Folia Biologica et Oecologica 16: 5–11 (2020)

Acta Universitatis Lodziensis

Impaired base excision repair is related to the pathogenesis of non-alcoholic fatty liver disease

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ABSTRACT

Non-alcoholic fatty disease (NAFLD) is a liver disorder that affects up to 30% of the population, mainly in Western countries. It is estimated that up to 75% of NAFLD patients will develop a more aggressive form of the disease, non-alcoholic steatohepatitis (NASH). NAFLD can lead to fibrosis and liver failure; however, it is difficult to diagnose NAFLD due to its non-specific symptoms. Unfortunately, there is no treatment available for this disease. The risk factors of NAFLD are obesity and insulin resistance (IR). The molecular factors that seem to play an important role in the pathogenesis of NAFLD are oxidative stress as well as impaired DNA damage repair processes; a great body of evidence confirms an association with the base excision repair (BER) pathway. The activity of BER is decreased in patients with NAFLD and in animal models of this disease. In order to better understand the underlying basis of the disease, knowledge should be broadened in the area of DNA repair in NAFLD.

KEYWORDS: DNA repair, non-alcoholic fatty liver, base excision repair

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition that affects up to 30% of the population around the world, most prevalently in Western countries. It is estimated that up to 75% of people suffering from simple fatty liver disease will develop a more aggressive form of the disease, non-alcoholic steatohepatitis (NASH), which can lead to a cirrhosis, primary hepatocarcinoma or liver failure. However, it is difficult to diagnose the NAFLD due to the non-specific symptoms of the disease. NASH is characterised by the occurrence of inflammation and fibrosis in the liver (Abd El-Kader and El-Den Ashmawy 2015; Paschos and Paletas 2009).

Little is known about the molecular mechanisms involved in the pathogenesis of NAFLD. However, the current literature shows that NAFLD is associated with increased production of reactive oxygen species (ROS) and with elevated levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG), which is characteristic of DNA damage caused by increased

DOI: https://doi.org/10.18778/1730-2366.16.01

oxidative stress. Mitochondria are essential organelles for cell survival, mainly due to their function as energy producers through the electron transport chain (ETC). On the other hand, ETC generates ROS and may contribute to increased oxidative stress. Oxidative stress and mitochondrial DNA (mtDNA) damage are linked and affect each other. ROS produced due to the electron transport chain may target mtDNA. In effect, damaged mtDNA needs to be repaired, mainly through the base excision repair (BER) pathway in mitochondria (Masarone *et al*. 2018; Yang *et al*. 2019).

A growing number of reports, which have been presented in the review, indicate that oxidative stress as well as impaired processes responsible for repairing DNA damage can play important in the development of NAFLD. The main factor that links DNA repair and NAFLD is an increased level of oxidative stress followed by an elevated level of oxidative damage in patients. In cases of faulty DNA repair, these lesions cannot be repaired (Nagahashi *et al*. 2016). The most important aspect of further research is the absence of a treatment that could prevent liver failure and eventually the death of the patient.

The pathophysiology of non-alcoholic fatty liver disease

NAFLD is a pathological condition in which fats build up in the liver; it can progress to a more aggressive form of the disorder, NASH. It can lead to cirrhosis and may develop into primary liver cancer (Pinter *et al*. 2016). Unfortunately, the disease has non-specific symptoms like a feeling of discomfort in the upper right side of the abdomen; often, there are no symptoms until considerable liver cell damage occurs. One of the risk factors is obesity; the prevalence in obese population is in the range of 57–74% (Sharma *et al*. 2015). Moreover, up to 75% of people suffering from NAFLD develop NASH (Chitturi *et al*. 2018). As there are no approved medicines to treat NAFLD (Wong and Singal 2019), doctors recommend vitamin E supplementation and losing weight as well as medicines for type 2 diabetes such as pioglitazone (Bril *et al*. 2018).

NAFLD is a hepatic disorder characterised by triglyceride (TG) accumulation in hepatocytes. It is caused by an imbalance between lipogenesis or fatty acid uptake and fatty acid removal, e.g. via mitochondrial fatty acid oxidation (Caligiuri *et al*. 2016). In the case of NASH, hepatic inflammation, hepatocellular ballooning and often fibrosis can occur (Cohen *et al*. 2011). The hypothesis explaining the pathogenesis of NAFLD assumes that (i) hepatic steatosis is a result of insulin resistance (IR), and (ii) the progression to NASH is associated with oxidative stress, lipid peroxidation, cytokine production or mitochondrial dysfunction (Day and James 1998).

