ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 6, pp. 816–821. © Pleiades Publishing, Ltd., 2010.

EXPERIMENTAL ARTICLES

Optimization of PCR in Application of Hot Start *Taq* DNA Polymerase for Detection of *Erwinia amylovora* with Primers FER1-F and FER1-R[1]

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Received December 23, 2009

Abstract—There are two approaches in detection of bacterium *Erwinia amylovora* by PCR. One is based on detection of plasmid pEA29 and the other is based on detection of a chromosomal DNA sequence, specific for *E. amylovora*, in a sample. Since pathogenic strains without pEA29 have been isolated from the environment, methods based on this plasmid have been compromised and PCR methods based on chromosomal DNA species specific sequences became only reliable methods. PCR method with chromosomal primers FER1-F and FER1-R is currently the most reliable method due to its high sensitivity and specificity. The goal of this research is to make a significant improvement of the method by optimization of PCR in application of hot start DNA *Taq* polymerase, instead of wax, to obtain a hot start reaction. This enzyme, which is currently widely applied, can provide simpler achievement of hot start, saving labor and time and decreasing possibility of cross contamination of samples. Experiments showed that simple replacement of a regular recombinant *Taq* DNA polymerase by a hot start *Taq* DNA polymerase leads to complete failure of the reaction. Many optimization experiments had to be carried out to obtain an operational and reliable PCR which simultaneously has high sensitivity and specificity. Content of the reaction mixture, as well as temperature and time parameters of PCR, were significantly changed to achieve proper optimization.

Keywords: hot start, PCR, molecular test, bacterium, chromosome, pEA 29, *E. amylovora*

DOI: 10.1134/S0026261710060147

INTRODUCTION

Erwinia amylovora is a gram negative bacterium which causes plant disease fire blight on pome trees and some other rosaceous plants. Molecular methods for detection of *E. amylovora* are assumed as the most appropriate assays. They can be highly sensitive, specific and rapid. Reliable results of these assays can be obtained for just one working day. For long time, the most applied molecular tests were PCR tests based on detection of plasmid pEA29 common for *E. amylovora* [1, 2]. That plasmid is present in the bacterium in more than one copy. This fact enables easier creation of a sensitive assay. However, some recent research showed that *E. amylovora* strains without plasmid pEA29 do exist in the nature compromising all assays based on pEA29 [3–5]. Some assays that are based on chromosomal species specific sequences avoid this problem, but it has been shown that they can lack sensitivity or specificity or both [6–9].

A highly sensitive and specific assay based on chromosomal species specific sequence was developed recently [10]. The sequence can be amplified in PCR by a pair of primers (FER1-F and FER1-R). It is a hot-start PCR reaction. The hot-start decreases amount of background product, which can decrease the yield of the desired product. Hot-start was achieved by application of wax and an ordinary *Taq* DNA polymerase. Preparation of wax and two reaction mixtures (separated by wax) introduces additional steps in the procedure that can increase possibility for cross contamination of samples and are more labor intensive and time consuming, especially if many samples are processed. By application of a hot-start *Taq* DNA polymerase these steps can be omitted. Procedure for preparation of reaction mixture with a hot-start enzyme is the same as for an ordinary PCR. Introduction of hot-start enzyme in PCR is a trend, because it is more convenient, especially in routine procedures with a large amount of processed samples. However, simple replacement of an ordinary *Taq* DNA polymerase with a hot-start *Taq* DNA polymerase is not possible. This replacement leads to complete failure of the reaction. Proper reaction, with high sensitivity and without meaningful background, is possible to achieve only with significant change in content of reaction mixture as well as temperature and duration of reaction cycling parameters.

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For routine detection of *E. amylovora* it is necessary to have highly standardized procedure which can provide consistent results. The goal of this research was optimization of reaction content and other parameters of PCR in application of hot-start *Taq* DNA polymerase for detection of *E. amylovora*. This optimization makes possible to have a highly standardized procedure in application of a more advanced way for achievement of hotstart.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. Bacterial strains were grown and DNA was isolated as already described in [10]. Actually, the material obtained during experiments described in that research [10] was saved in a freezer at –20°C and used in this work. So, this material was good for comparison of results obtained in earlier experiments and in this work because DNA source was the same. Various dilutions were obtained by diluting of frozen DNA.

