

# Biotechnology-Based Methods for the Detection, Enumeration and Epidemiology of Food Poisoning and Spoilage Organisms

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## Introduction

Methods based on biotechnology are being used increasingly to detect and enumerate both food poisoning and spoilage micro-organisms. In addition, it is possible to use such methods to understand the growth and physiology of such organisms in natural and man-made systems, to develop an understanding of their interaction with the environment and with each other and to discover much more about epidemiology, particularly of food-borne disease and intoxication. Clearly, the subject areas covered in this review are extremely broad. However, the methods in use and, in particular, the approach, have a commonality which cuts across the different areas, strengthening and building on developments which, at first sight, would seem to be unrelated.

## Microbial recovery and stress responses

The prelude to any method, biotechnology-based or otherwise, for the detection and enumeration of micro-organisms must address the problems

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Abbreviations: ATP, adenosine triphosphate; BRENDA, bacterial restriction endonuclease DNA analysis; DAB, diaminobenzidine; DEFT, direct epifluorescent filter technique; DIFT, direct immunofluorescent test; EIA, enzyme immuno-assay; ELISA, enzyme-linked immunosorbent assay; ECL, enhanced chemiluminescence; FMNH<sub>2</sub>, reduced flavin mononucleotide; FRET, fluorescence resonance energy transfer; HACCP, hazard analysis critical control point; HE, Hektoen enteric agar; HPA, hybridization protection assay; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride; PCR, polymerase chain reaction; p.p.b., parts per billion; p.p.m., parts per million; RFLP, restriction fragment length polymorphism; RPLA, reversed passive latex agglutination; rRNA, ribosomal RNA; SS, Salmonella Shigella agar; UHT, ultra-high temperature; UV, ultraviolet; VGBA, violet green bile agar; XLD, xylose lysine desoxycholate medium.

that are encountered in microbial recovery from the non-culturable to the culturable state. Inactivation of micro-organisms by physical or chemical agents invariably leaves a fraction of the population damaged and unable to grow or divide without an extended lag period (reviewed by Mackey, 1984). In particular, damage produced by chilling or freezing can be sublethal, especially to Gram-negative bacteria, and may be expressed in a number of different ways. For example, cells which survive have increased lag times, increased sensitivity to antimicrobial substances and reduced tolerance to fluctuations of pH and temperature. Such cells may also lose certain biological properties. Cold-stressed cells exhibit a variable response to the nutrient content of recovery medium. Thus cold-shocked cells of *Salmonella heidelberg* recover better on a minimal medium (Tang and Jackson, 1979) while freeze-injured cells of *Escherichia coli* recover better on a nutritionally rich medium (Straka and Stokes, 1959). There is evidence that the enhanced recovery of freeze-injured cells on a rich (compared with a minimal) medium is due to the growth stimulatory properties of some of the amino acids present in the rich medium (Kuo and Macleod, 1969) and to the chelation of toxic trace elements in the distilled water used in preparation of the medium by amino acids (Macleod, Kuo and Gelinis, 1967). The reverse phenomenon—of better recovery on minimal medium rather than rich medium—has been observed in cells of freeze-dried and freeze-thawed *Salmonella* spp. This has been called 'minimal medium recovery' (Gomez *et al.*, 1973; Takano, 1975; Tang and Jackson, 1979) and is usually found in cells grown in minimal medium and stressed during the exponential phase of growth. In addition, such recovery has been found in ultraviolet (UV)-irradiated strains of *E. coli*, which are deficient in excision-repair, and also in heat-injured *Salmonella typhimurium* (Ganesan and Smith, 1968; Gomez *et al.*, 1973). Sharma, Barfknecht and Smith (1982) showed that the minimal-medium recovery of UV-irradiated cells involved repair of DNA damage by a *recA*-dependent pathway. The poor recovery of UV-irradiated cells on rich growth medium was due to inhibition of this repair mechanism by amino acids. Mackey and Derrick (1986) found that cold-shocked *S. typhimurium* displayed minimal-medium recovery but that addition of catalase to the rich medium restored counts to the level found on the minimal medium. Since hydrogen peroxide could not be detected in the minimal medium but was present at concentrations between 12 and 38  $\mu\text{mol l}^{-1}$  in the rich medium, the minimal medium recovery phenomenon appears to be related to sensitivity to hydrogen peroxide. Prior exposure of growing cells to 30  $\mu\text{mol l}^{-1}$  of hydrogen peroxide abolished this sensitivity to rich medium following cold shock, suggesting that *S. typhimurium* contains an inducible system which protects the cells against oxidative stress.

Similar results have been shown for cells of *Campylobacter jejuni*. In this case the cells damaged by heating and freezing showed increased sensitivity to antibiotics (Humphrey and Cruickshank, 1983, 1985; Ray and Johnson, 1984; Humphrey, 1986a) and hydrogen peroxide or oxygen radicals (Humphrey, 1986a, 1988), and were less able to tolerate elevated temperatures for growth, since a significant fraction of a population of injured cells died when they

were inoculated into culture broth (Ray and Johnson, 1984; Humphrey, 1986a), this loss of viability being greater at 43°C than at 37°C (Humphrey, 1986a). Humphrey also found that, at the higher incubation temperature, damaged cells were able only to repair lesions caused by short exposures to high or low temperatures (Humphrey, 1986b). Cells exposed to either heating or freezing were progressively less able to grow at 43°C, particularly on selective media (Humphrey, 1989). As might be expected, this reduced the recovery of damaged cells from naturally and artificially contaminated samples, although the isolation rate was increased by pre-enrichment in basal or selective media at 37°C for 4 h. Alternatively, with membrane filtration or surface plating techniques the inclusion of agents that quench toxic derivatives of oxygen was more important.

Due to increasing concern about detection and enumeration of *Listeria monocytogenes* after injury, extensive and detailed studies have begun to examine both heat and freeze damage (Golden, Beuchat and Brackett, 1988a). Substantial populations of viable cells of all four strains examined were injured by heating at 52°, 54° and 56°C, as evidenced by increased sensitivity to NaCl when plated on tryptose phosphate recovery agar. Similarly, up to 80% of cells were not recovered when held at -18°C for 14 days. It is apparent that the ability of various selective media to recover injured *L. monocytogenes* needs to be investigated further (Golden, Beuchat and Brackett, 1988b).

Such effects, especially these increased lag times, have important practical implications in a number of areas of microbiology, which must be understood before the methods of studying the state of the cells can be discussed.

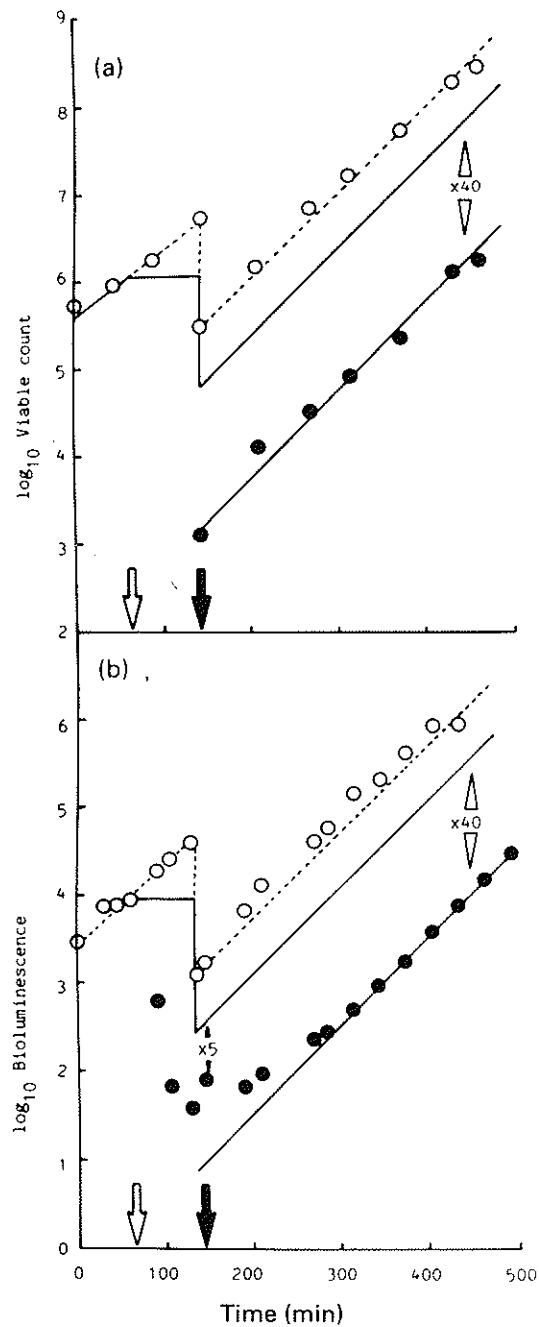
In the food industry (reviewed by Busta, 1976) the lag before growth occurs is used to extend the shelf-life of foods by delaying the time when spoilage or poisoning organisms reach unacceptable levels. The length of the lag can be increased by putting the damaged cells under conditions which delay their recovery. In food and other industries the detection of micro-organisms as rapidly and sensitively as possible is, of course, especially important. Most classical techniques of detection and enumeration depend upon growth of the micro-organisms, often on media solidified with agar (reviewed by Ray and Speck, 1978; APHA, 1985). More recently, growth in liquid media, for example in detection by impedance (Eden and Eden, 1984), has been used more frequently. Such methods may fail to detect damaged organisms, which show a lag before growth. Often the lag is extended by use of a selective medium, since such media invariably contain agents (for example, antibiotics, surface active agents, etc.) which inhibit the growth of competing organisms. Ideally, since a very small proportion of a natural microbial flora may represent say, a food poisoning bacterium, all the competing organisms, but none of the target organisms, should be inhibited by the selective agents. In general, although microbiologists often behave as if this were true, it is very rarely so. For example, a standard and well-used selective medium such as Baird-Parker agar (Baird-Parker, 1962) which was developed for the isolation and enumeration of coagulase-positive staphylococci, depends on the inhibitory action of tellurite and sodium pyruvate with egg yolk emulsion as a

diagnostic agent. After incubation for 24 h at 37°C on such a medium, *Staphylococcus aureus* produces black, shiny, convex colonies 1.0–1.5 mm in diameter, surrounded by a zone of clearing 2.5 mm in width. However, it is well known that some strains only produce a well-defined clearing after 36 h. In addition, *Staphylococcus saprophyticus* also produces a colony surrounded by a similar clearing, although the colonies are more irregular than those of *Staph. aureus*, while micrococci, yeasts and *Bacillus* spp. grow but produce different coloured colonies (Oxoid Manual, 1982). It is apparent that one colony of *Staph. aureus* in 1000 other colonies will be difficult to detect. The situation becomes even more difficult if the cells of the target organism have been damaged, since they often become sensitive to the selective agents. Van Netten *et al.* (1990) have investigated the enumeration of sublethally injured (heated at 60°C) *Staph. aureus* in foods in detail and have suggested that it is better to incubate plates of Baird–Parker at 42°C, preferably in combination with a solid medium repair, because this provides moderately anaerobic conditions of incubation. *Staph. aureus* which had been injured with acid were not quantitatively recovered by this method but could be completely restored by solid medium repair for at least 6 h at 23°C on tryptone soya peptone yeast extract egg yolk pyruvate agar.

The difficulty of recovering damaged cells on selective media has become particularly well known in the case of *Salmonella*, where in the standard isolation procedure (which involves enrichment in selective growth media) the cells are first resuscitated for 24 h in a non-selective medium. Use of such a method extends the minimum time of detection of *Salmonella* from 3 days to 4 and adds greatly to the cost of production and storage space for those companies (for example, chocolate manufacturers) operating a positive release system. A number of studies have devised procedures and/or methods to aid the recovery of damaged *Salmonella*. For example, Strantz and Zottola (1989) developed a modified plating technique for the recovery and enumeration of stressed *S. typhimurium*. This involved spreading onto tryptone soya agar and standing at room temperature for 4 h before overlaying with XLD, HE or SS and incubating at 37°C for 24 h. Without the solid medium, only 1% of cells were recovered after injury by freezing, blending in whole egg or by heating at 54°C.

It is apparent, therefore, that damaged cells are difficult to detect on selective media. This applies not only to heat damage but also to damage with chemicals. For example, spores of *Clostridium bifermentans* treated with hypochlorite took longer to produce colonies than undamaged spores, and the more severe the damage the longer the lag before growth and colony formation occurred (Waites and Bayliss, 1980).

Tsuchido, Aoki and Takano (1989) used permeability to a fluorescent dye (1-*N*-phenylnaphthylamine) to demonstrate damage to a permeability barrier in the outer membrane during the heating of cells of *E. coli* to 55°C. For repair of the permeability barrier, RNA, protein and lipid synthesis were required, together with an energy source. Studies by Jassim *et al.* (1990) have utilized bioluminescence to detect both bacterial freeze injury and the rate of



**Figure 1.** A comparison between viable plate count (a) and *in vivo* bioluminescence (b) for *S. typhimurium* LT2 subjected to freeze injury.  $\circ$ , control cultures without freeze injury. The solid line represents the control adjusted to subtract the growth obtained during the freeze period of the experimental sample.  $\bullet$ , experimental samples subjected to freezing at  $-20^{\circ}\text{C}$  and subsequent thaw. Open arrow indicates samples frozen; solid arrow indicates samples thawed.

recovery. Using *S. typhimurium* expressing the *lux* gene system from *Vibrio fischeri*, cells have been damaged by freezing to  $-18^{\circ}\text{C}$ . Real-time measurements of *in vivo* bioluminescence have revealed a major population of bacterial cells that retain functional intracellular biochemistry, but are defective in their ability to replicate after freeze-injury (*Figure 1*). Immediately after the freeze/thaw cycle the difference in bioluminescence between control and experimental samples was typically only fivefold compared to a fortyfold reduction in viable cell count.

