DNA cloning
Volume II

a practical approach

Edited by
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Cover illustration. The design for the cover was based on Figure 2 Chapter 2 Volume I, showing a map of λgt11; Figure 10 Chapter 4 Volume II, showing crown gall tumour on *Nicotiana tabacum*; and Figure 4 Chapter 6 Volume II, showing peroxidase stained cells.

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Preface

This is the second volume in this series describing practical aspects of DNA cloning techniques. There can be no doubt of the importance of such techniques in bringing about the information explosion that has occurred in Molecular Biology over the past decade. The first volume concentrated on developments that have occurred with cloning systems using *Escherichia coli* as the host organism, and in particular looked at innovations that have been adopted over the past three or four years. The second volume looks at a diversity of systems that are used alongside *E. coli* to allow the cloning and expression of genes in a variety of other prokaryotic and eukaryotic cells. In the previous book, I recommended that newcomers to the field might first want an overview before reading such a book as this which is primarily a laboratory handbook. The texts that I recommended would be an equally appropriate introduction to this volume, namely 'Recombinant DNA: A Short Course' by Watson, Toose and Kurtz (Scientific American Books, New York, 1983); 'Principles of Gene Manipulation' by Old and Primrose (Blackwell, Oxford, 1985); or the one by myself 'Gene Cloning: The Mechanics of DNA Manipulation' (Chapman and Hall, London and New York, 1984). The laboratory manual 'Molecular Cloning' by Maniatis, Fritsch and Sambrook (Cold Spring Harbor Laboratory, New York, 1982) is an additional, invaluable source of protocols for a variety of molecular cloning techniques using *E. coli*. I hope that the cloning techniques described within the first volume will indeed extend and complement the excellent manual of Maniatis and his co-authors as was our intention. The topics covered in Volume 1 include the use of bacteriophage lambda vectors that permit the direct selection of recombinants in library building and the use of lambda vectors for cDNA cloning. Plasmid vectors that direct the synthesis of fusion proteins are also described. These have recently come into their own as a means of providing antigenic material in order to raise antibodies against the products of cloned genes. This can of course be turned around, and libraries of DNA can be constructed in these vectors for screening using available antibodies as probes. Another imaginative group of vectors, the pEMBL plasmids, can be propagated as single-stranded DNA in bacteria carrying F-factors super-infected with the male-specific phage f1. Two approaches to the mutagenesis *in vitro* of DNA carried in 'single-stranded vectors' are then described. Methods for the high efficiency transformation of *E. coli* with naked DNA are presented in considerable detail. In the final chaper of the first volume we begin to consider other host organisms, but stay with Gram negative bacteria in a discussion of vectors that have a broader host range.

In this second volume, alternative bacterial hosts are explored; the first chapter dealing with *Bacilli* and the second chapter with *Streptomycetes*. Chapter 3 is the first encounter with a eukaryotic system, and contains a succinct account of the powerful methods of cloning in yeast. Molecular biologists interested in introducing foreign genes into plants using vectors derived from Ti plasmids have to master a variety of techniques. In Chapter 4 the techniques for working with *E. coli* and *Agrobacteirum tumefaciens* as well as with plant material are described in detail. The discovery that the phenomenon of hybrid dysgenesis in *Drosophila* can be mimicked by the micro-injection of P-elements has lead to the use of these transposons as vectors. The art of achieving the germ line transformation of *Drosophila* is presented in Chapter 5. In the final three

chapters of the book, we turn towards mammalian cell systems. Some tricks for efficient gene transfer into cultured cells are given in Chapter 6, whereas Chapters 7 and 8 look specifically at two viral vector systems using Vaccinia Virus and Bovine Papilloma Virus, respectively. This by no means represents a comprehensive cover of the field of animal cell host-vector systems. This field is evolving so rapidly, however, that in a short while a third volume might be appropriate to cover this area more thoroughly.

As before, I hope that the community of Molecular Biologists will find these volumes useful. My thanks go to all the authors for their willing participation in this project, for producing their manuscripts so promptly, and for being so tolerant of the interfering editor.

