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ORIGINAL PAPER

Genome-based reclassification of *Anoxybacillus kamchatkensis* Kevbrin et al. 2005 as a later heterotypic synonym of *Anoxybacillus ayderensis* Dulger et al. 2004

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Abstract

In this study, we aimed to clarify the taxonomic positions of *Anoxybacillus kamchatkensis* DSM 14988^T and *Anoxybacillus ayderensis* AB04^T using whole-genome phylogenetic analysis, biochemical and chemotaxonomic characteristics. In phylogenetic trees drawn using whole-genome sequences and 16S rRNA gene sequences, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T clade together and showed high sequence similarity (99.6%) based on 16S rRNA gene. The average amino acid identity, average nucleotide identity and digital DNA–DNA hybridization values between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were found to be greater than the threshold values for species demarcation. Most phenotypic and chemotaxonomic features between both species were almost identical except for a few exceptions. The present results show that *A. kamchatkensis* DSM 14988^T is a later heterotypic synonym of *A. ayderensis* AB04^T.

Keywords *Anoxybacillus kamchatkensis* · *Anoxybacillus ayderensis* · Genome-based reclassification

Introduction

The genus *Anoxybacillus* is separated from the genus *Bacillus*, and was first described by Pikuta et al. (2000) with *Anoxybacillus pushchinoensis* DSM 12423^T as the type species (Pikuta et al. 2000). At the time of writing, this genus comprised 24 species with validly published names (https://lpsn.dsmz.de/genus/bacillus). The taxonomy of *Anoxybacillus* members was mainly elucidated by 16S rRNA gene sequence analysis and DNA–DNA hybridization (DDH) methods. However, it is widely known that the identification power of 16S rRNA gene analysis is generally limited, and the reconstitution of DDH is quite difficult and sometimes varies depending on the method used by the laboratory. Using metrics such as digital DDH, average nucleotide identity (ANI) and average amino acid identity (AAI) associated with the whole-genome sequence, phylogeny is an important tool for the identification of prokaryotic taxa (Orata et al. 2018) and is currently used extensively for reclassification of many bacterial taxa (Liu et al. 2019; Rao et al. 2022).

The type strain AB04^T of *Anoxybacillus ayderensis* was isolated from hot spring in Turkey by Dulger et al. (2004) and described as validly named species based on a polyphasic taxonomic approach. *Anoxybacillus kamchatkensis* DSM 14988^T was isolated from an unnamed hot spring in the Kamchatka Peninsula by Kevbrin et al. (2005) and was validated in IJSEM (Validation List No. 109; Euze´ 2016). In the original article, Kevbrin et al. (2005) proposed *A. kamchatkensis* DSM 14988^T as a new species in the genus *Anoxybacillus* based mainly on DNA–DNA hybridization value between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T, *A. pushchinoensis* K1^T, *A. flavithermus* DSM2641^T, *A. gonensis* G2^T. However, during our genome-based analysis, we observed that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T shared similar features; as a result, we attempted to clarify the relationship between that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T through genomics-based methods, biochemical and chemotaxonomic characteristics. The data presented in this study

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provide evidence that *A. kamchatkensis* DSM 14988^T is later heterotypic synonym of *A. ayderensis* AB04^T.

Materials and methods

In the original article, Kevbrin et al. (2005) stated that *A. ayderensis* AB04^T were obtained from the NCIMB (National Collection of Industrial Food and Marine Bacteria) collection. Therefore, in this study, we purchased *A. ayderensis* AB04^T from NCIMB and *A. kamchatkensis* DSM 14988^T from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Two type strains were grown on trypticase soy agar (TSA) incubated at 50 °C for 24 h. The API 20E, API 50CH strips and Vitek2 Bacilli Identification Card (BCL) microtest systems (bioMérieux) were used to evaluate the biochemical properties of *A. ayderensis* AB04^T and *A. kamchatkensis* DSM 14988^T according to the manufacturer's instructions. Polar lipids of *A. ayderensis* AB04^T and *A. kamchatkensis* DSM 14988^T were extracted from 100 mg freeze-dried cells using the two-stage method described by Tindall (1990a, b). The method was performed using two-dimensional thin-layer chromatography (TLC) in the first direction with chloroform:methanol:water (65:25:4, v/v) and the second direction with chloroform:methanol:acetic acid:water (80:12:15:4, v/v). In the analysis of lipids, molybdophosphoric acid, molybdenum blue, ninhydrin, a-naphthol were used for total lipids, phospholipids, aminolipids and glycolipids, respectively (Tindall et al. 2007). Respiratory quinone analysis was performed using a high-performance liquid chromatography instrument as described by Collins (1985).

