SGM SPECIAL LECTURE

Challenging food microbiology from a molecular perspective

Gordon S. A. B. Stewart

Tel: +44 1159 516165. Fax: +44 1159 516162. e-mail: Gordon.Stewart@Nottingham.ac.uk

Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK

1997 Colworth Prize Lecture

(Delivered at the 137th Meeting of the Society for General Microbiology, 25 March 1997)

Keywords: bioluminescence, *lux*, bacteriophage, RpoS, injury

Overview

Two key themes within food microbiology are bacterial detection and control. There is a raft of sub-headings under each of these themes, but in the last decade molecular approaches within each have made a significant contribution to the field. This is a personal review of the author's past and present contributions and future ideas for challenging food microbiology from a molecular perspective.

Bacterial bioluminescence has played a major role in the studies covered within this review. Cloned in 1983, the *lux* operon from *Photobacterium fischeri* provides a dual-function reporter that has contributed to both the major themes of detection and control. Under constitutive expression light production depends upon the intracellular energy status as reflected by levels of reduced flavin mononucleotide ($FMNH_2$). Major food-poisoning bacteria engineered to constitutively express either the entire *lux* operon, or just the *luxAB* luciferase in conjunction with an exogenous fatty aldehyde, provide valuable tools for interrogating the effect of inimical processes. Cellular damage can be visualized with high sensitivity in real time using photon imaging systems. As a reporter of gene expression, *lux* also provides a unique tool with which to probe the molecular dynamics associated with bacterial stress adaptation and virulence gene activation. Studying stress adaptation in particular has led to the development of a new hypothesis that seeks to explain the differential sensitivity of exponential-phase and stationary-phase cells to inimical processes.

The *lux* gene reporter

Until the advent of genetic engineering the study of luminous bacteria was a relatively esoteric branch of microbiology that nevertheless slowly and methodically provided a substantial foundation of understanding for the biochemistry and genetics of light emission. In 1983, however, the laboratories of Thomas Baldwin (Texas A & M University) and Michael Silverman (University of California, La Jolla) cloned the *Vibrio harveyi* and *P. fischeri* luciferase genes into *Escherichia coli*. Since the original cloning, the DNA sequence information is now available for *lux* genes from a number of bioluminescent bacteria including *P. fischeri* (Foran & Brown, 1988; Baldwin *et al.*, 1989), *Photobacterium leiognathi* (Baldwin *et al.*, 1989; Illarionov *et al.*, 1990), *V. harveyi* (Cohn *et al.*, 1985; Johnston *et al.*, 1986), and *Photorhabdus luminescens* (Johnston *et al.*, 1990; Szittner & Meighen, 1990; Xi *et al.*, 1991). Such information provides easy access to the cloning of the genes via PCR (Hill *et al.*, 1991) and their subsequent introduction into normally dark terrestrial bacteria.

From the early days of cloning bacterial luciferase into *E. coli* the continually improving techniques of gene engineering have allowed a remarkable diversity of bacterial genera to be transformed into a bioluminescent phenotype. In our laboratories at Nottingham we have obtained stable bioluminescence in enteric bacteria, *Bacillus* spp., *Listeria* spp., *Staphylococcus* spp., *Erwinia* spp., *Aeromonas* spp., *Enterococccus* spp., lactic acid bacteria and the *Pseudomonadaceae*. With the inclusion of anaerobes (Phillips-Jones, 1993), it appears that all bacterial genera will eventually prove amenable to bioluminescent transformation.

Bioluminescence has been termed 'living light' (Campbell, 1986) and this is a particularly helpful concept since it underlines the relationship between metabolism and light emission. The light-emitting reaction involves an intracellular, luciferase-catalysed oxidation of $FMNH_2$ and a long-chain aliphatic aldehyde, such as dodecanal, by molecular oxygen:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + \text{light}$$

Since $FMNH_2$ production depends upon functional electron transport, only live cells can produce light. It is this relationship between cellular viability and light that

endows bioluminescence with the power to report on bacterial injury and recovery, to respond quickly to the activity of antibacterial agents and to signal the detection of live bacterial pathogens. It is a tool, therefore, that is enabling researchers to take a new look at persistent problems within food microbiology.

Bacterial viability

The importance of *in vivo* bioluminescence as a reporter of cellular viability has been the subject of several reviews (Stewart, 1990, 1993; Stewart & Williams, 1992, 1993; Stewart *et al.*, 1991, 1993, 1996, 1997a; Hill *et al.*, 1993a, b; Ellison *et al.*, 1994b). Given that the production of light from recombinant bacteria containing the *lux* genes depends upon a functional intracellular biochemistry, it can be established that any substance or environment that impairs that biochemistry, and thus compromises cellular viability, will lead to a reduction in light emission.

Shimon Ulitzur was the first to exploit the industrial potential of bioluminescent bacteria. His work with *Photobacterium phosphoreum* (Ulitzur, 1981) led to the development of the Microtox system (Microbics) for detecting environmentally toxic agents. The killing effect of any antimicrobial agent can be measured in real time as a decrease in light output, which reflects a decline in functional intracellular biochemistry. While the Microtox system has significant and useful applications, it is limited by the fact that the test organism is a marine bacterium that has limited relevance in many industrial settings. It is the ability to transfer bioluminescence into those bacteria which are either particularly sensitive to specific antimicrobial agents or specifically relevant to an industrial process that will allow the full potential of on-line microbial assays to be realized. For example, Walker *et al.* (1992a, b) have shown how bioluminescent *Listeria monocytogenes* can provide a 5 min assay to determine the efficacy of industrial biocides in on-site cleaning regimes. Fig. 1 indicates how closely bioluminescence data, obtained in a few minutes, reflects 18 h viable count data. With the development of surface-attached cells and biofilms from bioluminescent bacteria (Walker *et al.*, 1993, 1994; Dhir & Dodd, 1995) we can now more accurately reflect the nature of microbial contamination and growth in industrial environments (Stewart *et al.*, 1997a).

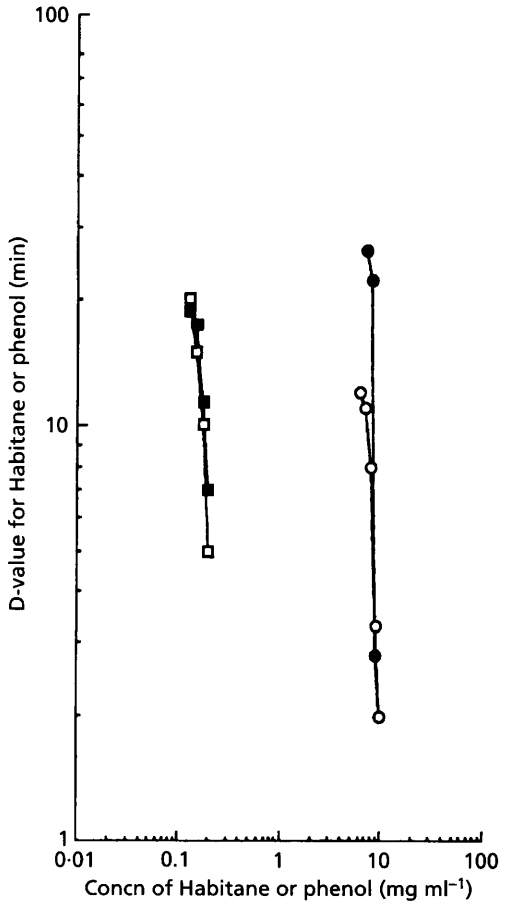

Fig. 1. The relationship between D-value for *L. monocytogenes* 23074(pSP4) and either phenol (circles) or Hibitane (squares) concentration as measured by viable count (filled symbols) and bioluminescence (open symbols).

