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EXPERIMENTAL ARTICLES

Characteristics of Microspheres Formed in PCR with Bacterial Genomic DNA or Plasmid DNA as Templates

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Abstract—Earlier, we discovered that, along with linear DNA fragments, nano- and microparticles of DNA and their aggregates are formed in the PCR with yeast genomic DNA used as a template and gene-specific or partially complementary primers. The size of the microparticles (microspheres) varied in the range of 0.5 to 3–4 μm. Only thermostable *KlenTaq* polymerase but not *Taq* polymerase could effectively generate microspheres. In this work, we demonstrate that *KlenTaq* polymerase can produce microspheres of variable size (1 to 7 μm in diameter) if genomic DNA of the bacterium *Acidithiobacillus ferrooxidans* and partially complementary primers are present in the PCR mixture. Conditions for generation of DNA microparticles in PCR with *Taq*-polymerase and bacterial genomic DNA as template were also elaborated. It was also found that mainly large microspheres of up to 7 μm accumulated in PCR with plasmid DNAs used as templates and gene-specific primers in the presence of *KlenTaq* polymerase or mixtures of *KlenTaq* and *Pfu* polymerases. Besides, small aggregates, as well as linear branched structures and three-dimensional conglomerates of fused microspheres, were also revealed in the PCR mixtures. UV absorption spectra of native DNA microspheres and microspheres that had undergone heating at 93°C were registered. The key role of Mg^{2+} cations in the formation and stabilization of the microsphere structure was established.

Key words: polymerase chain reaction, *KlenTaq* polymerase, *Taq* polymerase, DNA microspheres, fluorescent primers, fluorescent dyes, epifluorescence microscopy.

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Previously, we have established the formation of DNA microparticles in the course of polymerase chain reaction (PCR) [1, 2]. Microparticles and their aggregates were produced, along with routine double-stranded DNA fragments, in the final stages of PCR [3] that employed gene-specific and/or partially complementary oligonucleotide primers and yeast genomic DNA as a template. Microparticles were detected using the epifluorescence microscopy technique. Microparticles were visible owing to the use of primers fluorescently labeled at the 5'-end with fluorescein thiocyanate (FITC), 6-carboxyfluorescein (FAM), or 6-carboxytetramethylrhodamine (TAMRA). Otherwise, microparticles were stained upon PCR completion with acridine orange (AO), propidium iodide (PI), or diamidino-2-phenylindole (DAPI) [1, 2].

An indispensable requirement for microparticles generation in PCR was the use of a thermostable *KlenTaq* polymerase [4]. No microparticles were reliably detected when *Taq* polymerase was employed with the yeast DNA template.

The results of our preliminary studies indicate that microspheres are formed as a result of condensation of double-stranded and single-stranded DNA fragments, which accumulate in the course of the thermal cycles of the reaction and aggregate into microspheres at the final stages of PCR. The main product of PCR, double-stranded DNA flanked with primers, is the major component of microspheres. We are still investigating the details of the microsphere formation process. The phenomenon of DNA microsphere formation is an example of self-assembly of biopolymers in the course of biosynthesis.

A large number of studies have been devoted to the problem of DNA condensation. Double-stranded DNA and RNA molecules are known to condense and form structures of toroidal and rod-like shapes in water solutions in the presence of multivalent cations, such as the polyamines spermine and spermidine, or trivalent cobalt hexamine ions ($Co(NH_3)_6^{3+}$ [5–8]. The mechanism of ligand-induced condensation is rather well understood [9, 10].

In contrast to trivalent cations, divalent cations, including Mg^{2+}, are unable, according to available data, to induce condensation of linear double-stranded DNA molecules. Mn^{2+} cations are an exception, being capable of inducing toroidal condensation of supercoiled (but not linear) plasmid DNA in water solutions [11].

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Double-stranded DNA also condenses in the presence of polycations, such as poly-L-lysine, poly-L-arginine, etc. [12, 13]. Recenly, cationic lipids and cationic amphiphilic L-helical oligopeptides have been used to produce condensed DNA forms [14, 15]. Tight complexes of plasmid DNA with cationic lipids or peptides exhibit higher transfection efficiency than pure DNA. These complexes are considered to be promising vectors in gene therapy for gene delivery into human cells [14, 15].

DNA microparticles formed in the course of PCR are remarkable for their high stability in water [1, 2]. However, the microparticles dissociate easily in the presence of low concentrations of EDTA (1 mM) [1, 2]. This phenomenon indicates the importance of Mg^{2+} cations of the PCR buffer solution for formation of microspheres and stabilization of their structure. The microparticles discovered by us are of a unique micro- and ultrastructure. Under an electron microscope, DNA microparticles appear as microspheres with numerous thorns. The electron-dense nature of microspheres indicates that DNA fragments comprising them are bound with metal cations (K^+ and Mg^{2+}).

In the present work, we continued the study of DNA microsphere formation in PCR. In particular, plasmid templates and gene-specific primers were used for generation of microparticles. Employment of plasmid DNA in PCR with *KlenTaq* polymerase surprisingly produced large microspheres (up to 7 μm in diameter) and aggregates of such microspheres. In the case of bacterial genomic DNA used as template, *KlenTaq* polymerase produced microspheres of variable size, between 1 and 7 μm. The PCR regime for microsphere production by another enzyme, *Taq* polymerase, was optimized. This paper describes the new types of microspheres and conditions of their formation.

MATERIALS AND METHODS

DNA templates used in the work. Pure genomic DNA of the gram-negative bacterium *Acidithiobacillus ferrooxidans* was isolated by us previously [16].

All plasmid DNAs were obtained with the use of a Wizard kit according to the manufacturer's protocols (Promega, United States). Plasmid pBlueScriptIIsk+ and its derivatives with inserted IS-elements pBS::IST2, pBS::ISAfe1, and pBS::ISAfe600 [16], as well as plasmids pET23 and pET14b (Novagen, United States) (all from our collection) were used in this work. pACT2 plasmid DNA [17] was obtained from D. Kokoev (Institute of Bioorganic Chemistry, Russia). Plasmid DNA preparations (about 500 ng/ml) were diluted 100- and 1000-fold with water beforehand. Aliquots of 1 μl of the diluted samples were used for 50 μl of PCR mixture.

