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Site-Specific Integrative Elements of Rhizobiophage *16-3* Can Integrate into Proline tRNA (CGG) Genes in Different Bacterial Genera

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The integrase protein of the *Rhizobium meliloti* 41 phage *16-3* has been classified as a member of the Int family of tyrosine recombinases. The site-specific recombination system of the phage belongs to the group in which the target site of integration (*attB*) is within a tRNA gene. Since tRNA genes are conserved, we expected that the target sequence of the site-specific recombination system of the *16-3* phage could occur in other species and integration could take place if the required putative host factors were also provided by the targeted cells. Here we report that a plasmid (pSEM167) carrying the *attP* element and the integrase gene (*int*) of the phage can integrate into the chromosomes of *R. meliloti* 1021 and eight other species. In all cases integration occurred at so-far-unidentified, putative proline tRNA (CGG) genes, indicating the possibility of their common origin. Multiple alignment of the sequences suggested that the location of the *att* core was different from that expected previously. The minimal *attB* was identified as a 23-bp sequence corresponding to the anticodon arm of the tRNA.

Site-specific integration is an efficient way to introduce new genetic information into the chromosome of the cell in a targeted fashion and ensure stable maintenance of the feature gained. Integrative vectors developed by utilizing site-specific integration elements from temperate bacteriophages (i.e., *attP* and the integrase gene combined) provide tools for this technology. Since the presence of the appropriate target sequence (*attB*) in the recipient chromosome and specific host factors are required for the integrative process, utilization of a particular integrative recombination system is frequently restricted to the host range of a given phage. A number of known eubacterial temperate phages, such as L5 (37), HP1 (24), *16-3* (35), RP3 (21), ϕU (50), P4 (38), Mx8 (28), ϕSfi21 (7), ϕ10MC (22), T12 (31), A2 (2), mv4 (16), Ms6 (19), and VWB (51), use tRNA genes as target sequences for integration into the host chromosomes. There are some plasmids with integrative functions, such as pSAM2 (30), pSE101 (5), pSE211 (6), and pSLP1 (52), whose target sites are also within tRNA genes. The SSV1 virus of the thermophilic archaeon *Sulfolobus shibatae* can also integrate into a tRNA (40, 41). Considering the conservative nature of tRNAs, one can expect that the target sites for a particular integrative system could occur in several bacterial species at various levels of relatedness. This view was confirmed by well-documented examples: the site-specific recombination systems of Ms6, mv4, VWB, pSAM2, pSE101, and pSLP1 were shown to function in closely related bacteria (3, 5, 19, 39, 51, 52). Moreover A2 provides an example of integration in both gram-negative and gram-positive species (2).

The temperate bacteriophage *16-3* of *Rhizobium meliloti* 41 has been studied thoroughly. In addition to genetic and physical characterization of its genome (10, 12, 14), the central region of the phage chromosome (GenBank accession no. AJ131679) was investigated in detail. This part carries the *c* repressor gene essential for lysogenic development (8, 9, 11, 15, 33, 34) and the site-specific recombination system (13, 32, 35, 44) controlled by the *c* gene (48). The *attP* site and the genes responsible for the integration and excision functions (*int* and *xis*) have been identified (12, 13, 44). The sequence of the *attB* region (GenBank accession no. Z22146) indicated that identical sequences of 51 bp could be found in both the bacterial and phage *att* regions (13) overlapping the 3′ end of a putative proline tRNA (CGG) gene. Due to this identity, the nucleotide sequence of the proline tRNA (CGG) gene is not altered upon *16-3* integration (35). The core regions of *att* sites were expected to be within the 51-bp sequence where strand exchanges take place during recombination.

Previously, the *16-3* integrative system could be used for genetic modifications only if the integrase protein was provided in *trans* by a helper phage (26) or a cloned phage fragment (17). Separation of the integrase and excisionase functions led to the construction of a new integrative vector family (44). In both the theoretical sense and the practical sense, we thought it important to determine whether the proline tRNA (CGG) genes in bacteria other than *R. meliloti* 41 could serve as target sites for integration of the *16-3* site-specific recombination system.

