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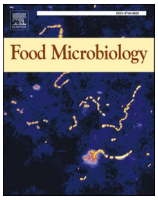

Genomic rearrangements in the *aspA-dcuA* locus of *Propionibacterium freudenreichii* are associated with aspartase activity

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ABSTRACT

Propionibacterium freudenreichii is crucial in Swiss-type cheese manufacture. Classic propionic acid fermentation yields the nutty flavor and the typical eyes. Co-metabolism of aspartate pronounces the flavor of the cheese; however, it also increases the size of the eyes, which can induce splitting and reduce the cheese quality. Aspartase (EC 4.3.1.1) catalyzes the deamination of aspartate, yielding fumarate and ammonia. The aspartase activity varies considerably among *P. freudenreichii* strains. Here, the correlation between aspartase activity and the locus of aspartase-encoding genes (aspA) and dcuA encoding the C4-dicarboxylate transporter was investigated in 46 strains to facilitate strain selection for cheese culture. Low aspartase activity was correlated with a particular genomic rearrangement: low in vitro aspartase activity always occurred in strains with gene clusters aspA - dcuA where the dcuA was frameshifted, producing a stop codon or was disrupted by an ISL3-like element. The low aspartase activity could be due to the protein sequence of the aspartase or a dysfunctional DcuA. The highest values of aspartase activity were detected in strains with aspA1 - aspA2-dcuA with a DcuA sequence sharing 99.07 – 100% identity with the DcuA sequence of strain DSM 20271 T and an additional C4-dicarboxylate transporter belonging to the DcuAB family.

1. Introduction

Propionibacterium freudenreichii is the crucial ripening bacterium in the production of Swiss-type cheese. This bacterium is responsible for the characteristic nutty flavor and the typical eyes (Fröhlich-Wyder et al., 2021). During cheese production, *P. freudenreichii* primarily metabolizes lactate to pyruvate. Approximately two-thirds of that pyruvate is subsequently reduced to propionate via the Wood-Werkman cycle (which includes the formation of the dicarboxylic acids oxaloacetate, malate, fumarate, and succinate), and this gives the typical nutty flavor of Swiss-type cheese. The characteristic eye formation is created when the remaining third of the pyruvate is converted to acetate and CO_2.

P. freudenreichii strains can co-metabolize aspartate with lactate (Crow, 1986) by aspartase (also called aspartate ammonia-lyase; EC 4.3.1.1), which catalyzes the deamination of aspartate, yielding fumarate and ammonia. This reaction induces the production of succinate (via fumarate) and ammonia, and it interacts with the fermentation of lactate to propionate, acetate, and CO_2 (Crow, 1986; Thierry et al., 2011). An increase in succinate enhances the conversion of pyruvate into acetate and CO_2 rather than into propionate to maintain the cell redox balance (Fröhlich-Wyder et al., 2017). The ability to use aspartate enhances the growth rate and yield of *P. freudenreichii* (Fröhlich-Wyder et al., 2001; Piveteau et al., 1995).

Variations in the production of acetate, propionate, succinate, ammonia, and CO_2 influence the flavor and maturity of the cheese, the size of the eyes, and the height of the cheese loaves (Fröhlich-Wyder

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et al., 2001). The level of aspartase activity is strain-dependent and covers a wide range (Blasco et al., 2011; Turgay et al., 2011), with the lowest value of the aspartase activity reported as 0.1% of the highest value (Turgay et al., 2011). Strains of *P. freudenreichii* with a high ability to use aspartate are rightly feared by cheese makers who produce the long-ripened varieties. Indeed, these strains are a potent cause of late and uncontrolled fermentation and hence, may cause cracking due to excessive CO_2 production and increased maturity during the ripening process. However, culture strains with a high ability to use aspartate also shorten the cheese-ripening time and contribute to a more pronounced flavor because of the enhanced ammonia and succinate production (Fröhlich-Wyder et al., 2002, 2017). Thus, for Swiss-type cheeses with short ripening times, the aspartase activity is indeed a desirable feature for characterizing a *P. freudenreichii* strain.

When classifying aspartase activity into high and low, we refer to our well-studied reference strains FAM14176, FAM14177, FAM14217, and FAM14218 from the strain collection of Agroscope (Bern, Switzerland) (Turgay et al., 2011). The highest activity (166.014 ± 0.500 μmol min^{-1} mg^{-1} protein) was detected in a previous study (Turgay et al., 2011), who also reported aspartase activity values of <1% for the FAM14176 and FAM14177 strains (low aspartase-active reference strains) and 19–25% for the FAM14217 and FAM14218 strains (high aspartase-active reference strains) with respect to the highest activity.

The complete Wood-Werkman cycle (reduction of pyruvate to propionate) has been reconstructed based on the genome sequence of *P. freudenreichii* strain CIRM-BIA1 (GeneBank: FN806773.1), and the cycle enzymes have been annotated (Falentin et al., 2010). Analysis of the genomic data indicates that two adjacent genes encode aspartase: *aspA1* (PFREUD_16320) and *aspA2* (PFREUD_16330). Only 4 bp downstream of *aspA2* is *dcuA* (PFREUD 16340) encoding for a C4-dicarboxylate transporter (Fig. 1) (Falentin et al., 2010). Similarly is this genome arrangement in *P. freudenreichii* strain DSM 20271T (GeneBank: NZ_CP010341.1) with two adjacent codirected aspartase encoding genes (RM25_RS07640, RM25_RS07645) and downstream the gene encoding an anaerobic C4-dicarboxylate transporter (RM25_RS07650) (Koskinen et al., 2015). An expanded genome sequence analysis of 20 *P. freudenreichii* strains by Deptula et al. (2017) showed a diverse gene arrangement with one, two, or three aspartase-encoding genes per strain (described in Supplemental file 23 of Deptula et al. (2017)).

The gene product of *dcuA* belongs to the family of DcuAB transporters, subdivided into DcuA and DcuB subgroups. They are present only in anaerobic and facultative anaerobic bacteria capable of fumarate respiration. Other C4-dicarboxylate carriers, DctA, DcuC, CitT and DctPCM are known transporting C4-dicarboxylates from the periplasm across the inner membrane into bacteria (Janausch et al., 2002). Only one additional gene (CIRM-BIA1: PFREUD_03570, DSM 20271T: RM_RS01630) is annotated as C4-dicarboxylate transporter in the genome of the strain CIRM-BIA1 and of the strain DSM 20271T. It belongs to the DcuAB family and is located at a different locus than the aspartase encoding genes. Studies of the C4-dicarboxylate transporters in *Campylobacter jejuni* revealed that DcuA and DcuB are involved in the uptake of aspartate under low-oxygen conditions while the secretion of succinate appeared to be mediated by DcuB. The *dcuA* is located in one operon together with *aspA*, while *dcuB* is located in a single-gene operon (Wösten et al., 2017).

In the present study, we investigated the possibility of predicting low or high aspartase activity of a strain based on information on the organization of the aspartase-encoding genes and *dcuA*. The additional gene encoding a C4-dicarboxylate transporter of the DcuAB family is also taken into account in our investigation. Finding a correlation between phenotype and genotype would allow pre-selection of strains based on genomic information, thereby reducing the amount of laboratory screening required. Overall, the aim of the study was to provide a more in-depth understanding of the genetic basis of strain-dependent aspartase activity in *P. freudenreichii*.

Our strategy was to first sequence the *aspA-dcuA* locus of 46 *P. freudenreichii* strains (Fig. 1). Aspartase activity was measured in cell-free extracts of the 46 strains, followed by comparison of the aspartase activity of the strains versus the number of aspartase-encoding genes and the presence and length of the *dcuA* and the presence of other annotated C4-dicarboxlate transporter genes. In the third step, three genes encoding three different aspartase alleles were cloned, heterologously over-expressed, purified, and assayed for their enzymatic activity. Of these three genes, one corresponded to the low aspartase-active strain FAM14176 and the other two corresponded to the sequences of the two aspartase-encoding genes of the high-aspartase activity strain FAM14217 (Turgay et al., 2011). Finally, we verified whether both aspartase-encoding genes in these two strains were transcribed under culture conditions used for aspartase activity measurements.

2. Material, methods, and experimental procedures

2.1. P. freudenreichii strains

A collection of 46 *P. freudenreichii* strains was used in this study: two strains, CIRM-BIA1 (DSM 4902) and DSM 20271^T, ordered from the DSMZ-German Collection of Microorganism and Cell Cultures GmbH (38124 Braunschweig, Germany) (Table 1) and 44 strains originated from the strain collection of Agroscope. The strain diversity was verified by typing each strain with a multiple-locus variable number of tandem repeat analysis (MLVA). The strains were further discriminated using insertion sequence-restriction fragment length polymorphism (IS-RFLP) (Turgay et al., 2011) (results not shown).

