Review Article

lux genes and the applications of bacterial bioluminescence

GORDON S. A. B. STEWART¹* and PAUL WILLIAMS²

¹Department of Applied Biochemistry and Food Science, University of Nottingham, Faculty of Agriculture and Food Sciences, Sutton Bonington, Leicestershire LE12 5RD, UK

²Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham, UK

Introduction

Luminous bacteria constitute some of the most fascinating subjects in microbiology and are much more prevalent than is frequently appreciated. They are found in marine, freshwater and terrestrial environments and can be most easily isolated as saprophytes growing on dead fish or meat. Fig. 1(a) shows the bioluminescence emission from a fresh fish-market sprat imaged using a photon video camera. This light, which can be obtained from almost any fish, is derived from bioluminescent bacteria. The diversity of these micro-organisms is such that only a very small percentage have been studied biochemically and even fewer have been the subject of genetic analysis. There may, therefore, be much yet to discover about the diverse biochemistry and genetics of bacterial bioluminescence. Nevertheless, through the techniques of genetic engineering, the phenomenon of bacterial bioluminescence can now be captured and applied within any bacterial species from several rather different perspectives. It provides a real-time non-invasive reporter for measuring gene expression, a sensitive marker for bacterial detection and a measure of intracellular biochemical function, i.e. as a holistic determinant of cellular viability. This review aims to provide the necessary background to examine recent developments in these areas and thereby encourage a growing awareness for the multi-faceted nature of *in vivo* bioluminescence.

The biochemical and genetic basis of bioluminescence

The light-emitting reaction involves an intracellular, luciferase-catalysed, oxidation of the reduced form of

flavin mononucleotide (FMNH₂) and a long-chain aliphatic aldehyde, such as dodecanal, by molecular oxygen:

Therefore, from a biochemical viewpoint, bacterial bioluminescence requires oxygen, a source of energy, a luciferase enzyme and a long-chain fatty aldehyde.

Bacterial luciferase [alkanal monooxygenase (FMN-linked); alkanal, reduced-FMN: oxygen oxidoreductase (1-hydroxylating, luminescing); EC 1.14.14.3] is a heterodimeric enzyme for which the subunits α (≈ 40 kDa) and β (≈ 37 kDa) are coded by the *luxA* and *luxB* genes respectively. Full DNA sequence information is available for these genes from a number of species including *Vibrio fischeri* (Foran & Brown, 1988; Baldwin *et al.*, 1989), *Photobacterium leiognathi* (Baldwin *et al.*, 1989; Illarionov *et al.*, 1990), *Vibrio harveyi* (Cohn *et al.*, 1985; Johnston *et al.*, 1986), and *Xenorhabdus luminescens* (Johnston *et al.*, 1990; Szittner & Meighen, 1990; Xi *et al.*, 1991). Such information provides easy access to the cloning of the genes via the polymerase chain reaction (Hill *et al.*, 1991).

As the first four amino acid residues of the α and β subunits are identical in both *V. harveyi* and *V. fischeri*, it has been suggested that *luxA* and *luxB* result from a tandem duplication of an ancestral gene, a view supported by data from Foran & Brown (1988). The active form of luciferase is an $\alpha\beta$ dimer and the active site is confined primarily to the α subunit; the specific role of β is unknown but absolutely essential for bioluminescence activity (Cohn *et al.*, 1985). Recently, LuxAB fusion proteins have been produced that retain biological function (Boylan *et al.*, 1989; Escher *et al.*, 1989; Kirchner *et al.*, 1989; Almashanu *et al.*, 1990; Hill *et al.*, 1991). These fusion proteins have particular value in optimizing expression in recombinant bacterial systems

* Author for correspondence. Tel. (0602) 484848; fax (0509) 673917.
Abbreviation: KHL, *N*-(β -ketocaproyl)homoserine lactone.

Fig. 2. Organization and function of *lux* genes cloned from *V. fischeri*. Arrows denote operons containing *lux* genes; the leftward arrow marks operon L (containing *luxR*) and the rightward arrow marks operon R. The molecular masses (kDa) of the *lux* gene products and their functions are shown below the gene designations. (Adapted from Silverman *et al.*, 1989.)

and one (Hill *et al.*, 1991) has been used to construct a highly bioluminescent derivative of *Bacillus subtilis* (Jacobs *et al.*, 1991).

