**Tandem Multiplication of the IS*26*-Flanked Amplicon with the *bla*SHV-5 Gene within Plasmid p1658/97.**

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The IncF plasmid p1658/97 (~125kb) from *Escherichia coli* isolates recovered during a clonal outbreak in a Warsaw, Poland, hospital in 1997 contains the extended-spectrum β-lactamase (ESBL) gene *bla*SHV-5, originated from the *Klebsiella pneumoniae* chromosome. A region containing the *bla*SHV-5 gene is flanked by two IS*26* copies and its copy number multiplies spontaneously within p1658/97 and RecA-deficient *E. coli* strains. Here we demonstrate that the amplified IS*26*-*bla*SHV-5 units were arranged in tandems, containing up to more than 10 units, which could raise ceftazidime MICs for host strains from 4μg/ml to more than 128μg/ml. Successive deletions within p1658/97, located outside the amplifiable module and encompassing even as little as ~15% of the plasmid, blocked the amplification. Moreover, the complementing re-introduction of the deleted fragments *in trans* did not restore the process. Similarly, insertions of a 1-kb DNA fragment into the amplicon inhibited its self-multiplication ability. The module was able to transmit into another IS*26*-containing plasmid by recombination. The results prompted us to speculate that local DNA structure, especially favourable in p1658/97, might have been responsible for the IS*26*-*bla*SHV-5 multiplication ability.

Mobile genetic elements (MGEs), such as plasmids, phages and transposable elements largely fuel the dynamics of acquired antimicrobial resistance in pathogenic bacteria, being responsible for mobilization of natural resistance genes and their horizontal dissemination (Bennett, 2004; Frost et al., 2005; Carattoli, 2009). Of note also is their role in raising resistance levels conferred by particular genes, which is usually achieved by delivery of a strong promoter by an insertion sequence (IS) integrating in the gene’s vicinity (**Depardieu et al., 2007**) or the gene copy multiplication along with the carrier MGE. Such multiplication may result from gene transfer into a multicopy plasmid (Wu et al., 1995), or its replicative duplication within a plasmid (Francia & Clewell, 2002) or to different plasmids (Spies et al., 1983). Recently these phenomena have been exemplified by genes encoding newer types of β-lactamases, such as extended-spectrum β-lactamases (ESBLs), AmpC-type cephalosporinases or various carbapenemases in Gram-negative bacteria (Bertini et al., 2007; Poirel et al., 2007; Poirel et al., 2008; Jacoby 2009).

One of the MGEs often associated with mobile β-lactamase genes (*bla*) is IS*26* of the IS*6* family (Mahillon & Chandler 1998). Two IS*26* copies, forming composite transposon-like structures, may surround genes of a CTX-M-type ESBL (*bla*CTX-M-3a), CMY-like AmpC (*bla*CMY-13), a carbapenem-hydrolyzing oxacillinase (*bla*OXA-58) (Miriagou et al., 2004; Bertini et al., 2007; Literacka et al., 2009) or ESBL BES-1 (Bonnin et al., 2012). Such structures have been always observed in the case of mobile *bla*SHV genes, encoding one of the essential β-lactamase families with broad- and extended-spectrum enzymes (Bradford, 2001). The IS*26* elements have mobilized the natural broad-spectrum β-lactamase *bla*SHV gene(s) from the *Klebsiella pneumoniae* chromosome, thus starting the spread of *bla*SHVs among plasmids and Gram-negative organisms, and their evolution towards ESBL-encoding genes (Ford & Avison, 2004; Haeggman, 2010). However, the IS*26*–*bla*–IS*26* segments, including those with *bla*SHV genes, have been frequently found not to be flanked by direct repeat (DR) sequences, indicating the *bla* gene transfer to proceed rather by IS*26*-mediated recombination than transposition (Miriagou et al., 2005; Bertini et al., 2007; Literacka et al., 2009). The ubiquitous presence of IS*26* in chromosomes and plasmids of Gram-negative bacteria greatly facilitates such events.