The measurement of antibiotic potency is an equally effective target for bioluminescent bacteria. We have had a particular interest in assessing the potential of lactic acid bacteria to 'sense' the presence of antimicrobial substances in milk (Ahmad & Stewart, 1991). Ultimately, the objective must be to transfer the *lux* genes into those bacteria that actually constitute the fermentation starter culture. Such recombinant microorganisms would then reflect the characteristics of the starter exactly, yet would represent a test reagent that would not be utilized for any manufacturing process.

Antimicrobial agents may also be deliberately added to foods as preservatives or to specifically combat the growth of pathogens. Evaluating the biological activity, *in situ*, of such agents, either singly or in combination, is extremely difficult using plate count assays, and is impossible in real time. Bioluminescence can be used to study the efficacy of preservatives in foods. For example, Nisaplin, a commercial preparation of the food-grade antibiotic nisin, caused a decrease in the bioluminescence produced by *lux* recombinant *Lactococcus* (*Streptococcus*) *lactis* subsp. *diacetylactis* F7/2. This work led us to develop a new way of visualizing in two dimensions the preservative action of shelf-stable foods (Reid *et al.*, 1993). Using bioluminescent *Salmonella typhimurium* incorporated into thin overlay gels and a Hamamatsu Argus 100 Vim3 photon video camera, we have monitored the preservative potential across the surface of Mortadella, German salami, Italian salami and Bierwurst (Reid *et al.*, 1993). The low surface pH of the German salami (4·6) was profoundly antimicrobial, as shown by the loss of bioluminescence; however neither the Mortadella nor Bierwurst showed any significant potential to inhibit the metabolic activity of the challenge organism. Studies employing photon counting imaging demonstrate the potential of this

technique to obtain near-instantaneous images of dynamic events that previously have been impossible to visualize. The technique is not only a powerful research tool but the ease of use, the low cost in terms of manpower and consumables, allied to the speed of visualization could be relevant in the future to quality-control laboratories within the food industry.

Bacterial injury

From the above it can be seen that stress responses which affect either directly or indirectly the production of $FMNH_2$ can be monitored by *lux* recombinant bacteria as changes in light output per cell. Photon emission is the result of a complex series of chemical reactions and there is no suggestion that light output may be directly correlated with a specific intracellular intermediate. There is, however, every indication that global changes in intracellular biochemistry that reflect detrimental environmental conditions can be monitored in real time as qualitative and perhaps quantitative changes in bioluminescence. The application of this provides a tool with which to probe in real time the recovery of micro-organisms from sub-lethal injury, a condition of considerable importance to the enumeration of micro-organisms in food systems. Ellison *et al.* (1994a, b) have developed the use of bioluminescence for the study of both freeze- and heat-induced injury. Working predominantly with *S. typhimurium*, we have shown that data obtained in real time by bioluminescence measurement is equivalent to, and in most cases indistinguishable from, data returned some 18 to 24 h later from viable counts.

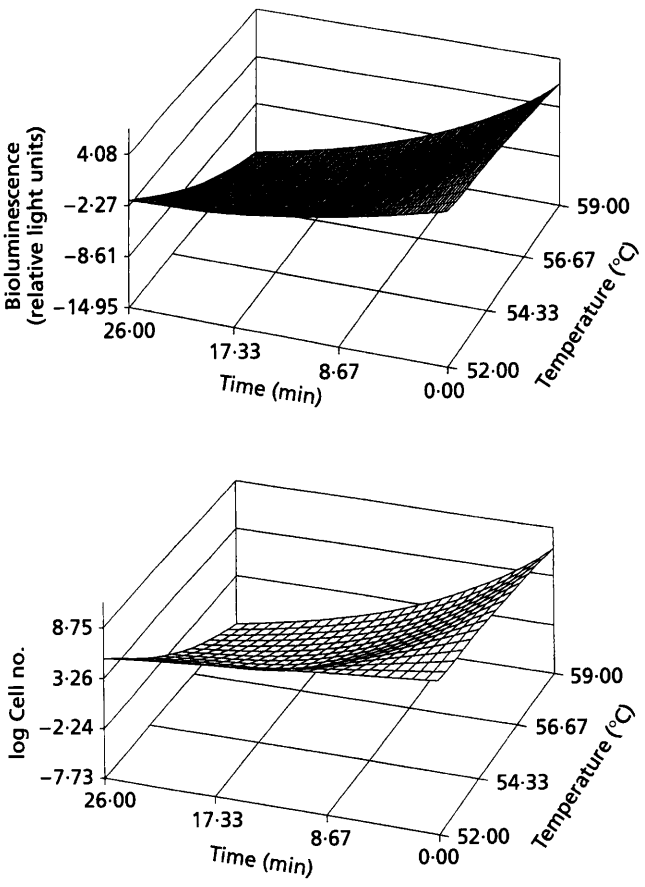

Fig. 2. Quadratic surfaces specifying the survival of *S. typhimurium* with time of exposure to different temperatures in the range 52–59°C. The upper surface was obtained using bioluminescence as a measure of recovery and growth while the lower surface was determined from viable plate counts.

Recent results obtained on the survival and recovery of *S. typhimurium* from heat injury underline this relationship. Fig. 2 shows the quadratic surface specifying the survival of *S. typhimurium* with time of exposure to different temperatures in the range 52–59°C. The upper surface was obtained using bioluminescence as a measure of recovery and growth while the lower surface was determined from viable plate counts. Both surfaces are essentially superimposable and this has important implications. At present the survival of pathogenic organisms in response to changes in a_w, pH and temperature is determined in axenic culture and usually in a liquid shake flask. This is not, of course, how bacteria grow in the real environment where they are typically associated with surfaces and are in dynamic competition with a heterogeneous microflora, but it represents an expedient determined from the practicalities of classical microbiology. It is singularly difficult to determine the growth of a target micro-organism in a complex mixture of bacteria using plate count techniques; bioluminescence, however, once it can be shown to accurately reflect plate count data, offers a simple solution to this complex problem. We believe that the data supporting the validity of bioluminescence as a measure of bacterial survival and growth is now sufficient to depart from the continuing need to provide these data in parallel. The challenge now is to use bioluminescence to monitor bacterial survival in situations where plate counts are impractical. The opportunity is available to monitor specific bacterial pathogens in real food systems where both the food matrix and the intrinsic microflora may significantly alter survival characteristics; the objective of such studies is to assist in the provision of safer food.

