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EXPERIMENTAL ARTICLES

Dormant State and Phenotypic Variability of *Staphylococcus aureus* and *Corynebacterium pseudodiphtheriticum*

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Abstract—Ability to produce dormant forms (DF) was demonstrated for non-spore-forming bacteria *Staphylococcus aureus* (a nonpathogenic strain) and *Corynebacterium pseudodiphtheriticum* (an organism of the normal oropharyngeal flora). The salient features of the sthaphylococcal and corynebacterial DF were (1) prolonged (4 months) preservation of viability; (2) resistance to damaging factors (heat treatment); and (3) specific morphology and ultrastructure. The optimal conditions for DF formation were (1) transfer of stationary-phase cultures into saline solution with $CaCl_2$ (10–300 mM) (for *S. aureus*); (2) growth in SR1 synthetic medium with fivefold nitrogen limitation (for *C. pseudodiphtheriticum*); and (3) incubation with $(1–5) \times 10^{-4}$ M of C_{12}-AHB, an alkylhydroxybenzene akin to microbial anabiosis autoinducers. Increase of C_{12}-AHB concentration to 7×10^{-4}–2×10^{-3} M resulted in "mummification" of cells with irreversible loss of viability without autolytic processes. Germination of dormant forms was followed by increasing of phenotypic variability, as seen from (1) diversity of colony types and (2) emergence of antibiotic-resistant clones on selective media. The share of kanamycin-resistant *S. aureus* variants was most numerous (0.002–0.01%) in 4-month DF suspensions in SALINE with $CaCl_2$. In the *C. pseudodiphtheriticum* DF produced under the effect of C_{12}-AHB, the share of kanamycin-resistant variants was also found to increase. These data point to an association between the emergence of antibiotic-resistant variants of bacteria and their persistence in dormant state mediated by starvation stress and regulated by AHB.

Keywords: dormant state, dormant cells, phenotypic variation, antibiotic resistance, alkylhydroxybenzenes
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The interest to dormant forms of non-spore-forming bacteria has attracted great attention in view of their long-term survival in nature under the conditions unsupportive for the growth [1, 2 and references therein], the persistence of pathogenic microorganisms in host and environment, and the phenomenon of antibiotic-resistant persister cells [3–5]. Various non-spore-forming gram-negative and gram-positive bacteria, including nonpathogenic species (relatives of pathogenic microorganisms) has been demonstrated to form differentiated cystlike dormant cells (CLC) [6–8]. Under laboratory conditions, the efficient CLC formation occurs under the imbalance of nutrients in media or in response to starvation stress and is controlled by extracellular microbial autoregulators, which in a number of bacteria belong to alkylhydroxybenzenes (AHB). CLC are probably formed by many, if not all bacteria [1]. The existence of dormant cells of nonpatogenic species or strains of a certain morphological type suggests the existence of such forms in their pathogenic relatives. Thus, ovoid cells with all the features of dormant cells initially found in *Mycobacterium smegmatis*, a saprotrophic analogue of the tuberculosis bacterium [7], were subsequently found in *Mycobacterium tuberculosis* cultures grown under similar conditions [9].

As for *Staphylococcus aureus*, the subpopulation of viable and metabolically inactive cells, having some attributes of dormant forms, was revealed in biofilms [10]. Other studies demonstrated that the preservation of viability by *S. aureus* cells depended on starvation conditions (limitation of C, N, or P sources or all of them) and on the density of cell suspensions [11]. Existence of the dormant forms capable of prolonged survival and especially the issue on regulation of their formation remains insufficiently studied for staphylococci and particularly for corynebacteria.

Entering a new growth cycle of germinating dormant forms is accompanied by phenotypic variation of developing populations, which may be observed as differences in colony morphology or in the physiological characteristic of the variants growing under selective

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conditions. Pronounced phenotypic variability was shown during germination of morphologically different dormant forms: CLC, anabiotic cells obtained by AHB treatment, and, to a lesser extent, bacillary endospores [1]. Emergence of the variants with different resistance to antimicrobial agents shown for *Pseudomonas fluorescens, P. aurantiaca*, and *Mycobacterium smegmatis* [7, 12] is one of the manifestations of phenotypic variability in germinating CLC. Association between the state of growth arrest (including the cells in biofilms) and emergence of the variants resistant to peroxide or kanamycin was discussed in [13, 14]. Resistance of persister cells to antibiotics has also been explained as a consequence of their being in a non-dividing state. It is, however, still unclear whether they ensure long-term survival [5].

Antibiotic resistance and emergence of strains resistant to antimicrobial agents became an urgent problem in the treatments of infectious diseases. For example, many publications deal with the propagation of methicillin-resistant *S. aureus* strains (MRSA) [15]. The described small colony variant (SCV) of *S. aureus* differs from the dominant type in small colony size, their weak pigmentation, increased resistance to antibiotics, and higher capacity for persistence in the host organism. This variant is responsible for chronic and recurrent infections [16 and references therein]. Moreover, the SCV has higher capacity for formation of antibiotic-resistant biofilms [17]. Investigation of the modes of persistence and emergence of antibiotic resistance is also important for other pathogenic and opportunistic bacteria, including *Corynebacterium* species [18, 19].

The goal of the present work was therefore to investigate the regulation of *S. aureus* and *Corynebacterium pseudodiphtheriticum* transition to a dormant state (using nonpathogenic strains as models), depending on the trophic conditions and the presence of AHB, as well as the intrapopulation phenotypic variation (including emergence of antibiotic-resistant variants) during germination of dormant forms.

MATERIALS AND METHODS

Subjects of study were gram-positive non-spore-forming bacteria *Staphylococcus aureus* 209P (nonpathogenic strain) and *Corynebacterium pseudodiphtheriticum* 090497 (an organism of the normal oropharyngeal flora) from the collection of Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations. The chemical analogue of microbial regulators, C_{12}-AHB (Sigma), belonging to alkylresorcinols, was used as an anabiosis autoinducer. It was added to bacterial cultures in micromolar concentrations as ethanol solutions (ethanol concentration in cell suspensions was 0.05% v/v).

The cultures of *S. aureus* and *C. pseudodiphtheriticum* were grown in Luria–Bertani broth (LB). Corynebacteria were also grown in SR1 medium containing the following (g/L): glucose, 20; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; KNO_3, 1; K_2HPO_4, 0.5; $CaCO_3$, 3; tap water; pH 7.2. Bacteria were grown at 30°C in 250-mL flasks with 50 mL of the medium on a shaker (140–160 rpm). The inoculum was added to the optical density of the culture 0.2 (Specord UV VIS, Carl Zeiss Jena, $\lambda = 650$ nm, $l = 1$ cm).

