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Genomic flank-sequencing of plasposon insertion sites for rapid identification of functional genes ☆

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Abstract

Plasposons are modified mini-Tn*5* transposons for random mutagenesis of Gram-negative bacteria. Their unique design allows for the rescue cloning and sequencing of DNA that flanks insertion sites in plasposon mutants. However, this process can be laborious and time-consuming, as it involves genomic DNA isolation, restriction endonuclease treatment, subsequent religation, transformation of religated DNA into an *Escherichia coli* host, and re-isolation as a plasmid, which is then used as a template in sequencing reactions with primers that read from the plasposon ends into the flanking DNA regions. We describe here a method that produces flanking DNA sequences directly from genomic DNA that is isolated from plasposon mutants. By eliminating the need for rescue cloning, our protocol dramatically reduces time and effort, typically by 2 to 3 working days, as well as costs associated with digestion, ligation, transformation, and plasmid isolation. Furthermore, it allows for a high-throughput automated approach to analysis of the plasposome, i.e. the collective set of plasposon insertion sites in a plasposon mutant library. We have tested the utility of genomic flank-sequencing on three plasposon mutants of the soil bacterium *Collimonas fungivorans* with abolished ability to degrade chitin.
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Keywords: Chitinase; *Collimonas fungivorans*; Genomic sequencing; Mini-Tn*5* transposon; Plasposome; Plasposon

1. Introduction

Mini-Tn*5* transposons (de Lorenzo et al., 1990) are valuable tools for the identification and analysis of functional genes in Gram-negative bacteria. They are stripped-down derivatives of Tn*5*, consisting in their basic form of a selectable gene in between two inverted repeats, usually on a suicide plasmid that also carries a transposase gene. Upon introduction into the host bacterium, the selectable gene, most often coding for an antibiotic resistance, is lost unless a transposition event takes place which involves expression of the transposase gene and stable integration of the DNA fragment in between and including the two inverted repeats into a random site on the host's chromosome. The transposase gene is lost with the suicide plasmid, which minimizes the occurrence of secondary

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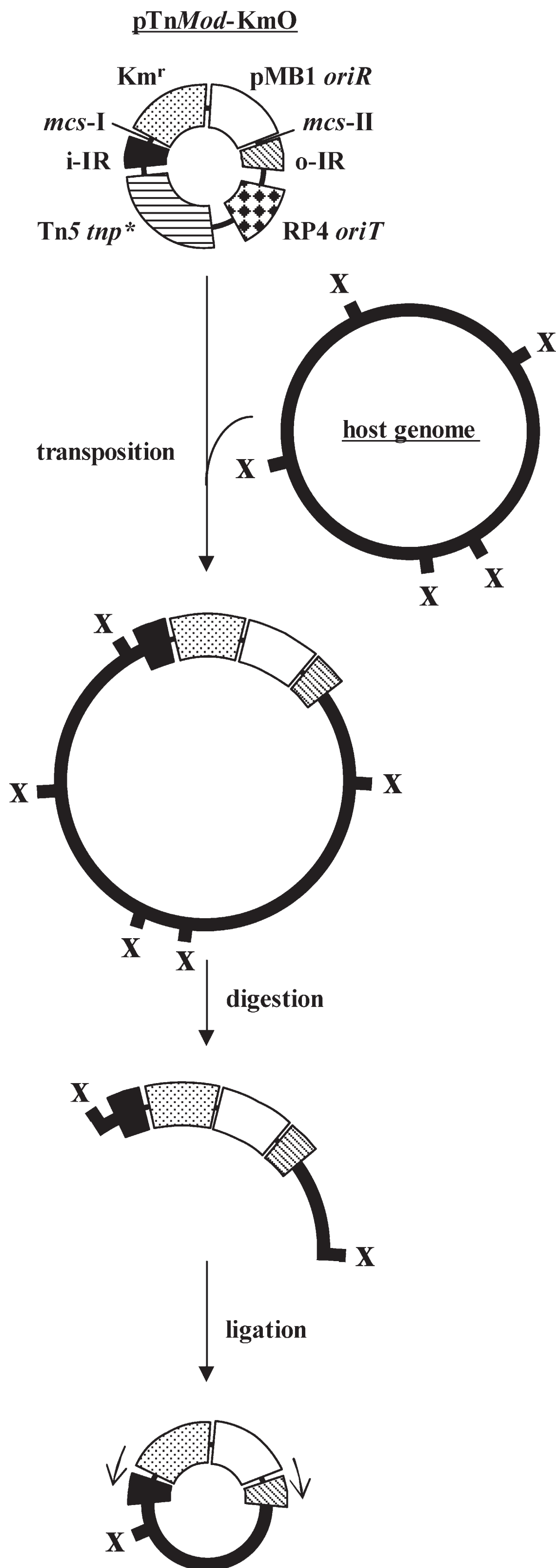

transposition events. The popularity of mini-Tn*5* transposons lies with the great ease and high efficiency of creating large libraries of transposon mutants which can be screened for loss-of-function or gain-of-function phenotypes.

