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The nickel resistance determinant cloned from the enterobacterium *Klebsiella oxytoca*: conjugational transfer, expression, regulation and DNA homologies to various nickel-resistant bacteria

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***Klebsiella oxytoca* strain CCUG 15788, isolated from a mineral oil emulsion tank in Göteborg, Sweden, was found to be nickel-resistant (tolerating 10 mM $NiCl_2$ in non-complexing mineral-gluconate media; inducible resistance). The nickel resistance determinants were transferred by helper-assisted conjugation to various strains of *Escherichia coli* and *Citrobacter freundii* and expressed to between 5 and 10 mM $NiCl_2$. A 4.3 kb *Hin*dIII fragment was cloned from the genomic DNA of *K. oxytoca*. Ligated into the vector pSUP202, the fragment caused constitutive nickel resistance (of up to 3 or 10 mM Ni^{2+}) in various *E. coli* strains. After cloning into the broad host range vector pVDZ'2 the fragment even expressed low nickel resistance in the transconjugant of *Alcaligenes eutrophus* AE104. With the 4.3 kb *Hin*dIII fragment as a biotinylated DNA probe it was shown by DNA–DNA hybridization that the nickel resistance determinant resides on the chromosome of *K. oxytoca* and not on its circular plasmid pKO1 (160 kb) or linear plasmid pKO2 (50 kb). Nickel resistance strongly correlated with the presence of the 4.3 kb *Hin*dIII fragment in the transconjugants. No homologies were detected when the nickel resistance determinants of other well-known nickel-resistant bacteria, such as *A. eutrophus* CH34 or *A. denitrificans* 4a-2, were used as target DNA. Among the 60 strains examined, positive signals only appeared with the 3.1 kb DNA fragment from *A. xylosoxydans* 31A and the genomic DNA of two enterobacterial strains (5–1 and 5–5) isolated from nickel-rich soil in New Caledonia.**

Keywords: DNA–DNA homologies, *Klebsiella oxytoca*, nickel resistance, transconjugants

Introduction

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 *Alcaligenes* group such as *Alcaligenes eutrophus* CH34 (Mergeay *et al.* 1978), *A. denitrificans* 4a-2 (Timotius & Schlegel 1987), *A. xylosoxydans* 31A (Schmidt & Schlegel 1989) and *A. eutrophus* KTO2 (Schmidt *et al.* 1991). Further strains apparently closely related to strain CH34 were isolated from nickel-ore-containing mine refuse heaps in Belgium and Zaïre (Diels & Mergeay 1990). In most of these bacteria the genetic information determining nickel resistance is localized on plasmids. These plasmids are self-transmissible to other members of the *Alcaligenes* group and to pseudomonads, but not to Enterobacteriaceae. The resistance determinants of some of these strains have already been studied by molecular genetic analysis, and the resistance genes, designated the *cnr* operon, of *A. eutrophus* CH34 have been cloned, sequenced and characterized (Siddiqui *et al.* 1989, Liesegang *et al.* 1993). The genes from *A. xylosoxydans* 31A, called the *ncc* operon (Schmidt *et al.* 1991) and *nre* operon (T. Schmidt, unpublished data) as well as from *A. denitrificans* 4a-2 (Kaur *et al.* 1990) were also characterized. The *cnr* operon-encoded nickel and cobalt resistances are inducible properties (Siddiqui & Schlegel 1987, Siddiqui *et al.* 1988). Nickel resistance was shown to be due to an energy-dependent specific efflux mechanism (Sensfuss & Schlegel 1988, Varma *et al.* 1990).

Nickel-resistant bacteria from ecosystems, which are naturally loaded with high concentrations of nickel salt, about 10 000 p.p.m. Ni, have been encountered recently (Schlegel *et al.* 1991). Some plants growing on metalliferous soils rich in nickel, cobalt and chromate take up

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nickel and accumulate it in stems and leaves. Among about 170 nickel-hyperaccumulating plants the most outstanding example is the New Caledonian tree *Sebertia acuminata*, which can accumulate more than 1% (dry weight) nickel in the leaves and about 25% in its blue-green latex. In soils under the nickel-hyperaccumulating trees the number of nickel-resistant bacteria almost equalled the total number of viable bacteria. Many of the 200 bacteria isolated tolerated up to 30 mM Ni^{2+}.

Studies on the diversity of mechanisms, regulation and host specificity of heavy metal resistance prompted us to examine bacteria isolated from a mineral oil emulsion in water and to screen for nickel-resistant bacteria. A 150 m^3 central tank containing an oil emulsion, which is used for cooling and lubrication in processes such as turning, grinding and cutting of metal tools, had been studied with respect to the dominant bacterial flora and the succession of species (Mattsby-Baltzer *et al.* 1989). The most dominant bacterium was *Pseudomonas pseudoalcaligenes* (more than 10^8 c.f.u. ml^{-1}). After this bacterium had luxuriantly grown, *Klebsiella oxytoca* appeared and obviously utilized the metabolic and autolytic products of *P. pseudoalcaligenes*. Among four identified isolates, kindly provided by Inger Mattsby-Baltzer, Göteborg, Sweden, only the strain of *K. oxytoca* turned out to have significant metal resistance properties. Except for a very early report (Smith 1967) nickel-resistant enterobacteria have not been described. Therefore, we studied *K. oxytoca* in some detail. The strain turned out to carry one circular plasmid and one linear plasmid, and to function as a donor of metal resistance characters when mating with some Enterobacteriaceae as recipients. The present paper describes the physiological properties of this bacterium, the resistance to nickel and copper salts, the localization of the genetic information for nickel resistance on the chromosome or the plasmids, and the relationship to other nickel resistance genes with respect to DNA–DNA homologies. Furthermore, using a DNA probe of the nickel resistance determinants of *K. oxytoca*, we screened 23 of the New Caledonian strains for homologies. The results of a few strains, which gave positive signals, were incorporated into the present paper. Strains 12-1, 18-1, 22-1 and 32W-2 were isolated from gluconate–nickel plates (Schlegel *et al.* 1991) and are not enterobacteria. Strains 5-1 and 5-5 were isolated by enrichment culture designed for selecting highly nickel-resistant enterobacteria.

