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Integration of Narrow-Host-Range Vectors from *Escherichia coli* into the Genomes of Amino Acid-Producing Corynebacteria after Intergeneric Conjugation

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Conjugative transfer of mobilizable derivatives of the *Escherichia coli* narrow-host-range plasmids pBR322, pBR325, pACYC177, and pACYC184 from *E. coli* to species of the gram-positive genera *Corynebacterium* and *Brevibacterium* resulted in the integration of the plasmids into the genomes of the recipient bacteria. Transconjugants appeared at low frequencies and reproducibly with a delay of 2 to 3 days compared with matings with replicative vectors. Southern analysis of corynebacterial transconjugants and nucleotide sequences from insertion sites revealed that integration occurs at different locations and that different parts of the vector are involved in the process. Integration is not dependent on indigenous insertion sequence elements but results from recombination between very short homologous DNA segments (8 to 12 bp) present in the vector and in the host DNA. In the majority of the cases (90%), integration led to cointegrate formation, and in some cases, deletions or rearrangements occurred during the recombination event. Insertions were found to be quite stable even in the absence of selective pressure.

Coryneform bacteria are gram-positive, nonsporulating soil microorganisms widely distributed in nature. Species of the genera *Corynebacterium* and *Brevibacterium* are of special interest for the industrial production of amino acids, nucleotides, and other compounds (22). In the last 10 years, efficient vector and transfer systems have been developed and a variety of genes involved in amino acid biosynthesis have been cloned (21, 43). Conjugal transfer of plasmids from *Escherichia coli* to coryneform bacteria (31) is the most efficient method to introduce heterologous DNA into *Corynebacterium* and *Brevibacterium* strains, reaching transfer frequencies of up to 10^{-2} per final donor colony, with 10^7 to 10^8 transconjugants per mating assay. Restriction systems in coryneform bacteria impairing intergeneric conjugation have been shown to be stress sensitive and can be inactivated by short stress treatments (30, 32). Gene disruption and gene replacement techniques have been applied to selectively inactivate genes in corynebacteria by the use of transformation techniques (20, 40) or by conjugation (33, 34) with mobilizable *E. coli* plasmids carrying internal fragments of the genes. In addition, because of their high frequency of conjugal transfer, corynebacteria are suitable candidates in which to search for and apply mobile elements for mutagenesis. Information is emerging about the existence of such mobile elements (insertion sequence [IS] elements or transposons) in corynebacteria. Vertès et al. (41) described the presence in *Corynebacterium glutamicum* of the element IS*31831* and used it to construct two artificial transposons (Tn*31831* and mini-Tn*31831*) which were functional in a *Brevibacterium flavum* strain (39). A new IS element (IS*1206*) from *C. glutamicum* has been described by Bonamy et al. (5), and a different one (IS*13869*) has been isolated from *Brevibacterium lactofermentum* (7). In addition, an extensive survey on the occurrence of IS elements in coryneform bacteria, conducted with an IS entrapment vector based on the *Bacillus subtilis sacB* gene, indicated that at least three different classes of IS elements are present in coryneform bacteria (16). Very recently, a transposable element from *Corynebacterium xerosis* was shown to be active in *C. glutamicum* after introduction of the delivery vector by intergeneric conjugation (38).

Insertional mutagenesis by illegitimate recombination was observed in *Rhodococcus fascians* (9) and in mycobacteria (17); however, in both cases, the plasmid used contained host DNA fragments. In the course of plasmid transfer experiments intended to evaluate the applicability of known transposons in coryneform strains, we discovered that the *E. coli* mobilizable plasmids pSUP1021 (35), pSUP2021 (35), pSUP301 (36), and pK18mob (33), all of which are based on classical narrow-host-range plasmids and carry the origin of transfer (*ori*T) of plasmid RP4, became integrated into the genomes of different coryneform bacteria. We describe the molecular mechanism of this integration and the possible use of these vectors in mutagenesis assays.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *B. lactofermentum* 13869, *C. glutamicum* 13032, and *B. lactofermentum* R-31 (a strain that has a high efficiency of transformation) were used as recipient strains in conjugation assays, whereas *E. coli* S17-1, which contains a derivative of the conjugative IncP plasmid RP4 (8) integrated into the chromosome, was used as the donor.

E. coli strains were grown on Luria-Bertani medium (a complex medium) or VB (37) minimal medium at 37°C. The media used for growth of corynebacteria were trypticase soy broth (a complex medium), trypticase soy agar (trypticase soy broth with 2% of agar), and minimal medium for corynebacteria (MMC [18]). MMC containing gluconic acid (0.1%) as the sole carbon source (instead of glucose) was used when required. The following supplements were added to MMC medium: *meso*-diaminopimelic acid (0.1 mM), L-homoserine (0.2 mM), and L-threonine (0.15 mM final concentration); other amino acids were used at 0.3 mM. Antibiotics used for plasmid selection were kanamycin (50 μg/ml for *E. coli*; 30 μg/ml for corynebacteria), ampicillin (100 μg/ml), chloramphenicol (100 μg/ml for *E. coli*; 10 μg/ml for corynebacteria), hygromycin (100 μg/ml), and tetracycline (50 μg/ml for *E. coli*; 10 μg/ml for corynebacteria).