Cystlike dormant cells (CLC) were obtained according to the previously developed protocols [6, 12], in (1) long-incubated cultures (up to 4 months) grown in modified media with C : N imbalance, and (2) suspensions of early stationary-phase cells collected by centrifugation (3000 *g*, 15 min) and resuspended in saline solution (0.9% NaCl) with or without $CaCl_2$ (10–300 mM, pH 7.25).

Anabiotic dormant cells were obtained by adding ethanol solutions of C_{12}-AHB to the stationary-phase bacterial cultures to the final concentration of 5×10^{-5}–4×10^{-4} M.

Total cell number (TCN) in the cultures was determined by direct microscopic counts in 20 small squares (25 μm^2 in a Goryaev chamber.

The number of colony-forming units (CFU/mL) was determined by plating aliquots of 10^N-fold diluted bacterial cultures on agar media (1.5% agar) with subsequent incubation for 3–5 days at relevant temperatures.

Most probable number (MPN/mL) of viable cells was determined by the growth of cultures after inoculations with consequently tenfold diluted cell suspensions (50 μL + 450 μL of the medium in multiwell plates). For CFU and MPN tests, liquid and solid media prepared from the standard or fivefold diluted LB broth were used.

Heat resistance of vegetative cells and dormant forms was determined by enumerating the cells retaining their viability (by CFU or MPN titers) after heating the cell suspensions (0.7 mL) in an ultrathermostat at 45–75°C for 5–10 min.

Resistance to kanamycin was determined by enumeration of surviving cells (CFU/mL) after addition of this antibiotic (0.5–10 μg/mL) to bacterial cultures and subsequent incubation for 3 h at 28°C.

Phenotypic variability was assayed by the appearance of colony morphology variants differing from the dominant type in shape, consistency, and pigmentation. Antibiotic-resistant variants were revealed on solid media supplemented with kanamycin (2–200 μg/mL). The variability index was calculated as a share of the nondominant colony types among the total colony number.

Microscopy was carried out using light microscopes Zetopan (Reichert, Austria) and Axioplan (Carl Zeiss, Germany). The cells were stained with (1) Live/Dead Baclight kit® L-13152 (Molecular Probes) to differentiate between alive and dead cells, (2) propidium iodide (PI, 3 μM) to reveal damaged

and dead cells, and (3) the CTC tetrazolium dye (1–3 μM) to reveal respiratory activity. Cell suspensions with the dyes were incubated in the dark for 10 min at 37°C. At least 20 fields of view were examined, with at least 1000 cells counted.

For **electron microscopy,** the cells were collected, fixed with 1.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times with the same buffer, and postfixed with 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration, the material was embedded in Epon 812. Ultrathin sections were contrasted for 30 min with 3% uranyl acetate solution in 70% ethanol and stained with lead citrate by Reynolds (20°C, 5 min). The sections were examined under a JEM-100B electron microscope (JEOL, Japan) at 80 kV.

Statistical analysis was carried out using the standard criteria: mean-square deviation within a group, Student's *t*-test, and correlation between two groups of data. The difference between groups of data was considered reliable at $P < 0.05$.

RESULTS

Regulation of Dormant Cells Formation by S. aureus and C. pseudodiphtheriticum

Trophic regulation. To confirm the ability of *S. aureus* and *C. pseudodiphtheriticum* to produce dormant forms, both the previously developed and new approaches based on starvation stress in bacterial cells were used [11, 12].

S. aureus. The protocol for obtaining CLC included transfer of the cells grown in LB broth to the early stationary phase into saline solution with 10–300 mM $CaCl_2$ and their incubation for 4 months at room temperature. Under these conditions, an insignificant decrease in total cell numbers, a decrease in CFU numbers by two orders of magnitude (LB agar), and emergence of numerous refractive cells (up to 70–80% of the population), which is characteristic for CLC, were observed (Fig. 1a). In the control variants (*S. aureus* cultures grown in LB broth and incubated for 4 months), the CFU number decreased by four orders, while CLC were not produced in noticeable amounts. The presence of $CaCl_2$ in saline solution had no effect on the numbers of viable CLC (10^7–10^8 CFU/mL) and on the ratio of CLC (by CFU) to the total cell number in stationary-phase cultures (1–3%). Incubation in saline with 10 or 100 mM $CaCl_2$ was, however, the optimal variant for production of heat-resistant cells (60°C, 10 min).

C. pseudodiphtheriticum. The efficient yield coccoid CLC of *C. pseudodiphtheriticum* occurred in post-stationary phase cultures (1 month) grown in SR1 medium with fivefold decreased concentration of the nitrogen source (KNO_3, 0.2 g/L). Incubation of post-stationary phase cultures for 3 months (Fig. 1b) did not result in decreased cell numbers, while the viable

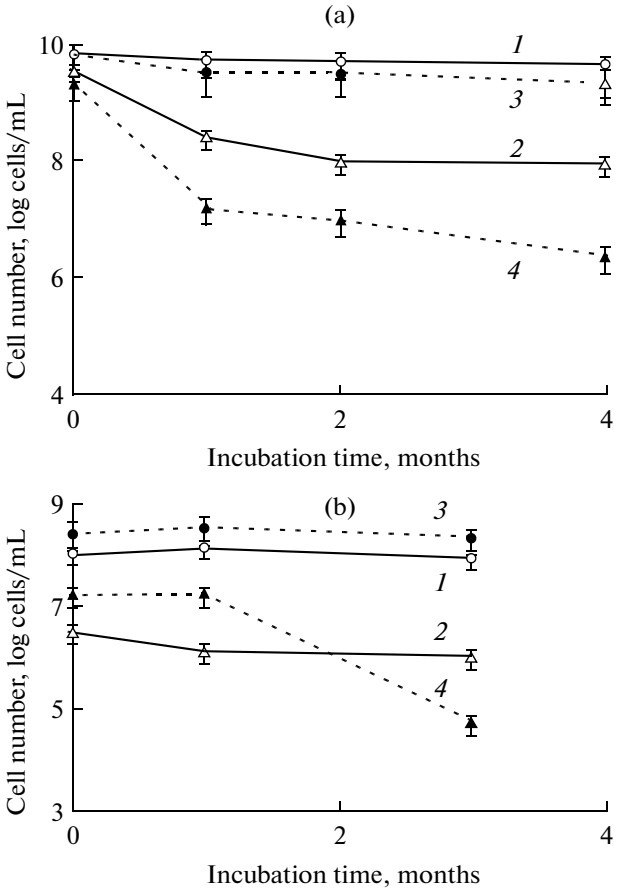

Fig. 1. Total cell number (TCN, cells/mL) and viable cell titer (logN, CFU/mL) in incubated cultures and cell suspensions of *S. aureus* and *C. pseudodiphtheriticum*. (a). *S. aureus*: suspensions in saline solution with 100 mM $CaCl_2$, TCN (*1*) and CFU (*2*); cultures grown in LB broth (control), TCN (*3*) and CFU (*4*). (b). *C. pseudodiphtheriticum*: cultures grown in SR1 medium with 5-fold N limitation, TCN (*1*) and CFU (*2*); cultures grown in full-strength SR1 medium (control), TCN (*3*) and CFU (*4*).

