FEBS Letters 579 (2005) 1124–1128 FEBS 29250

Mutational analysis of the role of calcium ions in the *Lactobacillus reuteri* strain 121 fructosyltransferase (levansucrase and inulosucrase) enzymes

L.K. Ozimek[a,b], G.J.W. Euverink[a,b], M.J.E.C. van der Maarel[a,c], L. Dijkhuizen[a,b,*]

[a] *Centre for Carbohydrate Bioengineering, TNO – University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands*
[b] *Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands*
[c] *Innovative Ingredients and Products Department, TNO Nutrition and Food Research, Rouaanstraat 27, 9723 CC Groningen, The Netherlands*

Received 24 September 2004; revised 16 November 2004; accepted 23 November 2004

Available online 19 January 2005

Edited by Judit Ovádi

Abstract Bacterial fructosyltransferase enzymes belonging to glycoside hydrolase family 68 (GH68) are not known to require a metal cofactor. Here, we show that Ca^{2+} ions play an important structural role in the *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes. Analysis of the *Bacillus subtilis* Lev 3D structure [Meng, G. and Futterer, K. (2003) Nat. Struct. Biol. 10, 935–941] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Characterization of site-directed mutants in the putative Ca^{2+} ion-binding sites of *Lb. reuteri* Lev and Inu revealed that the Inu Asp520 and Lev Asp500 residues play an important role in Ca^{2+} binding. Sequence alignments of family GH68 proteins showed that this Ca^{2+} ion-binding site is (largely) present only in proteins of Gram-positive origin.

Keywords: Fructosyltransferase; Levansucrase; Inulosucrase; Mutagenesis; Calcium ion; *Lactobacillus reuteri*

1. Introduction

Bacterial fructosyltransferase (FTF) enzymes are found in Gram-negative and Gram-positive bacteria (see CAZY database: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). They convert sucrose into fructan polysaccharides, in most cases with β2,6 glycosidic bonds (a levan). A few FTF enzymes of Gram-positive bacteria synthesize inulin, with β2,1 glycosidic bonds [1–3]. Previously, we have characterized the *Lactobacillus reuteri* strain 121 levansucrase (Lev; E.C. 2.4.1.10) [4] and inulosucrase (Inu; E.C. 2.4.1.9) [5] enzymes, and identified the catalytic triad in both enzymes [6]. These two FTF enzymes are very closely related (86% similarity and 56% identity over 768 amino acids), both depending on Ca^{2+} ions for activity (albeit to a different extent) and displaying unusually high temperature optima (about 50 °C) (see Section 3). They differ most clearly in fructans synthesized, levan and inulin. Fructan synthesis by FTF enzymes of this (probiotic) *Lb. reuteri* strain is of strong interest for food and nutrition applications.

*Corresponding author. Fax: +31 50 363 2154.
E-mail address: l.dijkhuizen@biol.rug.nl (L. Dijkhuizen).

Analysis of the first high-resolution (1.5 Å) 3D structure of a *Bacillus subtilis* Lev [7] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Amino acid residues involved in this putative Ca^{2+} ion-binding site are conserved in most of the FTF proteins from Gram-positive bacteria, but not in all members of family GH68 (bacterial FTF and invertase enzymes) [7] (see Section 3). In *B. subtilis* Lev, Asp339 was suggested to make a most important contribution to Ca^{2+} binding. Data for FTF Asp339 mutant enzymes have not been reported yet. In the present study, we have analyzed the (differences in) sensitivity of the *Lb. reuteri* strain 121 Lev and Inu enzymes for Ca^{2+} ions and EDTA. Moreover, the suggested role in Ca^{2+} binding of residues Asp500 (Lev) and Asp520 (Inu), equivalent to Asp339 in *B. subtilis* Lev, was probed by site-directed mutagenesis.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen) was used for expression of wild type (WT) and mutant *Lb. reuteri* 121 *ftf* genes, inulosucrase (*inu*; GenBank Accession No. AF459437) and levansucrase (*lev*; GenBank Accession No. AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. Plasmid carrying *E. coli* strains were grown at 37 °C on LB medium [8] supplemented with 100 μg/ml ampicillin and 0.02% (w/v) arabinose for *ftf* gene induction. WT and mutant proteins were expressed in *E. coli* as constructs with a C-terminal truncation of 32 amino acid residues, and a C-terminal poly-histidine tag [2].

