MASARYK UNIVERSITY

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FUNCTIONAL ROLE OF SULFATE-REDUCING BACTERIA IN THE DEVELOPMENT OF BOWEL DISEASES IN HUMAN AND ANIMALS

Habilitation thesis

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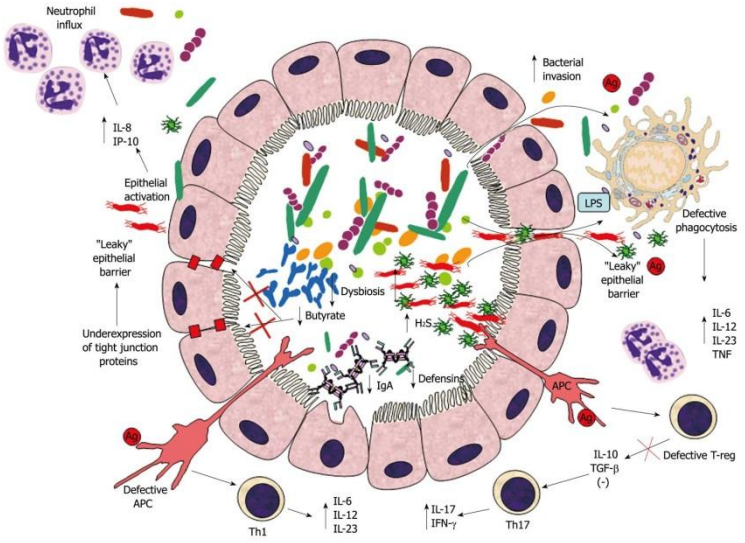
„*The method is the first and the main thing... From the method depends on the severity of the study. The method holds the fate of the study in hand*.“

I. P. Pavlov (1849 – 1936)



The proposed mechanism of pathogenesis of inflammatory bowel disease at elevated levels of sulfate-reducing bacteria (the scheme is modified from Fava and Danese, 2011)

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ABSTRACT

This habilitation thesis is focused on the study of physiological, biochemical and pathogenic properties of sulfate-reducing bacteria (SRB) isolated from human and animal feces. As a consequence of this work, a conception of the functional role of SRB in the intestinal microbiota based on the ability of these microorganisms in the process of dissimilatory sulfate reduction to produce toxic metabolites that are a prerequisite for colon disease was developed. The intestinal SRB, isolated from healthy and individuals with ulcerative colitis, were not differ statistically based on its physiological and biochemical properties. The ratio of dominant SRB, *Desulfovibrio* and *Desulfomicrobium* genera, was 93:7% in the feces of healthy people and 99:1% in the feces of people with ulcerative colitis. The main criteria for assessing the development and progression of an inflammatory process involving the intestinal SRB is: a change in microbial and colonic pH, bacterial enzyme activity, sulfate reduction, SRB growth rate and sulfate reduction intensity, hydrogen sulfide and acetate concentration in the feces. Prevention of this process can be accomplished by inhibiting two enzymes of the dissimilatory sulfate reduction process, particularly sulfite reductase and lactate dehydrogenase, which are the most sensitive side in intestinal SRB metabolism pathway.

The experimental results described in this work may be useful for a more detailed study of an inflammatory bowel disease with using a therapeutic strategy. Determining the number of SRBs and the hydrogen sulfide and acetate concentration allowed the development of basic criteria for assessing the aggressiveness of these bacteria, the toxic products of their metabolism to the intestinal mucosa as well as assessing the level of risk of the disease and the course of the inflammatory process. The work can be scientifically beneficial mainly because the results described are important for the study of ulcerative colitis in animal models, especially for the study of mechanisms of action of antimicrobials with prophylactic or therapeutic target of specific components involved in the pathogenesis of the disease. The study of the stability of selected strains of bacteria on antimicrobial and newly synthesized substances and also their influence on the physiological and biochemical properties of SRB allows the search for new and promising drugs. Such studies are promising in developing methods of preventing bowel disease.

Key words: sulfate-reducing bacteria, *Desulfovibrio piger*, *Desulfomicrobium orale*, hydrogen sulfide, toxicity, bowel disease.

CONTENTS

INTRODUCTION

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that metabolize sulfates as the terminal acceptor of electrons to hydrogen sulfide in the process of „dissimilatory sulfate reduction“ (or dissimilatory sulfate respiration). For this process, SRB uses exogenous electron donors, such as organic compounds and molecular hydrogen (Postgate, 1984). Depending on the SRB genera, organic compounds are oxidized incompletely to acetate (acetogenic SRB) or completely to carbon dioxide (Barton & Hamilton, 2010).

SRB are widespread in anaerobic areas of soils, wetlands, marine and fresh water, they are available in microbiocenosis large intestine of humans and animals (Postgate, 1984; Barton & Hamilton, 2010). Acetogenic SRB and their final products of metabolism (including, hydrogen sulfide and acetate) are often found in the feces of people with bloody diarrhea (Pitcher *et al.*, 1996, 1998) and the mono- and polymicrobial infections of the gastrointestinal tract (McDougall *et al.*, 1997; Loubinoux *et al.*, 2003; Rowan *et al.*, 2009). It is believed that SRB can cause frequent defecation, weight loss, increased intestinal permeability (Pitcher *et al.*, 2000; Cummings *et al.*, 2003; Rowan *et al.*, 2009). The species and quantitative composition of the SRB on the surface of the intestinal mucosa are differs from those microorganisms in the lumen (Macfarlane *et al.*, 1999; Gibson *et al.*, 1991). Such genera of SRB, *Desulfovibrio*, *Desulfomicrobium*, *Lawsonia*, *Bilophila*, are the most isolated from the intestines of healthy and sick people and animals (Gibson *et al.*, 1991; Barton & Hamilton, 2010; Brenner *et al.*, 2005).

In recent years, the number of cases of inflammatory bowel disease, including ulcerative colitis is growing, the cause of the occurrence of which is still unknown. This disease is mainly distributed in people aged 15 to 30 years, although there is evidence of cases of disease in people aged 50–70 years, which can be in potential risk group. Frequent cases of the disease recorded in both developed and underdeveloped countries (Lakatos *et al.*, 2006; O'Connor *et al*., 2011; Low *et al.*, 2013). Ulcerative colitis suffer not only people but also animals: horses (Scott, *et al*., 2014) and cows (Braun *et al*., 2015), pigs (Thomson, 2009; White, 2016), dogs (Cerquetella *et al*., 2010), cats (Heilmann & Suchodolski, 2015), rodents (mice and rats) (Lee *et al*., 2014; Dugani *et al*., 2016). The functional role of SRB in the development of inflammatory bowel disease, including ulcerative colitis and etiology of these diseases is less explored, the mechanisms of the disease has not been fully elucidated.

The fundamental basis of the thesis is research approach based on identifying the basic mechanisms of inflammatory bowel disease, based on a study of the functional role of SRB; to develop the basic evaluation criteria of aggressiveness of these microorganisms, and toxicity of their products of metabolism for the intestinal mucosa. This work is focused on theoretical and experimental study of mechanisms of inflammatory bowel disease under the influence of SRB and based on a comprehensive study of their physiological and biochemical characteristics, implementation of kinetic analysis of enzyme involved in process of dissimilatory sulfate reduction.

The aim of this work was to create and justify the functional role of acetogenic sulfate-reducing bacteria in the development of bowel disease in humans and animals based on the physiological and biochemical properties of its dominant genera in the gut.

To achieve of this purpose, the following tasks were:

1. Investigation of the composition of intestinal microbial communities in healthy people and patients with ulcerative colitis, isolate and identify by morphological, physiological, biochemical and genetic characteristics sulfate-reducing bacteria;

2. Determinatoin of the biochemical characteristics of the isolated bacteria, in particular kinetic analysis of main enzymes involved in the process of the transporting substances, dissimilatory sulfate reduction, oxidation of lactate to acetate, and antioxidant protection;
3. Investigation of the physiological characteristics of SRB, parameters of biomass accumulation and process dissimilation sulphate under the influence of different values of pH, concentration of hydrogen sulfide, electron acceptors and donors, and to carry out the kinetic analysis of these parameters;
4. The creation a model of ulcerative colitis in rats using isolated strains of sulfate-reducing bacteria;
5. Molecular and genetic construction of SRB which can utilize hydrogen sulfide and its metabolizing into cysteine;
6. Identification of the main criteria for assessing the aggressiveness of SRB, toxicity of their metabolic products for the colon mucosa, and establishment of functional role of bacteria in the disease development.

1. THE SCIENTIFIC NOVELTY AND CONCEPTIONS OF THE RESEARCHES

The conception of the basic developmental mechanisms of inflammatory bowel diseases, including ulcerative colitis, was created based on the physiological and biochemical characteristics of acetogenic SRB. The main provisions of the conception are:

- Acetogenic representatives of the *Desulfovibrio* and *Desulfomicrobium* genera are dominant among SRB in the intestines of healthy people and patients with colitis. Increasing the number of SRB and concentration of the main metabolic products (hydrogen sulfide and acetate) in the consumption of exogenous electron acceptor (sulfate) causes changes in the structure of the microbiota of the intestine, including decreasing of the number of autochthonous representatives of bacterial genera: *Bifidobacterium* and *Lactobacillus*, increasing the number of elective and opportunistic bacteria genera: *Bacteroides*, *Escherichia*, *Clostridium* and *Proteus*, changing the ratio of intestinal microbiota groups, sustainability index and frequency of detection.
- Genetically constructed strain of *D. piger* Vib-7, containing the *CysK* gene, able to metabolize hydrogen sulfide to non-toxic cysteine, which contributes to reduce of the toxic effects of this compound on the colon mucosa. Using of this strain was demonstrated the influence of dissimilatory (not assimilatory) process of sulfate reduction on the occurrence of inflammation in the intestines of animals.
- Inflammatory bowel disease can develop by such mechanism: the increased number of acetogenic SRB, due to exogenous acceptors (sulfate), activates and accelerates the process of dissimilatory sulfate reduction, accumulation of hydrogen sulfide and acetate. Acetate causes lowering pH in intestinal lumen to 6.8, showing a synergistic effect and increases the negative impact of toxic hydrogen sulfide on the mucous membrane and the colon cells, leading to changes in species diversity of autochthonous microflora, contributes to the destruction of the protective mucous layer of intestinal cells with subsequent development of inflammatory process, the withering away of the epithelial cells and the occurrence of ulcerative colitis.

The number of *Lactobacillus* and *Bifidobacterium* bacterial genus in the feces of people with ulcerative colitis was reduced by 6–7 orders, but the numbers of facultative (*Bacteroides*, *Escherichia*, *Enterococcus* genera) and conventionally pathogenic (*Clostridium* and *Proteus* genera) microflora were increased by 1–4 orders. The number of acetogenic SRB, *Desulfovibrio* and *Desulfomicrobium*, genera were also significantly (by 5 orders) increased. The ratio of these bacterial genera was 93:7% in healthy people and 99:1% in people with UC. The isolated and described dominant SRB in the large intestine by morphological, physiological, biochemical and genetic characteristics were identified as the *Desulfovibrio* and *Desulfomicrobium* genera. Based on statistical analysis methods, including cluster, the affinity of SRB isolated from healthy people and patients with UC was shown.

Using the model selected strains, *D. piger* Vib-7 and *D. orale* Rod-9, the physiological characteristics of growth and process of dissimilatory sulfate reduction in these bacteria were determined. The main enzymes and kinetic parameters of transporting substances, process of dissimilatory sulfate reduction, metabolize of lactate to acetate in the SRB cells were characterized. The highest enzymatic activities was determined at temperature (+35 °C) and pH (pH 7.0–8.5). These data is consistent with the the colon condition which is optimal for the growth of these microorganisms. Hydrogen sulfide affects not only the representatives of the autochthonous intestinal flora, but also completely inhibit the growth of its producers (*D. piger* Vib-7) and their process of sulfate reduction in concentration of 7 mM H_2S. Based on the simulation of the electron

acceptor and donor on the SRB growth, optimal concentrations (10.5 mM and 53.4 mM, respectively) were determined. Under these conditions, their biomass was increased in 2.5 times and the maximum amount (6.06±0.59 mM) of hydrogen sulfide was accumelated. It can be one of the prerequisites of inflammation process.

The model of ulcerative colitis in rats using acetogenic SRB was created based on study of their physiological and biochemical characteristics. It is possible to define the role of these microorganisms and possible mechanisms of the colon diseases, assess the aggressiveness of SRB and toxicity of final products of their metabolism on the formation of ulcers. Final SRB metabolites cause changes in the colon lumen conditions, such as pH and reducing the amount of lactic acid microorganisms. Acetate produced by SRB shows synergistic effect and enhances the negative effect of hydrogen sulfide on the mucous membrane, which leads to inflammation followed its complications. According to the model of ulcerative colitis in rats using a strain of genetically constructed *D. piger* Vib-7M containing gene *CysK*, able to metabolize hydrogen sulfide to non-toxic compounds (cysteine). A decisive role in the inflammatory process plays SRB with excessive production of hydrogen sulfide, which has toxic effects on epithelial cells and leads to the formation of ulcers.

The practical significance of the researches. The described physiological and biochemical properties of metabolic processes of acetogenic SRB from intestine of animals and humans allow to assess their ability to induce inflammatory bowel disease. Data on changes in the number of SRB, their proportion in the feces of humans, and production of hydrogen sulfide can be used for predicting of the course of bowel inflammation. The indicators for determining of the aggressiveness of SRB were proposed based on toxicity of their metabolic products for the intestinal mucosa. New scientific approaches and criteria for evaluating of bowel inflammation progress, allowing determination of the level of risk of the disease with the aim of early diagnosis and timely predicting were proposed. The results of the work are recommended for developing prevention methods and using of antimicrobial compounds for the treatment of intestinal diseases. A collection of SRB strains isolated from human intestine was created and complemented by genetically constructed *D. piger* Vib-7M strain which produce of cysteine as the end product of sulfate reduction but non-toxic hydrogen sulfide.

The practical value of the results is implementated and applicated at the Laboratory of Anaerobic Microorganisms at the Department of Experimental Biology of Faculty of Science at Masaryk University. The study is used in pedagogical process in teaching courses „General Microbiology", „Bacterial Physiology", „Human Physiology", „Human Pharmacology and Toxicology", „Veterinary Microbiology", „Molecular Biology".

Personal contribution of the author. The habilitation thesis is an independent work of the author. Based on the results obtained in the study of physiological and biochemical characteristics of sulfate-reducing bacteria, the concept and hypotheses by the topic of the dissertation were created. The author himself made information retrieval and conducted an analytical review of the literature. The methodology was substantiated and the main experimental studies and the statistical processing, analysis and summarizing of the results were carried by author personally as well as the articles for printing were prepared. SRB strains were isolated from human intestine, their physiological and biochemical properties were studied, and the cluster, correlation, cross-correlation analysis and the analysis of variance of parameters of dissimilatory sulfate reduction process in SRB, kinetic analysis of the process, were carried. Based on isolated bacteria, a model of ulcerative colitis in rats was created by the author.

The discussion of the directions of the research and the main results of the research was carried out jointly with a scientific consultant Assoc. Prof. Monika Vítězová, Ph.D. The modeling of physiological and biochemical parameters of SRB was carried out jointly with Dr. Mark Bolis from Mario Negri Institute of pharmacological research (Milan, Italy). Together with Assoc. Prof. Milan Bartoš , Ph.D., the molecular and genetic studies and identification of SRB were carried out, and described in joint scientific publications.

Approbations the results of the habilitation thesis. The results of the habilitation thesis were presented at the international scientific conferences „Youth and Progress of Biology" (Lviv, Ukraine 2012); International Medical Congress of Students and Young Scientists (Ternopil, Ukraine 2011, 2012); 73 University Scientific Conference „Achievements of Modern Medicine" (Danylo Halytsky Lviv National Medical University, Lviv, Ukraine 2012); International Congress of Medical Sciences (Sofia, Bulgaria 2014; 2015); Czech-Slovak International Conference „Květinův den" (Brno, Czech Republic, 2013, 2014, 2015); International scientific conference „Actual problems of Biophysics" (Lviv, 2014); International scientific-practical conference „Current problems of modern biology, animal husbandry and veterinary medicine" (Lviv, Ukraine 2014); XIII National Scientific Conference of Young Scientists „Young scientists in solving current problems of biology, animal husbandry and veterinary medicine" (Lviv, Ukraine 2014), Proceedings of International PhD Students Conference – MendelNet (Brno, 2017, 2018). The results of the thesis are described in 30 scientific papers in foreign scientific journals registered in the scientific-metric database (Web of Science, Scopus, PubMed, Google Scholar, etc.).

The main methods used in this thesis. SRB strains isolated from human and animal feces were used in this work. Isolated strains have been identified based on their physiological and biochemical properties as described in the paper by Kushkevych (2013) as well as on the basis of sequential analysis of the 16S rRNA gene (Kushkevych et al., 2014). The strains have been kept in a collection of microorganisms in the Laboratory of Anaerobic Microorganisms at the Department of Experimental Biology at Masaryk University in Brno.

The ecological-trophic groups of microorganisms of intestinal microflora were determined in the stool samples of the people. Determination of the number of microorganisms was carried out by ten-fold dilutions with followed screening on elective nutrient media, according to conventional methods (Egorov, 1976; Gerhardt, 1984). To determine the number of SRB and the isolation of their pure cultures, isolated colonies were multiple replanting in the appropriate modified selective liquid and agar Kravtsov-Sorokin medium (Sorokin, 1966). Pure cultures of microorganisms were isolated by Koch method. Cultivation was for 10 days at a temperature of +37°C under the fixed anaerobic conditions using boxes with oxygen absorbing of generators (Genbox anaer Biomerieux, France). Phenotypic studies of pure SRB cultures were carried out by generally known methods (Postgate, 1984; Rozanova et al. 1989; Stebbings et al., 2002). Electron microscope examination of SRB cell morphology was conducted by Reynolds method (Reynolds, 1963). Samples were microscopic using transmission electronic microscope (PEM-100). The concentration of sulfate ions was determined by turbidometric method (Kolmert et al. 2000), sulfide ions by spectrophotometric methods (Cline 1969), and lactate level by the dehydratation reaction using Lactate Assay Kit (Sigma-Aldrich, USA). Accumulation of acetate during the growth of SRB was determined using a commercial reagent of Acetate Assay Kit (Sigma-Aldrich Co. LLC, USA).

To determine the phylogenetic identity of SRB, the molecular and genetic identification using analysis of 16S rRNA gene full sequences was carried out. Isolation and purification of DNA carried out using biomass of three daily cultures of SRB by using „QIAmp DNA Mini Kit

(QIAGEN, Germany)". Amplification of 16S rRNA gene fragments was performed by W.G. Weisburg et al. (1991) and D.H. Pershing (2011) using pairs of universal primer: 8FPL (5′-AGT-TTG-ATC-CTG-GCT-CAG-3′), 1492RPL (5′-GGT-TAC-CTT-GTT-ACG-ACT-T-3′), 8FPL (5′-AGT-TTG-ATC-CTG-GCT-CAG-3′) and 806R (5′-GGA-CTA-CCA-GGG-TAT-CTA-AT-3′) (Eurofins Scientific, Luxembourg). To purification of amplicons, the "MinElute Gel Extraction Kit" (QIAGEN) was used. Sequencing was performed using the "Genetic Analyzer" (Life Technologies Corporation, USA) and reagents "BigDye Terminator v3.1 Cycle Sequencing Kit" (Applied Biosystems, USA). Analysis of the obtained sequences of 16S rRNA gene with homologous nucleotide sequences of the genes deposited in database GenBank, was performed using BLASTN program (www.ncbi.nlm.nih.gov/nblast). The sequences of gene fragments of 16S rRNA SRB were deposited in the GenBank database as the numbers: KT881309, KT989311 – KT989316.

To determine the enzymatic characteristics of intestinal SRB, the cell-free extracts were obtained. The concentration of protein in the cell-free extracts was determined by Lowry method (Lowry 1951). Enzymatic activity was determined by following methods: ATPase (Flynn et al. 2001; Rathbun & Betlach 1969), ATP sulfurylase (Ravilious et al. 2013), APS reductase (Peck et al. 1965), sulfite reductase, desulfoviridin, desulforubridin (Kobayashi et al. 1972; 1974; Lee et al. 1973), pyrophosphatase (Akagi & Campbell 1963), hydrogenase (Yu & Wolin 1969), lactate dehydrogenase using reagents "Cytotoxity Assay Kit" (Roche Diagnostics GmbH, Germany), pyruvate:ferredoxin oxidoreductase (Pieulle et al. 1995; Zeikus et al. 1977), phosphotransacetylase (Shimizu et al., 1969), acetate kinase (Rose et al. 1954; Mannens et al. 1988). Catalase activity was determined spectrophotometrically (Biospectrophotometer, Eppendorf, USA) by the reaction of decomposition of hydrogen peroxide (Luck 1963; Goldblith & Proctor 1950), superoxide dismutase by inhibiting reduction of nitroblue tetrazol (Beavchamp & Fridovich 1971). One unit (U) of enzyme activity was such amount of enzyme, which forms 1 micromol of primary product per minute.

A kinetic analysis of enzymatic reactions was performed in the standard incubation medium with changed the physical and chemical conditions of such parameters: incubation time, substrate concentration, temperature and pH. The following kinetic parameters of enzymatic reactions: initial (immediate) reaction rate (V_0), maximum reaction rate (V_{max}), the maximum amount of the reaction product (P_{max}) and the reaction time (half saturation period, τ) were determined. Amount of reaction product was calculated stoichiometric as described by Keleti (1988). Kinetic parameters: Michaelis constant (K_m) and maximum rate of reaction of the product accumulation was identified in Lineweaver–Burk coordinates (Lineweaver & Burk 1934). To analyze of the kinetic mechanisms of substrate consumption, the initial (instantaneous) reaction rate under standard conditions with different concentration substrate was determined.

To investigate the role of SRB in the development of colon diseases and creating a model of ulcerative colitis, in total 45 laboratory rats were used. The animals were divided into three groups (15 animals in each group): the first group was used as control; second and third experimental group for creating a model of ulcerative colitis. The control group of animals received a standard certified feed for laboratory rats (PK-120-1, Laboratorsnab). Animal of second group received standard feed and 1 ml modified Kravtsov-Sorokin medium every day. The third group received standard animal feed, 1 ml suspension of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium orale* Rod-9 (ratio 1:1) bacterial strains in Kravtsov-Sorokin medium (bacterial concentration was 3 mg/mL). Quantitative and qualitative analysis of the intestinal microflora of experimental animals was determined after 5, 10, 15, 20, 25 days from the start of the experiment. The identification of isolated cultures of microorganisms was carried out by morphological, tinctirial, cultural, biochemical properties using

test systems API 20E (bioMerieux, France). After incubation, the number of microbial colonies was counted and expressed as an index log10 CFU/gram of wet weight of the sample. Microflora of the lumen colon was evaluated by an Sustainability Index (*SI*, %) and frequency detection (P_i) (Odum 1986; Klymiuk 1995). The release and concentration of H_2S of 1 g wet weight faeces calculated as was described in the papers (Ohge et al. 2003; Levitt et al. 2003). Acetate concentration in the samples was determined colorimetrically using reagents of Acetate Colorimetric Assay Kit (Sigma-Aldrich, USA).

For molecular and genetic construction of SRB strain, which would be able to cysteine synthesis, *CysK* gene from *E. coli* K-12 genome was cloned and transformed in SRB cells by modified method (Hanahan 1983; Seidman 1994). For cloning of the gene, the commercial reagents "HotStar Master Mix Taq polymerase" (QIAGEN), UDG-glycosylase (New England Biolabs) and a pair of primers: direct CysK-F (5'-CGAGGCAGATCTTAG-3') and reverse CysK-R (5' – CGAGGCAGATCTCTCGA-GTAG-3') (Generi-Biotech). Regime of thermal cycling: 2 min (+37°C), initial denaturation (15 min, +95°C), 35 cycles (10 s, +95°C), 20 s (+ 50°C) and 30 s (+72°C), 5 min (+72°C) and final cooling (to +10°C). PCR products analyzed by electrophoresis in 1.5% PAA gel with using ethidium bromide (Sigma-Aldrich, USA) and field strength of 5 V/cm. As molecular weight marker was used 100 bp ladder (New England Biolabs, USA). Isolation and purification of DNA fragments from agarose carried out by centrifugation (14,000 g) through aerosol filters and using "MinElute Gel Extraction Kit" (QIAGEN, USA). PCR products transferred to SRB by using reagents "AccepTorTM Vector Kit" (Novagen, USA). Plasmid DNA of SRB was isolated using reagents "QIAprep Spin Miniprep Kit" (QIAGEN). The presence of prebuilt *CysK* gene in the plasmid was confirmed by PCR using "Maxima^{TM} Probe qPCR Master Mix 2X" (Fermentas, Lithuania) and a pair of universal primers: direct M13F20 (5'-CTAACGACGGCCAG-3') and reverse M13R (5'-CAGGAAACAGCTATGAC-3') (Applied Biosystems, USA). The sequence of the cloned *CysK* gene in the plasmid confirmed by sequencing. The presence of the *CysK* gene was also determined by activity of *O*-acetylserine(thiol)lyase (Fimmel & Loughlin 1977) and cysteine concentration in the medium which was determined as the previously described in the paper (Gaitonde 1967).

The basic statistical parameters including arithmetic mean (M) and standard error (m) of the arithmetic mean (M ± m) were calculated. To assess of the reliability of the differences between the statistical characteristics of alternative of data, ratio Student's coefficient was calculated (Bailey 1995). All research results processed by variation statistics methods. The difference of the parameter of the reliability of $P \leq 0.05$ was considered as an authentically. The value of the statistical reliability of the parameters using Fisher test (F-test) was tested. Statistical analysis of the results was carried out by cluster, multivariate variance, correlation and cross-correlation analysis using of software packages: Statistica 6.1 (www.statistica.software.informer.com) and Origin 7.0 (www.originlab.com). The absolute value of the linear correlation coefficient (*r*) was calculated by the method of Pearson. Cross-correlation graphical data converted to digital using the software package GRAPH DIGITIZER (www.getdata-graph-digitizer.com). Obtaining equations of functions approximation of the experimental data was performed by least squares (Chen & Popovich 2002). The share impact on the accumulation of SRB biomass (η^2, %), Fisher's coefficients (*F* practical, *F* critical) and the reliability of the impact (Lapach et al. 2001). The obtained time series of the process of sulfate reduction was determined using a STATGRARNICS software package (www.statgraphics.com).

2. DIVERSITY OF MICROBIAL COMMUNITIES IN THE INTESTINE

The study of intestinal microbial communities in healthy people and patients with UC showed the changes in the ratio of main genera of autochthonous and facultative microbiocenosis (Fig. 1). The number of representatives of the autochthonous bacterial flora, particularly *Lactobacillus* and *Bifidobacterium* genera, in patients with UC were by 6–7 orders lower, and facultative and conditionally pathogenic bacteria, including genera *Bacteroides*, *Escherichia*, *Enterococcus*, and *Proteus* were by 1–4 orders higher compared with microflora of healthy people. The content of bacteria capable to sulfate reduction (particularly, *Clostridium* and SRB) in stool samples of patients with UC was greater, respectively, by 4 and 5 orders, compared with samples of healthy. The number of methanogens microorganisms was also decreased by 2 orders (Fig. 1*A*), which is consistent with the literature data on the substrate competition of these microbial groups (Gibson et al. 1993).

The different ratio in the members of the dominant SRB morphotypes in the feces of healthy people and patients with UC was determined. Two morphotypes of SRB colonies were detected: large colonies **I** (ø 2–3 mm vibrios cell shape) and small colonies **II** (ø to 1 mm, short rods). In the feces of healthy people, the vibrios SRB (93%) were dominated, while amount of SRB of rod shape was only 7%. In patients with colitis, the ratio (in %) of these morphotypes was 99:1. The concentration of hydrogen sulfide in the feces of persons with UC was 2 times higher, compared with healthy and dependent on the number of SRB. The culture of SRB isolated from patients with UC produced by 1.5–2 times higher of this metabolite than isolates from healthy (Fig. 1*B*). It is consistent with the literature (Florin et al., 1990).

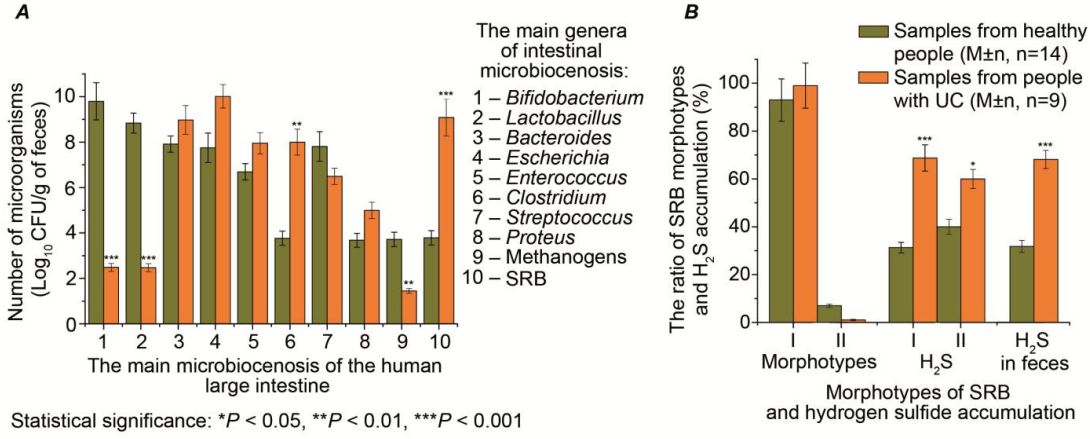

Fig. 1. Changes in the number of the main microbial genera in the intestine of healthy people and patients with UC (*A*), the ratio of SRB morphotypes isolates and accumulation of hydrogen sulfide (*B*).

It is known that hydrogen sulfide is in the ionized, non-volatile and volatile state (S^-, HS^- and H_2S) (Roediger *et al.*, 1993; Levitt *et al.*, 1999). M. D. Levitt et al. found that 95% of total sulfide in the intestine is absorbed, and only 5% in feces is bound form with other compounds (Levitt *et al.*, 1999; 2002). H_2S concentration in feces is largely dependent on its ability to interact with other substances than stand by bacteria in the intestinal lumen (Ohge *et al.*, 2003). Some researchers reported that total number of SRB was not increased in the feces of people with UC, but the ratio of their species was only changed. They were aggressive and intense growing in the culture medium. The level of hydrogen sulfide production in feces was increased by 28% (Willis *et al.*, 1997).

Since we have established a significant amount of SRB and high concentrations of H_2S in the faeces of patients with UC, 157 SRB colonies were selected and analyzed, including 79% (124 colonies) belonged to morphotype **I** and 21% (33 colonies) belonged to morphotype **II**. Isolates from the patients with colitis and healthy people did not differ in morphology and size of their cells.

For further phenotypic identification, 20 isolates of bacteria were selected, including 10 of them from patients with UC and 10 from healthy people. All bacteria were catalase positive and able to reduction of nitrate to nitrite, but not form indole, and also produced a significant amount of hydrogen sulfide. As a source of carbon and electron donor they metabolized lactate, pyruvate, malate, citrate, ethanol and glucose and not used formate, propionate, methanol, glycerin, oleate, stearate and benzoate. Growth of the isolates was observed in the medium with H_2 and CO_2 + acetate in the presence. Intestinal SRB assimilated organic acids, alcohols and some amino acids (alanine, aspartate, glutamate). The type of their growth was hemolitoheterotrophic. Maximum biomass of SRB (3.89±0.35 g/l) and their production of hydrogen sulfide (3.23±0.29 mM) was determined on the 6^{th} day of cultivation (time of achievement of stationary growth phase) at +35 °C and pH 7.0–8.0, which correspond to conditions of existence of SRB in the gut. SRB actively dissimilate of sulfate and accumulate of hydrogen sulfide produced, while lactate oxidized uncomplete to acetate. Based on morphological, cultural, physiological and biochemical research (by Bergey's Manual of Determinative Bacteriology, 1994) isolated vibrios shaped of SRB (Vib-1, Vib-2, Vib-3, Vib-4, Vib-5, Vib-6, Vib-7, Vib-8, Vib-9, Vib-10) was previously identified as the *Desulfovibrio* genus, and short rod shaped of SRB (Rod-1, Rod-2, Rod-3, Rod-4, Rod-5, Rod-6, Rod-7, Rod-8, Rod-9, Rod-10) as the *Desulfomicrobium* genus.

The length of lag phase, generation time (T_d) and the maximum growth rate (μ_{max}) strains isolated from patients and healthy people was calculated (Table 1). As seen from the results, the generation time was less (1.73±0.15 h) in the *Desulfovibrio* strains rom patients with UC compared with species of the *Desulomicrobium* genus (2.17±0.23 h). The maximum growth rate in the desulfovibrios was also higher (0.058±0.007 h^{-1}). It may indicate the intense growth of these bacteria in the gut. As for the *Desulfomicrobium* bacterial strains, they are able to multiply faster in healthy people, the generation time of microorganisms was 1.93±0.17 h and maximum growth rate was 0.052±0.004 h^{-1}.

Table 1. The kinetic parameters of growth of the strains of *Desulfovibrio* and *Desulfomicrobium* genus isolated from healthy people and patients with UC

SRB strains	Duration of lag phase (hour)	Time of generation T_d (hour)	Maximal speed of the growth μ_{max} ($hour^{-1}$)
Desulfovibrio isolated from			
patients with UC	5.21±0.49	1.73±0.15	0.058±0.007
healthy people	5.46±0.51	1.77±0.19	0.056±0.009
Desulfomicrobium isolated from			
patients with UC	4.02±0.35	2.17±0.23	0.046±0.002
healthy people	5.08±0.46	1.93±0.17	0.052±0.004

The kinetic parameters of growth of SRB from patients and healthy people did not differ statistically and consistent with the literature, where were also not found physiological differences between intestinal SRB isolated from patients with UC and healthy people (Levine et al., 1998). Based on the sequences of 16S rRNA gene analysis, the detailed identification of SRB was carried

out. The nucleotide similarity of 20 studied SRB with strains deposited in GenBank, determined by the results of the analysis and presented some of them in Table 2.

For bacteria of *Desulfovibrio* was found that the nucleotide sequence of 7 strains (Vib-1, Vib-2, Vib-3, Vib-4, Vib-7, Vib-8, Vib-10) had homology of 99% with the nucleotide sequence of the strain of *Desulfovibrio piger* ATCC 29098 deposited in the GenBank database by accession number NR 041778.1. As for the strains of *Desulfovibrio* sp. Vib-5, Vib-6 and Vib-9, these sequences data are not allowed to determine their species membership, but only on their genera level, because nucleotide sequences have homology of 95–96% to referent strain *D. piger* ATCC 29098, that indicates they probable belong to other species. The nucleotide sequence of 16S rRNA gene of the strain Rod-9 of *Desulfomicrobium* genus was 99% homologous to *Desulfomicrobium orale* strain NY677 (AJ251629.1) deposited in the GenBank database. For the other 9 strains of *Desulfomicrobium* genus, their species membership could not determine according to the sequences analysis, because a percentage of homology to the representatives of the *D. orale* species was only 96–98%, they were only identified to genus (data not shown in table). The obtained nucleotide sequence fragments of 16S rRNA gene of identified *Desulfovibrio piger* cultures been deposited in the GenBank database under the corresponding numbers (KT989311), Vib-2 (KT989312), Vib-3 (KT989313), Vib-4 (KT989314), Vib-7 (KT881309), Vib-8 (KT989315), Vib-10 (KT989316).

Table 2. The results of sequence analysis of 16S rRNA gene of SRB strains isolated from human intestine

Strains and access number in GenBank	Length of the gene fragment (bp)	Reference strains in GenBank and accession number	Identity (%)
Vib-7 KT881309	1370	*D. piger* ATCC 29098 NR 041778.1 *D. fairfieldensis* ATCC700045 *D. desulfuricans* Essex 6 NR 104990.1	99 96 95
Vib-10 KT989316	734	*D. piger* ATCC 29098 NR 041778.1 *D. desulfuricans* Essex 6 NR 104990.1 *D. legallii* strain H1 NR 108301.1	99 95 94
Vib-1 KT989311 Vib-2 KT989312 Vib-3 KT989313 Vib-4 KT989314 Vib-8 KT989315	742 742 743 736 748	*D. piger* ATCC 29098 NR 041778.1 *D. desulfuricans* Essex 6 NR 104990.1 *D. legallii* strain H1 NR 108301.1 *D. intestinalis* KMS2 NR 026413.1	99 94–95 93–94 93–94
Vib-5	748	*D. piger* ATCC 29098 NR 041778.1 *D. desulfuricans* Essex 6 NR 104990.1 *D. intestinalis* KMS2 NR 026413.1	96 95 93
Vib-6 Vib-9	722 742	*D. piger* ATCC 29098 NR 041778.1 *D. piger* ATCC 29098 NR 041778.1	95 96
Rod-9	1470	*D. orale* NY677 AJ251629.1 *D. orale* DSM 12838 CP014230.1 *D. orale* JCM 17150 NR 113205.1	99 99 99

Based on the results of sequence analysis of 16S rRNA gene, the phylogenetic analysis was carried out and dendrogram showing the affinity of the *D. piger* Vib-7 and

D. orale Rod-9 strains with other members of the *Desulfovibrio* and *Desulfomicrobium* genera was built (Fig. 2). As shown in the dendrogram, bacteria *D. piger* Vib-7 belonged to one cluster with *D. piger* ATCC 29098, *D. desulfuricans* Essex 6, *D. fairfieldensis* ATCC700045, *D. intestinalis* DSM 11275, which were also isolated from the intestine of humans and animals. As for bacteria *Desulfomicrobium orale* Rod-9, they form a single cluster with group *D. orale* strains.

From the literature it is known that *D. piger* belong to SRB, which are the commensal microflora in human intestine (Gibson *et al*., 1991, 1993; Loubinoux *et al*., 2003). In general, bacteria of *Desulfovibrio* genera are most common among members of SRB in the feces of people with inflammatory bowel disease (Tee *et al*., 1996; McDougall *et al*., 1997; Cummings *et al*., 2003). Isolated and identified in this study bacteria *D. piger* were similar by the genetic, physiological and biochemical characteristics to *Desulfomonas pigra*, which was isolated by W.E. Moore in 1976 from feces for the first time (Moore *et al*., 1976) and subsequently reclassified as *D. piger* (Loubinoux *et al*., 2002). A typical strain isolated from human faeces is *D. piger*, which is represented in various collections ATCC 29098, HAQ-6, EBA23-28 (Brenner *et al*., 2005). As for bacteria *Desulfomicrobium orale*, they often isolated from oral cavity of people with gum problems, gum bleeding and periodontal disease (Langendijk *et al*., 2001).

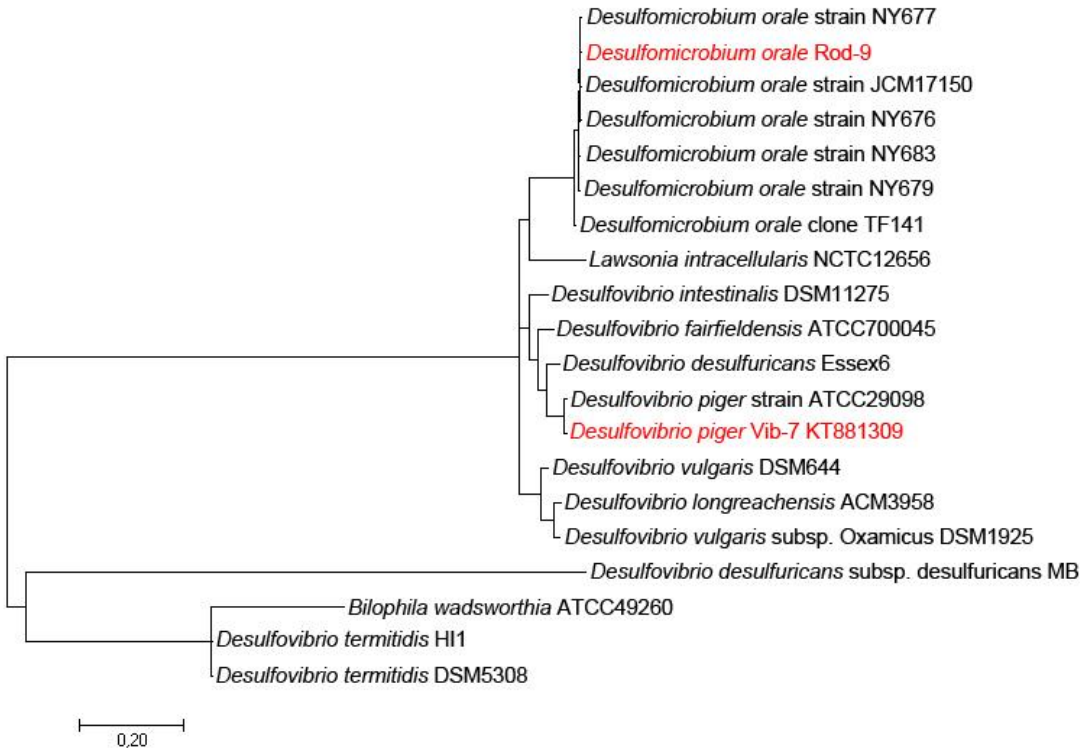

Fig. 2. Dendrogram of phylogenetic analysis of relationship sequences of 16S rRNA gene of *Desulfovibrio* and *Desulfomicrobium* genera with SRB isolated from intestine.

Comment: The dendrogram was constructed by Neighbor Joining methods, the scale indicates the genetic distance between the species.

Thus, according to the phylogenetic identification on the basis of sequences analysis of 16S rRNA gene of 20 strains of SRB isolated from human intestine was found that the nucleotide sequence of SRB strains had 99 % homology with sequences strain of *Desulfovibrio piger* ATCC 29098 (NR 041778.1) and *Desulfomicrobium* sp. strain Rod-9 belongs to the representatives of the *D. orale* NY677 species (AJ251629.1).

To study the role of SRB in causing ulcerative colitis, the determined parameters of dissimilatory sulfate reduction, including bacterial growth, their consumption of sulfate and lactate, production of hydrogen sulfide and acetate were used as well as a statistical study of the multidimensional dataset was carried out. Using method of cluster analysis, the SRB strains isolated from patients with colitis and healthy people were combined in groups by their physiological and biochemical parameters of dissimilatory sulfate reduction process.

As seen from the obtained results of cluster analysis (Fig. 3), strains of the SRB isolated from patients with UC formed two separate clusters, which were distant by Euclidean distance (I and III) as well as formed by bacteria *Desulfovibrio piger* (Vib-1, Vib-2) and representatives of the *Desulfomicrobium* genus (Rod): Rod-1, 2, 5. It can be explained by the fact that the SRB strains belonging to different genera.

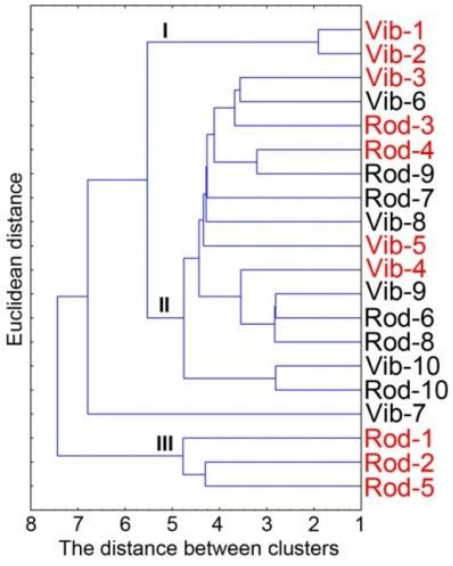

Fig. 3. Dendrogram of SRB affinity based on physiological and biochemical parameters of dissimilatory sulfate reduction.

Comment: **red color** on the correlogram indicates the SRB strains isolated from samples of patients with colitis and **black color** from healthy people. Roman numerals indicate the grouping of bacterial isolates in clusters.

The cluster II, which consists of separate not clearly defined subclusters, included strains of bacteria Rod and Vib, both from healthy people and patients with UC. Conventionally, this cluster might call “transitional”, the ratio of bacteria isolates from healthy people and patients in the cluster was 9:5, respectively.

Based on a detailed cluster analysis for each of physiological and biochemical parameters separately, the ratio of SRB isolated from patients with colitis and healthy people and their distribution in clusters was established. The differences between bacteria of different genera were in the fact that determining factors of the process of dissimilatory sulfate reduction for *Desulfovibrio* genus, which led to the emergence of symptoms of colitis, were parameters such as: biomass accumulation and production of hydrogen sulfide and acetate. For the *Desulfomicrobium* genus, all parameters has influenced, although most clear division by the symptoms of the bowel inflammation led parameters such as production of acetate and hydrogen sulfide, dividing bacteria to separate two clusters, which included those that were mainly isolated from healthy people and patients with colitis.

These data contribute to a detailed understanding of the degree of affinity between intestinal SRB. The defining parameters playing a significant role in colitis development are products dissimilatory sulfate reduction in the gut. This, in turn, is the result of revenues intestinal agents (sulfates and volatile organic acids, including lactate), which lead to the emergence the colitis symptoms.

To establish the role of final products of SRB metabolism and patterns of development symptom of colitis under their actions, and the statistical relationship between the variables of dissimilatory sulfate reduction, the correlation and cross-correlation analysis were carried out (Bailey, 1995, Chen & Popovich, 2002). Analyzing the parameters of biomass accumulation and products of sulfate reduction was found positive cross-correlation (Fig. 4).

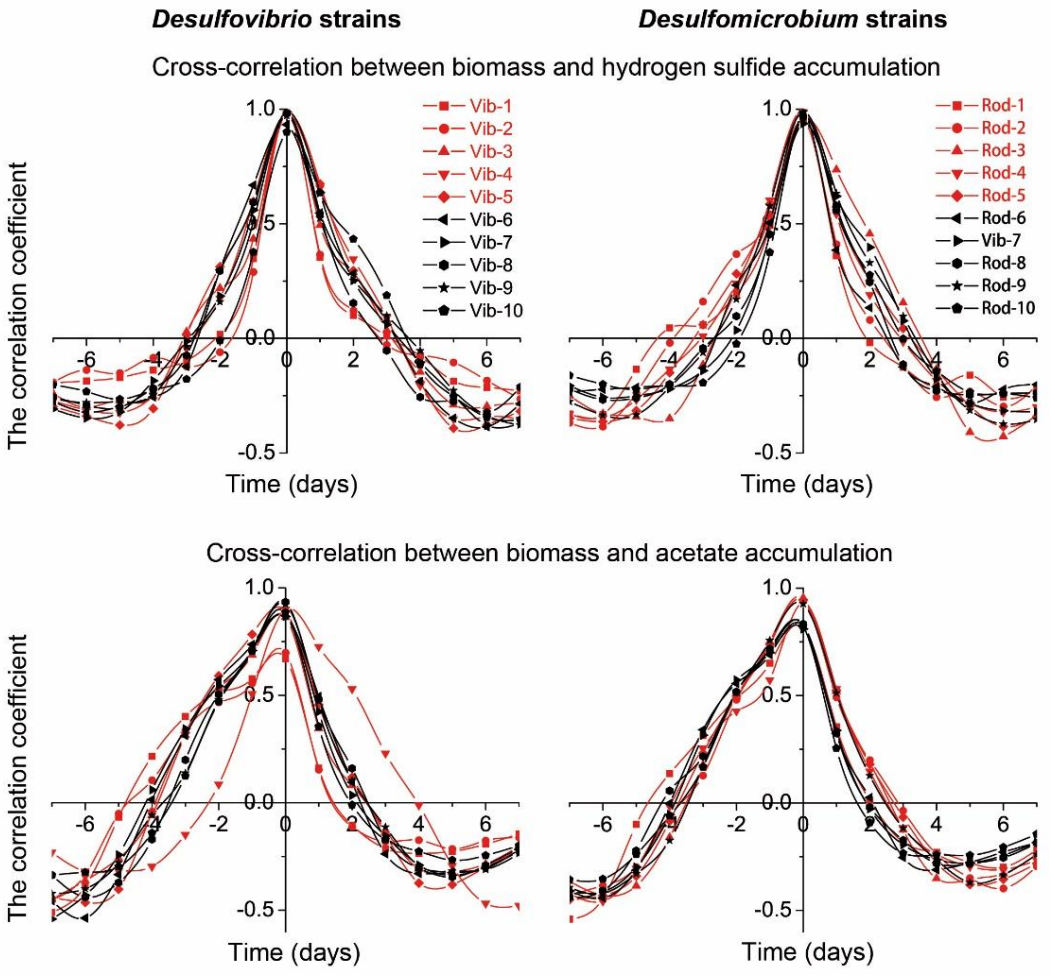

Fig. 4. The cross-correlograms based on parameters of SRB biomass accumulation and final products of sulfate reduction.

Comment: **red color** (here and thereafter) on the correlogram indicates the SRB strains isolated from samples of patients with colitis and **black color** from healthy people.

The correlation coefficients of parameters of dissimilatory sulfate reduction for all SRB strains varied in time and closely related in magnitude and sign between the parameters of biomass and hydrogen sulfide production. Based on these data, it can be argued that the mechanisms and rate of hydrogen sulfide production in the intestine by studied bacterial isolates are interrelated, but not identical, because the landslides in biomass accumulation values on 3–4 days were found in representatives of *Desulfomicrobium* genus isolated from patients with UC. Temporary slight shift of some correlation coefficients for SRB from patients with UC, depicted on cross-correlogram in different directions, indicate significant stable cross-correlation relationships between parameters of sulfate dissimilation.

Based on the cross-correlation between the biomass and the amount of acetate was found differences compared with the previous couple of parameters: the value of biomass and hydrogen sulfide. Time landslides and changes of correlation coefficients for values of biomass in both bacterial genera were oserved at 3–5 days, while for acetate at 1–3 days. The production of acetate was significantly different from the production of hydrogen sulfide in time. A similar pattern was

observed at 2–3 days for all SRB strains of *Desulfomicrobium* genus isolated from patients with colitis. Significant advance of values of acetate production and biomass accumulation of *D. piger* Vib-4 strain isolated from patient with colitis compared to other strains on 1–4 day was oserved. Obviously, such advance in acetate production compared to the biomass accumulation of SRB isolated from patients with UC can be caused by the action of acetate on the bacterial growth because during the accumulation of acetate occur a decrease in pH, which can affect the growth of SRB.

Cross-correlation analysis was conducted to establish time changes between parameters of consumption of lactate and sulfate, production of acetate and sulfide in the studied SRB strains from healthy people and patients with UC (Fig. 5).

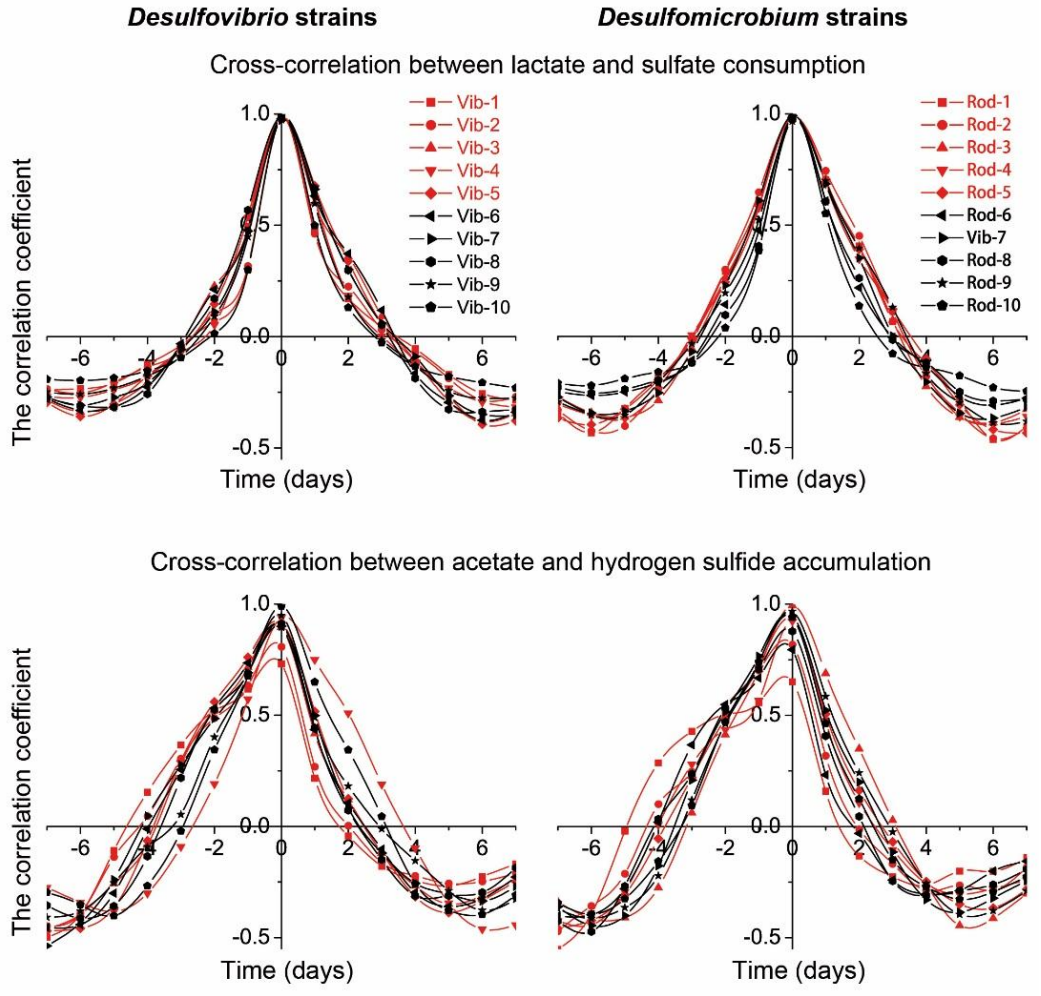

Fig. 5. The cross-correlograms based on parameters of electron donor/acceptor consumption and final products of sulfate reduction.

For both genera of SRB, a positive correlation between the studied parameters was confirmed. The final stage of the dissimilatory sulfate reduction described by changes in indicators of reaction products is similar in strains of both genera. There no significant differences between the parameters of linear correlations in SRB from healthy people and patients with UC. The correlation parameters of final products of sulfate reduction (acetate and hydrogen sulfide) on the cross-

correlograms showing a significant landslide of correlation coefficients on 1–3 days for mostly representatives of *Desulfovibrio* isolated patients. The landslides correlation coefficients for bacteria of *Desulfomicrobium* were different. It may be due, on the one hand, the non-linear nature of changes of the parameters or instability period, and on the other hand, the periodic presence of other components. Another explanation of landslides may be different time limits of metabolite production in dissimilatory sulfate reduction process: the difference between the amount of hydrogen sulfide and acetate varies during 1–2 days. Due to the synergistic interaction, acetate enhances the action of hydrogen sulfide on the intestinal cells, and exactly this factor can cause the disease development.

A cross-correlation analysis of the dynamics of joint metabolism process in intastinal SRB isolated from healthy people and patients with colitis given more opportunity to understand time mechanisms of sulfate reduction process by species of different *Desulfovibrio* and *Desulfomicrobium* genera. The determined analogy in time between the dynamics of energy metabolism and process of dissimilatory sulfate reduction affirmed the universality of the process in the studied bacteria. From the literature it is known that the relationship between time changes of these parameters may occur periodically (Chen & Popovich, 2002).

