

Nickel-Resistance-Based Minitransposons: New Tools for Genetic Manipulation of Environmental Bacteria

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Received 5 October 2000/Accepted 27 November 2000

The *ncc* and *nre* nickel resistance determinants from *Ralstonia eutropha*-like strain 31A were used to construct mini-Tn5 transposons. Broad host expression of nickel resistance was observed for the *nre* minitransposons in members of the α , β , and γ subclasses of the *Proteobacteria*, while the *ncc* minitransposons expressed nickel resistance only in *R. eutropha*-like strains.

Several nickel resistance determinants have been identified in *Ralstonia eutropha* (*Alcaligenes eutrophus*) (24) strains isolated from different biotopes heavily polluted with heavy metals. The *cnr*YXHCBA operon of *R. eutropha* CH34 plasmid pMOL28 (12), which mediates medium levels of nickel resistance (up to 10 mM) and cobalt resistance, is the most thoroughly studied determinant (3, 11, 17, 18, 20). The resistance mechanism mediated by *cnr* is inducible and is due to an energy-dependent efflux system driven by a chemo-osmotic proton-antiporter system (6, 18, 22, 23). A 14.5-kb *Bam*HI fragment of plasmid pTOM9 from *R. eutropha*-like strain 31A (*Alcaligenes xylooxidans* 31A) (10) and a similar *Bam*HI fragment of plasmid pGOE2 from *R. eutropha*-like strain KTO2 were also found to encode Ni resistance. On both fragments a locus mediating high-level nickel resistance (up to 20 to 50 mM) and a locus mediating low-level nickel resistance (3 mM) were identified and designated *ncc* and *nre*, respectively (15, 16). The *ncc*YXHCBA determinant, which except for the *nccN* gene is very similar to *cnr*, causes high levels of nickel and cobalt resistance and a low level of cadmium resistance in *R. eutropha*. Neither *cnr* nor *ncc* is expressed in *Escherichia coli*. On the other hand, the 1.8-kb *nre* locus causes low levels of nickel resistance in both *Ralstonia* and *E. coli* (16). An *nre*-like determinant, which could be expressed in *E. coli* and *Citrobacter freundii*, was also found in *Klebsiella oxytoca* CCUG15788 (19, 20).

Recently, amplified ribosomal DNA restriction analysis was used to determine the phylogenetic position of zinc- and nickel-resistant *Ralstonia*-like strains (2). The *ncc* operon was found in many nickel-resistant *R. eutropha*-like strains and in environmental strains in the direct vicinity of the genus *Burkholderia* (2), a member of the β subclass of the class *Proteobacteria* like the genus *Ralstonia*. This might indicate that *ncc* has range of expression broader than the genus *Ralstonia*.

Heavy metal resistance markers with broad host expression ranges have been shown to be useful for genetic manipulation of *Pseudomonas* strains potentially designated for environmen-

tal release (14). Broad-host-range expression of *ncc-nre* was recently confirmed by Dong et al. (7), who found *ncc-nre*-based Ni resistance in *Comamonas*, *Sphingobacterium heparinum*, flavobacteria, and even gram-positive bacteria related to *Arthrobacter*. However, it was not clear from this study which of the Ni resistance determinants was responsible for the broad-host-range Ni resistance. In addition, plasmid instability problems were encountered with some of the transconjugants. In order to study the range of expression of *ncc* and *nre* and to develop new tools for genetic manipulation of environmental bacteria, which are not based on antibiotic resistance markers, the Ni resistance markers were introduced into mini-Tn5 transposon vectors. The new *nre*-based minitransposons were found to have a broad expression range and were successfully used for constructing Ni-resistant transconjugants of plant-associated bacteria belonging to families of the α , β , and γ subclasses of the class *Proteobacteria*, including plant-associated endophytic bacteria with potential to improve phytoremediation strategies (C. Lodewyckx, S. Taghavi, M. Mergeay, J. Vangronsveld, H. Clijsters, and D. van der Lelie, submitted for publication).

