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A Stable Plasmid Vector and Control of Its Copy Number in *Bacillus brevis* 47, a Protein-Producing Bacterium

HIDEO YAMAGATA,* KAZUKO NAKAGAWA, NORIHIRO TSUKAGOSHI, AND SHIGEZO UDAKA

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

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A low-copy-number plasmid vector, pHY481, was constructed by combining a macrolide resistance gene of a *Staphylococcus aureus* plasmid with a cryptic plasmid found in a *Bacillus brevis* strain isolated from soil. The plasmid introduced into *B. brevis* 47, an extensively investigated protein-producing bacterium, was maintained very stably in the absence of selective antibiotics. A *Bacillus megaterium* α-amylase gene subcloned into pHY481 was retained much more stably in *B. brevis* 47 than one subcloned into a plasmid of *S. aureus* origin. *B. brevis* 47 mutants were also isolated in which the copy number of pHY481 was amplified about 10-fold. The copy number of pHY481 with the inserted amylase gene also increased in the mutants. As a result, a severalfold-higher amount of the enzyme was produced in the mutants compared with that produced in wild-type *B. brevis* 47. Thus, the plasmid vector constructed here and the copy-number mutants of *B. brevis* 47 are useful for cloning foreign genes and performing genetic engineering in the protein-producing bacterium.

Bacillus brevis is widely distributed in nature and its production of antibiotics such as gramicidin S and tyrocidine has long been investigated (6). Recently, a group of *B. brevis* strains that accumulate a large amount of protein in the medium was isolated from soil (12). *B. brevis* 47, one of the isolated strains, secretes vast amounts of protein into the medium. The amount of protein accumulated in the medium reaches 12 mg/ml under optimal growth conditions (7). Protein thus secreted consists mainly of two proteins with approximate molecular weights of 150,000 and 130,000 which are indistinguishable from two major proteins found in the outer two protein layers of the *B. brevis* 47 cell wall (13). Since *B. brevis* 47 is the only microorganism known that secretes such vast amounts of protein into the medium, it seems to be a useful organism for studying the mechanism of protein secretion. It may be possible to synthesize and accumulate foreign proteins in vast amounts with the aid of genetic engineering in this organism.

Recently, two plasmids of *Staphylococcus aureus* origin, pHW1 (4) and pUB110 (3), were introduced into *B. brevis* 47 by the Tris-polyethylene glycol method, a unique method for transforming this organism with plasmid DNA (9). These plasmids, however, were found to be unstable in *B. brevis* 47 in the absence of a selective drug, which is especially disadvantageous for prolonged and large-scale cultivation of the plasmid-harboring cells. In the search for plasmids naturally harbored in *B. brevis* species, we found a 1.6-megadalton plasmid in *B. brevis* 481, one of the protein-producing bacteria (12). This plasmid, pWT481, was introduced into *B. brevis* 47 by cotransformation and indirect selection, using pHW1 as an indicator plasmid. pWT481, a relatively low-copy-number plasmid, was retained very stably in *B. brevis* 47 in the absence of a selective drug, whereas pHW1 was unstable under the same growth conditions (14). In this study we constructed a very stable plasmid vector by inserting the macrolide resistance gene of pHW1 into one of *Hin*dIII sites of pWT481 without inactivating the pWT481 genes required for its stable maintenance in *B. brevis* 47. The constructed plasmid, pHY481, could be easily introduced into *B. brevis* 47 by using the Tris-polyethylene glycol method for selecting erythromycin-resistant transformants. The usefulness of pHY481 as a stable plasmid vector in *B. brevis* 47 is shown. Also, the copy number of the plasmid could be increased by introducing it into *B. brevis* 47 mutants.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. brevis* 47 is a protein-producing bacterium isolated by Udaka (12). *B. brevis* 47-5, a uracil-requiring derivative, was used in this study. Plasmids pHW1, pUB110, and pWT481 were described previously (3, 4, 14).

Media. T2 medium was used to grow *B. brevis* 47. The medium contained 10 g of polypeptone (Daigo Eiyo Kagaku), 5 g of meat extract (Wako Pure Chemical Industries), 2 g of yeast extract (Difco Laboratories), 10 g of glucose, and 0.1 g of uracil per liter. The pH was adjusted to 7 with NaOH. Solid medium contained 1.5% agar.

