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Characterization of PCR Products from Bacilli Using Electrospray Ionization FTICR Mass Spectrometry

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A procedure for rapid purification of polymerase chain reaction (PCR) products allowing precise molecular weight determination using electrospray ionization-Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry is described. PCR amplification utilized the DNA polymerase from *Pyrococcus furiosus* (Pfu) which, unlike Taq, does not incorporate a nontemplated terminal deoxyadenosine phosphate. An 89-base pair nucleotide portion of the spacer region between the 16S and 23S ribosomal rRNA genes was amplified from the genome of three members of *Bacillus cereus* group and a 114 nucleotide region from the *Bacillus subtilis*. PCR involves polymerization of nucleotide precursors using two oligonucleotide primers and an amplification enzyme, as well as the presence of metal ions. Mass spectrometric analysis greatly benefits from removal of the oligonucleotide primers (15- and 17-mers in this instance) and nucleotide precursors since they adversely affect sensitivity and metal ion adduction results in an inaccurate molecular weight determination. In the presence of guanidinium hydrochloride, the PCR products bind preferentially to a silica resin, allowing removal of other components (i.e., dNTP's, primers, and salts). Further removal of metal ions was accomplished using a microdialysis device, allowing samples to be pumped through a hollow cellulose fiber with an external countercurrent flow of 2.5 mM ammonium acetate. Prior to injection into the mass spectrometer, the sample buffer was adjusted to 50 vol % acetonitrile, 25 mM piperidine, and 25 mM imidazole, which enhanced signal intensity. The molecular weights of the PCR products determined by nucleotide sequence and MS analysis were in excellent agreement, and several PCR products were analyzed where mass differences corresponding to single base substitutions could be accurately assigned. These assignments were possible due to the high mass precision, accuracy, and resolution FTICR inherently affords. This constitutes the first report demonstrating the ionization and detection of PCR products by mass spectrometry with mass precision and accuracy for assigment of such modifications or substitutions.

The polymerase chain reaction (PCR) is widely used in biology,[1] including such diverse fields as microbiology, medicine, and forensics. The rapid amplification and selective nature of this reaction has led to its use as a means of generating informative nucleic acid fragments from small quantities of DNA or RNA for detection or comparison of different genetic loci. Using PCR, deletion of a large portion of a gene, as observed in Duchenne's muscular dystrophy,[2] is readily determined. However, relatively small changes in the coding region of genes are difficult to discern using conventional separation techniques (e.g., agarose gel electrophoresis). For example, inherited disorders such as sickle cell anemia,[3] Tay-Sach's disease[4] and neurofibromatosis 2,[5] result from simple substitution of a single base pair or the deletion/

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addition of a few base pairs which results in the production of a nonfunctional protein.

The steps in a PCR analysis generally include isolating the template DNA from the sample matrix, amplifying a targeted DNA region, and determining the molecular weight of the products. Currently, the molecular weight is determined by relative electrophoretic mobility of the PCR products using conventional agarose gel electrophoresis,[6] which generally takes several hours (depending upon the size of the PCR product and the nature of the modifications). The quality of the "molecular weight" determination and limited resolution provided by agarose gel electrophoresis can restrict the utility of PCR in situations where single base deletions, additions, or substitutions are of interest.

To determine deletions or additions of single bases, the use of high-resolution polyacrylamide gel electrophoresis is required, which significantly lengthens the analysis time. The potential of modern liquid chromatography, and particularly capillary electrophoresis, for more rapid analysis has been demonstrated; however, even using the highest resolution separation methods, the precision is inadequate to differentiate single base substitutions.[7,8] The recourse in this case is sequencing the PCR product, which requires further enzymatic manipulations and an electrophoretic separation requiring on the order of several hours.

The use of mass spectrometry (MS) has advanced rapidly over the past decade due to the advent of electrospray ionization (ESI)[9] and matrix-assisted laser desorption/ionization (MALDI)[10] for the analysis of high molecular weight gene products.[11,12] However, the relative success of MS for analysis of larger nucleic acids has been much more modest. Until recently, mass spectrometry has been restricted to low molecular weight synthetic oligonucleotides.[13−17] One major reason for the limited application of MS for larger nucleic acids (e.g., PCR products) is the highly anionic phosphate backbone of DNA. The high affinity of this backbone for positively charged metal ions substantially reduces sensitivity, potentially introduces error into the mass measurement, and can preclude the analysis of larger oligonucleotides.[18,19] Although analysis of DNA by MALDI was originally thought to be insensitive to cation adduction, a recent report using delayed-ion extraction clearly illustrated that cation adduction is problematic.[20] Additionally, the preferential ionization of low molecular weight oligonucleotide species relative to those of high molecular weight can also cause difficulties;[21] since PCR reaction mixtures contain nucleotides, oligonucleotide primers, and buffers, the PCR products constitute only a minor component of this mixture. Thus, some cleanup of the PCR reaction is highly beneficial or essential prior to mass spectrometric analysis, and the effectiveness of the cleanup step will likely define the utility of mass spectrometric analysis.

