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Heat Resistance of *Desulfotomaculum nigrificans* Spores in Soy Protein Infant Formula Preparations†

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The heat resistance of *Desulfotomaculum nigrificans* spores was determined in soy protein infant formula preparations. Methods of sporulation were developed and evaluated. *D. nigrificans* spores of highest heat resistance were produced in a 40% infusion of spent mushroom compost. Fraction-negative $D_{121°C}$-values obtained in modified soy formula were 25.8 min for spores of ATCC 7946 produced at 55°C and 54.4 min for an isolate designated RGI 1, which was sporulated at 66°C. From the fraction-negative *D*-values, *z*-values were obtained of 6.7°C for ATCC 7946 and 9.5°C for RGI 1. Survivor-curve $D_{121°C}$-values were 5.6 min for ATCC 7946 and 2.7 min for RGI 1 sporulated at 55°C and heated in modified soy formula. Corresponding $D_{121°C}$-values in Butterfield phosphate buffer (pH 7.2) were 3.3 min (ATCC 7946) and 1.1 min (RGI 1). The *z*-values generated from survivor-curve *D*-values were similar to those obtained by using fraction-negative procedures. In all instances the inactivation kinetics appeared to be linear. The isolate designated RGI 1, when sporulated at 66°C and heated in a modified infant soy formula, exhibited an extraordinary heat resistance far in excess of previous reports.

Desulfotomaculum nigrificans (formerly *Clostridium nigrificans*) is a thermophilic, anaerobic sporeformer that uses sulfates, sulfites, and reducible sulfur compounds as terminal electron acceptors, resulting in H_2S production. *D. nigrificans* has a temperature range for growth of 30 to 70°C and an optimum temperature of 55°C (2). In foods, *D. nigrificans* is responsible for sulfur stinker spoilage in canned products. Virtually no work has been done on the heat resistance of *D. nigrificans* spores, although a $D_{120°C}$-value of 2.0 to 3.0 min is commonly accepted in the industry when comparing the heat resistance of spores from various species. No specific sporulation procedure for heat resistance studies could be found in the literature, although Lin and Lin (3) apparently obtained spores using liver broth. Our research was first directed towards determining adequate sporulation procedures for *D. nigrificans*. The second objective was to determine the heat resistance of *D. nigrificans* in soy protein infant formula preparations (soy formula).

MATERIALS AND METHODS

Cultures. Two strains were used to produce spore crops: ATCC 7946, obtained from the American Type Culture Collection, and a natural isolate, designated RGI 1. Stock cultures were kept in ISF broth (see below) at 55°C and transferred every 2 to 4 weeks.

Sporulation. A spent mushroom compost infusion was developed as a sporulation medium. Spent mushroom compost was obtained from the Lehmann Mushroom Company, St. Paul, Minn. This medium was autoclaved for 20 min at 121°C, incubated for 24 h at 55°C, reautoclaved, reincubated, filtered through cheesecloth, reautoclaved, and inoculated. Inoculum was added to the top of freshly heated and cooled flasks. Individual spore crops were prepared as follows.

(i) 7946. A 20% infusion of spent mushroom compost was prepared and sterilized in 500-ml portions. After the filtering procedure, the volume was readjusted to 500 ml. After completion of the sterilization procedure, the medium was tempered to 55°C, transferred to a sterile 500-ml flask, inoculated with 1.0 ml of a 24-h culture of *D. nigrificans* ATCC 7946 grown in BETI broth (see below), overlaid with Vaspar (50% paraffin, 50% mineral oil), and incubated for 14 days at 55°C.

(ii) RGI 66. A 40% mushroom compost infusion was prepared in the same manner as 7946. After tempering, the infusion was inoculated with 5.0 ml of a 7-day-old culture of RGI 1 grown in ISF broth, overlaid with Vaspar, and incubated at 66°C for 15 days.

(iii) RGI 55. A 50% infusion was prepared as for 7946, except the infusion was not diluted back to the original volume. After tempering, 450 ml of infusion was inoculated with 50 ml of RGI 1 (grown in BETI broth), transferred every 48 h for three successive transfers, overlaid with Vaspar, and incubated at 55°C for 14 days.