DNA techniques and transformation of *E. coli* and corynebacteria. Plasmid DNA of *E. coli* was isolated by the alkali lysis method (4) or the boiling method

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TABLE 1. Strains and plasmids used in this work

Strain, plasmid, or phage	Description[a]	Source or reference
E. coli S17-1	Mobilizing donor strain; *hsd pro recA*	36
E. coli DH5α	F^- RecA *endA gyrA96 thi-1 hsdR17* Δ(*lacZYA-argF*)	14
E. coli WK6mutS	Δ(*lac-proAB*)*galE strA mutS*215::Tn*10*/F′ *lacIZ*ΔM15 *proAB*$^+$	R. Zell
B. lactofermentum 13869	Type strain; yellow	ATCC[b]
B. lactofermentum R-31	Mly^r Aec^r; white	28
C. glutamicum 13032	Type strain	ATCC
C. glutamicum TRA-1	Leu auxotrophic mutant obtained with pSUP1021; Km^r	This work
C. glutamicum TRA-20	Met auxotrophic mutant obtained with pSUP1021; Km^r	This work
C. glutamicum TRA-2	Prototrophic strain obtained with pSUP301; Km^r	This work
C. glutamicum TRA-3	Ile auxotrophic mutant obtained with pSUP301; Km^r	This work
C. glutamicum TRA-4	Prototrophic strain obtained with pSUP301; Km^r	This work
C. glutamicum TRA-7	Trp auxotrophic mutant obtained with pSUP301; Km^r	This work
C. glutamicum TRA-8	Prototrophic strain obtained with pSUP301; Km^r	This work
C. glutamicum TRA-9	Prototrophic strain obtained with pSUP301; Km^r	This work
C. glutamicum TRA-12	Prototrophic strain obtained with pK18mob; Km^r	This work
B. lactofermentum TRA-50	Prototrophic strain obtained with pSUP301; Km^r	This work
Plasmids or phage		
M13K07	Helper phage	24
pUC18 and pUC19	2.7 kb; *E. coli* cloning vectors; *lacZ*α Ap^r	Laboratory stock
pUC118 and pUC119	3 kb; phagemid vectors (pUC derivatives) used in sequencing; Ap^r *lacZ*	44
pBluescript KS and SK	3 kb; phagemids derived from pUC; Ap^r	Stratagene
pECM1	10.6 kb; *E. coli-Corynebacterium* mobilizable plasmid; Km^r Cm^r	31
pSUP301	5 kb; *E. coli* mobilizable vector (Mob of plasmid RP4); replicon pACYC177 (Ap^r Km^r)	36
pSUP1021	11.7 kb; *E. coli* mobilizable vector (Mob of plasmid RP4); replicon pACYC184 (Tc^r Cm^r) and transposon Tn*5* (Km^r)	35
pSUP1021Δ*Pst*	9.2 kb; pSUP1021 derivative with deletion of the functional transposase gene of Tn*5* by partial digestion with *Pst*I; Tc^r Cm^r Km^r	This work
pSUP1021ΔIS	8.1 kb; pSUP1021 derivative with deletion of both transposase genes from Tn*5*; Tc^r Cm^r Km^r	This work
pSUP2021	13 kb; *E. coli* mobilizable vector (Mob of plasmid RP4); replicon pBR325 (Ap^r Cm^r Tc^r) and Tn*5* (Km^r) into the *tet* gene (Tc^s)	35
pK18mob	3.8 kb; *E. coli* mobilizable vector (Mob of plasmid RP4); pBR322 replicon; Km^r *lacZ*	33
pBL1	4.5 kb; endogenous cryptic plasmid from *B. lactofermentum* ATCC 13869	29
pULM21	12 kb; in vitro-formed recombinant plasmid containing the entire pBL1 replicon and a 7.5-kb fragment from pSUP1021 which contains the entire Tn*5* transposon (Km^r)	This work
pSUP301-3	8.2 kb; obtained by plasmid rescue from *C. glutamicum* TRA-3 by *Eco*RI digestion	This work
pSUP301-8	6.9 kb; obtained by plasmid rescue from *C. glutamicum* TRA-8 by *Eco*RI digestion	This work
pSUP301-9	24 kb; obtained by plasmid rescue from *C. glutamicum* TRA-9 by *Eco*RI digestion	This work
pK18mob-12	6.2 kb; obtained by plasmid rescue from *C. glutamicum* TRA-12 by *Eco*RV digestion	This work
pUL609M	8.0 kb; bifunctional *E. coli-Corynebacterium* vector; Km^r Hyg^r Ap^r	23
pUL609M-ile	10.9 kb; pUL609M derivative containing the *ilvA* gene from *C. glutamicum* in a 2.9-kb *Hin*dIII fragment; Hyg^r Ap^r	This work

[a] Abbreviations: Mly, methyllysine; Aec, *S*-aminoethylcysteine; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Hyg, hygromycin.
[b] ATCC, American Type Culture Collection.

of Holmes and Quigley (15). Plasmids from corynebacteria were isolated by a modified alkaline lysis method (26), except that treatment with lysozyme was extended up to 3 h. Chromosomal DNA of corynebacteria was prepared according to the method of Altenbuchner and Cullum (1). Restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, and other modification enzymes were used as recommended by the manufacturers. DNA restriction fragments were isolated from agarose gels by using the Geneclean Kit (Bio 101 Inc., La Jolla, Calif.).

Transformation of *E. coli* strains was carried out by the RbCl method (14), and corynebacteria were transformed by electroporation (10).

Conjugal transfer protocol. Mobilization of plasmids from *E. coli* to coryneform strains was done essentially as described by Schäfer et al. (31), except that the donor-recipient ratio was 1:1 and 5×10^9 cells of each strain were used. Transconjugants were selected on trypticase soy agar medium containing 50 μg of nalidixic acid and 30 μg of kanamycin per ml. The presence of cointegration was analyzed further by growing the Km^r transconjugant colonies in complex media with chloramphenicol or tetracycline.

Hybridization experiments. To confirm the integration of the mobilizable plasmids, total DNAs of transconjugant clones were isolated and digested with selected restriction enzymes. Restriction fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane (Amersham). Labelling and hybridization of DNA were performed with the nonradioactive DNA labelling and detection kit from Boehringer GmbH (Mannheim, Germany) according to the manufacturer's specifications.