Linear DNA fragments produced in PCR were purified by electrophoresis in 0.8% agarose gel followed by elution from the gel [18].

PCR procedure. An MJ Research PTC-200 thermocycler (United States) was used for PCR. The reaction mixture (50 μl) contained a corresponding PCR buffer, a mixture of four dNTPs (0.2 mM each), primer oligonucleotides (10–15 pmol each), 1–10 ng genomic or plasmid DNA, and 2.5 U of *Taq* polymerase or 10 U of *KlenTaq* polymerase. *KlenTaq* polymerase buffer contained 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 3.3 mM $MgCl_2$, and 0.01% Tween 20. *Taq* polymerase buffer contained 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, and 1.5–2.0 mM $MgCl_2$. For each PCR experiment, there was a negative control (the reaction in the absence of DNA template).

Taq polymerase was kindly provided by L.I. Patrushev (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Russia); *KlenTaq* polymerase, by K.B. Ignatov (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Russia). An Encyclo PCR kit (Evrogen, Russia), containing a mixture of DNA polymerases, 10-fold concentrated buffer with Mg^{2+}, and dNTP mixture, was also used in the work.

The PCR conditions were as follows: denaturation at 94°C for 30 s, annealing at 53–65°C (depending on the pair of primers used) for 30 s, and synthesis at 72°C for 0.5–1.5 min. The number of cycles varied between 30 and 38 depending on the DNA template and is indicated further in the text.

Primer oligonucleotides Apdir (5') AATCAGTGAGGCACCTATCTC and Aprev (5') TGAAGATCAGTTGGGTGCACG or their fluorescently labeled derivatives TAMRA-Apdir and FAM-Aprev were used for PCR amplification of the central part of the β-lactamase gene (*bla*). Primers were designed proceeding from the *bla* gene nucleotide sequence in plasmid pACT2 (GenBank accession number, U29899).

Fluorescently labeled primers were synthesized by Syntol company, Russia. Routine primers were synthesized by ZAO Evrogen, Russia.

PCR amplification of the insertion sequences IST2, ISAfe1, and ISAfe600 present in the genomic DNA of various *A. ferrooxidans* strains and of the same insertion elements cloned into plasmid pBlueScriptIIsk+ was performed using primers E1.1f + E1.2r and E2.1f + E2.2r, respectively [16].

E1.1f: (5') TCGTCGGATTGAGTGGGTAG;

E1.2r: (5') TCGTCATTTCAAGTGGGTAG;

E2.1f: (5') GAGCTATAGTCAAATCTGGTGTATG;

E2.2r: (5') GCTATGCTCGAAAGTGGTGTATG.

Standard PCR products (linear DNA fragments), as well as plasmid and genomic DNAs, were analyzed by electrophoresis in 0.8–1.0% agarose gel in Tris–acetate buffer (TAE) with ethidium bromide [18].

DNA microparticles were isolated and analyzed as described previously [1].

Upon PCR completion, the reaction mixture with fluorescently labeled primers was diluted 8–10 fold with distilled water and centrifuged for 3 min at

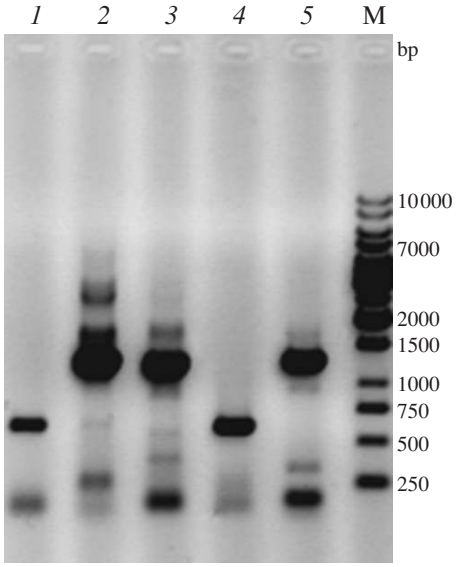

Fig. 1. Electrophoresis of linear DNA fragments produced in PCR (37 cycles) with genomic DNA of *A. ferrooxidans* strains (*1*) TFO, (*2*) TFBk, (*3*) TFL-2, (*4*) TFNd, and (*5*) ATCC19859 in the presence of an Encyclo enzyme mixture (Evrogen) and primers E1.1f + E1.2r complementary to terminal inverted repeats (TIRs) of insertion sequences ISAfe1 and ISAfe600. "M" designates markers of DNA fragment length (1 kb DNA ladder).

10000 rpm (a MiniSpeen centrifuge, Eppendorf, Germany). The supernatant was carefully removed, and the precipitate of microparticles was washed twice with distilled water and finally suspended in 40 μl of water. After suspending the precipitate on a vortex shaker or by pipetting, 3–5 μl aliquots of each sample were applied to a slide and examined under an epifluorescence microscope.

Staining of DNA microspheres with fluorescent dyes. When routine primers were used, microparticles were stained with intercalating fluorescent dyes as described elsewhere [2], usually with propidium iodide (PI). In some cases, acridine orange (AO) or diamidino-2-phenylindole (DAPI) were used.

Fluorescence microscopy of the samples. Microparticle preparations were studied using an Olympus CK40 epifluorescent microscope (Germany). ×20 and ×40 objectives and the light filters 41004 Chroma Texas Red (excitation band, 532–588 nm; emission band, 645/75 nm) and DMB2 Olympus (excitation band, 460–490 nm; emission above 515 nm (515 Long Path)) were used. Images were recorded with an Olympus DP50 digital color camera coupled to a personal computer (Pentium 5 processor).

DNA microparticles were treated with *Micrococcus* nuclease as described previously [2].

DNA microsphere digestion by restriction endonucleases. The water suspension of microspheres obtained in PCR (one tenth of the 50 μl of the PCR mixture) was supplemented with 10× (R + BSA) buffer (Fermentas, Lithuania) and water to the final volume of 15 μl and with 1 μl of the corresponding enzyme solution. The reaction was carried out at 37°C for 2 h. The whole sample was used for electrophoresis.

UV-absorbance spectra of DNA microparticles were recorded on a U-3210 spectrophotometer (Hitachi, Japan) as described earlier [2].

