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Rapid Isolation and Sequencing of Purified Plasmid DNA from *Bacillus subtilis*

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We report two methods for isolation of plasmid DNA from the gram-positive bacterium *Bacillus subtilis*. The protoplast alkaline lysis procedure was developed for general use, and the protoplast alkaline lysis magic procedure was developed for isolation of DNA for sequencing. Both procedures yielded large amounts of high-quality DNA in less than 1 h, while current protocols require 4 to 7 h to perform and give lower yields and quality. Plasmid DNA was obtained from strains containing either high- or low-copy-number plasmids. In addition, the procedures were easily adapted to yield large amounts of plasmid DNA suitable for sequencing from another gram-positive organism, *Staphylococcus aureus*. Further, we demonstrated that neither chloramphenicol, used for plasmid selection, nor the mutation *recE4* reduced plasmid DNA yield from the strains we examined.

One of the major difficulties in applying molecular biological techniques developed for *Escherichia coli* to *Bacillus subtilis* and other gram-positive bacteria is obtaining sufficient amounts of high-quality plasmid DNA in a timely fashion. The alkaline lysis method (5, 10) and the boiling method (8), both derived from *E. coli* plasmid purification procedures, have been adapted for use with *B. subtilis* (6), but these procedures are time consuming and the plasmid DNA must subsequently be purified by CsCl-ethidium bromide dye–buoyant density centrifugation (15) to obtain the high-quality plasmid DNA required for many molecular biological techniques, such as sequencing. Another problem with these procedures is that nonspecific cleavage of both chromosomal and plasmid DNAs causes a background smear on agarose gels. Elimination of much of the background smearing without the need for CsCl-ethidium bromide purification has been achieved with a recently published procedure (4). Unfortunately, this procedure required approximately 7 h and it was demonstrated only for a high-copy plasmid.

It was our goal to develop a rapid plasmid purification procedure to confirm the sequences of over 60 different oligonucleotide-directed mutations we had generated on low-copy-number plasmid pAMY10. The mutations were first identified by sequencing DNA isolated from *E. coli*. It was necessary to verify that the mutations were still present after they had been introduced into *B. subtilis* by transformation following several genetic manipulations. Previously, these mutations were confirmed by transforming plasmids containing the mutations back into *E. coli* before resequencing, since an efficient method for sequencing plasmid DNA isolated from *B. subtilis* was not available. To avoid these extra steps, we developed the protoplast alkaline lysis (PAL) and protoplast alkaline lysis magic (PALM) miniprep procedures for *B. subtilis*. The PALM miniprep differs from the PAL procedure in that it takes advantage of the new technology developed by Promega Corporation, Madison, Wis. Use of the Promega Magic Miniprep resin and column filter to replace the last steps of the PAL miniprep yielded plasmid DNA, isolated from *B. subtilis*, suitable for sequencing. We also demonstrated in this study that with a few modifications these procedures work well for another gram-positive organism, *Staphylococcus aureus*.

It was reported that plasmid copy number in *B. subtilis* is reduced when cultures are grown in the presence of chloramphenicol for plasmid selection, resulting in a lower plasmid yield (6). We investigated this assertion by isolating plasmid DNA from *B. subtilis* 168 carrying plasmid p5′αB10, which encodes genes that confer resistance to both chloramphenicol and kanamycin. We also investigated whether a *recE4* mutant strain of *B. subtilis* deficient in homologous recombination and SOS repair gives lower plasmid yields than a wild-type strain, as had been previously reported (6).

MATERIALS AND METHODS

Growth conditions. The bacterial strains and plasmids used are described in Table 1. Unless otherwise noted, *B. subtilis* strains were grown in Luria-Bertani (LB) medium (15) plus drug for plasmid selection and harvested in the early stationary phase. Strains containing pAMY10 were grown to 125 Klett units, the end of exponential growth. All other *B. subtilis* strains were grown to 150 Klett units, which is about 1.5 h after the beginning of the stationary phase, except those which were harvested as noted (see Fig. 2). *B. subtilis* was also cultured in CHT50 (1), a supplemented minimal medium, and A3 medium (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.).

