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RESEARCH ARTICLE

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Conjugative plasmid mediated inducible nickel resistance in *Hafnia alvei* 5-5

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Abstract *Hafnia alvei* 5-5, isolated from a soil-litter mixture underneath the canopy of the nickel-hyperaccumulating tree *Sebertia acuminata* (Sapotaceae) in New Caledonia, was found to be resistant to 30 mM Ni^{2+} or 2 mM Co^{2+}. The 70-kb plasmid, pEJH 501, was transferred by conjugation to *Escherichia coli*, *Serratia marcescens*, and *Klebsiella oxytoca*. Transconjugant strains expressed inducible nickel resistance to between 5 and 17 mM Ni^{2+}, and cobalt resistance to 2 mM Co^{2+}. A 4.8-kb *Sal–Eco*RI fragment containing the nickel resistance determinant was subcloned, and the hybrid plasmid was found to confer a moderate level of resistance to nickel (7 mM Ni^{2+}) even to *E. coli*. The expression of nickel resistance was inducible by exposure to nickel chloride at a concentration as low as 0.5 mM Ni^{2+}. By random Tn*phoA′*-1 insertion mutagenesis, the fragment was shown to have structural genes as well as regulatory regions for nickel resistance. Southern hybridization studies showed that the nickel-resistance determinant from pEJH501 of *H. alvei* 5-5 was homologous to that of pTOM9 from *Alcaligenes xylosoxydans* 31A.

Keywords Transconjugant · Nickel-resistant bacteria · Inducible nickel resistance · Tn*phoA′*-1 insertion mutagenesis

Abbreviations *MIC* Minimal inhibitory concentration · *CFUs* Colony-forming units · *ars* Arsenite · *cnr* Cobalt and nickel resistance · *czc* Cadmium, zinc and cobalt · *ncc* Nickel and cobalt · *nre* Nickel resistance · Tn Transposon

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Introduction

The population of nickel-resistant bacteria in heavy-metal-containing mine drainage and cooling waters of the metal-processing industry is higher than in typical municipal drainage. The predominant bacteria found in such nickel-polluted environments are *Ralstonia metallidurans* strain CH34 (formerly *Alcaligenes eutrophus* CH34) [1], *A. denitrificans* strain 4a-2 [28, 31], *A. xylosoxydans* strain 31A [20, 21, 22], *Ralstonia* sp. strain KTO2 [21, 31], and *Klebsiella oxytoca* CCUG 15788 [29]. Some of these bacteria have been studied in detail, and some of the nickel-resistance determinants have been cloned and sequenced. Among the bacterial nickel-resistance genes, those of *R. metallidurans* CH34 have been studied in detail [3, 9, 11, 15]. Strain CH34 is a facultative chemolithotroph that harbors two large metal-resistance plasmids. One plasmid, pMOL28 (180 kb), hosts an operon responsible for inducible resistance to cobalt and nickel (*cnr*), and encodes for an energy-dependent efflux system [23, 24, 30]. The plasmid is self-transmissible in intrastrain matings and has been transferred to other strains of *R. eutropha* and cured strains. The phenotypes of transconjugants are characterized by the ability to tolerate 3 mM $NiCl_2$ and 5 mM $CoCl_2$ on solid media and in liquid media [28]. The plasmid *cnr* determinant was cloned previously, and the plasmid containing an 8.5-kb *Eco*RI–*Pst*I fragment complemented the nickel-sensitive Tn5 mutant of *R. eutropha* with full nickel and cobalt resistance [9, 16]. The hybrid plasmid also conferred nickel and cobalt resistance to *R. eutropha* H16, *R. hydrogenophilus*, *Pseudomonas putida*, and *P. oleovorans*. Seven ORFs for *cnr* genes were found: *cnrC*, *cnrB*, and *cnrA* encode potentially structural proteins; *cnrH*, *cnrR* and *cnrY* are probably involved in the regulation of expression [9]. The other plasmid, pMOL30 (238 kb), contains the operon for resistance to cobalt, zinc, and cadmium (*czc*), which is carried out by a chemiosmotic proton-antiporter-mediated efflux of cations [14, 17].

Stoppel and Schlegel [28] used the soil from the rhizosphere of the New Caledonian tree *Sebertia acuminata*, a nickel-hyperaccumulating plant, to isolate 56 nickel-resistant bacteria. These strains were studied by DNA-DNA hybridization with the nickel-resistance determinants from strain CH34, *Alcaligenes denitrificans* strain 4a-2, *A. xylosoxydans* strain 31A, and *Klebsiella oxytoca* CCUG 15788 as DNA probes. Some of the nickel-resistant strains were identified as *Acinetobacter* sp., *Pseudomonas mendocina*, *Comamonas* sp., *Hafnia alvei*, *Burkholderia* sp., *Arthrobacter aurescens*, and *A. ramosus* strains. On the basis of their hybridization signals, the nickel-resistance determinants of the strains could be grouped into four classes: (1) *cnr*/*ncc*-type determinants had strong homologies with the *cnr* operon and the *ncc* (nickel-cobalt-cadmium) operon; (2) *crn*/*ncc*/*nre*-type determinants had strong homologies with both the *ncc*/*cnr* operon and the *nre* (nickel resistance determinant) operon; (3) *K.-oxytoca*-type determinants had strong homologies with nickel-resistance determinants of *K. oxytoca* and weak hybridization signals with the *nre* operon; (4) the others had no hybridization reaction with any of the DNA probes used [28]. *Hafnia alvei* 5-5, isolated from a soil sample obtained in New Caledonia, was selected by aerobic enrichment culture designed for nickel-resistant Enterobacteriaceae [27, 28]. This bacterium was found to be highly nickel-resistant and moderately cobalt-resistant.