Based on these data we can generalize and assert that the mechanism sulfate reduction process in different instestinal SRB genera is almost identical. Differences in the sulfate reduction were observed for parameters of production of acetate and hydrogen sulfide. Consequently, we can assert that the decisive role in causing symptoms of colitis belongs to this metabolite. They can manifest their combined or synergistic effect (potentiating effect) on the intestinal cells. On the basis of the synergistic action of acetate may occur such processes as: the effect on the mechanisms of hydrogen sulfide binding, its distribution in the lumen, and withdrawal or absorption in different intestinal parts. It can affect on the biochemical and physiological parameters of intestinal cells and cause the changes microbiocenosis in general. The acceleration of the absorption or increase permeability histohematogenous barriers to hydrogen sulfide under the influence acetate can cause inhibition of the enzymes, including rhodanese (also known as thiosulfate thiotransferase or thiosulfate cyanide transsulfurase) that can render high concentrations of this compound (Rowan *et al*., 2009). Synergic interaction of these substances may be as the result of acetate and hydrogen sulfide effect on various functionally important biological substrates, membrane and cytoplasmic receptors, and ionophores (Lodish *et al.*, 2000). The potentiation (synergy) of acetate and hydrogen sulfide can increase the expressiveness level of their side effects with the further development of inflammation and its complications (Roediger *et al*., 1993; Florin *et al*., 1991; Aslam *et al*., 1992).

Besides microbiological component, particularly studied above parameters of dissimilatory sulfate reduction in SRB, the physical and chemical parameters affect also on intestinal inflammatory processes. To investigate the influence of physical and chemical factors (temperature and pH) on the biomass accumulation, dispersion analysis, which allow for the impact of these independent factors, was carried out (Liubischev, 1986). To exclude from consideration the impact of the natural increase in the number of bacteria in the growth (caused by the time factor), all effects of stimulation was assessed on the regression line. Using this method of analysis is appropriate for small samples and carried out with using a sample dispersion, which is called dispersion measurements (Bailey, 1995).

To establish the total impact of physical and chemical factors (temperature and pH, and other uncontrollable (unaccounted) factor) on the growth of strains of the *Desulfovibrio* and *Desulfomicrobium* genera isolated from healthy people and patients with UC, dispersion analysis, which allowed to take into account the percentage of these factors in one system, was carried out (Table 3).

Table 3. Two-factor dispersion analysis of temperature and pH influence on SRB biomass accumulation

Factor of influence		Indicators of analysis of variance			
		Strains of *Desulfovibrio* genus		Strains of *Desulfomicrobium* genus	
		UC	Healthy	UC	Healthy
Share of influence (η^2, %)	t	60.58	61.35	45.3	49.03
	pH	38.39	37.18	51.21	48.28
Not included factors (%)		1.03	1.47	3.45	2.69
Fisher coefficient (*F* practical)	t	235.60	167.11	52.58	72.87
	pH	149.30	101.29	59.39	71.77

Comments: Fisher coefficient (*F* critical) for all parameters is 3, the reliability of influence (*p*) is 0.99.

Two-factor dispersion analysis of influence of temperature and pH on biomass accumulation of bacterial strains, *Desulfovibrio* and *Desulfomicrobium* genera, gave an opportunity to identify the proportion of these factors. As shown in the Table 3, share of the influence of temperature and pH, respectively, were 60:38% for bacteria of the *Desulfovibrio* genus and 50:50% for *Desulfomicrobium* genus. Unaccounted growth factors (biomass accumulation) of *Desulfomicrobium* were in 2.5 times more compared to the strains of the *Desulfovibrio* genus. The obtained data show the significance of the differences between the averages of variable factors. It is known that during the development of bowel inflammation, the temperature increases, which in turn obviously leads to a more intensive growth of the *Desulfovibrio* genus (share effect of temperature was 60%), that is why they are often found as dominant at various inflammatory processes. On the basis of dispersion analysis, the level of variability rate of growth (biomass accumulation) was determined that are not due to changes in its level caused by the presence or absence of experimental influences. The start of the inflammatory process caused by any other factors exogenous or endogenous nature contributes to the bacterial growth of dominant *Desulfovibrio* genus in the gut and leads to intensive production of hydrogen sulfide. The changes in pH are probably due to an increase in the production of acetate in SRB. Such conditions may provide progression from further complications.

3. BIOCHEMICAL CHARACTERISTICS OF *DESULFOVIBRIO PIGER* VIB-7 AND *DESULFOMICROBIUM ORALE* ROD-9

In the previous chapter, the influence of basic metabolites of dissimilatory sulfate reduction by SRB from human intestine on the emergence of inflammatory processes was studied. The differences in the sulfate reduction to hydrogen sulfide and metabolize of lactate to acetate was found. For a detailed study of the kinetics of these changes, two model SRB strains, *D. piger* Vib-7 and *D. orale* Rod-9, was selected, because they are typical representatives of SRB which are often isolated from human intestine. These microorganisms exhibit high metabolic activity, the most biomass accumulation, production of hydrogen sulfide and acetate. The process of dissimilatory sulfate reduction is very complicated and a large number of enzymes are involved (Phartiyal et al., 2006; Czechowski et al., 1990; Furdui et al., 2000; Nicolet et al., 1999 and others). However, no information on the enzymatic properties of SRB, including the *Desulfovibrio* and *Desulfomicrobium* genera, isolated from the intestine.

In this chapter, the main stages of intestinal SRB metabolism and related enzymes involved in the dissimilatory sulfate reduction, including in the process of transporting substances, dissimilatory sulfate reduction to hydrogen sulfide, oxidation of lactate to acetate, were characterized (Table 4).

The maximum value of enzymatic activity in *D. piger* Vib-7 and *D. orale* Rod-9 strains was determined at incubation temperature +35ºC and pH in range 8.0–8.5 that was consistent with the conditions of the human colon and, obviously, caused by adaptive mechanisms of SRB. The initial and maximum speed of enzymatic reactions, the maximum amount of reaction products were by 1–2 orders higher in bacteria *D. piger* Vib-7 compared to *D. orale* Rod-9. This indicates a more intense metabolism in bacteria of the *Desulfovibrio* genus, because they are often found as the dominant representatives in the intestine of humans and animals.

Transportation of sulfate ions and organic compounds to the cell cytoplasm of SRB occurs by the mechanism of active transport with using ATP energy (Barton & Hamilton, 2010), that is, as an electron donor and acceptor can compete by these systems. Constants of inhibition of ATPase by ouabain were 22.9±4.6 and 15.8±2.6 mM, respectively, for *D. piger* Vib-7 and *D. orale* Rod-9. Obviously, the kinetic parameters ATPase of SRB depend on activity of ATP sulfurylase that competes with the enzyme for ATP in transportation process of sulfate. Equally important transport function belongs to hydrogenases, which is involved in the transfer of hydrogen through the plasma membrane in dissimilatory sulfate reduction process. The high activity of this enzyme and its maximum rate was found in both SRB strains. These parameters were the highest compared with other enzymatic activity of sulfate dissimilation process, which may be due to excessive formation of hydrogen in the cytoplasm.

It should be noted that the value of inorganic pyrophosphatase activity in *D. piger* Vib-7 and *D. orale* Rod-9 were, respectively, 24.27±2.47 and 8.16±0.82 U×mg protein^{-1}, and the maximum rate of reaction was 3.86±4.24 and 13.74±1.32 μmole×min^{-1}×mg^{-1}. Obviously, this enzyme is involved not only in sulfate reduction but also in exchange of ATP, since, as already mentioned, for ATPase of studied microorganisms is also detected high value of activities and the maximum rates of the reactions.

Thus, transport processes in the SRB cells were quite active and although competition by ATPase interrelated by substrate specificity, which enters the cell, and rate of its metabolism.

Table 4. Enzymatic characteristics of the intestinal SRB strains

The kinetic characteristics of enzymes (EC number)		SRB strains	
		D. piger Vib-7	*D. orale* Rod-9
Transportation of substances			
ATPase (EC 3.6.1.3)	A (U×mg^{-1} of protein)	16.11±1.87	7.31±0.98
	V_0 (μmole×min^{-1}×mg^{-1})	15.95±1.58	10.69±0.93
	V_{max} (μmole×min^{-1}×mg^{-1})	36.10±2.87	16.64±1.73
	K_m (mM)	2.24±0.21	2.06±0.18
Hydrogenases (EC 1.12.99.6)	A (U×mg^{-1} of protein)	1421.4±123.7	568.7±45.6
	V_0 (μmole×min^{-1}×mg^{-1})	205.67±18.91	58.16±5.38
	V_{max} (μmole×min^{-1}×mg^{-1})	2500±219	1111±107
	K_m (mM)	864±73	669±62
Reduction of sulfate to hydrogen sulfide			
ATP sulfurylase (EC 2.7.7.4)	A (U×mg^{-1} of protein)	2.26±0.231	0.98±0.0082
	V_0 (μmole×min^{-1}×mg^{-1})	5.48±0.57	4.12±0.38
	V_{max} (μmole×min^{-1}×mg^{-1})	4.87±0.55	2.11±0.22
	K_m (mM)	1.98±0.21	1.07±0.12
APS reductase (EC 1.8.99.2)	A (U×mg^{-1} of protein)	0.34±0.029	0.11±0.012
	V_0 (μmole×min^{-1}×mg^{-1})	0.675±0.062	0.231±0.022
	V_{max} (μmole×min^{-1}×mg^{-1})	0.862±0.084	0.282±0.027
	K_m (mM)	4.33±0.47	3.57±0.32
Sulfite reductase (EC 1.8.99.1)	A (U×mg^{-1} of protein)	0.032±0.0026	0.028±0.0022
	V_0 (μmole×min^{-1}×mg^{-1})	0.351±0.033	0.138±0.012
	V_{max} (μmole×min^{-1}×mg^{-1})	0.067±0.0053	0.045±0.0039
	K_m (mM)	3.53±0.334	3.86±0.341
Oxidation of lactate to acetate			
Lactate dehydrogenase (EC 1.1.1.27)	A (U×mg^{-1} of protein)	0.472±0.037	0.153±0.014
	V_0 (μmole×min^{-1}×mg^{-1})	0.114±0.012	0.026±0.022
	V_{max} (μmole×min^{-1}×mg^{-1})	1.20±0.11	0.65±0.053
	K_m (mM)	0.83±0.07	1.54±0.14
Pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1)	A (U×mg^{-1} of protein)	1.24±0.127	0.48±0.051
	V_0 (μmole×min^{-1}×mg^{-1})	4.15±0.43	1.37±0.12
	V_{max} (μmole×min^{-1}×mg^{-1})	2.54±0.261	0.89±0.092
	K_m (mM)	2.72±0.283	2.55±0.245
Phosphotransacetylase (EC 2.3.1.8)	A (U×mg^{-1} of protein)	1.19±0.122	0.37±0.041
	V_0 (μmole×min^{-1}×mg^{-1})	5.68±0.58	2.14±0.23
	V_{max} (μmole×min^{-1}×mg^{-1})	2.73±0.31	0.98±0.089
	K_m (mM)	3.36±0.35	5.97±0.62
Acetate kinase (EC 2.7.2.1)	A (U×mg^{-1} of protein)	1.52±0.163	0.46±0.044
	V_0 (μmole×min^{-1}×mg^{-1})	6.16±0.63	1.39±0.14
	V_{max} (μmole×min^{-1}×mg^{-1})	3.12±0.32	1.03±0.098
	K_m (mM)	2.54±0.26	2.68±0.25

To determine, which one of metabolic processes are proactive: dissimilatory sulfate reduction to hydrogen sulfide or lactate to acetate and which enzymes of these reactions would be "sensitive area", the enzymatic activities of these two processes were determined and the kinetic analysis of the enzymatic reactions was carried out (Fig. 6).

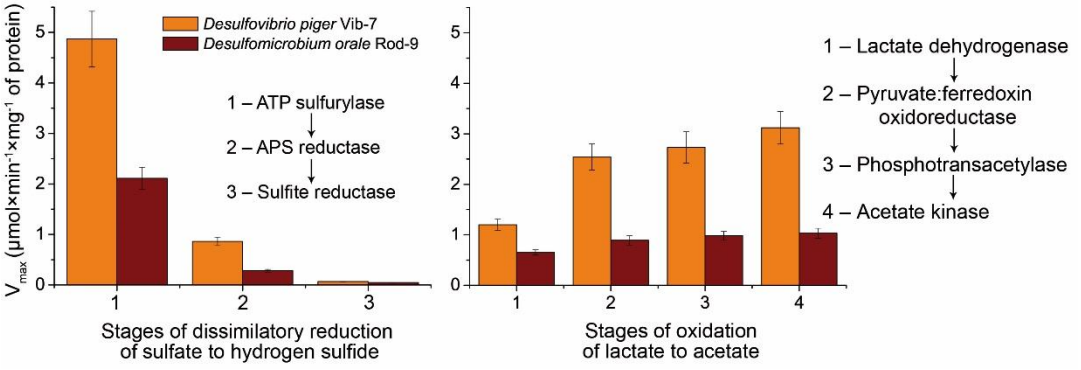

Fig. 6. The comparison of processes of sulfate reduction and lactate oxidation.

According to the dynamics of the maximum rate (V_{max}) and the time (τ, half saturation period) of enzymatic reactions, the intestinal SRB can oxidize of lactate and produce of acetate faster (total process time is 20–27 min) compared with dissimilatory sulfate reduction and accumulation of hydrogen sulfide (the process is 51–58 min). It is confirmed in previous calculated kinetic parameters of consumption of acceptor/donor of electrons and the production of the final products of intestinal SRB metabolism. Maximum rates of consumption of sulfate and lactate, production of hydrogen sulfide and acetate by bacteria *D. piger* Vib-7, respectively, were 0.315±0.028, 1.082±0.103, 0.510±0.043, 1.193±0.117 h^{-1}. A similar pattern was found for the strain of bacteria *D. orale* Rod-9. The sensitive area of sulfate reduction was dissimilatory sulfite reductase, which slowed this process almost in 10 times. For the oxidation of lactate to acetate, the "sensitive area" was observed at the initial stage of the process: lactate dehydrogenase. The determined low activity sulfite reductase consistent with the literature (Kobayashi *et al*., 1972, 1974; Ogata *et al.*, 1981; Czechowski *et al*., 1990).

Thus, based on studied enzymatic parameters was found that the production of acetate is 2 times faster compared to hydrogen sulfide and the consumption of lactate compared to sulfate. The study of compounds, which can inhibit sulfite reductase and lactate dehydrogenase in SRB, is promising to create methods and drugs to prevent and reduce the risk of bowel inflammation. Accordingly, SRB, dissimilating sulfate and oxidizing lactate, will not be able to metabolize and produce high concentrations of hydrogen sulfide and acetate. The perspective is to create methods for diagnosis of ulcerative colitis based on the determination of the main activity of these enzymes. Comprehensive study of enzymes of dissimilatory sulfate reduction and sulfide accumulation enables the formation of a holistic understanding involvement of these systems of SRB in maintaining ion homeostasis and their effects on the intestinal cells.

4. PHYSIOLOGICAL CHARACTERISTICS BACTERIA *DESULFOVIBRIO PIGER* VIB-7

Since based on biochemical studies was found high kinetic parameters of studied enzymes in *D. piger* Vib-7 strain and given the fact that the properties of intestinal SRB are still unexplored, this chapter describes of sulfate dissimilation under different conditions of cultivation. The influence of medium pH, different concentrations of sulfide, electron donor and acceptor in this complex and multistage process was studied.

Optimal parameters of *D. piger* Vib-7 growth, their consumption of sulfate and lactate, accumulation of hydrogen sulfide and acetate depending on the acidity of the medium was studied (Kushkevych, 2014). The maximum values of the process were determined by the optimal pH 7.0-8.0, which is consistent with the results of the activity of enzymes described in the previous chapter. Under these conditions, the highest growth rate (0.047–0.050 h^{-1}), the production of hydrogen sulfide (0.035–0.036 h^{-1}) and acetate (0.102–0.104 h^{-1}) were detected (Table 5). Decrease of pH < 7 or increase of pH > 9 caused inhibition of sulfate reduction process. The pH in the colon humans is 8–9, which apparently causes intensive growth of microorganisms and therefore their production of sulfide. Decrease of pH to 6–7 is critical for colon, because neutral or slightly acidic pH can cause of development of conditional pathogens (Nugent *et al.*, 2001).

Table 5. Kinetics of *D. piger* Vib-7 bacterial growth under the effect of pH medium and hydrogen sulfide

Effect	Duration of lag phase (hour)	Time of generation T_d (hour)	Maximal speed of accumulation μ_{max} ($hour^{-1}$)		
			Biomass	Hydrogen sulfide	Acetate
pH medium					
4	47±4.5	27±2.3	0.020±0.0019	0.001±0.0001	0.065±0.0055
5	14±1.3	3±0.28	0.026±0.0023	0.010±0.0015	0.092±0.0088
6	5.8±0.4	2±0.22	0.038±0.0034	0.024±0.0021	0.094±0.0091
7	6.6±0.5	1.8±0.15	0.047±0.0045	0.036±0.0033	0.102±0.0098
8	6.2±0.5	1.7±0.12	0.050±0.0049	0.035±0.0029	0.104±0.0095
9	6.7±0.6	2±0.23	0.039±0.0033	0.032±0.0025	0.089±0.0081
10	24±2.3	4±0.37	0.014±0.0011	0.0083±0.0009	0.070±0.0062
Hydrogen sulfide (mM)					
0	6.58±0.61	1.79±0.14	0.49±0.042	0.034±0.0036	0.103±0.009
1	6.65±0.65	2.37±0.22	0.37±0.036	0.025±0.0022	0.093±0.0088
2	7.72±0.73	3.31±0.29	0.25±0.022	0.013±0.0011	0.079±0.0072
3	8.05±0.76	6.20±0.57	0.11±0.001	0.004±0.0001	0.064±0.0057
4	10±1.11	15.21±1.55	0.01±0.0001	0.001±0.0001	0.052±0.0046
5	15±1.23	15.29±1.61	0.01±0.0001	0	0.031±0.0025

No less important factor that influenced on the *D. piger* Vib-7 growth and their sulfate reduction is hydrogen sulfide. Despite the fact that *D. piger* Vib-7 is a producer of sulfide, but its

additional adding in the medium in a concentration of 1 mM caused inhibition of bacterial growth and reduced of sulfide and acetate production in the bacteria by 21% compared with the control. The maximum growth rate under these conditions was also decreased by 25% compared with the control medium without introducing hydrogen sulfide. Increasing concentrations of hydrogen sulfide to 6 mM caused a significant decrease in the maximum rate of growth and the production of final products of intestinal SRB metabolism (Table 5). Complete inhibition of bacterial growth and sulfate reduction process was determined at the concentration of 7.0 mM hydrogen sulfide. It is known that the concentration of free (unbound) sulfide in the lumen of the human colon is in the micromolar range and sulfide ions in millimolar concentrations (1.0–2.4 mM), because it is bound with a large number of feces components (Blachier *et al.,* 2010). Hydrogen sulfide is cytotoxic for intestinal cells of mice in the concentration range of 0.2–1.0 mM (Deplancke *et al.,* 2003) and human intestines in 0.3–3.4 mM (Florin *et al.*, 1991; Pochart *et al.*, 1992; Magee *et al.*, 2000). However, the mechanisms of protection of intestinal cells under prolonged exposure to this toxic substance are still not fully elucidated.

The integrity of the gut epithelial layer is preserved through detoxification mechanisms (Picton *et al.*, 2002) or permanent removal of damaged cells (Attene-Ramos *et al.*, 2006). Thus, hydrogen sulfide was toxic not only to gut microflora and its cells, but also for its producers, as described in the literature (Beauchamp *et al.*, 1984). The effect of electron acceptor and donor on the growth rate and the dissimolatory sulfate reduction was determined (Table 6). It helped clarify which one of these paramters will play a more significant role in the development of diseases.

Table 6. Kinetics of *D. piger* Vib-7 growth under the effect of electron acceptor and donor

Electron acceptor				Electron donor			
Sulfate (mM)	Lag-phase (hour)	Generation time T_d (hour)	μ_{max} ($hour^{-1}$)	**Lactate** (mM)	Lag-phase (hour)	Generation time T_d (hour)	μ_{max} ($hour^{-1}$)
0.87	38.2±3.5	16.5±1.5	0.009±0.0001	**4.45**	36.6±3.7	14.5±1.35	0.009±0.008
1.75	5.9±0.46	4.3±0.44	0.02±0.001	**8.9**	7.1±0.66	3.6±0.33	0.03±0.001
3.5	6.4±0.62	1.8±0.15	0.05±0.004	**17.3**	6.4±0.60	1.8±0.12	0.05±0.004
7.0	7.4±0.73	1.1±0.10	0.08±0.007	**35.6**	4.9±0.43	1.1±0.10	0.08±0.007
10.5	3.3±0.31	1.3±0.12	0.06±0.005	**53.4**	3.1±0.29	1.3±0.11	0.07±0.005
17.5	5.5±0.59	1.6±0.14	0.05±0.005	**89.0**	5.4±0.51	1.5±0.13	0.06±0.004

The greatest amount of hydrogen sulfide (6.06±0.59 mM) at the presence of 10.5 mM sulfate and acetate (21.10±2.16 mM) at the presence of 53.4 mM lactate was accumulated in the cultivation medium. Under these conditions, the highest growth rate (0.080 h^{-1}) was observed at the electron acceptor concentration of 7 mM and electron donor of 35.6 mM. Obviously, sulfate and lactate determined concentrations lead to stimulating the SRB growth in the gut, their production of sulfide and acetate, which will increase the risk of inflammation.

The intensity of the sulfate reduction process may also depend on the number of SRB. The increase in SRB biomass to 1 mg/ml led to a decrease in the length of the exponential growth phase and the process of dissimilation sulfate. At the introduction of 1.0 mg/ml of the cell, the growth phase duration (t_e) was 12 hours. Under these conditions, the greatest value of absolute growth rate (0.020±0.002 mg/ml×h) was detected; while the maximum intensity of the sulfate reduction process achieved at the point (t_e) after 12 hour of cultivation (Fig. 7).

The highest specific growth rate of *D. piger* Vib-7 and parameters of sulfate dissimilation (μ_{max}) was detected in the exponential growth phase in a range of critical points of the growth from T_1 to T_e. Increasing the number of SRB in the range of 0.5–1.0 mg/ml (x_0) led to an increase in the absolute growth rate of *D. piger* Vib-7 and stimulate of the sulfate reduction process. Reducing the duration of these processes (t_e) indicates the intensive production of hydrogen sulfide and, accordingly, the occurrence of sudden flares of inflammation. In general, 1 mg of SRB cells in 1 ml was titer 10^6 CFU/ml. It is obviously critical to the emergence of symptoms of colitis.

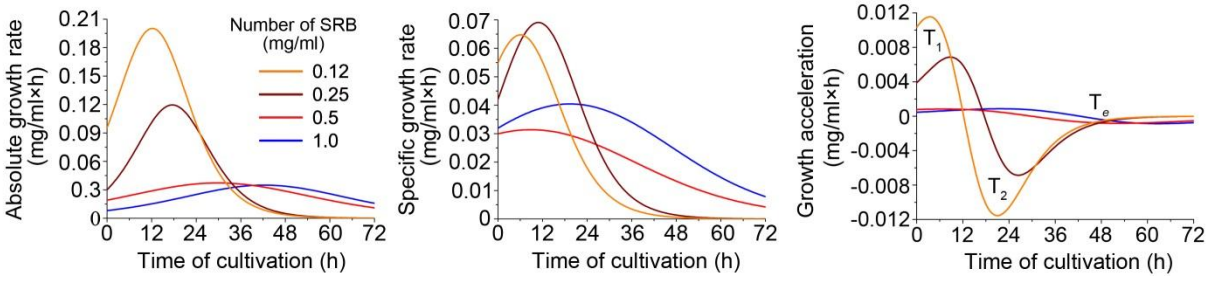

Fig. 7. Kinetics of *D. piger* Vib-7 growth depending on number of their cells.

Thus, the optimum parameters, providing the greatest rate of SRB growth are: pH 7.0–8.0 (rates of growth 0.047–0.050 h^{-1}, production of hydrogen sulfide 0.036 h^{-1}, and acetate 0.102–0.104 h^{-1}), 7.0 mM electron acceptor and 35.6 mM electron donor. The intensive accumulation of hydrogen sulfide in concentrations of 1 mM inhibited the maximum growth rate by 24%, and sulfate reduction process in SRB by 26%. Increasing the number of SRB in the range of 0.5–1.0 mg/ml led to a reduction lag- and exponential growth phase; stimulate growth and rate of the dissimilatory sulfate reduction process. Determination of the kinetics of SRB growth and their sulfate reduction process are particular importance for predicting the risk of occurrence of symptoms and duration of the bowel disease.

5. ETIOLOGICAL ROLE OF SULFATE-REDUCING BACTERIA IN THE DEVELOPMENT OF COLON DISEASES

Inflammatory bowel disease, including ulcerative colitis, is complex multifactorial diseases mostly unknown etiology. The development of the pathology is detailed studied on animal models of inflammation, inducing it's by chemical substances such as dextran sulfate, acetic acid, carrageenan, sodium lignosulfonate or amylopectin sulfate (Nell *et al.*, 2010; Barnett *et al.*, 2011; Perse *et al.*, 2012; Low *et al.*, 2013). In this chapter, using SRB, ulcerative colitis in rats (*in vivo*) was experimentally induced and assessed ulceration level in different parts of the intestine. The determined concentrations of hydrogen sulfide and acetate in feces samples taken from different parts of the intestine of rats correlated with the level of ulceration. Analysis of the microflora of the distal intestine of rats was carried out. The microflora of the first (control) group of animals during twenty-five days was not significantly changed. The significant changes in microbiota in animals of the second and third group on 25 day of experiment were observed. Under these conditions, the number of microorganisms, their ratio, index and incidence of sustainability was significantly changed. Number microbial genera *Bifidobacterium* and *Lactobacillus* was decreased by 90%, while the number of bacterial genera of *Clostridium* (71%), *Escherichia* (79–94%), *Proteus* (63%), *Klebsiella* (80%), *Staphylococcus* (43%) and SRB (95%) were increased for a specified number of percents. It is obvious that these microorganisms play an important role in the development of inflammatory bowel disease. Dysbiosis of intestinal microflora may indicate the initial stages of inflammatory bowel disease and other pathological processes.

The concentration of sulfide and acetate were increasing directly proportional to the duration of the experiment. Increase in the concentration of these compounds by 32 and 65%, and 35% and 69%, respectively, in the second and third group of animals compared to controls was observed at the 5^{th} day. On the 25^{th} day, the levels of hydrogen sulfide and acetate in the colon was increased by 88 and 95%, respectively, (2^{nd} group of animals) and by 93 and 97% (3^{rd} group of animals). A large number of ulcers (78±7.50%) in the descending colon, compared with other parts of the colon of rats, were detected in this period in the experimental animals. The highest level of ulceration was found in animals of third group compared with the second group. Obviously, SRB showed high aggressiveness on the colon of rats, compared with their own microflora, causing intensive development of inflammatory bowel disease including ulcerative colitis.

The genetically-modified strain of *D. piger* Vib-7M, containing *CysK* gene transformed from *E.coli*, was constructed. It is able to convert toxic hydrogen sulfide to cysteine through activity of *O*-acetylserine(thiol)lyase (97.56 U×mg of protein^{-1}), which uses it as a substrate. This bacterial strain is able to utilize its own produced and exogenous hydrogen sulfide, released by other SRB. The mutant strain of SRB accumulate biomass by 36% more than wild type and 5.38±0.52 mM of cysteine at 84th hour of cultivation. Using this strain, assimilatory sulfate reduction way was modeled.

Daily adding 1 ml of *D. piger* Vib-7M (3 mg/ml) suspension in the medium in the diet to rats with colitis helped restore the diversity of the autochthonous microbiota (Fig. 8). The number of lactic acid bacteria of *Lactobacillus* and *Bifidobacterium* genera was increased by 93%, accordingly, the number of representatives of facultative and conditionally pathogenic microflora, including *Bacteroides*, *Escherichia*, *Enterococcus* and *Proteus* was decreased. The number of SRB which able to produce hydrogen sulfide was significantly (98%) decreased. It can be caused by competition for the acceptor. Indeed, as already noted, a mutant strain capable with higher speeds (1.5 times) using sulfate, compared with strains that are in the intestine of animals with colitis.

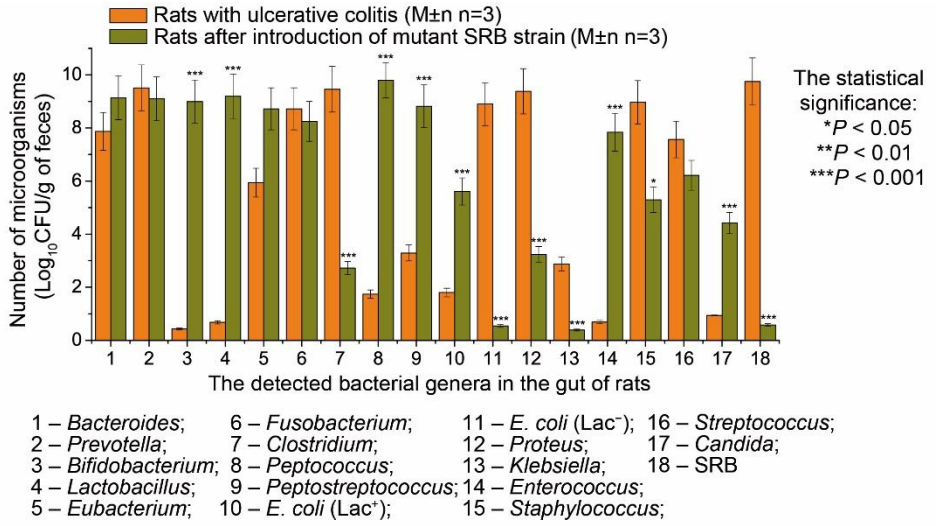

Fig. 8. The microbiota of large intestine in rats under conditions of ulcerative colitis induced by SRB and after the introduction of the *D. piger* Vib-7M strain being able to utilize of hydrogen sulfide.

The titer of these bacteria on the 25th day amounted to 3.8×10^5 CFU/g of feces, and hydrogen sulfide levels decreased by 96% compared to the control group animals that were not treated. Using this *D. piger* Vib-7M strain experimentally was confirmed that the development of inflammatory diseases involved bacteria which are able to dissimilatory sulfate reduction but not assimilatory.

Thus, based on studied SRB strains, a model of intestinal inflammation in rats was created and proved their functional role in the development of the disease. Introduction SRB suspension in the gastrointestinal tract of rats causes changes in the structure of its microbiota. The highest accumulation levels of hydrogen sulfide, which is toxic for cells, and acetate was found in the descending colon. It is evidenced most frequent cases of inflammation in these areas are the large intestine of animals and humans. Using SRB constructed strain that can metabolize hydrogen sulfide to cysteine, competing with other SRB by the substrate, experimentally proved and justified that the main role in the inflammatory process belongs to SRB which exercising dissimilatory sulfate reduction.

6. SUMMARIZING

Inflammatory bowel diseases, including ulcerative colitis, are complex and multifactorial. People with infections of the gastrointestinal tract often show an increased number of SRB.

The physiological and biochemical characteristics of SRB the under different conditions, consumption of sulfate and lactate, production of hydrogen sulfide and acetate, functional role of these microorganisms in disease development were studied. It was shown that ulcerative colitis in humans is caused by changing the qualitative and quantitative levels of the intestinal microbiota representatives. In people with ulcerative colitis is reduced by 6–7 orders of the bacterial genus of *Lactobacillus* and *Bifidobacterium* and increased by 1–4 orders of representatives of optional and conditional pathogenic microflora. The increased by 5 orders of the SRB number in the intestine of people with colitis was detected. The ratio of SRB genera, *Desulfovibrio* and *Desulfomicrobium*, is 93:7% in healthy and 99:1% in people with colitis. The dominant of SRB in the intestine of healthy and people with UC are members of the *Desulfovibrio* genus. It is known that these bacteria often form a microbial biofilm on the mucosa of the colon of humans and animals (Macfarlane *et al.*, 2007; Gibson *et al*., 1991).

Based on cluster analysis of physiological and biochemical parameters, the differences between SRB isolated from patients with UC and healthy people were investigated. These differences were in the fact that for bacteria of the *Desulfovibrio* genus determining factors of dissimilatory sulfate reduction process, which led to the emergence of symptoms of colitis, were biomass accumulation, production of hydrogen sulfide and acetate. For species of the *Desulfomicrobium* genus were all influential parameters, although most significant division on grounds of illness led parameters such as: production of acetate and hydrogen sulfide. Thus, we can argue that the defining parameters, playing a significant role in causing colitis, are products dissimilatory sulfate reduction (hydrogen sulfide and acetate). This is the result of revenues sulfate in large intestine and volatile organic acids, including lactate, which lead to the strengthening of the process and the emergence of symptoms of colitis.

A cross-correlation analysis of the dynamics of metabolic processes in studied SRB isolated from patients with colitis and healthy was carried out and has allowed more understand the time mechanisms of the sulfate reduction process in the species of different genera of *Desulfovibrio* and *Desulfomicrobium*. The analogy was observed between the dynamics in time of energy metabolism and process of dissimilatory sulfate reduction. It was provided an opportunity to confirm the universality of the process in the bacterial strains. As a result of joint action of hydrogen sulfide and acetate on intestinal cells can occur potentiation effect (synergistic effect) than separately effects of these substances. The basis of the synergistic action of acetate may be processes such as: effect on the mechanisms binding of hydrogen sulfide, its distribution in the bowel lumen and deactivation or absorption in different parts of the intestine. During the development of bowel inflammation, increasing temperature, it in turn, obviously, leads to a more intensive growth of the *Desulfovibrio* genus (share effect of temperature is 60%). The launch of inflammation process caused by any other factors exogenous or endogenous nature and contributing to an increase in growth rates among the dominant SRB of *Desulfovibrio*, leads to an intense production of hydrogen sulfide and increase the pH causes the intensive accumulation of acetate. Consequently, these conditions can provide of the progression of the disease with its further complications.

Sulfate-reducing bacteria isolated from feces of patients with UC and healthy people were not statistically different by their physiological and biochemical properties. Among the studied bacteria, the greatest concentration of hydrogen sulfide (3.23 ± 0.29 and 3.14 ± 0.27 mM) and acetate (15.87 ± 1.46 and 16.24 ± 1.54 mM) was producted by *D. piger* Vib-7 and *D. orale* Rod-9. Therefore, they

were selected as model objects for studying of biochemical and physiological characteristics as well as their etiological role in the development of inflammatory bowel disease.

The maximum of enzymatic activities for both strains was observed at a temperature of +35ºC and pH 7.0–8.5. Obviously, such conditions ensure their intensive development in the gut. The initial and maximum speed of enzymatic reactions in *D. piger* Vib-7 was in 2 times higher than in *D. orale* Rod-9. Thus, bacteria of *Desulfovibrio*, dissimilating sulfate and oxidizing lactate faster, can be more aggressive and play a pathogenic role in the development of intestinal diseases. Based on the research of enzymatic parameters, the advance in the production of acetate compared to hydrogen sulfide was detected. The inhibition of two enzymes of SRB, sulfite reductase and lactate dehydrogenase, is a promising in development of the method for preventing and reducing of the risk of inflammation in the intestine.

Given the fact that the kinetic parameters of enzymes of sulfate reduction are higher in bacteria *D. piger* Vib-7, they were selected to study of their physiological characteristics under different conditions. The highest growth rate (0.047–0.050 h^{-1}), production of hydrogen sulfide (0.035–0.036 h^{-1}) and acetate (0.102–0.104 h^{-1}) at optimum pH 7.0–8.0 was determined. It is consistent with the results of enzymatic activities of studied SRB. However, intensive production of hydrogen sulfide and its accumulation in 1 mM concentration can inhibit by 24% of maximum growth rate and by 26% of sulfate reduction in SRB. Increasing the number of SRB in the range of 0.5–1.0 mg/ml led to a reduction lag- and exponential growth phases; stimulate growth and speed of the process of dissimilatory sulfate reduction. All these factors are involved in development of inflammatory processes and their subsequent complications and the occurrence of UC.

Injection of sulfate-containing medium or SRB suspension in the gastrointestinal tract caused significant changes in the structure and sustainability of intestinal microbiota of rats. It corresponds to research of intestinal microflora of people with colitis. Number of bacterial genera of *Lactobacillus* and *Bifidobacterium* were decreased by 93–95%, but the number of bacteria *Clostridium* (73%), *Escherichia* (96%), *Staphylococcus* (45%), *Proteus* (67%) genera and SRB (97%) were increased. Perhaps, SRB with other intestinal microbial groups can compete by substrates, particularly volatile organic acids, and metabolic products of normal microflora as well as show the impact on obligate microflora (*Lactobacillus* and *Bifidobacterium*) and inhibit their development under effect of hydrogen sulfide. The highest accumulation levels of hydrogen sulfide and acetate found in the descending colon, where they grow in direct proportion to the duration of dosing sulfate-containing medium or suspension of SRB. These data are consistent with the results of bowel ulceration in third animal group, where the ulceration was significantly higher than in the second group. Obviously, SRB contribution in the development of the disease is much greater than their induced own endogenous of SRB in rats.

On the basis of these results, the generalized scheme mechanisms of inflammation development involving SRB with the subsequent emergence of ulcerative colitis and bacteremia was created (Fig. 9). The increased number of SRB ($\times 10^8$) in the intestine causes competition with methanogens by molecular hydrogen as evidenced their decrease number by 66%. Under these conditions, SRB intensely attached to epithelial cells, forming a biofilm together with *Bacteroides* and *Clostridium*. These studies are consistent with data from the literature on the formation of resistant biofilms formed by SRB (Macfarlane *et al.*, 2007; Newton *et al.*, 1998).

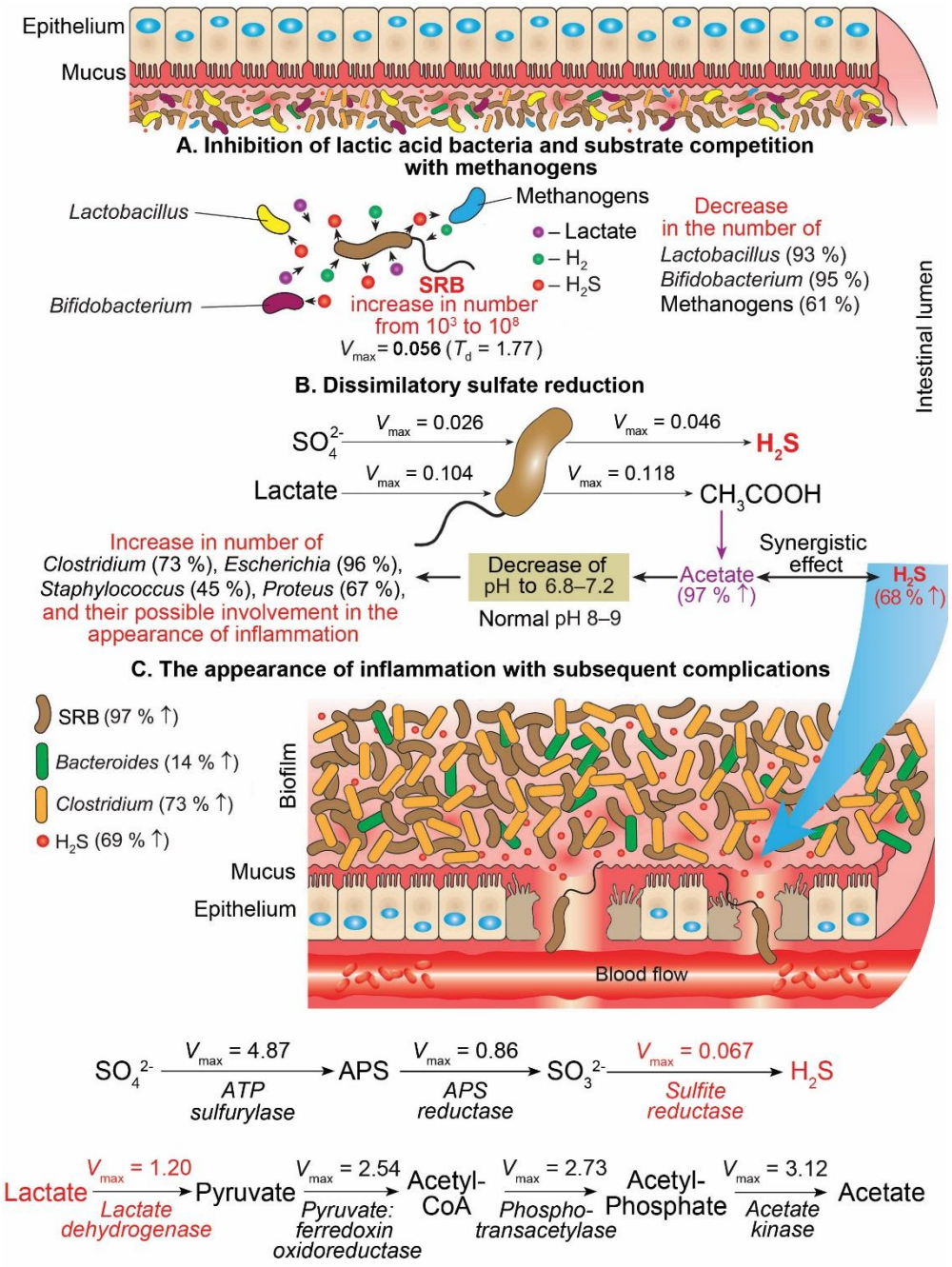

Fig. 9. The scheme of mechanisms of inflammation process development involving SRB with the subsequent emergence of ulcerative colitis and bacteremia.

Dissimilatory sulfate reduction process and production of aggressive metabolites for microorganisms (acetate: 97% and hydrogen sulfide: 68%) leads to a change in pH from slightly alkaline (pH 8–9) to neutral or even slightly acid (pH 6.8–7.2) in the large intestine. It can cause changes in microbiota and, accordingly, the growth of facultative and opportunistic microorganisms. Under these conditions, after reduced number of obligate microflora, SRB firmly attached to the mucosal cells and begin to rapidly destroy the protective mucous layer of the intestine. In the current biofilm clostridia, which are resistant to SRB and are also able to produce

hydrogen sulfide. Clostridia in this biofilm can metabolize more complex organic compounds, including mucins layer containing polysaccharides, to volatile organic compounds and release sulfated residues, which SRB reduce to hydrogen sulfide and acetate.

Violation of the intestinal barrier layer by SRB and the loss of the epithelial cells ability to utilize of aggressive toxic metabolites leads to inflammation of the further formation of ulcers. Acetate is produced faster but may be partially neutralized in the lumen intestine, enabling of SRB to produce of sufficient amount of hydrogen sulfide, because the alkalinity of the colon allows quite a long time to maintain homeostasis of intestinal lumen. Under these conditions, the intestinal lumen, obviously, decreases the concentration required chelating compounds to hydrogen sulfide, which leads to a lesser extent its binding and neutralization. Accordingly, permanently attached SRB have toxic affect on cells of the intestine and thereby destroying the epithelium, causing inflammation of the appearance of ulcers.

The hypothetical mechanisms of colitis development under the influence of SRB and their dissimilatory sulfate reduction was confirmed by constructed mutant strain, which was capable to metabolize sulfate and hydrogen sulfide to cysteine.

Stability studies of mutant strain *D. piger* Vib-7M that can convert hydrogen sulfide to cysteine and the likelihood of its reversion to wild type show that the genetically modified strain was stable to reversion (3% revertants). Mutant strain competing for substrate with other intestinal SRB and actively growing faster, metabolizing sulfate and lactate, can convert hydrogen sulfide to untoxic cysteine. It was recommended for use in the study of inflammatory diseases of created animal models.

It was proved that in the development of colitis is required exactly free hydrogen sulfide in a high concentration. Constructed mutant strain allowed modeling "assimilation sulfate reduction", which are also capable of many members of the intestinal microflora (*Clostridium*, *Escherichia*, *Salmonella*) and prove that their number does not affect the formation of ulcers. The effect of these microorganisms is based on the production of secondary toxins that can not be produced for some time and wait for the time of their induction. Hydrogen sulfide is the primary metabolite of SRB and formed in the sulfate reduction, therefore, the development of inflammation depends on the intensity of dissimilatory sulfate reduction. Using "cysteine" mutant SRB strain, experimentally proved and justified that the main role in the inflammatory process belongs to SRB, which are present in the gut, scilicet, the appearance of inflammatory process causing bacteria, which capable to dissimilatory sulfate reduction, but not assimilatory. Constructed mutant strain is a valuable and indispensable to study the factors involved in the pathogenesis of inflammatory bowel disease, and essential for use in therapeutic research.

A new created animal model of inflammatory diseases based on SRB is scientifically valuable in the research stages of the disease with its subsequent complications. Specificity of the pathogenesis of inflammatory bowel disease is complex, and the etiology of ulcerative colitis can be even caused affect of genetic, microbiological, environmental and other factors. These results are important for the prevention of diseases of the gastrointestinal tract, since, for the first time, the detailed information of the etiological role of SRB in causing intestinal diseases is described, which are valuable for their diagnosis.

Sulfate-reducing bacteria are probably involved in the occurrence of pathological states such as: increased intestinal permeability, frequent bowel movements, weight loss, and general malaise development. Based on the number of SRB, production of hydrogen sulfide and acetate, their aggressiveness and toxicity of metabolic products for intestinal mucosa was assessed. The described results are new and made it possible to create models of colitis involving SRB, based on which was developed the main evaluation criteria of progress inflammation: changes in the composition of

microflora and pH of the colon, changes in enzymes of dissimilatory sulfate reduction, changes in the rate of SRB growth and intensity of sulfate reduction, changes in the concentration of hydrogen sulfide and acetate in faeces. Understanding the role of SRB and their contribution to the development of diseases, it is necessary to adjust the amount and value of SRB in the large intestine and, consequently, decrease the production of sulfide and acetate. The described experimental results are important for further careful study of the mechanisms of inflammatory bowel disease with the use of appropriate therapeutic strategies, for studying the mechanisms of action of antimicrobial drugs with a prophylactic or therapeutic purpose of specific components involved in the pathogenesis of the disease. The study of selected strains of bacteria resistance to antimicrobial and new synthesized chemotherapy drugs based on the research of patterns of course process of occurrence of ulcerative colitis with considering the complex of physiological, biochemical, enzymatic characteristics of SRB, enables the search for new effective and harmless to the patient's drug-inhibitors. Such research is also perspective to develop methods for diagnosis and prevention of inflammatory bowel disease.

CONCLUSIONS

1. Based on experimental and theoretical approaches and studies of physiological and biochemical characteristics of dominant intestinal acetogenic sulfate-reducing bacteria, the concept of their functional role in the development of diseases of the large intestine in animals and human, was created.

2. The changes of hunam intestinal microbiota, including bacteria of *Bifidobacterium* and *Lactobacillus* genus, were demonstated. Their number in people with ulcerative colitis was reduced (2.49±0.22 and 3.47±0.33 $\log_{10}$CFU/g of feces). Species of the facultative and conditionally pathogenic microflora in the intestines of these patients was increased by 1–4 orders and SRB was also increased by 5 orders, compared to the healthy.

3. On the basis of morphological, physiological, biochemical, molecular and genetic features, the isolated bacteria were identified as species of *Desulfovibrio* and *Desulfomicrobium* genera. SRB from the intestine of healthy people and with UC statistically did not differ on physiological and biochemical properties. The dominated genera among SRB were *Desulfovibrio* (93%) and *Desulfomicrobium* (7%) in feces of healthy people and their ratio in patients with colitis was 99:1%, respectively.

4. The determining factors of dissimilatory sulfate reduction process, which lead to the appearance of symptoms of colitis was the SRB biomass accumulation, their production of hydrogen sulfide and acetate, most important of which are the final products. Share the influence of temperature and pH on the growth of the *Desulfovibrio* genus was, respectively, 60 and 38% and leads to the launch of the inflammatory process. The affiliation to genera of SRB was slightly affects on the differences in the mechanisms dissimilatory sulfate reduction and the emergence of symptoms of colitis.

5. Based on the determined enzymatic parameters of strains of *D. piger* Vib-7 and *D. orale* Rod-9, the advancing production of acetate in 4 times compared with hydrogen sulfide (time advance was 19:5 min) was determined for the first time. It leads to potentiation of the effect and increases the aggressiveness of these metabolites. Sensitive area of the sulfate dissimilation process is sulfite reductase and lactate dehydrogenase for lactate oxidation.

6. The optimal parameters of the environment, providing the greatest rate of growth of SRB isolated from the intestine are: 7.0–8.0 pH, concentrations of 7.0 mM sulfate and 35.6 mM lactate. Hydrogen sulfide in concentrations of 1 mM inhibits the maximum growth rate (by 24%) and the process sulfate reduction (by 26%). Increased SRB biomass (to 0.5–1.0 mg/ml) leads to reduction of lag-phase and exponential phase by 12 hours, to stimulate growth speed and the process of dissimilatory sulfate reduction.

7. For the first time, a model of ulcerative colitis in rats using strains of *D. piger* Vib-7 and *D. orale* Rod-9 was created and their etiological role in the development of disease was demonstrated. Introduction suspension of the strains in the gastrointestinal tract changes the structure of the microbiota, sustainability index and their frequency detection. An increase in the production of aggressive metabolites: acetate (97%), hydrogen sulfide (68%) compared with control was detected in experimental animals. It changes the parameters of pH in the colon of rats from 8–9 to 6.8–7.2.

8. In intestine of rads with UC, the decrease in number of the *Bifidobacterium* and *Lactobacillus* (93–95%), *Peptococcus* (81%) genera and increase bacteria of the *Clostridium* (73%), *Escherichia* (96%), *Proteus* (67%), *Klebsiella* (86%), *Staphylococcus* (45%) genera and SRB (97%) were detected. It is consistent with data on intestinal microflora people with UC. Increased levels of hydrogen sulfide and acetate, respectively, 88 and 95% and ulceration (78%) are found in the descending colon of animals with colitis.

9. The mutant strain of SRB *D. piger* Vib-7M, containing *CysK* gene, which encodes the enzyme *O*-acetylserine(thiol*)*lyase, and is able to metabolize toxic hydrogen sulfide to cysteine, was constructed. Daily adding suspensions of *D. piger* Vib-7M in ration of rats with colitis contributes the restore the population of lactic acid bacteria, reducing the number of representatives of facultative and conditional pathogens and SRB, which able to produce hydrogen sulfide. The concentration of hydrogen sulfide in the feces is reduced by 95% and the number of ulcers by 72%, compared to animals that did not receive in the diet of the mutant strain.

10. The main criteria for evaluating the development and course of inflammatory process involving SRB is changes: composition of the microflora and pH of the large intestine, the activities of enzymes of dissymilatory sulfate reduction, growth rate of SRB and intensity of sulfate reduction, the concentration of hydrogen sulfide and acetate in feces. Prevent of this process can be achieved by inhibiting two enzymes sulfate reducrion process, sulfite reductase and lactate dehydrogenase, which is most sensitive areas in metabolism of studied SRB.

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Workplace: Department of Experimental Biology (Section of Microbiology), Faculty of Science at Masaryk University (MU), Kamenice 753/5, 625 00 Brno, Czech Republic
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University Degrees:

2006 Master study programme at Microbiology Department, Biology Faculty, Ivan Franko L'viv National University (LNU), Ukraine (diploma with honours).
2010 Postgraduate study program at LNU. Ph.D. thesis in Microbiology: "Influence of heavy metals salts on physiological and biochemical characteristics of sulfur cycle bacteria".
2016 Doctor of Biological Sciences (Habilitated Doctor in Microbiology): "The functional role of sulfate-reducing bacteria in the development of bowel diseases in human and animals". Zabolotny Institute of Microbiology and Virology of NAS of Ukraine.
2017 Position of Associate Professor at Department of Experimental Biology at MU.

Employment Positions:

2017 – so far Position of Associate Professor at Department of Experimental Biology (Section of Microbiology) at MU.
2015 – 2016 Assistant Professor at Department of Experimental Biology at MU.
2013 – 2015 Postdoc at Department of Human Pharmacology and Toxicology at University of Veterinary and Pharmaceutical Sciences (UVPS) Brno, Czech Republic.
2011 – 2012 Lector at Department of Microbiology, Virology and Immunology at Danylo Halytsky Lviv National Medical University, Ukraine.
2007 – 2010 Assistant Professor at Microbiology Department at LNU, Ukraine.

Research Stays:

2018 Internship in Laboratory of Pathogenic Microorganisms of Fishes at the Department of Microbiology and Ecology at University of Valencia, Spain (1 month).
2016 Visiting prof. Galina O. Iutynska, Dr.Sc., Corresponding Member of the NAS of Ukraine, Zabolotny Institute of Microbiology and Virology of NAS of Ukraine (1 week).
2013 Research Internship in the Laboratory of Molecular Biology at the Department of Molecular Biochemistry and Pharmacology at Institute of Institute pharmacological research Mario Negri, Milan, Italy (3 months). Detailed information on website: http://www.vfu.cz/informace-o-univerzite/vita-universitatis/pdf/vu_2014_2.pdf (page 19).
2008 III Summer School «Molecular microbiology and biotechnology», Odessa, Ukraine (2 weeks).

Pedagogical Activities:

From 2016 Practical courses: General Microbiology and Bacterial Physiology. Supervisor of Microbiology Diploma Thesis and Bachelor Thesis at the Faculty of Science at MU.
From 2014: Lector of 2 subjects at the Faculty of Pharmacy at UVPS (General Biology and Microbiology and practical classes for both Czech and foreign students).
From 2013: Supervisor of 4 master program students at the Department of Human Pharmacology and Toxicology, Faculty of Pharmacy at UVPS.
From 2007: Supervisor of 7 Microbiology Diploma Thesis and 3 Bachelor Thesis (10 of them have successfully defended) at the Microbiology Department LNU, Ukraine.

Scientific and Research Activities:

- Molecular microbiology – especially biochemistry of sulfate-reducing bacteria (SRB) and their enzymes of dissimilatory sulfate reduction under the effect various compounds.
  - High level of knowledge – applicant graduated in Microbiology and awarded by Certificate in molecular biology (Italy, 2013).
- Physiology and Biochemistry of intestinal SRB – especially activity of their enzymes.
  - Advanced level of knowledge – applicant established this research direction at Department of Experimental Biology, Faculty of Science at MU in 2016.

Commissions of Trust

From 2014: Editorial board member and reviewer of three international journals: *American Journal of Infectious Diseases and Microbiology*, *American Journal of Microbiology and Biotechnology*, *International Journal of Biological Sciences and Applications*.

Ad-hoc referee of more than 3 scientific journals (incl. *Archives of Medical Science*).

Publications, Citations:

Total publications 57 (https://www.researchgate.net/profile/Ivan_Kushkevych)
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Research monographs, chapters in collective volumes and any translations thereof:
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Education:

2017 Position of Associate Professor at Department of Experimental Biology at MU.
2016 Doctor of Biological Sciences (Habilitated Doctor in Microbiology).
2013 Postdoc at Department of Human Pharmacology and Toxicology at UVPS Brno (3 years).
2010 Philosophy Doctor in Microbiology at LNU, Ukraine.
2006 Master's degree in Microbiology at LNU, Ukraine.

Pedagogical Activities:

Microbiology, Physiology of Microorganisms (practical classes), supervisor of diploma thesis.

