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Isolation and Characteristics of a Skatole-Producing *Lactobacillus* sp. from the Bovine Rumen†

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A bacterium that is capable of decarboxylating indoleacetic acid to skatole (3-methylindole) has been isolated from an L-tryptophan enrichment of bovine rumen fluid. The bacterium is a gram-positive, nonmotile, nonsporeforming rod. It is an obligate anaerobe, and strains predominantly produce D-(−)-lactic acid, with smaller amounts of L-(+)-lactic acid and acetic acid, from sugar. All four strains isolated gave a negative reaction to the indole test because they cannot form skatole directly from tryptophan. This is the first report of indoleacetic acid decarboxylation to skatole in pure culture and the demonstration of skatole production by a *Lactobacillus* species.

Skatole (3-methylindole) is a common metabolite found in the feces of all mammalian and avian species. Its occurrence also has been shown in the bovine rumen (17, 26). When the etiology of bovine pulmonary edema and emphysema induced by L-tryptophan administration into the rumen was elucidated, skatole was found to be the metabolite responsible for the syndrome (8, 9, 27). Although the exact mechanism by which skatole is produced from tryptophan by bacteria has not been determined, recent investigations (26) have shown that, in long-term in vitro incubations of rumen fluid with L-tryptophan, skatole production occurred as a two-step process involving (i) conversion of L-tryptophan to indoleacetic acid (IAA) and (ii) decarboxylation of IAA to skatole. The subsequent separation of a mixed ruminal culture that showed only decarboxylase activity toward IAA indicated that more than one bacterial species was involved in the production of skatole by this process (26). Decarboxylation of IAA to skatole was postulated long ago (1); however, it has never been demonstrated in a pure culture (24). The present investigation was concerned with the isolation and characteristics of a *Lactobacillus* species from rumen fluid with the capacity to decarboxylate IAA to skatole.

MATERIALS AND METHODS

Culture methods and media. Anaerobic culture and roll tube techniques used were essentially those described by Hungate (15) as modified by Bryant (4). The medium (PYG-RF) used to initially maintain the bacterium is described in Table 1. In early attempts at isolation of a bacterium that would produce skatole, 0.5% L-tryptophan was added to this medium. The additional amino acid, however, was not essential for the maintenance of the bacterium and was subsequently deleted. Rumen fluid, obtained from fistulated steers fed an alfalfa-grass hay diet, was clarified by passage through cheesecloth, centrifuged at 20,000 × *g* for 30 min, bottled, and autoclaved at 15 lb/in^2 for 20 min. The clarified rumen fluid was recentrifuged at the same speed prior to its use. Medium ingredients, minus the sodium carbonate and cysteine-sulfide solutions, were combined, the pH was adjusted to 6.5 with 1.0 N NaOH, and the medium was brought rapidly to a boil under oxygen-free CO_2. The flask was stoppered, wired down, and autoclaved at 121°C for 20 min. After autoclaving and cooling, previously prepared sterile, CO_2-equilibrated 8% sodium carbonate and 2.5% cysteine-sulfide solutions were aseptically added to the medium. The medium was tubed under a 100% CO_2, oxygen-free gas phase in 9.0-ml quantities (18 by 150-mm tubes). The final pH of the medium was 6.7. The IAA medium used to demonstrate decarboxylase activity is also described in Table 1 and was prepared as indicated above, except that the IAA was dissolved in 1.0 ml of 0.1 N NaOH prior to its addition into the medium. Previous work of Rosenberger (20) indicated that skatole production by clostridial species was inhibited by cysteine; therefore, ascorbic acid was initially substituted as a weak reducing agent. Subsequent work showed that cysteine-sulfide did not inhibit skatole production by the isolated strains, and it was routinely added to the medium, along with the ascorbic acid.

