Genetic inactivation of the H^+-translocating NADH:ubiquinone oxidoreductase of *Paracoccus denitrificans* is facilitated by insertion of the *ndh* gene from *Escherichia coli*

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Abstract The H^+-translocating NADH:ubiquinone oxidoreductase (NDH1) is probably an obligatory enzyme in *Paracoccus denitrificans* and disruption of its genes may be lethal to this organism. In order to overcome this problem and delete the *nqo8* and *nqo9* genes of NDH1, it was necessary to render the enzyme non-essential. This was achieved by constructing a deletion plasmid in which most of the coding regions of *nqo8* and *nqo9* were replaced by the *ndh* gene of *Escherichia coli* that encodes an alternative NADH:ubiquinone oxidoreductase (NDH2), and a kanamycin resistance gene. Subsequent homologous recombination gave rise to a mutant the membranes of which catalyzed rotenone-insensitive NADH oxidation, but which did not oxidize deamino-NADH. Hence, this mutant expressed active and membrane-bound NDH2, and lacked NDH1 activity.

Key words: NDH1; NDH2; Complex I; Respiratory chain; Membrane protein

1. Introduction

The H^+-translocating NADH:ubiquinone oxidoreductase (also called NDH1 in bacteria and complex I in mitochondria) is the first and largest enzyme of the respiratory chain [1–3]. Mitochondrial complex I is composed of more than 40 different protein subunits while NDH1 of *Paracoccus denitrificans* and *Escherichia coli* may contain 14 subunits [4,5]. Despite having about half the molecular mass of complex I and a third of its subunits, the *P. denitrificans* NDH1 contains the same set of redox centres [6,7]. Furthermore, the EPR spectra of all the detectable Fe-S clusters in *P. denitrificans* NDH1, unlike those of *E. coli*, are very similar to the respective clusters in complex I [5–8].

Many bacteria and fungi contain a smaller and simpler NADH:ubiquinone reductase which does not translocate protons across the membrane [9], e.g. NDH2 of *E. coli* [10]. However, such an enzyme was not detected in *P. denitrificans* [9]. Due to this, and since *P. denitrificans* is non-photosynthetic and does not ferment, NDH1 appears to be an obligatory enzyme in this bacterium.

The *nqo8* and *nqo9* genes encode subunits of NDH1 that are homologous to ND1 and TYKY of complex I, respectively [11]. These proteins probably play a central role in the function of the enzyme and deletion of their genes, a prerequisite for their mutagenesis, may render NDH1 inactive and hence to be lethal to the cell. A solution to this problem was found and the deletion of *nqo8* and *nqo9* of *P. denitrificans* was achieved by replacing them with *ndh*, the *E. coli* gene that encodes NDH2 [12].

2. Materials and methods

2.1. Materials

Deamino NADH (nicotinamide hypoxanthine dinucleotide, reduced form) was purchased from Sigma, and NADH from Boehringer.

2.2. Bacterial strains and plasmids

The bacteria and plasmids used in this work are listed in Table 1. *E. coli* XL-1 blue strain was purchased from Stratagene, and the pUC19, as well as the kanamycin resistance marker (GenBlock) were from Pharmacia.

2.3. Membrane preparation

Cells for membrane preparations were grown in a malate-containing medium and membranes were prepared essentially as described previously [13]. Bacteria were grown either in 100 ml cultures in 250 ml flasks under vigorous shaking, or as a 16 l culture in a fermenter under high aeration. After the lysozyme and osmotic shock treatments special care was taken to collect only the cytoplasmic membrane fraction, the thin coloured layer on top of the pellet, without cell debris and unbroken cells.

2.4. Assays of enzymatic activities

NADH and deamino-NADH oxidase activities in the membrane samples were assayed polarographically as previously described [13].

2.5. Miscellaneous

Oligonucleotides were custom-synthesized by Pharmacia. Gene amplification by PCR was carried out using Vent DNA polymerase (New England Biolabs). Plasmids and PCR products were purified using Wizard preps, either Mini or PCR (Promega). Genomic DNA from *P. denitrificans* was purified as described in [14].

