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Effect of Indoleacetic Acid and Related Indoles on *Lactobacillus* sp. Strain 11201 Growth, Indoleacetic Acid Catabolism, and 3-Methylindole Formation†

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A study was conducted to determine the activity of the 3-methylindole (3MI)-forming enzyme in *Lactobacillus* sp. strain 11201. Cells were incubated anaerobically with 17 different indolic and aromatic compounds. Indoleacetic acid (IAA), 5-hydroxyindoleacetic acid, 5-methoxy-3-indoleacetic acid, indole-3-pyruvate, or indole-3-propionic acid induced 3MI-forming activity. The highest total enzyme activity induced by IAA was observed in cells incubated with an initial concentration of 1.14 mM IAA. Peak activity of the 3MI-forming enzyme occurred 4 h after bacteria were incubated with either 0.114 or 1.14 mM IAA. Enzyme activity peaked earlier (2 h) and disappeared more rapidly at 5.7 mM IAA than at other concentrations of IAA. The effects of IAA and 3MI on the growth of *Lactobacillus* sp. strain 11201 and formation of 3MI from IAA also were determined. Bacterial growth and 3MI formation from IAA were reduced in medium containing exogenous 3MI. The growth depression observed in medium containing 5.7 mM IAA appears to be due to the toxicity of 3MI rather than IAA. The formation of 3MI in this ruminal *Lactobacillus* sp. is mediated by an inducible enzyme, and as 3MI accumulates, bacterial growth and rates of 3MI formation from IAA are reduced.

Yokoyama and co-workers (30, 31) isolated a ruminal *Lactobacillus* species that catabolizes indoleacetic acid (IAA) to 3-methylindole (3MI). A metabolite of 3MI has been shown to be pneumotoxic to ruminants, horses, and rats (3–7). Toxicity of 3MI is not limited to mammals. 3MI has been shown to lyse protozoal (9) and bacterial cells (1, 8, 28). In a recent study, *Desulfobacterium indolicum* was isolated with indole as a sole source of carbon for growth. This species was shown to catabolize many indolic compounds but was completely lysed in 65.5 μg of 3MI per ml. The effect of 3MI or IAA on *Lactobacillus* sp. strain 11201 has not been reported.

Naturally occurring acute bovine pulmonary edema and emphysema result from ruminal formation of 3MI after cows are abruptly switched from poor quality feed to lush, rapidly growing pasture (3–7). This syndrome can be reproduced experimentally by giving an oral dose of tryptophan which is catabolized to IAA by a number of bacterial species and subsequently decarboxylated to 3MI by *Lactobacillus* species. The quantity and nutrient composition of forage fed to cows for 3 weeks prior to an oral dose of tryptophan will alter ruminal production of 3MI (17, 23). The increased capacity of ruminal microorganisms to produce 3MI is not known but could be related to changes in relative bacterial populations (i.e., increased number of *Lactobacillus* cells) or a metabolic change within the 3MI-producing bacteria. The effect of diet on changes in bacterial numbers is well known (18–21). A better understanding of factors that change the capacity of *Lactobacillus* species to produce 3MI is important for the development of more effective means of controlling outbreaks of acute bovine pulmonary edema and emphysema. The objectives of this study were to determine activity of the 3MI-forming enzyme in cells incubated with indolic or aromatic compounds and to determine the effects of IAA and 3MI on the growth of *Lactobacillus* sp. strain 11201.