2.2. Screening of genes encoding aspartase and C4-dicarboxylate transporter

Previous high-throughput Illumina sequencing at the University of Bern has provided complementation of the background information for selected Agroscope strains with their genome sequences. The Illumina sequence of the 44 sampling strains was therefore available in our genome database. Ten of these strains were further sequenced using PacBio technology. An insight was given into the existence of C4-dicarboxlyate transporter-encoding genes and the arrangement of the genes around the locus of the aspartase-encoding genes. The presence of

Fig. 1. Illustration of the genomic neighborhood of *aspA1*, *aspA2,* and *dcuA* between *fdxA* (ferredoxin family protein) and *pimH* (internal membrane efflux protein), based on the example of *Propionibacterium freudenreichii* strain CIRM-BIA1 (GeneBank: FN806773.1). The locus comprises *gya* (glyoxylate reductase), *fumC* (fumarate hydratase class-II), * = ORF (hypothetical protein), *aspA1*, *aspA2* (aspartase), *dcuA* (C4-dicarboxylate transporter), *int* (integrase), and *tnpA* (transposase IS3/IS911). Small arrows indicate the annealing regions of the primers used in this study (numbers refer to the primers listed in Table 2). Primers 1 and 2: for *fdxA-pimH* fragment to study the genomic arrangement of *aspA1, aspA2,* and *dcuA* of the sampling strains. Primers 3–6: for amplification of *aspA1* and *aspA2* to study the protein activity of the two aspartase alleles, aspA1 and aspA2.

Table 1
Propionibacterium freudenreichii strains isolated from milk or cheese from various manufactories were included in this study. Except for DSM 4902, all strains originated in Switzerland. (SG = St-Gall, FR = Fribourg, TG = Thurgau, BE = Berne, NW = Nidwald, OW = Obwald, ZS = Central Switzerland, and n. t. = not traceable). The accession numbers of the sequences comprising the *aspA-dcuA* locus are listed below.

Reference strain from collection of Agroscope, Bern, Switzerland			
Strain	Isolation Source	published in	GenBank accession number
strains with low aspartase activity			
FAM14176[1]	Appenzeller cheese manufactory, Niederbühren, SG	Turgay et al. (2011)	OK490935
FAM14177[1]			OK490936
strains with high aspartase activity			
FAM14217[2]	Emmentaler cheese manufactory, n. t.	Turgay et al. (2011)	OK490940
FAM14218[2]			OK490941
Strains from collection of Agroscope, Bern, Switzerland			
Strain	Isolation Source	Reference	
FAM14221	Emmentaler cheese manufactory, n. t.	Turgay et al. (2011)	OK490942
FAM14222			OK490943
FAM14184	Gruyère cheese manufactory, Cottens, FR	Turgay et al. (2011)	OK490937
FAM14193	Appenzeller cheese manufactory, Frittschen, TG	Turgay et al. (2011)	OK490938
FAM14197[3]	Appenzeller cheese manufactory, Sax, SG	Turgay et al. (2011)	OK490939
FAM19030	Emmentaler cheese manufactory, Aarwangen, BE		OK490955
FAM19031			OK490956
FAM19032			OK490957
FAM19033			OK490971
FAM23877	n. t., Bussy sur Moudon, VD	Fessler (1997)	OK490970
FAM15078		Fessler (1997)	OK490970
FAM23863	Pilot plant cheese manufactory Agroscope		OK490964
FAM23864			OK490965
FAM23866			OK490966
FAM23867			OK490967
FAM23868			OK490968
FAM23869[4]			OK490969
FAM19034	Emmentaler cheese manufactory, Melchnau, BE		OK490958
FAM22019[5]	Sbrinz cheese manufactory, Obbürgen, NW		OK490976
FAM22020			OK490977
FAM22021			OK490959
FAM23854			OK490963
FAM23855			OK490980
FAM19028	Emmentaler cheese manufactory, Oschwand, BE		OK490954
FAM23848	Sbrinz cheese manufactory, Rain, LU		OK490978
FAM23850			OK490979
FAM23851[1]	Sbrinz cheese manufactory, St. Nicklausen, OW		OK490960
FAM23852			OK490961
FAM23853			OK490962
FAM19014	Emmentaler cheese manufactory, Uettligen, BE	Turgay et al. (2011)	OK490949
FAM19015		Turgay et al. (2011)	OK490950
FAM19019		Turgay et al. (2011)	OK490972
FAM19020[5]		Turgay et al. (2011)	OK490973
FAM19022		Turgay et al. (2011)	OK490974
FAM19023		Turgay et al. (2011)	OK490951
FAM19024		Turgay et al. (2011)	OK490975
FAM19025		Turgay et al. (2011)	OK490952
FAM19026		Turgay et al. (2011)	OK490953
FAM15061[3]	n. t., Herder, TG	Fessler (1997)	OK490944
FAM15113	n. t., Bodenberg, ZS	Fessler (1997)	OK490946
FAM15415[4]	DSM 4902	Falentin et al. (2011)	OK490948
FAM15414	DSM 20271	Koskinen et al. (2015)	OK490947

[1-5] Strains which could not be discriminated by MLVA and IS-RFLP.

multiple transposase genes and the similarity of the collocated aspartase-encoding genes led us to use a long read sequencing technology to characterize the locus of the aspartase-encoding genes and *dcuA* in the 46 strains investigated in this study. The sequence flanked by the genes *fdxA* (ferredoxin family protein) and *pimH* (internal membrane efflux protein) (Fig. 1) was amplified, sequenced, and annotated, as described below.

2.2.1. Extraction of genomic DNA

For DNA extraction, the bacteria were grown anaerobically in lactate broth (pH 6.8) at 30 °C for 48 h, as described in Turgay et al. (2011). The liquid culture (1–2 mL) was pelleted by centrifugation (10 min, 18600×*g*) at room temperature (RT). The pellet was resuspended in 1 mL NaOH (0.05 M), incubated for 10 min at RT, and centrifuged (18600×*g*, 10 min, RT). All subsequent steps are described in Turgay et al. (2016). With NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) DNA was quantified.

2.2.2. Primer design

The primers in this study were designed between *fdxA* and *pimH* of strain DSM20271^T (Genbank: NZ_CP010341) and CIRM-BIA1 (GenBank: FN806773.1). Fig. 1 shows a schematic overview of the primer locations. Appropriate primers were designed in conserved regions using the CLC Main Workbench 20.0 software tools and Primer Express Software V3.0.1 (ThermoFisher Scientific). The oligonucleotides were synthesized by Microsynth (Microsynth AG). PacBio fusion primers were ordered from IDT (Integrated Technologies, 3001 Leuven, Belgium). All primers are listed in Table 2.

2.2.3. PacBio amplicon sequencing

For AmpliconSeq with PacBio technology, the designed primers flanked the locus harboring the aspartase-encoding genes and *dcuA*, annealing on *fdxA* and on *pimH* (primers 1 and 2 in Fig. 1). The first PCR was performed in a reaction volume of 25 μL containing 30–40 ng genomic DNA, 0.5 mM of each PacBio fusion primer, and Platinum™ SuperFi™ PCR MasterMix (Invitrogen by Thermo Fisher Scientific, Zug, Switzerland). The reaction was conducted in a MiniAmp Thermal Cycler (Applied Biosystems by ThermoFisher Scientific) under the following conditions: initial denaturation at 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 58.1 °C for 10 s, 72 °C for 5 min, and 72 °C for 10 min. Agilent 2100 bioanalyzer using the Agilent DNA 12000 Kit (Agilent Technologies AG, Basel, Switzerland) was used for visualization. After PCR purification using the QIAquick PCR Purification Kit (Qiagen), the Qubit™ dsDNA HS Assay Kit (Invitrogen by Thermo Fisher Scientific) was used for their quantitative determination. A second PCR was conducted with one ng of amplicon in 30 μL containing Platinum™ SuperFi™ PCR MasterMix, and PacBio BUP containing barcoded universal primers (Pacific Biosciences, Menlo Park, CA, USA). The PCR was initiated at 98 °C for 30 s, followed by 15 or 25 cycles with 98 °C for 15 s, 72 °C for 5 min 15 s, and 72 °C for 10 min. The amplicons were visualized as described above and cleaned up using AMPure Beads (Beckman Coulter, Brea, CA, and the USA). The Qubit™ dsDNA HS Assay Kit

Table 2
Primers used in this study. PacBio fusion primers: 1, 2. Primers No. 1–6 refer to positions in Fig. 1. Primers No. 7–12 to positions in Fig. 5.

