A New ICEclc Subfamily Integrative and Conjugative Element Responsible for Horizontal Transfer of Biphenyl and Salicylic Acid Catabolic Pathway in the PCB-Degradating Strain Pseudomonas stutzeri KF716

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Abstract: Integrative and conjugative elements (ICEs) are chromosomally integrated self-transmissible mobile genetic elements. Although some ICEs are known to carry genes for the degradation of aromatic compounds, information on their genetic features is limited. We identified a new member of the ICEclc family carrying biphenyl catabolic bph genes and salicylic acid catabolic sal genes from the PCB-degrading strain Pseudomonas stutzeri KF716. The 117-kb ICEbph-salKF716 contains common core regions exhibiting homology with those of degradative ICEclc from P. knackmussii B13 and ICEXTD from Azotobacter sp. CIB. A comparison of the gene loci collected from the public database revealed that several putative ICEs from P. putida B6-2, P. alcaliflava JABI, P. stutzeri AN10, and P. stutzeri 2A20 had highly conserved core regions with those of ICEbph-salKF716, along with the variable region that encodes the catabolic genes for biphenyl, naphthalene, toluene, or phenol. These data indicate that this type of ICE subfamily is ubiquitously distributed within aromatic compound-degrading bacteria. ICEbph-salKF716 was transferred from P. stutzeri KF716 to P. aeruginosa PAO1 via a circular extrachromosomal intermediate form. In this study, we describe the structure and genetic features of ICEbph-salKF716 compared to other catabolic ICEs.

Keywords: horizontal gene transfer; ICEbph-sal; ICEclc; ICEXTD; integrative and conjugative elements; polychlorinated biphenyls; Pseudomonas stutzeri; salicylic acid

1. Introduction

Integrative and conjugative elements (ICEs) are mobile genetic elements of bacteria that are excised from the chromosome, transferred to other bacteria via conjugation, and reintegrated into the chromosome. They often carry cargo genes involved in antibiotic resistance, pathogenicity, heavy metal resistance, nitrogen fixation, or aromatic ring catabolism to impart beneficial traits to bacteria [1]. To date, a limited number of ICEs are known to carry cargo genes for the catabolism of aromatic pollutants, of which ICEclc is one of the best-characterized [2]. ICEclc contains cargo genes that encode the ortho-cleavage

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of chlorocatechol (clc genes) and aminophenol catabolism (amm genes). This element was originally identified in the 3-chlorobenzoic acid-degrading bacterium Pseudomonas knackmussii B13. Almost identical copies have been found on the chromosome of Burkholderia xenovorans LB400 (designated ICEecl-LB400) [5] and P. aeruginosa JB2 (ICEecl-JB2) [4]. The structure of ICEecl was compared with other conserved ICEs from five bacterial strains, but none of them contained a gene encoding the aromatic ring degradation pathway [5]. ICEXTO from Azotobacter sp. CIB is another member of the ICEecl family, which is well characterized in terms of the function of cargo genes and the transferability by conjugation [6]. A notable feature of ICEXTO is that it carries gene clusters for both aerobic and anaerobic degradation of xylene and toluene.

