Cross-complementation between the products of the genes P1 and ORF6 of *Mycoplasma pneumoniae* subtypes 1 and 2

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The genes P1 (MPN141) and ORF6 (MPN142) are essential for the successful colonization of the human respiratory tract by *Mycoplasma pneumoniae*. Both genes are located in the P1 operon, which consists of three genes. The P1 gene is the second gene in the operon, followed by the ORF6 gene. The P1 gene contains two (RepMP2/3, RepMP4) and the ORF6 gene one (RepMP5) specific repetitive DNA sequence, of which seven to nine similar but not identical copies are dispersed on the genome. Despite this large potential pool for genetic variation, *M. pneumoniae* isolates from patients contain only one of two distinct combinations of the genes P1 and ORF6. To analyse the functions of the repetitive DNA sequences, two 'new' combinations of the genes P1 and ORF6 were constructed, keeping the P1 gene constant but exchanging RepMP5 copies of the ORF6 gene. *M. pneumoniae* was transformed with these constructs and the transformants were tested for their ability to grow and survive under *in vitro* conditions and in guinea pigs. The two transformants colonized the respiratory tract of guinea pigs and showed no obvious differences in their growth behaviour compared to *M. pneumoniae* isolates from patients. The results indicate that the subtype-specific combinations of the repetitive elements in the P1 and ORF6 genes are not essential for the successful adherence of *M. pneumoniae* to host cells and the colonization of the respiratory tract of guinea pigs.

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INTRODUCTION

Mycoplasma pneumoniae is a small wall-less bacterium with a genome size of about 816 kbp (Himmelreich *et al.*, 1996) and a proposed coding capacity for 688 ORFs (Dandekar *et al.*, 2000). This bacterium is host dependent in nature, but it can be grown in the laboratory without a host cell in a rich medium supplemented with serum (Hayflick, 1965). *M. pneumoniae* is the causative agent of a primary atypical pneumonia in humans (Jacobs, 1991; Principi & Esposito, 2001; Rosengarten *et al.*, 2000; Taylor-Robinson, 1996). Epidemic outbreaks were registered in time-intervals of 3–7 years followed by endemic phases in the epidemiology of *M. pneumoniae* infections (Jacobs *et al.*, 1996; Lind *et al.*, 1997). The circulation of different subtypes in the human population and therefore the time-dependent subtype-specific immune response of the hosts were discussed as possible reasons for the unique epidemiological course of

respiratory diseases due to *M. pneumoniae* (Jacobs *et al.*, 1996). Genetic differences of *M. pneumoniae* patient isolates were first described by Su *et al.* (1990). They used the gene P1 (MPN141), which encodes the main adhesin P1 (Inamine *et al.*, 1988; Su *et al.*, 1987) as a discriminating probe. Based on sequence differences of the P1 gene and restriction fragment length polymorphism of genomic DNA, the species *M. pneumoniae* was divided into two subtypes. The prototype of subtype 1 became the strain *M. pneumoniae* M129 (ATCC 29342), of which the genome has been completely sequenced (Himmelreich *et al.*, 1996), and the strain *M. pneumoniae* FH (ATCC 15531) represents subtype 2. Several subtyping analyses with patient isolates collected in different countries during the past 30 years confirmed the original observations that, based on the gene P1, all isolates can be classified as either subtype 1 or 2, although minor sequence differences in the P1 gene could be found in a few isolates (Dorigo-Zetsma *et al.*, 2001; Dumke *et al.*, 2003; Kenri *et al.*, 1999). Only recently, attempts were made to find additional genetic markers for subtyping purposes (Dumke *et al.*, 2003). The results of this study indicated a remarkable genetic stability among

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the 115 *M. pneumoniae* isolates from patients collected in different countries over a period of 30 years. So far, in addition to the P1 gene, only the genes encoding the proteins P65 (MPN309) (Proft *et al.*, 1995), P40 and P90 have turned out to be useful for subtyping. P40 and P90 are the products of the ORF6 gene. Both are surface exposed and probably arise by post-translational cleavage, since the nucleotide sequence of the ORF6 gene predicts an 130 kDa protein (Franzoso *et al.*, 1993; Sperker *et al.*, 1991). P40 and P90 are probably identical with the proteins B and C missing in certain avirulent, cytoadherence-negative mutants of *M. pneumoniae* (Hansen *et al.*, 1979; Hu *et al.*, 1984; Krause & Balish, 2004). Cross-linking experiments indicated that P40 and P90 form a protein complex together with the P1 adhesin (Layh-Schmitt & Herrmann, 1994; Layh-Schmitt *et al.*, 2000). It is specifically inserted into the region of the membrane that forms the terminal attachment organelle, an asymmetric extension of the bacterium, also denoted the tip structure. The proper formation of the attachment organelle is essential for adherence of *M. pneumoniae* to its host cell. Mutations in the ORF6 gene result in an avirulent, cytoadherence-negative phenotype and a random distribution of the P1 protein over the whole surface of *M. pneumoniae* instead of the concentration of P1 at the tip structure as seen in wild-type strains (Baseman *et al.*, 1982).

The ORF6 gene is part of the P1 operon (Fig. 1), which consists, in the following order, of the genes ORF4 (MPN140),

Fig. 1. Scheme of the P1 operon and of ORF6 genes. (a) Organization of the P1 operon of *M. pneumoniae* M129. The P1 operon of *M. pneumoniae* FH is organized in the same order. (b) The three different ORF6 genes used in this study are enlarged. The vertical dotted lines in the ORF6 genes show the positions of the unique restriction sites for the endonucleases *Bpl* and *BstEII*. The black arrow points to the site where the proteolytic cleavage into P40 and P90 takes place. Genome position and origin of the copy give the nucleotide positions of the ORF6 gene and of RepMP5 copies on the genome of *M. pneumoniae* M129. Differences in the DNA sequences are indicated by different shades of grey. For details see Fig. 5.