Classic PCR					
Target	Primer	Sequence (5' -3')[a]	source	Amplicon size (bp)	No.
fdxA	fer1-PacBio	5AmMC6tggatcacttgtgcaagcatcacatcgtagCGGATGGATGTAGAGGGT	this study	7872–10181	1
pimH	entS-8F-PacBio	5AmMC6gcagtcgaacatgtagctgactcaggtcacCGTGTTGTTCATGGTGTT			2
aspA1	CIRM-BIA1_aspA1-F	atgagcacacgtatcgaagtgg	this study	1470	3 4
	CIRM-BIA1_aspA1-R	tcagcgctcatccagcgc			
aspA2	CIRM-BIA1_aspA2-F	atgtccacgcgtacagaagaa	this study	1473	5
	CIRM-BIA1_aspA2-R	ctaggcctcgtgactgac			6
aspA	14176p_aspA-F	atgagcacacgtatcgaagtg	this study	1473	
	14176p_aspA-R2	ctagccttcggggccga			
Tagged RT-PCR					
Target	Primer	Sequence (5' -3')[a]	source	Amplicon size (bp)	
dcuA	tag-dcuA-4	AGTGGTACACGCAGAGTACTTcagggatactgctggat	this study		7
aspA1, *aspA2*	tag-AspA501-RNA	AGTGGTACACGCAGAGTACTTttggtcgactgcgactt	this study		8
	tag	AGTGGTACACGCAGAGTACTT	Aquena and Spira, 2003		9
aspA2-dcuA	AspA2-3	ccggagctcgtcagtca	this study	1038	10
aspA1	RNA-PFS_aspA1-F	atgagcacacgtatcgaagtg	this study	449	11
aspA1-aspA2	AspA500-RNA	acctacctcaacgacgtgat	this study	995	12

[a] Capital letters indicate the tag-sequence.

revealed amplicon concentrations in the range of 0.682–17.4 ng μL^{-1}. The samples (30–35 μL) were sent for PacBio sequencing to the University of Lausanne (Genomic Technologies Facility, University of Lausanne, 1015 Lausanne, Switzerland). There, the 46 PCR amplicons were quantified using a fluorometric method (Qubit Life Technologies). Their sizes were evaluated with a bioanalyzer (Agilent Technologies). An equimolar mix of all amplicons was then processed into a sequencing library with the PacBio SMRTbell Template Prep Kit 1.0 (Pacific Biosciences), according to the manufacturer's recommendations. The library was sequenced on a Sequel instrument (Pacific Biosciences) on a v3 SMRT cell for 20H with a 4H pre-extension, using the v3.0/v3.0 chemistry. Circular Consensus Sequences (CCS) were generated using the pbccs package (v. 4.0.0) in conda (v. 4.8.3) (see script in Supplemental file 1), with default parameters (Minimum Number of Passes = 3, Minimum Predicted Accuracy = 0.99). Primers were trimmed from the CCS using the PacBio-adapted dada2 pipeline (Callahan et al., 2019).

Qualitative best consensus sequences were obtained by first hybrid-assembling the CCS in combination with Illumina short reads (sequenced earlier by Microsynth or University of Bern, Switzerland) using Unicycler (v. 0.4.8) (Wick et al., 2017), and finally polished with Pilon (v. 1.23) (Walker et al., 2014). For isolates (n = #4) where no Illumina short reads were available, the CCS were first assembled using wtdbg2 (v. 2.5) (Ruan and Li, 2020) and finally polished with minimap2 (v. 2.17) (Li, 2018). The final sequence for the investigated region was extracted from the assembly using the forward and reverse primers as references. The final reference sequences were annotated using pgap with the option "ignore-all-errors" (version 2020-07-09.build4716 or 2020-09-24.build4849) (Tatusova et al., 2016) (see script in Supplemental file 1).

2.2.4. Data analysis

Amplicon sequences were visualized with CLC Main Workbench Version 20.0 software. The number, length, and order of the genes between *fdxA* and *pimH* were compared for all studied strains.

All protein aspartase and DcuAB sequences were aligned and pairwise compared using CLC Main Workbench Version 20.0.

2.3. Aspartase activity assays

2.3.1. Cloning and preparation of expression vectors

The functional annotation of the aspartase-encoding genes was confirmed by cloning and over-expressing three of them. The aspartase-encoding genes (*aspA1*, *aspA2*) from strains CIRM-BIA1 and FAM14176 (*aspA*) were subjected to heterologous expression using the DNA of these two strains as a template for amplification. PCR was conducted using a Biometra ^{T}Gradient instrument (Biometra, ^{T}Gradient, Biometra GmbH, 37079 Göttingen, Germany) in 25 μL consisting of 1 μL template DNA, 1 U AmpliTaq Gold (Roche Diagnostics AG, Rotkreuz, Switzerland), 2.5 μL 10 × PCR buffer containing 15 mM $MgCl_2$ (Roche Diagnostics), 0.5 μL PCR nucleotide mix (10 mM; Promega AG, Dübendorf, Switzerland), and 1 μM forward and reverse primers (Table 2: No. 3 & 4, No. 5 & 6). PCR amplification was initiated at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 60.5 °C (*aspA1*) or 58.2 °C (*aspA2*) for 30 s, 72 °C for 3 min, and 72 °C for 7 min. The *aspA* was amplified with the primers 14176p_aspA-F and 14176p_aspA-R2 in 25 μL using the Expand Long Template PCR System (Roche Diagnostics, Indianapolis, and the USA). The PCR amplification was initiated at 95 °C for 10 min, followed by 35 cycles of 93 °C for 15 s, 60.5 °C for 30 s, 68 °C for 2 min, and 68 °C for 7 min. The amplicons were visualized with an Agilent 2100 bioanalyzer using the Agilent DNA 7500 Kit (Agilent Technologies AG). Each of the PCR products of *aspA*, *aspA1,* or *aspA*2 was cloned into pEXP5-NT/TOPO vectors according to the manufacturer's protocol and transformed into One Shot TOP10 competent *E. coli* (ThermoFisher Scientific). Plasmids were isolated with a QIAprep® Spin Miniprep Kit (QIAGEN). The sequence of the plasmid inserts was verified by Sanger sequencing (Fasteris SA, Plan-les Ouates, Switzerland).

2.3.2. Recombinant aspartase production in E. coli

E. coli BL21 (DE3) harboring the pEXP5-NT/*asp*A1, pEXP5-NT/*asp*A2, or pEXP5-NT/*aspA* plasmids were grown in 200 mL LB broth supplemented with ampicillin (100 μg mL^{-1}) at 37 °C on a rotary shaker (220 rpm). When the culture reached an optical density of 0.4 at 600 nm, 1 mM of isopropyl β-D-1-thiogalactopyranoside was added to induce the expression of the cloned gene. The culture was then further incubated at 27 °C for 4 h on the shaker. Bacterial cells were harvested by centrifugation (3000×*g*, 10 min, RT), washed twice with 20 mM sodium phosphate (pH 7.4), and frozen at −20 °C. The recombinant His-tagged proteins were purified using a Protino Ni-TED 1000 kit (Macherey-Nagel, Düren, Germany) by suspending the cells in 500 μL 1 × LEW buffer provided by the kit, adding approximately 0.4 g glass beads (212–300 μm diameter), and homogenizing with an Omni Bead Ruptor (6 m s^{-1} for 45 s). The homogenate was centrifuged (13,000×*g*, 10 min, RT), the pellet was resuspended in 500 μL 1 × LEW buffer, homogenized, and re-centrifuged. The supernatant was pooled with the previous one and applied to Protino Ni-TED 1000 prepacked columns to purify the His-tagged proteins according to the kit's instructions. The buffer of the eluted proteins was changed to 20 mM sodium phosphate (pH 7.4) using PD-10 columns (VWR, Dietikon, Switzerland). The protein

concentration was determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific). The purity of the purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by colloidal Coomassie blue staining.

2.3.3. Determination of the recombinant aspartase activity

The enzyme assays were performed in total volumes of 2 mL, consisting of 50 mM sodium phosphate (pH 6.0, pH 6.8, or pH 7.4), 7.5 mM L-aspartate, and 5 mM $MgCl_2$. The assays were performed at RT, and the absorption at 240 nm was determined at 5 min intervals for 30 min using a PerkinElmer spectrophotometer. All measurements were done in triplicate. The amount of fumarate produced per min was calculated with a molar extinction coefficient of 2530 M^{-1} cm^{-1} (Emery, 1963).

2.3.4. Quantification of aspartase activity in wild-type P. freudenreichii strains

2.3.4.1. Preparation of cell-free extracts. Each *P. freudenreichii* strain was cultured at 30 °C for 72 h in 50 mL lactate broth pH 6.8 (Turgay et al., 2011). The bacterial cells were then harvested by centrifugation (3000×*g*, 10 min, RT), washed twice with 20 mM sodium phosphate (pH 7.4), and finally suspended in 500 μL 20 mM sodium phosphate (pH 7.4). The cells were disrupted with approximately 0.4 g glass beads (212–300 μm diameter, 6 m s^{-1} for 45 s) using an Omni Bead Ruptor (Labforce AG, Muttenz, Switzerland). The extract was cleared by centrifugation (13,000×*g*, 10 min, 4 °C). Bacterial metabolites were removed by applying each extract to a PD-10 column (VWR, Dietikon, Switzerland). The total cell proteins were eluted with 20 mM sodium phosphate (pH 7.4) according to the manufacturer's instructions. The total cell protein content of the eluted fraction was determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific). The aspartase activity was analyzed in two cell-free protein extracts unless otherwise indicated.

