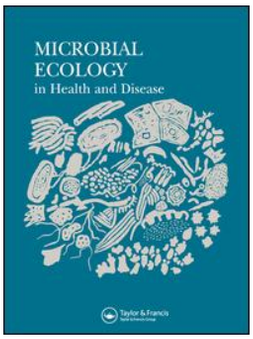

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In Vitro Studies on Reuterin Synthesis by *Lactobacillus reuteri*

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Lactobacillus reuteri resides in the gastrointestinal ecosystem of humans and other animals. In an earlier report we showed that this enteric species converts glycerol into reuterin—a substance with broad-spectrum antibacterial activity. In this report we show that reuterin also has antimycotic and antiprotozoal activity. A minimum inhibitory concentration (MIC) assay was developed and used to study the conditions under which reuterin is synthesised. The results show reuterin to be synthesised *in vitro* under pH, temperature and relative anaerobic conditions similar to those believed to exist in the regions of the gastrointestinal ecosystem inhabited by *L. reuteri*. It was also demonstrated that *L. reuteri* cells are stimulated to produce reuterin when permitted to interact directly with a variety of other microorganisms. A symbiotic relationship between *L. reuteri* and its host is postulated and discussed.

KEY WORDS—*Lactobacillus reuteri*; Reuterin; Antimicrobial substance; Symbiont.

INTRODUCTION

Since the turn of this century enteric lactobacilli have been implicated as contributors to healthy gastrointestinal functions in humans and other animals.[1-6] Their existence in relatively high numbers has been documented, their activities in this ecosystem have been discussed[4,5,7,11,12,15,17,18,20,21,22,24,27] and evidence continues to accumulate that certain species confer benefits to the host. Recent reports, for example, have shown that dietary *Lactobacillus* therapy affords protection from colon cancer for human populations on Western diets,[10] that *L. acidophilus* supplemented in the diet of rats reduces the incidence of dimethylhydrazine-induced large bowel tumours,[8] that feeding viable *L. acidophilus* with milk to healthy volunteers reduces the faecal concentrations of bacterial enzymes known to catalyse conversion of pro-carcinogens to proximal carcinogens,[9] and that pigs treated with certain strains of *L. acidophilus* have considerably lower levels of serum cholesterol than do their untreated counterparts.[6]

Our laboratories recently discovered that another prominent enteric species, *Lactobacillus reuteri*, is able to convert glycerol into a broad-spectrum bactericidal substance, termed reuterin.[1]

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Although we are not able at this time to ascertain whether or not reuterin plays a role in the gastrointestinal ecosystem, we are able to determine the *in vitro* conditions under which this substance is produced. The results of these studies are presented in this report.

MATERIALS AND METHODS

General experimental format

In most experiments described in this report approximately 10^6 CFU/ml *L. reuteri* 1063 cells were inoculated together with approximately 10^7 CFU/ml of *Escherichia coli* K12 cells into basal medium (BM) containing 40 mM glycerol and co-cultured under anaerobic conditions at 37°C. Samples were removed at intervals, centrifuged and the supernatant fractions analysed for reuterin using the following minimum inhibitory concentration (MIC) assay.

MIC assay procedure

E. coli K12 was used as the susceptible test culture according to the following protocol: overnight cultures of *E. coli* K12 were harvested by centrifugation, washed twice with sterile 0·05 M sodium phosphate buffer (pH 7·5), suspended in this

buffer and adjusted to $A_{420\,nm}=0{\cdot}20$ using a Spectronic 70 spectrophotometer. This suspension was diluted 1:100 and 0·1 ml aliquots (containing 10^4 CFU) used to inoculate 1 ml of the MIC assay medium, which is the BM medium described below with 20 mM glucose replacing glycerol. Filter-sterilised samples (1 ml) to be tested for reuterin were added to 1 ml of the MIC assay medium and vortexed to obtain a 1:2 dilution. Serial (and staggered) dilutions were made as required, the cultures incubated for 24 h at 37°C and examined for growth. Relative reuterin concentrations (units reuterin/ml) were calculated as the reciprocal of the sample dilution preceding the dilution permitting visible growth of the indicator cells.

