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High-Frequency Electroporation and Maintenance of pUC- and pBR-Based Cloning Vectors in *Pseudomonas stutzeri*

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Abstract. A number of *Escherichia coli* cloning vectors, based on ColE1-like replicons, were shown to be maintained in *Pseudomonas stutzeri* ATCC 17588. A restrictionless mutant of *P. stutzeri* was isolated, and this strain was used to develop an efficient electroporation system. With the *E. coli* cloning vector pHSG298, transformation frequencies of up to 2×10^7 transformants/μg DNA were achieved. This frequency is comparable to that obtained for $CaCl_2$-mediated transformation of *E. coli*; thus, direct cloning of DNA into *P. stutzeri* is feasible. As will be discussed, this may prove useful for cloning DNA from high mol% G + C genera in cases in which *E. coli* is not a suitable heterologous cloning host.

Pseudomonas stutzeri is a nonfluorescent denitrifying pseudomonad, of minor clinical importance [10], that is widely distributed in soil and water [13]. Phylogenetically, it is grouped with the fluorescent pseudomonads and enterics in the γ-3 subdivision of proteobacteria [25]. An investigation into genetic transfer in *P. stutzeri* revealed that 10 of 17 strains tested could be naturally transformed with homologous chromosomal DNA [3]. Further studies of natural transformation in *P. stutzeri* have been largely restricted to strain JM300 and its derivatives [11, 12, 20]. One study assayed plasmid transformation with a variety of broad-host-range plasmids and one based on a ColE1-like replicon. No natural (or artificial) transformation was detected except when homologous chromosomal DNA was cloned into one of the broad-host-range plasmids [4]. Only one other strain of *P. stutzeri* has been developed genetically, namely, strain ZoBell (formerly *P. perfectomarina* [6]), which has been used extensively to investigate the genetic basis of denitrification [23]. Strains JM300 and ZoBell can exchange chromosomal markers by natural transformation, which suggests a close relationship [19]. In both of these strains, plasmids based on ColE1-like replicons have been shown to be incapable of replication [23, 24]. We report here that plasmids based on ColE1-like replicons can replicate in *P. stutzeri* ATCC 17588. We have developed a high-efficiency electroporation protocol to introduce such plasmids; this potentiates direct cloning of DNA into *P. stutzeri*.

Methods and Materials

Growth conditions, bacterial strains, and plasmids. Unless stated otherwise, cultures were grown on Z media [22] at 37°C. Xgal media contained 16 g/L casein hydrolysate, 10 g/L yeast extract, 5 g/L NaCl, 50 m*M* 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, and 40 m*M* isopropyl-β-D-thiogalactopyranoside. Where appropriate, media were supplemented with 100 μg of streptomycin (Sm)/ml or 50 μg of kanamycin (Km)/ml. Bacterial strains and plasmids are given in Table 1.

Genetic techniques. Conjugal transfer was achieved by mixing equal amounts ($\sim 1 \times 10^9$ colony-forming units) of fresh donor and recipient cells in 0.9% (wt/vol) NaCl and spotting the mix onto a Z agar plate. After incubation for 4 h at 37°C, the mating was diluted and plated onto appropriate selective media.

Plasmid DNA was extracted from JM109 cultures by alkaline lysis [2]. This DNA was used for electroporation experiments except in one case in which the plasmid DNA used was extracted from JM109 with a Qiagen ion-exchange tip (Qiagen Inc., Chatsworth, California) according to manufacturer's instructions. DNA concentration was estimated by serially diluting linearized plasmid DNA and comparing the fluorescence with a serially diluted lambda DNA standard [17]. Restriction digests were performed according to manufacturer's instructions.

Optimized electroporation of *Pseudomonas stutzeri*. Electroporation was performed with a Bio-Rad Gene Pulser and cuvettes with a 0.1-cm electrode gap. Used cuvettes were recycled by boiling in distilled water for 30 min followed by propylene oxide gas sterilization. All the growth from a 15-h plate culture was harvested, resuspended in 1 ml of ice-cold 10% glycerol (in distilled

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Table 1. Bacterial strain and plasmids

Strain/plasmid	Relevant genotype/phenotype[a]	Source
Escherichia coli		
JM109	*hsdR17* (r_K^- m_K^+)	[26]
Pseudomonas stutzeri		
ATCC 17588 (UQM782)	type/wild strain	UQM[b]
JMP2536	UQM782 Sm^r	This report
JMP2537	UQM782 Sm^r res−	This report
Plasmids		
pRK2013::Tn*813*::Tn*5-crt*+	*tra*+, ColE1 replicon	[16]
pHSG298	Km^r, ColE1 replicon	[21]
pJP116	Ap^r Km^r *crt*+, ColE1 replicon	[14]

[a] *hsdR17* (r_K^- m_K^+), host-determined restriction and modification; Sm^r, streptomycin resistance; *res*−, restrictionless; *tra*+, conjugal transfer ability; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; *crt*+, carotenoid biosynthetic genes of *Rhodobacter sphaeroides*.

