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Physical mapping of the *Mycoplasma pneumoniae* genome

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ABSTRACT

In order to study the genome organization of Mycoplasma pneumoniae a cosmid library of M.pneumoniae DNA was established using a newly designed cosmid vector (pcosRW2). From this library 32 overlapping clones were isolated covering a contiguous 720 kbp DNA segment representing about 90% of the genome assuming a genome size of about 800 kbp.

INTRODUCTION

Mycoplasma pneumoniae exhibits several features that make it an appealing model for studying certain aspects of microbiology. Besides the general advantage of the mycoplasmas of having the smallest bacterial genome with a size of 800 kbp (1) Mycoplasma pneumoniae offers some specific attractions: as the causative agent of primary atypical pneumonia in humans (2), it is a good model for analyzing the relationship between a pathogenic bacterial surface parasite and its eucaryotic host.

So far it has not been possible to apply the tools of classical genetics such as transformation, transduction or conjugation to mycoplasmas to study the bacteria-host relationship. Only recently progress was made in this respect when a transposon was introduced into the genome of Mycoplasma pulmonis via transformation (3) and that of Mycoplasma hominis via conjugation (4).

An alternative way of performing genetic analysis was made possible by the in vitro recombinant DNA technology which permits the analysis of cloned genes in heterologous systems.

As a first step towards a detailed genetic analysis of M. pneumoniae we undertook to establish a cosmid gene library of M. pneumoniae DNA in Escherichia coli. To ensure the completeness of this gene bank, we attempted to map as many overlapping clo-

nes as necessary in order to cover the whole genome. Theoretically, in a library of 200 clones 30-40 cosmids should be sufficient for that purpose assuming a genome size of about 800 kbp and an average insert size of 40 kbp per cosmid.

MATERIALS AND METHODS

Growth of M. pneumoniae

M. pneumoniae strain M129 in the 18th broth passage (a gift from I. Kahane, Jerusalem) was grown in modified Hayflick medium (5) at 37°C using 75cm^2 cell culture flasks. After colour change of the medium from red to yellow, cells were harvested by centrifugation at 12.500 x g for 15 min at 4°C, washed once in PBS (150 mM NaCl - 10 mM sodium phosphate buffer, pH 7.4), and suspended in 1/10 the original volume of PBS.

DNA isolation

25 ml of M. pneumoniae culture (equivalent to 2 x 10^{10} cells) were centrifuged, washed in PBS and resuspended in 2-3 ml 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.3% SDS, 20 µg/ml Proteinase K. The solution was incubated at 50°C for 2 hours, extracted twice with phenol and once with 1:1 chloroforme/phenol. Then the aqueous phase was dialyzed against 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.

Southern blotting

The selected DNA was analyzed on agarose gels varying from 0.3 to 1%. After electrophoresis the gels were soaked in 0.25 M HCl for 15 min. The DNA was then denatured in the presence of 0.5 M NaOH, 1.5 M NaCl for 20 min and neutralized in 1 M Tris-HCl pH 8.0, 3 M NaCl for 30 min. The DNA was transferred to nitrocellulose filters (Schleicher & Schüll BA 85) according to Maniatis (6). The filters were dried and baked at 80°C for 2 hours.

Radioactive labeling of nucleic acids

Nicktranslation was done according to Rigby et al.(7), 5'- and 3'- labeling was performed following the method of Maniatis (5).

DNA sequence analysis

DNA sequences were determined by the dideoxy-chain-termination method of Sanger (8). The procedure was modified as indicated by Chen and Seeburg (9).

Computer analysis

DNA sequences were analysed with the Biological Sequence Analysis program at the German Cancer Research Center, Heidelberg.

