Novel Activities of Glycolytic Enzymes in *Bacillus subtilis*

INTERACTIONS WITH ESSENTIAL PROTEINS INVOLVED IN mRNA PROCESSING*[S]

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Glycolysis is one of the most important metabolic pathways in heterotrophic organisms. Several genes encoding glycolytic enzymes are essential in many bacteria even under conditions when neither glycolytic nor gluconeogenic activities are required. In this study, a screening for *in vivo* interaction partners of glycolytic enzymes of the soil bacterium *Bacillus subtilis* was used to provide a rationale for essentiality of glycolytic enzymes. Glycolytic enzymes proved to be in close contact with several other proteins, among them a high proportion of essential proteins. Among these essential interaction partners, other glycolytic enzymes were most prominent. Two-hybrid studies confirmed interactions of phosphofructokinase with phosphoglyceromutase and enolase. Such a complex of glycolytic enzymes might allow direct substrate channeling of glycolytic intermediates. Moreover we found associations of glycolytic enzymes with several proteins known or suspected to be involved in RNA processing and degradation. One of these proteins, Rny (YmdA), which has so far not been functionally characterized, is required for the processing of the mRNA of the glycolytic *gapA* operon. Two-hybrid analyses confirmed the interactions between the glycolytic enzymes phosphofructokinase and enolase and the enzymes involved in RNA processing, RNase J1, Rny, and polynucleotide phosphorylase. Moreover RNase J1 interacts with its homologue RNase J2. We suggest that this complex of mRNA processing and glycolytic enzymes is the *B. subtilis* equivalent of the RNA degradosome. Our findings suggest that the functional interaction of glycolytic enzymes with essential proteins may be the reason why they are indispensable. *Molecular & Cellular Proteomics 8: 1350–1360, 2009.*

Glycolysis is a central metabolic pathway that appeared early in the evolution of life (1). Major functions of the glycolytic pathway are the generation of precursors for anabolic reactions and the conservation of energy that is needed to fuel all other cellular processes. The glycolytic pathway is conventionally divided into two parts: (i) the upper part, also referred to as the preparatory phase of glycolysis because the reactions of this part consume energy to convert the incoming sugars to triose phosphates, and (ii), the lower part or payoff phase that is characterized by the net gain of energy and the formation of reduction equivalents. Although glycolysis is highly conserved from archaea and bacteria to man not all organisms use this pathway for the oxidization of glucose. *Escherichia coli* is able to oxidize glucose via glycolysis, but the Entner-Doudoroff and the pentose phosphate pathways may replace the preparatory phase. In contrast to *E. coli*, the Entner-Doudoroff pathway is not present in *Bacillus subtilis* (2). Interestingly the enzymes of the upper glycolytic part seem to be completely absent in many archaea (3, 4). These few examples show that there is a high plasticity in how archaea and bacteria can feed glucose into the triose phosphate part of glycolysis. This plasticity is in good agreement with the observation that the genes encoding the enzymes of the upper glycolytic part are less conserved than those for the enzymes of the lower part (3). The great importance of the enzymes of the three-carbon part of glycolysis is underlined by the fact that two of the enzymes, *i.e.* glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] and phosphoglycerate kinase (PGK), catalyze reactions of the Calvin cycle. Moreover the PGK and the enolase (ENO) are among the about 30 proteins that are universally conserved in all organisms that have been sequenced so far (MicrobesOnline). Thus, these two enzymes seem to be of key importance for all extant life!

A systematic inactivation of *B. subtilis* genes revealed that 271 of the ~4100 genes are essential (5). Surprisingly several of these essential genes encode glycolytic enzymes. It turned out that the two genes *pfkA* and *fbaA* encoding 6-phosphofructokinase (PFK) and fructose-1,6-bisphosphate aldolase

[1] The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; ENO, enolase; PFK, 6-phosphofructokinase; FBA, fructose-1,6-bisphosphate aldolase; TPI, triose-phosphate isomerase; PGM, phosphoglycerate mutase; B2H, bacterial two-hybrid; SPINE, Strep-protein interaction experiment.
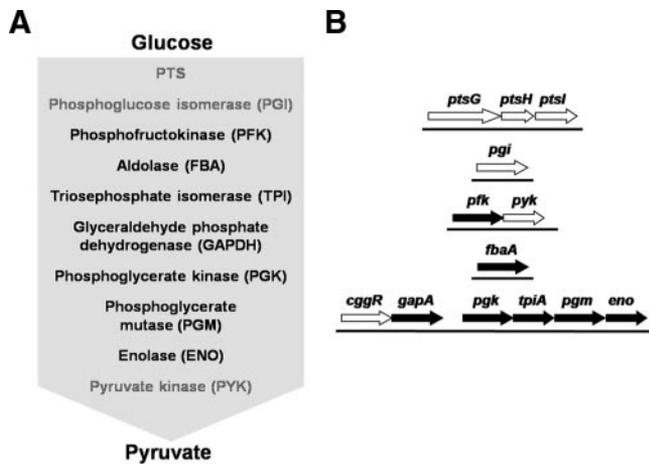
FIG. 1. **Schematic illustration of glycolysis and the glycolytic genes of *B. subtilis*.** *A*, glycolytic enzymes involved in glucose catabolism. Enzymes encoded by non-essential and essential genes are depicted in *gray* and *black*, respectively. The abbreviations for the essential glycolytic enzymes are indicated. *PTS*, phosphotransferase system. *B*, genetic organization of the glycolytic genes of *B. subtilis*. Non-essential genes are illustrated by *white arrows*, and essential genes are illustrated by *black arrows*.