Most investigations of bioluminescent bacterial systems have been based upon two genera, *Photobacterium* and *Vibrio*. Engebrecht *et al.* (1983) first identified the enzymic and regulatory functions necessary for expression of a bioluminescent phenotype and determined the key aspects of genetic organisation (Engebrecht & Silverman, 1984). In addition to *luxA* and *luxB*, the *lux* operon typically contains three further structural genes *luxC*, *luxD* and *luxE* that encode the subunits of a fatty acid reductase. There appears to be a universal conservation of the order of these genes in *lux* operons, as defined in Fig. 2. The remaining *lux* genes, *luxR* and *luxI*, are involved in the regulation of *lux* expression, an aspect which has been the subject of extensive review (Meighen, 1988, 1991; Silverman *et al.*, 1989). A number of additional *lux* genes (*luxF*, *luxG*, *luxH* and *luxY*) of unknown function have recently been identified and are reviewed by Meighen (1991).

Most micro-organisms lack the genetic blueprint for luciferase and fatty acid reductase but they can supply FMNH₂; so, for a dark bacterium such as *Escherichia coli* to become bioluminescent, all that is required is the genetic transfer of the genes for luciferase and fatty acid reductase. In practice, the long-chain aldehyde can be provided exogenously to bacterial cultures expressing only the luciferase component of the *lux* operon. Although the apparent *K_m* for aldehyde is increased by some 1000-fold over an *in vitro* enzyme assay, the compound can be supplied in sufficient excess to ensure that it is non-limiting in the bioluminescent pathway (Blisset & Stewart, 1989). With this addition, the genetic components necessary for bioluminescence are reduced to only 2 kb of DNA, comprising the *luxA* and *luxB* genes. Providing that these genes are expressed and that the luciferase is sufficiently stable, the potential exists to confer a bioluminescent phenotype on any prokaryotic organism. For eukaryotic micro-organisms the firefly luciferase (*luc*) (Tatsumi *et al.*, 1988; Gould & Subramani, 1988; Kricka, 1988; Alam & Cook, 1990) and

Fig. 1 (facing page). Photographs taken using a Hamamatsu Argus 100 Vim 3 photon video camera. (a) Light emission from saprophytic bioluminescent bacteria on the surface of a sprat. (b) A microscopic image of a *V. fischeri* microcolony upon which has been superimposed a photon counting image of bacterial bioluminescence (blue, red and pink indicate increasing light intensity, respectively); three centres of light induction can be seen with a fourth in the top-left corner and these reflect the mechanism of autoinduction for bioluminescence in this bacterium (see text). (c) Agr-dependent regulation of *eta-luxAB* expression in *S. aureus* strains (Sheehan *et al.*, 1992). (d) Two spread plates of *E. coli*[pSB237] which requires the autoinducer KHL for bioluminescence; the plate on the right has been inoculated in the centre with *Erwinia carotovora* and the production of autoinducer by this bacterium activates bioluminescence in surrounding *E. coli* cells, also shown in the accompanying 3-D light intensity map of both plates. (e) 23 cells of *Pseudomonas aeruginosa* deposited on a nitrocellulose filter and grown on a lawn of *E. coli*[pSB237]; KHL production from the *P. aeruginosa* colonies has activated bioluminescence in adjacent *E. coli* cells, quantified in the accompanying 3-D light intensity map. (f) A slice of Italian salami overlayed with a thin agarose gel containing bioluminescent *Salmonella typhimurium* is shown under normal room illumination on the left; the right-hand image is a photon counting image that provides a 2-D map of the preservative action of the salami on the overlayed *Salmonella*.

other eukaryotic luciferases (Wood *et al.*, 1989) are available. However, *luxAB* fusion genes have also been expressed in yeast and plant cells (Kirchner *et al.*, 1989).

Cloning vectors and transposons

It is clearly impractical to review this area exhaustively; nevertheless, there is merit in highlighting a number of constructs that serve as illustrations of methodology.

