One step assembly of multiple DNA fragments with a designed order and orientation in *Bacillus subtilis* plasmid

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Received August 19, 2003; Revised and Accepted September 16, 2003 DDBJ/EMBL/GenBank accession no. AB117035

**ABSTRACT**

A universal method to reconstitute sets of genes was developed. Owing to the intrinsic nature of the plasmid establishment mechanism in *Bacillus subtilis*, the assembly of five antibiotic resistance genes with a defined order and orientation was achieved. These five fragments and the plasmid have three-base protruding sequences at both ends. The protruding sequences are designed so that each fragment is ligated once in a row according to the pairing. Ligation by T4 DNA ligase in the presence of 150 mM NaCl and 10% polyethylene glycol at 37°C yielded high molecular tandem repeat linear form DNA. This multimeric form of DNA was preferentially used for plasmid establishment in *B.subtilis*. The method, referred to as Ordered Gene Assembly in *B.subtilis* (OGAB), allows for the design of multiple fragments with very high efficiency and great fidelity.

**INTRODUCTION**

Complete sequences of genomic DNA from a number of species of all three kingdoms have unveiled much information on genome structure as well as the function of gene products (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome). The DNA cloning technology developed in the last century has led to great success in DNA information on genome structure as well as the function of gene products (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome). The DNA cloning technology developed in the last century has led to great success in DNA manipulation (1,2). According to the cloning protocol using, for example, an *Escherichia coli* plasmid, the cloned DNA comes from DNA treated by physical shearing or digested with appropriate sequence-specific endonucleases. Once these primary clones are obtained, sub-cloning is merely a question of combinatorial PCR technology. The handling of a large number of only relevant genes becomes important (3,4). When these genes are fortuitously included in a single continuous DNA segment such as the genes for the biosynthesis of ML-236B (compactin), a substrate for pravastatin (5), special assembly technology may not be necessary. In most other cases, however, relevant genes necessary for particular bioprocesses are dispersed along the genome. A few examples for gene assembly have been reported to date (6–8). In the synthesis of 6-deoxyerythronolide B, an erythromycin intermediate, in *E.coli*, genes encoding DEBS1 + TE, DEBS2 and DEBS3 were collected from *Saccharopolyspora erythraea* and the sfp gene was obtained from *Bacillus subtilis* (6). A *Sacharomyces cerevisiae* strain was converted to a producer of hydrocortisone, which is the major adrenal glucocorticoid of mammals and an important intermediate of steroidal drug synthesis (7). Among 13 genes engineered for the synthesis of hydrocortisone, eight from mammals were progressively introduced into the yeast. The assembly of >300 kb segments from a *B.subtilis* (*natto*) in the genome of *B.subtilis* 168 converted strain 168 to a *natto* producer (8). Although individual relevant *natto* genes were not clearly identified, the work indicated that a very complicated bioprocess could be realized by progressive gene transfer in different species. The DNA transfer of a candidate for a symbiotic island in bacterial genomes among which a 611 kb segment was found in a *Mesorhizobium loti* strain (9) is the largest assembly conducted by nature to date. The chemical synthesis of long DNA by a rather complicated method is an alternative for gene assembly (10). Despite these pioneering approaches, effective and versatile methods to assemble a number of genes have been limited.

The *E.coli* host may not be suitable for assembling a number of genes in one plasmid. *Escherichia coli* employs only circular form DNA (cfDNA) as a structural requirement when being transformed. Therefore, preparation of the cfDNA prior to transformation is critical (Fig. 1). cfDNA prepared with more than two fragments is the result of two self-contradictory ligation reactions, which are inter- and intramolecule ligation. Inter-molecule ligation results in a preferential alignment of all the fragments in a row, but intramolecule ligation must be followed to form cfDNA. As conditions are significantly different for these two reactions (11–13), conducting both types of ligation in the same reaction tube is difficult. Furthermore, the preparation of cfDNA in one in vitro ligation becomes inefficient as the number of fragments increases. To avoid these problems, genes are delivered separately by more than two compatible plasmids (6), or integrated at different loci of the genome sequentially (7). The use of multiple plasmids, however, accordingly requires more selection markers (6). The design and actual construction of the DNA is boring and time-consuming.