2.3.4.2. Determination of aspartase activity in cell-free extracts. The reactions were performed in a total volume of 200 μL consisting of 50 mM sodium phosphate (pH 6.0, pH 6.8, or pH 7.4), 10 mM L-aspartate, 5 mM $MgCl_2$, and 20 μL cell-free extract. The tubes were incubated at 37 °C for 2 h, the reaction was stopped by adding 20 μL 5 M perchloric acid. The samples were filtered through disposable filter holders (0.45 μm pore size). The organic acids present in the assays were separated on an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Cressier, Switzerland) protected with a cation H + Microguard cartridge at a flow rate of 0.6 mL min^{-1} at 65 °C and detection at 210 nm. The mobile phase was 3.8 mM H_2SO_4. Fumarate was used as standard. The activity was monitored as fumarate formation in a reaction volume of 200 μL per min per mg protein (fumarate•200 μL^{-1} min^{-1}•mg^{-1} protein). The peak area was determined using Chrom-Card v2.3 software (Thermo Electron S.p. A., Milan, Italy).

2.4. Assessment of the polycistronic mRNA containing aspA1, aspA2, and dcuA genes/tagged reverse transcription PCR (tagged RT-PCR)

We used strains FAM14217 and FAM19025 as examples to determine whether both *aspA1* and *aspA2* were transcribed under the cultivation conditions used for the analysis of strain-dependent aspartase activity. We also examined whether the genes *aspA1*, *aspA2,* and *dcuA* were transcribed into a single polycistronic mRNA. We minimized the loss of long transcripts and preserved their quality by adopting the method described by Aguena and Spira (2003), with some modifications, to avoid exhaustive DNase treatment after RNA extraction. False-positive results due to residual DNA were avoided by removing the hybrid primer used for reverse transcriptase PCR, as well as its leftovers not incorporated into the first cDNA, by ultrafiltration and RNaseH treatment. The hybrid primer comprises one end with 21 bases (tag) corresponding to a unique sequence (which does not anneal to the targeted genome) and the other end with a sequence homologous to the target gene. In the subsequent PCR (with the generated cDNA as template), the hybrid primer is replaced with the anchored reverse primer (tag) corresponding to the unique sequence so that only cDNA is amplified (Aguena and Spira, 2003).

The two strains were cultivated under the same conditions as used for determination of the aspartase activity. From each strain, 2 × 1 mL of bacterial culture was pelleted (18,600×*g*, 5 min, 4 °C) and pooled in a total of 200 μL TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) containing 3 mg lysozyme (Merck, Dr. Grogg Chemie AG, Stettlen, Switzerland) and transferred to DNA low-binding tubes (DNA LoBind Tube, Eppendorf AG, 22331 Hamburg, Germany). After incubation (10 min, 37 °C) and centrifugation (10,000×*g*, 5 min, RT), the supernatant was discarded and the pellet was incubated (5 min, RT) in 750 μL QIAzoL (Qiagen, Germany). After adding 150 μL chloroform, vortexing (15 s), incubating (3 min, RT), and centrifugation (12,000×*g*, 15 min, and 4 °C), 350 μL supernatant was transferred to a 2 mL tube from the ET® RNA Tissue Mini Kit (QIAGEN Instruments AG, Hombrechtikon, Switzerland) for further processing with the included DNase using the BioRobot EZ1 Advanced XL workstation (BioRobot® QIAGEN). A second DNase treatment (DNA-free™ Kit, Ambion, ThermoFisher Scientific, Zug, Switzerland) was performed on 44 μL RNA to remove residual DNA prior to complementary DNA (cDNA) production. The RNA was quantified using the Qubit™ RNA HS Assay kit (Invitrogen by ThermoFisher Scientific).

The cDNA synthesis was performed with 25 ng RNA using ThermoScript™ RT-PCR System according to the manufacturer's instructions (Invitrogen by Life Technologies). The samples were incubated with the hybrid primers (Table 2, Fig. 5) annealing on *dcuA* (primer: tag-dcuA-4), on *aspA2* and *aspA1* (primer: tag-AspA501-RNA) (Table 2: Primer-No. 7–8).

Transcription of *aspA1* was examined with the primer tag (Primer-No. 9) and RNA-PFS-aspA1-F (Primer-No. 11) and the cDNA generated before with the "*aspA1* and *aspA2*" hybrid primer (Primer-No. 8). Co-transcription of *aspA1* with *aspA2* was examined using the primer tag (Primer-No. 9) and AspA500-RNA (No. 12) with the cDNA generated with the "*aspA1* and *aspA2*" hybrid primer (Primer-No. 8). Co-transcription of *aspA2* with *dcuA* was examined with the primer tag (Primer-No. 9) and AspA2–3 (Primer-No. 10) and the cDNA generated with the "*dcuA*" hybrid primer (Primer-No. 7). The PCRs were conducted in 25 μL containing 1 U AmpliTaq Gold (Roche Diagnostics AG), 2.5 μL 10 × PCR buffer containing 15 mM $MgCl_2$ (Roche Diagnostics), 0.5 μL 10 mM PCR nucleotide mix (Promega AG), 1 μM forward and reverse primer, and 1/25 of the template cDNA. No RNA (but H_2O) was included as a PCR control. Since primer tag is used for PCR corresponding to a unique sequence (which does not anneal to the targeted genome). The PCRs were initiated at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min. The amplicons were visualized with an Agilent 2100 bioanalyzer using an Agilent DNA 7500 Kit.

3. Results

3.1. Locus of the aspartase-encoding genes and dcuA

The *fdxA-pimH* fragments (Fig. 1) in the 46 strains (Table 1) were analyzed to determine the number of aspartase-encoding genes and the presence or absence of *dcuA* (GenBank accession numbers are shown in Table 1). For each strain, we assessed the locus from *fumC* to the gene encoding aspartate/glutamate racemase (if present) to establish the number of genes and their respective lengths and copy numbers. Strains with similar characteristics were grouped as described in Fig. 2. The length of *fumC* was identical in all strains (1425 bp). An open reading frame (ORF) encoding a hypothetical protein with a size between 363 and 447 bp was found downstream of *fumC*, followed by one or two

