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Nanoarchaeum equitans and *Ignicoccus hospitalis*: New Insights into a Unique, Intimate Association of Two Archaea▽

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***Nanoarchaeum equitans* and *Ignicoccus hospitalis* represent a unique, intimate association of two archaea. Both form a stable coculture which is mandatory for *N. equitans* but not for the host *I. hospitalis*. Here, we investigated interactions and mutual influence between these microorganisms. Fermentation studies revealed that during exponential growth only about 25% of *I. hospitalis* cells are occupied by *N. equitans* cells (one to three cells). The latter strongly proliferate in the stationary phase of *I. hospitalis*, until 80 to 90% of the *I. hospitalis* cells carry around 10 *N. equitans* cells. Furthermore, the expulsion of H_2S, the major metabolic end product of *I. hospitalis*, by strong gas stripping yields huge amounts of free *N. equitans* cells. *N. equitans* had no influence on the doubling times, final cell concentrations, and growth temperature, pH, or salt concentration ranges or optima of *I. hospitalis*. However, isolation studies using optical tweezers revealed that infection with *N. equitans* inhibited the proliferation of individual *I. hospitalis* cells. This inhibition might be caused by deprivation of the host of cell components like amino acids, as demonstrated by ^{13}C-labeling studies. The strong dependence of *N. equitans* on *I. hospitalis* was affirmed by live-dead staining and electron microscopic analyses, which indicated a tight physiological and structural connection between the two microorganisms. No alternative hosts, including other *Ignicoccus* species, were accepted by *N. equitans*. In summary, the data show a highly specialized association of *N. equitans* and *I. hospitalis* which so far cannot be assigned to a classical symbiosis, commensalism, or parasitism.**

The concept of symbiosis originally encompassed all long-term relationships of different organisms in close proximity (28). Those associations could be mutualistic, commensalistic, or parasitic. However, for many types of interactions between organisms, assignment to these simple categories is not possible. Symbiotic systems are often very difficult to cultivate in the laboratory and therefore hard to analyze (2). In addition, interactions between many organisms turned out to be dynamic and to change among mutualism, commensalism, and parasitism (25).

The first and so far only known intimate association of two archaea was described in 2002 by Huber et al. (12). It consists of the designated "host" *Ignicoccus hospitalis*, a member of the crenarchaeal order Desulfurococcales, and the "symbiont" *Nanoarchaeum equitans*. *I. hospitalis* is an anaerobic, hyperthermophilic coccus growing strictly chemolithoautotrophically by reduction of elemental sulfur with molecular hydrogen as an electron donor (24) and fixation of CO_2 via a novel CO_2 fixation pathway (15). Like cells of the other described *Ignicoccus* species, *I. hospitalis* cells exhibit a unique morphology; they lack an S layer and are the only archaea which are surrounded by an outer membrane exhibiting a unique protein and lipid composition (4, 16, 22, 26). *N. equitans* was identified as the first representative of a novel archaeal kingdom, the Nanoarchaeota (12, 13, 31). However, the exact branching point within phylogenetic trees of this organism is dependent on the molecule investigated and an object of ongoing scientific discussion (3, 5, 6). For clarification, the identification and characterization of further members of this widely distributed group (11, 20) might be crucial. *N. equitans* cells are tiny cocci with a diameter of 350 to 500 nm attached to the surface of *I. hospitalis* (12). Various attempts to cultivate *N. equitans* in the absence of its host failed (13). With about 490 kb, *N. equitans* has one of the smallest genomes known so far, lacking nearly all known genes for lipid, cofactor, amino acid, or nucleotide biosynthesis (31). Lipid analyses of *N. equitans* and *I. hospitalis* revealed that *N. equitans* derives all of its membrane lipids from its host (16). However, still very little is known about its physiology and the interaction with *I. hospitalis*.

In this study, we investigated the growth characteristics, interactions and mutual influence of *N. equitans* and *I. hospitalis*. The data shed light on a unique system which combines characteristics of symbiosis, commensalisms, and parasitism.

MATERIALS AND METHODS

Strains and culture conditions. *I. hospitalis* strain KIN4/I^T, *I. islandicus* strain Kol8^T, *I. pacificus* strain LPC33^T, *Pyrodictium occultum* strain PL19^T, *Pyrococcus furiosus* strain F1^T, and the coculture of *I. hospitalis* and *N. equitans* were routinely grown at 90°C in 120-ml serum bottles containing 20 ml of synthetic-seawater medium (0.5× SME medium [13] pressurized with H_2-CO_2 [80/20, vol/vol], 3×10^5 Pa). *Pyrobaculum arsenaticum* strain PZ6^T and *Thermoproteus* strain CU1 were cultivated at 90°C in 0.37× SME medium pressurized with

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H_2-CO_2 (80/20 [vol/vol], 3×10^5 Pa). All strains were obtained from the culture collection of the Lehrstuhl für Mikrobiologie, University of Regensburg.

Physiological tests for *I. hospitalis* and the coculture. Determination of growth at different NaCl concentrations was conducted in standard 0.5× SME medium with various NaCl concentrations (0 to 7% NaCl, 0.2% [wt/vol] steps). Different pH values (pHs 3.5 to 8, in 0.5-U steps) were adjusted with H_2SO_4 or NaOH in 0.5× SME medium without $NaHCO_3$ to avoid its buffering effect. The pH was checked before and after incubation to ensure that it remained unchanged during incubation. Temperature ranges were determined by cultivating the cells at 60 to 100°C (5°C steps).

