An approach to characterizing uncultivated prokaryotes: the Grey Lung agent and proposal of a Candidatus taxon for the organism, ‘Candidatus Mycoplasma ravipulmonis’

Harold Neimark,1 Deborah Mitchelmore2 and Ronald H. Leach2†

Author for correspondence: Harold Neimark. Tel: +1 718 270 1242. Fax: +1 718 270 2656.

An approach to characterizing uncultivated bacteria which combines a PFGE procedure for obtaining purified full-length chromosomes with PCR amplification is described. Isolated chromosomes from uncultivated organisms provide a specifically identifiable source material for hybridization, amplification and cloning. The availability of purified chromosomes for DNA amplification should aid in examining the diversity of microbial populations and should also reduce the possibility of forming hybrid DNA artifact molecules. The approach is illustrated by isolating the chromosome of the uncultivated agent of rodent Grey Lung disease and using the purified chromosomes to amplify and directly sequence the evolutionarily conserved 16S rRNA gene. The Grey Lung agent (GLA) contains a 650 kb chromosome and is shown to be a Mycoplasma sp. located phylogenetically in the hominis group of mycoplasmas. If a simple genomic lesion(s) is responsible for the unculturability of GLA, it is conceivable that complementation with DNA from a close relative could permit growth on artificial media.

Keywords: ‘Candidatus Mycoplasma ravipulmonis’, Grey Lung disease

INTRODUCTION

Uncultivated bacteria have long been recognized in natural environments, and similarly, pathogenic or commensal bacteria that have defied all efforts at cultivation inhabit specialized niches in animal or plant hosts. Uncultivated bacteria have been observed in a wide variety of hosts including protozoa (34), insects (38), other invertebrates including marine bryozoa (44), vertebrates (5, 14) and plants (36, 16). Molecular methods have permitted identification of uncultivated bacteria from some of these habitats but obstacles to characterizing uncultivated bacteria remain [for a review see (1)].

Here we describe an approach to characterizing uncultivated bacteria, which combines a PFGE procedure for obtaining purified full-length chromosomes (27) with PCR amplification. Isolated chromosomes from uncultivated organisms provide a specifically identifiable source material for hybridization, amplification and cloning. The utility of this approach is illustrated by isolating the chromosome of the uncultivated agent of Grey Lung disease of mice and using the purified chromosomes, for example here, to amplify and directly sequence the evolutionarily conserved 16S rRNA gene. The Grey Lung agent (GLA) contains a 650 kb chromosome and is shown to be a Mycoplasma sp. located phylogenetically in the hominis group of mycoplasmas. If a simple genomic lesion(s) is responsible for the unculturability of GLA, it is conceivable that complementation with DNA from a close relative could permit growth on artificial media.

The Grey Lung agent (GLA), initially described as the ‘Grey Lung virus’, causes a pneumonia that appeared spontaneously in mice during passage of respiratory tract materials from different mammalian sources (2). Its pathogenicity seems to be confined to mice and, to a lesser degree, other rodents (2). GLA appears to be strictly pneumotropic with no evident effects on other organs; its name derives from the characteristic greyish-pink oedematous consolidation produced in the lungs (2, 12, 31). The histopathologic picture shows mononuclear ‘cuffing’ of bronchioles and pulmonary blood vessels. Numerous pleomorphic particles, 300–600 nm in diameter, are evident in Giemsa-stained...
impression smears of infected lung. Grey Lung disease is not known to occur naturally in mice, but a similar form of pneumonia occurs in laboratory and wild rats, and is caused by an aetiologial agent(s) closely resembling the GLA (3, 10, 30, 41). Originally regarded as a virus because of its filterability, its resistance to antibacterial agents (including penicillin, sulfonamide and chloramphenicol), and its failure to grow in bacteriological culture media (2, 4), GLA was later recognized by Marmion and colleagues to possess some of the characteristics of a mycoplasma (13, 23). Extensive efforts during the past 30 years to cultivate the GLA from infectious lung materials have failed to demonstrate growth or even survival of the agent in vitro (21; Leach, unpublished data). No active immunity nor any serological response has been detected in infected mice or in Grey Lung-immunized mice or rabbits by use of standard serological methods (2, 11); more recent attempts to detect antibody to the GLA (or to Mycoplasma pulmonis or Mycoplasma arthritidis) in infected mice by ELISA, colony-immunofluorescence, or by an immuno-binding assay (W18) also have failed (21). The enzootic chronic pneumonia agent from laboratory rats also resembles GLA in its failure to elicit an immune response in infected animals (3, 11, 41).

