ACTA ABP BIOCHIMICA POLONICA

Vol. 68, No 2/2021
301–307
https://doi.org/10.18388/abp.2020_5574

Regular paper

Isolation and identification of guaiacol-producing *Alicyclobacillus fastidiosus* strains from orchards in Poland

Marzena Połaska, Agnieszka Dekowska and Barbara Sokołowska✉

Department of Microbiology, Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology – State Research Institute, Warsaw, Poland

The genus *Alicyclobacillus* comprises a group of Gram-positive, endospore-forming, and thermo-acidophilic bacteria. Some of the *Alicyclobacillus* species are categorized as spoilage bacteria due to their ability to produce off-flavor compounds (e.g. guaiacol and halophenols) that adversely affect the taste and aroma of beverages. In our study, *Alicyclobacillus* species isolated from Polish orchard soils and fruits were subjected to 16S rDNA sequencing and *rpoB* gene RFLP analysis. The results showed that the isolated strains belong to the species *A. acidoterrestris* and *A. fastidiosus*. Additionally, a fragment of the *vdc* operon, which is responsible for guaiacol production, was amplified and subjected to RFLP analysis. The resulting RFLP patterns allowed classifying five strains as *A. acidoterrestris* type II, while the patterns of another three strains showed no similarity to the patterns of any reference strains used for comparison. The reference strain *A. fastidiosus* DSM 17978 did not give any PCR product in this reaction, nor showed guaiacol production. All the three isolated strains of *A. fastidiosus* were subjected to a series of tests, including determination of the biochemical profile using API 50CH tests and determination of the ability to produce guaiacol at 20°C, 25°C, and 45°C. All *A. fastidiosus* strains exhibited similar morphological and biochemical properties as the strain described in the literature. However, our strains, unlike the strain described previously, were able to produce guaiacol at all tested temperatures, and could therefore be included in the group of spoilage species. To date, this study is the first to report the isolation of *A. fastidiosus* from Poland and is the second such report in the world.

Keywords: *Alicyclobacillus fastidiosus*, *Alicyclobacillus acidoterrestris*, thermo-acidophilic bacteria, guaiacol; RFLP, *vdc* operon, *rpoB* gene

Received: 12 December, 2020; revised: 17 February, 2021; accepted: 18 February, 2021; available on-line: 10 May, 2021

✉e-mail: barbara.sokolowska@ibprs.pl
Accession numbers: KY088041, KY088042, KY088043, KY088044, KY088045, KY088046, KY088047, MW332524
Abbreviations: API, analytical profile index; ATCC, American Type Culture Collection; BLAST, Basic Local Alignment Search Tool; CIM, Culture Collection of Industrial Microorganisms; DSMZ, German Collection of Microorganisms and Cell Cultures GmbH; IFU, International Federation of Fruit Juice Producers; PCR, polymerase chain reaction; RFLP, Restriction fragment length polymorphism; WGS, Whole Genome Sequencing

INTRODUCTION

The genus *Alicyclobacillus* comprises Gram-positive, thermo-acidophilic, nonpathogenic, and spore-forming bacteria. *Alicyclobacillus* species possess unique fatty acids (cyclohexane or cycloheptane) in their cell membrane (Deinhard *et al.*, 1987a; Deinhard *et al.*, 1987b). These thermo-acidophilic bacteria were first isolated from different hot springs in Japan (Uchino & Doi 1967). *Alicyclobacillus* species are also found in soil which acts as the main reservoir of these bacteria (Goto *et al.*, 2008; Groenewald *et al.*, 2008; Wang *et al.*, 2010). In addition, the soil is an important source of fruit contamination by *Alicyclobacillus* spores, which are transmitted by wind or during the harvesting period. For several years, the fruit juice industry has been affected by the problem of deterioration of fruits by *Alicyclobacillus* species (Sokołowska *et al.*, 2020).

The metabolic products of these bacteria, which include halophenols (2,6-dibromophenol and 2,6-dichlorophenol) and guaiacol, are responsible for the formation of unusual medical, phenolic off-flavor and for the adverse changes noted in the taste and aroma of the fruit juice products (Pettipher *et al.*, 1997; Orr *et al.*, 2000). Furthermore, *Alicyclobacillus* spores are heat-resistant and can therefore survive pasteurization. The thermal and acid resistance of *Alicyclobacillus* is the result of the unique composition of fatty acids in the cell membrane of these organisms. These fatty acids contribute to increased packing of lipids in the membrane core and the stabilization of the membrane structure of the bacteria, which in turn allow them to tolerate high temperatures and highly acidic conditions (Kannenberg *et al.*, 1984; Chang *et al.*, 2004; Karavaiko *et al.*, 2005; Ciuffreda *et al.*, 2015).

Thus far, 26 species of *Alicyclobacillus* have been identified. Eight of these – *A. acidiphilus*, *A. acidoterrestris*, *A. daucy*, *A. herbarius*, *A. cycloheptanicus*, *A. pomorum*, *A. contaminans*, and some strains of *A. hesperidum* – were reported to have the ability to produce guaiacol (Deinhard *et al.*, 1987a; Deinhard *et al.*, 1987b; Wisotzkey *et al.*, 1992; Albuquerque *et al.*, 2000; Goto *et al.*, 2002; Matsubara *et al.*, 2002; Goto *et al.*, 2003; Goto *et al.*, 2007; Jiang *et al.*, 2008; Guo *et al.*, 2009; Nakanano e*t al.*, 2015). Among all the *Alicyclobacillus* species, *A. acidoterrestris* was found to be the most prevalent cause of pasteurized beverages spoilage (Durak *et al.*, 2010; Sokołowska *et al.*, 2016).

