Note

Construction of a Vector Plasmid for Use in *Gluconobacter oxydans*

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A host vector system in *Gluconobacter oxydans* was constructed. An *Acetobacter-Escherichia coli* shuttle vector was introduced with the efficiency of $10^4$ transformants/µg of DNA. Next, aiming for a self-cloning vector, we found a cryptic plasmid (which we named pAG5) of 5648 bp in *G. oxydans* strain IFO 3171, and sequenced the nucleotides. The plasmid seemed to have only one open reading frame (ORF) for a possible replication protein. Shuttle vectors of *Gluconobacter-E. coli* were constructed with the plasmid pAG5 and an *E. coli* vector, pUC18.

**Key words:** *Gluconobacter*; host-vector system; cryptic plasmid

*Gluconobacter oxydans* is a microorganism of industrial importance. Many compounds, such as acetic acid, d-gluconic acid, ketogluconic acids, l-sorbose, and dihydroxyacetone, are produced with *G. oxydans.* For efficient production, strain improvement has been investigated, including genetic engineering. Some vector systems in *G. oxydans* with a broad-host-range plasmid or a cryptic plasmid have been reported. Transformation of *G. oxydans* by conjugation and electroporation has been described. Recently, we found that *G. oxydans* strain ATCC 621 produces xylitol from d-arabitol efficiently. A host-vector system might lead an improvement of the xylitol production of this strain. A self-cloning vector is preferable for industrial use. Several native plasmids in *Gluconobacter* strains have been reported, but the structures of those plasmids were not fully investigated. In this paper, we describe a new host-vector system in *G. oxydans* ATCC 621. First, introduction of an *Acetobacter-E. coli* shuttle plasmid, which we developed earlier, showed that the *E. coli* bla marker and the lac promoter could be used in this strain. Thereafter, for self-cloning, a cryptic plasmid was obtained from *G. oxydans* strains. The structure of the plasmid was found by analysis of the nucleotide sequence. Vectors using this plasmid were constructed and introduced into the strain.

*G. oxydans* ATCC 621 was grown in YPG medium, containing 0.5% yeast extract, 0.2% peptone, and 3% glucose. An *Acetobacter-E. coli* shuttle plasmid, pSA19, was used. Transformants were selected as colonies resistant to ampicillin (100 µg/ml). Plasmids were introduced into the *G. oxydans* cells by electroporation. The conditions of electroporation were: ten treatments with a pulse of 14 kV/mm, 400 µF, with SSH-10 (Shimadzu, Japan). pSA19 was thus introduced into ATCC 621 cells. The transformation efficiency was $10^4$ μg of DNA, almost the same as that in *Acetobacter xylinum* BPR 2001.

As a demonstration, expression of the XDH gene of *Morganella morgani* was investigated. The xylitol dehydrogenase (XDH) gene was amplified by PCR, with the chromosomal DNA of *M. morgani* ATCC 25829, and a pair of primers designed from the known sequence (Genbank accession No. L34345): CGGGAATTCGATATCATTTTAATGAA and GGCCTCGAGATCATATCCGCGCATAGA. The amplified XDH gene was ligated into pSA19 at the *EcoRI* and *BamHI* sites under the lac promoter, and then introduced into the strain. The reaction of XDH was done in a mixture containing 100 mM Tris HCl (pH 6.0), 50 mM d-xylulose, and 2 mM NAD, with the supernatant of sonicated cells as the enzyme solution. XDH activity was measured as the decrease of OD$_{340}$ and expressed as the specific activity per milligram of protein.

Although *G. oxydans* ATCC 621 has some XDH activity (0.20 U/mg protein), activity was increased in the transformant (0.79 U/mg protein). This result shows that foreign genes can be expressed in *G. oxydans* under the lac promoter, as was observed in *Acetobacter* species also. Therefore, with the vector, it is possible to investigate production by introduction of some genes into *G. oxydans*.

It is very important to develop a "self-cloning" strain for commercial production. A self-cloning strain is considered to be as safe as non-recombinant strains. Therefore, to use one for development of a self-cloning vector, cryptic plasmids were screened in some *G. oxydans* strains. No plasmid was found in strain ATCC 621, but a plasmid of 5.5 kb was found in IFO 3171, and we named it pAG5 (Fig. 1). Some large plasmids have been reported, and were found in strains IFO 3250 and IFO 3292 in this study.

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Fig. 1. Restriction Map and Structure of Plasmid pAG5.

Nucleotides are numbered from the HindIII site. An open reading frame, which encodes a putative replication protein, is indicated by the arrow. An AT-rich region, a putative replication starting site, is indicated as a closed box. Restriction sites: E; EcoRI, H; HindIII. K; KpnI, Sm; SmaI, Sp; SphI, and X; XhoI. The complete nucleotide sequence was deposited in the DDBJ nucleotide sequence database under the accession number AB086443.

we chose pAG5 as the most suitable for construction of a vector.

The nucleotide sequence was analysed and deposited in the DDBJ nucleotide sequence database under accession no. AB086443. The plasmid consisted of 5648 bp, and the structure is shown in the figure. A BLAST homology search showed that this plasmid was not similar to any other reported plasmids. A region of 136 bp in nucleotide positions 4965-5100, which was highly AT-rich (78%), was found; it may be a replication origin. Only one open reading frame, which coded a polypeptide of more than 150 amino acids, was present at nucleotide positions 3489-2467. The coded polypeptide contained a Leu-His-Val-His-Ala-Ile-Val sequence, which resembled the sequence Ala-His-Trp-His-Ala-Glu-Arg, found in the putative replication protein of pAH4, and the consensus sequence Ala-His-Uue-His-Ala-Uue-Uue (Uue represents a hydrophobic amino acid residue) in Rep proteins. Therefore, we supposed that this protein was the replication protein of pAG5.

The plasmid pAG5 was ligated with an E. coli vector pUC18 at the HindIII site, and the resulting plasmid was named pSG8. pSG8 was further digested with EcoRI and then ligated to itself. The plasmid, named pSG6, had the structure of pSG8 with the deletion of about 2 kb portion between two EcoRI sites. Both pSG8 and pSG6 were introduced into strain ATCC 621 with the same efficiency as that of pSA19. The copy numbers of these plasmids were ten per genome when estimated by the method of Projan et al. Because no ampicillin-sensitive colonies were found when the transformants were cultured for 10 days in the absence of ampicillin (total, 50 generations) by repeated inoculation (1%) into fresh medium every 2 days, both plasmid in G. oxydans ATCC 621 seemed to be stable. Therefore, with this cryptic plasmid, a “self” vector in G. oxydans could be constructed.

References

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