NEW MODIFICATION OF CULTIVATION MEDIUM FOR ISOLATION AND GROWTH OF INTESTINAL SULFATE-REDUCING BACTERIA

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Abstract: Different genera of sulfate-reducing bacteria (SRB) are always detected in the large intestine of humans and animals with diseases like an ulcerative colitis and Crohn's disease or even cancer. The final metabolism product of these anaerobic microorganisms is hydrogen sulfide which is known as a toxic substance and can lead to damage of epithelial cells of the bowel in high concentration. Some genera of the intestinal SRB included to the *Desulfovibrionaceae* family are hard to cultivate or even uncultivable. Isolation of these genera is also complicated because there are others satellite microorganisms. Up to now, Postgate's medium and other media do still not solve the cultivation problem and are created generally for *Desulfovibrio* species from nature environment but not for SRB species from the intestine. The object of our research was to modify the principle of isolation of intestinal SRB and cultivation medium based on their physiological and biochemical properties. Thus, there is no selective medium for intestine SRB which would improve cultivation and isolation of these important microorganisms. New created medium can be useful for more opportunities of intestinal SRB cultivation and understanding their involvement in inflammatory bowel diseases.

Key Words: sulfate-reducing bacteria, *Desulfovibrio*, cultivation media, modification of conditions, bacterial growth.

INTRODUCTION

Inflammatory bowel disease (IBD) including ulcerative colitis (UC) or Crohn's disease is characterized by chronic inflammation of the gut in genetically susceptible individuals of unknown etiology (Podolsky 2002, Schirbel and Fiocchi 2010). One of the hypothesis is, that UC is caused by the toxic molecule of hydrogen sulfide (H_2S). This compound in high concentration can lead to damaging of epithelial cells of human and animal large intestine (Kushkevych 2014a).

In persons, with rheumatic diseases, and with ankylosing spondylitis, etc. are often found sulfate-reducing bacteria (SRB) (Barton and Hamilton 2010, Sekirov et al. 2010), which in the increased numbers of them and intense process of dissimilatory sulfate reduction in the gut can cause these inflammatory bowel diseases (Loubinoux et al. 2002). Moreover, the increased number of SRB was found in feces from people with ulcerative colitis compared with healthy individuals (Gibson et al. 1991, 1993a, Levitt et al. 1999, Macfarlane et al. 2000). There is also an assumption that intestinal SRB can cause some forms of cancer of the rectum through the production of hydrogen sulfide which can affect the intestinal epithelial cells (Levitt et al. 1999). Because of this, SRB is important to study more in detail.

Sulfate-reducing bacteria are anaerobic microorganisms, which use sulfate as an electron acceptor in the process of dissimilatory sulfate reduction. This process is also called "dissimilatory sulfate respiration". To obtain energy and sulfate reduction, the electron donor is also necessary. Such electron donors in large intestinal can be lactate, ethanol, butyrate, succinate, acetate, propionate, pyruvate and some amino acids or even molecular hydrogen (Gibson et al. 1993b). All of these electron donors are the products of fermentations of following microorganisms, including genera *Clostridium*, *Escherichia*, *Saccharomyces*, *Bacteroides*, *Fusobacterium*, *Butyrivibrio*, and other. Described microbial genera can produce not only electron donors for SRB but also other important

biologically active substances, including vitamins or amino acids. On the other hand, the final product of SRB metabolism and their sulfate dissimilation is hydrogen sulfide.

SRB, especially *Desulfovibrio* genus, have been studied for over a century because of their ubiquity and their important roles in chemical processes and elemental cycles (Voordouw 1995). Also, *Desulfovibrio* genus is the most common species of SRB and its species are most often isolated from the large intestine of human and animals (Gibson et al. 1988, Moore et al. 1991).

Isolation of SRB from the mixture of human and animals' microbiota and their cultivation is also difficult. Some species of SRB such as *Bilophila wadsworthia* and *Lawsoni intracellularis* are uncultured. Other genera of the *Desulfovibrionaceae* family also grow poorly in cultivation medium or are uncultured. However, in our previous research, two genera of SRB isolated from human intestine and grew up well in cultivation medium were identified and described (Kushkevych 2013, Kushkevych et al. 2014d). It is known that in the intestine can be other genera of SRB and their species but isolation of which is complicated because not all intestinal SRB species can grow in classic Postgate medium or other media for cultivation of natural SRB isolated from the environment.

The aim of our research was to modify isolation conditions and create the optimal medium for cultivation of intestinal SRB based on their biochemical and physiological properties and conditions present in the large intestine of human and animals.