16S rDNA gene is an essential tool in taxonomy, especially considering the large and still-growing database of 16S rDNA sequences, derived from diverse microorganisms. However, it does not always allow distinguishing closely related species; in addition, most microorganisms have several non-identical copies of this gene in their genome, which makes PCR-RFLP analysis difficult. Thus, we decided to include the *rpoB* gene in our taxonomy studies. *RpoB* encodes the β-subunit of bacterial RNA polymerase. This is a housekeeping gene that occurs in the genome in one copy, is widely used in taxonomy,

and has already been used to identify and differentiate *Alicyclobacillus* strains (Dekowska *et al.*, 2018).

To date, there have been no reports on the guaiacol-producing ability of *A. fastidiosus*. In *A. acidoterrestris*, guaiacol production is determined by the *vdc* operon consisting of three genes: *vdcB*, *vdcC*, and *vdcD* (Matsubara *et al.*, 2003). The presence of this operon was confirmed in *A. acidoterrestris*, *A. acidophilus*, and *A. herbarius*. The operon sequence has been proven to be a useful marker in differentiating *Alicyclobacillus* strains that are capable of producing guaiacol (Dekowska *et al.*, 2018).

In this study, we aimed to confirm the presence of *Alicyclobacillus* bacteria in Polish fruit orchards and characterize the three atypical *A. fastidiosus* strains that were isolated for the first time from the orchards in the Mazovia region of Poland.

MATERIALS AND METHODS

Bacterial strains

The bacteria tested in the study were isolated from the samples taken from the top layer of soil and from the apple and pear fruits that were hand-collected from various orchards in the Mazovia region of Poland. All isolated strains were deposited in the Culture Collection of Industrial Microorganisms (CIM) at the Institute of Agricultural and Food Biotechnology (collection number in World Federation for Culture Collections WDCM—IAFB 212).

The following reference strains were used in the study: *A. acidoterrestris* ATCC 49025, *A. acidoterrestris* DSM 2498, *A. acidiphilus* DSM 14558, *A. herbarius* DSM 13609, *A. hesperidum* DSM 12489, *A. acidocaldarius* DSM 446, and *A. fastidiosus* DSM 17978. These strains were obtained from the American Type Culture Collection and Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH.

Alicyclobacillus species isolation and differentiation from other thermo-acidophilic bacterial species

The taint-producing *Alicyclobacillus* species was isolated using the International Federation of Fruit Juice Producers (IFU) Method No. 12 (IFU Method No. 12, 2004/2007). Twenty soil samples and 20 fruit samples were taken manually from the five orchards in the Mazovia region for the study. The fruit or soil samples (10 g) were mixed with 90 ml of BAT broth (Merck, Darmstadt, Germany) and were heat-treated at 80°C for 10 min. This high-temperature treatment eliminated other vegetative microbial contaminants and induced the germination of spores. Following the heat treatment, the samples were incubated for 5 days at 45°C. Then, a loop of suspension was transferred onto BAT agar (pH 4.0±0.1) (Merck), and the plates were incubated for 3 days at 45°C. The cell morphology of all the isolated strains was studied by Gram staining and observation under an Olympus CX40 microscope (oil immersion, ×1500 magnification). Then, single colonies were spread-plated onto Plate Count Agar (PCA, pH 7.0±0.2) (Merck) to exclude the presence of *Bacillus*. In addition, the colonies assumed to be belonging to the genus *Alicyclobacillus* were spread-plated onto BAT agar and incubated at 65°C for 3 days. The presumptive strains were confirmed as taint-producing *Alicyclobacillus* if they could not grow on the BAT agar medium at a temperature of 65°C (Goto, 2007; IFU Method No. 12, 2004/2007).

According to the IFU Method No. 12, the isolated strains that potentially belonged to the *Alicyclobacillus* family were tested for the ability to produce guaiacol at 45°C (Niwa & Kuriyama, 2013). A 24-h plate culture of *Alicyclobacillus* species capable of causing spoilage was added to 1 ml BAT broth with vanillic acid (100 mg/l) and incubated for 3–4 h at 45°C. After incubation, guaiacol was detected through its oxidation by peroxidase (50 µl of 0.01% peroxidase in 500 µl of phosphate buffer (pH 6.0±0.1)), in the presence of hydrogen peroxide (50 µl of 0.5% H_2O_2). The reaction resulted in the formation of brown-colored 3,3'-dimethoxy-4,4'-biphenoquinolone.

An additional confirmatory test was performed for differentiating *A. acidoterrestris* from other *Alicyclobacillus* species, according to the method described by Baumgart's team (Baumgart *et al.*, 2003). The presumptive *A. acidoterrestris* colonies isolated from the BAT agar plates were streaked onto erythritol agar (1.0 g yeast extract, 10.0 g erythritol, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.015 g bromophenol blue, 18.0 g agar, 1000 ml deionized water, and 1 ml solution of trace minerals consisting of 0.066 g $CaCl_2 \cdot 2H_2O$, 0.018 g $ZnSO_4 \cdot 7H_2O$, 0.016 g $CuSO_4$, 0.015 g $MnSO_4 \cdot H_2O$, 0.018 g $CoCl_2 \cdot 6H_2O$, 0.01 g H_3BO_3, 0.03 g Na_2MoO_4, and 100 ml deionized water) and were incubated at 45°C and 65°C for 3 days.

