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The "pH optimum anomaly" of intracellular enzymes of *Ferroplasma acidiphilum*

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ABSTRACT

A wide range of microorganisms, the so-called acidophiles, inhabit acidic environments and grow optimally at pH values between 0 and 3. The intracellular pH of these organisms is, however, close to neutrality or slightly acidic. It is to be expected that enzymatic activities dedicated to extracellular functions would be adapted to the prevailing low pH of the environment (0-3), whereas intracellular enzymes would be optimally active at the near-neutral pH of the cytoplasm (4.6-7.0). The genes of several intracellular or cell-bound enzymes, a carboxylesterase and three α–glucosidases, from *Ferroplasma acidiphilum*, a cell wall-lacking acidophilic archaeon with a growth optimum at pH 1.7 (Golyshina *et al*., 2000), were cloned and expressed in *E. coli*, and their products purified and characterized. The *Ferroplasma* α–glucosidases exhibited no sequence similarity to known glycosyl hydrolases. All enzymes functioned and were stable *in vitro* in the pH range 1.7-4.0, and had pH optima much lower than the mean intracellular pH of 5.6. This “pH optimum anomaly” suggests the existence of yet undetected cellular compartmentalization providing cytoplasmic pH patchiness and low pH environments for the enzymes we have analysed.

INTRODUCTION

Certain microorganisms, the extremophiles, inhabit environments characterized by harsh physico-chemical conditions. Extremophiles may be categorized into two general classes, according to their cytoplasmic milieu. In some environments, for example those characterized by extremes of temperature or pressure, the prevailing extreme environmental parameter(s) extend into the intracellular milieu, and the intracellular functions are therefore exquisitely adapted to such conditions. In other environments, however, for instance those characterized by extremes of pH or salinity/osmolality, or the presence of organic solvents, high levels of irradiation, extremophiles invest considerable metabolic resources to exclude, resist and/or compensate for the extreme parameter, and to create and maintain intracellular conditions more typical of non-extremophiles (mesophiles). Their cytoplasmic conditions are therefore distinct from those of the habitat, and intracellular functions are not adapted to the prevailing extreme environmental conditions.

Acidic environments, both natural and man-made, occur in many locations in the biosphere, and the acidophilic microbes that inhabit them have important ecological roles and make key contributions to the biogeochemical cycles. Moreover, acidophilic microbes have become a focus of interest for biotechnological applications (Matin, 1999; Edwards *et al*., 2000; Johnson and Hallberg, 2003; Kinnunen *et al*., 2003). Of particular interest among the acidophiles are representatives of three distinct archaeal families of the order *Thermoplasmatales*, namely *Thermoplasma acidophilum and T. volcanium* (Darland *et al*., 1970; Searcy, 1976; Segerer *et al*., 1988), *Picrophilus oshimae* and *P. torridus* (Schleper *et al*., 1995, 1996) and *Ferroplasma acidiphilum* and *"F. acidarmanus"* (Golyshina *et al*., 2000; Edwards *et al*., 2000; Pivovarova *et al*., 2002; Dopson *et al*., 2004), that grow optimally between pH 0 and 2, the lowest for all acidophiles. These archaeal acidophiles maintain an intracellular pH of 4.6-5.6 (Searcy, 1976; Van de Vossenberg *et al*., 1998b; Macalady *et al*., 2004). Intuitively, this would suggest that the biochemical machinery of the acidophile cell, separated from the external environment by a

cytoplasmic membrane exhibiting low proton permeability, would be optimised for this near-neutral-slightly acidic pH, whereas secreted enzymes having extracellular roles should function optimally at the very low pH values characteristic of the external environment (Van de Vossenberg *et al*., 1998a; Matin, 1999; Santos *et al*., 2004).

We have tested this postulate by determining the pH optima of a number of intracellular or cell-bound enzymes from *Ferroplasma acidiphilum* Y (DSM 12658^T), a cell wall-lacking acidophilic archaeon with a growth optimum of pH 1.7 isolated from a pyrite-leaching bioreactor fed with pyrite ores from Bakyrtchik, Kazakhstan (Golyshina *et al*., 2000). Surprisingly, the pH optima of all enzymes examined were low and corresponded more to the pH of the extracellular environment rather than that of the intracellular milieu.

RESULTS

The enzymes we selected were glycosidases/glycosyltransferases and a carboxylesterase, all of which play central roles in metabolism: the former participate in carbohydrate metabolism, energy processing, and glycosylation of lipids (most *Ferroplasma* lipids are glycosylated (Batrakov *et al*., 2002)) and other molecules, whereas the latter is involved in the synthesis of cofactors and precursors of macromolecules, as well as in energetic processes. Such enzymes are also ubiquitous, and therefore suitable for comparative analyses, and exhibit potential for biocatalysis applications (see Bornscheuer, 2002, and Panke and co-authors, 2004).

We extracted genomic DNA from *F. acidiphilum*, and constructed a genomic expression library using the bacteriophage lambda-based ZAP vector. The library was subsequently screened for the production of a number of hydrolytic enzymes, and three distinct clones expressing α-glucosidase activities were identified. The DNA inserts from these clones were sequenced and expressed. The expressed proteins were sequenced from their N-termini and

their sequences aligned with the ORFs on the corresponding cloned fragments (αGluFa, GlyFa1 and GlyFa2; database accession numbers: AJ717661, AJ850916 and AJ850917, respectively).

In addition, the gene of a carboxylesterase was specifically targeted on the basis of homology to known carboxylesterases of other members of the *Thermoplasmatales,* and was selectively amplified from *F. acidiphilum* genomic DNA by polymerase chain reaction (PCR), and cloned in pET31b vector. This enzyme, EstFa (AJ850914), was also expressed in *E. coli* and purified both from *E. coli* and *F. acidiphilum*, to ensure identity of the native and recombinant forms.

The protein sequences were compared with those in public databases, and the enzymes were characterized in terms of activities, stabilities, substrate ranges and catalytic parameters.

***Functional assignment of* Ferroplasma *α–glucosidases lacking homology to known glycosyl hydrolases*.**

Sequence analysis of the 308 aa-long esterase, EstFa [MW 34,734; pI 5.91), revealed it to be very similar to the homologous proteins predicted from the genomes of "*F. acidarmanus*" (94% protein similarity), *P. torridus* DSM 9790 (65%) and *Thermoplasma volcanium* GSS1 (61%). It belongs to ester hydrolase family IV of the Arpigny and Jaeger classification (1999), according to the conserved motifs surrounding the catalytic residues of the family IV (/GDSAG/, /DPL/, /HGS/). The catalytic triad was deduced to be formed by S156, D251 and H281.