An alternative method is provided in this study using strain *B.subtilis* 168 as a host. The key principle of the reported method is that competent *B.subtilis* cells have different

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Nucleic Acids Research, Vol. 31 No. 21 © Oxford University Press 2003; all rights reserved
requirements from *E. coli* cells. This is based on unique mechanisms for DNA uptake and plasmid establishment (14). Double-stranded DNA is cleaved by a nuclease on the surface of the competent cell. The DNA is processed into single-stranded DNA from the cleavage position and actively incorporated into the cell by competent complex through the cell wall and membrane. Number of competent complex was estimated at about 50 per competent cell. The highly recombinogenic single-stranded DNAs simultaneously incorporated inside the cell form double-stranded DNA by pairing. The partial double-stranded molecule thus formed, is repaired to give an intact double-stranded circular DNA that starts replication as a plasmid (15). This unique mechanism, completely different from that of *E. coli*, is regulated by a number of intrinsic competence genes (16). There is no absolute requirement for cfdNA, because the cfdNA is cleaved during transformation. Long repeated linear multimer form DNA (lfdDNA) is more efficient than monomer cfDNA for the pairing of two independently incorporated single-stranded DNAs in *B. subtilis* as indicated in Figure 1.

In this paper we report the construction of a high molecular weight lfdDNA comprised of as many as six fragments and one plasmid vector by *in vitro* ligation in one test tube. The order and orientations of all the component fragments were designed with end structures generated using the restriction endonuclease SfiI (17). Successful ligations of the pairing of 3 nt protruding with variations in the SfiI recognition sequence 5'-GGCCNNNN/NGGCC-3' are presented. The ligation product transformed competent *B. subtilis* 168 with significantly high efficiency; however, it never gave the correct plasmid in *E. coli*. This method is referred to as Ordered Gene Assembly in *B. subtilis* (OGAB).

**MATERIALS AND METHODS**

**Bacterial strains and media**

A restriction-modification deficient mutant strain *B. subtilis* RM125 (18,19) was used. *Escherichia coli* TOP10 [F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str') endA1 nupG] was used for cloning of the PCR fragment into the pCR-TOPO-X vector. *Escherichia coli* JA221 (F- hsdR hsdM+ trp leu lacY recA1) is routinely used in this laboratory as a host for general molecular cloning. Luria–Bertani (LB) broth was used for both *E. coli* and *B. subtilis*. Bacteria were grown at 37°C. The antibiotics kanamycin at 25 µg/ml and ampicillin at 50 µg/ml were used for selection of *E. coli* transformants. Antibiotics used for the selection of *B. subtilis* transformant were: blasticidin S 500 µg/ml, chloramphenicol 5 µg/ml, erythromycin 5 µg/ml, neomycin 3 µg/ml, spectinomycin 50 µg/ml and tetracycline 10 µg/ml. These antibiotics were purchased from Sigma (St Louis, MO), except for blasticidin S, which was from Funakoshi (Tokyo, Japan).

**DNA manipulation**

The preparation of plasmid from *E. coli* and *B. subtilis* was carried out using alkaline-sodium dodecyl sulfate (1,20). A large amount of plasmid DNA from *E. coli* was prepared using a Qiagen Plasmid Midi Kit (Qiagen, CA). The restriction endonuclease SfiI was purchased from New England BioLabs (MA). Other restriction endonucleases and T4 DNA Ligase were purchased from TOYOBO (Osaka, Japan). PCR for DNA cloning was performed using Ex-Taq Hot start version (Takara Bio, Otsu, Japan). Cloning of the PCR product in the plasmid pCR-XL-TOPO was done with a TOPO XL PCR Cloning Kit (Invitrogen, CA). *Escherichia coli* were transformed using the competent cell method described by Mandel and Higa (21).

