**New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*.
IV. The nucleotide sequence of pHY300PLK and some
properties in relation to transformation**

BY HIROMI ISHIWA and HARUE SHIBAHARA-SONE

*Yakult Central Institute for Microbiological Research,
1796 Yaho, Kunitachi, Tokyo 186*

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ABSTRACT

The whole nucleotide sequence of pHY300PLK (Ishiwa and Shibahara 1985a) has been determined. pHY300PLK consists of 4870 bp and contains an RNA primer for ori-177 and three open reading frames corresponding to Tc^R, Ap^R and Rep- α 1. Twenty-three unique restriction endonuclease recognition sites were found. We have confirmed that ORF α 1 of pHY300PLK is the plasmid replication gene. A rescue phenomenon has been found with respect to multiple infection by pHY300.2PLK (*rep*⁻) and pUB110. A series of experiments indicated that the oligomer form, pHY300.2PLK, was rescued by the monomeric form, pUB110. Additionally, some useful information for using pHY300PLK as a shuttle vector in molecular cloning studies is presented. (Key words: shuttle vector; *E. coli*; *B. subtilis*; tetracycline; ampicillin; kanamycin; rescue; MIC).

1. INTRODUCTION

In the hope of advancing the host-vector systems of *B. subtilis*, we undertook the construction of a new series of chimeric plasmids using the parental plasmids, pACYC177 of *E. coli* (Chang and Cohen 1978) and pAM α 1 of *Streptococcus faecalis* (Clewell *et al.* 1975; Ishiwa and Tsuchida 1984).

One of the smallest hybrids, designated pHY300PLK can replicate and express the Tc^R gene in both *E. coli* and *B. subtilis* (Ishiwa and Shibahara 1985a). pHY300PLK contains the Tc^R gene, the Ap^R gene, two replication origins, one for *E. coli* and one *B. subtilis*, and a polylinker. Due to a copy number mutation, large amounts of plasmid DNA can be collected from small volumes of *E. coli* culture. In an *E. coli* *rec*⁺ strain, the oligomer forms of the plasmid are produced, which are effective in transforming competent cells of *B. subtilis* (Canosi *et al.* 1978; de Vos *et al.* 1981).

In addition to analyzing the nucleotide sequence of the small plasmid pHY 163PLK (2.5 kbp), which is derived from pHY300PLK (Ishiwa and Shibahara

Abbreviations: Ap, ampicillin; Km, kanamycin; Tc, tetracycline; kbp, kilobase pairs; ORF, open reading frame; ori, origin of DNA replication; PLK, polylinker; ^R, resistance; ^S, sensitivity; MIC, minimum inhibitory concentration; ccc, covalently closed circular; oc, open circular; [], indicates plasmid-carrier state.

1985b), we recently completed determination of the nucleotide sequence of Rep- α 1, one of the replication origin regions of pAM α 1.

This paper concerns the construction of the whole nucleotide sequence of pHY300PLK. The nucleotide sequence of this shuttle vector for *E. coli* and *B. subtilis* consists of 4870 bp and has been found to contain an RNA primer for ori-177 and three open reading frames which correspond to Tc^R, Ap^R and Rep- α 1. Twenty-three unique recognition restriction endonuclease sites have been found in pHY300PLK.

2. MATERIALS AND METHODS

(a) *Bacterial strains and plasmids*

The *E. coli* K12 strain C600 F⁻ *thi-1 thr-1 pro leuB6 lacY1 tonA21 supE44 hsdR hsdM* λ^- (Maniatis *et al.* 1982) and the *B. subtilis* strain 1012 *hsrM1 leuA8 metB5* (Ikawa *et al.* 1981) were used as host cells for the shuttle vector pHY300PLK (Ishiwa and Shibahara, 1985a). The *recA1* gene was introduced into *E. coli* strain C600 by conjugation with *E. coli* strain RY107 Hfr *recA1* Δ (*gal-bio*) described by Miller (1972). *B. subtilis* strain 1012 and *E. coli* strain RY107 were kindly provided by H. Saito (University of Tokyo) and T. Sako (Yakult Central Institute), respectively.

(b) *Cell growth, preparation of plasmid DNA and transformation procedures of B. subtilis*

These procedures were conducted as described previously (Ishiwa and Shibahara, 1985a).

