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EXPERIMENTAL ARTICLES

Determination of the Sensitivity of Bacteria to Barium Ions, a Taxonomic Marker of the Genus *Pseudomonas*

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Abstract—Comparative efficacy of the determination of the sensitivity of bacterial cells to barium ions was evaluated on a synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and AGV agar. The synthetic nutrient medium developed for this study contained L-proline and L-glutamine as the sole nitrogen and carbon source, which promoted growth of all *Pseudomonas* strains and ensured the minimal level of barium binding. The sensitivity of 80 strains belonging to 11 *Pseudomonas* species, including the type strains, as well as of 80 strains of 22 other bacterial species, was studied. The sensitivity of bacteria to barium ions was determined by using serial dilutions of barium chloride in the nutrient medium. The highest level of analytical sensitivity of pseudomonads to barium ions was determined on the synthetic nutrient medium: the minimal inhibitory concentration (MIC) values of barium chloride ranged from 0.5 to 6 g/L, the MIC_{90} value was 2 g/L. At the same time, 86.1% of all strains of fluorescent *Pseudomonas* species produced fluorescein on the control $BaCl_2$-free synthetic nutrient medium. For representatives of other genera grown on all the studied nutrient media, the MIC values of barium chloride ranged from 20 to 50 g/L. The proposed method for determination of the sensitivity of bacteria to barium ions using the synthetic nutrient medium with 6 g/L of barium chloride as a criterion for the classification of barium-sensitive strains to the genus *Pseudomonas* is suitable for standardization.

Keywords: sensitivity of bacteria to barium ions, synthetic nutrient medium, taxonomic marker, *Pseudomonas*.

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The unique selective sensitivity of bacteria of the genus *Pseudomonas* (previously the first rRNA homology group) to barium ions was first described by usin 1985 [1]. Based on this sensitivity, a method for identification of bacteria of the genus *Pseudomonas* was developed [2, 3]. Fish meal hydrolysate agar manufactured by the Dagestan Research Institute of Nutrient Media was used. The minimal inhibitory concentration (MIC) value of 2 g/L was used as a criterion of the sensitivity of pseudomonads to barium ions (as barium chloride or barium nitrate). The results obtained by other authors studying a wide spectrum of pseudomonads confirm the selective sensitivity of the *Pseudomonas* species to barium ions [4, 5]. The sensitivity of bacteria to barium chloride and barium nitrate was studied using meat-peptone agar (the manufacturer was not specified). It was established that the range of MIC values of barium chloride for pseudomonads of the first rRNA homology group was 0.5–10 g/L and 20–50 g/L for other genera. The authors advised to use the MIC value of barium chloride of 10 g/L as a criterion of sensitivity.

Hence, there is no standard method for evaluation of bacterial sensitivity to barium ions, as various nutrient media manufactured from natural products such as fish and meat hydrolyzates and peptones with different compositions of barium-binding substrates are used, resulting in different levels of analytical sensitivity and evaluation criteria.

The goal of the present work was to develop and experimentally validate the method (suitable for standardization) for determination of bacterial sensitivity to barium ions as a taxonomic marker for bacteria of the genus *Pseudomonas*.

MATERIALS AND METHODS

The subjects of study were 80 strains of 11 *Pseudomonas* species and 80 strains of 22 other bacterial species. The type strains *P. putida* CIP 52191^T (ATCC 12633), *P. alcaligenes* CIP 101034^T (ATCC 14909), *P. luteola* CIP 102995^T (JCM 3352), *P. oryzihabitans* CIP 102996^T (JCM2952), and *P. fragi* CIP 55.4^T (ATCC 4973) were obtained from the bacterial collection of the Pasteur Institute, Paris; the type strains *P. fluorescens* IMV 4125 (ATCC 13525), *P. chlororaphis* subsp. *chlororaphis* IMV 4139 (ATCC 9446), *P. chlororaphis* subsp. *aureofaciens* IMV 4133 (ATCC 13985), *P. chlororaphis* subsp. *aurantiaca* IMV 387 (ATCC 49054), *P. mendocina* IMV 4172 (ATCC 25411), *P. stutzeri* IMV 4136 (ATCC 175887), and *P. pseudoalcaligenes* IMV 4134 (ATCC 17440) were