PCR procedure. The total volume of the reaction mixture was 25 μl. The primers used in this reaction were FER1-F and FER1-R (they enable amplification of a 1269 bp long sequence in an *E. amylovora* specific PCR assay) which were already described in [10]. The reaction mixture contained: 5 nmol of each dNTP; 10 pmol of primer FER1-F (5'-AGCAGCAATTAATGGCAAGTATAGTCA-3'); 10 pmol of primer FER1-R (5'-AATTTAATCAGGTCACCTCTGTTCAAC-3'); 2.5 μ l of 10 × PCR buffer (200 mM Tris-HCl (pH 8.3 at 25°C); 200 mM KCl; 50 mM $(NH_4)2SO_4$; 1.2 mg/ml BSA); 2.5 μl of 25 mM $MgCl_2$; 0.5 μl of TrueStart *Taq* DNA polymerase (5 units/μl; Fermentas – catalogue number #EP0611); 10 μl of DNA sample; and double distilled H_2O to 25 μl. The mixture was covered with a drop of PCR oil.

PCR buffer was obtained by addition of 0.72 mg of BSA to 0.6 ml of 10 × PCR buffer, which was supplied with TrueStart *Taq* DNA polymerase by the manufacturer.

The reaction conditions were as follows: initial denaturation for 4 min at 95°C; 10 s at 94°C, 10 s at 62°C, 90 s at 72°C (35 cycles); and the final extension step for 5 min at 72°C.

These reaction conditions are valid for thermal cyclers with the tube control mode, where the specified temperatures are maintained inside the test tube according to an appropriate algorithm, but not for thermal cyclers in which the specified temperatures are maintained in the heating block. The tube control mode is employed in eppended in thermal cyclers and as well as in those of many other menufactures (GeneAmp PCR System, 2000, Perkin Elmer; UNOII, Biomejra; Omni–E, Hybrid, etc.).

TrueStart *Taq* DNA polymerase (which is designed for Hot start PCR) is inactive at room temperature, avoiding extension of non-specifically annealed primers or primers dimmers. The enzyme is instantly activated during the initial denaturation step of PCR. So, initial denaturation step (4 min at 95°C) is essential for enzyme activity. PCR product was analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 μl/ml of ethidium bromide.

PCR sensitivity test. Sensitivity was determined with cell lysate serial tenfold dilutions of *E. amylovora* strain NCPPB 595, corresponding to CFU concentrations of 6.1×10^6 to 6.1×10^3 CFU/ml and dilutions corresponding to 3×10^2, 1.5×10^2 and 61 CFU/ml. Ten microliters of these DNA dilutions were included in each PCR. Negative control was 10 μl of H_2O.

A sensitivity assay in presence of plant (apple) extract was performed with dilutions obtained by mixing 5 μl of plant extract with 5 μl of cell lysate serial tenfold dilutions corresponding to CFU concentrations of 12.2×10^6 to 12.2×10^3 CFU/ml and dilutions corresponding to 6×10^2, 3×10^2 and 122 CFU/ml (after mixing with the plant extract, the resulting dilutions were the same as in the test without plant extract). Negative control was 5 μl of H_2O mixed with 5 μl of plant extract. Ten microliters of so obtained mixture was added to each PCR. The plant extract was obtained as described in [15].

PCR specificity tests. Specificity test was carried out with all bacterial strains that were performed in paper already presented by Obradovic et al. [10] (74 various strains). These tests were carried out to find out if specific 1269-bp PCR product can be obtained in the reaction with *E. amylovora* DNA only, and not with DNA of any other species. In specificity tests, 10 μl of dilutions of bacterial DNAs corresponding to 6×10^6 CFU/ml (6×10^4CFU/reaction), were used as potential templates.