(FBA) of the upper part and the five genes *tpiA, gapA, pgk, pgm*, and *eno* encoding the triose-phosphate isomerase (TPI), GAPDH, PGK, phosphoglycerate mutase (PGM), and ENO of the lower part of glycolysis are essential in *B. subtilis* (see Fig. 1) (5, 6). This is a striking observation because the systematic gene inactivation was performed in complex growth medium (Luria-Bertani medium containing glucose) in which neither glycolysis nor gluconeogenesis are expected to be necessary. Glycolytic enzymes are also essential in bacteria belonging to other phylogenetic branches. In *E. coli*, the aldolase FBA, GAPDH, ENO, and PGK are encoded by essential genes (7), and in *Corynebacterium glutamicum* the genes for six (FBA, TPI, GAPDH, PGK, PGM, and ENO) of the nine glycolytic enzymes are essential (8).

These observations suggest that glycolytic enzymes play an essential role in the bacterial cell that may not be related to their enzymatic activity! It is thus tempting to speculate that these enzymes might have functions in addition to their catalytic role in metabolism. Such enzymes with additional functions that are not directly related to their primary (enzymatic) tasks in metabolism are collectively termed moonlighting proteins (9). It is well established that several glycolytic enzymes in eukaryotic organisms have moonlighting functions (10, 11). In eukaryotes the hexokinase, GAPDH, ENO, and the lactate dehydrogenase are active in transcriptional regulation (10). Moreover the two isoforms of ENO encoded by *ENO1* and *ENO2* are involved in vacuolar protein traffic and in mitochondrial tRNA import, respectively, in yeast (12, 13). In addition, GAPDHs from all domains of life are able to cleave RNA *in vitro* (14). In *E. coli*, ENO is part of a complex mRNA degradation machinery, the degradosome. The degradosome is a large multiprotein complex that consists of the major endori-

bonuclease RNase E, the polynucleotide phosphorylase, the ATP-dependent RNA helicase RhlB, and ENO (15). The role of ENO within the complex has been enigmatic until recently. This enzyme plays a crucial role in the regulation of the *ptsG* mRNA stability in response to metabolic stress. Thus, glycolytic activity might be linked to RNA metabolism by a regulatory protein-protein interaction via ENO (16). In contrast to *E. coli* and eukaryotes, much less is known about moonlighting functions of glycolytic enzymes in *B. subtilis*. A recent work shows that there might be a link between enzymes of the lower glycolytic part and DNA replication in *B. subtilis* (17).

We are interested in basic metabolism and its regulation in *B. subtilis*. Among the genes encoding glycolytic enzymes, those for the glucose transporter, *ptsG*, and for the GAPDH, *gapA*, are only expressed in the presence of glucose (Fig. 1) (18). The *gapA* gene is the second gene of the hexacistronic *cggR gapA pgk tpi pgm eno* operon (the *gapA* operon). In the absence of glucose or other sugars, expression of the *gapA* operon is repressed by CggR, the product of the first gene (19). Interestingly the intracellular amount of GAPDH exceeds that of CggR by a factor of 100 even though the two proteins are encoded by consecutive genes in one operon! This differential expression of the two genes is largely caused by an mRNA cleavage event between the *cggR* and *gapA* open reading frames resulting in a substantial stabilization of the mature *gapA* transcript (18, 20). The RNase responsible for this processing event has so far not been identified.

Recently we have established that a class of metabolic enzymes, the trigger enzymes, have moonlighting activities in the regulation of gene expression in *B. subtilis* and many other bacteria (21, 22). In this work, we wanted to define why the genes encoding glycolytic enzymes are essential in *B. subtilis*. We considered the idea that these enzymes might be involved in essential interactions in the cell most attractive. Indeed we found that PFK and ENO interact with the essential RNase J1 and with a novel protein that is encoded by the essential gene *ymdA*. In addition, the polynucleotide phosphorylase PnpA and RNase J2 interact with glycolytic enzymes. RNases J1 and J2, PnpA, and YmdA are involved in RNA metabolism in *B. subtilis* suggesting the formation of a complex equivalent to the *E. coli* degradosome. Moreover it turned out that several glycolytic enzymes, *i.e.* PFK, ENO, and PGM, form a complex *in vivo*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*B. subtilis* 168 (*trpC2*, laboratory collection) was used for the purification of glycolytic enzymes with their potential interaction partners. *B. subtilis* GP193 (*trpC2 ΩymdA*::pGP774(p$_{xylA}$-*ymdA cat*)) was used to test the effect of the YmdA depletion on the *gapA* operon mRNA processing. *E. coli* DH5α, XL1-Blue, and BTH101 (23, 24) were used for cloning experiments and bacterial two-hybrid (B2H) analyses. *B. subtilis* was grown in LB and minimal medium containing succinate and glutamate/ammonium as basic sources of carbon and nitrogen, respectively (25). The media were supplemented with auxotrophic requirements (at 50 mg/liter) and glucose (0.5% (w/v)). *E. coli* was grown in LB medium.

LB and sporulation plates were prepared by addition of 17 g of Bacto agar/liter (Difco) to LB and sporulation, respectively (21, 23).

DNA Manipulation—Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (23). Restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen). *Pfu* DNA polymerase was used for the polymerase chain reaction as recommended by the manufacturer. All primer sequences are provided in supplemental Table 1. DNA sequences were determined using the dideoxy chain termination method (23). Chromosomal DNA of *B. subtilis* was isolated as described previously (21).

Transformation and Phenotypic Analysis—Standard procedures were used to transform *E. coli* (23), and transformants were selected on LB plates containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml). *B. subtilis* was transformed with plasmid DNA according to the two-step protocol (21). Transformants were selected on SP plates containing 2 μg/ml erythromycin plus 25 μg/ml lincomycin.

