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New facultative lithoautotrophic nitrite-oxidizing bacteria

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Abstract. A nitrite-oxidizing bacterium was isolated from soil of the Old Botanic Garden in Hamburg and of a cornfield near Uxmal in Yucatan (Mexico). The cells are 0.5–0.8 × 1.2–2.0 µm in size, pleomorphic, mostly pear-shaped and motile by means of one subpolar to lateral flagellum. Intracytoplasmic membranes are present as caps of flattened vesicles or membrane vesicles in the central region of the cell. The organism grows best mixotrophically with a doubling time of 10 h to 18 h. The growth rate under heterotrophic conditions is slower than under mixotrophic conditions but higher than under lithoautotrophic conditions. The DNA G + C content of the DNAs of strain X14 and strain Y is 61.2 mol% and 61.6 mol%, respectively. Both strains share 100% DNA homology. *Nitrobacter winogradskyi* is moderately related only to the two isolates, showing an average DNA homology value of 36%. The phenotypic and genotypic differences from *N. winogradskyi* support evidence that more than one species of *Nitrobacter* exists. For *Nitrobacter X14* and *Nitrobacter Y* the name *N. hamburgensis* is proposed.

Key words: New *Nitrobacter*, better heterotrophic than lithoautotrophic growth – Morphology – Physiology – Taxonomy

The taxonomy of the nitrite oxidizing bacteria is based on their shape, size and arrangement of intracytoplasmic membranes (Watson and Waterburry 1971; Watson et al. 1981). *N. winogradskyi* was established as the only species of the genus (Winogradsky 1892, Watson et al. 1981). The former species *N. agilis* was reassigned to the species *N. winogradskyi* after investigation of morphology, ultrastructure and G + C content (Watson and Mandel 1971). Immunological studies supported this conclusion (Kalthoff et al. 1979).

For many years, nitrifying organisms of the genus *Nitrobacter* were regarded as obligate lithoautotrophs. However, Smith and Hoare (1968), Bock (1976) and Kalthoff et al. (1979) demonstrated that some *Nitrobacter* strains are able to grow heterotrophically as well. This study describes two facultative lithoautotrophic strains of *Nitrobacter* which – in contrast to *N. winogradskyi* – grow better heterotrophically than autotrophically. In former studies one of these strains was designed *Nitrobacter X14* (Sundermeyer and Bock 1981; Seewaldt et al. 1982).

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Materials and methods

Isolation. Nitrobacter X14 was isolated from soil of the Old Botanic Garden in Hamburg (Sundermeyer and Bock 1981) and *Nitrobacter Y* from soil of a cornfield near Uxmal in Yucatan (Mexico). The cells were grown at 30°C in an enrichment medium containing mineral salts (see below) and 2 g $NaNO_2$/l (*Nitrobacter X14*) or 2 g $NaNO_2$/l + 0.015 g peptone/l (*Nitrobacter Y*). When the nitrite was oxidized fresh nitrite medium was supplied with a 5% (v/v) inoculum. After the 3rd passage when the nitrite was consumed, serial dilutions of the culture were transferred to fresh media. Pure cultures of *Nitrobacter X14* were obtained by serial dilution in mineral nitrite medium. *Nitrobacter Y* could not be isolated by the technique described. This nitrite oxidizing organism only grew in the presence of a heterotrophic contaminant which was identified as *Geodermatophilus*. The contaminant was not able to survive starvation which is typical for *Nitrobacter* (Bock 1972). Therefore the isolation of *Nitrobacter* was started after a resting period of two months. Serial dilution in mixotrophic medium led to pure cultures of *Nitrobacter Y*. Cultures were checked for heterotrophic contaminants according to Steinmüller and Bock (1976). In addition, cells were examined routinously by phase contrast and electron microscopy.