Cosmid cloning

M. pneumoniae DNA was partially digested by restriction endonuclease EcoRI (Boehringer) and the DNA-fragments were dephosphorylated by calf intestinal phosphatase (Boehringer) under conditions as recommended by the manufacturer. Following size selection by sucrose gradient (15-40%) centrifugation, the restriction fragments were ligated into the cosmid vector pcosRW2. A standard ligation mixture contained 4μg (=1pmol) vector DNA, 1μg M. pneumoniae DNA and 6 Weiss units of T4-Ligase in a volume of 20μl. After incubation at 16°C for at least 12 h, an aliquot of the ligation mixture was packaged in vitro and infected using E.coli HB101. Packaging and infection was done according to Maniatis (6). Transfected cells were selected on agar plates containing 50 μg/ml ampicillin. The efficiency was up to 5 x 10^4 clones per μg Mycoplasma DNA.

Screening of cosmids

Selected cosmid clones were grown overnight in 96-well microtiter plates in Standard I nutrient broth (Merck) containing 50 μg/ml ampicillin at 37°C. Using a replicating block, cells were transferred to nitrocellulose filters which were placed on agar plates containing ampicillin (50 μg/ml). Following overnight incubation at 37°C, the filters were transferred in 10% sodium dodecyl sulfate, then the DNA was denatured in 1.5 M NaCl, 0.5 M NaOH and neutralized in 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0. After drying and baking at 80°C for 2 h the filters were incubated at 37°C for 1 h in 50 mM Tris-HCl pH 8.0, 1 M NaCl, 1mM EDTA, 0.1% sodium dodecyl sulfate and used for hybridization.

The original clones were stored in microtiter plates in StI Medium containing ampicillin and 20% glycerol at -70°C. From this stock, cosmids were transferred into StI-medium with 10 μg/ml tetracycline (see Results) using sterile toothpicks and propagated at 37°C.

Strains and plasmids

The E. coli strain HB101 (F^-, recA13, rpsL20, supE44) (10) was

used for transformation with plasmid DNA as described in Hanahan (11) and for cosmid transfection and propagation. The cosmid pcosRW2 was constructed from pcos2EMBL (12) and pSPT18 (13) (for details see Results).

Restriction enzyme digests were performed under conditions recommended by the manufacturer (Boehringer or Biolabs).

Oligonucleotides

SP6TW (5'-CACATACGATTTAGGTGACAC-3')

T7RW (5'-CTGGCTTATCGAAATTAATACG-3')

NOTI (5'-AATTGGCCATAGCGGCCGCGAATTCGCGGCCGC-3')

NOTII (5'-AATTGCGGCCGCGAATTCGCGGCCGCTATGGCC-3')

MP16S (5'-CTTTAGCAGGTAATGGCTAGAG-3')

The oligonucleotides were synthesized by a Biosystems 380A machine according to the phosphoramidite method using a solid carrier (14). Purification of the oligonucleotides was done as described by Ferreti (15).

Hybridization

After pre-hybridizing the nitrocellulose 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) at 37°C for at least 4 hours, the hybridization was performed in 50% formamide, 5x SSC by adding the appropriate ^{32}P-labelled RNA probe and incubating at 46°C overnight. The filters were washed twice with 2x SSC, 0.1% SDS at 68°C for 30 min and at least once for one hour at 68°C in 1x SSC, 0.1% SDS. Autoradiographic signals were obtained by exposing X-ray films (Kodak) overnight to the filters.

In vitro transcription

This was done according to Melton (16) except that the DNA concentration was 50 µg/ml. In general, T7-RNA transcripts were synthesized more efficiently than SP6-transcripts. In a standard assay, 10^5-10^7 Cerenkov counts were incorporated into RNA per µg cosmid DNA dependent on the cosmid and the quality of the DNA preparation. In most cases, relative crude preparations from 2 ml culture volumes (minipreps) could be used for transcription. However, some cosmid templates had to be purified by cesium

chloride density gradient centrifugation in order to obtain adequate results.