David M. Glover

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Abbreviations

APRT	adenine phosphoribosyl transferase
ars	autonomously replicating segment
BPV-1	bovine papillomavirus type 1
BUdR	5-bromodeoxyuridine
C23O	catechol 2,3-oxygenase
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyltransferase
CEF	chick embryo fibroblasts
CHO	Chinese hamster ovary
Cm	chloramphenicol
CRM	cross-reacting material
CTAB	cetyl triethylammonium bromide
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
d.s.	double-stranded
DTT	dithiothreitol
EtBr	ethidium bromide
FCS	foetal calf serum
βgal	β-galactosidase
gdDNA	gapped duplex DNA
GH	growth hormone
HA	haemagglutinin
HBS	Hepes-buffered saline
HbsAg	hepatitis virus surface antigen
HGT	high gelling temperature
HSV-1	herpes simplex virus type 1
IF	interferon
IMP	inosine monophosphate
IPTG	isopropyl-1-thio-β-D-galactoside
Km	kamamycin
LGT	low gelling temperature
LTR	long terminal repeat
2ME	2-mercaptoethanol
MEM	Eagle's minimal medium
MES	2[N-morphino]ethone sulphonic acid
MMTV	mouse mammary tumour virus
MSV	Moloney murine sarcoma virus
NPTII	neomycin phosphotransferase
NRDC	National Research and Development Corporation
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PBS	phosphate-buffered saline
PEG	polyethylene glycol
RNP	ribonucleoprotein
RSV	Rous sarcoma virus
SAM	S-adenosyl-L-[methyl-^{3}H]methionine

SB	simple transformation buffer
SDS	sodium dodecylsulphate
Sm	streptomycin
s.s.	single-stranded
SSC	standard saline citrate
Su	sulphonamides
T-DNA	transforming DNA of the Ti-plasmid
TBS	*Tris*-buffered saline
Tc	tetracycline
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
TFB	transformation buffer
Ti plasmid	tumour inducing plasmid
TK	thymidine kinase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Contents

CHAPTER 1

Bacillus Cloning Methods

KIMBER G. HARDY

1. INTRODUCTION

The potential advantages of *Bacillus* as a bacterial host for cloned DNA include its ability to secrete proteins and the wide range of very different species which can be used. A secreted protein may be easier to recover and purify than an intracellular protein, and in addition, may already be correctly folded, in contrast to an intracellular product for which a step for correct folding must form part of the purification process. The various *Bacillus* species differ greatly from each other in terms of their optimal growth temperatures (there are many thermophilic strains, for example) in whether or not they are obligately aerobic or facultatively anaerobic, and in their proteolytic activities.

Much progress has been made towards the realisation of the potential advantages of *Bacillus* since 1978 when the first *B. subtilis* cloning experiments were reported (1 – 3). Several plasmids have been tried as vectors and a number of promoters have been used to express several mammalian or viral genes. The products of some foreign genes have been secreted from *Bacillus* (4,5). Of course, not all gene cloning experiments in *Bacillus* are designed to express animal or viral genes; cloning experiments can tell us much about the bacterium itself, for example about the mechanisms involved in sporulation (6).

This chapter is written for those who are familiar with basic cloning techniques as applied to *Escherichia coli* and who may wish to begin cloning with *Bacillus*. The difficulties encountered when using *Bacillus* as a host, have included plasmid instability (both loss of the entire plasmid and genetic rearrangements) and the high protease levels of certain strains. In addition, plasmid vectors are not as well developed as they are for *E. coli* and, in particular, the strong controlled promoters, such as *trp*, *tac* and λpL which have proved so useful for expressing foreign genes in *E. coli* do not have such well developed counterparts in *Bacillus*. Problems associated with plasmid instability may be caused by recombination between homologous regions (7) and can sometimes be overcome by avoiding duplicated sequences in constructs. Degradation of the products of cloned genes may be prevented by using a non-proteolytic species such as *Bacillus sphaericus*.

2. STRAINS AND PLASMIDS

2.1 Bacterial strains

B. subtilis strains commonly used as hosts for recombinant plasmids are shown in *Table 1*. Strain BR151 is perhaps the most commonly used host. Almost all

Table 1. *Bacillus* Strains used for Cloning.

Strain	*Characteristics*	*Reference*
BR151	trpC2, metB10, lys-3	15
YB886	trpC2, metB10, xin-1, SPβ$^-$	16
MI112	leuA8, arg-15, thr-5, recE4, r$^-$, m$^-$	12
MI119	leuB6, trpC2, r$^-$, m$^-$	12
MI120	leuB6, recE4, r$^-$, m$^-$	12
CU403	thyA, thyB, metB, divIVB1	17
BD170	trpC2, thr-5	9
BD224	trpC2, thr-5, recE4	9

the standard laboratory strains of *B. subtilis* 168 have two prophages in their chromosomes, PBSX and SPβ (8). When PBSX is induced, functional phage particles are not produced; the phage particles contain only bacterial DNA (9). SPβ produces functional particles, but it is difficult to induce. Strains have been made which lack these prophages (e.g., YB886, a derivative of BR151, *Table 1*).