(c) *Enzymes*

Restriction endonucleases were purchased from Boehringer-Mannheim (FRG), Bethesda Research Laboratories (Maryland, USA), New England Biolabs Inc. (Massachusetts, USA), Takara Shuzo (Kyoto, Japan), Toyobo Co. Ltd., (Osaka, Japan) and Nippon gene (Toyama, Japan). Enzymatic reactions were carried out under the conditions recommended by the suppliers. T4 DNA ligase, T4 DNA polymerase, and *Xho*I linker (d(CCTCGAGG)) were from Takara Shuzo with the experimental conditions used being those described by Maniatis *et al.* (1982).

(d) *Nucleotide sequence determination*

The nucleotide sequence was determined by the dideoxy chain termination method (Sanger 1981) as previously described (Ishiwa and Shibahara 1985b).

(e) *Determination of MIC values*

(i) Overnight cultures grown in L-broth (Lennox, 1955) were diluted to 10^6 cells/ml, and 0.1 ml portions were inoculated into tubes containing 1-ml of the indicated media with serial tetracycline concentrations of the ranging from 0 to 200 μg per/ml. MIC values were determined as the lowest drug concentration that prevented visible growth after incubation at 37°C for 24 h.

(ii) Overnight cultures grown in L-broth were diluted to 10^6 cells/ml, and 10 μl portions were inoculated on the surface of L-agar plates containing serial concentrations of tetracycline ranging from 0 to 300 μg /ml. MIC values were determined as the lowest drug concentration preventing visible growth after incubation at 37°C for 24 h.

The MIC values determined on plates are useful for transformation experiments.

(f) *Mutagenesis of B. subtilis*

Because *B. subtilis* strain 1012 exhibits a high basal level of tetracycline resistance when grown on plates, this strain was not a suitable transformational host strain for *pHY300PLK* which encodes tetracycline resistance gene as the selective marker. Therefore, *B. subtilis* strain 1012 was mutagenized with introsoguanidine (Millar 1972) and two tetracycline sensitive clones, ISW1213 and ISW1214 were selected by means of the replica plating method among the 3000 colonies. The MIC value and the frequency of reversion to tetracycline resistance were determined for each clone.

The strain ISW1214 showed a higher sensitivity to Tc and a lower reversion frequency than ISW1213 (data not shown). The Tc^R gene in *pHY300PLK* was expressed well in *B. subtilis* ISW1214. The MIC value of *B. subtilis* ISW1214[*pHY300PLK*] was about 300 times higher than that of *B. subtilis* ISW1214 (Table 1 b).

Table 1. *Comparison of MIC values for Tc among four strains of B. subtilis. The dosages of Tc used in this experiment were 0, 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6, 12.5, 25, 50, 75, 100, 150, 200 and 300 μg /ml for both L-broth and L-plates*

Strain	Plasmid	Tc (μg /ml)	
		liquid culture ^{a)}	plate culture ^{b)}
1012	—	6	25
ISW1213	—	1.5	6
ISW1214	—	0.1	0.8
ISW1214	<i>pHY300PLK</i>	100	300

3. RESULTS

(a) *The nucleotide sequence of the replication function region in pHY300 PLK*: Restriction map analyses have indicated that pUB110, pBC16 and pAM α 141, each isolated from different gram-positive genera, have a common replication function region (Perkins and Youngman 1983). The whole nucleotide sequence of the pUB110 was presented by McKenzie *et al.* (1986). The region, derived from pAM α 1, having replication function in pHY300PLK was estimated to reside between the *Hae*III site (1668) and the *Acc*I site (3041) by means of deletion and insertional inactivation experiments (Ishiwa and Tsuchida 1984; Ishiwa and Shibahara 1985a, b). The nucleotide sequence in this region was determined by the dideoxy chain termination method using several restriction sites. The sequencing strategy is shown in Fig. 1. One large open reading frame (ORF α 1) was found. The nucleotide sequence is presented in Fig. 2. Surprisingly, comparison of the large ORF α of pUB110 and ORF α 1 of pHY300PLK showed that the two ORF's are identical without one base substitution. Predicted amino acid sequences are not added here because the protein α predicted from the ORF α of pUB110 has already been described and discussed by McKenzie *et al.* (1986).

