A new insertion sequence IS1452 from *Acetobacter pasteurianus*

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A new insertion sequence element, IS1452, was found to be associated with inactivation of the alcohol dehydrogenase by insertion in the *adhS* gene encoding subunit III of the three-component membrane-bound alcohol dehydrogenase complex in *Acetobacter pasteurianus*. Cloning and sequencing analyses of the mutated subunit III gene locus revealed that IS1452 was inserted at or near the ribosome-binding sequence of *adhS*. Analysis of transcription using the chloramphenicol acetyltransferase gene as the reporter indicated that IS1452 abolished transcription of *adhS* by separating its promoter from the subunit III structural gene. IS1452 was 1411 bp in length and had a terminal inverted repeat of 21 bp. IS1452 contained one long ORF of 416 amino acids rich in basic amino acids. This protein showed homology with a putative transposase, Tra1, of IS701 isolated from the cyanobacterium *Calothrix* species PCC 7601. Like IS701, IS1452 was found to generate a 4 bp direct repeat at the site of insertion upon transposition. The target site specificity was rather strict, and a CTA(A or G) sequence appeared to be preferentially recognized. Transposition of IS1452 was replicative, since it was accompanied by an increase in the copy number of IS1452. Several strains belonging to the genus *Acetobacter* also contained IS1452 at varying copy numbers from one to more than ten. These observations suggest that IS1452 is one of the insertion sequences that are responsible for genetic instability leading to deficiencies in various physiological properties in acetic acid bacteria.

Keywords: *Acetobacter pasteurianus*, insertion sequence, alcohol dehydrogenase, genetic instability

INTRODUCTION

Acetic acid bacteria are Gram-negative obligately aerobic bacteria consisting of two genera, *Acetobacter* and *Gluconobacter*. They are characterized by their ability to oxidize ethanol into acetic acid and their resistance to acetic acid and ethanol (De Ley *et al.*, 1984). Because of these characteristics, *Acetobacter* spp. are used for the industrial production of vinegar. We have studied the biochemistry and genetics of ethanol oxidation by acetic acid bacteria through purification, characterization and gene cloning of membrane-bound alcohol dehydrogen-

ase (ADH) (Kondo *et al.*, 1995; Takemura *et al.*, 1993b; Tamaki *et al.*, 1991; Tayama *et al.*, 1989), membrane-bound aldehyde dehydrogenase (ALDH) (Fukaya *et al.*, 1989a, b) and terminal ubiquinol oxidase (Fukaya *et al.*, 1993). ADH of *Acetobacter pasteurianus* NCI 1380 consists of three components, a 78 kDa alcohol dehydrogenase (subunit I), a 48 kDa cytochrome *c* (subunit II), and a 20 kDa protein (subunit III). During these studies, we have found that insertion of a 1665 bp insertion sequence (IS), IS1380, into a TCGA sequence within the cytochrome *c* gene inactivates the ethanol-oxidizing ability (Takemura *et al.*, 1991). This type of mutation was observed in most spontaneous mutants found to be deficient in ethanol oxidation. In addition, the copy number of IS1380 in cells of strain NCI 1380 was about 100, and this IS was distributed in several *Acetobacter* spp. at a similar high copy number. These observations indicate that the observed genetic instabili-

Abbreviations: ADH, membrane-bound alcohol dehydrogenase; CAT, chloramphenicol acetyltransferase; IS, insertion sequence.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is D63923.

ties in ethanol oxidation may be explained in terms of IS1380 insertion into the cytochrome *c* gene.

In addition to the genetic instability in ethanol oxidation in *Acetobacter* spp., spontaneous mutations resulting in deficiency in acetic acid resistance (Ohmori *et al.*, 1980) and cellulose formation (Cook & Colvin, 1980; Steel & Walker, 1957) arise at high frequencies. Investigations have revealed that insertion sequences, such as IS1031s (from IS1031A to IS1031D) and IS1032, participate in the inactivation of several genes (Coucheron, 1991, 1993; Iversen *et al.*, 1994). It is thus apparent that multiple insertion sequences play a major role in generating spontaneous mutations leading to deficiencies in various physiological properties.