MATERIAL AND METHODS

Object of the study

Strains of SRB were isolated and identified from the large intestine of rats and have been kept in the collections of microorganisms in the Laboratory Anaerobic Microorganisms of Department of Experimental Biology at Masaryk University (Brno, Czech Republic). Other strains of SRB, *Desulfovibrio piger* were isolated from the healthy human large intestine as described previously (Kushkevych, 2013, Kushkevych et al. 2014b) and have also been kept in the same collection.

New modification of the medium for intestinal SRB growth, based on composition of well-known media described below and necessary conditions for SRB in bowel was created.

Media

Postgate's medium B which is a general-purpose medium for detecting and cultivating *Desulfovibrio* and *Desulfotomaculum* (pH was between 7.0 and 7.5).

Postgate's medium C which is a clear medium for biomass culture of *Desulfovibrio* (pH 7.5).

Postgate's medium E which is for isolation of pure cultures (pH 7.6).

The medium of Baars which gives a considerable precipitate after sterilization what is no problem in crude culture (pH 7.0–7.5).

Isolation of intestinal SRB

Samples were cultivated in sterile Eppendorf tubes full of liquid medium (pH 8.8 and flushed with N_2 to attain anaerobic condition) in the thermostat at +37 °C. Each positive SRB suspension was diluted in the same modified agar medium at temperature +43–45 °C and poured into plastic bags with a volume of 20 mL.

After cultivation, the black colonies grown in deep of the agar medium were selected and suspended in sterile saline (0.9% solution of NaCl). Suspensions with isolated colonies were pipetted (100 μL) in the standard media: one with sulfate (concentration 3.5 mM), one with elemental sulfur (without sulfate ions), and another medium without sulfate to be sure that the selected microorganisms belong to the SRB. Selected SRB was transferred to tempered Eppendorf tubes with the liquid medium. These procedures were 3–5 times repeated to selective and obtain pure cultures of intestinal SRB.

The contribution of new modified medium was verified by comparison of intestinal SRB growth rate in this medium and in media described above as well as the diversity of these microorganisms.

RESULTS AND DISCUSSION

As a result of our research is a comparison of a composition of different media for cultivation of SRB of various genera. It is known that mesophilic strains of SRB can grow up at temperature optimum around +30 °C but they can tolerate and grow up to +42 °C (Postgate 1984). On the other hand, the thermophilic strains are able to grow between temperatures from +50 to +70 °C (Kluyver and Baars 1932).

These microorganisms can tolerate pH from 5 to 9.5 and a wide range of osmotic conditions which all depend on the environment where they live (Postgate 1984). However, the pH range in the large intestine of humans or animals is limited and depend on many factors, including composition and enzymatic activity of intestinal microorganisms, substrates which they use, and the process of digestion and quality of consumed food. Basically, the pH in the human colon can be from neutral to alkaline (pH 7.6–8). Despite the wide range of temperatures of environmental SRB, their intestinal species always grow at temperature +37 °C what is a consistent temperature of the warm-blooded animal species and humans.

The composition of different cultivation media and composition of modified medium for isolation intestinal SRB is presented in Table 1.

Table 1 Composition of different cultivation media.

Salts (gram per liter)	Baars	Postgate B	Postgate C	Postgate E	Modified
Na_2SO_4	1	–	4.5	1	3
KH_2PO_4	–	0.5	0.5	0.5	0.3
K_2HPO_4	0.5	–	–	–	0.5
NH_4Cl	1	1	1	1	1
$CaCl_2 \times 6H_2O$	0.1	–	0.06	1	0.06
Yeast extract	–	1	1	1	1
Sodium Citrate $\times 2H_2O$	–	–	0.3	–	0.3
Sodium lactate	5	3.5	6	–	6
$MgSO_4 \times 7H_2O$	2	2	0.06	2	0.1
$CaSO_4$	–	1	–	–	–
Ascorbic acid	–	0.1	–	0.1	0.1
Thioglycolic acid	–	0.1	–	–	–
$(NH_4)_2SO_4$	–	–	–	–	0.2

The concentration of sulfate in the intestine depends on its introduction with food. Oxidized forms of sulfur including sulfate and sulfite are present in such food as commercial bread, dried fruits and vegetables, nuts, fermented beverages and brassica vegetable is sulfate mainly in the free anionic form. About 2–15 mM of sulfate is introduced with foods in human gastrointestinal tract every day. However, the concentration of sulfate ions in the feces is much lower and it is about 0.26 mM/day. It was also observed that 95% of sulfate is absorbed in the gastrointestinal tract and only 5% is in the remaining and detected in the feces (Florin et al. 1993). Other researchers have reported that absorption of sulfate by the human gastrointestinal tract is believed to be badly (Goodman and Gilman 1975, Wilson 1962). Apparently, such a concentration of sulfate (0.26 mM = 0.025 g/L) is sufficient for growth of SRB. This concentration was increased for initiation of SRB growth rate. Thus, the final concentration of the main acceptor in the modified medium is 3 g/L which corresponds 22.69 mM.