Given that the *lux* operons from *V. fischeri* and *V. harveyi* were the first to be cloned (Engebrecht *et al.*, 1983; Cohn *et al.*, 1983), the majority of vector constructs employ *lux* DNA from these sources. The question of equivalence between the various bacterial *lux* genes is nevertheless important from an application point of view. The key difference between those luciferases for which gene sequences are presently available is their temperature stability. The cloned enzyme from *V. fischeri* is stable in *E. coli* at 30 °C but quickly loses activity at 37 °C (Friedland & Hastings, 1967; Sakharov *et al.*, 1988; Ahmad & Stewart, 1991). The enzyme from *V. harveyi*, by contrast, is stable in *E. coli* at 37 °C and that from *X. luminescens* is stable at 42 °C (Szittner & Meighen, 1990). The recent proliferation of *luxAB* fusion genes, uniquely of *V. harveyi*, has also introduced a variation in temperature stability; typically, the fusion derivatives have an optimum temperature some 7–10 °C below wild-type (see Meighen, 1991, for review). The choice of enzyme must be made with care, therefore, with due regard to the recombinant microbial growth temperature. However, it can be very convenient to irreversibly denature bacterial luciferase *in vivo* by mild temperature shock, particularly during studies that seek to explore gene expression in a cyclical manner.

Sevigny & Gossard (1990) have developed the *Vibrio harveyi luxAB* genes for the selection of plasmid recombinants as an alternative to the ubiquitous *lacZ* α complementation system developed by J. Messing and co-workers (Vieira & Messing, 1982; Messing, 1983; Norrander *et al.*, 1983). A synthetic DNA sequence containing a multiple cloning site (MCS) was inserted into the *luxB* gene without disrupting the reading frame. Despite the insertion, this construct retains the capacity to provide *E. coli* with a bioluminescent phenotype in the presence of aldehyde. Insertion of cloned DNA into the MCS results in disruption of *luxB* expression and loss of bioluminescence. Advantages include, firstly, the ability to select recombinants in any *E. coli*, or indeed any enteric that can replicate the plasmid, since no special genetic background is required; secondly, it obviates the need for costly chemicals, such as IPTG and X-Gal.

A number of *lux* transposon vectors have been developed. Engebrecht *et al.* (1985) introduced the entire

lux operon from *V. fischeri* into the transposon mini-Mu to derive a mini-Mulux. This transposon induces mutations by insertional inactivation and, in many instances, target gene expression can be monitored as a function of bioluminescence (McCarter & Silverman, 1989). A Tn5-*lux* transposon has been developed by Boivin *et al.* (1988) to introduce constitutive *luxAB* expression into a variety of Gram-negative bacteria including *Pseudomonas*, *Agrobacterium* and *Rhizobium*. A mini-Tn5 *luxAB* transposon has been described by De Lorenzo *et al.* (1990). Devised to generate gene fusions, it has so far been used only in *Pseudomonas putida*. Wolk *et al.* (1991) have developed a Tn5-based *V. fischeri luxAB* transposon for cyanobacteria and have isolated mutants responding rapidly to nitrogen deprivation with increased bioluminescence. A *proU-luxAB* fusion has been cloned into Tn10 (Park *et al.*, 1989; Park, 1990) as a derivative of pNK861 (Way *et al.*, 1984), having the transposase gene located outside the transposon and inducible by IPTG. Finally, derivatives of the *Streptomyces* transposon Tn4556 (a member of the Tn3 family) have been developed with a *V. harveyi* luciferase reporter cassette (Sohaskey *et al.*, 1992). These Tn*lux* transposons (Tn5351 and Tn5353) insert randomly into the *Streptomyces* chromosome and fuse the *luxAB* genes to a variety of transcription units. Colonies which glow brightly enough to be seen with the unaided eye have been generated with this system and some clearly show developmental regulation of light expression. It is clear that the development of *lux* transposition systems will continue to advance as the ease and sensitivity of bioluminescence detection becomes more widely recognized. In particular, it will be interesting to await the development of a *lux* version of Tn917, analogous to Tn917*lac* (Perkins & Youngman, 1986), which can be used on bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (Mengaud *et al.*, 1991).

Promoter probe vectors employing *lux* genes have been developed for both Gram-positive and Gram-negative bacteria. Using *luxAB* from *V. harveyi*, Farinha & Kropinski (1990) have constructed a broad-host-range plasmid, pQF70, that is suitable for the detection and analysis of promoters in a wide range of Gram-negative bacteria. A promoter probe vector for Gram-positive bacteria has recently been described by Park *et al.* (1991). Both these vectors require the addition of a long-chain aldehyde (decanal or dodecanal) to bacterial cultures prior to measuring bioluminescence. There are, however, examples of promoter probe vectors that use the entire *lux* pathway including the aldehyde synthesis genes, as for example pUCD615 (Rogowsky *et al.*, 1987). Given that expression of *V. harveyi luxA* and *luxB* genes on separate replicons still provides an active bacterial luciferase (Gupta *et al.*, 1985), it is interesting to note that

probes based on the use of only *luxA* or *luxB*, complemented by the alternate gene either in the chromosome, F' or a compatible plasmid, have yet to be developed.

