JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1976, p. 432-436

Vol. 4, No. 5
Printed in U.S.A.

Effect of Chilling on the Survival of *Bacteroides fragilis*

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Received for publication 4 June 1976

Factors affecting the susceptibility of *Bacteroides fragilis* subsp. *fragilis* to low temperature were examined. Predetermined numbers of cells were spread on agar media or suspended in enriched Trypticase soy broth and exposed to low temperature under both aerobic and anaerobic conditions. Exposure of 18-h growth of a freshly isolated *B. fragilis* strain to 4°C aerobically or anaerobically resulted in a loss of at least 50% viability after 12 h. *B. fragilis* cells in early growth (6 h) were more tolerant to exposure at 4°C than older cells (18 h). When the freshly isolated strain was repeatedly subcultured in the laboratory it was uniformly more cold tolerant than fresh clinical isolates. The incorporation of 1.0 M sucrose and 5 mM magnesium chloride into liquid media partially alleviated the lethal effects of cold temperature on *B. fragilis* subsp. *fragilis*.

Recent advances in techniques for anaerobic microbiology have contributed significantly to the increased recognition of anaerobes as a cause of human infections. For rapid and accurate diagnosis of anaerobic infections, proper handling of clinical specimens during collection, transport, storage, and culture is essential. However, there seems to be no consensus among authors of various manuals of anaerobic microbiology (2, 3, 7) as to the temperature at which such specimens should be stored. Finegold et al. (3) recommended that if the culture could not be set up within 2 h, specimens should be refrigerated to slow down the growth of facultative species which might be present, whereas others (2, 7) warned that specimens should not be exposed to chilling. This confusion seems to have stemmed from the lack of sufficient information on the effect of chilling on anaerobes in general.

Traci and Duncan (11) demonstrated that *Clostridium perfringens* was highly susceptible to cold shock even under anaerobic conditions, whereas *Bacteroides convexus* (*Bacteroides fragilis* subsp. *fragilis*) was reported to be inactivated progressively at 4°C in the presence of atmospheric oxygen (12).

In the present investigation, we studied the effects of chilling on *B. fragilis* subsp. *fragilis*, one of the most common anaerobes of clinical importance (6), and analyzed factors affecting their cold susceptibility.

MATERIALS AND METHODS

Organisms. *Bacteroides fragilis* subsp. *fragilis* strain 8044, a clinical isolate obtained from J. Vice, Loyola University Medical Center, was used throughout this study. In certain experiments, *B. fragilis* subsp. *fragilis* strains 8268, 7728, 6880, and 2701, all clinical isolates at Loyola University Medical Center, were also used. Immediately after isolation, colonies of each strain were placed in a number of tubes containing 1.0 ml of defibrinated sheep blood and frozen at −60°C for storage. When needed, tubes were removed from storage and thawed. These organisms will be referred to as freshly isolated *B. fragilis*.

Media. Enriched Trypticase soy broth (enriched TSB) contained Trypticase soy broth (BBL) supplemented with 0.5% yeast extract (BBL), 0.05% hemin (Eastman Organic Chemicals Div., Eastman Kodak Co.), and 0.0005% menadione (Sigma Chemical Co.). Enriched Trypticase soy agar (enriched TSA) was prepared by adding 1.5% agar (Difco Laboratories) to the enriched TSB. Mueller-Hinton agar (Difco) and Schaedler agar (BBL), both supplemented with yeast extract, hemin, and menadione as before, as well as blood agar (BBL) were also used in certain experiments. For assay of protection against killing at 4°C, sucrose (Merck & Co., Inc.), calf serum (Flow Laboratories, Inc.), calcium chloride (J. T. Baker Chemical Co.), and magnesium chloride (J. T. Baker Chemical Co.) were added to enriched TSB.

Effect of low temperature on the survival of *B. fragilis*. The effect of low temperature on the survival of *B. fragilis* was studied both on solid media and in liquid media.