The GLA characterized here has been maintained by mouse serial passage for more than 50 years, and aside from the agents of leprosy and syphilis, is one of the oldest examples of an uncultivated bacterial pathogen.

METHODS

Mouse inoculation and infectivity-titration procedures. These were carried out with the technical collaboration of David Conway. White Schofield or BALB/c mice housed under specific pathogen-free conditions in the animal facility of the Central Public Health Laboratory, Colindale, UK, were used in all experiments; all-male or all-female mice were used for individual experiments. Mice under light anaesthesia (Halothane) were inoculated with one drop in each nostril of an infected-lung suspension or lung-wash fluid (see below). Development of pulmonary disease was observable at post-mortem examination within about 12 weeks with high-titre inocula, or longer for lower-titre inocula. Some animals showed transient or permanent external signs of illness, in the form of increased respiration or physical appearance, but others showed no overt signs and remained relatively normal despite the fact that this infection does not resolve naturally and the lung lesions persist throughout the animal’s lifetime. The agent can thus be maintained by occasional mouse-to-mouse passages at intervals of many months or even years.

High-infectivity suspensions of the GLA were prepared by lightly homogenizing small portions (10–100 μl) of infected-lung tissue in Hartley’s broth (Oxoid; 5–10%, w/v) and using either the whole suspension or the supernate obtained after low-speed centrifugation. Clearer suspensions, relatively free of mouse tissue cells, could be obtained by employing a lung-washing method (39); this entails inserting a tracheal cannula into a mouse killed by cervical dislocation and gently inflating the lungs with 0.5–1 ml Hartley’s broth, followed by withdrawal of the resulting lung-wash fluid for use as an infective suspension. By either of these means, Grey Lung suspensions with high mouse-infectivity titres (up to 108 ml−1) could be obtained. Infectivity levels of suspensions were titred by inoculating dilutions into mice and subsequent post-mortem examination for characteristic ‘Grey Lung’ consolidation.

Electron microscopy. Electron microscopy was carried out by Kitty Plaskitt at the John Innes Institute, Norwich, UK. Lung samples for electron microscopy were fixed in glutaraldehyde, post-fixed in 1% osmic acid, dehydrated in ethanol, and embedded in araldite; sections were stained with uranyl acetate and lead acetate. Microscopy was done with a Siemens 101 or 1A microscope.