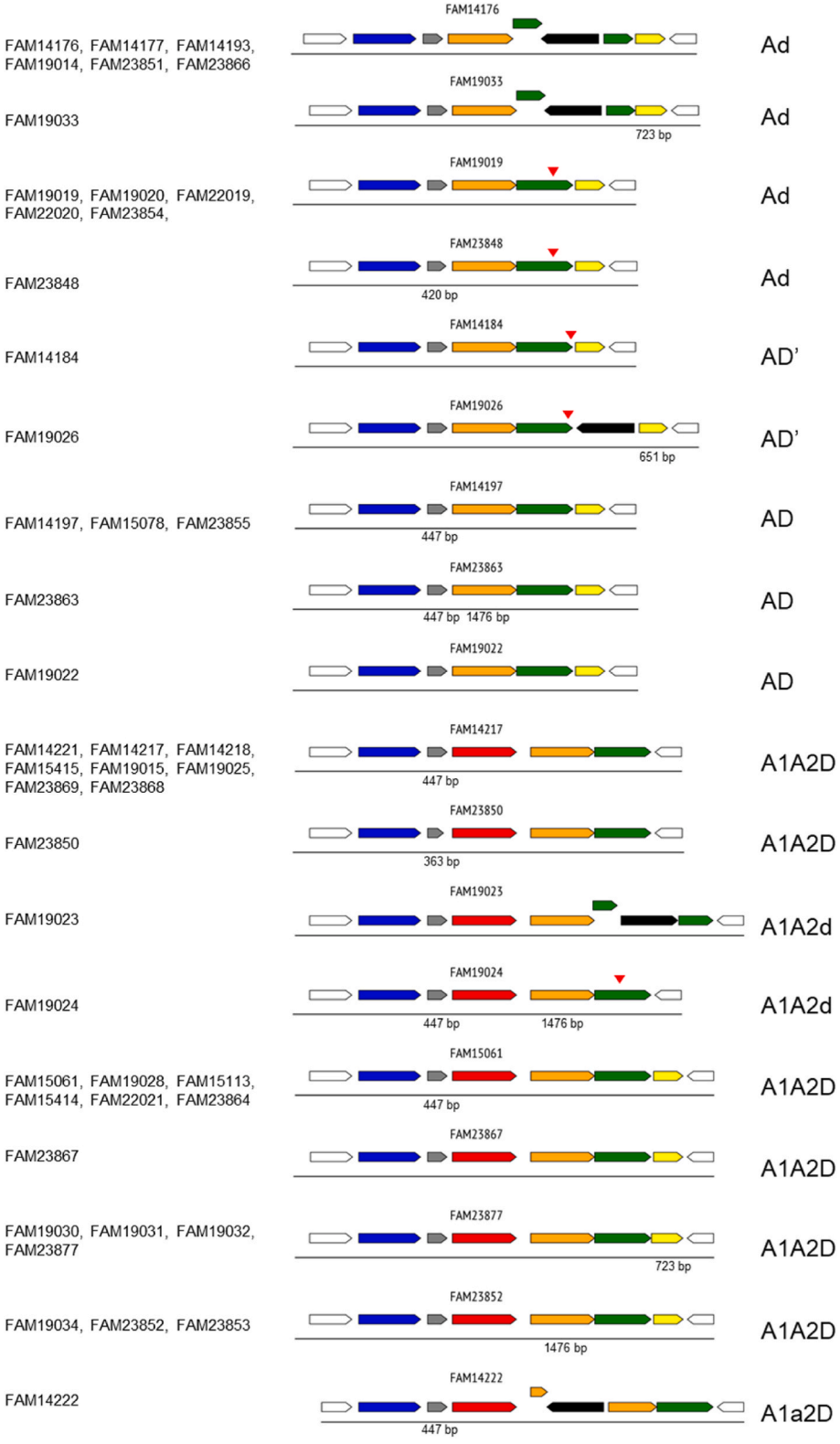

Fig. 2. Genomic rearrangements. Visualization of genomic rearrangement in the *aspA* locus in *Propionibacterium freudenreichii* strains depicted from *fumC* to the racemase-encoding gene (if present). Blue: *fumC* = 1425 bp, gray: ORF encoding a hypothetical protein = 444 bp, orange: aspartase-encoding gene = 1473 bp (*aspA* or *aspA2*) next to *dcuA*, red: aspartase-encoding gene *aspA1* = 1470 bp, green: *dcuA* = 1296 bp, yellow: aspartate/glutamate racemase-encoding gene = 675 bp, black: ORF encoding ISL3-like element ISPfr1 family transposase = 1308 bp (ORF in FAM19026 encodes ISL3-like element ISPfr2 family transposase = 1326 bp). Gene lengths are as described above unless otherwise indicated. Red triangle: frameshift mutation. Grouping according to copy number and arrangement of the aspartase-encoding genes and *dcuA*: Ad = strains with one aspartase-encoding gene and with *dcuA* with frameshift mutation or with *dcuA* disrupted by an ISL3-like element ISPfr1 family transposase: AD' = strains with one aspartase-encoding gene and the *dcuA* = 1287 bp lacking the last eight nucleotides: AD = strains with one aspartase-encoding gene and with *dcuA* = 1296 bp: A1A2D = strains with two aspartase-encoding genes and with *dcuA* = 1296 bp: A1A2d = strains with two aspartase-encoding genes and with *dcuA* with frameshift mutation or with *dcuA* disrupted by an ISL3-like element ISPfr1 family transposase: A1a2D = consisting of strain FAM14222 in which the *aspA2* is disrupted by an ISL3-like element ISPfr1 family transposase.

aspartase-encoding genes, depending on the strain. The *dcuA* gene occurred in all our strains. By contrast, the presence of the gene encoding an aspartate/glutamate racemase family protein downstream of *dcuA* was strain-dependent; it was identified in 34 strains and had a length of 651 bp (in FAM19026), 675 bp, or 723 bp (Fig. 2).

The strains were further distinguished based on their aspartase-encoding genes and *dcuA* (Fig. 2 for the Ad-, AD'-, AD-, A1A2D-, A1A2d-, and A1a2D-strains). The Ad-strains (13 strains) are strains with one aspartase-encoding 1473 bp *aspA* gene (denoted by uppercase A) and with a *dcuA* disrupted by an ISL3-like element ISPfr1 family transposase or with a *dcuA* exhibiting a frameshift mutation producing a stop codon as a result, generating 999 bp ORFs (denoted by lowercase d). The low aspartase-active reference strains FAM14176 and FAM14177 belong to the Ad-strains.

The AD′-strains (2 strains) are strains with *aspA* (as described above) and a 1287-bp *dcuA* lacking the last eight nucleotides (denoted by uppercase D′). The AD-strains (5 strains) are strains with *aspA* (1473 bp) and with a 1296-bp *dcuA* (denoted by uppercase D). The AD-strain FAM23863 harbors a 1476-bp *aspA*. The A1A2D-strains (23 strains) are strains with two aspartase-encoding genes, *aspA1* (1470 bp) and *aspA2* (1473 bp) and with a 1296-bp *dcuA*. Three of the A1A2D-strains (FAM23852, FAM23853, and FAM19034) harbor a 1476-bp *aspA2*. The high aspartase-active reference strains FAM14217 and FAM14218 belong to the A1A2D-strains. The A1A2d-strains (2 strains) comprise *aspA1-aspA2-dcuA* with a *dcuA* exhibiting a frameshift mutation producing a stop codon as a result, generating a 792-bp ORF (FAM19024) or with a *dcuA* disrupted by an ISL3-like element ISPfr1 family transposase (FAM19023). The label A1a2D-strain stands for FAM14222 in which the *aspA2* is disrupted by an ISL3-like element ISPfr1 family transposase (denoted by lowercase a2).

3.2. C4-dicarboxylate transporter

Genome analysis revealed that in addition to *dcuA* (see above) 34 Agroscope strains harbour a second gene encoding an anaerobic C4-dicarboxylate transporter. It belongs to the DcuAB family and has a size of 1317 bp, and is also present in the genomes of strains CIRM-BIA1 (PFREUD_03570) and DSM 20271T (RM_RS01630). In the genome sequence of strain FAM19022, parts of this gene (219 bp, 1104 bp) were found on two scaffolds flanked by ORFs encoding for a transposase, indicating a disruption in this C4-dicarboxylase transporter-encoding gene. No additional anaerobic C4-dicarboxylate transporter-encoding gene was found in the genome annotation of ten Agroscope strains (FAM14184, FAM14193, FAM14217, FAM14218, FAM14221, FAM14222, FAM19014, FAM19025, FAM19026 and FAM23866). A third C4-dicarboxylate transporter-encoding gene with the size of 1368 bp was found only in strain FAM19022, belonging to the DcuC family.

3.3. Protein sequence comparison of the aspartase-encoding genes and anaerobic C4-dicarboxylate transporter encoding genes

We performed a pairwise comparison of the protein sequences of the aspartase-encoding genes of all strains (Table S1 in the Supplemental material). The evaluation revealed 31 different protein sequences in 46 examined *P. freudenreichii* strains. They shared 87.58–98.16% identity. The different protein sequences were given numbers and assigned to their strains in Fig. 3 (indicated in brackets). The protein sequences of the two aspartase-encoding genes within a strain were never 100% identical; their identity was in the range of 84.49–95.52%, depending on the strain. Neither was an aspartase protein sequence from a strain with only one aspartase-encoding gene 100% identical to one from a strain with two aspartase-encoding genes. However, strains harboring one aspartase-encoding gene with identical (100% identity) aspartase protein sequences were detected: protein sequence 1 was found in 13 strains and protein sequence 2 in two strains. Similarly, gene clusters *aspA1-aspA2* were concordant between strains in their aspartase protein sequences.

The DcuA sequences of the A1a2D-, A1A2D- and AD-strains consist of 431 amino acid residues. Based on the alignment of their DcuA sequences, two groups became apparent, group CIRM-BIA1-DcuA and group DSM 20271^T-DcuA (alignment in the Supplemental material file 4). The DcuA sequences of the strains DSM 20271^T and CIRM-BIA1 differed in 121 amino acid residues (71.93% identity). Group CIRM-BIA1-DcuA comprises 10 DcuA sequences sharing 99.77–100% identity with the DcuA sequence of strain CIRM-BIA1. Group DSM 20271^T-DcuA comprises 19 DcuA sequences sharing 99.07–100% identity with the DcuA sequence of strain DSM 20271^T. In the same Group DSM 20271^T-DcuA fitted the DcuA sequences of the two AD′-strains (FAM14184, FAM19026) sharing 97.91% identity with the DcuA sequence of strain DSM 20271^T.

The protein sequences of the second gene encoding an anaerobic C4-dicarboxylate transporter of the DcuAB family consist of 438 amino acid residues. They share 99.77–100% identity. Only four protein sequences differ in one amino acid residue (from strains FAM14197, FAM15061, FAM15078, and FAM23853). At position 187, isoleucine is replaced by valine. The C4-dicarboxylate transporters were assigned to their strains in Table 3.

3.4. Aspartase activity of recombinant aspartase AspA, AspA1, and AspA2

Three genes encoding for three different aspartase alleles were cloned. The *aspA1* and *aspA2* of strain CIRM-BIA1 (Fig. 3: Protein sequences (17–18)) were used to investigate whether both gene products were enzymatically active. In addition, *aspA* of FAM14176 whose adjacent *dcuA* was interrupted by an ISL3-like element (Fig. 3: Protein sequences (1)) was cloned in order to analyze whether it has lost its activity. After heterologous gene expression in *E. coli*, the purified recombinant proteins were studied at pH 6.0, 6.8, and 7.4 (Fig. 4). All three recombinant aspartase enzymes catalyzed pH-dependent catabolism of aspartate to fumarate. The activity of the three enzymes was highest at pH 6.8. In comparison of the enzymes to each other, AspA showed the lowest activity.