2.2. Molecular techniques

Alignments of FTF and invertase enzymes of family GH68 (CAZY, URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) were constructed using the Clustal X (1.5b) program. Using site-directed mutagenesis, the *Lb. reuteri* 121 Lev Asp500 and Inu Asp520 amino acids were replaced by Asn and Ala residues. Single mutations were introduced in the *inu* and *lev* genes using the "megaprimer" method [9] and were confirmed by sequencing. PCRs with *Pwo* polymerase (Roche biochemicals) used plasmid pBAD/myc/his/C containing the *lev* or *inu* genes as templates. All PCR products were digested with *Nco*I and *Bgl*II and ligated into the pBAD/myc/his/C vector, downstream of an inducible arabinose promoter and upstream of a His tag. For site-directed mutagenesis (synthesis of "megaprimers") the following oligonucleotides were used in PCRs: AD520A, 5′-CCAATGGTAAGCGCTGAAATTGAG-3′ (Inu D520A); BD500A, 5′-GGCTAGTGCTGAAGTTGAACGAC-3′ (Lev D500A); BD500N-I, 5′-CGTTTGGTCGTTCAACTTCATTACTAGCCATC-3′ (Lev D500N); AD520N, 5′-CAATGGTAAGCAATGAAATTGAG-3′ (Inu D520N).

0014-5793/$30.00
doi:10.1016/j.febslet.2004.11.113

Additionally, two flanking primers were used in all reactions: pBADRV-I, 5′-TCTGAGATGAGTTTTTGTTCGG-3′; pBADFD, 5′-TCCTACCTGACGCTTTTTATCG-3′. The underlined codons indicate mutations introduced; -I: antisense primer.

2.3. Purification of FTF proteins

All proteins produced were expressed in His-tag versions and purified by Ni–NTA affinity chromatography as described [2]. MilliQ water was used in all purification steps to minimize the calcium concentration in protein samples. However, no metal ion chelators were added. Purity was checked by SDS–PAGE. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

2.4. FTF enzyme activity assays

Activity variations with temperature (22–57 °C) were determined in 25 mM sodium acetate buffer, pH 5.4, with 100 mM sucrose (and Ca^{2+} ions as indicated), using purified *Lb. reuteri* 121 enzymes, WT Inu (2.9 μg/ml protein) and Inu mutants D520N, A (7.2 and 104 μg/ml, respectively), WT Lev (4.5 μg/ml protein) and Lev mutants D500N, A (13.8 and 21.6 μg/ml, respectively). At the highest temperatures (Lev mutants 45–55 °C, Inu mutants 50–57 °C), 5-fold higher protein concentrations were used. After preincubation of the assay mixture at the assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose released from sucrose [10]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). Experiments were performed in duplicate. Effects of EDTA were analyzed by determining temperature optima of Inu and Lev enzyme activity using the standard assay but with the reaction buffer containing 1 mM EDTA instead of Ca^{2+} ions.

2.5. FTF affinity for calcium-ion binding

Lev and Inu enzyme activities were determined as described above with $CaCl_2$ at concentrations of 0 (calcium-free buffer, prepared with MilliQ water) to 10 mM. The data obtained were used to estimate the Ca^{2+} binding affinity of the proteins. The Sigma Plot (version 8.0) program was used for curve fitting of data, with the standard Michaelis–Menten equation: $[y = (ax)/(b + x)]$. In this formula, y is the specific enzyme activity [U/mg], x is the calcium concentration [mM], a is the V_{max} and b is the apparent K_d (K'_d) [mM of calcium].

2.6. Thermostability of Inu and Lev enzymes

The WT Inu (2.9 μg/ml protein) and WT Lev (4.5 μg/ml protein) enzymes were incubated at a range of temperatures (37–60 °C) for 30 min with 1 mM calcium or in calcium-free buffer. The remaining enzyme activities were assayed at 37 °C according to the standard procedure (see Section 2.4). Samples preincubated in the presence and absence of calcium were assayed in buffer containing 1 mM calcium or in calcium-free buffer, respectively.