In this paper we report that a 23-bp sequence, only partially overlapping the 51-bp *attB-attP* homology region, constitutes a

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minimal *attB*. We have found that a plasmid containing the *16-3* integrative elements was able to integrate into the chromosomes of various bacteria of biotechnological and agricultural importance. Sequence determinations revealed that the target sites in these species are within putative proline tRNA (CGG) genes which might have a common origin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain DH5α (23) was used in all cloning experiments and served as the host for donor plasmids used to conjugate into the recipient bacterial strains, including *R. meliloti* 41, *R. meliloti* 1021, *Rhizobium fredii* USDA205, *Rhizobium leguminosarum* biovar Viciae TOM, *Rhizobium trifolii* ANU843, *Rhizobium galegae, Rhizobium* sp. strain NGR234, *Bradyrhizobium japonicum* USDA110, *Azorhizobium caulinodans* ORS571, *Agrobacterium tumefaciens* GV2260, *Agrobacterium rhizogenes* 1724, and *Rhodobacter sphaeroides* 2.4.1.

E. coli was grown in Luria broth at 37°C (42), and all the other bacteria were grown in yeast-tryptone broth (YTB) (1% tryptone, 0.1% yeast extract, 0.5% sodium chloride, 1 mM magnesium chloride and 1 mM calcium chloride, pH 7.0) at 28°C. For plating bacteria, both Luria broth and YTB were supplemented with 1.5% agar, resulting in Luria agar and YTA, respectively.

Triparental mating. The triparental mating method was used to transfer the different plasmids resident in *E. coli* into the recipient bacteria listed above. Helper bacteria were *E. coli* harboring either pRK2013 (18) or pCU101 (49), depending on the origin of the *mob* region carried by the given plasmid in the donor. The helper plasmid, providing transfer function, is indicated in the case of each experiment. One-milliliter portions from each cultured bacterium (optical density at 600 nm, 2.0) were mixed, and cells were collected by brief centrifugation and resuspended in 3 ml of YTB. After a brief centrifugation, the cells were resuspended in 100 μl of YTB and loaded onto a 0.2-μm-pore-size nylon filter (Sartorius, Göttingen, Germany) which was placed onto the surface of YTA plate. Cells were washed from the filter after overnight incubation at 28°C and spread on selective plates (YTA supplemented with either kanamycin or tetracycline or both) to obtain single colonies. Tetracycline was used at a concentration of 15 μg/ml, while the concentration of kanamycin was 30 μg/ml in the cases of *E. coli* and *R. sphaeroides*, 100 μg/ml in the case of *A. tumefaciens*, and 400 μg/ml in the cases of the other bacteria.

DNA procedures. Basic DNA manipulations and molecular techniques were employed as described previously (42). Total bacterial DNA was prepared by a method described previously (4). Briefly, lysozyme-treated cells were disrupted by alkaline lysis, followed by digestion with proteinase K (15 min at 50°C). Proteins were eliminated by phenol extraction, and 1 volume of ethyl ether was added to eliminate polysaccharides. After centrifugation, the lower phase was collected and DNA was precipitated with propanol. Extractions of DNA from agarose gels were done with a QIAEX II gel extraction kit (Qiagen). DNA was labeled by nick translation in the presence of [α-^{32}P]dATP. Southern hybridization was performed as described previously (42, 45). PCR-mediated DNA amplifications were carried out using *Taq* polymerase (Promega or Sigma) to generate DNA fragments for cloning. After 30 cycles of 1 minute at 94°C, 1 min at 55°C, and 1 min at 72°C, the PCR mixtures were extracted with phenol and precipitated in ethanol. The DNAs were then resuspended in Tris-EDTA buffer and digested with the appropriate restriction enzyme(s) to generate the required ends of the fragments. The DNA fragments were purified before cloning by isolating them from agarose gels. Nucleotide sequence determination was performed by the dideoxy chain termination method (43) with a TaqTrack sequencing kit (Promega).

