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Self-sufficient cytochrome P450 monooxygenases from *Bacillus thuringiensis:* amplification and comparative sequence analysis

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Four primers targeting the self-sufficient cytochrome P450 monooxygenase gene in the *Bacillus cereus* group were designed in this study. PCR was carried out using these primers, and *Bacillus thuringiensis* wild type strains C32 and 1.2 as well as the reference strain HER1410 were proven to possess this gene. Our results also showed that *B. thuringiensis* C32 possesses two different copies of this gene. Phylogenetic analysis and comparative sequence analysis were performed in order to evaluate the differences between the sequences of *B. thuringiensis* C32, 1.2 and HER1410 as well as those from the public databases. The sequence of *B. thuringiensis* HER1410 clustered with that of *B. cereus* 14579^T, and the sequences of *B. thuringiensis* C32 and 1.2 clustered with that of *Bacillus weihenstephanensis* KBAB4. The sequences of both *B. thuringiensis* Al Hakam and *B. thuringiensis* serovar *konkukian* 97–27 did not cluster with strains C32, 1.2 and HER1410. The diversity of CYP102A in *B. thuringiensis* strains implies that this species represents an excellent potential resource of different self-sufficient cytochrome P450 monooxygenases.

Key words: cytochrome P450, self-sufficient cytochrome P450 monooxygenase, *Bacillus thuringiensis*, *Bacillus cereus* group, *Bacillus megaterium*, CYP102A

INTRODUCTION

The cytochromes P450 (CYP) comprise a superfamily of haem-thiolate enzymes of which over 2700 members are currently known from all three domains of life, and their main function lies in the Phase 1 oxidative metabolism of many organic chemicals of diverse structure, both exogenous and endogenous. Bacterial P450s form a large proportion of the superfamily and, according to the systematic nomenclature, these have been designated families CYP101 and above, where CYP184 represents the current limit of the named bacterial P450 families [1]. From the industrial point of view, P450 enzymes are interesting targets for the production of fine chemicals, including pharmaceuticals [2, 3]. Many bacterial P450 enzymes are involved in oxidative biotransformations of natural compounds or man-made chemicals such as herbicides. Of particular interest are P450s involved in the degradation of recalcitrant pollutants [4, 5]. In order to change the characteristics of P450s, the construction of chimeric proteins started a few years ago. These chimeric sequences can display functional properties characteristic of the parents or acquire entirely new functions [6–9].

The CYP102A subfamily represents a unique group of bacterial self-sufficient cytochrome P450 monooxygenases (SSP450). Members of the CYP102A subfamily are in several ways unique. They are natural fusion enzymes of approximately 117–119 kDa comprised of the N-terminal monooxygenase domain and a FAD and FMN containing a diflavin reductase domain. Due to their structural organisation, CYP102A members exhibit extraordinary activities in comparison to other P450 monooxygenases. These exceptional activities make them promising candidates to be used in industrial applications. Up to now cloning and characterisation of only four members of this subfamily have been published – CYP102A1 from *Bacillus megaterium* [10], CYP102A2 and CYP102A3 from the *Bacillus subtilis* strain 168 [11, 12] and CYP102A5 from *Bacillus cereus* ATCC 14579^T [13]. In total, 14 different members of the CYP102A subfamily (CYP102A1–CYP102A14) are known to date (http://drnelson.utmem.edu/biblioE.html#102).

In this paper, we describe the construction of the primers, amplification and comparative sequence analysis of a fragment of the SSP450 genes from three *Bacillus thuringiensis* strains.

MATERIALS AND METHODS

Bacterial strains, morphological characterization and maintenance of the culture

Bacterial strains C32, 1.2 and *B. thuringiensis* HER1410 were used for this study. The reference strain *B. thuringiensis* HER1410 was kindly provided by Prof. R. Daugelavičius (Vilnius University). The wild type strains C32 and 1.2 were obtained from soil, isolated as described previously [14], and selected for appearance of parasporal inclusions by phase-contrast microscopy. The cultures were cultivated and maintained on nutrient agar.

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DNA extraction

Bacterial genomic DNA was extracted from fresh cell culture (after cultivation on nutrient agar for 12 hours at 30 °C) using the Genomic DNA Purification Kit (Fermentas) according to the manufacturer's instructions.