Plasmid analysis. Overnight cultures in T2 medium were diluted to an optical density of 2 at 660 nm with the same medium. Plasmid DNAs were prepared from 1.5 ml of the diluted cultures by the alkaline extraction method of Birnboim and Doly (1) and subjected to 1% agarose gel electrophoresis and staining with ethidium bromide (8).

α-Amylase assay. α-Amylase activity was assayed as described by Fuwa (2). A 2-ml portion of 0.5% soluble starch in 50 mM acetate buffer (pH 6.0) was mixed with 1 ml of enzyme solution diluted appropriately with the same buffer. After incubation at 40°C for various periods, the reaction was stopped by mixing 0.2 ml of the reaction mixture with 5 ml of 0.17 mM I_2-KI solution. One unit of enzyme reduced 1% of the A_{700} in 1 min. Intracellular amylase was assayed after cells were disrupted by sonication.

Detection of α-amylase-positive colonies. Colonies were grown on T2 agar plates containing 0.3% soluble starch and then stained with 0.25% I_2–2.5% KI solution. The colonies producing α-amylase were surrounded by a transparent zone.

RESULTS AND DISCUSSION

Insertion of a macrolide resistance gene of pHW1 into pWT481 and selection of stable plasmids. The linear form of

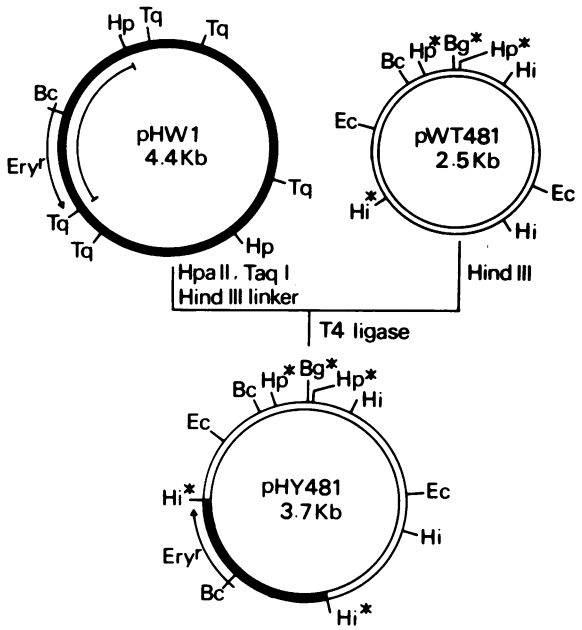

FIG. 1. Construction of pHY481 (restriction map of pHW1 as given by Horinouchi and Weisblum [4]). Restriction sites: Tq, *Taq*I; Hp, *Hpa*II; Bc, *Bcl*I; Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII. Restriction sites on pWT481 (14) and pHY481 marked with asterisks can be used as cloning sites. Abbreviations: Ery^r, erythromycin resistance; Kb, kilobase pairs. Arrows indicate the direction of transcription.

pWT481 DNA generated by partial digestion with *Hin*dIII was used as a vector DNA so that one of the three *Hin*dIII sites of the plasmid could be used as a cloning site. pHW1 DNA was cleaved with *Taq*I and *Hpa*II, and a 1.2 kilobase-pair fragment carrying the macrolide resistance gene (4) was isolated by agarose gel electrophoresis. The isolated fragment (0.2 μg) was treated with T4 DNA polymerase in the presence of four deoxyribonucleoside triphosphates to fill in cohesive ends; it was then attached to synthetic *Hin*dIII linkers and ligated with 0.24 μg of the linear form of pWT481 DNA (Fig. 1). The ligated DNA was extracted with phenol and used to transform *B. brevis* 47 by the Tris-polyethylene glycol method (9) to erythromycin resistance. Approximately 1,000 transformant colonies obtained on T2 plates supplemented with erythromycin (10 μg/ml) were replica plated onto erythromycin-free plates. This replica plating was repeated twice. Colonies were then replicated onto erythromycin-containing plates. About one-fourth of the colonies grew in the presence of the antibiotic. Plasmid DNAs were extracted from eight of these clones by the method of Birnboim and Doly (1) and analyzed for insertions. All of the plasmid DNAs analyzed had an insertion of the 1.2-kilobase-pair fragment in the same *Hin*dIII site (indicated by asterisks in Fig. 1), suggesting that the insertion of this fragment into other sites inactivated genes necessary for the stable maintenance of the plasmid. One of the plasmids obtained was designated pHY481 and used for further experiments. A restriction map of pHY481 is shown in Fig. 1.

