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Molecular and Genetic Characterization of an *Alcaligenes eutrophus* Insertion Element

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An insertion element, ISAE*1*, was discovered during the molecular analysis of mutants defective in the autotrophic growth (Aut$^-$) of *Alcaligenes eutrophus* H1-4, a mitomycin C-generated derivative of strain H1. ISAE*1* is 1,313 bp long, has 12-bp nearly perfect inverted terminal repeats, and contains an open reading frame that has a coding capacity of 408 amino acids. Direct repeats of 8 bp were generated by insertion of ISAE*1* into chromosomes or plasmids. Most insertions were found in the AT-rich target sites. The distribution of ISAE*1* is limited to *A. eutrophus* H1 (ATCC 17698) and H16 (ATCC 17699). Variants with newly transposed copies of ISAE*1* could be isolated at an elevated frequency by changing the growth conditions.

Insertion sequence (IS) elements are stretches of DNA that only encode genes related to their own transposition (for a review, see reference 11). IS elements from various prokaryotes are generally 800 to 2,500 bp long and have nearly perfect inverted terminal repeats ranging in length from 8 to 41 bp. IS elements, upon insertion, can generate direct repeats of their target DNA sequences. The number of duplicated target DNA sequences, ranging in length from 2 to 13 bp, is characteristic of the element. Transposition of an IS element can lead to insertional inactivation of a gene or an operon and thus enable the identification of the transposed IS element (11, 12).

Alcaligenes eutrophus is a gram-negative facultative chemolithoautotrophic (autotrophic) bacterium that can oxidize hydrogen gas to generate energy for carbon dioxide fixation. Hydrogen oxidation is carried out by two nickel-containing hydrogenase systems, a soluble NAD^+-reducing hydrogenase (Hos) system and a membrane-bound particulate hydrogenase (Hop) system (23, 24). Large plasmids (350 to 450 kb) carrying structural genes of both hydrogenases have been identified in several *A. eutrophus* strains (8, 15, 27). The plasmids found in *A. eutrophus* H1 and H16 are, respectively, pAE1 and pHG1 (15, 27). Mutants defective in either the soluble or the particulate hydrogenase but not both retain the ability to grow autotrophically (5, 13, 27). Tn*5* mutagenesis was performed with Hos$^-$ strain H1-4 to facilitate the isolation of Hop$^-$ mutants. Physical analyses of two of the Hop$^-$ mutants derived from strain H1-4 revealed that DNA unrelated to Tn*5* had been inserted into the DNA involved in the expression of the particulate hydrogenase. The new insertions were found to be caused by a new IS element, ISAE*1*, whose characteristics are reported in this communication.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions; Tn*5* mutagenesis; and enzyme assays. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* was grown on Luria-Bertani medium (22) at 37°C, and *A. eutrophus* was grown on TY medium (3) at 30°C. For autotrophic growth conditions, cells were grown on HUM medium (16) and incubated in an atmosphere of 80% hydrogen–10% oxygen–10% carbon dioxide at 30°C. Tn*5* mutagenesis was performed by conjugation between *E. coli* SM10-1011 (25) and *A. eutrophus* H1-4 (nalidixic acid resistant), and transconjugants were selected on TY plates containing 200 μg of kanamycin per ml and 100 μg of nalidixic acid per ml as previously described (5). HUMf (HUM medium supplemented with 1% fructose) was used as a minimal medium for identifying auxotrophic mutants, which represented approximately 1.3% of the Kan^r transconjugants. FMM medium (17), containing formic acid as the carbon source, was used for screening putative mutants with a defective carbon dioxide fixation system (Cfx$^-$). The activities of the particulate hydrogenase were determined by measuring the hydrogen-dependent methylene blue reduction of partially purified membrane fractions as previously described (5). Clones of *E. coli* DH5α harboring recombinant pUC or pBlueScript plasmids were identified on Luria-Bertani agar plates supplemented with 100 μg of ampicillin per ml, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml, and 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Difco Laboratories (Detroit, Mich.).