The extent of the effect of mild heating of *S. typhimurium* has recently been related to the DNA replication velocity (Mirhabibollahi and Davies, 1990) while the presence of iso-ascorbic acid has been shown to increase the bacteriocidal effect of mild heat on *Salmonella thompson* and a number of other micro-organisms (Mackey and Seymour, 1989).

It must be apparent that recovery from damage is usually detected by the ability to grow and form visible colonies on selective agar media in comparison to the number of cells forming colonies on non-selective agar. However, such studies provide no indication of the mechanism of recovery nor of the steps involved. In addition, the success of such resuscitation media can only be determined by the increased ability to form colonies. Further understanding of the mechanisms involved in damage and recovery are urgently required.

### Antibody-based assays

Detection of micro-organisms and their toxins by antibody-based probes (*see* Chu *et al.*, 1989) has a long history of development, although the commercial success of such methods has been less than might have been expected. Since toxins are difficult to detect by other rapid methods and bioassays are time-consuming, labour intensive and often inaccurate, there has been some successful commercialization of antibody-based methods for their detection. Commercial kits are available, for example, for enterotoxin A for *Staph. aureus* and for different mycotoxins, while monoclonal antibodies for the detection of type B *Clostridium botulinum* toxin, produced both in axenic culture and in an inoculated model-cured meat system, have also been developed (Gibson *et al.*, 1988). Using 38 strains of *C. botulinum* and 10 of *Clostridium sporogenes*, 8/9 cultures and 73/101 slurry samples were positive, including 28 at or below the detection limit of  $20 \text{ LD}_{50} \text{ ml}^{-1}$ . No false positives were found with type A, C, D, E or F or *C. sporogenes* NCTC 3807, although detection of type B needed two antibodies. However, the efforts of the food industry are directed at preventing the growth of, and toxin formation by, this organism (for example, by hazard analysis and determination of critical control points; Waites, 1988). Commercial testing for its toxins, which would never be found in any statistically relevant end-product testing scheme, has never been proposed.

Detection of enterotoxin A (from *Staph. aureus*) by immunoassay is of particular interest, since it is much more heat resistant than the bacterium and can therefore be present in a food in the absence of viable cells (reviewed by

Stannard *et al.*, 1987). In addition, less than 1 µg may have an emetic effect and efforts have been made to detect as little as 0.125–0.250 µg per 100 g of food (Reiser, Conaway and Bergdoll, 1974). A recent collaborative study between several laboratories, comparing three enzyme-linked immunosorbent assay (ELISA) systems for detection of the toxin in sausage extracts (Schönwälder *et al.*, 1988) concluded that for factory use and for normal food control a simpler qualitative method using polyclonal antibodies ((Fey ELISA) (Fey, Pfister and Rugg, 1984; Fey, 1987) resulting in a YES/NO answer seemed sufficiently reliable. Only well-trained staff would be able to perform the quantitative ELISAs properly and this might lead to variable results in routine food industry laboratories. A similar comparison of reversed passive latex agglutination (RPLA) (Shingaki *et al.*, 1981; Igarashi *et al.*, 1985), plate ELISA (Notermans *et al.*, 1983; Wieneke and Gilbert, 1987) and kit ELISA (Fey, Pfister and Rugg, 1984) carried out by Berry *et al.* (1987) showed that the ELISA kit was the most sensitive method (0.01–0.1 µg per 100 g) with good to excellent specificity, but was labour intensive and laborious. In contrast, the RPLA test was easy to carry out, but its sensitivity was insufficient to detect enterotoxin in all foods found to be positive with the ELISA kit. Although neither method contained reagents for the detection of enterotoxin E (because of its infrequent occurrence), used side by side, the two kits were found to complement each other. Previous work on detection of enterotoxin produced by *Staph. aureus* isolates had already shown that the method needs some level of expertise (Gibbs, Patterson and Harvey, 1978; Harvey, Patterson and Gibbs, 1982). In the study by Schönwälder and his collaborators (1988) it was apparent that the extraction procedures needed to be optimized for different foods. Nevertheless, the methods used were able to detect as little as 0.125 µg per 100 g of food within ripened and unripened sausages. Other studies with an indirect double-sandwich ELISA using monoclonal antibodies for detection of staphylococcal enterotoxins A, B, C and D in foods have allowed 0.1 µg toxin per 100g of food to be detected with horseradish peroxidase (Lapeyre, Janin and Kareri, 1988). This group concluded that the method was a more reliable test than isolation of *Staph. aureus*. In addition, recent work has used commercially produced systems, such as the RPLA kit, to detect staphylococcal enterotoxins in dairy products (Rose, Bankes and Stringer, 1989) and, by centrifugation of microtitration plates, the time for the assay has been reduced from 20–24 h to 4 h (Bankes and Rose, 1989).

A comparison of methods for the detection of enterotoxin A from *Clostridium perfringens* has also been carried out by Berry *et al.* (1987). These workers compared an ELISA method developed by Bartholomew *et al.* (1985) (*see also* Wimsatt, Harmon and Shah, 1986) with a reversed passive agglutination test produced in Japan but available from Oxoid Ltd (Code No. DR930). Of 274 faecal specimens from 55 separate outbreaks of *C. perfringens* food poisoning, 93% gave the same results in both tests. The discrepancy which arose in 20 of the specimens was due to very low toxin levels ( $\leq 7$  ng per g faeces), which was close to the limit of detection for both methods. However, this discrepancy is unlikely to be of practical significance since,

with confirmed outbreaks of food poisoning, the majority of faecal specimens collected within 2 days of the onset of symptoms have toxin levels  $>1 \mu\text{g g}^{-1}$  (Bartholomew *et al.*, 1985). Berry and his co-workers concluded that the RPLA test was simple, rapid, sensitive, specific and required no specialized equipment. A similar conclusion was reached by Harmon and Kautter (1986), using culture supernatants and faecal specimens as test materials.

Similar and even more detailed studies have been made to develop methods for the detection of a number of different mycotoxins. This area has been reviewed by Candlish, Smith and Stimson (1990), who examined the development of monoclonal antibodies for detection of aflatoxins from academia through to commercial production. For comparison with bioassay methods and the use of tissue culture for the detection of mycotoxins, the reader is referred to Buckle and Sanders (1990) and Robb, Norval and Neill (1990), respectively. A recent example of an antibody-linked probe for the detection of mycotoxins is the monoclonal antibody developed for the trichothecene mycotoxin, diacetoxyscirpenol, which was able to detect  $16 \text{ ng ml}^{-1}$  (Hack, Klaffer and Terplan, 1989). However, this was less sensitive than the polyclonal antibodies developed earlier (Klaffer, Märtlbauer and Terplan, 1988; Mills *et al.*, 1988), which had been used for detection of diacetoxyscirpenol in wheat.

Interestingly, work has also been carried out on the development of enzyme-linked assays to detect microbial enzymes which can lead to food spoilage, even after the loss of viability of the organisms that have produced them. One example of such studies is the development of antibody-linked detection methods for the heat-stable proteases produced by pseudomonads, such as *Pseudomonas fluorescens*. Such enzymes can cause the spoilage of milk heated at ultra-high temperatures (UHT) and subsequently packaged aseptically before storing at ambient temperatures for as long as 12 months. Under such conditions enzyme concentrations as low as  $1 \text{ ng ml}^{-1}$  of UHT milk are sufficient to render the milk unacceptable within 6 months (Mitchell and Ewings, 1985). Clements *et al.* (1990) utilized an inhibition enzyme-linked immunoabsorbent assay to detect  $0.24\text{--}7.8 \text{ ng}$  of proteases per ml of UHT milk within 6 h. Previous work also used a sandwich-type enzyme-linked assay (Birkeland, Stepaniak and Sorhaug, 1985). However, proteases produced by *P. fluorescens* are not immunologically homogenous (Jackman, Bartlett and Patel, 1983; Symons *et al.*, 1985), although the extent of the homogeneity is not yet known. Monoclonal antibodies able to cross-react with a number of proteases have been developed.

In addition to the detection of toxins and spoilage enzymes, many investigators have developed antibody-linked probes for the detection of micro-organisms important in food and agriculture, e.g. for moulds such as *Brochothrix thermosphacta*, for *L. monocytogenes* and, especially, for *Salmonella*.

Dewey *et al.* (1990) have developed a monoclonal-antibody-ELISA and DIP-STICK immunoassay for the mould, *Penicillium islandicum*, in rice grains. This species causes one type of yellowing in stored milled rice and has been associated with toxicity, particularly in South-East Asia (Tsunoda,



1953), the symptoms including liver lesions, cirrhosis and primary liver cancer (Moreau, 1979). In the study by Dewey and her collaborators, all inoculated grains and approximately 90% of grains in naturally infected samples from Indonesia were positive. These results [and those obtained for discoloured grains from both Indonesia (32%) and the Philippines (14%)] were higher than those obtained by direct plating of surface-sterilized grains. In addition, the antibody showed strong binding to mycelia but not to mature conidia, suggesting that the assay could be used to detect active growth, which could be differentiated from the mere presence of spores. Notermans *et al.* (1987) have also developed a relatively specific antiserum able to recognize only species of *Penicillium* and *Aspergillus* and have compared ELISA with colony counts for the detection of moulds in spices and nuts (Notermans *et al.*, 1987). Such antibody-linked probes may also lead to a redevelopment of the Howard Mould Count (Howard, 1911), which is a direct microscopic method of determining the extent of fungal hyphae contaminating such products as tomato ketchup. The method is slow, laborious and inaccurate. Robertson, Patel and Sargeant (1988) have developed an immunogen cocktail to produce an antibody for *Aternaria alternata*, *Botrytis cinerea*, *Fusarium solani*, *Rhizopus stolonifer* and *Mucor piriformis*. Although the antibody also cross-reacted with mould species of the same genera and with moulds of some other genera which were not present in the cocktail, there was little cross-reaction with tomato.

With regard to detection of spoilage bacteria by antibody techniques, there has been little commercial pressure to develop such methods. However, Alcock *et al.* (1990) have produced a polyclonal antibody against *B. thermosphacta* which causes spoilage of meat and British fresh sausages stored at refrigeration temperatures. This antibody failed to cross-react with closely related species such as *Kurthia zopfii* and *L. monocytogenes* and has been used to detect the sites at which the organisms grow in sausages (*see p. 24*).

It would be expected that most effort to develop antibody-linked probes would be put into the detection of bacteria which are of particular concern to the food industry because of public concern about their presence in foods. Both *L. monocytogenes* and *Salmonella* come into this category. Recently, McLaughlin and Pini (1989) used monoclonal antibodies in a direct immunofluorescent test (DIFT) to make a presumptive identification of *L. monocytogenes* in food. Using fluorescein isothiocyanate as a marker,  $<10^2$  cells per g of soft cheese were detected in  $<2$  h. The method made a presumptive identification of  $>90\%$  of strains, and less than 10% of strains of other species were misidentified when cultured on modified McBride agar or on Blood agar. An earlier study had indicated the potential of such methods by developing monoclonal antibodies against flagellar antigens of *L. monocytogenes* and using them in an enzyme immuno-assay (EIA) (Farber and Speirs, 1987). More recently Blumer and Brinkman (1989) developed a monoclonal antibody-based ELISA method for *Listeria* spp. which was able to detect  $10^6$  or more cells. These workers found some influence of the food product and/or competitive microbes on the assay, which detected *Listeria innocua* as well as *L. monocytogenes*. However, the test was not restricted to a certain growth

phase and no false positives or negatives were found. Mattingly *et al.* (1988) also developed a monoclonal antibody-based enzyme-linked immunosorbent assay which was positive with  $2.5 \times 10^4$  *Listeria* cells.

