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Construction and detection of bioluminescent strains of *Bacillus subtilis*

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N. COOK, D.J. SILCOCK, R.N. WATERHOUSE, J.I. PROSSER, L.A. GLOVER AND K. KILLHAM. 1993. Bioluminescence (*lux*) genes from *Vibrio fischeri* and *V. harveyi* were introduced into *Bacillus subtilis* on a plasmid vector and by chromosomal integration. The plasmid-bearing strain was highly luminescent and stable under antibiotic selection, but luminescence was lost in the absence of selection and following sporulation and germination. The chromosomally marked strains emitted less light but were found to be stable without the requirement for antibiotic selection and following sporulation and germination. Individual luminescing colonies of both *B. subtilis* strains could be detected against a high background of non-bioluminescent indigenous soil microbial colonies on agar plates using a charge-coupled device camera. These bioluminescent Gram-positive strains could be of value in studies concerning the survival and spread of genetically-modified micro-organisms in soil environments.

INTRODUCTION

Bacillus subtilis is an aerobic Gram-positive endospore-forming micro-organism commonly found in soil and associated water sources (Priest 1989). Along with other members of the genus *B. subtilis* is used extensively in the industrial production of enzymes, biochemicals, antibiotics and insecticides (Harwood 1992) and genetically-modified strains may be candidates for release into the environment. There is currently much interest in the possible release of genetically-modified micro-organisms (GMMs) into natural environments to perform beneficial tasks such as biological pest control and biodegradation of toxic wastes. Confidence in the release of GMMs requires information on their potential survival in natural ecosystems and the possible dissemination of their novel genetic information amongst the indigenous microbial population. A major method of acquiring this information involves the marking of model microbial strains with an easily detectable phenotype, prior to controlled experimentation designed to monitor survival and/or gene transfer (Pickup *et al.* 1991). A recent development for monitoring micro-organisms in natural environments involves detection of luminescence, conferred by the introduction of bioluminescence (*lux*) genes into *Escherichia coli* (Rattray *et al.* 1990), *Pseudomonas* spp. (de Weger *et al.* 1991; Amin-Hanjani *et al.* 1993) and *Xanthomonas campestris* (Shaw *et al.* 1992). Luminescent micro-organisms can be detected by several means, including visual identification, luminometry, X-ray film-imaging, charged-coupled device (CCD) imaging, PCR analysis and gene probing (Rattray *et al.* 1990; de Weger *et al.* 1991; Grant *et al.* 1991; Gray *et al.* 1992; Shaw *et al.* 1992). Luminescence offers considerable advantages over other marker systems, in that it quantifies the metabolic activity of the cell (Rattray *et al.* 1992; Meikle *et al.* 1992), and can provide a means of *in situ*, non-extractive detection in environmental samples (Rattray *et al.* 1990; de Weger *et al.* 1991; Silcock *et al.* 1992).

As yet environmental studies using the *lux* system have been carried out only with Gram-negative, non-spore-forming organisms. The Gram-positive *Bacillus* species have special characteristics, such as sporulation and germination, and merit specific attention. Previous work on bioluminescent bacilli (Carni *et al.* 1987; Karp 1989; Jacobs *et al.* 1991) has been concerned with gene expression and cell physiology. Some of the constructs used are unsuitable for detection in natural environments, in particular because of instability and low levels of light output. This report describes the construction and characterization of bioluminescent strains of *Bacillus* designed specifically for environmental detection, and their use in viable cell enumeration following inoculation into soil.

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MATERIALS AND METHODS

Bacterial strains and plasmids

Indigenous soil bacteria were isolated from a sandy loam of the Craibstone series (pH 6·5; organic C, 2·47%; cation exchange capacity, 7·4 C mol kg^{-1}).

Plasmid pUCD607, which contains the *lux* CDABE genes from *Vibrio fischeri* (Shaw and Kado 1986); plasmid pCSS114, which carries the *V. harveyi luxAB* genes expressed from the *B. subtilis veg* II promoter (Karp 1989); plasmid pHV1431, a stable high copy number (approx. 150 copies per cell) *B. subtilis* cloning vector (Janniere *et al.* 1990) and plasmid pJHOX1 (Todd *et al.* 1986) were used. Plasmid pSP167 was constructed previously in this laboratory.

Rifampicin-resistant mutants of *B. subtilis* NCTC 3610 were obtained following growth and continued subculture in LB broth containing (g l^{-1}); tryptone, 10; yeast extract, 5; NaCl, 5 and progressively higher concentrations of rifampicin. This led to the selection of a strain (3610R) which was chromosomally resistant to 100 μg ml^{-1} rifampicin.

The construction of plasmid pOTH951 is shown in Fig. 1. Subcloning was performed in *E. coli* HB101. As pOTH951 does not contain a Gram-negative origin of replication, the ligation mix from which it was formed was transformed directly into *B. subtilis* NCTC 3610 protoplasts by the method of Chang and Cohen (1979). Transformants were selected by growth on regeneration medium containing 2 μg ml^{-1} chloramphenicol. Supercoiled pOTH951 DNA was then prepared and transformed into *B. subtilis* 3610R, as described above.