Plasmids. Plasmid pSEM167 and pSEM168 carry *attP* and the *int* gene in a natural context in different vectors, and the transcription of *int* gene is driven by a *tac* promoter (44). pSEM166 was created by inserting the *Sal*I-*Acc*65I fragment (both ends of which were made blunt) of pSEM164 (44) into the blunt-ended *Eco*RI site of pLAFR1 (20). To construct pSEM210, the *oriT* region of plasmid pCU1 from pUC1813-NoriT (25) was isolated as a *Hin*dIII-*Bam*HI fragment and inserted into *Hin*dIII-*Bam*HI-digested pLG338 (47), resulting in pSEM143. Next, a synthetic 23-bp *attB* site,

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5′-GTACCGCACCACGTTCGGGACGTGGGGG-3′
    3′-GCGTGGTGCAAGCCCTGCACCCCCTTAA-5′
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was created by annealing two oligonucleotides, and the fragment was inserted into the *Acc*65I-*Eco*RI-cut pSEM143. Plasmid pSEM252 was created by inserting the PCR product into the *Eco*RI (blunt-ended) site of pSEM143, which was obtained by amplification of the region from nucleotide 476 to 737 (numbered according GenBank accession no. AJ131679). using pSEM167 DNA as a template and the primer pair 5′-GGTCGTTTTTATTGCCGTGG-3′ and 5′-ATCT AGAAGTAAATCATTGCCGTAAT-3′.

Sequence analysis. Sequence analyses were performed using the programs of the Genetics Computer Group (Madison, Wis.) Wisconsin package version 9.1. BLAST (36) and FASTA (1) were used to search for similarity with sequences in the GenBank, EMBL, SWISSPROT, and PDB databases.

Testing sequences for *attB* function with cointegrate formation between two plasmids. The two plasmids were transferred sequentially into the cell by triparental mating. First plasmids containing *attB* or the *attB*-like sequences were introduced, and then the plasmid containing *attP* and the constitutively expressed *int* gene was transferred by another mating. These plasmids were constructed with care to avoid any homology between them. Colonies were selected following the second mating on a plate containing two kinds of antibiotics (kanamycin and tetracycline), each selecting for the presence of one of the plasmids. Cells were cultured from the colonies, and plasmid DNAs were purified. Competent *E. coli* cells were transformed with a small amount (5 ng) of DNA to disfavor transformation of a cell with more than one plasmid. Plasmid DNAs purified from double-resistent colonies were digested with relevant restriction enzymes.

RESULTS

The site-specific integration system of phage *16-3* is functional in bacteria belonging to several different genera. The integration of pSEM167 into the chromosome of *R. meliloti* 41 was demonstrated previously, and for detection of the integration various experimental setups were established (44). Since the target site of integration has been located within the proline tRNA gene, it was reasonable to expect that the required target sequence in other bacterial species could be also present, because of the conservative nature of tRNA genes. However, the observation that pSEM167 failed to integrate in *E. coli* despite the accumulation of the Int protein, even when the cognate *attB* was supplied, drew our attention to the possible requirement for an appropriate host factor(s) (44). We expected that only close relatives of *R. meliloti* may have both the target site and functional host factor(s), and bacterial species from the alpha subdivision (according to the classification of the National Center for Biotechnology Information [NCBI] taxonomy database), particularly from the *Rhizobiaceae*, were selected to test for functional *16-3* site-specific recombination.

Integration of pSEM167 into the bacteria listed in Materials and Methods was tested. The plasmid was introduced into the recipient by triparental mating (helper, pRK2013). Transconjugants were selected for kanamycin resistance, and single colonies were isolated for further investigations. A subset of the results can be seen in Fig. 1. With the exceptions of *R. galegae* and *R. sphaeroides*, integration of the plasmid occurred in all of the bacteria tested.

The integration target sites in different bacteria are within proline tRNA genes. To determine target site sequences for pSEM167 integration in different bacteria, a plasmid pair was constructed. pSEM166 carried the *int* gene of phage *16-3* expressed constitutively; it provided the integrase function in *trans*. The other plasmid, the *attP* donor for the *attP-attB* reaction was pSEM252. This plasmid was unable to replicate in bacteria other than *E. coli* due to the narrow host range of its replication origin. It is worth noting that the inability of pSEM252 to replicate in the recipient bacteria was essential in the plasmid-rescuing experiments (to reduce background) and was why we had to change our experimental setup from using pSEM167 to using the plasmid pair of pSEM166 and pSEM252.

