APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1989, p. 778–787
0099-2240/89/040778-10$02.00/0

Vol. 55, No. 4

Microcolony Epifluorescence Microscopy for Selective Enumeration of Injured Bacteria in Frozen and Heat-Treated Foods

UBALDINA M. RODRIGUES AND ROHAN G. KROLL*

Department of Microbiology, AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT, United Kingdom

Received 21 July 1988/Accepted 3 January 1989

A rapid (<6 h) method for selectively enumerating coliforms, pseudomonads, and staphylococci has been developed which involves counting microcolonies grown on the surface of polycarbonate membranes under selective conditions. The method was not directly applicable to foods containing injured bacteria due to the poor formation of or an inability to form microcolonies under selective conditions. However, the introduction of a 3- to 5-h resuscitation step in tryptone soya broth allowed the method to give reliable estimates of these organisms in a variety of frozen and heat-processed foods. Under nonselective conditions, i.e., for total counts, the microcolony method enabled a rapid count to be made of viable bacteria in heat-treated foods, but these results were also made more consistent by the introduction of a resuscitation step. This method makes results from these foods available far faster than conventional enumeration methods.

Many types of physical or chemical stresses are used in the food and other industries to eliminate or control the growth of microorganisms in products. This often results in the sublethal injury of cells which are unable to grow under certain environmental conditions and renders the cells susceptible to secondary stresses such as the selective agents commonly used in media for their enumeration (9). These organisms can pass undetected during routine microbiological examinations but, because they are capable of recovery and growth following the removal of the stress, they can represent a considerable health hazard (2). It is thus essential to use enumeration media that allow the growth of both injured and noninjured cells (4, 15). However, this is commonly not possible, and a preincubation step in a nonselective medium must be included to allow recovery before exposing the cells to the selective media (13, 15).

With perishable items like foods, it is also extremely important that more rapid methods of analysis be developed to allow faster product release to the market place and identification of hygiene problems. One such method is the direct epifluorescent filter technique (DEFT; 16–18), which can be used to give a very rapid (<0.5 h) and reliable estimate of total bacterial counts in a variety of raw foods. However, the method cannot be used with foods that have been heat treated because of unreliable differential staining of heat-treated bacteria. Furthermore, DEFT cannot discriminate between the types of bacteria present. Membrane filter methods would seem to be particularly suitable for the enumeration of injured organisms because of the ease with which, once they are concentrated on the surface of the membrane, transfer from nonselective to selective media can be made. This has been exploited for the enumeration of specific types of organisms by the hydrophobic membrane filter method (8), but despite many advantages, such as convenience, this method can hardly be described as rapid.

Recently, we described a method which combined aspects of both the DEFT and hydrophobic membrane filter method approaches (22). This microcolony epifluorescence microscopy (MEM) method involved filtering a nonenzyme detergent-treated sample and transferring the membrane carrying the bacteria to the surface of a selective agar. The membranes were incubated for 3 h (for media selective for gram-negative bacteria) or 6 h (for gram-positive bacteria), after which the membranes were stained with acridine orange and the microcolonies (μ-colonies) that had formed were counted by using epifluorescence microscopy. This method was shown to reliably estimate the numbers of pseudomonads, coliforms, or staphylococci in pure cultures and in a variety of raw foods within one working day. However, some variability was observed in results from frozen foods, mainly due to a slight undercounting in a few samples. This was likely because of sublethally injured bacteria having insufficient time to repair and form μ-colonies or because they were completely unable to do so on selective media. It could be predicted that similar problems might occur with heat-treated foods, but there are no data concerning this at present.

This paper examines the ability of heat- and freeze-injured bacteria to form μ-colonies on some selective media and the use of a resuscitation step prior to enumeration by MEM and plate count methods. The possibility of using the MEM method for selective enumeration in frozen and processed foods is investigated.

MATERIALS AND METHODS

Bacterial cultures and growth and injury conditions. Pure cultures of *Escherichia coli* NCDO 902, *E. coli* NCDO 2328, *Pseudomonas fragi* NCDO 2755, *Pseudomonas stutzeri* HRI B414 (M. R. W. Griffiths, Hannah Research Institute, Ayr, Scotland), *Micrococcus* sp. strain M3 (laboratory of the author), *Staphylococcus aureus* NCDO 949, and *S. aureus* NCDO 1022 were maintained on slopes of nutrient agar (NA; Oxoid Ltd., London, England) by storage at 5°C after overnight growth at 30°C. Broth cultures were obtained by overnight growth in yeast dextrose broth (1% Peptone, 0.8% beef extract, 0.5% NaCl, 0.5% glucose, 0.3% yeast extract, [pH 6.8]) which were then inoculated into 100 ml of yeast dextrose broth in 250-ml conical flasks which were shaken continuously in an Orbital Mixer (Denley Instruments) (approximately 100 rpm) at 30°C. At intervals of up to 6 h samples were removed for freezing or heat treatment. The former treatment was performed by freezing broth cultures

* Corresponding author.

