Cloning of the complete *Mycoplasma pneumoniae* genome

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ABSTRACT
The complete genome of Mycoplasma pneumoniae was cloned in an
ordered library consisting of 34 overlapping or adjacent cos-
mids, one plasmid and two λ phages. The genome size was determi-
ned by adding up the sizes of either the individual unique EcoRI
restriction fragments of the gene bank or of the XhoI fragments
of genomic M. pneumoniae DNA. The values from these calculati-
ons, 835 and 849 kbp, are in good agreement. An XhoI restriction
map was constructed by identifying adjacent DNA fragments by
probing with selected cosmid clones.

INTRODUCTION
During the last few years techniques which permit sequencing at

a megabase scale have been developed and improved. Therefore the

DNA sequence analysis of bacterial genomes now is a realistic

project for smaller research teams to perform within a reasonab-

le time.

Among the smallest bacterial genomes are those of the Mollicutes

ranging from 700 - 1600 kbp (1). Mainly because of the relative

small size of its genome - 800 kbp (1) when compared to 4.7 Mbp

for Escherichia coli (2) - we have chosen Mycoplasma pneumoniae

for defining the minimal genetic requirement of a self-replica-

ting cell. M. pneumoniae, the causative agent of atypical pneu-

monia in humans, may also be useful for analyzing the interacti-

on between a human pathogenic bacterial surface parasite and its

eukaryotic host cell.

A genome of 800 kbp may code for as many as 600-800 proteins

(3). It should be possible to map and identify a considerable

proportion of the corresponding genes with known functions by

techniques like cross hybridization using DNA or RNA probes de-

rived from conserved bacterial genes, or random DNA sequencing

and computer aided search for homology to cloned and sequenced
bacterial genes. In addition, the functions of certain genes can
be elucidated by complementation of mutants by cloned M. pneumo-
niae DNA in homologous and heterologous systems (4). This publi-
cation describes the construction of a complete cosmid gene lib-
rary of the M. pneumoniae genome in E. coli to provide the basis
for comprehensive genome analysis including DNA sequence deter-
mination.

MATERIALS AND METHODS
Strains and plasmids, growth of M. pneumoniae, DNA isolation,
radioactive labelling, cosmid cloning and mapping procedures we-
re described elsewhere (5).
Restriction enzymes
Restriction endonucleases were from Boehringer or New England
Biolabs and used as recommended by the manufacturers.
Lambda cloning
M. pneumoniae DNA was digested to completion with endonuclease
XhoI and ligated to XhoI cleaved λZAPII arms (6) under conditi-
ons recommended by the supplier (Stratagene cloning systems). In
vitro packaging was performed using the Gigapack plus extract
(Stratagene). E. coli XL1-Blue cells (6) were infected with the
phage and selected on NZY plates containing X-Gal and IPTG.
Recovery of DNA fragments from agarose gels
Restriction fragments were eluted from agarose gels using the
Gene clean kit (Bio 101 Inc.) according to the instructions of
the supplier.
Gel electrophoresis and Southern blotting
Separation of high-molecular weight M.pneumoniae DNA was perfor-
med by field inversion gel electrophoresis (FIGE) using an EMBL
CS130 FIGE/OFAGE apparatus with 1% agarose gels (15x 10cm). Ap-
proximately 5μg XhoI digested M.pneumoniae DNA was loaded into a
10cm wide slot (see Fig.5). The gel was run with a pulse time
ranging from 6 to 7 seconds for 48h at 80V in 0.5x TBE (1x TBE
is 90mM Tris, 90mM boric acid, 2mM EDTA) at 4°C.
Smaller restriction fragments (up to 40 kbp) were separated by
direct electrophoresis on 0.4% agarose gels (23x 20cm). Approxi-
mately 25μg DNA was loaded into a 15cm wide slot. The gel was

run for 96h at 70mA in running buffer (36mM Tris, 10mM Na_2EDTA, 30mM NaH_2PO_4) at 4°C (7). The buffer was exchanged daily.
Following ethidium bromide staining, the gels were transferred to nitrocellulose (BA85, Schleicher and Schuell) or nylon membranes (Biodyne, Pall) according to the instructions of the suppliers. The filters were cut into 0.5cm wide strips for hybridization probing with the cosmids which were ^{32}P-labelled by nick-translation (ca. 10^8 cpm/μg DNA).

