Auxin biosynthesis and denitrification in plant growth promoting bacteria

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Abstract

An indole-3-pyruvate decarboxylase (=IPDC) is known to be involved in the production of indole-3-acetic acid (=IAA) in the root associated *Enterobacter cloacae* strain FERM BP-1529 and in *Azospirillum brasilense*. Segments of the IPDC gene could be amplified by PCR from several different enterobacterial species which convert tryptophan to IAA. Five of the cloned segments were sequenced. All sequences showed strong homology to the IPDC-gene but were significantly different from the published sequence from *A. brasilense* Sp 245. Conserved region, detected in the alignment of the IPDC-gene segments were used as primers for PCR to identify this gene in *Zea mays*. An amplification product and a positive hybridization signal were the first genetical hints for the existence of this gene in higher plants. As the ability to produce the plant hormone was found not to be limited to nitrogen fixing plant growth promoting bacteria, but occurred also in the non-nitrogen fixing, free living *Klebsiella aerogenes*, the bacterial production of IAA might have other functions than promoting plant growth.
To study the distribution of the denitrification ability in the genus *Azospirillum* by DNA-hybridization, oligonucleotides were synthesized for the genes for the dissimilatory cytochrome cd_1 nitrite reductase (*nirS*), the N_2O reductase (*nosZ*) and the dissimilatory Cu-nitrite reductase (*nirK*). These primers were used for PCR with the different *Azospirillum* species. In case of *A. irakense* KA3 an amplificate was obtained with the *nirK* oligonucleotides. Cloning, sequencing and hybridization of the fragment established the identity of the segment as part of the Cu-nitrite reductase gene, the occurrence of which was unknown for *Azospirillum*. In case of *A. brasilense* Sp7, a segment of the cytochrome cd_1 nitrite reductase gene, *nirS*, and a segment of the N_2O reductase gene, *nosZ*, could be amplified by PCR with strong sequence homology to the corresponding genes from *P. stutzeri*. Whereas the *nosZ* segment cross-hybridized also with DNA from all other *A. brasilense* and *A. lipoferum* strains investigated and with *A. halopraeferens*, the *nirS* segment gave only positive hybridization signals for *A. brasilense* Sp7 and strain Cd. *nirS* could be localized on a cosmid of a genomic gene bank of strain Sp7.

Production of the auxin indole-3-acetic acid by species of the Enterobacteriaceae.

Among free living, root associated and symbiotic bacteria, the production of the plant hormone indole-3-acetic acid (=IAA) is catalyzed either by (i) a tryptophan (=Trp) monoxygenase and an indole-3-acetamide hydrolase as in the phytopathogenic *Pseudomonas syringae* (Yamada et al. 1985), (ii) a nitrilase as in *Alcaligenes faecalis* (Kobayashi et al. 1993), (iii) an unspecific Trp side chain oxidase as in *Pseudomonas fluorescens* (Oberhänsli et al. 1991), or by (iv) an indole-3-pyruvate decarboxylase (=IPDC) as in *Enterobacter cloacae* strain FERM BP-1529 isolated from cucumber (Koga et al. 1991) and in *Azospirillum* (Bothe et al. 1994, Costacurta et al. 1994) where anthranilate is probably involved in the regulation of the IAA biosynthesis (Zimmer et al. 1991). Other strains of *Enterobacter* were described to be incapable in IAA formation (Koga et al. 1991). However, studying the ability of IAA formation among different species of the *Enterobacteriaceae*, also other *Enterobacter cloacae* strains (NCIMB11461 and NCIMB11463), *E. agglomerans* strains (strain 333 and 339, from Prof. W. Kling-

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müller), *Pantoea agglomerans* IMET11328 (from Dr. S. Ruppel/Dr. C. Scholz), *Klebsiella aerogenes* DSM681 and *K. oxytoca* DSM3539 were found to convert Trp to IAA in amounts comparable to *Azospirillum* (Zimmer et al. 1994). In contrast, *E. coli* K12 was incapable in this conversion (Zimmer et al. 1994). As it was intended to identify the conserved regions of the IPDC known to be involved in the IAA production in *E. cloacae* FERM BP-1529, oligonucleotide primers were synthesized for different regions of the corresponding gene. One pair of these oligonucleotides allowed to amplify a segment of the predicted size by PCR with DNA of each of the seven IAA producing enterobacterial species but not with *E. coli* K12 indicating that the IPDC gene is spread among several species of the *Enterobacteriaceae* (Zimmer et al. 1994).

