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Natural Competence in *Thermoanaerobacter* and *Thermoanaerobacterium* Species▽†

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Low-G+C thermophilic obligate anaerobes in the class *Clostridia* are considered among the bacteria most resistant to genetic engineering due to the difficulty of introducing foreign DNA, thus limiting the ability to study and exploit their native hydrolytic and fermentative capabilities. Here, we report evidence of natural genetic competence in 13 *Thermoanaerobacter* and *Thermoanaerobacterium* strains previously believed to be difficult to transform or genetically recalcitrant. In *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, natural competence-mediated DNA incorporation occurs during the exponential growth phase with both replicating plasmid and homologous recombination-based integration, and circular or linear DNA. In *T. saccharolyticum*, disruptions of genes similar to *comEA*, *comEC*, and a type IV pilus (T4P) gene operon result in strains unable to incorporate further DNA, suggesting that natural competence occurs via a conserved Gram-positive mechanism. The relative ease of employing natural competence for gene transfer should foster genetic engineering in these industrially relevant organisms, and understanding the mechanisms underlying natural competence may be useful in increasing the applicability of genetic tools to difficult-to-transform organisms.

The genera *Thermoanaerobacter* and *Thermoanaerobacterium* contain bacteria which are thermophilic, obligate anaerobes that specialize in polysaccharide and carbohydrate fermentation, producing primarily L-lactic acid, acetic acid, ethanol, CO_2, and H_2 (24, 27, 49). Taxonomically, they are distinguished from other anaerobic thermophilic clostridia by the ability to reduce thiosulfate to hydrogen sulfide or elemental sulfur (21). The majority of characterized *Thermoanaerobacter* and *Thermoanaerobacterium* strains have been isolated from hot springs and other thermal environments (20–22, 38, 47); however, they have also been isolated from canned foods (4, 10), soil (48), paper mills and breweries (41, 43), and deep subsurface environments (5, 13, 35), suggesting a somewhat ubiquitous environmental presence.

Representatives of the *Thermoanaerobacter* and *Thermoanaerobacterium* genera have been considered for biotechnological applications, such as conversion of lignocellulosic biomass to ethanol (8, 27) or other fuels and chemicals (3, 24). However, the branched fermentation pathways of these organisms generally require modification for industrial application. Several studies have investigated manipulating bioprocess and growth conditions to alter end product ratios and yields, but this has not resulted in reliable conditions to maximize the yield of a single end product (18, 25). Genetic engineering is likely necessary for commercial application of *Thermanaerobacter* or *Thermoanaerobacterium* species (26, 27, 44). As genetic systems for these bacteria have emerged (28, 45), increased product yields have been demonstrated by gene knockout of L-lactate dehydrogenase (9, 14), phosphotransacetylase and acetate kinase (40), and hydrogenase (39). Despite this recent progress, genetic transformation is still considered the greatest barrier for engineering these organisms (44).

In contrast, some of the bacteria most amenable to genetic manipulation are those exhibiting natural competence; for example, work with the naturally competent *Streptococcus pneumoniae* first established DNA as the molecule containing inheritable information (42). Naturally competent organisms are found in many bacterial phyla, although the overall number of bacteria known to be naturally competent is relatively small (16).

The molecular mechanisms of natural competence are often divided into two stages: early-stage genes that encode regulatory and signal cascades to control competence induction, and late-stage genes that encode the machinery of DNA uptake and integration (16). The Gram-positive late-stage consensus mechanism for DNA uptake and assimilation, elucidated primarily through work with *Bacillus subtilis*, occurs through several molecular machinery steps. First, DNA is believed to interact with a type IV pilus (T4P) or pseudopilus that brings it into close proximity of the cell membrane. The precise mechanism of this phenomenon is unclear; although components of the T4P in both Gram-positive and Gram-negative bacteria have been shown to bind DNA (7, 19), in specific studies, a full pilus structure has been either not observed or shown not to be essential during natural competence (6, 36). Two proteins, ComEA and ComEC, are then involved in creation and transport of single-stranded DNA across the membrane, where it is subsequently bound by CinA-localized RecA and either integrated into the genome or replicated at an independent origin, as for plasmid DNA (6).

Here, we report that several *Thermoanaerobacter* and *Thermoanaerobacterium* strains are naturally competent, characterize growth conditions conducive to natural competence, and identify genes in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 required for competence exhibition.