MALDI has recently demonstrated potential for the characterization of different PCR products, including those amplified from human and bacterial DNA.[22−24] In one study, the differentiation of PCR products (75- and 78-mers) of the cystic fibrosis transmembrane conductance regulator (CFTR) allowed determination of the 3-base pair (bp) deletion in cystic fibrosis patients.[23] In another study, the normal polymorphic alleles of the carbonic anhydrase gene were differentiated.[24] Homozygotes and heterozygotes were differentiated by the presence of either a 133- or 152-mer or both.[24] In a third recent study, MALDI was used to identify *Legionella pneumophila*, the causative agents of Legionnaires' disease.[22] Identification was based on detection of two PCR products (a 108-mer from the 5S rRNA gene and a 168-mer from the mip gene).[22] Although MALDI has made significant progress in the past year in the analysis of PCR products, the limited resolution has precluded precise characterization of the PCR products of this size (e.g., for sequence variations).

Recently, we and others have reported the analysis of PCR products using electrospray ionization (ESI)-MS.[21,25] In both initial studies, the preparation of the crude PCR reaction prior to ESI-MS analysis was time consuming. In our initial report, ESI was used with a high-resolution Fourier transform ion cyclotron mass spectrometer (FTICR-MS) to investigate 105- and 106-bp PCR products of the spacer region between the 16S and 23S rRNA genes of two members of the *Bacillus cereus* group of bacteria. Our results yielded a mass precision and accuracy comparable with gel electrophoresis.[21] The relatively low mass precision and accuracy was attributed to spectral complexity arising from nontemplated 3′ addition of deoxyadenosine phosphate (dA), residual cation adduction, and the resulting low signal-to-noise ratio (S/N). Elimination of nontemplated dA was addressed in another study using MS by incorporating restriction sites in the primers with subsequent endonuclease digestion.[25] This demonstrated that ESI-MS could provide high mass accuracy and precision for the individual strands of the resulting shorter 57-bp PCR product. However, this approach requires additional enzymatic manipulations and incurs a considerable lengthening of sample preparation time (as much as 1 day or more).

In general, the PCR amplification of any genomic region, whether bacterial or human, presents similar challenges (i.e., the same type of product in a similar reaction mixture). Thus, while this report focuses on bacterial identification, the analytical

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methods described are generally applicable for PCR products. For the model systems studied, the conserved portions of the spacer region between the 16S and 23S rRNA genes vary in size and sequence for different species of bacilli. PCR, using appropriate primers, generates an 89-mer for the *Bacillus cereus* group (*B. anthracis*, *B. cereus*, and *Bacillus thuringiensis*) and a 114-mer for a more distantly related organism (*Bacillus subtilis*).[26] A new procedure was developed to eliminate nontemplate dA addition during PCR and to rapidly and efficiently remove metal ions, amplification enzyme, primers, and nucleotide precursors before MS analysis. High-resolution mass spectra were obtained using ESI-FTICR-MS, a method shown to provide accurate and precise molecular weight determinations after amplification, without need for further enzymatic manipulations. Most importantly, we show that single-base substitutions in the PCR products are readily detected.

EXPERIMENTAL SECTION

Materials and Methods. *Bacterial Strains. B. cereus* (BC64) was obtained from the American Type Culture Collection (Rockville, MD), *B. anthracis* (Δ-Sterne) was obtained from Stephen Lepla (Fort Detrick, MD), *B. thuringiensis* (4C2) was obtained from the Bacillus Stock Center (Columbus OH), and *B. subtilis* (W23) was kindly donated by G. C. Stewart (School of Veterinary Medicine, Kansas State University).

DNA Preparation. After a 12−16-h growth in nutrient broth at 37 °C, with vigorous shaking, the cultured cells were centrifuged into a pellet at 1000*g*. The pellet was washed twice with 10 mL of 1 mM Tris-EDTA-HCl (pH 8.6). Three steps of rapid freezing and thawing were used to lyse the bacterial cells followed by a 30-min incubation with RNase (40 mg/mL) at 37 °C and a 30-min incubation with protease K (100 mg/mL) at 37 °C. These steps were followed by three phenol/chloroform (50:50) extractions. A final chloroform extraction was performed to remove remaining phenol, followed by ethanol (100%) precipitation of the DNA in the presence of 0.3 M sodium acetate. The preparation yield was determined using UV absorbance at 260 nm, and purity estimated via the 260/280 ratio.

Polymerase Chain Reaction. PCR was carried out in 100-μL volumes. The reaction buffer consisted of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-10, and 10 mg of BSA. The reaction also contained the following components: 250 mM concentrations of each dNTP, 100 pmol of each primer, 3.5 mM $MgCl_2$, ∼500 ng of bacterial genomic DNA, and 2.5 units of Pfu DNA polymerase. Approximately 35 μL of mineral oil was placed on the surface of the reactions to prevent evaporation. The primers KF6 (5′-CAAGGCATCCATCGT-3′) and KF9 (5′-GTGTTCTTTGAAAACTAG-3′) correspond to positions 35−21 of the 23S rRNA gene and positions 91−108 of the 16S/23S rRNA spacer region, respectively, of *B. cereus* group. These primers also correspond to positions 35−21 of the 23S rRNA gene and 91−109 of the 16S/23S rRNA spacer region in the *B. subtilis* species. The reaction cycles for PCR consisted of a denaturation at 95 °C for 1 min, annealing at 50 °C for 3 min, and a 2-min extension step at 72 °C preceded by a 2-min ramp. A total of 40 cycles were performed, followed by a 10-min final extension at 72 °C.

