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Isolation of *Bacillus thuringiensis* strains from Saudi Arabia soil and study of their potential efficiency against the lepidopteran pest *Ephestia kuehniella*

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Abstract

Insect pests represent a major threat to food crops and human health, and therefore have to be combated in several ways, including chemical methods. However, researchers demonstrated that these molecules are dangerous for the farmers, consumers and the environment in general. For this reason, scientists permanently searched environment friendly alternatives such as the use of the bacterium *Bacillus thuringiensis* classified as one of the best insect pathogens. This microorganism is known by its ability to produce two types of insecticidal proteins, Vegetative insecticidal proteins (Vip) and delta-endotoxins produced during vegetative and sporulation stages of growth, respectively.

In the present study, 15 *B. thuringiensis* strains were isolated from soil collected from different regions in Saudi Arabia (Al Baha, Jeddah, Khulis and Yanbu). *B. thuringiensis* isolates were then classified according to the shape of their parasporal crystals identified under microscope and proteins content of these crystals. Delta-endotoxins efficiency of the different isolates was investigated and promising strains were identified as very active. After 5 days-treatment, *B. thuringiensis* isolates 14 and 7 killed *Ephestia kuehniella* larvae with low LC_{50} of about 59.18 and 65.67 µg/cm^2, respectively.

The results described in the present study proved that the new *B. thuringiensis* isolates could be of a great interest in the control of lepidopteran pests by using their delta-endotoxins in bioinsecticide formulations.

Keywords: *Bacillus thuringiensis*; Delta-endotoxins; Biocontrol; *Ephestia kuehniella*

1. Introduction

Over the past decades, protecting the environment has increasingly become a major global concern. In agriculture, it is undeniable that agricultural expansion and productivity must be achieved through optimal management of pests and weeds [1, 2]. Thus, to protect crops and to increase the productivity of the agricultural and forestry sectors, chemical insecticides have been used extensively [3]. However, growing public concerns about the potentially harmful effects of these chemical molecules on the environment and humans have led the scientific community to seek alternatives to chemical control [4, 5]. One of the ways of protecting consumers and their environment is the partial or total substitution of chemical pesticides by biopesticides. In this context, the world market for biopesticides continues to increase in parallel with a decline in sales of chemical pesticides, and substances based on *Bacillus thuringiensis* (*B.*

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thuringiensis) occupied the first place since decades. In fact, since 2006, this entomopathogenic bacterium represents 70% of the world market for microbial biopesticides [6].

In various countries, large quantities of bioinsecticides based on *B. thuringiensis* are used each year in the agricultural sector to fight against crop insects such as the polyphagous pest *Spodoptera littoralis* and the stored products pest *Ephestia kuehniella* (*E. kuehniella*).

Due to its interest in pest's management control programs, *B. thuringiensis* has been the subject of numerous studies around the world. This Gram positive entomopathogenic bacterium produces various toxins and metabolites that can have different industrial applications, such as delta-endotoxins and Vip proteins.

Delta-endotoxins, known as first generation bioinsecticides, are crystalliferous proteins encoded by *cry* genes and produced during sporulation phase of *B. thuringiensis* [7]. These Cry toxins have been widely used as a substitute for chemical insecticides in the control of insect pests attacking crops and vectors of diseases.

Second generation bioinsecticides, which are toxins secreted by the bacterium, are called Vip "Vegetative Insecticidal Proteins" [8]. These proteins are known by their specific activity against larvae of Coleoptera (Vip1-Vip2) and Lepidoptera (Vip3) and are expressed during the vegetative phase of growth of many strains of *B. thuringiensis*. The use of Vip toxins supplemented the insecticidal activity of the Cry proteins and allowed to overcome resistance emergence. Indeed, some studies have shown that Vip3 and Cry proteins do not show sequence homology and do not recognize the same receptors in the intestinal epithelial cells of target larvae [9].