RESULTS

Restriction endonuclease EcoRI analysis of genomic M. pneumoniae DNA had shown that the largest EcoRI DNA fragment was about 32 kb long with only a few other fragments between 20 and 30 kb. We therefore decided to size fractionate M. pneumoniae DNA for cloning by partial digestion with endonuclease EcoRI rather than with a statistically more frequently cutting enzyme such as endonuclease Sau3A. The advantage of using the enzyme EcoRI instead of Sau3A is that the degree of overlap between two cosmid clones can be seen readily by an EcoRI restriction analysis of the cloned DNA. A disadvantage is that two adjacent large EcoRI fragments might exceed the packaging capacity of phage λ, and cosmids carrying either one of these fragments would not be identified as adjacent.

Construction of the cloning vector pcosRW2

The cloning vector had to fulfill the following criteria:

1) Effective cloning of EcoRI restriction fragments into cosmids.

2) Synthesis of hybridization probes specific to the ends of the cloned DNA.

3) Excision of the entire DNA insert cloned in EcoRI and subsequent modification of the cosmid clones, for instance by inserting new genetic markers (i.e. presence of a unique restriction site following cloning of foreign DNA).

4) Possibility of setting up restriction maps of individual cosmid clones quickly and simply.

Since no vector meeting all these requirements was available for our purposes, the cloning vector pcosRW2 was constructed as follows:

The 2.75 kb EcoRI fragment of pcos2EMBL containing a gene coding for tetracycline resistance and two phage λ cos sites was cloned into the unique PvuII site of the plasmid pSPT18. In order to prevent restoration of an intact EcoRI or PvuII restriction site during ligation, the EcoRI generated fragment was modified first

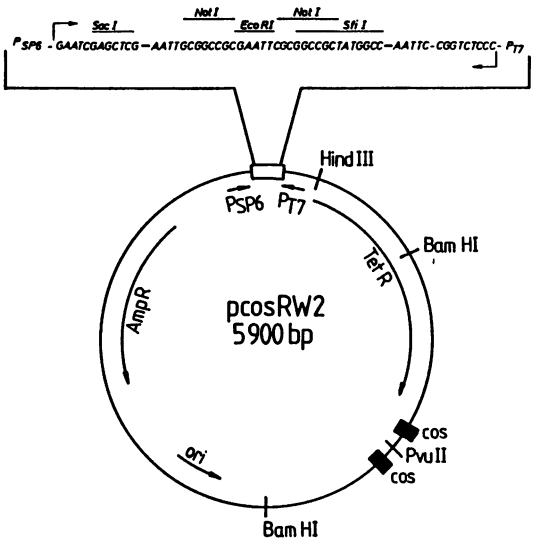

Figure 1: Map of pcosRW2

by converting its sticky ends to blunt ends by repair synthesis and second by subcloning it into the HindII site of the pSP65 polylinker (Melton et al. 1984). After the cutting off of pSP65 with endonucleases SmaI and PstI and removing the protruding 3'-ends of the PstI cut by S1 nuclease treatment, the thus modified pcos2EMBL EcoRI fragment could be ligated into the PvuII site of pSPT18 without restoring an EcoRI or PvuII restriction site. Finally the polylinker of pSPT18 was replaced by a linker of our choice. The structure of pcosRW2 is shown in fig.1.
This cosmid vector combines all the above-mentioned prerequisites. It has the unique EcoRI cloning site and the intact cloned insert can, in most cases, be cut out with endonuclease NotI, since M. pneumoniae DNA has only two NotI sites (unpublished data). The SfiI site allows insertion of additional DNA (up to the packaging capacity of phage λ), as a SfiI site occurs probably only once in the M. pneumoniae genome (unpublished data). Furthermore, cleavage of the SfiI site linearizes the cosmid. Any labelled probe hybridizing against the vector part of the cosmid can be used to map restriction fragments according to the method of Smith and Birnstiel (17). And finally, SP6 and T7 polymerases

will synthesize RNA probes which are specific to the ends of the cloned DNA.

Gene bank of M. pneumoniae DNA

Total M.pneumoniae DNA was digested with restriction endonuclease EcoRI. The reaction kinetics were monitored by taking samples at different times.

