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ORIGINAL PAPER

Bicarbonate is a stimulus in the inter-species induced sporulation of strict anaerobic *Syntrophomonas erecta* subsp. *sporosyntropha*

Chenggang Wu · Guishan Zhang · Xiaoli Liu · Xiuzhu Dong

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Abstract Previously *Syntrophomonas* species had been described as the bacteria those did not form spores, however, in our previous studies, a newly isolated *S. erecta* subsp. *sporosyntropha* JCM13344^T was found to form spores in the co-culture with methanogens, while not in mono-culture or in co-culture with sulfate reducer. In this study, we examined the sporulation stimulus conferred by methanogens in the co-culture. By reducing bicarbonate in mono-culture and sulfate-reducing co-culture, we could induce *S. erecta* subsp. *sporosyntropha* JCM13344^T to form spores, so that bicarbonate at lower concentration was determined as another stimulus for sporulation. Based on the substrate degradation by strain JCM13344^T in different concentration of bicarbonate vs at different pHs, it was suggested that bicarbonate could stimulate the sporulation by mediating a nutrient deprivation but not pH drop. To further confirm the sporulation potential of this group of bacteria, *spo0A* fragments were amplified from strain JCM13344^T as well as all the recognized *Syntrophomonas* species, confirming that they were members of the spore-forming group. Since sporulation is a kind of response of spore-forming bacteria to environmental stresses, the observation in this work implies that stresses can be created even between the mutual beneficial partners, in this case, inducing sporulation.

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C. Wu · G. Zhang · X. Liu · X. Dong (✉)
State Key Laboratory of Microbial Resources,
Institute of Microbiology, Chinese Academy of Sciences,
No. 3A, Datun Road, Chao Yang District,
Beijing 100101, People's Republic of China
e-mail: dongxz@sun.im.ac.cn

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Introduction

Syntrophomonas species are a group of obligate anaerobic bacteria. They had been described as a non-spore forming genus (Lorowitz et al. 1989; Zhao et al. 1993) until the spore-forming species were proposed (Wu et al. 2006a, b). *Syntrophomonas* species have a very restrict substrate range—fatty acids with 4–18 carbon atoms in the consortia with methanogens or sulfate reducing bacteria, those use the H_2 released from fatty acid degradation as energy source meanwhile removing the inhibitory effect of H_2 on *Syntrophomonas* species (McInerney et al. 1981). This kind of consortia is a special example of beneficial synergetic interactions among different species in microbial communities; however, in recent study we found a spore-forming bacterium *S. erecta* subsp. *sporosyntropha* JCM13344^T, and it formed spores only when co-cultured with its metabolic partner methanogen (Wu et al. 2006a). Strain JCM13344^T degraded fatty acids with 4–8 carbon atoms into acetate in the co-cultures with a methanogen, and could grow in monoculture with crotonate as substrate.

Spore formation is a very complex biological process involving many genes, among those, *spo0A* encodes a universal regulator Spo0A, which is conserved in all the bacterial species that are known to form spores (Brown et al. 1994). It is well known that sporulation is a kind of response by microorganisms to stress environments, such as nutritional deprivation (Matsuno et al. 1999). However, it is not known whether stress can be created as well in a syntrophic consortium such as the *Synthophomonas* spp. - methanogens association. To gain insight of the sporulation-induced

mechanism by the beneficial partners, in this study, we determined the possible stimulus for sporulation of *S. erecta* subsp. *sporosyntropha* and showed that bacteria in the genus *Syntrophomonas* as a group may all have the potential to sporulate under stress conditions.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study were listed in Table 1. The pre-reduced media were prepared according to McInerney et al. (1979). Pure cultures of *Syntrophomonas* spp. were maintained by subculturing on DSMZ medium 213 but omitting rumen fluid, with 20 mM crotonate instead of butyrate as substrate (Beaty and McInerney 1987). Methanogens and *Desulfovibrio* G11 were grown in a basal medium as described previously (McInerney et al. 1979) under a gas phase of H_2/CO_2 (80:20, 1.25×10^5 Pa), except 20 mM sodium sulfate was supplemented.

The media were dispensed into 18 × 150 mm anaerobic tubes or 100 ml serum bottles sealed with butyl rubber stoppers. All inoculations and transfers were done with syringes and needles. Incubation was at 37°C and in dark unless indicated.