Due to the damages that it caused in stored products, *E. kuehniella* was used in different studies related with *B. thuringiensis* toxins. *E. kuehniella* is a cosmopolitan specie, known as the flour moth or "Mediterranean flour moth" and belonging to the Lepidoptera order. The larva, generally whitish in color turning slightly pinkish with a dark head, is 15 to 20 mm long after full development. The adult, which is 20 to 25 mm in size, has greyish forewings with fused black dots in the tips and whitish hindwings. The larva of *E. kuehniella* caused damage mainly on flour, but also on cereal grains, semolina, cookies, pasta and even dried fruits [10].

The aim of the present research was to isolate *B. thuringiensis* strains, to investigate their potential in producing Cry proteins and to test the efficiency of these toxins in controlling pests such as lepidopteran insects.

2. Material and methods

2.1. *Bacillus thuringiensis* isolation and growth conditions

Soil samples were collected from different areas in Saudi Arabia, from Jeddah, Khulis, Al Baha and Yanbu, by using sterilized spatula to collect sample from soil from 1.5 to 2 cm below the surface. Samples were then saved in sterilized plastic bags with proper labeling and stored at 4°C until use.

The sixteen soil samples collected were used to isolate spore-forming bacteria by the acetate selection method described by Travers et al. [11]. In fact, one gram of each soil sample was suspended in 10 mL of LB broth supplemented with 250 mM Sodium Acetate (pH 6.8). After incubation at 30°C with shaking at 200 rpm for 4 h, cultures were heated at 80°C for 15 min to kill vegetative cells. From each sample, 100 µL were spread on T3 agar plates then incubated for 72 h at 30°C. This technique abled the purchase of spores that germinated, multiplied and entered in sporulation phase.

To confirm the presence of spores and crystals related to the presence of *B. thuringiensis* isolates, pure colonies obtained on T3 agar plates were investigated under light microscope. Then, each parasporal crystal forming isolate was stored and considered as *B. thuringiensis*.

2.2. Crystal morphology investigation

To investigate *B. thuringiensis* crystal morphology, each isolate was grown on T3 agar plates and incubated at 30°C for 96 h. Cultures were then analyzed by light microscopy (Nikon E-100 Eclipse) after staining with Coomassie blue solution to know the crystal morphology [12]. In fact, the smear was prepared on glass slide and after being heated, the sample was stained and washed then observed through light microscope to identify the shape of the crystals.

2.3. *B. thuringiensis* crystal purification, protein quantification and electrophoresis

After total sporulation on T3 medium, spore-crystal mixture of each *B. thuringiensis* isolate was suspended in the crystal purification solution (2 mL NaCl, 1 M; Triton X-100, 0.1%). The pellet was harvested by 20,000×g centrifugation for 10 min then suspended in cold distilled water (2 mL). After 6 times washing of the pellet in these conditions, it was suspended in 1 mL NaOH 50 mM, incubated for 3 h at 37°C then a centrifugation was applied at 20,000×g for 5 min as described by El Khoury et al. [13].

The concentrations of *B. thuringiensis* solubilized crystal proteins were measured with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard then analyzed by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (10% SDS-PAGE) and visualized by Coomassie blue staining [14].

2.4. Bioassays against *E. kuehniella* larvae

Bioassays were done using the lepidopteran insect *E. kuehniella* known as a stored products pest. For that, a free ingestion technique was used to evaluate the toxicity to *E. kuehniella* larvae of *B. thuringiensis* delta-endotoxins according to the technique described by different studies [15-18]. Using semolina as diet and different toxin concentrations, each test was replicated three times in the presence of a negative control set. Experiments were done under same conditions of temperature (23°C), relative humidity (65%) and photoperiod (18 h light/6 h dark). Mortality was recorded after treatment (3 and 5 days) as lethal concentrations (LC_{50}) calculated by probit analysis [19].

3. Results and discussion

3.1. Soil samples collection and *B. thuringiensis* isolation

Sixteen soil samples were collected from different regions in Saudi Arabia, from Al Baha, Jeddah, Khulis and Yanbu. Using the acetate selection method, spore-forming isolates were obtained from all the soil samples and the higher frequency of spore-forming isolates was obtained using the soil collected from Khulis. After bacteria isolation from soil samples, about 100 white rough pure colonies were investigated by light microscopy to search the presence of parasporal crystals that confirmed the ability of the isolated strains to produce delta-endotoxins characterizing *B. thuringiensis* from all the other sporulating bacteria. And surprisingly, this type of isolates was found only in the case of the soil collected from Khulis. In the contrast of the other soil samples, those of Khulis were collected from farms in which different plants were cultured and the probability to have insect pests is high making possible the presence and the multiplication of *B. turingiensis* bacterium. Similar observations were reported by Martin and Travers (1989) [20] and in the total 15 isolates were identified as *B. thuringiensis* and were investigated in the present work.

