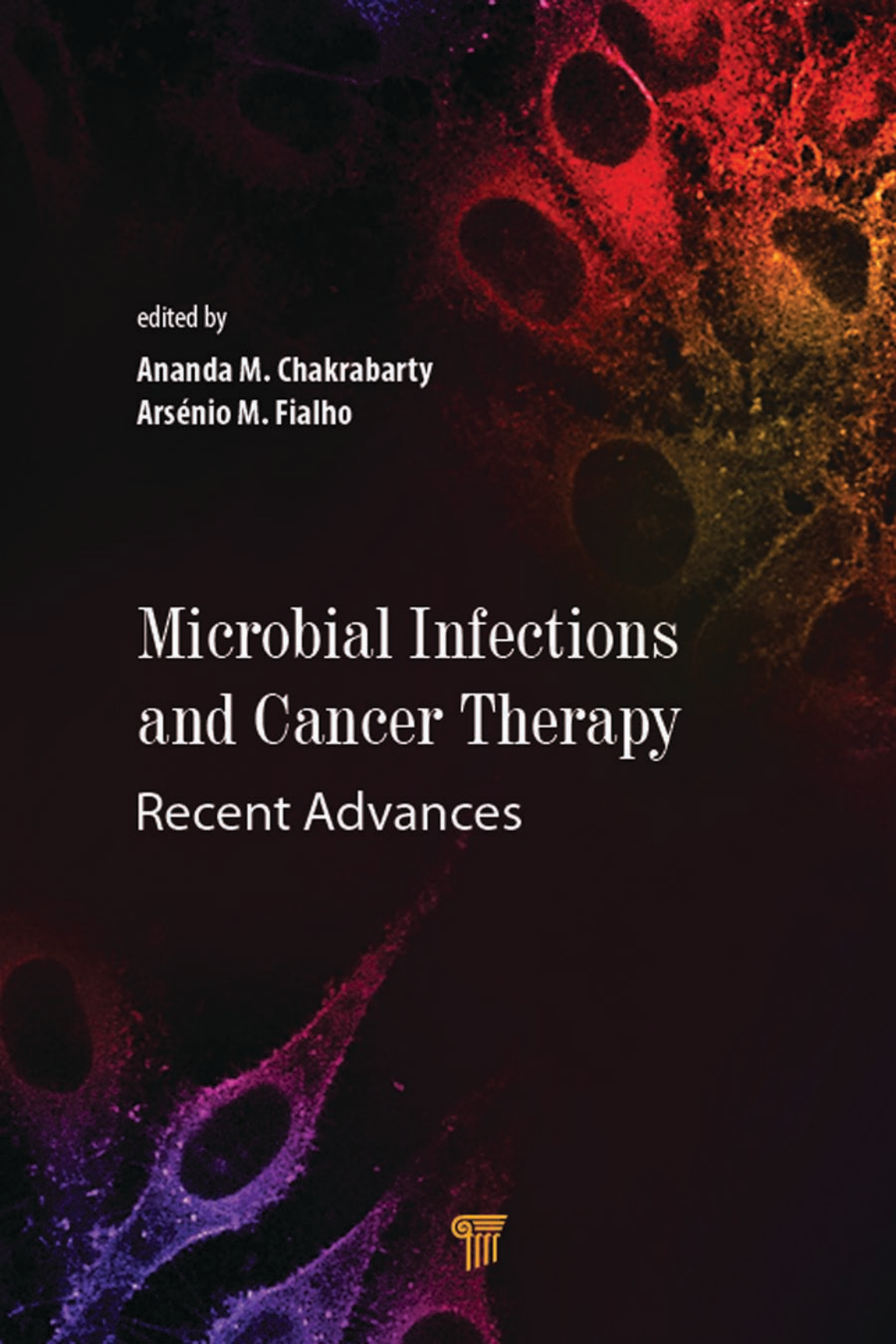
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