Previously we analyzed *Escherichia coli* clonal isolates with the IncF plasmid p1658/97 encoding ESBL SHV-5 and recovered in 1997 in a hospital in Poland. Some of the isolates were hyper-resistant to ceftazidime when compared with others (MICs, 128 and 4μg/ml, respectively), which correlated with the increase in the *bla*SHV-5 copy number within plasmid DNA (**Pałucha et al., 1999)**. Because the low-level-resistant isolates segregated the hyper-resistant variants spontaneously, we have analyzed the IncF plasmid p1658/97 in order to elucidate the nature of the mechanism of hyper-resistance to ceftazidime.

# MATERIALS AND METHODS

**Plasmids and bacterial strains.** The origin of the IncF plasmid p1658/97 (with FIB and FII replicons) was reported previously (Pałucha et al., 1999); sequence coordinates (in bp) are according to its annotated sequence (GenBank accession No. AF550679) (Zienkiewicz et al.,2007). The IncL/M plasmid pSEM was described previously (Villa et al., 2000). Plasmids constructed and used in this work are listed in Table 1. The *cat* gene cassette for plasmid constructs was generated by PCR with the pACYC184 (Chang & Cohen, 1978) DNA template (Table 2). The following *E. coli* strains were used: DH5α - F-(Φ80 *lac*ZΔM15) *rec*A1*end*A1 *gyr*A96 *thi*1 *hsd*R17 (r-k m-k) *sup*E44 *rel*A1 *deo*R Δ*lac*(ZYA-*arg*F)U169 (Hanahan, 1983) and BW25113 – (*ΔaraBADAH33 ΔlacZ4787::rrnB-3 lacIq LAM- rph-1 Δ(rhaD-rhaB) 568 hsdR514*) (**Datsenko & Wanner, 2000)**. Electrocompetent E. coli were prepared and electrotransformed according to Sambrook & Russell, 2001 in GenePulser apparatus (BioRad, Hercules, USA).

**Assays for IS*26*-*bla*SHV-5 amplification – plate and disc tests.** In the plate test, 3 ml of LB broth with 100µg/ml ampicillin (Sigma, St. Louis, MO) was inoculated with a single bacterial colony and incubated overnight at 37˚C. Then 100μl of the culture or its dilution was plated on LB agar with 30 or 128μg/ml ceftazidime (CAZ; GSK, Stevenage, United Kingdom) and without the antibiotic. Concentration of CAZ 128μg/ml was applied according to MIC value of the “resistant” group, while 30μg/ml was chosen arbitrary as an intermediate value higher than MIC 4μg/ml for “susceptible” group (Pałucha et al., 1999). In the disc test, overnight cultures were plated on LB plates without antibiotic and then home-made discs with 30 or 128μg CAZ (10mm in diameter; 3MM Whatman paper) were put onto the plates (in some cases the CAZ amount of 50μg was used). *E. coli* colonies appearing inside growth inhibition zones were observed. In both tests, the presence of multiplied IS*26*-*bla*SHV-5 modules was confirmed via restriction analysis of plasmid DNA with SwaI (Fermentas, Vilnius, Lithuania). Intensity of DNA bands produced by amplified units was compared with those of the non-amplified ones in agarose gels; quantification was performed densitometrically.

**Generation time estimation.** The rate of growth of*E. coli* culture was monitored by measurement of optical density (OD600). The generation time was measured as a time of OD600 value doubling (between OD600=0.3 and OD600 =0.6). We measured that until reaching the stationary phase bacteria divide ca. 10 times. The cultures which had to be cultivated for 70 generations were continuously grown for 7 days, each day being 1000x diluted.

