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Isolation and Purification of Sulfate-Reducing Bacteria

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This chapter, I dedicate to all scientists who have interests in the research on the physiology and biochemistry of this promising and heterogeneous group of microorganisms, the sulfate-reducing bacteria.

Author.

Abstract

Sulfate-reducing bacteria (SRB) are a widespread group of microorganisms that are often isolated from the anoxygenic environments (lake depths, soil, or swamps), and they are also present in the human and animal intestines. This group is often detected in patients with inflammatory bowel disease, including ulcerative colitis. That is why new rapid methods for their isolation, purification, and identification are important and necessary. In this chapter, the methods of mesophilic SRB isolation from various environments are described. Particular attention is paid to the purification of mesophilic SRB since they can be in close interaction with other microorganisms (*Clostridium*, *Bacteroides*, *Pseudomonas*, etc.), which are their frequent satellites. Moreover, the main methods of mesophilic SRB identification based on their morphological, physiological, biochemical, and genetical characteristics are presented.

Keywords: sulfate-reducing bacteria, sulfate, sulfite agar, hydrogen sulfide, isolation, identification

1. Introduction

Sulfate-reducing bacteria (SRB) are a heterogeneous group of microorganisms which is widespread in anaerobic places where sulfate-containing compounds are present [1–5]. These microorganisms use sulfate ions, which are reduced to hydrogen sulfide in the process called “dissimilatory sulfate reducing” or “sulfate respiration.” In this process, sulfate is a terminal electron acceptor [1, 2, 6–8]. For the implementation of dissimilatory sulfate reduction, exogenous electron donors are necessary [3, 4].

Molecular hydrogen is the main electron donor for all SRB, but commonly used electron donors are also lactate, acetate, pyruvate, ethanol, fatty acids, amino acids, dicarboxylic acids, and other organic compounds [9, 10]. Depending on the species of SRB, organic compounds can be oxidized completely to carbon dioxide or incompletely with the formation of acetate [11]. The SRB can also use ammonium salts as nitrogen sources [3, 11]. SRB species can assimilate molecular nitrogen [3]. So, SRB are widespread in the following areas as lake depths, soils, swamps [1, 3], and biogas

plant [12–14] and also present in the human and animal intestines [15–19]. The main species of intestinal SRB, *Desulfovibrio* genus, are often isolated from patients with inflammatory bowel disease (IBD) and healthy subjects [15, 20–25]. Other species of SRB, *Desulfomicrobium*, *Desulfobulbus*, *Desulfobacter*, *Desulfomonas*, and *Desulfotomaculum* were also seldom isolated from human and animal feces [1, 23, 26].

An increased number of SRB are often detected in patients with periodontitis [18]; inflammatory bowel diseases, including ulcerative colitis; and many other diseases [27–31]. Some scientists also suggest that SRB may be the cause of some forms of colon cancer, given the fact that these microorganisms produce hydrogen sulfide affecting the intestinal cell metabolism causing various diseases [32, 33]. That is why the isolation of SRB new strains, their purification from other microorganisms, and study of SRB cultural, physiological, biochemical, and genetical properties in detail are necessary.

It should be also noted that many species may be uncultured, so it is important to apply molecular and genetic methods such as Illumina sequencing. This method can give a clear picture of SRB diversity in the detected sample. However, in this chapter, the focus will be on isolation, purification, and cultivation of cultured mesophilic SRB species.

The goal of chapter is to describe:

- Methods of sample selections from water, soil, swamp, and feces of human or animals and from biopsy material
- Media, isolation, purification, and cultivation conditions
- Morphological diversity and physiological and biochemical properties
- Identification based on physiological and biochemical properties and sequence analysis of the 16S rRNA gene
- Generalization of this research

2. Selection of samples

As was noted, the SRB can be present in a sulfate-rich environment. The samples selected from the different ecotopes should be directly placed in anoxic modified Postgate liquid medium [3]. The composition of the medium and conditions of selections is described in Section 3.

2.1 Samples from environment (water, soil, swamp, and environmental surfaces)

One milliliter of water (or 1 g of swamp, soil, and metal rust) should be suspended in 9 ml of anoxic Postgate liquid medium. The tubes should be brim-filled with medium and closed to provide anaerobic conditions. Another option to provide anaerobic conditions is to add in tube 1 ml sterile liquid paraffin. The schema of sampling is presented in **Figure 1**.

2.2 Samples from feces of human or animals

It is thought that the species of SRB, their composition, and the number found in the intestinal lumen differ from that of the composition and number of

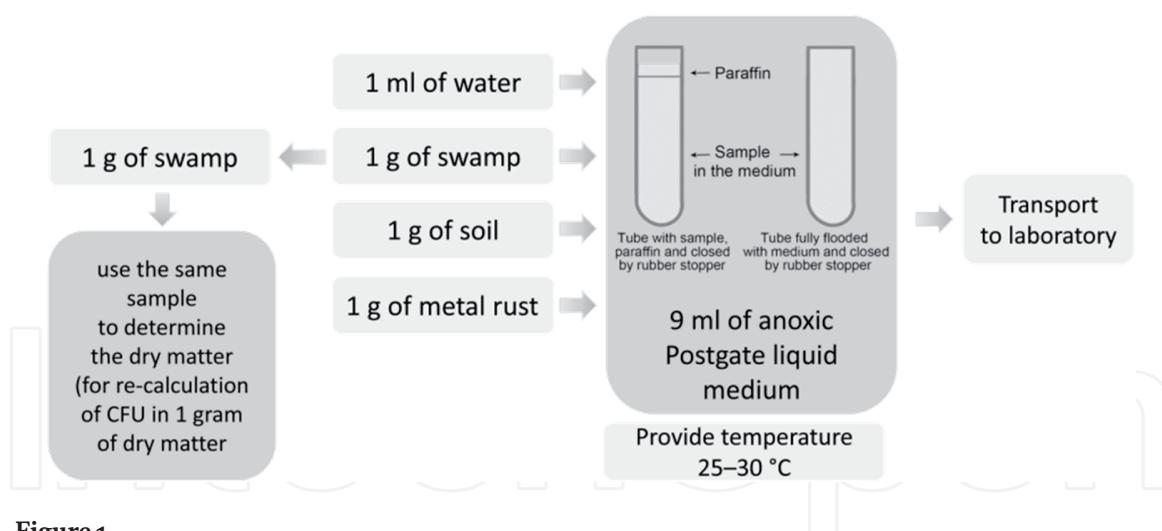


Figure 1.
 The scheme of sampling from different environmental biotopes.

microorganisms on the surface of the intestinal mucosa [2, 9, 28, 34]. Similar to environmental samples (see **Figure 1**), fecal samples from human or animals should be fresh and directly suspended in anoxic modified Postgate liquid medium (pH 7.5, temperature to +37°C). One gram of feces suspends in 9 ml of the modified Postgate liquid medium [3, 35]. The same quantity of feces should be taken for determining the dry matter and recalculation of colony-forming units (CFU) per 1 g of dry matter. Before this procedure, the medium should be heated in thermostat to +37°C temperature. If the samples from domestic or wild birds (chickens, geese, ducks, etc.), the temperature of medium should be +40°C.

