Antonie van Leeuwenhoek **64**: 121–135, 1993.

Genetic organization of *Acetobacter* for acetic acid fermentation

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Received 6 August 1993; accepted in revised form 14 September 1993

Key words: acetic acid resistance, *Acetobacter*, alcohol dehydrogenase, aldehyde dehydrogenase, insertion sequence, spontaneous mutation

Abstract

Plasmid vectors for the acetic acid-producing strains of *Acetobacter* and *Gluconobacter* were constructed from their cryptic plasmids and the efficient transformation conditions were established. The systems allowed to reveal the genetic background of the strains used in the acetic acid fermentation. Genes encoding indispensable components in the acetic acid fermentation, such as alcohol dehydrogenase, aldehyde dehydrogenase and terminal oxidase, were cloned and characterized. Spontaneous mutations at high frequencies in the acetic acid bacteria to cause the deficiency in ethanol oxidation were analyzed. A new insertion sequence element, IS*1380*, was identified as a major factor of the genetic instability, which causes insertional inactivation of the gene encoding cytochrome *c*, an essential component of the functional alcohol dehydrogenase complex. Several genes including the citrate synthase gene of *A. aceti* were identified to confer acetic acid resistance, and the histidinolphosphate aminotransferase gene was cloned as a multicopy suppressor of an ethanol sensitive mutant. Improvement of the acetic acid productivity of an *A. aceti* strain was achieved through amplification of the aldehyde dehydrogenase gene with a multicopy vector. In addition, spheroplast fusion of the *Acetobacter* strains was developed and applied to improve their properties.

ADH – membrane-bound alcohol dehydrogenase; ALDH – membrane-bound aldehyde dehydrogenase; IS – insertion sequence; NTG – *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PQQ – pyrroloquinoline quinone

Introduction

The acetic acid bacteria are Gram-negative and obligately aerobic bacteria consisting of three genera, i.e., *Acetobacter*, *Gluconobacter* and *Frateuria* according to the current edition of Bergey's Manual (De Ley et al. 1984a, 1984b; De Ley and Swings 1984; Swings et al. 1984), all of which are characterized by the strong activities to oxidize alcohols and sugars. Because of these activities, *Acetobacter* strains have been used since historic ages for the vinegar fermentation, while the oxidative conversion of D-sorbitol to L-sorbose by *Gluconobacter* strains is integrated in the process of vitamin C production. In addition, another characteristic ability of a member of this group, cellulose formation by *Acetobacter xylinum* , has recently been examined for production of bacterial cellulose as a new industrial material (for a review, see Cannon & Anderson 1991). Marked genetic instability of these physiological activities has been recognized, which can cause even taxonomical confusions in these industrially important organisms. In fact, many acetic acid producers probably having almost identical ge-

netic constitution are frequently given different names of species or subspecies. Despite the significance in both basic understandings and practical strain breeding, very little has been known about their genetic background mainly due to the absence of recombination systems.

For the purpose of introducing the recombinant DNA techniques for genetic analyses and strain improvement of this group of bacteria, we have developed efficient host-vector systems, which allow us to clone and characterize various *Acetobacter* genes. This review summarizes progresses in genetic analyses of the acetic acid bacteria, mainly focusing on the *Acetobacter* strains used for acetic acid fermentation. Recent advances on the cellulose formers of this group can be found in another review (Cannon & Anderson 1991).

Development of the genetic systems for DNA manipulation in acetic acid bacteria

Observations on the gene exchange in the acetic acid bacteria were very scarce until recently. No exchange by means of intrinsic conjugation nor phages has yet been observed, while a broad host range plasmid in Gram-negative bacteria was known to induce conjugation in the *Acetobacter* at low frequency. The absence of the efficient systems for genetic analyses of the acetic acid bacteria prompted us to develop host-vector systems to apply recombinant DNA techniques.

Plasmid vectors

Plasmids of the acetic acid bacteria attracted much attention as possible candidates responsible for triggering the genetic instability. Screening of the indigeneous plasmids has also been conducted to develope vectors for DNA manipulation. A variety of plasmids have been reported from many *Acetobacter* and *Gluconobacter* strains (Ohmori et al. 1982; Masuda et al. 1983; Valla et al. 1983, 1986; Inoue et al. 1985; Okumura et al. 1985a; Teuber et al. 1987b; Fujiwara et al. 1989, 1992; Qazi et al. 1989; Grones et al. 1991; Takeda & Shimizu 1992). Our extensive screening also revealed the presence of cryptic plasmids with different sizes and restriction patterns among most strains of *Acetobacter* and *Gluconobacter* (Fukaya et al. 1985a). For example, 27 among 33 *Acetobacter* strains and 23 among 36 *Gluconobacter* strains were found to contain a single or multiple species of plasmids, whose sizes ranged between 0.9–17 megadaltons (Md). More than 90% of the isolates from a vinegar factory possessed plasmids of various sizes. Although some of these plasmids were proposed to associate with the unstable physiological phenotypes such as cellulose synthesis (Valla et al. 1983,1987) and glucose oxidation (Qazi et al. 1989), no substantial evidence to confirm their functions has been obtained.

