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ScienceDirect
Plasmid 58 (2007) 51–60
PLASMID
www.elsevier.com/locate/yplas

Isolation and characterization of a new plasmid pSpnP1 from a multidrug-resistant clone of *Streptococcus pneumoniae*

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Received 17 November 2006, revised 11 December 2006
Available online 2 February 2007
Communicated by Manuel Espinosa

Abstract

A novel *Streptococcus pneumoniae* plasmid (pSpnP1; 5413 bp) has been isolated from the multidrug-resistant clone Poland^{23F}-16, and its complete nucleotide sequence has been determined. Sequence analysis predicted seven co-directional open reading frames and comparative analyses revealed that plasmid pSpnP1 is different to pDP1, the only previously described pneumococcal plasmid, whereas it is highly similar to pSt08, a plasmid from *Streptococcus thermophilus*. A double-stranded origin for replication similar to the replication origin of the pC194/pUB110 family was located upstream of the putative *rep* gene (*orf2*). It also contained a 144-bp region with over 60% identity to the single-stranded origin type A of the *Streptococcus agalactiae* plasmid pMV158/pLS1. Detection of single-stranded DNA by Southern blot analysis indicated that pSpnP1 replicates via a rolling circle mechanism. Interestingly, the product of *orf1* has a putative Zonular occludens toxin conserved domain present in toxigenic strains of *Vibrio cholerae*. Real-time PCR assays revealed that this ORF was expressed. Hybridization experiments showed that the pSpnP1 replicon was unusual among other examined antibiotic-resistant pneumococcal clones, although the recombinant plasmids based on pSpnP1 were able to replicate in *Bacillus subtilis* and *Lactococcus lactis*.

Keywords: *Streptococcus pneumoniae*; Cryptic plasmid; Rolling circle replication; DNA sequence; Zonular occludens toxin domain

1. Introduction

Streptococcus pneumoniae is a major Gram-positive human pathogen and the leading cause of illnesses such as pneumonia, bacteremia, and meningitis, and of non-invasive infections, such as otitis media and sinusitis. The first isolated pneumococcal plasmid, named pDP1, was found in derivatives of a type 2 *S. pneumoniae* strain that had been isolated by Avery in 1916 (Smith and Guild, 1979). Despite considerable efforts to identify plasmids in the pneumococcus, only small cryptic plasmids showing homology to pDP1 have been discovered in clinical isolates of pneumococci in Italy (Coratza et al., 1983), South Africa (Chalkley and Koornhof, 1988), Australia (Berry et al., 1989),

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doi:10.1016/j.plasmid.2006.12.006

Germany (Sibold et al., 1991), and Spain (Sibold et al., 1991; Muñoz et al., 1999). Four virtually identical, cryptic pneumococcal plasmids have been sequenced so far, i.e., pDP1 (3161 bp) and pSMB1 (3162 bp) (Oggioni et al., 1999), pPR3 (3160 bp) (Schuster et al., 1998), and pRMG1 (3160 bp) (Muñoz et al., 1999). The host range of pDP1 and its relatives include *Streptococcus gordonii*, *Streptococcus agalactiae*, *Enterococcus faecalis* and *Bacillus subtilis* (Oggioni et al., 1999). The scarcity of naturally occurring plasmids in *S. pneumoniae* is not yet understood in view of the demonstrated capacity of pneumococci to accept and transfer plasmids originally isolated from other species (Barany and Tomasz, 1980; Stassi et al., 1981). However, despite the low frequency of plasmid-containing pneumococci (Berry et al., 1989; Sibold et al., 1991) two strains harboring two plasmids at the same time have been described and in both cases, a pDP1-related plasmid was accompanied of an uncharacterized small cryptic plasmid (Sibold et al., 1991).

