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Production and Isolation of Reuterin, a Growth Inhibitor Produced by *Lactobacillus reuteri*†

TODD L. TALARICO,[1] IVAN A. CASAS,[2] TUNG C. CHUNG,[1] AND WALTER J. DOBROGOSZ[1]*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695,[1] and Pro Biologics International, Inc., Raleigh, North Carolina 27612[2]

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***Lactobacillus reuteri* is a prominent member of the *Lactobacillus* population in the gastrointestinal ecosystem of humans, poultry, swine, and other animals. Reuterin is a newly discovered, broad-spectrum antimicrobial substance produced by this species during fermentation of glycerol. In this report, we describe procedures for (i) producing reuterin in sufficient amounts to isolate from a fermentation mixture and (ii) isolating this substance by high-performance liquid chromatography. By using uniformly labeled [^{14}C]glycerol, reuterin was identified as a product of glycerol fermentation associated with the production of β-hydroxypropionic acid and trimethylene glycol.**

Axelsson and co-workers (L. Axelsson, T. C. Chung, W. J. Dobrogosz, and S. E. Lindgren, submitted for publication) and Chung and colleagues (T. C. Chung, L. Axelsson, S. E. Lindgren, and W. J. Dobrogosz, submitted for publication) reported the discovery of a broad-spectrum antimicrobial substance termed reuterin produced by *Lactobacillus reuteri*. *L. reuteri* resides in the gastrointestinal tract of healthy humans and animals (2, 5) and is believed to function as a symbiont in the enteric ecosystem. Synthesis of such an antimicrobial substance by an enteric resident raises a number of interesting questions and possibilities as to the role these residents may play in the health of the host. Yet little is known about *L. reuteri* and less about reuterin except that it is produced specifically from glycerol by anaerobic resting cells under physiological conditions of temperature and pH. Preliminary investigations indicate that it is a low-molecular-weight, neutral, water-soluble, nonprotein material that has antibacterial, antimycotic, and antiprotozoal activity.

The culture conditions described for reuterin production in other reports (Axelsson et al., submitted; Chung et al., submitted) yield only relatively small quantities of this substance. The objectives of the present study were twofold: first, to develop a protocol that would enable us to obtain relatively large amounts of reuterin, and second, to develop a procedure to isolate and purify reuterin. Both objectives were accomplished, and reuterin is now available in sufficient amounts to initiate studies on its molecular structure. In addition, by using uniformly labeled [^{14}C]glycerol in these studies, we characterized the reaction pathway that is associated with reuterin production.

MATERIALS AND METHODS

Growth of cells. *L. reuteri* 1063 was maintained at 37°C overnight in lactobacillus carrying medium containing 20 mM glucose (Axelsson et al., submitted). A 1% inoculum from an overnight culture was added to fresh medium and grown for 16 h at 37°C in standing (still) culture, collected by centrifugation at 4,000 × *g*, washed twice with 50 mM sodium phosphate buffer (pH 7.5), and used for production of reuterin. Stock cultures of *L. reuteri* 1063 were maintained at −70°C in lactobacillus carrying medium-glucose containing 12.5% glycerol as a cryoprotectant as previously described (2).

Production and assay of reuterin. Reuterin was produced by a modification of the system reported by Chung et al. (submitted) and Axelsson et al. (submitted). A 16-h-old overnight culture of *L. reuteri* cells (50 mg [dry weight]) was washed as described above, suspended in 5 ml of 250 mM glycerol in distilled water, and incubated under anaerobic conditions (nitrogen sparging) at 37°C. In some studies, 2 M glucose was added together with the glycerol. After incubation, the cells were harvested by centrifugation at 4,000 × *g* for 10 min and discarded. The supernatant fraction was filtered through an Acrodisc filter (0.45-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at 4°C in a sterile container for subsequent assay or purification. Reuterin was assayed by an MIC procedure with *Escherichia coli* K-12 as the indicator strain as described elsewhere (Chung et al., submitted). Relative quantitation was based on this assay with units of reuterin per milliliter calculated as the reciprocal of the dilution preceding that which allowed growth of *E. coli* under standardized conditions.

