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EXPERIMENTAL ARTICLES

Colony Structure of a Consortium of Nitrifying Bacteria

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Abstract—Colonies produced by a consortium of nitrifying bacteria were studied using light and electron microscopy. The colonies were obtained by direct plating of inoculum from a two-stage nonsterile chemostat fermentor and by repeatedly passing the microbial community of the fermentor through selective media containing ammonium or nitrite. The colonies studied can be characterized by a specific combination of six types of cells differing in their ultrastructure and spatial location within the colony. The types of cells occurring within a given colony were found to depend on the nitrogen compound present in the medium. As a result of our study, morphological features of colonial bacterial communities were identified. The proposed approach can be viewed as a method to describe microbial associations and communities.

Key words: electron microscopy, colony architectonics, nitrifying bacteria.

Pure microbial cultures are rarely found in natural environmental conditions, and most of the ecological niches are occupied by specific microbial communities. The investigation of a community by isolating pure cultures and describing their properties is a notoriously hard problem. For example, the nitrifying bacteria, known to play an important part in the nitrogen cycle, are very difficult to study because of their slow growth and very small colony size (less than 100 μm in diameter). The severe problems encountered in the isolation of pure cultures of nitrifying bacteria offer an explanation for the fact that only a very limited number of strains of these microorganisms have been studied to date [1].

A different approach to community analysis, used in studies of biofilms, relies on data yielded by electron microscopy and does not require the isolation of pure cultures. In particular, by using electron microscopy methods, it has been established that the biofilms occurring in wastewater treatment plants are composed of several bacterial species. A strong correlation was found between the amount of organic material in sewage waters and the morphological and ultrastructural features of the biofilms. These features were further used as indicators of the physiological and ecological conditions in the biofilm [2]. Electron microscopic study of a microbial community from the mouth cavity grown as a biofilm revealed nine constituent microbial species which exhibited noticeable spatial differentiation across the biofilm [3].

It follows that, despite their relative complexity, electron microscopic methods can facilitate the investigation of microbial communities by making manifest the cytomorphological spatial heterogeneity of individual microbial associations and can shed new light on the diversity and ecology of microorganisms.

We are not aware of any previous electron microscopic studies of intact communities of nitrifying bacteria. The purpose of this work was to investigate, without isolating pure cultures, the colonies formed by a consortium of bacteria responsible for the process of nitrification in a two-stage nonsterile chemostat fermentor.

MATERIALS AND METHODS

Cultivation of the communities of nitrifying bacteria in liquid media. The nitrifying bacterial culture studied was the result of seven years of cultivation in a nonsterile two-stage chemostat fermentor. The inoculum was taken from an aeration tank of a water treatment installation in a chemical plant, where the nitrification process was effective [4].

The nutrient medium by Engel and Alexander [1] composed of (g/l) NH_4HCO_3, 2.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.004; $FeSO_4 \cdot 7H_2O$, 0.005 was used. A slight modification of the original medium consisted of replacing ammonium sulfate with bicarbonate, which concurrently served as a carbon source. The pH in the first and second stages of the chemostat fermentor was automatically maintained in the ranges of 7.2–7.4 and 7.8–8.0, respectively, by titration with a 0.03 M solution of KOH. The temperature of the culture was not controlled and remained at room level (18–22°C). The dilution rate was 0.02 h^{-1}. Under such conditions, the major part of the ammonium nitrogen was already utilized in the first stage; the nutrient supplied to the second stage contained no more than 20 mg/l of ammonium and up to 260 mg/l of nitrite, formed as a result of the activities of the microbial community in the first stage.