The construction of plasmids pVIT5111 and pRNW111 is shown in Figs 2 and 3, respectively. These plasmids were designed to integrate into the *B. subtilis* chromosome.

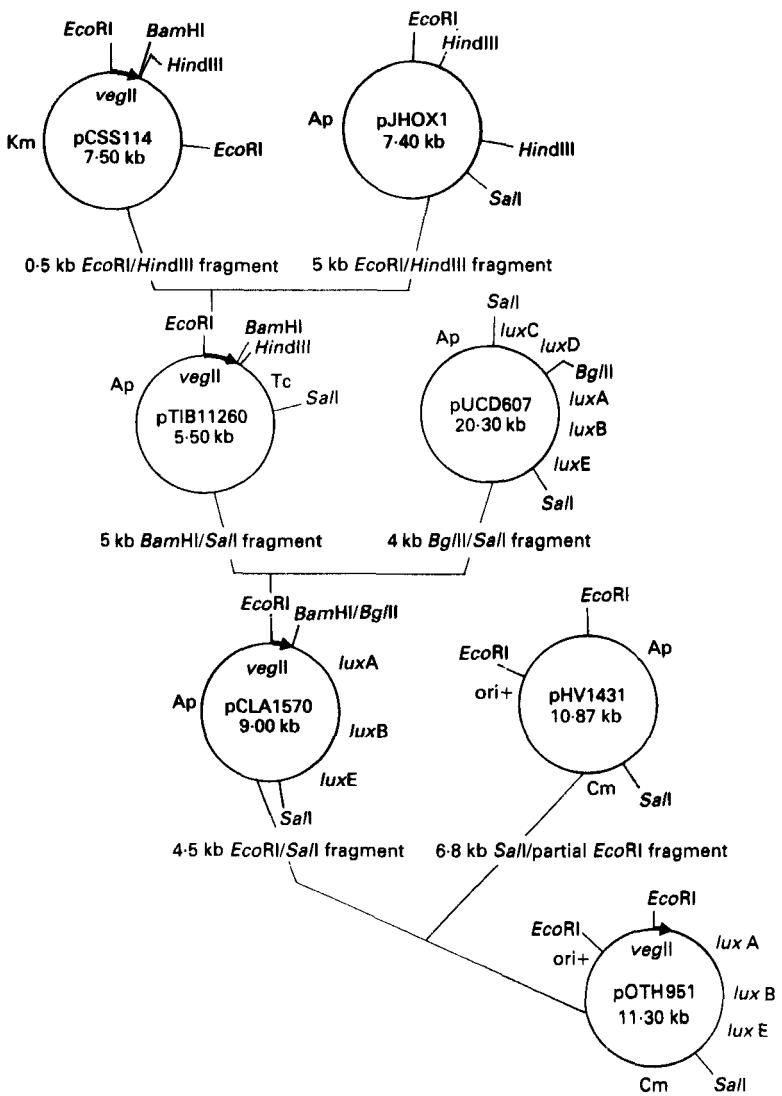

Fig. 1 Construction of plasmid pOTH951, which contains the *lux* ABE genes from *Vibrio fischeri* under the control of the *Bacillus subtilis veg*II promoter. Only the relevant restriction sites are indicated and maps are not drawn to scale. Ap, Ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Tc, tetracycline resistance; ori−, Gram-negative origin of replication; ori+, Gram-positive origin of replication

