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Homologies among three *Bacillus licheniformis* plasmids and molecular characterization of their replication module

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Abstract

To assess to what extent three *Bacillus licheniformis* plasmids had the same molecular organization a physical map of the 9.34, 8.40 and 7.90 kb plasmids was achieved by using seventeen restriction enzymes. Southern hybridization was performed on plasmids using restriction fragments of the smallest plasmid as probes. Data from different hybridization patterns show a close homology among the three plasmids hypothesizing a similar molecular organization. The lack of plasmid diversity observed, seem to support the hypothesis of a similar phylogeny among these plasmids.

This investigation provides more information concerning phylogeny, interrelationships and level of diversity among *Bacillus* plasmids and a molecular characterization of three plasmids useful for the construction of cloning vectors.
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Introduction

Plasmids are key players in the team of mobile genetic elements that fuel bacterial adaptability and diversity. Therefore, plasmid-encoded genes represent a pool of mobile DNA that contributes significantly to the genetic adaptation of natural microbial communities. Horizontal transfer of genes has a major impact on the adaptability of bacteria, exemplified by the dissemination of antibiotic resistance genes (O'Brien et al., 1985; Belliveau et al., 1991). Other compounds exert different selective pressures in the environment, and plasmids carrying genes for functions such as heavy metal resistance and catabolic pathways are often found (van der Meer et al., 1992; Veal et al., 1992). However, there remains a general lack of information about plasmid distribution, their

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phylogeny and interrelationships, and plasmid diversity in relation to natural selection pressures (Burton et al., 1982; Ogunseitan et al., 1987). Studies in pursuit of such knowledge are needed to understand the role of plasmids in the flow of genetic information in natural bacterial communities. Only one species, *Escherichia coli*, has been surveyed for the full diversity of its plasmids. To understand whether the high degree of diversity found in *E. coli* is typical of this bacterial species, some authors (Zawadzki et al., 1996) have investigated the diversity in naturally occurring plasmids of closely related species of *Bacillus*. They assayed the relatedness among the plasmids and showed that cryptic plasmids from *Bacillus* species are far less diverse than the plasmids from other species. With this in mind the aim of this study is to determine the level of homology among three plasmids of *Bacillus licheniformis* strains isolated from the same sample of pasture-land soil during a previous investigation (Parini et al., 1991). We carried out the present study on three arbitrarily chosen strains, particularly *B. licheniformis* FL5, FL7 and FL11 strains each harboring one plasmid. By Southern hybridization experiments we assessed to what extent these plasmids had the same molecular organization. Moreover, in this report we describe the characterization of the plasmids by construction of restriction maps in order to develop cloning vectors for the application of recombinant DNA technology. At last the replication module of the three plasmid was putatively identified and the sequence of pFL7 module was characterized.

Materials and methods

Bacterial strains

Plasmid investigation was carried out on *B. licheniformis* strains FL5, FL7 and FL11. *E. coli* DH5α was used as the recipient strain for transformation experiments. *B. licheniformis* strains were grown at 45°C in TSB (Tryptic soy broth), while *E. coli* was grown at 37°C in Luria-Bertani (LB) medium. For solid media, 15 g of agar was added per litre of liquid medium.

Isolation of plasmids

Plasmid DNA was isolated according to the procedure described by Ish-Horowicz and Burke (Sambrook et al., 1989) with centrifugation to equilibrium in a CsCl-EtBr density gradient.

Physical analysis of plasmids

Purified plasmid preparations were digested with the following restriction enzymes: *Acc*I, *Ava*I, *Bam*HI, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*II, *Hind*III, *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sph*I, *Xba*I, and *Xho*I. Enzymes, buffers and protocols were supplied by Amersham Pharmacia biotech. All digests were separated by electrophoresis on agarose gels in Tris–acetate buffer. For restriction mapping, the plasmids were subjected to simple, double and triple digestions with several enzymes. Molecular sizes of the plasmid fragments were determined against DNA ladders (Roche Molecular Biochemicals).