DNA biochemistry. Plasmid profiles of *A. eutrophus* were obtained by an in-well lysis procedure (9). Plasmid DNA of *A. eutrophus* was purified by CsCl-ethidium bromide gradient centrifugation as described by Chow et al. (5). The genomic DNA was prepared by a CTAB (cetyltrimethylammonium bromide) extraction procedure (1). *E. coli* plasmid DNA was isolated by the quick boiling method (22). Standard DNA manipulation procedures, including restriction endonuclease digestion, agarose gel electrophoresis, ligation, and Southern transfer to Hybond-N (Amersham, Buckinghamshire, England), were performed in accordance with manufacturer instructions or as described by Sambrook et al. (22). Enzymes were supplied by Boehringer Mannheim GmbH Biochemical (Mannheim, Germany) and GIBCO/Bethesda Research Laboratories (BRL). DNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by use of nick translation kits (BRL) or multiprime DNA labeling systems (Amersham). DNA hybridizations were performed at 65°C with 6× SSC (1× SSC is 0.15 M

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TABLE 1. Bacterial strains and plasmids used

Strain	Plasmid	Relevant characteristics or genotype[a]	Reference or source
Alcaligenes eutrophus			
H1	pAE1	Aut$^+$ Hop$^+$ Hos$^+$ Cfx$^+$; wild type	ATCC 17698; 27
H1-4	pAE1-4	Aut$^+$ Hop$^+$ Hos$^-$ Cfx$^+$; derived from strain H1 by mitomycin C treatment	27
H1-6	None	Aut$^-$; plasmid cured; derived from strain H1	27
H16	pHG1	Wild type	ATCC 17699; 15
ATCC 17707		Wild type	ATCC 17707
CH34		Wild type	18
Alcaligenes latus			ATCC 29714
Alcaligenes denitrificans			ATCC 15173
Bradyrhizobium japonicum			Strain 110
Escherichia coli			
SM10	pSUP1011	RP4-2-Tc::Mu for Tn*5* mutagenesis	24
DH5α		F$^-$ *hsdR17 recA1 supE44 endA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* φ80d*lacZ*ΔM15	BRL
	pBR322::Tn*5*		2
	pUC19	Ap^r *lacPOZ'*; multiple cloning sites	29
	pBlueScript II	*lacPOZ'*; multiple cloning sites; M13 *ori*	Stratagene
	pCA4-7	Cosmid clone carrying 13.1 kb of DNA from pAE1	5 (see Fig. 1B)
	pE2	12-kb *Eco*RI DNA insert, partially overlapping pCA4-7	5 (see Fig. 1B)
	pE3	0.25-kb *Eco*RI fragment of pCA4-7	This study
	pCA218	3.4-kb *Sal*I plasmid fragment from strain WW4-1	This study
	pCK1	2.6-kb *Eco*RI fragment of pAE1	This study
	pCK3	12-kb *Eco*RI fragment of pAE1	This study
	pCK9	16-kb *Eco*RI fragment of pAE1	This study
	pCK10	1.5-kb *Eco*RI plasmid fragment from strain WW4-1	This study
	pCK11	10-kb *Eco*RI fragment of strain H1	This study

[a] Aut, ability to grow autotrophically; Hop, particulate hydrogenase activity; Hos, soluble hydrogenase activity; Cfx, ability to grow on FMM plates.

NaCl plus 0.015 M sodium citrate)–0.025% nonfat milk–50 μg of salmon sperm DNA per ml and followed by sequential washes of blots at 65°C with 2× SSC–0.5% SDS (sodium dodecyl sulfate) and 1× SSC–0.5% SDS.

DNA sequence determination. The *Eco*RI DNA fragments containing ISAE*1* from strains H1 and H16 were cloned into the *Eco*RI site of either pBlueScript II (Stratagene, La Jolla, Calif.) or pUC19 vector (29) and are listed in Table 1. The newly found transposed ISAE*1* copies were also cloned as *Eco*RI DNA fragments from strains WW4-1 and WW4-4-1. Their corresponding target DNAs were also cloned as *Eco*RI fragments from pCA4-7 (the 0.25-kb *Eco*RI fragment; see Fig. 1B) and genomic DNA of strain WW4-4. The DNAs to be sequenced were constructed by creating unidirectional exonuclease III-generated deletions in pCK10 with the Erase-a-Base System (Promega) in accordance with the instructions of the manufacturer. The sequences of both strands were determined. The chain termination method was done essentially in accordance with the instructions in the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio), with [α-^{35}S]thio-ATP (800 Ci/mmol; New England Nuclear). M13 universal and reverse primers were used for inserts in pUC19. T3, T7, SK, and KS primers (Stratagene) were used for inserts in pBlueScript II.

A computer-assisted homology search was performed with a computer connected to the GenBank Service when sequence data banks and the FASTA program (20) for DNA sequence and protein similarity searches were accessible.

Determination of the target duplication. Cloned plasmid DNA containing an intact copy of ISAE*1* was doubly digested with *Eco*RI plus either *Sal*I or *Xho*I and hybridized to an L- or an R-end probe. The L- and R-end probes were derived from pCK10 in which nested deletions had been made to generate DNA templates for sequence analyses. These L- and R-end probes contained only 200 to 300 bp of ISAE*1* terminal sequences. The hybridization bands corresponding to the L- and R-end junctions were then subcloned into pBlueScript II. Since the *Sal*I and *Xho*I sites are 124 and 286 bp from the L and R ends, the DNA sequences of the insertion junctions were obtained by reading through the L- or R-end sequence of ISAE*1* to the neighboring sequence with a universal or reverse primer.