Reuterin production was inhibited by adding 300 mM neutralized semicarbazide hydrochloride (Sigma Chemical Co., St. Louis, Mo.) to the reuterin production system containing 250 mM glycerol in 1 M sodium phosphate buffer (pH 7.5).

Metabolism of radiolabeled glycerol. A 5-μCi sample of uniformly labeled [^{14}C]glycerol (171 mCi/mmol; Radiochemical Centre, Amersham, England) was added to 2 ml of the reuterin production system described above. Nonlabeled carrier glycerol was added to achieve a wide range of glycerol concentrations. The production systems were then purged with nitrogen and incubated for 2 h. After removal of cells by centrifugation, supernatant fractions were filtered through a 0.45-μm-pore-size filter and stored at 4°C until assayed. Samples were placed in 20-ml glass scintillation vials containing 7 ml of Scintiverse E (Fisher Scientific Co., Raleigh, N.C.). Scintillation counting was done with a Packard Tri-Carb liquid scintillation spectrometer.

HPLC isolation of reuterin. Reuterin was separated from other components in the reaction mixture with a C_{18} resin: μBondapak column (300 by 4.6 mm; Waters Associates,

* Corresponding author.

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Inc., Milford, Mass.) in series with a Zorbax ODS column (250 by 4.6 mm; Dupont Instruments) effected separation. A flow rate of 1 ml of filtered (0.45-μm-pore-size filter; Gelman) distilled deionized water min^{-1} was delivered with a pump (110 A; Beckman Instruments, Inc., Fullerton, Calif.). Injections of 20 μl were made with a Beckman 210 injector. Products were detected with a Waters 410 differential refractometer at a sensitivity of 8× and a scale factor of 5. Chromatograms were recorded on a strip chart recorder (BD40; Kipp & Zonen). Glycerol (Fisher), 1,3-propanediol (Aldrich Chemical Co., Inc., Milwaukee, Wis.), and β-hydroxypropionic acid (β-HPA) were used as standards for peak confirmation. β-HPA was synthesized by the method of Read (6) and confirmed by Fourier transform infrared (FTIR) spectroscopy. The above separation method was scaled up to a semipreparative level with an Altex Ultrasphere-ODS column (10 by 300 mm). The chromatography system described above was used with the exception of a 250-μl sample loop and a refractometer sensitivity setting of 4×. Fractions were collected manually after detection.

An analytical high-performance liquid chromatography (HPLC) system with superior peak resolution was obtained by utilizing an Aminex HPX 87H column (Bio-Rad Laboratories, Richmond, Calif.) (3). The HPLC equipment was the same as described above; the solvent system consisted of a 65:35 mixture of filtered deionized water and HPLC-grade acetonitrile (Fisher) containing 0.01 N H_2SO_4. A flow rate of 0.4 ml min^{-1} was maintained at ambient temperature.

FTIR analysis of samples. HPLC peak identification was confirmed by FTIR analyses of collected components. Samples were lyophilized to dryness, suspended in HPLC-grade methanol (Fisher), evaporated onto a sodium chloride absorption cell (Sergent Welch), and subjected to FTIR analysis on a Perkin-Elmer 1550 FTIR spectrometer. The data were analyzed on a Perkin-Elmer 7500 data system.