cell number was maintained at 10^6 CFU/mL. The same prolonged incubation of corynebacteria grown in unlimited SR1 medium (the control) resulted in a decrease in the number of cells forming colonies on LB agar by three orders of magnitude, although the total cell number did not change. Small, non-refractive cells predominated after prolonged (3 months) incubation of the control variant.

The total cell numbers in post-stationary (3–4 months) cultures of *S. aureus* and *C. pseudodiphtheriticum* was high and the share of intact cells (with green and yellow-green fluorescence in the Live/Dead test) constituted 41–77% of the total cell number, being although almost two orders of magnitude higher than the CFU titer. Using MPN test made it possible to increase 30-fold the efficiency of detection of viable dormant cells reverting to growth in full-strength (for

Table 1. Increased efficiency (*N*-fold) of detection of viable dormant forms by application of MPN and modified media

Total cell number*, cells/mL	Number of living cells**, cells/mL	CFU/mL	Medium (in MPN tests)	Viable cell titer, MPN/mL	*N*, times
CLC of *S. aureus* (incubation in saline solution with 100 mM $CaCl_2$, 4 months)					
$(9.0 \pm 1.9) \times 10^9$	$(7 \pm 1.3) \times 10^9$	$(1.0 \pm 0.1) \times 10^8$	LB broth	3.0×10^9 $(1 - 5) \times 10^9$	30
CLC of *C. pseudodiphtheriticum* (SR1 medium, N limitation, 3 months)					
$(1.4 \pm 0.4) \times 10^8$	$(5.8 \pm 1.2) \times 10^7$	$(1.5 \pm 0.4) \times 10^6$	Fivefold diluted LB broth LB broth	4×10^7 $(1 - 7) \times 10^7$	27

* According to microscopic counts in a Goryaev chamber.

** According to the Live/Dead test.

Table 2. Dose-dependent effects of the chemical analogue of anabiosis autoinducer C_{12}-AHB on the stationary-phase *S. aureus* cells (incubation for 1 month)

C_{12}-AHB, M	Cell number**		Cell share*, %		Transition of most of the population into
	Alive cells/mL	Viable	Culturable	Nonculturable	
1×10^{-4}	2×10^8	1.2×10^8 CFU/mL	60	40	Dormancy with preserved colony-forming capacity
3×10^{-4}	2×10^8	2.8×10^7 CFU/mL	14	86	Dormancy with lost colony-forming capacity
7×10^{-4}	6×10^7	0 CFU/mL 2×10^3 MPN/mL	0 0.03	100 99.7	Nonculturable state***
2×10^{-3}	<detection limit	0 CFU/mL 0 MPN/mL	0 0	100 100	Post-anabiotic mummified state

* 1 month after AHB addition.

** According to direct microscopic counts with Live/Dead.

*** Part of the population was capable of growth in LB broth.

S. aureus) or fivefold diluted LB broth (for *C. pseudodiphtheriticum*) (Table 1).

Thus, we selected the conditions favorable for the formation of *S. aureus* and *C. pseudodiphtheriticum* dormant cells, including those which could not be enumerated by CFU counting. To improve the efficiency of detection of dormant forms, approaches were used based on prevention of substrate-accelerated death (by diluting the medium for CLC germination) and abolishment of oxidative stress (by culturing under static conidtions).

Regulation of anabiotic cell formation by AHB. Treatment of actively growing *S. aureus* cells (mid-log phase) with C_{12}-AHB (1×10^{-4}–3×10^{-4} M) resulted in a drastic, irreversible decrease of the CFU titer (Fig. 2a, curve *3*). Addition of the same AHB concentrations to early stationary-phase cultures resulted in the formation of anabiotic dormant cells preserving their colony-forming capacity for up to two months (Figs. 2a, 2b). Thus, physiological competence of cells to specific concentrations of microbial autoregulators (AHB) is important for realization of the programs for cell survival or death.

Effect of C_{12}-AHB on the stationary-phase *S. aureus* cultures. Depending on its concentration, C_{12}-AHB caused transition of cells to (1) the state of metabolic dormancy (anabiosis), when most cells preserved their colony-forming capacity (at 1×10^{-4} M AHB); (2) the nonculturable state, when the capacity for colony formation under standard conditions was reversibly lost (3×10^{-4} M); and (3) the post-anabiotic state with formation of mummified cells irreversibly losing their capacity to revert to growth (7×10^{-4}–2×10^{-3} M) (Table 2). Preservation of the colony-forming capacity in a course of incubation of *S. aureus* cultures after AHB addition was dose-dependent. Thus, in a narrow range of C_{12}-AHB concentrations, from 0.7 to 1×10^{-4} M, a relatively stable CFU titer was maintained. At 7×10^{-4} M AHB, CFU titer decreased by four orders of magnitude after 7 days and to zero after a month. In the variants with millimolar AHB concentrations, $(1–2) \times 10^{-3}$ M, CFU titer dropped rapidly, reaching zero after 3 h (Fig. 2b). The decrease in the number of viable *S. aureus* (CFU and MPN counts), after addition of C_{12}-AHB at concentrations over $7 \times$

10^{-4} M to the stationary culture was accompanied by increased ratios (up to 100%) of the cells permeable to DNA-binding stain PI, which penetrate only through damaged cell membranes (Fig. 2, curve *2*).

In the case of early stationary-phase *C. pseudodiphtheriticum* cultures, a similar concentration-dependent effect of C_{12}-ANB was observed. In the concentration range of $(1–2.5) \times 10^{-4}$M, AHB induced formation of viable CLC (3×10^7 CFU/mL, 3 months of incubation). At concentrations of 5×10^{-4} M and higher, treatment with C_{12}-AHB resulted in a loss of colony-forming capacity in most of the cells in the population ($10^4–10^5$ CFU/mL, 0.01–0.1% of the CFU in the stationary phase culture).

