Specific Oligonucleotide Primers for Detection of Lecithinase-Positive *Bacillus* spp. by PCR

H. SCHRAFT AND M. W. GRIFFITHS*

Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 24 June 1994/Accepted 24 October 1994

An assay based on the PCR has been developed to facilitate detection and identification of *Bacillus cereus* in foods. Three primers for the PCR have been designed within the sequence for cereolysin AB, a cytolytic determinant that encodes lecithin-hydrolyzing and hemolytic activities of *B. cereus*. With the PCR and hybridization, the specificity of the primers was tested with 39 isolates of the *B. cereus* group, with 17 other *Bacillus* spp., and with 21 non-*Bacillus* strains. Results demonstrate a high specificity of the three oligonucleotides for isolates of the *B. cereus* group. With a combined PCR-hybridization assay, the detection limit for *B. cereus* in artificially contaminated milk was 1 CFU/ml of milk.

Bacillus cereus is known as a causative agent for food-borne gastroenteritis. Illness occurs in two different forms, emetic and diarrheal syndromes, which are caused apparently by two different toxins (11). The organism is also recognized as a spoilage organism in milk and milk products. *B. cereus* is a sporeformer and as such is widespread in the environment. This enhances both the risk of contamination during food production and the risk of recontamination of processed, heat-treated foods. Certain strains are capable of growth at refrigeration temperatures, and spores of the species can survive pasteurization. Therefore, for effective monitoring of *B. cereus* contamination, detection methods must be capable of identifying the organism in processed foods as well as in ingredients and in the processing environment.

Present detection methods for *B. cereus* rely on standard plate counting. Hydrolysis of lecithin (egg yolk reaction) is a major criterion for detection and identification of *B. cereus* on plating media, since most strains of the *B. cereus* group (e.g., *B. cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis*) possess lecithinase activity. Selective media contain polymyxin B to inhibit growth of gram-negative bacteria and mannitol and egg yolk to identify suspect colonies (17). *B. cereus* does not ferment mannitol, and most strains develop a positive egg yolk reaction. This reaction is catalyzed by phosphatidylcholine hydrolase, a phospholipase C that hydrolyses lecithin (16). Detection of low numbers of *B. cereus* is especially difficult if the foods are heavily contaminated with other microorganisms. Therefore, PCR amplification using specific primers would facilitate direct detection of *B. cereus* in foods. At present, no sequence data which could serve to design specific primers for *B. cereus* are available, and on the basis of 16S sequencing data, it has been suggested that members of the *B. cereus* group represent a single species (1). In the present study, primers have therefore been designed within the coupled sequences for phospholipase C and sphingomyelinase of *B. cereus* (7). Since activity of these enzymes includes hydrolysis of lecithin, these primers were expected to amplify DNA of isolates of the *B. cereus* group. The specificity of the primers was tested with isolates of the *B. cereus* group, with other *Bacillus* spp., and with various non-*Bacillus* strains. Furthermore, a combination

of PCR and hybridization was used to detect *B. cereus* in artificially contaminated milk.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. A total of 56 *Bacillus* strains were used in this study (Table 1). Twenty of the 35 *B. cereus* strains were isolates from outbreaks of food poisonings and were kindly provided by E. C. D. Todd, Bureau of Microbial Hazards, Health and Welfare Canada, Ottawa. Furthermore, 13 milk isolates and two ATCC strains were tested. For *B. mycoides* and *B. thuringiensis*, which are closely related to *B. cereus*, an ATCC strain as well as a milk isolate was analyzed. Three further milk isolates had been classified as *Bacillus circulans*, and one isolate was *Bacillus pasteurii*. For the remaining *Bacillus* spp., only ATCC cultures were tested. All milk isolates had been identified with the API 50 CHB and API 20E test systems (bioMérieux Canada Inc., St-Laurent, Quebec, Canada). In addition, 21 isolates of other bacterial species were included in the study.

Hydrolysis of lecithin. Degradation of lecithin (egg yolk reaction) was detected on *B. cereus* selective agar (Oxoid) supplemented with egg yolk emulsion (50 ml/liter). The plates were incubated at 30°C for 24 to 48 h.