RESULTS

Formation of Microparticles in PCR Using Bacterial Genomic DNA as a Template

In our earlier experiments, yeast genomic DNA was used as a template in PCR [1, 2]. Among the tasks of the present work was the study of the possibility of microparticle formation in the process of amplification of bacterial genomic DNA. Bacterial genomes are known to be considerably smaller than the genomes of yeast and to contain less noncoding DNA, which includes DNA repeats (IS-elements, transposons, etc.). Besides, bacterial genes lack introns.

We possess a collection of genomic DNA of various strains of the gram-negative bacterium *A. ferrooxidans*, as well as primers partially complementary to the terminal inverted repeats (TIR) of the insertion elements ISAfe1 and ISAfe600 [16] occurring in the chromosomes of these strains. This allowed us to set up experiments on PCR amplification of these IS-sequences.

Five independent *A. ferrooxidans* strains [16] and primer oligonucleotides E1.1f and E1.2r were used in the experiment. PCR was performed with an enzyme mixture of a Evrogen kit and, in parallel, with *Taq* polymerase. Primers were annealed at 62°C, and synthesis was carried out at 72°C for 1.5 min. Upon PCR completion, linear DNA fragments were analyzed by electrophoresis in agarose gel and the NA remaining in the reaction mixture were stained with PI.

In the experiment with the enzyme mixture from Evrogen, fluorescent microspheres were detected in only two samples out of five (samples number 2 and 3, containing genomic DNA of strains TFBk and TFL-2, respectively) and only after 37 thermal cycles. The results of electrophoresis of the PCR products obtained in this experiment are presented in Fig. 1.

As can be seen from Fig. 1, DNA fragments about 1250 bp long accumulated in the course of PCR of samples number 2, 3, and 5. The length of these fragments corresponds to the length of the internal part of the ISAfe1 insertion sequence [16]. The largest amount of linear PCR products, as well as the largest amount of microparticles (1.4×10^5 per 50 μl of the reaction mixture) was found in sample number 2, where DNA of strain TFBk was used as the template. Sample number 3 (DNA of strain TFL-2 as the template) contained a similar amount of linear products but five times less microspheres. Interestingly, their size was significantly larger than the size of the microspheres revealed earlier in PCR with yeast DNA [1, 2]. According to our mea-

surements, the diameter of the microspheres varied in the range of 1 to 7 μm (see Fig. 2).

In sample number 5 (DNA of strain ATCC19859) linear PCR product was somewhat less abundant than in sample number 2, and no microparticles were detected (Fig. 1).

In samples number 1 (DNA of strain TFO) and 4 (DNA of strain TFNd) a 600-bp fragment accumulated in a less significant amount; and no microparticle formation occurred. Therefore, microparticles are formed at the final PCR stages when a high synthesis level of linear DNA fragment has been achieved.

In parallel experiments with *Taq* polymerase we also recorded the formation of fluorescent microparticles in two samples (DNA of strains TFBk and ATCC19859); however, they could be detected only after 38 thermal cycles (data not shown). The amount of microparticles was low (about 1×10^4 per 50 μl of the reaction mixture), which is more than an order of magnitude lower than in the corresponding sample amplified with an Encyclo enzyme mixture obtained from Evrogen. The diameter of microparticles (microspheres) generated by *Taq* polymerase was evaluated as 1 to 3 μm (data not shown). No further studies of these microparticles were performed.

Amplification of β-Lactamase Gene in PCR with Plasmid pACT2 DNA as the Template

A priori, one could assume the nature of template DNA to be an important factor in accumulation of single-stranded DNA fragments in the course of PCR and the following self-assembly of the fragments into spherical structures. Plasmid pACT2 of strain *Saccharomyces cerevisiae* (pACT2)(pGBT9) was used to test this supposition [17]. Primers Apdir and Aprev, complementary to the *bla* gene harbored by plasmid pACT2 were synthesized earlier, as well as their fluorescently labeled derivatives [2].

The following conditions were used for PCR amplification of the *bla* gene with primers TAMRA-Apdir + FAM-Aprev and *KlenTaq* polymerase: annealing at 60°C; synthesis for 1 min; and the number of cycles, 30. The mixture of the same composition lacking template DNA was used as the control.

Electrophoresis of PCR products revealed accumulation under the given conditions of a 750-bp DNA fragment (data not shown), which well corresponded to the size of the *bla* gene; an insignificant amount of a DNA fragment of the same size was synthesized in the control sample, which was due to *Taq* polymerase contamination with *Escherichia coli* DNA.

Spherical microbodies, primarily large ones, 5–7 μm in diameter (Fig. 3), were detected by fluorescence microscopy. Smaller microspheres of 2–3 μm were also present in all samples in a low amount. The microspheres could be very well seen under white light illumination (Fig. 3a). Under green light, microspheres fluoresced intensely in the red range of the spectrum (Fig. 3b). No microparticles were revealed in the control sample (without a template). The number of microspheres in the samples depended on the template concentration and reached 8×10^4 per 50 μl of the reaction mixture.

