# Acetate kinase Activity and Kinetic Properties of the Enzyme in *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 Intestinal Bacterial Strains

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**Abstract:** Activity of acetate kinase in cell-free extracts and individual fractions and the kinetic properties of the enzyme obtained from the *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal bacterial strains were presented at the first time. The highest activity of the enzyme was measured in the cell-free extracts (1.52 ± 0.163 and 0.46 ± 0.044 U × $mg^{-1}$ protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively) compared to other fractions. The specific activity of acetate kinase in the extracts of both bacterial strains was determined at different temperature and pH. Analysis of the kinetic properties of the purified acetate kinase was carried out. The acetate kinase activity, initial (instantaneous) reaction rate ($V_0$) and maximum rate of the acetate kinase reaction ($V_{max}$) in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal bacterial strains were defined. Michaelis constants ($K_m^{Acetyl\ phosphate}$ and $K_m^{ADP}$) of the enzyme reaction (2.54 ± 0.26 and 2.39 ± 0.24 mM for *D. piger* Vib-7 as well as 2.68 ± 0.25 and 2.47 ± 0.27 mM for *Desulfomicrobium* sp. Rod-9, respectively) were calculated. The described results of acetate kinase, an important enzyme in the process of organic compounds oxidation and dissimilatory sulfate reduction would be perspective and useful for clarification of the etiological role of these bacteria in the development of inflammatory bowel diseases in humans and animals.

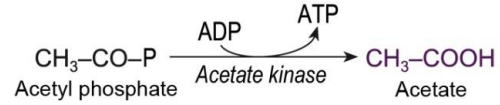
**Keywords**: Acetate kinase, inflammatory bowel diseases, kinetic analysis, sulfate-reducing bacteria.

## INTRODUCTION

Intestinal sulfate-reducing bacteria consume the organic compounds as a carbon source and electron donor in the process of dissimilatory sulfate reduction [1]. The species of *Desulfovibrio* and *Desulfomicrobium* genera oxidize these compounds incompletely to acetate *via* pyruvate and other intermediates [2]. The process of organic compounds oxidation is a complex and multistage that provides the bacterial cells with energy. The lactate is the most common substrate used by the species belonging to the intestinal sulfate-reducing bacteria [3].

In previous researches, it was demonstrated that the lactate consumption by the intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 and acetate accumulation in cultivation medium [4]. Lactate oxidation to acetate occurs with the intermediate compounds formation: pyruvate, acetyl-CoA and acetyl phosphate [1, 2, 5].

Acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) plays a significant role in energy production and catalyzes the formation of acetate from acetyl phosphate. This enzyme is involved in the synthesis of most of the ATP formed catabolically [1, 6, 7]:




Lactate oxidation to acetate occurs together with the concurrent reduction of sulfate to sulfide [1, 2]. In the presence of lactate and sulfate in the human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolism products, acetate and hydrogen sulfide, which are toxic and mutagenic to epithelial intestinal cells [3, 8]. The increased number of the sulfate-reducing bacteria and intensity of dissimilatory sulfate reduction in the gut can cause inflammatory bowel diseases of humans and animals [3, 8-10].

Acetate kinase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* has never been well-characterized. In literature, there are some data on acetate kinase in various organisms as well as in the sulfate-reducing bacteria isolated from environment [6, 11-18]. However, the data on the activity and the kinetic properties of this enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. has not been reported yet.

The aim of this work was to study acetate kinase activity in cell-free extracts of intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 and to carry out the kinetic analysis of enzymatic reaction.

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## MATERIALS AND METHODS

Objects of the study were sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and identified by the sequence analysis of the 16S rRNA gene [4, 19].

