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Evolution of a chlorobenzene degradative pathway among bacteria in a contaminated groundwater mediated by a genomic island in *Ralstonia*

Tina Andrea Müller,[1] Christoph Werlen,[1] Jim Spain[2] and Jan Roelof van der Meer[1]*

[1]*Swiss Federal Institute for Environmental Science and Technology (EAWAG), Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland.*

[2]*Air Force Research Laboratory, AFRL/MLQL, 139 Barnes Drive, Tyndall AFB, FL 32403, USA.*

Summary

The genetic structure of two *Ralstonia* spp., strain JS705 and strain JS745, isolated from the same groundwater aquifer, was characterized with respect to the degradation capacities for toluene and chlorobenzene degradation. Cosmid library construction, cloning, DNA sequencing and mating experiments indicated that the genes for chlorobenzene degradation in strain JS705 were a mosaic of the *clc* genes, previously described for *Pseudomonas* sp. strain B13, and a 5 kb fragment identical to strain JS745. The 5 kb fragment identical to both JS705 and JS745 was flanked in JS705 by one complete and one incomplete insertion (IS) element. This suggested involvement of the IS element in mobilizing the genes from JS745 to JS705, although insertional activity of the IS element in its present configuration could not be demonstrated. The complete genetic structure for chlorobenzene degradation in strain JS705 resided on a genomic island very similar to the *clc* element (Ravatn, R., Studer, S., Springael, D., Zehnder, A.J., van der Meer, J.R. 1998. Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *J Bacteriol* 180: 4360–4369). The unique reconstruction of formation of a metabolic pathway through the activity of IS elements and a genomic island in the chlorobenzene-degrading strain JS705 demonstrated how pathway evolution can occur under natural conditions in a few 'steps'.

Received 9 August, 2002; accepted 1 November, 2002. *For correspondence. E-mail vdmeer@eawag.ch; Tel. (+41) 1 823 5438; Fax (+41) 1 823 5547.

Introduction

Horizontal gene transfer is widely documented as one of the main mechanisms responsible for the evolution of new characteristics in microorganisms. Evidence for horizontal gene transfer comes mainly from three types of experiments: (i) laboratory 'mating' experiments with isolated pure cultures of microorganisms or deliberately released plasmid donating bacteria into more complex communities; (ii) studies of various types of mobile DNA and demonstration of the mechanistic details of transfer machinery; and (iii) indirectly inferred evidence from sequence comparisons among different isolated microorganisms. For example, Herrick *et al.* (1997) studied the naphthalene degradation characteristics of bacterial isolates in the same area. By phylogenetic comparisons, they concluded that the allele for the *nahAc* gene in seven isolates was incongruent to that for 16S rRNA, suggesting recent horizontal transfer of the *nah* genes. Similarly, by comparing 16S rDNA sequences and *tfdA* genes from bacteria degrading 2,4-dichlorophenoxyacetic acid (2,4-D), it was concluded that (some) *tfdA* genes must have been transferred by horizontal movement (McGowan *et al.*, 1998). Movement of genes for atrazine degradation was inferred from sequence comparisons of bacteria isolated at various geographic regions (de Souza *et al.*, 1998). Also, more or less accidentally, Peters *et al.* (1997) discovered the occurrence of a plasmid for phenol degradation that was identical to a plasmid from a strain introduced deliberately into a river basin to speed up phenol degradation after industrial spills.

One of our main interests has been the study of the formation of new catabolic pathways in bacteria. Such bacteria might provide evidence for evolutionary mechanisms that took place in the relatively recent past and also that the formation of a new catabolic pathway might involve the capture or recombination of foreign DNA fragments into one host. Degradation of chloroaromatic compounds would be a reasonable choice to test this hypothesis, as chloroaromatic compounds in general are difficult to degrade for microorganisms, and only a few

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organisms can profit from these substances as carbon source. This would provide a unique carbon source for the selection of new bacterial genotypes in the environment. This hypothesis has remained essentially correct, although it has become clear that chlorinated compounds of natural origin are more abundant than previously assumed. Therefore, we and others have had to assume that several of the capabilities for degradation of (parts of) chloroaromatic structures, such as chlorocatechols, must have existed among bacteria long before the introduction of chloroaromatic compounds by humans.

There is good experimental evidence now from different environmentally isolated bacteria to demonstrate that bacteria indeed seem to have captured or recombined existing DNA fragments from different bacterial sources into a new host (or 'singular' DNA structure). Yet, to the best of our knowledge, there is no study reporting the acquisition of a (non-self-mobilizable) DNA fragment with 100% identity between two or more bacterial strains isolated from one and the same site, which would make a perfect case for the hypothesis that evolution of catabolic pathways proceeds by exchange of gene building blocks (van der Meer, 1997; Arber, 2000). For example, bacteria degrading the herbicide 2,4-D often display similar gene clusters for 2,4-D degradation, almost like mosaic structures composed of a few gene blocks, which seem to have been assembled into different viable structures (Fulthorpe *et al.*, 1995; Leveau and van der Meer, 1997). Similar *cba* genes for 3-chlorobenzoate degradation were shown to be independently captured by the same insertion sequence (IS) element in two bacterial strains isolated at geographically distinct places (Di Gioia *et al.*, 1998). Bacteria degrading the herbicide atrazine often have the same pathway as *atz* genes (de Souza *et al.*, 1998; Martinez *et al.*, 2001). In many cases, bacteria degrading chlorobenzenes display a very typical combination of genes for a toluene type of multicomponent aromatic ring dioxygenase, dihydrodiol dehydrogenase, and genes for chlorocatechol degradation (van der Meer *et al.*, 1991; Beil *et al.*, 1999). This was of particular interest to our study, as the formation of a viable chlorobenzene pathway would involve only a 'single' evolutionary step of putting together existing genes for chlorocatechol and toluene degradation (although this step might have required different mechanistic 'steps'). Unfortunately, genetic analysis of the chlorobenzene pathway has come from single isolates, from which it has not been possible to trace the natural donor organisms for each of the pathway parts. Therefore, we were interested to see whether the formation of a chlorobenzene pathway could be traced directly at one single environmental site and would allow a more detailed reconstruction of evolutionary events.