Culture media and bacterial strains used

The co-culture BM contained (g/L): casein hydrolysate, vitamin-free (Difco), 3; ammonium citrate, 1·9; citric acid, 0·63; KH_2PO_4, 12·6; $MgSO(7H_2O)$, 0·2; pH adjusted to 7·0 prior to sterilisation and 40 mM glycerol added after sterilisation. In some experiments Muller-Hinton (Difco) medium was used supplemented with 40 mM glycerol or glucose. In others, Thioglycoloate (Difco) medium was used also supplemented with 40 mM glycerol or glucose. *Trypanosoma cruzi*, a protozoan species, was cultured in Yaeger's LIT medium which contained (g/L): NaCl, 4; KCl, 0·4; Na_2HPO_4, 8; glucose, 2; calf serum (heat inactivated) 100 ml; 10 per cent haemoglobin solution, 20 ml; 5 per cent ox liver infusion (Oxoid) 100 ml; tryptose (Difco), 5; pH adjusted to 7·3. The medium was filter sterilised. The yeast and fungal strains were grown and tested in Yeast Nitrogen Base (Difco) medium.

Lactobacillus reuteri 1063 was recently isolated from swine intestines and classified as described by Axelsson *et al.*[2,28] The *L. reuteri* type strain, DSM 20016 (and ATCC 23273), and other bacterial strains used in this study were obtained from departmental culture collections (Departments of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden and N.C. State University, Raleigh, NC, USA). Yeast and fungal strains obtained from Dr T. Mitchell (Duke University, Durham, NC, USA) are also maintained in these collections. The protozoal species, *T. cruzi*, was obtained from Dr John Roberts and is maintained in the Zoology Department collection at NC State University. Unless otherwise indicated, lactobacilli were grown in Lactobacillus Carrying Medium (LCM) or MRS medium containing 20 mM glucose as previously described.[1,2] Generally, all strains were grown as still cultures in 10 ml of medium. Anaerobic cultures were maintained in tightly sealed tubes sparged with sterile 95 per cent N_2–5 per cent CO_2. Aerobic cultures were maintained by vigorous shaking.

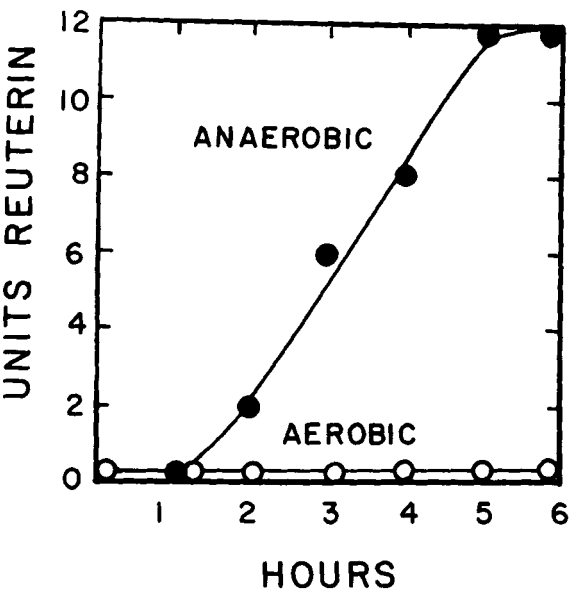

Figure 1. Production of reuterin under aerobic and anaerobic conditions. The standard co-culture conditions were used as described in materials and methods. Approximately 10^6 CFU/ml of *L. reuteri* 1063 and 10^7 CFU/ml *E. coli* K12 were co-cultured in the BM containing 40 mM glycerol. One co-culture (10 ml in a 125 ml flask) was aerated by rapid shaking (○); the other (10 ml in a 18 ml screw cap test tube) was sparged for 1 min as required with N_2-CO_2 to produce an anaerobic environment (●). Samples were removed as indicated and assayed for reuterin using the MIC assay

RESULTS

Resting cells of *L. reuteri* 1063 co-cultured with *E. coli* convert glycerol into reuterin, a broad-spectrum antimicrobial substance.[1] The data summarised in Figure 1 show that anaerobic conditions are required for production of reuterin. On the other hand, production of this antibiotic occurs over a broad range of pH values. Production of reuterin is coincident with maintenance of viability of both the *E. coli* and the *L. reuteri* 1063 cells under these conditions (Figure 2a). The highest production occurs in the pH 6–8 range but is still appreciable at values as low as pH 5 and as high as pH 9 (Figure 2b). Commensurate with the likelihood that it is an enzyme-catalysed process, reuterin production increases as the co-culture incubation temperature is