In this report, we demonstrate that inducible nickel- and cobalt-resistance determinants located on plasmid pEJH501 of *H. alvei* 5-5 hybridize with nickel-resistance genes from *A. xylosoxydans* 31A and *K. oxytoca*. Analysis of Tn*phoA'-1* insertional mutants and of subclones carrying a portion of the resistance determinant demonstrates that the hybrid plasmid, containing a 4.8-kb *Sal*I–*Eco*RI fragment, confers nickel resistance even to *E. coli*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1 and Table 2. *K. oxytoca*, *E. coli*, and *S. marcescens* strains were grown in Luria-Bertani (LB) medium. *H. alvei* 5-5 was isolated and cultured in Tris-gluconate-media as described previously [9, 11]. For maintenance of plasmid markers, antibiotics of filter-sterilized solutions were added, as appropriate, to the following final concentrations: ampicillin (Ap) 100 μg/ml, kanamycin (Km) 30 μg/ml and tetracycline (Tet) 12.5 μg/ml for *E. coli*. Clones of *E. coli* DH5α harboring recombinant pUC plasmids were identified on LB agar plates containing 100 μg Ap/ml , 40 μg5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml and 0.2 mM isopropyl-β-D-thiogalactopyranoside (Duchepa, the Netherlands).

Analytical-grade $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, and $ZnCl_2$ (Sigma, USA) were prepared as 1.0 M stock solutions and sterilized by autoclaving. Resistance to heavy-metal salts was examined in Tris mineral medium supplemented with 0.3% (w/v) gluconate as carbon source [9].

Determination of minimal inhibitory concentration

To determine the lowest concentration of metal salts at which no colony-forming units (CFUs) were observed, cells were grown for 18 h in Tris-gluconate medium at 30 °C (*H. alvei* and *K. oxytoca*) or at 37 °C (*E. coli* and *S. marcescens*). The cells were streaked onto Tris-gluconate agar plates containing various concentrations of metal chlorides. The plates were then incubated at 30 °C and at 37 °C and inspected for growth for up to 4 days.

Table 1 Bacterial strains used in this study. *WT* Wild-type, *r* resistant, *s* sensitive

Strain	Relevant phenotype	Reference or source
Klebsiella oxytoca		
15788	WT, Ni^r, plasmids pKO1 (160 kb) and pKO2 (linear, 50 kb)	[29]
S69	WT, Ni^s	Dr. U. Obst, Wasserforschung, Mainz
M673	Transconjugant of S69 and M622, Ni^r	This study
JCCM1665	WT, Ni^s, type strain	[29]
Hafnia alvei		
5-5	WT, Ni^r	[19]
ATCC13337	WT, Ni^s	
M622	Ni^r, Triple auxotrophic (Trp^-, Leu^-, His^-) mutant of *H.alvei* 5-5	This study
M625	Derivative of M622, containing pULB113	This study
Escherichia coli		
DH5α	*Sup*E44 *lac*U169 (ø80*lac* ZM15) *hsd*R17 *rec*A1 *end*A1 *rel*A1	Gibco BRL
H11	WT, Ap^r, Km^r	
LE392	F– *hsd*S574 *sup*E44 *sup*F58 *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55	Promega
BW13745	DE3 (*lac*)X74 *pho*A532e	[12]
CSH52	*thi*, *pro*, *ara*, *rec*A, Str^r,Su^o	[13]
M661	Transconjugant of H11 and M622, Ni^r	This study
CM214	Carrying plasmid pULB113	[8]
Serratia marcescens		
S15	WT, Ni^s	Dr. U. Obst, Wasserforschung, Mainz
M672	Transconjugant of S15 and M622, Ni^r	This study
Ralstonia metallidurans		
CH34	WT, Ni^r, plasmids pMOL28 and pMOL30	[11]
AE104	Cured mutant of *R. metallidurans* CH34, Ni^s	[11]
Phage		
28	b221 cl857 Pam3	[6]

