

Radio-responsive recA promoter significantly increases TNF alpha production in recombinant clostridia after 2 Gy irradiation

Citation for published version (APA):

Nuyts, S., Van Mellaert, L., Theys, J., Landuyt, W., Bosmans, E., Anne, J., & Lambin, P. (2001). Radio-responsive recA promoter significantly increases TNF alpha production in recombinant clostridia after 2 Gy irradiation. *Gene Therapy*, 8(15), 1197-1201. <https://doi.org/10.1038/sj.gt.3301499>

Document status and date:

Published: 01/08/2001

DOI:

[10.1038/sj.gt.3301499](https://doi.org/10.1038/sj.gt.3301499)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

Taverne

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.



RESEARCH ARTICLE

Radio-responsive *recA* promoter significantly increases $TNF\alpha$ production in recombinant clostridia after 2 Gy irradiation

S Nuyts^{1,2}, L Van Mellaert¹, J Theys¹, W Landuyt², E Bosmans³, J Anné¹ and P Lambin⁴

Laboratories of ¹Bacteriology, Rega Institute, Katholieke Universiteit Leuven, and ²Experimental Radiobiology, University Hospital Gasthuisberg, Leuven; ³DiaMed EuroGen, Tessenderlo, Belgium; and ⁴Department of Radiation Oncology, RTIL, Academic Hospital Maastricht, University Maastricht, Maastricht, The Netherlands

One of the major problems with gene therapy today is the lack of tumour specificity. The use of anaerobic apathogenic clostridia as a gene transfer system can target anoxic areas within the tumour. These bacteria can be genetically modified to express therapeutic proteins such as $TNF\alpha$ locally in the tumour. As shown in our results, ionising irradiation can be used in clostridia to activate genes encoding cytotoxic agents under control of a radiation-inducible promoter. A 44% significant increase ($P < 0.05$) in $TNF\alpha$ secretion was seen 3.5 h after a single dose of 2 Gy. A second dose of 2 Gy was also capable of repeating gene activation and gave

a significant increase of $TNF\alpha$ production of 42% ($P < 0.05$). These results provide evidence that spatial and temporal control of gene expression can be achieved using a radio-inducible promoter. Repetitive gene activation was feasible with a second dose of 2 Gy, indicating that fractionated radiotherapy could lead to repeated gene induction resulting in prolonged and enhanced protein expression. Gene targeting by ionising radiation could thus provide a new means of increasing the therapeutic ratio in cancer treatment. Gene Therapy (2001) 8, 1197–1201.

Keywords: Clostridium; radio-inducible promoter; radiotherapy; *recA*; $TNF\alpha$

Introduction

Approximately 30% of cancer-related deaths are caused by local tumour failure suggesting that improving local control has the potential to improve the survival of one-third of all cancer patients. Many strategies to improve local tumour control are currently under investigation. Promising strategies seem to be those that combine existing therapeutic modalities with new developments including combining ionising irradiation with gene therapy.^{1–9}

This combination appears particularly promising as therapeutic genes can be chosen which have a radiosensitizing effect, thereby improving local tumour eradication. An example of this is the gene encoding the cytokine $TNF\alpha$. This protein has direct cytotoxic effects on some tumour cells,¹⁰ can activate a cellular immune response¹¹ and can cause destruction of tumour microvasculature.¹² Moreover, $TNF\alpha$ shows synergistic or additive cell killing in combination with radiation.¹³

A major obstacle in gene therapy is the specific targeting of therapeutic products to the tumour cell while concurrently leaving normal tissue unaffected. Tumour-specific gene expression can be achieved either by targeting the delivery system (transductional targeting) or

by limiting expression of the gene to the tumour cells (transcriptional targeting). One way to address the latter strategy is the use of radiation-inducible promoters to spatially and temporally target gene expression.^{1–9} For example, when the radio-responsive *Egr1* promoter was used to regulate expression of $TNF\alpha$ in human xenografts, an increase in tumour cure was noted without increasing normal tissue toxicity.^{2,3}

The combination of gene therapy with radiotherapy thus appears to be a promising approach to increase the therapeutic ratio of cancer therapy. However, an important limitation in this strategy remains the lack of specificity of the vectors used to deliver the therapeutic gene to the tumour.