(i) Survival of *B. fragilis* on solid media. For determination on solid media, *B. fragilis* was inoculated on enriched TSA plates to approximately 150 cells/plate. A bacterial suspension was appropriately diluted in enriched TSB, and 0.1 ml was spread with a sterile bent glass rod on the surface of the plates. Sets of inoculated plates were placed at a specified temperature both under aerobic and anaerobic conditions. Unless otherwise stated, anaerobic experiments were performed in GasPak (BBL) anaerobe jars. To avoid unnecessary exposure to oxygen, a

separate jar was used for each pair of plates to be removed at different time periods. The GasPak (BBL) anaerobe jars were placed at 37°C for 1 h to obtain complete anaerobiosis, as indicated by complete reduction of the methylene blue indicator, prior to the initiation of the experiment. At appropriate intervals, duplicate plates were removed from each set and incubated anaerobically in the GasPak (BBL) anaerobe jars at 37°C. Colonies were counted 24 h later.

(ii) Survival of *B. fragilis* in liquid media. To test the effect of chilling on *B. fragilis* suspended in liquid media, 50-ml portions of enriched TSB were dispensed into 60-ml squatt dropping bottles. A sufficient number of bottles was prepared so that each bottle would be sampled only once before discarding. Bottles to be used for anaerobic cultures were preincubated overnight at 37°C in a GasPak (BBL) anaerobe jar, while the set to be used for aerobic cultures was stored in an incubator at 37°C. Both sets were allowed to come to room temperature before use. Each bottle was inoculated with 0.5 ml of a diluted culture of *B. fragilis* to obtain a final concentration of approximately 10^5 cells/ml. The anaerobic cultures were immediately flushed with 10% CO_2 + 90% N_2 (anaerobe grade; Benster Specialty Gas Supply Co.) and tightly stoppered, while the aerobic cultures were covered with sterile aluminum foil. All bottles were then placed at the specified conditions. Samples were taken at the designated time periods, serially diluted in enriched TSB, and plated on enriched TSA. The plates were incubated anaerobically at 37°C for 24 h, and colonies were counted as before.

Effect of subculture on cold tolerance of *B. fragilis*. The difference of the cold susceptibility between freshly isolated and repeatedly transferred *B. fragilis* was studied. *B. fragilis* reisolated from the stock that had been frozen in blood immediately after initial isolation was referred to as freshly isolated in our experiments. The same strain of *B. fragilis* transferred 15 times at weekly intervals on blood agar media was referred to as the subcultured strain.

RESULTS

The changes in viability of *B. fragilis* during exposure to chilling (4°C) under aerobic conditions are shown in Fig. 1. The *B. fragilis* cells placed on enriched TSA or in enriched TSB were rapidly killed at 4°C, but there was essentially no loss of viability at 25°C. Similar rapid loss of the viability of *B. fragilis* was observed at 4°C but not at 25°C, when enriched TSA was substituted by other common agar media (Fig. 2). Although data are not shown here, other strains (8268, 7728, 6880, and 2701) of *B. fragilis* exhibited a similar behavior.

To clarify whether this inactivation of *B. fragilis* exposed to atmospheric oxygen at 4°C was due to the lethal effect of a higher oxygen concentration dissolved in the cold media or due to injuries induced by cold temperature, we determined the loss of viability of *B. fragilis* exposed to low temperature (4°C) under both aerobic and anaerobic conditions. It appears that the inactivation of *B. fragilis* was a result of cold injury, since loss of viability occurred at 4°C either in the presence or absence of oxygen (Fig. 3). Under aerobic conditions, cells suspended in liquid media (Fig. 3B) appeared to be more susceptible ($P < 0.005$) to cold temperature than those plated on solid media (Fig. 3A). However, under anaerobic conditions there seemed to be no significant difference in the extent of killing of *B. fragilis* between solid and liquid media (Fig. 3).

