Applied and Environmental Microbiology, Nov. 1996, p. 4229–4232 Vol. 62, No. 11
0099-2240/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Epidemiological Typing of *Bacillus* spp. Isolated from Food

HEIDI SCHRAFT,[1] MARINA STEELE,[2] BRUCE McNAB,[2] JOSEPH ODUMERU,[2]
and MANSEL W. GRIFFITHS[1]*

Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1,[1] and Agriculture and Food Laboratory Services Centre, Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, Ontario N1H 8J7,[2] Canada

Received 15 March 1996/Accepted 4 September 1996

Biotypes, fatty acid profiles, and restriction fragment length polymorphisms of a PCR product (PCR-RFLP of the cereolysin AB gene) were compared for 62 isolates of the *Bacillus cereus* group. Eleven isolates originated from various foods, and 51 isolates were obtained from pasteurized milk which had been processed by two different dairies. The isolates were clustered into 6 biotypes, 10 fatty acid groups, or 7 PCR-RFLP clusters. Isolates with mesophilic or psychrotrophic characteristics were preferentially distributed into specific fatty acid or PCR-RFLP groups ($P = 0.004$). Unique fatty acid clusters were predominantly found in milk samples of each dairy ($P < 0.0001$), suggesting that certain dairy plants may harbor plant-specific *B. cereus* which might constantly contribute to postpasteurization contamination.

Improving microbial safety and extending the shelf life of pasteurized milk and related products have always been an important concern to the dairy industry. A major factor limiting realization of these goals is microorganisms surviving the pasteurization process and/or contributing to postpasteurization contamination. *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* are frequently found in pasteurized milk, causing spoilage because of the production of lipases and proteases. They can also exhibit a health risk to the consumer since they produce enterotoxins (5, 9). It has been proposed to merge *B. cereus*, *B. thuringiensis*, and *B. mycoides* into one single species. DNA-DNA hybridization experiments and pulsed-field gel electrophoresis of chromosomal DNA demonstrated a very close genetic relationship among the three species (1, 4). Furthermore, the 16s rRNA sequences of these species are more than 99% similar (1). In the present article, *B. cereus*, *B. thuringiensis*, and *B. mycoides* are, therefore, not distinguished and are referred to as *B. cereus.*

Many dairy scientists believe that contamination of pasteurized milk with *Bacillus* spp. results from spores that were present in the raw milk and survived the pasteurization process (7, 14). However, other data in the literature suggest that postpasteurization contamination might significantly contribute to *Bacillus* counts in pasteurized milk (5, 6). This controversy can partly be related to the lack of adequate typing methods for *B. cereus*. Ternström et al. (15) described an attempt to use numerical phenotypic analysis for characterization of dairy *Bacillus* spp. However, the authors did not show any relationship between *Bacillus* isolates from raw and pasteurized milk. Väisänen et al. (17) used fatty acid analysis and phages to type dairy *B. cereus* isolates. Although they found both techniques useful for typing of *B. cereus*, the authors did not systematically compare *B. cereus* isolated throughout the processing lines of single dairies. Thus, their data do not allow conclusions about the origin of *B. cereus* found in pasteurized milk. The objective of the present study was to evaluate the feasibility of three typing techniques for *B. cereus*. The techniques chosen were biotyping, analysis of cellular fatty acids (CFA), and restriction fragment length polymorphisms of PCR products (PCR-RFLP). To allow direct comparison, the three techniques were applied to a defined collection of *B. cereus* strains. Because a large proportion of the *Bacillus* strains studied were isolated from pasteurized milk, additional epidemiological data for dairy *Bacillus* spp. were also acquired.

Bacterial strains. A total of 62 isolates of *B. cereus* were used. Eleven of these isolates, originating from food-borne *B. cereus* outbreaks and from randomly collected milk samples, were obtained from our culture collection. The remaining 51 isolates were cultured from 23 retail cartons (250 ml and 500 ml) of pasteurized milk that had been processed by two different dairies. Isolation procedures followed the methods described in the Association of Official Analytical Chemists' *Bacteriological Analytical Manual* (2), except that *B. cereus* selective agar (Oxoid) supplemented with polymyxin B (Oxoid) and egg yolk emulsion (Oxoid) was used as selective plating medium.

