

Mini-review

Bacterial targeted tumour therapy-dawn of a new era

Ming Q Wei ^{a,b,*}, Asferd Mengesha ^c, David Good ^{a,b}, Jozef Anné ^d^a Department of Medicine, University of Queensland, Prince Charles Hospital, Brisbane, Qld 4032, Australia^b Division of Molecular Medicine and Gene Therapy, Griffith Institute for Health and Medical Research, G16, Gold Coast campus, Griffith University, Qld 4222, Australia^c Maastrro Lab, UNS50/23, University of Maastricht, Maastricht, The Netherlands^d Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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Abstract

Original observation of patients' spontaneous recovery from advanced tumours after an infection or a "fever" inspired extensive research. As a result, Coley's toxin for the therapy of sarcomas and live *Bacillus Calmette–Guerin* (BCG) for bladder cancer were born. In addition, three genera of anaerobic bacteria have been shown to specifically and preferentially target solid tumours and cause significant tumour lyses. Initial research had focused on determining the best tumour colonizing bacteria, and assessing the therapeutic efficacy of different strategies either as a single or combination treatment modalities. However, although clinical trials were carried out as early as the 1960s, lack of complete tumour lyses with injection of *Clostridial* spores had limited their further use. Recent progress in the field has highlighted the rapid development of new tools for genetic manipulation of *Clostridia* which have otherwise been a hurdle for a long time, such as plasmid transformation using electroporation that bore the problems of inefficiency, instability and plasmid loss. A new *Clostridium* strain, *C. novyi*-NT made apathogenic by genetic modification, is under clinical trials. New genetic engineering tools, such as the group II intron has shown promise for genetic manipulation of bacteria and forecast the dawn of a new era for a tumour-targeted bacterial vector system for gene therapy of solid tumours. In this review we will discuss the potential of genetically manipulated bacteria that will usher in the new era of bacterial therapy for solid tumours, and highlight strategies and tools used to improve the bacterial oncolytic capability.

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1. Brief history of using bacteria for the treatment of solid tumours

The original observation of tumour spontaneous recovery and regression of certain cancer patients

* Corresponding author. Address: Department of Medicine, University of Queensland, Prince Charles Hospital, Brisbane, Qld 4032, Australia. Tel.: +61 7 3139 4552; fax: +61 7 3359 2173.

E-mail address: d.wei@uq.edu.au (M.Q. Wei).

from concurrent bacterial infections was made over 300 years ago [1]. Since then, numerous case reports, review papers and book chapters on the subject have been published, a significant number of them were peer reviewed [2–7], including about 1000 cases by the year 1987 and mean 10 reports per year by 2003 [8].

As early as the year 1813, Vautier reported that tumours regressed in his patients, who had suffered

from gas gangrene. We know now that gas gangrene is caused by Clostridial infection [9]. In 1868, Busch was the first to undertake an effort, at a small scale, to inoculate erysipelas to treat cancer patients with inoperable tumours even before the streptococcal aetiology of erysipelas was known [10]. This effort was repeated by Fehleisen in 1882 [11] who eventually sorted out the aetiology of erysipelas was *Streptococcus pyogenes*. In 1890, William Coley, a New York physician took advantage of the previous work and started a large-scale effort trying to benefit his cancer patients. He began inoculating *S. pyogenes* to his patients with inoperable tumours [12]. Unfortunately, the results were not as effective as he expected [12,13]. To enhance the toxicity of *S. pyogenes*, he included a Gram-negative strain, *Serratia marcescens*. This inclusion ultimately turned out to be a crucial step towards the accomplishment of reproducible therapeutic effects in the clinic, a degree of success far beyond those who had gone before him [14,15]. It was this combination of Gram-positive, heat-killed *Streptococcus* plus the Gram-negative, heat-killed *S. marcescens* that we commonly refer to as “Coley’s toxins”, which were widely used in treating patients with sarcomas until 1963 [12–15]. This work played a major role in stimulating the emergence of the burgeoning fields of bacterial tumour therapy and tumour immunology.

In 1935, Connell used sterile filtrates from *Clostridium histolyticum* to treat advanced cancers and attributed the observed tumour regression to the production of proteolytic enzymes [16]. Thereafter, several strains of Clostridia have been tested extensively in animal models as well as human trials, but none showed significant therapeutic effects. Thus, the field of bacterial cancer therapy was stagnant until 1976, when Morales, Eidinger and Bruce reported on successful treatment of superficial bladder cancer (SBC) with bacillus Calmette–Guerin (BCG) [17]. Today, BCG has become the treatment of choice for high risk, superficial bladder cancer in most countries, with an increasing rate of treating approximately 1 million patients per year [18].

