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EXPERIMENTAL ARTICLES

Free Radicals in Mercury-Resistant Bacteria Indicate a Novel Metabolic Pathway

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Abstract—A mercury resistant-soil bacterium P.10.15, identified as a close relative of *Pseudomonas veronii*, was shown to accumulate a specific compound in the stationary phase of growth. This compound is converted to a long-lived free radical under oxidizing conditions, as registered by its EPR signal at room temperature. The compound was purified by ion-exchange and gel-filtration chromatography and identified by mass spectroscopy, 2D NMR, and EPR as a trisaccharide β-D-Gl*cp*NOH,CH_3-(1⟶6)-α-D-Gl*cp*-(1⟶1)-α-D-Gl*cp*, or, in other words, as 6-O-(2-deoxy-2-{*N*-methyl}hydroxylamino-β-D-glucopyranosyl)-α-α-trehalose, previously discovered in *Micrococcus luteus (lysodeikticus)* and named lysodektose. It is suggested that the compound is a novel intermediate of a previously unknown basic metabolic pathway of trehalose transformation in bacteria, a potential target for antibacterial drug development.

Key words: mercury, trehalose, *Pseudomonas*, radicals.

Soil bacteria usually detoxify divalent mercury ions Hg^{++} by enzymatic reduction to volatile Hg^0 [1] using NADPH and thiol compounds as electron donors. As we reported earlier [2–4], some bacteria contain highly reactive reducing hydroxylamine derivatives, which, in the presence of oxidizing agents, are converted into long-lived free radicals easily detectable at room temperature. As a part of the program "Microbial Biodiversity as a Function of Soil Contamination by Mercury," we have investigated these long-lived radicals in mercury-resistant (Hg-R) bacteria from soils of Germany and Kazakhstan [5, 6]. A radical-forming compound from a Hg-R strain proved to be structurally similar to a trisaccharide described earlier, lysodektose [3], suggesting that these compounds are indicative of the existence of a previously unknown metabolic pathway of trehalose derivatives. Therefore, the present work was aimed at the purification and characterization of the compound that is oxidized with the formation of long-lived free radicals in Hg-R bacteria.

MATERIALS AND METHODS

Bacterial *Pseudomonas* sp. strain P.10.15 isolated from soil was grown aerobically for 20 h at 28°C with shaking in a nutrient broth containing 10 g/l peptone, 5 g/l yeast extract, and 5 g/l NaCl. The strain was also capable of growth in the presence of 10 mg/l mercuric chloride. The taxonomic affiliation of the bacterial strain was determined by sequencing its 16S rRNA gene [7–9].

The radical-forming compound was extracted from bacterial pellets with 50% methanol. Formic acid was added to bring the extract to pH 4. The extract was then loaded onto a column packed with the SP Sephadex cation exchange resin, and the compound of interest was eluted in a gradient of 0–0.1 M ammonium formate (pH 6.5). The fractions containing the radical-forming compound were further purified by passage through Dowex 1 × 4 anion exchange resin, followed with an octyl Sepharose column. The eluate was adjusted to pH 4 by formic acid, loaded on an SP Sephadex column, and eluted with a gradient of 0–0.1 M NaCl. The fractions showing EPR spectra were concentrated to 1–3 ml and desalted by gel filtration on an 80-cm-long Sephadex G-10 column with conductometric detection. The fractions of interest were lyophilized and redissolved in 0.6 ml D_2O for NMR spectroscopy, after which they were transferred in methanol for mass spectroscopy.

Free radical formation was induced by the addition of potassium ferricyanide to 0.01 M. The samples were immediately transferred into a quartz cuvette of a RE-1306 radiospectrometer. The spectra were recorded at room temperature. Mn^{++}/MgO was used as an external standard, with the third and fourth components of its spectrum flanking the signal of the studied compound and H_{3-4} = 8.69 mT. NMR spectra were obtained on a DRX-500 spectrometer (Brucker) using COSY, ROESY, and TOCSY methods.