We first constructed shuttle vectors composed of an *Escherichia coli* vector pACYC177 (Ap^r, Km^r) and cryptic plasmids pTA5001A (23.5 kilobases (Kb)) and pTA5001B (23 Kb) which had been isolated from *Acetobacter aceti* No.1023 (Okumura et al. 1985a). The copy numbers of the shuttle vectors in the *A. aceti* host were estimated to be approximately 1 – 3. We also constructed a series of shuttle vectors by ligating another *Acetobacter* plasmid pMV102 (2.3 Kb and the copy number of about 15) with various *E. coli* plasmids (Fukaya et al. 1985b). Drug resistance genes derived from *E. coli* plasmids as selective markers, such as resistance to ampicillin, kanamycin, chloramphenicol and tetracyclin, were successfully expressed in the acetic acid bacteria. A series of the hybrid plasmids derived from pMV102 were maintained quite stably without the selective pressure by corresponding drugs. Further improved vectors such as pNK7 (Tamaki et al. 1991), pMV24 (Fukaya et al. 1989b) and pMVC1 (Fukaya et al. 1990) were also constructed from pTA5001A and pMV102. The β-isopropylmalate dehydrogenase (*leuB*) gene complementing the *leuB* mutation of *E. coli* was cloned from *A. aceti* No.1023 and introduced onto some of these vectors, which allowed the selection of leu^+ transformants from leu^- *A. aceti* hosts (Okumura et al. 1988).

Phages were detected in several *Gluconobacter* strains and the vinegar producers (Schocher et al. 1979; Jucker & Ettlinger 1981; Robakis et al. 1985a, 1985b; Teuber et al. 1987a; Stamm et al. 1989; Wünsche et al. 1983a, 1983b), but none has been

used for construction of vectors for acetic acid bacteria.

Genetic transformation systems

The conjugational transfer of drug resistance genes from *E. coli* to *Gluconobacter suboxydans* by using a hybrid plasmid of RP4::Mu *cts* was one of the few early examples of gene exchange in the acetic acid bacteria, but the transfer frequency was extremely low (5×10^{-10}) (Murooka et al. 1981). Similar conjugational transfer systems were developed by using broad host-range vectors for Gram-negative bacteria as vectors (Chu et al. 1985; Inoue et al. 1985; Valla et al. 1986, 1989; Cleton-Jansen et al. 1991; Condon et al. 1991; Fjaervik et al. 1991; Grones et al. 1991, 1993; Grones & Turna 1992).

In 1985, we first developed a genetic transformation system of *A. aceti* No.1023 (Okumura et al. 1985a). A proline auxotrophic and streptomycin resistant mutant of *A. aceti* No.1023 was treated with 100 mM $CaCl_2$ at 0° C and then transformed with chromosomal DNA fragments from the parental strain. The *pro*$^+$ transformants appeared on streptomycin-containing plates at a frequency of up to 10^2 per μg DNA. We next examined transformation of Ca-treated cells of strain No.1023 with plasmid DNAs composed of pACYC177 from *E. coli* and the cryptic plasmid, pTA5001A or pTA5001B, from this strain and obtained ampicillin resistant transformants with a maximum frequency of 10^3 per μg DNA. Further improvement of the transformation conditions by adding polyethyleneglycol 4000 (35%) increased the transformation efficiency by a factor of about ten. The maximum frequency of approximately 10^5 transformants per μg plasmid DNA was achieved by using these improved conditions (Fukaya et al. 1985c).

Gene disruption depending on the strong recombination activity of the *A. aceti* hosts was demonstrated by using the cloned *leuB* gene (Okumura et al. 1988). The kanamycin resistance gene was inserted within the cloned *leuB* gene on a plasmid and the linearized DNA fragment was used for transformation of the wild-type *A. aceti* strain. All the Km^r transformants thus obtained were found to be Leu$^-$, indicating the inactivation of the chromosomal *leuB* gene through integration of the disrupted sequence by homologous recombination. A recombination-negative mutant of *A. aceti* No.1023 was recently obtained by gene disruption with the cloned *recA* gene from the same strain (Tayama et al. 1993). The mutant showed similar phenotypes to those of *E. coli recA* strains such as increased sensitivity to UV irradiation and decreased homologous recombination, which will be useful to improve the stability of vectors as well as cloned genes.

Restriction by hosts is one of the important factors to determine the transformation efficiency. When a shuttle vector DNA replicated in *E. coli* was used for transformation of *A. aceti* No.1023, the transformation efficency was lower than that with the DNA prepared from *A. aceti* by about one tenth. A restriction enzyme *Aaa*I, an isoschizomer of *Xma*III and *Eco*52I, was found in No.1023 (Tagami et al. 1988).

Many other host-vector systems by a similar $CaCl_2$ transformation method have also been developed in the acetic acid bacteria (Fukaya et al. 1985d; Fujiwara et al. 1989, 1992; Takemura et al. 1991; Takeda & Shimizu 1992). Recently electroporation has been shown to be effective for transformation of *A. xylinum* (Wong et al. 1990; Hall et al. 1992). These systems have made it possible to elucidate the genetic background of *Acetobacter* through cloning of the genes involved in not only the acetic acid fermentation but also the cellulose production (Valla et al. 1989; Saxena et al. 1990, 1991; Wong et al. 1990; Brede et al. 1991; Fjaervik et al. 1991) and others.

Spheroplast fusion

Spheroplast fusion is expected to be useful for breeding of microbial strains, especially when multiple genes are involved in the expression of a phenotype of practical usefulness. We developed a spheroplast fusion method for *Acetobacter* strains (Fukaya et al. 1989c). Spheroplasts of *A. aceti* No. 1023 were prepared by the treatment with lysozyme in the presence of 0.8 M sucrose as an osmotic stabilizer at a maximum frequency of 90%, and were re-

