THE Mycoplasma pneumoniae ORIGIN OF DNA REPLICATION: A STUDY OF ITS ABILITY TO FUNCTION IN Escherichia coli

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LANGTUO DENG

The *Mycoplasma pneumoniae* Origin of DNA Replication: A Study of Its Ability to Function in *Escherichia coli*

by

(C) Langtuo Deng (B.Sc.)

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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September 1987

St. John's Newfoundland

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ISBN 0-315-50496-X

DEVOTED TO MY LOVING FAMILY,

MY BELOVED TEACHERS,

AND

MY UNDERSTANDING FRIENDS !

ABSTRACT

The initiation of chromosomal DNA replication from *oriC* in *E. coli* is a very complicated and well regulated process, involving at least 8 proteins (DnaA, DnaB, DnaC, DnaG, ssb, HU proteins, RNA polymerase, and gyrase). Mycoplasmas are a large, divergent group distinctive from other bacteria, whose study is expected to yield new insight into the universality of current ideas concerning the mechanism and regulation of DNA replication. To this end, an attempt has been made to clone the replication origin (*oriC*) of *Mycoplasma pneumoniae* in *E. coli* using a marker rescue method with a Km^r gene fragment as the marker. A total of 8 ligations and transformations were performed but no transformant was obtained even though a variety of conditions were tried in these experiments. In order to determine the effectiveness of the technique used in the experiments, an internal positive control experiment was carried out. In this experiment equal genomic amounts of plasmid pUC12 DNA (completely digested with *Eco*R1) and *M. pneumoniae* DNA fragments (partially digested with *Eco*R1) were mixed, dephosphorylated, and ligated with the Km^r gene fragment. From 7 experiments about 160 Km^r transformants were obtained, and all but one were also Ap^r in phenotype, which means that they had been transformed by plasmids containing the replication origin from pUC12. The plasmid extracted from the only Km^rAp^s transformant was analysed by restriction mapping and Southern blot transfer and DNA hybridization. The components of the plasmid were identified and a possible mechanism is proposed by which the *M. pneumoniae* DNA fragment is progressively lost from the original plasmid. The DNA replication origin of this plasmid was identified to be the pUC12 replication origin.

The possibility that there exists a different type(s) of DNA replication system and apparatus in mycoplasmas from that in *E. coli*, and the implications of this in view of the evolutionary distance between the genus *Mycoplasma* and other bacteria such as *E. coli* are discussed.

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Patricia Hempstead, Assistant Professor of Biology, for her constant guidance, supervision, enthusiastic encouragement and academic help throughout the two years and also for the financial support provided from her grants.

I would like to express my special gratitude to Dr. Ban Younghusband, Associate Professor of Medicine, Dr. Eric A. Barnsley, Professor of Biochemistry, Dr. John Robinson, Assistant Professor of Biochemistry, and Dr. Willie Davidson, Associate Professor of Biochemistry, for all their good advice and enthusiastic technical help in the work as well as spiritual and academic support during these two years.

I am grateful to all the lecturers who have jointly given the Molecular Biology courses for their kind academic help.

I am also grateful to the staff in the Biology Department and the secretaries in the general office for their enthusiastic service and help.

I would like to thank the Dean of the Graduate Studies for providing a graduate fellowship in the two years of my graduate program.

I also want to thank Jeffery Paynter and June Stewart, former technicians in this laboratory, for their kind technical help and assistance.

Finally, I would like to express my thanks to Shawn Regular, Sandra

Luscombe and Betty Hatfield, honors and graduate students in this laboratory, for the good cooperation and the friendship they have brought to me.

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ABBREVIATIONS

Ap: ampicillin

ARS: autonomously replicating sequence

ATP: adenosine 5'-triphosphate

BAP: bacterial alkaline phosphatase

bp: base pairs

BSA: bovine serum albumin

CIP: calf intestine phosphatase

DNA: deoxyribonucleic acid

DTT: dithiotheitol

EDTA: disodium ethylenediamine tetraacetate

EGTA: ethyleneglycol-bis-(b-amino-ethylether)-N, N'- tetraacetic acid

EtBr: ethidium bromide (2,7-diamino-10-ethyl-9-phenyl- phenanthridinium bromide)

Expt: experiment

h: hour, hours

HMW: high molecular weight

Km: kanamycin

KDa: kilo dalton

MDa: million dalton

nm: nanometre

NaOAc: sodium acetate

No. number

ORF: open reading frame

PEG: polyethylene glycol

PAGE: polyacrylamide gel electrophoresis

RF: replicative form

RNA: ribonucleic acid

RNase A: ribonuclease A

SDS: Sodium dodecyl sulfate

RNX: reaction

TEMED: N, N, N', N'-tetramethylethylene diamine

Temp: temperature

Tris: tris(hydroxymethyl)aminomethane

tRNA: transfer RNA

Tet: tetracycline

U: unit

μg: microgram

μl: microliter

UV: ultraviolet

vol: volume

Chapter 1

INTRODUCTION

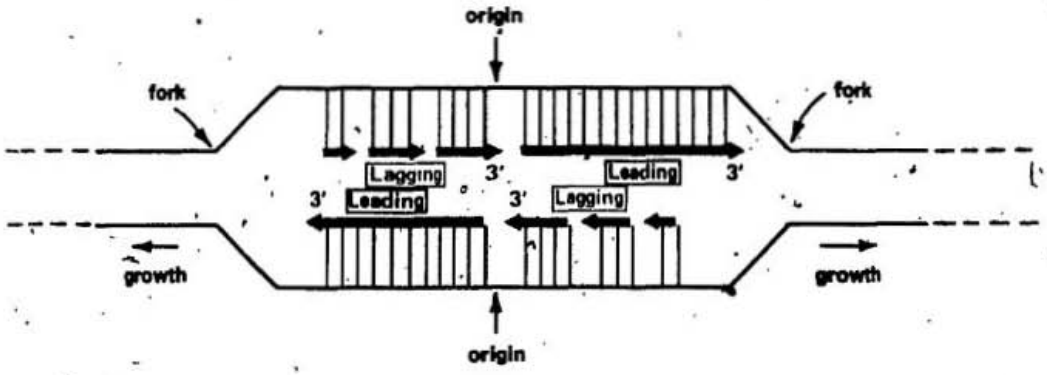
1.1. BACTERIAL DNA REPLICATION AND CELL CYCLE CONTROL

Each bacterial cell has a single, circular, double stranded DNA molecule, called a chromosome. A round of chromosome replication begins with the initiation of DNA synthesis at a specific site, called the origin of chromosomal DNA replication (*oriC*). The initiation of replication is a very complicated and well-regulated process which is not fully understood, but the following are current views (see Fig. 1, 2 and 3) based on the research mostly on a Gram negative bacterium, *Escherichia coli*. The nascent strands synthesized in 5'-3' direction are called leading strands, *i.e.* the strand made in the overall direction of polymerization, while the lagging strands are synthesized later, opposite to the overall direction of polymerization (Fig. 1).

Much experimental evidence suggests that the very earliest, *in vivo* replication from *oriC* is unidirectional and that the first leading strand is counterclockwise (Yoshimoto *et al.*, 1986a, 1986b) (clockwise is defined by the orientation of the standard *E. coli* genetic map, see Bachmann, 1983). In Fig. 3, transcriptions from P*ori*-1 and the *mio*C promotor are counterclockwise.

Fig. 1. Bidirectional fork movement from the origin.

Replication is semidiscontinuous: continuous on the leading strand and discontinuous on the lagging strand. The arrows of growth show the overall direction of polymerization of DNA. (Reprinted from Kornberg, 1981)


origin
fork
fork
Lagging
3'
Leading
3'
3'
Leading
3'
Lagging
growth
growth
origin

while transcription from P*ori*-r is clockwise.) The counterclockwise leading strand is considered to be more important than the clockwise leading strand, because after the duplex has been opened by replication of the first leading strand, the second leading strand can be initiated from several potential initiation sites, e.g. P*ori* (promotor within the origin), a second priming site, or even an Okazaki primosome assembly site (see below). The first leading strand is often simply referred to as the leading strand.

At initiation, the initiation proteins (DnaA, DnaB, DnaC, DnaG and RNA polymerase) interact with each other and *oriC* (Lother *et al.*, 1983; Marians, 1984) forming a complex, and opening the two strands. Then the primase/RNA polymerase starts to synthesize RNA. The transcription by RNA polymerase either activates the *oriC* by opening the two strands so as to expose a priming site, or the RNA transcript itself may be used as the primer (Kornberg, 1983). Most people believe that the exposed priming site is an n' site where the ϕx174-type primosome [which includes proteins i, n, n', n", DnaB, DnaC and DnaG (primase) proteins] assembles and starts primer synthesis. Seufert and Messer (1986), however, suggest that since no n' site has been found within the origin, an n' site is not involved in the initiation of the lagging strand but that the same set of pre-replisome proteins assembled at *oriC* is responsible for this initiation as well. Replication from an n' site is protein i-dependent and possibly involved in *s*table-*D*NA-replication (*sdr*). The pre-replisome (which includes DnaA, DnaB, DnaC, and DnaG proteins) would thus be responsible for the priming of both the leading strand and the lagging strands, *i.e.* Okazaki fragment initiation.

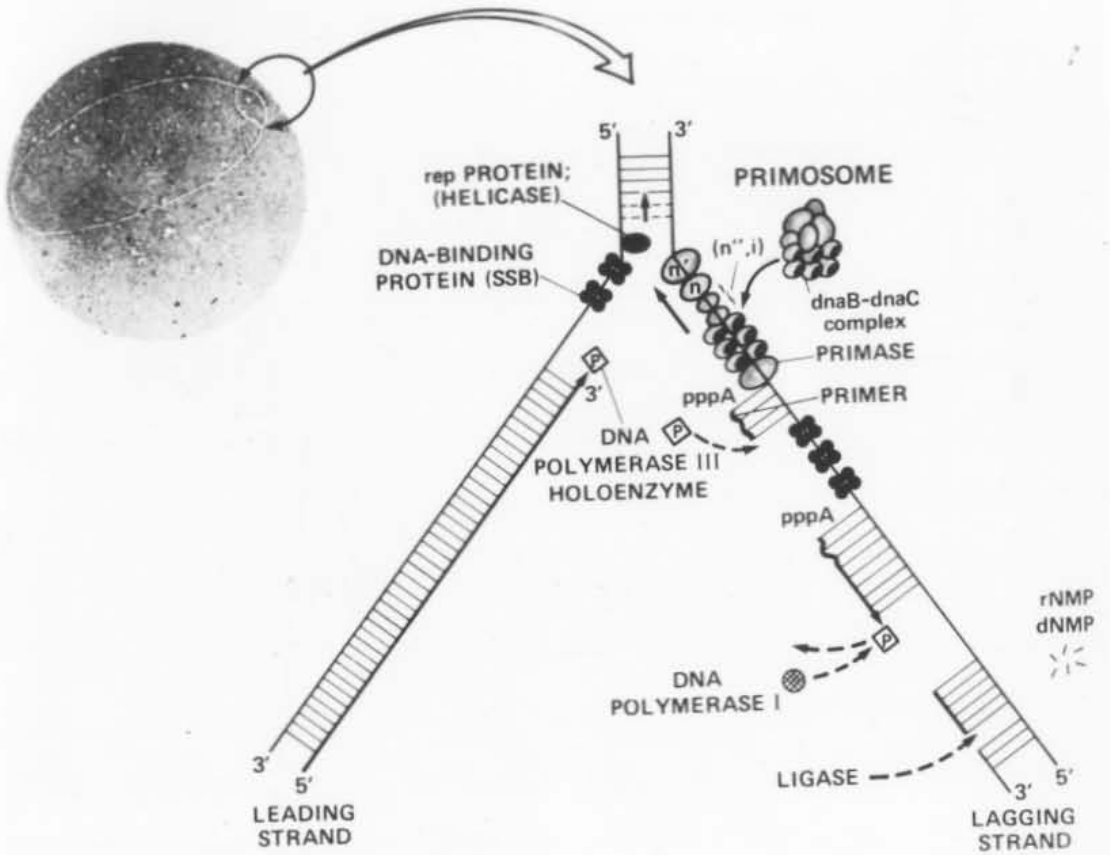
When the primer for the leading strand has been synthesized, DNA polymerase III holoenzyme will take over (after the processing or termination of the primer) and begin to synthesis the DNA leading strand. When both leading strands have been synthesized beyond the origin, two replicaton forks are established and replication continues bidirectionally until the round of DNA synthesis is completed.

During the replication process all the proteins and enzymes involved in replication congregate on or close to the forks. They function cooperatively and coordinately and the complex is called a replisome (Kornberg, 1982).

As for the synthesis of the lagging strand, the direction of its synthesis is opposite to the direction of fork movement. The primosome, (or pre-replisome), after laying down a short 5'-3' RNA primer, moves in a 3'-5' direction, the same as that of fork movement (Fig. 2). At intervals the primosome will stop to synthesize a short RNA primer fragment. DNA polymerase III holoenzyme then elongates the primers with DNA continuing to be synthesized in a 5' - 3' direction. When the DNA synthesized by the holoenzyme meets the primer of the previous Okazaki fragment, DNA polymerase I takes over, hydrolysing the primer and filling the gap by DNA synthesis. Finally, DNA ligase closes the last phosphodiester bonds between the synthesized short fragments producing a continuous strand (Kornberg, 1982). A primosome assembly site, when available as a single strand, has the potential for starting DNA synthesis and hence it is called a "single strand initiation site" (SSI). A phage M13 derivative, M13 ΔE101, can be used to clone and express the SSI. (Ray *et al.*, 1982)

Fig. 2. Scheme for enzymes operating at one of the forks in bidirectional replication of an *E. coli* chromosome or an *oriC*-plasmid

Processivity (lack of dissociation) of the DNA polymerase III holoenzyme in continuous chain elongation and of the primosome in short RNA primer formation for discontinuous synthesis assures rapid replication. (Reprinted from Kornberg, 1982)


5'
3'
rep PROTEIN;
(HELICASE)
PRIMOSOME
DNA-BINDING
PROTEIN (SSB)
(n'',i)
dnaB-dnaC
complex
PRIMASE
pppA
PRIMER
3'
DNA
POLYMERASE III
HOLOENZYME
pppA
rNMP
dNMP
DNA
POLYMERASE I
LIGASE
3'
5'
LEADING
STRAND
3'
5'
LAGGING
STRAND

The initiation of bacterial DNA replication is coupled to the growth rate and determines the cell cycle, however, the mechanism of this regulation has not been fully elucidated. The dominant belief is that the ratio of origins/cell mass determines the time of initiation. After a cell division, cells grow to increase their cell mass, and when the ratio is reduced below a threshold, replication is initiated. This is called the titration of cell mass hypothesis. Two types of model have been proposed for this hypothesis. One is the "initiator" or "autorepressor" model (Sompayrac and Maaløe, 1973), in which a putative initiator protein is continuously produced throughout the cell cycle, accumulating to a critical amount which then triggers the initiation of DNA replication. An autorepressor gene is on the same operon as the initiator gene, which functions is to keep the concentration of the initiator protein stable by negative feedback. The other model involves a repressor of DNA replication which is synthesized once at a fixed point per cell cycle and is diluted by the subsequent increase in cell volume. When the concentration of the repressor drops below the effective level, replication initiates (Pritchard *et al.*, 1969).

1.2. THE STRUCTURE AND FUNCTION OF oriC

Within the *oriC* of *E. coli* a 245 bp (+22 -- +267 bp) fragment was found to be an autonomous replication sequence (Oka *et al.*, 1980, 1984), called the minimal origin (Oka *et al.*, 1980, Tabata *et al.*, 1983). Even though the minimal origin is called simply "*oriC*" quite frequently, in this Thesis I will call it "mini-*oriC*" to distinguish it from the integral origin of the intact chromosome. According to Lother *et al.* (1985), the integral origin includes sequences flanking

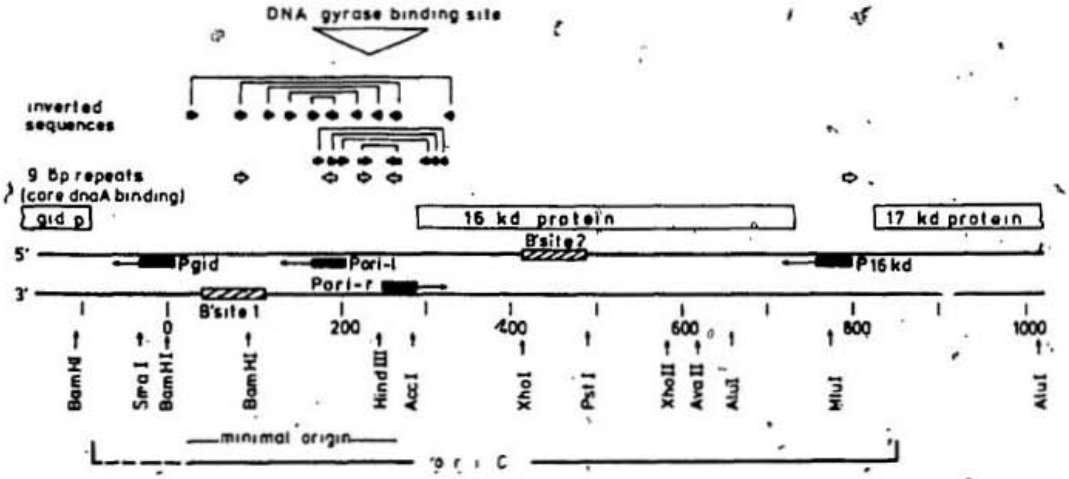
the mini-*oriC*, approximately 900 bp long, which are involved in the regulation of initiation. The right flanking sequence contains a *mioC* (*m*odulation of *i*nitiation at *oriC*) gene, coding for a 16 K Dalton protein (also called x-protein). The function of this protein is unknown, but the transcription of the gene is believed to be a very important regulatory element for the initiation from *oriC* (Tanaka *et al.*, 1985; Schauzu *et al.*, 1987). The essential features of the origin region are summarized in Fig. 3.

In order to study the mechanism and control of chromosome replication, minichromosomes were constructed by circularizing the *oriC* region or by the ligation of the *oriC* region with a DNA fragment (usually a marker gene) to produce an artificial plasmid in which autonomous replication starts only from the chromosomal origin of replication, *oriC*. In these experiments, the minichromosome is also called an "*oriC*-plasmid". The minichromosomes require the same set of gene products thought to be involved in chromosomal replication.

Six different species of *oriC* fragments from different Gram negative bacteria have been shown to function in *E. coli*. Five are from the same family as *E. coli*, the family *Enterobacteriaceae*: *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Erwinia carotovora*, and the other is a marine bacterium: *Vibrio harveyi* of the family *Vibrionaceae*. From analysis of the 6 origin sequences there has been proposed a general structure for *oriC*, and in particular a consensus sequence of mini-*oriC*. This conserved sequence and structure is called an *E. coli*-type origin and it has the following features.

Fig. 3. Structure of *oriC*

The outer limits of *oriC* are indicated by brackets. The highly conserved region (broken line) believed to be essential for the initiation of DNA replication is included. The outer limits of minimal origin (mini-*oriC*) are indicated by the ends of the thin line. Some restriction sites and coordinates are given for orientation. B' membrane binding sites (Jacq *et al.*, 1983) are indicated by hatched bars. Promotors are indicated by filled bars. P16Kd is the *mioC* promotor. Transcripts start at the base of the arrow. Open reading frames for proteins are indicated by open bars. The 9 bp repeats acting as core DnaA protein binding sites are shown as open arrows. The specific DNA gyrase binding site with its center is also indicated. Numbers are base pairs from the *Bam*H1 site and are counted in clockwise orientation. (Reprinted from H. Lother *et al.*, 1985)


DNA gyrase binding site
inverted sequences
9 bp repeats (core dnaA binding)
gid p
16 kd protein
17 kd protein
B'site2
5'
3'
Pgid
Pori-l
Pori-r
P16kd
B'site 1
0
200
400
600
800
1000
BamHI
SmaI
BamHI
BamHI
HindIII
AccI
XhoI
PstI
XhoII
AvaII
AluI
MluI
AluI
minimal origin
oriC

In the mini-*oriC* region very few deletions or insertions are found and the few which occur are mostly between +20 bp to +60 bp *i.e.* at the left border of mini-*oriC*. The mini-*oriC* is AT rich (56%), with an asymmetric nucleotide distribution. Mini-*oriC* contains completely conserved regions and regions of identical length but of different sequences. The conserved sequence regions cannot tolerate insertions or deletions nor can they tolerate base substitution; the non-conserved regions can tolerate base substitutions but not deletions or insertions. The former constitute recognition sequences for the initiation proteins, e.g. DnaA, DNA gyrase and RNA polymerase, and B' membrane binding sites; the latter sequences may function as "spacers" (Marians, 1984; Zyskind *et al.*, 1986).

The most important conserved sequence is a 9 bp repeat, TTATCCACA, called the *dnaA* box, which is the binding site for the DnaA protein. Four copies of it are found within mini-*oriC*, a fifth is located near the -35 box of the *mioC* promotor (Hansen *et al.* 1982). As discussed below, the DnaA protein is uniquely vital in the initiation and regulation of replication from *oriC*.