Spontaneous attenuation of chlorobenzene was observed previously in a site polluted with (among other compounds) chlorobenzenes (Nishino *et al.*, 1992; 1994; van der Meer *et al.*, 1998). Monitoring of chlorobenzene concentrations over a number of years showed substantial and consistent decreasing concentrations, which suggested active microbial degradation. Sampling at sites within and outside the plume of chlorobenzene pollution showed one major type of chlorobenzene-degrading organisms occurring very abundantly inside the plume but an absence of chlorobenzene-degrading organisms in groundwater outside the plume (van der Meer *et al.*, 1998). Initial results suggested that part of the genetic material for the chlorobenzene pathway was very similar to that of toluene-degrading microorganisms from the same site (van der Meer *et al.*, 1998). Here, we show by cosmid mapping, cloning and DNA sequencing that the main chlorobenzene degrader at this site, *Ralstonia* sp. strain JS705, carries a 5 kb region of five genes completely identical to those of the toluene degrader. The gene region was analysed further for the presence of mobile DNA elements that could provide evidence for the hypothesis that the genes had been mobilized from the toluene degrader. Interestingly, we could demonstrate that the chlorobenzene pathway of strain JS705 is located on the chromosome, but is part of a mobile genomic island similar to the recently described *clc* genomic island of *Pseudomonas* sp. strain B13 (van der Meer *et al.*, 2001). This shows for the first time that genomic islands can acquire catabolic gene blocks and subsequently distribute these in a microbial community.

Results

Characterization of the region for chlorobenzene metabolism in strain JS705

Ralstonia sp. strain JS705 was previously isolated from groundwater contaminated with chlorobenzene at Kelly Air Force Base (KAFB) as the main chlorobenzene-degrading microorganism in that area (van der Meer *et al.*, 1998). DNA hybridizations with gene probes for the *clc*-encoded chlorocatechol degradative pathway (Frantz and Chakrabarty, 1987) and for chlorobenzene dioxygenase (Werlen *et al.*, 1996) had shown that both *clc*-like and *tcbAB*-like genetic material were present in one contiguous region. Complete DNA sequencing of this region, retrieved and subcloned from a cosmid library, revealed the following gene organization (Figs 1 and 2). A gene for the chlorocatechol pathway regulator (*clcR*) was followed by the chlorocatechol 1,2-dioxygenase gene (*clcA*) as for the originally described *clcRABDE* cluster (Frantz and Chakrabarty, 1987). However, almost directly after *clcA*, another small open reading frame (ORF) was found that did not resemble *clcB*. In fact, this ORF appeared to be an internal part of an insertion element repeated further

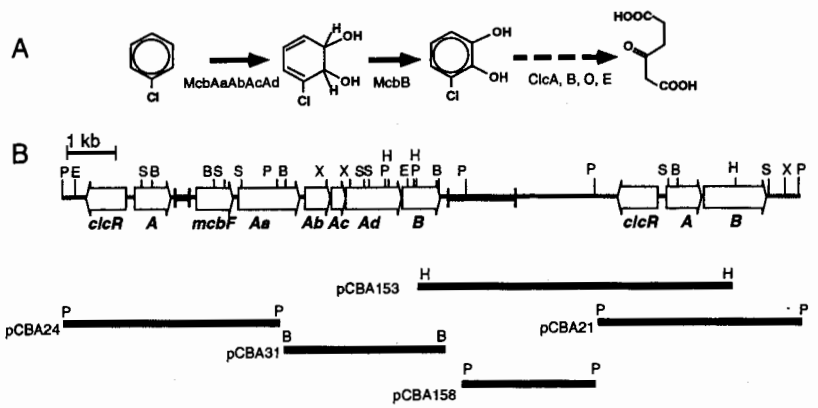

Fig. 1. Short overview of the chlorobenzene degradation pathway (A) and organization of the genes for chlorobenzene degradation in strain JS705 (B). Orientation and size of the different ORFs are indicated by open arrows. Gene assignments are given below. The solid black bars depict the positions of the left (partial) and right (intact) IS element. Single letters abbreviate restriction enzyme sites. The lower part of the figure shows some of the fragments cloned separately and their plasmid assignment. P, *Pst*I, E, *Eco*RI, S, *Sal*I, B, *Bam*HI, X, *Xho*I, H, *Hin*dIII.

downstream (Fig. 2). Downstream of this ORF, a set of genes was found for the aromatic ring dioxygenase and dihydrodiol dehydrogenase (here assigned the gene names *mcbAa*, *Ab*, *Ac*, *Ad* and *mcbB* for monochlorobenzene dioxygenase to express their similarities to the trichlorobenzene dioxygenase) (Werlen *et al.*, 1996) (Table 1). However, upstream of the *mcbAa* gene, we discovered an ORF with overall size of 831 bp and high sequence identity to *todF* (Menn *et al.*, 1991), encoding the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase. Downstream of the *mcbB* gene, putatively encoding a dihydrodiol dehydrogenase, a small sequence region was detected with identity to *todE*, encoding the methylcatechol 2,3-dioxygenase (Zylstra *et al.*, 1988). This *todE*-like sequence was interrupted by a different sequence, which strongly resembled a set of suspected insertion elements,