Instrumentation

The application of *lux* genes in any field of biology requires the availability of instrumentation for the visualization and quantification of photon emission. A failure to relate scientific objectives with appropriate instrumentation has probably limited the uptake of *lux* as a molecular biology tool. The most sensitive detector of photon emission available in most laboratories is the scintillation counter. A relatively unsophisticated instrument, without sample cooling, can be configured for the detection of less than 10 bioluminescent bacteria per ml of sample. To achieve this the coincidence counting, a standard feature of scintillation counting, must be disengaged, thus enabling the photon detectors to register every photon, not just those that arrive at the detectors with coincidence. Dedicated luminometer instruments may be less sensitive by an order of magnitude or so, but represent the most versatile and least costly detection devices. An evaluation of the performance of ten commercial luminometers has been undertaken by Jago *et al.* (1989).

One of the great attractions of bioluminescence is the ability to visualize light in two or even three dimensions. A number of photon counting imaging devices are available. Image Research's BIQ (Bioluminescence Image Quantifier; Cambridge, UK) can detect single bioluminescent *E. coli* cells (Mitchell, 1991). Similar performance can be achieved from Hamamatsu Photonic's (Enfield, UK) Argus 100 Vim 3 camera (Masuko *et al.*, 1991, and Fig. 1b). Using these devices, spatial information on gene expression within micro-colonies, colony differentiation during development, or bacterial/plant symbiosis/pathogenesis can be gained in real-time and in a non-disruptive manner. Examples of the unique value of photon imaging are included in subsequent sections of this review.

A reporter of gene expression

It is as a reporter of gene expression that bacterial luciferase has been most widely applied. First reviewed by Meighen (1984) there are now more than 40 examples of its use in this area. The vast majority are naturally concerned with monitoring the initiation of transcription, but one report has used the *V. harveyi lux* genes to provide quantitative data on transcription termination

Fig. 3. Response of RB1351 batch cultures to naphthalene. *Pseudomonas* sp. strain RB1351 was grown in LB broth at 25 °C with constant stirring. Naphthalene crystals were added at 1% (w/v) final concentration (arrow). Culture growth (■) was determined by using a Klett meter. ▲, Bioluminescence with naphthalene; ▼, bioluminescence without naphthalene. (Adopted from Burlage *et al.*, 1990.)

in *E. coli* (Peabody *et al.*, 1989) and one for tRNA-mediated nonsense suppression (Shultz & Yarus, 1990). For the remainder, examples have been chosen in this review that demonstrate the advantages of bioluminescence in being non-invasive and non-destructive and offering high sensitivity, in real-time analysis. Burlage *et al.* (1990) have used bioluminescence to monitor naphthalene catabolism in *Pseudomonas putida*. They describe the subcloning of the upper pathway promoter P_{nah} of NAH7 to form a fusion with the *luxCDABE* genes of *V. fischeri* and demonstrate the utility of this system for describing genetic expression in a unique manner, i.e. real-time analysis of a growing culture. Fig. 3 shows their data from a chemostat culture of *Pseudomonas putida* RB1351. Addition of either naphthalene or salicylate to the reaction vessel produced a bioluminescent response commensurate with activation of P_{nah} . They describe their construct as producing sufficient light for remote sensing by fibre optic or liquid-light pipe technology: thereby they highlight the narrow line between pure scientific understanding and industrial application that is so often seen in bioluminescence studies.

A landmark publication on the use of bioluminescence as a marker for gene expression appeared in *Plant Molecular Biology* (O'Kane *et al.*, 1988). Aladar Szalay's group were the first to report that bacterial bioluminescence could be detected and localized in single rhizobium-infected plant cells. They clearly demonstrated that the availability of reduced FMN and molecular oxygen, within bacteroides, was non-limiting for the bioluminescence reaction. Thus they confirmed that bioluminescence is a precise marker for *in vivo* bacterial gene expression, even in complex environments where bacteria reside within eukaryotic cells. The implications

Fig. 4. Use of a fusion to the *luxAB* reporter to monitor the time course of *sapA* transcription. Light emission from patches of *Streptomyces coelicolor* cells bearing plasmid pS950 containing the *sapA-luxAB* fusion was measured using a photometer. At each time point, a homogeneous portion of each patch (of uniform surface area) was removed from an agar plate and transferred to a glass scintillation vial containing 2 μ l n-decanal, the aldehyde substrate for luciferase. The patch was oriented such that its upper surface was facing the input window of the photometer. (Adopted from Guijarro *et al.*, 1988.)

of this work for clinical studies on macrophage engulfment of bacteria, in which the sustained biochemical viability of bacteria can be detected as light emission, have yet to be fully appreciated. O'Kane *et al.* (1988) were able to monitor both constitutive and *nifD* promoter-controlled luciferase gene expression and, by using photon counting video microscopy, they were able to localize luminescence within a single living plant cell.