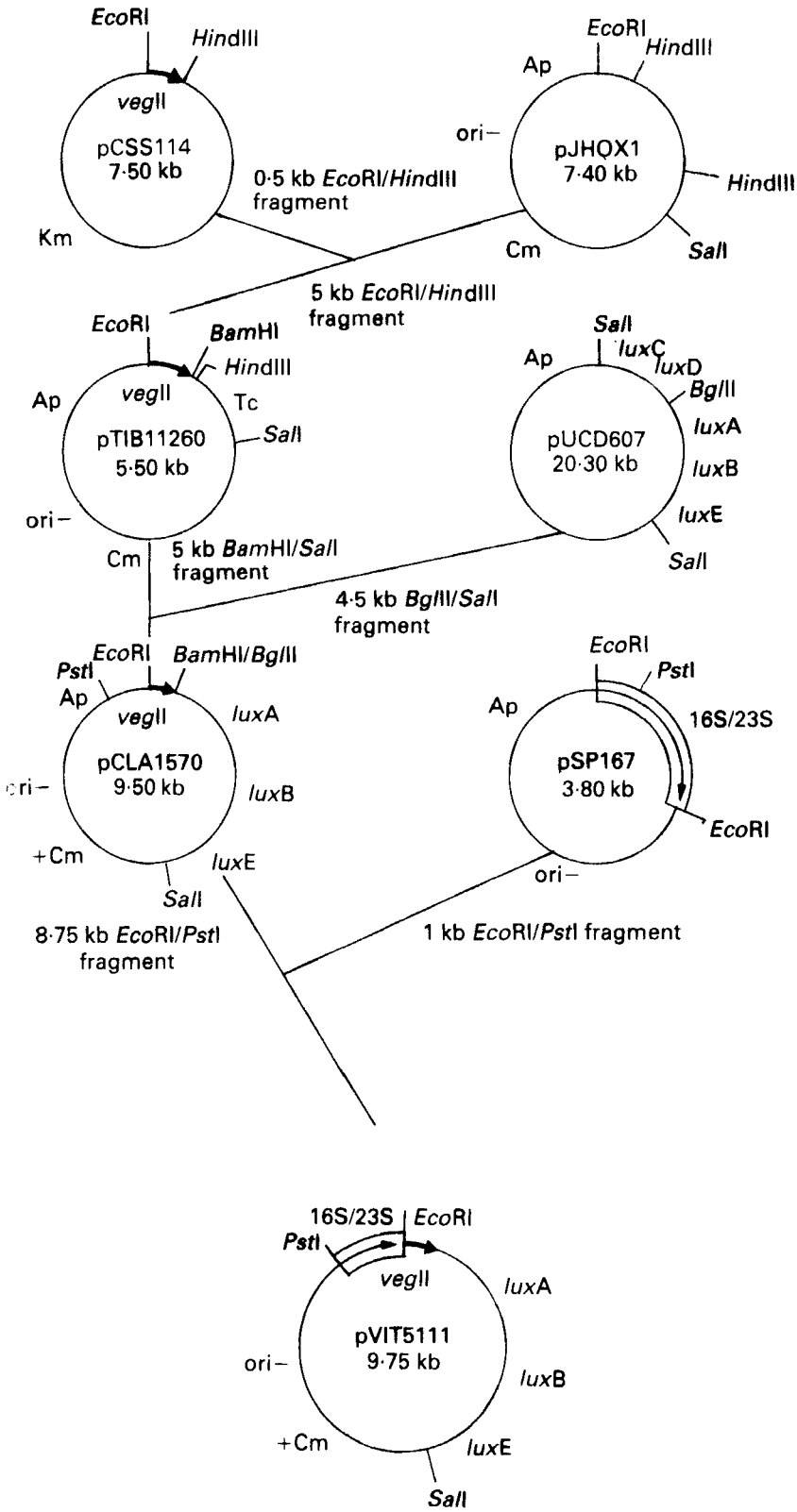

Fig. 2 Construction of plasmid pVIT5111, used to mediate the integration of *Vibrio fischeri lux* genes expressed from the *Bacillus subtilis veg*II promoter into the *B. subtilis* chromosome. Only the relevant restriction sites are indicated and maps are not drawn to scale. Ap, Ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Tc, tetracycline resistance; ori−, Gram-negative origin of replication; ori+, Gram-positive origin of replication