Culture conditions. Cells were grown in the following media: The basal mineral salt medium contained in 1 l water: 0.5 g NaCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.15 g KH_2PO_4, 0.003 g $CaCO_3$, 0.05 mg $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$, and 0.15 mg $FeSO_4 \cdot 7H_2O$. Depending upon the growth conditions the following compounds were added to 1 l of the basal medium: For autotrophic growth 2 g $NaNO_2$; for mixotrophic growth 2 g $NaNO_2$, 0.55 g Na-pyruvate, 1.5 g yeast extract (Difco, Detroit, MI, USA), and 1.5 g peptone (Difco); for heterotrophic growth (i) 0.55 g Na-pyruvate or 0.41 g Na-acetate or 1.4 g glycerol plus 1.5 g yeast extract (Difco), and 1.5 g peptone (Difco); (ii) 0.55 g Na-pyruvate plus 1.0 g vitamin-free Casamino acids (Difco). After sterilization the pH values of the media were 7.4–7.6. Stock cultures were grown under mixotrophic conditions and incubated at 28°C.

Electron microscopy. The electron microscopical methods were those described by Bock and Heinrich (1969). For the preparation of flagella mixotrophically grown cells were air dried and shadowed with cromium at an angle of 30°.

Isolation of DNA. 3 g of wet weight cells were suspended in 5 ml of a 1 M NaCl solution containing 0.3 mg proteinase K

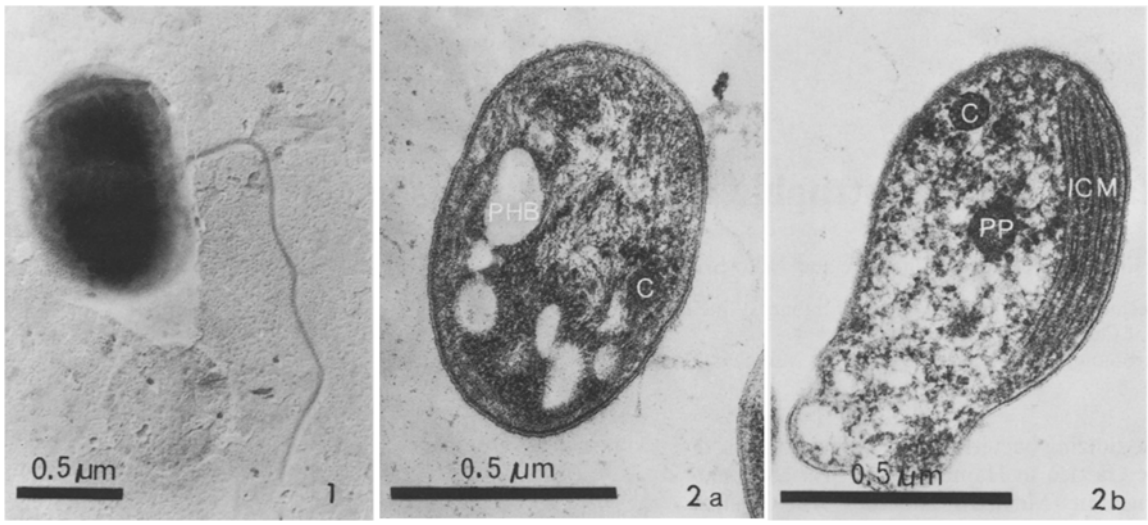

Fig. 1. Electron micrograph, showing a lateral inserted flagellum of *Nitrobacter hamburgensis* strain X14 (× 30,000)

Fig. 2a, b. Electron micrograph of thin sections of *Nitrobacter hamburgensis* (× 50,000). *ICM*, intracytoplasmic membranes; *C*, carboxysomes; *PHB*, poly-β-hydroxybutyrate granules; *PP*, polyphosphate granules; **a** strain *Nitrobacter Y*; **b** strain *Nitrobacter X14*

(Boehringer, Mannheim, Corp., New York, NY, USA). 0.2 ml of a 25% dodecylsulfate (SDS) solution were added to the cell suspension and immediately the cells were broken by passing them through a French pressure cell at 0.7 GPa. 10 ml of a 1 M NaCl and 2 ml of 25% SDS were added to the lysate which was then incubated for 10 min at 60° C. The procedures used in the isolation and purification of DNA have been described (Stackebrandt and Kandler 1979).