In the present study with *A. pasteurianus* NCI 1452, we found a new insertion sequence, named IS1452, in the ADH subunit III (*adhS*) gene in spontaneous mutants deficient in ethanol oxidation. Subunit III is required for enzyme activity of the three-component type ADH (Kondo *et al.*, 1995). This paper describes the characterization and sequencing of IS1452. It is present as multiple copies in *A. pasteurianus* and distributed in several *Acetobacter* spp. It is therefore likely that IS1452 is another type of IS that can cause spontaneous mutations at high frequencies in acetic acid bacteria.

METHODS

Bacterial strains and plasmids. All of the bacterial strains and plasmids used are listed in Table 1. *A. pasteurianus* NCI 1452, a restriction-deficient mutant, was isolated from *A. pasteurianus* NCI 1380 by NTG treatment (Takemura *et al.*, 1993b).

Media and culture conditions. YPG medium (pH 6.5) consisted of 5 g yeast extract (Wako Pure Chemicals), 2 g polypeptone (Wako), and 30 g glucose in 1 l water. Each strain of acetic acid bacteria was first cultured in a 50 ml test tube containing 5 ml YPG medium with shaking for 24–40 h at 30 °C. From 1 to 5 ml of the broth was inoculated into 100 ml YPG medium in a 500 ml shaking flask and further cultured with shaking at 30 °C. When necessary, ethanol at a final concentration of 3% (v/v) was added to the medium at the start of the culture. *Escherichia coli* strains were routinely cultured in Luria broth (Sambrook *et al.*, 1989). When necessary, ampicillin was added to the medium at a final concentration of 50 µg ml⁻¹.

DNA preparation and manipulation. Total DNA of acetic acid bacteria was prepared as described by Okumura *et al.* (1985). DNA–DNA hybridization was performed by standard methods (Sambrook *et al.*, 1989; Southern, 1975) with a nylon membrane (Hybond-N⁺; Amersham International). [α -³²P]dCTP at 3 kCi mmol⁻¹ (111 TBq mmol⁻¹) for the Multiprime DNA labelling system was purchased from Amersham. Restriction endonucleases, Klenow fragment, *Taq* DNA polymerase, T4 DNA polymerase and T4 DNA ligase were purchased from Takara Shuzo. *E. coli* was transformed as described by Hanahan (1983). *Acetobacter* strains were transformed by electroporation as described by Wong *et al.* (1990). Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) combined with the M13 cloning system (Yanisch-Perron *et al.*, 1985) on a DSQ-1 (Shimadzu) DNA sequencer. General recombinant DNA techniques were as described by Sambrook *et al.* (1989).

Determination of the target site of IS1452. The 3.5 and 4.0 kb *EcoRV* fragments, which had been shown by Southern hybridization to contain IS1452, were cloned from the total DNA of mutant m00-09 into the *SmaI* site of pUC18. The recombinant plasmids possessing these fragments were used as templates for amplification with *Taq* DNA polymerase by PCR of the region covering the target site of IS1452. Four primers used were: IS1452-N-primer (5'-CCGAATTCAGCGTCTCTTCAACAG-3', which extends from nt 98 to 77 of IS1452 (see Fig. 2), with three bases added to the 5'-end of the oligonucleotide to generate an *EcoRI* site); IS1452-C-primer (5'-AAAGCTTGCACAACGCAGACTACCC-3', which extends from nt 1299 to 1319, with four bases added to the 5'-end of the oligonucleotide to generate a *HindIII* site); M13-47 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'); and RV-M (5'-GAGCGGATAACAATTTACACAGG-3'). IS1452-N- and IS1452-C-primers were synthesized on an Expedite Nucleic Acid Synthesis System DNA synthesizer (PerSeptive Biosystems). M13-47 and RV-M were sequencing primers purchased from Takara Shuzo. The fragments amplified by PCR with the appropriate sets of primers were then cloned onto pUC18 and their nucleotide sequences covering both ends of IS1452 were determined. Five of each clone bearing the respective fragments were randomly picked up and independently sequenced.

