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SHORT COMMUNICATIONS

The Role of the Heterotrophic Bacteria Associated with the Cyanobacterium *Nostoc muscorum* in the Formation of Cadmium Sulfide

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Some heterotrophic microorganisms are able to form cadmium sulfide [1–3]. Sulfate-reducing bacteria and some yeasts produce CdS extracellularly. The yeasts *Schizosaccharomyces pombe* and *Candida glabrata* form intracellular CdS nanoparticles. *Klebsiella pneumonia* and *Clostridium thermoaceticum* form CdS crystallites on the cell surface. The deepwater marine bacterium *Pseudomonas aeruginosa* form CdS crystallites on the cell wall. The formation of CdS detoxifies Cd and enhances the tolerance of microorganisms to this heavy metal.

Recently, the formation of CdS crystallites has been observed in the slimy sheath of the cyanobacterium *Nostoc muscorum* [4]; however, neither the mechanism of CdS formation nor the source of sulfide ions have been revealed.

We suggested that CdS is formed in *N. muscorum* cultures with the involvement of heterotrophic bacteria that live in the slimy sheath of the cyanobacterium. To verify this supposition, we isolated such heterotrophic bacteria and investigated their ability to form H_2S, to bind Cd ions, and to tolerate the toxic action of these ions.

Heterotrophic bacteria were isolated from a culture of the *Nostoc muscorum* strain VKM-16, which was obtained from the All-Russia Collection of Microorganisms (VKM). The cyanobacterium was grown at room temperature in a modified C medium of Kratz and Myers containing 0.4 g/l K_2HPO_4. Heterotrophic bacteria were isolated from the *N. muscorum* culture incubated in the presence of 1 mM $Cd(NO_3)_2$ for 2 months. The isolation was carried out at 27°C using the C medium supplemented with 1 g of each glucose and peptone and solidified with 1.4% agar (the so-called Csp medium).

The ability of the bacteria to form H_2S was tested using indicator paper impregnated with lead acetate. Alternatively, H_2S was detected by growing bacterial cultures on Kligler iron agar, which turns black in the presence of H_2S.

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The effect of 1 mM $Cd(NO_3)_2$ on the growth of heterotrophic cultures in a Csp medium containing 0.04 g/l K_2HPO_4 and no EDTA was investigated by measuring culture turbidity at 600 nm in an SF-18 spectrophotometer (LOMO, Russia). The medium was inoculated with 1-day-old cultures.

Cadmium ions bound to bacterial cells were detected with dithizone, which forms the yellow–orange insoluble salt dithizonate in the presence of cadmium ions [5]. The control experiments showed that dithizone did not produce colored products in the media used in this work if they did not contain cadmium ions.

We succeeded in obtaining 16 heterotrophic isolates, 14 of which were tested for the ability to form H_2S in a liquid Csp medium supplemented with 0.005% of both cystine and cysteine. Of these strains, eight were found to be able to form H_2S with different intensities. Further studies were carried out with strains 1A and 2A, which were isolated from the *N. muscorum* culture that was not incubated with cadmium, and strains 1B and 2B, which were isolated from the *N. muscorum* culture incubated with 1 mM $Cd(NO_3)_2$ for 2 months. These strains showed good growth and intense formation of H_2S.

The colonies produced by strains 1A and 2A were orange, round, convex, shiny, smooth, and had a diameter of 3 mm. The colonies produced by strains 1B and 2B were colorless, round, convex, shiny, smooth, pinpoint, blue-fluorescent, and had a diameter of less than 1 mm. Cells were gram-negative short rods.

To investigate the effect of sulfur-containing amino acids on the formation of H_2S, 1-day-old cultures were inoculated into the C medium containing 40 mg/l K_2HPO_4 and 0.1 g/l Na_2EDTA and supplemented with 0.2 g/l of both glucose and peptone, 0.01 g/l yeast extract, and 0.01% of both cysteine and cystine (medium 1); 0.01% of both cysteine and cystine (medium 2); 0.4 g/l of both glucose and peptone and 0.01 g/l yeast extract (medium 3); and 0.4 g/l of both glucose and peptone (medium 4). After 1 day of incubation, only strain 2B showed the formation of H_2S, irrespective of the growth medium used. After the next 1 day of incubation, H_2S was detected in all of the four