Hybridization

After pre-hybridizing the nitrocellulose (or nylon) filters in 50% formamide, 5x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate pH 7.2), 5x Denhardt's solution (1x Denhardt's solution: 0.02 % Ficoll, 0.02% polyvinylpyrrolidine, 0.02% bovine serum albumin), 0.1% SDS and 100μg/ml denatured herring sperm DNA at 37°C for at least 4 hours, the hybridization was performed in 50% formamide, 5x SSC, 5x Denhardt's, 0.1% SDS and 10% dextran sulphate by adding the appropriate ^{32}P-labelled probe and incubating at 37°C overnight. The filters were washed three times for 30min with 1x SSC, 0.1% SDS at 68°C. Autoradiographic signals were obtained by exposing X-ray films (Kodak) overnight to the filters.

Preparation of the high-molecular-weight size standard

40μg of EcoRI linearized DNA from pSC101 (8) or pUC18 (9) was ligated at a concentration of 500μg/ml overnight at 16°C with 6 Weiss units of T4 DNA ligase. In the case of pUC18 the ligation product was partially digested with EcoRI to reduce the size of the highly polymerized concatamers to the range of 2.6 - 40 kbp. Approximately 0.5μg of DNA was loaded into a 5mm slot.

RESULTS

Cloning of the complete genome

In a previous publication we reported the cloning of about 90% of the M. pneumoniae genome (5).
At that time, attempts to clone the residual 10% had failed despite the screening of several thousand clones, a number more than sufficient from a statistical point of view (10). Since then, extended screening has led to the isolation of three more overlapping cosmids, pcosMPK4, pcosMPG7 and pcosMPGT9, however the two clones at the end, pcosMPGT9 and pcosMPE7, were neither
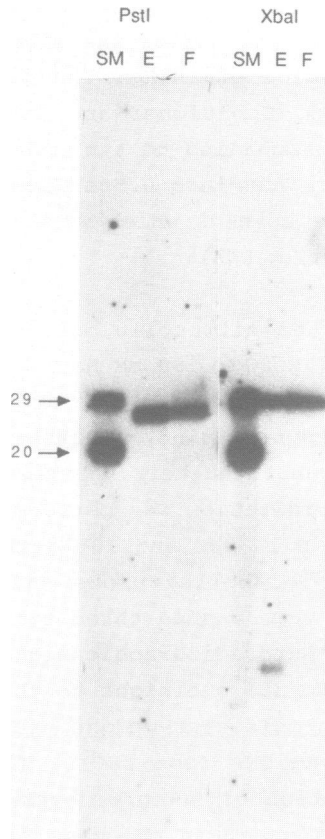
Figure 1: Restriction analysis of the region between pcosMPGT9 and pcosMPE7. <u>M. pneumoniae</u> DNA was digested with PstI and XbaI, separated on a 0.4% agarose gel, blotted and hybridized to probes which were specific for the ends of the cloned region. SM = size marker (λEMBL3x EcoRI; the cos site containing fragments of 9 and 20 kbp form the 29 kbp band); E = E7E (subclone of E7, see Fig.3); F = XhoI restriction fragment V (specific for the end of pcosMPGT9, see Fig.3). Sizes are in kbp.