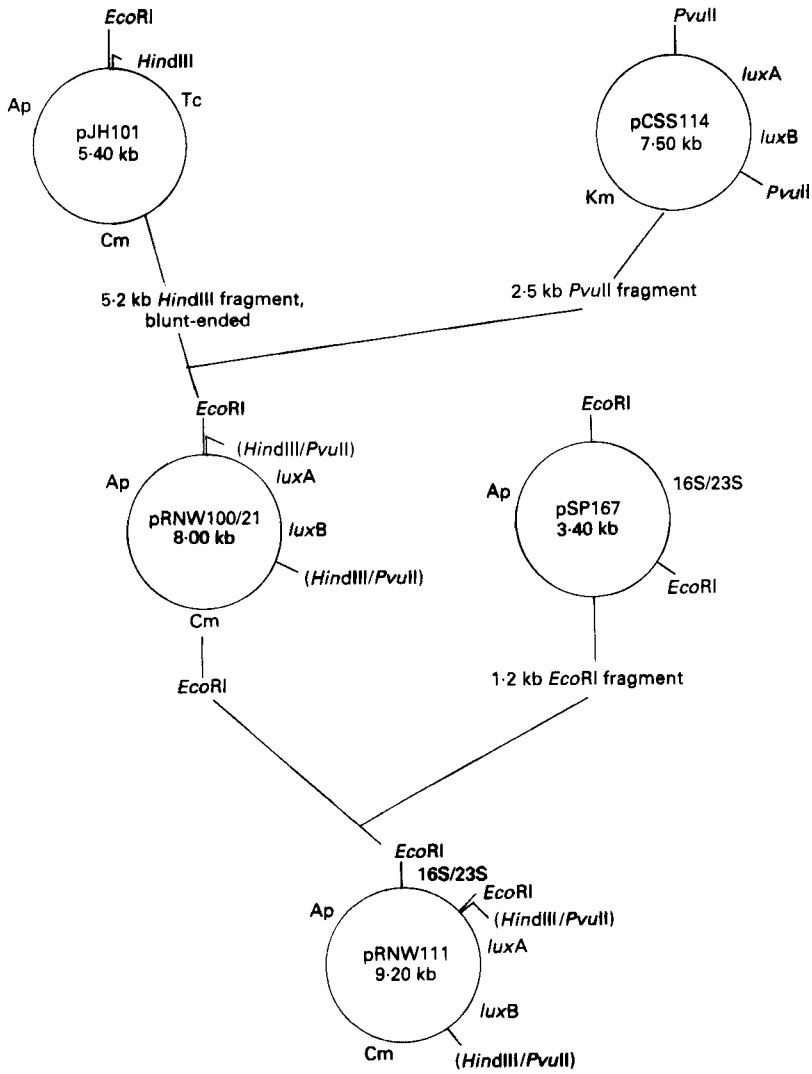

Fig. 3 Construction of plasmid pRNW111, used to mediate the introduction of *Vibrio harveyi lux* genes into the *Bacillus subtilis* chromosome and to allow their expression from an endogenous chromosomal rRNA operon promoter. Ap, Ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Tc, tetracycline resistance

They lack a Gram-positive origin of replication and can only be maintained in *B. subtilis* following integration mediated by recombination between homologous sequences on the plasmid and chromosome. Recombination was facilitated by incorporation of sequences of the 16S and 23S ribosomal RNA genes of *B. subtilis*, which are the target of and site of integration of these plasmids. Subcloning was performed in *E. coli* HB101, from which plasmid DNA was subsequently prepared by alkaline lysis and caesium chloride density gradient centrifugation (Maniatis *et al.* 1982). *Bacillus subtilis* 3610 was transformed with supercoiled plasmid DNA by the protoplast regeneration method (Chang and Cohen 1979). Transformants were selected on regeneration medium containing 2 μg ml^{-1} chloramphenicol. The bioluminescent strains of *B. subtilis* resulting from integration of pVIT5111 and pRNW111 were given the epithets VIN and PBC11, respectively.

Growth and light output in liquid culture

Erlenmeyer flasks (500 ml) containing 200 ml LB broth were inoculated with 1 ml of an overnight culture and incubated at 30°C. At hourly intervals, 1 ml samples were removed from the culture, diluted with saline phosphate buffer (15 mmol l^{-1}; pH 7) and 5 μl of 0·2% *n*-decyl aldehyde added. Following incubation for 10 min at ambient temperature, luminescence was measured with an LKB model 1251 luminometer with light output integrated over a 10 s period with continuous mixing. Light output is expressed as relative light units. The absorbance (A_{600}) of samples (1 ml) was measured and related to biomass concentration.

Visualization of luminescent colonies

Colonies were examined using a nitrogen-cooled slow-scan CCD (Wright Instruments Ltd., Enfield, UK), fitted with

a 50 mm lens and encased in a light-tight box. *Bacillus subtilis* pOTH951 and *B. subtilis* VIN were grown in 10 ml LB broth on an orbital shaker (150 rev min^{-1}) at 30°C to an optical density of 0·5 at 600 nm before serial 10-fold dilution in quarter-strength Ringer's solution. LB agar plates were inoculated with 100 μl of each dilution and incubated at 30°C overnight. Immediately prior to detection, 2 μl of 100% *n*-decyl aldehyde was applied to the inside surface of the lid of each Petri dish. For bright field images, plates were exposed to the CCD camera for 0·1 s, and for dark field images plates were exposed for periods ranging from 10 s to 15 min, depending on the level of luminescence. The final image was enhanced by removal of cosmic ray effects. For detection of bioluminescent *B. subtilis* colonies against a background of indigenous soil bacterial colonies, a 10 g sample of soil was suspended in 100 ml of Ringer's solution and then serially diluted by 10^4 in 10 ml volumes. Overnight cultures of *B. subtilis* were serially diluted to 10^{-8} in Ringer's solution. Each 10^{-2}–10^{-4} soil slurry dilution was inoculated with 10^{-6}–10^{-8} dilutions of either *B. subtilis* strain. Plates of LB agar were spread with 100 μl of each inoculated soil slurry dilution and incubated at 30°C for 24 h before imaging, as described above, by the CCD camera.

Determination of stability

A liquid culture (10 ml of LB broth) of the strain was grown overnight with antibiotic selection. A 1 ml sample of this culture was serially diluted and plated on selective and non-selective agar plates. A 10 μl volume of the overnight culture was used to inoculate fresh medium, both with and without antibiotic selection. This procedure was repeated for 12 d, or until no colonies were detected on selective plates.

Growth on minimal medium and sporulation

The ability of the strains to grow on minimal medium was examined by harvesting 1 ml of an overnight culture, washing in sterile distilled water and streaking the cell suspension on *B. subtilis* minimal medium (Anagnostopoulos and Spizizen 1961). The plates were incubated at 30°C for 48 h before inspection. The ability of the *B. subtilis* strains to sporulate was tested by batch growth in Schaeffer Sporulation Medium (SSM) (Priest 1989) followed by microscopic examination for spores.