In our laboratory, we use strictly anaerobic apathogenic *Clostridium* spp. to deliver the therapeutic proteins to the tumour. When *Clostridium* spores are injected intravenously into a tumour-bearing animal, a selective colonisation of hypoxic/necrotic tissues, with more than 10^8 CFU/g tumour is obtained.¹⁴ Moreover, the bacteria can be genetically modified to produce and secrete therapeutic proteins.^{14–18} To further increase the specificity of cytotoxic protein delivery and to achieve temporal control of protein expression, we have investigated the use of prokaryotic radio-induced promoters to control gene expression in *Clostridium*.^{19,20} Via the use of a reporter gene, we demonstrated that the *recA* gene, belonging to the SOS-repair system in bacteria, is induced by ionising irradiation.

In this report, we investigated whether ionising

Correspondence: S Nuyts, Department of Experimental Radiobiology/Bacteriology, Rega Institute, Minderbroedersstraat 10, 3000 Leuven, Belgium

Received 2 March 2001; accepted 23 May 2001

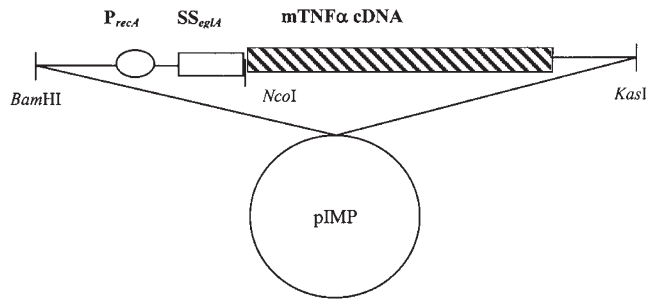


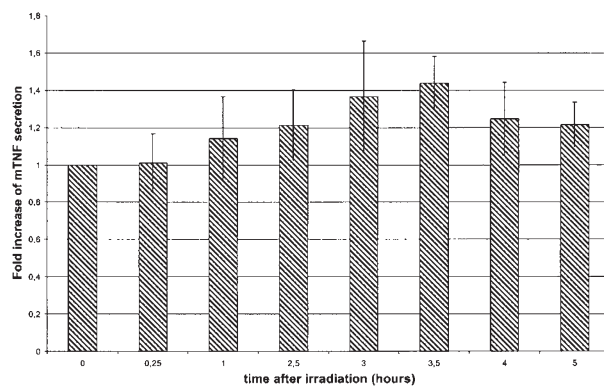
Figure 1 The shuttle vector pIMP contains mTNF α under regulation of the *recA* promoter. Schematic representation of the pIMP-*recA*-mTNF α shuttle vector. *P_{recA}*, promoter region of *recA*; *SS_{eglA}*, signal sequence of *eglA*. Restriction sites are indicated.

irradiation could increase the production of TNF α in *Clostridium* in the case of TNF α -cDNA placed under the control of the *recA* promoter. We measured the amount of TNF α production after single-dose irradiation and tested if gene activation could be repeated with a second radiation dose.

Results

ELISA analysis was used to quantify TNF α secretion by recombinant clostridia containing mTNF α -cDNA on the shuttle plasmid pIMP (Figure 1). Figure 2 shows the induction of TNF α production in recombinant clostridia containing the pIMP-*recA*-TNF α vector after a single dose of 2 Gy. The data are expressed as fold increase in secretion compared with unirradiated bacteria containing the same plasmid (see Materials and methods).