(b) *The whole nucleotide sequence of pHY300PLK*: The whole nucleotide sequence of pHY300PLK (Fig. 2) was constructed by combining the sequences of the three components; the nucleotide sequences of the replication function region derived from pAM α 1 in pHY300PLK (Fig. 1), pHY163PLK which is a

Fig. 1. Sequencing strategy of the *ori- α 1* region and the predicted ORF. A restriction enzyme cleavage map of the replication origin region derived from pAM α 1. The DNA fragments which we cloned into the sequencing vectors M13mp10, or mp11, and their sequences were determined using the dideoxy procedure. Arrows above a clone represent sequences determined from the coding strand. The scale at the top shows the number of base pairs, with position 1 being assigned to the first base pair of the *Hind*III recognition site in the polylinker. Below the restriction map, the *ori- α 1* region in the open box was sequenced in this experiment.

derivative plasmid of pHY300PLK (Ishiwa and Shibahara 1985b), and the Ap^R gene from pBR322 (Sutcliffe 1978). The Ap^R gene of pBR322 and pACYC177 originates from Tn3 (Chang and Cohen 1978; Sutcliffe 1978). The physical and genetic map of pHY300PLK is presented in Fig. 3. With the exception of *Cla*I, most of the tests for restriction enzyme susceptibility were carried out using the plasmid DNA extracted from *E. coli*. In the case of *Cla*I, DNA extracted from *B. subtilis* is sensitive to *Cla*I digestion while DNA extracted from *E. coli* K12 strain C600 is not. It is possible that the adenine residue in GATC portion of the ATCGATC, *Cla*I recognition site in pHY300PLK is methylated by the *dam* methylase of *E. coli* (Geier and Modrich 1979). In summary, twenty-three unique restriction endonuclease sites have been located on pHY300PLK. Their recognition sequences and locations at the nucleotide sequence level are illustrated in Fig. 2 and described in Table 2.

(c) *Analysis of the ORF in the replication function region*: The physical mapping of pHY300PLK showed that the *Nco*I² site was located inside of the ORF α 1 region. The *Nco*I² site (2583 in Fig. 2) in the ORF α 1 was interrupted by insertion of either one or two *Xho*I-linkers as described previously (Ishiwa and Shibahara 1985b), to produce pHY300.1PLK and pHY300.2PLK, respectively. The insertion of two *Xho*I-linkers in pHY300.2PLK created a new *Hae*III site in pHY300.2PLK. These plasmids were subsequently amplified in *E. coli* and tested for their ability to transform *B. subtilis* to Tc^R. However, no transformants were obtained in either case. These results strongly suggest that ORF α 1 is an essential region for plasmid replication in *B. subtilis* cells. Insertion of the *Xho*I-linkers should result in a frameshift which would lead to a shortening of ORF α 1, or should result in an insertion of several amino acids.

A similar experiment was carried out using the *Nsi*I site in the ORF α 1 of pHY300PLK. At the *Nsi*I site, the ORF α 1 was interrupted by insertion of a short fragment of λ DNA obtained by digestion with *Nsi*I, producing two new plasmids designated pHY300.8PLK and pHY300.9PLK. Both of these plasmids can replicate normally in *E. coli* but not at all in *B. subtilis*. Therefore, in accord with McKenzie *et al.* (1986), we also conclude that the ORF α 1 of pHY300PLK may be the plasmid replication gene (*rep*) in *B. subtilis*.

(d) *Molecular structure and transformation efficiency*: The transformation efficiency of plasmid DNA for *B. subtilis* strongly depends on the molecular structure of the DNA (Canosi *et al.* 1978). The relationship between the molecular structure and transformation efficiency of pHY300PLK was studied using *E. coli* and *B. subtilis* as recipients. It was not difficult to obtain various molecular structures of pHY300PLK since amplification of pHY300PLK in an *E. coli* *rec*⁺ strain results in the production of both monomer and oli-

Fig. 2(A)

gomer form cccDNA. Besides, it is highly yielded due to a copy-number mutation (Ishiwa and Shibahara 1985a, b).