Fermentation conditions. Standard fermentation assays for the pure culture of *I. hospitalis* and the coculture of *N. equitans* and *I. hospitalis* were conducted in 50-liter enamel-protected fermentors with a 1-liter preculture for inoculation (concentrations, 5×10^6 to 10^7 *I. hospitalis* cells ml^{-1} and about 2×10^6 to 5×10^6 *N. equitans* cells ml^{-1}) under the culture conditions described above. The coculture was purged with H_2-CO_2 (80/20 [vol/vol]) at a flow rate of 10 liters min^{-1} after the *Ignicoccus* cells had reached a density of about 10^6 cells ml^{-1}.

Light microscopy. Cells were routinely observed with an Olympus BX60 phase-contrast microscope with an oil immersion objective, UPlanF1 100/1.3, and epifluorescence equipment. Growth of *I. hospitalis* was monitored by direct cell counting using a Thoma chamber (depth, 0.02 mm). Due to the small cell diameter of *N. equitans* cells, their growth was monitored by counting the *N. equitans* cells attached to 50 *I. hospitalis* cells, as well as the free *N. equitans* cells compared to the *I. hospitalis* cells at a magnification of ×1,000. Live-dead staining was performed by using BacLight (Molecular Probes, Leiden, The Netherlands). Four-microliter culture samples were incubated with 1 μl 1:10-diluted BacLight reagent for 5 min at room temperature in the dark. The BacLight reagent employs two dyes which stain nucleic acids, i.e., SYTO 9 and propidium iodide. SYTO 9 (green fluorescent) penetrates intact and damaged (bacterial and archaeal) cell membranes. In contrast, propidium iodide (reducing the green SYTO 9 fluorescence to red) only penetrates damaged cell membranes. Thus, under UV light (excitation, 360 to 370 nm), cells with an intact membrane (living cells) exhibit a green color whereas cells with a damaged membrane (dead cells) stain red (19, 30).

Optical-tweezer experiments. From a coculture in the late exponential growth phase, single cells of *I. hospitalis* occupied by 0 to 10 *N. equitans* cells were isolated by the optical-tweezer technique (14), transferred into fresh medium, and cultivated for 4 weeks as described above. The culture vessels were checked for microbial growth by microscopy every 2 days.

Electron microscopy. Following cultivation in 0.5× SME medium inside the lumen of cellulose capillaries, cells of *I. hospitalis* with *N. equitans* were processed by high-pressure freezing, freeze substitution, and embedding in Epon as previously described (4, 26, 27). Digital electron micrographs of ultrathin sections, contrasted with uranyl acetate and lead citrate, were recorded using a slow-scan charge-coupled device camera (Tietz, Gauting, Germany) mounted on a CM12 transmission electron microscope (FEI Co., Eindhoven, The Netherlands), which was operated at 120,000 eV.

Production of ^{13}C-labeled cell masses. *I. hospitalis* cells were cultivated under autotrophic growth conditions in a 300-liter enamel-protected fermentor in the presence of 0.5 mM [1-^{13}C]acetate (Euriso-Top, Gif-sur-Yvette Cedex, France) (15). To avoid loss of acetate, the fermentation was performed without gas stripping. Cells were harvested at the end of the exponential growth phase of *I. hospitalis* to obtain distinct labeling patterns. This labeling experiment was repeated with the coculture of *I. hospitalis* and *N. equitans*, with the following modifications. To obtain maximal cell masses of *N. equitans*, the fermentor was gassed for 10 h after inoculation (10 liters min^{-1}) and the cells were harvested in the stationary growth phase of *I. hospitalis*.

Separation of free *N. equitans* cells. Free *N. equitans* cells were separated from *I. hospitalis* cells from the coculture by differential centrifugation (12).

Fractionation of cell material and separation of amino acids. Cells (wet weights: *I. hospitalis*, 3 g; *N. equitans*, 1 g) were fractionated, and isolated proteins were hydrolyzed as described by Jahn et al. (15). Amino acids were isolated by chromatographic procedures published earlier (9).

NMR spectroscopy. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 500.13 and 125.76 MHz, respectively, with a DRX500 spectrometer (Bruker Biospin, Rheinstetten, Germany) as previously described (8, 15). Tyr and Asp were dissolved in D_2O containing 0.1 M NaOD (pH 13); the other amino acids were dissolved in D_2O containing 0.1 M DCl (pH 1). ^{13}C enrichments were determined for individual positions by quantitative NMR spectroscopy (8).

Cross infection experiments. To test the possibility of serving as alternative host organisms for *N. equitans*, *I. islandicus* strain Kol8^T, *I. pacificus* strain LPC33^T, *Pyrodictium occultum* strain PL19^T, and *Pyrococcus furiosus* strain F1^T were mixed with the coculture of *I. hospitalis* and *N. equitans* in 0.5× SME medium (13) pressurized with H_2-CO_2 (80/20 [vol/vol], 3×10^5 Pa). *Pyrobaculum arsenaticum* strain PZ6^T and *Thermoproteus* strain CU1, which do not grow in 0.5× SME medium, were cocultivated with *I. hospitalis* and *N. equitans* in 0.37× SME medium. Several dilutions were used for each of the possible host organism to obtain cocultures with various proportions of *I. hospitalis* (with attached *N. equitans* cells) and the applied archaeal species. All cross infection experiments were carried out at 90°C.

Alternatively, infection experiments were carried out with purified *N. equitans* cells obtained from fermentations or from serum bottles. Such *N. equitans* cells were purified by separation from *I. hospitalis* cells by filtration (pore size, 0.4 μm), by Percoll or sucrose density gradients, or by differential centrifugation. To enforce infectiousness of the purified *N. equitans* cells, the medium with the tested host organism was supplemented with, e.g., 0.1% yeast extract, peptone, meat extract, *I. hospitalis* extract, supernatant of a grown *I. hospitalis* culture, 1 to 100 mM magnesium ions, or 0.25 to 5% carboxymethyl cellulose (to enhance the viscosity of the medium). Alternatively, the addition of the purified *N. equitans* cells to the potential hosts occurred in different growth phases of these organisms and with different concentrations of host and *N. equitans* cells.