3.2. Classification of *B. thuringiensis* isolates based on crystal morphology

When *B. thuringiensis* sporulated cultures were observed by light microscopy, the presence of free spores and crystals, released after complete lysis of the bacterium, was detected (Figure 1).

Table 1 *B. thuringiensis* crystal shapes under light microscopy

Isolate number	Description
1, 24	Bipyramidal, cuboidal, spherical
2, 4, 8	Bipyramidal, cuboidal
7, 14, 16	Bipyramidal (different sizes), cuboidal
9	Bipyramidal
15, 22, 23, 25	Bipyramidal, cuboidal, amorph
17, 18	Bipyramidal (different sizes), amorph

To study the crystal morphology of the newly isolated *B. thuringiensis* bacteria, sporulated cells were treated with Coomassie blue. Using this stain, crystal proteins were stained in dark-blue while the spores remain unstained as demonstrated in figure 1. Different crystal shapes were observed for the studied isolates including bipyramidal, amorph,

cuboidal and spherical. In some cases, the presence of different crystal shapes and sizes was detected for the same isolate (Figure 1; Table 1).

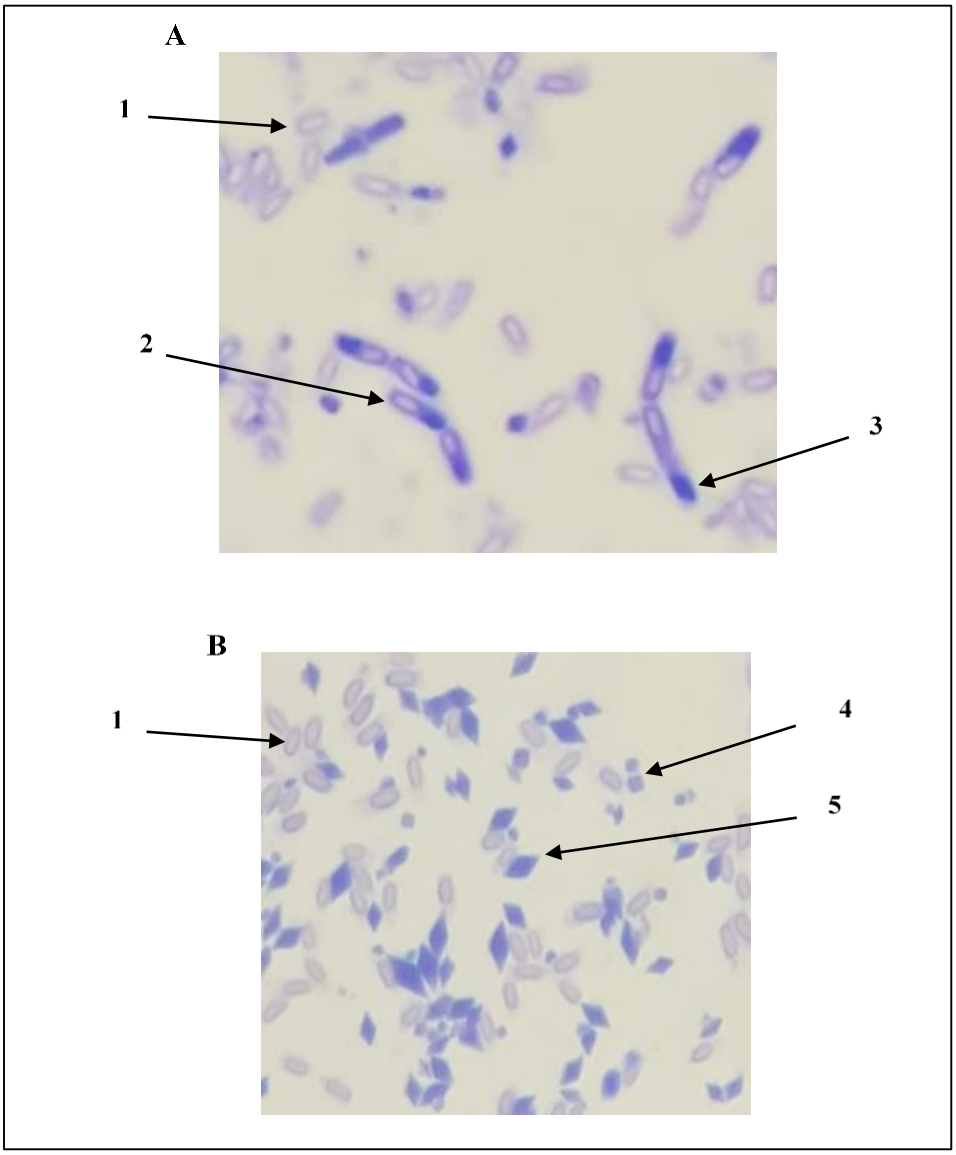

Figure 1 Light microscopy of *B. thuringiensis* isolate 7

(A) Sporulated *B. thuringiensis* cells before complete lysis; (B) Complete lysis of sporulated *B. thuringiensis* cells. 1, Free unstained spore; 2, Unstained spore (in the cell); 3, Bipyramidal crystal (in the cell); 4, Cuboidal crystal; 5, Bipyramidal crystal. Magnification: 100x

In Table 1, were summarized the different crystal shapes for the studied *B. thuringiensis* isolates. All the isolates contained bipyramidal crystals and were classified according to their crystal shapes into 6 classes (Table 1). Nair et al. [21] classified their *B. thuringiensis* collection using the same strategy based on the study of the morphology of the parasporal crystals.

The variation of the crystal shapes from one isolate to another indicated the presence of different *B. thuringiensis* strains in our new isolated collection and promised a variation in the activity spectra between these strains against lepidopteran pests.

3.3. δ-endotoxins profiling of *B. thuringiensis* isolates

To have an idea concerning the potency of our *B. thuringiensis* strains in the control of insect pests, a study to investigate their corresponding delta-endotoxins was conducted. For that, these proteins were extracted from the different isolates and their profiles and activities against Lepidoptera were investigated.