Amplification and cloning of 16S rRNA gene

Amplification of 16S rDNA was performed as described by Kuisiene et al. [15]. The 16S rDNA PCR product was extracted from agarose gel using DNA Extraction Kit (Fermentas). The purified PCR product was cloned into *Escherichia coli* DH5α using InsT/Aclone™ a PCR Product Cloning Kit (Fermentas). Recombinant clones were detected by blue / white screening [16]. Recombinant plasmid DNA was extracted as described by Birnboim & Doly [17]. The cloned 1.5 kb DNA fragments amplified in PCR were sequenced by automated DNA sequencing.

Construction of primers for SSP450 amplification

Sequences used for constructing the primers are listed in Table 1. The sequences were edited and PCR primers were designed using the SEQBUILDER and PRIMERSELECT components of LASERGENE 6 (DNASTAR). The sequences were aligned and analysed using MEGA 3.1 program [18].

Amplification of SSP450 from *B. thuringiensis* strains

Table 1. **Sequences used for constructing primers for the amplification of self-sufficient cytochrome P450 monooxygenases**

Species and strain	Accession number
B. anthracis str. 'Ames Ancestor'	AE017334: 2965672...2968869
B. anthracis str. Ames	AE016879: 2965544...2968741
B. anthracis str. Sterne	AE017225: 2966235...2969432
B. cereus ATCC 10987	AE017194: 3026594...3029791
B. cereus ATCC 14579^T	AE016877: 3189230...3192427
B. cereus E33L	CP000001: 3049859...3053056
B. thuringiensis str. Al Hakam	CP000485: 3037966...3041187
B. thuringiensis serovar konkukian str. 97–27	AE017355: 3058435...3061632
B. weihenstephanensis KBAB4	CP000903: 2982807...2986004

B – *Bacillus*

The primers P450Ox, BpgF, P450F and P450R, designed in this study, were used for the amplification of the SSP450 gene. The gene was amplified in 50 μl of reaction mixture containing PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer, 1.25 U recombinant *Taq*DNA Polymerase and 10 ng of bacterial genomic DNA. Amplification was conducted under the following conditions: initial denaturation at 95 °C for 2 min followed by 29 cycles each consisting of 95 °C for 1 min, 55 °C for 2 min and 72 °C for 3 °min with a final extension step at 72 °C for 7 min in an Eppendorf thermal cycler. The amplification products were analysed by electrophoresis through 1% agarose gel.

Sequence analysis

The sequences of SSP450 obtained in the present study were deposited in the GenBank (accession numbers EU591718-EU591720).

The sequences were edited and sequence similarity was determined using the SEQBUILDER and MEGALIGN components of LASERGENE 6 (DNASTAR). The sequences were aligned using the MEGA 3.1 program [18]. The size of 16S rDNA used for the alignment was 1307 nucleotides and that of SSP450 466 nucleotides. Phylogenetic trees were constructed by the neighbour-joining method using the MEGA 3.1 program [19]. Pairwise-deletion option was used. Bootstrap analysis of the neighbour-joining data, using 1000 resamplings, was carried out to evaluate the validity and reliability of the tree topology. The 16S rDNA tree was rooted using the AJ276351 sequence of *B. subtilis* DSM 10^T as an outgroup. The SSP450 tree was rooted using the J04832 sequence of *B. megaterium* isolate Fulco PB85 clone BM3-3[A, B] as an outgroup.

RESULTS AND DISCUSSION

Characterization of the bacterial strains

Strains 1.2 and C32 were isolated from soil. These strains formed endospores during a prolonged incubation on nutrient agar. Parasporal inclusions were detected in the cells using phase-contrast microscopy. It was hypothesized that strains 1.2 and C32 belong to the endospore-forming species *B. thuringiensis*. In order to test this assumption, the phylogenetic analysis of the 16S rRNA gene was carried out. The 16S rDNA sequences determined for strains 1.2 and C32 were of 1418 and 1465 nucleotide length, respectively. The sequences of strains 1.2 and C32 were most similar to that of *B. thuringiensis* NBRC 101235^T. The phylogenetic tree (Fig. 1a) shows the phylogenetic position of strains 1.2 and C32 among the closely related species of the *B. cereus* group. *Bacillus anthracis*, *B. cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *B. thuringiensis*, and *Bacillus weihenstephanensis* represent six related species, but only *B. thuringiensis* is known in this group to produce crystal proteins during sporulation. Thus, both the morphological examination and phylogenetic analysis showed strains 1.2 and C32 to belong to *B. thuringiensis*.