16S rRNA gene sequence analysis. DNA was prepared as previously described (21). Nearly complete 16S rRNA gene sequences were amplified by PCR using a standard protocol (7) and the following primer combinations. To screen for the presence of *N. equitans*, primer combinations 7mcF-1511mcR, 7mcF-1116mcR, 518mcF-1511mcR, and 518mcF-1116mcR were used (11). The archaeon-specific primer combination 8aF-1512uR (7) served as a positive control for these assays and for obtaining the 16S rRNA gene sequences of *Ignicoccus* subcultures. The PCR products were sequenced and compared to the 16S rRNA gene sequences of *N. equitans* or the three *Ignicoccus* species, respectively.

RESULTS

Effects of pH, temperature, and salt concentration. The temperature, pH, and salt concentration ranges and optima for the growth of *I. hospitalis* were determined (Table 1). No differences were found between cells grown in coculture with *N. equitans* and those cultivated in pure culture. Under optimal growth conditions (90°C, pH 5.5 to 6.0, 1.4% NaCl), *I. hospitalis* reached maximum concentrations of about 1×10^7 to 3×10^7 cells ml^{-1} and shortest doubling times of 50 to 60 min with or without *N. equitans*. Quite similar growth ranges (and optima) were obtained for *N. equitans* (Table 1), although for pH and salt tolerance smaller growth ranges occurred. Since *N. equitans* is strictly dependent on growing *I. hospitalis* cells, the possibility of an extended temperature range for *N. equitans* cannot be excluded.

Growth characteristics of the coculture of *I. hospitalis* and *N. equitans* under standard cultivation conditions in fermentors. Usually, the preculture for growth experiments in 50-liter fermentors consisted of about 5×10^6 *I. hospitalis* cells ml^{-1} with 20 to 40% of these cells being occupied by *N. equitans* (average, two to three *N. equitans* cells per occupied *I. hospitalis* cell). At a density of about 10^6 *I. hospitalis* cells ml^{-1}, usually after 8 h of incubation, a gassing rate of 10 liters min^{-1} H_2-

TABLE 1. Ranges (optima) of temperature, pH, and salt concentration for *I. hospitalis* (24) and the coculture of *I. hospitalis* and *N. equitans*

Cells	Temp (°C)	pH	NaCl concn (%)
I. hospitalis	75–98 (90)	4.5–7.5 (5.5)	0.2–5.5 (1.4)
Coculture			
I. hospitalis host	75–98 (90)	4.5–7.5 (5.5)	0.2–5.5 (1.4)
N. equitans	75–98 (85–90)	5–6.5 (5.5)	0.9–3.5 (1.4)

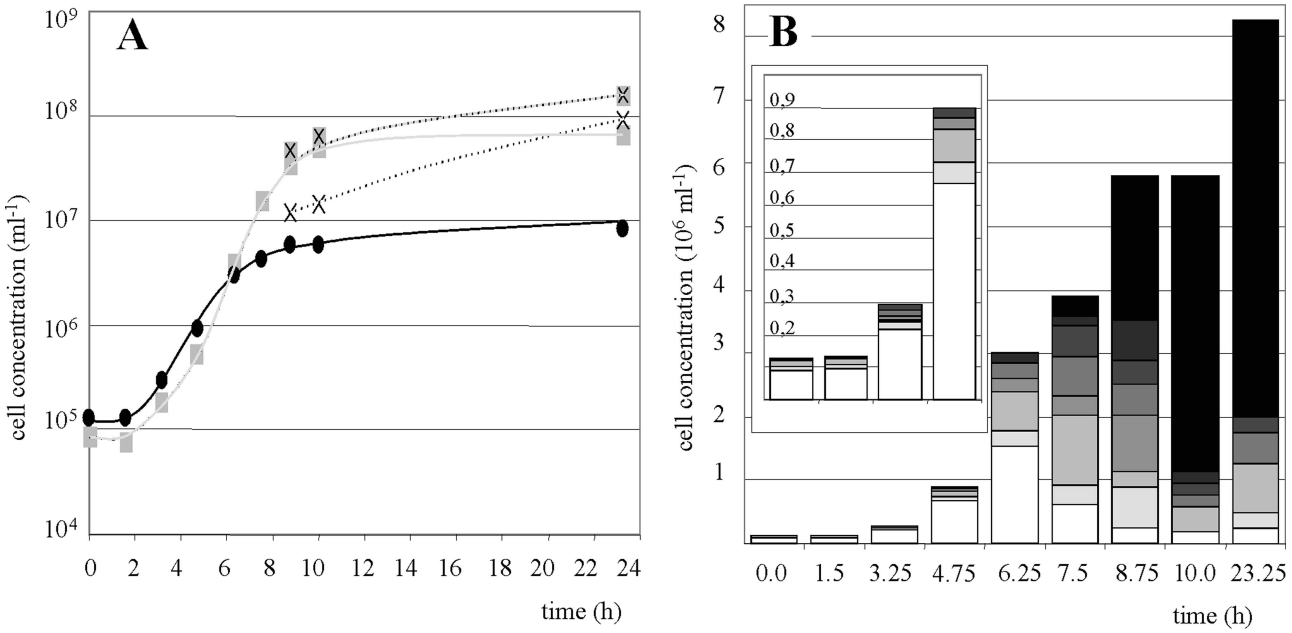

FIG. 1. Growth of the coculture in the fermentor. In the preculture, around 30% of the *I. hospitalis* cells were occupied by *N. equitans* cells (two to four per *Ignicoccus* cell). (A) Growth curves. *I. hospitalis* cells, ●; total *N. equitans* cells, shaded ×; attached *N. equitans* cells, plain shaded square; free *N. equitans* cells, unshaded ×. (B) Numbers of *N. equitans* cells (ranging from 0 to 10) attached to cells of *I. hospitalis* are represented by no shading ($n = 0$) increasing to the darkest shading ($n = 10$). The insert is an enlarged view of the cell concentrations within the first 5 h after inoculation.