Nucleotide sequence accession number. The nucleotide sequence of ISAE*1* (see Fig. 2A) has been deposited in GenBank under accession number M86608.

RESULTS AND DISCUSSION

Isolation of Hop$^-$ mutants. Tn*5* was introduced into *A. eutrophus* H1-4 by conjugation. Since parental strain H1-4 lacked soluble hydrogenase activity, mutants defective in either particulate hydrogenase activity or carbon dioxide fixation could not be grown autotrophically (Aut$^-$). Aut$^-$ mutants were picked for further analyses. Strain WW4-7 could not be grown on plates containing formate as a carbon and energy source (FMM plates), suggesting that the mutation affected genes involved in carbon dioxide fixation (10, 17). More than 80% of the Aut$^-$ mutants did not contain a plasmid, as shown by the in-well lysis procedure, and their genomic DNAs failed to show plasmid-specific hybridization to the cloned pAE1 DNA fragments. Moreover, none of these non-plasmid-containing mutants was able to revert back to Aut$^+$. These results indicate that megaplasmid pAE1-4 was cured from these Aut$^-$ mutants. One of the plasmid-cured strains, WW4-4, is shown as a representative strain in Fig. 1A (lanes 3 and 11) and Table 2. Strain H1-4 is

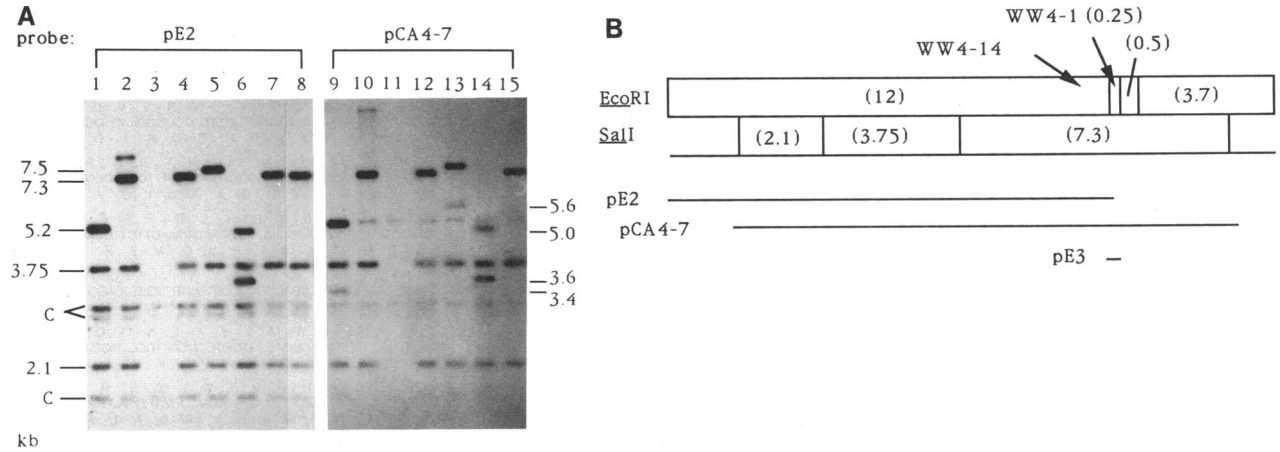

FIG. 1. Southern analysis of DNAs from Aut⁻ mutants of *A. eutrophus* H1-4. (A) Hybridization of ^{32}P-labeled pE2 or pCA4-7 to *Sal*I-digested genomic DNA from WW4-1 (lanes 1 and 9), WW4-3 (lanes 2 and 10), WW4-4 (lanes 3 and 11), WW4-5 (lanes 4 and 12), WW4-8 (lanes 5 and 13), WW4-14 (lanes 6 and 14), WW4-18 (lanes 7 and 15), and H1-4 (lane 8). C, homologous chromosome sequences showing hybridization to the probes. (B) Restriction endonuclease maps of pCA4-7 and pE2. Arrows indicate sites of ISAE*1* insertions. Numbers in parentheses are sizes of restriction fragments in kilobases.

similar to its parental strain, H1, in the high-frequency plasmid curing resulting from Tn*5* mutagenesis and selection (5).