Liquid media. A broth medium designated ISF broth was developed. This medium utilized a commercial infant soy formula (Isomil; Ross Laboratories, Columbus, Ohio) that was prepared according to di-

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rections on the container and modified by the addition of 0.05% ferric citrate. This medium was commonly distributed in either 10- or 20-ml amounts in test tubes (13 × 100 mm or 18 × 150 mm, respectively) containing a common 4-penny nail. Nails were not cleaned with acid because this did not influence growth. ISF broth and the nail were sterilized together at 121°C for 15 min. BETI (beef extract tryptone iron) broth was prepared by the method of Lin and Lin (3).

Solid media. A solid plating medium designated iso-sulfite agar (ISA) was developed which contained: 180 ml of Isomil concentrate, 200 ml of water, 0.5 g of sodium sulfite, 0.25 g of ferric citrate, and 6.0 g of agar. After sterilization at 121°C for 15 min and tempering to 55°C, thin (approximately 10 ml/plate) ISA plates were poured and overlaid with sulfite agar (Difco). *D. nigrificans* colonies appeared jet black after 48 h of incubation at 55°C under 55 cm Hg vacuum.

Buffers. Sorensen 0.067 M phosphate buffer was prepared by mixing 61.1 ml of a stock solution of 0.067 M disodium phosphate (Na_2HPO_4) with 38.9 ml of a stock solution of 0.067 M monopotassium phosphate (KH_2PO_4). Butterfield phosphate buffer (pH 7.2) was prepared by the method of Speck (4).

Fraction-negative procedures. Fraction-negative studies were performed by heating spores in ISF broth with the nail. To handle spores as anaerobically as possible, tubes were inoculated immediately after sterilization and tempering to 55°C and then were gassed with CO_2. Heating was done in screw cap tubes (13 × 100 mm; 8 per heating time) in miniature retorts (I. J. Pflug, Environmental Sterilization Laboratory, University of Minnesota). No lag-time correction was used because of the extended heating times. After heating, tubes were cooled in ice water, overlaid with Vaspar, preheated to 55°C in a water bath, and incubated at 55°C for 14 to 21 days. Tubes were counted as positive when blackening occurred. Initial spore numbers (N_0) were determined by heat shocking for 15 min at 100°C and performing a three-tube most-probable-number estimation in ISF broth.

Survivor curve procedures. Survivor curves were determined by modifying the procedures that were used for the fraction-negative experiments. Heating was in screw cap tubes (18 × 150 mm) with a lag-time correction of 2.0 min. Survivors were enumerated by using ISA plates. Initial spore numbers were determined by heat shocking a replicate tube (15 min at 100°C) and enumerating as above. Survivor curve studies in buffers were conducted in an identical manner.

Analysis of data. Fraction-negative *D*-values were calculated by the method of Stumbo et al. (5): $D\text{-value} = U/\log A - \log \ln (N/Q)$, where U is the heating time (minutes), A is N_0, N is the total number of replicates, and Q is the number of sterile replicates. *D*-values were calculated for each heating time, and the number of replicate *D*-values were averaged. All other *D*- and *z*-values were calculated by linear regression analysis of the data, using computer programs provided by I. J. Pflug.

RESULTS

With strains ATCC 7946 and RGI 1 we were unable to duplicate Lin and Lin's (3) success of sporulating *D. nigrificans* in liver broth. Unfortunately, it is impossible to determine whether 7946 was used by Lin and Lin. In addition, several other commonly used media and methods for the sporulation and growth of thermophilic anaerobes were examined as possible sporulation procedures for *D. nigrificans*. These included pea broth, thioglycolate broth, BETI broth, and a solid pea-based medium suggested by the National Canners Association. Efforts were also directed at examining the effects of size and age of inoculum on sporulation; no combination of inoculum size or age led to sporulation with these media. At the suggestion of G. M. Evancho (Campbell Institute for Food Research, Camden, N.J.), spent mushroom compost infusions were prepared as sporulation media. Tindalization was used because of the high load of thermophilic anaerobes in the mushroom compost. In general, sporulation in mushroom compost infusion produced approximately 10^6 heat-resistant spores per ml. *D. nigrificans* spores were not easily cleaned without loss of heat resistance and substantial numbers of an already low (10^6/ml) population. Consequently, spore crops were used directly without further treatment.

Figure 1 presents an example of a *D. nigrificans* survivor curve. Spore crop 7946 was heated in soy formula (ISF broth) at 121°C. The *D*-value was 5.6 min and was calculated by linear regression analysis of the data points excluding the N_0. The survivor curve was linear, had no shoulder or tail, and was representative of the other survivor curves. The exception was that some tailing was observed in other survivor curves (not shown).