The use of antibody-linked probes to detect *Salmonella* would be a review in itself (*see* Clayden, Alcock and Stringer, 1987). Although a monoclonal antibody (MOPC 467, from an IgA myeloma) to detect *Salmonella* was first reported by Robinson, Pretzman and Mattingly (1983), the technique only detected 94% of the serotypes examined. This was overcome by the addition of a second monoclonal antibody hybridoma, referred to as 6H4 (Mattingly, 1984). Using horseradish peroxidase and polycarbonate-coated metal beads, Mattingly and Gehle (1984) eliminated the antibody/microtitre plate coupling step from the assay. Subsequent research resulted in commercially available kits for the detection of *Salmonella* in foods. These include the Bio-Enzabead *Salmonella* screening kit (Organon Teknika Corp., Durham, USA) and the Bactelisa screening kit for *Salmonella* (Kirkegaard and Perry Laboratories, Maryland, USA). The Bio-Enzabead kit uses the monoclonal antibodies developed by Robinson, Pretzman and Mattingly (1983) and Mattingly (1984), while the Bactelisa kit utilizes a polyclonal antibody bound to a well in a microtitre plate, but both detect about  $10^6$  cells. Both kits require pre- and selective enrichments (*see* p. 4), although the Bio-Enzabead kit also requires a selective enrichment followed by centrifugation. Clayden, Alcock and Stringer (1987) found that, using chicken and beef slurry samples artificially inoculated with *Salmonella*, 95% of the chicken and 90.5% of the beef curry were positive by both the Bio-Enzabead ELISA and the AOAC cultural method (AOAC, 1984). However, 9.5% of the beef samples were positive by the Bio-Enzabead kit alone, while 5% of the chicken samples were positive only by the AOAC method, suggesting that false-negative results might have been produced by the Bio-Enzabead kit. In a previous comparison of the Bio-Enzabead ELISA methods and the standard cultural procedure, 18% and 1.7% of the samples were positive only by the Bio-Enzabead kit and cultural methods, respectively. Todd *et al.* (1986) suggested that these results could be attributed to competing bacteria. Similarly, D'Aoust and Sewell (1986) suspected the kit of producing 25% false positives due to competing bacteria, especially as a result of cross-reactivity with *Citrobacter*.

Other recent studies have included a comparison of the 'ELISA Screening Kit for Salmonella' with the 'Salmonella Bio-Enzabead Screen Kit' by Beckers *et al.* (1988). Compared with the standard plate count method, in which 32 samples were positive, the ELISA method produced 28 positives and the bead method 35. In addition, during the selective enrichment required to multiply the cells to a detectable level, problems were observed with the competing flora. Prusak-Sochaczewski and Luong (1989) have also reported an improved ELISA method for the detection of *S. typhimurium* which would detect  $5 \times 10^4$ – $10^5$  cells ml<sup>-1</sup> within 24 h. In addition, the combination of a fluorescent antibody-linked probe with a microcolony technique has been developed by Rodrigues and Kroll (1990). Of 101 raw meat samples, 96 produced the same results as with conventional plate counts, although there were six false positives and one false negative.

Holbrook *et al.* (1989) have also compared the standard plate count (Andrews, Poelma and Wilson, 1984) with four rapid antibody methods. They concluded that the Oxoid *Salmonella* Rapid Latex Test (Oxoid code FT203) was the best rapid method with the TECRA ELISA (Bioenterprises Pty. Ltd, Roseville, NSW, Australia) second, the EQUATE ELISA (Binax, Maine, Missouri, USA) third and an enrichment culture/immunodiffusion assay—the BioControl 1–2 test (BioControl Systems Inc., Bothell, Washington, USA), fourth. The EQUATE and Oxoid SRT detected 98% of the samples positive by plate counts, the TECRA 96% and BioControl 1–2, 92%. The BioControl 1–2 and EQUATE gave six false positives each, TECRA two and the Oxoid SRT nil. In addition, the traditional culture methods took >90 h to obtain a negative result, compared with 36–58 h for other methods.

### Hybridization assays

The near 4 million nucleotide bases in an average bacterial genome constitute the definitive marker of biological identity. In consequence, even closely related bacteria possess a unique signature of base sequences at specific loci and, if these unique differences can be identified, they provide genetic markers with which to categorize natural bacterial isolates.

As with other biotechnology-based methods, clinical microbiology has pioneered new detection and enumeration systems based on DNA or RNA hybridization (Walker and Dougan, 1989). Nevertheless there is now a rapidly growing interest in DNA probe technology from research groups and industrial laboratories concerned with improving both the rate and specificity of microbial detection in foods. In broad terms, nucleic acid hybridization can be used by food microbiologists to detect or enumerate specific bacteria from foods (usually post-enrichment) and, in addition, it provides a novel method of identifying the presence or absence of virulent or pathogenic strains within a genus.

## BACTERIAL DETECTION

### Salmonella

While it would be unrealistic to claim that at the present time *Salmonella* testing by DNA hybridization constitutes a substantial challenge to existing protocols (for example, this technology was not featured in a survey of rapid methods under evaluation in six industrial laboratories in 1987; Jarvis and Easter, 1987), progress is apparent. The thrust for development in this area is the lure of the potential market for a leading reagent in rapid *Salmonella* screening for the food industry. The US food industry tests for *Salmonella* at a level of about 15% of total microbial assays and with a consequent reagent market of some US\$40 million. With a comparable market existing in Europe, if even a fraction of assays could be secured by a new technology then the rewards would be significant.

Integrated Genetics of Framingham, Massachusetts have been a pioneering

company in the development of DNA-DNA hybridization tests for the presence of *Salmonella* in foods. Their Gene-Trak<sup>R</sup> system developed by Rene Fitts and his colleagues offers a salutary example of the complexities of the search for appropriate DNA sequences to use as probes. A library of genomic *Salmonella* DNA was prepared in a plasmid vector and individual clones systematically screened for cross-hybridization to other enteric bacteria (Fitts *et al.*, 1983; Fitts, 1985). Ten unique sequences appeared to be specific for *Salmonella* and two have formed the basis of a commercial product (Flowers *et al.*, 1987). As the first to the market in this new area of biotechnology, the performance of the Gene-Trak<sup>R</sup> test is clearly a useful guide to the prospects for this approach to microbial detection in the food industry. Emswiler-Rose, Bennett and Okrend (1987) have described a comparison of cultural methods and the DNA hybridization test for detection of *Salmonella* in ground beef. All samples which were *Salmonella*-positive by cultural methods were also positive by hybridization (5 out of 108). There was a small percentage (3.7%) of false-positive samples (4 out of 108) but no false negatives.

Sall *et al.* (1988) have recently presented the results of a year-long study of the performance of the Gene-Trak<sup>R</sup> *Salmonella* assay with samples provided in coded form as a quality control sample check programme from the American Association of Cereal Chemists (AACC). The study showed that the method detects a wide variety of *Salmonella* species, including an atypical lactose-positive strain that was missed by the majority of the other participants in the sample check service. This indicates that DNA hybridization technology can provide a fast screening programme (a saving of 1-2 days over conventional cultural methods) and can actually provide more accurate information. Izat *et al.* (1989) have described a comparison between the Gene-Trak<sup>R</sup> product and culture methods for the detection of *Salmonella* on poultry carcasses and in processing waters. In a series of five experiments a total of 169 broiler carcasses and chill-tank water samples were tested. In addition, three pre-enrichment/enrichment procedures were evaluated to establish an optimum protocol for the hybridization assay. The results from these experiments indicate that direct enrichment in selective cystine medium followed by a 24 h incubation in Gram-negative broth allowed for the recovery of 0.03 salmonellae ml<sup>-1</sup> from carcass rinse and from pre-chill water, using either the standard culture method or the hybridization assay. Given that the DNA hybridization assay can be completed in 48 h, these data present an encouraging prospective for increasing acceptance of such technology. Finally, although lacking a detailed follow-up in food trials, Tompkins *et al.* (1986) have used a similar strategy for identifying cloned chromosomal sequences specific to *Salmonella* spp.

Recently a new *Salmonella*-specific DNA probe has been reported (Tsen *et al.*, 1989). Described as a 1.8 kb *Hind*III DNA fragment from the chromosome of *S. typhimurium* with a low probability of homology to the fragments described by Fitts (1985), in a study of 134 *Salmonella* and 50 non-*Salmonella* isolates no false positives or false negatives were obtained. All of the *Salmonella* probes to date, however, require in the order of 10<sup>8</sup> salmonellae

for a positive assay result, and improvements in this sensitivity must be addressed. In addition, all of the studies reported have utilized either <sup>35</sup>S- or <sup>32</sup>P-labelled radionucleotides. Both the need for and prospects of non-radioactive probes will be considered at the end of this section.

#### *Listeria (detection of the genus)*

As for *Salmonella* spp., Gene-Trak<sup>R</sup> have developed a radioactive DNA probe-based assay which detects all species of the genus *Listeria* (Klinger and Johnson, 1988; Klinger *et al.*, 1988). Monoclonal antibodies against a genus-specific *Listeria* antigen have been used for an ELISA for the detection of the *Listeria* genus in food products (Butman *et al.*, 1988). These three monoclonal antibodies have recently been used to clone the gene encoding the 30 kDa surface antigen [Susan M. Pearson and Philip J. Warner (Cranfield Institute of Biotechnology), personal communication] and the corresponding DNA sequence may prove a valuable addition to the existing repertoire of *Listeria* probes.

Within the genus *Listeria*, only *L. monocytogenes* has been identified as providing concern as an aetiological agent of food poisoning and disease in humans. Even within this group, only certain so-called virulent strains are of particular concern, and these are typically identified as falling into two serotype groups: 4b and 1/2a. In the UK the total number of cases of listeriosis more than doubled between 1983 and 1988 (Richmond, 1990) and, although only four cases in the UK have been proven to be food-borne, the importance of monitoring the presence of *Listeria* in food now reflects an enhanced consumer awareness of the potential hazards of the bacterium, in particular to pregnant women and other immuno-compromised individuals.

Given the ubiquity of *Listeria* in the environment and the near impossibility of a total eradication from foods, processed or otherwise, it is desirable that known virulent derivatives be selectively enumerated and placed in balance with the total *Listeria* population. The detailed nature of *L. monocytogenes* pathogenicity is, as yet, unclear and this absence of knowledge limits the number of genetic targets that might be identified as suitable specific markers for virulent strain detection (the urgent need to extend research in this area has been recently highlighted; Richmond, 1990). Nevertheless, several groups have evaluated putative candidates for virulence specificity, and progress in this field, already significant, is developing rapidly.

Secreted polypeptides, such as haemolysins, toxins and siderochromes (ironophores), often play a role in bacterial pathogenesis. Thus genes encoding major extracellular products have been the first to be evaluated as genetic markers of virulent strains within *L. monocytogenes*.

*β*-haemolysin. Flamm (1986) described the cloning of a presumptive *β*-haemolysin gene from *L. monocytogenes* 10403A. This element has been used by Datta, Wentz and Hill (1987) in a colony blot DNA hybridization assay to evaluate its potential for selectively identifying *L. monocytogenes*. The authors describe results from the screening of 52 different strains of *Listeria* in which only DNA from *β*-haemolytic (CAMP-positive; a test

procedure used in the identification of *Streptococcus agalactiae*) strains of *L. monocytogenes* hybridized with the probe.

More recently, Flamm, Hinrichs and Thomashow (1989) have described the cloned gene as *msp* (major secreted polypeptide), encoding a 60 kDa secreted protein from *L. monocytogenes* having haemolytic activity. The authors confirm that the *msp* gene product is not listeriolysin-0 but definitive identification with  $\beta$ -haemolysin remains to be determined.

An interesting exchange of views has been reported between Datta and Swaminathan and Broome (Datta, 1989) following a review on listeriosis presented by the latter group (Gellin and Broome, 1989). In response to a suggestion that current DNA probe-based tests identify *Listeria* only at the genus level, Datta reaffirmed findings in relation to the presumptive  $\beta$ -haemolysin clone. In reply, however, Swaminathan and Broome refer to the original doctoral thesis of Flamm (1986) where considerable cross-reactivity within the genus was demonstrated. In this present discussion we do not wish to comment further, save to point out that these exchanges reflect the still considerable difficulties in defining protocols and conditions necessary for specificity in DNA hybridization tests targeted at genus and/or strain detection.

*Listeriolysin-0* ( $\alpha$ -listeriolysin). The thiol-activated haemolysin from *L. monocytogenes* (Parrisius *et al.*, 1986) is a prominent candidate among virulence factors that promote the invasive response. Mengaud *et al.* (1988) have reported the cloning and complete nucleotide sequence of the *hlyA* locus coding for the 504 amino acid listeriolysin-0 secretion product. More recently, the regulatory features that constitute the gene expression control function for *hlyA* have also been characterized (Mengaud, Vicente and Cossart, 1989). Mengaud *et al.* (1988) and Chenevert *et al.* (1989) have described the use of a 651 bp *Hind*III fragment internal to the *hlyA* open reading frame as a candidate for the specific detection of *L. monocytogenes* by DNA hybridization. Their careful studies based on Southern hybridization of *Hind*III digested chromosomal DNAs, have shown that under stringent conditions of hybridization the 651 bp probe only reacts with *L. monocytogenes*. Detailed studies on *Listeria* isolated directly from enrichment cultures of food homogenates, remain to be evaluated before the full potential of this probe can be considered. Clearly, however, a focus on the likely causative agents of virulence and pathogenicity is a promising route to species discrimination in this genus.

*Delayed hypersensitivity.* Antonissen *et al.* (1986) have described the purification of a delayed hypersensitivity-inducing protein from *L. monocytogenes*. The gene encoding this protein has subsequently been cloned from a virulent *L. monocytogenes* serotype 1/2a strain and used as a 1.1 kb DNA probe to screen *Listeria* strains by DNA colony hybridization (Notermans *et al.*, 1989). The work described showed an encouraging specificity for this probe in identifying virulent derivatives of the genus *Listeria*, and the apparent presence/absence of the gene rather than increasing sequence degeneracy

may make this gene a potential target for the polymerase chain reaction (PCR), considered in more detail in a subsequent section.

