Group II intron from *Pseudomonas alcaligenes* NCIB 9867 (P25X): entrapment in plasmid RP4 and sequence analysis

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***Pseudomonas alcaligenes* NCIB 9867 (strain P25X), which grows on 2,5-xyleneol and harbours the plasmid RP4, was mated with a plasmid-free derivative of *Pseudomonas putida* NCIB 9869, strain RA713, which cannot grow on 2,5-xyleneol. Some RA713 transconjugants, initially selected on 2,5-xyleneol, were found to carry RP4 plasmids that had acquired additional fragments (designated XIn) which ranged in size from 2 kb to approximately 26 kb. Instability of DNA inserts in RP4::XIn hybrid plasmids was observed. The smallest insert present in a stable RP4::XIn6 hybrid plasmid, termed XIn6, yielded multiple bands when it was used as a probe with digested P25X chromosomal DNA. Sequence analysis of XIn6 led to the discovery of an open reading frame with homology to the maturases of group II introns. The XIn6 insert also exhibited several features characteristic of a group II intron. These included the presence of the consensus sequence GUGYG at the 5' end and RAY at the 3' end of the intron. RNA secondary structure modelling of XIn6 also revealed the presence of perfectly conserved domains V and VI. Differences were detected in the XIn6 hybridization profiles of several P25X catabolic mutants that have lost the ability to grow on 2,5-xyleneol. In these mutants the loss of 2,5-xyleneol degradative ability could be due to genome rearrangements mediated by sequences related to the XIn6 group II intron. This is the first reported group II intron isolated from *Pseudomonas* spp. and the first time that the mobility of a bacterial group II intron has been demonstrated.**

Keywords: group II introns, *Pseudomonas alcaligenes*, 2,5-xyleneol degradation

INTRODUCTION

Group II introns are mainly found in the genomes of eukaryotic organelles in fungi and plants. Group II introns are of particular interest not only as ribozymes which catalyse their own splicing, but also as mobile genetic elements (reviewed by Lambowitz & Belfort, 1993; Michel & Ferat, 1995). All group II introns have a conserved secondary structure which consists of six double helical domains radiating from a central wheel. This conserved structure catalyses splicing via formation

of a lariat intermediate which closely resembles the intermediate formed during the splicing of nuclear pre-messenger introns. The core structure of group II introns contains very few conserved nucleotides and only the first few bases of the intron and some of those constituting the small domain V could be considered as characteristic of group II (Michel & Ferat, 1995). Using two degenerate primers which match these conserved regions to amplify DNA extracts by PCR, Ferat and his colleagues were able to detect group II introns in both cyanobacteria (*Calothrix*, *Anabaena*, *Nostoc*) and proteobacteria (*Azotobacter*, *Escherichia*) (Ferat & Michel, 1993; Ferat *et al.*, 1994), that is, for the first time in prokaryotes. Interestingly, all four introns identified in *E. coli* interrupt either proven or putative mobile elements. One of the four *E. coli* introns was independently identified using consensus sequences matching

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Abbreviations: EBS, exon-binding site; IBS, intron-binding site.

The GenBank accession number for the nucleotide sequence reported in this paper is U77945.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Reference/source
<i>Pseudomonas</i>		
<i>P. alcaligenes</i> NCIB 9867 (P25X)	Wild-type; 2,5-Xln ⁺	Hopper & Chapman (1971)
<i>P. alcaligenes</i> P25X11	EMS-treated derivative of P25X; 2,5-Xln ⁻	Poh & Bayly (1988)
<i>P. alcaligenes</i> S16	Spontaneous derivative of P25X; 2,5-Xln ⁻	Poh & Bayly (1980)
<i>P. alcaligenes</i> MC7	Mitomycin-C-treated derivative of P25X; 2,5-Xln ⁻	Poh & Bayly (1980)
<i>P. putida</i> RA713	Plasmid-free derivative of <i>P. putida</i> NCIB 9869 (P35X); 2,5-Xln ⁻ 3,5-Xln ⁻ ; Sm ^R	Jain <i>et al.</i> (1984)
<i>Escherichia coli</i>		
J53	F ⁻ <i>thi thr leu</i> ; host for plasmid RP4	Miller (1972)
JM109	F' <i>traD36 proAB lacI^aΔ(lacZ)M15 recA1 endA1 gyrA96 hsdR17 (r_K⁻ m_K⁺) thi supE44 relA1 Δ(lac-proAB)</i> ; host for pUC129 and its derivatives	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pUC129	3.2 kb Ap ^R cloning vector	Keen <i>et al.</i> (1988)
RP4	60 kb IncP broad-host-range plasmid; Ap ^R Km ^R Tc ^R	Datta <i>et al.</i> (1971)
RP4::Xln6	2.0 kb of P25X DNA inserted into RP4 near Ap ^R gene; smallest of RP4::Xln hybrid plasmids; does not confer growth on 2,5-xynol upon RA713	This study
pCC129X6	2.6 kb <i>Sma</i> I fragment containing part of Xln6 fragment and flanking RP4 sequences cloned into pUC129	This study

