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EXPERIMENTAL ARTICLES

Response of Bacteria to Earthworm Surface Excreta

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Abstract—Response of bacteria to the surface excreta of the *Aporrectodea caliginosa* earthworm was studied. The excreta were obtained by a 1 h incubation of the earthworms in petri dishes with subsequent collection of the slime. Both inhibition and stimulation of growth were revealed, as well as suppression of the respiratory activity of some bacterial species treated with *A. caliginosa* surface excreta. The organisms studied included various taxa of soil bacteria (19 strains), bacteria isolated from *A. caliginosa* intestine and excrements (82 strain), and 48 *Bacillus thuringiensis* strains. For the cultures of soil bacteria, the respiratory activity was determined using the formazan color reaction due to the activity of the respiratory cycle enzymes. Earthworm excreta caused a consistent 30–50% decrease of dehydrogenase activity in 13 out of the 19 cultures. Determination of the growth rates (derived from OD_{620} of cell suspensions) after 10 h of incubation revealed growth stimulation in 48 out of the 82 strains isolated from intestines and excrement. Other strains exhibited no reaction to the excreta. For 29 out of 45 *B. thuringiensis* strains, growth stimulation was observed, while growth of two strains was suppressed; other strains exhibited no reaction to the excreta. No relation was found between bacterial reaction to the excreta and their taxonomic position. These results correlate with the research, demonstrating antibacterial and antifungal activity of the extracts from the earthworm body and digestive tract. Thus, earthworms, apart from their medium-forming function, affect the formation of soil microbial communities by direct stimulation or suppression of specific microbial populations.

Key words: bacteria, earthworms, surface excreta, growth stimulation and suppression.

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Microorganisms are known to play a major role in soil processes; invertebrates are believed to act as regulators of microbial activity. The methods of interaction between these organisms include modification of the environment (fabric interactions) and "communication" by means of signal metabolites [1]. Although these types of interorganism interactions (including the interaction between earthworms and microorganisms) are poorly studied, they are widespread in soil.

Burrowing activity of earthworms results in changes of the microbial and invertebrate composition and of the enzymatic activity; it therefore affects the processes in the soil around the burrows [2–8]. The mechanisms of these phenomena are not known. Biochemical modification of the environment, resulting from the physiological activity of surface slimes and coprolites, is among the possible explanations. These substrates contain mucoproteids, products of nitrogen metabolism, and other complex organic compounds [9]. We have previously demonstrated the capacity of both the earthworm surface and intestinal excreta for activating microbial respiration and nitrification in soil; the required concentrations were too low to support these processes energetically [10]. Selective microbiocidal and microbiostatic activity of the earthworm tissue, homogenates against some phytopathogenic bacteria and fungi has also been observed [11, 12]. These facts, although not numerous, convince us of the physiological activity of the earthworm excreta. In the present paper, reaction of bacteria belonging to different taxa and isolated from different environments to earthworm surface excreta was investigated. This knowledge may provide important insights concerning the ecological aspects of the interaction between earthworms and microorganisms.

The goal of the present work was to characterize the effect of the *Aporrectodea caliginosa* earthworm excreta on pure bacterial cultures.

MATERIALS AND METHODS

Soil. The cultivated sod-podzolic soil under legume–cereal vegetation was collected at the long-term experimental site of the Department of Agrochemistry, Moscow State University (Moscow oblast, Chashnikovo Soil Ecology Center, Moscow State University). The total content of carbon in the soil was 1.72%, the content of nitrogen was 0.13%; pH of the water extract was 5.7.