Particularly in obese patients with NAFLD, there is an overload of TG in adipose tissue, which is an energy storage organ and is involved in the secretion of hormones, cytokines and chemokines (Kershaw and Flier 2004). Excess TG is converted into free fatty acids (FFA) that can enter the liver, which can result in peripheral IR (Boden 1997). Insulin, a lipolysis-inhibiting hormone, controls the release of FFA into the liver. Thus, IR leads to intense lipolysis of adipose tissue and, consequently, to an increased influx of FFA into the liver. Additionally, FFA may serve as ligands for Toll-like receptor 4, thus inducing cytokine production and eventually inflammation, which play important roles in NAFLD development (Shi *et al*. 2006). IR is an effect of an

increased level of cytokines, such as interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-α; high cytokine levels are correlated with a greater content of adipose tissue. NAFLD is more associated with visceral adipose tissue than with subcutaneous adipose tissue. (Parker 2018). Because of an excess of visceral adipose tissue, there is a dysregulation in a production of chemokines responsible for fatty acid oxidation (Rotter *et al*. 2003; Skurk *et al*. 2007). However, the liver can eliminate TG through secretion as very low-density lipoprotein (VLDL) or by performing a fatty acid oxidation. Both reduced oxidation and increased TG levels have a place in NAFLD pathogenesis (Koo 2013).

The fat accumulation in the liver may trigger cellular injury and death because of fatty acid intermediates (Lee *et al*. 1994; Tomita *et al*. 2014). Palmitic acid, a type of FFA, is able to cause inflammatory activation in endothelial cells (Maloney *et al*. 2009). Free cholesterol accumulation also induces lipotoxicity (Tomita *et al*. 2014). However, fatty acid oxidation can directly lead to the production of ROS (Neuschwander-Tetri 2010). Oxidative stress induces hepatocellular damage through mechanisms such as lipid peroxidation, which is responsible for activation of cell necrosis and the intrinsic pathway of apoptosis. In effect, it can trigger fibrosis (Koek *et al*. 2011). Palmitic acid can also activate the c-Jun N-terminal kinase (JNK) and the NF-κB proinflammatory pathways and may lead to mitochondrial dysfunction (Maloney *et al*. 2009). Additionally, free cholesterol induce the JNK pathway, thus generating a higher amount of ROS (Caballero *et al*. 2009). TG accumulation and steatosis can occur without liver damage, as shown by studies on genetic defects in diacylglycerol acyltransferase (DGAT), which catalyses the final step in TG synthesis, and in microsomal transfer protein (MTP), which influences VLDL synthesis (Liao *et al*. 2003; Monetti *et al*. 2007).

In pathophysiology of NAFLD also involves Kupffer cells, i.e. resident hepatic macrophages, which play a role in the activation of the macrophage M1 phenotype and altered activation of M2 macrophages (Stienstra *et al*. 2010). Toxic lipids accumulate in hepatocytes and may be phagocytised by Kupffer cells, which may indirectly lead to inflammation. Kupffer cells are involved in inflammation via inflammatory and apoptotic pathways mediated by NF-κB, which induce further release of proinflammatory cytokines (Seki *et al*. 2007).

Base excision repair pathway

Mitochondria are crucial in the pathogenesis of NAFLD. This statement has been confirmed by the relationship between NAFLD and the metabolic syndrome, which is tightly associated with mitochondrial dysfunction and oxidative stress (Kim *et al*. 2018; Mabalirajan and Ghosh 2013). The most important DNA repair pathway in mitochondria is BER, because it eliminates oxidative lesions (Alexeyev *et al*. 2013). BER recognises forms of oxidative, deamination, alkylation and abasic sites. which are not significant alterations to the DNA helix shape. It is divided into four steps (Figure 1): (i) recognition of the DNA damage, (ii) excision, (iii) synthesis and (iv) ligation of DNA. The first two steps are executed by DNA glycosylases, e.g. OGG1, MYH, NEIL1 and AP endonuclease (APE1) (Kim and Wilson 2012). DNA glycosylases are able to recognise and excise damaged bases, while the endonuclease cleaves the phosphodiester bonds. During synthesis, polymerase (POLG in mitochondria) inserts the correct nucleotide in the

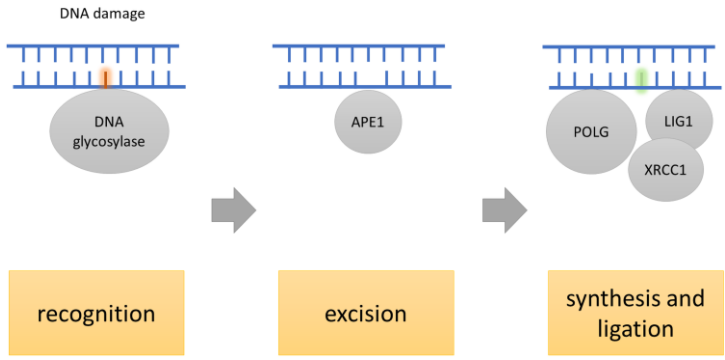

Figure 1. The scheme of base excision repair.

generated gap. The final step of BER in mitochondria, i.e. ligation, is performed by the complex of X-ray repair cross-complementing protein 1 (XRCC1) and DNA ligase III (LIG3) or ligase 1 (LIG1) (Chatterjee and Walker 2017; Kim and Wilson 2012). In the BER machinery, an important role is also played by structure-specific nucleases that remove 5' overhanging flaps and process the 5' ends of Okazaki fragments in lagging strand DNA synthesis. This nuclease is encoded by flap structure-specific endonuclease 1 (FEN1) (Kim and Wilson 2012).