Plasmid Constructions—Plasmids for the overexpression and purification of the glycolytic enzymes PFK, FBA, TPI, GAPDH, PGK, PGM, and ENO from *B. subtilis* were constructed as follows. The coding sequence of each gene was amplified by PCR with gene-specific primers (listed in supplemental Table 1) using chromosomal DNA of *B. subtilis* 168 as the template. The PCR products were digested with BamHI and SalI and cloned into the expression vector pGP380 (26). These plasmids allowed the expression of the glycolytic enzymes carrying an N-terminal Strep-tag. The resulting plasmids are listed in supplemental Table 2. To obtain the plasmids for the B2H analyses the coding sequences of the genes *pgi*, *pfkA*, *fbaA*, *tpiA*, *gapA*, *pgk*, *pgm*, *eno*, *pykA*, *pnpA*, *rnjA*, *rnjB*, *rnc*, and *ymdA* were amplified by PCR using chromosomal DNA of *B. subtilis* 168. The gene-specific primers are listed in supplemental Table 1. The PCR products were digested with XbaI and KpnI, and the resulting fragments were cloned into each of the four plasmids p25-N, pKT25, pUT18, and pUT18c (24, 27), which were digested with the same enzymes. The resulting plasmids used for the B2H analyses are listed in supplemental Table 2. All plasmid inserts were verified by DNA sequencing.

Construction of a B. subtilis Strain That Allows Controlled Depletion of ymdA—To express the *ymdA* gene under the control of the strongly regulated promoter of the *B. subtilis xyl* operon, plasmid pGP774 was constructed as follows. A 423-bp PCR fragment covering the region of the *ymdA* translational start was generated using the primers DH11 (5′-AAAGGATCCGCAACAACCAAGTTCATAGCAAG) and DH12 (5′-AAAGGATCCGCTTTCCATCTCTTCAATATGTTG). The resulting PCR product was digested with BamHI and ligated into plasmid pX2 (28) linearized with BamHI. The identity of the cloned insert was verified by sequencing. *B. subtilis* 168 was transformed with the resulting plasmid pGP774, and transformants were selected on SP plates containing chloramphenicol and xylose (1%, w/v). The resulting strain GP193 was unable to grow in the absence of xylose confirming that the *ymdA* gene is essential for *B. subtilis*.

Purification of Glycolytic Enzymes with Their Potential Interaction Partners from B. subtilis—*B. subtilis* 168 served as host for the overexpression of the glycolytic enzymes. A first preculture was grown in LB medium for 10 h at 28 °C. 4 ml of this preculture were used to inoculate 100 ml of CSE medium containing 0.5% glucose. This second preculture was grown overnight at 28 °C. Then we inoculated 1 liter of the same medium with the second preculture to an A_{600} of 0.1. This culture was grown at 37 °C until the A_{600} had reached the value of 1.0. Cells were harvested and lysed using a French press (20,000 p.s.i., 138,000 kilopascals; Spectronic Instruments). After lysis the crude extracts were centrifuged at 100,000 × g for 1 h. For purification of the Strep-tagged proteins the resulting supernatants

were passed over a Strep-Tactin column (IBA, Göttingen, Germany) (1.0-ml bed volume). The recombinant proteins were eluted with desthiobiotin (IBA; final concentration, 2.5 mM). After elution the fractions were tested for the desired protein using 12.5% SDS-PAGE. Protein concentrations were determined using the Bio-Rad dye binding assay with bovine serum albumin as the standard. To facilitate the isolation of glycolytic enzymes together with their potential interaction partners, cells expressing these enzymes were treated with formaldehyde (0.6%, w/v; 20 min) (26). After cross-linking, the cells were harvested and washed once with a buffer containing 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl. As control, we used the *B. subtilis* wild type strain carrying the empty vector pGP380 (26). The cells were disrupted using a French press, and the glycolytic enzymes were purified using a Strep-Tactin column as described above. Aliquots of the different fractions were subjected to SDS-PAGE and visualized by silver staining. Prior to electrophoresis, the protein samples were boiled for 20 min in Laemmli buffer to reverse the cross-links.

Protein Identification by Mass Spectrometry—Silver nitrate-stained gel slices were destained by incubation in 30 mM $K_3[Fe(CN)_6]$, 100 mM $Na_2S_2O_3$ until colorless and washed three times in water before processing gel slices as described previously (29). Briefly gel pieces were washed twice with 200 μl of 20 mM NH_4HCO_3, 50% (v/v) ACN for 30 min at 37 °C and dried by adding 200 μl of ACN two times for 15 min. Trypsin solution (10 ng/μl trypsin in 20 mM ammonium bicarbonate) was added until gel pieces stopped swelling, and digestion was allowed to proceed for 16–18 h at 37 °C. Peptides were extracted from gel pieces by incubation in an ultrasonic bath for 30 min in 40 μl of 0.1% (v/v) acetic acid followed by a second extraction with 40 μl of 50% ACN in 0.05% acetic acid. The supernatants containing peptides were collected, combined, ACN-depleted by evaporation, and transferred into microvials for mass spectrometric analysis. Peptides were separated by a non-linear water-acetonitrile gradient in 0.1% acetic acid on a PepMap reverse phase column (75-μm inner diameter × 150 mm; LC Packings, Idstein, Germany) with an MDLC (multidimensional liquid chromatography) nano-HPLC system (GE Healthcare) coupled on line with an LTQ (linear trap quadrupole) Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) operated in data-dependent MS/MS mode. Proteins were identified by searching all MS/MS spectra in .dta format against a *B. subtilis* protein database (4106 entries; extracted from SubtiList using SEQUEST (Bioworks 3.2/Sequest version 2.7 revision 11, Thermo Electron) on an IBM cluster with eight dual nodes. Initial mass tolerance for peptide identification on MS and MS/MS peaks were 10 ppm and 1 Da, respectively. Up to two missed tryptic cleavages were allowed. Methionine oxidation (+15.99492 Da) and propionamide modification on cysteine (+71.037109 Da) were set as variable modifications. Protein identification results were evaluated by determination of probability for peptide and protein assignments provided by PeptideProphet and ProteinProphet (Institute for Systems Biology, Seattle, WA) incorporated in the Scaffold software package release 2.01 (Proteome Software, Portland, OR). Proteins were identified by at least two peptides with peptide probability >90% reflecting protein probability of >99%.