Plasmid rescue technique. Total DNAs from Km^r coryneform transconjugants were digested with different restriction enzymes which do not cut the integrated mobilizable vector. The resulting DNA fragments were ligated and transformed into *E. coli* DH5α cells, with subsequent selection for resistance to kanamycin. Plasmid DNA was isolated from the *E. coli* Km^r transformants and analyzed.

DNA sequencing and sequence analysis. DNA fragments were subcloned in pUC118 and pUC119 or pBluescript KS and SK phagemids (Table 1). Single-stranded plasmid DNA was isolated after transformation of *E. coli* WK6mutS (Table 1) with the recombinant phagemids and the helper virus M13K07 (Table 1). α-^{35}S-dATP (>600 Ci mmol^{-1}) was purchased from Amersham. DNA sequence analysis was performed by the dideoxy chain termination method (27) with Sequenase (United States Biochemical Co.). DNA and protein sequences were analyzed with the DNAstar computer program (DNAstar Inc., Madison, Wis.).

RESULTS

Integration of mobilizable, nonreplicating vectors containing the Tn*5* transposon into the *C. glutamicum* and *B. lactofermentum* chromosomes. Our initial intention was to investigate the applicability of different known transposable elements in corynebacteria. For this purpose, we used mobilizable trans-

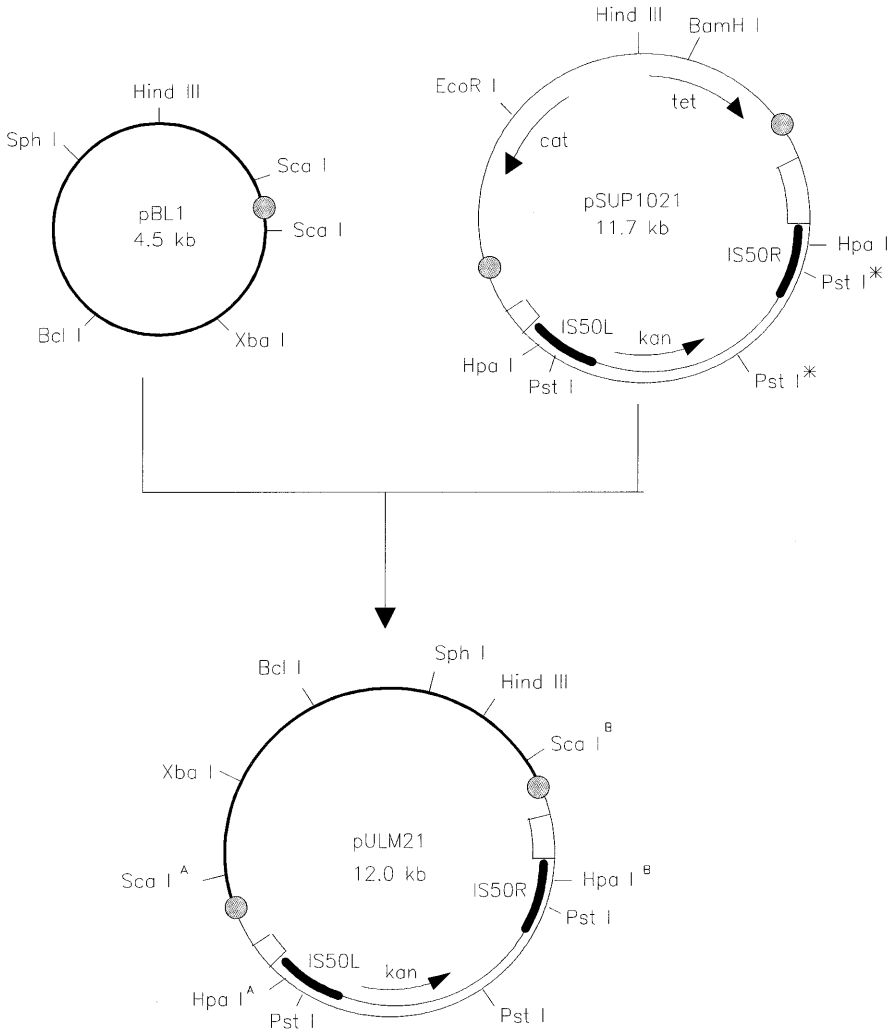

FIG. 1. Structure of plasmids pBL1, pSUP1021, and pULM21. pULM21 was obtained by in vivo recombination and reorganization involving the 8-bp sequence present in pBL1 and pSUP1021 (◯). The 2.5-kb *Pst*I* fragment from pSUP1021 was removed by *Pst*I partial digestion to form pSUP1021Δ*Pst*; the *Sca*I^{A}-*Hpa*I^{A} and *Sca*I^{B}-*Hpa*I^{B} fragments from pULM21 correspond, respectively, to the 0.85- and 1.1-kb fragments subcloned in pUC118 and pUC119 and sequenced. ▬, DNA from pBL1; ——, DNA from pACYC184; ●—●, *Tn5*; ▭, Mob fragment from RP4.

poson delivery vectors, such as the *E. coli* plasmid pSUP1021 (Table 1; Fig. 1), which carries the transposon Tn*5*. Plasmid pSUP1021 is based on the pACYC184 replicon, which is able to replicate in *E. coli* and closely related strains. This plasmid was transferred from *E. coli* S17-1 (36) to *C. glutamicum* 13032 as well as to *B. lactofermentum* 13869 and R31 by intergeneric conjugation. In each mating assay we found kanamycin-resistant transconjugants, which arose at a frequency of 5×10^{-8} per final donor colony (CFU) and with a delay of 2 to 3 days compared with transconjugants obtained with the mobilizable shuttle plasmid pECM1 (31). Similar results were obtained with plasmid pSUP2021 (Table 1), which is based on the narrow-host-range plasmid pBR325.