Sporulation test

For spore formation test, 0.5 ml monocultures or co-cultures of *Syntrophomonas* spp. at stationary phase were collected by centrifugation with 10,000*g* for 10 min, and washed with sterilized water for three times, and then re-suspended in 0.5 ml fresh medium and inoculated into 4.5 ml the same medium. All the procedures were carried out inside of an anaerobic glove box (Anaerobic system model 1028, Forma Scientific).

Cultures at different growth phases cells were stained with Gram staining and malachite green, and then examined under Olympus BH-2 microscope (Japan) and electron microscope (Hitachi H-600A). Before EM observation, the ultrathin section was stained with uranyl acetate and lead citrate according to Reynolds (1963). The average ratio of the sporulated cells was calculated from the counts of at least six random microscopic fields. Sporulated cultures were also verified by surviving the heating at 80°C for 15 min. The sporulation test was carried out in triplicate with three different batches of cultures.

Table 1 Bacterial strains used in this study

Strains	Source
Syntrophomonas cellicola JCM 13582^T	Our laboratory
S. erecta subsp. *erecta* DSM 16215^T	Our laboratory
S. erecta subsp. *sporosyntropha* JCM 13344 ^{T}	Our laboratory
S. curvata DSM 15682^T	Our laboratory
S. wolfei subsp. *wolfei* DSM 2245^T	DSMZ
S. wolfei subsp. *saponavida* DSM 4212^T	DSMZ
S. sapovorans DSM 3441^T	DSMZ
S. wolfei subsp. *methylbutyratca* JCM 14075^T	Our laboratory
Methanobacterium beijingense DSM 15999^T	Our laboratory
M. arboriphilicus DSM 1125^T	Our laboratory
M. formicicum DSM1535^T	DSMZ
Desulfovibrio strain G11	DSMZ
Streptococcus oligofermentans LMG 21535^T	Our laboratory

DSMZ Deutsche Sammlung von Mikroorganismem

Analytical procedures

Methane and short-chain fatty acids were detected with Gas Chromatograph (GC 14A, Shimadzu) as previously described (Zhang et al. 2004). The pH values of the media were measured by pH meter (Orion, Model 818). Cell growth was monitored by $OD_{600\ nm}$ measurement as well as acetate or methane production.

Extraction and purification of genomic DNA

Genomic DNA was extracted from syntrophic bacteria either in co-cultures or monoculture and from *Desulfovibrio* G11 by a method described by Marmur (1961), and DNA extraction from methanogens was as described by Jarrell et al. (1992).

PCR amplification and DNA sequencing

Degenerate PCR primers designed previously (Brown et al. 1994; Brill and Wiegel 1997) were used to amplify *spo0A* of *Syntrophomonas* strains. The primers lie within a short region of about 280 bp corresponding to the target region of *spo0A* (Brown et al. 1994). PCR was carried out as previous described (Brill and Wiegel 1997).

PCR products were purified using 3S spin Genomic DNA Minipreps kit (Shenergy Bilcolor) and cloned into pUCmT vector and then transformed into competent *Escherichia coli* DH5α as specified in the TA Cloning Kit (Invitrogen). The plasmids with inserts were purified using Montage Plasmids Miniprep96 kit (Invitrogen) and at least two clones per bacterial strain were sequenced using primer T7.

Phylogenetic analysis of partial *spo0A* genes

The partial *spo0A* gene sequences amplified from eight strains of described *Syntrophomonas* spp. were compared with those of ten spore-forming bacteria obtained from GenBank database. Sequences in a consensus length 269 bp were aligned using CLUSTAL X program (version 1.83) and the phylogenetic tree was constructed with neighbor-joining method implemented in MEGA3 program. The topology of the phylogenetic trees was evaluated by bootstrap analysis of 1,000 datasets.

Nucleotide sequence accession numbers

All the partial *spo0A* gene sequences determined in this study have been deposited in GenBank under accession nos. DQ486652–486653 and DQ388571–388576.

Quantitative real-time PCR determination of *spo0A* transcription

Total RNA was extracted from the cells in culture either buffered with 10 or 40 mM bicarbonate using a RNA miniprep kit (Invitrogen). Reverse transcription was performed using the RNA as template, random primers (Promega) and MMLV reverse transcriptase (Promega).