PAL miniprep procedure. Depending on the plasmid copy number, 5 (for high-copy plasmids)- to 10 (for low-copy plasmids)-ml culture volumes were centrifuged at 10,000 rpm for 5 min in a Sorvall SS34 rotor (or its equivalent) and the supernatant was removed by aspiration. Pellets were suspended by vortexing in 200 μl of SET (25% sucrose, 50 mM EDTA, 50 mM Tris-HCl [pH 8.0]) and 5 mg of lysozyme per ml of SET, transferred to a microcentrifuge tube, and incubated for 10 min at 37°C. A 350-μl volume of a fresh NaOH sodium dodecyl sulfate solution (0.2 N NaOH, 1% sodium dodecyl sulfate) was added, and the microcentrifuge tube was repeatedly inverted until the suspension cleared. To the cleared suspension was added 350 μl of a cold 3 M

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TABLE 1. Bacterial strains and plasmids

Strain (genotype)	Plasmid(s)	Approx copy no.	Phenotype[a]	Reference(s)
B. subtilis				
168 (*trpC2*)	pAMY10	10–15	Cm^r	3, 17
	p5′αB10	50	Cm^r Km^r	12
BRE (*trpC2 recE4 lys-3 amyR1*$^+$ *amyE*)	pAMY10	10–15	Cm^r	3, 17
	p5′αB10	50	Cm^r Km^r	12
S. aureus RN4220	pMJB179	15	Cm^r	9, 14

[a] Cm^r, chloramphenicol resistance (drug concentration, 10 μg/ml); Km^r, kanamycin resistance (drug concentration, 10 μg/ml).

K^+–5 M acetate solution. This was rapidly inverted for *S. aureus* or vortexed for 10 s at medium speed for *B. subtilis*. The suspension was centrifuged for 5 min at top speed in a microcentrifuge, and 750 μl of the supernatant was transferred to a new microcentrifuge tube. This supernatant fluid was extracted with 650 μl of cold phenol-chloroform-isoamyl alcohol (25:24:1) by vortexing for 1 min. The mixture was centrifuged for 5 min, and 620 μl of the aqueous phase was transferred to a new microcentrifuge tube, where it was extracted with 620 μl of cold chloroform-isoamyl alcohol (24:1) by vortexing for 30 s. The mixture was centrifuged for 3 min, and 550 μl of the aqueous phase was transferred to a new microcentrifuge tube. The plasmid DNA was precipitated by adding an equal volume of cold (−20°C) isopropanol and inverting it several times. The suspension was centrifuged for 5 min, and the isopropanol was removed by aspiration. The pellet was washed with 1 ml of 70% ethanol and centrifuged for 2 min. The ethanol was removed by aspiration. The pellet was dried under vacuum for 5 min and suspended in 50 μl of deionized H_2O containing DNase-free pancreatic RNase (20 μg/ml).

PALM miniprep procedure. For the PALM miniprep procedure, the PAL procedure was used through the phenol-chloroform-isoamyl alcohol extraction step. One milliliter of Magic Miniprep resin (Promega, Madison, Wis.), shaken before use, was added to the 620-μl aqueous phase, and the mixture was inverted several times. The resin solution was added to a 5-ml syringe column (Magic Miniprep column). The solution was pushed through the column filter with a plunger. The filter was washed with 2 ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl [pH 7.5], 5 mM EDTA diluted 1:1 with 100% ethanol). The column was transferred to a microcentrifuge tube and centrifuged for 20 s. The column was then transferred to a new microcentrifuge tube, and the plasmid DNA was eluted with 50 μl of deionized H_2O at 65 to 70°C by centrifugation for 5 s.

Modifications for *S. aureus*. *S. aureus* RN4220 was grown in LB medium plus drug in a test tube with vigorous overnight shaking. A 3-ml culture volume was harvested by centrifuging a 1.5-ml culture volume in a microcentrifuge tube for 1 min and aspirating the medium. Another 1.5-ml culture volume was added to the same microcentrifuge tube, and the procedure was repeated. The cell pellet was suspended in SET with 0.1 mg of lysostaphin per ml substituted for lysozyme. All of the other steps were performed in the same manner as with *B. subtilis* strains.

Enzyme digestions and agarose gel conditions. All restriction endonuclease reactions were carried out with 5 μl of miniprep DNA, 3.5 μl of deionized H_2O, 1 μl of 10× buffer, and 0.5 μl of enzyme (6 U of *Eco*RI or *Hin*dIII; Promega Corp.). The digestions were incubated for 1 h at 37°C. Five microliters of uncut plasmid DNA and the 10 μl from the restriction reactions were loaded into thin 1% agarose gels. The plasmid DNA was subjected to electrophoresis for 30 min at 80 V.

