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Cloning and Characterization of *Streptococcus mutans* LM7 Plasmid pAM7

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The 5.6-kilobase-pair cryptic plasmid, pAM7, of *Streptococcus mutans* LM7 was cloned into *Escherichia coli* plasmids or a shuttle plasmid to examine whether the plasmid encodes bacteriocin. Plasmid pAM7 encoded proteins with molecular weights of 30,000, 22,000, and 12,000, but none of them appeared to be bacteriocin.

Streptococcus mutans serotype c and e strains infrequently carry a small cryptic plasmid (5.6 kilobase pairs [kbp]) (13). Hansen et al. (9) characterized cryptic plasmid pVA318 of *S. mutans* V318 (serotype c/f) by cloning into *Escherichia coli*. This chimera was found to produce a pVA318-specific protein of approximately 20,000 molecular weight, having no bacteriocin activity against *S. mutans* or group A *Streptococcus* indicator strains (9). However, Caufield et al. (4, 5) found that the presence of 5.6-kbp plasmids was correlated with bacteriocin activity against *S. mutans* (mutacin). They classified the plasmid-positive strains into two groups (I and II) on the basis of bacteriocin profiles and restriction enzyme digests (4). For instance, UA140 and V318 strains belong to groups I and II, respectively (4). The 5.6-kbp cryptic plasmid in *S. mutans* LM7 (serotype e), first described by Dunny et al. (7), was designated pAM7 and classified in group I′ because, according to Caufield et al. (4, 5), the restriction digests of pLM7 differ slightly from those of the other plasmid-containing strains in group I. In the present study, we cloned and characterized plasmid pAM7 to determine its role in specifying bacteriocin activity. Strain LM7 is known to produce bacteriocins against most strains of *S. mutans* (8).

We will first describe the construction of the three chimeric plasmids with *E. coli* vector plasmids and pAM7. *S. mutans* LM7 was from the strain stock of Tokyo Dental College (Chiba, Japan). The cells were grown in Berman broth (2). Plasmid pAM7, purified as described previously (9), was linearized with *Hin*dIII, *Eco*RI, or *Pst*I as previously described (9). Each digested plasmid was ligated with a *Hin*dIII or *Pst*I digest of pBR322 or an *Eco*RI digest of pACYC184. After *E. coli* HB101 cells were transformed with the ligation mixture, strains carrying presumed plasmids were identified by their antibiotic resistance phenotypes. The desired plasmid DNAs were purified from representative transformants and designated pDS3, pTK4, and pST8 for *Hin*dIII, *Eco*RI, and *Pst*I insertions of pAM7, respectively. It is interesting that the pAM7 fragment of the chimeric plasmids from *E. coli* was digested in two (1.4 and 4.2 kbp) sites with *Eco*RI, although pAM7 from *S. mutans* was digested at a single site by *Eco*RI. The results indicate the appearance of an additional *Eco*RI site in the pAM7 fragment from transformed *E. coli*.

To transform *S. mutans* by a chimeric plasmid containing pAM7, we constructed a shuttle plasmid designated pNS83 (Fig. 1). A shuttle vector pVA856 (12) was kindly supplied by F. L. Macrina. Purified plasmid pVA856 was digested with *Hin*dIII, ligated, and transformed into *E. coli* HB101 to obtain a large fragment of *Hin*dIII-digested pVA856, designated pNM1. Plasmid pNM1 comprises an erythromycin resistance (Em^r) gene originally isolated from a group D streptococcal strain (12) and the chloramphenicol resistance (Cm^r) gene and replication region of *E. coli* plasmid pACYC184. pAM7 was digested with *Eco*RI and ligated with an *Eco*RI digest of pNM1. The transformants of *E. coli* HB101 were identified by Em^r and Cm^s phenotypes. An additional *Eco*RI site was also found in pNS83 from *E. coli* but not in that from *S. mutans* LM7. An *Eco*RI site on this plasmid could be inferred to be methylated in *S. mutans* but not in *E. coli*, accounting for this observation.