The relationship between non-alcoholic fatty liver disease and base excision repair

There is still little known about the relationship between NAFLD and DNA repair systems. However, the results of recent studies allow us to assume that fatty liver disease has a tight link with the BER pathway. These studies were conducted not only on NAFLD patients, but also on animal models mimicking human NAFLD, i.e. the high fat diet (HFD), methionine-choline-deficient diet (MCD) and fructose-rich diet models. The findings confirm that MCD upregulates gene expression of BER enzymes, i.e. DNA glycosylase and APE1, in mice and induces hepatic steatosis, confirming the link between diet and steatosis (Takumi *et al*. 2015). Furthermore, both MCD and HFD not only increase ROS production and oxidative damage DNA, but also reduce DNA repair by decreasing *MYH* expression (Gao *et al*. 2004). In addition, genetically modified *OGG1* knockout mice fed an HFD had impaired glucose tolerance and a higher plasma insulin level, as well as downregulation of carnitine palmitoyl transferase-1, important in fatty acid oxidation and associated with the development of NAFLD (Sampath *et al*. 2012).

Moreover, there are studies that confirm the appearance of single-nucleotide polymorphisms (SNPs), which can contribute to impaired BER in mitochondria (Czarny *et al*. 2018; Ibarrola-Villava *et al*. 2011; Lillenes *et al*. 2017; Popanda *et al*. 2013). The SNPs are broadly present in other diseases, mainly in neurodegenerative disorders and carcinomas. However, they should be evaluated in the context of NAFLD. The SNPs mentioned above have been found in such genes as *OGG1*, *MYH*, *POLG*, *POLB* (polymerase β) *NEIL1*, *APE1*, *FEN1*, *LIG1*, *LIG3* and *XRCC1*.

In terms of therapeutic approaches to NAFLD, interesting results have been obtained in a study that used pioglitazone as treatment method. This drug is

sometimes given to treat this disease, and has been beneficial in NASH patients. An HFD induced hepatic steatosis, but this effect was reversed by adding pioglitazone. Furthermore, treatment increased the expression level of *OGG1* and *MYH*, which could indicate that the improvement in the health of patients with steatosis may be related to an impact on DNA repair systems (Hsiao *et al*. 2008). Interestingly, in the liver of rats fed a high-fructose diet, an increase in mtDNA damage was found. The diet also caused a decrease in the expression level of DNA polymerase gamma and reduced mtDNA copy number (Cioffi *et al*. 2017).

There are also some research findings that support an association between NAFLD and other DNA repair mechanisms. MCD-fed wild type mice were compared to MCD-fed growth arrest and DNA damage-inducible gene (*Gadd45a*) knockout mice. This gene plays a role in DNA survival and repair, in both the BER and nucleotide excision repair (NER) pathways. Knockout mice had significantly more severe hepatitis and fibrosis, elevated expression levels of pro-inflammatory proteins as well as decreased TG levels in comparison to wild type mice (Tanaka *et al*. 2017). Obese patients with steatotic livers have elevated oxidative stress in the liver and, at the same time, a significant decrease in NER activity (Schults *et al*. 2012).

Summary

Accordingly to the latest literature, a growing body of evidence suggests that mitochondrial dysfunction may play an important role in NAFLD, which can be triggered by impaired mitochondrial genome stability. Increased ROS production and elevated oxidative stress in mitochondria contribute to the development and progression of NAFLD. Many reports have shown that an important factor in the pathogenesis of fatty liver disease is impaired DNA repair systems. Furthermore, the expression of genes involved in DNA repair is increased upon treatment of this disease. This review suggests the need for further research into the molecular processes underlying NAFLD, especially in context of DNA damage and repair. This could contribute to the development of an appropriate treatment for this disease.