PCR failure assay. Determination of the reason of the failure of PCR assay described by Obradovic et al. [10] (with TrueStart *Taq* DNA polymerase instead of an ordinary *Taq* DNA polymerase) was achieved with repeated PCR assays in which a particular reaction component was missed (cresol red, Tween 20, glycerol). TrueStart *Taq* DNA polymerase instead of an ordinary *Taq* DNA polymerase was used and 10 μl of bacterial DNAs *E. amylovora* strain NCPPB 595 corresponding to 6×10^4 CFU/ml (6×10^2 CFU/reaction) were added as potential templates. All other reaction components were included and assay was performed as it was already described in the original paper.

Length of extension step of the PCR. Verification of extension step length was performed in two PCRs with 10 μl of bacterial DNAs *E. amylovora* strain NCPPB 595 corresponding to 6×10^4 CFU/ml (6×10^2 CFU/reaction). The reaction conditions were as follows:

—initial denaturation for 4 min at 95°C; 10 s at 94°C, 10 s at 62°C, 30 s at 72°C (35 cycles); and the final extension step for 5 min at 72°C

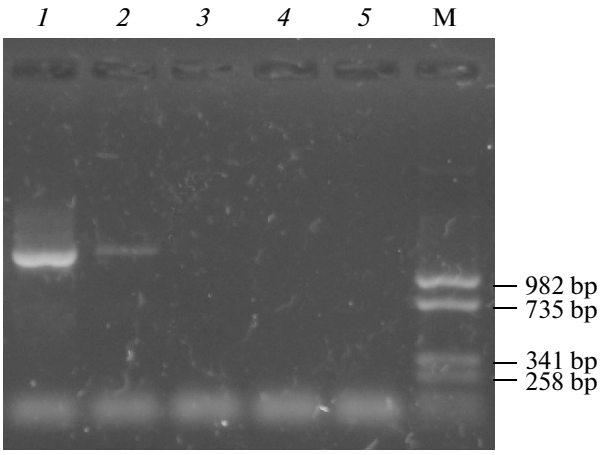

Fig. 1. PCR failure assay. Lanes: (*1*) PCR without Tween 20 and glycerol; (*2*) PCR without Tween 20; (*3*) PCR without cresol red; (*4*) PCR with all ingredients; (*5*) negative control (H_2O); (M) marker DNA.

—initial denaturation for 4 min at 95°C; 10 s at 94°C, 10 s at 62°C, 90 s at 72°C (35 cycles); and the final extension step for 5 min at 72°C.

Electrophoresis. PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 μg/ml of ethidium bromide. The product was placed into the gel directly from the test tube (there was no need for gel loading buffer) in the cases when PCR buffer contained glycerol and cresol red. In the cases when PCR buffer did not contained glycerol and cresol red, product was mixed with a 6 × loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 20% glycerol in water) and loaded into the gel.

All electrophoretic gels were photographed with a digital camera Canon model PowerShot S3 IS. All pictures were taken at highest resolution of the camera (2816 × 2112 pixels) and, when sensitivity of PCR was important, at exposition of 16 seconds and sensitivity of 400 ASA. Electrophoretic bands on photographs taken at so high sensitivity and long exposition cannot be seen clearly without an additional adjustment of brightness and contrast. So, after taking of photos, brightness and contrast was additionally adjusted on a computer with appropriate software (Microsoft Office Picture Manager).