To confirm the incorporation of pAM7 into pNS83, the plasmid-coded proteins were translated in vitro by using a cell-free coupled transcription-translation system derived from *E. coli* (Amersham DNA-directed translation kit). The results are shown in Fig. 2. No significant proteins were observed (Fig. 2, lane a) in the absence of plasmid as a negative control. With plasmid pACYC184 used as a positive control, on the other hand, several protein bands containing [^{35}S]methionine were observed (Fig. 2, lane b). On the basis of molecular weight estimations, the main band (26,000) was a product of the chloramphenicol resistance gene (chloramphenicol acetyltransferase). The product of the tetracycline resistance gene (molecular weight, 34,000) (3) was obscure. The remaining minor proteins (less than 20,000 molecular weight) may be due to incomplete translation or transcription of chloramphenicol acetyltransferase gene (3). The result for the gene products of pACYC184 is consistent with that obtained with maxicells (3). When pAM7 from *S. mutans* was used, at least three major proteins (molecular weights, 30,000, 22,000, and 12,000) were observed (Fig. 2, lane d). These proteins were all identified with the pNS83-coded proteins (Fig. 2, lane c), except for an extra protein (molecular weight, 29,000) which should be the product of the erythromycin resistance gene. Thus, it was concluded that pNS83 encodes the pAM7-coded proteins and is also able to be expressed in *E. coli*. The coding region of pAM7 is estimated to be about 2 to 3 kbp (including minor proteins), which corresponds to 36 to 54% of the total length of the plasmid. Since the plasmid is composed of a replication origin, promoters, and uncoding regions, in addition to the coding region, the coding region is roughly as small as one-half of the whole plasmid. Thus, most of the pAM7-

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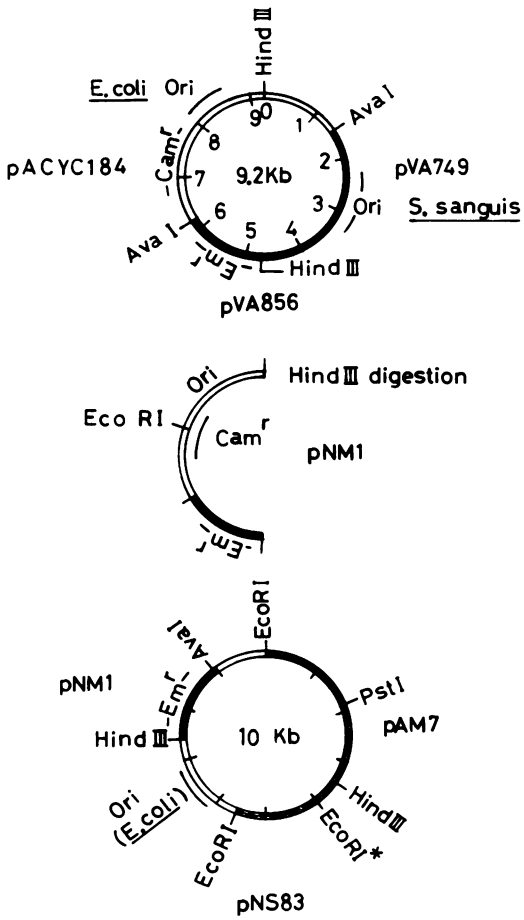

FIG. 1. Construction of shuttle plasmid pNS83. Plasmid pVA856 comprises *E. coli* plasmid pACYC184 and *S. sanguis* plasmid pVA749 (12). pVA856 was digested with *Hin*dIII, and the fragment carrying Em^r and Cm^r was circularized by ligation. The resultant plasmid was designated pNM1. pNM1 was digested with *Eco*RI and ligated into the *Eco*RI site of pAM7. The shuttle plasmid was designated pNS83. The open, thick, and dotted lines in the plasmids are regions derived from pACYC184, pVA749, and pAM7, respectively. In pNS83 isolated from *E. coli* HB101, the site indicated by *Eco*RI* was digested by *Eco*RI. Ori, Origin of replication; Kb, kilobases.

