Thermochimica Acta, 235 (1994) 153–160
Elsevier Science B.V., Amsterdam
SSDI 0040-6031(93)01546-F

Determination of the water necessary for survival of *Bacillus subtilis* vegetative cells and spores

Yoshimi Ishihara [a,*], Jiro Takano [a], Satoru Mashimo [a] and Masaichi Yamamura [b]

[a] *School of Science, Tokai University, 1117 Kaname, Hiratsuka-shi, Kanagawa 259-12 (Japan)*
[b] *Department of Molecular Life Science, School of Medicine, Tokai University, Bohseidai, Isehara-shi, Kanagawa 259-11 (Japan)*

(Received 22 June 1993; accepted 30 July 1993)

Abstract

To determine the minimal amount of water necessary for survival in *Bacillus subtilis*, bound and free water were measured by time domain reflectometry (TDR) and the gel–sol phase transition of bound water was measured by differential thermal analysis–thermogravimetry (DTA–TG). Both the vegetative and the spore form of *Bacillus subtilis* were dried at different temperatures. From the measurements, the relationship between survival of the bacteria and water can be summarized as follows. (1) The vegetative form of bacteria was still 100% viable even when 80% of bound water was removed. (2) The spore form contained only 20% of bound water, which was easily and quickly removed by drying, and resulted in loss of viability. (3) Phase transition between gel and sol phases of bound water occurred repeatedly in the dried vegetative 100%-viable bacteria, which still had 20% bound water. The bacteria were still viable after such measurements. (4) In the spore form, phase transition was not detected.

INTRODUCTION

Some species of bacteria, including *Bacillus* and *Clostridium*, produce spores in the stationary phase of their life cycle [1]. The change of form is to ensure survival during adverse conditions and, hence, the spores do not grow, but have high dormancy as well as extremely high heat resistivity as compared to their vegetative counterparts. The spores which are composed of a cort, cortex and core, are dehydrated from the cells and the spore's cortex contains very little water although the presence of this water assures spore viability. The factors which lead to spore formation are numerous [2–18], but the mechanism of spore formation is not yet clearly understood.

Usually, the spores are formed when there is a plentiful supply of water. The water in the spores is comprised of both free and bound water.

* Corresponding author.

Therefore, it is thought that the spores retain a vital amount of water which can be measured by several methods, including the difference in weight between a hydrated and a dry sample [19], the refractive index of the cells [20], and the dielectric loss and NMR absorption pattern of the cells [21]. It has been reported that bacterial spores have a low water content, as estimated from the isothermal adsorption curve for water vapour [22], and from the refractive index deduced using interference light microscopy and laser light-scattering photometry [23, 24]. But water in cells cannot be measured directly and, from these reports, bound and free water in cells cannot be fully distinguished. Maeda and Koga have measured the thermal proposals of water by differential scanning calorimetry (DSC) [25].

The water volume in spores is a reference of viability. In this study, the free and bound water and the phase transition were measured in the vegetative and spore forms with reference to survival during drying.

MATERIALS AND METHODS

Bacillus subtilis (globigii) was used throughout and was maintained using Bacto Nutrient Broth (Difco Laboratories) medium. To obtain a culture with nearly 100%-viable vegetative bacteria, the bacteria were cultured in Bacto Nutrient Broth for 10–12 h while shaking at 37°C.

100% viable spores were obtained when vegetative bacteria were kept at 37°C in Hanks medium (Nissui Co.) for 24 h and heated at 100°C for 10 min. The Wirtz method of staining [26] was used to distinguish the vegetative and endospore forms by microscopic examination. The number of bacteria and spores were measured by colony formation. To determine the survival of bacteria under dry conditions, briefly, about 100 mg (approx. 1×10^{11}) of bacteria were suspended in distilled water and placed in a desiccator at various temperatures (4, 25 or 37°C). The viability of the bacteria was determined at various intervals. When viable bacteria were counted, free and bound water were measured using the same bacteria. The time domain reflection (TDR) method [27–31], used here, measures free and bound water [32–35] in biological materials such as DNA and proteins; it is basically an application of a sonar principle. Briefly, high-frequency sound is transmitted and the sound is reflected by free and bound water in the materials. The reflection time depends on the restriction in mobility of water molecules. A faster reflection time indicates that water molecules are less mobile, which indicates bound water in biological materials. Free water is reflected slowly because the energy of sound is adsorbed in the movement of water molecules. As the state of free water differs from the state of bound water, the reflection time can be analysed independently. The calculated results presented here are expressed in percentages, with the vegetative form of the bacteria before drying being 100%.

