IS1236, a newly discovered member of the IS3 family, exhibits varied patterns of insertion into the *Acinetobacter calcoaceticus* chromosome

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Analysis of spontaneous mutations in *Acinetobacter calcoaceticus* revealed a 1237 bp insertion sequence named IS1236 and possessing a nucleotide sequence resembling those of members of the IS3 family. The chromosome of *A. calcoaceticus* strain ADP1 contains seven copies of IS1236 which appears to insert preferentially into *pobR*, the transcriptional activator of the structural gene for *p*-hydroxybenzoate hydroxylase. IS1236 creates tandem 3 bp DNA duplications flanking the sites of its insertion in *pobR*. Different duplication patterns are found following insertion of IS1236 into *pcaH*, a structural gene for protocatechuate 3,4-dioxygenase. Therefore the insertion properties of IS1236 appear to be influenced by its DNA target. Amino acid sequences associated with the apparent transposase function have been conserved in ORFB of IS1236 whereas the presumed DNA-binding helix–turn–helix region of IS1236 ORFA exhibits substantial amino acid sequence divergence from its IS3 counterparts. IS1236 ORFA and ORFB coding sequences overlap considerably, and sequence evidence indicates mechanisms for ORFB expression in IS1236 may resemble those employed by other members of the IS3 family. Portions of the IS1236 terminal repeats exhibit substantial sequence divergence from other members of the IS3 family, but evolution appears to have conserved a mechanism preventing expression of the insertion sequence genes as a consequence of transcriptional readthrough.

Keywords: *Acinetobacter calcoaceticus*, chromosome, genetic targets, insertion sequence, mutation

INTRODUCTION

The ease with which *Acinetobacter calcoaceticus* strain ADP1 undergoes natural transformation makes it a convenient system for analysis of spontaneous mutations. The metabolic pathway used by this bacterium for dissimilation of *p*-hydroxybenzoate is particularly useful for genetic investigation because it is possible to select for strains containing null mutations preventing metabolism of this compound *via* protocatechuate (DiMarco *et al.*, 1993a, b; Gerischer & Ornston, 1995). Investigation of such mutations has shown that they all alter DNA within

the 18 kbp *pca–qui–pob* supraoperonic cluster (Averhoff *et al.*, 1992; Elsemore & Ornston, 1994, 1995; Hartnett *et al.*, 1990; Kowalchuk *et al.*, 1994)(Fig. 1).

The most extensive investigation of this genetic system focused upon spontaneous mutations preventing expression of *pcaH* and *-G* (Gerischer & Ornston, 1995). Among 42 sequenced mutations, four were caused by the insertion sequence IS1236 as described in this paper. Other studies had developed procedures for characterizing mutations in either *pobA* (DiMarco *et al.*, 1993a) or *pobR* (DiMarco *et al.*, 1993b), so it was possible to assess the relative frequency with which IS1236 is the source of spontaneous null mutations in these genes. As described here, mutations caused by IS1236 in *pobR* differ from those caused by IS1236 in *pcaH* in both frequency

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Fig. 1. Chromosomal organization of genes associated with protocatechuate catabolism in *A. calcoaceticus*. Dashed horizontal arrows indicate directions of transcription. Vertical arrows show positions of IS1236 mutations that were sequenced as part of this study. The respective sizes of *pcaH*, *pcaG*, *pobR* and *pobA* are 726, 630, 816 and 1215 bp. Protocatechuate is converted to citric acid cycle intermediates by enzymes encoded by the *pcaI/FBDKCHG* operon (Kowalchuk *et al.*, 1994). *p*-Hydroxybenzoate hydroxylase, an enzyme that converts its substrate to protocatechuate, is encoded by *pobA* (DiMarco *et al.*, 1993a), and the transcriptional activator of this gene is *pobR* (DiMarco *et al.*, 1993b; DiMarco & Ornston, 1994). The ends of *pcaG* and *pobR* are separated by 7827 bp of DNA largely associated with catabolism of quinate and shikimate to protocatechuate (Elsemore & Ornston, 1994, 1995; Kloos *et al.*, 1995). Null mutations in *pcaB* prevent the growth of cells in the presence of protocatechuate or its metabolic precursors (Hartnett *et al.*, 1990). Growth media containing protocatechuate have been used to select from *pcaB* mutants strains containing secondary mutations blocking expression of *pcaH* and -G (Gerischer & Ornston, 1995); similar procedures with *p*-hydroxybenzoate-containing growth media allow selection of strains with secondary mutations blocking expression of either *pobA* (DiMarco *et al.*, 1993a) or *pobR* (DiMarco *et al.*, 1993b).

