

Lysogeny of *Streptococcus pneumoniae* with MM1 Phage: Improved Adherence and Other Phenotypic Changes

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Pneumococcal prophages are extremely frequent, but no role in pathogenesis has so far been attributed to them. We isolated a variant of phage MM1, named MM1-1998, from a serotype 24 strain of *Streptococcus pneumoniae*. We created three isogenic strain pairs (serotypes 3, 4, and 24) that differed only by the lysogenic presence of the MM1-1998 phage and did a phenotypic comparison. Lysogeny led to improved adherence to inert surfaces and pharyngeal cells compared to that with the cured variants of the strains. We found that lysogeny with MM1-1998 coincided with a more transparent phenotype and phage curing with more opaque colonies in all strain pairs, and we discovered that transparency was associated with more successful and stable lysogeny. Since transparency alone was possibly responsible for the adherence difference, we further compared the TIGR4 lysogen with an equally transparent variant of TIGR4 in order to reassess the role of phage or transparency separately. The results revealed that improved adherence was independently associated with lysogeny with the MM1-1998 phage. Other phenotypic differences such as faster growth, increased autolysis, and decreased intracellular hemolytic activity were more likely due to transparency. By improving the adherence of pneumococci, this prophage may contribute to their fitness and possibly to their persistence in humans.

Asymptomatic carriage in the nasopharynx, particularly in children, is believed to be the reservoir of *Streptococcus pneumoniae*, a strictly human pathogen. Under predisposing conditions, such as a viral respiratory infection, smoking, or certain chronic illnesses, pneumococci can provoke serious infections such as otitis media, pneumonia, bacteremia, and meningitis. The majority of colonizing pneumococci have been shown to contain prophages or remnants thereof when examined with a DNA probe specific for the major autolysin *lytA*, which hybridizes to lytic enzyme genes of temperate pneumococcal phages because of their relative sequence similarity (7, 27, 32, 35).

While apparent virulence factors such as toxins and secreted enzymes have been associated with prophages in *Staphylococcus aureus*, *Streptococcus pyogenes*, and many others (10, 42), the role of pneumococcal prophages remains so far unknown. Presently no obvious phage-encoded fitness-enhancing factors have been found in pneumococci, except perhaps the phage lysin itself. It is unlikely that the role of prophages in pneumococci is limited to genetic exchange and evolution, since the 10% diversity between the three currently sequenced pneumococcal strains was not due to the presence of phage (with the exception of a small, incomplete prophage-like element in the TIGR4 strain) (33). The pneumococcus is a naturally competent organism which can take up foreign DNA without the help of phages, and it contains many insertion elements and repeats which encourage recombinatorial events.

Two pneumococcal prophages, MM1 and EJ-1, have been sequenced entirely, and others have been sequenced partially (22, 24, 28, 29). In the following work, we will describe the isolation and sequencing of a variant of the MM1 phage,

named MM1-1998, from a Portuguese clinical strain, and studies of a possible role this prophage plays in colonization or pathogenesis, using a set of three different host strains lysogenized with MM1-1998.

MATERIALS AND METHODS

Bacterial strains and phages. Strains are listed in Table 1. For ease of understanding, we renamed two clinical strains (30): DCC1714 was changed to strain 7, and DCC1808 was changed to strain 8. Chemicals were purchased from Sigma (St. Louis, MO) if not otherwise noted. The MM1-1998 phage was induced with 0.1 μ g/ml mitomycin C in strain 8 (serotype 24) grown in brain heart infusion medium (BHI). After lysis, the culture was ultracentrifuged and the pellet was suspended in SM buffer (31). A stable unencapsulated “rough” mutant of strain 7 (serotype 3), named 7R, was selected after internalization by a macrophage (RAW 264.7) culture. Sandwich plaque assays were performed as described elsewhere (21) with modifications: blood agar was replaced with agar made from chemically defined streptococcal medium (36) plus 0.8% yeast extract (Difco), 0.5% choline, and 170 U catalase/ml agar. A cylinder of agar was picked at the edge of a plaque of MM1-1998 on 7R. The presence of MM1-1998 phage was tested by streaked colony blot of 50 to 100 single colonies (see below). A lysogenized colony was subcultured three times and named 7RM. The serotype 3 capsule was then reintroduced by transformation with DNA from strain 7 and visual phenotype screening (41). A mucoid colony was isolated, confirmed for MM1-1998 presence, and named 7M. Strain TIGR4 (serotype 4) was lysogenized without preselection, resulting in strain T4M. For the phenotypic comparison, we cured all three lysogenized strains of their phage by simply growing them on agar and analyzing a sufficient number repeatedly by colony blot for the loss of phage. The cured strains were named 7MC, 8C, and T4MC.

PFGE. Pulsed-field gel electrophoresis (PFGE) of pneumococcal strains was carried out according to a protocol described in detail elsewhere (9).

Phage DNA sequencing and restriction. The phage pellet was digested with DNase (5 μ g/ml) and RNase (200 μ g/ml) for 30 min at 37°C, followed by digestion with proteinase K (50 μ g/ml) for 15 min at 65°C. Phage particles were lysed with sodium dodecyl sulfate (0.5%) and EDTA (20 mM) and incubated for another hour at 65°C. DNA was extracted and shotgun cloned, resulting in overall 10 \times coverage of the phage genome.

DNA from induced phages from strains 7RM, 8, and T4M was digested with HindIII (New England Biolabs, Beverly, MA) and run on a 1% agarose gel for comparison.

Confirmation of phage insertion sites. Phage insertion sites *attL* and *attR* in strains 7M, 8, and T4M were amplified by PCR using primers based on the

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TABLE 1. Pneumococcal strains used in this study

Original name	Source or reference	Name in this publication	Serotype	Manipulation
DCC1714	A. Tomasz (30)	7	3	None
		7R	No capsule	Selection by macrophage internalization
		7RM	No capsule	7R lysogenized with MM1-1998 phage
		7M	3	7RM transformed with DNA of strain 7
		7MC	3	Selection of cured colony of 7M
DCC1808	30	8	24	None
		8C	24	Selection of cured colony of strain 8
TIGR4	American Type Culture Collection	TIGR4	4	None
		T4M	4	TIGR4 lysogenized with MM1-1998 phage
		T4MC	4	Selection of cured colony of T4M
		Tigr4OS	4	Small opaque variant of TIGR4, subcultured
		Tigr4OL	4	Large opaque variant of TIGR4, subcultured
		Tigr4T	4	Transparent variant of TIGR4, subcultured

published sequences of phage MM1 (GenBank no. AJ302074) and *S. pneumoniae* TIGR4 (GenBank no. AE005672) (22, 34) and were sequenced.

Growth curves and phenotype comparisons. Growth of isogenic strain pairs was compared in BHI, streptococcal chemically defined medium with 0.8% yeast extract and 0.5% choline, and in minimal essential medium (MEM) with 10% fetal bovine serum at 34, 37, and 40°C, starting at 10⁷ CFU/ml. The colony aspect was observed on Columbia blood agar and transparent BHI agar with ~5,000 U/plate catalase.