overlapping nor adjacent. At this point of the analysis it seemed unprofitable to continue just screening more clones. We decided rather to characterize the remaining gap between pcosMP-GT9 and pcosMPE7 first, using restriction analysis of genomic <u>M. pneumoniae</u> DNA to find one or more restriction fragments which would link the terminal cosmids. To probe the genomic restriction fragments on Southern blots it was unsatisfactory to use the
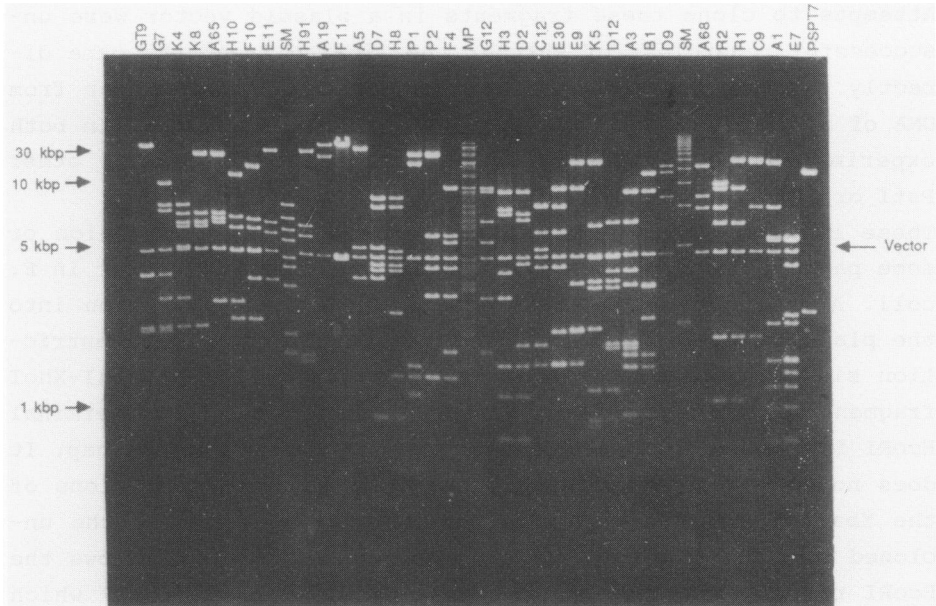
Figure 2: Restriction analysis of the 34 cosmids plus 1 plasmid (pSPT7) covering the complete genome of Mycoplasma pneumoniae except for 5.3 kbp cloned in λ (see Fig.3). The cosmids were digested with EcoRI and pSPT7 with XbaI and XhoI and the digests were separated on a 0.8% agarose gel. SM = size marker (left: λx BstEII; right: pUC18 polymers); MP = M.pneumoniae DNA x EcoRI

complete cosmids as probes since both contain repetitive elements (11) making it necessary to use DNA with sequences from only the gap proximal ends of the cosmid inserts.

For the end of pcosMPGT9, the probe used was an 8 kbp genomic DNA XhoI DNA fragment (fragment V, see below) which could be readily isolated from an agarose gel. It has been shown that the 6.7 kbp XhoI-EcoRI gap proximal fragment of pcosMPGT9 is part of this XhoI fragment and that the residual 1.3 kbp reaches the gap region. For a probe from pcosMPE7, its gap proximal 2.7 kbp EcoRI fragment was subcloned resulting in E7E. Southern blots of genomic DNA digested with several enzymes (BamHI, EcoRI, XhoI, HindIII, PstI and XbaI) were screened with these probes. Among them, a 27 kbp PstI fragment and a 29 kbp XbaI fragment reacted positively with both probes (Fig.1), strongly indicating that these fragments linked the terminal cosmids.

Attempts to clone these fragments in a plasmid vector were un-
successful regardless of whether the ligated fragments came di-
rectly from a restriction digest of total genomic DNA or from
DNA of appropriate size fractionated in an agarose gel. In both
experiments positive controls came from the cloning of other
PstI or XbaI fragments.

These results supported the conclusion that the gap region or
some part of it might not be clonable in a plasmid vector in E.
coli. Attempts to clone subfragments from the gap region into
the plasmid pSBO2 (12) which contains unique XbaI/XhoI restric-
tion sites resulted in pSPT7 which contains a 13 kbp XbaI-XhoI
fragment. This fragment overlaps 1.9 kbp of the 2.7 kbp terminal
EcoRI fragment of pcosMPE7 and reaches 11.1 kbp into the gap. It
does not bridge pcosMPGT9 and pcosMPE7. Since the positions of
the XbaI restriction sites in the cosmids were known, the un-
cloned region could not be larger than 5.3 kbp. Fig.2 shows the
EcoRI restriction pattern of the 34 cosmids and of pSPT7 which
was XbaI/XhoI digested.