Stability of pHY481 under nonselective growth conditions. *B. brevis* 47 harboring pHY481 was grown in T2 medium at 37°C, and the frequency of plasmid-harboring cells among total viable cells was determined periodically. Almost 100% of the cells retained pHY481 during growth for more than 50 generations (Fig. 2). In contrast, 99% of the cells harboring pHW1 lost the plasmid during growth under the same conditions. The stable maintenance of pHY481 was also observed when a foreign gene, the α-amylase gene of *Bacillus megaterium*, was subcloned into the plasmid; the *B. megaterium* α-amylase gene has been cloned and expressed in *Escherichia coli* by using pBR322 as a vector (pKN1; Fig. 3) in our laboratory (manuscript in preparation). The amylase gene could be introduced into *B. brevis* 47 by using the low-copy-number plasmids pWT481 and pHW1 as vectors (the procedures are diagramed in Fig. 3). pHY481 with the amylase gene (pHY482) was much more stable in *B. brevis* 47 than was pHW1 with the amylase gene (pKN11) (Fig. 4), although the insertion of the amylase gene appreciably reduced the stability of both plasmids.

Isolation of copy-number mutants of *B. brevis* 47. pHY481 DNA was mutagenized with hydroxylamine as described by Humphreys et al. (5) and used to transform *B. brevis* 47. Colonies of erythromycin-resistant transformants grown at 30°C were replicated onto T2 agar plates containing erythromycin (10 μg/ml) and then grown at 42°C. Colonies that showed temperature-sensitive growth were obtained with a frequency of 0.1%. Among the 20 temperature-sensitive colonies examined, 19 were temperature sensitive only in the presence of erythromycin and 1 was temperature sensitive regardless of the presence of erythromycin. Plasmid DNAs were extracted from two of the mutants of the former type and used to transform *B. brevis* 47. Transformants obtained at 30°C on T2 plates containing erythromycin (10 μg/ml) showed temperature-sensitive growth in erythromycin-containing medium, indicating that the mutations occurred in the plasmid. These mutant plasmids were named

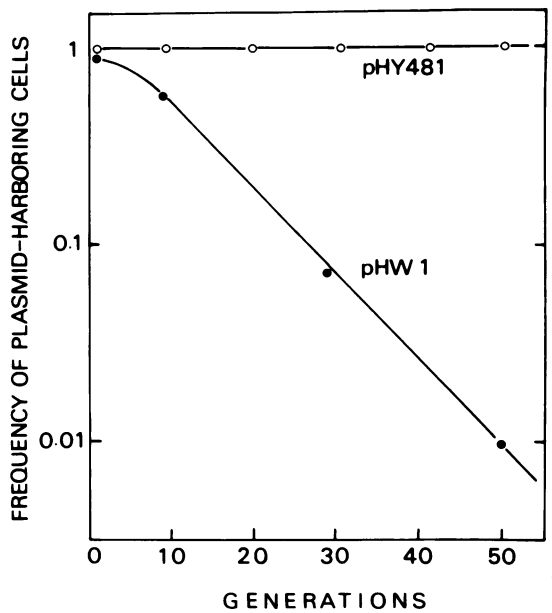

FIG. 2. Stability of pHY481 and pHW1 in *B. brevis* 47 under nonselective growth conditions. *B. brevis* 47 harboring pHY481 or pHW1 was grown in T2 medium at 37°C with shaking. When the bacterial growth reached the stationary phase, the culture medium was diluted 10^3-fold with the same medium, and incubation was continued. Samples were taken at intervals and spread onto T2 agar plates after appropriate dilution. The frequencies of plasmid-harboring cells were determined by replica plating colonies onto T2 agar plates containing erythromycin (10μg/ml).

pHY481ETS1 and pHY481ETS2. About 10^8 cells of *B. brevis* 47 harboring pHY481ETS1 were spread on T2 plates containing erythromycin (10 μg/ml) and incubated at 42°C for 48 h. Temperature-resistant revertants obtained with a frequency of 10^{-5} were analyzed for their plasmids by the alkaline extraction method (1). In about 10% of the revertant strains, the amount of plasmids was increased. One of the revertants with an increased plasmid copy number was designated *B. brevis* 47 Copl1 (copy-number mutant) and used for further experiments. The copy number of plasmid pHY481 in *B. brevis* 47 Copl1 increased about 10-fold compared with that observed in wild-type *B. brevis* 47 (Fig. 5). Similar results were obtained when the plasmid DNAs were analyzed by the NaCl-sodium dodecyl sulfate method described by Tanaka et al. (10). *B. brevis* 47 Copl1 harboring pHY481ETS1 was resistant to erythromycin at a concentration higher than 100 μg/ml at 42°C, whereas wild-type *B. brevis* 47 harboring pHY481ETS1 was sensitive to erythromycin at a concentration of 10 μg/ml at 42°C. Thus, in the Cop mutants, the amount of the temperature-sensitive erythromycin resistance gene product seemed to be increased as a result of a high copy number of the plasmid so that the temperature sensitivity of erythromycin resistance was suppressed.