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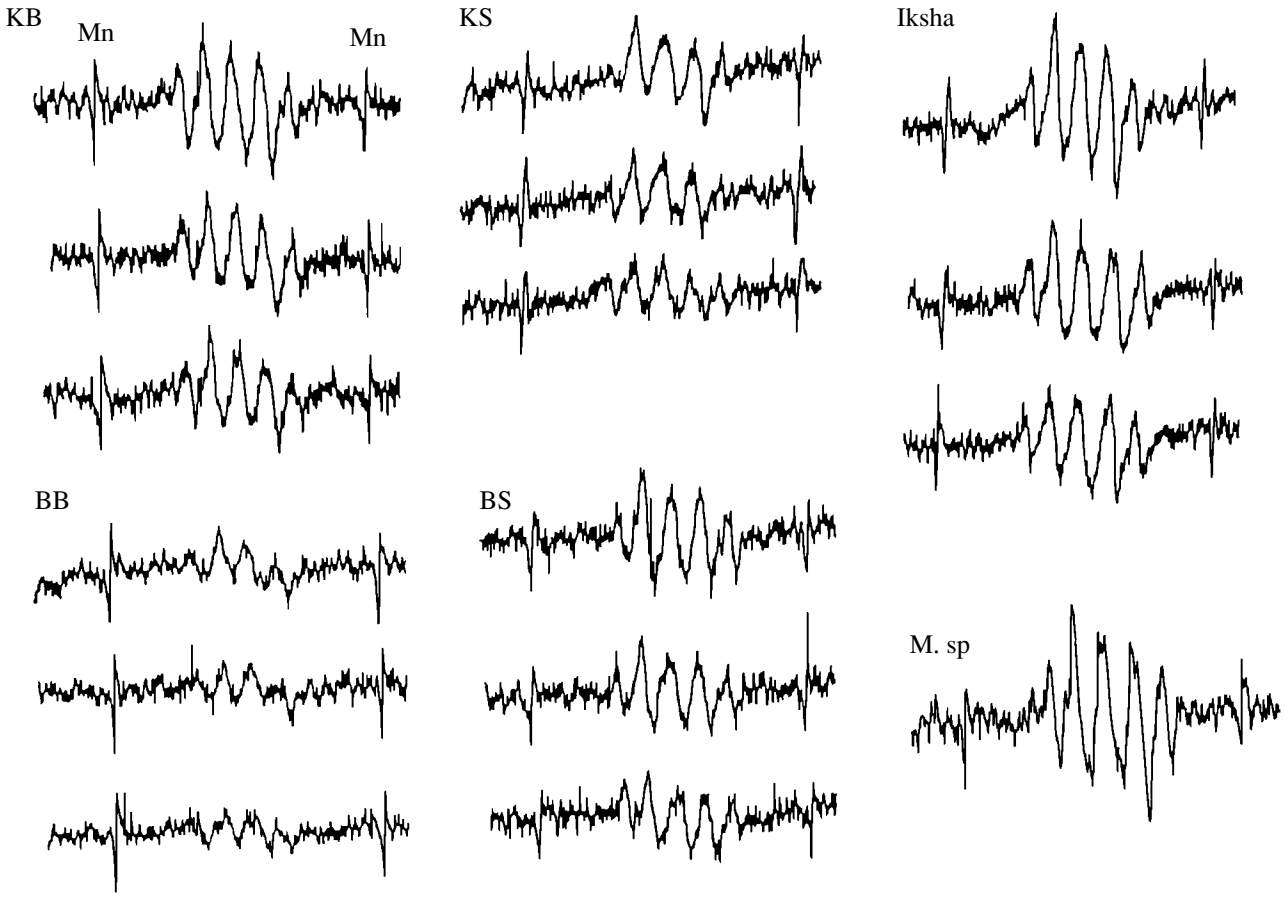


Fig. 1. EPR spectra of methanolic extracts from soil bacteria grown for three days at 30°C on nutrient agar. The extracts were concentrated so that 1 ml corresponded to 7 g of wet cell weight, and treated with 10 mM $K_3Fe(CN)_6$. KB, BB, and Iksha are soil samples; KS and BS, bottom sediment samples; M. sp, biomass of *Microbacterium* sp. AC-2050, an isolate from the rhizosphere of wild cereals from Tajikistan; Mn, the external standard.