Figure 1: Identification of conserved regions at the alignment of enterobacterial IPDC amino acid sequences

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E.agg.333     NGIAGSYAEYVPVVHIVGAPALTSQRKGELLHHTLGDGEFSHFMRMSAPVSVAQASLTPENALAEIDRVIEEVMYHSRPGYLLLPSDVA
E.agg.339     NGIAGSYAEYVPVVHIVGAPRLTSQRKGELLHHTLGDGEFCHFMRMSAPVSVAQASLTPENALAEIDRVIEEVMYSSRPGYLLLPSDVP
E.clo.1529    NGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGDGEFRHFYHMSEPITVAQAVLTEQNACYEIDRVLTTMLRERRPGYLMLPADVA
E.clo.11461   NGVAGSYAEYVPLLHIVGAPCSGVQQRGELLHHTLGDGDFHHFYRMSEPVTAARAILTAQNACYEIDRVLEVMLMQSRPGYLMLPADVA
E.clo.11463   NGIAGSYAEYVPLLHIVGAPCSGVQQRGELLHHTLGDGDFHPFYRMSESVTAARAILTAQNACYEIDRVLEVMLTQSRPGYLMLPADVA
K.aer.681     NGIAGSYAEHIPVLHIVGAPSTGAQQPGELLHHTLGDGDFPSFARMTEQITCSQALLTAGNAANEIDRVLRDMLTHHRPGYLIVPADVA
              **.****** .*..******    *. ***********.*  * .*.  .. ..* **  **  *****.  ..   *****..*.**.

E.agg.333     ALPVSTRAHALPARQPPFSPSSLEA
E.agg.339     ALPVSTRAHALPARQPPFSPSSLEAFTAAAQTQLRGANRVSLLADFLADRFKVKKALEQWMEEVPLGTSTLLMGKGLFNEQQAHFAGTY
E.clo.1529    KKAATPPVNALTHKQAHADSACLKAFRDAAENKLAMSKRTALLADFLVLRHGLKHALQKWVKEVPMAHATMLMGKGIFDERQAGFYGTY
E.clo.11461   KKPATPPVNALTTPPFPVNEACLNAFRQHAQAMLSVGSSVALLADFLAA-FGFIQMLQCWMTDQLPTHTTVILGKGIVKRKHGGFSYPY
E.clo.11463   KKPATPPVNALTIPPFPVNEACLNA
K.aer.681     R-AGTLPQPALRVEPPAVKPAC-RV
                 . .    **       .    *    *.   *  .   .******          *. *. .    .*...***.   ... *  *
                       <-----
E.agg.339     SGAASAPSTKEAIEGADVVITVGVKFTDTITAGFYQQRPSGNASTSALFAARAIRSSITGRSKNGQRDAPPPPLNLAFRPGRLILLNTT
E.clo.1529    SGSASTGAVKEAIEGADTVLCVGTRFTDTLTAGFTHQLTPAQTIEVQPRAARVGDVWFTGIPMNQAIETLVELCKQHVHAGLMSSSSGA
E.clo.11461   HGAGGQRGPVPAIEGADTVICIGTRFTDTVTAGFTQQFARERTIEIQPFAARVGDVWFSGIPMREAINALIPLVRARAHAAANHRPPQA
E.clo.11463                                                               VGDVWFSGIPMREAINALIPLVRARAYSAANHRPLQV
K.aer.681                                                                 VGDHWFSGVPMDNALAALMTLCPPWRRSAYA-SGRGA
               *...   .   ****** *. .* .****.**** .*      .      ***     ..* .       .          ..