### *Enterobacteriaceae*

Although *Salmonella* and *Listeria* represent the major targets for developing rapid detection assays, a considerable breadth of knowledge and expertise has been accumulated over the past decade on the use of nucleotide hybridization probes for the detection of a broad range of enteric bacteria. Enterotoxigenic *E. coli* (Moseley *et al.*, 1980, 1982; Hill, 1981; Echeverria *et al.*, 1982; Hill *et al.*, 1985; Ferreira *et al.*, 1986; Romick, Lindsay and Busta, 1987, 1989), *Proteus* spp. (Haun and Göbel, 1987), *Yersinia enterocolitica* (Hill, Payne and Aulisio, 1983; Jargow and Hill, 1986) and *Shigella* spp. (Boileau, D'Hauville and Sansonetti, 1984) have been successfully detected in laboratory-based assays.

### *Clostridium perfringens*

Cloning of the *C. perfringens* enterotoxin gene (van Damme-Jongsten, Wernars and Notermans, 1989; Iwanejko, Routledge and Stewart, 1989) has led to a recent application of a synthetic DNA probe, complementary to the toxin gene, for the detection of enterotoxigenic *C. perfringens* isolated from outbreaks of food poisoning (van Damme-Jongsten *et al.*, 1990).

## NON-RADIOACTIVE DETECTION METHODS

The introduction of radioactive labels into a food microbiology laboratory is anathema. Despite the relative success of Gene-Trak<sup>R</sup> products, in the majority of instances these have been taken up only in large and sophisticated laboratories servicing major manufacturers. There is no question that to reach a broad application, hybridization probes must employ non-radioactive labelling technology without significantly compromising sensitivity. Allied to developments in antibody probe technology, initial approaches have employed biotin/streptavidin-labelled DNA. Romick, Lindsay and Busta (1987, 1989) have used an enzyme-labelled probe to detect enterotoxigenic *E. coli* but observed some problems associated with contaminating bacterial flora and food matrix residues. Dovey and Towner (1989) have employed a biotinylated DNA probe to detect *E. coli* in artificially contaminated foodstuffs. A positive hybridization result could be clearly distinguished with 10 diverse foodstuffs at a contamination level of  $10^5$  *E. coli* per 100  $\mu$ l of diluted sample, representing  $2 \times 10^8$   $g^{-1}$  in the original food sample, indicating that for practical applications an overnight enrichment of the dilute suspension broth would be required. This, however, would be consistent with current practice.

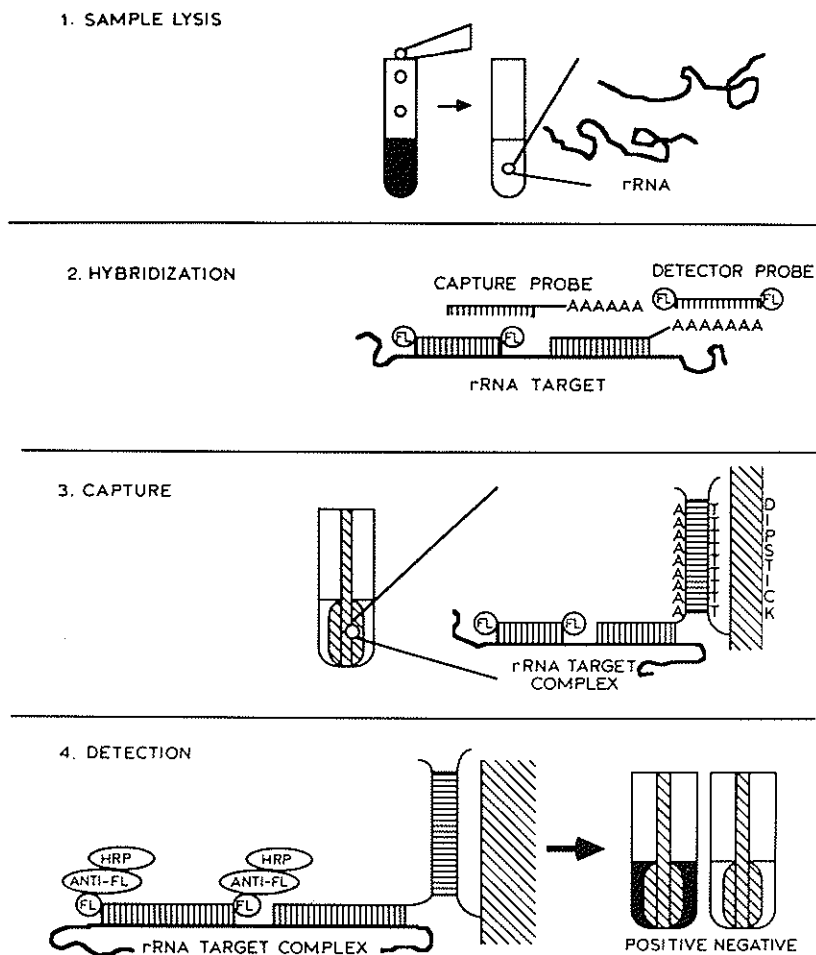


Figure 2. Hybridization assay for *Listeria* (adopted from King *et al.*, 1990).

King *et al.* (1989) have described a colorimetric nucleic acid hybridization assay for *Listeria* in foods. Based on the Gene-Trak<sup>R</sup> isotopic hybridization assay described earlier (Klinger *et al.*, 1988; Klinger and Johnson, 1988) it detects the *Listeria* genus by identifying unique regions of 16S rRNA. In the colorimetric assay two types of probes are employed, a fluorescein-labelled 'detector' probe and an unlabelled 'captor' probe (Figure 2). If *Listeria* is present in the test sample (20–24 h enrichment culture), hybridization of both detector and captor probes to specific 16S rRNA targets occur. Additionally, target complexes bind to a specially coated polystyrene 'dipstick'. After incubation of the dipstick with a rabbit antfluorescein antiserum conjugated



to horseradish peroxidase, samples are read at 450 nm in a spectrophotometer. Absorbance values of 0.1 or greater are considered positive.

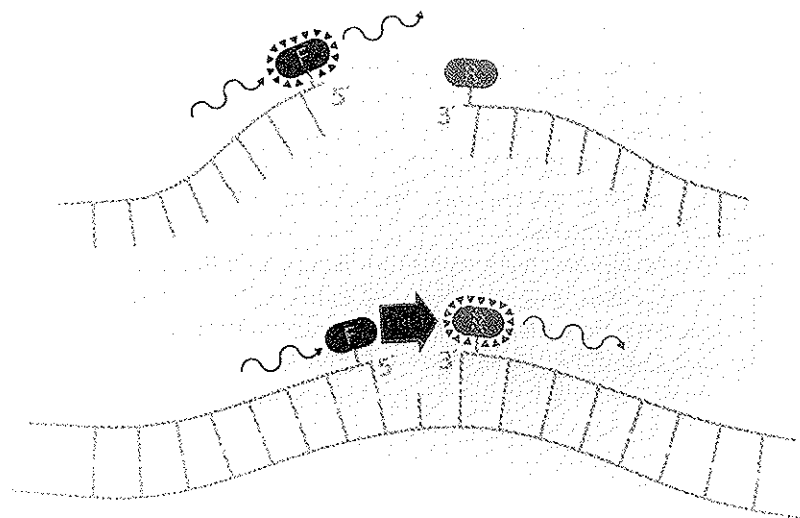
This dipstick approach was compared with culture results and had unconfirmed false-positive and false-negative rates of approximately 1.4–2.9% and 0.8–4.7%, respectively. These results are very encouraging and have subsequently been further extended (King *et al.*, 1990). If non-radioactive probes can be coupled to dipstick technology, then the future prospects for hybridization assays are considerable.

A number of new approaches to non-radioactive labelling appear particularly promising. Random primed DNA labelling with digoxigenin-dUTP followed by the detection of hybrids by enzyme immunoassay is a novel development marketed by Boehringer Mannheim for molecular biology applications. With a sensitivity of 0.1 pg of homologous DNA (12 h colour development), it will be of considerable interest to evaluate its performance in bacterial detection assays.

Enhanced chemiluminescence (ECL) constitutes a new development in DNA detection developed by Amersham International. A modified horseradish peroxidase is cross-linked to a single-stranded denatured DNA probe by using glutaraldehyde. The peroxidase complex bound to the probe stimulates the production of a peroxide ion which subsequently reacts with luminol reagent, resulting in a sustained emission of light. Claimed as conferring the highest sensitivity presently available from a non-radioactive detection system (Anon, 1989, 1990a; Pollard-Knight *et al.*, 1990), this system again awaits evaluation in bacterial detection assays.

An alternative luminescent assay is currently associated with a new DNA hybridization culture confirmation test for *Campylobacter* that will be marketed through Laboratory Impex Limited. Developed by Gen-Probe<sup>R</sup> it is termed the hybridization protection assay (HPA) and is centred around a highly chemiluminescent acridinium ester. The acridinium ester reacts with hydrogen peroxide under basic conditions rapidly to produce light. Acridinium *N*-hydroxysuccinimide esters are covalently attached to synthetic DNA probes and hydrolysis of the ester bond renders the acridinium permanently non-chemiluminescent. Gen-Probe<sup>R</sup> have devised a system whereby the acridinium ester is protected from hydrolysis when the DNA probe to which it is attached is hybridized with its target DNA. In this format, chemiluminescence is a direct measure of the degree of hybridization. Data on the effective detection of bacteria isolated from food samples remains currently 'in house' but, as a candidate for replacing radioactive labels, this system shows interesting potential. In particular, the Gen-Probe<sup>R</sup> format is part of a new generation of hybridization probes that may remove the necessity for binding the target nucleic acid to a solid matrix. Liquid phase hybridization assays should dramatically reduce the technical complexity of hybridization protocols.

Perhaps leading the new technology in this area is the application of non-radiative fluorescence resonance energy transfer (FRET) (Cardullo *et al.*, 1988). When two fluorophores, whose excitation and emission spectra overlap, are in sufficiently close proximity (5–7 nm), the excited-state energy



**Figure 3.** Schematic representation of the use of non-radiative fluorescence resonance energy transfer (FRET) to detect specific nucleic acid sequences. In the upper panel, two oligonucleotide sequences are in solution, one with fluorescein attached at the 5' end, the other with rhodamine attached at the 3' end. In this case the probes are too far apart for FRET to occur so that on excitation with blue light, only green, fluorescein fluorescence occurs. In the lower panel, the probes have hybridized to adjacent complementary sites separated by only a few bases. In this case, the probes are close enough for FRET to occur so that on exposure to blue light, instead of fluorescing green, the energy is transferred non-radiatively to the rhodamine, and red fluorescence is observed. (Courtesy of Dr David Wolf.)

of the donor molecule is transferred by a resonance dipole-induced dipole interaction to the neighbouring acceptor fluorophore. When the probes are in solution, there is no such energy transfer.

Application to nucleic acid hybridization works as follows. Two nucleic acid probes are required that hybridize to adjacent sequences on the target molecule. One may be labelled at the 3' end with rhodamine and the other with fluorescein at the 5' end (*Figure 3*). In the absence of target DNA sequences, the two probes are unassociated and the fluorescent labels are too far apart to allow FRET to occur. When the fluorescein is excited by blue light, only green fluorescence is obtained. Bound to a target sequence, however, (*Figure 3*) fluorescein and rhodamine become sufficiently close to allow FRET and, in consequence, excitation with blue light produces fluorescence not now in the green but at the red end of the spectrum. The proportion of red to green fluorescence emission, on excitation with blue light, is now a direct measure of the extent of hybridization. The technique requires no blotting or autoradiography and can be used to detect DNA or RNA in intact cells or liquid lysed cell suspensions. The simplicity of avoiding the time-consuming steps of immobilizing the target nucleic acid on a solid substrate, coupled with the specificity afforded by using two nucleotide probes and the end-user acceptability of non-radioactive labels, offers exciting new prospects for hybridization assays to detect and enumerate micro-organisms in foods.

### The polymerase chain reaction

In the past 5 years a new technique termed the polymerase chain reaction (PCR) has revolutionized gene engineering technology. PCR is an *in vitro* method for the enzymatic synthesis and amplification of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest on the target DNA. A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase, results in the exponential accumulation of a specific fragment whose termini are defined by the '5' end of the primers (Oste, 1988; Saiki *et al.*, 1988; Erlich, 1989; White, Arnheim and Erlich, 1989).

As indicated in the previous section, the sensitivity of existing DNA hybridization protocols based on single gene target sequences is low, requiring  $10^6$ – $10^8$  bacteria for a positive signal. Increased sensitivity can be obtained by using ribosomal RNA (rRNA) as a target sequence. For example, Haun and Göbel (1987) could detect as few as  $10^3$  bacteria in DNA/RNA dot blots. While such DNA/RNA probes provide a useful contribution to bacterial detection, they cannot detect and identify details of pathogenicity and virulence. It is, however, just such a generic mechanism for increased sensitivity that can be provided by PCR.