RESULTS

Upon the addition of potassium ferricyanide to methanolic extracts of the total biomass of soil microorganisms grown for three days at 30°C on nutrient agar at a density of about 1000 colonies per petri dish, we detected an electronic paramagnetic resonance (EPR) signal with a *g*-factor value of 2.005, identical to the signal observed in extracts of a pure culture of *Microbacterium* sp. AC-2050 kindly provided by L. Evtushenko (Fig. 1). However, no EPR signals were detected in concentrated methanolic extracts of the soil itself, most likely due to the very low fraction of free radical producers in the soil biota.

We inquired whether similar free radical-forming compounds were present in individual clones of Hg-R bacteria grown aerobically to the stationary phase. Of the fourteen clones analyzed, we found reliable signals in three, and an extremely strong signal in one clone, designated P.10.15 (Fig. 2). We therefore concluded that such radical-forming compounds are unlikely to represent a common mechanism of mercury resistance. Nevertheless, the compound from clone P.10.15 was remarkable because of the similarity of its EPR spectrum (a sextet with the 1 : 4 : 7 : 7 : 4 : 1 signal ratio and $g = 2.005$) to the spectrum of lysodektose nitroxyl radical from *Micrococcus luteus*.

To prove the nitroxyl nature of the compound from clone P.10.15, we thoroughly oxidized the cell extracts until the EPR signal disappeared, reduced the extracts with sodium borodeuteride, and oxidized them again with ferricyanide. From the new EPR spectrum (Fig. 2, D) it was evident that such treatment resulted in an introduction of deuterium in the position adjacent to the radical, a process characteristic of the nitron–nitroxyl–hydroxylamine rearrangement (Fig. 3). Prolonged incubation with an oxidizing reagent caused irreversible disappearance of the EPR signal.

Cells of clone P.10.15 were rods exhibiting typical gram-negative staining, although radical-forming compounds were never identified in Gram-negative bacteria before [3]. Therefore, we determined the taxonomic position of the strain by sequencing the 16S rRNA gene and comparing this sequence with known 16S rDNA

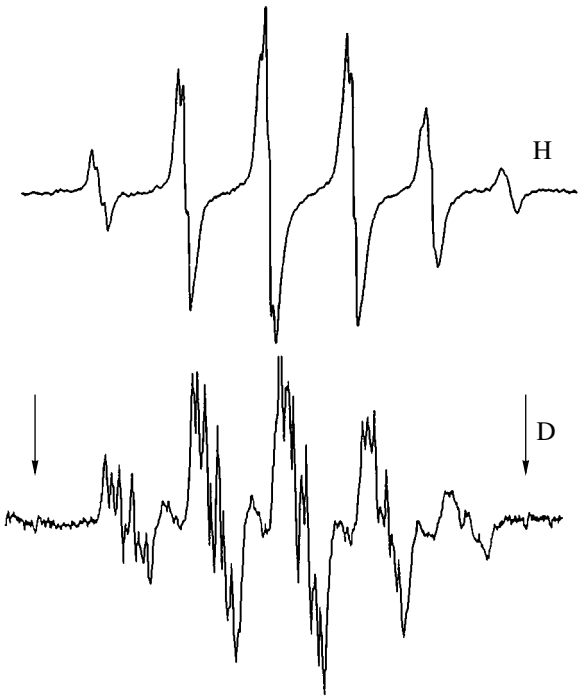
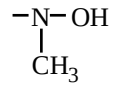


Fig. 2. EPR spectra of the radical-forming compound from the Hg-R bacterium *Pseudomonas* sp. P.10.15 in water after the addition of potassium ferricyanide. H, fully protonated starting material; D, monodeuterated compound. Arrows indicate the positions of signals from the Mn standard.

sequences. The clone was most closely related to *Pseudomonas veronii*, a species possibly infectious in mammals but beneficial in plants [7–9], which is in turn related to *Ps. syringae*, a very dangerous plant pathogen [10, 11].