Growth of *B. cereus* at 8°C. Growth curves for the 62 *B. cereus* isolates were determined with the multiwell photometric plate reader Bioscreen C (Labsystems Corp., Helsinki, Finland). Bacteria were grown for 18 h in brain heart infusion broth (Difco) at 30°C and were diluted in fresh brain heart infusion broth to an optical density at 600 nm (OD_{600}) of 0.005, and the suspensions were used to inoculate the multiwell plate of the Bioscreen C. The turbidity development (OD_{600}) of the suspended bacteria at 8°C was recorded for 144 h. Growth curves (i.e., turbidity development curves) were subsequently generated from the OD readings by using the computer software BIOLINK (Labsystems Corp.). Preliminary experiments had shown that growth velocity at 8°C can be used to divide mesophilic and psychrotrophic *B. cereus* strains by recording the detection time for each strain. The detection time is defined by the BIOLINK software as the time at which an OD of 0.05 is reached. The detection time of psychrotrophic *B. cereus* isolates is less than 100 h, whereas it is considerably higher than 100 h for mesophilic isolates.

Biochemical properties. Twenty-one biochemical variables of the 62 *B. cereus* isolates were determined by using the VITEK Jr. system (Biomérieux Vitek Inc., Hazelwood, Miss.) according to the manufacturer's instructions. From the biochemical patterns obtained, a binary profile was established for each isolate. The distances between these profiles were calculated by Jaccard's index [$d = 1 - c/(p + q + c)$], where c is the number of variables present in both strains, and p and q are the

* Corresponding author. Mailing address: Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Phone: (519) 824-4120, ext. 2269. Fax: (519) 824-6631. Electronic mail address: mgriffiths@aps.uoguelph.ca.

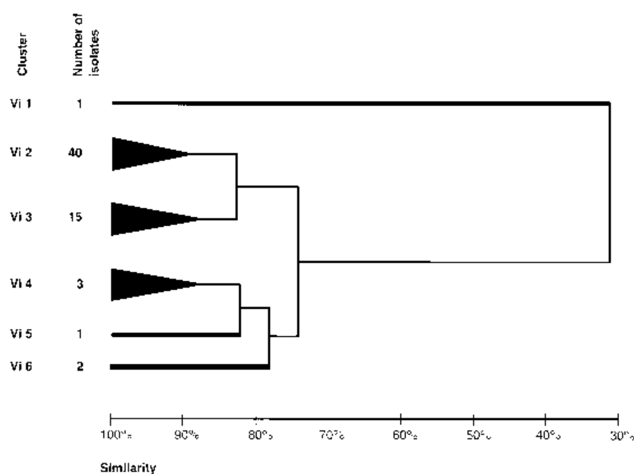

FIG. 1. Dendrogram for biochemical data of 62 *B. cereus* isolates. Constructed from 21 binary characteristics by using Jaccard's index and UGPMA.

numbers of variables present in each strain. A dendrogram (Fig. 1) was constructed from the resulting distance matrix by using the unweighted pair-group method with arithmetic averages (UGPMA [12]). Calculations were made with the computer programs MacDendro and MacMul (16).

PCR-RFLP. It has been demonstrated that the lecithinase and sphingomyelinase genes (*cerAB*) of *B. cereus* display genetic variations among different isolates (13). This diversity and its possible application for typing of *B. cereus* were further explored in the present study. A PCR product encompassing a 1.5-kb fragment of the *cerAB* gene was used to assess the genetic diversity of the 62 *B. cereus* isolates at that locus. PCR amplifications with primers specific for *cerAB* were performed as described previously (13). Ten-microliter aliquots of each amplicon were digested for 2 h with 10 U of the following restriction endonucleases: *Mnl*I, *Hga*I, *Sau*$_{96}$I (New England Biolabs Ltd., Mississauga, Ontario, Canada), *Pvu*II, and *Bst*XI (Boehringer, Mannheim, Germany). Reaction conditions were set as recommended by the manufacturers of the enzymes. RFLP profiles were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. A binary matrix of the profiles was used to calculate the distances between strains by Jaccard's index. A dendrogram (Fig. 2) was constructed from the distance matrix by UGPMA as described above.