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TABLE 1. Plasmids and strains used in this study

Plasmid/strain	Description[a]	Source/reference
pMU131	*T. saccharolyticum-E. coli* shuttle plasmid, Kan^r Amp^r	PCT/US2008/010545
pMQ87	Cloning plasmid for yeast homologous recombination, Gen^r, *ura*3	Presque Isle culture collection
pSGD8	L-*ldh* knockout plasmid with Kan^r Amp^r	24
pMU1966	*T. saccharolyticum* T4P knockout vector, Ery^r Gen^r, *ura*3	This study
pMU1967	*T. saccharolyticum comEA* knockout vector Ery^r Gen^r, *ura*3	This study
pMU1968	*T. saccharolyticum comEC* knockout vector Ery^r Gen^r, *ura*3	This study
pMU1969	*T. saccharolyticum recA* knockout vector Ery^r Gen^r, *ura*3	This study
TOP10	*E. coli* cloning strain	Invitrogen
DSM 8691	*Thermoanaerobacterium saccharolyticum* JW/SL-YS485	DSMZ
ALK2	*T. saccharolyticum* YS485 ΔL-*ldh* Δ*pta* Δ*ack*, Kan^r Ery^r	27
M1464	*T. saccharolyticum* YS485 Δ*tfp*, Ery^r	This study
M1465	*T. saccharolyticum* YS485 Δ*recA*, Ery^r	This study
M1466	*T. saccharolyticum* YS485 Δ*comEC*, Ery^r	This study
M1467	*T. saccharolyticum* YS485 Δ*comEA*, Ery^r	This study
ATCC 27405	*Clostridium thermocellum*	Lynd lab
DSM 8903	*Caldicellulosiruptor saccharolyticus*	DSMZ
ATCC 35047	*Thermoanaerobacter brockii*	ATCC
DSM 2246	*Thermoanaerobacter ethanolicus* JW200	DSMZ
DSM 11426	*Thermoanaerobacter mathranii*	DSMZ
ATCC 33223	*Thermoanaerobacter pseudethanolicus* 39E	ATCC
DSM 10170	*Thermoanaerobacterium aotearoense*	DSMZ
B6A	*Thermoanaerobacterium saccharolyticum* B6A	Paul Weimer
ATCC 49915	*Thermoanaerobacterium saccharolyticum* B6A-RI	ATCC
ATCC 7956	*Thermoanaerobacterium thermosaccharolyticum*	ATCC
ATCC 31960	*Thermoanaerobacterium thermosaccharolyticum* HG-8	ATCC
M0523	*Thermoanaerobacterium thermosaccharolyticum*	Mascoma
M0524	*Thermoanaerobacterium thermosaccharolyticum*	Mascoma
M0795	*Thermoanaerobacterium thermosaccharolyticum*	Mascoma
DSM 7097	*Thermoanaerobacterium xylanolyticum*	DSMZ
DSM 13642	*Thermoanaerobacterium zeae*	DSMZ

[a] Kan^r, kanamycin resistance; Amp^r, ampicillin resistance; Ery^r, erythromycin resistance; Gen^r, gentamicin resistance.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this study are listed in Table 1. The replicating shuttle plasmid pMU131 contains a thermostable kanamycin resistance (Kan^r) marker (28), the pUC origin of replication and ampicillin resistance marker, and a thermostable Gram-positive origin of replication isolated from a native plasmid of *Thermoanaerobacterium saccharolyticum* B6A-RI (N. Caiazza, A. Warner, and C. Herring, 19 March 2009, international patent application no. PCT/US2008/010545; N. Caiazza, C. Rice, A. Warner, D. Hogsett, and C. Herring, unpublished data; see Weimer et al. [47] for an earlier description of these native plasmids).

Media and growth conditions. All culturing of thermophilic bacteria was performed in modified DSMZ medium 122, containing per liter 5.0 g cellobiose, 1.3 g $(NH_4)_2SO_4$, 2.6 g $MgCl_2$ 6 · H_2O, 1.43 g KH_2PO_4, 1.8 g K_2HPO_4, 0.13 g$CaCl_2$ 2 · H_2O, 6.0 g Na-β-glycerophosphate, 0.00013 g $FeSO_4$ 7 · H_2O, 4.5 g yeast extract, 0.002 g resazurin, 0.5 g L-cysteine-HCl, and 10 g agarose for solid media. The pH was adjusted to 6.7 with 10 N NaOH or 72% (wt/vol) H_2SO_4 if necessary. Chemicals were obtained from Sigma-Aldrich, and yeast extract was obtained from BD Difco. Cultures were grown at 55°C, unless otherwise noted, in an anaerobic chamber (COY Labs, Grass Lake, MI). For selection of erythromycin-resistant colonies of *T. saccharolyticum*, a medium pH of 6.1 and an incubation temperature of 50°C were used. *Escherichia coli* was grown in LB medium with kanamycin at 50 μg/ml or gentamicin at 25 μg/ml for plasmid selection and maintenance. *Saccharomyces cerevisiae* was grown on solid CM minus uracil media for plasmid selection (37). Stock cultures of thermophilic strains were prepared from cultures grown to exponential or early stationary phase by the addition of 5% dimethyl sulfoxide (DMSO) and frozen at −80°C.