Another important conserved sequence is the GATC *dam* methylation site. There are 8 conserved GATC sites in the mini-*oriC* consensus sequence and it appears at a high frequency in the promotor and regulation regions of the *mioC* gene and the *dnaA* gene. Methylation of one or more of the GATC sites in the origin is very important for its function, especially *in vivo*. Messer *et al.* (1985) and Smith *et al.* (1985) have demonstrated that *oriC*-plasmids can only transform *dam* mutants at very low frequency or not at all. Possibly the methylation affects the recognition/binding of the initiation proteins. Methylation of GATC sites in

the promotor/regulation regions of the *mioC* and *dnaA* genes was found to enhance their expression (Braun and Wright, 1986; Schauzu *et al.*, 1987). Braun and Wright (1986) also suggest that *dam* methylation may have a role in "fine-tuning" the expression of *dnaA* during the *E. coli* cycle. Since the DnaA protein controls replication, its own synthesis would also be tightly controlled. They propose that the hemimethylated *dnaA* gene is less efficient in transcription than the fully methylated gene, such that hemimethylation resulting from gene duplication during replication would prevent an instantaneous doubling in the rate of *dnaA* expression.

Close to the left border within the mini-*oriC*, there is a conserved region of 50 bp in length containing three 13-mers. This region is supposed to be involved in interaction with DnaB protein with the assistance of DnaA and DnaC proteins (Funnell *et al.*, 1987) or involved in the translocation of the initial initiation complex (Messer, 1987).

Recently, Schauzu *et al.* (1987) confirmed *in vivo* the existence of two promotors, which were found *in vitro* by Lother and Messer (1981), within mini-*oriC* by S1 nuclease mapping: P*ori*-r and P*ori*-l. They were found to start divergent transcription *in vivo*: leftward (or counterclockwise) at position +178 for P*ori*-l and rightward (clockwise) at positions +294 and +304 for P*ori*-r. These transcripts were found to terminate after 100-150 bases, at terminators designated T*ori*-l and T*ori*-r. An additional promotor for rightward transcription within mini-*oriC* was recently located in the left half of the mini-*oriC* (Junker *et al.*, 1986), but its role and existence have not been well recognized. For simplicity it is not included in Fig. 3.

Transcription proceeds counter-clockwise from the *mioC* gene promotor and most transcripts enter or even go through mini-*oriC* (Junker *et al.*, 1986; Schauzu *et al.*, 1987). Tanaka and Hiraga (1985) replaced the *mioC* promotor with the *lac* promotor, and found that the *lac*-inducer IPTG induced transcripts that entered mini-*oriC* and negatively-regulated DNA replication from *oriC*. Stuitje *et al.* (1986), on the other hand, by dissecting the *mioC* promotor sequences, found that transcripts entering mini-*oriC* positively regulated replication. Specifically, they found that a terminator inserted to prevent the transcripts from entering *oriC* reduced the copy number of *oriC*-plasmids. When the *dnaA* box in the promotor was deleted, the transcripts entering *oriC* were not reduced significantly but the copy number of *oriC*-plasmids was reduced very obviously. These observations suggest that the binding of DnaA protein to the *dnaA* box of the *mioC* gene is essential to accomplish the actual activation event within *oriC*, and that the negative result of Tanaka and Hiraga (1985) resulted from a lack of DnaA binding (Stuitje, *et al.*, 1986).

The currently accepted view is that of a "positive action" by *mioC* transcripts. As for the mechanism of their function, there are two possibilities. First, the transcripts may be used as primers to initiate the counter-clockwise leading strand; then the P*ori*-r or even perhaps an Okazaki assembly site, opened by the transcription from *mioC*, would start the clockwise leading strand. In this case, P*ori*-l would only function as a backup promotor for *mioC*, a possibility which could explain the low level of replication obtained when the *mioC* gene was deleted. The other possibility is that the transcripts from *mioC* activate *oriC* by

opening the double strands, possibly facilitating DnaA binding (Schauzu *et al.*, 1987). In this case, the primers for the two leading strands would be synthesized from P*ori*-r and P*ori*-l, respectively.

Initiation may involve DNA gyrase, DnaA, DnaB, DnaC, DnaG proteins and RNA polymerase. The most important feature of *in vivo* initiation of DNA replication from *oriC* is that it is DnaA-dependent and protein synthesis-dependent (Lark *et al.*, 1963) and there is a requirement for RNA polymerase. The function of *oriC* involves two steps: to open the double strands and to begin to synthesize the leading strands. The mechanism of initiation involves many initiation proteins. Before initiation, gyrase may be needed to introduce negative supercoils to facilitate the binding of DnaB/DnaC to the DnaA, DNA complex (Funnell *et al.*, 1987) and would be continuously required to release the stress of positive supercoils produced by continuous unwinding during replication (Baker *et al.*, 1987). DnaA protein binds cooperatively to the 4 *dnaA* boxes within the mini-*oriC* and forms a multimeric complex (Fuller *et al.*, 1984). Fuller *et al.* (1984) examined DnaA protein binding by footprinting and suggested that the complex contains 20-40 monomers of DnaA protein with the DNA wound around in a specific way. This is consistant with the supposition of Funnell *et al.* (1987) inferred from the size measurement of the diameter of the DnaA-*oriC* complex. Afterwards, DnaB protein may join the DnaA protein in a reaction dependent on DnaC protein and apparently binds to a 50 bp conserved region containing three 13-mers close to the left border as deduced from studies involving *in vitro* assembly (Funnell *et al.*, 1987). The histone-like HU protein assists the initiation

(Baker *et al.*, 1987). Funnell *et al.* (1987) suggest that the HU protein as well as the temperature may cause the topological change enabling an alignment of DNA in which the prepriming proteins produce an active complex.

The *in vivo* DNA replication from *oriC* at the very earliest stage is commonly accepted as being unidirectional, and the first DNA strand synthesized is the counterclockwise leading strand. The electron microscopic observation by Yoshimoto *et al.*, (1986b) of early replication intermediates of an *oriC*-plasmid which contained the integral *oriC* region, provide evidence for such asymmetric replication from the integral *oriC* region at an early time. Baker *et al.*, (1987), however, found that the *in vitro* replication is different. By electron microscopic observation of the initiation complexes of *oriC*-plasmids established *in vitro* with different initiation proteins, they found that the DnaB protein functions as a helicase, starting bidirectional unwinding of the parental strands. When primase and DNA polymerase III are present together with DnaB and gyrase in the correct proportions, the *in vitro* replication is bidirectional. Maybe bidirectional replication is established simply by joining primase and DNA polymerase III to the DnaB proteins which are working in each direction. Baker *et al.* (1987) also suggest a reason for the difference in orientation between the *in vivo* and *in vitro* replication: *in vivo*, the transcription from the *mioC* promotor inhibits the rightward priming, but in the *in vitro* system RNA polymerase is absent and the *mioC* promotor is also absent from the *oriC*-plasmid.

In addition to the observation that DnaB unwinds bidirectionally, Baker *et al.* (1987) also found that when unwinding by DnaB is allowed to precede priming

and DNA synthesis, primase and the polymerase III holoenzyme do not exhibit a preference for starting at *oriC*. Thus it appears that the DnaA*DnaB*DnaC complex is responsible for the specificity of initiation at *oriC* in replication.

Recently, Seufert and Messer (1987) found that the DnaA protein can substitute for protein i to initiate replication of pBR322 and several other plasmids, or to stimulate replication of plasmids previously considered to be *dnaA* independent. When the normal priming site is deleted from pBR322 but the DnaA protein binding site remains, the DnaA protein becomes essential for replication. There is no analogous priming site (n' site) in the vicinity of *oriC* (Stuitje *et al.*, 1984), so Seufert and Messer (1987) suggested that the DnaA protein directs DnaB, DnaC and DnaG (primase) proteins to the *dnaA* binding site to form a pre-replisome and start priming for DNA synthesis. That is to say, the DnaA protein may function as a nucleation protein for assembling the pre-replisome complex and is capable of functioning on many different origins and the pre-replisome is responsible for the priming of both the leading strand and the lagging strand (Seufert and Messer, 1986, 1987).

1.3. THE REGULATION OF INITIATION AT oriC

The replication properties of *oriC*-plasmids have been extensively studied, based on the idea that their characteristics may reflect the mechanism and control of the chromosomal DNA replication. As mentioned above, this expectation has been supported by numerous findings that *oriC*-plasmids require a number of the same gene products for initiation of replication as the chromosome. On the other hand, it has also been found that *oriC*-plasmids are present in higher copy

numbers than the chromosome and are not stably maintained (Leonard and Helmstetter, 1986). However, *oriC*-plasmids were found to replicate synchronously with the chromosome in exponential-phase culture and Leonard and Helmstetter (1986) and Helmstetter and Leonard (1987) have suggested that this is because their replication frequency is governed by the same mechanism Helmstetter and Leonard (1987) suggest that this is so even with an *oriC*-plasmid which contains only 327 bp of the *oriC* region, which implies that the information in the mini-*oriC* is enough for control of the timing of the initiation of DNA replication.

Minichromosomes have also been used to study the incompatibility properties of *oriC*. Plasmids of the same type, that is, plasmids from the same compatibility group, are unable to coexist in the same bacterial cell. The mechanism for this incompatibility is related to the regulation of plasmid copy number. Stuitje *et al.* (1986) demonstrated that *oriC* is responsible for the incompatibility observed between *oriC*-plasmids. Two sequences, *incA* and *incB*, were found to be responsible for this feature. A pBR322 plasmid derivative, carrying both a 210 bp sequence overlapping the promotor of the *mioC* gene (*incB*) and the mini-*oriC* region (*incA*), exhibits strong incompatibility towards other *oriC*-plasmids. According to Stuitje *et al.* (1986), the expression of *incA* requires transcription into mini-*oriC*, and the role of *incB* in incompatibility is the titration of DnaA protein by binding to the *mioC dnaA* box, with RNA polymerase facilitating the titration. Thus the conditions for expression of incompatibility are nearly the same as the regulation of initiation. Perhaps they are two phenomena of one single event.

The stability of the minichromosome depends both on the copy number (Stuitje *et al.*, 1986) and the partition characteristic (Løbner-Olesen *et al.*, 1987). The latter may depend both on the membrane binding sites in *oriC* (Jacq *et al.*, 1983) and also on the size and conformation of the minichromosome which may influence the tightness of its binding to the membrane. What determines the copy number is not yet clear. It will be a function of the interval time between two initiations, which may in turn be decided by the time required for *oriC* methylation and for gyrase to introduce negative supercoils (Atlung *et al.*, 1987).

As for the regulation of the replication *per se*, it is not yet possible to synthesize a clear picture. DnaA protein is apparently the key factor in controlling the cell-cycle. Atlung *et al.* (1987) consider that the DnaA protein is a good candidate for the initiator in the "initiator accumulation" or "autorepressor" model (Sompayrac and Maaløe, 1973). There are several reasons for this belief. Firstly, the DnaA protein is specifically required for initiation of replication from *oriC*. Secondly, the *dnaA* gene is negatively autoregulated (Hansen *et al.*, 1987; Kücherer *et al.*, 1986). (In this case, the DnaA protein is both the initiator and also the autorepressor, a modification of the original Sompayrac and Maaløe model). Thirdly, when temperature-sensitive *dnaA* mutants are grown at intermediate temperature, *i.e.* when the DnaA protein is partially inactive, initiation starts at a decreased origin/mass ratio resulting in decreased *oriC*-plasmid copy number (Lycett *et al.*, 1980), while a cold-sensitive mutant has an increased ratio at low temperature (Kellenberger-Gujer *et al.*, 1978). These observations mean that the activity of DnaA protein can alter the origin/mass

ratio at initiation. Experiments by Atlung *et al.* (1987) show that overproduction of DnaA protein (obtained with plasmids carrying the *dnaA* gene coding sequence under the control of the heat-inducible λpL promotor) induced overinitiation. For minichromosomes, the copy number increases; for the chromosome, the gene dosage near the origin is increased, even though the overall ratio of DNA/cell mass does not alter. The overinitiation occurs very rapidly after the induction but then the initiation frequency reduces to nearly normal. This suggests that the concentration of DnaA protein also determines the ratio of *oriC*/cell mass at initiation.

The mechanism by which the DnaA protein regulates initiation by binding to the *mioC dnaA* box is still not well understood. Maybe it involves the interaction between the DnaA protein and RNA polymerase, since *dnaA* boxes are found close to the *mioC* promotor and also close to the P*ori*-l and P*ori*-r promotors (see Fig. 3). What is more, some *rpoB* (coding for the RNA polymerase β subunit) mutations were found to be able to suppress a *dnaA* mutation which, according to Bagdasarian *et al.* (1977), suggests the physical interaction between these two gene products. The experiment by Stuitje *et al.* (1986) shows that the binding of DnaA protein to the *dnaA* box near the promotor of the *mioC* gene is essential for the activating transcription to occur, but higher concentrations inhibit the transcription. Rokeach and Zyskind (1986) found that both increased and decreased levels of intracellular DnaA protein deceased the transcripts into *oriC*. These observations, together with the fact that the *dnaA* gene is autogenously regulated, provide us with an autoregulatory, homeostatic control model of DNA replication by DnaA protein.

The transcription from the *mioC* promotor into mini-*oriC* is responsive to changes in the intracellular concentration of other transcription and replication factors and ppGpp (guanosine 5'-diphosphate, 2'(3')-diphosphate) (Rokeach and Zyskind, 1986). The promotor of *mioC* has the same conserved region as the stable RNA promotors which are responsible for the stringent response (Travers, 1984; Lamond and Travers, 1985). The significance to replication of stringent regulation of a transcript required for initiation is that in the stringently regulated ribosomal system, promotor P1 is also under growth-rate-dependent control (Deneer and Spiegelman, 1987). Growth rate regulation of the *mioC* transcript could provide a mechanism whereby the rate of chromosomal duplication would be coupled to the growth rate. An additional way of coordinating replication with transcription and translation in *E. coli* may rely on the organization of operons such as the *rps-dnaG-rpoD* macromolecular synthesis operon, an operon which contains genes involved in translation (*rps*), transcription (*rpoD*) and replication (*dnaG*) (Lupski, *et al.*, 1984). The coupling of genes into such operons would also contribute to the coordination of replication and overall cellular growth.

1.4. E. coli IS CURRENTLY THE BEST HOST FOR CLONING FUNCTIONAL ORIGINS

E. coli is a very flexible organism for DNA replication. Apart from *oriC*, the *E. coli* chromosome contains several other potential origins, which are repressed under normal conditions. So-called stable DNA replication (*sdr*) mutations were found when *oriC* was deleted or when *dnaA* and/or *rnh* (Ribonuclease H) mutants were grown under non-permissive conditions (Kogoma, 1986). The *sdr*

replication is DnaA-independent and protein synthesis-independent (Kogoma and Meyenburg, 1983). Massy *et al.* (1984), by analyzing the relative copy numbers of various genes aound the chromosome in exponentially growing *sdrA* mutant cells with or without the *oriC* site, found the locations for several *sdr* initiation sites: *oriJ*, *oriX* and 2 or 3 *oriK* sites. Among these potential origins, only *oriJ* was known to be the origin of a defective prophage, *rac* (Diaz, *et al.*, 1979; Kaiser and Murray, 1979); the rest are sequences of unknown origin. Topoisomerase I, HU protein and RNase H are proteins which normally repress replication from origins other than *oriC* (Kogoma, 1986; Ogawa, 1985).

Many plasmids (of different incompatibility groups), viruses and autonomously replicating sequences (ARS) can be replicated in *E. coli*, involving many different mechanisms of initiation and replication. Thus, it is not strange that the *E. coli* chromosome has so many potential initiation sites, since any sequence with sufficient homology to one of these origins may constitute a potential origin. *E. coli* is also flexible in its transcription system. Genes from the Gram positive bacterium *Bacillus subtilis* can be expressed in *E. coli*, whereas *B. subtilis* does not express most genes from *E. coli* or other Gram positive organisms (Dubnau, 1983). The enzyme systems in *E. coli* demonstrate a great flexibility, possibly to increase its ability to adapt to different situations. As will be discussed in section 1.7, *E. coli* can express cloned mycoplasma genes. The conservation observed in transcription systems is encouraging to people searching for possible conservation between the two replication systems. In addition to transcriptional and replicational flexibility, *E. coli* is a well-studied organism. It

is easy to transform. It has many well-characterized strains with different phenotypes and many well-established vector-host systems available for different cloning purposes. Except for the *E. coli* origin itself, 5 origins from 5 other Gram negative bacteria have been expressed in *E. coli*, including one from a taxonomically distinct marine bacterium (see section.1.2.).

1.5. THE oriC OF Bacillus subtilis

Except for *E. coli*, the most extensively studied bacterium is *B. subtilis*, a Gram positive bacterium whose origin region has been identified and sequenced. From the sequence of the origin region, 6 open reading frames (ORF) have been deduced. The 6 ORFs have homology to 6 genes in the *rnp-dnaA-gyrB* region of the *E. coli* chromosome and they are organized in the same order (Ogasawara *et al.*, 1985). Based on this homology the 6 ORFs found in *B. subtilis* have been designated: *'rnpA'*, *'rpmH'*, *'dnaA'*, *'dnaN'*, *'recF'*, *'gyrB'*. On two regulation regions flanking the *dnaA* gene, there are 9 and 4 *dnaA* boxes, which are supposed to be part of two origins in the *B. subtilis* chromosome (Ogasawara, 1986). In *E. coli*, the mini-*oriC*, with 4 *dnaA* boxes, has apparently been translocated 44 Kb away from this region. Thus the origin of *B. subtilis* is considered to be a more primordial origin than that of *E. coli* (Ogasawara, 1986).

Levine *et al.* (1987) radioactively labelled the part of the *B. subtilis* chromosome that replicates first in a synchronous round of DNA replication and the results suggest that the *B. subtilis* chromosome may contain two origins. However, their exact roles *in vivo* during normal replication is not known. The origin region of *B. subtilis* cannot be rescued as an *oriC*-plasmid even in *B.*

subtilis itself (D. Dubnau, pers.comm.). The apparatus of *E. coli* and *B. subtilis* replication demonstrated some homology. The *dnaA* genes are highly homologous between these two bacteria (Ogasawara, *et al.*, 1985) and the *gyrA* gene from *B. subtilis* can complement a *gyrA* mutant of *E. coli* (Lampe and Bott, 1985).

1.6. METHODS USED FOR CLONING CHROMOSOMAL ORIGINS

Phage λ and F plasmid were used initially to isolate the origin of *E. coli* so as to characterize its organization. Miki *et al.* (1978) successfully isolated the origin of *E. coli* using phage λ. The basic principle is that the λ genome is inserted into the chromosome in the vicinity of the origin and then a series of λ transducing strains are isolated which can transduce various markers located in this region. Restriction enzyme digestion of these λ strains released a fragment which, by self-ligation and transformation into a suitable host, formed an autonomously replicating plasmid. Currently, there are 3 types of vectors used for the cloning of a functional origin, *i.e.* to clone the origin by expressing it.

The first vector system is a marker-rescue system. In this system the origin to be cloned becomes the vector. The principle of this system is that potential origin fragments are ligated with a marker (antibiotic resistance) gene. The hybrid DNA is transformed into host cells which are then plated on selective medium for the marker, and only an origin-containing transformant will grow. Plasmids extracted from the transformants are likely to consist of a cloned functional origin ligated to the marker gene which itself is not capable of autonomous replication. With this method, Diaz *et al.* (1978) successively cloned the *oriJ* origin of *E. coli*

K-12 using an ampicillin resistance marker gene and Zyskind *et al.* (1979) cloned the *oriC* of of *Salmonella typhimurium* using a kanamycin resistance marker gene.

The second vector system is for cloning a single strand initiation site (SSI site) (see section 1.1). This SSI-cloning vector is an M13 derivative, M13ΔE101, which has a large deletion in its complementary strand origin with an *Eco*R1 cloning site replacing the deletion. It is defective in replication from the single-stranded form to the double-stranded form (about 1/20 its normal activity), and can only form tiny plaques. When an SSI fragment is inserted into the *Eco*R1 site, its replication activity will be restored and large plaques are formed. With this vector, several SSI have been cloned, including SSI from bacteriophages G4 and ϕX174, the plasmid GolE1, the *E. coli* chromosome and even one from a region of the *his* operon from *S. typhimurium* (Ray, *et al.*, 1982; Nomura, *et al.*, 1982). It is assumed that any Okazaki primosome assembly site can function as an SSI, so any organism which has a similar mechanism and apparatus as *E. coli* should contain sequences capable of restoring efficient replication to M13ΔE101.

The third vector is pMK2004, a derivative of pBR322, which has three antibiotic resistance markers and whose replication depends on DNA polymerase I. In a *pol*A host, pMK2004 cannot replicate unless an *oriC* fragment is inserted into it. There is a possibility, however, that the restoration of replication results from the insertion of a *pol*A gene which compensates for the host mutation, so a further selection for sensitivity of the transformants to methylmethane sulphonate is neccessay to demonstrate that the host cell is still *polA*. Takeda *et al.* (1982)

successfully used pMK2004 to clone the *oriC* of a plant pathogen, ***Erwinia carotovora***, in ***E. coli***.

