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. EtOH = ethanol and Tet = tetracycline. Final concentrations of EtOH and Tet in cultures are presented in parentheses. Data are expressed as the average of duplicate samples.
but was lower with tetracycline than that in the controls. After 48 h of incubation, coprostanol production increased significantly in cultures with tetracycline at 50 \( \mu g/ml \), and became similar to that of the control after 72 h of incubation. Cultures with higher tetracycline (75 or 100 \( \mu g/ml \)), however, produced less coprostanol than did the control even after 96 h of incubation. Ethanol showed no effect on coprostanol production. The relationship between cholesterol reduction and tetracycline resistance was investigated. An attempt was made to search for a plasmid that might be responsible for tetracycline resistance and cholesterol-reducing activity. Accordingly, no plasmid was found in \( E. \) coprostanoligenes. The mechanism for tetracycline resistance of this bacterium, therefore, remains unknown.

**Effects of bile salts**

A primary bile salt, sodium taurocholate, and a secondary bile salt, sodium taurodeoxycholate, were added to the growth medium. An *in vivo* situation was mimicked by adding appropriate amounts of bile salts to reach the physiological concentrations of bile salts in human intestines. In bile, the concentration of bile salts is about 50 mM (Muraca et al. 1991). Cholate was added in the growth medium with a final concentration at 10 mM because a 5-fold dilution was assumed by the intestinal lumen fluid. Deoxycholate is formed by bacterial actions that occurs mostly in the large intestine after bile acid reabsorption; therefore, its concentration was set at 2.5 mM. Effects of these two bile salts on bacterial growth
Figure 11. Effect of bile salts on bacterial growth. Cell protein is a measure of growth. Media with cholate and deoxycholate are presented as + Cholate and + Deoxycholate, respectively. Data are expressed as the mean of triplicate samples ± standard error.

were determined (Figure 11). In the presence of bile salts, bacteria did not grow as well as did the control. The primary bile salt, cholate, showed a stronger inhibitory effect on growth than did the secondary bile salt, deoxycholate. When the concentration of cholate was decreased to 2.5 mM as that of deoxycholate, bacterial growth still was inhibited by cholate, indicating that the effect was not caused by higher concentration of cholate. Thus, effects of bile salts on bacterial growth need to be considered when bacterial doses are determined for animal feeding studies.

In summary, *E. coprostanoligenes* did not require cholesterol for growth and retained cholesterol-reducing activity in the absence of cholesterol. Lecithin was required for growth but excess lecithin in media did not improve bacterial growth.
Pyruvate, lactose, and most reducing agents improved bacterial growth and coprostanol production. The optimal pH range for bacterial growth and coprostanol production was 7.0 to 8.0. Addition of a buffer system to media was not necessary for bacterial growth but increased coprostanol production. The optimal incubation temperature was 37°C to 39°C. Most antibiotics inhibited bacterial growth and coprostanol production with the exception of tetracycline at a final concentration of 50 μg/ml. The resistance to tetracycline was dose-dependent. Ethanol as the solvent for dissolving tetracycline did not affect growth or coprostanol production at a final concentration up to 1.3%. The mechanism for tetracycline resistance is unknown. No plasmid was found in the bacterium. Bile salts slightly inhibited bacterial growth. The information on optimal conditions is necessary for future characterizations and applications of *E. coprostanoligenes*.

References Cited


A RESTING CELL ASSAY FOR CHOLESTEROL REDUCTASE ACTIVITY
IN EUBACTERIUM COPROSTANOLIGENES ATCC 51222

A paper accepted by Applied Microbiology and Biotechnology

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Jerry W. Young, Donald C. Beitz

Abstract

A resting cell assay was established to evaluate the cholesterol reductase (CR) activity of Eubacterium coprostanoligenes ATCC 51222. Cell suspensions from cholesterol-free media rapidly reduced cholesterol to coprostanol. Optimal assay conditions in a 1-ml reaction mixture were determined to be up to 1 h of incubation and up to 0.25 mg of bacterial protein per assay with at least 1 mM of cholesterol as substrate. The CR activity in cells decreased as a function of storage time at 22°C, 4°C, and -20°C. Filling the headspace of the reaction mixture with H₂ increased the CR activity about 20%. Optimal CR activity occurred at pH 7.5 in sodium phosphate buffer. Pyruvate and reducing agents in the buffer increased the CR activity. This study has validated assay conditions for determination of CR activity in resting cells of E. coprostanoligenes.
Introduction

It was established in the 1930s that cholesterol is reduced enzymatically to the saturated product coprostanol by intestinal bacteria (Schoenheimer 1931). The enzyme responsible for the reaction is now named cholesterol reductase (CR) (Dehal et al. 1991). Depending on diet composition, coprostanol can represent more than 50% of total fecal sterols of humans. Coprostanol, unlike cholesterol, is absorbed poorly by the human intestine (Bhattacharyya 1986), and any increase in formation of coprostanol could decrease absorption of cholesterol. Cholesterol-reducing bacteria have been isolated from the feces of rats (Eyssen et al. 1973), humans (Sadzikowsky et al. 1977) and baboons (Brinkley et al. 1982). These previously isolated bacteria, however, had growth characteristics that made common laboratory manipulations difficult. Growth requirements included the need for plasmalogen, which is found only in high concentrations in mammalian brain tissue, and the need for complete anaerobiosis. Further, those bacteria did not form colonies on solid plating media. Attempts to detect CR activity in extracts of those bacterial cells were unsuccessful (Björkhem et al. 1973). Recently, a unique cholesterol-reducing bacterium, designated *Eubacterium coprostanoligenes* ATCC 51222, that is much easier to grow in the laboratory was isolated (Freier et al. 1994). *E. coprostanoligenes* is a Gram-positive, anaerobic, nonfermentative bacterium that forms colonies on lecithin-containing solid media. It converts up to 90% of cholesterol to coprostanol in cholesterol-containing media, but it does not require plasmalogen or cholesterol for growth. Thus, *E. coprostanoligenes* seems
more amenable than previously studied cholesterol-reducing bacteria for applications in the food and pharmaceutical industries. An active, stable, cell-free enzyme preparation of *E. coprostanoligenes* would be even more ideal. The present study was undertaken to validate an assay for the CR activity of resting cells of *E. coprostanoligenes* as we attempt to define conditions leading to maximal production of CR activity.

**Materials and Methods**

**Growth of bacteria**

*E. coprostanoligenes* ATCC 51222 was grown in a cholesterol-free medium that contained the following components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g lactose, 5 g sodium pyruvate, 1 g CaCl$_2$·2 H$_2$O, 0.5 g sodium thioglycolate, 0.1 g lecithin (type IV-S, Sigma) and 1 mg resazurin. The pH was adjusted to 7.5 with 3 N KOH. The Hungate technique was used for the anaerobic preparation of media and handling of cultures (Holdeman et al. 1977). Fresh medium was inoculated with 1% of a 48-h culture and incubated at 37°C for 48 h.

**Preparation of cell suspensions**

After incubation for 48 h at 37°C, cultures were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. The cell pellets then were suspended in 25 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5)
containing 5 mM sodium thioglycolate and 45 mM sodium pyruvate. No precaution was taken to maintain anaerobiosis during preparation of cell suspensions.

**Preparation of micellar cholesterol substrate**

Cholesterol (58 mg), [1α, 2α-3H]-cholesterol (20 μCi) and lecithin (148.8 mg) were dissolved in chloroform (20 ml). The solution was evaporated to dryness at about 35°C. After the addition of PIPES buffer (20 ml) and glass beads, the solution was mixed vigorously in a vortex mixer until all lipid was dispersed. The lipid dispersion was sonicated for 5 minutes in ice by a sonifier. Residue was removed by centrifugation at 1,800 x g for 10 minutes, and the micellar solution was stored at 4°C. It contained 7.5 mM cholesterol (133 μCi/mmol) and 10 mM lecithin. These preparation procedures were modified from Dehal et al. (1991).

**Resting cell assay for CR activity**

The standard assay contained 1 ml of bacterial cell suspension (= 0.4 mg cell protein) in 25 mM PIPES buffer at pH 7.5, 45 mM sodium pyruvate, 5 mM sodium thioglycolate, 1.33 mM lecithin and 1 mM micellar cholesterol. Control assay mixtures contained bacterial suspension that had been boiled for 10 min. The assay was started by addition of micellar cholesterol. Each assay was conducted in triplicate, and results are expressed as mean ± standard error (SE). Student’s t test was employed to analyze the difference among treatments. Probability (P) less
than 0.05 was considered significant. After incubation at 37°C for 1 h with shaking, the assay mixture was extracted with 1:1:0.8 chloroform:methanol:H₂O (Bligh and Dyer 1959). The organic extract was concentrated under N₂ to about 100 μl. Sterols were separated on thin-layer chromatography (TLC) plates by using a 3:1 solvent system of hexane:ethyl acetate (v/v). Bands corresponding to cholesterol and coprostanol were scraped into scintillation vials, and radioactivity was determined by using a Beckman LS-1701 liquid scintillation counter. Under these assay conditions, one unit (U) of activity is equal to 1 nmole of coprostanol produced per hour, and specific activity is equal to U/mg protein.

**Protein determination**

Total cellular protein was determined by the Bradford method with microtiter plates (Redinbaugh and Campbell 1985). A portion of the cell suspension was mixed with 2 N NaOH and incubated at 37°C overnight to solubilize the protein. Bovine serum albumin, treated in the same manner, was used as a standard.

**Results**

**Effects of storage temperature and time on CR activity**

*E. coprostanoligenes* is an anaerobic bacterium and grows only in a pre-reduced, anaerobically sterilized (PRAS) medium. It can, however, survive exposure to air for at least 48 h (Freier et al. 1994). Therefore, no precautions
were taken to maintain anaerobiosis during preparation of the cell suspensions.

Assays for CR activity are not always conducted immediately after a suspension of bacterial cells is prepared. Therefore, effects of temperature (22, 4 and -20°C) and time (2 and 24 h) for storing cell suspensions after harvesting cells were determined (Figure 1). The CR activity of resting cells decreased over time at all temperatures tested. The decrease was especially evident at -20°C. Therefore, the resting cell assays were conducted immediately after a suspension of bacterial cells was prepared for all subsequent assays.

Figure 1. Effects of storage temperature and time on CR activity. After bacterial cells were harvested from the growth medium, the CR activity assays were conducted immediately (■) or after cell suspension were kept at room temperature (22°C), 4°C or -20°C for 2 h (□□) or 24 h (□□□). Standard assay conditions (see Materials and Methods) were used in this experiment. Data are expressed as the mean of triplicate assays + standard error.
Figure 2. Effects of the composition of headspace gases on CR activity. The headspace of the reaction mixture was filled with air, N\textsubscript{2} or H\textsubscript{2}. Standard assay conditions (see Materials and Methods) were used in this experiment. Data are expressed as the mean of triplicate assays + standard error.

**Effects of composition of headspace gases on CR activity**

H\textsubscript{2} and CO\textsubscript{2} are produced as headspace gases by *E. coprostanoligenes* during growth in PRAS medium (Freier et al. 1994). To determine effects of gas environment on CR activity of resting cells, the headspace above the reaction mixture was filled with either air (control), N\textsubscript{2} or H\textsubscript{2}. Data from the experiment are shown in Figure 2. Filling the headspace with H\textsubscript{2} significantly increased the CR activity above that with air (P < 0.01). Compared with the air control, filling
with N\textsubscript{2} also increased CR activity, but the increase was not as significant as with H\textsubscript{2} because of the variation in CR activity from N\textsubscript{2} treatment (P = 0.10). The headspace was not filled with N\textsubscript{2} or H\textsubscript{2} for all subsequent assays.

**Effects of buffers and pH on CR activity**

Usually, PIPES buffer at pH 7.5 was used in the resting cell assay. To determine the effects of different buffers and pH values on CR activity, the following buffers were tested within their effective buffering ranges: sodium acetate buffer (pH 4.5 to 6.0), PIPES buffer (pH 6.0 to 7.5), sodium phosphate buffer (pH 6.0 to 7.5) and TAPS (tris-[hydroxymethyl]-methylaminopropane sulfonic acid) buffer (pH 7.5 to 9.0). No CR activity was observed below pH 5.0 (Figure 3). Maximal CR activity was obtained at pH 7.5 with phosphate buffer. With PIPES buffer, pH 7.5 also was optimal for CR activity. With TAPS buffer, however, CR activity was substantial at pH values of 8.5 and 9.0.

**Effect of pyruvate on CR activity**

Addition of pyruvate to a growth medium containing cholesterol stimulated coprostanol production by *E. coprostanoligenes* (Freier et al. 1994). The effect of pyruvate on CR activity of resting cells was determined in the present study (Figure 4). The CR activity increased with increasing pyruvate concentration in the reaction mixture up to about 36 mM.
Figure 3. Effect of buffers (v, sodium acetate; □, sodium phosphate; ○, PIPES; △, TAPS) and pH on CR activity. Buffers were used at a final concentration of 25 mM. The pH was adjusted by KOH or HCl. The other assay conditions were as described in the text (see Materials and Methods). Data are expressed as the mean of triplicate assays ± standard error.