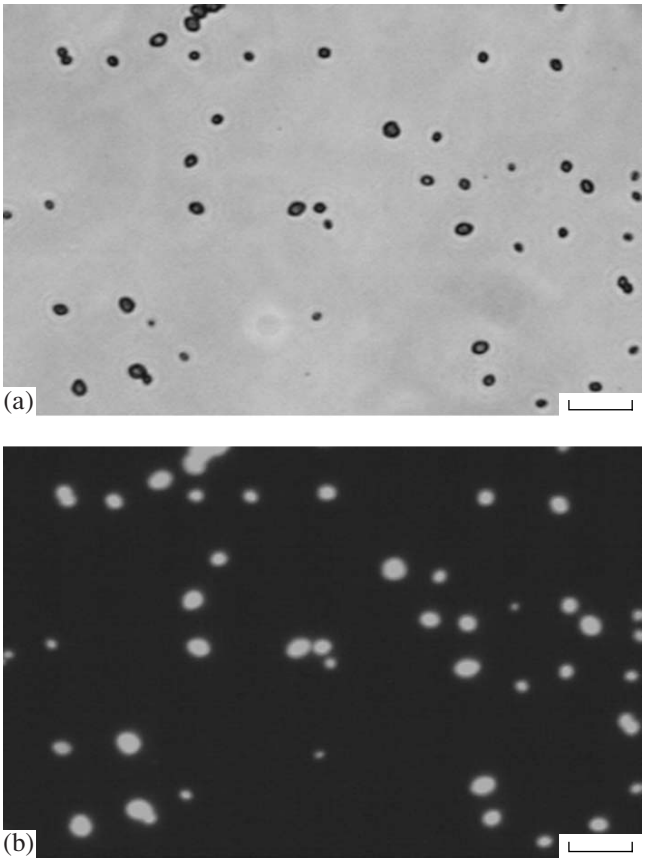

Fig. 2. DNA microspheres formed in PCR amplification (37 cycles) of genomic DNA fragments of *A. ferrooxidans* strain TFBk with an Encyclo enzyme mixture (Evrogen) and primer E1.1f + E1.2r. Microspheres were stained with PI are irradiated with (a) white or (b) green light. Bars, 20 μm.

Thus, large microspheres are produced in the course of PCR in the presence of plasmid templates.

Formation of Large Microparticles in PCR Amplification of the bla Gene in Plasmid Templates of Various Sizes and Structures

In our further experiments, a number of plasmids containing *bla* were studied. *KlenTaq* polymerase and plasmids pACT2, pET23, pET14, and pBluescriptIIsk+ were used in these experiments. All these plasmids contain the *bla* gene. The primer oligonucleotides used were Apdir + Aprev and their fluorescently labeled derivatives. PCR conditions were the same as in the previous experiment.

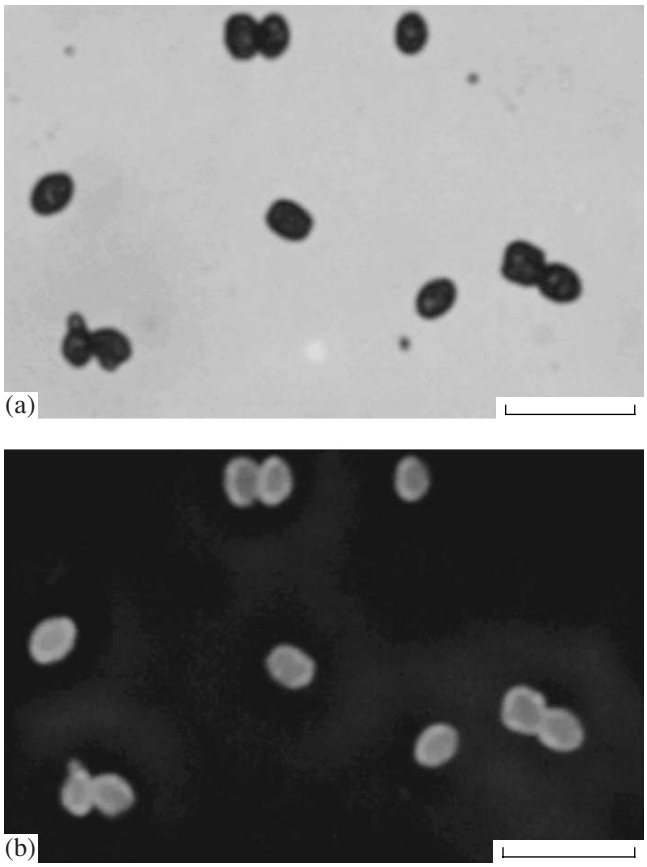

Fig. 3. Individual DNA microspheres produced in PCR (30 cycles) with pACT2 plasmid DNA as template and gene-specific fluorescently labeled primers TAMRA-Apdir + FAM-Aprev. Irradiation with (a) white and (b) green light. Bars, 20 μm.

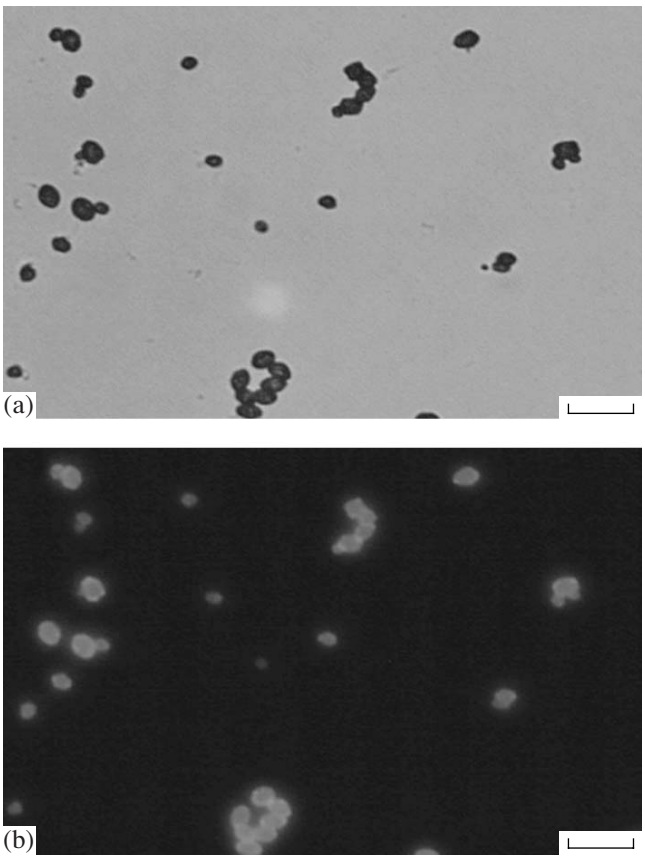

Fig. 4. Individual microspheres and small aggregates of fused microspheres formed in PCR (30 cycles) with pACT2 plasmid DNA as template and gene-specific primers Apdir + Aprev. The microspheres were stained with PI and irradiated with (a) white and (b) green light. Bars, 20 μm.

In the presence of all templates under study, accumulation of a 750-bp DNA fragment was observed (data not shown). The reaction mixtures were used to prepare specimens for fluorescent microscopy. Microspheres were stained with PI. DAPI staining was an option.