Scientific and Research Activities:

- Anti-inflammatory activity of chemical compounds, animal models of ulcerative colitis.
- Internal grant projects UVPS Brno ("Pharmaco-toxicological evaluation of newly synthesized (isolated) compounds as an integration tool for pre-clinical disciplines at VFU Brno" Project reg. No. CZ.1.07/2.3.00/30.00 (04/2013–04/2015)), and grant projects of West-Ukrainian BioMedical Research Center for young biomedical researchers in Ukraine (06/2007–06/2008).
- Active attendance at conferences (ICMS, Sofia, Bulgaria, 2014, 2015).

ATTACHMENT 1

Pages 41 – 50

Activity of Na^+/K^+-activated Mg^{2+}-dependent ATP-hydrolase in the cell-free extracts of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

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Activity of Na^+/K^+-activated Mg^{2+}-dependent ATP-hydrolase in the cell-free extracts of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

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Abstract

The aim of our work was to study Na^+/K^+-activated Mg^{2+}-dependent ATPase activity in cell-free extracts of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine, and to carry out the kinetic analysis of the enzyme reaction. The maximum ATPase activity for both bacterial strains at +35 ºC was determined. The highest activities of the studied enzyme in the cell-free extracts of *D. piger* Vib-7 at pH 7.0 and *Desulfomicrobium* sp. Rod-9 at pH 6.5 were measured. Based on experimental data, the analysis of kinetic properties of the ATP-hydrolase reaction by the studied bacteria was carried out. The enzyme activity, initial (instantaneous) reaction rate (V_0) and maximum rate of the ATPase reaction (V_{max}) was significantly higher in *D. piger* Vib-7 cells than in *Desulfomicrobium* sp. Rod-9. Michaelis constants (K_m) of the enzyme reaction for both bacterial strains were determined.

ATPase activity, hydrogen sulfide, intestinal microbiocenosis, bowel diseases, ulcerative colitis

Sulfate-reducing bacteria carry out the dissimilatory sulfate reduction during anaerobic respiration (Barton and Hamilton 2007). The final product of the sulfate reduction in the human intestine is hydrogen sulfide which is carcinogenic to its cells, and can cause inhibition of cytochrome oxidase, colonocytes oxidation of butyrate, destruction of epithelial cells, development of ulcers, and inflammation with subsequent development of colon cancer (Pitcher and Cummings 1996; Gibson et al. 1991; Cummings et al. 2003). The transport of sulfate ions and organic compounds in the cytoplasm of the bacterial cells occurs through active transport using ATP energy (Barton and Hamilton 2007). In this regard, it is very important to study the mechanisms of sulfate ions transport, enzymatic activity and kinetic properties of other ATP-dependent enzymes of sulfate-reducing bacteria from human intestine.

Plentiful data are available on the functions of biological membranes including the integral membrane protein ATP-dependent systems of transport ions (Lodish et al. 2000; Yuan et al. 2005; Tian et al. 2006). However, the ATPase activity of the sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. isolated from the human large intestine has not been studied yet. A comprehensive study of the functioning and role of Na^+/K^+-pump as a system of energy-dependent transport of different ions in the regulation of the dissimilatory sulfate reduction and accumulation of hydrogen sulfide will enable to form a holistic view on the participation of these systems in maintaining ion homeostasis of the sulfate-reducing bacteria cells.

The aim of our work was to study Na^+/K^+-activated Mg^{2+}-dependent ATPase activity in cell-free extracts of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine, and to carry out the kinetic analysis of the enzyme reaction.

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Materials and Methods

Bacterial strains

Objects of the study were sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine (Kushkevych 2013; Kushkevych et al. 2014). The strains have been kept in the collection of microorganisms at the Biotechnology Laboratory of the Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic).

Preparation of cell-free extracts

Cell-free extracts were prepared from the exponential phase of growth. The bacteria were grown anaerobically in nutrition-modified Kravtsov-Sorokin's liquid medium (Kushkevych 2013). The cold extraction buffer (50 mM potassium phosphate buffer, pH 7.5, 10^{-5} M EDTA (ethylenediaminetetraacetic acid) was added to centrifuged sedimented cells to bind heavy metal ions. A total of 10^{-5} M PMSF (phenylmethylsulfonyl fluoride) for the inhibition of proteases, which is effective at pH above 7.0, was added. After this procedure, a suspension of cells (150–200 mg/ml, which correspond to optical density 0.569–0.761) was obtained. The cells were homogenized using the ultrasonic disintegrator (Soniprep 150 Plus MSE Limited, United Kingdom) at 22 kHz for 5 min at 0 ºC. The soluble extracts constituted by the supernatant were used as the source of the enzyme. The soluble fraction was displaced into centrifugal tubes and cell-free extracts were separated from the cells fragments by centrifugation for 30 min at 14,000 × *g* and at 4 ºC. Pure supernatant was then used as cell-free extracts. The spinned cells fragments were used as sedimentary fraction. Protein concentration in the cell-free extracts was determined by the Lowry method (Lowry et al. 1951).

Measuring ATPase activity

The total Na^+/K^+-ATPase enzymatic activity of bacterial cell-free extracts was measured in the incubation medium (volume 1 ml) of the following composition: 120 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, 1.5 mM ATP, 1 mM EGTA, 20 mM Hepes-Tris-buffer (pH 7.4), 0.1 μM thapsigargin (selective inhibitor of Ca^{2+}/Mg^{2+}-ATPase E (C)) (Flynn et al. 2001). Calcium chelators EGTA were added for the binding of endogenous Ca^{2+} ions in the incubation medium. The enzymatic reaction was initiated by adding bacterial cell-free extracts (100 μl) in the incubation medium (the amount of the protein in the sample did not exceed 50–150 mg/ml). The incubation period was 1–20 min. The enzymatic reaction was stopped by adding 1 ml of cooled stop solution of the following composition: 1.5 M sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% trichloroacetic acid (pH 4.3). The suspension was centrifuged (1,600 × *g*, 10 min) and the concentration of inorganic phosphorus (P_i) was measured spectrophotometrically (Biospectrophotometer, Eppendorf, USA) in the obtained supernatant without protein. The amount of reaction products were determined by Rathbun and Betlach (1969) and expressed as μmol P_i/min·mg^{-1} protein. Magnesium-ATPase activity of the bacterial cell-free extracts was tested in the same incubation medium, in the presence of 1 mM ouabain (selective inhibitor of Na^+/K^+-ATPase). The Na^+/K^+-ATPase activity was calculated by the difference between the value of total ATPase and Mg^{2+}-ATPase activity. The standard incubation medium (without the adding of cell-free extract) of non-enzymatic hydrolysis of ATP was used in the experiments as a control. A mixture of bacterial cells in saline for measuring the amount of endogenous P_i in the bacterial suspension was also used as a control.

Kinetic analysis

The study of kinetic properties of enzymatic reactions Na^+/K^+-activated Mg^{2+}-dependent ATP-hydrolase reaction was performed in a standard incubation medium, which was modified by physical and chemical characteristics or composition of the respective components (incubation time, protein bacterial mixture in the sample, the concentration of ATP, $Na^+(K^+)$, Mg^{2+}, ouabain). All experiments to study the properties of the Na^+/K^+-ATPase reaction were performed using the initial rate V_0 (linear accumulation of product (P_i) in time). The kinetic indicators characterizing the reaction release P_i during Na^+/K^+-activated Mg^{2+}-dependent of ATP hydrolysis were the initial (instantaneous) reaction rate (V_0), maximum rate of the Na^+/K^+-ATPase reaction (V_{max}), maximum amount (plateau) of the reaction product (P_{max}) and the characteristic reaction time (time half saturation) τ were determined. The kinetic indicators that characterize the Na^+/K^+-activated Mg^{2+}-dependent ATP-hydrolase reaction (the constant activation of ions Mg^{2+}, K^+ (K_{Mg}^{2+}, K_K^+), Michaelis constant (K_{mATP}) and maximal rate of ATP hydrolysis by the Mg^{2+}, K^+ (V_{Mg}^{2+}, V_K^+) and ATP (V_{ATP}) were determined by the Lineweaver-Burk plot (Keleti 1988). The obtained concentration dependence of the rate of enzymatic reaction on the studied reagents (ATP and Mg^{2+}, Na^+ (K^+)) was constructed in the coordinates {1/V on 1/S}, where S is the concentration of the reagent (ATP, Mg^{2+} or Na^+ (K^+)), and V is the rate of enzymatic hydrolysis of ATP at a concentration of ATP, Mg^{2+} or Na^+ (K^+), respectively. In determining the effectiveness of the ouabain on ATPase activity (the constant of inhibition ($I_{0,5}$) and Hill coefficient (n_H)), the linearized curves of the concentration dependence were constructed in Hill coordinates {lg[(A_0–A)/A]; lg[I]} according to the empirical Hill's equation: $\lg[(A_0-A)/A] = -n_H \lg I_{0,5} + n_H \lg [I]$, where A_0 and A are the specific activity of the enzyme in the absence and in the presence of ouabain in a concentration I in medium.

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 methods of variation statistics using Student's *t*-test.

The significance of the calculated indicators of line was tested by Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ (Bailey 1995).

Results

ATPase activity and the effect of temperature and pH

The activity of Na^+/K^+-ATPase was studied in different fractions (Table 1). Our results revealed a high enzyme activity in all fractions (16.11 ± 1.87, 15.89 ± 1.72, 28.27 ± 2.53 $U \cdot mg^{-1}$ protein for cell-free extracts, soluble and sedimentary, respectively), which were obtained from *D. piger* Vib-7 cells. The activity was significantly lower ($P < 0.01$) in the fractions obtained from *Desulfomicrobium* sp. Rod-9.

Table 1. Na^+/K^+-ATPase activity in different fractions.

Fractions	Na^+/K^+-ATPase activity ($U \cdot mg^{-1}$ protein)	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
Cell-free extract	16.11 ± 1.87	7.31 ± 0.98
Individual fractions:		
soluble	15.89 ± 1.72	6.92 ± 0.86
sedimentary	28.27 ± 2.53	15.25 ± 1.62

The activity of the enzyme in cell-free extracts under the effect of temperatures and pH in the medium incubation was determined (Fig. 1). The Na^+/K^+-ATPase activity exhibited bell-shaped curves as a function of temperature and pH. A peak of the activity for both bacterial strains was determined at +35 ºC. An increase or decrease in temperature of the incubation led to a decrease of the activity of the enzyme. Similar peaks of the activity were also determined in extracts of *D. piger* Vib-7 at pH 7.0 and *Desulfomicrobium* sp. Rod-9 at pH 6.5.

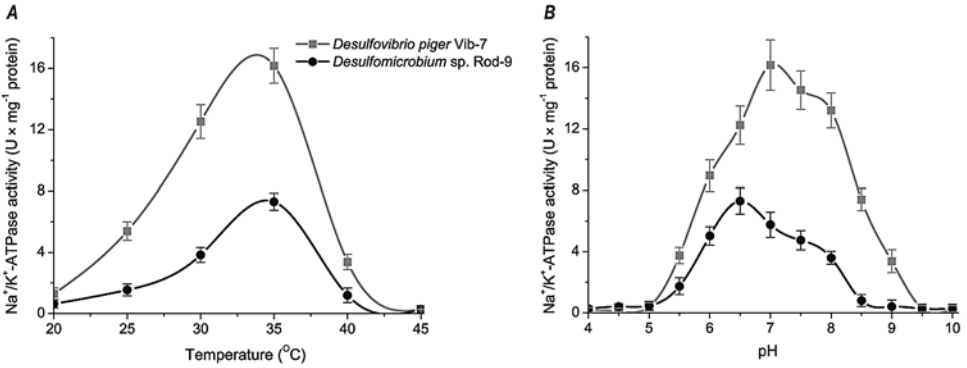

Fig. 1. Effect of temperature (A) and pH (B) on the Na^+/K^+-ATPase activity in the cell-free extracts

Kinetic analysis of the release of inorganic phosphorus in the ATP hydrolysis

To study the characteristics and mechanism of Na^+/K^+-ATPase, the initial (instantaneous) reaction rate (V_0), maximum (plateau) amount formation of product reaction (P_{max}) and reaction time (τ) was defined. To calculate the kinetic indicators of ATP hydrolysis which is

catalyzed by Na^+/K^+-ATPase, the dynamics of the product accumulation of ATP hydrolysis reaction was studied. For that, bacterial suspensions were incubated in a standard incubation medium for different periods of time (1–20 min). Our experimental data imply that the kinetic curves of ATP hydrolysis in both bacterial strains have tendency to saturation (Fig. 2).

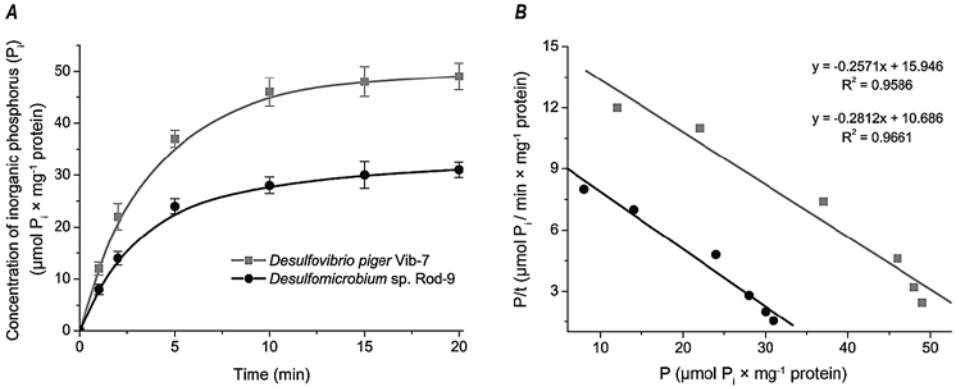

Fig. 2. Dynamics of inorganic phosphorus (P_i) release in the process of ATP hydrolysis (A) and linearization curves of inorganic phosphorus (P_i) accumulation in {P/t; P} coordinates (B) (M ± m, n = 5, $R^2 > 0.95$; $P < 0.02$).

The kinetic of ATP hydrolysis catalyzed in the bacterial cells was consistent with the zero-order reaction in the range 0–5 min (the graph of dependence of P on the incubation time is almost linear in this time interval). Therefore, the duration of incubation of bacterial cell extracts and accordingly the ATP hydrolysis was 5 min in subsequent experiments. The amount of P_i released by the ATPase from *D. piger* Vib-7 was higher compared to *Desulfomicrobium* sp. Rod-9 in the entire range of time factor. The main kinetic properties of the ATP hydrolysis reaction were calculated by the linearization of the data in the {P/t; P} coordinates (Fig. 2B).

Kinetic indicators of ATP hydrolysis in the cell-free extracts were significantly different ($P > 0.05$). Values of initial reaction rate for the enzyme reaction by the maximum amount of the reaction product (P_{max}) were calculated. The V_0 (15.95 ± 1.58 μmol P_i/min·mg^{-1} protein) for *D. piger* Vib-7 was 1.6 greater compared to *Desulfomicrobium* sp. Rod-9 (10.69 ± 0.93 μmol P_i/min·mg^{-1} protein). P_{max} was 62.02 ± 4.21 and 38.01 ± 2.81 μmol P_i·mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively. Based on these data, we assume that the transport of Na^+ and K^+ ions through the membrane is slower and less active in *Desulfomicrobium* sp. Rod-9 cells compared to *D. piger* Vib-7. However, they are characterized by almost the same reaction time, which was 3.89 ± 0.34 and 3.56 ± 2.28 min for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively.

Initial rate of ATP hydrolysis depending on protein concentration in incubation mixture

The dependence of the Na^+/K^+-ATPase activity on the protein concentration was studied in a range of concentrations from 15 to 150 mg protein/ml. A gradual increase in the concentration of protein in the bacterial cell-free extracts of the incubation medium increased the initial (instantaneous) rate of enzyme reaction; the V_0 reached its a maximum value at 115 μg/ml for extract of *D. piger* Vib-7 and 75 μg/ml for *Desulfomicrobium* sp. Rod-9 (Fig. 3).

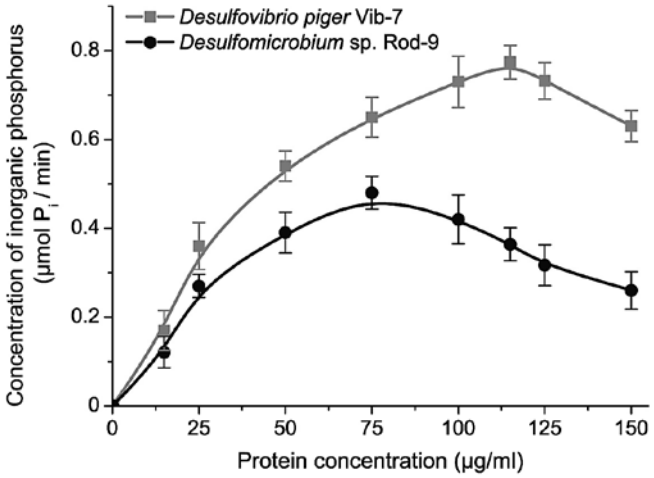

Fig. 3. Initial rate of ATP hydrolysis depending on protein concentration (M ± m, n = 5)

Similarly, the dependency of P_i accumulation depending on the protein content of bacterial cells in the incubation medium had the same character for both strains. However, the data imply that the initial reaction rate was significantly different ($P > 0.05$).

Kinetic analysis of ATP hydrolytic activity depending on ATP concentration

According to the results obtained for the extracts by ATP at concentrations ranging from 0.25 to 2.5 mM (at a constant concentration of 5 mM of Mg^{2+}), there was observed a monotonic increase in enzymatic activity of Na^+/K^+-ATPase to maximum values under substrate concentrations over 1.75 mM and after that activity was maintained on an unchanged (plateau) level (Fig. 4).

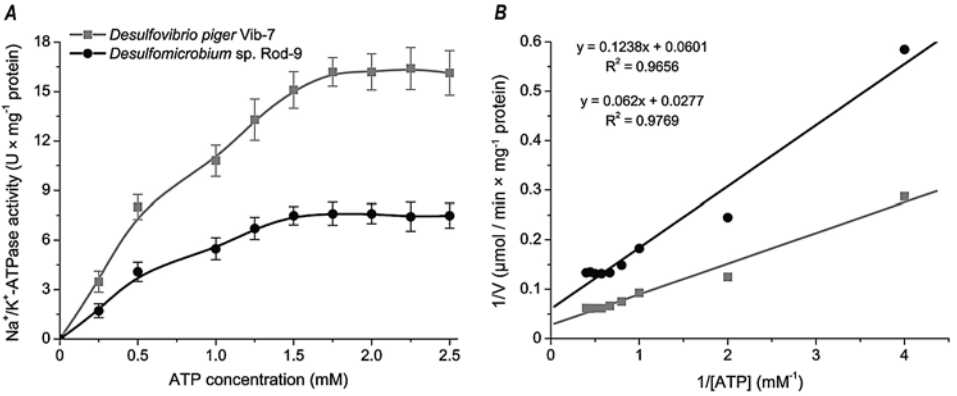

Fig. 4. ATP effect on the Na^+/K^+-ATPase activity (A) and the linearization of the concentration curves (B), which are shown in Fig. 4A, in the Lineweaver-Burk plot, where V is the rate of enzyme reaction (M ± m, n = 5; $R^2 > 0.95$; $F < 0.005$).

The enzyme activity in the *D. piger* Vib-7 was higher compared to the *Desulfomicrobium* sp. Rod-9 within the range of ATP concentrations. To elucidate the possible mechanism of changes of the enzymatic ATPase activity, the kinetic indicators of ATP hydrolysis were defined. The maximum rate of ATP hydrolysis was 36.10 ± 2.87 and 16.64 ± 1.73 μmol P_i/min·mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp., respectively. The value for *D. piger* Vib-7 was × 2 greater than for *Desulfomicrobium* sp. ($P < 0.001$). However, the Michaelis affinity constant (K_m) of the ATPase to ATP in the extract from *D. piger* Vib-7 (2.24 ± 0.21 mM) was not significantly different from *Desulfomicrobium* sp. Rod-9 (2.06 ± 0.18 mM). Thus, according to the obtained kinetic indicators of the ATP hydrolysis for both bacterial strains, we concluded that the activity of Na^+/K^+-ATPase, V_0 and V_{max} was significantly higher ($P < 0.001$) in *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9.

Kinetic analysis of ATP hydrolytic activity depending on Mg^{2+} ion concentration

Experiments focusing on the effect of Mg^{2+} ions on the ATPase activity were performed in a range of $MgCl_2$ concentrations from 0 to 7 mM (at constant ATP concentration of 1.5 mM) (Fig. 5).

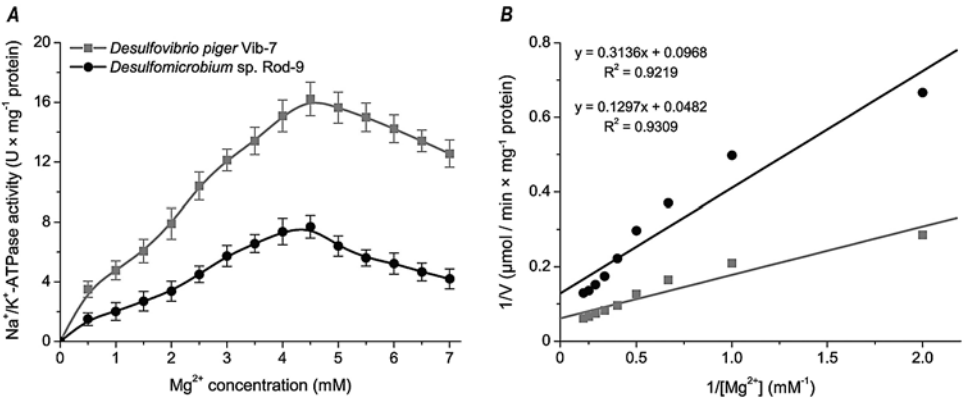

Fig. 5. Mg^{2+} ions effect on the Na^+/K^+-ATPase activity (A) and linearization of concentration curves (B), which are shown in Fig. 5A, in the Lineweaver-Burk plot, where V is the rate of the enzyme reaction (M ± m, n = 5; $R^2 > 0.9$; $P < 0.001$).

Enzyme activity depended on the concentration of Mg^{2+} ions in the incubation medium, and the activity curves had a typical bell-shape. Results presented in Fig. 5A indicate that the maximum value of activity was observed at 4.5 mM $MgCl_2$ for both bacterial strains. As shown in Fig. 5B, the curves of dose-dependent of Mg^{2+} ions effect on the ATPase activity in {1/V, 1/[Mg^{2+}]} coordinates differed by the tangent slope. The V_{max} (20.75 ± 2.3 and 10.33 ± 1.22 μmol P_i/min·mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively) were significantly different from each other ($P < 0.001$). The apparent activation constant of Mg^{2+} ions was 2.65 ± 0.21 mM for *D. piger* Vib-7 and 3.04 ± 0.29 mM for *Desulfomicrobium* sp. Rod-9.

Kinetic analysis of ATP hydrolytic activity depending on Na^+ and K^+ ion concentration

To study the effect of different concentrations of Na^+ and K^+ ions on the specific enzymatic activity of the Na^+/K^+-ATPase, NaCl was changed by isotonic KCl in the incubation medium

(the isotonic conditions of $Na^+ + K^+$ ions equal 150 mM). ATPase activity depended on concentration of Na^+ and K^+ ions and had a typical dome shape (Fig. 6).

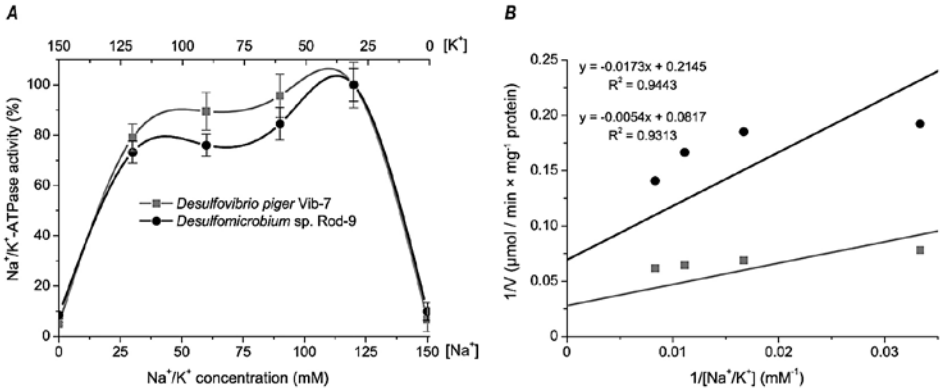

Fig. 6. Effect of Na^+ and K^+ ions (isotonic conditions of $Na^+ + K^+$ is 150 mM) on the Na^+/K^+-ATPase activity (A) and linearization of concentration curves (B), which are shown in Fig. 6A, in the Lineweaver-Burk plot, where V is the rate of the enzyme reaction (M ± m, n = 5; $R^2 > 0.9$; $P < 0.001$).

The optimal ion concentration ratio for the functioning of the enzyme is 125 Na^+ : 30 K^+ in incubation medium for both strains. In the absence of one of the ion species in the incubation medium, the Na^+/K^+-ATPase activity was not observed. The maximum rate of ATP hydrolysis (12.24 ± 1.29 and 4.66 ± 0.38 µmol P_i/min·mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively) was determined by Na^+ ions. The V_{max} in both strains was significantly different ($P < 0.001$). However, the apparent constants of activation of Na^+ and K^+ ions were close (15.12 ± 1.4 and 12.39 ± 1.25 mM) in both strains.

Thus, based on calculation of the data received from the kinetic indicators, we concluded that the enzyme activity is lower in the *Desulfomicrobium* sp. Rod-9 than *D. piger* Vib-7 strain. Perhaps, it is through reduced indicators of the enzyme rate. It can be assumed that the decrease in values of V_{max} in the *Desulfomicrobium* sp. Rod-9 can be a species-specific trait, which could be contributed to a lower Na^+/K^+ electrochemical gradient of the cytoplasmic membrane of the *Desulfomicrobium* sp. Rod-9 and a decrease in the number of the transported units (a decrease of their expression in the membrane) or a decrease in the enzyme rate. Perhaps, the decreased value of the apparent activation constants K_{Na}^+ in the *Desulfomicrobium* sp. Rod- 9 indicated the affinity increase of the Na^+/K^+-ATPase to sodium ions.

Analysis of the inhibition effect of ouabain on the ATP hydrolytic activity

As shown in Fig. 7, inhibition by ouabain is the same for both cell-free extracts. To clarify the indicators of the inhibition of Na^+/K^+-ATPase by ouabain, the linearization of the concentration curves in Hill's coordinates was implemented. Values of apparent inhibition constants ($I_{0.5}$) and Hill coefficient (n_H) did not differ significantly. The $I_{0.5}$ was 22.9 ± 4.6 µM and 15.8 ± 2.6 µM as well as n_H was 1.1 ± 0.12 and 0.99 ± 0.08, for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively. However, it should be noted that inhibition coefficient does not match to the apparent inhibition constant, which is determined when achieving a stationary level of inhibition of the enzyme.

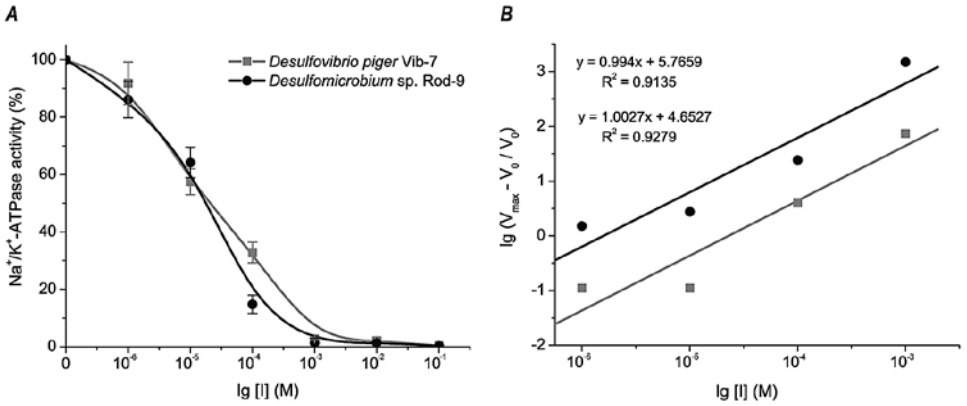

Fig. 7. Ouabain inhibited effect on the Na^+/K^+-ATPase activity (A) and linearization of concentration curves (B) in Hill's coordinates (M ± m, n = 5; $R^2 > 0.9$; $P < 0.001$).

Discussion

Except for ATP, magnesium ions are necessary for the functioning of Na^+/K^+-ATPase, where ions act as cofactors. The Mg^{2+} ions form Mg-ATP chelate complex, which is a substrate of enzymatic reaction. The Mg^{2+} ions interact with the phosphate group of ATP and polarize them, and thus facilitate the nucleophilic attack on the terminal γ-phosphate. The Mg^{2+} ions can also bind to the regulatory center of Na^+/K^+-ATPase (Yuan et al. 2005; Tian et al. 2006).

Na^+/K^+-activated Mg^{2+}-dependent ATPase combines transport, hydrolytic and receptor functions and specifically interacts with exogenous inhibitors in particular with glycosides or their endogenous counterparts (Lodish et al. 2000; Yuan et al. 2005; Tian et al. 2006). Ouabain is a steroid compound, which is a highly selective inhibitor of Na^+/K^+-ATPase. Ouabain enzyme binds to the outside of the cytoplasmic membrane. It is supposed that ouabain blocks an enzyme in the conformation of the $P\text{-}E_2$, which, in turn, prevents the further course of the catalytic cycle of the enzyme. In experimental conditions (insufficient time for binding ouabain in unsteady mode, antagonism of K^+ ions at its high concentration in samples) the coefficient inhibition does not match the apparent inhibition constant, determined in achieving the stationary level of inhibition the enzyme. Inhibition indicators characterize the highly sensitive phenotype of the Na^+/K^+-ATPase to ouabain under these experimental conditions of inhibition. Ouabain is defined by the similarity of patterns of the receptor sites, which is typical for all isoenzymes Na^+/K^+-ATPase (Yuan et al. 2005; Tian et al. 2006). Perhaps, Na^+/K^+-ATPase activity of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 can have the same receptors, which are characterized by sensitivity to inhibition.

The reduction of sulfate ions to hydrogen sulfide occurs as a result of the formation of many intermediate compounds. The sulfate reduction enzymes are located in the cytoplasm and peripheral plasma. The initial stages of the sulfate reduction include uptake of sulfate ions in the bacterial cells. The sulfate ions can be transported into the cells simultaneously with protons and some sulfate-reducing bacteria can absorb sulfate from the flow of sodium ions (Barton and Hamilton 2007). It is known that the center of the ATP hydrolysis is located on the cytoplasmic surface of the membrane (Yuan et al. 2005; Tian et al. 2006).

Guarraia and Peck (1971) determined the ATPase activity in both the soluble and

particulate fractions of the anaerobic sulfate-reducing bacterium, *Desulfovibrio gigas*. As the soluble ATPase was labile to storage, only the particulate enzyme was studied in detail. ATPase was stimulated by both Ca^{2+} and Mg^{2+}, but the magnitude of stimulation depended on pH. In the presence of Ca^{2+} the optimum pH was 6.5, whereas, in the presence of Mg^{2+} the pH optimum was 8.0. However, under optimum conditions, the activity was the same with both Mg^{2+} and Ca^{2+}.

Taking into consideration all of the obtained results, according to change of the hydrolase activity, the transport of Na^+ and K^+ ions by Na^+/K^+-ATPase in *Desulfomicrobium* sp. Rod-9 is slower and less intense compared to the *D. piger* Vib-7 strain. However, it is characterized by almost the same capacity. The Na^+/K^+-ATPase activity, V_0 and V_{max} were significantly higher in the *D. piger* Vib- 7 cells than in *Desulfomicrobium* sp. Rod-9.

The affinity of Na^+/K^+-ATPase to ATP is also the lowest in the *Desulfomicrobium* sp. Rod-9 compared to *D. piger* Vib-7. At the same time, the Mg^{2+}-binding site of Na^+/K^+-ATPase of the studied bacterial strains is native. Increasing adenosine triphosphate in the concentration range from 0.25 to 2.5 mM caused a monotonic increase in enzymatic activity to maximum values and after that activity was maintained on an unchanged (plateau) level. The increase in the affinity of enzyme activity to Na^+ ions in the cell-free bacterial extracts and maintaining the receptor properties of Na^+/K^+-ATPase to ouabain was observed.

The above described experimental data can be used for further clarification of the mechanisms of ion exchange membrane of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 as well as to study the transport mechanisms. According to these data, we can assume that the transports of sulfate ions into *D. piger* Vib-7 cells are much more intensive, and can lead, in turn, to an increase of the intensity of sulfate reduction and the accumulation of hydrogen sulfide, which is toxic for epithelial cells of the intestine and can cause bowel diseases, in particular ulcerative colitis both in humans and animals.

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ATTACHMENT 2

Pages 51 – 58

Activity of selected salicylamides against intestinal sulfate-reducing bacteria

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ORIGINAL ARTICLE

Activity of selected salicylamides against intestinal sulfate-reducing bacteria

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Key words: **salicylamides; *Desulfovibrio piger*; bowel diseases; ulcerative colitis; sulfate-reducing bacteria**

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Abstract

OBJECTIVES: The aim of our work was to evaluate effect of selected salicylamides on cell viability of sulfate-reducing bacterium *Desulfovibrio piger* Vib-7 isolated from the human large intestine, as well as to assess antimicrobial activity and biological properties of these compounds.
METHODS: Microbiological, biochemical, biophysical methods, and statistical processing of the results were used.
RESULTS: An antimicrobial activity and biological properties of salicylamides against intestinal sulfate-reducing bacteria was studied. Primary in vitro screening of the synthesized selected salicylamides was performed against *D. piger* Vib-7. Adding 0.37–1.10 μmol.L^{-1} (N-(4-bromophenyl)-5-chloro-2-hydroxybenzamide, 5-chloro-2-hydroxy-N-[4-(trifluoromethyl)phenyl]benzamide, 5-chloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide, 5-chloro-2-hydroxy-N-(4-nitrophenyl) benzamide and 4-chloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide) caused decrease in biomass accumulation by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The studied compounds completely inhibited the growth of *D. piger* Vib-7 under the effect of 30 μmol.L^{-1}. Moreover, addition of the compounds in the culture medium inhibited the process of dissimilation sulfate dose dependently. Treatment with salicylamides led to the bacterial growth inhibition which correlated with the level of inhibition of sulfate reduction. The data on relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides are consistent to our research in previous series of the biomass accumulation experiments.
CONCLUSIONS: A significant cytotoxic activity under the influence of salicylamides was determined. These results are consistent with a data on bacterial growth and inhibition process of dissimilation sulfate. The strongest cytotoxic effect of the derivatives was observed in compounds of 5-chloro-2-hydroxy-N-[4-(trifluoromethyl)phenyl]benzamide and 5-chloro-2-hydroxy-N-(4-nitrophenyl) benzamide which showed low survival and high toxicity rates.

Abbreviations
CFU - colony-forming unit
DMSO - dimethyl sulfoxide
IC_{50} - inhibitory concentration
KS - Kravtsov-Sorokin medium
MIC - minimal inhibitory concentration
OD - optical density
PBS - phosphate-buffered saline
SRB - sulfate-reducing bacteria
UC - ulcerative colitis

INTRODUCTION

The increased number of sulfate-reducing bacteria (SRB) and intense process of dissimilatory sulfate reduction in the gut is thought to be a significant risk factor of inflammatory bowel diseases in both humans and animals (Gibson *et al.* 1991, 1993; Kushkevych 2012a). These bacteria are often found in persons with rheumatic diseases, and with ankylosing spondylitis, etc. (Barton & Hamilton 2010). There is also an assumption that SRB can be responsible for some forms of cancer of the rectum through the formation of hydrogen sulfide, which affects the metabolism of intestinal cells and give rise to various inflammatory bowel diseases. The species of *Desulfovibrio* genus can cause bloody diarrhea, weight loss, anorexia, epithelial hyperplasia, abscesses and inflammatory infiltrates in animals and humans (Loubinoux *et al.* 2000, 2002). The increased number of SRB was found in feces from people with ulcerative colitis in comparison with healthy individuals (Cummings *et al.* 2003). The injection of these bacteria in hamster intestine caused infection clinically similar to human colitis (Pitcher *et al.* 1996; Cummings *et al.* 2003).

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon that affects up to 12 per 100,000 people in Western countries, mostly between 15 and 30 years of age (Rowan *et al.* 2009). The treatment of mild to moderate UC includes, in a first approach, sulfasalazine and mainly 5-aminosalicylate containing drugs which type and dosage depend on the location and severity of the disease. Other options of treatment include corticosteroids and immunosuppressants (for moderate to severe UC, with a high mortality disease) or probiotics (for improving the microbial balance) (Cummings *et al.* 2003; Kushkevych 2012b). Despite the bacterial nature of the disease, antibiotics failed in the treatment of UC so far. However, new antibacterial compounds with high specific effect against SRB could yield better efficiency in the treatment of this disease. At present, the effects of salicylamides on the intestinal sulfate-reducing bacteria *D. piger* have never been well-characterized and have not been studied yet.

The aim of our work was to evaluate effect of selected salicylamides on cell viability of sulfate-reducing bacterium *D. piger* Vib-7 isolated from the human large intestine, as well as to assess antimicrobial activity and biological properties of these compounds.

MATERIALS AND METHODS

Tested compounds

The studied salicylamides **1**–**5** were synthesized by means of microwave-assisted synthesis described in literature. The compounds were isolated and fully characterized (melting point, elemental analysis, infrared as well as ^{1}H and ^{13}C NMR spectroscopy) (Pauk *et al.* 2013). The compounds were kept in microtubes dissolved in dimethyl sulfoxide (DMSO) solution. The quantity of DMSO necessary to dissolve each compound was calculated previously so that the concentration of the component would be 30 $mmol.L^{-1}$. Afterwards it was diluted 4 times in a proportion 1:3 so that there would be 5 different concentrations of chemical compound: 0.37; 1.1; 3.3; 10 and 30 $mmol.L^{-1}$. The maximum concentration of DMSO in the assays never exceeded 0.1%.

Bacterial culture

The sulfate-reducing bacteria *D. piger* Vib-7 were isolated from the healthy human large intestine as described previously (Kushkevych 2013; Kushkevych *et al.* 2014). The strain has been kept in the collection of microorganisms at the Department of Molecular Biology and Pharmaceutical Biotechnology, Faculty of Pharmacy at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic).

Bacterial cultivation

The bacteria were grown for 72 hours at 37 °C under anaerobic conditions in nutrition modified Kravtsov-Sorokin's (KS) liquid medium (Kushkevych & Moroz 2012). Before bacterial passage in the medium, 0.05 $ml.L^{-1}$ of sterile solution of $Na_2S{\times}9H_2O$ (1%) was added. The sterile 10 $mol.L^{-1}$ solution of NaOH (0.9 $ml.L^{-1}$) 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 cooled to 30 °C. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Assay of bacterial cell concentration

About 1 mL of liquid medium without Mohr's salt (blank) was transferred into a plastic cuvette and taken to a biophotometer (Eppendorf®) for taring. Subsequently, 1 mL of bacterial suspension was transferred into another cuvette and taken again to the biophotometer for measuring at OD_{340}. Before SRB were used for the experiments, optical density (OD_{340}) was always measured to assure there was approximately the same amount of bacteria in each experiment. If it was necessary proper dilutions were made in order to obtain the desired concentration of the bacteria.

The best concentration of *D. piger* Vib-7 was assessed to be $5{\times}10^5$ $CFU.mL^{-1}$. Based on our previous work a correlation between OD_{340} and the amount of cells in the solutions measured in the biophotometer was concluded as:

$$y = 1.0\times10^{9}\,\chi - 6.0\times10^{6},$$

where y means the bacterial concentration and χ means the OD_{340} measured (Figure 1).

The biomass of the *D. piger* Vib-7 cells was calculated by the formula:

$$C = \frac{E \times n}{K},$$

where C – bacterial biomass ($mg.mL^{-1}$); E – extinction; n – dilution factor, times; K – coefficient of conversion, obtained gravimetrically.

Assay of sulfate, lactate sulfide and acetate in cultivation medium

The sulfate ion concentration in the medium was determined by the turbidimetric method after it had been precipitated by barium chloride. To stabilize the suspension, glycerol was used (Kolmert *et al.* 2000). Lactate concentration was measured through the dehydrogenation reaction using Lactate Assay Kit (Sigma-Aldrich, Catalog Number MAK064). Sulfide concentration in the culture medium was assayed by the spectrophotometric method as was described (Cline 1969). Accumulation of acetate ions in process of bacterial growth in the medium was determined using Acetate Assay Kit (Colorimetric, Catalog Number KA3764).

Tratment of bacterial culture

Bacterial culture of stationary phase of growth was centrifuged for 3 min at a rotation speed of 3,500 rpm. Supernatant was removed and replaced by new and fresh KS liquid medium where the bacterial precipitate was diluted. The bacterial suspension was mixed and OD_{340} was measured. Numbers and viabilities of the bacterial cells were determined by counting with a haemocytometer after staining with erythrosine B [0.1% erythrosine B ($w{\cdot}v^{-1}$) in phosphate-buffered saline (PBS), pH 7.2–7.4]. Unstained cells were considered to be viable.

The bacterial suspension (initial concentration $0.5\,mg.mL^{-1}$) was poured to the top in microtubes (280 µL) which contained samples + solvent control (DMSO) + sample control + blanks; it was prepared 3 times each sample as well as the controls so that the average of the results did not had a great discrepancy from the results independently (except in the 3 blanks). The sample controls contained only bacterial suspension and medium (free of tested compounds); and for the 3 blanks only medium.

Calculations were made to assess how much bacterial solution should be in each, based on the OD. Determination of biomass and concentrations of sulfate, lactate, acetate and sulfide in the culture medium under the treatment of 0.37; 1.1; 3.3; 10 and 30 $\mu mol.L^{-1}$ compounds after 12, 24, 36, 48, 60, 72 hours was carried out. During experiments, bacteria were grown at 37 °C under anaerobic conditions.

Analysis of viability of D. piger Vib-7 and cytotoxicity of the compounds

The bacterial suspension (5×10^{4} cells·well^{-1} in 200 µL culture medium) was filled to the top in 96-well plates in triplicate in the KS liquid medium (without Mohr's salt), treated with 0.37; 1.1; 3.3; 10 and 30 $\mu mol.L^{-1}$ compounds and incubated at 37 °C. Relative survival of *D. piger* Vib-7 cells and cytotoxicity of the compounds was determined by 36th hour of cultivation using a WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions (Zídek *et al.* 2014). The relative survival rate was calculated by the following equation:

$$\frac{(A_{Sample} - A_{Blank})}{A_{Control} - A_{Blank}},$$

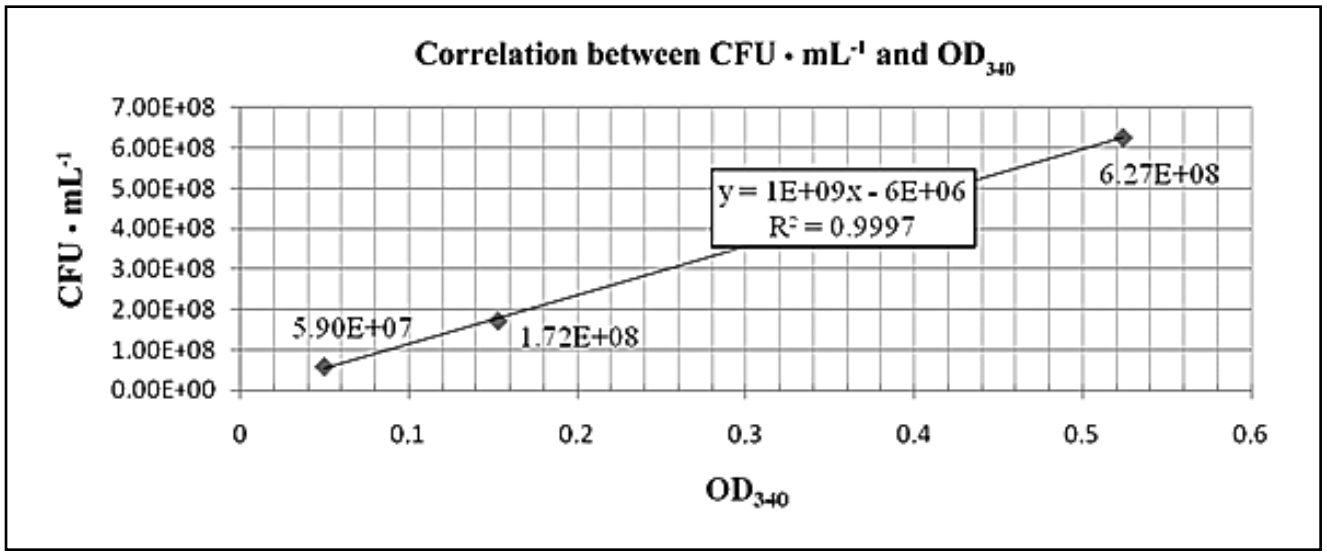

Fig. 1. The correlation between optical density and the amount of bacterial cells.

and multiplied by 100 for the result in percentage. The relative cytotoxicity rate was determined as described previously (Tengler *et al.* 2013). All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Statistical analysis

The statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by methods of variation statistics using Student *t*-test. Statistical significance was tested using the one-way analysis of variance with Dunnett's test and Tukey post-test for comparisons between the means, and differences between two conditions were retained for $p \leq 0.05$. Statistical significance was determined at levels of $p<0.05$, $p<0.01$, and $p<0.001$ (Bailey 1995).

RESULTS

The structure of the discussed salicylamides, their molecular weight and position of the lateral group are shown in Table 1.

The effect of the selected salicylamides in increasing concentrations on the growth of sulfate-reducing bacteria *D. piger* Vib-7 was studied. As shown in Figure 2, the highest level of bacterial biomass accumulation was found in the control KS medium (compounds-free group). Maximum biomass (3.88±0.36 mg.mL^{-1}) of the bacteria *D. piger* Vib-7 on the 60th hour of cultivation was accumulated. Exponential growth phase started from the beginning of bacterial cells seeding in the medium and continued until 48th hour, subsequently, the stationary growth phase started. Treatment with salicylamides in the concentration range between 0.37 to 30 μmol.L^{-1} led to decrease in biomass accumulation (Figure 2).

Under the effect of 0.37–1.1 μmol.L^{-1} **1**–**5** compounds, the growth on the 48th hour of cultivation was inhibited by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The highest level of growth inhibition was observed at higher concentrations (3.3–10 μmol.L^{-1}) of the compounds. Treatment with 30 μmol.L^{-1} of studied compounds completely inhibited the growth of *D. piger* Vib-7. Most likely, this concentration of salicylamides was the most toxic for bacterial cells, as a complete lack of the growth and statistically significant reduction in the initial biomass of the cells (initial seeding) was observed. Based on these data, we can assume that these compounds in concentration of 30 μmol.L^{-1} can cause the lysis of bacterial cells.

Sulfate-reducing bacteria consume the sulfate ions as electron acceptor in the process of dissimilatory sulfate reduction (Kushkevych 2012a, 2012b). The final metabolite of such a process is hydrogen sulfide, which accumulates in the medium. These bacteria also need organic compounds as electron donor (Kushkevych & Moroz 2012). In our previous studies, it was shown that the bacteria *D. piger* Vib-7 consumed sulfate and accumulated hydrogen sulfide in concentration of 2.31±0.21 mmol.L^{-1}. They use lactate as electron donor which oxides to acetate (Kushkevych 2013).

The effect of different concentration of salicylamides on the process of dissimilatory sulfate reduction in *D. piger* Vib-7 cells on the 36th of cultivation was studied. As shown in Figure 3, the addition of the compounds in the culture medium inhibits the process of dissimilation sulfate directly proportional to the increase in concentrations (0.37–30 μmol.L^{-1}).

Under these conditions, the utilization of sulfate and lactate was inhibited, hence the level of accumulation of hydrogen sulfide and acetate was reduced. These data are consistent with our research in previous series of the experiments. The percentage inhibition of sulfate reduction process correlates with the percentage of bacterial growth inhibition under the salicylamides treatment.

Tab. 1. Structure of the discussed salicylamides (Pauk *et al.* 2013); and minimal inhibitory concentration (MIC) and inhibitory concentration (IC_{50}) of compounds **1**–**5** and ciprofloxacin standard against intestinal sulfate-reducing bacteria *D. piger* Vib-7.

Comp.	R^1	R^2	MW	Name	MIC (μmol.L^{-1})	IC_{50} (μmol.L^{-1})
1	5-Cl	4-Br	326.57	*N*-(4-bromophenyl)-5-chloro-2-hydroxybenzamide	<0.37	>1.1
2	5-Cl	4-CF_3	315.67	5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide	<0.16	>0.28
3	5-Cl	3,4-Cl	316.57	5-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide	<0.25	>0.37
4	5-Cl	4-NO_2	292.68	5-chloro-2-hydroxy-*N*-(4-nitrophenyl)benzamide	<0.27	>0.32
5	4-Cl	3,4-Cl	316.57	4-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide	<0.24	>1.1
CPX	–	–	–	ciprofloxacin	>41	>68

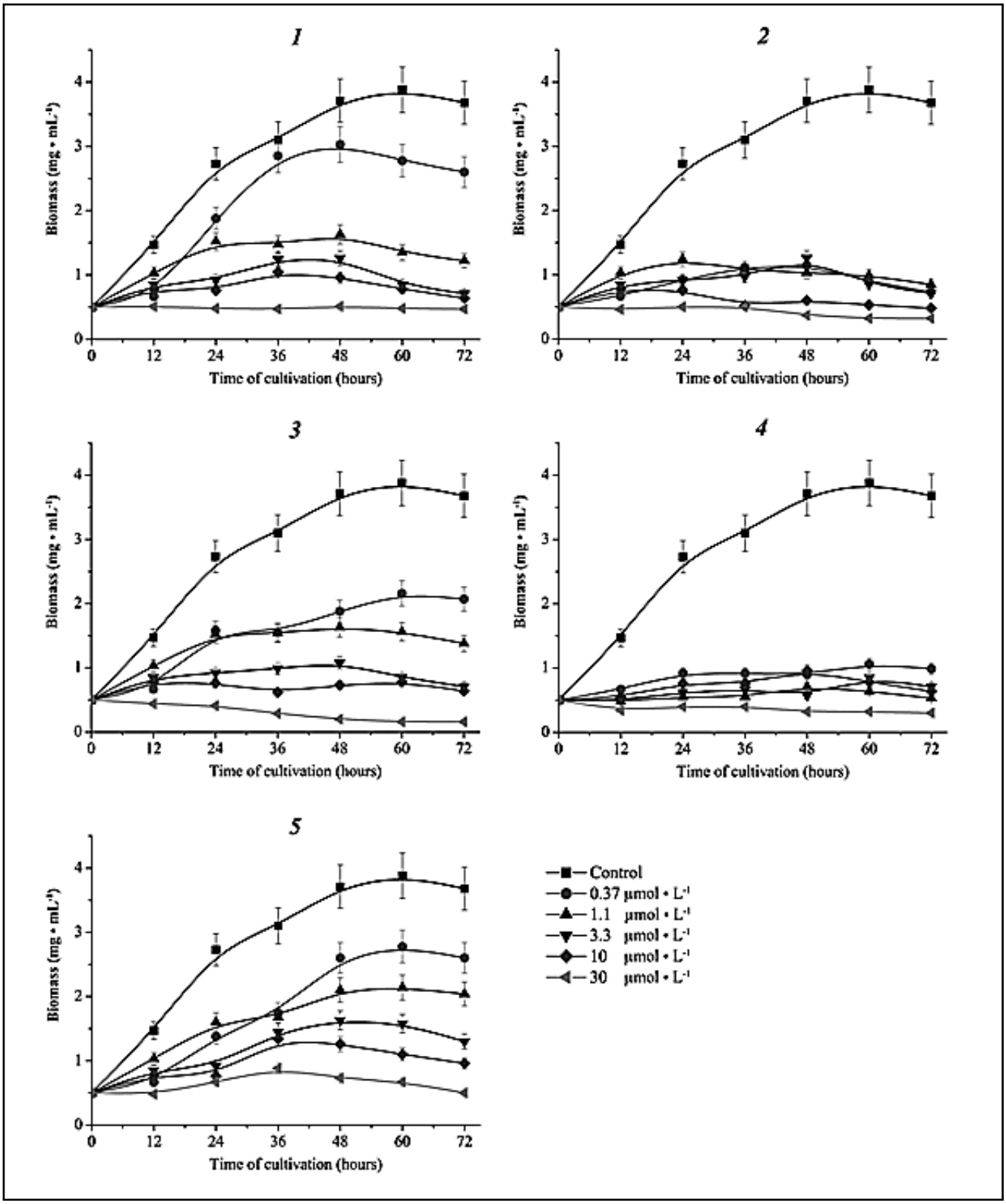

Fig. 2. Growth of *D. piger* Vib-7 cells under the effect of salicylamides in indicated concentrations.

The relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides using WST-1 method was studied. In this series of experiments, compounds **1**–**5** exerted cytotoxicity against these bacteria already in low (0.37–1.1 μmol.L^{-1}) concentrations (Figure 4).

The data on relative survival of *D. piger* Vib-7 cells and relative cytotoxicity under the salicylamides effect were in concert with the previously obtained results of bacterial growth and inhibition level of dissilitatory sulfate reduction process. Treatment with all tested com-

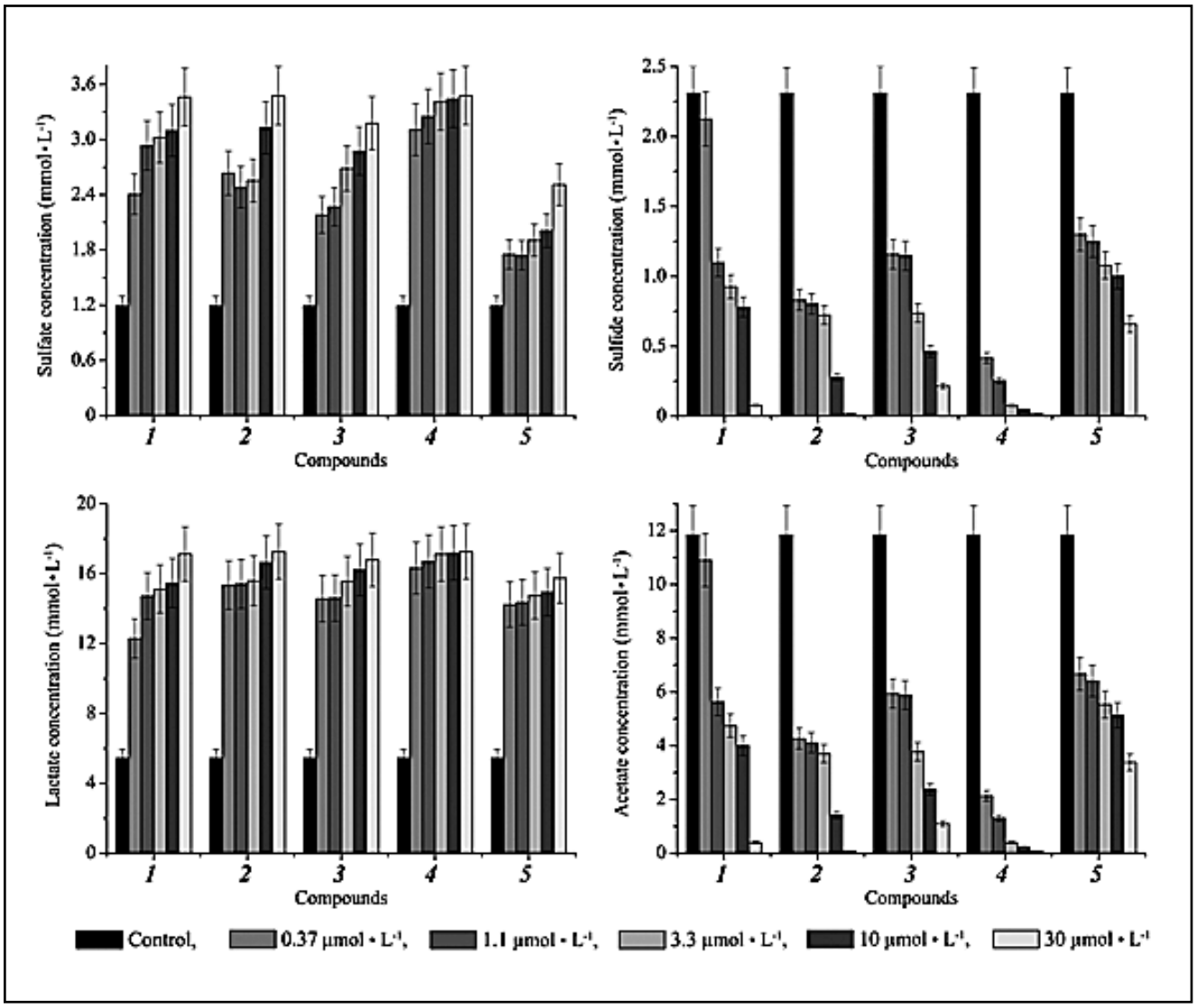

Fig. 3. Sulfate reduction of *D. piger* Vib-7 cells under the treatment of indicated doses of salicylamides.