Materials and methods

Strains and transconjugants

Strains and transconjugants used are summarized in Table 1. Cloning vectors and plasmids are listed in Table 2.

Media and growth conditions

Klebsiella and *Alcaligenes* strains were grown at 30 °C in Tris–mineral medium (Mergeay *et al.* 1985) supplemented

Table 1. Bacterial strains, mutants, and transconjugants

Species and strain	Relevant characteristics	Reference or source
A. eutrophus		
CH34	WT, Ni^r, plasmids pMOL28 (163 kb) and pMOL30 (238 kb)	Mergeay *et al.* (1985)
AE104	curing mutant of *A. eutrophus* CH34, Ni^s	Mergeay *et al.* (1985)
A. xylosoxydans		
31A	WT, Ni^r, plasmids pTOM8 (340 kb) and pTOM9 (200 kb)	Schmidt *et al.* (1991)
A. denitrificans		
4a-2	WT, Ni^r	Kaur *et al.* (1990)
K. oxytoca		
1665	WT, Ni^s	JCCM 1665
15788	WT, Ni^r, plasmids pKO1 (160 kb) and pKO2 (linear, 50 kb)	CCUG 15788 Mattsby-Baltzer *et al.* (1989)
Auxotrophic mutants of strain 15788		
M480	pro, leu, pKO1, pKO2	this study
M571	pro, leu, trp, pKO1, pKO2	this study
M578	pro, leu, trp, pKO1, pKO2, pULB113	this study
E. coli		
K12	WT, Ni^s	DSM 496
S17-1	Ni^s, *pro thi* mutant, *rec*A2 *tra*$^+$	Simon *et al.* (1983a)
JM109	Ni^s, *thi rec*A Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB *lacI*^{q}, *lacZ*ΔM15]	Yanisch-Perron *et al.* (1985)
CM214	Ni^s, F^-, *lac*, *pro*, *thi*A, *gal*E	Lejeune *et al.* (1983)
J53	Ni^s, *met*, *pro*	Datta *et al.* (1971)
C. freundii	WT, Ni^s	DSM 30040
New soil isolates		
Strain 5–1	WT, Ni^r	Schlegel *et al.* (1991)
Strain 5–5	WT, Ni^r	Schlegel *et al.* (1991)

WT, wild-type; Ni^s, nickel-sensitive; Ni^r, nickel-resistant.

Table 2. Vectors and plasmids

Plasmid	Antibiotic resistance(s)	Reference
pSUP202	Ap^r, Cm^r, Tc^r	Simon *et al.* (1983b)
pVDZ'2	Tc^r	Deretic *et al.* (1987)
pULB113	Ap^r, Km^r, Tc^r	van Gijsegem & Toussaint (1982)
pBluescript	Ap^r	Stratagene, Heidelberg, Germany

with gluconate (0.4% w/v) or substrates as indicated. Heavy metal chlorides were added before autoclaving, and the pH was adjusted to pH 7.0. In special experiments, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes)-buffered (50 mM) mineral media of the same composition were used.

Growth in liquid media

Cells were grown in Tris-buffered or Tes-buffered (*E. coli*) gluconate media contained in 300 ml Klett flasks; the vessels were agitated on a rotary shaker at 30 or 37 °C (*E. coli*). Turbidity was determined by using a Klett–Summerson photometer. Precultures were grown without added $NiCl_2$ (uninduced cells) or in the presence of the challenge nickel concentration used in the growth experiments. Two cultures containing the challenge nickel concentration were inoculated with induced or uninduced cells, respectively. A culture without added $NiCl_2$ served as a positive control. A comparable nickel-sensitive strain was incubated in the presence of the challenge nickel concentration as a negative control.

Estimation of the highest tolerable metal concentration

Cells were grown in liquid or on solidified Tris- or Tes-mineral medium supplemented with metal salts. The plates were incubated at 30 or 37 °C and were inspected at intervals. Instead of the minimal inhibitory concentration (MIC), the maximal tolerable concentration (MTC) of metal salts was measured, i.e. the concentration allowing growth without decreasing the number of c.f.u.s (Mergeay 1991).