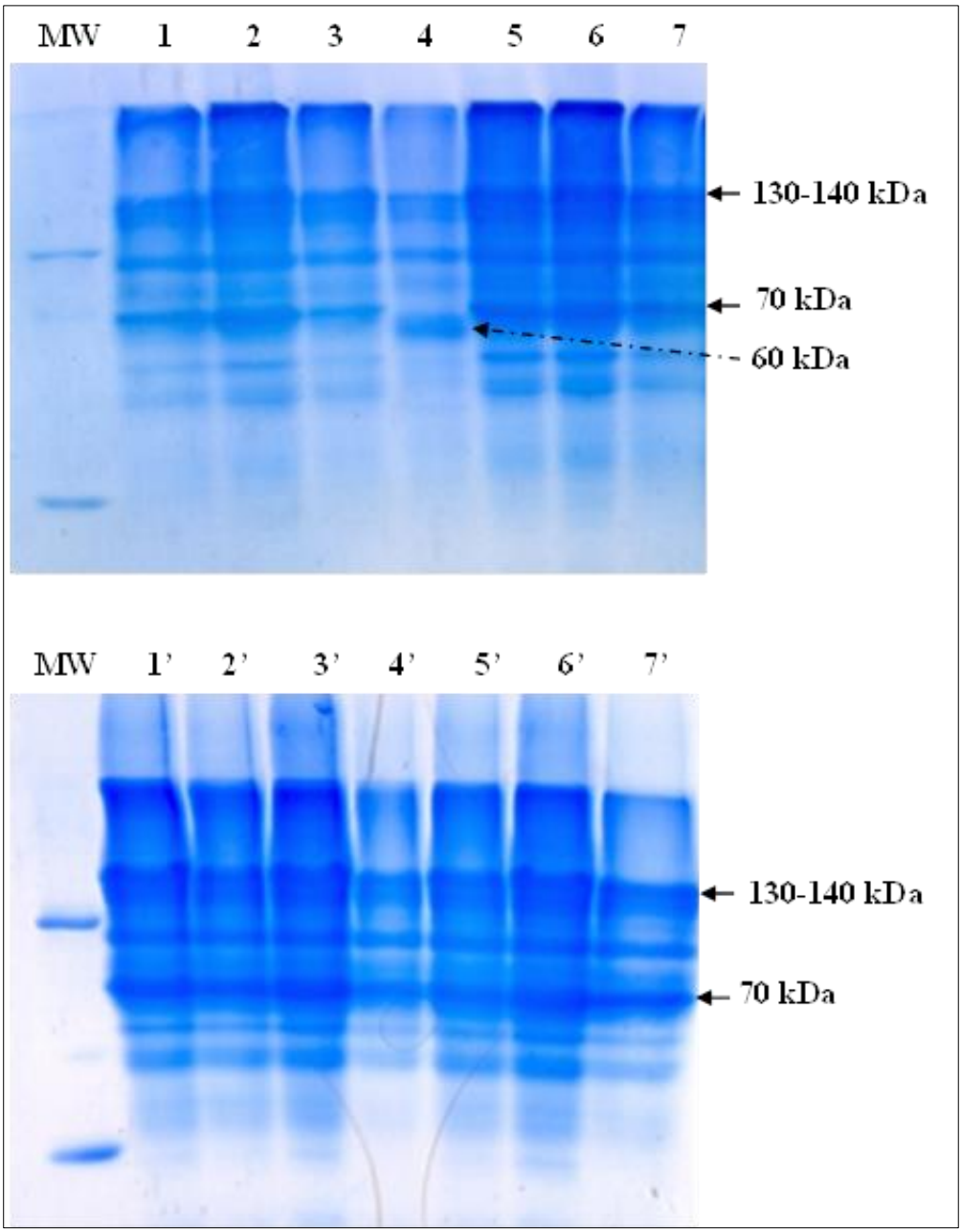

Figure 2 Analysis of *B. thuringiensis* inclusions content by SDS-PAGE

MW, Molecular weight marker; Lanes: 1-7, Crystal proteins extracted from isolates 1, 2, 4, 7, 8, 9 and 14, respectively; Lanes: 1' to 7', Crystal proteins extracted from isolates 15, 16, 17, 18, 22, 23 and 24, respectively.

The study of delta-endotoxins content of the different *B. thuringiensis* isolates was achieved by extraction of the parasporal crystals according to the method described by El Khoury et al. [13]. Then, these crystals were solubilized and proteins were separated by SDS-PAGE in the presence of a molecular weight marker.

After electrophoresis in denaturing conditions, the obtained gels showed that parasporal crystals of all the studied isolates contained delta-endotoxins having almost similar molecular weights with the exception of isolate 7 (Figure 2, lane 4) that demonstrated a profile slightly different from the others. In fact, we noticed that all the parasporal crystals contained delta-endotoxin proteins of about 130-140 kDa (Figure 2). Moreover, all the isolates presented a second delta-endotoxin protein with a molecular weight rounding the 70 kDa, with the exception of isolate 7 in which the second delta-endotoxin has a size of about 60 kDa. In all the cases, different degradation bands were obtained (Figure 2).

3.4. Insecticidal activity of *B. thuringiensis* δ-endotoxins against the lepidopteran pest *E. kuehniella*

The presence of delta-endotoxins in *B. thuringiensis* new isolates encouraged us to test their activity. For that, these proteins were tested against the Lepidoptera *E. kuehniella*. Our choice was based mainly on the fact that this insect was known by its damages on different stored products such as flour, semolina, pasta and cereal grains.

By mixing semolina with delta-endotoxins, this media was used to feed neonate larvae of *E. kuehniella* and after 3 and 5 days, mortality was recorded and LC_{50} concentrations were determined using probit analysis.