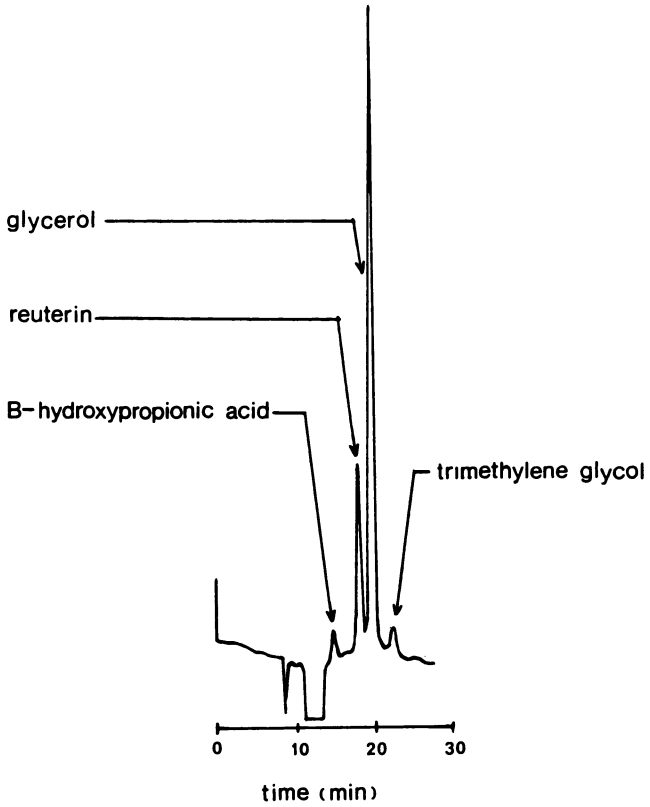

FIG. 1. Chromatographic separation of glycerol fermentation products from *L. reuteri* was accomplished by using an HPX 87H column with a 0.4-ml min^{-1} flow rate of a 65:35 mixture of water and acetonitrile containing 0.01 N sulfuric acid. The four major products present in the fermentation mixture are labeled on the chromatogram.

RESULTS

Production of reuterin. The coculture system containing both *L. reuteri* and *E. coli* cells as described by Chung et al. (submitted) yielded reuterin in concentrations of approximately 100 units ml^{-1}. We determined that a reaction system containing higher concentrations (10 mg [dry weight] ml^{-1}) of only *L. reuteri* cells incubated anaerobically in the presence of 250 mM glycerol produced reuterin in concentrations as high as 1,000 MIC units ml^{-1}. This reaction system was used to obtain the data described in this report.

Identification of glycerol fermentation products by HPLC. Separation of components present in these glycerol fermentations was accomplished initially with two C_{18} columns in series with a water mobile phase. Four major component peaks eluted within 30 min of injection at a flow rate of 1 ml min^{-1}. This separation was successfully scaled up to semipreparative chromatography. Collection of all four fractions and subsequent FTIR analysis led to identification of three of the components as glycerol, trimethylene glycol (TMG), and β-HPA. The fourth component, reuterin, could not be structurally confirmed by FTIR analysis alone. All fractions collected were assayed for inhibitory activity against *E. coli* (by the MIC assay), and only the reuterin fraction possessed activity.

Analytical HPLC with an HPX 87H column was performed to quantitate these four compounds (Fig. 1). A linear relationship was established between reuterin concentration (as measured by the MIC assay) and peak height as determined by HPLC. The HPLC assay was thus considered to be an accurate measure of reuterin concentration and was used in subsequent analyses.

Pure samples of the other components (TMG, β-HPA, and glycerol) found in fermentation supernatant fractions were tested for their ability to inhibit *E. coli* growth, and none was effective in the concentration ranges (as determined by HPLC) found in the reaction system supernatant fraction.

Other heterofermentative strains of lactobacilli have been reported to ferment glycerol to TMG and β-HPA (8), and it has been suggested that the formation of TMG and β-HPA (Fig. 2) proceeds through an aldehydic intermediate which may be trapped by semicarbazide hydrochloride (1). To establish the presence of an aldehydic intermediate during reuterin production, semicarbazide hydrochloride was added to *L. reuteri* cells fermenting glycerol in our standard reaction system. This addition eliminated reuterin production. Upon removal (by centrifugation) of the supernatant fraction containing the semicarbazide hydrochloride and subsequent addition of a glycerol solution, the cells regained the capability to produce reuterin, indicating that the action of the semicarbazide hydrochloride did not destroy the capability of the organism to produce reuterin. Control *L. reuteri* fermentations produced enough reuterin in the absence of semicarbazide hydrochloride to kill the producer cells, and reintroduction of glycerol with subsequent incubation resulted in no fermentation of the added glycerol.