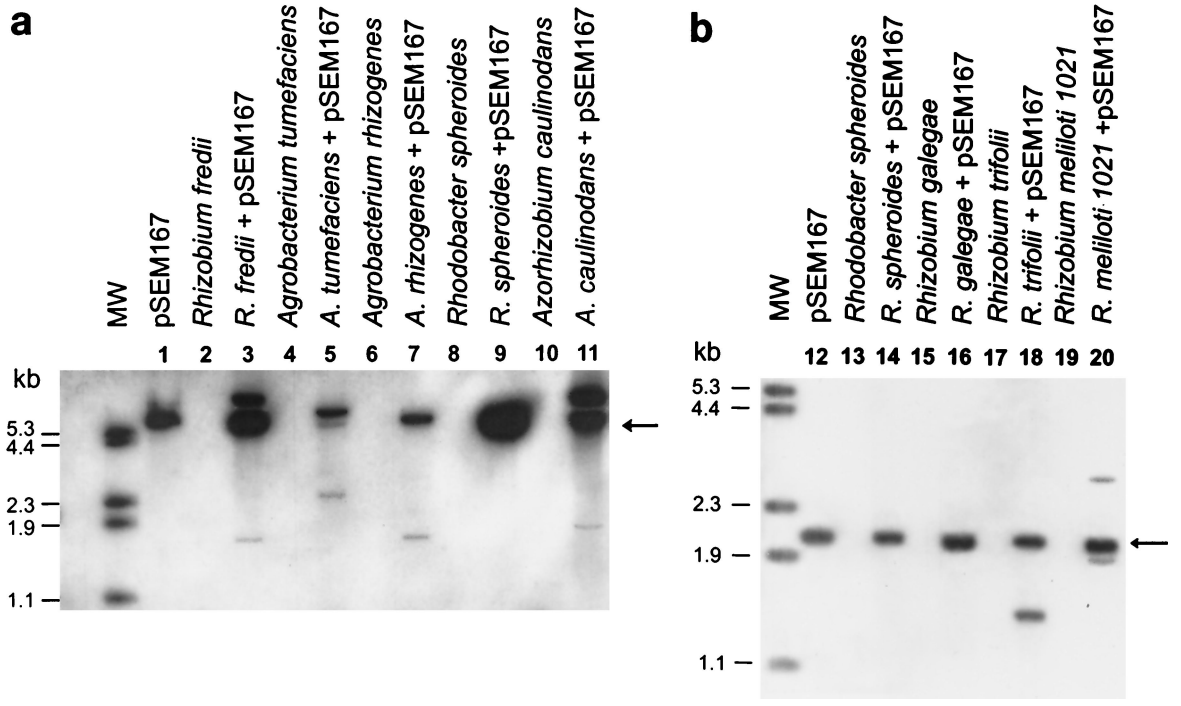

FIG. 1. Detection of the integration of pSEM167 into the chromosomes of different bacteria by Southern analysis. ^{32}P-labeled *Eco*RI-digested pSEM167 DNA (a) or the *Hin*dIII fragment of pSEM167 (containing the 3′ end of the *int* gene and the *attP* with its flanking regions) (b) was used as a probe, and the arrows indicate the positions of the bands obtained by self-hybridization of the entire plasmid (a) or its corresponding fragment (b). Lanes MW, molecular size marker made by mixing fragments derived from digestion of the *attP*-containing plasmid pSEM80 (44) with different restriction endonucleases. *Eco*RI digests (a) and *Bam*HI digests (b) of pSEM167 (lanes 1 and 12, respectively) and total DNAs isolated from a bacterium and its pSEM167-containing derivative are shown in pairs (lanes 2 to 11 and lanes 13 to 20, respectively). The appearance of two bands instead of or in addition to the one representing pSEM167 or its corresponding fragment (marked with arrows) indicates successful integration of the plasmid into the chromosome. We have noticed in a few cases that integration of pSEM167 resulted in the loss of extrachromosomal copies of the plasmid. This phenomenon has nothing to do with recombination. It is possibly due to the nature of the pCU1 replicon, and it may be derived from the interference between the integrated and extrachromosomal copies of the plasmid in controlling replication. This is the case in both *A. tumefaciens* (lane 5) and *A. rhizogenes* (lane 7). The complete loss of pSEM167 could be detected by *attP*-specific PCR in both bacteria. However, if not enough time was allowed to complete this process, the existence of the extrachromosomal copies of the plasmid in a population can be seen. This appears in lane 5 as a faint band in the correct position. In lane 7 one of the bands representing a plasmid-bacterial chromosome junction has the same size as pSEM167. It is not possible to tell whether the loss of the extrachromosomal copies of the plasmid is fully completed or the extent of loss is the same as seen in the case of *A. tumefaciens* in lane 5. In lane 18 only one additional band, representing a junction fragment, can be seen. However, the density of this band is significantly higher than the densities of bands representing junction fragments in other cases, and it seems likely that the band is a doublet containing both junction fragments. We were not able to detect integration of pSEM167 in *R. galegae* (lane 16) and *Rhodobacter sphaeroides* (lanes 9 and 14). Negative results were also obtained with these two species if *Pst*I or *Hin*dIII was used for digestion of DNAs in the Southern analyses (data not shown).

