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Genetic Characterization of Site-Specific Integration Functions of ϕAAU2 Infecting "*Arthrobacter aureus*" C70

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All the essential genetic determinants for site-specific integration of corynephage ϕAAU2 are contained within a 1,756-bp DNA fragment, carried on the integrative plasmid p5510, and are shown to be functional in *Escherichia coli*. One open reading frame, ORF4, encoding a protein of 266 amino acids was shown to represent the ϕAAU2 integrase. The nucleotide sequence of the ϕAAU2 attachment site, *attP*, and the *attB*, *attL*, and *attR* sequences in the host "*Arthrobacter aureus*" C70 were determined. Identical nucleotide sequences were shown to be responsible for the integration of p5510 in the chromosomes of *Corynebacterium glutamicum*, *Brevibacterium divaricatum*, and *B. lactofermentum*, and a sequence almost identical to *attB* was found to be present in these three strains. In contrast to other phage site-specific recombination systems, a plasmid encompassing only *int-attP* failed to integrate into the host chromosome. This led to the identification of an 800-bp noncoding region, immediately upstream of *int*, absolutely required for site-specific integration of p5510.

Genera *Arthrobacter*, *Corynebacterium*, and *Brevibacterium* belong to the group of coryneform bacteria from which several strains are industrially used for the production of certain amino acids (52). Most of the corynephages isolated to date originated from fermentation failures or soils and are virulent (19, 45, 47, 51). ϕAAU2 is a temperate phage isolated from soil that infects and lysogenizes "*Arthrobacter aureus*" C70 (24). Other known temperate corynephages have been isolated following UV or mitomycin induction of coryneform strains (20, 29, 30, 34, 42). Thus, a significant proportion of strains were shown to harbor prophages, but only for a few of them was the phage-host relationship studied (30, 42). The only well-documented temperate corynephage is phage β of the human pathogen *Corynebacterium diphtheriae* (18). Its integration occurred into either of two sites (*attB1* and *attB2*) located on the *C. diphtheriae* chromosome (36). These *attB* sites share a 96-bp sequence with the *attP* sites of β-related phages (37). Moreover, screening of a variety of *Corynebacterium* species including the soil isolate *C. glutamicum* ATCC 13032 with *attP* and *attB* probes showed that all of the species investigated contained at least one DNA fragment that hybridized with both of these probes (8, 9, 15). Besides this report, information on the molecular biology of lysogeny in coryneform bacteria remained scarce.

The present study reports on the phage-encoded site-specific recombination system of ϕAAU2, which has been pinpointed previously to a 5.25-kb *Bgl*II-*Eco*RV fragment on the phage genome (24). The phage attachment sites (*attP*, *attB*, *attR*, and *attL*) were sequenced, and a common sequence was identified. The ϕAAU2 integrase gene was also delineated by transposon mutagenesis, and its deduced protein sequence was shown to resemble the conserved C-terminal region of other members of the integrase family.

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MATERIALS AND METHODS

Bacteriophage, bacterial strains, and plasmids. The bacteriophage, bacterial strains, and plasmids used in this study are listed in Table 1. Growth conditions were as previously described (46). DNA manipulations, phage and bacterial chromosomal DNA extractions, transformation, electrophoresis, restriction endonuclease digestion, Southern blotting and hybridization have been reported previously (24). Exonuclease III deletions were obtained with the nested-deletion kit from Pharmacia (Orsay, France).

Construction of pBK271 and pBK276. pBK is a cloning vector derived from pBluescriptIISK$^+$, which contains an *aphIII* gene (48) instead of the *bla* gene (Fig. 1). The integrative functions of ϕAAU2 were obtained from p5510 (24) as a 2.2-kb *Bst*EII-*Eco*RV DNA fragment. This restriction fragment was blunted with Klenow polymerase and inserted in both orientations into the *Sma*I site of pBK, yielding pBK271 and pBK276. Orientation of DNA inserts is indicated in Fig. 5. As with p5510, these two plasmids do not replicate in corynebacteria.

DNA amplification. PCR-mediated amplification of the bacterial attachment site of "*A. aureus*" C70 was performed by using 1.0 μg of chromosomal DNA and 40 pM primers B1 (5′ GTAGTCCCGTGGCGCCG 3′) and B2 (5′ CGACC TCGCTGTGGCCG 3′). They are mapped in Fig. 3. The same strategy was used to amplify the *attB′* site from *C. glutamicum* RM3, by using primers B′1 (5′ TCTCCCGTGCCATGCCT 3′) and B′2 (5′ CACTTCGCTCCCCAAG 3′) (mapped in Fig. 4). Similarly, the *attR* and *attL* junction fragments from lysogenic strains and p5510 integrants of "*A. aureus*" C70 were amplified by using primer combinations B1-P2 (5′ GCTTTGCGTGAGGCGGC 3′) for amplication of *attR* and B2-P1 (5′ AGGCAAGTGGGAAGCGA 3′) for amplication of *attL*. Reactions were carried out for 35 cycles as follows: denaturation at 94°C for 1 min, primer annealing at 58°C for 3 min, and primer extension at 72°C for 1.5 min. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium).

DNA sequencing. DNA sequencing analysis was performed as described by Sanger et al. (40) by using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Template DNA was obtained from progressive exonuclease III derivatives of pBK271 and pBK276. In general, sequences were obtained by using primers which are designed to prime at the flanking ends of the pBSK multiple cloning site (Stratagene). Each residue was determined at least four times for each strand. Sequences located on either side of the attachment sites between ϕAAU2 and corynebacterial genomic DNAs were obtained by using primers P1 and P2. Database searches were performed by using FASTA (35) and BLAST (2) programs.