2.3 Samples from intestine (biopsy or sections of the large intestine of animals)

Intestinal SRB with other intestinal bacteria can form biofilms on the surface of the epithelial cells of the large intestine [34]. These biofilms include species of *Desulfovibrio* genus and the species of *Bacteroides*, *Pseudomonas*, *Clostridium*, *Escherichia*, or other intestinal microorganisms. Such biofilms are often resistant to antimicrobial substances [36]; therefore it is an interesting area of the study.

For isolation of SRB from biofilms, 10⁻⁵ M EDTA (ethylenediaminetetraacetic acid) should be added to the modified Postgate liquid medium for releasing SRB from a biofilm. A fresh piece of biopsy should be weighed, and its approximate square (in cm²) must be calculated and added to 9 ml of the modified Postgate liquid medium (pH 7.5, temperature to +37°C). This calculation of square must be done for recalculation of CFU of SRB released from cm² of a biofilm.

The same procedure can be applied for isolation of SRB from sections of the large intestine of animals.

3. Medium and cultivation conditions

The composition of modified liquid Postgate medium [3, 35] is the following (g/l): Na₂SO₄ (0.5); KH₂PO₄ (0.3); K₂HPO₄ (0.5); (NH₄)₂SO₄ (0.2); NH₄Cl (1.0); CaCl₂ × 6H₂O (0.06); MgSO₄ × 7H₂O (0.1); lactate, C₃H₅O₃Na (2.0); yeast extract (1.0); FeSO₄ × 7H₂O (0.004); sodium citrate, C₆H₅O₇Na₃ × 2H₂O (0.3); and distilled water (1 l).

Separated solutions: Mohr's salt solution [(NH₄)₂Fe(SO₄)₂ × 6H₂O] (10%) and Na₂S × 9H₂O solution (1%) and 10 M solution of NaOH must be sterilized separately.

The modified liquid Postgate medium and solutions of Mohr's salt, sodium sulfide, and sodium hydroxide should be sterilized in autoclave (20 min, at 1 atm.). The sterilization provides sterile conditions and partial release of oxygen from the medium. The solution of sodium sulfide is hydrolyzed to hydrogen sulfide during autoclaving.

After sterilization, 10 ml/l of sterile Mohr's salt solution and 0.05 ml/l of sterile solution of sodium sulfide must be added to the medium. The addition of a small quantity (one drop) of sodium sulfide solution to the medium makes visible a black ring which confirms interactions of hydrogen sulfide and free Fe^{2+} released from Mohr's salt.

A sterile ascorbic acid solution also must be added to the medium, but it cannot be sterilized by autoclaving because it may partially decompose and lose its properties for redox potential. So, 20% ascorbic acid solution should be filtrated through membrane filters (0.2 μm) and added directly to the medium after sterilization. The final concentration of ascorbic acid in the medium should be 0.1 g/l, and the redox potential of the medium must be around -100 mV . Solution of hydrogen sulfide added to medium can also decrease a redox potential [3].

The redox and anaerobic conditions can be controlled by sodium resazurin as an indicator. In addition, FeS reduced and Na_2S contained in the medium provides the necessary redox conditions for SRB cultures. The discoloration of sodium resazurin (redox potential of discoloration $E_h = -100\text{ mV}$) confirms the decrease of redox potential. A pH medium (7.5) provides by the addition of a sterile 10 M solution of NaOH.

The temperature of the media should be $+25\ldots+30^\circ\text{C}$ for environmental samples, and $+37^\circ\text{C}$ for intestinal samples ($+40^\circ\text{C}$ for samples from birds).

The tubes with samples must be completely filled up to the edges of the test tube with completed medium and closed with rubber stoppers. In another case, tubes can be filled up incompletely, but 1 ml of sterile liquid paraffin must be filled up to the top of the medium and closed with rubber stoppers.

As a control of the quality of the medium, known pure culture of SRB from some collections of microorganisms is recommended to also be used.

Cultivate in the thermostat at $+25\ldots+30^\circ\text{C}$, $+37^\circ\text{C}$, or $+40^\circ\text{C}$, depending on the origin of the sample, during for 1–5 days under anaerobic conditions. SRB from birds, animals, and humans mostly grow faster than environmental species.

Positive growth of SRB is indicated by observing a black FeS precipitate occurred in the bottom of the tube.

4. Isolation and purification of positive SRB samples

As already mentioned above, SRB are in close interactions with other microorganisms and can form biofilms in which they may be in a symbiotic relationship [34, 37]. Such microorganisms cooperating with SRB are often called satellite microorganisms [3]. Among the intestinal microorganisms, the species of the *Bacteroides*, *Pseudomonas*, *Clostridium*, and *Escherichia* genera are most often detected. Phototrophic green sulfur bacteria can make consortium with SRB [38]. On one hand, SRB produce hydrogen sulfide, and on the other hand, green sulfur bacteria oxidize hydrogen sulfide to molecular sulfur in the process of anoxygenic photosynthesis. Molecular sulfur may subsequently be oxidized to sulfate, in which SRB can be used as a final electron acceptor. Such an example of interaction can be consortium *Pelochromatium roseo-viridae* [11]. That is why it is important to purify the mixed cultures of SRB from satellite microorganisms which are very difficult to remove of during this process.

For obtaining pure cultures of SRB colonies, positive SRB samples (mixed SRB cultures) should be diluted (1,9) in a series of tubes (to 10^{-5}) containing the modified Postgate liquid medium. The scheme of the series of tubes is presented in **Figure 2**. Before it, the modified Postgate agar medium of the same composition like liquid should be prepared but in this case adds to the medium additional compounds: Na_2SO_3 (7.5 g/l) and microbiological agar (12 g/l). Sterilize it by autoclaving like Postgate liquid medium. Sodium sulfite in high concentration in medium inhibits most of intestinal species of *Enterobacteriaceae* family, including *Bacteroides*, *Pseudomonas*, and *Clostridium*, *Escherichia*, which can be satellites of SRB. The species of SRB are resisted to sulfite ions and can be used as an alternative electron acceptor [11] in the process of dissimilatory sulfate reduction since they have sulfite reductase activity [39].

The modified Postgate agar medium containing sodium sulfite (Na_2SO_3) after sterilization in autoclave should be cooled to $+40^\circ\text{C}$ and 10 ml/l of sterile Mohr's salt solution, 0.05 ml/l of sterile solution of sodium sulfide and ascorbic acid (0.1 g/l) added to the medium. These components must be thoroughly mixed in the flask and a sterile 10 M solution of NaOH added to provide accordingly a pH depending on the samples. To prevent the medium solidation, use a water bath to keep the temperature ($+40^\circ\text{C}$) at a constant level.

In total, 20 ml of warm modified Postgate agar medium spill in Petri palates and add to the medium 100 μl of each diluted suspension of a positive sample, thoroughly mix the suspension with the warm medium. The temperature should be according to the sample from where it was isolated.

