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Effect of Glutathione on Growth of the Probiotic Bacterium *Lactobacillus reuteri*

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Abstract—Glutathione (GSH) is an abundant nonprotein thiol that plays numerous roles within the cell. Previously, we showed that *Lactobacillus salivarius* has the capacity to mount a glutathione-mediated acid-tolerance response. In the present work we provide evidence of a requirement for GSH by *Lactobacillus reuteri* and have studied the role of GSH during cell growth. Medium supplementation with 0.5 mM GSH as the sole sulfur source enhanced cell growth, resulting in an increase in glucose consumption, and increased cell GSH and protein contents compared with levels seen in the absence of supplementation. Moreover, *L. reuteri* showed enhanced amino acid consumption when grown with 0.5 mM GSH. These findings indicate that glutathione is a nutrient for bacterial growth.

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Key words: amino acid consumption, bacterial growth, glutathione, *Lactobacillus reuteri*, protein content

Lactobacillus strains are Gram-positive lactic acid bacteria residing in the gastrointestinal tract (GIT) of humans and other animals [1]. In the past few decades, *Lactobacillus* species of the GIT have received a great deal of attention because the bacteria have health-promoting properties. These strains are commonly used as probiotics that confer health benefits when ingested in sufficient quantities. Both industrial and research laboratories have investigated the benefits provided by probiotics, the use of which in animal feed has also been linked to enhancement of disease resistance and improvement in health.

Lactobacillus strains are frequently confronted with nutritional, environmental, and oxidative stresses during fermentation. Such challenges may impair growth and metabolism and can seriously impact the economics of industrial processes. *Lactobacillus* strains are nutritionally fastidious, and cell culture is complex, requiring an elaborate culture medium, because the bacteria have limited biosynthetic abilities [2]. Such growth requirements are usually satisfied by the host (human, animal, or plant) *in vivo* and by the addition of complex substrates such as peptone, yeast extract, meat extract, or casein hydrolyzate to culture medium *in vitro* [3]. Amino acids promote the growth of *Lactobacillus* strains and increase fermentative activity and lactic acid production [4, 5]. In the present study we investigated the effects of glutathione (GSH) on *L. reuteri* growth in de Man–Rogosa–Sharpe (MRS) medium.

GSH is the most abundant intracellular nonprotein thiol in most organisms. GSH has been proposed to play a physiological role in many cellular processes, including amino acid transport, synthesis of proteins and nucleic acids, modulation of enzyme activity, metabolism of xenobiotics, detoxification of carcinogens, and elimination of reactive oxygen species [6-10]. However, little is known about the physiological function of GSH during growth of Gram-positive *Lactobacillus* species. We recently described a glutathione-mediated acid stress response of *L. salivarius* [11] suggesting that GSH might have multiple roles in Gram-positive lactobacilli. Thus, we considered that addition of GSH to medium might accelerate the growth of *L. reuteri*. We found that the bacterium is able to utilize GSH as a sulfur source, suggesting that medium supplementation with GSH may indeed be valuable.

Abbreviations: FAA, free amino acid; GIT, gastrointestinal tract; GSH, glutathione; MRS, de Man–Rogosa–Sharpe.
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MATERIALS AND METHODS

Bacterial strains and growth medium. *Lactobacillus reuteri* strain ATCC23272 was grown in MRS broth (Difco Laboratories, USA) at pH 6.5. Pre-cultures were grown overnight at 37°C with shaking (150 rpm), and 2 ml aliquots of these cultures were inoculated into 200 ml amounts of medium without (control) or with 0.5 mM GSH prior to further culture at 37°C for 10 h with shaking.

Analytical techniques. Bacterial growth was assessed by A_{600} measurements. Glucose content was measured enzymatically using a glucose assay kit (Sigma, USA). Determination of intracellular GSH concentration in *L. reuteri* has been previously described in detail [12]. At various intervals, culture samples were harvested by centrifugation (10,000*g*, 15 min, 4°C), and the cells were washed with 100 mM Tris-HCl buffer (pH 7.5). Cell-free extracts were obtained by freezing of the cells at –80°C, followed by resuspension in 500 µl lysis buffer (62.5 mM Tris-HCl, pH 6.8), addition of sterile glass beads (diameter 150-212 µm; Sigma), and mechanical disruption using the FastPrep-24 system (MP Biomedicals). Unbroken cells and the beads were removed by centrifugation (10,000*g* for 10 min at 4°C), and the supernatants were transferred to 1.5 ml Eppendorf tubes. Cell-free extracts and supernatants were stored at –80 and 4°C, respectively, until further analysis. Intracellular protein concentrations were determined using a BCA assay kit (Pierce, USA) employing BSA as a standard. Amino acid contents of supernatants were measured using a Hitachi Model L-8800A automated amino acid analyzer (Hitachi, Japan) after hydrolysis of supernatants in 6 M HCl for 24 h at 110°C under vacuum.

