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Short Communication

The Close Phylogenetic Relationship of *Nitrobacter* and *Rhodopseudomonas palustris*

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Abstract. The phylogenetic position of *Nitrobacter winogradskyi* and two other nitrite-oxidizing bacteria was elucidated comparing oligonucleotides of the 16S ribosomal RNA. *Nitrobacter winogradskyi* and the *Nitrobacter* isolate 'Yukatan' are genetically nearly identical; *Nitrobacter* isolate X14 is more distantly related. Phylogenetically, *Nitrobacter* is a member of a group of purple non-sulfur bacteria that is defined by various species of *Rhodopseudomonas*, *Rhodomicrobium vannielii*, *Rhodospirillum rubum* and their non-phototrophic relatives. *Nitrobacter* shares a high sequence similarity to *Rhodopseudomonas palustris*. These findings are in accord with several common taxonomic characteristics, and in addition support the conversion hypothesis for the origin of this group of chemolithotrophic bacteria.

Key words: *Nitrobacter winogradskyi* – *Rhodopseudomonas palustris* – Chemolithotrophy – Phylogeny – 16S ribosomal catalogues

The phylogenetic origin of chemolithotrophic bacteria is still a matter of speculation. According to the conversion hypothesis, all bacteria producing ATP by oxidative phosphorylation originated from photosynthetic ancestors many times in parallel (Broda 1970, 1971, 1978). This hypothesis is bases on the similarities of the electron-flow chain in photosynthesis and respiration, e.g. the presence of cytochromes, quinones, flavoproteins and non-heme Fe-S-proteins (Gaffron 1965; Olson 1970), and the connection of these chains to membranes (Broda 1978). Other authors favour hypotheses whereby the bulk of aerobic bacteria stem directly from anaerobic respiring bacteria, which, like other major groupings e.g. the anaerobic chemoorganotrophic bacteria, the phototrophic bacteria and the 'photergers' (halobacteria and relatives, Schwemmler 1979) originated from primitive porphyrin synthesizing bacteria (Sagan 1967; Margulis 1968, 1970; Schwemmler 1979). According to this view, the respiration chain and the electron-flow chain of photosynthesis evolved independently and the increasing number of redox compounds in electron-flow chains of aerobic respiring bacteria, as compared to those of anaerobic respiring bacteria (nitrate and sulfate respiration) reflects the course of the evolution of these systems.

The validity of the phylogenetic trees deduced from similarities in bioenergetic processes and metabolic pathways can now be tested; they can be compared to genealogical trees derived from comparison of primary structures of conserved and ubiquitously distributed macromolecules. As a consistant result of the sequencing studies (Schwartz and Dayhoff 1978; Hori and Osawa 1979; Dickerson 1980; Fox et al. 1980; Gibson et al. 1980), the aerobic chemoorganotrophic respiring bacteria were not found to form a coherent cluster. Various groups of aerobic Gram-negative bacteria evolved independently from each other within different lines of descent defined by purple sulfur- and non-sulfur phototrophic bacteria, respectively, while the aerobic Gram-positive bacteria evolved separately (Fox et al. 1980; Stackebrandt and Woese 1981). These findings support the conversion hypothesis.

As an extension of these studies we now present data on the phylogeny of the nitrite-oxidizing bacteria based on analysis and comparison of RNase T_1 resistant oligonucleotides of their 16S ribosomal RNA (16S rRNA).

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Material and Methods

Nitrobacter winogradskyi strain Engel and *Nitrobacter* strain 'Yucatan' (strain collection of E. Bock) were grown lithoautotrophically in 18 l of a mineral medium containing 0.5 g NaCl per 1,000 ml destilled water, 0.05 g $MgSO_4 \cdot 7\ H_2O$, 0.15 g KH_2PO_4, 0.003 g $CaCO_3$, 0.05 mg $(NH_4)_6MO_7O_{24} \cdot 4\ H_2O$, 0.15 mg $Fe\ SO_4 \cdot 7\ H_2O$, pH 7. The medium was supplemented with 0.2% and 0.02% $NaNO_2$ for *N. winogradskyi* and strain 'Yucatan', respectively. To obtain about the same cell yield as with *N. winogradskyi*, growing cells of strain 'Yucatan' were supplemented five times with 0.02% $NaNO_2$ every third day. Doubling time for the autotrophic growth was 13 h and longer. *Nitrobacter* strain X14 was grown mixotrophically in a 19 l batch of the above mineral medium, supplemented with 0.2% $NaNO_2$, 0.0055% sodium pyruvate, 0.015% yeast extract (Difco Lab., Detroit, MI, USA) and 0.015% Bacto-peptone (Difco), pH 7.5. Cell doubling time was 10–11 h.