TABLE 1

Relative relaxation strengths of free water and bound water in the bacteria determined by TDR: 100% was taken as the relaxation strength of free water and bound water in the vegetative form. Free water and bound water were measured in spores suspended for 0.5 and 2 h in nutrient broth

	Vegetative	Freeze-dried vegetative	Spore	Freeze-dried spore	Vegetative 0.5 h	Vegetative 2 h
Bound water (%)	100	0.81	22.32	0.36	28.75	92.25
Free water (%)	100	0	82.27	0	75.27	85.52

In bacteria, water in the gel phase interchanges with water in the sol phase depending on the surrounding temperature. This was measured by differential thermal analysis–thermogravimetry (DTA–TG). DTA–TG was performed at a heating rate of 1°C min^{-1} using a thermal analyser (DT-40, Shimadzu Co. Ltd.).

RESULTS

The 100% value of free water was 9.44×10^{-13} g per cell and that of bound water was 3.35×10^{-13} g per cell in vegetative cells. Free and bound water in spores was 82.27% and 22.32%, respectively, as compared with the vegetative form. When the vegetative form and spores were freeze-dried at −50°C and 200 bar, the viability was 100% in both cases; only a little bound water was detected but no free water could be measured (see Table 1).

Free and bound water in the bacteria were measured every 12 h for 96 h during drying at 4, 25 and 37°C. It was clear that as long as more than 20% bound water remained, viability was 100%, regardless of the drying temperature (Fig. 1A and 1B). However, if the bound water was less than 15%, no bacteria could survive.

Figure 2 shows the results for free and bound water in spores measured every 12 h for 96 h during drying at 4, 25 and 37°C. Because spores contained only 22% bound water at the start of drying, bound water was quickly lost with a corresponding loss of viability.

When vegetative bacteria that had los 80% of its bound water and were still alive, were measured by DTA–TG, repeated three times using the same sample, almost identical phase transitions occurred each time (see Fig. 3A). After measurements, viability was tested and found to be 100%. DTA–TG was performed using spores; the results are shown in Fig. 3C and 3D. An indistinct transition phase could be recognized in the first measurement, but no transition could be detected in the following two measurements.

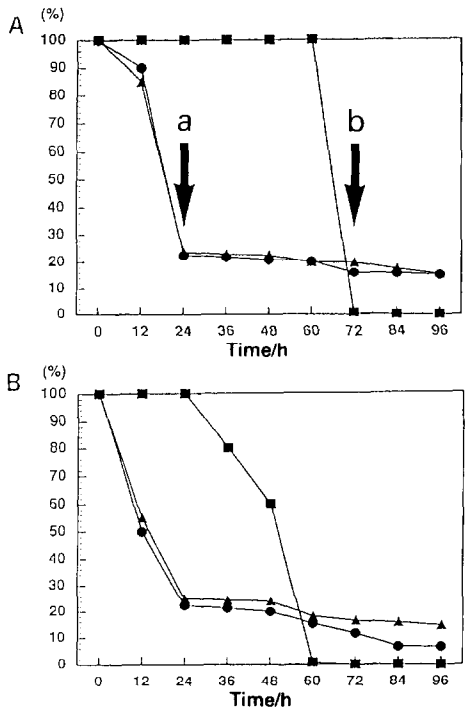

Fig. 1. Vegetative *Bacillus subtilis* (100 mg) was dried for up to 96 h in a desiccator at various temperatures: 4°C (A), 25°C (B) and 37°C (C). The relative relaxation strengths of free water (▲), bound water (●) and viability (■) of the bacteria were measured every 12 h.