Southern hybridization analysis

The restriction fragments obtained from the digestion of plasmid pFL7 with *Bam*HI, *Hind*II and *Hind*III were visualized by agarose gel electrophoresis and excised from the gel using the Nucleospin extraction kit (Macherey-Nagel). The separated fragments obtained were then DIG-dUTP labelled by random priming with a labelling kit (Roche Molecular Biochemicals) and used as probes in hybridization experiments. Plasmid pFL11 was digested by *Hind*III and *Pvu*II, while plasmid pFL5 was restricted by *Acc*I, *Ava*I, *Bam*HI, *Hind*II and *Eco*RI. The restriction fragments were separated by electrophoresis and transferred onto a Hybond-N nylon membrane (Amersham Pharmacia biotech) (Sambrook et al., 1989). Filter hybridization was performed according to the supplier's instructions with pre-hybridization and hybridization steps in 50% (wt/vol) formamide at 42°C and stringency washes in 0.1 × SSC at 68°C.

Molecular cloning, transformation and sequencing

The 1.55 kb *Hind*III-*Hind*II fragment of pFL7 was excised and cloned into the pGEM®-4z vector according to the manufacturer's recommendations (Promega). General cloning techniques were carried out as described by Sambrook et al. (1989). Transformants were selected for blue/white screening on X-gal/IPTG indicator plates containing ampicillin (100 μg ml^{-1}). The sequence of both the strands was determined with dideoxy chain terminator principle (Sanger et al., 1977), using the ABI Prism BigDye™ terminators technology in a ABI Prism™ 310 DNA sequencer (Perkin Elmer). The obtained sequence was analyzed using DNASIS software (Hitachi Software Engineering) for the

presence of open reading frames (ORFs). The predicted products of each ORF were used for similarity searching in protein databases by FASTA (European Bioinformatics Institute) and BLASTp (National Centre for Biotechnology Information).

Results and discussion

The results of this study provide information on the physical characteristics of three plasmids isolated from three different *B. licheniformis* strains. The analysis of the CCC DNA bands by electrophoresis on 0.8% agarose gel revealed the presence of three plasmids of 9.34, 8.40 and 7.90 kb designated pFL5, pFL11 and pFL7, respectively. The restriction enzyme digestion patterns of plasmids pFL5, pFL11 and pFL7 were examined by 17 restriction endonucleases and the number and size of generated fragments are summarized in Table 1.

The sum of the fragment size obtained from a given digestion was always close to the size of the undigested DNA, indicating that all major fragments were detected. The construction of physical maps for the plasmids was achieved by multiple digestions of the plasmids themselves and of isolated fragments: this approach allowed the unambiguous alignment of all restriction fragments (Fig. 1). Comparing the physical maps of these plasmids it was possible to note that several restriction sites were placed in the same position and this fact took us to presume a probable relationship among them. In order to confirm this hypothesis and to show the presence of a real homology among the three plasmid molecules, further investigation was undertaken by hybridization experiments. The smallest plasmid, pFL7, was *Bam*HI-*Hind*II-*Hind*III restricted and the purified fragments of 2.00, 1.55, 1.40, 1.15, 1.00 and 0.80 kb were used separately as probes. In Fig. 2 the results of the hybridization experiments are shown and a close homology between pFL5 and pFL7 is evident. Each probe matches pFL5 with the corresponding region hypothesizing a similar molecular organization of the two plasmids. This very close molecular correlation could presume a common origin from an ancestral plasmid, which by recombinative events diverged, giving origin to distinct entities of different size but with the same molecular organization.