Stability of *lux* phenotype through sporulation and germination

Erlenmeyer flasks (500 ml) containing 200 ml of SSM were inoculated with 1 ml of an overnight culture and incubated until the culture consisted only of spores. The culture was then serially diluted and 100 μl of each dilution was plated on LB agar and LB agar containing 10 μg ml^{-1} chloramphenicol. After overnight incubation at 30°C the colonies were counted and compared. Colonies arising on selective plates were examined for luminescence, either visually or by CCD imaging, after exposure to *n*-decyl aldehyde.

RESULTS

Bioluminescence of liquid cultures and colonies of *B. subtilis* 3610R containing pOTH951 grown under antibiotic selection was detectable visually in a darkened room. The plasmid conferred resistance to 40 μg ml^{-1} chloramphenicol and, under this selection, was maintained through 12 cycles of daily subculturing. Figure 3 shows the luminescence profile of *B. subtilis* 3610R pOTH951 during growth in batch culture. Under non-selective conditions, light emission declined after the early exponential phase, and subcultured *B. subtilis* cells lost pOTH951 by 30 generations. The maximum specific growth rate of *B. subtilis* 3610R pOTH951 was significantly lower than that of its parent strain *B. subtilis* 3610R, which had a growth rate identical to that of the original *B. subtilis* 3610 (Table 1). The reduction in maximum specific growth rate was most probably due to the metabolic burden imposed on the cell by the maintenance and expression of the *lux*-bearing plasmid. *Bacillus subtilis* 3610R pOTH951 was able to grow on minimal medium and sporulated both in the presence and absence of 40 μg ml^{-1} chloramphenicol. Rifampicin resistance was retained following sporulation and germination, but after germination of spores in selective medium only 2·5% of the population retained the luminescence phenotype, and only 0·001% of spores retained the luminescence phenotype after germination in non-selective medium (Table 2).

Table 1 Maximum specific growth rates of parental strains and luminescent strains of *Bacillus subtilis*

B. subtilis strain	Maximum specific growth rate (h^{-1})*	Variance	*P*†
3610	1·21	0·0006	
3610R	1·21	0·004	0·88
3610R pOTH951	0·62	0·003	0·0036
VIN	0·94	0·0027	0·015
PBC11	1·20	0·0016	0·91

* Maximum specific growth rates are the means from three replicate flasks.

† Values of *P* were determined using the Student *t*-test to compare the mean specific growth rate of each strain with that of the parental strain 3610R.

Table 2 Retention of bioluminescence after sporulation and germination of *lux*$^+$ *Bacillus subtilis* strains

B. subtilis strain	% luminescent cells after sporulation and germination
3610R pOTH951 + Cm	2·5
pOTH951 − Cm	0·001
VIN	100
PBC11	100

+ Cm, Spores produced in the presence of 40 μg ml^{-1} chloramphenicol; − Cm, spores produced in the absence of chloramphenicol.

The number of spores was determined by germination and growth on LB agar. Potential *lux*$^+$ spores were selected and enumerated on LB agar containing 10 μg ml^{-1} chloramphenicol; luminescence was confirmed by subsequent visual inspection or charged-coupled device imaging of the colonies. All chloramphenicol-resistant colonies proved to be luminescent.

Bacillus subtilis VIN, which carries chromosomally-integrated *V. fischeri luxABE* genes, expressed from the *veg*II promoter as in plasmid pOTH951, was resistant to 20 μg ml^{-1} chloramphenicol. Light emitted could not be detected visually, but was stable, the luminescence phenotype being retained through 12 cycles of daily subculturing in the absence of selection. The luminescence profile was characterized during non-selective batch culture only (Fig. 4). Light emission was lower than from *B. subtilis* pOTH951, probably reflecting the relative numbers of the *lux* genes in the two constructs, but otherwise the profiles were similar. The maximum specific growth rate of *B. subtilis* VIN was lower than that of its parent strain *B. subtilis* 3610 (Table 1), possibly due to the burden imposed by *lux* expression from the strong promoter (*veg*II). *Bacillus subtilis* VIN was able to grow on minimal medium and sporulate, and it retained its bioluminescent phenotype after spore germination (Table 2).

Bacillus subtilis PCB11, which contains the *V. harveyi luxAB* genes integrated into the chromosome and expressed from a chromosomal rRNA promoter, was resistant to 10 μg ml^{-1} chloramphenicol. As with *B. subtilis* VIN, light emitted could not be detected visually, but was stable throughout 12 cycles of daily non-selective subculturing. Figure 4 shows the luminescence profile of *B. subtilis* PBC11. The maximum specific growth rate of *B. subtilis* PBC11 was identical to that of the parent strain *B. subtilis* 3610 (Table 1), possibly due to lower expression of the *lux* genes, and consequent reduced metabolic burden, in comparison with other bioluminescent constructs. This strain was able to sporulate and grow on minimal medium and retained its bioluminescent phenotype after spore germination (Table 2).