Cells were harvested by centrifugation, washed one time with Hatefi buffer containing 0.05 M Tris-HCL, 0.66 M sucrose, 0.001 M histidine, pH 8.0 and immediately frozen at −70° C. Isolation of the 16S rRNA, digestion of the RNA with RNase T_1, labelling of 5′ ends with γ-^{32}P-ATP (Amersham-Buchler, Braunschweig, FRG), and polynucleotide kinase, fingerprinting of the oligonucleotides and the sequence determination followed the description of Stackebrandt et al. (1981). The calculation of the binary coefficients, S_{AB} values, has been described by Gibson et al. (1980).

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Table 1. Oligonucleotide catalogues. The sequences listed are found in the organisms as numbered (1) *Nitrobacter winogradskyi* strain Engel, (2) *Nitrobacter* strain X14. Modified nucleotides are indicated by a superscript dot

6-mers		*7-mers cont'd*		*9-mers cont'd*	
ACCACG	(2)	UAAUACG	(1,2)	AAACUUCAG	(2)
CACAAG	(1,2)	AAAUUCG	(1,2)	CUAACUUCG	(1,2)
ACCAAG	(1)	AAACUUG	(1)	UCCAACUUG	(1,2)
CCCUAG	(1,2)	CUCUUAG	(2)	AUCUUUACG	(2)
CCCAUG	(1,2)	UCCUUAG	(1,2)	UUUAAUUCG	(1,2)
UCCACG	(1,2)	CUUUAAG	(1,2)	UACCUUUUG	(1,2)
CUAACG	(1,2)	AUAUUCG	(1,2)	UUUUACCUG	(1,2)
AUCCAG	(2)				
UAAACG	(1,2)	*8-mers*		*10-mers*	
ACAAUG	(1,2)				
AAUACG	(2)	AACACCAG	(1,2)	CACAACCCAG	(1)
CCCUUG	(1,2)	ACCCCUAG	(1)	UCACACCAUG	(1,2)
UUCCCG	(1,2)	CUAACCCG	(1,2)	CAACCCCUAG	(2)
CCUCUG	(2)	CUACACUG	(2)		
UUCCAG	(1)	CCCUUACG	(1,2)	*11-mers*	
UCUCAG	(1,2)	CCACAUUG	(1,2)		
UCCAUG	(2)	AUACCCUG	(1,2)	CUCAACUCCAG	(1,2)
UCACUG	(2)	CUACAAUG	(1,2)	AACCUUACCAG	(1,2)
AUCCUG	(1,2)	AAUCACUG	(2)	CUUAACACAUG	(1,2)
ACUCUG	(2)	AAAUCCUG	(1)	CUACCAUUUAG	(1,2)
UAAUCG	(1,2)	UCCUCAUG	(1,2)		
AUACUG	(1,2)	AAUUACUG	(1)	*12-mers*	
AUAAUG	(1)	UCUUUAAG	(1)		
CCUUUG	(1,2)	AUUUAUCG	(1,2)	ACCUUCUCUUCG	(1,2)
		CUCUUUUG	(1,2)	UUUACUCACUAG	(1,2)
7-mers				*14-mers*	
		9-mers			
CAACCCG	(1)			CCCAAACUCCUACG	(1,2)
CAAACAG	(1,2)	CAACCCCCG	(1,2)	AUUAAAACUCAAAG	(1,2)
CAACUCG	(2)	CUCACCAAG	(1,2)		
CAAUACG	(1,2)	UACACACCG	(1,2)	*16-mers*	
UAACACG	(2)	CUACACACG	(1,2)	CAAAUCUCAAAAAACG	(1,2)
UAACAAG	(1,2)	UCACACCUG	(1)		
CAUUCCG	(1,2)	CACUCUAAG	(1,2)	*3'-end*	
CUCACUG	(1,2)	CUAAUACCG	(1,2)	AUCACCUCCUUUCU$_{OH}$	(1,2)

Results and Discussion

Table 1 lists the oligonucleotide catalogues for *Nitrobacter winogradskyi* and *Nitrobacter* strain X 14. The catalogue for *Nitrobacter* strain 'Yucatan' was found to be identical to that of *N. winogradskyi*, except for an additional oligonucleotide CAACCCCUAG. The S_{AB} values for the binary comparison of the catalogues together with those found between the catalogues of *Nitrobacter* strains and various other Gram-negative bacteria are presented in Table 2.