Another important factor for the SRB growth is present organic compounds which are electron donors in the process of sulfate reduction, carbon sources, and energy (Kushkevych et al. 2015a). The main electron donor in the large intestine is lactate which can be produced by lactic acid bacteria such genera as *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, and other. Their final product of metabolism, lactic acid, is used by intestinal SRB. As was mentioned by Younes et al. (1996), the concentration of

lactic acid in the large intestine is approximately 80 mM. However, the concentration of this electron donor in the modified medium is 6 g/L which corresponds 53.54 mM.

A varied microflora can be observed during cultivation anaerobically, but comparatively few characteristic SRB can be seen (Butlin et al. 1948). Unfortunately, the contaminations are very persistent and cannot be eliminated by repeated transfer of inoculum to fresh medium. Both Baars (1930) and Starkey (1938) were unlucky with their methods for isolations of pure SRB culture, even by addition 4 ml of 10% of H_2S to 60 mL of medium. The addition of 3% $Na_2SO_3 \times 7H_2O$ to the medium has been shown even in the Butlin's research with halophilic SRB as a positive inhibition for others persistent neighboring contaminating colonies. Thus, at the beginning of the isolation of intestinal SRB, 15 g of Na_2SO_3 (concentration 118.98 mM) in the medium was added for inhibition other representatives of intestinal microbiota. SRB, except for sulfate, can use also another electron acceptor (sulfite) which involved enzymes of sulfate reduction (Kushkevych 2014c, 2015b,c, Kushkevych et al. 2014d, Kushkevych and Fafula 2014e).

One of the main chemical properties of ascorbic acid is that it is a reducing agent. Also, Borsook et al. (1937) have observed in electrometric measurements of the oxidation-reduction potential rapid negative potential drifts of ascorbic acid in pH higher than 6.0. Another important compound which can be used to lower the redox potential is $Na_2S_2O_4$ (30 mg/L what is corresponds 0.17 mM) (Langendijk et al. 2001).

Escherichia coli is an only part of microbiota which is involved in the process of digestion and is able to a synthesis of biologically active substances, its absorption, and synthesis of some vitamins, including vitamin K. It can be also important for the cultivation and growth of intestinal SRB. The addition of 1 mg of vitamin K_1 to the liquid cultivation medium compensate the presence of vitamin K as it is in the large intestine. Others vitamins are compensated by the addition of yeast extract

The compounds used for the modified medium are mainly used from Postgate's medium C which is used for mass cultivation. K_2HPO_4 which is used by Baars in cultivation media for thermophilic strains of SRB was used in our cultivation with +37 °C (Baars 1930). Ammonium ions occurred in a colon is compensated in the medium by the $(NH_4)_2SO_4$ without using more chloride ions. Also, the concentration of hydrogen sulfide is lowered to balance a growth. As reducing agents to lower redox potential was used only ascorbic acid instead of ascorbic acid and thioglycolic acid. However, the medium for isolation was not used Postgate's medium E but modified Postgate's medium C only with the addition of agar.

For the presence of the intestinal SRB during isolation, 10 ml of 10% solution (w/L) of Mohr's salt $[(NH_4)_2Fe(SO_4)_2 \times 6H_2O]$ should be used and added after sterilization to media. This salt is easily dissociated and free Fe^{2+} interact with H_2S produced in the process of dissimilatory sulfate reduction. As a result, black FeS complex is formed and positive bacterial colonies are black colored. However, it is known that H_2S can produce not only SRB but this property have also sulfur-reducing bacteria, including species of *Desulfurella* and *Desulfuromonas* genera, which use elemental sulfur or in some case sulfate as an electron acceptor (Kushkevych, 2013). Other intestinal bacteria which can produce H_2S are species of *Clostridium*, *Salmonella*, *Enterococcus*, *Enterobacter*, *Klebsiella* genera, and numerous of *Bacteroides* species via the expression of the iron flavoprotein sulfite reductase (Linden 2014). Given this fact it is important to confirm that isolated colonies belong to SRB. The confirmation of this type is a cultivation of the isolates in both medium without and with sulfate, and also only with the elemental sulfur but without sulfate. The cultivation of intestinal SRB was carried at +37 °C and pH 7.6 which consistent with pH of human colon lumen (Charalambides and Segal 1992).