In contrast, GenBank and Pfam database searches revealed no significant similarity of the αGluFa [531 aa; MW 57,300 Da; pI 6.42] sequence to sequences of known glycosyl hydrolases. However, it exhibited high similarity to "uncharacterized membrane proteins" belonging to the COG1287 family found in almost all archaeal genomes (e.g. *A. fulgidus, Halobacterium sp.* NRC1*, M. jannaschii, Methanobacterium thermoautotrophicum, Thermoplasma volcanium, Pyrococcus horikoshii*), in the pathogenic bacteria *Helicobacter spp.* and *Campylobacter spp.,* and in the yeast *Saccharomyces cerevisiae*. Its highest similarity

(~96%) was to the provisionally annotated "PPE-repeat protein" of the "*F. acidarmanus*" unfinished genome sequence.

The second α-glucosidase activity was attributed to a 246 aa long protein, GlyFa1 [MW 28,316 Da; pI 9.53], which was relatively similar (up to 55% protein similarity, 40 % identity) to the dolichol phosphate mannosyltransferase-related proteins, from *T. acidophilum, T. volcanium*, *P. horikoshii* OT3, *P. furiosus*, *Aeropyrum pernix* K1 and *Pyrobaculum aerophilum* IM2.

The third α-glucosidase, GlyFa2 [361 aa; MW 40.505 Da; pI 6.42], clustered together with archaeal proteins annotated as "L-alanine-DL-glutamate epimerase and related enzymes" in the "*F. acidarmanus*" unfinished genome (100% protein sequence identity), O-succinylbenzoate-CoA synthase of *P. torridus* DSM 9790 (58% identity, 78% sequence similarity), probable N-acylamino acid racemase of *T. acidophilum* DSM 1728 and *T. volcanium* GSS1 (53% identity, 73% sequence similarity). Lower homology ranking was found to the predicted bacterial proteins N-acylamino acid racemase of *Oceanobacillus iheyensis* HTE831 (41% identity, 63% similarity), and O-succinylbenzoate-CoA synthase from *B. subtilis* 168, *Listeria monocytogenes* EGD-e, *Thermus thermophilus* HB27, and from about a dozen other *Bacteria* (38-39% identity, 58-60% similarity), but, interestingly, not from *Archaea*.

The enzymes have activity optima and are stable at extremely low pH values.

The enzymes expressed in *E. coli* and purified by standard procedures were inactive unless dialyzed against 10 mM sodium citrate buffer at pH values determined empirically to support the highest specific activities: these were pH 2.0 for EstFa and GlyFa1, 3.0 for αGluFa and 3.5-4.0 for GlyFa2. The EstFa esterase exhibited greatest activity at 50ºC with *p*-nitrophenyl propionate as substrate at pH values between 1.0 and 2.5; no activity was observed above pH 5.0 (Fig. 1). Native EstFa isolated directly from *F. acidiphilum* cell biomass exhibited properties identical to those of the recombinant esterase. EstFa showed the following substrate preferences: *p*-nitrophenyl acetate/*p*-nitrophenyl propionate>*p*-nitrophenyl butyrate>>*p*-nitrophenyl caprylate (k_{cat}/K_m from 64.2 to 0.6 $s^{-1}mM^{-1}$); it preferred triacetin over tributyrin

(k_{cat}/K_m: 21.3 and 3.1 $s^{-1}mM^{-1}$, respectively) and did not use triolein as the substrate (Table 1).

Maximal activities of the αGluFa, GlyFa1 and GlyFa2 α-glucosidases, with sucrose or maltose as substrates, were observed at pH values 2.5-3.0, 2.0, and 3.5-4.0, respectively (Fig. 1). The αGluFa and GlyFa1 enzymes exhibited more than 90% of maximal activities at pH values lower than 2.0, whereas the GlyFa2 exhibited minimal activity at pH values below 2.0; no activity was detected above 7.0 for αGluFa and GlyFa2, and above 5.5 for GlyFa1. All glucosidases had the following substrate preferences: sucrose>maltose>>maltotriose>>maltotetraose (k_{cat}/K_m from 293 to 0.1 $s^{-1}mM^{-1}$) (Table 2). All enzymes had temperature optima between 50 and 60°C, which is typical of enzymes of mesophilic organisms (Table 3). The acid tolerances of the *Ferroplasma* enzymes were estimated by incubating them for increasing periods of time at pH values ranging from 1.5 to 4.0 and measuring the residual activities. All were acid stable: $t_{1/2}$ values were 48 min at pH 1.7-2.0 and 50ºC for EstFa, 34 min at pH 2.5-3.5 and 60ºC for αGluFa, 23 min at pH 1.7-2.5 and 60ºC for GlyFa1, and 12 min at pH 3.5-4.0 and 60ºC for GlyFa2 (Table 3).

GlyFa1 and GlyFa2 are intracellular proteins, EstFa and αGluFa are membrane-localised.

The pH optima and acid stability of the enzymes studied would suggest that they are extracellular. In order to determine their cellular localizations, membrane, cytoplasmic and extracellular fractions of *Ferroplasma* cultures were obtained and assayed for esterase and α-glucosidase activities. Esterase activity was detected exclusively in the membrane fraction (53.9 ± 4.0 units/g of protein), whereas α-glucosidase activity was detected in both the membrane (1.6 ± 0.2 units/g of protein) and cytoplasmic (57.3 ± 2.7 units/g of protein) fractions. No hydrolytic activity was found in the concentrated culture supernatant of *F. acidiphilum* Y grown at 37ºC in 9K medium, pH 1.7 (Golyshina *et al*., 2000).

No convincing indications of signal peptide sequences of excreted proteins were found for any enzyme using the SignalP and Sigcleave tools. However, the search for potential membrane-spanning domains with the Tmpred tool (http://www.ch.embnet.org/software/TMPRED_form.html) revealed the existence of several such domains in αGluFa and suggested with a high probability that the region of the protein from aa position 30 to 283 may be located on the outside surface of the membrane. αGluFa also had a high similarity to "uncharacterized membrane proteins" belonging to COG1287, which is found in almost all archaeal genomes. The N-terminal part of EstFa was also predicted with a high probability to be membrane-anchored. EstFa has another potential membrane-spanning domain, aa 155-167, but the low probability of this prediction and the location of the catalytic triad (S155, D256 and H281 (Ferrer et al., unpublished)) were suggesting otherwise. No potential membrane-spanning domains were detected in the sequences of either GlyFa1 or GlyFa2 proteins.