Table 2 showed that, after 3 days of treatment, all crystal proteins were active against *E. kuehniella* with the exception of delta-endotoxins of the *B. thuringiensis* isolate 22 that was not active after this treatment duration. These results permitted the classification of *B. thuringiensis* isolates into three classes, class 1 with very high efficiency (Isolates 1, 7, 14 and 24), class 2 corresponding to isolates with less activity against *E. kuehniella* (Isolates 2, 4, 8, 9, 15, 16, 23 and 25) and class 3 with very low activity (Isolates 17 and 18), or without detected activity (Isolate 22).

Table 2 Toxicity of *B. thuringiensis* delta-endotoxins against *E. kuehniella* after 3 days of treatment

Classification	Isolate number	LC_{50} (μg/cm²)
Class 1	1	109.98 (+/- 33.17)
	24	122.24 (+/- 31.75)
	14	136.50 (+/- 36.81)
	7	140.77 (+/- 33.54)
Class 2	16	153.79 (+/- 36.35)
	4	163.98 (+/- 51.15)
	8	168.99 (+/- 59.43)
	25	169.95 (+/- 51.27)
	15	175.51 (+/- 51.27)
	2	175.57 (+/- 59.71)
	9	180.69 (+/- 51.12)
	23	185.52 (+/- 50.82)
Class 3	17	224.02 (+/- 99.63)
	18	289.11 (+/- 174.72)
	22	--------

To confirm these results, the bioassay test was extended to 5 days and results were confirmed for the majority of the isolates. For the isolate 18, previously classified in class 3, higher activity was detected allowing its classification in class 2. Moreover, the isolate 22 showing no activity against *E. kuehniella* after 3 days of treatment, was found to be active after 5 days (Table 3). Concerning isolates 18 and 22 showing much better activity against *E. kuehniella* larvae after 5 days treatment compared to that detected after 3 days, results can be explained by the lower activation rate of the protoxin in midgut larvae compared to that of delta-endotoxins of the other isolates. In fact, Bradley et al. [22] demonstrated that proteolysis of delta-endotoxins by protease midgut larvae is a key step in the mode of action of these toxins. Thus, after 5 days, activation of delta-endotoxins of *B. thuringiensis* isolates 18 and 22 occurred correctly and caused a decrease in the LC_{50} related with the increase of the activity against the tested Lepidoptera.

Delta-endotoxins of *B. thuringiensis* isolate 14 showed the most promising activity against the same Lepidoptera under same conditions with LC_{50} rounding the 59.18 μg/cm² which makes this isolate promising in controlling insect pests. This activity was higher than that of HD1 and BLB459 delta-endotoxins described by Boukedi et al. [16].

Table 3 Toxicity of *B. thuringiensis* delta-endotoxins against *E. kuehniella* after 5 days of treatment

Classification	Isolate number	LC_{50} (µg/cm²)
Class 1	14	59.18 (+/- 17.78)
	7	65.67 (+/- 23.45)
	24	66.73 (+/- 16.25)
	1	67.39 (+/- 26.68)
Class 2	4	74.95 (+/- 27.24)
	16	79.63 (+/- 20.33)
	2	83.75 (+/- 28.19)
	25	86.62 (+/- 42.00)
	18	90.13 (+/- 49.83)
	15	92.29 (+/- 18.16)
	8	99.29 (+/- 27.76)
	9	109.84 (+/- 30.59)
	23	115.65 (+/- 29.93)
Class 3	17	116.12 (+/- 35.09)
	22	168.99 (+/- 59.43)

4. Conclusion

The present study was undertaken to isolate and screen new *B. thuringiensis* strains for their aptitude to produce delta-endotoxins able to be used as biological agent to combat lepidopteran pests.

Fifteen *B. thuringiensis* strains were isolated from soil samples and classified according to different criteria such as parasporal crystal morphology, delta-endotoxins content and activity against the Lepidoptera *E. kuehniella*.

Promising results were obtained demonstrating that isolated *B. thuringiensis* strains are active against the stored products insect *E. kuehniella* and can be used in biological control programs against lepidopteran pests.

Compliance with ethical standards

Disclosure of conflict of interest

The authors: Kallaf, F.I., Dr. Boukedi H., Dr. Daâssi, D. and Pr. Abdelkefi-Mesrati L. have declared that there is no conflict of interest with the publication of this manuscript, institution as well as product that is mentioned in the manuscript.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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