The growth of *A. acidoterrestris* was confirmed if the bacterial colony showed a green color, accompanied by a change in the color of the medium from blue to yellow (green color was observed during incubation at 45°C). Furthermore, a lack of growth was observed at 65°C for this species. Other *Alicyclobacillus* species (e.g. *A. acidocaldarius*) showed blue colonies on erythritol agar due to the lack of acid formation from erythritol at both 45°C and 65°C.

Identification of bacterial isolates *via* 16S rDNA gene sequence analysis

The bacterial isolates that were assumed to be belonging to the *Alicyclobacillus* genus were subjected to 16S rDNA gene sequence analysis. DNA was isolated from the bacterial cells using Genomic DNA Purification Kit (EUR_X, Gdańsk, Poland) according to the manufacturer's instruction. The fragment of the 16S rDNA gene (1500 bp) was amplified and sequenced using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG) and Alicyc-16srev (5'-ACGGCTACCTTGTTACGACT). The composition of the PCR mixture was as follows: 5 ng of template DNA, 50 pM of each of the primers, 25 µl of DreamTaq™, and Green PCR Master Mix (Thermo Scientific, Wallham, USA) with water added to obtain a final volume of 50 µl. PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 35 s, elongation at 72°C for 1 min and 40 s, and the final elongation at 72°C for 2 min. The obtained sequences were assembled using Serial Cloner (Serial Basic) and compared to the GenBank database using BLAST. DNA sequences obtained during this study were deposited in GenBank, and their accession numbers are given in Table 1.

RFLP analysis of *rpoB* gene fragment and vdc operon fragment

The RFLP analysis was carried out as described by Dekowska's group (Dekowska *et al.*, 2018). Briefly, the *rpoB* gene fragment (1735 bp) was amplified using Gru3–Gru6 primers, the sequences of which were as follows:

Table 1. Alicyclobacillus strains isolated from the orchards in the Mazovia region of Poland.

Strains	Source of isolation	Species	CIM number	GenBank accession number
Strain 1	Soil from apple orchard	*Alicyclobacillus acidoterrestris*	KKP 3194	KY088041
Strain 2	Soil from apple orchard	*Alicyclobacillus acidoterrestris*	KKP 3195	KY088042
Strain 3	Apples	*Alicyclobacillus acidoterrestris*	KKP 3347	KY088043
Strain 4	Soil from apple orchard	*Alicyclobacillus acidoterrestris*	KKP 3348	KY088047
Strain 5	Apples	*Alicyclobacillus acidoterrestris*	KKP 3349	MW332524
Strain f1	Soil from pear orchard	*Alicyclobacillus fastidiosus*	KKP 3000	KY088044
Strain f2	Pears	*Alicyclobacillus fastidiosus*	KKP 3001	KY088045
Strain f3	Soil from pear orchard	*Alicyclobacillus fastidiosus*	KKP 3002	KY088046

Gru3: 5'-CGYGACGTDCACTAYTCBCACTA,
Gru4: 5'-GCCCANACYTCCATCTCRCCRAA,
Gru5: 5'-CGCGACGTACACTATTCGCACTA, and
Gru6: 5'-GCCCAAACCTCCATCTCACCAAA.

The primers Gru5 and Gru6 were nondegenerated versions of the primers Gru3 and Gru4, respectively. The nondegenerated primers were used for amplifying the *A. acidoterrestris* samples, while the degenerated ones were used for amplifying other samples. All the primers were designed based on the comparison of *rpoB* sequences isolated from several *Alicyclobacillus* species, *Bacillus subtilis*, and *Geobacillus stearothermophilus* published in GenBank.

The composition of the PCR mixture was as follows: 30 ng of template DNA, 40 pM of each of the primers, 25 µl of DreamTaq™, and Green PCR Master Mix (Thermo Scientific) with water added to obtain the final volume of 50 µl. PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C (for the Gru5 and Gru6 primers) or at 59°C (for the Gru3 and Gru4 primers) for 35 s, elongation at 72°C for 1 min 45 s, and the final elongation at 72°C for 2 min.

The *vdc* operon fragment (1810 bp) was amplified and sequenced using primers Vdc1fr (5'-AACGACGCAGGTGTGGAAAC) and Bur6 (5'-GTSGCRTCGAGAATCATCTTGTG). These primers were designed based on the raw genome sequences of *A. acidoterrestris* ATCC 49025 (Shemesh *et al.*, 2013) (GenBank accession number AURB01000113.1) and *A. herbarius* DSM 13609 (GenBank accession number AUMH01000032.1) and the sequence published by Matsubara (2003) (JP 2003000259-A12, 07-Jan-2003; GenBank accession number BD187778.1).

The PCR mixture contained 25 ng of template DNA, 15 pM of each of the primers, 25 µl of DreamTaq™, and Green PCR Master Mix (Thermo Scientific) with water added to obtain the final volume of 50 µl. PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 35 s, elongation at 72°C for 1 min 50 s, and the final elongation at 72°C for 2 min.

The PCR-RFLP assay was performed according to the following procedure. First, 8–12 µl of the PCR products was digested with endonuclease HphI (Thermo Scientific) in a volume of 20 µl. Following incubation at 37°C for 1–2 h and inactivation of the enzyme (10 min, 80°C), the samples were analyzed on a 2.5–3.0% agarose gel.

Growth profile

For determining the growth temperature range of the isolates, 20 µl of 18-h bacterial culture was added to 2 ml of BAT broth and incubated at different temperatures: 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, and 65°C. The optical density of the bacterial suspension was measured after 24 h, 48 h, and 72 h of incubation (McFarland Densitometer DEN-1B, Biosan, Riga, Latvia).