On the basis of their unique inorganic reductant the three *Nitrobacter* strains were expected to be closely related, as indeed they are. *N. winogradskyi* and strain 'Yucatan' cannot be separately classified on the basis of thier RNA catalogues. A similar high degree of identity in RNA catalogues has already been found between various strains of *Escherichia coli* (Uchida et al. 1974); this indicates that strain 'Yucatan' can be treated taxonomically as an obligate chemolithoautotrophic strain of *N. winogradskyi*. Strain X 14, on the other hand, is more distantly related to the other two *Nitrobacter* strains (S_{AB} values of 0.82 and 0.81). The genealogical differences found between strain X 14 and *N. winogradskyi* support the description of strain X 14 as a new species, which so far was based only on phenotypical differences (Bock et al., in preparation).

The comparison of the 16S rRNA catalogues of *Nitrobacter* with those from representatives of various other groups of Gram-negative eubacteria, e.g. purple sulfur- and non-sulfur bacteria (Gibson et al. 1980), enterobacteria (Uchida et al. 1974) and cyanobacteria (Bonen et al. 1979) reveals that *Nitrobacter* is a member of that group of

Table 2. S_{AB} values and total number of bases (in brackets) in oligonucleotides (hexamers and larger) common to the catalogues of *Nitrobacter* strains and various reference organisms, whose catalogues have been published previously (1) = Gibson et al. 1980; (2) = Uchida et al. 1974; (3) = Bonen et al. 1979

	Nitrobacter winogradskyi	*Nitrobacter* strain X 14
Nitrobacter winogradskyi	–	0.82 (455)
Nitrobacter strain X 14	0.82 (455)	–
Rhodopseudomonas palustris (1)	0.76 (430)	0.78 (444)
R. viridis (1)	0.53 (292)	0.54 (299)
Rhodomicrobium vannielii (1)	0.42 (247)	0.45 (267)
Rhodopseudomonas sphaeroides (1)	0.42 (231)	0.41 (229)
R. capsulata (1)	0.41 (229)	0.40 (228)
Rhodospirillum rubrum (1)	0.34 (183)	0.35 (195)
Chromatium vinosum (1)	0.35 (182)	0.39 (209)
Rhodopseudomonas gelatinosa (1)	0.24 (130)	0.26 (143)
Rhodospirillum tenue (1)	0.25 (133)	0.25 (144)
Escherichia coli (2)	0.25 (136)	0.25 (141)
Synechococcus 6301 (3)	0.23 (132)	0.24 (141)
Nostoc strain MAC (3)	0.22 (115)	0.22 (115)