P1 (=ORF5 (MPN141)) and ORF6 (MPN142). Repetitive DNA sequences are located within both the P1 gene and the ORF6 gene. P1 contains RepMP2/3 and RepMP4 while ORF6 contains RepMP5 (Colman *et al.*, 1990; Ruland *et al.*, 1990, 1994). Each of these repetitive sequences appears eight to ten times as similar copies dispersed on the genome of *M. pneumoniae* M129 (Himmelreich *et al.*, 1996; Ruland *et al.*, 1990). They vary in size and are about 1.5–1.8 kbp (RepMP2/3), 1.1–1.5 kbp (RepMP4) and 1.5–2 kbp (RepMP5) long. Most of the repetitive sequences consist of variable middle sections, which are bordered by two constant regions. Comparative sequence and PCR analyses of the P1 operon from 115 *M. pneumoniae* patient isolates had shown that there is a constant fixed subtype-specific combination of copies of RepMP2/3, RepMP4 and RepMP5. Although not proven, a switch from a *M. pneumoniae* M129-specific P1 or ORF6 gene to a *M. pneumoniae* FH-specific one could be explained by exchange of the subtype-specific RepMP copy by recombination between the constant regions (Ruland *et al.*, 1994). So far, *M. pneumoniae* patient isolates carrying the combination of a *M. pneumoniae* M129-specific P1 gene and a *M. pneumoniae* FH-specific ORF6 gene or vice versa have not been found (Dumke *et al.*, 2003), although a subtype switch would require such intermediates.

These results seem to indicate that the 'correct' combination of the P1 adhesin and ORF6 gene products is important for the adherence of *M. pneumoniae* to the host cells and the survival of the bacterium in its host environment, and that other combinations might be disadvantageous for the infection or the colonization process. To test this hypothesis, we constructed 'new' combinations of P1 and ORF6 genes and investigated the ability of *M. pneumoniae* transformants carrying these combinations to grow and survive in guinea pigs, which were introduced as suitable animal models for studying *in vivo* the consequences of a *M. pneumoniae* infection (Jacobs, 1991).

For our studies we used the cytoadherence-negative mutant *M. pneumoniae* B170 (ATCC 29343), a derivative of *M. pneumoniae* M129 (Lipman *et al.*, 1969). It possesses M129-specific P1 and ORF6 genes but has lost the ability to synthesize P40 and P90 (Sperker *et al.*, 1991) due to the deletion of one nucleotide (G) at position 1040 of the ORF6 gene (genome position 186787, P. C. Hu, personal communication). We complemented this mutant by introducing into its genome, via transposon, different ORF6 genes and compared the growth characteristics, expression of selected proteins, adherence properties and survival in an immunocompetent host of the resulting transformants with those of *M. pneumoniae* M129 and *M. pneumoniae* B170.

METHODS

Bacteria. *M. pneumoniae* reference strains M129 [ATCC 29342; subtype 1; broth passage no. 31] and FH [ATCC 15531; subtype 2 (Su *et al.*, 1990); broth passage no. 5] were cultivated as described

previously (Regula *et al.*, 2000) and stored at -80°C . *M. pneumoniae* B170 cells were grown non-adherently in 500 ml glass flasks. Frozen stocks of *M. pneumoniae* were diluted 1:10 with Hayflick medium and incubated at 37°C with gentle agitation for 96 h. The cells were harvested by centrifugation for 15 min at 8000 g at 4°C .

Isolation of genomic DNA, restriction analysis and Southern blot. These were done as described previously (Dumke *et al.*, 2003).

DIG labelling of probes and hybridization. The genomic DNAs from single clones were characterized by Southern blotting, using DIG-labelled probes. These were generated by incorporating Digoxigenin-11-dUTP (Roche) with Taq DNA polymerase (Promega) during polymerase chain reactions according to the manufacturer's recommendation. Two probes were synthesized, deriving either from IS256 or from the gentamicin-resistance gene of the transposon Tn400I_{mod} (Hahn *et al.*, 1996). The primer set 1 (Table 1) was used for amplification of a part of the IS element and the primer set 2 for amplification of a subfragment of the gentamicin-resistance gene.

PCR and sequencing. The DNAs for the detection of *M. pneumoniae*-specific gene sequences by PCR were routinely extracted with the QIAquick DNA mini kit (Qiagen) according to the manufacturer's recommendations. Most of the PCRs were done in a Mastercycler gradient (Eppendorf) in a final volume of 50 μl containing 5 μl $10\times$ DNA polymerase buffer, 100 mM each of dATP,

dGTP, dTTP and dCTP, 30 pmol of each primer, 1 μl DNA polymerase [either Taq DNA polymerase 2000 or 5000 U ml^{-1} (New England Biolabs), or DeepVent DNA polymerase 2000 U ml^{-1} (New England Biolabs)] and 0.05 μg genomic or cosmid DNA as template. For amplification of any template DNA, 35 cycles were run under the following conditions: (i) heating of the reaction mixture for 2 min at 95°C ; (ii) denaturation step during cycles for 30 s at 95°C ; (iii) primer hybridization for 30 s at $48\text{--}60^{\circ}\text{C}$ depending on the melting temperature of the primer; (iv) DNA synthesis for 45 s to 8 min at 72°C depending on the length of PCR product; (v) final extension for 10 min at 72°C . The PCR products were analysed in 1.5% agarose gels and purified with the High Pure Product purification kit (Roche).

The mutation in the ORF6 gene of *M. pneumoniae* B170 at gene position 1040 was confirmed by sequencing the relevant part of PCR products generated with primer set 3 or primer set 4. Primer set 11 was used for sequencing only.