### Bacterial Growth and Cultivation

Bacteria were grown in a nutrition-modified Kravtsov-Sorokin's liquid medium [4]. Before seeding bacteria in the medium, 0.05 ml/l of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. A sterile 10N solution of NaOH (0.9 ml/l) in the medium was used to provide the final pH 7.2. The medium was heated in boiling water for 30 min in order to obtain an oxygen-free medium, and then cooled to +30°C. The bacteria were grown for 72 hours at +37°C under anaerobic conditions. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

### Obtaining Cell-free Extracts

Cells were harvested at the beginning of the stationary phase, centrifuged and suspended in 100 ml of 50 mM Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.0 (henceforth referred to as Tris buffer), containing 1 mM ethylenediaminetetraacetate (EDTA). A suspension of cells (150–200 mg/ml) was obtained and homogenized using the ultrasonic disintegrator at 22 kHz for 5 minutes at 0 °C to obtain cell-free extracts. The homogenate was centrifuged for 20 min at 16,000 g to remove the cell debris. The pellet was then used as sedimentary fraction, and the supernatant obtained was termed the soluble fraction. The supernatant fluid and a Tris buffer wash of the pellet were subjected to a second centrifugation at 16,000 g for 40 min [20]. The soluble extract constituted by the supernatant was used as the source of the enzyme. A pure supernatant, containing the soluble fraction, was then used as cell-free extract. Protein concentration in the cell-free extracts was determined by the Lowry method [21].

### Assays for Acetate Kinase Activity

The acetate kinase activity was assayed colorimetrically as described previously in paper [17]. The reaction mixture of 1 ml contained 50 µmol imidazole buffer of pH 7.3, 800 µmol potassium acetate, 10 µmol ATP, 20 µmol $MgCl_2$, 200 µmol neutralized hydroxylamine and 0.5–1.0 U of acetate kinase dissolved in 0.1 M phosphate buffer of pH 7.4 containing 0.005 M cysteine. After incubation for l0 min at 29°C the reaction was stopped by the addition of 1.0 ml 10% trichloroacetic acid. For the immobilization screening and storage stability tests, 1.0 ml of the same reaction mixture was pipetted onto the enzyme-loaded glass beads. After reaction for 10 min at 29°C the glass beads were allowed to settle and 800 µl of the supernatant was added to 1.0 ml 10% trichloroacetic acid. For both assays colour was developed by adding 4.0 ml of 1.25% $FeCl_3$ in 1.0 M HCl and absorbance was measured at 510 nm. The enzyme was also purified as described previously in paper [6]. One unit of acetate kinase is defined as that amount of acetate kinase which forms one micromole of acethydroxamic acid per minute under the conditions described. Specific enzyme activity was expressed as U × $mg^{-1}$ protein. The specific activity of the studied enzyme in the cell-free extracts of both bacterial strains under the effect of different temperature (+20, +25, +30, +35, +40, +45°C) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) in the incubation medium was measured.

### Kinetic Analysis

Kinetic analysis of the enzyme reaction was performed in a standard incubation medium (as it was described above) with modified physical and chemical characteristics of the respective parameters (the incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the acetate kinase reaction are the initial (instantaneous) reaction velocity ($V_0$), maximum velocity of the reaction ($V_{max}$), maximum amount of the reaction product ($P_{max}$) and characteristic reaction time (time half saturation) $\tau$ were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing acetate kinase reactions such as Michaelis constant ($K_m$) and maximum reaction velocity of substrate decomposition were determined by Lineweaver-Burk plot [22]. For analysis of the substrate kinetic mechanism of acetate kinase, initial velocities were measured under standard assay conditions with different substrate concentrations. The resulting data were also analyzed by global curve fitting in SigmaPlot to model the kinetic data for rapid equilibrium rate equations describing ordered sequential, $V=(V_{max}\ [A]\ [B])/(K_A\ K_B+K_B\ [A]+[A]\ [B])$, and random sequential, $V=(V_{max}\ [A]\ [B])/(\alpha\ K_A\ K_B+K_B\ [A]+K_A\ [B]+[A]\ [B])$, kinetic mechanisms, where V is the initial velocity, $V_{max}$ is the maximum velocity, $K_A$ and $K_B$ are the $K_m$ values for substrates A and B, respectively, and $\alpha$ is the interaction factor if the binding of one substrate changes the dissociation constant for the other [23].

### Statistical Analysis

Kinetic and statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by the methods of variation statistics using Student *t*-test. The equation of the straight line that the best approximates the experimental data was calculated by the method of least squares. The absolute value of the correlation coefficient *r* was from 0.90 to 0.98. The statistical significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ [24].

## RESULTS AND DISCUSSION

Specific activity of acetate kinase, an important enzyme in the process of organic compounds oxidation in intestinal sulfate-reducing bacteria, was measured in different fractions obtained from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cells (Table **1**).

