Doctoral Thesis

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Author(s):
Knobel, Hans-Rudolf

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GENETIC STUDY OF BACTERIAL NITRILOTRIACETATE-DEGRADING ENZYMES

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH for the degree of Doctor of Natural Sciences

presented by Hans-Rudolf Knobel Dipl. Natw. ETH born on August 29, 1965 citizen of Altendorf (SZ)

accepted on the recommendation of Prof. Dr. A. J. B. Zehnder, examiner PD Dr. T. Egli, co-examiner Dr. B. Nörtemann, co-examiner

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SUMMARY

Two different bacterial enzymes are presently known to catalyze the first step in nitrilotriacetate (NTA) degradation. In obligately aerobic strains the oxygen-dependent NTA monooxygenase was detected, whereas the oxygen-independent NTA dehydrogenase was present in the facultatively denitrifying strain TE11. Until now, nothing was known about these proteins at the genetic level. Therefore, a genetic study of these bacterial NTA-degrading enzymes was performed.

A 6.2-kb DNA fragment containing the genes for the two-component nitrilotriacetate (NTA) monooxygenase of Chelatobacter heintzii ATCC 29600 was cloned and characterized by DNA sequencing and expression studies. The two genes - named ntaA (for component A of NTA monooxygenase) and ntaB (for component B) - were oriented divergently with an intergenic region of 307 bp. The ntaA and ntaB genes could be expressed in Escherichia coli DH5α and the gene products visualized after Western blotting and incubation with polyclonal antibodies against component A or B. By mixing overproduced NtaB from E. coli and purified component A from Chb. heintzii ATCC 29600, reconstitution of a functional NTA monooxygenase complex was possible. The deduced gene product of ntaA showed only significant homology to SoxA (of dibenzothiophene degradation) and to SnaA (of pristamycin synthesis); that of ntaB shared weak homologies in one domain with other NADH:FMN oxidoreductases.

Downstream of the ntaA gene, another open reading frame (ORF1) was found, which was characterized by DNA sequencing. The predicted gene product of this ORF1 with a size of 24.4 kDa has a DNA binding motif (helix-turn-helix) characteristic for the GntR family of bacterial regulatory proteins at its N-terminal end. Therefore, we believe that this ORF encodes a regulatory protein, which we think is involved in regulation of ntaA and ntaB gene expression. Between ntaA and ORF1 a possible terminator structure was observed, suggesting that ORF1 is independently transcribed from ntaA. To find out whether the ORF1 product is really the NTA-regulator, we tested if it could bind the intergenic region between the ntaA and ntaB genes, which would carry the
necessary promoter signals, in DNA binding experiments. Unfortunately, no clear data could be obtained so far, leaving the suspected role of ORF1 unclarified.

Hybridizations of genomic DNA of the strain *Chb. heintzii* TE6 with *nta* gene probes showed that a region homologous to the ORF1 and the *ntaA* gene, but not to the *ntaB* gene of *Chb. heintzii* ATCC 29600 was present. The *ntaA* region of strain TE6 is apparently not very strongly related to that of ATCC 29600, as judged from the differences in the physical maps. *Chelatococcus* strain TE2 appeared to contain regions highly homologous to that containing ORF1, *ntaA* and *ntaB* in *Chb. heintzii* ATCC 29600. Even some restriction sites in this area seem to be conserved. This strongly suggests that *ntaA* and ORF1 (and perhaps *ntaB*) are indeed present in strain TE2. Interestingly, the region hybridizing to ORF1 and *ntaA*, and that to *ntaB* are not adjacent on the DNA of *Ch. asaccharavorans* TE2. Somewhere near the beginning of an *ntaB* gene equivalent the homology with *Chb. heintzii* ATCC 29600 ends and continuous on another region. This other region could still contain a complete *ntaB* gene in strain TE2.

Furthermore, the NTA-degrading enzyme complex consisting of the NTA dehydrogenase (NTADH) and a nitrate reductase (NtrR) was investigated and further characterized in the denitrifying strain TE11. In this strain immunoblot studies demonstrated maximum levels of NtrR expression during denitrifying growth in the absence of oxygen. In cell-free extracts containing NtrR significant reduction of nitrate and chlorate was measurable indicating activity of a dissimilatory type of NtrR. Inductively coupled plasma mass spectrophotometer (ICP-MS) measurements of the pure NtrR enzyme solution indicated a molybdenum content of 1.3 atoms of molybdenum per subunit of NtrR. Furthermore, immunocytochemical labelling of NtrR and the enzyme NTA dehydrogenase (NTADH) clearly indicated that NtrR is associated or integrated in the cytoplasmic membrane whereas NTADH is located in the cytoplasm of TE11. These results strongly suggest that NtrR from the NTADH/NtrR enzyme complex is belonging to the dissimilatory type of nitrate reductases.
ZUSAMMENFASSUNG


einem unbekannten störenden Faktor in den Bindungsassays leider keine Aussagekraft.


Im weiteren, wurde der NTA abbauende Enzymkomplex des denitrifizierenden Bakterienstammes TE11 bestehend aus der NTA Dehydrogenase (NTADH) und einer Nitratreduktase (NtrR) untersucht und weiter charakterisiert. In diesem Bakterium zeigten Immunoblot-Studien eine maximale NtrR Expression während dem denitrifizierenden Wachstum von TE11 in Abwesenheit von molekularem Sauerstoff. In zellfreiem NtrR enthaltendem Extrakt wurde eine signifikante Reduktion von Nitrat und Chlorat gemessen, was auf eine dissimilatorische Funktion der NtrR hinweist. Messungen von reinen NtrR Enzymlösungen mit einem induktiv gekoppelten Plasma Massenspektrophotometer (ICP-MS) ergaben ein Molybdän Gehalt von 1.3 Molybdän Moleküle pro Untereinheit der NtrR. Ferner zeigte immunocytochemisches Markieren von NtrR und NTADH klar auf, dass NtrR mit der cytoplasmatischen Membran assoziiert oder in sie integriert ist, währenddem NTADH im Cytoplasma des Bakteriums TE11 lokalisiert ist. Diese Resultate lassen stark vermuten, dass die NtrR vom *in vitro* stabilen NTADH/NtrR Enzymkomplexes zum dissimilatorischen Typ der Nitratreduktasen gehört.