The ORF6 gene from *M. pneumoniae* M129 was amplified with primer set 5 (primer o.7179 contains an additional *Clal* restriction site). This PCR product was ligated to the expression unit of MPN531. The expression unit was amplified with primer set 6 (primer o.6531 contains an additional *PstI* restriction site). The PCR was done as described above except that pcosMPG12 (Wenzel & Herrmann, 1989) was used as template. The ligation product of the expression

Table 1. Primers

Primer set no.	Oligonucleotide	Sequence (5'→3')	Position*	Remarks
1	o.8186	TAA TCA ACT AAT GGA AAA TCA	203–223 in the IS	Tn4001 IS256 specific
	o.7252	TAC TCG ATT TTC TTC TCG	620–612 in the IS	Tn4001 IS256 specific
2	o.8089	ATG AAT ATA GTT GAA AAT GA	1725–1744	Tn4001 gent specific
	o.7181	TCA ATC TTT ATA AGT CCT T	3165–3146	Tn4001 gent specific
3	o.672	CCG GTG TAT GAA CCC CTG	186521–186538	Deletion ORF6 <i>M. pneumoniae</i> B170
	o.1935	TGC ACT AGG AAG GTA ATG	189506–189489	Deletion ORF6 <i>M. pneumoniae</i> B170
4	o.60f	TCA GTC AAG CGC CAA AAC G	185617–185635	Deletion ORF6 <i>M. pneumoniae</i> B170
	o.61r	TTC TGC TTG TCG GTG CCC C	187311–187329	Deletion ORF6 <i>M. pneumoniae</i> B170
5	o.7178	ATG AAA TCG AAG CTA AAG TTA AAA CG	185747–185772	ORF6 <i>M. pneumoniae</i> M129
	o.7179	TTA AGC CTT TTT AGG TGG TTG GAC	189403–189380	ORF6 <i>M. pneumoniae</i> M129
6	o.5781	CTA TTT TAT CCC CTT TCT TTG TTA ATA GGC	653950–653979	MPN531 expression unit
	o.6531	AAC TGC AGA ACC AAT GCA TTG GAA ATT ATG CCT CAA ACA C	654250–654232	MPN531 expression unit
7	o.782	ACC CCT CAT ACG AAC TCG	185922–185939	ORF6 <i>M. pneumoniae</i> FH
	o.457	CAC TTT CGT TTA CAA TCC C	187919–187901	ORF6 <i>M. pneumoniae</i> FH
8	o.7199	GGT CCG CTC AGC GTT	185994–185980	ORF6 <i>M. pneumoniae</i> B170/R, PCR1
	o.7393	GAA TTT TTC CAA GAG CTC TC	186588–186569	ORF6 <i>M. pneumoniae</i> B170/R, PCR1
9	o.7394	TTG ACG TTG TGG CCG ACC	187457–187440	ORF6 <i>M. pneumoniae</i> B170/R, PCR3
	o.7204	ACA CGG TCA CCC CAT CAT CCC C	187820–187799	ORF6 <i>M. pneumoniae</i> B170/R, PCR3
10	o.7396	GGA AAA AAC AAC GGC GGA	132530–132513	ORF6 <i>M. pneumoniae</i> B170/R, PCR2
	o.7395	GGC CAC TGA TTA AGT AAG C	133210–133192	ORF6 <i>M. pneumoniae</i> B170/R, PCR2
11	o.62f	CGG GTC AAG AAT CGA GTT GG	186393–186412	Deletion ORF6 <i>M. pneumoniae</i> B170
	o.64r	CCA TCT GGT GCA AGG CCT	186911–186894	Deletion ORF6 <i>M. pneumoniae</i> B170
12	o.922	GTT TCA CTG CTA TTG CCC	187131–187114	
	o.4581	ATG AAA GAG AGC AGC ACT	654097–654080	MPN531 expression unit specific
	o.3601	CCC CCA TCA CTG TCC AAC	187581–187564	ORF6 specific <i>M. pneumoniae</i>

*All positions for *M. pneumoniae* sequences are genome positions (Dandekar *et al.*, 2000).

unit and ORF6 gene was amplified by PCR, using primers o.6531 and o.7179, and subcloned into the *Clal* and *PstI* restriction sites of the vector pBC (Stratagene).

The ORF6 gene from *M. pneumoniae* FH was amplified with primer set 7.

The recombinant ORF6 gene was assembled by ligating three different PCR products, PCR1, PCR2 and PCR3. They were synthesized with primer set 8 (PCR1, primer o.7199 contains a *BlpI* restriction site), set 9 (PCR3, primer o.7204 contains a *BstEII* restriction site) and set 10 (PCR2) using the cosmids (Wenzel & Herrmann, 1989) pcosMPE07 (PCR1, PCR3) and pcosMPCO9 (PCR2) as templates. PCR2 and PCR3 were then ligated and the product used to amplify a PCR2/3 intermediate, which was ligated to PCR1. This new intermediate served as template for synthesizing, with primers o.7199 and o.7204, sufficient amounts of the final product. This DNA fragment, consisting in the 5'–3' direction of PCR1, PCR2 and PCR3, contained a *BlpI* restriction site near the 5' end and a *BstEII* site near the 3' end.

To prove the presence of Tn4001 in reisolates of *M. pneumoniae* from bronchial alveolar lavage fluids a specific part of this transposon was amplified with primer set 2. The stable mutation in the ORF6 gene of the reisolated mutants at genome position 186787 was confirmed by amplifying the relevant part of the gene with primer set 3 and sequencing with primer set 11.

Sequencing was done using the BigDye terminator cycle sequencing kit and the DNA sequencer ABI PRISM 377 or 373A (Perkin-Elmer Applied Biosystems) or by using external sequencing facilities (GATC Biotech).

Cloning of ORF6 genes

(i) **ORF6 of *M. pneumoniae* M129.** The purified PCR products of the expression unit of MPN531 and of the ORF6 gene were ligated with T4 ligase (New England Biolabs) and inserted into the unique *SmaI* site of the vector pBC (Stratagene), yielding the plasmid pIC1. The ORF6 gene together with the expression unit of MPN531 was transferred from pIC1 into the transposon Tn4001mod, which is part of the plasmid pKV74 (Hahn *et al.*, 1996). For this cloning step, the ORF6 gene was excised from pIC1 with the restriction enzymes *PstI* and *Clal* (New England Biolabs) and, after the sticky ends had been converted to blunt ends with the Klenow enzyme (AGS), ligated into the *BamHI* site of the vector pKV74. The sticky *BamHI* ends were also converted to blunt ends by the Klenow enzyme. The new plasmid was named pICT1.

