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Cloning of Genes Responsible for Acetic Acid Resistance in *Acetobacter aceti*

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Five acetic acid-sensitive mutants of *Acetobacter aceti* subsp. *aceti* no. 1023 were isolated by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Three recombinant plasmids that complemented the mutations were isolated from a gene bank of the chromosome DNA of the parental strain constructed in *Escherichia coli* by using cosmid vector pMVC1. One of these plasmids (pAR1611), carrying about a 30-kilobase-pair (kb) fragment that conferred acetic acid resistance to all five mutants, was further analyzed. Subcloning experiments indicated that a 8.3-kb fragment was sufficient to complement all five mutations. To identify the mutation loci and genes involved in acetic acid resistance, insertional inactivation was performed by insertion of the kanamycin resistance gene derived from *E. coli* plasmid pACYC177 into the cloned 8.3-kb fragment and successive integration into the chromosome of the parental strain. The results suggested that three genes, designated *aarA*, *aarB*, and *aarC*, were responsible for expression of acetic acid resistance. Gene products of these genes were detected by means of overproduction in *E. coli* by use of the *lac* promoter. The amino acid sequence of the *aarA* gene product deduced from the nucleotide sequence was significantly similar to those of the citrate synthases (CSs) of *E. coli* and other bacteria. The *A. aceti* mutants defective in the *aarA* gene were found to lack CS activity, which was restored by introduction of a plasmid containing the *aarA* gene. A mutation in the CS gene of *E. coli* was also complemented by the *aarA* gene. These results indicate that *aarA* is the CS gene.

Acetic acid bacteria, which are composed of two genera, *Acetobacter* and *Gluconobacter*, are unique microorganisms characterized by their strong ability to oxidize alcohols and sugars and high resistance to acetic acid. Recently, the biochemical background of ethanol oxidation has been elucidated by purification and characterization of membrane-bound alcohol dehydrogenase (ADH) and membrane-bound aldehyde dehydrogenase (ALDH), both of which are responsible for oxidation of ethanol into acetic acid (1, 2).

However, there have been few studies on the mechanism of resistance to acetic acid despite its basic and industrial interest. Yamada et al. (30) isolated *Gluconobacter* mutants that were sensitive to acidic pH. However, they did not analyze the acetic acid resistance of their mutants. Resistance to acetic acid does not always result from resistance to low pH. For example, strains capable of growing at low pH cannot grow at the same pH when adjusted with acetic acid. There may be some specific machinery to protect the cells from the toxicity of acetic acid.

To analyze acetic acid resistance in *Acetobacter* strains, we isolated acetic acid-sensitive mutants by mutagenesis. Using these mutants as hosts, we succeeded in cloning the DNA fragment that conferred distinct acetic acid resistance to the mutants and found that at least three genes are required for conferring acetic acid resistance. The nucleotide sequence of one of the genes revealed that it coded for citrate synthase (CS). This paper describes the cloning and characterization of genes responsible for acetic acid resistance in *Acetobacter* strains.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Acetic acid-sensitive mutants of *Acetobacter aceti* subsp. *aceti* no. 1023 were isolated by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG).

Media and culture conditions. *Escherichia coli* was grown in Luria broth (5). YPG medium and growth conditions for derivative strains of *A. aceti* subsp. *aceti* no. 1023 were as those described previously (22). Ampicillin and kanamycin were added, if necessary, at final concentrations of 50 and 80 μg/ml, respectively. Luria broth containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction of the *lac* promoter in *E. coli* JM109. The acetic acid resistance of the *Acetobacter* strains was judged by growth on YPG agar medium containing various concentrations of acetic acid. Ethanol oxidation was measured by assaying the amount of acetic acid in a liquid medium containing, per liter, 30 g of ethanol, 1 g of glucose, 5 g of yeast extract and 2 g of polypeptone (pH 6.0). Acetic acid was determined by titration with 0.1 N sodium hydroxide.

Isolation of acetic acid-sensitive mutants. Cells of *A. aceti* subsp. *aceti* 10-8 were treated with 30 μg of NTG per ml as described previously (21) and incubated on YPG agar medium for several days. Acetic acid-sensitive mutants were selected by replica plating on YPG agar medium containing 20 g of acetic acid per liter, which was the highest concentration allowing growth of the parental strain. About 0.1% of cells that grew after NTG treatment were acetic acid sensitive.

DNA preparation and manipulation. Total DNA and plas-

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics	Source or reference(s)
Strains		
E. coli		
HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rps-120 xyl-5 mtl-1 gyrA96 supE44 leuB6 thi-1*	3
JM109	*recA1* Δ(*lac-pro*) *endA1 gyrA96 thi-1 hsdR17 relA1* F′ *traD36 proAB lacI*^{q}*Z*ΔM15	31
IFO 3208	K-12, wild type	IFO
MOB150	*gltA lct-1 thi-1 lacY1 galK2 xyl-5 ntl-1 tsx-57 tfr-5 rpsL supE44 hsdR4*	28
A. aceti subsp. *aceti*		
No. 1023	Wild type	20
10-8	*pro* Ace^r; mutant derived from no. 1023	23
Plasmids		
pHC79	Ap^r Tc^r *cos*	14
pUC8, -9, -18, -19	Ap^r *lacZ′*	18, 31
pACYC177	Ap^r Km^r	4
pMV102	Cryptic plasmid of *A. aceti* subsp. *xylinum* NBI1002	8
pMV24	Ap^r; shuttle vector between *E. coli* and *Acetobacter* sp.	11
pMVC1	Ap^r *cos*; shuttle vector between *E. coli* and *Acetobacter* sp.	This study

[a] Ace^r, Able to grow in the presence of 20 g of acetic acid per liter; IFO, Institute for Fermentation, Osaka, Japan. pMVC1 (8.4 kb) was constructed by inserting pMV102 between the *Eco*RI and the *Cla*I sites of pHC79.

mid DNAs were prepared by methods described previously (22). Restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase, and T4 DNA polymerase were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan) and used as specified by the manufacturer. Agarose gel electrophoresis was carried out as described previously (22).

