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Short communication

Regulation of the Expression of the Catabolic Acetolactate Synthase by Branched Chain Amino Acids in *Serratia marcescens*

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In *Serratia marcescens*, acetolactate produced by the catabolic acetolactate synthase (ALS) is converted into acetoin, its physiological role of which is to maintain intracellular pH homeostasis. In this study, the expression mode of catabolic ALS by aeration and branched-chain amino acids was examined by the ELISA method. The amount of catabolic ALS decreased approximately 93% under aerobic conditions. We also showed that the expression of catabolic ALS decreased approximately 34% and 65% in the presence of 2.5 mM and 10 mM leucine, respectively. The repression of catabolic ALS by leucine has not been reported previously. In contrast to leucine, catabolic ALS levels increased approximately 13% and 38% by treatment with 2.5 mM and 10 mM isoleucine, respectively, while valine alone did not have any significant effect on the synthesis of catabolic ALS. The amount of catabolic ALS was also reduced to approximately 32% and 45% in the presence of 10 mM Leu+Ile and Leu+Ile+Val, respectively. The regulatory mode of the *Serratia* catabolic ALS suggests that catabolic ALS may also have a role in supplying acetolactate as an intermediate of valine and leucine biosynthesis in addition to the maintenance of internal pH.

Keywords: Catabolic ALS, ELISA, Leucine, *Serratia marcescens*.

Introduction

There are two different enzymes that can catalyze the formation of acetolactate in enteric bacteria (Hapern and Umbarger, 1959). One is the biosynthetic acetolactate synthase (ALS), which catalyzes the condensation of the hydroxyethyl-TPP carbanion intermediate with either pyruvate or α-ketobutyrate to form either of two physiologically significant acetohydroxy acid products, acetolactate and acetohydroxybutyrate, respectively (Tse and Schloss, 1993). The second enzyme is catabolic ALS, also known as the pH 6 acetolactate-forming enzyme, which catalyzes the condensation of the (hydroxyethyl-TPP) carbanion intermediate with only pyruvate to form acetolactate (Umbarger, 1978). Both the catabolic and anabolic ALS contain thiamine pyrophosphate (TPP), and they have 20 to 30% amino acids sequence similarity (Peng *et al.*, 1992). The anabolic and catabolic ALS differ in a number of important aspects such as optimum pH, requirement of flavin adenine dinucleotide (FAD) for activity, feedback inhibition by the end-products, and inhibition by several herbicides (Stormer, 1967; 1968; Holtzclaw and Chapman, 1975; Zahler *et al.*, 1990; Peng *et al.*, 1992; Sneop *et al.*, 1992). In some bacteria such as *Enterobacter aerogenes* and *Bacillus subtilis*, but not in *Escherichia coli* (Rossmann *et al.*, 1991), pyruvate can be converted into the neutral compound, butanediol, through the production of acetolactate by the enzymatic reaction of the catabolic ALS to maintain intracellular pH homeostasis (Johansen *et al.*, 1975; Tsau *et al.*, 1992). Since the optimum pH of the catabolic ALS from enteric bacteria is low (below 6.0), catabolic ALS is well suited in the fermentation pathway leading to acetoin and butanediol, and this fermentation pathway prevents further acidification of the growth medium (Johansen *et al.*, 1975). The expression of catabolic ALS is optimal in the presence of acetate, at low medium pH, and under anaerobic conditions (Magee and Kosaric, 1987). Purified catabolic ALS does not require FAD for its activity and is composed of large subunits only (Joo and Kim, 1998). This enzyme is not inhibited by the branched-chain amino acids and some herbicides such as sulfometuron methyl. Although intensive studies have been carried out with the biosynthetic ALS isozymes from enteric bacteria and

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plants, there is relatively little information available for the regulatory mode of the expression of the catabolic ALS from enteric bacteria. In this report, we have examined the regulation of the expression of the catabolic ALS from *Serratia marcescens* by aeration and branched-chain amino acids.

Material and Methods

Materials Protein A-Sepharose and protein molecular weight marker were purchased from Pharmacia Co.(Uppsala, Sweden); Cocarboxylase (TPP), flavin adenine dinucleotide (FAD), dithiothreitol (DTT), pyruvic acid, creatine, valine, isoleucine, leucine, Tween 20, bovine serum albumin (BSA), and Trizma base were purchased from Sigma Chemical Co. (St. Louis, USA); α-naphthol was purchased from Kanto Chemical Co. (Tokyo, Japan); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and o-phenylenediamine (OPD) were purchased from Gibco BRL Co. (Gaithersburg, USA). All other reagents used were of reagent grade.