(ii) **ORF6 of *M. pneumoniae* FH.** Plasmid pICT1 was digested with nucleases *BstEII* and *BlpI*, yielding a 10·8 kbp and a 1·8 kbp fragment. The 10·8 kbp fragment was isolated from a 0·8% agarose gel and ligated with a *BstEII/BlpI* DNA fragment carrying the FH-specific RepMP5 copy. This fragment was amplified with primer set 7 from the FH-specific ORF6 gene using genomic DNA of *M. pneumoniae* FH as substrate. The new plasmid was named pICT2.

(iii) **Recombinant ORF6.** The PCR product containing RepMP5 833 was synthesized as described. It was directly cloned into the unique *SmaI* site of the vector pBC. The resulting plasmid was called pIC3. The new recombinant ORF6 was excised from pIC3 with *BlpI* and *BstEII* and ligated into the plasmid pICT2, also treated with *BlpI* and *BstEII*, such that it replaced the FH-specific ORF6 gene, generating the new plasmid pICT3.

Transformation of *M. pneumoniae*. *M. pneumoniae* cells were transformed with the corresponding plasmids by electroporation as described previously (Hedreya *et al.*, 1993) and transformants were selected on PPLO agar plates containing gentamicin (80 µg ml⁻¹).

Colonies were picked from PPLO agar plates and grown in 1 ml modified Hayflick medium also containing gentamicin (80 µg ml⁻¹). These cultures were then used for inoculation of new expanded cultures or stored at –70 °C.

Filtration technique for *Mycoplasma*. To get single colonies from *M. pneumoniae* suspensions, the standard technique as described by Tully (1983) was used with the following modifications. The cells were passed sequentially through a 400 nm, a 220 nm and a 100 nm filter, plated on PPLO agar plates and incubated at 37 °C. Single colonies were picked after 10–14 days from plates and cultivated in liquid broth culture.

Haemadsorption test. The haemadsorption test was done as published (Krause *et al.*, 1982) with the modification that concentrates of human erythrocytes were diluted in Dulbecco's modified Eagle medium (Gibco) until the OD₆₀₀ was 0·8. Pictures were taken with an Olympus DP50 digital camera connected to a Zeiss Axiophot with the 20 × objective.

Polyclonal antibodies and preparation of antisera. The specific antisera against the P40 proteins of subtypes 1 and 2 were obtained by immunizing rabbits with the following peptides, which have been coupled to keyhole limpet haemocyanin (mcKLH, Pierce): pep 2528F CDESSWKNSEKTAEND (amino acid residues 275–290; specific for *M. pneumoniae* M129); pep 2527M CSDSSGSQGQG-TTDNKFQKY (amino acid residues 330–346; specific for *M. pneumoniae* FH – see Fig. 5). For coupling the peptides to mcKLH, a cysteine residue was added at the amino-terminus of each peptide. The peptides were synthesized by M. Ellis and T. Ruppert (ZMBH). The antisera were delivered from Peptide Speciality Laboratories. The antisera against P1 (Dumke *et al.*, 2003), P65 (Proft & Herrmann, 1994), P90 and P40 (Sperker *et al.*, 1991) have been described.

SDS-PAGE and Western blotting. The determination of the protein concentration, the SDS-PAGE and the Western blotting were carried out as published (Dumke *et al.*, 2003).

Animal experiments. Three male guinea pigs (450–600 g, Dunkin-Hartley, Charles River) per group were intranasally infected with an identical inoculum of a specific *M. pneumoniae* strain on the same day (Dumke *et al.*, 2004). The inoculum varied between groups from approximately 4 × 10⁶ to 6 × 10⁸ c.f.u. suspended in 300 µl PBS. The infected animals were kept separately in air-conditioned boxes with filter systems (Biozone) until they were sacrificed. The experiments with the different transformant groups were organized in such a way that the groups were infected one after the other to avoid any cross-contamination. Ten days after infection, the animals were killed by heart puncture under anaesthesia (Jacobs, 1991). The trachea was prepared and a small catheter device was introduced into the trachea drainage hole. For bronchoalveolar washing we instilled approximately 10 ml PPLO broth. The regained solution (approx. 3 ml) was used to isolate *M. pneumoniae* from the lower respiratory tract of the animals.

Two hundred microlitres of the infection suspensions and of the bronchial alveolar lavage fluids were spread on PPLO agar and incubated for at least 3 weeks at 37 °C. In the case of negative results the remaining bronchial alveolar lavage fluid (stored at –20 °C) was concentrated by centrifugation (8000 g, 10 min), and the sediments were resuspended in 500 µl PPLO broth and incubated in the same way. In parallel, 100 µl concentrated bronchoalveolar washing fluid was added to 900 µl PPLO broth and incubated at 37 °C until the colour of the medium changed from red to orange, or for at least 3 weeks. For molecular characterization selected colonies were picked and incubated in 1·5 ml PPLO broth at 37 °C.

RESULTS

Complementation of *M. pneumoniae* B170 with the ORF6 gene of *M. pneumoniae* M129

First of all, we resequenced the ORF6 gene of our *M. pneumoniae* B170 clone and confirmed that it still had the frame-shift mutation in the ORF6 gene. Western blotting analyses with P40- and P90-specific polyclonal antibodies (Sperker *et al.*, 1991) supported the DNA sequencing results, showing that neither P40 (Fig. 2a, lane 6) nor P90 (data not shown) was synthesized in the cytoadherence-negative mutant *M. pneumoniae* B170.