E.agg.339     HCRWSRDNSLGQYAFWQQIQDSLQPGDVLVAEQGTACFAAAALNLPQDLQFVVQPLWGSIGYTLAA
E.clo.1529    IPFPQPDGSLTQENFWRTLQTFIRPGDIILADQGTSAFGAIDLRLPADVNFIVQPLWGSIGYTLAA
E.clo.11461   ARAPVHQGTLNQETLWQTVQQFIRPDDILLADQGTAAFGAASLRLPAGAELLVQPLWGSIGYTLAA
E.clo.11463   RHAPVHQGTLNQETLWQTVQAFIRPDDILLADQGTAAFGAASLRLPAGAELLVQPLWGSIGYTLAA
K.aer.681     EAEEGPEGELTQKNFWATVQGPLRPGDIILADQGTAAFGIAAVKLPSEASLIVQPLWGSIGYTLAA
                . * *  .*  .*  ..*.*...*.***. *.    . **    ..**************
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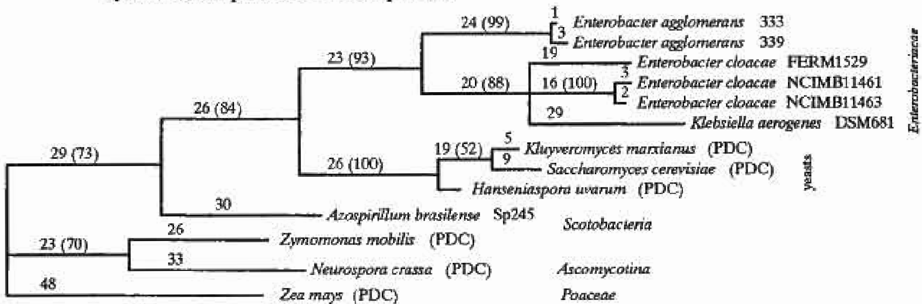
Sequences are from *Enterobacter agglomerans* 333 (*E.agg*.333, this work), *E. agglomerans 339* (*E.agg*.339, this work), *E. cloacae* FERM BP-1529 (*E.clo*.1529, translated from EMBL-No. D90214), *E. cloacae* NCIMB11461 (*E.clo*.11461, this work), *E. cloacae* NCIMB11463 (E.clo.11463, this work) and *Klebsiella aerogenes* (*K.aer*.681, this work). An asterisk indicates identical residues in all five sequences and a point indicates conservative changes.

Identification of conserved regions in the IPDC-sequences. After cloning of the IPDC segments from *E. agglomerans* 333 and 339, *E. cloacae* NCIMB11461 and NCIMB11463 and from *K. aerogenes* DSM681 the origin of the segments from these strains was successfully verified by DNA-hybridization (Zimmer et al. 1994). In order to identify conserved regions in the IPDC-gene, the segments were sequenced completely or partially (EMBL No. X80713, X80714, X80715, X80716 and X80717). As it was intended using such regions to detect the gene also in other bacterial families or even in plants, the

deduced amino acid sequences were aligned and homologous and conservative amino acid exchanges were calculated by the program CLUSTAL V (Fig. 1). All sequences showed significant homology to the published IPDC-gene of *E. cloacae* FERM BP-1529, establishing that the amplified segments code for the same enzyme (Fig. 1). New oligonucleotide primers were synthesized against the identified conserved regions in the IPDC-sequence (Fig. 1). Using these primers it was possible to amplify a segment of the predicted size from *Zea mays* DNA by PCR, which positively cross-hybridized with the *Enterobacter* IPDC-segments resulting in a weak hybridization signal (data not shown). This is the first genetical indication of the presence of the IPDC gene in a higher plant.

Figure 2: Tree and distant matrix of the IPDC amino acid sequences of *Enterobacteriaceae* and *Azospirillum* compared to PDC sequences.



