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Full Length Research Paper

Screening of new isolates of *Bacillus thuringiensis* (Bt) and cloning of the *cry* genes

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Nine new indigenous isolates of *Bacillus thuringiensis* (Bt) were characterized for their colony type, crystal inclusion and toxicity analysis with *Helicoverpa armigera* Hubner and *Spodoptera litura* Linn. Genomic deoxyribonucleic acid (DNA) isolated from all the new isolates were subjected to screening for *cry1, cry2, cry4, cry10* and *cry11* genes and predicted possible potential DNA amplicons were cloned and sequenced. Partial *cry1* gene fragment (~1.5 kb) amplified by degenerate primers and about 450 bp DNA fragment amplified by *cry10* gene specific primers from two isolates T109 and T136 were cloned in to T/A cloning vector. DNA sequencing of about 1.5 kb amplicon showed 99% homology to the holotype sequence of *cry1Ac1.* Nucleotide sequence of about 450 bp fragments of isolate T109 and T136 showed homology to a hypothetical protein and serine/threonine phosphatase respectively.

Key words: *Bacillus thuringiensis* (Bt), cloning, *cry* genes, polymerase chain reaction (PCR), toxicity analysis.

INTRODUCTION

Food security is one of this century's key global challenges and ever increasing world population demands increased food production in order to feed its predicted nine billion people by 2050. There is a pressing need for the 'sustainable intensification' of global agriculture in which yields are increased without adverse environmental impact and within the land available for cultivation. Insect damage to food crops is devastating and biopesticides [*Bacillus thuringiensis* (Bt)] have proved beneficial over conventional chemical pesticides in controlling them. The cry toxins constitute a family of related proteins that can kill insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, and Mallophaga, as well as other invertebrates (Schnepf et al., 1998; Feitelson et al., 1999). Diversity and activity of Bt isolates have a relationship with geographical origin of samples (Armengol et al., 2007). However Bt shows great genetic and molecular diversity even in isolates from the same division but of different soil samples.

The polymerase chain reaction (PCR) method is proven to be a powerful tool which allows quick, simultaneous screening of many Bt samples (Porcar and Juarez-Perez, 2003), identification of specific insecticidal genes carried by different Bt strains and classification of *cry* genes (Carozzi et al., 1991; Ben-Dov et al., 1997). A two-step PCR strategy allows the ability to further clone and sequence genes for which no specific primers are available, but a variable region exists between two conserved regions (Juarez-Perez et al., 1997).

Screening of more number of Bt isolates is relevant for identifying new *cry* genes from new isolates of Bt. New gene sequences encoding more active toxins could be used for developing better versions of transgenic crop plants. So, the present study was undertaken with the objectives of characterization of new isolates of Bt, screening by PCR for *cry* gene content, cloning and sequencing of DNA fragments amplified from new isolates of

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Bt.

MATERIALS AND METHODS

Bacterial strains, plasmids

New isolates of Bt (T33, T46, T96, T109, T110, T136, T153, T183 and T191) and reference strain, Bt subsp kurstaki (HD1) and Bt subsps isrelensis (Bti) used in this study were obtained from the collection of Bt strains maintained by the Department of Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India. The reference strain, HD1 was originally obtained from Bacillus Genetic Stock Centre, Ohio state university, Columbus, Ohio, USA. The T/A cloning vector, pTZ57R/T used in the present study was purchased from Fermentas, INC.

Characterization of indigenous isolates of Bt

The Bt isolates streaked on T_3 agar plates were incubated at 30°C for two to three days. Colony morphology was observed for the single colonies developed on T_3 agar plates. To analyze the crystal morphology, new isolates were grown in T3 media till 90% cell lyses and stained with 0.133% Coomassie brilliant blue G250 in 50% acetic acid permanent staining solution and observed for crystal morphology under light microsope. Toxicity analysis of new isolates of Bt against *Helicoverpa armigera* and *Spodoptera litura* was carried out as described by Patel et al. (1968). The spore-crystal mixture was isolated from the three new isolates of Bt and a reference strain, HD1 and Bti, as described by Laemmli (1970).

