Conjugative Transfer and Expression of Genes Coding for Periplasmic Nitrate Reductase in the Purple Bacterium *Rhodospirillum rubrum*

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Plasmid pFR400, a derivative of the vector plasmid pPHU231 containing the structural genes of the periplasmic nitrate reductase (*nap* genes) of *Rhodobacter sphaeroides* DSM158 (F. Reyes *et al.,* Molec. Microbiol. **19,** 1307–1318 [1996]) was transferred by conjugative mating to a streptomycin-resistant strain of the nitrate reductase-negative nonsulfur purple bacterium *Rhodospirillum rubrum* S1. Transconjugant cells of the latter bacterium, identified by their resistance to streptomycin and tetracycline and by their plasmid pattern, contained an active nitrate reductase which, like other periplasmic bacterial nitrate reductases, was not repressed by ammonium.

Introduction

Among the nonsulfur purple bacteria, nitrate reducing strains are mainly found within the genera *Rhodobacter* and *Rhodopseudomonas* (Klemme, 1979; Klemme *et al.,* 1980; McEwan *et al.,* 1984; Martinez-Luque *et al.,* 1991). The few reports of nitrate reduction in members of the genus *Rhodospirillum* (Katoh [1963a, b]; Brown and Herbert [1977]) were either not confirmed by other investigators or were based only on incidental growth experiments without demonstration of nitrate reductase activity *in vitro.* According to our own experience, none of 5 wild type strains of *R. rubrum* is able to reduce nitrate. In view of the fact that, in some *Rhodobacter capsulatus* strains, nitrate reductase genes are located on endogenous plasmids (Willison, 1990; Koch and Klemme, 1994) it was of interest to see whether *R. rubrum* cells can be transformed to a NO_3^- reduction-positive state by conjugative introduction of nitrate reductase genes.

Materials and Methods

R. rubrum S1 (DSM 467); *R. rubrum* K100, a Sm-resistant spontaneous mutant of strain S1; *E. coli* C600(RP4) and *E. coli* SM10 (a genetically modified strain bearing the *tra* genes of the broad host range plasmid RP4 in its chromosomal DNA) are kept in the culture collection of the institute. Plasmid pFR400 (a gift of Dr. C. Moreno-Vivian, University of Cordoba, Spain) is a derivative of the mobilizable cloning vector pPHU231 containing a 6.8 kb *Pst*I-fragment of the DNA of *Rhodobacter sphaeroides* DSM158 with the structural genes (*nap* genes) of the periplasmic nitrate reductase (Reyes *et al.,* 1996). pFR400 contains a Tc-resistance site as a selection marker.

If not otherwise stated, phototrophic bacteria were grown photosynthetically at 30 °C in a malate-ammoniumsulfate medium supplemented with 0.05% [w/v] yeast extract (Kern *et al.,* 1994). Agar plates were incubated aerobically in the dark or anaerobically in the light by using a GasPak jar (BBL Microbiology Systems, Becton Dickinson, Heidelberg). Antibiotic resistances were selected by using PY medium (see Kern *et al.,* 1994) supplemented with the appropriate antibiotics (Km: 50 μg/ml; Sm: 200 μg/ml; Tc: 5 μg/ml). *E. coli* strains were cultivated aerobically at 37 °C in LB medium (see Kern *et al.,* 1994). Protein assays and tests for nitrate reduction were performed as described by Witt and Klemme, 1991). Plasmid contents of bacterial strains were analyzed by agarose gel electrophoresis (Eckhardt, 1978).

Results and Discussion

To test the ability of *R. rubrum* K100(Sm^R) to act as a recipient strain, conjugation experiments with *E. coli* C600 harbouring the broad host range plasmid RP4 were conducted. By using a filter mating technique (Kern *et al.,* 1994). *R. rubrum*

Abbreviations: DMSO, dimethylsulfoxide; DSM, German Collection of Microorganisms and Cell Cultures; Km, kanamycin; *R., Rhodospirillum; Rb., Rhodobacter;* Sm, Streptomycin; Tc, Tetracycline.