Seven strains lost more than 90% of their ability to reduce methylene blue under hydrogen gas and were considered to be defective in particulate hydrogenase activity (Hop⁻; Table 2). Hybridization of Tn*5* probes (pBR322::Tn*5*) to the plasmids resolved by the in-well lysis procedure indicated that four plasmids from the seven Hop⁻ mutants contained Tn*5*. These four strains could revert back to autotrophy at a frequency comparable to the estimated frequency of excision of Tn*5* from *E. coli* (2).

Discovery of ISAE*1*. In a previous report, two overlapping clones carrying genes responsible for the expression of particulate hydrogenase activity were cloned from pAE1 (5). These two clones, pCA4-7 and pE2, were used as hybridization probes to investigate whether any of the newly isolated Hop⁻ mutants had insertions in this region of pAE1 DNA (Fig. 1A). The 7.3-kb *Sal*I DNA hybridization band of parental strain H1-4 (lanes 8 and 15) was missing and, instead, two new *Sal*I hybridization bands appeared in the genomes of strains WW4-1 (lanes 1 and 9), WW4-8 (lanes 5 and 13), and WW4-14 (lanes 6 and 14). This result suggested that the 7.3-kb *Sal*I DNAs of these strains were interrupted by DNA insertions with at least one internal *Sal*I recognition site. The different hybridization patterns of strain WW4-8 (lanes 5 and 13) were a result of Tn*5* insertion into the 7.3-kb *Sal*I DNA fragment, because the two new fragments also hybridized to the Tn*5* probe (data not shown). Mutations found in strains WW4-1 and WW4-14 could not have resulted from Tn*5* insertions, because the sum of the two new hybridization bands (8.6 kb) was much smaller than the calculated size of the 7.3-kb *Sal*I DNA fragment plus a 5.7-kb Tn*5* insertion (14). Furthermore, the two new hybridization bands did not hybridize to the Tn*5* probe (data not shown). Strains WW4-1 and WW4-14, in contrast to strains with mutations caused by Tn*5* insertions (e.g., strain WW4-8), were not found to regain autotrophy. Many Nif⁻ mutants obtained after Tn*5* mutagenesis have been reported to have resulted from the transposition of an endogenous insertion element of *Rhizobium meliloti* (21). It is likely that the new mutations found in strains WW4-1 and WW4-14 were also due to the transposition of an endogenous insertion element of *A. eutrophus*. This is the first IS element found in *A. eutrophus* H1; mercury-resistant transposons have been reported for strain CH34 (7). This new element is named ISAE*1*.

TABLE 2. Phenotypes of Aut⁻ mutants derived from *A. eutrophus* H1-4

Bacterial strain	Phenotype	Presence of plasmids	Tn*5* insertion site	Reversion frequency[a]
WW4-1	Hop⁻	+	Chromosome	$\ll 1.35 \times 11^{-10}$
WW4-3	Hop⁻	+	Plasmid	1.50×10^{-8}
WW4-4[b]	Aut⁻	−	Chromosome	$\ll 3.80 \times 10^{-10}$
WW4-5	Hop⁻	+	Plasmid	1.92×10^{-7}
WW4-7	Cfx⁻	+	Chromosome	7.69×10^{-10}
WW4-8	Hop⁻	+	Plasmid	4.08×10^{-8}
WW4-14	Hop⁻	+	Chromosome	$\ll 5.50 \times 10^{-10}$
WW4-15	Hop⁻	+	Chromosome	3.36×10^{-8}
WW4-18	Hop⁻	+	Plasmid	2.70×10^{-8}

[a] Calculated as the number of cells grown on HUM medium (autotrophic conditions) or FMM medium per viable count on HUMf medium.

[b] One of the 36 non-plasmid-containing mutants derived from strain H1-4.

Cloning of ISAE*1*. The 3.4-kb *Sal*I DNA fragment (Fig. 1A, lane 9) showing homology to pCA4-7 was cloned from plasmid DNA isolated from strain WW4-1. The resulting clone, pCA218, contained a portion of the newly transposed ISAE*1* and its target DNA. To obtain an intact copy of transposed ISAE*1* in strain WW4-1, we used the insert of pCA218 to screen the library constructed from the *Eco*RI-digested plasmid DNA of strain WW4-1. The resultant clone, with a 1.5-kb *Eco*RI insert, was named pCK10. DNA from strain H1 showed five *Eco*RI bands (0.25, 2.6, 10, 12, and 14 kb) hybridizing to pCK10 (data not shown). Clone pCK10 should therefore contain the transposed copy of ISAE*1* observed in strain WW4-1 plus its target sequence, the 0.25-kb *Eco*RI DNA fragment. The 0.25-kb *Eco*RI DNA fragment was subcloned from pCA4-7 and designated pE3 (Fig. 1B). The nucleotide sequences of pCK10 and pE3 were determined and compared. The nucleotide sequence of pE3

showed very extensive homology (83%) to those of the uptake hydrogenase genes of *Rhodocyclus gelatinosus* and other hydrogen-utilizing bacteria (15a, 28). The ISAE*1* insertion thus leads to the interruption of the particulate hydrogenase gene in mutant strain WW4-1. The DNA sequence present in pCK10 and absent from pE3 is considered to be derived from ISAE*1* and is shown in Fig. 2A. An 8-bp duplication, present only once in pE3, was found flanking ISAE*1* in pCK10 (see below for details).