Biofilm assays. (i) Microtiter plates. PVC U-bottom 96-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), disinfected with 70% ethanol, were inoculated with a 1:50 dilution of overnight cultures in fresh BHI. Plates were covered with a lid and closed with parafilm. After 24 h of incubation at 37°C with 5% CO₂, plates were emptied by inversion and washed twice with distilled water (dH₂O). Wells were then stained for 30 min with 0.2% crystal violet (CV) in dH₂O and washed again three times with dH₂O. Remaining CV stain was dissolved in ethanol, which was transferred to a flat-bottom polypropylene plate to measure the absorbance at 595 nm.

(ii) Tubes. Fifteen-milliliter polypropylene tubes were inoculated with 10 ml BHI and one loopful of frozen glycerol stock. Cultures were grown for 24 h at 37°C with 5% CO₂ and discarded. Tubes were gently rinsed once with dH₂O, stained with 1 ml 1% CV in dH₂O for 15 min, and again rinsed five times. Photographs were taken of the dried tubes lying on a light box.

Cell culture adherence assays. The human pharyngeal cell line Detroit 562 (American Type Culture Collection, Manassas, VA) and the human bronchial cell line HBE4-E6/E7 were seeded in 24-well plates and grown to confluence (~5 × 10⁵ cells). Wells were washed once with phosphate-buffered saline (PBS), and *S. pneumoniae* in cell culture medium was added at a multiplicity of infection of 1 (for 8, 8C, T4M, Tigr4T, and T4MC) or 50 (for 7M and 7MC). After 2 h, the supernatant of half of the wells (total titer) was removed and kept on ice, and the cells were detached with 500 µl 0.25% trypsin/0.02% EDTA in PBS for 10 min and added to the supernatants. The other half of the wells (adherence titer) was emptied and washed three times with PBS, and cells were detached with 100 µl trypsin/EDTA and added to 900 µl PBS. Cells in all samples were lysed with a sonicator rod for 10 s at a low power setting which does not affect pneumococci. Each adherence titer was divided by the average of the whole-well total titers of the experiment. To further investigate the capsule suppression in adhering 7M colonies, assays were repeated without serum or with the addition of 1.6% bovine serum albumin. Colony aspect was also examined after strain 7M was grown in spent or fresh cell culture medium.

Mouse model of nasal colonization. The mouse model of nasal colonization is described in detail elsewhere (16, 17). Swiss CD-1 (8 versus 8C) or BALB/c mice (T4M versus T4MC) were inoculated with 10⁶ CFU bacteria in 10 µl sterile saline. Bacterial titers in retrograde nasal washes were determined after 48 h.

Scanning electron microscopy. Sterile 12-mm round coverslips were coated with 200 µl BHI agar containing catalase. Colonies of T4M, T4MC, and Tigr4T were grown on these for 15 h and fixed with 2.5% glutaraldehyde in cacodylic acid. For bacteria adhering to PVC, 10-mm PVC squares were added to an overnight culture of T4M and T4MC in BHI. The coupons were gently rinsed in sterile water and fixed. Coverslips and PVC coupons were postfixated in 1% OsO₄. After dehydration through 70% to 100% ethanol, the samples were either critical

point dried or hexamethyldisilazane dried, either sputter coated with gold or carbon coated, and viewed in a LEO 1550 field emission scanning electron microscope.

Colony blots, dot blots, and Southern blots. Colony blots were carried out either by direct lift on HATF nitrocellulose membranes (Millipore, Bedford, MA) or by lifting streaked subcultures from agar plates, using strains 7 and 8 as controls. For dot blot experiments of T4M, eight single opaque and eight transparent colonies were cultured, pelleted, lysed, and vacuum blotted on Hybond-N⁺ nylon membranes (Amersham Biosciences, Piscataway, NJ). For Southern blots, DNA was vacuum blotted to Hybond-N⁺ membranes. As probes, we used the purified PCR products of the MM1 *cl* repressor gene, the pneumococcal house-keeping gene of D-Ala D-Ala ligase (*ddl*), or *lytA*, the major autolysin. Probes were labeled, hybridized, and detected with the ECL Direct Nucleic Acid Labeling and Detection Systems kit (Amersham Biosciences, Piscataway, NJ).

Sequencing of *cap3A* of rough serotype 3 mutants. Single rough colonies were picked and boiled in dH₂O for 10 min and used as PCR templates. *cap3A* was amplified by PCR using primers located just outside the gene (GenBank accession no. Z47210) (2) and sequenced.

Quellung reaction. The presence of a serotype 3 capsule was tested by Quellung reaction with serotype-specific rabbit antiserum (Miravista Diagnostics, Indianapolis, IN).

Antibiotic susceptibility. MICs were determined by the broth dilution method in Müller-Hinton broth with 2.5% lysed horse blood (1) for gentamicin, ampicillin, levofloxacin (Ortho McNeil, Raritan, NJ), azithromycin (Pfizer, New York, NY), streptomycin, novobiocin, and rifampin. Susceptibility against clindamycin, nalidixic acid, and erythromycin was tested with the disc diffusion method (BBL, BD, Franklin Lakes, NJ).

Mutation frequency. Cells (10⁹ CFU) of all strains were spread on BHI agar containing catalase and 1 µg/ml rifampin and were incubated at 37°C with 5% CO₂. Colonies were counted after 48 h.

Hemolysis assay. Hemolytic activity was measured as described previously (6). To account for different growth characteristics of the strains, hourly 1-ml samples of log phase to early stationary-phase cultures were analyzed for titer and the presence of hemolytic activity in supernatant and pellet. Pellets were lysed with 35 µg of the phage lysis Cpl-1 (17). Maximum activity was divided by the bacterial titer at that time point (in CFU) for strain comparison.

Nucleotide sequence accession number. The sequence of phage MM1-1998 is deposited in GenBank under the accession number DQ113772.

RESULTS

Isolation and sequencing of prophage MM1-1998. We isolated a prophage with apparent similarity to the published MM1 phage from strain 8 (formerly DCC1808), a penicillin-sensitive serotype 24 isolate from a surveillance study of day care centers in Portugal (30). HindIII digestion of mature phage DNA showed a restriction pattern highly related to the published pattern of MM1, except for one major band of 7.2