MATERIALS AND METHODS

Bacteria and medium. *Lactobacillus* sp. strain 11201 (Virginia Polytechnic Institute Culture Designation, VPI 11201) was obtained from M. T. Yokoyama, Michigan State University, East Lansing, Michigan, and maintained on 2% agar slants prepared in bacterial medium described below. Initial growth of the bacteria was slow and variable when the bacteria were transferred directly from agar slants. Therefore, bacteria were transferred and grown in liquid medium prior to being used as a source of experimental inoculum. The medium consisted of 3% glucose, 1.5% yeast extract, 3% Proteose Peptone no. 3 (Difco Laboratories, Detroit, Mich.), 0.003% Tween 80 (M. T. Yokoyama, personal communication), and minerals (31) prepared anaerobically by the methods of Hungate (12, 15, 18). Medium (300 ml) was prepared in 500-ml round-bottomed flasks. The flasks were stoppered with black rubber stoppers and securely wired in place, or 10 ml of medium was pipetted into borosilicate test tubes (14 by 150 mm) and the tubes were stoppered with black rubber stoppers and placed in a test tube press (Bellco Glass, Inc., Vineland, N.J.) to be autoclaved. A 0.5-ml inoculum, when added to fresh medium, resulted in an initial protein concentration of 10 to 14 μg of microbial protein per ml before incubation and growth. Bacterial cultures were grown anaerobically at 39°C for 24 h unless stated otherwise.

Measurement of enzyme activity. *Lactobacillus* sp. strain 11201 was grown at 39°C in control medium to late log or early stationary phase (20 to 22 h) and harvested by centrifugation. The cells were transferred to 300 ml of control medium described above containing 0, 0.114, 1.14, or 5.7 mM IAA. Fractions (30 ml) were collected at 0, 1, 2, 3, 4, 8, 12, and 24 h after cells were transferred to fresh medium containing the four levels of IAA. Collected samples were chilled to 4°C, and the cells were harvested by centrifugation

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(10,000 × g for 15 min). An anaerobic environment was maintained throughout all procedures either by keeping cells in stoppered containers or by purging the containers with a continuous flow of oxygen-free carbon dioxide.

A detailed description of the enzyme assay has been given previously (16). Briefly, the reaction mixture contained enzyme (50 μg of protein per ml from pellet of sonicated cells), 1 mM IAA, 10 mM pyruvate, 5 mM threonine, 5 mM serine, 1 mM dithiothreitol, and 1 mM sodium dithionite. All components were combined and preincubated anaerobically for 20 to 25 min at 39°C without any loss of activity. The enzyme reaction was initiated by the addition of IAA, and the mixture was incubated for 25 min. Radioactive 3MI formed from [2-^{14}C]indoleacetic acid was quantified following separation by reverse-phase high-pressure liquid chromatography.

To determine whether enzymatic formation of 3MI was induced by indolic and aromatic compounds, the following compounds (1.14 mM), which are all commercially available (Sigma Chemical Co., St. Louis, Mo.), were individually added to the medium prior to autoclaving: cinnamic acid, *p*-coumaric acid, *p*-cresol, *p*-3,4-dihydroxyphenylacetic acid, ferulic acid, hydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, indole, IAA, indoleacrylic acid, indole-3-aldehyde, indole-3-propionate, indole-3-pyruvate, 3MI, tryptophan, tryptophol, and vanillic acid. The bacterial cells were grown for 20 to 22 h in control medium and transferred to medium containing one of the 17 compounds for 2 h. Cells were then collected by centrifugation, and 3MI-forming enzyme activity was evaluated.

Effects of IAA and 3MI on microbial growth. Medium containing three concentrations of IAA (0, 1.14, and 5.7 mM) was prepared. Inoculated medium was incubated for 8, 16, 24, and 32 h, and the accretion of microbial protein and 3MI was measured. 3MI concentrations were determined by gas chromatography (6). Microbial protein concentrations were determined in the resuspended bacterial pellet by the method of Lowry et al. (22). Since 3MI reacts with the Lowry reagents, 3MI was first extracted: bacterial cells from 2 ml of culture were collected by centrifugation (10,000 × g for 15 min), and the pellet was suspended in 6 ml of methanol and centrifuged again. The methanol wash was repeated three times prior to protein determination.