Isolation of a triple auxotrophic mutant

A triple auxotrophic mutant of *K. oxytoca* 15788 was isolated after nitrite mutagenesis and by exposing the cell population to three cycles of the ampicillin counter-selection technique; the Holiday method was employed for screening. The protocol of Drews (1983) was used. The first cycle resulted in a pro^- mutant (M478), the second cycle in a pro^- leu^- mutant (M480) and the third cycle in a pro^- leu^- trp^- mutant (M571); the latter mutants were chosen as donors for conjugational transfer of nickel and copper resistance markers, either without (M480 and M571) or with helper plasmid pULB113 (M481 and M578).

Conjugation

Conjugations were performed on solidified nutrient broth medium as described previously (Schmidt *et al.* 1991). The desired transconjugants were selected on appropriate selective media. In order to differentiate between *K. oxytoca* and *E. coli* the Fluorocult medium (Merck, Darmstadt, Germany) was used. This medium contains 4-methylumbelliferyl-β-D-glucuronide (MUG). β-D-glucuronidase metabolized MUG to glucuronic acid and the fluorescent 4-methylumbelliferon. Since *E. coli* in contrast to *K. oxytoca* is able to form β-D-glucuronidase, irradiation with light of a wavelength of 357 nm causes only colonies of *E. coli* to fluoresce while colonies of *K. oxytoca* do not (Feng & Hartman 1982).

Transformation of E. coli

E. coli S17-1 was grown in Luria–Bertani medium in the presence of 20 mM $MgCl_2$ at 37 °C to approximately 40 Klett units. Competence of cells was obtained by using the calcium chloride procedure (Hanahan 1983, Sambrook *et al.* 1989). Transformants were selected as described in Results.

Isolation of genomic DNA and plasmid DNA

Isolation of genomic DNA was essentially as described by Ausubel *et al.* (1987). Plasmid DNA of *K. oxytoca* was isolated by the method of Kado & Liu (1981) as modified by Nies *et al.* (1987). The alkaline lysis method (Sambrook *et al.* 1989) was employed for isolation of vector DNA.

DNA–DNA hybridization

The respective fragment was isolated from the agarose gel by using the Qiaex gel extraction kit (Diagen, Hilden, Germany). The Nick translation kit (Gibco/BRL, Eggenstein, Germany) was used for labeling the designated probe with biotin-16-dUTP. Target DNA was blotted to nylon membranes by the Southern blot procedure. Hybridization conditions were essentially those as described by Oelmüller *et al.* (1990), except that the hybridization roll-incubator was used. The BlueGene kit (Gibco/BRL) was employed for detection of the biotinylated probe.

Chemicals

The sources of the chemicals and enzymes used were the same as described by Schmidt *et al.* (1991).

Results

All physiological studies on *K. oxytoca*, provided by Dr I. Mattsby-Baltzer and registered in the Culture Collection of the University of Göteborg under strain number CCUG 15788, were done in parallel with *K. oxytoca* strain 1665 of the JCCM as reference strain and as internal standard. Both strains were able to grow aerobically on nutrient broth as well as on mineral media containing fructose,

glucose, sucrose, lactate, acetate or benzoate as substrates. Under anaerobic conditions the strains grew in the presence of 0.5% (w/v) nutrient broth and 1% (w/v) glucose with gas formation; while strain 1665 grew as a homogeneous suspension, strain 15788 formed flocs and pellets. All differences between these strains were in the range of species variability.

Resistance to heavy metals

On solid Tris-buffered gluconate media, which do not significantly complex or precipitate heavy metals (Mergeay *et al.* 1985), *K. oxytoca* 15788 tolerated up to 10 mM $NiCl_2$ or 1 mM $CuCl_2$. On media without added chloride (chloride salts replaced by sulfates), 5 mM $AgNO_3$ were tolerated also. Strain 15788 did not grow at 0.6 mM $CoCl_2$, 1 mM $CdCl_2$, 2 mM $ZnCl_2$, 0.03 mM $HgCl_2$ or 5 mM $CuCl_2$. In contrast, *K. oxytoca* strain 1665 did not grow in the presence of 0.8 mM $NiCl_2$, 1.0 mM $CoCl_2$, 1.0 mM $CdCl_2$, 2.0 mM $ZnCl_2$, 0.5 mM $CuCl_2$ and 0.5 $AgNO_3$. On the basis of these minimal inhibitory concentrations, strain 15788 has to be assigned to the high-level nickel-resistant, medium-level copper-resistant and medium-level silver-resistant bacteria. Nickel resistance without concomitant cobalt resistance is seldom, although not unique, at least among the *Alcaligenes* or *Pseudomonas* species so far studied.

Regulation of the expression of nickel and copper resistance

When the wild-type strain 15788 or its auxotrophic derivative (pro^- leu^-) M480 were grown in liquid media containing 5 mM Ni^{2+}, the beginning of the exponential growth phase depended on the conditions of pregrowth. Cells pregrown in nickel-free medium had after transfer to pass a lag time of about 12 h, while cells pregrown in the presence of 5 mM Ni^{2+} passed a lag phase of only 2–3 h (Figure 1). Thus, nickel resistance is an inducible property in *K. oxytoca*. However, the duration of the lag phase depended on the nickel concentration employed for pregrowth in a pronounced manner. In contrast, *A. eutrophus* CH34 was already fully induced, when pregrown at 0.5 mM Ni^{2+}, and started to grow exponentially within the first 30 min after transfer to 3 mM Ni^{2+} (Siddiqui *et al.* 1988). *K. oxytoca* reached full induction only after pregrowth at the challenge concentration.