*Phenotype designations: 2,5-Xln⁺, ability to grow on 2,5-xynol; 2,5-Xln⁻, no growth on 2,5-xynol; 3,5-Xln⁻, no growth on 3,5-xynol.

domain V to search the databases (Knoop & Brennicke, 1994; Knoop *et al.*, 1994). The database search also yielded putative group II intron sequences in plasmid-borne insertion sequence elements in *Agrobacterium tumefaciens* and rhizobia as well as a strain of *Yersinia pseudotuberculosis* (Knoop & Brennicke, 1994). Recently, group II introns were found in a region involved in the conjugative transfer of a *Lactococcus lactis* conjugative plasmid (Mills *et al.*, 1996) as well as a chromosomally located sex factor which controls conjugation in *L. lactis* (Shearman *et al.*, 1996). A transposon isolated from *Clostridium difficile* was recently shown to harbour a group II intron (Mullany *et al.*, 1996). Group II introns thus appear to have a widespread distribution in the prokaryotic world although the true extent of their occurrence in prokaryotes has yet to be determined.

Pseudomonas alcaligenes NCIB 9867 (strain P25X) was isolated from river mud in Hull, UK; it is able to degrade 2,5-xynol, 3,5-xynol and *m*-cresol via the gentisate pathway (Hopper & Chapman, 1971). Spontaneous loss of the ability of P25X to degrade 2,5-xynol was reported to occur at a frequency of 3–5% and this rate of loss could be increased to 40–50% in the presence of mitomycin C or by growth at 42 °C (Poh & Bayly, 1980). This high frequency of loss could be explained if the genes for 2,5-xynol degradation were carried on a plasmid or on a mobile genetic element. Strain P25X was found to harbour an approximately 33 kb plasmid, pRA2; however, all P25X mutants which had lost the ability to grow on 2,5-xynol were found to carry an

identical plasmid (C. L. Poh, unpublished results). To investigate the presence of mobile genetic elements in P25X, the plasmid RP4 was used as a target DNA in conjugative transposition experiments. Here, we report the *in vivo* construction of several RP4::Xln hybrid plasmids and the discovery that the smallest of these hybrid plasmids, RP4::Xln6, harbours a group II intron, the first to be reported in *Pseudomonas*. Although several eukaryotic group II introns are capable of homing (i.e. converting intron-less alleles to the intron-plus state) as well as transposing to new insertion sites (Mueller *et al.*, 1993; Sellem *et al.*, 1993; Moran *et al.*, 1995), the mobility of bacterial group II introns has not yet been demonstrated before. In this report, we also explore the possibility that the genes for 2,5-xynol degradation are localized in the vicinity of group II introns.

METHODS

Bacterial strains and plasmids. The *Pseudomonas* and *Escherichia coli* strains, as well as the plasmids used and constructed during the course of this study, are described in Table 1.

Media and culture conditions. LB agar and LB broth were prepared according to Miller (1972). Media containing aromatic compounds as sole carbon sources were made by adding the aromatic compound to the basal minimal medium (Hegeman, 1966) to a final concentration of 2.5 mM. *P. alcaligenes* P25X and *P. putida* RA713 transconjugants harbouring RP4::Xln hybrid plasmids were maintained on minimal agar plates containing 2,5-xynol as the sole carbon

source. *P. putida* RA713 harbouring RP4::Xln6 and *E. coli* hosts containing RP4 and its derivatives were maintained on LB agar containing kanamycin (Km, 50 µg ml⁻¹) and tetracycline (Tc, 30 µg ml⁻¹) while *E. coli* hosts containing derivatives of pUC129 were maintained on LB agar containing ampicillin (Ap, 100 µg ml⁻¹). Streptomycin (Sm) was used at a final concentration of 500 µg ml⁻¹. All *Pseudomonas* spp. were grown at 32 °C; *E. coli* was grown at 37 °C.

