APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1989, p. 1653–1655
0099-2240/89/061653-03$02.00/0

Vol. 55, No. 6

Complete Nucleotide Sequence and Characterization of a Cryptic Plasmid from *Lactobacillus helveticus* subsp. *jugurti*

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Received 18 October 1988/Accepted 28 February 1989

A small cryptic plasmid, pLJ1, was isolated from *Lactobacillus helveticus* subsp. *jugurti* and was cloned into *Escherichia coli* HB101 by using pBR329 as a vector. Plasmid pLJ1 was 3,292 base pairs long and had single restriction endonuclease sites for *Pvu*II, *Kpn*I, *Ava*II, *Acc*I, *Hin*dIII, and *Eco*RI. In a maxicell system, pLJ1 produced a protein of about 41 kilodaltons.

Lactobacillus helveticus subsp. *jugurti* is an industrially important microorganism in dairy fermentation. Several groups have reported the presence of plasmid DNA in *Lactobacillus* strains (1, 3, 4). In *L. helveticus* strains, *N*-acetyl-D-glucosamine metabolism and slow production of lactic acid have been reported to be encoded by a specific plasmid (9). However, most *Lactobacillus* plasmids are cryptic.

We have isolated a small cryptic plasmid, pLJ1, from *L. helveticus* subsp. *jugurti* to construct a cloning vector for transformation of *L. helveticus* subsp. *jugurti*. For construction of a cloning vector, a selection marker is essential in addition to a replicon. However, there are no reports of an antibiotic resistance gene in *L. helveticus* subsp. *jugurti*. Consequently, for the expression of antibiotic resistance markers such as chloramphenicol or erythromycin resistance genes on cloning vectors, a promoter capable of functioning in *L. helveticus* subsp. *jugurti* is required.

We characterized pLJ1 to detect a plasmid-encoded protein and determined its nucleotide sequence to obtain a promoter for construction of a potential cloning vector.

L. helveticus subsp. *jugurti* SBT2161, harboring a single small plasmid, pLJ1, was isolated from our stock cultures. It was maintained by biweekly transfer at 37°C in lactobacilli MRS broth (Difco Laboratories, Detroit, Mich.). *Escherichia coli* HB101 (5) was used for transformation, and *E. coli* CSR603 (7) was used for the maxicell experiment. *E. coli* strains were grown in LB medium (5) by incubation at 37°C. When appropriate, the medium was supplemented with ampicillin (50 μg/ml), tetracycline (10 μg/ml), or chloramphenicol (25 μg/ml). The plasmids used were pBR329 (2) and pUC18.

Cells for plasmid extraction were harvested by centrifugation from *L. helveticus* subsp. *jugurti* SBT2161 overnight cultures grown in lactobacilli MRS broth at 37°C. The cell pellet was washed and suspended in 1 M sucrose–10 mM Tris maleate (pH 6.5). After 5 min of incubation at 37°C, *N*-acetylmuramidase SG (Dainippon Pharmaceutical Co., Ltd., Japan) was added to a final concentration of 50 μg/ml. The mixture was then incubated at 37°C for 30 min. The cells were lysed by the addition of 2 volumes of alkali-sodium dodecyl sulfate solution (0.2 N NaOH, 1% sodium dodecyl sulfate) and incubation at 37°C for 15 min. Chromosomal DNA was precipitated with 1.5 volumes of 3 M sodium acetate-acetate buffer (pH 4.8) and centrifuged. The supernatant was extracted sequentially with phenol saturated with 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0) and chloroform-isoamyl alcohol (24:1, vol/vol). Plasmid DNA was precipitated with 2 volumes of ethanol and purified by centrifugation in cesium chloride-ethidium bromide density gradients.

pLJ1 was inserted into the *Hin*dIII and *Eco*RI sites of pBR329, and the resulting plasmids were named pLHP100 and pLEP100, respectively. Since the insertion into the *Hin*dIII and *Eco*RI sites of pBR329 resulted in the loss of expression of tetracycline and chloramphenicol resistance, respectively, transformants harboring pLHP100 or pLEP100 could be detected on selection plates. They were stably maintained in *E. coli* HB101.