The bph genes, which are responsible for the co-metabolic degradation of polychlorinated biphenyls (PCBs), are widely distributed among both Gram-negative and Gram-positive bacteria [7]. Some bph genes are known to be horizontally transferred via mobile genetic elements. The first reported ICE carrying bph genes is Tn4371, found in the chromosome of the Gram-negative bacterium Cupriavidus oxalaticus A5 [8], with a total length of 61.8 kb. The chromosome of the Gram-negative bacterium Acidovorax sp. strain KKS102 contains ICEKKS102.4677, which belongs to the Tn4371 family [9]. ICEKKS102.4677 is known to transfer to a wide variety of bacteria across species and genera via a circular intermediate. Recently, we reported the entire genomes of ten PCB-degrading bacteria isolated from biphenyl-contaminated soil in Kitakyushu, Japan [10]. Among them, we detected ICEs carrying the bph gene from nine strains. ICEKbh-KF708 from Cupriavidus basilensis KF708 and ICEKbh-KF712 from Comamonas testosteroni KF712 are Tn4371 type ICEs, where ICEKbh-KF708 is almost identical to ICEKKS102.4677. A 483-kb plasmid pKF715A carrying the bph gene and salicylic acid catabolic sal gene was detected from P. putida KF715, which could be transferred and integrated into the chromosome of P. putida AC30 or KT2440, and then maintained as ICEKbh-salKFI15 [11]. Six Pseudomonas strains (P. abietaniphila KF701, P. aeruginosa KF702, P. putida KF703, P. furukawaii KF707 (formerly P. pseudoalcaligenes KF707), P. toytomimensis KF710, and P. stutzeri KF716) carry an ICEKbh-sal element with sizes ranging from 117 kb to 130 kb, and integrate at the 3’ end of the tRNA-Gly(CCC) gene. It is obvious from their highly conserved sequences that ICEKbh-sal8 were generated via horizontal gene transfer. These ICEs carrying bph genes play an important role in the degradation of PCBs in the environment. Precise information on their structure and function will be important to understand the adaptation of their host strains to environmental niches and to design bioremediation processes using PCB-degrading bacteria. Here, we investigated the gene structure of ICEKbh-salKFI16 in comparison with other catabolic ICEs and demonstrated that ICEKbh-salKFI16 can be transferred via a circular intermediate form.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Pseudomonas stutzeri KF716 (NBRC 110668), which has the ability to utilize biphenyl (Bph+) and salicylic acid (Sal+), was isolated from the soil in Kitakyushu, Japan [10]. P. aeruginosa PAO1 (NBRC 106052), which has kanamycin resistance (KmR), was obtained from the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC, Tokyo, Japan). For the growth of Pseudomonas strains, a basal salt medium containing (in grams per liter) K2HPO4, 4.3; KH2PO4, 3.4; (NH4)2SO4, 2.0; MgCl2·6H2O, 0.34; MnCl2·4H2O, 0.001; FeSO4·7H2O, 0.0006; CaCl2·2H2O, 0.026; and Na2MoO4·2H2O, 0.002 (pH 7.0) was used. The bacterial strains were grown by shaking at 120 rpm at 30 °C. For DNA isolation or freezing stock preparation, Luria–Bertani (LB) medium (Bacto Tryptone, 10 g; yeast extract, 5 g; and NaCl, 10 g/L, pH 7.0) was used.

2.2. Sequence Annotation and Computational Analysis

The sequence of ICEKbh-salKFI16 was determined as previously reported [10,12]. The complete nucleotide sequence of ICEKbh-salKFI16 has been deposited in DDBJ/ENA/GenBank under accession no. LC469614. The sequences were annotated using the NCBI Prokary-
otic Genome Annotation Pipeline (PGAP) [13] and Rapid Annotations using Subsystems Technology (RAST) server v.2.0 [14]. The coding genes were identified using BLAST and BLASTX searches [15]. Sequence comparisons were performed using EasyFig v.2.1 [16], and a map was generated using the drawGeneArrows3 program (http://www.ige.tohoku.ac.jp/joho/labhome/tool.html, accessed on 28 November 2021). GC content and identity between genes were calculated using GENETYX version 15 (Genetyx Co. Ltd., Tokyo, Japan).

2.3. Detection of Target DNA by Polymerase Chain Reaction

Genomic DNA was extracted using a Genomic-tip 20/G (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Amplification of genes was performed in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and PCR conditions were performed in a 25 µL reaction mix containing 12.5 µL Gene RED PCR Mix Plus (Nippon Gene Co. Ltd., Toyama, Japan) at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 60 s, with a final extension of 5 min at 72 °C. See Table S1 for primer sequences. PCR products were detected by agarose gel electrophoresis according to a standard procedure. The sequences of PCR products were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3500 Genetic Analyzer.

