



A chemotaxonomic and phylogenomic re-evaluation of the genus *Alicyclobacillus*: A proposal for reclassification of about half of the species into the new genus, *Paenalicyclobacillus* gen. nov., with an emended description of the genus *Alicyclobacillus*, and recognition that the names *Alicyclobacillus tengchongensis* and *Alicyclobacillus montanus* are later heterotypic synonyms of *Alicyclobacillus tolerans*

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ABSTRACT

The genus *Alicyclobacillus* represents a group of mostly acidophilic, meso-thermophilic bacteria, which obtained their name through the biosynthesis of uncommon, alicyclic fatty acids (FAs), i.e., ω -cyclohexyl or ω -cycloheptyl FAs. However, there are also species belonging to this genus that do not possess this trait. In addition, the difference of up to 9 % of the 16S rRNA gene sequences of 33 type strains compared with the first isolated type species, *A. acidocaldarius* DSM 446^T, exceeds the 5.5 % threshold, which is typically used for the demarcation of a genus. This suggested that the taxonomy of the genus required a re-examination. We performed a phylogenomic and chemotaxonomic survey of the genus *Alicyclobacillus*. Phylogenetic trees based on the 16S rRNA gene and comparison of whole proteomes reconstructed from their genomes reveal that the genus *Alicyclobacillus* is reflected by two distinct clades; one represented by 14 type strains with a >94.1 % 16S rRNA gene similarity to *A. acidocaldarius* DSM 446^T, and a second clade (composed of 14 type strains including *A. cycloheptanicus*) with a similarity of 91–94 %. This distinction fits well with their reported ability to produce alicyclic FAs, also revealed by the detection of two putative biosynthetic gene clusters (BGC) encoding their biosynthetic pathway in their genomes. The first BGC is responsible for the production of cyclohexane carboxylic acid-CoA and comprises five genes encoding proteins acting as an adjusted side branch of the shikimate pathway. The putative second BGC, responsible for production of cycloheptane carboxylic acid-CoA, comprises six genes encoding proteins acting as a side branch of the catabolic phenylacetic acid pathway. All *Alicyclobacillus* species of the first clade produce ω -cyclohexyl FAs and their genomes possess the corresponding BGC. In the second cluster, some species produce ω -cyclohexyl FAs, some produce ω -cycloheptyl FAs, whilst quite some members are not producing any alicyclic FAs. This corresponds to the presence or absence of the two BGCs in their genomes. We propose to reclassify all species of the second clade as members of a new genus, *Paenalicyclobacillus* gen. nov. and show that both *Alicyclobacillus tengchongensis* and *Alicyclobacillus montanus* are later heterotypic synonyms of *Alicyclobacillus tolerans*.

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Introduction

Alicyclobacillus is an intriguing genus of bacteria due to its distinctive features and diverse ecological presence. These strictly aerobic, meso- to thermophilic, Gram-stain positive, spore-forming, rod-shaped bacteria belong to the phylum Bacillota class Bacilli, order Caryophanales, family Alicyclobacillaceae, and have proven to possess a remarkable ability to thrive in acidic environments. Originally discovered in a hot spring (i.e. the type species, *A. acidocaldarius*; Darland and Brock, 1971), *Alicyclobacillus* spp. have since been found in soil, and other natural habitats, and acidic drinks such as fruit juices and ciders, which they may spoil because mild pasteurization techniques do not deactivate their spores.

An intriguing aspect of the physiology of *Alicyclobacillus* spp. is their uncommon and specific lipid distribution. In fact, the genus name derives from the presence of ω -alicyclic fatty acids (FAs) as major component of the cell membrane of many members and serves also to

reflect the previous classification as *Bacillus* (Wisotzkey et al., 1992). However, not all *Alicyclobacillus* spp. produce ω -cyclohexyl FAs, some produce ω -cycloheptyl FAs, while other species produce none (see Ciuffreda et al., 2015 for an overview; Table 1). These ω -alicyclic FAs are thought to stabilize the membranes of bacteria by reducing the fluidity in membranes and increasing lipid packing and lipid chain order. Molecular dynamics simulations have suggested that ω -alicyclic FAs might protect the membrane of thermo-acidophilic bacteria from high-temperature and low-pH conditions through a “dynamical barrier” that limits lipid diffusion and transmembrane diffusion of undesired ions and molecules (Poger and Mark, 2015).

Here we describe how the presence of two distinct Biosynthetic Gene Clusters (BGCs) in the genomes of 28 *Alicyclobacillus* type strains is related to the production of alicyclic FAs and how this differs from other bacterial groups. This phylogenomic and chemotaxonomic survey also indicates that taxonomically the *Alicyclobacillus* genus should be

Table 1
Alicyclobacillus type strains and some of their properties.

Species	DSM #	Origin	Opt. T (°C)	Opt. pH	Ali-FA ^a	Genome Size (Mb)	Reference(s)
Clade 1							
<i>A. acidiphilus</i> TA-67 ^T	14558	Acidic beverage	50	3.0	C6	3.9	Matsubara et al. (2002)
<i>A. acidocaldarius</i> 104-1A ^T	446	Acidic hot spring	45	3–4	C6	3.2	Darland and Brock (1971); Wisotzkey et al. (1992)
<i>A. acidocaldarius</i> subsp. <i>ritmannii</i> MRI ^T	11297	Geothermal soil	63	4.0	C6	n.d.	Nicolaus et al. (1998)
<i>A. acidoterrestris</i> GD3B ^T	3922	Garden soil	42–53	4.5	C6	4.3	Deinhard et al. (1987a); Wisotzkey et al. (1992)
<i>A. dauci</i> 4F ^T	28700	spoiled fruit juice	40	4.0	C6	4.9	Nakano et al. (2015)
<i>A. fastidiosus</i> S-TAB ^T	17978	Apple juice	40–45	4.0–4.5	C6	6.0	Goto et al. (2007)
<i>A. fodiniaquatilis</i> G45-16 ^T	–	Acidic water of copper mine	40	3.5	C6	5.5	Zhang et al. (2015)
<i>A. fructus</i> FSL-W10-0059 ^T	112028	Mixed fruit juice concentrate	60	4.0–4.5	C6	2.9	Roth et al. (2021)
<i>A. hesperidum</i> FR-11 ^T	12489	Solfataric soil	50	4.0	C6	2.9	Albuquerque et al. (2000)
<i>A. hesperidum</i> subsp. <i>aegles</i> FR-12 ^T	11985	Geothermal soil	45–50	3.5	C6	2.9	Goto et al. (2023)
<i>A. mali</i> FSL-W10-0018 ^T	112016	Pasteurized pear juice	60	3.0–4.5	C6	2.8	Roth et al. (2021)
<i>A. sacchari</i> RB-718 ^T	17974	Liquid sugar	45–50	4.0–4.5	C6	2.9	Goto et al. (2007)
<i>A. sendaiensis</i> NTAP-1 ^T	17614	Soil	55	5.5	C6	2.8	Tsuruoka et al. (2003)
<i>A. suci</i> VF-FSL-W10-0049 ^T	112017	Apple juice	50	3.5–4.0	C6	4.0	Roth et al. (2021)
<i>A. vulcanalis</i> CsHg2 ^T	16176	Geothermal pool	55	4.0	C6	3.0	Simbahan et al. (2004)
Clade 2							
<i>A. aeris</i> ZJ-6 ^T	–	Copper mine drainage	30	3.5	–	n.d.	Guo et al. (2009)
<i>A. cellulositrophicus</i> Sueoka ^T	107339	Steamed cedar chips	55	4.8	C6	2.7	Kusube et al. (2014)
<i>A. contaminans</i> 3-A191 ^T	17975	Soil of a crop field	50–55	4.0–4.5	–	3.3	Goto et al. (2007)
<i>A. curvatus</i> ALEF1 ^T	–	Acid mine drainage sediment	30	3.5	–	6.0 ^b	Jiang et al. (2022)
<i>A. cycloheptanicus</i> SCH ^T	4006	Soil	48	3.5–4.5	C7	3.6	Deinhard et al. (1987b); Wisotzkey et al. (1992)
<i>A. disulfidooxidans</i> SD-11 ^T	12064	Waste water treatment sludge	35	1.5–2.5	C6	n.d.	Dufresne et al. (1996); Karavaiko et al. (2005)
<i>A. ferrooxydans</i> TC-34 ^T	22381	Solfataric soil	28	3.0	–	5.0	Jiang et al. (2008)
<i>A. herbarius</i> CP-1 ^T	13609	dry flower of <i>Hibiscus</i>	55–60	4.5–5.0	C7	3.3	Goto et al. (2002)
<i>A. kakegawensis</i> 5-A83J ^T	17979	Soil of a crop field	50–55	4.0–4.5	C7	3.4	Goto et al. (2007)
<i>A. macrosporangioides</i> 5A-239-20-A ^T	17980	Soil of a crop field	50–55	4.0–4.5	–	3.8	Goto et al. (2007)
<i>A. mengziensis</i> S30H14 ^T	–	Acid mine drainage sediment	30	4.5	- ^c	5.3	Jiang et al. (2022)
<i>A. montanus</i> USBA-GBX-503 ^T	–	Acidic hot spring	45	3.0	- ^d	3.0	López et al. (2018)
<i>A. pomorum</i> 3A ^T	14955	Mixed fruit juice	45–50	4.0–4.5	–	3.4	Goto et al. (2003)
<i>A. shizuokensis</i> 4-A336 ^T	17981	Soil of a crop field	45–50	4.0–4.5	C7	3.6	Goto et al. (2007)
<i>A. tengchongensis</i> ACK006 ^T	25924	Soil of a hot spring	45	3.2	C7	2.9	Kim et al. (2014)
<i>A. tolerans</i> K1 ^T	16297	oxidizable lead–zinc ores	38–42	2.0–2.7	C6	3.2 ^e	Karavaiko et al. (2005)

^a C6 = production of ω -cyclohexyl FAs; C7 = production of ω -cycloheptyl FAs; – = no production of ω -alicyclic FAs. The underlined composition has been reexamined in this study.

^b This genome is currently under investigation by NCBI because it is potentially contaminated.

^c No ω -alicyclic FAs were reported by Jiang et al. (2022). However, they reported the same “summed feature 8” including C_{18:1 ω 7c} and/or C_{18:1 ω 6c} representing ca. 5 % of the FAs. The same “summed feature 8” was reported for *A. acidocaldarius* DSM 446 in a relative abundance of 71 %. It has been well documented that this species contains the C₁₇ ω -cyclohexyl FA, which co-elutes with the C_{18:1 ω 7c} in the MIDI system used for FA identification. Hence, we must conclude that *A. mengziensis* in all probability produces the C₁₇ ω -cyclohexyl FA as a minor FA.

^d The reported FA data is probably incorrect. López et al. (2018) report for *A. montanus* and two reference strains the C_{18:1 ω 7} FA as major FA and the absence of ω -cyclohexyl FAs. One of these reference strains is *A. acidocaldarius* DSM 446. The reported absence of ω -cyclohexyl FAs in this species is in strong contradiction with data reported in the literature (at least five other studies report their presence, typically in the order of 80–90 % of total FAs), whereas unsaturated FAs are highly uncommon in *Alicyclobacillus* spp. A possible reason might be the use of the MIDI system for FA identification or contamination of the cultures with other bacteria.

^e This study. Key: n.d. = not determined, i.e., the genome has not been sequenced.

reclassified in two individual genera. A new taxonomy for this group is then proposed.

Methods

Cultivation

Five strains were chosen for reanalysis of their fatty acid profiles to verify already published data. The majority of the growth media used for cultivation of the strains contained mineral salts, and yeast extract. Glucose, starch, carboxymethylcellulose and FeSO₄ were also added as ingredients to some of them. Strain *Alicyclobacillus tengchongensis* DSM 25924^T was grown in YSG medium (<https://mediadive.dsmz.de/medium/1805>), pH 3.7 at 50 °C. Strain *Alicyclobacillus tolerans* DSM 16297^T was cultured in Mannig medium, pH 2.5 with FeSO₄ (<https://mediadive.dsmz.de/medium/1023>) at 37 °C. Medium 1648 (<https://mediadive.dsmz.de/medium/1684>), pH 4.8 and 50 °C was chosen for strain *Alicyclobacillus cellulosityticus* DSM 107339^T. Strains *Alicyclobacillus shizuokensis* DSM 17981^T and *Alicyclobacillus cycloheptanicus* DSM 4006^T were grown in medium 13, pH 4 at 45 °C (<https://mediadive.dsmz.de/medium/13>). Cultures were harvested in the transit of the late exponential phase to the beginning stationary phase. The cells of all strains were harvested by centrifugation at 10000 rpm for 20 min. After the liquid was removed the cells were lyophilized overnight.

Fatty acid analysis and identification

Whole freeze-dried cells of *A. cellulosityticus*, *A. tengchongensis*, *A. tolerans*, *A. cycloheptanicus*, and *A. shizuokensis* were acid hydrolyzed in 1.4 N HCl in methanol by refluxing for 3 h, using the method described by Sinninghe Damsté et al. (2011). The mixture was centrifuged and the pH of the supernatant was adjusted to 4 by addition of 2 N KOH solution. The extract was methylated with diazomethane in diethyl ether and subsequently fractionated over a small column of activated Al₂O₃ with dichloromethane as the eluent. The FAs (as their methyl ester derivatives) were analyzed by gas chromatography (GC) with a flame ionization for determination of FA abundances and GC-mass spectrometry (MS) for identification. The alicyclic fatty acid methyl esters were identified by their characteristic mass spectra and relative retention time data.

DNA extraction and whole genome sequencing

Genomic DNA from *Alicyclobacillus tolerans* strain DSM 16297^T was extracted from the same batch of cell material used for FA analysis using the Qiagen Powersoil Pro extraction kit with some modifications. In short, freeze-dried biomass was rehydrated in CD1 buffer overnight at room temperature, after which manufacturer's instructions were followed except from an incubation at 4 °C for 10 min after adding CD2 buffer. After the extraction, DNA was further cleaned with Quantabio sparQ PureMag Beads (Beverly, MA, US). Magnetic beads after the clean-up were re-extracted with the Qiagen Powersoil Pro extraction kit and cleaned again with the Quantabio sparQ PureMag Beads. Finally, both DNA extracts were combined and re-cleaned and concentrated with the Quantabio sparQ PureMag Beads once again to a final volume of 20 µL.

DNA was sequenced by using long-read sequencing technology from Oxford Nanopore Technologies. An amplification-free long-read sequencing library was prepared by using v14 library prep chemistry and R10.4.1 flow cells by Plasmidsaurus. The genome was assembled as follows: (1) Removal of the bottom 5 % worst fastq reads via Filtlong v0.2.1 (default parameters); (2) Downsample the reads to 250 Mb via Filtlong to create a rough sketch of the assembly with Miniasm v0.3; (3) Using information acquired from the Miniasm assembly, re-downsample the reads to ~100× coverage with heavy weight applied to removing low quality reads; (4) run Flye v2.9.1 assembly with parameters selected

for high quality ONT reads; (5) polish Flye assembly via Medaka v1.8.0 using the reads generated in step 3. The genome was annotated by using Bakta v1.6.1, contig analysis was performed by Bandage v0.8.1, and genome completeness and contamination assessed by CheckM v1.2.2.

Phylogenetic, phylogenomic, and bioinformatic analyses

Phylogenetic analysis was performed with 16S rRNA gene sequences from all studied type strains. Some relevant non-type strains were also included in the analysis. Reconstruction of the phylogenetic tree was conducted with Mega 7 (Kumar et al., 2016) using the maximum-likelihood method. Evolutionary distances were calculated according to the General Time Reversible (GTR) model (Nei and Kumar, 2000). Accession numbers are given in Table S1.