The results of the microscopic analysis were as follows:

(1) All samples contained abundant large individual microspheres. The size of the microspheres was as shown in Fig. 3, their diameter varying between 5 to 7 μm. In white light, PI-stained microspheres were dense, smooth-faced, with characteristic dark-brown pigmentation. In green light, they fluoresced with red and in the blue light, with green (only in the case where one of the primers used was FAM-Aprev; data not shown). The glow was evenly spread over the surface of the microspheres.

(2) The number of microspheres in the samples varied between 1×10^4 and 8×10^4 per 50 μl of the reaction mixture and depended on the concentration of the template DNA; no direct relationship between the microsphere titer and DNA template concentration was revealed.

(3) In addition to large microspheres, smaller microbodies 2–3 μm in diameter were frequently observed in the samples. In some samples, along with spherical microparticles, a rather large number of microparticles of irregular shape was observed; these were aggregates of two or more fused microparticles (Fig. 4).

Thus, in the case of circular plasmid templates of varying structure, large microspheres are formed on all occasions in PCR with gene-specific primers.

Formation of Microspheres in PCR with Plasmid DNA and Primers Complementary to Terminal Inverted Repeats (TIRs) of IS Elements

In the next series of experiments, plasmids derived from plasmid pBlueScriptIIsk+ and carrying *Acidithiobacillus ferrooxidans* insertion sequences IST2, ISAfe1, and ISAfe600 [16] were used as templates. These IS elements are of different lengths (1400, 1250, and 600 bp, respectively), and this allowed us to explore whether the size of the generated microspheres

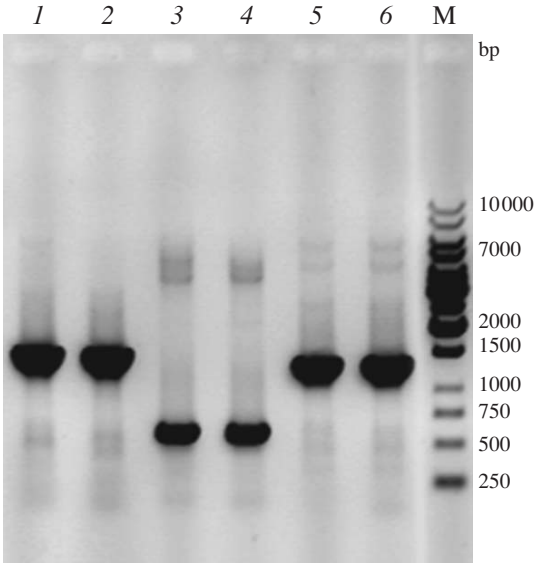

Fig. 5. Electrophoresis of double-stranded DNA fragments produced by PCR amplification (30 cycles) with a Evrogen enzyme mixture and gene-specific primers E2.1f + E2.2r and E1.1f + E1.2r. The templates were plasmid DNAs originating from plasmid pBluescriptIIsk+ and containing IS elements (*1, 2*) IST2, (*3, 4*) ISAfe600, and (*5, 6*) ISAfe1. M are markers of DNA fragment length.

depends on the length of the PCR product, determined by the primer pair used.

To amplify the IST2 element, the gene-specific primers E2.1f + E2.2r were used; for ISAfe1 and ISAfe600 elements, another pair of primers, E1.1f + E1.2r, was employed. Amplification was performed with *KlenTaq* polymerase under standard conditions: annealing at 63°C; synthesis at 72°C for 1.5 min; and the number of cycles, 30.

Data on electrophoresis of linear products are presented in Fig. 5. As seen from the picture, specific DNA fragments of the size corresponding to IS elements accumulated during PCR; the background signal was low.

Specimens of PI-stained microsphere suspensions were examined under a fluorescent microscope. Large microspheres were observed in all samples. The size and morphology of microspheres resembled those of microspheres obtained by β-lactamase gene amplification. The number of microspheres in the sample did not exceed 6–8 × 10^4. The microspheres fluoresced with red light intensely when irradiated with a green beam. Interestingly, in addition to individual microspheres, two–three linear aggregates of a bizarre shape were observed in every examined sample (Fig. 6a). In the aggregates, microspheres formed a chain branching at several points.

Thus, the size of microspheres and their morphology does not depend on the length of the linear PCR product.

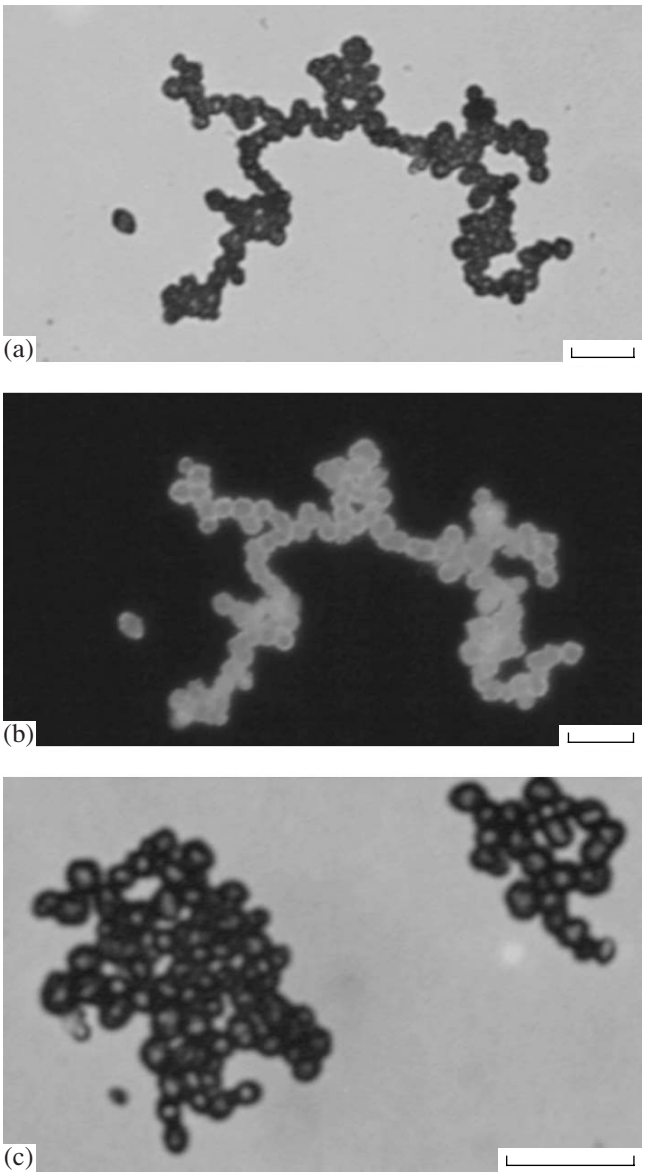

Fig. 6. (a, b) Chains of dragon-like aggregates of microspheres generated in PCR (30 cycles) with pBS::ISAfe1 plasmid DNA and gene-specific primers E1.1f + E1.2r in the presence of *KlenTaq* polymerase and (c) Three-dimensional aggregates of microspheres formed in PCR with pBS::ISAfe600 plasmid DNA and gene-specific primers E1.1f + E1.2r with a Evrogen enzyme mixture. Microspheres were stained with PI and irradiated with (a and c) white and (b) green light. Bar, 20 μm.