Sequencing. All sequencing reactions were carried out by the dideoxy-chain termination method of Sanger et al. (16) with a Sequenase II kit (U.S. Biochemical Corp., Cleveland, Ohio) and [^{35}S]dATP (Amersham Corp., Arlington Heights, Ill.). Denaturation and annealing of primers to double-stranded DNA were carried out by two methods: the standard alkaline denaturation method described in the Sequenase II kit instructions, with 30 min of annealing incubation at 37°C, and a detergent-boiling method.

The more rapid detergent-boiling method was originally modified from a method used to anneal primers to polymerase chain reaction products for sequencing (2, 4). Plasmid DNA, either digested for linearization or undigested, was prepared by the detergent-boiling method. A 40-μl volume of plasmid DNA isolated by the PALM procedure was digested with *Hin*dIII until linearized (60 min) and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Linearized and nonlinearized plasmid DNAs were precipitated by addition of 0.1 volume (4.5 μl) of 3 M sodium acetate (pH 5.0) and 2 volumes (100 μl) of 100% cold ethanol and then incubated for 15 min in a dry-ice–ethanol bath. The precipitated DNA was collected by centrifugation for 15 min. The plasmid DNA pellet was washed with 200 μl of 70% ethanol, centrifuged for 5 min, and dried under a vacuum for 5 min. The pellet was suspended in 6 μl of distilled H_2O–2 μl of 5× Sequenase buffer (U. S. Biochemical Corp.)–1 μl of primer–1 μl of 4% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.). The primer was annealed by boiling for 3 min and snap-cooled on powdered dry ice. Upon thawing, sequencing reactions were performed as described above.

RESULTS

PAL and PALM procedures. After 10 min of incubation in SET-lysozyme, nearly all of the thick peptidoglycan layer covering *B. subtilis* was removed, resulting in protoplast formation. Increasing the number of cells extracted to obtain plasmid DNA from strains carrying low-copy plasmids presented no apparent problems. We used up to 25 ml of a dense culture for the procedure and saw no decrease in plasmid purity and even observed increased efficiency of protoplast formation with increased cell density. Yield with the PAL or PALM procedure was dependent primarily on plasmid copy number. Yields of up to 15 μg were obtained when isolating *B. subtilis* plasmid p5′αB10 and *S. aureus* plasmid pMJB179, and yields of 4 μg and up were obtained from low-copy *B. subtilis* plasmid pAMY10.

The PAL and PALM procedures required only 45 to 60 min to perform. Even though the procedures are rapid, they do not sacrifice purity for speed. Plasmid DNA isolated by the PAL procedure was easily digested (Fig. 1) and was used successfully to transform *B. subtilis* and *S. aureus*, although it was not readily sequenced. Plasmid DNA isolated by the PALM procedure can be digested, employed in transformation, and also sequenced with a high degree of reproducibility (Fig. 1; see Fig. 4). The PAL procedure has the advantage of larger yields of plasmid DNA than the PALM procedure (Fig. 2) and does not require the purchase of Promega Magic Miniprep resin and filters, but the PALM procedure yields DNA of greater purity. Plasmid DNA isolated with the PALM procedure also appeared to contain less chromo-

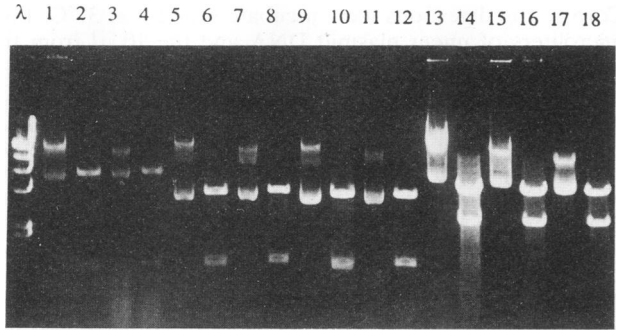

FIG. 1. Plasmid DNA isolated by the PAL and PALM procedures. Plasmid DNA fragments were separated by 1% agarose gel electrophoresis. Odd-numbered lanes contained undigested plasmid DNA, and even-numbered lanes contained digested plasmid DNA. Lanes: λ, lambda DNA digested with *Hin*dIII; 1, 2, 5, 6, 9, 10, 13, 14, 15, and 16, plasmid DNA isolated by the PAL procedure; 3, 4, 7, 8, 11, 12, 17, and 18, plasmid DNA isolated by the PALM procedure; 1 to 4, *B. subtilis* 168(pAMY10); 5 to 8, *B. subtilis* 168(p5′αB10); 9 to 12, *B. subtilis* BRE(p5′αB10); 13 to 18, *S. aureus* RN4220(pMJB179); 2, 4, 6, 8, 10, and 12, plasmid DNA digested with *Eco*RI; 14, 16, and 18, plasmid DNA digested with *Hin*dIII. Vortexing was used in the PAL procedure to isolate plasmid DNA in lanes 13 and 14 but not that in lanes 15 and 16.