To determine the site of insertion in mutants with altered phenotype, several methods are available to obtain the DNA sequences flanking the mini-Tn*5* transposon insertion site. The transposon itself can be used as a probe in a Southern blot analysis on digested genomic DNA from the mutant strain, followed by cloning, subcloning, and sequencing of the hybridizing fragment. Alternatively, the genomic DNA is digested with an enzyme that does not cut in the transposon, followed by religation and an inverse PCR using primers that target the plasposon ends and face outward from the transposon. The resulting amplicon can then be cloned and sequenced. Another PCR-based method involves a two-round PCR reaction on genomic DNA using primers specific to the end of the transposon and arbitrary primers, followed by DNA sequencing of the resulting PCR amplicon (e.g. O'Toole and Kolter, 1998). All of these methods can be quite laborious and time-consuming, especially if there are several mutants to be analyzed.

Fig. 1. Principle of plasposon technology. Depicted is pTn*Mod*–KmO, a typical plasposon (Dennis and Zylstra, 1998). It harbors several modules (not to scale): a kanamycin resistance gene (Km^r), an origin of replication (pMB1 *oriR*), an origin of transfer (RP4 *oriT*) which allows delivery to the host by conjugation, a transposase gene (Tn*5 tnp*∗), inside/outside inverted repeats *i*-IR and *o*-IR, and two multiple cloning sites (*mcs*-I and -II). The pMB1 *oriR* origin is pUC-derived and allows for replication in *E. coli* hosts such as DH5α and JM109. The original plasposon collection includes derivatives with different origins of replication (e.g. pSC101 or R6K; the latter allows replication only in *E. coli* hosts expressing the π protein, e.g. CC118 λpir) or with genes conferring resistance to other types of antibiotics (e.g. chloramphenicol, streptomycin, gentamycin, tetracycline, and trimethoprim). The plasposon is delivered to the target by electrotransformation or by conjugation using a helper strain such as *E. coli* HB101 (pRK2013). Once inside the target, Tn*5 tnp*∗ gene activity invokes transposition of the DNA fragment between and including the inverted repeats on the plasposon to the host genome. Since the introduced plasposon cannot replicate in the target, acquisition of antibiotic resistance by the host can be used to screen for this integration event. To analyze the DNA that flanks the plasposon insertion site in a mutant strain, the original protocol calls for isolation of genomic DNA and incubation with a restriction enzyme (X) that does not cut in the transposed part of the plasposon. The digested chromosomal DNA is then religated to produce a circular, self-replicating entity conferring antibiotic resistance which is electrotransformed into an appropriate *E. coli* host, and re-isolated as a plasmid for use as template DNA in a sequencing reaction with primers (shown as arrows) that are designed against the ends of the plasposon and facing outwards. The protocol described in this paper allows for sequencing the DNA that flanks plasposon insertion sites using the same primers but with genomic DNA as template instead.

Plasposons (Dennis and Zylstra, 1998) are derivatives of mini-Tn*5* transposons that were designed to facilitate the process of retrieving DNA that flanks transposon insertion sites. They contain a conditional origin of replication in between the inverted repeats in addition to a selectable marker gene (Fig. 1). The basic protocol is based on rescue cloning which involves digestion of total genomic DNA, self-ligation, and transformation into a permissive *Escherichia coli* host (Dennis and Zylstra, 1998). Subsequently, the plasposon can be isolated and used in a sequence reaction with primers facing outward from the plasposon (Fig. 1).

Plasposons have been used with great success in the generation of mutant libraries of several α-, β-, and γ-Proteobacteria (Table 1). Since the original publication by Dennis and Zylstra, several derivatives of the original plasposons have become available and been used successfully (Bolton and Woods, 2000; Burtnick et al., 2001; Husken et al., 2001; Widada et al., 2001; Larsen et al., 2002; van Diepeningen, 2002; Chang et al., 2003; Kholti et al., 2003; Hunt et al., 2004; Maxson and Darwin, 2004; Enos-Berlage et al., 2005; Leveau and Lindow, 2005; Venecia and Young, 2005). Modifications include a hyperactive transposase gene for increased transposition efficiency (Larsen et al., 2002; Enos-Berlage et al., 2005) and the incorporation of a promoterless reporter gene directly downstream one of the inverted repeats for measuring the expression of plasposon-affected gene(s) (Bolton and Woods, 2000; van Diepeningen, 2002; Enos-Berlage et al., 2005; Venecia and Young, 2005).

