0273-2289/92/3435—0383$02.00

Plasmids from Thermophilic *Clostridia*

Scientific Note

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Index Entries: Thermophilic *Clostridia;* plasmids; cloning; shuttle-vector; transformation.

INTRODUCTION

Clostridia present an area of great potential interest to biotechnology, because among their metabolic products are some prospective biofuels (ethanol, butanol, acetone, hydrogen), useful organic acids (acetic, propionic, butyric, and so forth), and technologically important enzymes (cellulases, hemicellulases, and so on). Particular attention is paid to the thermophilic species of these bacteria according to the following: First, under thermophilic conditions, the realization of technological processes is made easier (lower oxygen solubility, lower demands for sterility, higher metabolic rates), and second, many enzymes isolated from thermophilic species feature a notable thermostability. At the same time, *Clostridia* possess some disadvantages that prevent their use in biotechnology: relatively lower growth rates, strict observance of anaerobiosis, and lower yield of necessary products. One of the ways to overcome these disadvantages may be to construct new strains with desirable properties by means of genetic engineering methods (*1,2*).

Today the solution of this problem is impossible because appropriate cloning vectors are absent and convenient systems for genetic engineering manipulation in *Clostridia* are not developed. The aim of this short review is to present a generalization of data on the availability of plasmids in

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Table 1
Plasmids from Thermophilic *Clostridia*

Bacterium	Strain	Source	Plasmid	Size, kb	Reference
C. thermocellum	F7	Soil, silt	PCT1[a]	25–30	(4)
			pFA7[a]	25	(5)
C. thermocellum	F7	Soil, silt	pFB7	50	(5)
			pFA1	25	(5)
C. thermocellum	F1	Soil, silt	pFB1	50	(5)
		From	pNB1	5.27	(6)
C. thermosaccharolyticum	DSM571	collection	pNB2	1.88	(6)
C. thermosaccharolyticum[b]	106	Soil, compost	pTA106	1.5	(7)
			pTA688S	2.3	(7)
C. thermosaccharolyticum[b]	688	Soil, compost	pTA688L	7.2	(7)
C. thermosaccharolyticum[b]	729	Soil, compost	pTA729	1.5	(7)
		From	Not yet		
C. thermosaccharolyticum	?	collection	named	30	(8)
C. thermosaccharolyticum	?	?	pLJ88	Large	(9)

[a]It is likely to be one and the same plasmid.
[b]These strains are poorly characterized. As the authors (7) noted, their phenotypes are similar to that of *C. thermosaccharolyticum* ATCC 31925.

thermophilic *Clostridia,* including a description of their properties as well as attempts to construct convenient vectors on their basis. Our own experimental data from studying plasmids from *C. thermosaccharolyticum* DSM 571 will also be listed.

RESULTS AND DISCUSSION

At the present time, plasmids are found in a limited number of strains of thermophilic *Clostridia.* The first plasmids from thermophilic anaerobes, which are close to a clostridial type, were isolated as recently as 1984. The strains B6A, DM24D, R7D, and F266B, which were isolated from geothermal springs and attributed to genus *Thermoanaerobium, Thermoanaerobacter,* and *Thermobacteroides,* contain one plasmid each: pDP5010 (2.3 kb), pDP5009 (2.3 kb), pDP5016 (1.8 kb), and pDP5012 (2.3 kb), respectively (3). As to proper thermophilic clostridial bacteria, plasmids have been found in some strains of *Clostridium thermocellum, Clostridium thermoaceticum,* and *Clostridium thermosaccharolyticum,* among which there are both well-characterized strains (from collections) and more recently isolated ones (Table 1).*

*The works dealing with investigation of plasmids from mesophilic *Clostridia* (such as *C. perfringes*) are not a subject of our consideration.

Methods of Isolation of Plasmids

Disintegration of the clostridial cell wall is achieved by treatment with lysozyme or sodium dodecyl sulfate (SDS) in an alkali medium. For the isolation of plasmids, both alkali lysis and heat denaturation were applied (*10–14*). The usage of various isolation methods of plasmid DNA is likely to be the reason that different results were obtained by different authors. Thus, we have found two plasmids in the cells of *C. thermosaccharolyticum* DSM 571 (*6*) that were not detected earlier (*15*). We used Birnboim-Doly's method, in which the cell wall was disintegrated by lysozyme, and the separation of plasmid and chromosomal DNA was performed by alkali denaturation of the latter at pH 12–12.5. The Kado method was also used, (*15*), in which the cells were broken up by treatment of alkali SDS and the separation of two kinds of DNA was carried out only by electrophoresis. In the work (*5*), plasmids approaching 25 kb were isolated by Birnboim-Doly's methods, whereas larger plasmids (nearly 50 kb) were isolated by a modification of the method of soft isolation of plasmid DNA (*14*). The latter method is useful for the isolation of large plasmids from bacterial cells with high extracellular nuclease activity (of which *Clostridia* are relatives). Thus, the choice of the method of plasmid DNA isolation used for the screening of thermophilic *Clostridia* depends on the availability of plasmids within the organisms.