Promoter assays. Cell extracts were prepared with an ultrasonifier (Branson Sonifier cell disrupter 250) from mid- or late-exponential-phase cells suspended in 10 mM potassium phosphate buffer (pH 6.0). Chloramphenicol acetyltransferase (CAT) activity was measured as described by Shaw (1975). We used pMVC18, an *Acetobacter*–*E. coli* shuttle-plasmid carrying a promoterless CAT gene, for promoter-probing experiments (Takemura *et al.*, 1993b). DNA fragments to be tested were prepared from pADSm911 containing the originally cloned IS1452 sequence and they were inserted in the *SmaI* site on pMVC18 for the promoter assays. Plasmids pMVC18-m1 and pMVC18-m2 (see Fig. 4) contained the 0.16 kb *SphI*–*NheI* fragment of pADSm911, which had been blunt-ended by T4 DNA polymerase, in the *SmaI* site of pMVC18 in the same orientation as and the opposite orientation to that of the CAT gene, respectively. Similarly, pMVC18-m3 and pMVC18-m4 were constructed using the 0.66 kb *SphI* fragment, and pMVC18-m5 and pMVC18-m6 were constructed using the 0.5 kb *NheI*–*SphI* fragment. For construction of pMVC18-m7 and pMVC18-m8, the 1.6 kb *SphI*–*SplI* fragment was obtained by PCR with the *SphI*-side-primer [5'-GTGGATCCGCTTA-TGCCCCGGCCAAGGCCAGTC-3', which extends nt 1–31 of the *adhS* gene (Kondo *et al.*, 1995), with two bases added to the 5'-end of the oligonucleotide and with two bases changed (underlined) to change the *SphI* site into a *Bam*HI site], and the *SplI*-side-primer (5'-GACAGGGGCACGTACGGCAATCAG-TTTCATTCAG-3', which extends from nt 201 to 168). Plasmid pMVC18-ADH, containing the promoter of *adh* at the *SmaI* site (Takemura *et al.*, 1993b), was used as a positive control of this assay.

Computer-aided analysis of nucleotide and amino acid sequences. The DNA sequence was analysed by using the GENETYX sequence analysis program (Software Development). DNA and amino acid sequence homology searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service (Altschul *et al.*, 1990).

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics*	Reference/source
Strains		
<i>Acetobacter pasteurianus</i>		
NCI 1452	Sm ^r , met, thermophilic, restriction-deficient mutant of NCI 1452	Takemura <i>et al.</i> (1993a, b)
m00-09	Reduced ADH activity (subunit III deficient); spontaneous mutant of NCI 1452	Kondo <i>et al.</i> (1995)
m00-12, m00-14, m12-10, m12-20	Reduced ADH activity (subunit III deficient); spontaneous mutants of NCI 1452	This work
NCIB 7214	Wild-type	NCIB
<i>Acetobacter aceti</i>		
No. 1023	Thermophilic	Ohmori <i>et al.</i> (1980)
IFO 3284	Wild-type	IFO
<i>Acetobacter xylinum</i> NCIB 11664	Wild-type	NCIB
<i>Acetobacter liquefaciens</i> IFO 12388	Wild-type	IFO
<i>Acetobacter Hansenii</i> NCIB 8746	Wild-type	NCIB
<i>Acetobacter diazotrophicus</i> ATCC 49037	Wild-type	ATCC
<i>Acetobacter polyoxogenes</i> NCI 1028	Wild-type	Entani <i>et al.</i> (1985)
<i>Gluconobacter oxydans</i> ATCC 19357	Wild-type	IFO
<i>Gluconobacter suboxydans</i> IFO 12528	Wild-type	ATCC
<i>Gluconobacter asaii</i> IFO 3276	Wild-type	IFO
<i>Gluconobacter cerinus</i> IFO 3267	Wild-type	IFO
<i>Escherichia coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pUC18	Ap ^r , <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pMV24	Ap ^r , <i>lacZ'</i> ; <i>Acetobacter</i> - <i>E. coli</i> shuttle vector	Fukaya <i>et al.</i> (1989b)
pMVC18	Ap ^r , <i>Acetobacter</i> - <i>E. coli</i> shuttle vector containing a promoterless CAT gene	Takemura <i>et al.</i> (1991)
pADSm911	Ap ^r , containing an IS1452-inactivated <i>adhS</i> gene on pUC18	This work

* Ap^r, ampicillin resistance; Sm^r, streptomycin resistance.