To determine the growth pH range of *A. fastidiosus*, the pH of the BAT broth was adjusted by adding 1 M sulfuric acid to achieve the following values: 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5. The medium was inoculated with 18-h bacterial culture and incubated at an optimal temperature of 40°C for 24 h, 48 h, and 72 h. Following each incubation period, the optical density of the bacterial suspension was measured.

Assessment of the guaiacol-producing ability of *A. fastidiosus* isolates at three different temperatures

The ability to produce guaiacol was tested using the peroxidase method (Niwa & Kuriyama, 2013). The f1, f2, and f3 strains of *A. fastidiosus* were cultivated on BAT agar plates for 72 h at three different temperatures: 20°C, 25°C, and 45°C. Briefly, the BAT broth medium supplemented with vanillic acid (100 mg/l) was inoculated with a loop of harvested cells of *A. fastidiosus*. The medium was incubated for 7 h at 20°C, 25°C, and 45°C. If the guaiacol was present, the color change of the medium containing vanillic acid and the bacterial strain was observed after adding an appropriate amount of phosphate buffer, peroxidase, and hydrogen peroxide.

Biochemical profile

The ability of the *A. fastidiosus* isolates to produce acid from various carbohydrates and their derivates (heterosides, polyalcohols, uronic acids) was tested at the optimum growth temperature – 40°C. The API 50 CH (bioMerieux. Marcy l'Etoil, France) test kits supplemented with medium (0.5 g yeast extract, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 1

ml 0.75% bromophenol blue solution as an indicator of acidification, 1000 ml deionized water, and 1 ml solution of trace minerals consisting of 0.066 g $CaCl_2 \cdot 2H_2O$, 0.018 g $ZnSO_4 \cdot 7H_2O$, 0.016 g $CuSO_4$, 0.015 g $MnSO_4 \cdot H_2O$, 0.018 g $CoCl_2 \cdot 6H_2O$, 0.01 g H_3BO_3, 0.03 g Na_2MoO_4, and 100 ml deionized water) were used for this analysis (Deinhard *et al.*, 1987a; Deinhard *et al.*, 1987b).

RESULTS AND DISCUSSION

Identification of *Alicyclobacillus* bacteria based on culture-dependent methods

The samples of the top layer of soil as well as the apple and pear fruits collected from the orchards in the Mazovia region, one of the biggest fruit-producing regions of Poland, were screened for the presence of taint-producing *Alicyclobacillus*. Eight isolated bacterial strains were characterized as Gram-positive, spore-forming bacilli and were found to be able to grow on BAT agar at 45°C, whereas at the same temperature they could not grow on PCA. Based on these observations, they were classified as the members of *Alicyclobacillus* genus. Furthermore, these isolates could not grow at 65°C, which is a characteristic of presumptive taint-producing *Alicyclobacillus* species (IFU Method No. 12, 2004/2007). All the eight isolated strains exhibited the ability to produce guaiacol at 45°C.

According to Baumgart and others (Baumgart *et al.*, 2003), the species *A. acidoterrestris* has the ability to utilize erythritol as a carbon source for acid formation, which is indicated by the growth of yellow-green colonies on erythritol agar with bromophenol blue. In the present study, five acidophilic strains – strain 1, strain 2, strain 3, strain 4, and strain 5 – isolated from apples and soil samples collected from apple orchard were able to grow on erythritol agar and formed yellow-green colonies, whereas three acidophilic strains – strain f1, strain f2, and strain f3 – isolated from pears and soil samples collected from pear orchard formed blue colonies on this medium. Taking these results into account, we can assume that strains 1–5 belong to the species *A. acidoterrestris*, whereas strains f1, f2, and f3 do not belong to this species although they showed guaiacol-producing ability.

Sequence analysis and PCR-RFLP

The 16S rDNA sequence analysis indicated that strains f1, f2, and f3 probably belong to the species *A. fastidiosus*. The 16S rDNA sequences of these isolates showed the highest identity (>99%) to those of the strain *A. fastidiosus* DSM 17978 (GenBank accession number AB264021) and two other *A. fastidiosus* strains (NR_041471 and NR_114208). On the other hand, strains 1–5 were identified as *A. acidoterrestris* (>99% identity to *A. acidoterrestris*).

RFLP analysis was performed for the *rpoB* gene fragment of all the analyzed *Alicyclobacillus* strains, and their patterns were compared to those of the following reference strains: *A. acidoterrestris* DSM 2498, *A. acidoterrestris* ATCC 49025, *A. acidiphilus* DSM 14558, *A. herbarius* DSM 13609, *A. hesperidum* DSM 12489, *A. acidocaldarius* DSM 446 (Fig. 1a), and *A. fastidiosus* DSM 17978 (Fig. 1b). The RFLP patterns of strains f1 and f3 were identical to that of *A. fastidiosus* DSM 17978, while the pattern of strain f2 was not identical but similar to that of the mentioned strain. Based on the comparison of the RFLP patterns of all *A. acidoterrestris* strains and isolates f1, f2, and f3, we excluded the possibility that strains f1, f2, and f3 belong to *A. acidoterrestris* species, whereas the rest of the analyzed strains were found to show identical patterns as *A. acidoterrestris* type II ATCC 49025 (Fig. 1a and 1b). So far, two types (type I and type II) of *A. acidoterrestris* strains have been reported (Durak *et al.*, 2010, Osopale *et al.*, 2016, Dekowska *et al.*, 2018). In this study, we observed only type II *A. acidoterrestris*. Dekowska's team (Dekowska *et al.*, 2018) analyzed 60 *A. acidoterrestris* species isolated from Polish fruits and fruit products and showed that the distribution of these species was quite even with 27 belonging to type I and 33 to type II.