3. Results and discussion

The NDH1 of *P. denitrificans* is an attractive model system for site-specific mutagenesis studies of complex I, and a prerequisite for such studies is cloning and disruption of the chromosomal copy of the gene of interest. In the present case such an approach requires that *P. denitrificans* will survive the inactivation of NDH1 by employing an alternative NADH:ubiquinone reductase, or a metabolic pathway that does not depend on NDH1. However, this precondition may not be easily fulfilled by *P. denitrificans* which is a non-photosynthetic bacterium that does not ferment and lacks NDH2 [9]. Nonetheless, it was anticipated that inactivation of NDH1 would lead to expression of an NDH2-like enzyme in *P. denitrificans*. That anticipation was based on the presence of alternative pathways in the respiratory chain of *P. denitrificans* at

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Abbreviations: NDH, NADH:quinone oxidoreductase (NADH dehydrogenase); MDH, (*S*)-Mandelate dehydrogenase

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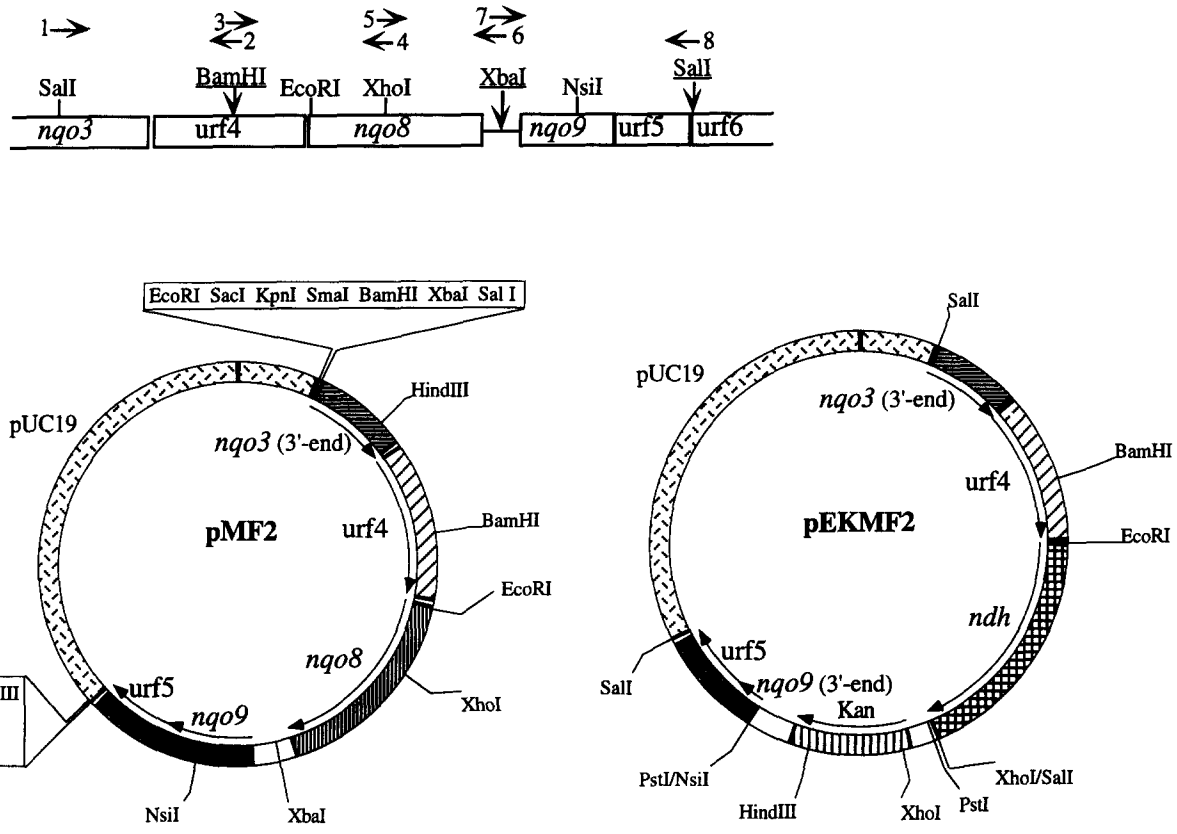

