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Characterization of Fusants between Thermophilic *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*

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고온성 *Clostridium thermocellum*과 *Clostridium thermohydrosulfuricum*의 융합체의 특성

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ABSTRACT: Intraspecific and interspecific protoplast fusions in/between *C. thermocellum* and *C. thermohydrosulfuricum* were studied. Protoplast fusions were well induced in 30-40% PEG solution, however, their fusion frequencies were low level of 1.2×10^{-7} for intraspecific fusion of *C. thermocellum*, 6.7×10^{-7} for *C. thermohydrosulfuricum*, and 4.2×10^{-7} for interspecific fusion between above two *Clostridia*, respectively. Most fusants were unstable and segregated after 3 subcultures. Relatively stable intraspecific *C. thermocellum* fusant FTT17, intraspecific *C. thermohydrosulfuricum* fusant FSS22 and interspecific fusant FTS3, which were stable after several subcultures, were selected and properties of fusants were further investigated. Phenotypes of the fusants were similar with wild types mostly in cellular morphology, carbon source assimilation and enzyme activities. However they were differed in assimilation of pyruvic acid and sorbitol as carbon source. The DNA contents of fusants were slightly increased compared with wild types. Ethanol production by intraspecific and/or interspecific fusants was not increased, however, acetic acid production as byproduct was decreased or not detected, which indicates that industrial thermophilic anaerobes can be improved by means of protoplast fusion of two strains.

KEY WORDS □ *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum*, **protoplast fusion, fusant.**

The thermophilic anaerobic *Clostridium thermocellum* has received considerable attention for its ability of direct conversion of cellulosic biomass to ethanol (Kim *et al.*, 1987; Lee *et al.*, 1987). However, the low yield of ethanol has confined the usability of above strains for practical process for ethanol production. Coculture of *C. thermocellum* with *Clostridium thermohydrosulfuricum*, that has higher ethanol productivity but no utilizability of cellulosics, can substantially increase ethanol yield from cellulosic biomass by the synergistic action of both microorganims (Ng *et al.*, 1981). Genetic manipulation, such as the interspecific protoplast fusion of above two strains, may improve the direct fermentation system (Lee *et al.*, 1988), which would overcome the technical difficulty of coculture of above two strains.

Protoplast fusion has been extensively used as the gene manipulation technique for the improvement of strain along with the gene cloning technique. However the studies on protoplast fusion and transformation systems related with anaerobes were rarely carried out (Allcock *et al.*, 1982; Heefner *et al.*, 1984;

Minton *et al.*, 1983; Yoshino *et al.*, 1984). Recently Chen *et al.* (1987) reported intergeneric protoplast fusion of rumen anaerobes, *Fusobacterium varium* and *Enterococcus faecium*. Furthermore the transformation systems or fusion system of thermophilic anaerobic *Clostridia* have not been investigated. This may be due to the difficulty of manipulation of the cells which require the obligate anaerobic growth conditions and the lack of fundamental studies on thermophilic anaerobes.

In this work, the possibilities of strain improvement and transfer of genetic materials through protoplast fusion in genus thermophilic *Clostridia* have been investigated. Induction of autolysis and autoplast formation of anaerobic *C. thermohydrosulfuricum* (Kim *et al.*, 1989) and protoplast formation and regeneration of thermophilic *C. thermocellum* and *C. thermohydrosulfuricum* (Kim *et al.*, 1990) were investigated in our previous work. In this work, intraspecific protoplast fusion in *C. thermocellum* and in *C. thermohydrosulfuricum* and interspecific protoplast fusion between *C. thermocellum* and *C. thermohydrosulfuricum* were performed, and characteristics of the fusants were investigated.

MATERIALS AND METHODS

Strains and media

Clostridium thermocellum ATCC 27405 and *Clostridium thermohydrosulfuricum* ATCC 33223 were used, and properties of antibiotic resistant mutants are described in Table 1. The media used were same as described in our previous work (Kim *et al.*, 1990).