Construction of primers for the amplification of SSP450

Amino acid sequence identity among P450 proteins is often less than 20%. The regions with the highest homology (potential targets for the construction of the primers) comprise:

1. The heme binding loop, containing the most characteristic P450 consensus sequence (F-G/S-X-G-X-H/R-X-**C**-X-G/A) with the absolutely conserved cysteine that serves as a ligand to the heme iron.

2. The conserved E-X-X-R motif probably needed to stabilise the core structure through a salt bridge.

3. The consensus sequence A/G-G-X-E/D-**T** (with the absolutely conserved threonine) considered as P450 signature which is thought to play a role in oxygen activation through proton transfer [20].

The P450 motif F-G/S-X-G-X-H/R-X-**C**-X-G/A, located in the heme binding loop, was F-G-N-G-Q-R-A-C-I-G in all the examined sequences. The latter sequence represents the signature motif of CYP102 [1]. The forward primer BpgF was designed that targeted this signature motif (Table 2, Fig. 2).

The consensus sequence A/G-G-X-E/D-**T**, involved in the oxygen activation, was A-G-H-E-T in all the examined sequences. The forward primer P450Ox was constructed, which targeted this sequence (Table 2, Fig. 2).

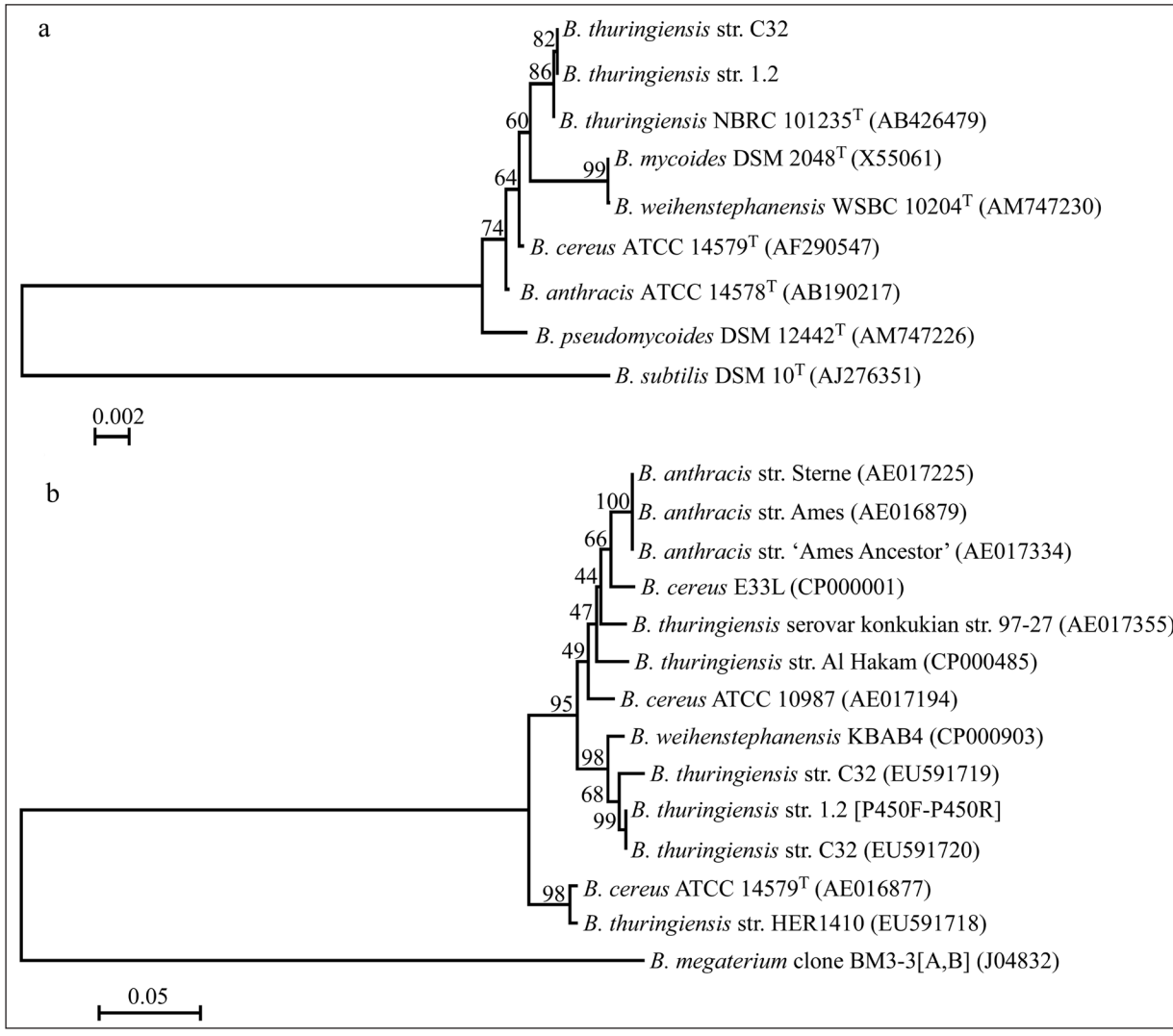

Fig. 1. Phylogenetic analysis of a) strains 1.2 and C32 on the basis of 16S rRNA gene sequences and b) fragment (466 nt) of self-sufficient cytochrome P450 monooxygenase gene. The numbers at the nodes represent the percentage of bootstrap values obtained from 1000 samplings. Only the most significant values are shown. Bars, 0.002 (a) and 0.05 (b) nucleotide substitution per site

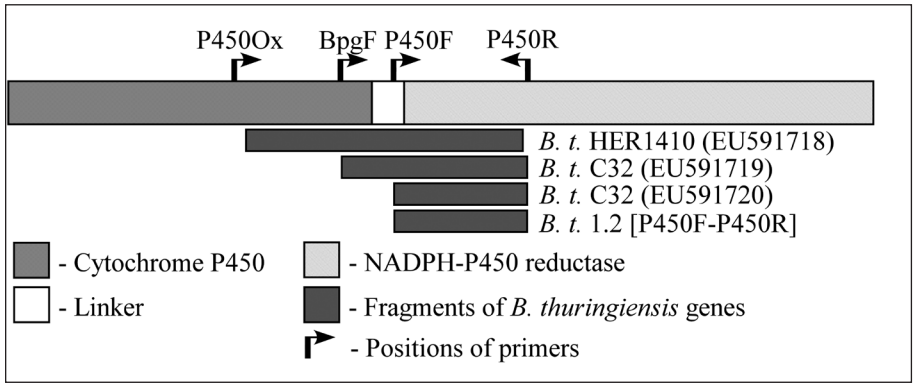

Fig. 2. Schematic representation of the positions of primers and the fragments of *B. thuringiensis* self-sufficient cytochrome P450 monooxygenase genes obtained using these primers

Table 2. **List of primers designed for the amplification of self-sufficient cytochrome P450 monooxygenases**

Primer	Target	Sequence of the primer
P450Ox	Oxygen activation site (P450 domain)	GGC ATG AGA CAA CAA GTG GA
BpgF	Heme binding loop (P450 domain)	GGT CAG CGA GCA TGT ATC
P450F	Linker between P450 and reductase domains	GCG CCT ACA GAG GAG AAA C
P450R	Reductase domain	CTT CAT CGC ATC AGA CCA CA