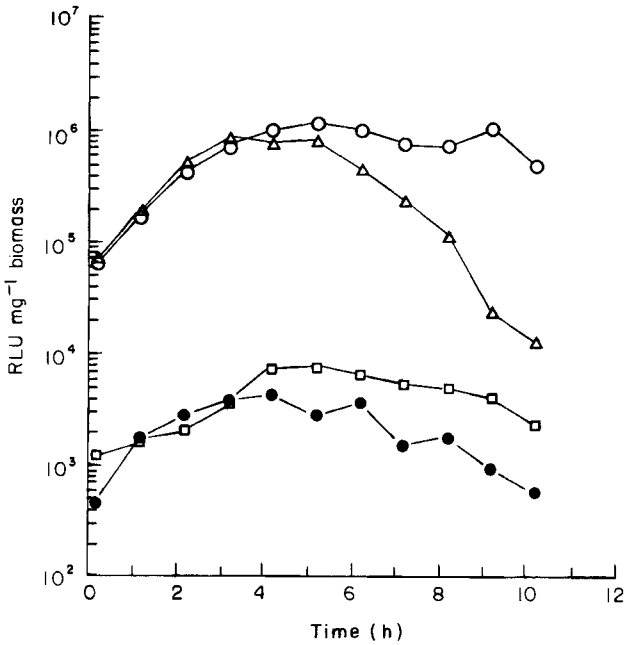

Fig. 4 Light emission during the growth of *Bacillus subtilis* 3610R (OTH$^-$) (△), *B. subtilis* 3610R pOTH951 (OTH$^+$) (○), *B. subtilis* VIN (□) and *B. subtilis* PBC11 (●). Light emission is represented as a function of cell density. *Bacillus subtilis* 3610R pOTH951 was grown in LB broth plus 40 μg ml^{-1} chloramphenicol; *B. subtilis* 3610R, VIN and PBC11 were grown in LB broth

Visualization and detection of luminescent *B. subtilis* inoculated into soil

Figure 5a shows a bright field image of *B. subtilis* pOTH951 and Figs 5b, c and d show dark field images produced after 1, 10 and 30 s exposure, respectively. Each individual colony was clearly and distinctly visible through luminescence. Figure 6a shows a bright field image of *B. subtilis* pOTH951 colonies among a high background of chloramphenicol-resistant indigenous soil bacterial colonies on an agar plate. It was difficult to identify and enumerate the colonies of the introduced strain. However, with luminescence induced by application of the substrate *n*-decyl aldehyde and visualized by 5 min exposure to the CCD camera, the colonies of *B. subtilis* pOTH951 could easily be distinguished from those of the indigenous microorganisms (Fig. 6b).

Dark field images of colonies of the chromosomally bioluminescent *B. subtilis* strains VIN and PBC11 could be obtained after 2 and 30 min exposure, respectively. Detection of VIN and PBC11 among soil bacterial colonies on non-selective plates is shown in Figs 6c, d and 6e, f, respectively. Although it is virtually impossible to distinguish the introduced strains amongst the overgrowth of indigenous soil microbial colonies, they can readily be identified after CCD imaging.