Filled with medium and suspension Petri plates introduce into an anaerobic box with oxygen uptake generators for anaerobiosis. Mohr's salt in the agar medium allows to detect black colonies of SRB since as a result, FeS was formed by hydrogen sulfide bacterial production that caused black-colored colonies. Cultivate in the thermostat at the appropriate temperature. The black colonies will be visible in 1–5 days in the deep of agar medium depending on sample and its dilution.

The black colonies obtained from Petri palates cut from agar and suspend in modified liquid Postgate medium. Cultivate in the thermostat at the appropriate temperature. The formation of black sediment (FeS precipitate) will be visible in the tube (about in 1–3 days). This sediment confirms sulfate reduction and

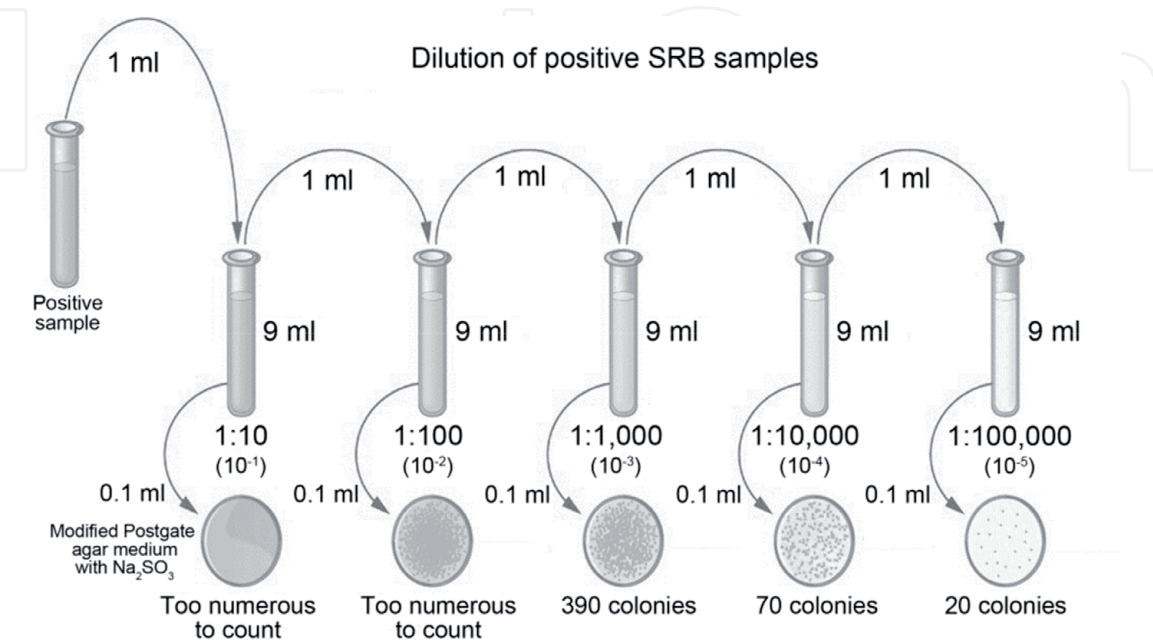


Figure 2.
The scheme of dilution of positive SRB samples (mixed cultures).

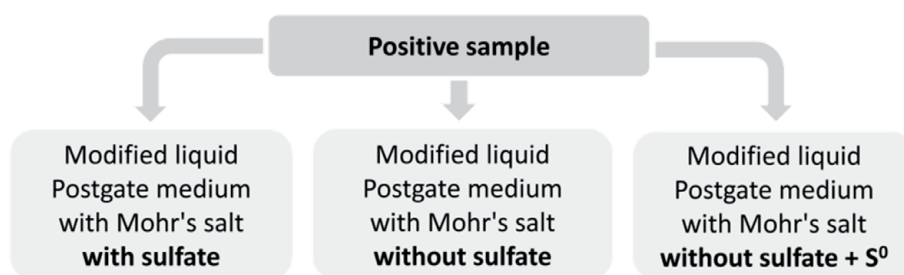


Figure 3.
The scheme of the confirmation that the isolates belong to the SRB.

production of hydrogen sulfide, which interact with Fe^{2+} from Mohr's salt, and FeS precipitate is formed. However, hydrogen sulfide can also be produced by species of *Clostridium*, *Salmonella*, and other intestinal bacteria. Moreover, some sulfur-reducing bacteria in the same case can also use sulfate as an electron acceptor [11]. To be sure that the selected microorganisms are not sulfur reducers or other bacteria capable of hydrogen sulfide production, the liquid media following the composition should be prepared: first modified liquid Postgate medium with sulfate (concentration 3.5 mM), second the same medium but without sulfate, and third the same medium but without sulfate containing molecular sulfur (0.5 g/l). The scheme of the confirmation that the isolates belong to the SRB is presented in **Figure 3**.

The grown black sample should be mixed and 100 μl of bacterial suspension pipetted into Eppendorf tubes (volume 1.5 ml) with 900 μl of liquid media by the scheme (**Figure 3**). Pipette 200 μl of sterile liquid paraffin on the surface of the media with suspension, and close a cap of Eppendorf tubes. Cultivate in thermostat.

If the sample after cultivation forms a black sediment in the modified liquid Postgate medium without sulfate ions that contained molecular sulfur, it means that isolates in a positive sample can belong to the sulfur-reducing bacteria (not SRB).

If the sample after cultivation does not form black sediment (FeS precipitate) in modified liquid Postgate medium without sulfate and the same medium without sulfate ions that contained molecular sulfur, but bacterial growth is observed in the medium with sulfate, it means that isolates in a positive sample belong to the SRB.

The positive sample with SRB culture should be diluted in the modified liquid Postgate medium and again seed each dilution in agar medium containing sodium sulfite (see **Figure 2**). This procedure must be repeated 3–5 times for full purification of SRB from other bacterial satellites.

After that, to check the purity of the SRB cultures from satellites, other additional tests are necessary. These additional tests are bacterial growth on the growth on different nonselective media (meat peptone agar; wort agar; starch-and-ammonia agar; Giltay's, Baalsrud's, and modified Postgate medium). Growth of SRB should be positive only in modified Postgate medium.

5. Morphological diversity: physiological and biochemical properties

The SRB cells are spherical, oval, rod-shaped, spiral, or vibrio-shaped with a diameter of 0.4–3.0 μm . The cells can be either single or in pairs or aggregates also may form a single row of multicellular filaments [1, 3]. Most cells of SRB genera are Gram-negative, although the filamentous and spore-forming microorganisms are Gram-positive. The SRB genera are anaerobes [11]. Morphology of SRB cells can be studied by using the light microscope, phase-contrast microscopy, or electronic microscopy.

