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Protoplast Formation and Regeneration of Dehydrodivanillin-Degrading Strains of *Fusobacterium varium* and *Enterococcus faecium*

WEI CHEN, KUNIO OHMIYA,* AND SHOICHI SHIMIZU

Department of Food Science and Technology, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

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Two strains of rumen anaerobes isolated from dehydrodivanillin-degrading cultures were identified as *Fusobacterium varium* and *Enterococcus faecium*. These organisms degraded dehydrodivanillin synergistically to 5-carboxymethylvanillin and vanillic acid. Specific conditions for protoplast formation and cell wall regeneration for both bacteria were determined, under strictly anaerobic conditions, to be as follows. (i) The cell wall of each bacterium in yeast extract medium was loosened by adding penicillin G during early log-phase growth. (ii) The cell wall of *F. varium* was lysed by lysozyme (1 mg/ml) in glycerol (0.2 M)-phosphate buffer (0.05 M; pH 7.0). The addition of NaCl (0.08 M) with lysozyme was necessary for lysis of *E. faecium* in this solution. Almost all cells were converted to protoplasts after 2 h of incubation at 37°C. (iii) Regeneration of both protoplasts was 20 to 30% on an agar-containing yeast extract medium.

The formation of bacterial protoplasts and the regeneration of their cell walls are essential features in the initial steps of development of either a protoplast transformation or a fusion system for strain improvement. Specific conditions for protoplast formation have been developed in plant cells (10, 23, 25), basidiomycetes (6), fungi (22), and many bacteria (4, 5, 8, 12, 15, 16, 18, 19, 24, 27), including aerotolerant anaerobes such as *Clostridium* spp. (1, 11, 14). However, there has been negligible information on protoplast formation and regeneration of rumen anaerobes under strictly anaerobic conditions.

In our previous report (2) dehydrodivanillin (DDV)-degrading activity was found in the anaerobic culture broth of microflora originating from pig and cow feces, pond mud, and cow rumen fluid. The activity of the organisms from rumen fluid was enhanced by repeated addition of DDV, a lignin-related compound, to the culture broth. Potentially, DDV-degrading activity can be greatly improved with the development of genetic transfer systems for these bacteria, since lignin degradation has been enhanced in *Streptomyces* spp. by use of protoplast fusion techniques (21).

In this paper, we describe the isolation and identification from acclimated culture broth of two strains of rumen microorganisms which degraded DDV synergistically under strictly anaerobic conditions. We report also a procedure for protoplast formation in both microorganisms and the regeneration of their cell walls under strictly anaerobic conditions.

MATERIALS AND METHODS

Microorganisms. Rumen fluid microflora, acclimated on DDV as described earlier (2), were used to isolate anaerobes with DDV-degrading activity.

Chemicals. DDV was synthesized strictly following the method of Elbs and Lerch (3); its quality and purity were checked by gas chromatography, mass spectrometry, high-performance liquid chromatography, and thin-layer chromatography, and it was used as the carbon source for the microorganisms. Chicken egg white lysozyme, muramidase, β-amylase, β-glucuronidase, ampicillin, and bacitracin were purchased from Sigma Chemical Co. Other agents and their sources were as follows: pronase (Kaken Chemical Co.), Zymolyase-5000 (Seikagaku Kogyo Co.), lipase (Amano Pharmaceutical Co.), crystalline penicillin G potassium and streptomycin sulfate (Meiji Seika Co.), and kanamycin sulfate (Takeda Chemical Industries Co.). All other chemicals were analytical-grade commercial products.

Media. Yeast extract medium (2) containing minerals, cysteine, Na_2S, Na_2CO_3, and DDV was used to cultivate the organisms. A series of peptone yeast broths (Scott Laboratories) and media for the API-20 STREP SYSTEM (Analytab Products, Inc.) were used to identify isolates of rods and cocci, respectively.