To determine the effect of added 3MI on bacterial growth and formation of 3MI from IAA, medium with and without 5.7 mM IAA was prepared, containing 0.19, 0.38, 0.76, 1.53, 3.05, and 6.11 μmol of 3MI per ml. The 24-h accretion of microbial protein was measured in medium without IAA. In medium containing 5.7 mM IAA, the concentration of 3MI formed from IAA was determined by subtracting the amount of 3MI in the uninoculated medium from the amount in the incubated cultures.

3MI formation at different growth stages was evaluated by adding IAA to active cultures at 0, 12, 24, and 36 h of incubation. IAA (21 mg/ml) was dissolved in methanol, and 5.7 mM IAA was obtained by adding 0.05 ml of methanol solution per ml of medium. Cultures were incubated for an additional 24 h after the addition of IAA. Data reported were collected from two replicate experiments with triplicate values within each growth experiment and in duplicate for the data reported on enzyme activity. Means and standard errors of the means were calculated (26).

RESULTS

Enzyme activity. Bacteria were grown in control medium for 20 to 22 h and then transferred to fresh medium containing four concentrations of IAA (0, 0.114, 1.14, and 5.7 mM). No 3MI-forming activity was found in cells from control medium (Fig. 1). Peak 3MI-forming activity was found at 4 h in medium containing 0.114 and 1.14 mM IAA. In medium containing 5.7 mM IAA, enzyme activity peaked at 2 h and dropped to 0 by 8 h. Figure 1 represents total culture activity rather than specific activity (nanomoles of 3MI per minute per milligram of protein).

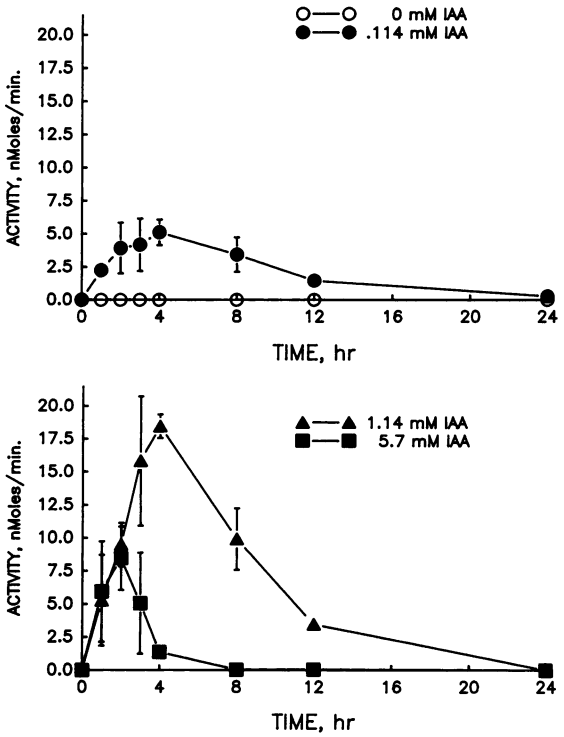

FIG. 1. Induction over time of 3MI-forming enzyme activity with four concentrations of IAA (0, 0.114, 1.14, and 5.7 mM).

Once maximal activity was reached, enzyme activity decreased over time. The loss of activity was not due to dilution caused by an increase in uninduced bacteria. Minimal microbial protein accretion occurred during the first 12 h after cells were transferred to experimental medium. A doubling of microbial protein was the maximum accretion of protein observed between 12 and 24 h of incubation. Plotting data as specific activity did not change the shape of the curves shown in Fig. 1.

Accumulation of 3MI increased from 0.92 to 3.44 μmol/ml between the third and fourth hour in the induction medium containing 5.7 mM IAA (Fig. 2). By 12 h, 93% of the IAA was converted to 3MI in these cultures. At the start of incubation, the concentration of microbial protein was between 200 to 240 μg/ml in this experiment. At this elevated concentration of cells, microbial protein does not increase for the first 12 h (data not shown). After 12 h, a doubling in microbial protein is observed only in cultures without IAA. Maximal 3MI formation occurred at 2 h in 0.114 and 1.14 mM IAA medium with 84 and 70% conversion of IAA to 3MI, respectively. Enzyme activity was observed to decrease as 3MI accumulated at 3 h in 5.7 mM IAA and at 4 h in 0.114 and 1.14 mM IAA (Fig. 1). Washing cells in buffer to remove 3MI did not restore enzymatic activity.