The whole-genome sequences of *A. kamchatkensis* DSM 14988^T (JACDUV000000000) and *A. ayderensis* AB04^T (JXTG00000000) are available from NCBI Database (https://www.ncbi.nlm.nih.gov/genome/). The 16S rRNA gene sequences were retrieved from the draft genome sequence of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T using RNAmmer (version 1.2) (Lagesen et al. 2007) and were deposited under accession number OP218102 and OP218102, respectively.

The 16S rRNA gene sequence similarity values between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were calculated using the pairwise alignment tool available on the EZBioCloud server at https://www.ezbiocloud.net/tools/pairA.

The 16S rRNA gene sequences of closely related type strains were retrieved from EzBioCloud server at https://www.ezbiocloud.net/ (Yoon et al. 2017a) and edited by using the Bioedit software (Hall, 1999). CLUSTAL_W (Thompson et al. 1994) software was used to perform the multiple sequence alignment of 16S rRNA gene sequences). Maximum-likelihood (Felsenstein 1981), neighbor-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971) algorithms implemented in MEGA software V 7.0 was used to reconstruct the phylogenetic trees based on 16S rRNA gene sequences (Kumar et al. 2016). Kimura's two-parameter model (Kimura 1980) was used to calculate the evolutionary distance matric. Tree topologies were assessed by bootstrapping method with 1000 replicates (Felsenstein 1985).

The phylogenetic analysis of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was performed using the type strain genomes server pipeline at website https://tygs.dsmz.de/ Meier-Kolthoff and Göker (2019). The digital DNA–DNA hybridization (dDDH) value between the draft genome sequences of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was calculated with the Formula 2 of the online Genome-to-Genome Distance Calculator at http://ggdc.dsmz.de/distcalc2.php (Meier-Kolthoff et al. 2013). OrthoANIu algorithm and an ANI calculator server (www.ezbiocloud.net/tools/ani) were used to calculate average nucleotide identity (ANI) values for evaluating the genetic relationship between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T (Lee et al. 2016; Yoon et al. 2017b). TYGS web server (https://tygs.dsmz.de/) was used to construct a phylogenetic tree based on whole-genome sequences Meier-Kolthoff and Göker (2019) and CompareM tool calculated the amino acid identity (AAI) value at https://github.com/dparks1134/CompareM.

Results and discussion

The phylogenetic analysis based on whole-genome sequences has clarified the taxonomic inconsistence of prokaryotic taxa; as a result, several bacterial species have been reclassified (Orata et al. 2018). In the present study, the taxonomic relationship of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was re-evaluated using whole-genome phylogenetic analysis, biochemical and chemotaxonomic features.

In the original study, Kevbrin et al. (2005) stated that *A. kamchatkensis* DSM 14988^T had the highest 16S rRNA gene sequence similarity (99.2%) with *A. ayderensis* AB04^T. Despite high 16S rRNA gene sequence similarity, they reported 35% DNA–DNA hybridization value between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T which was below the threshold value (70%) for species delineation (Wayne et al. 1987). In our study, the whole-genome sequences of *A. kamchatkensis* DSM 14988^T (JACDUV000000000) and *A. ayderensis* AB04^T (JXTG00000000) were retrieved from NCBI Database and we obtained the 16S rRNA gene sequences from these genomes of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T.