The ability to generate EPR spectra was quickly lost during purification of the target compound, probably because of removal of reducing agents and reaction with the oxygen in air. In the final preparation, which contained less than 10% of the total activity found in the crude extract, we identified three compounds with the same trisaccharide carbohydrate backbone but differing in the moiety adjacent to the heteroatom in the C2 position of the C ring. As the ^{1}H NMR spectra show (Fig. 4), the dominant compound, whose content was estimated from the intensity of signals from protons (marked in the figure by a dotted line), constitutes about 80% of the total. However, modern two-dimensional NMR techniques allow detection of long-range interactions between different atoms in a molecule and thus filter out the signals from contaminating species. The characteristics of the major component shown in the table identify it as a trisaccharide in which two glucose rings A and B are connected as in trehalose, and one hydroxyl in the ring C is replaced with a heteroatom at C2 as



The major peaks in the mass spectrum had m/z = 518, m/z = 519, and m/z = 504 and probably corresponded, respectively, to the methylamine form and methoxy and hydroxy derivatives substituted at C2 in the C ring; however, the full form with m/z = 534 was also present (Fig. 5). Variations in the relative amounts of these forms in this preparation and in the lysodektose preparations analyzed earlier likely explain some differences in NMR parameters for the C2 atom and the substituent at C2 in the carbohydrate ring C.

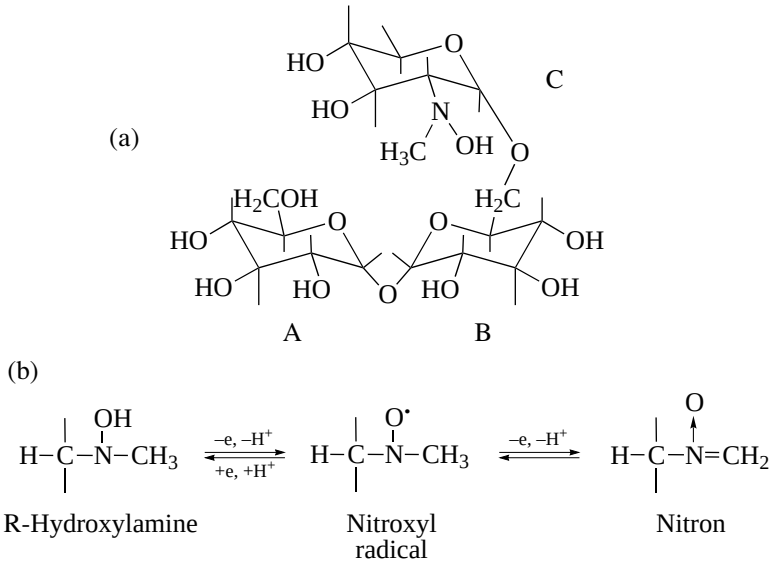


Fig. 3. Structure of the compound studied (A) and the scheme of its redox transformations induced by potassium ferricyanide (B).