CO_2 (80/20 [vol/vol]) was applied. Figure 1A shows the growth curves of the *I. hospitalis* cells, as well as the numbers of attached, free, and total *N. equitans* cells. Typically, a lag phase of about 90 min occurred for both organisms. In the exponential growth phase, only a minor portion of the *I. hospitalis* cells (20 to 30%) was occupied by *N. equitans* (one to three *N. equitans* cells per *I. hospitalis* cell, Fig. 1B, 0 to 4.75 h). At concentrations of 2×10^6 to 5×10^6 cells ml^{-1}, *I. hospitalis* slowly transited into the stationary growth phase. Interestingly, *N. equitans* showed different growth characteristics. During the exponential growth of *I. hospitalis*, the growth curve of *N. equitans* paralleled that of its host. When *I. hospitalis* transited into the stationary growth phase, *N. equitans* exhibited increased cell proliferation (doubling time, 45 min) for a further 4 h. As a consequence, the number of *N. equitans* cells per occupied *I. hospitalis* cell, as well as the number of occupied *I. hospitalis* cells in general, significantly increased (Fig. 1B, 6.25 to 8.75 h). At the end of the stationary growth phase, more than 80% of the *I. hospitalis* cells were covered with at least 10 *N. equitans* cells (Fig. 1B, 10 and 32.25 h). In addition, the strong gas stripping yielded large amounts of free *N. equitans* cells emerging in the stationary growth phase of *I. hospitalis*. The number of attached *N. equitans* cells did not decrease during the formation of the free cells, resulting in a significantly increased total number of *N. equitans* cells. Finally, attached *N. equitans* cells reached a concentration of about 10^8 cells ml^{-1} and free *N. equitans* cells reached a concentration of about 1×10^8 to 2.5×10^8 cells ml^{-1}.

Influence of the gassing rate on the occurrence of free *N. equitans* cells. In contrast to the standard cultivation experiment (10 liters min^{-1} H_2-CO_2, 80/20 [vol/vol]), a reduced gassing rate of only 1 liter min^{-1} H_2-CO_2 (80/20 [vol/vol]) resulted in a 10-fold smaller amount of free *N. equitans* cells while the concentration of *I. hospitalis* cells and attached *N. equitans* cells remained unchanged. Detachment of *N. equitans* cells from their host cells due to turbulence alone appears to be insufficient to explain the large amounts of free *N. equitans* cells at high gassing rates. An explanation might be the increased availability of molecular hydrogen or CO_2. To test this assumption, the concentrations of both gases were reduced during gas stripping while simultaneously the amount of N_2 was increased. Finally, the fermentors were gassed with a mixture of N_2-H_2-CO_2 (95/3/2 [vol/vol]) with high gassing rates (10 liters min^{-1}) resulting in an identical CO_2 supply and an even lower hydrogen supply, compared to the low gassing rates (1 liter min^{-1}) obtained with H_2-CO_2 (80/20 [vol/vol]). Interestingly, the same amounts of free *N. equitans* cells were obtained as in the cultivations with high H_2-CO_2 gassing rates (80/20 [vol/vol], 10 liters min^{-1}). Furthermore, no effects on the growth rates and final yields of *I. hospitalis* cells and attached *N. equitans* cells were detectable for all of the variations of gassing rates and gas compositions applied in these experiments. This result demonstrates that an excess supply of H_2 or CO_2 does not cause an increased amount of free *N. equitans* cells. In contrast, it strongly indicates that the removal of H_2S, the major metabolic end product of *I. hospitalis*, gives rise to this enhanced proliferation.

Ratio of dead to living *I. hospitalis* and *N. equitans* cells during the fermentation process. As expected, during the exponential growth phase of *I. hospitalis*, the amount of living (green) cells was at least 10 times larger than the amount of dead (red) cells (Fig. 2A). After *I. hospitalis* entered the stationary growth phase (Fig. 2B), the number of living cells slowly began to decrease. However, cell proliferation of *N.*