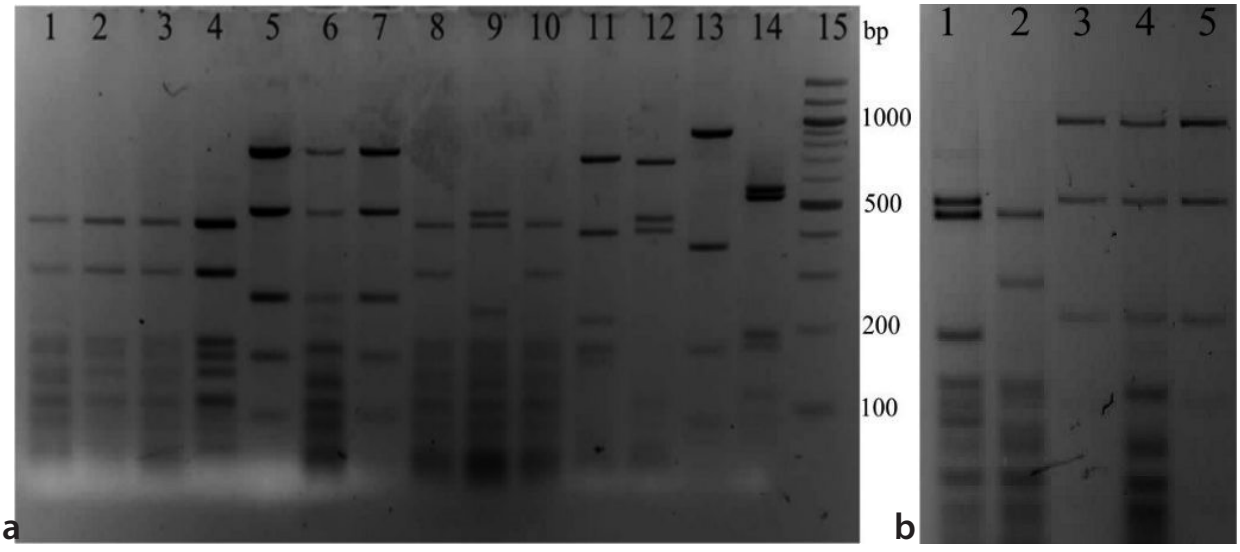

Figure 1a. RFLP patterns of the *rpoB* gene isolated from the analyzed strains.
Lane 1: *A. acidoterrestris* strain 1, lane 2: *A. acidoterrestris* strain 2, lane 3: *A. acidoterrestris* strain 3, lane 4: *A . acidoterrestris* strain 4, lane 5: *A. fastidiosus* strain f1, lane 6: *A. fastidiosus* strain f2, lane 7: *A. fastidiosus* strain f3, lane 8: *A. acidoterrestris* strain 5, lane 9: *A. acidoterrestris* type I DSM 2498, lane 10: *A. acidoterrestris* type II ATCC 49025, lane 11: *A. acidiphilus* DSM 14558, lane 12: *A. hesperidum* DSM 12489, lane 13: *A. herbarius* DSM 13609, lane 14: *A. acidocaldarius* DSM 446, lane15: DNA molecular weight marker λ/AvaII.

Figure 1b. RFLP patterns of the *rpoB* gene isolated from the analyzed strains.
Lane 1: *A. acidoterrestris* type I DSM 2498, lane 2: *A. acidoterrestris* type II ATCC 49025, lane 3: *A. fastidiosus* DSM 17978, lane 4: *A. fastidiosus* strain f2, and lane 5: *A. fastidiosus* strain f3.

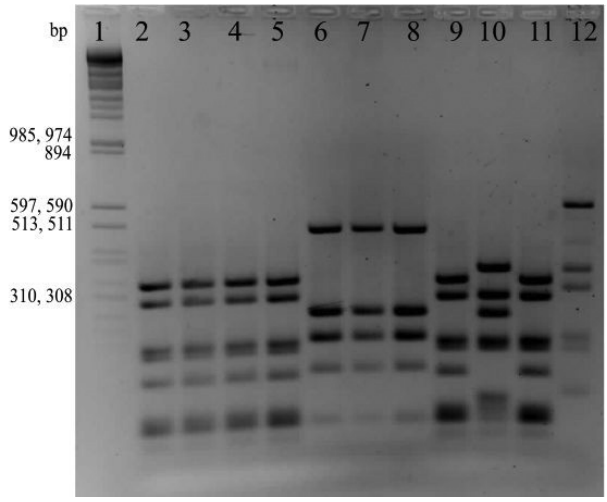

Figure 2. RFLP analysis of the *vdc* operon fragment isolated from the analyzed strains.
Lane 1: DNA molecular weight marker λ/AvaII, lane 2: *A. acidoterrestris* strain 1, lane 3: *A. acidoterrestris* strain 2, lane 4: *A. acidoterrestris* strain 3, lane 5: *A. acidoterrestris* strain 4, lane 6: *A. fastidiosus* strain f1, lane 7: *A. fastidiosus* strain f2, lane 8: *A. fastidiosus* strain f3, lane 9: *A. acidoterrestris* strain 5, lane 10: *A. acidoterrestris* type I DSM 2498, lane 11: *A. acidoterrestris* type II ATCC 49025, and lane 12: *A. acidiphilus* DSM 14558.