Among these three currently used vector systems, the marker-rescue system is the simplest and the most straightforward one. My aim was to clone a functional *oriC*, so the marker-rescue cloning method was choosen.

1.7. MYCOPLASMA IS A DIVERGENT GROUP WITH UNIQUE CHARACTERISTICS

Mycoplasmas, belonging to the class Mollicutes, are the smallest and the simplest self replication prokaryotes. They have the smallest genome size among self replication organisms with as little a molecular weight as 500 M Dalton of DNA (Morowitz, 1976), which may code for approximately 650 cistrons, very close to the theoretically estimated minimal number (approximately 400) of essential proteins for a free-living microorganism (Morowitz, 1967, 1984). Because of the small genome size it is not strange that mycoplasmas have complex nutritional requirements and are poor in energy-yielding mechanisms. They have a restricted number of ribosomal RNA cistrons, transfer RNA species and a limited number of modified bases in their tRNAs (for review, see Stanbridge and Reff, 1979 and Razin, 1985). The dependence of mycoplasmas on complex growth media and their poorly defined metabolic pathways have hampered the selection of auxotropic mutants. The lack of Knowledge of their genetic system, lack of practical research method have prevented genetic studies on mycoplsmas. Mycoplasma genomes generally have a very low G+C content. With the exception of *Mycoplasma pneumoniae* and some *Anaeroplasma* spp., the G+C values of all

mycoplasmas, particularly those of bovine and caprine origin, exhibit values as low as 24 mol% (Razin and Tully, 1983) values which are slightly lower than the theoretical minimum of 26 mol% G+C required to code for proteins with a normal amino acid composition (Elton, 1973; Rogers, *et al.*, 1984). DNA-DNA hybridization between mycoplasmas and eubacteria has shown very little homology, a result which was instrumental in putting an end to the heated controversy centered around the notion that mycoplasmas are L-phase variants of present day eubacteria (Razin, 1969). The data from DNA-DNA hybridization between different species of the group have also indicated wide genetic heterogeneity of the mycoplasmas as a whole, and a marked heteogeneity within some established genera (Razin, 1969; Sugino, *et al.*, 1980; Aulakh, *et al.*, 1983). The discovery that these organisms are generally poorly endowed with repair mechanisms for ultraviolet light damaged DNA (Ghosh, *et al.*, 1977) and their marked heterogeneity has resulted in the idea that they are a rapidly evolving group (Woese, 1987). Because of these unique features many microbiologists take mycoplasmas to be distinctive and highly unusual bacteria that are distantly related to eubacteria. The sequence analysis of 5S rRNA and 16S rRNA genes, however, suggests that they are Gram positive bacteria (Rogers, *et al.*, 1985, Rasmussen, *et al.*, 1987). Despite their apparently mundane genealogy, the rRNAs of mycoplasmas are definitely unique. Mycoplasma rRNA sequences change more rapidly than those of eubacteria. More importantly, certain positions that tend to be invariant in other prokaryotes show significant variations in mycoplasmas (Woese, 1987). Because of the difficulties in the application of classical genetic techniques to, and in the transformation of, mycoplasmas, their genetics is not

application of classical genetic techniques to, and in the transformation of, mycoplasmas, their genetics is not well understood. Limited work has been done with several species, but it does not neccessarily mean that the results can be applied to other species within this group.

A small amount of research relating to DNA metabolism has been done with some species other than *M. pneumoniae*. Extrachromosomal circular duplex DNA molecules were found to exist in some *Mycoplasma* species (Morowitz, 1969), and these plasmids are cryptic and no function has as yet been assigned to them. Recent searches, however, have failed to confirm the presence of plasmids in *Mycoplasma* species (R. Herrmann, pers. comm.).

Membrane attachment of DNA replication has been reported (Smith and Hanawalt, 1968) as has the general DNA repair capacity of the organisms (Ghosh, *et al.*, 1977). Although some recombination involving transformation has been reported, the transformation efficency is too low to be used as a routine tool (S. Rottem, pers. comm.). Several *Spiroplasma* species have been found to contain three DNA polymerase activities (Charron, *et al.*, 1982); from *M. orale* and *M. hyorhinis*, only a single activity has been reported (Mills, *et al.*, 1977). The DNA polymerase activity purified by Boxer and Korn (1979) from *M. orale* appeared to contain only a single peptide and was devoid of exo- and endo-deoxyribonuclease (DNase) activities. Since the fidelity of this enzyme resembles that of *E. coli*, the nature of proof-reading in mycoplasmas is not clear.

In 1982, Nicolau and Rottem obtained expression of a β-lactamase gene in

pBR322 (Nicolau, and Rottem, 1982). The expression of cloned mycoplasma genes in *E. coli* has been demonstrated using either monoclonal antibodies or polyclonal antisera (Mouches, *et al.*, 1984; Kawauchi, *et al.*, 1986; Trevino, *et al.*, 1986). This indicates that the *E. coli* transcription and translation machinery can recognize the promotor and translation control sequences of mycoplasma DNA. This implies that the transcription and translation apparatus is conserved despite the divergent evolution that has occurred. However, none of these experiments have been performed with *M. pneumoniae*.

The mycoplsmas are a genetically divergent group and our knowledge about their molecular genetics is incomplete. The taxonomic distance between *E. coli* and *M. pneumoniae* is relatively large and it is possible that despite the observations discussed in the preceeding paragraph, the *M. pneumoniae* origin might not be expressed in *E. coli*. However, the possibility of there being an ARS from mycoplasma DNA that could function in *E. coli* cannot be ruled out on theoretical grounds. If any ARS could be obtained, that sequence might give us some information about the replication of mycoplasmas. If no ARS can be obtained, this result would also give us some idea about the evolutionary distance between *E. coli* and mycoplasmas, and the degree of conservation of replication machinery and sequences across such distances.

The reason why the particular species *M. pneumoniae* was used as the subject of this investigation is that *M. pneumoniae* is a pathogen of humans and animals that has been extensively studied both physiologically and biochemically. In addition, *M. pneumoniae* shares with other *Mycoplasma* (and *Ureaplasma*)

In addition, *M. pneumoniae* shares with other *Mycoplasma* (and *Ureaplasma*) spp. the smallest genome size known among self-replicating organisms (0.8×10^6 bp), (Morowitz, 1976). Identifying its origin and studying its replication apparatus will help us to build a replication model involving fewer complexities than in the eubacterial systems currently under study and perhaps determine the minimal essential genes needed for DNA replication in a self-replicating organism.

Chapter 2

MATERIALS AND METHODS

2.1. MATERIALS

All the restriction enzymes used were purchased from GIBCO/BRL Life Technologies, Inc., Burlington, Ontario. The T4 DNA ligase, BAP, BSA, pBR322 and DNA electrophoresis standards were also purchased from BRL. The ATP, agarose, ampicillin, kanamycin, tetracycline, bromphenol blue, EDTA, EGTA, Ficoll, glycerol, lysozyme, polyethelene glycol (PEG) 8000, polyvinyl-pyrollidone, proteinase K, RNase A, spermine, SDS, tris-base (Trisma) and tRNAs were all purchased from Sigma Chemical Company, St. Louis, MO, USA. Isopropanol, isoamylalcohol, xylene cyanol, and urea were purchased from BDH Chemicals, Dartmouth, Nova Scotia. Dithiotheitol (DTT), Triton X-100, acrylamide, bisacrylamide and TEMED were obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario. Formamide was from Fisher Scientific, Dartmouth, Nova Scotia. CIP was purchased from Boehringer Mannheim, Dorval, Quebec. Microbiological media components were purchased from Difco Laboratories (BDH Chemicals, Dartmouth, Nova Scotia). DE-81 cellulose ion exchange paper was purchased from Whatman Ltd., Maidstone, England. Sephadex G-50 (fine) was purchased from Pharmacia (Canada) Inc., Dorval, Quebec.

The purchased Km^r gene fragment [Pharmacia (Canada) Inc., Dorval, Quebec] was obtained as a purified fragment containing *Eco*R1 "sticky ends". The Km^r gene fragment which was prepared by author from pUKn (section 3.2.3) was completely digested with *Eco*R1 and recovered from a low temperature melting gel as described in section 2.3.4.1.

2.2. GROWTH OF CELLS

2.2.1. STRAINS OF CELLS

The sources, relevant genotype and references for the *E. coli*, *Mycoplasma* species and plasmids used in these experiments are listed in Tables 1, Table 2 and Table 3 respectively.

Table 1. STRAINS OF *E. coli* K-12

Strain	Genotype	Source
RRI	F⁻, *pro, leu, thi, lac Y, hsdR, hsdM, ara14, gal2, xyl5, mtl1, supE44 endo*I⁻	Rodriquez and Tait (1983)
RL108	F⁺, *leu, met,* r_k^-, m_k^+, *tet*ʳ, *recA56,*	Hines and Ray (1980)

Table 2. MYCOPLASMA SPECIES

Strain	Source
Mycoplasma pneumoniae FH	M. F. Barile[a]
M. fermentans PG-18	M. F. Barile[a]
M. gallisepticum PG-31	ATCC[b]
M. arginini G 230	ATCC[b]

a. M. F. Barile, Food and Drug Administration, Bethesda, Maryland, 20205 USA.
Reference: O'Brien *et al.* (1981)

b. ATCC: American Type Culture Collection, Rockville, Maryland, USA.

Table 3. PLASMID SPECIES

Plasmid	Relevant genotype	Reference
pUC12	*Ap*^{r}, *lacZ* α-complement fragment	Vieira and Messing (1982)
pBR322	*Ap*^{r}, *Tet*^{r}	Sutcliffe (1978)
pUKn	*Km*^{r}, *Ap*^{r}	This thesis
pOX	*Km*^{r}	This thesis
pOY	*Km*^{r}	This thesis

2.2.2. MEDIA AND GROWTH CONDITIONS

2.2.2.1. *Mycoplasma pneumoniae*

A. Standard Mycoplasma Medium (SMM): Standard mycoplasma medium (Whitcomb, 1983) consisted of 1.5% mycoplasma broth base (BBL, Fisher Scientific, Dartmouth, Nova Scotia) supplemented with 0.002% phenol red (Flow Laboratories, Inc. Burlington, Ontario), 0.5 % glucose, 500 U/ml penicillin (Flow Laboratories Inc., Burlington, Ontario), 10% yeast extract solution (25%, GIBCO/BRL Life Technologies, Inc., Burlington, Ontario.), 20% fetal calf serum (Bocknek, BDH Chemicals, Dartmouth, Nova Scotia), pH 7.5. Final medium was prepared by the aseptic addition of remaining components to a 2.2% solution of broth base that had been titrated to pH 7.7 and autoclaved.

B. Mycoplasma wash solution: 0.25 M NaCl, 20 mM Na_2HPO_4-HCl·pH 7.0, 10 mM Mg_2SO_4

C. Growth and Harvesting of *Mycoplasma pneumoniae* cells: All *M. pneumoniae* cultures were grown in tissue culture flasks of the appropriate size (GIBCO/BRL Life technologies, Inc. Burlington, Ontario). Starter cultures of 10 mls of SMM were inoculated with 100 µl of cell stock stored at -80^{0}C and incubated at 37^{0}C for about three days until mid-log phase (medium yellow-orange in colour). Log-phase starter cultures were used to inoculate 90-100 mls of medium. Cells adhering to the surface of the flask were removed by gentle shaking and with a sterile rubber scraper. Cultures were incubated at 37^{0}C until mid-log phase (medium yellow-orange in colour).

The cells were collected at 4^{0}C by centrifugation at 12 000 x g for 20 minutes, washed twice with mycoplasma wash solution, and once with TE (50/10) (50 mM Tris-HCl pH 7.5, 10 mM EDTA). Washed cells were resuspended in about 40 µl TE (50/10) and transferred to a 1.5 ml microfuge tube. An additional 1 ml of TE (50/10) was used to rinse the centrifuge tube and the two suspensions were combined. Cells were washed twice more with TE (50/10), resuspended in 100 µl TE (50/10) and frozen at -20^{0}C.

2.2.2.2. *Escherichia coli*

A. Growth of *E. coli* for Plasmid Extraction: Bacteria were grown overnight to stationary phase in Luria Broth (titrated to pH 7.5 instead of pH 7.0) (Miller, 1972) supplemented with glucose (0.2 %) and antibiotics appropriate for the plasmid. Cultures were shaken at 37^{0}C until they reached the stationary phase. In these experiments the final concentrations of kanamycin, ampicillin and tetracycline were respectively 40µg/ml, 50µg/ml and 20µg/ml.

2.3. DNA PURIFICATION METHODS

2.3.1. MATERIALS

TE buffer (10/1): 10 mM Tris-HCl pH 8.0, 1 mM EDTA

TE/SDS buffer: 0.1% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

1% SDS in TEE : 1% SDS, 50 mM Tris-HCl pH 8, 10 mM EDTA, 2 mM EGTA.

RNase A buffer: 10 mg/ml Ribonuclease A (RNase A) in 0.1 M sodium acetate, 0.3 mM EDTA pH 4.8, heated to 80^{0}C for 10 min, and stored at -20^{0}C.

Proteinase K: 20 mg/ml, in TE / SDS, incubated at 30^{0}C for one hour and stored at -80^{0}C.

KP buffer: 10 mM potassium phosphate pH 7.0, 150 mM NaCl.

TEN buffer: 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 mM NaCl.

DNA wash solution: 1.5 M NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4.

Isopropanol.

2.3.2. Extraction of *Mycoplasma pneumoniae* Chromosomal DNA

The washed *M. pneumoniae* cells prepared according to the procedure described in 2.2.2.1. were lysed by the addition of 1 ml of 1 % SDS in TEE and, after gentle inversion, 5.5 μl proteinase K were added. The tube was inverted several times to mix the contents and incubated at 55^{0}C for 20 min. RNase A

(5.5 μl) was added followed by incubation at 37^{0}C for one hour. SDS was precipitated by the addition of 100 μl of 5 M potassium acetate (untitrated), incubation on ice for 30 min, and centrifugation for 10 min in a microfuge (Fisher). The pellet was discarded. The supernatant was gently extracted with an equal volume of chloroform/isoamylalcohol (24:1, v/v) for 15 min at room temperature. The two phases were separated by centrifugation in a microfuge for 2 min. The aqueous (top) layer was removed and extracted two more times. Sodium acetate buffer (3 M, pH 4.8, 45 μl) was added and the tube was filled with 95% ethanol to precipitate the DNA. A sterile pasteur pipette flame-sealed at the tip was used to pick up and wash the recovered DNA in 70 % ethanol. The DNA was dissolved in 450 μl TE (10/1).

Isopropanol precipitation was used as a final clean-up step, in which 50 μl 3M sodium acetate (pH 4.8) and 500 μl isopropanol were added and the contents mixed. The precipitated DNA was lifted out, washed in 70 % ethanol and dissolved in TE (10/1). The final volume of TE (10/1) used depended on the yield of DNA.

The DNA concentration was determined quantitatively by electrophoresing 1 μl DNA solution on an agarose gel. Lambda bacteriophage DNA digested with *Hind* III standards was electrophoresed on the same gel for comparison. EtBr was included in the buffer of the gels. The intensity of the bands was compared, and DNA concentration of samples was calculated from the DNA amount as estimated by comparison with the λ DNA standard. Alteratively, the DNA concentration was determined by measuring the absorbance at 260 and 280 nm of DNA samples

diluted as appropriate with KP buffer using a Shimadzu Double-Beam Spectrophotometer, UV-200 (Bausch and Lomb).

2.3.3. EXTRACTION OF PLASMID DNA FROM *E. coli*

2.3.3.1. MINISCEEN PREPARATION

The procedure of Rodriguez and Tait (1983) was followed.

2.3.3.2. LARGE SCALE PLASMID PREPARATION

Plasmid DNA was prepared using either an alkaline lysis method (Rodriguez and Tait, 1983) or a rapid boiling method (Gold, 1984), which is the modified method of Holmes and Quigley (1981).

2.3.3.3. PURIFICATION BY CsCl CENTRIFUGATION

The plasmid DNA isolated as described in 2.3.3.2. was further purified by CsCl centrifugation. To plasmid DNA dissolved in 200-1,000 μl TE (10/1) buffer was added 6 mls of saturated CsCl solution (Miller, 1972) and 1 ml of EtBr (3 mg/ml, in TEN), and the refractive index of the solution was adjusted to 1.3900$\pm$ 0.0005 by the addition of TEN buffer. The tubes were completely filled with mineral oil and centrifuged at 38,000 r.p.m. and 20^{0}C for 40 h in a 50 Ti rotor in an L5-50B ultracentrifuge [Beckman Instruments (Canada), Toronto, Ontario].

After centrifugation, aliquots of 15 drops were collected by puncturing the bottom of the tube. The plasmid-containing fractions were identified by gel electrophoresis of samples from each fraction. The plasmid-containing fractions were pooled, the EtBr was removed by extraction with approximately equal volumes of 2-butanol until the colour completely disappeared, and then the

solution was extracted once more. After extraction of EtBr, the plasmid DNA was recovered by precipitation by one of the following methods.

Method <1>: To the above solution 1.6 volumes of TE (10/1), 1/10 volume of 4 M ammonium acetate and 2.5 volumes of ethanol were added. The DNA solution was allowed to precipitate at -20^{0}C overnight. The pellet was collected by centrifugation at 12,000 x g for 30 min at 4^{0}C. The pellet was dissolved in 400-500 μl TE (10/1) and the precipitation as indicated above was repeated. The final pellet, after being dried briefly *in vacuo*, and being dissolved in an appropriate amount of TE (10/1), was stored at 4^{0}C.

Method<2>: The volume of DNA solution after extraction of EtBr was measured. To each 0.5 ml, 1.1 mls of water and 3.2 mls of 95% ethanol were added. The pellet was collected by centrifugation for 5 min and washed with 70% ethanol once or twice before being dissolved in TE (10/1).

2.3.4. PURIFICATION OF DNA FRAGMENTS FROM AGAROSE GELS

2.3.4.1. DNA PURIFICATION FROM LOW MELTING TEMPERATURE AGAROSE GELS

A low melting temperature agarose gel (Sigma, Type VII) with a large well of appropriate size to contain the DNA sample to be loaded, was prepared. To make the large wells, the teeth of a standard comb were joined with tape. The electrophoresis was carried out at approximately 2.5 volts/cm for 18 h in the dark. The gel was examined under U.V. light with only one edge being exposed. The band of interest was marked and cut out of the gel.

The excised piece of gel was placed in 4 volumes of TE (10/1) and heated to 65^{0}C until completely melted (approximately 30 min). The DNA solution was extracted twice with an equal volume of phenol and once with phenol/chloroform (1:1, v/v). Following each extraction the phases were separated by centrifugation into aqueous (top) and organic (bottom) phases with a white agarose and protein layer in between.

The final aqueous phase was transferred to a clean tube, the salt concentration was adjusted to 0.3 M sodium acetate by adding 1/9 volume of 3 M NaOAc (pH 4.8), and the DNA was precipitated by adding 2 volumes of 95 % ethanol. After brief drying *in vacuo* the DNA pellet was dissolved in TE (10/1).

2.3.4.2. DNA PURIFICATION FROM AGAROSE GELS WITH DE 81 FILTER PAPER

Electrophoresis and band identification were carried out in the same way as described in 2.3.4.1. A thin slice of gel was cut from in front of the band of interest (taking the DNA migration direction as forward). A piece of DE 81 cellulose ion exchange paper (Whatman) sized to fit exactly the cross-section of the gel was inserted vertically into the well against the cut edge closest to the fragment to be recovered. The DE 81 paper was fixed firmly in position by the insertion of a slice of clean gel cut from the end of the gel. The DNA band immediately behind the band of interest was also cut out and replaced with a clean gel slice.

The gel was put back into the chamber and the buffer was added back without covering the upper surface of the gel. Electrophoresis was carried out at 7

volts/cm for 5-6 min. The gel was examined under U.V. light to be sure that all the DNA of interest was bound to the DE 81 paper.

The DE 81 paper was divided between two microfuge tubes and to each tube 300 μl of DNA wash solution was added. The paper was broken up with a syringe needle, the tubes were vortexed and incubated at 37^0C for 15 min.

Holes were punched through the top and through the bottom of the microfuge tubes with a syringe needle. The tubes were placed in the top of a Falcon 2063 centrifuge tube (Becton Dickson Labware, Beckton Dickinson and Company, Oxnard, CA USA) and centrifuged briefly to force the DNA wash solution into the Falcon tubes. The DE 81 paper was transferred to two clean microfuge tubes, to be washed once more and the wash solution collected as before into the same two Falcon tubes.

The combined DNA wash solutions were transferred to two 1.5 ml microfuge tubes. To each tube 0.6 ml of isopropanol was added and the DNA was allowed to precipitate at -20^0C overnight. The precipitated DNA was collected by centrifugation for 5 min in a microfuge. The pellet was washed with ice-cold 70% ethanol 3-4 times, dried *in vacuo* at room temperature, and dissolved in about 20 μl TE (10/1).

2.4. GEL ELECTROPHORESIS OF DNA SAMPLES

2.4.1. MATERIALS

Double dye solution (DDye):
0.1 % Bromphenol blue, 0.1 % xylene cyanol, 50 % glycerol, 50 mM EDTA, 5 mM Tris-HCl pH 7.5

Reaction stop mix (RSM):
30% (w/v) urea, 10% (v/v) glycerol, 0.025% (w/v) bromphenol blue, 0.025% (w/v) xylene cyanol, 0.5% (w/v) SDS.