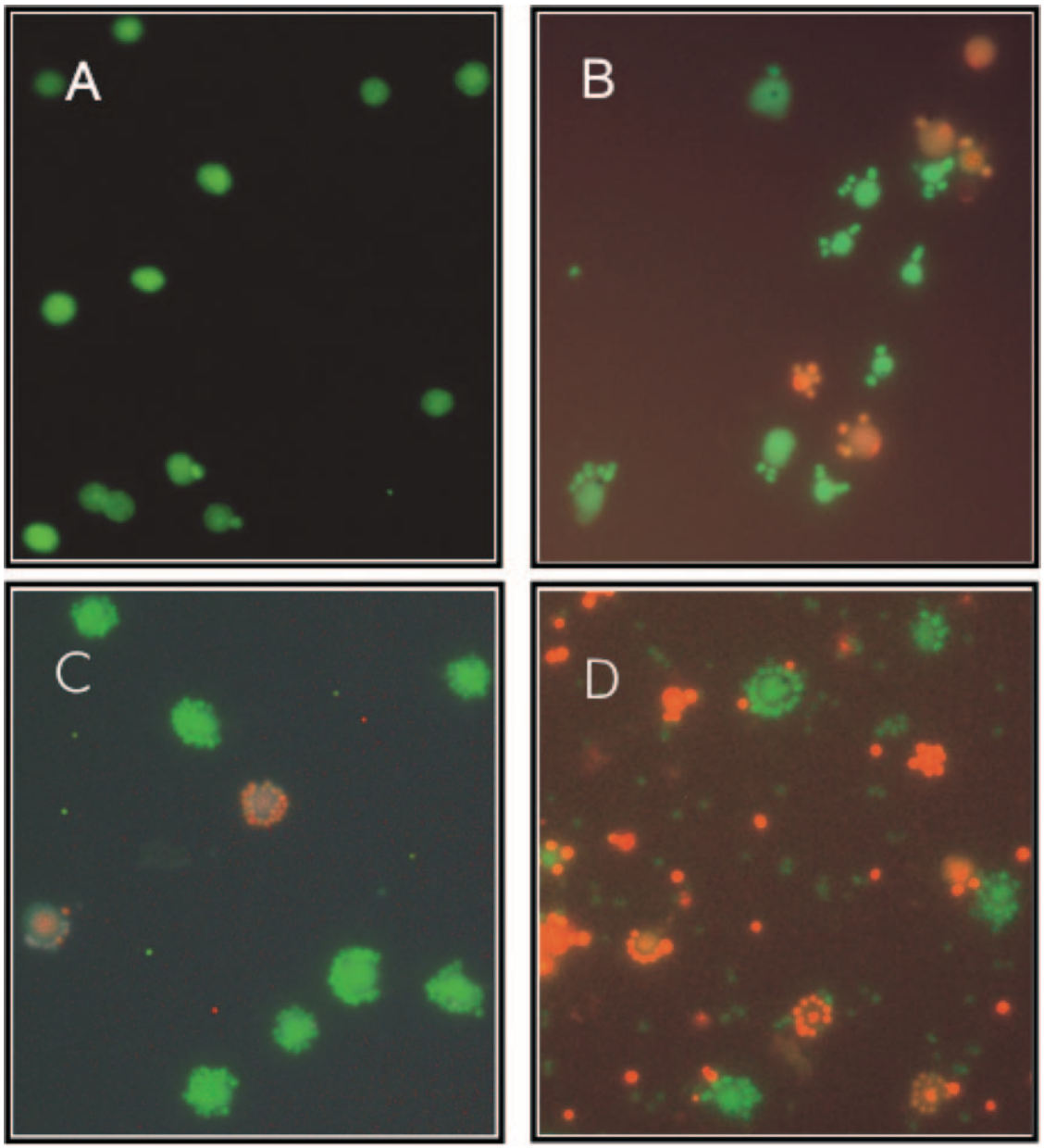

FIG. 2. Epifluorescence microphotographs of the coculture at different growth phases after staining with BacLight. Dead cells stained red, and living cells stained green. (A) At 3.25 h after inoculation, exponential growth phase. (B) At 7.5 h after inoculation, transition into the stationary phase. (C) At 10 h after inoculation, stationary phase. (D) At 23 h after inoculation, stationary phase.

equitans continued and about 3 h later, nearly all of the *I. hospitalis* cells were densely covered with *N. equitans* cells (Fig. 2C). At the end of the stationary growth phase of *I. hospitalis*, a ratio of living to dead cells of about 1:1 could be seen (Fig. 2D). To our surprise, *N. equitans* cells attached to an *I. hospitalis* cell always showed the same BacLight staining as their host cell (Fig. 2A to D), demonstrating again a strong dependence and interaction of the two partners. About 40 to 60% of the free *N. equitans* cells stained green, suggesting that they do not immediately lose their viability after detaching from the host cells. Further experiments with *I. hospitalis* grown in pure culture revealed that, at all phases of growth the ratios of living to dead cells were identical with *I. hospitalis* grown in the coculture with *N. equitans* (data not shown).

Inhibiting effects of *N. equitans* on *I. hospitalis*. (i) Fermentation experiments. In contrast to standard fermentation experiments, cultivations were carried out with atypical precultures in which the majority (more than 90%) of the *I. hospitalis* cells was occupied by at least 10 *N. equitans* cells. This resulted in significant differences in the growth curves (Fig. 3A and B). The lag phase of *I. hospitalis* seemed to be extended (6 h instead of 1.5 h in the standard cultivation experiment). However, Fig. 3B demonstrates that this effect was caused by the inability of occupied *I. hospitalis* cells to divide; only *I. hospi-*