The real-time PCR primers were designed based on *spo0A* (DQ388573) and 16S rRNA (DQ086234) gene sequences of *S. erecta* subsp. *sporosyntropha*. spo0AF (5′-AGTACCTG CGTGATGCTATTATGCTG-3′) and spo0AR (5′-TCCCC TATCCCATGCCAATTC-3′) were used for quantifying *spo0A* mRNA (Amplicon of 161 bp); 16SrRNAF (5′-GGT GTAGCGGTGAAATGCGTAGAA-3′) and 16SrRNAR (5′-ATACCTCCCACACCTAGTATCCATC-3′) were used for quantifying 16S rRNA. The 161 bp amplicon of 16S rRNA gene served as the internal biomass standard.

Quantitative real-time PCR reactions were performed as following. Each reaction mixture contained 12.5 μl SYBR green master 2 × mix (Applied Biosystems), 1 μl of cDNA, 100 nM of each primer and distilled water to make a final volume of 25 μl. The PCR cycle started with 2 min at 50°C for optimal AmpErase uracil-*N*-glycosylase enzyme activity, and then followed by denaturation at 95°C for 10 min and 40 cycles of amplification as follows, 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. Real-time PCR was carried out by using ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). *Streptococcus oligofermentans* was used as *spo0A*-null control of the non-spore forming bacterium. All reactions were run in triplicate.

Results and discussion

Methanogens acted as the essential organisms to induce sporulation of *S. erecta* subsp. *sporosyntropha*

Since sporulation of *S. erecta* subsp. s*porosyntropha* JCM13344^T was observed only in co-culture with *Methanobacterium formicicum* DSM1535^T, but not in mono-culture, to determine whether the presence of methanogens was essential for its sporulation, a sulfate-reducing consortium as an alternative butyrate-utilizing co-culture was constructed by mixing the pre-grown *S. erecta* subsp. *sporosyntropha* and *Desulfovibrio* G11 at a volume ratio of 1: 2. As comparison, methanogenic co-cultures were constructed with *M. arbophilicum* DSM 1125^T or *M. beijingense* DSM15999^T as the partner organisms instead of *Desulfovibrio* G11. The results showed that while no spores were formed in *Desulfovibrio* G11 co-culture (Fig. 1a), about 29% sporulated cells were observed in the methanogenic co-cultures and the sporulated cells were also confirmed by surviving of pasteurization (80°C for 15 min). This experiment indicated that the presence of methanogens was essential to induce the sporulation of *S. erecta* subsp. *sporosyntropha*, but not the substrates, as butyrate was used in the sulfate-reducing co-culture as well.

Syntrophism is defined as that the cooperation of partners depends entirely on each other to perform the metabolism (Schink 1997), while sporulation is a well-known stress-responding morphological development of the bacteria with endospore formation potentials. The observation in this work implies that the beneficial friends can generate stresses under some circumstances as well.

Lowered bicarbonate induced sporulation of *S. erecta* subsp. *sporosyntropha*

To get insight of the substances that may stimulate the sporulation of *S. erecta* subsp. *sporosyntropha*, the differences between its methanogenic and sulfate reducing co-cultures were compared. First, the different products, H_2S versus CH_4 on sporulation of strain JCM13344^T were simply added in the gas phase of the methanogenic and sulfate-reducing co-cultures, respectively. Second, the diffusible substance(s) produced by methanogens on the sporulation were determined by addition of spent culture of *M. formicicum* into the co-culture of JCM13344^T with *Desulfovibrio* G11. However, no spores were observed for strain JCM13344^T in the co-culture with *Desulfovibrio* G11 even after 45 days incubation (data not shown). These suggested that neither the gas products (CH_4 vs. H_2S) nor the possible extracellular factor(s) of the partner (data not