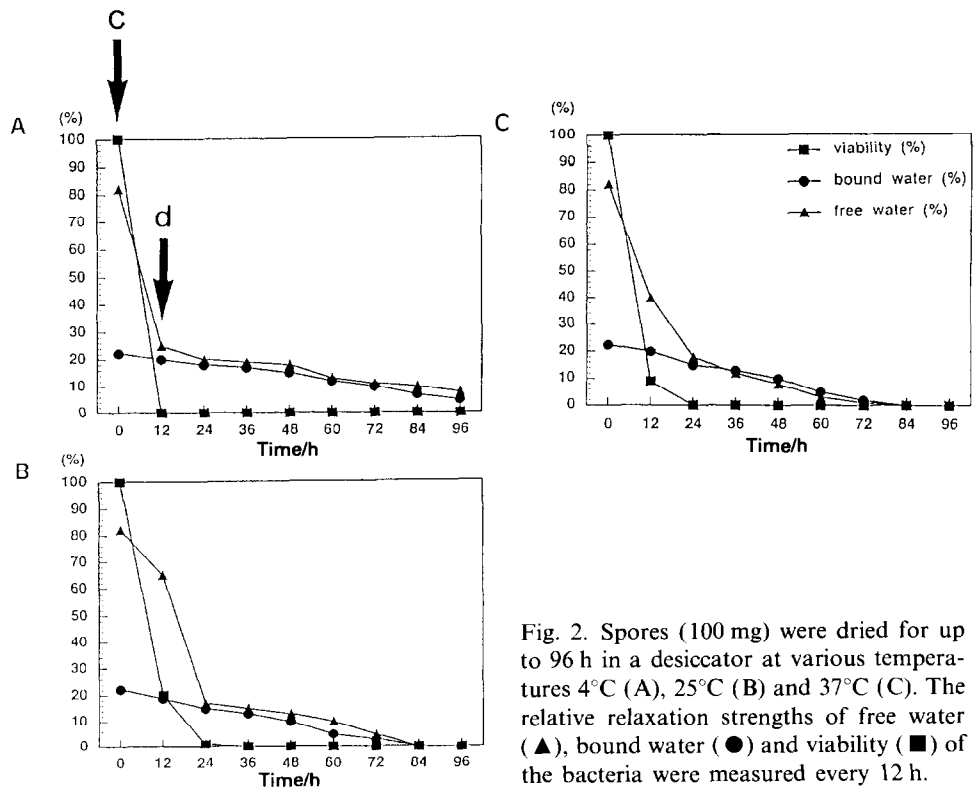

Fig. 2. Spores (100 mg) were dried for up to 96 h in a desiccator at various temperatures 4°C (A), 25°C (B) and 37°C (C). The relative relaxation strengths of free water (▲), bound water (●) and viability (■) of the bacteria were measured every 12 h.