RESULTS

PCR failure assay. Application of TrueStart *Taq* DNA polymerase in PCR assay described by Obradovic et al. [10], instead of an ordinary *Taq* DNA polymerase, for detection of *E. amylovora* caused complete failure of the reaction. A PCR failure assay was performed for determination of the reason of the failure (Fig. 1). Assay without cresol red also failed, indicating that cresol red is not a reason for the failure. Exclusion of Tween 20 enabled synthesis of the specific

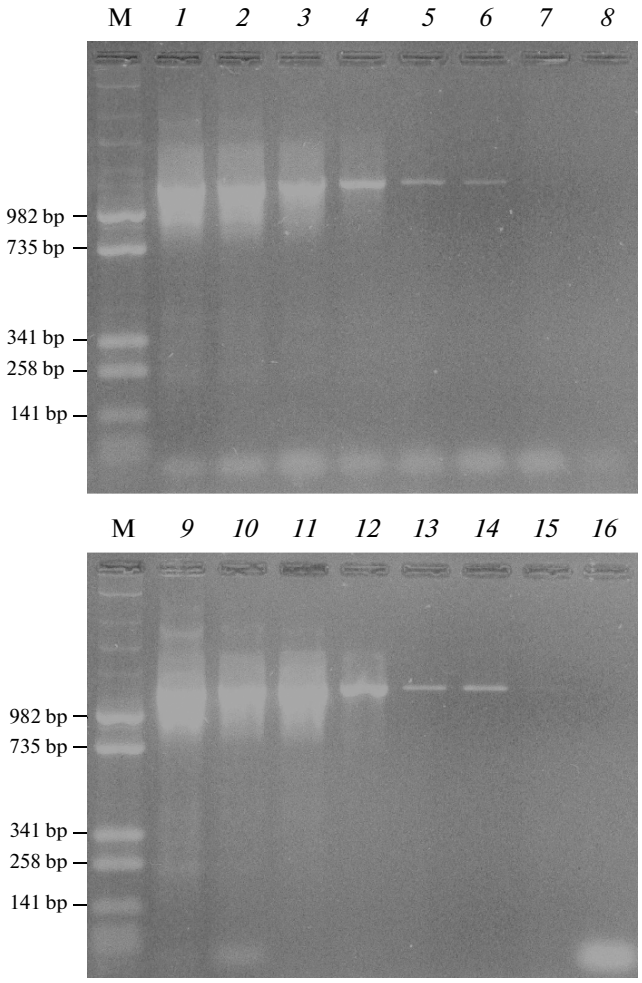

Fig. 2. Sensitivity of *E. amylovora* detection by polymerase chain reaction. All concentrations are expressed as CFU/reaction. Lanes: (M) marker DNA; (*1*) 6.1×10^4; (*2*) 6.1×10^3; (*3*) 6.1×10^2; (*4*) 61; (*5*) 3; (*6*) 1.5; (*7*) 0.6; (*8*) negative control (H_2O); (M) marker DNA; (*9*) 6.1×10^4 with plant extract; (*10*) 6.1×10^3 with plant extract; (*11*) 6.1×10^2 with plant extract; (*12*) 61 with plant extract; (*13*) 3 with plant extract; (*14*) 1.5 with plant extract; (*15*) 0.6 with plant extract; (*16*) negative control (H_2O) with plant extract.

product of the reaction. However, the yield of the product was low. It was significantly lower than in a control reaction. This indicated that there is another component which inhibited the reaction. In a reaction without Tween 20 and glycerol the yield of the product was high.

Sensitivity of the PCR. PCR sensitivity assay was carried out with various concentrations of *E. amylovora* DNA and as well as in the presence of plant (apple) extract (Fig. 2). The amounts of DNA added to the reactions of sensitivity assay corresponded to 6.1×10^4, 6.1×10^3, 6.1×10^2, 61, 3, 1.5, and 0.6 CFU/reaction. The highest consistent sensitivity in the reactions without plant extract was 1.5 CFU/reaction. The same result was obtained in

the reactions with plant extract. However, intensity of electrophoretic bands obtained in presence of plant extract was weaker. Actually, the highest sensitivity achieved was 0.6 CFU/reaction, but the reproducibility of this result was just 50% in the reactions without plant extract, and 25% in the reactions with plant extract. At 0.6 CFU/reaction, the 1269 bp *E. amylovora* specific band was very weak and sometimes was detectable only by photographing. Application of the camera for detection of so low amount of targets was particularly important in the presence of plant extract, when intensity of the band was significantly lower.