Comparison between pFL7 and pFL11 revealed undoubtedly a high relationship, but two regions of pFL11, the 1.9 kb *Hind*III-*Hind*III and 0.58 kb *Hind*III-*Pvu*II fragments, did not show homology with any of the probes tested. We could also presume that pFL11 is closely related to pFL7 and pFL5 and that probably it has had the same origin, but during its own evolutionary course, more drastic changes occurred. These events result in a molecule with a different region from that represented by the 2.0 kb probe and with an inserted fragment of

Table 1. Numbers and size of fragments generated by different endonuclease digestions of the three plasmids of *Bacillus licheniformis* strains

Restriction enzymes	Size of fragments (kb)		
	pFL5	pFL7	pFL11
*Acc*I	4·74+4·60 (2)	3·21+2·72+1·97 (3)	7·68+0·72 (2)
*Ava*I	4·39+3·28+1·67 (3)	4·66+2·02+1·22 (3)	3·15+2·83+2·42 (3)
*Bam*HI	9·34 (1)	7·90 (1)	8·40 (1)
*Bgl*II	9·34 (1)	7·90 (1)	8·40 (1)
*Cla*I	9·34 (1)	7·90 (1)	8·40 (1)
*Dra*I	6·88+2·06+0·4 (3)	5·35+2·09+0·46 (3)	ND[a]
*Eco*RI	4·48+4·86 (2)	6·77+1·13 (2)	8.40 (1)
*Eco*RV	0	7·90 (1)	0
*Hind*II	4·60+3·94+0·80 (3)	3·55+3·55+0·80 (3)	6·38+1·75+0·27 (3)
*Hind*III	3·15+2·29+2·14+1·12+0·64 (5)	5·20+2·70 (2)	3·73+1·90+1·42+1·35 (4)
*Kpn*I	0	7·90 (1)	0
*Pst*I	5·69+2·33+1·32 (3)	4·44+3·46 (2)	8·40 (1)
*Pvu*I	7·12+2·22 (2)	4·70+3·20 (2)	4·20+3·25+0·95 (3)
*Pvu*II	6·03+2·18+1·13 (3)	5·91+1·99 (2)	5·90+1·69+1·23+0·52 (4)
*Sph*I	0	0	8·40 (1)
*Xba*I	0	7·90 (1)	8·40 (1)
*Xho*I	9·34 (1)	7·90 (1)	0

The number of restriction sites are indicated in brackets.
[a]ND = not determined.