Of 17 indolic compounds tested (Table 1), 5 induced

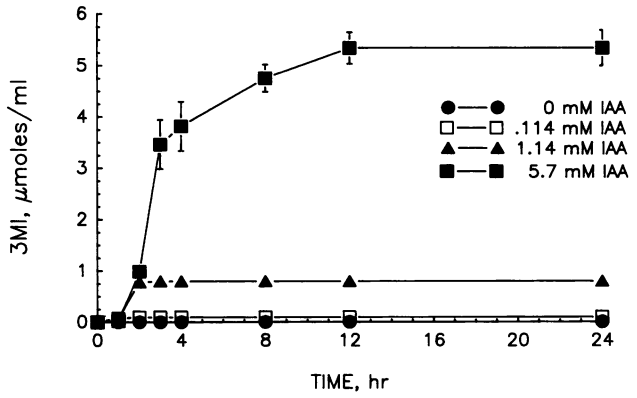

FIG. 2. Concentrations of 3MI in the induction medium containing four concentrations of IAA with *Lactobacillus* sp. strain 11201.

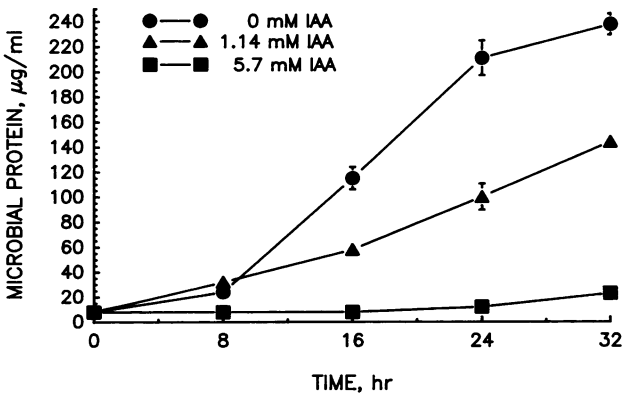

FIG. 3. Effects of IAA concentrations on microbial protein accretion over time with *Lactobacillus* sp. strain 11201.

3MI-forming enzyme activity. 5-Hydroxyindoleacetic acid and 5-methoxyindoleacetic acid were twice as effective as IAA as inducers of 3MI-forming activity. Both indole-3-pyruvate and indole-3-propionate were weak inducers of 3MI-forming activity.

Effects of IAA and 3MI. The lag phase of growth for *Lactobacillus* sp. strain 11201 was 8 h in control medium (Fig. 3). Exponential growth occurs between 8 and 24 h in control cultures. Microbial protein concentration did not increase in medium containing 5.7 mM IAA. Microbial protein in medium containing 1.14 mM IAA was only 60 to 65% of the protein generated in the control cultures.

As expected, no 3MI was produced in control medium. By 8 h, 4.0 to 4.1 μmol of 3MI per ml was produced in 5.7 mM IAA and 0.92 μmol of 3MI per ml was produced in 1.14 mM IAA (data not shown). The concentration of 3MI remained unchanged from 8 to 32 h. Although starting substrate concentrations were different, 70 to 72% of the IAA was converted to 3MI in both cultures. Initially, we attempted to monitor growth by using the increase in optical density at 610 nm, but this was not possible, because as 3MI accumulated, the medium first turned milky white and then crystals of 3MI began to form. In 5.7 mM IAA medium, optical density had peaked by 8 h yet no increase in microbial protein was evident. It was therefore necessary to measure microbial protein by using the Lowry procedure after 3MI was removed.