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Folia Biologica et Oecologica 16: 12–21 (2020)

Acta Universitatis Lodziensis

Differentiation of *Bacillus anthracis* and other *Bacillus cereus* group bacterial strains using multilocus sequence typing method

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ABSTRACT

The study describes the preparation of the phylogenetic differentiation of *Bacillus cereus* strains. The *Bacillus cereus* group of bacteria is very important for human and animal health. The multilocus sequence typing scheme has been used to present this group of bacteria's phylogenetic relationship and structure. The MLST system was established using 60 isolates of *B. anthracis*, *B. cereus sensu stricto*, *B. thuringiensis*, and transitional environment strains of *Bacillus spp.* As a negative control, five strains of *B. subtilis* and *B. megaterium* were used. Primers for amplification and sequencing were designed to target highly conserved internal fragment of seven housekeeping genes: *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi*. A total of 22 different sequence types (STs) were distinguished. Analysis of the sequence data showed that all of the *Bacillus cereus* strains are very closely related. The MLST scheme exhibited a high level of resolution that can be used as an excellent tool for studying the phylogenetic relationship, epidemiology, and population structure of the *Bacillus cereus* group strains. The MLST method additionally allows us to define the phylogenetic relationship between very closely related strains based on a combination of the sequences of all seven alleles fragments and each of them separately. Thus, this genetic investigation tool is very useful in epidemiological investigation of potential military/ bioterrorist use of *B. anthracis*.

KEYWORDS: *Bacillus cereus* group, multilocus sequence typing, sequencing, house-keeping genes, phylogenetic differentiation, BioNumerics

Introduction

Bacterial strains from the *Bacillus cereus* group belong to the Bacillaceae family (Ludwig *et al*. 2009). This group comprises six species of bacteria: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, and

DOI: https://doi.org/10.18778/1730-2366.16.02

B. pseudomycoides, which are very important for medical (biological) and economic reasons. The *Bacillus cereus* group contains nonpathogenic strains found in humans and animals (saprophytic environmental strains), industrial use strains, and very pathogenic strains, which can be used as a biological weapon agent. *B. cereus* is an opportunistic human pathogen commonly present in soil and water. Due to its ubiquitous nature, *B. cereus* can cause contamination in the pulp and paper and the dairy industry. Additionally, *B. cereus* strains can cause various infections in immunodeficient patients and patients after surgery, including endocarditis, cutaneous infections, septicemia, pneumonia (Hoffmaster *et al*. 2006), or emetic and diarrheal food poisoning (Kim *et al*. 2010). *B. thuringiensis* is a pathogen for insects *Diptera sp.*, *Lepidoptera*, and *Coleoptera sp.* (Elleuch *et al*. 2014) and an opportunistic pathogen for human. *B. thuringiensis* is used in agriculture as a source of natural insecticides in tomatoes, cucumbers, or cabbages cultivation (Frederiksen *et al*. 2006). *B. weihenstephanensis* has been described recently due to its ability to grow in very low temperatures (+4°C to +7°C). As a separate species in the *Bacillus cereus* group, it was identified based on the 16S rRNA and cold-shock protein gene *cspA* sequences (Thorsen *et al*. 2006). Additionally, some strains of *B. weihenstephanensis* possess genes for enterotoxin *NheA* and emetic toxin cereulide (Svensson *et al*. 2007; Økstad and Kolstø 2011), like *B. cereus*. For *B. mycoides* and *B. pseudomycoides* strains, a different rhizoidal type of growth was observed (Sorokin *et al*. 2006). *B. anthracis* is a Gram-positive, spore-forming, aerobic, rod-shaped bacterium. It is the most dangerous human and animal pathogen, causes the very high lethality zoonotic disease anthrax, which can be acquired in four ways: inhalation (50–80% mortality even with treatment), cutaneous, gastrointestinal, and injection. The pathogenicity of *B. anthracis* depends on two virulent factors encoded by genes located on two plasmids pXO1 (anthrax toxin) and pXO2 (poly-γ-d-glutamic acid capsule) (Brossier and Mock 2001; Gierczyński 2010). Changes in the genome (addition or loss of the pathogenicity) can result from the conjugation (horizontal gene transfer) process, which has been described in *Bacillus* strains (Singh *et al*. 2013). Owing to its spores being very easy to produce, transport, and disperse, *B. anthracis* is potentially useful as a biological warfare agent, classified as a group of dangerous pathogens by the Centers for Disease Control and Prevention (2017).

Numerous methods have been used to perform genetic differentiation of *Bacillus cereus* group strains. Recently, the most popular were: restriction enzyme analysis – pulsed-field gel electrophoresis (REA-PFGE) (Helgason *et al*. 2000b; Tenover *et al*. 1995, 1997), amplified fragment length polymorphism (AFLP) (DeVos *et al*. 1995; Ticknoe *et al*. 2001), 16S and 23S rRNA analysis (Cherif *et al*. 2003; Soufiane and Côté 2013), and multilocus enzyme electrophoresis (MEE) (Helgason *et al*. 2000a; Selander *et al*. 1986). Even though those methods are advantageous, the results are very arduous to standardise and compare among different laboratories. In 1998 Maiden's team developed a novel molecular method based on sequencing internal fragments of the chromosome, enabling genetic characterisation of multiple bacterial strains in one experiment (Maiden *et al*. 1998).