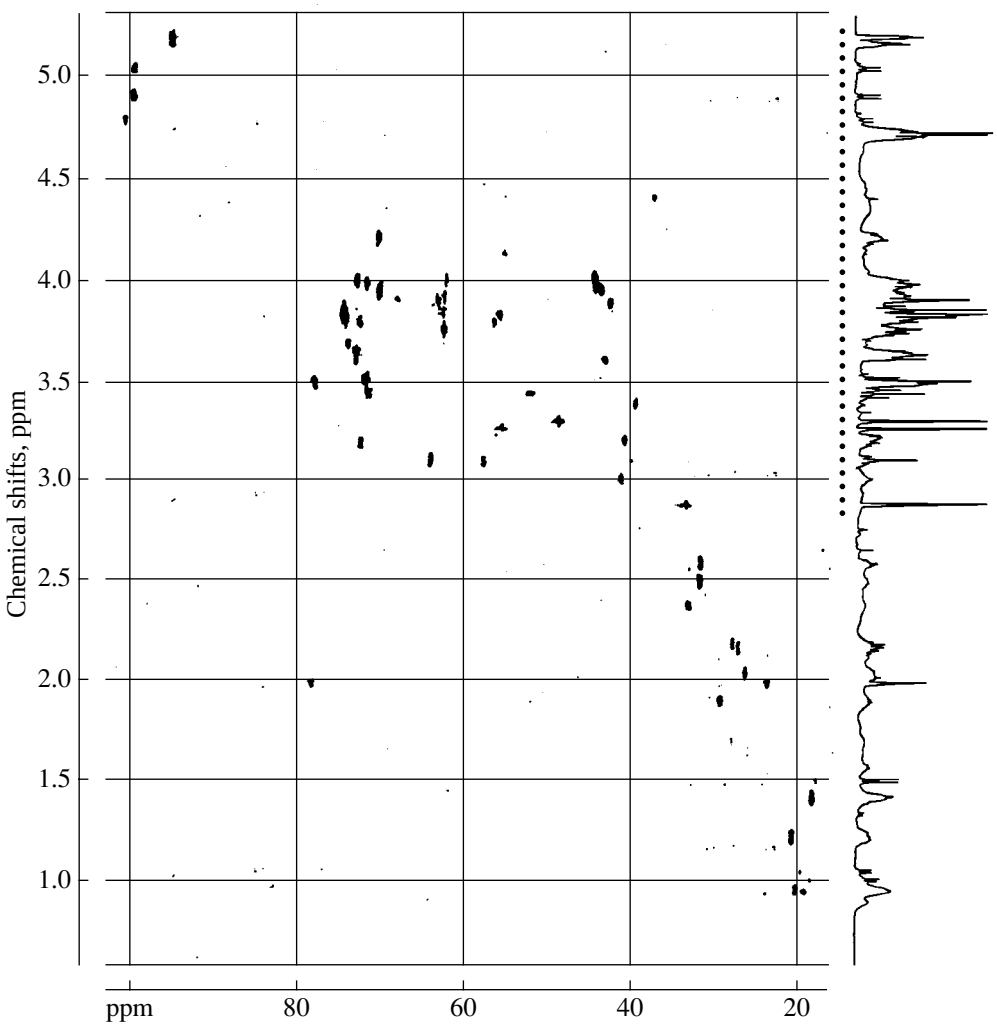


Fig. 4. Two-dimensional $^{1}H–^{13}C$ NMR spectra (HSQC) of the radical-forming compound from the Hg-R bacterium *Pseudomonas* sp. P.10.15 in D_2O. The linear part of the ^{13}C NMR spectrum was eliminated. The region of signals due to the protons of the investigated trisaccharide (2.8–5.2 ppm) is marked with a dotted line.