Efficiency of Various DNA Polymerases in Microsphere Formation during PCR Amplification of Plasmid Genes

Further on, we studied microsphere formation by various DNA polymerases and their mixtures in PCR amplification of plasmid genes.

Three plasmids pBS::IST2, pBS::ISAfe1, and pBS::ISAfe600 were used in these experiments. The

DNA polymerases employed were (1) *KlenTaq* + *Pfu* mixture, 50 : 1, (2) an Encyclo enzyme mixture obtained from Evrogen (*KlenTaq* + minute amounts of *Pfu*), (3) *KlenTaq*, (4) *Taq*, and (5) *Pfu*. Primers complementary to TIRs of IS elements (see above) were used.

Amplification was performed under standard conditions: annealing at 63°C; synthesis at 72°C for 1.5 min (in the case of *KlenTaq* polymerase, the time of synthesis was 40 s); and the number of cycles, 30. Upon PCR completion, reaction mixtures were analyzed by a standard procedure; microparticles were stained with PI.

Electrophoresis pictures did not differ significantly from those presented in Fig. 5. Accumulation of considerable amounts of target linear DNA fragments was observed in all samples. However, in samples where *Pfu* polymerase was employed, the intensity of corresponding bands was low (data not shown).

The data of fluorescent microscopy study are described below.

Mixture of *KlenTaq* + *Pfu* (50 : 1). In three samples (with three independent templates), a large amount of microspheres was revealed; aggregates were also present. Microspheres morphology and size did not differ from those already described. At high magnification (40× objectives) under white and, especially, green light some microparticles were clearly seen to consist of several separate subparticles. Thus, the products of amplification by a *KlenTaq* + *Pfu* (50 : 1) mixture did not differ significantly in their characteristics from *KlenTaq* polymerase products.

An Encyclo enzyme mixture from Evrogen. All three samples contained appreciable amounts of microspheres. Most microparticles were grouped into large and medium-size three-dimensional conglomerates (Fig. 6b). The ratio between single microspheres and microspheres incorporated into conglomerates varied significantly. Samples obtained with plasmids pBS::IST2 and pBS::ISAfe1 mainly contained individual microspheres. On the contrary, a small number of single microspheres was observed in a sample derived from plasmid pBS::ISAfe600, most of them occurred in conglomerates. At high magnification (40× objectives) in white light the microspheres and their conglomerates resembled coffee beans.

Thus, the production of large conglomerates of microspheres was a characteristic specific to the Encyclo enzyme mixture from Evrogen.

***KlenTaq* polymerase.** All three templates produced a considerable amount of large microspheres. They were mainly individual structures, although small aggregates were also observed. Thus, shorter synthesis time (40 s instead of 1.5 min) did not cause any changes in microsphere formation.

***Pfu* polymerase.** No microspheres were observed in all three samples, probably due to insufficient accumulation of the linear PCR product.

***Taq* polymerase.** With all three templates, unique huge microspheres were formed. Description and analysis of such microspheres is a matter of a separate publication.

Formation of Microspheres in PCR with Linearized Plasmid DNA

In the experiments described above, circular plasmid DNAs of a known structure were used as templates. It remained unclear whether microsphere formation would occur with linearized plasmid templates.

Plasmid pBlueScriptIIsk+ and its linearized form obtained by *Eco*RV digestion were used. Linearized plasmid sample was diluted 100-fold with distilled water. Either 1 or 2 μl aliquots of the diluted plasmid were used per 50 μl of PCR reaction mixture. The primers used were Apdir + Aprev. PCR was carried out under standard conditions (30 cycles, 1.5-min synthesis).

Considerable amounts of a 750-bp DNA fragment accumulated during PCR in all samples (data not shown).

Presence of microspheres was detected by microscopy under white light (unstained particles) and after staining with PI. It was found that microspheres were present in all samples in the same concentration. Unstained microspheres under white light appeared semi-transparent, were hard to focus at, and it was thus difficult to estimate their size. PI staining resulted in a dark-brown color and contrast. Under green light, the microspheres fluoresced intensely red. No differences in size or density was revealed between microspheres formed on circular and linearized plasmid DNAs. Therefore, microspheres are also generated in PCR amplification with linearized plasmids.

UV-Absorbance Spectra of the Microspheres

We studied UV spectra of the DNA microspheres to evaluate the feasibility of using UV spectroscopy for control of the purity of DNA and its quantitative determination in microspheres.

Large microspheres were obtained in amplification of plasmid pBS::ISAfe1 template with either Apdir + Aprev or E1.1f + E1.2r primers. The microspheres were not stained and were studied under white light.

The recorded UV spectra of native microspheres in water only distantly resembled those of NA; and the absorbance peak at 260 nm was either indistinct or completely absent. The spectra were characterized by a significantly increased baseline level in the region between 300 and 340 nm, which was probably due to light scattering by microparticles [19]. A typical UV spectrum of native microparticles is presented in Fig. 7a (curve *1*). To record the spectrum, suspensions from four tubes were pooled together into one sample.