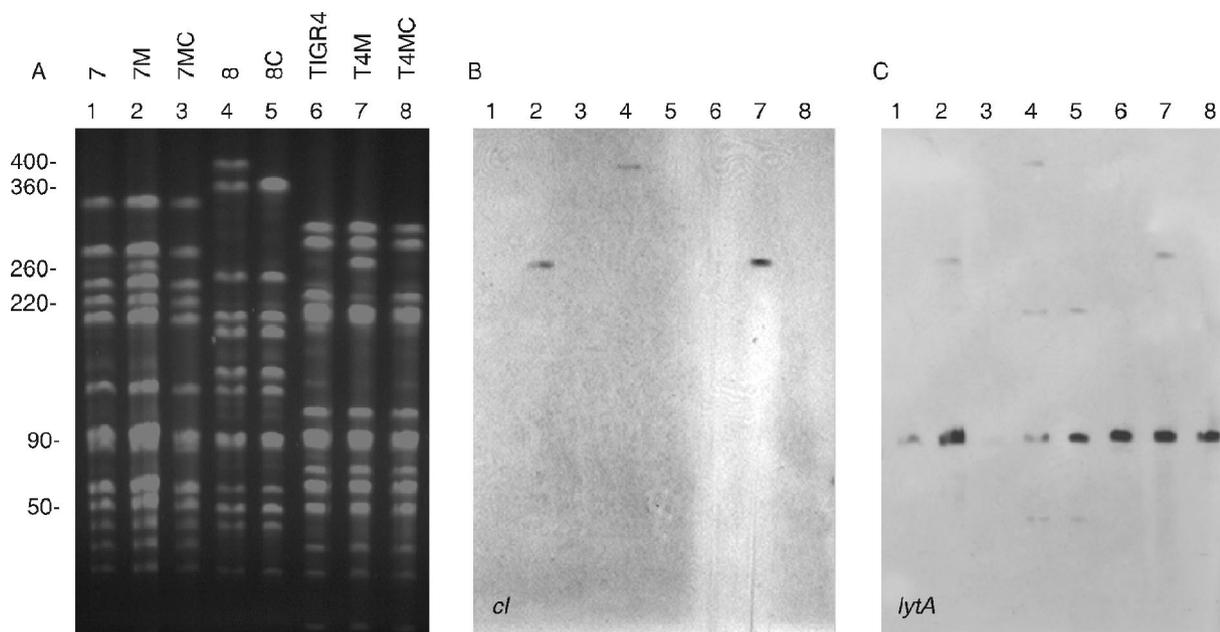


FIG. 1. Pulsed-field electrophoresis (PFGE) and Southern blots. A. PFGE of the strains used in this study, showing 40-kb band shifts associated with lysogeny or curing of MM1-1998. B. Southern blot of the PFGE hybridized with a *cI* (major phage repressor) probe, which binds to all three lysogenized strains. C. Southern blot hybridized with a *lytA* (autolysin) probe. All strains (except for 7MC, which is rather weak) show a strong signal around 90 kb, the presumed location of *lytA*; the lysogenized strains show another weak signal colocalizing with the *cI* probe. Strains 8 and 8C have additional signals, suggesting additional prophages. Lane numbers correspond to the strains detailed in panel A.

kb, which was approximately 1.5 kb shorter than that of MM1 (23). The phage DNA was shotgun cloned and sequenced, resulting in an overall 10 \times coverage of the phage genome. Alignment of the sequence with that of MM1 showed virtual identity (>99.8%), and the new phage was named MM1-1998.

The genome length of MM1-1998 was 38,893 bp, while that of MM1 is 40,248 bp long. It appeared that the difference in length of 1,355 bp was due to one small insertion of 57 bp in *orf25* and one deletion of 1,413 bp in *orf47* in MM1-1998. The insertion, comprised of a 41-bp repeat and 16 additional unique nucleotides, leads to a premature stop in *orf25*. *orf47*, the putative phage antireceptor gene in the original MM1, has four-and-a-half repeats of a 471-bp sequence that codes for many collagen-like G-X-Y amino acid repeats. In MM1-1998, only one-and-a-half of these repeats remain, reducing the predicted size of the protein from 1,989 to 1,518 amino acids.

Other than the two indels, there were only 36 mostly silent single-nucleotide substitutions throughout the rest of the genome (<0.2%), located mainly in the packaging cluster ($n = 7$), head assembly ($n = 13$), and the tail protein region ($n = 15$).

Construction of three isogenic strain pairs. Phage MM1-1998 was used to lysogenize strains TIGR4 (serotype 4) and 7 (serotype 3), resulting in strains T4M and 7M. To lysogenize the mucoid strain 7, we first selected an acapsular (rough) variant of strain 7 (7R), infected it with MM1-1998 (7RM), and reintroduced the capsule by transforming it with genomic DNA of strain 7 (7M). Phages were then cured from all three strains, resulting in 8C, 7MC, and T4MC (Table 1).

Presence and absence of the bacteriophage was tested repeatedly by streaked colony blots (blots from repicked, streaked colonies; data not shown), and results were confirmed by pulsed-field electrophoresis (PFGE) of *Sma*I-digested genomic DNA, fol-

lowed by Southern blotting using the PCR product of the major phage repressor gene *cI* as a probe. Figure 1A shows the PFGE pattern and Southern blots of strains 7, 7M (lysogenized), 7MC (cured), 8 (original lysogenized), 8C (cured), TIGR4, T4M (lysogenized), and T4MC (cured).

Lysogeny of strain 7 with the MM1-1998 bacteriophage (39 kb) resulted in the appearance of a new band of \sim 260 kb in 7M and no disappearance, but a possible relative weakening, of a band \sim 220 kb in size. Curing the phage from strain 8 resulted in the loss of an \sim 400 kb fragment in 8C and possibly in a more intensively stained \sim 360-kb band. Lysogeny of TIGR4 resulted in a band shift from \sim 220 kb to \sim 260 kb and the persistence of a weak 220-kb fragment. The unique bands in the lysogenized strains 7M, 8, and T4M hybridized to the *cI* repressor probe (Fig. 1B, lanes 2, 4, and 7). We considered the disappearance of \sim 40 kb from the PFGE band patterns and the absence of a signal for *cI* (the lack of which precludes lysogeny) as proof of the entire phage being cut out or cured in strains 8C, 7MC, and T4MC (lanes 3, 5, and 8).

When the same membrane was probed with a PCR product of *lytA*, the major autolysin of *S. pneumoniae*, all strains contained a 90-kb fragment that hybridized to the probe (Fig. 1C). The MM1-1998 lysogens (lanes 2, 4, and 7) had an additional, weaker band colocalizing with the *cI* signal, suggesting the presence of phage lysins, and strains 8 and 8C (lanes 4 and 5) had two more weak signals at around 200 and possibly 40 kb, presumably from additional, less inducible phages (27).

To test whether the MM1-1998 phage had remained unchanged in all three lysogens, it was induced with mitomycin C, and purified phage DNA was subjected to HindIII digestion. Since the large amount of capsular polysaccharide of the mucoid strain 7M could not be well separated from the phage

DNA and interfered with digestion and gel electrophoresis, we induced strain 7RM (the nonencapsulated precursor of 7M) and analyzed its phage DNA instead. Restriction patterns (data not shown) indicated that phage genomic DNA from strains 8 and T4M were identical, but that DNA from 7RM was approximately 0.5 kb shorter in one band (6.7 versus 7.2 kb). We suspected that this could be a further deletion in *orf47*, which is located in that band. Amplification and size comparison of *orf47* indeed showed a shorter product in phage DNA from 7RM (data not shown), possibly the deletion of the last remaining 471-bp repeat sequence.

The insertion site of MM1-1998 in all three lysogens was between genes SP1563 and SP1564, an oxidoreductase family protein and a protein of unknown function, as shown previously for MM1 (12).