Many mutations causing sporulation deficiency (and in certain cases a concommitant reduction in protease activities) also result in the inability to become competent for transformation by DNA. However, although the cells of such strains cannot be transformed, it is possible to transform protoplasts made from them. Mutants of *B. subtilis*, a highly proteolytic organism, have been made which lack certain proteases, but none of these have such low levels of protease as are found in other species such as *B. sphaericus, B. freundenreichii* or *B. coagulans*. In order to transform species of *Bacillus* other than *subtilis* it is necessary to work with sphaeroplasts.

The *recE4* allele (10) greatly reduces but does not completely eliminate homologous recombination; plasmids carrying a chromosomal homologue can be unstable even in a *recE4* strain (11). Both RecE4$^+$ and RecE$^-$ strains transform equally well with plasmid DNA (2).

The restriction system of *B. subtilis* 168 and its derivatives has only a low activity against unmodified plasmid DNA. Although the transformation efficiency of an unmodified plasmid is often the same as that of the modified form, instances have been reported where the transformation frequency in an r$^+$ strain is 10% or 0.2% of that in an r$^-$ strain (11–13). In practice many cloning experiments have been successfully carried out using r$^+$ strains of *B. subtilis*. The *B. subtilis* restriction system also operates against phage DNA introduced by transfection, but is not active against chromosomal DNA introduced by transformation (14).

The Bacillus Genetic Stock Centre (Director, D.H. Dean, Department of Microbiology, The Ohio State University, 484 W 12th Avenue, Columbus, OH 43210) provides a valuable service in keeping a collection of strains and plasmids, and also publishes a useful catalogue of strains, plasmids and phages. Another good source of information about strains is the Monograph on *Bacillus* by Gordon *et al.* (15). Many species other than *B. subtilis* are listed together with their reference numbers as they appear in the American Type culture Collection.

Table 2. Plasmids for Cloning in *Bacillus*.

Plasmid	*Source*	*Mol. wt.* (x 10^{-6})	*Markers*[a]	*Reference*
pBC16	*Bacillus cereus*	3.0	*Tc*	34
pAB124	*B. stearothermophilus*	2.9	*Tc*	35
pPL10	*B. pumilus*	4.4	bacteriocin	36
pPL7065	*B. pumilus*	4.6	bacteriocin	37
pIM13	*B. subtilus*	1.5	*Em*	38
pLS28	*B. subtilus (natto)*	4.1	–	40
pBS1	*B. subtilus*	5.5	–	34
pFTB14	*B. amyloliquefacians*	5.3	–	40
pUB110	*Staphylococcus aureus*	3.0	*Km (Nm)*	41
pE194	*S. aureus*	2.4	*Em*	20,41
pC194	*S. aureus*	1.8	*Cm*	12,21,42
pT127	*S. aureus*	2.9	*Tc*	12
pC221	*S. aureus*	3.0	*Cm*	12
pC223	*S. aureus*	3.0	*Cm*	12
pUB112	*S. aureus*	3.0	*Cm*	12
pSA501 (pS194)	*S. aureus*	2.8	*Sm*	41–43
pSA2100 (pSC194)	*S. aureus*	4.7	*Cm,Sm*	41–43

[a]Resistance markers are *Tc*, tetracycline, *Cm*, chloramphenicol; *Sm*, streptomycin; *Km*, kanamycin; *Tp*, trimethoprim; *Ap*, ampicillin; *Em*, erythromycin; *Nm*, neomycin. Auxotrophic markers are *trp*, tryptophan biosynthesis; *leu*, leucine biosynthesis; *ilv*, isoleucine-valine biosynthesis.

Table 3. Recombinant Plasmids used for *Bacillus* Cloning.

