Symbiobacterium thermophilum is a unique syntrophic bacterium that exhibits marked growth only in coculture with a cognate Bacillus sp. In this study, we found that the bacterium is capable of marked mono-growth when supplied with CO₂ or bicarbonate. The evidence suggests that the genetic defect for carbonic anhydrase in this bacterium is a reason for the syntrophic property based on CO₂ requirement.

**Key words:** Symbiobacterium thermophilum; microbial commensalism; carbon dioxide; carbonic anhydrase; yadF

Symbiobacterium thermophilum is a rod-shaped thermophilic bacterium isolated from compost. The organism is characterized by a unique growth dependence on commensalism with Bacillus sp. strain S, which was isolated from compost along with it. While *S. thermophilum* does not grow or show significantly impaired growth in a pure culture, it effectively proliferates up to 5 × 10⁸ cells/ml when it is co-cultivated with *Bacillus* strain S. In the coculture, which is usually performed by stationary incubation at 60°C, first the growth of *Bacillus* occurs, and then *S. thermophilum* commences to grow. The evidence obtained from our recent taxonomical and ecological study strongly suggests that *S. thermophilum* and its relatives are unusual in terms of phylogeny but have widespread occurrence, and that they have not been identified because they cannot be obtained as pure cultures.

We are interested in the molecular basis that underlies the commensalism between *S. thermophilum* and *Bacillus* strain S. Our previous study, using an originally designed dialysis culture vessel, revealed that the growth of *S. thermophilum* occurs even when it is cultured separately from the *Bacillus* cells by a dialysis membrane. It indicated that the growth factor for *S. thermophilum* is a dialyzable substance(s) and is probably produced by the *Bacillus* strain, but the growth-promoting activity in the culture supernatant of *Bacillus* strain S was weak and not reproducibly fractionated by any chromatography.

Apart from the culture supernatant of the *Bacillus*, we examined various culture conditions and noticed that cultivation under a CO₂-supplied condition enabled *S. thermophilum* to grow in the absence of *Bacillus*. Figure 1A shows the mono-growth of *S. thermophilum* in LB liquid medium (containing, tryptone [DIFCO, Detroit, MI] 10; yeast extract [DIFCO] 5 and NaCl [Kokusan, Tokyo] 5 [all the constituents in grams per liter]; pH 7.6) introduced with CO₂-containing atmosphere. Pure cells of *S. thermophilum* obtained from the dialysis culture with *Bacillus* strain S, as described previously were inoculated to give an initial cellular concentration of approximately 1 × 10⁴ cells/ml, and was cultured at 60°C for 5 d with continuous aeration (50 ml/min) with N₂ gas that carried CO₂ at 0, 0.1, and 1.6%. The growth of *S. thermophilum* was measured by a quantitative PCR technique described previously. The result showed that the introduction of 0.1 or 1.6% CO₂ with N₂ gas markedly promoted the growth of *S. thermophilum*, yielding 3 × 10⁷–5 × 10⁷ cells/ml, while the introduction of pure N₂ gas did not. Similar growth promotion was observed by supplying bicarbonate to culture media (data not shown).

Using a closed cultivation system, we examined the threshold CO₂ concentration critical for the growth of *S. thermophilum*. The culture was performed for 72 h at 60°C in an airtight 0.5-liter Erlenmeyer flask with two side arms, which contained 200 ml LB medium supplied with various concentrations of sodium bicarbonate. The atmosphere was flushed several times and filled with pure N₂ gas. Subsequently, after inoculation of *S. thermophilum* cells (1 × 10⁴ cells/ml), 0.5 ml atmosphere was collected and examined for CO₂ concentration by gas chromatography. Figure 1B shows the cellular concentration of *S. thermophilum* yielded after 72 h cultivation as a function of initial atmospheric CO₂ concentration. The result indicates that the threshold CO₂ concentration for the initiation of proliferation lies between 0.014 and 0.017% in the anaerobic atmosphere. A similar study using synthetic air (N₂:O₂ = 9:1) did not give clear results as for the threshold concentration due to slow and impaired growth of *S. thermophilum*. It