Cloning of *attP* of ϕAAU2 and *attR*, *attL*, and *attB* from "*A. aureus*" C70 and other coryneform bacteria. The genetic functions for integration of phage ϕAAU2 were previously shown to be localized on a 5.25-kb *Bgl*II-*Eco*RV phage DNA fragment, which was cloned into the integrative plasmid p5510. *attP* was assigned to a 1-kb *Sal*I subfragment (24), which was subcloned into pBluescript-IISK$^+$ (pBSK), giving pBA643 (Fig. 2). Integration of plasmid p5510 occurred within a 0.9-kb *Eco*RI fragment on the "*A. aureus*" C70 chromosome. The absence of *Eco*RI sites on p5510 was used to rescue the plasmid together with its right- and left-hand chromosomal junctions from a p5510 integrant of "*A. aureus*" C70, following the transformation of the ligation mixture obtained from an *Eco*RI digestion of "*A. aureus*" C70 genomic DNA into *Escherichia coli* DH5α (Fig. 2). Several Kan^r clones, harboring a 9.4-kb plasmid named pRESA, were

TABLE 1. Bacterial strains, phages, and plasmids

Phage, strain, or plasmid	Description	Source or reference
φAAU2	Temperate	24
Bacteria		
"*A. aureus*" C70	Soil isolate	Prof. Gounot[a]
C. glutamicum RM3	ATCC 13032 r^- m^-	41
B. divaricatum	ATCC 14020	American Type Culture Collection
B. lactofermentum BL15	ATCC 21086 r^- m^-	4
E. coli DH5α	*supE44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	17
E. coli NM522	*supE thi*Δ(*lac-proAB*) *hsd*Δ*5* (F′ *proAB*$^+$ *lacI*^{q}ZΔM15)	27
E. coli MC4100 (Mucts) (MudIIPR13)	*araD139* Δ(*argF-lac*)*U169 rspL150*(Sm^r) *relA1 flbB5301 pstF25 deoC1* (Mucts), bearing MudIIPR13 (*lacZ′YA* Cm^r) integrated in the chromosome	38
E. coli BL21 (DE3)	*hsdS gal* (λcI*ts*857 *ind*-1 *Sam*-7 *nin*-5 *lacUV5* T7 gene *1*)	43
Plasmids		
pJRD16905	Kan^r Amp^r Tet^r	46
pACYC184	Cm^r Tet^r	6
pBluescriptIISK$^+$	*lacZ* Amp^r	Stratagene[b]
p5510	p25435 Δ*Sma*I, 5.25-kb *Bgl*II-*Eco*RV φAAU2 Kan^r *attP int*	24
pRESA	p5510 *Eco*RI rescue from "*A. aureus*" Kan^r *attR attL* (9.4 kb)	This study
pRESBd	p5510 *Bam*HI rescue from *B. divaricatum* Kan^r *attR′ attL′* (13.55 kb)	This study
pRESB1	p5510 *Bam*HI rescue from *B. lactofermentum* BL15 Kan^r *attR′ attL′* (13.55 kb)	This study
pRESC	p5510 *Bam*HI rescue from *C. glutamicum* RM3 Kan^r *attR′ attL′* (13.55 kb)	This study
p5510Mu1	p5510::MudIIPR13 (+50 bp)	This study
p5510Mu2	p5510::MudIIPR13 (+1755 bp)	This study
pBA643	pBluescriptIISK$^+$, 1.0-kb *Sal*I fragment, Amp^r *attP*	This study
pBA860	pBluescriptIISK$^+$, 0.7-kb *Sal*I-*Eco*RI fragment, Amp^r *attR* "*A. aureus*"	This study
pBA627	pBluescriptIISK$^+$, 1.2-kb *Sal*I-*Eco*RI fragment, Amp^r *attL* "*A. aureus*"	This study
pBA1579	pBluescriptIISK$^+$, 0.413-kb PCR product carrying *attB* from "*A. aureus*" chromosome	This study
pBK	Derivative of pBluescriptIISK$^+$ Kan^r Amp^s	This study
pBK271	pBK, 2.2-kb *Bst*EII-*Eco*RV Amp^r *attP int*	This study
pBK276	Opposite orientation from pBK271	This study
pBK1403, -1422	ExoIII[c] derivatives of pBK271	This study
pBK966	pBK271 Δ*Apa*I	This study
pBK1286	pBK966 Δ*Sal*I	This study
pBK1503	pBK1400 Δ*Sal*I	This study
pAC1579	pACYC184, 0.413-kb *Hin*dIII-*Bam*HI fragment derived from pBA1579 and carrying "*A. aureus*" *attB*	This study

[a] Lyon, France
[b] La Jolla, Calif.
[c] ExoIII, exonuclease III.

obtained (Fig. 2). This plasmid hybridized to both p5510 and a 0.9-kb *Eco*RI chromosomal fragment from "*A. aureus*" C70 and was shown by restriction analysis to be inserted within the *attP* region. The junctions, arbitrarily named *attL* and *attR*, were subcloned from pRESA into pBSK, giving pBA627 and pBA860, respectively (Fig. 2). The comparison of the *Sal*I-*Bst*EII profiles of p5510 and pRESA showed that integration resulted in the disappearing of a 216-bp *Bst*EII-*Sal*I fragment from the φAAU2 genome. It was therefore concluded that the *attP* site is part of this 216-bp fragment. In order to characterize the "*A. aureus*" C70 attachment site (*attB*), the DNA regions corresponding to *attP*, *attL*, and *attR*, present in pBA643, pBA627, and pBA860, respectively, were sequenced and their sequences were compared (Fig. 3). This allowed the designing of two primers, B1 and B2, complementary to *attR* and *attL*, which were used to amplify the corresponding 413-bp region from the chromosome of "*A. aureus*" C70 by PCR (Fig. 2 and 3). The resulting PCR product was introduced in the *Eco*RV site of pBSK, and five recombinant plasmids were sequenced. All showed the same sequence, and one was designated pBA1579 (Fig. 3). The strategy to rescue p5510 integrated in the chromosome in other coryneform bacteria was similar to that described for "*A. aureus*" C70, except that chromosomal DNAs were digested with *Bam*HI. Thus, plasmids pRESB1, pRESC, and pRESBd were obtained from *Brevibacterium lactofermentum* BL15, *C. glutamicum* RM3, and *B. divaricatum* ATCC 14020, respectively. Nucleotide sequences of right (*attR′*) and left (*attL′*) junctions between phage and bacterial DNAs were compared with the *attP* fragment and shown to be identical between the three strains tested (data not shown). PCR amplification of their common bacterial attachment site (*attB′*) was performed with the chromosome of *C. glutamicum* RM3, by using primers B′1 and B′2 (Fig. 4).