**Bacillus subtilis vector plasmid for gene assembly**

The *E. coli–B. subtilis* shuttling vector plasmid pGETS103 constructed in this laboratory was employed first (22). As the copy number of pGETS103 was maintained at about 10 per cell, a copy number controllable derivative, pGETS109 (Fig. 2A), was constructed. The theta-type replication plasmid pTB522 from *B. stearothermophilus* (23) was digested with EcoRI and MluI. The fragment containing the repA gene, which includes the replication functions, blunt-ended, was cloned between the HindIII and BglII sites, dephosphorylated and orientations of all the component fragments were designed

**Figure 1.** The shuttle plasmid transforms *E. coli* and *B. subtilis* by a different mechanism described in the text. *Escherichia coli* accepts only cfDNA. *Bacillus subtilis* preferentially takes up more than a dimer to establish a mechanism described in the text.
pBRDHRenAT1F constructed in E.coli by selection with ampicillin required a selection marker for shuttling in B.subtilis. A tetracycline resistance gene prepared from pBEST305 (27) with EcoRI and PstI digestion was cloned into the SmaI site of pBRDHRenAT1F after being blunted. The tetracycline-resistant pBRDHRenAT1F, referred to as pGETS109 (DDBJ accession number AB117035) hereafter, was isolated from E.coli, and when introduced into B.subtilis rendered cells resistant to tetracycline. pGETS109S\textasciicircum{I}fil (Fig. 2A) was obtained by insertion of an S\textasciicircum{I} adaptor generated by annealing the two synthetic DNA oligomers 5'-AGC\textasciicircum{I}TCGGCCACTCGGCGGTACGATATGAA-3' (VShKpn-1F) and 5'-AGCTGGCCGATGGCCGGTACC-GGCCGATTTGGCGG-3' (VShKpn-1R) into the unique HindIII site of pGETS109. pGETS109 recognizes the sequence underlined and provides three protruding nucleotides at the 3' end italicized. pGETS109 replicates also in B.subtilis, and when introduced into E.coli with a medium copy number above 20 due to the nature of pBR322. The copy number of pGETS109 and its derivative in E.coli remained as low as one and is increased when the transformant is cultured in the presence of isopropyl 1-thio-\beta-D-galactoside (IPTG) at a final concentration of 1 mM (data not shown).

**Fragments prepared with S\textasciicircum{I}I**

Five antibiotic resistance genes with S\textasciicircum{I}I sites at both ends were prepared separately via a PCR-mediated amplification method. The blasticidin S resistance gene (erm) was prepared from pCISP310 (28). The erythromycin resistance gene (erm) was prepared from pCISP311 (28) and the neomycin resistance gene (neo) from pBEST305 (27). The PCR was programmed for one cycle at 98°C for 5 min, followed by 30 cycles at 98°C for 20 s, 60°C for 30 s and 72°C for 60 s per 1 kb amplification. The primers were: bsr 5'-GGCCGACTCGCGGATCCTATGGAA-3' (Bsr-S\textasciicircum{I}fil1F) and 5'-GGCCGACTCGCGGATCCTATGGAA-3' (Bsr-S\textasciicircum{I}fil2F); cat 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Cat-S\textasciicircum{I}fil1R) and 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Cat-S\textasciicircum{I}fil2R); neo 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Neo-S\textasciicircum{I}fil1F) and 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Neo-S\textasciicircum{I}fil2F); spc 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Spc-S\textasciicircum{I}fil1F) and 5'-GGCCGACCTCGCGGATCCTATGGAA-3' (Spc-S\textasciicircum{I}fil2F). The copy number of pGETS109 and its derivative in B.subtilis remained as low as one and is increased when the transformant is cultured in the presence of isopropyl 1-thio-\beta-D-galactoside (IPTG) at a final concentration of 1 mM (data not shown).

**In vitro ligation**

Two ligation systems were adopted. The first is available commercially to produce cfDNA ligation products for transforming E.coli. We chose Takara ligation kit Ver. 1 commonly used for the construction of E.coli plasmids. Ten microliters of the mixture was added to 40 μl of solution A and 10 μl of solution B, according to the instruction manual. The ligation solution was then incubated at 16°C for 30 min. The second is the ‘linear form ligation’ system developed by Hayashi et al. (13). Ligation with this system generates high molecular weight tandem repeat iFDNA. A 2-fold higher concentration (2×) of buffer [132 mM Tris–HCl (pH 7.6), 13.2 mM MgCl₂, 20 mM dithiothreitol, 0.2 mM ATP, 300 mM NaCl, 20 % (w/v) polyethylene glycol 6000 (Wako pure chemical, Japan)] was prepared and used. There was no inhibitory effect on the transformation of E.coli by the iFDNA ligation buffer.