The resistance to copper is differently regulated in *K. oxytoca*. Both, cells pregrown in an 0.5 mM Cu^{2+}-containing Tris-gluconate medium and cells grown without added copper salt started exponential growth within about 1 h after inoculation when transferred to 0.5 or 1.0 mM $CuCl_2$ media. Thus, copper resistance can be regarded as a constitutive property in the *K. oxytoca* strain.

The presence of plasmids

Plasmid DNA analysis by the method of Kado & Liu (1981) and agarose gel electrophoresis revealed the presence of two plasmids in *K. oxytoca* 15788; both appeared

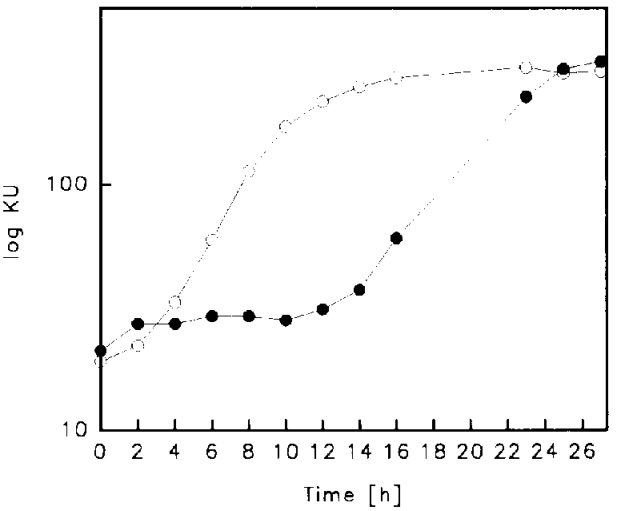

Figure 1. Growth curves of *K. oxytoca* 15788. Cells were grown in Tris-buffered mineral medium containing 5 mM $NiCl_2$. ○, *K. oxytoca* pregrown in the presence of the challenge concentration of $NiCl_2$; ●, cells pregrown without added $NiCl_2$.

to be cccDNA. By using pHG1 (Gerstenberg *et al.* 1982) and pMOL28 (Mergeay *et al.* 1985), whose size had been determined by contour length measurements, as well as plasmids pRmMVIII (1–4) of *Rhizobium meliloti* MVIII (Simon *et al.* 1983a) as standards the size of one plasmid was determined to be 160 kb and the size of the other about 12 kb. They were called pKO1 and pKO2, respectively. The application of pulsed field gel electrophoresis to the DNA of *K. oxytoca* revealed the presence of a linear plasmid of about 50 kb. As a mutant derivative of the wild-type strain did not show either the small plasmid pKO2 or the linear plasmid, the assumption suggested itself that pKO2 and the linear plasmid are identical. After separation of plasmids pKO1 and pKO2 by conventional electrophoresis, the putative 12 kb cccDNA plasmid pKO2 was excised from the gel. Digestions of the isolated plasmid with two restriction endonucleases yielded nine and 12 fragments, respectively, which were between 1 and 20 kb in size. The sum of the fragment sizes was about 45 kb. This finding caused us to deduce the identity of pKO2 and the linear plasmid.

Genetic localization of resistance properties

In order to find out whether nickel and copper resistance reside on the chromosome, two strategies were employed: conjugational transfer to appropriate recipients as well as plasmid curing. To facilitate the selection of transconjugants a double-auxotrophic mutant (*pro*, *leu*) and a triple auxotrophic mutant (*pro*, *leu*, *trp*) of *K. oxytoca* 15788 were isolated (see Methods). By mating with *E. coli* CM214, the plasmid pULB113 was introduced into both donor strains using the genes for resistance to kanamycin, ampicillin and tetracycline as markers for selection of the pULB113-harboring derivatives. The following species were chosen as possible recipients of the nickel and copper

Table 3. Nickel-resistant transconjugants of various *E. coli* strains and *Citrobacter*

Transconjugants examined (strain number)	Donor strain	Recipient strain	Maximally tolerable concentrations (mM)			
			Ni^{2+}	Co^{2+}	Cd^{2-}	Zn^{2+}
M582	M480	*E. coli* K12	5	< 1	< 0.6	< 2.5
M593	M578	K12	5	< 1	< 0.6	< 2.5
M586	M571	JM109	5	< 1	< 0.6	< 2.5
M572	M480	JM109	5	< 1	< 0.6	< 2.5
M591	M571	J53	5	< 1	< 0.6	< 2.5
M595	M571	S17-1	3	< 1	< 0.6	< 2.5
M597	M571	*Citrobacter*	10	< 1	< 1	< 2.5
Recipients themselves		*E. coli* K12	< 1	< 1	< 0.6	< 2.5
		JM109	< 1	< 1	< 0.6	< 2.5
		S17-1	< 1	< 1	< 0.6	< 2.5
		Citrobacter	< 1	< 1	< 1	< 2.5

All transconjugants showed only the plasmid present in the recipient. The DNA from all transconjugants gave positive signals, when hybridized with the 4.3 kb *Hin*dIII fragment biotinylated DNA probe, while recipients did not.

resistance determinants: *C. freundii*, *E. coli* strains K12, J53, S17-1, JM109 and WK6, *E. aerogenes*, *S. marcescens*, *K. oxytoca* 1665, *A. eutrophus* wild-type strains N9A and H16, and *A. eutrophus* AE104. These bacteria were used as native strains or as streptomycin–nalidixic acid double-resistant derivatives. As shown in Table 3, nickel-resistant transconjugants could only be isolated with *E. coli* strains K12, JM109, J53 and S17-1, and *C. freundii* as recipients. The transfer frequency in matings of the double- and the triple-auxotrophic donor strains was equally low, e.g. 4×10^{-8} and 1×10^{-7} with *E. coli* JM109 as the recipient. The transconjugants had not received the donor plasmids pKO1 or pKO2, and copper resistance was not transferred.