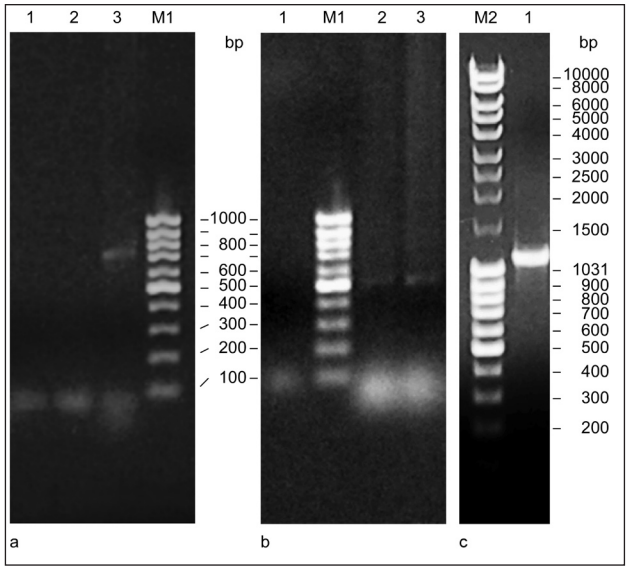

Fig. 3. Agarose gel electrophoresis of PCR products obtained using primers targeting self-sufficient cytochrome P450 monooxygenase gene: a) PCR products using BpgF–P450R. Lanes: 1 – HER1410; 2 – strain 1.2; 3 – strain C32; b) PCR products using P450F–P450R. Lanes: 1 – HER1410; 2 – strain 1.2; 3 – strain C32; c) PCR products using P450Ox–P450R. Lane 1 – HER1410. Lanes: M1 – GeneRuler™ 100-bp DNA Ladder (Fermentas); M2 – MassRuler™ DNA Ladder Mix (Fermentas)

The third forward primer P450F was designed based on the sequence of the linker between P450 and reductase domains (Table 2, Fig. 2). The conservative sequence A-P-T-E-E-K-L was identified in this linker. Only for *B. weihenstephanensis* KBAB4 the difference in one nucleotide resulted in I instead of T and for *B. cereus* ATCC 14579^T in E-D instead of E-E.

The reverse primer P450R was designed on the basis of the analysis of reductase domain (Table 2, Fig. 2). The highly conservative sequence M-W-S-D-A-M was identified in all the examined sequences. This sequence was used to construct the primer P450R.

Thus, four primers were constructed in this study. All three forward primers could be used in pair with the reverse primer.

Amplification of SSP450

Genomic DNA of *B. thuringiensis* strains 1.2, C32 and HER1410 was used for the amplification of CYP102A. The pairs of primers P450F–P450R, BpgF–P450R and P450Ox–P450R were tested.

PCR using the pair of primers P450F-P450R was successful for strains 1.2 and C32. PCR resulted in a fragment ~500 bp in length. We could not obtain PCR product from the genomic DNA of HER1410 (Figs. 2, 3b). PCR using the pair of primers BpgF-P450R was also unsuccessful for strains HER1410 and 1.2. A fragment ~720 bp in size was obtained for strain C32 (Figs. 2, 3a). PCR using the third combination of primers (P450Ox–P450R) resulted in a fragment ~1100 bp in length for strain HER1410, but was unsuccessful for strains C32 and 1.2 (Figs. 2, 3c).

It should be noted that at least one amplification was successful for every strain. Thus, it may be clearly stated that genes of SSP450 were detected in strains 1.2, C32 and HER1410.

Comparative sequence analysis of SSP450 from *B. thuringiensis* C32, 1.2 and HER1410

The amplified fragments of SSP450 were sequenced. The obtained sequences were subjected to a comparative sequence analysis. Phylogenetic analysis of these sequences was also carried out.

Comparative analysis showed that the sequence of HER1410 differed from the sequences of strains C32 and 1.2. Phylogenetic analysis confirmed these results. The sequence of HER1410 was more closely related to that of *B. cereus* ATCC 14579^T representing a distinct cluster in the phylogenetic tree (Fig. 1b). The sequences of strains C32 and 1.2 clustered with that of *B. weihenstephanensis* KBAB4. It should be noted that the sequences of both *B. thuringiensis* Al Hakam and *B. thuringiensis* serovar *konkukian* 97–27 did not cluster with strains C32, 1.2 and HER1410 and were included into the cluster with strains of *B. anthracis* as well as some *B. cereus* strains. Interestingly, CYP102A5 was identified in *B. cereus* ATCC 14579^T, and CYP102A9 in *B. weihenstephanensis* KBAB4 (http://drnelson.utmem.edu/biblioE.html#102). We hypothesize that strain HER1410 has CYP102A5-like, genes and strains C32 and 1.2 contain CYP102A9-like genes and / or those highly similar to P450s. According to the database, CYP102A8 has been found in *B. thuringiensis* serovar *konkukian* 97–27. Taken together, these data imply that SSP450s of *B. thuringiensis* vary from strain to strain and does not depend on the species. The implication is that *B. thuringiensis* represents an excellent potential resource of the different SSP450s.