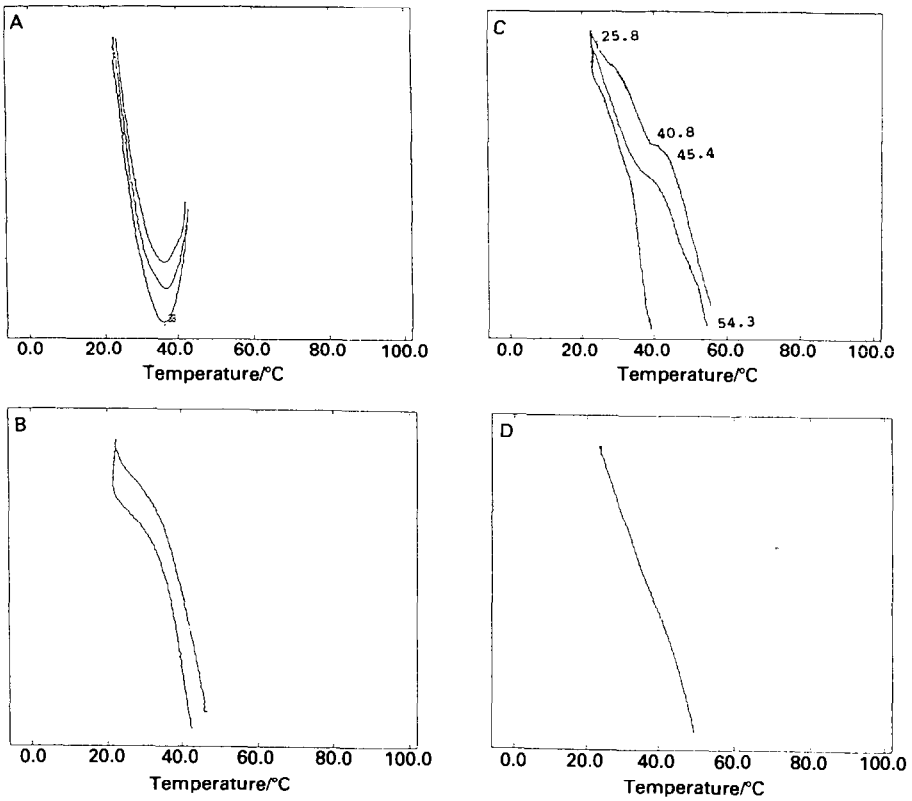

Fig. 3. DTA–TG measurements, in triplicate, for: A, *Bacillus subtilis* vegetative form dried for 24 h at 4°C (Fig. 1A, arrow a); B, *Bacillus subtilis* vegetative form dried for 72 h at 4°C (Fig. 1A, arrow b); C, *Bacillus subtilis* spores, not dried (Fig. 2A, arrow c); D, *Bacillus subtilis* spores dried for 12 h at 4°C (Fig. 2A, arrow d).

The vegetative form was no longer viable but retained 15% bound water and had no phase transition (Fig. 3B).

DISCUSSION

To the best of our knowledge, this is the first report describing the minimum essential bound water and the phase transition of water with exclusive reference to viable *Bacillus subtilis* (globigii).

Spores are formed when harsh conditions are encountered, such as a lack of nutrition for survival. They are, in fact, able to survive even if temperatures reach 100°C. It was surprising to find that the vegetative form survives better than spores in dry conditions. This may be because of the bound water retained in the spores which can easily be removed, because only a minimum amount of essential bound water is present. This lack of bound water is a disadvantage during dryness, but has the advantage of providing insulation in the presence of water. Spores, therefore, can survive higher surrounding water temperatures such as 100°C [36–38].