In all studies listed in Table 1, the original protocol of rescue cloning was used to determine into what DNA locus plasposons had inserted. While it is true that plasposons in comparison to the original mini-Tn*5* transposons greatly facilitate the characterization of insertion sites, they still have some disadvantages. First is the considerable amount of time and effort to obtain the desired result: starting with isolated genomic DNA, it takes only 1 to 2 h for the digestion and 1 to 16 h to religate, but it takes at least two over-nights to complete the transformation and plasmid isolation steps, making this a two- or three-day protocol. A second disadvantage is that the method is not amenable for high-throughput. With the protocol described above, it is impractical for one person to handle more than a dozen samples per day. Also, due to the need for a transformation step, the protocol cannot be automated easily. A final drawback relates to the digestion of the genomic DNA: if a plasposon integrates into a very small restriction fragment, only a small portion of the flanking DNA sequence can be retrieved and read, whereas if integration occurs into a very large restriction fragment, transformation into *E. coli* may become problematic. Either problem can be anticipated by performing parallel digestions of the same genomic DNA with different restriction enzymes. However, this means an increase in the work load per plasposon mutant strain to be analyzed.

We describe here a one-step alternative for the original protocol of rescue cloning to retrieve DNA sequences flanking plasposon insertion sites. Our method is based on the demonstrated ability to use bacterial genomic DNA as a template in a sequencing reaction (Heiner et al., 1998). Thus, with sequencing primers that target the ends of a plasposon and face outward, one can obtain DNA sequences flanking the insertion site directly from genomic DNA. This eliminates the need for digestion, religation, transformation, and re-isolation, which reduces the handling time per mutant by 2 to 3 working days. As a proof-of-principle, we analyzed the insertion sites in three plasposon mutants of the chitinolytic bacterium *Collimonas fungivorans* Ter331 by genomic flank-sequencing, and compared the results with those obtained using the method of rescue cloning.

2. Materials and methods

C. fungivorans Ter331 has been described elsewhere (de Boer et al., 2004). A spontaneous rifampicin-resistant mutant of Ter331 (*C. fungivorans* Ter331R3) was used as a recipient in conjugation with donor strain *E. coli* (pTn*Mod*–KmO*lacZ*) and helper strain *E. coli* (pRK2013) (Figurski and Helinski, 1979). Plasposon pTn*Mod*–KmO*lacZ* (van Diepeningen, 2002) is a derivative of pTn*Mod*–KmO (Dennis and Zylstra, 1998) (Fig. 1), modified to contain a promoterless *lacZ* gene inserted into the *Not*I site of multiple cloning site *mcs*-II, with its 5′-end proximal to the outside inverted repeat. Rifampicin- and kanamycin-resistant Ter331R3 transconjugants were isolated and screened on chitin/yeast agar plates (de Boer et al., 2004) containing per liter 1 g KH_2PO_4, 5 g NaCl, 2 g colloidal chitin, 0.1 g yeast extract, 20 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 20 g agar. Genomic DNA was isolated from selected mutants using an Ultra Clean Soil DNA Kit (Mobio, Carlsbad, CA) or a Blood and Cell Culture DNA Kit (Qiagen, Hilden, Germany), and digested with *Pst*I (Amersham Life Sciences, Piscataway, NJ) for Southern blot analysis using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland). The hybridization probe was a 2.1-kb *Xba*I fragment of pTn*Mod*–KmO*lacZ*, containing the KmO cassette. The *Pst*I-digested chromosomal DNA of mutants 17H10, 18D9, and 23B7 was religated with T4 DNA ligase (New England Biolabs, Beverly, MA) and used to