**DNA purification and cloning.** Plasmids were purified using the Plasmid Midi AX DNA kit (A&A Biotechnology, Gdańsk, Poland). Larger DNA amounts for sequencing and manipulations were prepared by the alkaline lysis [Birnboim & Doly, 1979), followed by the CsCl-EtBr gradient ultracentrifugation (Sambrook et al., 1989). All enzymes used for DNA manipulations were from Fermentas (Vilnius, Lithuania).

**PCR.** All PCRs were performed in standard cycling conditions; primers are listed in Table 2.

**Pulsed-field gel electrophoresis** **(PFGE).** DNA samples mixed with 6 x Loading buffer (Fermentas) were loaded on the gel. PFGE was performed in 0.8 % Prona LE agarose gels (Prona, Madrid, Spain), using a CHEF MAPPER apparatus (Bio-Rad Laboratories, Hercules, CA). The running conditions were as follows: switching range, 0.1-5.0s; temperature, 14°C; running time, 10h; voltage, 6V cm-1; angle, 120°.

**qRT-PCR analysis.** The multiplication of the amplicon sequence encoding *bla*SHV-5 gene within the p1658/97 plasmid was measured by QPCR carried on 7500 Real Time PCR System (Applied Biosystems, Carlsbad, USA). The 20 μl reaction mixture contained: 1x SYBR Green mix (AmpliQ real-time SYBR GREEN KIT, Novazym, Poznań, Poland), primers (400 nM each) and appropriate amount of plasmid DNA (90, 30 or 10 ng, each in duplicate). The program used: 95°C for 4 min; (95°C for 15 sec, 60°C for 1 min)x40; followed by melting curve readings.

Primers, listed in Table 3, were designed in the program Primer Express 2.0.0 (Applied Biosystems), supplied with the apparatus. After the reaction the efficiency of each primers pair was estimated and the average efficiency was calculated. Obtained Ct values for each gene were normalized to one amount of matrix (30 ng) and the geometric averages of Ct values was calculated, one for both reference genes, the second for both studied genes. The number of studied genes relative to reference gene was calculated as follows:

*studied gene number*

*reference gene number*

*(average efficiency)*

*=*

*- (Ct studied-Ct ref )*

The error of final value was calculated according to standard error propagation rules.

