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Peculiarities of the DNA of MM1, a temperate phage of *Streptococcus pneumoniae*

Summary. The abundant presence of temperate phages in the chromosomes of clinical isolates of *Streptococcus pneumoniae* has been well documented. The genome of MM1, a temperate phage of pneumococcus, has been isolated as a DNA-protein complex. The protein is covalently bound to the DNA, was iodinated in vitro with Na¹²⁵I, and has an *Mr* of 22,000. Electron microscopy and enzymatic analyses revealed that the MM1 genome is a linear, circularly permuted, terminally redundant collection of double-stranded DNA molecules packaged via a headful mechanism. The location of the *pac* site appears to be downstream of the terminase, between *orf32* and *orf34* of the MM1 genome. [Int Microbiol 2004; 7(2):133–137]

Key words: pneumococcus · DNA-protein complex · *pac* site · temperate phages

Introduction

Infectious diseases are currently the third leading cause of death in the USA, and *Streptococcus pneumoniae* is the most frequent cause of pneumonia and blood stream infections in the elderly, and one of the main causes of middle-ear infections in children. In some parts of the United States, resistance to penicillin in pneumococcus is as high as 20 to 40% [13]. Pneumococcal phages were first isolated in the mid-1970s [12,23]; since then, only 12 different phages have been identified and/or characterized [8,9]. Pneumococcal phages belong to four different morphological families and include lytic and temperate phages [8]. Lysogeny in pneumococci freshly isolated from humans was first documented by Bernheimer [2], who showed that about half of pneumococci recovered from pediatric patients and one third of isolates from adults yielded bacteriophages [1]. Moreover, recent surveys using the *lytA* gene as a probe have shown that many isolates contain prophages or some remnants of phage genomes [17]. Bernheimer [1] showed that 18 out of 20 multiply antibiotic-resistant pneumococcal strains of type 19A,

first isolated in South Africa, were lysogenic whereas the other two strains lysed with mitomycin C, which suggested the presence of defective phages. She also showed that only one out of three antibiotic-sensitive 19A strains isolated from the same location was lysogenic. However, the indicator strain used in this assay was different from that employed for the drug-resistant strains. This leaves open the possibility of the presence of different phages in that analysis.

Several years ago, a phage named MM1 was isolated, and partially characterized [9], from the multi-resistant 23F Spanish pneumococcal clone (Spain^{23F}-1) which is, together with South African strains, one of the best examples illustrating the rapid spread of drug resistance worldwide [15]. Microbial pathogens have developed a great variety of strategies to guarantee their own survival and expansion and, as already documented for many other bacterial species, phages might be important vehicles to introduce new factors that microorganisms can eventually use to cause infections and diseases [14]. Recently, a molecular analysis of MM1 was accomplished [16]. In this study, we report some peculiarities of the DNA of the first temperate phage of pneumococcus that has been completely sequenced.

Materials and methods

Bacteria, bacteriophage, and growth conditions. *Streptococcus pneumoniae* 949 strain, a member of the Spain^{23F}-1 clone, was grown in C medium [11] supplemented with yeast extract (0.8 mg/ml; Difco laboratories) (C+Y medium) at 37°C without shaking. Growth was monitored with a Hach 2100N nephelometer. Phage MM1 was induced from the lysogenic strain 949. At a cell concentration of 1.2×10^8 colony-forming units (CFU)/ml, mitomycin C was added to a final concentration of 75 ng/ml, and the culture was incubated in the dark at 37°C until lysis occurred. The phages were precipitated with 0.5 M NaCl and 10% (w/v) polyethylene glycol 6000 and purified in a two-step CsCl gradient as previously described [6].

SDS-PAGE. SDS-polyacrylamide (10%) gel electrophoresis was done as described previously [22]. One part of the gel was stained with Coomassie blue and the other was dried for autoradiography.

DNA isolation. Pneumococcal DNA [5], proteinase K-treated MM1 DNA, and DNA-protein complexes [19] were prepared as previously described.