Fig. 1. Amplification and cloning of *nqo8* and *nqo9* of *P. denitrificans*, and their deletion construct. (Upper) Amplification reactions of four fragments within the *NQO* gene cluster [11,18]. The arrows indicate oligonucleotides primers, the sequence of which is presented in Table 2. The newly introduced restriction sites are underlined. (Lower) Plasmids pMF2 (6.10 kbp) and pEKMF2 (6.94 kbp), and selected restriction sites within them. *XhoI/SalI* and *PstI/NsiI* indicate restriction sites that were eliminated by ligation of compatible ends. See text and Table 1 for further details.

the levels of both ubiquinone and cytochrome *c* [15], and the presence of NDH2 in many other bacteria [9]. It may be noted here that the presence of NDH2 in *E. coli* membranes does not obstruct studies of NDH1 because the latter enzyme oxidises both NADH and deamino-NADH, while NDH2 does not oxidise deamino-NADH [10].

Two subunits of NDH1 were chosen for this study, the bacterial counterparts of ND1 and TYKY [2]. Their respective genes, *nqo8* and *nqo9*, are located in the middle of the *NQO* operon [11], and they were amplified from genomic DNA of *P. denitrificans* by PCR (Fig. 1). Oligonucleotide primers for these reactions were designed according to published sequences [11,16], and they included three new restriction sites which were introduced in order to simplify cloning and future manipulation of the genes (Fig. 1, Table 2). The amplified segments were subcloned into pUC19 and the resulting plasmid was named pMF2 (Fig. 1).

The initial inactivation constructs were prepared for either

Table 1
Bacterial strains and plasmids

	Relevant characteristics	Relevant characteristics
Bacteria		
P. denitrificans		
Pd9220	Δ*cta*DI,Δ*cta*DII,(Km^S)	[23]
Pd92-223	Pd9220 derivative, $NDH1^-$, $NDH2^+$, Δ*nqo8-nqo9*::*ndh*-Km^R	This work
E. coli		
SM10	Km^R,*thi,thr,leu,lacY,tonA,phx,supE*, RP4-2 integrated (Tc::Mu)	[24]
XL-1 blue	*recA1,endA1,gyrA96,thi-1,hsdR17,supE44,relA1,lac*,[*F' proAB,lacI*^{q}*ZDM15*,Tn*10* (tet^r)]	[25]
Plasmids		
pUC19	Amp^R,*lacZ'*	[26]
pSUP202	*mob*,Cm^R,Amp^R,Tc^R	[24]
pSUP202mp7	pSUP202 derivative, Tc^R::Sm^R as a *Bam*HI-*Hin*dIII fragment	M. Raitio, unpublished
pMF2	pUC19 derivative with the PCR-amplified *urf4-nqo8-nqo9-urf5* and flanks as a *Sal*I fragment in mcs	This work
pEKMF2	pMF2 derivative, Δ*nqo8-nqo9*::*ndh*-Km^R	This work

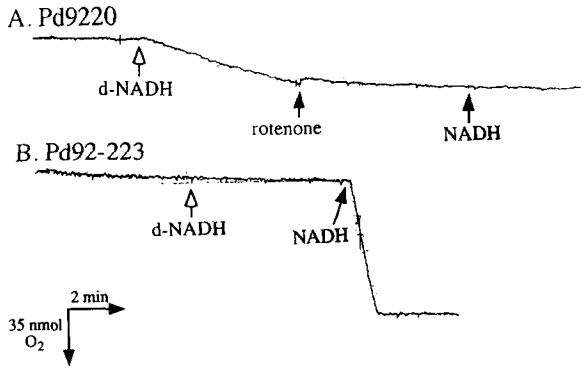

Fig. 2. NADH and deamino-NADH oxidase activities in isolated membranes of the two strains of *P. denitrificans*. (A) Parent strain Pd9220; (B) Pd92-223, the strain lacking *nqo8* and *nqo9*. Oxygen uptake rates were assayed polarographically in the presence of 50 mM Tris-HCl pH 7.5. About 0.3 mg protein was present in each assay. The additions, where indicated, were 200 nmol NADH or deamino-NADH (d-NADH), or 15 nmol rotenone.