Confirmation of these *in silico* predictions was subsequently obtained by a proteome analysis of the membrane and cytoplasmic fractions of *F. acidiphilum*. Of 10 abundant proteins from the membrane fraction (Fig. 2A) that were well resolved on the 2-D gel, excised, and partially sequenced, two were unambiguously identified as EstFa (spot 3) and αGluFa (spot 6). The other eight were highly similar to the predicted permease COG0730, a membrane protease subunit/stomatin/prohibitin homolog, acetyl-CoA acyltransferase COG0183, acyl-CoA synthase COG0318 [both of which are potentially involved in the reductive acetyl-CoA pathway of CO_2 fixation (Tyson *et al.*, 2004)], predicted transcriptional regulator COG0640, NADH dehydrogenase CPG 0650, hypothetical protein, and medium-chain acyl-CoA ligase COG0318, found in the sequenced genomes of *Thermoplasmatales* (Table 4).

Proteins GlyFa1 (spot 18) and GlyFa2 (spot 14) were unambiguously identified in the 2-D gel of the cytoplasmic fraction (Fig. 2B). Other major protein spots identified (Table 4) were

homologs of predicted proteins in *Thermoplasmatales*, namely glycine/serine hydroxymethyltransferase COG0112, methionine aminopeptidase COG0024, pyruvate kinase COG0469, glucosyl transferase COG1215, 3-hydroxyacyl-CoA dehydrogenase COG1250, thermosome COG0459, and phosphoenolpyruvate carboxykinase (PEP-carboxylase) COG1274, the enzyme expressed at higher levels (up to 10-fold) in autotrophically grown microbes and potentially involved in inorganic carbon fixation (Hügler *et al.*, 2003; Ettema *et al*., 2004). These results provide definitive evidence that EstFa and αGluFa are membrane-associated enzymes and GlyFa1 and GlyFa2 are intracellular cytoplasmic proteins. None of the studied enzymes were secreted into the extracellular medium. While the possibility remains that the catalytic domain of αGluFa functions in the extracellular milieu, those of the other enzymes clearly do not. Their pH activity optima are several units lower than the mean pH of the cytoplasm in which they function: this is the "pH optimum anomaly" of these *Ferroplasma* enzymes.

DISCUSSION

F. acidiphilum is an acidophilic, mesophilic and chemolithoautotrophic archaeon within the order of *Thermoplasmatales*: it grows in the pH range of 1.3 to 2.2, with an optimum at pH 1.7, has temperature optimum at about 37° C, gains energy by oxidizing ferrous iron and fixes inorganic carbon. In this communication, we have posed the question: do the pH optima of *Ferroplasma* enzymes reflect the extreme (extracellular milieu) or non-extreme (intracellular milieu) pH values prevailing in the environments in which they function? To answer this question, we have cloned and expressed the genes of a number of enzymes from *Ferroplasma*. Until now, the functional characterization of enzymes from this archaeon has been limited to aspartyl-tRNA synthetase and AAA ATPase (Tumbula-Hansen *et al*., 2002; Santos *et al*., 2004). Although many enzymes, such as glycosidases, from archaea have been readily produced in *E. coli*, the functional expression of archaeal genes is generally problematic for a number of reasons (Allers

and Mevarech, 2005). Thus, apart from the transcriptional peculiarities of archaea, their high proportion of As and Ts in the third codon position, especially in the A- and T-rich genomes of methanogens (<48 molGC%), *Sulfolobales* (about 30-40 molGC%), *Thermoplasma* (38-46 molGC%), *Picrophilus* and *Ferroplasma* (36 molGC%), poses a major obstacle for expression of archaeal genes in bacteria, in which codons like AGG, AUA, AGA, etc. are relatively rare (Lange and Ahring, 2001). However, despite the anticipated problems of expression of *Ferroplasma* genes in *E. coli*, the only difficulty we experienced was lack of enzyme activity in standard buffers of close to neutral pH.

From the expression library of the *Ferroplasma* genome we retrieved three α-glucosidases that exhibit no significant homology to known glycosyl hydrolases. Proteins homologous to αGluFa, GlyFa1 and GlyFa2 predicted from the genome sequences of other archaea have been previously annotated to have different functions to those experimentally deduced in the present study, reflecting the inadequacy of homology-based genome annotations. Although *F. acidiphilum* is generally unable to utilize sugars as carbon sources, it is conceivable that the cloned α-glucosidases may be involved in glycosylation of membrane lipids, the major fraction of which is represented by caldarchaetidylglycerol glycoside and triglycoside (Batrakov *et al.*, 2002).

All of the enzymes studied here, including the carboxylesterase obtained by PCR amplification of its gene and subsequent cloning of the PCR product, were found to be cell associated: two were cytoplasmic and two were membrane-bound. Counter-intuitively, all exhibited optimal activities and high stabilities at pH values substantially lower than the mean pH of 5.6 of the cytoplasm (Macalady *et al.*, 2004), and two of the four functioned best at 3 pH units lower (i.e. at a 1000-fold higher concentration of protons), values that are more typical of the external medium in which the organism grows, and that might be expected for excreted enzymes. These findings stand in stark contrast to those of earlier studies on other microbes, which demonstrated that extracellular enzymes (or extracellular domains of enzymes)

functioning in an acidic environment exhibit *in vitro* activity optima at low pH values, whereas intracellular enzymes from the same organisms have *in vitro* pH optima close to that of the cytoplasm or slightly basic (5.5-9.0) (Visser *et al*., 1996; Matzke *et al*., 1997; Sievers *et al*., 1997; Seow *et al*., 1998; Macalady *et al*., 2004; Schäfer *et al.*, 2004).