Bacterial detection

The detection of bacteria by classical plate count techniques is discriminatory, effective and inexpensive. It has kept food production under a high degree of microbiological safety for many decades and yet it is under continual challenge quite simply because it takes too long. In 1987 Shimon Ulitzur and Jon Kuhn described a new method for bacterial detection that brought together the old arts of microbiology and the new tools of gene engineering (Ulitzur & Kuhn, 1987).

At the 19th Annual Meeting of the British Medical Association held in Glasgow (Scotland) in July 1922, a discussion on the nature of bacteriophage was presented

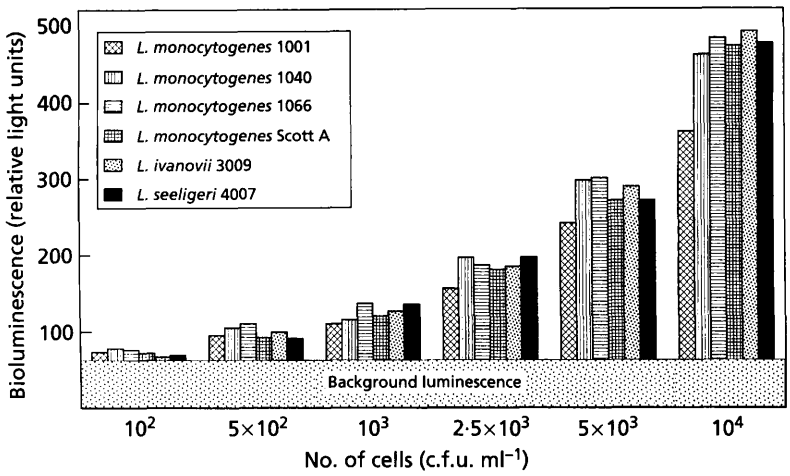

Fig. 3. Detection of *Listeria* spp. using the bioluminescent reporter bacteriophage A511::*luxAB*. Cultures were diluted to the concentrations shown, infected with 3×10^8 A511::*luxAB* ml^{-1}, incubated at 20°C for 130 min and assayed for bioluminescence. Background luminescence (approx. 60 relative light units per 10 s) is shown as a stippled area.

by F. d'Hérelle. Although originally recognized by F. W. Twort in 1915, this paper (d'Hérelle, 1992) nevertheless defined the key elements of the bacteriophage and its bacterial host specificity. This understanding of bacteriophage/bacteria specificity had, therefore, some 60 years of scientific maturity underpinning the concept that for any target pathogen a bacteriophage could be identified that would infect only the target, or at worst the target and a few closely related species. The innovation brought to this knowledge by Jon Kuhn was that if such a bacteriophage were genetically engineered to contain the genes for bacterial bioluminescence it could express these genes only after successful infection and only if its target was metabolically functional, i.e. 'alive'. The sensitivity with which light can be measured, coupled with the relative speed of bacteriophage infection promised significant reagent opportunities for detecting important bacterial pathogens in all fields of microbiology. Since the original concept was defined, the gene engineered into bacteriophage for pathogen detection has shifted from luciferase to *ina* (ice nucleation; Wolber & Green, 1990), but there remains a role for bioluminescence in recombinant bacteriophage designed for bacterial monitoring.

Engineering the bacterial bioluminescence *lux* genes into bacteriophage that infect a broad range of enteric bacteria could provide a reagent for a near on-line enteric test. In concept, the test would require no more than 1 h to perform, would not demand major capital equipment, would be compatible with operation by factory personnel and would allow an accurate assessment of the level of enteric micro-organisms in raw materials, product (during manufacture), floor swabs, equipment swabs and the end product. Such a test already shows promise in prototype form and would be timely because of the increasing emphasis by food companies on the Hazard Analysis Critical Control Points (HACCP) system (Savage, 1995). By definition, this approach depends upon the ability to rapidly monitor what are perceived as critical points in a manufacturing process by chemical, physical or microbiological indicators. At present, the first two have advantages because they can be rapidly and easily performed during production while microbiological examination is retrospective, requiring at least 8 h (and usually 24 h) incubation. Recombinant lux^+ bacteriophage have already been shown to detect enteric indicator bacteria in real food systems without enrichment or recovery in an assay time of 1 h and with a detection limit of 10^4 g^{-1} (Kodikara, 1991).

Since 1991 the opportunities for developing a near on-line test for enteric indicator organisms have remained industrially dormant. However, there are new indications that the concept of the *lux* recombinant bacteriophage may see a resurgence of interest. The work of Sarkis *et al.* (1995) on L5 luciferase reporter mycobacteriophage describes the detection of fewer than ten cells of *Mycobacterium smegmatis* in 40 h. These *lux* recombinant mycobacteriophage can also provide a procedure to discriminate between drug-sensitive and drug-resistant strains of *M. smegmatis*, thus providing novel tools for the rapid identification and classification of anti-mycobacterial agents.

The construction of *lux* recombinant bacteriophage capable of infecting and thereby detecting *Listeria* is another example of the use of *lux* recombinant bacteriophage that we have been exploring at Nottingham for several years. One of the limitations that we initially faced was that all known bacteriophage for *Listeria*, most of which derive from the phage typing collections (generously made available to us by Jocelyne Rocourt, Institut Pasteur, Paris, France and Janos Durst, Public Hygiene Institute, Szolnok, Hungary) are lysogenic, have a very restricted host range, and could fail to detect natural lysogenic isolates of *Listeria* from food or environmental sources. In 1990, Loessner & Busse published the discovery of a lytic bacteriophage (A511) specific for *Listeria* and he began a series of communications and visits to Nottingham that have recently led to the construction of a *lux* reporter phage for the rapid and sensitive detection of viable *Listeria* cells (Loessner *et al.*, 1996). Fig. 3 illustrates the efficacy of the polyvalent but *Listeria*-specific A511::*luxAB* bacterio-

phage in detecting a wide range of different listeriae. We await with interest the application of this research.

A broader perspective on detection

There is no single all-embracing technology to deliver rapid bacterial detection. One of the problems associated with *lux* as a reporter for detection, for example, is the cost of engineering bacteriophage. In considering the advantages of bacteriophage as having bacterial specificity and in trying to avoid the complexities of gene engineering we have recently developed a new technology which is based on the use of classical culture media and is termed 'phage lytic cycle' technology or the 'phage amplification assay' (Stewart *et al.*, 1997b). The principle of the approach (Fig. 4) is to use bacteriophage to reflect the presence of target bacteria. Bacteriophage replicate hundreds of times faster than bacteria; if amplified in a suitable bacterial host a single bacteriophage will reach detectable levels in 3–4 h. By adding pathogen- or target-specific bacteriophage to a clinical or food sample, all the target bacteria are rapidly infected. A critical component of the method then allows for the destruction of all bacteriophage that have not infected a bacterial host and subsequent neutralization of the virucidal effect.