Bacterial conjugation by membrane filter mating. Plasmid RP4 and its hybrid derivatives were introduced into *Pseudomonas* by membrane filter mating as described by Bagdasarian *et al.* (1981). Equal volumes of donor and recipient cells were filtered through a 25 mm diameter membrane filter with pore size of 0.45 µm. The filter was placed on an LB agar plate and incubated for 2 d at 32 °C. The filter was then washed with sterile minimal medium and the cells plated out onto selection plates.

DNA manipulations. Plasmid DNA was prepared by alkaline lysis (Birnboim & Doly, 1979). For large-scale preparations, plasmid DNA was further purified through a caesium chloride/ethidium bromide density gradient as described by Sambrook *et al.* (1989). Genomic DNA was prepared according to the method described by Ausubel *et al.* (1987).

Recombinant plasmids were constructed by standard protocols (Sambrook *et al.*, 1989). Labelled probes for Southern hybridization analysis were made with horseradish peroxidase labelling reactions using the enhanced chemoluminescence (ECL) direct nucleic acid labelling system from Amersham.

DNA sequencing and computer analysis. Nested deletions of the pUC129 clone, pCC129X6, were generated using the double-stranded nested deletion kit (Pharmacia). Nucleotide sequencing was carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and the Applied Biosystems DNA Sequenator ABI 373 (Applied Biosystems/Perkin Elmer) on double-stranded templates. Sequencing of deletion fragments in pUC129 were carried out using either the pUC18 forward (5'-TGTAACGACGGCCAGT-3') or reverse (5'-CAGGAAACAGCTATGAC-3') primers. To complete the sequence of Xln6, a series of 18-mer primers were used to 'walk' along RP4::Xln6 in both the forward and reverse orientations.

The nucleotide sequences and deduced amino acid sequences were analysed using DNASIS and PROSIS (Hitachi Software Engineering) respectively. The BLAST program at the National Centre for Biotechnology Information (NCBI) was used to search for DNA and amino acid homologies in the databases.

RESULTS

In vivo construction of RP4::Xln hybrid plasmids

The broad-host-range plasmid RP4 was introduced into *P. alcaligenes* P25X by conjugation with *E. coli* J53 carrying RP4. Transconjugants were selected on 2,5-xyleneol plates containing Km. Analysis of a few transconjugants which grew on 2,5-xyleneol showed the presence of enlarged RP4 plasmids. When the additional fragments from the RP4 hybrid plasmids were used in Southern hybridization with P25X genomic DNA, multiple hybridizing bands were observed. No hybridization was observed between RP4 and the P25X genomic DNA. *P. alcaligenes* strains carrying these RP4::Xln hybrid plasmids were then used as donors in

Fig. 1. Agarose gel electrophoresis of plasmid RP4 and several RP4::Xln hybrid plasmids digested with *Pst*I. λ DNA restricted with *Hind*III served as the size marker (lane M).

further conjugation experiments with *P. putida* RA713, a plasmid-free, Sm^R-derivative of *P. putida* NCIB 9869 (P35X) which did not express the genes for the gentisate (2,5-dihydroxybenzoate) pathway (Jain *et al.*, 1984). RA713 transconjugants that could grow on 2,5-xyleneol plates supplemented with Km and Sm were selected. Transconjugants appeared at a low frequency of approximately 1×10^{-8} . The ability to grow on 2,5-xyleneol was found to be unstable in some of the transconjugants (four of the nine transconjugants obtained lost the ability to grow on 2,5-xyleneol upon subsequent passages) but the loss of this ability did not lead to a loss of any of the antibiotic resistance markers encoded by RP4 (i.e. Ap, Km and Tc). Plasmids isolated from the RA713 transconjugants showed the presence of additional fragments ranging from approximately 2 kb in RP4::Xln6 to about 26 kb in RP4::Xln1 (Fig. 1).

Subcloning and sequencing of the insertion fragment in RP4::Xln6

Restriction analysis of RP4::Xln6 showed that a 2 kb insertion, designated as Xln6, was located around coordinate 0 of the RP4 plasmid (Lanka *et al.*, 1983) close to the Ap^R gene. Southern hybridization analysis revealed that the sequences related to the Xln6 fragment were repeated numerous times in the P25X genome (Fig.