Recent understanding of the pathophysiology of solid tumours has shed more lights on the growth of solid tumours and their unique microenvironment. Most importantly, we start to dissect the associated difficulty in developing successful cancer therapies. All solid tumours undergo angiogenesis, as tumour cells grow much faster than cells making up the

blood vessel, which results in biological changes and adaptive metabolisms, i.e. formation of defective vessels, appearance of apoptotic and necrotic cancer tissues, and emergence of hypoxic areas and heterogeneous tumour cell populations [19].

In this review we will discuss the potential of genetically manipulated bacteria that will usher in the new era of bacterial therapy for solid tumours, and highlight the importance of novel genetic strategies to improve the bacterial oncolytic capability and genetic engineering tools to advance the efficiency of bacterial modification.

2. Tumour microenvironment creates an ideal condition for the growth of anaerobic and some facultative anaerobic bacteria

The unique solid tumour micro-milieu, though, provides a haven for anaerobic bacteria. Anaerobic and facultative anaerobes tested so far fell into three classes. (1) The lactic acid, Gram-positive anaerobic bacteria; (2) the intracellular, Gram-negative facultative anaerobes, and (3) the strictly anaerobic, Gram-positive saccharolytic/proteolytic Clostridia (Table 1).

The members of *Bifidobacterium* genus are Gram-positive, anaerobic, non-motile and non-sporulating. Three species were the focus of studies, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium adolescentis*. These are common flora of human intestine and have been used as probiotics and dairy fomenters for many years [20,21]. As these are commonly used as probiotic agents, we have regarded bifidobacteria as “safe bacteria” and studied for their efficacy in the prevention and treatment for a wide range of animal and human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer [20]. Bifidobacteria may exert protective intestinal actions through various mechanisms, and represent promising advances in the fields of prophylaxis and therapy. The initial “proof of concept” for the use of Bifidobacteria in cancer therapy was demonstrated in the 1980s when mice implanted with Ehrlich ascites tumours were treated with suspension of Bifidobacteria. Intravenous injection of 5×10^6 colony forming units (cfu) per mouse led to tumour colonisation (tumour size of 15 mm or bigger in diameter) [22]. The bacteria were shown to be highly selective and localised primarily within the tumour, with virtually no bacteria in other organs

Table 1

Three classes of anaerobic and facultative anaerobes have been tested for anticancer therapy

Class	Species	Features	Advantages	Disadvantages
Class I: Bifidobacteria	<i>B. longum</i>	Gram ⁺ non-motile	Non-pathogenic present in common	No obvious oncolytic effect
	<i>B. adolescentis</i> <i>B. infantis</i>	obligate anaerobes	intestinal flora, Have been used in human for many years Probiotic bacteria Can be used for intravenous or oral administration Expression of recombinant protein	Non-spore former More susceptible to non-permissive conditions More difficult to store and handle
Class II: Facultative intracellular Bacteria	<i>Salmonella</i>	Gram ⁻ facultative anaerobes	Attenuated vaccine strain has been proved safe clinically in human, Biochemistry pathways and genomes are well characterized	Intracellular bacteria, thus may have difficulty to infect and lyse quiescent cell
	<i>S. typhimurium</i>	Agent for intestine infection	Auxotrophic isolates for solid tumours have intrinsic antitumour activity	Have a tumour to normal tissue ratio of 1000:1, therefore a significant number of bacteria colonize normal organs Cell wall components are immunogenic
	<i>Listeria</i> <i>L. monocytogenes</i> <i>E. coli</i>	Gram ⁺ , facultative anaerobes Gram ⁻ , facultative anaerobes	Grow under aerobic and anaerobic conditions, thus can target both large and small tumours, enter professional antigen presenting cells and induce strong innate immune response Have the potential as a vaccine vector for tumour therapy Biology is well studied and known	Virulence factors exist, especially LPS in the bacterial cell wall, thus safety is an issue when large amount of bacteria are delivered Virulence factors exist, such as LPS
Class III: Strictly Anaerobic bacteria	<i>Clostridium</i>	Gram ⁺ , strictly anaerobes	Spore former	Some strains are pathogenic
	Proteolytic		Spores are stable, easy to produce and economic to use	Some strains are difficult to manipulate genetically
	<i>C. sporogenes</i>	normal habitat in the soil, aquatic sediments, and intestinal tract of	Clostridial spores can be delivered non-invasively and systemically, i.e. intravenous injection	Only colonize in large tumours with area of hypoxia/necrosis
	<i>C. novyi</i>	both animals and humans		Oncolysis interrupted at the rim causing incomplete tumour lysis
	<i>C. butyricum</i>		Have shown extensive oncolytic ability	
	<i>C. acetobutylicum</i>		Spores are non-immunogenic and can be repeatedly delivered	
	<i>C. oncolyticum</i> <i>C. beijerinckii</i>		Oncolysis occurs irrespective of tumour cells' heterogeneity or growth status	