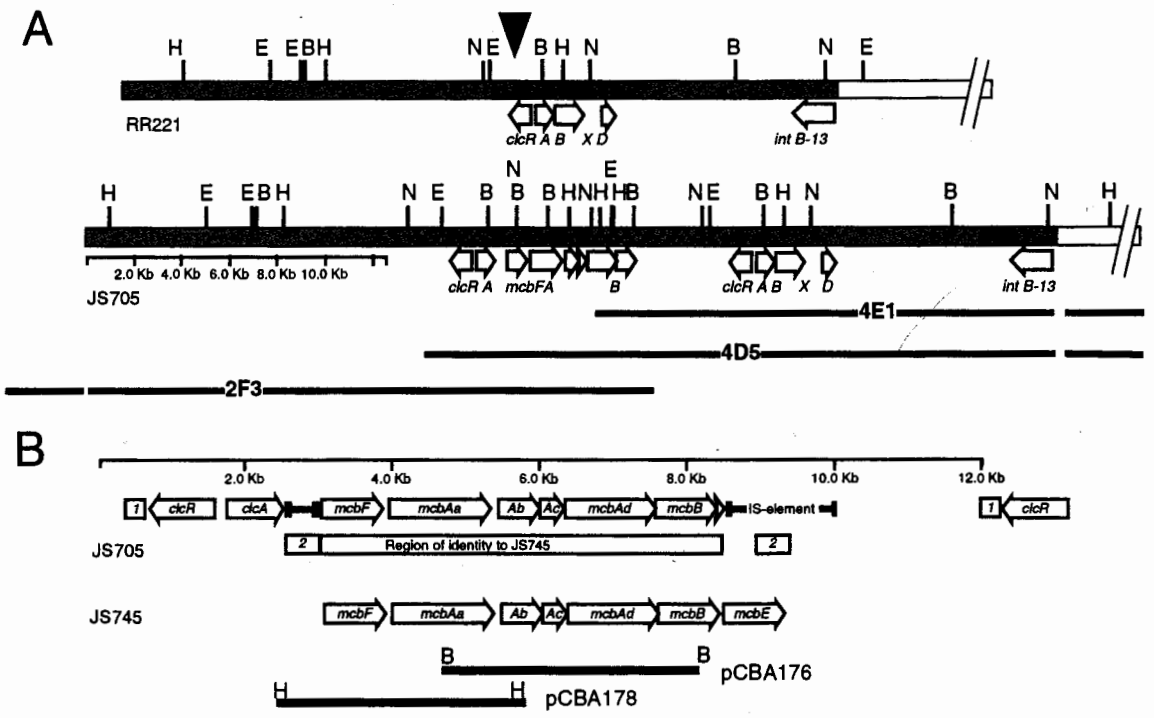

Fig. 2. Genetic organization of the *clc mcb* gene region of strain JS705 in comparison with that of the *clc* genes on the *clc* genomic island (A) and with that of the *mcb* gene region in strain JS745 (B).
A. The relevant area of the genomic islands and the cosmids used for mapping the area. The black triangle indicates the proposed insertion site for the *mcb* genes.
B. The *clc mcb* gene organization with gene assignments (sizes and orientations of genes as arrows), plus the duplicated regions upstream of *clcR* (indicated as box 1) and the duplicated parts of the insertion element (box 2). The region of identity among JS705 and JS745 is indicated. Bars below the JS745 region indicate the fragments cloned from JS745 for sequencing. B, *Bam*HI, E, *Eco*RI, H, *Hin*dIII, N, *Nhe*I.

Table 1. Percentages of identical nucleotides between proposed ORFs of the chlorobenzene gene cluster and major relatives in GenBank.

ORF (gene name)	Position	Proposed function	Major relative	Organism	Accession number	Percentage identity	Reference
clcR	687–1571c	LysR-type transcriptional activator	*clcR*	*Pseudomonas putida* (pAC27)	PSECLCR	99% (884/885)[a]	Coco *et al.* (1993)
			clcR	*P. aeruginosa* JB2	AF087482	99% (883/885)	Hickey *et al.* (2001)
clcA	1741–2523	Chlorocatechol 1,2-dioxygenase	*clcA*	*P. aeruginosa* JB2	AF164958	99% (780/783)	Hickey *et al.* (2001)
				P. putida (pAC27)	PLMCLC	99% (780/783)	Frantz and Chakrabarty (1987)
mcbF	3036–3866	2-hydroxy-6-oxo-2,4-heptadienoate hydrolase	*todF*	*P. putida* DOT	PPUY18245	89% (737/827)	Mosqueda *et al.* (1999)
mcbAa	3960–5315	Large subunit terminal oxygenase	*todC1*	*P. putida* F1	PSETODC1C	90% (1195/1319)	Zylstra and Gibson (1989)
mcbAb	5426–5989	Small subunit terminal oxygenase	*tcbAb*	*Pseudomonas* sp. strain P51	PSU15298	89% (507/564)	Werlen *et al.* (1996)
mcbAc	6000–6323	Electron transfer component, ferredoxin	*tecA3*	*Burkholderia* sp. strain PS12	BSU78099	90% (293/324)	Beil *et al.* (1997)
mcbAd	6323–7555	Reductase	*todA*	*P. putida* strain DOT-T1	PPUY18245	85% (1028/1200)	Mosqueda *et al.* (1999)
mcbB	7552–8379	*Cis*-toluene dihydrodiol dehydrogenase	*todD*	*P. putida* F1	PSETODC1C	87% (719/818)	Zylstra and Gibson (1989)
IS-ORF	8574–9818	Transposase		*Pseudomonas pseudoalcaligenes* sp. strain JS45	AF028594	99% (1503/1515)	Davis *et al.* (2000)
		Transposase	*tnpA*	*Pseudomonas* sp. ADP (pADP-1)	U66917	98% (1495/1515)	Martinez *et al.* (2001)
	10100–11080	None	nt11008–11986	*Xylella fastidiosa*	AE003995	73% (382/523)	
	11418–12050	None	nt 12–643	*P. aeruginosa* JB2	AF087482	98% (620/632) 8 gaps	
clcR'	12270–12483c	Regulatory gene	*clcR*	*P. aeruginosa* JB2	AF087482	99% (192/193)	Hickey *et al.* (2001)

a. Numbers within brackets indicate the number of identical nucleotides per number of nucleotides in the overlapping region.

