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Complete nucleotide sequence and determination of the replication region of the sporulation inhibiting plasmid p576 from *Bacillus pumilus* NRS576

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Abstract

Large plasmids, presumably replicating via the theta mechanism, have been identified in numerous gram-positive bacteria. However, their characterization is rather poor and predominantly limited to those harbored by some (opportunistic) pathogenic bacteria. Here we determined the DNA sequence of the 43.3 kb plasmid p576 from *Bacillus pumilus* strain NRS576, the first *B. pumilus* theta-replicating plasmid sequenced. Plasmid p576 has a modular structure, but surprisingly, it does not seem to encode a Rep protein found on most theta-replicating plasmids. However, a ~1 kb region was identified showing homology with the Rep-independent replication region of *Bacillus subtilis* plasmid pLS20, and we demonstrated that this region is sufficient for autonomous replication. The plasmid contains various large direct repeat sequences. A likely function could be attributed to at least 15 putative p576 genes. Some of these are predicted to be involved in stable maintenance of the plasmid; others are likely to encode proteins involved in conjugation. p576 also carries a *rap–phr* cassette whose possible function is discussed.

Keywords: *Bacillus pumilus*; Plasmid; Conjugation; DNA replication; Sporulation

1. Introduction

Circular plasmids are commonly present in gram-positive and -negative bacteria. Based on their mode of replication, they can be grouped into two classes. One group replicates via the rolling-circle mechanism and the other via the theta mechanism. Generally, small (<10 kb) and large plasmids replicate via the rolling circle and theta mechanism of replication, respectively (for review see, Meijer et al., 1998). Various large theta-replicating plasmids from gram-negative bacteria have been studied extensively. However, although numerous large plasmids have been identified in gram-positive bacteria, they are rather poorly characterized, with a few exceptions (Titok et al., 2003; Lioy et al., 2010). Many large plasmids carry conjugation genes permitting them to be transferred horizontally, sometimes even to distantly related species. In addition, rolling circle replicating plasmids, which on their own cannot be transferred horizontally, can often be transferred (via the process termed mobilization) when co-resident with a large conjugative theta-type plasmid. Thus, together with bacteriophages and transposons, conjugative plasmids form part of a large prokaryotic mobile gene pool or "mobilome" that, in combination with (homologous) recombination, can enable rapid and profound prokaryotic genomic rearrangements (for review see, Frost et al., 2005; Gogarten and Townsend, 2005). This warrants a better understanding of gram-positive theta-replicating plasmids. Our attention was caught by a publication describing the presence of a naturally occurring 45 kb plasmid, p576, in the *Bacillus pumilus* strain NRS576 (Lovett and Bramucci, 1974). No complete or partial DNA sequence of any *B. pumilus* theta-replicating plasmid is available. In addition, the presence of p576 appears to inhibit sporulation of its host (Lovett, 1973). As a first impetus to improving our knowledge of this gram-positive plasmid, we

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determined its sequence. Analysis of the p576 sequence revealed that it has a modular structure, as generally observed for plasmids. A function could be attributed to at least 15 putative p576 genes. Some of these putative genes are predicted to be involved in stable maintenance of the plasmid. Others, which are located in a putative large operon, are predicted to encode essential conjugation proteins. In addition, we identified a *rap–phr* cassette that might be involved in its associated phenotypic trait to inhibit sporulation. Finally, we determined the minimal replication region and show that it is sufficient to drive replication in *Bacillus subtilis*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were grown in Luria–Bertani (LB) medium or on 1.5% LB agar plates (Sambrook et al., 1989). When appropriate, media or agar plates were supplemented with the following antibiotics: ampicillin (100 μg/ml), erythromycin (1 and 150 μg/ml in *B. subtilis* and *E. coli*, respectively). Insert-containing derivatives of pBluescript KS+ were selected on LB agar plates supplemented with IPTG and Xgal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) to final concentrations of 1 mM and 40 μg/ml, respectively.

2.2. Plasmid p576 isolation and other DNA techniques

Plasmid p576 used for cloning and direct sequencing was isolated by isopycnic cesium chloride ethidium bromide gradients (Sambrook et al., 1989). Other DNA techniques were performed using standard molecular methods (Sambrook et al., 1989).

2.3. Subcloning and sequence determination of plasmid p576

Two different approaches were used to clone p576 fragments in pBluescript KS+ vector. In the first approach, HindIII-digested fragments of p576 were cloned in the corresponding site of pBluescript KS+. Five out of the nine p576 HindIII fragments were cloned. In the second approach, purified p576 DNA was partially digested with Sau3A and then fragments ranging in size between 0.6 and 2 kb were isolated from agarose gels. Next, these fragments were cloned in the pBluescript KS+ vector. To avoid cloning of multiple fragments in the same vector, the following strategy was followed. The p576 Sau3A fragments were treated with Klenow enzyme in the presence of GTP and ATP nucleotides. Similarly, SalI linearized pBluescript was treated with Klenow enzyme in the presence of nucleotides CTP and TTP. Next, a ligation mixture of these partially filled-in fragments was used to transform competent *E. coli* XL1-Blue cells. Finally, plasmid DNA was isolated from 100 white transformants grown on IPTG/Xgal/ampicillin complemented agar plates and the borders of the cloned inserts were sequenced using universal primers M13Rev (5′-CAGGAAACAGCTATGACC-3′) and M13Fw-21 (5′-TGTAAAACGACGGCCAGT-3′). The DNA sequences obtained were then used to design primers (purchased from Isogen Life Science, The Netherlands), which were used in a subsequent round of DNA sequencing using Maxi-Prep purified p576 as template DNA. p576 was then sequenced to completeness in additional rounds by the primer walking strategy. Sequencing was performed by capillary electrophoresis on an ABI PRISM 3730xl equipment (Applied Biosystems) with Big-Dye Terminators v3.1.

2.4. Sequence analysis

DNA sequences were analyzed and assembled using the Lasergene software package from DNASTAR, Inc (Madison, WI, USA). Putative genes were initially identified using the

Table 1
Bacterial strains and plasmids used in this study.

Strains	Properties	Reference/source
B. pumilus		
NRS576	Natural isolate harboring plasmid p576	(Lovett, 1973), BGSC[a]
B. subtilis		
168	*trpC2*	BGSC[a]
E. coli		
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F′[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	(Bullock et al., 1987)
Plasmids		
pBluescript KS+	High-copy *E. coli* cloning vector	Stratagene (USA)
pMTL23ET	pMTL23 derivative containing erythromycin resistance gene from pE194 (cloned in *Cla*I site) and *E. coli rrnB* terminator (cloned in *Eco*RI site)	Laboratory stock
pNRS100E_A/B	pMTL23ET containing 1054 bp minimal replication region from p576 (positions 19975–21028). A and B correspond to different orientations of the insert	This study

[a] BGSC: Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus, OH; USA.