Some species of SRB have single flagellum or more flagella depending on the genus. A simple, qualitative, and rapid method for detecting bacterial flagella and their shape, length, curvature, arrangement, and number on the cell is Hardy Diagnostics Flagella Stain (HDFS) [40, 41]. In 1937, Ryu developed this method, and later Kodaka et al. further described it [42, 43]. This test is especially useful in taxonomy and identifying characteristic about SRB motile, and more recently, anaerobic bacteria. Due to their narrow diameter, SRB flagella cannot be seen with a light microscope. The method of flagella stain can provide viewing SRB flagella by employing a crystal violet in an alcoholic solution as the primary stain. The alcoholic solution evaporates and leaves a precipitate around the flagella during the staining procedure and in increasing its apparent size.

In addition to the cell morphology and the presence of flagella, the following physiological characteristic, which is no less important, is also the formation of spores. However, among the heterogeneous quantity of SRB, the species of *Desulfotomaculum* genus can sporulate. To determine the ability of the SRB cells to sporulate, 1 ml of 72 h pure culture of SRB grown in modified liquid Postgate medium should be heated at +80°C for 15 min and then 100 µl of bacterial suspension pipetted into epindorph (volume 1.5 ml) with 900 µl of liquid media, and add 200 µl of sterile liquid paraffin on the surface of the media with suspension, and close the cap of Eppendorf tubes. Cultivate in thermostat. Thermoresistant forms of the *Desulfotomaculum* genus can be observed by FeS precipitate in the Eppendorf tubes. The SRB spores can be also additionally detected by a staining method for endospore. This method was published by Dorner [44] and later modified by Schaeffer and Fulton [45]. The modified process is simpler and faster and commonly used to differentiate bacterial endospores from other vegetative cells. It is also used to differentiate spore-forming bacteria from nonspore-forming [45].

Other physiological and biochemical characteristics which are important for identification are the determination of SRB growth at various pH and temperature, biomass accumulation, sulfate/lactate consumption, hydrogen sulfide and acetate production, catalase test, indole test, nitrate reduction, carbohydrate fermentation, gas production, and desulfovirdin test (Figure 4).

The effect of acidity (pH) is one of many important environmental factors which can be used for physiological characteristics of new SRB strains. The decreasing and

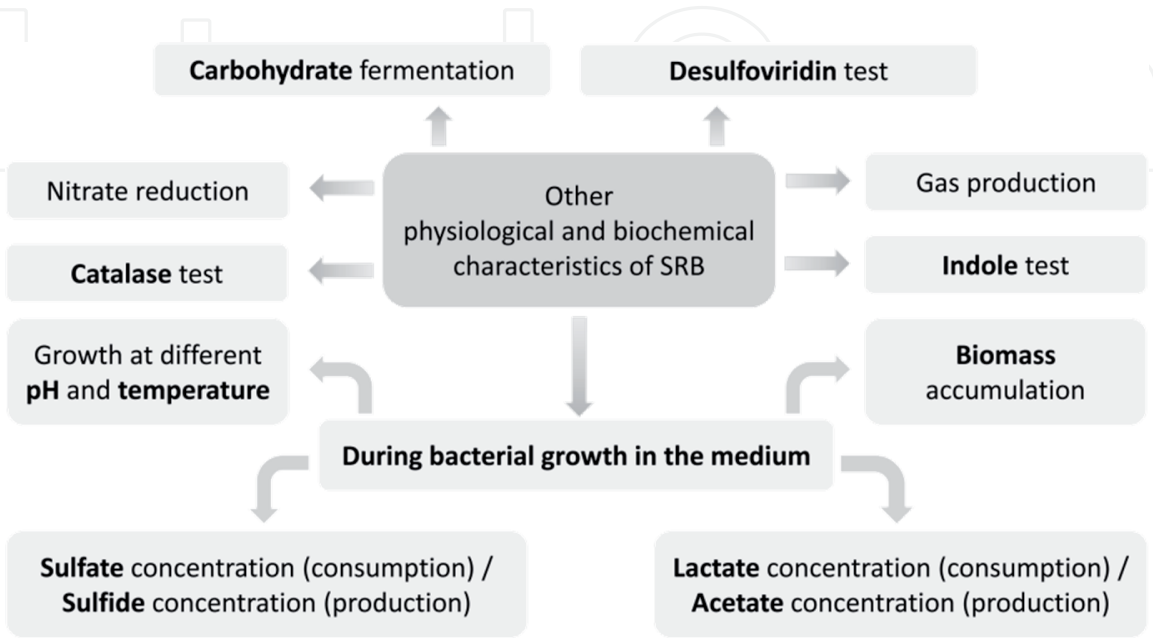


Figure 4.
The scheme of other physiological and biochemical characteristics.

increasing acidity of the medium can lead to the decrease of the SRB growth rate and hydrogen sulfide production [25]. Furutani and Schindler reported that the process of dissimilatory sulfate reduction was significantly slowed at low pH [46]. The increasing of the pH medium until 9.0–10.0 also caused growth inhibition of the studied bacteria [25]. To test the pH effect on the SRB growth, the modified liquid Postgate medium (*without Mohr's salt*) with different pH 3, 4, 5, 6, 7, 8, 9, and 10 is necessary to be prepared. Inoculation (initial concentration) of bacterial cells should be not less than 10%. After cultivation in the thermostat (24–36 h), biomass accumulation can be determined and compared in which the value of pH is optimum for SRB growth.

Most of the species of SRB are mesophilic microorganisms and live at a temperature from +20 to +40°C. Some SRB species can be also thermophilic microorganisms, e.g., *Thermodesulfobacterium* genus (*T. thermophilum*, *T. hveragerdense*, *T. commune*, and others) [11]. However, this chapter is focused on isolation and purification of mesophilic SRB. Similar to the case with test pH effect, the optimum of temperature for SRB growth is necessary to be determined. Inoculate bacterial cells (10%) in the modified liquid Postgate medium (*without Mohr's salt*), and cultivate at different temperatures (+4, +14, +20, +35, +45°C). After cultivation (24–36 h), biomass accumulation can be determined and compared in which the value of the temperature is optimum for SRB growth.

Biomass accumulation of the SRB cells in liquid medium can be measured by the photometric method by using a spectrophotometer, but the medium cannot contain Mohr's salt, since FeS precipitate makes it impossible [26, 47].

The cultivation of SRB in anaerobic, microaerophilic, or aerobic conditions allows testing their viability and resistance to molecular oxygen. However, SRB are anaerobes, but some of them may have high activity of antioxidant enzymes, catalase, and superoxide dismutase [1, 3].

Sulfate consumption as a terminal acceptor and determination of its concentration in the medium during SRB growth is important for observing and understanding more the process of dissimilatory sulfate reduction. The sulfate concentration in the medium (*without Mohr's salt*) can be assayed by the turbidimetric method by precipitation with barium chloride. For stabilizing the suspension, glycerol should be used [48].