Construction of Ni resistance minitransposons. The *ncc* operon of pTOM9 was cloned in pUC18/*Not*I as a 8.1-kb *Bam*HI-*Pst*I fragment, resulting in pMOL1522 (*E. coli* CM2395). Plasmid pMOL1522 was digested with *Not*I, and the *ncc*-containing *Not*I fragment was subsequently cloned in the unique *Not*I site of pUTmini-Tn5-Km1 (4). This resulted in plasmid pUTminiTn5-Km1/*ncc* (pMOL1524 in *E. coli* CM2428).

In order to construct an *nre*-based mini-Tn5 transposon vector, it was necessary to inactivate the *Not*I site in *nreB*. PCR mutagenesis performed with primers *nre-Pst*I (sense) and *nre-Not*I (antisense) and with primers *nre-Not*I (sense) and *nre-Eco*RI (antisense) (Table 1) was used to change the *Not*I site with the sequence GCGGCCGC into GCGCAGC. This resulted in two PCR fragments that were approximately 1.6 and 1.1 kb long, respectively. Subsequently, these fragments were mixed and joined by using a PCR-based ligation strategy and were amplified with primers *nre-Pst*I (sense) and *nre-Eco*RI (antisense). This resulted in a 2.7-kb *Pst*I-*Eco*RI fragment with the mutated *nre* operon. The mutation did not affect the amino acid sequence of the NreB protein, since GCC and GCA both

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TABLE 1. Primers used for PCR mutagenesis and amplification of the *nre* region^a

Primer	Start position	End position	Sequence (5'-3')	Comment
<i>nre-PstI</i> (sense)	1477	1510	CGC <u>CTG CAG</u> CGC AGA CCG TGG CGG CAG CGG CGC C	
<i>nre-PstI</i> (antisense)	4183	4160	AAA <u>CTG CAG</u> CCC GGA TTG AAA ATG CGA CTC ATG	AAA CTG CAG was added at the 5' end
<i>nre-EcoRI</i> (antisense)	4189	4160	AAA <u>GAA TTC</u> CCC GGA TTG AAA ATG CGA CTC ATG	AAA was added at the 5' end
<i>nre-SfiI</i> (sense)	2234	2286	GGA GAG CGC CGT GAC CCA GGC gAA GAA GGC gCT GGT GCA TGA CCA TAT CGA CC	Mutations (C to G) are indicated by lowercase g
<i>nre-SfiI</i> (antisense)	2286	2234	GGT CGA TAT GGT CAT GCA CCA GcG CCT TCT TcG CCT GGG TCA CGG CGC TCT CC	Mutations (G to C) are indicated by lowercase c
<i>nre-NotI</i> (sense)	3107	3164	GAA GGG ACT GCT GGC GCT GAA TCT TGC CGC GGC aGC AGC CAG CGC CAT GGT GAT CGT G	Mutation (C to A) is indicated by lowercase a
<i>nre-NotI</i> (antisense)	3164	3107	CAC GAT CAC CAT GGC GCT GGC TGC tGC CGC GGC AAG ATT CAG CGC CAG CAG TCC CTTC	Mutation (G to T) is indicated by lowercase t

^a The positions of the relevant restriction sites used for cloning of the amplified PCR fragments are underlined in the primer sequences. Sequence positions are based on the *nre* sequence (GenBank accession number L31491 [10]).

encode alanine. The 2.7-kb *PstI-EcoRI* fragment was subsequently cloned into pUC18/*NotI*, resulting in plasmid pMOL1525 (*E. coli* CM2438). Plasmid pMOL1525 was digested with *NotI*, and the *nre*-containing *NotI* fragment was cloned in the unique

NotI site of pUTmini-Tn5-Km1. This resulted in plasmid pUTminiTn5-Km1/*nre(NotI)* (pMOL1527 in *E. coli* CM2442).