The rumen fluid-glucose-cellobiose-starch (GCS-RF) medium (6), modified by the addition of 0.7% yeast extract, hemin, and menadione, is currently used to maintain the isolated strains (Table 1). Better

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TABLE 1. *Culture media for study of the skatole-producing strains*

Component	% in medium		
	PYG-RF	IAA	GCS-RF
Peptone (Bacto)	0.5		
Trypticase (Bacto)			0.5
Glucose	0.5		0.05
Cellobiose			0.05
Soluble starch			0.05
Yeast extract (BBL)	0.3	0.2	0.7
IAA[a]		0.2	
Ascorbic acid		0.2	
Hemin-menadione solution[b]	1.0	1.0	1.0
Mineral solution I[c]	8.3	8.3	3.75
Mineral solution II[c]	8.3	8.3	3.75
Clarified rumen fluid	20.0		30.0
Resazurin solution (0.1%)[b]	0.1	0.1	0.1
Sodium carbonate (8%)[d]	5.0	5.0	5.0
Cysteine-sulfide (2.5%)[d,e]	2.0	2.0	2.0
Agar (optional)	2.0	2.0	2.0
Distilled water	To 100 ml	To 100 ml	To 100 ml
Gas phase	CO_2	CO_2	CO_2

[a] Dissolved in 1.0 ml of 0.1 N NaOH prior to addition into medium.
[b] Prepared as described previously (14).
[c] Mineral solution I: 0.6% K_2HPO_4; mineral solution II: 0.6% KH_2PO_4–0.6% $(NH_4)_2SO_4$–1.2% NaCl–0.245% $MgSO_4$–0.159% $CaCl_2 \cdot H_2O$.
[d] Aseptically added to medium after autoclaving and cooling.
[e] Prepared as described by Caldwell and Bryant (7).

growth of the strains is obtained in this medium than in PYG-RF medium.

Isolation procedures. The skatole-producing bacterium was isolated from rumen fluid obtained from a rumen-fistulated steer fed an alfalfa-grass hay diet, maintained at Pullman, Wash. The rumen fluid was strained through cheesecloth, transported to the laboratory in vacuum bottles, and used directly in incubations without further processing. About 50 ml of the strained rumen fluid was incubated anaerobically under oxygen-free CO_2 with 0.5 g of either L-tryptophan or IAA, previously dissolved in 2.0 ml of 0.1 N NaOH. Incubation was for 5 days at 37°C, during which time a strong, pungent odor characteristic of skatole became evident in the incubation flasks.

Inocula (2.0 ml) from the incubation flasks were transferred to prereduced tubes of PYG-RF medium containing 0.5% L-tryptophan, with transfers made every 3 to 5 days. All further attempts at isolation were made from these PYG-RF cultures or cultures on slants of the same medium. Well-grown cultures in PYG-RF medium were serially diluted to 10^8 in the same medium, and 1.0 ml of inoculum from the 10^4 to 10^8 dilutions was used for roll tubes of PYG-RF agar medium. Roll tubes were incubated at 37°C for 7 to 10 days, after which time well-isolated colonies from the 10^6 to 10^8 dilutions were picked under oxygen-free CO_2 and inoculated into PYG-RF medium. After 2 days of growth, cultures picked to PYG-RF medium were checked for skatole production by aseptically transferring 1.0 ml of inoculum into IAA medium, which was then incubated for 3 days. Cultures in IAA medium were then rapidly screened for skatole production either by the detection of the characteristic odor of the metabolite in tubes opened under CO_2 or by the presence of skatole crystals that precipitated out of the medium. PYG-RF cultures that demonstrated a positive skatole production in IAA medium were retained, diluted, and inoculated into roll tubes as previously described. The procedure was repeated until a pure culture was obtained. The isolation of the skatole-producing bacterium was based on the similarity of colony type and cell morphology in two consecutive roll tube subcultures and the demonstration of decarboxylase activity in IAA medium.

Determination of skatole. Skatole was detected in cultures by its characteristic odor and by gas-liquid chromatography and thin-layer chromatography as previously described (2, 26), with authentic skatole (Sigma Chemical Co.) as the standard.

Characterization tests. The methods used for the biochemical, physiological, and metabolic characterization of the strains were described previously (14).

RESULTS

Isolation. The original mixed cultures obtained from the enrichment incubations and maintained in PYG-RF medium produced indole, indolepropionic acid, IAA, and skatole from L-tryptophan as determined by thin-layer chromatography with authentic compounds. The predominant metabolite produced was indole. Large-inoculum transfers (2 to 3 ml) were required to maintain skatole production, and the decarboxylation reaction could not be enhanced even with the addition of 0.2% IAA. Repeated

attempts to isolate a pure culture by picking all of the colonies from 10^8 and 10^9 dilutions in roll tubes of this medium were unsuccessful. However, mixed cultures were obtained from picks out of roll tubes inoculated with a 10^4 dilution. These cultures decarboxylated IAA to skatole, but did not produce IAA, indole, indolepropionic acid, or skatole from L-tryptophan.

