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New Cryptic Plasmid of *Bacillus subtilis* and Restriction Analysis of Other Plasmids Found by General Screening

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A new cryptic plasmid, pTA1030 (4.5 megadaltons, copy number 16), was characterized by restriction analysis, together with some other plasmids of *Bacillus subtilis*.

Since Cohen et al. (4) succeeded in constructing biologically functional recombinant plasmids in vitro, bacterial host-vector systems have been developed mainly with *Escherichia coli*. In contrast, there have been only a few reports on useful vector plasmids in *Bacillus subtilis* (5, 8, 14, 27). Most of the plasmids originated in *Staphylococcus* (5, 8, 14), except for pLS102, derived from a cryptic plasmid of *B. subtilis* (27). As for the plasmids of *Bacillus* species, several plasmids have been reported in *B. pumilus* (17-19, 21), *B. megaterium* (11), *B. subtilis* (2, 16, 25, 26), and *B. cereus* (2). However, the biological functions of most plasmids have not been determined, except pPL10 and pPL7065 which determine bacteriocin production in *B. pumilus* (20, 21), and pBC7 and pBC16 which determine bacteriocin production and tetracycline resistance, respectively, in *B. cereus* (2).

To find useful vectors for *B. subtilis*, we screened all our laboratory collections of *B. subtilis* strains for the presence of plasmids. Sixty-seven strains were cultivated, each in 100 ml of nutrient broth with shaking at 37°C. Late-logarithmic-phase cells were collected and lysed with lysozyme and sodium dodecyl sulfate, and cleared lysates were obtained according to the method of Guerry et al. (9) with slight modifications. DNAs in the cleared lysate were precipitated by ethanol and subjected to electrophoresis on 0.7% agarose gels according to Sharp et al. (23). Twenty strains among 67 were found to harbor plasmids, but none of those strains showed clear drug resistances or bacteriocin production.

Plasmids were prepared from 1-liter cultures of those 20 strains, by precipitation of plasmid DNA in the cleared lysate with 10% polyethylene glycol and centrifugation in cesium chloride-ethidium bromide, according to Humphreys et al. (13). Plasmids were cleaved with restriction enzymes *Eco*RI (purified according to Tanaka and Weisblum [28]), *Bam*HI (Boehringer Mannheim), and *Hin*dIII (Miles Laboratory and Boehringer Mannheim), under the reaction conditions of Gryczan et al. (7). Molecular weights of restriction fragments were determined by electrophoresis on 1.25% agarose gels, using *Eco*RI fragments (10) and *Hin*dIII fragments (30) of phage λ DNA as standards of molecular weight. Twenty plasmids were classified into six classes according to their molecular weights and restriction patterns (Table 1). Plasmids in each class showed homogeneity, manifesting the same molecular weight of each corresponding fragment. Some of the plasmids in five classes (1 to 5) have been reported by Tanaka et al. (25, 26). However, some strains were newly found to contain class 1 plasmids pTA1015 (IAM1028), pTA1012 (IFO3021), pTA1014 (IFO 3336), pTA1016 (IAM 1071), and pTA1019 (IAM 1230) and class 2 plasmids pTA1020 (IAM 1076) and pTA1023 (IAM 1246).

Class 6 plasmids pTA1030 (IAM 1113) and pTA1031 (IAM 1075) were new plasmids. These plasmids were cleaved by *Eco*RI at four sites (Fig. 1a) and by *Hin*dIII at two sites, and they had no cleavage site by *Bam*HI. Molecular weights of corresponding restriction fragments were exactly the same between two plasmids. The molecular weights of pTA1030 and pTA1031 were estimated to be 4.48×10^6 from the sum of the molecular weights of restriction fragments. The molecular weight of pTA1030 was determined from the contour length under electron microscopy (15) to be $4.55 \pm 0.26 \times 10^6$, as compared to pSC101 (5.8×10^6) (3). *B. subtilis* IAM 1113 harboring pTA1030 was cultured in the presence of [*methyl*-^{3}H]thymidine, and labeled DNAs in cell lysate, prepared by successive actions of lysozyme, RNase A, pronase, and sodium *N*-lauroyl sarcosinate (26), were subjected to cesium chloride-ethidium bromide centrifugation. A satellite peak of covalently closed circular plasmid DNA, distinct from a large chromosomal DNA peak, was observed. The amount of covalently closed circular plasmid DNA was found to be 3.7% of chromosomal DNA, from radioactivities of both peaks. A minimal copy number of pTA1030 was estimated to

TABLE 1. *Classification of B. subtilis plasmids detected in this study*

Class[a]	Plasmid[b]	Host strain	Mol wt[c] (10^6)	No. of cleavage sites		Mol wt of cleavage fragments[d] (10^6)
1	pTA1015	IAM 1028	3.55	*Eco*RI	1	3.52
				*Bam*HI	2	2.50, 1.04
				*Hin*dIII	4	2.21, 0.66, 0.42, 0.29
2	pTA1020	IAM 1076	4.04	*Eco*RI	2	2.27, 1.75
				*Bam*HI	1	4.05
				*Hin*dIII	5	1.37, 1.04, 0.92, 0.42, 0.29
3	(pTA1040) pLS13	IAM 1232	4.80	*Eco*RI	4	2.32, 1.23, 0.99, 0.28
				*Bam*HI	0	
				*Hin*dIII	3	2.00, 1.72, 1.05
4	pTA1050	IAM 1261	5.13	*Eco*RI	3	2.25, 1.65, 1.25
				*Bam*HI	0	
				*Hin*dIII	5	1.66, 1.60, 0.94, 0.62, 0.29
5	pTA1060	IFO 3022	5.28	*Eco*RI	1	5.24,
				*Bam*HI	1	5.34
				*Hin*dIII	6	1.42, 1.37, 1.22, 0.64, 0.43, 0.17
6	pTA1030	IAM 1113	4.48	*Eco*RI	4	2.11, 1.05, 1.00, 0.29
				*Bam*HI	0	
				*Hin*dIII	2	2.55, 1.96