**RESULTS AND DISCUSSION**

**Arrangement and number of** IS*26*-*bla*SHV-5 **amplified units in p1658/97.** In a previous study we have analyzed the IncF plasmid p1658/97 (Zienkiewicz et al.,2007). Its full sequence allowed identification of an IS*26*–*bla*SHV-5–IS*26* segment, derivative of 7,997-bp *K. pneumoniae* chromosome fragment (Wu et al., 2009), almost identical (~99%) to those in other enterobacterial plasmids – pSEM, pIP1202 and pHNM1 and a class 1 integron with aminoglycoside resistance gene cassettes, also flanked by two IS*26* copies (Villa et al.,2000; Welch et al., 2007; Garza-Ramos et al., 2009)*.* The IS*26*–*bla*SHV-5–IS*26* segment, but not integron containing one could amplify in the RecA-independent manner (Zienkiewicz et al.,2007). Since the *bla*SHV-5 gene amplification had been noticed, though not studied, in *K. pneumoniae* (Xiang et al., 1997), we searched for IS*26*-*bla*SHV-5 module amplification in hyper-resistant colonies, hypothesizing a mechanism similar to that previously observed in *Acinetobacter baumannii* for the IS*26*–*bla*OXA-58 module (Bertini et al., 2007). In most cases of DNA amplification, the resulting units are tandemly arranged (Romero & Palacios, 1997). This was observed in case of the IS*26*-*bla*OXA-58 module’s duplication in *A. baumannii*; in the resulting tandem, the fragments with *bla*OXA-58 were separated by a single IS*26* (Bertini et al., 2007). In this study, the amplification was examined in *E. coli* DH5α (*recA*-), transformed with p1658/97 and challenged by 30 or 128μg/ml CAZ concentrations in the plate test.The presence of multiplied IS*26*-*bla*SHV-5 modules was analyzed by SwaI digestion of plasmid DNA (Zienkiewicz et al.,2007). The scheme of the plasmid region is presented in Fig. 1a. SwaI cuts IS*26* at one site, yielding a 8,817-bp fragment out of the IS*26*-*bla*SHV-5-IS*26* segment, and a 7,442-bp fragment out of the integron-containing locus. In agarose gels, the ~8.8-kb band was significantly more intense than the ~7.5-kb band in plasmids from hyper-resistant colonies when compared with those from the low-level resistant ones, suggesting amplification of the IS*26*-*bla*SHV-5-IS*26* module but not of the IS*26*-integron-IS*26* module. Similar ~8,8-kb band of augmented intensity was obtained after NcoI digestion of plasmids isolated from hyper-resistant *E. coli;* NcoI recognizes one site inside the IS*26*-*bla*SHV-5-IS*26* segment (Fig. 1b). In order to reveal the arrangement and roughly evaluate numbers of amplified units, plasmids from hyper- and low-level-resistant colonies were digested with AvaIII and BcuI and run by PFGE (Fig. 1c and Fig. 1d). Both enzymes cut p1658/97 outside the IS*26*-*bla*SHV-5-IS*26* region (Fig. 1a), yielding fragments: 12,450bp (AvaIII) or 23,870bp (BcuI) in plasmid with the single IS*26*-*bla*SHV-5-IS*26* module. In case when IS*26*-*bla*SHV-5 modules form tandems, AvaIII and BcuI digests should yield fragments sized according to the rule: 12,450bp + (n–1) x 8,817bp for AvaIII or 23,870bp + (n–1) x 8,817bp for BcuI, where “n” means the number of amplicons, and 8,817bp is the length of the single amplicon unit (IS*26*-*bla*SHV-5). Instead of the 12,450-bp and 23870-bp fragments, plasmids from hyper-resistant colonies produced few longer ones with sizes fitting to the formula (Fig. 1a), which evidenced tandems composed of IS*26*-*bla*SHV-5 units. Plasmids from colonies grown on 30μg/ml CAZ had 1-2 up to 11 amplicons at least; the most intense AvaIII band was of ~65kb, thus containing seven units. In BcuI digestion, the most intensive bands were ~50-70 kb, thus containing 4-6 amplicon units. We observed that in repeated isolations and digestions of plasmids from other colonies collected from plates with CAZ 30μg/ml, the number of amplicons differed, but still the range of 4-7 unit was observed. This may be explained by spontaneity of the amplification process. Plasmids from colonies grown on CAZ 128μg/ml produced ladders of amplicon bands starting with size of over 100kb (11 units or more; data not shown) delineating the positive correlation between resistance level and the number of amplified units; however, populations of hyper-resistant bacteria contained heterogeneous pools of plasmids, varying in amplicon numbers. Interestingly, results of qRT-PCR analysis of plasmids from colonies grown on 30μg/ml CAZ showed that in average there was 23±0.5 times more amplicon-derived products than those of the plasmid-located reference sequence. This might be due to the presence of intermediates of the amplification process, probably in circular forms of a single or tandemly arranged IS*26*-*bla*SHV-5 unit(s), transiently existing in bacterial cell. When plasmid isolated from colonies grown without antibiotic pressure were used as a template in qRT-PCR analysis the average number of the amplicon sequences was equal to that of the reference sequence.

###### Amplification stability.

###### In order to check for the amplification reversibility, ten hyper-resistant E. coli DH5α colonies were selected from the inhibition zone around the CAZ 50μg disc (Fig. 2A), and verified by SwaI to contain plasmids with amplified IS26-blaSHV-5 units. LB broth cultures inoculated with such single colonies were grown for ~10 generations without antibiotic and subjected to the disc test; no growth inhibition zone around the CAZ disc was observed (Fig. 2B). However, when the hyper-resistant cultures were cultivated further for ~60 generations the inhibition zone re-appeared, with a diameter similar to that of low-level-resistant bacteria (Fig. 2C). This result indicated that upon prolonged culturing without antibiotic pressure, the majority of cells returned to the low-level resistance phenotype, confirmed by the SwaI plasmid analysis (data not shown).