When the latter mixed cultures were used, skatole production was demonstrated in IAA medium after 3 to 5 days of incubation. Because of its insolubility, the skatole frequently precipitated out of the medium if left standing at room temperature. In roll tubes of IAA medium incubated for 10 to 14 days, crystalline spicules of skatole were often observed around colonies. Despite the voluminous production of skatole in IAA medium, growth was very poor, and attempts to isolate the bacterium from roll tubes were hindered by low numbers and the minute colonies formed. Skatole was also apparently toxic to the bacterium in that the transfer of either liquid cultures showing precipitated skatole or single colonies from roll tubes showing skatole spicules into PYG-RF medium did not grow. For this reason, single colonies picked from roll tube dilutions of the mixed skatole-producing cultures first had to be inoculated into PYG-RF medium, and the resultant PYG-RF cultures were then checked for skatole production by transfer into IAA medium.

Isolation of strains was accomplished by growing skatole-positive cultures in PYG-RF medium, transferring into IAA medium with overnight incubation, and then reinoculating roll tubes of IAA medium containing 0.1% glucose. The cultures obtained showed a similar colony type in two consecutive agar subcultures, homogeneous cellular morphology, and decarboxylation of IAA to skatole.

Characteristics of the bacterium. The bacterium is a nonmotile, gram-positive, nonspore-forming, short rod (1.9 by 0.7 μm) that occurs as singles, pairs, and short chains of from four to six cells under microscopic examination of PYG-RF cultures (Fig. 1). It is usually irregular and pleomorphic in shape. Deep colonies in PYG-RF medium roll tubes after 7 days of incubation are lenticular and cream to white in color, with an entire margin and smooth surface. Surface colonies are opaque white and slightly convex with an entire edge. The strains are obligate anaerobes and will not grow if the resazurin indicator in the medium is oxidized. They will not grow aerobically in petri dishes of PYG-RF medium, nor have they become aerotolerant after 2 years of cultivation. They grow slowly in PYG-RF and GCS-RF medium, with a maximum optical density at 600 nm of about 0.2 to 0.4 after 4 days of incubation. Growth in PYG-RF medium occurs at 37°C, but not at 15 or 45°C. Initial growth in PYG-RF and GCS-RF media appears as a white swirl; however, after 3 to 5 days of incubation, a ropy sediment that is white turning to brown in color becomes apparent. There is no gas production. The strains grow poorly in IAA medium, but produce large amounts of skatole. In PYG-RF medium, the strains produce predominantly D-(−)-lactic acid, with 7 to 27% L-(+)-lactic acid and small amounts of acetic acid. The ratio of lactate to acetate varies from 7:1 to 1.5:1 with the different strains. Carbohydrate substrates fermented by the strains are shown in Table 2.

All strains demonstrate positive arginine hydrolysis. Gelatin, milk, meat, nitrate reduction, catalase, and ammonia tests are negative for all strains. Bile, lecithinase, and lipase activities were also negative. Although the strains produce skatole from IAA, they cannot convert L-tryptophan to indole, IAA, or skatole. In IAA medium after 3 to 5 days of incubation, however, a strongly positive reaction to the indole test is evident.

Intramuscular injection of 0.2 ml of either PYG-RF or IAA medium cultures of the bacterium into five mice, with observation for 10 days, indicated no pathogenicity. Similarly, intraperitoneal injection of 0.5 ml of supernatant obtained from either centrifuged PYG-RF or IAA medium cultures into five mice, with observation for 10 days, indicated no toxicity.