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obtained from the culture collection of the Zabolotnyi Institute of Microbiology, National Academy of Sciences of Ukraine, Kiev; the type strain *P. aeruginosa* GISK 190154 (ATCC 10145) was obtained from the Tarasevich State Institute of Standardization and Control of Biomedical Preparations, Moscow. In addition to the above-listed type strains, *P. aeruginosa* GISK 190127 (ATCC 27853) and 22 strains of *P. aeruginosa*, including 12 strains (109-N, 152-N, 156-N, 157-N, 160-N, 235-N, 330-N, 431-N, 558-N, 560-N, 662-N, and 731-N) isolated from the Neva River, 25 strains of *P. putida* (8 strains of *P. putida* biovar A, 3-N, 4-N, 5-N, 7-N, 9-N, 11-N, 12-N, 13-N and 2 strains of *P. putida* biovar B, 2-N and 6-N, were isolated from the Neva River), 12 strains of *P. fluorescens* (strains 356-N, 64-N, 176-N, 117-N, 128-N, and 363-N were isolated from the Neva River), as well as *P. stutzeri* 390-N, *P. alcaligenes* (18R-N and 19M-N), and *P. pseudoalcaligenes* (10-N and 16-N), were studied. Strains *P. alcaligenes* 79-K, *P. pseudoalcaligenes* 6897-K, *P. luteola* 22-K, and other *Pseudomonas* strains were isolated from clinical specimens at the Laboratory of Bacteriology of the Kirov Military Medical Academy, St. Petersburg.

Members of the species *Achromobacter xylosoxidans, Burkholderia cepacia, Acinetobacter baumannii, Alcaligenes faecalis, Bordetella bronchiseptica, Stenotrophomonas maltophilia, Elizabethkingia meningoseptica, Shewanella putrefaciens, Klebsiella pneumoniae, Raoultella planticola, Kluyvera ascorbata, Enterobacter cloacae, Serratia marcescens, Hafnia alvei, Salmonella typhimurium, Citrobacter freundii, Shigella sonnei, Morganella morganii, Escherichia coli, Providencia rettgeri, Aeromonas caviae, Aeromonas hydrophila*, and *Vibrio metschnikovii* (1–5 strains of each species), including the type strains *B. cepacia* IMV 4137 (ATCC 25416) and *S. maltophilia* IMV 4131 (ATCC 13637) from the culture collection of the Zabolotnyi Institute of Microbiology, National Academy of Sciences of Ukraine, Kiev, as well as *A. faecalis* GISK 242531 (ATCC 8750), *A. baumannii* GISK 030171 (ATCC 1530), *H. alvei* GISK 245530 (ATCC 13337), and *S. marcescens* GISK 220015 (ATCC 138801) from the Tarasevich State Institute of Standardization and Control of Biomedical Preparations, Moscow, were studied. Bacteria of the species *S. putrefaciens, A. caviae, A. hydrophila*, and *V. metschnikovii* were isolated from the samples collected in the Neva River; strains of other species were isolated from clinical specimens. All of the above-listed bacterial strains are stored in the working collection of the author at the Department of Microbiology, Kirov Military Medical Academy, Saint-Petersburg. Bacterial strains were identified using the methods and criteria described in [6–8].

The reagents used for the preparation of the synthetic nutrient medium were amino acids L-proline (Merck, United States; Reanal, Hungary) and L-glutamine (Merck, United States), bacto agar (Difco, United States), and barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) according to the State Standard (GOST) 4108-72.

Bacteria were grown on the nutrient FMH agar (Research and Production Center of Genetically Engineered Preparations, Obolensk, Russia), Mueller–Hinton agar (Hi-Media, India), and AGV medium (Scientific Production Association "Nutrient Media", Makhachkala, Russia). These media were used according to the manufacturers' instructions and sterilized at 121°C for 20 min. Then, the melted agarized medium was supplemented with weighed portions of barium chloride (0.25– 50 g/L) and dispensed into sterile petri dishes (nutrient medium with barium ions). Some of the medium did not contain barium chloride (nutrient medium without barium ions).