Bacterial culture conditions and preparation of crude extracts *Serratia marcescens* was grown anaerobically for 24 h in 1-L culture flasks containing 1L of a minimal medium supplemented with various concentrations of branched-chain amino acids, ranging from 2.5 to 10 mM. The minimal medium contained 22 mM potassium phosphate monobasic, 51 mM potassium phosphate dibasic, 8 mM ammonium sulfate, 0.4 mM magnesium sulfate, and 0.5% glucose. Cells were harvested by centrifugation (15,000 $\times$ *g*, 30 min), and wet cells (2.1 g) were resuspended in standard buffer [20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, 10 mM $MgCl_2$, and 10% glycerol]. The cell suspension was subjected to ultrasonic treatment for 30 s and 5 min intervals on ice for 15 cycles. After centrifugation at 15,000 $\times$ *g*, the supernatants were pooled.

Enzyme assay and protein determination The activity of catabolic ALS was assayed at pH 5.5 using the method of McEwen and Silverman (1980). One unit of enzyme activity was defined as the formation of 1 μmole acetolactate per min, and specific activity was expressed as U/mg protein at standard conditions. The amount of protein was determined by the Lowry method (1951).

Enzyme purification and preparation of antibodies Catabolic ALS was purified using ammonium sulfate fractionation, DEAE-Sepharose, Phenyl-Sepharose, and hydroxylapatite chromatography (Joo and Kim, 1998). Polyclonal antibodics against catabolic ALS were prepared from rabbit and IgG was purified using a Protein A-Sepharose.

ELISA assay Test samples were serially diluted two-fold in 0.1 M sodium carbonate buffer (pH 9.5), and 200 μl of sample was added to each well of a 96-well flat-bottom polystyrene plate and then incubated for 16 h. After being washed with PBS containing 0.05% Tween 20 (PBST), each well was blocked with 2% BSA for 30 min and then washed briefly with PBST. Two hundred μl of the primary antibody (1:1000 diluted) was added to each well, and the plate was incubated for 2 h. The wells were washed three times with PBST and 200 μl of HRP-conjugated goat anti-rabbit IgG (1:1000 diluted) was added to each well. After incubation for 1 h, the wells were washed with PBST and 100 μl of o-phenylenediamine solution was added, followed by incubation for 30 min. The colorimetric response was measured by an ELISA reader at a wavelength of 450 nm. All incubations were performed at 37°C. A known amount of purified catabolic ALS was used as the standard (Joo and Kim, 1998).

Results and Discussion

Effect of aeration When assayed at pH 5.5, anabolic ALS activity was below 10% of its activity at pH 7.5. Therefore, the total ALS activity at pH 5.5 was mainly due to catabolic ALS. We examined the total ALS activity and amount of catabolic ALS in cells grown in a minimal medium containing 0.5% glucose under aerobic or anaerobic conditions. The total ALS activity and the amount of catabolic ALS were much higher in cells grown anaerobically than in those grown aerobically. The total ALS activities were 91.7 and 9.0 U/g cell, and the amounts of the catabolic ALS measured by the ELISA method were 1.73 and 0.12 mg/g cell in cells grown anaerobically and aerobically, respectively (Table 1). The data suggest that reduction of the total ALS activities by aeration was closely related to the decrease in the production of catabolic ALS, and this result was consistent with the previous report that anaerobic conditions were optimal for the induction of catabolic ALS (Magee and Kosaric, 1987).

Effect of branched-chain amino acids We examined the effect of branched-chain amino acids on the amount of catabolic ALS by the ELISA method expressed in a minimal medium containing 0.5% glucose under anaerobic conditions. The total ALS activity decreased as leucine concentration increased, whereas it increased as isoleucine concentration increased, and valine did not have any significant effect on the total ALS activity (Fig. 1 and Table 2). When the amount of catabolic ALS was measured by ELISA, it reduced to 1.14 or 0.61 mg/g cell in the presence of 2.5 or 10 mM leucine, respectively. In contrast,

Table 1. The effect of aeration on the expression of catabolic ALS. The amount of catabolic ALS in the crude extract from the cells grown anaerobically in a minimal medium containing 0.5% glucose was designated as 100% of the amount of catabolic ALS. The values are the averages of three independent experiments.