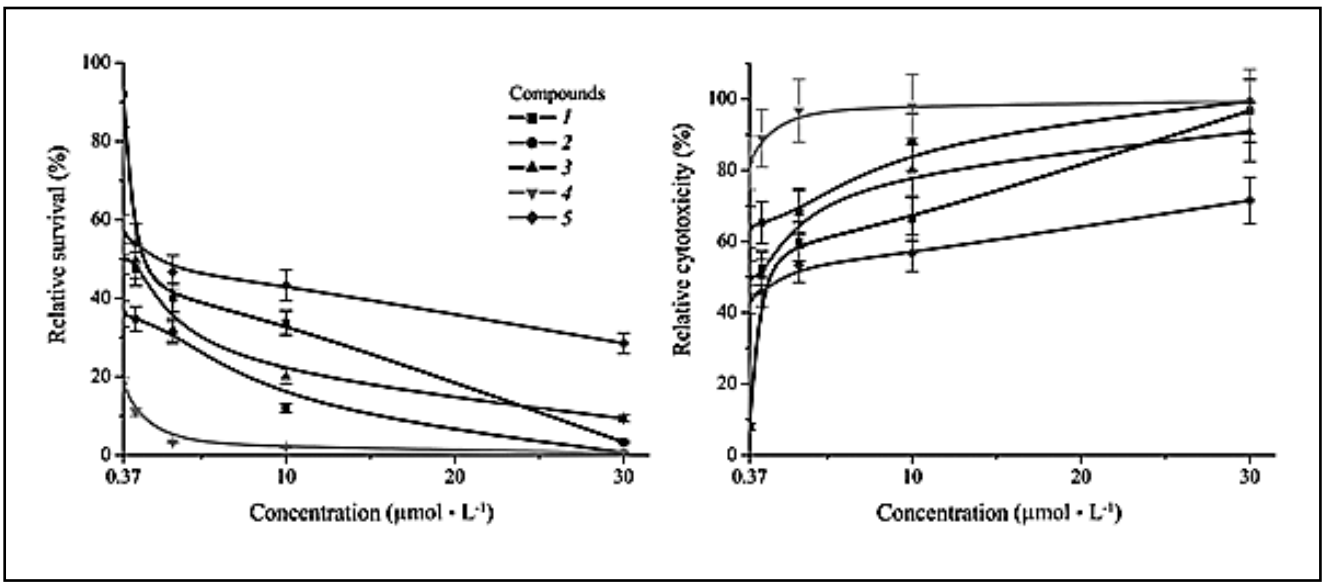

Fig. 4. Relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides.

pounds led to a significant dose-dependent cytotoxicity, with the strongest effect observed in compounds **4** and **5**.

Since some of salicylamides (such as **4**, **2**, or **3**) have a pronounced antimicrobial effect against *D. piger* Vib-7 (combination of low survival and high toxicity rates) they could be considered as promising agents against the growth of this type of bacteria.

Based on obtained results, the minimal inhibitory concentration (MIC) and inhibitory concentration (IC_{50}) of salicylamides against intestinal sulfate-reducing bacteria were established. As shown in Table 1, the MIC for all compounds was lesser 0.37 μmol.L^{-1}.

Regarding IC_{50}, for the compounds **1** and **5**, it was over 1.1 μmol.L^{-1}. On the other hand, for the compounds **3** and **4** the IC_{50} was over 0.37 and 0.32 μmol.L^{-1} respectively. Concerning IC_{50}, only for the compound **2** was over 0.28 μmol.L^{-1}, while all other compounds had shown IC_{50} over 30 μmol.L^{-1}.

DISCUSSION

Sulfate-reducing bacteria *Desulfovibrio* genus belongs to the intestinal microbiota of humans and animals (Kushkevych 2012b). They are anaerobic microorganisms, dissimilating sulfate as an electron acceptor and organic compounds as an electron donor and carbon source in the process of "dissimilatory sulfate reduction" (also known as "dissimilatory anaerobic sulfate respiration") (Kushkevych 2012a). Lactate is the most common substrate used by the species belonging to the intestinal sulfate-reducing bacteria. The species of *Desulfovibrio* oxidize lactate incompletely to acetate. Lactate oxidation to acetate occurs together with the concurrent reduction of sulfate to sulfide (Barton & Hamilton 2010). The presence of lactate and sulfate in the human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolic products, acetate and hydrogen sulfide, which are toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher *et al.* 1996; Rowan *et al.* 2009). There is also an assumption that sulfate-reducing bacteria can cause some forms of cancer of the rectum through the formation of hydrogen sulfide.

In the light of our results it can be stated that almost all of salicylamides in concentrations above 10 μmol.L^{-1} have an interesting antimicrobial activity against *Desulfovibrio piger*. This activity was concentration-dependent, with the strongest effect in 30 μmol.L^{-1} concentrations. Tested compounds can be considered as good alternative for the treatment of colitis or colorectal cancer although it should be taken in account that these compounds can be aggressive also to commensally bacteria and even to other parts of the human body. This should be a concern to be clarified in the near future with complementary assays.

Based on all obtained results in our study we can conclude that compounds **1**–**5** in concentration 0.37–1.1 μmol.L^{-1} inhibited the growth on the 48th hour of cultivation by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The highest level of growth inhibition was observed at higher concentrations (3.3–10 μmol.L^{-1}) of the compounds. These results are consistent with a data on inhibition process of dissimilation sulfate. A significant cytotoxic activity under the influence of salicylamides was determined. The strongest cytotoxic effect was observed in compounds **4** and **5**. Derivatives **4**, **2**, and **3** showed low survival and high toxicity rates, and thus are interesting for further studies.

Understanding the role of sulfate-reducing bacteria in colonic conditions would be enhanced by the ability to inhibit the number of the sulfate-reducing bacteria and/or reduce the production of sulfide and acetate. This would help to clarify the factors influencing sulfide production in the human and animal colon.

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ATTACHMENT 3

Pages 59 – 64

Activity and kinetic properties of phosphotransacetylase

from intestinal sulfate-reducing bacteria

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Regular paper

Activity and kinetic properties of phosphotransacetylase from intestinal sulfate-reducing bacteria

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Phosphotransacetylase activity and the kinetic properties of the enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. has never been well-characterized and has not been studied yet. In this paper, the specific activity of phosphotransacetylase and the kinetic properties of the enzyme in cell-free extracts of both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal bacterial strains were presented at the first time. The microbiological, biochemical, biophysical and statistical methods in this work were used. The optimal temperature and pH for enzyme reaction was determined. Analysis of the kinetic properties of the studied enzyme was carried out. Initial (instantaneous) reaction velocity (V_0), maximum amount of the product of reaction (P_{max}), the reaction time (half saturation period, τ) and maximum velocity of the phosphotransacetylase reaction (V_{max}) were defined. Michaelis constants (K_m) of the enzyme reaction (3.36 ± 0.35 mM for *D. piger* Vib-7, 5.97 ± 0.62 mM for *Desulfomicrobium* sp. Rod-9) were calculated. The studies of the phosphotransacetylase in the process of dissimilatory sulfate reduction and kinetic properties of this enzyme in intestinal sulfate-reducing bacteria, their production of acetate in detail can be perspective for clarification of their etiological role in the development 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.

Key words: sulfate-reducing bacteria, phosphotransacetylase, kinetic analysis, inflammatory bowel diseases

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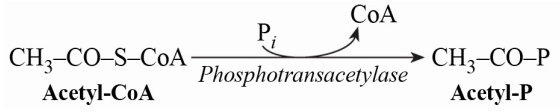
INTRODUCTION

Dissimilatory sulfate-reducing bacteria reduce inorganic sulfate or other oxidized sulfur forms to sulfide (Barton & Hamilton, 2010). This bacteria are heterotrophs and therefore, require an organic carbon source. In the case of *Desulfovibrio* and *Desulfomicrobium* genera, this carbon source can be supplied by simple organic molecules such as lactate, pyruvate, and malate. These are subsequently oxidized to acetate with the concurrent reduction of sulfate to sulfide (Rowan *et al.*, 2009; Kushkevych, 2012a).

The process of organic compounds oxidation is a complex and multistage that provides the bacterial cells with energy (Kushkevych, 2012b). The lactate is the most common substrate used by the species belonging to the sulfate-reducing bacteria (Barton & Hamilton, 2010). This compound is oxidized to acetate *via* pyruvate (Sadana, 1954; Kushkevych, 2012a).

In our previous researches, we have demonstrated that lactate was oxidized incompletely to acetate by the intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 (Kushkevych, 2013). Lactate oxidation to acetate occurs with the intermediate compounds formation: pyruvate, acetyl-CoA and atsetylfosfat (Kushkevych, 2012a).

One important step in this degradative pathway involves the transfer of an acetyl group from acetyl-S-CoA to orthophosphate to form acetyl-PO_4. The acetyltransferase catalyzing this reaction is phosphotransacetylase (acetyl-S-CoA: orthophosphate acetyltransferase, EC 2.3.18) (Kushkevych, 2012a):



In the presence of sulfate, lactate in human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolism product, hydrogen sulfide, which is toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher & Cummings, 2003; Rowan *et al.*, 2009; Gibson *et al.*, 1991; Kushkevych, 2012a). 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 (Cummings *et al.*, 2003; Gibson *et al.*, 1991; Loubinoux *et al.*, 2000; Kushkevych, 2012b).

As far as it is aware, phosphotransacetylase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* has never been well-characterized. In literature, there are some data on phosphotransacetylase in various organisms as well as in the sulfate-reducing bacteria isolated from environment (Goldman, 1958; Reichenbecher & Schink 1997; Robinson & Sagers, 1972; Sadana, 1954; Shimizu, *et al.*, 1969). However, the data on activity and the kinetic properties of this enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. has not been reported yet.

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Abbreviations: EDTA, ethylenediaminetetraacetate; SRB, sulfate-reducing bacteria

The aim of this work was to study phosphotransacetylase 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.

The aim was accomplished using microbiological, biochemical, biophysical methods, and statistical processing of the results; the obtained data were compared with those from the literature.

MATERIALS AND METHODS

Objects of the study were sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the healthy human large intestine and identified by the sequence analysis of the 16S rRNA gene (Kushkevych, 2013; Kushkevych *et al.*, 2014).

Bacterial growth and cultivation. Bacteria were grown in a nutrition-modified Kravtsov-Sorokin's liquid medium (Kushkevych & Moroz, 2012). 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 the 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 (Robinson & Sagers, 1972). 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 a cell-free extract. Protein concentration in the cell-free extracts was determined by the Lowry method (Lowry *et al.*, 1951).

Assays for phosphotransacetylase activity. The phosphotransacetylase activity was assayed by measuring acetyl-P arsenolyzed in the presence of CoA as described previously in paper (Shimizu *et al.*, 1969). A reaction mixture, containing 6 μmoles of acetyl-P, 15.8 nmoles of CoA (5 Lipmann units), 5 μmoles of cysteine, 20 μmoles of Tris-HC1 (pH 8.0), 50 μmoles of potassium arsenate (pH 8.0) and the enzyme in a final volume of 1 ml was incubated at 25°C for 12 min. The enzyme was diluted 10–250 times with 50 mM Tris-HC1 (pH 8.0) before incubation, and 50 μl of the solution were added to the mixture. One unit of phosphotransacetylase is defined as the amount of the enzyme which catalyzes the decomposition of μmole of acetyl-P under the specified conditions. 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 phosphotransacetylase 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) τ were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing phosphotransacetylase reactions such as Michaelis constant (K_m) and maximum reaction velocity of substrate decomposition were determined by Lineweaver-Burk plot (Keleti, 1988). For analysis of the substrate kinetic mechanism of phosphotransacetylase, initial velocities were measured under standard assay conditions with different substrate concentrations. The resulting data were also analyzed by global curve fitting in SigmaPlot (Systat Software, Inc.) 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, $V=(V_{max}\ [A]\ [B])/(a\ K_A\ K_B+K_B\ [A]+K_A\ [B]+[A]\ [B])$, 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 a is the interaction factor if the binding of one substrate changes the dissociation constant for the other (Segal, 1975).

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's *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 significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ (Bailey, 1995).

RESULTS AND DISCUSSION

Specific activity of phosphotransacetylase, an important enzyme in the process of organic compounds oxidation in sulfate-reducing bacteria, was measured in different fractions obtained from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cells (Table 1). Results of our study showed that the highest specific activity of the enzyme was detected in cell-free extracts (1.19 ± 0.122 and 0.37 ± 0.041 U × mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). The slightly lower values of activity of phosphotransacetylase were determined in the soluble fraction compared to the cell-free extracts. Its values designated 0.87 ± 0.091 U × mg^{-1} protein for *D. piger* Vib-7 and 0.32 ± 0.036 U × mg^{-1} protein for *Desulfomicrobium* sp. Rod-9. The enzyme activity in sedimentary fraction was not observed.

The effect of temperature and pH of the reaction mixture on phosphotransacetylase activity in the cell-free extracts of the sulfate-reducing bacteria was studied (Fig. 1). The maximum specific activity for both bacterial strains was determined at 30…35°C. The highest enzyme

Table 1. Phosphotransacetylase activity in different fractions obtained from the bacterial cells

Sulfate-reducing bacteria	Specific activity of the enzyme (U × mg^{-1} protein)		
	Cell-free extract	Individual fractions	
		Soluble	Sedimentary
Desulfovibrio piger Vib-7	1.19 ± 0.122	0.87 ± 0.091	0
Desulfomicrobium sp. Rod-9	0.37 ± 0.041***	0.32 ± 0.036**	0

Comment: The assays were carried out at a protein concentration of 41.17 mg/ml (for *D. piger* Vib-7) and 38.12 mg/ml (for *Desulfomicrobium* sp. Rod-9). Enzyme activity was determined after 10 min incubation. Statistical significance of the values M ±m, n = 5; $^{**}P < 0.01$, $^{***}P < 0.001$, compared to *D. piger* Vib-7 strain.

activity of phosphotransacetylase for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was measured at pH 7.5…8.5. The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH.

Thus, temperature and pH optimum of this enzyme was 30…35ºC and pH 7.5…8.5, respectively. An increase or decrease in temperature and pH led to a decrease of the activity of studied enzyme in the cell-free bacterial extracts of the sulfate-reducing bacteria.

To study the characteristics and mechanism of phosphotransacetylase reaction, the initial (instantaneous) reaction velocity (V_0), maximum velocity of the reaction (V_{max}), maximum amount of reaction product (P_{max}) and reaction time (τ) were defined. Dynamics of reaction product accumulation was studied for investigation of the kinetic parameters of phosphotransacetylase (Fig. 2).

Experimental data showed that the kinetic curves of phosphotransacetylase activity have tendency to saturation (Fig. 2A). Analysis of the results allows to reach the conclusion that the kinetics of phosphotransacetylase activity in the sulfate-reducing bacteria was consistent to the zero-order reaction in the range of 0–3 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore, the duration of the incubation of bacterial cells extracts was 5 min in subsequent experiments.

Amount of product of phosphotransacetylase reaction in the *D. piger* Vib-7 was higher (15.43 ± 1.61 μmol × mg^{-1} protein) compared to the *Desulfomicrobium* sp. Rod-9 (4.56 ± 0.47 μmol × mg^{-1} protein) in the entire range of time factor. The basic kinetic properties of the reaction in the sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (Fig. 2B, Table 2).

The kinetic parameters of phosphotransacetylase from both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different. Values of initial (instantaneous) reaction velocity (V_0) for the enzyme was calculated by the maximal amount of the product reaction (P_{max}). As shown in Table 2, V_0 for phosphotransacetylase reaction was slightly higher (5.68 ± 0.58 μmol × min^{-1} × mg^{-1} protein) in *D. piger* Vib-7 compared to *Desulfomicrobium* sp. Rod-9 (2.14 ± 0.23 μmol×min^{-1}×mg^{-1} protein). In this case, the values of the reaction time (τ) were more similar for the studied enzyme in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains. Based on these data, there is an assumption that the *D. piger* Vib-7 can consume lactate ion much faster in their cells than a *Desulfomicrobium* sp. Rod-9. Moreover, this hypothetical assumption can be also confirmed by obtained data on maximal velocities of accumulation of the final reaction products, where V_{max} for enzyme reaction in *D. piger* Vib-7 were also more intensively compared to *Desulfomicrobium* sp. Rod-9 (Table 3).

The kinetic analysis of phosphotransacetylase activity dependence on concentration of substrate (acetyl-CoA) was carried out. The increasing of acetyl-CoA 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 3.0 mM. (Fig. 2C). Clearly, the enzyme was saturated with substrate and the higher concentrations (3.0–5.0 mM acetyl-CoA) did not affect its activity, so the activity was maintained on unchanged (plateau) level.

Curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (Fig. 2D). The basic kinetic parameters

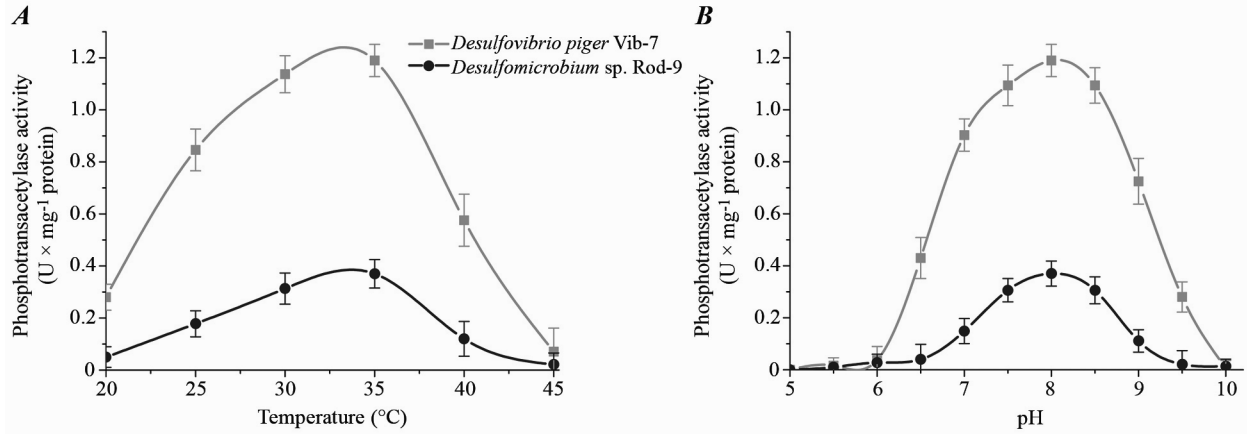


Figure 1. The effect of temperature (A) and pH (B) on the phosphotransacetylase activity in the cell-free extracts of the intestinal sulfate-reducing bacteria

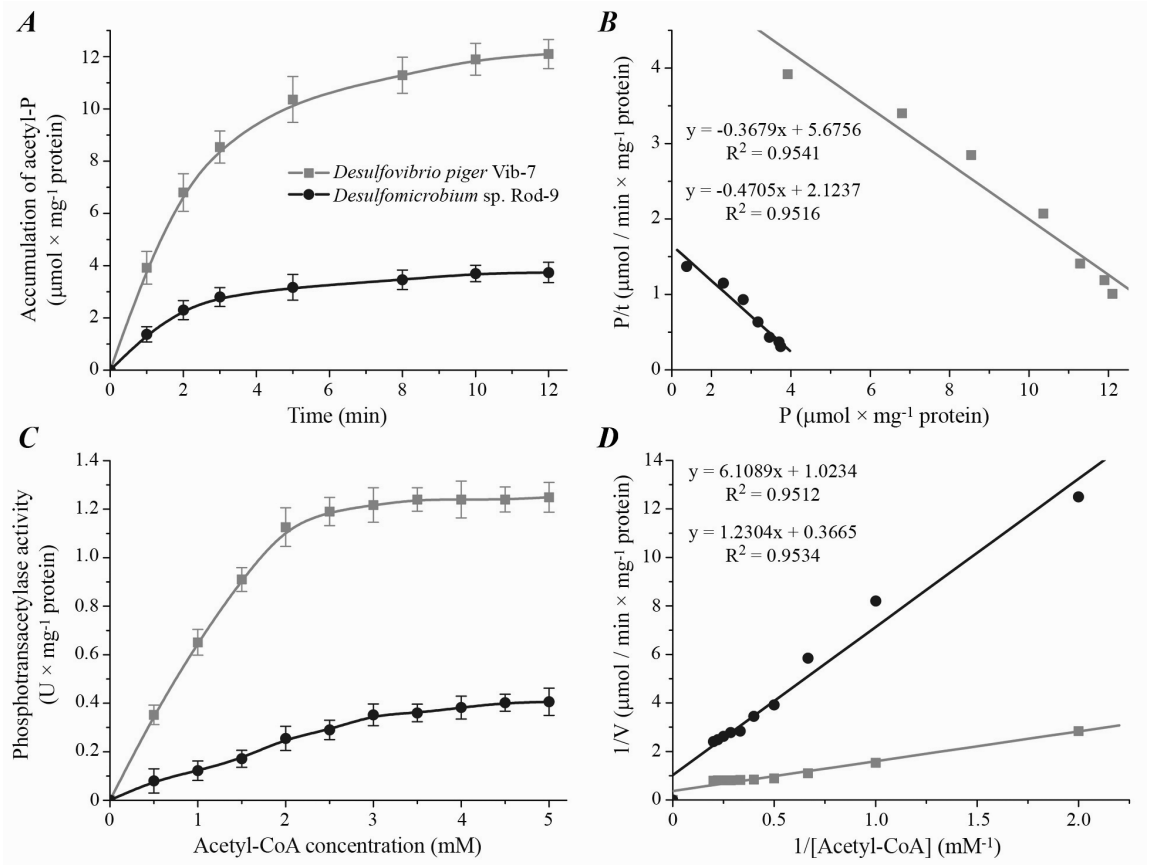


Figure 2. Kinetic parameters of phosphotransacetylase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9
(**A**) dynamics of product accumulation (M± m, n = 5); (**B**) linearization of curves of product accumulation in {P/t; P} coordinates (n = 5; $R^2 > 0.95$; $F < 0.02$); (**C**) the effect of different concentrations of substrate (Acetyl-coenzyme A) on the enzyme activity (M±m, n = 5); (**D**) linearization of concentration curves, which are shown in Fig. 2C, in the Lineweaver-Burk plot, where V is velocity of the enzyme reaction and [Acetyl-coenzyme A] is substrate concentration (n = 5; $R^2 > 0.95$; $F < 0.005$).

of phosphotransacetylase 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-CoA in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different from each other ($P < 0.001$). The values of K_m were also quite different for acetyl-CoA (3.36±0.35, 5.97±0.62 mM) in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains, respectively.

The described results of the phosphotransacetylase activity and the kinetic properties of the enzyme in cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains are new and have never reported in the literature before. These obtained studies were differed significantly from previously described by Sadana (1954). A soluble enzyme system from *Desulfovibrio desufuricans* which catalyses the conversion of two moles of pyruvate to one mole of acetyl phosphate, one mole of ethyl alcohol, and two moles of CO_2 was described. The system required inorganic phosfate for pyruvate dissimilation. Pyrophosphate and arsenate could replace inorganic phosphate. The reaction was most rapid at pH 6.4. The optimum phosphate concentration was 12 M. The requirement of phosphate for the metabolism of pyruvate by the bacterial extract suggests the formation of acetyl coensyme A as an intermediate which is converted to acetyl phosphate and coenzyme A in the presence of inorganic phosphate by transacetylase. The effect is not due to hydrolysis of pyrophosphate to inorganic phosphate since the extracts show no appreciable pyrophosphatase activity (Sadana, 1954).

The phosphotransacetylase activity was also studied in crude extracts of *Escherichia coli* K-12 by Goldman in 1958. A little later, in 1969, Shimizu *et al.* have obtained and purified the phosphotransacetylase from crude extracts of *Escherichia coli* B with the use of ammonium

Table 2. Kinetic parameters of the phosphotransacetylase 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)	5.68±0.58	2.14±0.23**
P_{max} (μmol × mg^{-1} protein)	15.43±1.61	4.56±0.47***
τ (min)	2.72±0.29	2.12±0.22

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; $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the *D. piger* Vib-7 strain.

Table 3. Kinetic parameters of phosphotransacetylase reaction

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
$V_{max}^{Acetyl\text{-}CoA}$ (μmol × min^{-1} × mg^{-1} protein)	2.73 ± 0.31	0.98 ± 0.089***
$K_m^{Acetyl\text{-}CoA}$ (mM)	3.36 ± 0.35	5.97 ± 0.62*

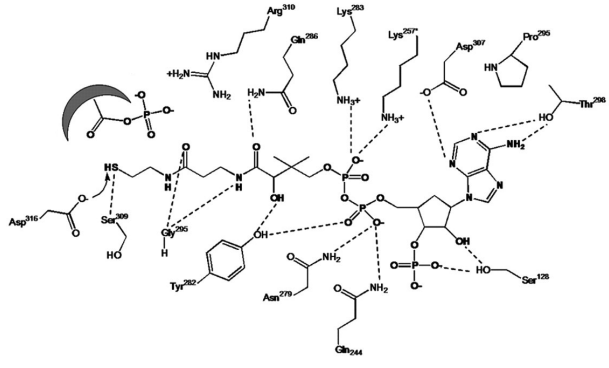
Comment: V_{max} is maximum velocity of the enzyme reaction; K_m is Michaelis constant which was determined by substrate (acetyl-CoA). Statistical significance of the values M ± m, n = 5; *$P < 0.05$, ***$P < 0.001$, compared to the *D. piger* Vib-7 strain.

sulfate as a stabilizer. The purified enzyme was homogeneous in ultracentrifugal analysis and molecular weight was tentatively estimated from the s value. K_m values of this enzyme were 3×10^{-3} and 4×10^{-3} M for acetyl phosphate and 3.2×10^{-4} M for CoA (Shimizu *et al.*, 1969). The values of calculated K_m of acetyl-CoA for phosphotransacetylase reaction in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were slightly different to those previously described by Shimizu *et al.* (1969)

Robinson and Sagers (1972) have measured the activity of phosphotransacetylase from *Clostridium acidiurici*. Authors showed that the phosphotransacetylase has two properties not observed for this enzyme in other bacteria: it required a divalent metal for activity, and it was not subject to uncoupling by arsenate. The enzyme was obtained in highly purified form, with a specific activity 500-fold higher than crude extracts (Robinson & Sagers, 1972).

The described data were also different from results described by Reichenbecher and Schink (1997) for phosphotransacetylase activity from the *Desulfovibrio inopinatus*. Authors demonstrated that the activity was 44 mU (mg protein)$^{-1}$ in extracts of ethanol-grown cells, while it was below 3 mU (mg protein)$^{-1}$ after growth with hydroxyhydroquinone or lactate (Reichenbecher and Schink,1997).

Lawrence and co-authors (2006) have reported structural and functional studies suggesting a catalytic mechanism for the phosphotransacetylase from *Methanosarcina thermophila*. Two crystal structures of phosphotransacetylase from the methanogenic archaeon *M. thermophila* in complex with the substrate CoA revealed one CoA (CoA1) bound in the proposed active site cleft and an additional CoA (CoA2) bound at the periphery of the cleft. Kinetic and calorimetric analyses of site-specific replacement variants indicated that there are catalytic roles for Ser309 and Arg310, which are proximal to the reactive sulfhydryl of CoA1. The reaction is hypothesized to proceed through base-catalyzed abstraction of the thiol proton of CoA by the adjacent and invariant residue Asp316, followed by nucleophilic attack of the thiolate anion of CoA on the carbonyl carbon of acetyl phosphate. Authors have proposed the mechanism of the reaction catalyzed by phosphotransacetylase from *M. thermophila* (Lawrence *et al.*, 2006):



Scheme of the mechanism of the phosphotransacetylase reaction (by Lawrence *et al.*, 2006, modified)

Perhaps, the proposed mechanism for phosphotransacetylase from the methanogenic archaeon *M. thermophila* and the description given by Lawrence and co-authors (2006) may be similar to the studied enzymes from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. However, to confirm this hypothetical assumption, the enzyme should be purified from cell-free extracts to study its structure and properties in detail.

CONCLUSIONS

The phosphotransacetylase, an important enzyme in process of dissimilatory sulfate reduction and lactate oxidation in sulfate-reducing bacteria, carries out the central step in oxidative decarboxylation of acetyl-CoA to acetyl-P.

The enzyme activity, V_0 and V_{max} were significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. However, Michaelis constants for acetyl-CoA were quite higher (5.97 ± 0.62 mM) in *Desulfomicrobium* sp. Rod-9 strain compared to *D. piger* Vib-7. The maximum enzyme activity for both strains was determined at +30…35ºC and at pH 7.5…8.5. The kinetic parameters of enzyme reaction are depended on the substrate concentration.

The studies of the phosphotransacetylase in the process of dissimilatory sulfate reduction and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal strains, their production of acetate and hydrogen sulfide in detail can be perspective for clarification of their etiological role in the development of the humans and animals bowel diseases. Data on the activity and kinetic properties of this enzyme in the strains can be useful to predict the velocity of the accumulation of the final products of metabolism of these bacteria, hydrogen sulfide and acetate, which are formed in the process of dissimilatory sulfate reduction. Assessing rate of formation of these dangerous products in the gut, we are able to predict their toxicity and occurrence of bowel diseases.

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ATTACHMENT 4

Pages 65 – 73

Kinetic Properties of Pyruvate Ferredoxin Oxidoreductase of Intestinal Sulfate-Reducing Bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9.

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ORIGINAL PAPER

Kinetic Properties of Pyruvate Ferredoxin Oxidoreductase of Intestinal Sulfate-Reducing Bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

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Abstract

Intestinal sulfate-reducing bacteria reduce sulfate ions to hydrogen sulfide causing inflammatory bowel diseases of humans and animals. The bacteria consume lactate as electron donor which is oxidized to acetate *via* pyruvate in process of the dissimilatory sulfate reduction. Pyruvate-ferredoxin oxidoreductase activity and the kinetic properties of the enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. have never been well-characterized and have not been yet studied. In this paper we present for the first time the specific activity of pyruvate-ferredoxin oxidoreductase and the kinetic properties of the enzyme in cell-free extracts of both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal bacterial strains. Microbiological, biochemical, biophysical and statistical methods were used in this work. The optimal temperature (+35°C) and pH 8.5 for enzyme reaction were determined. The spectral analysis of the purified pyruvate-ferredoxin oxidoreductase from the cell-free extracts was demonstrated. Analysis of the kinetic properties of the studied enzyme was carried out. Initial (instantaneous) reaction velocity (V_0), maximum amount of the product of reaction (P_{max}), the reaction time (half saturation period) and maximum velocity of the pyruvate-ferredoxin oxidoreductase reaction (V_{max}) were defined. Michaelis constants (K_m) of the enzyme reaction were calculated for both intestinal bacterial strains. The studies of the kinetic enzyme properties in the intestinal sulfate-reducing bacteria strains in detail can be prospects for clarifying the etiological role of these bacteria in the development of inflammatory bowel diseases.

Key words: kinetic analysis, inflammatory bowel diseases, pyruvate ferredoxin oxidoreductase, sulfate-reducing bacteria

Introduction

Intestinal sulfate-reducing bacteria are often isolated from the gut of healthy humans and persons with ulcerative colitis and inflammatory bowel diseases (Gibson *et al.*, 1991; Barton and Hamilton, 2010). A greater number of these bacteria is found mainly in sick people (Cummings *et al.*, 2003; Gibson *et al.*, 1991). In the presence of sulfate, lactate in human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolism product, hydrogen sulfide, which is toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher and Cummings, 2003; Gibson *et al.*, 1991; Kushkevych, 2012a). The increased number of sulfate-reducing bacteria and the intensity of dissimilatory sulfate reduction in the gut can cause inflammatory bowel diseases of humans and animals (Cummings *et al.*, 2003; Gibson *et al.*, 1991; Kushkevych, 2012b).

Lactate is the most common substrate used by the species belonging to the sulfate-reducing bacteria (Kushkevych, 2012a). This compound is oxidized to acetate *via* pyruvate. The type of enzyme present in these microorganisms appears to be a pyruvate-ferredoxin oxidoreductase, as can be deduced from the low potential electron carriers, ferredoxin and flavodoxin, which serve as electron acceptors for the enzyme (Akagi, 1967; Hatchikian *et al.*, 1979; Guerlesquin *et al.*, 1980). In strict anaerobes microorganisms, pyruvate is oxidatively decarboxylated by pyruvate oxidoreductase (EC 1.2.7.1). Pyruvate ferredoxin oxidoreductase catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 (Akagi, 1967; Barton and Hamilton, 2010; Kushkevych, 2012a).

The reaction of this enzyme has been most extensively studied in the forward (oxidative decarboxylation) direction beginning with a series of seminal studies published in 1971 by Raeburn and Rabinowitz which have isolated and characterized pyruvate-ferredoxin oxidoreductase. They have also demonstrated that low potential electron donors, like reduced ferredoxin, can drive the reductive carboxylation of acetyl-CoA (Raeburn and Rabinowitz, 1971).

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As far as we are aware, pyruvate-ferredoxin oxidoreductase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* sp. has never been well-characterized. In the literature there are a lot of data on pyruvate-ferredoxin oxidoreductase in various organisms as well as in sulfate-reducing bacteria isolated from environment (Akagi, 1967; Barton and Hamilton, 2010; Hatchikian *et al.*, 1979; Furdui *et al.*, 2000; Garczarek *et al.*, 2007; Guerlesquin *et al.*, 1980; Zeikus *et al.*, 1977; Raeburn and Rabinowitz, 1971; Uyeda and Rabinowitz, 1971; Ma *et al.*, 1997; Meinecke, *et al.*, 1989; Pieulle *et al.*, 1995). However, data on the activity of this enzyme from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* sp. have not yet been reported.

The aim of this work was to study pyruvate-ferredoxin oxidoreductase 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.

The aim was accomplished using microbiological, biochemical, biophysical methods, and statistical processing of the results; the obtained data were compared with those from the literature.

Experimental

Materials and Methods

The objects of the study were sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and identified by sequence analysis of the 16S rRNA gene (Kushkevych, 2013; Kushkevych *et al.*, 2014).

Bacterial growth and cultivation. Bacteria were grown in a nutrition-modified Kravtsov-Sorokin's liquid medium (Kushkevych, 2013). Before seeding bacteria in the medium, 0.05 ml/l of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. A sterile 10 N 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, suspended in 10 mM Tris-HCl buffer in a 1/1 ratio (w/v) at pH 7.6, and disrupted using a Manton-Gaulin press at 9000 psi. The extract was centrifuged at 15,000 *g* for 1 h; the pellet was then used as sedimentary fraction, and the supernatant obtained was termed the soluble fraction (Gavel *et al.*, 1998). The soluble extract constituted by the supernatant was used as the source of the enzyme. This extract was subjected to further centrifugation at 180,000 *g* for 1 h to eliminate the membrane fraction. 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 (Lowry *et al.*, 1951).

Assays for pyruvate-ferredoxin oxidoreductase activity. The pyruvate-ferredoxin oxidoreductase was assayed and purified as described in paper (Pieulle *et al.*, 1995). The enzyme activity was routinely determined spectrophotometrically by following the reduction of methyl viologen as previously described (Zeikus *et al.*, 1977). All enzyme assays were performed under anaerobic conditions at +35°C using serum-stoppered cuvettes. Samples of enzyme were made anaerobic by flushing the solution with argon as previously reported (Fernandez *et al.*, 1985). The reaction mixture containing 50 μmol Tris-HCl (pH 8.5), 10 μmol sodium pyruvate, 0.1 μmol sodium coenzyme A, 2 μmol methyl viologen and 16 μmol dithioerythritol, in a final volume of 1.0 ml, was bubbled with argon for 20 min and the cell was then incubated at +30°C. The reaction was started by injection of pyruvate-ferredoxin oxidoreductase into the assay cuvette using a gastight syringe and the absorbance at 604 nm was followed. Rates of methyl viologen reduction were calculated using an absorption coefficient of 13.6 $mM^{-1} \times cm^{-1}$. A regenerating system was used to determine the K_m for coenzyme A as previously described (Meinecke *et al.*, 1989). One unit of enzyme activity was defined as the amount of enzyme, which catalyzes the oxidation of 1 μmol of pyruvate or the reduction of 2 μmol of methyl viologen per min under the specified conditions. Specific enzyme activity was expressed as $U \times mg^{-1}$ protein. Michaelis constant (K_m) for pyruvate-ferredoxin oxidoreductase reaction has been determined by substrate (pyruvate and coenzyme A). In order to maintain the concentration of oxidized ferredoxin, a recycling system consisting of spinach ferredoxin-NADP reductase (5 μg/assay) (Sigma) and $NADP^+$ (5 mM) was used. The overall rate was measured by the appearance of NADPH. The activity of the studied enzyme in the cell-free extracts of both bacterial strains at different temperature (from +20°C to +45°C) and pH (in the range from 5.0 to 10.0) in the incubation medium was measured. Spectral analysis of the purified enzyme was carried out as previously described (Pieulle *et al.*, 1995).

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 (incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the pyruvate-ferredoxin oxidoreductase 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) were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing pyruvate-ferredoxin oxidoreductase reactions such as Michaelis constant (K_m) and maximum reaction velocity of substrate decomposition were determined by Lineweaver-Burk plot (Keleti, 1988). For analysis of the substrate kinetic mechanism of pyruvate-ferredoxin oxidoreductase, initial velocities were measured under standard assay conditions with different substrate concentrations. The resulting data were also analyzed by global curve fitting in SigmaPlot (Systat Software, Inc.) 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 α is the interaction factor if the binding of one substrate changes the dissociation constant for the other (Segal, 1975).

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 significance of the calculated parameters of line was tested by Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ (Bailey, 1995).

Results and Discussion

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

Results of our study showed that the highest specific activity of the enzyme was detected in cell-free extracts (1.24 ± 0.127 and 0.48 ± 0.051 $U \times mg^{-1}$ protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). The slightly lower values of activity of pyruvate-ferredoxin oxidoreductase were determined in the soluble fraction compared to cell-free extracts. Its values designated 1.11 ± 0.114 $U \times mg^{-1}$ protein for *D. piger* Vib-7 and 0.37 ± 0.033 $U \times mg^{-1}$ protein for *Desulfomicrobium* sp. Rod-9. The enzyme activity in sedimentary fraction was not observed.

The effect of temperature and pH of the reaction mixture on pyruvate-ferredoxin oxidoreductase activity in the cell-free extracts of the sulfate-reducing bacteria was studied (Fig. 1). The maximum specific activity for both bacterial strains was determined at +35°C. The highest enzyme activity of pyruvate-ferredoxin oxidoreductase for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was measured at pH 8.5.

Thus, temperature and pH optimum of this enzyme was +35°C and pH 8.5, respectively. An increase or decrease in temperature and pH led to a decrease of the activity of studied enzyme in the cell-free bacterial extracts of the sulfate-reducing bacteria. The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH.

Next task of this study was to carry out a spectral analysis of the purified pyruvate-ferredoxin oxidoreductase from the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. The absorption maxima were 317 and 423, 316 and 425 nm for pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively (Fig. 2). Ten-minute incubation of the enzyme with 0.75 mM sodium pyruvate led to a slight decrease in absorption maxima. The same peaks of absorption as without addition of sodium pyruvate were observed. However, the significant decrease in absorption spectra after the addition of 0.75 mM sodium pyruvate and 0.1 mM coenzyme A

Table I
Pyruvate-ferredoxin oxidoreductase activity in different fractions obtained from the bacterial cells

Sulfate-reducing bacteria	Specific activity of the enzyme ($U \times mg^{-1}$ protein)		
	Cell-free extract	Individual fractions	
		Soluble	Sedimentary
Desulfovibrio piger Vib-7	1.24 ± 0.127	1.11 ± 0.114	0
Desulfomicrobium sp. Rod-9	0.48 ± 0.051**	0.37 ± 0.033***	0

Comment: The assays were carried out at a protein concentration of 43.57 μg/ml (for *D. piger* Vib-7) and 41.94 μg/ml (for *Desulfomicrobium* sp. Rod-9). Enzyme activity was determined after 20 min incubation. Statistical significance of the values $M \pm m$, $n = 5$; **$P < 0.01$, ***$P < 0.001$, compared to *D. piger* Vib-7 strain

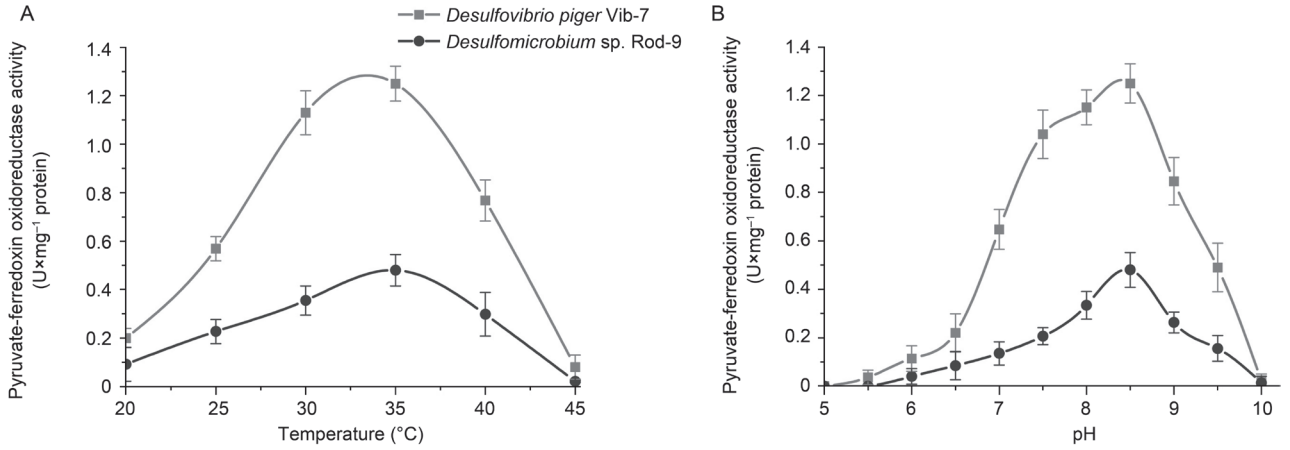


Fig. 1. The effect of temperature (A) and pH (B) on the pyruvate-ferredoxin oxidoreductase activity in the cell-free extracts of the sulfate-reducing bacteria

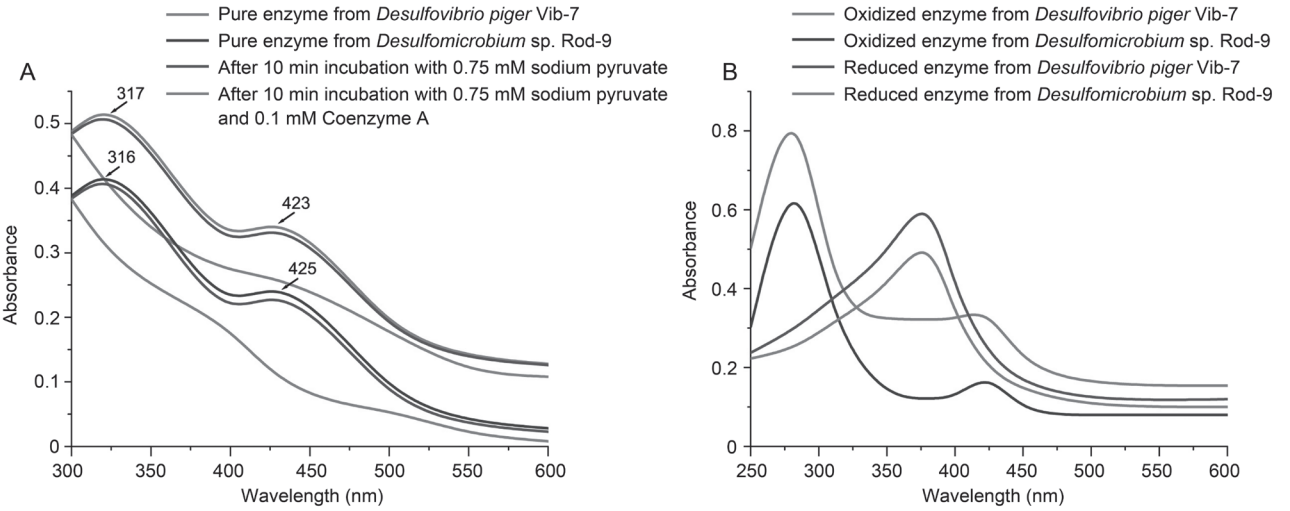


Fig. 2. Absorption spectra of pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. The serum-stoppered cuvette contains 3 μM of pure enzyme in 50 mM Tris-HC1 (pH 8.5) under argon at +35°C, final volume, 1 ml (A). The spectra were recorded in a final volume of 1 ml in a serum-stoppered cuvette of path length 1 cm under argon. Spectrum of the oxidized enzyme and spectrum of the reduced enzyme after injection of 2 μl dithionite (150 mM) (B)

in the incubation medium was registered. The absorption peaks was no observed (Fig. 2A). The spectroscopic analyses of oxidized and reduced pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains were also carried out (Fig. 2B).

Similar data on the absorption spectra of pyruvate-ferredoxin oxidoreductase from *Desulfovibrio africanus* were obtained by Pieulle *et al.* (1995). The authors described the ultraviolet-visible spectrum of studied enzyme which was typical of an iron-sulfur protein with a broad absorbance band around 400 nm and a shoulder in the 315 nm region (Pieulle *et al.*, 1995). Iron and acid-labile sulfide content, as well as the absorption coefficient at 400 nm suggest the presence of six [4Fe-4S] clusters per molecule of enzyme. The absorption band at 400 nm was partially bleached after addition of dithionite; this indicates only partial reduction of the protein, if one considers that full reduction of iron-sulfur clusters should lead to about 50% decrease of the absorption band. Pyruvate reduced the enzyme slightly, whereas pyruvate and CoASH produced a more pronounced reduction of the protein than that obtained with dithionite (Pieulle *et al.*, 1995).

To study the characteristics and mechanism of pyruvate-ferredoxin oxidoreductase reaction, the initial (instantaneous) reaction velocity (V_0), maximum velocity of the reaction (V_{max}), maximum amount of reaction product (P_{max}) and reaction time (τ) were defined. Dynamics of reaction product accumulation was studied for investigation of the kinetic parameters of pyruvate-ferredoxin oxidoreductase (Fig. 3).

Experimental data showed that the kinetic curves of pyruvate-ferredoxin oxidoreductase activity have a saturation tendency (Fig. 3A). Analysis of the results allows to reach the conclusion that the kinetics of pyruvate-ferredoxin oxidoreductase activity in the

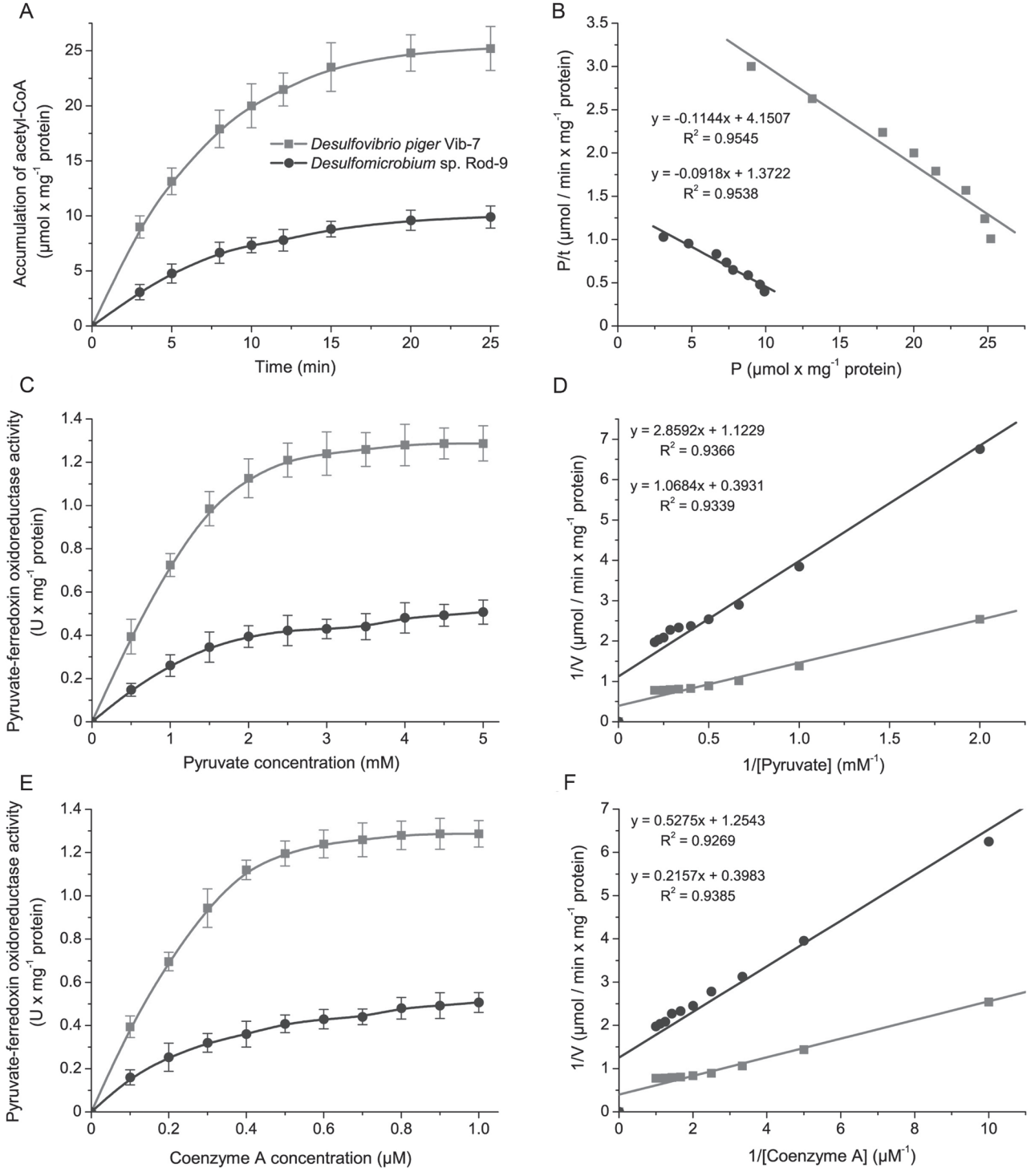


Fig. 3. Kinetic parameters of pyruvate-ferredoxin oxidoreductase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: A – dynamics of product accumulation (M ± m, n = 5); B – linearization of curves of product accumulation in {P/t; P} coordinates (n = 5; $R^2 > 0.95$; $F < 0.02$); C, E – the effect of different concentrations of substrate (pyruvate and coenzyme A) on the enzyme activity (M ± m, n = 5); D, F – linearization of concentration curves, which are shown in fig. 3C, E, in the Lineweaver-Burk plot, where V is velocity of the enzyme reaction and [Pyruvate] or [Coenzyme A] is substrate concentration (n = 5; $R^2 > 0.9$; $F < 0.005$)

sulfate-reducing bacteria was consistent to the zero-order reaction in the range of 0–10 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of bacterial cells extracts was 10 min in subsequent experiments.

The amount of the product of pyruvate-ferredoxin oxidoreductase reaction in the *D. piger* Vib-7 was the higher (36.28 ± 3.59 μmol × mg^{-1} protein) compared to the *Desulfomicrobium* sp. Rod-9 (14.95 ± 1.48 μmol × mg^{-1} protein) in the entire range of time factor. The basic kinetic properties of the reaction in the

Table II
Kinetic parameters of the pyruvate-ferredoxin oxidoreductase 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)	4.15 ± 0.43	1.37 ± 0.12***
P_{max} (μmol × mg^{-1} protein)	36.28 ± 3.59	14.95 ± 1.48**
τ (min)	8.74 ± 0.88	10.89 ± 1.11

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 < 0.01, ***P < 0.001, compared to the *D. piger* Vib-7 strain.

Table III
Kinetic parameters of pyruvate-ferredoxin oxidoreductase reaction

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
$V_{max}^{Pyruvate}$ μmol × min^{-1} × mg^{-1} protein)	2.54 ± 0.261	0.89 ± 0.092***
$K_m^{Pyruvate}$ (mM)	2.72 ± 0.283	2.55 ± 0.245
V_{max}^{CoA} (μmol×min^{-1}×mg^{-1} protein)	2.51 ± 0.248	0.81 ± 0.076***
K_m^{CoA} (μM)	0.54 ± 0.052	0.42 ± 0.044

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

sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (Fig. 3B, Table II).

The kinetic parameters of pyruvate-ferredoxin oxidoreductase from both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different. Values of initial (instantaneous) reaction velocity (V_0) for the enzyme was calculated by the maximal amount of the product reaction (P_{max}). As shown in Table II, V_0 for pyruvate-ferredoxin oxidoreductase reaction was slightly higher (4.15 ± 0.43 μmol × min^{-1} × mg^{-1} protein) in *D. piger* Vib-7 compared to *Desulfomicrobium* sp. Rod-9 (1.37 ± 0.12 μmol × min^{-1} × mg^{-1} protein). In this case, the values of the reaction time (τ) were more similar for the studied enzyme in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains. Based on these data, it may be assumed that the *D. piger* Vib-7 can consume lactate ion much faster in their cells than a *Desulfomicrobium* sp. Rod-9. Moreover, this hypothetical assumption can be also confirmed by obtained data on maximal velocities of accumulation of the final reaction products, where V_{max} for enzyme reaction in *D. piger* Vib-7 were also more intensively compared to *Desulfomicrobium* sp. Rod-9 (Table III).