First pSEM166 was introduced and made resident in each species where integration of pSEM167 was detected previously, and then pSEM252 was introduced into these bacteria by triparental matings (helper, pCU101). pSEM252 could be maintained only if it was integrated into the chromosome. Since pSEM252 was constructed so that it did not have *Eco*RI restriction sites, digestion of a bacterial chromosome carrying the integrated pSEM252 with *Eco*RI resulted in a specific fragment containing the entire pSEM252 plasmid flanked by sequences of the integration site. Competent *E. coli* cells were transformed with self-ligation mixtures of *Eco*RI digests of different bacterial chromosomes, and selections for the antibiotic resistance of pSEM252 were made. The sequences of the target regions were determined for each bacterium from which the recombinant pSEM252 derivatives could have been obtained. The alignment of these sequences is shown in Fig. 2. Interestingly, we have found two different sequences in *A. caulinodans* into which pSEM252 was able to integrate. The presence of the two different target sites was confirmed by sequencing the PCR products obtained with primer pairs designed specifically according to the sequences flanking the tRNA genes shown in Fig. 2.

From the high homology found between the sequences, we can conclude that the integration target sites for *attP* of *16-3* are within (putative) proline tRNA (CGG) genes.

A 23-bp sequence constitutes the minimal *attB*. The 51-bp homology region between *attP* and *attB* of *R. meliloti* is located from position 29 to 79 according to the base numbering in Fig. 2. Despite this homology, we have never been able to detect the integration of any plasmid containing *attP* in the absence of integrase, even if selection was made for nonreplicative plasmids. Since site-specific integration could take place if the 51-bp region was supplied on a plasmid as *attB*, the region between positions 44 and 72 (the site of an imperfect inverted repeat) was thought to contain the *att* core region and hence the integrase binding sites (13, 35). Interestingly, the sequence