The only bacteriophage that remain are those that have been protected by their target bacteria. These continue to replicate until, after some 30–40 min, new bacteriophage are produced as the host bacteria lyse. These new bacteriophage can be amplified very rapidly by introducing a small number of their host bacteria, typically as a non-pathogenic variant. If there were no target bacteria in the original sample, there will be no phage amplified and therefore no phage to detect at the end of the assay. Even a single live target bacterium, however, can be detected in only a few hours by this procedure.

At present there are prototype assays available for *Pseudomonas aeruginosa* and for *Mycobacterium tuberculosis* (Graham, 1996). The phage amplification assay for the latter organism was independently invented by Stuart M. Wilson (London School of Hygiene & Tropical Medicine). One of the more interesting aspects of this technology is that it has no expensive instrumentation associated with the end-point assay. In this way it provides an opportunity to offer rapid microbiology to Third World laboratories.

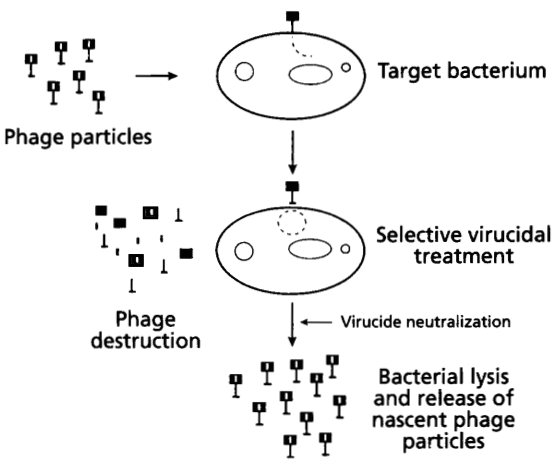

Fig. 4. Schematic diagram of the phage amplification assay.

Adding specificity to the ATP hygiene assay

The ATP assay is the only current example of 'on-line' microbiology. It is used predominantly as a hygiene audit system and is being integrated into HACCP procedures where it is applied as a critical control point monitor (Siragusa *et al.*, 1995). Although in the laboratory it is possible to generate a standard curve where ATP correlates with bacterial numbers, the ubiquity of ATP as cellular energy currency means that in practice there are relatively few applications where ATP is used to specifically detect bacteria. While ATP assays will not replace classical culture-based assays (we think it will always be necessary to derive a bacterial colony for detailed serological typing, etc.) there are real opportunities to limit the extent of samples passed through a full culture-screening procedure.

A significant opportunity for the future may be the provision of pathogen specificity to the ATP assay. Loessner *et al.* (1995) recently described the cloning of three *Listeria*-bacteriophage-encoded lysins which can be overexpressed in *E. coli* to very high levels. The cloned lysins are extremely active in lysing all listerial cells when added exogenously, but have no effect on other Gram-positive or Gram-negative bacteria outside the *Listeria* genus. The potential to use such a lysin to specifically release intracellular ATP from *Listeria* within a complex mixture of bacteria is apparent and the experimental validity of this concept is indicated in Fig. 5(a) and (b) (Stewart *et al.*, 1996). The bacteriophage lysin PLY118 lyses a culture of *Listeria* within a few minutes (Fig. 5a) and this lysis is, indeed, the basis for the specific detection of *Listeria* by coordinate ATP assay (Fig. 5b).

It was unexpected that a bacteriophage lysin would have genus specificity; rather, one would expect either a broad-range lysozyme-type activity or a highly restricted activity analogous to that of the bacteriophage host range. The speed of action of the lysin is such that an entire population of target bacteria are lysed within a few minutes. Application of a cloned and purified protein lysin with bacterial genus specificity should constitute a highly significant step change in ATP-based bacterial assays.

The history of invention frequently catalogues the emergence of concepts from different people at almost simultaneous times. It is as if there is a 'time' for an inventive process to emerge and this is certainly true of rapid methods in microbiology. In addition to the co-invention of the phage amplification assay described

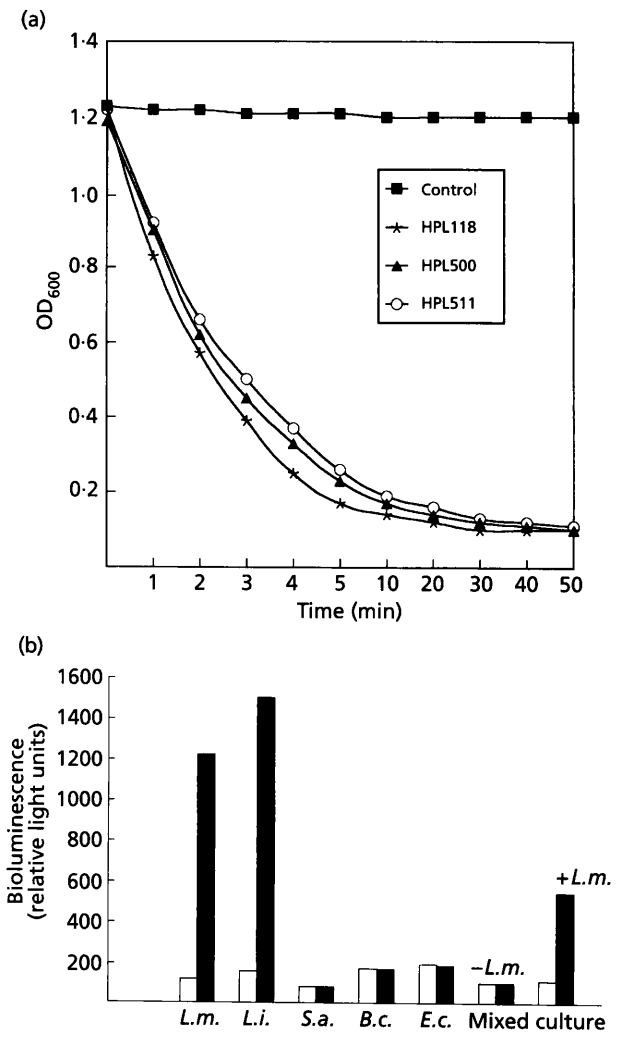

Fig. 5. (a) Lysis of *L. monocytogenes* cells by different *Listeria* bacteriophage endolysins. Cultures were harvested by centrifugation, resuspended in Tris/HCl buffer (pH 8·0) and mixed with small amounts of the recombinant endolysin proteins. The subsequent decrease in optical density of the cell suspensions was determined photometrically at 600 nm. (b) Specific lysis of *Listeria* cells by cloned endolysin HPL118 and subsequent detection of released ATP using a firefly luciferase-luciferin assay. *L.m., L. monocytogenes; L.i., Listeria ivanovii; S.a., Staphylococcus aureus; B.c., B. cereus; E.c., E. coli.* The mixed culture contained all of the above strains except (on left) or including (on right) *L. monocytogenes*. Bacterial cultures were diluted to approximately 10^7 c.f.u. ml^{-1}, mixed with 5 units endolysin (black bars) incubated for 10 min at room temperature and assayed for released ATP. Controls (white bars) had no lysin added.