B2H Assay—Primary protein-protein interactions were identified by B2H analysis (24). The B2H system is based on the interaction-mediated reconstruction of adenylate cyclase (CyaA) activity from *Bordetella pertussis* in *E. coli*. The CyaA enzyme consists of two complementary fragments, T18 and T25, that are not active when physically separated. Fusion of these fragments to interacting proteins results in functional complementation between the T18 and T25 fragments and the synthesis of cAMP. cAMP production can be monitored by measuring the β-galactosidase activity of the cAMP-catabolite gene activator protein-dependent promoter of the *E. coli lac* operon. Thus, a high β-galactosidase activity reflects the interac-

tion between the hybrid proteins. Plasmids pUT18 and p25-N allow the expression of proteins fused to the N terminus of the T18 and T25 fragments of the CyaA protein, respectively, and the plasmids pUT18C and pKT25 allow the expression of proteins fused to the C terminus of the T18 and T25 fragments of the CyaA protein, respectively (24, 27). The plasmids pKT25-zip and pUT18C-zip served as positive controls for complementation. These plasmids express T18-zip and T25-zip fusion proteins that can associate because of the leucine zipper motifs resulting in an active CyaA enzyme and a high β-galactosidase activity. The plasmids constructed for the B2H assay (see supplemental Table 2) were used for cotransformations of *E. coli* BTH101, and the protein-protein interactions were then analyzed by plating the cells on LB plates containing ampicillin (100 μg/ml), kanamycin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml), and isopropyl β-D-thiogalactopyranoside (0.5 mM), respectively. The plates were incubated for a maximum of 36 h at 30 °C.

Northern Blot Analysis—To test the effect of the YmdA depletion on the *gapA* operon mRNA processing we used *B. subtilis* GP193 in which the *ymdA* gene is expressed under control of a xylose-inducible promoter. This strain was grown overnight at 37 °C in LB containing 0.25% glucose and 1% xylose. This preculture was used to inoculate two cultures containing 20 ml of LB to an A_{600} of 0.1. One culture contained 1% xylose to allow the expression of the *ymdA* gene. Both cultures were grown to an A_{600} of 1.5–2.0. The cells were then washed twice in fresh LB and used to inoculate two main cultures (100 ml each). One culture contained 1% xylose to induce *ymdA* expression. The cells were grown to an A_{600} of 0.5–0.8 and harvested for RNA isolation. Preparation of total RNA and Northern blot analysis were carried out as described previously (18). Digoxigenin (DIG) RNA probes were obtained by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) using PCR-generated DNA fragments as templates. The primer pair gapfor/gaprev (30) was used to amplify a DNA fragment specific for *gapA*. The reverse primer contained a T7 RNA polymerase recognition sequence. *In vitro* RNA labeling, hybridization, and signal detection were carried out according to the instructions of the manufacturer (DIG RNA labeling kit and detection chemicals; Roche Diagnostics). The sizes of the RNA molecular weight marker I (Roche Diagnostics) were as follows: 6.9, 4.7, 2.6, 1.8, 1.5, 1.0, 0.57, 0.48, and 0.31 kb.

RESULTS

Identification of Potential Interaction Partners of the Essential Glycolytic Enzymes in B. subtilis—To isolate the glycolytic enzymes together with their potential interaction partners we used the *Strep-protein interaction experiment* (SPINE) method (26). Briefly the bait proteins were fused to a Strep-tag, and the fusion proteins were expressed in *B. subtilis*. *In vivo* protein complexes were cross-linked by formaldehyde and purified by affinity chromatography, and the cross-links were broken. Finally the proteins were analyzed by SDS-PAGE, and the interaction partners were identified by mass spectrometry. Those potential interaction partners that were detected with 10 or more peptides were regarded as significant in this study.

This procedure was performed for PFK, FBA, TPI, GAPDH, PGK, PGM, and ENO. As an example, Fig. 2 shows the purification of TPI with its potential interaction partners. Only those proteins are shown for which at least 10 different peptides were identified (see supplemental Table 3). As can be
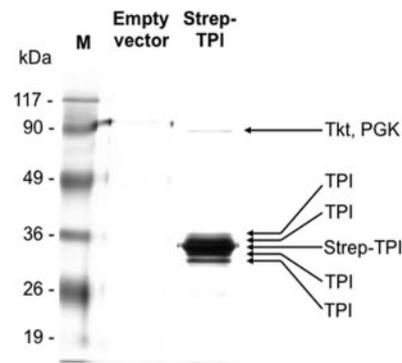
FIG. 2. **Identification of potential interaction partners of the TPI.** The protein complex was isolated from the *B. subtilis* wild type strain 168 carrying either pGP380 (empty vector) or the vector pGP89 (expressing TPI). All strains were grown in CSE-Glc minimal medium containing glucose and glutamate as carbon and nitrogen sources, respectively. 15 μl of the first elution fractions from each purification were loaded on a 12.5% SDS-polyacrylamide gel. Protein bands were visualized by silver staining. *M*, prestained protein molecular mass marker (Fermentas). *Tkt*, transketolase.

seen, the application of the purification scheme to a control strain carrying the empty vector did not reveal appreciable protein enrichment/purification. In contrast, distinct bands in addition to the bait protein (Strep-TPI) were observed in the strain carrying the expression vector. These bands were identified as PGK, TPI, and transketolase. For PFK we identified 180 proteins that were cross-linked and co-purified with the bait. Of these, 48 proteins are encoded by essential genes (see supplemental Table 3). However, many of these potential interaction partners were identified only with a few peptides. 25 potential interaction partners, among them six essential proteins, exceeded the threshold of 10 peptides (see below; Table I). Unfortunately for FBA, no purification was achieved. For GAPDH, 59 proteins were co-purified with the bait, but only the chromosomally encoded GAPDH itself was above the threshold.