In the present study, we determined the pairwise nucleotide sequence alignment (16S rRNA gene sequence obtained from genomes) between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was 99.6% with a mismatch of six nucleotides. In addition, in the present study, we reconstructed the phylogenetic trees based on 16S rRNA gene sequences using the acquired 16S rRNA gene sequences from the genome of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T. We determined that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T clustered together in the neighbor-joining phylogenetic tree with high bootstrap resampling values of 97% (Fig. 1). Topologies of phylogenetic trees built according to the maximum-likelihood and maximum-parsimony algorithms also supported the results of the neighbor-joining algorithm (Figs. S1, S2). Further, in the phylogenomic tree (Fig. 2), *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T formed a robust branch different from other type strains of this genus with high bootstrap resampling values of 97%. The ANI value between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was 97.58% which was greater than the threshold value (95–96%) for species demarcation Richter and Rossello´-Mora´ (2009), confirming that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were highly phylogenetically closely related. The calculated AAI value between the *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was 98.0% and this value is also clearly above the suggested cut-offs for species delineation (AAI > 95%) (Luo et al. 2014), confirming that they belong to the same species. Also, digital DNA–DNA hybridization (DDH) analyses indicated that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T exhibited 78.5% dDDH value which is higher than the cut-off (70%)

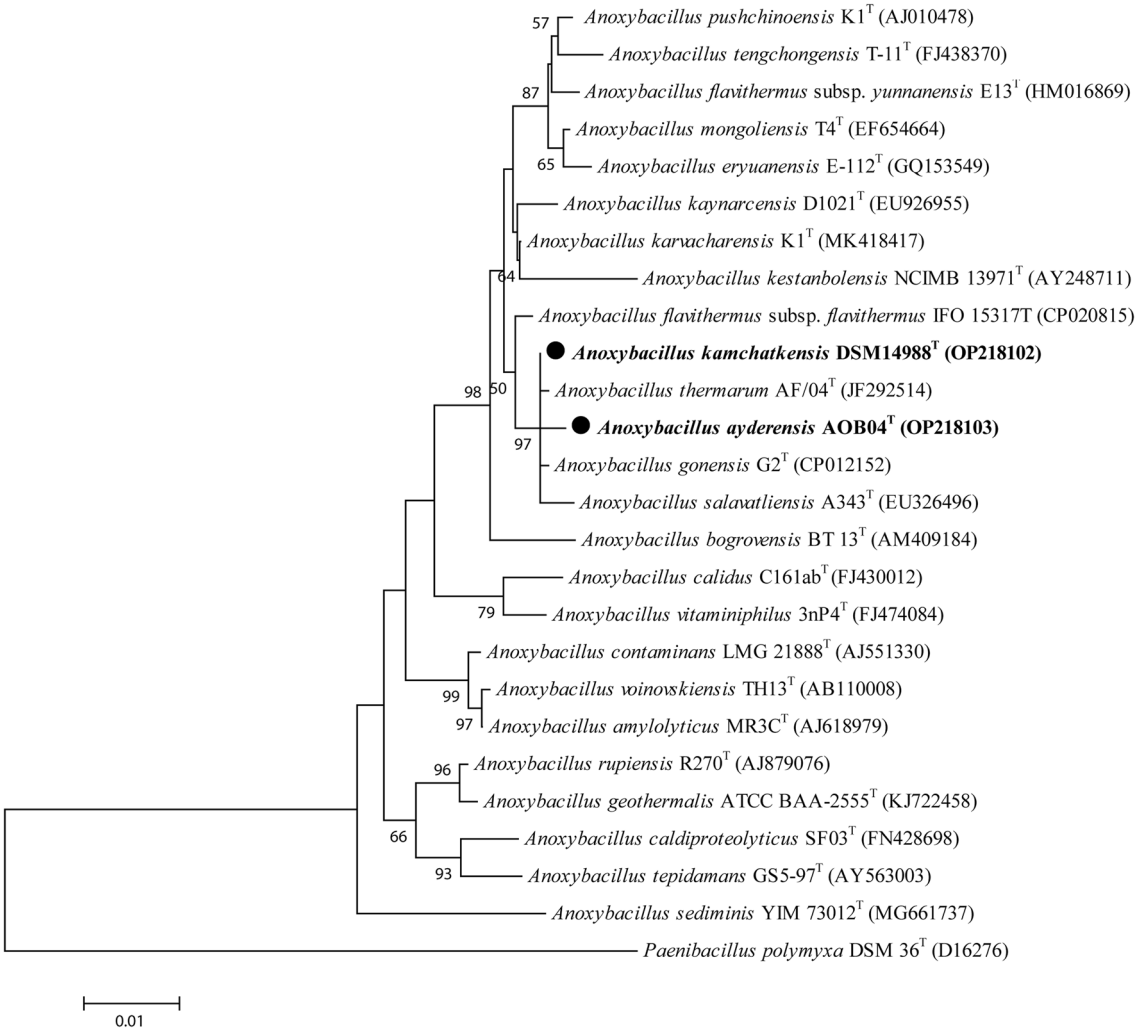

Fig. 1 Neighbor-joining (NJ) tree constructed based on 16S rRNA gene sequences available from the GenBank database. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Bar, 0.01 represents substitutions per nucleotide position. *Paenibacillus polymyxa* DSM 36^T used as the outgroup

