Production of Skatole and para-Cresol by a Rumen
Lactobacillus sp.†

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The objective of this study was to examine the substrate specificity of several ruminal strains of a Lactobacillus sp. which previously was shown to produce skatole (3-methylindole) by the decarboxylation of indoleacetic acid. A total of 13 compounds were tested for decarboxylase activity. The Lactobacillus strains produced p-cresol (4-methylphenol) by the decarboxylation of p-hydroxyphenylacetic acid, but did not produce either o-cresol or m-cresol from the corresponding hydroxyphenylacetic acid isomers. These strains also decarboxylated 5-hydroxyindoleacetic acid to 5-hydroxyskatole and 3,4-dihydroxyphenylacetic acid to methylecathol. Skatole and p-cresol were produced in a 0.5:1 ratio, when indoleacetic acid and p-hydroxyphenylacetic acid were combined in equimolar concentrations. Competition studies with indoleacetic acid and p-hydroxyphenylacetic acid suggested that two different decarboxylating enzymes are involved in the production of skatole and p-cresol by these strains. This is the first demonstration of both skatole production and p-cresol production by a single bacterium.

The isolation of several ruminal strains of a bacterium with the ability to produce skatole (3-methylindole) has been reported previously (22, 24). In contrast to other skatole-producing bacteria (12), these ruminal strains do not produce skatole directly from L-tryptophan, but produce it by the decarboxylation of indoleacetic acid (IAA). Based on their physiological, biochemical, and metabolic characteristics, these ruminal strains were assigned to the genus Lactobacillus (24). The significance of skatole production in the rumen is that this metabolite has been identified as being responsible for tryptophan-induced, acute bovine pulmonary emphysema (8, 9, 23). Recent studies (7) suggest that skatole is also the cause of the naturally occurring form of this bovine respiratory disease.

Recognizing that bacteria which degrade tryptophan often are capable of degrading other aromatic amino acids, the objective of this study was to examine the ability of these ruminal Lactobacillus strains to decarboxylate the intermediate metabolites and derivatives of other aromatic amino acids. Our data show that besides skatole, these ruminal Lactobacillus strains also produce p-cresol (4-methylphenol), 5-hydroxyskatole, and methylecathol by the same mechanism of decarboxylation.

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MATERIALS AND METHODS

Culture methods and media. The skatole-producing Lactobacillus strains were designated by their Virginia Polytechnic Institute and State University Anaerobe Laboratory numbers (strains 11198, 11199, 11200, and 11201) and were maintained on 2% agar slants of glucose cellobiose starch rumen fluid medium (4), modified as previously described (24). The slants were held at 4°C, and cultures were transferred at monthly intervals. A modification of the MRS Lactobacillus medium of deMan et al. (10) sustained the growth of these strains in the absence of clarified rumen fluid. This medium was modified by the deletion of Lab-Lenico (Oxoid), the substitution of the mineral ingredients (except MnSO₄.4H₂O) with mineral solutions I and II, and the addition after autoclaving of an 8% sodium carbonate solution and a 2.5% cysteine sulfide solution (5). The ruminal Lactobacillus strains grown in this medium were used as a source of inoculum in all of the studies. Anaerobic cultivating procedures were essentially those described by Hungate (13), as modified by Bryant (3).

The basal medium used to test for the decarboxylation of IAA has been described previously (24); this medium contained 0.2% yeast extract, 0.2% ascorbic acid, and 0.2% IAA as its principal ingredients. All of the other compounds tested for decarboxylase activity were substituted for IAA at equimolar concentrations; 1 ml of a well-grown 3-day-old modified MRS culture was aseptically inoculated into 9.0 ml of test medium, and incubation was at 39°C for 72 h.

Substrates. Besides IAA and para-hydroxyphenylacetic acid (p-HPAA), 11 other compounds were
tested for possible decarboxylation by the ruminal Lactobacillus strains. These were 5-hydroxyindoleacetic acid, ortho-hydroxyphenylacetic acid, meta-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, 3,4-dimethoxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, para-fluorophenylacetic acid, ortho-nitrophenylacetic acid, para-nitrophenylacetic acid, and phenylacetic acid. All of these compounds were from Sigma Chemical Co.