Cloning of nickel resistance genes

DNA of *K. oxytoca* containing its native plasmids and in addition pULB113 was isolated and digested with *Hin*dIII endonuclease. The resultant fragments were cloned into the *Hin*dIII site of the vector pSUP202. Hybrid plasmids were transferred to *E. coli* S17-1 by transformation. The clones containing the hybrid plasmid were screened on Tris-mineral agar containing 3 mM $NiCl_2$. One nickel-resistant clone was found among 300 recombinants. Isolation and *Hin*dIII digestion of the hybrid plasmid from this clone yielded a 4.3 kb *Hin*dIII fragment, designated KOHI4.

Expression of the 4.3 kb Hin*dIII fragment in* E. coli *and* A. eutrophus

In *E. coli* strains the 4.3 kb *Hin*dIII fragment caused nickel resistance up to 3 mM $NiCl_2$ on Tris-buffered or 10 mM $NiCl_2$ on Tes-buffered solid media, respectively. Since plasmid pSUP202 is not replicated in *A. eutrophus* strains, the two possible orientations of the 4.3 kb *Hin*dIII fragment were cloned into the mobilizable broad host range vector pVDZ'2. Hybrid plasmids were transformed in *E. coli* strains S17-1 and JM109, and transferred to *A. eutrophus* AE104 by conjugation. The transconjugants AE104 (pVDZ'2::KOHI4) tolerated nickel concentrations as high as 3 mM. The orientation of the fragment within the vector plasmid did not significantly influence the MTC.

In liquid media both *E. coli* and *A. eutrophus* harboring fragment KOHI4 tolerated only lower concentrations of $NiCl_2$ about a quarter of the nickel concentrations tolerated on solid media.

Constitutive expression of the Hin*dIII fragment in* E. coli

In order to examine the expression of fragment KOHI4 within *E. coli* in liquid media the fragment was inserted into the *Hin*dIII site of vector pBluescript in two orientations and transferred to *E. coli* JM109 by transformation. Growth experiments were performed using the two strains of *E. coli* JM109, one harboring the fragment in one and the other in the opposite orientation. Precultures were grown in Tes-buffered media without and in the presence of 2 mM $NiCl_2$. The challenge concentration in the growth experiment was also 2 mM $NiCl_2$. The growth curves (Figure 2) were found to be almost identical regardless whether induced or uninduced cells were used for inoculation. Furthermore, the orientation of fragment KOHI4 in pBluescript did not influence the regulation of expression. The results indicate that the nickel resistance determined by fragment KOHI4 was constitutively expressed in *E. coli*.

Localization of nickel resistance genes

Using the 4.3 kb *Hin*dIII fragment as a biotinylated DNA probe in DNA–DNA hybridization experiments we tried to answer the question whether the nickel resistance genes of *K. oxytoca* are localized on the chromosome or on one of the plasmids. The isolated intact plasmids pKO1 and pKO2 did not show any signal. The genomic DNA of the nickel-sensitive strain *K. oxytoca* 1665 digested by *Hin*dIII endonuclease did not show any signal either. The

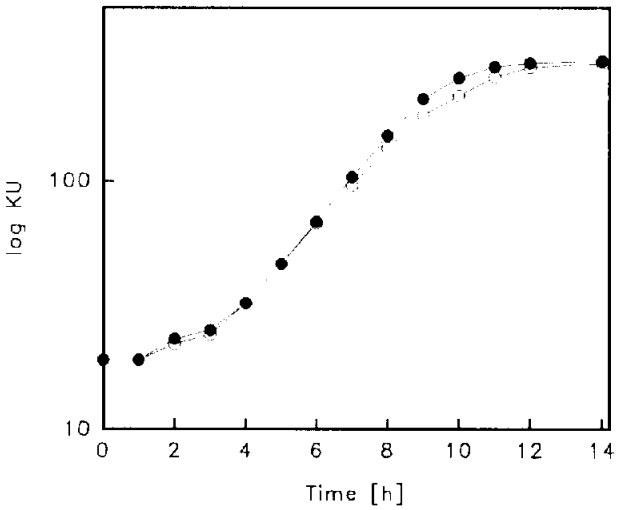

Figure 2. Growth curves of *E. coli* JM109 harboring fragment KOHI4. Cells were grown in Tes-buffered mineral medium containing 2 mM $NiCl_2$. ○, *E. coli* JM109 (pBluescript::KOHI4) pregrown in the presence of the challenge concentration of $NiCl_2$: ●, cells pregrown without added $NiCl_2$. Identical growth curves were obtained with a strain harboring fragment KOHI4 in pBluescript in the opposite orientation.

genomic DNA of *K. oxytoca* 15788, digested with *Eco*RI or *Hin*dIII endonuclease and separated by agarose gel electrophoresis, showed a very strong signal (Figure 3, lane 1). These results indicate that the nickel resistance determinants of *K. oxytoca* 15788 reside on the chromosome. It may be remarked that in the course of these experiments we were irritated by a faint signal detected (Figure 3, lane 2) in the *Hin*dIII-digested plasmid DNA of *K. oxytoca* 15788 isolated by the procedure of Kado & Liu (1981). The observation is easily understandable considering that the plasmid preparations obtained by this method are usually contaminated with traces of chromosomal DNA.