Silica Resin Cleanup. The removal of primers, nucleotides, and enzyme from the PCR reactions was performed using the QIAquick PCR purification kit (Qiagen, Inc. Chatsworth, CA) with some modifications. Briefly, 250-μL volumes of the PCR were combined with 1.25 mL of a chaotropic salt (guanidinium hydrochloride) containing binding buffer. The resulting volume was centrifuged for 1 min through an immobilized silica phase at 10000 *g*. Binding of the larger (over 65 nucleotides in length) polymeric nucleic acids to the silica resin is favored in the presence of higher concentrations of guanidinium hydrochloride. The chaotropic salts were removed with 0.75 mL of 1 mM ammonium acetate in 90:10 ethanol/water and centrifugation for 1 min at 10000*g*. Two further cleanups were performed using 0.75 mL of 70% ethanol at 10000*g* for 1 min to remove excess salts and a 1 min "dry" centrifugation step to remove remaining ethanol. The products were eluted with 50 μL of elution buffer containing 1 mM triethylamine hydrochloride (pH 8.5−9.0), 50 nM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) by centrifuging for 1 min at 10000*g*. The presence and purity of the products was determined using 3% agarose gel electrophoresis (3 h at 90 V). Relative amounts of product were determined from fluorescence intensity on an agarose gel. Known quantities of double-stranded DNA (ranging from 5 to 254 ng) were electrophoresed on the same gel as the PCR products. Estimates of the quantity of product are based on comparative fluorescence between the "standard curve" and the PCR products.

Microdialysis Cleanup. A key improvement in the quality of the PCR samples in this work was due to the use of a new microdialysis procedure, described elsewhere.[27−29] Briefly, a regenerated cellulose hollow fiber (200-μm-i.d. microdialysis tubing having 13 000 molecular weight cutoff) was used with a countercurrent flow of dialysis buffer (2.5 mM NH_4OAc) that was continuously introduced through the annual space between the dialysis tube and a large concentric Teflon tube sheathing the dialysis tubing. The sample was injected into the dialysis tube by a Harvard syringe pump and the dialyzed sample was collected in a microcentrifuge tube. The sample flow rate inside the dialysis tube was 2 μL/min, and the dialysis buffer flow rate was 0.5 mL/min.

Mass Spectrometry. The ESI Fourier transform ion cyclotron resonance mass spectrometer used for the present study has been described in detail elsewhere.[30] Briefly, the PCR products were introduced into the mass spectrometer at a flow rate of 0.3 μL/min in a pulled glass capillary with a coaxial sheath gas (SF_6) to suppress corona discharge. A source potential of ∼2.3 kV was applied to produce a stable negative ion current. To enhance ion accumulation, broad-band noise wave forms were applied from *m/z* 1000 to 3500 at 10 V peak-to-peak.[31−33] Prior to injection, the sample contained a final concentration of 50 vol % acetonitrile, 25 mM piperidine, and 25 mM imidazole.

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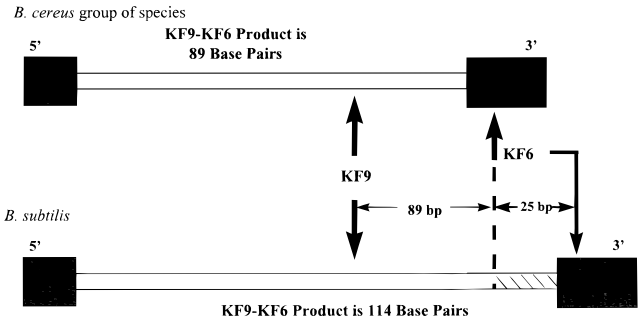

Figure 1. Schematic of the PCR products amplified from the 16S/23S rRNA gene spacer region using the primer set labeled KF9 and KF6. The *B. cereus* group produces an 89-bp PCR product while the *B. subtilis* produces a 114-bp PCR product.

RESULTS

PCR Amplification Scheme. The DNA polymerase from *Thermus aquaticus* (Taq) is the most widely used enzyme for PCR amplifications; however, the fidelity of this enzyme has been sacrificed due to a lack of proofreading activity which can result in an increased error rate.[34] Taq has been reported to incorporate an additional dA residue on the 3′ ends of a blunt-ended double-stranded PCR product,[34] which complicates spectral interpretation. As already demonstrated, one can address this problem by introducing a restriction site on each primer, followed by enzymatic cleavage, but this substantially increases the time and complexity of the overall analysis.[25] Recently, the DNA polymerase from *Pyrococcus furiosus* (Pfu) has been shown to possess superior thermostability and 3′−5′proofreading ability,[35] which generates a product without the additional nucleotide on the 3′ end. Therefore, the Pfu DNA polymerase was used in the current work rather than Taq (which was used in our initial study).[21] No adenylation of the 3′ end of the double-stranded products was observed (vide infra) using Pfu, which was confirmed by high-resolution polyacrylamide gel electrophoresis.