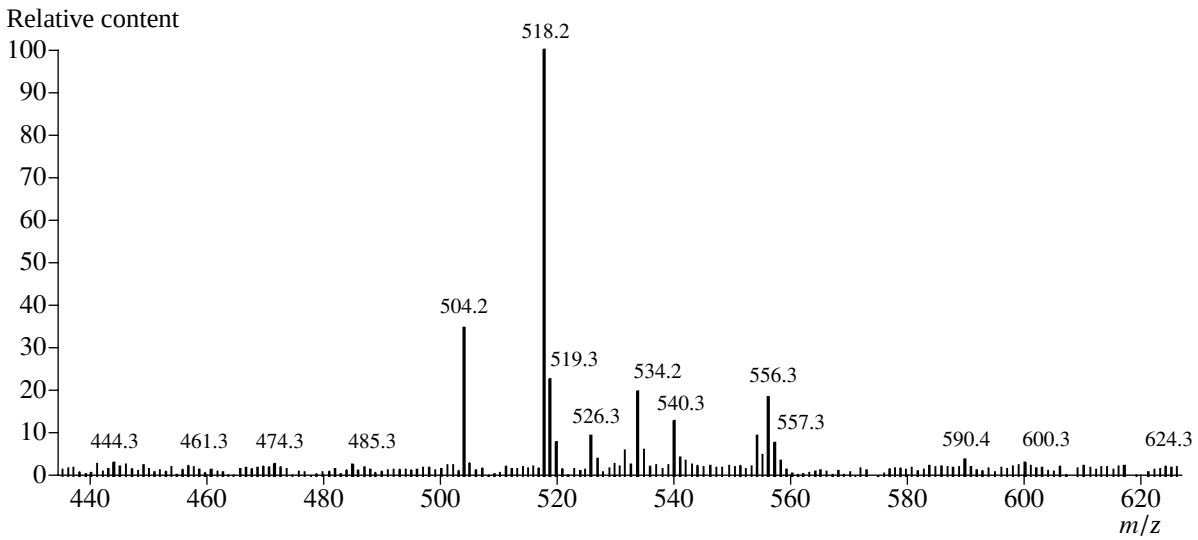


Fig. 5. Mass spectrum of the radical-forming compound from the Hg-R bacterium *Pseudomonas* sp. The spectrum was recorded on an LCQ mass spectrometer (Finnigan MAT).

NMR data (chemical shifts, ppm; and *J* constant, Hz) and the presumable structure of the radical-producing compound from the mercury-resistant bacterium *Pseudomonas* sp. P.10.15

Residue	Nucleus	^{13}C	^{13}C (lit.)	^{1}H	^{1}H (lit.)	*J* constant, Hz
A	1	94.8	94.7	5.15	5.19	d; 4.0 (1–2)
	2	72.3	72.3	3.63	3.63	dd;10.0 (2–3)
	3	73.9	73.8	3.85	3.84	t; 10.0 (3–4)
	4	71.0	71.0	3.45	3.76	ddt; 9.0 (4–5)
	5	72.3	72.9	3.78		
	6	61.9	61.9	3.96		
	6'			3.75		
B	1	94.8	94.7	5.20	5.19	d; 4.0 (1–2)
	2	72.3	72.3	3.65	3.65	dd; 10.0
	3	73.9	73.9	3.85	3.86	t; 10.0 (3–4)
	4	71.0	71.0	3.50	3.56	dd; 9.0 (4–5)
	5	72.3	71.9	4.00		
	6	69.7	69.0	4.22		
	6'			3.92		
C	1	99.8	101.4	4.86	4.85	d; 8.5 (1–2)
	2	63.6	47.6	3.04	2.61	dd;10.0 (2–3)
	3	73.8	73.5	3.82	3.81	dd; 9.0 (3–4)
	4	71.0	71.4	3.50	3.44	dd; 10.0 (4–5)
	5	77.3	76.8	3.50		
	6	61.9	62.2	3.90		
	6'			3.75		
	Nme	33.2	34.1	2.84	2.91	

β-D-Glc*p*NOH,CH_3-(1⟶6)-α-D-Glc*p*-(1⟶1)-α-D-Glc*p*
(C) (B) (A)

Note: lit., data from [2]; d, doublet; t, triplet.

The compound was eluted from a G-10 gel-filtration column in the fraction corresponding to $(V_t - V_0/V_e - V_0) = 0.5$, which translates to an organic salt molecular weight of 300 Da. However, nitrous compounds are notorious for their slowed elution in unbuffered solutions due to interactions with the anion-exchange groups of the resin. An increase in the ionic strength of the buffer (0.1 M NaCl) shifted the coefficient to 0.23, corresponding to a molecular weight of about 600 Da.

Therefore, we conclude that the radical-forming compound from *Pseudomonas* sp. strain P.10.15 is identical to the trisaccharide lysodektose from *M. luteus* [3].