Thus, depending on their concentration and the physiological age of recipient cells, microbial autoregulators (AHB) control transition of staphylococcal and corynebacterial cells into a state of metabolic dormancy (anabiosis) with the preservation of their colony-forming capacity.

Properties of the Dormant Cells of S. aureus and C. pseudodiphtheriticum

S. aureus. Prolonged (up to 4 months) preservation of viability, as was determined by CFU titers on solid media ($4 \times 10^7–1.1 \times 10^8$ cells/mL) and in some variants by MPN ($0.8–1.2 \times 10^9$ cells/mL), was an important feature of *S. aureus* dormant forms (DF) (Table 1). Direct microscopic tests with CTC confirmed the absence of respiratory activity, which indicated suppression of the metabolic processes.

Heat resistance, which developed in the course of their maturation during prolonged incubation, was an indicator of the general resistance of *S. aureus* DF to unfavorable factors (Fig. 3a). Thus, for suspensions incubated in saline solution with 100 mM $CaCl_2$ for 2 and 4 months, the viable cells after 10-min heating at 55–60°C were single and $3.2–5.6 \times 10^7$ CFU/mL, respectively. The latter value was 32–56% of the CFU titer of unheated suspensions. Importantly, while CFU number of 2-month suspensions heated at 55–60°C (10 min) dropped drastically, heating to 65–75°C (10 min) did not cause a decrease in colony-forming capacity (Fig. 3a). This phenomenon requires further investigation.

S. aureus dormant cells (incubation in saline solution with 100 mM $CaCl_2$, 4 months) had the ultrastructural organization typical of cystlike cells of other bacteria [1, 6–8]. They were characterized by thickened cell walls with laminated structure, fine-grained texture of the cytoplasm, and compaction of the nucleoid (Fig. 4). Apart from single cells, aggregates of two cells bound by a common layer of the thickened cell wall were observed (Fig. 4). Live/Dead test revealed both the intact cells with green or yellow-green fluorescence and unstained cells in CLC suspensions, indicating heterogeneity of the population

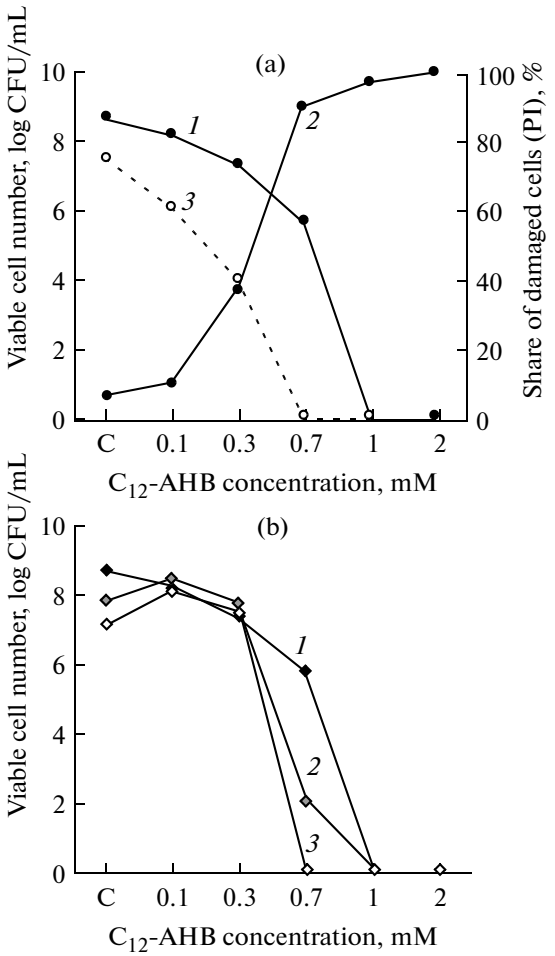

Fig. 2. (a). Concentration-dependent effect of C_{12}-AHB on formation of *S. aureus* antibiotic cells. Number of viable cells (logN, CFU/mL) 3 h after C_{12}-AHB addition to the stationary-phase (*1*) and linear-phase (*3*) cultures; share of damaged cells (logN, CFU/mL) by PI test in the stationary-phase culture (*2*). (b). Number of viable cells (logN, CFU/mL) 3 h (*1*), 7 days (*2*), and 1 month (*3*) after C_{12}-AHB addition to the stationary-phase culture. AHB was not added to the control variants (C).

of dormant forms in its reaction to the dye components, as well as the preservation of the barrier function of the CLC membranes.

C. pseudodiphtheriticum. Combined tests—plating on solid media (1.5×10^6 CFU/mL) and MPN assay in 5-fold diluted LB broth (MPN = $1–7 \times 10^7$ cells/mL)—revealed prolonged preservation of viability of the dormant cells in 3-month cultures grown in SR1 medium with nitrogen limitation (Fig. 1b, Table 1). According to the CFU and MPN titers, the levels of viability for anabiotic dormant cells produced after addition of C_{12}-AHB (2.5×10^{-4} M) and incubated for 3 months were of the same order of magnitude ($3–4 \times 10^7$ cells/mL). Tests with CTC did not reveal the respiratory activity of these dormant cells.