A single dose of 2 Gy showed a 1.44 (± 0.15 s.d.) fold increase of TNF α production compared with unirradiated bacteria ($P < 0.05$, Student's *t* test, two-paired samples for means). This maximum induction of 44% was observed 3.5 h after irradiation and declined thereafter. At earlier time intervals, no significant increase in TNF α



Induction factor	1.00	1.01	1.14	1.21	1.37	1.44	1.25	1.21
\pm standard deviation	± 0.00	± 0.16	± 0.22	± 0.19	± 0.30	± 0.15	± 0.19	± 0.12

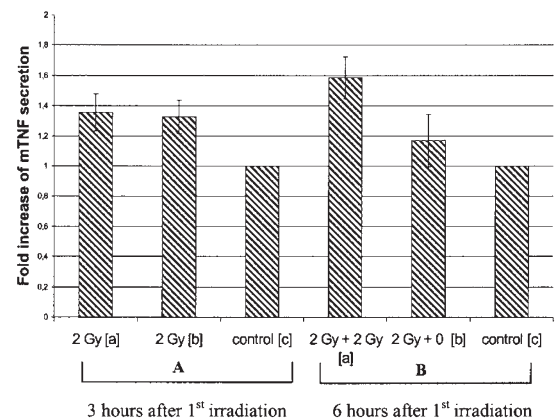
Figure 2 Induction of mTNF α in recombinant clostridia after a single dose of 2 Gy. Fold increase of mTNF α secretion in *Clostridium acetobutylicum* DSM792 pIMP-*recA*-mTNF α 15 min, 1 h, 2.5 h, 3 h, 3.5 h, 4 h and 5 h after a single dose of 2 Gy. The bars represent data from three independent experiments. Vertical bars represent standard deviation. Induction factors and standard deviations are represented in the table.

secretion was seen, but there was a trend to increase secretion reaching a significant level after 3.5 h. These data demonstrate that the *recA* promoter can give a significant increase of TNF α production by recombinant clostridia after a single dose of 2 Gy.

In patient treatments, radiotherapy is typically given in multiple fractionated doses. Therefore, we examined if the radio-inducible promoter could be reactivated with a second irradiation. The data presented in Figure 3A represent the induction of TNF α 3 h after a first dose of 2 Gy. A 1.33 to 1.36 (± 0.11 – 0.12 s.d.)-fold increase was seen, which corresponds with the 37% increase in TNF α production seen in Figure 2. TNF α induction 3 h after a second dose of 2 Gy for sample [a] and a mock irradiation for sample [b] is shown in Figure 3B. The higher level of TNF α production seen after 2 \times 2 Gy fractions in comparison to 1 \times 2 Gy, indicated that the promoter can be reactivated at a clinically relevant dose. Overall, two fractions of 2 Gy resulted in a 1.59 (± 0.14 s.d.)-fold increase in TNF α production which was significant, compared with no irradiation ($P < 0.02$, Student's *t* test) and with a single dose of irradiation (1.17 (± 0.18 s.d.)-fold increase), ($P < 0.05$, Student's *t* test). The 17% increase in sample [b] (Figure 3B) is the remaining induction effect from the first irradiation indicating that at this time interval, an induction of TNF α secretion as a result of the first irradiation remains. These data indicate that the *recA* promoter in *Clostridium* can be reactivated by a second dose of radiotherapy.

Discussion

Several previous studies have employed radiotherapy to spatially and temporally control gene expression using the *Egr1* promoter in almost each case.^{1–9} However, in



Induction factor	1.36	1.33	1.00	1.59	1.17	1.00
\pm standard deviation	± 0.12	± 0.11	± 0.00	± 0.14	± 0.18	± 0.00

Figure 3 Induction of mTNF α in recombinant clostridia after 2 \times 2 Gy. (A) Fold increase of mTNF α secreted by *C. acetobutylicum* 3 h after a first dose of 2 Gy ([a]) and [b]). (B) Fold increase of mTNF α secreted by *C. acetobutylicum* after a second dose of 2 Gy ([a]) in comparison with one dose of 2 Gy ([b]), 6 h after the first irradiation, 3 h after the second. Vertical bars represent standard deviation. Induction factors and standard deviations are represented in the table. All data are the result of three independent experiments.