To prove that the cytoadherence-negative phenotype is caused only by the frame-shift mutation in the ORF6 gene we complemented *M. pneumoniae* B170 with the intact ORF6 gene from *M. pneumoniae* M129. The gene was integrated into the mutant genome via the modified transposon Tn4001, which is part of the plasmid pKV74 (Hahn *et al.*, 1996). This vector contains the unique restriction sites for the endonucleases *Eco*RI and *Bam*HI. The ORF6 gene of *M. pneumoniae* M129 was amplified by PCR and fused to the expression unit of MPN531 (*clpB*) also amplified by PCR. The expression unit was defined as the DNA segment beginning at the first nucleotide in front of the start codon of MPN531 and ending 313 nucleotides further upstream (genome position 653950–654263). The transcriptional start point of this gene (Weiner *et al.*, 2000) and its relative transcription rate (Weiner *et al.*, 2003) were determined experimentally. We selected this expression unit, because *clpB* is well transcribed and translated (Ueberle *et al.*, 2002) and can be up- and down-regulated by changes in temperature (Weiner *et al.*, 2003). This promoter fusion was necessary, because the ORF6 gene, as part of an operon, does not have its own promoter (Fig. 1). The amplified and modified ORF6 gene was ligated first into the *Bam*HI site of the plasmid pBC,

generating the plasmid pIC1, and from there transferred into the transposon Tn4001 (see Methods). This new plasmid was named pICT1. After confirming the correct nucleotide sequence of the modified ORF6 gene, *M. pneumoniae* B170 was transformed with pICT1 by electroporation. Transformants, now called *M. pneumoniae* B170/M, which could grow in the presence of gentamicin ($80 \mu\text{g ml}^{-1}$) either in liquid medium or on agar plates, were filter cloned by standard methods and tested (i) for the presence of Tn4001 sequences, (ii) for adsorption to plastic surfaces and erythrocytes and (iii) for synthesis of the products of the intact ORF6 gene, P40 and P90. The results for transformants *M. pneumoniae* B170/M5, *M. pneumoniae* B170/M19 and *M. pneumoniae* B170/M31 are shown as examples (Figs 2a, 3a and 4c).

Southern blotting analysis confirmed the presence of Tn4001 in all transformants by identification of the IS element and of the gentamicin-resistance gene. We used the endonuclease *Bgl*II for restriction of the genomic DNA. Since it did not cut the plasmid pICT1, the gentamicin- and IS-specific probes recognized the same DNA fragments (Fig. 3a). The sizes of the reacting DNA fragments of individual transformants were different, indicating that the sites of insertion of the transposon into the genomes of these transformants were not identical. The site of insertion was determined precisely for *M. pneumoniae* B170/M31. This was done by digesting the complete genomic DNA with restriction endonuclease *Hind*III, cloning the DNA fragment which contained the transition between the end of the transposon and the genomic DNA into the vector pBC and sequencing the relevant DNA region. The transposon was inserted within MPN285 between nucleotides 340652 and 340653. This gene encodes a *prfB* homologue. It is a member of a family of restriction enzymes and obviously nonessential, since *M. pneumoniae* B170/M31 shows normal growth. Western blotting analyses with

Fig. 2. Western blots of total protein extracts from cloned transformants of *M. pneumoniae* B170. After 10% SDS-PAGE and transfer to nitrocellulose membranes the blots were incubated with antisera specific to the proteins P1 and P40 from either *M. pneumoniae* M129 or FH. In each panel, lanes 1–3 contain samples of cloned transformants and lanes 4–6 contain positive and negative control samples derived from *M. pneumoniae* M129 (lane 4); *M. pneumoniae* FH (lane 5) and *M. pneumoniae* B170 (lane 6). (a) Lane 1, *M.p.* B170/M5; lane 2, *M.p.* B170/M19; lane 3, *M.p.* B170/M31. (b) Lane 1, *M.p.* B170/F3; lane 2, *M.p.* B170/F8; lane 3, *M.p.* B170/F12. (c) Lane 1, *M.p.* B170/R1; lane 2, *M.p.* B170/R2; lane 3, *M.p.* B170/R3. *M. pneumoniae* B170/F8 (b, lane 2) is a revertant which again synthesized the *M.p.* M129-specific P40.

Fig. 3. Southern blots of genomic DNAs from cloned transformants of *M. pneumoniae* B170. The genomic DNAs were digested with restriction enzyme *Bgl*II (a, d) or *Dra*III (b, c), separated on 0.8% agarose gels and transferred to a nylon membrane. Hybridizations were done with DIG-labelled PCR-fragment specific either for the IS element or for the gentamicin-resistance gene. The concentration was 8 ng per ml of hybridization solution. (a) Lane 1, plCT1; lane 2, *M.p.* B170; lanes 3–5: *M.p.* B170/M5, *M.p.* B170/M19, *M.p.* B170/M31. (b) Lane 1, plCT2; lane 2, *M.p.* B170; lanes 3–5, *M.p.* B170/F1, *M.p.* B170/F5, *M.p.* B170/F3. (c) Lane 2, *M.p.* B170; lanes 3–5, *M.p.* B170/fr1, *M.p.* B170/fr2, *M.p.* B170/fr4. (d) Lane 1, plCT3; lane 2, *M.p.* B170; lanes 3–5, *M.p.* B170/R1, *M.p.* B170/R2, *M.p.* B170/R3.

polyclonal antisera against P40 (Fig. 2a, lanes 1–3) and P90 (data not shown) proved the synthesis of the corresponding proteins. These two proteins must have been translated from the newly inserted ORF6 gene since the frame-shift mutation in the original ORF6 gene was still present. The sequence analysis of a PCR fragment amplified with two primers (set 3) binding at a unique position (o.1935) near the end of the P1 gene of *M. pneumoniae* B170 and

downstream of the position of the frame-shift mutation in the ORF6 gene (o.672) confirmed the frame-shift mutation in the original ORF6 gene. Antisera against P1 (Fig. 2a) and P65 (data not shown) proved that both proteins were also expressed in all three transformants. The regained ability of *M. pneumoniae* B170/M31 to adhere to surfaces (glass, plastic) and erythrocytes (Fig. 4c) was in agreement with the Western blot results.

Selection of RepMP5 copies for constructing 'new' ORF6 genes

After proving that *trans*-complementation with the ORF6 gene products is in principle possible, we constructed two 'new' P1–ORF6 combinations composed of the *M. pneumoniae* M129-specific P1 gene complemented with either the *M. pneumoniae* FH-specific ORF6 gene (Ruland *et al.*, 1994), or a RepMP5-specific copy occurring outside the P1 operon of *M. pneumoniae*. These combinations have, so far, not been detected in *M. pneumoniae* isolates (Dumke *et al.*, 2003).