To separate monomeric and oligomeric forms, the plasmid pHY300PLK DNA extracted from *E. coli* C600 *recA1* was purified first by CsCl-ethidium bromide equilibrium density gradient centrifugation, followed by sucrose

Fig. 2(B)

Fig. 2. Nucleotide sequence of pHY300PLK. Three ORFs, $\alpha 1$, Ap^R , and Tc^R , and the RNA primer for ori-177 are shown. The four functional regions are surrounded by a line. The amino terminal and carboxy terminal ends are indicated at the start and the end, respectively. The start codons of the ORFs are predicted only on the basis of the start codon resulting in the largest ORF. However, it is unlikely that the ATG at 2687 is the start codon for the ORF $\alpha 1$ for reasons described by McKenzie *et al.* 1986. The GTG at position 2618 appears more likely to be the starting codon. SD indicates a potential Shine-Dalgarno sequence (Shine and Dalgarno 1975). The sequence from 3532 (*HincII*) to 4123 (*BanI*) presented in this figure has been determined by Sutcliffe (1978). About half of the present nucleotide sequence has been previously reported as pHY163PLK (Ishiwa and Shibahara 1985b).

density gradient centrifugation of the cccDNA fraction. The monomer and oligomer forms were separated by fractionation of the sucrose gradient and identified by agarose gel electrophoresis (Fig. 4A). A portion of every other fraction was diluted and used to transform either *E. coli* or *B. subtilis* to Tc^R . The following results were observed. Monomeric pHY300PLK exhibits a high

Table 2. *Unique restriction enzyme recognition sites in pHY300PLK. Twenty-three unique restriction enzyme recognition sites in pHY300PLK are listed. The fourth column indicates the site of the first nucleotide of the recognition sequence. (see Fig. 2 and 3)*

Enzyme	Sequence	Site	Enzyme	Sequence	Site
1. <i>Hind</i> III	AAGCTT	1	13. <i>Bal</i> I	TGGCCA	1178
2. <i>Xba</i> I	TCTAGA	6	14. <i>Hpa</i> I	GTTAAC	1295
3. <i>Bgl</i> II	AGATCT	11	15. <i>Nsi</i> I	ATGCAT	2133
4. <i>Sal</i> I	GTCGAC	21	16. <i>Alf</i> II	CTTAAG	3094
5. <i>Bam</i> HI	GGATCC	27	17. <i>Aat</i> II	GACGTC	3153
6. <i>Ava</i> I	CCCGGG	32	18. <i>Apa</i> LI	GTGCAC	3404
7. <i>Sma</i> I	CCCGGG	32	19. <i>Pvu</i> I	CGATCG	3704
8. <i>Eco</i> RI	GAATTC	37	20. <i>Mst</i> I	TGCGCA	3851
9. <i>Bst</i> EII	GGTAACC	136	21. <i>Bgl</i> I	GCCCTTCGGC	3952
10. <i>Eco</i> RV	GATATC	874	22. <i>Ban</i> I	GGTGCC	4123
11. <i>Cla</i> I	ATCGAT	1037	23. <i>Nsp</i> V	TTCGAA	4383
12. <i>Bss</i> HII	GCGCGC	1087			

frequency of transformation in *E. coli* but shows only very low frequency transformation in *B. subtilis*. In contrast, the oligomeric form has a high transformation frequency in *B. subtilis* but only a low frequency in *E. coli* (Fig. 4 A, B).

Although pHY300.2PLK DNA itself was incapable of transforming *B. subtilis* to Tc^R, as described in section (c), it could transform *B. subtilis* if the cells were co-transformed with the monomer form of pHY300PLK. For instance, when the oligomer form pHY300.2PLK (*rep*⁻) and monomer pHY300PLK were applied together for transformation of *B. subtilis* then 50 times more transformants were obtained than when pHY300PLK monomer was used alone. This suggests that both plasmids could be introduced into the competent cells and that a replicon carrying the Tc^R gene was formed through interaction between the two plasmids.

(e) *Further studies of rescue phenomena with respect to multiple infection by pHY300.2PLK and pUB110.* To study the above rescue phenomena in more detail *B. subtilis* cells were co-transformed with pUB110 (Km^R) monomer DNA and a mixture of monomer and oligomer form of pHY300.2PLK (Tc^R). To characterize more clearly the *B. subtilis* transformants obtained by co-transformation, the transformants were selected on three different plates: Tc(20 µg/ml), Km(20 µg/ml) and Tc(20 µg/ml) + Km(20 µg/ml).