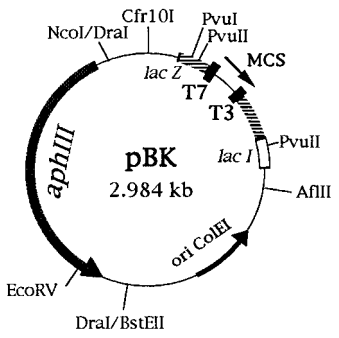


FIG. 1. Restriction map of pBK. Multiple cloning site (MCS): *Kpn*I-*Apa*I-*Dra*II-*Xho*I-*Hin*cII-*Acc*I-*Sal*I-*Cla*I-*Hin*dIII-*Eco*RV-*Eco*RI-*Pst*I-*Sma*I-*Bam*HI-*Spe*I-*Xba*I-*Not*I-*Eag*I-*Bst*XI-*Sac*II-*Sac*I.

Protein expression in the bacteriophage T7 promoter-RNA polymerase system. Plasmids pBK276 and pBK271 and derivatives thereof were introduced into *E. coli* BL21 (DE3) (43). To specifically label the products encoded by genes cloned downstream of the φ10 promoter, a slight modification of the protocol of Tabor and Richardson (44) was used. Induction was performed for 30 min at 37°C, using 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Rifampin was then added to 600 μg ml^{-1}, and cells were incubated for an additional 10 min at

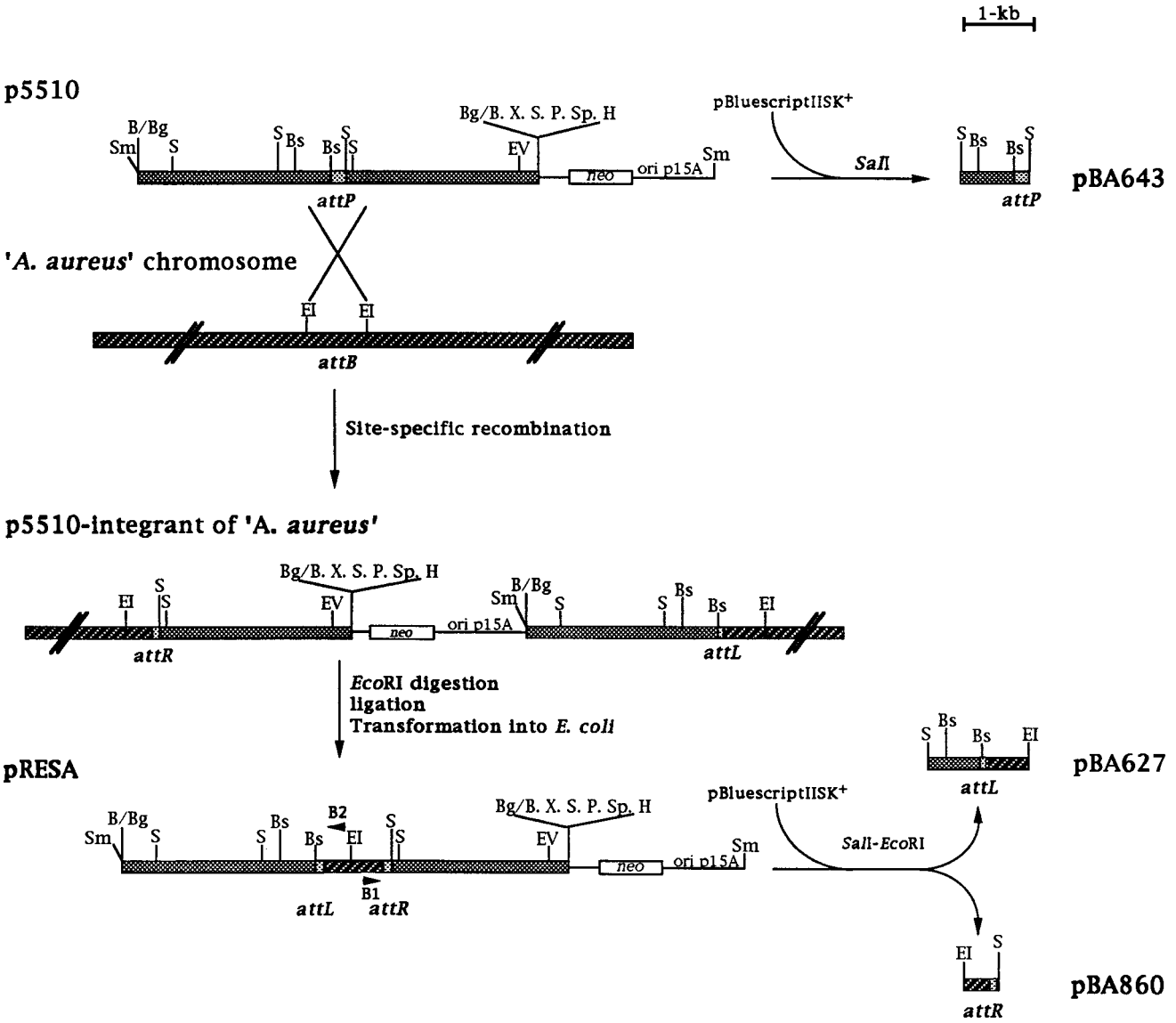


FIG. 2. Schematic presentation of p5510 site-specific integration into the "*A. aureus*" C70 chromosome and *Eco*RI rescue of the p5510 integrant (for a detailed explanation, see Materials and Methods). Darkly shaded bars, ϕAAU2 5.25-kb *Bgl*II-*Eco*RV fragment; lightly shaded bars, 216-bp *Bst*EII-*Sal*I *attP* fragment; hatched bars, bacterial chromosomal region containing *attB*. B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

42°C followed by 20 min at 37°C. Protein labelling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), staining, and autoradiography of the gel have been described elsewhere (46).

MudIIPR13 insertional mutation. Mutagenesis was performed as reported previously (38), and insertion sites were localized by restriction analysis and DNA sequencing.

Nucleotide sequence accession numbers. The nucleotide sequence of the 1,756-bp ϕAAU2 integration region and of the *attB* regions of ϕAAU2 in "*A. aureus*" and in *C. glutamicum* have been deposited in GenBank under accession numbers X89830, X89849, and X89850, respectively.