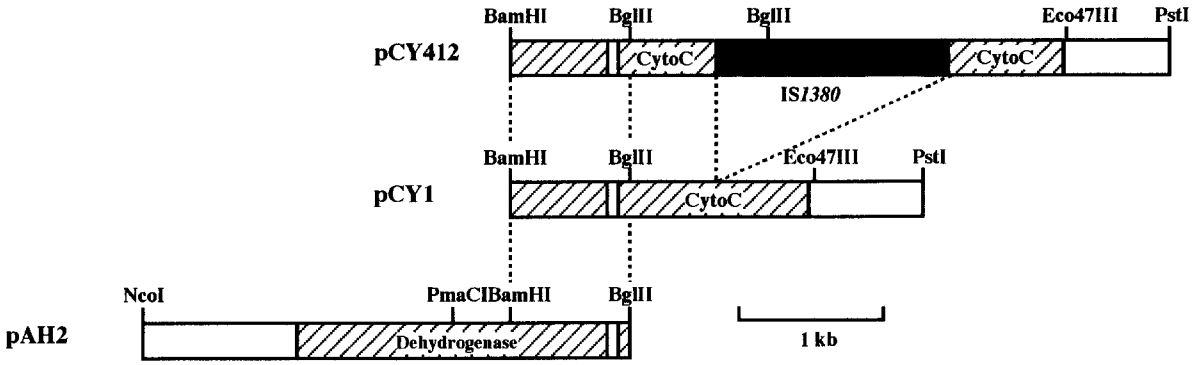

Fig. 1. Restriction maps of the ADH gene cluster from parental and mutant strains of *A. pasteurianus* (Takemura et al. 1993b). pCY412 and pCY1 contain *Pst*I-*Bam*HI fragmens, which code for the cytochrome *c* subunit and a COOH-terminal portion of a 75 kDa (dehydrogenase) subunit. pAH2 contains *Nco*I- *Bgl*II fragment, which codes for the 75 kDa subunit. Shaded boxes indicate the 75 kDa subunit and the cytochrome *c* subunit, respectively. Filled box indicates the insertion sequence, named IS*1380*.

generated on an agar plate containing 0.5 M sorbitol and 0.5–4% gelatin at a frequency of 15%. The spheroplast fusion was enhanced by adding polyethyleneglycol 4000 (40%) and $CaCl_2$ (50 mM) to the fusion mixture. The fusion frequency between auxotrophic mutants was about 5×10^{-5} under the optimum conditions. Intergeneric spheroplast fusion of *Gluconobacter oxydans* and *Corynebacterium* strains was reported recently (Verma et al. 1992).

Cloning and characterization of genes involved in acetic acid fermentation

The principal process in vinegar fermentation by the acetic acid bacteria is ethanol oxidation, which consists of two successive oxidation reactions catalyzed by two membrane-bound dehydrogenases, i.e., alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Ameyama & Adachi 1982a, 1982b). Electrons generated from these reactions are transferred to oxygen through the electron transport system integrated in the cell membrane, in which several cytochromes and ubiquinone are involved as the terminal oxidase system (Matsuhita et al. 1992). Development of the host-vector systems for the acetic acid bacteria enabled to clone and identify the genes encoding these dehydrogenases as well as components of the electron transfer systems in the acetic acid fermentation.

Cloning of the ADH gene

ADH from the acetic acid bacteria was purified as a complex of several protein subunits. Matsushita et al. (1992) reported that the purified ADH preparation from *A. aceti* contained three subunits, a 72 kilodaltons (kDa) protein possibly corresponding to the catalytic dehydrogenase subunit, cytochrome *c* of 50 kDa, and an unidentified protein of 15 kDa. The enzyme of an industrial vinegar producer for the submerged fermentation process, *Acetobacter polyoxogenes* NBI1028 (Entani et al. 1985), was purified as a complex of the 72 kDa dehydrogenase subunit and cytochrome *c* of 44 kDa, but it did not contain the third subunit (Tayama et al. 1989). We cloned the both dehydrogenase and cytochrome *c* genes of NBI1028 by using a DNA fragment that was amplified with two DNA segments as the primers designed according to the partial amino acid sequence of the dehydrogease (Tamaki et al. 1991). Sequencing analysis revealed that they are clustered with the same transcriptional polarity and probably co-transcribed as a single operon. The same gene organization was found in *A. pasteurianus* (Fig. 1) (Takemura et al. 1991, 1993b) and an *A. aceti* strain (Inoue et al. 1989, 1990, 1992). It is noteworthy that the genes encoding methanol dehydrogenase and cytochrome *c* in a methylotrophic bacterium are arranged in a quite similar manner (Harms et al. 1987). The predicted amino acid sequence of the 72 kDa subunit (738 amino acid residues) of *A.*