2.4. Conjugal Transfer of ICE

Transfer of the Bph$^+$ phenotype by conjugation into the recipient cells was carried out through filter mating. Donor and recipient cells were grown overnight in LB agar medium, and both cultures were suspended in 1 mL of LB broth. The cell suspensions (0.5 mL each) were mixed and placed on a nitrocellulose filter (0.45 µm, Merck Millipore, Bedford, MA, USA), and placed on an LB agar plate at 30 °C for 16 h. After incubation, the cells on the filter were suspended in sterilized saline, further diluted, and inoculated onto basal salt medium plates containing 30 µg of kanamycin, providing solid biphenyl in the inverted lid to select transconjugants. Conjugative transfer frequencies were calculated as the number of transconjugant cells per number of donor bacterial cells present in each mating.

3. Results and Discussion

3.1. Detection of ICE$_{bph-sal}$KF716

A detailed analysis of the genome sequence of _P. stutzeri_ KF716 revealed a genomic island of 117,300 bp (Figure 1), named ICE$_{bph-sal}$KF716. ICE$_{bph-sal}$KF716 is located at the 3’ end (attB site) of tRNA$^{Gly}$($^{CCC}$). The left end (attL) of ICE$_{bph-sal}$KF716 is formed by 18 bp of tRNA-$^{Gly}$ (TTCCCTTCGCCCGCTCCA), and the right end (attR) is formed by a repetition of these 18 bp (Figure 2). We previously confirmed that this 18 bp direct repeat sequence is located on the border of the conserved ICE region and the non-conserved region of the chromosome [10]. The complete sequence of ICE$_{bph-sal}$KF716 was submitted to the PGAP and RAST pipeline for annotation, and 112 ORFs were identified. Annotation was refined manually and compared with pKF715A [11], ICEclc [2], and ICE$_{XTD}$ [6]. ICE$_{bph-sal}$KF716 is a mobile genetic element that shares a core region with ICEclc and ICE$_{XTD}$, which have been shown to impart aromatic compound degradation genes to bacteria. The genetic maps of ICE$_{bph-sal}$KF716, ICEclc, and ICE$_{XTD}$ are shown in Figure 1, while their attL and attR are shown in Figure 2. The genes encoded by ICE$_{bph-sal}$KF716 are listed in Table S2.
Figure 1. Gene map of ICEclc, ICEbph-salKF716, and ICEXTD. Regions with nucleotide identity above 64% are connected by red (forward) or blue (reverse) windows using a color intensity gradient based on identity scores of BLASTn comparison. 1: tRNA-Gly (partial); 2: int genes; 3: traI gene; 4: traG gene; 5: VirB4 components of the type IV secretory pathway; 6: VirD4 component of the type IV secretory pathway; 7: pilL; 8: inrR; 9: parB; 10: parA; 11: clc genes; 12: amn genes; 13: bph genes; 14: sal genes; 15: putative multi-drug efflux pumps; 16: tod genes; 17: mdb genes; 18: putative benzoate transporter; 19: bss genes; 20: bbs genes.

**attL**

TTCCCTTGCCTCGCTCCA (KF716)  
TTCCCTTCTACCCGCTCCA (B6-2)  
TTCCCTATCGCGCTCCA (JAB1)  
TTCCCTTCGCCGGCTCCA (AN10)  
TTCCCTTCGCCGGCTCCA (2A20)  
TTCCATTCCCAGCGCTCCA (ICE_{XTD})  
GTTCCTTTCCGGCTCCA (ICE_{clc})

**attR**

TTCCCTTGCCTCGCTCCA (KF716)  
TTCCCTTCTACCCGCTCCA (B6-2)  
TTCCCTATCGCGCTCCA (JAB1)  
TTCCCTTCGCCGGCTCCA (AN10)  
TTCCCTTCGCCGGCTCCA (2A20)  
TTCCATTCCCAGCGCTCCA (ICE_{XTD})  
GTTCCTTTCCGGCTCCA (ICE_{clc})

Figure 2. Direct repeat sequences flanking ICE_{bph-sal}, ICEclc, ICE_{XTD}, and other putative ICEs. The attL and attR correspond to the sequence of the 5' and 3' termini of the respective ICE.