RESULTS AND DISCUSSION

Identification of the core sequence in "*A. aureus*" C70 and sequence features of *attP*. Analysis of the integration site of p5510 in the chromosome of "*A. aureus*" C70 revealed the presence of distinct *attP*, *attL*, *attR*, and *attB* sites which were shown to share a 9-bp sequence, 5′-GTT(py)(py)CATA-3′ (Fig. 3). PCR amplification of *attR* and *attL* junction fragments was performed by using chromosomal DNAs isolated from several lysogenic strains of "*A. aureus*" C70 and p5510 integrants as templates. DNA products of identical sizes were generated in all PCRs, suggesting that the same *attP* and *attB* sites had been used for integration of p5510 and ϕAAU2 (data not shown). The presence of 2 bp separating the 3- and 4-bp identical regions was found in *attP* and *attR* and in *attL* and *attB* and is thus not due to a mutation. Hence, the recombination between the phage genome and the host chromosome must occur in the identical 3-bp 5′GTT3′ segment and not in the 4-bp region. This 9-bp segment is called the core region (the central base was designated 0). Core sizes are usually larger but vary from one phage to another (22, 28, 49). Nevertheless, a 3-bp core has been reported for the *Streptomyces* temperate phage ϕC31, in which it is proposed to be the 3-bp 5′TTG3′ sequence (39). This is far different from the best known corynephage, β, whose genome contains a 96-bp homology with the *C. diphtheriae* chromosome (37). The core is

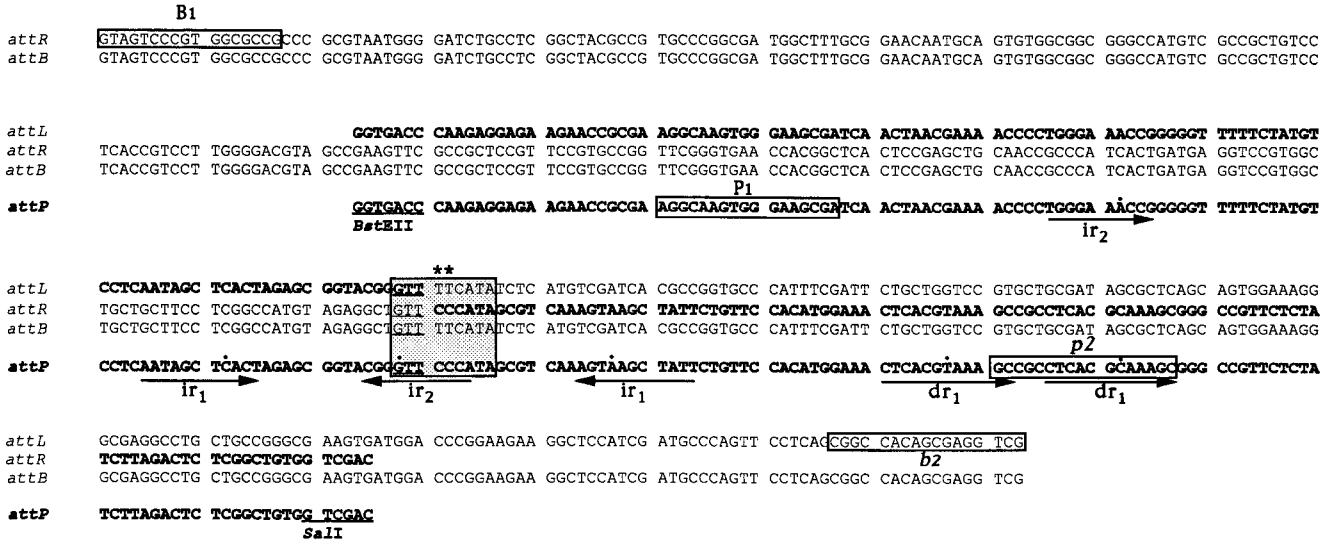


FIG. 3. Nucleotide sequence comparisons between *attL*, *attR*, and *attB* regions from "*A. aureus*" and φAAU2 *attP*. Phage sequences are boldfaced. The 9-bp imperfect homology is boxed and shaded, and the two mismatches are indicated (asterisks). The 3-bp segment where exchange occurs is underlined. Amplification of the "*A. aureus*" C70 *attB* fragment was carried out by using primers B1 and B2, derived from sequence B_1 and the complement of b2, respectively. Nucleotide sequences of *attR* and *attL* were determined by using primers P1 and P2, derived from sequence P_1 and the complement of p2. Direct repeat, dir1, and inverted repeats, ir1 and ir2, present in the vicinity of the 9-bp core are indicated. Dots, mismatches.

located within a relatively AT-rich DNA region, a common feature for core regions of many other temperate phages. In fact, the *attP*-containing 216-bp *Bst*EII-*Sal*I fragment (hereafter "*attP* fragment") has an A+T content of 48%, in contrast to the A+T content in both 800-bp flanking regions, which is 38% (data not shown). The sequence analysis of the *attP* fragment revealed one direct repeat (12 bp, one mismatch) and two inverted repeats (11 bp, one mismatch, and 10 bp, one mismatch) in the vicinity of the identity segment (Fig. 3). The 11-bp inverted-repeat sequences were located symmetrically to the 5′GTT3′ core. None of these sequences was identified on *attB*. Such features are typical of other site-specific systems of recombination and may represent potential binding sites for a protein(s) involved in the integration of φAAU2. The *attB* site described here could be the only, or at least a preferred, site used for φAAU2 integration. In fact, restriction profiles of over 100 integrants obtained in this work were analyzed by using different restriction enzymes and blotted with φAAU2 DNA (data not shown). The same junction fragments were revealed, corresponding to those previously described for φAAU2 lysogens (24). Since we have not deleted this site, we could not exclude the existence of secondary chromosomal attachment sites used in the absence of the main site. The *attB* region from "*A. aureus*" C70 did not show significant homology to tRNA-specifying DNAs, which constitute preferential insertion sites for many temperate phages (5). In contrast, a partial potential open reading frame (ORF) was identified within the 413 bp of the PCR-amplified *attB*-containing fragment and specifies a protein of at least 137 amino acids (data not shown). Database searches showed significant similarity to the product of the *ipa44* gene from *Bacillus subtilis* (35% identity, 60% similarity). Although the function of *ipa44* is unknown, it is located between two genes involved in sugar degradation (14). Similarity was also observed with the N-terminal region of various bacterial monooxygenases (data not shown). Nevertheless, the hypothesis that "*A. aureus*" C70 DNA could integrate into a gene encoding a monooxygenase is unlikely, since the homology does not include the very conserved active site reported for monooxygenases (23).