1x Tris-borate buffer (TBE):
0.089 M Tris-base, 0.089 M boric acid
0.002 M EDTA, pH 8.0.

1x Tris-acetate buffer (TAE):
0.04 M Tris-acetate, 0.02 M EDTA, pH 8.0.

Molecular weight standards:
λDNA digested with *Hind* III
123 bp ladder
DNA high molecular weight standard (HMW)
ϕx 174 RF DNA digested with *Hae* III

2.4.2. AGAROSE GEL ELECTROPHORESIS

Agarose gel stocks were prepared at 1.1 times final concentration in water and autoclaved. Prior to pouring, gel stocks were melted in a boiling water bath. The molten gel was immediately mixed with 10x TBE or 10x TAE buffer in the volume ratio of 9 : 1. EtBr (2 mg/ml) was added: 1 μl/4 mls (gel+buffer) for minigels or 1 μl/5.3 mls of (gel+buffer) for standard gels.

The DNA samples were mixed with approximately 0.3 vol RSM or DDye prior to loading onto a gel. All the molecular weight standard DNAs were used

following the manufacturer's instructions. The amounts of DNA standard loaded per well varied from experiment to experiment but the *Hind* III digested λ DNA marker was always added at 325 ng per well.

Minigels were usually run at 7.5 volts/cm, but occasionally at 5-6 volts/cm for better resolution. The standard gel was usually run at 7.5-8.5 volts/cm unless otherwise specified.

2.4.3. POLYACRYLAMIDE GEL ELECTROPHORESIS

All reagents were prepared according to Maniatis *et al.* (1982) and polyacrymide gel electrophoresis was carried out in an SE 600 vertical slab gel electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, U.S.A.).

Gel preparation also followed Maniatis *et al.* (1982) with the exception that the gel solution was degased before ammonium persulfate and TEMED (Bio-rad) were added. Before the sample was loaded, the gel (14.5 cm long, 1.5 mm thick) was run at 200 volts for 30 min to remove non-polymerized acrylamide. After loading, the DNA was electrophoresed at 75 volts for about 7-9 h, depending on the sizes of the DNA fragments to be separated.

After electrophoresis, the gel was stained in 2μg/ml EtBr solution for 20 min with occasional agitation. Distilled water was used to remove the excess EtBr.

2.5. MOLECULAR HYBRIDIZATION

2.5.1. NICK TRANSLATION OF DNA PROBE

DNA probes were labelled with a Nick Translation Kit (N-5000, Amersham Corporation, Oakville, Ontario) following the manufacturer's instructions. The unincorporated α-^{32}P-dCTP was separated from the labelled probe by passage through a Sephadex G-50 column equilibrated with elution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA). Aliquots were collected into microfuge tubes which were counted in a Beckman LS-3150T liquid scintillation counter (Beckman Instruments, Inc. Palo Alto, California, USA). The fractions containing the first peak were pooled and the DNA was precipitated and redissolved in TE (10/1).

The incorporation of α-^{32}P-dCTP was determined by measuring the trichloroacetic acid-precipitable radioactivity of the pooled, concentrated DNA.

2.5.2. TRANSFER OF DNA TO NITROCELLULOSE MEMBRANES

2.5.2.1. SLOT BLOT

Slot blotting of DNA onto nitrocellulose membranes was carried out with the Minifold II apparatus (Schleicher and Schuell, Inc., Keene, N.H, USA). The appropriate amount of DNA sample for one slot was dissolved in 100 μl of TE/SDS buffer, denatured by adding 1/10 volume of 3 M NaOH and incubated at 60-70^{0}C for 1 hour. After being cooled to room temperature, one volume of 2 M NH_4OAc pH 7.0 was added and the sample was loaded by aspiration onto the filter.

After the filter was air dried with a heat lamp it was baked *in vacuo* at 80^0C for 2 h and then stored at 4^0C.

2.5.2.2. SOUTHERN BLOT

The denaturation and Southern blotting of DNA onto nitrocellulose membranes was carried out with a BRL DNA/RNA blot transfer system (GIBCO/BRL Life Technologies, Inc., Burlington, Ontario) following the manufacture's instructions. DNA to be transferred was denatured but not depurinated prior to transfer.

2.5.3. HYBRIDIZATION

2.5.3.1. MATERIALS

1 x SSPE buffer: 180 mM NaCl, 20 mM $NaHPO_4$ PH 7.4
1 mM EDTA.

1 x Denhardt's solution:
0.02 % each of Ficoll, polyvinyl-pyrollidone and bovine serum albumen in 0.2 x SSPE.

Prehybridization mixture:
50 % formamide, 2 x Denhardt's, 0.1 % SDS, 5 x SSPE, 100μg/ml tRNA.

2.5.3.2. PROCEDURE FOR DNA HYBRIDIZATION

The DNA hybridization followed the method published by Schleicher and Schuell (Barinaga *et. al*, 1981). The baked nitrocellulose filter was prehybridized at 42^0C for 5 min in a heat sealable bag with 100 μl of prehybridization mixture per cm^2 of nitrocellulose filter. Contents of the bag were mixed by rotation on an inclined rotary table. Usually about 10^6 CPM of probe was denatured by the addition of 1/10 volume of 1 M NaOH and incubation at 37^0C for 5 min before

addition to the bag containing the filter and prehybridization mixture. The bag was resealed and the DNA hybridization was carried out at 42^{0}C overnight with continuous mixing on the rotary table. After hybridization, the nitrocellulose filter was washed twice with 250 mls of 1 x SSPE/0.1% SDS for 30 min at 42^{0}C, the filter was then washed twice more for 45 min at 50-55^{0}C. The washed filter was blotted dry, wrapped in Saran wrap and placed in a film holder. The film was exposed at -80^{0}C for 5-8 days with X-ray film (Kodak, X-Omat AR film) and an intensifying screen (Dupont par-speed el) and then developed.

2.6. RESTRICTION NUCLEASE DIGESTION AND LIGATION OF DNA

2.6.1. RESTRICTION NUCLEASE DIGESTION

All restriction enzymes were purchased from GIBCO/BRL (GIBCO/BRL life Technologies, Inc. Burlington, Ontario), and, unless otherwise specified, the conditions of the restriction digestions were either according to Rodriguez and Tait (1983) or following the manufacturer's instructions. When neccessary, the enzymes were diluted with restriction enzyme dilution buffer: 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM EDTA pH 7.5, 1 mM dithiothreitol, 50 % (v/v) glycerol, 0.5 mg/ml BSA. Restriction nuclease digestions were terminated either by heating at 65^{0}C for 5-10 min or by the addition of RSM (Section 2.4.1).

2.6.2. DEPHOSPHORYLATION OF DNA

DNA fragments were dephosphorylated with either bacterial alkaline phosphatase (BAP) or calf intestine phosphatase (CIP). Unless otherwise indicated, the proportion of enzyme to DNA ends in the reactions was calculated following the manufacturer's instructions. Unless otherwise indicated, the pH was adjusted to pH 8.0, the enzyme added and the mixture incubated. CIP treatment was carried out at 37^0C for 30 min and stopped by heating at 65^0C for 45 min. If after CIP treatment the DNA concentration was too low for the ligation, reprecipitation was carried out. BAP treatment was at 65^0C for 1 hour, and was stopped by the extraction of protein with chloroform:isoamylalcohol (24:1, v/v). After three extractions, the DNA was precipitated from the aqueous phase by the addition of 1/9 volume of 3 M NaOAc (pH 4.8) and 2 volumes of 95% ethanol. The DNA pellet, dried *in vacuo*, was dissolved in TE (10/1) and its concentration determined by electrophoresis.

2.6.3. DNA LIGATION

2.6.3.1. CALCULATION OF END CONCENTRATION AND FRAGMENT RATIO

Principles: All the variations of fragment concentration and ratio were based on data or principles from the following papers or considerations. For example:

According to Maniatis *et al.* (1982), when a fragment has $j/i > 1$ its self-ligation is dominant over its ligation with another fragment, where $j = (3/2\pi lb)^{3/2}$, and is the effective concentration of one end of the DNA molecule in

the immediate neighbourhood of the other end of the same molecule, "l" is the length of DNA in cm, "b" is the length of a randomly coiled segment of DNA, and "i" is the total end concentration in the ligation.

Thus, for the first experiment in Table 4., the value j of the desired hybrid fragment (a mycoplasma DNA fragment ligated with a Km^r gene fragment) j_{hyb} was calculated and the value of total initial "i" was set equal to the calculated j_{hyb}. The hybrid molecule was idealistically supposed to be composed of one fragment of Km^r gene and one fragment of mycoplasma DNA. According to Rodriguez and Tait (1983), in practice, it is only when $j/i > 2\text{-}3$ that the self-ligation becomes dominant. In the first experiment in Table 4. the ratio $j_{hyb}/i = 1$ was chosen because of the belief that when the ligation starts the "i" will decrease and the ratio of j/i will increase. Also in this experiment a ratio of $i_{PH}/i_{Km^r} = 1/2$ was chosen, based on the belief that increasing the dephosphorylated fragment relative to the phosphorylated fragment increases the probability of forming the desired hybrids without the loss of as many fragments as increasing the phosphorylated fragment would do.

In experiments 2-4 in Table 4. different variations of experiment 1 with respect to the total concentration of DNA fragments and their ratio were tried.

King and Blakesley (1986) recommended that the vector/insert ratio should be 3/1 with the vector dephosphorylated, and the total end concentration, "i", should be 80 fmol/20 μl ligation solution. Those conditions were basically followed in experiments 5-8 in Table 4.

An article entitled "Ligation theory and Practice Part II" published in BRL-Focus (Vol.2, No.3, p.1, 1979) made recommendations about concentrations and fragment ratios. According to the data in this article, 238 ng of mycoplasma DNA and pUC12 DNA mixture (the average molecular weight of the DNA fragments in the mixture is estimated to be approximately 13 x 10^6 KDa), should be ligated with approximately 120 ng Km^r gene fragment in 100 μl ligation buffer. These were the conditions used in the experiments listed in Table 5. with some variations, especially in the ligation volume.

2.6.3.2. DNA LIGATION

T4 DNA ligase was used in this study. The specific ligation conditions used, such as ligation buffer, temperature, time, ATP concentration, the concentration of DNA ends, and the ratio of different DNA fragments varied widely and are reported for each experiment in Chapter 3.

When ligation was carried out at 13^0C, the ligation reaction was incubated in a refrigerated water bath; when it was carried out at 24^0C, it was incubated in an ordinary water bath.

The composition of the two buffers used are given below:

Ligation buffer A: 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermine.

Ligation buffer B: 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% (w/v) PEG 8000, 1 mM DTT.

2.7. TRANSFORMATION OF E. coli

2.7.1. PREPARATION OF COMPETENT CELLS

Competent cells were prepared by the method of Dagert and Ehrlich (1979). The competent cells were either used after 1-3 h incubation or their competence was tested after 1-3 h incubation and, if high enough (more than 4000 transformants per 1 ng of pUC12), the cells were used after 24 h incubation.

2.7.2. TRANSFORMATION

DNA samples to be transformed were usually diluted 5-10 times with TE (10/1) or 0.1 M $CaCl_2$ before addition to the cell suspension. More dilute DNA aliquots (10 µl) were diluted with 20 µls of 0.1 M $CaCl_2$ before addition to 100-200 µl cells and the mixture was incubated on ice for 45-60 min. When kanamycin or ampicillin resistance was used as the selective marker, the transformed cells were diluted with 1.5 mls of Luria Broth and incubated for 30 min at 37^0C in a shaking water bath. The cells were pelleted by centrifugation. The supernatant was decanted leaving the last 1-2 drops to resuspend the pellets before spreading on selective plates. When tetracycline resistance was used as the selective marker, transformed cell suspensions were plated directly onto selective plates without expression. Selective plates consisted of Luria Broth solidified with 1.5% agar and supplemented with 0.2% glucose and antibiotics as appropriate. The concentrations of the antibiotics in selective media were 40µg/ml, 50µg/ml and 20µg/ml for kanamycin, ampicillin and tetracycline, respectively.

All plates were incubated at 37^0C overnight. Positive (pUC12, pUKn or

pBR322 DNA) and negative (0.1 M $CaCl_2$) controls were included in each transformation experiment.

Chapter 3
RESULTS

3.1. PRINCIPLES AND BRIEF SUMMARY OF THE EXPERIMENTS AND RESULTS

In these experiments the marker rescue method, using a kanamycin resistance (Km^r) gene as the marker gene to be rescued, was used in an attempt to clone a functional ARS from *M. pneumoniae* DNA. *M. pneumoniae* chromosomal DNA was partially digested with *Eco*R1 and ligated with the purified Km^r gene fragment. The ligation reaction mixtures were used to transform competent *E. coli* RR1 cells, and Km^r transformants were selected. Eight ligation and transformation experiments involving a variety of conditions were performed, and no Km^r transformants were obtained. On the other hand, in a positive control experiment with pUC12 as a known origin source, numerous Km^r transformants were obtained. In order to eliminate or reduce the differences in ligation and transformation between these two experiments., an internal control experiment was designed. In this experiment, the partially *Eco*R1-digested *M. pneumoniae* DNA and pUC12 DNA (containing an Ap^r gene) completely digested with *Eco*R1 were mixed in a 1:1 genomic ratio. The mixture was ligated with the Km^r gene fragment and then the ligation reaction mixture was used to transform *E. coli* RR1 competent cells. Seven transformations were carried out from which

about 150 transformants were obtained. All except one were Km^rAp^r in phenotype, which means they are pUC12-Km^r transformants. The plasmid extracted from the only Km^rAp^s transformant was analysed by restriction endonuclease mapping and DNA blot hybridization. Its structure has been deduced and the origin sequence cloned has also been identified as the origin of pUC12.

3.2. CONSTRUCTION OF pOX BY MARKER RESCUE

3.2.1. PARTIAL DIGESTION OF *M. pneumoniae* CHROMOSOMAL DNA

The principle used for attempting to clone the mycoplasma origin of DNA replication was marker (Km^r) rescue. The Km^r gene fragment was ligated with mycoplasma chromosomal DNA fragments and the mixture used to transform *E. coli* RR1 cells, with Km^r transformants being selected. Such Km^r transformants should contain a plasmid which is composed of the Km^r gene fragment and a DNA fragment from *M. pneumoniae* capable of functioning as a replication origin in *E. coli*.

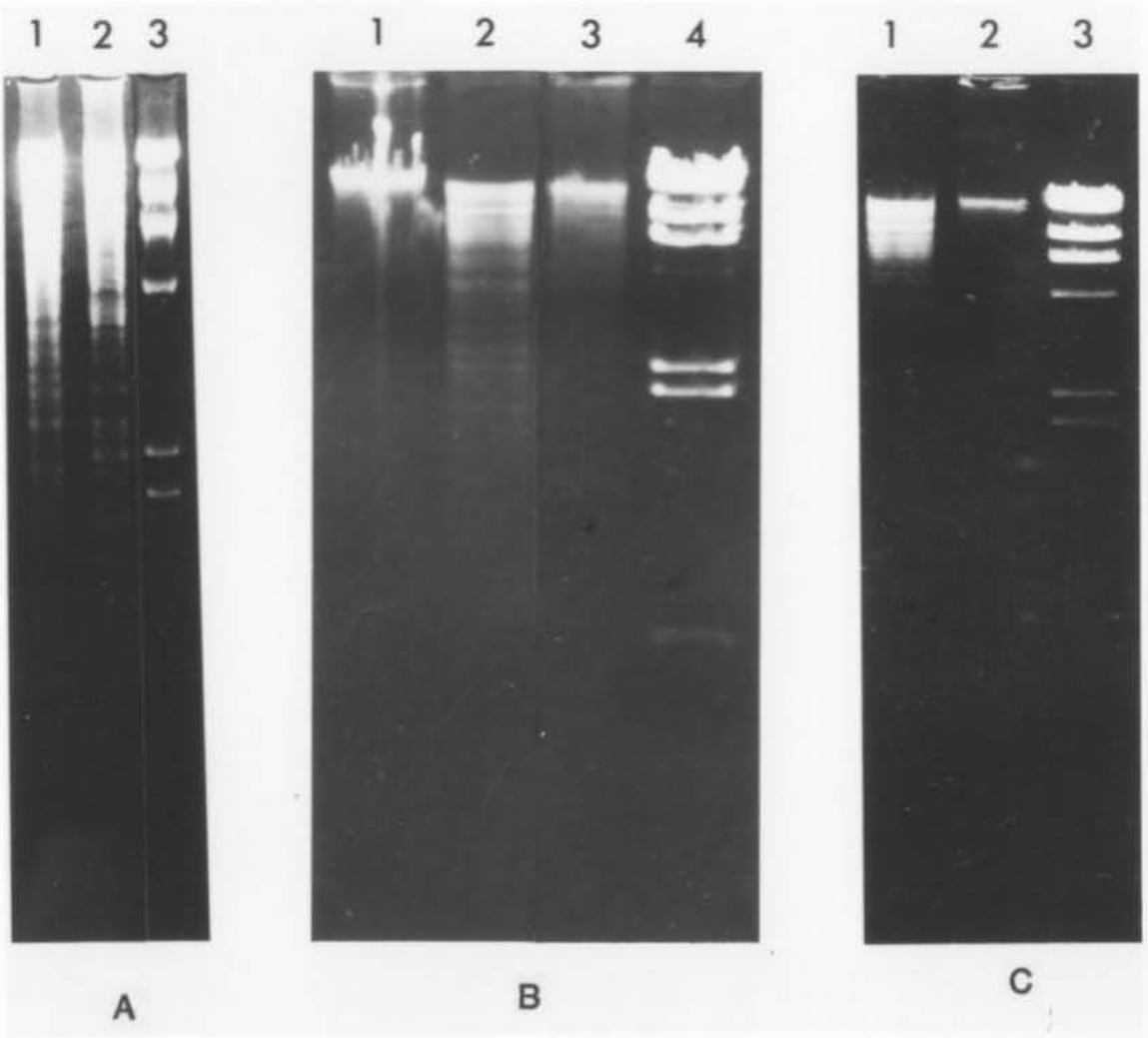
Before ligation, *Mycoplasma pneumoniae* DNA was partially digested with *Eco*R1 to avoid cutting all fragments within the origin at an *Eco*R1 site, should there be one there. Partial digestion products were examined by agarose gel electrophoresis and the average fragment size was determined from the gel pattern to be about 20 Kb. Fig. 4A, lane 2 shows the electrophoresis pattern of completely digested *M. pneumoniae* chromosomal DNA and Fig. 4B,

Fig. 4. Characterization of *M. pneumoniae* DNA

A. *Eco*R1 complete digestion of *E. coli* (lane 1) and *M. pneumoniae* (lane 2) DNA. Lane 3 is λ DNA digested with *Hin*dIII molecular weight marker.

B. *Eco*R1 partial digestion and ligation of *M. pneumoniae* DNA.
Lane 1: undigested *M. pneumoniae* DNA.
Lane 2: *M. pneumoniae* DNA partially digested with *Eco*R1.
Lane 3: *Eco*R1 digested *M. pneumoniae* DNA ligated with the Km^r gene fragment
Lane 4: λ DNA digested with *Hin*dIII.

C. *Eco*R1 partial digestion and ligation of *M. pneumoniae* DNA. The average fragment size is estimated to be 20 Kb.
Lane 1: *M. pneumoniae* DNA partially digested with *Eco*R1.
Lane 2: *Eco*R1 digested *M. pneumoniae* DNA ligated with the Km^r gene fragment
Lane 3: λ DNA digested with *Hin*dIII.


1 2 3
1 2 3 4
1 2 3
A
B
C

lane 2 and Fig. 4C, lane 1 show the pattern of partially digested *M. pneumoniae* DNA. Fig. 4B, lane 3 and Fig. 4C, lane 2 also demonstrate that partially digested *M. pneumoniae* DNA is capable of being ligated, indicating that the digestion products are not inhibitory to the ligation reaction.

3.2.2. LIGATION AND TRANSFORMATION

The first stage in the experiment was to attempt to recover the mycoplasma origin of DNA replication by direct ligation with the Km^r gene, followed by transformation of *E. coli* competent cells. Eight ligations and transformations were done with the ligation conditions varied widely as shown in Table 4. Each time competence was tested prior to transformation and usually was approximately $4x10^6$ transformants per μg of pUC12 or pBR322 DNA. None of these experiments resulted in a single Km^r transformant.

3.2.3. CONSTRUCTION OF PLASMID pUKn

In order to determine whether the Km^r gene fragment is capable of being rescued by an exogenous origin sequence, a positive control experiment was carried out. In this experiment pUC12 DNA was used as the source of DNA replication origin. It was completely digested with *Eco*R1 before ligation with the Km^r gene and the ligation reaction mixture was used to transform *E. coli* cells. In this experiment, a 20 μl ligation mixture was used. It contained 36 ng of pUC12 and 20 ng of the Km^r gene (the approximate molar ratio of pUC12/Km^r gene is 1). For one Km^r selection plate, 5 μl of this ligation mixture, diluted with TE (10/1) buffer, was added to 200 μl of competent cells. Half of the ligation mixture yielded 122 Km resistant transformants.