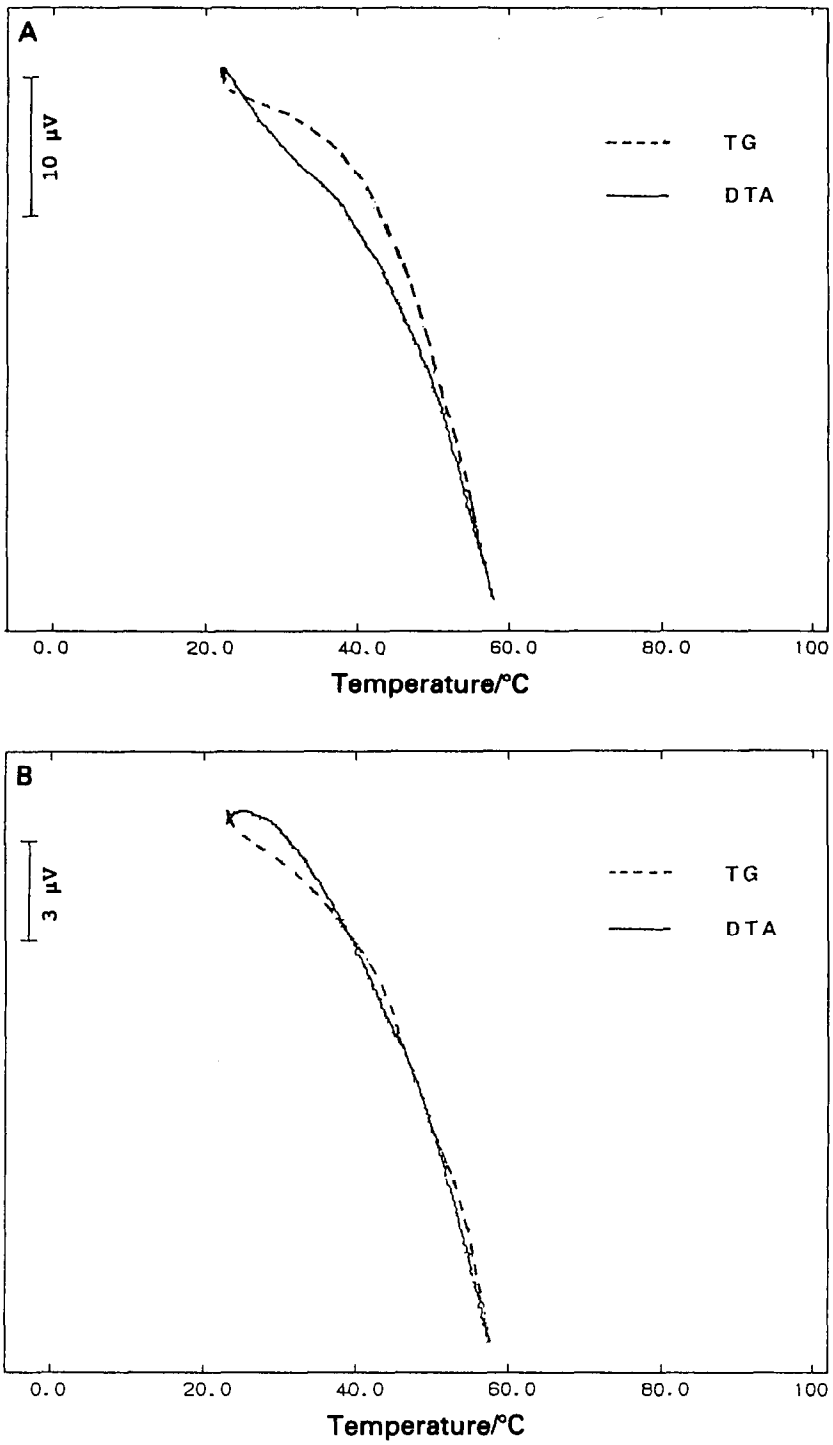

Fig. 4. A. DTA (—) and TG (– – –) curves of the freeze-dried *Bacillus subtilis* vegetative form. B. DTA (—) and TG (– – –) curves of the freeze-dried *Bacillus subtilis* spores.

The 20% level of bound water in spores is curiously identical to the lowest amount of bound water in the dried but living vegetative form. It may be possible that 20% can be defined as the essential level of bound water for survival. To be precise, with 20% bound water, bacteria were 100% viable but no viable bacteria were present with 15% bound water.

The gel–sol transition is clearly demonstrated in the vegetative form (Fig. 3A), but not in the spore form (Fig. 3C and 3D). It is interesting to note that the transition temperature is 37°C. This transition is most likely due to the gel–sol phase of bound water, not the membrane lipids and phospholipids, because the transition temperature of single-layer lipids is 23°C [39–42].

When the bacterial form and the spores were freeze-dried, although almost all the bound and free water were removed (see Fig. 4), the bacteria remained viable. This is because, at extremely low temperatures, the organic molecules within the bacteria are not denatured and keep their structure intact. Therefore, when thawing begins, water can penetrate whenever possible and reinstate the function of the molecules. The transition no longer exists when viability is lost. The minimal amount of essential bound water may thus be necessary to maintain the flexibility of membranes, even in dryness.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Nadia El Borai (M.Sc.) for correcting the English text as well as giving valuable advice.