Introduction of pHY482 into a copy-number mutant of *B. brevis* 47. Since *B. brevis* 47 Copl1 retained plasmid pHY481ETS1 very stably, the curing of the plasmid was unsuccessful. Therefore, pHY482 was introduced into this mutant as follows. *B. brevis* 47 Copl1 harboring pHY481ETS1 was transformed by pHY482 DNA, and transformants resistant to erythromycin (2 mg/ml) at 42°C were selected (at an erythromycin concentration of 2 mg/ml, the growth of *B. brevis* 47 Copl1 harboring pHY481ETS1 was

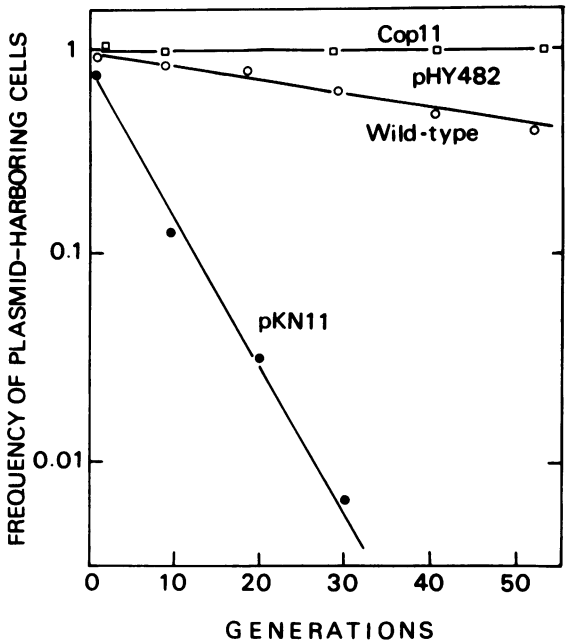

FIG. 4. Stability of pHY482 and pKN11 in *B. brevis* 47 under nonselective growth conditions. The stability of pHY482 in *B. brevis* 47 and in its copy-number mutant Copl1 and the stability of pKN11 in *B. brevis* 47 were examined as described in the legend to Fig. 2. In addition, amylase-producing colonies were determined as described in the text. All erythromycin-resistant colonies were amylase-positive.

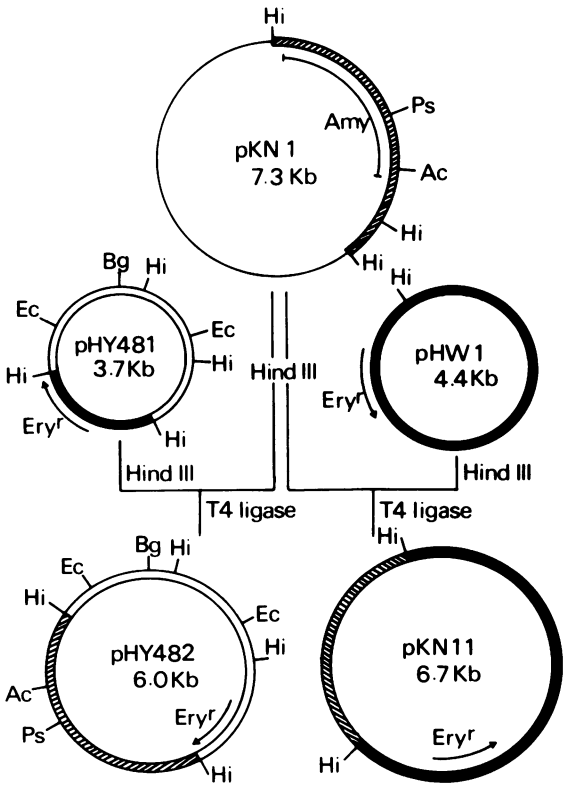

FIG. 3. Construction of pHY482 and pKN11. pKN1 is pBR322 carrying the *B. megaterium* α-amylase gene. Hatched bars indicate *B. megaterium* DNA fragments containing the α-amylase (Amy) gene. Restriction sites: Ps, *Pst*I; Ac, *Acc*I. Other abbreviations and symbols are given in the legend to Fig. 1.