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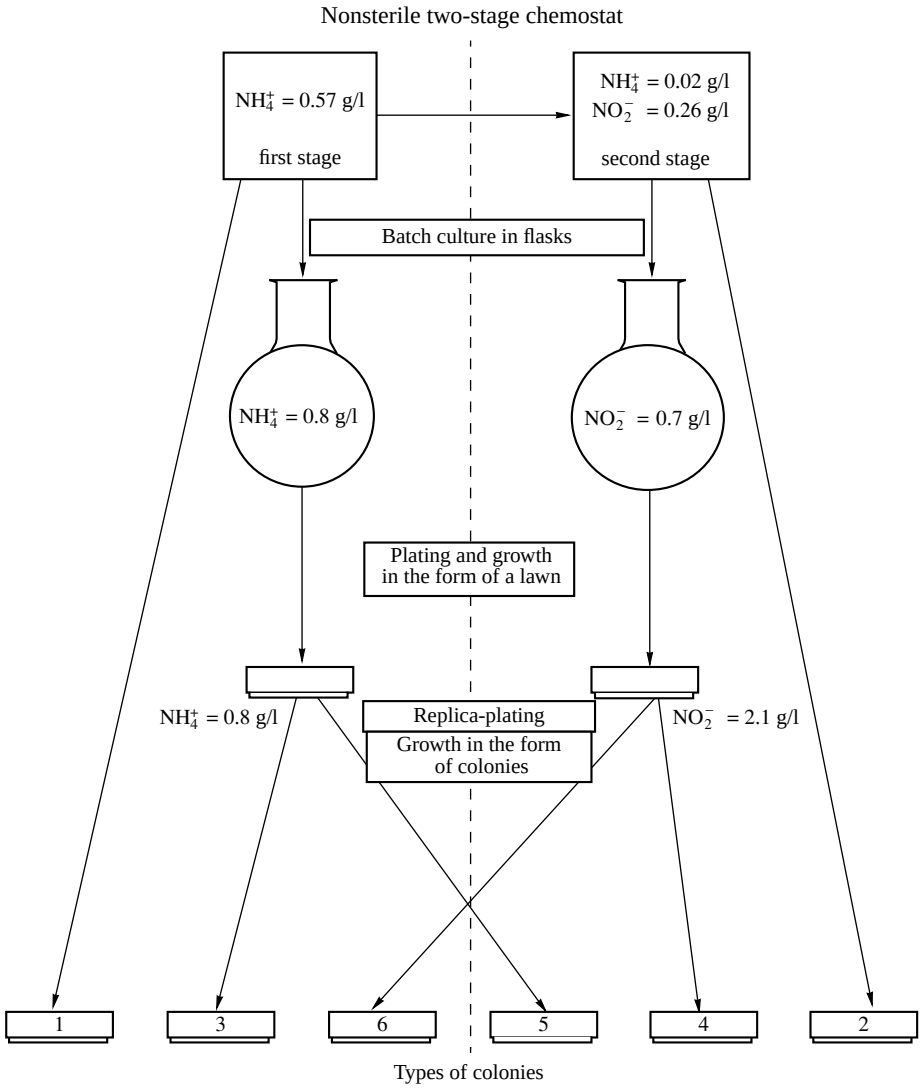

Fig. 1. Sketch of the entire procedure used to obtain colonies of the nitrifying community on solid nutrient media.

Samples of microorganisms were withdrawn from the chemostat and subjected to a series of transfers in a batch culture grown in flasks on a shaker. This was done to obtain a standardized inoculum for colony growth experiments. The nutrient medium used in the course of the culture transfers was the same as during the continuous-flow cultivation. Because the nonsterile culture in the reactor was a bacterial community that oxidized ammonium in the first stage and nitrite in the second stage, nitrogen was supplied as $(NH_4)_2SO_4$ (3.0 g/l) when microorganisms from the first stage were used for inoculation and as $NaNO_3$ (1.0 g/l) for microorganisms originating from the second chemostat stage.

Growing colonies of the nitrifying community on solid nutrient media. Colonies of the community under investigation were grown on media similar to those used in the chemostat and batch cultures, except that the concentrations of $(NH_4)_2SO_4$ and $NaNO_2$ were 3.0 g/l, and agar was added (20 g/l).