---

1 To whom correspondence should be addressed. Tel: +81-466-84-3937; Fax: +81-466-84-3935; E-mail: ueda@brs.nihon-u.ac.jp
was observed previously that *S. thermophilum* shows better growth in an anaerobic condition than in an aerobic one. In the bicarbonate-supplied culture, we observed that the CO$_2$ concentration increased in the atmospheric space increased along with the growth of *S. thermophilum* (Fig. 1C). This result indicates that the supply of CO$_2$ or bicarbonate is required only for the initial growth phase and that the bacterium can grow on CO$_2$ generated by its own metabolic activity in the later growth phase. The above result strongly suggests that CO$_2$ generated by the metabolic activity of *Bacillus* strain S induces the proliferation of *S. thermophilum*. This is consistent with our previous speculation that the growth-promoting activity is not specific to *Bacillus* strain S but quite general to bacteria. But, the cellular yield achieved by the introduction of CO$_2$ (1×10$^7$–3×10$^7$ cells/ml) was about 10% of the full growth yield achieved by cocultivation with *Bacillus* strain S (approximately 5×10$^8$ cells/ml). This suggests that an additional factor(s) is required in order to grow the organism fully.

Currently, we speculate that two more events are carried out by *Bacillus* strain S to fully support the growth of *S. thermophilum*. One is elimination of a self-inhibitory metabolite(s) produced by *S. thermophilum* and the other is supply of a putative peptidic substance (unpublished result). Since the latter or its alternative appears to be contained in LB medium, we assume that the former factor is responsible for the above 10-fold difference in cellular yield. A dialysis culture that eliminated the putative self-inhibitory metabolite achieved a cellular yield relevant to that obtained in co-culture with *Bacillus*.

In addition, the above observation indicates that the anaerobic condition is essential for a marked effect of CO$_2$. We speculate that an anaerobic environment created by the growth of *Bacillus* is also a critical factor for the growth of *S. thermophilum*. *S. thermophilum* does not grow in shaking culture even when it is cocultivated with *Bacillus* (our unpublished observation). Based on all these observations, we deduce that *S. thermophilum* requires a complex condition for effective proliferation.

Recently, several studies have revealed that mutants for carbonic anhydrase of *Ralstonia eutropha* and *Escherichia coli* show strict growth dependence on CO$_2$. Carbonic anhydrase is a widespread enzyme that catalyses conversion between CO$_2$ and bicarbonate. The enzyme has three classes, which have no significant sequence similarity. The recent completion of genomic sequencing of *S. thermophilum* revealed that the organism does not retain proteins that show similarity to any class of carbonic anhydrase. In fact, we could not detect carbonic anhydrase activity in the cell extract of *S. thermophilum* by the method described by Alber and Ferry. This raises the possibility that the CO$_2$ dependence of *S. thermophilum* described above is ascribed to a deficiency of the enzyme.

To examine whether the deficiency of carbonic anhydrase confers a syntrophic phenotype similar to that observed in *S. thermophilum*, an *E. coli* mutant for carbonic anhydrase was cocultured with *Bacillus subtilis*. The culture was performed at 37°C with shaking (90 rpm) in a 200 ml LB liquid medium prepared in an
Fig. 2. CO₂-Dependent Growth of a yadF Mutant of E. coli.

A. Cellular yields achieved under various CO₂-supplying culture conditions. The yadF mutant was monocultured (closed squares) or cocultured with B. subtilis (closed triangles) under ambient air in an air-tight flask containing 200 ml LB medium. Monoculture of the yadF mutant was also performed in LB medium containing 1 mM sodium bicarbonate under ambient air (closed circles) or LB medium under ambient air that contained approximately 2.0% CO₂ (open circles). As a control, yadF+ strain (refer to text) was also monocultured in LB medium under an ambient air condition (open squares). The bacterial cells were grown in a top-sealed Erlenmeyer flask incubated at 37°C for 18 h with moderate shaking (90 rpm). Cellular concentration was measured by colony counting on LB solid medium containing 20 µg/ml streptomycin.