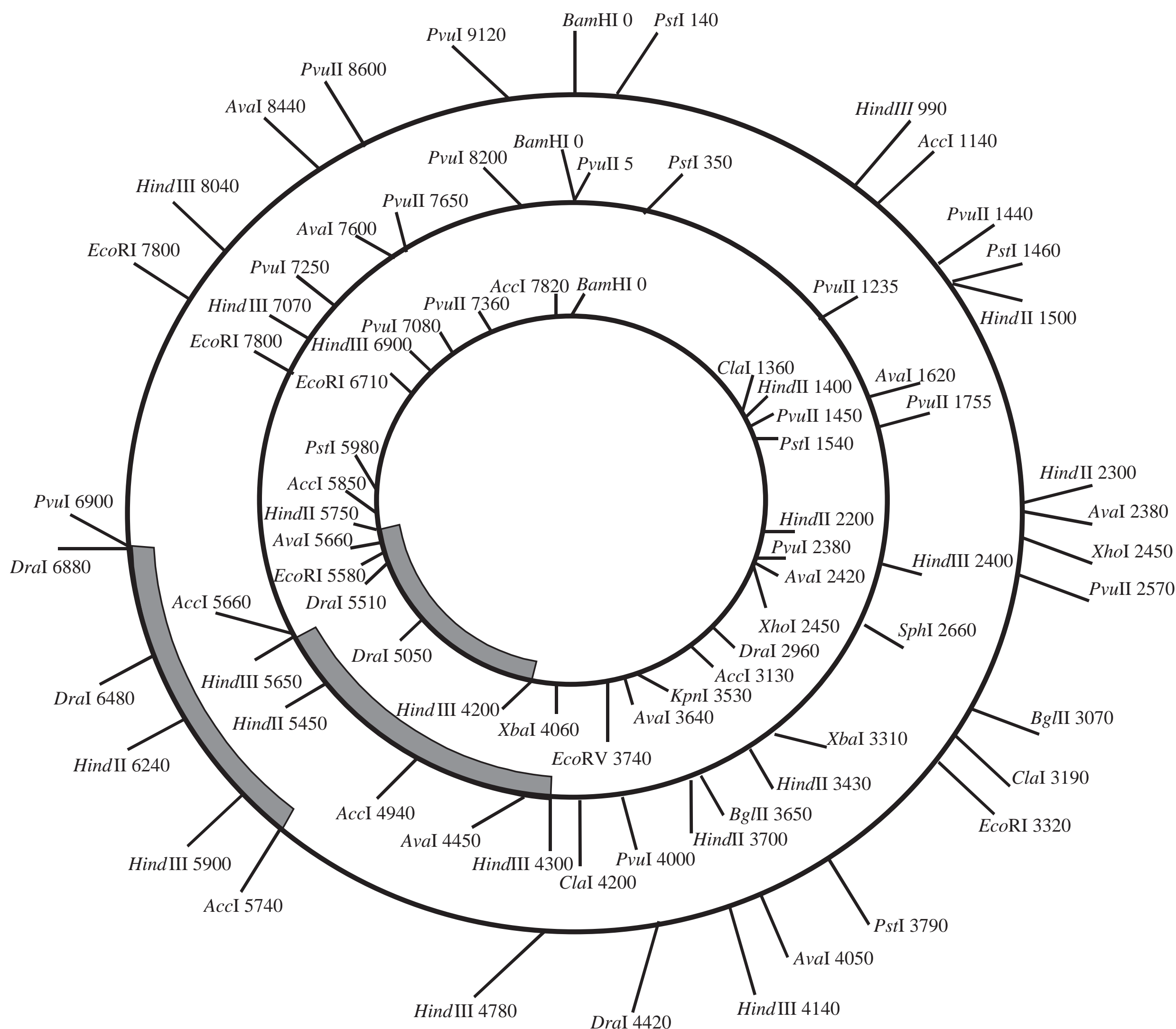

Figure 1. Restriction enzyme cleavage site maps of pFL5 (outer; 9.34 kb), pFL11 (middle; 8.40 kb) and pFL7 (inner; 7.90 kb) of *Bacillus licheniformis* strains. Shaded boxes represent the regions containing the putative replication functions.

0.58 kb between the 1.15 and 1.00 kb probes. To check the molecular organization of these plasmids further hybridizations were carried out using the 2.19 kb *Hind*III-*Pvu*II fragment of pMP3 containing the replicative functions (Parini et al., 1991) as a probe. A strong homology was detected with the 1.55 kb *Hind*III-*Hind*II fragment of pFL7 and with the corresponding 1.35 kb HindIII-HindIII fragment of pFL11. In pFL5 the hybridization signal was detected both with 0.50 kb *Acc*I-*Hind*II and with 1.56 kb *Hind*II-*Eco*RI fragments. In order to better define the region of homology the 1.56 kb *Hind*II-*Eco*RI fragment was further restricted by *Dra*I. As shown in Fig. 1 the regions containing the putative replicative functions are located in the same position and in pFL5 this region is sited in the 0.74 kb *Acc*I-*Dra*I fragment. Similar replicative functions and the same localization on the molecule, would strengthen our hypothesis still further on the common origin of these three plasmids. To confirm that plasmid replicative functions lay in these regions, the 1.55 kb *Hind*III-*Hind*II fragment of pFL7 was cloned and sequenced. *In silico* analysis of the sequence showed the presence of one open reading frame (ORF-FL7) codifying for a putative protein of 340 amino acids (accession number AJ495760) showing an high degree of similarity with the replication protein of plasmid p1414 from *B. subtilis* (85% of aminoacidic identities). A very high similarity was also found with the unique Rep protein from *B. licheniformis* plasmid available, pBL2 (Mason et al., 2002). In fact, the only partially sequenced replication protein of pBL2 has shown only one out of 130 amino acids different from the