3.2. Core Region of ICE_{bph-sal}KF716

The attL gene was found to be followed by a phage-related integrase (intL) gene, which shares an identity with that of ICEclc or ICE_{XTD} carrying catabolic genes of aromatic compounds. ICE_{bph-sal}KF716 contains core regions that are significantly similar to the core regions of ICEclc [2] and ICE_{XTD} [6] (Figure 1). The ORFs in the core region of ICE_{bph-sal}KF716 were 55–83% identical to those of ICEclc, and 57–93% identical to those of ICE_{XTD}. The ORFs display the same genetic order in the ICE_{bph-sal}KF716 element, and belong to the type IV secretion system (T4SS). Three factors have been recognized as important for DNA transfer by T4SS in Gram-negative and Gram-positive bacteria. These are: murein hydrolase [17], which is involved in controlled local degradation of the
peptidoglycan, making space for the formation of a mating channel; the VirB4 ATPase, which provides energy for the translocation: and the VirD4 coupling protein, which links the DNA transfer intermediate to the mating channel [18]. In the ICE\textsubscript{bph-sal}KF716 element, we identified the \textit{virB4} gene (KF716ICE\_480; the locus tags are listed in Table S2), \textit{virD4} gene (KF716ICE\_650), and putative murein hydrolase (KF716ICE\_670).

The \textit{parA} and \textit{parB} genes (KF716ICE\_960, KF716ICE\_940) encoding the replication partition proteins present 100 kb and 99 kb downstream of the \textit{attL} site, respectively, were proposed to act as a stabilization system for the maintenance of mobile elements in the bacterial genomes [19]. Near \textit{parB}, there is a gene encoding putative integrase regulator (KF716ICE\_890) a homolog of InrR involved in the regulation of the expression of integrase on ICE\textit{clc} [20]. Relaxase (\textit{traI}, KF716ICE\_370) which binds and nicks excised circular ICE at the origin of the transfer [21], TraG protein (\textit{traG}, KF716ICE\_410) [22], a component of the mating pair formation system, and pilin protein (\textit{pilL}, KF716ICE\_700) were also identified in the core region of ICE\textsubscript{bph-sal}KF716.

The homology of the integrase-encoding \textit{int} gene between ICE\textsubscript{bph-sal} and ICE\textsubscript{clc} was lower (66%) than that in the core region. The integration site of ICE\textsubscript{bph-sal} was tRNA-Gly(CCC), whereas that of ICE\textsubscript{clc} was different in tRNA-Gly(GCC). In contrast, the homology of the \textit{int} gene between ICE\textsubscript{XTD} and ICE\textsubscript{bph-sal}KF716 was 70%; the integration site of ICE\textsubscript{XTD} was tRNA-Gly(CCC), which was identical to that of ICE\textsubscript{bph-sal}KF716. The sequence of \textit{attL} and \textit{attR} at the end of ICE was five bases different between ICE\textsubscript{clc} and ICE\textsubscript{bph-sal}KF716, and one base difference between ICE\textsubscript{XTD} and ICE\textsubscript{bph-sal}KF716 (Figure 2). The amino acid sequences of these ICE integrases may reflect different recognition of the integration site.