Table 1. *Strains used*

Strains	Properties	Reference
C. thermocellum ATCC 27405		
CTRF 7	rif^r em^s	NTG mutant
CTEM 2	rif^s em^r	NTG mutant
C. thermohydrosulfuricum ATCC 33223		
CSRF 1	rif^r em^s	NTG mutant
CSEM 3	rif^s em^r	NTG mutant

rif; rifampicin, em; erythromycin

Mutant selection

Antibiotic resistant mutants were selected to be used as selection marker for protoplast fusion by treating with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as follows; 5 m*l* of culture broth in exponential growth phase was condensed by centrifugation (3,000×g, 20 min), and the separated cells were washed twice with 0.1 M Tris-HCl buffer (pH 7.5), thereafter, treated with 100 μg/m*l* of NTG for 30 min. The NTG-treated cells were washed with the same buffer to eliminate residual mutagen, and then cultivated at 60°C for 4 days in CM3 agar medium containing 0.1 μg/m*l* of rifampicin or 1 μg/m*l* of erythromycin. Rifampicin-resistant and erythromycin-sensitive mutant was selected from the colony which can grow in rifampicin-containing medium but not in erythromycin-containing medium. On the other hand, the colony which can grow in erythromycin-containing medium but not in rifampicin-containing medium was selected as erythromycin-resistant and rifampicin-sensitive mutant.

Protoplast formation and regeneration

The procedures of protoplast formation and regeneration of *C. thermocellum* and *C. thermohydrosulfuricum* were same as described in our previous work (Kim *et al.*, 1990).

Protoplast fusion

3 m*l* of sample from each protoplast suspension was gently mixed in a sterile tube under oxygen-free nitrogen atmosphere. The tube was then plugged with rubber stopper, and centrifuged at 3,000×g for 10 min. The protoplasts were resuspended in 0.2 m*l* of TMG buffer (100 mM Tris-HCl, 20mM $MgCl_2$, 0.5 M glycerol, pH 7.5). And then, 0 - 0.8 m*l* of 60% PEG-4000 solution in the same buffer was gently added under the mild shaking for homogenization. Protoplast aggregation and fusion in PEG solution was observed under a phase-contrast microscope. After 5 - 20 min treatment in PEG solution at room temperature, the protoplast suspension was diluted 100 folds with TMG buffer. The dilute suspension was directly inoculated on the regeneration medium containing 0.2 μg/m*l* of rifampicin and 50 μg/m*l* of erythromycin, and incubated for 7 - 10 days at 60°C. The colony grown above medium was directly selected as fusant.

Ethanol and acetic acid analysis

The amount of ethanol and acetic acid produced in medium were analysed by the gas-liquid chromatography (GLC). Before injection of the sample into GLC, the centrifuged supernatant was mixed with 1 M phosphoric acid at the ratio of 9:1, and 0.5% of isopropanol as final concentration for internal standard material. 5 μ*l* of sample was injected into GLC. The GLC (Pye Unicam series 304) was operated at following conditions; stainless steel column (2 mm×2.7 m) packed with Porapak Q (100–200

mesh), FID detecter, column temperature 180 °C, injector temperature 230 °C, detector temperature 250 °C, carrier gas (N_2) flow rate 30 m*l*/min, hydrogen flow rate 33 m*l*/min, air flow rate 330 m*l*/min, chart speed 2.5 mm/min, and Pye Unicam PU 4810 integrator were used.

Determination of DNA content

DNA content was determined by the method using diphenylamine (Giles and Meyers, 1965). Salmon sperm DNA (Sigma Co.) was used as DNA standard material. The cells grown for 3 days were lysed in lysis solution which contained 1.5 mg/m*l* of pronase E (Sigma Co.) and 0.1% SDS in TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8.0).

Determination of enzyme activities

CMCase activity was determined by the method of Mandels *et al.* (1976). β-Amylase activity was assayed by measuring the reducing sugars which were liberated during reaction on soluble starch. The reaction mixture consists of 2 m*l* of 1% soluble starch dissolved in 0.1 M sodium acetate buffer (pH 6.0) and 0.5 m*l* of enzyme solution. After incubation at 60 °C for 1 hour, the released reducing sugar was measured by the dinitrosalycilic acid method (Miller, 1959). Glucoamylase activity was measured after incubating the reaction mixture contained 2 m*l* of 1% soluble starch in 0.2 M sodium acetate buffer (pH 4.8) and 0.5 m*l* of enzyme solution at 60 °C for 1 hour. The released glucose was assayed by enzymatic PGO method (Sigma Co., 1990). And protease activity was measured by Folin-Ciocalteu method (Colowick and Kaplan, 1957).