Figure 4. Effect of pyruvate on CR activity. Except pyruvate concentrations, standard assay conditions (see Materials and Methods) were used in this experiment. Data are expressed as the mean of triplicate assays ± standard error.
Effects of reducing agents on CR activity

Sodium thioglycolate normally was used as a reducing agent in the buffer for the resting cell assay, and it caused about 20% increase in the CR activity. Other reducing agents, including β-mercaptoethanol, dithiothreitol, glutathione (reduced form) and L-cysteine, were compared for their effects on CR activity of *E. coprostanoligenes* (Table 1). Among them, β-mercaptoethanol and sodium thioglycolate had the greatest stimulatory effect (P < 0.01); however, all the reducing agents except L-cysteine were stimulatory to CR activity (P < 0.05).

<table>
<thead>
<tr>
<th>Reducing agenta</th>
<th>CR (U/mg protein)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>363 ± 7.9c</td>
</tr>
<tr>
<td>Sodium thioglycolate</td>
<td>439 ± 8.2d</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>449 ± 4.1d</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>413 ± 24.0d</td>
</tr>
<tr>
<td>Glutathione (reduced form)</td>
<td>412 ± 16.1d</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>380 ± 9.9c</td>
</tr>
</tbody>
</table>

a The final concentration of reducing agents was 5 mM in 25 mM PIPES buffer at pH 7.5. The control contained no reducing agents. Except reducing agents, the standard assay conditions (see Materials and Methods) were used in this experiment.

b Mean of triplicate assays ± standard error.

c,d Means with different superscripts are different (P < 0.05).
Validation of the assay was achieved by measuring the effects of incubation time, cell protein and cholesterol concentrations on CR activity of resting cells (Figure 5a-c). The time course of cholesterol conversion to coprostanol is shown in Figure 5a. The reaction was linear up to 1 h of incubation. The dependence of CR activity on bacterial cell protein concentration is illustrated in Figure 5b. Coprostanol production as a function of increasing cell protein was linear up to 0.25 mg bacterial cell protein in the assay. Figure 5c shows the relationship between cholesterol substrate concentration and coprostanol production. Maximal CR activity was achieved at about 1 mM of cholesterol in the reaction mixture.

Discussion

The present study provides the follow-up data on factors that affect the conversion of cholesterol to coprostanol by resting cells of \textit{E. coprostanoligenes} after the initial report on isolation of the bacterium (Freier et al. 1994). Cell suspension of \textit{E. coprostanoligenes} catalyzed a rapid enzymatic reduction of cholesterol to coprostanol, with a rate as high as 1200 nmole/h·mg protein. There were no significant differences in the CR activities of cultures from growth medium with or without cholesterol (data not shown). Resting cells from cholesterol-free medium possessed CR activity, which indicates that CR is synthesized constitutively and not inducibly by \textit{E. coprostanoligenes}. 
Figure 5a-c. Effects of incubation time, cell protein and cholesterol concentration on CR activity. a. Incubation time. The reaction mixture contained 0.4 mg bacterial cell protein and 1 mM cholesterol substrate. b. Bacterial cell protein concentration. The reaction mixture was incubated for 1 h with 1 mM cholesterol substrate. c. Cholesterol substrate concentration. The reaction mixture contained 0.4 mg bacterial cell protein and was incubated for 1 h. Except conditions indicated above, other assay conditions were as described in the text (see Materials and Methods). Data are expressed as the mean of triplicate assays ± standard error.
The CR activity declined over time after cells were harvested from cultures. This decline in activity occurred even though *E. coprostanoligenes* survives exposure to air for up to 48 h and even though there is no significant difference in CR activity of resting cells whether the cell suspension was prepared aerobically or anaerobically. The instability of CR activity is similar to that of both mammalian and microbial 3-oxo-Δ^4^-steroid 5β-reductase activity (Björkhem et al. 1973). Keeping cells at -20°C for 24 h almost completely destroyed their cholesterol-reducing ability. Loss of cell membrane integrity through freezing and thawing might account for this loss of CR activity because a great loss of CR activity occurred when *E. coprostanoligenes* was disrupted by sonication, passage though a French pressure cell or enzymatic digestion (unpublished data).

The CR activity of *E. coprostanoligenes* was increased by filling the headspace of the reaction vessel with H₂ in the resting cell assay (*P* < 0.01). Hydrogen gas has been reported to stimulate 16-dehydroprogesterone and progesterone reductases of *Eubacterium* sp. strain 144 (Glass and Burley 1985) and the deoxycorticosterone-21-dehydroxylase activity of *E. lentum* VPI 11122 (Feighner and Hylemon 1980). Hydrogen gas was thought to be an exogenous electron donor for the reduction reaction because hydrogenase activity was detected in both organisms. Whether hydrogenase activity is present in *E. coprostanoligenes* was not determined.

Effects of buffers and pH on CR activity were intriguing. Optimal CR activity occurred at pH 7.5 in both sodium phosphate and PIPES buffers. Higher
pH values could not be tested with the same buffer systems because of their ineffective buffering capacity over pH 7.5. In TAPS buffer, however, CR activity was inhibited at pH 7.5 but recovered at higher pH. The dramatically different responses to different buffers and pH values also were reported in other studies about steroid double bond-reductases in *Eubacterium* (Glass et al. 1991; Watkins and Glass 1991). Evidently, different chemical compositions of the buffer systems contribute to the difference in enzyme activities at the same pH. We suggest use of sodium phosphate buffer at pH 7.5 in further studies of CR activity in *E. coprostanoligenes*.

Pyruvate increases coprostanol production by *E. coprostanoligenes* in cholesterol-containing growth media (Freier et al. 1994). In the present study, pyruvate also stimulated the CR activity of *E. coprostanoligenes* in the resting cell assay. Pyruvate might act as a cofactor in cholesterol reduction because pyruvate stimulated 16-dehydroprogesterone reductase activity in both resting cells and cell extracts of *Eubacterium* sp. strain 144 (Glass and Burley 1985; Watkins and Glass 1991). More definitive data on the interrelationships of pyruvate and CR will become available when activity can be assayed in cell extracts of *E. coprostanoligenes*.

The mechanism of cholesterol reduction has been investigated by a number of researchers. Two pathways, direct and indirect, have been proposed for the conversion (Figure 6). The direct pathway was supported by the fact that no significant change in the labelling pattern of suitably marked cholesterol took place
Figure 6. Proposed reaction pathways for enzymatic conversion of cholesterol to coprostanol (Björkhem and Gustafsson 1971). I. Direct pathway. II. Indirect pathway: 1, oxidation; 2, nonenzymatic isomerization; 3, reduction; 4, reduction.

(Rosenfeld and Gallagher 1964). This pathway, however, was later questioned on the ground that intermediate cofactors may be involved in removing and restoring the label (Björkhem and Gustafsson 1971). Work with *Eubacterium* sp. ATCC 21408 confirmed the presence of the intermediates, $\Delta^4$-cholestenone and coprostanone, during the cholesterol-to-coprostanol conversion (Parmentier and Eyssen 1974). Ren (1991) observed that *E. coprostanoligenes* also reduced cholesterol by an indirect pathway. In the present study, substantial amounts of 4-cholesten-3-one and trivial amounts of coprostanone were detected in addition to coprostanol in the resting cell assay (data not shown). No intermediates were detected in the actively growing culture, however, when cholesterol was included in the growth medium. Coprostanol was the only measurable end product of
cholesterol reduction by *E. coprostanoligenes* in 24-h cultures (Freier et al. 1994).

The exact mechanism of cholesterol reduction by *E. coprostanoligenes* can be studied only after pure preparations of CR become available.

Results of the present study indicate that CR activity in *E. coprostanoligenes* can be maximized by careful selection of assay conditions. We have validated the assay conditions for determination of CR activity in resting cells of *E. coprostanoligenes*. Knowledge of optimal assay conditions are required to proceed toward purification of CR from this bacterium.

**Acknowledgments**

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CHARACTERIZATION OF A CHOLESTEROL-REDDUCING ANAEROBE,

*EUBACTERIUM COPROSTANOLIGENES ATCC 51222*

A paper prepared for submission to *Journal of Bacteriology*

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**Abstract**

The present study characterized *Eubacterium coprostanoligenes* ATCC 51222 from its electron microscopic structure and DNA composition to the mechanism of cholesterol reduction, substrate specificity, and effects of several cofactors and inhibitors. *E. coprostanoligenes* was shown to be a Gram-positive coccobacillloid bacterium. The cells were 0.5 to 0.7 μm in diameter and 0.7 to 1.0 μm in length and occurred both singly and in pairs. The bacterium reduced cholesterol to form coprostanol through an indirect pathway, and it seems to have a broad specificity for substrates. The bacterium converted all detected reaction intermediates to coprostanol, and it metabolized pregnenolone, progesterone, androstenediol, and etienic acid. In resting cell assays, either NADH or FAD increased cholesterol reductase activity, but activity was inhibited when NADH and FAD were added together. Iodoacetate, NaCN, and NaN₃ did not affect enzyme activity significantly, whereas EDTA inhibited activity, and HgCl₂ abolished activity completely. The enzyme activity was found to be associated with bacterial cells,
and attempts to achieve active cell-free extracts were not successful because a great loss of activity occurred after the bacterial cells were disrupted. Addition of different cofactors did not recover the cholesterol reductase activity. The genomic expression library of *E. coprostanoligenes* was constructed in an expression vector, bacteriophage λ vector λgt11. Several efforts were made to develop a screening technique for detection of positive clones for cholesterol reductase. The cholesterol oxidase gene (*cho*) was considered a possible probe, but the preliminary experiment did not show hybridization of the gene with the genomic DNA of *E. coprostanoligenes*. Studies are still in progress to develop ways to isolate the enzyme or the gene.

**Introduction**

A novel cholesterol-reducing anaerobic bacterium, *Eubacterium coprostanoligenes* ATCC 51222, has been isolated recently (Freier et al. 1994). This bacterium is of great interest because of its potential use to decrease serum cholesterol concentrations in hypercholesterolemic individuals and to decrease cholesterol contents of foods. Elevated plasma cholesterol is a major risk factor for atherosclerosis that results in coronary heart disease (CHD), which remains a leading cause of human mortality in Western countries (Thom et al. 1992). Dietary cholesterol has been of concern because of its potential to increase serum cholesterol concentration (Fraser 1994). Lowering plasma cholesterol concentrations has been shown to decrease the risk of CHD death and nonfatal
CHD events (Scandinavian Simvastatin Survival Study Group 1994). Decreasing of daily intake of dietary fat and cholesterol has been recommended to decrease plasma cholesterol and CHD risk in populations (Report of the Nutrition Committee 1993).

Cholesterol-reducing bacteria convert cholesterol to coprostanol, which is absorbed poorly from the gastrointestinal tract (Bhattacharyya 1986). Therefore, a novel method has been proposed to decrease cholesterol contents of foods and to lower elevated plasma cholesterol concentration by using cholesterol reductase or cholesterol-reducing bacteria (Dehal 1991). Cholesterol-reducing bacteria have been isolated from feces of rats (Eyssen et al. 1973), humans (Sadzikowsky et al. 1977), and baboons (Brinkley et al. 1982). All these previously isolated bacteria, however, had characteristics that made common laboratory manipulations difficult. The recent isolate, *E. coprostanoligenes*, has many unique characteristics that make the bacterium more amenable for future applications in food processing and pharmaceutical industries (Freier et al. 1994). *E. coprostanoligenes* reduces more than 90% of cholesterol to coprostanol in growth media but does not require cholesterol for growth. It grows under anaerobic conditions but does not require anaerobiosis for cholesterol reduction. A resting cell assay has been developed to analyze the cholesterol reductase activity of *E. coprostanoligenes* (Li et al. 1995b). Oral administration of *E. coprostanoligenes* has caused a significant decrease of plasma cholesterol concentrations in hypercholesterolemic rabbits (Li et al. 1995a).
Although foods treated directly with *E. coprostanoligenes* may not be readily accepted for human consumption, a pure preparation of cholesterol reductase from the bacterium would be more ideal to the treatment of foods and hypercholesterolemic individuals. Mass production of cholesterol reductase for commercial use would be more feasible economically if the gene(s) encoding cholesterol reductase from *E. coprostanoligenes* could be inserted into faster growing *Saccharomyces cerevisiae*, *Escherichia coli*, or other appropriate hosts. Another cholesterol metabolizing enzyme, cholesterol oxidase, converts cholesterol to 4-cholesten-3-one through 5-cholesten-3-one as an intermediate (Smith and Brooks 1977). Cholesterol reductase catalyzes the same reactions initially (Ren 1991). The cholesterol oxidase genes (*cho*) have been isolated from *Streptomyces* and *Brevibacterium* species (Murooka et al. 1986; Fujishiro et al. 1990). Extensive homology was found between *cho* genes from these two microorganisms (Ohta et al. 1991), and it was concluded that the genes are clearly from a common ancestor. It is hypothesized in this study that the genes encoding cholesterol reductase and cholesterol oxidase would share some homology because cholesterol reductase and cholesterol oxidase both must bind cholesterol and show similar catalytic functions. This hypothesis provides a potential method to isolate the cholesterol reductase gene from *E. coprostanoligenes* by using a *cho* gene as a probe.