E. coli strains were transformed as described by Hanahan (13). *Acetobacter* strains were transformed as described previously (10).

Construction of a cosmid bank of *A. aceti* subsp. *aceti* no. 1023 in *E. coli*. Total DNA of *A. aceti* subsp. *aceti* no. 1023 was partially digested with *Sau*3AI to yield fragments predominantly larger than 20 kilobase pairs (kb). The digested total DNA was ligated to pMVC1 that had been digested with *Bam*HI and dephosphorylated. The ligation mixture was packaged, and the packaging mixture was used for construction of a cosmid bank in *E. coli* HB101 as described previously (11).

Identification of an acetic acid resistance gene. A total of 1,500 Ap^r *E. coli* HB101 colonies of the cosmid bank were divided into 150 pools (each pool contained 10 transformants), and DNAs from each pool were introduced into acetic acid-sensitive mutants by transformation after linearization by digestion with *Pst*I to facilitate efficient recombination because the transformation efficiency was extremely low (less than 10 transformants per μg of DNA) when a covalently closed circular DNA was used as a donor DNA. The linearized DNA was transformed into acetic acid-sensitive mutants. After transformation and incubation in YPG medium overnight, transformants were plated on YPG agar supplemented with 20 g of acetic acid per liter, which completely inhibited growth of the mutants. Then each plasmid DNA from individual members of the pools that allowed growth on the medium described above was treated in the same way to identify plasmids that carried acetic acid resistance genes. Acetic acid-resistant transformants were obtained at an efficiency of about 50 to 100/μg of DNA of a plasmid that complemented the mutations.

For subcloning, the plasmid DNA complementing the mutations was digested with appropriate restriction endonucleases and ligated to pUC18 digested with the same restriction endonuclease. The resultant recombinant plasmids were then linearized with restriction endonucleases and introduced into the mutants to identify a region complementing their mutations.

Insertional inactivation by integration of the Km^r gene. To determine a region responsible for acetic acid resistance, we carried out insertional inactivation experiments as follows. We first constructed chimeric plasmids, which consisted of the vector, the cloned DNA fragments, and the fragment containing the Km^r gene (the *Hae*II fragment). The Km^r gene fragment was produced by digestion of *E. coli* plasmid pACYC177 with *Hae*II and inserted at various restriction sites in the cloned DNA fragment. These plasmids were digested with a restriction endonuclease that did not digest the *Hae*II fragment or its adjacent region, and the resultant linearized plasmid DNA was introduced by transformation into the acetic acid-resistant parental strain, 10-8. Transformants were selected on YPG agar supplemented with 80 μg of kanamycin per ml. We analyzed all Km^r transformants by Southern blot hybridization, using the *Hae*II fragment as well as the cloned DNA fragment as probes to confirm that the *Hae*II fragment was integrated into the correct position on the chromosome.

For example, to examine whether the *Eco*RV site on pAR144 depicted in Fig. 1 is involved in resistance, pAR144 DNA was digested with *Eco*RV, which cleaved it at a single site, and then ligated with the *Hae*II fragment, which was treated with T4 DNA polymerase to make both ends flush, and attached to *Bam*HI linker with T4 DNA ligase. The chimeric plasmid thus constructed in *E. coli* was digested with *Eco*RI to produce a linear form, and the linearized DNA was introduced into acetic acid-resistant strain 10-8. The transformation efficiency was about several hundred Km^r transformants per microgram of DNA. Total DNAs of the resultant Km^r transformants were digested with various restriction endonucleases and analyzed by Southern hybridization. When the *Hae*II fragment was used as the probe, a distinct hybridization signal was detected in the DNA of the transformants, whereas no hybridization was observed with the total DNA of acetic acid-resistant host 10-8. The size of the hybridized fragment coincided with that of the *Hae*II fragment when total DNA of the transformant digested with *Bam*HI was used as a target DNA. In addition, when the insert DNA of pAR301 was used as the probe, the hybridized band was larger than that obtained by using total DNA of the host as a target DNA; its size corresponded exactly to that expected as a result of integration of the *Hae*II fragment at the target position. On the basis of these results, we confirmed that the *Hae*II fragment was integrated in the correct position.

The Km^r transformants thus obtained were then replica plated on YPG agar medium containing various concentrations of acetic acid for examination of the extent of their acetic acid resistance.

TABLE 2. Physiological properties of mutants of *A. aceti* subsp. *aceti* 10-8 unable to grow in the presence of 5 g of acetic acid per liter[a]

Strain	Resistance to acetic acid (g/liter)[b]	Sp act (U/mg of protein)	
		ADH	ALDH
10-8	20	0.40	2.3
AS5	<5	0.53	2.4
AS8	<5	0.41	2.6
AS10	<5	0.64	3.1
AS13	<5	0.40	2.4
AS81	<5	0.41	2.8

[a] All strains were resistant to propionic acid at 8 g/liter and to fluoroacetic acid at 13 mM, had a pH range for growth of 3.1 to 7.2, and were able to produce acetic acid by oxidation of ethanol.
[b] Expressed as maximum concentration that allowed growth.