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Table 1. Bacteria studied in the work

Class	Species	Source of isolation
Alphaproteobacteria	*Agrobacterium tumefaciens* (*Rhizobium radiobacter*) 11*	*Aporrectodea caliginosa* intestine
	Brevundimonas diminuta E699	*Eisenia fetida* intestine
Betaproteobacteria	*Achromobacter xylosoxidans* 2	*Eisenia fetida* intestine
	Alcaligenes faecalis subsp. *parafaecalis* E588	*E. fetida* excrements
	Alcaligenes sp. TUT1002 1	*Eisenia fetida* intestine
	Delftia acidovorans 14	*Aporrectodea caliginosa* intestine
Gammaproteobacteria	*Aeromonas* sp. 616	*Aporrectodea caliginosa* intestine
	Schineria larvae 5	*Eisenia fetida* intestine
	Serratia marcescens E658	*E. fetida* excrements
	Stenotrophomonas maltophilia 12	*Aporrectodea caliginosa* intestine
	Pantoea sp. E680	*E. fetida* excrements
	Pseudomonas sp. 610–1	*Aporrectodea caliginosa* intestine
Actinobacteria	*Microbacterium paraoxydans* 597-3	*Aporrectodea caliginosa* intestine
	Micrococcus luteus 522-2	*Eisenia fetida* intestine
Bacilli	*Bacillus mojavensis*/*Bacillus subtilis* 523-1	*Eisenia fetida* intestine
	Paenibacillus sp. 598	*Aporrectodea caliginosa* intestine
	Sporosarcina sp. 2216.15.2 502-2	*Lumdricus terrestris* intestine
Flavobacteria	*Chryseobacterium* sp. 495	*Lumdricus terrestris* intestine
Sphingobacteria	*Sphingobacterium* sp. 590-2	*Aporrectodea caliginosa* intestine
	82 unidentified bacterial strains	*Aporrectodea caliginosa* intestine
Bacilli	46 *Bacillus thuringiensis* strains	Soil, sod, and insect larvae

* Only the strains exhibiting 99–100% similarity to respective collection species were used.

Earthworms. The *Aporrectodea caliginosa* earthworm used in the work, dwells in the 0–20 cm horizon of the investigated soil. The earthworms were collected in spring 2006 and maintained at 12–15°C in retainers with soil and tree waste.

Bacteria. Gram-positive and gram-negative bacteria from the collection of the Department of Soil Biology, Moscow State University were used. Some of the bacteria (19 strains) had been previously isolated from the soil and *A. caliginosa* intestine and excrement by plating on R2A agar [13]. Bacteria were identified by PCR amplification of the 16S rRNA and sequencing of obtained clones in the Institute of Microbiology, Biozentrum, Technical University of Braunschweig, Germany (Table 1). Unidentified strains of gram-positive and gram-negative bacteria (with different colony morphotypes) isolated from *A. caliginosa* intestinal walls and fresh excrements were also used. *Bacillus thuringiensis* strains were isolated from various soils, sod, and insect larvae by plating on a Luria medium with glucose [14]. In order to achieve sporulation, the cultures were then grown on a Luria medium with glucose for three days at 30°C. Smears were then prepared, fixed by flaming, stained with Amido Black (15 g/l), washed, and dried. The smears were then stained with Ziehl basic fuchsin (9.5 g/l), washed, and dried. The vegetative cells were stained blue–violet or mauve, the terminal endospores were either yellowish or reddish, and the toxin crystals were blue.

Cultivation of bacteria. Bacterial cultures were grown in a shaker (180 rpm) at 20–22°C for 24 h in microbiological trays with a liquid glucose–peptone–yeast medium (PYG) containing the following (g/l of tap water): yeast extract, 5; peptone, 20; glucose, 40.

Obtaining the surface excreta. Reaction of the bacteria to the earthworm surface excreta was determined. Prior to obtaining the excreta, 30 adult worms were placed on wet filter paper for 2 h in order to clear their digestive tracts of soil particles. The worms were then incubated for an hour in a sterile petri dish. After removing the earthworms, the slime was washed off with 15 ml of distilled water. An aliquot of the resulting suspension was stored at –18°C; the remnant was centrifuged for 3 min at 8000 g in order to remove soil particles, as well as fungi and bacteria. For final sterilization, the supernatant was filtered through a 0.22 μm membrane filter. Sterility was determined by plating an undiluted filtrate on PYG agar with subsequent 7-day incubation at 20–22°C. No bacteria or fungi were revealed. Thus, sterile and nonsterile suspensions of the

Table 2. Characterization of the *A. caliginosa* excreta

Excreta	C, μg/ml	N, μg/ml	S, μg/ml	Bacteria, cells/ml	Fungal spores, cells/ml
Sterile	8.2	1.4	8.6	–	–
Nonsterile	7.6	2.0	6.8	$3.8 \pm 0.3 \times 10^4$	$5.1 \pm 0.7 \times 10^3$

earthworm surface excreta were obtained. Prior to testing, they were stored at –18°C.