3. Results and discussion

3.1. Effects of Ca^{2+} ions and EDTA on Lb. reuteri 121 FTF enzyme activity

Plots of *Lb. reuteri* 121 Inu and Lev specific activity (initial rates) versus temperature in the presence or absence of Ca^{2+} (or EDTA, data not shown) revealed interesting similarities and differences between the two proteins (Fig. 1). Without addition of EDTA or Ca^{2+}, the *E. coli* produced and purified Lev enzyme lost activity at temperatures above 40 °C and was completely inactive at 50 °C, whereas Inu activity decreased above 45 °C and was completely inactive at 55 °C. Addition of 1 mM EDTA strongly reduced the activities of both enzymes. At room temperature, there was no effect of EDTA on Inu activity whereas a 60% reduction in Lev activity occurred. The negative effect of EDTA strongly increased with temperature, reaching 65% (Lev) and 40% (Inu) reduction in activities at 30 and 37 °C, respectively, the temperature optima for activity in the presence of EDTA (data not shown). With additional Ca^{2+} ions (1 mM) present, the Lev and Inu enzymes both showed considerably enhanced activities at higher temperatures, Inu now displaying an optimum at 55 and Lev at 45 °C (Fig. 1). The presence of (extra) Ca^{2+} ions thus appeared essential, especially at higher temperatures, to prevent inactivation of both the Lev and Inu enzymes (Fig. 1), most likely by temperature-dependent unfolding (see below). These effects of calcium ions and EDTA on both *Lb. reuteri* FTF proteins are in agreement with previously published observations for *B. subtilis* Lev [11–14] and *Streptococcus salivarius* FTF [15–17]. Our observations thus suggest that Ca^{2+} ions play an important structural role in these bacterial FTF enzymes and promote the *Lb. reuteri* Lev and Inu enzyme activity at elevated temperatures. Both *Lb. reuteri* WT FTF enzymes showed a high-affinity for Ca^{2+} binding, with K'_d values of 6.9 μM (Lev at 45 °C) and 0.48 μM (Inu at 50 °C). By comparison, a somewhat lower affinity for Ca^{2+} binding (K_d of 18 μM) has been reported for the *S. salivarius* Lev [18].

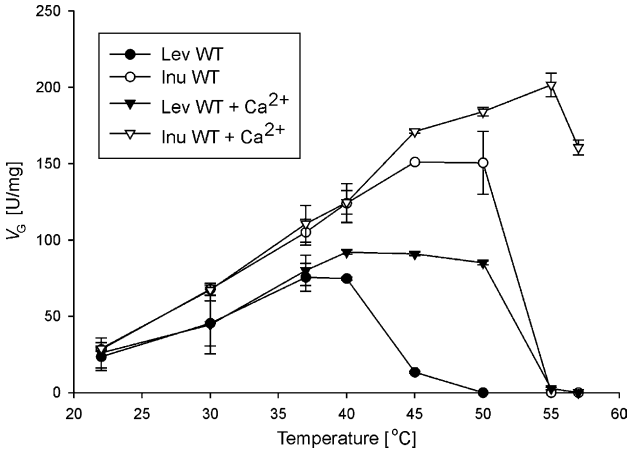

Fig. 1. Relationship between the specific activities (V_G) of the *Lb. reuteri* 121 Inu and Lev wild type enzymes and incubation temperature, measured in the absence or presence of 1 mM Ca^{2+} ions.

Chambert and Petit-Glatron [11] have shown previously that proper folding of Lev of *B. subtilis* depends on the presence or absence of a metal chelator. Our data show that also the two FTF enzymes of *Lb. reuteri* are affected in a similar way by the presence and absence of calcium ions and the chelator EDTA.