Southern blot hybridization. Southern blot hybridization was performed by the standard method (17). Probe DNA was prepared with the biotin labeling kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the membrane was treated as instructed by the manufacturer.

Enzyme assays. Membrane-bound ADH and membrane-bound ALDH activities in the cellular extracts of *Acetobacter* strains, which were prepared by methods described previously (11), were measured and expressed as described by Ameyama and Adachi (1, 2). CS and succinate dehydrogenase activities were determined as described by Srere (26) and Veeger et al. (27), respectively. Protein was determined by the method of Lowry et al. (16).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (15), using a 4 to 20% linear gradient of polyacrylamide. For low-molecular-weight proteins, the method of Fling and Gregerson (7) was also used. Proteins were visualized by staining with Coomassie brilliant blue. Standard proteins for molecular weight determination were obtained from Pharmacia (Uppsala, Sweden).

DNA sequencing. The nucleotide sequence of the 1.6-kb *Sph*I fragment containing the *aarA* gene was determined by the dideoxynucleotide triphosphate chain termination method of Sanger et al. (25), using M13mp18 or M13mp19 phage (18), a Takara sequencing kit, and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). A DNA sequencer (model 370A; Applied Biosystems, Inc., Foster City, Calif.) was also used to determine part of the sequence.

Computer analysis of the DNA sequence. The DNA sequence was analyzed by using the GENETYX sequence analysis program (Software Development Co., Ltd., Tokyo, Japan).

RESULTS

Isolation of acetic acid-sensitive mutants. Acetic acid-sensitive mutants of *A. aceti* subsp. *aceti* no. 1023 were selected after NTG treatment by replica plating on YPG agar medium containing 20 g of acetic acid per liter. In the first cycle of isolation, 23 acetic acid-sensitive colonies were isolated. However, the sensitivity of most mutants easily reverted to acetic acid resistance during several transfers of culture. After several cycles of isolation, we selected five stable mutants, designated AS5, AS8, AS10, AS13, and AS81. The frequency of reversion of each mutant was below 10^{-9}.

The physiological characteristics of the five mutants are shown in Table 2. These mutants showed properties similar to those of the parental strain 10-8 except for acetic acid resistance. All of the mutants possessed both ADH and ALDH activities at the same levels as those of the parental strain. They are not pH-sensitive mutants because the pH range allowing their growth, which was adjusted with HCl, was the same as that of the parental strain. Furthermore, since their resistance to other toxic chemicals, such as propionic acid and fluoroacetic acid, was the same as that of the parental strain, these mutants were considered to be sensitive only to acetic acid. These data also suggest that there is a specific machinery for protecting the cells from acetic acid.

Cloning of the acetic acid resistance gene. First, we tried to clone genes involved in acetic acid resistance by using two of the five mutants (AS5 and AS10) as described in Materials and Methods. Using AS5 as a host, we isolated three plasmids, designated pAR1611, pAR2424, and pAR2910. These three plasmids contained about a 30-kb insert DNA. When AS10 was used as a host, two of the same plasmids (pAR1611 and pAR2910) were selected. In addition, we found that these three plasmids also conferred acetic acid resistance on strains AS8, AS13, and AS81.

We next subcloned the insert DNA of pAR1611 by using pUC18 as a vector to identify the region essential to complement AS5 and AS10, because pAR1611 conferred acetic acid resistance on all five mutants. The mutations of AS5 and AS10 were complemented by a plasmid (pAR144) containing a 7.5-kb *Eco*RI fragment and by a plasmid (pAR248) containing a 6.7-kb *Bgl*II fragment, respectively. The DNA fragments cloned in pAR144 and pAR248 shared a common 1.5-kb region (Fig. 1). On the other hand, pAR301, containing a 8.3-kb *Pst*I fragment that covered most parts of the aforementioned *Eco*RI and *Bgl*II fragments, conferred resistance not only on AS5 and AS10 but also on the other three mutants (Table 3). The 8.3-kb *Pst*I fragment was confirmed to be derived from *A. aceti* subsp. *aceti* no. 1023 by Southern hybridization (data not shown).

The positions of mutations on the restriction map were determined by cloning six fragments (A to F; Fig. 1) and determining their abilities to confer acetic acid resistance on each mutant. Fragments A, B, and E complemented the mutations of AS5 and AS8, AS13, and AS81 and AS10, respectively (Table 3), suggesting that the acetic acid resistance gene(s) was located in this *Pst*I fragment of pAR301.

Determination of a region involved in acetic acid resistance by insertional inactivation. *A. aceti* subsp. *aceti* 10-8 has strong recombination activity; by use of this activity, a relatively simple technique for gene disruption or replacement has been developed (24). We applied this technique to determine a region associated with the resistance. The *Hae*II fragment containing the Km^r gene derived from pACYC177, which is expressed in *Acetobacter* strains (9), was used for this purpose. If a restriction site into which the Km^r gene has been inserted is located in a region essential for expression of acetic acid resistance, integration of the Km^r gene into this site is expected to influence expression of the resistance gene and, in some cases, cause inactivation of the gene.