Table 1
Plasposon technology applications

Plasposon	Organism	Mutant screening	Restriction enzyme(s)	Sequencing primer(s) (5′→3′)	Reference
pTn*Mod*–KmO	*Burkholderia cepacia* DBO1	N/a; plasposon proof-of-principle	*Bam*HI, *Pst*I	TTCCCGTTGAATATGGC (JD48), ACGCTCAGTGGAACG (JD45)	Dennis and Zylstra, 1998; Lutter et al., 2001
pTn*Mod*–KmO	*Pseudomonas putida* S12	Octanol tolerance; chlorampenicol resistance	*Bam*HI, *Pst*I, *Sst*I	JD48, JD45	Kieboom et al., 2001; Kieboom and de Bont, 2001
pTn*Mod*–KmO	*B. cepacia* IST408	Exopolysaccharide production	*Eco*RI	TTCCCGTTGAATATGGC (50KMR), CCTTTTTACGGTTCCTGGCCT (ORIR)	Moreira et al., 2003
pTn*Mod*–OKm	*Pseudomonas fluorescens* BF13	Growth on ferulic acid or vanillic acid	*Bam*HI, *Eco*RI, *Pst*I	Not applicable; subclone strategy	Civolani et al., 2000
pTn*Mod*–OKm	P. *fluorescens* NCIMB 10586	Mupirocin production	*Bam*HI	Not specified	Rangaswamy et al., 2002
pTn*Mod*–OKm	*Pseudomonas chlororaphis*	Production of *N*-acyl homoserine lactone and phenazine-1-carboxamide	*Pst*I, *Sal*I	GCCTTTTGCTCACATGTTCTTTCC (Ori5seq), CCCCGAGCTCTTAATTAATTTAAATC (Ori3seq)	Schmidt-Eisenlohr et al., 2003
pTn*Mod*–OKm	*Thiocapsa roseopersicina* BBS	Pigmentation	*Bam*HI, *Kpn*I, *Xba*I	Not specified	Kovacs et al., 2003
pTn*Mod*–OKm	*P. putida* RU-KM3_S	Hydantoin as nitrogen source	*Bam*HI, *Csp*451, *Pst*I	TTTACGGTTCCTGGCCTTT (prGM11), TGAGACACAACGTGGCTTTC (prGM12)	Matcher et al., 2004
pTn*Mod*–OKm	*Sphingomonas paucimobilis* UT26	Degradation of γ-hexachlorocyclohexane	*Bam*HI, *Nco*I	GCTGGCCTTTTGCTCAC, TTGAGACACAACGTGGC	Endo et al., 2005
pTn*Mod*–OKm′	*Agrobacterium tumefaciens* C58	Biofilm formation	*Bam*HI	Not specified	Ramey et al., 2004
pTn*Mod*–OKm′	*Pseudomonas* sp. BW11M1	Bacteriocin production	*Pst*I	TCTGGCTGGATGATGGGGCG (pseu-596), CGGTTCCTGGCCTTTTGCTGG (pseu-597)C	Parret et al., 2003; Estrada de los Santos et al., 2005
pTn*Mod*–OTc	*Burkholderia vietnamiensis* CEP040	Sensitivity to polymyxin B; secretion deficiency	*Bgl*II, *Sal*I	Not specified	Fehlner-Gardiner et al., 2002; Fehlner-Gardiner and Valvano, 2002
pTn*Mod*–OTc	*P. fluorescens* ATCC 17400	Haemin auxotrophy	Not specified	Not specified	Baysse et al., 2001; Baysse et al., 2003
pTn*Mod*–RTp′	*Burkholderia multivorans* ATCC 17616	Auxotrophy	*Bam*HI, *Eco*RI, *Sph*I	TTGAACGTGTGGCCTAAGCGAGC (Tntpow2)	Komatsu et al., 2003
pTn*Mod*–RTp’	*Burkholderia cenocepacia* K56-2	Plant tissue water soaking	*PstI*	GGTACCGTCGACATGCATGG (RTp1), CAGTGCAAATTTATCCTGTG (RTp2)	Engledow et al., 2004
pTn*Mod*–RKm	*Hyphomicrobium chloromethanicum*	Growth on chloromethane	*Sph*I	Not specified	Borodina et al., 2004
pTn*Mod*–RKm′	*Yersinia enterocolitica* JB580v, *Y. enterocolitica* pGY100	Phospholipase activity	*Eco*RI	CCCCGAGCTCTTAATTAA (KM1), GAACACTTAACGGCTGAC (KM2)	Petersen and Young, 2002; Young and Young, 2002

electrotransform *E. coli* EP-Max 10B competent cells (Bio-Rad, Hercules, CA) with a Gene Pulser Xcell Microbial System (Bio-Rad) according to the manufacturer's instructions. From the resulting transformants, plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen), then digested with *Pst*I and analyzed on agarose gel. Plasmid DNAs were also used as template DNA in a sequencing reaction using primer LAC (5′- CG ACGGCCAGTGAATTCCC -3′) or PP1 (5′- TGCA TGGCGCGCCGGCGA -3′). LAC is reverse complementary to the 5′ end of *lacZ*, while PP1 targets multiple cloning site *mcs*-I facing away from the kanamycin resistance gene. The same primers were used to directly sequence on genomic DNA isolated from plasposon mutants by the Blood and Cell Culture DNA Kit (Qiagen). Primer PP1 consistently failed to produce good-quality sequences on genomic DNA template. We therefore designed and used a partially overlapping primer, PP1-31 (5′- GTGGC CAGATCTGATCAAGAG -3′), which gave good results. Primer M13for(-47) (5′- CGCCAGGGTTTTCCCAGT CACGAC -3′) was tried as an alternative to LAC and it produced sequences of comparable quality. BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA) was used to sequence 2 to 3 μg of genomic DNA with 10 pmol primer in a total volume of 30 μl. An initial denaturation step at 95 °C for 4 min was followed by 50 cycles of denaturation (30 s at 95 °C), annealing (1 min at 55 °C), and extension (2 min at 60 °C). Sequencing reactions were purified from excess primer and nucleotides by Sephadex columns (Sigma-Aldrich, St. Louis, MO) and run on an AB3730 automated sequencer (Applied Biosystems). Sequence quality was assessed by Phred analysis software (CodonCode Corp., Dedham, MA) and by visual inspection of the chromatograms. DNA sequences were analyzed using Lasergene software (DNASTAR, Madison, WI). Promoter searches were performed using Softberry's BPROM (www.softberry.com). BLAST searches were done at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