was found after 96 h following injection. Bacteria within the tumour at 1 h were present at 10^2 cfu/g. tumour tissues, which increased to 10^6 cfu/g. by day 7. However, no antitumour effects were documented in this study [22]. Subsequent study also demonstrated that *B. adolescentis* markedly prevented the occurrence and development of colorectal carcinoma and induced tumour apoptosis *in vivo* in animal models [23]. The shortcomings of using Bifidobacteria for cancer therapy are several, primarily due to the facts that they are non-spore formers, and more susceptible to non-permissive conditions, thus more difficult to store and handle. Current studies are primarily in China

and Japan using Bifidobacterium as a gene delivery vehicle [23].

Apart from the obligate anaerobic non-sporulating Bifidobacteria, the Gram-negative facultative anaerobic *Salmonella typhimurium* has also been exploited as a potential anticancer agent and offered an alternative approach to selectively target solid tumours. Their tumour colonization properties were known half a century ago [24,25]. However, most *Salmonella* strains are pathogenic due to substantial immunostimulation produced by *Salmonella* lipopolysaccharide (LPS) and other virulence factors. To overcome the pathogenicity, *S. typhimurium* was genetically attenuated by deleting *purI* and

msbB. The latter gene is responsible for the terminal myristalization of lipid A [26]. Genetic disruption of this gene results in a stable non-conditional mutation which lowers TNF α induction, thereby minimizing the risk for a subsequent septic shock. The deletion of *purI* creates a requirement for an external source of adenine. The low toxicity profile of the *purI*[−], *msbB*[−] strain has been demonstrated in different animal models. The potential of this strain to function as antitumour agent was first reported by Pawelek in 1997, in a study where attenuated *S. typhimurium* injected into tumour-bearing animals were shown to replicate preferentially in tumours [26]. In addition to selective tumour accumulation, its inherent antitumour activity was demonstrated in a number of tumour models. Attenuated *Salmonella* has been further modified to express a variety of genes coding for therapeutic proteins such as cytosine deaminase (CD), tumour necrosis factor (TNF α), mitomycin C, herpes simplex virus thymidine kinase (HSV-tk) and colicin E3 [27–31]. Mice treated with such recombinant strains demonstrated remarkable tumour regression compared to the wild type. Recently, attenuated *Salmonella choleraesuis*, capable of preferentially colonizing and amplifying within tumours was reported as an anticancer agent [32]. This vaccine strain of *S. choleraesuis* is capable of delaying tumour growth and enhancing survival in both subcutaneous tumour and experimental metastatic models. Furthermore, this strain was shown to transfer gene encoding therapeutic protein at the tumour site using eukaryotic expression vector [33,34]. Double auxotroph mutant strain of *Salmonella* (*S. typhimurium* A1), which has a requirement for amino acids Leu and Arg, developed to investigate tumour targeting, have also proved very successful at tumour inhibition and regression [35]. Analogous to anaerobes, *Salmonella* have also been used in conjunction with other anti-cancer agents such as chemotherapy and radiotherapy. Such combination therapy appeared to result in better tumour control and/or supra-additive antitumour effects [36].

So far two clinical trials have been reported since the development of attenuated *Salmonella* as a tumour targeting agent [37,38]. The first phase I clinical trial consisted of 26 patients with either metastatic melanomas or metastatic renal cell carcinoma [37]. The results established a maximum tolerated dose of 3×10^8 cfu/m². Dose related elevated level of pro-inflammatory cytokines such as IL1 β , TNF α and IL6 in the circulation was