To produce colonies with a stable morphology, lawns were grown on solid nutrient media, and then individual colonies were obtained by replica-plating. In all, six variants of colony growth conditions were used (Fig. 1): (1) Inoculum from stage 1 of the chemostat was plated onto solid medium with NH_4^+; (2) Inoculum from stage 2 of the chemostat was plated onto solid medium with NO_2^-; (3) A culture obtained from the first chemostat stage, upon a series of passages in flasks with medium containing NH_4^+, was plated onto solid medium with NH_4^+; (4) A culture obtained from the second chemostat stage, upon a series of passages in flasks with medium containing NH_4^+, was plated onto solid

Table 1. Some morphological features of colonies observed under a light microscope

Colony feature	Colony type			
	1, 3*	2, 4*	5	6
Diameter, mm	1	2–3	2–3	1
Edge	Clear-cut, slightly undulated	Scalloped	Scalloped	Clear-cut, slightly undulated
Periphery (aureole)	None	Thin layer, mat	Thin layer, lustrous	None
Center	Convex, mat, slightly rugged	Convex, lustrous, fairly rough	Convex, lustrous, rugged	Convex, mat, slightly rugged

* Morphological features of colonies in variants 1 and 3 and 2 and 4 are identical.

medium with NO_2^-; and (5, 6) Two complementary colonial cultures obtained by plating cultures passed through a medium with ammonium onto a medium with nitrite and vice versa.

As a result, colonies of nitrifying communities were obtained for comparative analysis, differing with regard to the inoculum used and the nitrogen source available in the liquid and solid media.

Visual examination of colonies was performed under a Jenavert microscope (Carl Zeiss, Jena) in reflected light at an up to 100× magnification.

Preparing the material for electron microscopic studies. Colonies enclosed in agar capsules were initially fixed in a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C. The second fixation was in a 1% solution of osmium tetroxide in the same buffer, followed by a contrasting fixation in a 3% solution of uranyl acetate in 30% ethanol. Ethanol and acetone were used in increasing concentrations for sample dehydration. A mixture of Epon 812 and Araldite M (Serva) epoxy resins was used to embed the colonies. Ultrathin sections of three to four colonies for each test variant were obtained with a Reichert UM-03 (Austria) ultramicrotome. Sections were additionally contrasted with a 0.5% water solution of lead isocitrate and examined under a JEM-100C (Japan) electron microscope at an accelerating voltage of 80 kV.

RESULTS

Light Microscopy of Colonies

A certain colony type, readily recognized by visual inspection, was characteristic for each variant of plating on solid media (Table 1).

Electron Microscopic Study of Colonies

Cytomorphological diversity of bacteria in the nitrifying community. Six types of cells were identified by the analysis of the ultrastructure of characteristic colonies (Fig. 2). The bacteria differed in their shape, size, cell wall structure, presence of reserve substances, and occurrence of a microcapsule and its structure. Type I cells are gram-positive cocci, 0.5–0.7 μm in diameter. Type II cells are small rods, gram-positive, about 1 μm in length and 0.2–0.3 μm in diameter. Type III cells are gram-negative rods, about 2.5–3.0 μm in length and 0.5 μm in diameter, containing inclusions of poly-β-hydroxybutyrate. Type IV cells are gram-negative coccobacilli with a diameter of about 0.5 μm and a fibrillar microcapsule. The fibrils are comparable in length with the cell diameter and stick out perpendicular to the cell surface. The DNA often occurs in a compact state and appears as electron-dense regions stretched along the long cell axis. The cells contain electron-dense inclusions. Type V cells are irregularly shaped, gram-negative, and, as a rule, contain no inclusions of reserve substances. The cells are kept together in groups by a multilayered capsule. Type VI is represented by gram-negative electron-dense pleomorphic bacteria.

The combinations of cells comprising the colonial populations are listed in Tables 2 and 3.

Spatial distribution of cells in colonies. By using large-area ultrathin sections of whole colonies with intact spatial arrangement of cells in our electron microscopic studies, we managed to explore the spatial distribution of different morphological types of bacteria across the colony volume. The distribution of cells in the colonies studied is diagrammed in Fig. 3.