The Km^r gene was inserted in 18 restriction sites located in the *Pst*I fragment of pAR301 (Fig. 1). Acetic acid resistance, oxidation of ethanol, and ADH and ALDH activities of the transformants were examined. Integration of the Km^r gene into eight sites (Fig. 1) abolished acetic acid resistance, but with no other phenotype alteration. We could not obtain Km^r transformants when the Km^r gene was inserted into two sites (*Cla*I and *Pma*CI). The sites at which integration of the Km^r gene caused a remarkable decrease in resistance were

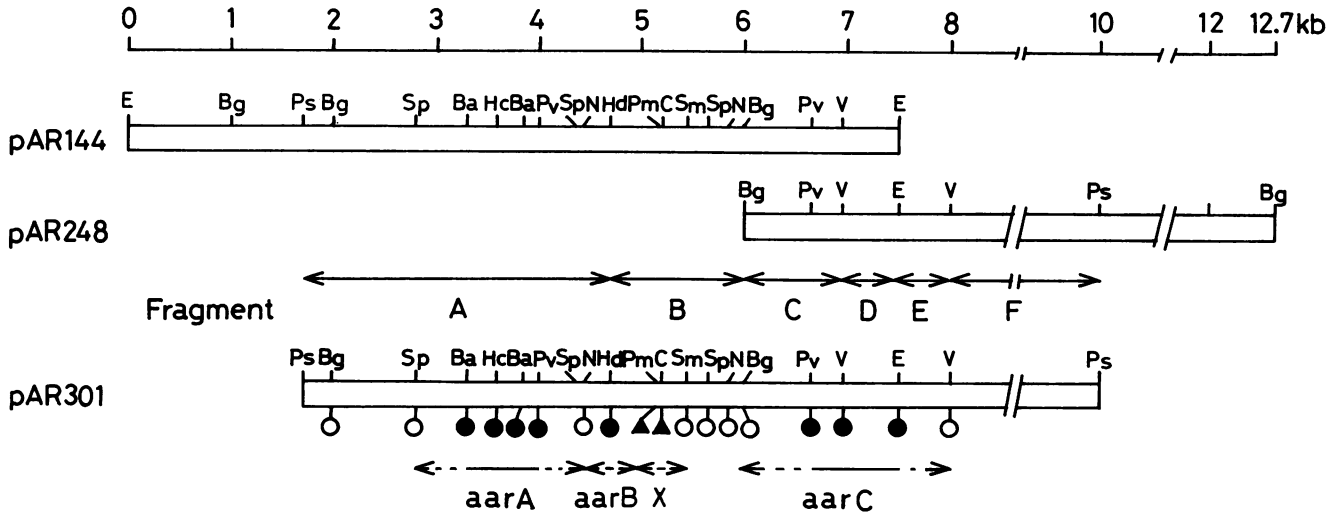

FIG. 1. Restriction maps of regions of *A. aceti* subsp. *aceti* no. 1023 DNA that complement acetic acid sensitivity mutations and the effect of integration of *Hae*II fragment on acetic acid resistance of the host. The *Hae*II fragment containing the Km^r gene derived from pACYC177 was inserted into various restriction sites (indicated by horizontal lines) in the insert DNA on pAR301 and integrated into the chromosome of acetic acid-resistant strain 10-8. ○ and ●, Acetic acid-resistant and -sensitive phenotypes, respectively, of Km^r transformants obtained by integration of the *Hae*II fragment; ▲, no Km^r transformants were obtained by transformation with the DNAs containing the *Hae*II fragment at these sites. *aarA*, *aarB*, and *aarC* are genes predicted by in vitro protein synthesis experiments. Restriction sites: Ba, *Bal*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Hc, *Hinc*II; Hd, *Hin*dIII; N, *Nsi*I; Pm, *Pma*CI; Ps, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I; Sp, *Sph*I; V, *Eco*RV.

clustered and divided in three regions. These three regions were in good agreement with the results of complementation experiments in which fragments A, B, and E were identified, which were designated *aarA*, *aarB*, and *aarC*, respectively. The *Cla*I and *Pma*CI sites were tentatively included in gene X because disruption of the sequence around these sites brought about a disadvantageous, presumably lethal, effect on the host, and its involvement in acetic acid resistance remains unclear. Oxidation activity and ADH and ALDH activities were not influenced by the integration in any of the Km^r transformants. From these results, we concluded that acetic acid resistance genes, which are composed of at least three genes, were located within the 8.3-kb insert.

To confirm this conclusion, we transformed the additional eight acetic acid-sensitive Km^r transformants described above with pAR311, which contained the 8.3-kb *Pst*I fragment at the *Pst*I site of pMV24, and Ap^r Km^r transformants were selected. After the presence of both plasmid pAR311 and the Km^r gene on the chromosome had been confirmed by Southern hybridization, the acetic acid resistance of each transformant was examined by the plate assay method. All of the transformants grew on medium containing 20 g of acetic acid per liter; i.e., their resistance levels were the same as that of the parental strain. All of these data are consistent with the conclusion that the genes required for conferring acetic acid resistance are contained in the 8.3-kb *Pst*I fragment.

TABLE 3. Complementation of mutations of acetic acid-sensitive mutants by various DNA fragments

Strain	Complementation by[a]:								
	pAR144	pAR248	pAR301	Fragment					
				A	B	C	D	E	F
AS5	+	−	+	+	−	−	−	−	−
AS8	+	−	+	−	+	−	−	−	−
AS10	−	+	+	−	−	−	−	+	−
AS13	+	−	+	−	+	−	−	−	−
AS81	+	−	+	−	+	−	−	−	−

[a] +, Acetic acid resistance; −, acetic acid sensitivity.