In the construction (described below) of an endonuclease XhoI
restriction map of the M. pneumoniae genome based on the cosmid
collection we correlated the XhoI fragments in genomic DNA di-
gests with those in the cosmids. Only one, a 4 kbp fragment
(fragment X, see below), could not be attributed to correspond-
ing cosmids. Therefore this fragment was assumed to be located
in the gap region between XhoI fragments A and V. This assumpti-
on was verified by probing Southern blots of XbaI and PstI di-
gested M. pneumoniae DNA with the genomic 4 kbp XhoI fragment as
a labelled probe. This probe recognized only the 27 kbp PstI and
29 kbp XbaI fragments.

Attempts to clone the XhoI fragments V and X into either a low
or high copy number plasmid failed. These results, indicating
that the fragments can not be cloned into an E.coli plasmid vec-
tor, suggest that the presence of fragment X and a 1.3 kbp part
of fragment V in an E. coli cell is inhibitory to its continued
growth. Therefore we used a vector system in which cell survival
is not required and the chance of cloning the missing fragments
should be greater. Using the λZAPII system (6), which has the
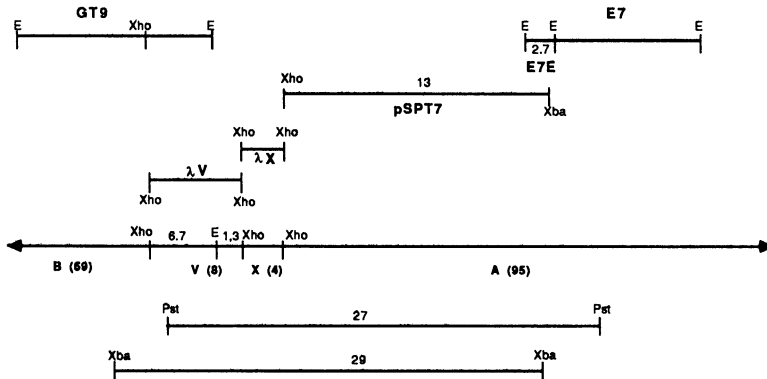advantage that its maximum cloning capacity of 10 kbp would ac-

Figure 3: Restriction map of the pcosMPGT9/pcosMPE7 region. The numbers indicate the size of the fragments in kbp. The map shows the position of the λ clones λV, λX and of pSPT7. A, B, V and X represent the corresponding XhoI restriction fragments (see Table 1). E = EcoRI

cept only the four smaller XhoI fragments, it was possible to clone fragments V and X to give λV and λX. This closed the gap in the region between pcosMPGT9 and pSPT7. Fig.3 shows the restriction map and the position of the clones in the region of the original gap.

Fig.4 shows the final alignment of the 34 cosmids, one plasmid and two λ recombinants which cover the complete genome. Compared to the recently published cosmid map (5) one clone – pcosMPB11 – had to be eliminated since it proved to be a mixture of two DNA regions, one overlapping with pcosMPR2 in two EcoRI fragments and the other with pcosMPGT9 in three EcoRI fragments (see also discussion).

Size of the M.pneumoniae genome

Our data allow estimations of the size of the M. pneumoniae genome by calculation of the sum of the sizes for the fragments generated by restriction endonuclease EcoRI and XhoI digests.