3.2. Effects of Ca^{2+} ions on thermostability of Lb. reuteri 121 FTF enzymes

The Inu and Lev proteins were incubated at a range of temperatures for 30 min, followed by determination of the remaining activity at 37 °C. A drastic loss of Inu activity was observed at temperatures above 45–50 °C, the presence of Ca^{2+} ions providing clear protection (Fig. 2A). Rather different profiles were obtained for the Lev protein (Fig. 2B). Following incubation of Lev at temperatures of 50–60 °C, 50–80% of activity was recovered at 37 °C. Also in this case the presence of Ca^{2+} ions provided protection. A clear reduction in Lev activity (initial rates) was observed at higher temperatures (Fig. 1), but the Lev protein apparently suffered no irreversible damage and recovered activity upon subsequent incubation at 37 °C

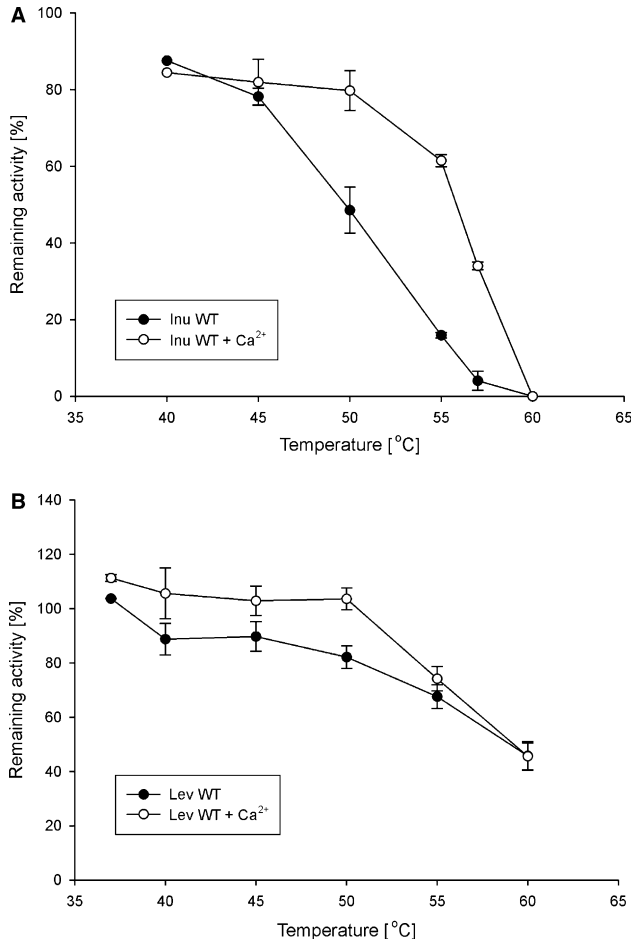

Fig. 2. Thermostability of the *Lb. reuteri* 121 Inu (2.9 μg/ml protein) (A) and Lev (4.5 μg/ml protein) (B) wild-type enzymes, measured in the presence and absence of 1 mM Ca^{2+} ions. The relationship between the temperature of pre-incubation (for 30 min) and the remaining specific activity (V_G, measured at 37 °C), is shown.

(Fig. 2B). We speculate that this involved a reversible protein unfolding–refolding process. In contrast, the damage of higher temperatures to the Inu protein is more drastic, with no such strong recovery apparent (compare Figs. 1 and 2A).