PFGE. All steps were carried out with ice-cold reagents: centrifugations were at 4 °C. Lung-wash fluids from 12 to 22 mice, 4–8 weeks post-infection (titre 108–1010 ml−1), were pooled and centrifuged at 1500 g for 3 min and the resulting supernatant was centrifuged at 17000 g for 30 min. The pellet containing the GLA was resuspended in 20 ml PBS and centrifuged again at 17000 g for 30 min. The final pellet was resuspended in 100–200 μl PBS buffer (0.1 M Tris, 10 mM disodium EDTA, 0.1 M NaCl, pH 8.0). From electron microscopy studies or other experience, these buffers are known to be suitable for suspending GLA cells. The suspension was quickly warmed to 37 °C and mixed with an equal volume of previously melted agarose (10 mg InCert grade agarose (FM C) ml−1 TES buffer kept at 40 °C). Aliquots of this mixture were rapidly dispensed into a plastic mould, the mould was chilled, and the resulting 25 μl blocks were extruded into lysis buffer (0.5 M disodium EDTA, 1% SDS and 1 mg proteinase K ml−1) and incubated at 52 °C for 48–72 h to release GLA chromosomes. (Many walled bacteria must be treated with wall lytic enzymes such as lysozyme and mutanolysin to obtain satisfactory lysis with detergents.) The agarose blocks were washed in TE (10 mM Tris, 1 mM disodium EDTA, pH 8.0) three times for at least 15 min each, resuspended in TE and gamma-irradiated (12 500 rad) to produce one (on average) random double-strand break per chromosome (28). The use of gamma irradiation to produce linearized chromosomes for PFGE was also devised independently by Van der Bliek et al. (40). Blocks were then subjected to PFGE, the DNA bands were stained with ethidium bromide and photographed, and the chromosome size was estimated by comparing DNA band mobilities to yeast chromosome size markers as described previously (28).

Chromosome isolation, PCR amplification and DNA sequencing. Rigorous precautions were taken to avoid PCR contamination. Deionized water for solutions from a flushed apparatus was filtered through a 0.22 nm filter and solutions made with this water were filter sterilized immediately after preparation. Chromosome bands were cut from gels with a flame sterilized scalpel and the DNA extracted from the gel with glass powder (Biot101) and eluted with 0.5×TE. Controls were gel slices cut from unused lanes. 16S rRNA genes were amplified (3 cycles: 94 °C, 1 min; 55 °C, 1 min; 72 °C 3 min, with a 10 min extension for the last cycle) using two conserved 16S rRNA sequence primers: 8F (5'-AGA GTTGTACMTGCGCTACG-3') and 1492R (5'-CGGTT-ACCTTGTATGACGCT-3'). The 50 or 100 μl reaction mix contained 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl2; 200 μM each of dATP, dCTP, dGTP and dTTP; 50 mM each primer and 2.5 U Taq polymerase (PerkinElmer). The PCR products were subjected to electrophoresis, the band was extracted from the gel with glass powder or a spin-column (Qiagen), and the DNA was sequenced.
Characterizing uncultivated prokaryotes

sequenced directly with a dye terminator cycle sequencing core kit and an automated sequencer (Applied Biosystems). Amplification and sequencing primers were 8F, 339R, 519R, 536F, 786F, 907R, 1175F and 1492R (8, 20, 43).

Phylogenetic analysis. 16S rRNA gene sequences were obtained from the GenBank database. Alignments of 16S rRNA sequences were performed with the program MULTALIN (http://www.toulouse.inra.fr/mutalin.html) and further adjusted manually. Alignments were aided by comparison to the secondary structure models of the 16S rRNA from the Gram-positive bacterium Bacillus subtilis (42) and from Mycoplasma hyopneumoniae (15). Phylogenetic analysis of the sequence alignment was by the neighbour-joining method of the program MEGA, version 1.01 (19).

Nucleotide sequence accession numbers. The following Mycoplasma species 16S rRNA gene sequences were used in this study: Mycoplasma hominis PG21T, accession no. M24473; Mycoplasma mobile 163K², M24480; Mycoplasma mycoides subsp. mycoides UM30847, M23943; Mycoplasma orale CH19299T, M24659; M. pulmonis PG34¹, M23941; and Mycoplasma sualvi Mayfield B², M23936.

RESULTS AND DISCUSSION

Electron microscopic studies of lung sections (Fig. 1) show numerous extracellular organisms in the alveoli only of infected lungs and demonstrate that the agent present has the morphology of a wall-less prokaryote which closely resembles earlier descriptions of the 'Grey Lung virus' (10, 11): the pleomorphic organisms lack a cell wall, are bound by a single unit membrane (110 Å), and the size is about 650 nm in diameter. Furthermore, the infectious cells pass 0.45 µm filters.