The performed PCR assay targeting the *vdc* operon fragment resulted in a product of about 1800 bp for all the tested strains, regardless of whether they were classified as *A. acidoterrestris* or *A. fastidiosus*. A band of identical size was obtained for both reference strains of *A. acidoterrestris*, but not for the reference strain *A. fastidiosus* DSM 17987. The strain *A. fastidiosus* DSM 17987 probably carries a defective version of the operon as Wang and coworkers (Wang *et al.*, 2021) obtained a PCR product for this strain using primers designed for *vdcC* gene, whereas they did not observe guaiacol production. This suggests that whole-genome sequencing or further study on this particular region is required to shed more light on this issue.

The RFLP analysis of the *vdc* operon fragment showed that the patterns obtained for the environmental samples classified as *A. acidoterrestris* were identical to those of *A. acidoterrestris* type II ATCC 49025, while the patterns obtained for the environmental samples classified as *A. fastidiosus* were identical to each other and showed no similarity to the patterns of any reference strain. The strain *A. fastidiosus* DSM 17978 produced no band in the PCR assay and, therefore, it was excluded from the RFLP analysis (Fig. 2).

Sequences of the analyzed *vdc* fragment (GenBank accession number MH938036) were found to be identical to those of all three strains of *A. fastidiosus* isolated in this study, and they showed 80%, 76%, and 71% identity to the sequences of *A. acidoterrestris* ATCC 49025, *A. acidiphilus* NBRC 100859, and *A. herbarius* DSM 13609, respectively (BLAST search against nucleotide and WGS database). The BLAST search revealed no sequence with more than 80% identity to that of the analyzed samples.

Morphological and physiological characterization of *A. fastidiosus*

Strains f1, f2, and f3 stained Gram-positive and produced flat, creamy-white colonies. When viewed under the microscope, they appeared as rods of a length of 4.0–4.5 μm and a width of 0.8–1.0 μm. The growth temperature range of all these strains was found to be 20–55°C with optimal growth noted at 30–45°C. All three strains of *A. fastidiosus* grew in the pH range of 3.0–5.0 with an optimum of 3.0–3.5. These results differ from the optimum values reported by Goto's group (Goto *et al.*, 2007) because in their research the optimum pH was 4.0–4.5 and the optimum temperature was 40–45°C.

Furthermore, the examined strains f1, f2, and f3 exhibited a common characteristic: they were able to produce guaiacol from vanillic acid at a temperature of 20°C, 25°C, and 45°C and at an optimal pH of 3.0–3.5. Another important biochemical feature of these strains was the lack of acid formation from erythritol. This was evidenced by the absence of a color change of the medium containing erythritol and bromophenol blue. However, all the isolated strains of *A. acidoterrestris* were able to use erythritol as a carbon source for the formation of acids. The phenotypic characteristics of the isolated *A. fastidiosus* strains and their comparison with the characteristics reported by Goto and coworkers (Goto *et al.*, 2007) are shown in Table 2.

The classification of strains f1, f2, and f3 as *A. fastidiosus* was strongly supported by DNA sequencing analy-

Table 2. Phenotypic characteristic of *A. fastidiosus* strains f1, f2, f3 and *A. fastidiosus* described by Goto and others = *A. fastidiosus* DSM 17978 (Goto *et al.*, 2007).

Characteristic	*A. fastidiosus* strains			*A. fastidiosus* described by Goto and others (Goto *et al.*, 2007) = *A. fastidiosus* DSM 17978
	f1	f2	f3	
Growth temperature (°C) range optimum	20-55°C 30-45°C	20-55°C 30-45°C	20-55°C 30-45°C	20-55°C 40-45°C
Growth pH range optimum	3.0-5.0 3.0-3.5	3.0-5.0 3.0-3.5	3.0-5.0 3.0-3.5	2.5-5.0 4.0-4.5
Guaiacol production	+	+	+	-
Acid production from:				
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	+
L-Arabinose	+	+	+	+
D-Ribose	+	+	+	n
D-Xylose	+	+	+	+

L-Xylose	-	-	-	-
D-Adonitol	-	-	-	n
Methyl-β-D-xylopyranoside	-	-	-	+
D-Galactose	+	+	+	+
D-Glucose	+	+	+	n
D-Fructose	+	+	+	n
D-Mannose	-	-	+	n
L-Sorbose	-	-	-	-
L-Rhamnose	-	w	+	+
Dulcitol	-	-	-	n
Inositol	-	-	-	+
D-Mannitol	+	+	+	+
D-Sorbitol	-	-	-	-
Methyl-α-D-mannopyranoside	-	-	-	-
Methyl-α-D-glucopyranoside	-	-	-	-
N-Acetylglucosamine	-	-	-	n
Amygdalin	-	-	-	-
Arbutin	-	-	-	-
Esculin ferric citrate	+	+	+	n
Salicin	w	w	-	-
D-Cellobiose	+	+	+	-
D-Maltose	w	+	+	-
D-Lactose (bovine origin)	-	-	-	-
D-Melibiose	-	-	-	+
D-Saccharose (sucrose)	+	+	+	-
D-Trehalose	+	+	+	+
Inulin	-	-	-	-
D-Melezitose	-	-	-	-
D-Raffinose	-	-	-	+
Amidon (starch)	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	-	-	-	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	+
D-Tagatose	-	-	-	+
D-Fucose	-	-	-	+
L-Fucose	-	-	-	+
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	n
Potassium gluconate	-	-	-	n
Potassium 2-ketogluconate	-	-	-	n
Potassium 5-ketogluconate	+	+	+	-

(+) positive reaction, (-) negative reaction, (w) weekly positive reaction, and (n) no information.