***attB* in other coryneform bacteria.** p5510 integrative plasmid was previously shown to integrate in the chromosomes of *B. lactofermentum* BL15 *C. glutamicum* RM3 (24) and recently in *B. divaricatum* ATCC 14020 with an efficiency of about 10^4 integrants μg of DNA^{-1}, similar to that reported for the above strains. The common *attB′* site observed for these three strains was amplified. *attB′* and *attB* from "*A. aureus*" C70 were found

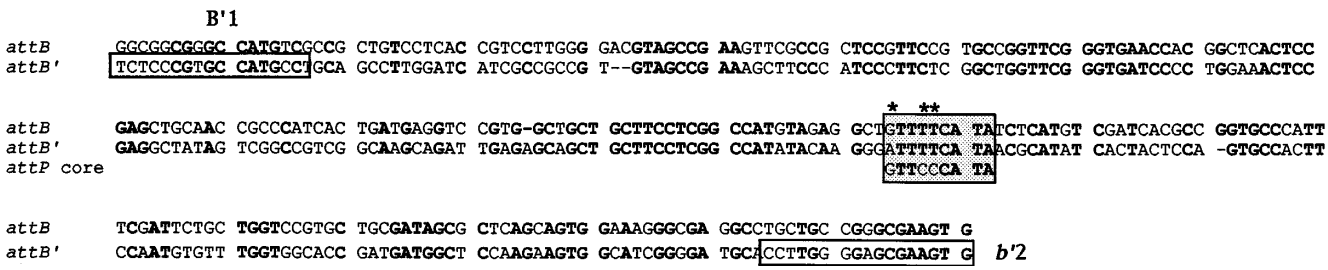


FIG. 4. Nucleotide sequence comparisons between the attachment sites from "*A. aureus*" C70 (*attB*), *C. glutamicum* RM3 (*attB′*), and the 9-bp core from φAAU2. Sequences were aligned manually, and gaps were introduced to maximize homology. Identical nucleotides are boldfaced. The core is boxed and shaded, and mismatches are indicated (asterisks). Amplification of the *attB′* fragment from *C. glutamicum* RM3 was carried out by using primers B′1 and B′2, derived from the boxed sequences B′1 and the complement of b′2.

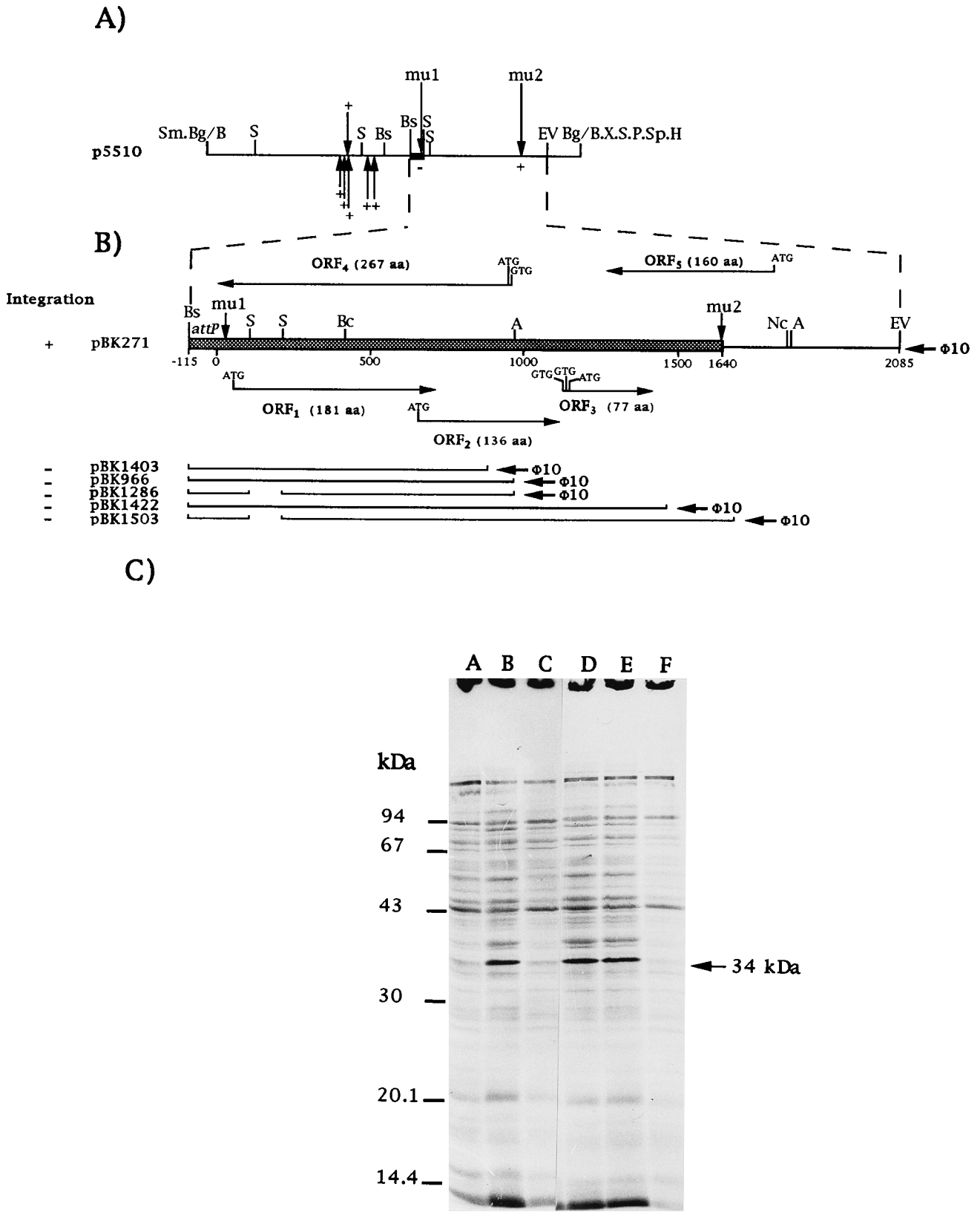


FIG. 5. Location of ϕAAU2 integrative functions on p5510. (A) MudIIPR13 mutagenesis of p5510. Insertion points are indicated by vertical arrows. +, efficiency of integration of 10^4 integrants μg DNA^{-1}; −, total loss of integration. (B) Restriction map of the ϕAAU2 region encoding integrative functions. The *attP*, mu1, and mu2 insertion points are indicated. Exonuclease III derivatives and their integration behavior are indicated on the left. The direction of transcription from the T7 ϕ10 promoter is indicated on the right. The sizes of the polypeptides potentially encoded by the ORFs are given in amino acids (aa). A, *Apa*I; Bc, *Bcl*I; Nc, *Nco*I. Other restriction enzyme abbreviations are the same as those for Fig. 2. (C) Synthesis of pBK271 and derivative polypeptides in the T7 expression system of Tabor and Richardson (44). SDS-polyacrylamide gels of [^{35}S]methionine-labelled polypeptides encoded by the cloned ϕAAU2 sequences were autoradiographed, by using a 16-h exposure. Lanes: A, pBK276; B, pBK271; C, pBK1403; D, pBK966; E, pBK1422; F, pBK. The positions of molecular mass markers are indicated on the left. The molecular mass of the ORF4 product is indicated on the right.