above, the use of bacteriophage lysins for the near on-line detection of pathogens has also had another protagonist in Mike J. Gasson (BBSRC Institute of Food Research, Norwich). He has recently reviewed the lytic systems in lactic acid bacteria and their bacteriophage (Gasson, 1996), but the application potential of bacteriophage lysins as an adjunct to the ATP assay was described previously only in patent form (European patent application 92303533.1). Our hope now is to bring together all of the European expertise in this field so as to bring the concept through to real and useful assays.

Innovation in PCR

PCR has still to come of age in terms of a widespread application in microbial detection for food and related industries. One problem with the currently available technology is that PCR can only interrogate a sample for a preselected target organism. It would be preferable to determine if *any* contaminating bacteria were present in a sample, followed by identification.

Conventionally, PCR is carried out on a sample using primers chosen to amplify a particular target gene sequence within a given organism; hence a sample can only be interrogated for a preselected target organism. Thus, when the identity of one or more organisms in a sample is to be established, it is necessary to guess or assume what sequences/organisms may be present, identify and obtain specific primers and then conduct the PCR. The ideal situation would be to have a set of primers that would provide an amplified DNA product from any bacterium in a sample and where some variable feature of the amplified product could give information on the identity of the unknown. As a concept this has been considered previously with ribosomal DNA primers but there is no generic solution to the ideal at this point. Working on the cold shock genes of *L. monocytogenes* we have discovered that homologues of *E. coli cspA* (Jones & Inouye, 1994) and the related family are very widely distributed in prokaryotes (K. P. Francis, S. Scherer & G. S. A. B. Stewart unpublished). Although the homologue of the cold shock gene *cspA* was detected in various isolates of Antarctic psychrotrophs, representing both Gram-positive and Gram-negative bacteria, by Ray *et al.* (1994) there have been no other suggestions that this gene family is ubiquitous in prokaryotes.

We have used two universal primers, 5′-GGTAAAGT-AAAAAGCTTCAAC-3′ and 5′-GTAACGTTAGAAG-CTTGTGGTCC-3′, to amplify a family of PCR products (representing the cold shock gene family) from all bacteria so far tested. Cloning of PCR products from bacteria including *L. monocytogenes* have confirmed that the amplified DNAs are indeed from homologues of *cspA*. Fig. 6 indicates a parsimony analysis of the sequences obtained from 31 different bacteria. We have, therefore, a primer set that provides an amplified product if there is any bacterial contaminant present in the sample; in all cases controls have no amplified DNA (K. P. Francis & G. S. A. B. Stewart, unpublished).

There is one additional and rather significant element to the cold shock primer story. There are currently no rapid methods available to interrogate a sample for psychrotrophic *Bacillus* spp. The spoilage of milk and other dairy products by psychrotrophic *Bacillus cereus* is, however, a particular problem to an industry worth annually some £3·5 billion in the UK alone. In col-

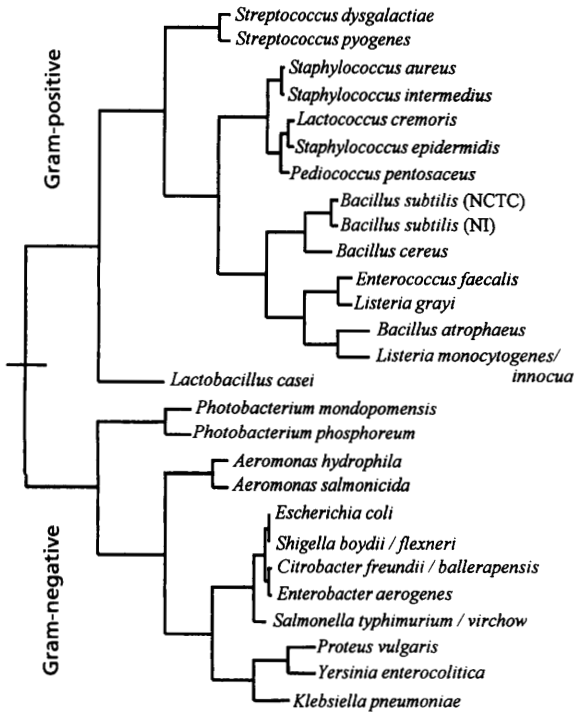

Fig. 6. Dendrographic representation of PILEUP data derived from alignment of partial bacterial major cold shock protein DNA sequences. Vertical line distances indicate closeness of sequence homologies.

laboration with Siegfried Scherer (Technische Universität München, Germany) we have discovered that the DNA encoding the major cold shock protein homologue known as *cspA* in *B. cereus* shows conserved nucleotide differences, at particular positions in the sequence, between psychrotrophic and mesophilic strains. Determining whether the psychrotrophic sequence is present in a sample may be carried out as an integral part of the PCR amplification reaction (K. P. Francis and others, unpublished). In particular, the probe cleavage amplification techniques such as the system marketed by PE Applied Biosystems under the trade mark TaqMan are ideally suited to this diagnostic opportunity. In principle it should be possible to screen a sample quantitatively for the presence of psychrotrophic *Bacillus* spp. in 40 min.

Studying bacterial survival and growth *in vivo*

Bioluminescence, as displayed by the expression of bacterial luciferase in target bacteria, has its own place in the future of microbiology and rapid methods, a future that has been amply discussed and exemplified in recent literature (e.g. Stewart *et al.*, 1996). There is one remarkable new discovery, however, that for the first time allows for the real-time imaging of bacteria *in situ* during a developing infection. The breakthrough comes from the work of Contag *et al.* (1995) who demonstrated that the light from a recombinant *S. typhimurium* strain expressing the *lux* operon could be 'seen' by a photon imaging camera through the skin and fur of a living mouse. The opportunity to literally see a developing infection and to monitor in real time the effect of anti-infective therapies (antibiotics etc.) is a fundamental advance in the development of disease models. There is a special challenge here for the commercial provision of *lux*-tagged bacteria and for the development of more cost-effective imaging systems to open up the use of this new technology. It will be fascinating to see if the real potential of this new approach can be fully realized.