now described in different bacteria (Fig. 3, Table 1). The largest ORF contained in this IS-like region covered 1245 bp and may encode the transposase. Downstream of this putative IS element, a region was located with resemblance to a DNA sequence from *Xylella fastidiosa* and, after that, to *Pseudomonas aeruginosa* sp. strain JB2. Further downstream, a second copy of the *clcR* and *clcA* genes was found, 99.5% identical to the 'left' copies and in exactly the same gene order (Fig. 1). Actually, the duplicated region was larger than just the ORF for *clcR* (Fig. 4). The second copy of *clcA* was followed by the *clcB* gene, coding for the chloromuconate cycloisomerase. Only a very short sequence of duplication was detected between *clcB* and the sequence downstream of the left *clcA* copy (Fig. 4A). We did not sequence further downstream of *clcB*, but the presence of restriction sites was exactly as described for the *clcABDE* gene cluster of *Pseudomonas* sp. strain B13 (Frantz and Chakrabarty, 1987).

No specific attempts were made in this study to characterize and prove any of the gene functions assigned to the *clc* and *mcb* genes on the basis of comparisons with other similar systems (Table 1). However, we feel relatively confident that the *clc* and *mcb* genes indeed encode the chlorobenzene pathway in strain JS705. First, because the percentage sequence identities between the *clc* and *mcb* genes of strain JS705 and other related systems were extraordinarily high and each ORF appeared intact. Secondly, because transfer of the *clc* and *mcb* gene regions to *Ralstonia eutropha* JMP289 resulted in a chlorobenzene degradative phenotype in that strain (see below).

The mcb *genes between strains JS705 and JS745 are 100% identical*

Ralstonia sp. strain JS745 is a toluene- and benzene-degrading bacterium that was isolated from the same site as strain JS705 (van der Meer *et al.*, 1998). From previous hybridizations and restriction mappings, we suspected strain JS745 to have been a possible 'donor' for the *mcb* genes of strain JS705. A cosmid library was constructed with JS745 total DNA and probed for the *mcb* genes of strain JS705. Positively hybridizing cosmid clones were retrieved, and the regions with identity to *mcb* were subcloned and sequenced (Fig. 2). As suspected, strain JS745 indeed contained a gene region with 100% sequence identity to the *mcb* gene region of strain JS705. The region in strain JS745 encompassed basically the same gene organization, i.e. *mcbF-Aa-Ab-Ac-Ad-B*. However, downstream of *mcbB*, a *todE*-like gene was found, which continued throughout the point at which the *todE*-like ORF was interrupted in strain JS705 (Fig. 5). Interestingly, the sequences near the point of

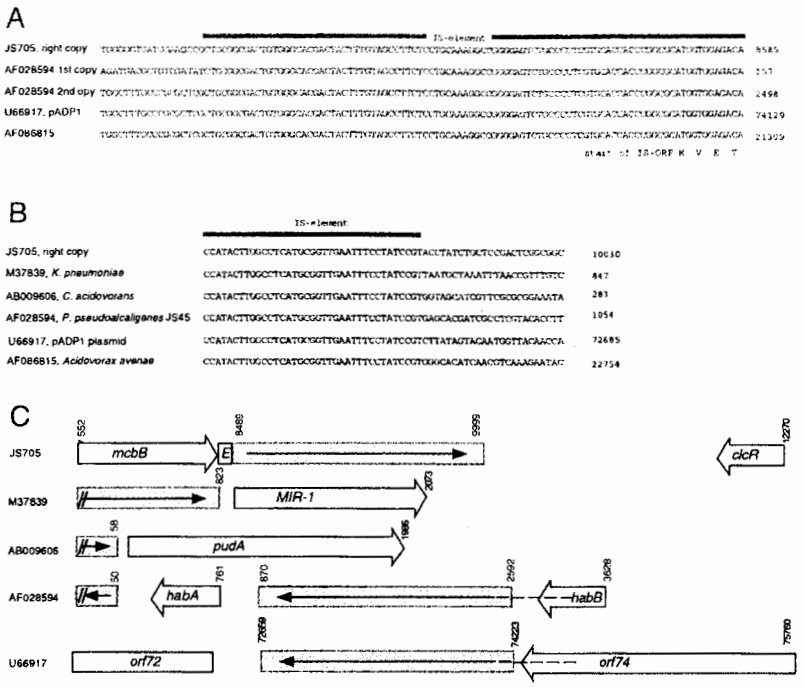

Fig. 3. Boundaries of the insertion element. Sequence comparisons between the right copy of the IS element of strain JS705 and related IS elements from other bacteria allow identification of a possible left end boundary (A) and right end boundary (B).
C. Schematic organization of the nearby genetic configuration of the IS elements in the different strains. Numbers refer to nucleotide numbering in the original GenBank accessions as indicated.

interruption in the *mcbE* ORF were not identical between JS705 and JS745. Upstream of *mcbF*, the region of identity with JS705 stopped relatively soon (Fig. 5A), and no evidence for other sequences related to the *clc* genes of strain JS705 or the IS element in the *clc* and *mcb* gene region of strain JS705 was obtained by Southern hybridizations.