**Search for amplification factors.** The essentially RecA-independent IS*26*-*bla*SHV-5 amplification prompted us to look for plasmidic factor(s) involved in this process. The IS*26* transposase seemed to be a good candidate, however, as four identical full length IS*26* encoding genecopies are present in the p1658/97 sequence, the IS*26*s could act also as homologous repeats in a recombination-based mechanism (Romero & Palacios, 1997). Moreover, other recombinases are numerously encoded by p1658/97 (Zienkiewicz et al. 2007) so their participation in amplification process should be considered.

###### i/ deletion approach. Six plasmid derivatives containing the IS26-blaSHV-5 amplicon and different p1658/97 fragments were constructed; their sequences covered 100% of the wild-type plasmid altogether (Fig. 3). Additionally, for one of the constructs (pXB), a variant with two IS26-blaSHV-5 copies in tandem was obtained (pXB2A). The amplification ability of the constructs was analyzed in the RecA-deficient (DH5α) backgrounds by the disc test and SwaI analysis. The majority of strains with the p1658/97 derivatives produced no hyper-resistant colonies inside the CAZ inhibition zones, regardless of the RecA status. The exceptions were E. coli with constructs pFBAMP and pFMAMP; however, sequence analysis of plasmids from their in-zone colonies revealed lack of the IS26-blaSHV-5 amplification but a deletion of a fragment adjacent to the repA gene, increasing the plasmid copy number (data not shown). These results revealed that none of the constructs was able to amplify the amplicon, including pXH which contained ~85% of p1658/97 with all its recombinase genes and mobile elements. Interestingly, in the case of plasmid pXB2A with two tandem units, the negative results indicated that the initial duplication did not stimulate further amplification, even if the RecA is present. This observation was in opposition to that on the aforementioned plasmid NR1, in which duplication of the IS1-flanked region was essential for its further RecA-mediated amplification (Perlman & Sticgold, 1977; Peterson & Rownd, 1985b).

**ii/ complementation approach.** The lack of amplification in all of the deletion mutants incited us to perform an experiment, in which two plasmids with complementary p1658/97 parts were introduced into *E. coli* DH5α simultaneously (Fig. 4). Four pairs of plasmids were analyzed, and in each of these one construct contained the part with the amplicon (“tested” plasmid), while the second one carried the missing set of p1658/97 genes (“auxiliary” plasmid). It should be noted that two pairs comprised 100% of the p1658/97 sequence. The disc and plate tests did not yield any hyper-resistant colonies, even in the case of the pair pXH + pXB-FIBamp::*cat*, for which plasmid cointegrates were observed (data not shown).

**iii/ insertion approach.** The results of the deletion and complementation approaches indicated that factors essential for the IS*26-bla*SHV-5 amplification were not proteins and suggested a role of local DNA structure (nearby or inside the amplicon). To verify this hypothesis, three p1658/97 derivatives containing the *cat* cassette (1,015bp) were constructed (Fig. 5). In construct p1658/97I::*cat*, the cassette was introduced between the amplicon genes *orf39* and *orf40*, enlarging it by ~11%. In p1658/97*orf42*::*cat*, the gene replaced *orf42* inside the amplicon, lengthening it only by ~1% (the *orf42* hypothetical product has no domains indicative for DNA or protein binding). The p1658/97O::*cat* construct had the insertion outside the amplicon, 176bp downstream of IS*26*. Considering also the possible involvement of gyrase, the insertion constructs and p1658/97 were tested in *E. coli* DH5α (RecA- and gyrase-deficient) and BW25113 (RecA- and gyrase-proficient). Hyper-resistant colonies appeared only when the two strains had either the intact p1658/97 or p1658/97O::*cat*, with similar amplification frequency. Therefore, the manipulations inside IS*26*-*bla*SHV-5inhibited its amplification but the insertion outside had no any detrimental effect. These observations have rather supported the DNA structure hypothesis, while the lack of differences between DH5α and BW25113 has excluded the role of gyrase. Several reports described similar topological effects on the behaviour of small plasmids (Lovett et al., 1993; Bi et al., 1995; Bi & Liu, 1996; Oussatcheva et al.,2004; Pavlicek et al., 2004); however, to our knowledge, no data on molecules comparable in size to p1658/97 have been published so far. The only study on a DNA fragment exceeding 10kb has been that on the IS*1*-flanked transposon Tn*2901* in the *E. coli* chromosome; the RecA-independent step of its tandem amplification was stimulated by insertion of factor F into the chromosome but beyond Tn*2901* (Clugston & Jessop, 1991).