For one calculation every unique EcoRI restriction fragment which has been cloned in the cosmid collection was defined and its size was measured by agarose gel electrophoresis using appropriate size markers. A total number of 145 fragments was counted from an agarose gel like in Fig.2. For an exact size determination, different agarose concentrations were applied: a
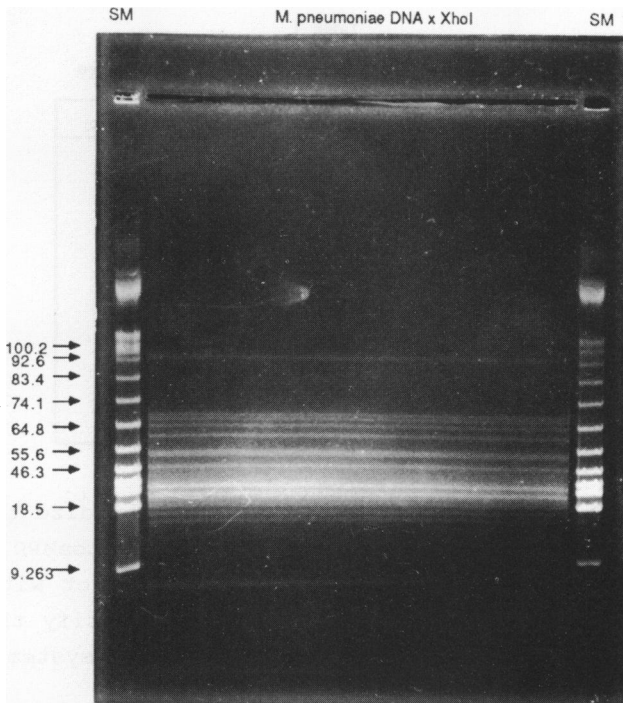
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            ⟵── λV── λX ── pSPT7⟶

    G7─ GT9─┴─E7─ A1 ─ C9 ─ R1 ─ R2 ─A68 ─ D9─B1─A3
    K4                                               │
    │                                               D12
    K8                                               │
    │                                               K5
    A65                                              │
    │                                               E9
    H10                                              │
    │                                               E30
    F10                                              │
    │                                               C12
    E11                                              │
    │                                               │
    H91─ A19─F11─A5─D7─ H8 ─ P1 ─P2─F4─G12─H3 ─D2
      └ pSPT2 ┘   └ pSPT13 ┘
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Figure 4: Schematic diagram of the cosmid map. The pairs pcosMP-
A5/F11 and pcosMPA19/H91 are linked by the cloned Bam HI frag-
ments of pSPT13 and pSPT2, respectively. pcosMPGT9 and pcosMPE7
are linked by the λ clones λV, λX and by the plasmid pSPT7.

0.4%-agarose gel for fragments between 5 and 28 kbp (= the lar-
gest EcoRI fragment, see also pcosMPF11) and a 1.2%-agarose gel
for fragments smaller than 5 kbp. The size markers used were:
phage λ DNA x BstEII digest covering sizes from 117 to 8453 bp,
multimers of the linearized plasmids pUC18 (monomer 2686 bp) or
pSC101 (monomer 9363 bp) (see Figures 2, 6). All markers had si-
zes which were defined from sequence data. The sum of the sizes
of all 145 EcoRI fragments including the fragment in the gap re-
gion is 835 kbp as a measure for the size of the total genome.
Fragments smaller than 300 bp were not taken into account.
The second calculation used restriction fragments from genomic
DNA of M. pneumoniae. Pilot experiments tested several restric-
tion enzymes which were predicted to cut a DNA with 41%- GC con-
tent infrequently (13). Among them AvrII, RsrII and XhoI gave
about 20-30 restriction fragments. XhoI was chosen because the
restriction pattern appeared to be the simplest one. Again, de-
pending on the fragment lengths, different gels were used for
sizing: 0.6% and 0.4% agarose gels for fragments up to 30 kbp
and a field inversion gel (14) for the larger ones (Fig.5). The
sizes of the 25 XhoI fragments range from 95 kbp to 1.3 kbp
(Table 1). The estimate of genome size according to this calcu-
lation is 849 kbp which is in good agreement with the value de-
rived from EcoRI fragments.

