Molecular and biochemical analysis of the system regulating the lytic/lysogenic cycle in the pneumococcal temperate phage MM1

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Abstract

The temperate phage MM1 forms stable lysogens in Streptococcus pneumoniae. We report here the first characterization of the lysogenic control region in Pneumococcus which contains two functional divergent promoters (PR and PL). MM1 encodes a 14-kDa cI protein (CI) that appears to be responsible for maintaining the lysogenic state in Pneumococcus since it prevents elongation of the transcripts controlled by PR and PL.

Keywords: Phage repressor; Lytic/lysogenic cycle; Temperate phage; Pneumococcus

1. Introduction

Streptococcus pneumoniae (the Pneumococcus) is a Gram-positive human pathogen and the leading cause of pneumonia, meningitis and bloodstream infections in the elderly and one of the main responsible of middle ear infections in children. A better knowledge of the molecular biology of Pneumococcus has been achieved through the study of pneumococcal phages [1]. The abundance of temperate phages in clinical isolates of Pneumococcus was suggested some years ago [2] and it recently proposed that they account for as much as 75% of the samples analyzed [3].

We had isolated and partly characterized a temperate phage (MM1), belonging to the Siphoviridae family, from a clinical isolate (strain 949) of the multiply antibiotic-resistant Spain 23F-1 S. pneumoniae clone [4]. This epidemic clone is the best illustration of rapid spread of drug resistance [5], in this case originally detected in Spain and then rapidly disseminated to other parts of the world. Most recently, we have dissected the entire nucleotide (nt) sequence of this temperate phage, as well as the description of its open reading frames (ORFs), the genome organization, and functional assignments of gene products, based on N-terminal amino acid sequencing and sequence similarities [6]. Temperate phages regulate their developmental fate by encoding a product (named CI) that works as a transcriptional regulator. There are only few data available on the commitment between lytic and lysogenic state in Gram-positive bacteria [7]. We report here the first molecular approach to characterize the elements involved in the regulation of the lysogenic state in Pneumococcus.

2. Materials and methods

2.1. Bacteria, bacteriophages, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. S. pneumoniae was grown in C medium [8] supplemented with yeast extract (0.8 mg ml⁻¹; Difco Laboratories) (C+Y medium) at 37°C without shaking and the growth was monitored with a Hach 2100N nephelometer. Escherichia coli was grown in Luria Bertani (LB) medium at 37°C with shaking. Phage MM1 was induced from the lysogenic strain 949. At a cell concentration of 1.2×10⁸ colony-forming units (CFU) ml⁻¹, mitomycin C was added to a final concentration of 75 ng ml⁻¹, 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 (w/v) and purified in a two-step CsCl gradient as previously described [6].

2.2. DNA preparation, transformation procedures and recombinant DNA techniques

The preparation of pneumococcal DNA has been described elsewhere [9]. Protein-free MM1 DNA was obtained by treatment of purified phage preparations with sodium dodecyl sulfate (SDS) and proteinase K and DNA-protein complexes were isolated as previously described [9]. Plasmid DNA was extracted from E. coli by the rapid alkaline method [10]. DNA restriction or amplification reactions (PCR) amplifications were performed using 2 U of AmpliTaq DNA ligase (Amersham-Pharmacia-Biotech., AP) were used as recommended by the suppliers. Polymerase chain reaction (PCR) amplifications were performed using 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA), 1 µg of chromosomal DNA (or 0.1 µg of phage DNA), 1 mM of each deoxynucleoside triphosphate, and 2.5 mM of MgCl2 in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G+C content of the corresponding oligonucleotide. The oligonucleotides used are reported in Table 1. Transformation of E. coli DH5α was carried out by the RbCl method [10]. Transformants were selected on LB plates containing ampicillin (100 µg/ml) and containing 0.6 g of chromosomal DNA (or 0.1 µg of phage DNA), 1 µM of each deoxynucleoside triphosphate, and 2.5 mM of MgCl2 in the buffer recommended by the manufacturer. Non-digested murein was separated by centrifugation at 12,000×g for 15 min, and the digested (solubilized) substrate was quantified by measuring the reactivity in the supernatant. One unit of lytic activity was defined as the amount of enzyme that catalyzed the hydrolysis (solubilization) of 1 µg (715 cpm) of cell wall material in 15 min at 37°C.

2.4. Miscellaneous methods

N-terminal amino acid sequence analyses were carried out according to a published procedure [13]. SDS–polyacrylamide gel electrophoresis (PAGE) was performed on gels containing 12.5% (w/v) polyacrylamide and the gels were stained with Coomassie blue.