For PGK, no distinct protein bands were obtained with the SPINE procedure suggesting that this enzyme interacts with many partners. Therefore, the interaction partners of PGK were isolated without *in vivo* cross-linking. With all bait proteins, we obtained multiple potential interaction partners that had been cross-linked to the glycolytic enzymes. PGK seems to interact with a wide variety of proteins. In total, we identified 186 potential interaction partners for PGK. 44 potential partners of PGK, among them seven essential proteins, yielded at least 10 different peptides in mass spectrometry. With PGM as the bait, only PGM itself and PFK were co-purified in significant amounts. Finally 110 potential partners were identified for ENO; among them were only five proteins above the threshold. Three of these proteins are encoded by essential genes.

The essential proteins that were co-purified with the glycolytic enzymes and that were detected in high abundance are listed in Table I. Interestingly the glycolytic enzymes them-

TABLE I

Essential proteins that interact with glycolytic enzymes

The table lists all those proteins encoded by essential genes that were abundant in the SPINE analysis (≥10 identified peptides). The first column gives the name of the interacting proteins, and the second column gives the glycolytic enzymes with which these proteins eluted.

Protein	Bait enzyme	Function
Basic carbon metabolism (glycolysis, pentose phosphate pathway)		
PFK	PFK, PGM	Phosphofructokinase
TPI	TPI	Triose-phosphate isomerase
GAPDH	PFK, GAPDH, PGK	Glyceraldehyde-3-phosphate dehydrogenase
PGK	TPI, PGK	Phosphoglycerate kinase
PGM	PGM	Phosphoglycerate mutase
ENO	PGK, ENO	Enolase
Tkt	TPI	Transketolase
Translation		
FusA	PFK	Elongation factor G
RplE	PGK	Ribosomal protein L5
RpsB	PGK	Ribosomal protein S2
Tsf	PFK, ENO	Elongation factor Ts
GltX	PFK	Glutamate-tRNA synthetase
Transcription		
RpoB	ENO	RNA polymerase, β subunit
Lipid biosynthesis		
PlsX	PGK	Acyl carrier protein:phosphate acyltransferase
Other functions		
YmdA (Rny)	PGK	RNA processing factor, previously unknown
YurU	PFK	Involved in the synthesis of Fe-S clusters

selves form the largest group of these proteins. The glycolytic enzymes were all cross-linked to their own variant carrying a Strep-tag. This observation is in good agreement with the fact that these enzymes form dimers or tetramers (31). In addition, several of the glycolytic enzymes were co-purified with multiple other glycolytic enzymes. This finding suggests the existence of a complex of glycolytic enzymes in the cell (see below). Moreover transketolase, an enzyme of the pentose phosphate pathway that feeds into the lower part of glycolysis, was co-purified with TPI. Together with PFK, we also purified two translation elongation factors, glutamyl-tRNA synthetase, and a protein involved in the biosynthesis of iron-sulfur clusters, whereas two ribosomal proteins were cross-linked to PGK. Finally ENO was found to interact with the translation elongation factor Ts and the β-subunit of the RNA polymerase (RpoB), and PGK was co-purified with an essential factor of lipid biosynthesis (PlsX) and a so far unknown protein, YmdA (see below).

Analysis of Primary Protein-Protein Interactions among the Glycolytic Enzymes of B. subtilis—The SPINE approach is highly efficient to isolate protein complexes, but it does not allow determination of the primary interactions in a protein complex that is composed of more than two proteins. We used the B2H system to analyze the primary protein-protein interactions among the glycolytic enzymes. In the B2H system, the T25 and the T18 fragments of the catalytic domain of the *B. pertussis* adenylate cyclase were fused to full-length copies of all glycolytic enzymes of *B. subtilis*. The leucine zipper of the yeast GCN4 transcription factor served as a
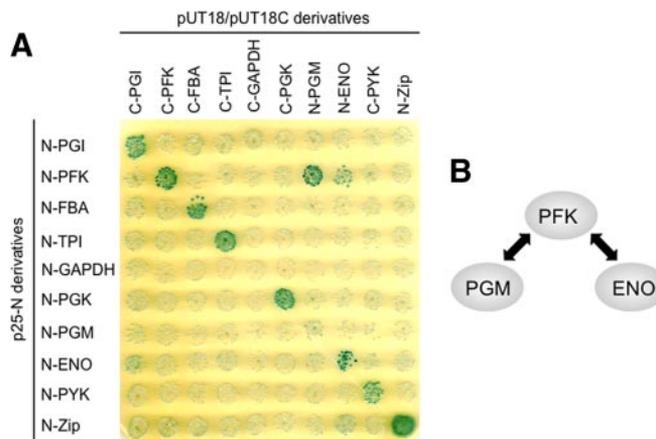

FIG. 3. **Analysis of interactions among glycolytic enzymes of *B. subtilis*.** *A*, B2H analysis to identify interactions among the glycolytic enzymes of *B. subtilis*. All genes were cloned in the plasmids pUT18, pUT18C, and p25-N. Plasmids pUT18 and pUT18C allow the expression of the glycolytic enzymes fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid p25-N allows the expression of the glycolytic enzymes fused to the N terminus of the T25 domain of the adenylate cyclase. The *E. coli* transformants were incubated for 48 h at 28 °C. *B*, schematic summary of the outcome of the B2H analysis. *PYK*, pyruvate kinase; *PGI*, phosphoglucose isomerase.

control (24). The results of the B2H analysis are shown in Fig. 3. As expected, the leucine zipper of GCN4 showed strong self-interaction but failed to interact with any of the glycolytic enzymes. With the exception of the GAPDH, all glycolytic enzymes gave positive signals when they were fused to both

the N- and C-terminal domains of the *B. pertussis* adenylate cyclase. This observation corresponds to the formation of dimers (or larger oligomers) and is in good agreement with the results of the SPINE assay and the published evidence (31). The GAPDH did not interact with any other protein in the B2H assay. This might indicate that the GAPDH fusion proteins are not correctly folded or that they have lost their potential to interact with other proteins. In contrast, we observed direct interactions between PFK and both PGM and ENO. Thus the B2H analysis supports the hypothesis of a glycolytic complex in *B. subtilis*.