Growth characteristics and microscopy. In all tested media and at different temperatures, lysogenized strain 8 grew faster than 8C and T4M grew faster than T4MC, but 7M grew slower than 7MC. The growth differences were due to variations in the lag phase, since in mid-logarithmic phase the generation times of each pair were fairly equal. After overnight growth, the density of all lysogenized strains was lower than that of cured strains, either due to phage lysis or increased autolysis of the strains.

Gram-stained bacterial preparations and bacteria mixed with India ink did not reveal differences between isogenic strains.

Antibiotic susceptibilities. The three strain pairs showed different susceptibilities to antibiotics. All derivatives of strain 7 were resistant to macrolides (erythromycin and azithromycin) and clindamycin, which is classically due to the presence of an *erm*-encoded methylase. While there were differences between the antibiotic MICs for isogenic strain pairs in the range of 1 to 2 dilutions, there was no clear shift in antibiotic susceptibility present in all three that could suggest an influence of the prophage. We found an isolated large difference in streptomycin MICs between strains 8 (>256 $\mu\text{g/ml}$) and 8C (16 $\mu\text{g/ml}$) which remains largely unexplained.

Mutation rates. We determined the mutation frequency by counting rifampin-resistant colonies after 48 h of growth on agar containing 1 $\mu\text{g/ml}$ rifampin. The results were in the 10^{-8} range for all strains and showed no significant difference between isogenic strain pairs.

Adherence to inert surfaces (plastic). We used a simple assay of adherence to an abiotic surface that has been described as a screening tool for early steps in biofilm formation in other bacteria (25). Crystal violet staining of rinsed PVC microtiter plates, in which the six strains (three lysogenized and three cured) had grown for 24 h in BHI medium, clearly showed that the three lysogenized strains adhered significantly better to the plastic than the cured strains (Fig. 2A), despite having a lower density after overnight growth. This difference was also seen in polypropylene tubes after overnight growth (Fig. 2B). Scanning electron microscopic images of T4M and T4MC adhering to PVC coupons showed no obvious differences in bacterial surface structures (Fig. 2C). Small protruding appendages, possibly pili-related structures, were seen in both isogenic strains.

Adherence to cells in culture. We also performed adherence assays on two different cell lines, a neoplastic human pharyn-

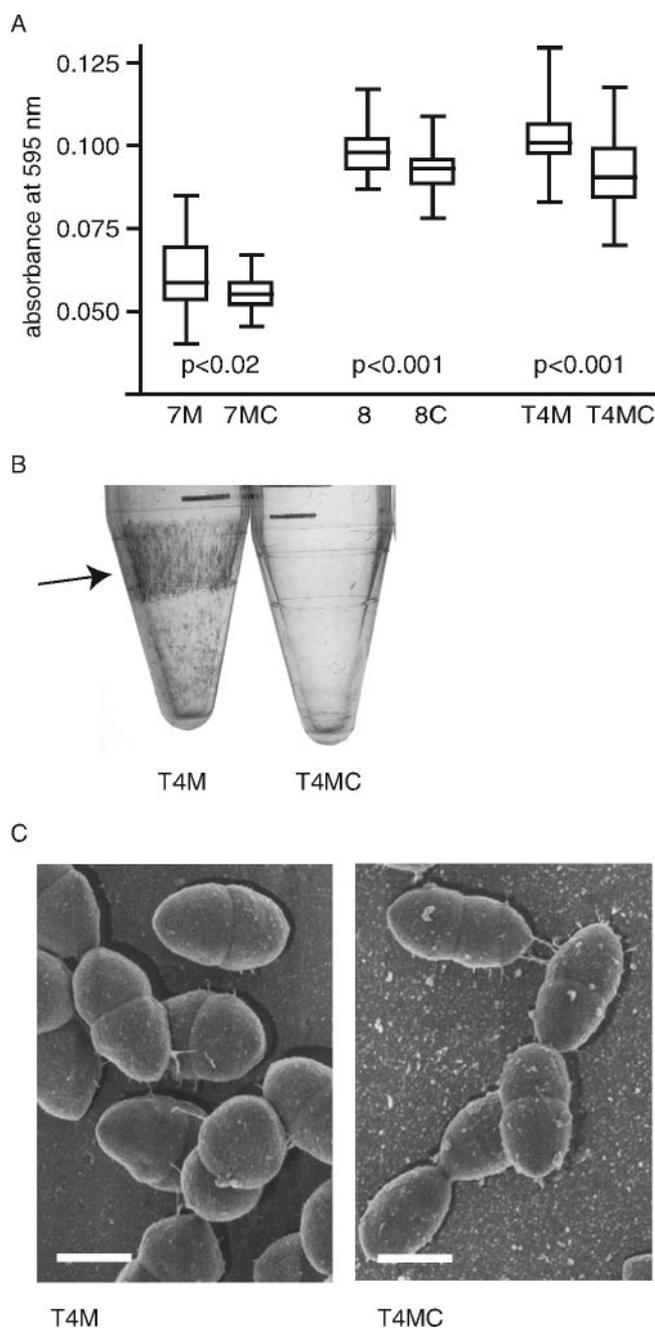


FIG. 2. Adherence to inert surfaces. A. Adherence to PVC microtiter plate wells after 24 h of incubation. Surface-attached cells were stained with crystal violet and quantified by solubilizing the dye in ethanol and determining the absorbance at 595 nm. All three lysogenized strains adhered significantly better. Boxes show medians and quartiles, and whiskers show range. B. Crystal violet (CV) staining of emptied and rinsed polypropylene tubes which had contained overnight cultures. The arrow points to a zone of CV-stained adhering bacteria that was visible for T4M but not for T4MC. C. Scanning electron microscopy of T4M and T4MC pneumococci adhering to PVC coupons. Both appear to have pili-like protrusions, but no obvious surface structure can be seen. Bars represent 500 nm.

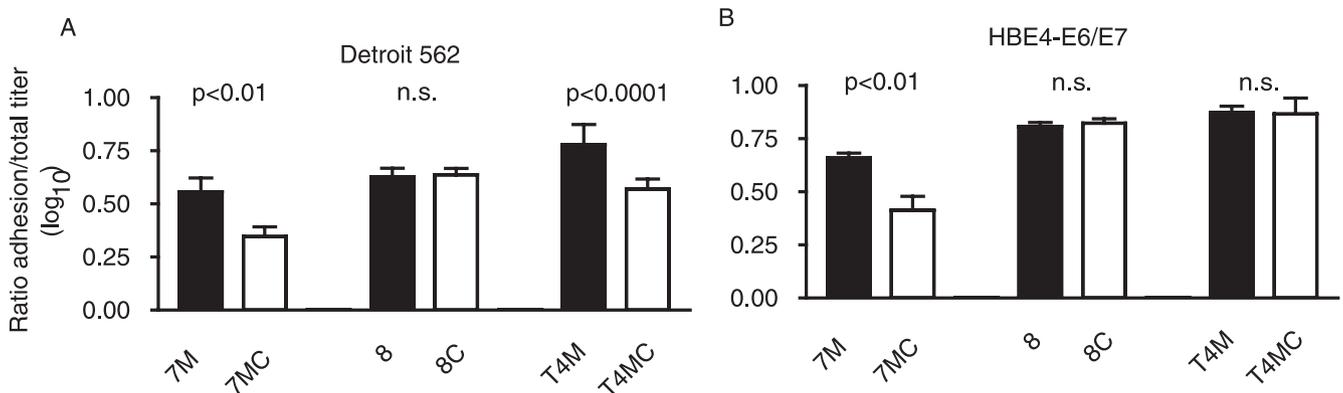


FIG. 3. Adherence to cells in culture. A. Human pharyngeal cell line Detroit 562. Both 7M and T4M adhere significantly better than their cured counterparts. B. Bronchial cell line HBE4-E6/E7. Only 7M adheres significantly better than its cured variant. n.s., not significant.