RESULTS AND DISCUSSION

To assess whether GSH functioned as a nutrient source, *L. reuteri* was incubated in medium supplemented with 0.5 mM GSH or control unsupplemented medium (Fig. 1, a and b). Culture in the presence of 0.5 mM reduced GSH markedly increased both growth rate and glucose consumption compared with growth in GSH-free medium. Growth promotion may be attributable to the fact that the GSH tripeptide *per se* can serve as an endogenous source of amino acids for bacterial growth

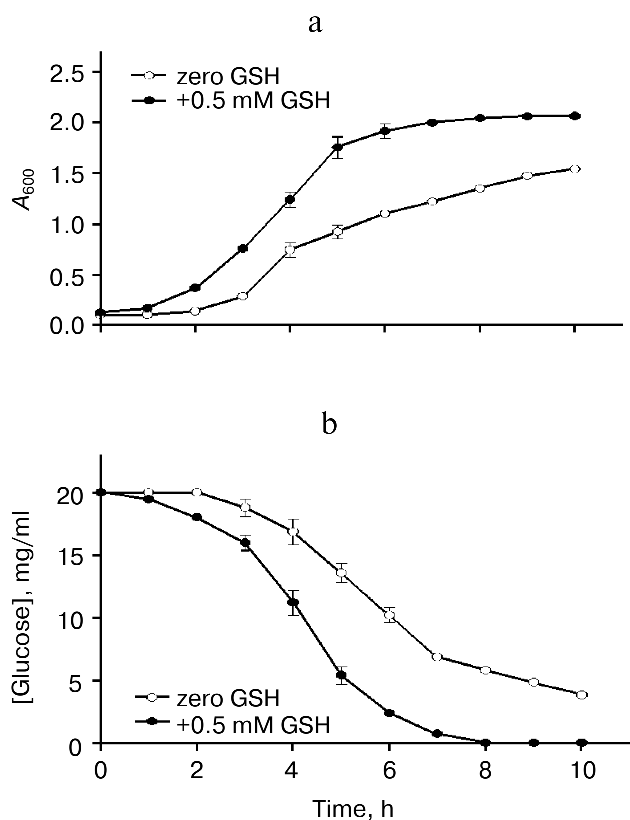

Fig. 1. Comparative growth rate (a) and glucose concentration in *L. reuteri* (b) growing in unsupplemented medium and medium supplemented with 0.5 mM GSH. Each number represents the mean ± SD of three replicates.