The 2.6-, 10-, 12-, and 14-kb *Eco*RI fragments showing homology to pCK10 were cloned from the genomic library of strain H1 and designated pCK1, pCK11, pCK3, and pCK9, respectively (Table 1). These four clones should contain copies of ISAE*1* in the genome of strain H1.

General features of ISAE*1*. ISAE*1* is 1,313 bp long and has 12-bp (11 of 12) nearly perfect terminal inverted repeats that show no significant sequence homology to termini of other known prokaryotic insertion elements. The terminal repeats can be further extended to 24 or 43 bp with 6 or 18 mismatches, respectively (Fig. 2B). A restriction map is shown in Fig. 2C. The ends with *Hin*dIII and *Xho*I restriction sites are designated the L and R ends, respectively. A search of the GenBank data base failed to reveal any known transposable element or DNA sequence that shared significant homology with ISAE*1*. ISAE*1* does not contain consensus sequences, such as the binding sites for integration host factor or DnaA protein found in a few *E. coli* insertion elements (2, 11).

ISAE*1* contains two open reading frames (ORFs), each spanning the entire element in opposite orientations. When the first methionine residue is taken as the translational initiation codon, ORF1, starting from the 64th nucleotide from the L end, is predicted to code for a protein of 45.7 kDa with 408 amino acids (Fig. 2A). An *E. coli*-type promoter is located upstream of ORF1; however, sequences resembling a typical ribosome binding site cannot be found (19). The −35 and −10 regions of the putative promoter, 5′-TTGA AT-3′ and 5′-TATCGA-3′, respectively, lie within the L-end inverted repeat (Fig. 2A). The methionine codon of ORF2 is only 14 nucleotides away from the R end. ORF2 contains no in-frame termination codon within the coding capacity of ISAE*1*. This result suggests that the protein product of ORF2, if expressed, can be influenced by the target sequence and with a variable C-terminal amino acid sequence. None of the remaining predicted ORFs has the capacity to code for more than 100 amino acids, and none is preceded by recognizable promoter sequences. The primary amino acid sequences of the two largest ORFs were compared with those deposited in the Swiss-Prot and EMBL libraries. Weak homology, 23.4% identical and 56.9% conserved amino acids, was found between the C-terminal 313 amino acids of ORF1 and a 414-amino-acid ORF of IS*1096* of *Mycobacterium smegmatis* (6). This ORF of IS*1096* was suggested to encode a transposase (TnpA; 6) because of its homology to the Tn*3926* ORF-encoded transposase. The facts that ORF1 is preceded by promoter sequences and that the ORF1 product shows homology to TnpA encoded by IS*1096* lend support to the idea that the product of ORF1 is a candidate protein for transposase. On the other hand, homology was not found between the ORF2 product and proteins encoded by other insertion elements. The existence and possible functions of ORF2 remain to be elucidated.

Distribution of ISAE*1*. The internal fragment (*Hin*dIII-*Xho*I fragment; Fig. 2C) of ISAE*1* was used as a probe for determining the copy number and distribution of ISAE*1* in various hydrogen-utilizing bacteria. Genomic DNA of each