At present, we do not know the reason for the "pH optima anomaly" described here, but several possibilities suggest themselves. *Ferroplasma* is a cell wall-lacking microbe, and it is conceivable that a pH gradient might exist not only across the membrane, but also across the adjoining membrane:cytoplasm interface layer. If so, some enzymes may be confined to this more acidic layer, or other, as yet unknown, highly acidic compartments of the cell (ultrastructural studies on thin sections of *Ferroplasma* have not so far revealed any organelles or cellular compartments, the cell staining with fluorescent pH indicator dyes did not reveal those, either; unpublished). Alternatively, intracellular *Ferroplasma* enzymes with low pH optima might function as multi-enzyme complexes possessing overall pH optima closer to neutrality. Another possibility is that a low pH optimum is a reflection of another, presently unknown enzyme property selected by the *Ferroplasma* habitat. Although, at present we do not know what proportion of the intracellular enzymes of *F. acidiphilum* are active at low pH, our study strongly suggests that the number of "acidic" processes in acidophiles may be much higher than expected thus far.

EXPERIMENTAL PROCEDURES

***Materials*.** *p*-nitrophenyl esters ranging from acetate to caprilate, triacyglycerols, Fast Blue RR, α-naphthyl acetate, sucrose, maltose, TEMED and ammonium persulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA); acrylamide and bisacrylamide (37.5-1) was purchased from Carl Roth GmbH (Karlsruhe, Germany); molecular mass markers for SDS-PAGE were obtained from Novagen (Madison, USA); DNA restriction and modification enzymes were from New England Biolabs (Beverly, USA); lambda DNA was from Promega

(Mannheim, Germany); DNase I grade II was from Boehringer Mannheim (Mannheim, Germany); chromatographic media and LMW calibration kit for native electrophoresis were from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). All operations were performed at 4°C. Unless mentioned otherwise, the standard buffers used in the present study were 100 mM sodium citrate buffer, pH 2.0 (for EstFa and GlyFa1 enzymes), 3.0 (αGluFa enzymes), or 4.0 (for GlyFa2 enzyme).

Cloning, expression and purification of EstFa, αGluFa, GlyFa1 and GlyFa2 from F. acidiphilium strain Y^T (DSM 12658). An expression library of *F. acidiphilum* genomic DNA was established using the bacteriophage lambda-based ZAP Express Kit from Stratagene (La Jolla, USA) and the resulting phage suspension was used to infect the *E. coli* XL1-Blue MRF' host strain. Infected cells were mixed with NZY soft agar containing 0.2% (w/v) sucrose and 100 μM $FeCl_2$ and poured on 22.5 x 22.5 cm plates containing normal NZY bottom agar containing the same supplements. Plates containing about 10000 phage clones were incubated overnight and then overlaid with 50 ml of iodine solution (Sigma). Positive clones exhibiting a violet halo were picked, purified by dilution and re-plating, and converted to phagemids. Phagemids pBKαGluFa, pBKGlyFa1, and pBKGlyFa2, were maintained and expressed in *E. coli* strain XLOLR. Cells were grown in liquid LB medium containing 100 μM $FeCl_2$ and 50 μg/ml kanamycin.

The gene for esterase (EstFa) was PCR amplified using oligonucleotides EstFaFNsi 5'A**AT GCA T**TT AAT GAA TAT GGT AGA TCC GG and EstFaRXho 5'-A**CT CGA G**CT ATA AAT AGT CAG GAA TAA TCC TGG (endonuclease sites for *Nsi*I and *Xho*I are shown in bold). Amplification conditions were as follows: 95°C – 120 s, 30x[95°C – 45 s, 50°C – 60 s, 72°C – 120 s], 72°C – 500 s. The ca. 930 bp *est*Fa PCR product was purified by agarose gel electrophoresis, extracted with QiaExII Gel Extraction Kit (Qiagen, Hilden, Germany), ligated with the pCR2.1 plasmid using the TOPO TA Cloning kit (Invitrogen, California, USA), as recommended by the supplier, and electroporated into *E. coli* DH5α electrocompetent cells

(Invitrogen). Positive clones were selected on LB agar supplemented with kanamycin (50 µg/ml) and X-gal (5 mg/ml). The plasmid pCREst harbouring the PCR-amplified DNA fragments were isolated using Plasmid Mini Kit (Qiagen), and sequenced using universal oligonucleotide primers. The fragment containing the coding sequence for EstFa was excised from this plasmid by *Nsi*I and *Xho*I endonucleases, gel-purified as above and ligated (14°C, 16 hrs), with the pET-31b(+) plasmid (Novagen) that had been pre-digested with same endonucleases and dephosphorylated with shrimp alkaline phosphatase (Roche, Basel, Switzerland). The ligation mixtures were transformed into *E. coli* DH5α electrocompetent cells (Invitrogen) that were plated on LB agar supplemented with 50 µg/ml ampicillin. Plasmid pET-31b(+)Est, was subsequently isolated and then introduced into *E. coli* Origami (DE3)pLysS expression host by heat-shock transformation, according to the suppliers' protocols (Novagen). The transformation mixtures were plated on LB agar supplemented with chloramphenicol (34 µg/ml), ampicillin (50 µg/ml) and kanamycin (15 µg/ml).

For the expression of EstFa, αGluFa, GlyFa1 and GlyFa2, the corresponding *E. coli* strains were grown overnight at 37°C in LB liquid medium containing 100 µM $FeCl_2$ and appropriate antibiotics (see above). Overnight cultures were diluted 10-fold with fresh pre-warmed LB medium, incubated for 12 hours, after which isopropyl-β-D-galactopyranoside (IPTG) (2mM) was added. Incubation was continued for a further 2 hrs and then cells were harvested, resuspended in standard buffer containing one protease inhibitor cocktail tablet and DNase I grade II (Roche; Basel, Switzerland), incubated on ice for 30-45 min, and then sonicated for a total of 4 min. The soluble fraction was separated from debris by centrifugation (10,000 g, 30 min, 4°C), dialyzed overnight against the same buffer, and concentrated by ultrafiltration on a Centricon YM-10 membrane (Amicon, Millipore; Billerica, USA) to a total volume of 1000 µl. Enzymes were purified on a HiPrep 16/10 SP XL (Amersham Pharmacia Biotech) column equilibrated with standard buffer. The enzymes were eluted with a 0 to 1 M NaCl linear gradient.

Peak fractions containing active enzymes were collected, concentrated by ultrafiltration on a Centricon YM-10 membrane to a total volume of 1000 µl, and applied to a Superose 12 HR 10/30 gel filtration column pre-equilibrated with the above buffers containing 150 mM NaCl. Separation was performed at 4°C at a flow rate of 0.5 ml/min. The purified recombinant enzymes were dialyzed *vs.* buffer A and stored at –20°C, at a concentration of 10 µM.