Electron microscopy. Electron microscopic specimens of the isolates were prepared as follows. One drop of pure culture broth of rods or cocci was spread on a finder grid covered by Formvar film; excess sample volume was removed with filter paper. The organisms were negatively stained with phosphotungstic acid solution (0.3%; pH 7.1). Observation of the specimens was performed with a Hitachi HU-12A electron microscope.

Assay for DDV degradation. DDV-degrading activity of the microorganisms was determined by measuring the A_{360} with a dual-beam spectrophotometer (Jasco UVIDEC-610B) equipped with an integrating sphere unit, TIS-341 (2), and assayed by high-performance liquid chromatography (Jasco, BIP-I, UVIDEC-100V, SIC chromatocorder 11), using a Finepak SIL $C_{18}S$ column with eluting solvent, methanol-water (1:1) (flow rate, 1 ml/min; pressure, 100 kg/cm^2; UV at 280 nm). Thin-layer chromatography (Kieselgel 60 F254; E. Merck AG) was carried out with developing solvent, *n*-propanol–33% ammonia water (8:2).

Isolation and identification of metabolites in mono- and co-culture. Isolates of rods and cocci were mono- and cocultivated in yeast extract medium (2) (1 liter) containing DDV (400 mg/liter) for 1 week at 37°C in a jar fermentor under strictly anaerobic conditions. Compounds derived from DDV were extracted from the broth at 95°C with ethyl acetate. Extracts were analyzed by gas chromatography/mass spectrometry as described earlier (2).

* Corresponding author.

TABLE 1. Characterization of a DDV-degrading bacterium according to the *Anaerobe Laboratory Manual* (7)[a]

Characteristic/test	Result
Catalase	−
Milk	−
Nitrate	−
Bile growth	+
Gelatin	−
Indole	−
H_2S	−
Hippurate	−
Lactate→propionate	−
Pyruvate→propionate	−
Threonine→propionate	−
Propionic acid	tr
Lactic acid	Minor
Arabinose	−
Duicitol	−
Erythritol	−
Esculin (hydrolysis)	−
Galactose	−
Glycogen	−
Inositol	−
Maltose	−
Mannose	Weak
Melibiose	−
Raffinose	−
Ribose	−
Starch (pH)	−
Sucrose	−
Tween 80	−
Xylan	−
Acetic acid	Major
Butyric acid	−
Amygdalin	−
Cellobiose	−
Dextrin	−
Esculin (pH)	−
Fructose	Weak
Glucose	Weak
Glycerol	−
Lactose	−
Mannitol	−
Melezitose	−
Pectin	−
Rhamnose	−
Salicin	−
Starch (hydrolysis)	−
Trehalose	−
Xylose	−

[a] Identified as *F. varium*, a gram-negative rod with negative motility from cow rumen.

Protoplast formation of both isolates. Each bacterium stored on agar-yeast extract medium was cultivated overnight in liquid yeast extract medium (5 ml) and transferred into fresh medium. After 2 to 3 h of cultivation at 37°C, penicillin G (1,000 U) was added and incubation was continued for 1 h. Cells were harvested by centrifugation at 2,000 × *g* for 30 min, washed with a hypertonic solution of glycerol (0.2 M) and phosphate buffer (0.05 M; pH 7.0) prepared under an O_2-free CO_2 atmosphere, and suspended in the same solution (1 ml). Filter-sterilized lysozyme (final concentration, 1 mg/ml) was added to the cell suspension of *Fusobacterium varium*, while lysozyme (1 mg/ml) and NaCl (0.08 M) were added to the *Enterococcus faecium* cell suspension. Thereafter, the suspensions were incubated at 37°C for 2 to 3 h. Protoplast formation was monitored by measuring the turbidity decrement at 660 nm and by observing samples under a phase-contrast microscope.