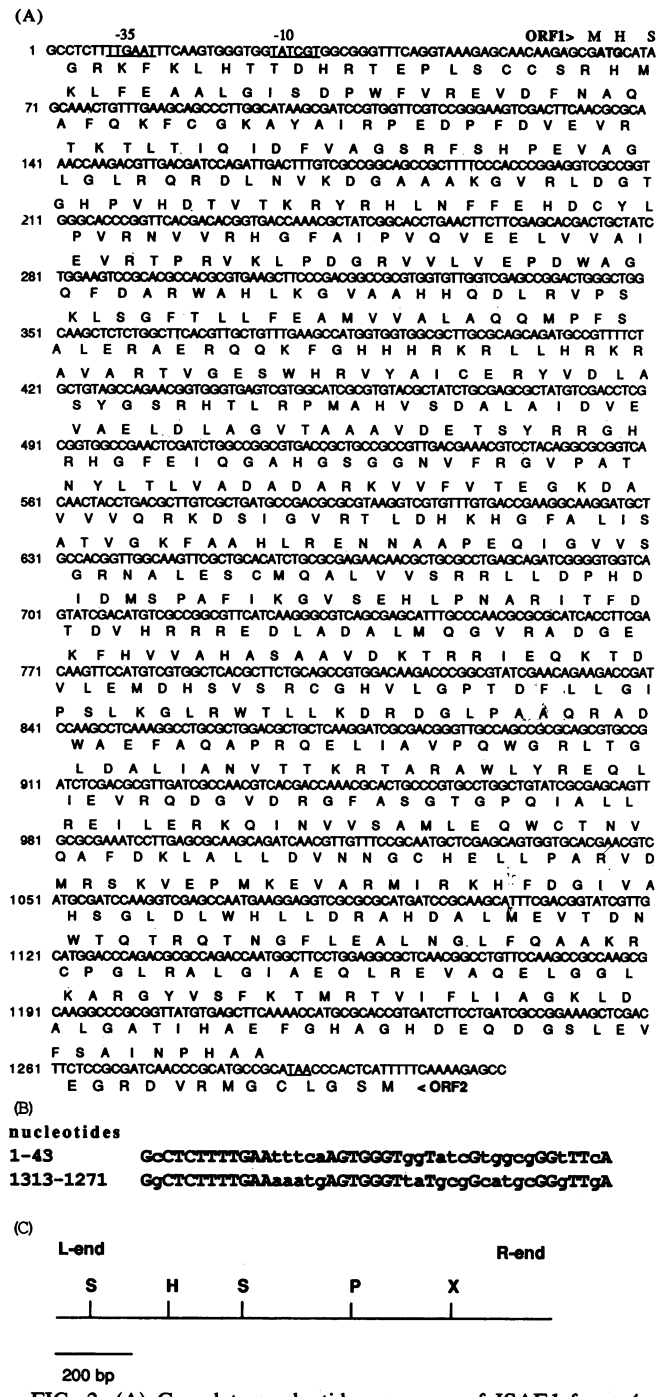

FIG. 2. (A) Complete nucleotide sequence of ISAE*1* from *A. eutrophus*. The sequence of the sense strand for ORF1 is presented and does not include the target duplication found in pCK10. The presumed translational initiation site for ORF1 (nucleotides 64 to 67) is printed in boldface type, and the termination codon (nucleotides 1287 to 1290) is underlined. Also shown are the −35 and −10 regions of a putative promoter for ORF1. The derived amino acid sequence is given in the one-letter code. The directions of the putative ORFs are indicated by short arrowheads. (B) Sequences of the inverted termini. Mismatched bases are presented in lowercase letters. (C) Restriction endonuclease map of ISAE*1*. Restriction endonuclease sites (S, *Sal*I; H, *Hin*dIII; P, *Pst*I; X, *Xho*I) were derived from the nucleotide sequence.

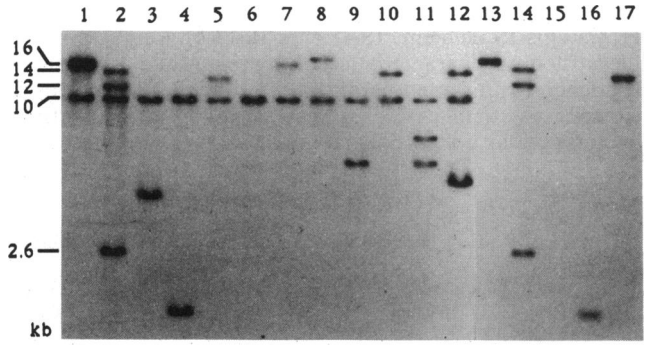

FIG. 3. Distribution and copy number of ISAE1. Genomic DNAs (lanes 1 to 12) and plasmid DNAs (lanes 13 to 17) were digested with *Eco*RI and probed with a labeled *Xho*I-*Hin*dIII fragment of ISAE1. Lanes: 1, H16; 2, H1; 3, H1-4f, derived from a 4-year-old H1-4 frozen stock; 4, WW4-1; 5, WW4-14; 6, H1-6; 7 and 8, WW4-4-1 and WW4-4-2, respectively, both derived from a freeze-thaw treatment of strain WW4-4; 9 to 12, four colonies derived from a 4-year-old H1-6 frozen stock; 13, pHG1; 14, pAE1; 15, pAE1-4; 16, a plasmid of strain WW4-1; 17, a plasmid of strain WW4-14.