Natural genetic competence. Natural competence transformations were conducted in an anaerobic chamber by inoculation of 10 ml of medium with 1 to 3 μl of a frozen stock culture. After mixing, 1-ml aliquots were transferred to tubes containing 250 ng DNA suspended in 10 mM Tris buffer, pH 8.0, at a concentration of approximately 50 ng/μl. pMU131 plasmid DNA prepared in *E. coli* TOP10 (Invitrogen, Madison, WI) was used for natural competence tests of different species. Different types of DNA used to transform *T. saccharolyticum* were prepared as described in Results. The tubes were then incubated at 55°C for 16 to 18 h to an optical density at 600 nm (OD_{600}) of 0.6 to 1.0. Dilutions of the transformation culture were mixed with liquid agar at 55°C containing the appropriate antibiotic concentration, poured into petri dishes, allowed to solidify at room temperature, and incubated at 55°C in a moisture-retaining container until colony formation. Negative controls were performed by the exclusion of DNA. Putative transformants were tested for the presence of the kanamycin marker via PCR with primers X00860 and X00861, and 16S sequencing was performed to confirm culture identity with primers X00050 and X00051 (Table 2).

Transformation frequency during batch growth. Exponentially growing *T. saccharolyticum* cells were diluted to an OD_{600} of 0.03 in fresh medium, and each hour 1-ml subcultures were mixed with 250 ng pMU131 DNA and grown under the same conditions as the main culture. After an hour of incubation, 2 units of DNase (New England Biolabs, Ipswich, MA) was added to the subcultures to hydrolyze free DNA, and the mixture was incubated for an additional hour to allow expression of the kanamycin resistance marker. Subcultures were then diluted and plated in nonselective and kanamycin-containing solid media to determine the transformation frequency.

Plasmid and knockout strain construction. Plasmids were constructed by *S. cerevisiae*-based *in vivo* recombination cloning (37) using the *S. cerevisiae-E. coli* shuttle plasmid pMQ87. Knockout plasmids were isolated by miniprep (Qiagen, Germantown, MD) in *E. coli* TOP10 cells prior to transformation in *T. saccharolyticum*. Primers used to construct knockout plasmids are shown on plasmid maps in Fig. S1 in the supplemental material and the primer sequences in Table 2.

Nucleotide sequence accession number. The sequences reported in this paper have been deposited in the GenBank database (accession no. GU479453 [T4P region], GU479454 [*comEA* region], GU479455 [*comEC* region], and GU479456 [*cinA recA* region]).

RESULTS

Determination of natural competence. To transform *T. saccharolyticum* JW/SL-YS485, we previously used a hybrid chemical-electrotransformation protocol (28) that includes incubation with isonicotinic acid hydrazide to weaken cell walls, cell