Fig. 2. Hybridization profiles of strain P25X wild-type, and P25X catabolic mutants P25X11, S16 and MC7, which have lost the ability to degrade 2,5-xynolol (Poh & Bayly, 1980; 1988), using a 484 bp internal fragment Xln6 (nucleotides 796–1280), obtained from a nested deletion clone of pCC129X6, as a probe against genomic digests. Note the absence of an approximately 9.4 kb hybridization band in all the catabolic mutants (marked A) and the presence of an additional 1.5 kb hybridization band in P25X11 (marked B). Hybridization conditions were as follows. The blot was placed in a hybridization buffer containing 0.5 M NaCl and 10 ng probe ml⁻¹ at 42 °C overnight. A high-stringency wash was used by making the primary wash buffer (containing 6 M urea and 0.4% SDS) in 0.1 × SSC from a 20 × SSC stock (0.3 M trisodium citrate, 3 M NaCl; pH 7.0). λ DNA restricted with *Hind*III served as the size marker (lane M).

2). Attempts to subclone the entire Xln6 fragment into pUC129 failed. However, we managed to subclone a 2.6 kb *Sma*I fragment which comprised 1.3 kb of the Xln6 fragment as well as 1.3 kb of surrounding RP4 sequences into pUC129. The sequence data generated from this clone allowed us to synthesize primers to further sequence the remainder of the Xln6 fragment using RP4::Xln6 as the template.

Sequence analysis of Xln6 and its identification as a group II intron

The entrapment of Xln6 in RP4 was suggestive of mobility, but a nucleotide sequence database search with BLAST (Altschul *et al.*, 1990) showed that Xln6 did not have any significant homology to known mobile genetic elements. Identification of flanking RP4

sequences indicated that the Xln6 insert spanned 1919 bp. Nucleotide sequence analysis of Xln6 revealed an open reading frame (ORF) which spanned nucleotides 429–1900 (Fig. 3) and could potentially encode a 56175 Da protein of 490 amino acid residues.

The ORF-encoded protein of Xln6, designated OrfX6, was found to have extensive similarity to the ORF-encoded maturases of eukaryotic group II introns (Lambowitz & Belfort, 1993; Michel & Ferat, 1995) and recently identified bacterial group II introns (Ferat & Michel, 1993; Ferat *et al.*, 1994; Mills *et al.*, 1996; Mullany *et al.*, 1996; Shearman *et al.*, 1996). About a quarter of the known group II introns potentially encode proteins called maturases (Michel & Ferat, 1995) and most of them include a domain with obvious homology to the polymerase domain of reverse transcriptases (Michel & Lang, 1985). The Xln6-encoded maturase, OrfX6, contained all seven polymerase-like domains conserved among retroelement reverse transcriptases (RT1 to RT7) (Fig. 4) (Xiong & Eickbush, 1990). A domain Z was detected at the N-terminal end of the Xln6 maturase. This domain is normally found upstream of the reverse transcriptase domain in retroid elements devoid of long-terminal repeats (LTRs) (Xiong & Eickbush, 1990). A domain X, which had been suggested to be involved in binding of the intron RNA during reverse transcription and splicing (Mohr *et al.*, 1993), was found downstream of the Xln6 reverse transcriptase domains.

The Xln6 intron boundaries showed the strongly conserved consensus sequence GUGYG at the 5' end and RAY at the 3' end of the intron (Michel *et al.*, 1989). The 3' end of the Xln6 intron could also be folded into perfect domain V and VI RNA secondary structures characteristic of group II introns (Fig. 5a) (Michel *et al.*, 1989). Domain VI contained a bulging adenine residue seven bases from the 3' splice site which has been demonstrated to constitute a 2' to 5' lariat branch point in several group II introns (Michel *et al.*, 1989; Michel & Ferat, 1995). Relevant domain V features of a bulging CG dinucleotide and a purine-rich terminal loop of four nucleotides (Michel *et al.*, 1989; Knoop & Brennicke, 1994) were also observed in the Xln6 group II intron.

A combination of comparative analysis (Michel *et al.*, 1989) and computer modelling allowed us to identify an exon-binding site loop (EBS1) (5'-ACAAATC-3') in domain I of the Xln6 group II intron. A search of the RP4 sequences upstream of the intron revealed an intron-binding-site-like (IBS1-like) sequence motif (5'-TGTTTTG-3') which could potentially base-pair with the EBS1 loop (albeit with one mismatch).