The final product of the dissimilatory sulfate reduction process is hydrogen sulfide, which can be measured in the culture medium (*without Mohr's salt*) by a photometric method based on the reaction of sulfide and n-aminodimethylaniline with the methylene blue formation [49]. The concentration of hydrogen sulfide is determined by calibration curve. The data on the concentration of hydrogen sulfide, produced by the isolates, is supposed to help in establishing and assessing a toxicity effect of hydrogen sulfide on the epithelial cells of the human intestine. Such studies might help in predicting the development of diseases in 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.

In the dissimilatory sulfate reduction process, SRB use exogenous electron donors. Molecular hydrogen is a universal electron donor for intestinal SRB [23, 37]. These bacteria are in close interaction with each other. It was established that SRB can completely displace methanogenic microorganisms of the intestine in the process of H₂ competition [9]. This competition for molecular hydrogen between SRB and methanogens largely depends on the presence and quantity of sulfate in the gut [9]. Adding sulfate and sulfated mucopolysaccharides to fecal suspensions which contain metabolically active products of the SRB stimulates the formation of hydrogen sulfide and inhibits the intensity of the methanogenesis [1, 14]. Except H₂, the second important electron donor is lactate, which SRB can oxidize incompletely to acetate or completely to CO₂.

The determination of lactate concentration can be carried out through dehydrogenation of lactate reaction by lactate dehydrogenase in the presence of NAD^+ , with formation of pyruvate and NADH. Another method for measurement of lactate concentration is the use of lactate assay kit (Sigma-Aldrich, Catalog Number MAK064). Acetate accumulated during lactate incompletely oxidizing in the process of bacterial growth can be determined by using the acetate assay kit (Colorimetric, Catalog Number KA3764) or by titration.

Simple catalase test on modified Postgate surface agar cultures can be carried out by adding a drop of 10% H_2O_2 solution over the colonies. Another way is adding 5 drops of 10% H_2O_2 solution in 1 ml of a modified liquid Postgate medium. If the culture is catalase positive, the bubbles are formed.

The indole production test can be carried out by using a 24-h liquid culture with nitric acid and isoamyl alcohol reagents (Salkowski's reaction).

Adding sodium nitrate (5%) to modified liquid Postgate medium can be used for testing nitrate reduction. Nitrites can be tested by using a naphthylamine-sulfanilic acid reagent on 24-h cultures.

The ability of SRB strains to metabolize except lactate or H_2 other electron donors and a carbon source is also necessary to test. With this purpose, formate, propionate, pyruvate, fumarate, malate, methanol, citrate, ethanol, acetate, glycerol, glucose, oleate, stearate, and benzoate should be added separately in modified liquid Postgate medium but without electron donor (lactate) and carbon source. A final concentration of each compound should be 1%. Glucose and pyruvate fermentation in the liquid medium can be analyzed by acidity (pH reaction) and pH indicators. This test confirms that SRB isolated strains are capable to *chemolithoheterotrophic* growth. In addition to organic acids with different carbon chain lengths and alcohol, the strains can also assimilate some amino acids.

Gas production can be observed in deep culture Postgate agar in the tubes.

The desulfovibrin production is a very important factor for identification of *Desulfovibrio* and *Desulfomonas* genera. The presence of this protein in bacterial cells can be examined by using ultraviolet (UV) light on Postgate agar surface cultures after treatment with a 1 N NaOH solution. Desulfovibrin will be green in UV light.

6. Identification based on physiological and biochemical properties and sequence analysis of the 16S rRNA gene

Identification of the SRB by morphological, physiological, and biochemical characteristics can be conducted according to Bergey's Manual of Determinative Bacteriology (ninth edition, 1994), where SRB belong to the seventh group and are called "dissimilatory sulfate- or sulfur-reducing bacteria" [11]. This group is divided into four subgroups (Figure 5).

However, more modern and complex classification of SRB is published in Bergey's Manual of Systematic Bacteriology (2005), where SRB are divided into different classes, for example, class IV, *Deltaproteobacteria*, including order II, *Desulfovibrionales*; family I, *Desulfovibrionaceae* (genus I. *Desulfovibrio*); or family II, *Desulfomicrobiaceae* (genus I. *Desulfomicrobium*) [10]. For details identification based on physiological and biochemical characteristics is necessary to use both Bergey's manuals.

As was mentioned above, the representatives of *Desulfovibrio* genus are often found in the animal and patients with IBD and healthy subjects, because it is necessary to pay attention to the more detailed steps for identification of the second subgroup where this genus belongs (see Figure 4).

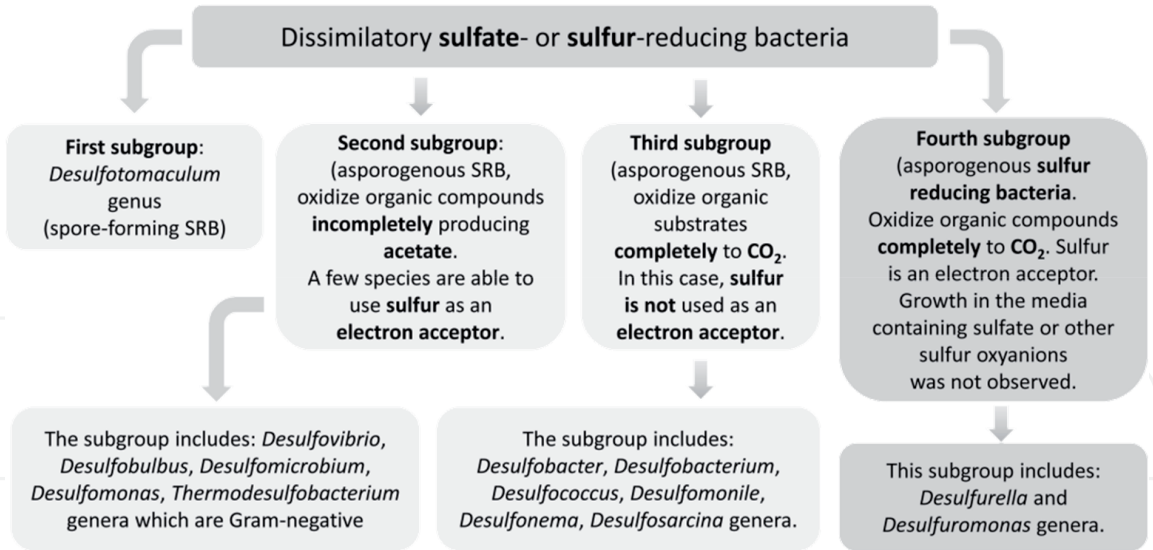


Figure 5. The scheme of classification of dissimilatory sulfate- or sulfur-reducing bacteria group, according to Bergey’s manual of determinative bacteriology (ninth edition, 1994) [11].

Features	Dvi	Dmo	Dbu	Dmi	Tdb
Spiral or vibrio-shaped cells	+	—	—	—	—
Oval or rod-shaped cells	—	+	+	+	+
Movement with the polar flagella	+/—		+/—	+	+/—
Optimal temperature range					
+25...+40°C	+	+	+	+	—
+65...+70°C	—	—	—	—	+
Ability of bacteria to grow in the presence of sulfate					
H ₂ + CO ₂ + acetate as a carbon source	+	+	+	+	+
Lactate	+	+	+	+	+
Propionate	—	—	+	—	—
Desulfovibrin	+	+	—	—	—
Notes: the feature is presence “+” or absence “—”.					