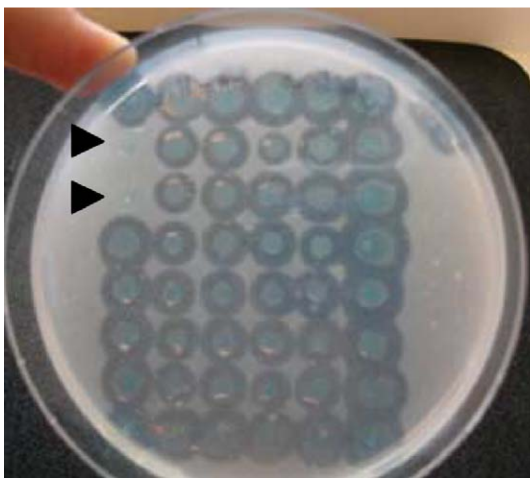

Fig. 2. Chitin–agar plate with 48 clones of the *C. fungivorans* Ter331::Tn*Mod*–KmO*lacZ* plasposon mutant library. Two clones (indicated by the black arrowheads) did not produce a cleared zone on the colloidal chitin surrounding the colony and were scored negative for chitinolytic activity.

3. Results

3.1. Plasposon library construction and screening

Plasposon pTn*Mod*–KmO*lacZ* was used to construct a mutant library of the chitinolytic bacterium *C. fungivorans* Ter331. In total, 2016 mutants were isolated and screened for their ability to clear colloidal chitin on chitin–agar plates (Fig. 2). Three mutants (17H10, 18D9, and 23B7) showed completely abolished chitinolytic activity (Fig. 2) and were chosen for further study. Southern blot analysis of *Pst*I-digested genomic DNA revealed a single insertion of the KmO*lacZ* cassette into the chromosome of each of these mutants (not shown). The sizes of the hybridizing *Pst*I fragments were different (i.e. >10, 8.5, and 5.9 kb, respectively) suggesting that the site of insertion was different for each mutant strain. Given that the KmO*lacZ* cassette is approximately 5.2 kb, we predicted the sizes of *Pst*I fragments into which the plasposon had inserted in mutants 17H10, 18D9, and 23B7 to be >4.8, 3.3, and 0.7 kb, respectively.

3.2. Rescue cloning and sequencing of plasposon insertion sites

Inserted KmO*lacZ* cassettes and their flanking sequences were recovered from all three mutants, as described in Materials and Methods, by religation of *Pst*I-digested genomic DNA, transformation into *E. coli*, and re-isolation as plasmids pTn*Mod*–KmO*lacZ*–17H10*Pst*I,–18D9*Pst*I, and –23B7*Pst*I, respectively. Restriction analysis of these plasmids revealed linear *Pst*I fragments with expected sizes of >10, 8.5, and 5.9 kb, respectively (not shown). Plasmids pTn*Mod*–KmO*lacZ*–17H10*Pst*I, –18D9*Pst*I, and –23B7*Pst*I were each used as template DNA in two separate sequencing reactions (one with primer LAC, the other with primer PP1; see Materials and Methods) in order to obtain DNA sequences flanking either side of the KmO*lacZ* cassette. For each plasmid, the two resulting sequences were joined to reconstruct the original DNA sequence as it occurs on the genome sequence of wild-type *C. fungivorans* Ter331. The results are summarized in Fig. 3.