RESULTS AND DISCUSSION

Selection of antibiotic resistant mutants

Both wild type strains, *C. thermocellum* and *C. thermohydrosulfuricum,* showed resistance to streptomycin (1 mg/m*l*), ampicillin (0.5 mg/m*l*), and nalidixic acid (1 mg/m*l*). However, they were sensitive to rifampicin at the concentration of 0.05 μg/m*l*, and to erythromycin at the concentration of 1 μg/m*l*. By the treatment of NTG, three rifampicin-resistant and erythromycin-sensitive mutants (CTRF2, CTRF5 and CTRF7) were obtained from *C. thermocellum.* On the other hand, three rifampicin-resistant and erythromycin-sensitive mutants (CSRF1, CSRF2 and CSRF3) were obtained from *C. thermohydrosulfuricum.* And also, one erythromycin-resistant and rifampicin-sensitive mutant (CTEM2) form *C. thermocellum* and four erythromycin-resistant and rifampicin-sensitive mutants (CSEM1, CSEM2, CSEM3, and CSEM4) from *C. thermohydrosulfuricum* were obtained. The mutants (CTRF7, CTEM2, CSRF1 and CSEM3), which showed the most steady resistance against rifampicin or erythromycin, were used for protoplast fusion.

Effect of various fusogen on protoplast fusion

Table 2 shows the effect of polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP) and calcium chloride as fusogen on intraspecific and interspecific protoplast fusion in/between *C. thermocellum* and *C. thermohydrosulfuricum,* in which protoplast fusion was induced for 5 min at different concentration of fusogen. Consequently, PEG was identified to be the most preferable fusogen among them. Intraspecific protoplast fusion between *C. thermocellum* mutants (CTRF7 and CTEM2) was most well induced in 40% PEG solution. On the other hand, intraspecific protoplast fusion between *C. thermohydrosulfuricum* mutants (CSRF1 and CSEM3) was maximally induced in 30% PEG solution. And interspecific protoplast fusion between *C. thermocellum* CTRF7 and *C. thermohydrosulfuricum* CSEM3 was also most well induced in 30% - 40% PEG solution. Whilst the mixture of PEG and calcium chloride, calcium chloride, and PVP were found to be not efficient as fusogen. Moreover, intraspecific protoplast fusion between Gram negative *C. thermocellum* mutants (CTRF7 and CTEM2) was hardly induced in above fusogens except PEG.

Fusion frequency

Fusion frequencies of intraspecific and interspecific protoplast fusions are shown in Table 3. The intraspecific fusion frequencies between *C. thermocellum* CTRF7 and CTEM2, and between *C. thermohydrosulfuricum* CSRF1 and CSEM3 were about 1.2×10^{-7} and 6.7×10^{-7}, respectively. And interspecific fusion frequency between *C. thermocellum* CTRF7 and *C. thermohydrosulfuricum* CSEM3 was about 4.2×10^{-7}. Above frequencies are much low compared with those frequency between anaerobic *F. varium* and *E. faecium* with 0.9 -1.3×10^{-5} (Chen *et al.*, 1987).

Intraspecific protoplast fusion frequency between Gram negative *C. thermocellum* CTRF7 and CTEM2 was appeared to be lower than those of Gram positive *C. thermohydrosulfuricum* CSRF1 and CSEM3, which may be due to the structural difference of cell wall between Gram positive and negative *Clostridia.* The low fusion frequencies of above strains are also considered due to the decrease of regeneration frequency to about 100 folds with the treatment of high

Table 2. *Effect of fusogens on intraspecific and interspecific protoplast fusion*

Fusogens	Fusion frequency ($\times 10^8$)		
	Intraspecific CTRF7 × CTEM2	Intraspecific CSRF1 × CSEM3	Interspecific CTRF7 × CSEM3
PEG 4000 0%	—	—	—
10%	—	—	2
20%	1	2	7
30%	1	73	12
40%	3	26	12
50%	—	4	—
PEG 40% + 100 mM $CaCl_2$	—	2	1
$CaCl_2$ 1M	—	5	5
PVP-40 30%	—	—	—