DISCUSSION

A number of *Lactobacillus* species have been isolated from the rumen and include *L. acidophilus, L. bifidus* (*Bifidobacterium*), *L. brevis, L. bucherni, L. casei, L. cellobiosus, L. fermentum, L. lactis, L. plantarum, L. ruminis,* and *L. vitulinus* (3, 21). The occurrence of these species in the rumen is generally associated with diets high in soluble carbohydrates (e.g., grain, milk, fresh forages) and sometimes with lowered ruminal pH. Under these conditions, the lactobacilli can constitute a significant proportion of the total bacterial population (16). Significant numbers of *L. vitulinus,* however, have been found in the rumen of cattle consuming either lush alfalfa or ladino clover with little change in ruminal pH (5). Based on their characteristics, however, none of these species appears to correspond to the skatole-producing strains isolated. Examinations of *L. vitulinus* from M. P. Bryant's collection (strains B62, T-185, and GA-1) show that these strains do not possess IAA decarboxylase activity.

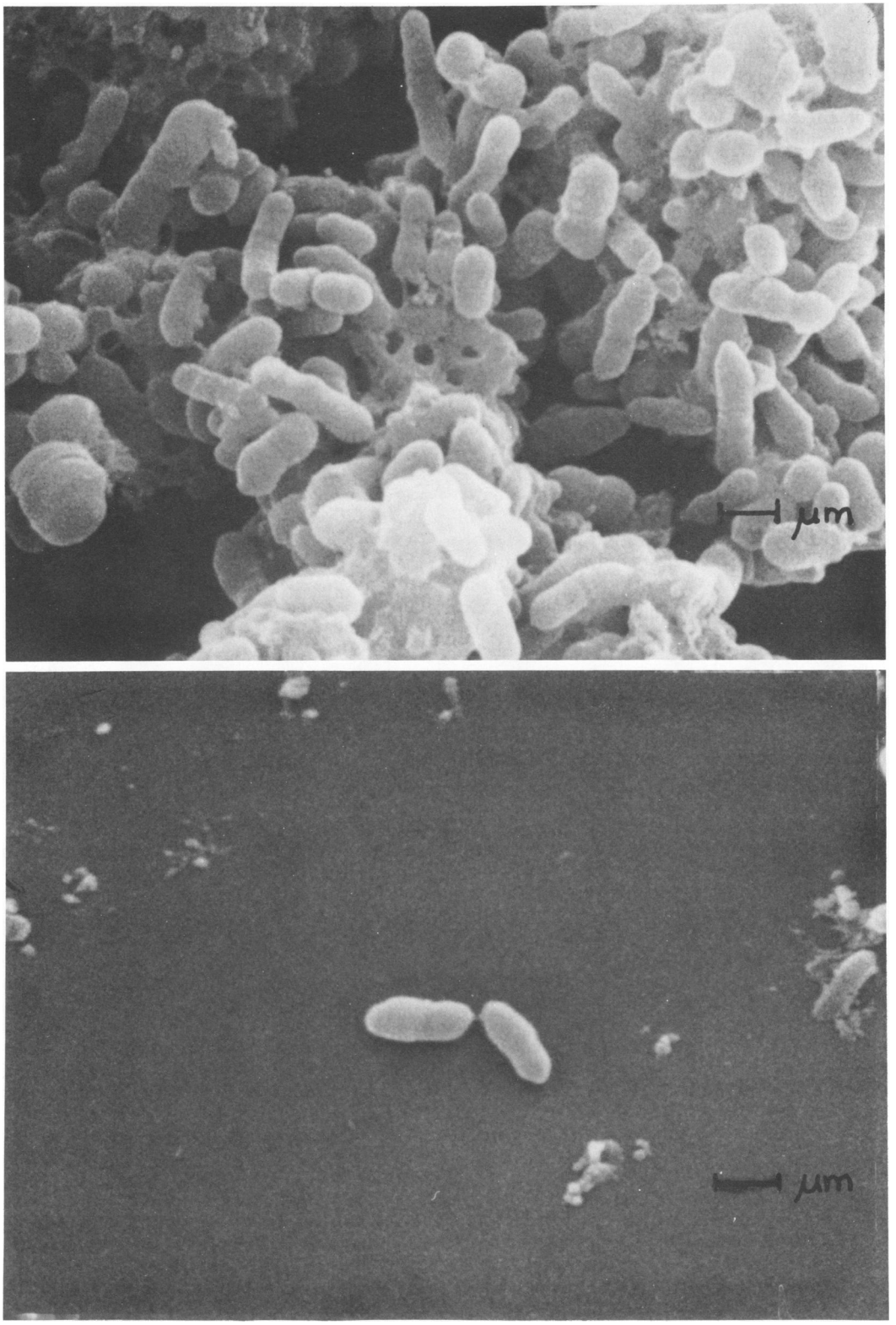

FIG. 1. *Scanning electron micrographs of skatole-producing rumen isolate. Bar = 1 µm.*