nqo8 alone, or both *nqo8* and *nqo9*. The former was carried out by inserting a kanamycin resistance gene into the *Xho*I site of *nqo8*, and the latter by replacing the *Xho*I-*Nsi*I fragment of pMF2 with the antibiotic resistance gene. The *Sal*I fragment of each construct was subcloned into the suicide plasmid pSUP202 mp7, transferred to the *E. coli* donor strain SM10 which was then subjected to conjugation with Pd9220. The resulting kanamycin-resistant *P. denitrificans* colonies were tested for sensitivity to streptomycin, which would indicate that crossover events had occurred on both sides of the kanamycin marker. Three out of more than 400 kanamycin-resistant colonies were sensitive to streptomycin, and they were picked out and analysed at the DNA and protein levels. Membranes prepared from these bacteria oxidised deamino-NADH and NADH at similar rates, and both activities were sensitive to rotenone. This indicates that NDH1, but not an NDH2-like enzyme, was active in these bacteria (not shown). These results, together with the Southern blotting (Fig. 3C), indicate that those colonies incorporated the suicide plasmid into a different site of the bacterial chromosome, and hence they are 'false positives'. In light of these results it was concluded that NDH1 may be an obligatory enzyme in *P. denitrificans* and cells in which its genes are deleted are not viable.

A possible way to inactivate an obligatory enzyme is to render it non-essential by introducing a bypassing system, and a suitable candidate in this case is NDH2 of *E. coli*. To this end, the *ndh* gene which encodes NDH2 [12] was amplified from genomic DNA of *E. coli* (strain HB101) and slightly modified in order to construct a fusion protein that contains the first three amino acids of *nqo8*. Hence, the 5′-end of the amplified *ndh* carried an EcoRI site which was placed just downstream the first TTG codon of *ndh* (primer no. 9), and the reverse primer (no. 10) carried an *Xho*I site downstream the stop codon. The latter was inserted in order to facilitate ligation to the *Sal*I site at the 5′-end of the kanamycin marker (Fig. 1). Subcloning the amplified *ndh* and the kanamycin resistance gene into pMF2 gave rise to pEKMF2, a plasmid that lacks most of the coding region of both *nqo8* and *nqo9* (Fig. 1). Following this, the *Sal*I fragment of pEKMF2 was subcloned into pSUP202mp7, and subsequent conjugation with Pd9220 and selection were carried out as before. Kanamycin-resistant and streptomycin-sensitive clones were picked, and studies on one such clone, Pd92-223, are presented below.

Membranes prepared from Pd92-223 exhibited a high rate of NADH oxidase activity which was insensitive to rotenone (not shown), and did not oxidise deamino-NADH (Fig. 2B). This is typical of *E. coli* NDH2, and strongly suggests that NDH1 was inactive. Furthermore, NADH:ferricyanide oxidoreductase activity was observed in these membranes, but no deamino-NADH oxidation by ferricyanide (results not shown). This rules out the possibility that a small isoform

Table 2
Oligonucleotides that were used as PCR primers in this study

No.	Sequence (5′→3′)
1	GACGAGGTCGACATCGATCA
2	GGGGATCCGCCTCGAAACTCA
3	GAGGCGGATCCCCAGGATTT
4	ACGCCGTAGACCTCGAGAGA
5	TCTCTCGAGGTCTACGGCGT
6	TCTATGGATTGTTGGCGAGGA
7	AATCTAGACAGTGCGGAACA
8	TGGTCGACATCGGTCATGGACTTGT
9	GGAATTCACTACGCCATTGAAAAAG
10	ACCTCGAGTTAATGCAACTTC

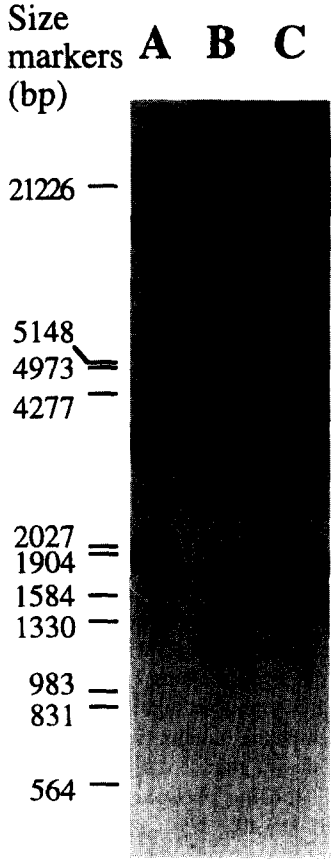

Fig. 3. Analysis of three *P. denitrificans* strains by Southern blotting. Genomic DNA samples were digested by *Hin*dIII plus *Xho*I, and hybridization was carried out using the 'lower region' probe which includes *nqo9* and urf5. (A) Parent strain Pd9220; (B) deletion strain Pd92-223; (C) 'false positive' strain from a clone obtained with the Δ*nqo8nqo9*::km deletion construct. See text for further details.