Primers and digoxigenin-labelled probes. On the basis of the published DNA sequence of cereolysin AB (GenBank accession number M24149), three primers (Pf, Pr, and Cr) were used to perform PCRs (Table 2). The primer sets Pf-Pr and Pf-Cr generated PCR products of 411 and 1,461 bp, respectively (Fig. 1).

To be used as an internal hybridization probe for the Pf-Cr PCR product, the primer Pr was labelled at the 3' end with digoxigenin (DIG)-dUTP with the DIG oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). A long hybridization probe was labelled directly with DIG-dUTP by use of a PCR DIG labelling mix (Boehringer Mannheim) in a second PCR amplification of the Pf-Cr PCR product with the primer set Pf-Pr. The reaction was run in a 100- μ l volume with the PCR conditions described below.

DNA extraction. Total genomic DNA was isolated from the bacterial strains by phenol-chloroform extraction. Bacteria were cultured in brain heart infusion broth (30°C, 18 to 48 h). To harvest the cells, 3 ml of the broth culture was centrifuged (14,000 $\times$ g, 10 min). After washing with 0.85% sterile NaCl, the bacteria were suspended in 400 μ l of sucrose solution (6.7% [wt/vol]), 25 μ l of lysozyme (10 mg/ml) was added, and the mixture was incubated for 30 min at 37°C. Afterwards, 50 μ l of 20% (wt/vol) sodium dodecyl sulfate (SDS) was added, and the mixture was incubated for 30 min and digested with 5 μ l of proteinase K solution (20 μ g/ml) at 37°C for 30 min. Genomic DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol. After adding 0.1 volume of 3 M sodium acetate, DNA was precipitated in ethanol (15), air dried, and dissolved in 80 μ l of sterile water. The minigel method (15) was used to quantify the extracted DNA.

PCR amplification. PCRs were carried out in 50- μ l volumes which consisted of 33 μ l of ultrapure sterile water, 0.5 μ l of 10% dimethyl sulfoxide, 5 μ l of a 10 $\times$ reaction buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl), 5 μ l of each deoxynucleoside triphosphate (0.1 mM), 0.75 μ l of each primer (0.1 μ g/ μ l), and 5 μ l of phenol-chloroform-extracted DNA (4 ng/ μ l). These mixtures were held at 70°C for 3 to 5 minutes, 1 U of *Taq* DNA polymerase (Boehringer Mannheim) was added, and the reaction mixes were overlaid with 45 μ l of mineral oil. Thirty-five amplification cycles were performed in an automated DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.) with the following parameters: denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 1 min. During the last cycle, the elongation temper-

* Corresponding author. Phone: (519) 824 4120, ext. 2269. Fax: (519) 824 6631. Electronic mailing address: MGriffiths@APS.uoguelph.ca.

TABLE 1. Bacterial strains

Species	Total no. of strains	Source
<i>Bacillus</i> spp.	56	
<i>Bacillus cereus</i>	35	ATCC 14579, NCTC 11145, 20 strains isolated from outbreaks of food poisonings (foods, feces, vomiting), 13 milk isolates
<i>Bacillus mycoides</i>	2	ATCC 6462, 1 milk isolate
<i>Bacillus thuringiensis</i>	2	ATCC 10792, 1 milk isolate
<i>Bacillus amyloliquefaciens</i>	1	ATCC 23842
<i>Bacillus brevis</i>	1	ATCC 8246
<i>Bacillus circulans</i>	4	ATCC 4513, 3 milk isolates
<i>Bacillus coagulans</i>	1	ATCC 7050
<i>Bacillus lentus</i>	1	ATCC 10840
<i>Bacillus licheniformis</i>	1	ATCC 14580
<i>Bacillus megaterium</i>	1	ATCC 14581
<i>Bacillus pantothenicus</i>	1	ATCC 14576
<i>Bacillus pasteurii</i>	1	Milk isolate
<i>Bacillus polymyxa</i>	1	ATCC 842
<i>Bacillus psychrosaccharolyticus</i>	1	ATCC 23296
<i>Bacillus pumilus</i>	1	ATCC 72
<i>Bacillus subtilis</i>	2	ATCC 6633, ATCC 6051
Other bacteria	21	
<i>Clostridium perfringens</i>	3	NCTC 8798, Food Science Guelph ^a
<i>Staphylococcus aureus</i>	4	Food Science Guelph
<i>Staphylococcus epidermidis</i>	1	Food Science Guelph
<i>Lactobacillus brevis</i>	1	ATCC 13648
<i>Listeria monocytogenes</i>	3	ATCC 13313, ATCC 19113, Scott A
<i>Lactococcus lactis</i>	1	Food Science Guelph
<i>Streptococcus faecalis</i>	1	Food Science Guelph
<i>Escherichia coli</i>	1	ATCC 10789
<i>Enterobacter aerogenes</i>	1	Food Science Guelph
<i>Proteus vulgaris</i>	1	Food Science Guelph
<i>Klebsiella pneumoniae</i>	1	Food Science Guelph
<i>Serratia marcescens</i>	1	ATCC 8100
<i>Pseudomonas aeruginosa</i>	1	ATCC 27853
<i>Leuconostoc lactis</i>	1	ATCC 19256