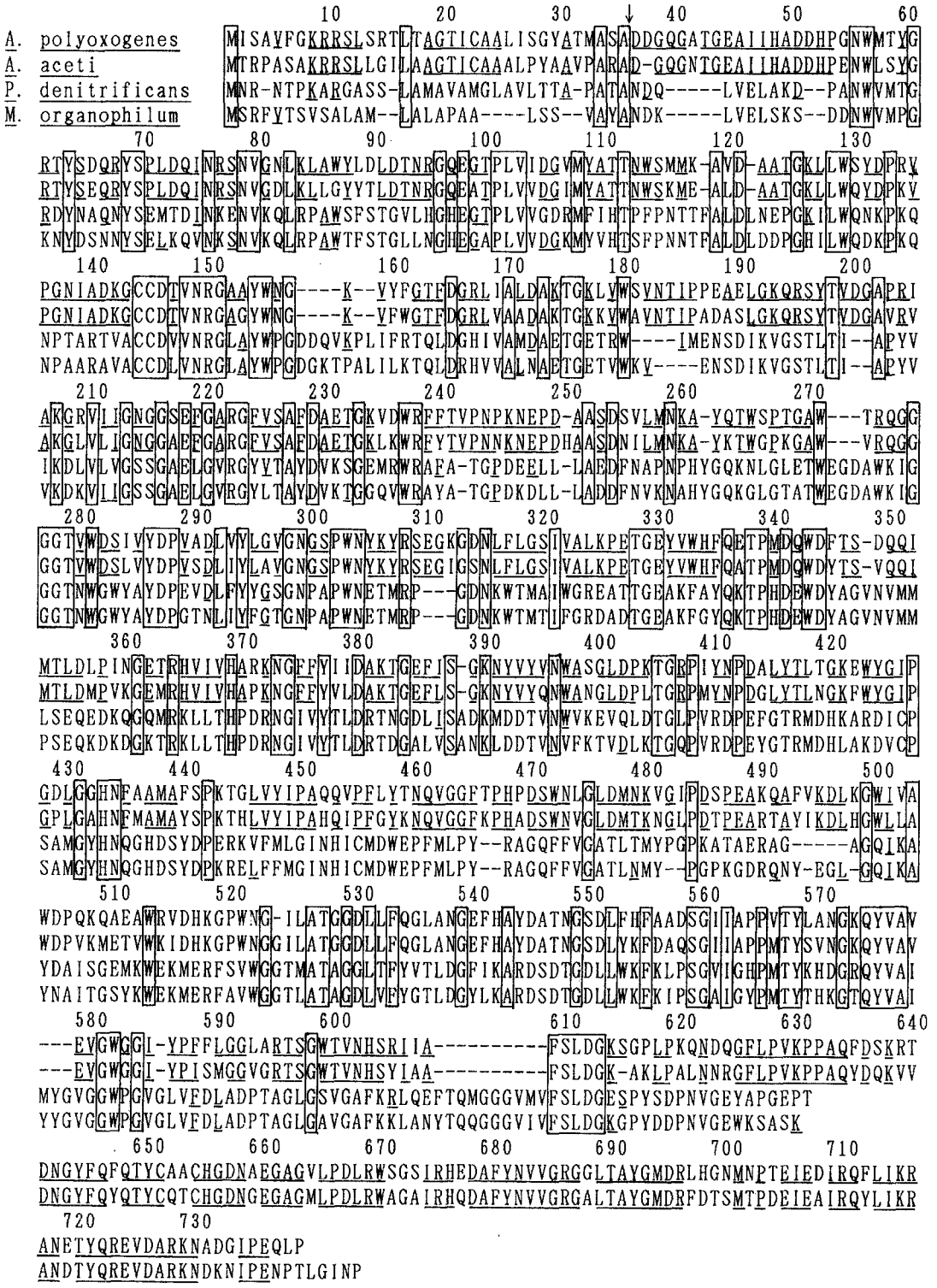

Fig. 2. Alignment of amino acid sequences of the 72 kDa subunit from *A. polyoxogenes* and *A. aceti*, methanol dehydrogenase from *P. denitrificans* and *M. organophilum* (Takemura et al. 1991). Numbers above the amino acid sequence are based on the sequence of the 72 kDa subunit of *A. polyoxogenes*. Amino acids identical in four enzymes are boxed and those identical to that of *A. polyoxogenes* are underlined. Broken lines in amino acid sequences are gaps introduced to obtain maximal matching. Arrow indicates the cleavage site of leader peptide.

polyoxogenes, containing 35 amino acid residues at its NH_2-terminus as a signal peptide, showed high similarity of 77% identity with that of *A. aceti* (Inoue et al. 1989), and about 30% identity with methanol dehydrogenases (Harms et al. 1987; Machlin & Hanson 1988) from methylotrophic bacteria (Fig. 2), all of which are considered to be the PQQ-containing enzymes. In addition, the 72 kDa ADH subunit was found to possess a typical consensus sequence for haem-binding, suggesting that the enzyme is a quino-haem protein (Groen et al. 1986).

The 44 kDa cytochrome *c* subunit (Tamaki et al. 1991), consisting of 468 amino acid residues with a signal peptide of 23 amino acid residues at its NH_2-terminus, showed considerable similarity to the corresponding subunit of ADH from other strains, such as the 45 kDa subunit gene of *A. aceti* (Inoue et al. 1989, 1990, 1992) and the cytochrome *c-553* of *G. suboxydans* (Takeda & Shimizu 1991, 1992, 1993). The 44 kDa cytochrome *c* of *A. polyoxogenes* contained three possible haem-binding sequences.

A plasmid containing both the dehydrogenase and cytochrome genes restored the ADH activity in the spontaneous mutant deficient in ethanol oxidation of *A. aceti* No.1023, while a plasmid carrying only the dehydrogenase gene failed (Tamaki et al. 1991). This result demonstrated that the 44 kDa cytochrome c subunit is a functionally essential component for ethanol oxidation in the acetic acid bacteria.

Matsushita et al. (1989) observed that lowering the external pH of *Gluconobacter suboxydans* in growth caused distinct increase in the celular contents of the cytochrome *c* subunit of ADH. Various external conditions might affect the dehydrogenase activity through regulating expression of the genes encoding the dehydrogenase components. We recently analyzed apparent induction of the ADH activity by ethanol in *A. pasteurianus* NCI1380 (Takemura et al. 1993b). When the strain was cultured in the presence of ethanol, the ADH activity was enhanced more than 10 folds, but the amounts of the immunologically reactive dehydrogenase subunit were almost the same in both the induced and uninduced cells. It was also observed that the greater part of the dehydrogenase subunit produced in the presence of ethanol was localized in the membrane fraction of cells, which was contrasting to the localization in the soluble fraction in the uninduced cells. This incorrect localization of the dehydrogenase subunit might cause the low ADH activity in the uninduced cells. The ADH gene cluster cloned from the strain had two different promoters upstream of the dehydrogenase gene, and determination of the transcription initiation point by primer extension revealed that the promoter 59 bp upstream from the ATG start codon was used in the presence of ethanol, while the other (232 bp upstream the ATG codon) was used in the absence of ethanol in the medium. The relationship between the promoter switching and the different localization of the gene product is not clear.