As a result, new strains of intestinal SRB from the animals' large intestine were isolated. Based on their growth in different media and modified medium as well as their physiological and biochemical properties, it was confirmed and identified that all isolates belong to the SRB group. The perspective of our research is studies of physiological and biochemical properties of these isolates in detail. Moreover, the testing activity of different new synthesized compounds against intestinal SRB with their subsequent application as promising drugs for the treatment of bowel diseases is also perspective. Because in our previous research, newly synthesized salicylamide derivatives showed inhibition effect against intestinal SRB and process of dissimilatory sulfate reduction (Kushkevych et al. 2015d, 2016).

CONCLUSION

Based on the studied biochemical and physiology properties of intestinal SRB and their condition in the large intestine and comparing different media for cultivation of SRB, the modified optimal medium was created. The modified medium contains a bigger concentration of sulfate for higher growth rate. For inhibition of persistent contaminating colonies in crude culture was used sulfite, which helped with an isolation of SRB colonies without intestinal bacterial satellites, which can be species of *Bacteroides*, *Pseudomonas*, *Clostridium* genera or other microorganisms.

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REFERENCES

Baars, E.K. 1930. *Over Sulfaatruductie Door Bakterien*. Delft: W. D. Meinema.

Barton, L.L., Hamilton, W.A. 2010. Sulphate-Reducing Bacteria. *Environmental and Engineered Systems*, Cambridge: Cambridge University Press, pp. 553.

Borsook, H., Davenport, H.W., Jefreys, C.E.P., Warner, R.C. 1937. The oxidation of ascorbic acid and its reduction *in vitro* and *in vivo*. *The Journal of Biological Chemistry*, 117: 237–279.

Butlin, K.R., Mary, E. Adams, Margaret, T. 1949. The isolation and cultivation of sulphate-reducing bacteria. *Microbiology*, 3(1): 46–59.

Charalambides, D, Segal, I. 1992. Colonic pH: a comparison between patients with colostomies due to trauma and colorectal cancer. *The American Journal of Gastroenterology*, 87(1): 74–8.

Florin, T.H.J., Goretski, S., Neale, G., Cummings, J.H., 1993. Sulfate in food and beverages. *Journal of Food Composition and Analysis*, 6(2): 140–151.

Gibson, G.R., Mc Farlane, G.T., Cummings J.H. 1988. Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *Journal of Applied Bacteriology*, 65: 103–111.

Gibson, G.R., Cummings, J.H., Mc Farlane, G.T. 1991. Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. *FEMS Microbiology Ecology*, 86: 103–112.

Gibson, G.R., Macfarlane, S., Macfarlane, G.T. 1993a. Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. *FEMS Microbiology Ecology*, 12: 117–125.

Gibson, G.R., Macfarlane, G.T., Cummings, J.H. 1993b. Sulphate-reducing bacteria and hydrogen metabolism in the human large intestine. *Gut*, 34: 437–439.

Goodman, L.S., Gilman, A. 1976. *The pharmacological basis of therapeutics*. 5th ed., New York: Macmillan.

Kluyver, A.J., Baars J.K. 1932. Microbiology – on some physiological artefacts. *Proceedings of the Royal Society of Amsterdam*, 35: 370–8.

Kushkevych, I.V. 2013. Identification of sulfate-reducing bacteria strains of human large intestine, *Studia Biologica*, 7(3): 115–124.

Kushkevych, I.V. 2014a. Etiological role of sulfate-reducing bacteria in the development of inflammatory bowel diseases and ulcerative colitis. *American Journal of Infectious Diseases and Microbiology*, 2(3): 63–73.

Kushkevych, I.V., Bartos, M., Bartosova, L. 2014b. Sequence analysis of the 16S rRNA gene of sulfate-reducing bacteria isolated from human intestine. *International Journal of Current Microbiology and Applied Sciences*, 3(2): 239–248.

Kushkevych, I.V. 2014c. Activity and kinetic properties of adenosine 5′-phosphosulfate reductase in the intestinal sulfate-reducing bacteria. *Microbiology and Biotechnology*, 2(26): 54–63.

Kushkevych, I., Fafula, R., Parak, T. and Bartos, M. 2014d. 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. *Acta Veterinaria Brno*, 84(1): 3–12.