The tree was calculated by PAUP (Vers. 3.0) using a CLUSTAL V alignment of the part of the protein sequence where all 13 sequences were available (see Fig. 1). The values above the branches are the branch lengths and the probability of the grouping in brackets (in %). The amino acid sequence of the pyruvate decarboxylase (PDC) from *Zea mays* (EMBL No. P28516) was used as an outgroup for the calculation of the tree. The abbreviations of the species are as given in Fig. 1. The sequence of *Azospirillum brasilense* Sp245 (A.bras.245, translated from EMBL No. L26240) and the fungal pyruvate decarboxylase sequence of *Kluyveromyces marxianus* (*K.mar.*(PDC), EMBL No. L09727) are included in the distance matrix.

Relation of enterobacterial IPDC- and fungal PDC-sequences to the corresponding A. brasilense *gene.* The tree calculation made for the enterobacterial IPDC-sequences is in good correlation with the systematical relationship of the strains (Fig. 2) with the exception that the *Klebsiella aerogenes* sequence showed a closer distance in the matrix to the *E. cloacae* sequences than the *Enterobacter agglomerans* segments. Unexpectedly, the published sequence of *A. brasilense* Sp245 (Costacurta et al. 1994) is significantly different from the enterobacterial sequences. It is even more distant from the enterobacterial IPDC sequences than the pyruvate decarboxylase (PDC) of yeasts, described to catalyze the related but different decarboxylation of pyruvate to acetaldehyde (Fig. 2). Therefore the enzyme encoded by the published IPDC-sequence of *Azospirillum* can have a distant specificity spectrum than the enzymes from the other organisms and possibly has some additional physiological functions.

Role of IAA production in soil bacteria. In contrast to the *Enterobacter* strains, *Klebsiella oxytoca* and *K. aerogenes* are described as free living non-associative organisms. The ability of IAA-formation and the presence of sequences homologous to the IPDC-gene in these soil bacteria causes some doubt that IAA production has only a role in the bacteria-plant interaction. An alternative function might be the use of Trp as a carbon source or the decreasement of growth inhibition caused by high concentrations of Trp, an idea forwarded for *Azospirillum* (Bar and Okon, 1992).

Distribution of genes for denitrification among *Azospirillum* species.

In addition to nitrogen fixation and the production of phytohormones some associative bacteria might affect plant growth by denitrification. In this reaction, nitrate is reduced via nitrite to the gaseous nitrogen compounds NO, N_2O and N_2 and is lost as a nitrogen source for plants. Therefore, the non denitrifying strains of the root associated bacterium *Azospirillum* are believed to be more beneficial for plant growth promotion. However, nitrite, which is the first intermediate in this pathway, has been shown to mimic effects of IAA in several plant tests for auxins (Zimmer et al. 1988). There is a controversal discussion about the ability of denitrification in the different strains and species of *Azospirillum*. Whereas most *A. lipoferum* and many *A. brasilense* strains were reported to be denitrifiers (Tarrand et al. 1978, Neyra et al. 1977), *A. irakense* and *A. amazonense* strains were originally described as non denitrifying species (Khammas et al. 1989, Magalhaes et al. 1983). Later on, denitrification was found also in *A. amazonense* QR242 and Y1, but the activity was lower than in the *A. lipoferum* and *A. brasilense* strains (Neuer et al. 1985). The physiological conditions for the dissimilatory reduction of nitrite to NO were intensively studied for *A. brasilense* Sp7, where it is catalyzed by a cytochrome cd_1 containing enzyme (for a review see Hartmann and Zimmer 1993). Whereas the reduction of nitrate, nitrite and N_2O is coupled to an energy formation at the respiratory chain and supports growth of *Azospirillum* under anaerobic conditions, *Azospirillum* does not grow reducing NO to N_2O (Voßwinkel et al. 1991).

Isolation of nitrite reductase and N_2O reductase gene segments. A genetical approach served to solve the question, which *Azospirillum* species and strains are denitrifiers. As it was aimed to identify the involved genes in the different species by DNA hybridization, suitable gene probes had to be isolated. Therefore oligonucleotides were synthesized against conserved regions in the published DNA-sequences of (i) the cytochrome cd_1 nitrite reductase gene of *Pseudomonas aeruginosa* NCTC6750, (ii) the Cu-nitrite reductase of *Alcaligenes faecalis* S-6, *Pseudomonas aureofaciens* ATCC13985 and *Pseudomonas aeruginosa* G-179, (iii) the Cu-containing N_2O reductase from *Pseudomonas stutzeri* ZoBell, *Pseudomonas aeruginosa* NCTC6750 and *Alcaligenes eutrophus* H16.