The clc *and* mcb *genes of strain JS705 are part of a genomic island similar to the* clc *element of* Pseudomonas *sp. strain B13*

As strain JS705 contained *clc* genes practically indistinguishable from the *clc* genes of *Pseudomonas* sp. strain B13, we investigated whether they would perhaps be part

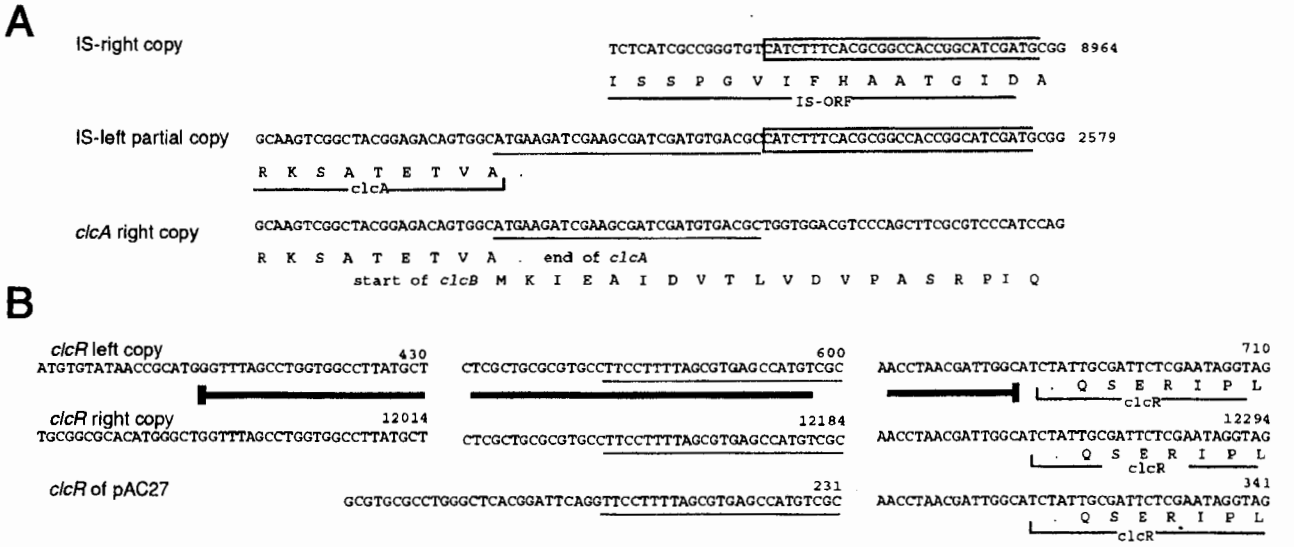

Fig. 4. Details of the duplications and boundaries near the IS fragments in the JS705 configuration.
A. Connection between the left *clcA* gene and the IS left partial copy, and a comparison with the intact *clcA–clcB* sequence and the complete IS right copy.
B. Duplicated sequences upstream of both copies of *clcR* on JS705 and in comparison with the known *clcR* sequence of *P. putida* pAC27 (see Table 1).

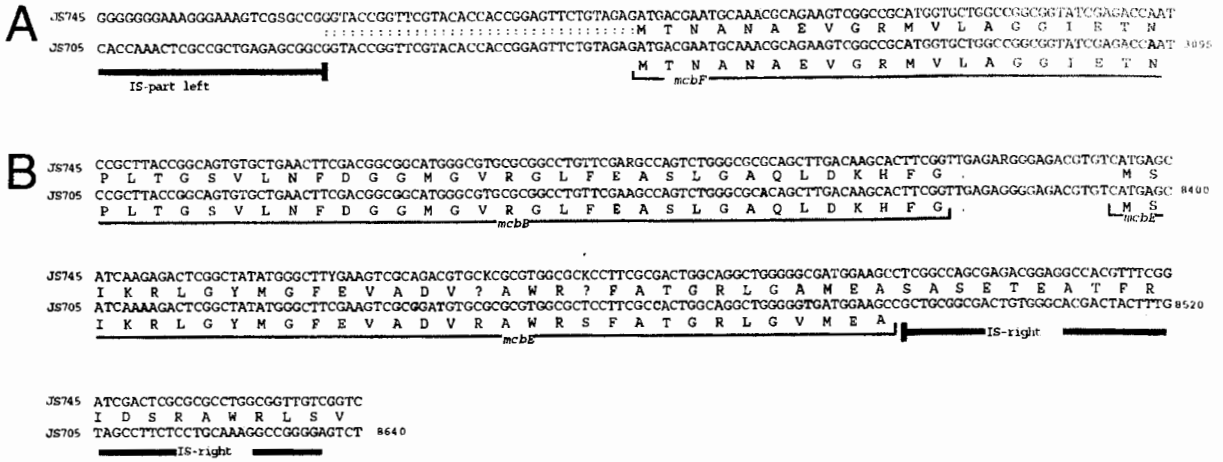

Fig. 5. Comparison of left (A) and right (B) ends of the identical DNA region between strains JS705 and JS745. Non-identical residues between JS705 and JS745 are printed in bold.

of a similar genetic element. The *clc* genes of *Pseudomonas* sp. strain B13 were recently found to be part of an integrative and conjugative element that we have designated the *clc* genomic island (Ravatn *et al.*, 1998a; van der Meer *et al.*, 2001). Southern hybridization of total DNA from strain JS705 with a gene probe for the *intB13* integrase gene was indeed positive (Fig. 6). Positively hybridizing cosmids to the *intB13* gene probe were retrieved from the JS705 cosmid library, and physical maps were constructed (Fig. 2). In fact, the physical maps were identical to those of the corresponding regions on the *clc* genomic island of strain B13 up to the point of the *mcb* genes in JS705. This strongly suggested that the *clc* and *mcb* genes in strain JS705 were part of an integrative element similar to that of the *clc* genomic island in strain B13.

