Direct transformation of site-saturation libraries in *Bacillus subtilis*

Neelam S. Amin, Stephanie Wong, and Volker Schellenberger
Genencor International, Palo Alto, CA, USA

BioTechniques 35:1134-1140 (December 2003)

Bacillus subtilis is a commonly used screening host for protein production and engineering because of its ability to secrete large quantities of proteins. However, direct transformation of site-saturation libraries or products of site-directed mutagenesis in this organism is highly inefficient because of the limited development of molecular cloning tools (1). Thus, libraries and mutant plasmids are first constructed in *Escherichia coli* and then introduced into *B. subtilis*. This procedure is followed because of the limited efficiency of in vitro plasmid ligation, which results in incompletely ligated products containing nicks and monomeric DNA, which can effectively transform *E. coli* but not *B. subtilis*. This indirect approach presents numerous limitations, including the inability to use desired plasmid systems because of their toxicity in *E. coli* (2), greater library bias by going through two hosts, the need for longer protein engineering and development times, and the inability to make high-throughput cloning a robust process.

The use of plasmid multimers for transforming *B. subtilis* has been described in the literature (3,4). However, multimeric plasmids are not generally formed during commonly used mutagenesis procedures. An alternative method that allows the generation of plasmid libraries in *B. subtilis* is plasmid marker rescue, but it too has its limitations, as it requires a two-plasmid system (5). Despite much work in the area, there is a lack of methods for the easy generation of libraries and mutants in *B. subtilis*. In particular, it would be desirable to eliminate the use of *E. coli* and directly introduce libraries and mutants into *B. subtilis* to produce proteins of interest. Here we describe the direct transformation of *B. subtilis* with site-saturation libraries created by modifying the QuikChange™ (QC) site-directed mutagenesis kit conditions (Stratagene, La Jolla, CA, USA) (6). In particular, we utilized the enzyme mixture provided in the QuikChange multi site-directed mutagenesis kit (QCMS; Stratagene), which includes a thermostable DNA polymerase and an enzyme(s) to seal nicks within double-stranded DNA, presumably a thermostable ligase.

To determine if we could create *B. subtilis* libraries without an intermediate host such as *E. coli*, we constructed site-saturation libraries using the QC kit under standard and modified conditions, and transformed the reaction products directly into *B. subtilis*. The libraries were created at the first amino acid position of an alkaline serine protease gene from *Bacillus lentus* under the regulation of the *B. subtilis aprE* promoter. In these experiments, the *B. subtilis* high-copy replicating plasmid pVS02 [6-kb *E. coli* and *B. subtilis* shuttle plasmid (4) containing a pUB110 origin of replication and a chloramphenicol resistance marker] was used.

First, the site-saturation library SW1 was constructed using the QC kit under standard conditions. In particular, 50- to 100-ng template plasmid pVS02**,** 1 µL primer A1NNS-FP (25 µM), 1 µL primer A1NNS-RP (25 µM), 1.5 µL dNTPs (QC kit), 5 µL 10× QC reaction buffer, 39.5 µL deionized water, and 1 µL of *PfuTurbo*® DNA polymerase (QC kit), were used in a 50-µL QC reaction, according to the manufacturer's instructions. The thermal cycler program used was one cycle at 95°C for 2 min, followed by 17 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min. *Dpn*I digestion was performed twice by addition of 1 µL *Dpn*I from the QC kit followed by incubation at 37°C for 1–2 h to remove the template DNA. Template DNA was obtained from *E. coli dam*+ strains such as TOP10 (Invitrogen, Carlsbad, CA, USA) or XL1-Blue (6), as the DNA must be methylated to allow *Dpn*I digestion; alternatively, in vitro methylation can be used. A template alone (i.e., no primer) control was carried through all of the steps of the experiment, to monitor the efficiency of *Dpn*I digestion. The QC product was assessed by running 5 µL of the reaction on a 0.8% agarose E-gel® (Invitrogen). Finally, 5 µL of the reaction were transformed into *B. subtilis* (see legend to Table 2). The SW1 library constructed using such standard QC conditions resulted in only three *B. subtilis* colonies upon transformation in the comK strain NSA40, whereas a typical *E. coli* transformation under similar conditions would yield 10–1000 colonies (Stratagene QuickChange manual; http://www.stratagene.com/manuals/