The genome sequence data (see Table S1 for genome assembly numbers) were uploaded to the Type (Strain) Genome Server (TYGS; <https://tygs.dsmz.de>) for a whole genome-based taxonomic analysis (Meier-Kolthoff and Göker, 2019). Precise distances were calculated using the genome blast distance phylogeny approach (GBDP). The resulting intergenomic distances were used to infer a balanced minimum-evolution tree via FastME 2.1.46.1 (Lefort et al., 2015). Branch support was inferred from 100 pseudo-bootstrap replicates each. The tree was rooted at the midpoint (Farris, 1972) and visualized with Mega 7 (Kumar et al., 2016).

Average nucleotide (ANI) values were calculated using the Jspecies web server (Richter et al., 2015), and the Average Amino acid Identity (AAI) calculator within the toolbox from the Kostas Lab (Rodriguez-R and Konstantinidis, 2016) was applied to construct the AAI distance matrix, which was subsequently used for hierarchical clustering of the input genomes.

Biosynthetic genes involved in the various biosynthetic pathways investigated were identified in the genomes of the studied *Alicyclobacillus* (see Table S1 for genome assembly numbers) and other species (see Table 2 for genome assembly numbers) with Position-Specific Iterated BLAST (PSI-BLAST) searches at the protein level using the NCBI Protein Reference Sequences database (Haft et al., 2023). An iteration process (1–3 steps) was conducted using well defined proteins of *Bacillus subtilis* DSM 10, *Streptomyces humidus* JCM 4386, *Halalkalibacterium halodurans* C-125, and *Alicyclobacillus kakegawensis* DSM 17979 as query sequences typically using minimal cut-off values of 1E-30 and 30 % sequence identity (Table S2).

Results and discussion

Phylogenomic data reveals two different clades in the genus *Alicyclobacillus*

Earlier published phylogenetic trees of the 16S rRNA gene of all type strains of the genus *Alicyclobacillus* (for one of the most recent ones see Jiang et al., 2022) clearly showed that this group falls apart into distinct clades. Reconstruction of a phylogenetic tree with the 16S rRNA gene sequences from all type strains of the species currently assigned to the genus *Alicyclobacillus* confirmed that several species are distantly related to the type species *Alicyclobacillus acidocaldarius* DSM 446^T and form separate clusters (Fig. 1). When the similarity of the 16S rRNA gene sequence of *A. acidocaldarius* DSM 446^T is compared with those of other species, we find that the sequences of the species in the first clade have a similarity >94 %, but that those of the species of the second clade (e.g., containing *A. cycloheptanicus*) have a similarity of only ca. 91–94 %. The 16S rRNA gene sequence similarities with species of the *Kyripidia*, *Tumebacillus*, and *Effusibacillus* genera, all also members of the *Alicyclobacillaceae*, are 87–89 %. A complication is that in clade 2 there are two branches that contain sequences that are labelled *A. tolerans* (Fig. 1).

This division of the *Alicyclobacillus* type strains into two main clades was further investigated by comparison of whole genome data. To this end, we acquired whole genome data of the type strain of *A. tolerans* K1^T

Table 2

Examples of the presence of individual “CHC-CoA” BGCs in diverse bacteria other than *Alicyclobacillus* spp. and the reported presence of CHC-FAs (as the % of total FAs) in these bacteria. NCBI locus tag numbers of putative homologues are indicated.

Species	Genome	Genes of CHC-CoA BGC					CHC-FAs
		<i>ansJ</i>	<i>ansK</i>	<i>ansL</i>	<i>chcA</i>	<i>ansM</i>	(%)
Terrabacteria group; Actinomycetota; Rubrobacteria; Rubrobacteriales; Rubrobacteraceae							
<i>Rubrobacter</i> ^b <i>calidifluminis</i> RG-1 ^T	GCF_028617075.1	PJB24_RS12510	PJB24_RS12515	PJB24_RS12520	PJB24_RS12525	- ^c	41 ^d
Terrabacteria group; Actinomycetota; Micrococcales; Microbacteriaceae							
<i>Cnuiabacter</i> ^e <i>physcomitrellae</i> XA ^T	GCF_002096055.1	B5808_RS21315	B5808_RS21320	B5808_RS12150	B5808_RS12155	B5808_RS12160	16 ^f
<i>Herbiconiux</i> ^g <i>ginsengi</i> wged11 ^T	GCF_900107435.1	BLW00_RS15600	BLW00_RS15605	BLW00_RS15610	BLW00_RS23815	BLW00_RS2382	42 ^h
<i>Curtobacterium</i> ⁱ <i>pusillum</i> ATCC 19096 ^T	GCF_013359865.1	HP507_RS09410	HP507_RS09410	HP507_RS09415	HP507_RS09420	HP507_RS09425	26-96 ^j
<i>Humibacter</i> ^k <i>albus</i> DSM18994 ^T	GCF_000421825.1	H623_RS0104585	H623_RS0104585	H623_RS0104580	H623_RS0104575	H623_RS0104570	21 ^f
Terrabacteria group; Actinomycetota; Micromonosporales; Micromonosporaceae							
<i>Rugosimonospora</i> ^l <i>acidiphila</i> Delta1 ^T	GCF_039543385.1	ABEA87_RS37420	ABEA87_RS37420	ABEA87_RS37425	ABEA87_RS37430	ABEA87_RS37435	24-46 ^m
<i>Actinocatenispora</i> ^o <i>thailandica</i> TT2-10 ^T	GCF_039527605.1	ABD196_RS10255	ABD196_RS10255	ABD196_RS10250	ABD196_RS10245	ABD196_RS10240	- ^p
<i>Micromonospora</i> ^q <i>wenchangensis</i> 2602GPT1-05	GCF_002210435.1	B5D80_RS24645	B5D80_RS24645	B5D80_RS24650	B5D80_RS24655	B5D80_RS24660	- ^r
Terrabacteria group; Actinomycetota; Actinomycetes; Propionibacteriales; Propionibacteriaceae							
<i>Propionibacterium</i> ^s <i>cyclohexanicum</i> TA-12 ^T	GCF_900111365.1	BMX93_RS07815	BMX93_RS07815	BMX93_RS07820	BMX93_RS07825	BMX93_RS07830	53 ^t
Terrabacteria group; Bacillota; Bacilli; Bacillales; Sporolactobacillaceae							
<i>Pullulanibacillus</i> ^u <i>pueri</i> YN3 ^T	GCF_014639255.1	IEV91_RS05415	IEV91_RS05410	IEV91_RS05405	IEV91_RS05395 ^v	IEV91_RS05400 ^v	- ^w
<i>Scopulibacillus</i> ^x <i>darangshiensis</i> DLS-06 ^T	GCF_004341035.1	EV207_RS12135	EV207_RS12130	EV207_RS12125	EV207_RS12115 ^v	EV207_RS12120 ^v	- ^y
Terrabacteria group; Bacillota; Bacilli; Bacillales; Paenibacillaceae							
<i>Cohnella</i> ^z <i>thermotolerans</i> DSM 17683 ^T	GCF_000429825.1	G485_RS0102695	G485_RS0102690	G485_RS0102685	G485_RS0102675 ^v	G485_RS0102680 ^v	- ^{aa}
<i>Paenibacillus</i> ^{ab} <i>glycinis</i>	GCF_009909185.1	GT019_RS03905	GT019_RS03900	GT019_RS03895	GT019_RS03858 ^v	GT019_RS03890 ^v	- ^{ac}
		GT019_RS25700	GT019_RS25705	GT019_RS25710	GT019_RS25720 ^v	GT019_RS25715 ^v	
Terrabacteria group; Bacillota; Clostridia; Eubacteriales							
<i>Sulfobacillus</i> ^{ad} <i>thermosulfidooxidans</i> CBAR-13	GCF_001280565.1	AOA63_RS12190	AOA63_RS12185	AOA63_RS12180	AOA63_RS12170 ^v	AOA63_RS12175 ^v	up to 70 % ^{ae}

^a The presence of the genes is indicated by the NCBI locus tags. Note that *ansJ* and *ansK* are often fused and, in these cases, have the same locus tag. The genes always occur in the same order except for *chcA* and *ansM*. ^b The occurrence of the CHC-CoA BGC is scattered in genomes of this genus; it was only detected in the genomes of two out of nine species (i.e. *R. calidifluminis* and *R. naiadicus*). ^c *ansM* was not detected. ^d Sinninghe Damsté et al. (2023). ^e *Cnuiabacter physcomitrellae* is the only species in the *Cnuiabacter* genus with an available genome. ^f Zhou et al. (2016). ^g The occurrence of the CHC-CoA BGC is common in the eight available genomes of the *Herbiconiux* species; it was only not detected in *H. gentiana*. ^h Behrendt et al. (2011). ⁱ The occurrence of the CHC-CoA BGC is scattered in genomes of the *Curtobacterium* genus; it was detected in *C. ammoniigenes*, *C. citreum*, *C. flaccumfaciens*, *C. luteum* and *C. pusillum* but not in *C. caseinilyticum*, *C. herbarum*, *C. oceanosedimentum*, *C. salicis* and *C. subtropicum*. ^j Suzuki et al. (1981); the variation depends on growth stage, temperature, and glucose concentration in the medium. ^k The occurrence of the CHC-CoA BGC is scattered in genomes of the *Humibacter* genus; it was detected in *H. albus* but not in *H. ginsenosidimutans*. This latter species also does not produce CHS-FAs (Choi et al., 2022). ^l *H. antri*, *H. ginsengiterrae*, and *H. ginsengisoli*, species for which no genome data is currently available, do produce CHC-FAs in the range 28–52 % (Zhou et al., 2016). ^m The CHC-CoA BGC is absent in the other available genomes of the *Rugosimonospora* genus; i.e. *R. africana*. ⁿ Monciardini et al. (2009). CHC-FAs were absent in *R. africana*, despite that *R. africana* and *R. acidiphila* share 99.6 % 16S rRNA gene sequence identity. ^o The occurrence of the CHC-CoA BGC is common in genomes of the *Actinocatenispora* genus; it was only not detected in *A. rupis* out of 4 described species with whole genome data. ^p Thawai et al. (2006); the FAs were identified by the MIDI system and not confirmed by GC–MS analysis. The unsaturated FA C_{18:1w7c} was reported as a minor FA (2 %) and may perhaps represent the C₁₇ CHC FA. ^q The occurrence of the CHC-CoA BGC is sparse in genomes of the *Micromonospora* genus; it was only detected in *M. coxensis*, *M. rosaria*, *M. carbonacea*, *M. haikouensis*, *M. antibiotica*, *M. humidisola*, *M. wenchangensis*, *M. humida* and *M. matsumotoense* out of >110 described species with whole genome data. ^r Ren et al. (2013) the FAs were identified by the MIDI system and not confirmed by GC–MS analysis. The unsaturated FA C_{18:1w9c} was reported as a major FA (19 %) component and could represent the C₁₇ CHC FA. ^s The occurrence of the CHC-CoA BGC is scattered in genomes of the *Propionibacterium* genus; it was detected in *P. cyclohexanicum* but not in *P. freudenreichii*, *P. acidifaciens*, *P. ruminifibrum*, and *P. australiense*. ^t Kusano et al. (1997). ^u The only other available genome (i.e., *C. camelliae*) also contains the CHC-CoA BGC. ^v Note that, in contrast to most other configurations, *chcA* occurs at the end of the BGC. ^w Niu et al. (2015); the FAs were identified by the MIDI system and not confirmed by GC–MS analysis. The unsaturated FA C_{18:1w7c} was reported as a major FA (19 %) component in strain. In the related *C. camelliae* this FA comprised 45 % of the total FAs (Niu et al., 2016). In other cases (e.g. Behrendt et al., 2011) it was shown that the use of the MIDI system can lead to a misassignment of the C₁₇ CHC FA as the unsaturated FA C_{18:1w7c}. ^x The only other available genome (i.e., *S. daqui*) also contains the CHC-CoA BGC. ^y Lee and Lee (2009). ^z There are currently 29 genomes of described species of this genus; only 5 contain the CHC-CoA BGC: *C. caldifontis*, *C. lubricantis*, *C. lupini*, *C. thermotolerans*, and *C. zeiphillai*. ^{aa} Kämpfer et al. (2006); the unsaturated FA C_{18:1w7c} was reported as a minor FA (4 %) component in this strain and may represent the C₁₇ CHC FA. No details on FA identification were provided. Johnson et al. (2021) reported the unusually high abundance of the unsaturated FA C_{18:1w6c} with the MIDI system for FA analysis without GC–MS confirmation. Again, this could be the C₁₇ CHC FA. ^{ab} The occurrence of the CHC-CoA BGC is scattered in genomes of the *Paenibacillus* genus; it was only detected in *P. glycinis*, *P. artemisiicola*, and *P. humicola* and *P. beijingensis* out of >250 described species with whole genome data. *P. glycinis* is remarkable since it contains two CHC-CoA BGCs; their protein sequences are quite different. There is one other *Paenibacillus* strain (R14; GCF_019431355.1) that also contains two intact CHC-CoA BGCs. ^{ac} Wang et al. (2021); the unsaturated FA C_{18:1w6c}/C_{18:1w7c} was reported as a minor FA (2 %) component in this strain and may represent the C₁₇ CHC FA because

only the MIDI system was used for FA identification.^{ad} The occurrence of the CHC-CoA BGC is scattered in genomes of the *Sulfobacillus* genus; it was only detected in the genome of one out of seven strains of *S. thermosulfidooxidans* and not in any other *Sulfobacillus* species.^{ae} Tsaplina et al. (1994).