The first account of the use of PCR to detect a virulence factor in pathogenic bacteria was presented by Olive, Atta and Setti, 1988. PCR amplification of the heat-labile toxin gene in toxigenic *E. coli* followed by detection of the amplified product, using a biotin-labelled probe, allowed detection of less than 1000 bacteria. This relatively early study employed the Klenow fragment of DNA polymerase to achieve the amplification cycles and this work has subsequently been repeated and extended using the polymerase enzyme now synonymous with PCR, namely the thermostable *Taq* polymerase (Olive, 1989). In this new study a single bacterium could be detected following 30 cycles of amplification. In a similar, though unrelated study, Furrer, Candrian and Lüthy (1990) have described the detection of as few as 20 *E. coli* of a strain producing heat-labile enterotoxin type 1 in water within 8–12 h by PCR.

No applications of PCR directly targeting the detection of bacteria in food systems have yet been described. Clinical applications predominate, with the detection of *Mycobacterium leprae* (Hartskeerl, De Wit and Klatser, 1989), *Bacterioides gingivalis* (Sorge *et al.*, 1989), *Legionella pneumophila* (Starnbach, Falkow and Tompkins, 1989) and *Eubacteria* (Rumore and Steinman, 1989). The specific detection of genetically engineered micro-organisms in the environment has also stimulated research into this application of PCR (Steffan and Atlas, 1988; Chaudhry, Toranzos and Bhatti, 1989). Despite the lack of data on food systems, it would seem that the detection of specific bacterial species or even serotypes in food should be no more challenging than the detection of a single bacterium in stool samples (Olive, 1989).

### Recombinant bacteriophage

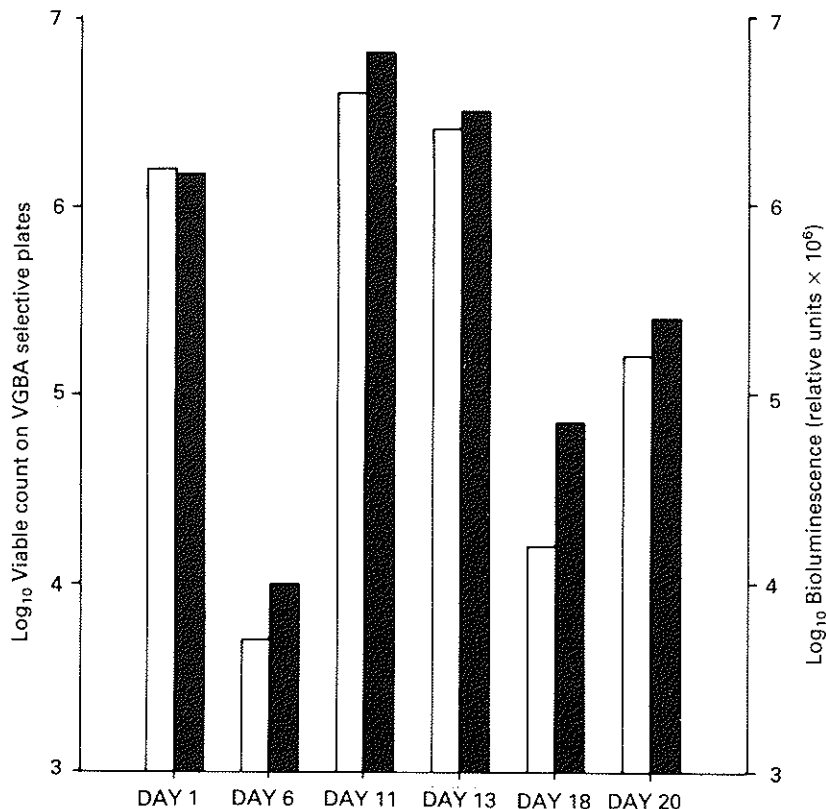
The use of antibodies, both polyclonal and monoclonal, and DNA hybridization, including PCR amplification, have evolved from a broad user base in the clinical sector to applications in the food industry. In 1987, however, an entirely new concept for bacterial detection was described (Ulitzur and Kuhn, 1987) which, having no prior art, has since developed in parallel for both clinical and industrial use. The principle of this new technology involves the introduction, via gene engineering, of *lux* genes into the genome of a bacteriophage.

The prokaryotic *lux* system encodes the biochemical (enzyme) components that provide a bioluminescent phenotype, and is found in a number of marine bacteria. The light-emitting reaction involves an intracellular luciferase-catalysed oxidation of FMNH<sub>2</sub> (reduced flavin mononucleotide) by molecular oxygen, with the concomitant oxidation of a long-chain aliphatic aldehyde such as dodecanal. The *lux* pathway encodes both the two subunits of the luciferase enzyme (*luxAB*) and the multisubunit components of a fatty acid reductase (*luxCDE*) (Meighen, 1988). In effect, however, only *luxAB* is required for the introduction of a bioluminescent phenotype because the long-chain aldehyde can be supplied in chemical form and is freely diffusible across the prokaryotic membrane (Blissett and Stewart, 1989). Bacteriophage genetically engineered to contain *luxAB* are dark because they lack the intracellular biochemistry necessary for light production. Infection of host bacteria by the phage, however, leads to the expression of host phage genes and, within 30–50 min, the additional *lux* genes. The result of phage infection is bioluminescent bacteria and the true potential of this system is that the entire evolutionary diversity of bacteria/bacteriophage coupling can be harnessed.

Ulitzur and Kuhn (1987) described the engineering of *Lambda* Charon 30 to contain a 9 kb DNA segment encoding the entire *lux* pathway from *V. fischeri* MJ1. As few as 10 *E. coli* cells could be detected after 100 minutes post-infection by the recombinant phage L28. A particularly significant point from these studies was the observation that these detection levels could be achieved directly in milk by the simple expedient of phage addition followed by a short period of incubation at 25°C.

Using further phage constructs obtained from Ulitzur, Kuhn and Suissa, 10<sup>2</sup> *S. typhimurium* have been detected using a simple luminometer (Stewart, Smith and Denyer, 1989). Although the detection of specific pathogens such as *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* will require a committed programme for the genetic engineering of host-specific bacteriophage, this technology offers the potential for rapid, user-friendly microbial testing, particularly pertinent to the food industry. The detection limits for specific pathogens in foods is in the order of 1 per 25 g, a level beyond the detection limit of direct bioluminescent bacteriophage assays. In consequence, there will still be a requirement to go through a recovery and enrichment protocol prior to the bacteriophage assay, but same-day testing seems possible.

The potential for a revolution in microbial testing can, however, be perceived with the near on-line detection of indicator micro-organisms. By definition, these are micro-organisms present in significant numbers within a food which, while not pathogenic, can be related through increasing count to the increasing probability of pathogen contamination. Recombinant *lux*<sup>+</sup> bacteriophages can detect such indicator bacteria without recovery or enrichment, provided that they are present in a food matrix at levels greater than 10<sup>3</sup> per g. Since the assay requires only 30–50 min, this allows the evaluation of indicator strains in less than an hour. An example of the use of this technology for the rapid evaluation of enteric bacteria on a factory surface of a meat-processing line is shown in *Figure 4* (Stewart, 1990). Using a pool of *lux*<sup>+</sup> recombinant enteric phages obtained from Ullitzur, Kuhn and Suissa, a comparison between standard plate count (18 h) and bioluminescence (50 min) has been determined that shows a striking correlation. Given the simplicity of the assay and the time-scale of 1 h, this technology would seem to have the unique potential to be directly applicable to a hazard analysis critical control point (HACCP) system.



**Figure 4.** Determination of enteric bacteria on a factory surface of a meat-processing line enumerated by viable plate count on selective agar or by bioluminescence after a 50 min incubation with a prototype *lux*<sup>+</sup> enteric phage assay. □, Coliform counts cm<sup>-2</sup>; ■, bioluminescence. (Adopted from Stewart, 1990.)

***In situ* detection of micro-organisms—applications to foods**

The detection of micro-organisms in foods currently depends on the use of methods which involve maceration of the food to produce a homogeneous suspension which may then be analysed by plating out onto appropriate agars or by one of the modern alternatives. However, this destroys valuable information on where the micro-organisms grow in the food. Many foods are complex heterogeneous mixtures of components. At different sites within the food there will be variation in the levels of oxygen, pH, water activity ( $a_w$ ), nutrients, and in certain foods, preservatives. This will lead to a series of interconnected micro-environments, some of which will be preferential for microbial growth. Detection of such sites would give valuable information on how to reduce microbial growth, for example, by reduction of the supporting phase, targeting preservatives or identification of one component significantly contributing to contamination, with the resultant potential to reduce the risk of poisoning or extend shelf-life. Such detection would also allow the interaction of micro-organisms and food components to be studied, as well as microbe-microbe interactions.

The detection of micro-organisms *in situ* is an area which has been recognized as of significant importance in medicine for the rapid detection of infection, especially by organisms which are difficult to culture or require prolonged incubation. However, the use of this technique has only recently been acknowledged as of benefit to the food industry (Fernandes *et al.*, 1988; Dodd, 1990).

*In situ* detection of micro-organisms can be achieved by the microscopic examination of thin sections of tissue appropriately stained to allow the organisms to become apparent. A variety of classical methods exist for producing sections, especially where embedding procedures are employed. However, these techniques have the disadvantage that they may require some days to produce embedded material and the processes used can produce structural alterations and risk the shifting of components, particularly micro-organisms.

A useful alternative to these techniques is cryosectioning. This involves the rapid freezing of a specimen which is then sectioned at a low temperature (usually  $-20$  to  $-40^\circ\text{C}$ ). Sections can be transferred to microscope slides, stained using an appropriate stain and viewed microscopically. The production of a section using this technique is so rapid that sections can be produced in about 1 h. Most, if not all, of the staining techniques used for embedded specimens can also be used with cryosections, although these require more careful handling.

The advantage of the method, other than its rapidity, is that there is little alteration to the structure of the specimen, and the method has been used for some time to examine food structure (Flint, 1988).

## DETECTION OF MICRO-ORGANISMS BY STAINING

The successful detection of micro-organisms within sections depends on the use of a stain which will give good differential staining between the micro-organisms and the food components.

Brown and Hopps (1973) described a reliable Gram stain method for use with tissue sections. Although designed for embedded sections, this method was used by Fernandes *et al.* (1988) to examine the growth of spoilage organisms in cryosections of pâté. The study used the method in conjunction with an image analysis system to produce a semi-automated detection and quantification method for microbial growth. The main disadvantage of the Gram stain as a tissue stain is that the acetone removes fat from the sections and so will produce changes in sections of fatty foods.

The use of toluidine blue as a stain for the differentiation of food components is well recognized (Flint and Firth, 1988). Preece (1978) reviewed its use for detection of bacteria in sections of infected plant tissues. Dodd and Waites (1989) used cryosectioning and toluidine blue staining to look at the spoilage of British fresh sausages. In this study sausages stored under refrigeration were examined periodically by viable counting and cryosectioning until gross spoilage was evident. The main spoilage flora components by viable counting were found to be the Gram-positive rod, *Brochothrix thermosphacta*, and yeasts. These were clearly identifiable in stained cryosections at  $\times 100$  and  $\times 400$  magnification, the lower magnification allowing detection of colonies and the higher magnification the detection of single cells. *B. thermosphacta* could be clearly distinguished as long filamentous-like rods which are typical of the species. With increasing spoilage (as determined by viable counts) increasing contamination of the surface and subsurface region of the sausages occurred to a depth of 4 mm. However, even when gross spoilage of the surface occurred, after 13 days, there was no evidence of growth of the organisms at the centre of the sausage sections. This differential growth distribution, with preferential growth at or near the surface, clearly demonstrates the influence of micro-environments and shows how knowledge of the sites of microbial growth within a food would suggest possible mechanisms to reduce growth and hence extend shelf-life. It also indicates the problems that might occur in obtaining a representative viable count within such a food. In this study yeasts were detectable at a level of  $10^4 \text{ g}^{-1}$  and bacteria at  $10^5 \text{ g}^{-1}$  (as determined by viable count).

As well as giving good detection of the micro-organisms, which stain purple, the use of toluidine blue means that the various food components can be distinguished. This has obvious advantages if only one food component is the contaminating source.

Fox *et al.* (1989) used the same techniques to look at the development of starter culture colonies in the fermentation of red meat. Using a commercial starter (Lactostart) a successful fermentation, as measured by decrease in pH and viable counts of the natural spoilage flora (*Pseudomonas* and *B. thermosphacta*) and an introduced challenge organism (*Staph. aureus*), was

obtained after 48 h at 30°C. A progressive development of the colonies from single cells at 3 h, to groups of cells at 9 h and microcolonies at 24 h was evident in stained samples. With progression of the fermentation and a decrease in the pH, staining of the muscle changed from blue to yellow, so that colonies were readily detected. There was an uneven distribution of starter culture, with some areas showing no visible growth of the organism. A similar irregularity of distribution was also observed in a commercially produced salami. In both instances the organisms tended to be present at the interfaces between pieces of tissue. These findings suggest that there is the potential to create micro-environments in such products, where pathogenic organisms would grow if inadequate mixing of the components occurs.

The use of toluidine blue staining and cryosectioning to look at microbial growth in other foods has also been reported by Charles, Dodd and Waites (1989), who examined rope production in bread, and Dodd (1990), for a variety of foods.