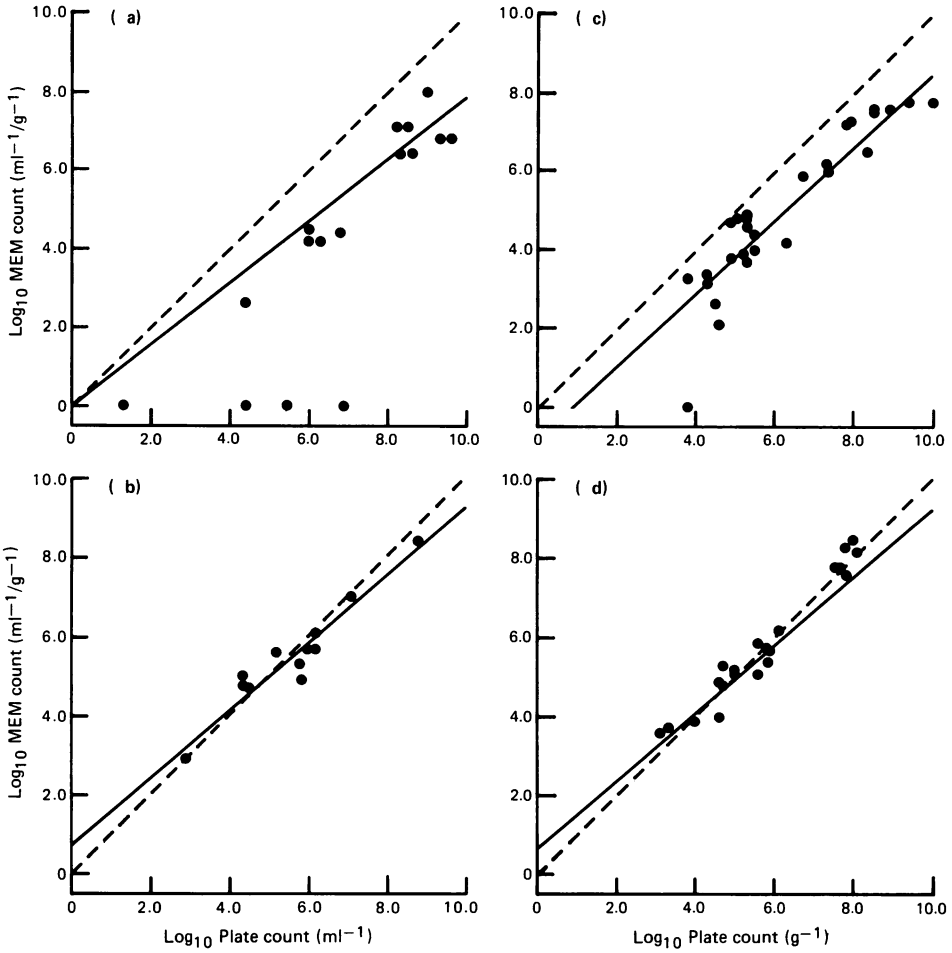


FIG. 1. Relationships between plate and MEM counts of freeze-injured cultures of *E. coli* NCDO 902 on ELSAB (a and b) and of frozen foods on NA (c and d). Samples were either nonresuscitated (*E. coli* [a]: m = 0.89, c = −0.09, r = 0.64; frozen foods [c]: m = 0.93, c = −0.81, r = 0.89) or resuscitated (*E. coli* [b]: m = 0.85, c = 0.73, r = 0.95; frozen foods [d]: m = 0.86, c = 0.64, r = 0.96). —, Regression line analysis values; ----, isoplate and MEM counts.

(5 ml) at −21°C for up to 7 days and thawing them at 22°C for approximately 15 min immediately prior to analysis. Heat injury was performed by completely immersing test tubes fitted with rubber bungs containing 10 ml of broth cultures for 15 min in a water bath. The different temperatures used ranged from 51 to 54°C for gram-negative bacteria and 53 to 63°C for gram-positive bacteria. Cultures were cooled immediately under running cold water and stored on ice for use within 2 h.

Food samples. A variety of frozen foods (mixed vegetables, peas, cauliflower, minced beef, braising steak, pork steaks) and precooked vacuum-packed meats (garlic sausage, ham, meat sausage, turkey ham, pork luncheon meat, chicken roll) were purchased from a local supermarket and examined on the day of arrival in the laboratory. Frozen samples were thawed at room temperature before analysis. In some experiments, the precooked meats were spiked with heat-treated cultures of *S. aureus* NCDO 949, *E. coli* NCDO 902, *P. fragi*, or *P. stutzeri* at cell densities ranging from 10^4 to 10^6 CFU g^{-1}. Portions (10 g) of each sample were weighed into stomacher bags and homogenized for 2 min in 90 ml of tryptone soya broth (TSB; Oxoid) at ambient temperature by using a stomacher (Colworth 100; Seward Ltd., London; 24). Samples were then filtered through 5.0-μm nylon mesh (18) to remove large particulate material.

Microbiological examinations. Samples were serially diluted in 9 ml of 0.1% peptone water (Oxoid). For total viable counts, dilutions were plated in NA and colonies were counted after 3 days of incubation at 30°C. Media used for selective enumeration of bacteria were enriched lauryl sulfate aniline blue agar (ELSAB; 27) for coliforms, Baird-Parker agar (BP; Oxoid) for staphylococci, pseudomonad selective agar C-F-C (PSA; Oxoid) for pseudomonads, and azide blood agar (ABA; Oxoid) for micrococci. Samples (0.1 ml) from dilutions were spread over the surface of these media by using an ethanol flamed-glass spreader. Plates of ELSAB and PSA were incubated for 24 h at 30°C and plates of BP and ABA for 48 h at 37°C.

Microcolony epifluorescence microscopy. The reagents and apparatus used were as described elsewhere (22). The enzyme detergent pretreatment step of DEFT (16–18) was not performed, and 2 to 10 ml of the appropriate dilution of pure culture or food homogenate was filtered under vacuum through 0.4- or 0.6-μm Nuclepore polycarbonate membranes, respectively. The membranes were removed from the filter towers and incubated on the surface of either nonselective (NA) or selective media as appropriate. Membranes transferred onto NA, ELSAB, and PSA were incubated at 30°C and those onto ABA and BP at 37°C. When they were incubated at 37°C, a few milliliters of sterile