Multilocus sequence typing is based on the analysis of nucleotide sequences of highly conserved fragments of a few housekeeping genes. Since 1998, the

MLST scheme was developed for several human bacterial pathogens including: *Clostridium botulinum* (Olsen *et al*. 2014), *Clostridium difficile* (Zhou Y. *et al*. 2014), *Corynebacterium diphtheriae* (Bolt *et al*. 2010), *Vibrio cholera* (Zhou H. *et al*. 2014), *Yersinia sp*. (Duan *et al*. 2014; Souza *et al*. 2013), *Bordetella sp*. (Diavatopoulos *et al*. 2005), *Haemophilus influenzae* (Skaare *et al*. 2014), and *Streptococcus pneumoniae* (Chang *et al*. 2014).

In this study, the MLST scheme for genetic differentiation and characterisation of *Bacillus cereus* group strains from the internal collection of the Biological Threats Identification and Countermeasure Centre of Military Institute of Hygiene and Epidemiology in Pulawy (BTICC MIHE) was performed.

Materials and methods

A total of 65 bacterial strains were analysed. Sixty of them were from the *Bacillus cereus* group strains, and five strains of *B. subtilis* and *B. megaterium* were used as negative control samples. All of them were part of an internal collection of the BTICC MIHE (obtained from the Institute of Medicine and Molecular Biology (IMBM) at the University of Scranton, Pennsylvania, USA; Veterinary Inspection, Łomża, Poland, Medical University of Lublin, Poland, and from the environment isolated by BTICC specialists) and have been identified before by various methods (microscopy, biochemical, immunological, PCR, real-time PCR, etc.) (Cieślik *et al*. 2015). The strains were isolated from animals, people, and the worldwide environment (Germany, China, France, USA). Tested groups of bacterial strains included 10 strains of *B. cereus*, 17 *B. thuringiensis*, seven *B. anthracis*, and 26 transitional environmental *Bacillus sp.* strains. Transitional strains gave positive results in the PCR reaction for *B. anthracis* chromosomal marker *Ba 813+*. The analysed strains are shown in Table 1.

This study includes selected fragments of seven housekeeping genes. The following genes were chosen: *glpF* (encoding glycerol uptake facilitator protein), *gmk* (guanylate kinase), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (AICAR transformylase), *pycA* (pyruvate carboxylase), and *tpi* (triosephosphate isomerase) (Priest *et al*. 2004). The primers used for amplification and sequencing of these fragments are shown in Table 2.

The primers were synthesised by Genomed JSC Poland, based on the sequence from the PubMLST Database (primers used for MLST of *Bacillus* genes) (PubMLST 2018).

The genetic material was isolated from fresh bacterial culture using the automatic isolation station QIAcube® (QiaGene Corp.®) using the QIAamp DNA Mini Kit (QiaGene Corp.®), according to the manufacturer's instructtions. Fresh cultures of tested strains were prepared on Columbia Agar plates containing 5% sheep blood (Graso Biotech, Poland), incubated at 37ºC for 24 hours under aerobic conditions. After incubation, the selected colonies (after morphological and microscopy evaluation) were transferred to the buffer from the isolation kit.

The amplification reactions were performed in a volume of 50 µl for 45 cycles. Reaction mixtures consisted of 5 µl of dNTP mix (starting concentration 2 mM each dNTP), 1 µl of each primer (starting concentration 10 mM), 5 µl of 10x PfuUltra II reaction buffer, 1 µl of PfuUltra II fusion HS DNA polymerase (Agilent Inc.®), and 50 ng of genomic DNA. The thermal profile comprised of the following steps: initial denaturation at 94°C for 1 min, denaturation at 94°C for

Table 1. The analysed strains of Bacillus sp.