Plasmid	*Mol. wt.* (x 10^{-6})	*Parental plasmids*	*Markers*[a]	*Reference*
pBD6	5.8	pSA501, pUB110	*Sm, Km*	2,44
pBD8	6.0	pSA2100, pUB110	*Sm, Cm, Km*	2,44
pBD9	5.4	pE194, pUB110	*Em, Km*	2,44
pBD64	3.2	pC194, pUB110	*Cm, Km*	44
pHV11	3.3	pC194, pT127	*Cm, Tc*	3
pHV41	4.5	pC194, pUB110, pBR322	*Cm, Km*	45
pLS103	5.0	pUB110, *B. pumilus trp* gene	*Km, trp*	1
pLS105	5.4	pUB110, *B. licheniformis trp* gene	*Km, trp*	46
pTL10	9.4	pLS28, *B. subtilis leu* and *Tp-r* genes	*leu, Tp*	47
pTL12	6.4	pLS28, *B. subtilis leu* and *Tp-r* genes	*leu, Tp*	47
pTB90	4.4	pTB19	*Km, Tc*	48
pPL608	3.5	pUB110, *B. pumilus cat-86* gene, 0.3 kb SP02 phage promoter	*Km, Cm*	49
pPL708	3.3	pPL608 with linker from phage M13mp7	*Km, Cm*	50

[a]The abbreviations used for markers are given in the footnote to *Table 2*.

2.2 Plasmids

Commonly used plasmids and vectors are listed in *Tables 2–4*. Many vectors are derived from plasmids originally found in *Staphylococcus aureus* and which were subsequently found to replicate in *Bacillus* (12). Some are closely related to plasmids found in *Bacillus* (19); for example, pUB110 and pBC16 have many

Table 4. Shuttle (or Bridge) Vectors Replicating in *E. coli* and *B. subtilis*.

Plasmid	*Mol. wt.* ($x\ 10^{-6}$)	*Parental plasmids*	*Markers*[a]		*Reference*
			B. subtilis	*E. coli*	
pHV14	4.6	pC194, pBR322	*Cm*	*Ap, Cm*	3
pHV33	4.6	pC194, pBR322	*Cm*	*Ap, Tc, Cm*	3
pHV23	6.1	pC194, pBR322, pT127	*Tc, Cm*	*Ap, Cm*	47
pOG2165	5.0	pC194, pUB110, *B. licheniformis* *pen-r* gene	*Ap, Cm*	*Ap, Cm*	51
pJK3	5.0	pBS161-1, pBR322	*Tc*	*Ap, Tc*	52
pCPP-3	3.7	pBR322, pUB110, pC194	*Km*	*Km*	53

[a]The abbreviations used for markers are given in the footnote to *Table 2*.

homologous regions (19). The pBC16 plasmid represents a common type in *Bacillus*; pBC16 and pAB124 are almost identical (19). Because of the considerable homology between many plasmids from Gram-positive bacteria, care must be taken when constructing cloning vectors to ensure that homologous regions are not repeated. It should be noted that several plasmids listed in *Table 3* have two or more origins of replication derived from their parent plasmids. This may cause some instability. The complete sequences of plasmids pC194 and pE194 have been published (20,21). The maps of these two plasmids together with that of pUB110, which form components of many of the most commonly used vectors, are shown in *Figures 1–3*.

Hybrid, or chimaeric, plasmids which can replicate in both *E. coli* and *B. subtilis* are especially useful vectors. These are listed in *Table 4*. It is often much simpler to carry out the initial cloning work using *E. coli* as a host, and then to transfer the final construction into *B. subtilis*.

There are numerous examples of plasmid instability, especially amongst plasmids derived from pC194 (2,22–24). The particular region of pC194 associated with instability may be a transposon-like element having an inverted repeat sequence (24). Small regions of homology which may provide foci for a high frequency of recombination (7,25) is another possible explanation for the instability of certain plasmids. Not all recombinant plasmids are unstable (see for example, 4). But because of the high frequency of rearrangements often seen in *B subtilis*, it is perhaps all the more important to ensure that the expression of foreign genes is well controlled, as variants which are not subject to growth inhibition by the foreign protein may quickly outgrow the parental strain. The difficulties arising from the recombination of plasmid promoters with their chromosomal homologues can be avoided by using promoters from unrelated species (the DNA of many *Bacillus* species have little homology with each other, 26) or from phages (see *Table 4*).

A number of *Bacillus* plasmids have been specially designed for cloning and selecting *Bacillus* promoters (for example, pCPP-3, *Table 4*).

2.3 Bacillus Phages and Phasmids

Several phage and plasmid vectors are being developed including those derived