Table 2 Plasmids usedin this study

Plasmid	Relevant phenotype	Reference
Cloning vectors		
pBluescriptII KS (+)	2.96 kb, Ap^r	Stratagene (USA)
pBR322	4.36 kb, Ap^r, Tc^r	Takara (Japan)
Plasmids		
KOHI4	4.3-kb *Hind*III fragment from *Klebsiella oxytoca* 15788 cloned into pBluescriptII KS(−); Ap^r	[29]
TBA9	14.5-kb *Bam*HI fragment from pTOM9 of *Alcaligenes xylosoxydans* 31A	[21]
TEC9	4.2 kb *Eco*RI fragment from pTOM9 of *A. xylosoxydans* 31A	[21]
PNRS148	4.8-kb *Sal*I–*Eco*RI fragment from PEJH501	This study
pEJH501	About 70 kb from *H. alvei* 5-5	This study
pEJH502	About 3 kb from *H.alvei* 5-5	This study
pHF69	DH5α, Ap^r, about 23 kb,*Bam*HI fragment from pEJH501 cloned into pBR322	This study
pHF24	DH5α, Ap^r, about 8.1 kb, *Bam*HI–*Eco*RI fragment from pHF69 cloned into pBR322	This study
pHF14	DH5α, Ap^r, about 4.8 kb, *Sal*I–*Eco*RI fragment from pHF24 cloned into pBR322	This study
pHF10	DH5α, Ap^r, about 3.1 kb, *Hind*III fragment from pHF14 cloned into pBluescriptII KS(+)	This study
pREC203	DH5α, Ap^r, about 1.9 kb, *Cla*I–*Eco*RI fragment from pHF14 cloned into pBluescriptII KS (+)	This study
pRCS208	DH5α, Ap^r, about 2.6 kb, *Sal*I–*Cla*I fragment from pHF14 cloned into pBluescriptII KS (+)	This study
pRSD401	0.8 kb, *Sma*I fragment from pHF14 cloned into pBluescriptII KS(+)	This study

Inducibility of nickel resistance

Precultures of strains were grown in Tris-gluconate medium containing either no $NiCl_2$ (uninduced cells) or 0.1–1 mM $NiCl_2$ (induced cells). Culture media containing high nickel concentrations (2–30 mM $NiCl_2$) were inoculated with induced or uninduced cells. Cultures were then incubated on a rotary shaker, and growth curves were obtained by measuring the turbidity spectrophotometrically at 600 nm.

Conjugation and genetic techniques

Conjugation was carried out as described previously [8, 21], and standard genetic techniques were used [5, 18]. For Tn*phoA′*-1 mutagenesis of cloned fragment, cells of *E. coli* BW13745 harboring an 8.1-kb *Bam*HI–*Eco*RI fragment in vector pHF24 were infected with λ*P*am::Tn*phoA′*-1 as described by Wilmes-Riesenberg and Wanner [32]. Tn*phoA′*-1 mutants were selected on LB agar containing kanamycin (30 μg/ml). The km^r colonies were washed off the plate, and the plasmid DNA of these mutants was used to transform *E. coli* DH5α. Tn*phoA′*-1-carrying plasmids were identified and analyzed for nickel-sensitive mutation.

For DNA hybridization, DNA restriction fragments were separated by horizontal electrophoresis in 0.8% (w/v) agarose gels and TAE buffer. The DNA was transferred overnight to a nitrocellulose or nylon membrane by capillary transfer and then fixed to the membrane by irradiation at 1,200 J with a UV cross-linker. The membranes were hybridized with biotinylated probes at 42 °C in hybridization buffer [0.5 M NaCl in cold hybridization buffer, 5% (w/v) blocking agent].

Results

Physiological characteristics of *Hafnia alvei* 5-5

H. alvei strain 5-5 is a newly discovered highly nickel-resistant bacterium. It was isolated after enrichment culture selective for *E.-coli*-type bacteria from a soil-litter mixture taken from underneath the canopy of the nickel-hyperaccumulating tree *Sebertia acuminata* in New Caledonia [19]. The isolate was able to grow in media containing nickel chloride up to 30 mM. The original selective medium contained 0.1% nutrient broth, 10 mM nickel, 1% lactose, 2% oxgall, 0.0013% brilliant green, pH 7.0–7.2, and aerobic conditions at 30 °C. The bacterium was identified by the Deutsche Sammlung von Mikroorganismen (DSM) and was assigned the number ID 94-268. The bacterium utilizes the following substrates as carbon and energy sources: gluconate, glucose, fructose, galactose, sucrose, lactose, xylose, arabinose, citrate, lactate, acetate, maltose, caproate, rhamnose, mannitol, CM-cellulose, soluble starch. It forms gas from glucose and is able to produce catalase and nitrate reductase. The bacterium is able to grow at 30, 35, 37, and 40 °C but not at 44 °C. It does not grow autotrophically and does not need any growth-factors.

Resistance to heavy metals

Cells were grown in the appropriate mineral medium and spread on minimal gluconate agar containing various concentrations of Ni^{2+}, and Co^{2+}. The Tris-gluconate medium was used to test for bacterial resistance since the buffer was neither utilized by the bacterium as a nitrogen or carbon source nor did it chelate or precipitate the metals tested at growth-inhibitory ion concentrations [11]. *H. alvei* 5-5 was found to be resistant to metal concentrations as high as 30 mM Ni^{2+} or 2 mM Co^{2+}. In contrast, *H. alvei* strain 13337, which is the type strain and which served as an internal standard, did not grow in the presence of 0.8 mM $NiCl_2$. On the basis of these minimal inhibitory concentrations (MICs), strain 5-5 has to be assigned to the high-level nickel-resistant and medium-level cobalt-resistant bacteria.