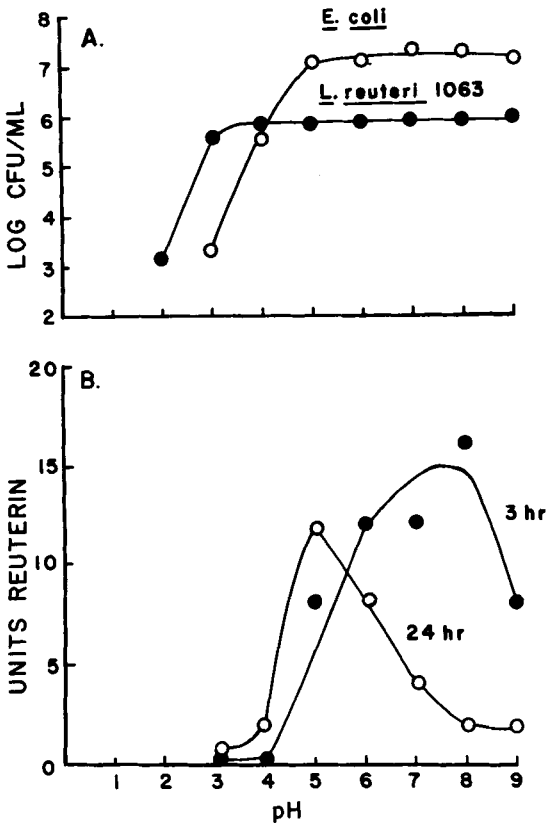

Figure 2. Affect of culture pH on reuterin production by *L. reuteri* 1063. Co-cultures were prepared as described in Figure 1 except that the pH of the medium was adjusted to the indicated values (using 10 N H_2 SO_4 or NaOH) prior to sterilisation. Viable counts for *E. coli* (○) and *L. reuteri* 1063 (●) were determined using MacConkey agar and LCM agar respectively, after 3 h incubation at the indicated pH values (A). Identical co-cultures were analysed for reuterin production after 3 h (●) and 24 h (○) incubation under these conditions (B)

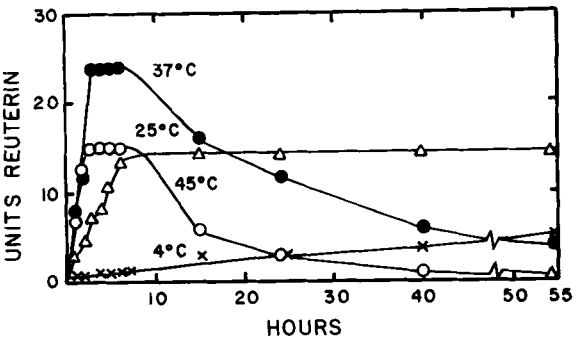

Figure 3. Effect of temperature on reuterin production. Co-cultures prepared as described in Figure 1 were incubated as indicated at 4°C (×), 25°C (△), 37°C (●) and 45°C (○). Samples were removed at the indicated times and assayed for reuterin

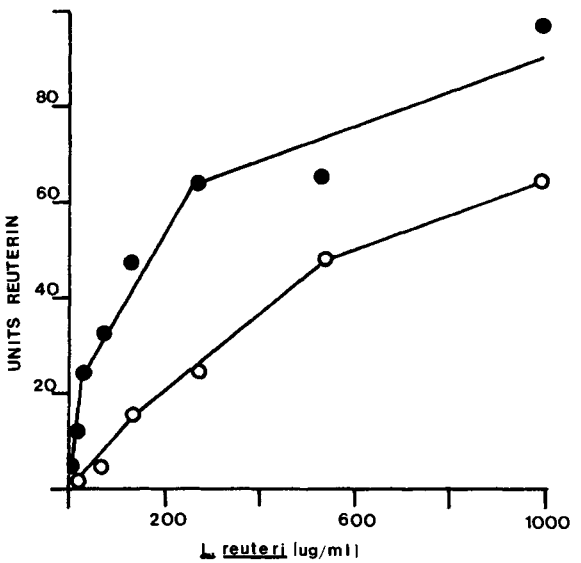

Figure 4. Effect of *L. reuteri* cell mass on reuterin production. Co-cultures of *L. reuteri* 1063 (●) and DSM 20016 (○) containing approximately 10^7 CFU/ml *E. coli* K12 were prepared as described in Figure 1 except that the *L. reuteri* cell mass was varied over a 10–1000 μg/ml range as shown. Reuterin was assayed after a 6 h incubation period

increased from 4°C to 45°C (Figure 3). Maximum production occurs between 37° and 45°C. Reuterin appears to be somewhat unstable at the higher temperatures (and the higher pH levels as seen in Figure 2) with little activity remaining under these conditions when measured at 55 h. At temperatures of 25°C or lower, reuterin appears to be stable.