[b] UQM indicates the University of Queensland Microbiology Culture Collection.

water), and pelleted by centrifugation at 12,000 *g* for 70 s. This washing procedure was repeated four times with a Pasteur pipette used to resuspend cell pellets. The final cell pellet was resuspended in 200 μl of ice-cold 10% glycerol. Aliquots (40 μl) were mixed with 1 μl of pHSG298 DNA (0.1 μg/μl) and transferred to a prechilled cuvette. Following a single pulse (25 kV/cm, 200 Ω, 25 μF), the cells were immediately resuspended in 5 ml SOC buffer [7] and incubated at 28°C for 60 min. Aliquots were then spread on Z agar plates containing Sm and Km. Colonies were counted after 3 days' incubation.

Results

Maintenance of plasmids based on ColE1-like replicons in *Pseudomonas stutzeri*. Recently, we used *P. stutzeri* as a heterologous expression host for the carotenoid biosynthesis genes of *R. sphaeroides* [16]. As an extension of these studies, we subsequently tried to obtain transpositions of transposon Tn*5-crt*+ into the *P. stutzeri* chromosome. For this we used plasmid pRK2013::Tn*813*::Tn*5-crt*+ which is based on a ColE1-like replicon, these replicons being commonly used as suicide vectors [8, 18]. JM109 pRK2013::Tn*813*::Tn*5-crt*+ was mated with *P. stutzeri* JMP2536, and exconjugants were selected on Z agar containing Sm and Km. Exconjugants, unlike the donors, were orange because of expression of the carotenoid gene cluster. However, the conjugal transfer frequency obtained (10^{-3} exconjugants/recipient) was much too high to be accounted for by transposition of the ~35 kilobase (kb) Tn*5-crt*+. Instead, the frequency suggested that the ColE1-like replicon was being maintained in *P. stutzeri*. This was supported by the ability to conjugally transfer the plasmid back to JM109 and by the invariable appearance of white Km-sensitive segregants when orange exconjugants were streaked on non-selective plates. As the vast majority of *Escherichia coli* cloning vectors are based on the ColE1-like family of replicons [1], we were interested to see whether any additional vectors could be maintained in JMP2536. As Km provided very clean selection in *P. stutzeri*, we attempted to electroporate JMP2536 with the Km vectors pHSG298 (pUC-based) and cosmid pJP116 (pBR-based). The *E. coli* electroporation protocol [7] was used except for the method of cell preparation (see Methods and Materials). Transformants were obtained with both pHSG298 (1×10^4/μg DNA) and pJP116 (40/μg DNA), the latter being orange because of expression of the carotenoid genes. The reason for the difference in electroporation frequencies was not clear, although the larger size of pJP116 (45 kb, compared with 2.7 kb for pHSG298) would have accounted for this at least in part. When cell/DNA mixes were not exposed to an electric pulse, no transformants resulted. The pigmentation of pJP116 transformants provided a ready marker to assay plasmid stability. JMP2536 pJP116 was subcultured at 24-h intervals in 10 ml Z broths for 7 days, and then cells were plated out on Z Sm plates. Some 11% (32/297) of resultant colonies were white and, upon patching, Km sensitive. Thus, pJP116 was quite stable in JMP2536. To confirm the maintenance of pHSG298 in *P. stutzeri*, plasmid DNA was isolated by the alkaline lysis technique. Yields obtained were very poor. When an entire extract was electrophoresed, plasmid DNA was visible but very faint. This barrier was circumvented by electroporating the plasmid DNA back into JM109, which resulted in many thousands of transformants. Estimation of the electroporation frequency was precluded by the very low concentration of the pHSG298 DNA. The presence of pHSG298 was confirmed by extracting plasmid DNA from six transformants and digesting with *Hin*dIII to reveal the expected bands of 2.1 and 0.6 kb. Plasmid pHSG298 is an α-complementation vector, so, as an additional test of its presence, 200 transformant colonies were patched onto Xgal plates containing Km. All patches turned blue.

Isolation of a restrictionless mutant of *P. stutzeri*. It seemed probable that the electroporation of

Table 2. Time constants and electroporation frequency

Time constant (ms)	Frequency (transformants/μg DNA)
2.5	1.2×10^5
5	2.0×10^5
10	1.2×10^6
15	1.0×10^6
20	1.4×10^6

JMP2536 with pJP116 had selected restrictionless mutants. To test this, a white Km-sensitive segregant was isolated from a JMP2536 pJP116 culture growing on nonselective media and electroporated with pHSG298 DNA. The transformation frequency of $2 \times 10^5/\mu$g DNA represented a 20-fold increase compared with the frequency of $1 \times 10^4/\mu$g obtained in the parallel experiment with strain JMP2536. This putatively restrictionless strain (JMP2537) was used in all further electroporation experiments.