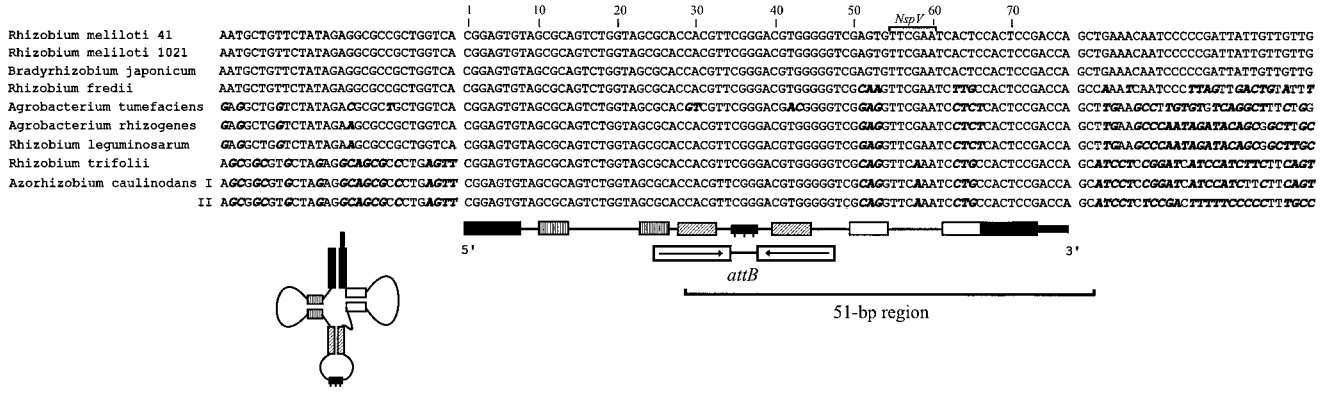

FIG. 2. Alignment of sequences of different species containing and flanking the target sites for *16-3* site-specific integration. Bases which differ from the base found in the appropriate positions in *R. meliloti* are indicated by boldface italics. Below the sequences a schematic tRNA and its alignment are shown. The 51-bp sequence identical in *R. meliloti* 41 *attB* and *16-3 attP* is represented with a bar. The location of the 23-bp minimal *attB* site is also indicated. The open boxes mark the possible core binding site of the *16-3* Int protein, and the arrows within the boxes indicate the palindromic nature of the sequence. The sequences shown in this figure are shorter than the sequences deposited in GenBank. Their accession numbers are as follows: *R. meliloti* 41, Z22146 (35); *R. meliloti* 1021, AF268600; *B. japonicum*, AF268604; *R. fredii*, AF268606; *A. tumefaciens*, AF268605; *A. rhizogenes*, AF268601; *R. leguminosarum*, AF268602; *R. trifolii*, AF268603; *A. caulinodans* I, AF268607; *A. caulinodans* II, AF268608.

homology between the target genes of different bacteria is not the greatest in this region. Although the base differences did not significantly change the palindromic feature of the sequences, the high level of variability in the target sequences still made us reluctant to assign the *attB* core function to the region.

The region from position 25 to 47 also consists of an imperfect inverted repeat which partially overlaps the 51-bp homology region and corresponds to the stem-and-loop regions of the anticodon arm of the tRNA. pSEM210 carrying this 23-bp-long region was constructed and tested for *attB* function. Triparental matings were used to introduce pSEM210 into *R. meliloti* 41 (helper, pCU101) and pSEM168 into *R. meliloti* 41 harboring pSEM210 (helper, pRK2013). Cointegrate formation between the two plasmids confirmed that the 23-bp region was sufficient for a functional *attB* (Fig. 3). When we attempted to shorten further the 23-bp target site by testing for *attB* function DNA fragments obtained by annealing of synthetic oligonucleotides representing variants of the 23-bp target site truncated at either end and inserted into pSEM143, we were not able to detect integration (data not shown). It seems likely that the 23-bp sequence is the core region, and practically, it could be considered a minimal *attB*.

DISCUSSION

This study demonstrates that the site-specific recombination system of phage *16-3* can function in several bacterial species other than *R. meliloti*, the native host of the phage. Integration of the plasmid-borne *attP-int* elements into the genome of a given bacterium confirms the existence of both a functional target site (*attB*) in the chromosome and a putative cognate host factor(s) required for recombination.

The taxonomic relationship, based on the sequences of 16S rRNA genes, between most of the bacteria used in our tests has been determined (29). The phylogenetic relationship among the species in which integration of the *16-3* site-specific recombination was able to function (29) suggests that a common ancestor having the appropriate integration target site along with the gene(s) for the required host factor(s) could have existed. The observation that neither *R. galegae* nor *R. sphaeroides* could support the integration of pSEM167 suggests that the target site or the required host factor(s) or both could have been either lost or significantly altered by a mutation(s).

The similarity (not shown) between the phylogenetic tree based on the sequences of the target proline tRNA (CGG) genes and their surroundings and the phylogenetic tree based on the 16S rRNA sequences (29) is consistent with the common origin of the proline tRNA (CGG) genes which harbor the target site for *16-3* integration.