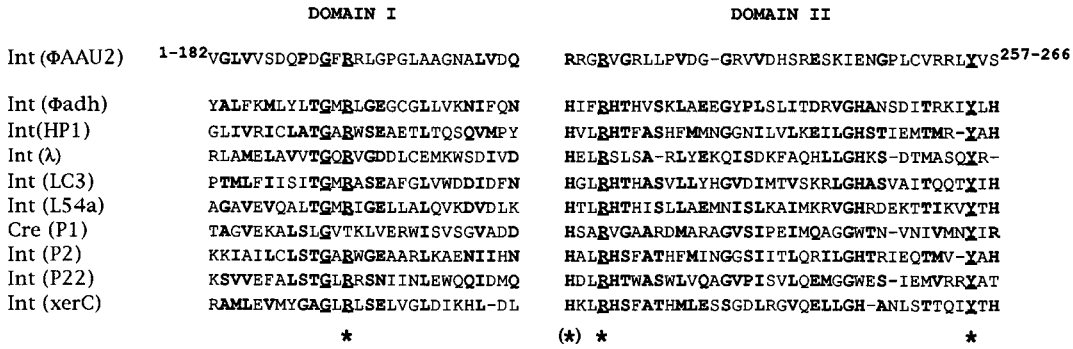


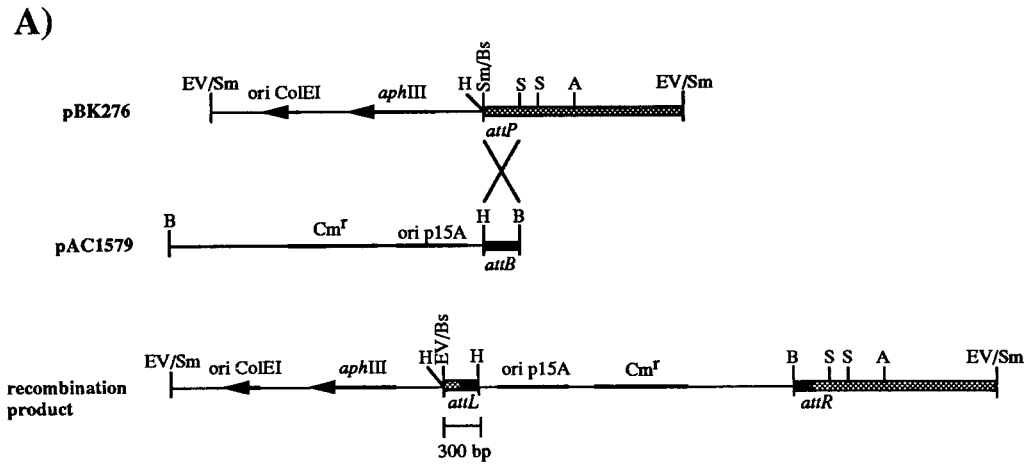
FIG. 6. Amino acid sequence comparison of the C-terminal parts of integrases of the Int family and the ϕAAU2 Int protein. The positions of both domains in the ϕAAU2 sequence are indicated. The four invariant amino acids in the Int family are indicated (asterisks). Identical and conserved amino acids are boldfaced; identical residues are also underlined. Conserved amino acids are those reported by Dayoff et al. (11). Gaps (dashes) have been introduced to maximize homology. Protein sequence sources: *Lactobacillus* bacteriophages ϕadh and LC3 (13, 26), *Staphylococcus* bacteriophage L54a (50), XerC (10), P2 (53), and *Haemophilus* bacteriophage HP1 (49). The sequences of the Cre recombinase of bacteriophage P1 and bacteriophages λ and P22 are those reported by Argos et al. (3).

to have homology, especially in the 9-bp region previously shown to be involved in the site-specific integration of p5510 into "*A. aureus*" C70 (Fig. 4). Only one mismatch was found at the first position of the sequence (G-to-A transition). Consequently, this transition represented a third mismatch between *attP* and *attB′* sequences in the 9-bp region mentioned above (Fig. 4). The three mismatches were flanking the sequence 5′TT3′ in the four attachment sites, suggesting that the 3-bp 5′GTT3′ sequence where exchange is proposed to occur in "*A. aureus*" C70 was reduced to the two last bases 5′TT3′ in *C. glutamicum* RM3. Surprisingly, in spite of this reduction of size, efficiencies of integration of p5510 in the four strains remain identical to that in "*A. aureus*" C70 (10^4 integrants μg of DNA^{-1}). This result raises the possibility that the G nucleotide, in "*A. aureus*" C70 core may not play any role in the recombination process.

Location of integrative functions on p5510. The integration efficiency of MudIIPR13-mutated plasmids was studied in "*A. aureus*" C70 (Fig. 5A and B). Only p5510::mu1 was unable to integrate into the "*A. aureus*" C70 chromosome. The insertion was mapped on the *attP* fragment, downstream of the core and 50 bp upstream from the *Sal*I site. In most reported systems of site-specific recombination, integrative functions are tightly clustered, *int* and *attP* being separated by a few bases only (25). Therefore, the loss of integration of p5510::mu1 could result from the disruption of *int*. Moreover, p5510::mu2 being as efficient as p5510 for integration, we hypothesized that ϕAAU2 integrative functions lie between *Bst*EII and mu2 (Fig. 5A). This was confirmed since the 2.2-kb *Bst*EII-*Eco*RV fragment cloned in pBK, and yielding pBK271 and pBK276, results in the same efficiency of integration as the insert of p5510. The 2.2-kb insert was then sequentially deleted and sequenced. None of the derivatives deleted upstream of the Mu2 insertion point located at bp +1640, pBK1422, pBK966, or pBK1403 (Fig. 5B), were integrative. Thus, phage-encoded integration functions were shown to be located on a 1,756-bp fragment, extending from position −115 to +1640 (Fig. 5B).