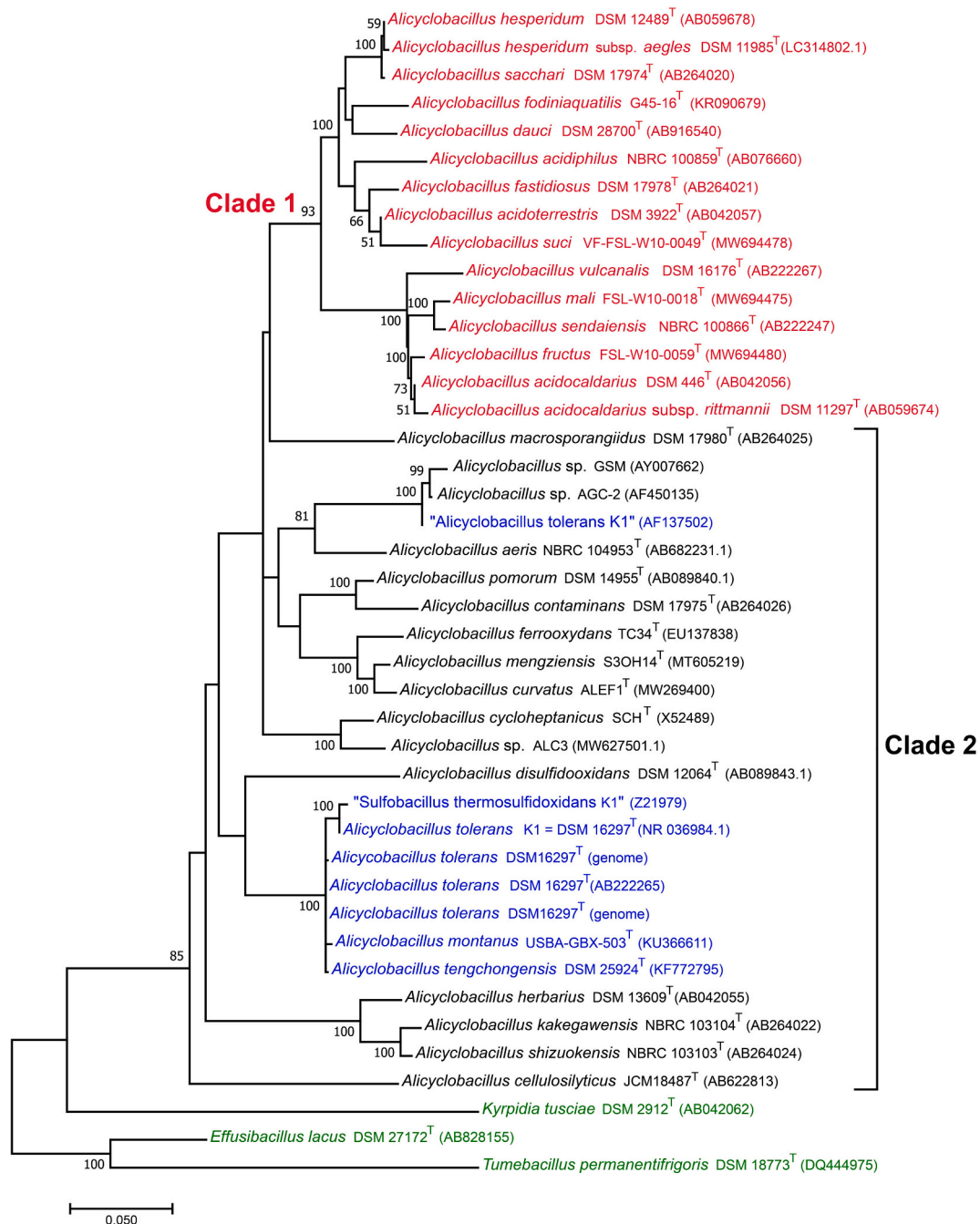


Fig. 1. Phylogenetic tree of the 16S rRNA gene sequences (composed of 1379 bp, base pairs) of all type strains of *Alicyclobacillus* spp. with an available whole genome (Table 1) and some additional relevant *Alicyclobacillus* spp. 16S rRNA gene sequences of other members of the *Alicyclobacillaceae*. The 16S rRNA gene sequences of other species of the *Alicyclobacillaceae* (i.e., *Tumebacillus* spp., *Effusibacillus* spp., and *Kyrpidia tusciae*) were used as outgroups (shown in green font). The sequences belonging to Clade 1 are colored in red. All the 16S rRNA gene sequences related to *A. tolerans* (see text for extensive discussion) falling in the remaining clade of *Alicyclobacillus* spp. are colored in blue; others are in black. The phylogenetic tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Evolutionary analyses were conducted in MEGA7. The accession numbers of the sequences used in the tree are also listed in Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(= DSM 16297^T), which was lacking up to now. Some characteristics of this genome are reported in Table S3. The phylogenetic position of the various *Alicyclobacillus* type strains were determined based on the proteome-based genome BLAST distance phylogeny by using the TYGS

platform (Meier-Kolthoff and Göker, 2019). The resulting phylogenomic tree (Fig. 2) shows that the type species of the genus *Alicyclobacillus*, *A. acidocaldarius*, forms a distinct clade of species, comprised of the same species as the Clade 1 in the 16S rRNA gene tree (cf. Figs. 1 and 2). The

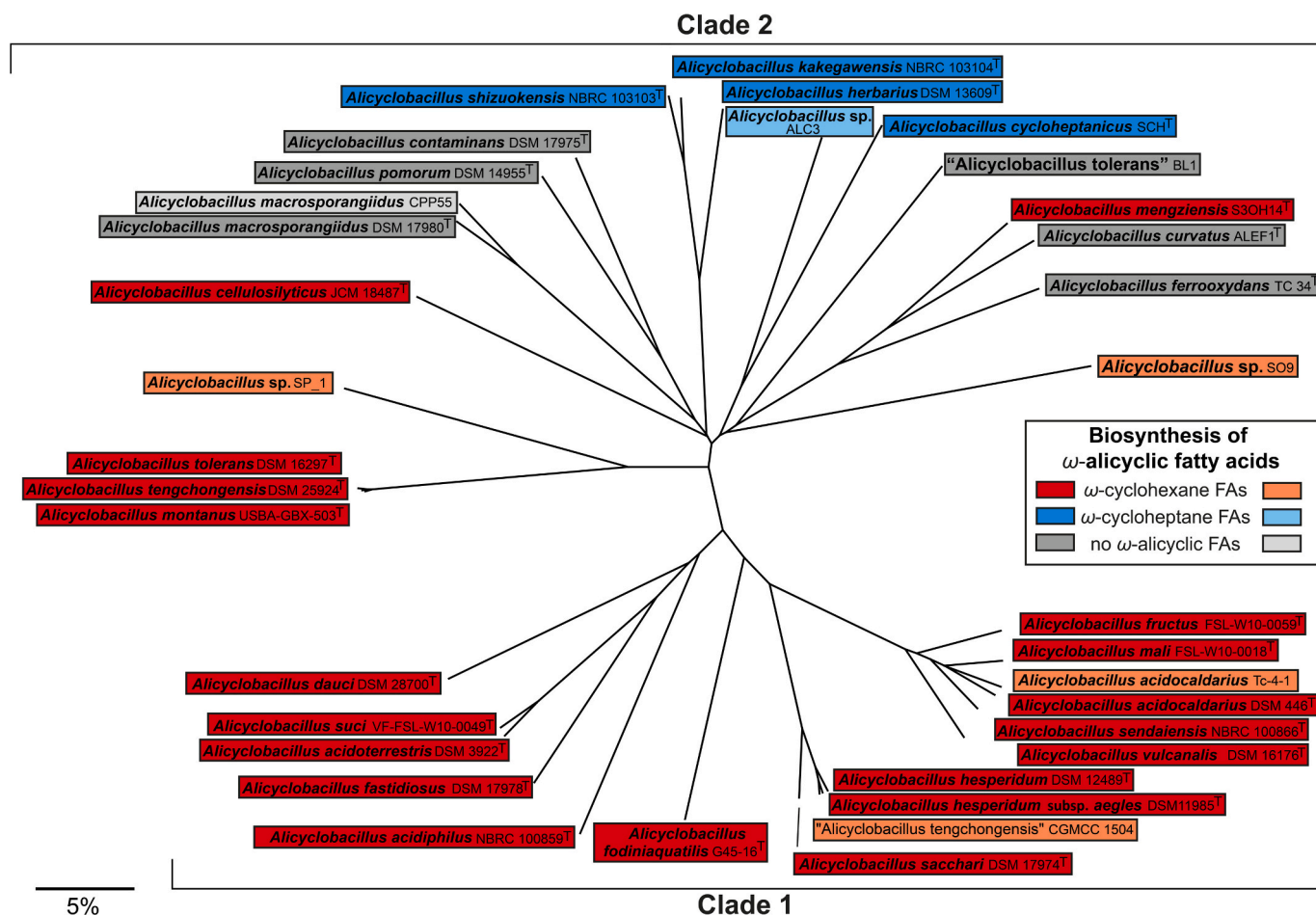


Fig. 2. Phylogenomic tree based on the whole proteome of all type strains of *Alicyclobacillus* spp. with an available whole genome. The tree reveals the clear distinction of the *Alicyclobacillus* spp. in different clades. The genome of “*Alicyclobacillus tolerans*” BL-1 (GCF_021739485.1), which has earlier mistakenly been labelled as *A. tolerans* (see text), has also been included. It was isolated from drainage of the the Çan coal mine (Biga Peninsula, Western Turkey). The ability to produce specific ω -alicyclic FAs of all *Alicyclobacillus* sp. is indicated by the color code (using the latest analysis and interpretations; see text). The faint colors indicate anticipated production only on basis of the presence or absence of CHC-CoA and CHP-CoA BGCs, not verified by actual FA analysis. “*Alicyclobacillus tolerans*” BL-1 likely does not produce any ω -alicyclic FAs since both the CHC-CoA and CHP-CoA BGCs are absent in its genome. Furthermore, its 16S rRNA gene is for ca. 99 % identical to that of *Alicyclobacillus* sp., the 16S rRNA gene (AY007662) of *Alicyclobacillus* strain GSM, which does not produce ω -alicyclic FAs (Yahya et al., 2008). Two unverified genome sequences from strains “*Alicyclobacillus tengchongensis*” CGMCC 1504”, and “*Alicyclobacillus curvatus* ALEF1” were also included for the sake of completeness in the analysis. Both have now been suppressed by NCBI due to its unverified source, but for the type strain of *A. curvatus* there is currently no other genome sequence available. The tree was inferred with FastMe 2.1.6.1 (Lefort et al., 2015) from GBDP distances calculated from genome sequences. The accession numbers of all the genomes used in the tree are listed in Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

second clade (Clade 2 in Fig. 2) is more heterogeneous. This analysis also revealed that *A. tolerans* K1^T, *A. tengchongensis* DSM 25924^T and *A. montanus* USB-GBX-503^T are closely related: the Average Nucleotide Identity (ANI) values of these genomes is ca. 99 % (Table S4). The two 16S rRNA gene sequences from the genome of *A. tolerans* K1^T matched that previously obtained from this type strain stored at DSMZ, unambiguously assessing the position of *A. tolerans* K1^T in the 16S rRNA gene tree. The reasons for the incorrect assignment of a second branch to *A. tolerans* in the 16S rRNA gene tree (Fig. 1) are discussed in the taxonomy section.

The annotated protein sequences coded by the genomes were also analyzed with a tool provided by Kostas Lab (<http://enve-omics.ce.gatech.edu/g-matrix/>) to generate an Average Amino Acid Identity (AAI) matrix (Table S4). These data also revealed that the species in Clade 1 are clearly separated from the other *Alicyclobacillus* species. For example, the AAI values of *A. acidocaldarius* in comparison with the other species falling in Clade 1 are 57–92 %, whilst those with the species of Clade 2 are 53–57 % (Table S4). The AAI matrix was also used for hierarchical clustering of the input genomes and the resulting tree

(Fig. S1) reveals the same two clusters as indicated in the phylogenomic tree.

Chemotaxonomical differences between the two different clades in the genus *Alicyclobacillus*

The existence of two different clades of *Alicyclobacillus* species fits well with their reported ability to produce ω -alicyclic FAs (and the presence of genes encoding their biosynthetic pathway; see below). All *Alicyclobacillus* species falling in Clade 1 are able to produce ω -cyclohexyl FAs (Fig. 2). In contrast, this trait is much less prominent in the Clade 2: some species produce ω -cyclohexyl FAs, some produce ω -cycloheptyl (C7) FAs, and quite some species are not producing any ω -alicyclic FAs at all (and do not possess the genes to do so; see below) (Fig. 2). Hence, the FA distributions do support, to some extent, the division of the *Alicyclobacillus* genus into two different clades. These differences in phenotypes probably have an underlying genetic reason. We, therefore, evaluated all available genomes of type strains of *Alicyclobacillus* in this respect.

A putative BGC for production of CHC-CoA in *Alicyclobacillus* species

Cyclohexane carboxylic acid CoA thioester (CHC-CoA) has been shown to act as the starter unit for the production of ω -cyclohexyl FAs in *A. acidocaldarius* and is derived from shikimate (Moore et al., 1993). Typically, the biosynthesis of such specific building blocks are encoded in the genome by so-called biosynthetic gene clusters (BGCs). BGCs can be defined as physically clustered groups comprised of ≥ 2 genes that together encode a biosynthetic pathway for the production of a specialized metabolite (Medema et al., 2015). In a number of *Streptomyces* spp. the BGC for the production of CHC-CoA has been well documented, and forms part of much larger BGCs that encode the biosynthesis of complex natural products such as ansatrienin and other mycotrienins, and asukamycins, which all contain a cyclohexyl moiety (e.g., Cropp et al., 2000; Skyrud et al., 2020). The “CHC-CoA” BGC constitutes 5 genes (*ansJ-ansM*, of which the first two in some species are fused, and *chcA*), which together encode the proteins of a complex eight-step pathway in which shikimate is converted to CHC-CoA (Fig. 3). Three genes comprising this BGC (*chcA*, *ansK* and *ansL*) have been extensively characterized (Wang et al., 1996; Skyrud et al., 2020). The gene *ansK* encodes shikimoyl-CoA synthetase, the first step in the eight-step pathway (Skyrud et al., 2020; Fig. 3). The gene *chcA* encodes an

$\Delta^{1,2}$ -enoyl-CoA reductase, proposed to catalyze three separate $\Delta^{1,2}$ -bond reductions in the pathway from shikimate to CHC-CoA (Reynolds et al., 1992; Wang et al., 1996). The *ansL* gene was shown to encode a 3-hydroxy-CHC-CoA dehydratase that promotes a non-redox dehydration reaction in an FAD-dependent manner (Skyrud et al., 2020), the penultimate step in the pathway (Fig. 3). The other two genes (*ansJ* and *ansM*) are less well characterized. *AnsJ* is a protein homologous to 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (AroA), catalyzing a conversion step in the shikimate pathway (Fig. 3). Attempts to isolate this enzyme were unsuccessful (Skyrud et al., 2020) and its anticipated role in the production of CHC-CoA through dehydration of shikimate (Fig. 3; two steps) remains elusive. *AnsM* has a clear sequence homology to various 2,4-dienoyl CoA reductases (Cropp et al., 2000), including the FadH of *Escherichia coli*, which catalyzes a $\Delta^{4,5}$ -bond reduction of a dienoyl CoA substrate, generated in the degradation of unsaturated FAs with *cis* double bonds (He et al., 1997). A similar reduction probably occurs in the fifth step of the CHC-CoA biosynthesis (Fig. 3). Skyrud et al. (2020) showed that all five genes were required to allow *E. coli* to produce CHC-CoA.

