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Analysis of proteins responsive to acetic acid in *Acetobacter*: Molecular mechanisms conferring acetic acid resistance in acetic acid bacteria

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Abstract

Acetic acid bacteria are used for industrial vinegar production because of their remarkable ability to oxidize ethanol and high resistance to acetic acid. Although several molecular machineries responsible for acetic acid resistance in acetic acid bacteria have been reported, the entire mechanism that confers acetic acid resistance has not been completely understood. One of the promising methods to elucidate the entire mechanism is global analysis of proteins responsive to acetic acid by two-dimensional gel electrophoresis. Recently, two proteins whose production was greatly enhanced by acetic acid in *Acetobacter aceti* were identified to be aconitase and a putative ABC-transporter, respectively; furthermore, overexpression or disruption of the genes encoding these proteins affected acetic acid resistance in *A. aceti*, indicating that these proteins are involved in acetic acid resistance. Overexpression of each gene increased acetic acid resistance in *Acetobacter*, which resulted in an improvement in the productivity of acetic acid fermentation. Taken together, the results of the proteomic analysis and those of previous studies indicate that acetic acid resistance in acetic acid bacteria is conferred by several mechanisms. These findings also provide a clue to breed a strain having high resistance to acetic acid for vinegar fermentation.

Keywords: ABC transporter; Acetic acid resistance; *Acetobacter*; Aconitase; *Gluconacetobacter*

1. Introduction

Acetic acid bacteria, particularly those belonging to the genera *Acetobacter* and *Gluconacetobacter* (De Ley et al., 1984; Swings, 1992; Yamada et al., 1997) are used for industrial vinegar production because of their remarkable ability to oxidize ethanol to acetic acid and high resistance to acetic acid. It is important to understand the mechanisms that underlie these two phenotypes from the viewpoints of industrial vinegar production and basic microbiology.

The molecular mechanism of ethanol oxidation has been extensively investigated in *Acetobacter* (Adachi et al., 1978; Ameyama et al., 1981; Matsushita et al., 1990, 1992; Kondo et al., 1995) and *Gluconacetobacter* (Fukaya et al., 1989a,b, 1993a; Inoue et al., 1989; Tayama et al., 1989; Tamaki et al., 1989, 1991; Thurner et al., 1997). It has been established that the oxidation of ethanol to acetic acid is carried out by a membrane-bound alcohol dehydrogenase (ADH) complex, membrane-bound aldehyde dehydrogenase (ALDH) complex, and ubiquinol oxidase.

On the other hand, the molecular machinery and mechanism that confer acetic acid resistance has not been thoroughly understood. In spontaneous mutants of *A. aceti* (Okumura et al., 1985) and *A. pasteurianus* (Takemura et al., 1991), a reduction in growth in the presence of acetic acid was associated with the loss of membrane-bound ADH activity. Later, Chinnawirotpisan et al. (2003) showed that the disruption of membrane-bound ADH gene resulted in a loss of acetic acid resistance in another strain of *A. pasteurianus*. A genetic approach using acetate-sensitive mutants identified a gene cluster consisting of *aarA*, *aarB*, and *aarC* genes as acetic acid resistance genes (Fukaya et al., 1990). Furthermore, it was revealed that *aarA* encodes citrate synthase that is involved in the TCA cycle, and *aarC* encodes a protein that is involved in acetate assimilation (Fukaya et al., 1993b); therefore, it is likely that these genes are responsible for acetic acid assimilation confer resistance. Recently, the presence of a proton motive force-dependent efflux system for acetic acid has been demonstrated in *A. aceti* (Matsushita et al., 2005); however, the molecular machinery

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that exhibits the transporter activity is yet to be identified. Although several molecular machineries that are involved in acetic acid resistance have been found in acetic acid bacteria, the entire mechanism that confers acetic acid resistance remains to be completely understood.

One of the most promising methods to elucidate the entire mechanism that confers acetic acid resistance in acetic acid bacteria is proteome analysis that may enable to find new molecular machinery involved in acetic acid resistance and is subsequently expected to provide an overview of the entire mechanism. In this review, we focus on the recent progress made in proteomic analysis of proteins induced by acetic acid in *Acetobacter* and overview the mechanisms that confer acetic acid resistance in acetic acid bacteria, based on the results of previous studies, including proteomic analysis.