H. alvei 5-5 and its triple auxotrophic mutant M622 were shown to contain three plasmids: two of the size of

Table 3 MICs of nickel and cobalt for transconjugants

Strain	MIC (mM)	
	Ni^{2+}	Co^{2+}
H. alvei		
5-5	30	2
M622	15	2
Recipients		
*E. coli*H11	1	0.5
S. marcescens S15	1	0.5
K. oxytoca S69	0.5	0.5
Transconjugants		
E. coli M661	17	2
S. marcescens M672	10	2
K. oxytoca M673	5	2

pULB113 (about 70 and 50 kb) and a very small plasmid (5 kb) (data not shown). Attempts to cure the *Hafnia* cells and isolate plasmid-free or nickel-sensitive cells were unsuccessful.

Characterization of transconjugants

To determine whether the nickel-resistance determinant of *H. alvei* 5-5 was located on a transmissible plasmid, agar mating experiments between *H. alvei* and many potential recipients were conducted (Table 3). To facilitate counterselection against the donor of *H. alvei* 5-5, we used a triple auxotrophic mutant (Trp^-, Leu^-, His^-) of *H. alvei*, *H. alvei* M622, as the donor strain. As a recipient strain AE104, which is the cured mutant of *R. metallidurans* CH34, was initially used; however, no nickel-resistant transconjugants were detected. Therefore, nickel-sensitive wild-type strains of *S. marcescens* S15, and *K. oxytoca* S69 (provided by Dr. U. Obst, Wasserforschung, Mainz) were used. In addition, the ampicillin-resistant strain H11 of *E. coli* (provided by Dr. J. Hacker, Universität Würzburg) and the type strain JCCM1665 of *K. oxytoca* were also tested. Selection for the transconjugants was carried out on minimal Tris-gluconate agar medium containing nickel (and ampicillin for the antibiotic marker of *E. coli* H11). This minimal medium eliminated the donor *H. alvei* M622, due to its amino acid requirements, and the presence of nickel (and Ap) ensured selection only of recipient cells containing a plasmid-borne nickel-resistance determinant. In all cases, the background frequency of nickel resistance of recipient strains in control crosses was less than 10^{-10} per recipient. The number of nickel-resistant transconjugants among about 60 screened potential recipients was very low (Table 3).

To confirm that a plasmid was transferred from *H. alvei* M622 to the transconjugant strains, lysates of the donor, recipients, and transconjugants were examined by agarose gel electrophoresis (Fig. 1). The gel pattern showed that transconjugants contained a single plasmid of about 70 kb that was identical to pEJH 501 from the donor strain *H. alvei* M622. Although in a few

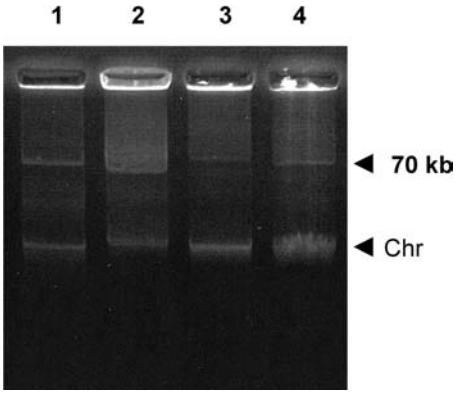

Fig. 1 Electrophoresis patterns of the triple-auxotrophic mutant M622 of the wild-type strain of *Hafnia alvei* 5-5 (*lane 1*) and the nickel-resistant transconjugant of *Escherichia coli* H11, M661 (*lane 2*); the nickel-resistant transconjugant of *Serratia marcescens* S15, M672 (*lane 3*); and the nickel-resistant transconjugant of *Klebsiella oxytoca* S69, M673 (*lane 4*). *Chr* Chromosome

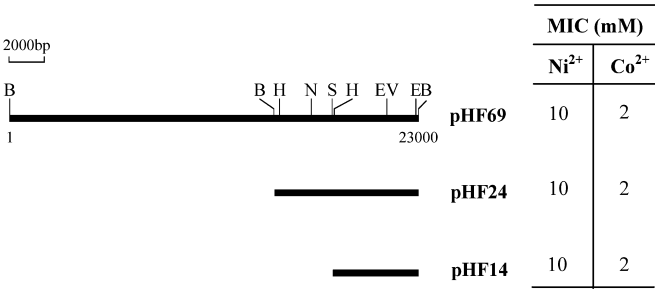

Fig. 2 Restriction map of the 23-kb *Bam*HI–*Bam*HI fragment of pHF69 and subclones pHF24 and pHF14 derived therefrom. On the *right* are the MICs of nickel chloride for the *E. coli* DH5α strains bearing these plasmids. Restriction sites: B, *Bam*HI; H, *Hind*III; S, *Sal*I; N, *Nco*I; E, *Eco*RI; EV, *Eco*RV

cases helper-unassisted transfer was successful, we introduced the RP4::mini-Mu plasmid pULB113 (Ap^r, Km^r, Tc^r) into strain M622 and thus obtained strain M625. With this strain as donor, screening for appropriate recipients was repeated, but we did not isolate more transconjugants than with M622 as donor.