Screening of Bt strains by PCR

Total genomic DNA from Bt strains *viz.* T33, T46, T96, T109, T110, T136, T153, T183 and T191 were extracted as described earlier by Kalman et al. (1993) and used as a template for the PCR amplification. The PCR was accomplished using an Eppendorf thermal cycler in 25 µl reaction volume containing 30 ng of total genomic DNA of Bt, 2.5 µl of 10X PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles. The primers and temperature profiles used for PCR for *cry1* and *cry2, cry4* genes were used as described by Ben-Dov et al. (1997) and for *cry10, cry11* as described by Ejiofor and Johnson (2002).

Cloning of DNA fragments from new isolates of Bt

The degenerate primers, JF and JR were used to amplify the partial *cry1* gene fragments (~1.5 kb) as described by Juarez- Perez et al. (1997) and *cry 4, cry 10* F & R gene specific primers were used to amplify *cry4* and *cry10* gene fragments as specified by Ejiofor and Johnson (2002). The PCR product was purified by column provided in the PCR cleanup kit, as per the manufacturer's instruction and ligated into T/A vector (pTZ57R/T, Fermentas, INC.) The ligated mixture was transformed into *Escherichia coli* as per the standard procedure (Sambrook et al., 1989). The transformed colonies of *E. coli* were screened by colony PCR with M13F and M13R primers for checking the presence of insert.

Nucleotide sequencing of recombinant clones

Nucleotide sequencing of DNA fragments cloned in pTZ57R/T was carried out by automated sequencing (Genei, Bangalore). Sequence data were generated using standard primer, M13F for ~450 bp amplicons of isolate T109 , T136 and three primers M13F, M13R and an internally designed primer in the known region of ~1.5 kb amplicon of *cry1* gene. The sequence obtained by cloned amplicons was subjected to homology analysis using Basic Local Alignment Search Tool (BLAST), programme of NCBI. 'BioEdit' programme was used to deduce amino acid sequence.

RESULTS

Characterization of indigenous isolates of Bt

Nine new isolates of Bt were observed for colony morphology on T_3 agar plates, two showed mucoid type and seven showed fried egg type (HD1 type) colonies. Further seven among nine isolates showed spherical inclusions and one each showed bipyramidal and cuboidal inclusions (Table 1). Toxicity analysis against neonate larvae of *Helicoverpa armigera* and *spodoptera litura* by artificial diet based bioassay, the nine new isolates of Bt showed high variations of toxicity ranging from zero to 100%. Only one of the four isolates of Bt (T191) showed 100% mortality as in the case of reference strain of Bt, HD1 whereas, the remaining three Bt isolates showed < 40% mortality.

Polymerase chain reaction (PCR) screening of new Bt isolates

Total genomic DNA was isolated from all the new isolates of Bt and subjected to screening for *cry1, cry2, cry4, cry10, cry11* genes with universal primers by PCR. Amplification of size ~270 bp (Figure 1) for *cry1* gene and ~450 bp (Figure 2) for *cry10* gene were observed whereas, *cry2, cry4 and cry11* genes with gene specific primers gave no amplification (Data not shown). Further *cry1* positive isolate was subjected to screening for its subfamily genes with a combination of a degenerate forward and gene specific reverse primers showed expected length amplicon for *cry1Ac* and non specific amplicons with others (Figure 3).

Cloning of DNA amplicons from new isolate of Bt

Partial *cry1* gene fragments of about 1.5 kb amplified by PCR from the genomic DNA of Bt isolate T191, using a set of forward and reverse degenerate primers; JF and JR; higher intensity nonspecific amplicon of ~1.7 kb amplified from Jf and JR from same isolate and ~450 bp DNA fragments amplified by *cry10* universal primers from isolate T109, T136 were further cloned separately into T/A vector. *E. coli* transformants were selected by blue-white screening and further confirmed by screening for the presence of target DNA insert by colony PCR with M13F and M13R primers. Automated DNA sequencing method was used to determine the nucleotide sequence

Table 1. Characterization of new isolates for colony and crystal morphology.