Characterization of the excreta. Aliquots of sterile and nonsterile excreta were lyophilized; the content of carbon, nitrogen, and sulfur was determined on a Vario EL III CHNS elemental analyzer (Elementar, Germany) at incineration temperature of 1150°C. This method permits determination of element content with 0.01% accuracy (information from the operating manual). The numbers of bacteria and fungi in nonsterile excreta were determined by direct fluorescence microscopy of acridine orange-stained samples [15].

Bacterial reaction to earthworm excreta. Reaction to the earthworm excreta was determined from their effect on dehydrogenase activity and on bacterial growth curves. To determine dehydrogenase activity, formazan formation was monitored [15]. For this purpose, 50 μl of liquid PYG medium, 50 μl of sterile or nonsterile excreta, and 10 μl of trimethyl tetrazolium chloride (0.9 μg/ml) were added to each cell of a microbiological tray. The cells were then inoculated with bacterial suspensions (6 μl). In the control, sterile distilled water was used instead of excreta. The experiments were carried out in five repeats. After three days of incubation, the degree of coloration was determined on a Tecan Sunrise Basic spectrophotometer at 492 nm. The Magellan 6.0 software package was used.

To investigate bacterial growth curves, sterile excreta (native and tenfold diluted) were used at concentrations of 7.6 and 0.76 μg C/ml, respectively. Distilled water was used for control. Effect on the growth curves was determined from the changes of optical density in the cells of microbiological trays. Each cell contained 50 μl of liquid PYG medium and 50 μl of a tested liquid; the cells were inoculated with 6 μl of bacterial suspensions and incubated for 72 h with shaking (180 rpm) at 20–22°C. Three times a day, OD_{620} was determined.

Parameters of microbial growth. The changes in optical density were used as an indicator of microbial growth. The parameters of the following logistic equation were used to describe microbial growth (optical density):

$$D = \frac{D_{\max}}{1 + \frac{D_{\max} - D_0}{D_0} e^{-R_{\max} t}},$$

where t is time; D is optical density at a given moment; D_0 is initial optical density; $R_{\max}$ is the maximal specific growth rate; and $D_{\max}$ is the maximal optical density during 70 h of incubation [16].

Statistical treatment of the data. The Statistica 7.0 software package was used to analyze the data. Using the ANOVA package, the variants of culture treatment were compared and the graphs were built. The parameters and graphs of the logistic growth equation were determined by nonlinear approximation using the least square method.

RESULTS

Characterization of the excreta. The C : N content of the native excreta was close to 4. A high sulfur content was revealed; its average concentration in sterile and nonsterile extracts was 7.7 μg/ml (Table 2). High amounts of sulfur-containing proteins and polysaccharides are probably present in the excreta; this finding correlates with the data on the composition of vermipeptides obtained from earthworm tissue homogenates [17].

Effect of excreta on dehydrogenase activity of bacteria isolated from the intestine and excrements of the *A. caliginosa* earthworm. Earthworm excreta had a reliable suppressive effect on 15 out of the 19 cultures; after 70 h, dehydrogenase activity decreased by 30–50%. For a given culture, the inhibitory effect of sterile and nonsterile extracts was the same. Four bacterial cultures exhibited a neutral reaction to excreta; no reliable difference was detected between the variants with sterile and nonsterile excreta. Among both the suppressed and insensitive bacteria, members of different taxa were present (Table 3).

Effect of excreta on growth of bacteria isolated from *A. caliginosa* intestine and excrements. Measurements of dehydrogenase activity provide information on the activity of cellular respiratory processes, but not necessarily on microbial growth. In the subsequent experiments, effect of earthworm excreta on bacterial growth (determined from changes in the optical density of the cultures) was investigated. A significantly broader range of bacteria was tested. The strains investigated included 82 bacterial strains isolated from the earthworm intestine and excrement and 45 *B. thuringiensis* strains isolated from soil, sod, and insect larvae. Since the previous experiment revealed no differences in the effect of sterile and nonsterile excreta, only sterile excreta were used. Native and tenfold diluted excreta were tested.