Bacterial control

As illustrated above, for some years we have had an interest in using *lux* as a marker for measuring the survival of bacteria subjected to inimical processes such as freezing and heating. Our initial objective was to validate the use of bioluminescence as a real-time reporter with equivalence to classical viable count data. From this position we wanted to measure the death, injury and recovery of sub-populations of food pathogens under conditions where the classical microbiology of plate counts were not applicable. The first objective was completed only in 1995 with the use of bioluminescence to model the thermal inactivation of *S. typhimurium* in the presence of a competitive microflora (Duffy *et al.*, 1995). Since then we have been addressing the question of the molecular basis for the increased resistance of a sub-population of *Salmonella* to heat and freeze injury when high levels of competitive bacteria are present. Our focus has been on the stationary-phase sigma factor RpoS and the effect of a competitive microflora on RpoS induction. Our initial assumption was that the competitive microflora would induce RpoS in a small sub-population of *Salmonella*, and in so doing the stationary-phase survival response would provide a molecular explanation for the observed enhancement in resistance to inimical processing. To measure the RpoS response specifically within a small sub-population of cells we used *lux* as a reporter of gene expression. RpoS is under complex control in *E. coli* and *Salmonella*; it is under transcriptional control, post-transcriptional control with respect to mRNA stability and post-translational control with respect to the role of chaperones in regulating protein folding and protein degradation (Hengge Aronis, 1996). A reporter that measures *rpoS* transcription would not therefore give any meaningful indication of the available intracellular levels of RpoS. Our reporter therefore measures the transcription of a virulence gene termed *spvA*, which is controlled by SpvR where *spvR* expression is under RpoS control (Swift & Stewart, 1994).

Using this *spvA::lux* bioluminescence reporter, we did indeed observe an early induction of RpoS in exponential cells of *Salmonella* challenged with competitor cells at levels of 10^6 or greater. Essentially the time for induction moves from the control time of 4·5 h to 27 min in the best case, where 10^8 competitors were added to a natural isolate of *Salmonella enteritidis*. Again, this early induction was dependent on the competitive

microflora being viable. Clearly, therefore, at least one of the major molecular indicators of the stationary phase is induced by the competitive microflora and consequently the dependent resistance mechanisms will also be induced.

There is a significant problem, however, in attempting to resolve the effect of the competitive microflora upon the resistance of the underlying population of *Salmonella* as the molecular induction of the stationary phase. This is that the time required for the *Salmonella* to acquire the very significant enhancement in resistance is very much shorter than the shortest time so far measured for the induction of RpoS. In respect of the experimental limitation, it appears that the acquisition of resistance is essentially instantaneous, providing the challenge is with living cells. It appears, therefore, that there are two components to the acquisition of resistance to these inimical processes that can be attributed to the presence of a competitive microflora. One is very rapid and may be biochemical in origin, and one is dependent upon the later induction of the stationary-phase sigma factor and the associated portfolio of resistance determinants.

The question that remains is 'what type of biochemical mechanism(s) could contribute to an instantaneous enhancement of resistance that translates into two orders of magnitude enhancement in survival?' We do not know the answer, but we have evolved a hypothesis (Dodd *et al.*, 1997). We consider that the observed differences in the resistance of stationary- and exponential-phase cells to the apparent lethal effects of freeze/thaw and heating at relatively low temperatures is not a direct measure of the inimical process. Rather, it results from self-destruction by the exponential-phase bacterial cells as a response to what is perceived by stationary-phase cells as only a sub-lethal stress. In effect, it is an artifact of current microbiology practice, which is to measure the effects of inimical processes on exponentially growing cells. Such cells are replicating at close to maximal rates and are metabolizing at elevated rates. A notionally sub-lethal inimical process disturbs cell growth but does not affect the metabolic rate. In consequence the cells develop an imbalance between anabolism and catabolism. This causes a burst of free radical production and it is this and not the inimical process *per se* that causes subsequent death.

In the 'real world' of microbiology, the rapidly dividing exponential-growth phase is not the norm. The enhanced resistance of bacteria in the quiescent state has long been recognized and there is no detailed understanding of the molecular mechanisms that underlie this. In addition, the empirical observations that the recovery of damaged cells is most efficiently achieved in very nutritionally restricted growth media, often with the addition of oxygen scavengers such as glutathione, is fully compatible with a hypothesis that imparts a self-destruction component on processes that place bacterial growth and metabolism in imbalance in an aerobic environment.

The implications for this hypothesis are that microbiologists may currently consider as lethal, inimical treatments that are actually sub-lethal; the true level of susceptibility is reflected by what we currently consider to be resistant populations, i.e. quiescent or stationary-phase cells. In consequence, models which predict bacterial survival against diverse inimical processes, and which are based on the study of rapidly growing and rapidly metabolizing exponential-phase cells, are potentially flawed. We are not implying by this that such models are intrinsically unsafe; in any event this is clearly not the experience of those models currently being used in the food industry. We are, however, saying that where the models explore levels of lethality that are close to the boundaries, there is scope for error. The drive in terms of processing and preservation is to move closer to the boundaries of microbial control using conditions that are approaching sub-lethal levels. To do this we must improve our understanding of the underlying mechanisms that impart lethality on a microbial population.

Cell–cell communication – new challenges in the molecular biology of food pathogens

The last few years have seen the emergence of a new field of molecular microbiology, that of cell–cell signalling using *N*-acylhomoserine lactones (*N*-AHLs) and more recently termed 'quorum sensing' (Swift *et al.*, 1996). In 1992 it was shown that the small molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) was responsible for the regulation of synthesis of the β-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid (carbapenem), in the terrestrial, plant-pathogenic bacterium, *Erwinia carotovora*. The significance of this discovery lay in the fact that OHHL had already been reported in the scientific literature several years earlier as the autoinducer of bioluminescence in the marine symbiont *P. fischeri*. In this organism, OHHL is produced by the action of the LuxI protein which is an autoinducer synthase. Even in the 1992 literature, OHHL-mediated autoinduction was considered to be uniquely connected with bioluminescence (the 'Lux' phenotype) in *P. fischeri*. However, the discovery of OHHL elaboration by a terrestrial plant pathogen suggested that production of this, and structurally similar molecules, might be far more widespread than originally supposed. To test this idea, a bioluminescence sensor system was developed and used to screen for OHHL production in the spent supernatants of a wide range of bacterial cultures, including strains of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Erwinia herbicola*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Proteus mirabilis* (Swift *et al.*, 1993).

To date, only a limited group of phenotypes are known to be regulated by *N*-AHLs. However, based on the results obtained using a *lux* sensor assay, a far larger spectrum of bacteria make molecules capable of activating expression of the bioluminescence genes. The phenotypes associated with *N*-AHL production are

physiologically diverse, including bioluminescence, synthesis of extracellular enzymes, virulence, antibiotic production and plasmid conjugal transfer. In some bacteria, it is known that multiple phenotypic traits are regulated by the same molecule; for example, in some strains of *E. carotovora*, OHHL regulates synthesis of multiple pectate lyases, polygalacturonase, cellulase, protease and a carbapenem antibiotic. In addition, it is now clear that OHHL is only one of a growing family of N-AHLs.