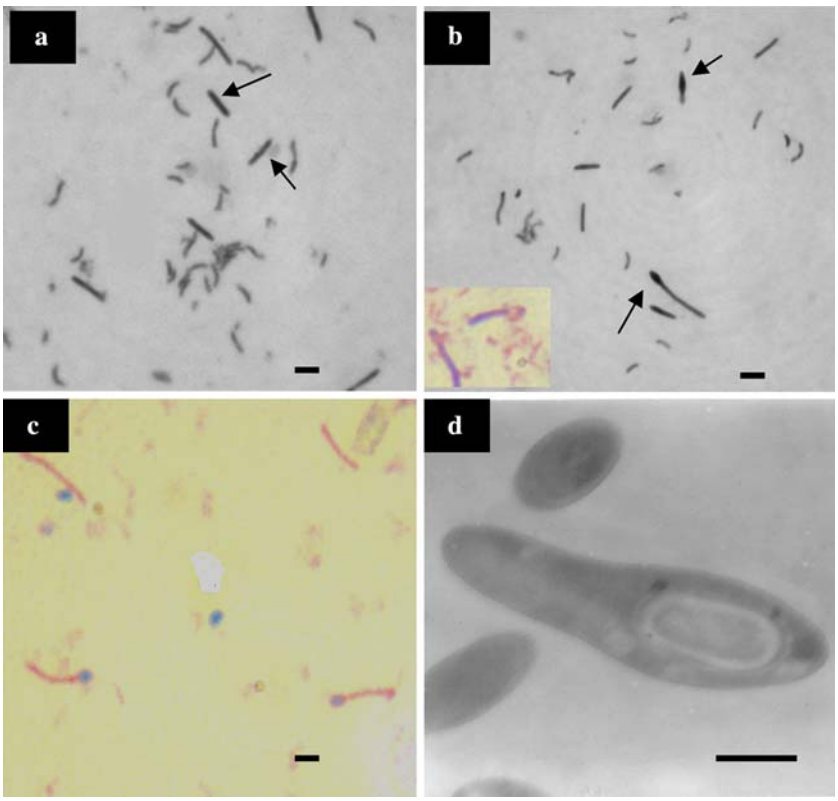

Fig. 1 Microphotographs of *S. erecta* subsp. *sporosyntropha* JCM13344^T grown in different concentrations of bicarbonate. **a** No spore in 40 mM bicarbonate in co-culture with *Desulfovibrio* sp. G11 under phase contrast microscope. *Arrow points* at cells of *Syntrophomonas erecta* subsp. *sporosyntropha*. **b** Forespores in 10 mM bicarbonate in co-culture with *Desulfovibrio* sp. G11 under phase contrast microscope. *Arrow points* at forespores-contained cells at the so-called clostridial stage of *S. erecta* subsp. *sporosyntropha*, and *inserted picture* is the Gram stained matured spore. **c** Malachite green stained spores in 10 mM bicarbonate monoculture with crotonate as substrate. **d** Transmission electron micrograph of the sporulated cells in 10 mM bicarbonate in co-culture with *Desulfovibrio* sp. G11. *Bar* equals 1 μm

shown) were the stimulus for the sporulaiton of *S. erecta* subsp. *sporosyntropha*.

When the methanogenic co-culture of *S. erecta* subsp. *sporosyntropha* degraded 20 mM butyrate, 10 mmol/l CH_4 was produced. This means that stoichiometrically 10 mM bicarbonate would be consumed in this co-culture as methanogens use bicarbonate (CO_2) for CH_4 formation as indicated in following equation: $2CH_3CH_2CH_2COO^- + HCO_3^- + H_2O \rightarrow 4CH_3COO^- + H^+ + CH_4$. While in sulfate-reducing co-culture, *Desulfovibrio* sp. G11 uses 20 mM sulfate instead of bicarbonate as electron acceptor for butyrate oxidation, and in monoculture crotonate is converted into butyrate and acetate apparently without bicarbonate consumed. Therefore, it is reasonable to postulate that lower bicarbonate can induce sporulation of *S. erecta* subsp. *sporosyntropha*. To test this postulation, we grew strain JCM13344^T in the medium with 30 and 10 mM bicarbonate (normally 40 mM used) in the co-culture with *Desulfovibrio* sp. G11, respectively. As expected, about 6 and 30% sporulated cells were observed at the stationary phase in 30 and 10 mM bicarbonate after 15 days, respectively (Fig. 1b, d).

Similarly by reducing $NaHCO_3$ from 40 to 30 mM and 10 mM in the medium, about 2 and 20% sporulated cells were also observed in the mono-culture of *S. erecta* subsp. *sporosyntropha* JCM13344^T growing on crotonate (Fig. 1c), respectively . These data demonstrated that $NaHCO_3$ was a sporulation associated factor for *S. erecta* subsp. *sporosyntropha*, and it induced the sporulation at lower concentration.

As the culture gas phase contains about 0.2 atm. CO_2, the threshold bicarbonate concentration to induce the sporulation of strain JCM13344^T has to include the bicarbonate portion originated from CO_2. On the basis of the dissociation constant (pKa) of HCO_3^- and H_2CO_3 (the form of CO_2 in water) as 6.38 at pH 7.4, the calculated CO_2-originated bicarbonate would be 4.0 and 4.94 mM when the medium contains 40 and 30 mM $NaHCO_3$, respectively. Thus the threshold bicarbonate concentration to induce the sporulation of strain JCM13344^T can be deduced between 34.94 and 44 mM.