most studies induction of gene expression is achieved using total doses of 20–50 Gy sometimes using fractions up to 5 Gy.^{3,12} Since a dose of 5 Gy is not used in a curative treatment setting, this high dose to achieve induction can be a limiting factor. Since the eukaryotic *Egr1* promoter is not functional in bacteria, we investigated the use of a prokaryotic radio-inducible promoter. In our study, significant induction of the *recA* promoter is achieved at only 2 Gy, which makes it more relevant for clinical use. A single dose of 2 Gy gives an increase of TNF α secretion of 44% 3.5 h after radiotherapy (Figure 2).

However, patients are not treated with a single dose of irradiation but with fractionated radiotherapy. This daily repeated small irradiation dose results in better tumour control for a given level of normal tissue toxicity than a single large dose. If fractionated radiotherapy is used, the first dose produces an increase of TNF α secretion of 33 to 36% 3 h after 2 Gy and an increase of 59% 3 h after a second dose of 2 Gy. Both inductions are statistically significant ($P < 0.05$ and $P < 0.02$, respectively). If we look at sample [b] which received a first dose of 2 Gy but did not receive a second irradiation, we see that at the second sample point, 6 h after the first dose (Figure 3B) there is still a 17% increase of extracellular TNF α activity, although not significant. This implies that of the 59% induction we measured after the second irradiation (sample [a]), 42% is the actual result of repeated gene activation and the residual 17% is still the effect of the first irradiation. This 42% induction after a second dose is in the range of induction values of 33–36% (± 11 –12%) obtained after the first dose.

These data provide the proof of principle that the *recA* promoter in *Clostridium* can be efficiently reactivated by a second dose of radiotherapy and that the degree of induction remains more or less the same as compared with single dose irradiation.

This will be important in achieving controlled high levels of therapeutic gene expression in the clinical setting. In patient treatments, daily radiation fractions would be capable of activating the radio-inducible promoter leading to a daily increase of TNF α of 44% locally in the tumour.

There is, however, a rapid decline in promoter activity. A maximum level of induction is achieved after 3.5 h, which drops back to half after 6 h. When we compared these kinetics with those of the *Egr1* promoter, the kinetics are quite similar.⁴ Joki *et al*⁴ measured a maximum induction 1–3 h after a single dose of 20 Gy, and this induction dropped to about 50% after 6 h, and to basal level after 12 h. Notwithstanding these rapid promoter kinetics, they proved a therapeutic benefit *in vitro* when using their radio-induced promoter.

Unirradiated clostridia produce TNF α because of basal activity of the *recA* promoter. Maximum levels of 6200 pg/ml TNF α were measured in untreated samples. This basal activity can be reduced by inserting an additional Cheo box in the promoter region.²¹ After irradiation of the recombinant clostridia, concentrations of 8800 pg/ml of mTNF α can be achieved (data not shown). Previous studies have reported that doses of hTNF α ranging between 10 and 1000 U/ml are cytotoxic to human tumour cell lines.¹³ Since mTNF α has a specific activity of about 10⁸ U/mg (data not shown), the concentration of 8800 pg/ml corresponds to 880 U/ml. This concentration should be sufficiently high enough to result in a cytotoxic

effect on human tumour cells indicating that *Clostridium* is capable of producing sufficient amounts of TNF α to have an antitumoural effect.