The region with the mutated *nreB* gene was also amplified as a 2.7-kb *PstI* fragment by using primers *nre-PstI* (sense) and

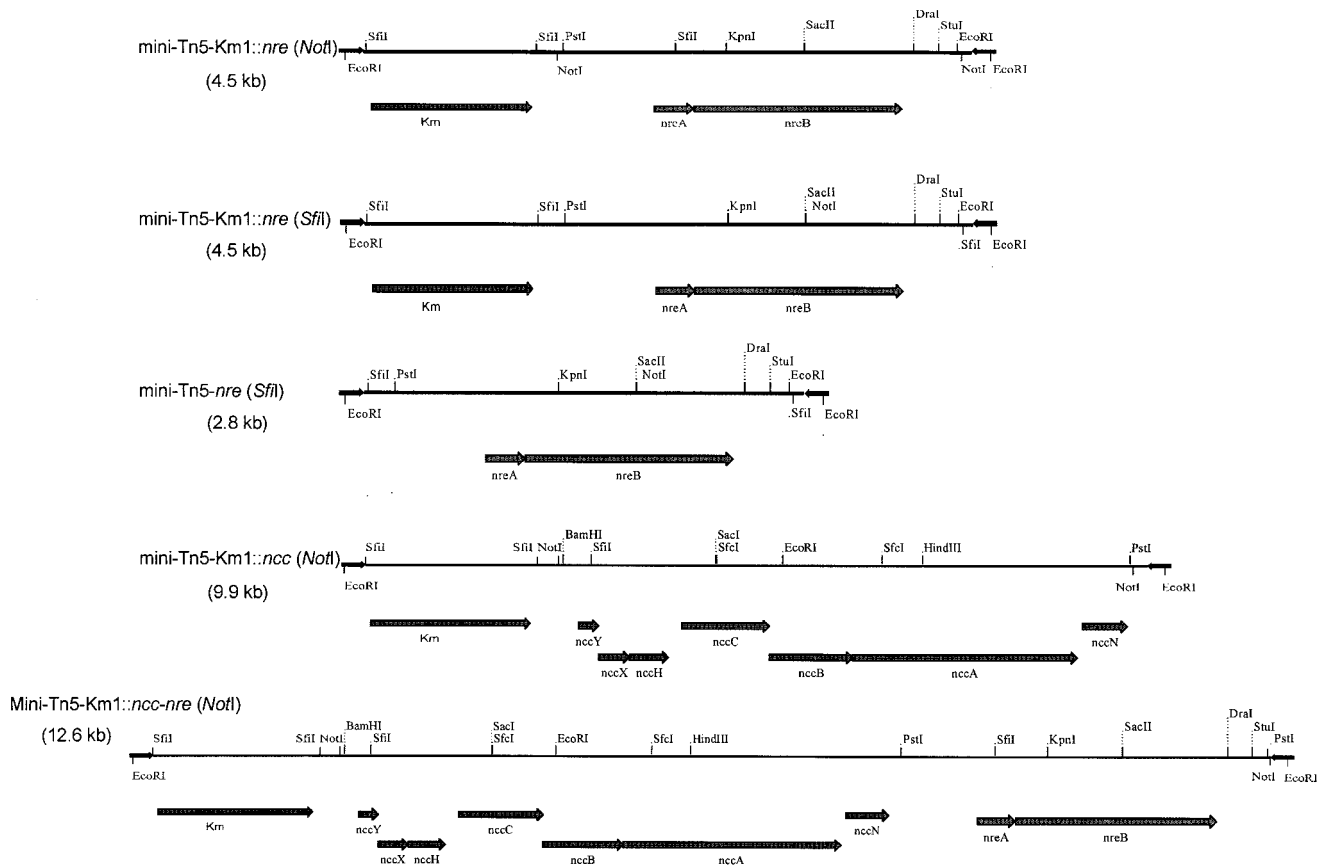


FIG. 1. Schematic representation of different mini-Tn5 Ni resistance transposons. The positions of the kanamycin resistance marker (Km), the Ni resistance determinants *ncc* and *nre*, the inverted repeats at the extremities of the minitransposons, and important restriction sites are indicated. The sizes of the minitransposons are given in parentheses.