TABLE 3. Frequency of ISAE1 transposition in strain WW4-4 under various growth conditions

Culture condition[a]	No. of independent single colonies picked	No. of variants observed[b]	Frequency of occurrence (%)[c]
Control	16	0	0.00
Freeze-thaw	16	3	18.75
37°C	7	1	14.30
Cm^r	15	3	21.00
Tet^r	16	3	18.75
Nov^r	13	2	15.40

[a] Bacteria were grown from independent single colonies, which had been pretreated under the following growth conditions. For the control, bacteria were grown at 30°C. For the freeze-thaw treatment, glycerol was added to stationary cultures to a final concentration of 50%, and then the cultures were stored at −70°C for 2 days. A portion of each frozen culture was inoculated into TY broth, grown at 30°C to the stationary phase, and then stored at −70°C. Single colonies were obtained by growing frozen cultures at 30°C. For the 37°C treatment, cultures were grown on TY plates at 37°C. For Cm^r, Tet^r, and Nov^r, colonies were obtained from TY plates containing 100 μg of chloramphenicol, 10 μg of tetracycline, and 200 μg of novobiocin per ml, respectively. Resistant colonies appeared at a frequency of approximately 1 in every 10^8 cells.

[b] Scored by Southern hybridization with the ISAE1 internal fragment as a probe.

[c] Expressed as the percentage of colonies showing different hybridization patterns for every colony picked for Southern hybridization analysis.

bacterial strain was digested with *Eco*RI because ISAE1 contained no *Eco*RI recognition site. Homology was only found in *A. eutrophus* type strains H16 and H1 (Fig. 3, lanes 1 and 2). A related sequence was not found in *A. eutrophus* ATCC 17707 and CH34 or other hydrogen-utilizing bacteria, such as *Alcaligenes latus*, *Alcaligenes denitrificans*, and *Bradyrhizobium japonicum* (data not shown). This result is consistent with the results of phylogenic studies showing that *A. eutrophus* H1 and H16 are closely related and that *A. eutrophus* ATCC 17707 and CH34, *A. denitrificans*, and *A. latus* differ greatly from the core group (26).

Four and three copies of ISAE1 were present in the genomes of strains H1 (Fig. 3, lane 2) and H16 (lane 1), respectively. The 10-kb *Eco*RI fragment was the only common hybridization band observed in strain H1, strain H16, and the plasmid-cured strain H1-6 (27) (lane 6). Therefore, the 10-kb *Eco*RI fragment contains the chromosomal copy found in the two type strains. The remaining copies of ISAE1 are located on large plasmids (lanes 13 and 14). The 16- and 16.5-kb *Eco*RI fragments of pHG1 were poorly resolved because of their large sizes (lane 13). The ISAE1 internal probe hybridized to two well-separated *Bam*HI bands of pHG1 (data not shown), confirming the fact that pHG1 contained two copies of ISAE1.

pAE1-4 was shown to be at least 50 kb smaller than pAE1 (5, 27). This 50-kb plasmid DNA was known to be rather deletion prone, as similar deletions were also observed in mutants derived from Tn*5* mutagenesis (5). The three plasmid-borne copies of ISAE1 were deleted in strain H1-4, because pAE1-4 did not show any homology to the ISAE1 probe (Fig. 3, lane 15). Recently, one of the three plasmid-borne copies of ISAE1 was mapped to the start site for plasmid deletions found in strain H1-4 and a Tn*5*-generated mutant (28a). Prokaryotic IS elements are known to promote deletions (11). Although there is no direct evidence to indicate that ISAE1 promoted the deletion, the fact that the deletion started at the first nucleotide of ISAE1 suggests that ISAE1 is likely to participate in generating the deletion. More experiments are needed to elucidate the role of ISAE1 in deletion formation.

Transposition of ISAE1. The presence of active IS elements can influence the stability of genomes via transposition or by providing substrates for homologous recombination (11). DNA was purified from cultures grown from streaked single colonies from 4-year-old frozen stocks and probed with the ISAE1 internal fragment to investigate the stability of ISAE1 in the *A. eutrophus* genome. The hybridization patterns of a few colonies derived from frozen stocks of strains H1-4 (Fig. 3, lane 3) and H1-6 (lanes 9 to 12) differed from that of strain H1-6 containing a single chromosomal copy (lane 6). Four different hybridization patterns were observed in colonies derived from the same frozen stock of strain H1-6 (lanes 9 to 12). A maximum of three copies of ISAE1, possibly resulting from two separated transposition events, were found (lanes 11 and 12 versus lanes 9 and 10). Variations in the hybridization pattern were not observed for frozen stocks of strains originally containing three (strain H16) or four (strain H1) copies of ISAE1. These observations suggest the existence of a copy number control mechanism that inhibits ISAE1 transposition as the copy number reaches three or four copies per genome.