Analysis of *L. reuteri* glycerol fermentation products. To obtain further information on this fermentation, we sus-

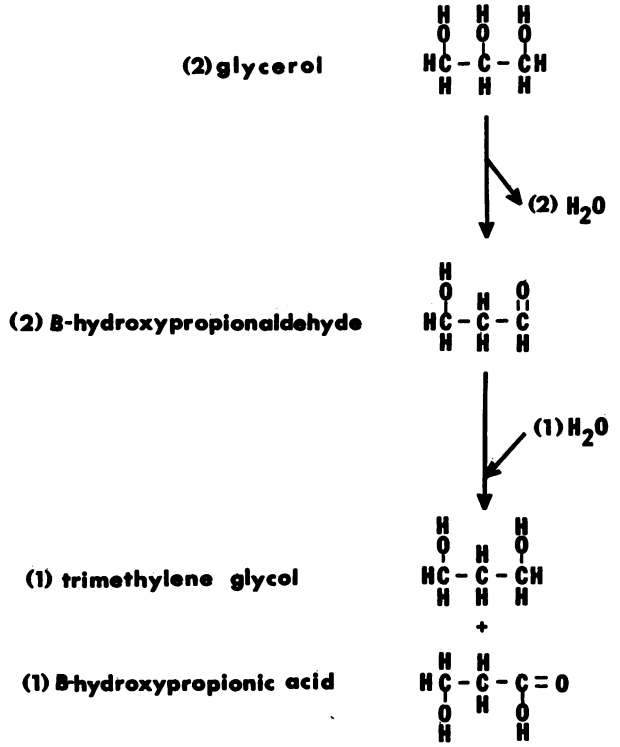

FIG. 2. Proposed pathway of glycerol fermentation in *L. reuteri*. Glycerol is converted to β-hydroxypropionaldehyde by a dehydrase. Two molecules of the aldehyde then undergo an aldehydic dismutation to form equimolar quantities of TMG and β-HPA.

pended 10 mg (dry weight) of washed *L. reuteri* cells ml^{-1} in glycerol and incubated them at 37°C for 1.5 h. Concentrations of 10, 25, 50, 100, 200, and 300 mM glycerol were used, each containing 5 μCi of uniformly labeled [^{14}C]glycerol. After incubation, cells were removed and the supernatant fractions were subjected to chromatography. Fractions were collected manually, and the radioactivity present in the samples was measured. Essentially all the radioactivity could be recovered in the four major components found in the supernatant (i.e., glycerol, reuterin, TMG, and β-HPA). Preliminary evidence indicated that 1 mol of glycerol is required to produce 1 mol of reuterin (data not shown), and the amount of reuterin present in fermentations was calculated based on this conversion. An increase in reuterin production occurred as the glycerol concentration increased (Fig. 3). TMG and β-HPA levels, however, remained constant at initial glycerol concentrations above 50 mM. Moreover, β-HPA and TMG were produced in equimolar amounts. A substantial portion of glycerol was not utilized when initial concentrations exceeded 50 mM. The above experiment was repeated in the presence of 20 mM glucose (Fig. 4). Reuterin concentrations were calculated based on a conversion ratio of 1 mol of glycerol to 1 mol of reuterin. Reuterin production was not affected by the presence of glucose when glycerol concentrations were above 100 mM, whereas at lower glycerol levels, reuterin production was slightly curtailed. The level of TMG was approximately doubled when glucose was present in the reaction system. The increase in TMG production correlated with a greater utilization of glycerol in this system. β-HPA production did not increase in the presence of glucose and, in fact, decreased slightly when compared with that of the glucose-free production system.

Production of reuterin as a function of incubation time. *L. reuteri* cells were suspended to concentrations of 2.5 and 4.5 mg of cells (dry weight) ml^{-1} in 200 mM glycerol and incubated. Samples were assayed by HPLC, and the results summarized in Fig. 5 show the relationship between reuterin production and incubation time. At both cellular concentrations, reuterin production attained a maximum after an incubation of approximately 2 h. Cells removed from the production systems after this time were not viable, indicating that reuterin had killed the producing organisms.