Table 4. Ligation conditions used in marker rescue method for cloning functional *M. pneumoniae* origin in *E.coli*

Expt. No.	ChDNA[a] (*M. pneu.*) ng/fmol	Km^r frag. ng/fmol	deP'd frag.[b]	ChDNA/ Km^r ratio[c]	RXN vol. μl	Concn of frag.[d] pM	Temp. ^0C	Time h	Buffer[e]	ATP mM
1	125/13.3	20/20	Km^r	0.67	10	3	13	24	A	0.5
2	250/26.7	80/80	Km^r	0.33	15	6	13	24	A	0.5
3	375/40	40/40	Km^r	1.00	15	4.6	13	24	A	0.5
4	250/26.7	60/60	Km^r	0.44	20	4	13	24	A	0.5
5[f]	750/80	20/20	ChDNA	4.0	20	4	24	4	B	1
6	750/80	20/20	ChDNA	4.0	20	4	24	4	B	1
7	500/53.3	40/40	None	1.33	20	4	24	4	B	1
8	250/26.7	60/60	None	0.44	20	4	24	4	B	1

a. The average molecular weight of the fragments of *M. pneumoniae* chromosomal DNA (ChDNA) partially digested with *Eco*R1 was approximately 15 Kb as determined by gel electrophoresis.

b. "deP'd frag." is an abbreviation for the "dephosphorylated fragment". The Km^r gene fragment (1.5 Kb, according to the information supplied by the manufacturer) used in these experiments was purchased from Pharmacia and had already been digested with *Eco*R1.

c. "ratio[c]" is the molar ratio of *M. pneumoniae* chromosomal DNA (ChDNA) fragments to Km^r fragment.

d. The concentration of total DNA fragments was calculated from the data in the ChDNA, Km^r and reaction mixture (RNX) vol. columns. The values of the total molar concentration of DNA fragments and the ratio of the two DNA fragments choosen for these experiments were based on the considerations discussed in section 2.6.3.1.

e.The composition of the two buffers used are given below:

Ligation buffer A: 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermine.

Ligation buffer B: 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% (w/v) PEG 8000, 1 mM DTT.

One unit of T4 ligase was used in each reaction.

f. Experiments 5-8 basically followed the optimal ligation conditions recommended by King and Blakesley (1986), which are: Buffer B, 1 mM ATP, 1 U T4 ligase, vector/insert molar ratio = 3/1, incubation at room temperature for 4 h, 3-5 x dilution before addition of the DNA to the competent cells. In their standard experiment, the vector was dephosphorylated.

Plasmids could be extracted from these transformants and the extracted plasmids transformed *E. coli* into Km resistance. The *Eco*R1 digestion of this plasmid produced 2 bands: one comigrated with the known Km^r gene fragment and the other comigrated with linear pUC12 (data not shown). This plasmid is therefore derived from the insertion of the Km^r gene into the *Eco*R1 site of pUC12, and was named pUKn.

The plasmid pUKn was subsequently used to produce a large amount of the Km^r gene fragment. Plasmid extracted by the large scale, rapid boiling method was completely digested with *Eco*R1 and the Km^r gene fragment was recovered from low temperature melting gel (see section 2.3.4.1.). The purity of this Km^r fragment was tested by transformation of *E. coli* with 10ng and 20ng of the Km^r gene fragment following self-ligation. No transformants were found.

3.2.4. LIGATION WITH AN INTERNAL CONTROL

In order to standardize the pUC12 origin and mycoplasma origin under the same ligation conditions, an internal control experiment was designed. In this experiment the *Eco*R1 partially digested mycoplasma DNA and completely digested pUC12 DNA were examined on a gel, mixed in a 1:1 genomic ratio, (20.8 μg of mycoplasma DNA mixed with 72 ng of pUC12) and then dephosphorylated with bacterial alkaline phosphatase (BAP). The size of pUC12 is 2680 bp and that of *M. pneumoniae* is assumed to be 0.8×10^6 bp (Morowitz, 1976). Following phenol extraction and recovery of the DNA, electrophoresis was carried out to determine the DNA concentration and to ensure small fragments had not been lost during these manipulations.

A total of 7 experiments were performed with variations in the ligation conditions used. The ligation and transformation conditions as well as the results are shown in Table 5. From these experiments, a total of 160 Km^r transformants were obtained. All except one were Km^rAp^r. A plasmid was extractable from the only Km^rAp^s transformant obtained. This transformant was determined to be *E. coli* strain RR1 and not a chance contaminant by examining its genotype (*pro, leu, lacY*). The plasmid which was isolated from the transformant, named pOX, could transform *E. coli* RR1 into Km^r. As shown in Fig. 5A, complete digestion of pOX with *Eco*R1 (Fig. 5A, lane 2) apparently produced 2 bands: one comigrated with the Km^r gene fragment (data not shown) and the other was 9.3 Kb in size. The intensity of the Km^r gene fragment was observed not to be in proportion to its size, an observation which led to the suggestion that the plasmid pOX contains multiple copies of the Km^r gene fragment.

3.3. SOURCE OF ORIGIN FRAGMENT IN PLASMID pOX

3.3.1. pOX CONTAINS AN *M. pneumoniae* DNA FRAGMENT

In order to determine the source of the putative origin, assumed to reside in the 9.3 Kb fragment, the following experiments were carried out. In these experiments, nick translation was used to label plasmid pOX with ^{32}P and the probe was used to hybridize with *E. coli* DNA, *M. pneumoniae* DNA, and DNA from several other species of mycoplasma which had been digested with *Eco*R1.

The result (shown in Fig. 6) was that pOX can hybridize with *M. pneumoniae* DNA (lane 1) and also with *E. coli* DNA (lane 5).

Table 5. Ligation conditions and transformants obtained using a mixture of pUC12 and *M. pneumoniae* DNA fragments

Expt. No.	Km^r gene[a] ng	fmol	ATP[b] mM	RXN vol. µl	Dil'n 2x[c]	Final vol. µl	Transf'd vol.[d] µl	No. transformants Km^r normal	Ap^r small[e]	Km^r normal	Ap^s small[f]
1	100	100	0.6[b]	50	+	100	100	32	0	0	3
2	150	150	1.0	75	--	75	50	8	6	1	0
3	150	150	1.0	75	+	150	40	8	3	0	0
4	150	150	1.0	45	--	45	45	33	3	0	0
5	150	150	1.0	45	+	90	50	23	0	0	0
6	150	150	1.0	35	--	35	30	22	0	0	0
7	150	150	1.0	35	+	70	50	16	1	0	0

All the reactions contained 238 ng (approximately 26 fmol) of the mixture of *Eco*R1 partially digested *M. pneumoniae* DNA and *Eco*R1 completely digested pUC12 in a 1:1 genomic ratio dephosphorylated with BAP (see section 2.6.2). Each reaction also contained 1.5 U T4 ligase and ligation buffer B, and were all incubated at 24^0C for 4 h. (King and Blakesley, 1986) (see Table 4.). The ratio of DNA fragments was choosen basically in accordence with the recommendations published in BRL-Focus, Vol.2, No.3, p.1 (1979) (See section 2.6.3.1).

a. In the first experiment the purchased Km^r gene fragment was used, in experiments 2-7 the Km^r fragment used was that prepared from pUKn (see section.2.3.4.2).

b. In the first experiment, the ATP concentration of 0.6 mM was choosen to give the molar ratio of ATP/fragment used by King and Blakesley (1986). In the later experiments an ATP concentration of 1.0 mM was used throughout.

c. Those experiments indicated with a plus-sign were diluted with an equal volume of buffer containing ATP (to maintain a constant ATP concentration) after 2 h incubation in order to decrease "i" and increase j_{hyb}/i, so as to maximize the yield of fully circularized hybrids (see section 2.6.3.1.). This procedure apparently increased the yield of transformants.

d. Because of the complexity of the experiments, only the portion indicated of each ligation reaction mixture was used for transformation. For transformation, 10 μl of ligation mixture were diluted with 20 μl of 0.1 M $CaCl_2$, added to 200 μl of competent cells and applied to a Km selective plate after incubation at 37^0C in nonselective medium. The plates were incubated at 37^0C overnight and the transformed colonies were counted on 3 consecutive days. All the Km^r transformants were tested on a plate containing kanamycin and ampicillin.

e. In this column are noted transformants which were of obviously smaller colony size than normal. Some of the small transformant colonies were morphologically altered and showed semi-resistance to ampicillin.

f. The 3 Km^rAp^s transformants noted in this column grew as very tiny colonies. They were slow growing, recultured with difficulty and genetically unstable. No plasmids were recoverable from them.

Fig. 5. Digestions of plasmid pOX DNA with different restriction endonucleases

A. Electrophoresis on 0.4% agarose gel, 25 volts for 16 h.
- Lane 1. pOX DNA digested with *Sma*I
- Lane 2. pOX DNA digested with *Eco*R1
- Lane 3. High molecular weight standard*
- Lane 4. λ digested with *Hin*dIII marker DNA#
- Lane 5. pOX DNA digested with *Bam*HI
- Lane 6. pOX DNA digested with *Pst*I
- Lane 7. pOX DNA digested with *Hin*dIII

B. Electrophoresis on 1.5% agarose gel, 35 volts for 14.5 h.
- Lane 1. Km^r gene fragment digested with *Sau*3A
- Lane 2. pOX DNA digested with *Sau*3A
- Lane 3. pOX DNA digested with *Eco*R1
- Lane 4. λ digested with *Hin*dIII marker DNA#
- Lane 5. φX174 RF DNA digested with *Hae*III
- Lane 6. pOX DNA digested with *Hae*III
- Lane 7. pOX DNA digested with *Hin*dIII
- Lane 8. pOX DNA digested with *Pst*I
- Lane 9. pOX DNA digested with *Bam*HI

*The sizes of the 13 bands of DNA high molecular weight standard fragments are 43.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.1, 15.9, 12.2, 10.1, 8.6, 8.3 Kb.

#The sizes of the six visible λ *Hin*dIII DNA fragments are 23.1, 9.4, 6.7, 4.4, 2.3, 2.0 Kb.

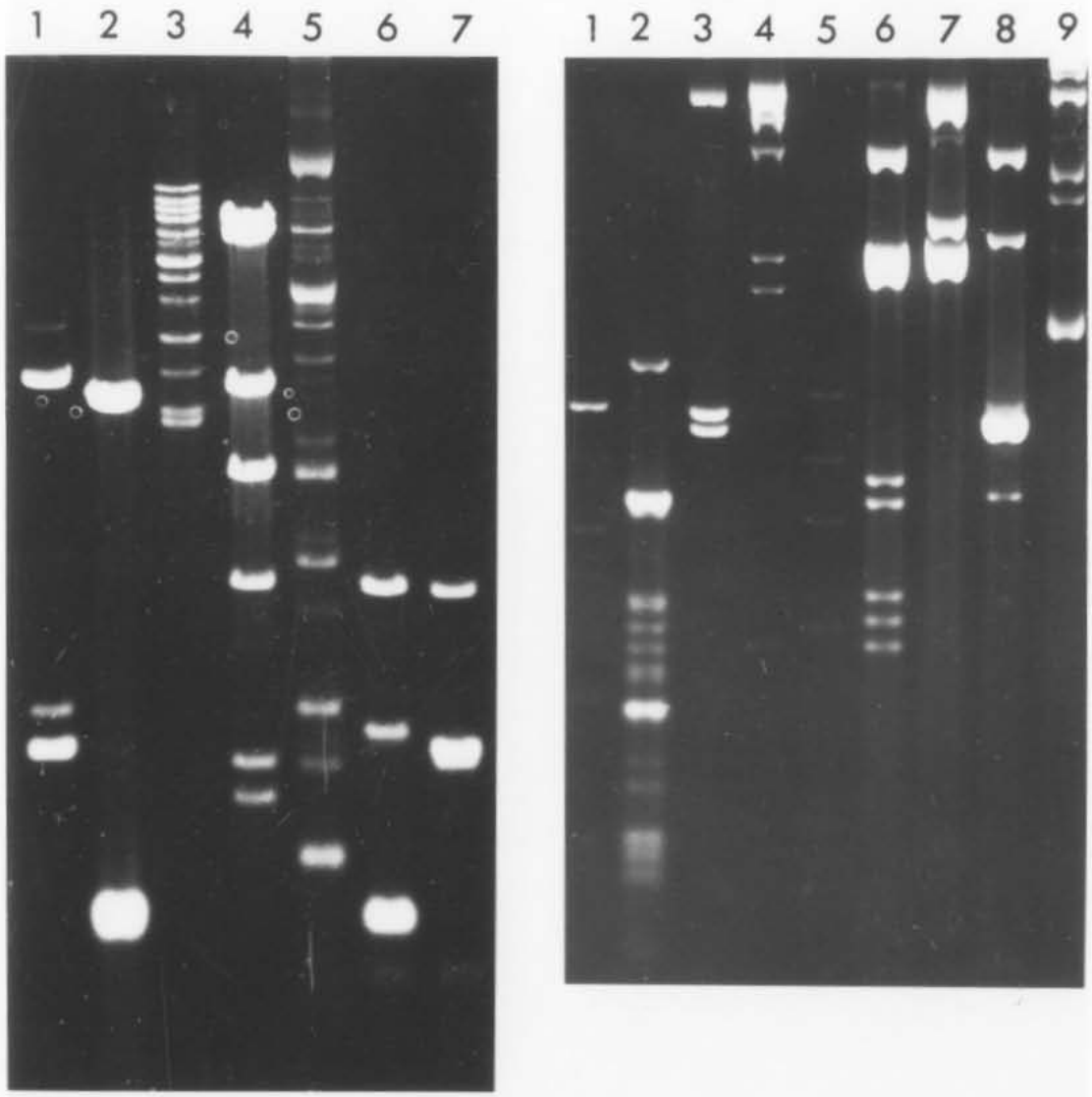
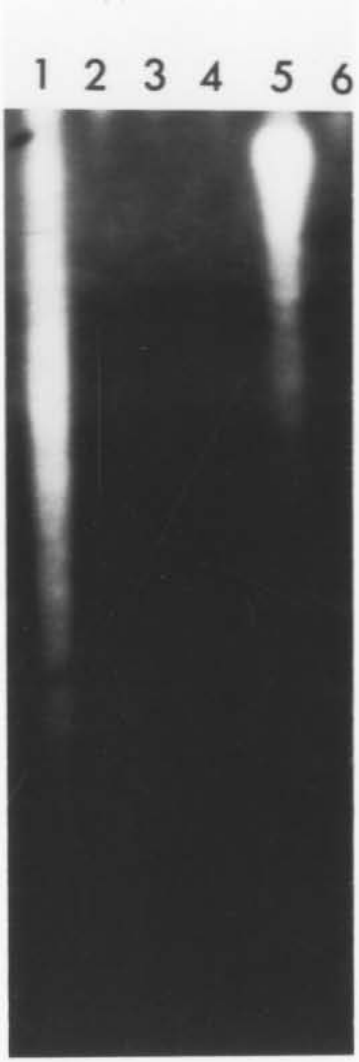

1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9
A
B

Fig. 6. Southern blot hybridization of chromosomal DNAs with pOX plasmid DNA as probe.

All the DNA species were completely digested with *Eco*R1 prior to separation on the agarose gel.

Lane 1: *M. pneumoniae* DNA, 3 μg.
Lane 2: *M. fermentans* PG-18 DNA, 3μg.
Lane 3: *M. arginini* DNA, 3 μg.
Lane 4: *M. gallisepticum* DNA, 3 μg.
Lane 5: *E. coli* DNA, 5 μg
Lane 6: calf thymus DNA, 20 μg

Hybridization was following the methods detailed in section 2.5.3 (50% formamide, 1 M Na^+ 42^0C overnight), using the higher stringency rewashing procedure (1 x SSPE/0.1% SDS for 45 min at 52^0C). The X-ray film (Kodak, X-Omat AR film)was exposed for 8 days.


1 2 3 4 5 6

The hybridization pattern produced, however, was a smear instead of distinct bands. After washing the filter using a higher stringency, no changes in intensity or pattern were found.

The possibility of contamination of the pOX probe (the purity of which had been examined by gel electrophoresis but which had not been purified by CsCl ultracentrifugation) with *E. coli* chromosomal DNA fragments was considered. Different restriction enzymes were tested (Fig. 5) to search for one which can cut pOX into several small distinct fragments, to be used to digest mycoplasma DNA prior to Southern blot hybridization. In this way it was hoped to produce distinct hybridization bands, and to be able to distinquish true homology from the results of possible contamination.

From Fig. 5A and Fig. 5B, it can be seen that among the enzymes tested *Sau*3A was the best candidate (Fig. 5B, lane 2) and it was used to digest the mycoplasma and *E. coli* DNAs. The DNA Southern blot hybridization analysis was carried out using the same pOX probe as used previously (Fig. 6). The results of the second hybridization are shown in Fig. 7. In Fig. 7 (Lane 2), the *M. pneumoniae* DNA digested with *Sau*3A and hybridized with the pOX probe produced a pattern similar to that found when the plasmid pOX DNA was digested with *Sau*3A (Fig. 5B, lane 2). This result means that pOX has homology with *M. pneumoniae* DNA. The *E. coli* DNA control also show some homology. There are very faint, low molecular weight bands in the *E. coli* DNA (Fig. 7, lane 8), which do not clearly show up in the photograph but which are very clear in the autoradiograph of the subsequent Southern blot transfer and DNA hybridizations.

In Fig. 5B, lane 3, it can be seen that the original small-sized band from pOX DNA digested with *Eco*R1 (see Fig. 5A, lane 2) has been resolved into two bands migrating close together. The larger band of the two comigrates with the Km^r gene fragment (compare lanes 1 and 3 in Fig. 5B). So the origin fragment must be either the largest (9.3 Kb) or the smallest (estimated to be 1.2 Kb) fragment. Again it can be seen that the intensity of the fragments is not in direct proportion to their size, the two small fragments having a higher intensity than expected from their sizes. This phenomenon can be seen in Fig. 9.

3.3.2. SEGREGATION OF PLASMID pOY

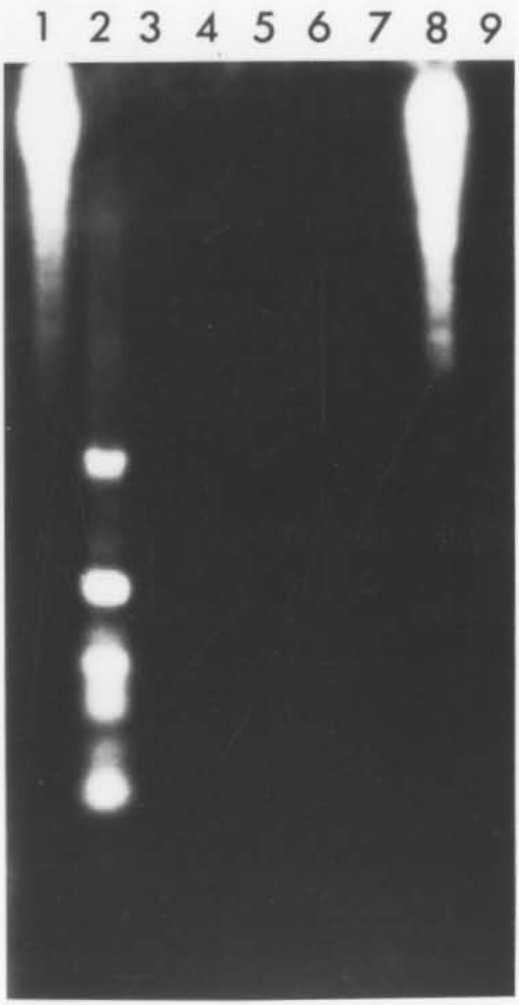
A large scale plasmid extraction using the rapid boiling method followed by CsCl ultracentrifugation was carried out to prepare more pOX plasmid DNA to prepare a new probe without *E. coli* DNA chromosmal contamination. A transformant colony was used as the parent cell for the culture. The extracted plasmid was digested with *Eco*R1 and examined on a gel. Surprisingly, the newly extracted plasmid had lost the 9.3 Kb fragment and only the two very close bands of approximately 1.3 Kb remained (Fig. 8, lane 6). The new plasmid was capable of transforming *E. coli* RR1 to kanamycin resistance. It was named pOY.

This phenomenon suggested that plasmid pOY was derived from plasmid pOX by spontaneous excision of the large DNA fragment. This was supported by other indirect evidence. Firstly, miniscreen plasmid DNA preparations from 6 independent pOX transformed *E. coli* colonies was carried out, one of which was found to be pOY. Secondly, although not examined directly, it appeared that small volume cultures prepared from independent transformant colonies (which

Fig. 7. Southern blot hybridization of different DNA species with plasmid pOX DNA as probe

Lane 1: *M. pneumoniae* DNA, 3 µg, digested with *Eco*R1
Lane 2: *M. pneumoniae* DNA, 3 µg, digested with *Sau*3A
Lane 3: *M. fermentans* PG-18 DNA, 3 µg, digested with *Eco*R1
Lane 4: *M. fermentans* PG-18 DNA, 3 µg, digested with *Sau*3A
Lane 5: *M. gallisepticum* DNA, 3 µg, digested with *Eco*R1
Lane 6: *M. gallisepticum* DNA, 3 µg, digested with *Sau*3A
Lane 7: *M. arginini* DNA, 3 µg, digested with *Eco*R1
Lane 8: *E. coli* (RL108) DNA, 5 µg, digested with *Eco*R1
Lane 9: calf thymus DNA, 20 µg, digested with *Eco*R1

Hybridization was following the methods detailed in section 2.5.3 (50% formamide, 1 M Na^+, 42^{0}C overnight), using the higher stringency washing procedure (1 x SSPE/0.1% SDS for 45 min at 55^{0}C).
The X-ray film (Kodak, X-Omat AR film) was exposed for one week.