observed that are associated with clinical toxicity in patients receiving 10^9 cfu/m². However, no patients exhibited tumour regression and only three patients had tumours containing viable bacteria. The unexpected feature of this trial was the failure of *Salmonella* to fully colonize human tumours, unlike the observation made with murine models, in which bacterial distribution appeared to be throughout the tumour tissue. This may indicate that the factors that allow efficient tumour colonization may differ among species and needs to be determined cautiously. Based on clinical data generated with the parent strain, another pilot clinical trial was conducted in 2003. This study investigated an intratumoural injection of attenuated *Salmonella* expressing the *Escherichia coli* CD gene (3×10^6 – 3×10^7 cfu/m²) in three refractory cancer patients, followed by administration of 5-fluorocytosine. No significant adverse events related to the treatment were reported. Two patients had evidence of bacterial colonization of the tumour that persisted for at least 15 days after the initial injection. Conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) as a result of CD expression was demonstrated in these two patients [38]. However, both clinical studies failed to demonstrate significant clinical efficacy, presumably due to insufficient tumour colonization. Other shortcomings of using *Salmonella* for cancer therapy are that their cellular walls containing LPS and their endotoxins can be rather toxic to humans and they are facultative, thus also colonise non-tumour tissues in a ratio of tumour to normal tissue 1000:1, likely causing side-effects. However, although *S. typhimurium* does not specifically target hypoxic regions, they can be modified as such that they are attracted to essential metabolites. Thus, the targeting mechanisms of facultative anaerobes can be fine-tuned, and developed to be used to prevent metastases [39,40].

The third genus investigated as a tumour targeting vector system is the strictly anaerobic, sporulating Gram-positive bacteria represented by *Clostridium*. This genus is one of the largest genera comprising of about 80 species. Up to 10 species of Clostridia have been studied and as strictly anaerobic bacteria they all require an anaerobic environment to grow but their oxygen tolerance and biochemical profile varies considerably among different species. Clostridial spores had been used to induce tumour lysis following intravenous delivery and shown a distinct advantage over *Bifidobacterium* and *Salmonella* in terms of easy production, hardy storage and impres-

sive oncolytic effects [41]. Both proteolytic and saccharolytic Clostridia have been tested for cancer therapy.

3. Clostridial spores for oncolytic therapy

Parker and colleagues [42] were the first to show Clostridial oncolysis (they called it liquefaction at the time) and tumour regression in mouse tumours by injecting Clostridial spore suspension into transplanted mouse sarcomas. The exquisite tumour selectivity of the system was further demonstrated by Malmgren and Flanigan [43] who intravenously administered *Clostridium tetani* spores to tumour-bearing mice resulting in death from tetanus toxin within 48 h with no apparent toxicity to healthy control animals without tumour. This observation indicated that the spores germinated exclusively within the tumours, thereby producing and releasing toxins that resulted in animal death. Apparently, it would be difficult to use pathogenic strains for cancer therapy in humans. The isolation of a non-pathogenic strain of *Clostridium butyricum* M-55 overcame the impasse. Intravenous administration of these bacteria into mice bearing Ehrlich ascites tumours resulted in tumour colonization and extensive oncolysis [44]. These results were confirmed in other studies using a variety of rodent tumour models and other non-pathogenic strains. Möse further proved that the growth of anaerobic bacteria in tumours of various animal species was the prerequisite for oncolysis as aerobic spore forming bacteria, such as genus *Bacillus* did not show similar oncolytic effect under comparable experimental settings [45]. Möse and Möse demonstrated the non-pathogenic nature of *C. butyricum* M-55 by injecting 10^{6-10} cfu of spore suspension into themselves. Subsequent clinical trials in human patients with these doses also demonstrated safety, with only occasional low grade fever [46,47]. In one of the trials, intracarotid administration of non-pathogenic *C. butyricum* M-55 spores resulted in liquefaction of glioblastomas. The oncolysis was obvious and near complete one week after injection and glioblastomas were converted into a brain abscess which required surgical intervention. Forty nine patients were treated in similar manner. The rate of recurrence, however, remained uninfluenced and none of the patients showed improvement in their life span [48,49]. Further studies also established critical parameters for Clostridia-mediated oncolysis, the optimal tumour size of 3 cm^3 (2 g of tumour

weight), a spore dose of 10^{6-9} cfu, and modes of spores delivery of either intratumoural or intravenous injection [50–53]. However, though tumour destruction was apparent in these studies, a curative outcome after a single spore administration was meagre. In fact, in most cases the animals did not survive extensive oncolysis and died. Moreover, significant difference has been documented among different animal models where murine tumours appeared more susceptible, while rat and rabbit tumours were more refractory [50–54].