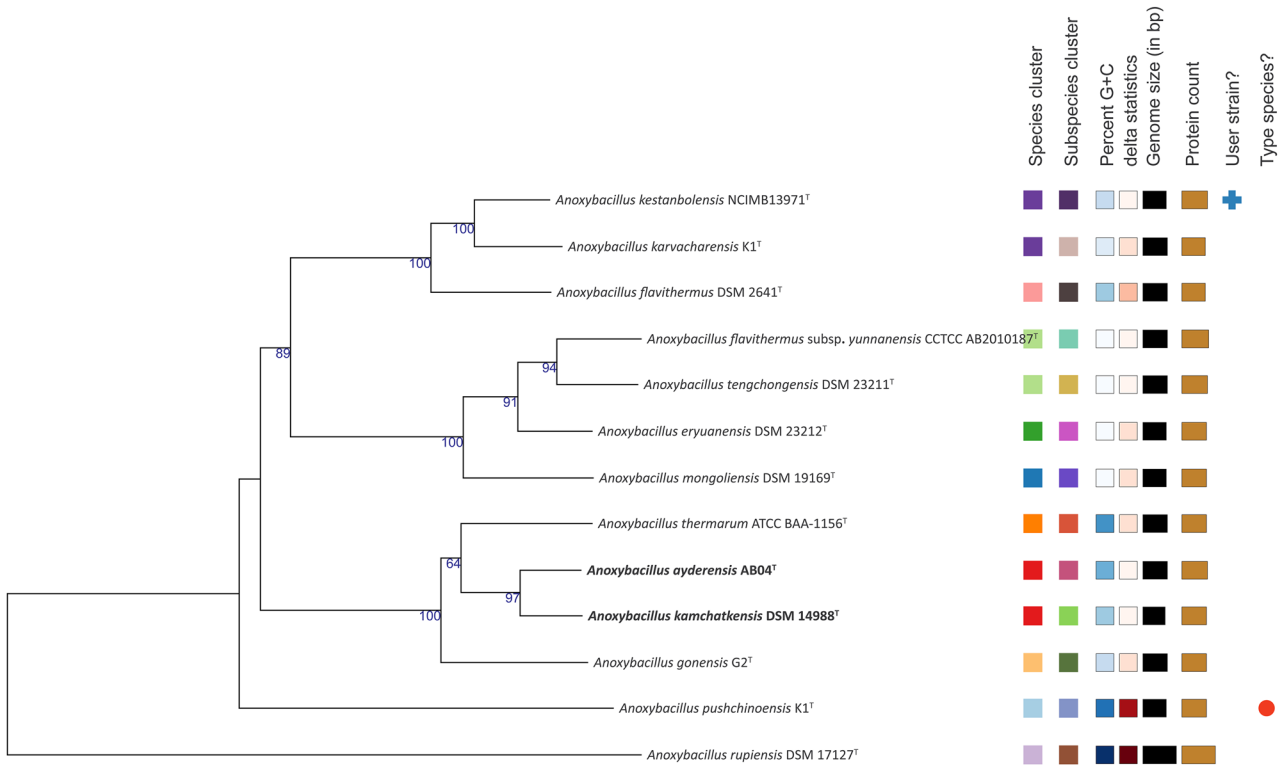

Fig. 2 Phylogenetic tree based on whole-genome sequences of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T and related reference strains. The tree was inferred with FastME 2.1.6.1 (Lefort et al. 2015) from genome blast distance phylogeny (GBDP) distances calculated from genome sequences using the TYGS server (https://tygs.dsmz.de) (Meier-Kolthoff and Göker 2019) The branch lengths are scaled in terms of GBDP distance formula d5. The numbers at branches are GBDP pseudo-bootstrap support values ≥ 64% from 100 replications with an average branch support of 97.7%. The tree was rooted at the midpoint (Farris, 1972)

used to classify bacterial strains to the same species (Wayne et al. 1987), further confirming that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T should belong to the same genomic species. Also, AAI, ANI and dDDH values between A. kamchatkensis DSM 14988^T, A. ayderensis AB04^T and closely related type strains are given in Table 1.

In addition, this conclusion has been also confirmed by a comparison of phenotypic and chemotaxonomic features between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T. In API 20E, API 50CH and Vitek2 BCL system, *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T shared similar biochemical features with few exceptions (Table 2). For example, acid production from L-arabinose, D-xylose, D-mannose, *N*-acetyl-glucosamine, xylitol, gentiobiose and β-xylosidase were negative for *A. kamchatkensis* DSM 14988^T, while positive for *A. ayderensis* AB04^T. L-aspartate arylamidase and β-glucosidase were positive for *A. kamchatkensis* DSM 14988^T, while negative for *A. ayderensis* AB04^T. Both