While testing for the presence of bacteriophages on antibiotic-resistant clones of *S. pneumoniae*, we found a new plasmid (pSpnP1) in the multidrug-resistant strain Poland^{23F}-16 (MIC values: penicillin 8 μg ml^{-1}; erythromycin >8 μg ml^{-1}; tetracycline 16 μg ml^{-1}; chloramphenicol 16 μg ml^{-1}; cefotaxime 8 μg ml^{-1}) (McGee et al., 2001). This strain (also designated as PMEN-16), is a widespread clone belonging to the collection of the Pneumococcal Molecular Epidemiology Network (PMEN). PMEN-16 was identified in a molecular epidemiological survey of penicillin-resistant pneumococci in Poland (Overweg et al., 1999; McGee et al., 2001) and recently it has been recognized as one of the seven multiresistant international clones responsible of the increase in penicillin resistance observed in this country (Sadowy et al., 2006). In this report, the complete nucleotide sequence of pSpnP1, a 5413-bp cryptic plasmid isolated from *S. pneumoniae*, and unrelated to the only previously sequenced pneumococcal plasmid pDP1, was determined. The sequence comparison identified pSpnP1 as a new member of the pC194/pUB110 family of rolling circle replicating plasmids (Khan, 1997).

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

S. pneumoniae Poland^{23F}-16 (Overweg et al., 1999) and other multidrug-resistant pneumococcal clones belonging to the PMEN were kindly provided by R.R. Reinert (National Reference Center for Streptococci, Aachen, Germany). *S. pneumoniae* R6 laboratory strain (Hoskins et al., 2001) was used as host for pSpnP1, pLS1 or other plasmids derived from them. Pneumococcal cells were grown either in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), in C medium (Lacks and Hotchkiss, 1960) supplemented with 0.08% yeast extract (C + Y) or in brain-heart infusion broth (BHI) at 37 °C without shaking. Tetracycline at 1 μg ml^{-1} and spectinomycin at 100 μg ml^{-1} were added to the media when required.

Escherichia coli DH10B (Live Technologies) was used as recipient for pUC18/pUC19 plasmid constructs and preparation of sequencing templates. It was cultivated in Luria–Bertani (LB) broth at 37 °C under agitation or LB broth solidified with 1% (w/v) agar (Sambrook and Russell, 2001). Ampicillin (100 μg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 40 μg ml^{-1}) were used when required.

Lactococcus lactis MG1363 (Gasson, 1983), *B. subtilis* YB886 (Love et al., 1985) and the *E. coli* strains C600 and HB101 (Sambrook and Russell, 2001) were used as hosts for the recombinant plasmid derivative of pSpnP1 (see below). *L. lactis* was grown in M17 medium (Terzaghi and Sandine, 1975) supplemented with 1% glucose at 30 °C without shaking, and *B. subtilis* was grown in LB broth at 37 °C with vigorous shaking. The respective media were solidified by the addition of 1% agar for plating. Spectinomycin was used at final concentrations of 50 μg ml^{-1} for *B. subtilis* and *E. coli*, and of 100 μg ml^{-1} for *L. lactis*.

The pUC18 and pUC19 plasmids (Yanisch-Perron et al., 1985) were used for cloning and sequencing pSpnP1 fragments into *E. coli*. The 4408-bp rolling circle plasmid pLS1 (Lacks et al., 1986) was a gift from M. Espinosa (CSIC, Spain). Plasmid pR412 (Martin et al., 2000) provided by J.P. Claverys (CNRS, France) was used for PCR-amplification of the *add9* gene for spectinomycin resistance, using the oligonucleotide primers 5′-GGTTG GCTGATAAGTCCCCGG-3′ and 5′-CACATAGATG GCG TCGCTAGTA-3′.

2.2. DNA techniques and transformation procedures

General DNA techniques were performed as described elsewhere (Sambrook and Russell, 2001). Plasmid extraction from *S. pneumoniae* was carried out by using the Qiagen® Plasmid Midi Kit following the suppliers' manual with minor modifications. The restriction enzymes *Dra*I, *Hin*dIII and *Pst*I (Boehringer–Mannheim, Germany) were used to generate fragments of the pSpnP1 plasmid. Restriction fragments were purified using the Geneclean II Kit (Bio 101), and cloned into pUC18 or pUC19 using T4 DNA ligase (New England Biolabs, USA). Plasmids of *E. coli* and *B. subtilis* were isolated using the High Pure Plasmid Isolation Kit (Roche