### 3.3. Variable Region of ICE\textsubscript{bph-sal}KF716

ICE\textsubscript{bph-sal}KF716 contains at least four variable regions (VR1–VR4) that are deficient in ICE\textsubscript{clc} and ICE\textsubscript{XTD} (Figure 1). Variable region 1 (VR1) located near the \textit{attL} site contains the biphenyl catabolic \textit{bph} gene cluster with a total length of 11.2 kb, as well as a salicylate catabolic \textit{sal} gene cluster with a total length of 11.5 kb. The \textit{bph} gene cluster was found to be located just downstream of the \textit{int} gene, followed by the \textit{sal} gene cluster approximately 6 kb further downstream. The \textit{bph} and \textit{sal} genes encode for the degradation of biphenyl and salicylic acid to TCA cycle intermediates via an initial oxygenation step, followed by a \textit{meta}-cleavage pathway. As shown in Figure 1, the \textit{bph} gene cluster in VR1 shares 59–79% identity at the nucleotide sequence level with the toluene catabolic \textit{tod} gene cluster located in the variable region of ICE\textsubscript{XTD} in the opposite direction. The relationship between the \textit{bph} gene of \textit{P. furukawai} KF707 and the \textit{tod} gene of \textit{P. putida} F1 has been reported in our previous paper [23]. The two variable regions (VR2 and VR3) are located at the midst of ICE\textsubscript{bph-sal}KF716. The GC content of these regions was lower than that of the other regions (Figure 3), and these ORFs (from KF716ICE\_570 to KF716ICE\_630 and from KF716ICE\_710 to KF716ICE\_740) were encoded in the opposite direction compared with ORFs in the surrounding core regions (Table S2), suggesting that these regions are inserted from the other genetic elements or chromosomes through horizontal gene transfer. VR2 contained ORFs coding for several hypothetical proteins with unknown functions, whereas VR3 is likely to be an insertion sequence since it includes an ORF identified as transposase (KF716ICE\_720). The other variable region (VR4) located near the \textit{attR} site contained the gene cluster coding for a putative ABC-type multi-drug efflux pump (from KF716ICE\_1060 to KF716ICE\_1110), with a total length of 6.6 kb that is deficient in ICE\textsubscript{clc} and ICE\textsubscript{XTD}. The substrate of this transporter was not identified due to the lack of reliable homologous genes whose functions have been elucidated.
Figure 3. GC content of ICE_{bph-sal}KF716. The horizontal bars labeled VR1 to VR4 represent four variable regions.

3.4. Comparison of ICE_{bph-sal}KF716 and Other Putative ICEs

A search of the public database using BLAST revealed that an almost identical core region to that of ICE_{bph-sal}KF716 was found in other putative ICEs. The identities were higher than those of ICE_{clc} or ICE_{XTD}. Putative ICEs were identified from the genome sequences of *P. putida* B6-2 (accession number: NZ_CP015202) [24,25], *P. alcaliphila* JAB1 (CP016162) [26], *P. stutzeri* AN10 (NC_018028) [27], and *P. stutzeri* 2A20 (KT935509) [28]. They are flanked by directed repeat sequences corresponding to the *attL* and *attR* sites (Figure 2).