species were shown positive for ONPG hydrolysis, tryptophan deaminase, nitrate reduction, α-glucosidase, Leucine-arylamidase, phenylalanine arylamidase, tyrosine arylamidase, Ala-Phe-Pro-Arylamidase, acid production from maltotriose, esculin ferric citrate, D-galactose, D-glucose, D-fructose, D-mannitol,

Table 1 AAI, ANI and dDDH values between *A. kamchatkensis* DSM 14988^T, *A. ayderensis* AB04^T and closely related type strains

	A. kamchatkensis DSM 14988^T			*A. ayderensis* AB04^T		
	AAI	ANI	dDDH	AAI	ANI	dDDH
A. gonensis G2^T (JRZG00000000)	96.12	94.58	58.00	96.17	94.75	59.00
A. kamchatkensis DSM 14988^T (JACDUV000000000)	–	–	–	98.00	97.58	78.50
A. ayderensis AB04^T (JXTG00000000)	98.00	97.58	78.50	–	–	–
A. thermarum AF/04^T (JXTH00000000)	96.17	94.88	60.50	96.36	94.75	62.20
A. salavatliensis DSM 22626T (JANGZY000000000)	96.30	94.45	57.10	96.37	94.50	57.50

Table 2 The biochemical characteristics of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T

	A. kamchatkensis DSM 14988^T	*A. ayderensis* AB04^T
β-Xylosidase	–	+
L-Aspartate arylamidase	+	–
β-Glucosidase	+	–
α-Glucosidase	+	+
Tyrosine arylamidase	+	+
Phenylalanine arylamidase	+	+
Leucine-arylamidase	+	+
Tryptophan deaminase	+	+
Lysine decarboxylase	–	–
Ornithine decarboxylase	–	–
Alanine arylamidase	–	–
α-Galactosidase,	–	–
L-Pyrrolydonyl arylamidase	–	–
L-Lysine arylamidase	–	–
L-Proline arylamidase	–	–
α-Mannosidase	–	–
Glycine arylamidase	–	–
Acid production from		
L-Arabinose	–	+
L-Xylose	–	+
L-Mannose	–	+
L-Galactose	+	+
L-Glucose	+	+
L-Fructose	+	+
L-Mannitol	+	+
L-Maltose	+	+
L-Sucrose	+	+
L-Trehalose	+	+
L-Arabinose	–	–
L-Ribose	–	–
L-Xylose	–	–
L-Lactose	–	–
L-Melibiose	–	–
L-Sorbitol	–	–