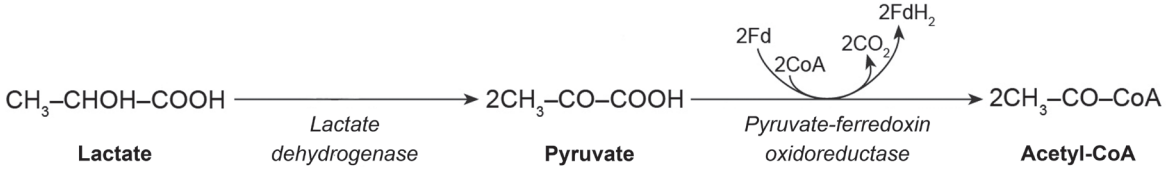
The kinetic analysis of pyruvate-ferredoxin oxidoreductase reaction depending on concentration of substrate (pyruvate and coenzyme A) was carried out. The increasing pyruvate concentrations from 0.5 to 5.0 mM and coenzyme A concentrations from 0.1 to 1.0 μM caused a monotonic rise of the studied enzyme activity and the activity was maintained on unchanged level (plateau) under substrate concentrations over 5.0 mM and 1.0 μM, respectively. (Fig. 3C, E). Curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (Fig. 3D, F). The basic kinetic parameters of pyruvate-ferredoxin oxidoreductase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were identified by linearization of the data in the Lineweaver-Burk plot (Table III).

Calculation of the kinetic parameters of enzyme activity indicates that the maximum velocities (V_{max}) of pyruvate and coenzyme A in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different from each other. However, it was observed a correlative relationship between $V_{max}^{Pyruvate}$ and V_{max}^{CoA} in both intestinal bacterial strains. Michaelis constants (K_m) of pyruvate-ferredoxin oxidoreductase reaction were identified for pyruvate and coenzyme A. The values of K_m were quite similar for pyruvate (2.72 ± 0.283, 2.55 ± 0.245 mM) and coenzyme A (0.54 ± 0.052, 0.42 ± 0.044 μM) in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains, respectively.

The obtained parameters of pyruvate-ferredoxin oxidoreductase reaction in *D. piger* Vib-7 are consistent with previously described data by Pieulle *et al.* for the activity of pyruvate-ferredoxin oxidoreductase from *D. africanus.* The apparent K_m for pyruvate and coenzyme A were also 2.5 mM and 0.5 μM, respectively and the V_{max} values were 10240 min^{-1} and 5890 min^{-1}, respectively. The apparent K_m for methyl viologen was found to be 0.5 mM in the presence of 10 mM and 0.1 mM of pyruvate and CoASH, respectively. Kinetics studies done with the enzyme and a slight decrease in the affinity for pyruvate and in the catalytic activity (K_m of 5.5 mM and V_{max} of 4810 min^{-1}) were reported (Pieulle *et al.*, 1995).

Furdui and Ragsdale (2000) have described the pyruvate-ferredoxin oxidoreductase from the *Clostridium thermoaceticum.* The Michaelis-Menten parameters for pyruvate synthesis by the enzyme were: V_{max} 1.6 unit/mg, $K_m^{Acetyl\text{-}CoA}$ 9 μM. The intracellular concentrations of acetyl-CoA, CoASH, and pyruvate were also measured (Furdui and Ragsdale, 2000).

Pyruvate-ferredoxin oxidoreductatse, an important enzyme in process of dissimilatory sulfate reduction and organic compounds oxidation in sulfate-reducing bacteria, carries out the central step in oxidative decarboxylation of pyruvate to acetyl-CoA (Kushkevych, 2012a):



Garczarek *et al.* (2007) have purified this enzyme from *Desulfovibrio vulgaris* Hildenborough as part of a systematic characterization of as many multiprotein complexes as possible for this organism (Garczarek *et al*., 2007).

Thus, based on the obtained studies results and according to the kinetic parameters of pyruvate-ferredoxin oxidoreductatse reaction for both bacterial strains, we have concluded that the enzyme activity, V_0 and V_{max} were significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. However, Michaelis constants were quite similar for pyruvate (2.72 ± 0.283, 2.55 ± 0.245 mM) and coenzyme A (0.54 ± 0.052, 0.42 ± 0.044 μM) in both bacterial strains. The maximum enzyme activity for both strains was determined at +35°C and at pH 8.5. These data correspond to conditions which are present in the human large intestine from where the bacterial strains were isolated. Perhaps such conditions favor intensive development of the *D. piger* and *Desulfomicrobium* sp. bacterial strains in the gut. The kinetic parameters of enzyme reaction are depended on the substrate concentration. The studies of the pyruvate-ferredoxin oxidoreductatse in the process of dissimilatory sulfate reduction and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal strains, their production of acetate in detail can be a perspective for clarification of their etiological role in the development 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.

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ATTACHMENT 5

Pages 74 – 79

Antimicrobial effect of salicylamide derivatives against intestinal sulfate-reducing bacteria

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Original Research Article

Antimicrobial effect of salicylamide derivatives against intestinal sulfate-reducing bacteria

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ABSTRACT

Sulfate-reducing bacteria (SRB) are most likely involved in both the initiation and maintenance of inflammatory bowel disease (IBD); unfortunately present antibacterial chemotherapeutics used in the treatment of IBD have been ineffective. Thus, the antimicrobial activity of salicylamide derivatives against two different genera of intestinal SRB, *Desulfovibrio* and *Desulfomicrobium*, was investigated. Six 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl] alkanoates and three 2-hydroxy-*N*-[(2*S*)-1-oxo-1-(phenylamino)alkan-2-yl]benzamides showed MIC values in the range from 0.22 to 0.35 μM against *Desulfovibrio piger* Vib-7 and in the range from 0.27 to 8.52 μM against *Desulfomicrobium* sp. Rod-9, while MIC values of ciprofloxacin were 41.2 μM and 39.3 μM. The highest potency against the two strains was observed for 4-chloro-*N*-{(2*S*)-1-[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl}-2-hydroxybenzamide (MIC 0.22 μM and 0.27 μM). 4-Chloro-2-[(4-nitrophenyl)carbamoyl]phenyl (2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-methylbutanoate showed high activity against *D. piger* Vib-7 (MIC = 0.26 μM), while 4-chloro-2-[(4-methylphenyl)carbamoyl]phenyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(1*H*-indol-2-yl)propanoate expressed high activity against *Desulfomicrobium* sp. Rod-9 (MIC = 0.31 μM). Structure–activity relationships are discussed.

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Introduction

Ulcerative colitis (UC) is one of the two major forms of idiopathic inflammatory bowel disease (IBD) (Cummings et al., 2003). Both acute and chronic forms of the illness affect the colon and rectum and can be a highly disabling condition (Barton and Hamilton, 2010). This disease is more common in North America and Western Europe with the increasing incidence in Asia. The reported incidence is 1.2–20.3 cases per 100,000 persons per year, and the prevalence is 7.6–245 cases per 100,000 per year (Feuerstein and Cheifetz, 2014). Ulcerative colitis usually has a relapsing/remitting pattern and current medical approaches focus on treating active disease to address symptoms, to improve the quality of life and thereafter to maintain remission. Bloody diarrhoea, an urgent need to defecate and abdominal pain are the main symptoms of active disease or relapse. The treatment chosen for active disease depends not only on clinical severity, but also on the extent of disease and the person's preference (Loubinoux et al., 2000, 2002a,b; Kornbluth and Sachar, 2010). Conventional drug therapy for UC involves the use of 5-aminosalicylates (the mainstay of treatment for mild to moderate disease), corticosteroids (for patients who failed 5-aminosalicylates therapy and for acute episodes), azathioprine/6-mercaptopurine, cyclosporine and anti-tumour necrosis factor therapy (Lissner and Siegmund, 2013).

Several reports suggested the possible involvement of sulfate-reducing bacteria (SRB), a group of phylogenetically diverse anaerobic microorganisms, in both the initiation and maintenance of the disease (Loubinoux et al., 2000, 2002a,b; Zinkevich and Beech, 2000; Cummings et al., 2003). SRB such as *Desulfovibrio* and *Desulfomicrobium* genera, are normal inhabitants of the human and animal large intestine, capable of dissimilatory sulfate reduction (Gibson et al., 1991, 1993; Kushkevych, 2012a,b; Kushkevych and Moroz, 2012). Most of the SRB utilize sulfate or other sulfur compounds such as thiosulfate, sulfite and sulfur as terminal electron acceptors. The main product of SRB metabolism, hydrogen sulfide, is a compound that may act through inhibition of butyrate oxidation, the main energy source for colonocytes. In addition it is cytotoxic, mutagenic and cancerogenic to epithelial intestinal cells. All these properties of hydrogen sulfide lead to the damage of the epithelial barrier function resulting in inflammatory responses characteristic for IBD (Pitcher and Cummings, 1996; Zinkevich and Beech, 2000). Therefore the association between SRB and inflammatory bowel diseases, such as ulcerative colitis, was hypothesized (Loubinoux et al., 2002a,b; Rowan et al., 2009; Kushkevych, 2014). Unlike Crohn's disease, ulcerative colitis occurs only in the large bowel, where bacteria amount is greater than in the rest of the gut and also where the rate of passage of material is characterized by slow movement of digestive materials (Cummings et al., 2003).

An antibiotic for animal colitis, in order to be effective, should have activity against gut anaerobes. Such antibiotics that specifically target Gram-negative facultative species are not successful in IBD (Cummings et al., 2003). The benefits of antibiotic therapy in UC are mediated by different mechanisms such as decreasing the concentration of luminal bacteria, altering the composition of gut microflora, decreasing bacterial tissue invasion and decreasing bacterial translocation and systemic dissemination. Antibiotics have been prescribed for UC, however they have been largely ineffective. Therefore, it is necessary to study new antibacterial compounds in order to improve the treatment and discover alternative therapeutics (Garud and Peppercorn, 2009).

In the previous studies it was found that salicylamide-like compounds can be considered as promising antimicrobial agents (Vinsova et al., 2007; Imramovsky et al., 2009a,b, 2011; Pauk et al., 2013; Zadrazilova et al., 2015a,b). Therefore this study focused on the investigation of the antimicrobial activity of selected derivatives of 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl]alkanoates and 2-hydroxy-*N*-[(2*S*)-1-oxo-1-(phenylamino)alkan-2-yl]benzamides against two different genera of SRB, *Desulfovibrio* and *Desulfomicrobium*, is a follow-up paper to the previous contributions. The investigated salicylamide derivatives showed high potency against different bacterial strains as was published recently (Pauk et al., 2013; Zadrazilova et al., 2015a). Both SRB are Gram-negative strictly anaerobe genera. *Desulfovibrio piger* is usually considered as a commensal bacterium in humans. More recently, *D. piger* has attracted more interest as it was found to be the most prevalent species of SRB in faeces of patients with inflammatory bowel disease (Holt et al., 1994; Barton and Hamilton, 2010).

Materials and methods

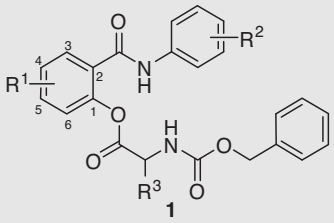
Tested compounds

The discussed salicylamide derivatives (see Table 1) were synthesized previously (Pauk et al., 2013) by means of microwave-assisted synthesis and rearrangement described in literature (Imramovsky et al., 2006, 2009a, 2010, 2011; Pauk et al., 2013). The compounds were fully characterized by melting point, CHN analyses, IR and NMR spectroscopy (Pauk et al., 2013).

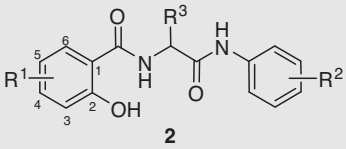
In vitro *antibacterial susceptibility testing*

The synthesized compounds were evaluated for *in vitro* antibacterial activity against the intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 that were isolated from the healthy human large intestine as described previously (Kushkevych, 2013; Kushkevych et al., 2014). The strains have been kept in the collection of microorganisms at the Department of Molecular Biology and Pharmaceutical Biotechnology of the Faculty of Pharmacy at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic). Ciprofloxacin (Sigma–Aldrich) was used as the standard. Prior to testing, each strain was passaged onto nutrition modified Kravtsov-Sorokin's (KS) agar medium (Kushkevych and Moroz, 2012). Bacterial inocula were prepared by suspending a small portion of bacterial colony in sterile KS liquid medium (pH 7.5). The cell density was adjusted to 0.5 McFarland units using a densitometer (Densi-La-Meter, LIAP, Latvia). The final inoculum was made to a 1:20 dilution of the suspension with KS liquid medium. Before bacterial passage in the medium, 10 mL/L of sterile Mohr's salt solution [$(NH_4)SO_4Fe(SO_4)_2{\cdot}6H_2O$] (10%) for detecting colonies of the

Table 1 – Structures of tested compounds 1a–f and 2a–c (Pauk et al., 2013); calculated values of Clog *P*; and *in vitro* antibacterial activity (MIC) of salicylamides compared to ciprofloxacin (CPX) standard against intestinal sulfate-reducing bacteria.



1



2

Comp.	R^1	R^2	R^3	Clog *P*[a]	MIC [μM]	
					DVP	DMS
1a	4-Cl	4-CH_3	(*S*)-Bn	6.5605	0.31	0.34
1b	4-Cl	4-CH_3	(*R*)-CH_2-indolyl	6.0835	0.28	0.31
1c	4-Cl	4-Br	(*S*)-CH_2-cHx	8.2553	0.35	0.39
1d	4-Cl	4-NO_2	(*S*)-iso-Pr	5.7077	0.26	6.98
1e	5-Cl	4-Br	(*S*)-iso-Pr	6.5333	0.29	0.33
1f	5-Cl	3,4-Cl	(*S*)-Bn	7.5008	0.33	0.37
2a	4-Cl	4-Br	(*S*)-iso-Pr	5.7186	0.27	8.52
2b	4-Cl	3,4-Cl	(*S*)-iso-Pr	6.2517	0.22	0.27
2c	4-Cl	3,4-Cl	(*S*)-Bn	6.7417	0.24	0.33
CPX	–	–	–	–	41.2	39.3

[a] ChemBioDraw ver. 13 (CambridgeSoft – PerkinElmer, Cambridge, MA, USA).
DVP = *Desulfovibrio piger* Vib-7, DMS = *Desulfomicrobium* sp. Rod-9.

sulfate-reducing bacteria was added. As a result, FeS was formed by the bacterial cells that caused black coloured colonies. The compounds were dissolved in DMSO (Sigma), and the final concentration of DMSO in the KS liquid medium did not exceed 0.1% of the total solution composition. The final concentrations of the evaluated compounds ranged from 100 to 0.05 μM. The medium dilution micro-method modified according to NCCLS guidelines (CLSI, 2012, 2014) in KS medium was used to determine the minimum inhibitory concentration (MIC). Drug-free controls, sterility controls and controls consisted of KS medium and DMSO alone were included. Petri plates were introduced into an anaerobic box with oxygen uptake generators (GENbox anaer, France) for anaerobiosis. The determination of results was performed visually after 72 h of static incubation in the darkness at 37 °C under anaerobic conditions. The MICs were defined as the lowest concentration of the compound at which no visible bacterial growth was observed. The results are summarized in Table 1.

Results

The studied compounds can be divided into two groups based on their chemical structure: Group I includes *N*-protected amino esters of *N*-phenylsalicylamides **1a–f**, and Group II includes compounds **2a–c** that can be named as diamides, since they contain two amidic moieties. The activity of both groups of salicylamide derivatives against sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were compared with the effect of ciprofloxacin as a clinically used drug. The *in vitro* antibacterial activity of compounds was expressed as the minimum inhibitory concentration (MIC) that is defined for bacteria as a 90% (IC_{90}) or greater reduction of growth in comparison with the control. The MIC/IC_{90} value is routinely and widely used in bacterial assays, being a standard detection limit according to the Clinical and Laboratory Standards Institute (CLSI, 2012, 2014). In the case of potency against *D. piger* all the compounds showed a narrow range of the MICs from 0.22 to 0.35 μM, while the activity against *Desulfomicrobium* sp. ranged from 0.27 to 8.52 μM. Nevertheless, the potency of all discussed compounds was much higher against both genera than that of ciprofloxacin (MIC 41.2 μM or 39.3 μM). All the results are listed in Table 1.

Lipophilicity is the most frequent physicochemical parameter employed in structure–activity relationship analysis. In a number of studies examining the biological activity of potential drugs, the relationship between lipophilicity and/or other descriptors (*e.g.*, electronic parameters or molar volume of substituents) and their potency have been investigated. In the present study the calculated lipophilicity (Clog *P* values), see Table 1, was found as the main parameter influencing general antibacterial potency. Clog *P* value is the logarithm of *n*-octanol/water partition coefficient based on established chemical interactions.

Within Group I, 4-chloro-2-[(4-nitrophenyl)carbamoyl]phenyl (2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-methylbutanoate (**1d**) showed the highest activity (MIC = 0.26 μM) against *D. piger*, while compound **1c** (R^1 = 4-Cl, R^2 = 4-Br, R^3 = -CH_2-cHx) demonstrated the lowest activity (MIC = 0.35 μM). Within Group II 4-chloro-*N*-{(2*S*)-1-[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl}-2-hydroxybenzamide (**2b**) was found to be the most effective compound with MIC = 0.22 μM. Fig. 1 illustrates the dependence of the antibacterial activity against *D. piger* expressed as log(1/MIC) of all the tested compounds on the lipophilicity expressed as Clog *P*. Activity within Group I decreases with the lipophilicity increase almost linearly

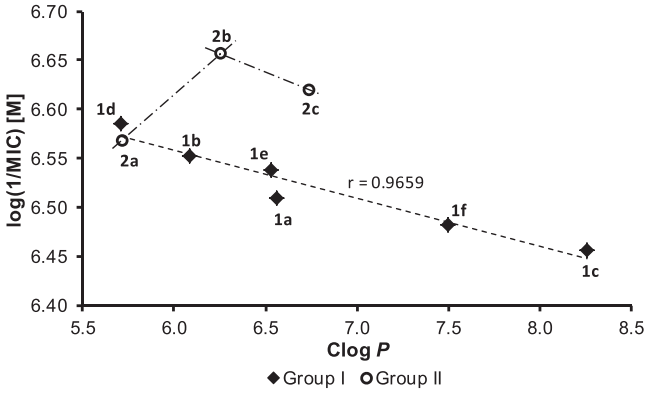


Fig. 1 – Dependence of antibacterial effect of tested compounds 1a–f (Group I) and 2a–c (Group II) against *Desulfovibrio piger* Vib-7 expressed as log(1/MIC [M]) on lipophilicity.

($r = 0.9659$, $n = 6$), while for Group II biphasic course can be found with the lipophilicity optimum Clog *P* = 6.25 (compound **2b**, R^1 = 4-Cl, R^2 = 3,4-Cl, R^3 = iso-Pr). It can be stated that diamides of Group II of comparable lipophilicity in the range of Clog *P* 6.2517–6.7417 can be considered more potent against *D. piger* than esters of Group I.

Based on the obtained MIC values of compounds from Groups I and II, it can be stated that there is no significant difference between the potency of both groups against *Desulfomicrobium* sp. Rod-9, see Table 1 and Fig. 2, where the dependence of the antibacterial activity against *Desulfomicrobium* sp. expressed as log(1/MIC) on lipophilicity is illustrated. Compounds **2b** (R^1 = 4-Cl, R^2 = 3,4-Cl, R^3 = iso-Pr) and 4-chloro-*N*-{(2*S*)-1-[(3,4-dichlorophenyl)amino]-1-oxo-3-phenylpropan-2-yl}-2-hydroxybenzamide (**2c**) showed the highest potency (MIC = 0.27 μM and MIC = 0.33 μM) within Group II. Within Group I, 4-chloro-2-[(4-methylphenyl) carbamoyl]phenyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(1*H*-indol-2-yl)propanoate (**1b**), 5-chloro-2-[(4-bromophenyl)carbamoyl]phenyl (2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-methylbutanoate (**1e**) and 4-chloro-2-[(4-methylphenyl)

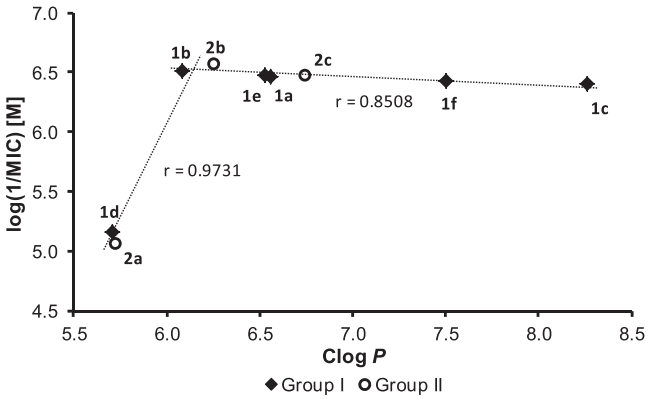


Fig. 2 – Dependence of antibacterial effect of tested compounds 1a–f (Group I) and 2a–c (Group II) against *Desulfomicrobium* sp. Rod-9 expressed as log(1/MIC [M]) on lipophilicity.

carbamoyl]phenyl (2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-phenylpropanoate (**1a**) demonstrated similar efficiency in the MIC range from 0.31 to 0.34 μM. In general, based on the observation from Fig. 2, it can be stated that the antibacterial activity against *Desulfomicrobium* sp. is connected with the lipophilicity in the range of Clog *P* approx. from 6.1 to 6.8. The biphasic dependences of activity on lipophilicity can be found for both groups of salicylamide derivatives. The lowest activity was determined for the least lipophilic compounds **1d** (R^1 = 4-Cl, R^2 = 4-NO_2, R^3 = iso-Pr; MIC = 6.98 μM, Clog *P* = 5.7077) and **2a** (R^1 = 4-Cl, R^2 = 4-Br, R^3 = iso-Pr; MIC = 8.52 μM, Clog *P* = 6.7417), from which the activity sharply increased to compound **2b** ($r = 0.9731$, $n = 4$), where an optimum lipophilicity can be found (Clog *P* = 6.25). With a subsequent increase of the lipophilicity, the activity slightly decreased ($r = 0.8508$, $n = 7$) to compounds **1f** (R^1 = 5-Cl, R^2 = 3,4-Cl, R^3 = Bn; MIC = 0.37 μM, Clog *P* = 7.5008) and **1c** (R^1 = 4-Cl, R^2 = 4-Br, R^3 = -CH_2-cHx; MIC = 0.39 μM, Clog *P* = 8.26) with the highest lipophilicity, see Fig. 2.

Discussion

Thus, based on the MIC values, it can be concluded that diamide **2b** is the most potent compound against both SRB genera. From Group I, ester of *N*-phenylsalicylamides **1d** is the most potent against *D. piger* Vib-7, while compounds **1b**, **1e** and **1a** showed an acceptable activity against *Desulfomicrobium* sp. Rod-9. Based on these results and the limited number of the compounds it is not possible to assess if R^1 substitution in $C_{(4)}$ or $C_{(5)}$ is more advantageous. Rather electron-withdrawing R^2 substituent and less lipophilic (isopropyl, benzyl) R^3 substituent seem to be also more favourable for higher activity. Compound **2b** is much more potent than any compound from Group I. These results are opposite to the results of the recently published study (Pauk et al., 2013), where just *N*-protected amino esters of *N*-phenylsalicylamides showed high activity against *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Clostridium perfringens*, *Pasteurella multocida* and, overall, were more effective than diamides. However, these microorganisms were more resistant to the effect of the compounds compared with *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. On the other hand, it is important to note that diamides were found to have bactericidal effect against three clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. aureus* ATCC 29213 as the reference and quality control strain (Zadrazilova et al., 2015a). None of above discussed compounds did not show any effect against typical Gram-negative bacterial strains, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella*, *Proteus* or *Helicobacter* (Zadrazilova, 2013; Zadrazilova et al., 2015a,b).

Salicylanilides show broad spectrum of antimicrobial activities, because due to their structure they can affect a wide range of targets, although exact mechanisms have not been elucidated. For example, they inhibit the two-component regulatory systems of bacteria, bind to protein kinase epidermal growth factor receptor, inhibit interleukin-12p40 production, inhibit bacterial enzymes, such as transglycosylases, D-alanine-D-alanine ligase, isocitrate lyase and methionine aminopeptidase or destruct the cellular proton gradient due to

their function as proton shuttles (Zadrazilova, 2013; Zadrazilova et al., 2015a,b). Nevertheless, such sensitivity of studied intestinal SRB to salicylamides derivatives can be caused by the peculiarities of their metabolism including their physiological, biochemical, cytological and ecological properties (Barton and Hamilton, 2010; Kushkevych, 2012a,b).

Despite the fact that both evaluated bacteria belong to the same group and even physiological subgroup, it is known that *Desulfovibrio* species is different from *Desulfomicrobium* in biochemical and physiological properties. Genera *Desulfovibrio* and *Desulfomicrobium* have the same basic structure of bacterial cell wall (peptidoglycan), but *Desulfovibrio* have such phenotypic features as the presence of desulfoviridin, cytochrome c_3 and menaquinone MK-6. *Desulfomicrobium* contains desulforubidin, not desulfoviridin, and also the structure of cytochrome is different. The activity, structure and properties of vital enzymes of *Desulfovibrio* and *Desulfomicrobium* genera differ (Holt et al., 1994; Brenner et al., 2005; Barton and Hamilton, 2010).

Based on above-mentioned facts, the inhibitory effect of salicylamides **1a–f** and **2a–c** can be obviously caused by their action on the process of dissimilatory sulfate reduction in both *Desulfovibrio* and *Desulfomicrobium* genera and their growth and production of hydrogen sulfide. However, the difference in these parameters may be due to different mechanisms of sulfate transport, the presence in their cells of different transport systems or, even, enzymes providing this process. Relative survival in *D. piger* Vib-7 or *Desulfomicrobium* sp. Rod-9 cells and antimicrobial effect under the influence of salicylamides derivatives **1a–f** and **2a–c** is different. It is due to the chemical structure of these compounds (diamides were described as bactericidal agents) and the genus characteristics of *Desulfovibrio* and *Desulfomicrobium*.

Conclusions

Compounds of Group 1 **1a–f** (2-(phenylcarbamoyl) phenyl *N*-[(benzyloxy)carbonyl]alkanoates) and Group II **2a–c** (2-hydroxy-*N*-[(2*S*)-1-oxo-1-(phenylamino)alkan-2-yl]benzamides) were found to inhibit the intestinal bacterial growth of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. 4-Chloro-*N*-{(2*S*)-1-[(3,4-dichlorophenyl)amino]-3-methyl-1-oxobutan-2-yl}-2-hydroxybenzamide (**2b**) demonstrated the highest potency against both strains: MIC = 0.22 μM against *D. piger* and MIC = 0.27 μM against *Desulfomicrobium* sp. 4-Chloro-2-[(4-nitrophenyl)carbamoyl] phenyl (2*S*)-2-{[(benzyloxy)carbonyl]amino}-3-methylbutanoate (**1d**) showed high activity against *D. piger* Vib-7 (MIC = 0.26 μM) and 4-chloro-2-[(4-methylphenyl)carbamoyl] phenyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(1*H*-indol-2-yl) propanoate (**1b**) showed high activity against *Desulfomicrobium* sp. Rod-9 (MIC = 0.31 μM). Lipophilicity was recognized as a significant parameter affecting biological activities. It is not possible to assess if R^1 substitution in $C_{(4)}$ or $C_{(5)}$ is more advantageous, but in general for both SRB strains rather electron-withdrawing R^2 substituent and less lipophilic (isopropyl or benzyl) R^3 substituent are also more favourable for higher activity. Based on the results it can be hypothesized that salicylamide derivatives interact with enzymatic systems of the bacteria affecting vital cell functions, and diamide **2b** could probably serve as bactericidal active agent. Therefore, salicylamide derivatives seem to be promising candidates of potential agents with activity against sulfate-reducing bacteria.

Conflict of interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

Acknowledgements

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ATTACHMENT 6

Pages 80 – 85

Kinetic properties of growth of intestinal sulphate-reducing bacteria isolated from healthy mice and mice with ulcerative colitis

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Kinetic properties of growth of intestinal sulphate-reducing bacteria isolated from healthy mice and mice with ulcerative colitis

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Abstract

Inflammatory bowel disease including ulcerative colitis are complex multifactorial diseases of unknown aetiology. Sulphate-reducing bacteria are often associated with the occurrence of the disease. The physiological properties of intestinal sulphate-reducing bacteria including kinetic characteristic of their growth have never been reported. The aim of this research was to evaluate the presence of sulphate-reducing bacteria isolated from the intestines of mice, study their growth, calculate and compare the kinetic growth properties on the model of dextran sulphate sodium induced ulcerative colitis in the mice. The number of viable intestinal sulphate-reducing bacteria from the bowel lumen of mice with ulcerative colitis was higher ($P > 0.05$) by 22% at 12 h of cultivation compared with cultures of sulphate-reducing bacteria from the bowel lumen of healthy mice. The sulphate-reducing bacteria from mice with colitis also had a slightly higher generation time (14.29 h) and exponential growth phase (22.24 h) compared with cultures from healthy mice. The time of lag-phase was 2 × shorter ($P > 0.01$) in the cultures of sulphate-reducing bacteria from mice with ulcerative colitis. The described research is new and important for the prediction of the sulphate-reducing bacteria number in the gut and their rate of dissimilatory sulphate reduction. The kinetic characteristic of their growth is important for further clarification of the mechanisms of sulphate reduction and accumulation of hydrogen sulphide, which is toxic for epithelial cells of the intestine and can cause bowel diseases both in humans and animals, in particular ulcerative colitis.

Intestinal microbiota, growth rate, hydrogen sulphide, bowel diseases

Sulphate-reducing bacteria (SRB) are widespread in anaerobic areas of soils, wetlands, fresh and marine waters and available in the microbiota of the large intestine of humans and animals (Barton and Hamilton 2010). These microorganisms metabolize sulphate as an electron acceptor to hydrogen sulphide. The sulphate dissimilation process is called the "dissimilatory sulphate reduction" or "sulphate respiration" (Kushkevych 2016a,b). For this process, SRB needs exogenous electron donors, including organic compounds or molecular hydrogen. Dependent on SRB genera, organic compounds are oxidized incompletely to acetate (acetogenic SRB) or completely to carbon (IV) oxide (Barton and Hamilton 2010).

The intensity of sulphate reduction in SRB and, accordingly, the accumulation of hydrogen sulphide in high or toxic concentrations in the intestines can lead to the development of various diseases (Kushkevych 2014a). Hydrogen sulphide is the final product in the sulphate reduction process of SRB metabolism. At high concentrations, this final metabolite

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http://actavet.vfu.cz/

is toxic and carcinogenic for the intestinal cell and can cause inhibition of cytochrome oxidase, colonocytes oxidation of butyrate, destruction of epithelial cells, and development of ulcers and inflammation with subsequent development of ulcerative colitis (Pitcher et al. 1996; Gibson et al. 1991; Cummings et al. 2003; Kushkevych 2015c,d).

Acetogenic SRB and their final products of metabolism (including hydrogen sulphide and acetate) are often found in the faeces of people with bloody diarrhoea (Pitcher et al. 1996) and the mono and polymicrobial infections of the gastrointestinal tract (McDougall et al. 1997). It is believed that SRB can cause frequent defecation, weight loss, and increased intestinal permeability (Kushkevych 2016b). The species and quantitative composition of the SRB on the surface of the intestinal mucosa are different from those microorganisms in the lumen (Macfarlane et al. 2000, 2007; Zinkevich et al. 2000; Kushkevych 2015e). Such genera of SRB, Desulfovibrio, Desulfomicrobium, Desulfobacter, Desulfobulbus, Desulfotomaculum, Lawsonia and Bilophila, are the most isolated from the intestines of healthy and ill humans and animals (Gibson et al. 1991, 1993; Kushkevych 2016b).

The number of cases of inflammatory bowel disease (IBD) including ulcerative colitis (UC) is growing. The cause of the occurrence is still unknown (Cummings et al. 2003). This disease is mostly observed in the human population of the age group of 15–30 years; although there is clear evidence of this disease occurring in the human population of the age group of 50–70 years, which can be labelled as a potential risk group. Frequent cases of this disease are recorded in both developed and underdeveloped countries (Garud and Peppercorn 2009). Ulcerative colitis is diagnosed not only in people but also in animals; e.g., horses, cows, pigs, dogs, cats, and rodents (mice and rats). However, the functional role of SRB in the development of inflammatory bowel disease including ulcerative colitis and their participation in the mechanisms of the disease have never been studied properly. The physiological properties of intestinal SRB including the kinetic characteristic of the SRB growth (doubling time, absolute and relative (specific) growth rate, the maximum growth rate and time of lag-phase) and their comparison between healthy mice and mice with induced ulcerative colitis, have never been reported.

The aim of our research was to induce ulcerative colitis in mice using dextran sulphate sodium, accumulate the SRB cultures from the intestines of healthy mice and of mice with ulcerative colitis, isolate and identify SRB cultures to study their growth, calculate and compare the kinetic growth properties.

Materials and Methods

Manipulation with animals

Male C57Bl/6 mice (20 g ± 2 g) were obtained from the Animal Breeding Facility of Masaryk University (Brno, Czech Republic). They were kept under standard conditions (22 ± 2 °C, 50 ± 10% relative humidity) and alternating 12 h light/dark cycles. The animals had access to a standard diet and drinking water *ad libitum*. Manipulations with the animals were carried out according to the bioethical rules as per the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" adopted in Strasbourg in 1986. The study was also approved by the "Commission for the Protection of Animals against Cruelty" and the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic.

In total, 6 animals in two groups (4 + 2 animals in the first and second group, respectively) were randomly separated and used in this experiment. In the dextran sulphate sodium (DSS) group (n = 4), colitis was induced by administering 5% (w/v) DSS (MP Biomedicals, Illkirch-Graffenstaden, France; MW 36.000–50.000 Da) in drinking water for 7 days. The mice in the intact group (n = 2) received drinking water only. On the last day of experiment, the animals were killed by decapitation under isoflurane anaesthesia. The isolated distal colonic segments were selected for the analysis of qualitative and quantitative composition of intestinal microflora of both groups of the animals.

Accumulation of intestinal SRB

In total, 100 mg of faeces were taken from healthy mice and mice with UC. A 5 mm-piece of the intestine was also taken for studying SRB which can be in biofilms from both groups of animals. All samples were suspended in tubes (volume 1.5 ml) with pre-heated (36.6 °C) liquid modified Postgate's medium I (Postgate 1984) of the following composition (g/l): Na_2SO_4 (4.0), KH_2PO_4 (0.3), K_2HPO_4 (0.5), $(NH_4)_2SO_4$ (0.2), NH_4Cl

(1.0), $CaCl_2 \times 6H_2O$ (0.06), $MgSO_4 \times 7H_2O$ (1.0), $C_3H_6O_3$ (6 ml), yeast extract (1.0), $Na_3C_6H_5O_7 \times 2H_2O$ (0.3). Additional solutions (g/l) were prepared separately using deionized water: sodium pyruvate (2 g in 100 ml of H_2O, 100 ml), $NaHCO_3$ (2 g in 50 ml of H_2O, 50 ml), $Na_2S_2O_4$ (30 mg in 1 ml of H_2O, 1 ml), Mohr's salt solution ($(NH4)SO_4Fe(SO_4)_2 \times 6H_2O$) (10%) 10 ml, $Na_2S \times 9H_2O$ (1%) 3 µl. The medium and solutions were sterilized separately. Each of these 5 solutions was added to the medium. To adjust the pH value on 8.5 sterile 10N solution of NaOH was used. The redox and anaerobic conditions were controlled by resazurin sodium (Oxoid, BR 0055B) as an indicator. In addition, reduced FeS and Na_2S contained in the medium provided the necessary redox conditions for SRB cultures. The discoloration of resazurin sodium (redox potential of discoloration Eh = -100 mV) confirmed the decrease of the redox potential. The SRB suspensions were cultivated at 36.6 °C under anaerobic conditions for 48 h. The tubes were filled with medium and closed by a rubber plug to provide anaerobic conditions. The positive cultures of SRB created a black sediment because Mohr's salt solution was added to the medium. As a result, FeS was formed by the bacterial cells that caused the black sediment.

Isolation and identification of intestinal SRB pure cultures

For isolation of the SRB pure cultures, each positive suspension with SRB was diluted to 10^{-12} in a series of tubes containing the liquid medium I. To obtain colonies for isolation, the same Postgate's agar (12 g/l) medium I was prepared. To prevent solidification, the medium I in Erlenmeyer flask (500 ml) was placed in water bath tempered to 45 °C. To obtain isolated SRB colonies, 20 ml of agar medium I + 100 µl of each dilution suspension were mixed and consequently poured to Petri dishes. After cooling down, the dishes were placed into an anaerobic box with oxygen uptake generators (GENbox anaer, bioMérieux, France) for anaerobiosis. The cultivation was carried out in incubator ES 120 (Nüve, Turkey) operated at 36.6 ± 0.5 °C. Black colonies were visible on the agar medium after 24 h of cultivation.

Isolated colonies were taken in the liquid medium I and grown for another 24 h. To make sure the samples were pure culture, this process was repeated × 2–3. The identification of SRB was carried out as described previously (Kushkevych 2013; Kushkevych et al. 2014b). The SRB isolates were identified as Desulfovibrio genus. Additional tests of the identification (including morphological characteristics, physiological and biochemical properties) showed that no other genera were present. The obtained SRB cultures were kept in the Laboratory of Anaerobic Microorganisms of the Section of Microbiology and Molecular Biotechnology at the Department of Experimental Biology at the Masaryk University (Brno, Czech Republic).

Testing of bacterial growth by the Bioscreen method

For the testing of bacterial growth, the suspensions of SRB and pure culture of Desulfovibrio genus isolated from 2 healthy mice (5 isolates from the faeces + 5 isolates from the intestinal surface which were in biofilm) as well as from 4 mice with UC (5 isolates from the faeces + 5 isolates from the intestinal surface).

Grown SRB in the liquid medium I without Mohr's salt solution were diluted in the liquid fresh medium I (transmittance was 90 at λ = 620 nm). Consequently, 320 µl of each suspension obtained were pipetted to the wells (total volume 350 µl) of multiwell plates. To provide anaerobic condition, sterile paraffin oil (30 µl) was added to each well. As a control, the medium without any sample (320 µl) + 30 µl of sterile paraffin oil was used. For statistical evaluation, each sample was pipetted into 5 wells. For the study of bacterial growth, the Bioscreen C (Oy Growth Curves Ab Ltd., Finland) was used. Bioscreen was set at a temperature of 36.6 °C, measuring at a 30-min interval within the 24 h of measurement.

Calculation of growth indices and statistical analysis

The growth indices of SRB were characterized by the following basic constants (Widdel 2010): generation time (G), absolute growth rate (R), relative (specific) rate (μ), the maximum growth rate (μ_{max}) and duration of the lag-phase (L).

Generation time (G) was defined as the time (t) per generation of one bacterial cell (N = number of SRB cells in the interval time), in practice, it is the duration of the cell cycle of one cell:

$$G = \frac{1}{R} = \log 2 \cdot \frac{t - t_0}{\log N - \log N_0}$$

where t_0 is initial time ($t_0 = 0$) and N_0 is initial number of bacterial cells ($N_0 = 5.69$ $\log_{10}$CFU·ml^{-1}). The absolute growth rate (or doubling rate) was calculated by the number of divisions (generations) per unit time. The absolute growth rate (R) was defined as the number of cells formed per time:

$$R = \frac{N}{t} = \frac{1}{\log 2} \cdot \frac{\log N - \log N_0}{t - t_0}$$

The relative (specific) growth rate (μ) was determined by the absolute growth rate related to the population size (number cells formed per time and existing number of cells, N):

$$\mu = \frac{dN}{dt} \cdot \frac{1}{N} = \frac{\mathrm{In}\, N - \mathrm{In}\, N_0}{t} = 2.3 \cdot \frac{\log N - \log N_0}{t - t_0}$$

Maximum growth rate was considered as the maximum value of relative (specific) growth rate. Time of lag-phase was calculated by formula: $L = t_k - t_e$, where t_k was the duration of the experiment, and t_e was the duration of the exponential growth phase:

$$t_e = \frac{1}{\log 2} \cdot \frac{\log N - \log N_0}{R} = \frac{1}{\log 2} \cdot G \cdot (\log N - \log N_0)$$

Kinetic and statistical calculations of the results were carried out using the software MS Office and Origin computer programs. Using the experimental data, the basic statistical parameters (mean: M, standard error: m, M ± m) were calculated. The research results were treated by methods of variation statistics using Student's *t*-test. The significance of the calculated indicators of line was tested by Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ (Bailey 1995).

Results

The first stage of our research was to compare the kinetic properties of SRB isolated from the lumen (faeces) of both experimental groups. The number of viable intestinal SRB from the bowel lumen of mice with UC was higher ($P > 0.05$) by 22% at 12 h of cultivation compared to cultures of SRB from the bowel lumen of healthy mice (Fig. 1A). These data correlate with specific maximal growth rate (μ_{max}) which was also higher ($P > 0.05$) by 13% in SRB from mice with UC. The specific maximal growth rate of the SRB from both groups was achieved at 12–12.5 h (Fig. 1B). These microorganisms also had a slightly higher generation time (14.29 h) and exponential growth phase (22.24 h) compared to the cultures from healthy mice (Table 1). It should be noted that the time of the lag-phase was × 2 shorter ($P > 0.01$) in the cultures of SRB from the faeces of mice with UC. That may indicate adaptive capacities of the SRB and their participation in the development of UC. The intestinal SRB from the faeces of healthy mice and mice with UC achieved the exponential phase after an adaptation period (3.49 and 1.76 h, respectively) in the lag phase. The stationary phase was achieved after 20.51 and 22.24 h, respectively (Table 1).

Sulphate-reducing bacteria can be found not only in the faeces or in the intestinal lumen but they can also be in interaction with other intestinal microorganisms in the biofilms. These biofilms are often formed by the SRB with species of Clostridium, Bacteroides or Escherichia genera. However, the features of the formation of these biofilms and the interaction of SRB with other bacterial genera are still unexplored.

Table 1. Kinetic properties of SRB cultures isolated from mice.

Suspension of SRB from		t_e (h)	Lag-phase (h)	G (h)	Absolute rate (number of cells/hour)	Specific rate (number of cells/hour)	
						Average	μ_{max}
Faeces	healthy	20.51	3.49	13.13	0.076 ± 0.023	0.052 ± 0.016	0.063 ± 0.011
	UC	22.24	1.76	14.29	0.069 ± 0.007	0.048 ± 0.005	0.071 ± 0.002
Surface (biofilm)	healthy	19.74	4.26	12.72	0.079 ± 0.009	0.054 ± 0.062	0.081 ± 0.014
	UC	21.21	2.79	15.48	0.065 ± 0.023	0.045 ± 0.014	0.060 ± 0.005

Comment: t_e is duration of the exponential growth phase (hours), G is generation time (hours), μ_{max} is maximum growth rate, SRB: sulphate-reducing bacteria, UC: mice with ulcerative colitis

Cultures of SRB from the intestinal surface of the biofilm of healthy mice and of mice with UC were accumulated in the medium and the kinetic properties of their growth were studied (Fig. 1C). The growth of SRB cultures from the bowel surface of both groups of mice was almost the same until 4.5 h of cultivation. The level of growth of SRB from the biofilm of mice with UC was increased ($P > 0.05$) within the time interval of 4.5–12.5 h and after that they were released from the biofilm of healthy mice. The specific maximal growth rate was higher ($P > 0.05$) by 26% in SRB from healthy mice compared to mice with UC (Table 1). However, maximal growth rate of SRB from the biofilm of healthy mice was achieved earlier at 1.5 h, whereas μ_{max} of SRB from the biofilm of mice with UC was achieved later at 9.5 h of cultivation (Fig. 1D). These results correlated with specific means and absolute growth rates which were also higher ($P > 0.05$) by 17–18% in SRB from healthy mice. The generation time (12.72 h) and

exponential growth phase (19.74 h) of these microorganisms were shorter ($P > 0.05$) compared to the cultures of SRB from mice with UC. It should be noted that the time of the lag-phase in SRB from surface was × 1.5 shorter ($P > 0.05$) in the cultures of SRB from mice with UC. It correlated with the lag-phase in the SRB isolated from the bowel lumen of mice with UC. The stationary phase of both SRB cultures was achieved after 20 h of cultivation.

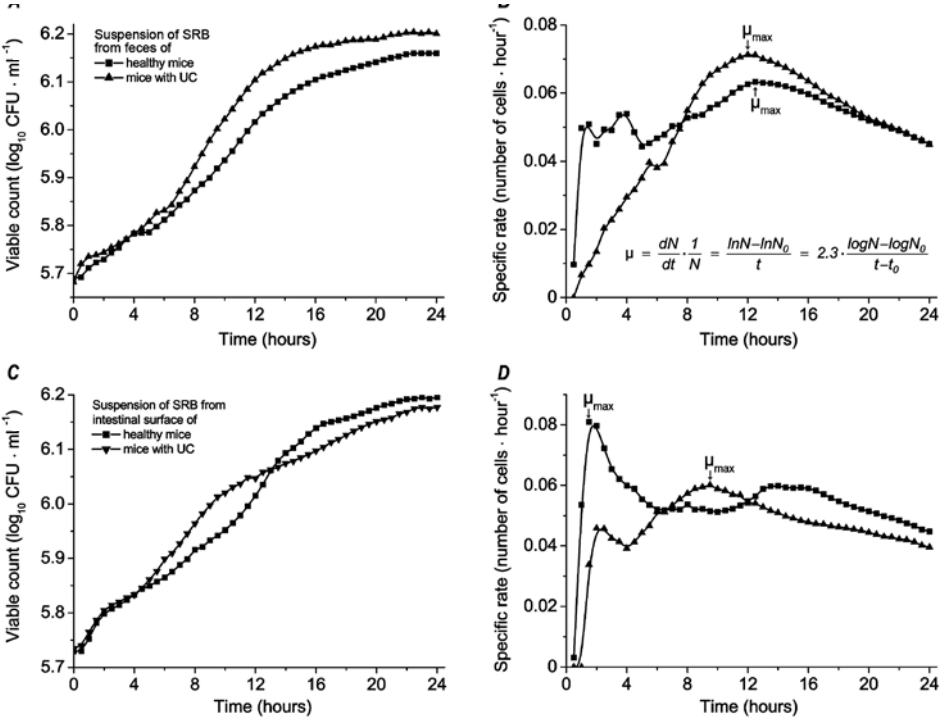

Fig. 1. Physiological indices of sulphate-reducing bacteria isolated from mice: bacterial growth (*A*, *C*) and specific growth rate (*B*, *D*), the arrows indicate the maximum rate of the growth and CFU is colony-forming units (M ± m, n = 5)

Discussion

Inflammatory bowel disease including ulcerative colitis are complex multifactorial diseases of unknown aetiology (Cummings et al. 2003). However, the aetiological role of the sulphate-reducing bacteria Desulfovibrio genus in the development of these inflammatory diseases was demonstrated in our previous research (Kushkevych 2014a). Ulcerative colitis was experimentally induced in the animals by application of sulphate-reducing bacteria. The initiation of the animals' own potential intestinal microflora of sulphate-reducing bacteria was observed in those animals which obtained a dose of sulphate containing medium. The changes in the colonic microbiota were observed in the animals which received a dose of SRB suspension. The bacteria belonging to the normal colonic microbiota were associated with the aetiology of inflammatory bowel disease and ulcerative colitis. The concentration of sulphide and acetate in faeces from different sections of the large intestine was determined. The level of ulcerations in the second and third group of sick animals under the specific conditions was demonstrated (Kushkevych 2014a).

The increasing number of viable intestinal SRB was also described previously in several researches published by Gibson et al. (1991, 1993), Kushkevych (2015a), Cummings et al. (2003), and Pitcher et al. (1996). The increasing specific maximal growth rate (μ_{max}) of SRB from mice with UC can lead to intensive sulphate reduction (Kushkevych 2014c). The increased concentration of sulphate in the intestine can lead to an increase in the specific maximal growth rate of the SRB and the production of hydrogen sulphide and acetate in high concentrations.

The reduction of sulphate ions to hydrogen sulphide occurs as a result of the formation of many intermediate compounds (Postgate 1984). The sulphate reduction enzymes are located in the cytoplasm and peripheral plasma. The initial stages of sulphate reduction include the uptake of sulphate ions in the bacterial cells. The sulphate ions can be transported into the cells simultaneously with protons and some sulphate-reducing bacteria can absorb sulphate from the flow of sodium ions (Barton and Hamilton 2007). It is known that the centre of adenosine triphosphate (ATP) hydrolysis is located on the cytoplasmic surface of the membrane. In another one of our studies, the ATPase activity in cell-free extracts of the sulphate-reducing bacteria isolated from the human large intestine was demonstrated. The maximum ATPase activity for SRB strains at +35 °C and at pH 7.0 was described (Kushkevych et al. 2015).

The described research is novel and important to predict the SRB number in the gut and their speed of dissimilatory sulphate reduction. The kinetic characteristic of their growth is important for further clarification of the mechanisms of sulphate reduction and accumulation of hydrogen sulphide, which is toxic for epithelial cells of the intestine and can cause bowel disease both in humans and animals, in particular ulcerative colitis. These results could be particularly useful for the study of IBD and its therapeutic strategy. These data are also indispensable for application into mechanistic details that will facilitate better preclinical drug/therapy design to target specific components involved in the disease pathogenesis.

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ATTACHMENT 7

Pages 86 – 95

Production of biogas: relationship between methanogenic and sulfate-reducing microorganisms

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Production of biogas: relationship between methanogenic and sulfate-reducing microorganisms

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Abstract: The production of high-quality methane depends on many factors, including temperature, pH, substrate, composition and relationship of the microorganisms. The qualitative and quantitative composition of methanogenic and sulfate-reducing microorganisms and their relationship in the experimental bioreactors has never been studied. The aim of this research was to characterize, for the first time, the diversity of the methanogenic microorganisms and sulfate-reducing bacteria, and study their relationship and biogas production in experimental bioreactors. Amplification of 16S rRNA gene fragments was carried out. Purified amplicons were paired-end sequenced on an Illumina Mi-Seq platform. The dominant morphotypes of these microorganisms in the bioreactor were homologous (99%) by the sequences of 16S rRNA gene to the *Methanosarcina*, *Thermogymnomonas*, *Methanoculleus* genera and *Archaeon* deposited in GenBank. Three dominant genera of sulfate-reducing bacteria, *Desulfomicrobium*, *Desulfobulbus* and *Desulfovibrio*, were detected in the bioreactor. The phylogenetic trees showing their genetic relationship were constructed. The diversity and number of the genera, production of methane, hydrogen sulfide and hydrogen in the bioreactor was investigated. This research is important for understanding the relationship between methanogenic microbial populations and other bacterial physiological groups, their substrate competition and, in turn, can be helpful for controlling methanogenesis in bioreactors.

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Keywords: biogas, methane production, methanogenic microorganisms, sulfate-reducing bacteria

1 Introduction

Bioenergy production from agricultural, municipal, and industrial waste is efficiently accomplished through anaerobic digestion to biogas. Biogas produced usually contains 55 – 70% of methane (CH_4) and 30 – 45% of carbon dioxide (CO_2) and it is stored in gas holders and subsequently used as a potential source of energy [1-4].

Anaerobic fermentation is a natural process in which microorganisms convert biodegradable substrate into biogas. It occurs in marshes, wetlands, river sediments, and also in the digestive tract of ruminants [5,6]. The microorganisms are also active in landfills where they are responsible for biodegradable waste decomposition [2,5,7]. Biogas is the final product of anaerobic metabolism. The process occurs in an anaerobic environment through the consecutive biochemical breakdown of polymers to methane and carbon dioxide [8, 9]. This is a result of the metabolism of different microorganisms which include fermentative microbes (acidogens); hydrogen-producing, acetate-forming microbes (acetogens); and methane-producing microbes (methanogens) [10-13].

Recently, the interest in anaerobic fermentation is mainly focused on its use in the economic recovery of biogas from industrial and agricultural surpluses [14-16].

On the basis of homologous sequence analysis of 16S rRNAs, methanogens have been classified into one of the three primary kingdoms of living organisms: the *Archaea* [10, 14, 17]. Recombinant DNA technology is one of the most powerful techniques for characterizing the biochemical and genetic regulation of methanogenesis [18]. This necessitates the selection of genetic markers, an efficient genetic transformation system, and a vector system for genetic recombination as prerequisites [19]. Anaerobic methane generation systems are known as methane bioreactors. The production of methane and growth of methanogenic microorganisms in the bioreactors depend

on many factors, including temperature, pH, substrate quality, composition of specific groups of microorganisms and their accumulation of the toxic metabolic products. One of such final products of sulfate-reducing bacteria metabolism is hydrogen sulfide produced in the process of dissimilatory sulfate reduction [6, 7, 20-22].

It is known that hydrogen sulfide is toxic for living organisms and can inhibit enzymes of different groups of microorganisms [6, 20]. The sulfate-reducing bacteria can compete with the methanogens for substrate components, in this case for molecular hydrogen, and produce hydrogen sulfide in high concentration in bioreactors. It, in turn, can inhibit the growth of methanogenic microorganisms and their process of methanogenesis.

Recent progress in the molecular biology of methanogens is reviewed, new digesters are described and improvements in the operation of various types of bioreactors are also discussed. However, the prevalence of the methanogenic populations and sulfate-reducing bacteria in methane bioreactors, their diversity and relationship has never been studied properly.

The aim of this research was to characterize the diversity of the methanogenic microorganisms and sulfate-reducing bacteria using amplification of gene fragments and Illumina sequencing, and to study their relationship and production biogas in the experimental bioreactors.

2 Materials and Methods

2.1 Batch anaerobic fermentation tests

A total of six laboratory batch fermenters of volume 120 liters each were used. Fermenters were equipped with a low speed agitator (operated at 60 rpm, 1 min agitation time, 60 min rest), temperature control, monitoring of pH, and sampling valve. On the first day of experiments, all fermenters were filled up with inoculum substrate and maize silage, in ratio 35:65% (v/v). Inoculum and maize silage was collected at a full scale biogas plant in Čejč, Czech Republic. This biogas plant processes as feedstock maize silage and liquid pig manure and it is operated in the mesophilic temperature regime (39°C). Inoculum characteristics were pH=7.3±0.1; DM 3.84%±0.3%; ODM 72.71%±0.4%. For maize silage, dry matter (DM) was 39.15%±0.5 and organic dry matter (ODM) was 99.21%±0.6%. Fermenters were set to 39°C ± 0.1°C. The hydraulic retention time was 26 days. During the fermentation, the quality of biogas was determined daily using X-am® 7000 gas analyzer (Drägerwerk AG & Co. KGaA Germany). Concentration of CH_4, CO_2, H_2S and H_2 in the biogas produced was determined. Quantity of biogas produced was measured by the gasometer PREMGAS BK G4 (Elster, Germany) and converted to standard conditions (T_0 = 273 K, p_0 = 101325 Pa).