**Transformation of B.subtilis**

Competent B.subtilis cells were prepared using the two-step culture method developed by Anagnostopoulos and Spizizen (30). Typically, DNA is added to 100 μl of TFII including competent B.subtilis cells and kept at 37°C for 30 min. Outgrowth at 37°C on addition of LB medium (300 μl) for 1 h allows expression of the tetracycline resistance gene. The culture was spread on LB plates containing tetracycline for selection.

**RESULTS**

**Design of six-fragment assembly**

The assembly is done through two separate steps. The first step is the ligation of multiple fragments in vitro and the second step, transformation using the ligation product. The five
antibiotic resistance gene cassettes B, C, E, N and S, and the pGETS109S®I vector (V) were prepared so as to be ligated in a row according to the designed pairing (Fig. 3). Ligation of fragment E and pGETS109S®I was done as a two-fragment assembly for comparison. The fragment E used in this experiment was designed separately as shown in Figure 2. None of these fragments self-circularizes. To accelerate correct pairing, irregular pairing was eliminated by 5 min of heat treatment at 50°C immediately before ligation. Theoretically, the products elongate by ligation in the defined order and orientation until the least concentrated fragment is consumed. Thus, the initial amount of all the fragments was adjusted to an equal molar concentration (~30 fmol/μl). Ligation was carried out under two different sets of

Figure 2. (A) Structure of the shuttle vectors pGETS109 and pGETS109S®I described in Materials and Methods. (B) SfiI fragments prepared for gene assembly. Genes encoding resistance to blasticidin S (B), chloramphenicol (C), erythromycin (E), neomycin (N), spectinomycin (S) and tetracycline (T) are designed to have 3’ protruding nucleotides provided by SfiI digestion as indicated. (C) Structure of assembled genes in this study. The erythromycin resistance gene (E) fragment is pivotal.
conditions; to stimulate linear formation (LF) and to allow circular formation (CF). Ten microliters of this mixture (3 fmol/μl each) was added to 11 μl of 2× LF ligation buffer, and 1 μl of T4 DNA Ligase (4 Weiss unit/μl) was added. After a reaction at 37°C for 30 min, ligation products were subjected to pulsed-field gel electrophoresis as shown in Figure 4. Most products of the LF ligation were resolved as a ladder with variable sizes in the high molecular weight range. Their sizes were consistent with those expected from the tandem repeat unit indicated in Figure 3. In contrast, products of the CF ligation migrated as mixtures of fractions with variable sizes and forms.

Establishment of the assembled unit in *B. subtilis*

Seven microliters of the LF product or 20 μl of the CF product was used to transform competent *B. subtilis* RM125 cells by the standard method. The LF product for the two-fragment ligation yielded more than 2500 tetracycline resistant colonies. By the same transformation procedure, the LF product for the six-fragment ligation yielded 426. The CF product, however, yielded only one tetracycline-resistant colony with the six-fragment assembly, compared with 223 colonies with the two-fragment assembly (Table 1). The relevant antibiotic resistances were examined for 100 randomly selected colonies. It was shown that 98% of six fragments and 100% of the two fragments had the expected antibiotic resistance.

Plasmids prepared from 12 two-fragment colonies were the result of subcloning of the E fragment into pGETS109Sfl (Fig. 5A). Plasmids prepared from 12 six-fragment clones with the appropriate antibiotic resistance trait were analyzed using SflI. If the six fragments were correctly ligated and established using the *B. subtilis* transformation mechanism, then digestion by SflI would produce all fragments at an equal molar ratio. Results shown in Figure 5A indicated that all had an identical structure to that of the expected plasmid.
assembly by *E. coli* was unsuccessful. We refer to the method employed here as OGAB. Two additional experiments, the assembly of four fragments were carried out to compare, in quality and effectiveness, the OGAB method with engineering in *E. coli*.