The Essential Protein YmdA Is Involved in the Maturation of the gapA Operon mRNA—Using the SPINE approach, we identified several essential interaction partners for the glycolytic enzymes. Among these proteins was a protein of unknown function, YmdA (see Table I). YmdA contains KH (ribonucleoprotein K homology) and HD (His-Asp-containing phosphohydrolase) domains. Both domains are found in many nucleic acid-binding proteins and are characteristic for a family of metal-dependent phosphohydrolases (32, 33). It was therefore hypothesized that YmdA might act as an RNase (34). In addition, the RNases III (Rnc), J1 (RnjA), and J2 (RnjB) were present in a complex with PFK, and the phosphonucleotide phosphorylase PnpA was identified as an interaction partner of ENO (see supplemental Table 3). This coincidence suggested that YmdA might indeed be involved in mRNA processing.

Because none of the known *B. subtilis* RNases is responsible for the endonucleolytic processing of the *gapA* operon mRNA, we considered the possibility that YmdA might be required for this RNA maturation event. To test this hypothesis, we analyzed the effect of a YmdA depletion on the *gapA* operon mRNA maturation. For this purpose we used the *B. subtilis* strain GP193 in which the *ymdA* gene is expressed under control of a xylose-inducible promoter. The strain was grown in the presence and absence of xylose. Growth in the absence of xylose resulted in a depletion of the YmdA protein. The effect of the YmdA depletion on the *gapA* operon mRNA maturation was monitored by Northern blot analysis using *cggR*- and *gapA*-specific riboprobes. The primary and the mature transcripts of the hexacistronic *gapA* operon are shown in Fig. 4A. First we analyzed the transcription of the *gapA* operon in the presence of xylose reflecting the wild type situation. As expected, two mRNAs of 2.2 and 1.0 kb were detected using the *cggR* probe (Fig. 4B). The 2.2-kb mRNA represents the primary transcripts of the *gapA* operon, whereas the faint 1.0-kb mRNA corresponds to the processed *cggR* transcript (Fig. 4B). These results are in good agreement with the previous transcriptional analysis of the *gapA* operon (18, 20). The depletion of YmdA resulted in a significant accumulation of the 7.2- and 2.2-kb primary transcripts, whereas the 1.0-kb *cggR* mature transcript was barely detectable (Fig. 4). These results clearly demonstrate that the processing of the *gapA* mRNA is severely impaired upon YmdA depletion.
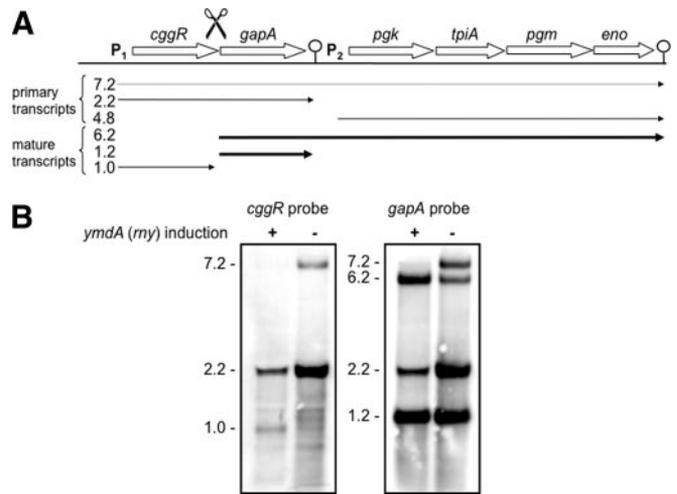
FIG. 4. **Effect of Rny depletion on the maturation of the *gapA* operon mRNA.** *A*, schematic illustration of the primary and mature transcripts of the *gapA* operon. The first promoter (P_1) of the *gapA* operon is located in front of the *cggR* gene, and the second promoter (P_2) is located between a terminator and the *pgk* gene. The cleavage site of the *gapA* operon mRNA is located at the end of the *cggR* open reading frame. *B*, Northern blot analysis to test the effect of Rny depletion on the maturation of the *gapA* operon mRNA. The *rny* gene was placed under control of a xylose-inducible promoter. The strain was grown in LB medium in the presence (+) and in the absence (−) of xylose. Primary and mature transcripts of the *gapA* operon were detected with *cggR*- and *gapA*-specific riboprobes.

Using the *gapA*-specific riboprobe three mRNAs of about 6.2, 2.2, and 1.2 kb were detected when the cells were grown in the presence of xylose (Fig. 4B). The intense 6.2- and 1.2-kb mRNAs correspond to the mature *gapA pgk tpiA pgm eno* and the *gapA* transcripts, respectively. The 2.2-kb transcript represents the primary *cggR gapA* mRNA that was also detected with the *cggR*-specific riboprobe (Fig. 4B). Again these results are in good agreement with previous observations (18, 20). As shown in Fig. 4B, the effect of a YmdA depletion on the maturation of the *gapA* operon mRNA using the *gapA*-specific riboprobe is quite similar to the results obtained with the *cggR*-specific riboprobe. As a consequence of the YmdA depletion, the mature 6.2-kb transcript was less intense than in the presence of the YmdA. Moreover the unprocessed hexacistronic *cggR gapA pgk tpiA pgm eno* transcript of the *gapA* operon significantly accumulated due to the depletion of the YmdA protein. These results unequivocally confirm the implication of YmdA in the processing of the *gapA* mRNA. Therefore, we suggest redesignating the *ymdA* gene as *rny* and the gene product as Rny.