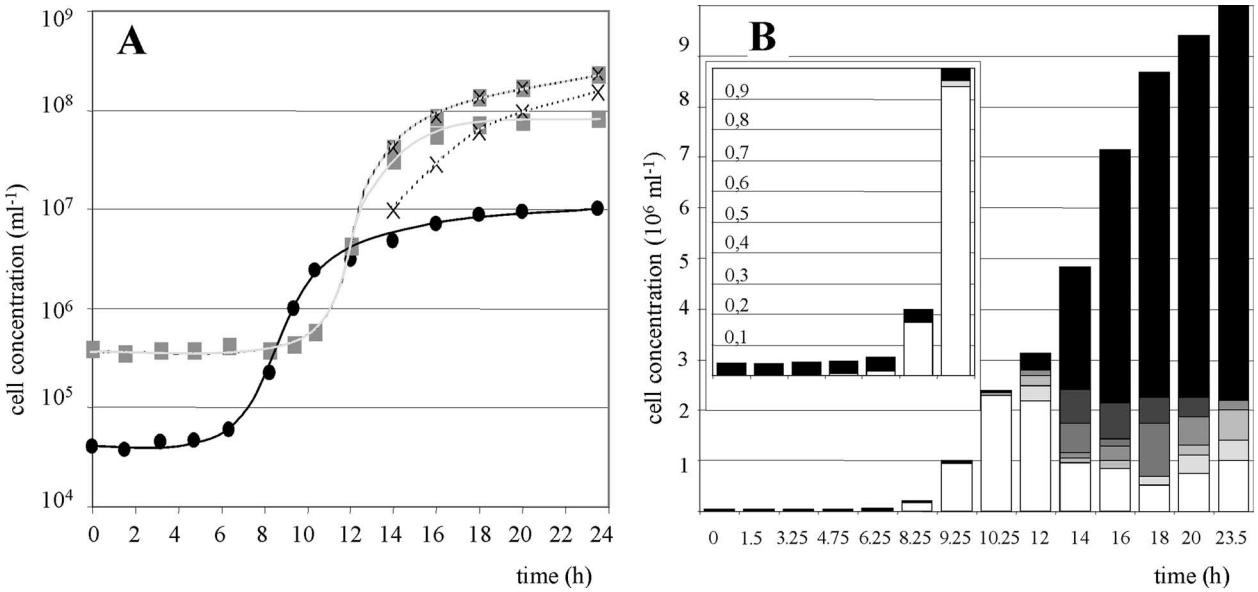

FIG. 3. Growth of the coculture in the fermentor. In the preculture, nearly all *I. hospitalis* cells were occupied by at least 10 *N. equitans* cells. (A) Growth curves. *I. hospitalis* cells, ●; total *N. equitans* cells, shaded ×; attached *N. equitans* cells, plain shaded square; free *N. equitans* cells, unshaded ×. (B) Numbers of *N. equitans* cells (ranging from 0 to 10) attached to cells of *I. hospitalis* are represented by no shading ($n = 0$) increasing to the darkest shading ($n = 10$). The insert is an enlarged view of the cell concentrations within the first 10 h after inoculation.

talis cells without *N. equitans* cells proliferated. Simultaneously, the number of highly occupied *I. hospitalis* cells remained unchanged until 10.25 h after inoculation. Since in the beginning the number of nonoccupied *I. hospitalis* cells was too low to have an apparent influence on the total number of *I. hospitalis* cells, this gave the impression of an extended lag phase. No actual differences in the doubling time for dividing *I. hospitalis* cells compared to the standard cultivation could be detected.

In contrast, *N. equitans* cells exhibited a true extended lag phase. They did not proliferate at all until the *I. hospitalis* cells reached the stationary growth phase. Then they grew exponentially with a doubling time of 45 min up to the same final cell densities as in the standard culture (Fig. 3A).

(ii) Optical-tweezer experiments. The fermentation experiments led to the impression that only free or weakly occupied *I. hospitalis* cells are able to divide. To test this assumption, *I. hospitalis* cells with 0, 1, 2, 3, 4, 5, 6, or 10 attached *N. equitans* cells were isolated with the optical tweezers and incubated in fresh medium (10 replicates each; incubation time, 3 weeks). A cell able to reproduce resulted in a culture with about 1×10^7 cells ml^{-1} within 1.5 days. As shown in Table 2, occupation by *N. equitans* clearly inhibited the proliferation of *I. hospitalis* cells. Compared to *N. equitans*-free *I. hospitalis* cells, cells occupied by one or two *N. equitans* cells showed a reduced ability to proliferate (60 to 30%). No *I. hospitalis* cell with more than two attached *N. equitans* cells was able to proliferate and form a subculture. Furthermore, only one out of the six subcultures that evolved from occupied cells formed a coculture of *N. equitans* and *I. hospitalis*. This observation was confirmed by PCR experiments with primers specific for the 16S rRNA gene of *N. equitans*. With the exception of the latter culture, no PCR amplification product of *N. equitans* was discovered in the remaining five subcultures.

Electron microscopy. In ultrathin sections (Fig. 4), cells of *I. hospitalis* and *N. equitans* showed the typical structural features already described (12, 13, 24, 26). The *N. equitans* cell is a small coccus, 0.4 μm in diameter, surrounded by a continuous sheet of an S layer, while the *I. hospitalis* cell is a large coccus, about 2.5 μm in diameter, surrounded by an irregular cytoplasmic membrane, a periplasmic space (30 to 400 nm in width in uninfected cells), and an outer membrane. Figure 4A and B revealed that at the attachment site of *N. equitans*, the cytoplasmic membrane and the outer membrane of *I. hospitalis* cells were in close contact (Fig. 4, white arrows). The periplasmic space was atypically narrow (<30 nm) and did not contain any of the periplasmic vesicles found in other parts of the same cell (Fig. 4B) and in all intact *Ignicoccus* cells (i.e., in pure culture and not lysed, with an intact cell envelope; 24, 26). Between the cells, there was a gap, approximately 20 to 50 nm

TABLE 2. Numbers of *I. hospitalis* cultures and *I. hospitalis* and *N. equitans* cocultures that developed from 10 individual separation experiments using the optical tweezers[a]

No. of attached *N. equitans* cells/separated *I. hospitalis* cell	No. of cultures grown from 10 separated *I. hospitalis* cells	No. of isolates that remained cocultures
0	6	
1	3	0
2	3	1
3	0	0
4	0	0
5	0	0
6	0	0
10	0	0

[a] The *I. hospitalis* cells were occupied with 0 to 10 *N. equitans* cells.