Two integration target sites were identified in *A. caulino-*

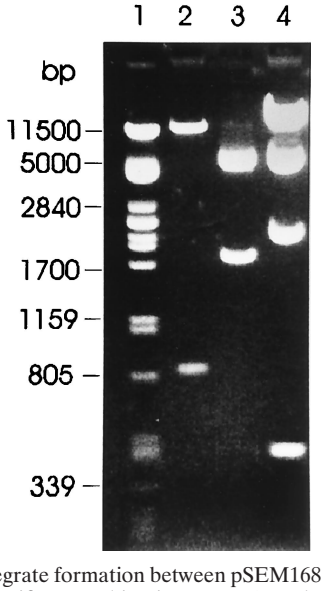

FIG. 3. Cointegrate formation between pSEM168 and pSEM210 as a result of site-specific recombination. Lane 1, molecular size marker (*Pst*I-digested λ DNA). Lanes 2 to 4, *Eco*RI digests of pSEM168 (lane 2), pSEM210 (lane 3), and the cointegrate of the two plasmids (lane 4).

dans; however, we were not able to prove the simultaneous presence of the two integrated plasmids by Southern analyses using either *Eco*RI (Fig. 1, lane 11) or *Bam*HI (data not shown) restriction endonuclease for digestion of total DNA. It might be possible that integration of the plasmid in two copies at different locations of the host chromosome results in lethal deletion due to recombination between the two integrated, identical sequences. Sequence comparison of the two putative tRNA genes and their flanking regions (Fig. 2) indicated that the two genes and 30 nucleotides at their 5′ ends were identical. Although 8 nucleotides following the 3′ ends of the tRNA genes were also identical, homology between the rest of the determined sequences was less than 30%. We think that the second copy of this putative proline tRNA gene could have originated from a duplication; however, a horizontal transfer cannot be ruled out.

Inspecting the sequences of the target genes obtained from different species showed full identity between positions 25 and 47, which corresponded to the minimal *attB* site, with the exception of *A. tumefaciens*. The target site in *A. tumefaciens* differs in four positions from the other target sites (Fig. 2). These changes did not change the palindromic nature of the sequence, and hence the anticodon loop of the tRNA was not destabilized. Integration of pSEM167 into the genome of *A. tumefaciens* means that recombination between the *16-3 attP* and the *attB* core regions does not need perfect homology. However, it was shown previously in the case of λ phage integration that perfect homology between *attB* and *attP* was required in the region where strand exchanges occurred; otherwise, the recombination process was aborted (27). If we suppose that the mechanism of site-specific recombination requires a similar condition in the case of the *16-3* system, the region where strand exchange could occur (overlap sequence) is restricted to between positions 31 and 41, since these regions are perfectly identical both in *A. tumefaciens* and in all of the other species tested. Indeed, sequence data obtained by determining the sequence of the fragment rescued from *A. tumefaciens* confirmed the above assumption, since the bases that differ between the *attB*s of *R. meliloti* and *A. tumefaciens* (at positions 29 to 30 and 42 to 43) were found on different junction fragments. The overlap sequence could be even shorter, considering that its length varied between 6 and 8 bp within the λ integrase family (46).

The possibility to extend further the host range of the *16-3* integration system is indicated by the proline tRNA (CGG) gene found in the *Deinococcus radiodurans* genome, the sequence of which has been determined recently (53). The sequence homology between the proline tRNA (CGG) genes of *D. radiodurans* and *A. tumefaciens* is 93%; moreover, they are fully identical between positions 17 and 67, including the region of the target sequence required for integration of the *16-3* integrative unit. The relationship between the *Agrobacterium* and *Deinococcus* genera is regarded as extremely distant (based on the classification of the National Center for Biotechnology Information taxonomy database). We think that there could be many other genomes, at least in the prokaryotic world, which could be targeted by the *16-3* integrative system, particularly if the supply of a host factor(s) can be solved.

It is worth mentioning that all of the bacteria in which functioning of the *16-3* site-specific recombination system has been proven have both scientific and biotechnological interest due to their role in either symbiotic nitrogen fixation (*Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species) with different plants or construction of transgenic plants via *Agrobacterium* species.

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