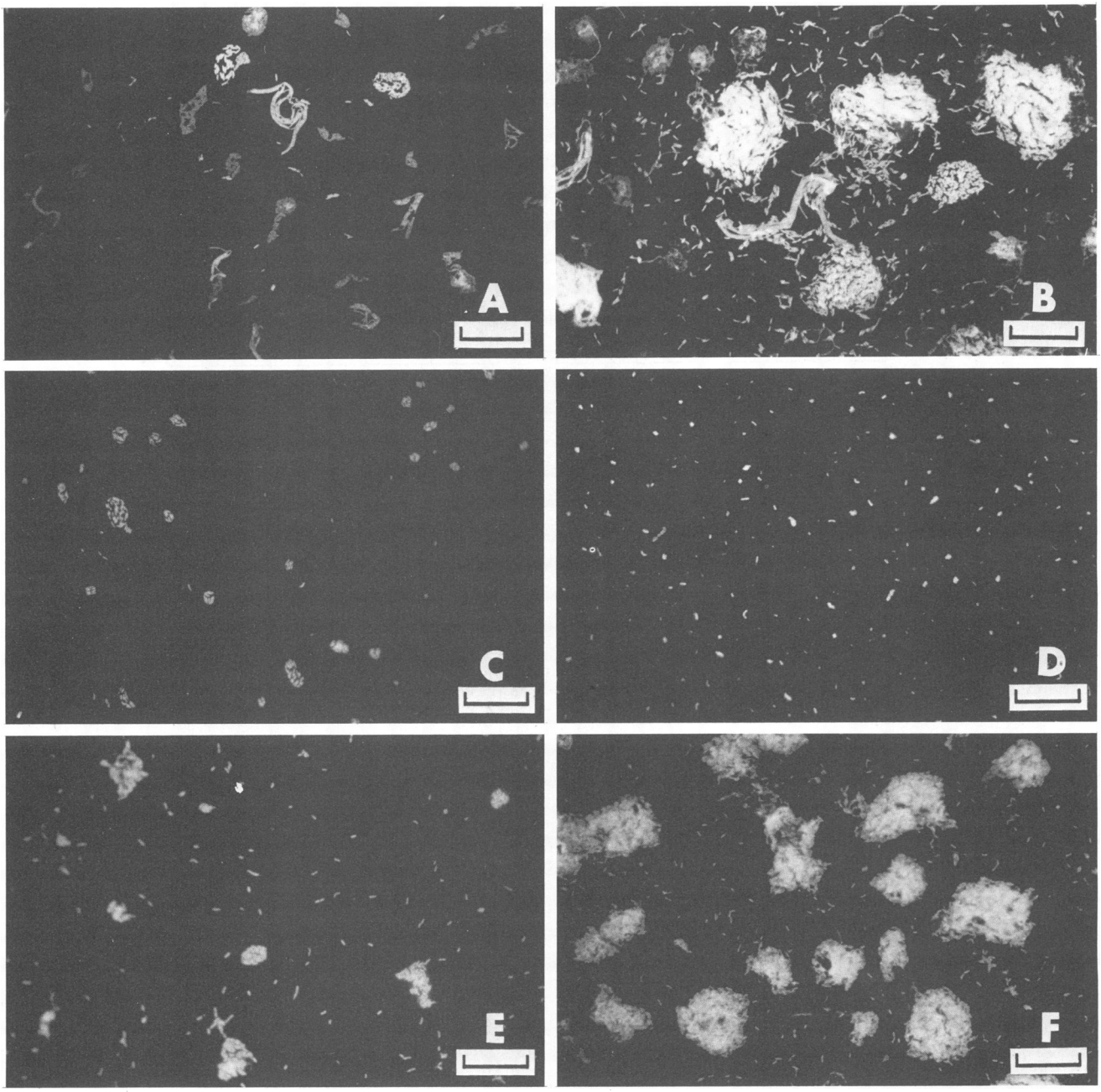
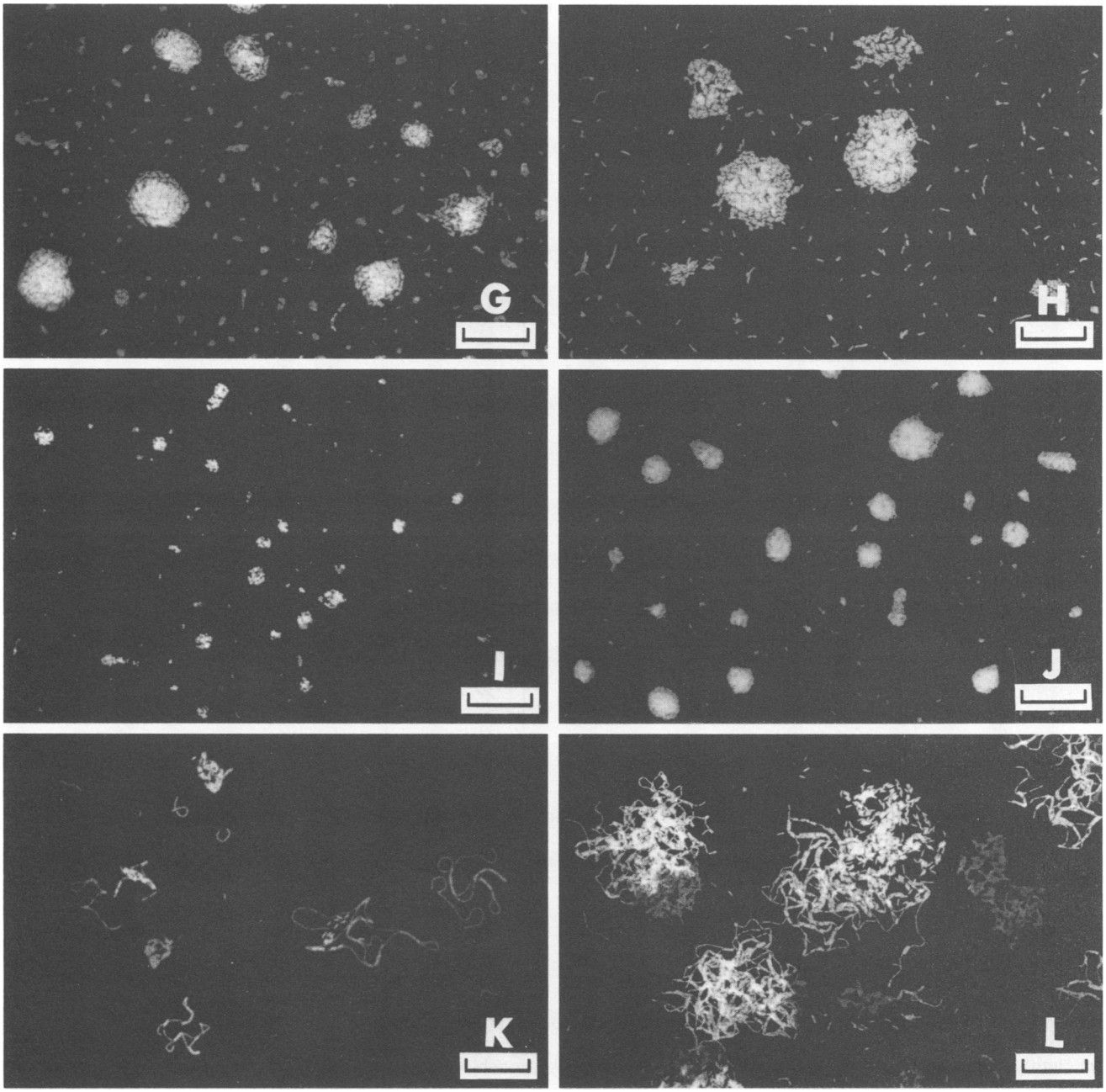


FIG. 2. Photomicrographs of μ-colonies taken with Kodak ASA 400 black and white film (10- to 15-s exposure). (A) Nonresuscitated sample of frozen pork grown on NA. (B) Sample from frozen pork resuscitated for 3 h in TSB with μ-colonies grown on NA. (C) Culture of *P. fragi* which had been heat treated at 52°C for 15 min. μ-Colonies formation from nonresuscitated sample grown on NA. (D) *P. fragi* as in panel C, but grown on selective medium (PSA). (E) Culture of *E. coli* NCDO 902 heat treated at 51°C for 15 min. μ-Colony formation from nonresuscitated sample grown on ELSAB. (F) Culture of *P. fragi* heat treated at 51°C for 15 min. μ-Colony formation from resuscitated (5 h in TSB) sample grown on NA. (G) *P. stutzeri* as in panel F, but grown on PSA. (H) *E. coli* as in panel E, but resuscitated in TSB for 5 h and grown on ELSAB. (I) Nonresuscitated culture of *S. aureus* NCDO 949 heat treated at 53°C for 15 min, grown on BP medium. (J) *S. aureus* as in panel I, but resuscitated for 5 h in TSB. (K) Nonresuscitated sample from pork luncheon meat grown on NA. (L) Pork luncheon meat as in panel K, but resuscitated for 5 h in TSB. Bar, 22 μm.

distilled water was placed in the lids of the inverted plates to avoid drying of the membranes. After 6 h of incubation, membranes were remounted in the filter towers and stained with acridine orange by the DEFT procedure (20). The membranes were mounted and examined by epifluorescence microscopy by using either an ×10 or ×40 objective. Individual cells, groups of four cells or less, irregularly shaped colonies, or μ-colonies showing abnormal growth were not included in the count. Only μ-colonies which had properly developed and which were uniformly round and compact were counted (22).