Follow-up studies shed more lights on these puzzling outcomes. It was believed that colonisation and growth of the organism did lead to the destruction of significant portion of the tumour; invariably however, regrowth could occur from an outer rim of viable tumour cells that usually remained [55]. From these observations it was concluded that spore treatment using wild-type Clostridia were not sufficient to eradicate solid tumours that eventually lead to another approach where Clostridial spore were given in conjunction with other anticancer therapy (combination therapy) [55–58]. In this context different therapeutic modalities have been explored in an attempt to enhance the tumour targeting and efficacy of clostridium-based cancer therapy [56–59]. *Clostridium oncolyticum* M-55 was combined with an existing radiofrequency therapy developed for solid tumour. The hypothesis was that short-termed radio-frequency could warm up the tumour, damage tumour cells, thereby promoting area of necrosis which in turn creates a favourable microenvironment for *C. oncolyticum* M-55 to germinate. Radiofrequency therapy was applied locally in 861 mice bearing three different types of neck tumours i.e.: Ehrlich solid carcinoma, Harding–Passey-melanoma, and fibrosarcoma induced by methylcholanthrene. In all cases, tumour oncolysis as a result of *C. oncolyticum* M-55 were significantly enhanced by short-termed radio-frequency therapy, which warmed the tumours up to $42\text{--}44\text{ }^{\circ}\text{C}$ [56]. Further experiments also tried to establish the optimal combination. Both Ehrlich adenocarcinoma and Harding–Passey-melanoma were implanted on the neck of mice. Pretreatment with radiofrequency therapy was carried out followed by *i. v.* administration of *C. oncolyticum* M-55 spores. In this study a clear dependence on the time interval between the two therapies was evident. Tumour oncolysis was most significant when spores were administered 12 h after initial radio-frequency therapy though Clostridia spores regerminated more quickly in rap-

idly growing Ehrlich adenocarcinoma than in the slowly growing Harding–Passey-melanoma [57–59](Table 2).

To treat the slowly growing Harding–Passey-melanoma, a combined treatment modality consisting radiofrequency, administration of *C. oncolyticum* M-55 spores and local X-irradiation was studied. This combination had resulted in a cure rate of approximately 20%. However, although the survival time is significantly higher, relapses at the sites of transplantation eventually killed the rest of the animals. Interestingly, a second and third round repeat of the three fold-combined therapies increased the rate of tumour free animals, suggesting repeated treatment could result in cure at a much higher percentage [58,59].

Another approach to enhance the oncolysis effect of *C. oncolyticum* M-55 was made by decreasing the oxygen concentration in the air that the tumour-bearing animals breathe to 11–12%. This combination increased the oncolysis-effect of the bacteria significantly, resulting in microscopically complete oncolysis. Furthermore, 30% of animals were cured completely of their tumours [59]. However, clinical use of these strategies did not generate expected result. Consequently, there were very few publications in the 80's. In a summarised review of 34 years of work comprising 1316 gliomas (508 glioblasto-

mas = 39%) patients, Dr. Heppner reported in 1986 that in addition to the operative removal and postoperative X-ray therapy, various attempts have been undertaken to prevent recurrences. These include intracerebral application of Cobalt60 (6 patients); locally applied antimitotic agents (76 patients); bacterial liquefaction induced by intracarotid administration of *C. butyricum* M-55 spores (Oncolysis, 67 patients); circumscribed heating of the extirpation cavity with metal and high-frequency electromagnetic field (85 patients); vaporization of the tumour bed with the defocused CO₂- or Neodymium–YAG-Laser beam (177 patients). Complete tumour cure was achieved only in a single case. These results clearly demonstrate that none of the procedures were effective. It was concluded by the authors that bacterial oncolysis therapy with *C. oncolyticum* M-55 in combination with periodic postoperative radio-frequency locally in the excision cavity of the tumour, might justify cautious optimism about future developments of effective therapy for glioblastomas [60].

More recently, Professor Vogelstein and coworkers directly compared 26 species of commonly used anaerobic bacteria from the genera of *Bifidobacterium*, *Lactobacillus*, and *Clostridium* on colorectal cancer xenograft and found that these strains behaved quite differently in their tumour colonisa-