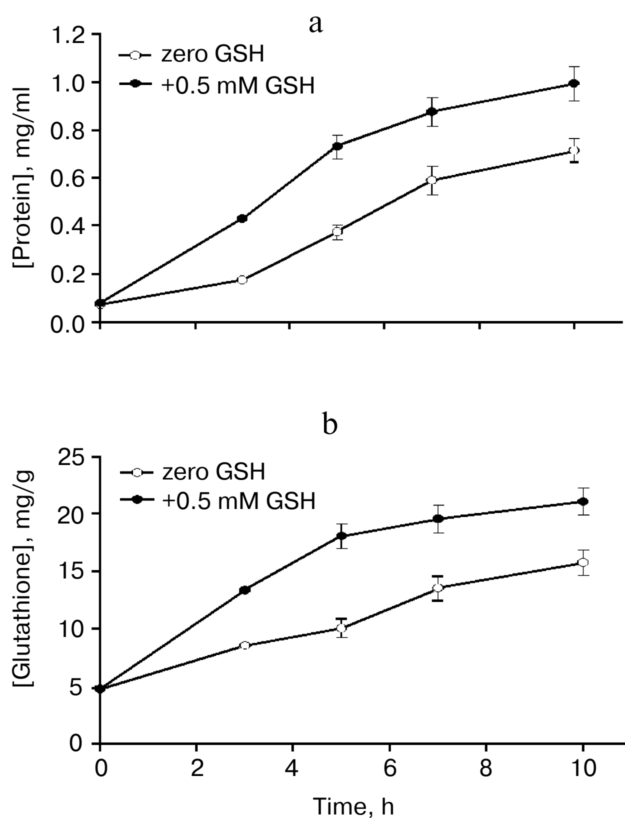

Fig. 2. Time course of soluble protein (a) and GSH (b) accumulation during culture of *L. reuteri*. Each number represents the mean ± SD of three replicates *L. reuteri* after growth in unsupplemented medium and medium supplemented with 0.5 mM GSH.

and maintenance, as *L. reuteri* can successfully transport GSH. On the other hand, it is known that GSH is involved in *L. salivarius* resistance to acid stress [11]. Therefore, GSH could protect the cells from these adverse conditions (and not only as endogenous source of amino acids) resulting in a higher growth. In addition, glucose is used by *L. reuteri* as a carbon source to increase biomass. The greater glucose consumption seen when cells were grown in medium supplemented with GSH is just related to the higher growth in the presence of GSH.

Changes in intracellular GSH and soluble protein levels during 10 h of growth in GSH-free and GSH-containing media are shown in Fig. 2, a and b. Glutathione is an important precursor of macromolecular synthesis and can also yield glutamate, glycine, ammonia, H_2S, and pyruvate. Several possible byproducts and intermediates of fermentation may stimulate cell growth. It has been shown that application of GSH to the plant *Matricaria chamomilla* significantly increased plant height and dry weight [13], being thus similar to our finding that medium supplementation with 0.5 mM GSH markedly increased *L. reuteri* growth (see above). Acceleration of growth in the presence of GSH may be explained by the fact that GSH is a reservoir of reduced sulfur, thus containing cysteine, which can induce production of GSH-synthesizing enzymes [14]. The final GSH level in cultures growing in 0.5 mM GSH were about 1.34-fold that of the GSH-free control after 10 h of growth, indicating that glutathione metabolites are rapidly absorbed by *L. reuteri* and are used to enhance bacterial growth and GSH biosynthesis. Furthermore, we observed a difference in protein content between cells grown with or without 0.5 mM GSH. The stimulation of *L. reuteri* growth by GSH thus results in increased protein content. It is possible that GSH pro-