DNA sequence analysis of the ϕAAU2 integrative fragment. Four complete ORFs, encoding putative proteins of 180, 135, 76, and 266 amino acids, were identified on the 1,756-bp DNA fragment (Fig. 5B). Since ORF1, ORF2, and ORF3 are not preceded by an obvious ribosome binding site, their biological significance may be questioned. Two possible translational start sites GTG (+811) and ATG (+778) are present upstream of ORF4. The most probable start codon is represented by GTG (+811), as it is preceded by an appropriately spaced ribosomal binding site, in contrast to ATG (+778). Assuming this, it would indicate that the deduced product of ORF4 is a basic protein with a molecular weight and a pI of 28,700 and 9.0, respectively. The amino acid sequences of the four putative proteins were compared with those of proteins in databases. No significant similarity was found for the ORF1, ORF2, ORF3, or ORF4 products. As ϕAAU2 integrase could not be identified through protein comparisons, deletions were performed on the 1,756-bp fragment in order to inactivate its integration properties (Fig. 5B). Nonintegrating derivatives fell into two classes. The former, represented by pBK1403, pBK1286, and pBK1503, disrupts ORF4, suggesting that it may encode ϕAAU2 integrase. The protein product of ORF4 was then compared with site-specific recombination proteins and showed similarity to some members of the Int-related family described by Argos et al. (3). These proteins show, despite their functional conformity, little similarity to each other, which is limited to two domains of the C-terminal portion, believed to be involved in the recognition of the DNA target (31–33). The first domain includes a conserved arginine (1), also present in ORF4 (domain 1, ϕAAU2 positions 182 to 208) (Fig. 6). In the second domain, three amino acid residues, histidine, arginine, and tyrosine, are highly conserved (1, 3, 7, 12, 13, 16). The amino acid sequence of the deduced ORF4 product matched two of these three strongly conserved amino acids (domain 2, ϕAAU2 positions 220 to 258) (Fig. 6). The third conserved amino acid in the Int family (H-308 for λ) seems to be replaced by an arginine residue at the corresponding position in the ϕAAU2 ORF4 product (Fig. 6). Moreover, similarities were also present at 13 other positions in the ϕAAU2 sequence (Fig. 6). On the bases of the involvement of ORF4 in integration, its location, and the homology of its encoded polypeptide with integrases, we assume that ORF4 encodes ϕAAU2 Int recombinase. The second class of derivatives inactivating the integrative properties of the 1,756-bp integrative fragment including pBK966 and pBK1422 do contain *attP*-ORF4. Thus, the presence of a 826-bp noncoding region upstream of ORF4 appears to be absolutely required for integration and may possibly contain transcriptional elements involved in expression or stability of *int*. This requirement remains unusual, in that most reported phage integrative plasmids are based only on *attP-int* (12, 13, 21).

Analysis of encoded polypeptides. In order to determine whether the various cloned fragments in plasmids pBK276 and pBK271 and its derivatives were capable of expressing proteins in *E. coli*, these plasmids were introduced in *E. coli* BL21



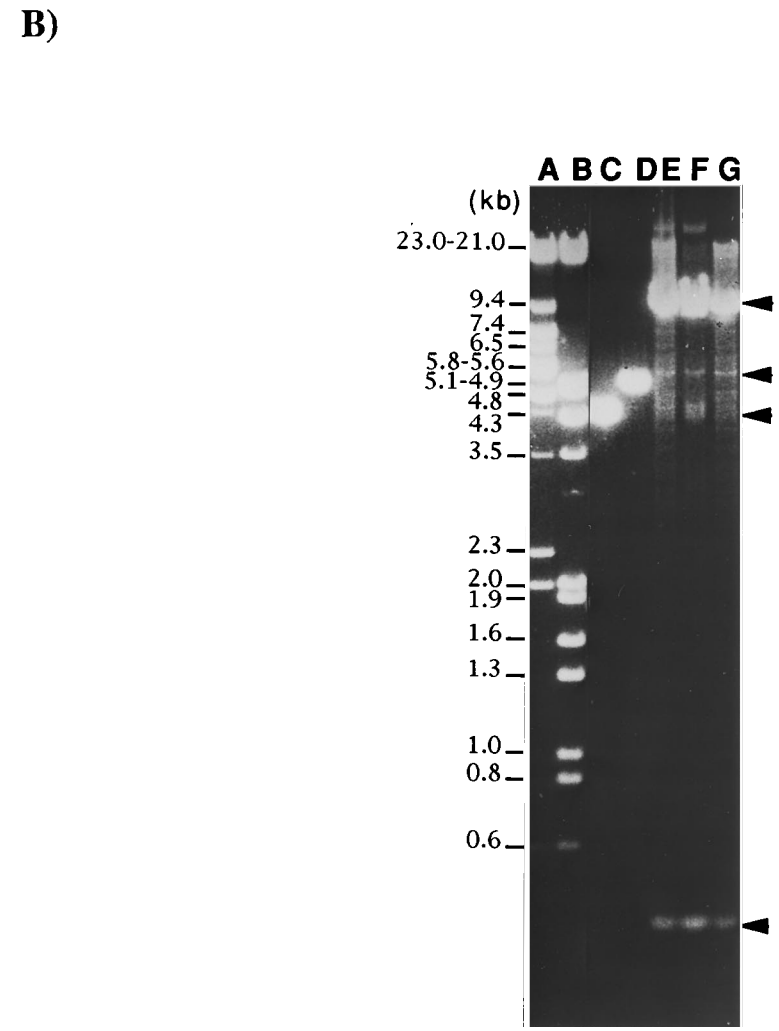


FIG. 7. Site-specific recombination in *E. coli* between plasmids bearing ϕAAU2 integrative functions (pBK276) and *attB* from "*A. aureus*" C70 (pAC1579). (A) Schematic presentation of the recombination product accumulated after site-specific recombination between pBK276 and pAC1579. The 2.2-kb fragment bearing ϕAAU2 integrative functions is shaded. Restriction enzyme abbreviations are the same as those for Fig. 2 and 5. (B) Lanes A and B, molecular size markers; lanes C to G, *Hin*dIII digests of pAC1579, pBK276, and three Kan^r recombinant clones, respectively. Molecular size markers consist of a mix of *Eco*RI and *Hin*dIII digests of λ DNA (lane A) and a double *Hin*dIII-*Eco*RI digest of λ DNA (lane B). Highest and lowest arrowheads, *Hin*dIII fragments contained in the recombinant clones (lanes E to G); other arrowheads, linear *Hin*dIII digests of pAC1579 and pBK276.