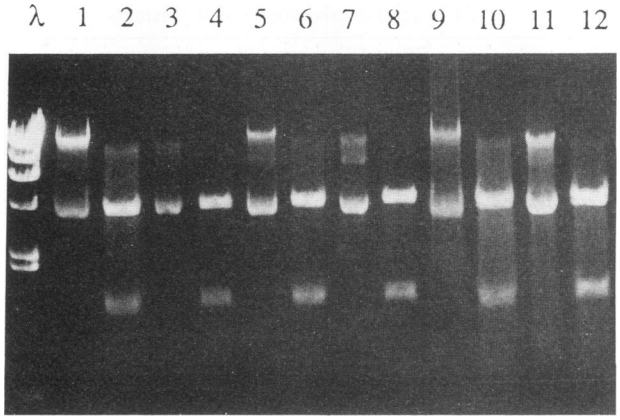

FIG. 3. Effect of culture age on p5′αB10 recovery from *B. subtilis* 168 cells, visualized in a 1% agarose gel. Odd-numbered lanes contained undigested plasmid DNA, and even-numbered lanes contained digested plasmid DNA. Lanes: λ, lambda DNA digested with *Hin*dIII; 1, 2, 5, 6, 9, and 10, plasmid DNA isolated by the PAL procedure; 3, 4, 7, 8, 11, and 12, plasmid DNA isolated by the PALM procedure; 1 to 4, cells harvested in the late exponential growth phase; 5 to 8, cells harvested in the early stationary phase; 9 to 12, cells harvested in the late stationary phase.

somal DNA contamination than did plasmid DNA isolated by the PAL procedure. *S. aureus* chromosomal DNA contamination was reduced in both the PAL and PALM procedures by rapidly inverting and not vortexing the microcentrifuge tube following potassium acetate addition (Fig. 1, lanes 13 to 16). Although vortexing caused increased chromosomal DNA contamination in *S. aureus*, it had little to no effect on chromosomal DNA contamination for *B. subtilis* and it effectively increased plasmid DNA yield. Vortexing was employed in all plasmid DNA isolations in this study, except those noted in Fig. 1.

Medium and growth stage effects. To determine whether medium type plays a role in plasmid purity and yield, *B. subtilis* 168 (p5′αB10) was cultured in three different media: CHT50 (a supplemented minimal medium), Difco antibiotic medium 3 (complex medium), and LB (complex) medium. Cells cultured in LB medium produced larger plasmid DNA yields than did cells cultured in A3 or CHT50 medium, while no difference in background smearing, as seen by visualization on agarose gels (Fig. 2), or ability to be sequenced (data not shown) was detected. The presence of chloramphenicol for selection of plasmid p5′αB10 in *B. subtilis* 168 had no effect on the yield of plasmid DNA compared with the same strain and plasmid grown with kanamycin for plasmid selection (Fig. 2).

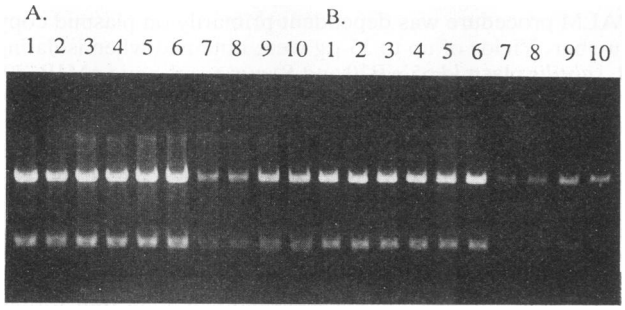

FIG. 2. Effects of bacterial strain differences, antibiotic selection conditions, and medium type on isolation of p5′αB10, visualized in a 1% agarose gel. Sets: A, PAL procedure-isolated DNA; B, PALM procedure-isolated DNA. Lanes: 1 and 2, strain 168 grown in LB medium (kanamycin); 3 and 4, strain BRE grown in LB (kanamycin); 5 and 6, strain 168 grown in LB (chloramphenicol); 7 and 8, strain 168 grown in A3 (kanamycin); 9 and 10, strain 168 grown in CHT50 (kanamycin).