^a Food Science Guelph, strains kindly provided by L. J. Harris, Department of Food Science, University of Guelph, Canada.

ature was held for a total of 4 min. Five microliters of the PCR amplifications was run on submarine 1.3% (wt/vol) agarose gels in Tris-acetate buffer (1.5 h at 40 V), stained with ethidium bromide, and photographed on a UV transilluminator.

Dot blot, Southern blot, and hybridization of PCR products. To prepare dot blots, a 10- μ l aliquot of the PCR amplifications was denatured in boiling water for 10 min, cooled on ice, and mixed with 20 μ l of 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Subsequently, the DNA was blotted onto a positively charged nylon membrane (Boehringer Mannheim) with a Hybri Dot well filtration manifold (GIBCO BRL, Gaithersburg, Md.). Southern blots were prepared by vacuum transfer (15). After fixing the DNA to the membranes by UV cross-linking for 5 min, prehybridization and hybridization were carried out as recommended by Boehringer Mannheim (3). With the oligonucleotide probe Pr, hybridizations were carried out at 50°C (4 h); for the PCR-labelled probe Pf-Pr, the hybridization temperature was 65°C (18 h). Hybridization signals were developed with Lumigen purified protein derivative, and chemiluminescence was detected with a BIO Bioview Biomedical Image Quantifier (Cambridge Imaging Ltd., Cambridge, United Kingdom).

Analysis of PCR products by restriction endonuclease. Six samples yielding PCR products with the primer set Pf-Cr were chosen to analyze the products with the restriction endonuclease *Taq*I. On the basis of the published sequence, the Pf-Cr PCR product contains one *Taq*I recognition site (Fig. 1). A 5- μ l aliquot of each PCR amplification was digested with 10 U of *Taq*I (Boehringer Mannheim) at 65°C for 1 h. Digestions were run on agarose gels and photographed as described above.

Detection of *B. cereus* in milk. Pasteurized milk samples were spiked with serial

dilutions of a suspension of *B. cereus* ATCC 14579. Counts of *B. cereus* in the spiked samples were determined by spread plating on *B. cereus*-selective agar (Oxoid) supplemented with egg yolk emulsion (50 ml/liter) and polymyxin B (100,000 U/liter).

Five milliliters of each sample was centrifuged (4,800 $\times$ g, 4°C, 30 min), the supernatant was removed, and the pellet was suspended in 400 μ l of 1 M Tris buffer (pH 7.6). To extract the bacterial DNA, suspensions were treated with lysozyme, proteinase K, and SDS as described by Flemming et al. (5). Crude DNA extracts were purified by centrifugation through a Sephadex G-75 (Sigma) spin column (5), and DNA was precipitated in ethanol as described above. The DNA was dissolved in 38 μ l of sterile water, and the whole sample was used for PCR with primer set Pf-Cr. A 10- μ l aliquot of the PCR amplifications was analyzed by agarose gel electrophoresis, and a 40- μ l aliquot was used for dot blot hybridization with the internal oligonucleotide probe Pr.

RESULTS

Results and correlation for egg yolk reaction, PCRs, and dot blot hybridizations are summarized in Table 3.