3.5. Aspartase activity from cell-free extracts

The aspartase activity assay required a cell-free extract protein concentration $\geq$1 mg mL^{-1}. Data from cell-free extracts below this concentration were excluded. This was the case for one of the three protein extracts of strain FAM22019 and for two of the three protein extracts of the strains FAM23850, FAM23851, FAM23855, and FAM23868. No aspartase activity was detected at pH 6.8 for the protein extracts from the strains FAM22020 and FAM23851. However, activity was detected at pH 7.4 in the protein extract from strain FAM23851 and in the protein extracts of both strains at pH 6.0. The results were shown by plotting the aspartase activity data of the strains in increasing order (Fig. 3 and Supplementary file 1).

Aspartate was also catabolized to fumarate in a pH-dependent reaction in this assay (Fig. 3 and Supplementary file 3). Fig. 3 shows that the Ad-strains had low aspartase activity. The A1A2D-strains had the highest values. We classified strains as having high or low aspartase activity according to the low aspartase-active reference strains FAM14176 and FAM14177 and to the high aspartase-active reference strains FAM14217 and FAM14218. The values of the high aspartase-active strains FAM14217 and FAM14218 were only 15.7% and 18.9% of the highest values at pH 6.0, 5.4% and 4.6% of the highest values at pH 6.8, and 3.4% and 3.5% of the highest values at pH 7.4, respectively. The aspartase activity values were higher in other A1A2D-strains at pH 6.8 and pH 7.4, as well as in two AD-strains (FAM23863 and FAM14197), than in the strains FAM14217 and FAM14218. The Ad-strains reached a maximum of 3.5%, 0.3%, and 0.3% of the highest measured aspartase activity values at pH 6.0, 6.8, and pH 7.4, respectively. Fig. 3 (and Supplementary file 3) shows the different ranges of aspartase activity values for the individual strains. Depending on the pH, a few A1A2D-strains had a lower aspartase activity than the reference strains FAM14217 and FAM14218 or even showed an aspartase activity in the range of the Ad-strains (A1A2D-strains FAM23850 and FAM23868).

3.6. Transcription of co-directional genes aspA1, aspA2, and dcuA

The transcription of the genes *aspA1, aspA2,* and *dcuA* was investigated in the strains FAM14217 and FAM19025 with tagged RT-PCR. The cDNA was generated with two hybrid primers comprising one end with a unique sequence (which does not anneal to the targeted genome) and the other end targeting *aspA1* and *aspA2* or *dcuA*. We took advantage of

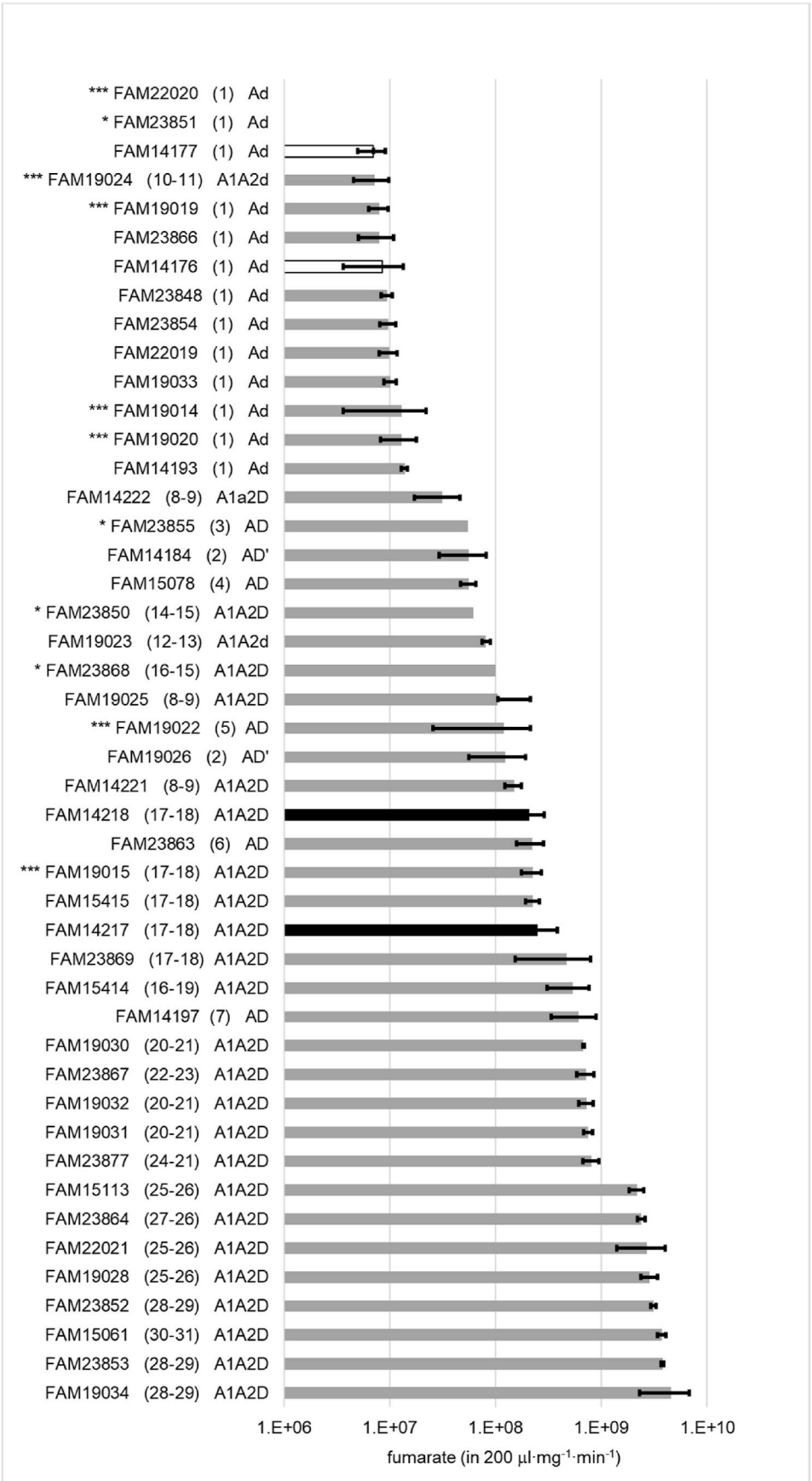

Fig. 3. Aspartase activity from cell-free extracts. Strain-dependent aspartase activity at pH 6.8 from 46 *Propionibacterium freudenreichii* strains. ***: n = 3 protein extracts, *: n = 1, without *: n = 2. Identical protein (100% identity) sequences are indicated with numbers in brackets. Equal numbers mean that the protein sequence has 100% identity. The gene organization of the labeled Ad-, AD'-, AD-, A1a2D, A1A2d-, and A1A2D-strains is described in Fig. 2. White bars: reference strains known as low aspartase-active strains, black bars: reference strains known as high aspartase-active strains. The horizontal axis is logarithmically scaled.

Table 3
The order and the labeling of the strains corresponds to the labels in Fig. 3 (aspartase activity from cell-free extracts). The identical protein sequences of the aspartase encoding genes (in brackets) and the gene arrangement of the locus of the aspartase encoding genes and *dcuA* are indicated. The presence of the C4-dicarboxylate transporter of the *aspA-dcuA* operon of the AD-, AD'-, A1a2D- and A1A2D-strains are assigned to the strains in the second and third column separated in two groups based on their protein sequences. The presence of the second annotated C4-dicarboxlate transporter is plotted in the fourth column. DcuC was only annotated in strain FAM19022. FAM14176, FAM14177: reference strains known as low aspartase-active strains, FAM14217, FAM14218: reference strains known as high aspartase-active strains.