Kushkevych, I.V., Fafula, R. V. 2014e. Dissimilatory sulfite reductase in cell-free extracts of intestinal sulfate-reducing bacteria. *Studia Biologica*, 8(2): 101–112.

Kushkevych, I., Bolis, M., Bartos, M. 2015a. Model-based Characterization of the Parameters of Dissimilatory Sulfate Reduction Under the Effect of Different Initial Density of *Desulfovibrio piger* Vib-7 Bacterial Cells. *The Open Microbiology Journal*, 9: 55–69.

Kushkevych, I.V. 2015b. Kinetic Properties of Pyruvate Ferredoxin Oxidoreductase of Intestinal Sulfate-Reducing Bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp Rod-9. *Polish Journal of Microbiology*, 64(2): 107–114.

Kushkevych, I.V. 2015c. Activity and kinetic properties of phosphotransacetylase from intestinal sulfate-reducing bacteria. *Acta Biochimica Polonica*, 62(1): 103–108.

Kushkevych, I., Kollar, P., Suchy, P. Parak, T., Pauk, K., Imramovsky, A. 2015d. Activity of selected salicylamides against intestinal sulfate-reducing bacteria. *Neuroendocrinology Letters*, 36(1): 106–113.

Kushkevych, I., Kollar, P., Ferreira, A.L. Palma, D., Duarte, A., Lopes, Maria M., Bartos, M., Pauk, K., Imramovsky, A., Jampilek, J. 2016. Antimicrobial effect of salicylamide derivatives against intestinal sulfate-reducing bacteria. *Journal of Applied Biomedicine*, 14(2): 125–130.

Langendijk, P.S., Kulik, E.M., Sandmeier, H., Meyer, J., Van Der Hoeven, J.S. 2001. Isolation of *Desulfomicrobium orale* sp. nov. and *Desulfovibrio* strain NY682, oral sulfate-reducing bacteria involved in human periodontal disease. *International Journal of Systematic and Evolutionary Microbiology*, 51: 1035–44.

Levitt, M.D., Furne, J., Springfield, J., Suarez, F., Demaster, E. 1999. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *The Journal of Clinical Investigation*, 104: 1107–1114.

Linden, D.R. 2014. Hydrogen Sulfide Signaling in the Gastrointestinal Tract. *Antioxidants & Redox Signaling*, 20(5): 818–830.

Loubinoux, J., Bronowicji, J.P., Pereira, I.A. 2002. Sulphate-reducing bacteria in human feces and their association with inflammatory diseases. *FEMS Microbiology Ecology*, 40: 107–112.

Macfarlane, S., Hopkins, M.J., Macfarlane, G.T. 2000. Bacterial growth and metabolism on surfaces in the large intestine. *Microbial Ecology in Health and Disease*, 2: 64–72.

Moore, W.E.C., Moore, L.H., Ranney, R.R., Smibert, R.M., Burmeister, J.A., Schenkein, H.A. 1991. The microflora of periodontal sites showing active destructive progression. *Journal of Clinical Periodontology*, 18: 729–739.

Podolsky, D.K. 2002. Inflammatory bowel disease. *The New England Journal of Medicine*, 347: 417–429.

Postgate, J.R. 1984. *The sulfate-reducing bacteria.* Cambridge: Cambridge University Press.

Rey, F.E., Gonzalez, M.D., Cheng, J.Y., Wu, M., Ahern, P.P., Gordon, J.I. 2013. Metabolic niche of a prominent sulfate-reducing human gut bacterium. *Proceedings of the National Academy of Sciences of the USA*, 110: 13582–13587.

Schirbel, A., Fiocchi, C. 2010. Inflammatory bowel disease: Established and evolving considerations on its etiopathogenesis and therapy. *Journal of Digestive Diseases*, 11: 266–276.

Sekirov, I., Russell, S.L., Antunes, L.C.M., Finlay, B.B. 2010. Gut Microbiota in Health and Disease. *Physiological Reviews*, 90: 859–904.

Starkeyr, L. 1938. A study of spore formation and other morphological characteristics of *Vibrio desulphuricans*. *Archives of Microbiology*, 9: 268.

Voordouw, G. 1995. The genus *Desulfovibrio* – The centennial. *Applied and Environmental Microbiology*, 61: 2813–2819.

Wilson, T.H. 1962. *Intestinal absorption*. Philadelphia: WB Sauders, pp. 134–137.

Younes, M, Lechago, L.V., Somoano, J.R., Mosharaf, M., Lechago, J. 1996. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. *Cancer Research*, 56: 1164–1167.