Yiu (1985) used fluorescence microscopy to detect the presence of the microflora in several cheese varieties. In this study, although cryosectioning was used to produce the sections, the samples were first fixed using glutaraldehyde, and some embedded sections were also used. Acridine orange or calcofluor white allowed detection of the moulds in surface-ripened cheeses, and acriflavin or acridine orange were used to show the presence of the streptococci and lactobacilli in cheddar cheese. Acridine orange had the advantage that it also allowed the cheese microstructure to be investigated.

#### DETECTION OF SPECIFIC MICRO-ORGANISMS *IN SITU*

Most of the methods described above have the disadvantage that only limited identification of the organisms observed is possible, primarily on the basis of morphology and correlation with viable counts, although the Gram stain allows a further distinction between Gram-positive and Gram-negative organisms.

Detection of a particular micro-organism in a section depends upon the use of specific probes, usually an antibody linked directly or indirectly to an enzyme or fluorochrome. Such methods are used clinically to look for specific disease-causing organisms in tissue specimens, but the use of these techniques for detecting spoilage or food-borne disease-causing organisms in food systems is only now receiving attention. The method has great potential as antibodies to many important food-borne disease-producing organisms already exist.

Alcock *et al.* (1990) have used a polyclonal antibody to detect the spoilage organism *B. thermosphacta* both in cryosections of model gel systems and in cryosections of sausages. In this work both fluorochrome detection, using acridine orange, and enzymic detection, using horseradish peroxidase developed with diaminobenzidine (DAB), have been used to visualize the bound antibody. Of these the peroxidase/DAB system has proved the most successful as the brown colour reaction is readily visible, even in sausage sections counter stained with toluidine blue to visualize the sausage components and



non-antigenic cells. This work is progressing to use both polyclonal and monoclonal antibodies to other micro-organisms and monoclonal antibodies to specific food components, so that both the organism and the component on which it is growing can be identified specifically.

Another interesting possibility for use with sections is that of *in situ* hybridization, which allows specific DNA and RNA sequences to be detected. With the development of specific probes this might enable not just the species of bacterium to be identified, but also whether it contained a particular gene, for example, a toxin gene. Although much of the original work in this area has been carried out using radioactively labelled probes, non-radioactive *in situ* hybridization systems are being developed which use enzymes or fluorochromes for the detection system (Scherthan, Kioschis and Zankl, 1990), similar to those used for antibody-linked detection. This means that the sites of hybridization could be viewed directly, providing a much clearer and more rapid detection of the presence of bacteria carrying specific sequences within a section.

#### DETECTION OF VIABLE CELLS *IN SITU*

One disadvantage of most of the staining techniques described so far is that they do not allow differentiation of live and dead cells. This may be an important consideration in studies which are not looking at the successive development of colonies in a food product but are looking for the presence of organisms in a sample.

Acridine orange has long been accepted as an indicator of viability of cells, live cells fluorescing orange under UV light and dead cells fluorescing green. This is the basis of all detection in the direct epifluorescent filter technique (DEFT) (Pettipher *et al.*, 1980). However, recent reports indicate that this system may be unreliable after certain processes, and dead cells may still appear orange (Pettipher and Rodrigues, 1981; Betts *et al.*, 1988).

The use of INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride] staining as a means of viable cell determination has been examined by a number of workers (Zimmerman, Iturriaga and Becker-Birck, 1978; Betts, Bankes and Banks, 1989), and a study using INT staining with specific fluorescent antibody staining has been reported (Baker and Mills, 1982). Whether such stains would prove successful for detection of cells in sections remains to be determined.

One interesting possibility in this area is the potential for use of the *in vivo* bioluminescent bacterial *lux* system as a marker of viability. As an active metabolism is needed for the production of bioluminescence (*see* the following section), then only actively metabolizing cells would be 'luminescent'. Using the light-detection capacity of the Hamamatsu Argus 100 Vim 3 camera linked to a microscope, such light-producing cells should be detectable *in situ*. This could be done either by incorporation of *lux*-carrying bacteria into foods, or by using the *lux*-based phage detection system described previously, specific organisms could be detected in sections of commercially produced foods. Work towards these ends is currently being developed in our laboratories.

### The genetic engineering of micro-organisms to provide novel environmental monitors

Micro-organisms are sensitive biological indicators of toxic agents. Increasingly, therefore, they are being employed as microbial sensors, and both the relevance and specificity of these sensors is being tuned by gene engineering techniques. A significant component within the genetic construction of new indicator strains is the introduction of a bioluminescent phenotype into normally dark terrestrial micro-organisms (Stewart, 1990).

Shimon Ulitzur was the first to exploit the potential of bioluminescent bacteria for detecting environmentally toxic agents. His work with *Photobacterium phosphoreum* led to the development of the Microtox system (Ribo and Kaiser, 1987; Kaiser and Ribo, 1988). With gene engineering, however, those micro-organisms which are either particularly sensitive to specific antimicrobial agents, or specifically relevant to an industrial process, can be harnessed. Applications for such recombinant bacteria extend across the entire spectrum of antimicrobial substances.

#### BIOCIDE MONITORING

Biocides are widely employed for microbiological control in manufacturing, environmental, engineering, service and other industries as preservative or disinfectant agents. Their application is often complex, and adverse situations can lead to their depletion through chemical decomposition, dilution and inactivation. Under these circumstances, monitoring of biocide levels is essential to ensure adequate protection, and continuing biocide presence and efficacy is usually confirmed by microbiological challenge testing (Stewart, Jassim and Denyer, 1990). Currently, such tests employ conventional culture techniques for determining microbial survival and are therefore not amenable to immediate on-site testing. Bioluminescent micro-organisms offer the potential for rapid biocide testing, provided that a clear correlation between cell viability and bioluminescence can be established.

In principle, any micro-organism can be converted to a bioluminescent phenotype and so, clearly, those organisms which form the current basis for biocide evaluation tests (e.g. *Table 1*) may be genetically engineered accordingly.

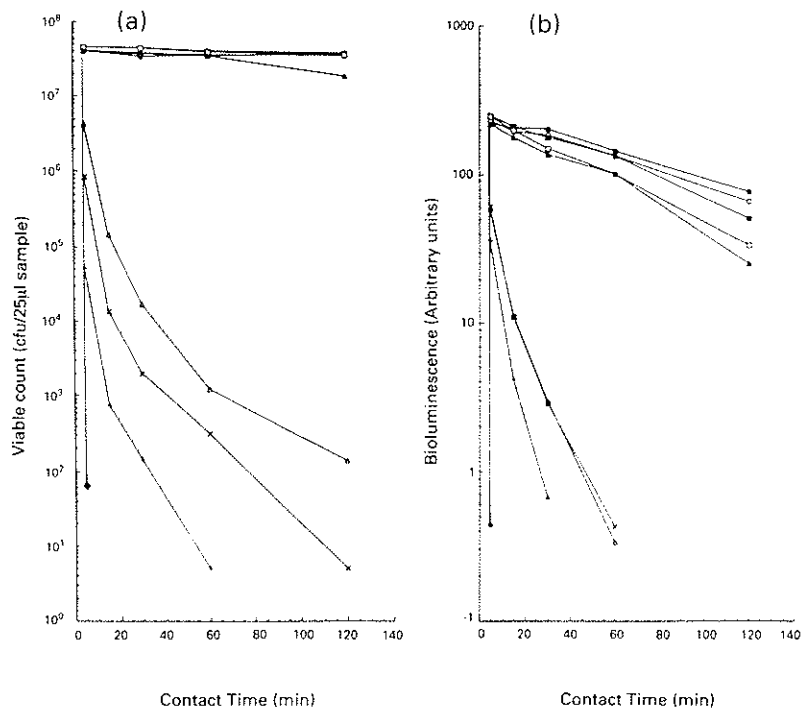
**Table 1.** Examples of biocide test organisms as employed in disinfectant assay BS 6905 (1987)

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|   |
|---|
| <i>Escherichia coli</i> NCTC 8196       |
| <i>Pseudomonas aeruginosa</i> NCTC 6749 |
| <i>Proteus vulgaris</i> NCTC 4635       |
| <i>Staphylococcus aureus</i> NCTC 4163  |

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For *E. coli* NCTC 8196, the kinetics of cell death following biocide action are clearly reported by bioluminescence and can be used to establish the decimal reduction time (*D*-value) at specified concentrations (*Figures 5, 6*) from which the concentration exponent for individual biocides (Hugo and

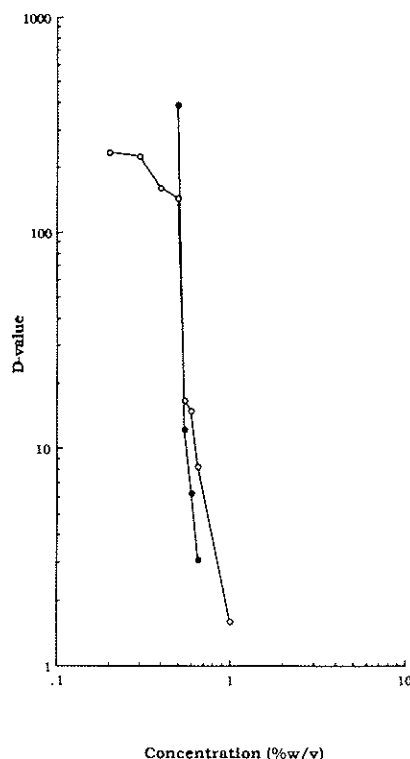


**Figure 5.** The effect of phenol concentration on (a) viable counts and (b) bioluminescence. Phenol concentration (% w/v): ●, 0; ○, 0.2; ■, 0.3; □, 0.4; ▲, 0.5; △, 0.55; ×, 0.6; +, 0.65; ◆, 1.0. (Adapted from Jassim *et al.*, 1990.)

Denyer, 1987) can be determined (Jassim *et al.*, 1990). Good agreement between bioluminescence and viable count data exists over in-use bactericidal concentration ranges.

The sensitive and early reporting of biocide-induced injury by bioluminescence has been demonstrated using biocide analogues of galactose (e.g. dichlorophenol galactoside and tribromophenol galactoside). These 'warhead delivery' molecules are actively transported into *E. coli* on the *lac* permease and the biocide released by intracellular hydrolysis (Denyer, Jackson and Al-Sagher, 1990). When tested in a bioluminescent phenotype, intracellular injury can be readily identified by a decrease in bioluminescence at concentrations showing no corresponding early decrease in viability (S.P. Denyer, personal communication).

In practical terms, bioluminescent *E. coli* provide a means of establishing the antibacterial efficacy of a biocide preparation within 5–10 min (Stewart, Smith and Denyer, 1989). Conventional microbiological techniques would require in excess of 18 h to provide equivalent data, and so, for the first time, there is the opportunity to perform rapid on-site evaluation of biocide activity in such areas as process water control and water-holding tanks.



**Figure 6.** Logarithmic relationship between phenol concentration and decimal reduction time (*D*-value) as calculated from viable count (●) and bioluminescence (○) measurements (adapted from Jassim *et al.*, 1990).

#### ANTIBIOTIC DETECTION

*lux* Expression elements functional in lactic acid bacteria have been constructed to provide novel reagents for the detection of antimicrobial activity in milk (Ahmad and Stewart, 1988). Inhibition of starter culture bacteria may be caused by antibiotics, detergents, disinfectants or bacteriophage, and although, in many cases, the precise identification of the inhibitory substance is not required, there is a need for a test which reports their presence more rapidly than the current and extensively used disc and dye reduction assays (Harrigan and McCance, 1976). Bioluminescent *Lactobacillus casei* has been used successfully in a prototype system to detect low levels of antibiotic activity within 60 min (Ahmad and Stewart, 1990). A bioluminescent starter culture assay system would be used as a test reagent, which would not itself be utilized for any manufacturing process but which could screen for any antimicrobial activity detrimental to culture growth and viability. Recently, Hofi *et al.* (1989) have described the inhibition of growth of *Lactobacillus diacetylactis* by insecticide residues and, although their work employed non-recombinant organisms, the potential to extend their studies into the

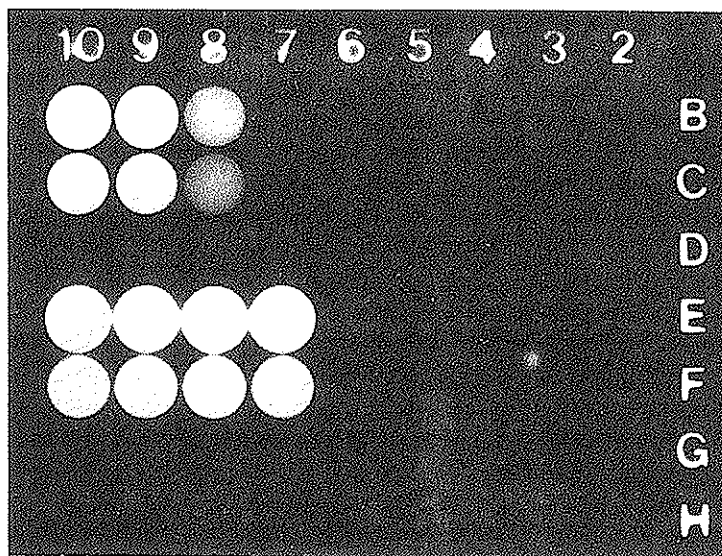
monitoring of such residues in foods by using bioluminescent lactic acid bacteria is clear.