The putative ICE from *P. putida* B6-2 (tentatively designated as ICE_{bph-sal}B6-2) has the closest relationship with ICE_{bph-sal}KF716; it carries a *bph-sal* gene cluster that shares 91–100% identity with that of ICE_{bph-sal}KF716, as well as highly conserved core regions (Figure 4). *P. putida* B6-2 is capable of degrading various polycyclic aromatic hydrocarbons [25]. Biphenyl (*bph*), salicylic acid (*sal*), and ferulic acid (*fcs, ech*), as well as downstream benzoic acid (*ben*) and protocatechueic acid (*pca*) catabolic gene clusters were identified on the genome sequence of *P. putida* B6-2, of which *bph* genes and *sal* genes are located in the ICE (Figure 4). The other putative ICE integrated in the genome of *P. alcaliphila* JAB1 (tentatively designated as ICE_{bph-sal}JAB1) is the second-closest ICE to ICE_{bph-sal}KF716; in this, the *bph-sal* gene cluster and benzoate catabolic *bza* gene cluster are included in the variable region (Figure 4). It is likely that inversion in the variable region, together with a part of the core region, occurred in the ICE_{bph-sal}JAB1. In addition, *sal*:bza and bza:*sal* fusion gene clusters were found, in which parts of the *sal* genes and the *bza* genes were replaced with one another. This inversion at the *sal*:bza locus was also detected in ICE_{bph-sal}KF702 of *P. aeruginosa* KF702, as described in our previous paper [10]. In addition to the well-characterized Tn4371 and ICE_{KKS102}4677, ICEs that carry the *bph* gene cluster included ICE_{bph-sal}KF701, ICE_{bph-sal}KF702, ICE_{bph-sal}KF703, ICE_{bph-sal}KF707, and ICE_{bph-sal}KF710 from five biphenyl/PCB-degrading strains isolated from Kitakyushu, Japan [10]. ICE_{bph-sal}KF716 shares a core region and *bph-sal* catabolic genes with these ICE_{bph-sal} elements, but lacks the *bza* gene, which encodes the benzoic acid degradation pathway. The comparison of the overall structure revealed that ICE_{bph-sal}B6-2 from *P. putida* B6-2, and ICE_{bph-sal}JAB1 from *P. alcaliphila* JAB1 are also members of the ‘ICE_{bph-sal} family’ (Figure 4). In particular, ICE_{bph-sal}B6-2 is more closely related to ICE_{bph-sal}KF716 as it lacks the *bza* gene. Since *P. alcaliphila* JAB1 was isolated in the Czech Republic, and *P. stutzeri* KF716 was isolated from Japan [10], it appears that ICE_{bph-sal} are globally distributed.
A BLAST search identified the other putative ICEs carrying a highly conserved core region with that of ICE\textsubscript{bph-sal}KF716 (Figure 5). The genome of \textit{P. stutzeri} AN10 contained the putative ICE (tentatively designated ICE\textsubscript{nah}AN10) carrying the naphthalene catabolic \textit{nah} genes \cite{29,30} that share more than 70–90% homology with the \textit{nah} operon on plasmid NAH7 at the nucleotide sequence level. The aerobic degradation pathway of naphthalene consists of an upper pathway that transforms naphthalene to salicylic acid and pyruvic acid, and a lower pathway that transforms salicylic acid to TCA cycle intermediates. The \textit{nah} lower operon coding for the lower pathway of ICE\textsubscript{nah}AN10 shares 97–100% identity with the \textit{sal} genes of ICE\textsubscript{bph-sal}KF716. In this context, it is likely that the \textit{nah} upper operon is replaced by the \textit{bph} genes in ICE\textsubscript{bph-sal}KF716, and, conversely, the \textit{bph} genes are replaced by the \textit{nah} upper operon in ICE\textsubscript{nah}AN10. It has been confirmed that many putative mobile protein genes are present on ICE\textsubscript{bph-sal}8 \cite{10}, which may act to replace the \textit{bph} genes and the \textit{nah} upper operon. The part of ICE\textsubscript{nah}AN10, including the core region and genes coding for putative multi-drug efflux pumps, is perfectly identical (100%) to that of ICE\textsubscript{bph-sal}KF716, showing a very close relationship between the two ICEs. In fact, our previous paper described that the \textit{nah} lower operon of \textit{P. stutzeri} AN10 and the \textit{sal} gene of \textit{P. furukawai} KF707 are highly conserved \cite{31}.

The putative ICE from \textit{P. stutzeri} 2A20 (tentatively designated ICE\textsubscript{phe-xy}2A20) carried the \textit{tou} genes coding for multicomponent toluene monooxygenases, \textit{phe} genes coding...
for phenol meta-cleavage pathway, and xyl upper and lower operons coding for toluene-xylene catabolic genes [28] in the variable regions. The core regions of ICE\textsubscript{phe-xyl}2A20, including partial phe genes, overlapped with the sal gene share 91–97% identity with those of ICE\textsubscript{phe-sal}KF716. A comparison of the variable regions of ICE\textsubscript{phe-xyl}2A20 and ICE\textsubscript{phe-sal}KF716 strongly indicates that the substitution occurred between the biphenyl catabolic bph genes and toluene monooxygenase tou genes together with the part of the phe genes. Although ICE\textsubscript{nah}AN10 and ICE\textsubscript{phe-xyl}2A20 do not carry bph genes, the evolutionary relationship of ICE\textsubscript{bph-sal}KF716 with ICE\textsubscript{nah}AN10 or ICE\textsubscript{phe-xyl}2A20 was closer than that of ICE\textsubscript{clc} or ICE\textsubscript{XTD}, as judged from the nucleotide sequence identity level. Their homologies of the core regions with ICE\textsubscript{bph-sal} were higher (77–100% identity between ORFs) than those of ICE\textsubscript{clc} and ICE\textsubscript{XTD}, indicating that these ICEs are more closely related to ICE\textsubscript{bph-sal}KF716. The identity of ICE\textsubscript{bph-sal}KF716 with ICE\textsubscript{nah}AN10 or ICE\textsubscript{phe-xyl}2A20 varies depending on the regions, where major parts including integrase (\textit{int}) gene were highly conserved (90–100% identity), as shown in Figure 5. Although the variable regions among ICE\textsubscript{bph-sal}KF716, ICE\textsubscript{nah}AN10, and ICE\textsubscript{phe-xyl}2A20 are different, they commonly possess genes for aromatic compound meta-cleavage pathways. ICE\textsubscript{bph-sal}KF716, ICE\textsubscript{nah}AN10, and ICE\textsubscript{phe-xyl}2A20 are all ICEs found in \textit{P. stutzeri}. This species is known to exhibit phenotypical diversity in the ecosystem and can adapt to the environmental niche [32], suggesting that it is particularly preferable as a host for this ICE subfamily.