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P.putida  MDH,  189-213   LHPRWSLDFVRHGMPQLANFVSSQT
E.coli    NDH2, 224-247   LPPRISAA-AHNELTKLGVRVLTQT
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Fig. 4. Alignment of a segment of the membrane binding domain of (*S*)-madelate dehydrogenase (MDH) from *P. putida* [22] with a segment of NDH2 from *E. coli*.

of NDH1, resembling the one found in *Neurospora crassa* [17], was active in the membranes of Pd92-223.

Analyses at the DNA level were performed by Southern blotting using genomic DNA samples that were digested with *Hin*dIII plus *Xho*I. Two digoxigenin-labelled probes were prepared for this analysis by PCR. The upper-region probe included *nqo8* and part of urf4, and it was synthesized using primer nos. 3 and 6 (Table 2). The lower-region probe included *nqo9* and urf5, and was synthesized using primers no. 7 and 8 (Table 2). Due to the presence of both *Hin*dIII and *Xho*I sites in the kanamycin marker (Fig. 1, pEKMF2), the sizes of the fragments that should hybridize to the lower-region probe are 1803 bp in Pd9220, and 1214 bp in Pd92-223. The results of the Southern blot are presented in Fig. 3, and they clearly demonstrate that in Pd92-223 (lane B), *nqo8* and *nqo9* were replaced by *ndh* plus the kanamycin marker. The additional band of about 3100 bp that is visible in lanes A and C of Fig. 3 may have arisen from incomplete digestion of *nqo8* by *Xho*I, resulting in a fragment extending from the *Hin*dIII site in *nqo3* to the *Xho*I site in urf6 [11]. The results of a parallel hybridization experiment in which the upper-region probe was used were in full agreement with the conclusion that Pd92-223 is a true deletion strain with regards to *nqo8* and *nqo9* (not shown). A false positive strain from the initial deletion experiments (see above) is also shown in Fig. 3 (lane C). The large hybridizing fragment, in addition to the wild-type fragments, indicates that in this case a segment of the suicide plasmid that contains the kanamycin-resistance gene and the 3'-end of *nqo9* was incorporated into the genome, but at a different site that does not affect the *NQO* gene cluster.

Several attempts have been made to complement the deletion in Pd92-223 using broad-host-range expression plasmids, but thus far they all failed. However, preliminary experiments to replace the chromosomal copy of the kanamycin-resistance gene in Pd92-223 with a fragment containing *nqo8*, *nqo9* and a streptomycin resistance gene appear to be successful (not shown).

An additional interesting outcome of the present study is the assembly of NDH2 of *E. coli* in *P. denitrificans* as a membrane enzyme that preserves its substrate specificity (Fig. 2B). It may be noted that hydropathy analysis of the *ndh* gene product according to Kyte and Doolittle [18] does not reveal any hydrophobic segments that are long enough to form a transmembrane α-helix (not shown). Previous studies, however, have shown that isolation of NDH2 requires detergent extraction, and the phospholipid content of the purified enzyme is high [19,20]. Hydrophobic interaction with the membrane is also observed when NDH2 is expressed in *P. denitrificans* because the NDH2 activity in Pd92-223 was not affected by washing the membranes in the presence of 200 mM NaCl, nor was it sensitive to the presence of 500 mM KCl prior and during the assay (results not shown). This observation renders support to the possibility that the interaction of NDH2 with the membrane stems from a protein structure that penetrates into the lipid bilayer rather than from a post-translation modification such as acylation or from an electrostatic interaction with the membrane surface.