Plasmid DNA preparations were obtained from 50 randomly chosen Km^r transconjugants of *C. glutamicum* 13032, *B. lactofermentum* 13869, and *B. lactofermentum* R-31, and the absence of autonomously replicating vector molecules was proven by agarose gel electrophoresis and Southern hybridization. We could detect the presence of the indigenous plasmid pBL1 only in *B. lactofermentum* 13869 (Fig. 1) (29) and Km^r transconjugants derived from this bacterium. In one case, the isolated plasmid did not show the expected size for pBL1 but a larger derivative named pULM21 (see below) (Table 1) was found.

Total DNAs from different Km^r *C. glutamicum* 13032 transconjugants obtained with plasmids pSUP1021 and pSUP2021 were isolated, digested with selected restriction enzymes, and hybridized against labelled plasmid DNA. Integration of the plasmids could be confirmed in all transconjugants, and different patterns of bands were found in different transconjugants.

Around 90% of the transconjugants obtained with pSUP1021 and pSUP2021 were also Cm^r, a result which we interpreted as being due to cointegration events mediated by the transposase of transposon Tn*5*. To examine this hypothesis, plasmid pSUP1021 was partially digested with *Pst*I to remove a 2.5-kb *Pst*I fragment from the 3′ end (IS*50R*) of Tn*5*, which contains the intact gene encoding the transposase, generating the plasmid pSUP1021Δ*Pst* (see the legend to Fig. 1); an additional pSUP1021 derivative plasmid (pSUP1021ΔIS) (Table 1) from which both transposase genes were deleted (IS*50R* and IS*50L*) was built and used in further experiments (data not shown). Plasmids pSUP1021, pSUP1021Δ*Pst*, and pSUP1021ΔIS

were used in conjugation assays with *B. lactofermentum* and *C. glutamicum* strains as recipients. Surprisingly, similar numbers of transconjugants were obtained with all the plasmids, indicating that a functional Tn*5* transposase is dispensable for integration.

C. glutamicum 13032 harbors an IS element termed IS*Cg1* which is present in four to seven copies in the chromosome (16). We assumed that this IS element might become involved in the integration by jumping from the chromosome into the plasmid and undergoing further recombination into the chromosome. Chromosomal DNA of 12 *C. glutamicum* transconjugants was therefore probed with labelled IS*Cg1* DNA. The IS*Cg1* pattern observed in all transconjugants analyzed, however, was identical to that observed in the wild-type strain, suggesting that this particular IS element is not involved in plasmid integration (data not shown).

In order to investigate whether transposon sequences are involved in the integration process, we mobilized the *E. coli* plasmids pSUP301 and pK18mob (Table 1), which are based on pACYC177 and pBR322, respectively. Although these plasmids do not carry any transposon sequences, integration events took place. The number of transconjugants obtained with pSUP301 was slightly higher than the numbers obtained with pSUP1021 and pSUP2021, with a yield of 25 to 30 transconjugants per mating assay with *C. glutamicum* and around 15 transconjugants when *B. lactofermentum* was used as the recipient. One to three transconjugants were obtained per mating assay when plasmid pK18mob was used. Again we could not detect any free plasmid DNA in the transconjugants. Hybridization experiments with total DNA of transconjugants obtained with both plasmids and labelled plasmid DNA revealed that the plasmids had become integrated at different sites in the genome (Fig. 2B). In addition, there was no detectable change in the pattern of IS*Cg1* elements in *C. glutamicum* or *B. lactofermentum* transconjugants compared with the pattern in the wild-type strains (Fig. 2C).

Plasmids pSUP1021, pSUP301, and pK18mob integrate by recombination between very short homologous DNA sequences. To investigate the mechanism of integration of pSUP1021, we analyzed plasmid pULM21. This plasmid, which was isolated from a unique *B. lactofermentum* 13869 transconjugant, is composed of the entire pBL1 plasmid and a fragment of pSUP1021 with the transposon Tn*5* and adjacent DNA regions (Fig. 1). This plasmid was probably generated by integration of pSUP1021 into pBL1 and subsequent deletions. The DNA regions of pSUP1021 and pBL1 flanking the Tn*5* in pULM21 were isolated as 0.85- and 1.1-kb *Sca*I-*Hpa*I fragments and subcloned into the sequencing vectors pUC118 and pUC119. Sequence analysis revealed the presence of a direct repeat of 8 bp at both borders to pBL1 (5′-CATCGCAG-3′). In pSUP1021, this sequence is present twice and is adjacent to the ends of the Mob fragment (Fig. 1). This result indicates that recombination occurred between short, 8-bp homologous sequences present on both pSUP1021 and plasmid pBL1 of *B. lactofermentum* 13869.

Three different insertions of plasmid pSUP301 were recovered from the chromosomes of *C. glutamicum* TRA-3, TRA-8, and TRA-9 (Table 1) by cleavage with *Eco*RI (a site for which is absent in pSUP301), religation, and subsequent transformation into *E. coli* DH5α. The isolated plasmids were termed pSUP301-3, pSUP301-8, and pSUP301-9, and they carried chromosomal DNA inserts of 3.2, 1.9, and 19.0 kb, respectively. The integration and the strategy for plasmid rescue corresponding to pSUP301-3 and pSUP301-8 are shown in Fig. 3A and B, respectively. Plasmids pSUP301-3 and pSUP301-8 were extensively mapped, and fragments close to the border of pSUP301 were subcloned in pUC118, pUC119, or pBluescript and sequenced. In the case of pSUP301-3, the sequence analysis revealed that pSUP301 became integrated into the chromosome by recombination between 11-bp sequences present in the Mob site of plasmid pSUP301 and in the 3′ region of the *ilvA* gene (encoding the enzyme threonine dehydratase), which had been previously cloned and sequenced by Mökel et al. (25), generating the 11-bp direct repeat at the integration site (Fig. 3A; Fig. 4B). In the case of pSUP301-8, integration involved a 12-bp sequence present in the Mob site of pSUP301 (Fig. 3B; Fig. 4C), 0.7 kb from the integration site of pSUP301-3. The chromosomal DNA sequence present in pSUP301-8 was compared with sequences stored in the GenBank library, and significant homology was found with the gluconate permease gene (*gntP*) from *B. subtilis* (11).