geal cell line (Detroit 562) and a human bronchial cell line that is immortalized with papilloma virus genes (HBE4-E6/E7) (37). To adjust for the significant growth differences described above, we divided the adhering bacterial titers (expressed as log₁₀ CFU) by the average total titer (also as log₁₀ CFU) of several wells at the end of incubation. Two of the lysogenized strains, 7M and T4M, adhered significantly better to Detroit 562 cells than their cured counterparts, whereas there was no difference between strains 8 and 8C (Fig. 3A) and between the unencapsulated type 3 mutants 7R and 7RM (data not shown). On HBE4-E6/E7 cells, only 7M adhered better than the cured isogenic strain 7MC (Fig. 3B).

We observed a striking difference in colony phenotype after adherence of the mucoid serotype 3 strains 7M and 7MC to the cell cultures. The majority (65 to 99%) of 7M colonies had become rough, whereas more than 99% of 7MC colonies remained mucoid/smooth. 7M colonies from the assay supernatant, from fresh or spent MEM with or without serum, or from an adherence assay without serum produced only smooth colonies. Serum or bovine serum albumin appeared to be necessary, together with the presence of cells, to induce the rough phenotype and the adherence difference.

Using the Quellung reaction, we found that the capsule was absent from the rough colonies of 7M after adherence. Mutations in *cap3A* (also called *cap3D*), the first gene of the serotype 3 capsular cassette encoding a UDP-glucosyltransferase, have been reported to be responsible for the rough phenotype. We sequenced this gene in strains 7, 7R, 7RM, and 7M and in 6 rough colonies of 7M after adherence to cells in culture. In all rough variants (i.e., all strains but 7 and 7M) we found an identical T→C nucleotide change leading to a conservative amino acid change (Y255H) within the putative active enzyme site (2).

Nasal colonization in mice. Strains 8 versus 8C and T4M versus T4MC were tested in a mouse model of nasal colonization (16, 17). Although in both comparisons the lysogenized strain showed a higher median bacterial titer in the nasal washes 48 h after inoculation, neither of the results reached significance because of the wide titer ranges (strain 8 median [range] in log₁₀ CFU/10 μl nasal wash, 3.3 [2.5 to 4.4]; 8C, 2.9 [1.9 to 4.6]; T4M, 2 [0.9 to 2.6]; T4MC, 1.5 [1.0 to 2.6]).

Prophage cure rates. To determine the prophage cure rate in bacteria growing in BHI or MEM and on agar or in cell culture assays, we tested 50 to 100 colonies of each lysogenized strain by streaked colony blot, assuming again that the absence of a signal for the *cI* repressor and consequently the absence of the repressor itself rules out lysogeny. We determined that

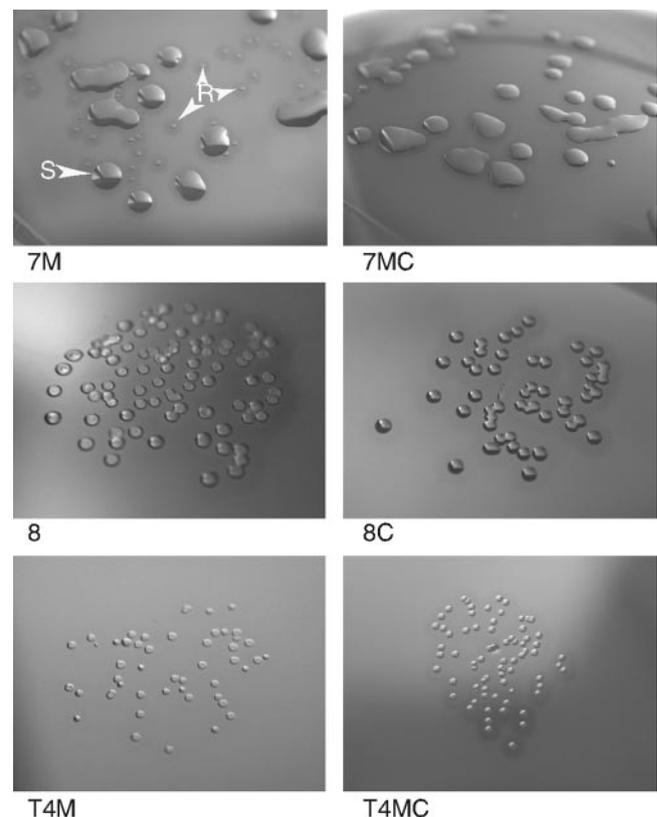


FIG. 4. Aspect of fresh colonies on Columbia sheep blood agar after adherence to Detroit 562 cells. Around 75% of 7M colonies are rough (R), whereas all colonies of 7MC remain smooth (S). Colonies of 8 and T4M are more heterogeneous than their cured counterparts 8C and T4MC. The domed colonies correspond to those that are phage cured.