Enzyme assays. Unless indicated, enzymatic activities were routinely measured by incubating purified enzymes (50 nM) with a substrate at various concentrations in standard buffer. All values were determined in triplicate and corrected for autohydrolysis of the substrate. The hydrolysis of the *p*-nitrophenyl esters was quantified spectrophotometrically after 2 min incubation, according to the method previously described (Ferrer *et al*., 2004); the hydrolysis of triglycerides was determined, using 0.01 M NaOH as titrant and 0.09% (v/v) acetonitrile as cosolvent, in a pH-stat (Mettler, model DL50), as described by San Clemente and Vadegra (1967). The specific activities are expressed in µmol of *p*-nitrophenoxide or free fatty acid released min-1 mg protein.

Hydrolytic activity using maltooligosaccharides was assayed by measuring by HPLC the reducing sugars released from 1% (w/v) substrate solution. The reaction mixture (1 ml of 100 mM buffer) contained 1% (w/v) of the substrate. The reaction was allowed to proceed for 10 min, and then stopped by heating 15 min at 80ºC, prior to analysis by HPLC on a 4.6 x 250 mm Lichrospher-NH_2 column (Merck). Acetonitrile:H_2O, 75:25 (v/v), was used as the mobile phase at 0.7 ml/min, at 25°C. Detection was performed with a refraction index detector (Varian). The hydrolytic activity towards sucrose was determined by measuring the release of reducing sugars from 1% (w/w) substrate solutions (50 µl) using the dinitrosalicylic acid (DNS) method (Summer and Howell, 1935). The microplate was incubated at 200 rpm for 10 min in an orbital shaker. Then, 50 µl of 10 g/l dinitrosalycilic acid were added to each well, heated at 85ºC for 30 min and cooled to room temperature. Finally, to each well with 150 µl water were added and absorbance at 540 nm was measured. A calibration curve was obtained with a 2 g/l glucose solution. All

enzyme reactions were determined to be linear with respect to time and protein concentration. Sample blanks were used to correct for spontaneous release of reducing sugar. One enzyme unit was defined as that liberating 1 µmol of glucose (or equivalent reducing groups) per minute.

Standard assays and kinetic analysis. Unlike otherwise indicated, the standard assays were performed at 50°C (for esterase) or 60ºC (for α-glucosidases) in standard buffer supplemented with sucrose (for α-glucosidase), and *p*-nitrophenyl propionate (*p*-NPP) (for esterase). Kinetic parameters (k_{cat} and K_m) were determined at 50 or 60ºC. Substrate concentration was varied in the range 0.1 – 20.0 mM and the activity was measured using the standard assays. Kinetic parameters were calculated fitting the initial rate values to the Hanes transformation of the Michaelis-Menten equation.

Preparation of extracellular, membrane and cytoplasmic proteins of F. acidiphilum for two-dimensional gel electrophoresis and activity analysis. Cells of *F. acidiphilum* Y were grown at 37ºC in 9K medium, pH 1.7, as described by Golyshina et al. (2000). The culture supernatant (1000 ml) was separated from the cells by centrifugation (4500 g x 20 min). The supernatant was concentrated by ultrafiltration in a Centricon YM-3 membrane to a final volume of 25 mL, and further dialyzed against 100 mM sodium citrate (pH 3.0). The harvested cells were resuspended in 5 mL 100 mM sodium citrate (pH 3.0), incubated at 37 °C in a shaking water bath for 2 h, and sonicated on ice for 15 min at 50% power and a duty cycle of 5 in a Branson Sonifier. The membrane and cytoplasmic fractions were separated by centrifugation at 30 000 g x 30 min at 4ºC. Cytoplasmatic proteins were dialyzed vs. 100 mM sodium citrate (pH 3.0), and stored at –20°C, until use. The membrane fraction was prepared by the sarkosyl method of Filip et al. (1973). Briefly, the membrane fraction was prepared as above, resuspended in an equal volume of buffer, 100 mM sodium citrate (pH 3.0), containing 2% (w/v) sodium-lauryl sarcosinate, 150 mM NaCl, and incubated at 37 °C for 1 h to facilitate membrane solubilization.

To precipitate proteins, 2 volumes of equilibrated phenol (AppliChem GmbH, Darmstadt, Germany) were added to 1 volume of sample, and the suspension was vigorously vortexed,

incubated on ice for 10 min and centrifuged (16000 x g, 15 min, 4°C). The top aqueous phase was removed, 2 volumes of distilled water were added, and the mixture was vortexed, incubated on ice for 10 min and centrifuged (4000 x g, 15 min, 4°C). The aqueous phase was removed and the step repeated. Then 1 ml of ice-cold acetone was added. Tubes were inverted several times, incubated on ice for 10 min and centrifuged (16000 x g, 15 min, 4°C). The liquid phase was removed and the remaining pellet air-dried for 5-10 min. Pellets were suspended in solubilization solution [7M urea, 2M thiourea, 4% CHAPS (BIOMOL GmbH, Hamburg, Germany), 20 mM Trizma Base (Sigma), 30 mM DTT (Carl Roth), 1 Complete Mini protease inhibitor cocktail tablet (Roche, Germany)] and stored at -70°C until use.

Two-dimensional gel electrophoresis and analysis of 2-D gel images. Two-dimensional gel electrophoresis was performed as described previously (Heim *et al*., 2003). Briefly, approximately 200 µg of protein was applied to 24 cm pH 3-7NL IPG strips (ReadyStrip™, Bio-Rad, CA, USA) and fractionated by isoelectric focusing on a Protean IEF Cell (Bio-Rad) at a maximum voltage of 10000 V for approximately 320 KVh according to the following program: 50 V, 100 Vh; 300 V, 800 Vh; 600 V, 2000 Vh; 2500 V, 5000 Vh; 7500 V, 30000 Vh; 10000 V until the end of run. The strips were then loaded on 1.5 mm thick 10-15% SDS-polyacrylamide gels and run overnight on a Hoefer DALT system (Amersham Biosciences). The gels were then fixed with 10% trichloroacetic acid and Coomassie-stained. Digitized images of Coomassie stained 2-D gels were acquired by scanning. Each sample was analysed in triplicate gels. Protein spots were excised from preparative gels stained with Coomassie Brilliant Blue G250. *In situ* trypsin digestion (sequencing grade modified trypsin, Promega, Madison, WI, USA) and peptide extractions were performed as described previously (Hale *et al*, 2000; Wissing *et al*., 2000). Peptide samples were eluted from ZipTips® U-C18 (Millipore, Bedford, MA, USA) using 1.5 µl of saturated α-cyano-4-hydroxycinnamic acid (Sigma) and analysed by protein sequence using Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometry (MS/MS peptide sequencing). Q-TOF analysis was performed to conveniently obtain the exact sequences of

internal fragments of peptides. Sequences of up to 30 amino acids were obtained from a tryptic digest of a protein from the 2-D gels.