	Culture condition	Total unit (U/g cell)	Catabolic ALS (mg/g cell)	Relative amount (%)
Minimal	Anaerobic	91.7 ± 4.4	1.73 ± 0.09	100
	Aerobic	9.0 ± 0.5	0.12 ± 0.01	7

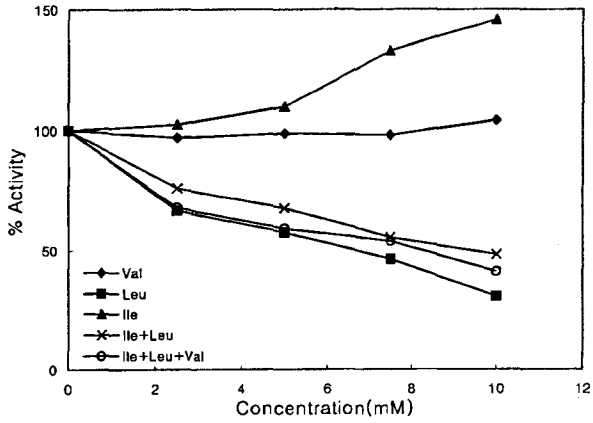

Fig. 1. The effect of the branched-chain amino acids on the expression of the total ALS activities in *Serratia marcescens*. Cells were grown anaerobically in a minimal medium containing 0.5% glucose supplemented with each branched-chain amino acid ranging from 2.5 to 10 mM. The total ALS activity in the crude extract from the cells grown in a minimal medium containing no branched-chain amino acid was designated as 100% of the total ALS activity.

Table 2. The effect of exogenous supplementation with the branched-chain amino acids on the expression of catabolic ALS. The amount of catabolic ALS in the crude extract from the cells grown in a minimal medium containing 0.5% glucose supplemented with no branched-chain amino acids was designated as 100% of the amount of catabolic ALS. The values are the averages of three independent experiments.

Branched chain amino acids	Concentration (mM)	Total unit (U/g cell)	Catabolic ALS (mg/g cell)	Relative amount (%)
None	–	91.7 ± 4.4	1.73 ± 0.09	100
Valine	2.5	101.9 ± 5.9	1.68 ± 0.09	97
	10.0	98.3 ± 7.9	1.86 ± 0.04	107
Leucine	2.5	80.3 ± 5.3	1.14 ± 0.10	65
	10.0	37.8 ± 3.5	0.61 ± 0.05	35
Isoleucine	2.5	106.0 ± 7.0	1.95 ± 0.07	112
	10.0	124.1 ± 7.5	2.39 ± 0.20	138
Ile + Leu	2.5	69.9 ± 5.8	1.32 ± 0.23	76
	10.0	44.4 ± 3.4	1.18 ± 0.16	68
Ile + Leu + Val	2.5	58.9 ± 3.2	1.27 ± 0.17	73
	10.0	40.9 ± 2.4	0.96 ± 0.02	55

it increased to approximately 1.95 or 2.39 mg/g cell in the presence of 2.5 or 10 mM isoleucine, respectively. The amount of the catabolic ALS was also reduced to approximately 1.18 or 0.96 mg/g cell in the presence of 10 mM Leu+Ile or Leu+Ile+Val, respectively (Table 2). The extents of repression were relieved approximately 33% and 20% by the combination of 10 mM Leu+Ile and Leu+Ile+Val, respectively, when compared with that of 10 mM leucine alone. These data suggest that leucine and isoleucine regulate the expression of catabolic ALS in opposite direction in *S. marcescens*. It has been reported that the *ilvGMEDA* operon of *S. marcescens* is regulated by leucine-mediated attenuation (Hsu *et al.*, 1985). With regard to this, the positive regulation of the catabolic ALS expression by isoleucine might be due to the isoleucine-mediated attenuation mechanism, which needs to be verified. The negative regulatory role of leucine for catabolic ALS is unusual because leucine has been known as a negative regulator of the anabolic ALS isozymes (LaCara and DeFelice, 1979; Hauser and Hatfield, 1983; 1984; Hsu *et al.*, 1985). Thus, we suggest that catabolic ALS may also have a role in supplying acetolactate as an intermediate of valine and leucine biosynthesis in addition to the maintenance of internal pH. This supposition is further supported by the observation that *S. marcescens* grew well under aerobic conditions in a minimal medium supplied with glucose as the sole carbon source, even though the anabolic ALS isozymes involved in the biosynthesis of the branched-chain amino acids are expressed in small amounts under these conditions (Joo and Kim, 1998).

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