In mating experiments between strain JS705 as donor and *R. eutropha* JMP289 as recipient, we tested whether the *clc* and *mcb* genes were transmissible. Upon selection for growth on chlorobenzene, transconjugants of *R. eutropha* JMP289 were detected at a frequency of 2.5×10^{-8} per recipient. Southern hybridization of *R. eutropha* JMP289 transconjugants with a 16S rDNA probe proved that the *clc* element had indeed travelled to this new host (Fig. 6). In addition, digestion of the chromosomal DNA with *Eco*RI and hybridization with the *intB13* gene probe demonstrated that the element most likely integrated into the chromosome and was not maintained as a plasmid or covalently closed intermediate. Hybridization with a probe for the *intB13* gene to the cosmid library resulted in the recovery of two positively reacting cosmids. A region of 900 bp of the *intB13* gene was sequenced directly on this cosmid using two primers (RR305 and RR308) developed on the basis of the *intB13* sequence and sequence of the integration site (Ravatn *et al.*, 1998b). The derived sequence of this region of the cosmid from JS705 was 100% identical to that of *intB13* of the *clc* element of *Pseudomonas* sp. strain B13 (not shown) (Ravatn *et al.*, 1998b). Restriction mapping (Fig. 2A) revealed a different pattern of sites upstream of the integration site (which is formed by the gene for glycine tRNA) in strain JS705 as in strain B13. Furthermore, the transfer experiment showed that the genetic information for chlorobenzene

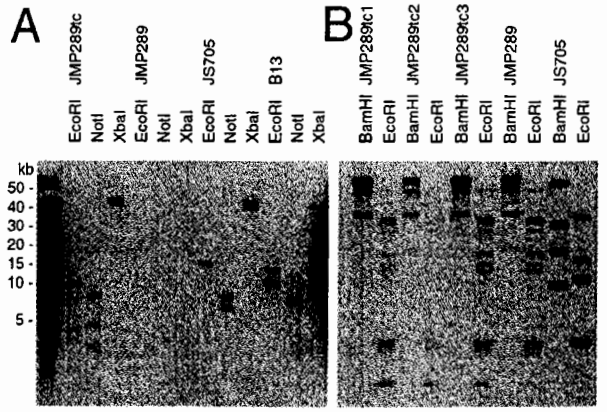

Fig. 6. Southern hybridizations demonstrating the transfer of the *clc* element from strain JS705 into *R. eutropha* strain JMP289.
A. Blot of an agarose gel separated by field inversion gel electrophoresis and hybridized with the insert of plasmid pRR165, containing the *intB13* gene of strain B13. Note that strain B13 has two copies of the *clc*-element.
B. Blot of a normal agarose gel hybridized with the 1.5 kb *Eco*RI insert of plasmid pCBA117 containing a 16S rDNA fragment of strain JS705 (van der Meer *et al.*, 1998). Note the identical banding patterns for three independent transconjugants (JMP289tc1 to 3) and strain JMP289, but the differences from those of strain JS705. Autoradiograms were recorded digitally on a Molecular Dynamics laser densitometer with the program IMAGEQUANT, transferred as TIFF files and printed from ADOBE ILLUSTRATOR.

metabolism resides on the transferred element, as the recipient *R. eutropha* JMP289 cannot metabolize chlorobenzene or chlorocatechols. This makes it very likely that the *clc* and *mcb* genes are indeed responsible for a functional chlorobenzene metabolic pathway.

Strain JS745 does not carry the gene for the intB13 *integrase or the IS element of strain JS705*

In another set of hybridization experiments, we answered the following questions: (i) does strain JS745 carry the *intB13* integrase gene; (ii) does strain JS745 carry DNA highly similar to the putative IS element located near the *mcb* genes in strain JS705; and (iii) is a similar IS element present on the *clc* genomic island of strain B13? In all these cases, the outcome was negative. As the IS element in strain JS705 was present only in the one complete and one partial copy near the *mcb* genes, but not anywhere else on the genome, these results indicated that the origin of the IS element seems not to lie in strain JS705, strain JS745 or the *clc* element itself.

Functionality of the IS element of JS705

To test the possible functionality of the insertion element present within the *clc* and *mcb* gene region, we performed the following experiments. The IS element was cloned in various combinations with the other internal left part plus a gene for kanamycin resistance on a mobilizable but non-replicative plasmid in *R. eutropha* JMP289. In none of the tested plasmids could the occurrence of kanamycin resistance in *R. eutropha* JMP289 higher than background be attributed to IS-mediated integration of the kanamycin resistance gene. Close inspection of the DNA sequence of the putative insertion element indicated that its orientation is most likely outwards to that required for effective transposition and that the small left part cannot contribute to transposition (Figs 1, 3 and 4). Therefore, we assume that the current organization of the intact insertion element copy and the remnant IS copy is not providing active transposition of the genes lying in between.

Purely on the basis of sequence comparison, the insertion element was highly identical to an insertion sequence present in *Pseudomonas pseudoalcaligenes* JS45, on the pADP1 plasmid (for atrazine degradation) and various other atrazine-degrading strains, and in *Klebsiella pneumoniae* (Fig. 3, Table 1), which suggests that the insertion sequence is capable of transposing in these bacteria. Comparisons of all these insertion sequences led to a proposed consensus end for this type of element although, strictly speaking, the start of the IS element can still not be defined clearly (Fig. 3A and C). If the ends of the IS element are defined as such, no target site duplication takes place (Fig. 3A and B).