Strains	group DSM20271^T-DcuA	group CIRM-BIA1-DcuA	second C4-dicarboxylate transporter
FAM22020 (1) Ad			x
FAM23851 (1) Ad			x
FAM14177 (1) Ad			x
FAM19024 (10–11) A1A2d			x
FAM19019 (1) Ad			x
FAM23866 (1) Ad			
FAM14176 (1) Ad			x
FAM23848 (1) Ad			x
FAM23854 (1) Ad			x
FAM22019 (1) Ad			x
FAM19033 (1) Ad			x
FAM19014 (1) Ad			
FAM19020 (1) Ad			x
FAM14193 (1) Ad			
FAM14222 (8–9) A1a2D		x	
FAM23855 (3) AD	x		x
FAM14184 (2) AD′	x		
FAM15078 (4) AD	x		x
FAM23850 (14–15) A1A2D		x	x
FAM19023 (12–13) A1A2d			x
FAM23868 (16–15) A1A2D		x	x
FAM19025 (8–9) A1A2D		x	
FAM19022 (5) AD	x		
FAM19026 (2) AD′	x		
FAM14221 (8–9) A1A2D		x	
FAM14218 (17–18) A1A2D		x	
FAM23863 (6) AD	x		x
FAM19015 (17–18) A1A2D		x	x
FAM15415 (17–18) A1A2D		x	x
FAM14217 (17–18) A1A2D		x	
FAM23869 (17–18) A1A2D		x	x
FAM15414 (16–19) A1A2D	x		x
FAM14197 (7) AD	x		x
FAM19030 (20–21) A1A2D	x		x
FAM23867 (22–23) A1A2D	x		x
FAM19032 (20–21) A1A2D	x		x
FAM19031 (20–21) A1A2D	x		x
FAM23877 (24–21) A1A2D	x		x
FAM15113 (25–26) A1A2D	x		x
	x		x
FAM23864 (27–26) A1A2D			
FAM22021 (25–26) A1A2D	x		x
FAM19028 (25–26) A1A2D	x		x
FAM23852 (28–29) A1A2D	x		x
FAM15061 (30–31) A1A2D	x		x
FAM23853 (28–29) A1A2D	x		x
FAM19034 (28–29) A1A2D	x		x

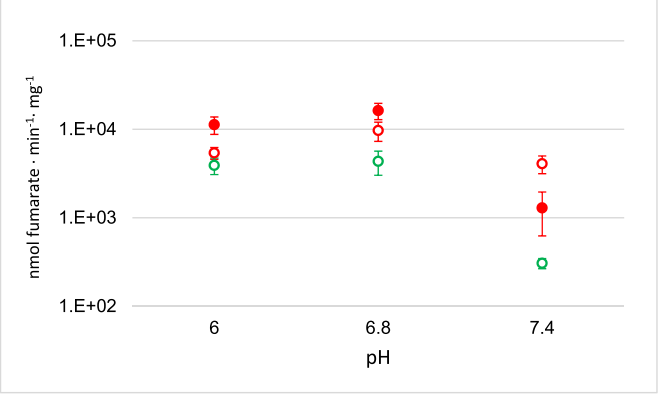

Fig. 4. Aspartase activity of recombinant aspartase. Aspartase activity at pH 6.0, pH 6.8, and pH 7.4 of recombinant aspartase AspA (green circle) from strain FAM14176, AspA1 (red circle), and AspA2 (full red circle) from *Propionibacterium freudenreichii* strain CIRM-BIA1. The vertical axis is logarithmically scaled.

the high similarity of the collocated aspartase-encoding genes by designing primers with a sequence homologous to *aspA1* and to *aspA2* (Primer: tag-AspA501-RNA, No. 8 or AspA500-RNA, No. 12) (Fig. 5). With the obtained cDNA (with primer tag-AspA501-RNA, No. 8), a subsequent PCR was performed replacing the hybrid primer with the anchored reverse primer tag and with primer RNA-PFS-AspA-F (No. 11), or with primer AspA500-RNA (No. 12). A PCR product of 452 bp (with primer tag + RNA-PFS-AspA-F, No. 11) resulted of both strains and confirmed the transcription of *aspA1*. With the obtained cDNAs of FAM14217 and FAM19025 (with primer tag-dcuA-4, No. 7), the subsequent PCR generated an amplicon of about 1080 bp (with primer tag and AspA2-3, No. 10), demonstrating that *aspA2* and *dcuA* were present in the same molecule of mRNA. We thus could show that both neighboring aspartase-encoding genes *aspA1* and *aspA2* were transcribed. However, they were not transcribed in one mRNA, as the expected PCR product (with primer tag + AspA500-RNA, No. 12) of approximately 995 bp covering the transition region from *aspA1* to *aspA2* could not be detected.

4. Discussion

The aspartase activity of *P. freudenreichii* is an important feature in cheese production, as the ability to use aspartate increases the growth rate of *P. freudenreichii*, while the co-metabolism of aspartate and lactate influences the sensory quality of the cheese. The aspartase activity varies considerably in cell-free extracts of *P. freudenreichii* strains (Blasco et al., 2011; Turgay et al., 2011). In this study, we assessed whether

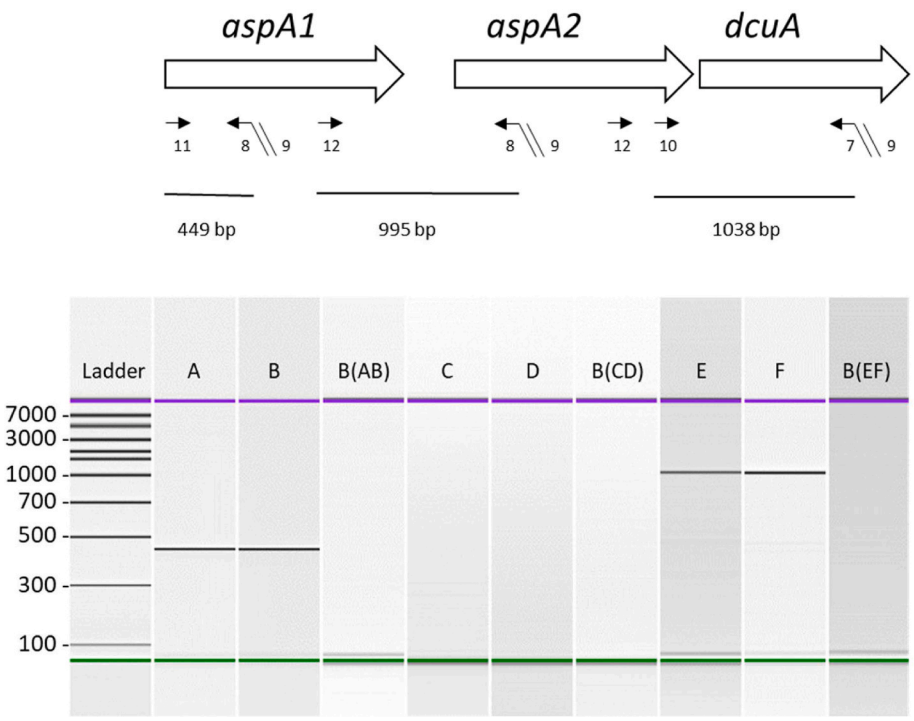

Fig. 5. Transcription analysis of the *aspA1-aspA2-dcuA* gene cluster with tagged RT-PCR. The upper part illustrates a scheme of the *aspA1-aspA2-dcuA* gene cluster of strain FAM14217 and FAM19025. Small numbered arrows indicate the annealing regions of the primers used for the tagged RT-PCR performance. The PCR products were analyzed with Agilent 2100 bioanalyzer software and DNA 7500 chips. FAM14217, lane A, C, and E. FAM19025, Lane B, D and F. PCR-Blanks in lane B(AB), B(CD) and B (EF). PCR products of 452 bp (A, B) were obtained with primers 9 and 11 with cDNA synthetized using tagged primer 8. No PCR product resulted with the primers 9 and 12 with cDNA synthetized using tagged primer 8. PCR products of 1084 bp (E) and 1075 bp (F) were obtained with primers 9 and 10 with cDNA synthetized using tagged primer 7.

DNA-based prediction of aspartase activity might reduce the burden of experimental screening by comparing the aspartase activity from cell-free protein extracts from 46 strains with the gene organization at the locus containing aspartase-encoding genes and the *dcuA* transporter gene. The study in our sampling of 46 strains revealed a diverse gene rearrangement, in agreement with Deptula et al. (2017), whose expanded genome sequence analysis of 20 *P. freudenreichii* strains revealed the presence of two aspartase-encoding genes in the majority of the strains. They found that only one gene coded for aspartase in three strains (JS4, JS9, and JS25), while the second aspartase gene was disrupted by a transposase in two strains (JS8 and JS18). Three strains (JS, JS11, and JS13) had a transposase inserted between the two aspartase-encoding genes. One strain (JS17) harbored three aspartase coding genes, two adjacent and the third one resulting from a transposase-mediated duplication (see Supplemental file 23 of Deptula et al. (2017)).

The aspartase activity data at pH 6.0, 6.8, and 7.4 from our 46 strains confirmed that all Ad-strains had at least 4.5 times lower aspartase activity than the high aspartase-active reference strains FAM14217 and FAM14218 (A1A2D). Under all three pH conditions, the lowest values of aspartase activity were recorded for the Ad-strains and the highest values for the A1A2D-strains.

We favored the data at pH 6.8, as the three aspartase isozymes studied by heterologous gene expression had the highest aspartase activity at pH 6.8. This pH may also correspond to the activity in the living cell, as the intracellular pH is assumed to be around pH 7 (Crow, 1987). Our data indicate that pre-selection of strains with low aspartase activity (Ad-strains) based on genomic data is feasible. The use of key target genes as screening opportunities has already been shown by Abeijón Mukdsi et al. (2014). We could not prove with our assay that the level of aspartase activity depends on the number of aspartase-encoding genes, since the aspartase activity values of the AD-strains with these gene organizations varied across a large range. However, we showed that *dcuA* is crucial.