In vitro and *in vivo* experiments are planned to prove that an additional increase in TNF α of 44% can lead to an increase in cell kill. However, when we look at some dose–response curves for TNF α , we can expect that an increase in TNF α concentration of 44% can lead to an increase in cell kill.⁵

Spatial and temporal control of gene expression by ionising irradiation is a relatively new and promising concept. The system we describe exploits the benefits of a tumour-specific vector in combination with radiotherapy which triggers and increases gene expression. Since therapeutic agents such as TNF α can be highly toxic, temporal control of protein expression can be very beneficial. In patient treatments, physicians will know from what time-point TNF α will be present in the tumour. TNF α will be expressed locally in the tumour, mainly in the hypoxic regions. However, TNF α shows a reduced cytotoxicity to hypoxic cells, because the mechanism of direct cytotoxicity may involve hydroxyl radical production.²² However, since TNF α is a small and compact molecule, it will diffuse and will also reach the more oxygenated cells. Moreover, in addition to a direct cytotoxic effect, TNF α also has an indirect antitumour effect. In combination with irradiation, TNF α causes occlusion of tumour microvessels, without significant normal tissue damage.¹² TNF α gene therapy targeted by ionising radiation results in an amplified bystander effect, resulting in extensive necrosis distal to thrombosed tumour vessels.

Fractionated radiotherapy can provide a method to repeat gene induction resulting in enhanced and prolonged gene expression. Since anaerobic bacteria like *Clostridium* may colonise other hypoxic/necrotic tissues besides tumoral, such as for instance abscesses and infarcted tissues, spatial control of gene expression would be advantageous. Further benefiting the treatment plan, *Clostridium* can be completely eradicated by the administration of antibiotics,²³ suggesting that complete control of gene expression can be obtained; radiation will switch it on, and, if necessary, antibiotics can switch it off.

The use of a prokaryotic radio-induced promoter to temporally and spatially control gene expression is not only limited to *Clostridium*-mediated therapy for cancer. Recently, genetically engineered *Salmonella*^{24,25} and *Bifidobacterium longum*²⁶ have shown that these bacteria also have the desirable properties of an antitumour vector. Both strains provide selective colonisation of solid tumours and can express proteins. Since the SOS-repair system in bacteria is highly conserved, the *recA* promoter can probably act as a radio-induced promoter in other bacteria to control gene expression after radiotherapy. The combination of radiotherapy, which preferentially kills well-oxygenated cells, with *Clostridium* (or analogous)-mediated protein delivery, which targets the hypoxic cells, provides new possibilities for future cancer therapy.

Materials and methods

Bacterial strains, media and culture conditions

Clostridium acetobutylicum DSM792 was grown in 2 \times YT medium²⁷ at 37°C in an anaerobic incubator (model 1024;

Forma Scientific, Marietta, OH, USA) with 90% N₂ and 10% H₂ and palladium as the catalyst.

For primary vector constructions, *Escherichia coli* TG1²⁸ was used. This strain was grown in Luria-Bertani broth at 37°C. *E. coli* ER2275 was used for *in vivo* methylation of plasmid DNA before electroporation of *C. acetobutylicum*.²⁹ The *E. coli*/*C. acetobutylicum* shuttle plasmid pIMP was used as cloning vector.³⁰ Media were supplemented, when applicable, with erythromycin (25 µg/ml) or ampicillin (50 µg/ml).

Plasmid construction, transformation procedures

The *recA* promoter was isolated as previously described.²⁰ The mTNFα cDNA was available on plasmid pIG2mTNF (Innogenetics, Gent, Belgium). The signal sequence of the *eglA* promoter was used to obtain secretion of TNFα.¹⁵

The promoter and signal sequence were cloned upstream of the mTNFα cDNA after introducing unique *NcoI* and *KasI* restriction sites in the pIMP vector using the 'Quickchange Site-directed Mutagenesis kit' (Stratagene, La Jolla, CA, USA) (Figure 1).

E. coli was transformed using chemically competent cells obtained with the RbCl method. Transformation of *C. acetobutylicum* DSM792 was carried out as recently published.³¹

All general DNA manipulations in *E. coli* were carried out as described by Sambrook *et al.*²⁸

Irradiation

Bacteria were grown until early log phase (OD_{600nm} = ± 0.3). At this time-point cultures were divided into two sets, one of which was exposed to radiation while the other was mock-irradiated and used as a control. Bacteria were exposed to 2 Gy with a ⁶⁰Cobalt unit at a dose rate of 0.9 Gy/min. After irradiation, bacteria were incubated anaerobically at 37°C and samples were taken at different time intervals after exposure.