### 3.5. Excision and Formation of Circular Intermediate Form of ICE\textsubscript{bph-sal}KF716

ICEs integrated into the chromosome can be excised from the chromosome to produce a circular form, and the host genome was repaired upon excision (Figure 6a). Attempts were made to detect the extrachromosomal circular form of ICE\textsubscript{bph-sal}KF716 from total DNA isolated from \textit{P. stutzeri} KF716 grown on biphenyl. The amplicons corresponding to the \textit{attP} site (R1 in Figure 6a) of excised circular forms of ICE\textsubscript{bph-sal}KF716, \textit{attB} sites (R2) of excised closed forms of chromosome, and \textit{attL}/\textit{attR} sites (R3 and R4) of the integrated form were detected using PCR (Figure 6b). It has been reported that tRNA-Gly(CCC) locus is the insertion site of ICE\textsubscript{bph-sal}KF716 [10]. DNA sequences of these amplicons matched the expected sequences compared to the total genome sequence of \textit{P. stutzeri} KF716 [12].

![Figure 6. (a) Schematic representation of formation of circular form and integrated form of ICE\textsubscript{bph-sal}KF716. The horizontal bars labeled R1 to R4 are the DNA regions amplified by PCR. Primers attL1 and attR1 (sequences given in Table S1) were used to amplify R1 including \textit{attP} site of ICE\textsubscript{bph-sal}KF716. Primers attL1 and attL2 were used to amplify R2 including \textit{attB} site. Primers attL1 and attL2 were used to amplify R3 including \textit{attL} site. Primers attR1 and attR2 were used to amplify R4 including \textit{attR} site. (b) Detection of circular form and integrated form of ICE\textsubscript{bph-sal}KF716 in wild type \textit{P. stutzeri} KF716 by PCR. Lane 1, DNA ladder marker; Lane 2, R1 (\textit{attP}); Lane 3, R2 (\textit{attB}); Lane 4, R3 (\textit{attL}); Lane 5, R4 (\textit{attR}).](image-url)
3.6. Transfer and Integration of ICE_{bph-sal}KF716