Detection of nickel resistance determinants in transconjugant strains

Nickel-resistant transconjugants derived from matings with *K. oxytoca* strains M571 and M578 as donors and *C. freundii* and *E. coli* strains K12, J53, S17-1 and JM109 as recipients were investigated for the presence of the 4.3 kb *Hin*dIII fragment KOHI4. Genomic DNA and (if feasible) plasmid DNA of donors, recipients and transconjugants was isolated and digested with *Hin*dIII. The restricted DNA was separated by electrophoresis and blotted onto nylon membranes. Hybridization with the biotinylated fragment KOHI4 as DNA probe was performed under stringent conditions allowing DNA–DNA hybridization only in case of homology higher than 95%. Figure 3 shows hybridization signals with the DNA of the donor strains (lanes 8, 9 and 14) and the DNA of the transconjugant strains (lanes 5, 7, 12, 13, 15 and 19). Hybridization of the genomic DNA of the wild-type strain *K. oxytoca* 15788 and of the isolated fragment KOHI4 (lanes 1 and 3) served as positive controls. No signals were detected with the genomic *Hin*dIII-digested DNA of the recipient strains and the *Hin*dIII- or *Pst*I-digested lambda DNA (lanes 4, 6, 11, 14 and 18). Thus, the expression of the nickel resistance determinants of *K. oxytoca* in *E. coli*

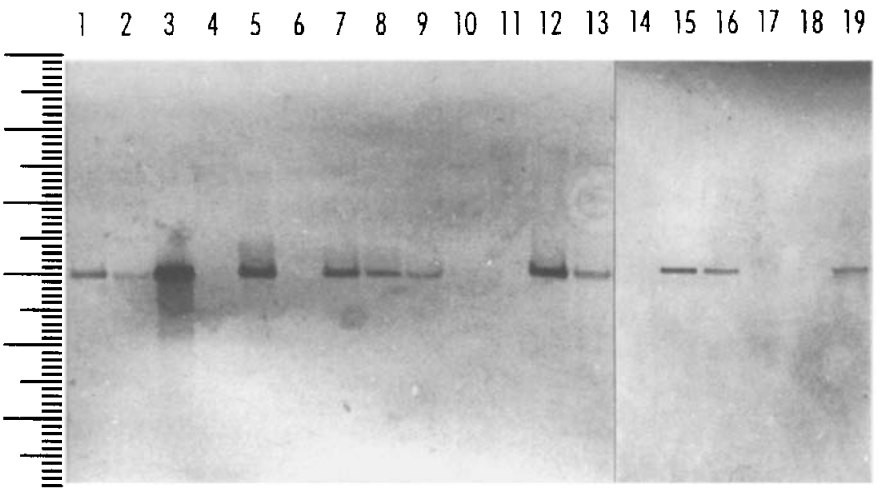

Figure 3. DNA–DNA hybridization of genomic or plasmid DNA of donor, recipient and transconjugant strains with the 4.3 kb *Hin*dIII fragment KOHI4 as a biotinylated DNA probe. DNA was isolated as described in Materials and methods and digested with *Hin*dIII. Restriction fragments were electrophorized in Tris-acetate-buffered 0.7% agarose for 2 h at 60 V, blotted onto a nylon filter and probed with the biotinylated fragment KOHI4. Hybridization signals were detected with the genomic DNA of the wild-type strain *K. oxytoca* 15788 (lane 1), the donor strains M571 and M578 (lanes 8 and 16), and the transconjugant strains M581, M582, M586, M595 and M597 (lanes 5, 7, 12, 15 and 19). Signals corresponding to the same fragment size but of lower intensity were obtained with the plasmid DNA of *K. oxytoca* 15788, the donor strain M571 and the transconjugant strain M581 (lanes 2, 9 and 13). No signals were detectable with the genomic DNA of the recipients, *E. coli* strains K12, JM109, J53 and S17-1, and *C. freundii* (lanes 4, 6, 11, 14 and 18) as well as with *Hin*dIII- or *Pst*I-digested lambda DNA (lanes 10 and 17). The isolated fragment KOHI4 served as positive control (lane 3).

strains and *Citrobacter* was found to be strongly correlated with the presence of the 4.3 kb *Hin*dIII fragment.