2.2 Determination of the physical and chemical characteristics

The physical and chemical characteristics of fermenter content at each bioreactor, including temperature, pH, redox potential, dry matter content, organic dry matter content, biogas composition, sulfate content and acetate content were determined. DM content was determined by oven drying at 105±5°C followed by cooling in a desiccator and weighing until a constant weight. EcoCELL 111 (BMT Medical Technology Ltd., Czech Republic) was used in accordance with the Czech Standard Method (CSN EN 14346 2007) [23]. ODM content was determined by incineration of the samples in a muffle furnace at 550°C ± 5°C in accordance with the Czech Standard Method (CSN EN 15169 2007) [24], using a muffle furnace LMH 11/12 (LAC, Ltd., Czech Republic). The pH, and redox potential were determined by using pH/Cond meter 3320 (WTW GmbH, Germany) in accordance with the standard (CSN EN 12176 1999) [25]. Temperature of samples was determined by using the high accuracy PT100 RTD thermometer HH804U (OMEGA Engineering, INC., USA). Acetate content was determined using capillary electrophoresis Ionosep 2005 (RECMAN, Czech Republic). For determination of sulfate concentration, the spectrophotometer DR 3800 (Hach Lange GmbH, Germany) was used.

2.3 Isolation of DNA from collected samples

For DNA isolation samples from all 6 reactors were taken. These samples were combined and then examined. The QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH, Germany) is designed for rapid purification of total DNA from stool samples and was used for DNA extraction from biogas digester samples. DNA extraction was performed in accordance to the manufacturers' instructions, with minor adjustments as described below. Briefly, 100 mg of sample was washed with 1.4 mL of ASL buffer (QIAGEN GmbH, Germany), heated at 95°C for 10 minutes. After centrifugation, an InhibitEX tablet was put in the supernatant to remove impurities and PCR inhibitors. After thorough centrifugation, 200 µL of the supernatant was added to 15 µL of proteinase K, and 200 µL of buffer

AL (QIAGEN GmbH, Germany) was added. The mixture was heated to 70°C for 10 minutes and 200 µL of absolute ethanol was added. The sample mixture was then passed through the QIAamp kit column, followed by two washes with buffers AW1 and AW2 (QIAGEN GmbH, Germany). The DNA was eluted in a volume of 200 µL of elution buffer.

2.4 Amplification of gene fragments and Illumina sequencing

Amplification of 16S rRNA gene fragments was carried out using universal primers for amplification of the V3 and V4 variable regions of the 16S rRNA [26]. The primers were flanked by molecular barcodes for sample identification. The PCR reaction was prepared using Maxima™ Probe qPCR Master Mix (Thermo Fisher Scientific, USA) with cycling conditions as follows: 95°C for 10 min. followed by 30 cycles of incubation at 94°C for 30 s, 60°C for 30 s and 72°C for 120 s, and a final extension step at 72°C for 2 min. PCR products were visualized using electrophoresis on 1.5% agarose gels and purified from the gel using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). DNA was quantified using the Quant-iTPicoGreen dsDNA Assay (Thermo Fisher Scientific, USA) and equimolar amounts of the PCR products were pooled together.

Purified amplicons were paired-end sequenced on an Illumina Mi-Seq platform. QIIME data analysis package was used for 16S rRNA data analysis [27]. Quality filtering on raw sequences was performed in accordance to base quality score distributions, average base content per read and GC distribution in the reads. Chimeras and reads that did not cluster with other sequences were removed. The obtained sequences with qual scores higher than 20 were shortened to the same length of 350 bp and classified with RDP Seqmatch with an operational taxonomic unit (OTU) discrimination level set to 97%. The relative abundance of the taxonomic groups was calculated to the microorganisms detected in this study.

Sequences were compared using the BLAST feature of the National Center for Biotechnology Information (NCBI) [28]. The sequences were uploaded to Mega7 for comparative genomic analyses [29]. Alignments of sequences were performed in Mega7 using Clustal W and clustering was performed by the neighbor-joining method [30].

The research results were analyzed using software packages Statistica12 (www.statistica.software.informer.com) and Origin7.0 (www.originlab.com). The basic statistic parameters (arithmetic average (M) and standard error (m) of the arithmetic average, M ± m) were calculated using the experimental data. For estimation of the validity of difference between the statistical characteristics of the data, Student's index was calculated. The difference was valid when $P<0.05$ [31, 32].

3 Results

The physical and chemical characteristics of samples taken on the 14th day from the experimental bioreactors, operated at temperature 39°C±0.5, were pH (7.2±0.1), redox (-5.6±0.2), total solids (3.76%±0.3), volatile solids (70.85%±0.9). Biogas composition during the tests is shown in figure 1. Concentration of acetic acid ranged from 460 to 490 mg/L and concentration of sulfate ranged from 305 to 333 mg/L, while the sulfate concentration in fermenters on the first day of experiment ranged from 380 to 450 mg/L. The results of our research show that methane was intensively produced for the first 12 days, after this time, the methane production achieved plateau and was almost unchanged (Fig. 1). This can be explained by the fact that the methanogenic microorganisms can achieve the stationary growth phase after 12 days. As growth slowed, methane production (concentration) achieved a stable level (46.2–49.3%$_{vol}$) during the 15–26 day period. An interesting pattern was observed in the production of hydrogen and hydrogen sulfide. The level of hydrogen rapidly grew the first 5 days and after that it was reduced. This can be explained by the fact that both hydrogenotrophic methanogens and sulfate-reducing bacteria consume this simple molecule. This is also evident when isolated microorganisms are examined and compared. Both acetotrophic and hydrogenotrophic metanogens were isolated. Sulfate-reducing bacteria can use hydrogen as an electron donor in the process of

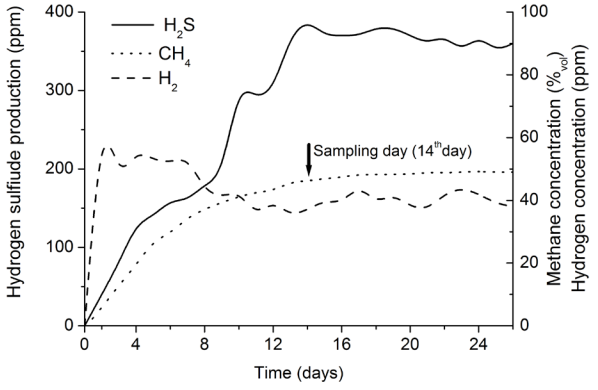

Fig. 1: Methane, hydrogen and hydrogen sulfide production in the biogas generated during fermentation test

dissimilatory sulfate reduction. The final product of this process is hydrogen sulfide. The highest concentration of hydrogen sulfide was achieved on the 14th day, which can confirm the high number of sulfate-reducing bacteria (1050 OUT·mL^{-1}) in the bioreactor. Different studies have found both unionized and total hydrogen sulfide concentrations important in inhibition of the SRB bacteria and methanogens. However, our results do not confirm the inhibitory effect of H_2S at maximal concentration 390 ppm, as evident by the biogas production trend. The distribution of main genera in the bioreactor was investigated using amplification of 16S rRNA gene and Illumina sequencing. To clarify the genetic relationship of the methanogenic and sulfate-reducing populations of microorganisms in the bioreactor, sample of 16S rRNA gene sequences were compared with sequences of different strains from GenBank. The genomic sequences of the microorganisms are available in GenBank under accession no. KY172649, KY172662, KY172650, KY194790, KY172816, KY172822, KY172821, KY172646, KY172643, KY172641, KY172640, KY172645, KY172824, KY172823, KY126837. The obtained sequences were compared with reference strains using nucleotide Blast:Search. The sequences of the 16S rRNA gene of the methanogens were homologous (98–99%) to genera of *Methanosarcina*, *Thermogymnomonas*, *Methanoculleus*, and *Archaeon* (Table 1). The sequences of sulfate-reducing bacteria were homologous (98–99%) to the genera of *Desulfomicrobium*, *Desulfobulbus* and *Desulfovibrio* (Table 2). It should be noted that most described sequences of the methanogenic strains and sulfate-reducing bacteria in GenBank are identified only to the domain, kingdom, family or genus and, in some cases, to species. Most of them are uncultured (www.ncbi.nlm.nih.gov/genbank).

Based on all of the 16S rRNA gene sequences of methanogens and sulfate-reducing bacteria from the bioreactor, a phylogenetic tree demonstrating their genetic relationship was built (Fig. 2, 3). The detected genera of methanogens were homologous with *Methanosarcina mazei* strain GS14-2aM, uncultured

Table 1: The results of sequence analysis of the 16S rRNA gene of methanogenic microorganisms

Detected sequences and their accession number in GenBank (length of the gene fragment)	Reference strains in GenBank	Accession number	Identity (%)
Sequence 1 KY172649 (424 bp)	*Methanosarcina mazei* strain GS14-2aM 16S rRNA gene, partial sequence	KX826992.1	99
	Methanosarcina sp. 1H1 gene for 16S rRNA, partial sequence	LC170394.1	99
	Uncultured *Methanosarcina* sp. clone T6190SA18-18 16S rRNA gene, partial sequence	KU355742.1	99
Sequence 2 KY172662 (422 bp)	Uncultured archaeon clone g10-56 16S rRNA gene, partial sequence	JX576125.1	98
	Uncultured *Thermoplasmata* archaeon clone g8-4 16S rRNA gene, partial sequence	JX576112.1	98
	Uncultured *Methanomassiliicoccus* sp. clone LZNG25 16S rRNA gene, partial sequence	JX456453.1	98
Sequence 3 KY172650 (422 bp)	Uncultured *Thermogymnomonas* sp. partial 16S rRNA gene, isolate OTU_11	LT624815.1	99
	Uncultured *Thermogymnomonas* sp. partial 16S rRNA gene, isolate OTU_4	LT624808.1	99
	Uncultured archaeon partial 16S rRNA gene, clone AKA055	LN874207.1	99
Sequence 4 KY194790 (423 bp)	Uncultured *Thermoplasmatales* archaeon partial 16S rRNA gene, isolate OTU_9	LT624813.1	99
	Uncultured *Thermoplasmatales* archaeon partial 16S rRNA gene, isolate OTU_6	LT624810.1	99
	Uncultured archaeon clone WWA-D10 16S rRNA gene, partial sequence	KM870439.1	99
Sequence 5 KY172816 (420 bp)	*Methanoculleus* sp. strain Biowerk_c-HAW 16S rRNA gene, partial sequence	KX619406.1	99
	Methanoculleus bourgensis isolate BA1 genome assembly, chromosome: I	LT549891.1	99
	Methanoculleus sp. MAB1 isolate MAB1 genome assembly, chromosome: chrI	LT158599.1	99

Table 2: The results of sequence analysis of the 16S rRNA gene of sulfate-reducing bacteria

Detected sequences and their accession number in GenBank (length of the gene fragment)	Reference strains in GenBank	Accession number	Identity (%)
Sequence 1 KY172822 (465 bp)	*Desulfovibrio desulfuricans* strain E4 16S rRNA, partial sequence	KJ459863.1	99
	Desulfovibrio desulfuricans strain E2 16S rRNA, partial sequence	KJ459861.1	99
	Uncultured bacterium partial 16S rRNA gene, clone 48h8	HG531899.1	99
Sequence 2 KY172821 (465 bp)	Uncultured *Desulfovibrio* sp. clone MFC-2-L19 16S rRNA gene, partial sequence	JX944554.1	99
	Uncultured bacterium clone MFC4P_127 16S rRNA gene, partial sequence	JF309175.1	99
	Desulfovibrio simplex strain JCM 16812 16S rRNA gene, partial sequence	NR_113296.1	99
Sequence 3 KY172646 (466 bp)	*Desulfobulbus* propionicus strain DSM 2032 16S rRNA gene, complete sequence	NR_074930.1	99
	Desulfobulbus propionicus DSM 2032, complete genome	CP002364.1	99
	Uncultured bacterium clone B01 16S rRNA gene, partial sequence	EU136253.1	99
Sequence 4 KY172643 (466 bp)	*Desulfobulbus* sp. canine oral taxon 078 clone OC011 16S rRNA gene, partial sequence	JN713241.1	96
	Uncultured *Desulfobulbus* sp. partial 16S rRNA gene, isolate OTU 265	LT625152.1	96
	Uncultured *Desulfobulbus* sp. partial 16S rRNA gene, isolate OTU 199	LT625082.1	96
Sequence 5 KY172641 (466 bp)	Uncultured *Desulfomicrobium* sp. gene for 16S rRNA, partial sequence, clone:3CP(-)_3	AB908615.1	96
	Uncultured *Desulfomicrobium* sp. gene for 16S rRNA, partial sequence, clone:3CP(-)_2	AB908614.1	96
	Uncultured *Desulfomicrobium* sp. gene for 16S rRNA, partial sequence, clone: 3CP(+)_10	AB908535.1	96
Sequence 6 KY172640 (466 bp)	*Desulfobulbus* sp. Prop6 16S rRNA gene, partial sequence	KU845305.1	99
	Uncultured bacterium clone 02d01 16S rRNA gene, partial sequence	GQ138500.1	99
	Uncultured bacterium clone 04c04 16S rRNA gene, partial sequence	GQ134634.1	99
Sequence 7 KY172645 (466 bp)	Uncultured *Desulfobulbus* sp. partial 16S rRNA gene, isolate OTU 265	LT625152.1	94
	Uncultured *Desulfobulbus* sp. partial 16S rRNA gene, isolate OTU 199	LT625082.1	94
	Desulfobulbus sp. oral taxon 041 clone WWP_SS1_G06 16S rRNA gene, partial sequence	GU398208.1	94
Sequence 8 KY172824 (466 bp)	Uncultured sulfate-reducing bacterium clone 2R1V11 16S rRNA gene, partial sequence	EF592786.1	93
	Uncultured delta proteobacterium clone 2R1U31 16S rRNA gene, partial sequence	EU104788.1	93
	Uncultured sulfate-reducing bacterium clone 2R1V05 16S rRNA gene, partial sequence	EF592783.1	92
Sequence 9 KY172823 (443 bp)	Uncultured *Desulfovibrionales* bacterium clone Flu2 26 16S rRNA gene, partial sequence	JQ701289.1	84
	Desulfovibrio sp. S4 gene for 16S rRNA, partial sequence	LC186051.1	83
	Uncultured *Desulfovibrio* sp. gene for 16S rRNA, partial sequence, clone: LR333B-40	LC001349.1	83
Sequence 10 KY126837 (465 bp)	Uncultured *Desulfovibrio* sp. clone MFC-2-L19 16S rRNA gene, partial sequence	JX944554.1	95
	Desulfovibrio simplex strain JCM 16812 16S rRNA gene, partial sequence	NR_113296.1	95
	Desulfovibrio intestinalis partial 16S rRNA gene, strain JG-G12	AJ295680.1	95

archaeon clone g10-56, uncultured *Thermogymnomonas* sp. isolate OTU_11, uncultured *Thermoplasmatales* archaeon isolate OTU_9, *Methanoculleus* sp. strain Biowerk_c-HAW (Fig. 2). Another phylogenetic tree was built for 16S rRNA gene sequences of sulfate-reducing bacteria which were homologous with *Desulfovibrio desulfuricans* strain E4 16S, uncultured *Desulfovibrio* sp. clone MFC-2-L19, *Desulfobulbus propionicus* strain DSM 2032, *Desulfobulbus* sp. clone OC011, uncultured *Desulfomicrobium* sp. clone: 3CP(-)_3, *Desulfobulbus* sp. Prop6, uncultured *Desulfobulbus* sp. isolate OTU 265, uncultured sulfate-reducing bacterium clone 2R1V11, uncultured *Desulfovibrionales* bacterium clone Flu2_26 (Fig. 3).

Percentage ratio of methanogenic and sulfate-reducing microorganisms was calculated by OUT·mL^{-1}

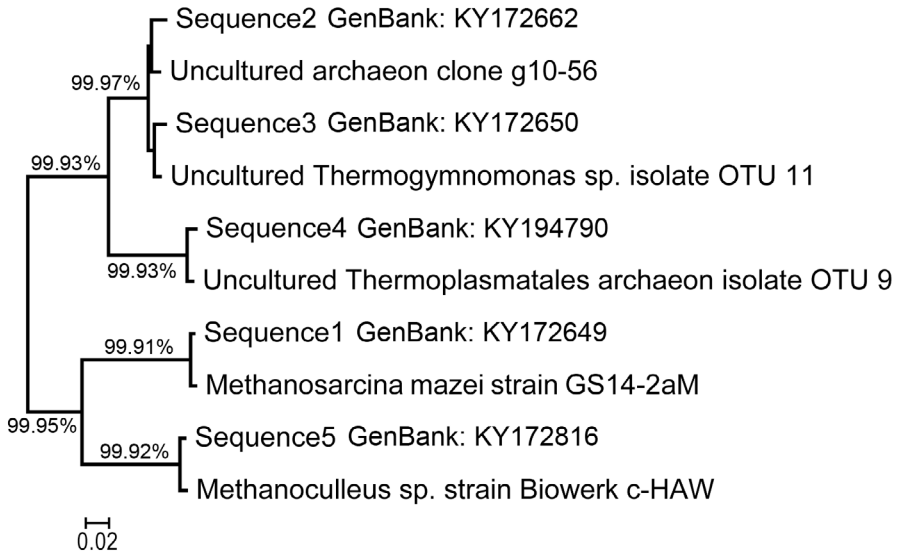

Fig. 2: Phylogenetic tree of relationship sequences of 16S rRNA gene of the methanogenic populations in methane bioreactor

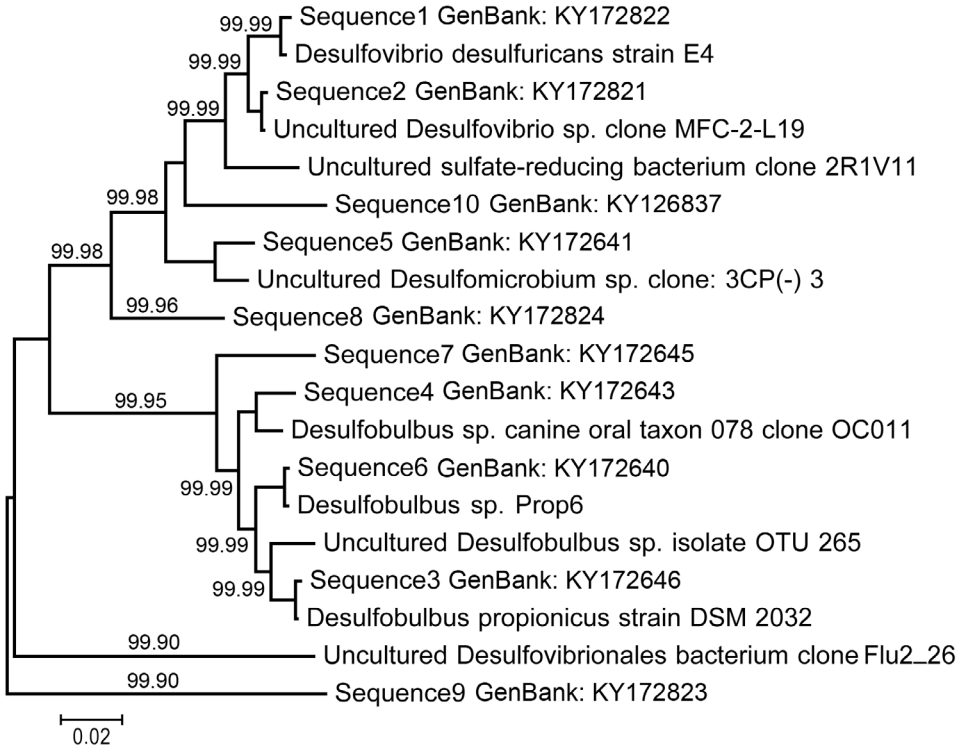

Fig. 3: Phylogenetic tree of relationship sequences of 16S rRNA gene of the sulfate-reducing bacteria in methane bioreactor

determined from Illumina sequencing (Fig. 4). Results indicate that three genera of both physiological groups of microorganisms were detected in the experimental bioreactors. The dominant genus of methanogens was *Methanosarcina*, which was 62% of all detected methanogens. Two other genera, *Thermogymnomonas* and *Methanoculleus*, were 16 and 2%, respectively, as well as other Archaeons which were not identified to the genus (20%). For sulfate-reducing bacteria, three genera were identified: *Desulfomicrobium*, *Desulfobulbus* and *Desulfovibrio* in percentage ratios of 48, 39.8, and 12%, respectively. Other sulfate-reducing bacteria (0.19%) were not identified to the genus.

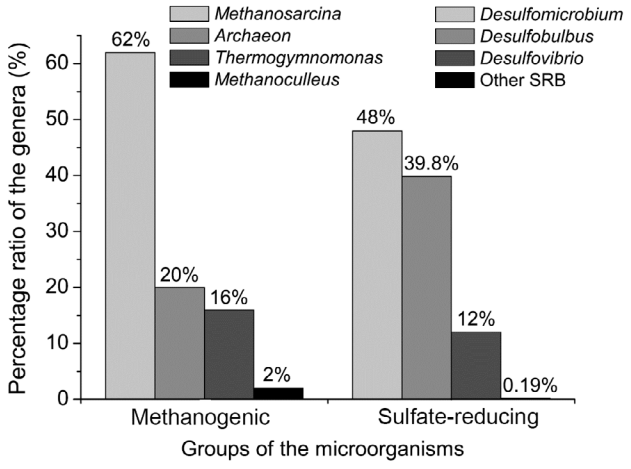

Fig. 4: Qualitative and percentage composition of methanogenic and sulfate-reducing microorganisms

4 Discussion

Methane is the final product of anaerobic metabolism carried out by communities of hydrolytic bacteria and fungi, acid-producing intermediary organisms, and finally, methanogenic microorganisms [17]. Methane-producing communities are very stable and resilient, but they are also complex and largely undefined. The results of our studies are consistent to other research described in literature [5, 14, 33, 34]. Production of methane depends on many factors, including physical (temperature) and chemical (pH), type of substrate, its concentration and accessibility for microorganisms, composition and the ratio of the microorganisms and their metabolic compounds in the bioreactors. Therefore, bioreactors are a complex fermentative system which includes various oxidations and reduction processes with changes in redox potential. Our research demonstrates that the intensive production of methane in the experimental bioreactor lasts the first 12 days and correlates with the accumulation of hydrogen sulfide. The highest concentration of hydrogen sulfide (390 ppm) was detected on the 14th day and it correlates with the titer of sulfate-reducing bacteria (1050 OUT·mL^{-1}). The sulfate-reducing bacteria (SRB) are a heterogeneous group of microorganisms which use sulfate as an electron acceptor in the process of dissimilatory sulfate reduction [35, 36]. The final product of this process is hydrogen sulfide [6, 20, 22, 37]. For sulfate reduction, SRB need exogenous electron donors, such as: organic compounds (e.g., lactate, propionate, butyrate, ethanol, etc.) and molecular hydrogen. Organic compounds for SRB can be simultaneously electron donors and carbon sources and oxidized completely (to CO_2) or incompletely (to acetate and CO_2) [6, 7]. SRB, oxidizing organic compounds incompletely, belong to the group called "Acetogenic sulfate-reducing bacteria" [7]. Detected SRB, *Desulfovibrio* (48%), *Desulfomicrobium* (39.8%), and *Desulfobulbus* (12%) genera, are acetogenic microorganisms which oxidize organic compounds, incompletely, to acetate and CO_2. Produced acetate is consumed by methanogens e.g., species of the *Methanosarcina* genus, which are dominant microorganisms (62%) in the bioreactor (see Fig. 4). Our results demonstrated the presence of acetotrophic and hydrogenotrophic methanogenic archaea. The species of the *Methanosarcina* genus can form multicellular colonies and are anaerobic methanogens. They are widespread in the rumen of cows, sheep, goats, deer, and the large intestine of humans [7]. Recently, there has been a study on *M. barkeri*, because this species has the enzyme methylamine methyltransferase, which catabolizes methylamines leading to methane production. *Methanosarcina* sp. possess all three known pathways for methanogenesis, and can utilize a broad spectrum of substrates, including hydrogen. All the other methanogens can utilize no more than two methanogenic substrates and possess a single pathway for methanogenesis [38]. It also has a number of distinct morphological forms, including single cells with and without a cell envelope, as well as multicellular packets and lamina [39].

The other dominant genus of microbial methanogens in the studied bioreactor was *Thermogymnomonas* (16%). The species of this genus were also isolated as a novel thermoacidophilic, cell wall-less archaeon from a solfataric field in Ohwaku-dani, Hakone, Japan. The cells were irregular cocci, sometimes lobed, club-shaped or catenated, and were highly variable in size, ranging from 0.8 to 8.0 μm in diameter [33]. Itoh et al. (2007) identified this strain as *Thermogymnomonas acidicola*. The strain grew at temperatures in the range 38–68°C (optimally at 60°C) and at pH 1.8–4.0 (optimally at around

pH 3.0). Strain IC-189T was an obligate anaerobic and heterotrophic microorganism, requiring yeast extract for growth. Yeast extract, glucose and mannose served as carbon and energy sources. Therefore, strain IC-189T represents a novel genus (order *Thermoplasmatales*) and species [33].

The *Methanoculleus* genus was found in the lowest number among all detected methanogens. The species of the genus were frequently described as playing an important role in different biogas reactor systems [34, 40, 41]. *Methanoculleus bourgensis* was always detected as the dominant in biogas systems. The prevalence of *M. bourgensis* in reactors performing syntrophic acetate oxidation under high ammonium concentrations [42, 43, 44], indicates the importance of this methanogen in corresponding communities. Isolation and/or cultivation of *M. bourgensis*, together with acetate-oxidizing bacteria [45] such as *Clostridium ultunense* [46], led to the assumption that syntrophic association may play an important role for members of the *Methanoculleus* genus [42, 47]. The 16S rRNA gene sequence analysis classified the isolate as a member of the species *M. bourgensis* with 99% sequence identity to the 16S rRNA gene of strain MS2T [48, 49]. Genomic DNA of strain BA1 was isolated and sequenced, applying the paired-end protocol on an Illumina MiSeq system [34, 48]. In our studies, using Illumina sequencing, the sequences belonging to this genus were the most often detected in all bioreactors.

The detected genera of methanogens and SRB described can compete by molecular hydrogen (Fig. 5). Hydrogen and CO_2 can be used by the methanogens for their growth and methane production and, simultaneously, SRB can also use H_2 as a simple electron donor. Therefore, competition for molecular hydrogen may occur between methanogens and SRB. Accordingly, decrease of H_2 was observed after the 6th day. Perhaps, the highest consumption of hydrogen by both microorganism groups occurred during this period because afterwards hydrogen levels stabilized. However, SRB produce hydrogen sulfide which can be toxic for methanogens, and may inhibit methanogenesis.

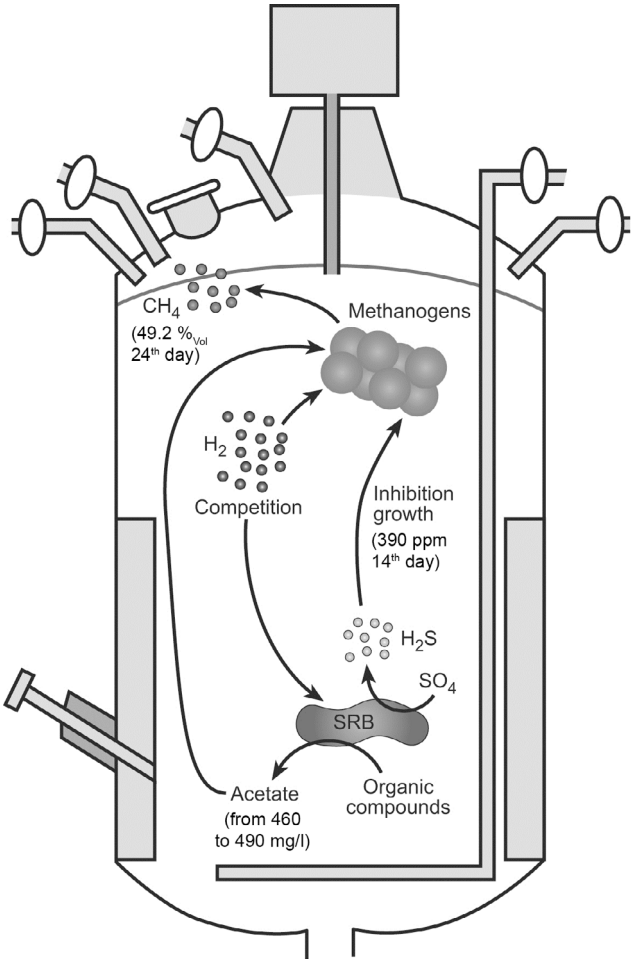

Fig. 5: The hypothetical scheme of the relationship between methanogenic and sulfate-reducing microorganisms

5 Conclusions

We conclude that studies of the diversity of methanogenic microorganisms under the influence of various factors in the bioreactors require further understanding of the process of biogas production. The sulfate-reducing bacteria can compete with the hydrogenotrophic methanogens for substrate components, in this case molecular hydrogen, and produce hydrogen sulfide in high concentrations in the bioreactors. This, in turn, can inhibit the growth of methanogenic microorganisms and their process of methanogenesis. It can cause unbalance of other microbial communities and instability of the fermentation process. However, this was not tested in our experiments at a maximum concentration of H_2S (390 ppm). The study of new isolates is still important for selecting the optimal conditions for methane production process.

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ATTACHMENT 8

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Effect of selected 8-hydroxyquinoline-2-carboxanilides on viability and sulfate metabolism of *Desulfovibrio piger*.

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Effect of selected 8-hydroxyquinoline-2-carboxanilides on viability and sulfate metabolism of *Desulfovibrio piger*

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ABSTRACT

An increased number of sulfate-reducing bacteria is often isolated from faeces of patients with gastrointestinal diseases, which can be the cause of the development of bowel inflammation. Frequent use of antibiotics causes the resistance of intestinal microorganisms and ineffective treatment of these diseases. The antimicrobial activity and biological properties of the selected ring-substituted 8-hydroxyquinoline-2-carboxanilides against *Desulfovibrio piger* Vib-7 were studied. The addition of these compounds in the cultivation medium inhibited the bacterial growth and the process of sulfate reduction dose-dependently. A significant cytotoxic activity under the influence of ring-substituted 8-hydroxyquinoline-2-carboxanilides was determined. The strongest cytotoxic effect of the derivatives was observed for compounds 8-hydroxy-*N*-(3-methoxyphenyl)quinoline-2-carboxamide and 8-hydroxy-*N*-(3-trifluoromethylphenyl)quinoline-2-carboxamide that caused a low survival of *D. piger* Vib-7 in concentration 17 μM and high toxicity rates.

Introduction

A high number of sulfate-reducing bacteria (SRB) and the intense process of dissimilatory sulfate reduction in the gut are thought to be significant risk factors of inflammatory bowel diseases in both humans and animals (Gibson et al., 1991, 1993; Kushkevych, 2016, Kushkevych et al., 2016). These bacteria are often found in patients with rheumatic diseases, ankylosing spondylitis, etc. (Barton and Hamilton, 2010). The species of *Desulfovibrio* genus can cause bloody diarrhea, weight loss, anorexia, epithelial hyperplasia, abscesses and inflammatory infiltrates in animals and humans (Loubinoux et al., 2000, 2002a,b). There is also an assumption that SRB can be responsible for some forms of cancer of the rectum through the formation of hydrogen sulfide that affects the metabolism of intestinal cells.

An increased number of SRB was found in faeces from people with ulcerative colitis in comparison with healthy individuals (Cummings et al., 2003). The injection of these bacteria in hamster intestine caused an infection clinically similar to human colitis (Cummings et al., 2003; Pitcher and Cummings, 1996). Using the model of laboratory rats, the etiological role of the cultures of SRB, *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, in the development of inflammatory processes in the intestine was examined. It was found that the introduction of pure cultures of SRB led to a high production of hydrogen sulfide and acetate in the intestine, and clinical manifestations similar to non-specific intestinal inflammation in humans appeared (Kushkevych, 2014).

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon that affects up to 12 per 100,000 people in Western countries, mostly between 15 and 30 years of age (Rowan et al., 2009). The treatment of mild to moderate UC includes, in the first instance, sulfasalazine and mainly 5-aminosalicylate containing drugs, the type and dosage of which depend on the location and severity of the disease. Other options of treatment include corticosteroids and immunosuppressants (for moderate to severe UC, with a high mortality) or probiotics (for improving the microbial balance) (Cummings et al., 2003; Kushkevych, 2016). Despite the bacterial nature of the disease, antibiotics have failed

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in the treatment of UC so far. However, new antibacterial compounds with high specific effect against SRB could yield better efficiency in the treatment of this disease.

Quinoline-based compounds have a wide range of promising biological properties (Cieslik et al., 2012; Jampilek, 2017; Jampilek et al., 2016, Kos et al., 2015; Mucaji et al., 2017; Musiol et al., 2006, 2007, 2008, 2010); therefore a special attention is paid to them at research and designing of new drugs (Cieslik et al., 2012; Jampilek et al., 2016; Kos et al., 2015; Musiol et al., 2006, 2007; Ranjith et al., 2017). This simple scaffold possesses unique physicochemical properties and provides a possibility of a great number of targeted modifications. Ring-substituted 8-hydroxyquinoline-2-carboxanilides were recently prepared (Kos et al., 2015) and published as compounds with noteworthy biological activities (Jampilek et al., 2016; Kos et al., 2015) based on the presence of an amide group and a hydroxy moiety in the quinoline scaffold (Gonec et al., 2012; Jampilek et al., 2016; Kos et al., 2015). Thus, in the context of the above-mentioned facts, the aim of this work was to evaluate viability of intestinal sulfate-reducing bacteria *Desulfovibrio piger* and parameters of their dissimilatory sulfate reduction (production of hydrogen sulfide and acetate as well as sulfate and lactate consumption) under effect of selected 8-hydroxyquinoline-2-carboxanilides (Kos et al., 2015).

Materials and methods

Tested compounds

The studied ring-substituted 8-hydroxyquinoline-2-carboxanilides were synthesized by means of microwave-assisted synthesis described recently. The compounds were isolated and fully characterized (melting point, elemental analysis, infrared as well as ^{1}H and ^{13}C NMR spectroscopy) (Kos et al., 2015). The compounds were kept in microtubes dissolved in dimethyl sulfoxide (DMSO) solution. The quantity of DMSO necessary to dissolve each compound was calculated previously to achieve the concentration of the component 30 mM. Afterwards it was diluted 4-fold in a proportion 1:3, and 5 different concentrations of the chemical compound – 5, 10, 15, 20, 25, 30 and 35 mM – were obtained. The maximum concentration of DMSO in the assays never exceeded 0.1%.

Bacterial culture and cultivation

The sulfate-reducing bacteria *D. piger* Vib-7 (GenBank: KT881309.1) were isolated from the healthy human large intestine as described previously (Kushkevych, 2013; Kushkevych et al., 2014). The strain has been kept in the collection of microorganisms at the Department of Experimental Biology, Faculty of Science at the Masaryk University (Brno, Czech Republic). The bacteria were grown for 36 h at 37 °C under anaerobic conditions in nutrition modified Postgate's liquid medium (Postgate, 1984). Before bacterial passage in the medium, 0.05 mM of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. The sterile 10 ml solution of NaOH (0.9 mM) 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 cooled to 30 °C. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Assay of bacterial cell concentration

The best concentration of *D. piger* Vib-7 was assessed to be 5×10^5 CFU/ml. Based on our previous work, the correlation between OD_{340} and the amount of cells in the solutions measured in the biophotometer was determined as $y = 1.0 \times 10^9 \chi - 6.0 \times 10^6$, where y means the bacterial concentration and χ means the OD_{340} measured as was described in our previous paper (Kushkevych et al., 2015a).

Treatment of bacterial culture

The bacterial culture of the stationary phase of growth was centrifuged for 3 min at a rotation speed of 3500 rpm. Supernatant was removed and replaced by a fresh liquid medium, where the bacterial precipitate was diluted. The bacterial suspension was mixed, and OD_{340} was measured. Numbers and viabilities of the bacterial cells were determined by counting with a haemocytometer after staining with erythrosine B [0.1% erythrosine B (w/v) in phosphate-buffered saline (PBS), pH 7.2–7.4]. Unstained cells were considered to be viable.

The bacterial suspension (initial concentration 0.5 mg/ml) was poured in microtubes (350 μl) that contained samples + solvent control (DMSO) + sample control + blanks; each sample as well as the controls were prepared in triplicate, so that the average of the results did not had a great discrepancy from the results independently (except in 3 blanks). The sample controls contained only bacterial suspension and medium (free of tested compounds); and for the 3 blanks only medium. Calculations were made to assess how much bacterial solution should be in each, based on the OD. The determination of biomass and concentrations of sulfate, lactate, acetate and sulfide in the culture medium under the treatment of 5, 10, 15, 20, 25, 30 and 35 μM compounds after 36 h was carried out. During experiments, bacteria were grown at 37 °C under anaerobic conditions.

Analysis of viability of D. piger *Vib-7 and cytotoxicity of compounds*

The bacterial suspension (5×10^4 cells/well in 300 μl culture medium) was filled in 100-well plates in triplicate in the Postgate's liquid medium (without Mohr's salt), treated with 5, 10, 15, 20, 25, 30 and 35 μM compounds and incubated at +37 °C. The relative survival of *D. piger* Vib-7 cells and the cytotoxicity of the compounds were determined at the 36th hour of cultivation using a WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The relative survival rate was calculated by the following equation: $(A_{Sample} - A_{Blank})/(A_{Control} - A_{Blank})$, and multiplied by 100 for the result in percentage. The relative toxicity rate was determined as described previously (Kos et al., 2015). All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Assay of sulfate, lactate, sulfide and acetate in cultivation medium

The sulfate ion concentration in the medium was determined by the turbidimetric method after it had been precipitated by barium chloride. To stabilize the suspension, glycerol was used (Kolmert et al., 2000). Lactate concentration was measured through the dehydrogenation reaction using Lactate Assay Kit (Sigma-Aldrich, Catalog Number MAK064). Sulfide concentration in the culture medium was assayed by the spectrophotometric method as was described (Cline, 1969). Accumulation of acetate ions in the process of bacterial growth in the medium was determined using Acetate Assay Kit (Colorimetric, Catalog Number KA3764).

Statistical analysis

The statistical calculations of the results were carried out using the software MS Office and Origin program. The research results were treated by methods of variation statistics using Student *t*-test.

Statistical significance was tested using the one-way analysis of variance with Dunnett's test and Tukey post-test for comparisons between the means, and differences between two conditions were retained for $P \leq 0.05$. Statistical significance was determined at levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ (Bailey, 1995).

Results

The structures of the selected and discussed ring-substituted 8-hydroxyquinoline-2-carboxanilides are shown in Table 1. A synthetic pathway and characterization were published by Kos et al. (2015).

The relative survival of *D. piger* Vib-7 cells and cytotoxicity of ring-substituted 8-hydroxyquinoline-2-carboxanilides were studied. In this series of experiments, compounds **1–7** exerted cytotoxicity against these bacteria already in low (5–35 μM) concentrations (Fig. 1).

Treatment with all tested compounds led to a significant dose-dependent activity with the strongest effect observed in compounds **3** (R = 3-OCH_3) and **6** (R = 3-CF_3). Since some of ring-substituted 8-hydroxyquinoline-2-carboxanilides (such as **3** or **6**) have a pronounced antimicrobial effect against *D. piger* Vib-7 (combination of low survival and high toxicity rates), they could be considered as promising agents against the growth of this type of bacteria.

The effect of different concentrations of ring-substituted 8-hydroxyquinoline-2-carboxanilides on the process of dissimilatory sulfate reduction in *D. piger* Vib-7 cells at the 36th h of cultivation was studied. As shown in Figs. 2 and 3, the addition of the compounds in the culture medium inhibits the process of dissimilation of sulfate directly proportional to the increase in concentrations (5–35 μM). Under these conditions, the utilization of sulfate and lactate was inhibited; hence the level of accumulation of hydrogen sulfide and acetate was reduced. These data are consistent with our research in previous series of the experiments. The percentage inhibition of sulfate reduction process correlates with the percentage of bacterial growth inhibition under the ring-substituted 8-hydroxyquinoline-2-carboxanilides treatment.

Based on the obtained results, the MIC and IC_{50} values of ring-substituted 8-hydroxyquinoline-2-carboxanilides against intestinal sulfate-reducing bacteria were established. As shown in Table 1, the MICs of all the compounds were ≤33 μM. The least potent compounds among the tested compounds for the bacterial strain were **2** (R = 2-OCH_3) and **5** (R = 3-Br), the MICs of which were 28 and 33 μM, respectively. The similar MICs were determined for compounds **1** (R = H) and **4** (R = 3-CH_3). It can be supposed that these compounds show the same cytotoxicity effect on sulfate-reducing bacteria *D. piger* Vib-7 cells. IC_{50} of compounds **4**, **6** and **7** was 10 μM. Compounds **2** and **5** had IC_{50} only 17 and 18 μM, which is consistent with the MICs of these compounds.

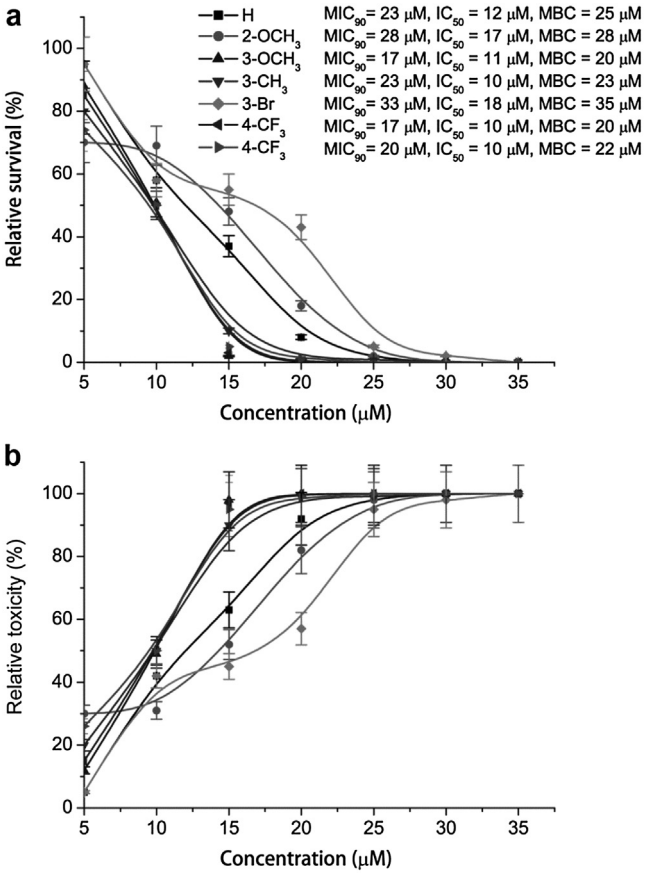
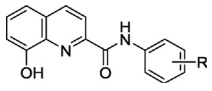


Fig. 1. Relative survival of *D. piger* Vib-7 cells (**a**) and toxicity (**b**) of ring-substituted 8-hydroxyquinoline-2-carboxanilides.

Table 1
Structures of ring-substituted 8-hydroxyquinoline-2-carboxanilides and *in vitro* antibacterial activity against *Desulfovibrio piger* Vib-7 (minimal inhibition concentration: MIC, half maximal inhibitory concentration: IC_{50}, minimal bactericidal concentration: MBC) of the compounds in comparison with ciprofloxacin (CPX).



Comp.	R	[μM]		
		MIC	IC_{50}	MBC
1	H	23	12	25
2	2-OCH_3	28	17	28
3	3-OCH_3	17	11	20
4	3-CH_3	23	10	23
5	3-Br	33	18	35
6	3-CF_3	17	10	20
7	4-CF_3	20	10	22
CPX	–	45	28	45

Discussion and conclusion

Sulfate-reducing bacteria of *Desulfovibrio* genus belong to the intestinal microbiota of humans and animals (Kushkevych, 2012, 2013). They are anaerobic microorganisms, dissimilating sulfate as an electron acceptor and organic compounds as an electron donor and carbon source in the process of "dissimilatory sulfate reduction" (also known as "dissimilatory anaerobic sulfate respiration") (Kushkevych, 2016). Lactate is the most common substrate used by the species belonging to the intestinal sulfate-reducing bacteria. The species of *Desulfovibrio* oxidize lactate incompletely to acetate. Lactate oxidation to acetate occurs together with the concurrent reduction of sulfate to sulfide (Barton and Hamilton, 2010). The presence of lactate and sulfate in the human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolic products, acetate and hydrogen sulfide, that are toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher and Cummings, 1996; Rowan et al., 2009). There is also an assumption that sulfate-reducing bacteria can cause some forms of cancer of the rectum through the

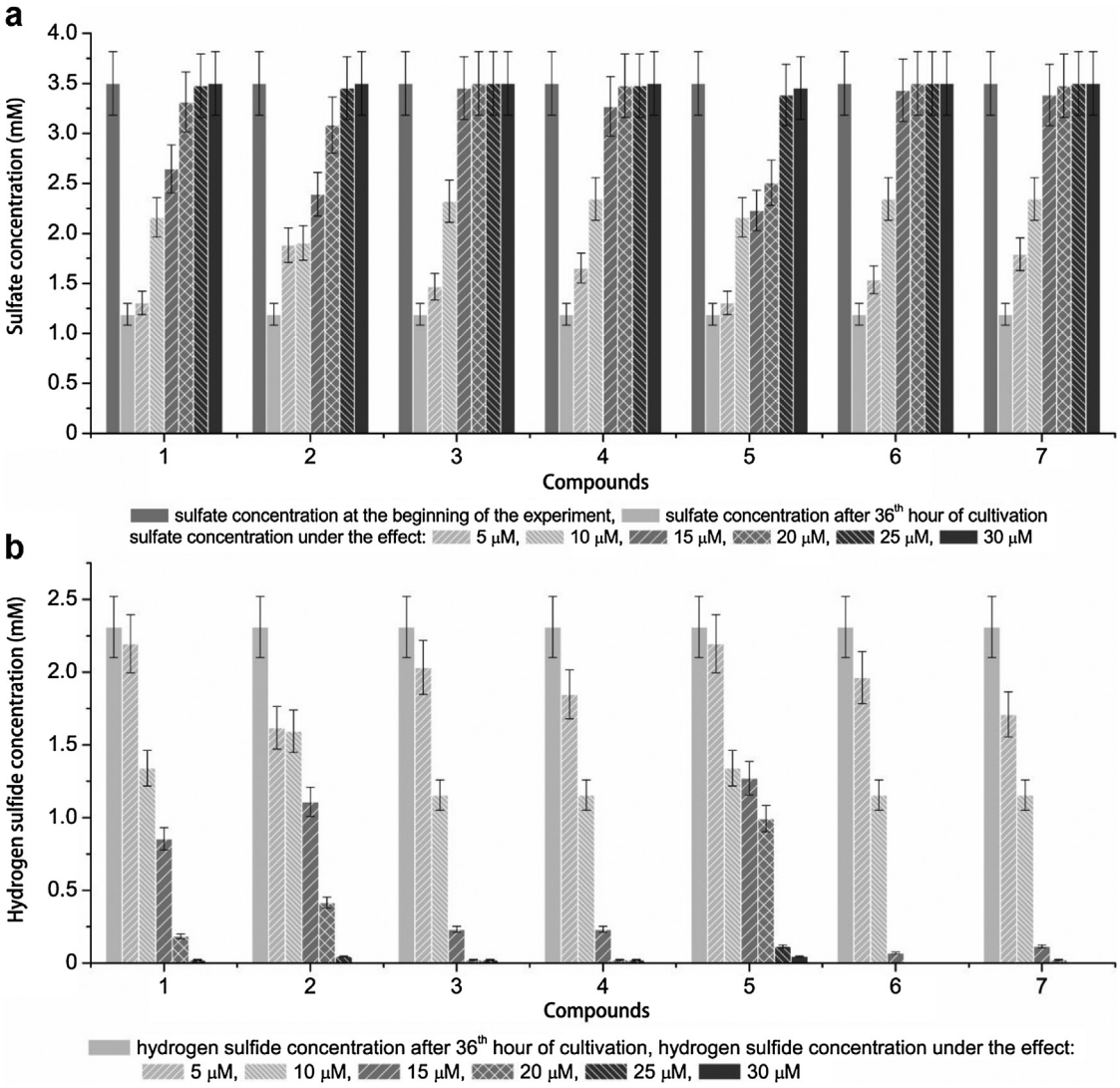


Fig. 2. Sulfate reduction of *D. piger* Vib-7 cells under the treatment of indicated doses of ring-substituted 8-hydroxyquinoline-2-carboxanilides: sulfate dissimilation (**a**), hydrogen sulfide production (**b**).

formation of hydrogen sulfide. In our previous studies, it was shown that bacteria *D. piger* Vib-7 consumed sulfate and accumulated hydrogen sulfide in concentration of 2.31 ± 0.21 mM (Kushkevych, 2013).

Based on all the obtained results in this study, it can be concluded that compounds **1**–**7** in concentrations 5–35 μM inhibited the growth and, accordingly, the process of dissimilatory sulfate reduction. This antimicrobial activity was concentration-dependent, with the strongest effect in 30 μM concentration. Similar effect was observed in our previous research for the activity of selected salicylamides against intestinal sulfate-reducing bacteria (Kushkevych et al., 2015a, 2016). Derivatives **1**, **2**, **4**, **5** and **7** showed cytotoxic affect at concentrations higher than 17 μM. The highest level of inhibition of this process and high activity rates were observed at concentration 17 μM of compounds **3** and **6** and thus are interesting for further studies. The cytotoxic effect of these compounds can be due to the inhibition of the enzymes of dissimilatory sulfate reduction (Kushkevych, 2015a,b; Kushkevych et al., 2015b), including sulfite reductase that is susceptible for various factors in intestinal sulfate-reducing bacteria *D. piger* Vib-7 (Kushkevych and Fafula, 2014). The tested compounds can be considered good alternatives for the treatment of colitis or colorectal cancer although it should be taken in account that these compounds can be aggressive also to commensally bacteria and even to other parts of the human body. This should be a concern to be clarified in the near future with complementary assays.

Despite the fact that evaluated bacteria *Desulfovibrio* species are heterogeneous group of microorganisms which are wide-spread in anaerobic areas of soils, wetlands, marine and fresh water, they are available in microbiota of large intestine of humans and animals. It is known that intestinal *Desulfovibrio* species are different from other SRB by their biochemical and physiological properties (Barton and Hamilton, 2010; Brenner et al., 2005; Holt et al., 1994). So, the mechanisms of the effect of studied compounds on these bacteria can also differ. These compounds may influence the synthesis of the bacterial cell wall (peptidoglycan), ribosomes, enzymes or other phenotypic features such as inhibition the synthesis of desulfoviridin, cytochrome c3 and menaquinone MK-6. Effect of studied compounds may depend on their activity, structure and properties.

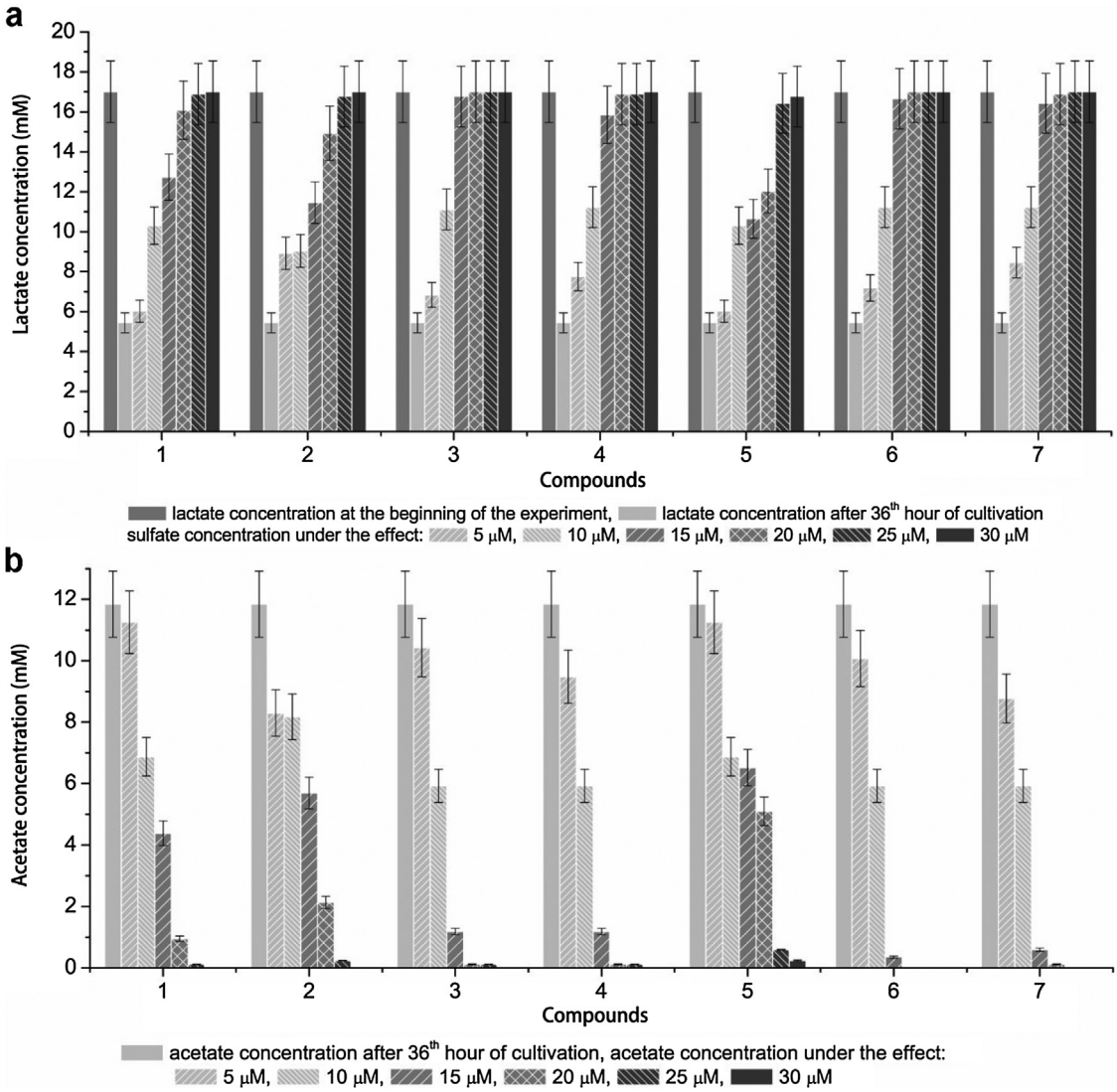


Fig. 3. The effect of ring-substituted 8-hydroxyquinoline-2-carboxanilides on lactate consumption (**a**) and acetate production (**b**) by *D. piger* Vib-7 cells.

The studied compounds are able to inhibit the number of sulfate-reducing bacteria and/or reduce the production of sulfide and acetate. This would help to clarify the factors influencing sulfide production in the human and animal colon.

Conflict of interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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ATTACHMENT 9

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The diversity of sulfate-reducing bacteria in the seven bioreactors

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ORIGINAL PAPER

The diversity of sulfate-reducing bacteria in the seven bioreactors

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Abstract
Anaerobic technology has a wide scope of application in different areas such as manufacturing, food industry, and agriculture. Nowadays, it is mainly used to produce electrical and thermal energy from crop processing, solid waste treatment or wastewater treatment. More intensively, trend nowadays is usage of this technology biodegradable and biomass waste processing and biomethane or hydrogen production. In this paper, the diversities of sulfate-reducing bacteria (SRB) under different imputed raw material to the bioreactors were characterized. These diversities at the beginning of sampling and after cultivation were compared. *Desulfovibrio, Desulfobulbus*, and *Desulfomicrobium* genus as dominant among sulfate reducers in the bioreactors were detected. The *Desulfobulbus* species were dominant among other SRB genera before cultivation, but these bacteria were detected only in three out of the seven bioreactors after cultivation dominant.