S/N	Isolate	Colony morphology	Crystal shape	Toxicity	
				S. litura (%)	*H. armigera* (%)
1	HD1	Fried egg	Bipyramidal and Cuboidal	100	100
2	Bti	Fried egg	Spherical and Irregular	40	40
3	T33	Fried egg	Spherical	15	20
4	T46	Mucoid	Spherical	20	20
5	T96	Fried egg	Spherical	30	40
6	T109	Fried egg	Cuboidal	30	30
7	T110	Mucoid	Spherical	30	30
8	T136	Fried egg	Spherical	30	30
9	T153	Fried egg	Spherical	20	10
10	T183	Fried egg	Spherical	20	20
11	T191	Fried egg	Bipyramidal	100	100
12	4Q7	-	No crystal	0	0
13	H_2O Control	-	-	0	0

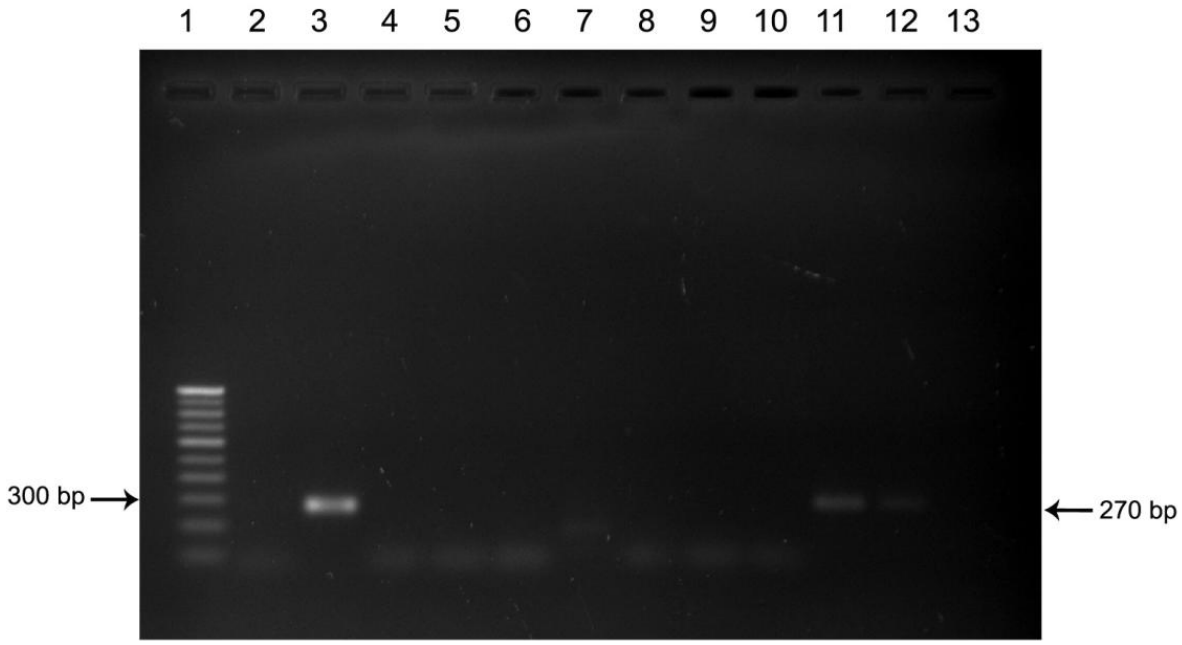

Figure 1. Agarose gel electrophoresis of PCR products amplified by primers specific for *cry1* gene. Lane 1, 100 bp marker; lane 2, new isolate T110 of Bt which is negative for *cry1*gene (namely : T110); lane 3, new isolate T191 of Bt which is positive for *Cry1* gene; lanes 4 to 10, new isolates of Bt which are negative for *cry1*gene (namely: T109, T183, T153, T136, T33, T46 and T96); lane 11, reference strain of Bt, HD1 (positive control); lane 12, reference strain of Bt, HD73 (positive control); lane 13: negative control.

of DNA inserts. The nucleotide data generated from ~1.5 kb insert of T191 isolate showed homology to holotype *cry1Ac1* gene from position 718 to 2282 of its ORF. Deduced amino acid sequence of the partial *cry1Ac* gene of the Bt isolate, T191 showed variation of one amino acid at position 235 (out of 521 residues) in comparison to *Cry1Ac1* (Figure 4). Insert DNA of ~1.7 kb from isolate T191 also showed homology to *Cry1Ac*. Insert DNA of size~450 bp from isolate T136 showed homology to serine/threonine specific protein phosphatase gene. Nucleotide data of ~450 bp fragment of T109 isolate did not show homology to any of the known genes, however deduced amino acid sequence showed homology to a hypothetical protein.