MICs of nickel and cobalt were tested to determine whether or not the plasmid was expressed similarly in the donor and the transconjugant strains. Transconjugant strain *E. coli* M661 expressed the same levels of nickel and cobalt resistance as the donor strain. Transconjugants *K. oxytoca* M673 and *S. marcescens* M672 were less resistant to nickel than the donor and were cobalt-resistant (Table 3).

Cloning and subcloning of the nickel-resistance determinant

Plasmids containing the nickel-resistance determinant were constructed by conventional techniques [5, 18], and their restriction maps are shown in Fig. 2. Plasmid

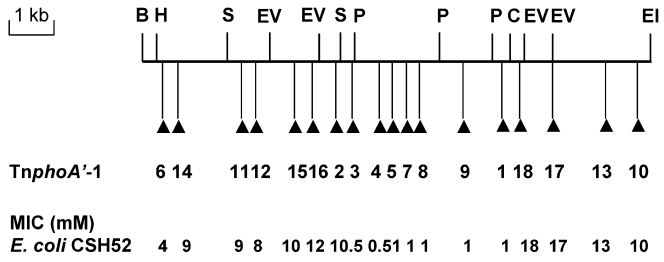

Fig. 3 Physical map of the nickel-resistance determinant with location of Tn*phoA′*-1 insertions. The *line* represents the 8.1-kb *Eco*RI–*Bam*HI fragment of pHF14. On the *bottom* are the insertion sites of Tn*phoA′*-1 in plasmids TM1–TM18 and the MICs of strain *E. coli* CSH52 bearing these plasmids. Restriction sites: B, *Bam*HI; H, *Hind*III; S, *Sal*I; EV, *Eco*RV; P, *Pst*I; C, *CLAI*; E, *Eco*RI

pHF69 was constructed by ligating the 23-kb *Bam*HI-digested DNA from pEJH501 with *Bam*HI-digested pBR322 and transforming the mixture into *E. coli* DH5α with selection for nickel (< 7 mM) resistance. Successful transformation indicated that the nickel resistance of *H. alvei* 5-5 functions in *E. coli*. Plasmid pHF24 was constructed by ligating an 8.1-kb *Bam*HI-*Eco*RI fragment from pHF69 into *Bam*HI–*Eco*RI-digested pBR322. Plasmid pHF14 was prepared by ligating a 4.8-kb *Sal*I–*Eco*RI restriction fragment from plasmid pHF24 into *Sal*I–*Eco*RI-digested pBR322.

Mapping of the nickel-resistance determinant by Tn*phoA′*-1 mutagenesis

To map the structural genes and the regulatory region of the nickel-resistance determinant, a series of independent Tn*phoA′*-1 insertions in those regions of plasmid pHF24 in *E. coli* BW13745 was isolated. To isolate mutants with plasmid insertion, kanamycin-resistant tansductants were selected after infecting cells with *λPam*::Tn*phoA′*-1 phages at a multiplicity of infection of 2.0. The resulting Tn*phoA′*-1-mutated hybrid plasmids from pools of several independent kanamycin resistants were transformed into *E. coli* CSH52 and screened for expression of kanamycin resistance as well as for nickel resistance. Among 165 Tn*phoA′*-1 hybrid pHF24 plasmids, 18 carried Tn*phoA′*-1 insertions within an 8.1-kb *Eco*RI–*Bam*HI fragment. The physical location and orientation of each insertion was determined by restriction endonuclease digestion and Southern analysis (Fig. 3).

Nickel sensitivity (MIC between 0.5 and 1.0 mM Ni^{2+}) resulted from each insertion within a 2.4-kb *Sal*I–*Pst*I fragment. These results suggest that nickel resistance is encoded by the region beginning at the site of Tn*phoA′*-1 in pHF24. The fact that insertions within the 2.4-kb *Sal*I–*Pst*I fragment also produce sensitivity to cobalt (MIC between 0.5 and 1.0 mM Co^{2+}) (data not shown) suggests that the genes for the two resistances probably use the same regulatory region. The insertions in TM17 and TM18, compared with nonmutated pHF24 plasmid, produced enhanced resistance to nickel. The insertion mutants exhibited resistance to 18 and 17 mM Ni^{2+}, respectively, which corresponds to about twice the control MIC. Furthermore, they expressed nickel resistance constitutively, as judged from comparing the growth rates of the mutants with that of the wild-type in the presence of 5 mM Ni^{2+} (data not shown). These results provide evidence for the location of a putative regulatory region between insertion sites TM1 and TM13.