Enzyme stability measurements. The stabilities of the EstFa, αGluFa, GlyFa1 and GlyFa2 enzymes at low pH were estimated by incubating the enzymes for increasing periods of time at pH values ranging from 1.5 to 5.0, and measuring the residual activity by the standard assays.

Other assays and methods. Protein concentrations were determined by the Bradford dye-binding method with a Bio-Rad Protein Assay Kit, using bovine serum albumin as standard (Bradford, 1976). SDS-PAGE and native electrophoresis were performed according to Laemmli (Laemmli, 1970).

Sequence analysis. The prediction of ORFs in the sequenced DNA fragments was done online (http://opal.biology.gatech.edu/GeneMark/heuristic_hmm2.cgi) using `GeneMark.hmm` tool for gene prediction in prokaryotes (Besemer and Borodovsky, 1999). The database search for homologous proteins/protein families was performed online using `blastX` and `blastP` tools with default settings at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul, *et al*., 1990) and in `Pfam` database (http://www.sanger.ac.uk/Software/Pfam/) (Bateman *et al*., 2004). Sequence alignments were then made using `ClustalW` online tool (www.ebi.ac.uk/clustalw) (Higgins *et al*., 1994). The enzymes were classified into respective families based on the conserved motifs found in the alignments and sequence similarities. The analysis of membrane-spanning domains was performed using the `Tmpred` tool at the EMBnet server (*http://www.ch.embnet.org/software/TMPRED_form.html*) (Hoffmann and Stoffel, 1993).

Accession numbers. The polypeptide sequences of, and genes coding for α-glucosidases (αGluFa, GlyFa1 and GlyFa2) and esterase (EstFa), have been submitted to the EMBL/DDBJ/GenBank databases under accession numbers AJ717661, AJ850916, AJ850917 and AJ850914, respectively.

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FIGURE LEGENDS

FIG. 1. pH-dependent activity curves for enzymes cloned from *F. acidiphilum*. The panels are: A: carboxylesterase EstFa; B: α-glucosidase αGluFa; C: GlyFa1; D: GlyFa2. Reactions were carried out in the following 100 mM buffer solutions: sodium citrate (○), sodium acetate (□), MES (morpholineethanesulfonic acid) buffer (△). 100% activity, determined by the standard assays, corresponds to k_{cat}/K_M values of 64.2 s^{-1} mM^{-1} for EstFa, 197 s^{-1} mM^{-1} for αGluFa, 95 s^{-1} mM^{-1} for GlyFa1, and 142 s^{-1} mM^{-1} for GlyFa2.

FIG. 2. 2-D protein gels of membrane-associated and cytoplasmic extracts of *F. acidiphilum* cells. A: membrane-associated extract; B cytoplasmic extract. Membrane and cytoplasmic proteins were isolated and analysed by 2-D SDS-PAGE and proteins of interest were cored and analysed by Q-TOF protein sequencing. The spots 3 and 6 on the membrane fraction gel (A) correspond to EstFa and αGluFa, respectively, the spots 14 and 18 of cytoplasmic proteome fraction (B) represent GlyFa2 and GlyFa1, respectively. Peptides corresponding to each marked spot are listed in Table 4.

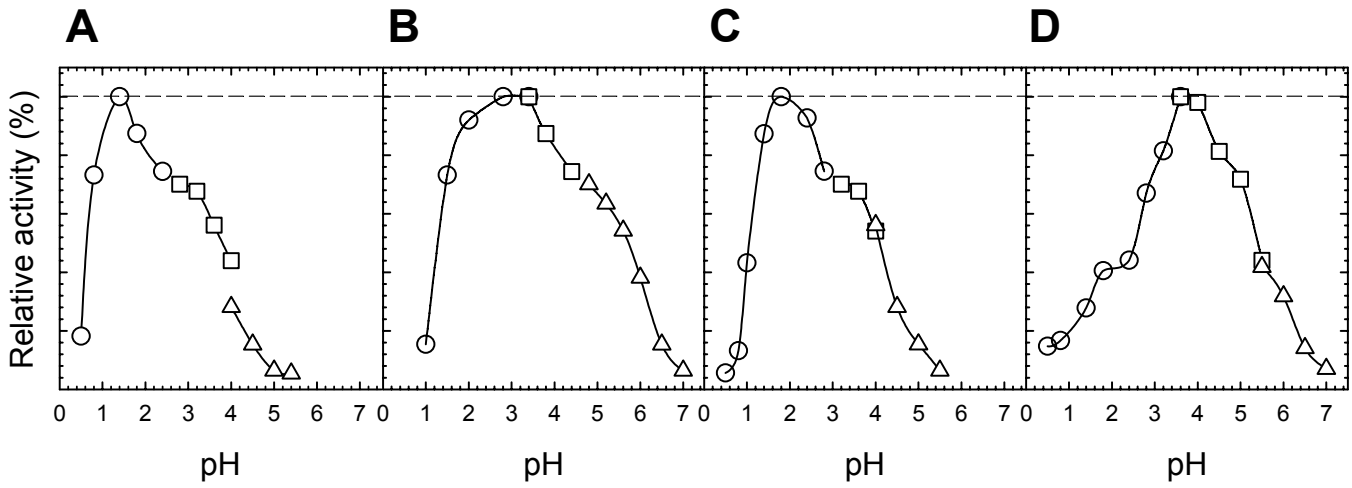

FIG. 1 Golyshina *et al*.