Cloning of the ALDH gene

The ALDH purified from *A. polyoxogenes* NBI1028 was composed of a 75 kDa (assumed as a catalytic subunit) and a 19 kDa subunits whose function has not been elucidated (Fukaya et al. 1989a). PQQ was detected in the purified enzyme preparation. The 75 kDa ALDH-subunit gene cloned from this strain was found to encode a protein of 773 amino acid residues containing a secretion signal sequence of 44 amino acid residues at its NH_2-terminus (Tamaki et al. 1989). The predicted amino acid sequence showed no homology with ADH nor with other quinoprotein dehydrogenases. The *E. coli lac* promoter was introduced upstream of the cloned ALDH gene on a multicopy vector pMV24 and was expressed in a spontaneous mutant of *A. aceti* deficient in both ADH and ALDH. As shown in Table 1, the *E. coli* promoter was functional in the *Acetobacter* host. The induced ALDH protein was localized in the membrane fraction and restored the cellular ALDH activity (Fukaya et al. 1989b). These results confirmed that the 75 kDa subunit is responsible for the ALDH activity. However, the ALDH protein produced in *E. coli* showed no enzymatic activity, even when the host cells were grown in the presence of PQQ, a prosthetic group of this enzyme (Tamaki et al. 1989). The ALDH protein produced in *E. coli* localized in the cell membrane but its size was larger than the

mature enzyme from *A. polyoxogenes*, suggesting that the 44 amino acid signal peptide was not processed in *E. coli*.

Cloning of the genes of terminal oxidase

Electrons generated in ethanol oxidation are transferred through the respiratory chain to the terminal oxidase via ubiquinone (Matsushita et al. 1992). *Acetobacter* strains are known to be divided into two groups on the basis of the terminal oxidases; one contains cytochrome a_1 and the other contains cytochrome *d* (Bächi & Ettlinger 1974). We purified the terminal oxidase of *A. aceti* No.1023, which is a cytochrome a_1 (*ba*)-type ubiquinol oxidase consisting of four subunits with a molecular weight of 72, 34, 21 and 13 kDa. The genes encoding these four components (*cyaA*, *B*, *C* and *D*) were cloned as a cluster with the same transcription polarity (Fukaya et al. 1993). Each of these genes showed great similarity in amino acid sequence to the corresponding subunits of the *E. coli o*-type ubiquinol oxidase and aa_3-type cytochrome *c* oxidase. Similar ubiquinol oxidase and its gene have been obtained from *A. polyoxogenes* (unpublished results), suggesting its wide distribution in the *Acetobacter* strains as a terminal oxidase for ethanol oxidation.

Genetic instability of acetic acid bacteria

Some types of spontaneous mutations are known to occur at very high frequencies in the acetic acid bacteria. Those are the mutations to cause loss (–) or acquisition (+) of various phenotypes, i.e., oxidizing activities of ethanol and acetate (–), similar activities to produce various oxidized products such as gluconate, ketogluconate and dihydroxyacetone (– and +), brown pigment formation (– and +), catalase activity (–), change in colony morphology and cellulose formation (– and +), most of which are considered to be the crucial taxonomical keys of this group (for a review, see Swings 1992). In partic-

Table 1. Characteristics of spontaneous mutants of *A. aceti* No. 1023.

Mutants group	Strains[a]	Genetic marker	Resistance to acetic acid[b]	Ethanol oxidizing ability[c]	Relative specific activity[d]	
					ADH	ALDH
Parents	No. 1023		r	++	91	103
	10–8	*pro*⁻	r	++	100	100
I	d–5		s	+	11	102
	10–815	*pro*⁻	s	+	9	47
	10–816	*pro*⁻	s	+	34	25
II	f–10		ss	–	0	98
	10–80	*pro*⁻	ss	–	0	79
	10–8201	*pro*	s	–	0	71
	10–8202	*pro*	s	–	0	92
	10–8206	*pro*⁻	ss	–	0	105
	10–8210	*pro*⁻	ss	–	0	108
	10–834	*pro*⁻	s	–	0	136
III	10–812	*pro*⁻	ss	–	0	2
	10–813	*pro*⁻	ss	–	0	5
	10–814	*pro*⁻	ss	–	0	5
IV	10–8204	*pro*⁻	s	–	54	103

[a] Strain 10–8 is a *pro*⁻ mutant of strain No. 1023. Strains d–5 and f–10 were derived from No. 1023 and all others were from 10–8.
[b] Acetic acid resistance is classified according to growth on agar medium containing acetic acid up to 5% (r), 2–3% (s) and 1.5% (ss), respectively.
[c] Ethanol oxidizing ability is classified according to the production of acetic acid more than 6% (++), 2–4% (+) and no production (–), respectively.
[d] Relative specific activities are calculated by assuming the activities in strain 10–8 to be 100.

ular, the ethanol oxidizing ability is used as a principal key to define the family *Acetobacteraceae*, which is composed of two genera, *Acetobacter* and *Gluconobacter*. Although previous reports on the spontaneous mutations were mostly based on the simple observations of the phenotypic changes during successive cultures, several experimental evidences proved the genetic instability of these phenotypes at unusually high frequencies in comparison to appropriate genetic markers as the control. Development of the DNA manipulation systems as well as identification of the genes involved in the ethanol oxidation described above have made it possible to reveal the mechanisms of the spontaneous mutations.