Total plate and MEM counts were made on dilutions of pure cultures which had been heat- or freeze-injured as described by using both nonselective (NA) and the various



selective media described. DEFT counts (20) were also made on samples that had been heat treated. In addition, samples (5 ml) were incubated with an equal volume of TSB for 3 or 5 h (freeze and heat stressed, respectively) at room temperature prior to analysis to determine the effects of a resuscitation period on μ-colony formation and plate counts. Similarly, food suspensions which had been prepared in TSB were analyzed immediately and after incubation for 3 h (frozen samples) or 5 h (processed foods) at room temperature before being plated on selective or nonselective media or examined for μ-colony formation on the different media.

Statistical analyses. The relationships between MEM and plate count results were determined by linear regression analysis of $\log_{10}$ transformations of the counts to give the intercept (c), slope (m), and correlation coefficient (r) for each set of data. The ideal correlation coefficient ($r = 1.0$) is indicated by the dotted line on the figures.

RESULTS

Both heat- and freeze-stressed bacteria, unlike noninjured cells, were often unable to form μ-colonies on the selective media. Even after long incubation periods of up to 24 h, μ-colony formation did not occur in some cases (results not shown). This indicated that for the MEM method to be of use, a resuscitation step prior to μ-colony formation under selective conditions was necessary for use with foods that might contain injured bacteria.

Experiments were carried out by using both freeze- and heat-injured pure cultures to determine the optimal resusci-

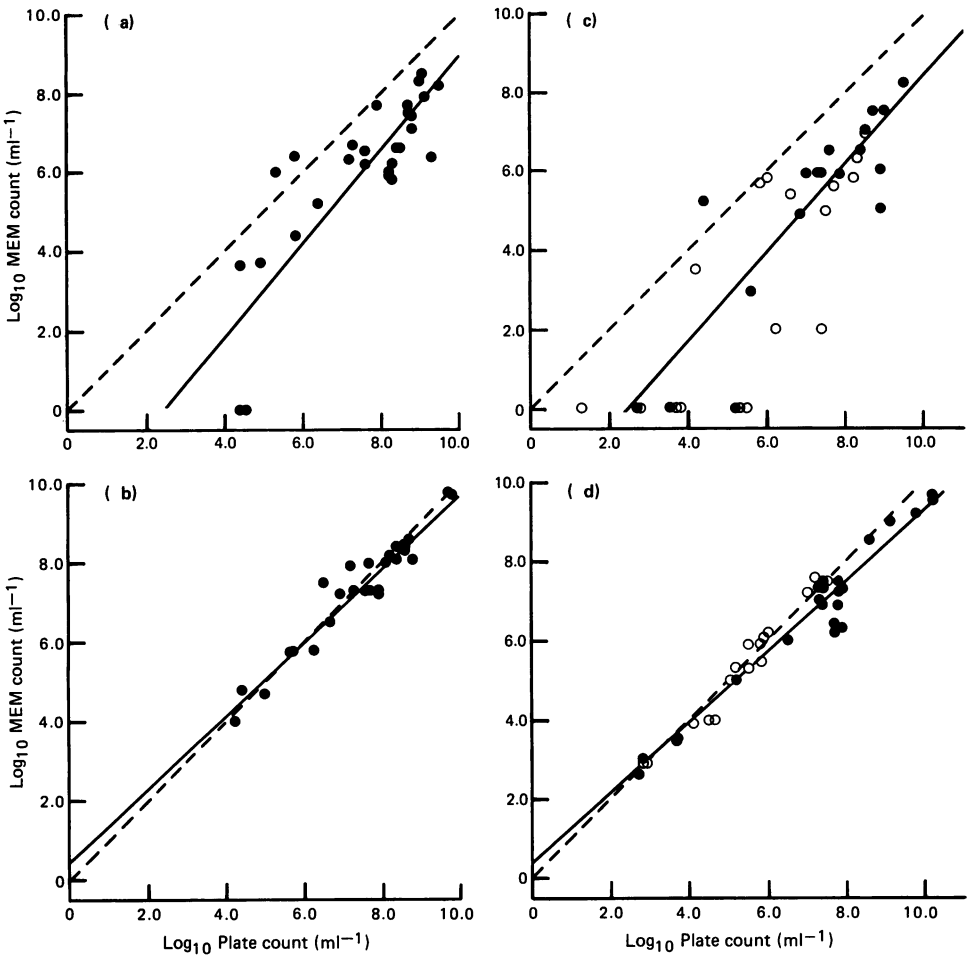


FIG. 3. Relationship between plate and MEM counts for heat-treated coliform and pseudomonad cultures. (a and c) Nonresuscitated; (b and d) resuscitated samples. a and b show total bacterial counts on NA. (a) m = 1.19, c = −2.93, r = 0.84. (b) m = 0.93, c = 0.4, r = 0.86. c and d show coliform ( ) or pseudomonad ( ) counts on ELSAB or PSA. Overall regression line data were (c) m = 0.96, c = 2.16, r = 0.69, nonresuscitated; and (d) m = 0.89, c = 0.36, r = 0.97). —, Regression line analysis values; ----, isoplate and MEM counts.

tation media, temperature, and duration for μ-colony formation from stressed cells. Buffered TSB consistently gave the best recovery of injured bacteria when compared with a number of other media. Resuscitation in liquid media (TSB) was superior to that in solid media (tryptone soya agar [TSA]) because the latter did not always resuscitate cells sufficiently for subsequent growth of μ-colonies and produced variable results (results not shown).

The optimal duration of the resuscitation step (up to 6 h) for the growth of μ-colonies varied according to the type and severity of the stress. Freeze injury was less severe and required a shorter resuscitation period (3 h). Heat injury required 5 h of resuscitation to give sufficient recovery for μ-colony formation. Resuscitation at room temperature (18 to 22°C) gave better results than at 30 or 37°C as highly variable or no resuscitation occurred at these temperatures as determined by MEM. In separate experiments, noninjured cells were subjected to the resuscitation procedures and no increase in cell numbers was observed when they were examined by plate count or the MEM methods (results not shown); however, the μ-colonies that formed appeared to be slightly larger in size.

A period of 6 h for the subsequent growth of μ-colonies after the resuscitation step was required for both the gram-positive and gram-negative injured bacteria; the 3-h period for noninjured gram-negative bacteria (22) was insufficient to give good μ-colony formation. The gram-negative organisms were more sensitive to the stresses than the gram-positive organisms, and this is probably the reason why the longer incubation period was necessary.

Freeze injury. The formation of μ-colonies and the relationship with plate count was not investigated with the pure cultures of the gram-positive organisms, as it is well known that these organisms are very resistant to freeze injury (25). Freezing the pseudomonads appeared to be very harmful, reducing the plate counts on PSA to undetectable levels. Even after a resuscitation step, none of the cells appeared to be recoverable (results not shown). The μ-colonies of nonresuscitated *E. coli* growing on ELSAB were fairly small and difficult to count. In some cases, no μ-colonies were detectable at all (Fig. 1a) and the MEM counts were generally lower than and had a poor correlation (r = 0.64) with the corresponding plate counts. The introduction of a resuscitation step allowed *E. coli* to form much larger μ-colonies on ELSAB and significantly improved the correlation of MEM counts with plate counts (r = 0.95; Fig. 1b).