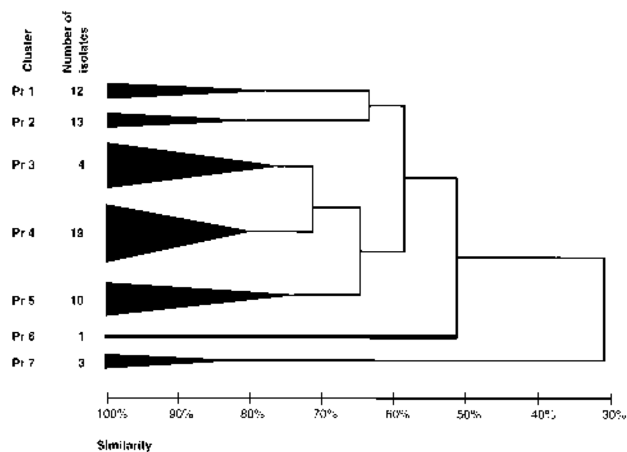

FIG. 2. Dendrogram for PCR-RFLP data of 62 *B. cereus* isolates. Constructed from 22 binary characteristics by using Jaccard's index and UGPMA.

Analysis of whole-cell fatty acid profiles with the MIDI system. Fatty acid methyl esters of *B. cereus* were analyzed by using the Microbial Identification System (MIS; Microbial ID, Inc. [MIDI], Newark, Del.). The analysis was conducted according to the instructions given by MIDI (10). Briefly, isolates were grown on Trypticase soy agar (BBL) for 24 ± 1 h at 28 ± 1°C. At that time, 45 ± 1 mg (wet weight) of cells was harvested from single colonies. Total fatty acids of the bacteria were converted to fatty acid methyl esters which were subsequently separated and quantified with a Hewlett-Packard 5890 series II gas liquid chromatography column equipped with a flame ionization detector. A dendrogram based on the fatty acid profiles was established for 137 *B. cereus* isolates (62 isolates described in this study plus 75 *B. cereus* isolates isolated from various foods) by using the MIS library generation software. This program calculates a similarity coefficient based on the Euclidean distances (ED) between pairs of isolates. Clustering of the isolates is determined by UGPMA (10, 12).

The phenotypic expression of relative amounts of fatty acids within bacterial cells depends on various factors, which include medium composition, growth temperature, and growth rate (8, 18). To determine an ED value that is not affected by such variations in CFA and that could therefore be used to obtain a reproducible grouping of the *B. cereus* isolates within the dendrogram, the variability of the fatty acid profile analysis was assessed by repeated testing of *B. cereus* ATCC 14579. Repeated testing was conducted during a 3-week period in the following manner. Two separate batches of medium plates were prepared independently and stored at 4°C. During each week, the test strain was subcultured daily onto fresh Trypticase soy agar plates. The second, fourth, and sixth subcultures were analyzed. Each of the subcultures tested was streaked onto duplicate plates of both batches of media. Each preparation of fatty acid methyl esters was split into two subsamples, and the fatty acid profiles were analyzed separately with the MIS. ED were calculated for the following datum pairs: (i) duplicate readings of 33 identical sample preparations, (ii) sample preparations from 33 identical subcultures streaked onto duplicate plates, (iii) sample preparations from 33 identical subcultures grown on two different batches of media, (iv) 43 pairs of sample preparations obtained from common subcultures grown during each week (e.g., second, fourth, or sixth subculture obtained in the 1st and 2nd, 2nd and 3rd, or 1st and

TABLE 1. Summary of ED for fatty acid profiles obtained by repeated measurements of *B. cereus* ATCC 14579

Treatment[a]	*n*	ED				
		Mean	SD	CV%[b]	Range	95th percentile
Duplicate readings of identical sample preparations	33	0.58	0.16	28	0.34–1.02	0.92
Duplicate plates	33	2.78	1.56	56	0.64–6.04	6.00
Two batches of media	33	3.47	1.82	52	0.89–6.73	6.70[c]
Common subcultures grown during 3 different wks	43	3.09	1.66	54	0.62–6.55	6.46
Serial subcultures grown during 1 wk	44	3.25	2.29	70	0.91–9.61	9.14

[a] Refer to the text for details of treatments.
[b] CV%, coefficients of variation.
[c] ED value used to group isolates into MIDI clusters.