Stability of reuterin at 37°C. Reuterin isolated by semipreparative chromatography, lyophilized to dryness, and suspended in water was tested for stability at 37°C at three pH values. Samples were adjusted to pH values of 2.0, 6.5, and 11.0 with 1 N sulfuric acid or sodium hydroxide. Each sample was divided into two equal portions, one placed at 37°C and the other at 0°C as a control since samples of reuterin stored at 0°C retained activity for more than 6 months. A portion of the samples was then chromatographed at various times, and a ratio of peak heights in each case was calculated as the percentage of reuterin remaining in the preparations. Reuterin incubated under acidic conditions degraded less rapidly than the corresponding neutral sample (Fig. 6). Reuterin exposed to pH 11 degraded immediately and could not be recovered by adjusting the pH to 5, indicating an irreversible degradation of the reuterin molecule upon exposure to an alkaline environment.

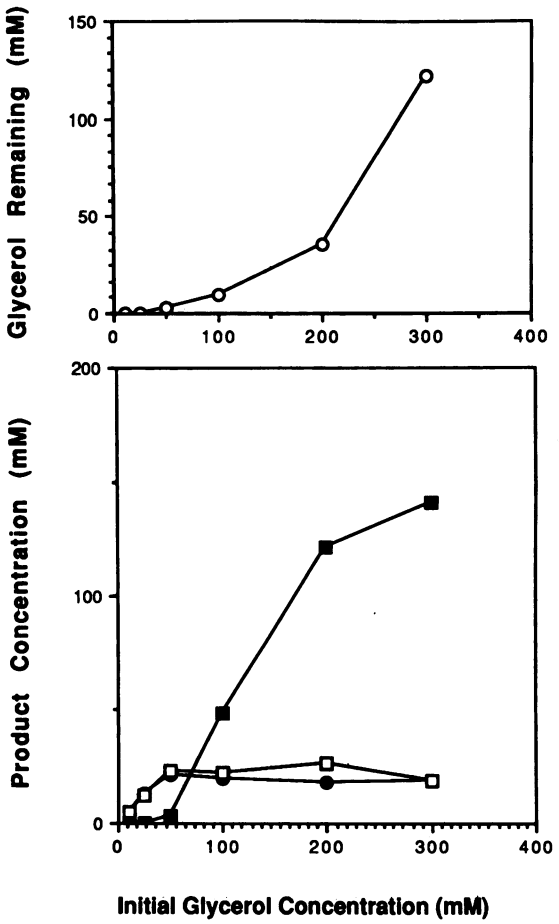

FIG. 3. *L. reuteri* fermentation products of [^{14}C]glycerol present in increasing initial concentration. Symbols: □, TMG; ●, β-HPA; ■, reuterin; ○, glycerol.