and mode of insertion. Analysis of the IS1236 nucleotide sequence reveals biochemical traits conserved as it diverged from other members of the IS3 family.

METHODS

Growth of cells, selection of mutant strains, and characterization of mutations. *Acinetobacter* strain ADP1 used in this investigation is the same as strain BD413 which was initially isolated after enrichment culture of a soil sample with butane 2,3-diol. The organism exhibits extraordinary competence as a recipient in natural transformation (Juni & Janik, 1969), and the capacity to donate DNA that replaces a recipient *trpE* allele through transformation has been taken as evidence that the DNA was derived from a member of the genus *Acinetobacter* (Juni, 1972). Classification of strains within the genus is complex and not completely resolved. According to some widely accepted classification schemes (Bouvet & Grimont, 1986; Bouvet *et al.*, 1990; Ino & Nichimura, 1989), strain ADP1 (or BD413) should not be designated *A. calcoaceticus*. Wiedmann-Al-Ahmad *et al.* (1994) have suggested that these schemes have placed relatively slight emphasis on environmental isolates and have raised the possibility that satisfactory methods for classification of all *Acinetobacter* strains are not yet available. Until these issues are fully resolved, we provisionally maintain the species designation *A. calcoaceticus* which has been consistently applied to strain ADP1 (or BD413) since its original isolation.

Previously described procedures were used for growth of cells and selection of mutant strains that failed to express either *pcaH* and -G (Gerischer & Ornston, 1995) or *pobA* (DiMarco *et al.*, 1993a, b). As described elsewhere, natural transformation was used to map mutations within 200 bp of known nucleotide sequence in *pcaH* and -G (Gerischer & Ornston, 1995), *pobA* (DiMarco *et al.*, 1993a) and *pobR* (DiMarco *et al.*, 1993b). PCR was used to amplify chromosomal DNA for direct sequencing (Gerischer & Ornston, 1995). Between 50 and 100 bp from each end of IS1236 was determined as part of the sequencing of each mutation.

Sequencing of IS1236. DNA fragments containing portions of IS1236 from either *pcaH3* or *pcaH10* (Gerischer & Ornston, 1995) were amplified by PCR, purified from small components by ultrafiltration using Ultrafree-MC Filter Units low-binding cellulose 30000 NMWL, type PLTK (Millipore), and treated

with the Klenow fragment of DNA polymerase to create blunt ends (Sambrook *et al.*, 1989). These fragments were cloned into the *Sma*I site of pUC19. The resulting clones were characterized by restriction analysis and nucleotide sequencing. Together with the results of the direct sequencing of insertion sites, this provided the complete IS1236 insertion sequence. Double-stranded plasmid DNA was sequenced by the dideoxy method using a Sequenase version 2.0 kit (USB) with either standard pUC sequencing primers or custom-designed primers (Gerischer & Dürre, 1993). Homology searches were performed with the BLAST network service (Altschul *et al.*, 1990).