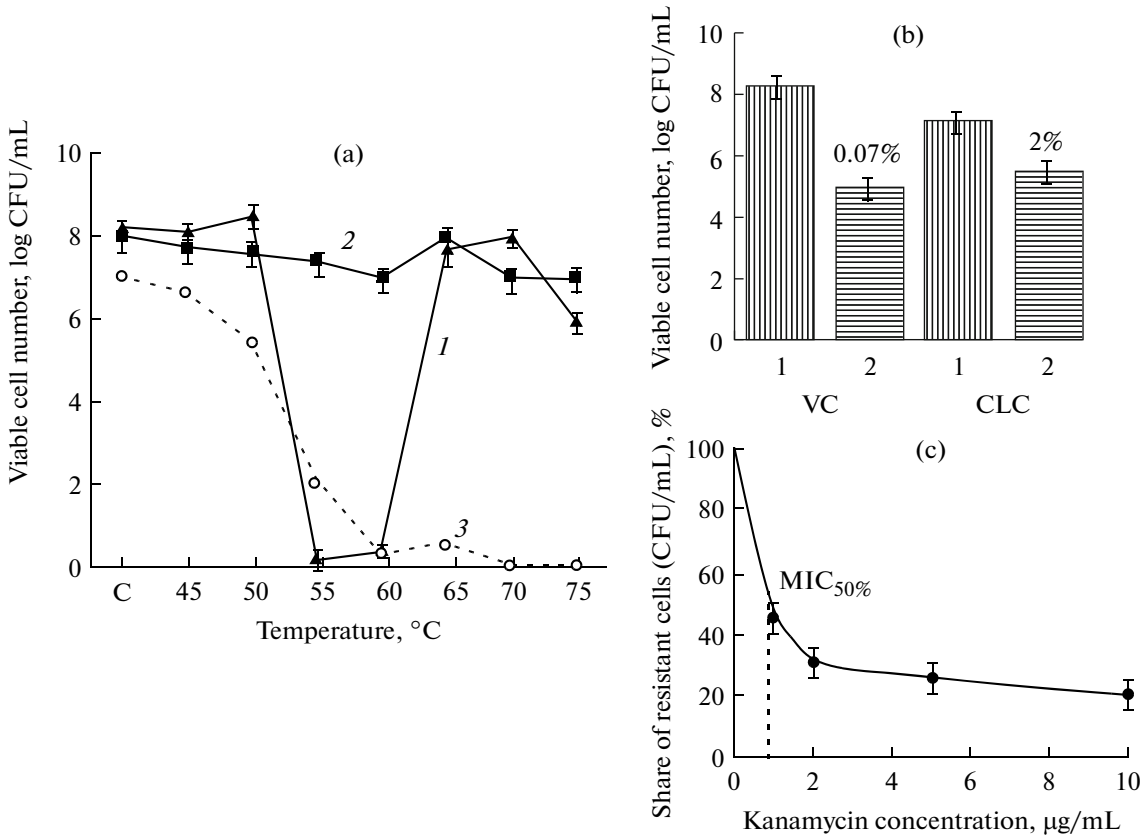

Fig. 3. Resistance of cystlike dormant cells of *S. aureus* and *C. pseudodiphtheriticum* to damaging factors (logN, CFU/mL). (a). Heat resistance (10 min at 45–75°C) of *S. aureus* cells, suspensions in saline solution with 100 mM $CaCl_2$ incubated for 2 (*1*) and 4 months (*2*), and linear-stage cells (*3*). Viability was determined by CFU titer (agar plating). C is the control before heat treatment. (b). Heat resistance of *C. pseudodiphtheriticum* anabiotic cells (addition of 2.5×10^{-4} M C_{12}-AHB, 2 months of incubation) and of the vegetative cells (VC) of the linear-stage cultures: number of viable cells before (*1*) and after (*2*) heat treatment (60°C, 5 min). Cell viability was determined by the MPN test (growth in 5-fold diluted LB broth). (c). Share of *C. pseudodiphtheriticum* anabiotic cells (C_{12}-AHB, 2.5×10^{-4} M, 2 months) resistant to kanamycin (0.5–10 μg/mL, 3 h of incubation). Viability was determined as CFU titer (plating on LB agar).

The dormant cells of *C. pseudodiphtheriticum* had a higher resistance to heating (5 min at 60°C) than the vegetative cells (Fig. 3b). Heat resistance exhibited 2% of the dormant forms (addition of 2.5×10^{-4} M C_{12}-AHB) and only 0.07% of the cells from the linear phase culture. Treatment of the DF suspensions with kanamycin (0.5–10 μg/mL) resulted in decreased CFU titers, with the $MIC_{50\%} = 0.7$ μg/mL (Fig. 3c). The sensitivity of *C. pseudodiphtheriticum* isolates to a broad spectrum of antibiotics, including aminoglycosides, is known to be an order of magnitude higher ($MIC_{50\%}$ for gentamycin is 0.06 μg/mL) [20].

The ultrastructural features of *C. pseudodiphtheriticum* dormant forms (Figs. 4e, 4f), distinguishing them from the vegetative cells (Fig. 4d), were greater cell wall thickness, its lamellar structure (especially noticeable in the forms obtained with 2.5×10^{-4} M C_{12}-AHB), compaction of the nucleoid, and granular texture of the cytoplasm. Coccoid cells predominated in the cultures of the DF obtained in the variant with nitrogen limitation (Fig. 4e).

Thus, the dormant forms of staphylococci and corynebacteria were characterized by (1) prolonged preservation of reproductive capacity; (2) metabolic activity not revealed experimentally; (3) increased resistance to damaging factors; (4) specific features of morphology and ultrastructure; and (5) formation in the cycle of culture development, under starvation stress, or at elevated AHB levels. The combination of these characteristics is necessary and sufficient for their description as dormant forms [21].

Phenotypic Variability in S. aureus and C. pseudodiphtheriticum Populations Developing from Germinated Dormant Forms

Plating of *S. aureus* and *C. pseudodiphtheriticum* CLC revealed a broadened spectrum of phenotypic variability in the first transfer. Antibiotic-resistant variants (clones) emerged when CLC suspensions were plated on selective media with antibiotics.