RESULTS AND DISCUSSION

Isolation and cloning of the mutated *adhS* gene

In our previous work (Kondo *et al.*, 1995), we demonstrated that the smallest 20 kDa subunit (subunit III) was essential for the enzymic activity of three-component ADH from *A. pasteurianus*. During this study, we characterized various spontaneous mutants which lacked the ethanol-oxidizing ability. One of the mutant strains, m00-09, lacked subunit III (*adhS*), as determined by immunoblotting with anti-subunit III antibody, and introduction of the *adhS* gene on a plasmid into mutant m00-09 caused production of subunit III (Kondo *et al.*, 1995). Hybridization of *EcoRV* digests of the chromosomal DNA from mutant m00-09 with the *adhS* probe revealed that the positive hybridization signal (6.5 kb) was larger than the expected 5.0 kb (Fig. 1a, b). This suggested that the mutation of strain m00-09 was due to an insertion and not a point mutation. The restriction map of the cloned 6.5 kb fragment showed that a 1.5 kb DNA sequence was inserted somewhere between the *SphI* and *PstI* sites in the first half of the gene (Fig. 1b).

We next examined the presence of the 1.5 kb fragment in the *adhS* gene sequence in other mutants deficient in production of subunit III. Four such mutants among our collection gave the same Southern hybridization pattern (Fig. 1a), suggesting that they had the same insertion as mutant m00-09.

Nucleotide sequence of IS1452

After determining the nucleotide sequence covering the ATG codon as well as the whole *adhS* gene, the 1.5 kb fragment was found to be inserted at or near the ribosome-binding sequence of *adhS* (Fig. 6) with a duplication of 4 bp (CTAG) at the target site. The inserted fragment was 1411 bp long and was bounded by a perfect 21 bp inverted repeat (Fig. 2). The nucleotide sequence of the coding region of *adhS* was the same as that determined for the parental strain, indicating that the mutant phenotype resulted from the insertion alone.

There is one long ORF within the insertion (ORF416) starting with ATG (nt 46–48) and terminating with TAA (nt 1294–1296), encoding 416 amino acids with a

Fig. 1. Detection of an insertion sequence in the *adhS* gene by Southern blot hybridization (a) and restriction maps of the *adhS* genes cloned from the parental strain (pAD5141) and mutant strain m00-09 (pADSm911) (b). (a) The *EcoRV*-digested chromosomal DNA from the parental strain gives a 5.0 kb positive signal when probed with a 32 P-labelled 0.7 kb *SphI* fragment (lane 1). Similar hybridization experiments with the chromosomal DNAs from mutant strains m00-09 (lane 2), m00-12 (lane 3), m00-14 (lane 4), m12-10 (lane 5) and m12-20 (lane 6) show a 6.5 kb signal, suggesting the presence of an IS of 1.5 kb. (b) The 6.5 kb fragment from mutant m00-09 was cloned and its restriction map was constructed. The precise insertion point of a 1.5 kb fragment adjacent to the *adhS* gene was determined by nucleotide sequencing. The arrows indicate the location and direction of *adhS*. The promoter of *adhS* is indicated by a circled 'p'. The probes used for hybridization are indicated by double heavy bars. Restriction enzymes: Ei, *EcoRI*; EV, *EcoRV*; Na, *NaeI*; Nc, *NcoI*; Nh, *NheI*; Pm, *PmaCI*; Ps, *PstI*; Sh, *SphI*; Sm, *SmaI*.

Fig. 2. Nucleotide sequence of IS1452 and deduced amino acid sequence of ORF416. Terminal inverted-repeat sequences are indicated by opposing arrows. Primer sequences for amplification of the target sites of IS1452 are indicated by dotted underlines.