Finally, a pK18mob insertion into *C. glutamicum* TRA-12 was analyzed by plasmid rescue with *Eco*RV (a site for which is absent in pK18mob), generating plasmid pK18mob-12 (Fig. 3C). This plasmid was unable to confer *lacZ*α complementation to the host strain *E. coli* DH5α. The plasmid was mapped and sequenced, and the insertion site in the *lacZ*α fragment was found to be close to the multiple cloning site of plasmid pK18mob. A 10-bp direct-repeat sequence was involved in the integration process (Fig. 4D). In this case, there was no significant homology between the chromosomal DNA insert in pK18mob-12 and any known gene or protein sequence.

These examples demonstrate that narrow-host-range plasmids from *E. coli* can integrate into the genomes of gram-positive coryneform bacteria by recombination between short (8- to 12-bp) homologous sequences.

Analysis of auxotrophic mutants generated by integration of mobilizable *E. coli* plasmids into the chromosomes of coryneform strains. Transconjugants of *C. glutamicum* obtained with plasmids pSUP1021, pSUP301, and pK18mob were checked for their ability to grow on minimal medium. Two clones with an integrated pSUP1021 were found to be auxotrophic; *C. glutamicum* TRA-1 and *C. glutamicum* TRA-20 required leucine and methionine for growth, respectively (Table 1). Plasmid rescue experiments with these mutants always failed. Among 200 transconjugants carrying pSUP301 in the chromosome, we found two auxotrophic mutants: *C. glutamicum* TRA-3 (Ile^-), and *C. glutamicum* TRA-7 (Trp^-). The stability of insertion mutations was quite high: 100 colonies from Leu^- and Ile^- *C. glutamicum* auxotrophs (TRA-1 and TRA-3) were analyzed after inoculation and growth to late exponential phase without selective pressure (about 30 generations), and 98 to 99% of the clones were still able to grow on complex medium supplemented with kanamycin.

In addition, two other clones could not grow on minimal medium; however, it was not possible to identify the deficiency in a normal auxanographic screening. *C. glutamicum* TRA-8, initially isolated as a prototroph (Table 1), contained plasmid pSUP301 integrated into the gluconate permease gene (*gntP*), as deduced from sequence homology with the *gntP* gene from *B. subtilis*. *C. glutamicum* TRA-8 was unable to grow in MMC containing gluconic acid as the sole carbon source, whereas the wild-type strain did grow. In addition, other *C. glutamicum* and *B. lactofermentum* prototrophic transconjugants (via pSUP301) were analyzed (Table 1; Fig. 2). In order to prove that the Ile^- phenotype of *C. glutamicum* TRA-3 was indeed caused by integration of pSUP301, an *ilvA* fragment from pSUP301-3 was used as a probe to isolate the entire *ilvA* gene from a *C. glutamicum* genomic library (digested partially with *Sau*3A) in pUC18; three clones were identified by hybridization. A 2.9-kb *Hin*dIII fragment containing the entire *ilvA* gene (25) was cloned into the *Hin*dIII site of the *E. coli*-coryneacterium shut-