S. aureus. Kanamycin-resistant variants had small colonies ($d < 0.5$–0.7 mm) with weaker pigmentation

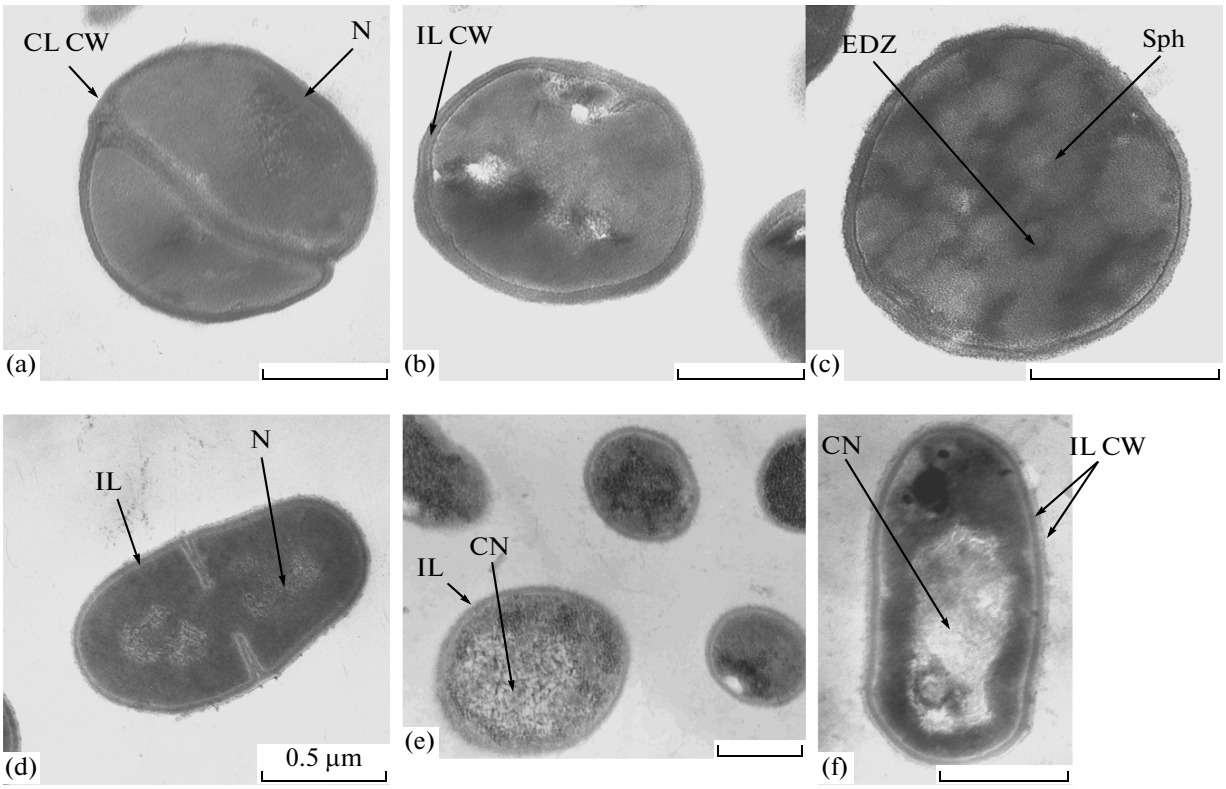

Fig. 4. Electron microscopy of *S. aureus* and *C. pseudodiphtheriticum* cells. Ultrathin sections. *S. aureus*: dormant forms in cell suspensions in saline with 100 mM $CaCl_2$, 4 months of incubation (a, b); micromummies after addition of C_{12}-AHB (1×10^{-3} M, 2 months of incubation (c). *C. pseudodiphtheriticum*: linear growth stage cells (d); dormant cells in the cultures grown in SR1 medium with N limitation, 3 months (e); anabiotic CLC formed after addition of C_{12}-AHB (2.5×10^{-4} M, 2 months of incubation) (f). Designations: CW, cell wall; CL, common CW layer; IL, individual CW layers; N, nucleoid; CN, compacted nucleoid; Sph, spherical granules in the cytoplasm; EDZ, electron-dense zones in the cytoplasm. Scale bar, 0.5 μm.

than in the colonies of the dominant phenotype. The highest share (0.002%) of the clones resistant to 50 μg/mL kanamycin was found in 4-month suspensions of CLC formed in saline solution with 10 mM $CaCl_2$. The highest number and share (0.01%) of the variants able to grow at lower kanamycin concentration (20 μg/mL) were observed for the CLC of the same incubation duration formed in suspensions with 300 mM $CaCl_2$ (Fig. 5a).

C. pseudodiphtheriticum. Plating of corynebacterial DF obtained by addition of 1×10^{-4} M–2.5×10^{-4} M C_{12}-AHB (optimal concentrations for the preservation of cell viability for up to 3 months) on selective media also revealed emergence of the variants growing on solid media with kanamycin (2 μg/mL). Most of these variants (up to 30% of the colony number on the medium without antibiotics) were found for the anabiotic cells formed by addition of 2.5×10^{-4} M C_{12}-AHB and incubated for 15–30 days.

The number and share of antibiotic-resistant persister cells in the cultures are known to depend on their physiological age (linear growth phase–stationary phase) [3–5]. It was therefore desirable to determine the number and share of kanamycin-resistant variants in the incubated (up to one month) cultures of anabiotic cells in comparison to the total number of viable cells (CFU) in the original, early stationary-phase cultures. An increase by five and more orders of magnitude in the number of kanamycin-resistant variants was observed in the populations grown from anabiotic cells (C_{12}-AHB, 1×10^{-4} and 2.5×10^{-4} M), compared to the control culture of the same incubation duration without AHB (Fig. 5b, columns 2). In the control cultures (without C_{12}-AHB) of both early stationary and post-stationary phase cells, antibiotic-resistant variants constituted a minor part (much less than 1%) of the total cell number, which was in agreement with the usual share of persistent cells [4]. The share of kanamycin-resistant variants (relative to the CFU titer in early stationary-phase cultures) revealed by plating anabiotic cells (C_{12}-AHB, 1×10^{-4} and 2.5×10^{-4} M) was, however, significantly higher (3–15%) (Fig. 5b, columns 3).

Emergence of the Sm type variants with small colonies ($d = 1$–1.5 mm) and of the mucoid M type with slimy colony consistency, which were both different