3.3. Sequence alignments of members of family GH68

Analysis of the *B. subtilis* Lev 3D structure provided evidence for the presence of a metal binding site, most likely for Ca^{2+} ions, showing (in) direct interactions with a number of amino acid residues [7]. This site is penta-bipyramidally coordinated with a water and the carbonyl oxygen of Leu308 on the apices, and with Asp339 Oδ1 and Oδ2, Asn310 Oδ1, Asp241 Oδ1 and Gln272 Oε1 on the equatorial positions. Alignment of all known family GH68 (a total of 22 bacterial FTF and invertase) enzymes revealed that only Asp339 is conserved in all sequences (data not shown). Ca^{2+} is coordinated by the *B. subtilis* Asp339 (conserved in all 22 GH68 sequences), present in the conserved DEIER motif, which also contains the general acid catalyst (Glu342 in the *B. subtilis* Lev; Glu523 and Glu503 in the *Lb. reuteri* Inu and Lev proteins, respectively) (Fig. 3) [6,7]. Alignment of the 15 known GH68 sequences from Gram-positive bacteria revealed that the five residues involved in calcium binding are conserved in most members of this group (Fig. 3). The *B. subtilis* Leu308 is conserved in 8 of these 15 family GH68 proteins of Gram-positive origin, e.g., in *Lb. reuteri* Lev (Leu431), but is present as a Trp, Thr or Tyr residue in the other 7 FTF proteins, e.g., in *Lb. reuteri* Inu (Trp486). The *B. subtilis* Gln272 is conserved in 13 FTF proteins of Gram-positive origin, except for the *Arthrobacter globiformis* beta-fructofuranosidase and the *Leuconostoc citreum* inulosucrase. The *B. subtilis* Asp241 and Asn310 are conserved within all these sequences of Gram-positive origin, except for the *A. globiformis* beta-fructofuranosidase and *Actinomyces naeslundii* FTF. Only Asp339 and none of the other residues forming the putative calcium-binding site is conserved in FTF proteins of Gram-negative origin. The putative Ca^{2+}-binding site in *B. subtilis* levansucrase thus is (largely) con-

```
SACB_BACSU   238  SSGDNHTLRDPH  265  TGTEDGYQGEE  305  TAELANGALG  337  VTDEIER
INU_LACRE    415  KGADNIAMRDAH  443  TGLEN-YQGED  483  RATWANAAIG  518  VSDEIER
LEV_LACRE    395  RENDDYCLRDPH  423  TGTED-YQSDD  463  LAGLANGALG  498  ASDEVER
SACB_STRMU   393  TGADNIAMRDPH  421  TGTEN-YQGED  461  RASWANAAIG  496  VSDELER
SACB_STRSA   440  DRTDNYCLRDPH  473  TGDEN-YQGEK  507  LASWANGSIG  542  VTDEVER
SACB_BACAM   238  TSGDNHTLRDPH  265  TGTENGYQGEE  305  DAELANGALG  337  VTDEIER
LEV_CLOAC    252  SSGDNHTLRDPH  279  TGTNDGYQGDT  320  DASLANGALG  352  VTDEIER
LEV_GEOST    238  SSGDNHTLRDPH  265  TGTEDGYQGEE  305  TAELANGALG  337  VTDEIER
LEV_LACJO    416  KGADNIAMRDAH  444  TGTEN-YQGDD  484  RAKWSNAAIG  519  VSDEIER
LEV_LACSA    457  RKNDDYCLRDPH  485  TGMED-YQSDD  525  LASLANGAIG  560  ASDEVER
LEV_PAEPO    250  VDMDNHTFRDPH  277  TGTETGYQGED  317  GAELANGALG  349  VTDEIER
UNK_BACLI    247  SSGDNHTMRDPH  274  TGTKTGYQGED  314  NASLANGALG  346  VTDEIER
FTF_ACTNA    350  ----GVNFRDPF  382  SAFVREQQYVD  430  GGYYQLANVG  463  VNDQTER
BFF_ARTGL    290  ----YYNFRDPF  321  SAMDRDE----  354  GATYQIGNVG  387  VTDQTER
INU_LEUCI    492  -QADMFTLRDPK  520  TGIYD-EASDQ  560  YSSTANGAIG  595  VTDEIER
                       : *        :.             .    . :*       ..*: **
```

Fig. 3. Partial amino acid sequence alignment of family GH68 proteins of Gram-positive bacteria. The 5 FTF amino acid residues forming the strongly conserved putative Ca^{2+} ion-binding site (*B. subtilis* numbering: Asp241, Gln272, Leu308, Asn310, Asp339; [7]) are shown in bold. The two FTF catalytic residues shown in this alignment, the transition state stabilizer (SACB_BACSUAsp247) and the acid/base catalyst (SACB_BACSU-Glu342) [6,7], are underlined. SACB_STRMU, *Streptococcus mutans* SacB; SACB_STRSA, *Streptococcus salivarius* SacB; SACB_BACSU, *Bacillus subtilis* SacB; INU_LACRE, *Lactobacillus reuteri* Inu; LEV_LACRE, *Lactobacillus reuteri* Lev; SACB_BACAM, *Bacillus amyloliquefaciens* SacB; LEV_CLOAC, *Clostridium acetobutylicum* levansucrase; LEV_GEOST, *Geobacillus stearothermophilus* levansucrase; LEV_LACJO, *Lactobacillus johnsonii* levansucrase precursor; LEV_LACSA, *Lactobacillus sanfranciscensis* levansucrase; LEV_PAEPO, *Paenibacillus polymyxa* levansucrase; UNK_BACLI, *Bacillus licheniformis* unknown protein; FTF_ACTNA, *Actinomyces naeslundii* fructosyltransferase; BFF_ARTGL, *Arthrobacter globiformis* beta-fructofuranosidase; and INU_LEUCI, *Leuconostoc citreum* inulosucrase. Asterisks (*) indicate conserved residues; (:) indicates that one of the following 'strong' group is conserved NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; (.) indicates that one of the following 'weaker' groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, NDEQK, NDEQHK, NEQHRK, FVLIM, HFY.