Discussion

Reconstruction of the evolutionary events leading to the formation of the chlorobenzene pathway genes in strain JS705

By characterizing two strains from the same contaminated groundwater, we were able to perform an unusual reconstruction of evolutionary events, which showed the effect of mobilization of gene cassettes on metabolic pathway formation. The reconstruction was also unusual, as only very few cases in the literature actually describe gene fragments of 100% identity assembled in different organisms that had been isolated from the same environmental site. As the strains analysed here were abundant at the site ($\approx 10^4$ cfu ml^{-1} or 1–10% of the total cfu count on tryptic soy agar; van der Meer *et al.*, 1998), our evolutionary reconstruction is not a description of two accidentally isolated strains, but a true reflection of an important event in that particular contaminated area. The outcome of these events was the gene organization for the chlorobenzene degradation pathway of *Ralstonia* sp. strain JS705. All the evidence obtained so far points to the following mechanistic steps. Some bacteria in the environment carry a genomic island, named the *clc* element, which consists of a self-transferable DNA region of ≈100 kb containing the genes for (among others) chlorocatechol degradation (Ravatn *et al.*, 1998a,b; van der Meer *et al.*, 2001). The element was first discovered in the bacterium *Pseudomonas* sp. strain B13, which was isolated in Germany (Dorn *et al.*, 1974) and is responsible for 3-chlorobenzoate degradation in this strain. In fact, the *clc* element turned out to be the mobile transferable unit used in many previous mating or breeding experiments with strain B13 as a donor strain (Reineke and Knackmuss, 1979; Ravatn *et al.*, 1998c). The main chlorobenzene degrader at the contaminated site in this study (strain JS705) appeared to carry a genomic island very similar to the *clc* element, except for the presence of an additional set of genes for the enzymes that catalyse the initial attack on the aromatic ring of chlorobenzene, insertion elements and a duplication of the genes *clcR* and *clcA*. As the *clc* genomic island is self-transmissable (Ravatn *et al.*, 1998c), this suggests that, at some point, a donor bacterium with the *clc* element (or a slightly different form) transferred the element to strain JS705. We discovered that a bacterium degrading toluene from the same subsurface site, *Ralstonia* sp. strain JS745, carried a 5 kb gene fragment 100% identical to that of JS705, encompassing the genes for an aromatic ring dioxygenase, dihydrodiol dehydrogenase and hydrolase. Within the level of accuracy of sequence determinations (99.9%), we can therefore conclude that the shared fragments between JS705 and JS745 were completely identical. As no *clc* element was present in JS745, this strongly suggests that the *mcb* genes were mobilized from

JS745 to JS705. However, at least one other donor bacterium must have existed that provided the insertion element, as this is not present in strain JS745, not present elsewhere in JS705 or on the *clc* element in strain B13 (unless the insertion element is a non-replicative element and was lost from JS745 upon its transposition). Furthermore, the structure of the *clc–mcb* genes in JS705 does not seem to be the result of a single insertion of a composite transposon, carrying two copies of an insertion element flanking the *mcb* genes. Rather, a series of events, possibly an insertion (of two IS copies flanking the *mcb* genes), duplication (of the *clcR-A* region) and deletion (of part of one IS element), may have taken place. We were not able to demonstrate activity of the IS element in its present configuration by the experimental approach taken here. Although these approaches have worked for us previously (Leveau and van der Meer, 1997), there may be several reasons why we could not detect IS transposition. First, the IS may be in the wrong orientation for mobilizing the gene cassette inserted by cloning. Secondly, the IS-ORF may actually need to be longer than present in JS705. At least for the (almost) identical ISs on plasmid pADP1 and in *P. pseudoalcaligenes* (Fig. 3), the ORF can start upstream of the boundaries that we defined here for the IS element. Still, we feel relatively confident that the IS element was active at some point, as it is present in several other bacteria (for example, see Fig. 3) and even in multiple copies (as on plasmid pADP1; Martinez *et al.*, 2001).

Sequence curiosities and effects on pathway assembly and function

Some sequence curiosities that we observed by comparing the structures and sequences of the *clc* and *mcb* gene regions may actually have had an important physiological implication for successful adaptation of the bacterium JS705 to chlorobenzene degradation. In the first place, the ORF for the catechol 2,3-dioxygenase (*mcbE*) was quite effectively disrupted during the capture and transposition events between strain JS745 and JS705. This has been observed in other chlorobenzene-degrading bacteria as well (van der Meer *et al.*, 1991; Beil *et al.*, 1999) and was always attributed to a malfunctioning of catechol 2,3-dioxygenase with chlorocatechols. However, more recent work has demonstrated the existence of catechol 2,3-dioxygenases that are not inhibited by chlorocatechols (Mars *et al.*, 1997; 1999). Furthermore, if there was a strong selective disadvantage against keeping a gene for catechol 2,3-dioxygenase, it would seem to be a secondary event (i.e. after acquisition of perhaps the complete gene) but not necessarily at the level of capture by the IS elements. Another curiosity was the finding that there is no second copy of *clcB* and that the ORF of the second *clcB* was also very nicely interrupted almost exactly after its start. At this point, there is no specific evidence to indicate that a second copy of chloromuconate cycloisomerase would lead to some selective physiological disadvantage.

We were also puzzled by the finding that the DNA sequence of the region near the end of *mcbB* and the beginning of *mcbE* was not completely identical between JS745 and JS705, whereas the other 5 kb of the shared region was 100% identical in nucleotide sequence. This divergence might be the effect of an accelerated genetic drift in unessential regions or result from disturbance of the local DNA equilibrium by the insertion events, as postulated by Ornston *et al.* (1990).