The present study was conducted to characterize further the properties of *E. coprostanoligenes*. The mechanism, substrate specificity, and cofactors and inhibitors of cholesterol reduction were investigated. Attempts were made to
develop active cell-free preparations and to isolate the cholesterol reductase
gene(s) from the bacterium. Such information is necessary for manipulating the
cholesterol-reducing system of *E. coprostanoligenes* for potential use in commercial
biotransformation processes.

**Materials and Methods**

**Bacterial cultivation**

A growth medium to culture *E. coprostanoligenes* contained the following
components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g
lactose, 5 g sodium pyruvate, 0.5 g sodium thioglycolate, 0.1 g lecithin (type IV-S,
Sigma), 0.1 g CaCl₂·2 H₂O, and 1 mg resazurin. Cholesterol (ash-free, Sigma
Chemical Co., St. Louis, MO) was added at a concentration of 0.02% in the
medium if coprostanol production in the actively growing culture was of concern.
Other steroids, including 5-cholesten-3-one, 4-cholesten-3-one, coprostanone,
pregnenolone (4-pregnen-3β-ol-20-one), progesterone (4-pregnen-3, 20-dione),
androstenediol (5-androst-3β, 17β-diol), and etienic acid (5-androsten-3β-ol 17β-
-carboxylic acid), were added in media instead of cholesterol for determination of
substrate specificity. The pH of the medium was adjusted to 7.5 with 3 N KOH.
The Hungate technique was used for the anaerobic preparation of media and
handling of the culture (Holdeman et al. 1977). Fresh medium was inoculated with
1% of a 2-d culture and incubated at 37°C for 2 to 5 d.
Electron microscopy

After 2 d of incubation at 37\(^{\circ}\)C, bacterial cultures were harvested by centrifugation at 10,000 x g for 15 min at 4\(^{\circ}\)C. The cell pellets then were washed three times and suspended in 25 mM PIPES (piperazine-N,N'bis-[2-ethanesulfonic acid]) buffer (pH 7.5). The bacterial suspensions were fixed in 4\% paraformaldehyde-3\% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4\(^{\circ}\)C, washed three times in the same buffer, and postfixed at 4\(^{\circ}\)C for 1 h in the same buffer containing 1\% osmium tetroxide. The prepared cells were washed three times in buffer, placed in a microcentrifuge tube containing 1\% agar (liquid), and pelleted. The pellet was dehydrated with a graded series of ethanol and embedded in LR white resin with constant agitation. The resin was polymerized overnight in an oven at 65\(^{\circ}\)C. Sections were cut with a Reichert Ultracut E ultramicrotome, placed on 200-mesh copper grids, poststained in a solution containing uranyl acetate and lead citrate, and viewed with a JEOL model 1200EX scanning transmission electron microscope operated at 80 kV.

Determination of coprostanol production in growing cultures

After incubation at 37\(^{\circ}\)C for up to 5 d, an aliquot of the bacterial cultures in cholesterol-containing media was taken through the lipid extraction procedure (Bligh and Dyer 1959). The amounts of residual cholesterol and coprostanol produced were determined by gas-liquid chromatography (GLC) by the method of Oles et al. (1990) with 5\(\alpha\)-cholestane as an internal standard. The lipid extract
from the samples was evaporated to dryness under a stream of N₂. Sterols in the
residue were etherified with 1,3-bis-(trimethylsilyl)trifluoroacetamide and assayed
with a 5830A Hewlett Packard gas chromatograph. The glass column, 0.91 m x 4
mm internal diameter, was packed with 3% SP-2250 on 100/120 Supelcoport
(Supelco Inc., PA). Operating conditions were: oven, 250-280°C; injector, 290°C;
flame ionization detector, 290°C; nitrogen flow rate, 20 ml/min. Thin-layer
chromatography (TLC) was performed also to determine the products of
cholesterol reduction. Sterols in lipid extracts were separated by TLC with
standards chromatographed on each plate by using hexane:ethyl acetate (75:25 v/v)
as a solvent. After spraying with a 0.2% ethanolic solution of 2,7-
dichlorofluorescein, sterols were identified under ultraviolet light.

Resting cell assays

Assays were conducted as described by Li et al. (1995b). Namely, bacteria
were cultured in cholesterol-free media for 2 d at 37°C before being harvested by
centrifugation. Then, the washed bacterial cells were suspended in 25 mM sodium
phosphate buffer at pH 7.5 with 45 mM sodium pyruvate and 5 mM sodium
thioglycolate. Different cofactors and metabolic inhibitors were added to reaction
mixtures respectively to determine their effect on cholesterol reductase activity of
the bacterium. Control assay mixtures contained bacterial suspension that had
been boiled for 10 min. Assays were started by addition of the [1α, 2α-³H]-micellar
cholesterol substrate. After incubation at 37°C, assay mixtures were extracted, and
organic extracts were concentrated under a stream of N₂ to about 100 µl. Sterols were separated by TLC with standards chromatographed on each plate. Corresponding bands were scraped into scintillation vials for determination of radioactivity.

Preparation of cell-free extract

Possible extracellular cholesterol reductase from *E. coprostanoligenes* cultures was prepared by precipitating the supernatant of 2-d cultures with 40% and then 65% ammonium sulfate. Precipitates were dissolved in 25 mM sodium phosphate buffer (pH 7.5) containing 45 mM sodium pyruvate and 5 mM sodium thioglycolate. Both the supernatant and the precipitate solution were dialyzed against the phosphate buffer overnight at 4°C. Cholesterol reductase activity in the preparation was determined by incubation with [1α, 2α-³H]-micellar cholesterol as substrate. A crude cell-free extract was prepared by disrupting bacterial cells with sonication, passage through a French pressure cell, freezing and thawing, liquid N₂ freezing and pulverizing in a mortar and pestle, or enzymatic digestion, followed by centrifugation at 10,000 x g for 20 min at 4°C. The supernatant was considered as the crude cell-free extract. The effect of disruption on cholesterol reductase activity was determined. Precautions were taken to keep anaerobiosis in the procedures by flushing the samples with N₂.
Protein determination

Total bacterial protein was determined by the Bradford method with microtiter plates (Redinbaugh and Campbell 1985). A portion of the cell suspension or enzyme preparations was mixed with an equal volume of 2 N NaOH and incubated at 37°C overnight to solubilize the protein. Bovine serum albumin, treated in the same manner, was used as a standard.

Isolation of genomic DNA

The genomic DNA of *E. coprostanoligenes* was isolated and purified by a procedure modified from Jarrell et al. (1992). The bacterial pellet was treated with liquid N₂. After the liquid N₂ had evaporated, the frozen cells were ground vigorously to a fine powder with a pestle, and 5 to 10 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 0.25 M sucrose were added immediately. When the slurry had thawed, sodium dodecyl sulfate (SDS; from a 10% w/v solution) was added to a final concentration of 1%. Proteinase K (Sigma, MO) (50 µg/ml) was added and the mixtures were incubated at 60°C for 30 min. Sodium chloride was added from a 5 M stock solution to a final concentration of 0.5 M, and the lysates were put on ice. After 1 h, the lysates were cleared by centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was decanted carefully, and the nucleic acids were precipitated with an equal volume of cold isopropanol. The DNA was collected by centrifugation and then dissolved in TE buffer at 60°C. After RNase treatment (50 µg/ml for 30 min at 37°C with DNase-
free RNase A), the DNA was extracted twice with an equal volume of phenol-
chloroform-isoamyl alcohol (25:24:1; v:v:v), followed by two extractions with
chloroform. After addition of NaCl to 0.5 M, the DNA was precipitated with 2.5
volumes of cold ethanol, collected by centrifugation, and dissolved in TE buffer.

**Determination of DNA base composition**

Genomic DNA was dissolved in 0.1 X SSC buffer (15 mM of NaCl and 1.5
mM of sodium citrate, pH 7.0). The melting temperature (Tm) of the DNA was
determined by the thermal denaturation method with genomic DNA of *E. coli* as a
control. The guanine plus cytosine (G + C) content was calculated with the
formula as follows: \((G + C)\% = (Tm - 53.9) \times 2.44\) (Mandel and Marmur 1968).

**Preparation of cho gene probe and Southern hybridization**

*Lactobacillus casei* LC102 carrying plasmid pNCO937, which contains the
*Streptomyces cho* gene, was kindly provided by G. A. Somkuti at the USDA, ARS,
North Atlantic Area Eastern Regional Research Center, Philadelphia,
Pennsylvania. *L. casei* was cultured in Lactobacilli MRS broth (Difco) with 20 mM
DL-threonine and chloramphenicol (15 µg/ml) at 37°C under 5% CO₂. Plasmid
DNA was isolated from *L. casei* by the procedures of Somkuti and Steinberg
(1986). Plasmid pNCO937 was confirmed by agarose gel electrophoresis and
purified further by QIAGEN plasmid protocol (QIAGEN Inc., CA). pNCO937 is
a 8.1-kb plasmid containing a *cho* gene within a 2.4-kb *Pst* I fragment (Somkuti, et
al. 1992). The plasmid was digested with \textit{Pst} I, and the \textit{cho} fragment was separated by agarose gel electrophoresis and recovered by using the QIAEX gel extraction kit (QIAGEN Inc., CA). The preparation of \textit{cho} DNA was used to synthesize the $^{32}\text{P}$-labeled probe by using Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemica Co., IN).

In addition to DNA from \textit{E. coprostanoligenes}, genomic DNA was isolated from \textit{E. coli} DH5$\alpha$, \textit{Eubacterium} sp. VPI 12708, and \textit{Brevibacterium sterolicum} ATCC 21387. The genomic DNA preparations were digested completely with \textit{EcoR} I and electrophoresed on a 0.7% agarose gel along with preparations of pNCO937, pNCO937 \textit{Pst} I digest, and DNA molecular weight markers. The DNA was transferred and immobilized on a nylon membrane by using a capillary transfer method. Southern hybridization of the $^{32}\text{P}$-labeled \textit{cho} probe to the immobilized DNA was conducted as described by Sambrook et al. (1989). After washing away the unhybridized $^{32}\text{P}$-label probes, the membrane was exposed to a Phosphor-Imager (Molecular Dynamics) to determine the results of hybridization.

\textbf{Construction of genomic expression library}

Genomic DNA of \textit{E. coprostanoligenes} was digested with \textit{EcoR} I for 5, 10, 20, 30, 40, 60, 80, 100, 120, and 240 min to obtain random sizes of DNA fragments. The partial digests were pooled and electrophoresed on a 0.6% low-melting temperature agarose gel, appropriate bands of DNA were sliced out, and DNA was purified from the gel slices (Sambrook et al. 1989). The DNA fragments of sizes
between 5 to 7 kb were used for library construction in the expression vector, bacteriophage λ vector λgt11, that accepts DNA inserts up to 7.2 kb. The DNA fragments were ligated with dephosphorylated λgt11 arms (Promega Co., WI) with T₄ ligase. Packaging of ligated DNA was fulfilled by incubating the ligation mixture with the Packagene Extract (Promega Co., WI). An aliquot of packaged phage was used for titration, which was conducted by using *E. coli* strain Y1090 as a host on LB plates with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the top agar. The number of plaques was counted, and the titers of the phage then were calculated. The rest of the packaged phage was mixed with chloroform and dimethylsulfoxide and stored at -70°C for future use.

**Results**

**Ultrastructure and genomic DNA composition**

Transmission electron micrographs (Figure 1A-B) showed that *E. coprostanoligenes* had a typical cell wall structure of Gram-positive bacteria. The bacterium was about 0.5 to 0.7 μm in diameter and 0.7 to 1.0 μm in length. Many cells existed in diploform, which was more obvious in the scanning electron micrograph (Figure 1C). The melting temperature for the genomic DNA was about 71°C, and the G + C content was calculated to be 41% (Figure 2).
Figure 1. Electron photomicrographs of *E. coprostanoligenes*. (A) Bar = 0.1 μm. (B) Bar = 0.2 μm. (C) Bar = 1.0 μm.
Mechanism of cholesterol reduction and substrate specificity

When *E. coprostanoligenes* was grown in cholesterol-containing media, the only sterols in cultures were the final product, coprostanol, and residual cholesterol determined both by GLC (Figure 3) and TLC (Figure 4). In resting cell assays with \([1\alpha, 2\alpha-^3\text{H}]\)-micellar cholesterol as substrate, however, in addition to a coprostanol band, 4-cholesten-3-one and coprostanone bands were observed clearly on TLC plates, although no 5-cholesten-3-one band was found (Figure 5). The corresponding bands were scraped and counted for radioactivity in a scintillation counter, and the final product, coprostanol, and the intermediates were quantified (Figure 6). Interestingly, the production rate for 4-cholesten-3-one was higher than
that of coprostanol, and coprostanone seemed not to accumulate in the reaction. When 5-cholesten-3-one, 4-cholesten-3-one, and coprostanone were added to the growth media, they all were converted to coprostanol by the bacterium (Figure 7). In addition to these cholesterol analogs, \textit{E. coprostanoligenes} also metabolized pregnenolone, progesterone, androstenediol, and etienic acid (Figure 8A-B).