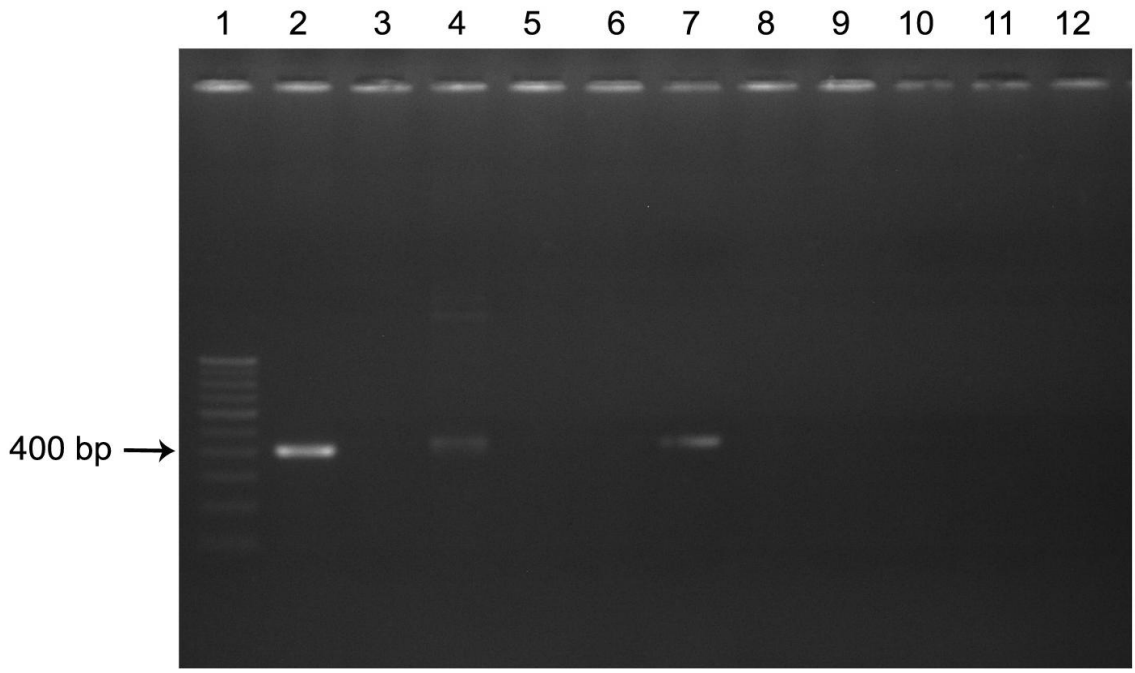

Figure 2. Agarose gel electrophoresis of PCR products amplified by primers specific for *cry10* gene. Lane 1, 100 bp marker; lane 2, Reference strain of Bt, Bti; lane 3, new isolates of Bt which is negative for *cry1* gene (namely: T33); lane 4, new isolates of Bt which is positive for *cry10* gene (namely: T109); lanes 5 and 6, new isolates of Bt which are negative for *cry10* gene (namely: T46, T96); lane 7, new isolates of Bt which is positive for *cry10* gene (namely: T136); lanes 8 to 11, new isolates of Bt which are negative for *cry10* gene (namely: T110, T153, T183, T191); lane 12, negative control.