2. Proteomic analysis of proteins induced by acetic acid in *Acetobacter*

Lasko et al. (1997) carried out the first analysis of proteins responsive to acetic acid by two-dimensional gel electrophoresis. They compared the relative protein patterns of *A. aceti* and *Gluconobacter suboxydans* during their growth in the presence and absence of acetate, and observed that eight proteins were overproduced specifically in response to acetate. Later, Steiner and Sauer (2001) analyzed global protein expression levels of *A. aceti* during adaptation to acetate by two-dimensional gel electrophoresis, and found that at least 50 proteins were specifically induced by adaptation to acetate. Based on the results of NH_2-terminal amino acid sequence analysis of the induced proteins, they suggested that membrane-associated processes were important for adaptation to acetate. These studies implied that two-dimensional gel electrophoresis is a useful method for elucidating the entire mechanism that confers acetic acid resistance; however, in these studies, the proteins responsive to acetic acid have not been identified, and their involvement in acetic acid resistance has never been examined.

We have recently analyzed proteins that were apparently induced by acetic acid in *A. aceti* by two-dimensional gel electrophoresis, and identified and characterized two of the most responsive proteins, thus demonstrating their involvement in acetic acid resistance (Nakano et al., 2004, 2006). The details of the studies are described below.

2.1. Procedure of two-dimensional gel electrophoresis

The experimental procedure is shown in Fig. 1. The strain used was *A. aceti* 10-8S2. Mutants that were sensitive to acetic acid with the loss of membrane-bound ADH activity appeared spontaneously from this strain during cultivation (Okumura et al., 1985), and the *aar* gene cluster was cloned from this strain (Fukaya et al., 1990). This strain was cultured with or without acetic acid. The pH of the medium was not adjusted because in vinegar fermentation, the pH decreases as the acetic acid concentration increases. The cultured cells were collected, broken, and fractionated by ultracentrifugation. The supernatant and precipitate fractions were referred to as soluble and membrane fractions, respectively, and these fractions were applied to two-dimensional gel electrophoresis (Nakano et al., 2004, 2006).

A comparison of the electrophoretic patterns of the soluble fractions prepared from cells cultured with and without acetic acid at the mid-log phase indicated that several proteins were apparently induced by acetic acid. Among the soluble fraction proteins, one protein with an apparent molecular mass of 100 kDa, named as S100, was selected for further analysis because its production was highly enhanced. In the membrane fractions, the amounts of several proteins also increased in response to acetic acid, and one protein with an apparent molecular mass of 60 kDa and named as AatA was selected for further analysis since its production was highly induced by acetic acid. These two proteins, i.e., S100 and AatA, were further analyzed to identify and characterize their functions.

2.2. Identification and characterization of the two selected proteins

2.2.1. Identification and characterization of S100

For identification of S100, a DNA fragment containing the gene encoding S100 was cloned using the oligonucleotides designed based on the NH_2-terminal amino acid sequence of S100, and the whole nucleotide sequence of the cloned fragment was determined. A single complete open reading frame (ORF) corresponding to S100 was present in the nucleotide sequence of the cloned fragment.

The deduced amino acid sequence is similar to *Escherichia coli* stress-responsive aconitase AcnA (Cunningham et al., 1997). Aconitase catalyzes the interconversion of citrate and isocitrate in the TCA cycle. In *A. aceti*, the aconitase activity at the mid-log phase increased approximately three-fold when acetic acid was added to the medium; this result was consistent with the result of protein profiles. To confirm that S100 is aconitase, a pACO300 plasmid was constructed by ligating the S100 gene with *E. coli–Acetobacter* shuttle plasmid vector pMV24 and introduced into *E. coli*. The *E. coli* transformant carrying pACO300 showed significantly higher aconitase activity (approximately 20-fold) than that of the strain carrying pMV24.

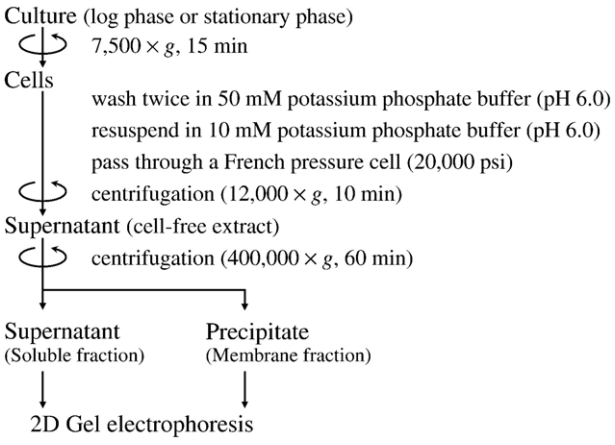

Fig. 1. Procedure of two-dimensional gel electrophoresis of soluble and membrane fractions prepared from *A. aceti* cells. 2D, two-dimensional.