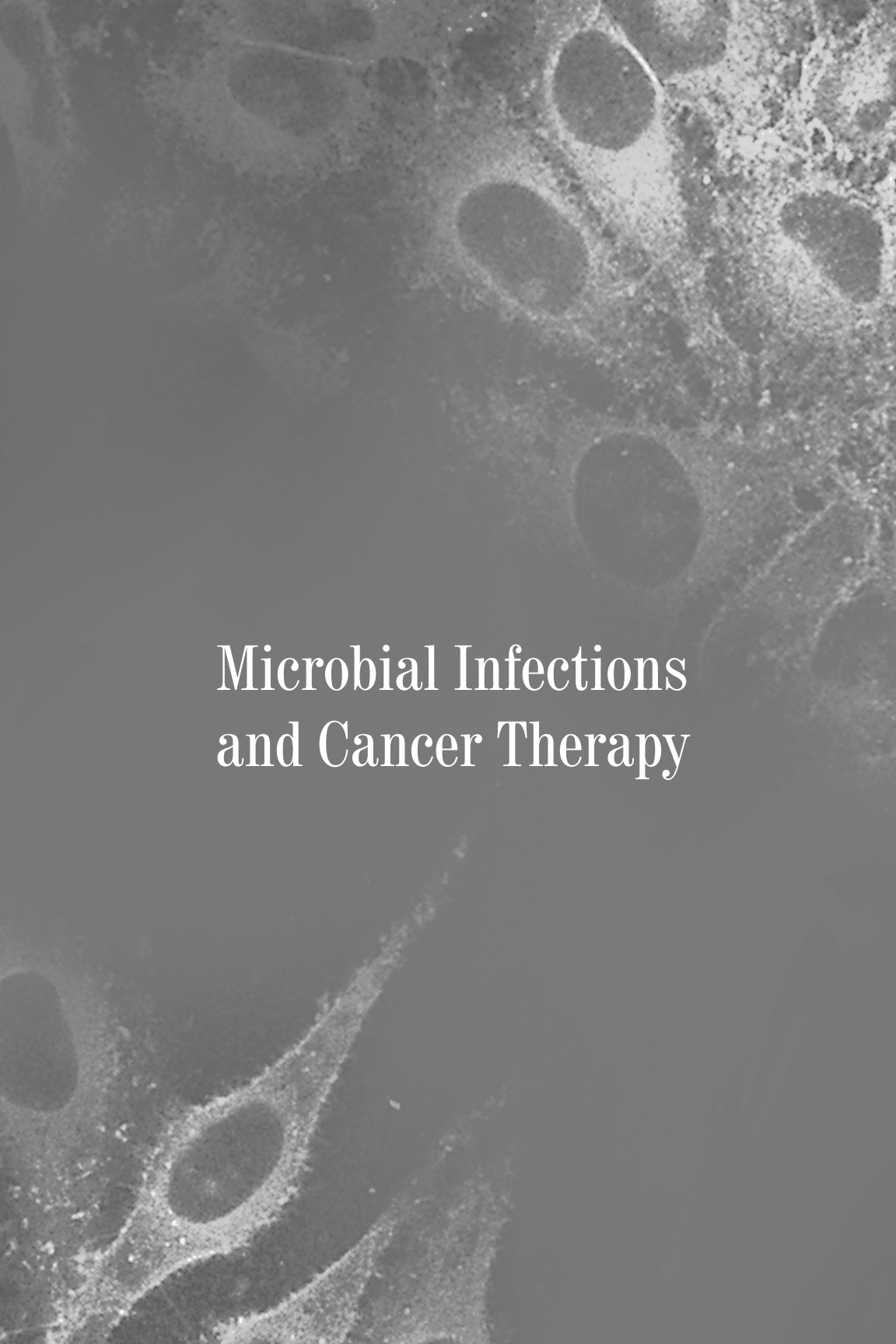
Microbial Infections and Cancer Therapy
Recent Advances