Table 1. Primer Pairs Used to Generate Site-Saturation Libraries

A1NNS-FP: 5′-Phosp-GCAGAAGTAACGACAATGNNSCAATCAGTGCCATGGGGA-3′
A1NNS-RP: 5′-Phosp-TCCCCATGGCACTGATTGSNNCATTGTCGTTACTTCTGC-3′
SW-84F-P: 5′-Phosp-AATATTCGTGGTGGCGCTAGCNNSGTACCAGGGGAACCATC-3′
SW-84R-P: 5′-Phosp-GATGGTTCCCCTGGTACSNNGCTAGCGCCACCACGAATAT-3′
SW-85F-P: 5′-Phosp-GTATTAGGGGCGAGCGGTNNSGGTTCGGTCAGCTCGATTG-3′
SW-85R-P: 5′-Phosp-AATCGAGCTGACCGAACCSNNACCGCTCGCCCCTAATAC-3′
SW-86F-P: 5′-Phosp-GGGGCGAGCGGTTCAGGTNNSGTCAGCTCGATTGCCCAAG-3′
SW-86R-P: 5′-Phosp-TTGGGCAATCGAGCTGACSNNACCTGAACCGCTCGCCC-3′
SW-87F-P: 5′-Phosp-TCAGGTTCGGTCAGCTCGNNSGCCCAAGGATTGGAATGG-3′
SW-87R-P: 5′-Phosp-CCATTCCAATCCTTGGGCSNNCGAGCTGACCGAACCTG-3′
SW-88F-P: 5′-Phosp-GTAGCGGCATCTGGAAATNNSGGTGCAGGCTCAATCAGC-3′
SW-88R-P: 5′-Phosp-GCTGATTGAGCCTGCACCSNNATTTCCAGATGCCGCTAC-3′
N = A, C, G, or T; S = G or C.

Table 2. Creation of Six Site-Saturation Libraries Directly in *Bacillus subtilis*

Library	Number of Colonies (Plasmid pVS08)	
	Strain NSA40	Strain NSA41
A1	>1000	>3000
F49	56	101
S99	850	562
S101	800	337
I105	>1000	478
S154	>1000	>2000
No DNA	0	0

All *B. subtilis* transformations were carried out in comK-induced competent strains NSA40 (*PxylA-comk-ble, ΔaprE, ΔnprE, Δisp, Δepr, Δbfp, sacUh32, spo*) or NSA41 (*PxylA-comK-ble, ΔaprE, ΔnprE, sacUh32, spo*), which were constructed as described previously (7,8). Cells were made competent by inducing expression of the competence gene *comK* using a xylose-inducible promoter PxylA, and 100 µL of NSA40 or NSA41 competent cells were transformed using 5 µL of each of the QC-modified reaction mixtures as described previously (7,8).

200518.pdf). Purification of the SW1 QC reaction product to remove any possibly inhibiting buffer and enzyme components also resulted in only one transformant (data not shown). These results were not surprising as the QC reaction produces monomeric DNA with nicks, which is not suitable for *B. subtilis* DNA transformation (3,9).

Subsequently, we tested the incorporation of a ligase during the QC reaction to seal nicks and possibly form plasmid multimers, which are known to transform *B. subtilis* very efficiently (3,9). We used the QCMS kit reagents, which contain an "enzyme blend" of the *PfuTurbo* thermostable polymerase as well as a thermostable nick-sealing enzyme(s). Using primers A1NNS-FP and A1NNS-RP (Table 1) and pVS02 as the template DNA, QCMS reagents, and QC cycling conditions, we created library SW2. This reaction included 50–100 ng template plasmid pVS02, 0.5 µL forward primer (25 µM), 0.5 µL reverse primer (25 µM), 1 µL dNTPs (QC kit), 2.5 µL 10× QCMS reaction buffer, 18.5 µL deionized water, and 1 µL of enzyme blend (QCMS kit), for a total volume of 25 µL. The cycling conditions and *Dpn*I digestion were performed as described for the QC reaction. Next, 5 µL of the reaction were transformed directly into the *B. subtilis* strain NSA40. A 1000-fold increase in the number of transformants was observed with library SW2 compared to SW1. These results indicate that by incorporating a nick-sealing enzyme into the QC reaction, the library DNA became more suitable for direct transformation of *B. subtilis*.