Hydrolysis of lecithin. All but one of the 35 *B. cereus* isolates as well as both *B. mycoides* isolates and both *B. thuringiensis* isolates showed a positive egg yolk reaction. Additionally, one *B. circulans* isolate was able to hydrolyze lecithin. Since both

TABLE 2. Primers for detection of egg yolk-hydrolyzing *Bacillus* spp.

Primer	Orientation	Position ^a	Sequence (5'-3')
Pf	Forward	250-275	GAG TTA GAG AAC GGT ATT TAT GCT GC
Pr	Reverse	638-658	CTA CTG CCG CTC CAT GAA TCC
Cr	Reverse	1685-1710	GCA TCC CAA GTC GCT GTA TGT CCA G

^a Position of primer within the nucleotide sequence of the cereolysin AB gene.

FIG. 1. Arrangement of primers (Pf, Pr, and Cr) for amplification of phospholipase C (*cerA*) and sphingomyelinase (*cerB*) genes by PCR and location of cutting site for restriction endonuclease *TaqI*. Numbers indicate the nucleotide positions as given by Gilmore et al. (7).

Staphylococcus aureus and *Clostridium perfringens* possess lecithinase activity, every isolate of these species was egg yolk positive. With the remaining strains, no egg yolk reaction could be observed.

Specificity of PCR. With both primer sets, all but one strain of the *B. cereus* group (i.e., *B. cereus*, *B. mycoides*, and *B. thuringiensis*) yielded a PCR product of the expected size (Fig. 2). With primer set Pf-Cr, one *B. cereus* isolate (4429/73D), which had been isolated from vanilla pudding, showed a product of approximately 2.5 kb in length (Fig. 2B, lane 4). The other non-*B. cereus* isolates with lecithinase activity, *B. circulans*, *C. perfringens*, and *S. aureus*, did not generate detectable PCR products; furthermore, no amplification was observed with egg yolk-negative isolates.

Hybridizations. To confirm the identity of the products, reactions run with primer sets Pf-Cr and Pf-Pr were dot blotted onto a nylon membrane and hybridized with the DIG-tailed oligonucleotide Pr and the DIG-labelled probe Pf-Pr. With these probes, each amplified DNA fragment, including the 2.5-kb PCR product of *B. cereus* 4429/73D, was confirmed to be the expected target sequence. None of the reactions with the other bacterial strains revealed a positive hybridization signal (Table 3). The PCR products of the six strains shown in Fig. 2 were tested additionally by Southern hybridization. With the DIG-labelled probe Pf-Pr, all six 411-bp products showed distinct hybridization signals (Fig. 3A). The large PCR products obtained with primer set Pf-Cr, including the 2.5-kb product of *B. cereus* 4429/73D, also hybridized with their long in-

ternal probe Pf-Pr. However, with the DIG-tailed internal oligonucleotide probe Pr, two of the large PCR products did not hybridize on Southern blots (Fig. 3B), although they consistently showed a positive hybridization signal on dot blots (Fig. 3C). When stringency was increased by raising the hybridization temperature to 65°C, the hybridization signals of these two PCR products (*B. cereus* NCTC 11145 and *B. cereus* 6A1045) were also negative on dot blots (Table 4).

Analysis of PCR products with restriction endonuclease. After amplification with primer set Pf-Cr and subsequent digestion with *TaqI*, four of the six samples showed the two expected fragments of approximately 0.3 and 1.1 kb. However, for the *B. mycoides* isolate, the smaller fragment was slightly larger (400 bp) than expected. Four restricted DNA fragments were obtained when the 2.5-kb product of the *B. cereus* isolate was digested with *TaqI* (Fig. 4).

Detection of *B. cereus* in milk. With a combined PCR-hybridization assay, *B. cereus* could be detected in milk samples containing less than 1 CFU/ml (Fig. 5). PCR fragments were visible on agarose gels when milk samples contained at least 10³ CFU/ml (data not shown). Uncontaminated milk samples constantly showed negative PCR and hybridization results.