To demonstrate the autonomous intercellular transfer of ICE_{bph-sal}KF716, we performed mating experiments using *P. stutzeri* KF716 as a donor strain. *P. aeruginosa* PAO1 was used as the recipient strain because it carries Km\(^R\) phenotype, and forms a green colony, which enables it to be distinguished from the donor strain. The donor strain was mated with the recipient strain, and transconjugants were selected based on their Km\(^R\) and Bph\(^+\) phenotypes. We observed the appearance of transconjugants, *P. aeruginosa* PAO1 acquiring Bph\(^+\), Sal\(^+\) and Km\(^R\) phenotypes. The transfer frequencies (transconjugants per donor cell) ranged from 6.2 \times 10^{-8} to 1.3 \times 10^{-7}, with 3.3 \times 10^{-7} average in eight replicates. The transconjugants grew in a liquid medium containing biphenyl and salicylic acid as the sole source of carbon (data not shown). To confirm the identity of the transconjugant cells, PCR was performed with genomic DNA using primers 27F and 907R that amplify the 16S ribosomal DNA (16S rDNA), and the DNA sequence of the amplicon matched the 16S rDNA of the recipient strain. The amplicons corresponding to the \(\text{attL}3\) and \(\text{attR}3\) (sequences were given in Figure 7a) two different sized amplicons were observed by electrophoresis (Figure 7b, Lane 3 and Lane 4) and by DNA sequencing when trying to obtain a fragment of R5 corresponding to the \(\text{attP}\) site or R6 corresponding to the \(\text{attB}\) site (R5 of excised closed form of chromosome were also detected from the transconjugants. DNA sequences of these amplicons matched the expected sequences compared to the \(\text{attP}\) site of ICE\({}_{\text{bph-sal}}\)KF716 or its insertion site on the *P. aeruginosa* PAO1 chromosome. At least two different sized amplicons were observed by electrophoresis (Figure 7b, Lane 3 and Lane 4) and by DNA sequencing when trying to obtain a fragment of R5 corresponding to the \(\text{attB}\) site or R6 corresponding to the \(\text{attL}\) site, respectively. This result indicates heterogeneity in the transconjugants, probably due to partial excision of ICE\(_{\text{bph-sal}}\)KF716 from chromosome. It has been reported that the transfer efficiency of ICEclc is extremely high (1.4 \times 10^{-2}) [20], whereas that of ICE\(_{\text{KKS102}}\)4677 is extremely low (5.8 \times 10^{-10}) [9]. The transfer efficiency of ICE\(_{\text{bph-sal}}\)KF716 was approximately 3.3 \times 10^{-7} in the intermediate of ICEclc and ICE\(_{\text{KKS102}}\)4677 and comparable with that of ICE\(_{\text{XTD}}\). The factors that govern the transfer efficiency of these ICEs remain to be elucidated, however many factors seem to involve the donor and recipient strains.

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Figure 7. (a) Schematic representation of the integration of ICE\(_{\text{bph-sal}}\)KF716 into chromosome of *P. aeruginosa* PAO1. The horizontal bars labeled R5 to R10 are the DNA regions amplified by PCR. Primers attL3 and attR3 (sequences were given in
Table S1) were used to amplify R5 including the attB site of the recipient strain. Primers attL1 and attL3 were used to amplify R6 including the attL site. Primers attR1 and attR3 were used to amplify R7 including the attR site. Primers bphL1 and bphR1 were used to amplify R8 including the bph gene. Primers salL1 and salR1 were used to amplify R9 including the sal gene. Primers MDL1 and MDR1 was used to amplify R10 including the putative gene for multi-drug efflux pump. (b) Detection of integrated from of ICEbph-salKF716 in transconjugant P. aeruginosa PA01 by PCR. Lane 1, DNA ladder marker; Lane 2, R1 (attP); Lane 3, R5 (attB); Lane 4, R6 (attL); Lane 5, R8 (bph); Lane 6, R9 (sal); Lane 7, R10 (multi-drug efflux pump); Lane 8, R7 (attR).

4. Conclusions

In this study, we investigated the structure and transfer of ICEbph-salKF716, which is integrated into the chromosome of the biphenyl/PCB-degrading bacterium P. stutzeri KF716. Comparison with putative ICEs from other related Pseudomonas strains suggested the existence of a new ICEclc subfamily that shares nearly identical core regions. Since ICEbph-salKF716 is the first member of this subfamily found, ICEbph-salKF716 represents the ICEclc subfamily which is involved in the horizontal transfer of various catabolic genes among Pseudomonas strains. The results presented in this study will provide new insights into the evolution of ICEs in the process of adaptation to environmental niches, as well as a basis for designing bioremediation processes using PCB-degrading bacteria.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9122462/s1, Table S1: PCR primers used in this study, Table S2: Genes encoded on ICEbph-salKF716.

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