To further test the modified QC method, we constructed six additional site-saturation libraries. We used a different template plasmid, pVS08 (5.5 kb; derivative of pVS02 containing a shortened *aprE* promoter), which expresses high levels of protease, causing toxicity in *E. coli*. We also tested if a slightly different comK *B. subtilis* strain NSA41 (Table 2) could be used for library transformation in addition to strain NSA40. Six libraries were made at amino acid positions A1, F49, S99, S101, I105, and S154 in the serine protease with the six primer pairs shown in Table 1 in a manner similar to library SW2 described above. Transformation of the library DNA into the *B. subtilis* strain NSA40 produced 56–1000 colonies, and 100–3000 colonies into strain NSA41 (Table 2). As the diversity in a site-saturation library is only 32 different codons, the number of transformants obtained represented an approximately 2- to 90-fold coverage of the theoretical diversity in each library. In the case of the library at position F49, which produced only 56 or 100 colonies, we observed that the reaction volume had been reduced during the cy-

Table 3. Sequence Analysis of Random *Bacillus subtilis* Clones from Six Site-Saturation Libraries

Clone (No.)	GCG → NNS Ala 1	TTT → NNS Phe 49	TCA → NNS Ser 99	TCG → NNS Ser 101	ATT → NNS Ile 105	TCA → NNS Ser 154
1	TTG (L)	ACC (T)	ATG (M)	GAG (E)	GTG (V)	CCC (P)
2	TAG (*)	TGC (C)	AGC (S)	ACG (T)	GTG (V)	CTC (L)
3	TGG (W)	CCG (P)	GCG (A)	WT	TGG (W)	AGG (R)
4	WT	WT	TGG (W)	GGC (G)	TTG (L)	ATC (I)
5	WT	CAC (H)	GGG (G)	GTG (V)	TGG (W)	CTC (L)
6	AAG (K)	GAC (D)	GAC (D)	TGG (W)	GTG (V)	CTC (L)
7	TAG (*)	ATC (I)	ACG (T)	GCG (A)	GTC (V)	TAG (*)
8	ACG (T)	GTC (V)	CTC (L)	CAG (Q)	TGC (C)	GAC (D)
9	AAC (N)	GTG (V)	GGG (G)	GCC (A)	GCG (A)	GTC (V)
10	GGG (G)	CGC (R)	ACC (T)	GCC (A)	TTG (L)	TTG (L)
11	CAG (Q)	ACC (T)	CAG (Q)	GGC (G)	GGG (G)	GGG (G)
12	CTG (L)	ACC (T)	TCG (S)	GGG (G)	AGC (S)	TAG (*)
13	ACG (T)	CGG (R)	ATG (M)	AGC (S)	GTG (V)	TCC (S)
14	TGC (C)	GAG (E)	CAC (H)	TAG (*)	GGG (G)	CGC (R)
15	AGC (S)	AAC (N)	AGC (S)	CGG (R)	TCG (S)	WT
16	WT	CAG (Q)	TCG (S)	CAG (Q)	AGG (R)	GTG (V)
17	TGC (C)	AAG (K)	TAG (*)	AAC (N)	AGG (R)	WT
18	CGC (R)	TGG (W)	AAC (N)	ATG (M)	TAG (*)	AAC (N)[a]
19	CTC (L)	TAG (*)	GAC (D)	AAG (K)	CGC (R)	WT
20	TGG (W)	GAG (E)	TTG (L)	ACC (T)	TGG (W)	AGC (S)[a]
21	AAG (K)	CAG (Q)		CTG (L)	AAC (N)	ACG (T)
22	CAC (H)	TGG (W)		GGC (G)	AAG (K)	ACC (T)
23	GGG (G)	CGC (R)		GCG (A)	TTG (L)	WT
24	AAG (K)			ACC (T)	TAG (*)	TTC (F)
Codons	15	16	15	16	13	17
Residues	11	13	11	12	10	10
WT codon	3	1	0	1	0	4