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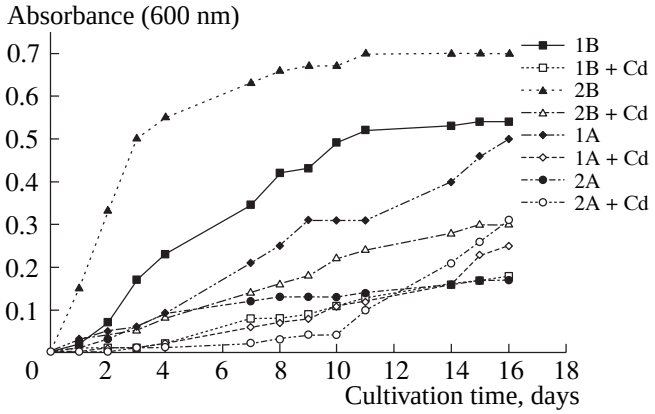
cultures, but strain 1A produced H_2S only when grown in medium 2. The production of H_2S by strains 2A, 1B, and 2B was most intense in media 1 and 2. The most intense formation of H_2S in a liquid Csp medium supplemented with 0.0025% of both cystine and cysteine was observed for strain 2B, while strain 1A did not produce H_2S in a this medium at all. It should be noted that the production of H_2S preceded the active growth of cells, which was most active in media 3 and 4 (these media contained two times as much glucose and peptone as media 1 and 2 did). All strains grew well on Kligler agar; however, only strain 2B produced hydrogen sulfide on this medium.

The effect of Cd on bacterial growth was studied using liquid and agar media. As can be seen from the figure, bacterial growth in liquid media in the presence of 1 mM $Cd(NO_3)_2$ had dynamics typical of batch cultivations. The growth of strains 1A, 1B, and 2B in the presence of Cd was characterized by slightly extended lag phases and lower growth rates than in the absence of Cd. Strain 2A began growing in the presence of Cd after a 10-day lag phase, but, by the end of cultivation, the biomass of this strain was higher than in the control. All of the four strains showed good growth on a Csp medium solidified by 1.2% agar and containing no EDTA. Namely, noticeable growth was observed 1 day after inoculation with 1-day-old cultures diluted by 10^3 and 10^4 times, while confluent growth was observed 3 days after such inoculation. When incubated for 3 days on the same agar medium containing 0.1 mM $Cd(NO_3)_2$, strains 1B and 2B showed confluent growth only in the case of inoculation with a 10^{-3} dilution. In the case of inoculation with a 10^{-4} dilution, these strains produced from 12 to 90 individual pinpoint colonies. Under the same conditions, strain 2A produced 200 and 90 colonies (when inoculated with the 10^{-3} and 10^{-4} dilutions). The poorest growth in the presence of Cd in early cultivation terms was exhibited by strain 1A (8 and 1 colony, respectively). However, after 6 days of cultivation in the presence of 0.1 mM $Cd(NO_3)_2$, the growth of all of the strains improved, so that even strain 1A produced, respectively, 200 and 46 colonies when inoculated with 10^{-3} and 10^{-4} dilutions. None of the strains could grow on an agar medium in the presence of 1 mM $Cd(NO_3)_2$.

Experiments with dithizone showed that cells of strain 2B grown on an agar medium containing 0.1 mM $Cd(NO_3)_2$ bind Cd ions.

Thus, some heterotrophic bacterial associated with the cyanobacterium are cadmium tolerant, which may be associated with their ability to produce hydrogen sulfide and to bind cadmium ions. The beneficial effect



The effect of cadmium on the growth of the heterotrophic bacteria (strains 1A, 2A, 1B, and 2B) isolated from *N. muscorum* cultures. Curves 1A, 2A, 1B, and 2B show the growth of the respective strains in the absence of Cd, whereas curves 1A + Cd, 2A + Cd, 1B + Cd, and 2B + Cd, show the same in the presence of 1 mM $Cd(NO_3)_2$.

of cysteine on the production of H_2S by heterotrophic bacteria suggests that they contain cysteine desulfohydrase. The formation of cadmium sulfide in the slimy sheaths of the cyanobacterium *N. muscorum* is likely due to the heterotrophic bacteria that live there. It is possible that the creation of an artificial association of the cyanobacterium with heterotrophic bacteria capable of sulfide production and tolerant of Cd ions will enhance the cadmium resistance of the cyanobacterium. Work along these lines is in progress in our laboratory.

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