DISCUSSION

The cytolytic determinant cereolysin AB (7), a coupled DNA sequence encoding for phospholipase C (*cerA*) and sphingomyelinase (*cerB*), has been chosen to design primers

TABLE 3. Correlation of lecithinase activity, PCR assay, and dot blot hybridization for 77 bacterial strains

Species	No. of strains			PCR with primer set:		Dot blot hybridization of products Pf and Cr with DIG-labelled probes	
	Total	Tested for hydrolysis of lecithin		Pf-Pr ^a	Pf-Cr ^b	Pr ^c	Pf-Pr ^c
		Positive	Negative				
<i>B. cereus</i>	35	34	1	+	+	+	+
<i>B. mycoides</i>	2	2		+	+	+	+
<i>B. thuringiensis</i>	2	2		+	+	—	—
<i>B. circulans</i>	4	1	3	—	—	—	—
Other <i>Bacillus</i> spp.	13		13	—	—	—	—
<i>S. aureus</i>	4	4		—	—	—	ND ^d
<i>C. perfringens</i>	3	3		—	—	—	ND
Other bacterial strains	14		14	—	—	—	ND

^a Detection (+) or absence (—) of a 0.4-kb product on an ethidium bromide-stained agarose gel was determined.

^b Detection (+) or absence (—) of a 1.46-kb product on an ethidium bromide-stained agarose gel was determined.

^c Positive (+) or absence (—) of a hybridization signal was determined.

^d ND, not determined.

FIG. 2. Gel electrophoresis of PCR products amplified with primer sets Pf-Pr (A) and Pf-Cr (B) from purified DNA of different *Bacillus* isolates. Lanes: 1, sample without DNA; 2, *B. cereus* 4094/73C (food isolate); 3, *B. cereus* 6A1054 (milk isolate); 4, *B. cereus* 4429/73D (food isolate); 5, *B. mycoides* ATCC 6462; 6, *B. thuringiensis* ATCC 10792; 7, *B. cereus* NCTC 11145; 8, 1-kb ladder (Bethesda Research Laboratories, Inc.).

that could be useful for detection of *B. cereus* in foods. Phospholipase C encoded by the gene *cerA* is responsible for lecithin degradation, a major criterion for detection and identification of *B. cereus*. Furthermore, synergism of phospholipase C and sphingomyelinase results in hemolysis, which is also widely used to identify *B. cereus* (17).

Three oligonucleotides were designed within the nucleotide sequence for cereolysin AB to be used either for two different PCR tests or for a combined PCR-hybridization assay. Searching the GenBank database (2) for complementary sequences revealed four records that showed 100% homology with the primers; the four genes encoded for phospholipase or sphingomyelinase or hemolysin of *B. cereus* (9, 10, 12, 18). No highly homologous sequences were found for other bacteria, indicating an excellent specificity of the primers. Consequently, the specificity of the three primers was tested with 39 isolates of the *B. cereus* group, with 17 other *Bacillus* spp., and with 21 non-*Bacillus* strains. PCR with the two primer sets and dot blot hybridizations were positive only with isolates of the *B. cereus* group. Positive results received with an egg yolk-negative *B. cereus* isolate indicate that these primers could also be specific for atypical *B. cereus*. Several other bacterial species can react with egg yolk: *S. aureus*, *C. perfringens*, *Serratia marcescens*, and *Proteus vulgaris*. With the *S. marcescens* and *P. vulgaris* used in the present study, no changes in the egg yolk-containing medium were observed; PCR and hybridizations were also negative. In contrast, precipitation of egg yolk was detected for *C. perfringens* and *S. aureus* isolates. Many *S. aureus* strains react with lecithin, and egg yolk reaction on Baird Parker agar is therefore used frequently to detect these organisms. The amino acid sequence of *C. perfringens* phospholipase C is significantly homologous to that of phospholipase C of *B. cereus*, but the encoding genes do not share extensive nucleotide sequence homology because the two species have different codon