[a]Two undesired mutations were observed: deletion of C in CAG of Gln 185 in clone no. 18 library S154; G inserted 11 bp upstream of TCA of S154 in clone no. 20 of S154. Letters in parentheses represent the single letter designation for amino acids encoded by the codon.

cling program, which was most likely caused by a faulty PCR tube. This produced concentrated buffer and enzyme components, which appeared to inhibit the *B. subtilis* transformation step, since 1392 colonies were obtained when a smaller amount of the reaction mixture (3 µL instead of 5 µL) was transformed into strain NSA41. Similarly, in other libraries, doubling the reaction amount transformed resulted in fewer colonies (data not shown). Although the present study tested transformation in comK strains due to the ease in their transformation, we expect the method to work in non-comK strains as well, since the mechanism of DNA uptake is similar in either strain.

The protease gene in several clones from each of the libraries was sequenced to test the efficiency of mutagenesis. We sequenced the entire open reading frame of the protease gene in 20–24 clones from each of the six libraries generated in NSA40 (Table 3). In general, we observed mutations at the expected positions in each of the libraries. Out of the possible 32 codons that are represented by the NNS codon, between 13 and 17 different codons (10–13 different amino acids) were observed in each library. In 139 clones that were sequenced, all amino acids except tyrosine were observed (four TAC codons were expected; it is possible that sequencing a larger number of clones would produce the expected number of tyrosines). In each of the libraries, the number of wild-type or parent codons observed was between zero and four (0%–17%). Overall, the libraries were diverse and contained a low number of wild-type transformants compared with those seen in *E. coli* (up to 40%).

It is likely that addition of other commercially available thermostable ligases to the QC reaction can be used instead of QCMS reagents, although it may be necessary to optimize the reaction conditions. Using *Taq* thermostable ligase in the QC reaction only resulted in a 4-fold increase in transformation efficiency, possibly because the reaction conditions were not optimized (data not shown). In addition, it is important that a nick-sealing enzyme is present during the QC reaction because, although addition of T4 DNA ligase after the QC reaction resulted in a 7- to 300-fold increase in transformation efficiency, the products obtained contain a duplication of the mutagenic primer (data not shown). Such duplications are expected upon postreaction ligation due to the complementary primers used in the reaction.

Most methods used for producing site-directed, deletion, insertion, and random (10) mutants do not generate plasmid multimers, so typical mutagenesis products cannot be efficiently transformed directly into *B. subtilis*. However, the addition of a nick-sealing enzyme during the QC reaction may aid formation of multimeric DNA, which is known to enhance the transformability of *B. subtilis* (3,9). For example, the QC and the QCMS kits by themselves were not useful in forming DNA suitable for *B. subtilis* transformation (data not shown for QCMS kit) presumably because the product of the QC reaction is monomeric and nicked and that of the QCMS reaction is single-stranded. Formation of plasmid multimers has been described by in vitro ligation of linear plasmids, but it requires very high DNA concentrations (3). Multimers can also be formed via a PCR-like reaction starting from two overlapping plasmid fragments as template (4). However, this process is rather mutagenic given the long extension cycles that are required. Although the state of the DNA produced by our mutagenesis method was not assessed, it is likely that an adequate number of plasmid multimers were formed, which resulted in a dramatic increase in the number of *B. subtilis* transformants.