Results of the study showed that the highest activity of the enzyme was measured in cell-free extracts ($1.52 \pm 0.163$ and $0.46 \pm 0.044$ U × $mg^{-1}$ protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). The slightly lower values of activity of acetate kinase were determined in

**Table 2. Kinetic parameters of the acetate kinase from intestinal sulfate-reducing bacteria.**

| Kinetic parameters | Sulfate-reducing bacteria | |
|---|---|---|
| | ***Desulfovibrio piger* Vib-7** | ***Desulfomicrobium* sp. Rod-9** |
| $V_0$ (μmol × $min^{-1}$ × $mg^{-1}$ protein) | 6.16 ± 0.63 | 1.39 ± 0.14*** |
| $P_{max}$ (μmol × $mg^{-1}$ protein) | 20.48 ± 2.51 | 6.74 ± 0.68*** |
| τ (min) | 3.33 ± 0.34 | 4.82 ± 0.49 |

**Comment**: $V_0$ is initial (instantaneous) reaction velocity; $P_{max}$ is maximum amount (plateau) of the product of reaction; τ is the reaction time (half saturation period). Statistical significance of the values M ± m, n = 5; ***P<0.001, compared to the *D. piger* Vib-7 strain.

**Table 3. Kinetic parameters of acetate kinase reaction.**

| Kinetic parameters | Sulfate-reducing bacteria | |
|---|---|---|
| | ***Desulfovibrio piger* Vib-7** | ***Desulfomicrobium* sp. Rod-9** |
| $V_{max}^{Acetyl\ phosphate}$ (μmol × $min^{-1}$ × $mg^{-1}$ protein) | 3.12 ± 0.32 | 1.03 ± 0.098*** |
| $K_m^{Acetyl\ phosphate}$ (mM) | 2.54 ± 0.26 | 2.68 ± 0.25 |
| $V_{max}^{ADP}$ (μmol × $min^{-1}$ × $mg^{-1}$ protein) | 3.05 ± 0.31 | 0.98 ± 0.095*** |
| $K_m^{ADP}$ (mM) | 2.39 ± 0.24 | 2.47 ± 0.27 |

**Comment**: $V_{max}$ is maximum velocity of the enzyme reaction; $K_m$ is Michaelis constant which was determined by substrate (acetyl phosphate and ADP). Statistical significance of the values M ± m, n = 5; ***P<0.001, compared to the *D. piger* Vib-7 strain.

The kinetic analysis of acetate kinase activity dependence on concentration of substrate (acetyl phosphate and ADP) was carried out. The increasing of acetyl phosphate and ADP concentrations from 0.5 to 5.0 mM caused a monotonic rise of the studied enzyme activity and the activity was maintained on unchanged level (plateau) under substrate concentrations over 2.5 mM (Fig. **2*C, E***). Curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (Fig. **2*D, F***). The basic kinetic parameters of acetate kinase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were identified by linearization of the data in the Lineweaver-Burk plot (Table **3**).

Calculation of the kinetic parameters of enzyme activity indicates that the maximum velocities ($V_{max}$) of acetyl phosphate and ADP in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different from each other. It was observed a correlative relationship between $V_{max}^{Acetyl\ phosphate}$ and $V_{max}^{ADP}$ as well as $K_m^{Acetyl\ phosphate}$ and $K_m^{ADP}$ in both intestinal bacterial strains. Michaelis constants ($K_m^{Acetyl\ phosphate}$ and $K_m^{ADP}$) of the enzyme reaction (2.54 ± 0.26 and 2.39 ± 0.24 mM for *D. piger* Vib-7 as well as 2.68 ± 0.25 and 2.47 ± 0.27 mM for *Desulfomicrobium* sp. Rod-9, respectively) were calculated.