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ORF416: 1 MIQDMHSGGTSVEETLEHARSLSAKDRMAFLPTQKRVVDSACAFDLVL-IGNEPRKTGMRAEAGDPGWRQALLG
Tral : 1 MVQPRPAAPTVEFVDEYCCQWYKSLFPDVRSE-APKYLHVGCISDLKRLTPEIAKIVGLNQQLHFLT
ORF416: 80 RGHWDADLRDVVRDYVIEHLGTEEGVLVIDETGFLKQASCGVGRQYTSAGKINCIGVFGAYSERGHAFIDRAL
Tral : 71 TSPWDIEKRLTLELILQVLKGRPIILLIDETGDKKRGSKTDYVQRQYIGNLGTONGIVAV-TVYGVFCGTF--PL
ORF416: 160 YLPKDMTSPKPERLKRANVPDDVFATPALASHMIESIAGVFFRWVAADSVYGVGDVE--RTLRRAGIGYLVGVGNH
Tral : 147 -LFEVY--K----PRERLQAGQYRTKPEIAAILIKKLQSMGPKFNLVLADSLYGSXKMFSLVDLMLAYIVAIRSNH
ORF416: 238 WFGSWATEPLIAGEAKDIAAGLPDQAMRLSAGHTKGERLYDWAYLPLADLDAEEFDCPIAGPWTGELLIRNADIADGL
Tral : 220 -----YVEILPRQHIQYLVKQKQFQVFSLSRENRFIRELIIPGKRGELRYWQITDPENLPDPT
ORF416: 318 AYFTTSPKQTTTQBLNVVEGTRMIREEGFETAKNEFGLOHNETRSMHGWHRHVSVMLAYAVMASRVYQANSLKPKKTQ
Tral : 279 TWYMSKYFDITPREVGNFYGLRTWVYGLKQSKNELGSDPLRTHYFDIEMRVNLYQLCLPVGWCIRSNCFSLHHNESQ
ORF416: 398 LRTQSLSAGPFRSGASS
Tral : 359 NLPHILGIMEMAGRTFLTIPV

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Fig. 3. Comparison of amino acid sequences of the putative transposases on IS1452 (ORF416) and IS701 (Tra1). Identical and conserved residues are indicated by asterisks and stops, respectively. The amino acids that probably form a DDE motif are indicated by underlined bold letters.

calculated molecular mass of 46 kDa. ORF416 shows considerable similarity in amino acid sequence to Tra1 (Fig. 3), a probable transposase of IS701 from the cyanobacterium *Calothrix* sp. PCC 7601 (Mazel *et al.*, 1991). These two proteins show about 30% identity in the primary sequence, especially in the NH₂-terminal regions. Both transposases contain a DDE motif (Fig. 3) which is conserved among bacterial IS transposases and retroviral/retrotransposon integrases as proposed by Kulkosky *et al.* (1992) and Grindley & Leschzinner (1995). Like other transposases (Galas & Chandler, 1989), ORF416 is rich in basic amino acids.

All of the above observations indicate that the DNA insert is a new IS element of *A. pasteurianus*. We named it IS1452 and registered it in the Plasmid Reference Center (Stanford, CA, USA) (Lederberg, 1987).

Promoter activity analysis of IS1452 and *adhS*

Insertion of IS1452 between the promoter region and the non-mutated coding region of the *adhS* gene in m00-09 suggested that it had interrupted expression of the *adhS* gene at the transcriptional level. Hence we assessed promoter activities of several regions in the 2.5 kb *SphI* fragment (Fig. 4) using the CAT gene on pMVC18 as a reporter gene. Previous work (Kondo *et al.*, 1995) has shown the presence of the promoter of *adhS* to be downstream of the *SphI* site. Recombinant reported constructs from pMVC18-m1 to pMVC18-m8 were introduced into *A. pasteurianus* NCI 1452 by electroporation (Wong *et al.*, 1990), and the CAT activities of the transformants are summarized in Fig. 4. The *adhS* promoter in pMVC18-m1 showed a one-order lower activity than the *adh* promoter in pMVC18-ADH as a positive control. It is apparent that transcription of the *adhS*-encoding sequence in pMVC18-m7 is almost completely abolished, due to a polar effect of IS1452 inserted between the promoter and the ATG start codon. The transcriptional read-through of the ORF416 sequence from the *adhS* promoter in pMVC18-m3 is detected, although the activity is somewhat reduced.

Fig. 4. Construction of plasmids for transcriptional assays and promoter activities of indicated fragments. The restriction map of the 2.5 kb *SphI* fragment including the *adhS* gene and IS1452 from *A. pasteurianus* m00-09 is shown at the top. Each fragment from m1 to m8 was inserted into the *SmaI* site of pMVC18 (Takemura *et al.*, 1993b), resulting in plasmids pMVC-m1 to -m8. Short arrows indicate the direction of transcription of the CAT gene. CAT activity of the transformants cultured in the presence of ethanol was assayed as described in Methods. pMV18-ADH is a positive clone control and pMV18 is the vector negative control. Restriction enzymes: Na, *NaeI*; Nc, *NcoI*; Nh, *NheI*; Sh, *SphI*; Sl, *SplI*.