In addition to the use of lactic acid bacteria, Ulitzur and Kuhn (1987) have described the detection of ampicillin at  $0.5 \mu\text{g ml}^{-1}$ , chloramphenicol at  $0.2 \mu\text{g ml}^{-1}$ , polymyxin-B at  $0.15 \mu\text{g ml}^{-1}$  and cephalosporin at  $0.05 \mu\text{g ml}^{-1}$  using bioluminescent *E. coli*.

#### HEAVY METAL DETECTION

Korpela and Karp (1988) have described the use of bioluminescent *E. coli* for the detection of the heavy metal, cadmium. These authors obtained a detection limit for the presence of  $\text{Cd}^{2+}$  ions of 47.5 p.p.b. using a 2 h measuring period. This value fits comfortably within the upper permissible values for Cd in soils used for food production (3 p.p.m.) and in sewage sludge to be used in agriculture (20–40 p.p.m.) (Giller and McGrath, 1989).

Bioluminescent *E. coli* have also been used to detect  $\text{Hg}^{2+}$  at concentrations of 5 p.p.m. in 15 min (Figure 7; Skelton and Stewart, unpublished results). Given the current EEC limits for Hg in agricultural soils (1 p.p.m.) and sewage sludges for land distribution (750–1200 p.p.m.), this detection limit is encouraging. Enhanced and specific sensitivity would be desirable,



**Figure 7.** A polaroid camera luminometer (Dynatech) was used to compare bioluminescent *E. coli*, with or without expression of *merT*, by a quaternary ammonium compound or by  $\text{HgCl}_2$ . Rows B and E, *E. coli* containing a *luxAB* expression plasmid; rows C and F, *E. coli* containing *luxAB* expression plasmid and a compatible *merT* expression plasmid; rows B and C columns 2–10, contain a quaternary ammonium biocide at serial twofold dilution, starting in column 2 at 1% v/v; rows E and F, columns 2–10, contain  $\text{HgCl}_2$  at serial twofold dilution, starting in column 2 at 100 p.p.m. Wells containing viable micro-organisms produce a white image on the film after a 5 min exposure. The bacteria were incubated with biocide or  $\text{HgCl}_2$  for 5 min prior to exposure.

however, and to this effect the *merT* gene (mercury transport) has been obtained from Dr T J Foster and expressed in a bioluminescent *E. coli* (Skelton and Stewart, unpublished results). In the event, the expression of *merT* did not increase the sensitivity with which Hg could be detected (Figure 7). There is, however, an alternative and more generic approach that could dramatically extend the use of recombinant bioluminescent bacteria in this type of assay.

Bacterial Hg resistance depends upon binding Hg to the *merR* repressor gene product and activation of expression (Brown, Lund and Nibriain, 1989). A genetic couple of the *merR* gene and accompanying rightward and leftward promoter so that the rightward promoter expressed *luxAB*, could allow very sensitive detection of Hg, based not on its cellular toxicity but on its activation of gene expression which, by definition, occurs at concentrations well below those that would lead to metabolic inactivation. Specificity would be provided by the Hg/*merR* interaction, sensitivity by the resulting increase in bioluminescence over background.

This type of genetic switch is applicable to the sensitive and specific detection of many industrial pollutants. The detection of aromatic hydrocarbons such as toluene and xylene, for example, could be via a *lux* couple to the *meta* pathway promoter from the TOL plasmids of *Pseudomonas* (Inouye, Nakazawa and Nakazawa, 1984). These pathways are substrate inducible so that, as for Hg, the presence of an aromatic hydrocarbon substrate would, at low concentrations, induce *lux* expression and hence a detectable bioluminescent phenotype.

The degradation pathways for many aromatic compounds proceed via synthesis and operation of oxygenase enzymes, thereby offering wide-ranging detection potential even amongst chemicals which would normally be considered antimicrobial (Bloomfield, 1990). In addition to the pseudomonads, this degradative capacity extends to species of *Acinetobacter*, *Moraxella* and *Nocardia* (Bloomfield, 1990), extending the potential for *in vivo* bioluminescent detection into other genera.

The advent of polymerase chain reaction technology (PCR) (*see p. 19*) allows the rapid isolation and cloning of a wide range of previously defined and sequenced promoters controlling the expression of catabolic or detoxification pathways. The potential exists, therefore, to harness current molecular understanding of genetic switching to the construction of gene switch/*lux* chimeras functional in bacteria such as *E. coli* or *Pseudomonas* spp. and with major potential for both research into gene control and as tools for toxicant monitoring.

#### BIOLOGICAL INDICATORS OF STERILIZER EFFICACY

Traditionally, the success of a sterilization process has been confirmed by the application of direct sterility tests on the final processed product, an approach recognized to suffer from major statistical limitations (Bryce, 1956; Brewer, 1957; Brown and Gilbert, 1977). For increased confidence, the indirect testing of sterilizers has become an integral part of any sterilization process,

affording both confirmation of correct operation and a measure of sterility assurance (Denyer, 1987). Methods of efficacy testing include instrumental, chemical and biological monitoring, the latter two approaches involve introduction of an indicator into a dummy article to measure sterilizing potential. The most realistic approach to sterilizer monitoring is offered by the use of live micro-organisms (biological indicators), an appropriate test organism requiring uniform and significant resistance to the sterilization protocol while demonstrating low pathogenicity. The use of biological indicators is strongly advocated by both the British Pharmacopoeia (1988) and United States Pharmacopoeia (1990) for the validation of all sterilization methods, and is recommended for incorporation into each sterilizing cycle in the case of gaseous sterilization, e.g. formaldehyde, ethylene oxide, which may suffer partial failures.

Biological indicators undoubtedly offer the most direct method for monitoring sterilizer performance but suffer from a reputation of intrinsic biological variability, principally dictated by inoculum history (Waites and Bayliss, 1980), strain selection and recovery methods (Greene, 1982; Hodges, 1982), and extended recovery and incubation periods of up to 7 days (United States Pharmacopoeia, 1990). There is undoubtedly a need for a rapid microbiological approach to sterilizer monitoring, in particular, for processes involving gaseous sterilization. Ideally, such an approach should provide a quantitative measure of the biocidal potential of the process within a period of only a few hours. In this way it should be possible to identify rapidly a flawed or faulty sterilization process, thereby conferring the ability to take corrective action immediately. This would reduce product wastage, improve sterilizer efficiency and enhance safety.

Although originally isolated from a Gram-negative marine bacterium, bacterial luciferase will function in Gram-positive bacteria. Genetic engineering is required to couple expression of the *lux* genes to appropriate Gram-positive promoters and to obtain stable insertion via Gram-positive replicons. Conversion of *Bacillus* spp. to a bioluminescent phenotype (Carmi *et al.*, 1987; Karp, 1989) is particularly interesting because many of these bacteria are able to undergo a biochemical differentiation and morphogenic change to produce heat-stable dormant endospores. Spores obtained from phenotypically bioluminescent vegetative cells are dark. This is not surprising since dormant bacterial spores exhibit no detectable metabolism, do not have detectable ATP or electron transport and hence have no energy source to drive the light reaction. The onset of electron transport and the initiation of metabolism is, however, a very early event during spore germination, a process which ultimately converts the spore back into a vegetative cell. For *lux*-containing spores, germination is accompanied by the emergence of bioluminescence, providing a sensitive real-time monitor of the germination and outgrowth process (Stewart, Smith and Denyer, 1989). Killed or injured spores that are unable to germinate can produce no light and hence those physical and chemical processes which are designed to kill bacterial spores can be effectively monitored within minutes in a simple luminometer (Hall, Harding and Waites, 1990).

A clear and powerful synergy exists between the need to develop biological monitors for rapidly evaluating sterilizer efficacy and the bioluminescence potential of recombinant bacterial spores. Innovation in this, as well as the other areas identified in this section, should provide major advances in microbial assays in the near future.

### **Molecular epidemiology**

The need to demonstrate the identity of two strains of the same species has been most clearly recognized in medicine in the epidemiology of disease. The same requirements are only now being identified in the food industry, although the potential benefits are enormous. The ability to trace end-product contamination specifically to a particular raw material or processing fault, the identification of critical control points in hazard analysis systems, even the ability to determine whether contamination has occurred in production or through consumer use, are all problems which can be addressed by this ability.

The more commonly used methods for strain differentiation, such as biotyping, phage typing and serotyping, have limitations which restrict the usefulness of the technique. The development of such typing schemes is time-consuming and, consequently, they have been developed for only a very limited number of species. The well-established phage typing schemes in *Salmonella*, for example, have taken some years to develop and the types needed are constantly expanding. With *S. typhimurium* strains isolated in 1988, the five most commonly reported phage types accounted for only around 45% of isolates, with over 70 other phage types being represented amongst the remaining isolates (Anon, 1990b). Thus, to satisfactorily phage type *S. typhimurium* a large number of different phage stocks would have to be maintained and used. With *Salmonella enteritidis* there is the opposite problem. Although the top five phage types accounted for around 94% of all isolates in 1988, 80% of these were accounted for by phage type 4 (Anon, 1990b) and thus there is a potential lack of discrimination within the system.

Many of these problems that exist for phage typing also exist for serotyping. With both systems there is the problem of new types arising which are not typable by the existing systems. With some species of bacteria for which typing schemes are less well developed, this means that there may be a high frequency of non-typables appearing, making the schemes unreliable.

The traditional methods also have the disadvantage that they may be slow to use; this is particularly so with biotyping, where a series of tests may take several days, and generally limits the number of strains that may be examined.

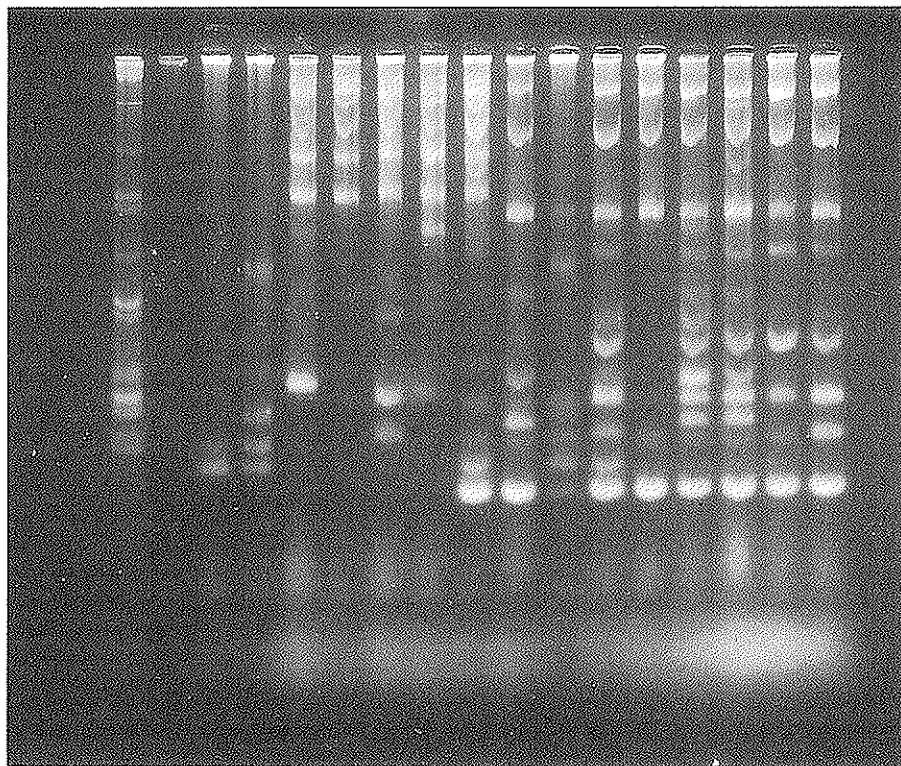
Molecular-based methods for strain differentiation provide a reliable and increasingly successful alternative to the traditional methods. In general, such alternatives are rapid, highly discriminating and applicable to a wide range of bacterial species. Their use and development have been led by clinical medicine, although the advantages of such methods are now being recognized by the food industry.