There was a good correlation (r = 0.89) between MEM counts and plate counts for total counts (NA) of frozen foods (Fig. 1c), although one sample with a plate count of 10^4 g^{-1} produced no μ-colonies. This perhaps reflected different

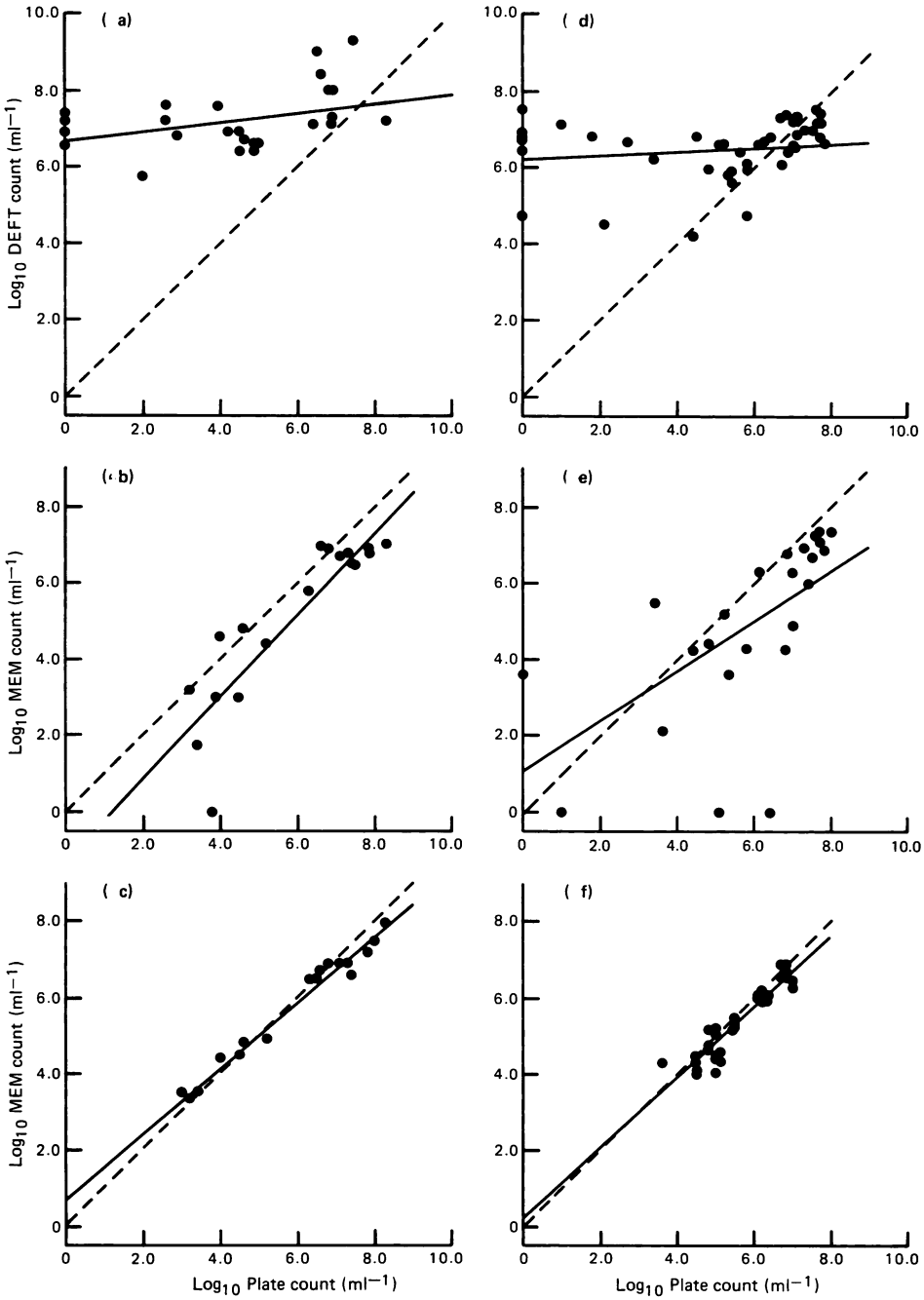


FIG. 4. Relationships between DEFT counts (a and d), nonresuscitated MEM counts (b and e) and TSB-resuscitated (5 h) MEM counts (c and f), and plate counts of heat-stressed *S. aureus* NCDO 949 grown on BP medium (a, b, c) and *Micrococcus* sp. strain M3 grown on ABA (d, e, f). The regression line data are (a) m = 0.13, c = 6.63, r = 0.40; (b) m = 1.07, c = −1.22, r = 0.88; (c) m = 0.86, c = 0.68, r = 0.98; (d) m = 0.06, c = 6.15, r = 0.22; (e) m = 0.66, c = 1.07, r = 0.62; (f) m = 0.93, c = 0.19, r = 0.93. —, Regression line analysis values; ----, isoplate and MEM counts.

flora in the different frozen foods. However, these MEM counts were generally $10^{0.5}$ to 10^{1} lower than the corresponding plate counts, and the μ-colonies were small, often looking like nongrowing clumps (Fig. 2A). The introduction of a 3-h resuscitation step in TSB increased the recovery of injured cells, and the μ-colonies produced were larger and easily countable (Fig. 2B), giving a very good correlation of MEM count with plate count (r = 0.96; Fig. 1d).

Heat injury and MEM counts. (i) Gram-negative bacteria. The ability of the MEM method to enumerate heat-injured cultures of the coliforms or pseudomonads on either nonselective (NA; Fig. 3a) or selective (PSA, ELSAB; Fig. 3c) media was not good, there being considerable variation in both counts, although the MEM counts on the selective media showed considerably more scatter. The pseudomonads were particularly badly affected by their selective media, and considerable underestimation by the MEM method occurred. Even when incubated on nonselective media, the heat-injured pseudomonads produced only small μ-colonies (Fig. 2C), some of which appeared to be like