Expression of the *aarA*, *aarB*, and *aarC* gene products in *E. coli*. As described above, the three genes *aarA*, *aarB*, and *aarC* were all required for expression of the acetic acid resistance phenotype. To determine whether proteins are encoded by these three regions, we tried to direct oversynthesis of the gene products under the control of the *lac* promoter in *E. coli* JM109. Various restriction fragments derived from the 8.3-kb fragment of pAR301 were ligated to the pUC vectors so as to express the gene in the form of a

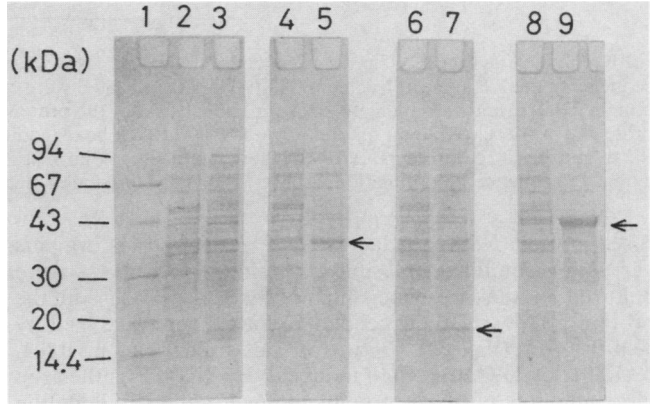

FIG. 2. SDS-PAGE analysis of cellular proteins prepared from *E. coli* JM109 transformants in the presence and absence of IPTG. Lanes: 1, molecular size markers; 2, uninduced JM109(pUC18); 3, induced JM109(pUC18); 4, uninduced JM109(pAR403); 5, induced JM109(pAR403); 6, uninduced JM109(pAR501); 7, induced JM109(pAR501); 8, uninduced JM109(pAR601); 9, induced JM109 (pAR601). The structures of pAR403, pAR501, and pAR601 are depicted in Fig. 3. Arrows indicate the oversynthesized proteins.

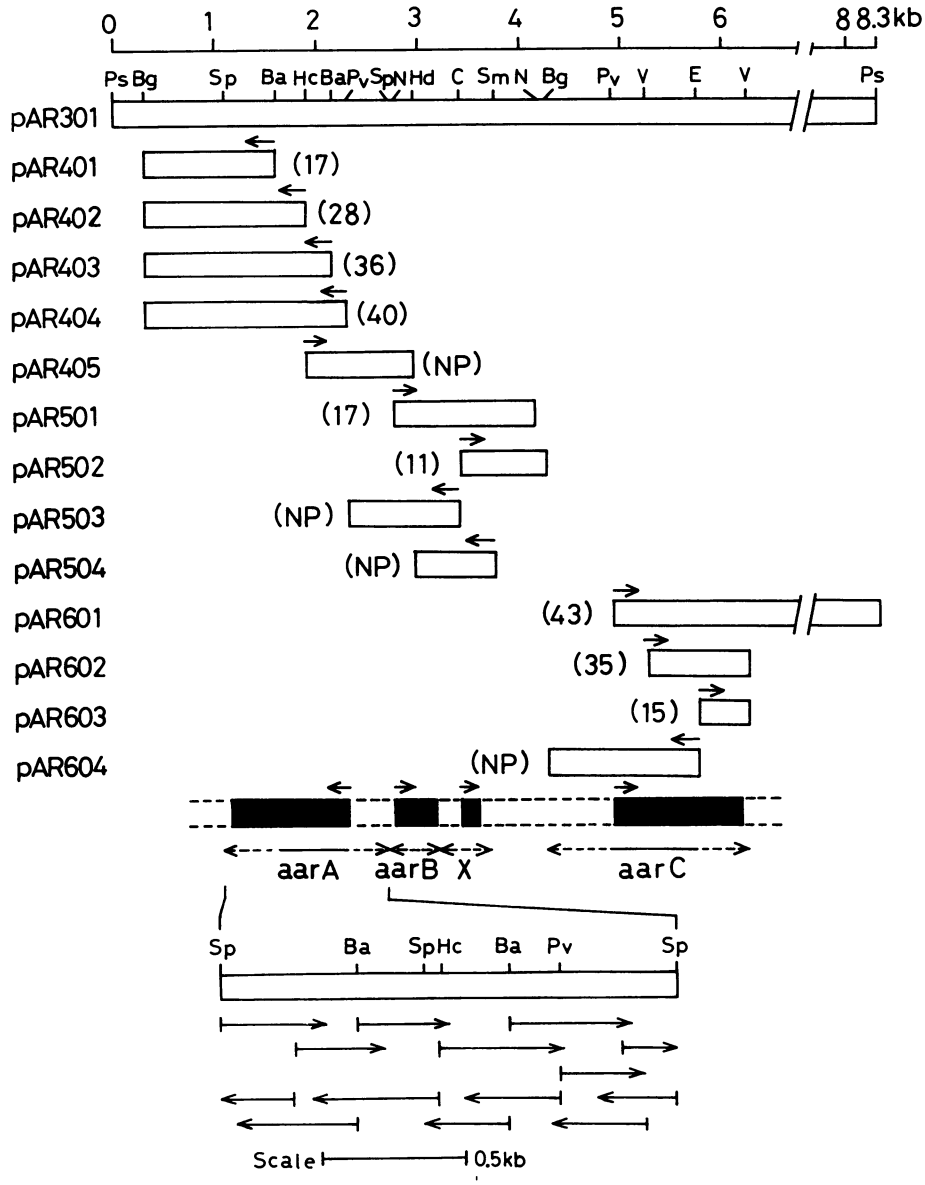

FIG. 3. Oversynthesis of proteins encoded by insert DNA of pAR301 in *E. coli* JM109 by use of the *lac* promoter and strategy for sequencing the 1.6-kb *Sph*I fragment. All fragments indicated were cloned in pUC8, -9, -18, or -19. Arrows indicate direction of transcription. Numbers in parentheses indicate sizes (in kilodaltons) of the proteins oversynthesized upon induction with IPTG. NP, No oversynthesis of proteins was observed upon induction with IPTG. Black boxes indicate locations of the putative coding regions estimated from the sizes of the oversynthesized proteins. Arrows below the black box corresponding to the putative ORF of the *aarA* gene indicate the orientation and extent of sequence determination. Abbreviations for restriction sites are defined in the legend to Fig. 1.