No.	Species	Strain
1.	*B. cereus*	ATCC 10876
2.	*B. cereus*	ATCC 13472
3.	*B. cereus*	ATCC 19637
4.	*B. cereus*	ATCC 23261
5.	*B. cereus*	F 17202
6.	*B. cereus*	F 17285
7.	*B. cereus*	T 7-101
8.	*B. cereus*	UM1
9.	*B. cereus*	UM2
10.	*B. cereus*	UW 85
11.	*B. thuringiensis*	ATCC 10792T
12.	*B. thuringiensis*	ATCC 33679
13.	*B. thuringiensis*	T07-128
14.	*B. thuringiensis*	H36
15.	*B. thuringiensis*	T 07-001
16.	*B. thuringiensis*	T 07-005
17.	*B. thuringiensis*	T 07-019
18.	*B. thuringiensis*	T 07-113
19.	*B. thuringiensis*	T 07-146
20.	*B. thuringiensis*	T 07-148
21.	*B. thuringiensis*	T 07-153
22.	*B. thuringiensis*	T 7-030
23.	*B. thuringiensis*	T 7-055
24.	*B. thuringiensis*	T 7-101
25.	*B. thuringiensis*	T 7-128
26.	*B. thuringiensis*	T07-151
27.	*B. thuringiensis*	T07-155
28.	*B. anthracis*	1153
29.	*B. anthracis*	1582
30.	*B. anthracis*	1583
31.	*B. anthracis*	1584
32.	*B. anthracis*	211
33.	*B. anthracis*	211Y
34.	*B. anthracis*	34F2
35.	*B. sp. Ba 813*+	#11 (9594(3)
36.	*B. sp. Ba 813*+	#12/S8553(2)
37.	*B. sp. Ba 813*+	#13 (PC1)
38.	*B. sp. Ba 813*+	#14
39.	*B. sp. Ba 813*+	#15 (11614-2)
40.	*B. sp. Ba 813*+	#16 (PJ572)
41.	*B. sp. Ba 813*+	#17 (094)
42.	*B. sp. Ba 813*+	#19 (T579-77)
43.	*B. sp. Ba 813*+	#21 (TII97-3)
44.	*B. sp. Ba 813*+	#22
45.	*B. sp. Ba 813*+	#22 (Br-13)
46.	*B. sp. Ba 813*+	#23 (III-4)
47.	*B. sp. Ba 813*+	#24 (II/B5)
48.	*B. sp. Ba 813*+	#25 (97-27)
49.	*B. sp. Ba 813*+	#28 (II)
50.	*B. sp. Ba 813*+	#28 (III)
51.	*B. sp. Ba 813*+	#29 (IV)
52.	*B. sp. Ba 813*+	#30 (1B)
53.	*B. sp. Ba 813*+	#31
54.	*B. sp. Ba 813*+	#25 (97-24)
55.	*B. sp. Ba 813*+	#6
56.	*B. sp. Ba 813*+	#S8553/2
57.	*B. sp. Ba 813*+	#7 (II/3)
58.	*B. sp. Ba 813*+	C9A
59.	*B. sp. Ba 813*+	Z 5 (1) Miazga
60.	*B. sp. Ba 813*+	ZL 2 B
61.	*B. subtilis*	ATCC 6633
62.	*B. subtilis*	UM1
63.	*B. subtilis*	UM2
64.	*B. megaterium*	UM1
65.	*B. megaterium*	UM2

Table 2. The primers used for amplification and sequencing chosen genes

glpF:	F - 5'GCGTTTGTGCTGGTGTAAGT3',	R - 5'CTGCAATCGGAAGGAAGAAG3';
gmk:	F - 5'ATTTAAGTGAGGAAGGGTAGG3',	R - 5'GCAATGTTCACCAACCACAA3';
ilvD:	F - 5'CGGGGCAAACATTAAGAGAA3',	R - 5'GGTTCTGGTCGTTTCCATTC3';
pta:	F - 5'GCAGAGCGTTTAGCAAAAGAA3',	R - 5'TGCAATGCGAGTTGCTTCTA3';
pur:	F - 5'CTGCTGCGAAAAATCACAAA3',	R - 5'CTCACGATTCGCTGCAATAA3',
pycA:	F - 5'GCGTTAGGTGGAAACGAAAG3',	R - 5'CGCGTCCAAGTTTATGGAAT3';
tpi:	F - 5'GCCCAGTAGCACTTAGCGAC3';	R - 5'CCGAAACCGTCAAGAATGAT3'.

20 sec, annealing for 30 sec at 60°C, and elongation for 20 sec at 72°C. After 45 cycles, the reaction was completed by the final elongation at 72°C for 5 min. PCR products were prepared for sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit and BigDye® XTerminator™ Purification Kit (Applied Biosystems™). DNA sequencing reactions were finalised using an ABI Prism® Genetic Analyzer 3100 sequencer (Applied Biosystems™).

Chromatograms and sequences were exported to BioNumerics Software (Applied Maths, BioMérieux Company) where they were edited, aligned, and compared with the *Bacillus cereus* group MLST profiles database [35] using the

recommended tools. Sequences of every fragment of the analysed gene were edited to a suitable length (*glpF* 381 bp, *gmk* 504 bp, *ilvD* 393 bp, *pta* 414 bp, *pur* 348 bp, *pycA* 363 bp, and *tpi* 435 bp). The allelic profile was arbitrarily assigned for all different sequences, and all identical sequences got the same value. Based on the characteristic profiles of the combination of seven loci, each strain was described by multilocus sequence type (MLST). The dendrograms, showing the genetic relationship between the *Bacillus cereus* group strains, are based on separate allelic profiles and were constructed using the unweighted pair group method with arithmetic mean (UPGMA).

Results

The length of the analysed fragments ranged between 348 bp (*pur*) and 504 bp (*gmk*). The average amount of GC bases in the analysed genes fragments ranged from 38.15% to 44.71%.