**Amplification in pSEM vs. p1658/97.** As it was mentioned above, the almost identical IS*26-bla*SHV-5-IS*26* segment (99% similarity within the entire length and 100% identity of IS*26* encoding sequences) was found in the IncL/M plasmid pSEM (Villa et al., 2000). For comparison purposes, we performed the disc and plate amplification tests with *E. coli* DH5α transformed with pSEM. In the plate test with varying CAZ concentrations, hyper-resistant colonies were observed only up to CAZ 50μg/ml. The presence of amplified the 8817bp segment within pSEM was verified by SwaI and NcoI digestions (data not shown). These results suggested that the pSEM structure context of the IS*26-bla*SHV-5 module might be not as favourable for the amplification as in p1658/97.

**In-trans mobility of the amplicon.** Apart from the in-plasmid amplification, we decided to analyze the ability of the *bla*SHV-5 containing region to transmit to another plasmid with and without IS*26*. The plasmid pACYC184::IS*26*, containing a single IS*26* copy, and the vector pACYC184 were used as recipients in the experiment, with p1658/97 and its amplification-deficient derivatives (pXH and pXB2A) being donors of the module. *E. coli* DH5α cells carrying donors were transformed with recipient plasmids, and cultured in LB broth with 30µg/ml CAZ and 10µg/ml tetracycline for ~70 generations. Subsequently, total plasmid DNA was purified and re-introduced into DH5α cells; selection of transformants was performed with both antibiotics. The transmission was observed only between p1658/97 and pACYC184::IS*26*; the SwaI analysis revealed that all transformants carried a pACYC184 variant with the complete IS*26-bla*SHV-5-IS*26* structure, pACYC184::AMP (Fig. 6).

Sequencing of the IS*26-bla*SHV-5-IS*26* regionin pACYC184::AMP did not show 8-bp DRs formed by IS*26* during transposition. Moreover, transposition of IS*6*-like elements, as IS*26* is, proceeds via cointegrate, which is then resolved by a resolvase (Mollet et al., 1983; Mollet et al., 1985; Mahillon & Chandler 1998; Doroshenko & Livshits, 2004) or, possibly, by RecA (Doroshenko & Livshits, 2004). The *in silico* analysis of the p1658/97 sequence did not reveal any putative resolvase gene, and the experiment was performed in the RecA-negative strain. These excluded transposition as the mechanism of the IS*26-bla*SHV-5 transmission. The lack of transmission from plasmids deficient in the *in cis* amplification (pXH and pXB2A) suggested the correlation between these two abilities. It should be stressed that IS*26-bla*SHV-5-IS*26* module present in pACYC184::AMP was not able to amplify.