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K100(RP4)-transconjugants (selected by their resistance to Km) were obtained at frequencies of 10^{-3} to 10^{-4} per recipient cell. Plasmid pFR400 (with a 6.8 kb *Pst*I-fragment of the DNA of *Rb. sphaeroides* DSM 158 containing the genes of the periplasmic nitrate reductase; Reyes *et al.*, 1996) was transformed to *E. coli* SM10. Due to integration of *tra* genes of the broad host range plasmid RP4 within its chromosomal DNA, the latter strain acts as host for mobilizable plasmids such as pFR400. *E. coli* SM10(pFR400)-transformants were now used as donor cells for matings with *R. rubrum* K100. *R. rubrum* colonies growing on selective agar plates (Sm: 200 μg/ml; Tc: 5 μg/ml) were picked as presumptive pFR400-transconjugants and subjected to agarose gel electrophoresis. Contrary to wild type strains of *R. rubrum* (including strain K100) containing only one endogenous 55 kb plasmid (Kuhl *et al.*, 1983; Kawamukai *et al.*, 1990), transconjugant strains showed two plasmid bands the upper one corresponding to the endogenous 55 kb plasmid, and the lower one corresponding to pFR400 (Fig. 1). When transconjugant clones were grown in the absence of Tc as selective agent, plasmid pFR400 was not stably maintained in the cell population.

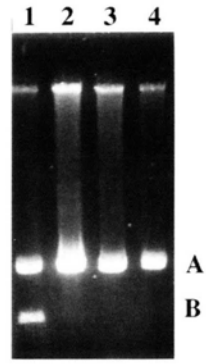

Fig. 1. Plasmid pattern in wild-type and transconjugant *R. rubrum* strains. Lane 1: *R. rubrum* K100-pFR400-transconjugant. Lanes 2–3: *R. rubrum S1. Lane 4: R. rubrum* K100. A: endogenous 55 kb plasmid of *R. rubrum;* B: pFR400.

Table I. Nitrite accumulation and cellular nitrate reductase activities in photosynthetic cultures of *Rhodospirillum rubrum* wild type K100 and a K100-transconjugant constructed by conjugational insertion of pFR400, a plasmid containing the structural genes (*nap* genes) of the periplasmic nitrate reductase of *Rhodobacter sphaeroides* DSM158.

	Wild type K100	K100-pFR400-transconjugant
Nitrate reductase activity in basal medium (A)	0	12–18
basal medium plus 10 mM KNO_3 (B)	0	30–40
basal medium plus 12.5 mM NH_4NO_3 (C)	0	40–60
Nitrite accumulation (μM) in medium C	0	60–110

For preparation of basal medium, the standard malate-ammoniumsulfate medium (Kern *et al.*, 1994) was modified by omitting ammoniumsulfate and adding 0.1% (w/v) yeast extract. Nitrate reductase activities (nmol/min* mg protein) were assayed at 30 °C as described by Witt and Klemme (1991). Data were obtained in three different experiments.

After 20 cultural transfers, less than 3% of the cells retained the Tc-resistance.

For analysis of nitrate reducing capacity, one *R. rubrum* transconjugant was chosen at random. To be sure that any nitrate reducing activity measured in cell suspensions of transconjugants was not due to contamination with other bacteria, the purity of cultures was carefully checked by streaking of samples on PY-agar plates. Contrary to the wild type strain K100, the transconjugant cells contained nitrate reductase activity up to 60 nmol/min * mg protein and excreted nitrite in nitrate-containing culture media up to growth inhibitory concentrations of about 100 μM NO_2^- and more (Table I). As shown for other bacterial periplasmic nitrate reductases (Reyes *et al.*, 1996), the nitrate reductase activity in transconjugant *R. rubrum* cells was not repressed by ammonium. The successful transfer and expression in *R. rubrum* of *nap* genes originating from another photosynthetic bacterium shows that the lack of nitrate reducing activity in wild type strains of the former organism is most probably due to the absence of nitrate reductase structural genes rather than to the absence of enzyme systems responsible for biosynthesis of the molybdenum-cofactor and/or for export of nitrate reductase subunits to the periplasmic space. If *R. rubrum* did not contain a molybdenum-cofactor processing system, one would expect the organism to be devoid of active molybdoenzymes (DMSO-reductase a.o.). However, wild-type

strains of this bacterium do contain a periplasmic DMSO-reductase (Sajitz *et al.*, 1993).

In view of the fact that possession of an active nitrate reductase (NIT^+ character) in purple bacteria, is normally found only in a fraction of newly isolated strains of a given species (for example, only about 40% of new isolates of *Rb. capsulatus* are NIT^+), it may be asked if this variability is due to gain and loss of nitrate reductase genes by horizontal gene transfer mediated by plasmids. Note in this connection that (i) the endogenous plasmid of *Rb. capsulatus* AD2 harbouring the *nap* genes of this organism can be transferred *via* conjugative mating to other purple bacteria (Koch and Klemme, 1994) and that (ii) even for photosynthesis genes, the possibility of horizontal gene transfer between different species of purple bacteria is being discussed (Nagashima *et al.*, 1993). The ecophysiological significance of conjugative gene transfer processes mediated by endogenous plasmids between different species of purple nonsulfur bacteria is now investigated in our laboratory.

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