There are fewer examples of *lux* as a monitor of gene expression in Gram-positive bacteria, a factor which undoubtedly reflects the fact that light emission from such bacteria is typically a hundredfold less than for the Gram-negative class. Notwithstanding, Schauer *et al.* (1988) and Guijarro *et al.* (1988) have used the *V. harveyi luxAB* genes to determine the timing and spatial localization of transcription of a *Streptomyces coelicolor* gene (*sapA*) encoding a 13 kDa spore-associated peptide. Fig. 4 shows their data for *sapA-luxAB* expression in colonies grown on solid sporulation medium and monitored for the induction of luciferase synthesis at daily intervals by the use of a luminometer. Luciferase synthesis commenced with the appearance of aerial mycelium formation, a finding in agreement with the time of appearance of the *sapA* transcript as determined by a nuclease protection-hybridization assay. Alan Schauer has continued to develop the application of *lux* genes in this area of research, as evidenced by the recent publication by Sohaskey *et al.* (1992) on the construction of plasmid- and transposon-based promoter probes for *Streptomyces* spp.

Carmi *et al.* (1987) were the first to use luciferase as a

marker for gene expression in *Bacillus* spp. The advantages of a non-disruptive indicator are particularly relevant for developmental systems such as sporulation where spore development is intrinsically dependent on the architectural integrity of the mother cell. Spores produced from *lux* recombinant bacilli are of intrinsic value as they remain dark until triggered into biochemical activity through germination. Hall *et al.* (1990) have used such spores to study their heat resistance, but there remain considerable opportunities to further understand the control of gene expression during germination and, more generally, bacterial morphogenesis.

One of the most persuasive reasons for using *lux* genes to mark expression, already noted in the work of O'Kane *et al.* (1988) but also in the pioneering work of Shaw & Kado (1986), is to monitor bacteria during host-symbiont or host-pathogen interaction. This would seem particularly important in clinical disease where the study of pathogenesis cannot be completed without reference to the influence of host-microbe interactions. An example where such potential may soon be realized is in the study of *Staphylococcus aureus eta* gene expression. The epidermolytic toxin ETA is the causative agent of staphylococcal scalded skin syndrome, a disease primarily of neonates that results in the upper layers of the epidermis peeling off. Although an assay procedure for the exfoliative toxin has been developed, its sensitivity and complexity cannot allow real-time analysis of toxin expression. However, the gene encoding ETA has been cloned and sequenced (O'Toole & Foster, 1987; Lee *et al.*, 1987; Sakurai *et al.*, 1988) and this has allowed the development of a chimeric gene fusion between the *eta* promoter region and the luciferase (*luxAB*) genes of *Vibrio fischeri* (Sheehan *et al.*, 1992). Transformation of this construct into accessory gene regulator positive (*agr*⁺) (*S. aureus* 8325-4) and *agr*⁻ (*S. aureus* ISP546) strains has shown that the expression of luciferase is 100-fold higher in *agr*⁺ than *agr*⁻ hosts, as shown in Fig. 1(c). Fig. 5 also indicates a growth phase dependence for *eta* expression with the rate of transcription of the *eta-luxAB* fusion increasing significantly during the transition from the exponential phase of growth to stationary phase. The effect of environmental parameters such as osmotic strength on *eta* promoter function are also easily assayable by bioluminescence (Sheehan *et al.*, 1992). While at present these studies explore the control of ETA expression *in vitro*, there is clearly a very exciting opportunity to explore in real time and with non-invasive photon video imaging the control of ETA production during the disease process in *in vivo* experimental models, such as that described for neonatal mice (Kinsman, 1986). Such studies might well hope to reveal subtle elements of host-microbe interactions that could themselves reflect essential triggers for microbial pathogene-

Fig. 5. Agr regulation of *eta-luxAB* expression. *Staphylococcus aureus* strains 8325-4 (*agr*⁺) and ISP546 (*agr*⁻) harbouring pSB100, an *eta-luxAB* fusion derivative, were grown in trypticase soy broth. ■, growth as measured by optical density; ▲, bioluminescence from 8325-4 *Agr*⁺; ▼, bioluminescence from ISP546 *Agr*⁻. (Adopted from Sheehan *et al.*, 1992.)

sis. The recent achievement of Jacobs *et al.* (1991) in overcoming the problems of expressing *luxAB* efficiently in Gram-positive bacteria simply serves to enhance this potential.