Figure 3: Alignment of the deduced amino acid sequence translated from the PCR segment of *Azospirillum irakense* KA3 and *Alcaligenes xylosoxidans* with the *nirK* oligonucleotide primers

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A.fa. GMVPWHVVSGMNGAIMVLPREGLHDGKGKALTYDKIYYVGEQDFYVPRDENGKYKKYEAPGDAYEDTVKVMRTLTPTHVVFNGAVGALTG
P.au. GMVPWHVVSGMNGALMVLPRDGLRDPQGKLLHYDRVYTIGESDLYIPKDKDGHYKDYPDLASSYQDTRAVMRTLTPSHVVFNGRVGALTG
P.ae. GMVPWHVTSGMNGAIMVLPRDGLKDHKGHELVYDKVYYVGEQDFYVPKDENGKFKKYESAGEAYPDVLEAMKTLTPTHVVFNGAVGALTG
A.xy. GMVPWHVTSGMSGTVMVLPRDGLKDPQGKPLHYDRAYTIGEFDLYIPKGPDGKYKDYATLAESYGDTVQVMRTLDAVHIVFNGKVGALTG
A.ir. GMVPWHVTSGMLRHIMVLPRDGLKDEKGKPIHYDRAYTIGEFDLYVPKDANGKYKDYESAGDSYADTGGGMKGLSH-HVVFNGAVGALTV
      ******* ***    +++++++** *   *  + **+ * +** * *+*     * +* *       +* *     *+ *   *+**** *****
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Sequences are from *Alcaligenes faecalis* S-6 (*A. fa.*, translated from EMBL-No. D13155), *Pseudomonas aureofaciens* ATCC13985 (*P.au.*, translated from EMBL-No. Z21945), *Pseudomonas aeruginosa* G-179 (*P.ae.*, translated from EMBL-No. M97294), *Alcaligenes xylosoxidans* NCIMB11915 (*A. xy.*, this work) and *Azospirillum irakense* KA3 (*A. ir.*, this work). Asterisk and plus indicate identical and similar amino acids, respectively, in all five sequences (amino acids regarded as similar are: S-T-A; L-V-I-M; K-R; D-E; Q-N; F-Y-W).

In case of the Cu-nitrite reductase oligonucleotides, a segment of the predicted size could be amplified by PCR from *Alcaligenes xylosoxidans* NCIMB11015 and unexpectedly also from *Azospirillum irakense* KA3. After cloning and sequencing of the segment, the sequence comparison established the identity as segments of the Cu-nitrite reductase gene (Fig. 3). DNA-hybridization studies among the different *Azospirillum* species revealed positive signals only in case of *A. irakense* (see Table 1) and therefore established the origin of the PCR product from this strain. Whereas *A. xylosoxidans* is known to have this enzyme (Smith and Tiedje 1992), the identification of the Cu-nitrite reductase in *Azospirillum irakense* KA3 is quite surprising, as it is described as a non denitrifying *Azospirillum* species (Khammas et al. 1989). Moreover, these are the first strong hints for the presence of the copper type enzyme in the genus *Azospirillum*, where up till-now only the cytochrome cd_1 containing nitrite reductase was found (Danneberg et al. 1986 and 1989, Lalande et al. 1987, Zimmer et al. 1984).