DISCUSSION

Light microscopic examination of colonies obtained from bacterial consortia that oxidize ammonium and consortia that oxidize nitrite and are grown on media with the corresponding nitrogen compounds (NH_4^+ or NO_2^-) showed that a number of features in these colonies differed. The most apparent one is the colony diameter, which correlates with other characteristics listed in Table 1. Small-sized colonies (1 mm) grew from inoculum obtained in an ammonium-oxidizing consortium (the first chemostat stage), and larger colonies (up to 3 mm) developed from inoculation material

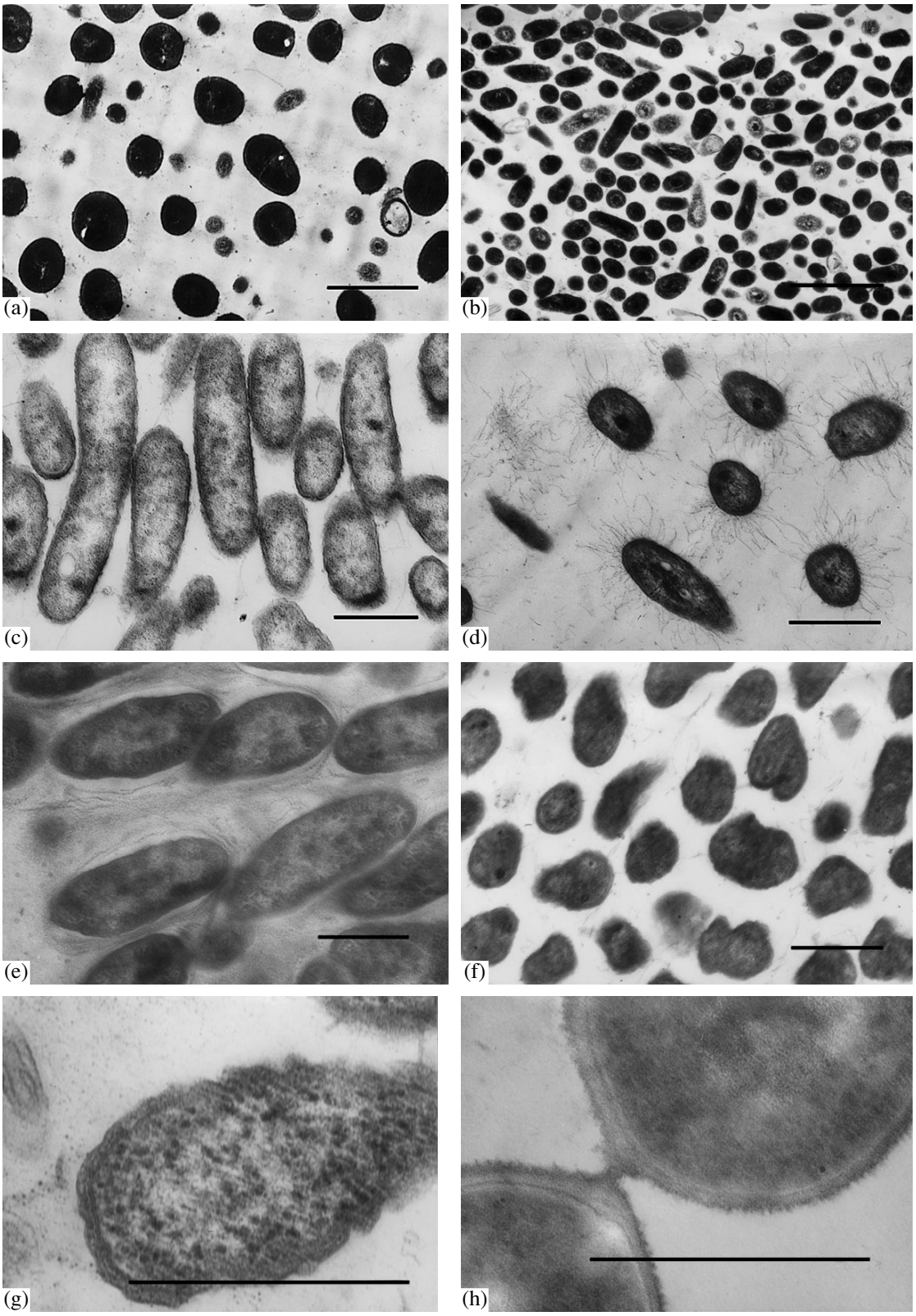

Fig. 2. Ultrastructure of bacteria in colonies of the nitrifying community, formed on solid nutrient media. The following morphological types of cells can be identified: (a) type I, (b) type II, (c) type III, (d) type IV, (e) type V, and (f) type VI (bar, 1 μm). The cell wall structures of gram-negative and gram-positive bacteria are shown in (g) and (h), respectively (bar, 0.5 μm).