DISCUSSION

Ps. syringae and related bacteria cause disease in many plant species. Apart from a number of environmental factors, the virulence of these bacterial agents depends on their ability to produce coronatine and similar compounds, which are synthesized under control of special plasmids and impair the hormonal state of the host plant [10–12]. *Ps. veronii* is believed to promote the development of beneficial microbial communities in the rhizosphere of some plants [8]. On the other hand, it is a suspected human pathogen causing a specific pathologic condition [9]. Can the free radicals described here also be virulence factors?

The RNO compound was found in *Pseudomonas* sp. cells in the stationary phase of growth but not in the logarithmic phase. Such accumulation dynamics are typical of the products of secondary metabolism, an idea also favored by the structural similarity to the well-known antibiotic streptomycin. However, the presence of such an unusual compound in such distantly related microorganisms as a gram-positive micrococcus and a gram-negative pseudomonad suggests that the RNO compound belongs to basic metabolites, which are normally never present in cells in high concentrations.

The postulated new metabolic pathway likely begins with trehalose, which by itself is an important factor for cellular protection against drying and other types of stress; in addition, trehalose dimycolate participates in the defense against bacteriophages. It is not clear at the moment whether the novel trehalose derivative is a final product or an intermediate of the synthesis of other compounds. If it is a final product, its possible function may be an increase in the antioxidant potential of the cytoplasm, or it may participate in the formation of protective compounds of lignin nature, since synthetic nitroxyls are known to become engaged in such free radical processes.

Trehalose and its derivatives are of long-standing interest for biologists and chemists [13]. This interest is still not waning owing to the role of trehalose in stabilizing microbial cells during stress [14, 15] and the functions of mycolate derivatives of trehalose both in the resistance of bacteria to viruses [16] and in the stimulation of nonspecific immunity in homoiothermal animals [17–19]. Similar to other bacteria-specific metabolic pathways, the metabolism of trehalose may be targeted during the development of antibacterial drugs [20]. We hope that the studies of biosynthesis and further metabolism of the radicals described in this paper may provide useful information for the search of novel antibacterial agents.

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REFERENCES

1. Osborn, A., Bruce, K., Strike, P., and Ritchie, D., Distribution, Diversity and Evolution of the Bacterial Mercury Resistance mer Operon, *FEMS Microbiol. Rev.*, 1997, vol. 19, pp. 239–262.