TABLE 2. Mutation and transconjugation frequencies of the strains used to test transfer and heterologous expression of *ncc*, *nre*, and *ncc* plus *nre*^a

Donor	Recipient	Frequency of transconjugation		Frequency of mutation	
		Ni resistant	Kanamycin resistant	Ni resistant	Kanamycin resistant
CM2676 (<i>nre</i> Km ^r)	AE104	9.2×10^{-5}		$<7.1 \times 10^{-10}$	2.4×10^{-8}
	DH10B	4.6×10^{-6}		$<4.2 \times 10^{-10}$	$<4.2 \times 10^{-10}$
	LMG2284	9.7×10^{-8}		$<1.8 \times 10^{-9}$	1.3×10^{-8}
	W1.2	7.1×10^{-10}		$<4.5 \times 10^{-10}$	$<4.5 \times 10^{-10}$
	LS2.4	5.8×10^{-8}		$<0.8 \times 10^{-9}$	5.7×10^{-8}
	A15	1.7×10^{-9}		$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
	VM0433	3.9×10^{-6}		$<2.5 \times 10^{-10}$	3.5×10^{-8}
	CM2677 (<i>nre</i>)	AE104	9.5×10^{-5}		
	DH10B	9×10^{-6}			
	LMG2284	1.1×10^{-7}			
	W1.2	$<4.5 \times 10^{-10}$			
	LS2.4	2.7×10^{-8}			
	A15	9.6×10^{-9}			
	VM0433	3.7×10^{-6}			
CM2536 (<i>ncc</i> Km ^r)	AE104	1.5×10^{-5}	8.2×10^{-6}		
	DH10B	$<4.2 \times 10^{-10}$	2.8×10^{-7}		
	LMG2284	$<1.8 \times 10^{-9}$	1.2×10^{-7}		
	W1.2	$<4.5 \times 10^{-10}$	1.3×10^{-9}		
	LS2.4	$<0.8 \times 10^{-9}$	2.3×10^{-7}		
	A15	$<1.3 \times 10^{-10}$	3.3×10^{-9}		
	VM0433	$<2.5 \times 10^{-10}$	1.4×10^{-7}		
	CM2520 (<i>ncc nre</i> Km ^r)	AE104	8.6×10^{-7}		
	DH10B	1.8×10^{-7}			
	LMG2284	1.4×10^{-8}			
	W1.2	1.1×10^{-9}			
	LS2.4	2×10^{-9}			
	A15	1.3×10^{-9}			
	VM0433	1.5×10^{-7}			

^a The strains used as recipients in the matings were *R. eutropha* AE104, *E. coli* DH10B (Gibco BRL), *H. seropedicae* LMG2284, *B. cepacia* W1.2 and LS2.4, *P. stutzeri* A15, and *P. putida* VMO433. *E. coli* S17-1 (λ pir) (5) containing pUTminiTn5-Km1:*ncc-nre* (strain CM2520), pUTminiTn5-Km1:*ncc* (strain CM2536), pUTminiTn5-Km1:*nre* (strain CM2676), and pUTminiTn5-*nre* (strain CM2677) were used as donors. Ni-resistant transconjugants were selected on 284 minimal medium containing 1 mM NiCl₂. Most of the kanamycin-resistant transconjugants were selected on 284 minimal medium (12) containing 100 mg of kanamycin per liter; the only exception was strain AE104, whose transconjugants were selected on 284 minimal medium containing 1,000 mg of kanamycin per liter.

nre-PstI (antisense). Subsequently, this fragment was cloned in the unique *PstI* site of pMOL1522. This resulted in a 10.8-kb *ncc-nre* fragment flanked by two *NotI* sites (plasmid pMOL1548 in *E. coli* CM2500). This fragment was subsequently cloned in the unique *NotI* site of pUTmini-Tn5-Km1, resulting in plasmid pUTminiTn5-Km1/*ncc-nre* (*NotI*) (pMOL1554 in *E. coli* CM2520).