Relationship of the Xln6 group II intron with 2,5-xynolol degradative genes

When the Xln6 group II intron was used as a probe against genomic digests of several P25X catabolic mutants which have lost their ability to degrade 2,5-xynolol, differences were observed in the hybridization

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                                                    IBS1-like
                                                    ----->
RP4 59542 ggtgatggag ccgggtgcag agccataagg ggcaagagac gaaagcccgg tttccgggct tttgttttgt 59472
Xln6 1 GTGCGCCAGG CATGGCGCGT TGCGCGTAAG CGCAACCCGC TTGGTTGTGG TGGCCAAGTG GGTGACTTGG
71 AGGTGAAAGT CCTCTACACA CCCGGCAAGG GGAAGTGCTA GCCGGAGGCA AGGGTGTGCG GGGTGACCGC
141 GAATCTGAAG GAAGCCCCGAG GCAAAATGCT GGCTTGACGA ACAGGAAGCG GATAGAGGCG GCACAGCGGG
EBS1
----->
211 GTGAGGTGGC ACAAATCGCC AAAGCCCAAT ACTTGACCGG AACGCTGTGA CGTAGATCCG ACAGGCATAA
281 GCAGGAAGGT CGCGCGAATT ACCCTGGGAG ATCTGTACGC TTTGGCACTG TGCTACCGCG CGTCGAAAGG
351 GGACGGGATG AGCGTGCGA AGTCAGCCGA AGCCGTAGTA AGTGGCGGTT AACCGCGCCA CCAAGGGCCG
421 AACAGTTTAT GCCGCCAGTA GCGCTGCGCG TCTCGTTGGT AACCGTAATG CAGAAATTTC CCACAGCGGA
LORF
491 AACTGTCATC CCGAATCCCG GCCAGAAGCC GAGGGTGATG CCTGACAGTG CAAAGGTGCC GCGGGCGTCA
561 GCGACGTGGA CGAACCGGGA GCCGGACACG CTGATGGAGC GGGTGCTTGC ACCGGCCAAC CTCAGGCGTG
631 CGTATCAACG CGTGGTTAGC AACAAAGGGG CTCGGGTGCG CGATGGCATG ACGGTGCGTG ACTTGGCGGG
701 CTACGTGAAA CAGTATTGGC CAACCTCAA GGCCAGGTTG CTGGCCGGTG AATACCATCC CCAAGCAGTG
771 CGAGCGGTTG AAATTCCCAA GCCCGAGGGC GGCACACGGC AACTGGGTAT CCCCAGCGTC GTGGATCGCC
841 TGATCCAGCA AGCACTGCAG CAGCAGCTCA CACCTATCTT CGACCCACTG TTCCTCGACT ACAGCTATGG
911 TTTTCGTCCG GGCAGAAGTA CGCATCAAGC CATCGAGATG GCTCGTGCTC ATGTGACAGC GGGACACCGC
981 TGGTGCGTGG AACTCGATCT GGAGAAGTTT TTCGATCGGG TCAATCACGA CATCTCATG GCCTGCATCG
1051 AACGTCGGAT CAAAGACAAG TGCGTACTCA GGCTTATTCG CCGCTACCTT GAAGCCGGGA TCATGTCGGG
1121 CGGTGTCGTC AGCCACCGGC AGGAGGGGAC GCCGCAAGGC GGCCCGCTTT CGCCGTTGCT GTCGAATATC
1191 CTGCTCGACG AACTCGACCG CGAGCTGGAG CGCGGGGGCC ATCGCTTCGT ACGTTATGCC GATGATCGCA
SmaI
1261 ACATCTATGT ACGCAGTCCC CGGGCCCGCG AACGGGTGTT GGTCAGCGTC GAGCGCTTCC TGAGAGAACG
1331 TCTGAAGCTG ACGGTGAACA GGAAGAAAAG CCAAGTGGCA AGGGCGTGGA AGTGCGACTA CCTAGGCTAT
1401 GGGATGAGCT GGCACCAGCA GCCAAGGCTG CGCGTGCGAA GGATGAGCCT AGACCGCTTA CGCGACCGGC
1471 TCAGAATGCT GCTGCGCAGC GTACGGGCTC GCAAAATGGC GACTGTCATC GAGCGGATCA ACCCCGTCCT
1541 GAGAGGCTGG GCTAGCTACT TCAAGCTCAG CCAGAGCAAG CGGCCGCTTG AGGAACTGGA TGGTTGGGTC
1611 AGGCACAAAC TCCGCTGCGT CATCTGGCGT CAATGGAAGC AGCCTCCAC GAGGCTGAGA AACTTGATGC
1681 GCCTAGGGTT GAGCGAGGAG CGTGCCAACA AGTCAGCCTT CAATGGCCGA GGTCCATGGT GGAATTCGGG
1751 AGCGCAACAT ATGAACTACG CGTGCCAAA GAAACTGTGG GACCGGCTCG GGCTGGTCTC GATACTGGAT
1821 ACGATTAAAC GGCTTAGCCG TAACCTGAAC CGCGGTGTAC GGAACCGTAC GCACGGTGGT GTGAGAGGAC
1891 GCGGGGTGTG AATCCGCCTC TACTCGATT acgccaagga cgagtttttag cggctaagg tgttgacgtg 59432 RP4
ORF ***