+, Positive; –, negative

amygdalin, salicin, D-cellobiose, D-maltose, D-sucrose, D-trehalose, D-melezitose, D-raffinose, starch, glycogen, and D-turanose. *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were shown negative for arginine dihydrolase, citrate utilization, urease, Voges–Proskauer, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide production, indole production (tryptophanase), growth in 6.5%NaCI, kanamycin resistance, oleandomycin resistance, L-lysine arylamidase, L-proline arylamidase, L-pyrrolydonyl arylamidase, α-galactosidase, alanine arylamidase, β-*N*-acetyl-glucosaminidase, cyclodextrin, ellman, methyl-D-xyloside, α-mannosidase, glycine arylamidase, palatinose, β-galactosidase, β-mannosidase, phosphoryl choline, pyruvate, acid production from inositol, sorbitol, glycerol, erythritol, D-arabinose, D-ribose, L-xylose, methyl-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl-alpha- D-mannopyranoside, methyl-a-D-glucopyranoside, arbutin, D-lactose, D-melibiose, inulin, D-lyxose, D-tagatose, D-L-fucose, D-and L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, and putrescine. Total number of phenotypic test performed using the API 20E, API 50CH and Vitek2 BCL system were 91. We observed differences only in 9 tests between *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T, accounting the value of dissimilarity only 10% which is lower than the threshold value (30%) and justifying the unification of species (Deb et al. 2020).

In the original articles, the polar lipids of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were not determined (Dulger et al. 2004; Kevbrin et al. 2005). In our study, the polar lipids found in *A. ayderensis* AB04^T were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), unidentified phospholipid-1 (PL1), unidentified amino phospholipid-1 (APL1) and unidentified amino phospholipid-2 (APL2) whereas *A. kamchatkensis* DSM 14988^T consist of PG, DPG, PE, PC, PL1, PL2, APL1 and APL2. Polar lipid composition showed very similar profile between two species (Fig. 3). The respiratory quinone of *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T was menaquinone MK-7. As is shown in Table 2 and Fig. 3, most phenotypic and chemotaxonomic features between them were almost identical except for a few exceptions. The disagreement for phenotypic and chemotaxonomic was probably due to their different ecological niches.

The present results, when evaluated together, show that *A. kamchatkensis* DSM 14988^T and *A. ayderensis* AB04^T were considered to belong to the same species. Therefore, according to phylogenetic analysis based on whole-genome sequences and rule 42 of the Bacteriological Code (Parker et al. 2019), we propose that *A. kamchatkensis* DSM 14988^T Kevbrin et al. 2005 should be reclassified as a later heterotypic synonym of *A. ayderensis* AB04^T Dulger et al. 2004. The type strain is AB04^T (=NCIMB 13972^T=NCCB 100050^T) and JW/VK-KG4 (=DSM 14988, =ATCC BAA-549) is an additional strain of *A. Ayderensis.*

Emended description of *A. ayderensis* Dulger et al. (2004)

The description is the same as given by Dulger et al. (2004) with the following modification.

Major polar lipids include phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine

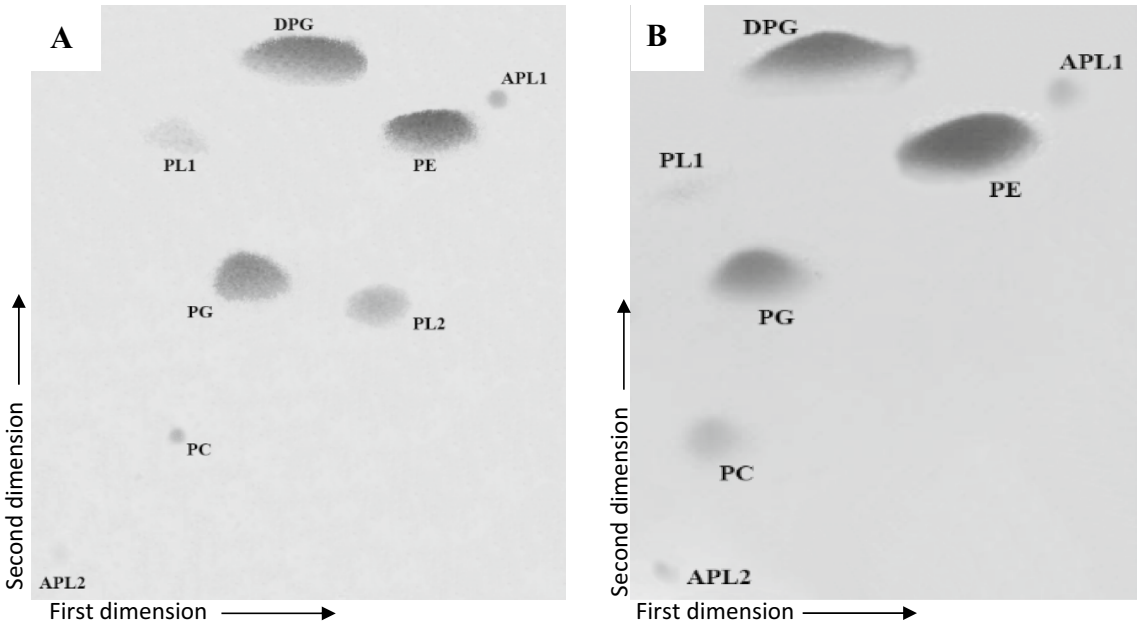

Fig. 3 Two-dimensional thin-layer chromatogram of polar lipids of **A** *A. kamchatkensis* DSM 14988^T and **B** *A. ayderensis* AB04^T. *DPG* diphosphatidylglycerol; *PG* phosphatidylglycerol; *PE* phosphatidylethanolamine; *PC* phosphatidylcholine; *APL* unidentified aminophospholipid; *PL* unidentified phospholipid

(PE), phosphatidylcholine (PC), unidentified phospholipid-1 (PL1), unidentified amino phospholipid-1 (APL1) and unidentified amino phospholipid-2 (APL2). The respiratory quinone is menaquinone MK-7. In API 20E, API 50CH and Vitek2 BCL system, the following activities were positive for ONPG hydrolysis, tryptophan deaminase, nitrate reduction, α-glucosidase, Leucine-arylamidase, phenylalanine arylamidase, tyrosine arylamidase, Ala-Phe-Pro-Arylamidase, acid production from maltotriose, esculin ferric citrate, D-galactose, D-mannitol, amygdalin, salicin, D-cellobiose, D-trehalose, D-melezitose, starch, glycogen, D -turanose, *N*-acetyl-glucosamine, xylitol, gentiobiose and β-xylosidase. Negative for arginine dihydrolase, citrate utilization, Voges–Proskauer, lysine decarboxylase, ornithine decarboxylase, growth in 6.5%NaCI, kanamycin resistance, oleandomycin resistance, L-aspartate arylamidase and β-glucosidase, L-lysine arylamidase, L-proline arylamidase, L-pyrrolydonyl arylamidase, α-galactosidase, alanine arylamidase, β-*N*-acetyl-glucosaminidase, cyclodextrin, ellman, methyl-D-xyloside, α-mannosidase, glycine arylamidase, palatinose, β-galactosidase, β-mannosidase, phosphoryl choline, pyruvate, acid production from inositol, sorbitol, glycerol, erythritol, D-arabinose, D-ribose, L-xylose, methyl-D-xylopyranoside, L-sorbose, dulcitol, D-sorbitol, methyl-alpha- D-mannopyranoside, methyl-a-D-glucopyranoside, arbutin, D-melibiose, inulin, D-lyxose, D-tagatose, D-L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, and putrescine. The DNA G + C content of the type strain AB04^T (= NCIMB 13972^T = NCCB 100050^T) is 41.83 mol%.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03201-4.

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Author contributions KIB designed the study. KIB, HIB and SC performed the genome analysis and analyzed the data. KIB, AN and AOB performed the phenotypic and chemotaxonomic analysis. KIB and AN wrote the manuscript. All the authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Funding I confirm that this declaration is accurate

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