fused protein with β-galactosidase. *E. coli* transformants carrying recombinant plasmids, obtained in this way, were cultured in the presence and absence of IPTG, and the protein profiles of their cellular extracts were analyzed by SDS-PAGE (Fig. 2). Transformants carrying pAR403, pAR501, or pAR601, when induced with IPTG, synthesized large amounts of fused proteins of 36, 17, and 43 kilodalton (kDa), respectively, which we assume were encoded by the insert DNA.

We observed that nine transformants having some insert DNAs on plasmids oversynthesized specific proteins when induced with IPTG (Fig. 3). On the basis of the sizes of the proteins and the orientations of the insert DNAs, we could predict the existence of four putative open reading frames (ORFs). Three of them corresponded well to the gene products of *aarA*, *aarB*, and *aarC* that were expected from the results of insertional inactivation. These results also clearly demonstrated that the putative ORF corresponding to gene X, which covered both the *Cla*I and *Pma*CI sites, coded for a protein different from that encoded by *aarB*, as expected.

Nucleotide sequence of the *aarA* gene. To analyze the role of each resistance gene, we determined the nucleotide sequence of the 1.6-kb *Sph*I fragment containing the *aarA* gene according to the sequence strategy shown in Fig. 3. The sequence (Fig. 4) comprised 1,624 nucleotides (nt); within this region was found a single ORF, which corresponded to the *aarA* gene product expected from the results of both

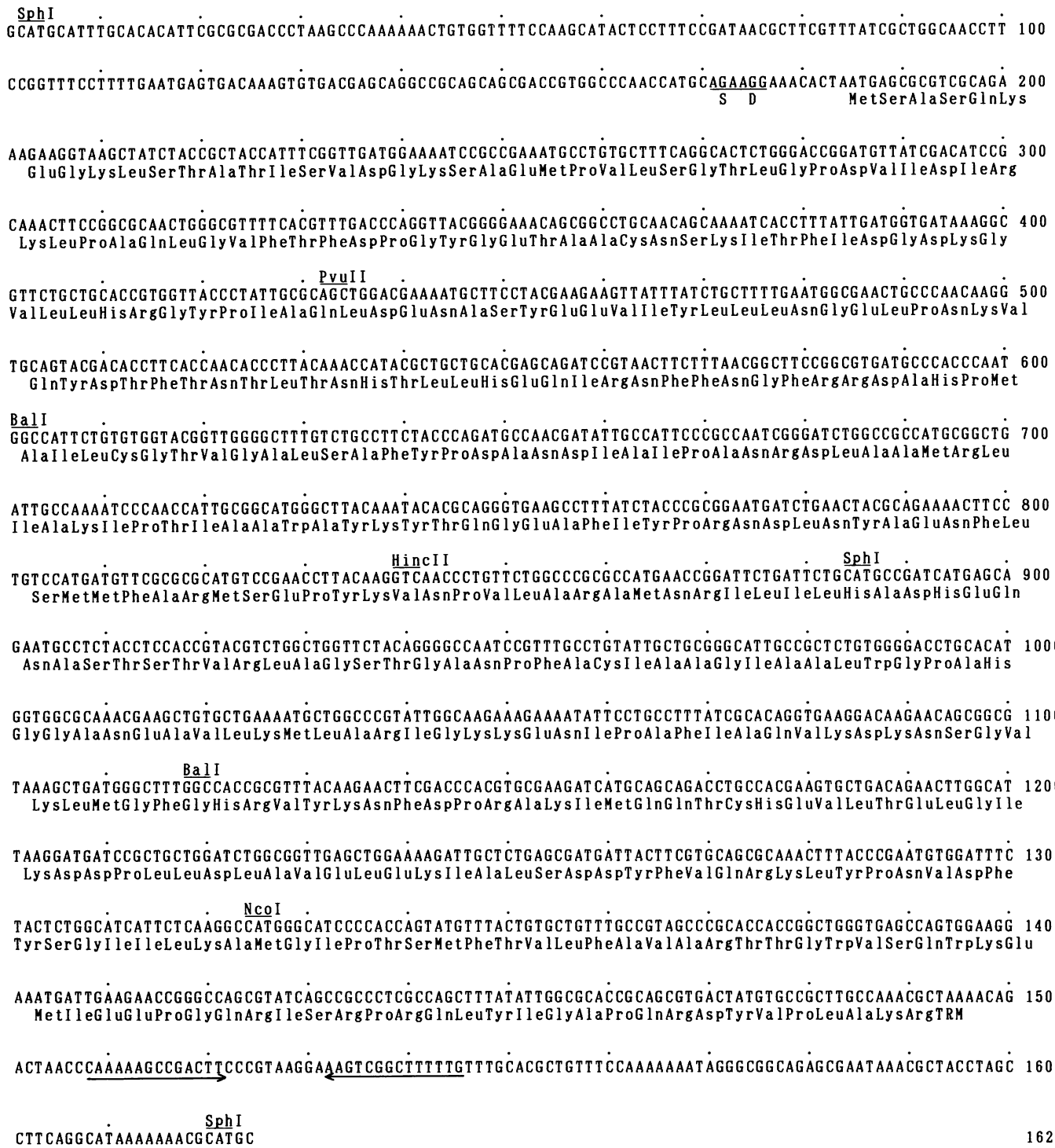

FIG. 4. Nucleotide and deduced amino acid sequences of the 1.6-kb *Sph*I fragment. The deduced amino acid sequence is shown below the nucleotide sequence. The putative Shine-Dalgarno (SD) sequence is underlined, and an inverted repeat downstream of the stop codon is indicated by arrows.