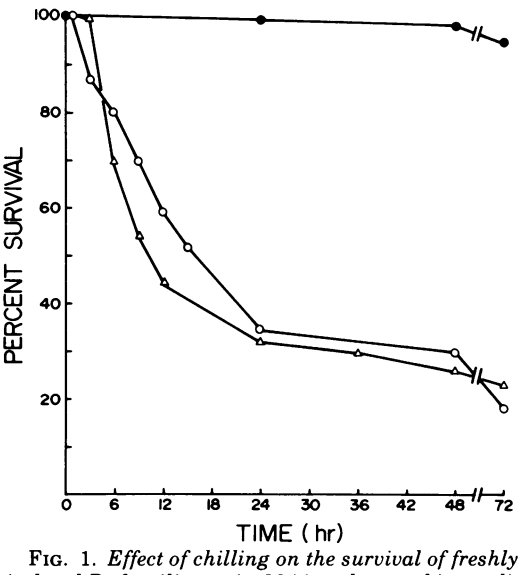

FIG. 1. *Effect of chilling on the survival of freshly isolated B. fragilis strain 8044 under aerobic conditions. Predetermined numbers of 18-h-old B. fragilis cells were plated on enriched TSA (○) or suspended in enriched TSB (△) and then exposed to chilling (4°C) for specified periods under aerobic conditions. The number of surviving cells after exposure to 4°C was determined as described in Materials and Methods. Duplicate sets of inoculated plates (enriched TSA) were left at 25°C (●) under aerobic conditions. In agreement with other workers (1, 8, 10), B. fragilis strain 8044 was shown to be highly tolerant to atmospheric oxygen at 25°C.*

The results of experiments to test the difference in cold susceptibility between 6- and 18-h-old *B. fragilis* cells are summarized in Fig. 4. Under all conditions tested, older cultures (18 h) were more susceptible to chilling than younger cultures (6 h).

The decrease in susceptibility of *B. fragilis* (18-h culture) to chilling after repeated transfers on blood agar medium is shown in Fig 5. *B. fragilis* cells transferred on blood agar more

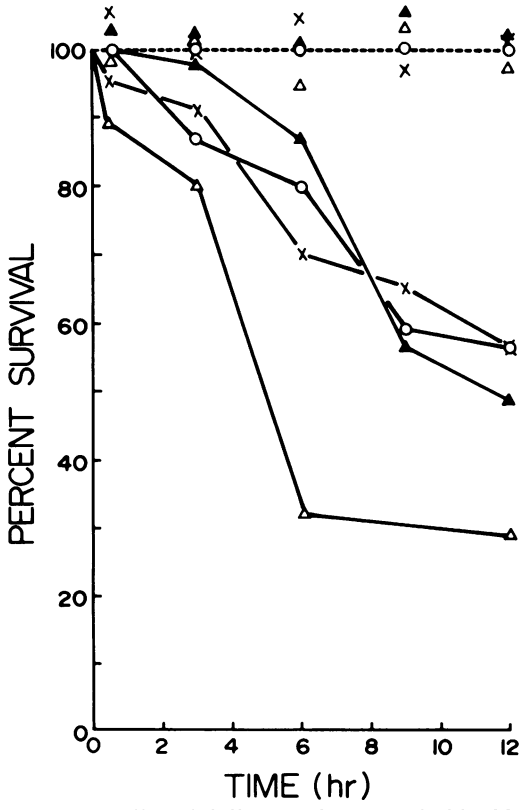

FIG. 2. *Effect of chilling on the survival of freshly isolated B. fragilis strain 8044. Predetermined numbers of 18-h-old cells were plated out on various solid media and exposed to 4 and 25°C for a specified time under aerobic conditions, and the numbers of surviving cells were determined as described in Materials and Methods. Symbols: ▲, Enriched Mueller-Hinton agar; ×, enriched Schaedler agar; ○, enriched TSA; △, commercial blood agar. Solid lines represent the exposure at 4°C, and the dotted line represents the exposure at 25°C.*