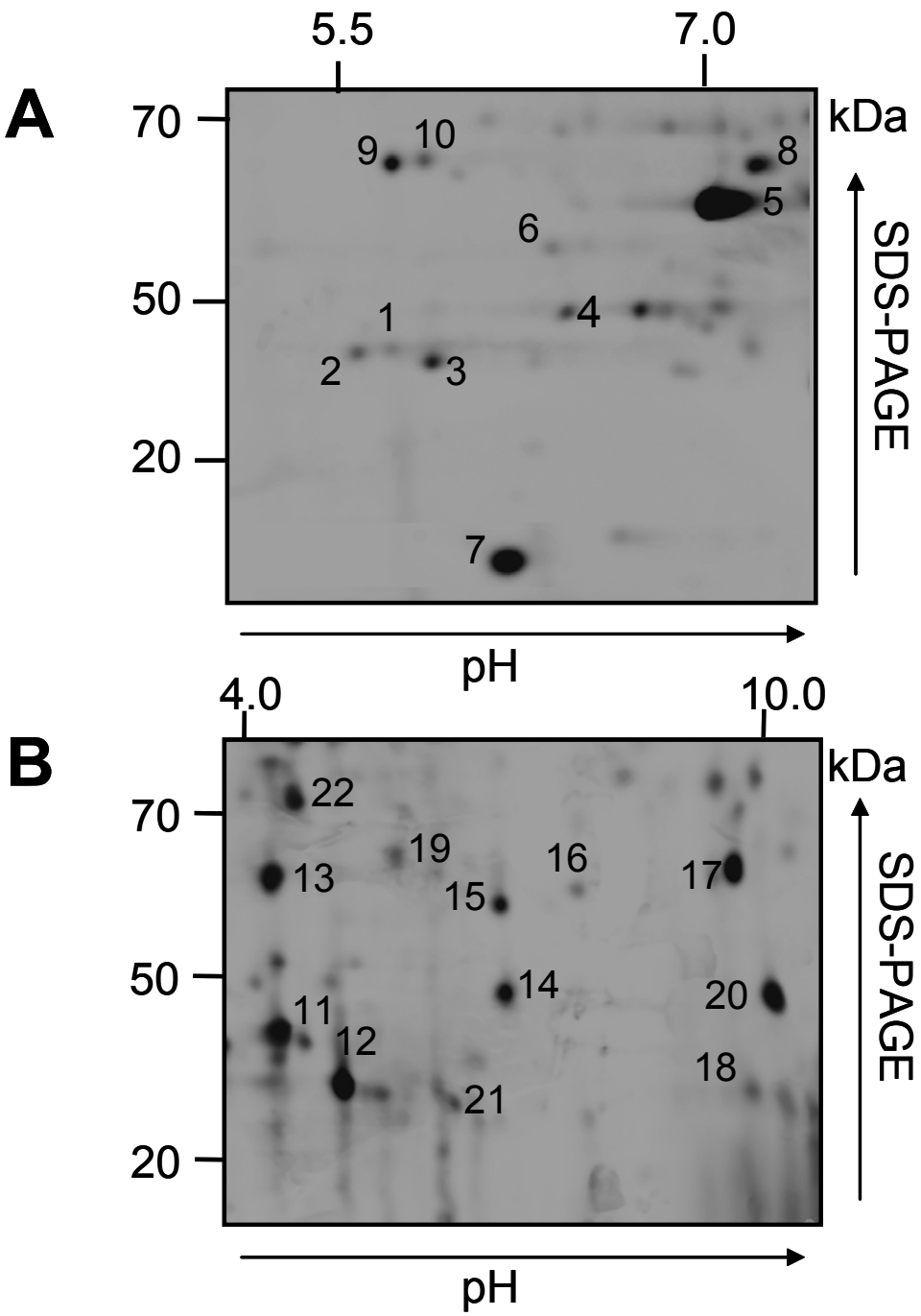

FIG. 2 Golyshina *et al*.

Table 1. Kinetic parameters [k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)] of EstFa carboxylesterase from *F. acidiphilum*

Substrate	K_m (mM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[a]
p-nitrophenyl acetate (*p*-NPA)	0.47 ± 0.15	25 ± 2	53.0 ± 6.4
p-nitrophenyl propionate (*p*-NPP)	0.45 ± 0.10	29 ± 2	64.2 ± 7.1
p-nitrophenyl butyrate (*p*-NPB)	0.60 ± 0.20	10 ± 1	16.7 ± 3.0
p-nitrophenyl caproate (*p*-NPC)	3.90 ± 1.00	2 ± 0.3	0.6 ± 0.1
Triacetin	2.20 ± 0.80	47 ± 3	21.3 ± 3.6
Tripropionin	3.50 ± 0.90	44 ± 3	12.7 ± 1.9
Tributyrin	7.40± 1.40	23 ± 2	3.1 ± 0.6
Triolein	–[b]	0	0

[a] Reaction conditions: $[E]_o$= 50 nM, [substrate] ranging from 0 to 20 mM, 100 sodium citrate buffer, pH 2.0, *T*= 50ºC. Calculated for molecular weight of 34734 Da for EstFa. The standard deviations refer to 95% confidence limit based in the curve fitting.
–[b] not determined.

Table 2. Kinetic parameters [k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)] of αGluFa, GlyFa1 and GlyFa2 α-glucosidases from *F. acidiphilum*

Substrate	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)[a]		
	αGluFa	GlyFa1	GlyFa2
Sucrose (α-D-Glc-(1→2)- α-D-Fru)	293.0 ± 44.0	142.0 ± 27.0	142.0 ± 30.0
Maltose (α-D-Glc-(1→4)-D-Glc)	197.0 ± 20.0	73.0 ± 12.0	101.0 ± 21.0
Maltotriose (α-D-Glc-(1→4)-D-Glc-(1→4)-D-Glc)	18.8 ± 1.8	28.0 ± 3.1	37.0 ± 4.3
Maltotetraose (α-D-Glc-(1→4)-D-Glc-(1→4)-D-Glc-(1→4)-D-Glc)	0.3 ± 0.1	0.6 ± 0.1	0.1 ± 0.01

[a] Data are means ± SDs

Table 3. Properties of the recombinant enzymes cloned from *F. acidiphilum*

Property	EstFa	αGluFa	GlyFa1	GlyFa2
Temperature optimum (ºC)	50	60	60	60
Optimum pH	2.0-3.5	2.5-3.0	2.0	3.5-4.0
Half life (min)	48 (pH 2.0; 50ºC) [a]	34 (pH 2.5; 60ºC) [a]	20 (pH 2.0; 60ºC) [a]	12 (pH 4.0; 60ºC) [a]
Apparent Mr (kDa)				
Native enzyme	35	57	56	80
Subunit [b]	36 (34.73)	57 (57.3)	28 (28.32)	42 (40.50)
p*I*	5.91	6.42	9.53	6.42

[a] After incubation at corresponding temperature and pH, the residual activities were measured in triplicate samples under the standard assay conditions.