Electron microscopy. To visualize the DNA-associated protein by electron microscopy, a DNA-protein-complex solution (0.4 µg/ml) was treated with 0.1% glutaraldehyde in 10 mM magnesium acetate buffer, pH 7.0, according to a procedure published previously [21]. To test whether the MM1 genome had redundant ends, 2 µg proteinase K-treated phage DNA were denatured with 100 mM NaOH at room temperature for 15 min and renatured with formamide (35%, final concentration) as previously reported [21].

Radioactive labeling of DNA-protein complexes with ¹²⁵I. DNA-protein complexes of MM1 were labeled with ¹²⁵I to high specific activity by the chloramine-T method [10]. In short, to 20 µg DNA-protein complex in 100 µl SSC (0.15 M NaCl, 0.015 M sodium citrate), 20 µl TNET

buffer [0.1 M Tris-HCl, pH 7.4, 2.5 M NaCl, 50 mM EDTA, 0.05% (v/v) Triton X-100] were added, followed by 7 µl Na¹²⁵I (100 mCi/ml) and 5 µl chloramine-T solution (1 mg/ml). The mixture was incubated with gentle shaking for a minute at room temperature and the reaction terminated by the addition of 10 µl each of sodium metabisulfite (6 mg/ml) and KI (1.4 g/ml). Afterwards, 1% SDS (final concentration) was added, and the mixture was heated at 65°C for 5 min and diluted with 100 µl TES buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1% SDS). The labeled complex was then chromatographed on a prepacked Sephadex G-25 column (Amersham-Pharmacia-Biotech, AP) equilibrated with TES buffer. The labeled fractions that eluted in the void volume were pooled, precipitated with ethanol and NaCl and dissolved in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA.

Results and Discussion

It was previously reported that MM1 DNA extracted from mature phage particles had been isolated as a DNA-protein complex [9]. To further study the characteristics of the protein associated with the DNA, the complex was labeled with ¹²⁵I and purified as described in Materials and methods. The radioactivity was mainly found in the protein, as based on the following: (i) SDS-PAGE of the complex yielded ¹²⁵I-labeling only at the top of the gel (Fig. 1A, lane 3), whereas no labeled band was found when proteinase K-treated complex was used (not shown); (ii) more than 95% of the ¹²⁵I-radioactivity was solubilized by proteinase-K treatment, whereas almost 100% of the label remained acid-insoluble after incubation with either pancreatic DNase or micrococcal nucle-