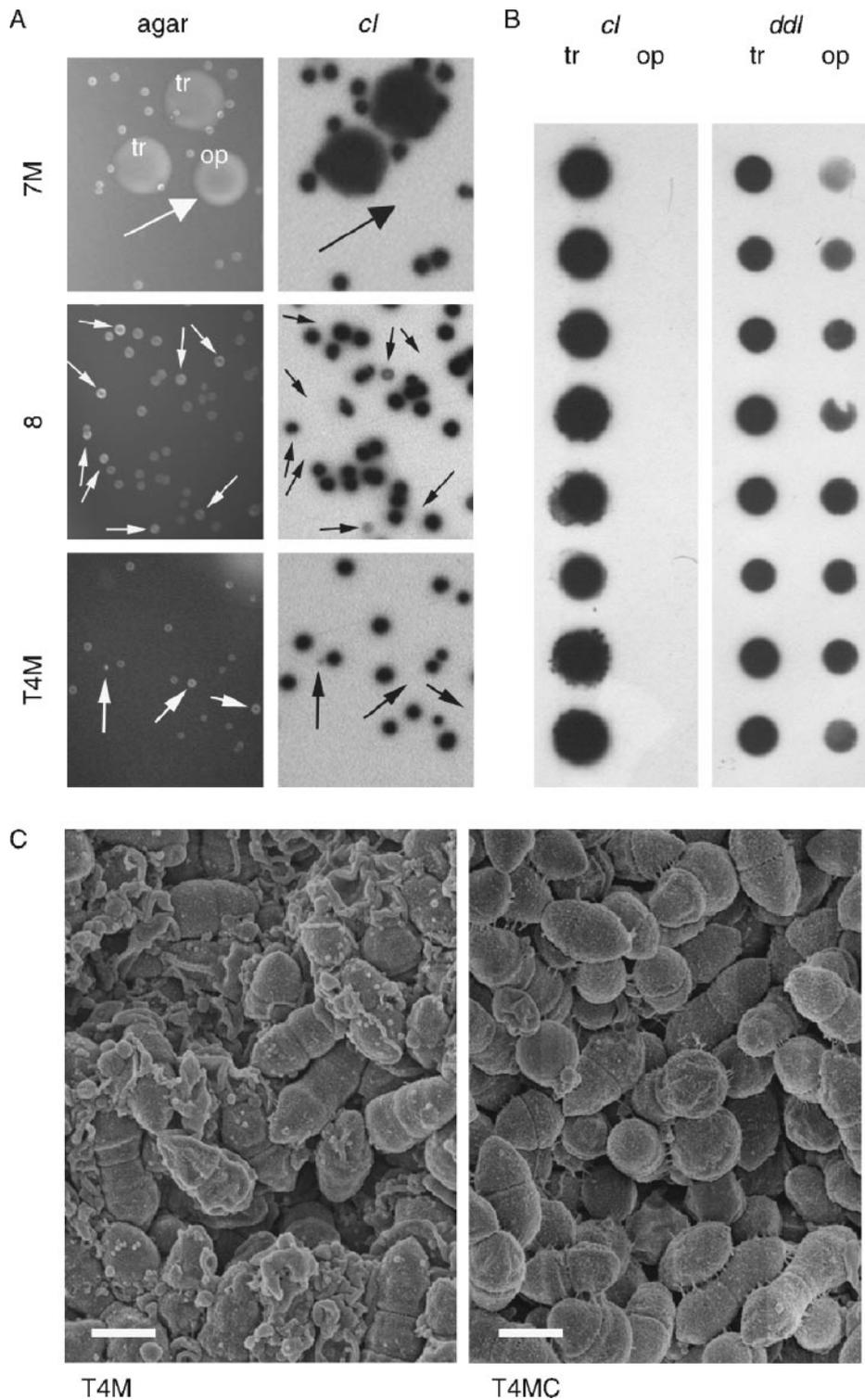


FIG. 5. Opaque colonies correspond to cured or curing colonies. A. Colony phase variation on transparent agar photographed with oblique transmitted light and direct colony blots probed with *cl*. Arrows point to opaque colonies which have a weak or no signal with *cl*. B. Dot blots of eight transparent (tr) and opaque (op) colonies of T4M grown in liquid culture and probed with *cl* or the housekeeping gene *ddl*. None of the opaque colonies showed a signal with *cl*, whereas they were positive for *ddl*, implying that they were cured of MM1-1998 phage. C. Scanning electron microscopy of a transparent T4M and an opaque T4MC colony surface. The surface of the T4M colony appears to contain more debris. Bars, 500 nm.

spontaneous cure (or loss of the phage without lysis) occurred in 1 to 10% of strain 8 and T4M colonies and in 40 to 65% of the 7M and 7RM colonies. The latter may be exaggerated by the slower growth of the lysogenized 7M and 7RM and may not represent the true cure rate. Strikingly, however, of the 7M colonies that converted to the rough phenotype after adherence to pharyngeal cells, less than 5% were cured.

Colony phenotype on blood agar or transparent agar. On blood agar, strains 7M and 7MC both produced mucoid, alpha-hemolytic colonies. As reported above, however, after adherence to cells the majority of 7M colonies appeared rough, i.e., unencapsulated (Fig. 4), which was not the case for 7MC or strain 7 (data not shown). Strain 8 grew as colonies of diverse size and aspect. Most were flat-looking/umbilicated and up to 10% were smooth/domed. Strain 8C grew as homogeneously smooth/domed colonies. The same was true for T4M, which grew as colonies of more mixed aspect, and T4MC, which had uniformly smooth, albeit tiny colonies. The phenotypic difference of the latter two strain pairs was independent of adherence to cells, but it disappeared after 16 h of incubation with the collapse of smooth/domed colonies. When tested with the *cI* probe, the domed colonies of strains 8 and T4M were all cured, corresponding to the above-reported 1 to 10% cure rate.

On transparent agar, we observed a difference in opacity corresponding to the presence of the phage (Fig. 5A). Around half of the mucoid 7M colonies were very transparent, almost “watery,” but no such colonies were seen in 7 or 7MC, which were generally more opaque. In addition, after adherence to pharyngeal cell cultures, 7M again produced a majority of rough, uniformly transparent colonies. When tested with the *cI* probe, both mucoid and rough transparent colonies revealed signals, whereas opaque mucoid colonies did not hybridize. In lysogenized strains 8 and T4M, a small fraction of colonies showed increased opacity (arrows), and these colonies had either no or a weak signal with the *cI* probe, suggesting that they were either cured or in the process of losing their phage. Since the opaque colonies of T4M lifted less efficiently on membranes, we performed a dot blot to rescreen a number of T4M colonies for the presence of phage. As shown in Fig. 5B, all colonies were positive for the control housekeeping gene *ddl*, but only DNA from the transparent ones hybridized with *cI*.

Scanning electron microscopy of several single colonies of T4M and T4MC after 15 h of growth on agar showed more debris on the surface of transparent colonies of T4M than on the opaque colonies of T4MC (Fig. 5C), suggesting the start of autolysis or phage lysis.

Phage versus transparency. We considered the possibility that the described adherence differences between lysogenized and cured strains might be due to the transparent phenotype rather than the presence of the phage itself. It has been shown previously that transparent-phase pneumococci adhere better to cells in culture (11), that transparent phenotypes display more choline on their surface (14, 18), and that pneumococcal phages bind to choline. It was therefore conceivable that MM1-1998 exclusively infects transparent-phase cells, and we thus inadvertently selected for transparency. Alternatively, it was possible that the opaque phase leads to phage curing and not vice versa.