The data summarised in Figure 4 show that reuterin production by *L. reuteri* 1063 and DSM 20016 increases as the cell mass increases in the standard co-culture system. These data, however, were difficult to evaluate on two accounts. First, it is known (data not shown) that reuterin concentrations in excess of 20 to 30 units/ml affect *L. reuteri* viability (for *E. coli* this occurs in the presence of only 4 to 5 units/ml). Secondly, in this experiment a variable was introduced which had not been addressed previously; namely, that the number of *L. reuteri* cells in the co-culture was being increased without proportionately increasing the number of *E. coli* cells. Our attempts to address this matter resulted in evidence that production of reuterin by *L. reuteri* is stimulated by direct interaction between the producer and the victim cells.

The results shown in Table 1 support this hypothesis. In this series of experiments reuterin

Table 1. Reuterin production by *L. reuteri* is stimulated by direct contact with *E. coli* cells

Incubation (h)	Units/ml reuterin produced		
	Direct co-culture*	Indirect co-culture†	*E. coli* omitted‡
0	0	0§ 0‖	0
1	16	— 1	—
2	24	— 4	—
3	32	— 6	—
4	32	— 8	—
5	32	— 8	—
6	32	4 8	8

*Co-cultures were prepared as described in materials and methods, and samples were removed at the indicated times and assayed for reuterin.
†Same conditions as above except that the *E. coli* were placed inside and the *L. reuteri* 1063 cells were placed outside a dialysis tube inserted into a test tube culture.
‡Same as * above except the *E. coli* K12 cells were omitted.
§Reuterin assayed from interior of the dialysis tubing.
‖Reuterin assayed from exterior of the dialysis tubing.

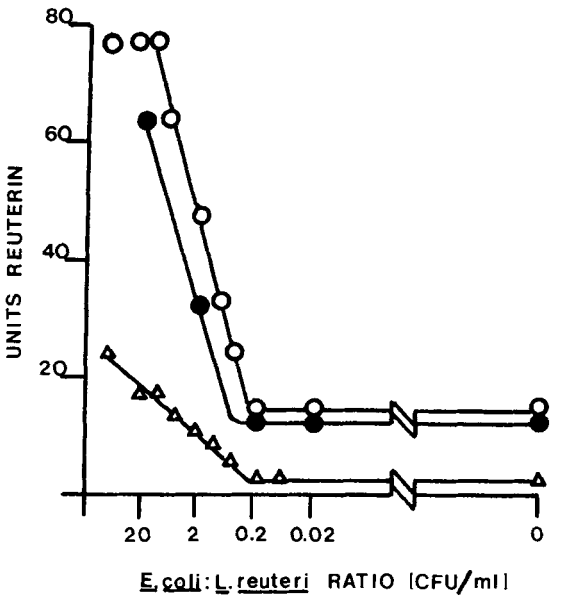

Figure 5. Heterologous stimulation of reuterin production by *E. coli* K12. Three co-cultures were prepared as described in Figure 1 containing the following concentrations of *L. reuteri* 1063 cells (CFU/ml): $4{\cdot}4 \times 10^6$ (△), $4{\cdot}4 \times 10^7$ (●) and $5{\cdot}2 \times 10^7$ (○). The ratio of *E. coli* CFU/ml to *L. reuteri* CFU/ml was varied as indicated from values of 0 to 50. Reuterin production in each culture was assayed after 6 h incubation under these conditions

production was followed over a 6 h period with the following alterations introduced into the standard co-culture system. In one set of experiments, the *E. coli* cells were omitted, in another set the *E. coli* cells were present as usual but separated from the *L. reuteri* cells by a dialysis membrane. The results obtained show that whereas *L. reuteri* itself is able to synthesise basal amounts of reuterin, production is significantly enhanced in the presence of the heterologous *E. coli* cells. It is also clear that this 'heterologous stimulation' does not occur when *L. reuteri* and *E. coli* are prevented from having a direct contact with each other by dialysis membrane separation. Inasmuch as oxygen has free passage across the dialysis membrane, this experiment appears to rule out the possibility that the heterologous stimulation is the consequence of reduced oxygen tension in the medium attributable to *E. coli* respiration. This heterologous stimulation occurs only if both the *E. coli* and the *L. reuteri* cells are viable. It is possible that the *E. coli* cells produce a non-dialysable 'nutrient' which stimulates reuterin production. This would appear an unlikely possibility but remains to be determined.