The synthetic nutrient medium used for assessment of bacterial sensitivity to barium ions was developed by us. The synthetic nutrient medium (pH 7 ± 0.2) contained the following (g/L): L-proline, 1.0–3.0; L-glutamine, 1.0–3.0; NaCl, 5.0; $MgCl_2 \cdot 6H_2O$, 0.05; KH_2PO_4, 0.05; K_2HPO_4, 0.1; bacto agar, 15.0; distilled water, 1 L; and barium chloride ($BaCl_2 \cdot 2H_2O$), 6.0–16.0.

Preparation and control of the synthetic nutrient medium. All ingredients, except for barium chloride, were dissolved during heating, and the medium was then boiled for 5 min. Then, part of the medium was dispensed into sterile petri dishes (nutrient medium without barium ions). The remaining hot medium was supplemented with the calculated amount of barium chloride according to the above protocol, agitated, and dispensed into sterile petri dishes (nutrient medium with barium ions). The petri dishes were stored at 4–8°C for 7 days. The biological control of the quality of the nutrient medium was performed when it was prepared for the first time and after changing any component of the medium. Strain *P. aeruginosa* ATCC 27853, used in bacteriological laboratories for controlling the accuracy of determination of the sensitivity of *Pseudomonas* species to antibiotics, was used as a control. Three different concentrations of barium chloride (0.5, 1.0, and 2.0 g/L) were added to the medium; the control medium did not contain barium chloride. The quality of the nutrient medium was deemed to have met the requirements if the control strain *P. aeruginosa* ATCC 27853 had the MIC value of barium chloride of 1 g/L, and if it grew well on the $BaCl_2$-free medium with fluorescein production.

Determination of bacterial sensitivity to barium ions on nutrient media. The cultures of gram-negative bacteria grown under aerobic conditions on FMH agar at 28°C for 18–24 h were used as inocula. The cells were collected with a loop (d = 2 mm) and resuspended in 0.2 mL of the sterile 0.85% NaCl solution in the wells of a sterile polystyrene plate. The petri dishes with the nutrient media (synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and AGV medium) containing different concentrations (0.25–50 g/L) of bar-

ium chloride, as well as the petri dishes with the control $BaCl_2$-free medium, were divided into 8 sectors which were labeled according to the strain numbers. The studied cultures were inoculated with a loopful of the suspensions from the plate wells (the inoculum dose was 1×10^7 CFU) in radial streaks onto the relevant sectors of the $BaCl_2$-containing media and the control $BaCl_2$-free medium. Inoculated plates were incubated at 28°C for 24 h. The MIC values of barium chloride (g/L) were determined if bacterial growth was detected on the control medium. Experiments with the synthetic nutrient medium were performed in triplicate.

Determination of the sensitivity of bacteria to barium ions as a taxonomic marker of the genus *Pseudomonas*. The studied cultures of gram-negative bacteria grown under aerobic conditions on nutrient media at 28°C for 18–24 h were collected with a loop (d =2 mm) and resuspended in 0.2 mL of the sterile 0.85% NaCl solution in the wells of a sterile polystyrene plate. Loopful of the bacterial suspension from the plate wells (the inoculum dose was 1×10^7 CFU) were used to inoculate the relevant sectors with the synthetic nutrient medium with $BaCl_2$ (6 g/L) (nutrient medium with barium ions) and without $BaCl_2$ (nutrient medium without barium ions; control) in radial streaks. The inoculated petri dishes were incubated under anaerobic conditions at 28°C for 24 h. Then, bacteria of the genus *Pseudomonas* were identified according to the absence of growth on the nutrient medium with barium ions and growth on the same nutrient medium without barium ions. At the same time, the yellow-green fluorescence on the bacterial lawn and the surrounding medium indicated the presence of fluorescent pseudomonads; bacteria capable of growing on both types of nutrient media belong to other species; bacteria incapable of growth on both types of nutrient media belong to auxotrophic strains of other species. The composition of the synthetic nutrient medium used in this study, as well as the methods used for its preparation and control, are described above.