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Microbial Infections and Cancer Therapy: Recent Advances

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Contents

Preface

The bacterial world is extremely diversified, evidencing the existence of bacteria able to successfully colonize the most varied environments, that is, from inhospitable places on the planet to their coexistence with humans. Such nature is based on the existence of unique and complex genetic systems, which is seen to be the key for the great success of their ubiquity. Nowadays, microbial biotechnology makes use of live microorganisms or derived products to find various industrial applications, particularly in health, food, and environment. Among those, in recent years, the use of pathogenic (attenuated) or non-pathogenic live bacteria and their purified products as new anticancer agents have gained prominence. In fact, based on a significant number of scientific publications, human clinical trials, and even clinical practice, it is found that bacteria can be successfully used as agents capable of stimulating the immune system and fight cancer. Furthermore, through genetic intervention, it is possible to modify bacteria and use them as gene delivery vehicles for anticancer proposals. In addition, it also deserves mentioning the fact that bacteria harboring this additional genetic information are able to show tropism, preferentially colonizing the tumor microenvironment. Besides live bacteria, the use of purified bacterial products as anticancer agents, namely proteins, peptides, and compounds derived from secondary metabolism, has also gained relevance. In this book, twelve chapters address the most recent developments regarding the success and limitations of the use of bacteria and their products as cancer therapeutic agents. Considering that we now face an era where the resistance of cancer cells to chemotherapy has become a global burden, the establishment of alternative anticancer therapies may add value in the definition of more efficient therapeutic protocols. As editors, we hope to be able to bring together in this book the most relevant and up-to-date information on this subject.

Ananda M. Chakrabarty
Arsénio M. Fialho
Autumn 2018

Chapter 1

Bifidobacterium as a Delivery System of Functional Genes for Cancer Therapy

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Bifidobacteria were first discovered in 1899 by Henri Tissier, a French pediatrician at the Pasteur Institute in Paris. They are gram-positive, anaerobic, catalase-negative, fermentative rods, which are often Y- or V-shaped. As an anaerobe, *Bifidobacteria* can germinate and proliferate in the hypoxic regions of solid tumors. Plasmids of *Bifidobacterium* encode many specific characteristics of *Bifidobacterium*, and they can also establish shuttle vectors to express exogenous genes. Previous studies exhibited anticancer effects of *Bifidobacteria* on many kinds of tumors. To date, only a few plasmids were found to replicate and express exogenous proteins in *Bifidobacterium;* besides the transformation efficiency and expression level were low in all cases. It can be foreseen that the *Bifidobacterium* will be the most important and perfect vector

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of anticancer gene in cancer gene therapy. The study on related plasmids of *Bifidobacterium* expression plasmids in *Bifidobacterium* for cancer gene therapy is summarized in this chapter, and the advantages and disadvantages of the use of *Bifidobacterium* as a delivery system of functional genes for cancer gene therapy are also discussed.

1.1 The Potential Superiority of *Bifidobacterium* as a Delivery System for Cancer Gene Therapy

1.1.1 The Biological Features of *Bifidobacterium* Associated with Cancer Gene Therapy

As a delivery system and compared with other bacterial genera, *Bifidobacterium* represents a promising biological tool for cancer therapy. *Bifidobacterium* is gram-positive, a strictly anaerobic bacterium with bifid morphology. Its anaerobic specialty is important for cancer therapy. Tissue oxygen electrode measurements taken in cancer patients show a median range of oxygen partial pressure of 10–30 mm Hg in tumors, with a significant proportion of readings below 2.5 mm Hg, whereas those in normal tissues range from 24 to 66 mm Hg [1]. Early in 1980, Kimura and colleagues showed that genus *Bifidobacterium* could germinate and grow in the hypoxic regions of solid tumors after intravenous injection. Contrastingly, the number of *Bifidobacterium* in normal tissues decreased and then disappeared [2]. Later, Yazawa *et al*. showed that wild-type and genetically engineered *B. longum* has the tumor specificity to localize and germinate in tumor regions [3]. Besides its anaerobic specialty, *Bifidobacterium* is known as a health-promoting and probiotic agent, playing an important role in the maintenance of a proper balance of normal intestinal flora [4]. Some bifidobacterial species are frequently used as the probiotic element in many functional foods, which means a high safety in administration. In early studies, *Bifidobacterium* itself also indicated the effect of cancer prevention [5, 6]. Jagveer *et al*. found that oral administration of *B. longum* exerted strong

antitumor activity, as showed by modulation of the intermediate biomarkers of colon cancer, and reduced tumor outcome [7].