Table 2
Clostridial strains tested for antitumour studies

Recombinant strain	Tumour model	Strategy/result	Reference
<i>C. histolyticum</i>	Mouse transplanted tumour	Intravenous injection of spores resulted in tumour liquefaction/lysis	[42]
<i>C. tetani</i>	Mouse transplanted tumour	Intravenous injection of spores resulted in death of mouse bearing tumour	[43]
<i>C. oncolyticum</i>	Mouse Ehrlich ascites tumour	Intravenous or intratumour injection of 10 ^{6–10} cfu of spores	[44]
<i>C. oncolyticum</i> (<i>sporogenes</i>)	<i>In vitro</i> study	Recombinant for <i>E. coli</i> colicin E3 for cancerostatic properties	[64]
<i>C. beijerinckii</i> (<i>acetobutylicum</i>)	EMT6 Mouse	Recombinant for NTR CDEPT strategy with CB1954, Nitroreductase activity detected in tumour lysate	[88]
<i>C. acetobutylicum</i>	Rhabdomyosarcoma	Recombinant for TNF- α Recombinant protein detected in tumour, but no control of tumour growth	[68]
<i>C. acetobutylicum</i>	Rhabdomyosarcoma	Recombinant for <i>E. coli</i> CD, CDEPT strategy, CD activity detected in tumour lysate	[88]
<i>C. sporogenes</i>	SCCVII tumours into syngeneic C3H/Km mice	Recombinant for <i>E. coli</i> CD, CDEPT strategy, Tumour growth delay	[65]
<i>C. acetobutylicum</i>	Rhabdomyosarcoma	Recombinant for IL-2, Enhanced antitumour effect	[71]
<i>C. sporogenes</i> and <i>C. novyi-NT</i>	Human colorectal carcinoma (HCT116)	Recombinant for NTR CDEPT strategy with CB1954, high level of colonization 10 ^{8–10} cfu/g tumour, Repeated treatment cycle, significant tumour growth delay	[66]
<i>C. novyi-NT</i>	B16 melanoma, HCT116 xenographs	Combination bacteriolytic therapy (COBALT), tumour regression and complete cure in a considerable proportion of mice that survived	[61]

tion, regermination and oncolysis [61]. Two strains of Clostridia, *Clostridium novyi* and *Clostridium sordellii* were shown to have a more significant antitumour effect than the rest as they are highly motile within tumours, which would ensure maximum spread of the bacteria (and therapeutic products) within the tumour, and was extremely sensitive to oxygen that would strictly limit its growth to tumour tissue, the only areas where hypoxia and necrosis exist. However, more than one third of mice died within 16–18 h following spores injection as a result of a lethal toxin produced by the strain. This lethal toxin (α) in *C. novyi* is located on a phage and was subsequently removed by heating. As a result, such an attenuated derivative named *C. novyi-NT* was created and shown to be safe. Despite the observed oncolysis and tumour growth retardation, however, *C. novyi-NT* treatment alone did not result in significant tumour control [62,63]. Consequently, a combined bacteriolytic therapy (COBALT) was proposed. Combination therapy including *C. novyi-NT* and conventional chemotherapeutic agents, such as dolastatin-10 and mitomycin C were tested. Intriguingly, the combination resulted in a significant antitumour effect, but in fact tumour lysis was too extensive so that severe systemic toxicities became evident and animals died as a consequence of toxæmia or due to the so called tumour lysis syndrome. In attempt to further reduce the observed toxicity, the group subsequently combined bacterial therapy with microtubule-interacting chemotherapeutic agents, including vinorelbine and docetaxel and demonstrated very promising results. A phase I trial of the combined approach in patients is in progress [www.clinicaltrials.gov/ct].

The same group also tried to combine bacterial therapy with radiation. In this context, three modes of radiotherapy were implemented combined with *C. novyi-NT* and showed an additive effect on tumour regression and long-term remission in several tumour models [63]. This radiation-enhanced effect of *C. novyi-NT* is believed to be the consequence that the bacteria attack those cells that are least sensitive to radiotherapy as radiation is more effective in rapidly proliferating and oxygenic cells.

4. Genetic engineering to enhance the capacity of tumour oncolysis

The advance of recombinant DNA technology reignited the field, enabling genetic improvement

of Clostridia's innate oncolytic capability. It provides a possible alternative to overcome the hitch of using wild type strains. Several strategies were employed, including genetic modification of strains to express prodrug converting enzyme, or other therapeutic proteins or cytokines. The first *Clostridium* to be genetically manipulated to express an exotic gene was *Clostridium sporogenes* (originally the *C. butyricum* M-55 strain, or the *C. oncolyticum*) [64]. Schlechte and Elbe tried to introduce a gene encoding Colicin E3, a bacteriocin produced by *E. coli*, with canceriostatic properties [64]. However, their report was not convincing to suggest that recombinant strains worked as expected. It was learned that *C. sporogenes* ATCC 13732 was not amenable for genetic transformation using the plasmid vectors and electrotransformation conditions routinely employed to introduce DNA into other Clostridial strains. Later on a transformation system based on a modified electroporation protocol has been described for *C. sporogenes* NCIMB 10696 [65], however, the frequency of transfer obtained is extremely low. As exogenous, secreted endonucleases represent a major impediment to DNA transfer, very recently a procedure based on conjugative transfer from *E. coli* donors was devised [66]. Using this methodology, it became possible to transfer plasmid vectors at higher frequencies.