Acetate kinase, discovered in 1944 by Lipmann (1944), is one of the earliest phosphoryl transfer enzymes recognized [25]. Following the first purification in 1954 from *E. coli* [17], the enzyme was the subject of investigations leading to two proposals for the catalytic mechanism: a direct in-line transfer of the γ-phosphoryl group of ATP to acetate [11, 18] or a triple-displacement mechanism involving two covalent phosphoenzyme intermediates [26]. The acetate kinase was also assayed and purified from cell extract of *Methanosarcina thermophila* by Ferry (2011). Cell extract from acetate-grown the cells was prepared for purification of acetate kinase [12]. This enzyme from *M. thermophila* was evaluated for detection of acetate in various biological fluids using the hydroxamate assay [14]. Acetate kinase had nearly an eightfold lower $K_m$ for acetate (<3 mM) than the wild type which was lower than those reported for commercially available acetate kinase from *Bacillus stearothermophilus* (120 mM) [16] or *E. coli* (7–300 mM) [13, 16] increasing the sensitivity [12].

The enzyme acetate kinase was also purified from *Desulfovibrio vulgaris* by a combination of ammonium sulfate precipitation, hydroxylapatite and dye-affinity chromatography. The specific activity in crude extract supernatant from *D. vulgaris* was 1.6 U/mg of protein [6, 15]. These data are consistent to the obtained results for the specific activity in cell-free extract from *D. piger* Vib-7 where the activity was 1.52 ± 0.163 U × $mg^{-1}$ protein.

Yu and coauthors (2001) have described two distinct forms of acetate kinase which were purified to homogeneity from a sulfate-reducing bacterium *Desulfbvibrio vulgaris* Miyazaki F [7]. The enzymes were separated from the soluble fraction of the cells. Total activity in the crude extract from *D. vulgaris* was 1.252 μmol/min. The dependence of initial velocity of AKI as measured by the rate of ATP formation on the ADP concentration under the fixed acetyl phosphate concentration of 2.5 mM. In the presence of fixed acetyl phosphate concentrations of 0.5, 1.0, 2.5, and 5.0 mM, the dependence of initial velocity of AK-I and AK-II on the ADP concentration was examined [7].

Acetate kinase from an thermophile, *B. stearothermophilus,* was purified and crystallized by

**Fig. (2).** Kinetic parameters of acetate kinase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: *A* – dynamics of product accumulation (M ± m, n = 5); *B* – linearization of curves of product accumulation in {P/t; P} coordinates (n = 5; $R^2 > 0.93$; F <0.02); *C*, *E* – the effect of different concentrations of substrate (acetyl phosphate and adenosine diphosphate) on the enzyme activity (M ± m, n = 5); *D*, *F* – linearization of concentration curves, which are shown in fig. 2*C*, *E*, in the Lineweaver-Burk plot, where V is velocity of the enzyme reaction and [Acetyl phosphate] or [Adenosine diphosphate] is substrate concentration (n = 5; $R^2 > 0.92$; F < 0.005).

Nakajima *et al*. (1978). This enzyme shared many common enzymatic properties with the counterpart from mesophiles, i.e. pH optimum, substrate specificity, requirement of metal ions and essential amino acid residues necessary for the catalytic activity. However, this enzyme was remarkably thermostable [16].

Thus, the acetate kinase, an important enzyme in process of dissimilatory sulfate reduction and lactate oxidation in sulfate-reducing bacteria, carries out the central step in the formation of acetate from acetyl phosphate.

## CONCLUSION

The acetate kinase activity, initial (instantaneous) reaction velocity and maximum velocity of the enzyme reaction were significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. The maximum enzyme activity for both strains was determined at +35ºC and at pH 8.0. The kinetic parameters of enzyme reaction depended on

substrate concentration. Michaelis constants for acetyl phosphate and ADP were quite similar (2.54 ± 0.26 and 2.39 ± 0.24 mM for *D. piger* Vib-7 as well as 2.68 ± 0.25 and 2.47 ± 0.27 mM for *Desulfomicrobium* sp. Rod-9, respectively) in both intestinal bacterial strains. The studies of the acetate kinase in the process of dissimilatory sulfate reduction and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, their production of acetate in detail may be perspective for clarification of their etiological role in the development in pathogenesis of the humans and animals bowel diseases. These studies might help in predicting the development of diseases of the gastrointestinal tract, by providing further details on the etiology of bowel diseases which are very important for the clinical diagnosis of these disease types.

## CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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## REFERENCES

[1] Barton LL, Hamilton WA. Sulphate-Reducing Bacteria. Environmental and Engineered Systems. Cambridge: Cambridge University Press, 2010.

[2] Kushkevych IV. Sulfate-reducing bacteria of the human intestine. I. Dissimilatory sulfate reduction. Studia Biologica 2012; 6(1): 149-80.