FIG. 3. (A) A Southern blot of PCR products amplified with primer set Pf-Pr was hybridized with the DIG-labelled probe Pf-Pr. (B) A Southern blot of PCR products amplified with primer set Pf-Cr was hybridized with the DIG-tailed oligonucleotide probe Pr. (C) A dot blot of PCR products amplified with primer set Pf-Pr was hybridized with the DIG-tailed oligonucleotide probe Pr. Lanes: 1, sample without DNA; 2, *B. cereus* 4094/73C (food isolate); 3, *B. cereus* 6A1054 (milk isolate); 4, *B. cereus* 4429/73D (food isolate); 5, *B. mycoides* ATCC 6462; 6, *B. thuringiensis* ATCC 10792; 7, *B. cereus* NCTC 11145; 8, DIG-labelled molecular weight marker VI (Boehringer Mannheim).

synonyms (14). Despite their positive egg yolk reactions, neither *C. perfringens* nor *S. aureus* yielded positive PCR results.

Variations in the nucleotide sequence of a given gene have been reported for strains of several bacterial species (4, 8, 13). Results of the present study provide three indications that isolates of the *B. cereus* group possess sequence variations within the nucleotide sequences for *cerA* and *cerB*. First, the primer set Pf-Cr that amplifies a 1.46-kb portion of cereolysin AB generated a substantially larger product from one *B. cereus*

TABLE 4. Dot blot hybridization of Pf-Cr PCR products with the internal oligonucleotide probe Pr at various temperatures

Strain	Dot blot hybridization at ^a :			
	50°C	55°C	60°C	65°C
<i>B. cereus</i> 4094/73C	+	+	+	+
<i>B. cereus</i> 6A1054	+	+	±	—
<i>B. cereus</i> 4429/73D	+	+	+	+
<i>B. mycoides</i> ATCC 6462	+	+	+	+
<i>B. thuringiensis</i> ATCC 10792	+	+	+	+
<i>B. cereus</i> NCTC 11145	+	+	±	—

^a Symbols: +, positive signal; ±, barely visible signal; —, absence of signal.

FIG. 4. Gel electrophoresis of PCR products amplified with primer set Pf-Cr after cutting with *Taq*I. Lanes: 1, *B. cereus* 4094/73C (food isolate); 2, *B. cereus* 6A1054 (milk isolate); 3, *B. cereus* 4429/73D (food isolate); 4, *B. mycoides* ATCC 6462; 5, *B. thuringiensis* ATCC 10792; 6, *B. cereus* NCTC 11145; 7, 1-kb ladder (Bethesda Research Laboratories, Inc.).

food isolate. Second, the position of the recognition site *Taq*I within the 1.46-kb PCR product amplified from the *B. mycoides* genome differed from that occurring within the products obtained by amplifying *B. cereus* and *B. thuringiensis* DNA. Third, Pf-Cr PCR products of *B. cereus* NCTC 11145 and a *B. cereus* milk isolate (6A1054) hybridized at 50°C with the internal oligonucleotide probe Pr on dot blots but not on Southern blots. When the stringency for dot blot hybridization was increased, no hybridization signal was detected for these two strains, while four other test strains hybridized well at high stringency. The disparity between the Southern and dot blot results may therefore be attributable to nucleotide sequence divergence within *cerA*. However, none of these sequence variations influenced the PCR assays or the results of the dot blot hybridizations.

A combined PCR-hybridization assay was applied to detect *B. cereus* in artificially contaminated milk samples. When 5 ml of milk was used to extract total bacterial DNA, the detection limit was 10^3 CFU/ml of milk for PCR products on agarose gels and approximately 1 CFU/ml of milk for hybridization assays. A similar detection limit was achieved by Furrer et al. (6), who could detect *Listeria monocytogenes* at a level of 1 CFU/ml milk with a PCR-hybridization assay.

The demonstrated specificity of the oligonucleotides used as PCR primers in this study and the results of experiments with

FIG. 5. Detection of *B. cereus* ATCC 14579 in artificially contaminated milk samples by dot blot hybridization of Pf-Cr PCR products with the internal oligonucleotide probe Pr. Dots: 1, 10^3 to 10^4 CFU/ml; 2, 10^2 to 10^3 CFU/ml; 3, 10 to 100 CFU/ml; 4, 1 to 10 CFU/ml; 5, 0.1 to 1 CFU/ml.

artificially contaminated milk samples provide the basis to develop a diagnostic assay for detection of *B. cereus* in food.

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