1.1.2 Endogenous Plasmids and Cloning Vectors in *Bifidobacterium*

To develop a cloning vector of *Bifidobacterium*, a comprehensive understanding of the replication mechanism and characterization of natural bifidobacterial plasmids is necessary. Plasmids in *Bifidobacterium* attracted great interest because they encode many special characters and play an important role in the research on genetics and construction of engineering strains for *Bifidobacterium*. *Bifidobacterium* with related plasmids not only revealed its own characteristics but also gained characters encoded by the plasmids, such as lactose catabolism, generation of bacteriocin, drug resistance and antibiotic resistance [8–10].

The majority of bifidobacterial strains do not harbor any plasmid. But if they do, they rarely contain more than one, which range in size from 1 to 19 kb [11]. Several plasmids have been isolated from 9 of the 32 species and 44 plasmids have been fully sequenced and annotated (Table 1.1). The majority of the sequenced plasmids were isolated from *B. longum*. Other sources are *B. asteroides*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. kashiwanohense*, *B. pseudocatenulatum*, *B. pseudolongum* subsp. *globosum* and *B. sp. A 24* (Table 1.1). Analysis of their replication (Rep) proteins has indicated that the majority of identifying bifidobacterial plasmids replicate by means of the so-called rolling circle mechanism (RCR), while other functions if encoded, remain to a large degree unknown [11, 12].

Several cloning vectors have been constructed with plasmids from *Bifidobacterium* and *Escherichia coli* and transformed into both of them by electroporation. In all cases, electroporation efficiency in *Bifidobacterium* was very low, and numerous attempts have been made to optimize it.

The nucleotide sequence of the *B. longum B2577* cryptic plasmid pMB1 was sequenced in 1996. Recombinant plasmids containing the pMB1 replicon could replicate in *B. animalis* MB209 [13]. Later, another pMB1-based vector pNC7 was successfully transformed into 10 bifidobacterial species [14]. In 1999, plasmid

pKJ50 was isolated from *B. longum* KJ and a shuttle vector constructed by cloning pKJ50 and a chloramphenicol resistance gene into pBR322 [15].

Table 1.1 Completely sequenced plasmids found in *Bifidobacterium* species

Species	Plasmid	Size (bp)	Accession no.
B. asteroides	pCIBAO89	2,111	NC_010908.1
B. bifidum	pB80	4,898	NC_011332.1
B. breve	pCIBb1	5,750	NC_002133.1
	pB21a	5,206	NC_010930.1
	pMP7017	190,178	GI:704484592
	pBR3	4,891	NZ_CP010414.1
B. catenulatum	pBC1	2,540	NC_007068.1
B. kashiwanohense	pBBKW-1	7,716	NC_021875.1
	pBBKW-2	2,920	NC_021876.1
B. longum	pKJ36	3,625	NC_002635.1
	pB44	3,624	NC_004443.1
	pNAC3	10,224	NC_004768.1
	PNAC2	3,684	NC_004769.1
	pNAC1	3,538	NC_004770.1
	pKJ50	4,960	NC_004978.1
	pMG1	3,682	NC_006997.1
	pTB6	3,624	NC_006843.1
	pBIF10	9,275	GI:73665544
	pFI2576	2,197	NC_011139.1
	p6043A	4,896	NC_010857.1
	p6043B	3,680	NC_010861.1
	pSP02	4,896	NC_019200.1
	pNAL8M	4,910	NC_025161.1
	pNAL8L	3,489	NC_025162.1
	pRY68	2,638	NZ_CP010454.1
	pDOJH10L	10,073	NC_004252.1
	pDOJH10S	3,661	NC_004253.1
	pBLO1	3,626	NC_004943.1

	p157F-NC1	4,895	NC_015053.1
	p157F-NC2	3,624	NC_015066.1
	p1-5B2	3,624	GI:822874551
	p1-5B1	3,919	GI:822874554
	p1-6B2	3,624	GI:822874529
	p1-6B1	3,919	GI:822874532
	p17-1B	3,919	GI:822874562
	p2-2B	3,624	GI:822874523
	p35B	3,624	GI:822874526
	p72B	3,624	GI:822874548
	pEK13	7,050	GI:822874540
	BLNIAS_P1	4,233	NC_017220.1
	BLNIAS_P2	6,230	NC_017222.1
B. pseudocatenulatum	p4M	4,488	NC_003527.1
B. pseudolongum subsp. Globosum	pASV479	4,815	NC_010877.1
B. sp. A24	pBIFA24	4,892	NC_010164.1