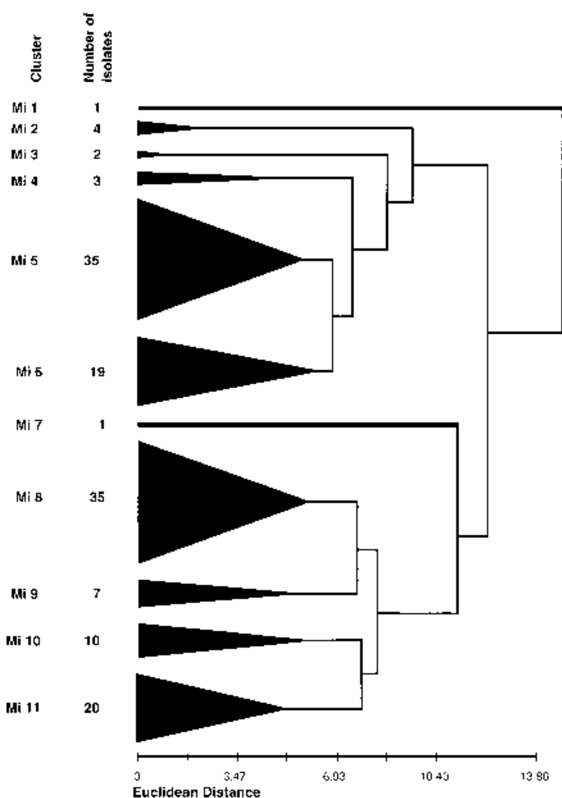

FIG. 3. Dendrogram depicting subgrouping and relative relatedness of 137 *B. cereus* isolates.

3rd weeks), and (v) forty-four pairs of sample preparations obtained from serial subcultures grown during each week (e.g., second and fourth, fourth and sixth, or second and sixth subcultures obtained in each week).

Table 1 summarizes the means, standard deviations, coefficients of variation, ranges, and 95th percentiles for the distributions of these ED. In this study, identical subcultures of the *B. cereus* isolates were used to generate the fatty acid profiles. The critical value observed with two batches of media (ED, 6.7) could, therefore, be used to group the 137 *B. cereus* isolates, resulting in 11 fatty acid clusters (Fig. 3). Although the MIS was originally designed for species identification, it has been suggested that it may also be valuable for epidemiological typing of bacteria (3, 11). Consequently, the datum library generated with 137 *B. cereus* isolates was used to evaluate the relationships of the 62 *B. cereus* isolates. The fatty acid profiles of these isolates were attributed to 10 (Mi 2 to Mi 11) of the 11 *B. cereus* fatty acid groups.

TABLE 2. Correlation between growth characteristics and MIDI clusters

Growth behavior	No. of isolates belonging to MIDI clusters		Total no.
	Mi 2 to Mi 6	Mi 7 to Mi 11	
Psychrotrophic	36	3	39
Mesophilic	1	22	23
Total	37	25	62

TABLE 3. Correlation between growth characteristics and PCR-RFLP clusters

Growth behavior	No. of isolates belonging to PCR-RFLP cluster			Total no.
	Pr 1 or Pr 2	Pr 3, Pr 4, or Pr 5	Pr 6 or Pr 7	
Psychrotrophic	10	26	3	39
Mesophilic	15	7	1	23
Total	25	33	4	62

Correlations. Comparison of growth characteristics and fatty acid profiles of the 62 isolates showed that psychrotrophic isolates were preferentially ($P < 0.0001$) grouped into fatty acid clusters Mi 2 to Mi 6, whereas mesophilic isolates belonged to fatty acid groups Mi 7 to Mi 11 (Table 2). A similar tendency ($P = 0.004$) was perceived when growth temperature and PCR-RFLP groups were compared (Table 3). No association could be observed among biotypes and growth characteristics, PCR-RFLP groups, or fatty acid groups. Evaluation of the fatty acid groups for the 51 *B. cereus* isolates from pasteurized milk revealed interesting tendencies. A majority of the isolates found in samples processed by dairy A belonged to the subgroup Mi 5, whereas isolates found in milk from dairy B belonged to groups Mi 6 and Mi 8 ($P < 0.0001$). Väisänen et al. (17) exploited phage typing, minimum growth temperature, and analysis of CFA for differentiation of dairy *B. cereus* isolates. They found a correlation of fatty acid composition and minimum growth temperature; however, they did not examine the fatty acid data with regard to further typing of the isolates. Our results confirm the observations regarding composition of CFA and ability to grow at low temperatures. Additionally, we found indications that psychrotrophic *B. cereus* strains with a specific CFA profile were predominantly found in pasteurized milk processed by one dairy, while they were less frequently found in milk from the other processing plant. This plant-specific distribution of *B. cereus* in pasteurized milk suggests that the strains may originate from common reservoirs within dairy plants or within certain farms supplying raw milk.