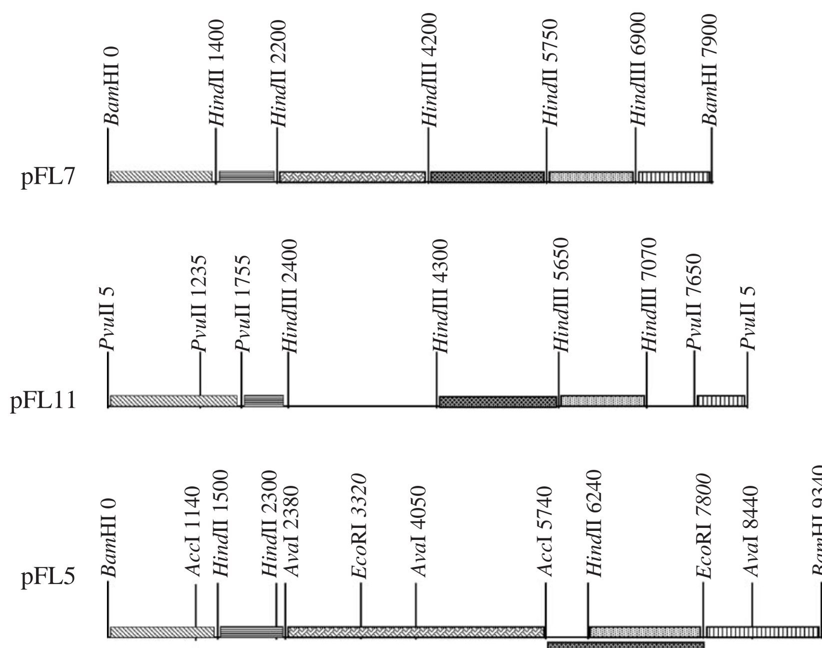

Figure 2. Comparison of *B. licheniformis* plasmids. Blocks of homologies are shown as shaded boxes. Each homology box in the first comparison with pFL7 is shaded differently to facilitate identification of the corresponding homology on the other plasmids.

Table 2. Conserved motives of the RCR plasmids REP proteins

		Motivo IV	**Motivo I**	**Motivo II**	**Motivo III**	Protein database accesion No.
pp	***Consensus*** →	C x x x x C P x C	F u T l T x x x	x p H u H u u u x	u x x Y u x K x x x	
pC194	*Staphylococcus aureus*	C K N R F C P V C	F L T L T T P N	N P H F H V L I A	M A K Y S G K D S B	QQSAEC
pFL7	Bacillus licheniformis	C K V R L C P M C	F L T L T V R N	H P H F H V L L P	I S K Y P V K D T D	AJ495760
pBL2	*Bacillus licheniformis*	Not available	F L T L T V R N	H P H F H V L L P	Not available	CAD22128
p1414	*Bacillus subtilis*	C K V R L C P M C	F L T L T V R N	H P H F H V L L P	I S K Y P V K D T D	NP_049435
pTA1060	*Bacillus subtilis*	C K V R L C P M C	F L T L T I R N	H P H F H V L I P	I S K Y I V K D T D	NP_053786
pTA1040	*Bacillus subtilis*	C K V R L C P M C	F L T L T I K N	H P H F H V L I P	I S K Y I V K D T D	NP_053774
pPL10	*Bacillus pumilus*	C K V R L C P M C	F L T L T V R N	H P H F H V L I P	I S K Y P V K D T D	NP_045751
pPL7065	*Bacillus pumilus*	- - - - - - - M C	F L T L T V K N	H P H F H V L I P	I S K Y P V K D T D	AAO72996
pFTB14	*Bacillus amyloliquefaciens*	C K V R L C P M C	F L T L T V R N	H P H F H V L L P	I S K Y P V K D T D	S01098
pBM14	*Bacillus mojavensis*	Not available	F L T L T V R N	H P H F H V L L P	Not available	CAD22142
			* * * * * *	* * * * * * *		