served within most of the family GH68 enzymes of Gram-positive origin and is missing in the FTF enzymes from Gram-negative bacteria. Previously, Ohtsuka et al. [19] and Hettwer et al. [20] have shown that the activity and stability of the levansucrases from the Gram-negative bacteria *Rahnella aquatilis* and *Pseudomonas syringae* are not affected by the presence of calcium or EDTA.

A low affinity Ca^{2+} binding site (K'_d of 1.25 mM, pH 7) has been mapped to Thr265 in the *B. subtilis* Lev [13]. This Thr265 hydroxyl hydrogen bonds to Asn310 Nδ2 [7] but is not involved directly in binding of calcium ions. Also the *B. subtilis* Thr265 residue is conserved in 13 out of the 15 GH68 proteins of Gram-positive bacteria, including both the *Lb. reuteri* 121 Inu (Thr443) and Lev (Thr423) (Fig. 3).

3.4. Site-directed mutagenesis of the Lb. reuteri FTF putative Ca^{2+}-binding site

The functional role of the conserved Asp339 residue in Ca^{2+} binding was studied by mutating the equivalent residues in the *Lb. reuteri* 121 Inu and Lev enzymes. Inu mutants D520N, D520A, and the Lev mutants D500N, D500A were constructed, expressed in *E. coli* and purified. The Lev and Inu mutants displayed interesting differences. In each case a strong reduction in activity was observed over the whole range of temperatures tested (compare Figs. 1 and 4). Especially Inu activity had become severely reduced. In both Inu and Lev, the Asp to Ala mutations caused a stronger reduction in activity than the Asp to Asn changes. The stimulatory effects of Ca^{2+} ions had become much smaller in the mutant enzymes, especially in case of the Asp to Ala mutations (compare Figs. 1 and 4). Not surprisingly, compared to an Asn residue, the introduction of an Ala residue at this position thus had a much stronger effect. The activities of mutants Lev D500N and D500A, however, were still clearly stimulated by Ca^{2+} ions (Fig. 4B).

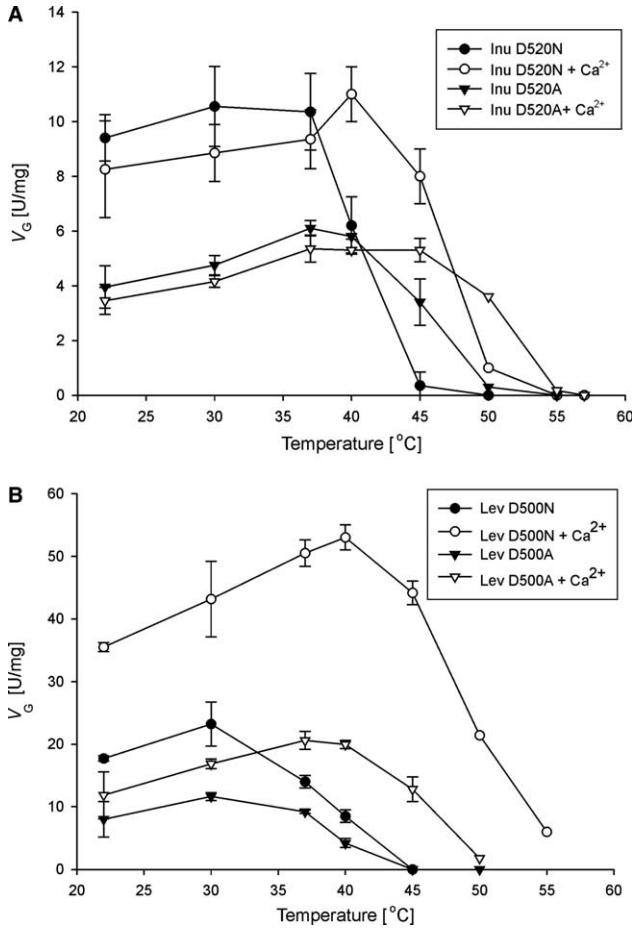

Fig. 4. Relationship between specific activities (V_G) of the *Lb. reuteri* 121 Inu D520N, (A) and Lev D500N, (B) mutant enzymes and incubation temperature, measured in the absence or presence of 1 mM Ca^{2+} ions.