The fact that the strains had such different levels of aspartase activity could reflect their protein sequences, as these differed in some amino acids. In all Ad-strains, the *aspA* gene coded the same protein sequence. We formed groups of Ad-, AD'-, AD-, A1A2d-, A1a2D, or A1A2D-strains based on gene organization and *dcuA* length. However, the 46 strains also varied in their aspartase protein sequences (Fig. 3). Examination of the three aspartase proteins showed lower activity for the AspA belonging to Ad-strain FAM14176 than for the AspA1 and AspA2 (protein sequence 17–18) from the A1A2D-strain FAM15415. The different aspartase activities resulting from the three isoenzymes indicated structure-function relationships. The impact of the protein sequence on aspartase activity was demonstrated by Murase et al. (1991), who used site-directed mutagenesis to show that replacement of Cys-430 with Trp residue in AspA from *E. coli* W enhanced the aspartase activity. The crucial residues that influence activity in the 31 different aspartase protein sequences we found within our 46 strains still require further exploration.

We also cannot yet explain the results for the two Ad-strains, FAM22020 and FAM23851, which had no detected strain-specific aspartase activity at pH 6.8. This lack of activity does not appear to be a consequence of unfavorable pH or an insufficient aspartase concentration in the protein extract, as the other Ad-strains with the same protein sequence showed aspartase activity at pH 6.8, and aspartase activity was detected in both FAM22020 and FAM23851 at pH 6.0. The low aspartase activity could reflect a low aspartase concentration in the total protein extract.

The role of the anaerobic C4-dicarboxylate transporter on aspartase metabolism must also be considered. In an elaborated study with *Campylobacter jejuni* Wösten et al. (2017) showed that anaerobic C4-dicarboxylate transporter of the DcuAB family contributes to the aspartate and succinate exchange in the cell under low-oxygen conditions. In *E. coli* and *C. jejuni*, DcuA is encoded in an operon with the gene for aspartase (Six et al., 1994; Wösten et al., 2017). Both genes are present in one operon, suggesting a functional link between aspartase and DcuA. With two of *P. freudenreichii* A1A2D strains we showed that *dcuA* is transcribed with *aspA2* as a polycistronic mRNA. Salgado et al. (2000) studied the distances between genes in *E. coli*. Namely, between neighboring genes in the same operon (knowing their functional relationships) and between neighboring genes in different transcription units. He demonstrated that genes within operons have much shorter intergenic distances than genes that are adjacent but not in the same operon (Saldago et al., 2000). Due to the same short intergenic distance

of one aspartase-encoding gene next to *dcuA*, this operon is also expected in our other *P. freudenreichii* strains.

In our assay for aspartase activity, the transport function of the detected C4-dicarboxylate transporters was not experimentally tested, as the assay was performed with total cell-free protein extracts.

A dysfunctional DcuA could possibly impair aspartase production but not its initiation. We detected aspartase activity in all strains; therefore, the initiation of aspartase production cannot depend on the functioning of DcuA. As it was shown in the study by Wösten et al. (2017) even when *dcuA* was knocked out, the *C. jejuni* mutant was able to utilize aspartate and secrete succinate. They demonstrated that an additional C4-dicarboxylate transporter DcuB also participates in the transport of aspartate into the bacterial cell, but considerably less than DcuA. DcuB was shown to be responsible for the transport of succinate to the environment, and when both genes *dcuA* and *dcuB* were mutated, neither succinate was excreted nor did the aspartate concentration decrease. We found in 36 out of the 46 studied strains an additional C4-dicarboxylate transporter belonging to the DcuAB family. No such transporter was detected in four A1A2D-strains (FAM14217, FAM14218, FAM14221, FAM19025), three Ad-strains (FAM14193, FAM19014, FAM23866), in both AD′- strains (FAM14184, FAM19026) and the A1a2D-strain (FAM14222). In one AD-strain (FAM19022) the gene was truncated by a transposase. In the study by Deptula et al. (2017) the genomes of six strains likewise did not harbour an additional C4-dicarboxylate transporter belonging to the DcuAB family (JS8, JS9, JS11, JS13, JS20, JS22). As aspartase activity was detected in the three Ad-strains (FAM14193, FAM19014, FAM23866) the initiation of aspartase production may also not depend on the function of the additional C4-dicarboxlate transporter of the DcuAB family.

In *C. jejuni* the regulation of *dcuA* and *dcuB* is controlled by RacR binding to the *aspA-dcuA* promoter (Wösten et al., 2017) and depends on the nature of other available electron acceptors (such as nitrate or trimethylamine *N*-oxide) (van der Stel et al., 2015). Further studies with *C. jejuni* have demonstrated that the addition of C4-carbon sources such as aspartate, succinate or fumarate to the culture medium had no effect on the transcription of *dcuA*, *dcuB* and *dcuC* (Wösten et al., 2017), i. e. no effect on the transcription of the *aspA-dcuA* operon. Nevertheless, the *dcuA* or *dcuB* mutants grew less than the wild type, especially the *dcuB* mutant, when aspartate was added to the medium. Furthermore, the regulation of *dcuA* transcription was assigned to the growth phase, as a 15-fold higher transcript level was observed in the grow phase compared to the stationary phase (Wösten et al., 2017). Extrapolating these observations to *P. freudenreichii* strains, one could hypothesize that Ad-strains grow less than AD-strains or than A1A2D-strains therefore that less *aspA-dcuA* is transcribed resulting in less aspartase. A benefit with the additional C4-dicarboxylate transporter (DcuAB) does probably not emerge from the data. For example the A1A2D-strains FAM14217 and FAM14218 (high aspartase-active reference strains) have no additional C4-dicarboxylate transporter (DcuAB), they grow well and produce high amount of succinate (Turgay et al., 2011). This cannot only be attributed to their different aspartase sequence. The strains FAM19015, FAM23869 and strain FAM15415 (CIRM-BIA1) have the same aspartase protein sequence, their aspartase activity values are in the same range and they have an additional C4-dicarboxlate transporter encoding gene (DcuAB). In *C. jejuni* DcuA and DcuB exhibited different functions (Wösten et al., 2017). It is possible that in *P. freudenreichii* the C4-dicarboxlate transporter from the *aspA-dcuA* operon is more general, comprising the properties of DcuA and DcuB of the species *C. jejuni*. The functions and importance of the different C4-dicarboxylate transporters in *P. freudenreichii* requires further research. However, it is clear from our data, that Ad-strains reveal lower aspartase activity than AD'-, AD-, A1a2D- and A1A2D-strains, independently of the presence of an additional C4-dicarboxylate transporter belonging to the DcuAB family. Based on the protein sequences, we divided the DcuA into two groups. The highest values of aspartase activity were detected in A1A2D-strains with the DcuA protein sequence of group DSM 20271^T-DcuA. Furthermore these strains harbour an additional C4-dicarboxlate transporter of the DcuAB family. The extent to which the highest aspartase activity values is due to the additional C4-dicarboxlate transporter, the protein sequence of DcuA, the protein sequences of the aspartases, or all of these together remains to be investigated.

The regulation of aspartase production in *P. freudenreichii* strains with one or two collocated aspartase-encoding genes also requires further investigation, as does the relevance of the second aspartase-encoding gene. Fumarate is vital as an electron acceptor under anaerobic conditions. The additional aspartase gene may allow a more balanced fumarate production. Our results for strain FAM14217 showed that *aspA1* and *aspA2* were transcribed from separate mRNAs and might be regulated separately. We showed that both proteins have a metabolic function. The example of the A1a2D-strain shows that *aspA1* is also expressed, as the measured aspartase activity cannot result from AspA2 because *aspA2* is disrupted by an ISL3-like element ISPfr1 family transposase.

We are aware that the strain-specific aspartase activity of *P. freudenreichii* is just one factor among many others. Other properties must also be considered when choosing a strain for cheese production and need experimental verification. However, the number of strains requiring testing can be reduced by first making a pre-selection based on genomic data.

5. Conclusion

The studied locus in 46 *P. freudenreichii* strains revealed a diverse gene arrangement around the aspartase-encoding gene. The highest values of aspartase activity were detected in strains with *aspA1-aspA2-dcuA* with a DcuA sequence sharing 99.07–100% identity with the DcuA sequence of strain DSM 20271^T and an additional C4-dicarboxylate transporter belonging to the DcuAB family. Both aspartase-encoding genes were transcribed separately and therefore might be regulated independently of each other. The extent to which both aspartase-encoding genes of *P. freudenreichii* strains are involved in aspartase activity remains to be investigated. Low strain-dependent aspartase activity was always observed in vitro at pH 6.8 in strains with *aspA-dcuA* with a *dcuA* with either a frameshift mutation producing a stop codon or with a disruption by an ISL3-like element with or without additional C4-dicarboxylate transporter belonging to the DcuAB family. Our assay could not discriminate whether the low aspartase activity values are due to the protein sequence of the aspartase present in the strains or to a dysfunctional DcuA. The localization of both genes in one operon suggests a functional connection between aspartase and DcuA. A pre-selection based on genomic data can be made for strains with low aspartase activity.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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