TABLE 2. Primers used in this study

Primer no.	5′–3′ sequence
X00004	GGGTTTATCGACCTTGGTTCGTGACATTGTGGGC
X00021	TGCTGCTTCTGTTCTTGACC
X00050	AGAGTTTGATCCTGGCTCAG
X00051	ACGGCTACCTTGTTACGACTT
X00861	ACCACCTATGATGTGGAACGGGAA
X00862	TTTCTCCCAATCAGGCTTGATCCC
X00957	GGGCATTTAACGACGAAACTGGCT
X00958	ACATCTGTGGTATGGCGGGTAAGT
X01177	GCTCATGAACCCAAAGTTGCAAAGC
X01178	CCCTCCTGCATTGCCTACAAAGTA
X08154	TGCTGTCAAGAGCTGTGTCCTCAT
X08155	AACTTCACTTCGCCAGCAGTTGTC
X08160	TTGATGGCACTTTGCTCCCTGTTG
X08161	CAGCCACACTAAATCCTGGGACAA
X08268	CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGGAGTCTTTCGCAATAAGAGGCAAC
X08151	GGTTTATCGACCTGCAACCCAGTCAATAATGAAGCTACTATCAA
X08269	TTGATAGTAGCTTCATTATTGACTGGGTTGCAGGTCGATAAACC
X08270	AGAGCCGCTGGATTTATCGTTGGATTAGTAACGTGTAACTTTCC
X08152	GGAAAGTTACACGTTACTAATCCAACGATAAATCCAGCGGCTCT
X08271	GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGCCGATACCGAATCAACCTGGA
X08272	CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGCAATTCTTGGCTCACATGGGCCTT
X08157	GGTTTATCGACCTGCATTTCTCCCACCGTCAATCCCAAGA
X08273	TCTTGGGATTGACGGTGGGAGAAATGCAGGTCGATAAACC
X08274	ACTACTTCTCCATCTGGCTGTCCATTAGTAACGTGTAACTTTCC
X08158	GGAAAGTTACACGTTACTAATGGACAGCCAGATGGAGAAGTAGT
X08275	GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCCCGAAACTGTCGTGCAATCATGGA
X08721	TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGATGAAACTGCTGTTGTTGGCGACC
X08722	GGTTTATCGACCTGCATAAACCCGACAATGATGCCGGTTG
X08723	CAACCGGCATCATTGTCGGGTTTATGCAGGTCGATAAACC
X08724	CAGGACTCTGCGATTGATTATCGGTTAGTAACGTGTAACTTTCC
X08725	GGAAAGTTACACGTTACTAACCGATAATCAATCGCAGAGTCCTG
X08726	CGGATAACAATTTCACACAGGAAACAGCTATGACCTTCCAGCTCCAATTGCACCAGATG
X08727	ATATGGCCTCTTAAATGGCGGTGC
X08728	TGCCAGAGCCACCAGCAATTTCAA
X08729	TTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGCGCGGCCAGCAATCTTGGTAAATA
X08730	GGTTTATCGACCTGCAAAATCCATTCCCAACAAGCGGAGC
X08731	GCTCCGCTTGTTGGGAATGGATTTTGCAGGTCGATAAACC
X08732	TGCCCAAGCCTTATGTCGCCATATTTAGTAACGTGTAACTTTCC
X08733	GGAAAGTTACACGTTACTAAATATGGCGACATAAGGCTTGGGCA
X08734	GTGAGCGGATAACAATTTCACACAGGAAACAGCTACGGGCATAATTTGTGAGCCATCCA
X08735	TTTCCGGGAGAGACAGAGGATGAA
X08736	TACTGCAGTTTACTGGGTCTTGTGGG

harvesting, washing, electropulsing, and an outgrowth period in fresh media prior to plating with a selective antibiotic. This protocol first came into question when a no-pulse control experiment yielded more transformants than one which included an electrical pulse. It was subsequently determined that the only essential step of the protocol was the cell outgrowth period, leading us to conclude that *T. saccharolyticum* JW/SL-YS485 is naturally competent.

Transformation with different DNA types. *T. saccharolyticum* was transformable by replicating plasmid and homologous recombination-based chromosomal integration vectors (Table 3), and like other naturally competent organisms, can be transformed with genomic DNA containing a selectable genotype (17, 23). pSGD8 (9; see also Fig. S1 in the supplemental material), a nonreplicating knockout vector containing 1.2 kb of upstream homology and 0.4 kb of downstream homology to the L-*ldh* locus, transformed *T. saccharolyticum* as circular DNA and after an AclI/EcoRI digestion that created a linear fragment. The linear digested plasmid was confirmed by agarose gel analysis to contain a fragment with the kanamycin resistance marker and flanking homology regions. Evidence of genome integration after transformation was determined by PCR (Fig. 1).

Natural competence occurs during exponential growth. No obvious induction event was required to bring *T. saccharolyti-*

TABLE 3. Transformation efficiency of *T. saccharolyticum* JW/SL-YS485 with different DNA types[a]

DNA type	Transformed cells per μg DNA	Transformed cells per μg Kan gene DNA
pMU131	2.5E+05	1.2E+06
ALK2 gDNA	2.0E+02	4.6E+05[b]
pSGD8	5.1E+04	2.2E+05
pSGD8 AclI/EcoRI[c]	5.7E+03	2.4E+04

[a] Transformation efficiency as a function of total DNA and of DNA encoding the kanamycin resistance marker.
[b] Estimate based on a genome size of 3.0 Mb.
[c] Plasmid digested to produce a linear DNA fragment containing the kanamycin resistance gene and flanking regions with homology to the L-*ldh* locus.