Protoplasts washed and diluted in the hypertonic solution or water were overlaid gently on yeast extract-agar medium with glycerol (0.2 M) and yeast extract-agar medium without glycerol, respectively, for cell wall regeneration and assessing osmotic fragility. Since regenerating protoplasts grew slowly, viable counts were taken when new colonies stopped appearing after 5 to 7 days of incubation. The frequencies of protoplast formation and regeneration were calculated by the equations $(X - Y)/X$ and $(X - Y)/Z$, respectively (see Table 3).

RESULTS AND DISCUSSION

Isolation and identification of rumen anaerobes with DDV-degrading activity. Rumen microflora acclimated on DDV were cultivated on agar slants containing yeast extract, revealing three kinds of colonies after incubation at 37°C for more than 24 h. Pale opaque colonies, consisting of rods and cocci, demonstrated DDV-degrading activity. Water drop-like colonies contained only short rods with little activity. White spread colonies, containing spirals, rods, and cocci, could degrade DDV to levels as high as pale opaque colonies. The organisms in a pale opaque colony were cultivated repeatedly on the agar slants, with the isolation of homogeneous cocci and rods. DDV-degrading activities of each isolate were lower than that seen during their cocultivation. Our interest was directed especially towards the growth of

TABLE 2. Characterization of a DDV-degrading bacterium by the API 20 STREP SYSTEM[a]

Test	Substrate	Enzyme activity or reaction	Characteristic
VP	Pyruvate	Production of acetone	+
HIP	Hippurate	Hydrolysis	+
ESC	Esculin	β-Glucosidase	+
PYRA	Pyrrolidonyl-2-naphthylamide	Pyrrolidonyl-arylamidase	+
α-GAL	6-Br-2-naphthyl α-D-galactopyranoside	α-Galactosidase	−
β-BUR	Naphthol AS.BI β-D-glucuronate	β-Glucuronidase	−
β-GAL	2-Naphthyl-β-D-galactropyranoside	β-Galactosidase	+
PAL	2-Naphthyl phosphate	Alkaline phosphatase	−
LAP	L-Leucyl-2-naphthylamide	Leucine arylamidase	+
ADH	Arginine	Arginine dehydrolase	+
RIB	Ribose	Production of acids	+
ARA	L-Arabinose		+
MAN	Mannitol		+
SOR	Sorbitol		−
LAC	Lactose		+
TRE	Trehalose		+
INU	Inulin		−
RAF	Raffinose		−
AMD	Starch (2)		+
GLYG	Glycogen		−

[a] Identified as *E. faecium*, a catalase-negative, gram-positive coccus with negative motility from cow rumen fluid.