Table 2. Types of cells occurring in colonies obtained from a two-stage nonsterile chemostat

Colony type	Cell type					
	I	II	III	IV	V	VI
1	+	++	+	+	–	–
2	+	+	++	–	–	–

Note: "+" means the presence of a given cell type; "++" means that the cells of a given type are predominant; "–" denotes absence of the given cell type.

Table 3. Types of cells in colonies obtained after passages on selective media

Colony type	Cell type					
	I	II	III	IV	V	VI
3	–	++	+	+	–	–
4	–	+	++	–	+	–
5	–	+	+	+	+	+
6	–	+	++	+	+	+

Note: "+" means the presence of a given cell type; "++" means that the cells of a given type are predominant; "–" denotes absence of the given cell type.

taken from the second chemostat stage. In this respect, there was no difference between the colonies grown directly from samples taken from the reactor or from the inoculum produced via a series of enrichment cultures. Based on this fact, it could be hypothesized that the colony size is not influenced by how the inoculum was obtained (a continuous-flow or a batch culture) but instead largely depends on whether it originated from a consortium responsible for nitrification of ammonium or nitrite.

However, the results obtained for colonies observed in cultivation variants five and six involving growth on the complementary media, fail to agree with this hypothesis. In this case, the factor governing colony size is not where the inoculation material originated (the first or the second stage of the chemostat) but in what form nitrogen was available in the solid nutrient medium.

This would be possible only if the inoculation material, irrespective of its origin, included all the major types of cells present in the community. Then, the morphological type of the colony (the structure of its central part and edges, the occurrence of an aureole on its periphery, etc.) would depend upon the source of nitrogen and would be determined by the ratio of cells able to proliferate under the given conditions. This explanation is also in agreement with the design of the cultivator employed, in which the two vessels (implementing the first and second chemostat stages) were not separated by a membrane impermeable to bacteria, so the cells could move freely across the entire cultivation space during the growth process. The functional and morphological differences between the first and second stage communities established in our study developed naturally as a result of the continuous one-way flow of the nutrient medium.

The results of the electron microscopic study of the ultrastructure of cells making up the colonies attest to the presence of all of their major types in both stages of the chemostat. We do not know if cell transition from one cell type to another is possible. We know, however, that if a certain type of cells occurs in colonies, then its ultrastructure is not affected either by the origin of the inoculum or by the source of nitrogen. For this reason, the caption for Fig. 2 does not specify the type of colonies whose ultrathin sections were used to take cell micrographs.

The occurrence of type I cells only in colonies produced by direct plating of a culture from the chemostat and their absence in colony variants obtained after growth of microorganisms on selective media could mean that type I cells correspond to a bacterial contaminant not directly related to the process of nitrification. The nutrients supporting the development of cocci in a nonsterile chemostat operating over a long period time could be provided by products excreted by the culture into the medium or by the biofilm (not studied in the present work) that covers internal fermentor surfaces and serves both as an autochthonous source of organic substrate and a niche for bacteria with other types of physiological activity.

It would be reasonable to suggest that long-term cultivation in a nonsterile chemostat reactor created a community in which the major process of nitrification was accompanied by other processes. One may also assume that these processes also occurred in the industrial aeration tank from which the inoculum was first taken. The corresponding cells fail to survive batch cultivation in selective media and, therefore, are not observed in colonies developed from the inoculum subjected to a series of transfers. Thus, by cultivating bacteria on strictly selective media in a liquid batch culture or in the form of a lawn, we were able to get rid of the accompanying nonspecific flora proliferating in the chemostat cultivator.