1 2 3 4 5 6 7 8 9

involves a relatively few generations of growth) gave smaller amounts of the pOY relative to pOX than did large volume cultures prepared from the same colony (which involves a larger number of generations). The amount of pOY in any one culture was variable, suggesting that excision, though possible, was not so frequent as to prevent the growth of a sufficiently large volume of culture essentially free of pOY for the preparation of relatively pure pOX. The assumed pOX preparation (Fig.5, line 3) was actually a mixture of pOy and pOX and pOY masked the 2:1 ratio of the 2 smaller fragments in pOX.

In order to confirm the supposition that pOY is derived from pOX rather than resulting from the coexistence of two plasmids (one pOY and the other a plasmid composed of the 9.3 Kb *M. pneumoniae* fragment) further *Eco*R1 digestions of pOX with gradually increasing amounts of enzyme (Fig. 8, lanes 3, 4, and 5) was carried out. Plasmid pOX was prepared by CsCl ultracentrifugation from a culture selected because it appeared to be derived from a colony that was not by chance producing plasmid pOY in detectable amounts and its purity was confirmed by gel electrophoresis. Fig. 8, lanes 3, 4, and 5 show that as the Km^r gene fragment (about 1.3 Kb as estimated from the migration rate in the electrophoresis gel) and the origin band (about 1.2 Kb) become more and more intense (these two bands are very faint and can only be seen in the original negative), the Km^r gene band remains approximately twice as intense as the origin band (Fig. 8, lane 5), while in lane 6 the two bands produced from pOY have the same intensity. *Eco*R1 digestion in variable amounts of EtBr was used in an unsuccessful attempt to produce a linear molecule with only one cut in the

plasmid DNA to see the total molecular weight of pOX. The extra bands in Fig. 9, lane 2 may be the result of cuts at *Eco*R1* sites. Fig. 9, lane 3 shows the double amount of Km^r gene fragment relative to the origin fragment. This suggests that two copies of the Km^r gene fragment may be present in plasmid pOX. This conclusion is consistant with the result of the series of partial *Eco*R1 digestions of pOX shown in Fig. 10, lane 5, in which the 4 large-sized bands of linear DNA in the 0.5 U *Eco*R1 digestion very likely represent the 9.3 Kb fragment, the 9.3 Kb fragment plus one small fragment, the 9.3 Kb fragment plus 2 small fragments and finally the 9.3 Kb fragment plus all 3 small fragments. The 2 copy insertion model can also explain the incompletely digested, highest molecular weight band (approximately 13.5 Kb) in the pOX *Sam*1 digestion (Fig. 5A, lane 1). Southern blot hybridization of *Eco*R1 digested pOX DNA with plasmid pOY DNA as a probe also shows a band estimated to have the same molecular weight as intact, linear pOX (data not shown). This suggests that pOY is a component of pOX, instead of resulting from the coexistence of two plasmids.

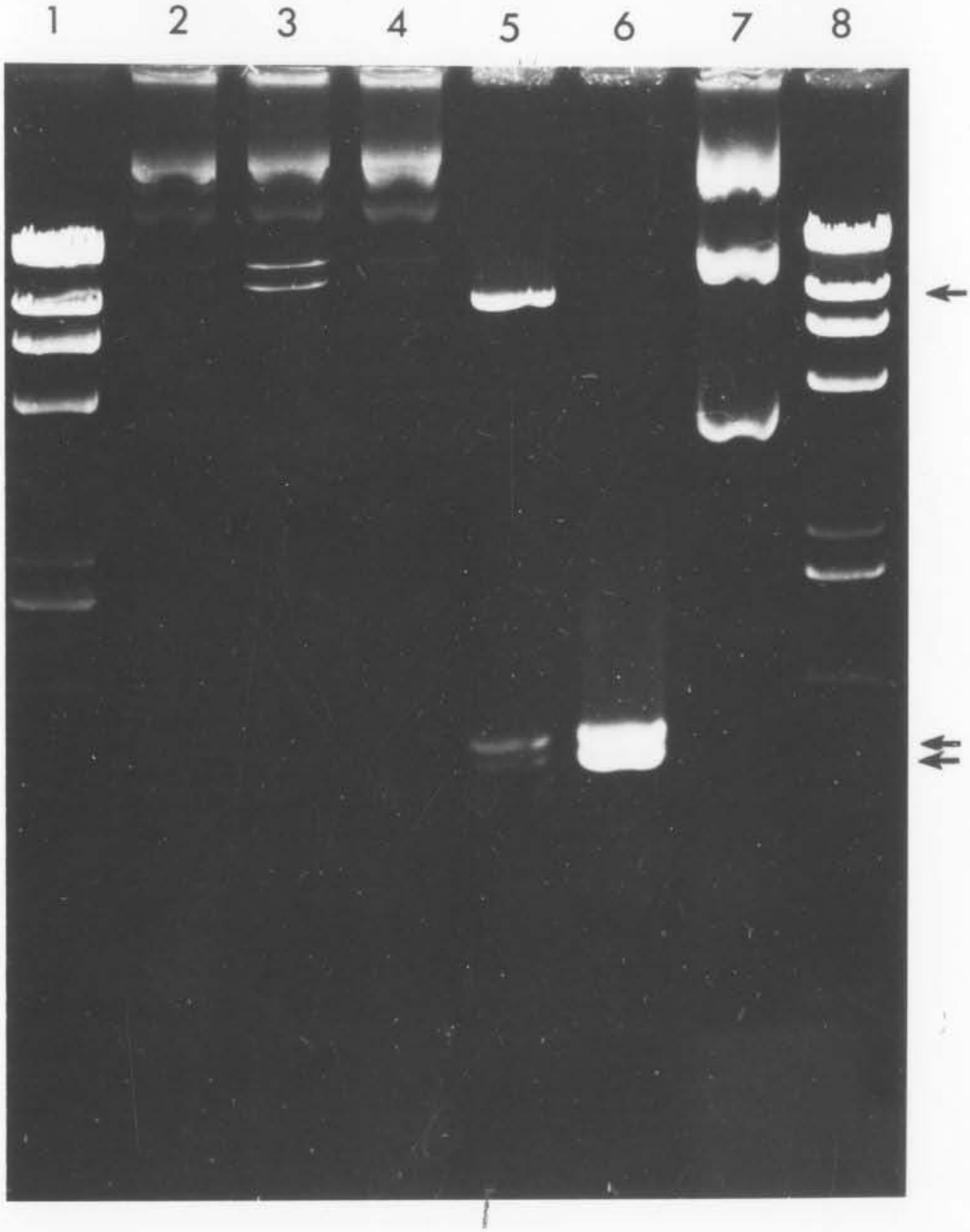
3.3.3. SOURCE OF ORIGIN IN pOY

To identify the source of the origin of pOY, 3 pieces of slot blot nitrocellulose filter were prepared, each with the same set of DNA samples. The 3 pieces of slot blot filter were hybridized separately with either pOX DNA (10^5 counts/min), pOY DNA (10^6 counts/min) or the Km^r gene fragment (10^6 counts/min) DNA probes. All the plasmid DNAs used in these hybridizations were purified by CsCl ultracentrifugation and the purity of the plasmids was examined on a gel. In Fig. 11 are shown the pattern of DNAs loaded (Fig. 11A) and the

Fig. 8. Analysis of the structure of plasmid pOX using partial *Eco*R1 digestion: Electrophoresis on 1% agarose gel, 50 volts for 3 h in TBE.

Lane 1. λ DNA digested with *Hin*dIII
Lane 2. pOX DNA digested with 0 U of *Eco*R1
Lane 3. pOX DNA digested with 0.5 U of *Eco*R1
Lane 4. pOX DNA digested with 0.13 U of *Eco*R1
Lane 5. pOX DNA digested with 2 U of *Eco*R1
Lane 6. pOY DNA digested with 2 U of *Eco*R1
Lane 7. pOY DNA digested with 0 U of *Eco*R1
Lane 8. λ DNA digested with *Hin*dIII

The pOX samples are 60 ng each well, pOY are 100 ng each well, and they were digested for 2.5 h. The 3 arrows indicate the positions of the 3 fragments produced by *Eco*R1 digestion of the plasmid pOX.


1
2
3
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7
8

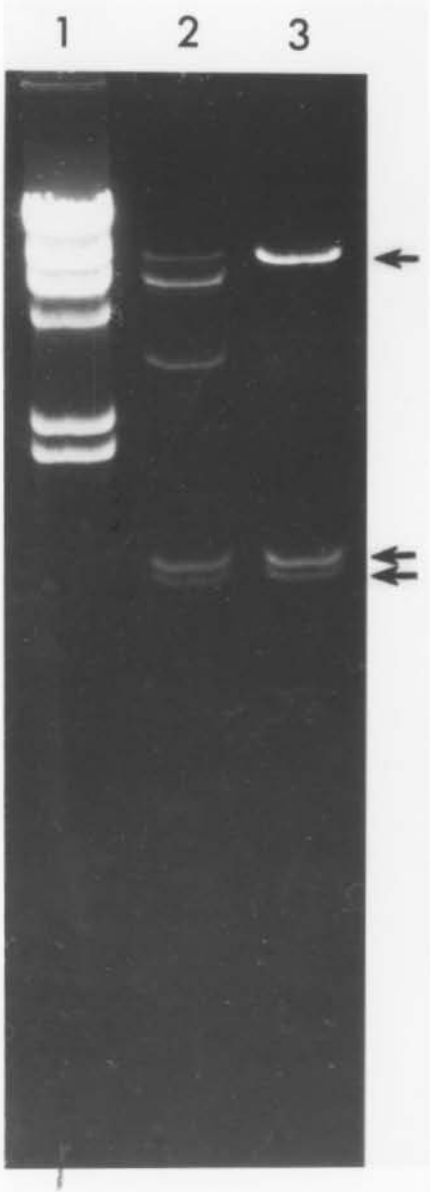
Fig. 9. Complete digestion of plasmid pOX with *Eco*R1 restriction endonuclease in the presence of EtBr.

Lane 1. λ DNA digested with *Hind*III

Lane 2. pOX DNA 22.5 ng, *Eco*R1 2.5 U, EtBr 0.7 μg/μl.

Lane 3. pOX DNA 22.5 ng, *Eco*R1 2.5 U, EtBr 0.5 μg/μl.

The 3 arrows indicates the positions of the 3 fragments produced by *Eco*R1 digestion of the plasmid pOX.


1
2
3

results of hybridization with the three probes (Fig. 11B-D). Comparing the result of *M. pneumoniae* DNA hybridized with the pOX DNA probe (Fig. 11B, slot 5) and hybridized with the pOY DNA probe (Fig. 11C, slot 5), we can see that pOX hybridizes with *M. pneumoniae* DNA and pOY does not.
This indicates that the homology of pOX to *M. pneumoniae* DNA was totally derived from the 9.3 Kb fragment, which is consistant with the results presented in the previous section.

The Km^r gene fragment can hybridize with itself and the Km^r gene-containing plasmids (Fig. 11D, slots 1, 2, 4, 12. Note that the DNA sample intended for slot 1 was misloaded into the slot one row above), and has no homology with any other DNA (also see below, Fig. 12B). Plasmid pOY, in addition to the expected hybridization (Fig. 11C, slots 1, 2, 4, 12), can hybridize with both *E. coli* DNA and plasmid pUC12 (Fig. 11C, slots 3, 9). The hybridization signal produced by pOX with pUC12 was weak and did not reproduce in Fig.11B, but it was detectable on the original X-ray film.

To obtain direct evidence for the source of the replication origin in plasmid pOX, another Southern blot hybridization was carried out with the pOY origin fragment itself as the probe (Fig. 12). For preparation of the probe, plasmid pOY DNA was completely digested with *Eco*R1, the fragments were separated on a gel and the origin fragment was recovered using DE 81 filter paper (see section 2.3.4.2.). The amounts of the DNAs loaded into the agarose gel prior to Southern blotting were in proportion to their genome size. After hybridization, the filters were washed with high stringency (50^0C).

Fig. 10. Analysis of the structure of plasmid pOX using partial *Eco*R1 digestion: Electrophoresis on 0.5% agarose gel, 25 volts for 17.5 h in TAE.

Lane 1. λ DNA digested with *Hin*dIII
Lane 2. pOX DNA digested with 0 U *Eco*R1
Lane 3. pOX DNA digested with 0.1 U *Eco*R1
Lane 4. pOX DNA digested with 0.25 U *Eco*R1
Lane 5. pOX DNA digested with 0.5 U *Eco*R1
Lane 6. pOX DNA digested with 1 U *Eco*R1
Lane 7. pOX DNA digested with 2 U *Eco*R1
Lane 8. pOY DNA digested with 2 U *Eco*R1
Lane 9. High molecular weight marker DNA
Lane 10. pOY DNA digested with 0 U *Eco*R1
Lane 11. λ DNA digested with *Hin*dIII

The pOX samples are 60 ng each well, pOY are 100 ng each well. The molecular weights of the marker DNA bands are indicated in the margin.

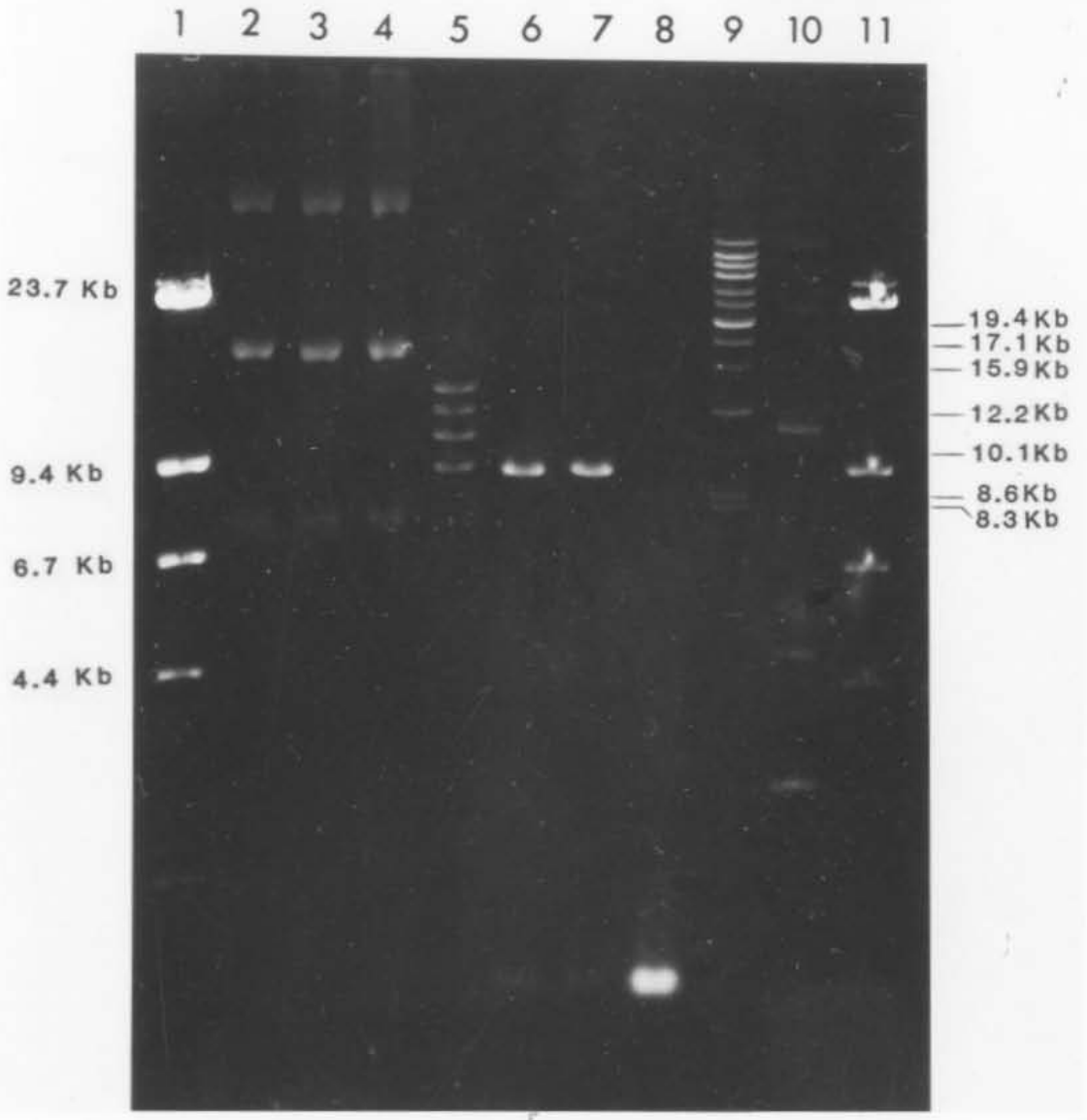

1 2 3 4 5 6 7 8 9 10 11
23.7 Kb
9.4 Kb
6.7 Kb
4.4 Kb
19.4Kb
17.1Kb
15.9Kb
12.2Kb
10.1Kb
8.6Kb
8.3Kb

Fig. 12A shows the EtBr stained gel prior to Southern blotting, illustrating the complete digestion of *M. pneumoniae* and *E. coli* chromosomal DNAs. The other samples are present in minute amounts and cannot be seen. From the autoradiograph of the DNA hybridization with the origin fragment as a probe (Fig. 12B), we can see that the origin probe was not completely pure but contained a small proportion of the Km^r gene (lanes 6 and 7).
This small amount of Km^r gene, however, does not interfere with the interpretation of the result and can be ignored. The absence of homology of the origin to the *M. pneumoniae* DNA and the presence of a strong homology to pUC12 DNA can clearly be seen. Since the DNA species were loaded into the gel in equal molar amounts, the equivalency of intensity of the bands (see lanes 1, 4, 5, 6, 8) suggests that the pOY origin fragment has a high homology to the pUC12 DNA. The supposition that the replication origin of pOY is derived from pUC12 and not from *M. pneumoniae* or *E. coli* DNAs can explain the observed hybridization of pOX and pOY to *E. coli* DNA, because pUC12 contains a partial *lacZ* gene which is also present in *E. coli* chromosomal DNA.

The supposition that the origin of pOY is derived from pUC12, on the other hand, presents a dilema as well. Plasmid pUC12 has only one *Eco*R1 site and the linear pUC12 DNA (2.68 Kb) is much larger than the origin fragment obtained (approximately 1.2 Kb). A possible resolution to this inconsistancy is to suggest that the pOY origin came from an *Eco*R1* fragment of pUC12. When the ion concentration in the buffer is reduced, *Eco*R1 may loose its specificity and only recognize the middle 4 bp of the 6 bp *Eco*R1 recognition sequence. The middle 4 bp of a normal *Eco*R1 site are called an *Eco*R1* site (Maniatis, *et al.*, 1982)

Fig. 11. Slot blot hybridization of chromosomal DNA with plasmids pOX, pOY and the Km^r gene fragment.

The loading pattern of the DNA samples in B to D followed the pattern shown in A except for the first slot in D, where the pOX DNA was inadvertently loaded into a slot one row above (indicated by an arrow) leaving the first slot empty.

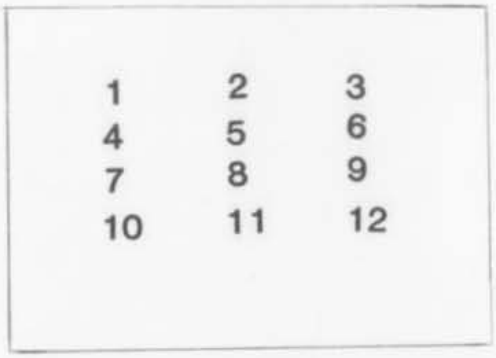
A. The species and amount of DNA samples
   1. pOX DNA, 1 ng
   2. pOY DNA, 1 ng
   3. pUC12 DNA, 1 ng
   4. pUKn DNA, 1 ng
   5. *M. pneumoniae* DNA, 1 μg
   6. *M. gallisepticum* DNA, 1 μg
   7. *M. fermentans PG-18* DNA, 1μg
   8. *M. arginini* DNA, 1 μg
   9. *E. coli* DNA, 4 μg
   10. *calf thymus* DNA, 20 μg
   11. λ DNA, 0.5 μg
   12. Km^r DNA, 0.5 ng

B. Hybridization of the slot-blot with plasmid pOX DNA as probe. 10^5 counts were used in this hybridization.

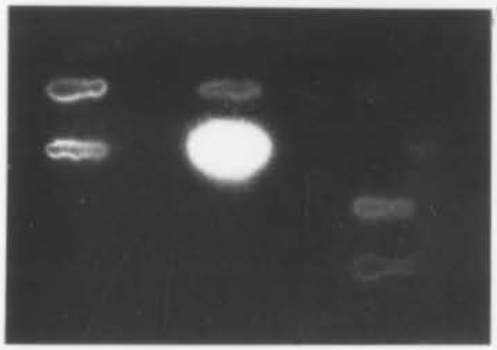
C. Hybridization of the slot-blot with plasmid pOY DNA as probe. 10^6 counts were used.