2. Biniukov, V., Kharatian, E., Ostrovsky, D., and Shashkov, A., Lysodektose—a New Trisaccharide from *Micrococcus lysodeikticus* Which Is Transformed into an Aminoxyl Free Radical with the Loss of an Electron, *Free Rad. Res. Commun.*, 1991, vol. 14, pp. 91–95.
3. Binyukov, V.I., Taptykova, S.D., and Ostrovskii, D.N., A Novel Type of Free-Radical Compounds from Cellular Extracts of Actinomycetes, *Mikrobiologiya*, 1986, vol. 52, pp. 342–344.
4. Stepanov, S., Biniukov, V., Kharatian, E., Shumaev, K., and Ostrovsky, D., Interconversion of Radical and Nitron Forms of Lysodektose—a New Trisaccharide from *Micrococcus lysodeikticus*, *BioFactors*, 1991, vol. 3, pp. 37–39.
5. Ostrovskii, D.N., Lysak, E.I., Demina, G.R., and Binyukov, V.I., Interaction of Bacteria with Mercuric Compounds, *Mikrobiologiya*, 2000, vol. 69, pp. 620–629.
6. Schloter, M., Lebuhn, M., Heulin, T., and Hartmann, A., Ecology and Evolution of Bacterial Microdiversity, *FEMS Microbiol. Lett.*, 2000, vol. 24, pp. 647–660.
7. Elomari, M., Coroler, L., Hoste, B., Gillis, M., Izard, D., and Leclerc, H., DNA Relatedness among *Pseudomonas* Strains Isolated from Natural Mineral Waters and Proposal of *Pseudomonas veronii* sp. nov., *Int. J. Syst. Bacteriol.*, 1996, vol. 46, pp. 1138–1144.
8. Adhikari, T., Joseph, C., Yang, G., Phillips, D., and Nelson, L., Evaluation of Bacteria Isolated from Rice for Plant Growth Promotion and Biological Control of Seedling Disease of Rice, *Can. J. Microbiol.*, 2001, vol. 47, pp. 916–924.
9. Cheuk, W., Woo, P., Yuen, K., Yu, P., and Chan, J., Intestinal Inflammatory Pseudotumour with Regional Lymph Node Involvement: Identification of a New Bacterium as the Aetiological Agent, *J. Pathol.*, 2000, vol. 192, pp. 289–292.
10. Ullrich, M., Bereswill, S., Volksch, B., Fritsche, W., and Geider, K., Molecular Characterization of Field Isolates of *Pseudomonas syringae* pv. *glycinea* Differing in Coronatine Production, *J. Gen. Microbiol.*, 1992, vol. 139, pp. 1927–1937.
11. Ullrich, M., *Pseudomonas syringae*—an Opportunistic Plant Pathogen and Saprophyte Controlled by Specific Environmental Factors, *Soc. Gen. Microbiol. 149th Meeting, Norwich, 2001, Abstracts*, p. 7.
12. Bender, C., Palmer, D., Penaloza-Vazquez, A., Rangaswamy, V., and Ullrich, M., Biosynthesis and Regulation of Coronatine, a Non-Host Specific Phytotoxin Produced by *Pseudomonas syringae*, *Subcell. Biochem.*, 1998, vol. 29, pp. 321–341.
13. Hunter, S., Barr, V., McNeil, M., Jardine, I., and Brennan, P., Trehalose-Containing Lipooligosaccharide Antigens of *Mycobacterium* sp.: Presence of a Mono-O-Methyltri-O-Acyltrehalose "Core", *Biochemistry*, 1988, vol. 27, pp. 1549–1556.
14. Muller, J., Boller, T., and Wiemken, A., Trehalose Becomes the Most Abundant Non-Structural Carbohydrate during Senescence of Soybean Nodules, *J. Exp. Bot.*, 2001, vol. 52, pp. 943–947.
15. Canovas, D., Fletcher, S., Hayashi, M., and Csonka, L., Role of Trehalose in Growth at High Temperature of *Salmonella enterica* Serovar *typhimurium*, *J. Bacteriol.*, 2001, vol. 183, pp. 3365–3371.
16. Besra, G., Khoo, K., Belisle, J., McNeil, M., Morris, H., Dell, A., and Brennan, P., New Pyruvylated, Glycosylated Acyltrehaloses from *Mycobacterium smegmatis* Strains, and Their Implications for Phage Resistance in Mycobacteria, *Carbohydr. Res.*, 1994, vol. 251, pp. 99–114.
17. Lima, V., Bonato, V., Lima, K., Dos Santos, S., Dos Santos, R., Goncalves, E., Faccioli, L., Rodrigues-Junior, J., and Silva, C., Role of Trehalose Dimycolate in Recruitment of Cells and Modulation of Production of Cytokines and NO in Tuberculosis, *Infect. Immun.*, 2001, vol. 69, pp. 5305–5312.
18. Madonna, G., Ledney, G., Elliott, T., Brook, I., Ulrich, J., Myers, K., Patchen, M., and Walker, R., Trehalose Dimycolate Enhances Resistance to Infection in Neutropenic Animals, *Infect. Immun.*, 1989, vol. 57, pp. 2495–2501.
19. Ryll, R., Watanabe, K., Fujiwara, N., Takimoto, H., Hasunuma, R., Kumazawa, Y., Okada, M., and Yano, I., Mycobacterial Cord Factor, but not Sulfolipid, Causes Depletion of NKT Cells and Upregulation of CD1d1 on Murine Macrophages, *Microbes Infect.*, 2001, vol. 3, pp. 611–619.
20. Sode, K., Akaike, E., Sugiura, H., and Tsugawa, W., Enzymatic Synthesis of a Novel Trehalose Derivative, 3,3'-Diketotrehalose, and Its Potential Application as the Trehalase Enzyme Inhibitor, *FEBS Lett.*, 2001, vol. 489, pp. 42–45.