Most *Bifidobacterium* are resistant to a wide range of antibiotics, including vancomycin, gentamicin, kanamycin, streptomycin and nalidixic acid [16]. The used selection markers in *Bifidobacterium* are genes conferring resistance to spectinomycin, erythromycin, chloramphenicol, or ampicillin [17–19].

1.1.3 Expression Plasmids in *Bifidobacterium* for Cancer Gene Therapy

Based on the research of the endogenous plasmids and cloning vectors in *Bifidobacterium*, many shuttle vectors encoding target genes were constructed. Efforts are focused mainly on the following three representatives: *B. longum, B. infantis*, and *B. adolescentis* [20]. The main expression plasmids used in *Bifidobacterium* for cancer gene therapy are discussed in the following text.

1.1.3.1 Plasmid pBLES100

pBLES100, which was constructed by cloning with a *B. longum* plasmid, pTB6, and an *E. coli* vector, pBR322, has been used as an

expression vector for several genes in cancer gene therapy. Yazawa *et al.* cloned a gene-encoding spectinomycin adenyltransferase AAD and the pBLES100 constructs were transferred directly into *B. longum*105-A or 108-A by electroporation. When these genetically engineered *Bifidobacterium* were introduced into tumor-bearing mice, bacteria were found only in the tumor environments [1]. Later, transformed *B. longum*105-A carrying pBLES100-AAD was also used in the gene therapy of chemically induced 7,12-dimethylbenz[a]anthracene (DMBA) rat mammary tumors. Consistent results were obtained demonstrating that transformed *B. longum* selectively colonize the tumors [2, 3].

In another study, Nakamura and colleagues constructed the plasmid pBLES100-S-eCD, which included the HU gene promoter and the gene encoding the cytosine deaminase from *E. coli* (converts the prodrug 5-fluorocytosine (5-FC) to the drug 5-fluorouracil (5-FU)). The results obtained suggest that the *B. longum* plasmid is an excellent gene delivery system and an effective candidate for enzyme/prodrug therapy [21, 22].

1.1.3.2 Plasmid pGEX-1LamdaT

Yi *et al*. successfully constructed a *B. infantis*/CD targeting the gene therapy system with a recombinant CD/pGEX-1LamdaT plasmid [23]. Experiments on the mice melanoma model showed that the tumor volume was significantly inhibited compared with controls after treatment with a combination of transfected *B. infantis* and 5-FC [24]. The engineered *B. infantis* containing the Herpes simplex virus—thymidine kinase (HSV-TK) gene was constructed by transformation of recombinant plasmid PGEX-1LamdaT [25]. Using the rat model of bladder tumors, Xiao *et al.* found that the rats treated with BI-TK/GCV group enhanced the efficacy of tumor growth compared with the normal saline control group [26]. Later, Zhou *et al.* demonstrated the efficacy and safety of the TK/GCV system for cancer therapy by intravenous administration [27].

1.1.3.3 Plasmids pBV220 and pBV22210

In our laboratory, a shuttle pBV220 was used to construct pBV220-endostatin and transformed the recombinant plasmid into *B. adolescentis* and *B. longum*. *B. adolescentis* with endostatin gene