In comparing different variants of colonies with respect to the assortment of cells they comprise, it should be noted that type II and III cells were present in all variants of the colonies studied and were therefore basic cell types. Type II was predominant in colonies functionally responsible for the oxidation of ammonium, and type III was predominant in colonies responsible for the oxidation of nitrite.

Other cell types occurred in only some colony variants. Type IV cells were observed, along with the basic types, in colonies grown on solid medium with NH_4^+ from inoculum from the first chemostat stage. This is an

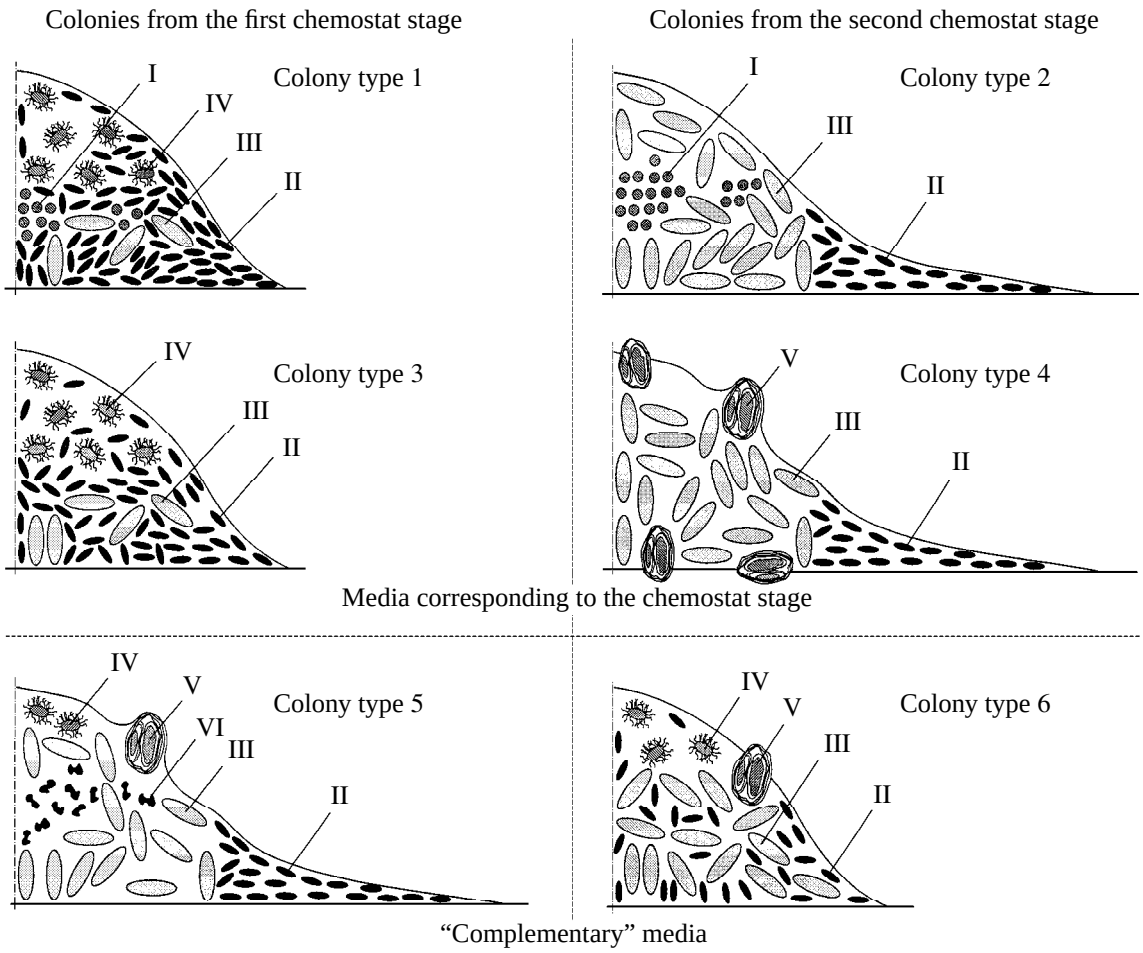

Fig. 3. Sketches of the spatial distribution of cells across the colony volume. I–VI are the six main types of cells.

indication that a key part in the oxidation of ammonium by an established community is played by type II and type IV cells (gram-positive bacteria and gram-negative coccobacilli having a fibrillar microcapsule, respectively).