Our attempts to quantitate this heterologous induction phenomenon are summarised in Figure 5. In these experiments a fixed number of *L. reuteri* 1063 cells were co-cultured in the presence of increasing numbers of *E. coli* cells yielding ratios of *E. coli* to *L. reuteri* 1063, based on CFU/ml, ranging from 0 to 50. In three separate experiments, each employing different levels of *L. reuteri* 1063, we see that reuterin is produced at a constant basal level until an *E. coli* to *L. reuteri* ratio of approximately 0·5 to 1·0 is reached. Thereafter, as this ratio increases, so also does the ability of *L. reuteri* cells to produce reuterin.

E. coli is not unique in its ability to stimulate reuterin synthesis. As seen in Table 2 a variety of bacterial species are capable of stimulating its production. A number of observations are noteworthy here. It can be seen that all species tested stimulate reuterin production under conditions which support their growth. All species grew on the Muller-Hinton or Thioglycolate media and stimulated reuterin production under these conditions. *Bacillus subtilis*, *Clostridium sporogenes*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Streptococcus cremoris* grew poorly on the glycerol BM, and under these conditions they were unable to stimulate reuterin production. Since *C. sporogenes* stimulated reuterin production under anaerobic

Table 2. Heterologous stimulation of reuterin production by various bacterial species

Heterologous species	Units/ml reuterin produced	
	GBM medium	GMH/GTG medium
None	8	0
Escherichia coli		
K12	32	32
431	64	32
73	64	32
P155	64	32
Salmonella typhimurium	64	32
Shigella sp.	64	32
Proteus sp.	32	32
Pseudomonas fluorescens	64	32
Staphylococcus epidermidis	32	32
Bacillus megaterium	12	32
Clostridium sporogenes	8	32
Pediococcus pentosaceus	8	24
Leuconostoc mesenteroides	8	24
Streptococcus cremoris	8	24

Co-cultures were prepared as described in Figure 1 except that the indicated species were compared to *E. coli* K12 for their ability to stimulate reuterin production in standard glycerol BM (GBM) (left column) and in glycerol Muller-Hinton (GMH) medium (right column). For *C. sporogenes* and *S. cremoris*, glycerol Thioglycolate (GTG) medium was used instead of Muller-Hinton medium. The *C. sporogenes* co-cultures were incubated in anaerobic jars using the BBL Gas-Pak system.

conditions (in the glycerol Thioglycolate medium), it would seem clear that this stimulatory effect is not the result of reduced oxygen tension brought about by the heterologous cells.

Two additional findings should be noted. First, the development of an MIC assay enabled us to determine relative sensitivity of various microorganisms to different concentrations of reuterin. The results of this survey are presented in Table 3. It can be seen that all microorganisms tested are sensitive to reuterin; this includes all bacteria, yeasts, fungi and protozoa examined to date. Generally 4 to 5 units/ml of reuterin suffice to completely prevent growth of the various species tested. The lactic acid group of bacteria appear to be slightly more resistant that other procaryotic species. The non-mycelial growing yeast strains were as sensitive as the procaryotes under these conditions. The mycelial yeast strain, *Saccharomycoides*, and the plant pathogenic fungus, *Fusarium*, were slightly less sensitive, but this was difficult to evaluate given the pellicle-like nature of surface mycelial growth exhibited by these species.

Secondly, we have developed a method for isolating and enumerating reuterin-producing *L. reuteri* cells from natural sources (unpublished results). This method is now being used to determine where and in what numbers these bacteria are to be found in the GI tracts of normal, healthy animals including humans. Thus far we have examined stomach and small intestinal tissues obtained from healthy piglets. A range of 10^3 to 10^6 CFU lactobacilli per cm^2 of tissue have been found with a relatively high percentage of these colonies exhibiting reuterin production (Pagano, Lecce and Dobrogosz, unpublished data).