For repetitive gene activation, bacteria were grown to early log phase. Cultures were then divided into three flasks and exposed to 2 Gy (flasks [a] and [b]) or mock-irradiated (flask [c]). Three hours after radiation, the culture was centrifuged (10 min, 10000 r.p.m., 4°C) and supernatant was taken for analysis. An interval of 3 h after irradiation was chosen because at this time-point the highest degree of induction was seen. Because in batch culture bacteria already reach stationary phase after ± 5 h, repetitive gene activation could not be tested at greater time intervals. Therefore, we chose to resuspend the bacteria in fresh medium to have actively dividing bacteria. Similarly, in the clinical setting, bacteria gain a continuous supply of nutrients which will result in a continuous growth of viable cells. The pellet was resuspended in fresh 2 × YT medium and allowed to regrow for 40 min. Flask [a] was irradiated a second time with 2 Gy, while flasks [b] and [c] were mock-irradiated. Again, 3 h after irradiation and incubation at 37°C, cultures were centrifuged and supernatant was sampled for analysis.

Each experiment was independently repeated three times.

Analysis of mTNFα production

The amount of TNFα secreted by recombinant clostridia was quantified using ELISA. Supernatant taken from irradiated and non-irradiated cultures was diluted 10-

fold in phosphate-buffered saline plus 7.5% bovine serum albumin and 100 µl aliquots were put in a 96-well microtiter plate. Further manipulations were done according to the manufacturer's protocol (DiaMed EuroGen, Tessenderlo, Belgium).

Concentrations of secreted mTNFα were calculated and compared for the irradiated and non-irradiated cultures. The level of radio-induced TNFα production was expressed as fold increase in mTNFα concentration of irradiated samples compared with the corresponding non-irradiated samples.

Acknowledgements

We acknowledge the financial support from 'Het Fonds voor Wetenschappelijk Onderzoek-Vlaanderen', 'Verkennde Internationale Samenwerking' and 'Het KU Leuven Onderzoeksfonds'. Sandra Nuyts is research fellow of 'IWT' (Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch onderzoek in de industrie). We wish to thank Raf Berghmans, DiaMed EuroGen, Tessenderlo, Belgium for providing the ELISA kits.

References

- Weichselbaum RR, Hallahan DE, Sukhatme VP, Kufe DW. Gene therapy targeted by ionizing irradiation. *Int J Radiat Oncol Biol Phys* 1992; **24**: 565–567.
- Weichselbaum RR *et al.* Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res* 1994; **54**: 4266–4269.
- Hallahan DE *et al.* Spatial and temporal control of gene therapy using ionizing irradiation. *Nate Med* 1995; **1**: 786–791.
- Joki J, Nakamura M, Ohno T. Activation of the radiosensitive *EGR-1* promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. *Hum Gene Ther* 1995; **6**: 1507–1513.
- Seung LP *et al.* Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer Res* 1995; **55**: 5561–5565.
- Takahashi T, Namiki Y, Ohno T. Induction of the suicide HSV TK gene by activation of the *Egr 1* promoter with radioisotopes. *Hum Gene Ther* 1997; **8**: 827–833.
- Manome Y *et al.* Transgene expression in malignant glioma using a replication-defective adenoviral vector containing the *Egr-1* promoter: activation by ionizing irradiation or uptake of radioactive iododeoxyridine. *Hum Gene Ther* 1998; **9**: 1409–1417.
- Kawashita Y *et al.* Regression of hepatocellular carcinoma *in vitro* and *in vivo* by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation. *Hum Gene Ther* 1999; **10**: 1509–1519.
- Marples B *et al.* Development of synthetic promoters for radiation-mediated gene therapy. *Gene Therapy* 2000; **7**: 511–517.
- Larrick JW, Wright SC. Cytotoxic mechanism of tumour necrosis factor-α. *FASEB J* 1990; **4**: 3215–3223.
- Old LJ. Tumour necrosis factor. *Science* 1985; **230**: 630–636.
- Staba MJ *et al.* Adenoviral TNF-α gene therapy and radiation damage tumor vasculature in a human malignant glioma xenograft. *Gene Therapy* 1998; **5**: 293–300.
- Hallahan DE, Beckett MA, Kufe D, Weichselbaum RR. The interaction between recombinant human tumor necrosis factor and radiation in 13 human tumor cell lines. *Int J Radiat Oncol Biol Phys* 1990; **19**: 69–74.
- Lambin P *et al.* Colonisation of *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumours. *Anaerobe* 1998; **4**: 183–188.
- Theys J *et al.* Stable *Escherichia coli*-*Clostridium acetobutylicum* shuttle vector for secretion of murine tumor necrosis factor alpha. *Appl Environ Microbiol* 1999; **65**: 4295–4300.