Copy number of IS1236. Southern blotting (Sambrook *et al.*, 1989) was used to estimate the copy number of IS1236 in *A. calcoaceticus* strain ADP1. The probe was the 1.5 kbp insert from plasmid pZR2018 which contains the first 1143 bp of IS1236 and a 442 bp fragment extending from position 27 to 468 in the nucleotide sequence of *pcaH*. *A. calcoaceticus* chromosomal DNA was digested with eight different restriction enzymes which do not contain predicted cleavage sites within IS1236. Restriction fragments were separated by agarose gel electrophoresis and blotted onto a nylon membrane. DNA fragments hybridizing with the *pcaH* portion of the probe were identified on the basis of the known 14 kbp nucleotide sequence of the *pcaI/FBDKCHGquiBCXA* cluster (Elsemore & Ornston, 1994, 1995; Kowalchuk *et al.*, 1994). Other hybridizing fragments were attributed to IS1236.

RESULTS

IS1236 is a member of the IS3 family of insertion sequences

The nucleotide sequence of IS1236 (Fig. 2) revealed characteristics identifying it as a member of the IS3 family of insertion sequences. A homology search among DNA segments with known nucleotide sequence showed that IS1236 most closely resembles IS3 and IS1133 found in six *Enterobacteriaceae* species (Chiou & Jones, 1993; Lawrence *et al.*, 1992). Greatest nucleotide sequence similarity was found in the ORFB sequences which exhibit identity exceeding 50% over aligned stretches of more than 500 residues. Properties shared by IS1236 and IS3 include (i) terminal inverted repetitions of about 40 bp ending in TGATC/GATCA, (ii) a small ORF (ORFA, occupying positions 73–363 in IS1236, Fig. 2) overlapped in part by

Fig. 2. Nucleotide sequence of IS1236 and the deduced amino acid sequences of ORFA and ORFB. Positions inferred to correspond to positions -35 and -10 with respect to the transcriptional start are overlined and underlined. A likely RBS for ORFA is shaded; there is no obvious RBS for ORFB. Double-lined arrows indicate the inverted repetitions at the termini of IS1236, and single-lined arrows mark an inverted repetition that may block translation of transcripts initiated outside the insertion element (Timmerman & Tu, 1985). Double-underlining marks a putative helix-turn-helix motif with a highly significant standard deviation score of 5.3 based on the Dodd-Egan weight matrix (Dodd & Egan, 1990). Acidic amino acid residues that appear to be conserved components of a catalytic triad conserved in IS3 family members and retroviral integrases are double-underlined and overlined (Fayet *et al.*, 1990; Polard & Chandler, 1995). Dashed arrows accompanied by lettered numerals depict a region of possible RNA secondary structure following a potential ribosomal frameshifting window ('AAAAAAA', shaded and underlined); assignment of these structures is based upon analogy with the corresponding portion of the IS3 nucleotide sequence (Sekine *et al.*, 1994). Potential initiation codons for IS1236 ORFB ('ATT' and 'GTG') are shaded, underlined and overlined. It should be noted that nucleotide sequence variations may be exhibited by different copies of IS1236 in the *A. calcoaceticus* chromosome.

a larger ORF (ORFB, occupying positions 306–1229 in IS1236) which is shifted -1 nucleotide in phase of translation, (iii) an inverted repetition that may block the RBS of ORFA (Timmerman & Tu, 1985), (iv) a putative helix-turn-helix motif in ORFA (Dodd & Egan, 1990; Prère *et al.*, 1990), and (v) nucleotide sequences that potentially form a RNA pseudoknot encompassing the end of ORFA (Fig. 2).

Frequency of mutations caused by IS1236 in different genes

Null mutations blocking metabolism of *p*-hydroxybenzoate could be caused by dysfunctions in either *pobR* or *pobA* which occupy about 2.2 kbp of chromosomal

DNA (Fig. 1). Of ten independently occurring null mutations isolated as part of this investigation, six were in the 816 bp *pobR*, and five of these were IS1236 insertions. None of the four mutations in the 1215 bp *pobA* was caused by IS1236 insertion. Analysis of 13 mutants independently derived as part of a separate study (Hartnett *et al.*, 1990) revealed six of seven *pobR* mutations caused by a 1.2 kbp insertion whereas such an insertion sequence appeared in only one of six *pobA* mutations. Preferential appearance of the 1.2 kbp insertion in *pobR* mutations (11 of 13) contrasts with its relatively low frequency in *pobA* mutations (1 of 10). IS1236 also is a relatively infrequent cause of mutations in the 1.4 kbp *pcaH* and -G region. Of 42 sequenced mutations in this region, only four were caused by IS1236, and all of these were in *pcaH*.