No transformants were obtained on Tc+Km plates. Therefore, complementation did not occur between the two replicons, nor was a cointegrate

Fig. 3. Physical and genetic map of pHY300PLK. The locations of the three ORFs and their coding directions are shown. Key restriction enzyme sites are indicated. Position numbers correspond to those of Fig. 1 and Fig. 2. Position 1 indicates the first base of the *Hind*III recognition sequence in the polylinker. The RNA primer for ori-177 in pHY300PLK corresponds to p15A. Each of the replication origins, ori-177 derived from pACYC177 (Selzer *et al.* 1983), and ori- α 1 derived from pAM α 1 (Scheer-Abramowitz *et al.* 1981) is also indicated with open arrows, respectively.

chimeric plasmid produced by recombination. Also, no transformants were observed on the Km plate. This means that monomer pUB110 was not rescued by pHY300.2PLK. Why the pUB110-Km^R gene was not recovered in the present series of experiments is not known. Transformants were, however, obtained on the plates supplemented with Tc. To determine the basis for recovery of Tc^R transformants, further investigation was conducted as follows.

(B)
Fig. 4

(B)
Fig. 5

Fig. 4. Sucrose gradient analysis of pHY300PLK cccDNA amplified in an *E. coli* C600 *recA1* host. (A) Neutral linear sucrose gradients from 20% to 5% in SSC buffer (total volume: 12 ml) were prepared in Hitachi SW40T tubes. 0.5 ml of DNA solution (c.a. 500 μ g DNA/ml) were layered on top of the gradients. The tubes were run of 16 h in a Hitachi SW40T rotor at 25,000 rpm at 15°C. 10 drop fractions were collected from the bottom. Every fractions was assayed by agarose gel electrophoresis. Agarose gel (0.7%) electrophoresis was run on 3 μ l of each fraction. (B) The biological activities of the fractionated DNAs were determined using *B. subtilis* ISW1214 and *E. coli* C600 as host strains. In the case of *B. subtilis*, 0.5 μ l of each fraction was diluted (1/100) and added to 50 μ l of competent cells. After a 30 min adsorption period, 100 μ l of L-broth was added to the adsorption mixture, followed by further incubation for 60 min to express the Tc^R gene. The transformation procedure for *E. coli* was similar to that for *B. subtilis* except that adsorption was carried out at 0°C for 10 min and 900 μ l of L-broth added instead of 100 μ l to allow for gene expression. Only small portions were plated to determine transformant cells. Open circles indicate Tc^R transformants of *B. subtilis*; closed circles indicate Tc^R transformants of *E. coli*.

Fig. 5. Sucrose gradient analysis of pHY300.2PLK cccDNA amplified in *E. coli* C600 *rec+* and plasmids rescued by co-transformation of fractionated pHY300.2PLK and the monomer form of pUB110 DNA. (A) Every fraction (3 μ l) contained dimeric form DNA (2xccc), with some fractions also containing tetramer (4xccc), trimer (not indicated) and monomer form (1xccc) as minor components. (B) The replication deficient plasmid dHY300.2PLK in *B. subtilis* was rescued by the monomer form of pUB110 DNA. The transformation procedure for *B. subtilis* was the same as that in Fig. 4(B) except that 1 μ l of monomer form pUB110 DNA was added to the adsorption tubes. The Tc^R transformants were determined as previously described.

Monomeric and oligomeric forms of *pHY300.2PLK* cccDNAs amplified in *E. coli* C600 *rec*⁺ strain were separated by sucrose density gradient centrifugation (Fig. 5A), and then a part of DNA in every other fraction was used with *pUB110* monomer DNA to transform *B. subtilis*. Tc^R transformants were obtained only for fractions containing the oligomeric form, especially the tetramer form of *pHY300.2PLK*, but not the fraction containing the dimeric form. However, the DNA concentration was lower in the tetramer fractions than in the dimer fractions (see the fraction 8–12 in Fig. 5A).

The plasmid DNAs were reisolated from twelve colonies randomly chosen from the several hundred Tc^R transformants obtained above. All of the plasmids showed a molecular size of about 5 kb which was similar to the size of the *pHY300.2PLK* monomeric molecule. Furthermore, these plasmids retained the ability to transform *E. coli* to Tc^R Ap^R and gained the ability to transform *B. subtilis* to Tc^R by themselves. By examination of the structure of the rescued plasmids, we found that all twelve plasmids had lost the *Xho*I site and regained the *Nco*I² site. A series of these experiments indicate that the tetramer DNA form (possibly the trimer, too) of *pHY300.2PLK* (*rep*⁻) was rescued by monomer form *pUB110* (*rep*⁺) (see fraction 4 in Fig. 5A and B). It is likely that the Tc^R-plasmid in transformants is produced by some kind of recombination in the homologous regions of the two replicons.