Spontaneous mutation to cause loss of ethanol oxidizing ability

Ohmori et al.(1982) first confirmed genetic instability of the ethanol oxizing ability in *A. aceti* No.1023, which was isolated from the surface culture in a traditional vinegar factory as a thermophilic strain (Ohmori et al. 1980). The strain showed a high fermentation activity even at 40° C, while the usual *A. aceti* strains lost the activity above 35° C. The strain also showed increased resistance to acetic acid (4.5%) at 30° C, which was distinctly higher than that of the usual strains (2.5%). A proline auxotrophic marker was added to the strain to follow genetic changes in these phenotypes. Prolonged submerged cultivation of the strain in the ethanol-containing liquid medium led to the appearance of the acetic acid sensitive mutants at high frequencies, most of which showed the total loss of the ethanol oxidizing ability. The number of the mutants began to increase concomitantly with cessation of the exponential growth phase followed by progressive decrease in the viable cell numbers, and the maximum population of the mutants in the survived cells exceeded 55%. All the mutants were confirmed to retain the genetic marker *pro*$^-$. The spontaneous mutation was apparently induced by ethanol, and no increase in the frequency of mutation was observed in the cultures using glucose as the carbon source. Similar instability was also observed with another thermophilic strain *Acetobacter pasteurianus* NCI1380, in which original methionine auxotrophy was used as a marker for the control (Takemura et al. 1991). The reversion rate of the spontaneous mutations in these strains was less than 10^{-9} in both strains.

Phenotypes and enzymatic activities of the spontaneous mutants derived from *A. aceti* No.1023 were analyzed as to their biochemical properties (Ohmori et al. 1982; Okumura et al. 1985b). Most of the mutants showed a simultaneous decrease in the acetic acid resistance, ethanol oxidizing ability and thermophilic property, suggesting the presence of some common mechanism involved in these phenotypes. ADH and ALDH are the two key enzymes responsible for the oxidation of ethanol to acetic acid. The spontaneous mutants could be classified into four groups according to these enzyme activities (Table 1). Most of the mutants (group I & II) showed a decrease in or the complete loss of the ADH activity, which corresponded to the level of cellular ethanol oxidizing activity. Almost complete loss of the ALDH activity in addition to complete loss of the ADH activity was observed in some other mutants (group III). Although the ADH activity seemed to determine primarily the cellular ethanol oxidizing abilities of these mutants, only one mutant (group IV) showed the complete loss of the ethanol oxidizing ability even when both the ADH and ALDH activities were retained. This mutant seems to be deficient in some other component essential for the ethanol oxidizing system in the cell. In the case of *A. pasteurianus* NCI1380, similar phenomena were observed, but all the mutants deficient in the ethanol oxidizing ability showed the complete loss of only the ADH activity (Takemura et al. 1991).

Genetic analysis of spontaneous mutants

Genetic instability was once supposed to correlate with the plasmids. For example, *A. aceti* No.1023 with marked instability mentioned above was found to possess three species of plasmids, pTA5001A (23.5 Kb), pTA5001B (23 Kb) and pTA5002 (about 80 Md) (Ohmori et al. 1982,

Okumura et al. 1985a). However, these three plasmids with their original sizes and restriction profiles were found in the spontaneous mutants, indicating null function of these plasmids.

On the other hand, insertion of some unknown DNA fragment into the chromosomal ADH genes was detected in the spontaneous mutants of *A. pasteurianus* NCI1380 deficient in ethanol oxidation (Takemura et al. 1991). Southern hybridization analyses by use of the cloned ADH gene cluster (Tamaki et al. 1991) as a probe revealed that the insertion occurred at a specific position in the coding region of the second ADH subunit, cytochrome *c* gene, which is an essential component for the dehydrogenase activity as described above (Fig. 1) (Takemura et al. 1991). Cloning and sequencing analyses revealed that the inserted sequence was 1665 bases in length with a terminal inverted repeat of 15 bases, which contained two long open reading frames (ORF) of 461 and 222 amino acids. These ORFs were overlapped and located on different DNA strands. Although these ORFs showed no homology with any protein registered in the DNA data banks, the longer ORF contained many basic amino acid residues (87 of total 461 residues) as observed with transposases of so-called insertion sequence (IS) elements. Since these properties are the typical ones of the IS elements, we named the sequence IS*1380*. The copy number of IS*1380* was estimated to be about 100. Recently, a similar transposable element has been found in a hyperreiterated DNA region of *Bradyrhizobium japonicum* (Judd & Sadowsky 1993).

A characteristic feature of IS*1380* is the relatively strict specificity for the target sequence. Although IS elements usually show little specificity for target sites of transposition, IS*1380* seems to require the TCGA sequence preferentially, because the insertion site in the cytochrome *c* subunit gene is only a single TCGA sequence and about 50% of the total copies of IS*1380* were inserted into the TCGA site (Takemura et al. 1991). This may explain the fact that all the spontaneous mutants examined were deficient in only the ADH activity. However, the finding that one mutant strain did not contain IS*1380* in the cytochrome *c* gene nor the ADH gene indicates the existence of some other mechanism associated with the loss of ethanol oxidizing ability.

Southern blot analysis carried out with IS *1380* as the probe showed that *A. aceti* No.1023 and several *Acetobacter* strains contained IS*1380* at high copy numbers, but *Gluconobacter* and *Frateuria* strains contained no DNA sequence having similarity to IS*1380*. The presence of a similar element in *A. aceti* No.1023 suggests that the genetic instablility of alcohol oxidizing ability and acetic acid resistance in this strain may also be explained in terms of a similar insertion event.

Recently, genetic analysis of cellulose biosynthetic ability of *Acetobacter xylinum* has been carried out extensively (for a review, see Cannon & Anderson 1991). A spontaneous mutation at high frequencies to cause the loss of cellulose formation was observed in this species (Valla et al. 1987; Asai 1968). Another insertion sequence element, IS*1031*, was discovered as an agent to cause inactivation of cellulose production in a spontaneous mutation in *A. xylinum* (Coucheron 1991, 1993). This finding of IS*1031* in the cellulose formers as well as wide distribution of *IS 1380* among various strains of *Acetobacter* suggest that transposition of IS elements is one of the major mechanisms responsible for the genetic instability of the acetic acid bacteria.