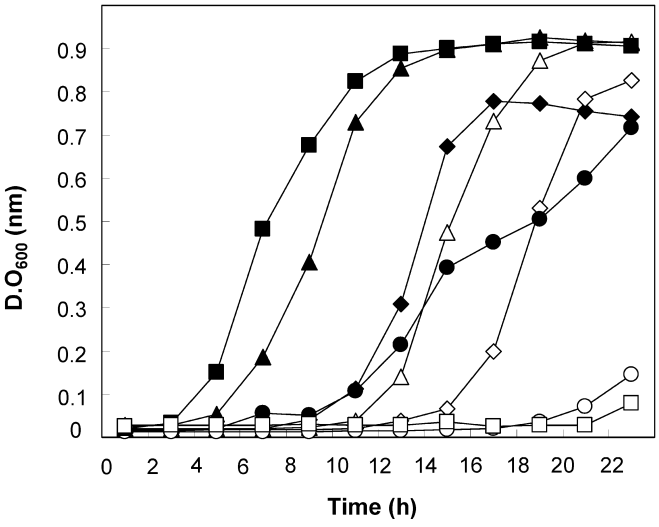

Fig. 4 Induction of nickel resistance in transconjugants. Strains were grown overnight in Tris-gluconate medium without (*open symbols*) or with (*closed symbols*) 1 mM nickel chloride. The cells were diluted in fresh medium, and 4 mM nickel chloride was added to the culture. *Diamonds* Wild-type strain of *H. alvei* 5-5, *triangles* nickel-resistant transconjugant of *E. coli* H11, M661, *circles* nickel-resistant transconjugant of *S. marcescens* S15, M672, *squares* nickel-resistant transconjugant of *K. oxytoca* S69, M673

Induction of nickel resistance

The growth rates of transconjugants and *E. coli* DH5α harboring pEJH501 from *H. alvei* M622 or hybrid plasmid were compared to determine whether nickel resistance was induced at the same levels as in the donor strain. When uninduced *H. alvei* M622 cells with pEJH501 were exposed to 4 mM $NiCl_2$, there was a delay of 14 h before growth commenced (Fig. 4). Pregrowth at 1 mM $NiCl_2$ resulted in a delay of 8 h, and the growth yield was not reduced. However, the induced transconjugants *E. coli* M672 and *K. oxytoca* M673 were able to grow with a shorter delay. These differences in the level of plasmid-mediated resistance may indicate that the interaction between the nickel-resistance determinant and chromosomal genes in *E. coli* M672, *K. oxytoca* M673 and *S. marcescens* M672 are different. The results indicated that $NiCl_2$ was an inducer of nickel resistance in the wild-type strains and transconjugants.

When the hybrid plasmid containing a 4.8-kb *Sal*I–*Eco*RI fragment was transferred to *E. coli* DH5α, the

resulting transformants showed an inducible resistance, although there was a longer delay before growth began (Fig. 5). These results indicated that all the information for nickel resistance was encoded within the 4.8-kb *Sal*I–*Eco*RI fragment. However, the transformants demonstrated a lower degree of resistance than in *H. alvei* M622 and transconjugants, suggesting that there are other loci on the plasmid required for full resistance.

Localization of the nickel-resistance determinant

To determine whether the nickel-resistance determinant is also localized on the chromosome, sequence homology of the plasmid pHF69 and genomic DNA of *H. alvei* 5-5 was investigated by DNA-DNA hybridization. By Tn*phoA′*-1 insertional analysis, the nickel-resistance determinant of plasmid pHF69 was shown to span nearly the entire length of a 4.8-kb *Sal*I–*Eco*RI fragment of pHF14. This fragment was isolated and used as a biotinylated DNA probe in DNA-DNA hybridization experiments under high-stringency conditions allowing hybridization between DNAs with an homology higher than 85%. As expected, plasmid pHF14-derived probe hybridized strongly with its own 4.8-kb *Sal*I–*Eco*RI fragment (Fig. 6). The genomic DNA of *H. alvei* 5-5, digested with *Eco*RI or *Sal*I endonuclease and separated by agarose gel electrophoresis, also showed a very strong signal. These results support the assumption that the nickel-resistance determinants of *H. alvei* 5-5 reside on both the plasmid and the genomic DNA.

Homologies between the nickel-resistance determinants of *H. alvei* 5-5 and *A. xylosoxydans* 31A

The hybrid plasmid pNRS148 probe was used to examine DNA homologies with the nickel-resistance determinants of other bacteria. DNA fragments containing nickel resistance in *A. xylosoxydans* 31A and *K. oxytoca* 15788 were separated by agarose gel electrophoresis and membrane blots were prepared. DNA-DNA hybridization experiments were carried out under high-stringency conditions. Positive signals were detected with the 14.5-kb *Bam*HI and 4.2-kb *Eco*RI fragments of pTOM9 from *A. xylosoxydans* 31A (Fig. 7). In addition to these signals, similar numbers of detectable signals and similarly sized corresponding DNA fragments were observed. These data suggest that the nickel-resistance determinant of the strains studied are very similar.