The occurrence of ω -cyclohexyl FAs is not limited to *Alicyclobacillus* spp. They are known to be produced by a variety of phylogenetically diverse thermo- and mesophilic bacteria: e.g., *Curtobacterium pusillum*

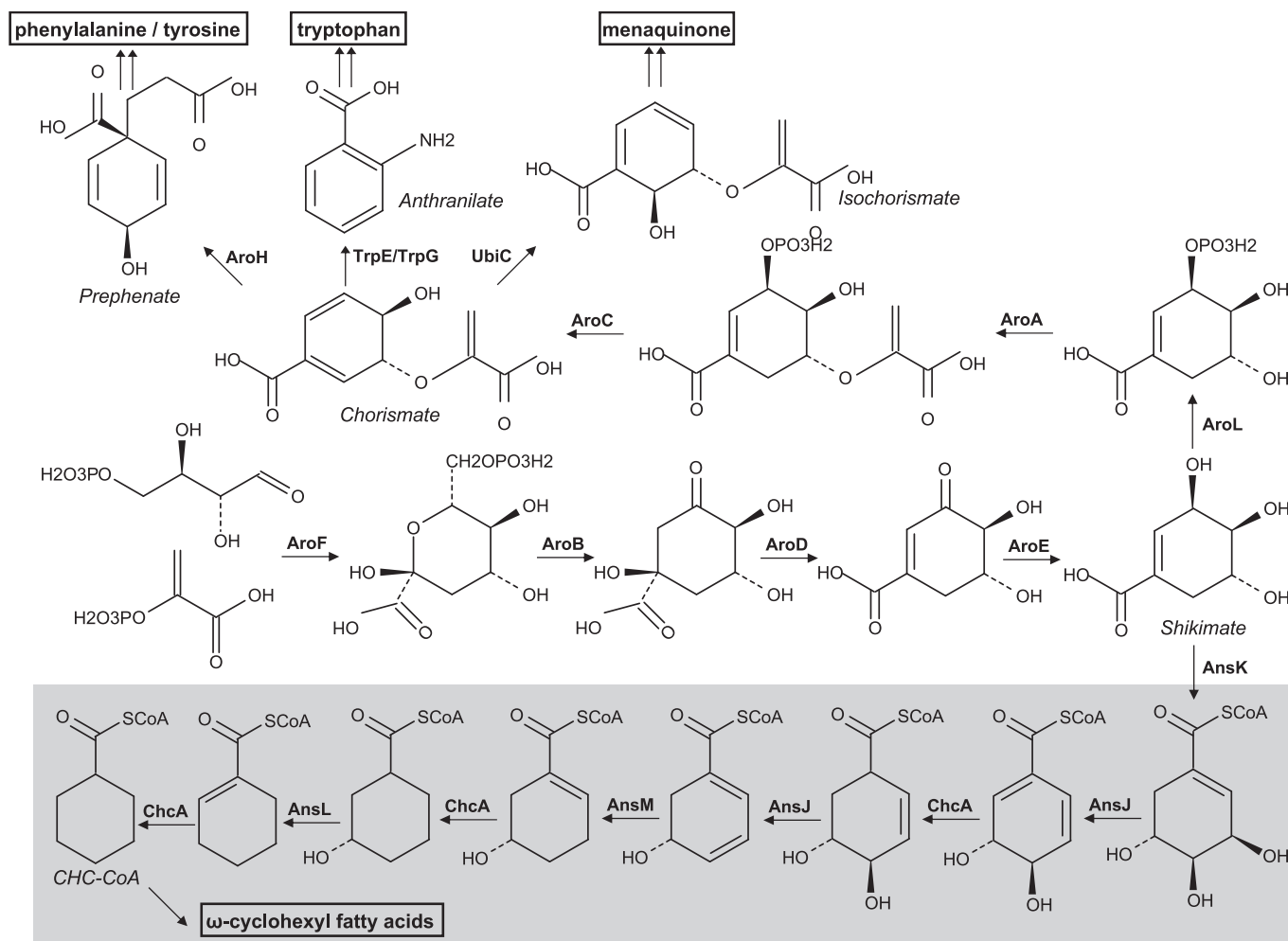


Fig. 3. Proposed biosynthetic pathway for the precursor of ω -cyclohexyl FAs, i.e. CHC-CoA in *Alicyclobacillus* spp. as a branch of the shikimate pathway. This pathway ultimately leads to the biosynthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, and menaquinone MK_{7,X} among other products (see for a recent review see Shende et al., 2024). In *Alicyclobacillus* Clade 1 and in some species of Clade 2 an important branching point occurs at shikimate; in a series of dehydration, reduction, and isomerization steps (indicated in the colored area), presumed to be catalyzed by five enzymes, shikimate is converted into CHC-CoA, an important building block for the biosynthesis of ω -cyclohexyl FAs (after Cropp et al., 2000; Skyrud et al., 2020). Note, however, although indicated in the pathway, the gene encoding *AnsJ* is apparently absent in all but one of the genomes of *Alicyclobacillus* spp. capable of biosynthesis of ω -cyclohexyl FAs (see text).

(Suzuki et al., 1981), *Propionibacterium cyclohexanicum* (Kusano et al., 1997), and *Rubrobacter calidifluminis* and *R. naiadicus* (Sinninghe Damsté et al., 2023). Sinninghe Damsté et al. (2023) showed that these two *Rubrobacter* species indeed contain a CHC-CoA BGC in their genomes, composed of four of the five genes, with only *ansM*, encoding an intermediate step in the production of CHC-CoA (Fig. 1), lacking for unknown reasons. To follow this up, we examined the occurrence of the CHC-CoA BGC in these and some other bacteria by PSI-BLAST searches using available genomes of bacteria. We detected this BGC in several species in the phyla *Actinomycetota* and *Bacillota* of the kingdom *Bacillati* (Table 2). The organization of these BGCs was like that initially discovered in *Streptomyces* spp. (Fig. 4a). For quite a number of species, the abundant production of ω -cyclohexyl FAs by these species has been reported (Table 2), again confirming the link between the CHC-CoA BGC and the production of these FAs. In other cases, the reported FA composition did not reveal the presence of ω -cyclohexyl FAs (Table 2). However, in many of these cases the identification of the FAs was not performed using characterization by mass spectrometry, resulting in suspicious assignments of FAs (see footnotes in Table 2).

Remarkably, in many cases, only a limited number of species of a genus contained the CHC-CoA BGC, whilst in the other species the BGC was absent (see footnotes in Table 2). This is in line with the study of Suzuki and Komagata (1983), who reported the FA profiles of 19 different strains of *Curtobacterium* spp. and only those belonging to *C. pusillum* produced ω -cyclohexyl FAs, most likely related to the presence/absence of the CHC-CoA BGC. This situation is like the one described for the *Alicyclobacillus* (Ciuffreda et al., 2015) and *Rubrobacter* (Sinninghe Damsté et al., 2023) genera, where only a limited number of species are able to produce ω -cyclohexyl FAs. In the case of the *Rubrobacter* spp., these were indeed the two species containing the CHC-CoA BGC in their genome (Sinninghe Damsté et al., 2023). The restricted occurrence of the CHC-CoA BGC suggests that the ability to synthesize ω -cyclohexyl FAs is easily lost and possibly gained during microbial evolution.

Next, we focused our attention on the potential presence of the CHC-CoA BGC in the genomes of the *Alicyclobacillus* type strains. PSI-BLAST searches of the proteins encoded by their genomes did reveal the presence of 4 of the 5 genes required for production of CHC-CoA and showed that they are organized in a BGC (Tables 3 and S5; Fig. 4a). The closest match within the *Streptomyces* group was with *S. humidus* (Fig. 4b). The CHC-CoA BGC was detected in 17 available NCBI reference genomes of *Alicyclobacillus* spp. (Table 3). Hence, the most plausible explanation for the biosynthesis of ω -cyclohexyl FAs in these species is the presence of this BGC in their genomes. However, the apparent absence of a homologue of the *ansJ* gene remains a challenging question because Skyrud et al. (2020) showed that all five genes (i.e., *ansJ-ansM* and *chcA*) were required to allow *E. coli* to produce CHC-CoA.

Inspection of the *Alicyclobacillus* genomes revealed that a fifth gene was always adjacent to the putative 4-gene BGC responsible for the formation of CHC-CoA. This is a gene (annotated as *aroF*) encoding a close homologue (57–67 % sequence homology at the protein level) of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, which catalyzes the first step of the shikimate pathway, i.e. aldol condensation of phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) to DAHP (Fig. 3). The end product of the shikimate pathway is chorismic acid, which acts as precursor for a wide range of aromatic compounds, like ubiquinones, and the aromatic amino acids, tyrosine, phenyl alanine, and tryptophan (Fig. 3). As the first enzyme of the shikimate pathway, the production of chorismic acid is controlled by regulating the activity of DAHP synthase by various feed-back mechanisms. For example, *E. coli* has three different genes encoding DAHP synthase iso-enzymes, which contain different regulatory domains responsive to cellular concentrations of tyrosin (*aroF*), phenylalanine (*aroG*), and tryptophan (*aroH*) (Byng et al., 1983). Only the *E. coli aroF* gene showed some similarity (i.e., a sequence homology of 17–21 % at the protein level; E values around 1E-100) with the *aroF* genes in the *Alicyclobacillus*

genomes. A much higher sequence homology (47–56 % at the protein level) was observed with the well characterized DAHP synthase of *Thermotoga maritima*, which is allosterically regulated by tyrosine (Cross et al., 2011), falling in the regulated Type 1 β DAHP synthases (Light and Anderson, 2013). Several (i.e., 2–4) copies of *aroF* genes are present in the *Alicyclobacillus* genomes (Table 3 and S5) and are, in most cases, phylogenetically defined by falling in specific clades. The group of *aroF* genes that encode DAHP synthases most closely related to that of *T. maritima* (50–56 % sequence similarity at the protein level, size 343–350 AA) occurs in all available genomes of *Alicyclobacillus* type strains. This group of *aroF* genes (designated as *aroF*-1 in Table 3) is always contained in a 10–11 gene containing operon composed mostly of genes encoding the enzymes required to produce aromatic amino acids and some genes of the of the shikimate pathway (e.g. Table S5). A similar operon, the so-called aromatic supra-operon, comprised of 12 genes, is characteristic for *Bacillus* spp. (e.g. Merino et al., 2008; see Table S5 for *B. subtilis*). Various *Alicyclobacillus* sp. contain (an) additional *aroF* gene(s) (listed as *aroF* 2–5; Table 3). The last group of DAHP synthases (*aroF*-5; Table 3) is of similar size as *AroF*-1 but the characteristic 65 amino acid N terminal extension shows no similarity with that of *AroF*-1. The *aroF* genes encoding these DAHP synthases form the first gene in the CHC-CoA BGC and only occur in *Alicyclobacillus* spp. that contain such a BGC (Fig. 4a; Table S5).

We can only speculate about the reason for the presence of the *aroF*-5 gene encoding DAHP synthase in the CHC-CoA BGC but it should be noted that the ω -cyclohexyl FAs often form >80 % of the FAs in *Alicyclobacillus* spp., and, hence, form an essential building block for the lipid membrane. In contrast to bacteria where CHC-CoA is only required for the production of complex natural products occurring in relatively low intracellular concentration, such as ansatrienin and other myco-terriens, and asukamycins in various *Streptomyces* spp. (e.g., Cropp et al., 2000; Skyrud et al., 2020), the branch point half-way the shikimate pathway resulting in production of CHC-CoA (Fig. 3) may thus be essential for growth of some *Alicyclobacillus* spp. The production of DAHP is therefore likely not only regulated by intracellular concentrations of aromatic amino acids but when the CHC-CoA BGC is turned on, production of DAHP would be essential. It is tempting to suggest that the entirely different 65 AA-long N terminal extension of this specific *aroF* gene plays a role in the allosteric control of this branching point. Further research is required to confirm this hypothesis. If confirmed, it would explain why in the genomes of *Alicyclobacillus* spp. a copy of the *aroF* gene is part of the CHC-CoA BGC.

The observation that the first gene in the CHC-CoA BGC of all *Alicyclobacillus* species that contain this gene operon is replaced (i.e., from an *aroA*-like to an *aroF*-like gene) leads to some further insight. Firstly, it indicates that the presence of *ansJ* (the *aroA*-like gene) is likely not essential for the biosynthesis of CHC-CoA because many *Alicyclobacillus* spp. can biosynthesize ω -cyclohexyl FAs without the presence of *ansJ* in the CHC-CoA BGC; only the genomes of *A. mengziensis* and *Alicyclobacillus* sp. SO9 contain *ansJ* (Table 3; Fig. 4a). Secondly, this observation questions the proposed function of the AnsJ protein in the dehydration of two intermediates in the CHC-CoA pathway (Fig. 3).

If we assume that the new putative CHC-CoA BGC is indeed an essential requirement for the biosynthesis of ω -cyclohexyl FAs, one may expect to see a good correspondence between the presence of this BGC in the genome and the production of ω -cyclohexyl FAs. This is indeed what is observed (Table 3): all species that fall in clade 1 possess the CHC-CoA BGC and produce ω -cyclohexyl FAs. In clade 2, the situation is more complicated. *A. cellulosityticus* has been shown to produce ω -cyclohexyl FAs (Table 1) but is missing the *aroF*-5 gene (Table 3) and the BGC is broken into two pieces. Re-analysis of the FA composition of *A. cellulosityticus* (see Table 4) confirmed, however, that it produces ω -cyclohexyl FAs. Hence, the presence of the *aroF*-5 gene is not essential for production of ω -cyclohexyl FAs, in line with its proposed “trafficking” function. Three other species (*A. mengziensis* S30H14^T, *A. montanus* USDA-GBX-503^T, and *A. tengchongensis* DSM 25924^T)