D. Hybridization of the slot-blot with the Km^r gene fragment DNA as probe. 10^6 counts were used.

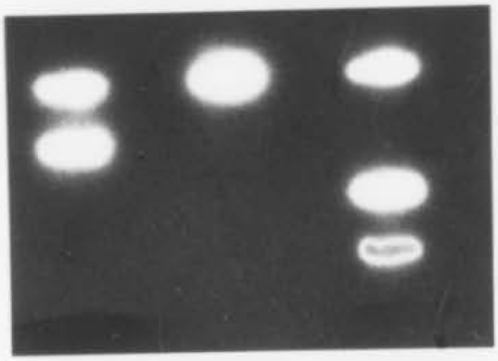
Hybridization was following the method detailed in section 2.5 (50% formamide, 1 M Na^+, 42°C overnight), using the higher stringency washing procedure (1 x SSPE/0.1% SDS for 45 min, at 55°C).
The X-ray film (Kodak, X-Omat AR film) was exposed for one week.



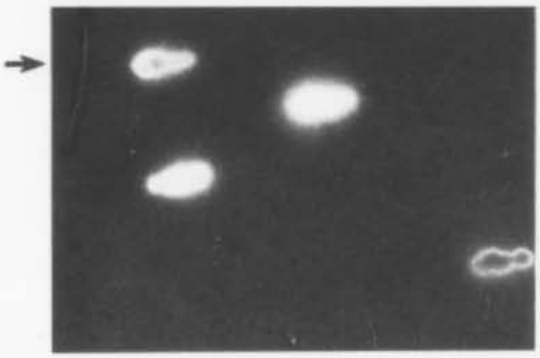
A



B



C



D

Fig. 12. Southern blot hybridization with the origin fragment from plasmid pOY as probe

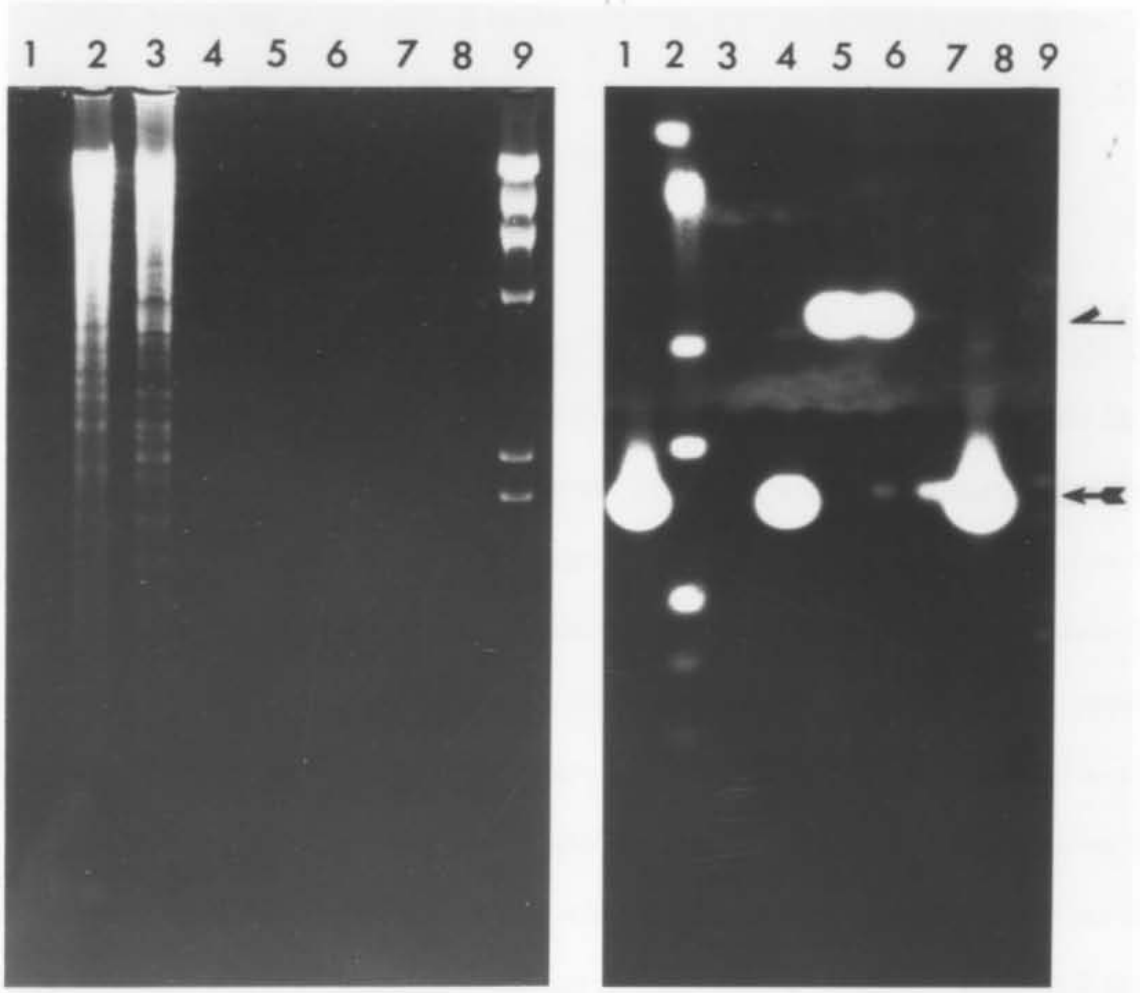
A. The EtBr stained agarose gel prior to Southern blotting
Lane 1. 1 ng origin fragment from pOY digested with *Eco*R1
Lane 2. 3.025 μg *E. coli* DNA digested with *Eco*R1 (complete digestion).
Lane 3. 0.625 μg *M. pneumoniae* DNA digested with *Eco*R1 (complete digestion).
Lane 4. 2 ng pOY DNA digested with *Eco*R1
Lane 5. 2 ng pUC12 DNA digested with *Eco*R1
Lane 6. 3 ng pUKn DNA digested with *Eco*R1
Lane 7. 1 ng Km^r gene digested with *Eco*R1
Lane 8. 1 ng origin fragment from pOY digested with *Eco*R1
Lane 9. λ DNA digested with *Hin*dIII

All the DNA samples were loaded in approximately equal molar amounts.

B. The autoradiograph obtained following Southern blotting and hybridization
3×10^6 cpm of probe was used.

The half arrow points to the position of the linearized pUC12; the full arrow points to the position of the origin fragment from pOY digested with *Eco*R1.

Hybridization was following the methode detailed in section 2.5 (50% formamide, 1 M Na^+, 42°C overnight), using the higher stringency washing procedure (1 x SSPE/0.1% SDS for 45 min, at 55°C).
The X-ray film (Kodak, X-Omat AR film) was exposed for one week.


1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
A
B

The pUC plasmids are derived from the ligation of a *Pvu*II/*Eco*R1 fragment of pBR322 (in which 3 *Eco*R1* sites are found) and a *Hae*II fragment of bacteriophage M13mp7 containing the *lacZ* gene α-fragment (Vieira and Messing, 1982). According to the pUC12 restriction map, cuts by *Eco*R1 at the unique *Eco*R1 site and the closest *Eco*R1* site of pUC12 would produce a fragment of the same size as the origin fragment of pOX and pOY (Fig. 13A), which would contain the functional origin of DNA replication..

To test this possibility, *Pvu*II and *Eco*R1 were used to double-digest pUC12 and pOY plasmids. For pUC12, three fragments of 136 bp, 180 bp and 2364 bp were predicted (Fig. 13A); for pOY, a 180 bp, which is the same fragment as the 180 bp pUC12 fragment, a 939 bp fragment and the Km^r fragment were expected (Fig. 13B). Following the double digestion, both an agarose gel electrophoresis (to resolve large-sized fragments) and a polyacrylamide gel electrophoresis (to resolve small-sized fragments) were carried out. The results, presented in Figs. 14 and 15, respectively, are consistant with the predicted result. Lane 4 in Fig. 14 shows the 2364 bp fragment of pUC12 digested with *Pvu*II and *Eco*R1; lane 5 shows the Km^r gene fragment (approximately 1350 bp) and the 939 bp fragment. In Fig. 15, lane 4 shows the 180 bp fragment of pOY produced by the double-digestion; lane 5 shows the 180 and 136 bp fragments of pUC12. The large-sized fragments are not well resolved because of overloading in order to show the small-sized bands. It is therefore concluded that the origin of plasmid pOY was derived from pUC12 DNA.

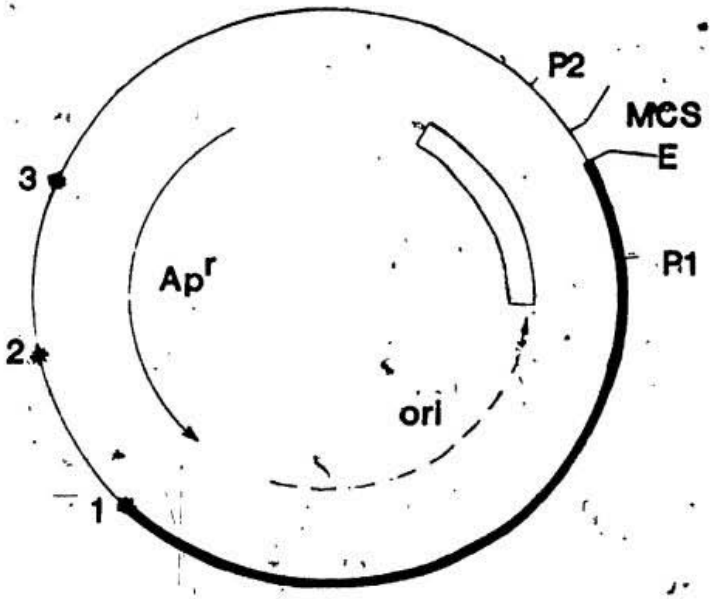
Fig. 13. Proposed derivation of the origin in plasmid pOY.

A. Map of plasmid pUC12
MCS: multiple cloning site.
P: *Pvu*II site
E: *Eco*R1 site
*: *Eco*R1* site
Open bar: bacteriophage M13mp *lacZ* gene insertion
Thick line: proposed fragment which becomes the origin of plasmid pOY.
The arrow with dotted line is the origin

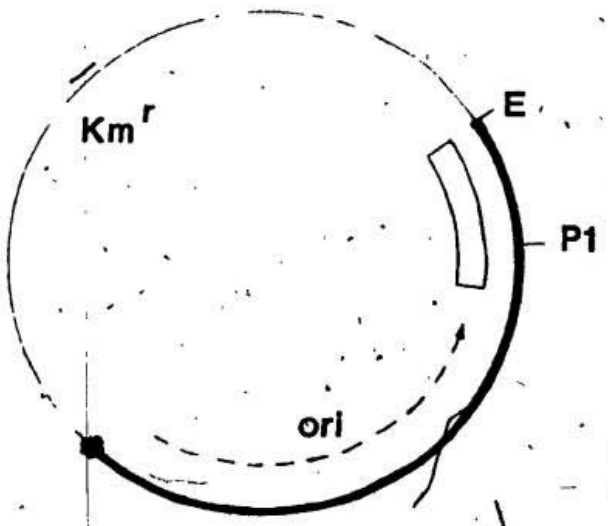
In pUC18, the 3 *Eco*R1* sites are located at 1569, 1874, 2130 bp respectively.
*Pvu*II and *Eco*R1 double digestion will produce 3 fragments: E-P fragment, 180 bp
P1-1*-2*-3*-P2, 2364 bp
P2-E, 136 bp

B. Proposed map of plasmid pOY.
Thick line: origin fragment.
Fine line: Km^r gene.
Open bar: part of the M13mp insertion from that shown in Fig. 8A.

After digestion with by *Pvu*II and *Eco*R1, pOY should produce 3 fragments:
E-P1 fragment, 180 bp
P1-E* fragment, 939 bp
E*-E fragment, Km^r gene



A



B

Fig. 14. Restriction digestion analysis of plasmids pOY and pUC12: Electrophoresis on 1% agarose gel, 40 volts for 3 h.

Lane 1. λ DNA digested with *Hin*dIII
Lane 2. φX174 DNA digested with *Hae*III marker
Lane 3. Km^r gene fragment
Lane 4. pUC12 DNA digested with *Pvu*II and *Eco*R1
Lane 5. pOY DNA digested with *Pvu*II and *Eco*R1
Lane 6. pUKn DNA digested with *Eco*R1
Lane 7. φX174 RF DNA digested with *Hae*III
Lane 8. λ DNA digested with *Hin*dIII

The numbers in the margin refer to the molecular weights (bp) of the molecular weight standard DNA fragments.

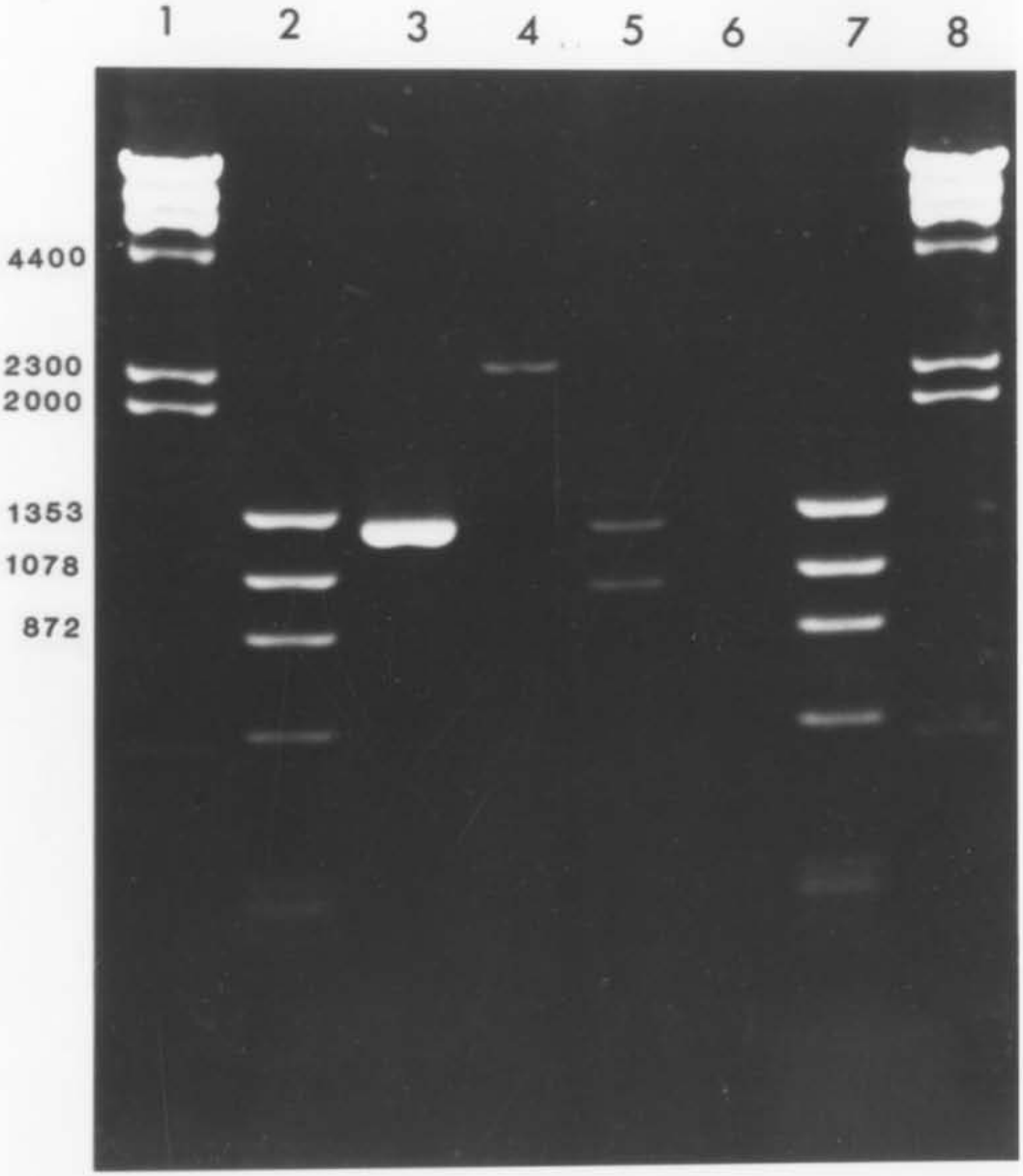
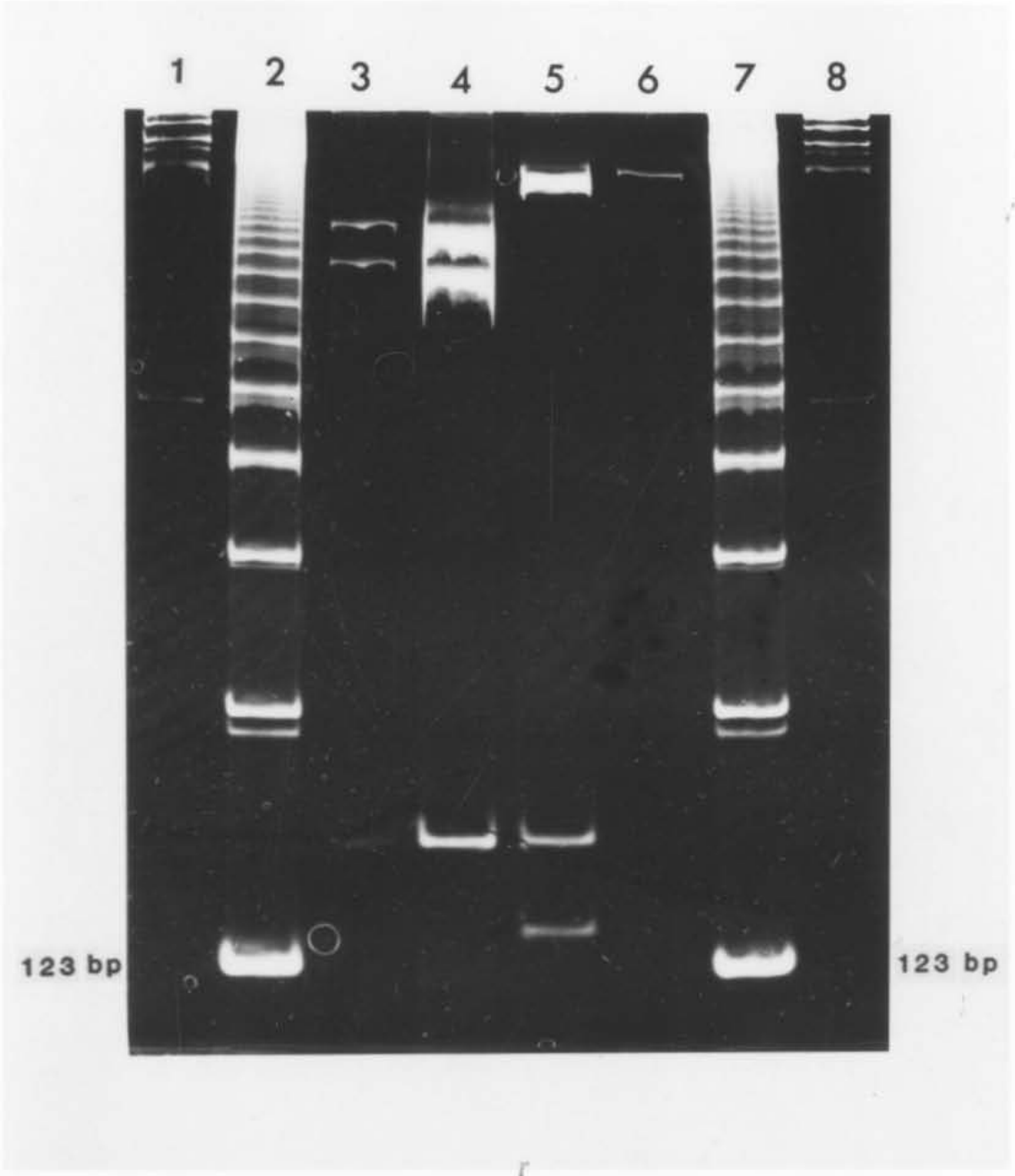

1
2
3
4
5
6
7
8
4400
2300
2000
1353
1078
872

Fig. 15. Restriction digestion analysis of plasmids pOY and pUC12. Electrophoresis on 7.5% polyacrylamide gel, 60 volts for 13 h.