	Distance from start of gene (bp)
<i>pobR990</i> <div> <div>→</div> <div>TGCACATCCCA-IS1236-cgaTTCAAGTGA</div> </div>	34
<i>pobR996</i> <div> <div>←</div> <div>AGCTCGGCACAT-IS1236-catTTACCCAAA</div> </div>	290
<i>pobR987</i> <div> <div>→</div> <div>AGCAAGATAATC-IS1236-atcTTCGCGTGA</div> </div>	420
<i>pobR989</i> <div> <div>→</div> <div>GATCGTGAAGTA-IS1236-gtgCAAATTGAA</div> </div>	518
<i>pobR991</i> <div> <div>←</div> <div>ACAGGGGCTTAC-IS1236-tgcCATTGCAGC</div> </div>	700
<i>pcaH10</i> <div> <div>←</div> <div>CCAAAGATGGTT-IS1236-gttTGCCGATTG</div> </div>	218
<i>pcaH2</i> <div> <div>←</div> <div>TATTTGGGGAac-IS1236-agCTTACGCTCA</div> </div>	20
<i>pcaH3</i> <div> <div>←</div> <div>TTGGGGAGCTTA-IS1236-gacGCTCAGCGT</div> </div>	25
<i>pcaH14</i> <div> <div>•←</div> <div>CAGGTCCCATGG-IS1236-cgaatggCGTAAC</div> </div>	450

Fig. 3. Target sites and modes of insertion of IS1236. The transcriptional orientation of IS1236 is indicated by arrows, and a 4 bp deletion in the left terminus of the *pcaH14* IS1236 is indicated by the asterisk. Nucleotide sequence newly acquired as a consequence of insertion is shown in lower-case and underlined.

Copy number

Digestion of the wild-type ADP1 chromosome with *Cla*I or *Xba*I produced seven DNA fragments that hybridized with the IS1236 portion of the probe whereas digestion with *Acc*I produced six such fragments, and four hybridizing chromosomal fragments were produced by *Eco*RI, *Hpa*II, *Nar*I, *Sal*I or *Sph*I. The largest hybridizing fragment produced by *Sph*I was 11 kbp. It therefore can be inferred that the chromosome of the *A. calcoaceticus* strain under investigation contains at least seven copies of IS1236. Some of these are separated by no more than 11 kbp.

Mode of IS1236 insertion

The genetic consequences of the IS1236 insertion mutations are summarized in Fig. 3. For the most part, the

insertion mutations are fairly evenly distributed throughout *pobR* and *pcaH*. Of the five IS1236 insertion mutations in *pobR*, three are oriented so that ORFA and ORFB are transcribed in the same direction as the mutated gene, and two carry IS1236 in the opposite orientation. The orientation of the four *pcaH* inserts is such that ORFA and ORFB transcription is opposed to the transcriptional direction of the mutated gene.

Insertion into *pobR* invariably produced a 3 bp direct repeat of the target DNA on either side of the insertion sequence, and the terminal inverted repetition of IS1236 was intact in *pobR* mutations (Fig. 3). Repetitions of 3 bp also flank the insert in *pcaH10*, but the other three *pcaH* mutants exhibited different patterns (Fig. 3). In two cases, newly acquired DNA flanking the insertion site could not be traced to a known genetic origin: insertion leading to *pcaH2* was accompanied by acquisition of 2 bp corresponding to AC on the 5' end of the insert and 1 bp corresponding to A on the 3' end of the insert; in *pcaH3*, 2 bp corresponding to CA lie at the 3' end of the insert. Most remarkably, the mutation leading to *pcaH14* resulted in a 6 bp direct repetition flanking the insert as well as deletion of the 4 bp from the right terminus of IS1236.