Figure 2 presents decimal reduction time curves plotted from the survivor curve data for spore crops 7946 and RGI 55. The heat resistance was less in buffer ($D_{121°C}$ = 3.3 and 1.1 min for 7946 and RGI 55, respectively) than in soy formula ($D_{121°C}$ = 5.6 and 2.7 min for 7946 and RGI 55, respectively). The inactivation kinetics were linear. The *z*-values (z = 7.7 and 6.9°C for 7946; z = 9.5 and 8.3°C for RGI 55) appear to be specific for the spore crop, regardless of the heating menstruum, and were similar to the *z*-value of 10°C frequently cited for bacterial spores. Spore crop RGI 66 could not be enumerated by using ISA plates; only fraction-negative experiments could be performed with these spores.

An extended heating time was required when using fraction-negative procedures before any negative tubes were obtained. As an example, for 7946 spores heated in ISF broth at 121°C,

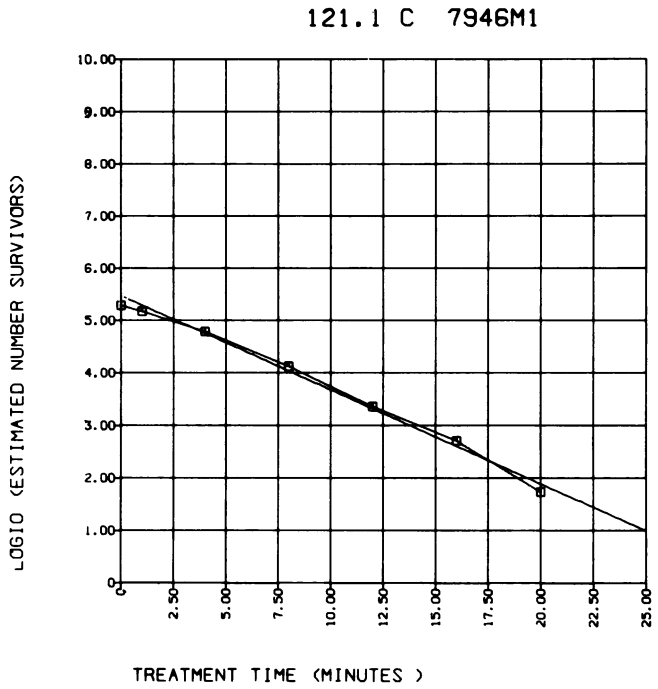

FIG. 1. *A typical survivor curve for ATCC 7946 heated at 121°C in ISF broth. Curve generated by computer. The terminology at the top of the figure is a descriptive code used to identify printouts.*

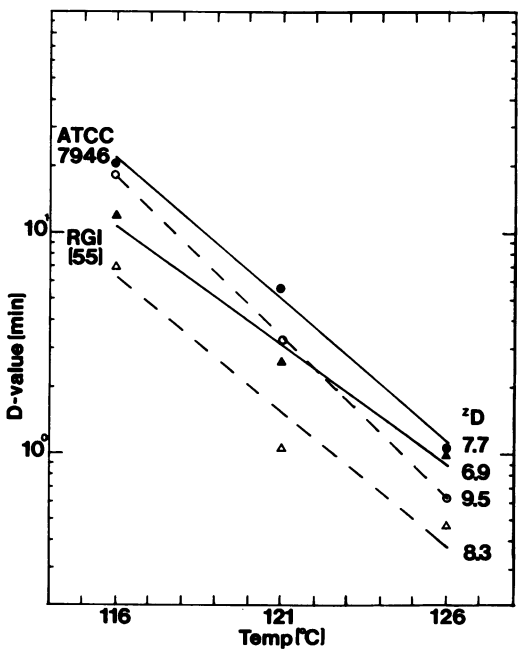

FIG. 2. *Survivor-curve decimal reduction time curves for ATCC 7946 and RGI 55 spores. ●, 7946 heated in ISF broth; ○, 7946 heated in Butterfield phosphate buffer (pH 7.2); ▲, RGI 66 heated in ISF broth; △, RGI 66 heated in Butterfield phosphate buffer (pH 7.2).*

129 min were required before negative tubes were obtained. The last heating time, 138 min, produced only four negative tubes out of eight heated tubes. Ideally, with fraction-negative data one would want all positive tubes for the first few heating times, all negative tubes for the last heating times, and fractions in between. Because of the extremely high heat resistance of these spores, this was not always possible to do.