Extraction of metabolites. Incubated cultures (10 ml) were acidified to pH 4.0 with concentrated HCl, transferred to 60-ml separatory funnels, and extracted twice with 30 ml of methylene chloride. Each methylene chloride extract was evaporated to near dryness with a vacuum rotary evaporator in a 30°C water bath, quantitatively transferred to a screw-capped vial with methylene chloride, and reconstituted to a volume of 3 ml. A 1-μl amount of this culture extract was used directly for an analysis by gas-liquid chromatography (GLC), or 25 μl of the culture extract was spotted onto plates for thin-layer chromatography (TLC). The presence of skatole, p-cresol, o-cresol, m-cresol, and methylbenzene in culture extracts was evaluated by GLC. Other possible metabolites were evaluated by TLC.

Competition studies. To determine whether the ruminal Lactobacillus strains could produce skatole and p-cresol simultaneously, we prepared a basal medium containing IAA and p-HPAA at equimolar concentrations (11 mM). Each strain was inoculated into this medium in triplicate, and these cultures were incubated at 39°C for 72 h. At 12-h intervals, 2 ml was removed aseptically under oxygen-free CO₂ from each tube, acidified with 2 drops of concentrated HCl, and extracted with 10 ml of diethyl ether. The ether phase was transferred to a screw-capped vial and evaporated under nitrogen to a volume of 3 ml, and 1 μl was analyzed by GLC, as described below.

To examine the effects of varying the molar ratio of IAA to p-HPAA on skatole production and p-cresol production, we prepared five batches of media which were similar to the basal medium described above. The concentration of p-HPAA was held constant in all media (11.0 mM), whereas the concentration of IAA was increased proportionately (i.e., 0, 5.5, 11.0, 16.5, and 22.0 mM) to provide molar ratios of IAA to p-HPAA of 0, 0.5, 1.0, 1.5, and 2.0. All media were processed identically, and preincubated media were placed into tubes in 9-ml quantities. The same growth culture was used to inoculate each series of media. After incubation for 72 h at 39°C, the inoculated cultures were extracted twice with methylene chloride and analyzed by GLC.

Rate of decarboxylation. To study the rate of decarboxylation of IAA and p-HPAA, two identical sets of basal media were prepared. The first set consisted of a control medium containing IAA at a concentration of 5.5 mM and a test medium containing 5.5 mM IAA plus 22.0 mM p-HPAA. The second set consisted of a control medium containing 5.5 mM p-HPAA and a test medium containing 5.5 mM p-HPAA plus 22.0 mM IAA. All four media were inoculated with the same growth culture and incubated at 39°C. At 3-h intervals, 2 ml of each inoculated medium was removed aseptically, extracted, and analyzed as described above for the competition studies.

GLC. A Hewlett-Packard model 5840A gas-liquid chromatograph with a hydrogen flame ionization detector was used for the analyses of skatole and p-cresol. A stainless steel column (2 m by 2 mm) packed with WAW-DMCS (60/80 mesh) and 21% Carbowax 4000 (Anspec Corp., Ann Arbor, Mich.) was used. The oven temperature was maintained isothermally at 190°C, and helium was the carrier gas (flow rate, 50 ml/min). The injector temperature was 250°C, the detector temperature was 250°C, the chart speed was 0.3 cm/min, and the attenuation was 128X. Under these conditions, p-cresol had a retention time of about 5.3 min, and skatole had a retention time of about 19.6 min. The concentrations of skatole and p-cresol in culture extracts were determined from standard solutions by using a microprocessor.

TLC. Methylene chloride extracts (25 μl) of certain test cultures were spotted onto precoated Silica Gel G plates (20 by 20 cm; E. Merck AG, Darmstadt, Germany). Known metabolites of decarboxylation were spotted onto the same plates, along with the corresponding parent compounds to obtain reference retardation factor (Rf) values. The solvent systems used were 2-propanol-ammonia-water (20:1:2, vol/vol) and 1-butanol-acetic acid-water (12:3:5, vol/vol). The development time was 3 to 3.5 h. Skatole, 5-hydroxy skatole, IAA, and 5-hydroxyindoleacetic acid were detected by spraying developed plates with Ehrlich reagent (16). Phenolic and catecholic metabolites were detected by spraying with diazotized sulfanilic acid reagent (19). Identifications of metabolites were made by comparing the Rf values of unknowns with the Rf values of the parent compounds and known metabolites, as well as by using the color reactions produced by the spray reagents.