Table 1. The differences between features of the second subgroup SRB genera according to Bergey’s manual of determinative bacteriology [11].

The second subgroup includes *Desulfovibrio* (Dvi), *Desulfomonas* (Dmo), *Desulfobulbus* (Dbu), *Desulfomicrobium* (Dmi), and *Thermodesulfobacterium* (Tdb) genera (Table 1).

Other SRB genera can be identified by Bergey’s manuals [10, 11]. However, for complete identification based on morphological, physiological, and biochemical properties, the molecular methods, in particular the sequence analysis of 16S rRNA gene, are also necessary to be applied [50]. Except sequence analysis of 16S rRNA gene, it is important to confirm the SRB species by using primers of functional genes of dissimilatory sulfate-reduction, such as *DsrAB* and *AprBA* (Table 2).

Further on the example of one isolate of intestinal SRB, identification based on sequence analysis of 16S rRNA gene by using the universal primers will be described. The schema of this identification is presented in Figure 6.

Functional genes	Primer sequence	Amplicon length (pb)
DsrAB gen		
DSR1F	5'-ACSCAYTGGAARCACG-3'	1900
DSR4R	5'-GTGTARCAGTTDCCRCA-3'	
AprBA gen		
aprB-1-FW	5'-TGC GTGTAYATHTGYCC-3'	1200–1350
aprA-5-RV	5'-GCGCCAACYGGRCCRTA-3'	

Table 2.
Primers designed based on functional gens of dissimilatory sulfate reduction, which can be used for amplification.

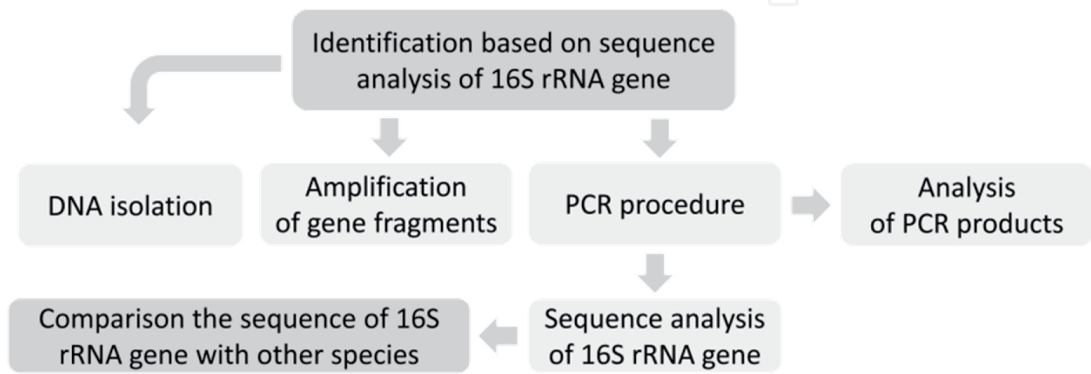


Figure 6.
The scheme of identification based on sequence analysis of 16S rRNA gene.

DNA isolation. Isolation and purification of DNA were carried out with a 72-h culture of SRB by using a “QIAmp DNA Mini Kit (QIAGEN), Cat. No 51304.” One single SRB colony was taken from modified Postgate agar medium and suspended in 50 µl of deionized water in a screw cap micro-centrifuge tube. The samples were boiled at 98°C for 5 min prior to being centrifuged for 5 min/14,000 g to settle cell debris. In total 2 µl of supernatant, containing the genomic DNA, were used for PCR amplification.

Amplification of gene fragments. Amplification of 16S rRNA gene fragments was carried out using the universal primers (Table 3) according to Weisburg et al. [51] and Persing [52].

PCR procedure. PCR was carried out on DNA isolated from SRB cells in a final volume of 20 µl consisting of 10.0 µl Taq PCR Master Mix Kit (Cat. No 201445), 0.1 µl of each primer, 0.1 µl uracil D-glycosylase (Cat. No. M0280 L), 7.7 µl deionized water, and 2.0 µl of DNA supernatant.

The amplicons were amplified by a preliminary incubation at 94°C for 5 min (initial denaturation) and then 34 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing of primers), and 72°C for 2 min (polymerization), using a

Primers	Sequence	Amplicon length (pb)
8FPL	5'-AGTTTGATCCTGGCTCAG-3' position 8–27	Approximately 1500
1492RPL	5'-GGTTACCTTGTTACGACTT-3' position 1510–1492	
806R	5'-GGACTACCAGGGTATCTAAT-3' position 806–787	Approximately 800

Table 3.
Universal primers for amplification of 16S rRNA gene fragments.

thermocycler (model MJ Research PTC-200, USA). After the last amplification cycle, the samples were incubated further at 72°C for 2 min for complete elongation of the final PCR products and cooled at 10°C.

Analysis of PCR products. Analysis of PCR products was carried out by electrophoresis in 1.5% agarose gel, with field strengths of 5 V/cm. Electrophoresis time was 40 min. The 100 bp ladder (Malamité, Czech Republic) was used as a size standard and molecular weight markers. Isolation and purification of fragments from agarose were performed by centrifugation of gel strips containing DNA through aerosol filters. For purification of SRB amplicons, the commercial kit from QIAGEN “MinElute Gel Extraction Kit” was used. The sequence was carried out using a “genetic analyzer” and reagents “BigDye Terminator v3.1 Cycle Sequencing Kit.” Search homologous deposited in the GenBank nucleotide sequence encoding the 16S rRNA gene, was performed using BLASTN and Blast2 programs.

The 16S rRNA gene amplicons which were used for sequence analysis were obtained by using the PCR method. The PCR products were separated by electrophoresis (**Figure 7**). Before sequence analysis the absorbance of amplicons (8FPL/806R, amplicon I about 800 bp; 8FPL/1492RPL, amplicon I about 1500 bp; 8FPL/806R, amplicon II about 800 bp; 8FPL/1492RPL, amplicon II about 1500 bp) was determined [50].