Figure 5: FIGE separation of large fragments from XhoI digested
M. pneumoniae genomic DNA on a 1% agarose gel. After determining
the size of the fragments, the gel was blotted and used for pro-
bing (Fig.6, part A). SM = size marker (pSC101 x EcoRI poly-
mers). Sizes are in kbp.

Endonuclease XhoI restriction map of the M. pneumoniae genome

A XhoI restriction map was constructed mainly by means of the
cosmid collection. Except for two cosmids (pcosMPE30, pcosMP-
D12), the XhoI sites were detected by comparing an EcoRI res-
triction digest with an EcoRI/XhoI double digest of the cosmids.
The disappearance of an EcoRI fragment and the appearance of two
smaller DNA fragments clearly demonstrated an XhoI site. In case
of pcosMPE30 and pcosMPD12, nucleases BamHI and HindIII, respec-
tively, were used because the EcoRI and EcoRI/XhoI restriction
patterns were indistinguishable in a standard agarose gel.
Cosmids containing one or more XhoI sites were ^{32}P-labelled by
nick-translation and used as probes for identifying adjacent
XhoI fragments in a Southern blot of XhoI digested genomic **M.**

Table 1

XhoI restriction fragments of Mycoplasma pneumoniae

Fragment	Size (kbp)	Fragment	Size (kbp)
A	95	N	24.5
B	69	O	24
C	68	P	22.5
D	66	Q	21.5
E	62	R	21
F	55	S	19.5
G	55	T	18
H	49	U	15
I	34	V	8
J	34	W	5
K	27.5	X	4
L	25	Y	1.3
M	25	Total	849

pneumoniae DNA. Cosmids with only one site hybridize to two XhoI fragments (for instance pcosMPD12, pcosMPD9, pcosMPD7 or pcos-MPG12) whereas clones containing two sites react with 3 fragments, like pcosMPH3 (Fig.6). In order to identify the genomic XhoI fragments unambiguously, two different gel systems and different agarose concentrations had to be used.

In a number of hybridizations several lighter bands appear (compare the cosmids pcosMPR2, pcosMPP1, pcosMPA5, pcosMPA19 and p-cosMPE7 in Fig.6). These cross-reactions are caused by one or more of at least five different repetitive DNA sequences (11,15, 16) which are present in the genome. In case of pcosMPB1 (two sites) only one strong band shows up. According to our interpretation this band represents two fragments of the same size. The missing third fragment is the smallest 1.3 kbp fragment Y which had run off the gel. Like all four fragments below 10 kbp, it was identified by direct comparison of XhoI digests of cosmids and genomic DNA in an agarose gel. Fig.7 shows the XhoI restriction map of the complete M. pneumoniae genome.