4. DISCUSSION

Comparison of the flanking regions of the drug resistance genes of pAM α 1 and pUB110: According to Perkins and Youngman (1983), the restriction maps of the two plasmids, *pAM α 1* and *pUB110* are superimposable over all areas except for the regions encoding the heterologous drug resistance genes. Therefore we became interested in the flanking regions of the drug resistance genes in the two replicons, *pUB110* and *pAM α 1*. Then, sequenced approximately 100 bp of the flanking region left of the Tc^R gene in *pAM α 1* (Fig. 6) which exists in *pAM α 1* but not in *pHY300PLK* (Ishiwa and Shibahara 1985a).

We compared the flanking regions of the drug resistance genes present in *pUB110* and *pAM α 1*. The relationship is illustrated in Fig. 6, where the short arrows represent 4 base pairs of direct repeats (GCCC) found in each replicon. Outside of these direct repeats, completely homologous sequences were found between the two replicons as indicated by the *. The homology of the two replicons indicates that these plasmids were established by the translocation of DNA fragments carrying the drug resistance genes from some other replicon into a common plasmid by independent events. However, the origins of the Km^R gene or the Tc^R gene are not yet known. From comparison of the two DNA sequences, we tentatively assume that the translocated region in *pAM α 1* is 1653 bp long and includes the ORF Tc^R between the direct re-

Fig. 6. Alignment of the flanking regions of the two drug resistance genes, each present in a different replicon. The flanking regions of the Tc^R gene carried by $pAM\alpha 1$ and the Km^R gene carried by $pUB110$ were aligned to identify common nucleotide sequences between the two replicons. Identical bases are marked with an *. The upper portion represents left side alignment, and the lower portion represents right side alignment of the flanking regions of the drug resistance genes. Two sets of direct repeats were discovered in each replicon and boxed. Long arrows under the sequences indicate the terminal inverted repeats found by computer analysis but the homologies of the left and right sides are weak with 24/52 in $pAM\alpha 1$ and 26/60 in $pUB110$ being identical. Only the black parts of the long arrows under the sequences indicate homologous sequences.

Fig. 7. A presumptive primary-plasmid including the "target sequence". The drug resistance genes and their flanking regions are removed from the two replicons.

peats, and that in $pUB110$ 1568 bp long including the ORF γ and the ORF Km^R (Fig. 6).

By inspection of the translocated region in $pAM\alpha 1$, inverted terminal repeats were found at both ends of this region although the homology was weak (23/52) (Fig. 6). However, in this region, one large ORF Tc^R was found.

In *Streptococcus faecalis*, two transposons, $Tn916$ (Tc^R) and $Tn917$ (Em^R)

have been reported (Franke and Clewell 1981; Shaw and Clewell 1985). Tn916 (Tc^R) is a transposon found on the chromosome of *Streptococcus faecalis*, but the Tc^R gene located within Tn916 does not hybridize with the Tc^R gene carried by pAM α 1 (Smith *et al.* 1981; Burdett *et al.* 1982). However, final conclusions as to the relationship of the two Tc^R genes should await completion of the nucleotide sequence analysis of Tn916. The possibility that Tn916 served as a donor of DNA to pAM α 1 has not been rejected completely yet. Unfortunately, the nucleotide sequence of Tn916 is not available at present.

Most transposable elements in procaryotes require specific DNA sequences as targets (Shapiro 1983). If the translocated regions are assumed to be transposons, the four base pair direct repeats observed in both pUB110 and pAM α 1 may correspond to the target sequences of the plasmid (Fig. 6 and Fig. 7).

The sequence structure as shown in Fig. 6 suggests that this supposedly translocated region in pAM α 1 might be a kind of transposon, but up to this point, we could not find any further conclusive evidences to support this possibility.

Using several cryptic plasmids found in Group D streptococci (Ishiwa unpubl.), we are planning experiments to find primary-plasmids in *Streptococci* spp.

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