By comparison of individual sequencing data from the amplicons 1–5, the following gene for 16S rRNA sequence of the total length 1370 bp was completed:

```
TTCGGTCCCGAGTAAAGTGGCGCACGGGTGAGTAACACGTGGATGATCTGCCTCTATGATGGGGATA
ACAGTTGGAACGACTGCTAATACCGAATACGCTCATGATGAACGTTGTGAGGAAAGGTGGCCTCTG
CTTGCAAGCTATCGCATAGAGATGAGTCCGCGTCCCATAGCTCGTTGGTGGGGTAACGGCCTACCA
AGGCAACGATGGGTAGCCGATCTGAGAGGATGATCGGCCACACTGGAAGTAAACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGT
GAGGGATGAAGGTCTTCGGATCGTAAACCTCTGTCAGAAGGGAAGAACTGGGGTGTCTTAATCAG
CATCCCACTGACGGTACCTTCAAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG
AGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGCGCACGTAGGCTGTTATGTAAGTCAGGGG
TGAAAGCCACGGCTCAACCGTGGAAGTGCCTTGATACTGCACGACTCGAATCCGGGAGAGGGTGG
CGGAATTCCAGGTGTAGGAGTGAAATCCGTAGATATCTGGAGGAACATCAGTGGCGAAGGCGGCCA
CCTGGACCGGTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGGATGCTAGATGTGCGGATGTATGTCTCGGTGTCGTAGTCAACGCGTTAA
GCATCCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAAGAAATTGACGGGGGCCGACAAAG
CGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATCTGGGGAAT
CCTCCCGAAAAGGAGGAGTGCCCTTCGGGGAGCCCCAAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATGCATAGTTGCCAGCAGGTAG
AAGCTGGGCACTCTATGCAGACTGCCCGGTTAACCGGGAGGAAGGTGGGGACGACGTCAAGTCAT
CATGGCCCTTACACCCAGGGCTACACACGTACTACAATGGCACGCACAAAGGGCAGCGATACCGTGA
GGTGGAGCCAATCCCAAAAAACGTGTCCAGTCCGGATTGCAGTCTGCAACTCGACTGCATGAAGTC
GGAATCGCTAGTAATTCGAGGTCAGCATACTCGGGTGAATGCGTTCGCGGCCCTTGTACACACCGCC
CGTCACACCACGAAAGTCGGTTTACCCGAAGCCGGGG
```

Black nucleotides are a summary from 1, 2, 3, and 5: the best sequence data (totally four sequencing). **Green nucleotides** are a summary from 3 and 4: good sequence data (two sequencing). **Red nucleotides** are the rest of 4: the worse sequence data because they were received from one sequencing only, but its quality was excellent. The obtained sequence BLASTN was analyzed. The highest homology of SRB colony was identified with *Desulfovibrio piger* ATCC 29098 from GenBank.

The obtained sequence results of SRB isolated colony were also compared by BLASTN analysis with the nucleotide sequences of 16S rRNA gene of other strains (**Table 4**).

Thus, the nucleotide sequence of the 16S rRNA gene of SRB has the highest homology (99%) compared to deposited nucleotide sequence *D. piger* ATCC 29098 (AF192152) in the GenBank database.

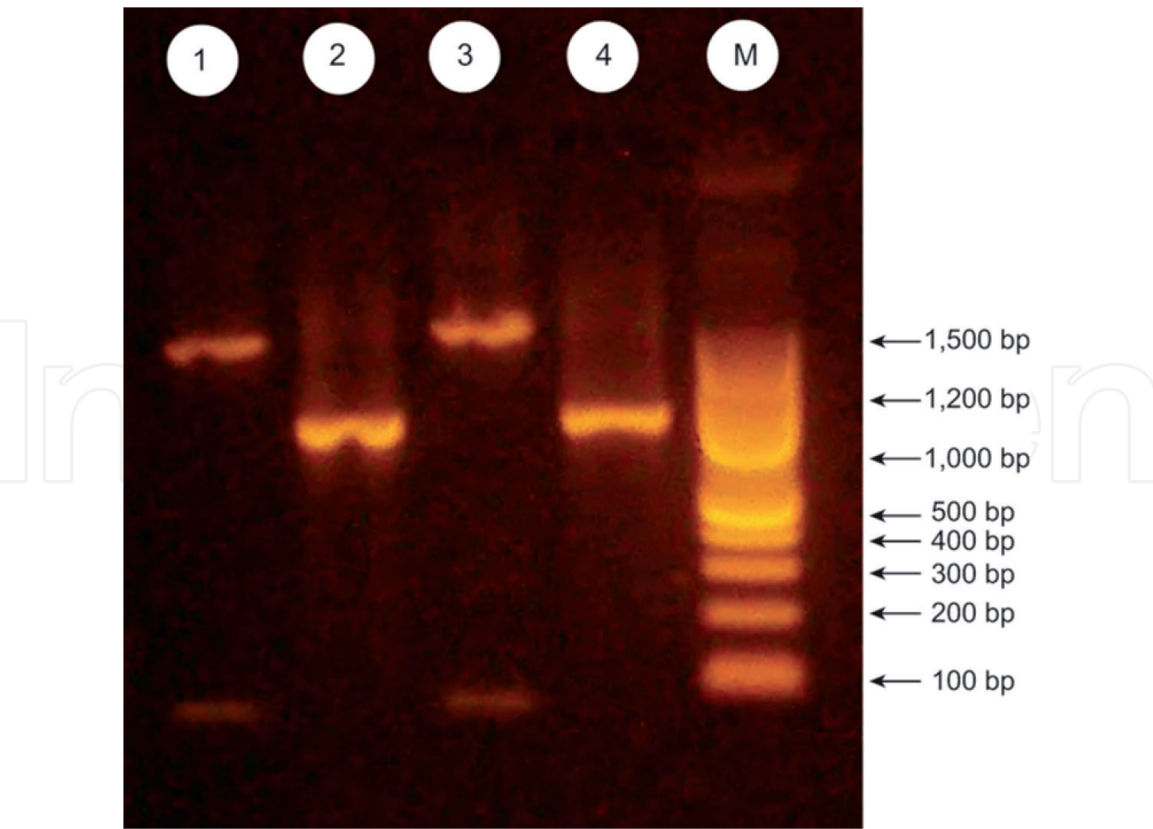


Figure 7.
The results of electrophoresis 16S rRNA PCR products (amplicons): 8FPL/1492RPL, 1500 bp (1); 8FPL/806R, 800 bp (2); 8FPL/1492RPL, 1500 bp (3); 8FPL/806R, 800 bp (4); M is marker (100 bp ladder).

SRB strains	Acc. No	Identities	Identity (%)
<i>Desulfovibrio piger</i> ATCC 29098	AF192152	1352/1368	99
<i>Desulfovibrio fairfieldensis</i> ATCC700045	U42221	1313/1374	96
<i>Desulfovibrio desulfuricans</i> Essex 6	AF192153	1299/1369	95
<i>Desulfovibrio intestinalis</i> DSM 11275	Y12254	1289/1373	94
<i>Desulfovibrio desulfuricans</i> MB	AF192154	1293/1373	94
<i>Bilophila wadsworthia</i> ATCC49260	L35148	1242/1369	91
<i>Desulfovibrio vulgaris</i> subsp. Oxamicus DSM 1925	AJ295677	1089/1195	91
<i>Desulfovibrio longreachensis</i> ACM 3958	Z24450	1253/1374	91
<i>Desulfovibrio termitidis</i> DSM 5308	X87409	1237/1372	90
<i>Lawsonia intracellularis</i> NCTC 12656	U30147	1215/1374	88
<i>Desulfovibrio vulgaris</i> subsp. vulgaris DSM 644	M34399	726/834	87

Table 4.
Comparing the resulting sequence of 16S rRNA gene with other *Desulfovibrio* species.