**Effects of cofactors and inhibitors**

Several potential cofactors and inhibitors were incorporated into resting cell assays to determine their effects on cholesterol reductase activity (Table 1). In addition to the final product, coprostanol, production of intermediates were shown also in the table. Among the cofactors used, NADH (β-nicotinamide adenine dinucleotide, reduced form) led to the highest cholesterol reductase activity, followed by FAD (flavin adenine dinucleotide) and NADP⁺ (β-nicotinamide adenine dinucleotide phosphate). When NADH and FAD were added together in the reaction mixture, an inhibitory effect was observed on cholesterol reductase activity. NADPH and NAD did not affect cholesterol reductase activity significantly. Among the inhibitors used, NaN₃, NaCN, and iodoacetate did not affect cholesterol reductase activity of \textit{E. coprostanoligenes}. EDTA (ethylenediaminetetraacetate) showed a strong inhibitory effect. HgCl₂ abolished the enzyme activity completely.
Figure 3. Chromatograms of GLC analysis. (A) Cholesterol-containing medium before bacterial inoculation. (B) One-day incubation with bacteria. (C) Two-day incubation with bacteria.
Figure 4. TLC analysis of *E. coprostanoligenes* culture in cholesterol-containing medium. The TLC plate was sprayed with ethanolic solution of 2,7-dichlorofluorescein, sterols were visualized under ultraviolet light. Lane 1 and 2, uninoculated culture; Lane 3 and 4, inoculated culture incubated for 5 d; Lane 5, standards.
Figure 5. TLC analysis of products from the resting cell assays with *E. coprostanoligenes*. The TLC plate was sprayed with ethanolic solution of 2,7-dichlorofluorescein, sterols were visualized under ultraviolet light. Lane 1 and 2, assays with killed (boiled) bacteria (controls); Lane 3, standards; Lane 4 and 5, assays with live bacteria.
Figure 6. Products of cholesterol reductase of *E. coprostanoligenes* in resting cell assays. Data are expressed as the mean of triplicate assays ± standard error.
Figure 7. TLC analysis of *E. coprostanoligenes* cultures with cholesterol and other steroids in the growth medium. The incubation time was 5 d. The TLC plate was sprayed with ethanolic solution of 2,7-dichlorofluorescein, sterols were visualized under ultraviolet light. Lane 1 and 2, uninoculated and inoculated cholesterol-containing culture, respectively; Lane 3 and 4, uninoculated and inoculated 5-cholesten-3-one-containing culture, respectively; Lane 5 and 6, uninoculated and inoculated 4-cholesten-3-one-containing culture, respectively; Lane 7 and 8, uninoculated and inoculated coprostanone-containing culture; Lane 9, standards.
Figure 8. TLC analysis of *E. coprostanoligenes* cultures with different steroids. Plates were sprayed with ethanolic solution of 2,7-dichlorofluorescein, steroids were visualized under ultraviolet light. (A) Lane 1 and 3, pregnenolone and progesterone standards; Lane 2 and 4, cultures with pregnenolone and progesterone. (B) Lane 1 and 3, androstenediol and etienic acid standards; Lane 2 and 4, cultures with androstenediol and etienic acid.
Table 1. Effects of cofactors and inhibitors on cholesterol reductase activity\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Product (nmole/h.mg protein)</th>
<th>Recovery\textsuperscript{b} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coprostanol</td>
<td>4-Chol-3-one</td>
</tr>
<tr>
<td>Control</td>
<td>592 ± 29</td>
<td>582 ± 13</td>
</tr>
<tr>
<td>Cofactors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>680 ± 2</td>
<td>234 ± 20</td>
</tr>
<tr>
<td>FAD</td>
<td>629 ± 5</td>
<td>589 ± 3</td>
</tr>
<tr>
<td>NADP\textsuperscript{+}</td>
<td>612 ± 4</td>
<td>481 ± 15</td>
</tr>
<tr>
<td>NADPH</td>
<td>599 ± 17</td>
<td>559 ± 10</td>
</tr>
<tr>
<td>FAD+NADPH</td>
<td>595 ± 70</td>
<td>505 ± 47</td>
</tr>
<tr>
<td>NAD\textsuperscript{+}</td>
<td>584 ± 15</td>
<td>483 ± 18</td>
</tr>
<tr>
<td>FAD+NADH</td>
<td>350 ± 28</td>
<td>246 ± 18</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaN\textsubscript{3}</td>
<td>615 ± 17</td>
<td>487 ± 12</td>
</tr>
<tr>
<td>NaCN</td>
<td>579 ± 24</td>
<td>583 ± 13</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>525 ± 13</td>
<td>601 ± 7</td>
</tr>
<tr>
<td>EDTA</td>
<td>181 ± 16</td>
<td>207 ± 15</td>
</tr>
<tr>
<td>HgCl\textsubscript{2}</td>
<td>3 ± 1</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are expressed as the mean of triplicate assays ± standard error.

\textsuperscript{b} Recovery (\%) = (amount of residual cholesterol + amount of products)/amount of added cholesterol substrate \times 100.

**Enzyme activity in extracellular fluid and cell-free extract**

No cholesterol reductase activity was found in extracellular broth before or after ammonium sulfate precipitations. Cholesterol reductase activity was associated with the bacterial cells. Great loss of cholesterol reductase activity occurred after the bacterial cells were disrupted by sonication, passage through a
French pressure cell, freezing and thawing, liquid N₂ freezing and pulverizing, or enzymatic digestion. Repeated trials resulted in the same observation. Crude cell-free extracts, prepared after two passages through a French pressure cell (16,000 psi), were studied further with addition of a variety of cofactors (Table 2). The cholesterol reductase activity decreased about 100 times as the cells are broken, and the activity could not be recovered by addition of cofactors.

### Table 2. Cholesterol reductase activity in cell-free extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coprostanol (nmole/h.mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before disruption²</td>
<td>1060.7</td>
</tr>
<tr>
<td>After disruption</td>
<td>11.1</td>
</tr>
<tr>
<td>Crude cell-free extract</td>
<td>2.3</td>
</tr>
<tr>
<td>Extract + H₂</td>
<td>3.5</td>
</tr>
<tr>
<td>Extract + FAD</td>
<td>4.0</td>
</tr>
<tr>
<td>Extract + FAD + H₂</td>
<td>5.0</td>
</tr>
<tr>
<td>Extract + NADH</td>
<td>2.1</td>
</tr>
<tr>
<td>Extract + NAD⁺</td>
<td>5.7</td>
</tr>
<tr>
<td>Extract + NADPH</td>
<td>3.9</td>
</tr>
<tr>
<td>Extract + FAD + NADPH</td>
<td>4.6</td>
</tr>
</tbody>
</table>

² Data are the average of two assays.

Bacterial cells were disrupted by two passages through a French pressure cell.

**Southern hybridization**

After hybridization with ^32^P-label cho gene, strong signals were observed for pNCO937 DNA and its 2.4-kb Pst I fragment. Weak signals were seen in the lane with DNA from *B. sterolicum* ATCC 21387. No signals, however, were detected in
lanes with genomic DNA of *E. coprostanoligenes*, *E.* sp. VPI 12708, or *E. coli* DH5α.

**Construction of genomic expression library**

With incorporation of IPTG and X-gal in the top agar, a blue-white color screen was possible to distinguish between the background nonrecombinant and recombinant phage. The nonrecombinant λgt11 phage had β-galactosidase activity that converts X-gal into a blue product under the induction of IPTG. The recombinants had received an insert in the phage genome and caused an insertional inactivation of β-galactosidase activity; thus, they could not convert X-gal to produce a blue product. Therefore, recombinants showed as white plaques, whereas nonrecombinants were blue. The titers for recombinants and nonrecombinants were $5 \times 10^5$ pfu/ml and $1 \times 10^4$ pfu/ml, respectively.

**Discussion**

*E. coprostanoligenes* cells appeared shorter than the cells of most *Eubacterium* spp. (Moore and Holdeman 1986). Many bacterial cells existed in diploforms in the cultures. Eyssen et al. (1973) reported similar observations with *Eubacterium* ATCC 21408 in young cultures. These cells might be in the process of dividing. The range for G + C content of DNA is 30 to 55% for *Eubacterium* spp. (Moore and Holdeman 1986). The G + C content (41%) of *E. coprostanoligenes* DNA was in the middle of this range.
The conversion of cholesterol to coprostanol by intestinal microorganisms has been reported to occur by two pathways (Figure 9). One, indirect pathway, involves the intermediary formation of 4-cholesten-3-one and coprostanone (Björkhem and Gustafsson 1971; Eyssen and Parmentier 1974; Parmentier and Eyssen 1974). In the other pathway, cholesterol is transformed into coprostanol by the direct reduction of the 5-6 double bond (Rosenfeld and Gallagher 1964).

Figure 9. Proposed reaction pathways for enzymatic conversion of cholesterol to coprostanol (Björkhem and Gustafsson 1971). I. Direct pathway. II. Indirect pathway: 1, oxidation; 2, isomerization; 3, reduction; 4, reduction.

In the beginning of the study with E. coprostanoligenes, it was thought that the bacterium reduces cholesterol to coprostanol in a direct pathway because no intermediates were observed in cultures with cholesterol. Later, by incubating the bacterium with a mixture of α and β isomers of [4-3H, 4-14C]-cholesterol, it was found that the final product, coprostanol, retained 97% of the tritium originally
present in cholesterol; the majority of this tritium (64%), however, was in the C-6 position in coprostanol, which showed that the conversion of cholesterol to coprostanol by *E. coprostanoligenes* involved the intermediate formation of 4-cholesten-3-one followed by the reduction of the latter to coprostanol (Ren et al. 1995). After the establishment of a resting cell assay for cholesterol reductase activity, it was possible to provide direct evidence of the cholesterol reduction pathway in *E. coprostanoligenes*.

In the current study, intermediates in cholesterol reduction were observed on TLC plates and quantified by determination of radioactivity. Isomerization of 5-cholesten-3-one to 4-cholesten-3-one must have been very rapid because no 5-cholesten-3-one accumulated in the reaction. Reduction of 4-cholesten-3-one to coprostanone might be a rate-limiting step in this pathway because 4-cholesten-3-one was formed in even greater amounts than was the final product, coprostanol. Reduction of coprostanone to coprostanol was rather fast because only little coprostanone accumulated in the reaction. *E. coprostanoligenes* converted 5-cholesten-3-one, 4-cholesten-3-one, and coprostanone, respectively, to coprostanol, validating the existence of the indirect pathway for cholesterol reduction in this bacterium and indicating that the bacterium has a broad substrate specificity. The broad specificity of the enzyme system on substrates was shown further by its ability to metabolize pregnenolone, progesterone, androstenediol, and etienic acid. These characteristics may be useful for future application of *E. coprostanoligenes* in the pharmaceutical industry.
Both NADH and NADPH have been suggested as cofactors for cholesterol reductase of plant origin (Dechal et al. 1991). In the current study, NADH also stimulated the cholesterol reductase activity in cells of *E. coprostanoligenes*. NADH increased coprostanol formation, probably through increasing the rate of 4-cholesten-3-one reduction because much less 4-cholesten-3-one accumulated in reactions in the presence of NADH (Figure 10).

![Figure 10](image)

**Figure 10.** Effect of NADH on cholesterol reductase activity in resting cell assays. The data are expressed as the mean of triplicate assays ± standard error.

FAD is a prosthetic group of most cholesterol oxidases that catalyze conversion of cholesterol to 4-cholesten-3-one (Kenney et al. 1979). Interestingly, FAD also showed a stimulatory effect on cholesterol reductase. The cholesterol reductase system could have used FAD as a cofactor for the first step in the
pathway, that is oxidation of cholesterol to 4-cholesten-3-one as in the cholesterol oxidase system.