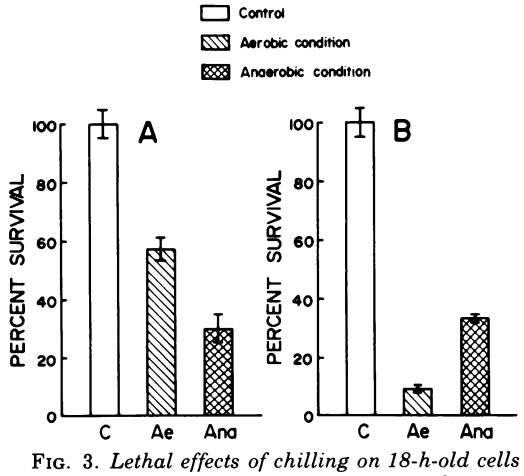

FIG. 3. *Lethal effects of chilling on 18-h-old cells of freshly isolated B. fragilis strain 8044 under aerobic and anaerobic conditions. Predetermined numbers of cells were either suspended in enriched TSA (A) or plated on enriched TSB (B) and exposed to 4°C under both aerobic and anaerobic conditions for 12 h; the numbers of surviving cells were determined as described in Materials and Methods. The controls refer to the number of B. fragilis cells inoculated at time 0. Mean and standard deviation are indicated in each bar.*

than 15 times became significantly ($P < 0.05$) more tolerant to cold temperatures than the fresh isolate.

The detrimental effect of chilling on *B. fragilis* was minimized when cells were suspended in enriched TSB supplemented with 5 mM magnesium chloride and 1.0 M sucrose (Table 1). Calf serum or divalent cations alone added to the enriched TSB did not protect *B. fragilis* from the lethal effect of low temperature.

DISCUSSION

Ideally, clinical specimens likely to contain anaerobes should be cultured immediately after collection from patients (2, 3, 7). However, it is often necessary to transport such specimens to laboratories or store them before they are actually processed. At the moment, there seems to be no concensus among authors of anaerobic laboratory manuals (2, 3, 7) as to the temperature at which these clinical specimens should be stored.

Our data (Fig. 3-5) show that *B. fragilis* was rapidly killed when exposed to chilling under most conditions tested. The killing of *B. fragilis* observed at 4°C does not seem to be due to the oxygen toxicity, because loss of viability occurred at 4°C either in the absence or presence of air (Fig. 3). Thus, it appears that chilling itself exerts a lethal effect on the anaerobes. Traci and Duncan (11) demonstrated earlier that at 4°C, 96% of an initial population of exponential phase *C. perfringens* was killed upon cold shock and 95% of the remaining population was killed within 90 min of continued exposure at 4°C under anaerobic conditions.

Ueno (12) successfully recovered *B. convexus* (*B. fragilis* subsp. *fragilis*) from human pus that had been refrigerated aerobically for as long as 3 weeks. Our data (Table 1) and those of Ueno (12) suggest that certain anaerobes may be able to survive at low temperature for long periods if favorable conditions are provided.

It has been shown that chilling of *Escherichia coli* (5), *Pseudomonas fluorescens* (*pyocyanea*) (4), and *Enterobacter* (*Aerobacter*) *aer-*

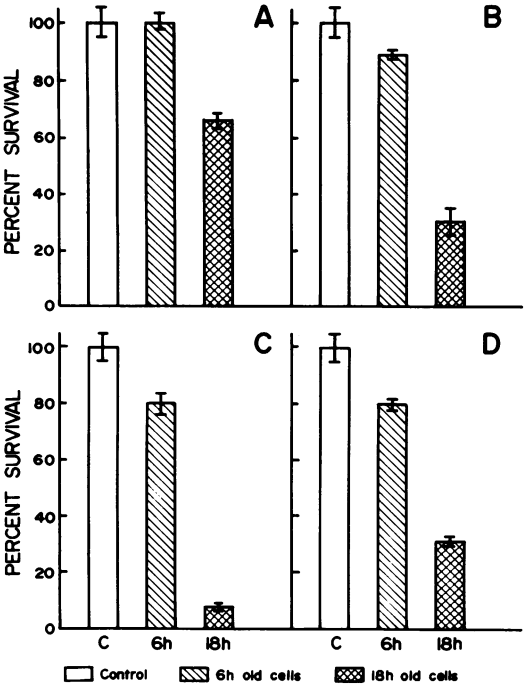

FIG. 4. *Effect of age of B. fragilis strain 8044 on the cold susceptibility. Predetermined numbers of 6- and 18-h-old B. fragilis (freshly isolated) were either plated on enriched TSA (A and B) or suspended in enriched TSB (C and D) and exposed at 4°C for 12 h, under either aerobic conditions (A and C) or anaerobic conditions (B and D). The numbers of surviving cells were determined as described in Materials and Methods. The controls represent the number of B. fragilis cells inoculated at time 0. Mean and standard deviation are indicated on each bar.*