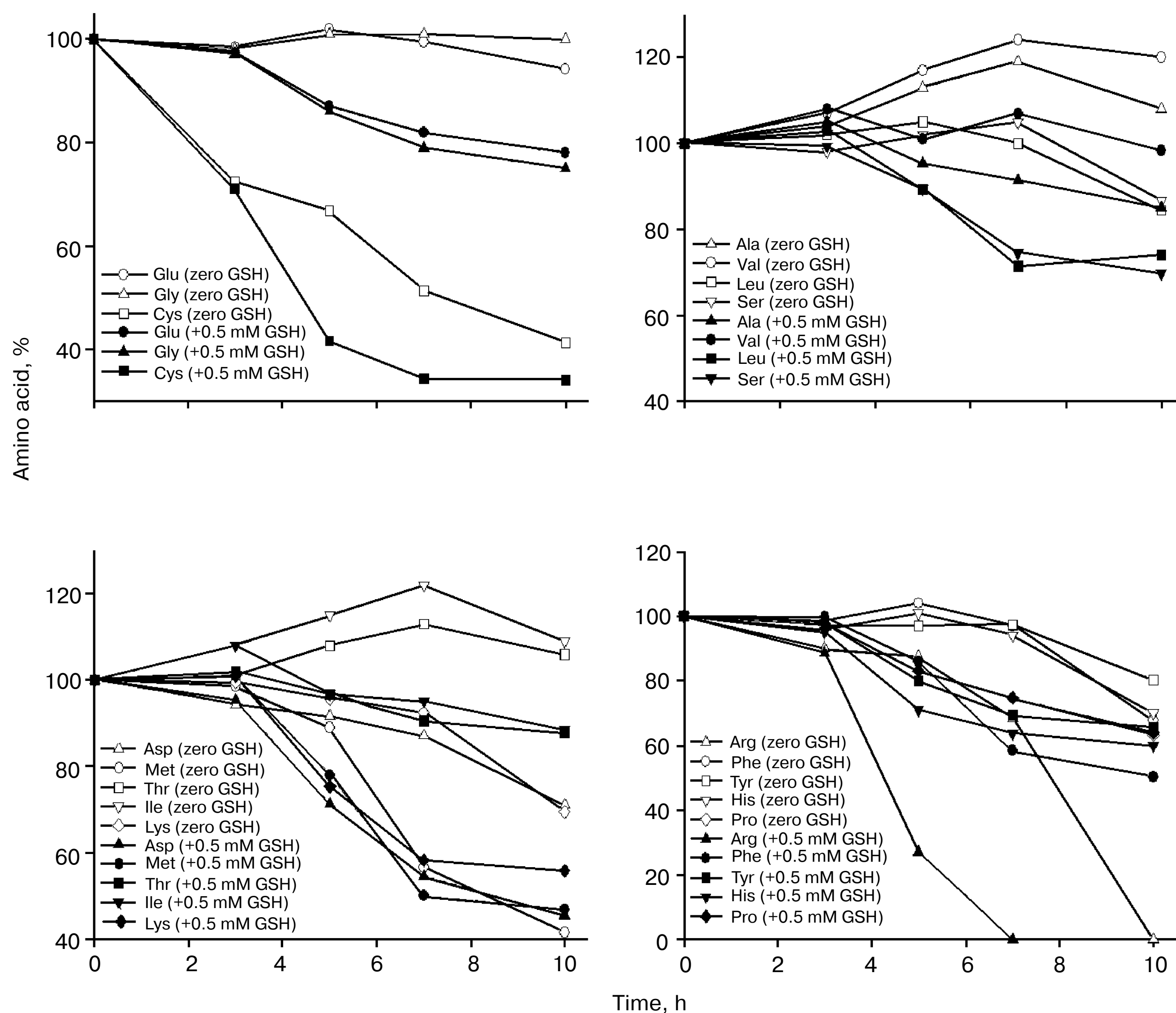

Fig. 3. Amino acid contents of *L. reuteri* after growth in unsupplemented medium and medium supplemented with 0.5 mM GSH.

tects the cells from acid stress through the cooperative induction of adaptive proteins and regulation of energy-metabolism related proteins. Although further experiments, including comparative proteomic analyses using *L. reuteri* cells containing or not-containing equivalent concentrations of GSH, will be needed to confirm these findings, our findings provide early insights into the physiological role of GSH in *L. reuteri*.

Supplementation with a large number of compounds, including all biological amino acids, is important for the effective growth of *Lactobacillus* strains. To investigate if GSH supplementation affected the free amino acid (FAA) uptake of *L. reuteri*, we measured utilization of 17 FAA during growth in medium without or with 0.5 mM GSH (Fig. 3). Supplementation significantly increased consumption of FAAs, suggesting that GSH facilitated uptake of medium FAAs by eliminating an unknown impediment to such uptake. Thus, the strain need not devote energy to the synthesis of amino acids, impacting beneficially on cell growth. Woolley et al. [15] showed that active peptides with particular sequences played a special role in metabolism and were not simply a readily available source of amino acids. Thus, supplementation with GSH permits the rapid growth of *L. reuteri*.

In the first 3 h of fermentation, FAA consumption was low both in medium without or with 0.5 mM GSH, but considerable FAA uptake was evident thereafter (Fig. 3). This suggests an early adaptation period followed by a strong demand for assimilable nitrogen. FAAs were consumed to a significantly greater extent in the presence of GSH than in the absence of this compound. The notable decrease in FAA medium content when 0.5 mM GSH was used as a supplement may be attributable to the expression of certain extracellular enzymes capable of hydrolyzing peptides or proteins. Such materials may thus be valuable supplements of *L. reuteri* growth medium, provided that the materials are small and easily digestible.

In conclusion, GSH seems to play a significant role in the growth of *L. reuteri*. Thus, GSH supplementation improved strain growth, glucose consumption, GSH level, and soluble protein and amino acid concentrations. Our results will be of importance to technologists who seek to maintain the metabolic viability and vitality of microbial strains exposed to environmental stresses associated with industrial fermentations.

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