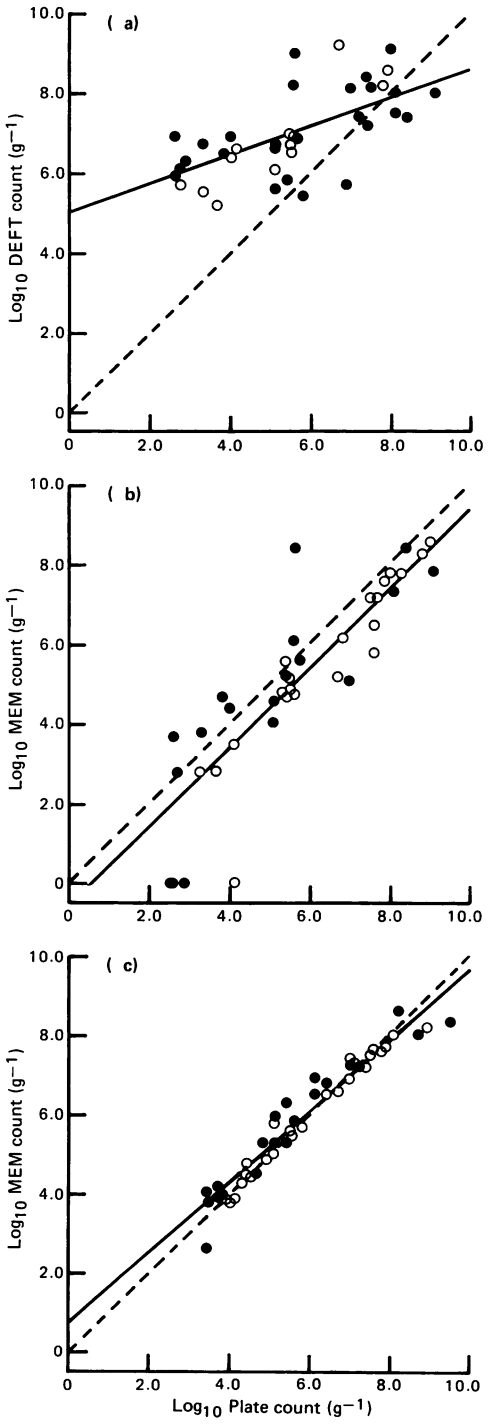


FIG. 5. Relationships between DEFT counts (a), nonresuscitated MEM counts (b) and resuscitated (TSB for 5 h) MEM counts (c), and total plate counts on NA with spiked ( ) or nonspiked ( ) samples of heat-processed vacuum-packed meats. Regression line data are (a) m = 0.36, c = 5.03, r = 0.66; (b) m = 1.0, c = 0.57, r = 0.86; (c) m = 0.89, c = 0.75, r = 0.95. —, Regression line analysis values; ----, isoplate and MEM counts.

nongrowing clumps and were not included in the MEM counts. On selective media, the injured pseudomonads were completely incapable of forming μ-colonies (Fig. 2D). Heat-injured *E. coli* also produced only small μ-colonies when grown on selective medium (Fig. 2E), but the selective agents in PSA appeared to have more drastic effects on injured cells than those in ELSAB. Naturally, the correlation between plate and MEM counts for nonresuscitated coliforms and pseudomonads was not good (r = 0.78 and 0.47, respectively).

Both coliforms and pseudomonads were able to form μ-colonies similar to those formed by noninjured cells if given the resuscitation period on nonselective (Fig. 2F) and their respective selective media (Fig. 2G and H). The relationships between MEM and plate counts under both nonselective and selective conditions were very good (Fig. 3b and d), with the correlation coefficients for coliform and pseudomonad counts being r = 0.96 and 0.98, respectively.

(ii) Gram-positive bacteria. DEFT counts of pure cultures of the heat-injured gram-positive organisms showed no relationship with plate count because of considerable overcounting in DEFT (Fig. 4a and d), apparently due to nonviable cells still fluorescing orange in DEFT.

When μ-colonies of *S. aureus* from nonresuscitated heat-injured cultures were grown on BP medium, a good relationship (r = 0.88) of plate and MEM counts was observed (Fig. 4b). However, again the μ-colonies that were produced were small and could be confused with nongrowing clumps (Fig. 2I). Counting such small μ-colonies was tedious, and nongrowing clumps could be mistakenly included in the count. Although a good relationship of MEM counts with plate counts was observed, this could not be recommended for routine use. Nonresuscitated colonies of micrococci were unable to consistently form μ-colonies on ABA (Fig. 4e), and a poor relationship of MEM count with plate count (r = 0.62) was seen, mainly due to MEM underestimating the plate count. Again, distinguishing the μ-colonies from nongrowing clumps was tedious and unreliable. However, the application of the resuscitation step greatly improved the relationships of MEM counts of staphylococci and micrococci with plate counts (Fig. 4c and f; r = 0.98 and 0.93, respectively). The μ-colonies produced were larger and well developed and were easily distinguishable from nongrowing clumps (Fig. 2J).

Food samples. The DEFT counts of heat-processed foods, which in some cases had been spiked with heat-treated cultures, overestimated the plate counts and gave a poor correlation with them (r = 0.66; Fig. 5a), again illustrating the inapplicability of DEFT to heat-treated samples.

The MEM counts on nonselective media (NA) showed a good relationship with plate counts (r = 0.86; Fig. 5b). However, the μ-colonies that formed were small, and some slight overcounting by MEM of spiked samples was observed (Fig. 5b), perhaps due to clumps that had formed when the pure cultures were grown being mistaken for μ-colonies. In a few samples, μ-colonies did not form, and the MEM counts grossly underestimated the plate counts (Fig. 5b). This may have been because of μ-colonies not having sufficient time to form (Fig. 2K). However, after resuscitation, the μ-colonies that formed were properly developed and easily counted (Fig. 2L). Over- or underestimation of counts by MEM did not occur, and the correlation with plate counts was very good (r = 0.95; Fig. 5c).

When the MEM counts on different selective media (ELSAB, PSA, BP) were performed (Fig. 6), the results obtained with pure cultures were confirmed. In all cases, the