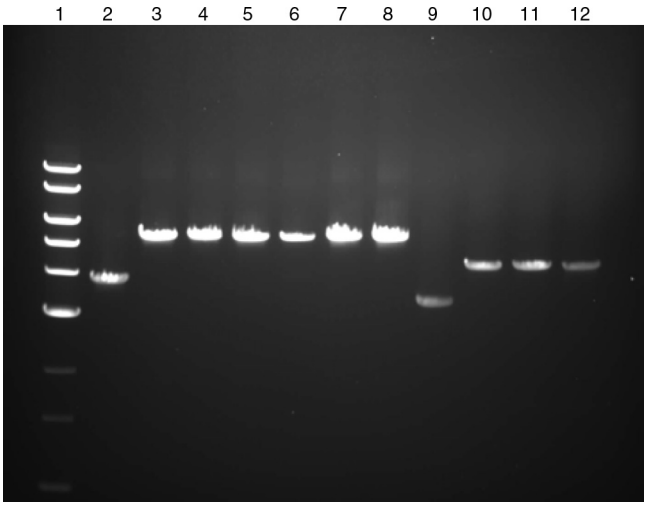

FIG. 1. PCRs used to confirm kanamycin marker integration in the *T. saccharolyticum* genome. Primers were external to homologous recombination regions for the L-*ldh* locus of pSGD8 and the *pta ack* Kan^r locus of ALK2. Lane 1, NEB 10-kb ladder; lane 2, wild-type with L-*ldh* external primers X01177 and X01178; lanes 3 to 5, Kan^r colonies transformed with the knockout plasmid pSGD8 with L-*ldh* external primers; lanes 6 to 8, Kan^r colonies transformed with restriction-digested pSGD8 linear vector with L-*ldh* external primers. Predicted sizes are 3,863 bp for wild type and 5,160 bp for kanamycin resistance marker integration. Lane 9, wild type with *pta ack* external primers X00004 and X00021; lanes 10 to 12, Kan^r colonies transformed with ALK2 genomic DNA with *pta ack* external primers. Predicted sizes are 3,209 bp for wild type and 4,245 bp for kanamycin resistance marker integration.

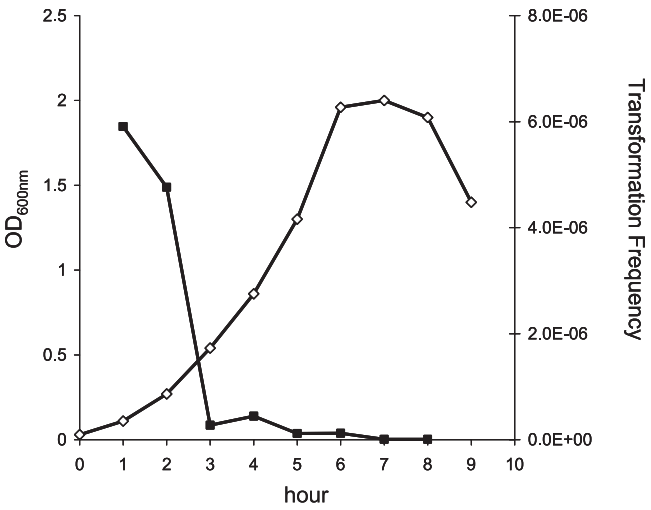

FIG. 2. Transformation efficiency of *T. saccharolyticum* JW/SL-YS485 during batch growth. ◇, optical density; ■, transformation frequency. Exponentially growing cells were transferred into fresh media at an initial OD of 0.03. To evaluate transformation efficiency, 1 ml of culture was transferred into a new tube containing 250 ng pMU131, incubated for 1 h before addition of DNase, and incubated for an additional hour to allow expression of the kanamycin resistance marker. Cells were then serially diluted on selective and nonselective media to determine transformation efficiency. Transformation efficiency data points are plotted at the time of DNase addition.

cum cells into the competent state beyond growth in a typical laboratory medium. Figure 2 shows the transformation frequency of *T. saccharolyticum* with the replicating plasmid pMU131 (see Materials and Methods) throughout batch growth. The transformation frequency was highest during early exponential growth and declined until reaching the stationary phase, in which the transformation frequency was below the limit of detection (8.0E−9 transformants per CFU). In this experiment DNA was incubated with cells for 1 h before DNase treatment to discern the effect of growth phase on transformation frequency, whereas for all other experiments described here cells were incubated with DNA for 16 to 18 h prior to plating on selective media (see Materials and Methods). In our hands, transformation efficiencies were highest when DNA was added at low initial cell densities (1E3 to 1E5 cells/ml), and cells were plated on selective medium prior to the onset of the stationary phase. Efficiencies were lowest when DNA was added at higher cell densities (1E8 cells/ml), and cells were plated after entering the stationary phase.