The low CAT activity from pMVC18-m5 may be due to the promoter for the ORF416 transposase protein. However, the low activity is in agreement with the observations that the expression of transposases is generally weak (Galas & Chandler, 1989). Although strong transcriptional activity in the opposite orientation to that of the transposases is observed in several IS elements, such as IS10R (Kleckner, 1989) and IS1186 (Podglajen *et al.*, 1994), this is not the case for IS1452. The fragment in pMVC18-m6 shows little or no outward promoter activity.

Replicative transposition of IS1452

Most IS elements are present at a multiple copy number in a cell (Galas & Chandler, 1989). To estimate the copy number of IS1452 in the parental strain (NCI 1452) and mutant strain (m00-09), we carried out Southern blot analysis against the restriction digests of total DNA of the two strains by using a 0.7 kb *NcoI* fragment as the probe (Fig. 1b). To obtain the precise copy number, we digested the chromosomal DNAs with three different restriction enzymes, *NcoI*, *EcoRV* and *SphI*. The *NcoI*-digest of the chromosomal DNA from the parental strain gave a single hybridization signal of 0.7 kb (Fig. 5, lane 1). The *EcoRV*-digest gave five signals (lane 2), indicating the presence of five copies of IS1452 in the parental strain, since *EcoRV* does not cleave the IS1452-encoding sequence. This was confirmed by the experiment with *SphI* which cleaves it into two; there are ten signals in the *SphI*-digest (lane 3). The copy number of IS1452 in strain NCI 1380, the parental strain of NCI 1452, was also determined to be five (data not shown).

On the other hand, the copy number of IS1452 in mutant m00-09 was calculated to be six, because the *EcoRV*-digest of total DNA from this mutant gave six positive signals (lane 4) and the *SphI*-digest gave 12 signals (lane 5). It is evident that transposition of IS1452 is accompanied by an increase in the copy number of the transposon. Since no change in electrophoretic mobilities of the original ten bands in the *SphI*-digests is

Fig. 5. Copy number analysis of IS1452 by Southern blot hybridization. The total DNAs from *A. pasteurianus* NCI 1452 (parental) and mutant strain m00-09 were probed with a ³²P-labelled 0.7 kb *NcoI* fragment within the IS1452 sequence. The total DNA from strain NCI 1452 (left panel) was digested with *NcoI* (lane 1), *EcoRV* (lane 2), and *SphI* (lane 3). The total DNA from mutant m00-09 (right panel) was digested with *EcoRV* (lane 4) and *SphI* (lane 5). Arrowheads indicate the bands observed only in the mutant strain.

observed, a newly copied IS1452 presumably inserts at the new site, whereas the template IS1452 remains at the original site. We therefore conclude that transposition of IS1452 is replicative. Replicative transposition was also observed for the four mutations by IS1452 deficient in production of subunit III as described above (data not shown).

Target site specificity of IS1452

To determine the target site specificity, we cloned the 3.5 and 4.0 kb *EcoRV* fragments giving a positive signal in DNA-DNA hybridization with the IS1452 sequence (Fig. 1b) as probe. Nucleotide sequencing of the regions containing the target sites showed that both contained a

Fig. 6. Determination of the target sites of IS1452 and the adjacent nucleotide sequences in *A. pasteurianus* m00-09. The target sites of IS1452 in the indicated fragments giving a positive signal by Southern hybridization are shown by underlined bold letters. Southern blot hybridization was carried out between the *EcoRV*-digested total DNA from mutant m00-09 and a 32 P-labelled 0.7 kb *NcoI* fragment within the IS1452 sequence. The IS1452 in the *adhS* gene was contained in the 6.5 kb fragment. Thin arrow indicates the NH₂-terminal region and its direction of *adhS*. A probable ribosome-binding (SD) sequence (Shine & Dalgarno, 1974) of *adhS* is marked. Heavy arrows indicate the direction of ORF416.

target site duplication of 4 bp, CTAA (Fig. 6). The duplication of CTAA is the same as that observed for the insertion of IS701 in the cyanobacterium *Calothrix* sp. (Mazel *et al.*, 1991). This is in agreement with the similarity of the putative transposases encoded by IS1452 and IS701, because the target repeat reflects the characteristics of transposases involved in generating staggered cuts as an early event in transposition. It is therefore apparent that the target site specificity of IS1452 may be relatively strict, and that a CTA(A or G) sequence may be preferentially recognized. The nucleotide sequences outside the IS1452 sequence suggested that the copies in the 3.5 and 4.0 kb fragments do not disrupt any ORFs.