**Conclusions.** This study analyzed in more detail an example of mobile elements flanked by IS*26*, often found to carry β-lactamase genes of high clinical and epidemiological relevance. Consistently with earlier observations (Miriagou et al., 2005; Bertini et al., 2007; Literacka et al., 2009) it did not copy by transposition but via IS*26*-mediated recombination. The IS*26-bla*SHV-5 module analyzed here showed the unusual ability of highly efficient self-amplification *in cis*, within the same plasmid molecule and *in* *trans*, to other molecules*.* Our main effort was to explain this phenomenon resulting in heterogeneous resistance of host *E. coli* strains to β-lactams, with the spontaneous emergence of hyper-resistant cells in bacterial cultures. We failed to reveal its actual mechanism but several lines of evidence suggested involvement of local DNA structure in a peculiar plasmid sequence context. However, considering the fragmentary character of the data and the scarcity of reports on topological aspects of gene amplification, the hypothesis needs further comprehensive studies.

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**TABLE 1**. Plasmids constructed and used in the study.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Plasmid** | | **Construction** | **Remarks** | |
| **Plasmids used in deletion approach** | | | | |
| **pRAMP** | BcuI-SnaBI fragment of p1658/97 (85091..99936) cloned into pR. Size, 28,436bp | | | FII replicon, an integrase gene, the IS*26*-*bla*SHV5 -IS*26* amplicon unit. |
| **pRFAMP** | BcuI-BcuI fragment of p1658/97 (85091..108960) cloned into pR. Size, 37,460bp | | | FII and FIB replicons, two IS*1* and the IS*26*-*bla*SHV5 -IS*26* amplicon unit. |
| **pXB** | XbaI-XbaI fragment of p1658/97 (82062..54780) self-ligated. Size, 98,208bp | | | FII and FIB replicons, whole operon of *tra* genes, four IS*1*, two put. transposons, four (all) IS*26* copies*,* an integrase gene flanked by truncated IS*26*, the IS*26*-*bla*SHV5-IS*26* amplicon unit. |
| **pXB2A** | Doubled XbaI-XbaI fragment of p1658/97 (82062..54780) self-ligated. Size, 107,025bp | | | pXB with two tandem amplicon units. |
| **pXH** | Xho-XhoI fragment of p1658/97 (41448..23250) self-ligated. Size, 107,312bp | | | FIB replicon, all mobile elements found in p1658/97, IS*26*-*bla*SHV5-IS*26* amplicon unit. |
| **pFMAMP** | MunI-MunI fragment of p1658/97 (82511..105517) self-ligated. Size, 23,007bp | | | FIB replicon, one IS*1*, the integrase gene flanked by truncated copy of IS*26,* the IS*26*-*bla*SHV5-IS*26* unit. |
| **pFBAMP** | BcuI-BcuI fragment of p1658/97 (85091..108960) self-ligated. Size, 23,870bp | | | FIB replicon, two IS*1,* the IS*26*-*bla*SHV5-IS*26* amplicon unit. |
| **Plasmid used in complementation approach** | | | | |
| **pR** | StuI-StuI fragment of p1658/97 (coordinates: 29356..42120) ligated with the *cat* cassette. Size, 13,590bp | | | FII replicon, "auxiliary" plasmid. |
| **pRX** | XhoI-XhoI fragment of p1658/97 (23250..41428) ligated with the *cat* cassette. Size, 19,074bp | | | FII replicon, "auxiliary" plasmid. |
| **p1658/97FIBamp::*cat*** | p1658/97 with the fragment 85178..102330 replaced by the *cat* cassette. Size, 109,354bp | | | p1658/97 without amplicon unit and FIB replicon, "auxiliary" plasmid. |
| **pXB-FIBamp::*cat*** | pXB with the fragment 85178..102330 (p1658/97 coordinates) replaced by the *cat* cassette. Size, 82,071bp | | | pXB without amplicon unit and FIB replicon, "auxiliary" plasmid |
| **pXH** | Described above in the table, used as a "tested" plasmid. | | | |
| **pFBAMP** | Described above in the table, used as a "tested" plasmid. | | | |
| **Plasmids used in insertion approach** | | | | |
| **p1658/97I::*cat*** | p1658/97 with the fragment 90107..90169 replaced by *cat*. Size, 126,444bp | | | *cat* gene introduced into the amplicon unit increasing its size about 10 %. |
| **p1658/97*orf*42::*cat*** | p1658/97 with the *orf42* fragment (92496..93640) replaced by *cat*. Size, 125,362bp | | | *cat* gene replacing *orf*42 within amplicon unit, decreasing its size about 1.3%. |
| **p1658/97O::*cat*** | p1658/97 with the fragment 95547..95594 replaced by *cat*. Size, 126,459bp | | | *cat* gene introduced outside of the amplicon, next to IS*26*. |
| **Plasmid used in in-trans mobility of the amplicon approach** | | | | |
| **pACYC184::IS*26*** | *bla*SHV-5-IS*26*-containing NcoI-NcoI fragment of p1658/97 with amplified units, cloned into pACYC184; *bla*SHV-5 removed by HpaI digestion and re-ligation. Size, 7,726bp | | | pACYC184 with the fragment of the amplicon unit with one IS*26*. |