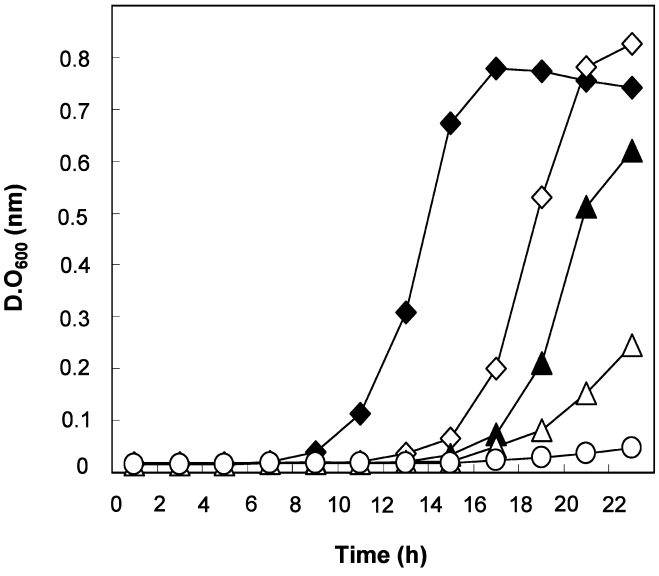

Fig. 5 Induction of nickel resistance in recombinant *E. coli* DH5α. Strains were grown overnight in Tris-gluconate medium without (*open symbols*) or with (*closed symbols*) 0.5 mM nickel chloride. The cells were diluted in fresh medium, and 4 mM nickel chloride was added to the culture. *Diamonds Hafnia alvei* 5-5, *triangles E. coli* DH5α with pHF14, *circles E. coli* DH5α with pBR322

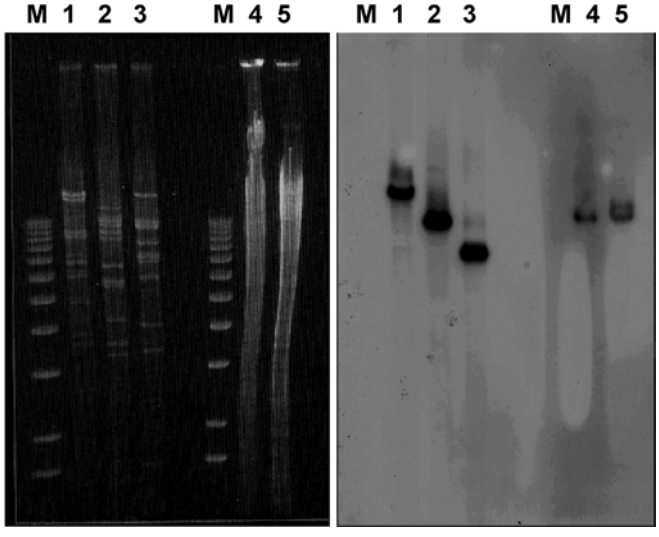

Fig. 6 Southern hybridization analysis of plasmid and chromosomal DNAs from *H. alvei* 5-5. The 4.8-kb *Sal*I–*Eco*RI fragment from pHF14 was used as a DNA probe. Plasmid DNA was digested with *Bam*HI (*lane 1*), *Eco*RI (*lane 2*), *Hind*III (*lane 3*). Chromosomal DNA was treated with *Sal*I (*lane 4*), and *Eco*RI (*lane 5*). *Lane M* 1-kb Ladder

Discussion

Bacteria resistant to nickel have so far been isolated from ecosystems polluted by heavy metals, such as waste waters, mine refuse, industrial composts, and cooling waters of the metal-processing industry. The bacteria identified belong to the *Ralstonia* groupand include *R. eutropha* CH34 [1], *A. denitrificans* 4a-2 [31], *A. xylosoxydans* 31A [31], *R. eutropha* KTO2 [21], and *K. oxytoca* CCUG 15788 [29]. In contrast, *H. alvei* 5-5 was isolated from soils under nickel-hyperaccumulating trees and selected by aerobic enrichment culture designed for nickel-resistant Enterobacteriaceae [28]. *H. alvei* 5-5 tolerated up to 30 mM $NiCl_2$; therefore, we decided to compare its nickel-resistance determinant

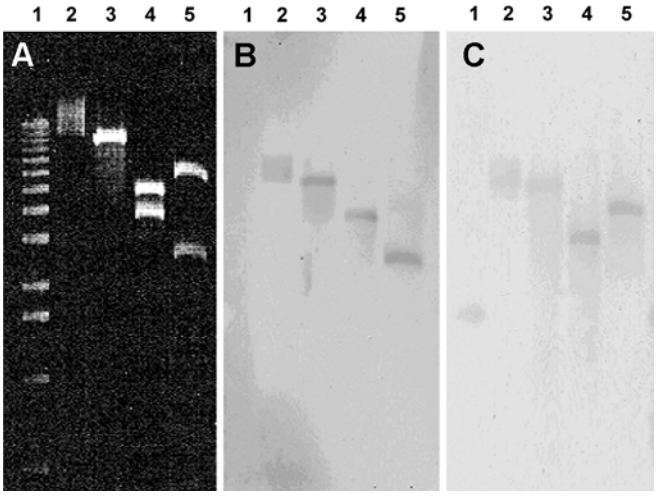

Fig. 7A–C Southern hybridization analysis of nickel-resistance determinant from *H. alvei* 5-5 with pTOM9 from *Alcaligenes xylosoxydans* 31A. DNA was undigested (*lane 2*) or digested with *Eco*RI (*lane 3*), *Sal*I and *Eco*RI (*lane 4*), *Cla*I and *Eco*RI (*lane 5*). *Lane 1* 1-kb Ladder. **A–C** Hybridized with a 4.8-kb *Sal*I–*Eco*RI probe from pHF14. **A** pHF 14, **B** 14.5-kb *Bam*HI fragment from pTOM9, **C** 4.2-kb *Eco*RI fragment from pTOM9

with that of strains from polluted ecosystems and which belong to the *Pseudomonas* group.