PCR products from three members of the *B. cereus* group of (*B. anthracis*, *B. cereus*, *B. thuringiensis*), as well as the more distantly related *B. subtilis* were investigated. PCR primers were designed to recognize areas of conserved sequence present in all four species. These products were amplified from the spacer region between the 16S and 23S rRNA genes, as shown in Figure 1. Existing sequence[36−38] indicates there is a difference of 25 bp's between the *B. cereus* group and *B. subtilis*. This polymorphism has been confirmed by conventional gel electrophoresis with members of the *B. cereus* group generating identical PCR products (89-mers) with strains of *B. subtilis* generating 114-mers.[26] While the mass difference between the *B. cereus* group species and the *B. subtilis* is large (∼15 500 Da), these regions were amplified to create a model system. Clearly, taxonomic differentiation between the *B. cereus* and the *B. subtilis* can be readily accomplished based on the known sequence information for this region.

To probe the variable 3′ end of the 16S/23S rRNA spacer region, PCR products have been generated for the 89- and 114-bp regions in the *B. cereus* group and *B. subtilis* species, respectively (see Figure 1). These products were amplified directly from the genomic DNA using Pfu DNA polymerase.[35] In order to bracket the informative spacer regions, the sense primer (KF9; refer to Figure 1) was designed to extend from position 90 in the *B. subtilis* spacer region to position 107. The antisense primer (KF6; see Figure 1) was designed to extend from position 21 to 35 of the 23S rRNA gene.

Analyte Purification Method. The ability to characterize PCR products by ESI-mass spectrometry with sufficient mass accuracy, precision, and resolution is largely dependent on the method by which the PCR products are isolated from the crude PCR reaction mixture and how the sample is desalted. The importance of PCR sample preparation prior to mass spectrometric analysis (or lack of) has been stated by Doktycz et al.; however, it was still unclear how to obtain quality mass measurements of PCR products.[39] The methods used to remove the PCR reaction components prior to MS analysis typically involve multiple ethanol precipitations or buffer exchanges with ammonium acetate or other "volatile salts" to remove adducting metal ions. At this stage, amplification enzyme, nucleotide precursors, and primers are also removed. Unfortunately, previously reported precipitation and buffer exchange methodologies require several hours and incur significant sample losses.[28] In this report, a more rapid alternative method has been utilized to prepare the products for mass spectrometry.

The nucleic acid was adsorbed onto a silica resin in the presence of a vast excess of chaotropic salts. The chaotropic salts were removed using ammonium acetate/70% ethanol washes. This simultaneously eliminates excess metal ions, low molecular weight nucleotide species, and the amplification enzyme. The lower molecular weight nucleic acid species (i.e., dNTP's and primers) are removed because they do not bind efficiently enough to the silica resin in the presence of a chaotropic salt. This procedure only requires ∼10 min and yields high analyte recoveries. However, further steps are necessary for the removal of metal ions to levels that facilitate ESI-MS. Recently, a novel microdialysis approach was developed which efficiently removes high concentrations of salt (>0.25 M NaCl) prior to infusion into the mass spectrometer.[27−29] The high efficiency of this desalting step results from the high surface area/volume ratio inside the tubing and the countercurrent flow of dialysis buffer, which serves to maintain the concentration gradient as the sample moves through the analysis tube. Efficient removal of buffer salts generally results in single-stranded products since salt is necessary to stabilize the secondary structure of DNA (in our previous study, only double-stranded products were observed, indicating residual salt as also evident from the broad peaks which yielded less accurate mass measurements). Furthermore, the introduction of piperidine and imidazole to the sample prior to infusion into the electrospray source effectively suppresses any effects due to residual salt.[40,41] The use of piperidine and imidazole has previously been shown to moderately shift charge states to higher m/z (i.e., charge-state reduction), which decreases the resolution requirements for mass

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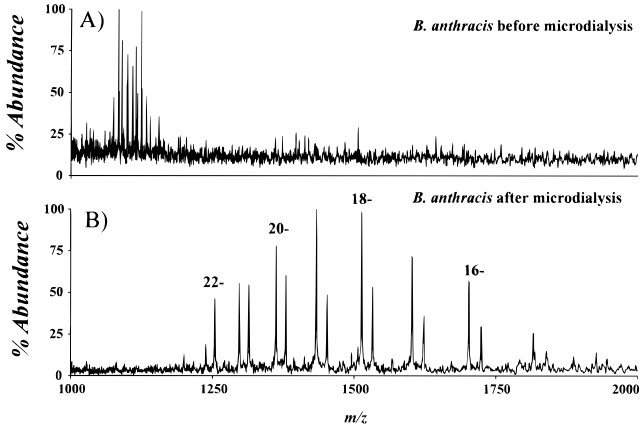

Figure 2. ESI-FTICR mass spectra of the 89-bp PCR product amplified from *B. anthracis*. (A) represents the spectra obtained after removal of reaction components and addition of acetonitrile, piperidine and imidazole, but without microdialysis. (B) shows the spectrum of the 89-bp product which was dialyzed prior to the addition of acetonitrile, piperidine, and imidazole. Note the shift in intensities to lower charge states (higher *m/z*) for the charge states of the individual strands of the product, as well as improved S/N.