Since 3MI accumulates in cultures containing IAA, an important unanswered question was whether the reduced growth was caused by IAA or the product, 3MI. The addition of 3MI to the control medium demonstrated that microbial protein accretion decreased with increasing concentrations of 3MI from 0 to 1.5 μmol per ml (Fig. 4). Above 1.5 μmol of 3MI per ml, microbial growth was minimal, with only a doubling of initial protein content. The production of 3MI from IAA decreased (Fig. 5) when cells were placed in 5.7 mM IAA medium containing exogenously added 3MI. The decreased production of 3MI followed a trend similar to that observed for the decrease in microbial protein in the presence of 3MI.

There was a difference in the capacity of the bacteria to produce 3MI during lag, exponential, and stationary growth phases. The addition of IAA to active cultures at 0 and 12 h (see Materials and Methods) resulted in 70 and 82% molar conversion of the IAA to 3MI, respectively. In contrast, the addition of IAA to 24- and 36-h-old control cultures resulted in 8 and 3% molar conversion of IAA to 3MI, respectively. The concentration of 3MI was measured 24 h after IAA was added to the cultures. Further evaluation revealed that copious amounts of 3MI were produced if 24-h-old cells were harvested and transferred at the same cell density as mature cultures to fresh medium containing IAA.

TABLE 1. Activity of 3MI-forming enzyme after treatment of *Lactobacillus* sp. strain 11201 with five indoles for 2 h

Inducer	Activity[a]	
	nmol/min	SEM
Indoleacetic acid	7.66	2.0
5-Methoxyindoleacetic acid	15.82	0.67
5-Hydroxyindoleacetic acid	13.44	0.37
Indole-3-pyruvate	0.23	0.02
Indole-3-propionate	0.06	0.01

[a] Microbial protein, 50 μg/ml in assay.

DISCUSSION

This work demonstrates that 3MI formation is not a constitutive function of *Lactobacillus* sp. strain 11201. Enzymatic formation of 3MI occurred only after bacteria were treated with IAA (or one of the indolic compounds listed in Table 1).

The structural similarities among the compounds that

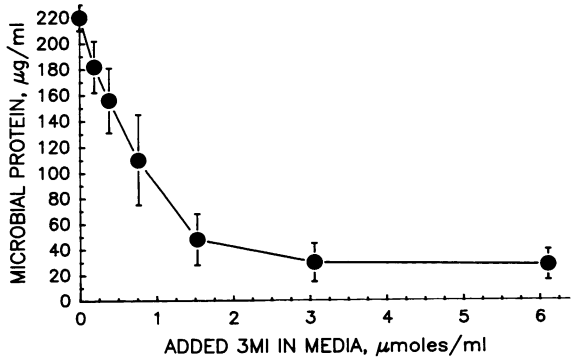

FIG. 4. Effects of increasing medium concentration of 3MI on *Lactobacillus* sp. strain 11201 protein accretion in basal medium incubated for 24 h.

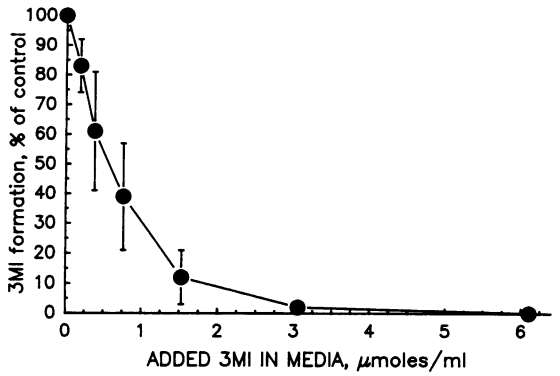

FIG. 5. Effects of increasing medium concentration of 3MI on the ability of *Lactobacillus* sp. strain 11201 to produce 3MI from IAA (5.7 mM IAA; incubated for 24 h).

trigger the bacteria to produce 3MI include an indole nucleus and a carboxyl group on the side chain. In most cases, enzymes are highly specific for substrate(s), and it is not surprising that this organism did not synthesize an enzyme for IAA when exposed to 3,4-dihydroxyphenylacetic acid and *p*-hydroxyphenylacetic acid. *Lactobacillus* sp. strain 11201 has previously been shown to decarboxylate the latter two compounds, and the inability to induce 3MI-forming activity supports earlier research suggesting that separate enzymes produce *p*-cresol and 3MI (30).