A reporter of density-dependent signal transduction

As shown above, expression of the *Staphylococcus aureus eta* gene is known to be dependent upon the accessory gene regulator locus *agr*. The *agrA* gene encodes a protein that has sequence homology with the response regulator proteins of two-component signal transduction systems with *agrB* as a putative histidine protein kinase (Stock *et al.*, 1989). The role of two-component signal transduction systems in the adaptive responses of prokaryotes to environmental stress is becoming increasingly evident (for reviews see Stock *et al.*, 1989, 1990; Miller *et al.*, 1989; Ronson *et al.*, 1987). The work of Sheehan *et al.* (1992), highlighted above, clearly indicates how *lux* gene fusions can aid in elucidating the mechanisms of these adaptive responses. It is just emerging, however, that the bacterial *lux* genes may have a much more profound role to play in this regard than a mere passive indicator of gene expression.

Induction of bioluminescence in the marine bacterium *Vibrio fischeri* lags behind cellular growth and may, therefore, be termed density dependent. This density dependence reflects the need to accumulate, to a threshold concentration, a small molecule produced and excreted by the cells and termed autoinducer. In *V. fischeri* this autoinducer is *N*-(β -ketocaproyl)homoserine lactone (KHL) (Eberhard *et al.*, 1981). The *luxI* gene

product (Devine *et al.*, 1988, and Fig. 2) is believed to be responsible for autoinducer synthesis since luminescence can be restored to *luxI*⁻ cells by addition of exogenous autoinducer (Engebrecht & Silverman, 1984). KHL is species specific, causing the induction of luminescence in *V. fischeri* and *V. logei* but in no other strains (Engebrecht *et al.*, 1983).

The mechanism of autoinduction of *lux* gene expression has been recently reviewed (Meighen, 1991). The autoinducer is produced at a low constitutive rate during the early stage of growth. When a sufficient level has accumulated, interaction with the *luxR* gene product (Fig. 2) stimulates transcription of the right operon which includes *luxI* and the *lux* structural genes (Devine *et al.*, 1989; Shadel & Baldwin, 1991). This leads to additional LuxI production and hence additional autoinducer, thus creating a positive feedback loop. Fig. 1(b) shows this process in action at the microscopic level. A micro-colony of *V. fischeri* is visualized by light microscopy and overlaid with a photon counting image of its natural bioluminescence. Three centres of bioluminescence can be seen which reflect three nucleation points where a single cell, in the centre of each light spot, has experienced a sufficient concentration of autoinducer to initiate autoinduction. The consequential increase in autoinducer triggers surrounding cells in a cascade event.

Although a fascinating example of gene expression control, such autoinduction has been seen as associated only with a few esoteric bioluminescent marine bacteria. However, Bainton *et al.* (1992a, b) have now reported that KHL is, in fact, produced by a diverse group of terrestrial bacteria and in one of these (*Erwinia carotovora*) it acts as a molecular control signal for the growth-phase-dependent expression of genes controlling carbenem antibiotic biosynthesis.

The key to understanding the significance of this resides in LuxR, the 28 kDa regulatory protein encoded by *luxR*. Mutational analysis of *luxR* has indicated that a region within the N-terminal domain of the LuxR polypeptide constitutes the autoinducer binding site (Slock *et al.*, 1990), which has recently been localized between $\alpha\alpha$ residues 79–127 (Shadel *et al.*, 1990). A second region, identified near the C-terminus of the polypeptide, is required for binding to the *lux* operator site, and a striking similarity has been noted to a 28 kDa protein of suspected regulatory function encoded upstream of the *E. coli* sequence encoding UvrC (Henikoff *et al.*, 1990). Both LuxR and UvrC-28K have C-terminal homology with UhpA, FixJ, ComA, NarL, DegU and UvrC-23K (UvrC-ORF2). These proteins are thought to function as transcriptional regulators, but their mechanisms of action have not been elucidated (Stock *et al.*, 1989). It is clear, however, from similarities in domain organization, that these regulators (including LuxR)

form part of a two-component response regulator superfamily (Deretic *et al.*, 1989; Stock *et al.*, 1989).