A genomic probe kit reported to be specific for mycoplasmal rRNA (Mycoplasma TC; Gen-Probe)
Fig. 2. Isolation of full-length chromosomes from the GLA by PFGE. Lung washings from uninfected and GLA-infected mice were prepared and subjected to PFGE as described in Methods. Lanes: 1 and 5, *Saccharomyces cerevisiae* YPH149 chromosome size markers; 2, lung-wash preparation from GLA-infected Schofield strain mice; 3, control lung-wash preparation from healthy Schofield strain mice; 4, preparation from GLA-infected BALB/c strain mice. Electrophoresis was in 1% agarose at 11°C with a current of 150 mA and a switching interval of 60 s for 18 h in a transverse-field gel electrophoresis apparatus.

was used to examine mouse lung-wash fluids (21); on high-titre infective preparations from consolidated lungs at 4–23 weeks post-infection, hybridization values of 7–39% were obtained as compared with negative values (<0·2%) for uninfected lung or early post-infection samples with no evident lung lesions (<3 weeks). These results provided the first molecular evidence for the mycoplasmal nature of the GLA and indicated that GLA is not a stable bacterial L-form. Thus, the GLA appeared to be an animal mycoplasma-like organism (21, 27) analogous to the uncultured plant pathogenic mycoplasma-like organisms (MLOs, now called phytoplasmas).

GLA suspensions embedded in agarose and then gamma-irradiated produced linear chromosomes which entered pulsed-field gels and migrated as a single, discrete DNA band (Fig. 2). Identical size bands were obtained with lung washings from infected BALB/c and Schofield strain mice; no DNA bands were detected in lung washings from healthy control mice. The GLA chromosome size is 650 kb. The DNA in unirradiated sample blocks did not travel appreciably into the gel which indicates that only GLA chromosomes which have sustained one DNA double-strand break migrate in the gel. Thus, the electrophoretic behaviour of the GLA chromosome is identical to that of circular prokaryotic chromosomes (28), and indicates that the GLA chromosome is circular.

The pulsed-field switching protocol used here separates DNA molecules up to about 1600 kb in size while larger DNA molecules form a band with a mobility near that of the largest yeast chromosome size marker; larger DNA molecules are resolved by employing longer interval switching protocols. In natural or man-made environments where several different microorganisms are present, chromosomes may be isolated by employing one or more different pulsed-field switching protocols. Although the possibility exists that more than one organism in a population could have exactly the same chromosome size, this possibility does not nullify the approach.

The GLA 16S rRNA gene was amplified from gel-purified chromosomes, sequenced directly in both directions and the sequence compared to the GenBank database. All the high score sequences found were from *Mycoplasma* species which affirms that the cell wall-less GLA is a mollicute and a member of the genus *Mycoplasma*. Several of the high-score *Mycoplasma* species were members of the hominis group of mycoplasmas (42), and the GLA 16S rRNA gene was found to contain the characteristic hominis group motif of a T residue at position 912 in the segment 907–915 (-AAACTTAAA-) [Escherichia coli numbering (7)] instead of the C residue found in virtually all eubacteria (42). Additionally, characteristic dominating residues which occur in the hominis

![Fig. 2](image-url)

![Fig. 3](image-url)

**International Journal of Systematic Bacteriology** 48

**Fig. 3.** Phylogenetic tree showing the relationship of GLA to selected members of the hominis group of mycoplasmas. The tree was estimated by the neighbour-joining method using 1321 positions in the 16S rRNA sequences from GLA and mycoplasmas obtained from GenBank. All alignment gaps and missing information sites were deleted before analysis. The sequence from *M. mycoides* subsp. *mycoides* UM30847 was used as the outgroup. Bootstrap confidence level percentages obtained from 500 resamplings of the data set are shown at several nodes.
group with no more than one exception (33, 42) were found at positions 157-164 (U-A), 158-163 (G-C), 746 (U) and 824-876 (C-G). However, position 610 was the dominating G common to mycoplasmas rather than a dominating U; also it is worth noting that the GLA 16S rRNA gene does not contain an additional loop-proximal nucleotide pair in the helix located between positions 416 and 427, a character which occurs in several members of the hominis group (42).