Digestion was terminated by an excess of EDTA and analyzed on a 0.4% agarose gel. Samples in which the main fraction of M. pneumoniae DNA migrated just above phage λ DNA were selected and pooled with more or less digested samples in order to include chromosomal DNA regions both with many and with few EcoRI restriction sites.

The restricted M. pneumoniae DNA was treated with calf intestinal phosphatase (CIP) and size fractionated on a sucrose gradient. Initially, the sucrose gradient was omitted but since some of our isolated clones had insertions which must have arisen by ligation of non-contiguous DNA fragments, size fractionation was included in all further DNA preparations.

Ligation, packaging and plating was performed as described under material and methods. The efficiency of plating varied between 1.5×10^4 and 5×10^4 clones per μg M. pneumoniae DNA, depending on the packaging extract. Clones were individually stored at -70°C in liquid media using microtiter plates.

For selection and propagation of E.coli cells carrying cosmid clones, it turned out to be essential to include tetracycline in the growth medium since in the presence of ampicillin only, we observed loss of the original cosmid and the appearance of smaller DNA molecules. Following this precaution, cosmids could be maintained without deletions as measured by restriction analysis.

Cosmid map

Two cosmids (pcosMPA3, pcosMPA5) with different EcoRI restriction patterns were chosen as starting points for the cosmid mapping.

Site specific RNA probes from each end of the insert were synthesized from the bacteriophage promoters SP6 and T7 respectively. The average size of the probes was about 1-2 kb. In some cases, when short probes were required, the cosmids were restric-

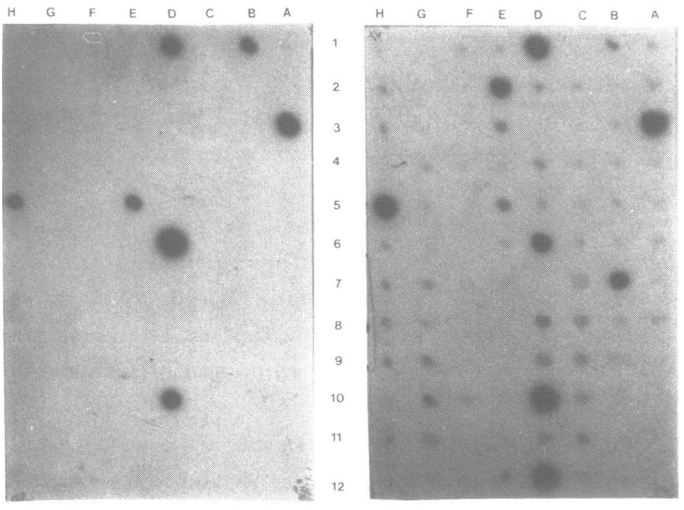

Figure 2: Search for overlapping cosmids. ^{32}p-labelled RNA probes synthesized from the SP6 and T7 promoters of pcosMPA3 were hybridized against 96 cosmid clones. From the positively reacting clones, pcosMPB1 (SP6) and pcosMPD12 (T7) were chosen for further analysis.

ted with endonucleases cutting downstream of the promoters.

After screening 200 randomly picked clones with site specific probes, four different hybridization patterns were obtained (fig.2):

1) no hybridization

2) hybridization with both probes

3, 4) hybridization with only one probe.

Clones reacting with one probe only were further analyzed by EcoRI restriction analysis in order to define the degree of overlap. Clones with the smallest overlap and the largest insert were selected and used for identification of the next overlapping cosmid clone, and so on.

In this way, most of the 32 clones shown in fig's.3 and 4 have been mapped, with the exception of the pair pcosMPK8/pcosMPE7, pcosMPH91/pcosMPA19 and pcosMPF11/pcosMPA5, which do not overlap.