RESULTS AND DISCUSSION

Comparative analysis of the sensitivity of 80 bacterial strains belonging to 11 *Pseudomonas* species to barium ions (as barium chloride) carried out using the synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and AGV medium revealed that the level of the analytical sensitivity of the synthetic nutrient medium was highest: the MIC range of barium chloride was 0.5–6.0 g/L; the MIC value for 90% of strains (MIC_{90}) was 2 g/L. All experiments were performed in triplicate with consistent and repeatable results. On FHM agar, the MIC values for pseudomonads ranged from 2 to 6 g/L, MIC_{90} = 4 g/L; on Mueller–Hinton agar, the MIC values ranged from 2 to 10 g/L, MIC_{90} = 6 g/L; and on the AGV medium, the MIC values ranged from 8 to 14 g/L, MIC_{90} = 10 g/L (Table 1). All the studied *Pseudomonas* strains grew on the synthetic nutrient medium without barium chloride (control) and on other $BaCl_2$-free media. The majority of the fluorescent *Pseudomonas* species (86.1%) actively produced fluorescein after 24-h incubation at 28°C on the $BaCl_2$-free synthetic nutrient medium. The high levels of analytical sensitivity and fluorescein production on the synthetic nutrient medium were due to the presence of the amino acids L-proline and L-glutamine. These amino acids promoted the growth of all pseudomonad strains irrespective of their profiles of amino acid utilization [9].

Comparative study of the sensitivity of 80 strains belonging to 23 species of other 22 genera of gram-negative bacteria (nonfermentative bacteria, *Aeromonas* species, vibrios, and enterobacteria) to barium ions on the synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and AGV medium revealed their high resistance to barium ions (with the MIC range of barium chloride from 20 to 50 g/L) on all the studied media (Table 2). Some strains were unable to grow on the control synthetic $BaCl_2$-free medium and were able to grow on other control media, which suggests that they are auxotrophic. When comparing the MIC range of barium chloride of pseudomonads to that of other bacteria grown on different nutrient media, the intermediate MIC ranges of barium chloride (which inhibit growth of all pseudomonads and do not inhibit growth of other bacteria) were determined: 6–16 g/L on the synthetic nutrient medium; 6–16 g/L on FMH agar; 10–16 g/L on Mueller–Hinton agar; and 14–16 g/L on the AGV medium. Therefore, the synthetic nutrient medium and FMH agar, rather than the AGV medium and Mueller–Hinton agar, are suitable for testing for *Pseudomonas* sensitivity to barium ions. However, preference should be given to the synthetic nutrient medium, which is most suitable for standardization, since it has fixed and reproducible composition, provides the highest analytical sensitivity and repeatability of results, and offers additional advantages. These include its ability to reveal fluorescein production, which confirms classification of the studied bacteria as pseudomonads without necessity of additional fluorescein production tests. Inhibition of bacterial growth on the synthetic nutrient medium containing 6 g/L of barium chloride is a criterion of the affiliation of the studied bacteria to the genus *Pseudomonas*.

We failed to detect any barium-sensitive strains among the studied 22 species, which indicates the specificity of this marker. The specificity and high taxonomic significance of the barium sensitivity test as a marker for bacteria of the genus *Pseudomonas* were confirmed by barium sensitivity of *P. oryzihabitans* (MIC value of barium chloride of 2 g/L) and *P. luteola* (MIC value of barium chloride of 1 g/L), which were

Table 1. Sensitivity of bacteria of the genus *Pseudomonas* to barium ions (as barium chloride) on the synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and the AGV medium at 28°C