To inactivate the *SfiI* site in *nreA*, we used a strategy similar to that used for mutation of the *NotI* site, except that primers *nre-PstI* (sense) and *nre-SfiI* (antisense) and primers *nre-SfiI* (sense) and *nre-EcoRI* (antisense) were used. The mutations did not affect the amino acid sequence of the NreA protein. A 2.7-kb *PstI-EcoRI* fragment with the mutated *nre* operon (*SfiI* site) was subsequently cloned in pUC18/*SfiI* (8), resulting in plasmid pMOL1526 (*E. coli* CM2440). Plasmid pMOL1526 was digested with *SfiI*, and the *nre*-containing *SfiI* fragment was cloned in *SfiI*-digested pUTmini-Tn5-Km1. This resulted in plasmids pUTminiTn5-Km1/*nre*(*SfiI*) (pMOL1529 in *E. coli* CM2446) and pUTminiTn5-*nre*(*SfiI*) (pMOL1528 in *E. coli* CM2444), in which the kanamycin resistance gene was replaced by *nre*. The restriction maps of the mini-Tn5 Ni resistance transposons are presented in Fig. 1.

Range of expression of Ni resistance. The range of expression of Ni resistance was examined for all mini-Tn5 Ni resistance transposons. To do this, the pUT-based constructs were

introduced into *E. coli* S17-1 (λ pir) (5) and subsequently transferred by conjugation into the nickel-sensitive strains *R. eutropha* AE104 (12), *E. coli* DH10B, *Burkholderia cepacia* W1.2 (isolated from wheat) and LS2.4 (isolated from lupine shoots) (a gift from K. Ophel-Keller), *Herbaspirillum seropedicae* LMG2284 (associated with rye grass) (1), *Pseudomonas stutzeri* A15 (associated with rice roots) (13, 25), *Azospirillum irakense* KBC1 (a rice endophyte) (9), and *Pseudomonas putida* VMO433. The last strain was isolated as an endophytic bacterium after surface sterilization of *Brassica napus* plants (Lodewyckx and van der Lelie, unpublished data). Transfer frequencies, as well as the appearance of nickel- and kanamycin-resistant mutants, were examined. The results are presented in Table 2.

Comparing the efficiencies of transfer of the minitransposons, we observed that *R. eutropha* AE104, *E. coli* DH10B, and *P. putida* VMO433 showed the highest transfer frequencies. The lowest transfer frequencies were observed for *B. cepacia* W1.2 and *P. stutzeri* A15; this might have been due to the presence of efficient restriction-modification systems present in these two strains.

No spontaneous Ni-resistant mutants were found for the strains used in the experiments; this is in contrast to the kanamycin-resistant mutants that were observed at low frequencies ($\sim 10^{-8}$) for most of the strains tested. This indicates that

TABLE 3. MICs of Ni in 284 minimal medium for wild-type strains and their Ni resistance transconjugants

Strain	MICs (mM) ^a				
	Wild type	MiniTn5-Km1:: <i>ncc</i> (<i>NorI</i>)	MiniTn5-Km1:: <i>nre</i> (<i>SfiI</i>)	MiniTn5:: <i>nre</i> (<i>SfiI</i>)	MiniTn5-Km1:: <i>ncc-nre</i> (<i>NotI</i>)
<i>Ralstonia metallidurans</i> AE104	0.6	20–40	3–4	3–4	>40
<i>Herbaspirillum seropedicae</i> LMG2284	0.4	0.4	3–4	3–4	2
<i>Burkholderia cepacia</i>					
W1.2	0.4	0.4	0.4	ND ^b	2
LS2.4	0.6	0.6	2	2	2
<i>Pseudomonas putida</i> VM0433	0.6	0.6	3–4	3	3
<i>Pseudomonas stutzeri</i> A15	0.6	1	3–4	2–3	3
<i>Escherichia coli</i> DH10B	≤0.6	≤0.6	3	2	2–3

^a The ranges of the MICs were determined with four individual transconjugants.

^b ND, not determined (no transconjugants available).

nickel resistance is a more reliable marker for selecting transconjugants than kanamycin.