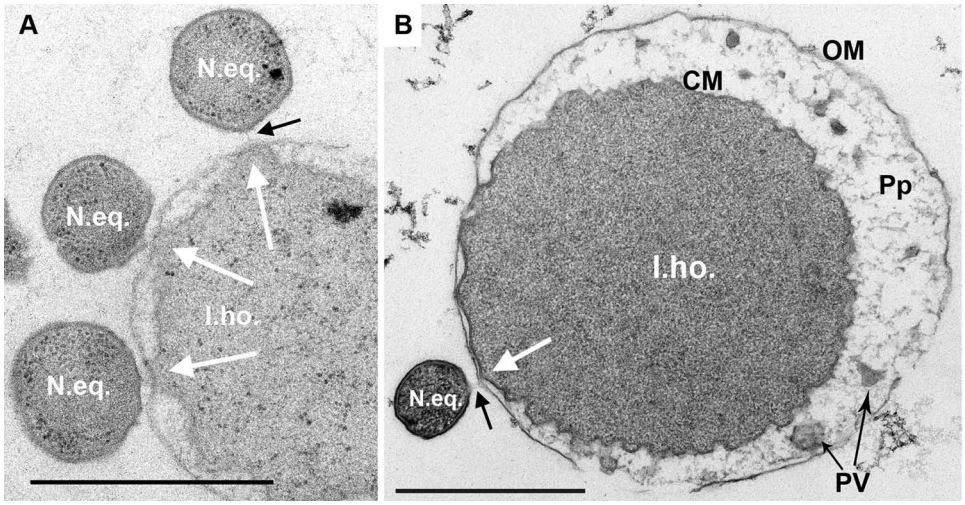

FIG. 4. Transmission electron micrographs of ultrathin sections of *I. hospitalis* and *N. equitans* following cryoprocessing as described in Materials and Methods. I.ho., *I. hospitalis* cell; CM, cytoplasmic membrane; OM, outer membrane; Pp, periplasm; PV, periplasmic vesicles; N.eq., *N. equitans* cell. White arrows point to the contact site where the *I. hospitalis* outer membrane is in close contact with the cytoplasmic membrane. Black arrows, fibrous material in the gap between the two cells. Bars, 1 μm.

wide, filled with an amorphous, fibrous biopolymer of unknown composition (black arrows).

Amino acid transfer from *I. hospitalis* to *N. equitans*. To investigate a possible amino acid transfer between *I. hospitalis* and *N. equitans*, long-term ^{13}C-labeling studies were performed by using a coculture grown in the presence of 0.5 mM [1-^{13}C]acetate. Free *N. equitans* cells were separated from *I. hospitalis* cells by differential centrifugation (*I. hospitalis* contamination, <0.1%). The cells were hydrolyzed under acidic conditions, and the amino acids were separated by chromatography. The incorporation of ^{13}C at specific carbon positions of amino acids was identified by ^{13}C NMR spectroscopy (Table 3).

The labeling patterns of all amino acids under study from *N. equitans* and the coculture were apparently identical (Table 3). Notably, the qualitative labeling patterns were also identical to the patterns observed in a labeling experiment where a pure culture of *I. hospitalis* was supplied with [1-^{13}C]acetate (15). In light of missing genes for enzymes involved in the biosynthesis of amino acids in *N. equitans* (31), it can be assumed that labeled amino acids made de novo from [1-^{13}C]acetate in *I. hospitalis* were transferred to *N. equitans*. In the case of any de novo formation of amino acids by *N. equitans*, we would have expected significant changes in the labeling patterns of the coculture and the *I. hospitalis* pure culture compared to the *N. equitans* fractions. The significantly increased ^{13}C enrichment of amino acids in the pure culture of *I. hospitalis* in comparison to amino acids from the coculture and the purified *N. equitans* cells can be explained easily by the different cultivation conditions in the two labeling experiments (see Materials and Methods; the high gassing rate necessary for the cultivation of the coculture leads to expulsion of the labeled acetate, which is quite volatile at 90°C and pH 5.5).

Cross infection studies. An important characteristic of many parasite-symbiont interactions is their host specificity. Therefore, a variety of archaea that grow under conditions identical or similar to those of *I. hospitalis* were tested as alternative

TABLE 3. ^{13}C enrichments at individual carbon positions of amino acids from *I. hospitalis* (15), from mixed cells of the coculture of *I. hospitalis* and *N. equitans*, and from purified *N. equitans* cells after cultivation in the presence of [1-^{13}C]acetate[a]

Amino acid	Position	Relative ^{13}C concn (%)		
		I. hospitalis	Coculture	*N. equitans*
Alanine	1	ND[b]	1.1	ND
	2	**16.8**	**3.3**	**3.1**
	3	3.3	1.2	1.1
Serine	1	ND	ND	ND
	2	**15.3**	**2.3**	**3.8**
	3	3.3	1.1	1.1
Aspartate	1	1.1	ND	ND
	2	**14.5**	**3.6**	**4.3**
	3	4.6	1.1	1.1
	4	1.1	ND	ND
Threonine	1	ND	1.7	ND
	2	**14.0**	**4.5**	**7.8**
	3	3.3	1.9	2.2
	4	1.1	1.1	1.1
Glutamate	**1**	**20.0**	**8.7**	**++**[c]
	2	3.3	1.9	1.1
	3	**16.9**	**8.2**	**7.8**
	4	5.7	2.3	1.8
	5	1.1	1.1	ND
Lysine	**1**	**22.6**	**5.6**	**10.7**
	2	76	2.2	2.8
	3	6.9	1.8	1.8
	4	**26.3**	**4.2**	**6.7**
	5	7.7	1.5	1.7
	6	1.1	1.1	1.1

[a] The values shown were determined from a single experiment by quantitative nuclear magnetic resonance spectroscopy. On this basis, we could not determine error margins. However, by comparison with other studies using quantitative nuclear magnetic resonance spectroscopy, the errors can be estimated to be around ±25% of the listed values. Due to possible different relaxation behaviors of the reference (i.e., unlabeled material) and the bioenriched samples, the errors can be even higher for quaternary carbon atoms (for example, C-1 of lysine). Strong ^{13}C enrichments are in boldface.

[b] ND, not detected.