Figure 3 shows the decimal reduction time curves plotted by using fraction-negative *D*-values; z_D-values were calculated as before. The inactivation kinetics appear to be linear up to 131°C. An extraordinarily high heat resistance was observed for RGI 66. At 121°C RGI 66 had a *D*-value of 54.4 min. (This *D*-value was obtained by using an inoculum diluted 1:10 in Isomil; the other *D*-values were obtained by using undiluted spore crops.) Even at 131°C a *D*-value of 4.8 min was observed. Spores from 7946 also exhibited extreme heat resistance with a *D*-value of 25.8 min at 121°C. The *z*-values (z = 9.5°C and 6.7°C for RGI 66 and 7946, respectively) were similar to those found in the survivor-curve studies.

A comparison of the heat resistance of *D. nigrificans* spores (7946) in various heating menstrua is presented in Table 1. *D*-values were obtained by using survivor-curve methodology. Since extraordinary heat resistance was ob-

served in ISF broth, two of the major ingredients of infant soy formula, sucrose and soy isolate, as well as other levels of solid content were compared with two buffers and distilled water. *D*-values for Butterfield buffer, Sorensen buffer, distilled water, and Sorensen buffer with added sucrose or soy isolate were essentially the same. ISF broth appeared to offer some protection when compared with the buffers. Solid content at the levels examined had no effect on heat resistance.

The extraordinary heat resistance observed for *D. nigrificans* spores was verified through the production and evaluation of four additional spore crops (Table 2). Again, unusually high resistance was obtained for all four new spore crops. The last two digits of the spore crop code refer to the incubation temperature used to produce the spore crop. Spores of high heat resistance were again obtained at an incubation temperature of 55°C during sporulation, as determined by RGI 2 $M_2$55 ($D_{121°C}$ = > 34.0 min) and RGI $M_4$55 ($D_{121°C}$ = 26.8 min).

DISCUSSION

Since *D. nigrificans* commonly inhabits soil and rumen content (1), and horse manure is a common component of mushroom compost, the usefulness of mushroom compost in a sporulation medium is not surprising. It is also consistent with the role of *D. nigrificans* as an important cause of spoilage in canned mushrooms (3). The thermophilic temperatures and potentially anaerobic conditions found in compost would appear to present an ideal ecological niche for *D. nigrificans*. The extremely high heat resistance of *D. nigrificans* spores in soy formula (ISF broth) has not been reported previously. It is apparently a function of the spores and not the heating menstruum (Table 1). Since the $D_{121°C}$-value of 54.4 min for RGI 66 was obtained by using an inoculum diluted 1:10 in Isomil, and this was consistent with the other *D*-values for RGI 66 (Fig. 3) obtained using undiluted spores, it would appear that at least 99.99% of the population had similarly high heat resistance. This is important because fraction-negative methodology would allow the expression of the extreme heat resistance found in a small minority of spores. Spores of *D. nigrificans* undoubtedly vary in heat resistance (Table 2); our data indicate that extreme heat resistance is possible and that infant soy formula allows the extreme heat resistance to be expressed.

The magnitude of the heat resistance for the *D. nigrificans* spores ($D_{121°C}$ of 54.4 min for RGI 66), although extremely large, has some precedent. Xezones et al. (7) demonstrated that a strain of *Clostridium thermosaccharolyticum*

TABLE 1. *A comparison of D-values for ATCC 7946 spores heated in different menstrua*

Heating menstruum	$D_{121°C}$-value (min)
Butterfield buffer (pH 7.2)	3.3
Sorensen buffer (0.067 M, pH 7.0)	3.2
Sorensen buffer plus 4% soy isolate	3.8
Sorensen buffer plus 4% sucrose	4.0
Distilled water	3.4
ISF broth (12% solids-normal)	6.0
ISF broth (18% solids)	7.6
ISF broth (24% solids)	5.6

TABLE 2. *Verification of D. nigrificans spore heat resistance*[a]

Spore crop code	*D*-value (min)
RGI 2 $M_2$55[b]	Est[c] > 34.0
RGI 2 $M_4$66[b]	Est > 34.0
RGI $M_4$55[b]	26.8
RGI $M_4$66[b]	15.6
7946	25.8
RGI 66	54.4
RGI 55	13.0

[a] All heating was done at 121°C by using fraction-negative methodology.

[b] Sporulation was as described in the text for ATCC 7946. Incubation temperature for the new spore crops was either 55 or 66°C, as designated by 55 and 66, respectively, in the spore code.

[c] Est, Estimated value.