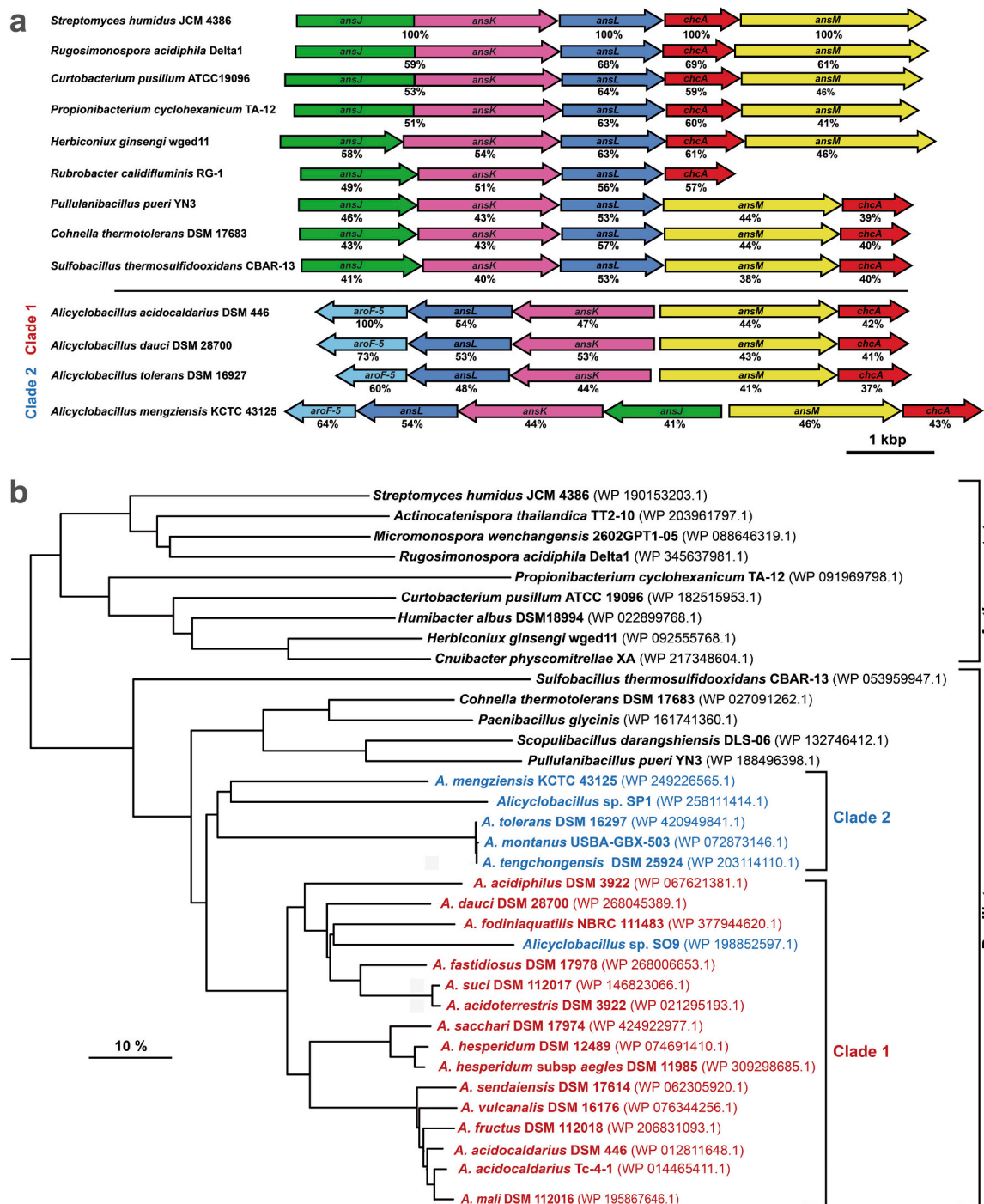


Fig. 4. Architecture of the CHC-CoA BGCs and phylogeny of one of the proteins encoded by this gene cluster. (a) Examples of the architecture of the CHC-CoA BGCs in genomes of various bacteria composed of five genes (*ansJ*, *ansK*, *ansL*, *chcA*, and *ansM*) encoding the enzymes catalyzing the biosynthesis of CHC-CoA. In *Streptomyces humidus* this BGC forms part of a much larger BGC (not shown) responsible for the biosynthesis of ansatrienin, comparable to that identified in *S. collinus* (Cropp et al., 2000). The CHC-CoA BGC occurs in a wide variety of bacteria (see for example Table 2). The *ansJ* and *ansK* genes are often fused but also occur as separate genes. *R. calidifluminis* is lacking the *ansM* gene but is still able to produce cyclohexane FAs (Sinninghe Damsté et al., 2023). The arrangement of genes in the CHC-CoA BGC is different in *P. pueri*, *C. thermotolerans*, and *S. thermosulfidooxidans*. In *Alicyclobacillus* spp. the organization of the CHC-CoA BGC is quite different because it lacks *ansJ* (except for *A. mengziensis*), which is replaced by an *aroF*-like gene and the arrangement of the genes is also different. The sequence similarity of protein homologues compared to those of *S. humidus* (based on a single PSI-BLAST) is shown below the genes. In case of the *aroF*-like genes, this comparison was made with the gene of *A. acidocaldarius*. The accession numbers of all sequences used in the figure and locus tags of the genes are listed in Table S5. (b) The phylogeny of the AnsM protein encoded by the largest gene of the CHC-CoA BGC. The species in the tree are those listed in Table 2 and all but one *Alicyclobacillus* spp. that possess a CHC-CoA BGC. AnsM of *A. cellulosilyticus* was excluded since the *ansM* gene was fragmented. The tree shows a clear separation of the AnsM sequences of the Actinomycetota and Bacillota. Within the later phylum, the *Alicyclobacillus* spp. form a distinct group that falls apart in Clade 1 and 2 as seen in other trees (Figs. 1 and 2). The accession numbers of the proteins used are given in brackets. The tree was constructed using software using the Fast Minimum Evolution method provided at the NCBI website after sequence alignments using a twofold PSI-BLAST search. All sequences had a query cover of 95–99 % with the query sequence (AnsM of *Streptomyces humidus*; WP_190153203.1) with an identity between 37 and 53 % (*E*-value 0.0). An oxidoreductase of *Alicyclobacillus* sp. SP1 (WP_258112405.1) was used as an outgroup (not shown in the tree). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

The presence and absence of genes of the shikimate pathway and of the CHC-CoA BGCs in NCBI reference genomes of type strains of *Alicyclobacillus* and some other relevant genomes of *Alicyclobacillus* spp. and the reported presence of alicyclic FAs in these bacteria. The NCBI locus tags for the genes and the accession numbers for the encoded proteins are listed in Table S5.

Species	Strain	Genes shikimate pathway ^a										Genes CHC-CoA BGC ^b							Alicyclic
		<i>aroF-1</i>	<i>aroF-2</i>	<i>aroF-3</i>	<i>aroF-4</i>	<i>aroB</i>	<i>aroD</i>	<i>aroE</i>	<i>aroK</i>	<i>aroA</i>	<i>aroC</i>	<i>aroF-5</i>	<i>ansL</i>	<i>ansK</i>	<i>ansJ</i>	<i>ansM</i>	<i>chcA</i>	FAs ^c	
CLADE 1																			
<i>A. acidiphilus</i>	DSM 14558 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. acidocaldarius</i>	DSM 446 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
	Tc-4-1	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	n.d.
<i>A. acidoterrestris</i>	DSM 3922 ^T	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. dauci</i>	DSM 28700 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. fastidiosus</i>	DSM 17978 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. fodiniaquatilis</i>	NBRC 111483 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. fructus</i>	DSM 112018 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. hesperidum</i>	DSM 12489 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. hesperidum subsp aegles</i>	DSM 11985 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. mali</i>	DSM 112016 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. sacchari</i>	DSM 17974 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. sendaiensis</i>	DSM 17614 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. suci</i>	DSM 112017 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
<i>A. vulcanalis</i>	DSM 16176 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
“ <i>A. tengchongensis</i> ”	CGMCC1504	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	n.d.
CLADE 2																			
<i>A. cellulolyticus</i>	JCM 18487 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	–	✓ ^d	✓	–	✓	✓	incomplete	C6
<i>A. contaminans</i>	DSM 17975 ^T	✓	✓	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. cycloheptanicus</i>	DSM 4006 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	✓ ^e	absent	C7
<i>A. curvatus</i>	ALEF1 ^T	✓	✓	✓	–	✓	✓	–	–	✓	✓	–	–	–	–	–	–	absent	–
<i>A. ferrooxydans</i>	DSM 22381 ^T	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. herbarius</i>	DSM 13609 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	✓ ^e	absent	C7
<i>A. kakegawensis</i>	DSM 17979 ^T	✓	✓	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	✓ ^e	absent	C7
<i>A. macrosporangii</i>	DSM 17980 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
	CPP55	✓	–	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	n.d.
<i>A. mengziensis</i>	KCTC 43125 ^T	✓	✓	✓	–	✓	✓	✓	✓	✓ ^d	✓	✓	✓	✓	✓	✓	✓	complete	C6 ^f
<i>A. montanus</i>	USBA-GBX-503 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6 ^f
<i>A. pomorum</i>	DSM 14955 ^T	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. shizuokensis</i>	DSM 17981 ^T	✓	–	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	✓ ^e	absent	C7
<i>A. tengchongensis</i>	DSM 25924 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6 ^g
<i>A. tolerans</i>	DSM 16297 ^T	✓	–	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	complete	C6
“ <i>A. tolerans</i> ”	BL-1	✓	–	✓	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>Alicyclobacillus</i> sp.	ALC3	✓	✓	–	–	✓	✓	✓	✓	✓	✓	–	–	–	–	–	✓ ^e	absent	n.d.
	SO9	✓	✓	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	complete	n.d.
	SP_1	✓	–	–	–	✓	–	✓	✓	✓	✓	–	✓	✓	–	✓	✓	incomplete	n.d.

^a The presence of the various genes responsible for encoding the proteins of the shikimate pathway are indicated. They are ordered according to the various steps in the pathway (see Fig. 3). Various *aroF* were detected. The *aroF-1* gene occurs in all *Alicyclobacillus* strains and is part of the so-called aromatic supra-operon (see text and Table S5). The *aroF-2* gene is not adjacent to any of the genes of the shikimate or follow-up pathways. The *aroF-3* gene always occurs in a small, 2-gene BGC together with a second copy of *aroH*, the gene encoding chorismate mutase (Fig. 3). The *aroF-4* gene is a bifunctional gene, probably a fusion of *aroF-4* and *aroH*. The *aroD* gene is a Type II. ^b Presence and absence of genes of the CHC-CoA BGC in order of appearance in the genomes (e.g. Fig. 4a). The gene *ansJ* is absent in all but one genome. The gene *aroF-5* is in most cases the starting gene of this cassette. If the five genes were detected the BGC is described as “complete”, if 1–3 genes are missing as “incomplete” and otherwise as “absent”. ^c The occurrence of ω -alicyclic FAs as described in Table 1 but adjusted to our new analyses and re-interpretations (see footnotes Table 1). ^d Two copies occur in the genome. ^e Note that, although these *chcA* gene are phylogenetically closely related to the other *chcA* genes, they are part of another BGC (see Table 5 and text). ^f reinterpreted as explained in footnotes of Table 1. ^g Kim et al. (2014) reported that *A. tengchongensis* produced cycloheptane FAs but our independent re-analysis revealed that it does not produce cycloheptane but cyclohexane FAs (see text and Table 4).

Table 4

The fatty acid composition (in % of sum; only relative abundances >0.5 % are given; tr denotes traces; relative abundances >10 % in bold figures) of five *Alicyclobacillus* spp. belonging to Clade 2 re-cultivated and analyzed after acid hydrolysis of whole cells.

Fatty acid ^a		<i>A. cellulosilyticus</i> DSM 107339 ^T	<i>A. tengchongensis</i> DSM 25924 ^T	<i>A. tolerans</i> DSM 16297 ^T	<i>A. cycloheptanicus</i> DSM 4006 ^T	<i>A. shizuokensis</i> DSM 17981 ^T
C15	iso	tr	tr	0.9	0.7	tr
	anteiso	tr	0.8	4.9	tr	–
	n-	tr	–	–	–	1.1
C16	iso	2.6	7.9	18.9	0.9	1.9
	n-	3.6	tr	1.6	–	2.6
	n- (uns)	tr	tr	0.7	–	tr
	ωC7	–	–	–	tr	1.2
C17	iso	tr	3.3	3.1	4.6	1.8
	anteiso	0.9	13.7	30.0	1.0	tr
	anteiso (uns)	–	–	1.5	–	–
	n-	0.9	tr	–	–	4.4
	ωC6	42.3	66.6	34.5	–	–
	ωC7	–	–	–	0.5	2.1
C18	iso	tr	tr	0.8 ^b	–	0.7
	n-	0.7	tr	0.6	–	0.5
	ωC6	–	tr	1.6	–	–
	ωC7	–	–	–	90.0	76.2
C19	ωC6	49.0	7.6	0.9	–	–
	ωC7	–	–	–	0.7	0.9
C20	ωC7	–	–	–	1.6	6.8

^a The fatty acid identity is revealed by the total number of carbon atoms followed by the identity of its carbon skeleton. Key: n- = normal, ωC6 = ω-cyclohexyl, ωC7 = ω-cycloheptyl, uns = double bond at unspecified position.

^b co-elutes with monounsaturated n-C18; the percentage given reflects the sum.

harbor the complete BGC (Table 3) but their reported FA distribution (Table 1) is not in line with that. *A. tengchongensis* has been reported to produce ω-cycloheptyl FAs (Kim et al., 2014). We reanalyzed the FA composition of *A. tengchongensis* DSM 25924^T, which is available in the DSMZ culture collection in contrast to the other two species. This revealed that *A. tengchongensis* does not produce ω-cycloheptane but ω-cyclohexane FAs (Table 4). The two other strains, *A. mengziensis* S30H14^T and *A. montanus* USBA-GBX-503^T, have been reported not to produce any ω-alicyclic FAs (Kim et al., 2014; López et al., 2018). However, we judge the reported FA composition of these two species to be incorrect (see footnotes in Table 1) and suppose that they also produce ω-cyclohexyl FAs. For *A. montanus* USBA-GBX-503^T this would be in line with its very close genetic compositional relationship to *A. tolerans* K1^T (e.g., many of their encoded protein coding sequences are identical). Hence, also in Clade 2, a 1:1 relationship between the presence of an (almost) complete CHC-CoA BGC and production of ω-cyclohexyl FAs is observed. It is remarkable that only 5 out of the 14 type strains of Clade 2 possess the CHC-CoA BGC, in contrast to all of the type strains of Clade 1 that do contain this BGC in their genomes. A phylogenetic tree of AnsM (Fig. 4b), the largest protein of this BGC (Fig. 4a), still shows a close relatedness in comparison to other ω-cyclohexyl FA-producing species. This suggests that the common ancestor of the *Alicyclobacillus* spp. contained a CHC-CoA BGC in its genome and that most species of Clade 2 have lost this BGC, as observed for other genera like *Curtobacterium* and *Rubrobacter* (Suzuki and Komagata, 1983; Sinninghe Damsté et al., 2023). Despite their close relatedness, the AnsM phylogeny (Fig. 4b) clearly supports the separation of the *Alicyclobacillus* spp. into the two clades, with the less characterized *Alicyclobacillus* sp. SO9 isolate as a notable exception.

Interestingly, five other species of Clade 2 still did contain one of the five genes of the *Alicyclobacillus* CHC-CoA BGC (i.e. the *chsA* gene) but these species do not produce ω-cyclohexyl FAs but instead produce ω-cycloheptyl FAs (Table 3). This observation directed us to the

detection of a putative gene BGC potentially responsible for the formation of cycloheptyl-CoA.

A putative BGC for production of cycloheptyl-CoA in Alicyclobacillus species

The biochemical pathway for the biosynthesis of ω-cycloheptyl FAs in *Alicyclobacillus* spp. has not been fully established. Moore et al. (1997) showed that cycloheptane carboxylic acid, probably as its CoA thioester, is the starter unit for ω-cycloheptyl FAs. This starter unit is most probably derived from phenyl alanine, also produced via the shikimate pathway (Fig. 5), or phenylacetic acid (PAA) as demonstrated by labeling experiments (Moore et al., 1997). This requires the expansion and subsequent hydrogenation of a 6-ring aromatic compound to the 7-membered cycloheptane carboxylic acid. Moore et al. (1997) proposed an oxidative ring-expansion of PAA to a seven membered carbon cycle intermediate followed by a series of double bond reductions and dehydrations resulting in the formation of the saturated cycloheptane carboxylic acid (Fig. 5). Teufel et al. (2010) elucidated the complete aerobic pathway for phenylacetate degradation operative in many bacterial species, including *E. coli*. The genes encoding the required enzymes often form a distinct BGC (Ferrández et al., 1998). Interestingly, all intermediates are processed as CoA thioesters formed in the first step through catalysis by PaaK, and the aromatic ring of phenylacetyl-CoA becomes activated to a ring 1,2-epoxide by a distinct complex of oxygenases (PaaABCDE) (Fig. 5). The reactive non-aromatic epoxide is thought to isomerize spontaneously to a seven-member O-heterocyclic enol ether. This intermediate or the 1,2-epoxide derivative is transformed by isomerase PaaG (Spieker et al., 2019) to the enol ether (Z)-2-(oxepin-2(3H)-ylidene)-acetyl-CoA (“oxepin-CoA”) (Fig. 5). This compound is prone to undergo hydrolytic ring cleavage catalyzed by PaaZ (Teufel et al., 2011). This enzyme consists of two domains: an enoyl-CoA hydratase domain (PaaZ-ECH), catalyzing the ring opening,