Motif II includes two hystidines which could be involved in biding of metal cofactor to the protein. Motif III contains a tyrosine that is used to create a covalent phosphotyrosine link betwen DNA and Rep protein and acts in the cleavage of a strand of DNA at the *dso*. Also Motif IV seems to be a metal-binding domain (del Solar et al., 1998).

corresponding internal region of pFL7 putative Rep. In the aminoacidic sequence codified by ORF-FL7, four motifs were moreover found, which are present in the same arrangement in Rep protein encoded by replicons that replicate by a rolling circle mechanism belonging to the family of pC194 from *Staphylococcus aureus* (del Solar et al., 1998) (Table 2).

In rolling circle replicating (RCR) plasmids, the replication module is constituted by the *rep* gene and an origin of replication located just upstream of or within it, called double strand origin (*dso*). Every replication protein can recognize its own *dso*, bind to it and make a cleavage into a specific conserved region inside *dso* called *nic*-site (Novick, 1989; del Solar et al., 1998).

An identical organization was present in pFL7. A region rich in inverted repeat sequences that contains 16 nucleotides identical to those of the *dso nic*-site of pC194 and many related plasmid

Table 3. Rep protein conserved motifs of pC194 family RCR replicons from *Bacillus*

Plasmid	Host	*dso* nic-site	Gene Bank accession No.
		↓	
pC194	*Staphylococcus aureus*	T T T C T T A T C T T G – A T A C	NC_002013
pFL7	***Bacillus licheniformis***	**T T T C T T A T C T T G – A T A C**	**AJ495760**
p1414	*Bacillus subtilis*	T T T C T T A T C T T G – A T A C	NC_002075
pTA1060	*Bacillus subtilis*	T T T C T T A T C T T G – A T A C	NC_001766
pTA1040	*Bacillus subtilis*	T T T C T T A T C T T G – A T A C	NC_001764
pPL10	*Bacillus pumilus*	T T T C T T A T C T T G – A T A C	NC_001858
pPL7065	*Bacillus pumilus*	T T T C T T A T C T T G – A T A C	AY230134
pFTB14	*Bacillus amyloliquefaciens*	T T T C T T A T C T T G – A T A C	X06242
		* * * * * * * * * * * * * * * *	

Double strand origin *nic*-site of RCR replicons from *Bacillus* belonging to pC194 family. The arrow indicates the site of cleavage by the replication protein.

from *Bacillus* species were detected just upstream the suggested *rep* gene (Table 3).

This is the first putative replication module identified and completely sequenced from a *B. licheniformis* plasmid. It shows the same organization and high level of sequence similarity with replication modules identified in plasmids isolated from the species more closely related to *B. licheniformis (B. amyloliquefaciens, B. mojavensis, B. pumilus* and *B. subtilis*).

Our data seem to confirm the low level of diversity found among the RCR plasmids of *B. subtilis* group (Zawadzki et al., 1996; Meijer et al., 1998), that is strongly in contrast with what was seen among *E. coli* plasmids. Previous studies have failed to find any host-benefiting functions of these plasmids and then Zawadzki et al. proposed that they must be considered truly parasitic and suggested therefore that the parasitic lifestyle of these plasmids depresses their diversity (Zawadzki et al., 1996).

Another possible explanation for the extremely low levels of diversity among *Bacillus* plasmids could be found in the nature of their host. *Bacillus* strains can be considered, in fact, "r-strategists", i.e. organisms which rely upon high reproductive rate and tend to prevail in situations which are not resource limited (Atlas and Bartha, 1987). This condition of relatively low selective pressure in their habitat could have led *Bacillus* species and also their plasmids to a slow phylogenetic diversification.

Further investigation are now in progress on other plasmids from *B. licheniformis* strains isolated from soil of different geographical areas to assess this hypothesis. Moreover, the determination of the entire sequence of these plasmids will now be pursued to elucidate gene/function and to develop cloning vectors.

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