As an effect of the analysis obtained by the BioNumerics software, 22 different STs profiles were observed, which had been previously identified in the pubmlst.org database (Table 1). Only once 10 STs (ST1, ST10, ST26, ST34, ST53, ST105, ST206, ST245, ST665, ST972) were identified, 13 strains were recognised to possess STs profile 15 (12 strains of *B. thuringiensis* and one of *B. cereus*), nine strains of transitional environmental strains of *Bacillus sp. Ba 813+* possess STs profile 109. All of the analysed full pathogenic *B. anthracis* strains have the same STs profile (ST3). However, the vaccination strain *B. anthracis 34F2* had a different one (ST1). The difference between them resides only in the *glpF* gene sequence. Furthermore, some of the transitional environmental strains of *Bacillus sp. Ba 813+* and *B. anthracis* have the same allelic types of sequences for *gmk* and *pta*.

After UPGMA clustering, four major groups were revealed (Figure 1). Cluster I is the most interesting because it includes all analysed *B. anthracis* isolates, both fully pathogenic, and vaccination strains (ST 3 and ST 1) and 13 transitional environmental *B. sp. Ba813+* strains (nine with ST109, two with ST163, and two with ST113). All the environmental strains had the same *gmk* and *pta* (excluding ST113 and ST163) genes like the analysed *B. anthracis* strains. Cluster II and III contains a mixture of *B. cereus*, *B. thuringiensis*, and transitional environmental strains, gained from reference collections and isolated from the environment. Cluster III is very interesting because it includes the *B. thuringiensis* strain from the ATCC collection (ST10), two *B. cereus* strains isolated from humans (ST56), and two environmental strains isolated from tooth pulp of dead horses (ST144). In Cluster IV, the one strain of *B. cereus* from the ATCC collection and eight isolates from the environment of *B. sp. Ba813+* were grouped.

Discussion

For many years, standard microbiology was the only bacterial identification method and bacteriological diagnostic in medicine. Biological tests with laboratory animals are still recommended by the World Health Organization as a reference method in diagnosis of anthrax (Turnbull 2008). The identification of *B. anthracis*, based on phenotypic features of strains isolated from material derived from the anthrax regarding affirmed cases, never caused a problem. However, after the 1960s, doubtful cases of anthrax diagnosis were described. In those cases, isolated strains from wounds considered anthrax related,