insertional inactivation and protein oversynthesis experiments. This ORF started at either ATG (nt 167 to 169) or ATG (nt 185 to 187) and terminated at TAA (nt 1493 to 1495). The ATG (nt 185 to 187) appeared to be functional, since a possible ribosome-binding sequence (12), AGAAGG, was present 8 nt upstream of this ATG codon. Taking this ATG as the start codon, we calculated that this ORF comprises a protein composed of 436 amino acid residues with a molecular size of 49.3 kDa. The overall G+C content of the coding sequence was 53 mol%; values for codon positions 1, 2, and 3 were 59, 41, and 61 mol%, respectively. Downstream of the stop codon was found an inverted repeat (nt 1508 to

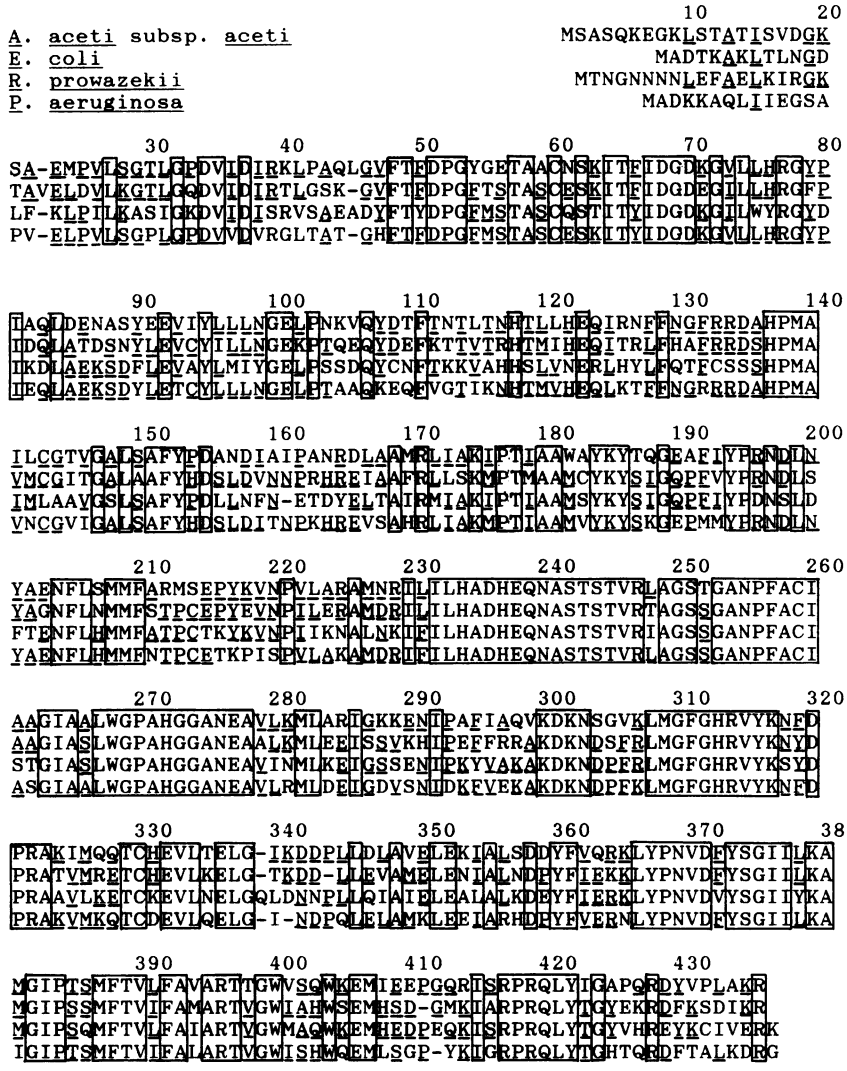

FIG. 5. Alignment of amino acid sequences of the ORF in the 1.6-kb *Sph*I fragment and CSs from *E. coli* (19), *R. prowazekii* (29), and *P. aeruginosa* (6). Numbers above the sequence are based on the sequence of the ORF within the 1.6-kb *Sph*I fragment. Amino acids identical in the four proteins are boxed; those identical in two or three proteins are underlined. -, Gap introduced to obtain maximal alignment.

1545), which might form a stem-loop structure acting as a transcription terminator; and its $-\Delta G$ was 28.6 kcal (ca. 119.7 kJ)/mol.

Computer analysis using the EMBL data base revealed that the ORF had significant similarity to CSs from *E. coli* (19), *Rickettsia prowazekii* (29), and *Pseudomonas aeruginosa* (6). There was a 64.4% match of amino acids with *E. coli* CS, a 56.8% match with *R. prowazekii* CS, and a 67.8% match with *P. aeruginosa* CS and many stretches conserved among these enzymes distributed over the sequence (Fig. 5). The molecular sizes of CSs of *E. coli*, *R. prowazekii*, and *P. aeruginosa* are 48.2, 49.2, and 47.6 kDa, respectively, which are similar to that of the expected *aarA* gene product. These results strongly suggested that the *aarA* gene codes for a CS.