We approached this question from two sides. First, we re-

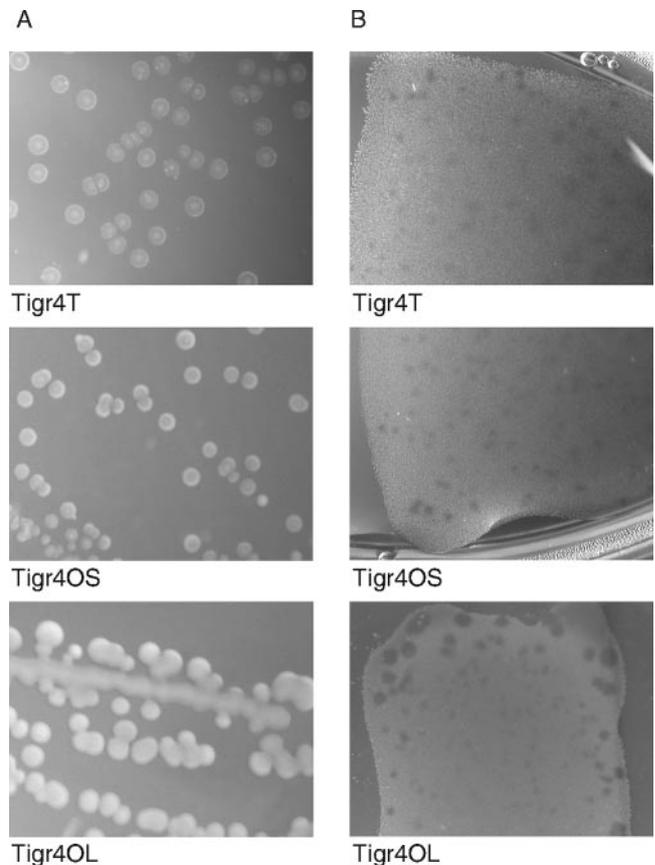


FIG. 6. Phenotypes of TIGR4 and phage plaques. (A) Colonies of Tigr4T (transparent), Tigr4OS (opaque small), and Tigr4OL (opaque large) on transparent agar, photographed with oblique transmitted light; (B) plaques formed by MM1-1998 on these variants. The number of plaques was roughly equal on all three. Plaques on Tigr4OL are noticeably larger and clearer than on the other two.

peatedly propagated transparent and opaque colonies of TIGR4, originally a mix of both, until we isolated colonies of three stable phenotypes: transparent (Tigr4T), opaque small (Tigr4OS), and opaque large (Tigr4OL) (Fig. 6A). MM1-1998 plaqued equally well on all three, refuting the hypothesis that it infects only transparent-phase bacteria, but plaques were noticeably larger and clearer on the opaque large Tigr4OL (Fig. 6B).

We next found that the selection of more transparent colonies from the edge of a plaque improved the chances of finding lysogenized bacteria compared to selection of randomly picked colonies. For Tigr4T, 3 of 13 more transparent colonies were *cI* positive, but only 1 of 35 random colonies were *cI* positive (Fisher's exact test, $P < 0.01$). For Tigr4OL, one of only two somewhat more transparent-looking colonies was positive, compared to none of 48 random colonies ($P < 0.04$). We need to add here that these more transparent colonies were only relatively more transparent than their preinfection phenotype, i.e., more transparent opaques were still quite opaque.

Repeated subculturing of lysogenized Tigr4T and Tigr4OL colonies further revealed a 0% cure rate in Tigr4T and a 96% cure rate in the very opaque Tigr4OL, suggesting that phage curing may be causally linked to opacity.

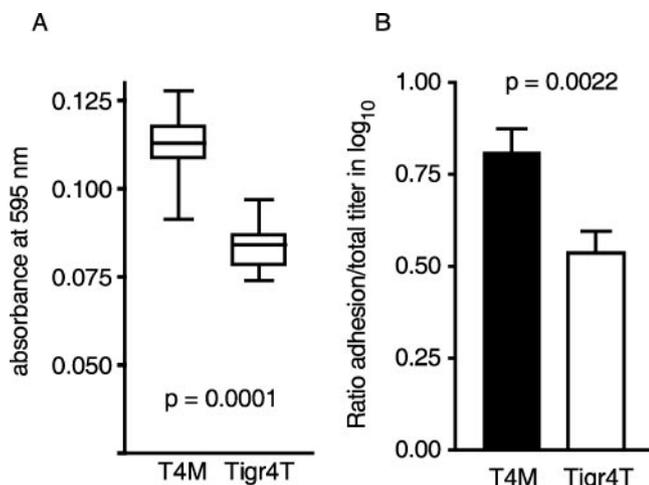


FIG. 7. Phenotypes independently associated with MM1 phage. Adherence of T4M to PVC microtiter plates (A) and to Detroit 562 pharyngeal cells (B) remains significantly stronger after adjusting for the effect of transparency.

The second approach to this question was to compare the nonlysogenized transparent Tigr4T in parallel with the lysogenized T4M, using the same adherence assays as those described above. As shown in Fig. 7, improved adherence to both PVC and pharyngeal cells remained clearly linked to the presence of MM1-1998. Growth curves of T4M and Tigr4T, however, no longer differed in speed or overnight density but closely followed that of T4M (data not shown).

HA. We observed that on blood agar plates which had been left at room temperature for several days, the area of hemolysis around streaks of cured strains was visibly larger than those around lysogens, and it was composed of several rings. This led us to perform a hemolysis assay to quantitate hemolytic activity (HA) (6). We found that intracellular HA (in the pellet) peaked in all strains just as they reached the maximum titer. But peak HA was higher in the cured 7MC, 8C, and T4MC strains than in their lysogenized counterparts (cured to lysogen ratios of 6.4, 1.4, and 5.1, respectively). On the other hand, extracellular HA (in the culture supernatant), which was at relatively low levels at all times, increased slightly in the lysogenized strains just after stationary phase was reached (cured to lysogen ratios of 0.4, 0.5, and 0.5, respectively). Comparing the equally transparent T4M and Tigr4T in order to correct for the effect of transparency alone, there was no longer a difference in the pellets (repeated ratio of 1), although there was again more HA in the supernatant of T4M than that of Tigr4T in early stationary phase (ratio of 0.3). Reduced intracellular HA was therefore related to the transparent phenotype, whereas the slight stationary-phase spilling of HA into the supernatant was attributed to the presence of MM1-1998.

DISCUSSION

To our surprise, the sequence of MM1-1998 phage genome was extremely similar to that of MM1. Previously, several isolates of the multidrug-resistant Spain23F-1 clone were shown to contain phage DNA with a highly similar restriction profile

(12). The host of the original MM1, Spain23F-1 strain 949, was isolated in Barcelona, Spain, in 1989. The host of MM1-1998, a penicillin-susceptible type 24 strain, was isolated in March 1998 in Lisbon, Portugal. Between these isolation dates and places lie almost a decade and 1,000 km. Theoretically, a single phage transmission may have happened between the two strains, with the prophage remaining dormant during the rest of the time. Contamination can be excluded, since we never had MM1 in our laboratory and the only Spain23F-1 isolate in our collection does not contain MM1.

Currently, a further MM1 variant can be identified in the unfinished sequence of a strain of the Spain23F-1 clone on the Sanger Institute's website (http://www.sanger.ac.uk/Projects/S_pneumoniae/). This pneumococcal strain (ATCC 700669) was isolated in 1984 in Spain. The total length of that phage is 39,307 nucleotides, which is 941 bp shorter than MM1. Alignment with MM1 and MM1-1998 showed that this difference is again due to the length of *orf47*, which contains two-and-a-half repeats of the 471-bp sequence coding for collagen-like G-X-Y amino acid repeats, i.e., two fewer than MM1. Other than the deletion, the MM1 phage in the Sanger Institute sequence shows 15 single-nucleotide changes compared with MM1. All of these are in the 36 nucleotide changes that distinguish MM1-1998 from MM1. Together, the three sequences show that MM1 prophage changes very little over time in the pneumococcal population despite switching hosts.