To simulate possible recombination events between the ORF6 gene and the repetitive elements, a set of scripts was created in the Perl programming language to simulate *in silico* all possible recombination events between the ORF6 gene and the repetitive elements. Two DNA fragments – one from ORF6, the other from a repetitive element – were assumed to be possible recombination sites if (i) the recombination site was longer than 20 bp (Cohan, 1994), (ii) there was a perfect match of at least 10 bp at either end of one recombination site, (iii) there was a

Fig. 4. Erythrocyte-binding assay. Colonies were incubated with human erythrocytes: (a) *M. pneumoniae* M129; (b) *M. pneumoniae* B170; (c) *M. pneumoniae* B170/M31; (d) *M. pneumoniae* B170/F3; (e) *M. pneumoniae* B170/fr4; (f) *M. pneumoniae* B170/R1. *M. pneumoniae* B170/fr1 and fr2 bind to erythrocytes like *M. pneumoniae* B170/fr4 (data not shown).

Fig. 5. Amino acid sequence comparison of the variable regions of the ORF6 gene products used in this study. The sequences were aligned using the computer program LASERGENE (DNASTAR). The P90 protein of *M. pneumoniae* M129 starts with arginine (R) at position 455 (see also the supplementary data files with the online version of this paper). The peptides used for generating specific antibodies against the P40 proteins of subtype 1 (M129, residues 275–290) and subtype 2 (FH, residues 330–346) are marked by ++ or small circles.

similarity of at least 90 % between the two fragments and (iv) the longest mismatch stretch was not longer than 3 bp. Next, all found recombination sites were permuted and the sequences were recombined at these sites, so that 5' and 3' regions came from the original ORF6 gene, and the central region from a repetitive sequence. Recombined sequences which contained a frame-shift or a stop mutation, or were more than 70 amino acids shorter than the original P1 protein, were rejected. Finally, a multiple alignment of all 601 resulting recombined sequences was done using the program CLUSTAL W. A recombined sequence contained in a cluster distant as far as possible from both M129- and FH-specific ORF6 gene sequences was chosen for further analysis. The central region of this sequence was derived from the repetitive element RepMP5 833, located on the genome between nucleotides 131796 and 133581 (Figs 1 and 5; for more details see the supplementary files with the online version of this paper).

Construction of 'new' ORF6 genes

The 'new' ORF6 genes were constructed by replacing a 1824 bp inner DNA fragment of the ORF6 gene of *M. pneumoniae* M129 located between the two unique restriction sites for the endonucleases *B*l*p*I (genome position nucleotide 185989) and *B*stEII (genome position nucleotide 187815). These two restriction sites are located outside of the variable RepMP5 repeat region within the conserved part of the ORF6 gene M129, the ORF6 gene FH and the RepMP5 833 copy (Fig. 1). The DNA manipulations were done with either plasmid pIC1 or pICT1 (see above), both carrying the ORF6 gene M129, by exchanging their *B*l*p*I/*B*stEII fragment (1824 bp) and inserting either the *B*l*p*I/*B*stEII fragment (1623 bp) from the ORF6 gene FH or the *B*l*p*I/*B*stEII fragment (1680 bp) from Rep MP5 833 (=ORF6 gene R, for details see Methods). The plasmids carrying the ORF6 gene FH were called pIC2 and pICT2, and the plasmids carrying the ORF6 gene R were called pIC3 and pICT3 (Table 2). The correctness of these gene constructions was confirmed by sequencing each of the newly constructed ORF6 genes. An amino acid sequence

Table 2. Plasmids

Plasmid	Comments/references
pIC1	ORF6 gene from <i>M.p.</i> M129 cloned in pBC under control of expression unit from MPN531 (<i>clpB</i>)
pICT1	ORF6 gene from <i>M.p.</i> M129 cloned from pIC1 in pKV74
pIC2	ORF6 gene from <i>M.p.</i> FH in pBC under control of the expression unit from MPN531
pICT2	ORF6 gene from <i>M.p.</i> FH cloned from pIC2 in pKV74
pIC3	'New' constructed ORF6 gene (containing RepMP5 833 from <i>M.p.</i> M129) under control of the expression unit from MPN531
pICT3	'New' constructed ORF6 gene cloned from pIC3 in pKV74
pKV74	Plasmid for transformation of <i>M.p.</i> (Hahn <i>et al.</i> , 1996)
pBC	Standard cloning vector (Stratagene)

comparison of the variable regions of the three ORF6 gene products used in this study is shown in Fig. 5.

After transforming *M. pneumoniae* B170 with pICT2 or pICT3 several transformants were isolated and tested for the same criteria as *M. pneumoniae* B170/M. *M. pneumoniae* B170 transformed with the ORF6 gene FH were named *M. pneumoniae* B170/F and those transformed with ORF6 gene R were named *M. pneumoniae* B170/R. Both *M. pneumoniae* B170/F and *M. pneumoniae* B170/R behaved like *M. pneumoniae* B170/M. They adhered to plastic and erythrocytes (Fig. 4d, f) and synthesized P40 as shown by Western blotting with subtype-specific antisera against P40 (Fig. 2b, c). The sera were directed against the peptide pepF2528 (=P40 FH-specific) or peptide pepM2527 (=P40 M129-specific). Since the ORF6 gene R also encodes the M129-specific peptide but not the FH-specific one, it reacts only with the M129-specific anti-P40 serum. Subtype-specific sera for P90 or for ORF6 gene R derived P40 and P90 were not available, but an antiserum against the non-variable region of P90 (Sperker *et al.*, 1991) recognized P90 of *M. pneumoniae* B170/M, B170F and B170R (data not shown). All transformants but one (Fig. 2b, lane 2), which gave positive results with anti-P40 antisera, had still the original frame-shift mutation in the ORF6 gene, as was shown by PCR amplification and sequence analyses of the relevant regions (data not shown). The single exception was *M. pneumoniae* B170/F8, protein extracts of which reacted positively (Fig. 2b, lane 2) with both the *M. pneumoniae* M129- and the *M. pneumoniae* FH-specific anti-P40 serum. Sequence analysis proved that this strain was a revertant carrying two intact ORF6 genes. In support of the data from Western blotting, we confirmed the transformation of *M. pneumoniae* B170 by identifying parts of the transposon Tn4001 in Southern blotting experiments as already described for *M. pneumoniae* B170/M (Fig. 3). Although the two tested ORF6 genes restored the ability of *M. pneumoniae* B170 to adhere to erythrocytes, we determined the site of insertion of the corresponding transposon, to ensure that no known cytoadherence-relevant gene had been inactivated. The insertion sites of the transposons in the genomes of the transformants used in