In colonies obtained in the fourth test variant (growth on solid medium with NO_2^- from inoculation material originating in the second chemostat stage), cells of type V appeared together with basic cell types. These cells, however, were not found in colonies grown in the second test variant (the second chemostat stage, with nitrogen supplied mostly as nitrite; note that it was this culture that served as the inoculum for all other colony variants associated with the oxidation of nitrite). This fact might signify that the fraction of cells with a layered microcapsule is insignificant in a continuous-flow culture, but such cells do accumulate as a result of culture transfers under batch cultivation conditions.

It can therefore be assumed that the key role in an established nitrite-oxidizing community is played by type III cells (gram-negative rods with inclusions of poly-β-hydroxybutyrate). Cells of type V (gram-negative, irregularly shaped, lacking inclusions of reserve substances, and kept in groups by a multilayered capsule) observed in colony variants that utilize nitrite are also likely to be associated functionally with the oxidation of nitrite.

The logically consistent picture outlined here is challenged by the results of the ultrastructural studies of the cells in colonies grown on complementary media (variants five and six). All such colonies are composed of similar and the most complete sets of cell types (II–V). The occurrence of the cell type VI (electron-dense pleomorphic bacteria) in colonies grown in the fifth test variant can be explained as follows. Irregularly shaped cells with electron-dense cytoplasm may be degrading cells. Pleomorphic forms are most often observed under nonoptimal growth conditions. It is not clear, however, from what type of cells they originated.

The spatial distribution of cells in ultrathin sections of the colonies was found to follow a certain pattern which is diagrammed in Fig. 3. It was established, for example, that the aureole around the colonies was formed by type II cells. Cells of type IV occupied the

central part of the colony facing the air and were never found in the colony next to the agar.

The fact that a given type of cell can be observed in an electron microscopic study is likely to imply that the fraction of such microorganisms in the colony and their functional significance are relatively high. It is worth mentioning that our data on the composition of the community are somewhat at odds with the conventional portrayal of nitrifying bacteria. First, we failed to observe any bacteria with an ultrastructure typical of nitrifiers (characterized by intracellular membrane formations). This is not at all unusual, because there are two genera, *Nitrosospira* and *Nitrospina*, whose representatives are physiologically indistinguishable from classical nitrifiers and at the same time lack intracellular membrane structures [5]. Second, the key role in the oxidation of ammonium by an established community is likely to be played by gram-positive bacteria, and, in the oxidation of nitrite, by gram-negative bacteria.

The consortium of microorganisms formed in an open system simulating the natural process of transformation of substances under a technogenic load can be regarded as a functionally connected community [6]. Despite the above-mentioned discrepancies, its is beyond any doubt that the primary function of the community studied is nitrification. The ammonium nitrogen, the only initial source of energy supplied, was present in the incoming nutrient solution at a high concentration (443 mg/l), and greater than 95% of it was eventually utilized.

Using conventional methods of light and electron microscopy, we were able to describe the morphological features of colonial communities of nitrifying bacteria in sufficient detail for their preliminary identification. In our study, we did not perform the microbiological isolation of pure cultures of the major cell types or biochemical tests aimed at a determination of the functional role of the identified cell types. However, based on the knowledge of the physiological requirements of the community members, determined by means of selective media and complementary plating, and taking into account the variety and the spatial distribution of cells across the colony, some new information can be gained, differing in nature from that yielded by microscopic studies of pure cultures.

We see that this approach, despite the relative complexity of the electron microscopic methods, can facilitate the investigation of microbial associations and provide original data on the spatial structure of colonies of microbial consortia. This approach can be regarded as a method to describe microbial associations and communities that does not involve their separation into pure cultures.

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