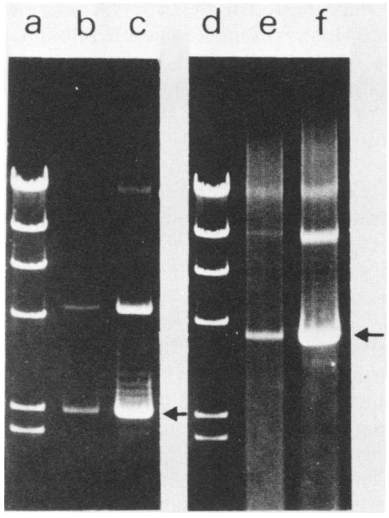

FIG. 5. Amplification of the copy number of pHY481ETS1 and pHY482 in *B. brevis* 47 Copl1. Wild-type *B. brevis* 47 harboring pHY481 (lane b) and pHY482 (lane e) and *B. brevis* 47 Copl1 harboring pHY481ETS1 (lane c) and pHY482 (lane f) were grown overnight in T2 medium at 37°C. Plasmids extracted from 0.4 ml of the cultures at an optical density of 2 at 660 nm were loaded onto each well of the agarose gel (see the text). Lanes a and d, λ DNA cleaved by *Hin*dIII. Arrows indicate position of the closed circular DNA of the plasmids.

TABLE 1. Amylase production by *B. brevis* 47 and its copy-number mutant harboring pHY482[a]

Host strain	Assay fraction	Amylase (U/ml) at:		
		6 h	24 h	46 h
B. brevis 47	Extracellular	3.1	11	4.5
	Intracellular	0.1	7	3.3
B. brevis 47 Copl1	Extracellular	25	55	17
	Intracellular	9	15	10

[a] Extracellular and intracellular amylase activities were determined as described in the text after growth at 37°C for the indicated periods in T2 medium supplemented with erythromycin (10 μg/ml) with vigorous shaking.

inhibited at 42°C, whereas the growth of wild-type *B. brevis* 47 harboring wild-type pHY481 or pHY482 was not inhibited). Such transformants bore plasmid pHY482. The copy number of plasmid pHY482 in these transformants increased about 10-fold compared with that observed in wild-type *B. brevis* 47 (Fig. 5). This indicated that the Copl1 phenotype is due to a chromosomal mutation. Plasmid pHY482 was also very stable in *B. brevis* 47 Copl1 under nonselective growth conditions (Fig. 4).

Increased amylase production in a copy-number mutant. Amylase production of *B. brevis* 47 Copl1 harboring pHY482 was compared with that of wild-type *B. brevis* 47 harboring pHY482. A severalfold increase in the amount of enzyme was observed in the mutant (Table 1). This supports the notion that the copy number of the plasmid is increased in the Copl1 mutant. A decrease in amylase activity after 46 h of cultivation may be due to the physical inactivation of the enzyme itself or to the protease formed by *B. brevis* 47.

As we have demonstrated, a newly constructed plasmid, pHY481, in combination with wild-type *B. brevis* 47 or its Copl1 mutant is useful as a vector for cloning foreign genes. Since its copy number in wild-type *B. brevis* 47 is relatively low, the plasmid may be used when the overproduction of gene products is harmful to the host cells. Among four *Hin*dIII restriction sites of pHY481, those marked by asterisks in Fig. 2 can be used as cloning sites. In addition, *Hpa*II and *Bgl*II sites (also marked by asterisks in Fig. 1) were shown to be convenient as cloning sites. This plasmid has no *Bam*HI, *Pst*I, *Sal*I, *Cla*I, *Kpn*I, or *Taq*I sites, so these sites can be easily introduced into this plasmid as unique cloning sites with the use of synthetic oligonucleotide linkers. We are currently attempting to construct an efficient secretion vector by inserting into this plasmid a strong promoter and signal sequence region of appropriate genes from *B. brevis* 47. Recently, a part of the cell wall protein gene of *B. brevis* 47 has been cloned into *E. coli* in our laboratory (11). Since the cell wall proteins are secreted into the medium in vast amounts, this gene should be useful for the construction of the secretion vector.

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