Figure 4: Alignment of the deduced amino acid sequence translated from the PCR segment of *Azospirillum brasilense* Sp7 amplified with the *nirS* oligonucleotide primers

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P.st. IYFERCAGCHGVLRKGATGKNLEPHWEKTEDGKKIEGGTLKLGTKRLENIIAFGTEGGMVNY--DDILTAEEINLMARYIQHTPDIPPEFSLQDMKDSWNLI
P.ae. IYFQRCAGCHGVLRKGATGKPLTPDITQ------------QRGQQYLEALITYGTPLGMPNWGSSGELSKEQITLMAKYIQHTPPQPPEWGMPEMRESWKVL
P.de. IYFERCAGCHGVLRKGATGKALTPDLTR------------DLGFDYLQSFITYGSPAGMPNWGTSGELTAEQVDLMANYLLLDPAAPPEFGMKEMRESWQVH
A.br. IYFQRCAGCHGVLRKGATAKNLEPDATK------------KLGQARLEKILAFGTE-GMPNF--EDILSKDEIKSLATYIQMTPDSPPEWGMTEMKNSWKVA
      *** ************** * * *                 *   *   **+*+  ** *+      *+ + +  +* **    *   ***+ + +*+ ** +

VPVERR--RQMNKVNLENVFAITLRDA---QLWDGDTHEIWKILDTGYAVHISRLSASGR-MSTPSAGWLTTIIDMWYPEPTTVATVRLGP-TRSVDVSKFKGYEDKY
VKPEDRPKKQLNDLDLPNLFSVTLRDAGQIALVDGDSKKIVKVIDTGYAVHISRMSASGRYLLVIGRDARIDMIDLWAKEPTKVAEIKIGIEARSVESSKFKGYEDRY
VAPEDRPTQQENDWDLENLFSVTLRDAGQIALIDGTTYEIKSVLDTGYAVHISRMSASGRYLFVIGRDGKVNMIDLWMKEPATVAEIKIGSEARSIETSKMEGWEDKY
IPPEKRPTKKMNNVNLDNVFSVTLRDAGKVALIDGDSKKIWNIVKSAYAVHISRWSASGRYVYVIGRDGKLDMIDLWMEKPEVVATIKTGLDARSVDTSKFKGYEDAY
+  * *      *   * *+*+++++++    * ** *  *  ++ + ******* ***** *           +**+*   *  ** +* *   **++ **  *+** *
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Sequences are from *Pseudomonas stutzeri* ZoBell (*P.st.*, translated from EMBL-No. M80653), *Pseudomonas aeruginosa* NCTC6750 (*P.ae.*, translated from EMBL-No. X16452), *Paracoccus denitrificans* (*P.de.*, translated from EMBL-No. U05002), and *Azospirillum brasilense* KA3 (*A. br.*, this work). Asterisk and plus indicate identical and similar amino acids, respectively, in all four sequences as described in the legend to Fig. 3.

The oligonucleotides for the cytochrome cd_1 nitrite reductase and the N_2O reductase allowed to amplify a segment of the predicted size by PCR in case of *Azospirillum brasilense* Sp7 and from *Pseudomonas stutzeri* which was used as a control strain. The amplification products were cloned and sequenced. Strong homologies were found to the published sequences in case of the nitrite reductase segment of *A. brasilense* and *P. stutzeri* (Fig. 4) and for the N_2O-reductase segments (39% identical and 43% similar residues, data not shown) establishing that the PCR products corresponded to the desired gene regions.

Table 1: Distribution of the nitrite- and nitrous oxide- reductase genes within the genus *Azospirillum*, monitored by DNA-hybridization.

strain	NO_2^--reductase		N_2O-reductase
	nirS	*nirK*	*nosZ*
A. brasilense Sp7	~20	-	>20
A. brasilense 7030	-	-	>20
A. brasilense Cd	>20	-	>20
A. lipoferum Sp59b	-	-	~18
A. amazonense Y1	-	-	-
A. halopraeferens AU4	-	-	~19
A. irakense KA3	-	5, 2.5	-