Although high copy number replicating plasmids are widely used for mutagenesis screens because they confer high protein expression levels, integrating plasmids are also used occasionally. Integrating plasmids do not contain an origin of replication and therefore require insertion into the host chromosome by recombination to be stably maintained. Using an integrating plasmid that contained two homology recombination regions (600–800 bp) flanking a gene of interest, we constructed a single site-saturation library using the mutagenesis method described in this study. Although the transformation efficiency was not as robust as that described for replicating plasmids, we still observed a 21-fold increase in the number of transformants obtained (data not shown). Thus, the modified QC mutagenesis method described in this study may be used for integrating plasmids in addition to replicating plasmids.

We describe a mutagenesis method that allows the direct transformation of libraries to *B. subtilis*, which is an excellent organism for protein engineering and production. This method bypasses the use of *E. coli* as a shuttle organism and consequently provides numerous benefits. First, it eliminates problems associated with sequences that will not replicate in *E. coli,* and therefore would result in a loss of diversity in the DNA library. Second, it allows the use of high copy number plasmids that are often deleterious to *E. coli* because of protein toxicity (e.g., pVS08). Third, it shortens the protein engineering time significantly. Finally, it makes high-throughput screening in the production host an option. Because of its ease of use and rapidity, this method will be broadly useful to researchers in the areas of directed evolution and lead candidate screening.

REFERENCES

1. **Ehrlich, S.D.** 1978. DNA cloning in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA *75*: 1433-1436.
2. **Strausberg, S.L., P.A. Alexander, D.T. Gallagher, G.L. Gilliland, B.L. Barnett, and P.N. Bryan.** 1995. Directed evolution of a subtilisin with calcium-independent stability. Biotechnology *13*:669-673.
3. **Mottes, M., G. Grandi, V. Sgaramella, U. Canosi, G. Morelli, and T.A. Trautner.** 1979. Different specific activities of the monomeric and oligomeric forms of plasmid DNA in transformation of *B. subtilis* and *E. coli*. Mol. Gen. Genet. *174*:281-286.
4. **Shafikhani, S., R.A. Siegel, E. Ferrari, and V. Schellenberger.** 1997. Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization. BioTechniques *23*:304-310.
5. **Contente, S. and D. Dubnau.** 1979. Marker rescue transformation by linear plasmid DNA in *Bacillus subtilis*. Plasmid *2*:555-571.
6. **Weiner, M.P., G.L. Costa, W. Schoettlin, J. Cline, E. Mathur, and J.C. Bauer.** 1994. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. Gene *151*:119-123.
7. **Diaz-Torres, M.R., V. Schellenberger, O.V. Selifonova, T.B. Morrison, and E.W. Lee.** 2002. Bacillus transformation, transformants

and mutant libraries. WIPO PCT Publication No. WO 0214490. 21 February, 2002.
8. **Hahn, J., A. Luttinger, and D. Dubnau.** 1996. Regulatory inputs for the synthesis of ComK, the competence transcription factor of *Bacillus subtilis*. Mol. Microbiol. *21*: 763-775.
9. **Canosi, U., G. Morelli, and T.A. Trautner.** 1978. The relationship between molecular structure and transformation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. Mol. Gen. Genet. *166*:259-267.
10. **Miyazaki, K. and M. Takenouchi.** 2002. Creating random mutagenesis libraries using megaprimer PCR of whole plasmid. BioTechniques *33*:1033-1038.

Received 5 June 2003; accepted 22 September 2003.

Address correspondence to Neelam S. Amin, Genencor International, 925 Page Mill Road, Palo Alto, CA 94304, USA. e-mail address: namin@genencor.com

Heparinase treatment of RNA before quantitative real-time RT-PCR

Mary Lynn Johnson, Chainarong Navanukraw, Anna T. Grazul-Bilska, Lawrence P. Reynolds, and Dale A. Redmer
North Dakota State University, Fargo, ND, USA

BioTechniques 35:1140-1144 (December 2003)