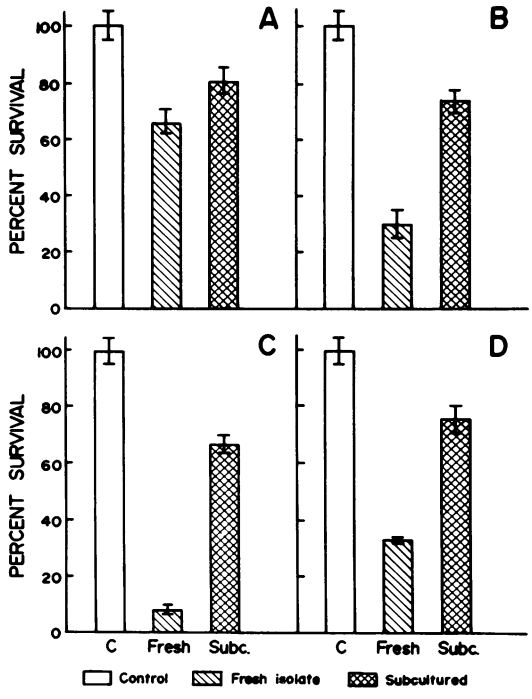

FIG. 5. *Effect of subculture on the cold suceptibility of B. fragilis strain 8044. Predetermined numbers of either freshly isolated or repeatedly subcultured B. fragilis (both were 18-h cultures) were either plated out on enriched TSA (A and B) or inoculated in enriched TSB (C and D) and exposed at 4°C under both aerobic (A and C) and anaerobic (B and D) conditions. The number of surviving cells after 12 h of exposure was determined as described in Materials and Methods. The controls represent the number of cells inoculated at time 0 (approximately 10^5 cell/ml final concentration). Mean and standard deviation are shown on each bar.*

ogenes (9) was accompanied by bacterial death. Strange and Dark (9) demonstrated that chilling was lethal to *E. aerogenes* to varying degrees, depending on the phase of growth, the diluent, and the concentration of bacteria. A leakage of endogenous constituents was thought to be responsible for the lethality in these organisms. Whether a similar mechanism may be responsible for the killing of *B. fragilis* and *C. perfringens* remains to be seen. In any event, the alleviation of the detrimental effects of chilling on *B. fragilis* subsp. *fragilis* strain 8044 by the addition of 1.0 M sucrose and 5 mM magnesium chloride (Table 1) may have important practical implications and merits further investigation.

TABLE 1. *Effect of selected compounds on the survival of B. fragilis at 4°C in enriched TSB*[a]

Stored in enriched TSB plus:	% Survival after 12 h
None	10
1 mM $MgCl_2$	4
5 mM $MgCl_2$	13
10 mM $MgCl_2$	6
5 mM $CaCl_2$	9
5 mM $MgCl_2$ + 0.1 M sucrose	11
5 mM $MgCl_2$ + 0.5 M sucrose	48
5 mM $MgCl_2$ + 1.0 M sucrose	97
5 mM $CaCl_2$ + 0.5 M sucrose	30
10% Calf serum	8
20% Calf serum	9
50% Calf serum	11

[a] Enriched TSB consisted of TSB supplemented with yeast extract (0.5%), hemin (0.05%), and menadione (0.0005%).

ACKNOWLEDGMENTS

This work was supported in part by a grant from Eli Lilly & Co., Indianapolis, Ind., and by general research grant no. RR05368 from the Stritch School of Medicine.

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