[b] The theoretical molecular masses of the proteins are shown in the brackets.

Table 4. Peptides of membrane-bound and cytoplasmic fractions from *F. acidiphilum* identified on 2-D gels (Fig. 2) by Q-TOF protein sequencing and `BlastP` search for "short, nearly exact matches". Boldface marks the corresponding proteins cloned from *F. acidiphilum*. E-values and mismatches are shown for corresponding top hit homolog from the GenBank.

Protein	Spot Nr.	Peptide Fragments Sequences	E-value	mismat-ches
Predicted permease, COG0730 [*Thermoplasmatales: Thermoplasma acidophilum, Thermoplasma volcanium, Picrophilus torridus, "F. acidarmanus"*]	1	EVPPETKPDYSTKLFQLTGS	2e-06	1
		YSSIYWYEGYINLFIMAATA	3e-09	0
		LLYYNSKRKEWGKVNEKSRR	1e-11	0
Membrane protease subunits, stomatin/prohibitin homologs [*Thermoplasmatales*]	2	REVLGKMSFDEILSEREKIGESA	1e-11	0
		LQDAMSRQALAERER	1e-04	0
		GKMVDAAKQYANN	3e-04	0
Carboxylesterase [*Thermoplasmatales*]; **Esterase from *F. acidiphilum*, EstFa**	3	IISIEYRLAPEHKFPDAFNDAYDSFHYIAKK	8e-23	0*
		HGSATDFEVSDGARNIV	2e-07	0*
Acetyl-CoA acyltransferase (COG0183) [*Thermoplasmatales*]	4	MANPKYSKFNMNVSYNMGL	2e-11	0
		LMSGKKLKEYGLK	5e-04	0
		GLSIGHPLGATGARIAGTLARTLE	1e-05	0
Acyl-CoA synthase/AMP-acid ligases II, COG0318 [*Thermoplasmatales* and *Sulfolofus solfataricus* strain P2]	5	SDTILSLIPFYHIWSWGSAFHAAYL	4e-17	0
		VGEMTPDGGIKILDRVKD	5e-09	0
		GNINKWWIPVE	9e-04	0
"PPE repeat protein", "*F. acidarmanus*"; **α-Glucosidase from *F. acidiphilum*, αGluFa**	6	LPQGNYSISSSIPGFQNYSSTI	1e-12	0*
		YYKYEVPVGFYNFAYSNAHY	1e-12	0*
		FYSGNGIHMATSGITTLL	0.010	0*
Predicted transcriptional regulator, COG0640 [*Thermoplasmatales*]	7	RIKVMKLLMESPKNAY	6e-06	1
		VQGQGYGKVYFMSPTLVNSIK	7e-09	1
NADH dehydrogenase, COG0650 [*Thermoplasmatales*]	8	KANIESRKGPSIFQPYYDLFK-	2e-12	0
		PWGMFNTFPLFFLNALTM;	6e-11	0
hypothetical protein [*Thermoplasmatales*]	9	RPWYNYKQPSMSSESDRDIL	2e-12	0
		SYMSTSVNSIPRD	2e-04	0
medium-chain acyl-CoA ligase, COG0318 [*Thermoplasmatales*]	10	RDDHIVSLSENGDYIKF	2e-08	0
		VGEMTPDGGIKILD	3e-05	0
Glycine/serine hydroxymethyltransferase, COG0112 [*Thermoplasmatales*]	11	MLNKSDEFEEDALFIR	1e-05	1
		PHKRYYQGNQIVDIIEDKV	4e-11	0
Spot 12: Methionine aminopeptidase, COG0024 [*Thermoplasmatales*]	12	VAEKAEQVIRDKGAVPS	2e-05	1
		MSDNATTIEVGNTGNYSDTIKT	4e-08	1
		DKVIAIEPFA	0.23	0
Transposase and inactivated derivatives, COG0675	13	YVKDHYETRELTELSSQMKEE	8e-11	1
		RLQRSLSRKARYVEGTN	9e-08	0
		IYYGIIKKNELLLIKNKKL	2e-06	2
"L-Ala-DL-Glu epimerase", COG4948, "*F. acidarmanus*"; **α-Glucosidase from *F. acidiphilum*, GlyFa2**	14	ISPFTTSFGTDVNKDVYVFKLEHNGITA	1e-18	0*
		IIYHSRLAKGLSTPLCLDESITSPE	2e-15	0*
Thermosome alpha subunit, COG0459 [*Thermoplasmatales*]	15	IVEASKSQDMAVGDGTTTV	3e-07	1
		KGIDDTVQYYLAKYGIYGVRRVK	3e-14	0
		RVVAITKEDGRYLPGGGA	3e-08	0
Pyruvate kinase, COG0469 [*Thermoplasmatales*]	16	MEKYNKKLGIMVDLKGPE	2e-08	0
		KYAVPTIVATQVLESMVNS	9e-09	1
Phosphoenol-pyruvate carboxykinase, COG1274 [*Thermoplasmatales*]	17	MLEPANIVKDTYISSLDEKN	3e-04	0
		PAGSKYSEAGIQITDNPYV	8e-10	0
		QLLSDDIIWMHAKSWAQGV	0.001	0
Dolychol-phosphate mannosyltransferase, [*Archaea*]	18	NDIVIASRYVKGGSTGNR	0.009	5*
α-Glucosidase from *F. acidiphilum*, GlyFa1		KYQLLFVDDN	no hits	*
ATP-Dependent DNA ligase [*Thermoplasmatales*]; **ATP-Dependent DNA ligase, *F. acidiphilum*, LigFa**	19	AFFSKDNADMVETAYNFHPDIGLI	9e-14	1**
		TRLSSDDSEEINKFFEQSIEDG	7e-13	0**
Glucosyl transferase, COG1215 [*Thermoplasmatales*]	20	KKNALVQGIKHVS	0.001	0
		RCAMYITDVVKPFMVSD	2e-09	0
3-hydroxyacyl-CoA dehydrogenase, COG1250 [*Thermoplasmatales*]	21	AVIGSGIMGQGIAQVF	1e-06	0
		FPMGPFELMDTIGLDTM	2e-09	0
Thermosome beta subunit, COG0459 [*Thermoplasmatales*]	22	GGQPIFILKEGTKRESGK	2e-08	0
		EERKIGDDYMTFVTGS	1e-07	0

* no mismatches with aa sequences of cloned peptides

** no mismatches with cloned ligase (Ferrer *et al*., unpublished).