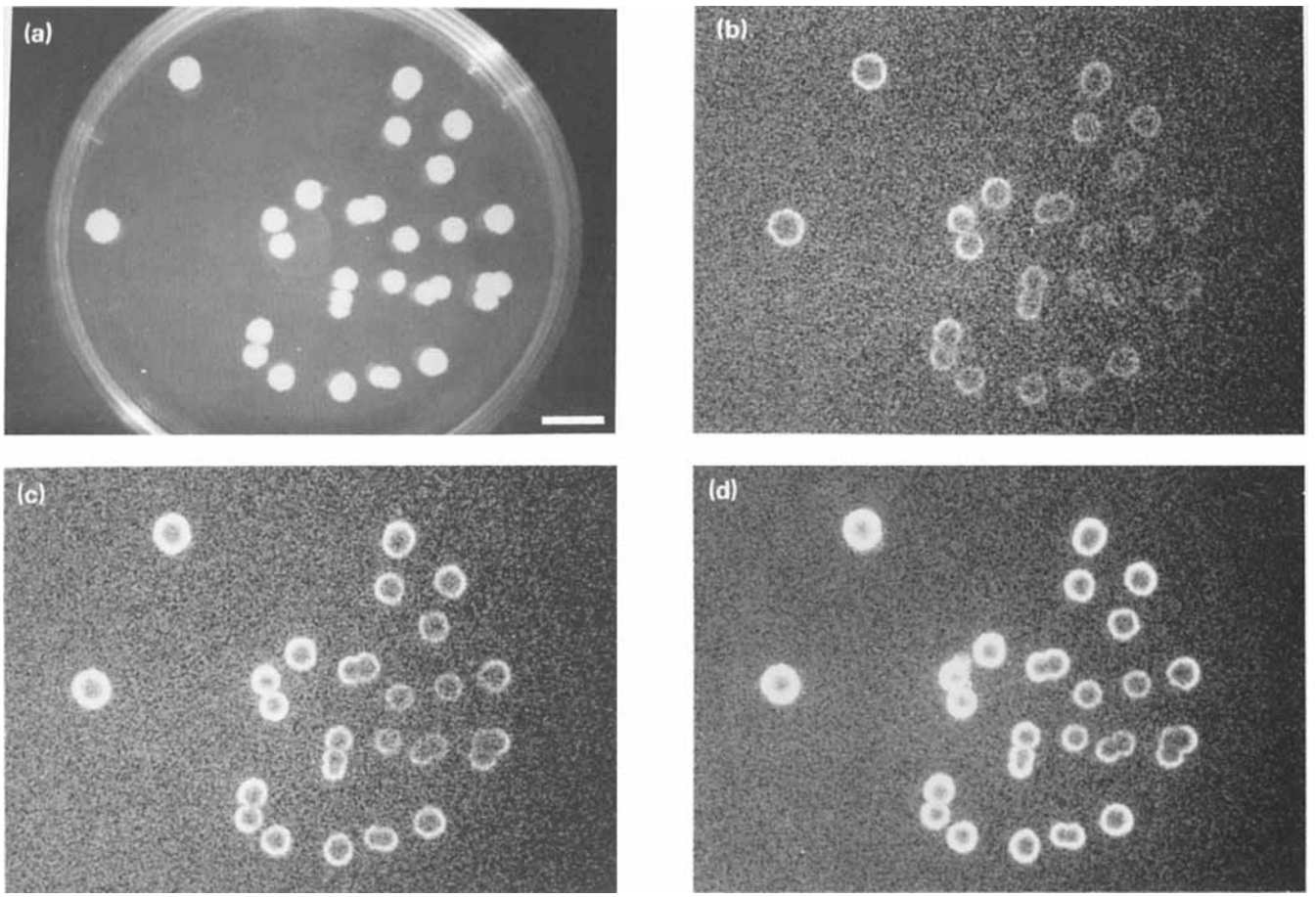

Fig. 5 Visualization of *Bacillus subtilis* 3610R pOTH951 colonies on LB agar (plus 40 μg ml^{-1} chloramphenicol) plates by charged-coupled device camera. (a) 0·1 s exposure in bright field; (b) 1 s exposure in dark field; (c) 10 s exposure in dark field; (d) 30 s exposure in dark field

DISCUSSION

The bioluminescent *B. subtilis* constructs described here have been designed for use in experiments involving the assessment of the potential for survival of, and gene transfer into the indigenous population from both plasmid-bearing and chromosomally marked micro-organisms introduced into soil. Among terrestrial micro-organisms, bioluminescence is possessed only by certain species of fungi (Campbell 1989) and a bacterial nematode symbiont (Schmidt *et al.* 1989). Consequently, there is no significant background amongst natural soil micro-organisms which could mask the detection of novel bioluminescent bacterial strains.

Bacillus subtilis strains carrying plasmid-encoded *lux* genes have been used in previous studies. Carni *et al.* (1987) used *lux*-fusion vectors to study the activity of various promoters but levels of light emission were low, while the *lux*-bearing promoter probe vectors constructed by Karp (1989) were structurally unstable in *B. subtilis*. Jacobs *et al.* (1991) constructed plasmids which provided high levels of luminescence but these were based on the vector pUB110 and such constructs can also be affected by structural instability (Ehrlich *et al.* 1986) and the *lux* genes were expressed from a promoter which required induction. The *veg*II promoter used in constructs described in this paper is derived (Peschke *et al.* 1985) from a gene which is transcribed throughout growth and sporulation (Ollington *et al.* 1981) and the plasmid construct used, pOTH951, is based on a vector, pHV1431 (Janniere *et al.* 1990), which is structurally stable in *B. subtilis*. Plasmid pOTH951 is, however, subject to segregational instability. The rifampicin-resistant *B. subtilis* 3610R was therefore chosen as its host so that, with potential loss of the plasmid in soil microcosm experiments (in which there was no antibiotic selection), some discrimination of the introduced *B. subtilis* cells may be allowed by the chromosomally-conferred rifampicin resistance. The loss of plasmid pOTH951