Homologies between the K. oxytoca *nickel resistance determinant and determinants of other bacteria*

The 4.3 kb *Hin*dIII fragment DNA probe was used to examine DNA homologies with the nickel resistance determinants of other bacteria. DNA fragments encoding nickel resistance in *A. eutrophus* CH34, *A. denitrificans* 4a-2, *A. xylosoxydans* 31A, as well as from the strains 32W-2, 12-1, 18-1 and 22-1 isolated from nickel-containing soils in New Caledonia, had been isolated (Stoppel 1992). The fragments and their source are listed in Table 4. With these DNA fragments as target DNA, the hybridization experiments were performed under conditions which allowed hybridization only in case of DNA–DNA homologies higher than 95%. No signals were detected with the fragments from the bacterial strains CH34, 4a-2 and the 8.8 kb *Pst*I subfragment (of TBA) from strain 31A (data not shown). A significant positive signal was found only with the 3.1 kb *Bam*HI–*Pst*I subfragment (of TBA) harboring the *nre* operon of strain 31A and (less pronounced) with the four nickel resistance fragments from the New Caledonian strains. Although the hybridization reaction with the 3.1 kb *Pst*I fragment was considered to be significant, the corresponding signal was of less intensity than the positive control. Therefore, with respect to the hybridization data obtained, the nickel resistance determinants of *K. oxytoca* were found to share strong homologies only with the *nre* operon from strain 31A which confers nickel resistance to *E. coli* strains (Schmidt 1992).

Further nickel-resistant bacterial strains isolated from habitats in Belgium, New Caledonia, Scotland, USA and Zaïre (altogether 57 strains) were investigated in order to determine homologies of their nickel resistance determinants with those of *K. oxytoca* 15788. Genomic and (if possible) plasmid DNA of these strains was isolated and digested with *Eco*RI. The restricted DNA was separated and probed with the biotinylated 4.3 kb *Hin*dIII fragment (KOHI4). Figure 4 shows an example of one of a total of seven blots treated in this manner. We failed to detect any signals (even in a less pronounced form) except with the DNA of the two New Caledonian enterobacterial strains. With genomic DNA of strain 5-1 and both genomic and plasmid DNA of strain 5-5 we obtained distinct signals indicating strong homologies (Figure 4, lanes 8, 9 and 10).

These results support our assumption that the *K. oxytoca* nickel resistance genes, carried by the 4.3 kb *Hin*dIII fragment, are representative for enterobacterial nickel resistance determinants and are different from the determinants such as *cnr* and *ncc* (and *czc*) which have so far been detected and expressed in members of *Alcaligenes* and *Pseudomonas*.

Discussion

The impulse to study a nickel-resistant isolate of *K. oxytoca* was provided by two unrelated circumstances, the observation of pronounced host specificities of nickel resistance determinants and the isolation of nickel-resistant bacteria from naturally nickel-loaden soil. (i) The nickel resistance determinants of several strains of *Alcaligenes* can be easily transferred—either in their native plasmids or as hybrid vectors—to species and strains of *Alcaligenes* or *Pseudomonas* and are expressed there. However, the native plasmids bearing the nickel resistance genes are usually not expressed in Enterobacteriaceae.

Table 4. DNA–DNA hybridization of the biotinylated 4.3 kb *Hin*dIII fragment (KOHI4) as DNA probe with DNA fragments carrying nickel resistance in various bacterial strains

Fragment	Size (kb)	Originating from strains	Location[a]	Hybridization signal	Isolation of fragment or reference
8.5 Kb *Eco*RI-*Pst*I (PPEI)		*A. eutrophus* CH34	Industry Liège, Belgium	none	Liesegang *et al.* (1993)
11.5 kb *Eco*RI		*A. denitrificans* 4a-2	Sewage plant in Dransfeld, Germany	none	Timotius & Schlegel (1987), Kaur *et al.* (1990) P. Kaur (unpublished data)
8.8 kb subfragment of the 14.5 *Bam*HI fragment (TBA)		*A. xylosoxydans* 31A	Industry, Holzminden, Germany	none	Schmidt *et al.* (1991)
3.1 kb subfragment of the 14.5 kb *Bam*HI fragment (TBA)				strong	
32W-2BA15	~ 15	32W-2	Garden soil, Monts des Koghis, New Caledonia	weak	Schlegel *et al.* (1991), Stoppel (1992)
12-1-EC7	~ 7	12–1	soils under nickel hyper-accumulating	weak	"
18-1-EC7	~ 7	18–1	tree, New Caledonia	weak	"
22-1-EC7	~ 7	22–1		weak	"

[a]Location where the nickel-resistant wild-type strains have been isolated from.