LB medium cultures of *B. subtilis* 168 containing high-copy-number plasmid p5′αB10 were harvested at three different periods during growth: exponential growth, the early stationary phase, and the late stationary phase. Of the three growth periods, the late-stationary-phase sample had the most chromosomal contamination, presumably as a result of increased nuclease activity, but this contamination was minimal (Fig. 3). For the low-copy *B. subtilis* plasmid, best results were obtained when the cells were harvested in the late exponential or early stationary phase.

Effect of the *recE4* mutation on plasmid yield. PAL and PALM plasmid DNA isolations were performed on wild-type *B. subtilis* 168 carrying the p5′αB10 plasmid and on *recE4* mutant *B. subtilis* BRE also carrying the p5′αB10 plasmid. Plasmid DNA yields were always essentially equal between strains 168 and BRE, the latter of which is deficient in homologous recombination and SOS repair (Fig. 1 and 2). Similar results were obtained when the same strains carried plasmid pAMY10 (data not shown).

Visualization of sequence data. Figure 4 contains sequences generated from *B. subtilis* and *S. aureus* double-stranded plasmid DNAs isolated by the PALM procedure. Plasmid DNA isolated from *B. subtilis* by the PAL procedure did not yield readable sequence ladders (set B), while plasmid DNA isolated by the PALM procedure produced easily readable ladders, although the bands were lighter for *B. subtilis* DNA then for *S. aureus* DNA. No consistent differences in sequence results were found when annealing the sequencing primer by either annealing method, alkaline denaturation or detergent boiling (sets C, D, and E). Little difference in the resulting sequence ladders was observed between DNA that was linearized by *Hin*dIII digestion and nonlinearized, supercoiled plasmid DNA (sets C and D).

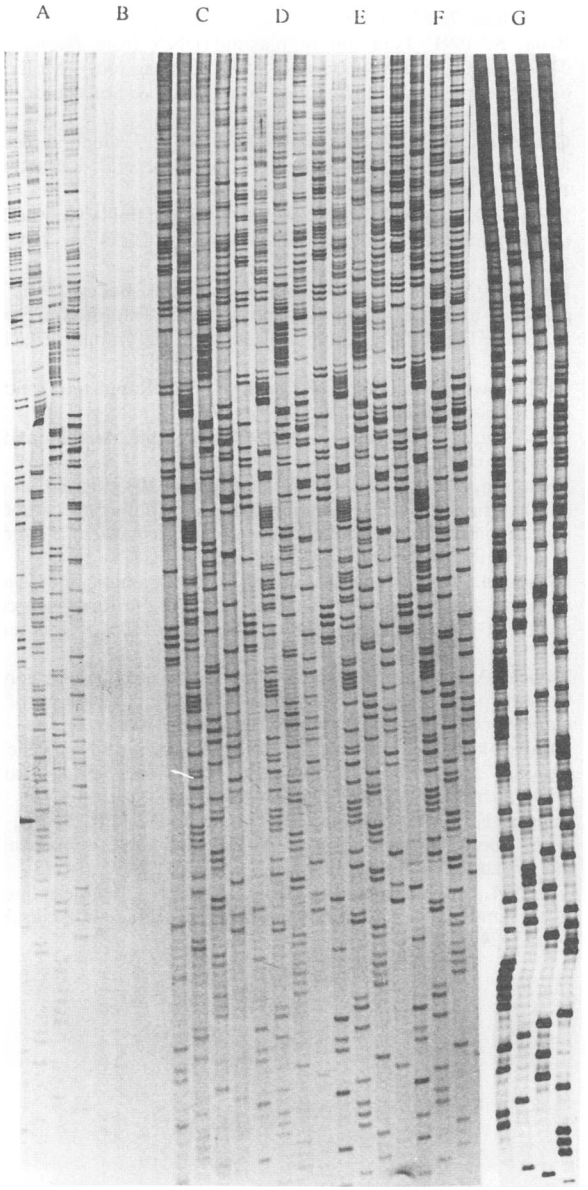

FIG. 4. Autoradiogram of sequence ladders from *B. subtilis* and *S. aureus* plasmid DNAs. XAR-5 film (Kodak, Rochester, N.Y.) was exposed for 48 h. Sets: A and C to G, plasmid DNA isolated by the PALM procedure; B, plasmid DNA isolated by the PAL procedure; A, *B. subtilis* 168(pAMY10); B to E, *B. subtilis* 168 (p5′αB10); F, *B. subtilis* BRE(p5′αB10); G, *S. aureus* RN4220 (pMJB179). Sets A, B, C, and F were sequenced by using linearized plasmid DNA and annealed by the detergent-boiling method; set D was sequenced by using nonlinearized plasmid DNA and annealed by the detergent-boiling method; sets E and C were annealed by the standard alkaline denaturation method.