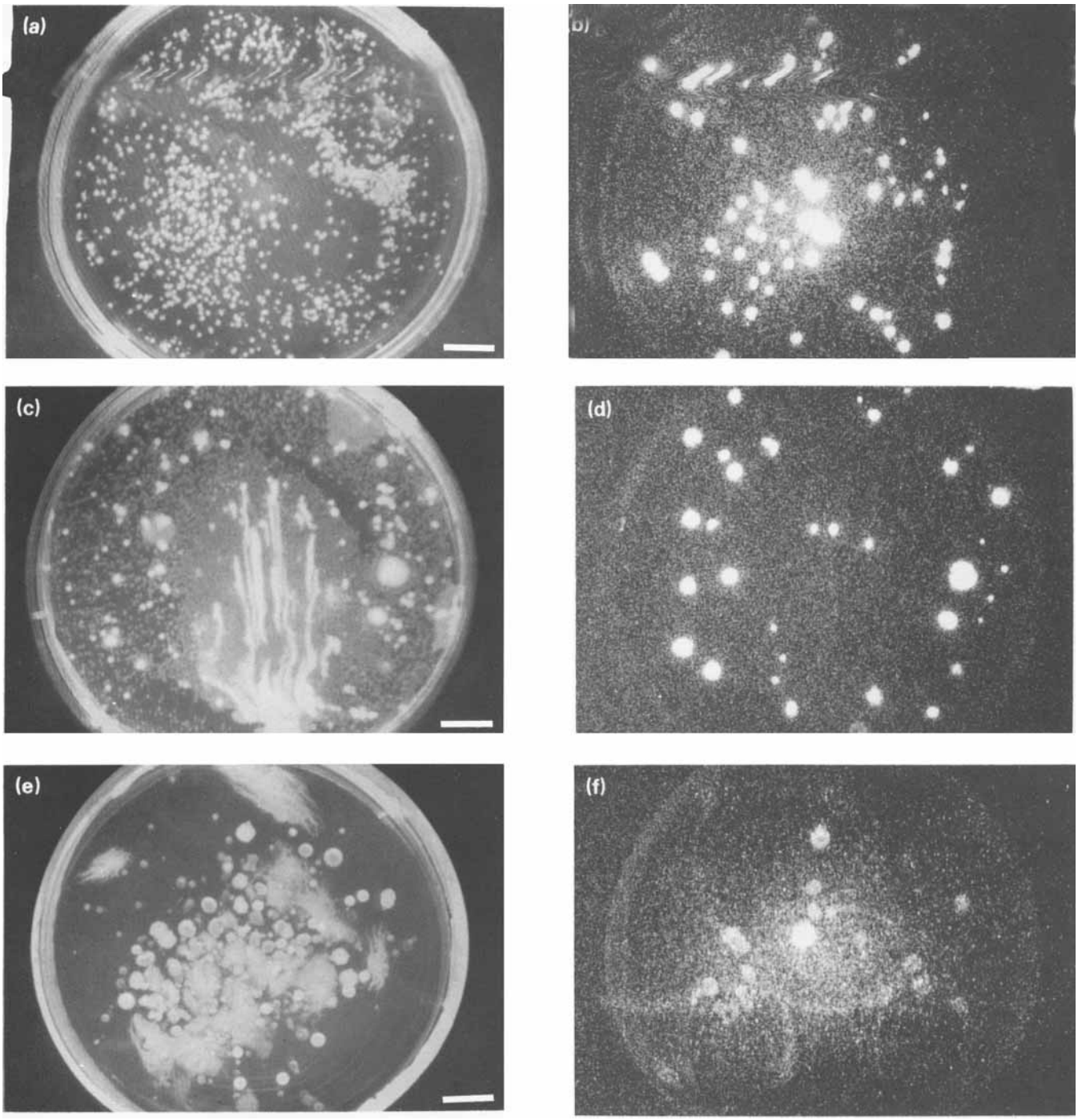

Fig. 6 Identification of *Bacillus subtilis* strains 3610R pOTH951 (a, b), VIN (c, d) and PBC11 (e, f) colonies among indigenous soil microbial colonies on LB agar plates (plus 40 μg ml^{-1} chloramphenicol for strain 3610R pOTH951). (a), (c) and (e) 0·1 s exposure in light field; (b) 30 s exposure; (d) 15 min exposure; (f) 30 min exposure in dark field. Scale bar represents 1 cm

during sporulation and germination is probably a consequence of partitional instability and may be an interesting aspect for further studies of plasmid stability under varying environmental conditions.

Ribosomal RNA gene sequences were chosen as the target of *lux* gene integration, as there are approximately 10 copies of the rRNA operon in the *B. subtilis* chromosome (Bott *et al.* 1984). It was considered that inactivation of one operon would not be detrimental to the cell. The chromosomally bioluminescent *B. subtilis* strains VIN and PBC11

were identical to the wild-type parent *B. subtilis* NCTC 3610 as regards growth requirements and sporulation proficiency. The maximum specific growth rates of PBC11 and 3610 were identical and, although the growth rate of VIN was somewhat less than that of the parent strain, subsequent studies have shown that it is no less viable after introduction into soil. The bioluminescent phenotype of these strains was retained through sporulation and germination, thus providing a reliable and constant means of identification as long as the strains survive within the environment.

The high sensitivity of the CCD camera enables visualization of individual bioluminescing cells of *Pseudomonas syringae* (Silcock *et al.* 1992). As Figs 4–6 demonstrate, CCD imaging also provides a precise and effective technique for distinguishing *lux*-marked colonies from other colonies which may have a similar morphology, even if they considerably outnumber the luminescent colonies on the plate. The facility of visualization of luminescent colonies by the CCD camera indicates the potential for rapid analysis of environmental samples, which would be desirable during experiments monitoring survival or gene transfer where large numbers of plates must be examined.

In conclusion, this study demonstrates the use of bioluminescence-based marker systems for the detection of *Bacillus* strains inoculated into soil, facilitating assessment of risks associated with environmental release of genetically modified Gram-positive micro-organisms.

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