Natural competence in related bacteria. To test whether the natural competence phenomenon was unique to *T. saccharolyticum* YS485 among related bacteria, 16 other strains were tested for the ability to be transformed with the replicating plasmid pMU131. No optimization of the transformation protocol was made beyond determination of the minimum concentration of kanamycin required to eliminate spontaneous colony formation. As shown in Table 4, a total of 13 strains exhibited natural competence, three of which were *Thermoanaerobacterium thermosaccharolyticum* strains isolated by Mascoma Corporation. Transformation frequencies ranged from 1.0E−3 to 1.9E−6 transformants per CFU. For each transformation, three colonies were checked for the presence of the kanamycin marker by PCR (Fig. 3), and a 16S sequence was amplified using universal primers, sequenced, and compared to that of the

TABLE 4. Transformation frequencies of *Thermoanaerobacter* and *Thermoanaerobacterium* bacteria[a]

Strain	Transformants per CFU	Kan (μg/ml)
Thermoanaerobacterium saccharolyticum JW/SL-YS485 DSM 8691	1.4E−04	200
Thermoanaerobacter ethanolicus JW200 DSM 2246	1.0E−03	1,000
Thermoanaerobacterium thermosaccharolyticum M0523	2.8E−04	200
Thermoanaerobacterium thermosaccharolyticum M0524	4.2E−05	200
Thermoanaerobacterium aotearoense DSM 10170	1.5E−04	1,000
Thermoanaerobacterium thermosaccharolyticum HG-8 ATCC 31960	1.2E−04	200
Thermoanaerobacterium saccharolyticum B6A	2.1E−04	200
Thermoanaerobacterium saccharolyticum B6A-RI ATCC 49915	1.7E−04	200
Thermoanaerobacterium thermosaccharolyticum M0795	7.1E−05	200
Thermoanaerobacterium xylanolyticum DSM 7097	1.6E−05	200
Thermoanaerobacterium thermosaccharolyticum ATCC 7956	1.2E−05	200
Thermoanaerobacter pseudethanolicus 39E ATCC 33223	6.3E−05	400
Thermoanaerobacter brockii ATCC 35047	1.9E−06	1,000

[a] Bacteria were transformed with the replicating plasmid pMU131 as described in Materials and Methods.

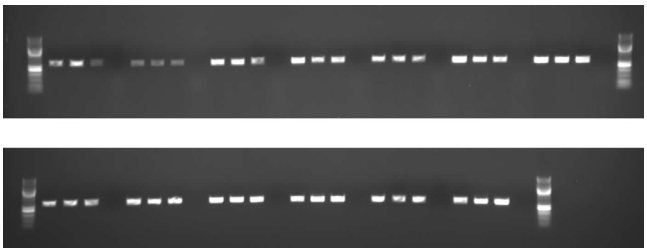

FIG. 3. PCRs with primers X00861 and X00862 of Kan^r colonies designed to amplify a 603-bp region in the kanamycin resistance marker. First and last lanes on each row were loaded with NEB 1-kb DNA ladder. Internal gel lanes are grouped four per strain: the first three per group are colonies transformed with pMU131, and the fourth is a reaction with cells from the same strain that was not transformed. Strain order is as follows: (first row, left to right) *Thermoanaerobacter brockii* ATCC 35047, *Thermoanaerobacter ethanolicus* JW200 DSM 2246, *Thermoanaerobacter pseudethanolicus* 39E ATCC 33223, *Thermoanaerobacterium aotearoense* DSM 10170, *Thermoanaerobacterium saccharolyticum* B6A, *Thermoanaerobacterium saccharolyticum* B6A-RI ATCC 49915, *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 DSM 8691, (second row, left to right) *Thermoanaerobacterium thermosaccharolyticum* ATCC 7956, *Thermoanaerobacterium thermosaccharolyticum* HG-8 ATCC 31960, *Thermoanaerobacterium thermosaccharolyticum* M0523, *Thermoanaerobacterium thermosaccharolyticum* M0524, *Thermoanaerobacterium thermosaccharolyticum* M0795, *Thermoanaerobacterium xylanolyticum* DSM 7097.