Distribution of IS1452 in other acetic acid bacteria

To investigate the distribution of IS1452 in other acetic acid bacteria, we performed Southern blot analysis against *EcoRV*-digested total DNAs from a number of acetic acid bacteria by using the 0.7 kb *NcoI* fragment in the IS1452 sequence (Fig. 1b) as probe; the results are shown in Fig. 7. *A. pasteurianus*, *Acetobacter aceti* and *Acetobacter Hansenii* were found to contain IS1452. In comparison with that of IS1380 (Takemura *et al.*, 1991), the copy numbers of IS1452 in these species were relatively low, varying from one to at least ten. IS1380 is present in several *Acetobacter* spp. at a high copy number of approximately 100. The distribution pattern of IS1452 among acetic acid bacteria is also different from that of IS1380 (Takemura *et al.*, 1991). *Acetobacter xylinum* and *Acetobacter polyoxogenes* possess IS1380 but do not possess IS1452. *A. Hansenii* does not possess IS1380 but possesses IS1452.

Comparison between IS1452 and IS1380

IS1452 of 1411 bp in length with a terminal inverted repeat of 21 bp encodes a putative transposase of 416 amino acids, and IS1380 of 1665 bp in length with a 15 bp terminal repeat encodes a putative transposase of 461 amino acids. The copy number of IS1452 is rather low, in comparison with that of IS1380 whose copy number is about 100. The former generates a 4 bp [CTA(A or G)] duplication at the site of insertion,

Fig. 7. Distribution of IS1452 in acetic acid bacteria. Southern blot hybridization of a 32 P-labelled 0.7 kb *NcoI* fragment within the IS1452 sequence against *EcoRV*-digested chromosomal DNA from each strain is shown. The presence (+) and absence (-) of IS1380 in each strain is indicated under the autoradiogram.

whereas the latter generates a different 4 bp duplication (TCGA). The difference in target site specificity might reflect the lack of amino acid sequence similarity. The wide distribution of both IS1452 and IS1380 among acetic acid bacteria suggests that they may have spread by horizontal gene transfer. Furthermore, the similarity in amino acid sequence of the putative transposases of IS1452 and IS701 and their same target specificity might suggest the possibility that horizontal gene transfer has occurred between *Acetobacter* and the cyanobacterium *Calothrix*.

Conclusions

Spontaneous mutations at high frequencies in strains of the genus *Acetobacter*, affecting various physiological properties such as acetic acid resistance (Ohmori *et al.*, 1982), ethanol oxidation (Ohmori *et al.*, 1982; Take-

mura *et al.*, 1991), and cellulose production (Cook & Colvin, 1980), have been observed. Our study on the genetic instability of ethanol oxidation in *A. pasteurianus* has led to the discovery of IS1452 which inserts in and disrupts the *adhS* gene, in addition to IS1380 which disrupts the cytochrome *c* gene (Takemura *et al.*, 1991). The present study showed that all of the five mutants deficient in production of subunit III contained IS1452 in the *adhS* gene. In addition, 10–60% of the alcohol dehydrogenase-deficient mutants obtained spontaneously from the culture in the late stationary phase contained IS1380 in the cytochrome *c* gene (unpublished results). The frequent transposition of these transposons observed as insertions in mutants deficient in ethanol oxidation suggests that these indigenous IS elements are responsible for the genetic instability. Although transposition of IS1380 seems to be induced in the late growth stage when the concentration of acetic acid in the medium is increased (Takemura *et al.*, 1991), the mechanism for induction of the transposition of IS1380 as well as IS1452 is still unclear.

In addition to IS1452 and IS1380 associated with inactivation of ethanol oxidation in *A. pasteurianus*, a family of IS1031 elements including IS1032 are known to inactivate cellulose and exopolysaccharide production in *A. xylinum* (Coucheron, 1991, 1993; Iversen *et al.*, 1994). The IS1031 family generate a 3 bp target sequence duplication (TNA, where N represents G, T or C) upon transposition. It is thus apparent that the genetic instability in the genus *Acetobacter* can be ascribed, in most cases, to transposition of multiple indigenous IS elements with multiple copies.

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Received 12 April 1996; revised 8 July 1996; accepted 9 September 1996.