Pseudomonas species	Number of strains	On the synthetic nutrient medium, the strains		MIC values of barium chloride (g/L) on nutrient media			
		Grow	Produce fluorescein	Synthetic nutrient medium*	FMH agar	Mueller–Hinton agar	AGV medium
P. putida biovar A	24	24	20	0.5–6	2–6	4–10	8–10
P. putida biovar B	2	2	2	2–6	4–6	6–8	10–12
P. aeruginosa	24	24	21	0.5–2	2–4	4	8
P. fluorescens	12	12	11	0.5–4	2–6	2–6	8–12
P. chlororaphis:							
subsp. *chlororaphis*	1	1	–	1	2	4	8
subsp. *aureofaciens*	1	1	1	0.5	2	2	8
subsp. *aurantiaca*	1	1	1	0.5	2	2	8
P. alcaligenes	4	4		0.5–1	4	2–4	8–10
P. pseudoalcaligenes	4	4		1–2	2–4	2	8
P. mendocina	1	1		1	2	4	8
P. stutzeri	2	2		2	4	4	8
P. fragi	1	1		4	6	6	14
P. oryzihabitans	1	1		2	2	4	8
P. luteola	2	2		1	2	4	8
Total: strains	80	80	56**				
%	100	100	86.1**				
MIC, g/L				0.5–6	2–6	2–10	8–14
MIC_{90}				2	4	6	10

* The experiments were performed in triplicate; **, of the fluorescent *Pseudomonas* species.

Table 2. Sensitivity of bacteria belonging to other genera to barium ions (as barium chloride) on the synthetic nutrient medium, FMH agar, Mueller–Hinton agar, and the AGV medium at 28°C

Genus, species	Number of strains	Growing on $BaCl_2$-free media		MIC values of barium chloride (g/L) on nutrient media			
		Synthetic nutrient medium	FMH agar, AGV medium, Mueller–Hinton agar	Synthetic nutrient medium	FMH agar	Mueller–Hinton agar	AGV medium
Achromobacter xylosoxidans	3	3	3	20–32	20–32	32	32
Burkholderia cepacia	5	5	5	20–32	20–32	32	32
Acinetobacter baumannii	5	5	5	32	32–40	50	32
Alcaligenes faecalis	1	1	1	40	50	50	50
Bordetella bronchiseptica	1	1	1	32	32	50	50
Stenotrophomonas maltophilia	5	–	5	–	40	50	50
Elizabethkingia meningoseptica	1	–	1	–	32	32	20
Shewanella putrefaciens	5	5	5	32	32	50	50
Klebsiella pneumoniae	5	5	5	40	40	50	40
Raoultella planticola	2	2	2	40	50	50	50
Kluyvera ascorbata	2	2	2	40	50	50	50
Enterobacter cloacae	5	5	5	40	50	50	50
Serratia marcescens	5	5	5	50	50	50	50
Hafnia alvei	5	5	5	40	40	50	50
Salmonella typhimurium	3	3	3	40	40	50	50
Citrobacter freundii	5	5	5	50	50	50	50
Shigella sonnei	3	–	3	–	32	50	32
Morganella morganii	3	–	3	–	50	50	50
Escherichia coli	3	3	3	32	20–32	50	50
Providencia rettgeri	2	2	2	40	50	50	50
Aeromonas caviae	5	5	5	40	50	50	50
Aeromonas hydrophila	3	3	3	40	50	50	50
Vibrio metschnikovii	3	–	3	–	20–32	32	32
Total: strains	80	65	80				
MIC, g/L				20–50	20–50	20–50	20–50

previously assigned to the genera *Flavimonas* and *Chryseomonas*, respectively; their affiliation to the genus *Pseudomonas* has long been a debatable issue [10–12]. At the same time, the specificity of this test requires further study and verification, since members of many genera belonging to various families and classes were not subjected to the barium sensitivity test.

In addition to the synthetic nutrient medium, unification of all stages of the study, as well as of assessment of the results obtained, are necessary for the standardization of this method for determination of bacterial sensitivity to barium ions.

As a result of this study, we propose a method for determination of bacterial sensitivity to barium ions as a taxonomic marker for the genus *Pseudomonas* (see

Materials and Methods). A patent for invention was issued for this method [13].

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