RESULTS

Decarboxylation of compounds. Of the 13 compounds examined, 4 were decarboxylated by the ruminal Lactobacillus strains to identifiable metabolites (Table 1). A strong odor, characteristic of skatole, was evident in all cultures in which IAA was the test compound. Because of its insolubility, skatole crystallized out of the medium as large, refractive flakes, even while the cultures were being incubated at 39°C, and also caused the cultures to turn milky white. The metabolite produced was identified as skatole by GLC and TLC. All of the strains decarboxylated 5-hydroxyindoleacetic acid to a metabolite identified by TLC as 5-hydroxyskatole. When the ruminal Lactobacillus strains were inoculated into a medium containing p-HPAA, a strong disinfectant type of odor, characteristic of p-cresol, was detected, concomitant with a perceptible darkening of the cultures from a bright yellow to a yellowish brown color, with a thin dark brown film at the meniscus. The metabolite produced was identified as p-cresol by GLC and TLC. Although p-HPAA was decar-
boxylated to p-cresol, none of the ruminal \textit{Lactobacillus} strains was able to decarboxylate \textit{m}-hydroxyphenylacetic acid to \textit{m}-cresol and \textit{o}-hydroxyphenylacetic acid to \textit{o}-cresol, as indicated by the lack of these metabolites in culture extracts when they were analyzed by GLC and TLC. The strains also decarboxylated 3,4-dihydroxyphenylacetic acid to 4-methylcatechol, but did not decarboxylate 2,5-dihydroxyphenylacetic acid, 3,4-dimethoxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylacetic acid, as indicated by the lack of detectable spots on TLC plates other than those of the parent compounds. Substitution of the hydroxyl group on the aromatic ring with a fluoro or nitro group (i.e., \textit{p}-fluorophenylacetic acid, \textit{o}-nitrophenylacetic acid, and \textit{p}-nitrophenylacetic acid) or elimination of the hydroxyl group (i.e., phenylacetic acid) resulted in no decarboxylation.

Decarboxylation by individual strains. When they were inoculated into medium containing 11 mM IAA, all of the ruminal \textit{Lactobacillus} strains showed about the same capability for decarboxylating this precursor to skatole. The mean efficiency of IAA conversion to skatole for the four strains was 51.5 ± 2.0%. The mean skatole concentration was 0.93 ± 0.05 mg/ml. When they were inoculated into medium containing 11 mM \textit{p}-HPAA, all of the strains again showed about the same capability for decarboxylating this precursor to p-cresol. The mean efficiency of \textit{p}-HPAA conversion to \textit{p}-cresol for the four strains was 49.2 ± 4.1%, and the mean \textit{p}-cresol concentration was 0.74 ± 0.06 mg/ml.

Simultaneous skatole production and \textit{p}-cresol production. Incubation of the ruminal \textit{Lactobacillus} strains in a medium containing both IAA and \textit{p}-HPAA in equimolar concentrations (11 mM) resulted in the production of both skatole and \textit{p}-cresol (Fig. 1). The decarboxylation reaction was completed after about 24 h of incubation, with no further increase in either the skatole concentration or the \textit{p}-cresol concentration. In contrast to the ratio of skatole concentration to \textit{p}-cresol concentration observed when IAA and \textit{p}-HPAA were incubated individually, a 0.5:1 skatole-to-\textit{p}-cresol conversion ratio was observed when IAA and \textit{p}-HPAA were combined. The combined concentrations of skatole and \textit{p}-cresol in these cultures did not exceed 1.26 ± 0.07 mg/ml.