sis, RFLP assay, and morphological characterization. The geographical and ecological distribution is considered an important source of information on spoilage species for the beverage industry. As far as we know, this study is the first to report the isolation of *A. fastidiosus* species from Polish pear orchard, and the second to report the isolation of these bacteria in the world. Strains f1, f2, and f3 were mostly characterized by similar morphological and biochemical properties as *A. fastidiosus* species described by Goto and others (Goto *et al.*, 2007). According to

these authors, *A. fastidiosus* species does not exhibit the ability to produce guaiacol. These results differ from those observed in the guaiacol production test carried out in the present study. The test showed that all the environmental strains of *A. fastidiosus* and *A. acidoterrestris*, as well as the reference strains of *A. acidoterrestris*, were able to produce guaiacol from vanillic acid at 45°C. Unlike the other spoilage bacteria belonging to the *Alicyclobacillus* genus, *A. fastidiosus* was proven to produce guaiacol after 7 h of incubation at a temperature of 20°C, which may pose a serious threat to the fruit juice industry. Thus far, only *A. acidoterrestris* species has been identified as capable to produce guaiacol at 21–25°C, which is an unfavorable temperature for the growth of most thermophilic species of *Alicyclobacillus* (Pettipher *et al.*, 1997, Orr *et al.*, 2000). According to Orr and coworkers (Orr *et al.*, 2000), guaiacol production by *A. acidoterrestris* can be noticed after 8 days of incubation in juice at 21°C. By contrast, Sokołowska's group (Sokołowska *et al.*, 2013) reported that *A. acidoterrestris* strains were able to produce guaiacol only after 34 days of incubation in juice at a temperature of 25°C. The species *A. fastidiosus* is closely related to *A. acidoterrestris*, which is also a guaiacol-producing bacterium, most commonly isolated from fruit raw materials and fruit products. In conclusion, we suggest that *A. fastidiosus* should be added to the group of spoilage species.

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

Albuquerque L, Rainey FA, Chung AP, Sunna A, Nobre MF, Grote R, Antranikian G, da Costa MS (2000) *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores. *Int. J. Syst. Evol. Microbiol.* **50**: 451–457. https://doi.org/10.1099/00207713-50-2-451

Baumgart J (2003) Media for the detection and enumeration of *Alicyclobacillus acidoterrestris* and *Alicylobacillus acidocaldarius* in foods. In *Handbook of culture media for food microbiology. Progress in industrial microbiology*. Corry JEL, Curtis GDW, Baird RM eds, pp 161–166. Elsevier

Chang S, Kang H (2004) *Alicyclobacillus* spp. in the fruit juice industry: history, characteristics, and current isolation/detection procedures. *Crit. Rev. Microbiol.* **30**: 55–74. https://doi.org/10.1080/10408410490435089

Ciuffreda E, Bevilacqua A, Sinigaglia M, Corbo R (2015) *Alicyclobacillus* spp.: New Insights on Ecology and Preserving Food Quality through New. Approaches. *Microorganisms* **3**: 625–640. https://doi.org/10.3390/microorganisms3040625

Deinhard G, Blanz P, Poralla K, Altan E (1987a) *Bacillus acidoterrestris sp.* nov., a new thermotolerant acidophile isolated from different soils. *Syst. Appl. Microbiol.* **10**: 47–53

Deinhard G, Saar J, Krischke W, Poralla K (1987b) *Bacillus cycloheptanicus sp. nov.,* a new thermoacidophile containing ω-cycloheptane fatty acids. *Syst. Appl. Microbiol.* **10**: 68–73

Dekowska A, Sokołowska B, Niezgoda J (2018) Genetic heterogeneity of *Alicyclobacillus* strains revealed by RFLP analysis of *vdc* region and *rpoB* gene. *BioMed Res. Int.* **5**: 1–12. https://doi.org/10.1155/2018/9608756

Durak MZ, Churey JJ, Danyluk MD, Worobo RW (2010) Identification and haplotype distribution of *Alicyclobacillus* spp. from different juices and beverages, *Int. J. Food Microbiol.* **142**: 286–291. https://doi.org/10.1016/j.ijfoodmicro.2010.07.003

Goto K (2007) Parameters for detection of *Alicyclobacillus* and test methods. In *Alicyclobacillus Thermophilic Acidophilic Bacilli*. Yokota A, Fujii T, Goto K eds, pp 49–77. Springer

Goto K, Matsubara H, Mochida K, Matsumura T, Hara Y, Niwa M, Yamasato K (2002) *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω-cycloheptane fatty acids, isolated from herbal tea. *Int. J. Syst. Evol. Microbiol.* **52**: 109–113. https://doi.org/10.1099/00207713-52-1-109

Goto K, Mochida K, Asahara M, Suzuki M, Kasai H, Yokota A (2003) *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess v-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. *Int. J. Syst. Evol. Microbiol.* **53**: 1537–1544. https://doi.org/10.1099/ijs.0.02546-0

Goto K, Mochida K, Kato Y, Asahara M, Fujita R, An SY, Kasai H, Yokota A (2007) Proposal of six species of moderately thermophilic, acidophilic, endospore-forming bacteria: *Alicyclobacillus contaminans* sp. nov., *Alicyclobacillus fastidiosus* sp. nov., *Alicyclobacillus kakegawensis* sp. nov., *Alicyclobacillus macrosporangiidus* sp. nov., *Alicyclobacillus sacchari* sp. nov. and *Alicyclobacillus shizuokensis sp.* nov. *Int. J. Syst. Evol. Microbiol.* **57**: 1276–1285. https://doi.org/10.1099/ijs.0.64692-0