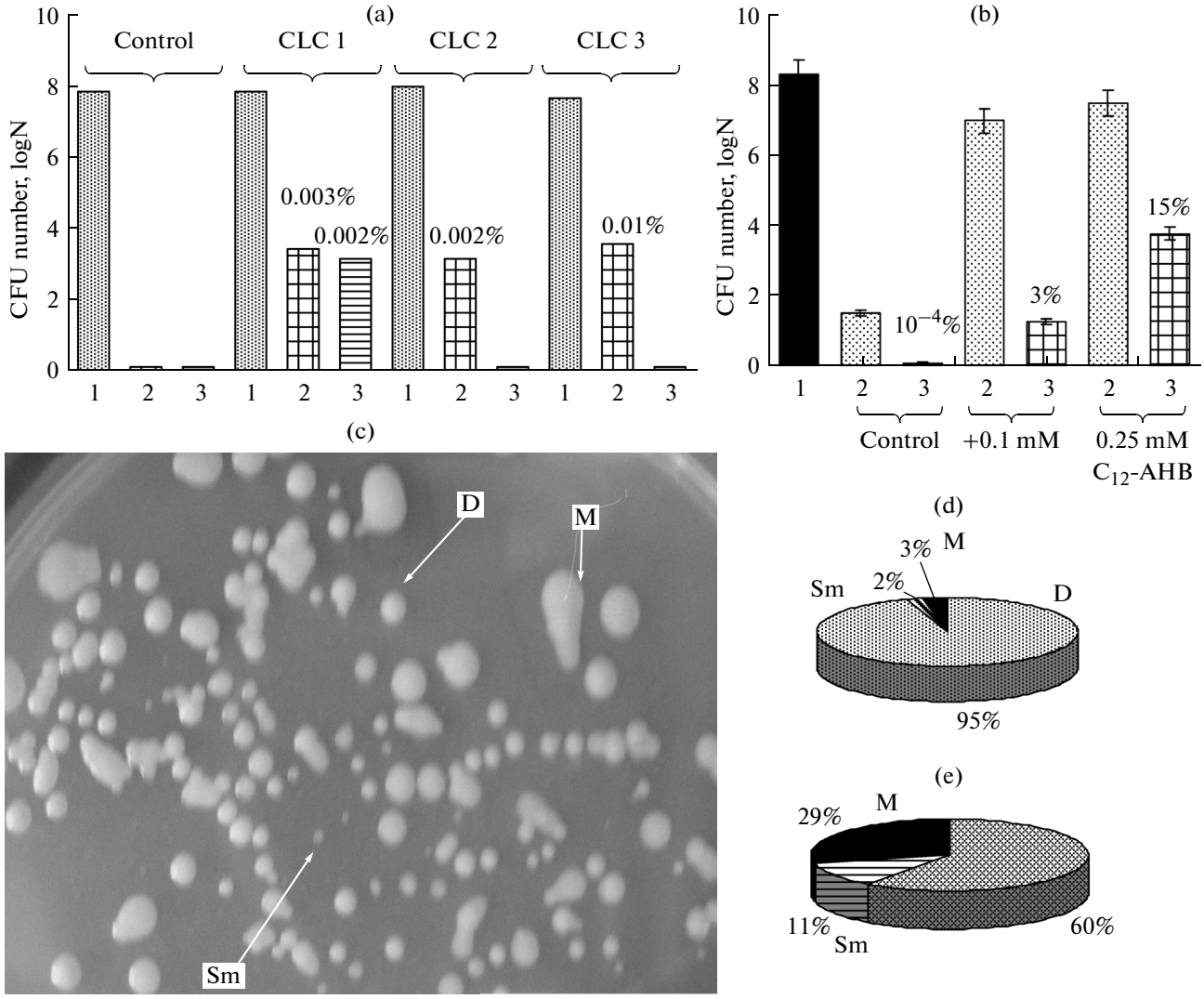

Fig. 5. Phenotypic variation after plating *S. aureus* and *C. pseudodiphtheriticum* dormant forms. (a) Emergence of antibiotic-resistant variants of *S. aureus* and *C. pseudodiphtheriticum*: number of viable cells (logN, CFU/mL) in *S. aureus* cell suspensions (4 months of incubation) in saline solution with 10 mM $CaCl_2$, CLC 1; 100 mM $CaCl_2$, CLC 2; 300 mM $CaCl_2$, CLC 3; the control is CFU number in suspensions immediately after transfer to saline without $CaCl_2$. CFU for plating on LB agar without kanamycin (1) and with 20 (2) and 50 μg/mL kanamycin (3). Percentage indicates the share of kanamycin-resistant variants. (b) Number of viable cells (logN, CFU/mL) in suspensions of *C. pseudodiphtheriticum* anabiotic dormant cells (15 days–1 month) obtained by addition of C_{12}-AHB (1×10^{-4} and 2.5×10^{-4} M). The controls were the cultures of the same incubation duration without AHB. CFU titer in the stationary-phase culture (5 days) plated on LB agar (1); on LB agar with 2 μg/mL kanamycin (2); and share of kanamycin-resistant variants of the CFU number in the stationary-phase culture (3). (c). Variations in colony morphology of the populations obtained from *C. pseudodiphtheriticum* CLC (SR1 medium, 5-fold N limitation, 3 months of incubation) in the first transfer on nonselective LB agar. Colonies of the D, M, and Sm types (c) and their share (% of the total colony number) in the populations obtained by plating the stationary-phase (5–7 days) cultures (d) and 3-month CLC suspensions (e).

from the dominant D variant, was another instance of phenotypic variation revealed by plating *C. pseudodiphtheriticum* CLC (SR1 medium, fivefold nitrogen limitation, 3 months of incubation) on nonselective medium (LB agar) (Fig. 5c). The frequency of emergence of the Sm and M variants (11 and 29%, respectively) depended on incubation time (3 months) and was higher than in the case of stationary cultures (5–7 days) grown in the same medium with nitrogen limitation. In the latter case, the total share of the minor colony types did not exceed 5% (Figs. 5d, 5e).

Thus, our results showed increased phenotypic variability, an important adaptive mechanism of bacteria, in *S. aureus* and *C. pseudodiphtheriticum* populations as a result of the dormant state induced by starvation stress or elevated AHB concentrations.

Properties of Irreversibly Inactivated S. aureus Micromummies

As mentioned above, special counting procedures and techniques resulted in high (but not 100%) efficiency of viable cell enumeration in CLC populations (Table 1). Loss of the colony-forming capacity by a part of the population resulted from their transition to a nonculturable state, in which the cells remained intact ("alive" according to Live/Dead staining). This state was especially characteristic of the anabiotic cells formed in the presence of C_{12}-AHB (Table 2). The C_{12}-AHB concentrations over a certain threshold (1×10^{-3}–2×10^{-3} M) resulted in complete loss of viability (CFU = 0, MPN = 0), while their external shape and was retained. This effect was caused by deep irreversible degradative changes of the cell membranes (Fig. 2, Table 2). Such cells were termed micromummies [22]. Micromummies of staphylococci preserved their cell wall and differed from CLC by the presence of spherical zones with low electron density within the cytoplasm (Fig. 4c), probably resulting from self-assemblage of membrane lipids dispersed by C_{12}-AHB. Destructive changes in the cytoplasmic membrane of micromummies were confirmed by high fluorescence intensity in PI-stained samples. No autolysed cells were found in staphylococci suspensions with 1×10^{-3}–2×10^{-3} M after 2 months of incubation.

Thus, millimolar AHB concentrations caused irreversible inactivation of *S. aureus* cells with their transition to the metabolically inert forms (micromummies), which were characterized by complete loss of viability and complete inhibition of autolytic processes.

DISCUSSION

Results of our study demonstrating the ability of non-spore-forming bacteria *S. aureus* and *C. pseudodiphtheriticum* to produce cystlike dormant forms broadens our concept of their survival strategies in natural systems under conditions not supporting growth. Similar dormant forms may be produced within a host organism, in the microzones where nutrient deficiency occurs or the concentration of regulatory factors, responsible for acquirement and maintenance of anabiosis, increases. Formation of dormant forms, which are able to revert to growth, may be associated with persistence, latent infection, or a carrier state.

Transition into a nonculturable state is one of the responses of pathogenic bacteria to starvation stress in diluted suspensions, e.g., in estuarine waters. In this state, the cells, while preserving their viability (potential proliferative activity) for some time, lose their ability to form colonies on solid media and are therefore not detected by the standard tests [23, 24]. Investigation of this state is therefore of importance for medical microbiology and sanitary and epidemiological monitoring.