When NADH and FAD were used together, however, cholesterol reductase activity was lower than that of the control. This observation might be explained by the possibility that NADH could have reduced FAD to form FADH$_2$ and NAD (Figure 11). Further, FADH$_2$ might have inhibited the production of 4-cholesten-3-one from cholesterol, and NAD might have inhibited the reduction of 4-cholesten-3-one to coprostanol. Therefore, the resultant effect would be less of the final product, coprostanol, produced in the reaction. This explanation assumes that NADH dehydrogenase is present in *E. coprostanoligenes*, but its presence has not been demonstrated. When NADPH and FAD were used together, no significant effects were observed on cholesterol reductase activity. FADH$_2$ might not be formed from NADPH, and NADPH itself did not affect cholesterol reductase activity either. The questions about how these cofactors work and what are the required cofactors for cholesterol reduction, however, can be answered only after a pure preparation of cholesterol reductase is available.

![Cholesterol pathway diagram](image)

Figure 11. Proposed effect of NADH-FAD on cholesterol reduction.
The results from inhibitor experiments are also interesting. An oxidizing agent, iodoacetate, and metal-complexing agents, $\text{CN}^-$ and $\text{N}_3^-$, did not affect cholesterol reductase significantly, whereas a metal-chelating agent, EDTA, significantly inhibited enzyme activity; and a heavy metal ion, $\text{Hg}^+$, abolished the activity completely. It seems that some metal ions such as those of Cu, Fe, Mo, Zn, and Mg may be required by the enzyme to reduce cholesterol to coprostanol. Again, however, elucidation of the mechanism of inhibition needs to wait until a pure preparation of the enzyme is available.

Trials to prepare active cell-free extracts were not successful because of the great loss of activity when the bacterial cells are broken. The only other reported attempt to detect cell-free activity with cholesterol-reducing bacteria was made by Björkhem et al. (1973), which also resulted in negative results. Several reasons could account for the loss of activity: (1) Cholesterol reductase may require an intact membrane to be functional, (2) Cholesterol reductase may be composed of multiple subunits that are separated during cell disruption, (3) Some required cofactor(s) may not be formed when bacterial cells are broken, and (4) Cholesterol reductase may be extremely sensitive to oxygen when it is freed from intact cells. Future experiments should take these reasons into account for preparation of cell-free extracts. Furthermore, cholesterol reductase does not seem to be a secretory protein. Its activity was associated with bacterial cells. The active site of the enzyme, however, may be located at the outer surface of the membrane, and substrate catalysis *in vivo* may occur externally to the cytoplasm because (a) the
rapid release of reduction products by resting cell action on cholesterol seemed to show a rapid and direct contact between the enzyme system and the substrate and (b) the enzyme activity could be measured by using whole cells, indicating that the substrate needs not intersect the cytoplasmic membrane for catalysis. Therefore, active cell-free enzyme preparation may be achieved by cell treatment with Triton X-100 or other nonionic detergents without cell disintegration. By using this method, Kreit et al. (1994) has extracted and purified cholesterol oxidase successfully from *Rhodococcus* sp. cells.

Cholesterol oxidase gene was considered as a probe to screen for cholesterol reductase gene. It is hypothesized that the genes encoding cholesterol reductase and cholesterol oxidase would share some homology because they both must bind cholesterol and show similar catalytic functions. This hypothesis provides a potential method to isolate the cholesterol reductase gene from *E. coprostanoligenes* without the purification of cholesterol reductase. Unfortunately, no hybridization signal was observed after genomic DNA of *E. coprostanoligenes* was hybridized with \(^{32}\)P-labeled *cho* gene. The conditions for Southern hybridization, however, were not optimized in the experiment because only weak signals were seen with genomic DNA from *B. sterolicum* ATCC 21387, which produces cholesterol oxidase and has a *cho* gene that has been reported to have extensive homology in DNA sequence with the *cho* gene from *Streptomyces* sp. (Ohta et al. 1991). Similar experiments should be conducted in the future with other cholesterol-related genes, in addition to *cho* gene, as probes. Potential candidates include genes for sterol carrier
protein, cholesterol 7α-hydroxylase, steroid 5α-reductase, and other cholesterol-metabolizing enzymes. With the genomic expression library available, discovery of an appropriate probe would lead to the cloning of the cholesterol reductase gene before the enzyme protein is purified.

Other potential methods to isolate cholesterol reductase and its gene(s) are under development. One method is to incorporate micellar cholesterol in the top agar when plating the genomic expression library with a lysogen such as *E. coli* strain Y1089. Production of the functional cholesterol reductase would convert cholesterol to coprostanol. Disappearance of cholesterol can be detected with a Sigma Cholesterol 100 Kit, which shows a pink color when cholesterol is present, and there is no color formation with coprostanol. Therefore, colonies or plaques with a colorless halo would be positive clones for cholesterol reductase. DNA inserts can be isolated from those clones, and the gene could be identified.

Another potential method is focusing more on the enzyme protein itself. An assumption needs to be made that cholesterol reductase protein binds the substrate cholesterol, even though the cholesterol-reducing activity is lost by a purification procedure. A whole cellular protein can be prepared and separated by native polyacrylamide gel electrophoresis. The proteins can be transferred to a nylon membrane. The immobilized proteins can be incubated with a micellar cholesterol solution. After washing away the unbound cholesterol, the membrane will be treated with agents that recognize cholesterol, such as Sigma Cholesterol 100 Kit or radiolabeled or fluorescent-labeled antibodies against cholesterol. The protein that
binds cholesterol could be isolated this way. Further investigation with this protein may lead to the isolation of active cholesterol reductase.

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HYPOCHOLESTEROLEMIC EFFECT OF

EUBACTERIUM COPROSTANOLIGENES ATCC 51222 IN RABBITS

A paper published in Letters in Applied Microbiology

L. Li, K.K. Buhman, P.A. Hartman and D.C. Beitz

Abstract

Recently, a unique bacterium, Eubacterium coprostanoligenes ATCC 51222, that reduces cholesterol to coprostanol was isolated. Because coprostanol is absorbed poorly, we hypothesized that oral administration of Eu. coprostanoligenes might decrease cholesterol concentration in blood because the microorganisms will decrease the absorption of endogenous and dietary cholesterol by conversion to coprostanol. To test the hypothesis, three adult New Zealand White rabbits received 4 ml of Eu. coprostanoligenes suspension (ca 2 x 10^7 cells ml^-1) daily per os for 10 days; three other adult New Zealand White rabbits received the same dosage of boiled bacterial suspension. Plasma cholesterol concentration of experimental rabbits (183.3 ± 11.0 mg dl^-1, mean ± S.E.) was significantly lower (P < 0.001) than that of controls (248.8 ± 12.3 mg dl^-1, mean ± S.E.). The coprostanol-to-cholesterol ratios in contents of digestive tracts of experimental rabbits were greater than those of controls. The data indicate that oral administration of Eu. coprostanoligenes caused a significant hypocholesterolemic effect in rabbits and that
this effect can be explained by the conversion of cholesterol to coprostanol in the intestine.

Introduction

Coronary heart disease is the leading cause of human mortality in the United States as well as in many other developed countries and is responsible for more deaths than all forms of cancer combined. For many years, it has been recognized that elevated serum cholesterol is a risk factor associated with atherosclerosis (Gotto et al. 1990). Numerous cholesterol-lowering drugs, including the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and drugs that increase net bile acid excretion, have been used to treat hypercholesterolemic individuals. The accompanying undesirable side effects of these drugs, however, have caused concerns about their therapeutic use (Erkelens et al. 1988).

The identification of bacteria that convert cholesterol to coprostanol has provided a potential method to naturally decrease serum cholesterol in humans. Coprostanol, a saturated product of cholesterol, is absorbed poorly from the gastrointestinal system (Bhattacharyya 1986). Cholesterol-reducing bacteria have been isolated from feces of rats (Eyssen et al. 1973), humans (Sadzikowski et al. 1977), and baboons (Brinkley et al. 1982). Most of these previously isolated bacteria were difficult to grow in the laboratory. Recently, a unique cholesterol-reducing bacterium designated *Eubacterium coprostanoligenes* ATCC 51222 was isolated and is much easier to grow in the laboratory (Freier et al. 1994). The
enzyme responsible for catalyzing the reduction of cholesterol to coprostanol has been named "cholesterol reductase" (Dehal et al. 1991).

Because coprostanol is absorbed poorly, we hypothesized that oral administration of *Eu. coprostanoligenes* would decrease cholesterol concentration in human blood because the microorganisms will decrease the absorption of endogenous (e.g. biliary) and dietary cholesterol by conversion to coprostanol in the small intestine. Therefore, the purpose of this study was to test the effect of orally administered *Eu. coprostanoligenes* on plasma cholesterol concentration by using rabbits as an animal model.

Materials and Methods

Animals and diets

Six adult New Zealand White rabbits were fed Purina Rabbit Chow with 0.05% cholesterol for 32 days and then 0.1% cholesterol for 57 days to raise their plasma cholesterol concentration. The dietarily induced hypercholesterolemic rabbits were divided randomly into two groups, the experimental group and the control group, with three rabbits in each group on day 50 of the 94-day experiment. The experimental group received 4 ml of *Eu. coprostanoligenes* suspension (ca 2 x 10^7 cells ml⁻¹) daily through stomach tubing for 10 days; the control group received the same dosage of killed (boiled) bacterial suspension.
Cultivation of bacteria

_Eu. coprostanoligenes_ was grown in the medium containing the following components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g lactose, 5 g sodium pyruvate, 0.5 g sodium thioglycolate, 0.2 g cholesterol (ash-free, Sigma Chemical Co., St. Louis, MO), 0.1 g lecithin (type IV-S, Sigma), 0.1 g CaCl$_2$·2H$_2$O and 1 mg resazurin. The pH was adjusted to 7.5 with 3 N KOH. The Hungate technique was used for the anaerobic preparation of media and handling of the culture (Holdeman _et al._ 1977). Fresh medium was inoculated with 1% of a 48-hour culture. After incubation for 48 hours at 37°C, the cultures were harvested by centrifugation (10,000 g, 10 min). The cell pellets were suspended in 25 mmol l$^{-1}$ PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5) containing 5 mmol l$^{-1}$ sodium thioglycolate and 45 mmol l$^{-1}$ pyruvate before being fed to rabbits.

Determination of plasma cholesterol concentrations

Blood samples were taken from the rabbits through the ear artery 2-3 times a week; ethylenediaminetetraacetic acid (disodium salt) was used as an anticoagulant. The plasma cholesterol concentrations were determined in triplicate enzymatically with a Sigma cholesterol kit (Cholesterol 100, Sigma).
Determination of coprostanol-to-cholesterol ratios in the contents of digestive tract

The contents of digestive tracts were collected from each rabbit at the end of the study. Lipids were extracted in duplicate (Lepage and Roy 1986), and the coprostanol-to-cholesterol weight ratios were determined in duplicate by gas-liquid chromatography by the method of Oles et al. (1990). The duplicate lipid extract from the samples was evaporated to dryness under nitrogen. Sterols in the residue were etherified with 1,3-bis-(trimethylsilyl)trifluoroacetamide. A 5830A Hewlett Packard gas chromatograph was used. The glass column, 0.91 m x 4 mm ID, was packed with 3% SP-2250 on 100/120 Supelcoport. Operating conditions were: oven, 250-280°C; injector, 290°C; flame ionization detector (FID), 290°C; nitrogen flow rate, 20 ml min⁻¹.

Statistical analysis

The data were analyzed by Student's t test for significance.

Results

Plasma cholesterol concentrations

The plasma cholesterol concentrations of the rabbits are shown in Figure 1. The bacterial administration started when the plasma cholesterol concentration reached about 175 mg dl⁻¹ at day 50. After 10 days of bacterial treatment, differences were observed between the two groups through the rest of the study.
The plasma cholesterol concentrations of the experimental (---) and control (-----) rabbits. Days of giving *Eu. coprostanoligenes* is indicated as Eubact. on the figure. Each data point represents the average of data from three rabbits. The plasma cholesterol concentration of the experimental group from day 61 through day 94 was 183.3 ± 6.8 mg dl⁻¹ (mean ± S.E.), which was significantly lower (*P* < 0.001) than that of the control group (248.8 ± 11.7 mg dl⁻¹).

The plasma cholesterol concentration of the experimental group from day 61 through day 94 was 183.3 ± 6.8 mg dl⁻¹ (mean ± S.E.), which was significantly lower (*P* < 0.001) than that of the control group (248.8 ± 11.7 mg dl⁻¹).

**Coprostanol-to-cholesterol ratios**

The coprostanol-to-cholesterol ratios of the contents of rabbit digestive tracts at the end of the experiment (day 94) are shown in Table 1. In the duodenum, coprostanol was not detectable in either the experimental group or the
control group. In the jejunum, the coprostanol-to-cholesterol ratio of the experimental group was significantly \((P = 0.05)\) greater than that of the control group. In the ileum, the ratio also was higher in the experimental group \((P = 0.08)\). The trend continued in the cecum and colon.

Table 1. Coprostanol-to-cholesterol weight ratios in contents of rabbit digestive tracts.