Specificity of the test. Some PCRs were carried out (74 reactions plus one positive control and a negative control reaction) to determine if any specific or non specific product is present in any of the reactions. In the reactions with any of *E. amylovora* strain only a specific 1269-bp PCR product was present. There was not any product in the reactions with any strain of species other than *E. amylovora*.

Length of extension step of the PCR. This experiment was performed to verify whether extension step of the reaction is enough long in the case of application of TrueStart *Taq* DNA polymerase. The amount of the product in the reaction with 90 seconds extension step was significantly higher than in the reaction with 30 seconds extension step (Fig. 3). The result showed that extension time which was sufficient in the case of a regular *Taq* DNA polymerase (30 seconds) was not sufficient for TrueStart *Taq* DNA polymerase.

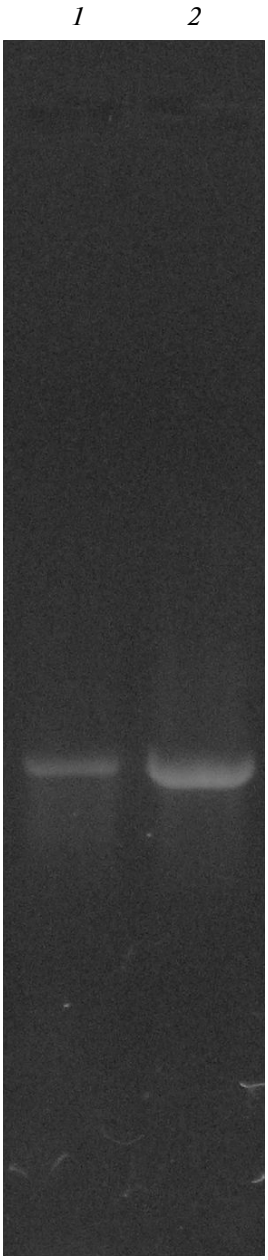

Fig. 3. Influence of PCR length of extension step on amount of the product. Lanes: (*1*) 30 seconds of extension time; (*2*) 60 seconds of extension time.

DISCUSSION

Application of primers FER1-F and FER1-R in a highly sensitive and specific PCR described by Obradovic et al. [10] enables detection of *E. amylovora* even if bacterium does not contain plasmid pEA29. It overcomes shortcomings that other methods suffer from. Very important point in that this redesigned procedure is hot start PCR. It has advantage providing less nonspecific products and thus higher sensitivity. As it is described in the original paper, a nonspecific product (about 600 bp long) can be present if hot start is missed. This product cannot be confused with specific product, because it is much shorter. However, synthesis of this nonspecific product can decrease sensitivity of the reaction.

Recently, hot start *Taq* DNA polymerase has intensively been replacing ordinary *Taq* DNA polymerase in PCR applications. Acquisition of hot start with hot start *Taq* DNA polymerase is very simple. This kind of polymerase has to be activated in PCR by heating of the reaction mixture in an initial denturation/enzyme activation step. Original PCR procedure with FER1-F and FER1-R primer attain hot start utilizing wax. Replacement of ordinary *Taq* DNA polymerase with a hot start enzyme would significantly simplify the procedure. This is particularly welcomed if a large amount of samples has to be processed. Every simplification of the procedure diminishes possibility of cross contamination of the samples. However, simple replacement of ordinary enzyme with hot start enzyme led to a complete failure of the reaction. Acquisition of hot start enzyme required a studious research to find the reason of the failure and as well as optimization of the reaction for application of new enzyme.