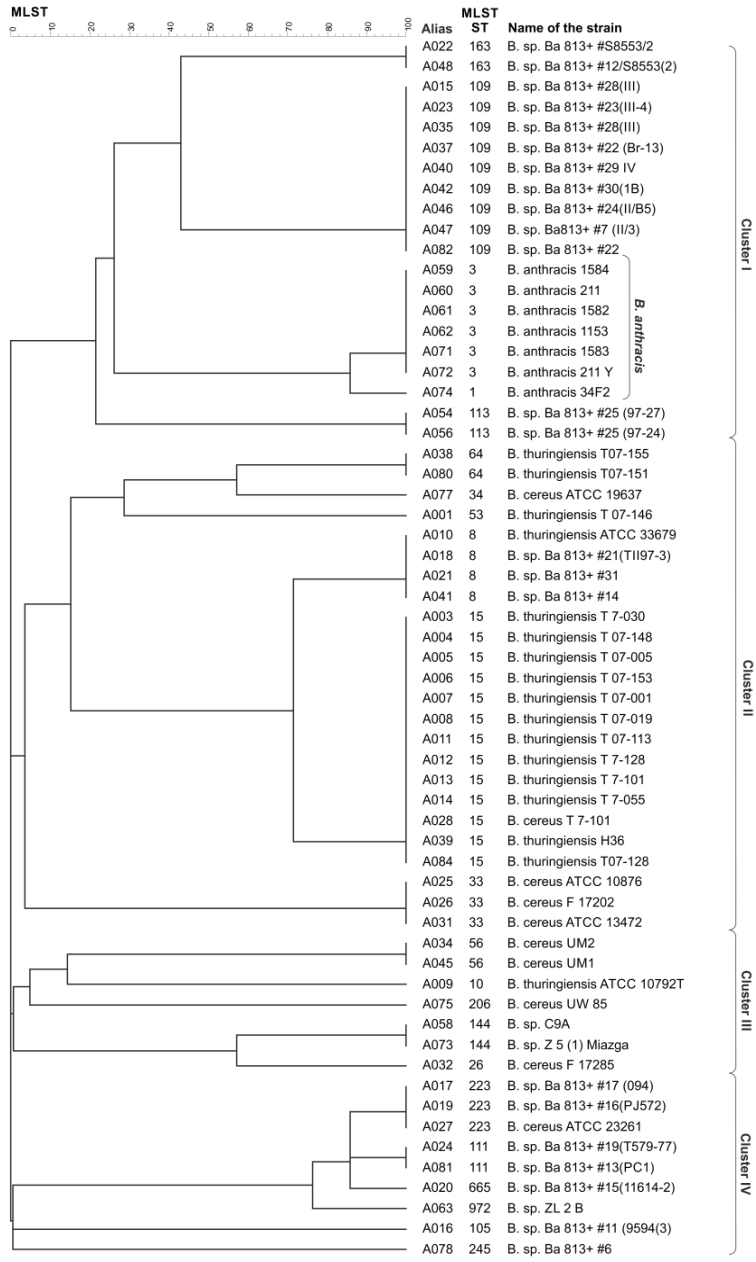

Figure 1. Phylogenetic differentiation of Bacillus cereus group strains clustered by UPGMA method

were identified as *B. cereus*. Those cases were interpreted as an environmental contamination, as *B. cereus* is present in the natural environment (Turnbull 1999). Owing to performed genetic studies, this hypothesis has been refuted.

The greater phenotypic identification problem is related to strains isolated from

the environment, which presents larger biochemical differentiation. Predisposing factors may include a long period of remaining in the spore form and a low protein content in the environment. The occurrence of the cell capsule, which is the characteristic structural element of *B. anthracis*, depended on the origin of the strains, was also observed. Strains without capsules were isolated from the environment more often than from living organisms (Turnbull 2008).

In relation to the advanced development of molecular biology techniques, phenotypic methods are increasingly being replaced by modern genetic tools. They allow for a rapid and sure analysis and reduce the risk of mistakes in the data interpretation. Furthermore, advanced bioinformatics programmes (e.g., BioNumerics®) are very useful in interpreting sequencing data from an experiment with a vast group of microorganisms.

In this paper, an MLST experiment based on seven conservative housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA* and *tpi*) was performed. Molecular typing and phylogenetic investigation concerned 60 *Bacillus cereus* group strains, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, and transitional environmental *Bacillus spp*. strains. This study aimed to identify the exact relationships among the various strains belonging to the common group of *Bacillus cereus*, including *B. anthracis*. Close relationships among these strains carry the large risk of their modification from the strains originally nonpathogenic form into a virulent form. In 2003, Radnegde et al. analysed the genetic homology between *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains isolated from several diverse places (environment, infected people and animals, and contaminated food) using amplified fragment length polymorphism (Radnedge *et al*. 2003). This study, employing the MLST technique, confirmed the large genetic homology of the strains comprehended by tests, which also characterised the various places of origin. Tests also showed very large genetic homology among *B. anthracis*, *B. cereus*, and *B. thuringiensis*. Very similar effects were obtained by Helgason's group in their studies on the different housekeeping genes (*adk*, *ccpA*, *ftsA*, *glpT*, *pyrE*, *recF*, and *sucC*) (Helgason *et al*. 2004).

Cluster I is very interesting from the epidemiological point due to its consistency in including all analysed *B. anthracis* strains and transitional strains isolated from the environment. The fact that all *B. anthracis* strains were grouped in one cluster has confirmed a very high level of genetic monomorphism. All of the full pathogenic strains showed the same sequence of analysed gene fragments and as a result, the same sequencing type. The vaccination strain *B. anthracis* 34F2 varied only in the sequence of the fragment of glycerol uptake facilitator protein gene. Unfolded differences caused the other ST profile classification.

Analysis of the individual alleles provided the information concerning full homology in the sequences of the *gmk* and *pta* genes among *B. anthracis*, and some transitional environment *Bacillus sp*. strains. This fact can support the contention that they possess a common ancestor (Patra *et al*. 1998; Ramisse *et al*. 1999). Full homology of the sequence of these genes was affirmed by Kim's team (Kim *et al*. 2005), which provided additional identifying sequences of these genes in some *B. cereus* and *B. thuringiensis* strains. However, similar to our results, Kim's team did not affirm the compatibility of the sequences in all analysed alleles among *B. anthracis* and other representatives of the *Bacillus*

cereus group. The examined group in the study included strains, which gave false-positive results in identifying *B. anthracis*. These results confirmed that MLST is a useful method to differentiate *Bacillus cereus* strains.

Multilocus sequence typing allows us to define the phylogenetic relationship between closely related strains based on the combination of the sequences of all seven alleles fragments and each of them separately. This characteristic is very important in epidemiological investigations on the potential military/bioterrorist use of *B. anthracis*. Using this method, the differentiation of *B. anthracis* from other *B. cereus* group strains after genetic modification, e.g., after cloning pXO1 and pXO2 plasmids, is possible. The MLST method is also very useful when the sequence of virulent genes (localised on plasmids) in material from people with anthrax symptoms can't be detected using typical molecular techniques (e.g., PCR). The false-negative results of PCR sometimes can be caused by the mutation changes in the target spaces during annealing (Turnbull *et al*. 1992).

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