The variability in ISAE1 hybridization patterns bears similarities to the situation with IS*892*, which hybridizes differently to DNAs extracted from frozen samples of an *Anabaena* sp. (4). The aforementioned frozen stocks have been through several temperature fluctuations (warming up from −70°C to room temperature). To further study the effects of temperature on ISAE1 transposition, we subjected colonies of the plasmid-cured strain WW4-4 (with one copy of ISAE1) to various temperature treatments. DNA was then purified from cultures of independent single colonies obtained from each treatment. The hybridization patterns for two representative variants, WW4-4-1 and WW4-4-2, derived from the freeze-thaw treatment are shown in Fig. 3 (lanes 7 and 8). New patterns of hybridization to ISAE1 were observed for 18.75 and 14.3% of the independent single colonies obtained from the freeze-thaw and 37°C treatments, respectively (Table 3). In contrast, the hybridization patterns for 16 independent single colonies constantly grown at 30°C remained unchanged. The new hybridization patterns

TABLE 4. Target duplications produced by insertions of ISAE1 in *A. eutrophus* strains

Strain	Source of insertion	Target duplication (5'-3')[a]	No. of A and T residues/8 residues
WW4-1	Plasmid (pCK10)	ATAAGAAT	7
H1	Plasmid (pCK1)	CAAAAACC	5
H1	Plasmid (pCK3)	ATAGAGAG	5
H16	Plasmid	ATAGAGAG	5
H1	Plasmid (pCK9)	CAACAAGA	5
H1	Chromosome (pCK11)	AATCTTTT	7
H16	Chromosome	AATCTTTT	7
H16	Plasmid	TAGTTTCA	6
WW4-4-1	Chromosome	TCTTTTCT	6

[a] Shown according to the orientation of insertion and listed as sequences adjacent to the L end.

observed after temperature treatments could have resulted from ISAE1 transposition or genome rearrangement unrelated to ISAE1. The new hybridization band appearing in strain WW4-4-1 (Fig. 3, lane 7) was cloned and sequenced. A new insertion site was found (Table 4), suggesting that ISAE1 transposition was responsible for the new hybridization pattern observed for strain WW4-4-1. New transpositions of ISAE1 were also observed more frequently for resistant colonies randomly picked from media containing chloramphenicol, tetracycline, or novobiocin (Table 3). Taken together, these results suggest that the transposition of ISAE1 is greatly influenced by growth conditions. Alternatively, higher copy numbers of ISAE1 are highly selected for in subpopulations grown under abnormal growth conditions.

Target sequence and specificity. A comparison between the nucleotide sequences of pCK10 and its target, pE3, revealed that an 8-bp duplication was generated at the insertion site. By Southern hybridization analysis, a DNA sequence homologous to pCK1, containing a 2.6-kb *Eco*RI insert, was found in a 1.3-kb *Eco*RI DNA fragment of pHG1 (data not shown). The DNA sequence of this 1.3-kb DNA fragment of pHG1 turned out to be almost identical to the sequence flanking ISAE1 of pCK1 (data not shown). The sequence of the 8-bp target duplication (5'-CAAAAACC-3') found in pCK1 was present only once in the 1.3-kb *Eco*RI DNA fragment of pHG1. The nucleotide sequences of newly transposed ISAE1 in strain WW4-4-1 (derived from freeze-thaw treatment of strain WW4-4) and its target were also determined. Again, only one copy of the 8-bp duplication was found in the target fragment.

The junction sequences of ISAE1 from strains H1 and H16 were analyzed (Table 4). The sequences of insertion junctions and of a few hundred base pairs surrounding the insertion site in pCK3 were identical to the sequence of the insertion found in the 16-kb *Eco*RI fragment of pHG1. The target sequence and surrounding sequences were also identical for the chromosomal copies of strains H1 and H16 (Table 3). An 8-bp target duplication was found for all of the ISAE1 insertion sites characterized. No target sequence specificity could be deduced from the nine (including two related) sites of insertion generated by ISAE1 in strains H1 and H16 (Table 4). All seven different insertions were found in the A- or T-rich target sites, and most of the insertion sites contained A · T pairs at one end.

Conclusions. An active insertion element of *A. eutrophus* was discovered to be transposed into a particulate hydrogenase gene and to result in the loss of autotrophy in strain H1-4. This IS element, ISAE1, is 1,313 bp long and has all the structural characteristics of a prokaryotic IS element. Evidence is presented here that the transposition of this IS element is stimulated or selected for by the growth of bacteria under unfavorable conditions.

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