Restriction Mapping

On the basis of electrophoretic analysis of plasmid DNA, the restriction maps were obtained for six plasmids: pNB1, pNB2, pTA106, pTA688S, pTA688L, and pTA729 (Fig. 1) (*6,7,16*).

Cloning

An attempt to develop the transformation system for thermophilic *Clostridia* was undertaken on the basis of plasmids pTA688S and pTA688L (Fig. 1) (*7*). Three cellulolytic strains, 088, 118, and 187B, were chosen as hosts. The most successful result was reached with plasmid pCS1 (Tc^r, Cm^r) obtained from pTA688S and *E. coli* plasmid pBR328 (Amp^r, Tc^r, Cm^r) under transformation of strains 008 and 118. The transformation frequency of the two strains with pSC1 was 80–140/μg of plasmid DNA. All the transformants grew at 60°C in K medium containing 10–100 μg/mL chloramphenicol (Cm). The resistance of the transformants to Cm, however, was easily lost by repeated cultivation at 60°C.

In our recent work (*16*), plasmids pNB1 and pNB2 were cloned into *E. coli* plasmid pUC18(Amp^r), via unique EcoRI and PstI sites. As a result, recombinant plasmids pNB3(Amp^r), pNB4(Amp^r), and pNB5(Amp^r) containing full-length insertion of both plasmids pNB1 and pNB2 were obtained (Fig. 2). Detailed comparison of restriction maps of pNB1 and pNB2

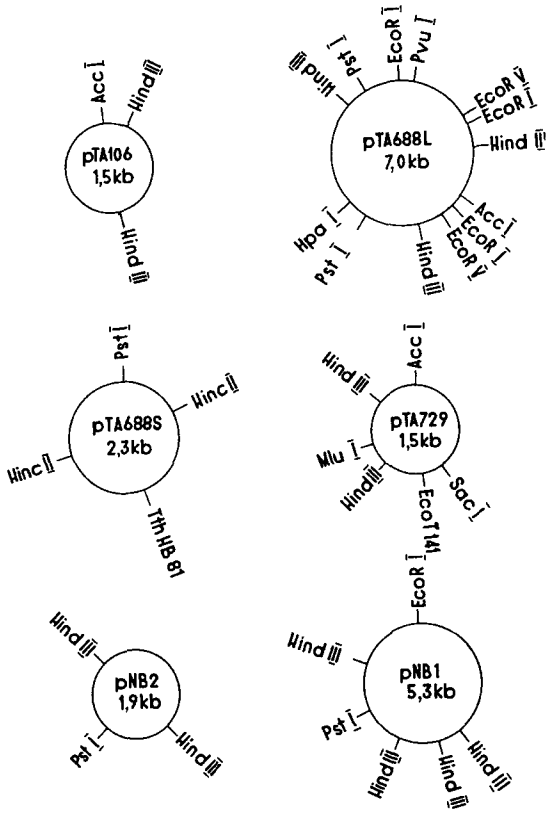

Fig. 1. Restriction maps of plasmids from thermophilic *Clostridia* (*6,7,16*).

reveals the similarity between these plasmids in the PstI-HindIII regions. The sequence analysis of 400-bp regions at PstI sites of pNB1 and pNB2 has shown their 100% homology. pNB2 (1.9 kb) is likely to be derived from its parent 5.3 kb pNB1 by in vivo deletion as a result of some recombination events.

Copy Number

The data on the copy number of thermophilic clostridial plasmids are presented only in our work (*6*). The copy number of pNB1 during cultivation of *C. thermosaccharolyticum* in medium without antibiotics is 1–2/cell. On selective media containing Km or Sm, the plasmid copy number per cell increases up to 5–10.

Antibiotic Resistance

Among plasmid carriers, the antibiotic resistance was found for *C. thermosaccharolyticum* DSM571 to kanamycin (Km) or streptomycin (Sm) in concentrations up to 100 *μg/mL* (*6*) and for *Clostridium* spp. nos. 106, 688, and 729 to 50–100 μg/mL Km or tetracycline (Tc) (*7*). We attempted

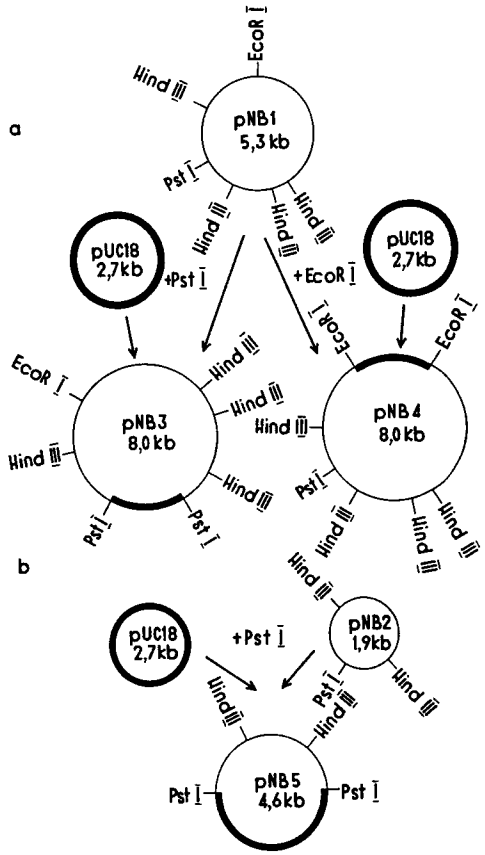

Fig. 2. Subcloning of plasmids pNB1 and pNB2 in pUC18 (*17*).