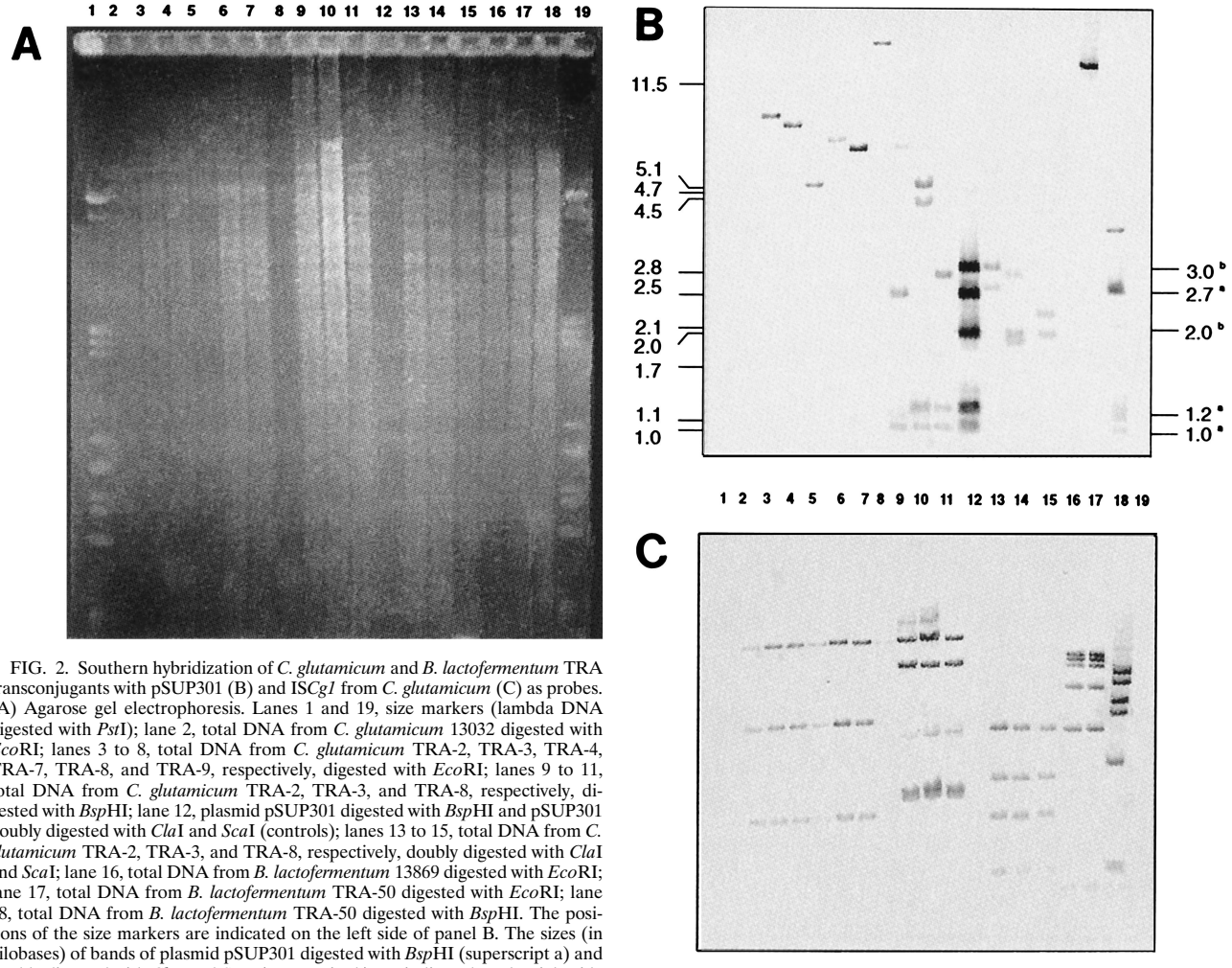

FIG. 2. Southern hybridization of *C. glutamicum* and *B. lactofermentum* TRA transconjugants with pSUP301 (B) and IS*Cg1* from *C. glutamicum* (C) as probes. (A) Agarose gel electrophoresis. Lanes 1 and 19, size markers (lambda DNA digested with *Pst*I); lane 2, total DNA from *C. glutamicum* 13032 digested with *Eco*RI; lanes 3 to 8, total DNA from *C. glutamicum* TRA-2, TRA-3, TRA-4, TRA-7, TRA-8, and TRA-9, respectively, digested with *Eco*RI; lanes 9 to 11, total DNA from *C. glutamicum* TRA-2, TRA-3, and TRA-8, respectively, digested with *Bsp*HI; lane 12, plasmid pSUP301 digested with *Bsp*HI and pSUP301 doubly digested with *Cla*I and *Sca*I (controls); lanes 13 to 15, total DNA from *C. glutamicum* TRA-2, TRA-3, and TRA-8, respectively, doubly digested with *Cla*I and *Sca*I; lane 16, total DNA from *B. lactofermentum* 13869 digested with *Eco*RI; lane 17, total DNA from *B. lactofermentum* TRA-50 digested with *Eco*RI; lane 18, total DNA from *B. lactofermentum* TRA-50 digested with *Bsp*HI. The positions of the size markers are indicated on the left side of panel B. The sizes (in kilobases) of bands of plasmid pSUP301 digested with *Bsp*HI (superscript a) and doubly digested with *Cla*I and *Sca*I (superscript b) are indicated on the right side of panel B. The 1.2-kb *Bsp*HI band (superscript a) and 2.0-kb *Cla*I-*Sca*I band (superscript b) from pSUP301 contained the origin of replication; this might be the reason why both bands were present in pSUP301-3 and pSUP301-8 (isolated by plasmid rescue from *C. glutamicum* TRA-3 and TRA-8) and absent in *C. glutamicum* TRA-2 (from which no plasmid was rescued). The *Bsp*HI, *Cla*I, and *Sca*I sites in pSUP301, pSUP301-3, and pSUP301-8 are indicated in Fig. 3.

tle vector pUL609M (Table 1). The new plasmid, pUL609M-ile, was used to transform the transconjugant *C. glutamicum* TRA-3. Clones were selected by their resistance to kanamycin (conferred by the integrated pSUP301) and hygromycin (conferred by pUL609M). Several Km- and Hyg-resistant colonies were analyzed in MMC, and in all cases Ile prototrophy was restored.

DISCUSSION

The persistence of distinct species in nature demands the presence of mechanisms limiting gene flow between species. The most efficient barriers seem to be directed not against the transfer of genetic information between species but against the establishment of new traits in a foreign genetic background. Genetic material that enters a new microbial host in which it is not able to replicate autonomously can (provided it escapes restriction) be maintained by integration into the genome. Integration can be accomplished by means of homologous recombination, which, however, requires sufficient sequence homology and therefore is a very strong barrier to interspecific gene transfer. For instance, for detection of homologous recombination, the minimum required sequence length with perfect homology has been determined to be 70 to 80 bp in *B. subtilis* (19) and 40 to 150 bp in *E. coli* (12, 42). Site-specific recombination processes are also known to be causes of integration of DNA into the host genome and are used, for example, by many temperate bacteriophages (6). An additional and less specific way in which foreign DNA can be acquired by a bacterial genome is through mobile elements that can generate cointegrates between incoming DNA and indigenous plasmids or the chromosome (3).