The products of PCR primed by the oligonucleotide pair P450F–P450R were completely identical in sequence. On the other hand, a comparative sequence analysis revealed differences between the two sequences of strain C32. The majority of the differences were concentrated downstream from the BpgF binding region (Fig. 4). The sequence, obtained with the pair of primers BpgF–P450R resembled more that of *B. weihenstephanensis* KBAB4 than the sequence obtained with the pair P450F–P450R (Fig. 4). These differences influenced on the composition of amino acids. Consequently, it may be

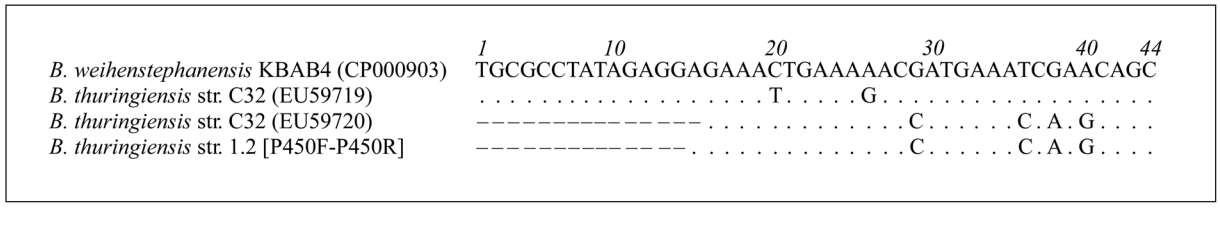

Fig. 4. Comparative analysis of the sequence located downstream the BpgF binding region. Dots indicate nucleotides identical to those of the self-sufficient cytochrome P450 monooxygenase gene of *Bacillus weihenstephanensis* KBAB4

concluded that the *B. thuringiensis* strain C32 possesses two different genes SSP450. To our knowledge, only the *B. subtilis* strain 168 possess two different copies of such monooxygenases – CYP102A2 and CYP102A3. Only one copy of these enzymes was identified in all the sequenced genomes of the *B. cereus* group.

In conclusion, in this study four primers were designed, which targeted the SSP450 gene in the *B. cereus* group. *B. thuringiensis* wild type strains C32 and 1.2 as well as the reference strain HER1410 were proven to possess this gene. Much more, our results have shown that strain C32 possesses two different copies of this gene. The diversity of CYP102A in *B. thuringiensis* strains implies that this species represents an excellent potential resource of different SSP450s.

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BACILLUS THURINGIENSIS SAVARANKIŠKOSIOS P450 CITOCHROMO MONOOKSIGENAZĖS GENO PAGAUSINIMAS IR LYGINAMOJI SEKŲ ANALIZĖ

Santrauka

Šiame darbe savarankiškosios citochromo P450 monooksigenazės geno sekų pagrindu buvo sukurti keturi pradmenys genui pagausinti *Bacillus cereus* grupėje. Naudojant šiuos pradmenis atlikta PGR reakcija. Nustatyta, kad *Bacillus thuringiensis* C32 ir 1.2 laukinio tipo kamienai bei HER1410 standartinis kamienas turi šios monooksigenazės geną. Be to, C32 kamienas turi dvi skirtingas šio geno kopijas. Siekiant įvertinti skirtumus tarp C32, 1.2 ir HER1410 kamienų sekų bei savarankiškosios citochromo P450 monooksigenazės geno sekų, esančių duomenų bazėse, buvo atlikta filogenetinė bei lyginamoji sekų analizės. *B. thuringiensis* HER1410 seka sudarė vieną grupę su *B. cereus* 14579^T seka, o *B. thuringiensis* C32 ir 1.2 sekos – su *Bacillus weihenstephanensis* KBAB4 seka. *B. thuringiensis* Al Hakam ir *B. thuringiensis* serovaro *konkukian* 97–27 sekos nesudarė grupės su C32, 1.2 ir HER1410 kamienais. *B. thuringiensis* CYP102A sekų įvairovė leidžia teigti, kad ši rūšis yra puikus potencialus įvairių savarankiškųjų citochromo P450 monooksigenazių šaltinis.