The reason for the loss of 3MI-forming activity in either the enzyme induction or culture study is not known. Possible reasons for the loss of activity over time during the induction study (Fig. 1) include enzyme turnover, inactivation, or other incapacitating processes, such as cell death. However, cell death alone would not explain the observed loss of activity. In an earlier study (16), 3MI was shown to be a noncompetitive inhibitor of the 3MI-forming enzyme. 3MI is very lipophilic, and once the product, 3MI, is present, washing cells to remove 3MI does not restore enzyme activity. Complete removal of 3MI by using organic solvents inactivates the enzyme.

Glucose concentration in medium also does not appear to play a critical role in loss of 3MI-forming activity. On the basis of work with mixed ruminal cultures (29), it was suggested that glucose may cause repression of the 3MI-forming enzyme analogous to glucose repression of tryptophanase (2, 10). In this present study, induction of the 3MI-forming enzyme was achieved at 55 mM glucose. More importantly, there was no difference in 3MI formation when *Lactobacillus* sp. strain 11201 was cultured with either 0 or 1% glucose (32). Therefore, medium glucose concentration does not significantly affect 3MI formation.

A moderate reduction in growth of *Lactobacillus* sp. strain 11201 was observed at pH 5.5, compared with pHs 6.5 and 7.5 (D. C. Honeyfield, unpublished data), but 3MI-forming enzyme activity at pH 5.7 was 82% of the activity at pH 7.5 (16). Although it has been reported (13) that a low pH reduces 3MI formation, the work was with mixed ruminal culture and the limiting factor probably was the conversion of tryptophan to IAA rather than a pH inhibition of 3MI formation. Therefore, it appears that a low pH may limit growth but not 3MI-forming activity.

Depletion of one or more essential medium components necessary for synthesis of the 3MI-forming enzyme could be the reason why the addition of IAA to 24- or 36-h-old cultures resulted in little 3MI formation. Accumulation of a 3MI-forming enzyme inhibitor is an alternate reason for the lower concentration of 3MI observed but should be considered only after a complete study of the components in the growth medium has been conducted.

The toxicity of 3MI to *Lactobacillus* sp. strain 11201 manifests itself by reducing microbial growth and decreasing the ability to produce 3MI. The toxicity of 3MI has been reported in other bacteria and protozoa (1, 8, 9, 28). Most notable is a recently identified bacterium, *D. indolicum*, isolated with indole as a sole source of carbon (1). This anaerobic species degrades many indolic compounds but not 3MI. Complete lysis of the cell occurred at 65.5 μg of 3 MI per ml. Although toxicity results, *Lactobacillus* cells are not lysed in the presence of 600 to 700 μg of 3MI per ml.

The toxicity of 3MI appears to be directly related to 3MI rather than IAA. An oxidation product of IAA, methylene oxindole, but not IAA has been shown to be toxic in other bacteria (11, 27). In plants and some bacteria, during the aerobic catabolism of IAA, an intermediate radical is produced via a one-electron oxidation of the indole ring (14). The possible toxicity of an intermediate radical to *Lactobacillus* sp. strain 11201 is not known.

The 3MI-producing enzyme may serve a useful function in *Lactobacillus* sp. strain 11201 in the rumen environment (6, 25), but why 3MI is produced or what benefit the bacteria derive from the reaction remain unanswered questions. The bacteria require IAA to induce the synthesis of the enzyme, but production of 3MI inhibits growth and is a noncompetitive inhibitor of the 3MI-forming enzyme (16). Not only is 3MI toxic to *Lactobacillus* sp. strain 11201, but also it is toxic to other microorganisms and the host ruminant (3–7, 24).

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