Determination of the G + C content of the DNA. The DNA G + C content was calculated from its thermal denaturation temperature. The melting profiles of DNA were determined in a microprocessor controlled recording spectrophotometer with an attached temperature programmer (Gilford Instruments Laboratories, Inc., modell 2600). The midpoint of the thermal denaturation Tm was determined as the maximum of the first derivative denaturation profile. DNA base composition was calculated according to De Ley (1970).

DNA-DNA renaturation. DNA was dialyzed against 2 SSC for 36 h at 30° C. DNA was sheared by passing the solution 3 times through a French pressure cell at 1.5 GPa, and the fragmented DNA was then centrifuged for 2 min at 10,000 × *g* and adjusted to E_{260} of 1.0. The determination of the renaturation rate was also done with the Gilford photometer. The four cuvettes contained respectively, 2 SSC as a blank, DNA solution of strain A, equal volume of DNA of strain B and a mixture of equal volumes of DNA of both strains. The reassociation reaction was started by quickly cooling to the optimal renaturation temperature (Gillis et al. 1970). Calculating from an average DNA G + C content of 61.2 mol% the optimal renaturation temperature was 78.1° C. The absorbance at 260 nm was measured for 30 min and the renaturation rates were determined according to De Ley et al. (1970).

Results and discussion

The nitrite oxidizing bacteria of the genera *Nitrobacter*, *Nitrococcus* and *Nitrospira* are classified mainly on the basis of morphological characteristics. Each genus has only one species (Watson et al. 1981). We support the existence of a new species of the genus *Nitrobacter* and propose that the strains until now designated *Nitrobacter X14* and *Nitrobacter Y* should be classified as a new species. As shown by light microscopy the cells of the new *Nitrobacter* and *N. winogradskyi* were both pleomorphic, mostly rod to pearshaped. Reproduction occurred by binary fission and by budding. The cell size was 0,5 − 0.8 × 1.0 − 2.0 µm. In contrast to the polar to subpolar flagellum of *N. winogradskyi*, *Nitrobacter X14* was motile by means of a lateral flagellum (Fig. 1) and *Nitrobacter Y* by means of a subpolar flagellum. On agar plates with yeast extract and peptone plus pyruvate *Nitrobacter X14* and *Y* formed yellowish-white tiny colonies of 0.1 mm in diameter within three weeks. Ultrathin sections showed that the organisms as well as *N. winogradskyi* are characterized by polar caps of flattened vesicles. In addition membrane vesicles may be present in the central region of the cell. Carboxysomes, poly-β-hydroxybutyrate and polyphosphate granules were typical inclusion bodies (Fig. 2). Like *Thiobacillus intermedius* (London and Rittenberg 1966) *Nitrobacter X14* and *Y* grew best mixotrophically. The shortest doubling time and the highest cell yield could be achieved with nitrite and yeast extract and peptone. In contrast to all known *N. winogradskyi* strains (Watson et al. 1981) the organism grew better heterotrophically than autotrophically. Heterotrophic growth was achieved on glycerol, acetate and pyruvate plus yeast extract and peptone. Acetate was a better energy and carbon source than pyruvate or glycerol. *Nitrobacter Y* could not grow mixotrophically with nitrite and pyruvate. The doubling times of autotrophically, heterotrophically and mixotrophically grown cells of *Nitrobacter X14* were 40, 20 and 10 h, respectively. *Nitrobacter Y* doubled within 18 h when grown mixotrophically with nitrite and yeast extract and peptone. No lithoautotrophic growth was achieved under normal oxygen partial pressures (air) but with reduced partial pressure (33% air, 66% N_2). As demonstrated by difference spectra high amounts of cytochromes of the c and a type were present in both organisms. The subunit composition of the carboxy-

somes of *Nitrobacter X14* was different from *N. winogradskyi*. Two of the minor polypeptides were lacking and a major subunit showed a significantly lower molecular weight (Ebert, personal communication). One of the main membrane proteins differed significantly in molecular weight (Milde, personal communication). In contrasts to *N. winogradskyi*, *Nitrobacter* strain X14 and Y possessed a plasmid of 75×10^6 Daltons (Kraft, personal communication).