Of the nine IS1236 mutations described in Fig. 3, only two (*pcaH2* and *pcaH3*) failed to give rise to revertants with detectable frequency. Since these two mutations do not include obvious target site duplications, their lack of reversion underscores the importance of flanking direct repeats for IS excision (Galas & Chandler, 1989). Reversion of *pcaH14* suggests that the right terminal TGAT sequence may not be required for excision of IS1236, but precise excision was not determined in this case. Precise excision of IS1236 from *pcaH10* was confirmed by direct sequencing of DNA from a revertant strain.

DISCUSSION

Characteristics shared by IS1236 and other members of the IS3 family of insertion sequences

Nucleotide sequence comparisons revealed that IS1236 is a member of the IS3 family (Galas & Chandler, 1989; Timmerman & Tu, 1985), with greatest sequence similarity extending over most of ORFB. ORFB amino acid sequences from IS1236 and *Escherichia coli* IS3 share 48% identity over 221 aligned residues, and conserved within this region is an apparent catalytic triad with invariant aspartate and glutamate residues (Fig. 2). Amino acid sequences containing this triad, believed to be catalytically active, are conserved among transposases of IS3 family members and retroviral integrases (Fayet *et al.*, 1990; Polard & Chandler, 1995).

Substantial divergence has occurred in the amino acid sequences associated with the helix-turn-helix motif in ORFA. IS1236 and IS3 share only 5 of 20 amino acids in this region, but the standard deviation of 5.3 determined with the Dodd-Egan weight matrix for this portion of IS1236 ORFA makes it likely that it conserves the DNA-binding function assigned to the helix-turn-helix motif in

Fig. 4. Divergence of inverted repetitions in IS1236 and IS3. (a) Aligned terminal repetitions. Double-lined arrows indicate potentially hybridizing nucleotides in the coding strand of the respective insertion sequences. Vertical lines mark identical nucleotides in the aligned sequences. Right terminal repeat: coding of IS1236 ORFB extends 24 nucleotides beyond the end of ORFB in the aligned IS3 sequence. Left terminal repeat: horizontal lines mark apparent -35 and -10 bp signal sequences for the start of transcription. Regions containing the -35 bp sequence appear to have been conserved, but DNA downstream from these regions has diverged. (b) Divergence of a hairpin loop that may prevent gene expression from readthrough transcripts. Analogous structures in IS1236 and IS3 may block RBSs (shaded sequences marked RBS) expressed as a consequence of transcriptional readthrough from upstream DNA. If the IS promoters were employed, the transcript would begin with the regions forming the loops which would not be formed (Timmerman & Tu, 1985). The respective IS1236 and IS3 hairpin loops have diverged so extensively that their -10 bp regions and RBSs occupy different positions and are protected by different oligonucleotide sequences. Nevertheless, the overall structural requirements for protection against transcriptional readthrough (Timmerman & Tu, 1985) have been maintained.

analogous regions of ORFA from other members of the IS3 family.

The nucleotide sequence in the region where ORFA and ORFB overlap (Fig. 2) suggests that the mechanism of IS1236 expression may be similar to that in the three best-characterized members of the IS3 family: IS911 (Polard *et al.*, 1991), IS150 (Vögele *et al.*, 1991) and IS3 (Sekine *et al.*, 1994). In these three IS elements, the transposase protein is believed to be an ORFA-ORFB fusion protein produced by a -1 translational frameshift between the overlapping reading frames. As shown in Fig. 2, IS1236 has a potential frameshift window, A AAA AAA, followed by a region of potential RNA secondary structure. The features are in an equivalent position to those shown to be involved in translational regulation in IS3 (Chandler & Fayet, 1993; Sekine *et al.*, 1994). Furthermore, in IS1236 a potential ORFB initiation codon, AUU, shown to be used in IS911 (Polard *et al.*, 1991), overlaps the ORFA termination codon in the region of potential RNA secondary structure. An analogous situation is found in IS3 where the RNA secondary structure has been shown to couple translation of ORFB to that of ORFA (Sekine *et al.*, 1994).