We therefore determined the CS activity in the *Acetobacter* mutants sensitive to acetic acid as a result of a defect of *aarA*. AS5, isolated by mutagen treatment, lost CS activity (Table 4). In addition, the Km^r and acetic acid-sensitive mutant, AS5K, generated by integration of the *Hae*II fragment into the *Hin*cII site (nt 839 to 844), also showed no detectable CS activity. Introduction of pAR406 and pAR416,

TABLE 4. Enzyme activities of acetic acid-sensitive mutants of *A. aceti* subsp. *aceti*

Strain	Resistance to acetic acid (g/liter)	Sp act (U/mg of protein)	
		CS	Succinate dehydrogenase
10-8	20	0.39	0.090
AS5	<5	<0.01	0.103
AS5K[a]	<5	<0.01	0.141
AS5K(pAR406[b])	20	4.5	0.121
AS5K(pAR416[c])	20	5.7	0.112

[a] Acetic acid sensitive, Km^r transformant induced by integration of the *Hae*II fragment into the *Hin*cII site within the *aarA* gene.

[b] Constructed by inserting the 1.6-kb *Sph*I fragment into the *Sph*I site of pMV24 in the orientation in which the possible Shine-Dalgarno sequence of the *aarA* gene was placed just downstream of the *lac* promoter on pMV24.

[c] Constructed the same way as pAR406 except that the 1.6-kb *Sph*I fragment was inserted into pMV24 in the opposite orientation.

which contained the 1.6-kb *Sph*I fragment in the *Sph*I site of pMV24 downstream of the *lac* promoter in both orientations, conferred CS activity as well as acetic acid resistance on the mutant. The CS activities of both transformants were about 10 times higher than that of the parental strain, probably because of a gene dosage effect.

The 1.6-kb *Sph*I fragment also conferred CS activity on an *E. coli gltA* (MOB150) mutant that had a defect in the CS gene when it was inserted in one orientation in which the possible Shine-Dalgarno sequence of the *aarA* gene was placed just downstream of the *lac* promoter of the vector (pAR406) (specific activity, 4.5 U/mg of protein, versus values of 0.12, <0.01, and <0.01 for each of three other strains [K-12, MOB150, and MOB150(pAR416)] analyzed).

From these results, we concluded that *aarA* coded for CS.

DISCUSSION

We have developed effective host-vector systems of *Acetobacter* strains (9, 10, 22, 24) that enable us to clone genes involved in acetic acid resistance. This is the first step in elucidation of the resistance mechanism.

Results of the complementation by the cloned fragment as well as the insertional inactivation indicate that cooperation of *aarA*, *aarB*, and *aarC* is necessary to confer acetic acid resistance on the host cell, since inactivation of one of the three genes completely abolished the resistance of the host. It is interesting that these three genes are clustered in the 8.3-kb *Pst*I fragment. These genes were suggested to encode a protein by the results of oversynthesis of the products in *E. coli* by use of the *lac* promoter, which corresponded well to the *aarA*, *aarB*, and *aarC* regions. The results of insertional inactivation and protein oversynthesis suggested that gene X is located adjacent to *aarB*, though whether it is involved in acetic acid resistance remains unclear because disruption of it is presumably lethal to the host cells.

As a first step in elucidating the function of each gene and the mechanism of resistance, we determined the nucleotide sequence of the *aarA* gene and found that the ORF encoded by it had significant similarity to the CSs from *E. coli* and other bacteria. We then confirmed that the *aarA* gene codes for CS by introducing the gene into *Acetobacter* and *E. coli* mutants defective in CS and measuring CS activity. We observed that the CS activity in the *Acetobacter* transformants was much higher than that of the parental strain and was not affected by the orientation of the 1.6-kb *Sph*I fragment with respect to the *E. coli* promoter. In contrast, when the *aarA* gene was placed in the direction opposite that of the *lac* promoter (pAR416), no CS activity was detected in *E. coli* cells that were defective in the CS gene. These results suggest that a promoter which functions in *Acetobacter* strains but not in *E. coli* is contained in the 1.6-kb *Sph*I fragment.

The involvement of CS in acetic acid resistance might be explained in terms of detoxification of acetic acid incorporated into a cell through assimilation via the tricarboxylic acid cycle or the glyoxylate cycle. Another possible explanation is that CS is not directly correlated with acetic acid resistance and simply serves as a member of the tricarboxylic acid cycle to supply sufficient ATP or a high extent of reducing power for maintaining a machinery for protecting cells from acetic acid. We are sequencing the other *aar* genes, which will give more information on the ORFs.

All of the mutants isolated in this study possess normal levels of ADH and ALDH activities and oxidize ethanol to acetic acid, indicating that the genes cloned in this study are not associated with ethanol oxidation. We previously observed that acetic acid-sensitive mutants appeared spontaneously at high frequency upon prolonged culture of *A. aceti* subsp. *aceti* no. 1023 (21). Analysis of the spontaneous mutants revealed that loss of ADH activity was always accompanied by a decrease in resistance (23). Although several features of the mutation suggested the involvement of a plasmid in expression of acetic acid resistance, we have so far failed to identify plasmids in the parental strain. Introduction of pAR301 carrying *aarA*, *aarB*, and *aarC* into these acetic acid-sensitive mutants did not complement the mutations (data not shown), suggesting the existence of another resistance mechanism that is linked with loss of ADH activity.

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