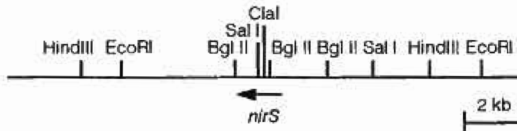
Genomic DNA, completely digested by *Eco*RI, was blotted onto nylon membrane. Hybridization was performed in 5 x SSC, 0.02% SDS at 68°C for 16h with digoxigenin labeled *nir*S-segments (cytochrome cd_1 nitrite reductase gene) from *Pseudomonas aeruginosa* DSM6195 and *A. brasilense* Sp7, a *nir*K-segment (Cu-nitrite reductase gene) from *Alcaligenes xylosoxidans* NCIMB11015 and *nos*Z-segments (nitrous oxide reductase gene) from *Pseudomonas stutzeri* ZoBell (Bothe et al. 1994) and *A. brasilense* Sp7. The sizes of the positive hybridizing signals on the Southern blot fragments are given in kb. (-) indicates no hybridization signal.

Study of the distribution of the denitrification genes in the genus Azospirillum. DNA-hybridization with the N_2O-reductase segment from *P. stutzeri* and *A. brasilense* revealed positive signals for all *A. brasilense* and *A. lipoferum* strains and for *A. halopraeferens* but not for *A. amazonense* and *A. irakense* (Table 1). In contrast, the cytochrome cd_1 nitrite reductase gene segment from *P. aeruginosa* and *A. brasilense* Sp7 hybridized only against digested DNA from *A. brasilense* Sp7 and the closely related strain *A. brasilense* Cd (Table 1). The fact, that *A. lipoferum* Sp59 and *A. amazonense* Y1 definitively known as denitrifiers from physiological investigations (Neuer et al. 1985) did not show any hybridization signal either with the two different *nirS* or the two different *nirK* probes, is unexpected and surprising. The segment of the nitrite reductase gene of *A. brasilense* Sp7 which was used as a probe could possibly represent a non conservative part of the gene which allowed to detect the gene only in the closely related species. Therefore this experiment will be repeated with a longer segment of the gene. The diverse nitrite reductases of *Azospirillum* possibly have different phylogenetic origins and may have been introduced into *Azospirillum* via conjugational transfer from different non related denitrifying soil bacteria. Interestingly, *A. brasilense* 7030, which is a mutant from *A. brasilense* Sp7 without the 115 MDa mega-

plasmid, did not show a hybridization signal with the *nirS*-probes (Table 1). This is a strong hint for a plasmid localization of *nirS* at least in *A. brasilense* Sp7 which could explain the differences in the distribution of the denitrification genes in the genus *Azospirillum*. However, it has to be studied in more detail, whether nitrite reductase genes are always localized on plasmids, which would explain the existence of non-denitrifying strains and closely related denitrifying strains.

Isolation of a cosmid clone carrying the nirS *gene from* Azospirillum. Aiming to obtain a longer segment of *nirS* for further hybridization studies, the PCR segment of the cytochrome cd_1 nitrite reductase gene from *A. brasilense* Sp7 was used for a screening of a genomic gene bank of *A. brasilense* Sp7, which was constructed cloning the genomic fragments after a partial *Sal*I digest into the broad host range vector pVK100. Among 500 clones one positive signal could be detected which allowed the localization of the *nirS* gene on the map of the corresponding cosmid pVI2 (Fig. 5).

Figure 5: Localization of the nitrite reductase gene *nirS* on the restriction map of pVI2, a cosmid of a genomic bank of *Azospirillum brasilense* Sp7



The cosmid pVI2 is a derivative of the broad host range vector pVK100, harbouring a fragment of the *A. brasilense* Sp7 genome partially digested by *Sal*I in the *Xho*I site of the kanamycin-resistance gene.

As the gene is located in the middle of the cloned segment, the cosmid probably contains the entire nitrite reductase cluster of *A. brasilense* Sp7. Therefore, the organisation of the genes and their regulation can be studied now. A subcloned *Bgl*II fragment of this cosmid, intragenic in the *nirS* gene, will be used to repeat the screening of the *Azospirillum* strains for the presence of the cytochrome cd_1 nitrite reductase gene. Moreover, transfer of this cosmid into weak denitrifying strains such as *A. brasilense* Sp245 and a study of the influence on the growth of the host plant will allow to elucidate the role of denitrification in the association.

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