For the adjacent clones pcosMPH91/pcosMPA19 and pcosMPF11 /pcos-

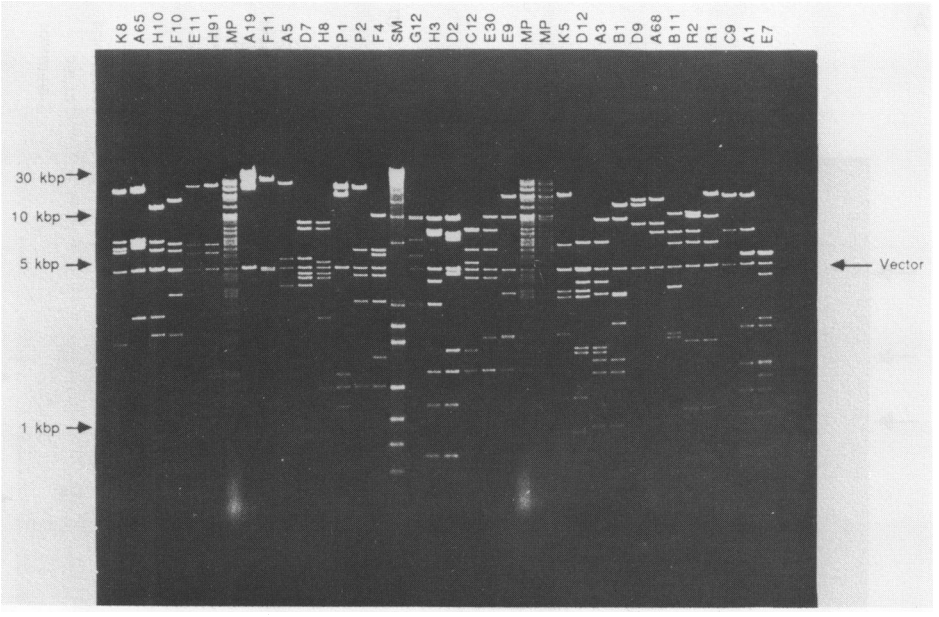

Figure 3: Restriction analysis of the 32 cosmids representing a contiguous DNA stretch of 720 kb. The cosmids were digested with EcoRI and separated on a 0.8% agarose gel. MP = M.pneumoniae DNA x EcoRI; SM = size marker.

MPA5 we showed that their terminal EcoRI fragments are adjacent, since pcosMPH91/pcosMPA19 are linked by a 4.1 kb BamH1 fragment and pcosMPF11/pcosMPA5 by a 30 kb Bam HI fragment. These two

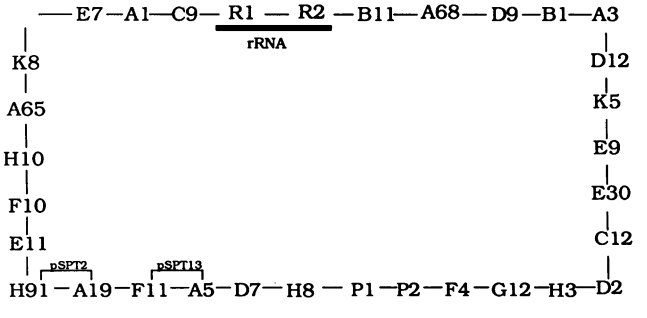

Figure 4: Schematic diagram of the cosmid map of M. pneumoniae. Cosmids pcosMPP1 and pcosMPP2 carrying the single rRNA operon are underlined. The pairs pcosMPA5/F11 and pcosMPA19/H91 are linked by the cloned BamHI-fragments of pSPT13 and pSPT2, respectively (see fig.5).