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Fig. 3. Nucleotide sequence of the Xln6 group II intron and flanking RP4 sequences (shown in lower-case letters). The RP4 sequence was numbered (in italics) according to Pansegrau *et al.* (1994). The ORF which encodes the maturase-related protein is enclosed in brackets. The stop codon is marked by asterisks. The *Sma*I site used for cloning is shown (the Xln6 fragment cloned in pCC129X6 was from nucleotides 1 to 1281). The nucleotide sequences which form the EBS1 loop in the Xln6 intron and the RP4 IBS1-like sequences upstream of the intron are overlined with dashed arrows.

profiles when compared with the wild-type P25X (Fig. 2). An approximately 9.4 kb hybridization band (A) was notably absent in all the catabolic mutants screened while in one mutant, P25X11, an additional 1.5 kb hybridization band was observed. The results strongly suggest that genomic rearrangements mediated by the Xln6 group II intron could have led to the loss of genes involved in the degradation of 2,5-xylanol.

In searching for nucleotide sequence homology with the databases, we found that the region of Xln6 which could fold into the domain VI RNA secondary structure (nucleotides 1871–1919) showed sequence identity, with only three point differences, with a noncoding region immediately downstream of the *p*-cresol hydroxylase gene (*pchF*) from both *Pseudomonas putida* NCIB 9869 (strain P35X) and *P. putida* NCIB 9866 (strain P24X) (Kim *et al.*, 1994) (Fig. 5b). Two of these three differences in the flanking *pchF* sequence consist of compensatory base substitutions which would still allow the sequence

to fold into a characteristic domain VI secondary structure.

DISCUSSION

Bacterial group II introns were first detected in cyanobacteria and proteobacteria, which include the presumptive ancestors of chloroplasts and mitochondria, respectively. However, since then, group II introns have been discovered in bacteria as diverse as *E. coli* (Ferat *et al.*, 1994), agrobacteria and rhizobia (Knoop & Brennicke, 1994), lactococci (Mills *et al.*, 1996; Shearman *et al.*, 1996) and *Clostridium difficile* (Mullany *et al.*, 1996). The GC content of all bacterial group II introns sequenced so far, including Xln6, reflects the GC content of their hosts, suggesting that these introns are not a recent introduction but, rather, are long-term residents of their bacterial hosts. Our finding of a group II intron in *P. alcaligenes* further extends the known distribution of these elements in