The nucleotide sequence of pLJ1 was determined by the dideoxy-chain termination method (8). pLJ1 was cloned into the *Hin*dIII and *Eco*RI sites of pUC18, and the deleted derivatives used as substrates in the sequencing reactions were obtained by the stepwise deletion method of Yanisch-Perron et al. (10). In this deletion method, however, pUC18 was used instead of a phage M13 derivative. A deletion kit (Takara Shuzo Co., Ltd., Japan), a sequencing kit (Takara Shuzo), and [α-^{32}P]dCTP (DuPont, NEN Research Products, Boston, Mass.) were used for stepwise deletion, sequencing, and DNA labeling, respectively.

For the detection of plasmid-encoded protein, a modification of the maxicell method of Sancar et al. (6) was used. *E. coli* CSR603 transformed with pLHP100, pLEP100, or pBR329 was irradiated with UV and incubated at 37°C with shaking in the presence of D-cycloserine (100 μg/ml) for 16 h. Proteins produced in the *E. coli* CSR603 were labeled with L-[^{35}S]methionine, extracted, and applied to a sodium dodecyl sulfate–10% polyacrylamide gel.

A restriction map of plasmid pLJ1 was constructed (Fig. 1). The *Pvu*II site of pLJ1 was arbitrarily chosen as the 0

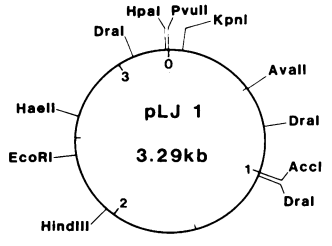

FIG. 1. Restriction map of *L. helveticus* subsp. *jugurti* plasmid pLJ1. The *Pvu*II site was arbitrarily chosen as the 0 reference point, and distances from it are indicated in kilobases (kb).

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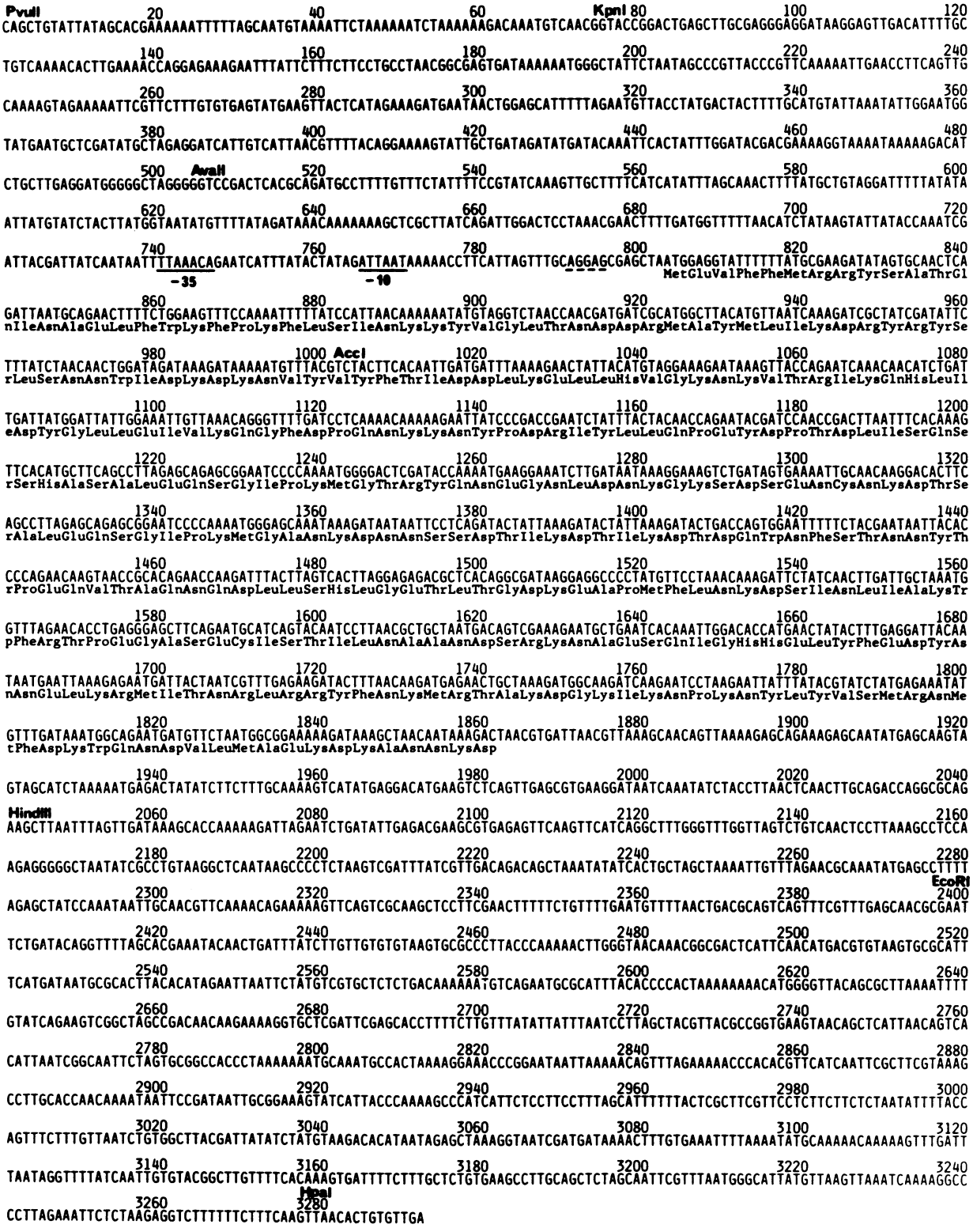