Genes involved in the resistance to acetic acid and ethanol

The resistance to acetic acid and ethanol are other crucial factors to determine the fermentation activity of the acetic acid bacteria, but very little has been revealed about their mechanisms. As described above, the spontaneous mutants of thermophilic *A. aceti* strains showed both the loss of the ethanol oxidizing activity and the decrease in the acetic acid resistance simultaneously, which indicates possible involvement of the membrane dehydrogenase activity in the resistance. However, multiple mechanims may be involved in the cellular acetic acid resistance, because different types of acetic acid sensitive mutants with intact ADH and ALDH activities can be obtained by NTG treatment. Gene cloning systems for the acetic acid bacteria were useful to

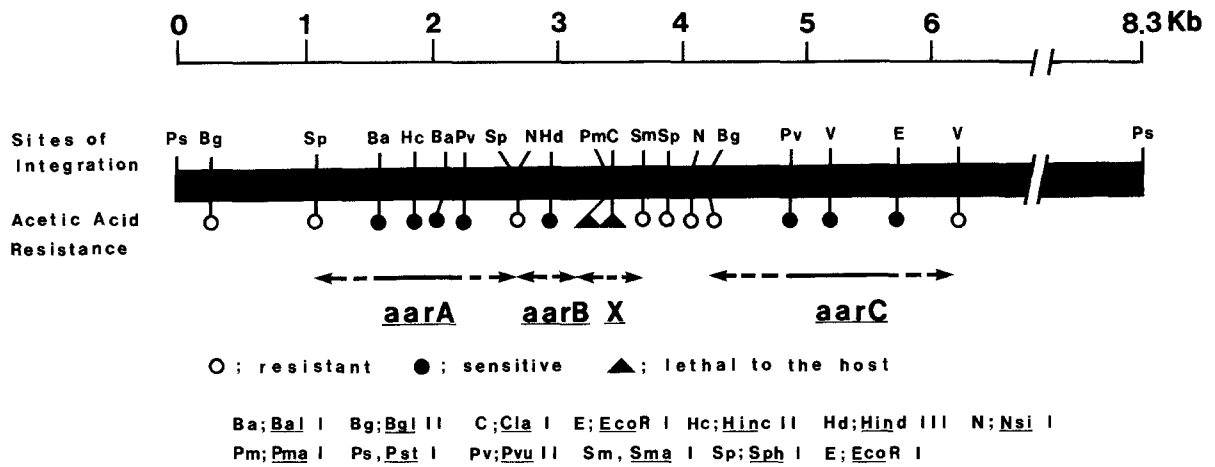

Fig. 3. Restriction maps of region of *A. aceti* No. 1023 DNA that complements acetic acid sensitivity mutations and the effect of integration of *Hae*II fragment harboring Km^r gene, derived from pACYC177, on acetic acid resistant host (Fukaya et al. 1990).

reveal new resistance mechanisms to not only acetic acid but also ethanol, as described below.

Acetic acid resistance gene cluster

Acetic acid sensitive mutants of *A. aceti* No.1023 were obtained after mutagenization with NTG by replica plating on the medium containing 2% acetic acid (Fukaya et al. 1990). Five sensitive mutants with sufficiently low reversion frequencies were selected, all of which possessed both the ADH and ALDH activities at the same level as the parental strain. These mutants were more sensitive to acetic acid than the spontaneous mutants lacking of the dehydrogenase activity. The increased sensitivity was observed only with acetic acid but not low pHs nor other chemicals such as propionic acid and monofluoro-acetic acid, indicating the presence of a specific machinery for protecting the cells from acetic acid. The 8.3 Kb DNA fragment which conferred the resistance to all the five mutants was cloned. An insertional inactivation technique (Okumura et al. 1988) with the wild-type strain was applied to identify genes on the cloned fragment which conferred the acetic acid resistance on the mutants. Briefly, the kanamaycin resistance gene derived from *E. coli* plasmid pACYC177 was inserted at various regions in the cloned 8.3-kb fragment and then integrated into the chromosome of the wild-type strain.

Table 2. Enzyme activities in acetic acid-sensitive mutants of *A. aceti.*

Strains	Resistance acetic acid (g/liter)	Specific activity (U/mg of protein)	
		Citrate synthase	Succinate dehydrogenase
10–8	20	0.39	0.090
AS5[a]	5	0.01	0.103
AS5K[b]	5	0.01	0.141
AS5K(pAR406[c])	20	4.5	0.121
AS5K(pAR416[d])	20	5.7	0.112

[a] The acetic acid sensitive mutant derived from strain 10–8 by NTG treatment. The *aar* genes were cloned to confer the resistance to this strain.

[b] An acetic acid sensitive strain derived by disruption of the *aarA* gene with the Km^r gene.

[c] A plasmid carrying the *aarA* gene under the control of the *E. coli lac* promoter on the vector pMV24.

[d] A similar plasmid carrying the *aarA* gene in the opposite direction.

According to the analyses, at least three genes, designated *aarA*, *aarB* and *aarC*, were identified to be the determinants responsible for the acetic acid resistance (Fig. 3). These *aar* genes could not complement the deficiency in acetic acid resistance in the spontaneous mutants of *A. aceti* No.1023. The *aarA* gene encodes a protein of 436 amino acids, whose sequence shows high homology with citrate synthase from *E. coli* and other bacteria (Ner et al. 1983; Wood et al. 1987; Donald et al. 1989). The identity was confirmed by the fact that the mutant lacked the synthase activity and introduction of the cloned aarA gene recovered the activity (Table 2). The *aarC* gene encodes a protein of 492 amino acids (Fukaya et al. 1993), which shows a significant homology with the *Neurospora crassa acu-8* gene product (Marathe et al. 1990) involved in the acetate utilization in this fungus. Disruption of the chromosomal gene in the *A. aceti* wild-strain caused marked decrease in the resistance as well as the inability to assimilate acetic acid, both of which were restored by introdction of the *aarC* gene. Although enzymatic activity of the *aarB* gene product and identity of *aarC* have not yet been revealed, these results indicate that one of the mechanisms of acetic acid resistance in *Acetobacter* is detoxification by efficient assimilation of acetic acid incorporated into the cells.