DISCUSSION

Advantages of the PAL and PALM procedures. The PAL and PALM procedures eliminated the long incubations required in past plasmid purification procedures, thus saving time and potentially reducing nuclease degradation of the plasmid DNA and chromosomal DNA. Past procedures typically yielded plasmid DNA that after restriction endonuclease digestion was nearly unrecognizable in agarose gels. This was due to low plasmid yields and to a heavy background smear, most likely resulting from chromosomal DNA contamination. In contrast, the PAL and PALM procedures prevent cell lysis during lysozyme treatment by using SET buffer, which contains a high concentration of sucrose. This, in turn, prevents premature cell lysis and exposure of chromosomal and plasmid DNAs to nuclease activity. Thus, protoplast formation prevented DNA degradation by keeping the cells intact and at the same time effectively removed the peptidoglycan layer. Once the peptidoglycan layer was removed, the protoplasts were easily and rapidly lysed. The result was a plasmid DNA purification procedure for gram-positive bacteria *B. subtilis* and *S. aureus* which was very rapid in comparison with past procedures (5 to 7 h) for *B. subtilis* (4, 6).

Plasmid DNA yields (4 to 15 μg) were higher than those previously obtained (0.5 to 5 μg) (6). High yields were attained in part by starting with larger cell pellets while not proportionally increasing the volume of the SET resuspension buffer (or any other solution), as has been done previously when larger cell pellets were extracted. A major benefit of the larger cell pellets was the ability to obtain sufficiently high yields of plasmid DNA to allow direct sequencing of low-copy plasmid pAMY10. The speed of the PALM procedure permitted us to efficiently confirm the sequences of over 60 mutations introduced into *B. subtilis* on low-copy plasmid pAMY10.

We found that for best results, when isolating plasmid DNA from strains containing low-copy plasmids, cells should be harvested before late stationary phase. Culture age was of less importance for isolation of high-copy plasmids. The increased smearing on agarose gels caused by digestion of chromosomal DNA as a result of cell harvesting in the late stationary phase had little or no effect on further manipulations of the plasmid DNA, including sequencing. We found no need to use minimal media to obtain plasmid DNA suitable for sequencing, as was reported in a recently published procedure (4). We tested three different media and found that all yielded plasmid DNAs of comparable quality, while LB produced the largest yields of plasmid DNA. Consequently, we routinely used LB, an easily prepared complex medium.

With slight modifications to remove *B. subtilis* spore coat proteins, the PAL procedure was used to isolate plasmid DNA from *B. subtilis* spores (11). It was digested easily by restriction enzymes and contained little nicked or linearized plasmid DNA. The previous procedure for plasmid isolation from spores was a long process requiring over 24 h (7, 13), but with the procedure reported here, plasmid DNA could be isolated in less than 3 h, 2 h to remove spore coat proteins and less than 1 h for the PAL procedure.

Sequencing. The PALM procedure yielded plasmid DNA that could be sequenced by employing either the alkaline denaturation or the detergent-boiling method for primer annealing, in contrast to a recently described method (4) for isolation of plasmid DNA suitable for sequencing from *B. subtilis* that required linearization of the plasmid DNA and annealing of the primer exclusively by a detergent-boiling method. Isolation of plasmid DNA by the PALM procedure also bypasses the requirement for plasmid linearization, thus eliminating the need for restriction endonuclease digestion and an organic extraction step. With the elimination of these steps, the detergent-boiling method becomes a quick procedure to anneal primers to double-stranded plasmid DNA, requiring only 45 min to precipitate and dry the plasmid DNA and 5 min to resuspend and anneal the primer. *B.*

subtilis plasmid DNA was isolated and sequencing reactions were performed in less than 2 h by the PALM procedure in combination with the detergent-boiling method for annealing primers. The detergent-boiling method could conceivably be employed for *E. coli* double-stranded DNA sequencing, but this has not been tried in our laboratory.

Plasmid yield. Plasmid DNA yield was dependent on plasmid copy number, the number of cells used, and the size of the plasmid. Contrary to previously published assertions (6), we found that the plasmid DNA yield from the strains we tested was affected neither by the presence of chloramphenicol in the growth media nor by the presence of the *recE4* mutation in the plasmid host strain.

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