the erythrocyte-binding assay (Fig. 4) were for *M. pneumoniae* B170/F3 in gene MPN506 (putative lipoprotein, unknown function) after nucleotide position 616708 and for *M. pneumoniae* B170/R1 within gene MPN077 (hypothetical ORF) after nucleotide position 113895. The results proved that a specific combination between a P1 and an ORF6 gene as seen in the prototype of subtypes 1 and 2 was not mandatory for the ability to adhere to erythrocytes.

Intranasal infection of guinea pigs with selected *M. pneumoniae* strains

To determine whether the transformed bacteria were able to colonize the host respiratory tract, we infected guinea pigs with selected transformants and screened for surviving bacteria in the bronchial alveolar lavage fluids.

In five sets of separate experiments three guinea pigs each were infected with a specific *M. pneumoniae* strain. The infection doses varied between 3.9×10^6 and 6.3×10^8 c.f.u. The following strains were tested for their ability to survive in the respiratory tract of the infected animals: (i) *M. pneumoniae* M129 (positive control for host colonization), expressing the characteristic subtype 1-specific P1 and ORF6 proteins (P40/P90); (ii) *M. pneumoniae* B170 (negative control for host colonization), unable to synthesize P40 and P90; (iii) three transformants of *M. pneumoniae* B170 containing the described ORF6 genes (Table 3), namely *M. pneumoniae* B170/M31 (clone 31, subtype 1-specific ORF6 gene), *M. pneumoniae* B170/F3 (clone 3, subtype 2-specific ORF6 gene) and *M. pneumoniae* B170/R1 (clone 1, RepMP5 833-derived ORF6 gene). Ten days after infection the bronchial alveolar lavage fluids were tested for viable *M. pneumoniae* strains surviving in the host environment, on agar plates and, in the case of negative results, enriched in liquid medium (Table 4).

As expected, the subtype 1 strain *M. pneumoniae* M129 was re-isolated on agar plates from the bronchial washing fluids of all infected guinea pigs and the mutant *M. pneumoniae* B170 was negative on direct agar cultivation, but also after enrichment and subcultivation, indicating that this strain was not able to colonize any of the three

Table 3. *M. pneumoniae* strains and mutants

Strain/mutant	Comments
M129 (ATCC 29342)	Subtype 1
FH (ATCC 15531)	Subtype 2
B170 (ATCC 29343)	Subtype 1, deletion in ORF6 gene (no expression of proteins P40 and P90)
B170-derived mutants*	
B170/M	<i>M.p.</i> B170 complemented with the ORF6 gene specific for <i>M.p.</i> M129
B170/R	<i>M.p.</i> B170 complemented with the 'new' ORF6 gene containing RepMP5 833 from <i>M.p.</i> M129
B170/F	<i>M.p.</i> B170 complemented with the ORF6 gene specific for <i>M.p.</i> FH
B170/fr	Re-isolates of <i>M.p.</i> B170/F from infected guinea pigs

*Individual clones received numbers e.g. *M. pneumoniae* B170/M31.

Table 4. Recovery of *M. pneumoniae* in bronchial washing fluids after intranasal infection of guinea pigs with mutants B170, B170/M31, B170/F3, B170/R1 and the control strain M129

Strain/mutant of <i>M. pneumoniae</i>	Infection dose (c.f.u.)	Animal	C.f.u. ml ⁻¹ on PPLO agar	Growth in PPLO broth
M129	6.3×10^8	1	4.1×10^2	ND
		2	4.6×10^2	ND
		3	3.0×10^3	ND
B170	8.7×10^7	1	no colonies	no growth
		2	no colonies	no growth
		3	no colonies	no growth
B170/M31	1.7×10^7	1	no colonies	no growth
		2	no colonies	+ *
		3	no colonies	+ *
B170/F3	3.9×10^6	1	3.5×10^1 †	ND
		2	5.0×10^0 †	ND
		3	2.5×10^1 †	ND
B170/R1	1.1×10^7	1	no colonies	+ ‡
		2	no colonies	+ ‡
		3	no colonies	+ ‡

ND, Not done.

*Confirmed by PCR and sequencing as B170/M.

†Confirmed by PCR and sequencing as B170/F.

‡Confirmed by PCR and sequencing as B170/R.

animals. Further colony growth on agar plates was detected in the bronchial fluids from guinea pigs which were infected with the transformant *M. pneumoniae* B170/F3. Growth of *M. pneumoniae* could be detected after incubating the bronchial alveolar fluids in liquid medium in two bronchial fluids of two guinea pigs infected with *M. pneumoniae* B170/M31 and in samples of three guinea pigs infected with *M. pneumoniae* B170/R1. These latter five positive cultures in PPLO broth were subcultured on PPLO agar. Twenty randomly picked *M. pneumoniae* colonies (except animal 2 from the guinea pigs infected with *M. pneumoniae* B170/F3, from which only five colonies were recovered), derived either from the primary agar plates or from subcultures of enriched PPLO broth cultures, were characterized by PCR, DNA sequencing, Southern blotting and their ability to adhere to erythrocytes. Compared with the initial inoculum we found no differences. All re-isolates had the one nucleotide deletion at position 1040 of the original ORF6 gene, still contained the inserted transposon with the specific new ORF6 sequences at the same genome position as determined by Southern blotting analysis and retained the ability to adhere to human erythrocytes. As examples, we show the results of the analyses of three isolates (*M. pneumoniae* B170/fr1, fr2 and fr4), which were recovered from *M. pneumoniae* B170/F3-infected guinea pigs (Figs 3c and 4e).