We also recorded UV spectra of microspheres heated at 94°C for 5 min with subsequent cooling to

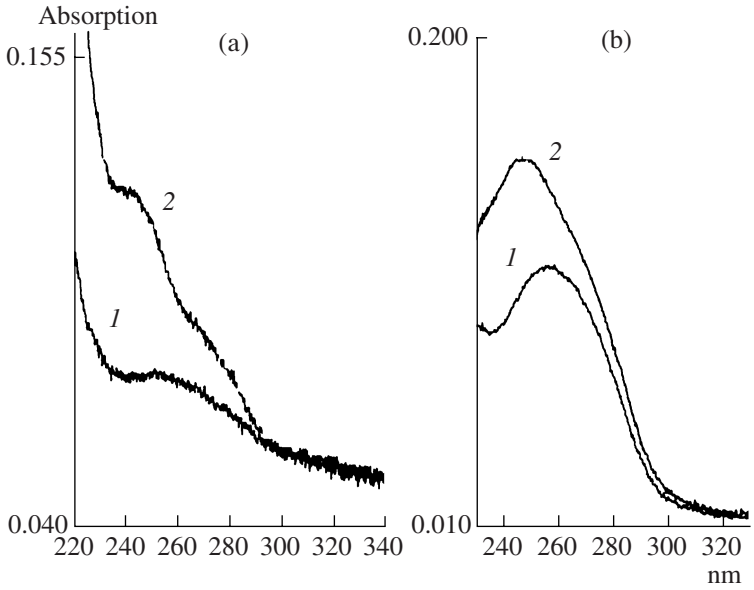

Fig. 7. (a) UV absorbance spectra of microspheres suspension in water and (b) products of their dissociation in 1× SSC buffer. Microspheres were obtained in PCR with pBS::ISAfe1 plasmid DNA as template with primers E1.1f + E1.2r. (*1*) spectrum before heating and (*2*) spectrum after heating at 94°C for 5 min. In variant (b), the control cuvette contained 1× SSC buffer.

room temperature (Fig. 7a, curve *2*). It can be seen that, after heating, the spectrum changed considerably, the shift of absorption maximum to the short-wavelength region indicating hyperchromatic effect in the region of 220–290 nm. In other words, the spectrum became even more "abnormal", atypical of NA. Similar abnormal UV spectra of heated DNA microparticles were obtained previously after PCR amplification of yeast genomic DNA [2]. As clarified in subsequent experiments employing epifluorescence and electron microscopy, the number of microparticles did not decrease upon heating; however partial melting of them occurred. The results of these experiments are subject of a separate publication.

Analysis of the data revealed inapplicability of UV spectroscopy of microparticle suspensions for quantitative determination of DNA content. The reason is the large microparticle size and high density of DNA packing in microparticles.

Dissociation (break-down) of Microspheres in the Presence of EDTA

Earlier, we have shown that DNA microspheres are destroyed (dissociated) in the presence of chelating agents (EDTA) [1, 2]. Larger microspheres also turned out to be sensitive to EDTA. We found out that in the presence of 50 and even 5 mM EDTA microspheres were quickly destroyed. The kinetics of the process could be studied in the presence of 1 mM EDTA, as in this case, the number of microspheres decreased by an order of magnitude over 10 min.

We also revealed that the addition of chelating agents (2 mM EDTA or 1× SSC buffer [18]) to microparticle suspensions generated samples adequate for quantitative DNA analysis. UV absorbance spectra of solutions of microspheres dissolved in 1× SSC are presented in Fig. 7b (curve *1*). These are spectra typical for NA. Heating of samples dissolved in 1× SSC at 94°C for 5 min followed by cooling to room temperature resulted in the hyperchromatic effect and a shift of the absorbance maximum to the short-wavelength region (Fig. 7b, curve *2*). Spectroscopy data allowed to quantify DNA amount in microparticles, which on average was 0.5 μg per 50 μl of the reaction mixture, or 12.5% of the yield of linear PCR products (about 4 μg).

Gel Electrophoresis of Microspheres in Agarose

We also studied the products of dissociation of microspheres in the process of electrophoresis. Standard water suspensions of the microspheres obtained in PCR amplification of various plasmid templates with different pairs of primers were used. Aliquots of the samples (5 μl, i.e., 1/10 of the microspheres formed in 50 μl of PCR mixture) were added to 1.0 μl of the 6× loading buffer for electrophoresis with dyes and EDTA (Fermentas, Lithuania) and applied to wells of a 0.8% agarose gel. Electrophoresis was performed in TAE buffer containing 1 mM EDTA [18].

The results of electrophoresis are presented in Fig. 8. Bands of high intensity, easily seen on the electrophoregram, correspond to the standard double-stranded DNA PCR product. Some minor bands of lower intensity corresponding to DNA of higher molecular mass (the nature of them is yet unknown) were also

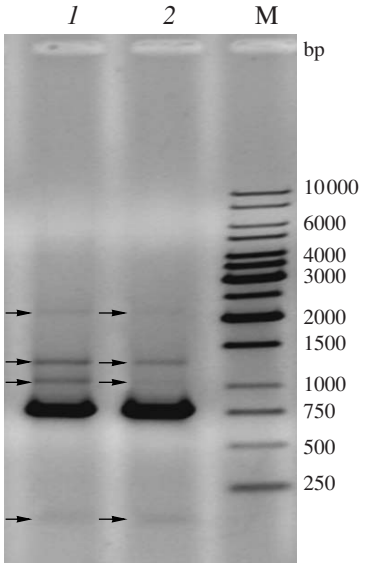

Fig. 8. Electrophoresis in 0.8% agarose gel of native microspheres obtained in PCR of (*1*) pBS::ISAfe1 plasmid and (*2*) a linearized form of pBlueScriptIIsk+ with *KlenTaq* polymerase and primers Apdir + Aprev.

present in the analyzed samples. Thus, in the process of electrophoresis, microspheres interact with the EDTA present in the loading buffer and dissociate. As follows from the data presented, linear double-stranded DNA flanked with primers is the major component of microspheres.

Effect of Nonspecific and Specific Nucleases on Large Microspheres

The effect of *Micrococcus* nuclease on microsphere samples was studied. The samples were obtained in PCR with plasmid pBS::ISAfe1 as the template and primers Apdir + Aprev.

As we had expected, aggregates of microparticles in samples degraded significantly, leaving only faint contours of microspheres with practically no fluorescence. Thus, the *Micrococcus* nuclease destroyed microspheres, which proves the DNA nature of the microsphere constituents.