Transconjugants were selected for kanamycin or nickel resistance (Table 2). The stabilities of the transconjugants were confirmed by growing them for more than 100 generations under nonselective conditions. Subsequently, the Ni resistance of these organisms was compared to that of the wild-type strains. As expected, both *ncc*- and *nre*-containing mini-Tn5 transposons gave Ni resistance in *R. eutropha* AE104, and the MICs on 284 gluconate medium (Lodewyckx et al., submitted) were 3 and 40 mM Ni for *nre* and *ncc*, respectively (Table 3).

For all of the other strains tested except *B. cepacia* W1.2, Ni resistance was observed when the *nre* determinant was present. For these strains miniTn5-Km1/*ncc*-containing transconjugants had to be selected for kanamycin resistance. The presence of *nre* resulted in MICs of Ni for Ni resistance on 284 minimal medium with an appropriate C source that varied from 2 to 3 mM depending on the bacterial species (Table 3). In all cases the presence of *nre* was confirmed by PCR (results not shown). No Ni resistance was observed for transconjugants containing *ncc*, and the presence of both *ncc* and *nre* in general did not increase the MIC for Ni resistance, as determined for *nre*. However, two exceptions were found: in *P. stutzeri* A15 the presence of *ncc* resulted in an increase in Ni resistance (MIC) from 0.6 to 1.0 mM, while *B. cepacia* W1.2 transconjugants showed Ni resistance only when both *ncc* and *nre* were present. The latter phenomenon might imply that both *ncc* and *nre* contribute to Ni resistance, but it is not clear in what way. Therefore, it can be concluded that in general broad-host-range Ni resistance is encoded by *nre* and that the *ncc* determinant is expressed only in *R. eutropha*-like strains. This implies that only the *nre*-based nickel resistance minitransposons, such as miniTn5-*nre*(*SfiI*), are suitable as broad-host-range selection markers for construction of antibiotic resistance-free but selectable strains belonging to the families of the α , β , and γ subclasses of the class *Proteobacteria*.

This work was financially supported by the European Commission and OVAM as an EFRO project.

We are grateful to K. Ophel-Keller and J. Balandreau for providing the *B. cepacia* strains used in this study and to J. Vanderleyden and M. Gillis for providing the *H. seropedicae* strain. We also thank T. Engelen and A. Bossus for technical assistance.