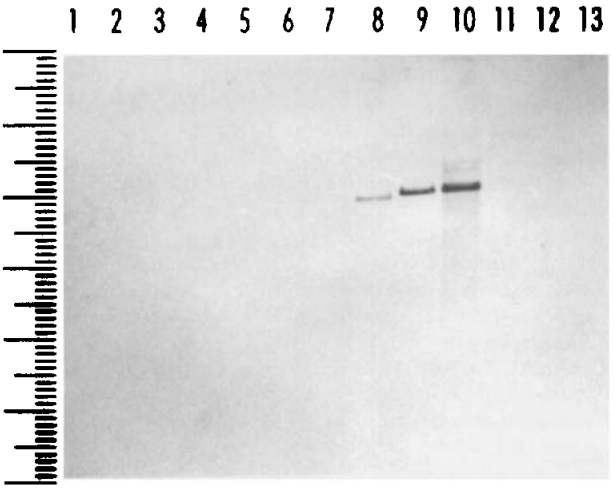

Figure 4. DNA–DNA hybridization of genomic or plasmid DNA from 57 nickel-resistant strains with fragment KOH14 as a biotinylated DNA probe (one of a total of seven blots is shown). The DNA was isolated as described, digested with *Eco*RI, electrophorized in Tris-acetate-buffered agarose (0.7%) for 2 h at 60 V, blotted onto a nylon filter and probed with the biotinylated fragment KOH14. Strong signals were detected with one fragment of the genomic DNA originating from the New Caledonian strains 5–1 and 5–5, and the plasmid DNA of strain 5–5 (lanes 8, 9 and 10). No signals could be shown with the DNA of other New Caledonian strains (lanes 11, 12 and 13), strains isolated in Zaïre (lanes 1–6) and *Hin*dIII-digested lambda DNA (lane 7). We also failed to detect any signal with the DNA from 48 other nickel resistant strains isolated from seven different geographic habitats (data not shown).

The characterization of *K. oxytoca* and its transconjugants is therefore of considerable interest. (ii) Two bacterial strains (5-1 and 5-5) were isolated from the nickel-rich soil under the canopy of nickel-hyperaccumulating trees (Schlegel *et al.* 1991) by enrichment culture under conditions which select for *E. coli* and some related bacteria. These strains lend themselves to comparison with enterobacterial strains isolated from anthropogenically polluted ecosystems.

Only recently, hybrid plasmids originating from the native nickel resistance plasmids of *A. eutrophus* KTO2 and *A. xylosoxydans* 31A were constructed which are expressed in *E. coli* (Schmidt *et al.* 1991), and the responsible genes, the *nre* operon, were cloned and sequenced (K. Lemke, unpublished data). We are just sequencing the nickel resistance determinant of *K. oxytoca*, the 4.3 kb *Hin*dIII DNA fragment, and wish to include the two strains 5-1 and 5-5 into this study. The question whether these enterobacteria-like bacteria share similar nickel resistance determinants and host specificities arises.

Within the present study we demonstrated that the nickel resistance determinants of *K. oxytoca* can be transferred by conjugation to various strains of *E. coli* and to *C. freundii*. Furthermore, a 4.3 kb *Hin*dIII fragment was cloned and transferred to various strains of *E. coli* where the genes are well expressed. With the *Hin*dIII fragment as a biotinylated DNA probe, about 60 strains of nickel-resistant bacteria were examined by DNA–DNA hybridization to detect homologies. These were only detected with the genomic DNA of the transconjugants, with the 3.1 kb fragment from *A. xylosoxydans* 31A harboring the *nre* operon and with genomic DNA of the New Caledonian strains 5-1 and 5-5 as target DNA. In other words: we regard the present results as compelling evidence that the *nre* operon of strain 31A and the resistance genes of *K. oxytoca* are representatives of nickel resistance genes mainly expressed in members of enterobacteria. This assumption is supported by the absence of detectable signals in the majority of the 60 strains studied which are clearly not enterobacterial strains.

The weak hybridization signals, which were obtained with the New Caledonian strains 12-1, 18-1, 22-1 and 32W-2 as target DNA, require a separate discussion. The signals appeared exclusively when the target DNA was offered in high copy number. The signals were only detected when the isolated nickel resistance fragments were used as target DNA; they were not detectable when the total genomic or the plasmid DNA was used. Furthermore, there were strong homologies between the *nre* operon (the 3.1 kb fragment of strain 31A) as DNA probe and the isolated nickel resistance fragments of four New Caledonian strains as target DNA. Based on this information we consider the weak hybridization signals between the *K. oxytoca* DNA probe and the nickel resistance determinants from the four New Caledonian strains are also significant.

The mechanism of nickel resistance determined by the *cnr* operon of *A. eutrophus* CH34 has been recognized to be due to energy-dependent specific efflux. The *cnr* operon shares high homology with the *czc* operon, coding for cobalt, zinc and cadmium resistance (Nies *et al.* 1987). The studies indicate that the efflux systems comprise a specific cation–proton antiporter, a cation binding subunit, a modifier protein and regulatory auxiliary proteins (Nies 1992).

The mechanism of nickel resistance determined by the 4.3 kb *Hin*dIII DNA fragment of *K. oxytoca* is not known. As data on the kinetics of nickel transport and on the proteins coded by the DNA fragment are so far lacking, we can only mention an alternative possibility of resistance, e.g. modification of the nickel uptake systems. Nickel is taken up in *A. eutrophus* by two systems. One is probably the magnesium carrier. Its affinity for nickel is low, but the transport capacity is high. The other system is a highly specific nickel carrier with high affinity for nickel, but with low transport capacity (Lohmeyer & Friedrich 1987, Eitinger & Friedrich 1991, Wolfram *et al.* 1991).

A similar high affinity nickel uptake system (*nik* operon) has recently been genetically studied in *E. coli* (Navarro *et al.* 1993). As the efficiency of these systems can be changed by mutation we envisage the possibility of profound changes of metal transport by substituting components of the carrier by other subunits resulting in a decrease of the transport capacity. The nickel resistance

system described in the present paper will possibly facilitate the exploration of alternative metal resistance systems.

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