Evidence for a Degradosome-like Protein Complex in B. subtilis—As presented above, the SPINE approach identified the mRNA processing factor Rny and the polynucleotide phosphorylase PnpA as potential interaction partners of the glycolytic enzymes PGK and ENO, respectively (supplemental Table 3). In addition, we found that Rny; the RNases III, J1,

FIG. 5. **Analysis of interactions among glycolytic enzymes and enzymes involved in RNA metabolism.** *A*, B2H analysis to identify interactions among the glycolytic enzymes and enzymes involved in RNA metabolism. All genes were cloned in the plasmids pUT18, pUT18C, p25-N, and pKT25. Plasmids pUT18 and pUT18C allow the expression of the selected enzymes fused either to the N or the C terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmids p25-N and pKT25 allow the expression of the selected enzymes fused either to the N or the C terminus of the T25 domain of the adenylate cyclase, respectively. The *E. coli* transformants were incubated for 48 h at 28 °C. *B*, schematic summary of the outcome of the B2H analysis.
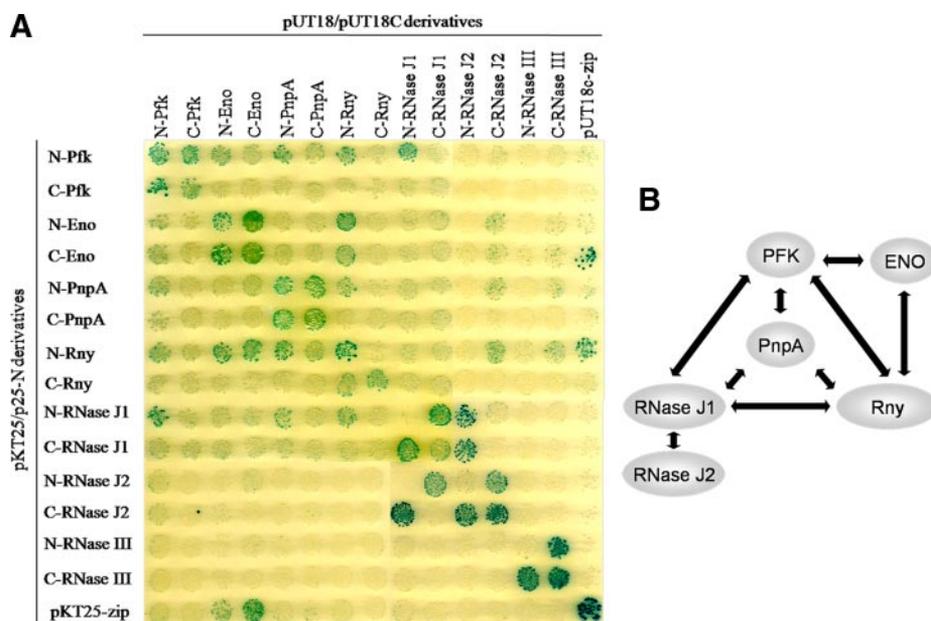
and J2; and PnpA were co-purified with PFK. Interestingly these proteins are all involved in RNA metabolism in *B. subtilis*, suggesting that there might be a direct link between carbon metabolism and RNA processing. In *E. coli*, ENO is part of the degradosome, a multiprotein complex consisting of the polynucleotide phosphorylase, RNase E, and the helicase RhlB (15). Nothing is known about the existence of such a protein complex in *B. subtilis*. Inspired by the co-purification of Rny, three RNases, and PnpA with glycolytic enzymes, we considered the possibility that a degradosome-like protein complex might also exist in *B. subtilis*.

Using the B2H system, we analyzed the primary protein-protein interactions between the bait proteins PFK, ENO, and PGK and their potential interaction partners PnpA, Rny, and the RNases J1, J2, and III. As shown in Fig. 5*A*, the B2H analysis confirmed the strong self-interactions of the glycolytic enzymes (see also Fig. 3). In addition, the B2H analysis also revealed strong self-interactions of PnpA, Rny, and the RNases J1, J2, and III, suggesting that these proteins dimerize or even multimerize *in vivo*.

The B2H experiment showed a direct and reciprocal interaction between PFK, ENO, and Rny. As observed with the SPINE approach, the B2H analysis confirmed that PFK directly interacts with the polynucleotide phosphorylase PnpA, Rny, and RNase J1 (supplemental Table 3 and Fig. 5*A*). Moreover direct and reciprocal interactions were observed between RNase J1 and PnpA, RNase J1 and Rny, the RNases J1 and J2, and Rny and PnpA (Fig. 5*A*). The B2H analyses did not support an interaction of Rny and ENO with PGK (data not shown), which was observed in the SPINE analysis (see Table I). Thus, the complex between Rny and PGK observed upon *in vivo* cross-linking might be the result of indirect interaction involving other proteins such as ENO and PFK. Finally RNase

III did not interact with any of the tested candidates in the B2H screen (see Fig. 5*A*).

Taken together, the results of the B2H analyses confirm the observations of the SPINE approach and clearly demonstrate that PFK, ENO, and the four proteins PnpA, Rny, and RNases J1 and J2, which are involved in RNA metabolism in *B. subtilis*, form a complex *in vivo* (Fig. 5*B*). This complex is very reminiscent of the *E. coli* degradosome because it contains four enzymes involved in RNA processing and the glycolytic enzyme ENO. The interaction of PFK and ENO with essential proteins that mediate mRNA processing might also account for the essentiality of the *pfkA* and *eno* genes, respectively.