DISCUSSION

The cytheadherence-negative mutant *M. pneumoniae* B170 was converted to a cytheadherence-positive strain by complementation with each of three different ORF6 genes.

These results confirmed that besides the P1 protein an expressed ORF6 gene is essential for cytheadherence and persisting survival of *M. pneumoniae* in a host environment and that *M. pneumoniae* strains with the same P1 protein but different expressed ORF6 genes show a colonization-positive phenotype in guinea pigs.

The number of recovered colonies after plating without prior enrichment in liquid medium is rather low (Table 4), but this is a general phenomenon of the fastidiously growing *M. pneumoniae* cells. For this reason it is recommended microbiological practice in diagnostics not only to spread infected samples directly on agar plates but also to enrich the bacteria in liquid medium. A positive result, the identification of an infectious *M. pneumoniae* is achieved if, with either procedure, bacteria were re-isolated. Applying these procedures we recovered the ORF6-complemented *M. pneumoniae* strains from almost all infected guinea pigs and confirmed by molecular methods that they were identical with the inoculum strains. In agreement with published data (Lipman *et al.*, 1969), the only strain that could not be recovered was the non-adhering *M. pneumoniae* B170. It was eliminated by the natural clearance of the bronchial tract within 10 days.

Due to the problems concerning the recovery of *M. pneumoniae* directly from samples of the bronchial washing fluids, plating efficiencies cannot be used to quantify differences in the ability of individual strains to adhere and to colonize the host. The re-isolation of *M. pneumoniae* from bronchial fluids on agar plates or in liquid medium is

therefore only a qualitative parameter for colonization. Since colonization is only one step in the interaction of *M. pneumoniae* with its host, further studies are needed to explore whether these transformants are still pathogenic and elicit comparable cellular and humoral host responses as the infectious subtype 1 and 2 strains (Jacobs, 1991; Jacobs *et al.*, 1988). We conclude from the result of the described experiments that *M. pneumoniae* strains carrying different gene P1/ORF6 combinations are able to colonize and to persist in a host organism and therefore could also act as true intermediates in a switch from subtype 1 to subtype 2 and vice versa.

In contrast to these findings are the results from analyses of the composition of the P1 operons from 115 *M. pneumoniae* strains collected from patients in different countries over a period of 30 years. They showed that only two subtype 1- and 2-specific combinations of the genes P1 and ORF6 could be identified (Dumke *et al.*, 2003). How can we explain the discrepancy between the results from the patient isolates and the *in vitro* generation of transformants with hitherto unknown combinations of P1 protein and ORF6 gene products? What is the function of the seven additional copies of RepMP5, which supply a potential reservoir – at least in theory – for generating many different ORF6 genes (Himmelreich *et al.*, 1996; Rocha & Blanchard, 2002)?

If we exclude the possibility that the isolation procedure for *M. pneumoniae* strains from patients is selective, then we should find more 'new' combinations of the P1 and ORF6 genes in the P1 operon carrying different repetitive DNA sequences, as we indeed observed in the described experiments (Dumke *et al.*, 2003). These 'new' combinations could be generated by homologous recombination leading, among others, to the same P1/ORF6 combination as in the transformants *M. pneumoniae* M129/F and M129/R. We explain the failure to detect such *M. pneumoniae* strains by the lack of an efficient system for homologous recombination.

We assume that the crucial enzyme for this reaction is RecA. *M. pneumoniae* contains the corresponding gene (MPN490), but in a transcriptome analysis of *M. pneumoniae* M129 (Weiner *et al.*, 2003) only low signals were obtained for a RecA-specific mRNA. In addition, RecA has not been identified in a proteome analysis combining 2D gel electrophoresis and mass spectrometry (Ueberle *et al.*, 2002) although the features of RecA, pI 9.77 and no predicted transmembrane segment, should have allowed the identification, unless the amount is too low for detection by standard 2D gel electrophoresis and mass spectrometry. However, recently RecA was identified in a total protein extract of *M. pneumoniae* FH by a proteome analysis, which was done without separation of the total protein extract into individual proteins prior to tryptic cleavage (Jaffe *et al.*, 2004). This method is more sensitive, since it avoids the considerable losses of proteins occurring during 2D gel electrophoresis and allows the application

of higher amounts of protein extracts. Therefore, the concentration of RecA in *M. pneumoniae* could be rather low. In agreement with these observations are the negative results from experiments aiming to construct mutants from *M. pneumoniae* by homologous recombination, which is in contrast to the results with the closely related species *Mycoplasma genitalium* (Dhandayuthapani *et al.*, 1999). The latter organism was successfully transformed by integration of foreign DNA via homologous recombination and its RecA was also identified on 2D gels of total *M. genitalium* cell extracts (Wasinger *et al.*, 2000).

Similar experiments as those carried out with the ORF6 gene should also be done with the P1 gene, although this will be more complicated, because this gene contains two repetitive DNA sequences, RepMP2/3 and RepMP4 (Fig. 1; Ruland *et al.*, 1990). In a first step, one of these sequences of a subtype 1-specific P1 gene could be exchanged with a subtype 2-specific one and the resulting *M. pneumoniae* strain tested for adherence, ability to infect hosts and its potential to serve as a true precursor for a complete subtype switch. Experiments along this line will help to explain whether the observed switch of the dominating subtype in epidemic outbreaks of *M. pneumoniae* appearing in intervals of 3–7 years is caused by conversion of one subtype to another one or by faster growth of one subtype in a mixture of both subtypes. Since the human immune defence would be primarily directed against the dominating infecting subtypes, the second subtype could grow uninhibited, at least for some time, and should become the dominating infectious subtype in the following years.

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