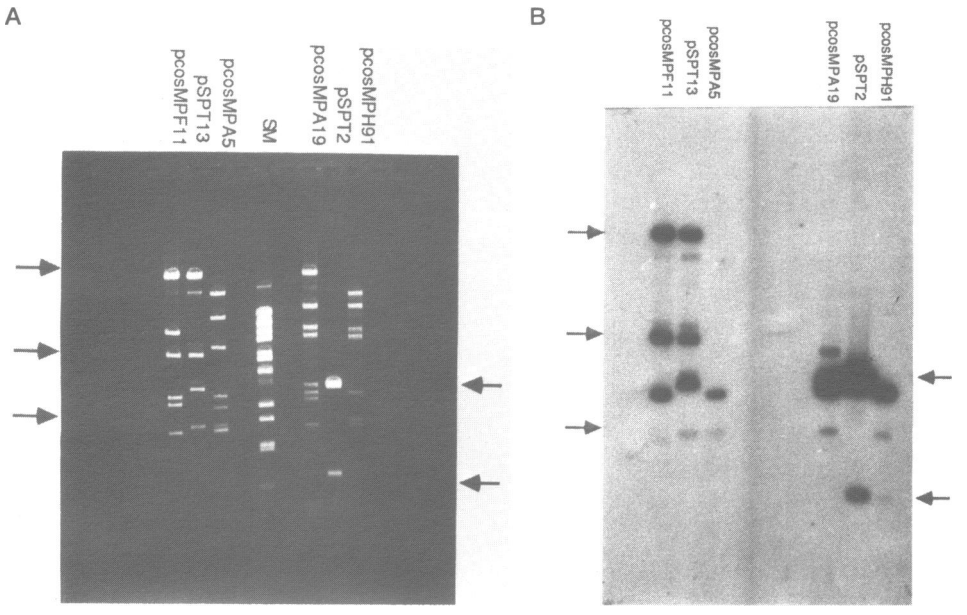

Figure 5A: Restriction analysis of the adjacent cosmid pairs pcosMPA5/F11 and pcosMPA19/H91 and their linking BamHI fragments cloned in pSPT13 and pSPT2. The DNA was double digested with BamHI and EcoRI and ran on a 0.8% agarose gel. Identical BamHI/-EcoRI restriction fragments are indicated by arrows. SM = size marker (λ DNA x BstEII).
Figure 5B: The gel of fig.5A was blotted on nitrocellulose and the filters were hybridized against nick-translated pSPT13 (left panel) and pSPT2 (right panel). Identical BamHI/EcoRI restriction fragments are indicated by arrows. Additional signals result from hybridization of the vector part of the probe (pSPT18) to pcosRW2 and pSPT18 itself. Some weaker bands are due to incomplete restriction digest (see fig.5A).

fragments were cloned into the BamHI site of pSPT18. They contain the same BamHI/EcoRI restriction sites as in the corresponding cosmid pairs (fig.5). By our cloning method it was not possible to find an overlapping cosmid clone for pcosMPF11/pcosMPA5, because the size of the adjacent EcoRI fragments (30 and 25 kbp) exceed the packaging capacity of phage λ.
The only gap remaining is between pcosMPE7 and pcosMPK8. We have not yet been able to define it by a restriction fragment to which both cosmids hybridize. Attempts to isolate cosmids which could link the two terminal clones have so far failed. Of sever-

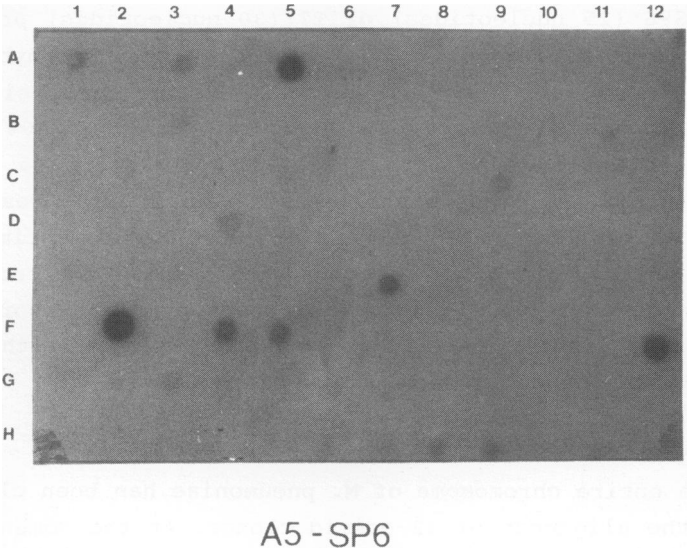

Figure 6: Identification of repetitive DNA elements. A ^{32}p-labelled RNA probe synthesized from the SP6 promoter of pcosMPA5 was hybridized against 96 cosmid clones. Compared with the analysis in fig.2 (left panel), an increased background is clearly visible.