Analysis of the growth curves of the pure cultures of unidentified bacteria revealed a spectrum of reactions.

Table 3. Effect of *A. caliginosa* surface excreta on dehydrogenase activity of bacteria isolated from sod-podzolic soil (average ± standard deviation, $n = 5$)

Bacteria	OD_{492}		
	Control	Nonsterile excreta	Sterile excreta
No effect			
Bacillus subtilis/*B. mojavensis* 523-1	4.64 ± 1.32	4.47 ± 0.93	4.92 ± 0.66
Delftia acidovorans 14	5.95 ± 1.29	4.35 ± 0.91	3.72 ± 0.48
Micrococcus luteus 522-2	6.36 ± 1.29	4.37 ± 0.75	4.51 ± 0.50
Sphingobacterium sp. 590-2	5.53 ± 1.14	4.40 ± 0.74	3.79 ± 0.31
Suppression of dehydrogenase activity*			
Achromobacter xylosoxidans 2	6.82 ± 1.07	4.58 ± 0.91	3.65 ± 0.24
Aeromonas sp. 616	8.17 ± 0.83	6.06 ± 1.02	4.04 ± 0.30
Agrobacterium tumefaciens 11	7.16 ± 1.16	3.75 ± 0.39	3.92 ± 0.32
Alcaligenes faecalis subsp. *parafaecalis* E588	6.18 ± 0.67	4.30 ± 0.77	4.24 ± 0.35
Alcaligenes sp. 99	6.65 ± 0.93	3.55 ± 0.80	3.52 ± 0.62
Brevundimonas diminuta E699	6.02 ± 1.00	4.01 ± 0.53	3.93 ± 0.54
Chryseobacterium sp. 495	6.84 ± 1.41	4.73 ± 0.54	4.00 ± 0.59
Microbacterium paraoxydans 597-3	6.63 ± 1.28	3.63 ± 0.46	3.75 ± 0.38
Paenibacillus sp. 598	8.36 ± 0.37	4.82 ± 1.15	4.22 ± 0.70
Pantoea sp. E680	6.77 ± 0.57	3.77 ± 0.34	4.29 ± 0.47
Schineria larvae 5	6.32 ± 1.21	3.75 ± 0.80	3.73 ± 0.06
Serratia marcescens E658	7.36 ± 0.67	4.43 ± 0.79	4.62 ± 0.45
Pseudomonas sp. 610-1	6.43 ± 0.87	3.87 ± 1.02	4.43 ± 0.48
Sporosarcina sp. 502-2	7.39 ± 1.10	3.65 ± 0.64	3.97 ± 0.65
Stenotrophomonas maltophilia 12	7.02 ± 1.24	4.20 ± 0.66	4.07 ± 0.41

* Statistically reliable difference from the control ($P < 0.05$).

The averaged graphs of each type of reaction are presented on Fig. 1. Suppressive, neutral, and stimulatory effects of the excreta were observed. Among the 82 strains studied, growth of only one was suppressed. For 15 strains, a neutral effect was observed. Growth of other bacteria was stimulated. The growth curves of bacteria stimulated by the excreta may be grouped into four types. Bacteria stimulated only by undiluted excreta (8 strains) belong to the first type (Fig. 1a). The second group (24 strains) comprises the cultures stimulated by native and tenfold diluted excreta to the same degree (Fig. 1b). Bacteria of the third group (17 strains) exhibited different patterns of stimulation depending on excreta dilution. Reaction to native extracts was stronger than to diluted ones; the reaction was detectable after 19 h of incubation (Fig. 1c). The fourth type of reaction differed from the three other ones; in this case, the excreta stimulated only the initial stages of growth, while the final yield (determined as the maximal optical density D_{max}) did not change. This reaction was found in 17 strains (Fig. 1d). Thus, the spectrum of bacterial reactions to earthworm surface excreta is very broad; the reaction occurs even at very low concentrations of the active substances (less than 0.8 μg C/ml and less than 0.14 μg N/ml).