The G + C content of the DNA of *Nitrobacter X14* was 61.2 mol%, that of strain *Y* was 61.6 mol%, while that of *N. winogradskyi* strain Engel was 61.7 mol%. These values are in the same range than those reported for various strains of *N. winogradskyi* (Watson and Mandel 1971). *E. coli*, used as a reference, had a DNA G + C content of 52.4 mol%. A DNA homology value of 100%, obtained under optimal reassociation conditions, showed the two strains of *Nitrobacter X14* and *Y* to be genetically indistinguishable. Lower DNA homology values of 34% and 38% were found between these strains and *N. winogradskyi* strain Engel. The DNA-DNA reassociation experiments clearly reveal that *N. winogradskyi* and *Nitrobacter X14* and *Y* are genetically so different that they have to be considered as two separate species. Steigerwalt et al. (1976) set the borderline for a genospecies at a homology value of 65% while Johnson (1973) considered 60% homology as the borderline. The homology value of 36% found between the two strains of *Nitrobacter* are definitely below this borderline and they are above the background level of the renaturation method, which is 20% to 30% (De Ley et al. 1970; Huß et al. 1982). Below this level no statement can be made on the degree of relatedness. Consequently, the two strains *Nitrobacter X14* and *Y* represents a new species of *Nitrobacter*, which is moderately related to *N. winogradskyi*. This finding is supported by results of phylogenetic studies (Seewaldt et al. 1982) using the 16 S ribosomal RNA as a marker. The similarity coefficient (SAB value) for the binary comparison of the oligonucleotide catalogues of the 16 S RNAs of these two species was 0.82. Similar values have been found between well defined species of other genera e.g. *Arthrobacter* and *Micrococcus* (Stackebrandt et al. 1980), *Streptomyces* (Stackebrandt et al. 1982) and *Staphylococcus* (Ludwig et al. 1981).

For taxonomic studies *Nitrobacter winogradskyi* strain Engel (Engel et al. 1954) was used because it is a typical *Nitrobacter* strain as shown by intensive physiological investigations and growth characteristics (Bock 1974, 1980). This strain is deposited in the Hamburg strain collection. The final species description of the new *Nitrobacter* strains X14 and Y will be presented as soon as the neotyp strain *Nitrobacter winogradskyi* ATCC 25391 is characterized, too. We propose to name the strains *Nitrobacter X14* and *Nitrobacter Y* "*Nitrobacter hamburgensis*" (ham'bur.gensis. L. adj. of Hamburg, named after the place were this organism was first isolated) with the following characters of the species: Cells are Gram-negative, pleomorphic, rod to pear-shaped with a size of $0.5-0.8 \times 1.0-2\,\mu m$. Reproduction by binary fission and by budding. Motile by means of a subpolar to lateral flagellum. Electron micrographs reveal intracytoplasmatic membranes forming caps of flattened vesicles under the cell wall or membrane vesicles in the central region of the cell. Colonies on agar plates are yellowish-white, 0.1 mm in diameter. Mixotrophic strict aerobic organism. Facultatively lithoautotrophic. Best growth mixotrophically with nitrite, plus yeasts extract and peptone. Heterotrophic growth. Slow growth under lithoautotrophic conditions. *Nitrobacter Y* is microaerophilic when grown lithoautotrophically preferring less than 6% (v/v) oxygen in the atmosphere. Optimal growth temperature between 25–30° C. The pH for optimal growth between 7.5–8.0. The DNA base composition is 61.4 mol% G + C. DNA-DNA hybridization experiments revealed a moderate relationship to *N. winogradskyi* strain Engel (36% using optimal reassociation conditions). Isolated from soil of the Old Botanic Garden in Hamburg and from soil of Uxmal in Yucatan (Mexico).

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