<table>
<thead>
<tr>
<th>Section</th>
<th>Experimental</th>
<th>Control</th>
<th>(P)-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.13 ± 0.04(^a)</td>
<td>0.01 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.21 ± 0.08</td>
<td>0.02 ± 0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>Cecum</td>
<td>0.46 ± 0.12</td>
<td>0.14 ± 0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>Colon</td>
<td>0.48 ± 0.10</td>
<td>0.33 ± 0.13</td>
<td>0.39</td>
</tr>
</tbody>
</table>

ND, not detectable.

\(^a\) Mean ± S.E., \(n = 3\).

Discussion

In this study, we showed that orally administration of \textit{Eu. coprostanoligenes} caused a significant decrease of plasma cholesterol concentration in dietarily induced hypercholesterolemic rabbits. The rabbits showed no adverse effects to being treated with the bacterial inocula. The mechanism by which \textit{Eu. coprostanoligenes} decreases the plasma cholesterol concentration relies on the fact that \textit{Eu. coprostanoligenes} converts readily absorbed cholesterol into poorly absorbed coprostanol (Freier \textit{et al.} 1994; Bhattacharyya 1986). Preliminary \textit{in vitro}
experiments showed that *Eu. coprostanoligenes* could survive in simulated stomach acid conditions for at least 2 hours and retain its cholesterol-reducing activity when transferred to the regular growth medium (data not shown). The sources of cholesterol available for absorption in the intestine include exogenous (dietary) and endogenous (e.g. biliary) cholesterol (Gotto *et al.* 1990). By conversion of cholesterol to coprostanol, *Eu. coprostanoligenes*, on one hand, could decrease the absorption of cholesterol so that less cholesterol will enter the body cholesterol pool, and, on the other hand, the bacterium may interrupt the enterohepatic circulation of biliary cholesterol so that the liver would partition more cholesterol into bile and less cholesterol into the bloodstream.

The hypocholesterolemic effect of *Eu. coprostanoligenes* continued for at least 34 days after the last bacterial feeding. Colonization of *Eu. coprostanoligenes* in the digestive tracts of the rabbits can explain that sustained effect. Significantly greater coprostanol-to-cholesterol ratios in the contents of digestive tracts of the experimental group at the end of the experiment suggested that the bacteria colonized there were reducing cholesterol to coprostanol actively. When the intestinal contents were inoculated into the growth medium, *Eu. coprostanoligenes* and its cholesterol-reducing activity were recoved from the experimental group but not from the control group (data not shown). The presence of other indigenous coprostanol-producing bacteria in the digestive tracts of rabbits accounts for the relatively great coprostanol-to-cholesterol ratios in the contents of cecum and colon of the control group (Eyssen *et al.* 1973; Sadzikowski *et al.* 1977; Brinkley *et al.*
These bacteria, however, exert little if any effects on cholesterol absorption because cholesterol absorption takes place primarily in the small intestine and not in the cecum and colon (Gotto et al. 1990).

In conclusion, oral administration of *Eu. coprostanoligenes* significantly decreased plasma cholesterol concentrations in hypercholesterolemic rabbits, and the hypocholesterolemic effect can be explained by the conversion of cholesterol to coprostanol in the intestine. These results suggest a potential application of *Eu. coprostanoligenes* in humans to decrease serum cholesterol in hypercholesterolemic individuals. Before a human study becomes feasible, however, more animal experiments are needed to test variables such as bacterial dosage, length of time of bacterial administration and interaction of bacterial dosage with blood cholesterol concentration.

Acknowledgments

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EFFECT OF ORALLY ADMINISTERED *Eubacterium coprostanoligenes* ATCC 51222 ON PLASMA CHOLESTEROL CONCENTRATION IN LAYING HENS

A paper prepared for submission to *Poultry Science*

Ling Li, Carol A. Baumann, Daryl D. Meling,
Jerry L. Sell, Paul A. Hartman, and Donald C. Beitz

Abstract

Thirty normocholesterolemic laying hens were used to investigate the effect of oral administration of *Eubacterium coprostanoligenes* on plasma cholesterol concentrations. Hens were divided randomly into three groups (active, inactive, and control) with 10 hens in each group. The active group received 0.5 ml of *E. coprostanoligenes* suspension (ca 2 x 10^7 cells/ml) daily for 4 wk; the inactive group received the same dosage of killed (boiled) bacterial suspension; and the control group received no supplemental bacteria. After bacterial feeding, the coprostanol-to-cholesterol ratio in feces of the active group was significantly higher than ratios of the inactive and control groups, indicating that *E. coprostanoligenes* was colonized in the intestine of hens and was converting intestinal cholesterol to coprostanol. Plasma cholesterol concentrations, however, were not affected by the bacterial treatment.
Introduction

Eggs are a popular and highly nutritious food throughout the world. The amount of cholesterol contained in egg yolk, however, has caused immense concerns about consuming eggs because of the adverse effect of dietary cholesterol on coronary heart disease (Fraser 1994; Pyörälä 1987). Great efforts have been made to decrease the cholesterol content of eggs through selective breeding and feeding or physical and chemical removal of egg cholesterol (Froning et al. 1990; Rossi et al. 1990; Michael Foods 1992). The "low-cholesterol" eggs are demanded by many consumers who love eggs as a food but are concerned about their daily cholesterol intake.

A newly isolated bacterium, *Eubacterium coprostanoligenes* ATCC 51222, converts cholesterol to coprostanol that is absorbed poorly by the gastrointestinal system (Freier et al. 1994). Previous studies have showed that oral administration of *E. coprostanoligenes* decreases the plasma cholesterol concentration significantly in hypercholesterolemic rabbits (Li et al. 1995). The hypothesis of this study is that *E. coprostanoligenes* will colonize in the intestine of laying hens after oral administration of the bacterium and that the bacterium in the intestine will convert exogenous (dietary) and endogenous (e.g., biliary) cholesterol to coprostanol so that less cholesterol will return to the liver through enterohepatic circulation. As a result, liver may partition more cholesterol into bile and less cholesterol into plasma. If plasma cholesterol was decreased markedly, less cholesterol probably would be incorporated into eggs of laying hens.
Materials and Methods

Bacteriology

*E. coprostanoligenes* was grown in the medium containing the following components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g lactose, 5 g sodium pyruvate, 0.5 g sodium thioglycolate, 0.2 g cholesterol (ash-free, Sigma Chemical Co., St. Louis, MO), 0.1 g lecithin (type IV-S, Sigma), 0.1 g CaCl₂·2 H₂O, and 1 mg resazurin. The pH was adjusted to 7.5 with 3 N KOH. The Hungate technique was used for the anaerobic preparation of media and handling of the culture (Holdeman *et al.* 1977). Fresh medium was inoculated with 1% of a 48-h culture. After incubation for 48 h at 37°C, cultures were harvested by centrifugation (10,000 x g, 10 min). The cell pellets were suspended in 25 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5) containing 5 mM sodium thioglycolate and 45 mM pyruvate before being fed to hens.

Management of hens

Thirty Leghorn laying hens were placed in individual cages and provided *ad libitum* water and a corn-soy diet that was adequate in all nutrients. The hens were allowed one week to acclimate to the environment before experimental challenges were carried out. They were divided randomly into three groups with 10 hens in each group. Hens in the active group were fed once daily a gelatin capsule containing 0.5 ml of *E. coprostanoligenes* suspension (ca 2 x 10⁷ cells/ml) for 4 wk; an inactive group received the same dosage of killed (boiled) bacterial suspension;
and a control group received no supplemental bacteria.

**Determination of plasma cholesterol concentrations**

Blood samples were taken from the hens via the wing vein once a week; ethylenediaminetetraacetic acid (disodium salt) was used as an anticoagulant. The plasma cholesterol concentrations were determined enzymatically with a Sigma cholesterol kit (Cholesterol 100, Sigma).

**Determination of coprostanol-to-cholesterol ratios in feces**

The feces were collected from each hen after the last bacterial feeding. Lipids were extracted (Lepage and Roy 1986), and the coprostanol-to-cholesterol ratios (w/w) were determined by gas-liquid chromatography by the method of Oles et al. (1990). The lipid extract from the samples was evaporated to dryness under nitrogen. Sterols in the residue were etherified with 1,3-bis-(trimethylsilyl)trifluoroacetamide. A 5830A Hewlett Packard gas chromatograph was used. The glass column, 0.91 m x 4 mm internal diameter, was packed with 3% SP-2250 on 100/120 Supelcoport (Supelco Inc., PA). Operating conditions were: oven, 250-280°C; injector, 290°C; flame ionization detector, 290°C; and nitrogen flow rate, 20 ml/min.

**Statistical analysis**

The data were analyzed by Student's $t$ test for significance.
Results

The plasma cholesterol concentrations of the three groups of hens are shown in Figure 1. Great fluctuation was observed with plasma cholesterol concentrations in all groups. No difference ($P > 0.05$) was observed among groups during the experiment. Table 1 shows the coprostanol-to-cholesterol ratios in the feces of hens at 1, 2, and 4 d after the last bacterial feeding. A significantly higher ratio was seen in the feces of the active group at 4 d after last bacterial feeding.

Figure 1. The plasma cholesterol concentration of laying hens. The time of bacterial treatment is indicated as "Eubacterial Feeding". "Control, Inactive, and Active" refer to groups that received no, killed, and live bacterial feeding, respectively, during the bacterial treatment. The data are expressed as Mean ± SE.
Table 1. Coprostanol-to-cholesterol ratios in the feces of laying hens\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Time\textsuperscript{b}</th>
<th>Active</th>
<th>Inactive</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.14 $\pm$ 0.02</td>
<td>0.14 $\pm$ 0.02</td>
<td>0.02 $\pm$ 0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.04 $\pm$ 0.01</td>
<td>0.02 $\pm$ 0.01</td>
<td>0.02 $\pm$ 0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.08 $\pm$ 0.02\textsuperscript{c}</td>
<td>0.03 $\pm$ 0.01\textsuperscript{d}</td>
<td>0.01 $\pm$ 0.003\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean $\pm$ SE, n=10.

\textsuperscript{b} Days after last bacterial feeding.

\textsuperscript{c,d} Means with different superscripts within a given time are different ($P < 0.05$).

Discussion

The role of intestinal bacteria in the regulation of cholesterol homeostasis has been studied by several investigators (Eyssen and Parmentier 1974; Martin et al. 1973; Tortuero et al. 1975; Wostmann 1973). Tortuero et al. (1975) studied the effect of cecectomy of laying hens on serum and egg cholesterol contents. The decrease in the number of the intestinal bacteria led to a significant increase in both serum and egg cholesterol concentrations. Eyssen and Parmentier (1974) did not observe a hypocholesterolemic effect after association of germ-free rats with a cholesterol-reducing bacterium, \textit{Eubacterium} ATCC 21408. The bacteria, however, were found to colonize only the cecum, where no cholesterol absorption takes place. Oral administration of \textit{E. coprostanoligenes} to dietary-induced hypercholesterolemic rabbits caused a significant decrease of plasma cholesterol concentration because the cholesterol-reducing bacteria were found to colonize in
the jejunum and ileum, which are major sites for cholesterol absorption (Li et al. 1995).

In the present study, the coprostanol-to-cholesterol ratio in feces of both the inactive and the active groups were high 1 d after bacterial feeding, which was caused by the coprostanol contained in the bacterial preparation. Bacterial suspensions from cholesterol-containing cultures usually contain substantial amounts of coprostanol because the growing bacteria actively reduce cholesterol in the medium to coprostanol during cultivation (Freier et al. 1994). At 2 d after the last bacterial feeding, the dietary coprostanol had been flushed out, and fecal coprostanol-to-cholesterol ratios started to show a difference among groups. By 4 d after the last bacterial feeding, a significantly higher fecal coprostanol-to-cholesterol ratio was observed in the active group than those in the inactive and the control groups on day 4. No difference was found between the inactive and the control groups. The results suggest that the higher coprostanol-to-cholesterol ratio in the active group was caused by the live bacterial feeding. *E. coprostanoligenes* must have inhabited the intestine of hens and reduced cholesterol therein to coprostanol. The anatomical sites of bacterial colonization, however, were not determined. Plasma cholesterol concentrations of hens were not affected by the bacterial feeding, even though oral administration of *E. coprostanoligenes* did cause a significant hypocholesterolemic effect in hypercholesterolemic rabbits (Li et al. 1995). Normocholesterolemic hens may have a mechanism to regulate the plasma cholesterol concentration so that it will not be subject to the changes of cholesterol
absorption in the intestine. Therefore, even though *E. coprostanoligenes* was reducing cholesterol to coprostanol and thereby caused less absorption of cholesterol in the intestine, the decreased absorption was not enough to reset the homeostasis for plasma cholesterol of hens.

![Graph showing the effect of culture temperatures on the growth and coprostanol production of *E. coprostanoligenes*](image)

**Figure 2.** Effect of culture temperatures on the growth and coprostanol production of *E. coprostanoligenes*. The cultures were incubated for 48 h. The data are expressed as the mean ± standard error of triplicate samples.