Lane 1. λ DNA digested with *Hin*dIII
Lane 2. 123 bp DNA marker ladder
Lane 3. pOY DNA digested with *Pvu*II and *Eco*R1
Lane 4. pOY DNA digested with *Pvu*II and *Eco*R1
Lane 5. pUC12 DNA digested with *Pvu*II and *Eco*R1
Lane 6. pUC12 DNA digested with *Pvu*II and *Eco*R1
Lane 7. 123 bp DNA marker ladder
Lane 8. λ DNA digested with *Hin*dIII


1
2
3
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5
6
7
8
123 bp
123 bp

Chapter 4

DISCUSSION

4.1. MYCOPLASMA DNA ORIGIN MAY NOT FUNCTION IN E. coli

The experiments described here strongly suggest that the mycoplasma origin of DNA replication may not function in *E. coli*, and that mycoplasma DNA may not contain any sequence homologous enough to be recognized in *E. coli* as a functional ARS sequence, because, had such a sequence been present, it would have had a good chance of being recovered in these experiments. This may be a reflection of the great taxonomic distance between these two organisms and a reflection of different replication systems.

In these experiments, aliquots of the *Eco*R1 digestion products of *M. pneumoniae* DNA were examined on a gel to ensure that only partial digestion had been achieved. This would ensure the preservation of any origin which contains an *Eco*R1 site. Aliquots of most of the ligation mixtures were also examined on gels and mycoplasma DNA was shown to be capabable of ligation. The fact that the extracted plasmid pOX was shown to have a mycoplasma DNA insert suggests that the ligation and transformation of mycoplasma DNA fragments can occur and that mycoplasma DNA is capable of being stably

transformed into *E. coli*. This is consistant with previous observations from this laboratory (Satishchandran and Barnsley, 1984).

When pUC12 was used as a control, a large number of transformants were obtained. When partially digested mycoplasma DNA was ligated with the Km^r gene fragment prior to transformation, no transformants were observed in a total of 8 experiments, even though a number of variations in the ligation conditions were tried.

In order to ensure equivalent conditions for the dephosphorylation, ligation, and transformation of the pUC12 origin fragment and the mycoplasma origin fragment, an internal control experiment was designed. In contrast to the pUC12 plasmid DNA, the mycoplasma DNA fragment carrying the origin is inevitably masked by the large number of non-origin mycoplasma DNA fragments. The *Eco*R1 partially digested mycoplasma DNA and *Eco*R1 completely digested pUC12 were therefore mixed in 1:1 genomic ratio, dephosphorylated, ligated with the Km^r gene and the mixture used to transform *E. coli* competent cells. From the 7 experiments of this type, a total of 160 Km^r transformants were obtained, all but one of which were also Ap^r which means that it was the pUC12 origin and not a mycoplasma origin that was recovered. Even the plasmid from the only Km^rAp^s transformant has proven to be the pUC12 origin. No mycoplasma origin was recovered. From these data we suggest that no single, contiguous sequence of mycoplasma DNA can function as an origin in *E. coli*.

Furthermore, recent Southern blot transfer and DNA hybridization

experiments have demonstrated that the *dnaB*, *dnaE*, *dnaG*, *dnaK* and *dnaZX* genes of *E. coli* have no homology with mycoplasma DNA (S. Regular and P. Hempstead, unpublished results). The products of those genes are important constituents of the DNA replication apparatus of *E. coli*. Although the *dnaA* gene has not as yet been examined, the complete lack of homology of those genes examined suggests either a totally different replication apparatus in mycoplasma, or virtually complete divergence of sequences due to the considerable taxonomic distance between mycoplasma and *E. coli*.

Some other possibilities, however, cannot be ruled out. Since the only enzyme I have used is *EcoR*1, it may cut in the replication origin of *M. pneumoniae* and hence reduce the number of intact functional origin fragments. Similarly, if the *EcoR*1 origin fragment of *M. pneumoniae*, on its own or after ligation, is larger than 20Kb, it will be transformed into the host cell with reduced efficiency. More restriction enzymes need to be tried in order to draw a more confident conclusion.

4.2. A POSSIBLE MECHANISM FOR THE SPONTANEOUS EXCISION OF THE MYCOPLASMA DNA FRAGMENT FROM pOX

When the spontaneous excision of the mycoplasma DNA fragment from pOX was first observed, the possibility that mycoplasma DNA is incompatible in *E. coli* was considered. Some genes from mycoplasma have, however, been transcribed and translated in *E. coli* without the help of an expression vector (Kawauchi, *et al.*, 1984; Mouches, *etal.*, 1984; Trevino, *et al.*, 1986) which spoke

for the opposite. Since 2 copies of the Km^r gene have been found in pOX and only a single Km^r gene in pOY, homologous recombination between these two Km^r genes appears to be a more reasonable explanation for this phenomenon. Such homologous recombination would result in the excision of the mycoplasma DNA fragment and also one copy of the Km^r gene as illustrated in Fig. 16. In this case the pOX must have two copies of the Km^r gene in the same orientation and separated by the origin fragment, with the other ends of the Km^r genes being ligated to the mycoplasma DNA fragment as shown in Fig. 13. If the 2 Km^r genes were oriented in an inverted arrangement, homologous recombination would result only in an inversion of the origin fragment, not an excision of the mycoplasma DNA fragment.

From the proposed structure of pOX (Fig. 13) it can be seen that incomplete digestion by *Eco*R1 could produce 4 species of 9.3 Kb-containing fragments: 9.3 Kb, 9.3 Kb + 1 Km^r gene, 9.3 Kb + 1 Km^r gene + the origin and 9.3 Kb + 2 Km^r genes + the origin. This result is consistant with the results shown in Fig. 10, lane 5, which shows 4 bands resulting from the incomplete digestion of pOX with *Eco*R1. General recombination between homologous sequences is a common event in living cells and the smaller pOY could have an advantage over pOX, for it has lost some of the replication burden, explaining why the proportion of pOY plasmid component increases with increased culture time.

In these experiments, there are still some questions to be answered. First, the *Eco*R1* end of the origin ligated with a *Eco*R1 site will produce another *Eco*R1* site with one terminal base pair different from the standard *Eco*R1 site

Fig. 16. Proposed Mechanism for Excision of pOY from pOX by Homologous Recombination

A. Putative structure of pOX

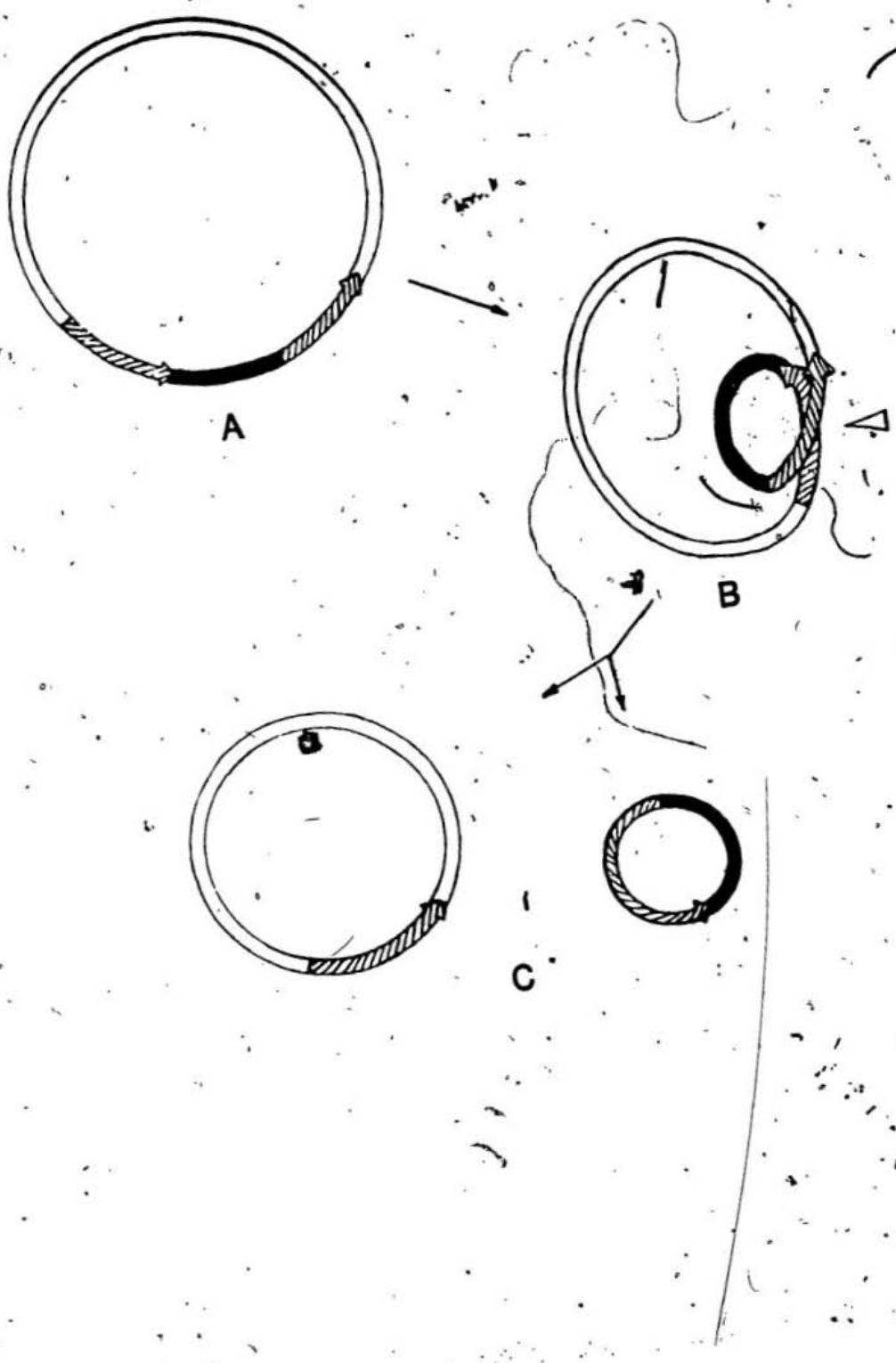
B. Homologous recombination between two Km^r genes

C. Recombination results in two circular DNA molecules. The smaller one is pOY, the larger one, lacking an origin, will be lost in subsequent cell divisions.

Hatched bar: Km^r gene, the arrow-head indicates the orientation.

Filled bar: origin fragment.

Open bar: *M. pneumoniae* DNA insert.


A
B
C

(Fig. 17). The plasmids pOX and pOY which should contain the new *Eco*R1* sites, however, can be digested to completion with approximately the normal amount of *Eco*R1 and at the normal rate. Is it a intrinsic feature of this new *Eco*R1* site or has it been corrected to a true *Eco*R1 site by the host cell? If it is the latter, then what was the selective pressure? The problem of whether or not the *Eco*R1* site has been repaired to an *Eco*R1 site can be solved by determining the sequence of the DNA in this region.

From the Southern blot transfer and DNA hybridization results pUC12 was found to have a certain amount of homology with *E. coli* DNA. In Fig. 12B, there are 7 bands of *E. coli* DNA which hybridize with the pUC12 replication origin (*i.e.* pOY origin) fragment.

The *E. coli* DNA was completely digested and most of the bands do not look like incomplete digestion products. What is the source of the observed homology? The presence of a *lac i, O, P* and *Z* fragment from pUC12 could explain the hybridization of some of the bands but it is difficult to explain all 7 bands in this way.

4.3. WHAT IS THE POSSIBLE REASON WHY THE M. pneumoniae ORIGIN MAY NOT FUNCTION IN E. coli?

Even though the mycoplasmas are assigned to the Gram positive bacteria according to their rRNA sequences, it appears that the mycoplasmas form a divergent group and are relatively distinct from the rest of the eubacteria (Razin, 1985; Woese, 1987). The evolutionary distance between the mycoplasmas and the eubacteria may be even greater than that between *E. coli* (as a representative of

Fig.17. The positions and sequences of 3 *Eco*R1* sites in pUC19 and 3 possible origin fragments with *Eco*R1* sites.

None of the 3 *Eco*R 1* ends can form an *Eco*R1 site after ligation with the *Eco*R1 end of the Km^r gene.

sites: 1569 1874 2130

```
      5'      3'  5'      3' 5'      3'
----------AAATTA--------TAATTG-------TAATTC------
----------TTTAAT--------ATTAAC-------ATTAAG------
origin
```

3 EcoR I* sites in pUC19

```
----------A 3'
----------TTTAA 5'
origin
```

```
-----------------------T 3'
-----------------------ATTAA 5'
origin
```

```
-----------------------------------T 3'
-----------------------------------ATTAA 5'
origin
```

3 possible origin fragments with EcoR I* ends

```
3'AATTC----------------------
   5'G----------------------
```

Km^r gene with EcoR I ends

Gram negative bacteria) and *B. subtilis* (as a representative of Gram positive bacteria). The results from the experiments reported here would suggest that this is particularly so with respect to the replication system. In addition to the evidence discussed in section 1.6., other reasons for this conclusion are as follows.

No homology has been found between mycoplasma DNA and the *dnaB, E, G, ZX, and K* genes of *E. coli* (S. Regular and P. Hempstead, unpublished results). Among these genes, *dnaB, E, G, ZX* produce important replication proteins, while *dnaK* is a heat shock gene and is conserved in both eubacteria and eukaryotes (Bardwell and Craig, 1984). Although the homology between the rRNA genes of *B. subtilis* and mycoplasma is relatively high (Razin, 1985; Woese, 1987), the mycoplasma 16s rRNA gene has many base alterations in the regions usually conserved between other eubacteria (Woese, 1987). Recently, *Mycoplasma mycoides* DNA has been shown to share similar features with the mitochondrial DNA of Neurospora, yeasts and mammals with respect to the reading of certain codons (Samuelsson *et al.*, 1987). Because the mitochondrion is considered to be derived from a primitive bacterium, the similarity between them suggests that the mycoplasma group is a primitive group among prokaryotes. This evidence suggests a clear distinction between the mycoplasmas and other eubacteria. On the other hand, *B. subtilis* retains considerable homology with *E. coli*. Its origin region has 6 open reading frames (ORF) which have homology to 6 genes of *E. coli* and have the same gene organizaton. The 6 genes of *E. coli* are involved in nucleic acid metabolism (*rnp*A), translation (*rpm*H), DNA replication (*dnaA, dnaN*), recombination (*rec*F) and DNA conformation (*gyr*B) (Ogasawara,

et al., 1985). The *dnaA* genes of the two bacteria have high homology and a gyrase gene fragment from *B. subtilis* can complement the *gyr*A mutant of *E. coli* (Lampe *et al.*, 1985). In addition, the *E. coli* and *B. subtilis* origins both contain multiple *dnaA* boxes and therefore may belong to the same *dnaA*-type origin (see below) with similar replication apparatus and initiation mechanism (Lother, 1985; Zyskind *et al.*, 1983; Ogasawara *et al.*, 1985).

The DnaA protein of *E. coli* has been found to be able to initiate replication of many plasmids (Seufert *et al.*, 1987) by binding to the *dnaA* box and directing DnaB, C, and G proteins to assemble into a pre-replisome. Such an origin, termed a *dnaA*-type, must contain the *dnaA* box, a primer initiation signal and a primer processing signal (either a termination signal or an RNase H cutting site) so that DNA polymerase III can continue synthesis from an RNA primer. In these experiments, however, no such origin has been found in *M. pneumoniae* DNA, which suggests that the origin in *M. pneumoniae* DNA may not be of *dnaA*-type.

Does *M. pneumoniae* share a similar overall DNA replication mechanism and exchangeable apparatus with *E. coli* but only the sequence specific initiation protein (DnaA equivalent) is different and recognizes a different specific initiation sequence? In this case the major possible reason for the failure to clone an *M. pneumoniae* origin could be that the sequence recognizing protein gene (*dnaA* equivalent) is located far away from the origin and the digested *M. pneumoniae* DNA always contains an origin fragment without the gene coding for the specific initiation protein. The origin fragment, not being recognized by DnaA protein, cannot function in the host cell only because of the lack of the corresponding

initiation protein. However, the heterology of the *dnaB, E, G and ZX* genes between the two bacteria suggests that the replication apparatus itself may have more extensive heterology than a difference between two initiation proteins. If the only difference between the DNA replication system of *M. pneumoniae* and that of *E. coli* lies in the initiation proteins, it might be possible to clone the *M. pneumoniae* origin by transforming ligation mixtures of *M. pneumoniae* DNA and a resistance gene fragment into *E. coli* which already carry one or more broad host range plasmid(s) resulting in the presence in the cells of an initiation protein(s) coded by the plasmid(s). If an initiation protein coded by the plasmid(s) can recognize the origin from *M. pneumoniae* and compensate for the missing "DnaA"-like function, the *M. pneumoniae* origin of DNA replication might then be functional in *E. coli*. Alternatively, it might be neccessary to clone *M. pneumoniae* DNA fragments ligated with a resistance gene directly into *M. pneumoniae* itself.

Another type of origin that functions in *E. coli* is the ColE1-type origin with a promotor for priming the leading strand and an 'n' site for initiating the lagging strand (Marians, 1984). The plasmids pBR322 and pUC12 have this origin derived from ColE1. In these experiments no such origin has been cloned from *M. pneumoniae* DNA and the plasmid pUC12 has no homology with the *M. pneumoniae* DNA, suggesting that the *M. pneumoniae* DNA does not have a ColE1-type initiation sequence.

In addition to the *E. coli* DnaA initiation origin and the ColE1 type origin, there are many other autonomously replicating sequences (ARS) which function

in *E. coli* (Kornberg, 1981; Scott, 1984). Some of them only need a portion of the replication apparatus of the host cell; some of them also encode proteins which are specific for their own replication. However, the functioning of these ARS (plasmids and phages) in *E. coli* usually involves a part of the whole replication system of the host cell. Since no ARS from *M. pneumoniae* was found that can function in *E. coli* it again implies that the difference between the two replication systems is extensive rather than only lying in the initiation protein itself.

As mentioned in section 1.2., *dam* methylation is essential for the functioning of *E. coli*-type origins. Four mycoplasma species have been tested for *dam* methylation by digesting the genomic DNAs with the restriction endonucleases *Sau*3AI and *Mbo*I. Of the 4 species tested, *M. arginini* and *M. fermentans* DNA are *dam* methylated, and *M. gallisepticum* and *M. pneumoniae* DNA are not methylated at *dam* sites (data not shown). If all the mycoplasma species examined share a similar replication mechanism and apparatus, *dam* methylation must not be essential, which supports the conclusion that the mycoplasma have a different replication initiation mechanism from that of *E. coli*; if *dam* methylation is essential for initiation in the *dam* methylation positive mycoplasmas, it means that there are different replication mechanisms within the mycoplasma group, providing additional evidence for wide divergence within the genus *Mycoplasma*.

That *M. pneumoniae* DNA is not methylated at the *dam* sites, suggests that the *M. pneumoniae* origin may represent an ancestral version of the *E. coli*-type *oriC* before *dam* methylation evolved, and that the *M. pneumoniae* origin, after

it is *dam* methylated, might be recognized by *E. coli* as an origin. This is not, however, likely to be the case because in these experiments the transformed cells were always incubated for 30 min in non-selective medium after transformation, which would allow the methylation of *dam* sites in the *M. pneumoniae* DNA to occur. The *dam* methylase can methylate all double stranded, non-methylated *dam* sites in incoming DNA (Adams, 1985). Since the *dam* methylase has no cognate restriction endonuclease in *E. coli* (Adams and Burdon, 1985) and the *E. coli* K-12 strain RR1, the host used in these experiments, lacks the only known restriction endonuclease in *E. coli* K-12, it is unlikely that there was extensive loss of transformed *M. pneumoniae* DNA sequences due to such restriction.

Also relevant to this discussion of why the *M. pneumoniae* origin fails to function in *E. coli*, is a consideration of the organization of the *B. subtilis* origin (section 1.5). Two origins have been found within the origin region of *B. subtilis*, but their *in vivo* roles are not yet clear. Levine *et al.* (1987) suggests that both origins of *B. subtilis* must be arranged in the correct order for it to function, and that this may be the reason for the failure of efforts to clone the *B. subtilis* origin as an *oriC*-plasmid in *B. subtilis* itself. In *E. coli*, the essential elements are clustered into a miniorigin. The *E. coli oriC*-plasmid, however, always shows some difference with respect to stability, copy number, partitioning, etc., from the chromosome, no matter how extensive the *oriC* fragment cloned. Except for the difference in absolute genome size between an *oriC*-plasmid and the chromosome, this suggests that there exist regulatory sequences located some distance from the miniorigin itself. If the essential sequences of the *M. pneumoniae* DNA origin are

distributed separately and cannot be cloned in one single fragment in an artificial plasmid after digestion and ligation, a functional origin can never be cloned with the method used in this experiment. A modification to the principle of these experiments may solve the problem, i.e. digesting and ligating mycoplasma DNA on its own before it is ligated with marker gene fragments. In the random ligation between mycoplasma fragments, a ligated fragment with 2 originally separated origin regions close to each other might be created that can be transformed and function as an intact origin.

This work is only the beginning of a study of mycoplasma DNA replication. There are further experiments worth doing, for example, to repeat these experiments but transform into host cells containing broad host range plasmids. The possibility of homology of the *E. coli dnaA* gene to *M. pneumoniae* DNA needs to be examined considering the important role the *dnaA* gene plays in determining the specifity of initiation at the origin. A further possibility is to attempt the cloning of a functional origin from *M. pneumoniae* as an *oriC*-plasmid in *M. pneumoniae* itself or in another mycoplasma species.

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