		Z	
<i>L. i. ltrA</i>	(11)	ISKNSQENIDEVFTLRYLLRPDIYVAYQNLVSNKGA	STKGILDDTAD
<i>P25X OrfX6</i>	(40)	VPAAATWTAEPDPTLMERVLAPANLRRAYQRVVSNKGA	PGAGDMTVADL
<i>E. coli</i>		(1) MTKKAFNIDKSLVVSATYRVKTSAGA	GIDKQSLADF
Consensus		-----AY-----GA-----	
		RT1	RT2
<i>L. i. ltrA</i>		GFSEEEKIKKIQLSKD-GTYYPQVRRMYIAKNSKMRPLG	IPFTFDKLI
<i>P25X OrfX6</i>		AGYVQYWP TLKARLLAGEYHPQAVRAVEI	PKPGGTRQLGIPSVVDLI
<i>E. coli</i>		DKRLVDNLYKIWNRLSSQSYFPAVKAIPAIPKLGGERIL	LGIPTVSDRIA
Consensus		-----G-Y-P--V--I-K-----R-LGIP--D--	
		RT2	RT3
<i>L. i. ltrA</i>		QEAVRTILESIYEPFVDSHGFRPQSRCHTALKTIKREFG	-GARNFVEG
<i>P25X OrfX6</i>		QQALQQQLTPIFDPLFSDSYGFRPGRSTHQAIEARAHVTA	GRHNCVEL
<i>E. coli</i>		QTVVKLAFEPQVEPHLADSYGYRBNKSALDAIGVTKRCKWYD	-WVLEF
Consensus		Q-----P-F--S--RP--S--A-----W--E-	
		RT3	
<i>L. i. ltrA</i>		DIKGGFDNIDVTLIGLINLAIKDKMSQLIYKFLKA	-GYLENWQYHKYS
<i>P25X OrfX6</i>		DLEKFFDRVNDILMACIERIKDKCVLRLLIRYLEA	-GIMGGVVSPPQE
<i>E. coli</i>		DIKGLFDNIPHELIMKAVDKHNPARWVKLYIQRLTAPVM	MDGEVRARTM
Consensus		D---FD--H--I--L-A-----	
		RT4	
<i>L. i. ltrA</i>		GTFPGGILSPLLANIYLHLELDFVLQKMKFDR	ESPERITPEYRELH[46]
<i>P25X OrfX6</i>		GTFPGGILSPLLSNILLDELDRELERGRH	-----
<i>E. coli</i>		GTFPGGVISPLLANLFMHYVDFDKLAKYYPKVP	-----
Consensus		GTFPGG--SPLL-N-----	
		RT5	RT6
<i>L. i. ltrA</i>		YVRYADDFIISVKGSKEDCQWIKQLKFIHNKLK	MELSEKTLITHS---
<i>P25X OrfX6</i>		FVRYADDANIYVRSRAGERVLV-SVERFLRERL	LKLTVNKKSQVAR---
<i>E. coli</i>		WYRYADDGILHCHS-EAEATEMR-EVLKRKF	SEGLMHPKTRVIYCKDG
Consensus		--RYADD-----K-----	
		RT7	domain X
<i>L. i. ltrA</i>		-----SQPARFLGYDIRVR[55] KYLIRSTDLEI	ITTYNSSELGICNYGL
<i>P25X OrfX6</i>		-----AWKCDYLGYSMSWH[23] RSVRARKMAT	VERINPVLGOWASYFKL
<i>E. coli</i>		SRKGDYEHTMFDPLGYTFRRR[35] RKRVDLSI	EQIAKWNPKLNGWINYGR
Consensus		-----LGY-----N--L-G--Y--	

Fig. 4. Protein alignment of the putative ORF1-encoded protein of Xln6, OrfX6, with prokaryotic group II intron-encoded ORFs from *E. coli* (Ferat *et al.*, 1994) and *Lactococcus lactis* (*L. i. ltrA*) (Mills *et al.*, 1996). Identical amino acid sequences are in bold letters. Domains conserved among intron-encoded ORFs are denoted by the lines above the alignment. RT1 through RT7 depict reverse transcriptase (RT)-like domains. Z refers to a domain of undetermined function in non-long-terminal-repeat retroelements (Xiong & Eickbush, 1990); the maturase-specific domain is designated X (Mohr *et al.*, 1993).

diverse bacterial groups. The possibility for intron dissemination via horizontal transfer exists in bacteria, as all the bacterial group II introns found so far are associated with insertion sequence elements (Ferat *et al.*, 1994; Knoop & Brennicke, 1994), a conjugative transposon (Mullany *et al.*, 1996), a lactococcal conjugative plasmid (Mills *et al.*, 1996) and a lactococcal chromosomal sex factor (Shearman *et al.*, 1996).

We believe that group II introns are indeed widespread in bacteria, but the search strategies employed thus far have not been exhaustive (Ferat & Michel, 1993; Michel & Ferat, 1995). Searching is difficult as the core structure of group II introns contains very few conserved nucleotides. A good example of the diversity of group II intron structures could be found in the chloroplast genome of *Euglena gracilis* (reviewed by Copertino & Hallick, 1993), where bona fide, although somewhat defective, group II introns coexist with numerous smaller introns that share the same lariat-forming mode of excision but lack any recognizable cognate of even domain V.

The mechanism for eukaryotic group II intron mobility is only beginning to be elucidated. It was suggested that the yeast mitochondrial group II intron, aI2, acted as a site-specific retroposon (Moran *et al.*, 1995) and its homing was dependent on both the reverse transcriptase domains as well as an endonuclease activity of the zinc finger domain (Zimmerly *et al.*, 1995). The yeast aI1 intron was recently shown to encode an analogous endonuclease specific for a different target site to the aI2 intron and more than half of aI1 molecules were reported to undergo complete reverse splicing *in vitro*, integrating linear intron RNA directly into DNA (Yang *et al.*, 1996).