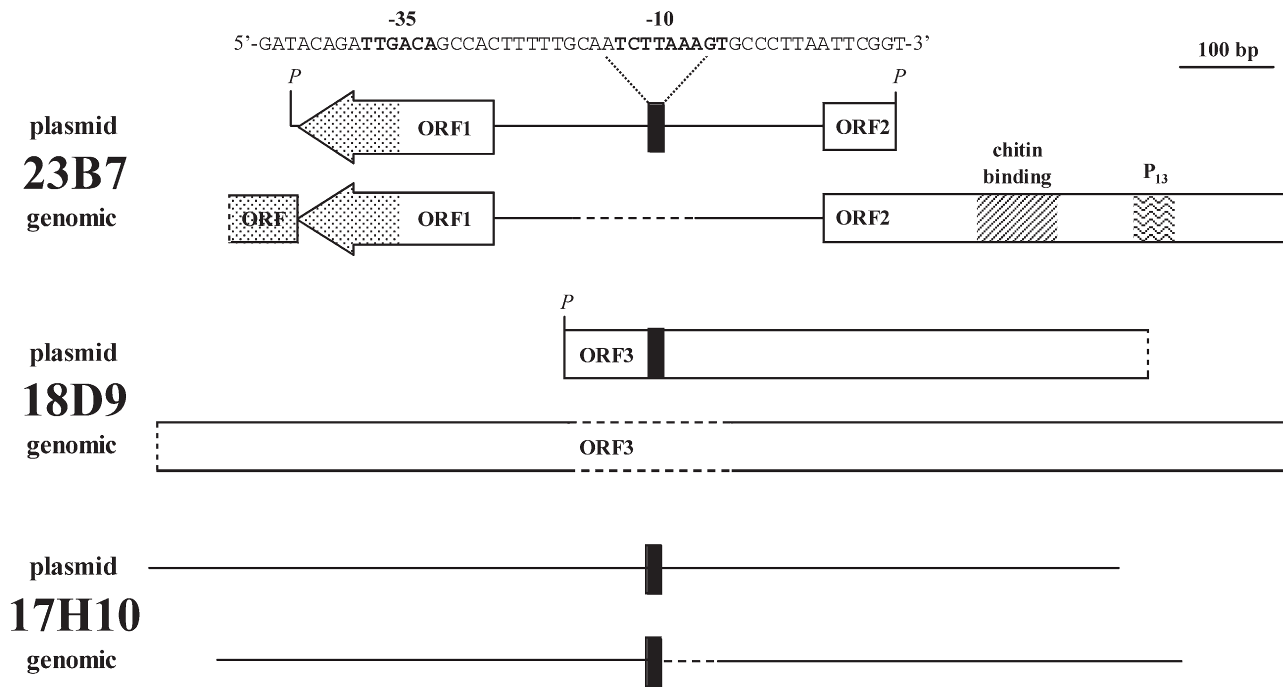

Fig. 3. Characterization of the plasposon insertion sites of chitinolytic mutants 23B7, 18D9, and 17H10 by DNA flank-sequencing on plasmids pTn*Mod*–KmO*lacZ*–23B7*Pst*I, –18D9*Pst*I, or –17H10*Pst*I, and on genomic DNA isolated from each of the mutants. The position of plasposon insertion sites is indicated by a black bar. Open reading frames are shown, as well as the position and sequence of the promoter region upstream of ORF2. Also shown are the location of the chitin-binding domain and proline-stretch in ORF2, as well as the region (grey area) in ORF1 and downstream ORF with homology to amino acid transporters. Broken lines represent regions for which no sequence data could be retrieved. *P*, *Pst*I restriction site. See text for further details.

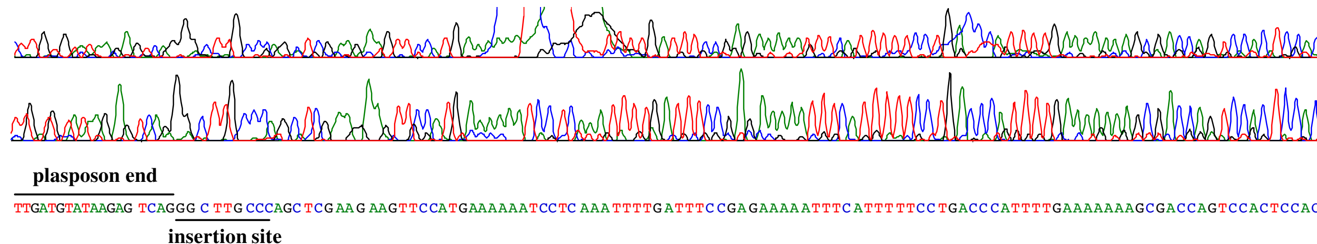

Fig. 4. Representative trace files obtained with sequencing primer LAC on genomic DNA from mutant 17H10 (top) or on plasmid pTn*Mod*–KmO*lacZ*–17H10*Pst*I (bottom). The corresponding DNA sequence is presented at the bottom. Indicated are the end of the plasposon and the site of insertion (GGCTTGCCC). Note that the peaks were lower for genomic DNA than for plasmid DNA. The anomaly in the genomic DNA sequence around positions 50–65 was probably due to residual dye terminator.

From pTn*Mod*-KmO*lacZ*-23B7*Pst*I, we recovered the nucleotide sequence of a 615-bp *Pst*I fragment, which is close to the size we had predicted (i.e. 0.7 kb). Using BLASTX, we identified a stretch of approximately 100 bp that upon translation showed significant homology (up to 72% identity) to predicted amino acid transporters from *Ralstonia*, *Burkholderia*, and other bacterial species. This region of homology was part of a small (201-bp) open reading frame, ORF1 (Fig. 3). We identified the start of a second open reading frame (ORF2) at the other end of the fragment, but the predicted translation product (26 amino acids) did not produce significant homology to proteins in the database. Interestingly, upstream of this ORF2, we located a putative promoter sequence (Fig. 3), the -10 region of which overlapped with the insertion site of the plasposon. This suggests to us that this promoter and the gene(s) it controls have an active role in the chitinolytic activity of *C. fungivorans* Ter331. Furthermore, in between the −35 and −10 regions of this promoter, Softberry software identified a putative binding site for the cAMP response protein CRP (Brown and Callan, 2004). Plasmid pTn*Mod*–KmO*lacZ*–18D9*Pst*I produced a 582-bp DNA sequence that was flanked on one side by a *Pst*I recognition site. A putative translation product over the entire length of this fragment (ORF3; 194 amino acids) showed a high degree of homology (up to 61% identical) to predicted periplasmic sugar-binding proteins of the ABC-type sugar transport system from several α-Proteobacteria and from *Vibrio* species *vulnificus* and *cholerae*. Much to our surprise, we did not find significant BLASTX or BLASTN hits (*E*-value <1) with the 1030-bp DNA sequence from plasmid pTn*Mod*–KmO*lacZ*–17H10*Pst*I. A remarkable feature of this DNA sequence was its G+C content (45%), which was much lower than that of the sequenced DNAs from mutants 18D9 (58%) or 23B7 (55%), and which was characterized by the presence of (up to 16 bp) A/T stretches (e.g. TTTTTTTATTTTTTTT).