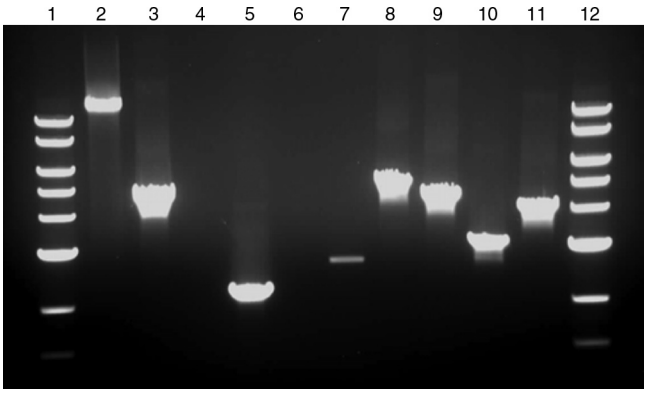

FIG. 4. PCRs used to confirm erythromycin marker integration in the *T. saccharolyticum* genome. Lane 1, NEB 10-kb ladder; lane 2, wild type with T4P external primers X08727, X08728, predicted size 12,981 bp; lane 3, M1464 with T4P external primers, predicted size 4,669 bp; lane 4, wild type with *cinA recA* downstream external primer X08736 and erythromycin internal primer X00957, no predicted band; lane 5, M1465 with primers X08736 and X00957, predicted size 2,191 bp; lane 6, wild type with *cinA recA* upstream external primer X08735 and erythromycin internal primer X00958, no predicted band; lane 7, M1465 with primers X08735 and X00958, predicted size 2,692 bp (internal and external primers were used to verify M1465, as the erythromycin gene replaced a similarly sized fragment of the *cinA recA* locus); lane 8, wild-type with *comEC* external primers X08160 and X08161, predicted size 5,217 bp; lane 9, M1466 with *comEC* external primers, predicted size 4,519 bp; lane 10, wild type with *comEA* external primers X08154 and X08155, predicted size 3,165 bp; lane 9, M1467 with *comEA* external primers, predicted size 4,187 bp; lane 12, NEB 10-kb ladder.

original starting culture. In no case was there evidence of spontaneously kanamycin-resistant colony formation or a transformable contaminant within the tested culture. *Thermoanaerobacterium zeae*, *Thermoanaerobacter mathranii*, *Caldicellulosiruptor saccharolyticus*, and *Clostridium thermocellum* were not transformed with this protocol. However, the ability of these strains to become naturally competent cannot be excluded based on this result, as several factors could result in a lack of transformants, such as the pMU131 resistance marker or replication origin not functioning in the host organism, an unmet condition for competence induction, or a native mechanism for limiting foreign DNA, such as a restriction or CRISPR system (29, 46).

Gram-positive competence homologues are required for natural competence. To begin elucidation of the natural competence mechanism in *T. saccharolyticum*, gene knockouts were made in loci with high similarity to genes involved in natural competence in other Gram-positive bacteria. Knockouts of a putative T4P locus (of which only one was identified on the genome), *comEA*, *comEC*, and a *cinA recA* locus were made using an erythromycin resistance marker. Deletions with chromosomal integration of both flanking regions were confirmed by PCR with primers external to the areas of homologous recombination (Fig. 4). The subsequent knockout strains were assayed for transformability with the replicating plasmid pMU131. As shown in Table 5, the ΔT4P, Δ*comEA*, and Δ*comEC* strains had transformation frequencies below the limit of detection, while the Δ*cinA* Δ*recA* strain had a 250-fold reduction in transformation efficiency compared to the wild type.

DISCUSSION

We were unable to identify previous reports of natural competence in members of the class *Clostridia*, although the 80 or so prokaryotic species known to be naturally competent are widely distributed phylogenetically (16). With 13 of the 15 tested *Thermoanaerobacter* and *Thermoanaerobacterium* strains demonstrating natural competence in this study, the phenomenon is apparently widespread among these organisms.

Most studied naturally competent bacterial species induce competence in response to external factors, such as pheromone density (quorum sensing) or stringent nutritional conditions; prominent examples include that of *Streptococcus pneumoniae* and *Bacillus subtilis* (11, 15). *T. saccharolyticum* falls within the smaller subset of studied bacteria, including *Acinetobacter calcoaceticus*, *Neisseria gonorrhoeae*, *Deinococcus radiodurans*, and the cyanobacteria *Synechococcus* and *Chlorobium*, that are naturally competent during the exponential growth phase without the requirement of special stimuli (16, 33). As seen in Fig. 2, the transformation frequency for *T. saccharolyticum* is highest during early growth in fresh medium

TABLE 5. Transformation frequencies of *T. saccharolyticum* JW/SL-YS485 and mutants

Strain	Description or genotype	Transformants per CFU[a]
JW/SL-YS485	Wild type	1.4E−04
M1464	ΔT4P (or1949 to or1961)	ND
M1465	Δ*cinA* Δ*recA* (or1848 and or1849)	5.7E−07
M1466	Δ*comEC* (or2075)	ND
M1467	Δ*comEA* (or2100)	ND

[a] ND, not detected, below detection limit of 5.4E−09.

and decreases toward zero as the stationary phase is reached. Further study of the physiology and regulation of natural competence in *T. saccharolyticum* will be required for a better understanding of how and why this organism enters the competent state.