- 16 Theys J *et al*. Specific targeting of cytosine deaminase to solid tumors by engineered *Clostridium acetobutylicum*. *Cancer Gene Ther* (in press).
- 17 Fox ME *et al*. Anaerobic bacteria as delivery system for cancer gene therapy: *in vitro* activation of 5-fluorocytosine by genetically engineered clostridia. *Gene Therapy* 1996; **3**: 173–178.
- 18 Lemmon MJ *et al*. Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. *Gene Therapy* 1997; **4**: 791–796.
- 19 Nuyts S *et al*. Increasing specificity of anti-tumor therapy: cytotoxic protein delivery by non-pathogenic clostridia under regulation of radio-induced promoters. *Anticancer Res* 2001; **21**: 857–862.
- 20 Nuyts S *et al*. The use of radio-induced bacterial promoters in anaerobic conditions: a means to control gene expression in *Clostridium*-mediated therapy for cancer. *Radiat Res* 2001; **155**: 716–723.
- 21 Nuyts S *et al*. Manipulation radio-inducibility of *recA* promoter in *Clostridium*. *Appl Environ Microbiol* (submitted).
- 22 Zimmerman RJ, Chan A, Leadon SA. Oxidative damage in murine tumor cells treated *in vitro* by recombinant human TNF. *Cancer Res* 1989; **49**: 1644–1648.
- 23 Theys J *et al*. Improvement of *Clostridium* tumour targeting vectors evaluated in rat rhabdomyosarcomas. *FEMS Immunol Med Microbiol* 2001; **30**: 37–41.
- 24 Platt J *et al*. Antitumour effects of genetically engineered *Salmonella* in combination with radiation. *Eur J Cancer* 2000; **36**: 2397–2402.
- 25 Pawelek JK, Low KB, Bermudes D. Tumor-targeted *Salmonella* as a novel anticancer vector. *Cancer Res* 1997; **57**: 4537–4544.
- 26 Yazawa K *et al*. *Bifidobacterium longum* as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. *Cancer Gene Ther* 2000; **7**: 269–274.
- 27 Oultram J *et al*. Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. *FEMS Microbiol Lett* 1988; **56**: 83–88.
- 28 Sambrook JE, Fritsch EF, Maniatis T. *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989.
- 29 Mermelstein LD, Papoutsakis ET. *In vivo* methylation in *Escherichia coli* by the *Bacillus subtilis* phage Φ 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC824. *Appl Environ Microbiol* 1993; **59**: 1077–1081.
- 30 Mermelstein LD, Welker NE, Bennett GE, Papoutsakis ET. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC824. *BioTechnology* 1992; **10**: 190–195.
- 31 Nakotte S, Schaffer M, Böhringer M, Dürre P. Electroporation of plasmid isolation from and plasmid conservation in *Clostridium acetobutylicum* DSM792. *Appl Microbiol Biotech* 1998; **50**: 564–567 .