Table 3. *Frequencies of intraspecific and interspecific protoplast fusion after treatment of 40% of PEG 4000 for 20 min.*

Strains	No. of regenerated colonies (a)	No. of fusants (b)	Fusion frequency (b/a)
Intraspecific			
CTRF7 × CTEM2	1.0×10^7	1.2	1.2×10^{-7}
CSRF1 × CSEM3	6.4×10^7	43.0	6.7×10^{-7}
Interspecific			
CTRF7 × CSEM3	3.1×10^6	1.3	4.2×10^{-7}

concentration of PEG, and due to the selection method of fusant in which only fusant having both rifampicin and erythromycin resistances was able to be selected.

Genetic stability of the selected fusants

18 strains of intraspecific fusants between *C. thermocellum* CTRF7 and CTEM2 (from FTT1 to FTT18), 35 strains of intraspecific fusants between *C. thermohydrosulfuricum* CSRF1 and CSEM3 (from FSS1 to FSS35) and 22 strains of interspecific fusants between *C. thermocellum* CTRF7 and *C. thermohydrosulfuricum* CSEM3 (from FTS1 to FTS22) were selected. Among them, 96% of intraspecific fusants of *C. thermocellum*, 86% of intraspecific fusants of *C. thermohydrosulfuricum*, and 90% of interspecific fusants of *C. thermocellum* and *C. thermohydrosulfuricum* were segregated after three subcultures, and then did not grow in the medium containing 0.2 μg/ml of rifampicin and 50 μg/ml of erythromycin. Among them, the most stable after five to seven subcultures (FTT17, FSS22 and FTS3) were further investigated to survey properties of the fusants.

Morphology and carbon source assimilation

All of the fusants were rod form and spore formation was observed under microscopic observation. According to Gram staining, the intraspecific *C. thermocellum* fusant FTT17 and interspecific fusant FTS3 are Gram negative like that of wild type of *C. thermocellum*, and intraspecific *C. thermohydrosulfuricum* fusant FSS22 is Gram positive like that of wild type of *C. thermohydrosulfuricum* as shown in Table 4. On the other hand, the tests of carbon source assimilation showed different pattern in ability of assimilation of sorbitol and pyruvic acid as carbon source, in which intraspecific *C. thermocellum* fusant FTT17 had the ability of assimilation of sorbitol, but intraspecific *C. thermohydrosulfuricum* fusant FSS22 and interspecific fusants FTS3 had not. On the other hand, FTT17 and FTS3 utilized pyruvic acid differ with FSS22. In comparison, the wild types of *C. thermocellum* and *C. thermohydrosulfuricum* possess the ability of utilization of sorbitol and pyruvic acid as carbon source. Only *C. thermocellum* utilized cellulose and produced characteristic yellow pigment in culture broth (Lee *et al.*, 1987), however, the fusants did not utilize cellulose and not produce pigment. Assimilation abilities of another carbon sources, including glucose, xylose, maltose, cellobiose and sucrose, did not show any significant difference in each others.

DNA content

As shown in Table 5, DNA contents of wild types

Table 4. *Characteristics of fusants*

Characteristics	*C. thermocellum*	*C. thermohydrosulfuricum*	FTT17	FSS22	FTS3
Morphology	rods	rods	rods	rods	rods
Gram stain	–	+	–	+	–
Spore formation	+	+	+	+	+
Assimilation of;					
glycerol	–	–	–	–	–
sorbitol	+	+	+	–	–
pyruvic acid	+	+	–	+	–
glucose	+	+	+	+	+
xylose	+	+	+	+	+
maltose	+	+	+	+	+
cellobiose	+	+	+	+	+
sucrose	+	+	+	+	+
cellulose	+	–	–	–	–
Pigment	yellow	–	–	–	–

FTT; CTRF7×CTEM2, FSS; CSRF1×CSEM3, FTS; CTRF7×CSEM3.