Reaction of *B. thuringiensis*. *B. thuringiensis* is a normal inhabitant of soil. It was used to determine whether the effect of earthworm excreta was species or strain-specific. A total of 45 strains were tested. Suppressive, neutral, and stimulatory effects of the excreta were revealed (Fig. 2). Growth of 2 strains was suppressed, 14 exhibited no reaction to the excreta. For

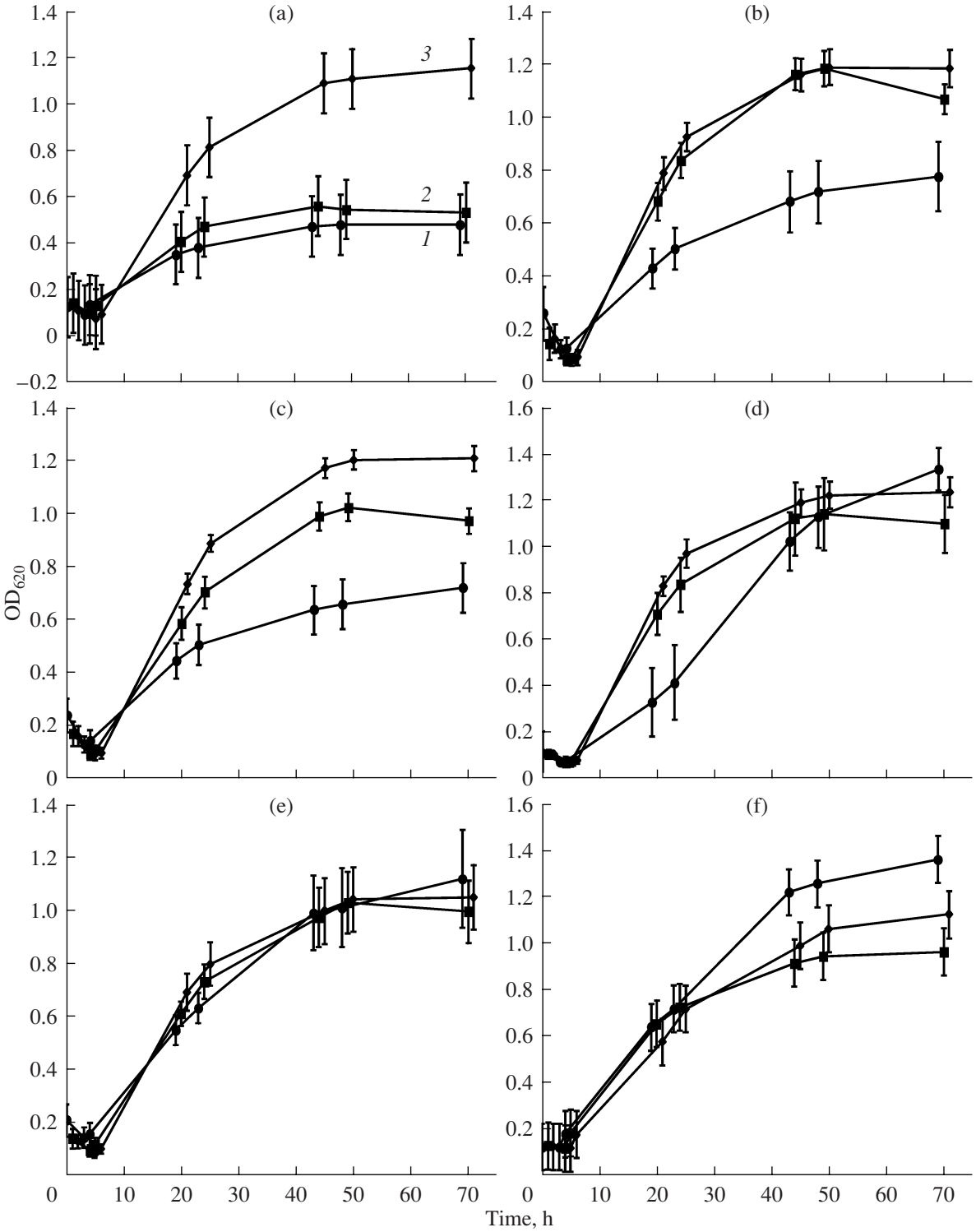

Fig. 1. Effect of excreta on the growth of bacteria isolated from *A. caliginosa* intestine: a, bacterial growth is stimulated only by native excreta; b, growth is equally stimulated by native and diluted excreta; c, the degree of growth stimulation depends on excreta dilution; d, excreta stimulate only initial stages of growth, while the final biomass yield does not change; e, excreta do not affect bacterial growth; f, excreta suppress growth. Control (*1*); tenfold diluted excreta (*2*); native excreta (*3*); confidence interval for the 0.95 significance level.