An anomaly which arose in this analysis was that the cosmid digests gave a total of 26 XhoI sites, including the one between λV and λX, whereas the genomic DNA digests showed only 25. XhoI digests of cosmids pcosMPA68 and pcosMPD9 showed a 4 kbp XhoI fragment which does not have its counterpart in the genomic digest. After isolating the fragment from an agarose gel and using
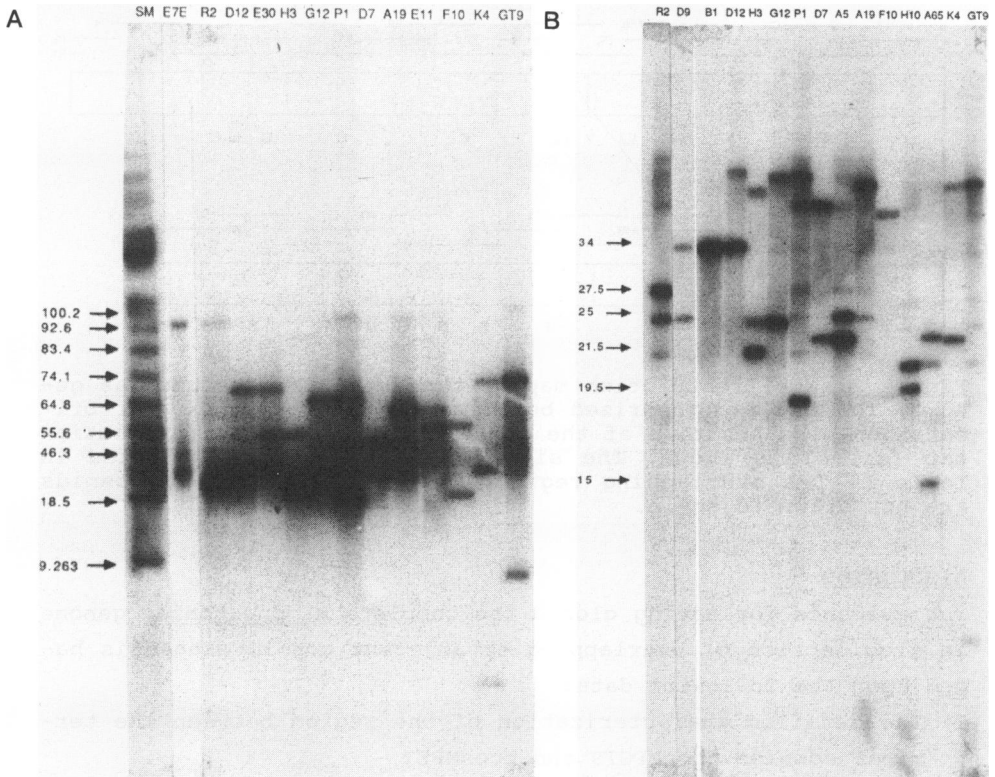
Figure 6: Southern blots of M.pneumoniae DNA digested with XhoI. In order to separate fragments larger than 40 kbp, a field inversion gel (FIGE) was run (part A). Fragments smaller than 40 kbp were separated by the gel system described in material and methods (part B). Both filters were cut into stripes and hybridized to a set of selected ^{32}P-labelled cosmids. The resulting XhoI restriction map is shown in Figure 7. SM = size marker (pSC101xEcoRI polymers). Sizes are in kbp.

it as a ^{32}P-labelled probe in a Southern blot against XhoI restricted M. pneumoniae DNA, it reacted only with the I/J double band (see Fig.7). This indicates strongly that either I or J possesses an additional XhoI site, which is protected in genomic DNA probably by methylation in vivo.

Our approach to use aligned cosmids from the gene library for constructing a restriction map appeared to be fast and simple and yielded unambiguous results.
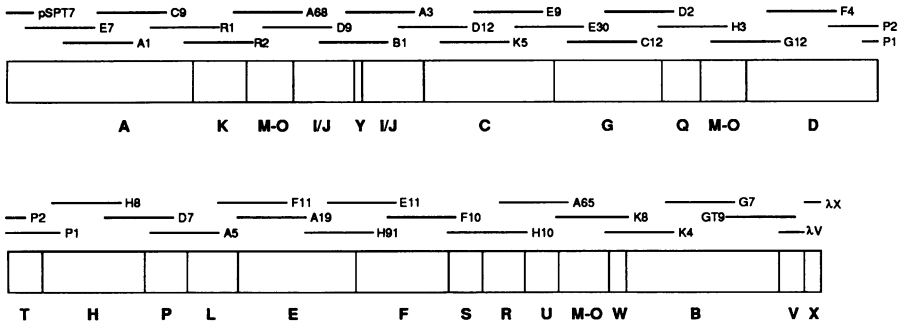
Figure 7: XhoI restriction map of the Mycoplasma pneumoniae ge-
nome. The map is linearized between fragments A and X. For ori-
entation, the position of the clones relative to the restriction
map (sites) is shown. The size of the fragments is listed in
Table 1. The overlapping regions between neighbouring cosmids
are not drawn to scale.

DISCUSSION

The evidence for having cloned the complete M. pneumoniae genome
in a collection of overlapping or adjacent cosmid clones is ba-
sed upon the following data:

1) the detailed characterization of the region between the ter-
   minal cosmids pcosMPGT9 and pcosMPE7,

2) the endonuclease XhoI restriction map and the identification
   of the corresponding restriction sites within the cosmids and

3) the consistency of the two independent genome size determina-
   tions, one based on data from the cloned DNA and the other on
   data from genomic DNA only.