So what is the significance of this in terms of developments in food microbiology? Quite simply, a key to bacterial growth and pathogenicity. It is clear that communication is a critical element for regulating the expression of bacterial virulence determinants. Break that communication by blocking the small molecule language and you should attenuate bacterial pathogenicity. This is a major new concept for the development of novel anti-infectives and, if successful, it will demand a new type of bacterial classification based on the molecular language used for communication. Companies with a focus on diagnostics would do well to watch this evolving research area closely. Finally, there are growing indications that communication may control cell growth and perhaps bacterial recovery from the so-called 'viable but non-culturable' (VNC) state. There is a serious possibility that the 'missing link' in the culture of VNCs is the lack of an appropriate signal molecule to initiate growth. Presumably good times ahead for the media company that first establishes this link unequivocally and adds the missing molecule to their proprietary recovery medium.

Acknowledgements

The author would like to extend his appreciation to all his research colleagues who, through their individual gifts, have made this research field so stimulating. Special thanks go to Sabah Jassim who contributed in important ways to the development of the phage amplification assay; Stephen Denyer who has been an equal partner in many of the applications for bioluminescence as a diagnostic tool; Will Waites, Chris Dodd, Cath Rees, Phil Hill, Sheila Godber and Dave Fowler who are very special colleagues in food microbiology and Paul Williams, Barrie Bycroft and George Salmond who are senior colleagues and friends in quorum sensing. The research from the author's laboratory described in this review was supported by the BBSRC, the Leverhulme Trust, the University of Nottingham, the MAFF, Amersham International, GlaxoWellcome, Unilever, the DoH, the DTI and the European Union.

References

Ahmad, K. A. & Stewart, G. S. A. B. (1991). The production of bioluminescent lactic acid bacteria suitable for the rapid assessment of starter culture activity in milk. *J Appl Bacteriol* **70**, 113–120.

Baldwin, T. O., Devine, J. H., Heckel, R. C., Lin, J. W. & Shadel, G. S. (1989). The complete nucleotide sequence of the *lux* regulon of *Vibrio fischeri* and the *luxABN* region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence. *J Biolumin Chemilumin* **4**, 326–341.

Campbell, A. K. (1986). Living light – chemi-luminescence in the research and clinical laboratory. *Trends Biochem Sci* **11**, 104–108.

Cohn, D. H., Mileham, A. J., Simon, M. I., Nealson, K. H., Raush, S. K., Bonam, D. & Baldwin, T. O. (1985). Nucleotide sequence of the *luxA* gene of *Vibrio harveyi* and the complete amino acid sequence of the alpha subunit of bacterial luciferase. *J Biol Chem* **260**, 6139–6146.

Contag, C. H., Contag, P. R., Mullins, J. I., Spilman, S. D., Stevenson, D. K. & Benaron, D. A. (1995). Photonic detection of bacterial pathogens in living hosts. *Mol Microbiol* **18**, 593–603.

Dhir, V. K. & Dodd, C. E. R. (1995). Susceptibility of suspended and surface attached *Salmonella enteritidis* to biocides and elevated temperatures. *Appl Environ Microbiol* **61**, 1731–1738.

Dodd, C. E. R., Sharman, R. L., Bloomfield, S. F., Booth, I. R. & Stewart, G. S. A. B. (1997). Inimical processes: bacterial self destruction and sub-lethal injury. *Trends Food Sci Technol* (in press).

Duffy, G., Ellison, A., Anderson, W., Cole, M. B. & Stewart, G. S. A. B. (1995). The use of bioluminescence to model the thermal inactivation of *Salmonella typhimurium* in the presence of a competitive microflora. *Appl Environ Microbiol* **61**, 3463–3465.

Ellison, A., Anderson, W., Cole, M. B. & Stewart, G. S. A. B. (1994a). Modelling the thermal inactivation of *Salmonella typhimurium* using bioluminescence data. *Int J Food Microbiol* **12**, 323–332.

Ellison, A., Cole, M. B. & Stewart, G. S. A. B. (1994b). The application of molecular biology to understanding the growth and survival of food poisoning and spoilage bacteria. In *Minimal Processing of Foods and Process Optimisation*, pp. 213–227. Edited by R. P. Singh & F. A. R. Oliveira. Boca Raton, FL: CRC Press.

Foran, D. R. & Brown, W. M. (1988). Nucleotide sequence of the *luxA* and *luxB* genes of the bioluminescent marine bacterium *Vibrio fischeri*. *Nucleic Acids Res* **16**, 777.

Gasson, M. J. (1996). Lytic systems in lactic acid bacteria and their bacteriophages. *Antonie Leeuwenhoek* **70**, 147–159.

Graham, J. (1996). Timely test spots TB 'in hours'. *New Sci* **151(2043)**, 21.

Hengge Aronis, R. (1996). Back to log phase – sigma(s) as a global regulator in the osmotic control of gene-expression in *Escherichia coli*. *Mol Microbiol* **21**, 887–893.

d'Hérelle, F. (1922). The nature of bacteriophage. *Brit Med J* **2**, 289–297.

Hill, P. J., Swift, S. & Stewart, G. S. A. B. (1991). PCR based gene engineering of the *Vibrio harveyi lux* operon and the *Escherichia coli trp* operon provides for biochemically functional native and fused gene products. *Mol Gen Genet* **226**, 41–48.

Hill, P. J., Denyer, S. P. & Stewart, G. S. A. B. (1993a). Rapid assays based on *in vivo* bacterial bioluminescence. *Eur Microbiol* **1**, 16–21.

Hill, P. J., Rees, C. E. D., Winson, M. K. & Stewart, G. S. A. B. (1993b). The application of *lux* genes. *Biotechnol Appl Biochem* **17**, 3–14.

Illarionov, B. A., Blinov, V., Donchenko, A. P., Protopopova, M. V., Karginov, V. A., Mertvetson, N. P. & Gitelson, J. I. (1990). Isolation of bioluminescent functions from *Photobacterium leiognathi*: analysis of *luxA*, *luxB*, *luxG* and neighbouring genes. *Gene* **86**, 89–94.

Johnston, T. C., Thompson, R. B. & Baldwin, T. O. (1986). Nucleotide sequence of the *luxB* gene of *Vibrio harveyi* and the complete amino acid sequence of the β-subunit of bacterial luciferase. *J Biol Chem* **261**, 4805–4811.

Johnston, T. C., Rucker, E. B., Cochrum, L., Hruska, K. S. & Vandegrift, V. (1990). The nucleotide sequence of the *luxA* and *luxB* genes of *Xenorhabdus luminescens* HM and a comparison of the amino acid sequences of luciferases from four species of bioluminescent bacteria. *Biochem Biophys Res Commun* **170**, 407–415.