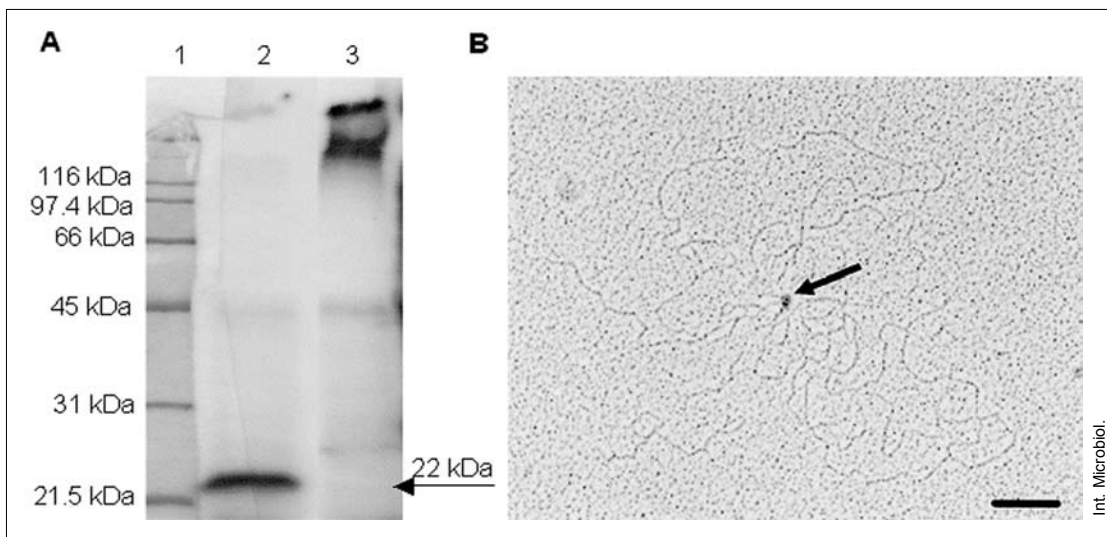


Fig. 1. SDS-PAGE of the ¹²⁵I-labeled DNA-protein complex of MM1 and its visualization by electron microscopy. (A) Labeling of the complex with ¹²⁵I was carried out as described in Materials and methods. The gel (except the standard markers, which were stained with Coomassie blue) was dried onto Whatman 3MM paper and exposed for autoradiography. Lane 3, ¹²⁵I-labeled DNA-protein complex without digestion with micrococcal nuclease. Arrow, M_r of the protein bound to the MM1 DNA (lane 2) released after the micrococcal nuclease treatment of the ¹²⁵I-complex. Molecular mass of the standard markers (lane 1) is given in kDa on the left. (B) Electron micrograph of the DNA-protein complex after treatment of MM1 DNA with 0.1% glutaraldehyde. The complex formed by several protein monomers is visualized as a dot marked with an arrow. Bar represents 250 nm.

ase. Electrophoresis of the ^{125}I -labeled complex treated with micrococcal nuclease showed a major radioactive peak corresponding to an *Mr* of 22,000 (Fig. 1A, lane 2). Furthermore, when the complex was denatured by boiling in SDS, and β -mercaptoethanol, or urea, and analyzed by SDS-PAGE, no radioactivity migrated beyond the top of the gel (not shown), indicating that the 22-kDa protein is covalently linked to MM1 DNA. This protein was made visible by treating the complex with glutaraldehyde, which cross-links the protein monomers, and examined by electron microscopy. The monomeric molecules of the DNA-protein complex appear as a dot in Fig. 1B.

Restriction enzyme digestions followed by heating and rapid cooling failed to provide evidence for the presence of cohesive ends. Electron-microscopy measurements of a score of MM1 DNA molecules using pUC19 as an internal control provided an estimated average length of $42,240 \pm 920$ bp. As the MM1 prophage is 40,248-bp long [16], it was assumed that the MM1 phage particle packaged about $5 \pm 2\%$ more

DNA than the genome unit, suggesting that MM1 has a redundant genome. Restriction endonuclease digestions of MM1 DNA with *AccI* or *HindIII* (Fig. 2A) revealed the presence of submolar fragments. These fragments may derive from the permuted terminal region of the MM1 DNA and would contain a specific site (*pac*) for initial recognition and cutting of phage concatemers sequentially packaged by the terminase enzyme [3]. It was also found that, for these restriction enzymes, the sizes of the submolar fragments agreed with those of the fragments predicted by computer analysis of the sequence. It is worth mentioning that submolar fragments are due to the peculiar headful packaging mechanism of this phage that generates the redundant genome, i.e. once the first *pac* site of the concatemer initiates the packaging process, the following encapsulated genome is excised downstream of the concatemer to the right of the subsequent *pac* site. These findings suggest that the *pac* site is located downstream of the gene encoding the large subunit of the terminase, between *orf32* and *orf34* [16]. Phage genes