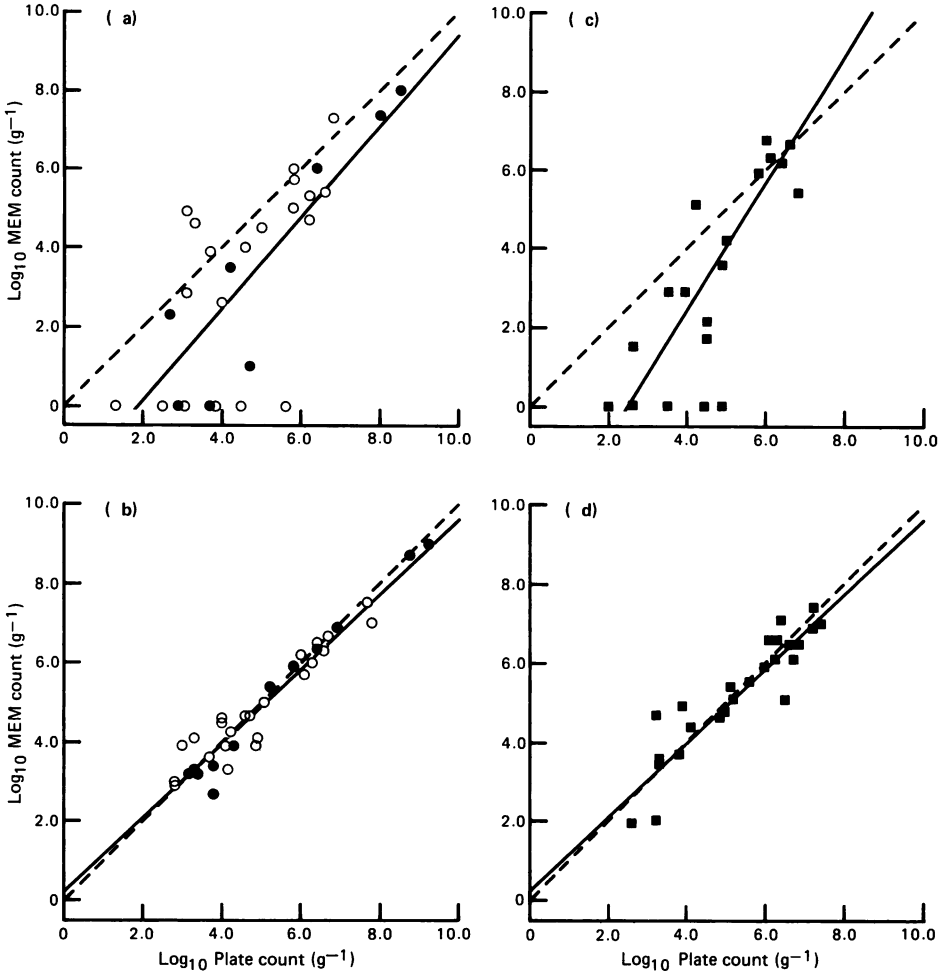


FIG. 6. Relationship between resuscitated (b and d) and nonresuscitated (a and c) MEM counts and plate counts of different bacterial types from vacuum-packed cooked meats. The media used were ELSAB for coliforms (●; a and b); PSA for pseudomonads (○; a and b), and BP medium for staphylococci (■; c and d). The regression line data were for coliforms: m = 1.5, c = −4.58, r = 0.95; for pseudomonads: m = 1.04, c = −1.41, r = 0.66; (b) for coliforms: m = 1.03, c = 0.37, r = 0.98; for pseudomonads: m = 0.83, c = 0.77, r = 0.94; (c) m = 1.59, c = −3.88, r = 0.81; (d) m = 0.89, c = 0.75, r = 0.97. —, Regression line analysis values; ----, isoplate and MEM counts.

MEM counts of nonresuscitated samples were unreliable, showing considerable variation and undercounting. Resuscitated cells in TSB greatly improved the relationship of MEM counts with plate counts, and very good correlations of specific counts of the different organisms in foods were obtained: r = 0.98, 0.94, and 0.97 for pseudomonads, coliforms, and staphylococci, respectively. It should be noted that staphylococci were not often detected in any of the foods examined, and the majority of the results were obtained with spiked samples.

DISCUSSION

Detection and enumeration of fluorescently labeled bacteria, bacterial spores, yeasts, and fungi is emerging as a powerful, rapid, sensitive, and accurate approach having many applications, e.g., for water, foods, and pharmaceuticals (5, 6, 10–12, 16–23). Many different fluorochromes have been used, but to date, acridine orange has been the most widely applied because of the differential staining that can occur, so that in DEFT "active" bacteria fluoresce orange and "inactive" bacteria fluoresce green (16–18). However, this relationship is so dependent upon the conditions of staining that the opposite relationship can be demonstrated (28). Other notable techniques to try to establish the activity of individual bacteria have exploited the reduction of tetrazolium dyes to give insoluble crystals of formazan (7, 26) or, most notably, the production of elongated cells on incubation with nalidixic acid, which inhibits cell division, and yeast extract (3, 23, 26), which enables direct viable counts to be made. In these methods, acridine orange is used to stain all the bacteria.

A profound problem with DEFT, which relies on differential staining by acridine orange, is that it cannot be used on heat-treated samples because nonviable gram-positive cocci fluoresce as viable (17). None of these methods can give an indication of the types of bacteria present (except by the use of fluorescently labeled antibodies), and both of these problems led us to develop our MEM method, which distinguishes viability by the ability of viable cells of form μ-colonies. It is therefore similar but not identical to the nalidixic acid method (3, 23, 26), because in this method active cells that are incapable of forming colonies can also be detected. The MEM method can also indicate the different types of bacteria by growing the μ-colonies under selective conditions.

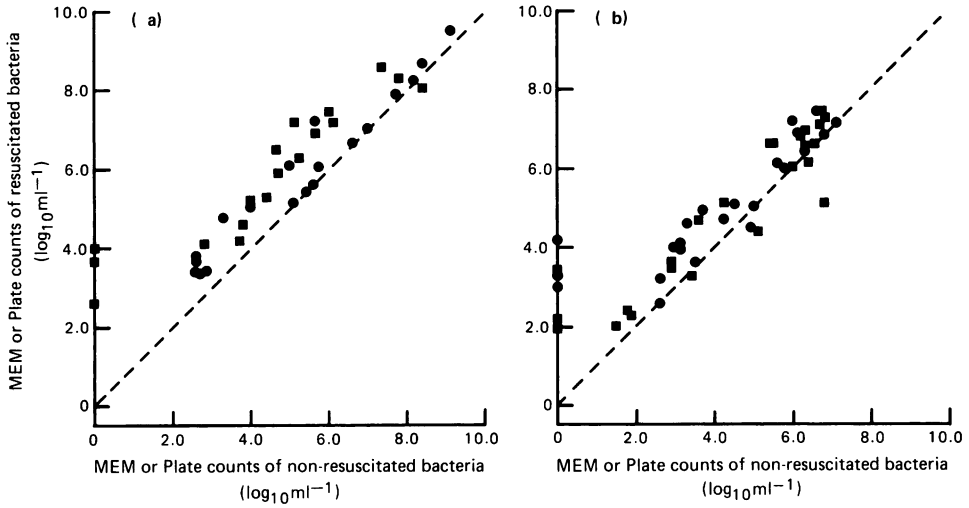


FIG. 7. MEM (■) and plate counts (●) of resuscitated against nonresuscitated noninjured bacteria to determine the degree of bacterial multiplication during the resuscitation stage (TSB, 5 h). (a) NA; (b) BP medium. —, Regression line analysis values; ----, isoplate and MEM counts.

We have shown here that the MEM method cannot be directly applied to enumerate pure cultures or samples from foods which contain heat- or freeze-injured bacteria (Fig. 1 through 6). However, the introduction of a resuscitation step allowed the reliable estimation of coliforms, pseudomonads, and staphylococci in such samples within 12 h, considerably faster than conventional plate counts, which require several days for results to be obtained.

TSA has been widely and successfully used as a resuscitation medium for injured cells. However, we could not exploit membrane collection of bacteria to enable easy transfer from solid resuscitation media (TSA) to selective media. Despite repeated attempts, resuscitation on TSA proved to be unreliable and sometimes ineffective (results not shown). We do not know the reason for this, but it could be related to limitations on nutrient diffusion from TSA through the membrane. TSB has also been widely used and proved highly successful with the MEM method. However, we were very concerned that in samples containing mixtures of injured and noninjured bacteria, some multiplication of the noninjured bacteria could occur to give artificially elevated plate and MEM counts, although our original observations suggested that this was not occurring. We therefore spent considerable effort comparing MEM and plate counts of both resuscitated and nonresuscitated noninjured bacteria. Some of these results (Fig. 7; NA and BP only) clearly show that the resuscitated counts are slightly higher (results on PSA and ABA were similar), in some cases by $10^{0.5}$ to 10^{1}. Thus, the MEM and plate counts from resuscitated samples could well be slightly higher than those actually present in the original sample. However, this is not greatly significant and must be a common feature when resuscitation in TSB is performed.