Although bacterial group II intron mobility has never been proven, few of the introns characterized revealed flanking exons potentially involved in DNA mobility (Knoop & Brennicke, 1994; Ferat *et al.*, 1994). The entrapment of the *P. alcaligenes* P25X Xln6 group II intron in RP4 demonstrated, for the first time, the mobility of a bacterial group II intron. It is interesting to note that an IBS1-like sequence could be detected in the target RP4 plasmid immediately upstream of the inserted Xln6 intron. All cases of spontaneous group II transposition investigated so far have turned out to occur immediately downstream of an IBS1-like sequence (Mueller *et al.*, 1993; Michel & Ferat, 1995).

The criterion for selection of the RP4::Xln hybrid plasmids was growth of the recipient *P. putida* RA713 on 2,5-xynol. The newly acquired 2,5-xynol degradative ability in RA713 carrying RP4::Xln6 was unstable. Analysis of RP4::Xln6 hybrid plasmids from RA713 hosts that failed to utilize 2,5-xynol showed that there was a reduction in size of the inserted fragment. Preliminary analysis of RP4::Xln6 had shown that the insert was 12.6 kb in size (Tham, 1993); however, upon subsequent passages, the plasmid was observed to have lost approximately 10.6 kb of DNA coinciding with the loss of the ability of the plasmid to confer growth on 2,5-xynol upon the host. The results indicated that in RA713 harbouring the stable form of RP4::Xln6, a rearrangement had taken place resulting in the loss of DNA which had enabled the host RA713 to grow initially on 2,5-xynol. Two possibilities could be offered regarding the mobility of the Xln6 intron. One, the Xln6 intron was associated with a mobile element which had transposed onto RP4 and later underwent further rearrangements, leaving the Xln6 intron on RP4. This is possible as some of the bacterial group II introns discovered have been associated with mobile elements (Ferat *et al.*, 1994; Knoop & Brennicke, 1994). Alternatively, the Xln6 intron itself could have pulled its flanking exons during its transposition into RP4 but these flanking regions were rather unstable and were concomitantly lost from the hybrid plasmid. In eukaryotic group II introns that have been shown to be mobile, transposition was reported to occur at a much lower frequency compared to homing (i.e. insertion into an intronless allele) (Michel & Ferat, 1995; Moran *et al.*, 1995). Intron homing was found to be accompanied by preferential transmission of markers flanking the intron (Michel & Ferat, 1995). However, for the yeast

group II introns al1 and al2, co-conversion associated with the transfer of the introns was reported to be limited to fewer than 100 bp in exon 1 and fewer than 23 bp in exon 3 (Moran *et al.*, 1995). Thus for a transposing Xln6 to bring along flanking exons which confer growth on 2,5-xynol would mean co-conversion of a fairly large fragment of DNA. This possibility, although unprecedented, should not be totally dismissed as no in-depth study of the mobility of bacterial group II introns has been carried out.

Even though RP4::Xln6 was unable to confer growth on 2,5-xynol upon RA713, some of the other RP4::Xln hybrid plasmids did. Efforts are under way to further characterize these hybrid plasmids and to investigate the relationship of group II introns and 2,5-xynol degradative genes. The 9.4 kb P25X DNA fragment which was found to be missing in all the 2,5-xynol-negative catabolic mutants (Fig. 2) is also being cloned and characterized. Results of Southern hybridization clearly showed that in these P25X catabolic mutants, genome rearrangements mediated by sequences related to the Xln6 group II intron had taken place. DNA sequences bounded by two Xln6 group II introns could have been deleted as a result of recombination between the introns. These DNA sequences could encode 2,5-xynol degradative genes. The presence of an additional 1.5 kb fragment in the DNA hybridization profile of mutant P25X11 (marked B in Fig. 2) when Xln6 was used as a hybridizing probe suggested the insertion of part of the group II intron into either the structural or regulatory genes of the gentisate pathway. Disruption of these genes could lead to the non-functioning of the gentisate pathway in the mutant.

A noncoding region immediately downstream of the

pchF gene that exhibited high homology with domain VI of the Xln6 group II intron and which could potentially fold into a characteristic domain VI secondary structure strongly suggests that a group II intron is associated with the *pchF* gene, which is involved in the degradation of *p*-cresol via the *ortho* pathway. This intron-like sequence, which is in an opposite orientation to the *pchF* gene and ends precisely at the *pchF* termination codon, has an unprecedented arrangement for introns and flanking sequences. The extent of the involvement of group II introns in biodegradative pathways clearly needs further investigation and it remains to be seen if group II introns play an important role in the evolution of catabolic functions, much like other mobile genetic elements.

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