A phylogenetic tree (Fig. 3) produced by a neighbour-joining method (19) shows GLA is related more closely to the hominis cluster than to any other recognized cluster in the hominis group, however the length of the GLA branch indicates that the GLA 16S rRNA gene has diverged considerably from the sequences in the hominis cluster. The similarity between the sequences from GLA and M. orale CH19299T, the closest mycoplasma sequence found, is only 87% while the similarity between GLA and M. pulmonis is just a little less at 84%; in contrast, the similarity between M. orale CH19299T and M. hominis PG21T, two members of the hominis cluster, is 94%. A phylogenetic tree with essentially identical structure (not shown) was produced with the maximum-likelihood program DNAML of the PHYLIP 3.5c package of programs (9).

Although the GLA 650 kb chromosome is small, it is still within the size range of 580–2200 kb known for cultured mollicutes (28, 35; P. Carle & H. Neimark, unpublished), so small genome size alone does not explain the inability to cultivate this wall-less prokaryote; all the other known members of the hominis group are cultivable.

If a simple genomic lesion(s) is responsible for the unculturability of GLA, it is conceivable that complementation with DNA from a close relative could permit GLA to grow on artificial media. This notion should be applicable to many other uncultivated bacteria.

The procedure described here permits isolation of individual chromosomes and amplification from these templates should obviate formation of hybrid molecule PCR artifacts (‘shuffled’ molecules). PCR products can usually be assumed to be homogeneous, but artifact DNA products can result when related target sequences are co-amplified; the artifact product is a chimeric molecule composed of parts of two different template sequences (6, 22, 24, 26, 32, 37). In the case of rRNA, secondary structure analysis does not necessarily detect chimeric structures (17). Of course, the use of any purified chromosome does not alter the fact that sequencing problems may occur where more than one rRNA operon exists and the operons contain alternate nucleotides at one or a few positions in the rRNA genes (33).

The single 650 kb chromosomal DNA band isolated and amplified here almost certainly came from the wall-less GLA, but conclusive evidence would require in situ hybridization to lung thin sections with a specific DNA probe.

The Grey Lung disease agent appears to be a newly recognized mycoplasma, still it and other uncultivated bacteria should not be given specific names only on the basis of 16S rRNA sequence data, since previously recognized species whose 16S rRNA has not been sequenced could contain the same sequence. In concurrence with guidelines suggested for recording the properties of uncultivated prokaryotes (25), we propose giving the GLA a Candidatus designation with the following description: ‘Candidatus Mycoplasma ravipulmonis’ (M.L. adj. ravus greyish; M.L. n. pulmo the lung; M.L. compound gen. n. ravipulmonis of a grey lung) [(Mollicutes) NC; NA; O. wall-less; NAS (GenBank no. AF001173), oligonucleotide sequence complementary to unique region of 16S rRNA 5'-CGCTTGCAACCCATTTGTAACCGC-3'; S (Mus musculus, lung); MJ].

ACKNOWLEDGEMENTS

This paper is dedicated to the late Dr D. G. ff. Edward, who instigated our first attempts (R.H.L., 1961) to confirm the mycoplasmal nature of the Grey Lung agent and whose enthusiasm for this question engendered our own persisting commitment to resolving it. We are grateful to David Conway for skilled collaboration in the nasal inoculation and lung washing of mice at the Central Public Health Laboratory and also to Kitty Plaskitt for her expertise in carrying out the electron microscopy and for providing electron micrographs. We also thank Christopher Lange for use of the gamma radiation source and PFGE equipment.

REFERENCES