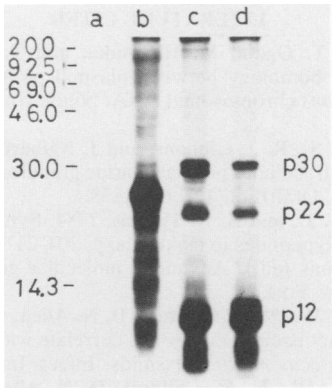

FIG. 2. Fluorograph, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, of [^{35}S]methionine-labeled proteins produced in a cell-free coupled transcription-translation system derived from *E. coli* (Amersham DNA-directed translation kit) by pAM7, pNS83, and pACYC184. Samples were run on 15% polyacrylamide slab gels, and the radiolabeled protein bands were detected by fluorography as described by Laemmli (11). Lanes: a, control; b, pACYC184; c, pNS83; d, pAM7. The positions and sizes (in thousands) of the molecular weight markers used are indicated on the left.

coding proteins are considered to be expressed in the transcription-translation system.

The bacteriocin activity from *S. mutans* or *E. coli* was examined by a drop or stab assay method described by Hamada and Ooshima (8). To extract the cellular contents of the transformants, cells were grown to stationary phase and harvested by centrifugation. The cells were concentrated 10 times and then disrupted in a French pressure cell. The resultant solutions were subjected to a drop assay for mutacin. A partially purified mutacin from a liquid culture of strain LM7 was used as a positive control for the drop assay. The indicator strains used were *S. mutans* HS6 (serotype a), BHT (b), GS5 (c), OMZ176 (d), OMZ175 (f), 6715 (g), *Streptococcus salivarius* IFO13956, and *Streptococcus pyogenes* Sv, which are all sensitive to the LM7 bacteriocins. These strains were kindly supplied from the culture collection of the Department of Microbiology, Okayama University Dental School.

The *E. coli* HB101 cells transformed with the three chimeric plasmids containing the pLM7 plasmid moiety (pDS3, pTK4, and pST8) exhibited no bacteriocin activity against any indicator strains aforementioned by either the drop or the stab assay method. When *S. mutans* LM7 was transformed with pNS83, as described by Kuramitsu and Long (10), and screened by Em^r phenotype, the transformants lost their resident pLM7 but still retained the bacteriocin activity. Although we attempted to cure pNS83 from strain LM7 by treating it with ethidium bromide or sodium dodecyl sulfate, none of them exhibited the Em^s phenotype. On the other hand, when strain GS5 was transformed with pNS83, the transformants exhibited no bacteriocin activity against any of the aforementioned indicator strains. The transformants were confirmed to contain pNS83 in intact form (three *Eco*RI sites) with no deletion. Thus, it is most likely that plasmid pAM7 encodes no bacteriocin.

Recently, Murchison et al. (14) demonstrated that when some strains of *S. mutans* were transformed with a shuttle plasmid, pYA629, which comprises plasmid pVA318 (classified under group II [4, 5]), all plasmid-containing strains except LM7 lost their resident cryptic plasmids. The results with pNS83 and those with pYA629 are consistent with one another. Curtiss (6) reported that loss or substitution of the cryptic plasmid by introduction of a shuttle plasmid, pYA629, does not change the type of bacteriocin activity expressed. Abiko et al. (1) found that a chromosomal DNA sequence from *S. mutans* AHT, GS5, LM7, and OMZ175 has homology with the DNA sequence of plasmid pVA318, although strains AHT and OMZ175 produced no bacteriocin in the present experimental conditions. Thus, we conclude that the 5.6-kbp cryptic plasmid encodes no bacteriocin activity.

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