We also studied the effect of restriction nucleases on the DNA in microspheres. We used microsphere samples obtained in PCR amplification of pBS::ISAfe1 and pBS::ISAfe2 templates with primers Apdir + Aprev. Restriction endonucleases *Eco*RI, *Eco*RV, and *Sca*I (Fermentas) were used in this experiment. The recognition sites of the restriction endonucleases *Eco*RI and *Eco*RV occur in the polylinker region of the template plasmids, and the recognition site of *Sca*I occurs in β-lactamase gene.

As follows from electrophoresis data, treatment with *Sca*I restriction endonuclease led to disappearance of the 750-bp fragment and the appearance of two new fragments 550 and 200 bp long (data not shown). *Eco*RI and *Eco*RV treatment did not alter the 750 bp long fragment.

Thus, specific restriction nucleases are capable of digesting the DNA of microspheres.

Large Microspheres Contain no Proteins

We performed an SDS-PAGE [20] study to find out whether admixture proteins were present in the samples of microspheres. The samples were obtained in PCR amplification with DNA of plasmid pBS::IST2 and primers Apdir + Aprev. A 1 μl aliquot of *KlenTaq* polymerase preparation was used as control.

Protein electrophoresis revealed a considerable amount of protein in the *KlenTaq* polymerase preparation (mainly BSA added for enzyme stabilization; data not shown). However, protein bands were hardly detectable in the specimens containing with water suspensions of microspheres. Thus, the proteins are not the structure-forming elements of the microspheres.

DISCUSSION

The data obtained in our present and previous studies [1, 2] allow the conclusion that the formation of DNA microparticles in the process of PCR is a regular phenomenon. They are formed irrespective of the template used, whether it is genomic DNA of yeast or bacteria or plasmid DNA. Microspheres are likely to be formed during amplification of any DNA fragment (any gene) located in a plasmid or a chromosome. In the present study, we used five independent pairs of oligonucleotide primers, and all of them yielded microspheres. *KlenTaq* polymerase, pure or with an admixture of *Pfu* polymerase, which possesses 3' ⟶ 5' exonuclease (proof reading) activity, is preferable for generation of microspheres. The reason why *Taq* polymerase is considerably less efficient in the formation of microspheres is yet unclear. However, we managed to demonstrate the possibility of the generation of microspheres in PCR with *Taq* polymerase and bacterial genomic DNA as template. The differences in properties of *KlenTaq* and *Taq* polymerases have been discussed previously [2].

Interestingly, in the case of using bacterial genomic DNA as a template and *KlenTaq* polymerase, microspheres are formed only after a large number of thermal cycles (37 or more), when considerable amount of the linear PCR product of PCR has already accumulated. When there was not enough linear PCR product (Fig. 1), microspheres were absent. To compare, amplification of yeast genomic DNA produced microspheres as early as upon 33 thermal cycles; meanwhile, the amount of the linear product was not as high as in the case with bacterial genomic DNA. A possible reason for this is the low frequency of repeats in bacterial genomic DNA as compared to yeast DNA.

As for the *Taq* polymerase, microspheres were detected only in two samples out of five and not earlier than after 38 thermal cycles. These two samples were characterized by a very high content of linear DNA fragments 750 bp long (data not shown). Thus, *Taq* polymerase is by far less efficient in microsphere production.

Quite a different picture was observed in the case with purified plasmid DNA, where large (5–7 μm) dense microspheres were produced as early as after 30 cycles. If the template was bacterial genomic DNA, microspheres of variable size, 1 to 7 μm, were generated. What factors govern the formation of DNA microspheres of specific size and density? We assume that the initial concentration of the sequence to be amplified in the template DNA is critical. In the case with purified plasmid DNA, the concentration of the DNA fragment to be amplified is by orders of magnitude higher than the concentration of the same fragment present in genomic (either yeast or bacterial) DNA.

As for the mechanism of microsphere assembly, special attention should be drawn to Mg^{2+} cations. They are absolutely essential in the process of formation of microsphere and stabilization of their structure. This conclusion follows from the observation that EDTA or sodium citrate, which bind Mg^{2+} ions, induce fast dissociation of microspheres into linear DNA fragments. Interestingly, Mg^{2+} cations are also absolutely required for DNA polymerase activity. Optimal magnesium concentration for *KlenTaq* polymerase is 3.5 mM; for *Taq* polymerase, it is a little lower, 1.5 mM. A question arises as to whether the difference in the ability of the two enzymes to form microspheres is due to different concentration of magnesium ions in the corresponding buffers.

In the literature, there are data on ligand-mediated condensation of DNA in water solutions in the presence of multivalent cations (polyamines, trivalent cobalt cations) with the production of toroid- and rod-like nanoparticles [5–8]. However, divalent cations, including Mg^{2+} ions, did not induce condensation of linear double-stranded DNA fragments [11]. Thus, the discovered microparticles represent a novel form of condensed DNA.

Electrophoresis of microspheres in TAE-buffer containing as little as 1 mM EDTA revealed that linear double-stranded DNA flanked with two primers, i.e. the standard PCR product, are the major product of microsphere dissociation. The nature of the other (minor) bands is to be explored. Experiments with restriction nucleases, which recognize double-stranded DNA targets, indicate that the microspheres consist of native linear DNA fragments. Single-stranded DNA fragments may be another component of the microspheres. Such fragments are formed in PCR as result, e.g., of synthesis on the initial template (i.e., plasmid or chromosomal DNA) and accumulate linearly. The length of single-stranded DNA fragments may considerably exceed the length of the amplicon. Therefore, a complex mixture of double- and single-stranded DNA molecules is present in PCR buffer at the final stages (cycles) of PCR. In the process of annealing the PCR mixture, single-stranded fragments may fold, forming nano-sized particles of compact three-dimensional structure (recently, we discovered particles of this kind by electron microscopy). Apparently, the role of Mg^{2+} cations is determined by the fact that they, as well as other multivalent cations, neutralize the negative charge of phosphate groups, thus reducing electrostatic repulsion between DNA molecules and promoting lateral condensation [13, 14] and the formation of tight three-dimensional nanostructures. Being divalent, Mg^{2+} cations may also simply hold together closely disposed DNA molecules. Further study of the mechanism of microsphere formation is currently in progress.

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