Ethanol resistance gene

A similar approach described above was taken to reveal the ethanol resistance in *A. pasteurianus* NCI1452, which can grow in the presence of 8% ethanol. An ethanol-sensitive mutant (MIC 3%) derived by NTG mutagenization was used as a host for cloning the resistance genes, and a gene which complemented the mutation was cloned from the parent (Takemura et al. 1993a). The cloned gene was found to code for a protein of 356 amino acid residues, whose sequence showed significant homology with the histidinolphosphate aminotransferase (HPAT) of *E. coli* and *S. typhimurium*, an enzyme involved in the histidine biosynthetic pathway (Carlomagno et al. 1988). The enzyme activity directed by the gene was confirmed in a HPAT-deficient *E. coli*, in which the cloned gene was expressed under the control of the *lac* promoter. Thus the gene was named *hisI*. Enzymatic analyses revealed that the enzyme activity of the mutant and the parent showed no difference, suggesting that the mutation did not occur in the *hisI* gene. However, introduction of *hisI* on a multicopy vector into the mutant caused increases in both the ethanol-resistance and HPAT activity. It is evident that *hisI* can suppress the ethanol-sensitive mutation, but the mechanism for involvement of its unexpected enzymatic activity in the suppression is not clear.

Application of the new genetic systems for breeding of *Acetobacter*

Since the metabolic pathway of the acetic acid fermentation is mainly composed of only two steps catalyzed by ADH and ALDH, it seems possible to improve the rate and yield of the fermentation through amplification of one or both of these dehydrogenase genes. We conducted this approach

Table 3. Comparison of acetic acid production between two transformants of *A. aceti* NBI2099.

	A. aceti NBI12099 (pMV24[a])	*A. aceti* (NBI2099 (pAL25[b])
Acetic acid productivity[c] (g/l/h)	1.8	4.0
Specific growth rated[d] (l/h)	0.072	0.142
Maximum acetic acid conc. (g/l)	68.4	96.6

[a] pMV24 is a plasmid vector for *Acetobacter.*
[b] pAL25 is a recombinant plasmid carrying the 75 kDa ALDH dehydrogenase gene on pMV24.
[c] Productivity at 20 g/l of acetic acid.
[d] Growth rate at 30 g/l of acetic acid.

with an isolate from a vinegar factory, *A. aceti* subsp. *xylinum* NBI2099 (Fukaya et al. 1989b), by introducing the ALDH genes from *A. polyoxogenes* cloned on a multicopy vector pMV24. The transformant containing the ALDH gene on the multicopy vector showed about two times higher ALDH activity than that of the transformant carrying the vector alone. As shown in Table 3, the growth rate and the maximum growth of the transformant harboring the ALDH gene on the plasmid were distinctly higher than those of the transformant harboring only the vector. Productivity of acetic acid by the transformant (specific volumetric production of acetic acid) at an acetic acid concentration of 20 g/liter was calculated to be 4.0 g per liter per hour, whereas that was 1.8 g of acetic acid per liter per hour. The maximum concentration of acetic acid produced by the transformant (96.9 g/liter) was about 1.4 times higher than that by the control (68.4 g/liter). The plasmid was stably maintained during the cultivation period. These clearly demonstrate that amplification of the ALDH gene is effective in improving the production of acetic acid from ethanol. Improvement of cellulose productivity of *A. xylinum* by gene amplification was also reported (Wong et al. 1990).

On the other hand, we examined another approach for breeding by spheroplast fusion (Fukaya et al. 1989c). Spheroplast fusion was conducted between two strains of *A. aceti*, i.e., one with the resistance to acetic acid and the other with the resistance to higher temperatures. The fusants showed various degrees of the resistance to acetic acid and higher temperature. One of the fusants was found to possess both the parental properties of resistance and to produce acetic acid from ethanol continuously under the conditions, which did not permit the growth of both parental strains. The results indicate that spheroplast fusion will be useful as a means of practical breeding of the *Acetobacter* strains.

Conclusions

The DNA manipulation techniques based on the host-vector systems here described were useful to identify a variety of genes in the acetic acid bacteria which encode essential components of the acetic acid fermentation. The results obtained through the analysis have now provided a substantial base to elucidate characteristic features of this group of bacteria. Promoter structures and mechanisms responsible for regulation of the genes by various environmental factors have not yet been sufficiently clarified. However, integration of ADH into the cell membrane seems to be controlled by the presence of ethanol in the medium, suggesting a new mechanism for induction of the enzyme activity in bacterial cells. Although rapid assimilation of acetate penetrating into the cells was shown to be one of the mechanisms for resistance to acetic acid, the decreased resistance in the spontaneous mutant deficient in the ethanol oxidation suggests another mechanism probably linked to the energy generating system in the cell membrane. IS elements were revealed to be one of the major factors responsible for the genetic instability in *Acetobacter*. However, it seems probable that a variety of IS elements with different insertional specificities are involved in the spontaneous mutations of other phenotypes. Another intriguing aspect is apparent induction of the spontaneous mutation associating with cessation of growth, which is now being recognized as a factor to control microbial flora in the ecosystem. These questions arised from the study of the strains of the acetic acid fermentation correlate at least partially with those for not only the cellulose-producing *Acetobacter* strains but also the methylotrophic bacteria and others. Better understandings of the genetic background are also expected to open new aspects in application of these industrially useful microorganisms.

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