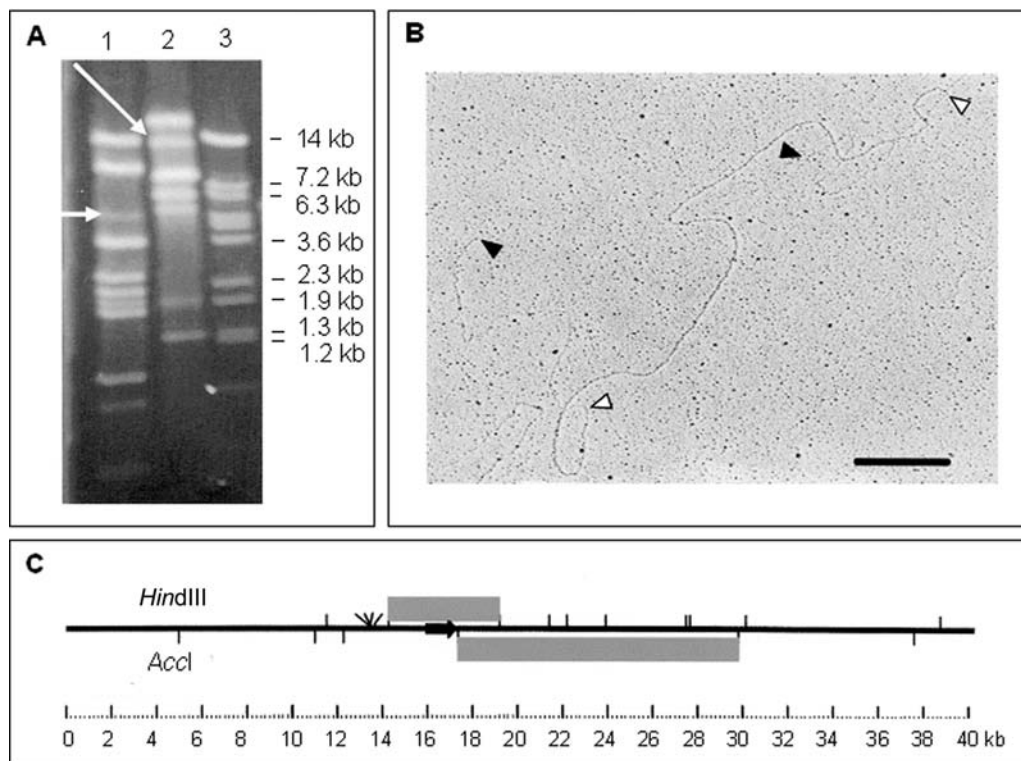


Fig. 2. Restriction endonuclease digestions of MM1 DNA and electron microscopy of renatured MM1 DNA. **(A)** Restriction endonuclease digestions of the MM1 DNA with *HindIII* (lane 1) and *AccI* (lane 2). Submolar fragments are marked with arrows. Molecular size of the standard markers (lane 3) is given in kb. **(B)** MM1 DNA preparation was denatured and renatured as described in Materials and methods. White and black arrows, double-stranded DNA regions and the ends of the single-stranded DNA regions, respectively. Bar, 400 nm. **(C)** Localization in the integrative state of the submolar fragments generated by the restriction enzymes *HindIII* and *AccI*, as shown in (A). Gray bars, submolar fragments; black arrow, gene coding for the large subunit of the terminase.

involved in DNA packaging are often located near the site for packaging, e.g. in phage P22 the *pac* site has been located within gene 3, coding for one element of the terminase [24]. These features also suggested that the MM1 genome is a terminally redundant and circularly permuted DNA generated by a headful packaging mechanism. To further address this point, MM1 DNA was denatured, renatured, and the re-associated molecules were analyzed by electron microscopy. Due to genome redundancy and permutation, all phage DNA molecules are not identical at their ends and, therefore, when non-identical genome strands are re-associated, chimeric DNA double-stranded molecules showing single-stranded ends with a variable size equivalent to the permutation length should be generated. As shown in Fig. 2B, the ends of the renatured molecules were single-stranded, as expected for a genome that is circularly permuted. Measurement of 20 complete DNA molecules revealed a circular permutation of about 50% of a genome unit, assuming that the concatemer formed during replication was built by 10 DNA units [3].