**TABLE 2**. Primers used in PCR study.

|  |  |  |
| --- | --- | --- |
| Name | Sequences 5`- 3` | Remarks |
| chlL | ATCCGCTTATTATCACTTATTCAGG | pACYC184 *cat* gene cassette for cloning |
| chlP | GGTGTCCCTGTTGATACCGG |
| CmLXhNh | ATGGCTAGCCTCGAGATCCGCTTATTATCACTTATTCAGG | *cat* gene cassette for pRX (XhoI overhangs) |
| CmPXhNh | AGTGCTAGCCTCGAGGGTGTCCCTGTTGATACCGG |
| RecFDn | TGACTCCAGCGCCCCCGTCAGGGATGACGGCTTCAGTGTAGGCTGGAGCTGCTTCG | *cat* gene cassette for the p1658/97*orf*42::*cat* |
| RecFUp | CTGTTAAACCCTGCCCGAAAGGGGGCGTAAGAGGGTTATATGAATATCCTCCTTA |
| OutAmpUp | GTTAACCGGTAATTATCGGTATGATAGCTTTGAGTTTATATGAATATCCTCCTTA | *cat* gene cassette for the p1658/97O::*cat* |
| OutAmpDn | ATAGGTGATCCTTTTCTCAGGGTTGTAATGCTCATCGTGTAGGCTGGAGCTGCTTCG |
| DeltaF | CCCGCTCTGCATACTGAACAACAGCCTGGTGCATGGGTGTAGGCTGGAGCTGCTTCG | *cat* gene cassette for the p1658/97-FIBamp::*cat* and pXB-FIBamp::*cat* |
| Deltaamp | GAAGGCCATCGGTGCCGCATCGAACGGCCGGTTGCGGTATATGAATATCCTCCTTA |

**TABLE 3**. Primers used in qRT-PCR analysis.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene | Name | Sequence (5'-3') | Amplicon length [bp] | Coordinates of amplified sequence |
| Reference 1  *aac(3)-Ia* | pR1aF | AGATCTCACTACGCGCCTGC | 180 | 83027…83207 |
| pR1aR | TCGGTCGTGAGTTCGGAGAC |
| Reference 2  *aac(6)-Ib* | pR2aF | ACCCAATCGGCTCTCCATTC | 180 | 83687…83867 |
| pR2aR | CTGAGCATGACCTTGCGATG |
| Studied 1  *orf*37 | pA1aF | GCCGGGTTGTGCAAATAGAC | 180 | 87575…87755  (within the amplicon sequence) |
| pA1aR | TGGCAATCTGTCGCTGCTG |
| Studied 2  *orf*39 | pA2aF | GCGCGTCGAGCATCAATAG | 180 | 89637…89817  (within the amplicon sequence) |
| pA2aR | CAGTTGTGCTGCTGGTGGTC |