Table 2 shows the effect of varying the molar ratio of IAA concentration to \textit{p}-HPAA concentration on skatole production and \textit{p}-cresol production. In the absence of IAA, the \textit{Lactobacillus} strains decarboxylated 61.8 ± 2.3% (mean ± standard error) of the \textit{p}-HPAA contained in the medium. The efficiency of decarboxylation of \textit{p}-HPAA to \textit{p}-cresol did not decrease appreciably regardless of increasing IAA concentrations in the media. The mean efficiency of conversion of \textit{p}-HPAA to \textit{p}-cresol for the five media was 59.5 ± 2.7%, and the mean \textit{p}-HPAA concentration

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Metabolite produced</th>
<th>R\textsubscript{t} values\textsuperscript{a}</th>
<th>Color reactions\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>Skatole</td>
<td>0.75 0.78</td>
<td>P→B</td>
</tr>
<tr>
<td>\textit{p}-HPAA</td>
<td>\textit{p}-Cresol</td>
<td>0.71 0.73</td>
<td>Y→P</td>
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<tr>
<td>5-HIAA\textsuperscript{c}</td>
<td>5-Hydroxyskatole</td>
<td>0.73 0.77</td>
<td>BG→B</td>
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<tr>
<td>3,4-DHPAA\textsuperscript{d}</td>
<td>4-Methylcatechol</td>
<td>0.70 0.79</td>
<td>LB</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The solvent systems used were as follows: solvent 1, 2-propanol-ammonia-water (20:1:2, vol/vol); solvent 2, 1-butanol-acetic acid-water (12:3:5, vol/vol).

\textsuperscript{b} Abbreviations: DSA, diazotized sulfanilic acid; B, blue; BG, blue-green; LB, light brown; P, pink; Y, yellow.

\textsuperscript{c} 5-HIAA, 5-Hydroxyindoleacetic acid.

\textsuperscript{d} 3,4-DHPAA, 3,4-Dihydroxyphenylacetic acid.

\textbf{FIG. 1.} Time sequence curves of \textit{p}-cresol production and skatole production by ruminal \textit{Lactobacillus} strains in medium containing both \textit{p}-HPAA and IAA. Strains were inoculated (1.0 ml) into medium (9.0 ml) containing 11 mM \textit{p}-HPAA and 11 mM IAA. Each point represents the mean of the four \textit{Lactobacillus} strains. Symbols: ●, \textit{p}-cresol; ▲, skatole.
was 0.90 ± 0.05 mg/ml. However, the efficiency of decarboxylation of IAA to skatole decreased from 55.0 ± 9.6 to 10.5 ± 2.7% as the concentration of IAA in the medium increased. Skatole concentration appeared to decrease only at the highest molar ratio of IAA to p-HPAA (i.e., 2.0); however, the total amount of skatole produced was not appreciably lower. The net effect was that the total combined concentration of skatole and p-cresol in the cultures was constant, with a mean of 1.36 ± 0.12 mg/ml and a ratio of skatole concentration to p-cresol concentration of 0.5:1.

Figure 2 shows the rate of decarboxylation of IAA to skatole in the presence and absence of p-HPAA during the first 12 h after inoculation. When p-HPAA was not included in the IAA medium, there was a lag of about 3 h before skatole was detected in cultures (Fig. 2, line A). However, the rate of decarboxylation of IAA to skatole was only slightly inhibited by the inclusion of p-HPAA in the IAA medium at four times the molar concentration of IAA (Fig. 2, line B). The rate of decarboxylation of p-HPAA, however, did not show a lag, and the p-cresol concentration rapidly increased in the cultures (Fig. 2, line B). The reciprocal experiment is shown in Fig. 3. When IAA was not included in the p-HPAA medium, decarboxylation of p-HPAA to p-cresol proceeded rapidly (Fig. 3, line A), and the rate was virtually unaltered despite the inclusion of IAA at four times the molar concentration of p-HPAA (Fig. 3, line B). The rate of decarboxylation of IAA to skatole again showed a lag of about 3 h, despite the higher concentration of IAA in the medium (Fig. 3, line B). The curves in Fig. 2 and 3 show that neither the rate of skatole production nor the rate of p-cresol production was inhibited appreciably by a higher concentration of the reciprocal precursor.

### DISCUSSION

There have been other reports of bacteria which degrade tryptophan to skatole and tyrosine to p-cresol. Previous studies by Fellers and Clough (12) indicated that Clostridium scatologenes produces skatole directly from tryptophan, and Elsden et al. (11) found that Clostridium difficile NCIB 10666 and C. scatologenes produce p-cresol from either tyrosine or p-HPAA. We examined C. difficile ATCC 9689 and C. scatologenes Prevot ATCC 25775 in our laboratory and confirmed that these strains produced these metabolites. However, contrary to the observations of Elsden et al. (11), C. scatologenes ATCC 25775 did not produce p-cresol from either tyrosine or p-HPAA, and neither C. difficile nor C. scatologenes decarboxylated IAA to skatole. Therefore, the ability of the ruminal Lactobacillus strains to produce both skatole and p-cresol, from IAA and p-HPAA, respectively, appears to be metabolically unique.