The observed copy number of seven for IS1236 in the examined *A. calcoaceticus* strain roughly matches the four to six IS3 copies observed in laboratory strains of *E. coli* (Galas & Chandler, 1989), but generalization on the basis

of this data would be unwise. The 71 *E. coli* natural isolates in the ECOR collection (Pennsylvania State University, USA) averaged significantly less in copies of IS3 (Sawyer *et al.*, 1987). Other investigators, working with different representatives of the same cell line as *A. calcoaceticus* ADP1, found respective IS1236 copy numbers of three and four (M. Stark & N. Kaplan, personal communication; Zhitomirsky *et al.*, 1994).

In accord with results generally observed with *E. coli* IS3, sites of IS1236 insertion in *pobR* are flanked by a 3 bp sequence repetition (Galas & Chandler, 1989). Only occasionally have variations been observed with *E. coli*: in one study, 2 out of 12 IS3 insertions resulted in 4 bp target site duplications (Spielmann-Ryser *et al.*, 1991).

Distinctive properties of IS1236

Behaviour of IS1236 upon insertion is not consistently similar to that generally observed with other IS3 family members. Three of the four IS1236 insertions in *pcaH* were not accompanied by acquisition of a flanking 3 bp nucleotide sequence repetition and, in one instance, insertion was accompanied by loss of 4 bp from the left terminus of IS1236 (Fig. 3). Other investigations (Machida & Machida, 1987) have shown that mutations within an insertion sequence affect the length of duplication introduced at its target site; the present study suggests that the target site itself may influence the length of the duplication.

The apparent presence of IS1236 in 11 of 13 spontaneous *pobR* mutations suggests that this gene is particularly susceptible to mutagenesis caused by the insertion sequence. This observation reinforces the conclusion that a general description of IS1236 behaviour will have to take into account properties of its target DNA. Although there is no obvious target site consensus sequence, in eight of the nine observed instances the 20 bp centred on the insertion site is relatively G + C rich (45–65 mol%) compared to the mutated gene.

Examination of the respective IS1236 and IS3 terminal repeats (Fig. 4) reveals substantial divergence. As shown in Fig. 4(a), the amino acid coding function in the IS1236 right terminal repeat extends eight amino acid residues beyond the end of this function in IS3. In the left terminal repeat, extensive nucleotide sequence divergence follows the conserved –35 bp sequence associated with transcriptional initiation. Fig. 4(b) demonstrates that, although the nucleotide sequences have diverged, they appear to have maintained analogous RNA hairpin structures that may protect ORFA expression against transcriptional readthrough (Timmerman & Tu, 1985). The trait appears to confer a significant selective benefit because it has been conserved despite extensive nucleotide sequence divergence.

Traits conserved by members of the IS3 family have been maintained despite substantial differences in the G + C content of divergent insertion sequences. Thus the G + C content of IS1236 is 42.2 mol%, in accord with the respective G + C contents of 42.1 and 42.5 mol% for the target genes *pcaH* and *pobR*. Such G + C contents are typical for *A. calcoaceticus* genes (Shanley *et al.*, 1994; White *et al.*, 1990) and well below those observed for genes from other organisms containing members of the IS3 family in their chromosomes (Galas & Chandler, 1989; Lawrence *et al.*, 1992). For example, a member of the IS3 family in *Mycobacterium smegmatis* has a G + C content of 63.4 mol% (Garcia *et al.*, 1994). Such findings indicate that the insertion sequences have diverged from a common ancestor to acquire the G + C content characteristic of their respective hosts.

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