al thousand clones tested, no clones reaching beyond pcosMPE7 and pcosMPK8 into the gap were isolated. Comparing the pattern of EcoRI restricted genomic M.pneumoniae DNA with that of the cosmid collection, all the larger EcoRI fragments seem to be cloned. Adding up the length of the EcoRI fragments, we end up with a cloned contiguous DNA stretch of 720 kbp which represents about 90% of the genome assuming a genome size of about 800 kb. As a reference point for future gene mapping experiments, the 9 kb EcoRI restriction fragment carrying the unique rRNA operon (18) was identified in pcosMPR1 and pcosMPR2 by Southern blot hybridization. As a probe we used an oligonucleotide (MP16S) which is specific for the 16S rRNA of M. pneumoniae (19).

Repetitive DNA elements

The background in hybridization experiments with T7 probes was always higher than in those with SP6 probes. This can be explained by the difference in length of the RNA probes, which is common to all cosmids, depending on whether they are transcribed

from the SP6 (25 nucleotides) or T7 (30 nucleotides) promoter. We were able to minimize this background by stringent hybridization conditions (46°C and 50% formamide), but surprisingly, a much more disturbing background appeared in about 20% of the clones which was independent of the kind of RNA probe (SP6 or T7, see fig.6). Follow-up experiments revealed two types of repetitive DNA elements, which appear at least 8 and 10 times respectively on the chromosome (accompanying paper). The problem of mapping false positive clones was overcome by using shorter RNA probes and by identifying all the cosmids containing the EcoRI fragments which carry the repetitive elements.

DISCUSSION

Almost the entire chromosome of M. pneumoniae has been cloned as shown by the alignment of 32 cosmid clones. At the moment we do not understand why we were unable to clone the genome completely.

Trivial reasons such as the size of the EcoRI fragments in the gap seem to be excluded by comparison of the EcoRI restriction pattern of genomic M. pneumoniae DNA with that of selected cosmids. In particular, the rather small size of the EcoRI fragments of pcosMPE7 contradicts such an argumentation. Our present working hypothesis is that this part of the genome can only be cloned with difficulty in E.coli and it might be helpful to use either a low copy number cosmid or a different host such as yeast.

For several reasons we are sure that the EcoRI fragments within individual cosmid clones are in the same order as in the genome and that mixing of fragments during the cloning procedure did not take place. Firstly, the restricted M. pneumoniae DNA fragments were not only dephosphorylated at their 5'-ends to prevent self-ligation, but also size-fractionated on a sucrose gradient. Secondly, for all but 3 of the 32 clones, identical or similar clones were independently isolated. And finally, hybridization of individual clones to a restriction enzyme XhoI digest of M. pneumoniae DNA revealed that the number and size of the cross-reacting XhoI fragments were in accordance, except for those clones carrying repetitive DNA elements. In these cases, the

same hybridization pattern of XhoI fragments was always observed.

The presented collection of cosmids will be helpful for many experiments, for example the determination of the genome size and the construction of physical maps of rarely cutting restriction enzymes such as EcoRI (appr. 120 sites) or XhoI (appr. 23 sites).

Beyond that, the establishment of a genetic map of conserved bacterial genes seems feasible. They can be identified by cross-hybridization, as exemplified by the ribosomal RNA operon.

Eventually, as soon as a transformation system for mycoplasmas is at hand, many more genes can be defined by gene rescue experiments. By applying these techniques, combined with selective DNA sequencing, it should be possible on a long term basis to identify many of the 600-800 genes of M. pneumoniae and to gain new knowledge of the organization of bacterial chromosomes.

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