Keywords Sulfate-reducing bacteria · Hydrogen sulfide · Bioreactors · Biogas

Introduction

Biogas is a mixture of CH_4 and CO_2 and other gases. This valuable product is result of conversion of the organic mass in the waste by anaerobic microorganisms in methanogenic bioreactors (McCarty and Smith 1986). Formation of biogas is mainly occurred in wetlands, marshes, and in the gastrointestinal system of ruminants (Krich et al. 2005). Landfilled food and wastes and another biomass may be degraded by anaerobic microorganisms in landfills. Potential source of renewable energy can be used through collected biogas (Wilkie 2008).

For feed of biogas bioreactor, various substrates are used. This substrate may differ by composition of organic and inorganic compounds. Sulfate-reducing bacteria (SRB) may out-compete methanogens for substrates (e.g., H_2, CO_2, and acetate) by growth in sulfate-rich maize silage or sludge in anaerobic environments, such as biogas bioreactors. Organic compound and molecular hydrogen is universal electron donor in dissimilatory sulfate reduction process as well as for methane formation. SRB consume hydrogen below a minimum threshold than methanogens in their hydrogen metabolism (Lovley 1985; Lovley and Ferry 1985; Kushkevych et al. 2017a).

Intensive consumption of substrate by SRB during sulfate reduction may cause a problem of a high concentration of hydrogen sulfide. This final product of SRB metabolism is toxic and can cause corrosion of metallic materials in cogeneration units of biogas plants (Koschorreck 2008). Therefore, a negative effect on microbial cells due to the precipitation of essential trace metals as metal sulfides can occur.

In any case, hydrogen sulfide toxicity is unlikely to be decoupled from competition between SRB and methanogens, and due to their more favorable growth and thermodynamic properties, SRB are considered to out-compete other anaerobes in the presence of excess sulfate. The diversity of SRB depending on various ratios and composition of substrates in operated bioreactors has never been studied yet. Such new data may be useful for regulation and controlling of the processes of methane production and for more clearly understanding of the mechanisms of substrates' transformation.

The aim of this research was to compare the diversity of sulfate-reducing bacteria populations in operated bioreactors with different input ratios of the initial substrate and to

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investigate the ability of SRB genera to grow in vitro and the change in its percentage ratio before and after cultivation as well as to create phylogenetic tree of SRB relationships in each bioreactor under these conditions.

Materials and methods

The three samples were collected from each different biogas plant reactors of volume ranged from 2500 to 3500 m^3 operated at about 40 ± 4 °C temperature depending on bioreactor and processing low solid suspension (liquid fermentation). Organic load rate was 3.5–5.5 kg org. mass/m^3 per fermenter and feed intervals were 80–100 kg/kWh_{el}.

The biogas plant reactors are located at Modřice, Bratčice, Pánov, Úvalno, Horní Benešov, Rusín, and Loděnice (Czech Republic). Hydraulic retention time (HRT) ranged from 18 to 20 days for one-stage biogas plant at Modřice and 25–35 days for primary reactor for six remaining biogas plants. The samples were taken directly from the sampling valve of the primary reactors into sterile-sampling vessels. After sampling, the samples were stored in thermos container and transported to the laboratory for further analysis. Each of the reactors processed a different type of substrate.

The type of substrate in bioreactor

The type of substrate is shown as the ratio (w/w) of fresh input substrate. Modřice (1): primary sludge, biological sludge (50:50), Bratčice (2): maize silage, whole crop silage, poultry litter (63:31:6), Pánov (3): maize silage, poultry litter (92:8), Úvalno (4): maize silage, sugar beet pulp, whole crop silage, cattle manure (44:44:6:6), Horní Benešov (5): maize silage, sugar beet pulp, whole crop silage, cattle manure, grass silage (29:39:12:15:5), Rusín (6): maize silage, sugar beet pulp (70 30), Loděnice (7): maize silage, sugar beet pulp (75:25).

Isolation of DNA and amplification of 16S rRNA gene fragments by Illumina sequencing

For DNA isolation, three samples were taken from all seven reactors and homogenized before sequencing. The QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH, Germany) is designed for rapid purification of total DNA from stool samples and was used for DNA extraction from biogas digester samples. DNA extraction was performed in accordance to the manufacturers' instructions, with minor adjustments as described below. Briefly, 100 mg of sample was washed with 1.4 mL of ASL buffer (QIAGEN GmbH, Germany), heated at 95 °C for 10 min. After centrifugation, an InhibitEX tablet was put in the supernatant to remove impurities and PCR inhibitors. After thorough centrifugation, 200 µL of the supernatant was added to 15 µL of proteinase K, and 200 µL of buffer AL (QIAGEN GmbH, Germany) was added. The mixture was heated to 70 °C for 10 min and 200 µL of absolute ethanol was added. The sample mixture was then passed through the QIAamp kit column, followed by two washes with buffers AW1 and AW2 (QIAGEN GmbH, Germany). The DNA was eluted in a volume of 200 µL of elution buffer.

Amplification of 16S rRNA gene fragments was carried out using universal primers for amplification of the V3 and V4 variable regions of the 16S rRNA (Nossa et al. 2010). The primers were flanked by molecular barcodes for sample identification. The PCR reaction was prepared using Maxima™ Probe qPCR Master Mix (Thermo Fisher Scientific, USA) with cycling conditions as follows: 95 °C for 10 min. followed by 30 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 120 s, and a final extension step at 72 °C for 2 min. PCR products were visualized using electrophoresis on 1.5% agarose gels and purified from the gel using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany). DNA was quantified using the QuantiTPicoGreen dsDNA Assay (Thermo Fisher Scientific, USA) and equimolar amounts of the PCR products were pooled together.

Purified amplicons were paired-end sequenced (10 nM sample DNA in 10 µl) on an Illumina Mi-Seq platform. QIIME data analysis package was used for 16S rRNA data analysis (Caporaso et al. 2010). Quality filtering on raw sequences was performed in accordance to base quality score distributions, average base content per read and GC distribution in the reads. Chimeras and reads that did not cluster with other sequences were removed. The obtained sequences with qual scores higher than 20 were shortened to the same length of 350 bp and classified with RDP Seqmatch with an operational taxonomic unit (OTU) discrimination level set to 97%. The relative abundance of the taxonomic groups was calculated to the microorganisms detected in this study.

Sequences were compared using the BLAST feature of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990). The sequences were uploaded to Mega7 for comparative genomic analyses (Kearse et al. 2012). Alignments of sequences were performed in Mega7 using Clustal W and clustering was performed by the neighbor-joining method (Larkin et al. 2007).

The sequences of their 16S rRNA gene and sequences of the strains from GenBank were compared to clarify the genetic relations of the SRB populations in the specific bioreactor. The genomic sequences of SRB are available in GenBank under accession no. MF991912.1, MF991913.1, KY285258, KY285259, MF991911.1, MF991932.1, KY285260, KY285261, KY290639, KY290609, KY290608, KY290610, KY290611, KY290612, KY290614, KY290617, KY290615, KY290636, KY290646, KY290647, KY290648,

KY290649, KY290651, KY290652, KY290653, KY290677, KY297012, KY295933, MF991933.1, KY295934, KY297013, MF991934.1, KY305420, KY305433, KY305431, KY305471, KY305532, KY305531, MF989223.1, KY305678, KY305658, KY305659, KY305660, KY305668, KY305679, KY305739, KY305738, KY305863, MF988750.1, KY305876, KY306668, KY306669, MF991936.1, KY306671, KY306670, MF988711.1, KY306672, KY306673, KY306674, KY306677, KY306676, KY306675, KY306678, MF991939.1, MF991938.1, MF988725.1.

To compare the genera of SRB diversity and their ability to growth in vitro, the same samples from each bioreactor were collected in liquid Postgate's medium C (Posgate, 1984) and cultivated under anaerobic conditions for 72 h. After cultivation, samples were sequenced as described above. The research results were analyzed using software package Origin7.0 (http://www.origin-lab.com).

Results and discussion

The main genera of SRB were investigated in different bioreactors. The most widespread genera were identified as *Desulfovibrio, Desulfobulbus, Desulfomicrobium*, and other SRB (Fig. 1). The most ratios of SRB genera, except the other ones, were detected in the bioreactor from Loděnice where two types of the substrate (maize silage, sugar beet pulp in the ratio 75:25) were used. On the other hand, other genera of SRB were detected only in the bioreactor located at wastewater treatment plant Modřice (12%) processed primary sludge and biological sludge (50:50) and in the bioreactor at Rusín (6%) processed maize silage, sugar beet pulp at ratio (70:30). However, *Desulfobulbus* genus was detected as a dominant genus of SRB in all bioreactors.

Two genera, *Desulfovibrio* and *Desulfobulbus* were both dominant SRB in cultivations from bioreactors at Modřice and Rusín. Also, single dominant *Desulfovibrio* genus was detected after sample cultivation from Úvalno (89%), Horní Benešov (87%), and Loděnice (81%). Although, the greatest percentage of *Desulfobulbus* genus isolated from Bratčice (98%) and Pánov (97%) after cultivation was observed and correlated with the same samples (without cultivation) isolated directly from bioreactors.

Phylogenetic tree of these isolates from each bioreactor was built (Fig. 2). Also, the phylogenetic tree of the samples after cultivation was built (Fig. 3) to observe diversity and ability to be cultivated of these bacteria. It should be mentioned that the most described sequences of SRB strains in GenBank are identified only to the domain, kingdom, family or genus, and in some cases, to species (http://www.ncbi.nlm.nih.gov/genbank). It is clearly visible that the greatest diversity of SRB is detected in a bioreactor from wastewater treatment plant located at Modřice.

Methane is the product of anaerobic metabolism carried out by communities of hydrolytic bacteria and fungi, acid-producing intermediary organisms, and finally, methanogenic *Archaea* (Zeikus 1977). Methane-producing communities are very stable and resilient, but they are also complex and largely undefined. The process of methanogenesis often collapses due to the high concentration of hydrogen sulfide produced by SRB (Weijma 2001). Methanogenic microorganisms are sensitive to this final

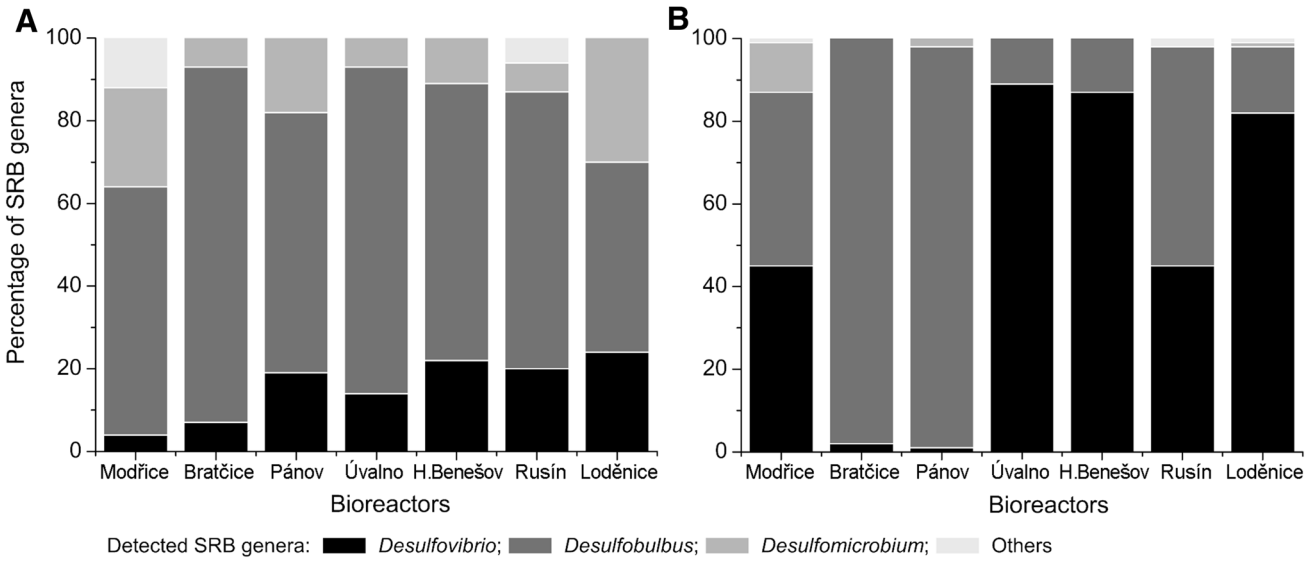

Fig. 1 Percentage ratio of sulfate-reducing bacteria genera in all analyzed bioreactors (**a**), diversity of sulfate-reducing bacteria populations observed after cultivation (**b**)

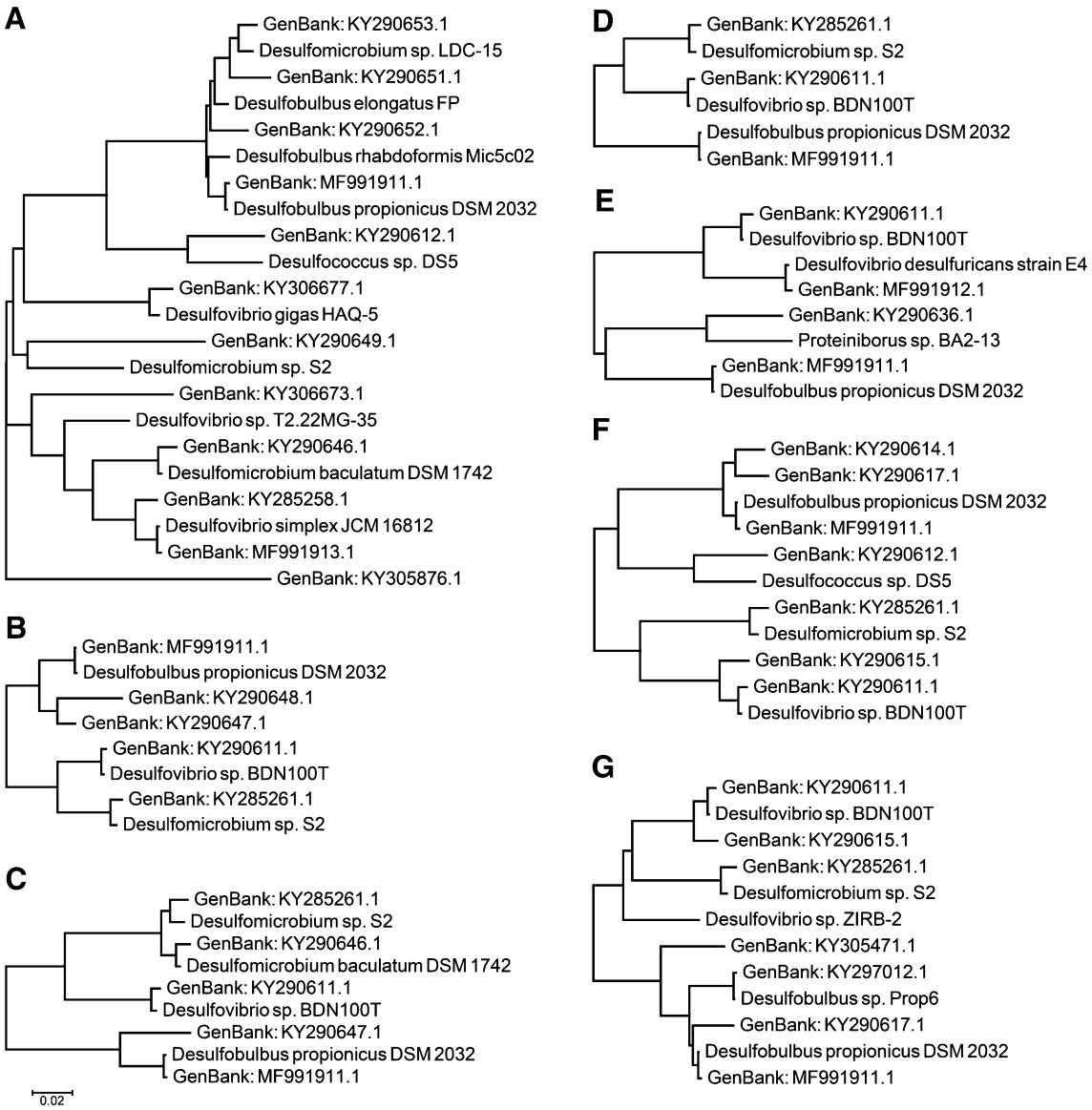

Fig. 2 Phylogenetic tree of sulfate-reducing bacteria relationships separately in each bioreactor: Modřice (**a**), Bratčice (**b**), Pánov (**c**), Úvalno (**d**), Horní Benešov (**e**), Rusín (**f**), and Loděnice (**g**)

metabolite of SRB and can compete with them for molecular hydrogen as the electron donor (Oremland and Polcin 1982).

SRB are a group of microorganisms which uses sulfate as an electron acceptor in their process of dissimilatory sulfate reduction (Postgate 1984; Barton and Hamilton 2010; Kushkevych 2015a, b; Kushkevych et al. 2015a, b, 2016, 2017b). Hydrogen sulfide is the final product of this process (Kushkevych 2016; Kushkevych et al. 2018). SRB need for sulfate reduction exogenous electron donors, such as lactate, propionate, butyrate, ethanol, and even molecular hydrogen. Organic compounds for SRB can be simultaneously electron donors and carbon sources and oxidized completely (to CO_2) or incompletely (to acetate and CO_2) (Barton and Hamilton 2010; Kushkevych 2016). SRB, oxidizing organic compounds incompletely, belong to the group called "Acetogenic sulfate-reducing bacteria" (Barton and Hamilton 2010). Detected SRB, *Desulfovibrio, Desulfomicrobium* and *Desulfobulbus* genera, are acetogenic microorganisms which oxidize organic compounds, incompletely, to acetate and CO_2. There was a wide diversity of SRB in the bioreactors. The bacterial populations found in all bioreactors examined were heterogeneous, encompassing an array of different genera and strains of acetogenic sulfate-reducing bacteria.

In our studies, using Illumina sequencing, the sequences of *Desulfobulbus* sp. were the most often detected in all bioreactors. This genus of SRB was frequently described in the other literature (Zellner et al. 1989; Lien et al. 1998). Zellner

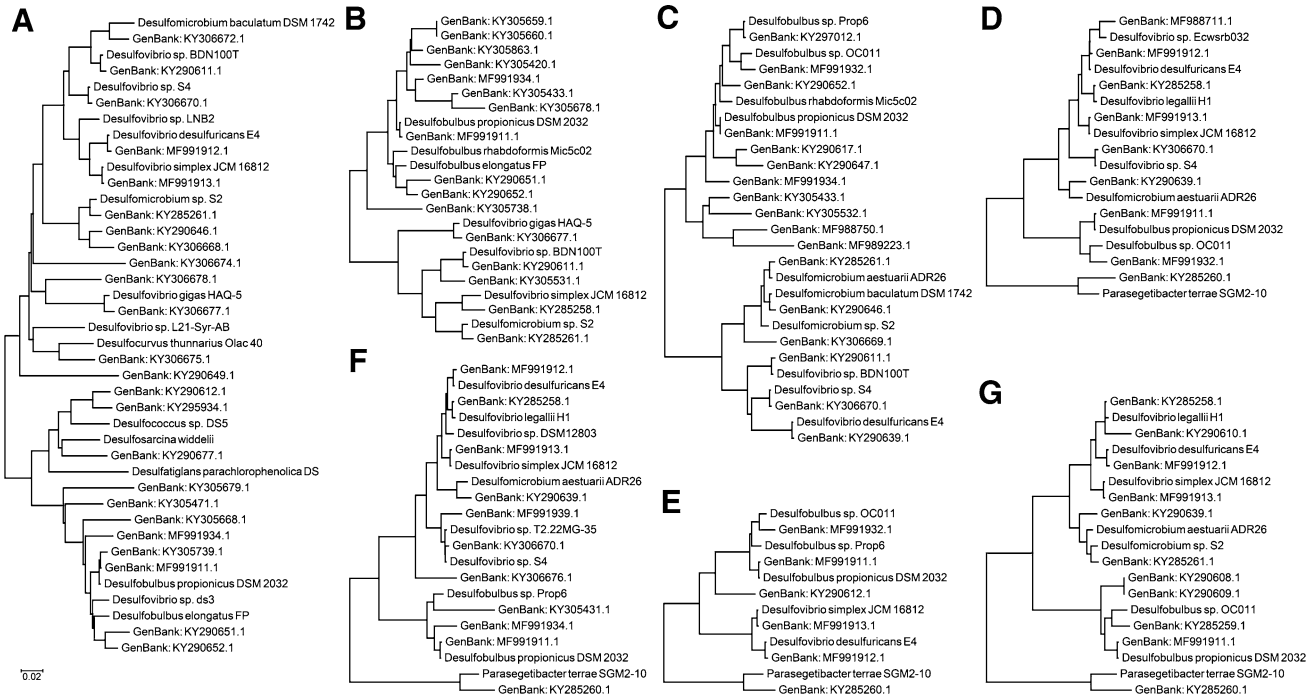

Fig. 3 Phylogenetic tree of sulfate-reducing bacteria relationships separately after cultivation from each bioreactor: Modřice (**a**), Bratčice (**b**), Pánov (**c**), Úvalno (**d**), Horní Benešov (**e**), Rusín (**f**), and Loděnice (**g**)

et al. (1989) which was mainly focused on *Desulfobulbus propionicus* has also observed a variety of SRB are similar to this genus. In our research, we have also observed widely diversity of SRB related to a genus of *Desulfobulbus propionicus* which is visible in our phylogenetic tree (Fig. 2).

Another dominant genus of microbial acetogenic SRB in the studied bioreactors was *Desulfovibrio* sp. which is the most widespread and known genus of the SRB (van Houten et al. 2009; Wawer et al. 1997). It was dominant genus only in three bioreactors after cultivation. But we can state that this genus of SRB is the most cultivable genus from all bioreactors compared to *Desulfobulbus* which was dominant only before cultivation (Fig. 1).

SRB with the lowest percentage in all samples was *Desulfomicrobium* genus. Despite its low percentage, this genus plays an important role in the environment with high heavy metal concentrations. This genus is mostly cultured at low temperature and shows heavy metal tolerant properties (Azabou et al. 2007).

The SRB populations depend on the different types and initial amount of substrate ratio in the bioreactors. Three dominant morphotypes of these bacteria in the bioreactors were homologous (99%) by the sequences of 16S rRNA gene to the *Desulfovibrio, Desulfobulbus* and *Desulfomicrobium* genera deposited in GenBank. Their genetic relationship can be shown by the phylogenetic trees and by comparison of the sequences 16S rRNA gene of these bacteria from methane bioreactors to the sequences of the strains from GenBank.

Conclusions

To conclude our research, changes of SRB genera profile, depending on ratios of substrates on the beginning of sampling and their ability to grow *in vitro*, were determined. Before cultivation, *Desulfobulbus* species was dominant (46–86%) in all biogas plants. The profile and number of this genus was changed after the cultivation and its biomass was increased by 97% and 98% in the samples from Bratčice and Pánov. Under these conditions, the most viable to grow were *Desulfovibrio* species (82–89%) detected in samples from Úvalno, Horní Benešov and Loděnice. It can be stated that agricultural biogas stations showed a comparable diversity of SRB, regardless of feedstock. On the contrary, the biogas treatment plant showed a higher diversity of SRB, due to a different type of substrate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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ATTACHMENT 10

Pages 108 – 114

Activity of ring-substituted 8-hydroxyquinoline-2-carboxanilides
against intestinal sulfate-reducing bacteria *Desulfovibrio piger*

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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Activity of ring-substituted 8-hydroxyquinoline-2-carboxanilides against intestinal sulfate-reducing bacteria *Desulfovibrio piger*

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Abstract *Desulfovibrio* genus is dominant among sulfate-reducing bacteria (SRB) in the large intestine of healthy people and animals. It is mostly isolated from patients with inflammatory bowel disease (IBD) and can be involved in the disease initiation. Primary in vitro screening of 8-hydroxyquinoline-2-carboxanilides was performed against *Desulfovibrio piger* Vib-7 representing SRB. The most effective compounds with MIC_{90}/MBC values in the range of 17–23 μM/20–23 μM, respectively, were substituted in C'$_{(3)}$ by CF_3, OCH_3, CH_3 and in C'$_{(4)}$ by CF_3. Their activity was twofold higher than that of ciprofloxacin. These compounds did not express any significant cytotoxic effect on THP-1 cells up to the tested concentration of 30 μM. The antibacterial efficacy of the most active C'$_{(3)}$-substituted compounds practically did not change with increasing compound lipophilicity, indicating that this position of substitution is favorable for significant antimicrobial effect, while the antibacterial activity of most of C'$_{(2)}$ and C'$_{(4)}$-substituted derivatives decreased linearly with increasing compound lipophilicity. In addition, the dependence of activity on electronic Hammett's σ parameter of the substituent R was quasi-parabolic for the most effective C'$_{(3)}$-substituted compounds.

Keywords 8-Hydroxyquinolines · Sulfate-reducing bacteria · Lipophilicity · Electronic parameter · Structure–activity relationships

Introduction

The species of *Desulfovibrio* genus are dominant among sulfate-reducing bacteria (SRB) and common inhabitants of the human and animal large intestine, capable of dissimilatory sulfate reduction (Gibson et al. 1991, 1993; Kushkevych 2015a, b; Kushkevych et al. 2015a, b; Wegmann et al. 2017). These microorganisms are the most isolated from patients with inflammatory bowel disease (IBD) and can be involved in the disease initiation caused by their main metabolite hydrogen sulfide, which is an inhibitor of butyrate oxidation in colonocytes. In addition, it is cytotoxic, mutagenic and cancerogenic to epithelial intestinal cells, which leads to the damage of the epithelial barrier function, resulting in inflammatory responses characteristic for IBD (Kushkevych et al. 2014; Pitcher and Cummings 1996; Zinkevich and Beech 2000). Therefore, the association between SRB and IBD, such as ulcerative colitis (UC), was hypothesized (Zinkevich and Beech 2000; Rowan et al. 2009; Loubinoux et al. 2002; Kushkevych 2014; Cummings et al. 2003). Over 1 million residents in the USA and 2.5 million in Europe are estimated to have IBD, with substantial costs for health care, whereas these estimates do not

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factor in the 'real' price of IBD, which can impede career aspirations, instil social stigma and impair quality of life in patients (Kaplan 2015). Unlike Crohn's disease, UC occurs only in the large bowel, where bacteria amount is greater than in the rest of the gut and also where the rate of passage of material is characterized by slow movement of digestive materials. Both acute and chronic forms of UC affect the colon and rectum and can be a highly uncomfortable condition (Cummings et al. 2003). UC usually has a relapsing/remitting pattern and current medical approaches focus on treating active disease to address symptoms, to improve the quality of life and thereafter to maintain remission. Diarrhea accompanied with blood, an urgent need to defecate and abdominal pain are the main symptoms of active disease or relapse. The reported incidence is 1.2 to 20.3 cases per 100,000 persons per year, and the prevalence is 7.6 to 245 cases per 100,000 per year (Feuerstein and Cheifetz 2014; Cesar da Silva et al. 2014).

The benefits of antibiotic therapy in UC are mediated by different mechanisms, such as decreasing the concentration of luminal bacteria, altering the composition of gut microflora, decreasing bacterial tissue invasion and decreasing bacterial translocation and systemic dissemination. Antibiotics have been prescribed for UC, however, they have been largely ineffective (Cummings et al. 2003; Garud and Peppercorn 2009). For example, the study of the in vitro activities of rifaximin and comparator agents against 536 anaerobic intestinal bacteria performed by Finegold et al. showed that the overall $MICs_{90}$ of rifaximin for 90% the tested strains were 338 µM, an activity equivalent to those of teicoplanin and vancomycin (Finegold et al. 2009). Nakao et al. (2009) tested the antimicrobial susceptibilities of 23 strains of *Desulfovibrio* spp. and found that they were susceptible to sulbactam-ampicillin, meropenem, clindamycin, and metronidazole with MIC_{90} corresponding to 17, 10, 0.45, and 1.46 µM, respectively. On the other hand, Lozniewski et al. (2001) tested the antimicrobial susceptibilities of 16 clinical isolates of *Desulfovibrio* spp. and found that these isolates were resistant to piperacillin-tazobactam, cefoxitin and cefotetan with MIC_{90} corresponding to 495, >600 and 111 µM, respectively. Therefore, it is necessary to study new antibacterial agents in order to improve the treatment and discover alternative therapeutics.

This paper follows our recently published articles dealing with the spectrum of biological activities of hydroxyquinoline-based compounds (Jampilek et al. 2005; Musiol et al. 2006, 2007, 2008, 2010; Mrozek-Wilczkiewicz et al. 2010; Gonec et al. 2012; Cieslik et al. 2012, 2015; Kos et al. 2015a; Jampilek et al. 2016). This study is focused on the investigation of the efficacy and searching for the structure–activity relationships within a series of 8-hydroxyquinoline-2-carboxanilides (Kos et al. 2015a) against *Desulfovibrio piger* Vib-7 representing SRB. *D. piger* is a Gram-negative strict anaerobe that is usually considered as a commensal bacterium in humans. More recently, it has attracted more interest as it was found to be the most prevalent species of SRB in feces of patients with IBDs (Kushkevych 2014; Barton and Hamilton 2010; Holt et al. 1994).

Material and methods

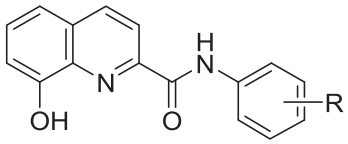
Synthesis

The discussed 8-hydroxyquinoline-2-carboxanilides **1–8c** (see Table 1) were synthesized previously (Kos et al. 2015a) by means of microwave-assisted synthesis. The compounds were fully characterized by melting point, infrared, nuclear magnetic resonance, and high-resolution mass spectrometry (Kos et al. 2015a).

In vitro antibacterial susceptibility testing

The synthesized compounds were evaluated for in vitro antibacterial activity against the intestinal SRB *Desulfovibrio piger* Vib-7 (Genbank: KT881309.1) that were isolated from the healthy human large intestine as described previously (Kushkevych 2013; Kushkevych et al. 2014). The strain has been kept in the collection of microorganisms at the Department of Molecular Biology and Pharmaceutical Biotechnology of the Faculty of Pharmacy at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic). Ciprofloxacin (Sigma-Aldrich) was used as the standard. Prior to testing, the strain at exponential phase growth was passaged onto nutrition modified Kravtsov-Sorokin's (KS) agar medium (Kushkevych and Moroz 2012). Bacterial inocula were prepared by suspending a small portion of bacterial colony in sterile KS liquid medium (pH 7.5). Before bacterial passage in the medium, 10 mL/L of sterile Mohr's salt solution $[(NH_4)SO_4Fe(SO_4)_2{\cdot}6H_2O]$ (10%) for visual detecting colonies of the SRB was added. A culture sample (10 mL) was centrifuged at 15,000 rpm/20 min using a bench top centrifuge (Model CR 4-12, Jouan Inc., Winchester, VA, USA). Following removal of the supernatant, the pellet was washed in fresh liquid KS and re-suspended in fresh supplemented KS (10 mL). The turbidity was adjusted to match McFarland standard No. 1 (5×10^6 cfu) with KS using a densitometer (Densi-La-Meter, LIAP, Latvia). The final inoculum was made to a 1:20 dilution of the suspension with KS liquid medium. The antimicrobial susceptibility of SRB was investigated in a 96-well plate format. In these experiments, sterile KS (300 µL) was added to all outer-perimeter wells of the plates to minimize evaporation of the medium in the

Table 1 Experimentally determined values of lipophilicity log *k*, predicted electronic Hammett's σ parameters of substituents R, in vitro antibacterial activity against *Desulfovibrio piger* Vib-7 (MIC, IC_{50}, MBC) of the compounds in comparison with ciprofloxacin (*CPX*) standard



Compounds	R^1	log k^a	σ^b	MIC_{90} [μM]	IC_{50} [μM]	MBC [μM]
1	H	0.7600	0	23	12	25
2a	2-OCH_3	0.7935	−0.28	28	17	28
2b	3-OCH_3	0.8164	0.12	17	11	20
2c	4-OCH_3	0.7129	−0.27	557	380	557
3a	2-CH_3	0.6944	−0.17	44	15	48
3b	3-CH_3	0.9686	−0.07	23	10	23
3c	4-CH_3	0.9521	−0.17	75	40	80
4a	2-F	0.6806	0.06	90	50	95
4b	3-F	0.9420	0.34	45	20	50
4c	4-F	0.8598	0.06	60	28	60
5a	2-Cl	0.9566	0.22	120	95	123
5b	3-Cl	1.1718	0.37	50	20	55
5c	4-Cl	1.1543	0.23	480	330	486
6a	2-Br	1.0536	0.22	150	103	150
6b	3-Br	1.2357	0.39	33	18	35
6c	4-Br	1.2347	0.23	337	225	340
7a	2-CF_3	0.9147	0.51	48	35	50
7b	3-CF_3	1.3206	0.43	17	10	20
7c	4-CF_3	1.3653	0.51	20	10	22
8a	2-NO_2	1.1277	0.77	197	134	202
8b	3-NO_2	0.9845	0.71	237	218	240
8c	4-NO_2	1.0495	0.78	223	165	225
CPX	–	–	–	45	28	45

[a] Experimental procedure described in Kos et al. (2015a)

[b] Predicted using sw. ACD/Percepta ver. 2012

test wells during incubation. Sample wells were composed of 100 μL of test compound dilution and 100 μL of the bacterial stock being tested against. The compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma), and the final concentration of DMSO in the KS liquid medium did not exceed 0.1% of the total solution composition. Dilutions of each compound were prepared in triplicate. The final concentrations of the evaluated compounds ranged from 100 to 0.05 μM. Plates were sealed with parafilm, introduced into an anaerobic box with oxygen uptake generators (GENbox anaer, France) for anaerobiosis. The determination of results was performed visually after 72 h of static incubation in the darkness at 37 °C under anaerobic conditions. In the process of bacterial growth, hydrogen sulfide was formed and interacted with Fe^{2+} from Mohr's salt. As a result, FeS was formed by the bacterial cells that caused black colored colonies that was interpreted as a presence of bacterial growth. The medium dilution micro-method modified according to NCCLS guidelines (CLSI 2012, 2014) in KS medium was used to determine the minimum inhibitory concentration (MIC_{90}), the inhibitory concentration (IC_{50}) and minimum bactericidal concentration (MBC). Drug-free controls, sterility controls and controls consisted of KS medium and DMSO alone were included. The MICs were defined as the lowest concentration of the compound at which no visible bacterial growth was observed. The IC_{50} values were defined as the compound concentration causing 50% inhibition of bacterial growth and the MBCs were defined as the lowest concentration of the compound at which an antimicrobial agent kills a bacteria (CLSI 2012, 2014). The results are summarized in Table 1.

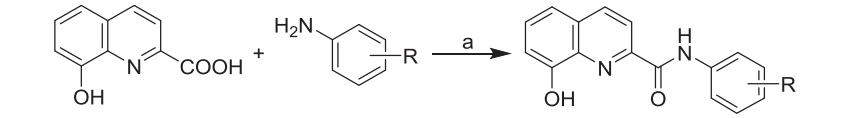
Results and discussion

In the previous studies it was confirmed that 2-substituted 8-hydroxyquinolines are promising antimicrobial agents (Jampilek et al. 2005; Musiol et al. 2006, 2010; Darby and Nathan 2010; Gonec et al. 2012; Cieslik et al. 2012; 2015; Kos et al. 2015a). Based on these observations, 8-hydroxyquinoline-2-carboxanilides prepared according to Scheme 1 and described recently (Kos et al. 2015a) were tested against *Desulfovibrio piger* Vib-7. The testing was performed according to Kushkevych et al. (2016).

The antibacterial potency of ring-substituted 8-hydroxyquinoline-2-carboxanilides was expressed as the MICs, IC_{50} values and MBCs, see Table 1. The activity of the most potent compounds **7b** (R = 3-CF_3; MIC = 17 μM, IC_{50} = 10 μM, MBC = 20 μM), **7c** (R = 4-CF_3; MIC = 20 μM, IC_{50} = 10 μM, MBC = 22 μM) and **2b** (R = 3-OCH_3; MIC = 17 μM, IC_{50} = 11 μM, MBC = 20 μM) was twofold higher that of the clinically used drug ciprofloxacin (MIC = MBC = 45 μM) that was used as the standard. Also other compounds such as **3b** (R = 3-CH_3; MBC = 23 μM), **1** (R = H; MBC = 25 μM), **2a** (R = 2-OCH_3; MBC = 28 μM), and **5b** (R = 3-Br; MBC = 35 μM) were effective in killing *D. piger* Vib-7. It is also important to note that no significant cytotoxic effect of these compounds on THP-1 cells up to tested concentration 30 μM was observed (Kos et al. 2015a).

The dependence of log(1/IC_{50}) on compound lipophilicity expressed by log *k* (Kos et al. 2015a) is shown in Fig. 1a. After exclusion of three derivatives: 4-OCH_3 (**2c**), which precipitated from testing solution due to limited

Scheme 1 Synthesis of ring-substituted 8-hydroxyquinoline-2-carboxanilides **1–8c**: Reagents and conditions: **a** PCl_3, chlorobenzene, microwave-assisted synthesis (Kos et al. 2015a)



R = H (**1**), OCH_3 (**2a-c**), CH_3 (**3a-c**), F (**4a-c**), Cl (**5a-c**), Br (**6a-c**), CF_3 (**7a-c**), NO_2 (**8a-c**)

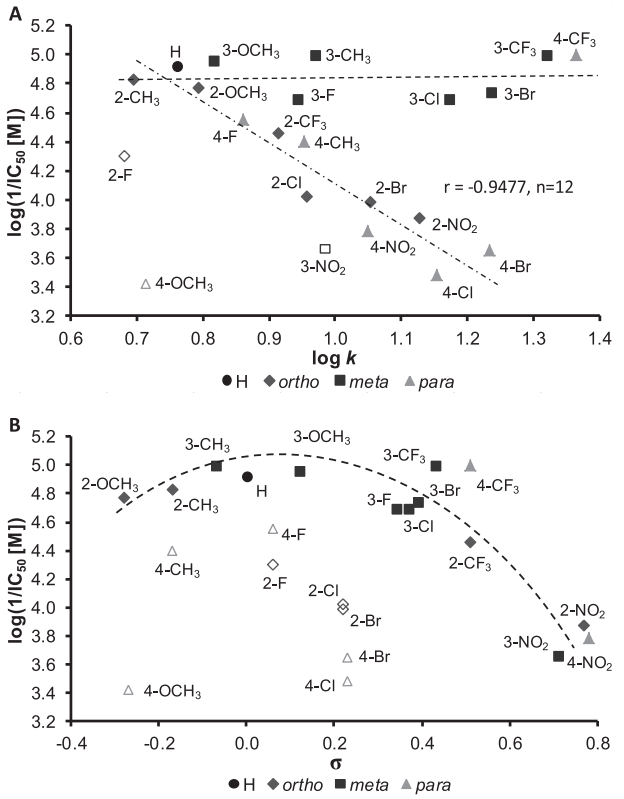


Fig. 1 Dependence of antibacterial activity against *Desulfovibrio piger* Vib-7 expressed as log(1/IC_{50}) [M] of tested compounds on lipophilicity expressed as log *k* (**a**) and on Hammett's σ constants of R substituent (**b**). (Compounds not included in individual SAR discussions are marked by empty symbol.)

aqueous solubility, and 2-F (**4a**) and 3-NO_2 (**8b**), showing lower activity than expected, the compounds can be divided into two groups. The antibacterial activity of nine derivatives with R = 2-CH_3 (**3a**), 2-OCH_3 (**2a**), 3-OCH_3 (**2a**), 3-F (**4b**), 3-CH_3 (**3b**), 3-Cl (**5b**), 3-Br (**6b**), 3-CF_3 (**7b**), and 4-CF_3 (**7c**) expressed by IC_{50} value was comparable with that of unsubstituted derivative **1** (R = H; IC_{50} = 12 μM) and varied in the range from 10 μM (3-CF_3 and 4-CF_3, 3-CH_3) to 20 μM (3-F and 3-Cl), while the antibacterial activity of other 12 tested (C'$_{(2)}$, C'$_{(4)}$-substituted derivatives and (**1**) compounds decreased linearly with increasing compound lipophilicity from log *k* = 0.6944 (2-CH_3, **3a**; IC_{50} = 15 μM) to log *k* = 1.2347 (4-Br, **6c**; IC_{50} = 225 μM) (r = −0.9477, n = 12). Thus, from seven C'$_{(3)}$-substituted compounds, six derivatives belonged to the set of the above-mentioned most active compounds, the antibacterial activity of which practically did not change with increasing compound lipophilicity, indicating that this position of substitution is favorable for significant antimicrobial effect.

The dependence of log(1/IC_{50}) on the Hammett's σ constants of the R substituent is shown in Fig. 1b. Except for derivatives with R = 4-OCH_3 (**2c**) and 4-CH_3 (**3c**) as well as derivatives with F, Cl, and Br substituents in positions C'$_{(2)}$ and C'$_{(4)}$, i.e., **4a**, **4c**, **5a**, **5c**, **6a**, and **6c**, for the remaining 14 compounds quasi-parabolic dependence of log(1/IC_{50}) on σ constants was estimated. However, it could be mentioned that within a narrow range of σ from 0.06 to 0.22/0.23, the activity of C'$_{(4)}$ halogen-substituted compounds decreased more sharply with increasing σ values than that of C'$_{(2)}$-substituted ones.

Similar trends as for the dependence of log(1/IC_{50}) on lipophilicity or on Hammett's σ constants can be found also for the dependences of the activity expressed as MIC or MBC values on both parameters; therefore, these dependences are not illustrated.

Summarizing, it can be concluded that generally for high antibacterial activity against *D. piger*, halogen substituent in the C'$_{(3)}$ position is favorable, whereby the highest antibacterial activity was exhibited by derivatives with R = 3-CF_3 and 4-CF_3, i.e., substituents that are known to promote electrostatic interactions with targets and improve the cellular membrane permeability of small molecules but also compounds with low lipophilicity (2-CH_3, 3-CH_3, 2-OCH_3, 3-OCH_3).

The estimated MIC values for *D. piger* (Table 1) are comparable with those estimated for the antimycobacterial activity of the tested compounds against *Mycobacterium tuberculosis* H37Ra ATCC 25177, *M. avium* complex CIT19/06 (clinical isolate) and *M. avium* subsp. *paratuberculosis* CIT03 (clinical isolate) (Kos et al. 2015a). It was found that with the exception of compounds with R = 4-OCH_3 (**2c**), 4-Cl (**5c**), 4-Br (**6c**), neither lipophilicity nor electronic properties of the R substituent nor the position of substitution exhibited any significant effect on the antitubercular activity against *M. tuberculosis*, and the 2-, 3- and 4-CF_3 (**7a–c**)-substituted derivatives belonged to the most active compounds with MIC = 24 μM. There were no any significant differences between antimycobacterial activities against *M. tuberculosis* and *M. avium* subsp. *paratuberculosis*. On the other hand, C'$_{(2)}$ and especially C'$_{(3)}$-

substituted derivatives expressed higher antimycobacterial activity against *M. avium* complex than C'$_{(4)}$-substituted ones, and antimycobacterial activity slightly increased with increasing lipophilicity (log *k* values) and electron-withdrawing effect of R substituents; the C'$_{(2)}$-substituted compounds with log $k > 0.8$ and Hammett's σ constants of R substituents > 0.1 and C'$_{(4)}$-substituted compounds with log $k < 0.8$ were found to be completely ineffective. However, the activity of compounds with potency against all three strains was achieved by the substitution of the C'$_{(3)}$ position of aniline. The significant bacterial/antimycobacterial activity of the studied compounds bearing an 8-hydroxyquinoline fragment and an amide moiety in their molecule is caused by the fact that these function groups are able to interact with a number of enzymes/receptors via hydrogen bonds and in this manner to affect the biological response (Pattabiraman and Bode 2011; Lavecchia and Di Giovanni 2013; Zumla et al. 2013). The significant contribution of the hydroxyl moiety in C$_{(8)}$ of quinoline to the antimycobacterial activity was reported by Gonec et al. (2012), who observed that its absence in quinoline-2-carboxanilides resulted in a decrease of antimycobacterial effect, while strengthening of antimycobacterial potency due to the presence of the hydroxyl moiety was observed with 1-hydroxynaphthalene-2-carboxanilides (Gonec et al. 2013) and 6-hydroxy-naphthalene-2-carboxanilides (Kos et al. 2015b) as compared to naphthalene-2-carboxanilides (Gonec et al. 2012).

Ring-substituted 8-hydroxyquinoline-2-carboxanilides were previously tested also as photosystem II (PS II) inhibitors (Jampilek et al 2016). The inhibition of photosynthetic electron transport (PET) in spinach chloroplasts by these compounds significantly depended on the position of substitution, and the inhibitory activity of C'$_{(3)}$-substituted compounds was by one or two orders higher than that of C'$_{(2)}$ and C'$_{(4)}$-substituted derivatives. For the most active compounds, the following IC_{50} values were observed: 2.7 µM (3-CH_3, **3b**), 2.3 µM (3-F, **4b**), 3.6 µM (3-Cl, **5b**), and 3.4 µM (3-Br, **6b**). However, it could be mentioned that the dependence of the PET-inhibiting activity on the lipophilicity of the compounds expressed by log *k* was quasi-parabolic for C'$_{(3)}$-substituted derivatives, while for C'$_{(2)}$ ones a slight increase and for C'$_{(4)}$ derivatives a sharp decrease of the activity were observed with increasing lipophilicity. Consequently, it could be assumed that for targeting the site of action in the photosynthetic apparatus suggested on the acceptor side of photosystem II between P680 and plastoquinone Q_B substitution in the C'$_{(3)}$ position is the most favorable.

Previously tested six 2-(phenylcarbamoyl)phenyl *N*-[(benzyloxy)carbonyl] alkanoates and three 2-hydroxy-*N*-[(2 *S*)-1-oxo-1-(phenylamino)alkan-2-yl]benzamides showed MIC values in the range from 0.22 to 0.35 µM against *D. piger* Vib-7 and in the range from 0.27 to 8.52 µM against *Desulfomicrobium* sp. Rod-9, while the MIC values of ciprofloxacin were 41.2 µM and 39.3 µM, respectively. Lipophilicity was recognized as a significant parameter affecting biological activities, and higher activity for both SRB was observed rather with electron-withdrawing R^2 substituent and less lipophilic (isopropyl or benzyl) R^3 substituent, and it was supposed that these derivatives interact with enzymatic systems of the bacteria affecting vital cell functions (Kushkevych 2015a, b; Kushkevych et al. 2015a, b; Kushkevych et al. 2016). This is in agreement with the presented results concerning the antibacterial activity of ring-substituted 8-hydroxyquinoline-2-carboxanilides against *D. piger* Vib-7. Thus, these results confirmed that the investigated compounds showed high efficiency not only against the aerobic microorganisms, but also against the anaerobic microorganism.

Conclusion

Primary in vitro screening of a prepared series of ring-substituted 8-hydroxyquinoline-2-carboxanilides was performed against *Desulfovibrio piger* Vib-7. The most effective compounds with MIC/MBC values in the range of 17–23 µM/20–23 µM, respectively, were as follows: 8-hydroxy-*N*-(3-trifluoromethylphenyl)- (**7b**), 8-hydroxy-*N*-(3-methoxyphenyl)- (**2b**) 8-hydroxy-*N*-(3-methylphenyl)- (**3b**), and 8-hydroxy-*N*-(4-trifluoromethylphenyl)quinoline-2-carboxamide (**7c**). Their activity was twofold higher than that of ciprofloxacin. The antibacterial efficacy of the most active C'$_{(3)}$-substituted compounds practically did not change with increasing compound lipophilicity, indicating that this position of substitution is favorable for significant antimicrobial effect, while the antibacterial activity of most of C'$_{(2)}$ and C'$_{(4)}$-substituted derivatives decreased linearly with increasing compound lipophilicity. In addition, the dependence of activity on electronic Hammett's σ parameter of the substituent R was quasi-parabolic for the most effective C'$_{(3)}$-substituted compounds. The most potent compounds did not express any significant cytotoxic effect on THP-1 cells up to the tested concentration of 30 µM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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ATTACHMENT 11

Pages 115 – 121

Cross-correlation analysis of the *Desulfovibrio* growth parameters of intestinal species isolated from people with colitis

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ORIGINAL ARTICLE

Cross-correlation analysis of the *Desulfovibrio* growth parameters of intestinal species isolated from people with colitis

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Abstract

Sulfate-reducing bacteria can be involved in inflammatory bowel disease. Cross-correlation parameters of their metabolic process in the gut have never been reported before. The aim of the research was to statistically (cross-correlation) evaluate the parameters of growth (biomass) of *Desulfovibrio* species from colitis people and healthy as well as to investigate the change in dissimilatory sulfate reduction of these bacterial strains. The microbiological, biochemical, chemical, and statistical methods were used in the research. The cross-correlation analysis indicates that the strains isolated from people with colitis shifted to the right side of Y axe by biomass accumulation, sulfate consumption, lactate oxidation as well as hydrogen sulfide and acetate production, compared with the strains isolated from healthy individuals. Different percentages were observed in shifting to the right side of Y axe: biomass accumulation 26%, sulfate consumption 1.5%, sulfide production 5%, lactate oxidation 3% and acetate production 12%. The biomass accumulation of intestinal sulfate-reducing bacteria and their hydrogen sulfide production are the main factors in inflammatory bowel disease development, including ulcerative colitis. Acetate production showed lesser influence while sulfate consumption and lactate oxidation are negligible factors in the inflammatory bowel disease.

Keywords Bowel diseases · Ulcerative colitis · Hydrogen sulfide · Sulfate consumption

Abbreviations

SRB	sulfate-reducing bacteria
UC	ulcerative colitis
IBD	inflammatory bowel disease
DSR	dissimilatory sulfate reduction
OD	optical density
PCA	principal component analysis

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Introduction

Inflammatory bowel diseases (IBD) in animals and humans is highly correlated with the presence of increased number of sulfate reducing bacteria (SRB); consequently also by their sulfate reduction process in the gut (Gibson et al. 1991, 1993; Kushkevych et al. 2015a; Kováč et al. 2018). This bacterial group is present very often in individuals diagnosed with other diseases, including rheumatic diseases and with ankylosing spondylitis (Barton and Hamilton 2010; Cummings et al. 2003; Pitcher and Cummings 1996). SRB consume sulfate as electron acceptor and convert it in metabolic pathways to final products, hydrogen sulfide. Hydrogen sulfide, which is toxic and beside the support of IBD development (including UC and Crohn's disease), it can also cause high risk for neurodegenerative illness (DNA damage and mutation, enzyme inhibition and mitochondrial respiration inhibition) (Attene-Ramos et al. 2006; Kováč and Kushkevych 2017; Kushkevych et al. 2015b, 2017b, 2018c, d).

Exogenic electron donors are necessary to provide sulfate reduction process, including organic compounds (e.g. lactate, propionate, ethanol, butyrate, etc.), which can play a role as the source of carbon and energy (Kushkevych et al. 2015a, b).

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Lactate is a universal electron donor for this bacterial group. These organic compounds are oxidized by SRB, depending on genera, to acetate (incompletely) or carbon (IV) oxide and water (completely). Among SRB species, *Desulfovibrio* genus is the most often detected in feces from the patients with bloody diarrhea, weight loss, anorexia, epithelial hyperplasia and abscesses (both is animals and humans) (Loubinoux et al. 2000, 2002a, b; Kushkevych et al. 2017a, 2018a, d).

There is a constant increase of people diagnosed with ulcerative colitis. According to statistical results IBD (including UC) rose up to 12 per 100,000 people in Western countries (Low et al. 2013; Rowan et al. 2009). The prevalence in Europe and North America is around 20 per 100,000 individuals, while in Asia and the Middle East is around 6. The majority of UC patients are between ages of 15 and 30 years; another group with higher prevalence are individuals aged 50 to 70 years. The influence of gender has not been observed in ulcerative risk. Economic and health care burdens are increasing due to the growing UC prevalence since the severity of these inflammations can be overviewed by the fact that they very often lead to severe flares that require hospitalization in up to 25% of patients (Low et al. 2013; Feuerstein et al. 2016). Ulcerative colitis development represents complex molecular etiology including genetic, microbial, environmental and other still unknown factors. Many studies including animal and human samples have been conducted for better understanding of IBD, though many its segments have not been explained yet (Low et al. 2013; Steenland et al. 2018).

Medication and dosage in medical treatment affect location and severity of UC disease (Gibson et al. 1993; Pitcher and Cummings 1996; Kushkevych et al. 2016, 2018d).

Intestinal SRB are the part of the whole gut system, especially due to their interaction with other microorganisms. Their process of sulfate reduction is biosystem with autoregulation whose survival and development are largely dependent on the fluctuations of different parameters (Brynjarsdottir et al. 2003). The subordination of the biological clock rhythm serves as a determining factor in the regulation of oscillatory dynamics of these processes, during the simultanious separation of bacterial cells (accompanied by the fluctuations of biophysical and metabolic parameters) (Chen and Popovich 2002). In previous studies, ten various *Desulfovibrio* sp. strains (colitis: $n = 5$; healthy: $n = 5$) were isolated from the human intestine and identified. The growth of these bacteria, their use of sulfate and lactate as well as the production of hydrogen sulfide and acetate were also studied (Kushkevych 2013).

The novelty of the study is the comparison of sulfate reduction parameters (sulfate and lactate consumption, production of hydrogen sulfide and acetate, as well as growth of the SRB strains isolated from healthy and people with UC evaluated by cross-correlation method have never been presented yet.

The aim of the research was to statistically (cross-correlation) evaluate the parameters of growth (biomass) of *Desulfovibrio* species from colitis people and healthy as well as to investigate the change in dissimilatory sulfate reduction of these bacterial strains.

Materials and methods

Bacterial culture and cultivation In total, 10 of sulfate-reducing bacteria *Desulfovibrio* strains, five of them isolated from the healthy human large intestine and five another isolated from people with colitis, were as described previously (Kushkevych 2013). The strains have been kept in the collection of microorganisms at the Department of Experimental Biology, Faculty of Science at the Masaryk University (Brno, Czech Republic). The bacteria were grown for 36 h at 37 °C under anaerobic conditions in modified Postgate's liquid medium (Kováč and Kushkevych 2017; Postgate 1984). The redox and anaerobic conditions were controlled by sodium resazurin (Oxoid, BR 0055B) as an indicator. In addition reduced FeS and Na_2S contained in the medium provided the necessary redox conditions for SRB cultures. The discoloration of sodium resazurin (redox potential of discoloration Eh = −100 mV) confirmed the decrease of redox potential.

Assay of bacterial biomass About 1 mL of liquid medium without Mohr's salt was transferred into a plastic cuvette and taken to a biophotometer (Eppendorf BioPhotometer®D30) for taring. Subsequently, 1 mL of bacterial suspension was transferred into another cuvette and taken again to the biophotom eter for measuring at OD $\lambda = 340$. Before SRB were used for the experiments, optical density (OD_{340}) was always measured to assure approximately the same amount of bacteria in each experiment (Kushkevych et al. 2015b).