Jones, P. G. & Inouye, M. (1994). The cold shock response – a hot topic. *Mol Microbiol* **11**, 811–818.

Kodikara, C. P., Crew, H. H. & Stewart, G. S. A. B. (1991). Near on-line detection of enteric bacteria using *lux* recombinant bacteriophage. *FEMS Microbiol Lett* **83**, 261–266.

Loessner, M. J. & Busse, M. (1990). Bacteriophage typing of *Listeria* species. *Appl Environ Microbiol* **56**, 1912–1918.

Loessner, M. J., Wendlinger, G. & Scherer, S. (1995). Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages – a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Mol Microbiol* **16**, 1231–1241.

Loessner, M. J., Rees, C. E. D., Stewart, G. S. A. B. & Scherer, S. (1996). Construction of luciferase reporter bacteriophage A511::*luxAB* for rapid and sensitive detection of viable *Listeria* cells. *Appl Environ Microbiol* **62**, 1133–1140.

Phillips-Jones, K. K. (1993). Bioluminescence (*lux*) expression in the anaerobe *Clostridium perfringens*. *FEMS Microbiol Lett* **106**, 265–270.

Ray, M. K., Sitaramamma, T., Ghandhi, S. & Shivaji, S. (1994). Occurrence and expression of *cspA*, a cold shock gene, in antarctic psychrotrophic bacteria. *FEMS Microbiol Lett* **116**, 55–60.

Reid, C. L., Waites, W. M. & Stewart, G. S. A. B. (1993). Microbial detection and two dimensional preservative mapping using *lux*-based bioluminescence. *Soc Appl Bact Tech Ser* **31**, 181–189.

Sarkis, G. J., Jacobs, W. R. & Hatfull, G. F. (1995). L5 luciferase reporter mycobacteriophages – a sensitive tool for the detection and assay of live mycobacteria. *Mol Microbiol* **15**, 1055–1067.

Savage, R. A. (1995). Hazard analysis critical control point – a review. *Food Rev Int* **4**, 575–595.

Siragusa, G. R., Cutter, C. N., Dorsa, W. J. & Koohmaraie, M. (1995). Use of a rapid microbial ATP bioluminescence assay to detect contamination on beef and pork carcasses. *J Food Prot* **58**, 770–775.

Stewart, G. S. A. B. (1990). *In vivo* bioluminescence: new potentials for microbiology. *Lett Appl Microbiol* **10**, 1–8.

Stewart, G. S. A. B. (1993). BIOSENSORS – bacterial luminescence: development and application. *Lancet* **341**, 279–280.

Stewart, G. S. A. B. & Williams, P. (1992). *lux* genes and the applications of bacterial bioluminescence. *J Gen Microbiol* **138**, 1289–1300.

Stewart, G. S. A. B. & Williams, P. (1993). Shedding new light on Food Microbiology. *ASM News* **59**, 241–246.

Stewart, G. S. A. B., Denyer, S. P. & Lewington, J. (1991). Microbiology illuminated: gene engineering and bioluminescence. *Trends Food Sci Technol* **2**, 7–10.

Stewart, G. S. A. B., Jassim, S. A. A. & Denyer, S. P. (1993). Engineering microbial bioluminescence and biosensor applications. In *Molecular Biology in Clinical Research and Diagnosis*, pp. 403–424. Edited by M. R. Walker. Oxford: Blackwell Scientific Publications.

Stewart, G. S. A. B., Loessner, M. J. & Scherer, S. (1996). The bacterial *lux* gene bioluminescent biosensor revisited. *ASM News* **62**, 297–301.

Stewart, G. S. A. B., Aldsworth, T. G., Sharman, R. L., Gibson, P. T. & Dodd, C. E. R. (1997). Bioluminescence – *lux* as an enabling tool for the microbiological analysis of food. In *Institute of Food Technology Basic Symposium Series*, pp. 265–288. New York: Marcel Dekker.

Swift, S. & Stewart, G. S. A. B. (1994). Luminescence as a signal of *spvA* expression. In *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, pp. 93–96. Edited by A. K. Campbell, L. J. Kricka & P. E. Stanley. Chichester: Wiley.

Swift, S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E. D., Chhabra, S. R., Hill, P. J., Throup, J. P., Bycroft, B. W., Salmond, G. P. C., Williams, P. & Stewart, G. S. A. B. (1993). A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**, 511–520.

Swift, S., Throup, J. P., Williams, P., Salmond, G. P. C. & Stewart, G. S. A. B. (1996). Quorum sensing: a population density component in the determination of bacterial phenotype. *Trends Biochem Sci* **21**, 214–219.

Szittner, R. & Meighen, E. (1990). Nucleotide sequence, expression and properties of luciferase coded by *lux* genes from a terrestrial bacterium. *J Biol Chem* **265**, 16581–16587.

Ulitzur, S. (1981). Analytical application of bacterial luminescence system. *J Clin Chem Clin Biochem* **19**, 582.

Ulitzur, S. & Kuhn, J. (1987). Introduction of *lux* genes into bacteria, a new approach for specific determination of bacteria and their antibiotic susceptibility. In *Bioluminescence and Chemiluminescence: New Perspectives*, pp. 463–472. Edited by J. Schölmerich, R. Andreesen, A. Karp, M. Ernst & W. G. Woods. Chichester: Wiley.

Walker, A. J., Holah, J. T., Denyer, S. P. & Stewart, G. S. A. B. (1992a). The antibacterial activity of Virkon measured by the colony growth and bioluminescence of *lux* recombinant *Listeria monocytogenes*. *Lett Appl Microbiol* **15**, 80–82.

Walker, A. J., Jassim, S. A. A., Holah, J. T., Denyer, S. P. & Stewart, G. S. A. B. (1992b). Bioluminescent *Listeria monocytogenes* provide a rapid assay for measuring biocide efficacy. *FEMS Microbiol Lett* **91**, 251–256.

Walker, A. J., Holah, J. T., Denyer, S. P. & Stewart, G. S. A. B. (1993). The use of bioluminescence to study the behaviour of *Listeria monocytogenes* when attached to surfaces. *Colloids Surf* **77**, 225–229.

Walker, A. J., Stewart, G. S. A. B., Shepperd, F., Bloomfield, S. F., Holah, J. T. & Denyer, S. P. (1994). Bioluminescence imaging as a tool for studying biocide challenge upon planktonic and surface attached bacteria. *Binary* **6**, 16–17.

Wolber, P. K. & Green, R. L. (1990). Detection of bacteria by transduction of ice nucleation genes. *Trends Biotechnol* **8**, 276–279.

Xi, L., Cho, K. W. & Tu, S. C. (1991). Cloning and nucleotide sequences of *lux* genes and characterisation of luciferase of *Xenorhabdus luminescens* from a human wound. *J Bacteriol* **173**, 1399–1405.