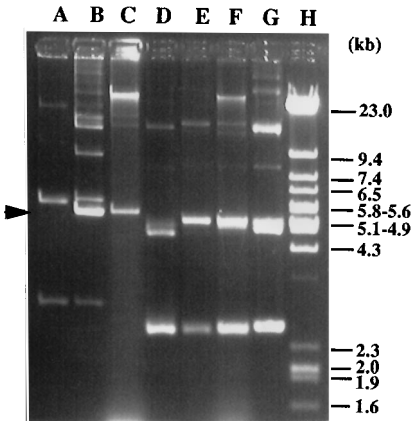


FIG. 8. Site-specific recombination in *E. coli* BL21 between plasmids bearing *attB* from "*A. aureus*" C70 (pAC1579) or ϕAAU2 integrative functions (pBK276) (lanes A to D) and pBK1422 (lanes D to G). Lanes A, D, and G, nondigested plasmids pBK271, pAC1579, and pBK1422, respectively. Lanes B and C, products of recombination between pAC1579 and pBK271, obtained in the absence or presence of IPTG, respectively (arrowhead). Lanes E and F, plasmids present after cotransformation of pBK276 and pBK1422 in the absence or presence of IPTG, respectively. Molecular size markers consist of a mix of *Eco*RI and *Hin*dIII fragments of λ DNA and a double *Hin*dIII-*Eco*RI digest of λ DNA (lane H).

(DE3). The transformants were tested for their capacity to produce labelled protein following transcriptional activation of the T7 promoter, located upstream of the plasmid inserts. No labelled polypeptide was detected by using plasmid pBK276, whereas by using plasmid pBK271 a unique 34-kDa polypeptide could be observed (Fig. 5C, lanes A and B). Taking the orientation of the insert into consideration, this protein may be the product of either ORF4 or ORF5 (Fig. 5B). Plasmids pBK966 and pBK1422, both containing ORF4, expressed the 34-kDa polypeptide, whereas no polypeptide was expressed by pBK1403, in which the 5′ end of ORF4 is deleted (Fig. 5B, lanes D, E, and C, respectively). The deduced protein product of ORF4 has a calculated mass of 28.7 kDa. The difference between the apparent and calculated masses (34 and 28.7 kDa, respectively) could indicate that the start of the structural gene might be located about 150 nucleotides upstream of its actual proposed start codon. Analysis of the sequence of the insert contained in pBK966 revealed the absence of this 150-nucleotide sequence. Assuming this, since pBK966 expressed the 34-kDa polypeptide, while it would not have the capacity to encode it, the mass determined by SDS-PAGE is overestimated. ϕAAU2 integrase is thus represented by a 28.7-kDa protein, encoded by ORF4.

Activity of ϕAAU2 integrative functions in *E. coli*. Two compatible plasmids, one carrying *attB* (pAC1579 [ori p15A, Cm^r]) and the other carrying ϕAAU2 integrative functions (pBK276 [ori ColE1, Kan^r]) were simultaneously transformed into the recombination-deficient *E. coli* DH5α. Clones selected for double resistance to chloramphenicol and kanamycin contained one new plasmid. Its restriction map suggested that site-specific integration had taken place between pAC1579 and pBK276, involving the previously described *attP* and *attB* sites (Fig. 7). Thus, *Hin*dIII digests of the recombination product revealed the presence of a 300-bp *attL* fragment, in agreement with the map of the expected plasmid (Fig. 7). Weak bands corresponding to free pBK276 were also visible (Fig. 7B, lanes E to G). Their presence may be explained by the 10-fold-higher copy number compared with pAC1579. In an analogous experiment, the phage integrative functions were located on pBK and *attB* was located on pACYC184. Cm^r and Kan^r transformants harbored plasmids corresponding to the pBK derivative and the recombination product. The presence of other DNA bands (Fig. 7B, lanes E and G) may be explained as the result of secondary integration events in newly generated *attL* and/or *attR* sites. Indeed, we have observed that pBK276 could form single, double, or triple integrants in "*A. aureus*" (data not shown). When a similar experiment was performed with the exonuclease III derivative pBK1403 instead of pBK276, *E. coli* Kan^r Cm^r clones were shown to harbor two free plasmids with no apparent accumulation of a recombination product (results not shown). This is in agreement with previous results which showed that this plasmid failed to integrate into the "*A. aureus*" C70 chromosome. These experiments indicated that all ϕAAU2 integrative determinants could be expressed in *E. coli*, in a recombination-proficient form.

Role of the 800-bp region upstream of *int*. pBK1422 is a derivative of pBK271, unable to integrate in the chromosome of "*A. aureus*" C70, while allowing the production of integrase in *E. coli*, under the control of the ϕ10 promoter (Fig. 5). In order to test if the lack of integration results from the deletion of the natural integrase promoter or transcriptional signals, we introduced pBK1422 and pAC1579 in *E. coli* BL21. Analysis of the integration events before and after T7 polymerase induction revealed no integrants (Fig. 8, lanes D to G), although the 34-kDa protein was produced, as illustrated in Fig. 5. An analogous experiment was conducted in the presence of pBK271, containing functional integrative functions, and pAC1579 (Fig. 8, lanes A to D). Recombination between the two plasmids was observed in the absence and presence of induction (Fig. 8, lanes B and C). From these results, we assume that the noncoding region upstream of the *int* gene contains some function necessary to recombination.

In summary, we characterized the integrative functions (*attP-int*) of the corynephage ϕAAU2. Further study of the region upstream of the *int* gene will help elucidate the mode of regulation of *int* expression. The wide integration spectrum of plasmid p5510, which extends to *C. glutamicum* strains, may allow wider application of this ϕAAU2-derived integrative vector.

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