Assay of sulfate, lactate sulfide and acetate The sulfate ion concentration in the medium was determined by the turbidimetric method after it had been precipitated by barium chloride as was described in the paper (Kolmert et al. 2000). Lactate concentration was measured through the dehydrogenation reaction using Lactate Assay Kit (Sigma-Aldrich, Catalog Number MAK064). Sulfide concentration in the culture medium was assayed by the spectrophotometric method as was described (Cline 1969). Accumulation of acetate ions in the process of bacterial growth in the medium was determined using Acetate Assay Kit (Colorimetric, Catalog Number KA3764).

All analyses were done during 6 days period; the analysis (biomass determination, sulfate consumption, lactate oxidation, and production sulfide and acetate) were performed each day.

Statistical analysis The correlation analysis in time has been carried out for the study of temporal correlations between the

growth of bacteria and their metabolic processes. The values of the defined function parameters, namely the bacterial growth, the sulfate consumption, lactate oxidation as well as hydrogen sulfide and acetate production by the *Desulfovibrio* strains were used in the correlation study. Overall differences of indicated above parameters were checked by principal component analysis (PCA), using SPSS 20 statistical software (IBM Corporation, Armonk, USA). Cross-correlation plots were built by software package Origin7.0 (www.origin-lab.com). Using the experimental data, the basic statistical parameters (mean: M, standard error: m, M ± m) were calculated. The accurate approximation was when $p \leq 0.05$ (Bailey 1995).

Results

Cross-correlation correlograms that corresponds with data present in Table 1 are shown in Figs. 1, 2 and 3. All figures show noticeable differences between healthy and colitis objects, though the most noticeable difference is observed in biomass accumulation (Fig. 1) and the smallest in acetate production (Fig. 3). The differences between analyzed physiological and biochemical properties of bacterial strains isolated from feces can be seen through curve time shifts of colitis samples in comparison with curves indicating results obtained from healthy individuals. The positive time shifts of colitis patients are observed in all figures, though differences in results are also obtained. More significantly expressed positive correlations for colitis samples can be observed in results for biomass, sulfide and sulfate; quite low positive correlations were found for lactate and acetate (Figs. 1, 2 and 3). Cross-correlation data between sulfate reduction parameters in *Desulfovibrio* isolates from healthy objects and people with colitis are shown in Table 1. From the Table 1 can be observed that noticeable ($p < 0.05$) differences exist between healthy and colitis objects in all measured parameters (biomass accumulation, sulfate and lactate consumption, sulfide and acetate production). The differences in overall cross-correlation factors are not very well noticeable (the differences are clearly observable in Fig. 4), but differences between the results in the middle of cross-correlation factors (the results over zero, where stability is assumed) between healthy and colitis objects in biomass accumulation was 26%, sulfate consumption 1.5%, sulfide production 5%, lactate oxidation 3% and acetate production 12%. The confirmation of these results is shown Fig. 4 by principal component analysis (PCA). According to Eigen values 3 groups are formed in PCA graph, showing differences between healthy and colitis individuals. The most remarkable differences are confirmed with separate PCA group differentiating biomass, sulfate and sulfide results of colitis samples from other samples.

Discussion

The SRB are wide-spread in the nature and sulfate rich environment, especially in different bioreactors where they interact with methanogenic microorganisms (Kushkevych et al. 2018b, c). Sulfate in food may play an important role in human metabolism. Free sulfate ions affect large bowel metabolism where it is reduced to hydrogen sulfide (the substance that is considered

Table 1 Data on cross-correlations between sulfate reduction parameters in *Desulfovibrio* isolates from healthy objects and people with colitis

Series Pair/Lag	Biomass		Sulfate		Sulfide		Lactate		Acetate	
	Healthy	Colitis	Healthy	Colitis	Healthy	Colitis	Healthy	Colitis	Healthy	Colitis
-7	−0.29	−0.21	−0.28	−0.21	−0.30	−0.26	−0.34	−0.31	−0.40	−0.39
−6	−0.33	−0.22	−0.28	−0.22	−0.35	−0.27	−0.36	−0.34	−0.47	−0.46
−5	−0.28	−0.23	−0.23	−0.23	−0.29	−0.27	−0.28	−0.27	−0.38	−0.40
−4	−0.18	−0.18	−0.14	−0.18	−0.17	−0.19	−0.15	−0.15	−0.13	−0.19
−3	−0.01	−0.09	0.04	−0.09	0.03	−0.05	0.08	−0.01	0.17	0.10
−2	0.29	0.04	0.22	0.04	0.21	0.13	0.32	0.20	0.47	0.42
−1	0.64	0.34	0.53	0.34	0.55	0.40	0.65	0.50	0.72	0.73
0	0.96	0.92	0.97	0.92	0.97	0.95	0.97	0.97	0.95	0.99
1	0.50	0.55	0.42	0.55	0.57	0.59	0.52	0.62	0.61	0.68
2	0.14	0.28	0.08	0.28	0.27	0.29	0.14	0.27	0.27	0.35
3	−0.09	0.09	−0.08	0.09	0.03	0.10	−0.08	0.03	−0.07	0.00
4	−0.22	−0.16	−0.19	−0.16	−0.17	−0.12	−0.21	−0.13	−0.29	−0.25
5	−0.28	−0.24	−0.24	−0.24	−0.28	−0.28	−0.30	−0.26	−0.38	−0.39
6	−0.29	−0.27	−0.24	−0.27	−0.34	−0.30	−0.30	−0.35	−0.37	−0.42
7	−0.25	−0.27	−0.23	−0.27	−0.33	−0.32	−0.27	−0.33	−0.30	−0.35

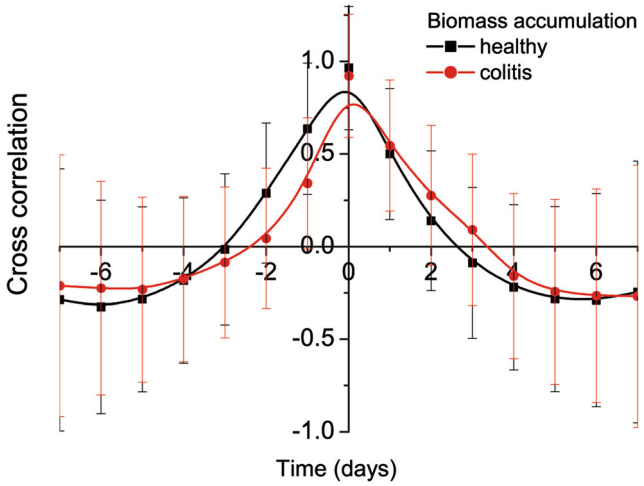

Fig. 1 Cross-correlation correlogram showing the bacterial growth (biomass accumulation) of the *Desulfovibrio* strains isolated from healthy people and with colitis. *each point represents one day measurement during six days period

potentially toxic to colonic epithelium. Sulfate concentrations are found in more than 200 individual foods (in concentrations: >10 μmol/g or 1 mg/g), including breads, soya flour, some dried fruits, some brassicas, and some sausages. Beers, ciders and wines are also considered sulfate rich sources (in concentrations: >2.5 μmol/mL or 0.25 mg/mL). The sulfate content of beer is commented in epidemiological observations which linked ingestion of beer with colorectal cancer (Florin et al. 1993).

The intestinal SRB metabolize sulfate free ions as an electron acceptor to hydrogen sulfide. This process of sulfate dissimilation is called the "dissimilatory sulfate reduction" or "sulfate respiration" (Kushkevych 2017a). For this process, SRB need organic compounds or molecular hydrogen which are exogenous electron donors. These organic compounds can be oxidized incompletely to acetate (acetogenic SRB, in particular *Desulfovibrio* species) or completely to CO_2 (Barton and Hamilton 2010). The sulfate reduction intensity by SRB and, accordingly, hydrogen sulfide production in high concentrations in the intestine can lead to the development of various diseases (Kushkevych et al. 2015a).

The cross-correlation method represents a generalization of standard linear correlation analysis. The strength of the correlation is measured by the correlation coefficient. Cross-correlation analysis is the most commonly used as multiple time series analysis. Cross-correlation gives a correlation between two time series or two waveforms. One series observations are correlated with other series observations (Bourke 1996). The cross-correlation data sets of two time-series test involves many calculations of the coefficient *r* by time-shifting the one data set relative to the other data set. The sift is called "lag", while the lag time is simply the sampling period of the two time-series data sets (Bourke 1996). As can be seen from correlograms (Figs. 1, 2 and 3) and Table 1, cross-correlation values close to 1 represent the strong relationship between two waveforms and weak relationship indicate correlation values close to zero. Negative correlation values, below zero, are emphasizing a lack of stability in the relationship (Chen and Popovich 2002; Bourke 1996). Different landslides of some processes are determined by a positive correlation coefficient value (Chen and Popovich 2002). The cross-correlation analysis is built from the correlograms for *Desulfovibrio* sp. strains. A correlogram is a sequence of values of the correlation coefficients.

It is well known that *Desulfovibrio* genus are the most often detected in people with IBD, including ulcer active colitis (Kushkevych 2015a, b; Kushkevych et al. 2015a, b). The presence of sulfate and lactate in the human intestine influences bacterial growth and the accumulation of their final metabolic products: acetate and hydrogen sulfide. Hydrogen sulfide is toxic, mutagenic same as cancerogenic for epithelial intestinal cells (Pitcher and Cummings 1996; Rowan et al. 2009). This final metabolite in high concentrations is also carcinogenic for intestinal cells and can cause inhibition of cytochrome oxidase, colonocytes oxidation of butyrate, destruction of epithelial cells, and development of ulcers and

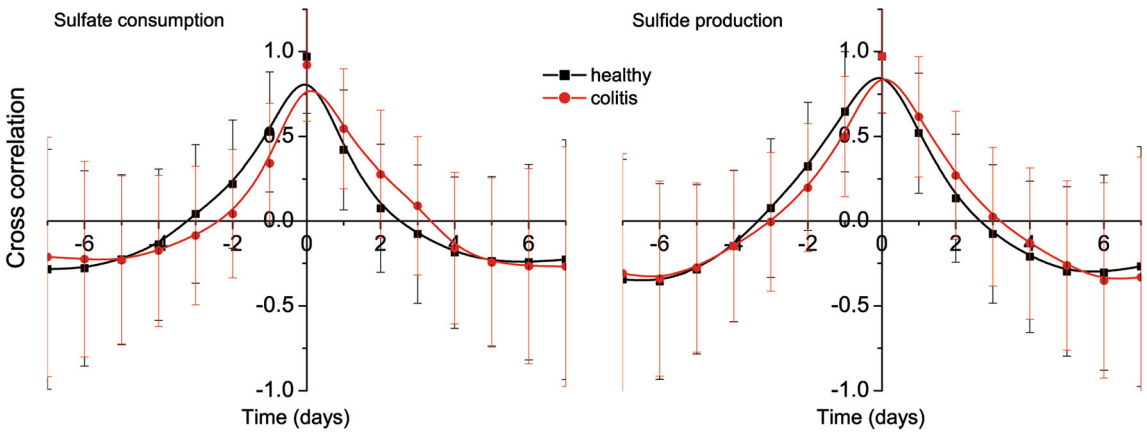

Fig. 2 Cross-correlation correlograms showing sulfate consumption and hydrogen sulfide production with time by bacterial *Desulfovibrio* strains, isolated from healthy and people with colitis. *each point represents one day measurement during six days period

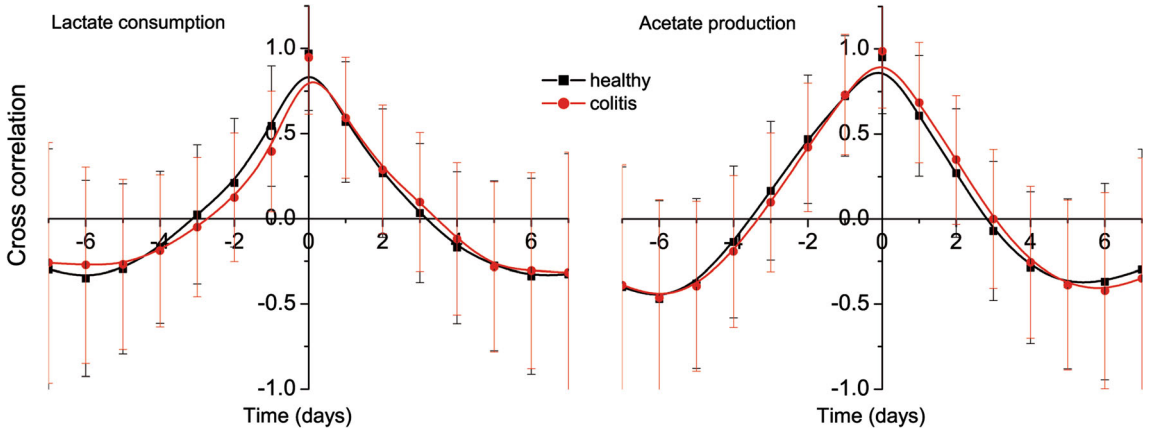

Fig. 3 Cross-correlation correlograms showing lactate consumption and acetate production with time by *Desulfovibrio* bacterial strains isolated from healthy and people with colitis

inflammation with subsequent development of ulcerative colitis (Pitcher and Cummings 1996; Gibson et al. 1991; Cummings et al. 2003; Kushkevych 2015c, d).

The illness development is caused by the increase amount of intestinal SRB and their production of hydrogen sulfide. The shift of the *Desulfovibrio* growth (biomass) and parameters of sulfate reduction to right side of Y axe (shown in Figs. 1, 2 and 3) can be a main cause disturbance of relationship of microbial populations in the gut. Consequently, the shift can indicate the development of IBD, including UC. On the other side, acetate produced by intestinal SRB can interact with hydrogen sulfide can increase its effect (synergic effect), leading to increased IBD risk. Attene-Ramos et al. (2006) published the research in which the authors are emphasizing that Na_2S is able to provoke genomic DNA damage in colonic cells (Attene-Ramos et al. 2006). A consensus and better understanding of the metabolic pathways involved in H_2S metabolism in colonocytes is still needed. Imbalance between the concentration of free sulfide in the large intestine mucosa and the metabolisms capacity of epithelial cells results in a loss of normal oxidative cell capacity. Endogenously produced H_2S plays a role in the neuromodulation of chloride secretion, in intestinal contractibility control, and on the large intestine nociception. Though, it is still not confirmed that H_2S is acting as a pro- or antinociceptive agent toward the large intestine. Mammalian cells use very simple sulfur-containing gas molecule due to the reminiscence of an ancestral function (Griesbeck et al. 2002).

Hence, the role of intestinal SRB in colonic conditions is important in better understanding of their ability to inhibit the

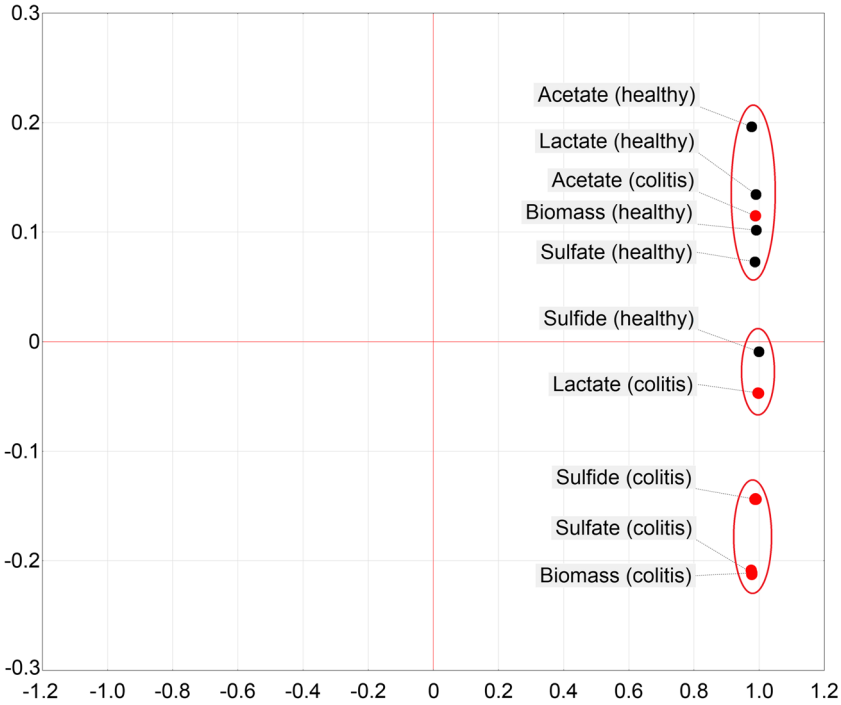

Fig. 4 Principal component analysis (PCA) of cross-correlation data between sulfate reduction parameters in *Desulfovibrio* isolates (data referring to Table 1). *each point represents one day measurement during six days period

production of hydrogen sulfide and acetate. Overall, all stated issues are emphasizing the factors influencing sulfide production in the human and animal colon.

Conclusions

Biomass accumulation of intestinal SRB and their hydrogen sulfide production are the main factors in IBD development, including UC. The statement is in accordance with observation from correlograms obtained by cross-correlation. Acetate production showed lesser influence. It should be also not underestimated the possibility of acetate production synergy with H_2S. According to gained results, sulfate consumption and lactate oxidation are negligible factors in IBD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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ATTACHMENT 12

Pages 122 – 130

Toxicity of hydrogen sulfide toward sulfate-reducing bacteria *Desulfovibrio piger* Vib-7.

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ORIGINALPAPER

Toxicity of hydrogen sulfide toward sulfate-reducing bacteria *Desulfovibrio piger* Vib-7

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Abstract

Sulfate-reducing bacteria (SRB) belonging to the intestinal microbiota are the main producers of hydrogen sulfide and their increasing amount due to the accumulation of this compound in the bowel are involved in the initiation and maintenance of inflammatory bowel disease. The purpose of this experiment is to study the relative toxicity of hydrogen sulfide and survival of *Desulfovibrio piger* Vib-7 through monitoring: sulfate reduction parameters (sulfate consumption, hydrogen sulfide production, lactate consumption and acetate production) and kinetic parameters of these processes. The research is highlighting the survival of intestinal SRB, *D. piger* Vib-7 under the influence of different hydrogen sulfide concentrations (1–7 mM). The highest toxicity of H_2S was measured in the presence of concentrations higher than 6 mM, where growing was stopped, though metabolic activities were not 100% inhibited. These findings are confirmed by cross correlation and principal component analysis that clearly supported the above mentioned results. The kinetic parameters of bacterial growth and sulfate reduction were inhibited proportionally with increasing H_2S concentration. The presence of 5 mM H_2S resulted in two times longer lag phase and generation time was eight times longer. Maximum rate of growth and hydrogen production was stopped under 4 mM, emphasizing the H_2S toxicity concentrations to be <4 mM, even for sulfide producing bacteria such as *Desulfovibrio*. The results are confirming H_2S concentrations toxicity toward *Desulfovibrio*, especially the study novelty should be emphasized where it was found that the exact H_2S limits (>4 mM) toward this bacterial strain inhabiting humans and animals intestine.

Keywords Hydrogen sulfide · Toxicity · Sulfate-reducing bacteria · Ulcerative colitis

Abbreviations

SRB Sulfate-reducing bacteria
UC Ulcerative colitis
IBD Inflammatory bowel disease
DSR Dissimilatory sulfate reduction
OD Optical density
PCA Principal component analysis

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Introduction

The main product of the sulfate-reducing bacteria metabolism is hydrogen sulfide (Barton and Hamilton 2010; Kushkevych et al. 2015a, b; Kushkevych 2015a, b). Hydrogen sulfide can be also created endogenously by trans-sulfurization and it is present in low (non-toxic) concentrations in the brain, heart, blood vessels, genitourinary and gastrointestinal tract (Pitcher and Cummings 1996; Attene-Ramos et al. 2006). Butyrate oxidation, which is the main source of energy for intestine colonocytes of humans and animals is inhibited by hydrogen sulfide higher concentrations (Cummings et al. 2003; Rowan et al. 2009). Intestinal ulceration in rats is caused by perfusion of colon by low concentrations of hydrogen sulfide. The literature data are indicating a connection between the sulfate reducing bacteria and the

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development of the inflammatory bowel diseases. It should be stressed out that the important factor is also the intensity of the hydrogen sulfide concentration produced in the colon lumen by these bacteria (Gibson et al. 1991, 1993; Loubinoux et al. 2000, 2002a, b; Griesbeck et al. 2002a, b; Kushkevych et al. 2016, 2018a, b, c, d, e; Kováč et al. 2018). H_2S production is higher in the distal intestine than in the proximal part (Attene-Ramos et al. 2006). The dense biofilms around ulcer are formed by sulfate reducing bacteria (Gibson et al. 1991, 1993; Pitcher and Cummings 1996; Loubinoux et al. 2000, 2002a, b; Griesbeck et al. 2002a, b; Cummings et al. 2003; Attene-Ramos et al. 2006; Rowan et al. 2009; Barton and Hamilton 2010; Kushkevych et al. 2015a, 2016, 2018a, b, c, d, e; Kováč et al. 2018). The fringe between the ulcer and the colonies of the bacteria depends on the following factors: immune status of the organism (host), the intestinal lumen pH and the availability of sulfate (Loubinoux et al. 2000, 2002a). The permeability of the epithelial barrier of the oral mucosa cells is increased by H_2S (Pitcher and Cummings 1996; Rowan et al. 2009; Blachier et al. 2010).

There are literature data on the effect of hydrogen sulfide on various microorganisms, including yeast, fungi, other environmental or intestinal bacteria (Beauchamp et al. 1984). However, there are no sufficient numbers of researchers focused on the influence of this compound toward its producers (e.g., SRB of *Desulfovibrio* genus). Since the sulfate-reducing bacteria are producers of hydrogen sulfide, it is very interesting to investigate their sensitivity to this highly toxic compound.

The aim of this work was to study the relative toxicity of hydrogen sulfide and survival of *Desulfovibrio piger* Vib-7 through monitoring: sulfate reduction parameters (sulfate consumption, hydrogen sulfide production, lactate consumption and acetate production) and kinetic parameters of these processes.

Materials and methods

Bacterial culture and cultivation

The objective of the study was the sulfate-reducing bacteria of the *D. piger* strain Vib-7 isolated from the human large intestine. The isolated strain was identified based on physiological and biochemical properties as described in the paper (Kushkevych 2013) and sequence analysis of 16S rRNA gene (Kushkevych et al. 2014). The GenBank accession number: KT881309.1. The strain was kept in the collection of microorganisms at the Laboratory of Anaerobic Microorganisms of Department of Experimental Biology at Masaryk University (Brno, Czech Republic).

Bacteria were grown in modified liquid Postgate's C medium with following composition (Postgate 1984) (g/l): Na_2SO_4 (0.5), KH_2PO_4 (0.3), K_2HPO_4 (0.5), $(NH_4)_2SO_4$ (0.2), NH_4Cl (1.0), $CaCl_2 \times 6H_2O$ (0.06), $MgSO_4 \times 7H_2O$ (0.1), $C_3H_5O_3Na$ (2.0), yeast extract (1.0), $FeSO_4 \times 7H_2O$ (0.004), $C_6H_5O_7Na_3 \times 2H_2O$ (0.3). The medium was heated in boiling water for 30 min to obtain an oxygen-free medium, and cooled to + 37 °C temperature. The final optimal pH 7.5 was provided by a sterile 1 M solution of NaOH (0.9 ml/l). The bacteria were grown for 72 h at 37 °C under anaerobic conditions. The tubes with strain were brim-filled with medium and closed to provide anaerobic conditions (Kováč and Kushkevych 2017).

Inoculation hydrogen sulfide and its determination

A sterile solution of $Na_2S \times 9H_2O$ with different concentration was added before bacterial seeding the liquid medium. The final concentration of hydrogen sulfide in the medium was 1.0; 2.0; 3.0; 4.0; 5.0; 6.0 and 7.0 mM. The *D. piger* Vib-7 growth (biomass) and their process of dissimilatory sulfate reduction (consumption of sulfate and lactate, production of hydrogen sulfide and acetate) under the effect of additional introduction of H_2S were studied. The medium without $Na_2S \times 9H_2O$ served as a control.

Bacterial biomass determination

About 1 mL of liquid medium without Mohr's salt was transferred into a plastic cuvette and taken to a biophotometer (Eppendorf BioPhotometer®D30) for taring. Subsequently, 1 mL of bacterial suspension was transferred into another cuvette and taken again to the biophotometer for measuring at OD $\lambda = 340$. Before SRB were used for the experiments, optical density (OD_{340}) was always measured to assure approximately the same amount of bacteria in each experiment (Kushkevych et al. 2017a).

Sulfate determination

The content of sulfate in the medium was determined by turbidimetric method right after seeding and after 24 h cultivation. The calibration curve has been constructed with the same process. Calibration solutions, has been prepared in distilled water at concentrations of a 2, 4, 8, 16, 24, 32, 40 and 48 µM sodium sulfate. A suspension of 40 mg/L $BaCl_2$ has been prepared in 0.12 M HCl. The resulting solution was mixed with glycerol in a 1:1 ratio. To the 1 mL of sample supernatant after centrifugation at 5000×*g* at 23 °C was added to 10 mL of prepared $BaCl_2$:glycerol solution and carefully stirred. The mixture has been let to stand for 10 min and right after that the absorbance has been measured at 520 nm (Spectronic Genesys 5). As a control, the

measurement was repeated in the same manner using a cultivation medium (Kolmert et al. 2000).

Hydrogen sulfide determination

Hydrogen sulfide was measured spectrophotometrically right after seeding and after 72 h of cultivation. Calibration solutions were prepared in distilled water at concentrations of 12.5, 25, 50, and 100 µM sodium sulfide. The calibration curve has been constructed with the same process. 1 mL of the sample was added to 10 mL of 5 g/L aqueous solution of zinc acetate. Right after, 2 mL of 0.75 g/mL *p*-aminodimethylaniline in a solution of sulfuric acid (2 M) was added. The mixture could stand for 5 min at room temperature. After that, 0.5 mL of 12 g/L solution of ferric chloride dissolved in 15 mM sulfuric acid was added. After standing another 5 min at room temperature, the mixture was centrifuged 5000×*g* at 23 °C. The absorbance of the mixture was determined to measure hydrogen sulfide at a wavelength of 665 nm by a spectrophotometer (Cecil Aquarius CE 7200 Double Beam Spectrophotometer) (Cline 1969).

Lactate and acetate determination

The measurement was repeated in the same manner using a cultivation medium and it served as the control sample (Bailey and Pluth 2013). Measurements of lactate concentration using Lactate Assay Kit (Sigma-Aldrich, Catalog Number MAK064) were carried out. Accumulation of acetate ions in the process of bacterial growth in the medium was determined using Acetate Assay Kit (Colorimetric, Catalog Number KA3764).

Statistical analysis

Using the experimental data, the basic statistical parameters (M—mean, m—standard error, $M \pm m$) have been calculated. The accurate approximation was when $p \leq 0.05$ (Bailey 1995). Statistical significance was measured with the use of principal component analysis (PCA) that gave overall differences among compared groups. Statistical analysis was done by SPSS 20 statistical software (IBM Corporation, Armonk, USA). Plots were built by software package Origin7.0 (http://www.origin-lab.com).

Results and discussion

Relative toxicity of hydrogen sulfide and survival parameters of *D. piger* Vib-7 (bacterial growth and sulfate reduction parameters: sulfate consumption, hydrogen sulfide production, lactate consumption, and acetate production) are shown in Figs. 1 and 2.

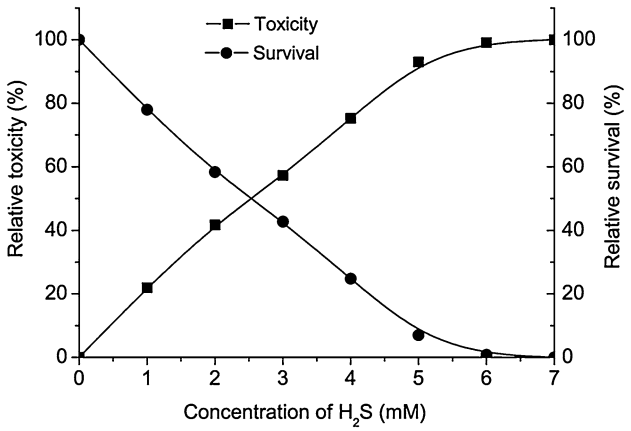

Fig. 1 Relative toxicity of hydrogen sulfide and survival of *D. piger* Vib-7

The addition of sulfide ions in concentrations of 1 mM to the growth media resulted in 21% relative toxicity and 78% survival of bacterial cells. The increase of sulfide ion concentrations from 2 to 3 mM increased relative toxicity to about 50%, same as the decrease of bacterial survival ($IC_{50} > 2$ mM) (Fig. 1). The observed trend in Fig. 1 corresponds to data dissimilatory sulfate reduction process (Fig. 2). The concentrations (> 3 mM) of sulfide ions lead to more significant trend in relative toxicity growth and decrease in bacterial survival, while the highest added (6–7 mM) concentrations possessed toxicity that almost totally reduced bacterial survival (Figs. 1, 2). However, the process of sulfate consumption (93%), lactate oxidation (94%), hydrogen sulfide production (91%) and acetate accumulation (91%) were not totally stopped under high sulfide ion concentrations (6–7 mM) that is indicating cell division inhibition, but not the end of metabolic processes.

The influence of hydrogen sulfide on *D. piger* Vib-7 growth and their sulfate reduction parameters was carried out by cluster analysis shown in Fig. 3. The cluster analysis grouped important metabolic parameters: growth and sulfate consumption belong to one cluster, which is connected with hydrogen sulfide production; the process of sulfate reduction to hydrogen sulfide needs exogenous electron donor (lactate) that is oxidized to acetate. The gained cluster analysis unambiguously supports this process.

Cross-correlation correlograms are shown in Figs. 4 and 5. The results are indicating both negative (between: biomass and sulfate, biomass and lactate, sulfate and sulfide, lactate and acetate) and positive (between: biomass and sulfide, biomass and acetate) correlations. The low sulfide concentrations (1–3 mM) did not affect significantly coefficients of correlation, measured at the time. On the contrary, higher

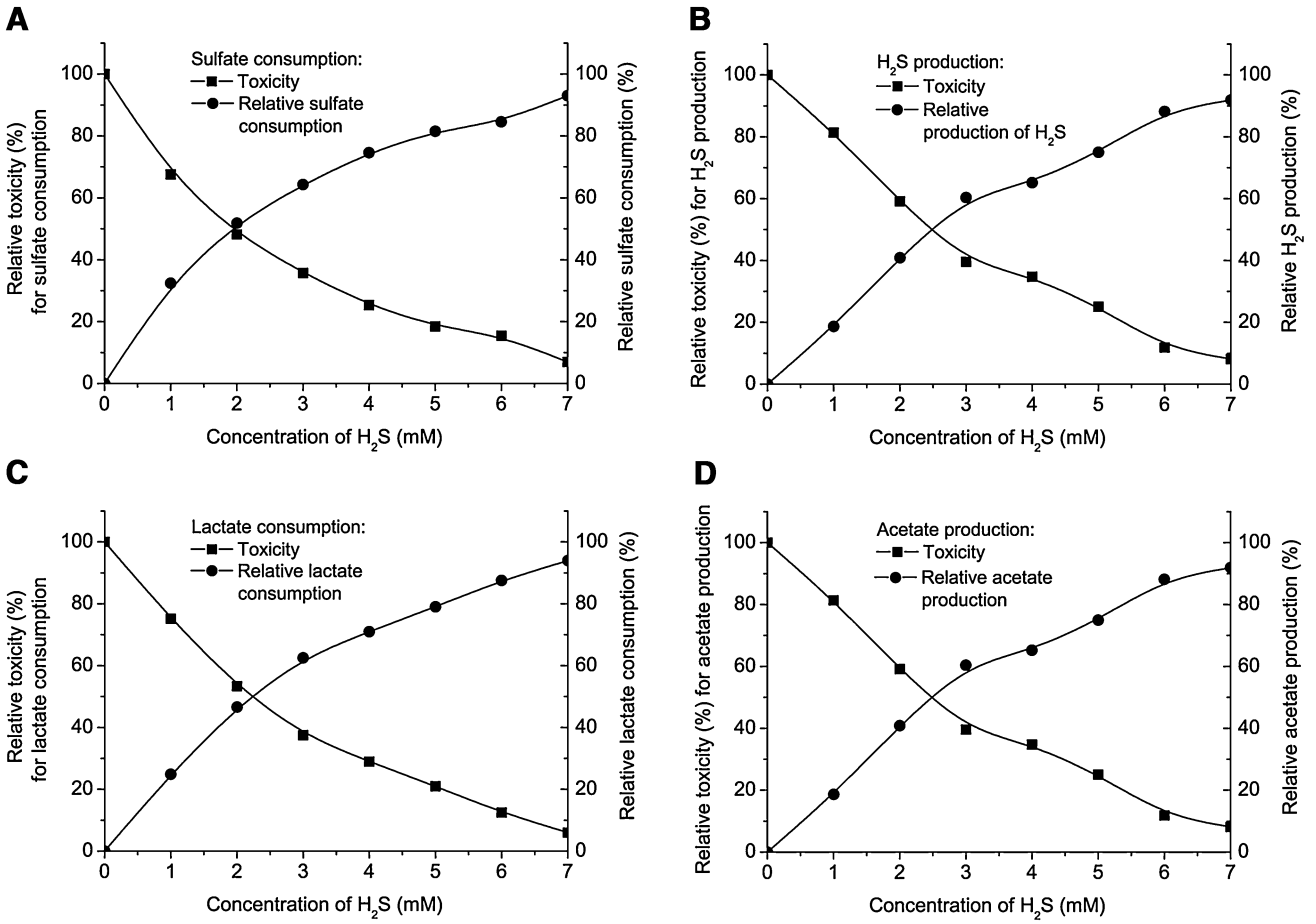

Fig. 2 Relative toxicity of hydrogen sulfide for sulfate reduction parameters: sulfate consumption (**a**), hydrogen sulfide production (**b**), lactate consumption (**c**), and acetate production (**d**)

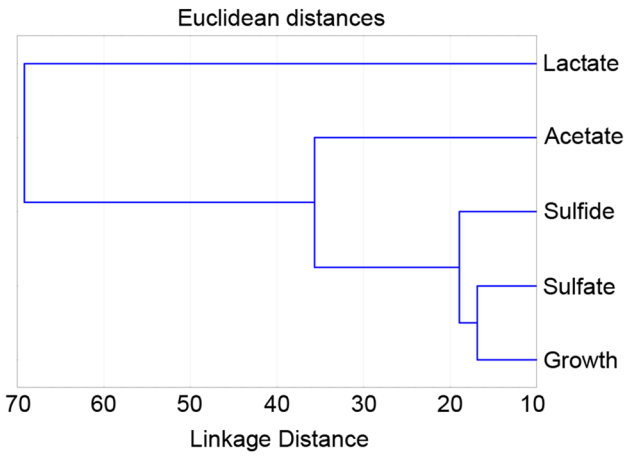

Fig. 3 Cluster analysis of *D. piger* Vib-7 growth and sulfate reduction parameters under effect of hydrogen sulfide

sulfide concentrations (from 4 to 5 mM) influenced significantly ($p < 0.05$) all measured parameters, beside lactate and acetate, in comparison with control samples (Fig. 4).

Reciprocal results are shown in Fig. 5, with the positive (between: sulfate and lactate, sulfide and acetate) and negative correlations (between: sulfide and lactate, sulfate and acetate), in comparison with the control samples. Sulfide concentrations affected most significantly ($p < 0.05$) sulfate consumption and lactate oxidation, same as sulfate consumption and acetate production. Contrarily, different H_2S concentrations did not have an impact on sulfide and lactate, though slight influence was noticed between sulfide and acetate (Fig. 5).

The results gained by cross correlation analysis were grouped by principal component analysis (PCA) as shown in Fig. 6. PCA analyses were done, including all measured parameters, but also separately for each parameter. PCA analysis that included all measured parameters made two clusters: the first cluster is formed by 1 mM, 2 mM and 3 mM; the second cluster is formed from 4 mM and 5 mM. These groups are clearly showing significant ($p < 0.05$) influence of sulfide when it is added, especially at higher concentrations (> 4 mM), compared with control samples (Fig. 6).

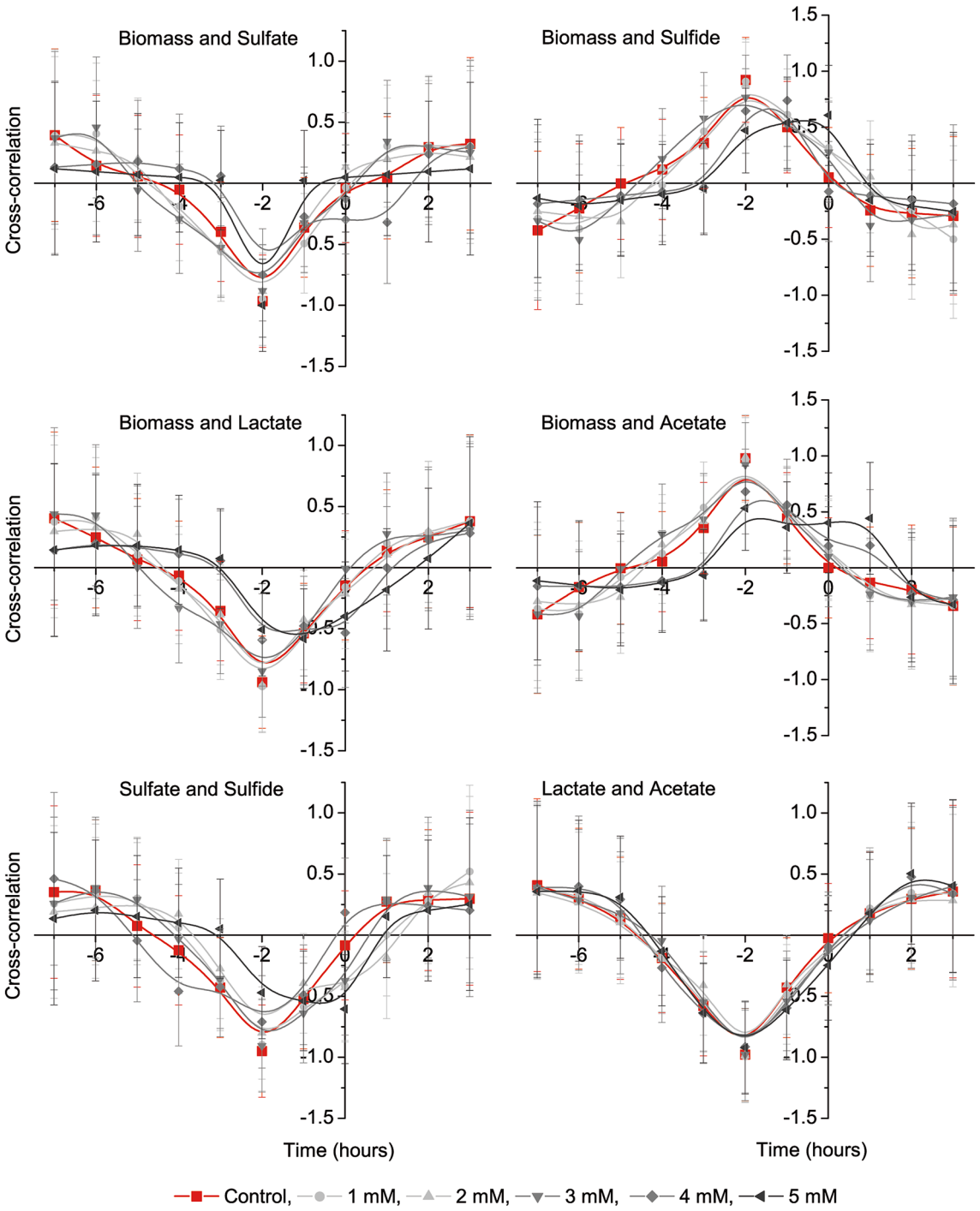

Fig. 4 Cross-correlation analysis between biomass accumulation and sulfate consumption, sulfide production, lactate oxidation and acetate accumulation as well as between sulfate and sulfide, same as between lactate and acetate

Kinetic parameters (lag phase, generation time, and the maximum rate of the bacterial growth, hydrogen sulfide and acetate production) of *D. piger* Vib-7 affected by the hydrogen sulfide addition, during 72 h of cultivation were calculated and presented in Table 1. Higher H_2S concentrations had high impact on all calculated parameters. The time of lag phase was two times longer in the presence of 5 mM H_2S, while generation time (time of division, T_d) was eight times longer. The maximum rate of the growth, hydrogen sulfide and acetate was calculated in control samples; the presence of H_2S resulted in smaller maximum

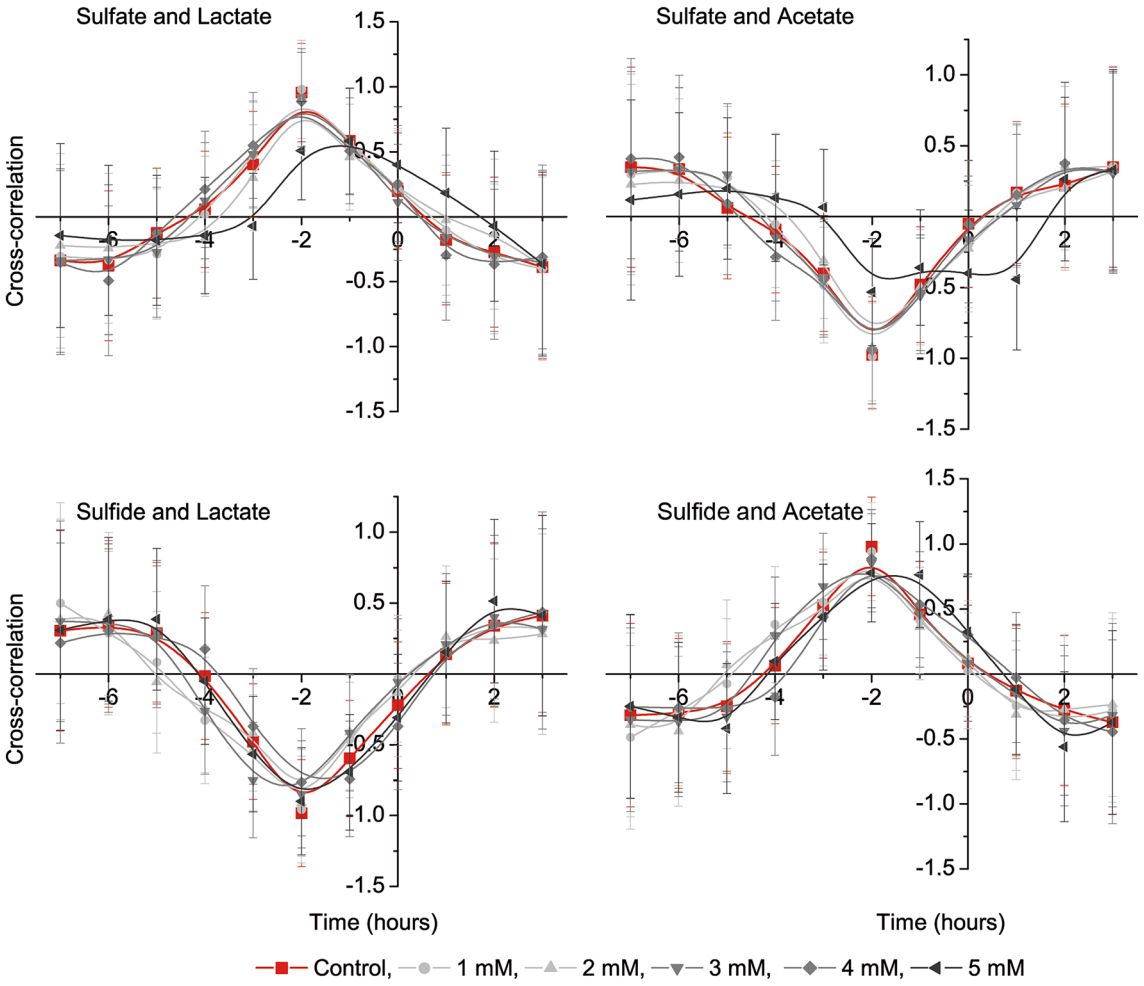

Fig. 5 Cross-correlation analysis between sulfate consumption and lactate oxidation, acetate production as well as sulfide and lactate, same as between sulfide and acetate

rate parameters where almost the absence of rates were observed in the presence of 4 mM and 5 mM.

Relative toxicity of hydrogen sulfide is obtained by our research, where it was found that the external H_2S addition inhibited the growth of *D. piger* Vib-7 and their process of sulfate reduction, including sulfate consumption, hydrogen sulfide production, lactate consumption, and acetate production, corresponding with bacteria survival. These findings were confirmed by cluster analysis, cross-correlation and principal component analysis, same as with kinetic parameters of *D. piger* Vib-7 growth under the effect of hydrogen sulfide during 72 h of cultivation.

Intestinal bacteria are sensitive to hydrogen sulfide, same as SRB, *D. piger* Vib-7, which are its producers, but at the same time their limiting factor for growing is higher H_2S concentrations. The lumen of the human large intestine is the point where hydrogen sulfide is present in millimolar concentrations (1.0–2.4 mM) (Blachier et al. 2010). Nevertheless, because of a large capacity of fecal components to bind the sulfide the concentration of free (unbound) sulfide is in the micromolar range (Pitcher and Cummings 1996; Attene-Ramos et al. 2006). Hydrogen sulfide can be formed by endogenous sulfur-containing compounds, including amino acids. The excessive concentrations of H_2S can severely inhibit cytochrome c oxidase, the terminal oxidase of the mitochondrial electron transport chain, and mitochondrial oxygen consumption. Colonocytes are able to metabolize H_2S and that represents an important feature for their resistance toward an excessive concentration of free luminal sulfide (Blachier et al. 2010). Prolonged excessive concentration of sulfide in the luminal content of the large intestine and detoxifying catalytic activities in the large intestine epithelium in the colon carcinogenesis have been according to the literature data scarcely investigated. The isolated bacteria *D. piger* Vib-7 can be considered as one of the very promising points for further studies. Na_2S at concentrations of 500 mol/L (and above) provoked genomic DNA damage in HT-29-Cl.16E colonic cells, according to Attene-Ramos

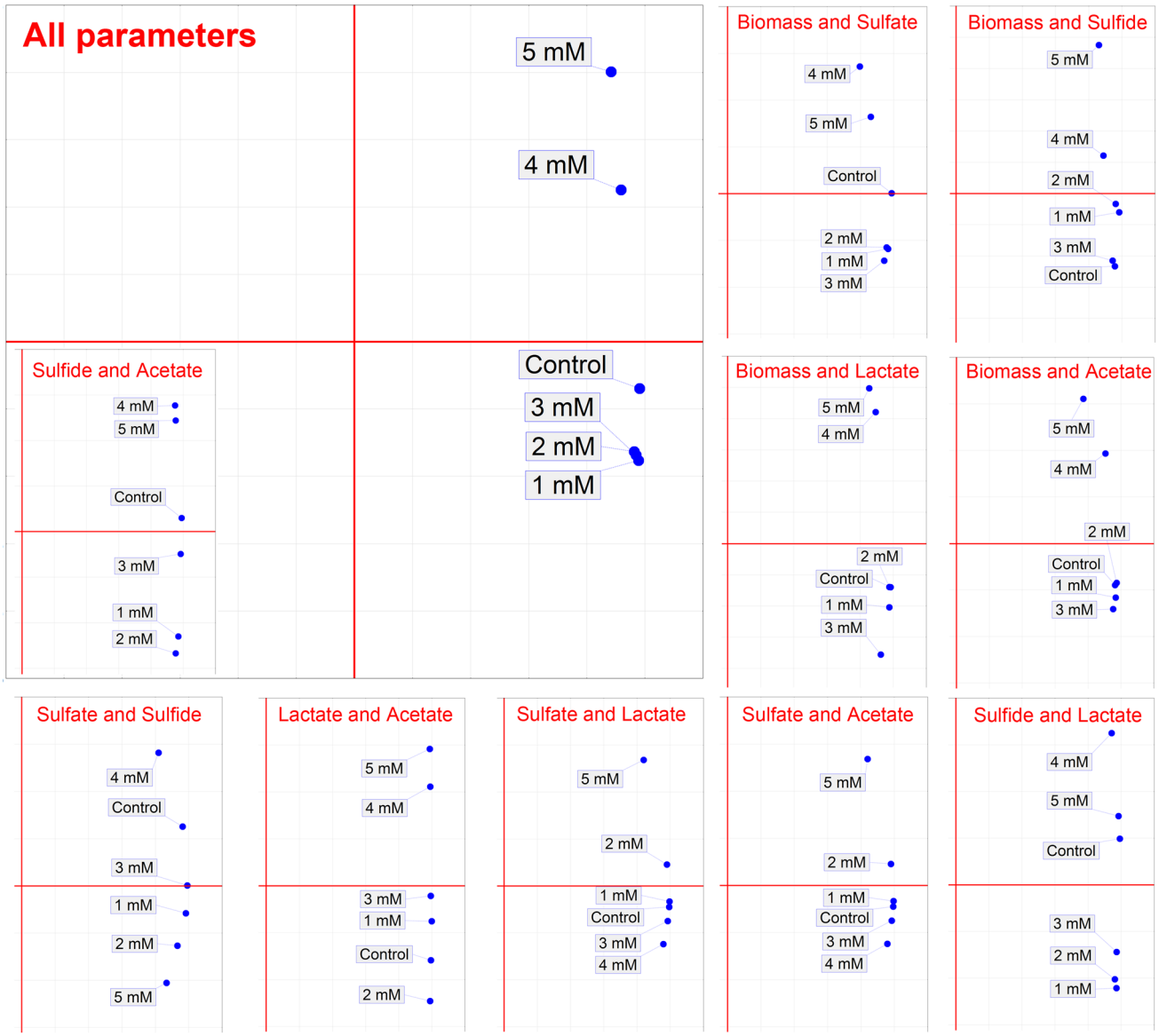

Fig. 6 Principal component analysis of the *D. piger* Vib-7 growth and the parameters of sulfate reduction under effect of hydrogen sulfide

Table 1 Kinetic parameters of *D. piger* Vib-7 growth under effect of hydrogen sulfide during 72 h of cultivation

H_2S (mM)	Lag phase (h)	Generation time T_d (h)	Maximum rate of production (μ_{max}, h^{-1})		
			Growth (biomass)	Hydrogen sulfide	Acetate
0	6.58 ± 0.61	1.79 ± 0.14	0.049 ± 0.0042	0.034 ± 0.0036	0.103 ± 0.009
1	6.65 ± 0.65	2.37 ± 0.22	0.037 ± 0.0036	0.025 ± 0.0022	0.093 ± 0.0088
2	7.72 ± 0.73	3.31 ± 0.29	0.025 ± 0.0022	0.013 ± 0.0011	0.079 ± 0.0072
3	8.05 ± 0.76	6.20 ± 0.57	0.011 ± 0,0001	0.004 ± 0.0001	0.064 ± 0,0057
4	10 ± 1.11	15.21 ± 1.55	0.001 ± 0.0001	0.001 ± 0.0001	0.052 ± 0.0046
5	15 ± 1.23	15.29 ± 1.61	0.001 ± 0.0001	0	0.031 ± 0.0025

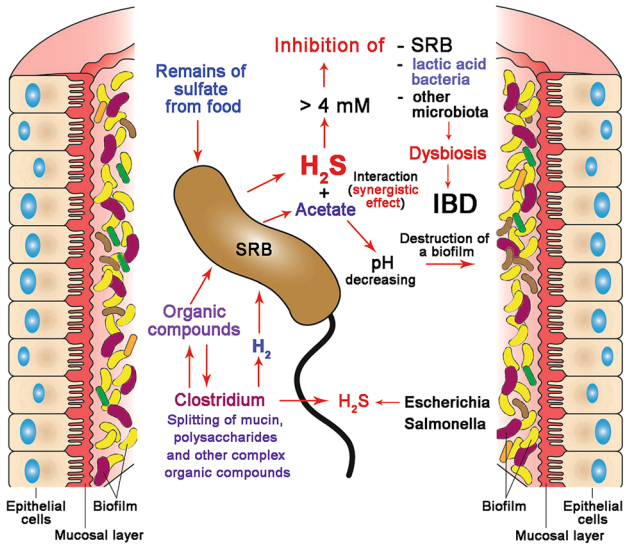

Fig. 7 Generalization schema of effect of H_2S on SRB and other microbial groups

et al. (2006); the authors in the research used the assay in which DNA repair was inhibited.

From the available data considering the effects of H_2S on the large intestine mucosa, it appears that this compound coming from the microbiota metabolic activity can interfere with the colonic epithelial cell metabolism. Both beneficial and deleterious effects of luminal sulfide are possible toward this intestinal epithelium. The generalization schema reflecting effect of H_2S on SRB and other groups of the microorganisms is shown in Fig. 7. On the other side, hydrogen sulfide can be produced by other microorganisms, such as *Clostridium* species, that can decompose more complex organic compounds (Černý et al. 2018). Consequently, formed compounds can be more readily used by *Desulfovibrio* bacteria strains as electron donors. Sulfides can possess advantage and disadvantage properties in dependence with: sulfide concentration inside the colonic lumen, substrate availability from exogenous (alimentary) and endogenous origins, metabolic capacity for the microbiota to produce H_2S, percentages of sulfide in free and bound forms, capacities of colonic epithelial cells to detoxicate and to use sulfide as an energy source, availability of anaerobic metabolic pathway (i.e., glycolysis) and capacity of colonic epithelial cells to rapidly adapt to an excess in luminal sulfide production (Attene-Ramos et al. 2006; Blachier et al. 2010).

When sulfide is present in excess, sulfide quinone oxidoreductase, sulfur dioxygenase, and rhodanese are likely to play an important role in the detoxication process, though consensus is still needed regarding the metabolic pathways involved. This assumption can be considered according to the present literature data. Endogenously produced H_2S is an important factor in the neuromodulation of chloride secretion. According to the present literature findings, it is not known if H_2S is a pro- or antinociceptive agent in the large intestine. The property of mammalian cells to metabolise sulfur containing gas molecule is an indication that is linked with bacteria and marine animals living in an environment rich in sulfuric compounds (Grieshaber and Völkel 1998; Griesbeck et al. 2002a, b). On the other hand, for the production of H_2S by SRB can be under the influence of sulfate concentrations in the intestine, present in the human diet. The concentration of sulfate in diets depends on food types (Florin et al. 1993). Consequently, sulfidogenic SRB can be a competitor with other microorganisms, especially with methanogens (Kushkevych et al. 2017b, 2018a, b, c, d, e). This kind of competition can inhibit methanogenesis that leads to more suitable conditions for SRB growth. Higher counts of SRB and their H_2S production can also be an important factor in the development of inflammatory bowel disease (Kushkevych et al. 2018a, b, c, d, e).

Conclusions

Accumulation of hydrogen sulfide in the intestine depends on its production by intestinal SRB which is the main producer of this compound. Hydrogen sulfide represents an important factor for the growth of SRB and their sulfate and lactate consumption. The highest relative toxicity of hydrogen sulfide and survival of *D. piger* Vib-7 was achieved at its concentrations ranging from 6 to 7 mM. Despite the fact, that the SRB can be capable to produce hydrogen sulfide, high concentrations are toxic to its producers. These findings are confirming H_2S concentrations toxicity toward *Desulfovibrio*. The gained results emphasized H2S (>4 mM) limits toward this bacterial strain inhabiting humans and animals intestine.

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Author contributions IK, DD, MV wrote the article. All authors contributed to the conception, design and critically revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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