FIG. 2. Complete nucleotide sequence of pLJ1. The deduced amino acid sequence of the unknown protein corresponding to the 1,059-bp open reading frame is shown. The putative promoter regions are underlined. The Shine-Dalgarno sequence is indicated by broken lines.

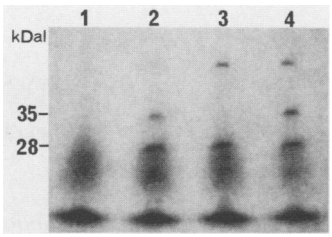

FIG. 3. Autoradiogram of L-[^{35}S]methionine-labeled proteins produced in *E. coli* CSR603 (lane 1) and *E. coli* CSR603 carrying pBR329 (lane 2), pLHP100 (lane 3), and pLEP100 (lane 4). Amp^r (28-kilodalton) and Tc^r (35-kilodalton) gene products from pBR329 were used as the molecular mass markers.

reference point. The plasmid had single restriction endonuclease sites for *Pvu*II, *Kpn*I, *Ava*II, *Acc*I, *Hin*dIII, and *Eco*RI.

The complete nucleotide sequence of pLJ1 was determined (Fig. 2). It was 3,292 base pairs (bp) long and had a low G+C content of 35.3%. A 1,059-bp open reading frame starting at ATG (position 803) and terminating at TAA (position 1862) was found. It was capable of coding for a 41,155-dalton protein of 353 amino acid residues.

A Shine-Dalgarno sequence, AGGAG, was observed 7 bp upstream from the initiation codon. In the promoter region, the −35 and −10 sequences were deduced to be TTAAACA (position 740) and ATTAAT (position 765), respectively.

In the maxicell analysis, *E. coli* CSR603 carrying pLHP100 or pLEP100 produced an unknown protein that was larger than the 35-kilodalton tetracycline resistance gene product used as a reference standard (Fig. 3). To confirm that the open reading frame produced the unknown protein, an *Ava*II-*Hin*dIII fragment of pLJ1, including the 1,059-bp open reading frame and the putative promoter region, was cloned into the *Eco*RV site of pBR329, after both ends were converted to flush ends, and was transformed into *E. coli* CSR603. In the maxicell analysis, the transformant produced a protein with the same R_f as the unknown protein (data not shown). The unknown protein, consequently, was considered to correspond to the product of the 1,059-bp open reading frame.

In this report, we have described some characteristics of the cryptic plasmid pLJ1. pLJ1 is small and contains unique restriction enzyme sites for cloning. It is stably maintained in *L. helveticus* subsp. *jugurti*, and its chimeric plasmids, pLHP100 and pLEP100, are also stably maintained in their host, *E. coli* HB101. In the maxicell analysis, an unknown protein was detected as a product of the 1,059-bp open reading frame on pLJ1. Since we have no system for the transformation of *L. helveticus* subsp. *jugurti*, it is not clear whether the open reading frame is required for replication. In general, however, a protein coded on a plasmid is not essential for the host. Therefore, if the protein were not required for replication, it should be possible to construct a cloning vector with a selection marker by inserting an antibiotic resistance gene downstream of the initiation codon of the open reading frame.

These characteristics will be of use in the construction of cloning vectors for *L. helveticus* subsp. *jugurti* or other lactic acid bacteria.

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