DISCUSSION

L. reuteri is a heterofermentative *Lactobacillus* resident in the GI tract of humans, swine, poultry and other animals.[2,13,14,28] Evidence is mounting that this species may be an enteric symbiont providing its host with certain benefits including, perhaps, a mechanism for regulating enteric microbiotic

Table 3. Sensitivity of selected procaryotes and eucaryotes to reuterin

Escherichia coli K12 (4)*	*Leuconostoc mesenteroides* (16)
Escherichia coli—	*Pediococcus cerevisiae* (12)
6 swine ETEC strains (4–5)	*Candida albicans*—
Salmonella typhimurium (4)	2 strains (2)
Pseudomonas fluorescens (5)	*Torulopsis glabrata* (4)
Proteus sp. (4)	*Saccharomyces cerevisiae* (12)
Shigella sp. (4)	*Saccharomycoides fibuligera* (16)
Bacillus megaterium (5)	*Aspergillus flavus* (8)
Clostridium sporogenes (5)	*Fusarium samfucienum* (36)
Staphylococcus epidermidis (5)	*Trypanosoma cruzi* (5)
Lactobacillus bulgaricus (9)	
Lactobacillus plantarum (12)	
Lactobacillus lactis (17)	
Lactobacillus acidophilus—	
6 strains (12–40)	

*Number in parentheses are the MICs for each species determined after 48 h incubation. All bacteria were grown in Muller-Hinton broth in still culture except for *C. sporogenes* and *S. cremoris* which were grown in Thioglycolate broth under anaerobic conditions. The yeasts and fungi were grown in Yeast Nitrogen Base broth at 30°C. The protozoan, *T. cruzi* was grown in LIT broth at 37°C.

populations and protection against potential pathogens. This viewpoint is based on firstly, discovery that *L. reuteri* produces reuterin, a potent, broad-spectrum bactericidal substance,[1] secondly, our findings presented in this report showing that reuterin is produced under conditions believed to exist in the gastrointestinal ecosystem, thirdly, the demonstration by Wadstrom *et al.*[28] that *L. reuteri* 1063 exhibits high surface hydrophobicity and adheres to isolated pig intestinal epithelial cells, and lastly that reuterin-producing *L. reuteri* can be isolated in relatively large numbers from the stomach, duodenum, jejunum, ileum and faeces of healthy piglets (Pagano, Lecce, Dobrogosz, unpublished observations). We can only speculate at this time concerning *in vivo* sources of glycerol for reuterin production. In this connection it has been reported that lipids are degraded throughout the GI tract including appreciable amounts even in the ileum.[19] Also both non-covalently and covalently bound lipids (neutral, glyco- and phospholipids) are found in mucus.[3] It is possible that host and/or microbial lipases generate glycerol for reuterin production from these and perhaps other lipid sources.

We reported earlier[1] that reuterin inhibits growth and is bactericidal to a variety of Gram-negative and Gram-positive bacteria. We now know that it is equally effective against lower eucaryotic genera of yeasts and fungi such as *Candida*, *Torulopsis*, *Saccharomyces*, *Saccharomycoides*, *Asperigillus* and *Fusarium* and the parasitic protozoan, *Trypanosoma cruzi*. Furthermore, we have shown (unpublished data) that it also inhibits replication of B2 and lambda phages in *L. plantarum* and *E. coli* respectively. Reuterin thus appears to be an antimicrobial, antiyeast, antifungal, antiprotozoan and antiviral agent. Further investigations along these lines examining the *in vivo* efficacy of the *L. reuteri*-reuterin system are ongoing.

The genetic factors and biochemical reactions involved in reuterin synthesis are also under investigation and progress is being made with respect to its isolation, purification and molecular characterisation. We know that it is not one of the end-products (i.e. lactic acid, acetic acid, hydrogen peroxide) traditionally associated with lactic acid fermentations and also that it is a low molecular weight, water-soluble, non-protein, neutral end-product associated with the 1,3-propanediol fermentation of glycerol (unpublished data) which is known to occur in other species of heterofermentative bacteria.[23,25,26]

Reuterin synthesis is stimulated by contact with heterologous cells. We hope to learn more about this interesting phenomenon in the future. As a working hypothesis we propose *L. reuteri* possesses a mechanism for 'sensing' increased numbers of heterologous cells, and that it responds to this stimulus by producing reuterin which proceeds to decrease this heterologous cell population without

seriously affecting the lactobacilli. We have shown in this and a previous report that lactic acid bacteria, including *L. reuteri* strains are considerably less sensitive to reuterin than are other cell types.[1] This differential sensitivity may prove to have ecological significance.

ACKNOWLEDGEMENTS

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