Fox and co-workers were the first to clone the CD gene of *E. coli* into a Clostridial expression vector and transform this into *Clostridium beijerinckii* [67]. High levels of the CD enzyme were detected in the bacterial medium and the sensitivity of murine EMT6 carcinoma cells to 5-FU increased 500-fold. Later on, our group have tested strains of non-pathogenic saccharolytic Clostridia including *C. beijerinckii*, *Clostridium acetobutylicum* and *C. butyricum* that have industry values [68]. *In vitro* analyses of *C. acetobutylicum* engineered to express CD showed biologically active protein in lysates and supernatants of the recombinant bacteria [68]. In addition, CD activity was detected in the tumour after intratumoural administration of recombinant *C. acetobutylicum* spores to WAG/Rij rats bearing rhabdomyosarcomas [69]. However, these past studies with genetically engineered saccharolytic Clostridia failed to produce antitumour activity when the relevant prodrug was injected systemically. Corresponding to CD, similar gene therapy approaches were performed using the *E. coli* NTR gene again in *C. beijerinckii*. Expression of the NTR gene by Clostridia *in vitro* enhanced tumour cell death by

CB1954 and NTR activity was detected in tumours following intravenous administration to mice bearing EMT6 tumours [70]. Besides to prodrug converting enzymes, therapeutic genes encoding the cytokine TNF α and IL2 have been introduced and tested for their anticancer efficacy in these strains [71,72].

Despite these encouraging data, animal studies with recombinant *C. beijerinckii* or *C. acetobutylicum* failed to produce antitumour activity when the prodrug was injected systemically. The lack of satisfactory tumour control when using such strains was explained by insufficient levels of therapeutics due to suboptimal tumour colonization of recombinant Clostridia. Indeed, upon systemic administration of spores, colonization levels of the saccharolytic Clostridia were shown to be 1000-fold lower compared to proteolytic *C. sporogenes* strains [73].

Similar to strategies used with saccharolytic strains, engineered *C. sporogenes* containing an expression plasmid for CD into tumour-bearing mice produce tumour-specific expression of prodrug converting enzyme. More importantly, a significant growth delay of the tumours was achieved by systemic administration of the prodrug (5-FC). The antitumour effect of 5-FC following a single intravenous injection of recombinant spore of *C. sporogenes* was equivalent or greater than that produced by maximum tolerated dose of 5-FU given by the same schedule [74]. More recently, *C. sporogenes* endowed with noble NTR, have been tested for its antitumour activity following repeated treatment cycles of Clostridium-directed enzyme prodrug therapy. This study clearly demonstrated the safety and prolonged antitumour efficacy of systemic administered spores of *C. sporogenes* expressing a novel nitroreductase enzyme [66]. In general, with genetic modifications bacterial oncolysis has resulted in high percentage of anti tumour effect for a range of solid tumours [41].

As described earlier all such Clostridial genetic engineering relied upon plasmid transfer, using either conjugative or electroporation protocols. However, plasmid-mediated genetic modification has drawbacks, as plasmid-associated antibiotic resistant genes could impede future clinical use. Furthermore, plasmid is not stable, and can be lost during bacterial growth. In contrary to plasmid based strain modification, a very recent development of a strain alteration was made possible by deleting gene coding for toxin using simple heat treatment [61]. This was possible as the toxin gene was encoded on

Phage. Chromosomally coded genes however, are not amenable for such modification. Apparently, novel and powerful genetic tool to manipulate Clostridial strains are exceedingly required.

5. Group II intron-based genetic manipulation

Group II intron is a new genetic element that was initially found in the mitochondrial and chloroplast genome of lower eukaryotes and plants and recent research has shown promise as a tool for bacterial genetic modification [75,76]. In 1993, the first bacterial group II intron was discovered [77]. Since then, more and more group II introns have been found in both Gram-negative and Gram-positive bacteria, accounting for about a quarter of sequenced bacterial genomes [78–80].