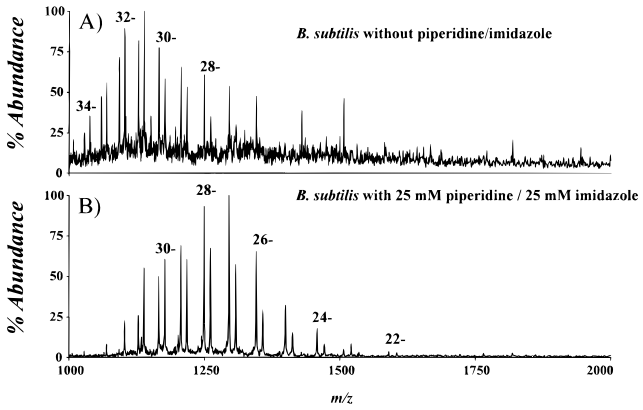

Figure 3. ESI-FTICR mass spectra obtained on the individual strands of the 114-bp product from *B. subtilis* after the removal of the reaction components and dialysis had been performed. In (A) piperidine and imidazole had not been added prior to ionization. In (B), the mixture of piperidine and imidazole had been added prior to analysis. Note the shift in intensities to lower charge states (higher *m/z*) as well as an improved S/N in (B).

spectrometry and allows more facile elucidation of single base deletions or additions.[40]

Figure 2 illustrates the importance of the microdialysis step prior to ESI-MS analysis. Figure 2A shows the ESI mass spectrum of the PCR product from *B. anthracis* after extraction from the crude PCR reaction mixture and addition of acetonitrile, piperidine, and imidazole (see Experimental Section). The signal-to-noise ratio of the single-stranded PCR product centered at m/z 1100 is poor due to cation adduction which precluded peak assignments. Figure 2B shows the ESI mass spectrum of the same PCR product after a 5-min microdialysis step. The spectrum shows both the sense and antisense strands of the PCR product (i.e., the single-stranded products), allowing facile mass determination of each strand. Thus, the 5-min microdialysis step (which can also be implemented on-line[27,28]) significantly improved the overall quality of the ESI mass spectrum of the PCR products. It should also be noted that the center of the charge-state distribution has shifted to higher m/z for the dialyzed sample but not for the raw sample, even though both contain piperidine and imidazole. This is consistent with previous results suggesting the first mechanism of piperidine and imidazole involves binding with cations in solution, which does not result in charge-state reduction.[40]

The addition of piperidine, imidazole, and acetonitrile to the solution containing the PCR products prior to infusion into the electrospray source was also found to enhance signal intensity. Figure 3 shows the ESI mass spectra of the 114-bp PCR product amplified from *B. subtilis*. Figure 3A shows the spectrum of the dialyzed sample with acetonitrile added, but *without* the addition of piperidine or imidazole. The spectrum is interpretable with resolution of the complementary strands; however, a relatively poor S/N ratio was observed. Figure 3B gives the mass spectrum for the same PCR product after addition of piperidine and imidazole showing the S/N ratio has been improved by over 1 order of magnitude and the maximum charge state has been shifted to a higher m/z. No fragmentation of the molecular ion was observed in the m/z range investigated (i.e., 800−2500). Clearly, single-stranded oligonucleotides of >100 bases are stable under analysis by ESI-MS. A likely reason for the greater stability of the single-stranded species is the use of gentler ESI interface conditions than in some previous work. Our ability to utilize gentler interface conditions is a likely result of more effective desalting. Metal ion adduction can be somewhat reduced by the use of harsher interface conditions, but this also promotes dissociation and ultimately limits application of ESI-MS to larger oligonucleotides.

Although the sample preparation has been altered to provide a more efficient removal of metal ions and low molecular weight nucleic acids, an additional attribute of the procedure is the decreased time required. Only six, 1-min steps are needed to use the preparative cartridges and the dialysis can be performed in less than 10 min or incorporated as an on-line step requiring less than 5 min.[27] The result is a rapid method requires less than 20 min from the crude PCR reaction mixture to the completed analysis.

Accurate Mass Measurement of PCR Products. The ESI-FTICR mass spectrum of the 89-bp product from *B. thuringiensis* (4C2) is shown in Figure 4 with only the single-stranded products observed. Cation adduction to the PCR product was not substantial, as shown by the inset for the 14− charge state. The mass spectrum also indicates that the Pfu DNA polymerase does not incorporate a nontemplated dA to the 3′ ends of the double-stranded blunt-ended PCR product (Figure 4). Clearly, if present, a single base addition or deletion would be readily detected. The expected mass, based on the reported sequence, is listed in Table 1 with the measured mass and its associated absolute mass difference (Δ).