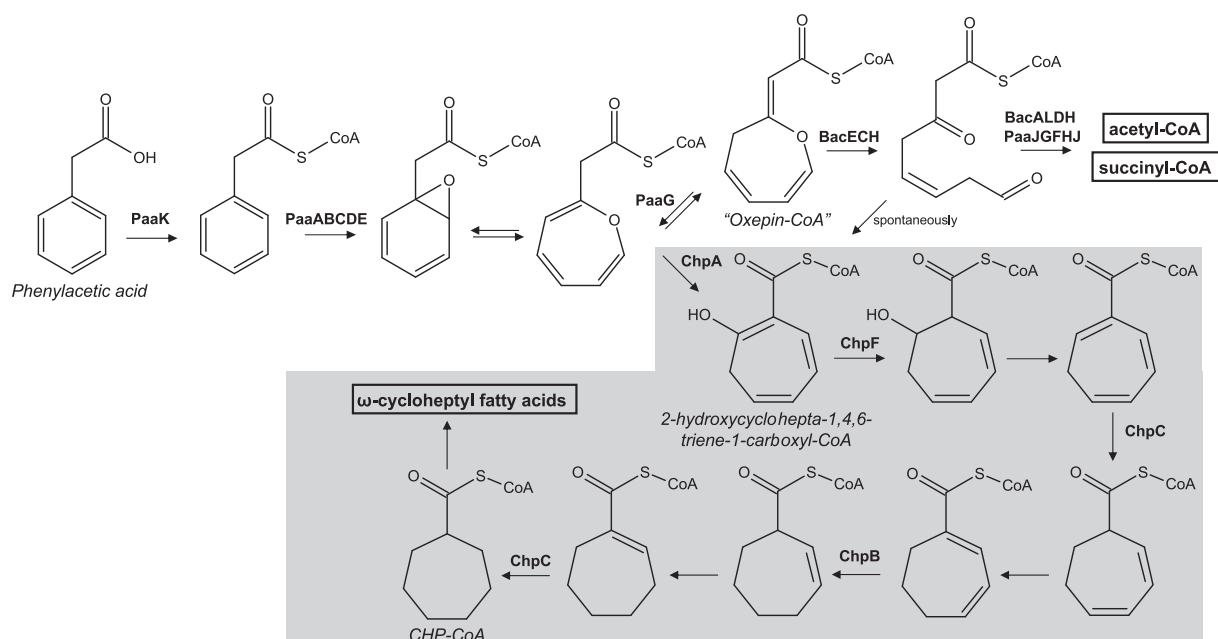


Fig. 5. Proposed biosynthetic pathway for the precursor of ω -cycloheptyl FAs, i.e. CHP-CoA in *Alicyclobacillus* spp., as a branch of the catabolic PAA pathway. This pathway ultimately results in the production of acetyl-CoA and succinyl-CoA via a large number of steps. In four *Alicyclobacillus* type strains of Clade 2 an important branching point occurs at the intermediate epoxide (or its isomerization product); in a series of dehydration, reduction, and isomerization steps (indicated in the colored area), presumed to be catalyzed by six enzymes, shikimate is converted into CHP-CoA, an important building block for the biosynthesis of ω -cycloheptyl FAs (after [Cropp et al., 2000](#); [Skyrud et al., 2020](#)). Note, that the assignment of the function of the six enzymes encoded by the CHP-CoA (see [Fig. 6](#)) is still hypothetical except for ChpC, which is a homologue of ChcA (see [Fig. 1](#)), which has been extensively characterized ([Reynolds et al., 1993](#); [Wang et al., 1996](#)). The exact function of ChpD and ChpE remains uncharacterized.

and an aldehyde dehydrogenase domain (PaaZ-ALDH) catalyzing oxidation of the resulting open-chain aldehyde, ultimately leading to the production of two acetyl-CoA and one succinyl-CoA (Teufel et al., 2010). However, if not directly oxidized, the highly reactive open-chain aldehyde spontaneously undergoes rapid intramolecular aldol condensation followed by dehydration, thereby producing 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA, featuring a seven membered carbon cycle. This has been proposed to represent a branching point in the catabolic pathway since this compound is thought to be the precursor for a variety of natural products including ω -cycloheptyl FAs (Teufel et al., 2011). However, the genes encoding the enzymes for the conversion of 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA to cycloheptanecarboxyl-CoA are currently unknown.

PSI-BLAST searches of the genomes of the *Alicyclobacillus* type strains revealed the presence of the (almost) complete catabolic pathway of PAA in all but six species examined (Table 5). The genes of this pathway are typically organized in an operon containing almost all the genes (Table S6), preceded by a homologue of *paaX*, which has been shown to act as a transcriptional regulator of this pathway in *E. coli* (Ferrández et al., 2000). There are a few differences in comparison with the operon described for *E. coli*. Firstly, the complex of oxygenases responsible for the epoxidation of phenylacetyl-CoA is formed by PaaABCD; the gene encoding PaaE was not detected in any of the genomes of *Alicyclobacillus* sp. Secondly, the gene encoding the bifunctional PaaZ was absent, as previously noted for many bacterial species (Teufel et al., 2011). These authors, however, identified a pseudohomologous gene in *Bacillus halodurans* (now *Halalkalibacterium halodurans*) and other *Bacillota*, designated *Bacillus*-ALDH (*Bac*ALDH), which likely fulfills the same role as PaaZ-ALDH. However, an apparent candidate gene encoding a protein catalyzing the preceding ring-cleavage step (as catalyzed by PaaZ-ECH) was not identified (Teufel et al., 2011). Re-examination of the *paa* gene operon of *H. halodurans* C-125 revealed an additional gene encoding an “enoyl-CoA hydrate-related protein” (Table S6), here designated as *Bacillus* ECH (*Bac*ECH), which we interpret as a likely candidate to fulfill

this role. Homologues of this *BacECH* gene with a sequence identity of 40–55 % at the protein level were also present in most of the genomes of *Alicyclobacillus* sp. containing a *paa* BGC (Table 5). Sometimes the *BacECH* gene was positioned adjacent to other *paa* genes (Table S6).

Our PSI-BLAST searches thus suggest the presence of a complete catabolic PAA pathway in most but not all *Alicyclobacillus* type strains. However, no relation is observed between the presence of the PAA pathway and the production of ω -cycloheptyl FAs (Table 5). Ten type strains of Clade 1, which are not producing ω -cycloheptyl FAs, are able to degrade PAA, which may lead to formation of oxepin-CoA, the proposed intermediate for the biosynthesis of ω -cycloheptyl FAs (Fig. 5). In Clade 2, only two *Alicyclobacillus* type strains lack the genes for the PAA pathway, but the remaining ones are producing ω -cycloheptyl FAs (4 species), ω -cyclohexyl FAs (4 species), or no acyclic FAs (3 species). Hence, although the presence of the PAA pathway seems essential for the ω -cycloheptyl FA producers, their production must be regulated in some other way.

The PSI-BLAST searches for PaaG identified a group of relatives with a sequence homology at the protein level of 34–41 % that were only detected in the *ω*-cycloheptyl FA-producing type strains, i.e. *A. cycloheptanicus* SCH^T, *A. herbarius* DSM 13609^T, *A. kakegawensis* NBRC 103104^T, and *A. shizuokensis* NBRC 103103^T. It was also identified in the genome of a non-type strain, i.e. *Alicyclobacillus* sp. ALC3 (Table 5). This strain was isolated from acid mine drainage and is phylogenetically most closely related to *A. cycloheptanicus* (Liu et al., 2021), and, hence, falls in Clade 2 (Fig. 2). The sequences of *A. herbarius* DSM 13609^T, *A. kakegawensis* NBRC 103104^T, and *A. shizuokensis* NBRC 103103^T were quite similar to each other (85–96 %), whilst that of *A. cycloheptanicus* SCH^T and *Alicyclobacillus* sp. ALC3 were less similar (63 %, Fig. 6a). These five sequences formed a distinct cluster in the PaaG phylogenetic tree (not shown) and all were annotated by JGI as “2-(1,2-epoxy-1,2-dihydroxyphenyl) CoA isomerase”. This would suggest that the substrate of this protein is the same as that of PaaG but the product is different. We hypothesize that this protein catalyzes the direct

Table 5

The presence and absence of genes of the catabolic PAA pathway and the CHP-CoA BGCs in NCBI reference genomes of *Alicyclobacillus* spp. and some other relevant genomes of *Alicyclobacillus* spp. and the reported presence of alicyclic FAs in these bacteria. The NCBI locus tags for the genes and the accession numbers for the encoded proteins are listed in Table S6.

Species	Strain	Genes catabolic PAA pathway ^a											Genes CHP-CoA BGC ^b						Alicyclic FAs ^c	
		<i>paaK</i>	<i>paaA</i>	<i>paaB</i>	<i>paaC</i>	<i>paaD</i>	<i>paaG</i>	<i>bacECH</i>	<i>bacALDH</i>	<i>paaJ</i>	<i>paaF</i>	<i>paaH</i>	<i>chpA</i>	<i>chpB</i>	<i>chpC</i>	<i>chpD</i>	<i>chpE</i>	<i>chpF</i>		
CLADE 1																				
<i>A. acidiphilus</i>	DSM 14558 ^T	–	–	–	–	–	–	–	✓	–	–	–	–	–	✓ ^d	–	–	–	absent	C6
<i>A. acidocaldarius</i>	DSM 446 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
	Tc-4-1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	n.d.
<i>A. acidoterrestris</i>	DSM 3922 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. dauci</i>	DSM 28700 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. fastidiosus</i>	DSM 17978 ^T	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. fodiniaquatilis</i>	NBRC 111483 ^T	–	–	–	–	–	–	–	✓	–	–	–	–	–	✓ ^d	–	–	–	absent	C6
<i>A. fructus</i>	DSM 112018 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. hesperidum</i>	DSM 12489 ^T	–	–	–	–	–	–	–	–	–	–	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. hesperidum subsp aegles</i>	DSM 11985 ^T	–	–	–	–	–	–	–	–	–	–	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. mali</i>	DSM 112016 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. sacchari</i>	DSM 17974 ^T	–	–	–	–	–	–	–	–	–	–	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. sendaiensis</i>	DSM 17614 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. suci</i>	DSM 112017 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
<i>A. vulcanalis</i>	DSM 16176 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6
“ <i>A. tengchongensis</i> ”	CGMCC1504	–	–	–	–	–	–	–	–	–	–	✓	–	–	✓ ^d	–	–	–	absent	n.d.
CLADE 2																				
<i>A. cellulosityticus</i>	JCM 18487 ^T	–	–	–	–	–	–	–	–	–	–	–	–	–	✓ ^d	–	–	–	absent	C6
<i>A. contaminans</i>	DSM 17975 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. cycloheptanicus</i>	DSM 4006 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	complete	C7
<i>A. curvatus</i>	ALEF1 ^T	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. ferrooxydans</i>	DSM 22381 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. herbarius</i>	DSM 13609 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	complete	C7
<i>A. kakegawensis</i>	DSM 17979 ^T	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	complete	C7
<i>A. macrosporangii</i>	DSM 17980 ^T	–	–	–	–	–	–	–	✓	✓	–	✓	–	–	–	–	–	–	absent	–
	CPP55	–	–	–	–	–	✓	–	✓	✓	–	✓	–	–	–	–	–	–	absent	n.d.
<i>A. mengziensis</i>	KCTC 43125 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6 ^f
<i>A. montanus</i>	USBA-GBX-503 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	C6 ^f
<i>A. pomorum</i>	DSM 14955 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>A. shizuokensis</i>	DSM 17981 ^T	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	complete	C7
<i>A. tengchongensis</i>	DSM 25924 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	C6 ^g
<i>A. tolerans</i>	DSM 16297 ^T	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	C6
“ <i>A. tolerans</i> ”	BL-1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–	absent	–
<i>Alicyclobacillus</i> sp.	ALC3	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓	complete	n.d.
	SO9	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	–	–	✓ ^d	–	–	–	absent	n.d.
	SP_1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓ ^d	–	–	–	absent	n.d.

^a The presence of the various genes responsible for encoding the proteins of the PAA pathway are indicated. They are ordered according to the various steps in the pathway (see Fig. 5). ^b Presence and absence of genes of the CHP-CoA BGC in order of appearance in the genomes (e.g. Fig. 6a). If the six genes were detected the BGC is described as “complete”, and otherwise as “absent”. ^c The occurrence of ω -alicyclic FAs as described in Table 1 but adjusted to our new analyses and re-interpretations (see footnotes Table 1). ^d Note that, although these *chpC* gene are phylogenetically closely related to the other *chpC* genes, they are part of another BGC where they are annotated as *chcA* (see Table 3 and text). ^f reinterpreted as explained in footnotes of Table 1. ^g Kim et al. (2014) reported that *A. tengchongensis* produced cycloheptane FAs but our re-analysis revealed that it does not produce cycloheptane but cyclohexane FAs (see text and Table 4).