Moreover, the average body temperature of chickens is about 42°C. The growth of *E. coprostanoligenes* at 41°C was about half of that at 39°C (Figure 2). Conversion of cholesterol to coprostanol by *E. coprostanoligenes* was also much less at the high temperature. Presumably, the influence of temperature on growth and coprostanol production in the intestine of hens would be similar to that observed in
Figure 2. Also, the fecal transit time of hens is shorter than that of rabbits. *E. coprostanoligenes* may need more time to efficiently reduce cholesterol to coprostanol. Selection of heat-resistant strains of *E. coprostanoligenes* and increasing the bacterial dosage should be considered in the future experiments with hens.

The effect of feeding *E. coprostanoligenes* on the cholesterol content in egg yolk was not determined in this study. No adverse effect of feeding *E. coprostanoligenes* was observed in laying hens on feed intake, egg production, body weight gain, and fecal consistency.

References Cited


FEEDING OF *EUBACTERIUM COPROSTANOLIGENES* ATCC 51222 TO GERM-FREE AND CONVENTIONAL MICE

A paper prepared for submission to *Applied and Environmental Microbiology*

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Abstract

This study investigated the possibility of using germ-free and conventional mice as animal models to study the effect of orally administered *Eubacterium coprostanoligenes* ATCC 51222 on serum cholesterol concentrations. It was found that *E. coprostanoligenes* from cholesterol-containing cultures survived treatment with a simulated stomach solution better than did cholesterol-free cultures. Germ-free mice had a serum cholesterol concentration that was two times higher than that of conventional mice, and their serum cholesterol also was more sensitive to dietary cholesterol. When fed a high cholesterol diet, germ-free mice increased their serum cholesterol concentration twice as much as did conventional mice. After oral inoculation of *E. coprostanoligenes*, the bacteria only lived temporarily in the intestine of germ-free mice. The positive oxidation-reduction potential in the intestine of germ-free mice might be responsible for the nonsurvival of the anaerobic bacterium. *E. coprostanoligenes* did not colonize in the intestine of
conventional mice either. The microflora in the intestine of conventional mice might have inhibited the growth and inhabitance of *E. coprostanoligenes*. Further studies need to be designed to make the digestive system of germ-free animals more suitable for growth of *E. coprostanoligenes*. Preinoculation of a nonpathogenic facultative bacterium such as Bacteroides into the digestive tract of germ-free animals may facilitate growth of the anaerobic bacterium.

**Introduction**

Elevated serum cholesterol concentration has been recognized as a major risk factor for the development of atherosclerosis that leads to coronary heart disease (CHD) (Gotto et al. 1990), which remains the leading cause of human mortality in developed countries (Thom et al. 1992). There is substantial evidence that lowering serum cholesterol concentrations will decrease the incidence of CHD (Lipid Research Clinic Program 1988). Clinical trials have shown that cholesterol-lowering therapy improves survival of patients with CHD (Scandinavian Simvastatin Survival Study Group 1994).

A newly isolated bacterium, *Eubacterium coprostanoligenes* ATCC 51222, converts cholesterol to coprostanol, which is absorbed poorly by the gastrointestinal system (Bhattacharyya 1986; Freier et al. 1994). Normally, coprostanol is excreted in the feces, and it is a major component of fecal sterols. Previous studies have showed that oral administration of *E. coprostanoligenes* decreased the plasma cholesterol concentration significantly in hypercholesterolemic rabbits (Li et al.
The observation has provided a potential way to decrease serum cholesterol concentrations without involvement of drugs, with which undesirable side effects have caused concerns about their therapeutic use (Erkelens et al 1988). Before the cholesterol-reducing bacterium can be used in clinical cases, however, many animal experiments have to be conducted to support the application.

The present study was designed to test the possibility of using mice as an alternative animal model to investigate the effect of feeding *E. coprostanoligenes* on serum cholesterol concentrations. The survivability of *E. coprostanoligenes* under stomach conditions was tested *in vitro* before the bacteria were fed to mice. The difference between germ-free and conventional mice was compared in terms of serum cholesterol concentrations, responses to dietary cholesterol, and colonization of *E. coprostanoligenes* after oral administration.

**Materials and Methods**

**Cultivation of bacteria**

*E. coprostanoligenes* was cultured in a growth medium containing the following components per liter: 10 g casitone (Difco, Detroit, MI), 10 g yeast extract, 5 g lactose, 5 g sodium pyruvate, 0.5 g sodium thioglycolate, 0.2 g cholesterol (ash-free, Sigma Chemical Co., St. Louis, MO), 0.1 g lecithin (type IV-S, Sigma), 0.1 g CaCl$_2$·2 H$_2$O, and 1 mg resazurin. Cholesterol was omitted to prepare a cholesterol-free medium. The pH of the medium was adjusted to 7.5 with 3 N KOH. The Hungate technique was used for the anaerobic preparation of
media and handling of the culture (Holdeman et al. 1977). Fresh medium was inoculated with 1% of a 48-h culture and incubated at 37°C.

Simulation of stomach conditions

Simulated stomach solution (pH 1.0) contained 0.1 M HCl, 50 mM NaCl, 5 mM KCl, 5 mM CaCl₂, and 0.01% pepsin (Tennant and Hornbuckle 1989). Bacterial cells harvested after 2 d of incubation in a cholesterol-containing or cholesterol-free media were suspended in three volumes of 25 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5) containing 5 mM sodium thioglycolate and 45 mM sodium pyruvate or the stomach solution. After 2 h of incubation, the mixtures were added to nine volumes of fresh cholesterol-containing media and incubated at 37°C. After 5 d of incubation, the bacterial growth and coprostanol production were determined. PIPES buffer treatment was used as a control.

Animals and diets

A total of 24 Swiss germ-free and conventional mice (Taconic Laboratories), 12 of each, were used in this study. Germ-free mice were living in a germ-free isolator and were fed Teklad mouse breeder sterilizable diet (Harlan Teklad Inc., WI) ad libitum. The serum cholesterol concentrations of mice were determined before the experiment started. A cholesterol diet (5%, dry matter basis) was made by dissolving cholesterol in ethyl ether and mixing with Teklad diet. After ether
was evaporated completely under $N_2$, the cholesterol diet was fed to mice for 1 wk to determine their response to dietary cholesterol intake in terms of serum cholesterol. After the serum cholesterol concentrations were back to the normal range, mice were divided randomly into 2 groups—experimental and control groups—with 6 mice in each group. The experimental group received 0.5 ml of active *E. coprostanoligenes* culture (~0.5 mg bacterial protein) *per os* daily for 1 wk. The control group received the same dose of killed (boiled) bacteria. The same design was used for conventional mouse experiment except that the conventional mice were fed a 0.5% cholesterol diet instead of regular chow when bacterial feeding started.

**Determination of serum cholesterol concentrations**

Blood samples were drawn weekly from the orbital sinus after mice were anesthetized by peritoneal injection of ketamine and rompun solution (Fort Dodge Laboratories, IA) or by inhalation of halothane (Halocarbon Laboratories, SC). The serum cholesterol concentrations were determined in triplicate enzymatically with a Sigma cholesterol kit (Cholesterol 100, Sigma).

**Determination of bacterial growth**

The total cellular protein concentration was used as a indicator of bacterial growth. Bacterial cultures were harvested by centrifugation at 10,000 x $g$ for 10 min at 4°C usually after incubation. The cell pellets then were washed once and
suspended in 25 mM PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) buffer (pH 7.5) containing 5 mM sodium thioglycolate and 45 mM sodium pyruvate. Total cellular protein was determined by the Bradford method with microtiter plates (Redinbaugh and Campbell 1985). A portion of the cell suspension was mixed with an equal volume of 2 N NaOH and incubated at 37°C overnight to solubilize the protein. Bovine serum albumin, treated in the same manner, was used as a standard.

Quantitation of coprostanol and cholesterol

Fresh fecal samples were collected from each mouse weekly to inoculate the cholesterol-containing medium for recovery of *E. coprostanoligenes*. Also, cholesterol-to-coprostanol ratios (wt/wt) of feces were determined by gas-liquid chromatography (Oles et al. 1990). Fecal lipids were extracted in duplicate as described by Lepage and Roy (1986). The duplicate lipid extract from the samples was evaporated to dryness under nitrogen. Sterols in the residue were etherified with 1,3-bis-(trimethylsilyl)trifluoroacetamide (Oles et al. 1990). A 5830A Hewlett Packard gas chromatograph was used. The glass column, 0.91 m x 4 mm internal diameter, was packed with 3% SP-2250 on 100/120 Supelcoport (Supelco Inc., PA). Operating conditions were: oven, 250-280°C; injector, 290°C; flame ionization detector, 290°C; nitrogen flow rate, 20 ml/min. At the end of the experiment, the mice were killed and the intestinal contents were inoculated into the cholesterol-containing medium for recovery of *E. coprostanoligenes*. The cholesterol contents
of liver and leg skeletal muscle from germ-free mice were determined also.

Statistical analysis

The data were analyzed by Student's $t$ test for significance.

Results

Survival of bacteria from simulated stomach conditions

Bacterial growth and coprostanol production were determined after treatment with the PIPES buffer (control) or the simulated stomach solution (Figure 1). Bacteria from cholesterol-containing cultures retained about 40% of growth and coprostanol production abilities when compared with controls. Bacteria from cholesterol-free cultures, however, only survived 10% after the treatment with simulated stomach conditions. Therefore, bacteria from cholesterol-containing cultures were fed to mice in this study.

Comparison of normal serum cholesterol concentrations

Normal serum cholesterol concentrations of germ-free and conventional mice were measured (Figure 2). It was found that serum cholesterol concentration of germ-free mice were two-fold higher than those of conventional mice.
Figure 1. Effect of simulated stomach conditions on bacterial growth (a) and coprostanol production (b). Cell protein is a measure of growth. Coprostanol production was measured as percentage of cholesterol in growth medium that was converted to coprostanol. Chol Culture and Chol-Free Culture refer to cultures with and without cholesterol, respectively. Data are expressed as the mean of triplicate samples ± standard error.
Figure 2. Serum cholesterol concentrations of germ-free and conventional mice. Data are expressed as the mean ± standard error of 12 mice.

Response to dietary cholesterol

Germ-free and conventional mice were fed 5% cholesterol diets for 1 wk, and the serum cholesterol concentrations were determined before and after the cholesterol feeding (Fig. 3). The serum cholesterol concentration increased by 130 mg/dL in germ-free mice, but only 62 mg/dL in conventional mice. Referring to the base values of serum cholesterol concentrations, however, the percentage of increase was similar in germ-free and conventional mice, which was about 70%. After the removal of cholesterol from the diet, serum cholesterol concentrations of conventional mice returned to normal. In germ-free mice, however, the serum cholesterol concentration declined but was still significantly higher than their normal value.
Figure 3. Effect of dietary cholesterol on serum cholesterol concentrations of germ-free and conventional mice. Mice were fed 5% cholesterol diet for the first week, and cholesterol was removed after the first week. Data are expressed as the mean ± standard error of 12 mice.

Effects of bacterial feeding on serum cholesterol concentrations

The serum cholesterol concentrations of germ-free and conventional mice are shown in Figure 4. In germ-free mice, after 1 wk of bacterial feeding, the serum cholesterol concentration of experimental mice tended to decrease to a greater extent compared with that of control mice. When the bacterial feeding stopped, however, no difference was observed between experimental and control groups afterwards. In conventional mice, the serum cholesterol concentration increased in the beginning of the experiment because mice were fed a 0.5% cholesterol diet. During the whole experiment, no significant difference ($P > 0.05$) was found in the serum cholesterol concentrations between experimental and control groups at any of measured times.
Figure 4. Effect of bacterial feeding on serum cholesterol concentrations of germ-free and conventional mice. Bacteria were fed between 3 to 4 wk. Conventional mice were fed a 0.5% cholesterol diet beginning at wk 2. Data are expressed as the mean ± standard error of 6 mice.

Coprostanol-to-cholesterol ratios in feces and fecal cultures

Coprostanol-to-cholesterol ratios in feces of germ-free and conventional mice are shown in Table 1. In the feces of germ-free mice, there was no coprostanol detectable before the feeding of *E. coprostanoligenes*. Coprostanol content was increased significantly (*P* < 0.05) in the feces of the experimental mice after being fed *E. coprostanoligenes* for 1 wk. It declined, however, 1 wk after the last bacterial feeding and was not detectable thereafter. In conventional mice, coprostanol was detectable, but the coprostanol-to-cholesterol ratios were low throughout the experiment. No differences were observed between the experimental and control groups (*P* > 0.05).
<table>
<thead>
<tr>
<th>Group</th>
<th>WK 1</th>
<th>WK 2</th>
<th>WK 3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>WK 4&lt;sup&gt;b&lt;/sup&gt;</th>
<th>WK 5</th>
<th>WK 6</th>
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<tr>
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<tr>
<td>Experimental</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.29&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>ND</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.07</td>
<td>ND</td>
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<tr>
<td>Conventional mice</td>
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<td></td>
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<tr>
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<td>0.04</td>
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<td>0.04</td>
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</table>

<sup>a</sup> Data are expressed as the average value of 6 mice.