3.3. Genomic flank-sequencing of plasposon insertions

The results from plasmids pTn*Mod*–KmO*lacZ*–17H10*Pst*I, –18D9*Pst*I, and –23B7*Pst*I were compared to those obtained from sequencing reactions using genomic DNA from mutant strains 17H10, 18D9, and 23B7. Applying the protocol described in the Materials and Methods section, we were able to produce high quality sequences (Fig. 4), with a Phred score of 20 for an average of 434 bp, compared to an average of 794 bp for plasmid DNA. Most sequencing reactions on genomic DNA did not allow us to read the DNA sequence immediately flanking the plasposon end, resulting in a gap in the reconstructed sequence (Fig. 3). On the other hand, more DNA information could be gathered with genomic DNA flank-sequencing due to the fact that *Pst*I sites were not endpoints for DNA sequence retrieval as they were for religated plasmids pTn*Mod*–KmO*lacZ*–17H10*Pst*I, –18D9*Pst*I, and –23B7*Pst*I. The extra sequence information allowed us to identify an additional small ORF downstream of ORF1, with homology to amino acid transporters from *Ralstonia*, *Burkholderia*, and other bacterial species, as well as an extension of ORF2 with 136 amino acids (Fig. 3). Interestingly, the extended ORF2 (162 aa) showed significant homology (57% identical) to chitinase pCA8 ORF from *Aeromonas* sp. 10S-24 (Ueda et al., 1998). Chitinase pCA8 ORF has two chitin-binding domains of type 3 at its N-terminal end. We identified one such domain (AWVASTAYTGGATVSYNGVNYKANWWTQGNNPSTNNGGSGTGQPWTI) at the N-terminal end of the ORF2 translation product. This portion of the predicted protein showed extensive homology to corresponding domains in chitinases from *Janthinobacterium lividum*, *Streptomyces*, *Burkholderia* and other bacterial species. Directly downstream of the chitin-binding domain of ORF2 we identified a region consisting of 13 proline residues in a row (P_{13}) which corresponds to a proline–threonine-rich region in pCA8 ORF linking the binding and catalytic domains. The region of ORF2 downstream of P_{13} was too short to be able to identify catalytic domain signatures typical for chitinases. Genomic flank-sequencing on DNA from mutant 18D9 also produced more DNA information, but mostly confirmed our earlier observation that the plasposon was inserted into a gene putatively coding for a periplasmic sugar-binding protein. No significant additional DNA information was obtained with genomic sequencing of mutant 17H10.

4. Discussion

Genomic flank-sequencing of plasposon insertion sites is a novel and powerful technique for the analysis of plasposon mutants. It is not necessarily meant to replace the original method of rescue cloning, because the Dennis and Zylstra protocol of digestion, religation, transformation and plasmid isolation (Dennis and Zylstra, 1998) still has the advantage that at the end of the procedure, in addition to a DNA sequence, one has in hand a DNA fragment that can be used for several follow-up purposes, e.g. for (sub)cloning or for use as a probe in Southern blotting. However, compared to the rescue cloning protocol, the direct sequencing method has several benefits, including a significant reduction in time and effort. One major advantage is high-throughput capability. The whole process – i.e. growing plasposon mutants, isolating

genomic DNA, and DNA sequencing – can be automated and performed in less than 24 h. This opens up for the first time the possibility to rapidly and cost-efficiently access and describe the plasposome, i.e. the collective set of plasposon insertion sites in a mutant library. The availability of plasposome sequence data greatly accelerates the interpretation of library screenings for plasposon mutants with altered phenotypes. Plasposome data can be used as a query to search databanks for DNA or protein homologies, and any locus of interest identified in this way can be instantly subjected to experimental study due to the availability of a plasposon mutant that is affected in that locus. This would be particularly useful in the study of bacteria for which a (partial) genome sequence is already available, because only little DNA sequence is needed to map a plasposon insertion onto the genome sequence.