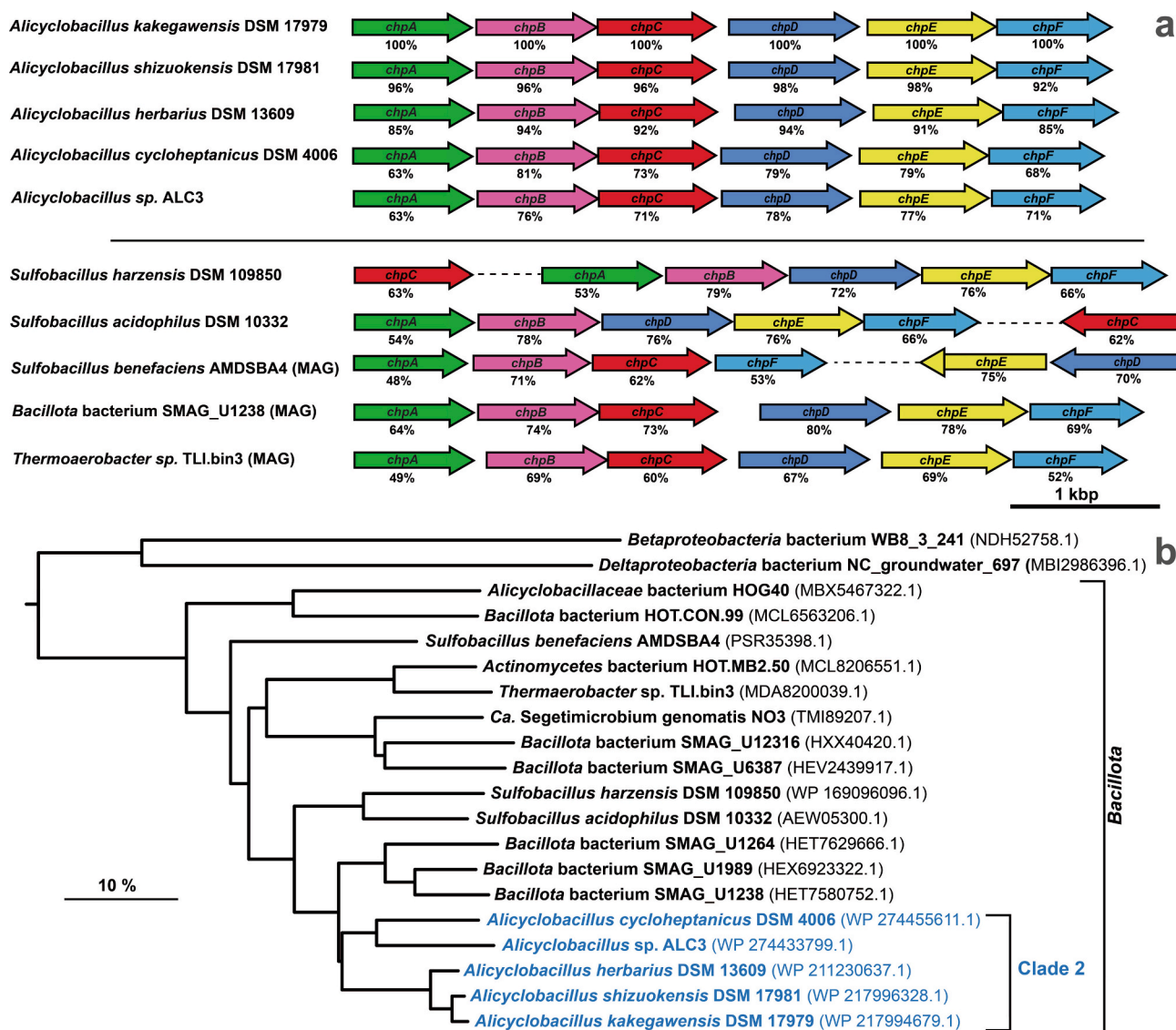


Fig. 6. Architecture of the CHP-CoA BGCs and phylogeny of one of the proteins encoded by this gene cluster. (a) Architecture of the proposed BGC for the biosynthesis of CHP-CoA. It is composed of six genes denoted as *chpA* - *chpF* as identified in the genomes of four *Alicyclobacillus* and two *Sulfobacillus* cultures which all six have been demonstrated to produce ω -cycloheptyl FAs (see text). The arrangement of genes in the CHP-CoA BGC is different in *Sulfobacillus* spp. because the *chpC* genes is not located in the BGC (indicated by the stippled line). The sequence similarity of protein homologues compared to those of *A. kakegawensis* (based on a single PSI-BLAST) (in %) is shown below the genes. The architecture of this BGC in the metagenome (MAG) of three other isolates is also shown. The accession numbers of all sequences used in the figure and locus tags of the genes are listed in Table S6. (b) The phylogeny of the ChpD protein encoded by the most conservative gene of the CHP-CoA BGC. The sequences in the tree comprise the five *Alicyclobacillus* spp. of Clade 2 that possess a CHP-CoA BGC (see Fig. 2) and a selection of other *chpD*-containing (meta)genomes from cultures and mostly isolates. Most of the latter ones contain a complete set of the 6 *chp* genes but not all of them (e.g. those of the *Proteobacteria*) and sometimes in a different arrangement (see panel a). The accession numbers of the proteins used are given in brackets. The tree was constructed using software using the Fast Minimum Evolution method provided at the NCBI website after sequence alignment using a single PSI-BLAST search. All sequence showed a query cover of 92–100 % with the query sequence (ChpD of *Alicyclobacillus kakegawensis*; WP_217994679.1) with an identity between 45 and 98 % (E-value 1E-87 - 0.0). A CoA transferase subunit A of *Actinomyces* *actinomyces* (WP_372503989.1) was used as an outgroup (not shown in the tree). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conversion of the epoxide or its isomerization product to 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA, the proposed starting substrate for synthesis of cycloheptyl (CHP)-CoA (Fig. 5). In the four genomes of ω -cycloheptyl FA-producing type strains a putative BGC was identified that starts with this gene, which we propose to call *chpA*, followed by five other genes (*chpBCDEF*; Fig. 6a, Table 5). The last gene of this BGC (*chpF*) was annotated as encoding an “NAD(P)-dependent oxidoreductase”, which may be responsible for the second step in the proposed pathway by reducing the 1,3-diketo isomerization product of the enol. The third gene of this BGC (*chpC*) is a close homologue of *chcA*, the gene that also occurs in the CHC-CoA BGC (Table 3) but phylogenetically

plots in a different cluster. We noted already that it was noteworthy that this gene, besides in all ω -cyclohexyl FA-producing strains, also occurred in the ω -cycloheptyl FA-producing strains but, in view of its function as an $\Delta^{1,2}$ -enoyl-CoA reductase, it likely also plays a role in reduction steps in the conversion of 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA to CHP-CoA, explaining its presence in the putative CHP-CoA BGC. This would assume that this enzyme would also reduce $\Delta^{1,2}$ -enoyl-CoA moieties of intermediates with a C7 instead of a C6 ring. The second gene of the presumed BGC, *chpB*, was annotated as a “NAD(P)-dependent 2,4-dienoyl-CoA reductase”, which probably catalyzes the hydrogenation of one of the two double bonds of a 2,4-dienoyl-CoA intermediate. The

function of the proteins encoded by the two remaining genes (*chpD* and *chpE*) remains enigmatic. They are both annotated as “CoA transferase” but more likely play a role in the isomerization of double bonds in the intermediates (Fig. 5). These proteins are highly specific, and their sequences are the most conservative of the six proteins encoded by the CHP-CoA BGCs (Fig. 6).

The presence of this additional BGC for the production of CHP-CoA in the four *Alicyclobacillus* type strains may be readily explainable. Teufel et al. (2011) proposed that ω -cycloheptyl FAs would be synthesized by the branching point in the PAA pathway created by the spontaneous conversion of the reactive open-chain aldehyde formed from oxepin-CoA to 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA (Fig. 5). However, it is hard to imagine that bacteria whose membrane FAs are for 72–87 % formed by ω -cycloheptyl FAs (Deinhard et al., 1987; Goto et al., 2002, 2007) would rely on such a spontaneous conversion, especially when they contain an enzyme (BacALDH) that converts the open-chain aldehyde to another product. Therefore, the availability of a CHP-CoA BGC, containing a gene encoding a protein (ChpA) to form 2-hydroxycyclohepta-1,4,6-triene-1-carboxyl-CoA directly seems a necessity, especially since many *Alicyclobacillus* spp. that do not biosynthesize ω -cycloheptyl FAs still process PAA via the open-chain aldehyde intermediate, a CHP-CoA BGC.

Using PSI-BLAST, we searched the NCBI database for other bacteria containing homologues to the conservative sequence of ChpD (Fig. 6b). This showed a high sequence homology of ChpD of the five type strains of the *Alicyclobacillus* Clade 2, suggesting a common ancestor. It also revealed the presence of a similar BGC in the genomes of the type species *Sulfobacillus harzensis* BGR 140^T and *S. acidophilus* DSM 10332^T (Fig. 6a), which strongly supports our interpretations. Both species, which like the *Alicyclobacillus* spp. also fall in the Phylum *Bacillota*, are one of the few identified bacterial species that also produce ω -cycloheptyl FAs (Zhang et al., 2021) besides some *Alicyclobacillus* spp. Interestingly, like in the Clade 2 *Alicyclobacillus* spp., most other *Sulfobacillus* spp. do not produce ω -cycloheptyl FAs but instead produce ω -cyclohexyl FAs, like *S. thermosulfidooxidans* CBAR-13 (Fig. 4b). The CHP-CoA BGC, fragmented into two pieces and differently organized was also detected in a metagenome-assembled genome (MAG) of the extreme acidophilic *S. benefaciens* obtained from a biofilm from an acid mine drainage (Fig. 6a) but, remarkably, not in any other of the MAGs of this species. The report on the isolated type strain of *S. benefaciens* unfortunately does not report its FA distribution (Johnson et al., 2008).

The ChpD phylogeny (Fig. 6b) identified also some other MAGs with a similar CHP-CoA BGC for species falling in the *Bacillota* (see Fig. 6 for two examples). In other cases, individual genes of the CHP-CoA BGC were identified in MAGs that were phylogenetically more remote from *Alicyclobacillus* sp. (e.g., the two proteobacterial MAGs in Fig. 6b), but not all six genes of the CHP-CoA BGC were detected. This suggests that, in contrast the biosynthesis of ω -cyclohexyl FAs, the biosynthetic capability for the production of ω -cycloheptyl FAs is scarcely distributed over the bacterial kingdom. The ability to synthesize ω -cycloheptyl FAs is apparently not easily transferable between different species through lateral gene transfer, even though the production of CHP-CoA is encoded by a readily transferable BGC. One observation seems to contradict this observation. A MAG of *A. macrosporangiidus* named HOT.CON.25 obtained from the sediment of the Lakelse Hot Springs (British Columbia, Canada) (NCBI RefSeq assembly GCF_023512275.1) contains a CHP-CoA BGC that is almost identical (>99 %) to that of the type strain of *A. herbarius* (ChpD sequence not shown in Fig. 6b). The ANI value of this MAG compared with the type strain of *A. macrosporangiidus* is 95.4 %, whilst that compared with *A. herbarius* is low, indicating that this MAG likely represents a new *Alicyclobacillus* species most closely related to *A. macrosporangiidus*. More detailed inspection of this MAG reveals that the presence of a CHP-CoA BGC potentially results from the transfer of a larger piece of DNA (K6T81_RS03840 - K6T81_RS04025) composed of ca. 26 genes, which are all almost identical to those *A. herbarius*. This observation would illustrate that, because all genes required for

production of CHP-CoA are organized in a BGC, the ability to produce alicyclic FAs may be exchanged by lateral gene transfer. So, perhaps lateral transfer of a CHP-CoA BGC is possible but only between closely related species. However, we cannot fully exclude the possibility of a contamination of the genome of the HOT.CON.25 MAG since according to check M the genome contamination is 7.6 %.

Implications for the taxonomy of *Alicyclobacillus* species

Phylogenomic data revealed that the type strains fall apart in different clades (1 and 2) that, based on various techniques, not all belong to one genus. Past and current chemotaxonomic data are in line with this. The species of Clade 1 all produce ω -cyclohexyl FAs as dominant membrane lipid constituents, in line with what has been described for the type species, *A. acidiphilus* (Wisotzky et al., 1992). This trait formed the basis for the name of this genus. Our detailed investigation of the genomes of these species identified a BGC probably responsible for the biosynthesis of CHC-CoA, the essential building block for the biosynthesis of ω -cyclohexyl FAs. All taxonomical descriptions of these species (Table 1) remain valid although the AAI value of the two subspecies of *A. hesperidium* is as high as 97 % (Table S4), indicating that they should be assigned to the same species.

A different picture emerges for the species comprising Clade 2 (Table 1). Genetically, they differ too much from the species in Clade 1 to carry the same genus name (Figs. 1 and 2). This is supported by the chemotaxonomical data; some produce ω -cyclohexyl FAs as dominant membrane lipid constituents, some ω -cycloheptyl FAs and others do not produce any acyclic FAs at all. This can all be directly linked to the presence/absence of the CHC-CoA BGC (Table 3), and its counterpart, a BGC composed of six genes, which we tentatively ascribe responsible for production of CHP-CoA, the building block for the biosynthesis of ω -cycloheptyl FAs (Table 5). We propose to create a new genus, *Paenalicyclobacillus*, to accommodate this (Table 6). The description of the new *Paenalicyclobacillus* spp. is provided in Table 7.

Strain *Alicyclobacillus tolerans* K1 was originally described as “*Sulfobacillus thermosulfidooxidans* subsp. thermotolerans”, but the name was never validly published and has therefore no standing in nomenclature. Tourova et al. (1994) published the 16S rRNA gene sequence type strain of the species *Sulfobacillus thermosulfidooxidans* (accession number Z21979 in GenBank). Two years later, the 1519 bp-long sequence entry was updated by changing one bp (Z21979.1), but it was also indicated that the sequence refers to bacterium K1, which was then listed as *Alicyclobacillus tolerans*. In 2005, the species description of strain *Alicyclobacillus tolerans* K1^T was formally described (Karavaiko et al., 2005), with reference to a new 1409-bp long 16S rRNA gene sequence (AF135502.2), which has subsequently been used in many studies as the marker gene for *Alicyclobacillus tolerans* K1^T. However, this sequence only showed a 93.9 % sequence identity with the former one (Z21979.1), which is rather surprising since Karavaiko et al. (2005) clearly stated that *A. tolerans* K1 was the starting material of their experiments to characterize this strain. Subsequently, Goto et al. (2007) in their work on several *Alicyclobacillus* spp. for reference purposes also obtained a 1488-bp long 16S rRNA gene sequence (AB222265.1) from *A. tolerans* K1^T deposited at DSMZ (DSM 16297^T), which shows a good match (Fig. 1) with the original sequence of *A. tolerans* K1^T. Finally, the reconstruction of the genome of *A. tolerans* K1^T (=DSM 16297^T) resulted in five full-length 16S rRNA gene sequences (two of them are included in Fig. 1) that matched very well the AB222265.1 sequence obtained by Goto et al. (2007). This discrepancy (i.e., the poor match of AF135502.2 with all other sequences; Fig. 1) cannot be caused by sequencing errors. It can only be explained by concluding that this sequence (AF135502.2) must have been derived from another strain that accidentally was mislabeled as *A. tolerans* K1. Dr. Tourova, one of the co-authors of the Karavaiko et al. (2005) paper, also feels this is the only plausible explanation (personal communication). This inaccuracy has led to substantial confusion in the scientific literature since in the meantime,

Table 6
Protologue table describing the newly proposed genus *Paenalicyclobacillus* in comparison with the genus *Alicyclobacillus*. The ICNP Guiding Code for Nomenclature was used. The individual *Paenalicyclobacillus* species are described in Table 7.

Genus name	<i>Alicyclobacillus</i>	<i>Paenalicyclobacillus</i>
Genus status	Emended description	gen. nov.
Genus etymology	<i>Al.i.cy.clo.ba.cil'lus</i> . G. adj. aliphos, fat; G.n.kykos, circle; L. masc. n. bacillus, a rod; N.L. masc. n. <i>Alicyclobacillus</i> , small rod containing circular fatty acids (ω -alicyclic fatty acids).	<i>Paen.a.li.cy.clo.ba.cil'lus</i> . Gr. adj. paene, almost; N.L. masc. n. <i>Alicyclobacillus</i> , a bacterial genus name; N.L. masc. n. <i>Paenalicyclobacillus</i> , almost <i>Alicyclobacillus</i> .
Description of the new genus and diagnostic traits	The description is as given for <i>Alicyclobacillus</i> (Wisotzkey et al., 1992) with a few modifications. Rod-shaped cells that are straight or nearly straight (0.3 to 0.8 by 2.0 to 4.5 μ m). Aerobic or facultatively anaerobic. Gram positive or gram variable. Endospores are formed under adverse environmental or nutritional conditions. One endospore per cell. Sporulation is tolerant of oxygen. Growth is obligately acidophilic and occurs at pH 2 to 6. Growth factors may or may not be required. The predominant membrane fatty acids are ω -cyclohexane fatty acids. The main isoprenoid quinone is menaquinone with seven isoprene units (MK-7). Hopanoids may be present; sulfonolipids are present. Growth temperatures range from less than 40 to 70 °C. The G + C content of the DNA ranges from 50 to 63 mol% (as determined from their genomes).	The description is identical to that for <i>Alicyclobacillus</i> except that the fatty acid composition is dominated by either ω -cycloheptane fatty acids, ω -cyclohexane fatty acids, or more conventional fatty acids. The G + C content of the DNA ranges from 45 to 63 mol% (as determined from their genomes).
Type species of the genus	<i>Alicyclobacillus acidocaldarius</i>	<i>Paenalicyclobacillus cycloheptanicus</i>
Specific epithet	<i>acidocaldarius</i>	<i>cycloheptanicus</i>
Species status	immutatus	comb. nov.
Species etymology	<i>a.ci.do.cal.dar'i.us</i> . M.L. n. <i>acidum</i> , acid; L. adj. <i>Caldarius</i> , pertaining to warm or hot; M.L. adj. <i>Acidocaldarius</i> , pertaining to acid thermal habitats	<i>cy.clo.hep.ta'ni.cus</i> . Gr.n. <i>kykos</i> circle, Gr.n. <i>hepta</i> seven; N.L. masc. Adj. <i>cycloheptanicus</i> , relating to ω -cycloheptane FAs in the membrane of the cells
Basonym	<i>Bacillus acidocaldarius</i> Darland and Brock, 1971	<i>Bacillus cycloheptanicus</i> Deinhard et al., 1988.
Other synonym	–	<i>Alicyclobacillus cycloheptanicus</i> Wisotzkey et al., 1992
Designation of the Type Strain	104–1A ^T	SCH ^T
Strain Collection Numbers	ATCC 27009 ^T = DSM 446 ^T	DSM 4006 ^T = ATCC 49028 ^T = IFO 15310 ^T = NBRC 15310 ^T
Type Genome	GCF_000024285.1	GCF_028751525.1
Genome status	Complete	Complete
Genome size (Mbp)	3.2	3.6
GC (mol%)	62.0	58.5
16S rRNA gene accession nr.	AB042056	X52489
Nagoya Protocol	No restrictions	No restrictions