Figure 5 shows the ESI-FTICR mass spectra of two double-stranded PCR products from *B. anthracis* and *B. cereus*. In this case, the occurrence of double-stranded products was attributable to the single dialysis step not effectively removing sufficient salt to denature the PCR product. Figure 5A shows the mass spectrum of the 89-bp double-stranded PCR product from *B. anthracis* with the corresponding mass measurement of 54 856.2 Da. This is in good agreement with the double-stranded mass (i.e., simple sum of the complimentary strands) obtained from the ESI-FTICR mass measurement of the single-stranded *B. anthracis* (Figure 2B and Table 1). Interestingly, the maximum intensities have shifted from m/z ~1400 to ~2400 for the double-

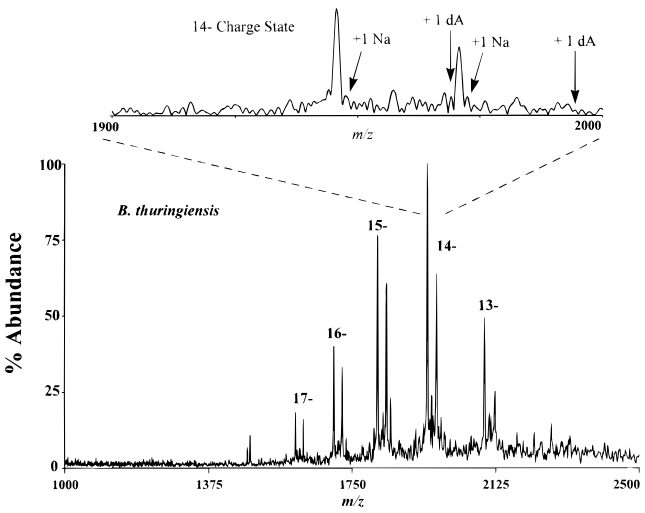

Figure 4. Mass spectrum of the single strands from the *B. thuringiensis* 89-bp product and expansion of the ESI-FTICR mass spectrum of the 14− charge state. The location of masses that correspond to either a sodium adduct or an additional dA residue for either strand is indicated. Note that no discernible masses are present for either a sodium adduct or an additional dA.

Table 1. ESI-FTICR Mass Measurement Results of PCR Products

bacterial strain	base pairs	strand[a]	mass (Da) pred[b]	measd	Δ[d]
B. subtilis	114	+	35 272.98	35 311.6 ± 3	+39
		−	35 027.80	34 986.6 ± 3	−41
		±	70 300.84	70 298.2[c]	−2.6
B. thuringiensis	89	+	27 618.94	27 603.8 ± 2	−15
		−	27 237.80	27 253.3 ± 2	+16
		±	54 856.74	54 857.1[c]	+0.4
B. anthracis	89	+	27 618.94	27 603.6 ± 2	−15
		−	27 237.80	27 253.3 ± 2	+16
		±	54 856.74	54 856.9[c]	+0.2
B. cereus	89	+	27 618.94		
		−	27 237.80		
		±	54 856.74	54 856.6 + 4	−0.14

[a] + is the sense strand, − is the antisense strand, and ± is the double-stranded product. [b] Average molecular mass based on sequence information in the literature. [c] Single-stranded PCR products were analyzed for all strains except BC64. The ± measured molecular mass is the simple sum of the single-stranded species. [d] Difference between measured mass and predicted mass (rounded to correct precision after subtraction).

stranded species when piperidine and imidazole are added to the solution. In our initial work,[21] a 105-bp product exhibited a maximum net charge of 42, which corresponds to ~2.5 bp/charge (i.e., every 2.5 bases average one charge) when piperidine and imidazole were not added. However, implementation of piperidine and imidazole to the solution of the 89-bp product (Figure 5A) resulted in a maximum net charge of ~22, which corresponds to ~4.0 bp/charge.

Figure 5B shows the ESI FTICR mass spectrum of the PCR product obtained from *B. cereus*. This spectrum yielded three *double-stranded* PCR products with the largest being the expected 89-bp species. The other two double-stranded products in the spectrum are attributed to artifacts of the PCR reaction and correspond to a single (heterodimer) and double base deletion (88-bp product), respectively. Thus, the unexpected products are lower in mass by 308.5 (for the heterodimer; i.e., the average of the two 88−89-bp species) and 617.1 for the 88-bp species. This