**Design of the four-fragment assembly**

Two sets of four fragments, BCE plus pGETS109SfI (B, C, E, V), and ENS plus pGETS109SfI (E, N, S, V) were prepared. The fragments B, C, N and S, and pGETS109SfI were the same as those prepared above, while the Es, EmR1st and EmRlast, were newly designed and prepared to allow extension by ligation (Fig. 2B). *In vitro* ligation of these fragments at equal molar concentrations was carried out with the CF or LF and the transformation of *E. coli* or *B. subtilis* was conducted. The numbers of transformants selected with tetracycline for *B. subtilis* and ampicillin for *E. coli* are summarized in Table 1. The relevant resistance to antibiotics of the *B. subtilis* transformants was verified for all 100 colonies examined. An SfI digestion analysis of the plasmid from 12 *B. subtilis* colonies randomly selected from the LF ligation verified that they were identical to the expected plasmid referred as pGETS-BCE or pGETS-ENS (Fig. 5A). The number of *B. subtilis* transformants with the four-fragment assembly obtained by the LF ligation increased compared with that of the six-fragment case. In contrast, the number of *E. coli* transformants with the CF product remained low. But the CF products of the four-fragment assembly in both cases actually produced *E. coli* transformants that had the expected plasmid. The number remained lower than for the two-fragment ligation. Because the *E. coli* plasmid does not start replication from a linear substrate, it is conceivable that CF ligation preferentially produced cDNA plasmid.

**Reassembly of the six-fragment construct**

The pGETS-BCENS prepared from *E. coli* was completely digested by SfI, and the fragments were reused to apply the OGAB method after phenol extraction and ethanol precipitation. This procedure assures a precise equal molar ratio of all the components assuming a constant recovery through the phenol and ethanol treatment. The DNA mixture was subjected to the OGAB method and the ligation products were confirmed (Fig. 4). Surprisingly, the reassembled LF products yielded more than 4000 tetracycline-resistant colonies in three independent experiments. The relevant antibiotic resistance and the plasmid structure of 12 representative clones were verified. This result strongly indicates that

**Table 1. Number of transformants formed using ligation products**

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<tbody>
<tr>
<td>Bacillus subtilis RM125</td>
<td>2539 (100/100)</td>
<td>1130 (100/100)</td>
<td>662 (100/100)</td>
<td>426 (98/100)</td>
</tr>
<tr>
<td>CF</td>
<td>223 (100/100)</td>
<td>77 (75/75)</td>
<td>15 (14/15)</td>
<td>2 (1/2)</td>
</tr>
<tr>
<td>Escherichia coli JA221</td>
<td>1 (1/1)</td>
<td>0</td>
<td>2 (1/2)</td>
<td>0</td>
</tr>
<tr>
<td>CF</td>
<td>127 (12/12)</td>
<td>32 (11/12)</td>
<td>22 (4/12)</td>
<td>11 (0/11)</td>
</tr>
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</table>

LF and CF indicate ligation conditions. V represents the pGETS109SfI vector. Transformants of *B. subtilis* RM125 were selected using tetracycline. *Escherichia coli* JA221 transformants were selected using ampicillin. Numbers in parentheses indicate (colony with resistance to relevant antibiotics/colony examined) for *B. subtilis* and (transformant with the correct plasmid/transformant examined) for *E. coli*. 

Digestion with EcoT14I and BglII verified the structure as well as order of all the SfI fragments presented in Figure 2C (data not shown). The representative clone is referred to as pGETS-BCENS (Fig. 2C). The two clones lacking resistance to chloramphenicol or erythromycin had an unusual structure that was not analyzed further.