Goto K, Nishibori A, Wasada Y, Furuhata K, Fukuyama M, Hara M (2008) Identification of thermo-acidophilic bacteria isolated from the soil of several Japanese fruit orchards. *Lett. Appl. Microbiol.* **46**: 289–294. https://doi.org/10.1111/j.1472-765X.2007.02307.x

Groenewald H, Gouws A, Witthuhn C (2008) Isolation and identification of species of *Alicyclobacillus* from orchard soil in the Western Cape, South Africa. *Extremophiles* **12**: 159–163. https://doi.org/10.1007/s00792-007-0112-z

IFU Method No 12 (2004/2007) International Federation of Fruit Juice Producers (IFU). *Method on the Detection of Alicyclobacillus in Fruit Juices*. IFU Paris

Kannenberg E, Blume A, Poralla AK (1984) Properties of ω-cyclohexane fatty acids in membranes**.** *FEBS* **172**: 331–334

Karavaiko GI, Bogdanova TI, Tourova TP, Kondrat'eva TF, Tsaplina IA, Egorova MA, Krasil'nikova EN, Zakharchuk LM (2005) Reclassification of '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' strain K1 as *Alicyclobacillus tolerans* sp. nov. and *Sulfobacillus disulfidooxidans* Dufresne et. al 1996 as *Alicyclobacillus disulfidooxidans comb.* nov., and emended description of the genus *Alicyclobacillus*. *Int. J. Sys. Evol. Microbiol.* **55**: 941–947. https://doi.org/10.1099/ijs.0.63300-0

Matsubara K (2003) A method for detecting and/or identifying guaiacol producing microorganism. *Patent no. JP2003000259A*

Matsubara H, Goto K, Matsumura T, Mochida K, Iwaki M, Niwa M, Yamasoto K (2002) *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, omega-alicyclic fatty acid-containing bacterium isolated from acidic beverages. *Int. J. Syst. Evol. Microbiol.* **50**: 451–457. https://doi.org/10.1099/00207713-52-5-1681

Nakano Ch, Takahashi N, Tanaka N, Okada S (2015) *Alicyclobacillus dauci* sp. nov., a slightly thermophilic, acidophilic bacterium isolated from a spoiled mixed vegetable and fruit juice product., *Int. J. Syst. Evol. Microbiol.* **65**: 716–722. https://doi.org/10.1099/ijs.0.068957-0

Niwa M, Kuriyama A (2013) *A. acidoterrestris* rapid detection kit. *Fruit Process.* **13**: 328–331

Orr RV, Shewfelt RL, Huang CJ, Tefera S, Beuchat LR (2000) Detection of guaiacol produced by *Alicyclobacillus acidoterrestris* in apple juice by sensory and chromatographic analyses, and comparison with spore and vegetative cell populations. *J. Food Protect.* **11**: 1517–1522

Osopale BA, Witthuhn CR, Albertyn J, Oguntoyinbo FA (2016) Culture dependent and independent genomic identification of *Alicyclobacillus species* in contaminated commercial fruit juices. *Food Microbiol.* **56**:21–28. http://dx.doi.org/10.1016/j.fm.2015.11.014

Pettipher GL, Osmundson ME, Murphy JM (1997) Methods for the detection and enumeration of *Alicyclobacillus acidoterrestris* and investigation of growth and production of taint in fruit juice and fruit juice-containing drinks. *Lett. Appl. Microbiol.* **24**: 185–189

Shemesh M, Pasvolsky R, Sela N, Green SJ, Zakin V (2013) Draft genome sequence of *Alicyclobacillus acidoterrestris* strain ATCC 49025, *Genome Announcement* **1(5)**: Article ID e00638–13. https://doi.org/10.1128/genomea.00638-13

Sokołowska B, Niezgoda J, Dekowska A, Porębska I, Nasiłowska J, Waldon-Wiewióra E, Kowalska M (2016) Incidence of *Alicyclobacillus* spp. in polish apple and dark berry juice concentrates and the ability of isolated *A. acidoterrestris* strains to spoilage of these juices. *Post. Nauki Technol. Przem. Rol.-Spoż.* **71**: 5–20 (in Polish)

Sokołowska B, Połaska M, Dekowska A (2020) *Alicyclobacillus* – still current issues in the beverage industry In *Safety Issues in Beverage Production*. Grumezescu AM, Holban AM, eds, pp 105–146. Elsevier (Woodhead Publishing)

Sokołowska B, Skąpska S, Sionek B, Niezgoda J, Chotkiewicz M (2013) *Alicyclobacillus acidoterrestris* – growth and production of odour compounds in apple juice *Post. Nauki Technol. Przem. Rol.-Spoż.* **68**: 19–37 (in Polish)

Uchino F, Doi S (1967) Acido-thermophilic bacteria from thermal waters. *J. Agric. Biol. Chem.* **31**: 817–822

Wang Y, Yue T, Yuan Y, Gao Z (2010) Isolation and identification of thermo-acidophilic bacteria from orchards in China. *J. Food Protect.* **73**: 390–394

Wang Z, Yue T, Yuan Y, Zhang Y, Gao Z, Cai R (2021) Targeting the vanillic acid decarboxylase gene for *Alicyclobacillus acidoterrestris* quantification and guaiacol assessment in apple juices using real time PCR. *Int. J. Food Microbiol.* **338**: 109006

Wisotzkey JD, Jurtshuk P, Fox GE, Deinhart G, Poralla K (1992) Comparative sequence analyses on the 16S rRNA (rRNA) of *Bacillus acidocaldarius, Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus gen.* nov. *Int. J. Syst. Bacteriol.* **42**: 263–269.