Our results demonstrate that an indigenous plasmid, pEJH501, coding for nickel resistance, is transferable to nickel-sensitive *E. coli* H11, and *S. marcescens* S15, in which it is expressed to the same degree as in the wild-type. The plasmid was also transferred by conjugation to *K. oxytoca* S69, but was not expressed effectively. The resistance to nickel determined by plasmid pEJH501 is inducible in the native host strain *H. alvei* M622 as well as the transconjugants. Transconjugants *S. marcescens* M672 and *K. oxytoca* M673 grewwith a shorter lag time than did *H. alvei* M622. These observations suggest that additional determinants for nickel resistance are located on the chromosome of recipients and that they may be amplified when strains are grown under selective conditions. In this regard, it is interesting to note that a plasmid-encoded inducible tellurite-resistance determinant was transferred from a *Ralstonia* strain to *E. coli*, in which a higher level of resistance was expressed than in the native host [7].

Plasmid DNA from lysates of transconjugant strains selected for nickel resistance was identical in mobility on agarose gels to the 70-kb plasmid from the donor strain (data not shown). Transconjugants selected for nickel resistance were also cobalt-resistant. Therefore, these results are analogous to those reported in *R. metallidurans* and *K. oxytoca*, in which resistance to these two metals is plasmid-mediated, genetically linked, and co-ordinately induced [1, 20, 21].

We isolated a 23-kb *Bam*HI fragment of pEJH501 that carries nickel resistance and is able to confer nickel resistance to nickel-sensitive *E. coli* DH5α. The 4.8-kb *Sal*I–*Eco*RI fragment resulted in inducible nickel resistance in *E. coli* DH5α, but required a longer lag time than in *H. alvei* M622. Thus, the additional gene products of the host cell are required for the initial response to nickel. Two Tn*phoA′*-1 insertions resulted not only in the loss of resistance but also in higher levels of resistance to nickel, which indicates the involvement of regulatory genes. The 4.8-kb *Sal*I–*Eco*RI fragment probably carries both structural and regulatory genes for nickel resistance.

Hybridization experiment with chromosomal DNA and a biotinylated 4.8-kb *Sal*I–*Eco*RI fragment probe resulted in the detection of a hybridization signal and thus indicated the presence of a DNA region on the chromosome which is homologous to that on the plasmid. We found additional gene loci on the chromosome of *H. alvei* 5-5 that determine a nickel resistance in *E. coli* DH5α (data not shown). These results are similar to those obtained in other studies on nickel resistance. The *ars* (arsenite-inducible operon) in the chromosome of *E. coli* displays strong homology, both in protein sequence and genetic organization, with the plasmid-borne arsenic-detoxication operon [2]. A chromosomal homolog of a plasmid-borne copper-resistance operon has been found in *Pseudomonas syringae* [10]. It has been proposed that the structure of the plasmid-borne, ATP-driven efflux pump may be related to the multiple-drug-resistance ATP-driven efflux pump that is amplified in mammalian cancer cells [25, 26]. Thus, in an analogous fashion, the high degree of nickel resistance (30 mM $NiCl_2$) of *H. alvei* wild-type strain 5-5 compared to the plasmid-containing transconjugants (< 15 mM $NiCl_2$) of nickel-sensitive recipients may be due to the presence of at least two nickel-resistance determinants, one on plasmid pEJH501 and one on the chromosome. To draw a final conclusion cured mutants of strain 5-5 are needed.

Hybridization experiments with the resistance determinant of *A. xylosoxydans* 31A and a biotinylated plasmid pHF14 probe resulted in identical numbers of hybridization signals and similarly sized corresponding DNA fragments. These results suggest that the nickel-resistance determinant of *H. alvei* 5-5 has a high degree of homology to the *ncc*/*nre*/*cnr* type [28]. The *ncc* system of *A. xylosoxydans* 31A confers a high level of resistance to nickel, as well as to cobalt and cadmium. The nucleotide sequence of the *ncc* locus revealed seven ORFs, designated *nccYXHCBAN*, the proteins of which share strong homology to proteins encoded by *cnrYXHCBA* [22]. In contrast, the *nre* system of *A. xylosoxydans* 31A confers a low level of nickel resistance, which is mediated by NreB [3, 22].

The 4.8-kb *Sal*I–*Eco*RI fragment has now been sequenced, and five ORFs were identified (manuscript in preparation). The sequence was submitted to the Gene Bank and was assigned the accession number AF322866.

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