## PLASMID PROFILING

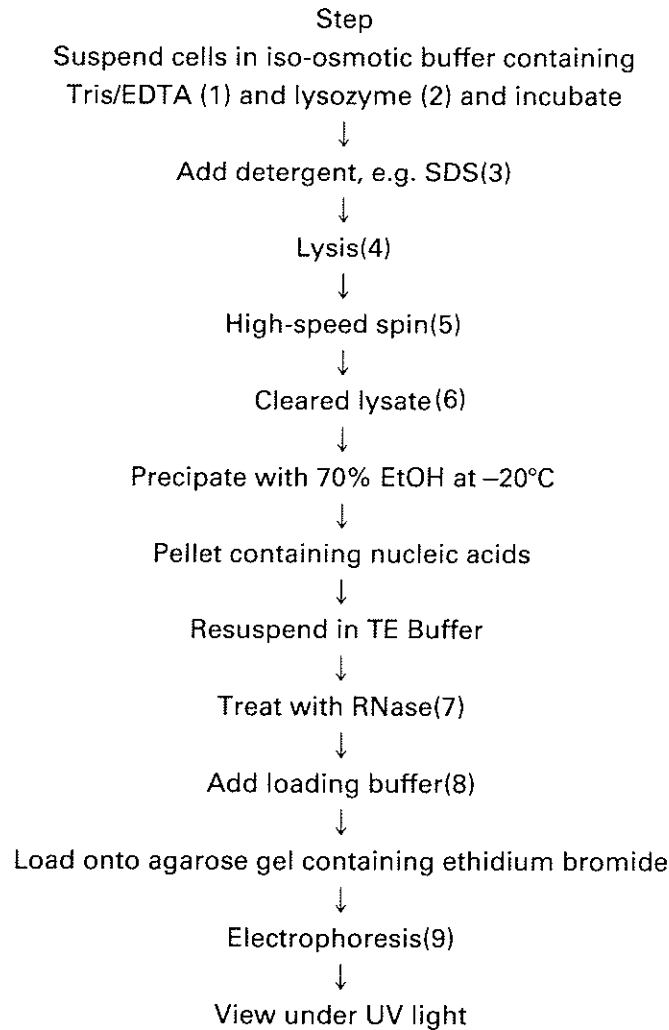
In addition to the chromosome, bacterial cells contain small DNA molecules called plasmids. These vary in size and number between different strains, and thus plasmid content can be used as a way of distinguishing between isolates of the same bacterial species.

In general, the technique consists of the preparation of a crude lysate containing the plasmid DNA and its separation on a size basis by agarose gel electrophoresis. This produces a series of bands, or a 'profile', which is characteristic of the strain (*Figure 8*). Profiles can be compared to determine the identity, or otherwise, of two isolates.



**Figure 8.** Plasmid profiles of *Staph. aureus* strains. Lane 1 (left of picture) shows an *E. coli* marker strain; lanes 2–17 are 16 isolates of *Staph. aureus*. Isolates in lanes 16 and 17 have the same profile and thus have a common source.

Generally, a small-scale method of plasmid isolation is used, requiring only a few colonies. A generalized procedure is shown in *Figure 9*. Numerous different isolation techniques have been devised which vary primarily in the growth and lysis conditions and the methods used to achieve a partial purification of the plasmid DNA. A useful review of methods is presented by Trevors (1985). Certain methods (Eckhardt, 1978; Birnboim and Doly, 1979;



#### Notes

- (1) Removes outer membrane in, for example, *E. coli*.
- (2) Causes partial digestion of peptidoglycan cell wall. For lysozyme-resistant species use other enzymes, e.g. lysostaphin for *Staphylococcus*.
- (3) Gently disrupts plasma membrane.
- (4) Solution becomes a clear semi-gel.
- (5) Removes high molecular weight chromosomal DNA and cell debris.
- (6) Contains plasmid DNA, RNA, carbohydrate, protein and some chromosomal DNA.
- (7) Removes RNA which may obscure low molecular weight bands.
- (8) Contains dye and 'heavy' molecule, e.g. sucrose or Ficoll.
- (9) Routinely overnight.

**Figure 9.** Generalized procedure for plasmid profiling (adapted from Dodd, 1988).

Kado and Liu, 1981) have a wide range of applicability and can be adapted for use with a variety of species.

The organisms for which plasmid profiling studies have been reported include: *C. perfringens* (Mahony *et al.*, 1986; Phillips-Jones, Iwanejko and Longden, 1989), *C. botulinum* (Strom, Eklund and Poysky, 1984), *Bacillus cereus* (Ellison, Dodd and Waites, 1989), *Staph. aureus* (McGowan *et al.*, 1979; Baumgartner, Nicolet and Eggimann, 1984; Noble and Rahman, 1986; Dodd *et al.*, 1987; Dodd, Chaffey and Waites, 1988), *Staphylococcus epidermidis* (Archer, Vishniavsky and Stiver, 1982; Archer *et al.*, 1984; Parisi, 1985; Degener *et al.*, 1987), *Lactobacillus* (Nes, 1983; Hill and Hill, 1986), *Salmonella* (Riley *et al.*, 1983; Bezanson *et al.*, 1985; Threlfall, Hall and Rowe, 1986; Threlfall *et al.*, 1986; Mitchell *et al.*, 1989), *E. coli* (Scheinbach and Hong, 1988; Senerwa *et al.*, 1989), Enterobacteriaceae (Tietze and Tschäpe, 1983), *C. jejuni* and other species (Ambrosio and Lastovica, 1983; Bradbury *et al.*, 1983; Bopp *et al.*, 1985), *Yersinia enterocolitica* and other species (De Grandis and Stevenson, 1982; Gemski *et al.*, 1986; Filippov *et al.*, 1990), *Lactococcus* (Davies, Underwood and Gasson, 1981; Anderson and McKay, 1983), *Listeria* (Fistrovici and Collins-Thompson, 1990), *B. thermosphacta* (Dodd and Waites, 1988), *Vibrio salmonicida* (Wiik *et al.*, 1989; Sorum *et al.*, 1990), *Propionibacterium* (Rehberger and Glatz, 1990).

In most instances, use of plasmid profiling has been to trace sources of infection in medical and veterinary epidemiology. Included in this is the detection of the sources of outbreaks of food-related disease caused by *Salmonella*. A number of reports have shown the value of the technique in this regard.

Several studies have demonstrated that plasmid profiling is a useful alternative to phage typing for *S. typhimurium* (Brunner *et al.*, 1983; Holmberg *et al.*, 1984). The technique was also used to examine an outbreak of food poisoning caused by *S. typhimurium* (Threlfall *et al.*, 1986) where, although beef was the food consumed, plasmid profiling demonstrated that contamination had occurred by its contact with raw chicken. Plasmid profiling was also used to trace the source of an outbreak of salmonellosis associated with imported French pâté which was caused by *Salmonella gold-coast*, for which no phage typing scheme was available (Threlfall, Hall and Rowe, 1986). Plasmid profile analysis has also been used to investigate outbreaks of food-borne disease caused by *Salmonella newport* (Riley *et al.*, 1983). Several studies have used the technique to determine subgroups within the same phage type, especially where the phage type is one commonly detected (Bezanson *et al.*, 1985; Whiley *et al.*, 1988; Mitchell *et al.*, 1989).

One interesting field of epidemiology is the use of plasmid profiles to differentiate strains of *V. salmonicida*. *V. salmonicida* is a very homogeneous species and the only tool to clearly differentiate between strains is that of plasmid profiles (Wiik *et al.*, 1989). In one study, Sorum *et al.* (1990) showed that strains of *V. salmonicida* isolated from Atlantic salmon off the Norwegian coast were of the same profiles as strains isolated from cod in a commercial fish farm. Moreover, the same strains could be traced in fish at several fish farms along the coast, all originating from one supplying farm.

The use of plasmid profiling in hazard analysis to identify critical control points in a production process is an application which has emerged recently (Dodd, 1988). In the HACCP system the identification of critical control points usually depends on the enumeration of viable counts of an organism at various sites in a process. However, not all strains of a particular organism may constitute a hazard. For example, not all strains of a species may produce a toxin, or have the same ability to cause food-related disease or spoilage. Some strains may be more resistant to those processes designed to eliminate them, such as heating or disinfection; thus, such strains would be more resistant to the critical control points. Detection of the sources of such strains and the processes allowing their survival would be more important than detection of those strains eliminated by the control points. Such strains can be differentiated by the use of typing methods and traced through a production process to discover their source.

One study which has demonstrated the use of this technique is that of Dodd, Chaffey and Waites (1988), which demonstrated the detection of endemic strains of *S. aureus* in a poultry processing plant. They showed that endemic strains within the processing plant could be distinguished from the natural *S. aureus* skin flora of the live birds by their plasmid profiles and that the same strains could be detected in the plant on successive visits several months apart. Reports of other workers (*see* Dodd, Mead and Waites, 1988) have demonstrated that the defeathering machinery allowed growth of endemic strains and increased contamination of the carcasses. Dodd, Chaffey and Waites (1988) confirmed these findings and showed that the endemic strains grew preferentially in one site within the machinery. Furthermore, although endemic strains had been suggested as being of human origin, coming from handlers and process workers (Kusch, 1977, Devriese, 1980), plasmid profiling showed that strains of the endemic types could be detected on the incoming birds as part of the normal skin flora, although at a very low frequency ( $\approx 4\%$ ) within the population. This study, together with previous work (Dodd *et al.*, 1987), thus demonstrated the usefulness of the technique in detecting the presence of endemic strains, in precisely identifying a site which is increasing contamination and in tracing the original source of a particular strain.

The use of plasmid analysis as a means of detecting resident populations of *Salmonella* and *E. coli* in food ingredients has also been suggested by Scheinbach and Hong (1988).

Often an organism found contaminating a final product may be detectable in more than one raw ingredient but only one of these sources may be responsible for the final contamination. Detecting the ingredient which has led to this contamination may be important in regulating the use of the contaminating material. Ellison, Dodd and Waites (1989) examined the contamination of pasteurized liquid whole egg with *B. cereus* and showed that, of the two egg sources used in liquid whole egg production (melange and shell egg), only one source (the melange) contributed the strains that produced heat-resistant spores that survived the pasteurization process. Thus, the level of contamination of the final product could be controlled by

controlling the contamination introduced by the one source material. This demonstrates the value of being able to detect the source of those strains capable of surviving a critical control point.

#### PLASMID FINGERPRINTING

Plasmid fingerprinting (Threlfall and Frost, 1990) is a refinement of plasmid profiling which may be used to produce a greater degree of distinction between strains. The technique involves the digestion of plasmid DNA with restriction endonucleases and the separation of the fragments to produce a 'fingerprint'. Molecular relationships between plasmids of the same size can be examined in this way, and this is particularly useful in strains where only one or two plasmids of similar molecular weights are present. Platt *et al.* (1986) described a strategy for the use of the technique with enterobacterial plasmids and Scheinbach and Hong (1988) used plasmid fingerprinting for further characterization of strains of *E. coli* isolated from foods. Platt *et al.* (1987) applied the technique to isolates of *S. typhimurium* to resolve discrepancies between phage typing, biotyping and plasmid profiling data. This discrepancy was due to the presence of two plasmids of a similar molecular weight in one strain. An interesting finding was that one of the plasmids, which conveyed several antibiotic resistances, was responsible for a change in the phage type of the organism, so that certain isolates appeared unrelated by phage typing. Restriction endonuclease digests have also been used as an adjunct to plasmid profiling in a number of studies to trace the source of *Salmonella* food-borne disease (Taylor *et al.*, 1982; Bezanson *et al.*, 1985; Whiley *et al.*, 1988), to demonstrate transfer of strains between animals and humans (Olsvik *et al.*, 1985), and to monitor the infection of raw milk from the environment with species of *Listeria* (Fistrovici and Collins-Thompson, 1990).

The use of labelled probes to determine the detailed molecular interrelationship between plasmids by colony or Southern hybridization is generally considered unnecessary in epidemiological studies and is used only rarely in some clinical studies, primarily to investigate the spread of particular antibiotic-resistance genes (Roussel and Chabbert, 1978).

#### CHROMOSOMAL DNA ANALYSIS

Restriction endonuclease digestion of chromosomal DNA (also termed BRENDA: bacterial restriction endonuclease DNA analysis) and separation of the fragments by gel electrophoresis produces a complex banding pattern which may be used to differentiate between strains, as with plasmid fingerprinting. The patterns produced are, however, much more complex and, depending upon the restriction enzyme used and its probable frequency of cutting, can produce a pattern of from 25 (Owen and Beck, 1987) to hundreds of bands. The use of such techniques as pulsed field gradient electrophoresis to enhance band resolution (Schwartz and Cantor, 1984; Smith *et al.*, 1987) and of computer, laser-analysed, densitometer scanning (Owen and Beck,

1987) can improve the ease with which such data can be readily compared.

The major advantage of the technique is that it can be used in those species of bacteria where the frequency of plasmid carriage is too low for plasmid profiling to be reliable, or where plasmid DNA is absent. This is the case with some species of *Salmonella*, where the majority of strains are plasmid-free (Threlfall and Frost, 1990), with *L. monocytogenes* (Facinelli *et al.*, 1988; Fistrovici and Collins-Thompson, 1990), and in instances where some *Salmonella* species carry serovar-specific plasmids which limit the use of plasmid profiling (Helmuth *et al.*, 1985).

Two main areas where the technique has been developed are those of *Campylobacter* (Owen, Beck and Borman, 1985) and *Salmonella typhi* (Maher *et al.*, 1986). The technique has been used in several studies with a number of organisms (Bjorvatn *et al.*, 1984; Bradbury *et al.*, 1984; Marshall *et al.*, 1985; Renaud *et al.*, 1988; Etienne *et al.*, 1990), although as yet it is not in routine use for epidemiological studies. For *Campylobacter* the method may be as important for species differentiation as for strain differentiation within a species (Collins and Ross, 1984).

The use of restriction fragment length polymorphism (RFLP) mapping, which uses a labelled chromosomal restriction fragment as a probe against digests of chromosomal DNA to produce a characteristic 'fingerprint' (as in human DNA 'fingerprinting'), has as yet received little attention as a means of strain differentiation. One study has examined the use of this technique for typing with a number of *Salmonella* species (Tompkins *et al.*, 1986).

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