[c] ++, high signal intensity.

hosts. Despite numerous and highly diverse assays (see Materials and Methods), it was not possible to reinfect *I. hospitalis* (and other archaeal) cells with separated *N. equitans* cells. Assuming the possibility that separated *N. equitans* cells lose their infectiousness, cross infection studies with the coculture and the alternative hosts were carried out. The following organisms were all able to grow in the presence of the *I. hospitalis-N. equitans* coculture without one species being suppressed: *Pyrodictium occultum*, *Pyrococcus furiosus*, *Pyrobaculum arsenaticum*, *Thermoproteus* sp. strain CU1/L1B, *I. pacificus*, and *I. islandicus*. Due to their characteristic morphology (with the exception of the *Ignicoccus* species), it was possible to distinguish between *I. hospitalis* and the other archaea by light microscopy. In all cross cultivation experiments, no *N. equitans* cell was found to be attached to any of the alternative hosts. To distinguish between *I. hospitalis* and *I. pacificus-I. islandicus* in the cross infection experiment, single cells occupied by one *N. equitans* cell were isolated via optical tweezers and subcultivated and the 16S rRNA sequences of the cultures were determined (two times six subcultures). All subcultures exhibited the 16S rRNA gene of *I. hospitalis*.

DISCUSSION

The physiological experiments described here demonstrate that major growth parameters of the *I. hospitalis* culture are not influenced by the presence of *N. equitans*. No differences between the pure culture and the coculture were detected concerning pH, temperature, or salt ranges; shortest doubling times; or final cell densities. Under standard cultivation conditions, the presence of *N. equitans* was neither beneficial nor harmful to the entirety of the *I. hospitalis* cells in the coculture, although the situation in the natural habitat might be different. Closer examination of the coculture under laboratory conditions revealed a highly differentiated picture of the mutual interactions. Densely occupied *I. hospitalis* cells are no longer able to proliferate, and in isolation experiments even one attached *N. equitans* cell significantly reduced the ability of *I. hospitalis* to reproduce. Comparative lipid analyses suggested that *N. equitans* derives its membrane lipids from *I. hospitalis* (16). Long-term labeling studies with [1-^{13}C]acetate exhibited identical ^{13}C-labeling patterns of amino acids from *N. equitans* and *I. hospitalis* cells, suggesting that *N. equitans* also obtains amino acids from its host. Both results are consistent with the genome analysis of *N. equitans* where very few genes for biosynthetic pathways like lipid or amino acid biosynthesis were identified (31). It is obvious that such a drain of cellular components must have some negative influence on the viability and/or proliferation of *I. hospitalis*. Although so far there is no direct evidence of how this transfer proceeds, the data presented here contradict the vesicle-based transport assumed earlier (31). The ultrathin sections showed that, at the contact site of *N. equitans* and *I. hospitalis*, the cytoplasmic membrane of the *I. hospitalis* cell is very close to its outer membrane (and therefore close to the *N. equitans* cell) without keeping the large periplasmic space between the inner and outer membranes typical of pure cultures of *Ignicoccus* (26). At the moment, one can only speculate about some sort of direct connection of the cytoplasms of the two organisms. However, this is in line with the results obtained by staining with BacLight, where attached *N. equitans* cells always exhibit the same color (= physiological status) as their host cell. In any case, our results rule out the assumption that *N. equitans* immediately kills its host cells and/or feeds on dead *I. hospitalis* cells, as is the case for some parasitic bacteria (e.g., *Bdellovibrio*, *Bacteriovorax*) (1, 29).

We showed that the increase in the total number of *N. equitans* cells caused by the large increase in free *N. equitans* cells after heavy gassing of the fermentor (12) is independent of the amount of molecular hydrogen (between 80 and 3% [vol/vol]) and/or CO_2 (between 20 and 2% [vol/vol]) in the gas mixture. Thus, the increase in the total number of *N. equitans* cells can be ascribed mainly to the stripping of H_2S, the major metabolic end product of *I. hospitalis* (24).

The presence of free and attached *N. equitans* cells in the coculture raises the question of their different functions. Attached *N. equitans* cells are obviously responsible for proliferation, an assumption which is supported by the fact that *N. equitans* cannot be cultivated without contact with living cells of *I. hospitalis* (12, 13). Similar to free cells of bacterial parasites like *Bdellovibrio* or *Micavibrio* (17, 29), the free cells of *N. equitans* could represent the infectious form. According to the BacLight staining experiments, they seem to be alive at least for a while after separation. In addition, it has been shown that *N. equitans* cells express extracellular appendages (13). Whether they are used for motility, attachment, or both (23) remains to be investigated. De novo infection of unoccupied *I. hospitalis* cells, probably by free *N. equitans* cells, indeed occurs in the coculture (Fig. 1 and 2A to C). So then why is it not possible to infect pure cultures of *I. hospitalis* with purified free *N. equitans* cells? One explanation could be that the free *N. equitans* cells are damaged during the purification process and therefore lose their infectiousness. Alternatively, one could speculate that *N. equitans* can only infect new host cells as long as they are associated with the "original" *Ignicoccus* cell, which would simultaneously imply a close proximity of the two *I. hospitalis* host cells. In the natural environment (e.g., in the sediment), such contacts may occur more often; there are data showing that a lot of (hyperthermophilic) organisms grow not only planktonically but also attached to several kinds of surfaces (23).

No infections of alternative host organisms could be obtained in cocultures. This result resembles the narrow host specificity of the obligate bacterial exoparasites *Vampirovibrio chlorellavorus* (host, *Chlorella* sp. [10]) or "*Micavibrio aeruginosavorus*" (host, *Pseudomonas aeruginosa* [18]). Common features of the bacterial exoparasites and *N. equitans* are adherence to the cell wall of the host, exploitation of the host cell leading to limited proliferation of the host, and obligate dependence on the presence of the host for proliferation. However, the fact that *I. hospitalis* and *N. equitans* build a stable coculture without sustained damage to the *Ignicoccus* population clearly distinguishes *N. equitans* from the bacterial parasites mentioned above. Therefore, we propose that the association of *I. hospitalis* and *N. equitans* represents a highly specialized system. Consequently, assignment to the classical category of symbiosis, commensalism, or parasitism might not be possible even in the future.

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