This research was funded by the Ontario Food Quality and Safety Research Fund, by the Dairy Farmers of Ontario, and by the Natural Sciences and Engineering Research Council of Canada.

H.S. was supported by a fellowship from the Commission for the Advancement of Young Scholars, Zürich, Switzerland.

REFERENCES

1. **Ash, C., J. A. E. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins.** 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. **41:**343–346.
2. **Association of Official Analytical Chemists.** 1992. Bacteriological analytical manual, 7th ed. Association of Official Analytical Chemists, International, Arlington, Va.
3. **Birnbaum, D., L. Herwaldt, D. E. Low, M. Noble, M. Pfaller, R. Sherertz, and A. W. Chow.** 1994. Efficacy of microbial identification system for epidemiologic typing of coagulase-negative staphylococci. J. Clin. Microbiol. **32:**2113–2119.
4. **Carlson, C. R., D. A. Caugant, and A. B. Kolstø.** 1994. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. **60:**1719–1725.
5. **Champagne, C. P., R. R. Laing, D. Roy, A. A. Mafu, and M. W. Griffiths.** 1994. Psychrotrophs in dairy products: their effects and their control. Crit. Rev. Food Sci. Nutr. **34:**1–30.
6. **Crielly, E. M., N. A. Logan, and A. Anderton.** 1994. Studies on the *Bacillus* flora of milk and milk products. J. Appl. Bacteriol. **77:**256–263.
7. **Griffiths, M. W., and J. D. Phillips.** 1990. Incidence, source and some properties of psychrotrophic *Bacillus* spp. found in raw and pasteurized milk. J. Soc. Dairy Technol. **43:**62–66.

8. **Juneja, V. K., and P. M. Davidson.** 1993. Influence of temperature on the fatty acid profile of *Listeria monocytogenes*. J. Rapid Methods Automat. Microbiol. **2:**55–71.
9. **Kramer, J. M., and R. J. Gilbert.** 1989. *Bacillus cereus* and other *Bacillus* species, p. 21–70. *In* M. P. Doyle (ed.), Foodborne bacterial pathogens. Marcel Dekker, New York.
10. **Microbial ID, Inc.** 1993. Microbial identification system operating manual, version 4. Microbial ID, Inc., Newark, Del.
11. **Mukwaya, G. M., and D. F. Welch.** 1989. Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. J. Clin. Microbiol. **27:**2640–2646.
12. **Romesburg, H. C.** 1984. Cluster analysis for researchers. Lifetime Learning Publications, Belmont, Calif.
13. **Schraft, H., and M. W. Griffiths.** 1995. Specific oligonucleotide primers for detection of lecithinase-positive *Bacillus* spp. by PCR. Appl. Environ. Microbiol. **61:**98–102.
14. **Sutherland, A. D., and R. Murdoch.** 1994. Seasonal occurrence of psychrotrophic *Bacillus* species in raw milk, and studies on the interactions with mesophilic *Bacillus* spp. Int. J. Food Microbiol. **21:**279–292.
15. **Ternström, A., A.-M. Lindberg, and G. Molin.** 1993. Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. J. Appl. Bacteriol. **75:**25–34.
16. **Thioulouse, J.** 1990. MacMul and GraphMu: two Macintosh programs for the display and analysis of multivariate data. Comput. Geosci. **16:**1235–1240.
17. **Väisänen, O. M., N. J. Mwaisumo, and M. S. Salkionja-Saonen.** 1991. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. J. Appl. Bacteriol. **70:**315–324.
18. **Welch, D. F.** 1991. Applications of cellular fatty acid analysis. Clin. Microbiol. Rev. **4:**422–438.