With the protocol reported here, the transformation efficiency of *T. saccharolyticum* JW/SL-YS485 with pMU131 was observed to be 1.4E−4 transformants per CFU. *Thermoanaerobacter ethanolicus* JW200 had the highest transformation frequency at 1.0E−3 transformants per CFU, while *Thermoanaerobacter brockii* had the lowest at 1.9E−6 transformants per CFU. DNA concentration, divalent cation concentration, pH, temperature, carbon source, exposure time to DNA, and the selective maker type have all been shown to influence transformation frequencies of other naturally competent organisms, such as *A. calcoaceticus* and *Thermus thermophilus* (17, 34). It is possible that many of these factors also influence transformation efficiency in *Thermoanaerobacter and Thermoanaerobacterium* strains and that the maximum transformation efficiencies remain to be determined. Nevertheless, with the efficiencies reported here, standard genetic manipulations, such as plasmid transformation, gene knockout, and gene integration, are easily performed, and transformation via linear DNA enables rapid PCR-based transformation strategies (12, 32).

The genome of *T. saccharolyticum* carries several genes that have homology to Gram-positive late-stage competence genes, including a 13-gene cluster with homology to type IV pilus (T4P) assembly genes which bind DNA during natural competence (6), *comEA* and *comEC* homologues, which are involved in DNA transport across the cell membrane (6), and *cinA* and *recA* homologues, which are thought to be involved in single-strand DNA protection and chromosomal integration after passage into the cytosol (2a). *cinA*, also referred to as colligrin or DNA damage/competence-induced protein, has been shown to mediate *recA* localization to the membrane when cells are in the competent state (30).

In *T. saccharolyticum*, homologues for T4P genes, *comEA*, and *comEC* are required for observable natural competence. This strongly suggests that natural competence occurs via a conserved Gram-positive mechanism involving these enzymes. Based on sequence similarity, the ΔT4P region (see Fig. S2 in the supplemental material) contains many T4P components, including putative traffic NTPases PilB and PilT (or1961 and or1960), pseudopilins PilE and PilV (or1958 and or1955), a prepilin processing peptidase, PilD (or1957), a polytopic membrane protein, PilG (or1959), and T4P or competence-associated proteins FimT, PilW, ComFB, PilM, PilN, and PilO (or1956, or1954, or1952, or1951, or1950, and or1949, respectively). The *comEA* and *comEC* genes of *T. saccharolyticum* are not located adjacent to other known competence genes, although the genetic organization at these two loci is conserved in other *Thermoanaerobacter* and *Thermoanaerobacterium* strains. The observed 250-fold drop in transformation efficiency of the Δ*cinA* Δ*recA* strain suggests that CinA, RecA, or both also play a role during natural competence, as has been shown with *B. subtilis* and *S. pneumoniae* (30, 50).

Model organisms such as *S. cerevisiae* and *E. coli* are often considered for lignocellulosic biofuel and biochemical production due to the relative ease of genetic engineering, even though they lack one or more of the traits required of an ideal biocatalyst, such as hydrolytic capabilities, high productivities, or broad substrate utilization (1). Organisms such as *Thermoanaerobacter* and *Thermoanaerobacterium* bacteria have inherent advantages relative to these model organisms, such as the ability to rapidly hydrolyze and ferment low-cost polysaccharides and sugars (2, 27) and the ability to grow at temperatures above 50°C, which could improve process metrics, such as fermentation heat load, microbial contamination, substrate solubility, and product recovery (44). Still, the absence or rudimentary status of genetic systems for such thermophilic anaerobes constrained their development as biocatalysts. The simple and powerful transformation system described here, along with recent genomic sequencing projects for several *Thermoanaerobacter* and *Thermoanaerobacterium* strains (DOE Joint Genome Institute [http://www.jgi.doe.gov/]), should greatly accelerate the pace and extent to which genetic manipulations can be made in these biotechnologically relevant organisms.

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