The clc *genomic island*

A very interesting discovery was the finding that other bacteria, directly isolated from the environment, carried a genomic island very similar to the *clc* genomic island of *Pseudomonas* sp. strain B13 (Ravatn *et al.*, 1998a,b; van der Meer *et al.*, 2001). The *clc* genomic island was the first description of a genomic island carrying functions to degrade aromatic chemicals (in this case 3-chlorobenzoate). In contrast to many pathogenicity islands, the *clc* genomic island is self-transmissable to other recipient bacteria but has a similar mode of integrating site specifically into one or two chromosomal target sites (Springael *et al.*, 2002). The target site of the *clc* genomic island is the gene for glycine tRNA (Ravatn *et al.*, 1998b). The finding that strain JS705 carried a transmissible integrative element very similar to the *clc* element strongly suggests that this type of element is more abundant in microbial communities and not necessarily limited to one or two specific isolates. Actually, the high percentage of nucleotide sequence identity between the *clc* genes of *Pseudomonas* sp. strain B13 and *P. aeruginosa* strain JB2 (Hickey *et al.*, 2001) may indicate that strain JB2 harbours a similar type of *clc* element as well. As the behaviour of promiscuous genomic islands is very intriguing but not very well understood, the findings of a *clc*-type genomic island in strains occurring in geographically distinct environments and its capability of picking up extra DNA while passing through a microbial community are important for widening our perspective on horizontal gene transfer.

Experimental procedures

Bacterial strains

Ralstonia sp. strains JS705 and JS745 were isolated from groundwater at Kelly Air Force Base, near San Antonio, Texas (van der Meer *et al.*, 1998), and use chlorobenzene and toluene as sole carbon and energy sources respectively. *Ralstonia eutropha* JMP289 was used as plasmid-free recipient strain for matings with *Ralstonia* sp. strain JS705 and carries

rifampin resistance (Don *et al.*, 1985). *Pseudomonas* sp. strain B13 (Dorn *et al.*, 1974) and *Pseudomonas putida* RR221 (Ravatn *et al.*, 1998a) are hosts for the *clc* genomic island (van der Meer *et al.*, 2001). *Escherichia coli* DH5α was used as host for plasmid clonings, *E. coli* HB101 (pRK2073) (Figurski and Helinski, 1979) was used as mobilizing donor in triparental matings, and *E. coli* XL1-blue MR (Stratagene) was used for the construction of cosmid libraries.

Plasmids

The most relevant plasmids that were constructed for subcloning purposes from the *clc* and *mcb* gene region of strain JS705 are drawn schematically in Fig. 1 and contain pUC19 (Yanisch-Perron *et al.*, 1985), pUC28 (Benes *et al.*, 1993) or pGEM5zf (Promega) as basis. Plasmids derived from the *mcb* gene region of strain JS745 are depicted in Fig. 2.

Molecular cloning techniques

All plasmid clonings were carried out according to standard procedures (Sambrook *et al.*, 1989). Cosmid libraries of strains JS705 and JS745 were made according to the procedures for DNA isolation, partial digestion, cosmid preparation, ligation and packaging and infection as described in the 'Supercos' and 'Packaging Extract' kits from Stratagene. Cosmid libraries were stored as organized individual clones at −80°C. Southern hybridizations were performed as described previously (Ravatn *et al.*, 1998a). Double-stranded DNA sequencing of the *clc* and *mcb* genes of strain JS705 was done using a Thermosequenase cycle sequencing kit (Amersham Pharmacia Biotech) with IRD700- and IRD800-labelled primers (MWG Biotech). Gels were run on a LiCOR IR^2 automated sequencer, and sequences were analysed and assembled by the programs DNASTAR (Lasergene) and ALIGNIR (LiCOR). Comparative sequencing of strain JS745 DNA was performed on single strands only. Database searching was performed using the subroutines BLAST (Altschul *et al.*, 1997) and FASTA (as part of the GCG package, version 8, Wisconsin Computer Group).

Bacterial matings

Matings between *Ralstonia* sp. strain JS705 and *R. eutropha* JMP289, or *Pseudomonas* sp. strain B13 and *R. eutropha* JMP289, were performed on nutrient broth (NB) agar plates (Biolife). Mating co-cultures were plated out on JS mineral medium (Spain and Nishino, 1987) with 5 mM 3-chlorobenzoate or without carbon source. Plates without carbon source were incubated in airtight glass jars with chlorobenzene supplied through the vapour phase. Transconjugants were retrieved after 5–7 days growth at 30°C, purified by restreaking on agar plates and cultivated in 10 ml of JS mineral medium with 5 mM 3-chlorobenzoate for DNA isolation and subsequent Southern analysis.

Activity assay of the insertion element

The activity of the insertion element of the *mcb clc* gene region was assessed by cloning different combinations of the left IS piece and/or the right IS element, flanking a kanamycin gene cassette on plasmid pSUP202 (Simon *et al.*, 1986). This vector is mobilizable to *Ralstonia* and *P. putida* but cannot replicate in those strains. The different constructs were mobilized into *R. eutropha* JMP289 by triparental mating as described elsewhere (Leveau and van der Meer, 1997), and transconjugant colonies of *R. eutropha* were selected for kanamycin resistance. Total DNA of kanamycin-resistant transconjugants was isolated, digested and hybridized with the 1.5 kb *NdeI–SphI* fragment of pCBA189, containing the IS DNA.

Accession number

The DNA sequence of the *clc* and *mcb* gene regions of strain JS705 is deposited at the EMBL database under accession number AJ006307.

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