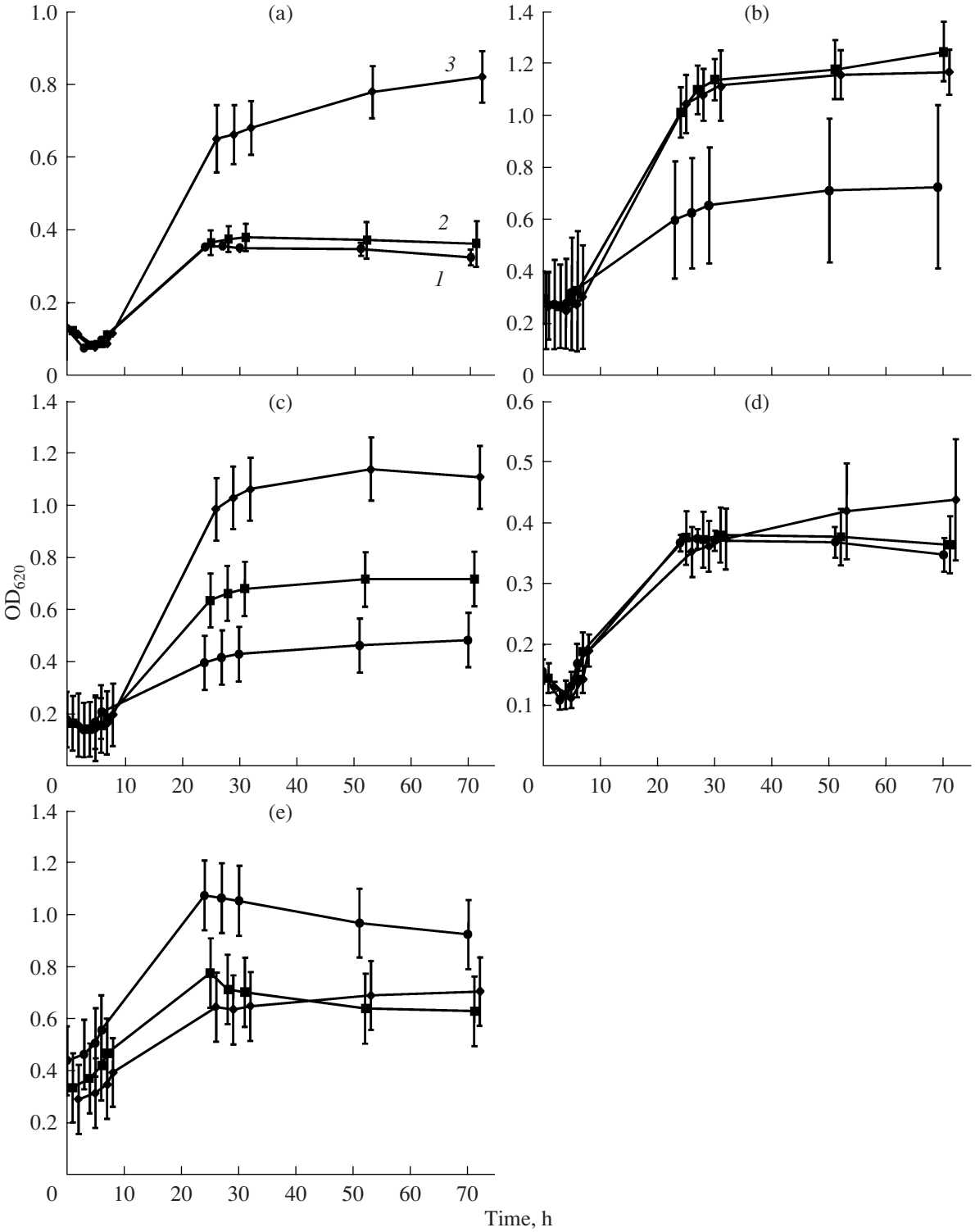

Fig. 2. Effect of *A. caliginosa* excreta on the growth of *B. thuringiensis* strains: a, bacterial growth is stimulated only by native excreta; b, growth is equally stimulated by native and diluted excreta; c, the degree of growth stimulation depends on excreta dilution; d, excreta do not affect bacterial growth; e, excreta suppress growth. Control (*1*); tenfold diluted excreta (*2*); native excreta (*3*); confidence interval for the 0.95 significance level.

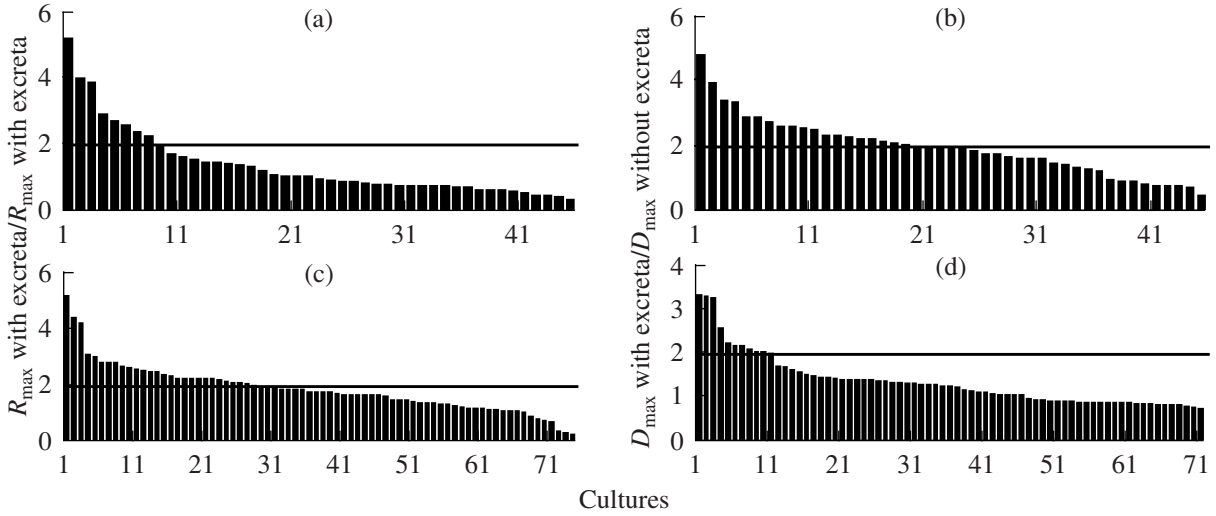

Fig. 3. Rank distribution of the ratios of specific growth rates (determined as optical densities) of the cultures and the ratios of maximal optical densities with and without excreta: a, ratios of specific growth rates (determined as optical densities) for *B. thuringiensis* strains; b, ratios of maximal optical densities for *B. thuringiensis* strains; c, ratios of specific growth rates (determined as optical densities) for the strains isolated from *A. caliginosa* intestine; d, ratios of maximal optical densities for the strains isolated from *A. caliginosa* intestine.

29 strains, growth was noticeably higher in the variants with excreta. Some strains (16) reacted only to the excreta containing 7.6 μg C/ml of active substances; 5 strains were equally stimulated by diluted and undiluted excreta. For 8 strains, a decreased effect of diluted excreta was observed.