Bainton *et al.* (1992*a, b*) have developed a recombinant derivative of the *lux* system (pSB237) in which the *luxR* and *lux* promoter regions from the *V. fischeri* operon are linked to the *luxA* and *luxB* genes from *V. harveyi*. The resultant vector provides a bioluminescent phenotype only in response to externally added KHL and dodecanal. Fig. 1(*d*) shows a combined photon video image of a lawn of *E. coli*[pSB237] and a similar lawn inoculated at the centre with *Erwinia carotovora*. The 3-D light intensity profile clearly shows the degree to which autoinducer production from *E. carotovora* stimulates bioluminescence from surrounding *E. coli*[pSB237] cells. This construct represents a new approach to the use of *lux* genes to probe gene expression. It enables diverse bacteria to be evaluated for their nascent production of autoinducer. Fig. 1(*e*) for example, shows the detection of KHL production from colonies of *Pseudomonas aeruginosa*: the implication is that, just as in *V. fischeri* and *E. carotovora*, this must be a molecular signal for density-dependent gene expression.

Transcription of *katE* (which encodes catalase) and *katF* (*rpoS*) in *E. coli* (Mulvey *et al.*, 1990) and the conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens* (Zhang & Kerr, 1991) are regulated by as-yet-unidentified, small diffusible factors, present in spent media from stationary phase cultures. Growth regulation of a *Salmonella* plasmid gene (*vsdC*) essential for virulence, displays density dependency and a starvation response totally analogous to KHL synthesis in *V. fischeri* (Fang *et al.*, 1991). In fact, Fang *et al.* (1991) hypothesize that 'an as yet unidentified coactivator molecule which rises in concentration during stationary phase is required for maximal *vsdC* activation'. While recognizing the importance of other microbial hormone systems (e.g. see Horinouchi & Beppu, 1992), as the archetypal mechanism for autoinducer control it would seem that the application of the *lux* operon and the associated *lux* genes for elucidating control of gene expression in pathogenicity, virulence, adaptation and secondary metabolism is only now about to begin.

A reporter for bacterial detection

An entirely new concept for bacterial detection was described by Ulitzur & Kuhn (1987). The principle of this new technology involves the introduction of the *lux* genes into the genome of a bacteriophage. Such bacteriophage 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 & Kuhn (1987) described the engineering of λ 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 100 min 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 from S. Ulitzur, J. Kuhn and M. Suissa, 10² *S. typhimurium* have been detected using a simple luminometer (Stewart *et al.*, 1989). Although the detection of specific pathogens such as *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* requires a committed programme for the genetic engineering of host-specific bacteriophage, this technology offers the potential for rapid, user-friendly microbial testing. In foods, the detection limits for specific pathogens is in the order of 1 per 25 g, a level beyond the detection limit of direct bioluminescent bacteriophage assays. In consequence, there will be a requirement to go through a recovery and enrichment protocol prior to the bacteriophage assay, but same-day testing seems possible.

A revolution in microbial testing can, however, be perceived with the near on-line detection of indicator micro-organisms (Kodikara *et al.*, 1991). 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*⁺ bacteriophages can detect such indicator bacteria without recovery or enrichment, provided the bacteria are present in a food matrix at levels greater than 10⁴ per gram (Fig. 6*a*). Since the assay requires only 30–50 min, this allows the evaluation of indicator strains in less than an hour. For samples that have intrinsically low enteric counts, a 4 h enrichment prior to the phage assay can allow detection of less than 10 enteric bacteria (Fig. 6*b*).

A reporter of cellular viability

The importance of *in vivo* bioluminescence as a reporter of cellular viability has been subject to several recent reviews (Stewart, 1990; Stewart *et al.*, 1991; Denyer *et al.*, 1992). 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

Fig. 6. The relationship between the mean enteric plate count per cm² and mean bioluminescence for 141 samples in a 1 h *lux*⁺ phage assay (a) and 77 samples after a 4 h enrichment prior to the phage assay (b). (Adopted from Kodikara *et al.*, 1991.)

impairs that biochemistry, and thus compromises cellular viability, will lead to a reduction in light emission.