Bicarbonate could mediate a nutrient poverty rather than pH drop as the stimulus for sporulation of *S. erecta* subsp. *sporosyntropha*

It is well accepted that starvation is the main stimulus for sporulation of endospore-forming bacteria. Whereas pH drop could also be indirectly related to sporulation through the stress generated from acidic products, like solvents accumulation by *Clostridium acetobutylicum* (Huang et al. 1986).

Bicarbonate is used as the routine culture buffer for *Syntrophomonas*, reducing its concentration in medium can lead to either pH drop or nutrition poverty, as many bacteria possess the CO_2-assimilation capacity. To determine whether reduced bicarbonate mediated a pH drop or starvation to induce sporulation of the bacterium, *S. erecta* subsp. *sporosyntropha* was grown in the monoculture either buffered with 40 mM or 10 mM bicarbonate, and the two media with different concentrations of bicarbonate were adjusted to pH of 7.0 and 6.4, respectively. Then both crotonate degradations and sporulation in the cultures were followed. Figure 2 showed that while the bacterium could degrade about 20 mM crotonate within 35 h in 40 mM bicarbonate under both pHs, it needed much longer time to complete the degradation in 10 mM bicarbonate, 54 h at pH 7 and more than 100 h at pH 6.4. This suggested that spores were only found in the culture of *S. erecta* subsp. *sporosyntropha* grown in 10 but not 40 mM bicarbonate could be attributed to mediating nutrient deprivation rather than pH drop. However the precise mechanism of bicarbonate mediated sporulation remains to be determined. Alternative chemicals, such as phosphate or PIPES were also tried to replace bicarbonate in this experiment, however the bacterium did not grow in both chemical buffered media, implying that bicarbonate may be involved in the metabolism.

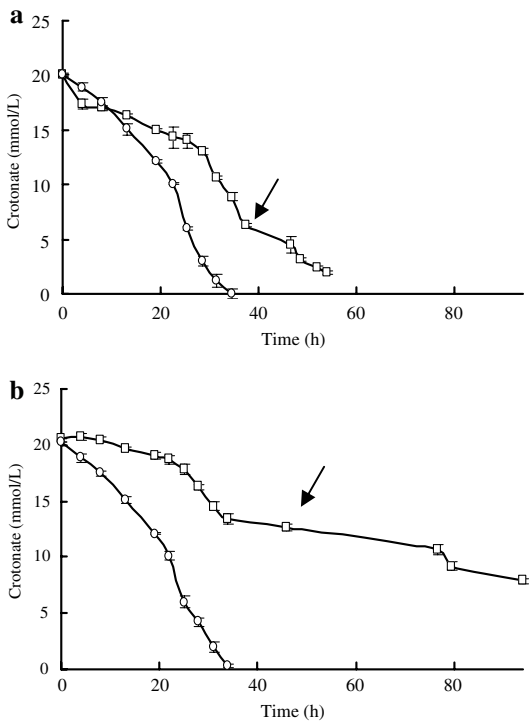

Fig. 2 Crotonate degradation of *S. erecta* subsp. *sporosyntropha* in the culture with different bicarbonate concentration and at pH 7.0 (**a**) and pH 6.4 (**b**). *Symbols, open square* crotonate degradation in 10 mM bicarbonate; *open circle* crotonate degradation in 40 mM bicarbonate. *Arrows* indicate the time point of forespores to be observed and mRNA extracted from the 10 mM bicarbonate culture

spo0A gene amplification from *Syntrophomonas* strains and expression in different concentration of bicarbonate buffered cultures

To test the genetic background of sporulation of *S. erecta* subsp. *sporosyntropha* and related members, *spo0A*, the universal regulator gene of sporulation was surveyed for all the *Syntrophomonas* strains by PCR amplification according to Onyenwoke et al. (2004). A fragment about 270 bp was amplified from all tested asporogenic and spore-forming *Syntrophomonas* strains. DNA sequence analysis indicated that they were closely related to the *spo0A* of