to find a relation between the presence of plasmids and antibiotic resistance. For this purpose, we have tested the Km and Sm resistance of *E. coli* JM109 harboring recombinant plasmids pNB3, pNB4, and pNB5 (Fig. 2). However, no resistance to Km and Sm, even at concentrations of 10 μg/mL, was detected. However, these data do not allow us to conclude unequivocally that the antibiotic resistance is not linked to either pNB1 or pNB2. For pNB2, however, an absence of such a relationship is confirmed by the determination of its complete nucleotide sequence (*see below*): No homologies have been found with known genes of Km and Sm resistance.

Nucleotide Sequence

The complete nucleotide sequence has been determined only for plasmid pNB2 in our recent works (*17,18*). This plasmid contains 1882 nucleotides, and the guanosine+cytosine (G+C) content is equal to 27.2%. Such G+C content is typical for clostridial DNA. A computer analysis of the obtained nucleotide squence of pNB2 was performed using the standard program PCGENE. As a result, three relatively long (more

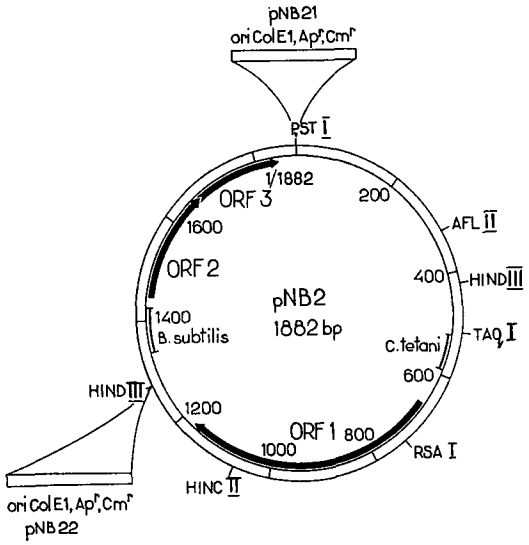

Fig. 3. Function maps of plasmid pNB2 and its derivatives pNB21 and pNB22 (*17*). Thick arrows show identified ORFs; thin lines identify homologies with nucleotide sequences of *C. tetani* and *B. subtilis*.

than 200 nucleotides) open reading frames (ORFs) were found (Fig. 3). Sizes of proteins encoded by these ORFs are equal to 30, 8, and 7 kDa, respectively. The second and the third ORFs are organized in operon with a strong bacterial promoter and corresponding sites of binding to ribosomes (Shine-Dalgarno sequence). Such structures were not revealed in front of the first ORF. A 20-nucleotide inverse repeat was found in the region 1300–1360. It is possible that the pNB2 *ori* is located in this region. Additional experiments on transformation of clostridial cells by plasmid pNB2 and its derivatives are necessary to confirm this supposition. Some homologies with nucleotide sequences of *C. tetani* and *B. subtilis* have been revealed (*17,18*).

The possibility of expression of two proteins encoded by second and third ORFs has been demonstrated in experiments with maxicells of *E. coli*. Eight- and 7-kDa proteins have been detected by using full-length insertion of pNB2 as well as by using its HindIII-PstI fragment (*17,18*). Potential shuttle-vectors for *Clostridia* were constructed by insertion of ColE1 replicon and antibiotic-resistant gene Amp^r (for *E. coli*) or Cm^r (for gram-positive bacteria) into HindIII and unique PstI sites of pNB2 (Fig. 3) (*17,18*). Currently, we are engaged in developing a transformation system for thermophilic *Clostridia* on the basis of pNB2-derived cloning vectors.

CONCLUSION

Thus, since the first publication (1987) on the detection of plasmids in thermophilic *Clostridia*, nearly 10 of their cryptic plasmids have been dis-

covered. Most of them are poorly characterized, but for pNB2, the complete nucleotide sequence has been obtained. On the basis of the most studied plasmids (pTA688L, pTA688S, pNB1, and pNB2), some shuttle-vectors have been constructed that could be expressed in several hosts. Unfortunately, the efficiency of the transformation of thermophilic *Clostridia* by these shuttle-vectors is still low. Therefore, special efforts should be made to construct more efficient systems of transformation. If success is achieved, it will open promising possibilities for genetic engineering manipulation with thermophilic *Clostridia* and other thermophilic gram-positive bacteria.

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