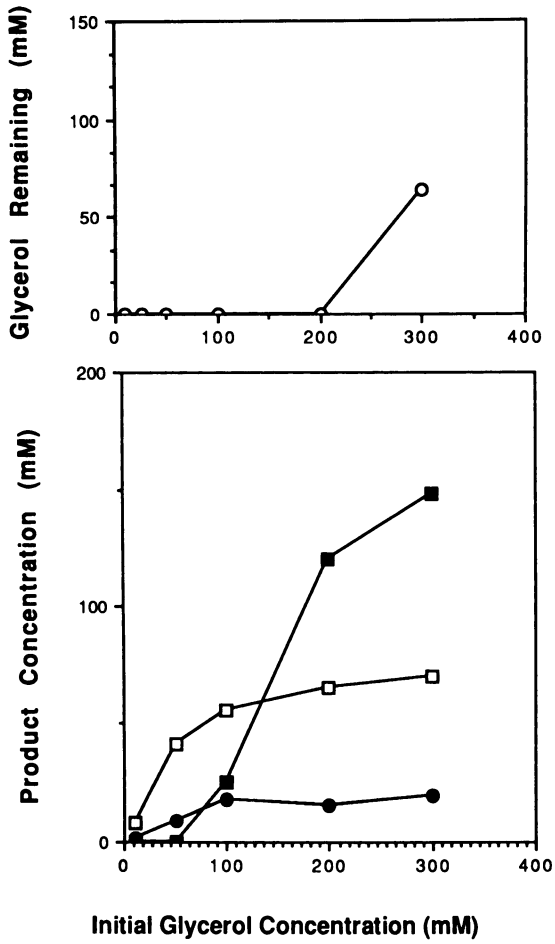

FIG. 4. *L. reuteri* fermentation products of [^{14}C]glycerol in the presence of glucose. Symbols: □, TMG; ■, reuterin; ●, β-HPA; □, glycerol.

DISCUSSION

Clostridium (4) and *Lactobacillus* (7, 8) species have been reported to ferment glycerol to TMG and β-HPA. This fermentation occurs in two steps (Fig. 2); the first step is catalyzed by glycerol dehydrase, which converts glycerol to β-hydroxypropionaldehyde; the second involves an aldehydic dismutation of β-hydroxypropionaldehyde. These reactions generate equimolar quantities of TMG and β-HPA (8). When glycerol fermentation occurs in the presence of growth substrates such as glucose, elevated levels of TMG are produced by *Klebsiella pneumoniae* (3) and *Clostridium* (4) and *Lactobacillus* (7, 8) species. In the presence of glucose and glycerol, some lactobacilli were found to ferment glycerol to β-hydroxypropionaldehyde, which functions as a hydrogen acceptor, resulting in TMG accumulation. Both systems operate simultaneously in the clostridia (4) and lactobacilli (7, 8), as evidenced by the low levels of β-HPA which are also present in fermentations containing both glycerol and growth substrates.

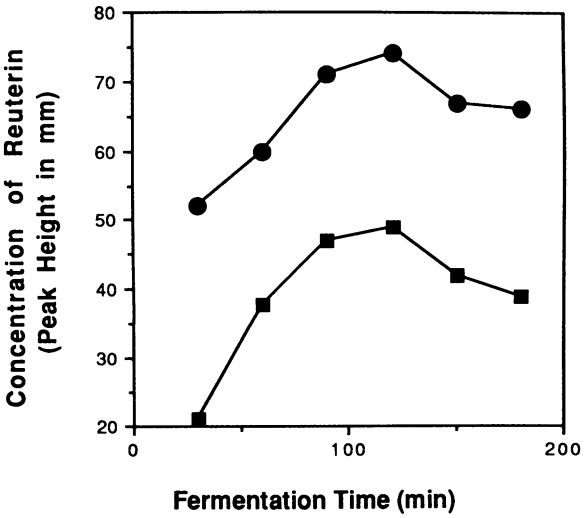

FIG. 5. Reuterin production with respect to time with *L. reuteri* cellular concentrations of 2.5 (■) and 4.5 (●) mg (dry weight) ml^{-1}.

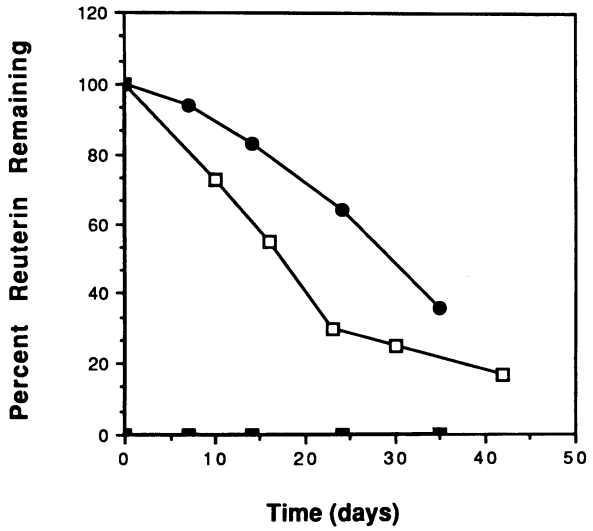

FIG. 6. Stability of reuterin was examined at 37°C with the pH of the solution adjusted to 2.0 (●), 6.5 (□), or 11.0 (■). The percentage of reuterin remaining is the ratio of reuterin peak heights of samples at the reported temperature and pH to those of a control held at 0°C for the indicated time.