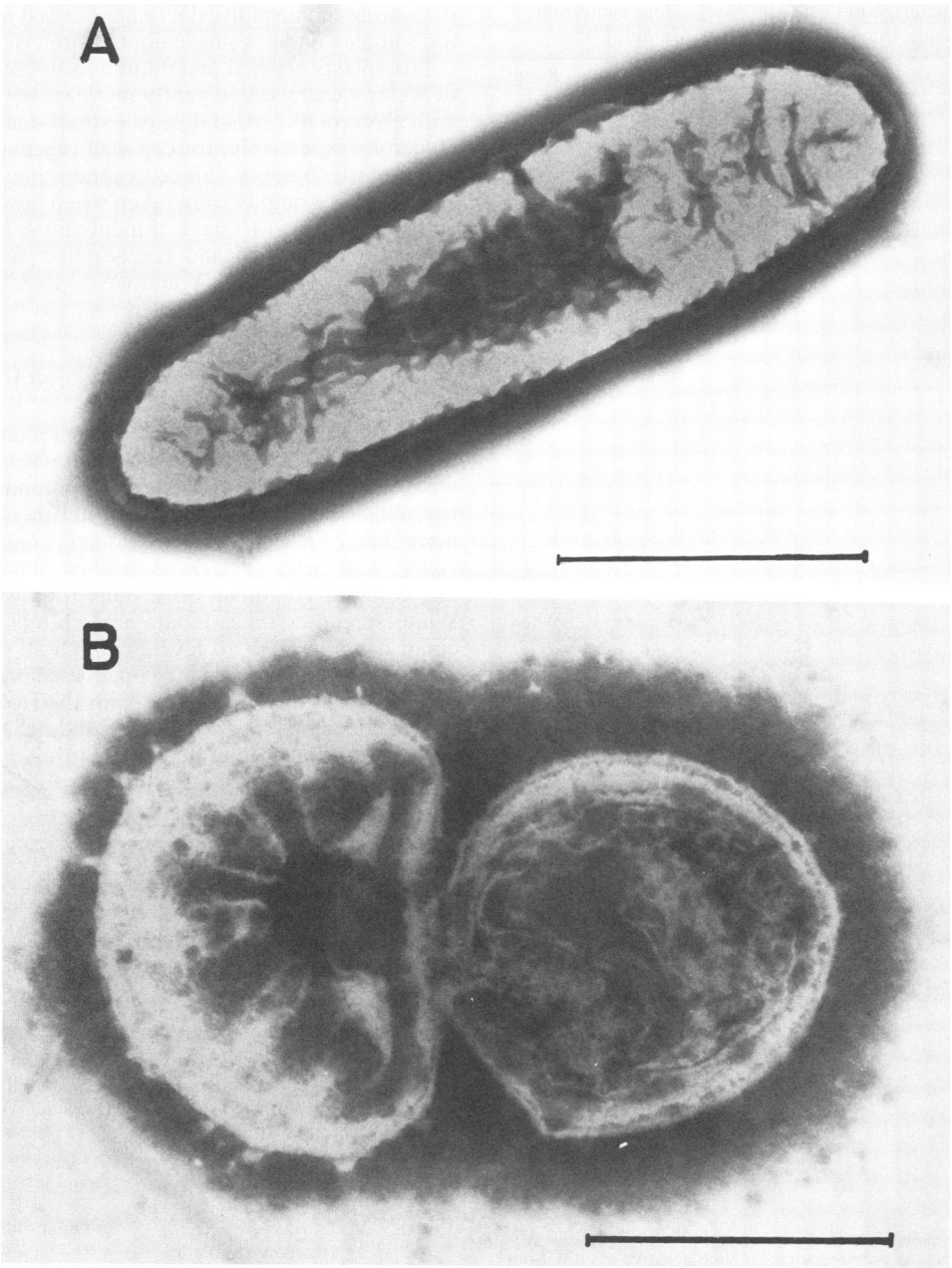

FIG. 1. Electron micrographs of *F. varium* (A) and *E. faecium* (B); both were stained with phosphotungstic acid solution. Bars, 1 μm.

new organisms with high DDV-degrading activities by using a cell fusion technique.

The rod was cultivated on a variety of media for characterization and identification (7). From the results listed in Table 1, it was identified as *F. varium*, a nonmotile gram-negative organism which can grow under strictly anaerobic conditions. The coccus was tested and identified by the API-20 STREP SYSTEM. The characteristics illustrated in Table 2 identified it as *E. faecium*. The electron micrographs of these bacteria are shown in Fig. 1.

Degradation of DDV by *F. varium* and *E. faecium*. Mono- and cocultivations of *F. varium* and *E. faecium* were performed in yeast extract medium (1 liter) containing DDV (400 mg). The extents of DDV degradation were about 3 and 10% after 1 week of cultivation of *E. faecium* and *F. varium*, respectively. The extent was increased with cocultivation to 30%, indicating a degree of synergism. Degradative intermediates of DDV were isolated and identified by gas chromatography/mass spectrometry. Those detected after cocultivation of the two organisms were dehydrodivanillic acid, dehydrodivanillic acid semialdehyde, vanillic acid, 5-carboxymethyl vanillin, 5-carboxyvanillin, and 4-hydroxy-3-methoxybenzyl alcohol, mostly similar to the products detected in the culture broth of rumen microflora acclimated on DDV as shown in a previous report (2). This indicates that *F. varium* and *E. faecium* are mainly responsible for the degradation of DDV in the acclimated culture. The only intermediates derived from DDV during cultivation of *E. faecium* were dehydrodivanillic acid semialdehyde and vanillic acid; those detected from *F. varium* culture broth

TABLE 3. Protoplast formation and regeneration in *F. varium* and *E. faecium*.