In many cases there is a close synergy between the potential of *lux* to monitor gene expression and as an environmental monitor of industrial effluents and toxicants. For example, rather than viewing the loss of light due to the gross disruption of biochemical processes by toxic agents, it is possible to be far more subtle. Many bacteria have evolved biochemical mechanisms to detoxify their growth environment. Typically, the genetic systems that encode the enzymes functional in such pathways are regulated and inducible by the presence of low levels of the relevant toxicant (the example of the naphthalene pathway in *P. putida* has been mentioned previously).

By definition such activation of gene expression occurs at concentrations of toxicant well below those that would lead to gross biochemical inactivation of cellular processes. For example, the detection of aromatic hydrocarbons such as toluene and xylene could be achieved by constructing a *lux* gene fusion to the *meta*-pathway promoter from the TOL plasmids of *Pseudomonas* (Inouye *et al.*, 1984). These pathways are substrate inducible so that the presence of an aromatic hydrocarbon substrate would, at low concentration, induce *lux* expression and hence a detectable bioluminescent phenotype.

Just such an approach has recently been described for the selective detection of mercury (Moelders, 1990). Bacterial Hg-resistance depends upon binding Hg²⁺ to the *merR* repressor gene product and activation of

expression (Brown *et al.*, 1989). A genetic couple of the *merR* gene and accompanying rightward and leftward promoter so that the rightward promoter expresses *luxAB*, allows very sensitive detection of Hg. According to the Genlux patent, bioluminescent biosensors containing the above recombinant organisms are absolutely specific for Hg and so provide rapid and unequivocal identification of Hg in heavy metal mixtures of variable composition without further processing. There is no contact between the sensor and the signal detector so they are useful for on-line application. In this particular case the Hg²⁺ detection limit is about 0.2 p.p.b. and is lower than that for most known methods, e.g. atomic absorption spectrometry.

The sensitivity advantage of this 'genetic switching' approach to the detection of toxic materials is particularly well illustrated by the use of Hg. Bioluminescent *E. coli* have previously been used to detect Hg²⁺ by gross biochemical damage and concomitant loss of light (Dodd *et al.*, 1991). In these studies a detection limit of 5 p.p.m. was achieved, some 25000-fold less sensitive than coupling detection to genetic switching.

The advent of polymerase chain reaction (PCR) technology (Saiki *et al.*, 1988) allows the rapid isolation and cloning of a wide range of previously defined and sequenced promoters controlling the expression of catabolic or detoxification pathways. It is now clear that PCR cloning strategies, hitherto used to develop genetic probes, provide biologically functional genes and gene control regions (Hill *et al.*, 1991). 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. with major potential both as tools for toxicant monitoring in water quality assessment and for relatively 'low-tech' simple assays for vitamins, minerals and amino acids – a potential that should have considerable interest to developing countries where 'high-tech' instrumentation cannot be afforded or supported.

One of the many beneficial consequences of using *luxAB* in reporter gene and promoter probe constructs is that the bioluminescent recombinant bacterial products may ultimately be used for purposes far removed from those which governed their original construction. For example, bioluminescent *Salmonella typhimurium*, originally constructed to explore gene regulation, has been applied to the study of preservative efficacy in foods. Current methods of microbial analysis disrupt the food matrix before bacterial enumeration and thus spatial information is lost. The potential now exists, however, to monitor the distribution of preservative concentration throughout a food matrix by either incorporating *lux* recombinant indicator bacteria into model foods and examining sections, or overlaying the indicator bacteria onto the surface of a food section. In either case, the effect of preservative action on light emission (and by inference cell viability) can be measured by photon video imaging. Fig. 1(f) shows such a system in operation on a slice of Italian salami (Reid, 1992). On the left is a photograph of the salami slice overlayed with a thin gelatin layer containing *lux*⁺ *S. typhimurium*. After a 1 h contact time, the bioluminescence remaining within the overlay was measured with a Hamamatsu Argus 100 Vim 3 photon video camera (right-hand image on Fig. 1f). The effect of preservative action upon the *S. typhimurium* is revealed as a 2-D map. Areas of high and low preservative action can be easily observed (low and high light respectively) and the spatial distribution of preservative action seen in a way hitherto impossible to achieve. Such *lux*-based systems may, therefore, be used in the future by microbiologists having no primary interest in molecular microbiology, but rather as a tool to enhance the confidence with which food safety assessments can be made.

It seems to the authors of this review that no other reporter system offers the long term depth of potential to microbiology than that presented by *in vivo* bioluminescence and the *lux* genes.

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