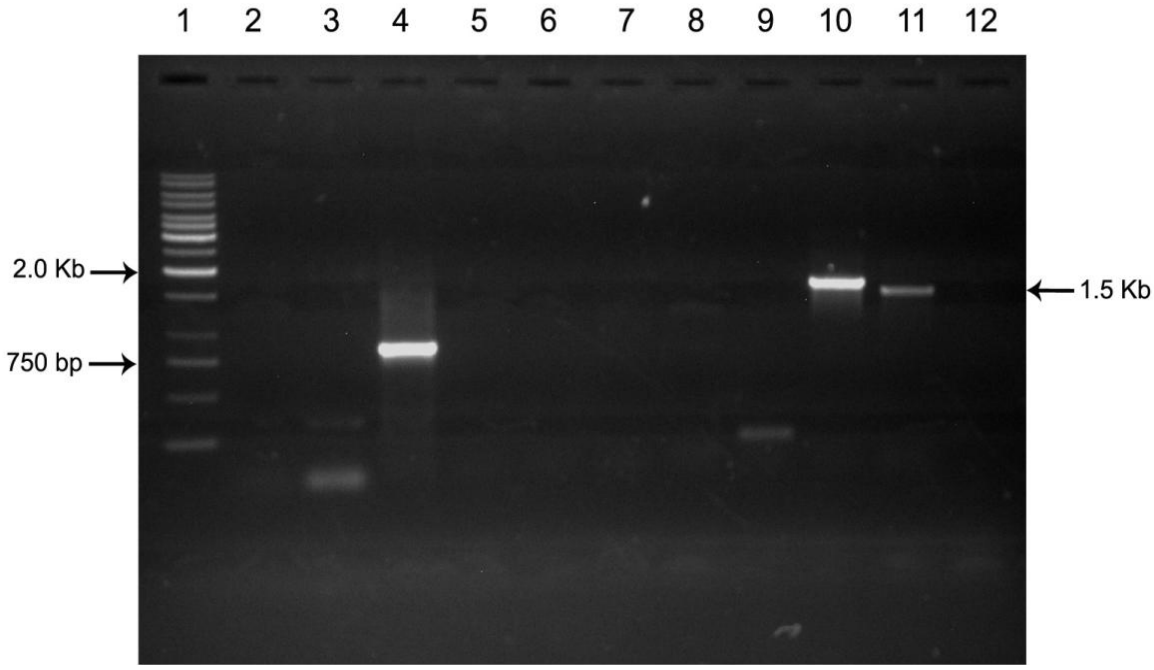

Figure 3. Screening of Bt isolate T191 for *cry1* sub family genes. Lane 1, 1 kb marker; lanes 2 and 3, no amplification by *cry1* sub- family primers (namely: J1Aa, J1Ab); lane 4, amplification by sub - family primer J1Ac; lanes 5 to 9, no amplification by *cry1* sub- family primers (namely: J1Ad, J1B, J1C, J1D, J1E); lane 10, amplification by sub - family primer J1F; lane 11, amplification by Family primer J1 Forward and Reverse; lane 12, negative control.

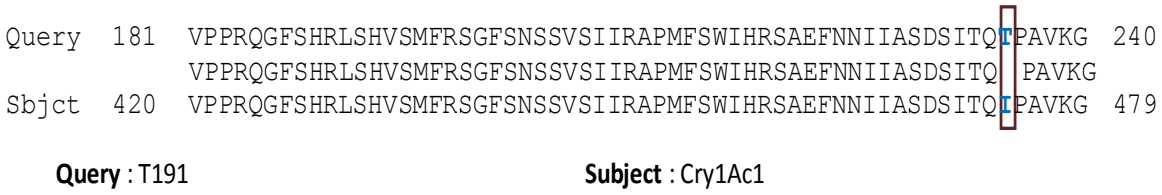

Figure 4. Homology between the deduced amino acid sequence of clone, T191 and Cry1Ac1.

DISCUSSION

Characterization of new isolates of Bt for colony morphology

Colony morphology can help to distinguish Bt colonies from other *Bacillus* species. The study on colony morphology of nine isolates of Bt showed two types of colonies that is, one typical, fried egg like as reported (Parry et al., 1983; Travers et al., 1987) earlier and another mucoid type having smooth surface with lustrous appearance.

Initial identification of Bt is mainly based on the presence of crystalline inclusions. The bright field microscopy is more useful than phase contrast microscopy for high throughput evaluation of bacterial colonies for the presence of crystals and also for identification of small crystals (Rampersad and Ammons, 2005). There is a striking correlation between the shape of the parasporal crystal and the spectrum of toxicity it displays; the lepidopteran-toxic crystals are bipyramidal, the dipteran toxic crystals are spherical, and coleopteran-toxic crystals are cuboidal in their shape (Chambers et al., 1991; Maher et al., 2004). Out of nine new isolates of Bt observed for crystal inclusion in present study, seven isolates produced spherical inclusions and one each of bipyramidal and cuboidal inclusion.