TABLE 2. *Fermentation of carbohydrate substrates by skatole-producing strains*[a]

Strain	Cellobiose	Esculin hydrolysis	Fructose	Glucose	Maltose	Mannose	Ribose	Salicin	Sorbitol	Sucrose	Xylose
A2 (VPI 11198)	S	+	A	W	W	W	–	W	–	–	–
A3 (VPI 11199)	S	+	A	W	A	A	S	W	S	S	S
A8 (VPI 11200)	S	+	A	W	W	W	–	–	–	A	–
A9 (VPI 11201)	S	+	W	W	W	W	S	S	–	–	–

[a] The basal medium was peptone-yeast extract (PY) (see reference 14, p. 124). Symbols: A, acid; W, weak; S, slight acid; +, positive; –, negative. The following sugars were negative for all strains: PY-NH_3, amygdalin, arabinose, erythritol, glycogen, inositol, lactose, mannitol, melezitose, melibiose, raffinose, rhamnose, starch, trehalose, and pectin.

Although this skatole-producing bacterium was isolated from the rumen, its habitat may be even more extensive. Unsuccessful efforts to isolate the bacterium directly from serially diluted rumen fluid indicate that it is not among the predominant rumen bacteria. Although skatole is periodically detected in rumen fluid, its concentration varies considerably and can be markedly increased, if at all, only by the administration of large amounts of L-tryptophan into the rumen (27). Recently, we have been able to demonstrate the decarboxylation of IAA to skatole by the mixed fecal microflora of domesticated (i.e., sheep, pigs, goats, cattle, chickens, horses, dogs, cats, and rabbits) and exotic animals (i.e., monkeys, deer, elephants, lions, and bison) and in human feces. Indeed, several intestinal bacteria, including *Bacteroides thetaiotaomicron, Citrobacter* sp., and *Escherichia coli,* will produce IAA from tryptophan (10, 23). Skatole production has also been demonstrated in the human small intestine (E. C. Horning and C. E. Dalgliesh, Biochem. J. **70**:13p, 1958), and the lactobacilli are a common component of the intestinal microflora (18).

There are few references in the literature concerning bacteria that produce skatole. Two species of the genus *Clostridium, C. scatologenes* (13) and *C. nauseum* (22), have been reported to produce skatole. Several clostridial strains resembling *C. scatologenes* have also been isolated from silage (20). The mechanism by which skatole was produced from tryptophan by these species was not elucidated. A pseudomonas species, isolated from soil, was reported to decarboxylate IAA to skatole as an intermediate step in the formation of catechol (19). Our studies (26) indicate that skatole is not degraded further to catechol by the present rumen strains.

Skatole has been shown to have a bacteriostatic effect on gram-negative enterobacteria, with species of the genera *Salmonella* and *Shigella* slightly more sensitive than the *Escherichia* and *Aerobacter* groups (25). In dilute solutions, skatole inhibited the growth and acid production of *L. acidophilus* (11). Skatole has also been shown to be toxic to rumen ciliated protozoa (12). In the isolation of the skatole-producing strains, no growth could be demonstrated in PYG-RF medium when inoculated with skatole-containing cultures. This suggests that skatole was toxic to the strains, which otherwise would grow in PYG-RF medium when transferred from cultures containing no skatole.

The detection of indole and skatole is routinely done by measuring the production of these compounds from tryptophan with *p*-dimethylaminobenzaldehyde. The present strains gave a negative reaction to this test because they cannot form skatole directly from tryptophan, requiring instead its immediate precursor, IAA, which is produced by other bacteria. If the indole test is used to demonstrate the presence of skatole, the possibility exists that other skatole-producing bacteria utilizing this mechanism would escape detection.

Based on their physiological, biochemical, and metabolic characteristics, the skatole-producing strains are assigned to the genus *Lactobacillus.*

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