The demonstration that the ruminal Lactobacillus strains are also capable of decarboxylating 5-hydroxyindoleacetic acid to 5-hydroxy- skatole and 3,4-dihydroxyphenylacetic acid to...
4-methylcatechol is not particularly surprising, since both of these compounds are the hydroxylated derivatives of IAA and p-HPAA. However, o-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid were not decarboxylated to o-cresol and m-cresol, respectively, suggesting that a hydroxyl group must be para to the acetyl group on the aromatic ring for decarboxylation to occur. Scheline (17) obtained similar results when he incubated the isomers of HPAA with rat cecal contents. This conclusion was further supported by the demonstration that 3,4-dihydroxyphenylacetic acid was decarboxylated to methylcatechol, but 2,5-dihydroxyphenylacetic acid was inactive. The substitution or elimination of the para hydroxyl group also resulted in no decarboxylase activity. An exception to these observations was 3-methoxy-4-hydroxyphenylacetic acid, in which a hydroxyl group is in the para position but the presence of an interposed methoxyl group apparently inhibited the reaction. Besides a possible steric involvement, the ability to effect decarboxylation could have been influenced by the solubilities of the parent compounds.

Although skatole and p-cresol were produced in similar amounts when IAA and p-HPAA were tested individually on an equimolar basis, the 0.5:1 ratio observed when IAA and p-HPAA were combined (Fig. 1) indicates that p-HPAA was decarboxylated twice as effectively as IAA. This difference in rate of decarboxylation could be explained by the inability of the ruminal Lactobacillus strains to take up IAA as rapidly as p-HPAA. The observed 3-h lag before skatole was detected in cultures, even in the absence of p-HPAA (Fig. 2 and 3), suggests this possibility.

Another explanation for this difference may be that the enzyme has a greater affinity for p-HPAA than for IAA, assuming that only one enzyme is involved.

The question as to whether different decarboxylating enzymes are involved in the production of skatole and p-cresol was not entirely resolved from the data. Decarboxylation of IAA to skatole was only slightly reduced by the presence of p-HPAA (Table 2 and Fig. 2), and the reduction was proportionately constant regardless of the IAA concentration (Table 2). Reciprocally, a high IAA concentration did not inhibit the decarboxylation of p-HPAA to p-cresol (Table 2), even when IAA was included at four times the molar concentration of p-HPAA (Fig. 3). Although inconclusive until cell-free extract studies have been conducted, these results suggest the involvement of two different decarboxylating enzymes, rather than a single enzyme that exhibits a low specificity. Indirect support of this two-enzyme hypothesis comes from our observation that C. difficile ATCC 9689 decarboxylates p-HPAA to p-cresol, but is unable to decarboxylate IAA to skatole (unpublished data).

Skatole and p-cresol have been detected in the rumen (14, 21, 25, 26), but their occurrence seems to vary with the conditions of ruminal fermentation. However, the ability of Lactobacillus sp. to produce both of these metabolites suggests that this bacterium could be responsible for both skatole production and p-cresol production in the rumen via the decarboxylation of IAA and p-HPAA. Short-term in vitro incubation studies with rumen fluid (18) show that IAA and p-HPAA are the predominant metabolites of tryptophan and tyrosine degradations. Other studies (1, 17, 20) show that the occurrence of skatole and p-cresol in the intestinal contents and feces of rats and pigs can result by the same mechanism of decarboxylation.

Both 5-hydroxy-skatole and 4-methylcatechol have been detected in human urine. Mori et al. (15) found high concentrations of 5-hydroxy-skatole in the urine of epileptic patients, and 4-methylcatechol has been reported in the urine of patients receiving large oral doses of L-3,4-dihydroxyphenylalanine (6). Both Scheline (17) and Bakke (2) have shown that 4-methylcatechol is produced when rat cecal contents are incubated with 3,4-dihydroxyphenylacetic acid. These observations and our results suggest a commonality in the production and occurrence of these metabolites.

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LITERATURE CITED