In the present study, efficacy of nine new isolates of Bt were compared with the reference strain, HD1 against the lepidopteran insects, *S. litura* and *H. armigera*. The mortality percentage showed high variations. Only T191 (which showed bipyramidal crystal inclusion) of the nine isolates of Bt showed 100% mortality to both species of larvae and remaining eight isolates showed less than 40% mortality on 7 DAT. All these results strengthened the results from crystal inclusion study and gave a direction to screen the less toxic isolates for dipteran specific genes as majority of them showed spherical inclusion. Another reference strain Bti used in this study showing 40% mortality to both the larvae led us to hypothesize that *cyt* genes present in this strain may be responsible for the mortality (Table 1). It was reported earlier that, cyt toxins synergize the toxic effect of some Cry proteins active against mosquitoes and also that of the Bin toxin produced by *B. sphaericus* (Wirth et al., 2001). Toxicity of *cyt* genes against lepidopteran larvae *Spodoptera frugiperda* has also been reported earlier (Jose et al., 2009).

Screening of new isolates of Bt by PCR

In the present study, nine new isolates of Bt were screened for the presence of *cry1*, *cry2 cry4*, *cry10* and *cry11* genes. One of the nine new isolate of Bt, T191 showed presence of *cry1* family genes which was further subjected to screening for presence *cry1* subfamily genes and the result was positive for *cry1Ac*. Interestingly a high intensity PCR *cry1Ac* product of unexpected size was obtained from *cry1F* sub-family primer. Furthermore all the nine new isolates were screened for the presence of dipteran specific *cry* genes (*cry4, cry10, cry11*). Among them two isolates (T109 and T136) showed near expected amplicon of size ~450 bp (expected 404 bp) with *cry10* gene specific primers and none of the isolates were positive for *cry4* and *cry11* genes.

Porcar et al. (2002) suggested that strains yielding unusual PCR products could be selected for further analyses leading to the identification and characterization of hypothetical novel *cry* genes.

Cloning of DNA fragments from new isolates of Bt

Nucleotide sequence data of DNA fragment amplified by *cry10* primer from isolate T109 did not show significant similarity with any of the sequence from database, but deduced amino acid sequence showed 43% homology to a hypothetical protein; whereas, nucleotide sequence data of *cry10* amplicon from isolate T136 showed 94% homology to serine/threonine protein phosphatase, which fall under the gene family reported to be associated with the Bti toxin resistance (Paris et al., 2012).

Nucleotide Sequence of ~1.7 kb DNA fragment amplified by *cry1F* sub-family gene specific primer showed 99% homology to holotype *cry1Ac1*. Further careful analysis of sequence revealed that the *cry1Ac* ORF contain a region 55% (11 oligomers out of 20) homology to the primer used, resulting in amplification of ~1.7 kb fragment of cry*1Ac* gene from *cry1F* subfamily

gene specific primer. Nucleotide sequence of partial *cry1* gene fragment (1.5 kb) amplified from an isolate T191 by degenerate primer from new Bt isolate, T191 showed 99% homology to *cry1Ac1* holotype. Deduced amino acid sequence showed one amino acid variation at position 235 in comparison to holotype *cry1Ac1*. Variation of even a single amino acid residue at certain positions of Cry proteins can remarkably influence the level of toxicity (Rajamohan et al., 1996). The mortality caused by the isolate of Bt, T191, in *H. armigera* and *S. litura* is found to be 100% as in case of reference strain, HD1. Lee et al. (1996) reported that Cry1Ac proteins differing at only two amino acids positions exhibited a tenfold difference in toxicity towards the gypsy moth *Lymantria dispar*.

Further screening of *Cry1, 2, 4, 10, 11* gene negative isolates for other dipterans and lepidopteron genes and expression studies of full length *Cry1Ac* gene from new isolate T191 of this study make the prospects of getting the novel toxic genes galore.

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