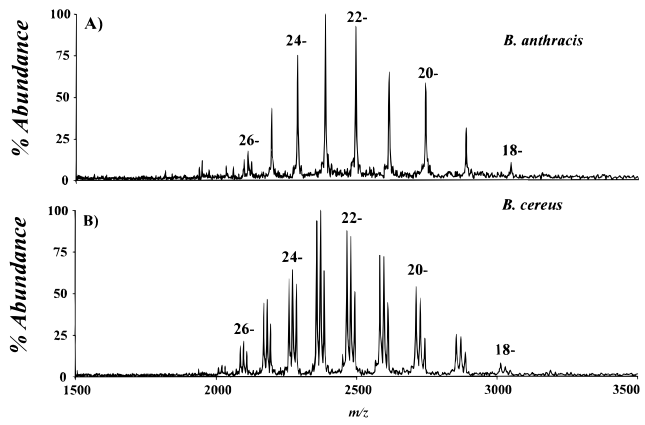

Figure 5. ESI-FTICR mass spectra of 89-bp double-stranded PCR products from (A) *B. anthracis* and (B) *B. cereus*. In both cases, the 18− through 26− charge states observed correspond to the double-stranded form of the products. Note that in (B) three double-stranded products are present (see text for explanation).

phenomenon was only observed for this sample, which was the only case studied where a mismatch between the primer and the genomic sequence was found.

DISCUSSION

The power of PCR to generate specific nucleic acids fragments from genomic DNA using primers flanking a targeted region is undeniable. Mass spectrometry has made significant progress in the analysis of macromolecules over the past decade and presents the possibility of rapid and accurate characterization of PCR products. While it is still uncertain what size PCR products will ultimately be amenable to MS, it is certain that MS offers the potential for rapidly obtaining information for the diagnosis of genetic disease, monitoring of natural or genetically modified organisms in the environment, or detection of pathogens in clinical samples.

The novel approach to PCR sample cleanup for MS described reduces the total analysis time to less than 0.5 h. This approach can undoubtedly be integrated and automated to further reduce analysis time. With the advent of rapid PCR amplifications, as many as 30 cycles can be completed in less than 10 min,[42] potentially allowing "real-time analysis" (e.g., monitoring of environmental bacterial communities). The discussion below includes the figures of merit for ESI-MS analysis of PCR products as compared to conventional techniques and the potential significance of PCR product characterization by high-resolution mass spectrometry. We also briefly discuss the fact that single-stranded PCR products are stable in the gas phase and that analysis of the single-stranded products allows single base substitutions to be identified.

Figures of Merit. The mass accuracy and precision obtainable by mass spectrometry are superior relative to electrophoretic techniques. Electrospray mass spectrometry readily affords mass measurement errors of less than 0.01% (i.e., 1 Da at 10 000), and 0.001% is achievable with similar mass precision. This high precision and accuracy substantially exceeds that demonstrated to date using MALDI-TOF mass spectrometry.[22−24] Thus, a single base substitution (the worst being an A to T polymorphism, which corresponds to a 9-Da difference) should be resolvable for PCR

(42) Wittwer, C. T.; Garling, D. J. *Biotechniques* **1991,** *10,* 76−83.

products of the size presented here. Since each single strand has a mass of ~35 000 Da, the mass error at worst (assuming 0.01%) would correspond to 3.5 Da, about one-third that of an A to T polymorphism. Therefore, single base substitutions should be readily distinguishable in large single-stranded DNA (>100 mer) using ESI-MS, and substitutions for species of 1 order of magnitude greater in length are potentially distinguishable.

The amount of material consumed in the analysis of nucleic acids using mass spectrometry still generally exceeds that of gel electrophoresis. In our first report,[21] it was estimated that the electrospray mass spectrum represented 5 pmol of a 105-bp product, which is ~3 orders of magnitude greater than that required for electrophoretic analysis. However, due to several changes made in sample preparation, all of the mass spectra presented in this report required the consumption of only 10 fmol of material, a significant improvement over the previous results (the amount of material injected was based on the fluorescence measurements prior to dialysis; therefore, any losses during the dialysis procedure are not reflected). Thus, taking into account all of the mechanisms for sample and/or ion loss, the amount of material represented in a single ESI mass spectrum is at the subfemtomole level, which is much less than picomole quantities generally produced by a single PCR preparation. Thus, not only can the characterization of PCR products be accomplished from the material produced in a single PCR amplification reaction, but this result also indicates that mass spectrometry can provide detection limits of the same order of magnitude as gel electrophoresis using existing technology.

Significance of Detecting Single-Stranded vs Double-Stranded PCR Products. While the microdialysis method was initially developed to desalt proteins and oligonucleotides, the simultaneous utility for assisting the denaturation of double-stranded DNA is of significance. Clearly, the results for the W23 strain from the *B. subtilis* (Figure 3) and other recent work,[43] shows that single-stranded oligonucleotides are not inherently unstable during ESI-MS. The significance of this result is that the characterization of base substitutions is much more readily discerned based upon accurate mass measurements of the two single-stranded DNA products (see below). It should be noted that the potential use of ESI-MS for DNA sequencing and other applications would be largely precluded if single-stranded DNA of greater than 100-mer size were inherently unstable.