**No six-fragment assembly in *E. coli***

Two-fragment ligation with CF produced 127 *E. coli* transformants selected using ampicillin (Table 1). They were the result of subcloning of the E fragment into pGETS109SfI (Fig. 5B). However, six-fragment ligation with CF yielded only 11 colonies on ampicillin. SfI digestion of these 11 plasmids showed, however, unusual structures (Fig. 5B). The pGETS-BCENS obtained through *B. subtilis* transformed *E. coli* normally and transformants selected using ampicillin stably harbored the plasmid (data not shown). These results strongly indicated that direct cloning of the six-fragment

**Figure 4.** Products of T4 DNA Ligase *in vitro*. (Left panel) A mixture of six fragments prepared separately. The mixture was adjusted to have an equal molar concentration. (Right panel) Products of complete SfI digestion of pGETS-BCENS. Theoretically, they are all equal in moles. In the lane LF, cDNA indicated by arrowheads is preferentially formed. In the lane CF, cfDNA is preferentially produced. Conditions for CHEF electrophoresis: 1% agarose, run in 0.5 TAE at 13°C, pulse time 6 s, electric field 4.6 V cm⁻¹.
the molar ratio is critical to produce linear multimer form DNA in vitro, and accordingly the products efficiently transform B. subtilis to yield the designed plasmid.

**DISCUSSION**

A novel method for the cloning of multi fragments was examined based on the intrinsic nature of competent B. subtilis cells. The efficiency of the OGAB method is demonstrated by the finding that assembled plasmids were obtained in all the experiments. Even the six-fragment assembly yielded a substantial number of clones whereas the E. coli system produced none. Plasmid establishment in B. subtilis requires concatamer form DNA as a donor for transformation (Fig. 1). The effectiveness of the multimeric form DNA is explained by the observation that competent B. subtilis is transformed not by a monomeric circular plasmid lacking a redundant sequence, but by a multimeric form having a redundant copy per molecule to repair the lost sequence after the DNA is taken up in the cell (15). The increased efficiency of these multimeric plasmids was probably due to the redundant sequences used for repair. The OGAB method requires function of recA gene of B. subtilis. Therefore, recA mutant host is inappropriate for plasmid construction. A copy number-controllable plasmid, pGETS109, was constructed as an alternative way to reduce the interaction between plasmids in B. subtilis. The copy number of pGETS109, normally one copy per cell, is increased by addition of IPTG when a large amount of plasmid DNA should be required.

It was shown that adjustment of the molar ratio is critical for obtaining clones efficiently. The formation of a high molecular weight product in the in vitro ligation is regulated by various factors. Excess fragments resulting from a slight molar ratio difference may inhibit formation of the higher molecular weight ligation product, due to two reasons: the concentration of all the substrate fragments at the beginning of the ligation reaction is sufficient to make equal the rate for all combinations of pairings, and as the reaction proceeds and the substrate concentration decreases at an equal rate, smaller amounts of fragments are consumed and the excess does not allow extension. Equal amounts of fragments allow the ligation reaction to continue near completion. The result is supported by the result of reassembly of six-fragment ligation and also consistent with the efficiency of plasmid reconstruction by digestion with BglI and ligation in E. coli (31).

SfiI recognizes the 8 nt sequence 5'-GGCNCNNN/NGGCC-3' (17) with 5 variable nt. The three-base staggerings formed by SfiI digestion provide at most 4³ = 64 cases of 3'-protruding ends. As combinations of pairings are given by 64/2 = 32, the assembly of up to 32 fragments can be designed in theory. Our preliminary experiment, however, suggested that the efficiency of ligation is likely sequence-dependent. For example, the pairing of 5'-AAA-3' with 5'-TTT-3' did not produce ligation products under the conditions employed. In the present study, we chose the base composition according to the putative rule that the 3 nt are comprised of two A/T and one C/G. Optimization of the in vitro ligation remains to be investigated with factors including concentration of DNA, DNA fragment size, temperature, choice and concentration of monovalent cation, and requirement for polyethylene glycol (11–13).

The OGAB method introduced in this study is an effective way to assemble a number of genes normally dispersed at multiple loci or diverged among different species. Several applications to various DNA segments from bacteria to eukaryotes are in progress. The combinational use of other SfiI-like enzymes such as BsaXI (32), Earl (32,33) and BstXI (32) would facilitate construction of a library with all combinations of orientation and order. In combination with gene or genome shuffling methods (34), the OGAB method would be of use to create more diversity for novel bioprocessing systems. It may be feasible to assemble a minimal gene set necessary for one organism (3,4).

**ACKNOWLEDGEMENTS**

This research was partially supported by the New Energy and Industrial Technology Development Organization (NEDO).