The *D. piger* belongs to sulfate-reducing bacteria which are usually considered as a commensal bacterium in humans [5, 10, 50]. More recently, *D. piger* has attracted more interest as it was found to be the most prevalent species of SRB in feces of patients with inflammatory bowel disease [20, 21, 25, 31]. The obtained bacterial strains have such phenotypic features as the presence of desulfovirlidin, cytochrome *c*₃, and menaquinone MK-6. They oxidize organic compounds incompletely to acetate [10, 11].

Moore W.E. found SRB for the first time in people’s feces and identified it as *Desulfomonas pigra* [53], which subsequently is reclassified as *Desulfovibrio piger* [16]. The described bacterial strains are similar to that of Moore et al. [53] except for the G–C content of the DNA, which is 64 mol%. Obligate anaerobic, sulfate-reducing, non-saccharolytic, non-proteolytic, nonspore-forming, and Gram-negative bacteria that are straight and vibrio-like and have rounded ends ($0.8\text{--}1.0 \times 2.5\text{--}10.0\text{ }\mu\text{m}$) [10]. These microorganisms use lactate, pyruvate, ethanol, and hydrogen as electron donors for sulfate reduction. They oxidize lactate and pyruvate incompletely to acetate. The optimum temperature for growth is $+37^{\circ}\text{C}$. Growth is not affected by 20% bile. Colonies on anaerobic blood agar are translucent, 2 mm in diameter, circular, and non-hemolytic. Cells contain desulfovibridin and cytochrome c_3 . These bacteria isolated from human specimens (feces, peritoneal fluids, and intra-abdominal collections). The type strain, isolated from human feces, is ATCC 29098 [10].

7. Generalization of the research

Taking into consideration all research described in the chapter, it is necessary to generalize that isolation of mesophilic SRB from environmental samples (water, soil, swamp, etc.) and intestinal samples can be similar, although swamps and feces are required to determine dry matter of the samples. It is important to purify a positive sample of SRB from other satellite microorganisms such as *Clostridium*, *Bacteroides*, *Pseudomonas*, etc. With this aim, obtained SRB mixed cultures should be 3–5 times repassed to the modified Postgate agar medium with sulfite which inhibit the growth other microorganisms. Ability to growth of SRB mixed culture with high sulfite concentration allows to eliminate (purify SRB) from other microorganisms which can be in close interactions with SRB. The cultivation conditions depend on sample from where it is isolated. The key criteria for identification based on physiological and biochemical characteristics are the morphology of bacterial

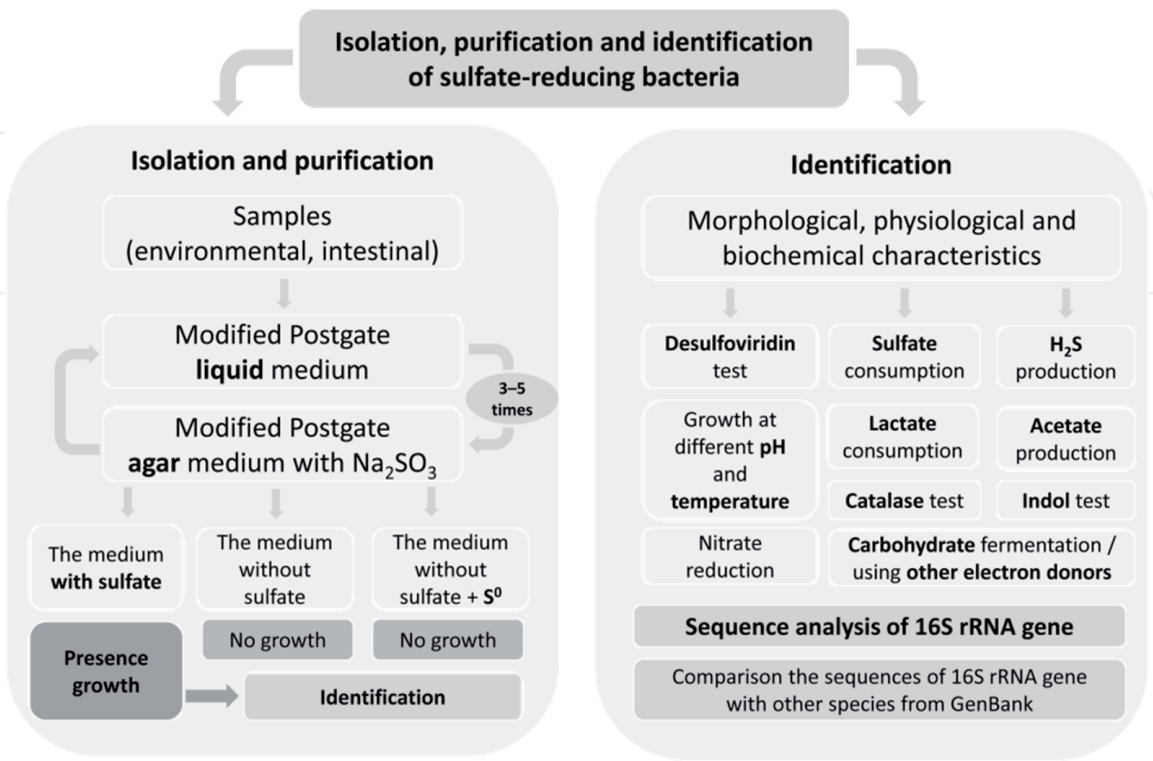


Figure 8. The general scheme of isolation, purification, and identification of mesophilic sulfate-reducing bacteria.

cells, ability to form of spores, sulfate reduction to hydrogen sulfide, lactate oxidation to acetate or CO₂, use of other organic compounds as an electron donor and carbon sources, etc. The general scheme of isolation, purification, and identification of mesophilic sulfate-reducing bacteria is presented in **Figure 8**.

For identifications of SRB based on morphological, physiological, and biochemical characteristics, two Bergey's Manuals [10, 11] are recommended. Moreover, all isolated SRB species should be confirmed by the sequence analysis of the 16S rRNA gene by using universal primers or primers of functional genes of dissimilatory sulfate-reduction, such as *DsrAB* and *AprBA*.

8. Conclusions

The methods of sample selections from water, soil, swamp, and feces of humans or animals and from biopsy material and the process of SRB isolation and purifications are similar, although cultivation conditions may differ. Identification based on physiological and biochemical properties is a complex process, and many other factors must be considered. For this identification, Bergey's Manuals are recommended to be used. The sequence analysis of the 16S rRNA gene should confirm the identification process based on physiological and biochemical properties.

It is of vital importance to obtain new strains of the SRB from various ecotopes and identify them and study their growth and physiological and biochemical properties. Aside from that, the process of dissimilatory sulfate reduction by SRB and the production of hydrogen sulfide should be investigated in order to clarify the etiological role of these bacteria in the nature and in the development of various diseases.

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Conflict of interest


The authors declare no conflict of interest.

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