In this paper, we show that mobilizable derivatives of the *E. coli* narrow-host-range plasmids pBR322, pBR325, pACYC177, and pACYC184 can be stably maintained in the gram-positive genera *Corynebacterium* and *Brevibacterium* by integration into the genome after transfer by intergeneric conjugation. Integration was accomplished via short homologous sequences of 8 to 12 bp in the vector and the recipient chromosomal or plasmid DNAs. In all the cases investigated, the short sequences used for recombination were different (Fig. 4), and insertion seems to occur at random sites in the chromosome, with different parts of the vector being involved. Given the minimal requirements for homologous recombination in other species, it seems unlikely that integration takes place by

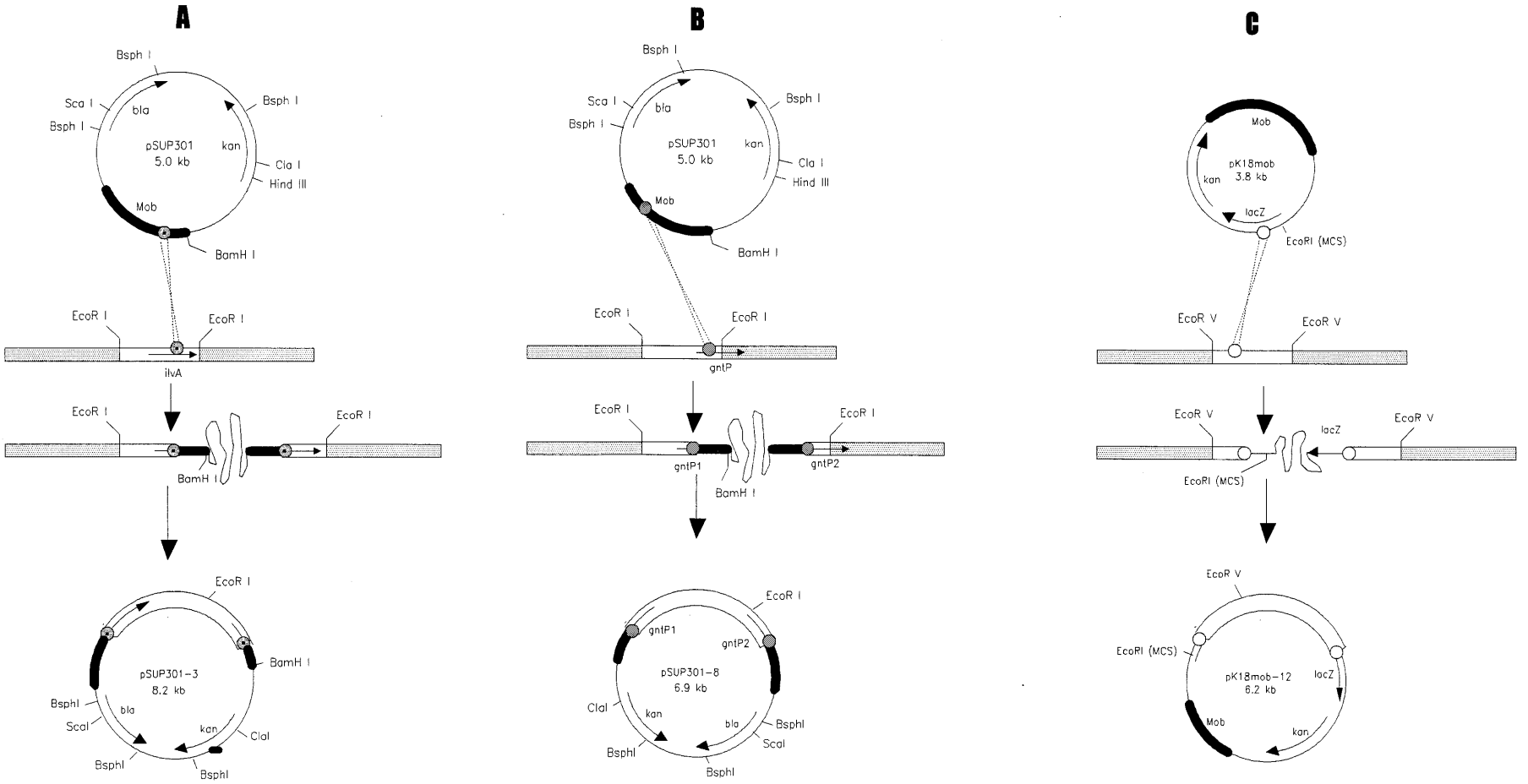

FIG. 3. In vivo integration of pSUP301 and pK18mob into the chromosomal DNA of *C. glutamicum* (▭). (A) Integration of pSUP301 into the *ilvA* gene. The plasmid rescued in *E. coli* contained a 3.2-kb fragment of exogenous DNA, and a direct repeat sequence of 11-bp was located at the ends of the fused sequences (⊙). (B) Integration of pSUP301 into the gluconate permease gene (*gntP*). The rescued plasmid contained a 1.9-kb fragment of exogenous DNA with a direct repeat sequence of 12 bp at the ends (◯). (C) Integration of pK18mob into the chromosome of *C. glutamicum*. The 2.4-kb exogenous fragment contained a 10-bp direct repeat sequence at the ends (○).The Mob fragment ( ) is indicated.