<sup>b</sup> From wk 3 to 4, the experimental group received 0.5 ml of live *E. coprostanoligenes* (~0.5 mg of bacterial protein) *per os* daily and the control group received the same dose of killed (boiled) bacteria.

<sup>c</sup> ND = Not detectable.

<sup>d,e</sup> Means with different superscripts within a given time are different (*P* < 0.05).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cholesterol (mg/g dry tissue)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Skeletal muscle</td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>3.95 ± 0.18</td>
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<tr>
<td>Control</td>
<td>3.36 ± 0.17</td>
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<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>Experimental</td>
<td>10.04 ± 0.58</td>
</tr>
<tr>
<td>Control</td>
<td>10.69 ± 0.43</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard error, *n*=6.
E. coprostanoligenes was not recovered either from the cultures of fresh feces during the experiment or from the cultures of intestinal contents of the mice at the end of the experiment. Cholesterol contents of skeletal muscle and liver of germ-free mice are shown in Table 2. No difference ($P > 0.05$) in cholesterol contents of either tissue was observed between the experimental and control groups.

**Discussion**

E. coprostanoligenes does not require cholesterol for growth but retains its cholesterol-reducing ability in the absence of cholesterol (Freier et al. 1994; Li et al. 1995b). It was planned originally to use bacteria from cholesterol-free cultures to feed mice. *In vitro* experiments, however, showed that bacteria from cholesterol-free cultures did not survive the treatment with the simulated stomach solution, whereas bacteria from cholesterol-containing cultures had about 40% of growth and coprostanol production ability after the stomach solution treatment. Bacteria growing in cholesterol-containing media may have formed some capsule-like structures that partially protected the bacteria from the strong acid treatment of stomach solution. Therefore, bacteria from cholesterol-containing cultures should be used to feed animals for a bacterial feeding study. It would be ideal if bacteria from cholesterol-free cultures could be sealed in some sort of acid-proof capsules before oral administration so that the carry-over effect of cholesterol from the bacterial preparation of cholesterol-containing cultures would not be of concern.
Germ-free mice had much higher serum cholesterol concentrations than did conventional mice when fed the same diet. Similar observations have been reported with other animals (Kellogg and Wostmann 1969a; Wostmann 1973). Absence of intestinal microflora is responsible for the higher cholesterol accumulation in germ-free animals. It has been found that conventional animals excrete 50 to 100% more neutral sterols through feces than do germ-free animals (Evrard et al. 1964; Kellogg and Wostmann 1969b; Eyssen et al. 1974). Furthermore, cholesterol was more efficiently absorbed from the intestine of germfree rats, and germ-free rats had a 2 to 3 times higher liver cholesterol content than did conventional rats when moderate amounts of cholesterol with the diet were fed (Wostmann 1973).

In the present study, germ-free mice were very sensitive to dietary cholesterol. Serum cholesterol concentrations were increased rapidly after initiation of cholesterol feeding. The amount of increase was double that of conventional mice. At 2 wk after replacement of the high cholesterol diet with a regular Teklad diet, serum cholesterol concentrations of conventional mice returned to normal. In germ-free mice, however, the serum cholesterol concentration declined but was still significantly higher than their normal value. Therefore, at least two mechanisms could account for the influence of the intestinal microflora on the cholesterol pools in the body: effects on elimination via direct microbial influences on bile acid excretion, and, indirectly, effects on dietary uptake of dietary cholesterol because of influences on the enterohepatic bile acid pools.
supporting absorption.

The results from the bacterial feeding study were somewhat disappointing. By using germ-free mice, the original attempt was to develop a monoassociated animal model with *E. coprostanoligenes* so that the mechanisms of the hypocholesterolemic effect can be investigated more specifically. After 1 wk of bacterial feeding, fecal coprostanol-to-cholesterol ratios were significantly higher in the experimental group than that in the control group, indicating that the bacteria did live in the digestive tract and reduced cholesterol to coprostanol. This effect, however, was only temporary because the ratios declined 1 wk after the last bacterial feeding, and coprostanol was not detectable thereafter as before bacterial feeding. This transient effect also was reflected in serum cholesterol concentrations. After 1 wk of bacterial feeding, the serum cholesterol concentration of the experimental group tended to decrease to a greater extent than that of controls. One wk after the last bacterial feeding, however, the serum cholesterol concentration were the same for both groups.

Negative results from recovery of *E. coprostanoligenes* in the fresh feces or the intestinal contents also suggested that the bacteria did not colonize the intestine. *E. coprostanoligenes* is an anaerobic bacterium and grows only in anaerobic conditions, although it survives exposure to air for at least 48 h (Freier et al. 1994). In germ-free animals, the digestive tract is not colonized with facultative or any other bacteria that usually consume oxygen and facilitate the growth of anaerobic bacteria. The presence of substantial oxygen in the digestive tract of
germ-free mice might cause the nonsurvival of *E. coprostanoligenes*.

Eyssen and Parmentier (1974) tried to establish a monoassociate with *Eubacterium ATCC 21408*, the first pure isolate of cholesterol-reducing bacterium, in the intestine of germ-free rats and failed because of a positive oxidation-reduction potential. The colonization of *E. ATCC 21408* was established by exposing rats to a *Clostridium* species first and then to *E. ATCC 21408*. The hypocholesterolemic effect, however, was not observed in gnotobiotic rats associated with *E. ATCC 21408* plus *Clostridium Cl-8* because the cholesterol-reducing bacterium was colonized in the cecum and the large intestine, sites from which no cholesterol absorption takes place (Eyssen and Parmentier 1974).

After oral administration of *E. coprostanoligenes* to rabbits, the bacteria colonized and reduced cholesterol in the jejunum and ileum, which are major sites for cholesterol absorption (Li et al. 1995b). A significant hypocholesterolemic effect was observed in dietary-induced hypercholesterolemic rabbits associated with *E. coprostanoligenes*. In the present study, *E. coprostanoligenes* failed to colonize the digestive tract of germ-free mice; therefore, the effect of the bacterium on serum cholesterol concentration could not be investigated. Future studies should focus on how to make the digestive system of germ-free animals suitable for growth of *E. coprostanoligenes*. Preinoculation of a nonpathogenic facultative bacteria such as *Bacteroides* into the digestive tract of germ-free animals may solve the problem. Only then would the study of impact of *E. coprostanoligenes* on serum cholesterol concentrations of germ-free animals be feasible.
Conventional mice were used also in this study because it was thought that the microflora in the intestine of conventional mice would facilitate the growth of *E. coprostanoligenes*. Surprisingly, the bacteria even did not show temporary growth in the intestine after oral inoculation. The fecal coprostanol-to-cholesterol ratios were low during the whole period of the experiment, and fresh fecal cultures did not result in recovery of the bacteria either. It seems that the environment in the intestine of mice is not suitable for the growth of *E. coprostanoligenes*. The microflora in the intestine of conventional mice may inhibit the growth and colonization of *E. coprostanoligenes*. Therefore, mice might not be a good animal model to study the effect of the bacterium on serum cholesterol concentration.

References Cited


Excess serum cholesterol is a major risk factor for the development of cardiovascular diseases. The identification of bacteria that convert cholesterol to coprostanol has provided a potential method to naturally decrease serum cholesterol in humans because coprostanol is absorbed poorly from the gastrointestinal system. In the present study, a novel cholesterol-reducing bacterium, *Eubacterium coprostanoligenes* ATCC 51222, was characterized and tested for its hypocholesterolemic effect in animal models.

*E. coprostanoligenes* did not require cholesterol for growth but retained its cholesterol-reducing ability. The enzyme, cholesterol reductase, seems to be synthesized constitutively by the bacterium. Lecithin was required for growth. Excess of lecithin in media, however, did not improve bacterial growth. Optimal growth and coprostanol production were achieved at pH 7.0 to 8.0 in the presence of pyruvate, lactose, and reducing agents in media incubated at 37°C to 39°C. Addition of a buffer system into media seemed unnecessary for bacterial growth but increased coprostanol production. Most antibiotics inhibit bacterial growth and coprostanol production at a final concentration of 50 μg/ml with the exception of tetracycline. The resistance to tetracycline was dose-dependent. Ethanol as a solvent for tetracycline showed no effect with a final concentration up to 1.3%. The mechanism for tetracycline resistance is unknown.

A resting cell assay was developed to evaluate the cholesterol reductase
activity of *E. coprostanoligenes*. Washed bacterial cells rapidly reduced cholesterol to coprostanol with micellar cholesterol as a substrate. Optimal assay conditions in a 1-ml reaction mixture were determined to be up to 1 h of incubation and up to 0.25 mg of bacterial protein per assay with at least 1 mM of cholesterol substrate. The cholesterol reductase activity in cells decreased as a function of storage time at 22°C, 4°C, and -20°C. Filling the headspace of the reaction mixture with H₂ increased the cholesterol reductase activity about 20%. Optimal cholesterol reductase activity occurred at pH 7.5 in sodium phosphate buffer. Pyruvate and reducing agents in the buffer increased the cholesterol reductase activity.

*E. coprostanoligenes* was shown to be a Gram-positive coccobacillloid bacterium by use of the electron microscope. The cells were 0.5 to 0.7 μm in diameter and 0.7 to 1.0 μm in length and occurred singly and in pairs. The bacterium reduced cholesterol through an indirect metabolic pathway. Intermediates, 4-cholesten-3-one and coprostanone, were detected in resting cell assays even though no accumulation of intermediates were observed in actively growing cultures with cholesterol. *E. coprostanoligenes* seemed to have a broad specificity for substrates. The bacterium converted all the intermediates to coprostanol, and it metabolized pregnenolone, progesterone, androstenediol, and etienic acid. In resting cell assays, NADH or FAD increased cholesterol reductase activity, but the activity was inhibited when NADH and FAD were added together to the reaction mixture. Iodoacetate, NaCN, and NaN₃ did not affect the enzyme activity significantly, whereas EDTA inhibited the activity, and HgCl₂ abolished the
cholesterol reductase activity completely.

The enzyme activity was found to be associated with bacterial cells. Attempts to achieve active cell-free extracts were not successful because a great loss of activity occurred after the bacterial cells were disrupted by sonication, passage through a French pressure cell, freezing and thawing, liquid N₂ freezing and pulverizing, or enzymatic digestion. Addition of different cofactors did not recover the cholesterol reductase activity. The genomic expression library of *E. coprostanoligenes* was constructed in an expression vector, bacteriophage \( \lambda \) vector \( \lambda gt11 \). Much effort was made to develop a screening technique for detection of positive clones for cholesterol reductase. The cholesterol oxidase gene (\( cho \)) was considered a possible probe, the preliminary experiment, however, did not show hybridization of the gene with the genomic DNA of *E. coprostanoligenes*. Other cholesterol-related genes should be considered as potential probes.

Effect of orally administered *E. coprostanoligenes* on plasma or serum cholesterol concentrations were investigated in rabbits, laying hens, and germ-free and conventional mice. *E. coprostanoligenes* caused a significant decrease of plasma cholesterol concentrations in dietary-induced hypercholesterolemic rabbits. The hypocholesterolemic effect was explained by the conversion of cholesterol to coprostanol by colonized *E. coprostanoligenes* in the intestine.

In laying hens, the bacterium did not grow well in the intestine because of the high body temperature of hens. Even though orally administered bacteria did convert cholesterol to coprostanol in the intestine, plasma cholesterol
concentrations of hens were not affected. Normocholesterolemic hens may have a mechanism to regulate the plasma cholesterol concentration so that it will not be subject to the changes of cholesterol absorption in the intestine, or the conversion of cholesterol to coprostanol in the intestine may not been enough to reset cholesterol homeostasis mechanisms of hens.

In germ-free mice, *E. coprostanoligenes* only survived temporarily in the intestine. The serum cholesterol concentrations were affected also transiently. The positive oxidation-reduction potential in the intestine of germ-free mice might be responsible for the dying-off of the anaerobic bacterium. *E. coprostanoligenes* did not colonize in the intestine of conventional mice either. The microflora in the intestine of conventional mice might have inhibited the growth and inhabitance of *E. coprostanoligenes*. Coinoculation of a nonpathogenic bacterium with *E. coprostanoligenes* in germ-free mice or modification of intestinal microflora in conventional mice would allow the colonization of the cholesterol-reducing anaerobic bacterium. Only then would the study of the impact of *E. coprostanoligenes* on serum cholesterol concentrations of mice be feasible.

In conclusion, *E. coprostanoligenes* has many unique characteristics that make the bacterium more amenable than previously isolated cholesterol-reducing bacteria to be used for decreasing cholesterol content of foods and for lowering serum cholesterol concentrations of hypercholesterolemic individuals. More studies need to be conducted, however, before the cholesterol-reducing bacterium can be applied in the food-processing and pharmaceutical industries.
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