[a] The following plasmids also belong to each class and show the same molecular weights and cleavage patterns by the three restriction enzymes (host strains are indicated in parentheses): class 1—pTA1012 (IFO 3021), pTA1014 (IFO 3336), pTA1016 (IAM 1071), pTA1019 (IAM 1230), (pTA1010) (IFO 3009) = pLS15, (pTA1011) (IFO 3013) = pLS17, (pTA1013) (IFO 3335) = pLS19, (pTA1017) (IAM 1143) = pLS24, (pTA1018) (IAM 1207) = pLS26; class 2—pTA1023 (IAM 1246), (pTA1021) (IAM 1114) = pLS28, (pTA1022) (IAM 1168) = pLS30; class 5—(pTA1061) (IFO 3215) = pLS12; class 6—pTA1031 (IAM 1075).

[b] Plasmids that are identical with pLS plasmids reported by Tanaka et al. (25, 26) are designated in parentheses. (See also footnote *a*.)

[c] Mean value of the sum of the molecular weights of restriction fragments.

[d] Molecular weights were estimated by agarose gel electrophoresis, using *Eco*RI fragments (10) (13.7×10^6, 4.67×10^6, 3.71×10^6, 3.57×10^6, 3.04×10^6, and 2.11×10^6) and *Hin*dIII fragments (30) (14.6×10^6, 5.84×10^6, 4.05×10^6, 2.67×10^6, 1.40×10^6, 1.21×10^6, and 0.34×10^6) of phage λ DNA as the standards.

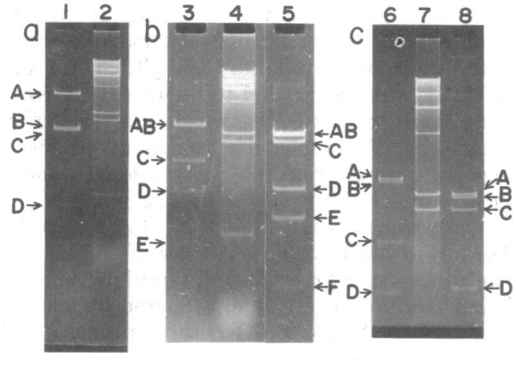

FIG. 1. *Agarose gel electrophoresis of restriction fragments of some plasmids. Electrophoresis was carried out on 1.25% gels at 60 V for 3 h (a and b) and at 36 V for 11 h (c). (1) EcoRI fragments of pTA1030; (3 and 6) HindIII fragments of pTA1050; (5 and 8) HindIII fragments of pTA1060; (2, 4, and 7) HindIII fragments of λ phage DNA.*

be 16 copies per chromosome, assuming that the molecular weight of the *B. subtilis* chromosome is 2×10^9. The ^{3}H-labeled pTA1030 was found to sediment with the same velocity as ^{14}H-labeled ColE1 DNA (molecular weight, 4.2×10^6 [1]) in neutral 5 to 20% linear sucrose density gradient centrifugation.

Class 4 plasmid pTA1050 and class 5 plasmid pTA1060 showed restriction patterns very similar to those of pLS14 (IAM 1261) and pLS11 (IFO 3022), respectively, except that the numbers of *Hin*dIII fragments of our plasmids were 5 and 6, respectively, whereas those of pLS plasmids were reported to be 4 and 5, respectively (26). The discrepancy seems to arise from the fact that two pairs of near bands, A (1.66×10^6) and B (1.60×10^6) of pTA1050 and A (1.42×10^6) and B (1.37×10^6) of pTA1060, often appear as single bands and can be resolved only after prolonged electrophoresis (Fig. 1b and c).

The IAM strains used in the present work

were obtained from the Institute of Applied Microbiology, The University of Tokyo, more than 20 years ago, and IFO strains were obtained from Institute of Fermentation, Osaka, in 1965 and 1969. Since then, these strains have been maintained as culture collections of our laboratory by periodical cultivation on nutrient agar slants, twice per year, and storing at 12°C. Thus it seems that the present plasmids are inherited very stably in the host strains.

In an attempt to attach a genetic marker to one of the cryptic plasmids, we constructed a composite plasmid pTA2010 (12) between *E. coli* plasmid RSF2124 (ampicillin resistance and colicin E1 productivity) and pTA1060 of *B. subtilis*. (The plasmid prefix of our laboratory was changed from pAT to pTA according to the assignment by the Plasmid Reference Center.) The composite pTA2010 cloned in *E. coli* conferred ampicillin resistance to the host (12), but it could not be introduced into a restrictionless and highly transformable mutant strain RM125 of *B. subtilis* 168 (29), in spite of many trials to get an ampicillin-resistant transformant (S. Horinouchi, T. Uozumi, and T. Beppu, unpublished data). This failure is not unexpected, because *E. coli* genes such as the ampicillin resistance gene of pBR322 (5) so far tested have been reported not to be expressed in *B. subtilis*, although some genes of *B. subtilis* can be expressed in *E. coli* (6, 22). To find a useful vector that originates in *B. subtilis*, more rigorous screening of *B. subtilis* strains, especially from natural sources, seems to be necessary.

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