Organism	Expt	Starting no. of cells (Z)	Total no. of colonies (X)[a]	No. of colonies from nonprotoplasts (Y)[b]	Protoplast formation frequency[c]	Regeneration frequency[d]
F. varium	1	6.5×10^6	1.8×10^6	2.1×10^4	9.9×10^{-1}	2.8×10^{-1}
	2	7.8×10^5	2.0×10^5	1.9×10^4	9.0×10^{-1}	2.3×10^{-1}
E. faecium	1	1.5×10^7	3.3×10^6	1.7×10^4	9.9×10^{-1}	2.2×10^{-1}
	2	1.3×10^6	3.1×10^5	6.1×10^4	8.0×10^{-1}	1.9×10^{-1}

[a] Total colonies include colonies from regenerated cells and nonprotoplasted cells.
[b] Colonies from nonprotoplasts were derived from lysozyme-treated cells which resisted lysis upon dilution in water.
[c] Protoplast formation frequency is based on the formula: $(X - Y)/X$.
[d] Regeneration frequency is based on the formula: $(X - Y)/Z$.

were vanillin, 5-carboxyvanillin, and 5-carboxymethyl vanillin. These intermediate compounds were not detected in either uninoculated DDV controls or inoculated non-DDV controls and might be expected to accumulate in the culture broths due to the slower modification of DDV by the organisms than of other undetected intermediates. Therefore, the detection of different intermediates in the culture broths of *F. varium* and *E. faecium* may indicate the presence of different enzyme systems in each organism for the degradation of DDV, with synergistic degradation of DDV during cocultivation. Volatile fatty acids such as acetic, propionic, isobutyric, butyric, and isovaleric acids, all of which were found in acclimated culture broth (2), were negligibly detected in any culture broth prepared by mono- or cocultivation of *E. faecium* and *F. varium*. This indicates that organisms other than these isolates would be expected to produce volatile fatty acids from the intermediates of DDV.

Protoplast formation of *F. varium* and *E. faecium*. The formation of protoplasts of *F. varium* and *E. faecium* was investigated under strictly anaerobic conditions. All steps for formation of protoplasts were carried out in a centrifuge tube (15 by 105 mm) into which O_2-free CO_2 gas was jetted during manipulation. An anaerobic state was maintained by plugging the tubes with butyl rubber stoppers during incubation and centrifugation. These conditions also allowed good growth of such strict anaerobes as *Ruminococcus albus* (17, 26).