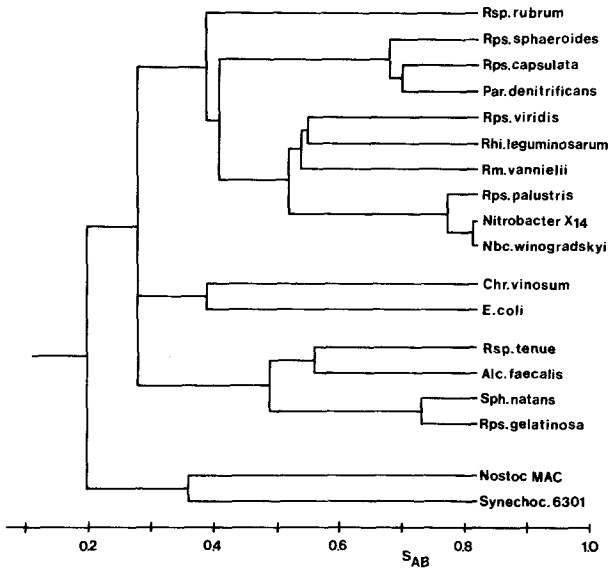

Fig. 1. Dendrogram of relationship showing the phylogenetic position of *Nitrobacter*. The figure was constructed by average linkage clustering (among the merged groups). Due to the omission of modified oligonucleotides smaller than hexamers in the calculation of S_{AB} values, the branching points within the dendrogram differ slightly from those published previously (Gibson et al. 1979; Fox et al. 1980). *Rsp* = *Rhodospirillum*; *Rps* = *Rhodopseudomonas*; *Par* = *Paracoccus*; *Rhi* = *Rhizobium*; *Rm* = *Rhodomicrobium*; *Nbc* = *Nitrobacter*; *Chr* = *Chromatium*; *E* = *Escherichia*; *Alc* = *Alcaligenes*; *Sph* = *Sphaerotilus*; *Synechoc* = *Synechococcus*

organisms, containing *Rhodopseudomonas sphaeroides*, *R. capsulata*, *R. palustris*, *Rhodospirillum rubum*, *R. viridis*, *Rhodomicrobium vannielii* and their non-phototrophic relatives, e. g. *Paracoccus denitrificans*, *Aquaspirillum itersonii*, *Rhizobium leguminosarum*, *Agrobacterium tumefaciens* and *Pseudomonas diminuta* (purple non sulfur group I, according to Gibson et al. 1980). Figure 1 is the dendrogram of relationship of *Nitrobacter* and various representatives of purple bacteria. Included are also some of those heterotrophic relatives, whose phylogenetic position has been presented previously (Fox et al. 1980), but whose RNA catalogues have not yet been published.

Nitrobacter is closely related to *Rhodopseudomonas palustris*. The S_{AB} values found between these organisms (average value of 0.77) are only slightly lower than those found between *N. winogradskyi* and *Nitrobacter* strain X 14 (0.82). Morphological and biochemical characteristics, e.g. reproduction by budding and a similar mol% G + C content of their DNA (Pfennig and Trüper 1974; Watson 1974) support the specific relatedness between *N. winogradskyi* and *Nitrobacter* strain X14. Further common properties are a negative Gram staining reaction, CO_2 fixation by the Calvin cycle, the presence of polyphosphate granules, storage of poly-β-hydroxybutyrate and an extensive intracytoplasmatic membrane-system paralleling the cytoplasmatic membrane. Moreover, *N. winogradskyi* is highly sensitive to visible light (Bock 1970) and furthermore, *R. palustris* like other purple non-sulfur bacteria, is able to grow aerobically in the dark.

The high phylogenetic relationship found between *Nitrobacter* and *R. palustris* is another example of the chemolithotrophic bacteria having evolved from the phototrophic bacteria. This has already been seen with *Paracoccus denitrificans*, a hydrogen-oxidizing, facultative chemolithotrophic species. Its phylogenetic position has been elucidated independently by sequencing studies on the 16S rRNA (Fox et al. 1980; Gibson et al. 1980), and cytochrome *c* (Ambler et al. 1979) and by the properties of cytochrome *c* (Dickerson 1980). According to the hypotheses of Margulis (1968, 1970) and Schwemmler (1979) *Nitrobacter* and *P. denitrificans* should have been grouped together more closely in one line of descent, which in addition should be clearly separated from other lines of descent, defined by phototrophic bacteria and by chemoorganotrophic bacteria. As demonstrated in Fig. 1, this is not the case. On the contrary, our results are in agreement with the conversion hypothesis of Broda (1971, 1978) whose arguments, e.g. the similarities in the electron-flow chains of phototrophic bacteria and aerobic respiring bacteria, are now seen to be valid.

According to Fox et al. (1980), S_{AB} values of 0.5 may be equalized with the onset of the aerobic earth atmosphere, i.e. about $1.0-1.2\times10^9$ years ago. Thus it follows from the rather high S_{AB} value found for the branching point of the *Nitrobacter* line of descent that this group is comparatively recent in evolutionary terms. It presumably evolved at the same time ($3-5\times10^8$ years ago), when the branching within other major groups of aerobic bacteria occurred, e.g., the enterobacteria, fluorescent pseudomonas, coryneform bacteria, actinomycetes and staphylococci (Fox et al. 1980; Stackebrandt and Woese 1981).

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