Comprehensive analyses of the mobile group II intron from the *ltrB* gene of *Lactococcus lactis* (*Ll.ltrB*) have revealed that such elements propagate into their specific target site via an RNA-mediated ‘retrohoming’ mechanism [81]. The retrohoming reaction of the *Ll.ltrB* intron is carried out by a ribonucleoprotein complex of an intron-encoded protein (IEP) and excised intron lariat RNA. During this process, target site recognition is achieved primarily by base-pairing between the excised intron lariat RNA and the target site DNA [82,83]. The IEP first recognizes a small number of fixed positions in the flanking regions of the DNA target site and facilitates local DNA unwinding, enabling the intron RNA to base pair to a 14- to 16-nucleotide target region. The inserted RNA is then reverse transcribed by the associated intron-encoded enzyme protein, producing a DNA insertion that inactivates the disrupted gene. Since the DNA target site is recognized primarily by base pairing of intron RNA and IEP recognition spots, the intron targeting sequences can be modified to insert into a specific DNA target site unlike other retro transposable elements such as retroviral and lentiviral vectors, which integrate gene into many different DNA sequences randomly.

The preferred positions recognized by the IEP are few and flexible; multiple intron target sites can be easily identified in any gene by an intron prediction program using a computer algorithm. Intron target specificity can therefore be re-programmed by modifying the DNA sequence encoding the appropriate part of the intron. More importantly, since most activities required to effect the *Ll.ltrB* intron’s site-specific integration are encoded within the intron, its mobility is essentially independent of host

factors. The modified group II introns have a wide host range and have been shown to be efficiently mobile in genomic DNA of both gram-positive and -negative bacteria such as *E. coli*, *Shigella flexneri*, *S. typhimurium*, *L. lactis*, *Staphylococcus aureus*, and *C. perfringens* [84–86].

In summary the relatively high frequency of stable gene inactivation observed in using intron-mediated technology represents a potentially major technical advance for the following reasons: (i) It has wide host range and can be further engineered to increase the frequency and efficiency of mobility; (ii) The insertion mechanism is independent of host-cell recombinant function; (iii) It can be reprogrammed to insert into any desired target sequence and develop new gene retargeting vectors; (iv) It has the ability to readily insert additional genetic markers into target gene; (v) It may have broad applications in a range of bacteria as well as higher organisms.

A good example is ClosTron, a Clostridial specific targetron has recently reported [87], opening a new era for genetic manipulation of Clostridial strains. In this study they have been able to demonstrate generation of five mutants in four Clostridial species including *C. acetobutylicum* and *C. sporogenes*, commonly used strains in anti cancer research. The procedure is highly efficient and reproducible, and that would revolutionize future Clostridia related cancer researches. Furthermore, the technology may also be used for ‘knock-in’ as well as for ‘knock-out’, providing the opportunity to introduce therapeutic genes into the genomes of those Clostridial strains being explored as antitumour agents. Due to its high frequency and site-specific insertion, targetron approach is a promising tool to facilitate construction of recombinant Clostridia carrying multiple heterogonous DNA insertions for expression from a single bacterial chromosome. This would open the possibility of using this technology to co-express therapeutic proteins with different therapeutic targets in the same bacterial cell, allowing their co-delivery into tumour cells. More intriguingly, intron insertion is stable. This stability is particularly important for anti cancer vectors, where consistent therapeutic gene expression maintained over time is desired.

6. Conclusion and future perspectives

It is only a matter of time when more and more bacteria will be manipulated and used to treat can-

cer and benefit humans. Anaerobic bacteria, such as Clostridia have now been convincingly shown to selectively colonise and regeminate in the hypoxic/necrotic regions of solid tumours and can be delivered systemically. A human trial of *C. novyi-NT* is underway. Currently we know that Clostridial cancer therapy has always resulted in oncolysis, but never complete. Furthermore, existing plasmid-based gene modification strategy harbours several safety concerns regarding possible horizontal plasmid transfer and spread of plasmid-associated antibiotic resistant genes. As a result novel method, such as using targetron has distinct advantages. This approach, would allow further improve the bacteria to satisfy a variety of requirements for clinical use. These include among others (i) targeting tumour tissues only and not anywhere else; (ii) elicit active immune response against the tumour so that they could kill off primary as well as metastases [88,89] and (iii) significantly reduce toxicity during bacterial spore delivery and tumour lysis. Once suitable strains of Clostridia are genetically modified, tumour lysing capacity confirmed, and safety verified, more clinical trials of such strains for the treatment of advanced solid tumours would be forthcoming. We are confident that current technologies are in place to achieve these goals.

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