The principle of genomic flank-sequencing is applicable to all types of plasposons and their derivatives, not just the plasposon used in this study, pTn*Mod*–KmO*lacZ*. Table 1 is a starting point for those interested in using plasposons and in analyzing plasposon mutants by genomic flank-sequencing. Indicated are not only combinations of plasposons and bacterial species that have already been shown to work successfully, but also the primers that were used in each of those studies to obtain flanking sequences from rescued plasmids. In principle, these primers should work for genomic flank-sequencing. However, we (see Materials and Methods) and others (Heiner et al., 1998) have noticed that not all primers work well on genomic DNA, and some testing and optimization may be necessary. Surprisingly, there seems to be no consensus among plasposon users on the primers that are best used for sequencing, despite the fact that most plasposons share a common backbone. While primers LAC and M13for(−47) are specific for p*Tn*Mod–KmO*lacZ*, primer PP1-31 targets multiple cloning site I and can therefore be used for genomic flank-sequencing of any of the plasposons described in the original Dennis and Zylstra publication, as well as many of their derivatives.

Few other studies have combined transposon mutagenesis with genomic flank-sequencing of insertion sites (Hoffman et al., 2000; Horecka and Jigami, 2000; Ruffin et al., 2000; Lee et al., 2003). This seems rather surprising given the real potential of genomic flank-sequencing of transposon insertion sites to contribute to the genome-wide characterization of transposon mutants (Hayes, 2003). For several completely sequenced bacteria, transposon mutant libraries are available and have proven to be important resources for the characterization of known or unknown genes and for the identification of essential genes (Hutchison et al., 1999; Geoffroy et al., 2003; Jacobs et al., 2003; Salama et al., 2004; Lewenza et al., 2005). For locating transposon insertion sites on the genome, methods other than genomic flank-sequencing were used in each case. They included direct sequencing of amplicons from a two-stage semidegenerate PCR on glycerol stocks (Jacobs et al., 2003), sequencing of (cloned) amplicons obtained by inverse PCR on cut and religated genomic DNA (Hutchison et al., 1999; Lewenza et al., 2005), sequencing of amplicons obtained by ligation-mediated PCR on genomic DNA (Geoffroy et al., 2003), and microarray-based mapping (Salama et al., 2004). It seems to us that direct genomic flank-sequencing would have been a cost-effective alternative in each case.

Plasposon technology has a proven record of being a reliable and efficient tool for the generation of insertion mutants, at least in α-, β-, and γ-Proteobacteria (Table 1). A vast number of other (mini)transposons is available (Haas et al., 1993; Goryshin et al., 2000; Rossignol et al., 2001; Hayes, 2003). Epicentre Biotechnologies even offers a kit with EZ-Tn5™ Transposome™ complexes for transposon mutagenesis of a wide range of microorganisms, including those for which transposon delivery by conjugation is not possible or inefficient. The choice of transposon will ultimately lie with the potential user, who initially will have to rely on literature to decide what technology best to use. It is unfortunate in this respect that negative experiences with transposons are usually not published. In one notable exception (Estrada de los Santos et al., 2005), it was recorded that a plasposon, pTn*Mod*–OKm', was preferred over EZ-Tn5™ Transposomes™ because of low transposition efficiency of the latter.

Plasposon mutagenesis has brought us closer to understanding the chitinolytic properties of *C. fungivorans* Ter331. The fact that we found three distinct mutants with completely abolished ability to produce halos on chitin agar plates indicates that *C. fungivorans* Ter331 possesses at least three loci that are absolutely required for chitinase expression. The putative chitinase gene that we identified in mutant 23B7 is our prime candidate responsible for the hydrolysis of chitin on agar plates. Interestingly, the presence of a CRP binding site in the promoter region of this gene would suggest that its expression is subject to catabolite repression. This fits well with an earlier observation that *C. fungivorans* Ter331 does not produce halos on chitin agar containing glucose or tryptic soy broth (de Boer et al., 2004). The translation product of the gene knocked out in mutant 18D9 showed amino-acid identity of up to 57% to bacterial periplasmic sugar-binding proteins of the ABC-type sugar transport system. Interestingly, the

corresponding gene for one of these proteins, found in *Jannaschia* sp. CCS1 (accession number EAM65674), is linked to genes that are predicted to code for a sugar isomerase and a *N*-acetylglucosamine kinase. Since chitin is a polymer of *N*-acetyl-glucosamine, we are now keen on testing the hypothesis that this aminosugar plays a role in the regulation of chitinolytic activity of *C. fungivorans* Ter331, as suggested by the inability of mutant 18D9 to hydrolyze chitin. As for mutant 17H10, the DNA sequences from both genome and rescued plasmid did not provide us with clues to explain the inability of this mutant to form halos on chitin plates. The high A+T content of the region into which the plasposon inserted may indicate the presence of regulatory sequences, such as UP elements required for efficient promoter activity (Gourse et al., 2000), but this remains speculative without additional DNA sequence information. We are currently in the process of retrieving DNA sequences further up- and downstream of the plasposon insertion site in this mutant, and are doing the same for mutants 23B7 and 18D9. In light of the results presented here, the most obvious tactic is a primer-walking approach using the protocol of direct genomic sequencing described in this study.

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