Analysis of the parameters of the logistic growth equation. The growth curves of 72 bacterial strains isolated from the earthworm intestine and of 45 *B. thuringiensis* strains are approximated by the logistic growth equation with the correlation coefficients from 0.85 to 0.99. Therefore the values of R (maximal growth rate measured as the rate of OD increase) and D_{max} (maximal optical density) obtained in different variants may be compared. Different cultures and bacterial groups isolated from different environments may be compared on the basis of the ratio between specific growth rates and maximal optical densities obtained with and without excreta. Analysis of the rank distribution of these ratios revealed that addition of the excreta resulted in doubling the specific growth rates in 18% of *B. thuringiensis* strains (Fig. 3a) and 38% of the cultures obtained from earthworm intestines (Fig. 3c). Moreover, addition of the excreta resulted in doubling the maximal optical density in 36% of *B. thuringiensis* strains (Fig. 3b) and 18% of the unidentified bacterial cultures (Fig. 3d).

DISCUSSION

This work is the first study of the effect of *A. caliginosa* earthworm surface excreta on bacteria isolated from different environments (soil, digestive tract, and insect larvae). Mucous excreta consist of mucoproteins, water-soluble compounds rich in nitrogen, protein, and free amino acids (asparagine, serine, and glycine), as well as ethanolamine. They have a neutral or weakly alkaline reaction and may decreas the acidity of near-burrow soil [18]. During a day, an earthworm can excrete as much as 88–279 μg N/g biomass with slime; this value depends on the earthworm species [9, 18]. Our findings are similar; during a day, one *A. caliginosa* specimen excretes 40 μg N/g biomass.

Together with coprolites and intestinal excretions, these slimes are delivered to the soil directly adjacent to the burrow and change its characteristics considerably. A 2-mm layer (drilosphere) is formed around the burrow, which differs in some respects from the surrounding soil. In this layer, an increased numbers of bacteria, decreased length of fungal mycelium, and increased length of actinomycete hyphae were revealed [5]. Humidity and the content of total carbon and nitrogen increased significantly compared to the surrounding soil (10 mm from the burrow wall) [7]. It is therefore reasonable to suggest that earthworms play an active part in the turnover of biogenic elements and regulate the drilospheric microbial community. However, the mechanisms of these phenomena are not known.

We have previously investigated the effect of excreta on the soil of microbial communities. Stimulation of nitrification and soil respiration was revealed after a single treatment with *A. caliginosa* excreta. During the first 2 hours after addition of the excreta, CO_2 emission from soil increased by 40%. The concentration of active substances in the excreta (calculated as carbon) was <8 μg C/ml. Addition of an equal amount (by carbon) of glucose did not affect respiration. Since the increase of C–CO_2 emission was not proportional to the amount

of carbon in the excreta, we suggested that the mechanism was related to the action of the physiologically active compounds within earthworm excreta on activity of specific groups of soil microorganisms [10]. In the present work, the effect of similar concentrations of earthworm excreta on pure cultures was tested; dynamics of dehydrogenase activity and culture growth were used as indicators. Suppression of hydrogenase activity in 80% of the cultures, together with no cases of stimulation, confirm the stress reaction hypothesis.

The effect of excreta on bacterial cultures varied from suppression to stimulation. The concentration of the active component in the excreta was very low (<8 μg C/ml, in some cases <0.8 μg/ml). Some cultures (19%) were found to react only to undiluted excreta. However, even tenfold dilution of the excreta did not change their effect on some bacteria: 42% of the strains were equally stimulated by diluted and undiluted excreta. This finding implies a threshold concentration of the active component in the excreta. Since their effect is not proportional to concentration, these substances do not act as growth substrates.

Since such a variety of effects was observed not only for different bacterial species, but for 45 strains of one species (*B. thuringiensis*), the effect of earthworm excreta is strain-specific.

The pattern of the biological action of earthworm excreta suggests that the earthworms are able to excrete physiologically active compounds from their surface. Other authors have also reported the biological activity of earthworms. For instance, the excreta were found to suppress growth of some phytopathogenic bacteria and fungi [11, 13].

Thus, earthworm excreta actively affect the microbial community of the burrows due to their selective effect on growth of individual microbial populations. Further studies of the composition and active concentrations of the excreta affecting the soil microbial communities are of interest. Such research is important to improve our understanding of the role of earthworms in ecosystems.

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