Cells of *F. varium* were grown in yeast extract medium at 37°C as the first step of the procedure. During early log phase (2 to 3 h of cultivation), penicillin was added to the culture broth at various concentrations and incubation was continued for various time periods. As the second step, the penicillin-treated cells were harvested by centrifugation at $2,000 \times g$ and suspended into 1 ml of the hypertonic solution containing glycerol (0.2 M), which can protect protoplasts from osmotic lysis. Based upon these results, >80% of log-phase cells were protoplasted by the preincubation of the cells with penicillin (200 U/ml) for 1 h and the incubation of the cell suspension with lysozyme (1 mg/ml) for 2 to 3 h at 37°C (Table 3). The concentrations of penicillin and lysozyme used in the present experiment are about 10 times higher than those used by Kaneko and Sakaguchi (9) to shorten the incubation time of cells to <10 h for fresh protoplast formation. In the case of *E. faecium*, a negligible number of cells was converted to protoplasts by the above procedure. Following the report of Metcalf and Deibel (13), early log-phase cells of *E. faecium* were incubated with lysozyme (1 mg/ml) following an addition of sodium chloride (0.08 M). However, immediate and extensive formation of protoplast was not observed in the present experiment. Protoplasts were formed with difficulty when NaCl (0.08 M) was added to the cell suspension of *E. faecium* before the addition of lysozyme. The addition of penicillin and glycine did not markedly influence the cocci either. Only with the simultaneous addition of lysozyme and NaCl to a cell suspension did effective formation of protoplasts occur, with a yield of about 80% after 2 h of incubation at 37°C. The ionic strength of the phosphate buffer (pH 7.0, 0.05 M)-NaCl (0.08 M) used in the present paper was 0.25, very close to that presented by Metcalf and Deibel (13) for their successful formation of protoplasts. However, the order of addition of lysozyme and NaCl to the cell suspension seems to be for unknown reasons very critical for the effective formation of *E. faecium* protoplasts. Phase-contrast microscopy showed that the *F. varium* protoplast was an ellipsoid with diameters of 2.5 and 2 μm, while the *E. faecium* protoplast was spherical with a diameter of 1 μm.

Regeneration of protoplasts. When an *F. varium* protoplast suspension was diluted with the hypertonic solution and overlaid on yeast extract-agar medium containing glycerol, most colonies appeared after 5 to 7 days of cultivation at 37°C. When the suspension was diluted with water and plated on yeast extract-agar medium, colonies formed after 2 to 3 days. These results suggest that protoplasted cells require a longer cultivation period to form visible colonies than that needed for lysis-resistant cells. This is reasonable since the protoplasted cells have to regenerate their cell wall before propagation. The frequencies of protoplast formation and regeneration of *F. varium* were about 9.0×10^{-1} to 9.9×10^{-1} and 2.3×10^{-1} to 2.8×10^{-1}, respectively (Table 3). In the case of *E. faecium*, the values were 8.0×10^{-1} to 9.9×10^{-1} and 1.9×10^{-1} to 2.2×10^{-1}, respectively.

The successful protoplast formation and regeneration in *F. varium* and *E. faecium* under strictly anaerobic conditions seem to be the first trial, though some *Enterococcus* species had been protoplasted (5, 13, 18, 24) under aerobic conditions. One problem for genetic studies on anaerobes is the difficulty in maintaining anaerobic conditions throughout various procedures. This could be solved by the modified Hungate method presented here, cultivating cells in a centrifuge tube which can easily be closed during incubation and centrifugation and opened to the jetting of O_2-free CO_2 during manipulation. Our experiments show that this simple technique is more effective than using an anaerobic box and is applicable to use with other anaerobes.

Usually, it is thought that protoplast formation of gram-negative organisms is more problematical due to the presence of outer membrane, and a lysozyme-EDTA method was developed successfully to protoplast the gram-negative ones (8, 20). However, lysozyme-EDTA did not affect *F. varium* cells from early log-phase (2 h) to stationary-phase

(12 h) cultures under anaerobic conditions. In contrast, it did cause a deep lysis of stationary-phase cells of *F. varium* within 30 min in the "open" or aerobic state, indicating that cell lysis in the anaerobic environments might be quite different and more complicated than that in aerobic conditions. Also, our experiments showed that early log-phase cells lysed more readily than mid-log-phase cells, which usually give the best protoplast yields in aerobic environments (20). The experiments with *E. facium* implied that lysis of cocci may be much more critical than that of rods and the optimal conditions may vary depending on the strains (5, 13, 18, 24). The critical factors for protoplast formation in *E. faecium* were the ionic conditions and growth phase of cells, as described in the text. The procedures for protoplast formation and regeneration of *F. varium* and *E. faecium* described in this report should enable genetic studies to be carried out in both organisms, using fusion and transformation techniques to enhance the degradation of lignin-related compounds under anaerobic conditions.

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