[3] Kushkevych IV. Sulfate-reducing bacteria of the human intestine. II. The role in the diseases development. Studia Biologica 2012; 6(2): 221-50.

[4] Kushkevych IV. Identification of sulfate-reducing bacteria strains of human large intestine. Studia Biologica 2013; 7(3): 115-24.

[5] Sadana JC. Pyruvate oxidation in *Desulphovibrio desulphuricans*. J Bacteriol 1954; 67(5): 547-53.

[6] Mannens G, Slegers G, Claeys A. Purification and immobilization of acetate kinase from *Desulfovibrio vulgaris*. Biotech Lett 1988; 10(8): 563-8.

[7] Yu L, Ishida T, Ozawa K, *et al*. Purification and characterization of homo- and hetero-dimeric acetate kinases from the sulfate-reducing bacterium *desulfbvibrio vulgaris*. J Biochem 2001; 129: 411-21.

[8] Pitcher MC, Cummings JH. Hydrogen sulphide: a bacterial toxin in ulcerative colitis? Gut 1996; 39: 1 - 4.

[9] Cummings JH, Macfarlane GT, Macfarlane S. Intestinal bacteria and ulcerative colitis. Curr Issues Intest Microbiol 2003; 4: 9-20.

[10] Gibson GR, Cummings JH, Macfarlane GT. Growth and activities of sulphate-reducing bacteria in gut contents of health subjects and patients with ulcerative colitis. FEMS Microbiol Ecol 1991; 86: 103-12.

[11] Blattler WA, Knowles JR. Stereochemical course of phosphokinases. The use of adenosine [gamma-(S)-$^{16}O$ $^{17}O$ $^{18}O$] triphosphate and the mechanistic consequences for the reactions catalyzed by glycerol kinase, hexokinase, pyruvate kinase, and acetate kinase. Biochem 1979; 18: 3927-33.

[12] Ferry JG. Acetate kinase and phosphotransacetylase. Methods Enzymol 2011; 494: 219-31.

[13] Fox DK, Roseman S. Isolation and characterization of homogeneous acetate kinase from *Salmonella typhimurium* and *Escherichia coli*. J Biol Chem 1986; 261: 13487-97.

[14] Iyer P, Ferry JG. Acetate kinase from *Methanosarcina thermophila*, a key enzyme for methanogenesis. Methods Biotech 2005; 17: 239-46.

[15] Mannens G, Slegers G, Lambrecht R. Immobilization of acetate kinase and phosphotransacetylase on derivatized glass beads. Enzyme Microb Technol 1987; 9: 285-90.

[16] Nakajima H, Suzuki K, Imahori K. Purification and properties of acetate kinase from *Bacillus stearothermophilus*. J Biochem 1978; 84: 193-203.

[17] Rose IA, Grunberg-Manago M, Korey SR, Ochoa S. Enzymatic phosphorylation of acetate. J Biol Chem 1954; 211(2): 737-56.

[18] Skarstedt MT, Silverstein E. *Escherichia coli* acetate kinase mechanism studied by net initial rate, equilibrium, and independent isotopic exchange kinetics. J Biol Chem 1976; 251: 6775-83.

[19] Kushkevych IV, Bartos M, Bartosova L. Sequence analysis of the 16S rRNA gene of sulfate-reducing bacteria isolated from human intestine. Int J Curr Microbiol Appl Sci 2014; 3(2): 239-48.

[20] Robinson JR, Sagers RD. Phosphotransacetylase from *Clostridium acidiurici*. J Bacteriol 1972; 112(1): 465-73.

[21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein determination with the folin phenol reagent. J Biol Chem 1951; 193: 265-75.

[22] Keleti T, Kramer M. Basic Enzyme Kinetics. Akademiai Kiado 1988.

[23] Segal IH. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. John Wiley & Sons: New York. 1975.

[24] Bailey NTJ. Statistical methods in biology. Cambridge: Cambridge University Press, 1995.

[25] Lipmann F. Enzymatic synthesis of acetyl phosphate. J Biol Chem 1944; 155: 55-70.

[26] Spector LB. Acetate kinase: A triple-displacement enzyme. Proc Natl Acad Sci USA 1980; 77: 2626-30.

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