REFERENCES

1 I.E. Young and P.C. Fitz-James, J. Biochem. Cyt., 6 (1959) 467.
2 J.W. Foster and F. Heiligman, J. Bacteriol., 57 (1949) 613.
3 J.W. Foster and F. Heiligman, J. Bacteriol., 57 (1949) 639.
4 W.A. Hardwick and J.W. Foster, J. Gen. Physiol., 35 (1952) 907.
5 N.D. Gray and R.C. Bard, J. Bacteriol., 64 (1952) 501.
6 D. Nakada, A. Matsushiro and T. Miwatani, Med. J. Osaka. Univ., 6 (1956) 1047.
7 R.E. Strange and F.A. Dark, J. Gen. Microbiol., 16 (1957) 236.
8 N. Grelet, J. Appl. Bacteriol., 20 (1957) 315.
9 N. Grelet, Ann. Inst. Pasteur, 81 (1951) 43.
10 N. Grelet, Ann. Inst. Pasteur, 82 (1952) 66.
11 H. Sugiyama, J. Bacteriol., 62 (1951) 81.
12 R.S. Hanson, V.R. Srinivasan and H.O. Halvorson, J. Bacteriol., 86 (1963) 45.
13 R.S. Hanson, J. Blickarska and J. Szulmajster, Biochem. Biophy. Res. Commun., 17 (1964) 1.
14 H.O. Halvorson, J. Appl. Bact., 20 (1957) 305.
15 H.M. Hakata and H.O. Halvorson, J. Bacteriol., 80 (1960) 801.
16 D.B. Rowley and H.R. Newcomb, J. Bacteriol., 87 (1964) 701.
17 A. Falaschi and A. Kornberg, J. Biol. Chem., 24 (1966) 1478.
18 R. Gardner and A. Kornberg, J. Biol. Chem., 24 (1967) 2383.

19 J.F. Powell and R.S. Strange, Biochem. J., 54 (1953) 205.
20 D.G. Waldham and H.O. Halvorson, Appl. Microbiol., 2 (1954) 333.
21 Y. Maeda, T. Fujita, Y. Sugiura and S. Koga, J. Gen. Appl. Microbiol., 14 (1968) 217.
22 B.J. Marshall and W.G. Murrell, J. Appl. Bacteriol., 33 (1970) 103.
23 K.F.A. Ross and F. Billing, J. Gen. Microbiol., 16 (1957) 418.
24 P.J. Wyatt, J. Appl. Bacteriol., 38 (1975) 47.
25 Y. Maeda and S. Koga, Netsusokutei, 8 (1981) 57.
26 P. Schaeffer, H. Ionesco and F. Jacob, C.R. Acad. Sci., 557 (1959) 249.
27 S. Kuwabara, T. Umehara and S. Mashimo, J. Phys. Chem., 92 (1988) 4839.
28 S. Mashimo, T. Umehara and S. Kuwabara, J. Phys. Chem., 93 (1989) 4963.
29 N. Asaka, N. Shinyashiki, T. Umehara and S. Mashimo, J. Chem. Phys., 93 (1990) 8273.
30 N. Shinyashiki, N. Asaka, S. Mashimo, S. Yagihara and N. Sasaki, Biopolymers, 29 (1990) 1185.
31 T. Umehara, S. Kuwabara, S. Mashimo and S. Yagihara, Biopolymers, 30 (1990) 649.
32 P.S. Belton, R.P. Jackson and K.J. Packer, Biochem. Biophys. Acta, 286 (1970) 16.
33 C.F. Hazlewood, D.C. Chang and B.L. Nichols and D.E. Woessner, Biophys. J., 14 (1974) 583.
34 I.D. Kuntz and W. Kawzmann, Adv. Protein Chem., 28 (1974) 289.
35 R. Cook and I.D. Kuntz, Ann. Rev. Biophys. Bioeng., 3 (1974) 95.
36 G. Alderton and N.S. Snell, Biochem. Biophys. Res. Commun., 10 (1963) 139.
37 W.G. Murrell and A.D. Warth, in L.L. Campbell and H.O. Halvorson (Eds.), Spores III, Am. Soc. Microbiol., Ann Arbor, 1965, pp. 1–24.
38 M.J.M. Michels and F.M.W. Visser, J. Appl. Bacteriol., 1 (1976) 14.
39 D. Chapman, in G.B. Ansell, J.N. Hawthorne and R.H.C. Dawson (Eds.), in Form and Function of Phospholipids, Elsevier Scientific Publishers, Amsterdam, 1973, p. 117–142.
40 P.W.M. Van Dijik, B. De Kruijiff, L.L.M. van Deenen, J. De Gier and R.A. Demel, Biochem. Biophys. Acta, 576 (1976) 455.
41 D.A. Wilkinson and J. F. Nagle, in C.G. Knight (Ed.), Liposomes, Elsevier/North-Holland Biomedical Press, Amsterdam, 1981, p. 273–298.
42 B.A. Smith and H.M. McConnell, Proc. Natl. Acad. Sci. USA, 75 (1978) 2759.