REFERENCES

- Baldani, J. I., V. D. L. Baldani, F. L. Olivares, G. Kirchof, A. Hartmann, B. Pot, B. Hoste, E. Falsen, K. Kersters, M. Gillis, and J. Döbereiner. 1996. Emended description of *Herbaspirillum*; inclusion of (*Pseudomonas*) *rubrisubalbicans*, a milk plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species. *Int. J. Syst. Bacteriol.* **46**:802–810.
- Brim, H., M. Heyndrickx, P. De Vos, A. Wilmotte, D. Springael, H. G. Schlegel, and M. Mergeay. 1999. Amplified rDNA restriction analysis and further genotypic characterisation of metal-resistant soil bacteria and related facultative hydrogenotrophs. *Syst. Appl. Microbiol.* **22**:258–268.
- Collard, J.-M., A. Provoost, S. Taghavi, and M. Mergeay. 1993. A new type of *Alcaligenes eutrophus* CH34 zinc resistance generated by mutations affecting regulation of the *cnr* cobalt-nickel resistance system. *J. Bacteriol.* **175**:779–784.
- de Lorenzo, V., M. Herrero, U. Jakubzik, and K. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
- de Lorenzo, V., and K. N. Timmis. 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5 and Tn10-derived mini-transposons. *Methods Enzymol.* **235**:386–405.
- Diels, L., Q. Dong, D. van der Lelie, W. Baeyens, and M. Mergeay. 1995. The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. *J. Ind. Microbiol.* **14**:142–153.
- Dong, Q., D. Springael, J. Schoeters, G. Nuyts, M. Mergeay, and L. Diels. 1998. Horizontal transfer of bacterial heavy metal resistance genes and its applications in activated sludge systems. *Water Sci. Technol.* **37**:465–468.
- Herrero, M., V. de Lorenzo, and K. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Khammas, K. M., E. Ageron, P. A. D. Grimont, and P. Kaiser. 1989. *Azospirillum irakense* sp. nov., nitrogen fixing bacterium associated with rice roots and rhizosphere soil. *Res. Microbiol.* **140**:679–693.
- Lemke, K. 1994. Schwermetallresistenz in den zwei *Alcaligenes*-Stämmen *A. eutrophus* CH34 und *A. xyloxydans* 31A: Transposonmutagenese, Klonierung und Sequenzierung. Ph.D. thesis. Universität Göttingen. Cuvillier Verlag Göttingen, Göttingen, Germany.
- Liesegang, H., K. Lemke, R. A. Siddiqui, and H. G. Schlegel. 1993. Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. *J. Bacteriol.* **175**:767–778.
- Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. Van Gijsegem. 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J. Bacteriol.* **162**:328–334.
- Qiu, Y. S., S. P. Zhou, X. Z. Mo, D. Wang, and J. H. Hong. 1981. Study of nitrogen fixing bacteria associated with rice root. *Acta Microbiol. Sin.* **21**:468–472. (In Chinese.)
- Sanchez-Romero, J. M., R. Diaz-Orejas, and V. de Lorenzo. 1998. Resistance to tellurite as a selection marker for genetic manipulations of *Pseudomonas* strains. *Appl. Environ. Microbiol.* **64**:4040–4046.
- Schmidt, T., R.-D. Stoppel, and H. G. Schlegel. 1991. High-level nickel resistance in *Alcaligenes xyloxydans* 31A and *Alcaligenes eutrophus* KTO2. *Appl. Environ. Microbiol.* **57**:3301–3309.
- Schmidt, T., and H. G. Schlegel. 1994. Combined nickel-cobalt-cadmium resistance encoded by the *ncc* locus of *Alcaligenes xyloxydans* 31A. *J. Bacteriol.* **176**:7045–7054.
- Sensfuss, C., and H. G. Schlegel. 1988. Plasmid pMOL28-encoded resistance

- to nickel is due to specific efflux. FEMS Microbiol. Lett. **55**:295–298.
18. **Siddiqui, R. A., K. Benthin, and H. G. Schlegel.** 1989. Cloning of pMOL28-encoded nickel resistance genes and expression of the genes in *Alcaligenes eutrophus* and *Pseudomonas* spp. J. Bacteriol. **171**:5071–5078.
 19. **Stoppel, R. D., M. Meyer, and H. G. Schlegel.** 1995. The nickel resistance determinant cloned from the enterobacterium *Klebsiella oxytoca*: conjugational transfer, expression, regulation and DNA homologies to various nickel-resistant bacteria. Biometals **8**:70–79.
 20. **Stoppel, R.-D., and H. G. Schlegel.** 1995. Nickel-resistant bacteria from anthropogenically nickel-polluted and naturally nickel-percolated ecosystems. Appl. Environ. Microbiol. **61**:2276–2285.
 21. **Taghavi, S., M. Mergeay, and D. van der Lelie.** 1997. Genetic and physical maps of the *Alcaligenes eutrophus* CH34 megaplasmid pMOL28 and its derivative pMOL150 obtained after temperature induced mutagenesis and mortality (TIMM). Plasmid **37**:22–34.
 22. **Tibazarwa, C., S. Wuertz, M. Mergeay, L. Wyns, and D. van der Lelie.** 2000. Regulation of the *cnr* cobalt and nickel resistance determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34. J. Bacteriol. **182**:1399–1409.
 23. **Varma, A. K., C. Sensfuss, and H. G. Schlegel.** 1990. Inhibitor effects on the accumulation and efflux of nickel ions in plasmid pMOL28-harboring strains of *Alcaligenes eutrophus*. Arch. Microbiol. **154**:42–49.
 24. **Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi.** 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. **39**:897–904.
 25. **You, C. B., X. Li, H. X. Wang, Y. S. Qiu, X. Z. Mo, and Y. L. Zhang.** 1983. Associative nitrogen fixation of *Alcaligenes faecalis* with rice plant. Biol. N₂ Fixation Newsl. Sydney **11**:92–103.