The ability to detect single-stranded (vs double-stranded) PCR products requires that the two strands be denatured in solution and that the complementary strands be resolved from one another. Since the sum of the mass of A and T (617.39 Da) is approximately the same as for C and G (618.38 Da), one can see that double-stranded DNA of specified length will have masses that are not significantly sequence dependent. In contrast, the masses of the corresponding single-stranded species are generally quite different. Figures 2B, 3B, and 4 clearly demonstrate that resolution of the two complementary strands can be accomplished, allowing redundant sequence-related information to be obtained directly from the mass measurement of each of the two strands. For example, the mass measurement of the individual single strands of *B. subtilis* (Figure 3B and Table 1) indicated that the sum of the two single strands added up to the expected molecular weight within experimental error. However, the mass measurements for the single strands were not in agreement with those calculated on the basis of the sequence of another strain, 168, reported in the literature.[36] The measured mass for the sense strand was determined to be ~39 Da higher than the predicted mass, and the antisense strand ~41 Da lower than the predicted mass. These mass discrepancies for the two strands clearly indicate a C to G switch between the two strands, which results in a 40.03-Da addition to one strand and a corresponding 40.03-Da subtraction in mass in the other.

The power of mass spectrometric analysis is readily evident by the fact that single base errors (or substitutions), such as the above example, can be readily detected and identified. Additionally, due to the more significant mass difference for the two strands and the relatively minor deviations that typically occur, the substitution can usually be easily assigned. As another example, mass discrepancies were also found for the *B. thuringiensis* product when the predicted mass of the single strands was compared to the expected masses for the single strands (Table 1). Again, the double-stranded mass (i.e., sum of the two single strands) matches that of the predicted double-stranded product. However, the ESI-FTICR mass measurements indicated that the sense strand is 15 ± 2 Da lower than predicted, and the antisense is 16 ± 2 Da higher than predicted, based on the reported sequence.[36] Since the mass precision of the FTICR measurement at this molecular weight is $\pm 2-3$ Da at worst, this apparent discrepancy almost certainly corresponds to a T to C switch (~ -15 Da) in one strand, which results in a corresponding A to G switch ($\sim +16$ Da) in the complementary strand. Thus, a single base substitution is effectively identified.

Analysis of the *B. anthracis* product also showed a mass difference for the single-stranded species. In this case, the double-stranded mass measurement [obtained from the sum of the single-stranded products (Figure 2B and Table 1) and the double-stranded spectrum (Figure 5A)] was determined to be 54 856.9 and 54 856.2 Da, respectively. These mass measurements both agree with the predicted mass of 54 856.74 Da to within 1 Da, which is within experimental error. However, inspection of the single-stranded masses again indicated the sense strand is 15 Da smaller than predicted and the antisense strand is 16 Da greater than predicted.[38] This anomaly is identical to that found for the *B. thuringiensis* product, which is not unlikely since they are both from the *B. cereus* group. Sequencing of this region would identify the source of this mass discrepancy for this PCR product. It should be noted that McLafferty and co-workers previously indicated the potential of ESI-FTICR for obtaining some sequence information for oligonucleotides of this size.[44,45]

The ESI-FTICR mass spectrum shown in Figure 5B illustrates another significant point even though the origin of the three double-stranded species is still under investigation. The resolving power of mass spectrometry should facilitate the analysis of a mixture of PCR products (e.g., simultaneous generation of several PCR products from a microbial community). The three double-stranded products in Figure 5B are completely resolved, each differing in mass by ~308 Da (i.e., approximately half the mass of either GC or AT base pair deletion). Generation of PCR products using multiple primer pairs that result in unique products with a single base pair difference between them, potentially allows

(43) Potier, N.; Dorsselaer, A. V.; Cordier, Y.; Roch, O.; Bischoff, R. *Nucleic Acids Res.* **1994,** *22,* 3895−3903.

(44) Little, D. P.; McLafferty, F. W. *J. Am. Chem. Soc.* **1995,** *117,* 6783−6784.

(45) Little, D. P.; Thannhauser, T. W.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995,** *92,* 2318−2322.

for direct measurement of the mixture and subsequent analysis by mass spectrometry (i.e., no front end separation). Again, more effective measurements would be afforded by the analysis of the corresponding single-stranded species.

CONCLUSIONS

A novel scheme for rapid purification of PCR products for analysis by electrospray ionization-MS has been developed. Accurate molecular weight determinations of several PCR products amplified from the *B. cereus* and *B. subtilis* group of organisms were obtained in a rapid fashion. Importantly, the results showed that single base substitutions from the expected products could be readily distinguished. These results serve to demonstrate the speed, mass accuracy, and precision of the PCR product isolation schemes used and the general applicability of this approach. Furthermore, the mass accuracy and precision reported indicates that identification of any single base substitution, which can be as small as 9 Da, is achievable for PCR products of the size studied. Further investigations are being undertaken aimed at further extending the size of PCR products amenable to this type of analysis. Coupling this approach with multistage mass spectrometry (i.e., dissociation studies) can potentially localize the sites of sequence variations.[44,45] The application of this technique to screen for mutations should allow the accurate and rapid evaluation of genetic variations. Also promising is the direct combination of this approach with high-speed PCR methods, where the high MS sensitivity could potentially be exploited to reduce the number of amplification steps required and to further speed analysis.

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