The lack of a transmembrane α-helix or any other membrane-spanning segment in an integral membrane protein is not unique, as shown by the 3-D structure of prostaglandin H_2 synthase [21]. Another example of a monotopic membrane protein is the (*S*)-mandelate dehydrogenase (MDH) from *Pseudomonas putida* the membrane binding domain of which was identified by homology search and mutagenesis [22]. In an attempt to identify a segment in NDH2 that may be involved in membrane binding, and perhaps also in ubiquinone binding, a homology search was carried out using the proposed membrane binding segment of MDH [22]. Interestingly, while the overall homology between these proteins is negligible, there is 32% identity between part of the putative membrane binding domain of MDH and the indicated segment of NDH2 (Fig. 4). Whether or not the NDH2 segment shown in Fig. 4 is indeed in contact with the membrane can now be tested experimentally.

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References

[1] Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) Eur. J. Biochem. 197, 563–576.
[2] Walker, J.E. (1992) Q. Rev. Biophys. 25, 253–324.
[3] Finel, M. (1993) J. Bioenerg. Biomembr. 25, 357–366.
[4] Yagi, T., Yano, T. and Matsuno-Yagi, A. (1993) J. Bioenerg. Biomembr. 25, 339–345.
[5] Leif, H., Sled, V.D., Ohnishi, T., Weiss, H. and Friedrich, T. (1995) Eur. J. Biochem. 230, 538–548.
[6] Albracht, S.P.J., Van Verseveld, H.W., Hagen, W.R. and Kalkman, M.L. (1980) Biochim. Biophys. Acta 593, 173–186.
[7] Meinhardt, S.W., Kula, T., Yagi, T., Lillich, T. and Ohnishi, T. (1987) J. Biol. Chem. 262, 9147–9153.
[8] Sled, V.D., Friedrich, T., Leif, H., Weiss, H., Meinhardt, S.W., Fukumori, Y., Calhoun, M.W., Gennis, R.B. and Ohnishi, T. (1993) J. Bioenerg. Biomembr. 25, 347–356.
[9] Yagi, T. (1991) J. Bioenerg. Biomembr. 23, 211–225.
[10] Matsushita, K., Ohnishi, T. and Kaback, R. (1987) Biochemistry 26, 7732–7737.
[11] Xu, X., Matsuno-Yagi, A. and Yagi, T (1993) Biochemistry 32, 986–981.
[12] Young, I.G., Rogers, B.L., Campbell, H.D., Jaworowski, A. and Shaw, D.C. (1981) Eur. J. Biochem. 116, 165–170.
[13] Finel, M. and Majander, A. (1994) FEBS Lett. 339, 142–146.
[14] Harms, N., De Vries, G.E., Maurer, K., Hoogendijk, J. and Stouthamer, A.H. (1987) J. Bacteriol. 169, 3969–3975.
[15] Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikström, M. and Saraste, M. (1989) EMBO J. 8, 3571–3579.
[16] Xu, X., Matsuno-Yagi, A. and Yagi, T (1992) Arch. Biochem. Biophys. 296, 40–48.
[17] Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B. and Weiss, H. (1989) Eur. J. Biochem. 180, 173–180.
[18] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[19] Jaworowski, A., Campbell, H.D., Poulis, M.I. and Young, I.G. (1981) Biochemistry 20, 2041–2047.
[20] Jaworowski, A., Mayo, G., Shaw, D.C., Campbell, H.D. and Young, I.G. (1981) Biochemistry 20, 3621–3628.
[21] Picot, D., Loll, P.J. and Garavito, M. (1994) Nature 367, 243–249.
[22] Mitra, B., Gerlt, J.A., Babbitt, P.C., Koo, C.W., Kenyon, G.L.,

Joseph, D. and Petsko, G.A. (1993) Biochemistry 32, 12959–12967.
[23] De Gier, J.W.L., Lübben, M., Reijnders, W.N.M., Tipker, C.A., Slotboom, D.J., Van Spanning, R.J.M., Stouthamer, A.H. and Van der Oost, J. (1994) Mol. Microbiol. 13, 183–196.
[24] Simon, R., Priefer, U. and Pühler, A. (1983) in: Molecular Genetics of Bacteria-Plant Interaction (Pühler, A. ed.) pp 98–106, Springer, Berlin.
[25] Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) Biotechniques 5, 376–379.
[26] Yanish-Perron, C., Vierra, J. and Messing, J. (1985) Gene 33, 103–119.