several bacterial 16S rRNA gene sequences have been published in Genbank, which showed a close relationship to AF137502.2 (see Fig. 1) and, therefore, these bacteria were identified as closely related to *A. tolerans* K1^T. Most confusing is the deposition of a genome derived from an *Alicyclobacillus* strain BL-1 (GCF_021739485.1), which was stored in Genbank under the name *Alicyclobacillus tolerans* on basis of the 100 % match with AF137502.2 of its partial 16S rRNA gene sequence (NZ_JAJFNK010000161.1; 1039 bp). Our whole genome data clearly show that this is a quite divergent strain in Clade 2 of the tree (Fig. 2). We recommend referring to this strain from now on as “*Alicyclobacillus* sp. BL-1”. Even more complicated is that after the formal description *A. tolerans* K1^T, two other strains have been formally described as *Alicyclobacillus tengchongensis* DSM 25924^T (Kim et al., 2014) and *Alicyclobacillus montanus* USBA-GBX-503 (Lopez et al., 2018). Their 16S rRNA gene sequences (NR_146350.1 and NR_178913.1, respectively) are 99.3 and 99.1 % identical to that of *A. tolerans* K1^T (AB222265.1), which was deposited at Genbank already in 2007 but were not considered in either Kim et al. (2014) and Lopez et al. (2018). Their genomes share ANI values of ca. 99 % with the genome of *A. tolerans* K1^T (Table S4; see also Fig. 2). In hindsight, these strains cannot be considered to represent new *Alicyclobacillus* species, even at the time of publication. In addition, both papers suffer from errors in the chemotaxonomical description. Kim et al. (2014) reported that the major FAs of *A. tengchongensis* DSM 25924^T are ω -cycloheptane C_{18:0}, isoC_{17:0}, and *ai*-C_{17:0}, whereas upon re-cultivation and re-analysis the major FAs turned out to be indeed *ai*-C_{17:0} but ω -cyclohexyl C_{17:0} instead of ω -cycloheptanyl C_{18:0} (Table 4). Lopez et al. (2018) reported the absence of ω -acyclic FAs in *A. montanus* USBA-GBX-503 but this identification is doubtful (see footnote d in Table 1). The genomes of both strains contain the CHC-CoA BGC (Table 3) but the CHP-CoA BGC is absent (Table 5), like in the closely

related genome of *A. tolerans* K1^T, indicating that these strains are likely producing ω -cyclohexyl FAs, which was confirmed in the case of *A. tengchongensis* experimentally (Table 4). In view of this history, it is most logical to consider *Alicyclobacillus tengchongensis* (Kim et al., 2014) and *Alicyclobacillus montanus* (López et al., 2018) as later heterotypic synonyms of *Alicyclobacillus tolerans* K1^T (Karavaiko et al., 2005) and should also be assigned to the species *Paenalicyclobacillus tolerans*.

Conclusions

- 1) A phylogenomic survey of the genus *Alicyclobacillus* based on both the 16S rRNA gene and the proteomes encoded by their genomes reveal that the genus is divided into two distinct clades that are genetically too far separated from each other to include all type strains in one genus.
- 2) This division follows to some extent their reported ability to produce alicyclic FAs. All *Alicyclobacillus* species of Clade 1 produce ω -cyclohexyl FAs. In Clade 2, some species produce ω -cyclohexyl FAs, some produce ω -cycloheptyl FAs, whilst other members are not producing any alicyclic FAs.
- 3) This corresponds to the presence or absence of the two tentatively assigned BGCs in their genomes. The first BGC is responsible for the production of cyclohexane carboxylic acid-CoA, an essential building block for the biosynthesis of ω -cyclohexyl FAs. It comprises five genes encoding proteins acting as an adjusted side branch of the shikimate pathway to convert shikimate into cyclohexane carboxylic acid-CoA. The putative second BGC is proposed to be responsible for the production of cycloheptane carboxylic acid-CoA comprises six genes encoding proteins acting as a side branch of the catabolic PAA pathway, where a seven-member O-heterocyclic enol ether

Table 7

Protologue table describing the species of the novel genus *Paenalicyclobacillus*. The ICNP Guiding Code for Nomenclature was used.

Nature of the type material	Strain	Strain	Strain	Strain	Strain	Strain	Strain
Genus name	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>	<i>Paenalicyclobacillus</i>
Species name	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus aeris</i>	<i>Paenalicyclobacillus cellulostycticus</i>	<i>Paenalicyclobacillus contaminans</i>	<i>Paenalicyclobacillus curvatus</i>	<i>Paenalicyclobacillus disulfidooxidans</i>	<i>Paenalicyclobacillus ferrooxydans</i>
Genus status	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.
Genus etymology	Paen.a.li.cy.clo.ba.cil'lus. Gr. adj. <i>paene</i> , almost; N.L. masc. n. <i>Alicyclobacillus</i> , a bacterial genus name; N.L. masc. n. <i>Paenalicyclobacillus</i> , almost <i>Alicyclobacillus</i>						
Type species of the genus	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>	<i>Paenalicyclobacillus cycloheptanicus</i>
Specific epithet	<i>cycloheptanicus</i>	<i>aeris</i>	<i>cellulostycticus</i>	<i>contaminans</i>	<i>curvatus</i>	<i>disulfidooxidans</i>	<i>ferrooxydans</i>
Species status	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.
Species etymology	cy.clo.hep.ta'ni.cus. Gr.n. <i>kyklos</i> circle, Gr.n. <i>hepta</i> seven; N.L. masc. adj. <i>cycloheptanicus</i> , relating to ω -cycloheptane FAs in the membrane of the cells	ae.ris. L. gen. neut. n. <i>aeris</i> of ore/copper	cel.lu.lo.si.ly'ti.cus. N.L. neut. n. <i>cellulosum</i> cellulose; N.L. masc. adj. <i>lyticus</i> , lysing; from Gr. masc. adj. <i>lytikos</i> , dissolving; N.L. masc. adj. <i>cellulostycticus</i> , cellulose-dissolving	con.ta'mi.nans. L. part. adj. <i>contaminans</i> contaminating, referring to contamination of fruit juice	cur. va'tus. L. masc. part. adj. <i>curvatus</i> bent, curved, referring to the curved shape of the cell	di.sul.fi.do.o.xi.dans. N.L. pres. part. <i>oxidans</i> , oxidizing; N.L. masc. part. adj. <i>disulfidooxidans</i> , disulfide-oxidizing bacterium	fer.ro.o'xy.dans. L. neut. n. <i>ferrum</i> , iron; Gr. masc. adj. <i>oxy</i> s, sharp, acid; N.L. inf. v. <i>oxydare</i> , to make acid, to oxidize; N.L. masc. part. adj. <i>ferrooxydans</i> , iron-oxidizing
Basonym	<i>Bacillus cycloheptanicus</i> Deinhard et al., 1988.	<i>Alicyclobacillus aeris</i> Guo et al. 2009	<i>Alicyclobacillus cellulostycticus</i> Kusube et al. 2014	<i>Alicyclobacillus contaminans</i> Goto et al. 2007	<i>Alicyclobacillus curvatus</i> Jiang et al. 2022	<i>Sulfobacillus disulfidooxidans</i> Dufresne et al. 1996	<i>Alicyclobacillus ferrooxydans</i> Jiang et al. 2008
Other synonym	<i>Alicyclobacillus cycloheptanicus</i> Wisotzkey et al., 1992	-	-	-	-	<i>Alicyclobacillus disulfidooxidans</i> Karavaiko et al. 2005	-
Designation of the Type Strain	SCH ^T	ZJ-6 ^T	Sueoka ^T	3-A191 ^T	ALEF1 ^T	SD-11 ^T	TC-34 ^T
Strain Collection Numbers	DSM 4006 ^T = ATCC 49028 ^T = IFO 15310 ^T = NBRC 15310 ^T	CGMCC 1.7072 ^T = NBRC 104953 ^T	DSM 107339 ^T = JCM 18487 ^T = KCTC 33007 ^T	DSM 17975 ^T = IAM 15224 ^T	CGMCC 1.17055 ^T = KCTC 43124 ^T	ATCC 51911 ^T = DSM 12064 ^T	DSM 22381 ^T = JCM 15090 ^T = CGMCC 1.6357 ^T
Type Genome	GCF_028751525.1	-	GCF_014647315.1	GCF_000429525.1	GCA_017298655.1	-	GCF_001399675.1
Genome status	Complete	-	Complete	Complete	Incomplete	-	Complete
Genome size (Mbp)	3.6	-	2.7	3.3	-	-	5.0
GC (mol%)	58.5	-	63.0	58.2	-	-	51.3
16S rRNA gene accession nr.	X52489	NR_116896.1	AB622813	AB264026	MW269400	NR_040944.1	EU137838
Description of the new taxon and diagnostic traits	The description is as given for <i>Alicyclobacillus cycloheptanicus</i> (Wisotzkey et al., 1992)	The description is as given for <i>Alicyclobacillus aeris</i> (Guo et al., 2009)	The description is as given for <i>Alicyclobacillus cellulostycticus</i> (Kusube et al., 2014)	The description is as given for <i>Alicyclobacillus contaminans</i> (Goto et al., 2007)	The description is as given for <i>Alicyclobacillus curvatus</i> (Jiang et al., 2022)	The description is as given for <i>Alicyclobacillus disulfidooxidans</i> (Dufresne et al., 1996)	The description is as given for <i>Alicyclobacillus ferrooxydans</i> (Jiang et al., 2008)
Nagoya Protocol	No restrictions	No restrictions	No restrictions	No restrictions	-	No restrictions	No restrictions
Species name	<i>Paenalicyclobacillus herbarius</i>	<i>Paenalicyclobacillus kakegawensis</i>	<i>Paenalicyclobacillus macrosporangiidus</i>	<i>Paenalicyclobacillus mengziensis</i>	<i>Paenalicyclobacillus pomorum</i>	<i>Paenalicyclobacillus shizuokensis</i>	<i>Paenalicyclobacillus tolerans</i>
Genus status	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.	nom. rev.
Genus etymology	Paen.a.li.cy.clo.ba.cil'lus. Gr. adj. <i>paene</i> , almost; N.L.						

(continued on next page)

Table 7 (continued)

Nature of the type material	Strain	Strain	Strain	Strain	Strain	Strain	Strain
Type species of the genus	masc. n. <i>Alicyclobacillus</i> , a bacterial genus name; N.L. masc. n. <i>Paenalicyclobacillus</i> , almost <i>Alicyclobacillus</i>						
Specific epithet	<i>Paenalicyclobacillus cycloheptanicus herbarius</i>	<i>Paenalicyclobacillus cycloheptanicus kakegawensis</i>	<i>Paenalicyclobacillus cycloheptanicus macrosporangiidus</i>	<i>Paenalicyclobacillus cycloheptanicus mengziensis</i>	<i>Paenalicyclobacillus cycloheptanicus pomorum</i>	<i>Paenalicyclobacillus cycloheptanicus shizuokensis</i>	<i>Paenalicyclobacillus cycloheptanicus tolerans</i>
Species status	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.	comb. nov.
Species etymology	her.ba'ri.us. L. masc. n. adj. <i>herbarius</i> herb, from which the organism was isolated	ka.ke.ga.wa.en'sis. N.L. masc. adj. <i>kakegawensis</i> pertaining to Kakegawa, a city in Shizuoka Prefecture, Japan, where the type strain was isolated	ma.cro.spo.ran.gi'i.dus. Gr. masc.adj. macros big; N.L. neut. n. sporangium sporangia; L. suff. -idus suffix expressing a quality or tendency; N.L. masc. adj. <i>macrosporangiidus</i> having large sporangia	meng. zi. en'sis. N. L. masc. adj. <i>mengziensis</i> , of Mengzi, China, referring to the source of the sample from which the type strain was isolated	po.mo'rum. L. gen. pl. n. <i>pomorum</i> , of fruits	shi.zu.o.ken'sis. N.L. masc. adj. <i>shizuokensis</i> pertaining to Shizuoka, a city in Shizuoka Prefecture, Japan, where the type strain was isolated	tol.er'ans. L. masc. part. adj. <i>tolerans</i> tolerant to changes in the growth temperature and pH
Basonym	<i>Alicyclobacillus herbarius</i> Goto et al. 2002	<i>Alicyclobacillus kakegawensis</i> Goto et al. 2007	<i>Alicyclobacillus macrosporangiidus</i> Goto et al. 2007	<i>Alicyclobacillus mengziensis</i> Jiang et al. 2022	<i>Alicyclobacillus pomorum</i> Goto et al. 2003	<i>Alicyclobacillus shizuokensis</i> Goto et al. 2007	<i>Alicyclobacillus tolerans</i> Karavaiko et al. 2005.
Other synonym	-	-	-	-	-	-	-
Designation of the Type Strain	CP-1 ^T	5-A83J ^T	5A-239-20-A ^T	S30H14 ^T	3-A ^T	4-A336J ^T	K1 ^T
Strain Collection Numbers	DSM 13609 ^T = IAM 14883 ^T = NRIC 0477 ^T	DSM 17979 ^T = IAM 15227 ^T	DSM 17980 ^T = IAM 15370 ^T	CGMCC 1.17050 ^T = KCTC 43125 ^T	DSM 14955 ^T = IAM 14988 ^T	DSM 17981 ^T = IAM 15226 ^T	DSM 116297 ^T = VKM B-2304 ^T
Type Genome	GCF_000430585.1	GCF_001552655.1	GCF_900116805.1	GCF_017298635.1	GCF_000472905.1	GCF_001552255.1	GCF_048984885.1
Genome status	Complete	Complete	Complete	Complete	Complete	Complete	Complete
Genome size (Mbp)	3.3	3.4	3.8	5.3	3.4	3.6	3.2
GC (mol%)	57.2	59.1	58.5	58.5	58.5	58.8	44.9
16S rRNA gene accession nr.	AB042055	AB264022	AB264025	MT605219	AB89840	AB264024	AB222265.1
Description of the new taxon and diagnostic traits	The description is as given for <i>Alicyclobacillus herbarius</i> (Goto et al., 2002)	The description is as given for <i>Alicyclobacillus kakegawensis</i> (Goto et al., 2007) with the exception that major FAs are ω-cycloheptyl C _{18:0} , ω-cycloheptyl C _{18:0} 2-OH and ω-cycloheptyl C _{20:0} .	The description is as given for <i>Alicyclobacillus macrosporangiidus</i> (Goto et al., 2007)	The description is as given for <i>Alicyclobacillus mengziensis</i> (Jiang et al., 2022)	The description is as given for <i>Alicyclobacillus pomorum</i> (Goto et al., 2003)	The description is as given for <i>Alicyclobacillus shizuokensis</i> (Goto et al., 2007) with the exception that major FAs are ω-cycloheptyl C _{18:0} , ω-cycloheptyl C _{18:0} 2-OH and ω-cycloheptyl C _{20:0} .	The description is as given for <i>Alicyclobacillus tolerans</i> (Karavaiko et al., 2005).
Nagoya Protocol	No restrictions	No restrictions	No restrictions	-	No restrictions	No restrictions	No restrictions

intermediate is ultimately converted into cycloheptane carboxylic acid-CoA, an essential building block for the biosynthesis of ω -cycloheptyl FAs. The identification of these BGCs was supported by their presence in other bacteria also producing ω -alicyclic FAs.

- 4) We propose to reclassify all species of the Clade 2 as members of a new genus, *Paenalcyclobacillus* gen. nov. Our investigations also reveal that both *Alicyclobacillus tengchongensis* and *Alicyclobacillus montanus* are later heterotypic synonyms of *Alicyclobacillus tolerans*.

CRediT authorship contribution statement

Jaap S. Sinninghe Damsté: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Laura Villanueva:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Rüdiger Pukall:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.syapm.2025.126651>.

Data availability

Data will be made available on request.

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