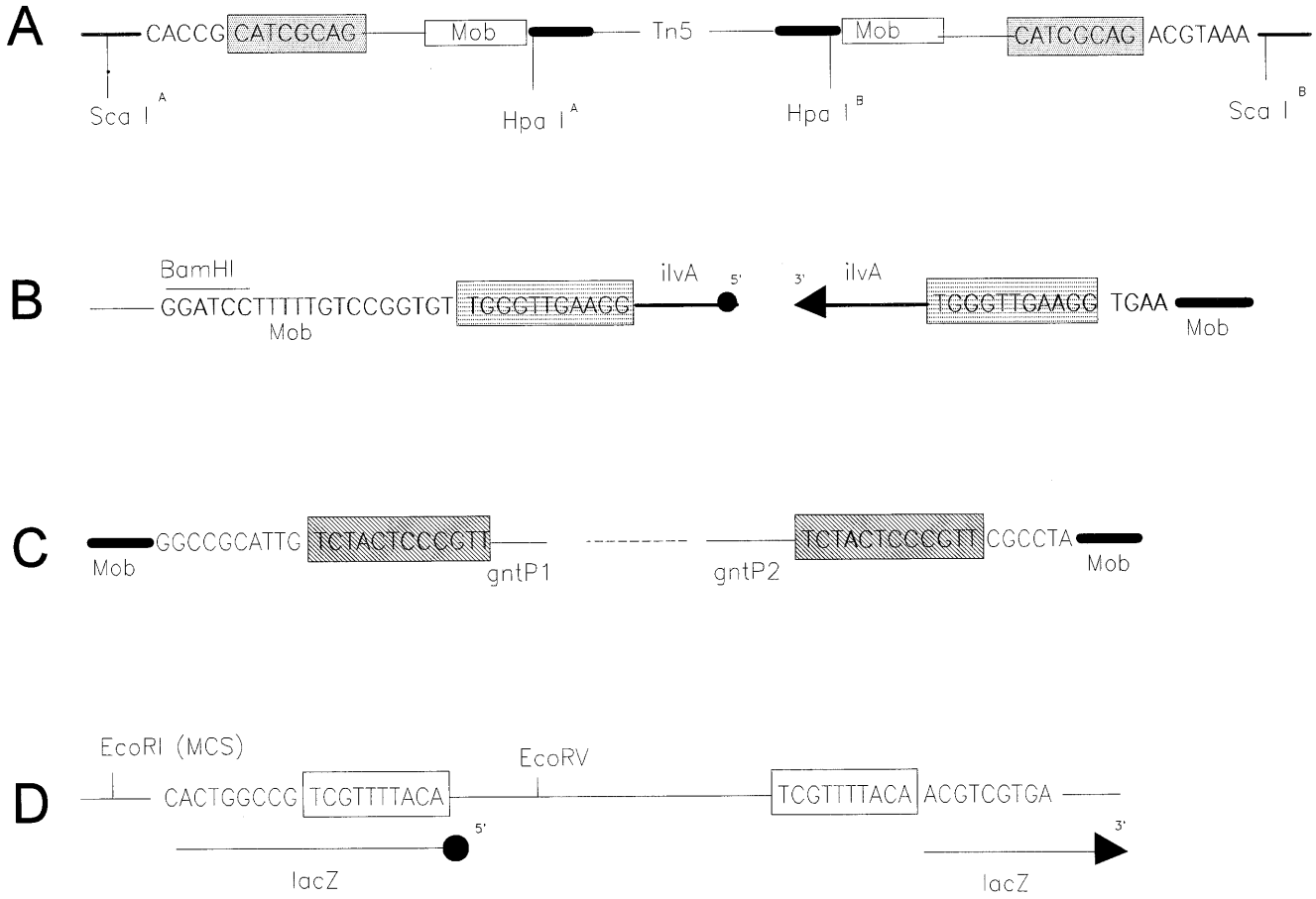

FIG. 4. Sequencing of the regions involved in plasmid integration described in this work. The boxes containing nucleotide sequences correspond to repeated DNA regions involved in integration. (A) Plasmid pULM21 (superscript letters A and B correspond to the subcloned fragments [see the legend to Fig. 1]); (B) plasmid pSUP301-3; (C) plasmid pSUP301-8; (D) plasmid pK18mob-12. MCS, multiple cloning site. The start of the gene (●) and the direction of the 3′ end of the gene after plasmid rescue (arrow) are indicated.

classical *recA*-mediated recombination. Site-specific integration could be excluded since integration occurred at different sites. In addition, the known IS elements present in the host strain did not participate in the process. We postulate that integration is mediated by an enzyme that recognizes very short homologous sequences. A similar mechanism of illegitimate recombination has been described in *Bacillus subtilis* with the temperature-sensitive plasmid pE194; in this case, the integration frequency of pE194 derivatives was also low (3×10^{-8}) and short stretches of homology (6 to 15 bp) were needed (2). It is noteworthy that transconjugants resulting from illegitimate recombination could be detected only after prolonged incubation and that they appeared at low frequencies of around 5×10^{-8}; however, we are now trying to optimize the process to obtain more transconjugants per assay.

We isolated different auxotrophic mutants, and in one case the mutation was further investigated: an isoleucine auxotroph was obtained by integration of plasmid pSUP301 into the *ilvA* gene of *C. glutamicum*. Integration of pSUP301 was proven to be the cause of auxotrophy by sequencing of the chromosomal DNA present in the rescued plasmid pSUP301-3 and by homologous complementation with the wild-type *ilvA* gene from *C. glutamicum*. In addition, the disruption of the chromosomal gluconate permease gene (*gntP*) in *C. glutamicum* TRA-8 by pSUP301 was verified by the inability of this strain to use gluconic acid as the sole carbon source. Several attempts to isolate plasmid DNA from other auxotrophic transconjugants by plasmid rescue techniques were unsuccessful. We assume that integration of the vector involved regions essential for its survival in the natural host, *E. coli* (see the hybridization in Fig. 2B), and/or that reorganization or deletion of essential plasmid fragments occurred during or after recombination.

It may also be important to take into account the mechanism described here in gene disruption and gene replacement studies which utilized mobilizable nonreplicative plasmids and internal fragments of corynebacterial genes. For example, Gubler et al. (13) used a pSUP301 derivative for gene disruption by homologous recombination in the corynebacterial host; it is advisable to analyze transconjugant clones carefully, since a low percentage may be due to illegitimate integration of the mobilizable plasmid, especially after prolonged incubation.

The results described herein would be of interest to workers in the field of coryneform bacteria, in which a limited number of systems capable of achieving gene tagging are available; this system will allow the integration of heterologous DNA into the chromosome and the isolation of stable auxotrophic mutants.

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