There have been few publications dealing with pneumococcal bacteriophages over the last 30 years. Pneumococcal bacteriophages were discovered in the 1970s, when phage studies led to new tools for molecular microbiology and genetics. Some difficulties that were encountered early with pneumococcal phages were unstable stocks, inconsistent plaques, variety of host range, and the apparent nontransformability of lysogens, which was later contested by the addition of external competence-stimulating peptide (20, 26, 27, 35). The recent finding that lysogenic bacteriophages are an integral part of many bacterial genomes, appear to be responsible for inter-strain genetic variation, and carry known or putative virulence factors has revived interest in phages. In pneumococci, however, lysogenic phages are extremely frequent but have not yet been shown to contribute to genetic variation, and no virulence factors have so far been associated with them. This study used *in vitro* lysogenization and the creation of three isogenic strain pairs to show that the presence of the prophage MM1-1998 induces modifications that may favor colonization or virulence.

The interpretation of the results suggests that MM1-1998 exerts a different influence on the serotype 3 strains than on the other two. Growth speed of the lysogenized and encapsulated type 3 strain 7M was reduced. Adhesion to plastic and both human cell lines was significantly improved in this strain compared to that of the phage-cured 7MC, and in the case of the adherence to cells, this was clearly due to the shutting down of the capsule, since there was no difference when the rough versions 7R and 7RM were compared. The switch to the rough phenotype required the presence of the bacteriophage and contact with the cells and either serum or albumin. Point mutations and sequence duplications in *cap3A* (also called *cap3D*) have been shown to be responsible for capsule-negative serotype 3 variants (2, 3, 19, 38). We found a new mutation in *cap3A* which was identical in all rough variants of strain 7,

suggesting the possibility of a recombination rather than a point mutation. Our results correlate with previous reports of type 3 capsule suppression in the context of adherence: an increase in rough colonies of three serotype 3 strains has been reported in Sorbarod-grown pneumococcal biofilms (38), and a recent publication showed downregulation of type 3 capsule during contact with cultured lung cells (13). However, in our study, capsule shutdown during adherence was clearly much more frequent when the MM1-1998 prophage was integrated. Cure rates in both lysogenized mucoid and rough serotype 3 mutants 7M and 7RM were very high (up to 65%). There was, however, an additional difference in transparency of the mucoid colonies (a very watery appearance) coinciding with lysogeny.

In the other two serotypes, 23 and 4, results showed increased adhesion of the lysogenized mutants to plastic and increased adherence of T4M only to pharyngeal cells, as well as no difference for either serotype on bronchial cells. We believe that the absence of a difference on pharyngeal cells between 8 and 8C may be due to the presence of possibly two more prophages in those strains. T4M and T4C are the best characterized strain pair among the three, with T4M containing only one phage, and they seem to show cell type specificity in the adherence assays. The capsule did not appear to be involved in types 23 and 4, since the observed phenotypes on agar, 90% domed/opaque and 10% flat/transparent, were present before and after adhesion, and there was no selection for the flat colony type. Less visible variations of capsule expression were not studied. Spontaneous cure rates of strains 8 and T4M were 1 to 10% in various conditions, much lower than of serotype 3 but still surprisingly high. High phage cure rates may be part of a regulatory system of *S. pneumoniae*, which supports lysogeny as long as it is useful during one stage of the life cycle but promotes curing when keeping a particular phage becomes unfavorable. We hypothesize that different effects of the phage on type 3 in contrast to types 4 and 23 depends to some extent on the capsule type, or possibly, since they included slower growth speed and very high phage cure rates, a regulatory mechanism that comprises type 3 capsule control.

Our investigation led to the discovery that lysogeny was associated with increased transparency. Transparent colonies, as first described by Weiser et al., appear more collapsed/flat than opaque colonies, and they show earlier autolysis but have identical doubling times and chain length (40). They were subsequently shown to possess greater amounts of teichoic acids and phosphorylcholine (14, 39), produced more autolysin and less PspA, appeared to adhere better to platelet-activating factor-transfected COS cells, and colonized rat pups more efficiently (11, 40). The underlying mechanism responsible for the many changes in phase variation remains unknown. We examined whether lysogeny with MM1-1998 caused transparency or vice versa. We discovered that MM1-1998 produced the same number of plaques in three TIGR4 variants of different transparency. This observation alone does not establish whether one phenotype was infected by more phage particles than the other. It appeared, however, that the choice of a lytic or lysogenic pathway after infection was influenced by the phase of the bacterium and that a selection for transparency may have happened at this stage during the search for lysogens. Plaques on the very opaque Tigr4OL were larger and clearer,

suggesting the lytic infectious pathway is favored over the lysogenic in the opaque-phase phenotype. This is supported by the infrequency of lysogens found at the edge of such a plaque. Studies with phage λ have shown that both the physiology of the host cell (growth in poor medium) and multiple-phage infection favor the lysogenic pathway (15). The very high cure rate of lysogenized Tigr4OL suggests that the opaque phase not only favors the lytic pathway but that it is also incompatible with stable lysogeny after it has been established.

After adjusting for the effect of transparency alone by comparing T4M to an equally transparent Tigr4T in this study, the difference in adherence to plastic and pharyngeal cells remained independently correlated to the presence of the MM1-1998 prophage.

Scanning electron micrographs of strains T4M and T4MC adhering to PVC coupons showed short piliform structures extending from both strains but no structural difference associated with the presence of MM1-1998 prophage. Two tail proteins of the *Streptococcus mitis* lysogenic bacteriophage SM1 have been shown to improve adherence of the bacteria to human blood platelets, and they were found to be surface associated. Since none of them had cell wall anchoring or transport signal features, it was hypothesized that they reach the surface either by phage lysis of a subset of cells or by the action of a holin without lysis (4, 5). A similar mechanism is possible for MM1-1998-mediated adherence. Preliminary comparison of surface protein preparations of T4M and T4MC did not reveal different profiles (data not shown). However, the increased spilling of hemolytic activity into the culture supernatant of lysogenized strains in early stationary phase supports the above hypothesis of phage protein translocation by lysis. Further mutagenesis studies of MM1-1998 genes may shed more light on how the prophage influences adherence. Previously, descriptions of MM1 and similar phages have focused on antibiotic-resistant pneumococci, where they are frequently but not invariably found (8, 12, 24). Our results showed that antibiotic resistance was not linked to the presence of MM1-1998 prophage.

The present study is the first attempt to lysogenize pneumococcal strains in vitro with a known bacteriophage in order to study the role of the phage in pathogenesis. We discovered that adherence to inert surfaces and specifically to pharyngeal cells is associated with the MM1-1998 prophage, which may confer an advantage in colonization of the human nasopharynx. MM1-like phages are frequent and have been described in multiresistant, globally spread clones (12). Adherence enhancement would contribute to the fitness of those strains and possibly to their persistence and spread. We furthermore found that the variation between transparent and opaque phases likely plays a role in successful lysogeny of MM1-1998, suggesting that the host bacterium can somewhat control its prophages.

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