Quantitative real-time reverse transcription PCR (RT-PCR) is a highly sensitive method for detecting changes in gene expression. Heparin was identified as an inhibitor of enzymatic reactions, similar to Moloney murine leukemia virus (MMLV) reverse transcriptase and *Taq* DNA polymerase reactions more than a decade ago (1–3). Most other inhibitors of RT-PCR may be removed from DNA or RNA during careful isolation of nucleic acids. Heparin presents a unique problem because it appears to co-purify with the RNA throughout numerous types of isolation procedures, even those using column purification (4). Methods for removing heparin from DNA and RNA using heparinase have been developed (2,3,5,6). Although RNA is much more susceptible to degradation than DNA during these treatments, the use of an RNase inhibitor (RNasin®; Promega, Madison, WI, USA) during the heparinase treatment appears to overcome the problems associated with using heparinase that is not certified RNase-free. Here we present a direct method for treating RNA samples with heparinase using the RNase inhibitor, buffer, and $MgCl_2$ from the TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA, USA) and then proceeding with quantitative real-time RT-PCR without further quantification or purification of the treated RNA.

For this study, RNA was isolated separately from the granulosa and thecal layers of sheep ovarian follicles. Granulosa cells were removed from the follicles using cell culture medium containing heparin (100 U/mL Dulbecco's modified Eagle's medium) (DMEM) so that the follicular fluid would not clot during the procedure, whereas thecal cell layers were dissected from the follicle without the use of heparin. Isolation of RNA from granulosa and thecal layers using Tri Reagent® (Molecular Research Center, Cincinnati, OH, USA) was similar, except that polyacrylamide carrier was used for the granulosa cells. The quality and quantity of the RNA, measured on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), were excellent for both cell types (Figure 1).

Triplicate 20-µL aliquots of RNA containing 30 ng RNA each were reverse-transcribed for each sample using the random primers kit and protocol. The RT preparation was subjected to quantitative real-time RT-PCR using the ABI PRISM® 7000 and the TaqMan Universal PCR Master Mix protocols (Applied Biosystems).

After discovering that the RNA from the thecal cells had vascular endothelial growth factor (VEGF) amplification and that RNA from granulosa cells had no VEGF amplification, the 18S rRNA amplification (TaqMan Pre-Developed Assay Reagents; Applied Biosystems), which is used as a positive control for eukaryotic gene expression and to normalize RNA concentrations, was performed on the granulosa samples to see if there was a problem with the RT reaction. The assay revealed that inhibition of the PCRs for the 18S rRNA assay was also present in granulosa cell RNA. An attempt to remove the unknown inhibitor(s) from the RNA using an RNA column purification clean-up procedure from Zymo Research (Orange, CA, USA) was unsuccessful.

Progressive dilutions of the RT of a thecal sample that amplified well during PCR with an RT from a sample of granulosa cell RNA that was PCR-inhibited resulted in the progressive inhibition of amplification of the thecal sample (Figure 2). To identify heparin as the inhibitor present in the RNA, heparin and/or polyacrylamide carrier

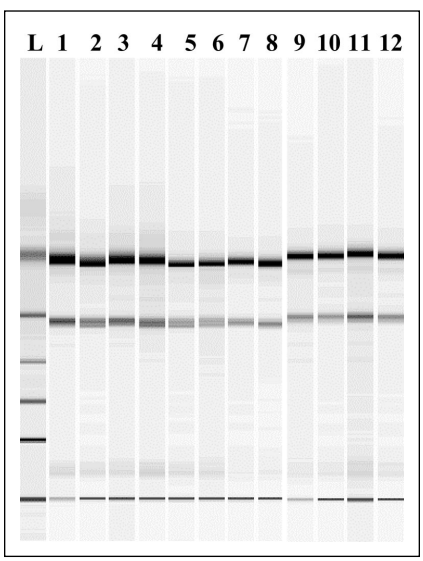

Figure 1. Computer-generated virtual gel image of total cellular RNA isolated from thecal and granulosa cells. Lanes 1–6 represent RNA from thecal cells, and lanes 7–12 represent RNA from granulosa cells. Lane L represents the RNA 6000 Ladder (Ambion, Austin, TX, USA) that is used as a quantification standard with the Agilent 2100 Bioanalyzer.