Optimization of *P. stutzeri* electroporation. Initial electroporation experiments with *P. stutzeri* followed the protocol used for *E. coli* [7] except for the method of cell preparation (see Methods and Materials). In summary, plasmid DNA was mixed with 40 μl cells and exposed to a pulse (12.5 kV/cm, 200 Ω, 5 ms, 25 μF) before being resuspended in 1 ml SOC medium. After stationary incubation at 37°C for 1 h, the cells were plated out on selective media. With plasmid pHSG298 and strain JMP2537, this procedure resulted in a transformation frequency of $2 \times 10^5/\mu$g DNA. To improve this frequency, parameters of the *E. coli* protocol were independently investigated. When the time constant was varied from 2.5 to 20 ms (Table 2), there was a modest increase in electroporation frequency between 5 and 10 ms with insigificant increases above this. The 5- and 10-ms time constants were compared at varying field strengths (Table 3). For both time constants, the yield increased as the field strength increased. However, the advantage of using a 10-ms time constant was negated by the arcing that invariably occurred with field strengths above 20 kV/cm. Thus, the highest frequency was obtained at a field strength of 25 kV/cm and a time constant of 5 ms. It should be noted, however, that arcing was still relatively frequent under these conditions and that, routinely, a field strength of 22.5 kV/cm would yield more reliable results. Investigation of various post-pulse incubation conditions (Table 4) revealed that they had only a small influence on

Table 3. Field strengths, time constants, and electroporation frequency[a]

Field strength (kV/cm)	Time constant	
	5 ms	10 ms
15.0	8.1×10^5	1.2×10^6
17.5	1.2×10^6	2.8×10^6
20.0	2.3×10^6	4.0×10^6
22.5	4.6×10^6	0[b]
25.0	6.0×10^6	0[b]

[a] Electroporation frequency given in transformants/μg DNA.
[b] Under these conditions, arcing invariably occurred.

Table 4. Post-pulse variables and electroporation frequency

Variable	Frequency (transformants/μg DNA)
Standard[a]	2.0×10^5
Z medium for outgrowth	1.5×10^5
5 ml outgrowth medium	3.0×10^5
Outgrowth at 28°C	3.0×10^5
Outgrowth shaking at 225 rpm	1.0×10^5
90 min outgrowth period	2.6×10^5

[a] See text for description of standard procedure.

electroporation frequencies. By combining the optimized pulse and post-pulse conditions, the electroporation frequency was increased to a maximum of 2×10^7 transformants/μg DNA. The same frequency was obtained when highly pure pHSG298 DNA, prepared with a Qiagen tip, was used.

Discussion

Plasmids based on ColE1-like replicons have a narrow host range [1]. Such plasmids (pBR-based) have been shown incapable of replication in the JM300 and ZoBell strain lines of *P. stutzeri* [23, 24]. Thus, it was somewhat surprising to find that *P. stutzeri* ATCC 17588 could maintain both pBR- and pUC-based ColE1-like replicons. However, it should be noted that a high level of intraspecific phenotypic variation exists within *P. stutzeri* as currently defined. This has led several authors to suggest that the group may contain two or more species [9, 10, 13]. Indeed, there is only 60% DNA homology between strain ZoBell and ATCC 17588 [6]. Interestingly, the JM300 and ZoBell strain lines cannot maintain ColE1-like plasmids but are naturally transformable [19], whereas ATCC 17588 is not nat-

urally transformable [3] but can maintain ColE1-like plasmids. The ability to maintain ColE1-like plasmids and the capacity for natural transformation may be useful characteristics for clarifying strain relationships within the *P. stutzeri* group.

We have demonstrated that *P. stutzeri* ATCC 17588 can maintain plasmids based on ColE1 replicons, these replicons forming the basis of most *E. coli* cloning vectors. Furthermore, these replicons can be introduced into *P. stutzeri* at a frequency comparable to $CaCl_2$-mediated transformation of *E. coli*. Therefore, direct cloning of DNA into *P. stutzeri* is feasible, obviating the need for broad-host-range cloning vectors. This will facilitate the genetic analysis of *P. stutzeri* for two reasons. Firstly, broad-host-range vectors are not well developed as cloning vectors compared with *E. coli* cloning vectors [15]. Secondly, the use of broad-host-range cloning vectors necessitates time-consuming gene transfer steps after the cloning steps are completed.

Direct cloning into *P. stutzeri* will, in some cases, provide a useful alternative to direct cloning into *E. coli*. It has been reported that a number of genes from genera such as *Rhodobacter, Bacillus, Myxococcus, Agrobacterium, Caulobacter,* and *Pseudomonas* are not expressed in *E. coli* [5]. In explanation, it was suggested that differences in mol % G + C may have given rise to differences in promoter specificity, ribosome structure, and mRNA secondary structure. *P. stutzeri* ATCC 17588 has a mol % G + C of 65 [13] compared with 48 for *E. coli* [5]. Thus, *P. stutzeri* may be useful for cloning genes from high mol % G + C genera such as *Rhodobacter, Myxococcus,* and *Streptomyces* when expression is not found in *E. coli*. For instance, in the field of bacterial photosynthesis, it is known that most *Rhodobacter* genes are not expressed in *E. coli* [5]. We have found that *P. stutzeri* expresses both the *R. sphaeroides* carotenoid gene cluster and the *pps* gene [16], and we are currently in the process of cloning the bacteriochlorophyll gene cluster directly into *P. stutzeri*.

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