At 50 °C, Inu mutant D520N was inactive in the absence of Ca^{2+}, with <1% of wild-type activity remaining in the presence of 1 mM Ca^{2+} (specific activity reduced from 180 to 1 U/mg) (Fig. 4A). Mutant D520A displayed a low activity at 50 °C in the absence of calcium (0.3 U/mg); its activity was clearly stimulated by the presence of calcium ions (3.6 U/mg). Mutant Inu D520N displayed a very severe reduction in affinity for Ca^{2+} binding, with a K'_d value of 790 μM at 50 °C (WT Inu at 50 °C: $K'_d = 0.48$ μM; approximately 1600-fold reduction in affinity). At lower temperatures, the Ca^{2+} dependency of Inu mutants D520N and D520A became reduced, also resulting in somewhat higher activities (11 and 6 U/mg, respectively). Inu WT incubated with 1 mM EDTA displayed the same relationship of activity with temperature as mutant D520N (without the presence of EDTA), albeit at a different level of activity (60 and 11 U/mg, respectively). Clearly, the mutations introduced in the Inu protein reduced its affinity for calcium binding, affecting both its stability and activity.

Characterization of Lev D500N revealed a clear but much smaller reduction in affinity for Ca^{2+} binding than observed for Inu D520N. Lev D500N displayed a K'_d value of 260 μM at 45 °C (Lev WT at 45 °C: $K'_d = 6.9$ μM; approximately 35-fold reduction in affinity). At 45 °C, Lev mutants D500N and D500A were inactive in the absence of Ca^{2+} ions, with, respectively, 45% and 15% of wild-type activity remaining in the presence of 1 mM Ca^{2+} (Fig. 4B). In the presence of 1 mM Ca^{2+}, the Lev D500N and D500A mutants displayed highest activity at 40 °C (53 and 19.5 U/mg, respectively). In the absence of Ca^{2+} ions, the optimal temperature for activity of the Lev D500N and D500A mutants was 30 °C (22.5 and 11 U/mg, respectively) (Fig. 4B). Lev WT enzyme exhibited the same relationship for activity with temperature in the presence of 1 mM EDTA (12 U/mg, data not shown) as mutant D500N.

4. Conclusions

The Inu and Lev enzymes studied in this paper have the same bacterial origin (*Lb. reuteri* 121) and share high sequence similarity. Nevertheless, they display interesting differences in thermostability and calcium dependency. Lev activity becomes reduced at relatively low temperatures already (Fig. 1), but this protein is clearly more resilient, largely recovering from activity loss during high temperature preincubations, even in the absence of calcium ions (Fig. 2). Mutations in the D520 (Inu) and D500 (Lev) residues in both cases resulted in strongly reduced activity levels (Fig. 4). Inu mutant D520N, however, was much more affected in its activity and affinity for calcium ion binding than Lev mutant D500N, with 1600 (at 50 °C) and 35-fold (at 45 °C) reduced K'_d values, respectively. The structural basis for these differences remains to be elucidated.

The data presented in this paper thus show that calcium ions have an important structural role in the *Lb. reuteri* 121 Lev and Inu proteins. Both proteins possess high-affinity Ca^{2+} binding sites. Residues D520 (Inu) and D500 (Lev), equivalent to D339 of *B. subtilis* levansucrase, are important for binding of the metal ion. The specific mutations D520N, D520A (Inu) and D500N, D500A (Lev) themselves, and/or the absence of Ca^{2+} caused by these mutations, may change the conformation of the strongly conserved DEIER motif, with the general acid catalyst [6,7], affecting catalysis and resulting in a strong reduction in FTF activities. Residues constituting this Ca^{2+} binding site [7] are completely conserved in most family GH68 enzymes from Gram-positive bacteria, suggesting that the stabilizing function of calcium ions is a general property of these proteins. This calcium-binding site appears to be absent in family GH68 enzymes of Gram-negative origin.