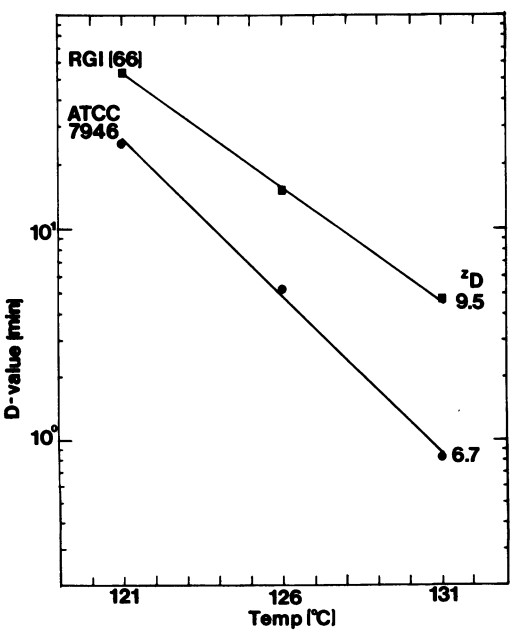

FIG. 3. *Fraction-negative decimal reduction curves for RGI 66 and ATCC 7946 spores heated in ISF broth.*

had a $D_{121°C}$-value in excess of 70 min in distilled water. This organism also is a thermophilic anaerobe. Warth (6) provided data indicating that bacterial sporeformers with higher optimum temperatures for growth (e.g., thermophilic anaerobes) produce spores of greater heat resistance than do sporeformers which grow optimally at lower temperatures. Whereas the high (55°C) optimum temperature of *D. nigrificans* would appear to favor highly resistant spores, incubation of sporulating cultures of *D. nigrificans* at above-optimum temperatures does not appear to play an essential role in developing extreme heat resistance (Table 2).

The *z*-values generated from survivor-curve *D*-values were similar to those obtained by using fraction-negative procedures (Fig. 2 and 3). These decimal reduction curves are linear, and it would appear that the observed extreme heat resistance at ultra-high temperatures is neither the result of an unusually large *z*-value nor the result of nonlinear kinetics at temperatures greater than 121°C. Since the influence of temperature on inactivation (*z*-value) was essentially the same regardless of spore crop, heating menstruum, or survivor enumeration procedure, it would appear that the inactivation event may be similar throughout the test temperature.

The reason for the difference in *D*-values between the survivor-curve (Fig. 2) and fraction-negative data (Fig. 3) was not readily apparent. The survivor-curve methodology includes transfers and use of a solid medium, whereas the fraction-negative methodology utilizes a liquid medium and no transfers. This may account for the differences observed, which include much higher *D*-values for 7946 when fraction-negative procedures were used compared with survivor-curve studies and the inability of RGI 66 spores to be enumerated on ISA plates.

Another explanation could be spore injury. Barach et al. (1) have demonstrated injury and repair of heated *Clostridium perfringens* spores. The relatively long (14-day) incubation time required for recovery of heat-stressed 7946 *D. nigrificans* spores in ISF broth compared with the 48 h used for recovery of spores using ISA may indicate that repair of injured spores occurred during extended incubation or that repair cannot occur in ISA.

The extremely heat-resistant spore crop, RGI 66, could not be enumerated by using ISA. In ISF broth, enumeration of RGI 66 spores receiving only a minimal heat shock required approximately 2 weeks, compared with 48 h for heat-shocked spores of 7946. During fraction-negative studies 7946 spores took approximately 14 days before growth could be observed visually. Perhaps two germination systems are involved, a fast, heat-labile system and a slower but extremely heat-resistant system. Under extreme heat stress the fast-germination system in 7946 spores would be destroyed, leaving only the slow, heat-resistant system, which could be expressed in ISF broth. The possibility then could exist that RGI 66 spores had only the slow, heat-resistant germination system present, which would mean a long incubation time regardless of the heat stress or enumeration procedure. Obviously, additional information must be collected before these hypotheses can be tested conclusively.

The extreme heat resistance of some of the laboratory-produced *D. nigrificans* spores in soy formula indicates the potential for sulfur stinker spoilage in products of this nature, as well as demonstrating that naturally occurring spores may be more heat resistant than previously thought. Work is needed to identify raw ingredients which may contain these spores. Current methodology utilizes a 48-h incubation for enumeration of *D. nigrificans* spores (4). Media and procedures may need to be reexamined in light of the lengthy incubation times needed for the recovery of the slow-growing, extremely heat-resistant strains described in this study.

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