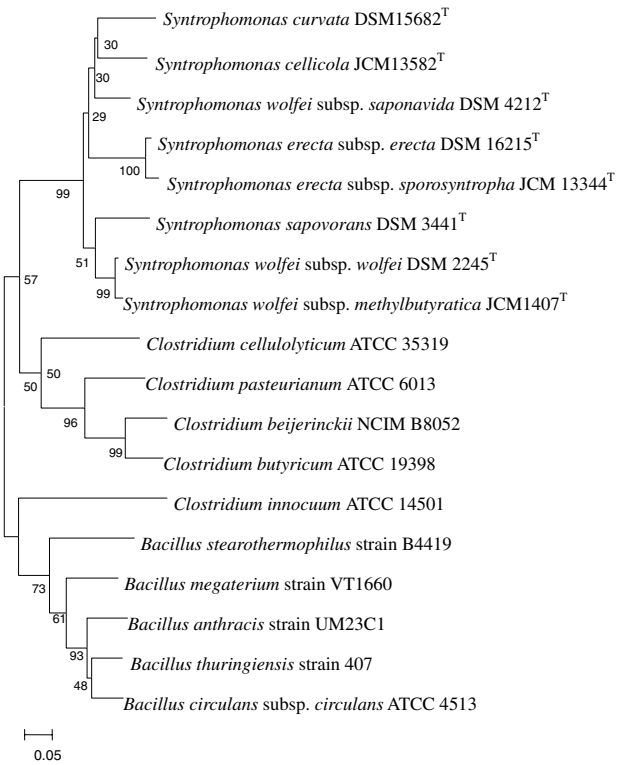

Fig. 3 Phylogenetic analysis of *Syntrophomonas* based on the *spo0A* gene sequences. The tree was constructed with 269 bp alignable sequences, using the neighbor-joining method with bootstrap values based on 1,000 replications. GenBank accession numbers of sequences from all of the species included in the analysis are as follows: U09970 (*Bacillus anthracis* strain UM23C1), U09973 (*B. circulans* subsp. *circulans* strain ATCC 4513), U09974 (*B. megaterium* strain VT1660), AJ002297 (*B. stearothermophilus* strain B4419), X80639 (*B. thuringiensis* strain 407), U09979 (*Clostridium beijerinckii* strain NCIMB 8052), U09980 (*C. butyricum* ATCC 19398), AJ288948 (*C. cellulolyticum* strain ATCC 35319), U09981 (*C. innocuum* ATCC 14501), U09982 (*C. pasteurianum* strain ATCC 6013).The number at each branch points is the percentage supported by bootstrap. *Bar* 5% sequence divergence

Clostridium and *Bacillus* strains (Fig. 3), the well-known spore-forming bacteria. However no such fragment was amplified from *Desulfovibrio* sp. G11 or the tested methanogens using the same protocol. The results indicated that all the *Syntrophomonas* spp. possessed the potential to form spores when growing under conditions that could induce sporulation.

To determine whether the *spo0A* gene of *S. erecta* subsp. *sporosyntropha* was differentially expressed in the cultures buffered with 40 and 10 mM bicarbonate, mRNAs of *spo0A* extracted from both cultures were quantified by means of quantitative real-time PCR using its 16S rRNA as an internal biomass standard. Total RNA was extracted from both cultures at late log phase as indicated in Fig. 2 for 10 mM bicarbonate culture, and at the end of degradation for 40 mM bicarbonate culture, respectively. The result showed no difference in *spo0A* gene expression under either bicarbonate concentrations (data not shown), although sporulation was observed only under the lower bicarbonate concentration. This result is not surprising, as *spo0A* is believed to be constitutively expressed in the vegetative and sporulated cells of *B. subtilis* and *Clostridium* spp. (Dürre and Hollergschwandner, 2004), and the sporulation is initiated by Spo0A in its activated, phosphorylated form (Chung et al. 1994), which is achieved by the multi-component phosphorelay (Burbulys et al. 1991).

Although *spo0A* gene homolog was detected in all the *Syntrophomonas* strains in this study, bicarbonate seemed to affect the sporulation differently for different species of this group in a dose-dependent manner. As for some *Syntrophomonas* strains, like *Syntrophomonas* (previously *Syntrophospora*) *bryantii* (Zhao et al. 1993; Wu et al. 2006b) and the newly described *S. cellicola* (Wu et al. 2006b), they formed spores under all the culture conditions with 40 mM bicarbonate while sporulated cells accounted for higher ratio under lower bicarbonate. Whereas some others like *S. erecta* subsp. *erecta* sporulated at very low ratio even in lowered bicarbonate (data not shown). These data indicated that the concentration threshold of bicarbonate to induce sporulation of *Syntrophomonoas* spp. was strain-specific.

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