References

[1] Sato, Y. and Kuramitsu, H.K. (1988) Sequence analysis of the *Streptococcus mutans Scrb* gene. Infect. Immun. 56, 1956–1960.

[2] van Hijum, S.A., Geel-Schutten, G.H., Rahaoui, H., van der Maarel, M.J. and Dijkhuizen, L. (2002) Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. Appl. Environ. Microbiol. 68, 4390–4398.

[3] Olivares-Illana, V., Lopez-Munguia, A. and Olvera, C. (2003) Molecular characterization of inulosucrase from *Leuconostoc citreum*: a fructosyltransferase within a glucosyltransferase. J. Bacteriol. 185, 3606–3612.

[4] van Hijum, S.A.F.T., Szalowska, E., Bonting, K., van der Maarel, M.J.E.C. and Dijkhuizen, L. (2004) Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. Microbiology 150, 621–630.

[5] van Hijum, S.A., van der Maarel, M.J. and Dijkhuizen, L. (2003) Kinetic properties of an inulosucrase from *Lactobacillus reuteri* 121. FEBS Lett. 534, 207–210.

[6] Ozimek, L.K., van Hijum, S.A.F.T., van Koningsveld, G.A., van der Maarel, M.J.E.C., Geel-Schutten, G.H. and Dijkhuizen, L. (2004) Site-directed mutagenesis study of the three catalytic residues of the fructosyltransferases of *Lactobacillus reuteri* 121. FEBS Lett. 560, 131–133.

[7] Meng, G.Y. and Futterer, K. (2003) Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. Nat. Struct. Biol. 10, 935–941.

[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[9] Sarkar, G. and Sommer, S.S. (1990) The megaprimer method of site-directed mutagenesis. Biotechniques 8, 404–407.

[10] van Hijum, S.A., Bonting, K., van der Maarel, M.J. and Dijkhuizen, L. (2001) Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol. Lett. 205, 323–328.

[11] Chambert, R. and Petit-Glatron, M.F. (1990) Reversible thermal unfolding of *Bacillus subtilis* levansucrase is modulated by Fe^{3+} and Ca^{2+}. FEBS Lett. 275, 61–64.

[12] Petitglatron, M.F., Grajcar, L., Munz, A. and Chambert, R. (1993) The contribution of the cell-wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. Mol. Microbiol. 9, 1097–1106.

[13] Petitglatron, M.F., Monteil, I., Benyahia, F. and Chambert, R. (1990) *Bacillus subtilis* levansucrase – amino-acid substitutions at one site affect secretion efficiency and refolding kinetics mediated by metals. Mol. Microbiol. 4, 2063–2070.

[14] Chambert, R. and Petit-Glatron, M.F. (1988) Secretion mechanism of *Bacillus subtilis* levansucrase: characterization of the second step. J. Gen. Microbiol. 134 (Pt 5), 1205–1214.

[15] Song, D.D. and Jacques, N.A. (1999) Purification and enzymic properties of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. Biochem. J. 341 (Pt 2), 285–291.

[16] Townsendlawman, P. and Bleiweis, A.S. (1991) Multilevel control of extracellular sucrose metabolism in *Streptococcus salivarius* by sucrose. J. Gen. Microbiol. 137, 5–13.

[17] Milward, C.P. and Jacques, N.A. (1990) Secretion of fructosyltransferase by *Streptococcus salivarius* involves the sucrose-dependent release of the cell-bound form. J. Gen. Microbiol. 136, 165–169.

[18] Jacques, N.A. (1984) Calcium dependence of the cell-associated fructosyltransferase of *Streptococcus salivarius*. Carbohydr. Res. 127, 349–355.

[19] Ohtsuka, K., Hino, S., Fukushima, T., Ozawa, O., Kanematsu, T. and Uchida, T. (1992) Characterization of levansucrase from *Rahnella aquatilis* Jcm-1683. Biosci. Biotechnol. Biochem. 56, 1373–1377.

[20] Hettwer, U., Gross, M. and Rudolph, K. (1995) Purification and characterization of an extracellular levansucrase from *Pseudomonas syringae* pv. phaseolicola. J. Bacteriol. 177, 2834–2839.