We showed that the production of β-HPA and TMG from glycerol by *L. reuteri* is similar to that observed in other lactobacillus and clostridium species. It was apparent from the radioisotope experiments that reuterin, a product associated with this fermentation, is derived either directly or indirectly from this glycerol fermentation. We found reuterin production to be inhibited by the addition of neutralized semicarbazide hydrochloride to the reaction mixtures. The semicarbazide is reported to complex with the β-hydroxypropionaldehyde intermediate (1). This process would explain the inhibition of reuterin synthesis by the semicarbazide. It also suggests that β-hydroxypropionaldehyde is a precursor to reuterin.

In other reports (Axelsson et al., submitted; Chung et al., submitted), reuterin production was observed in a complex coculture system. We established that reuterin can be produced by incubating high concentrations of *L. reuteri* cells alone with glycerol. To isolate the reuterin thus produced, an HPLC system utilizing two C_{18} columns was developed and then scaled up to semipreparative chromatography. The three major compounds produced were identified as reuterin, TMG, and β-HPA. Identification of these substances was based on coelution patterns of standards and FTIR analysis of collected fractions. FTIR analysis of reuterin alone was not sufficient to allow structural identification;

further analysis will be reported at a later date. A superior analytical separation was obtained on an HPX 87H weak ion-exchange column. Using this separation procedure, a linear relationship was established between reuterin concentration as measured by the MIC assay and reuterin peak height. This relationship holds over a wide range of reuterin concentrations. It was also utilized to study fermentation of [^{14}C]glycerol and reuterin stability over time. When the fermentation products formed from glycerol were analyzed with [^{14}C]glycerol, essentially all the ^{14}C was recovered in the three products and nonfermented glycerol, thus indicating that reuterin is a product of glycerol metabolism. β-HPA and TMG were produced in equimolar quantities and were the sole products formed at low glycerol concentrations under these conditions. Reuterin production was stimulated at glycerol concentrations greater than 50 mM coincident with maximal production of TMG and β-HPA.

When glucose was added to the fermentation, TMG production was elevated concomitant with a slight reduction in the amount of β-HPA produced, and these two products were no longer produced in equimolar quantities as was observed in fermentations lacking glucose. Similar results were obtained by Sobolov and Smiley (8) for other *Lactobacillus* strains. This increased level of TMG production is attributed to the use of the β-hydroxypropionaldehyde as a hydrogen acceptor resulting in production of increased levels of TMG. Our study indicates that such a system also functions when *L. reuteri* ferments glucose in the presence of glycerol. Production of reuterin was unaffected by the addition of glucose when initial glycerol concentrations were over 100 mM. At the lower initial glycerol concentrations, production of TMG apparently impedes formation of reuterin.

Reuterin production increased to a maximum after a 2-h incubation at 37°C. Since reuterin production was accompanied by production of β-HPA, resulting in the acidification of the production system, the stability of reuterin was examined in acidic and neutral conditions. Purified reuterin incubated at 37°C with the pH adjusted to 6.5 had a half-life of approximately 2 weeks. Reuterin incubated under acidic conditions had a half-life of approximately twice that of the neutralized reuterin. However, exposure of reuterin to basic conditions resulted in an immediate irreversible inactivation of the molecule. Storage of reuterin below 5°C at neutral and acidic pHs resulted in no appreciable breakdown over a 6-month period.

In this report, we described a simplified method of reuterin production as well as a procedure for the isolation of reuterin. These procedures will permit the isolation of milligram quantities of reuterin required for structural analysis of the molecule. This information along with that concerning intermediates involved in reuterin production will lead to an understanding of the mechanism of reuterin synthesis by *L. reuteri*.

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