B. Determination of threshold CO₂ concentration critical for the initiation of growth of the yadF mutant. The yadF mutant was monocultured in LB medium supplied with various concentrations of sodium bicarbonate under ambient air in the closed system. The cellular concentration yielded after 18 h cultivation at 37°C was plotted against the initial (0 h) CO₂ concentration in the atmospheric space.

C. The increase in atmospheric CO₂ along with the growth of the yadF mutant. The atmosphere of each culture shown in (A) was collected and examined for CO₂ content. The symbols are as described in (A).

Airtight 0.5-liter Erlenmeyer flask. Two carbonic anhydrase genes (cynT and yadF) exist in the E. coli genome, and it is known that yadF mutants require elevated atmospheric CO₂ concentration for their growth.⁷,⁸ As shown in Fig. 2A, the yadF mutant provided by J. Kato [yadF::Cm/mF(Sm)]⁹ did not grow in the monoculture in LB liquid medium under an ambient condition, but it showed marked growth when the atmosphere contained approximately 2% CO₂ gas or when the medium was supplied with 1 mM sodium bicarbonate. On the other hand, it was noteworthy that the yadF mutant showed marked growth under ambient air when it was cocultured with Bacillus subtilis ATCC6633. A similar result was obtained when the mutant was cocultured with a yadF+ parental strain [yadF::Cm/mF(Sm)-yadF]⁹ of E. coli (data not shown). The result indicates that the lack of carbonic anhydrase confers a syntrophic phenotype like that of S. thermophilum in E. coli.

Using the closed cultivation system described above, we examined the threshold CO₂ concentration critical for the growth of the yadF mutant. The culture was performed for 18 h at 37°C with shaking at 90 rpm. The atmosphere was ambient air, and the medium was supplied with various concentrations of sodium bicarbonate. As shown in Fig. 2B, more than 1.3% CO₂ in the initial atmosphere is required for the full growth of the mutant after 18 h incubation. The atmospheric CO₂ concentration reached over 15% in 18 h cultivation in every culture condition that enabled the yadF mutant to grow (Fig. 2C).

This evidence reinforces the notion that the CO₂-dependence of S. thermophilum is based on the deficiency of carbonic anhydrase. Presumably, carbonic anhydrase supplies bicarbonate to bicarbonate-dependent enzymes such as phosphoenol pyruvate carboxylase, acetyl-CoA carboxylase, and carbamoylphosphate synthase in non-phototrophic bacteria. The activities of these bicarbonate-dependent enzymes are essential for viability. The evidence will become more solid through study of the effect of the introduction of a carbonic anhydrase gene into S. thermophilum, but currently no genetic manipulation technique for this thermophilic bacterium is available.

Interestingly, there was a marked difference in the threshold CO₂ concentration between S. thermophilum and the yadF mutant of E. coli. This can be explained by the difference in the Km constant of some of the bicarbonate-dependent enzymes. Presumably, it is easy for S. thermophilum to obtain such a low concentration of bicarbonate from the environment. S. thermophilum retains an ABC transporter, possibly specified for bicarbonate transport (STH1969–1971).¹⁰ On the other hand, it can be difficult for E. coli to fulfill the requirement only by utilizing naturally-occurring bicarbonate. This might be the reason this organism retains carbonic anhydrase.
It is assumed that a relatively higher concentration of CO$_2$ and bicarbonate is widely available in the natural environment, as in commensal situations. This might make the carbonic anhydrase dispensable for some kinds of microorganisms living there. If this is the case, it is possible that there exist other microorganisms that also lack carbonic anhydrase but live in relatively high CO$_2$ environments. Such microbes, however, cannot be isolated under normal laboratory conditions since the ambient CO$_2$ and equilibrating bicarbonate are insufficient for the initiation of proliferation of those organisms. The CO$_2$-dependence might have a significant relationship with the issue of the unculturability of microorganisms.

Acknowledgments

We thank Dr. Junichi Kato and Dr. Shinichi Hashimoto for providing $yadF$ mutants and for helpful discussion. This study was supported by the grant-in-aid for scientific research (no. 14360059) and the 21st century COE program of MEXT, Japan.

References