was injected into the mice bearing Heps liver cancer. After determination of the expression of endostatin, the distribution and antitumor effect of transfected *B. adolescentis* were examined. At 168 hours after the third injection of *B. adolescentis* with endostatin gene, *B. adolescentis* cells were only found in the tumor tissues (Fig. 1.1). Our research group utilized a strain of *B. longum* as a delivery system to transport an endostatin gene that can inhibit growth of a tumor. The *B. longum* strain with the endostatin gene (*B. longum*-endostatin) was taken orally by tumor-bearing nude mice through drencher preparation. The results showed that *B. longum*-endostatin could strongly inhibit the growth of solid liver tumor in nude mice and prolong the survival time of such tumor-bearing mice. Furthermore, tumor growth was inhibited more efficiently when the *B. longum*-endostatin treatment included selenium (Se-*B. longum*-En) (Fig. 1.2). Se-*B. longum*-En also could improve the activities of NK and T cells and stimulate the activity of IL-2 and TNF-α in BALB/c mice [20]. Xu *et al.* constructed a new vector pBV22210-endostatin combining a chloramphenicol resistance gene and a cryptic plasmid pMB1 from a wild type *B. longum* strain, and *B. longum*-pBV22210-endostatin exhibited higher stability and stronger inhibitory effect on H22 liver tumor growth in xenografts models than the *B. longum*-pBV220-endostatin. Our results also indicated that the plasmid electroporated into *B. longum* was maintained stable in the absence of selective antibiotics and did not significantly affect biological characteristics of *B. longum*. These results suggested that pBV22210 may be a stable vector in *B. longum* for transporting anticancer genes in cancer gene therapy [28]. Besides the above, other genes were also successfully expressed by shuttle vectors in *Bifidobacterium*.

In addition, Cronin *et al*. constructed a reporter vector pLuxMC1, which is based on the plasmid pBC1 and the *luxABCDE* operon from pPL2lux. Later, they found it stably replicated in *B. breve* UCC2003. Thus, it can track the colonization potential and persistence of this probiotic species in real time [29]. Further studies showed that the *B. breve* harboring the plasmid expressing lux fed to mice bearing tumors were readily detected specifically in tumors, by live whole-body imaging, at levels equal to administration. The reporter gene expression was visible for at least two weeks in tumors [30]. Recently, this team revealed

the evidence, which furthermore underlined the significance of bioluminescent imaging and micro-computed tomography as tools to advance the application of vectors [31].

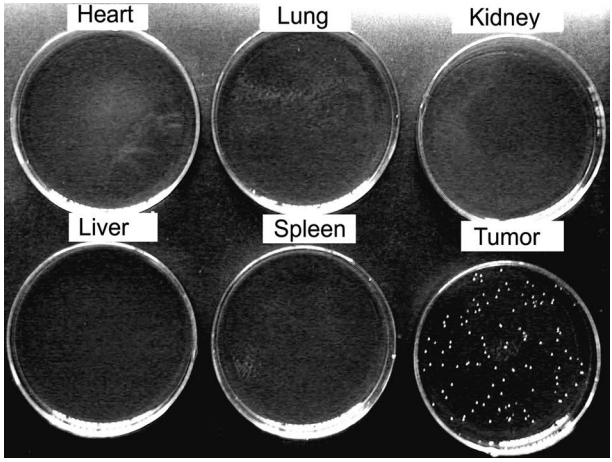

Figure 1.1 Comparison of the number of *B. adolescentis* carrying pBV220/endostatin plasmid in both tumors and normal tissues, after 68 hours of the third administration of 1×10^8 viable bacilli into tumor-bearing mice through tail vein for each time. After 72 hours of anaerobic cultivation, many colonies of *B. adolescentis* carrying pBV220/endostatin plasmid were observed in the tumor, but no colonies were found in normal tissues. Adapted from Ref. [52].

In another report, Guglielmetti *et al*. transformed the human intestinal bacterium *B. longum* with a vector (pGBL8b) containing the insect luciferase gene. The bioluminescent *B. longum* was used to test the efficacy of different carbohydrates to preserve cell physiology under acidic conditions. The results showed that bioluminescent *B. longum* harboring the pGBL8b plasmid is a valuable tool to study the physiological state of anaerobic bacterial cells under different environmental conditions [32].

Many studies have successfully constructed expression plasmids in *Bifidobacterium*, including those not for anticancer [33, 34]. But the accumulating evidence indicates the stability and expression level of these shuttle vectors, and the vectors were useful for further research and application of *Bifidobacterium* in cancer gene therapy.