PCR failure assay showed that the main reason for failure of the reaction when the ordinary *Taq* DNA polymerase was replaced with hot start TrueStart *Taq*

DNA polymerase was Tween 20 and also glycerol which partially inhibits the reaction. While Tween 20 significantly enhance product yield in the reaction with the ordinary enzyme, in reaction with hot start enzyme it has to be omitted. Actually, Tween 20 is also included in the buffer recommended by the manufacturer of ordinary enzyme, and it is omitted in the buffer recommended by the manufacturer of hot start enzyme. It has been well-known that Tween 20 can enhance yield of the PCR [10–12], but obviously it has to be omitted in the reactions with hot start TrueStart *Taq* DNA polymerase. Glycerol can improve product yield in reaction with the ordinary enzyme [11]. It was also included in the reaction because it eliminated the need for post-PCR manipulation (the reaction mixture could be loaded onto the gel without mixing with loading buffer). However, glycerol inhibits the reaction with hot start TrueStart *Taq* DNA polymerase and cannot be included in the reaction. Finaly, in PCR with hot start TrueStart *Taq* DNA polymerase was used buffer that was recommended by the manufacturer of the enzyme with addition of BSA. BSA did not inhibit the reaction and it has an important role because it prevents the effect of PCR inhibitors [13, 14]. These inhibitors have origin from the plant and they can be present into DNA sample after DNA extraction. Cresol red did not inhibit reaction with the hot start enzyme, but anyhow, it was omitted in the reaction. Without glycerol in the reaction, there was not any reason to include cresol red, which only with glycerol can eliminates the need for post-PCR manipulation.

PCR with hot start TrueStart *Taq* DNA polymerase provides the same sensitivity (consistent sensitivity 1.5 CFU/reaction and 0.61 CFU/reaction with reproducibility of 50%) as ordinary *Taq* DNA polymerase. Sensitivity of PCR with the ordinary enzyme in presence of plant extract was the same as without the extract. Sensitivity of PCR with the hot start enzyme in presence of plant extract was almost the same (consistent sensitivity 1.5 CFU/reaction and 0.61 CFU/reaction with reproducibility of 25%) as without the extract (consistent sensitivity 1.5 CFU/reaction and 0.61 CFU/reaction with reproducibility of 50%). However, intensity of electrophoretic bands was weaker in all reactions with the extract. Photographing of the gel with a digital camera adjusted at highest resolution, sensitivity and a long exposition greatly improve visibility of weak bands (some very weak bands are not visible by naked eyes and appear only on the photography). Photographs taken at such conditions have poor clarity (bands almost are not visible) until their additional adjustment of brightness and contrast on a computer with an appropriate software.

PCR with hot start TrueStart *Taq* DNA polymerase always gave product with every *E. amylovora* strain and did not give any product with any strain other than *E. amylovora*. Actually, this is the same specificity as in PCR with the ordinary enzyme.

According to the manufacturer of the enzymes, recommended extension time is 1 min for the synthesis of PCR fragments up to 2 kb for ordinary *Taq* DNA polymerase, and 1 min per 1 kb for hot start TrueStart *Taq* DNA polymerase. Verification whether extension time designed for ordinary enzyme is also good for hot start TrueStart *Taq* DNA polymerase shoved that hot start enzyme requires a longer extension time than the ordinary enzyme. That was the reason that extension time was increased from 30 seconds to 90 seconds.

Application of hot start TrueStart *Taq* DNA polymerase in PCR for detection of *E. amylovora* has some advantages and as well as disadvantages. The greatest improvement is that the procedure is significantly simplified. Avoiding of wax for obtaining of hot start, several steps in the procedure can be skipped (preparation of 2 separated reaction mixtures, insertion of those 2 reaction mixtures and wax separately into a reaction tube, heating of the wax, etc.). This is important because of time and labor saving. However, the most important advantage is that in simpler and shorter procedure possibility for sample cross contamination is greatly reduced.

Application of hot start TrueStart *Taq* DNA polymerase in PCR for detection of *E. amylovora* represents a significant contribution for fast and reliable detection. In spite of the fact that signal of electrophoretic band is weaker in a reaction with plant extract then without the extract, the signal is enough strong to provide very high sensitivity of the method. The most important is to follow instructions of the procedure, because any alteration of the procedure can lead to decreasing of sensitivity, specificity and even complete failure of the reaction. High standardization of the procedure is very important in every diagnostic test which is routinely applied. This research gave a contribution for standardization of the reaction with hot start TrueStart *Taq* DNA polymerase.

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