The reasons for the difficulties in cloning the region between
the terminal cosmids are still unknown. Since not only high copy
number plasmids but also low copy number vectors like pSC101
failed to replicate with the two XhoI fragments we assume that
they might code for sequences toxic to growth of E. coli. This
assumption is strongly supported by the fact that these frag-
ments could be cloned in phage λ for which replication is not
dependent on survival of the host bacterium which is usually ly-
sed as a result of infection. DNA sequence analysis of the re-
gion in question may help to explain the cloning difficulties.

As noted above, we had to delete the clone pcosMPB11 from our
previously published map (5), since it contained two DNA seg-

ments from different locations in the genome. It was one of
three cosmid clones - which we reported in our previous paper -
as not having other, independently isolated, identical or over-
lapping clones. In retrospect, the best argument for having clo-
ned a contiguous DNA fragment instead of a mixture of fragments
is the isolation of other identical or extensively overlapping
clones.

The introduction of pulsed field gel electrophoresis (PFGE) (17)
gives a powerful method for the size determination of complex
genomes. Originally, the size of various Mollicutes genomes has
been determined by electron microscopy (18) or by DNA renatura-
tion kinetics (1). The results indicated two size classes of
about 800 kbp and 1600 kbp length. Recently, Pyle et al. (19)
published results based on PFGE showing larger genome sizes than
anticipated for species in the former class, for instance 1140
kbp for Mycoplasma hyopneumoniae, J strain or 1240 kbp for My-
coplasma mycoides, subsp. mycoides, Y strain.

The discrepancies have not yet been resolved, though recently
the validity of PFGE for AT-rich DNA sequences (as most of the
Mollicutes genomes are) has been questioned by Maniloff and Pod-
dar (20,21). In case of M. pneumoniae our size determination ag-
rees fairly well with the value of 735 kbp derived from renatu-
ration kinetics. It should be mentioned that, as an exception
among the Mollicutes, M. pneumoniae has a GC-content of about
41% (22). Therefore M. pneumoniae DNA should behave "normally"
in a PFGE analysis. The size of the M. pneumoniae genome mea-
sured as a single, or two, fragments in PFGE was in the range of
800-850 kbp (L. Pyle and L. Finch, pers. communication) in good
agreement with our data.

The genome size determination by addition of the sizes of indi-
vidual restriction fragments is straight forward. Potential
sources of error could arise for calculations based on the res-
triction analysis of the cloned cosmids. Firstly, fragments in
overlapping clones showing a very similar size might not be re-
cognized as being different (for instance the largest EcoRI
fragments in pcosMPG12 and pcosMPF4 (Fig.2) are not identical).
Secondly, it is sometimes difficult to differentiate between a
double or a triplet band (for instance the heavy band in pcosMP-

E7 (Fig.2) just above the vector and the band in pcosMPD12 con-
taining the vector band are triplets). Smaller EcoRI fragments
(<300 bp) have been neglected but they are insignificant in the
overall size calculation.

The advantage of the calculation from the endonuclease XhoI ge-
nomic restriction analysis is the limited number of fragments so
that they can be distinguished for counting on a gel. An obser-
vation that sometimes the larger fragments seem not to be pre-
sent in equimolar amounts as judged from the staining is very
probably due to mechanical shearing of large DNA fragments (> 50
kbp). This would be minimized by release and digestion of the
DNA in agarose blocks (17).

Size determination for fragments larger than 40 kbp requires use
of PFGE gels because of the rapidly decreasing resolution in
conventional gel systems. In both systems it is also indispens-
able to have polymeric size markers from sequenced DNA to give
many reference points at close and defined distance. Even in the
worst case the error is then less than the difference between
two reference points. Therefore we believe that the error in our
calculation is less than 5% (or about 40 kbp). For a more pre-
cise determination, the DNA sequencing of the complete genome
will be necessary.

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