These results demonstrate that the phage genome contains a protein firmly bound to the DNA of the mature particle. Electrophoresis of the ¹²⁵I-labeled complex treated with micrococcal nuclease revealed a major peak of radioactivity corresponding to an *Mr* of 22,000. It has been proposed that terminal proteins (TPs) from several well studied viruses serve as primers for the initiation of linear DNA replication [7,18,21]. However, it has been reported that DNAs of adenoviruses, and S-1 and S-2 plasmids from maize pollen cells, which also contain TPs, are capable of integrating into and excising from chromosomal or mitochondrial DNA-like mobile genetic elements [20]. Furthermore, *Agrobacterium tumefaciens* offers an example in which free, linear double-stranded T-DNA molecules generated from the Ti plasmid become covalently linked to a protein (VirD2) before being transferred to the genome of the host plant [4]. Taking into account that MM1 has a redundant genome with variable ends and most probably replicates by the rolling circle mechanism, leading to the formation of concatemers, a role of the 22-kDa protein covalently linked to MM1 DNA in replication is unlikely. Repeated attempts to determine whether the TP is bound to one or both DNA ends have been unsuccessful. Concerning the physiological role of such TPs, it is tempting to speculate that they link to the monomeric mature DNA once the terminase has cut down the concatemer and, thus, the TP might be involved in the packaging of the virion. However, other roles for TPs, such as a protective effect on DNA ends just after the infection or a putative influence on the DNA circularization (length reduction) step that should precede phage integration, cannot be ruled out. Note that genome redundancy is a critical factor to guarantee the

integration of a complete copy of the phage genome. Redundancy facilitates a precise circularization by homologous recombination that is independent of the packaged-DNA length and of the nature of the terminal sequences. However, TP has to be removed during the circularization step to avoid any interference with the integration process.

In summary, because of the relevance of *S. pneumoniae* as a human pathogen and due to the abundant presence of temperate phages in the genomes of clinical isolates, a better knowledge of the molecular peculiarities of these phages might provide major tools to evaluate the involvement of pneumococcal phages in host virulence.

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Peculiaridades del DNA de MM1, un fago atemperado de *Streptococcus pneumoniae*

Resumen. La abundante presencia de fagos atenuados en el cromosoma de aislados clínicos de *Streptococcus pneumoniae* ha sido bien documentada. El genoma de MM1, un fago atemperado de neumococo, se ha aislado como un complejo de DNA-proteína. La proteína unida covalentemente al DNA, fue yodada in vitro con Na¹²⁵I, y tiene una Mr de 22.000. La microscopía electrónica y los análisis enzimáticos revelaron que el genoma de MM1 es una colección de moléculas lineales de DNA de doble cadena, permutadas circularmente y redundantes en la posición terminal, empaquetadas mediante un mecanismo de ensamblado de unidades genómicas discretas. También proponemos la localización del sitio *pac* en dirección 3' de la terminasa, entre *orf32* y *orf34* del genoma de MM1. [*Int Microbiol* 2004; 7(2):133–137]

Palabras clave: neumococo · complejo DNA-proteína · sitio *pac* · fagos atemperados

Peculiaridades do DNA de MM1, um fago atemperado de *Streptococcus pneumoniae*

Resumo. Tem sido documentada uma abundante presença de fagos atenuados nos cromossomas de isolados clínicos de *Streptococcus pneumoniae*. O genoma de MM1, um fago atemperado de pneumococo, tem sido isolado como um complexo DNA-proteína. A proteína, unida covalentemente ao DNA, foi iodada com Na¹²⁵I, e tem uma Mr de 22.000. A microscopia eletrônica e as análises enzimáticas revelaram que o genoma de MM1 é uma coleção de moléculas de DNA de dupla cadeia linear, permutadas circularmente, e redundantes na posição terminal, empacotadas mediante um mecanismo de ensamblado de unidades genômicas discretas. A localização do sitio *pac* parece estar na direção 3' da terminasa, entre *orf32* e *orf34* do genoma de MM1. [*Int Microbiol* 2004; 7(2):133–137]

Palavras chave: pneumococo · complexo DNA-proteína · sitio *pac* · fagos atemperados