In a few cases (Fig. 5, total counts of heat-processed foods; Fig. 4b, heat-injured *S. aureus*), the MEM method appeared to be able to be applied without resuscitation. However, in both cases resuscitation increased the correlation of MEM counts with plate counts significantly. BP agar is interesting because it is one of few very selective media that has been demonstrated to be effective in enumerating stressed cells (1). Pyruvate was proposed as the media component responsible because of the degradation of hydrogen peroxide. This was confirmed (14), and the addition of catalase has been reported to have a similar effect, removing the hydrogen peroxide produced by respiratory activity during the repair stages. The addition of catalase to any of the selective media used did not significantly enhance μ-colony formation (results not shown).

There are two other important facts to be borne in mind when considering the application of the MEM method. First, the selectivity of the counting can only be as good as the selectivity of the medium. Many selective media do not always suppress the growth of unwanted organisms and support the growth of the target organisms (22). Second, the sensitivity of the method needs to be increased. Although extremely good compared with other rapid methods of analysis, the sensitivity is at best just below 10^3 g^{-1} (Fig. 5b and 6b and d). Since these different types of bacteria often form only a proportion of the flora of a sample, the MEM method can, at present, be used only as a screening method to confirm the presence of these organisms at these levels. We are currently investigating ways to improve this aspect.

ACKNOWLEDGMENTS

We thank Anita Bayliss for her skilled assistance with some of this work and Ray Young (Photographic Section) for development of the photomicrographs.

LITERATURE CITED

1. **Baird-Parker, A. C., and E. Davenport.** 1965. The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat-treatment and after storage of frozen or dried cells. J. Appl. Bacteriol. **28**:390–402.
2. **Collins-Thompson, D. L., A. Hurst, and H. Kruse.** 1973. Synthesis of enterotoxin B by *Staphylococcus aureus* strain S-6 after recovery from heat injury. Can. J. Microbiol. **19**:1463–1468.
3. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3**:817–820.
4. **Corry, J. E. L., A. G. Kitchell, and T. A. Roberts.** 1969. Interactions in the recovery of *Salmonella typhimurium* damaged by heat or gamma irradiation. J. Appl. Bacteriol. **32:**

415–428.
5. **Denyer, S. P., and K. H. Ward.** 1983. A rapid method for the detection of bacterial contaminants in intravenous fluids using membrane filtration and epifluorescence microscopy. J. Parenter. Sci. Technol. **37**:156–158.
6. **Donnelly, C. W., and G. J. Baigent.** 1986. Method for flow cytometric detection of *Listeria monocytogenes* in milk. Appl. Environ. Microbiol. **52**:689–695.
7. **Dutton, R. J., G. Bitton, and B. Koopman.** 1983. Malachite Green-INT (MINT) method for determining active bacteria in sewage. Appl. Environ. Microbiol. **46**:1263–1267.
8. **Entis, P.** 1986. Membrane filtration systems, p. 91–106. *In* M. P. Pierson and N. J. Stern, (ed.), Foodborne microorganisms and their toxins: developing methodology. Marcel Dekker, Inc., New York.
9. **Jarvis, B., A. C. Rhodes, S. E. King, and M. Patel.** 1976. Sensitization of heat-damaged spores of *Clostridium botulinum* type B to sodium chloride and sodium nitrate. J. Food Technol. **11**:41–50.
10. **Kangatharalingam, N., and M. W. Ferguson.** 1984. A simple and rapid technique for fluorescence staining of fungal nuclei. Curr. Microbiol. **10**:99–104.
11. **Kelly, A. F., and R. G. Kroll.** 1987. Use of the direct epifluorescent filter technique for the enumeration of bacterial spores. J. Appl. Bacteriol. **63**:545–550.
12. **Koch, H. A., R. Bardler, and R. R. Gibson.** 1986. Fluorescence microscopy procedure for quantification of yeasts in beverages. Appl. Environ. Microbiol. **52**:599–601.
13. **Mackay, B. M., and C. M. Derrick.** 1982. A comparison of solid and liquid media for measuring the sensitivity of heat-injured *Salmonella typhimurium* to selenite and tetrathionite media, and the time needed to recover resistance. J. Appl. Bacteriol. **53**:233–242.
14. **Martin, S. E., R. S. Flowers, and Z. J. Ordal.** 1976. Catalase: its effect on microbial enumeration. Appl. Environ. Microbiol. **32**:731–734.
15. **Mosell, D. A. A., A. Veldman, and I. Eelderink.** 1980. Comparison of the effects of liquid medium repair and the incorporation of catalase in McConkey type media on the recovery of Enterobacteriaceae sublethally stressed by freezing. J. Appl. Bacteriol. **49**:405–419.
16. **Pettipher, G. L.** 1983. Review: the direct epifluorescent filter technique. Research Studies Press, Letchworth.
17. **Pettipher, G. L.** 1986. Review: the direct epifluorescent filter technique. J. Food Technol. **21**:535–546.
18. **Pettipher, G. L., and U. M. Rodrigues.** 1982. Rapid enumeration of microorganisms in foods by the direct epifluorescent filter technique. Appl. Environ. Microbiol. **44**:809–813.
19. **Pomroy, A. J.** 1984. Direct counting of bacteria preserved with lugol iodine solution. Appl. Environ. Microbiol. **47**:1191–1192.
20. **Rodrigues, U. M., and R. G. Kroll.** 1985. The direct epifluorescent filter technique (DEFT): increased selectivity, sensitivity and rapidity. J. Appl. Bacteriol. **59**:493–499.
21. **Rodrigues, U. M., and R. G. Kroll.** 1986. Use of the direct epifluorescent filter technique for the enumeration of yeasts. J. Appl. Bacteriol. **61**:139–144.
22. **Rodrigues, U. M., and R. G. Kroll.** 1988. Rapid selective enumeration of bacteria in foods using a microcolony epifluorescence microscopy technique. J. Appl. Bacteriol. **64**:65–78.
23. **Roszak, D. B., and R. R. Colwell.** 1987. Metabolic activity of bacterial cells enumerated by direct viable count. Appl. Environ. Microbiol. **53**:2889–2983.
24. **Sharpe, A. N., and A. K. Jackson.** 1972. Stomaching: a new concept in bacteriological sample preparation. Appl. Microbiol. **24**:175–178.
25. **Speck, M. L., and B. Ray.** 1977. Effects of freezing and storage on microorganisms in frozen foods: a review. J. Food Prot. **40**:333–336.
26. **Tabor, P. S., and R. A. Neihof.** 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl. Environ. Microbiol. **48**:1012–1019.
27. **Wright, R. C.** 1984. A new selective and differential agar medium for *Escherichia coli* and coliform organisms. J. Appl. Bacteriol. **56**:381–388.
28. **Yeh, T. Y., J. R. Godschalk, G. J. Olsen, and R. M. Kelly.** 1987. Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus ferrooxidans* on iron pyrite. Biotechnol. Bioeng. **30**:138–146.