3. Results and discussion

3.1. Characterization of the promoters controlling the lytic lysogenic cycle

The most left region (from nt 1 to 5013) of the MM1 prophage [6] encompasses the lysogenic control region, and contains four ORFs that are transcribed leftwards (int, orf1, orf2, and c1). The Int protein was identified as...
the MM1 integrase that belongs to the λ phage family of integrases [4]. The different functional clusters of the MM1 genome are apparently separated by intergenic regions that may contain promoter sequences and/or regions capable of forming stable stem-loop structures, most probably representing transcription terminators. An interesting finding was the existence of a 296-nt region located between genes cI and orf4 that contains two directly adjacent, outward-facing, putative promoter-like sequences (P_L: (2544/c) TTGTCA-19 nt-TAAAAT; P_R: (2696) AAGCTA-20 nt-TTAAAT) (/c means that the sequence is located in the complementary strand). Interestingly, this region contains three direct repeats of the sequence AA-CAACTAA, that most likely act as operators for regulatory purposes [6]. Moreover, this zone separates the two gene clusters/operons that constitute the MM1 genome and which are transcribed in opposite orientations. To test the role of this region in regulation we used pLSE4, a broad host range pneumococcal promoter-probe plasmid that uses the lytA gene coding for the major pneumococcal autolysin as reporter [14]. To clone the fragment containing the putative promoters in both orientations we prepared four oligonucleotides (Xba-prom1, Xba-prom2, Sph-prom1, and Sph-prom2) as depicted in Fig. 1. Each pair of oligonucleotides was designed to place P_L, the promoter of the cI gene (plasmid pP_L), or P_R, the promoter of orf4 (plasmid pP_R), upstream of lytA in the correct orientation to be independently tested. The accuracy of the constructions was confirmed by sequence determination. Extracts prepared from E. coli transformants containing either the resulting pP_R or pP_L plasmids were assayed on [3H]choline-labeled pneumococcal cell walls and the results demonstrated promoter activity for both P_R and P_L sequences (Fig. 1).

3.2. Expression of the CI protein

The first ORF downstream of P_L corresponds to gene cI that codes for a protein sharing extended similarity to well-characterized repressors identified in other temperate phages [6]. To further demonstrate that cI codes for a repressor protein, this gene was cloned into plasmid

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**Fig. 1. Cloning of the repressor cI gene and the promoters P_R and P_L controlling the lysogenic/lytic cycle.** At the top of the figure we show a schematic representation of the proposed organization of the genome of MM1 [6]. Genes involved in the lytic/lysogenic cycle are indicated in black and striped rectangles. Promoters were cloned upstream of the lytA reporter gene into plasmid pLSE4 (right), and the repressor cI gene was cloned into plasmid pUC18 (left). Abbreviations: H, HindIII; S, SphI; X, XbaI; Ap^R, Ln^R, and Te^R represent genes coding for resistance to ampicillin, lincomycin, and tetracycline, respectively.
pUC18 under the control of the \( P_{\text{lac}} \) promoter. To do that, the \( cI \) gene was amplified using primers MM1Rep5' and MM1Rep3', digested with HindIII and XhoI and ligated to pUC18 previously digested with the same enzymes to originate plasmid pREP. Crude sonicated extracts prepared from \( E. coli \) DH5\( \alpha \) containing the recombinant plasmid pREP induced with isopropyl-\( \beta \)-d-thiogalactopyranoside were analyzed by SDS-PAGE and revealed the presence of an overproduced 14-kDa protein (Fig. 2). The size of the protein corresponds to that deduced from the nucleotide sequence of \( cI \) (13 814 Da) and analysis of the N-terminal sequence of this protein yielded M-F-E-T-F-E-K that perfectly matched the seven first amino acid residues of the CI protein (not shown). Plasmid pREP was used to transform \( E. coli \) C600 (pPR) and \( E. coli \) C600 (pPL) to analyze the effect of CI on the promoters \( P_R \) and \( P_L \) by testing the inhibitory effect on the expression of the reporter lytA gene, that is under the control of either of these promoters, on the degradation of the labeled cell wall substrate. The results reported in Table 2 demonstrate that CI is a strong repressor of the activity of both promoters since residual activity of the amidase is only 2–3% of that found in the absence of the CI protein. To our knowledge, this is the first report on a lysogenic control protein in \( S. pneumoniae \) that should govern the genetic switch from the lysogenic to the lytic cycle.

Regulation of the lytic/lysogenic cycle of \( \lambda \) phage infecting \( E. coli \) has been analyzed in detail [15] whereas this type of regulation has been little studied in Gram-positive systems. An interesting and recent contribution to this topic has been the study on the regulation of lysogeny of \( Lactobacillus casei \) by phage A2 [7] where the activity of the CI protein regulates the lytic and lysogenic pathways of the A2 phage. We have demonstrated here that there are two promoter activities (\( P_R \) and \( P_L \)) in opposite orientations that are probably involved in the regulation of the lytic/lysogenic cycle of phage MM1. Besides, we have cloned and expressed here the product of gene \( cI \), highly similar to defined phage repressor proteins like the CI of \( \Phi \) NIH1.1 that infects \( Streptococcus pyogenes \) [16], that was capable of inhibiting the activity of \( P_L \).

The development of these molecular tools is essential to pave the way to study the competing transcription events occurring during the early decision for lysis or lysogeny in an important system like Pneumococcus where the presence of temperate phages appears to affect up to the 70% of clinical strains freshly isolated from humans.

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