Table 5. *DNA contents in cells of fusants*

Strains	Centent of DNA (mg/g of cell)
C. thermocellum	7.68
C. thermohydrosulfuricum	7.50
FTT 17	9.78
FSS 22	11.11
FTS 3	11.20

of *C. thermocellum* and *C. thermohydrosulfuricum* were measured to be 7.68 mg/g of the cell and 7.50 mg/g of the cell, respectively. And DNA contents of the fusants FTT17, FSS22 and FTS3 were measured to be 9.78 mg/g of the cell, 11.11 mg/g of the cell and 11.20 mg/g of the cell, respectively. DNA contents of fusants were increased in comparison with those of two wild types, which confirms that the fusants were really fused between two strains each other. The increase of DNA content of fusant was reported in other studies on protoplast fusion between *S. diastaticus* and *C. tropicalis* (Seu *et al.*, 1986) and on protoplast fusion between *H. anomala* var. *anomala* and *S. cerevisiae* (Chung *et al.*, 1987).

Enzyme activities of fusants

Phenotypic properties of three fusants on various enzyme activities are shown in Table 6. CMCase activity was expressed in intraspecific *C. thermocellum* fusant FTT17 and interspecific fusant FTS3 like wild type of *C. thermocellum*, and β-amylase and glucoamylase activities were expressed in intraspecific *C. thermohydrosulfuricum* fusant FSS22 and interspecific fusant FTS3 like wild type of *C. thermohydrosulfuricum*. These results indicate that genetic imformations of both *C. thermocellum* and *C. thermohydro-*

Table 6. *Enzyme activities in culture broth produced from fusants*

Strains	CMCase (mU/m*l*)	β-amylase (mU/m*l*)	Glucoamylase (mU/m*l*)	Protease (U/m*l*)
C. thermocellum	96.2	—	—	—
C. thermohydrosulfuricum	—	31.5	1.5	9.3
FTT 17	50.8	—	—	—
FSS 22	—	34.0	3.9	—
FTS 3	48.1	27.8	3.9	—

Table 7. *Concentration of ethanol and acetic acid produced from fusants*

Strains	Ethanol (%)	Acetic acid (%)
C. thermocellum	0.150	0.080
C. thermohydrosulfuricum	0.125	0.090
FTT 17	0.140	—
FSS 22	0.130	0.065
FTS 3	0.125	—

sulfuricum were introduced into interspecific protoplast fusant FTS3. On the other hand, Protease activity was shown in only *C. thermohydrosulfuricum*, but not in three fusants.

Ethanol and acetic acid production abilities

As shown in Table 7, The production abilities of ethanol of the fusants were similar to those of wild types of *C. thermocellum* and *C. thermohydrosulfuricum*, however, the intraspecific *C. thermocellum* fusant FTT17 and interspecific fusant FTS3 except intraspecific *C. thermohydrosulfuricum* fusant FSS22 did not show the ability of acetic acid production. Even FSS22 showed decreased production of acetic acid compared with wild type of *C. thermohydrosulfuricum*. These results indicate that the fusants may have considerable advantage of ethanol production preventing accumulation of undesirable acetic acid as byproduct. The usuable thermophilic anaerobic microorganism seems to be developed by selecting procedure for obtaining improved multifunctional fusants from two or three distinct strains.

적 요

고온 혐기성 *Clostridium thermocellum* 및 *Clostridium thermohydrosulfuricum*의 동종간 및 이종간 원형질체 융합을 시도하였다. 융합제로서 30-40% PEG-4000을 사용할 때 융합이 비교적 잘 되었으나, *C. thermocellum* 동종간 융합빈도는 1.2×10^{-7}, *C. thermohydrosulfuricum* 동종간 융합빈도는 6.7×10^{-7}, 두 균주 이종간 융합빈도는 4.2×10^{-7}으로 저조하였다. 대부분의 융합체는 불안정하여 3회 계대배양 후에는 segregation되었다. 수회의 계대배양 후에도 비교적 안정했던 동종간 융합체인 FTT17과 FSS22, 이종간 융합체인 FTS3을 선별하여 그 특성을 조사한 결과 세균의 형태와 탄소원 자화능, 효소활성 등은 대체로 원래의 야생주와 같았으나, sorbitol과 pyruvic acid의 자화능에 차이가 있었다. DNA 함량은 야생주에 비하여 증가되었다. 또한 에탄올 생산능은 큰 차이가 없었으나 부산물로서 초산생산능은 감소되거나 측정되지 않은 것으로 볼 때 원형질체 융합에 의한 고온 혐기성 직접발효균주의 개발의 가능성이 확인되었다.

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