In our studies, we reproduced the situation of transition to a dormant state, which was accompanied by (1) formation of cystlike cells capable of prolonged survival, which were found in many spore-forming and non-spore-forming bacteria [1, 6, 7], or (2) development of a nonculturable state in the course of prolonged incubation, as was described previously for *Micrococcus luteus* [25]. Starvation of *S. aureus*, which was achieved in the present work by transfer of the stationary-phase cells into saline solution with $CaCl_2$ (and subsequent incubation for up to 4 months), caused dormant forms (CLC) to develop. Dynamics of CFU titers in these suspensions, with their stabilization at 1% in 4-month cultures (Fig. 1) was similar, but not identical to that described in [11, 26]. In one of these works, transition of staphylococcal cells to a partially reversible nonculturable state occurred when the incubation temperature was decreased to 4°C [26], while in the other, a drastic drop in the CFU titer, associated with either a nonculturable state or cell death, was observed for *S. aureus* cultures developing under phosphorus or amino acid limitation [11]. In our experiments, 4-month starving suspensions of *S. aureus* dormant forms had CFU titer two orders of magnitude lower than the total number of viable cells Live/Dead test), which was comparable to the viable cell number determined by MPN (Table 1). For more accurate determination of DF abundance in environmental objects and biological material, a combined approach is desirable, including variations in the medium composition and independent methods of enumeration (Table 1 and results of our previous works [7, 9]).

Thus, survival of *S. aureus*, depending on starvation conditions, density of the cell suspensions, and incubation temperature, is achieved either by development of viable, metabolically inactive DF resistant to damaging factors (Fig. 1a, Table 1, Fig. 3a), which are ultrastructurally similar (Figs. 4a, 4b) to CLC of non-spore-forming bacteria [1], or by transition to a nonculturable state described in the works [11, 26].

Development of cystlike dormant forms and nonculturable cells shown for nonpathogenic *S. aureus* and *C. pseudodiphtheriticum* strains may be also a feature of opportunistic and pathogenic strains of these species. A similar phenomenon was shown for *Mycobaterium tuberculosis* and its saprotrophic relative *M. smegmatis* [9, 12]. It is therefore reasonable to ask whether the dormant and nonculturable cells, such as those found in staphylococci and corynebacteria, may act as persisters in the host organism. It should be noted that our results do not contradict the data on bacteria survival within the host organism depending on the nutritional conditions and cell density. Thus, in the cultures of staphylococci-infected adipocyte-like cells 3T3-L1, capacity of *S. aureus* for intracellular survival was higher at decreased glucose level (carbon limitation) and when the tester cells were infected with increased numbers of staphylococci cells [27]. Microscopic (including transmission electron microscopy)

detection of single, non-dividing *S. aureus* cells within the epithelial cells of nasal mucosa is also of interest in this respect [28]. While these cells probably had the ultrastructural organization of the dormant forms, insufficient resolution did not allow us to compare them to CLC (Fig. 5). In the case of another pathogenic bacterium, *Legionella pneumophila*, morphologically differentiated forms of a cystlike type were found within HeLa cells and in the macrophages [8]. Thus, the dormant and surviving forms of bacteria belonging to various morphological types may be the subject of research on bacterial symbionts or parasites in host tissues by direct high-resolution microscopy.

Since survival of bacteria in the environment or within a host organism depends on numerous and often indefinite factors, one more important result of the present work should be mentioned. Under equal conditions of the medium composition and cell density, transition of *S. aureus* and *C. pseudodiphtheriticum* into various dormant states (which preserve or lose their colony-forming capacity) was shown to be controlled by the concentration of microbial anabiosis autoinducers (C_{12}-AHB) (Table 2). Considering the possibility of AHB-mediated regulation of transition to a dormant state and its preservation in environmental objects, it should be noted that AHB of the alkylresorcinol class have been found in both modern and ancient buried or permafrost soils in concentrations of 10^{-4}–2×10^{-4} M (calculated for the content of available moisture) [unpublished data]. This range of concentrations was sufficient to induce transition of the cells in submerged laboratory cultures into a dormant state mediated by C_{12}-AHB (Fig. 2, Table 2). Nano- and submicromolar concentrations of structurally cognate compounds (long-chain alkylresorcinols) were found in blood plasma and adipose tissue [29, 30]. This level of alkylresorcinols is lower than the AHB concentrations required for transition of staphylococcal and corynebacterial cells into a dormant state in model experiments. In natural systems, biofilms, and microcolonies, concentrations of the factors responsible for intercellular communication, such as AHB, may be high enough for them to carry out their regulatory functions, including control of transition into a dormant (anabiotic) state [31]. Practical application of AHB, which have a broad spectrum of biological activity, may be promising [32], as well as their use for investigation of bacterial persistence in immunological research.

One more aspect of AHB practical application is base on their ability (in over-threshold concentrations) to induce transition of the vegetative cells into a mummified state, which is not accompanied by cell lysis and release of autolysis products (possible substrates for secondary microbial growth) into the medium. Due to this effect of AHB, their preparations may be promising as antiseptics and disinfection agents suitable for cold sterilization. Since the action of AHB is not species-specific [1], their efficiency against antibiotic-resistant bacterial variants, such as MRSA, is of interest.

In relation to the urgent issue of bacterial resistance to antibiotics [2–5, 14–19], it should be mentioned that CLC, similar to other dormant forms, are resistant to antimicrobial preparations and other damaging agents, as was shown in the present work (Fig. 3) and previously [1, 8, 9]. Detection of *S. aureus* forms capable of prolonged survival suggests the issue of the possible transformation of antibiotic-resistant persister cells into CLC or other morphological types of dormant cells. Another important aspect of this problem is association of the dormant state with enhanced phenotypic variety emerging in germinating DF. This may result in emergence of antibiotic-resistant variants of *S. aureus* and *C. pseudodiphtheriticum* (Fig. 5). A similar phenomenon was previously reported for *M. smegmatis* [7]. Some phenotypic characteristics of *S. aureus* variants emerging from plated CLC, such as colony size and pigmentation and kanamycin resistance, were similar to the SCV variant of staphylococci [16]. Additional tests are, however, required for the attribution of kanamycin-resistant variants to the SCV type. The presence of the M type with increased production of mucous material (Fig. 5c), apart from antibiotic-resistant variants, in *C. pseudodiphtheriticum* population, may result in higher capacity for adhesion and biofilm formation, which may result in additional antibiotic resistance [18].

In general, the results of the present work and of earlier research [1, 7, 12] pose the question of whether the population of dormant forms of opportunistic and pathogenic bacteria in environmental objects may act as a source of antibiotic-resistant clones. Detailed investigation of the mechanisms responsible for emergence antibiotic-resistant bacterial variants associated with the dormant state of the cells will be important for antibiotic treatment and may be the subject of further research.

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