Phylogenetic Distribution of the RNA Processing Factor Rny—The phylogenetic distribution of the novel processing factor Rny was analyzed by using the MicrobesOnline Web site for comparative genomics. The distributions of the polynucleotide phosphorylase and of enzymes of the E/G or the J1/J2 RNase families were included in this analysis (35).

As shown in Table II, the gene encoding Rny is conserved in the groups *Aquifex*, *Thermotoga*, *Deinococcus/Thermus*, the flavobacteria, and the ε-group of the proteobacteria. Moreover Rny is highly conserved within the firmicutes. The gene is present in all bacilli, lactobacilli, and the clostridia. Because Rny is an essential enzyme in *B. subtilis*, one may speculate that this enzyme might play an important role also in other bacteria that belong to the firmicutes. Despite its high conservation, the *rny* gene is absent in some members of the mollicutes. The complete absence of a homologous gene in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* might be due to a severe genome reduction that these two bacteria have experienced. Similarly to Rny, the polynucleotide phosphorylase is also less conserved among the mollicutes. Rny is barely conserved within the spirochaetes, the actinobacteria,

TABLE II

Phylogenetic distribution of proteins involved in mRNA processing

P, polynucleotide phosphorylase; E/G, RNases E, G, or E/G; J1/J2, RNases J1 or J2. The black dots indicate the presence of the enzyme in all representatives of the group, the gray dots indicate the presence of the enzyme in some representatives of a group, and a whithe dot indicates absence of the enzyme from a bacterial group.

Group	P	E/G	J1/J2	Rny
Aquifex	●	●	○	●
Thermotoga	●	●	○	●
Chloroflexus	●	◐	●	○
Chlamydiae	●	●	○	○
Deinococcus/ Thermus	●	○	◐	●
Flavobacteria	●	●	○	●
Spirochaetes	●	○	○	◐
Cyanobacteria	●	●	●	○
Actinobacteria	●	●	●	◐
Firmicutes: Bacilli	●	◐	●	●
Firmicutes: Lactobacilli	◐	○	●	●
Firmicutes: Clostridia	●	●	●	●
Firmicutes: Mollicutes	◐	○	●	◐
α-Proteobacteria	●	●	●	○
β-Proteobacteria	●	●	○	○
γ-Proteobacteria	●	◐	○	○
δ-Proteobacteria	●	◐	◐	◐
ε-Proteobacteria	●	○	●	●

and the δ-proteobacteria, and it is completely absent in the *Chloroflexus* group, the chlamydiae, the cyanobacteria, and the α–γ groups of the proteobacteria. Most of the groups listed in Table II that do not contain an Rny homologue possess at least one enzyme that belongs either to the E/G or the J1/J2 family of RNases. Interestingly some bacteria such as *Leptospira interrogans*, *Legionella pneumophila*, and *Stigma-*

tella aurantiaca belonging to the spirochaetes and proteobacteria, respectively, possess neither Rny nor an RNase that is homologous to the E/G or the J1/J2 RNases, suggesting that these bacteria might have other enzymes with so far not identified ribonucleolytic activities.

Taken together, the phylogenetic analysis shows that the equipment of bacteria with essential RNases is highly diverse. Moreover Rny is widely distributed among different phylogenetic branches, but it is highly conserved among the firmicutes.

DISCUSSION

Glycolysis and its variations such as gluconeogenesis are among the key metabolic pathways in most organisms. The importance of this pathway is underlined by the observation that several glycolytic enzymes are essential in many bacteria. The reason for this essentiality has so far remained unknown because the systematic gene inactivation experiments have been performed under conditions were neither glycolytic nor gluconeogenic activities are required (*i.e.* in LB medium containing glucose) (5). In this work, we tested the hypothesis that the glycolytic enzymes are involved in essential interactions. Indeed it turned out that all of the essential glycolytic enzymes of *B. subtilis* form complexes with essential proteins *in vivo* (see Table I). The most prominent class of essential interaction partners are glycolytic enzymes themselves suggesting the existence of protein complexes that perform glycolysis (see below). In addition, PFK, PGK, and ENO interact with proteins involved in translation and transcription. PFK was also crosslinked to YurU, a protein involved in the biosynthesis of iron-sulfur clusters. Moreover PGK was present in complexes with PlsX, a protein involved in lipid biosynthesis, and Rny (YmdA), a previously unknown protein that was identified as an essential RNA processing factor in this work. Although Table I lists only those essential proteins that were found in large abundance, there may be other essential proteins interacting with glycolytic enzymes that were present in smaller amounts in the purified complexes (see supplemental Table 3). Recently it has been reported that glycolytic enzymes are also linked to replication (17). Most interestingly, we observed interactions between glycolytic enzymes and replication proteins. The leading and lagging strand polymerases PolC and DnaE were found to be cross-linked to ENO and GAPDH, respectively, and the β-clamp of DNA polymerase III, DnaN, was present in a complex with PGK. These observations support the existence of a direct link between glycolysis and DNA replication that has been suggested based on genetic evidence (17). It is, however, unknown whether the observed interactions are (i) direct and (ii) relevant. The *in vivo* cross-linking approach used in this study does not allow distinguishing between primary interactions and those that are mediated by intermediary partners. However, there are indications that suggest that our observations are meaningful. First, the SPINE strategy has already successfully been applied to identify the regulatory

interaction between the transcription activator GltC and the glutamate dehydrogenase RocG (21). Second, the presence of glycolytic as well as degradosome-like complexes was verified by the two-hybrid analysis. This analysis does also provide insights into the primary interactions that may differ from the outcome of the SPINE experiment.

All studies at the global level may give valuable insights into the functions of so far unknown proteins. In this work, we focused our attention on Rny (YmdA), the only essential protein that interacts with glycolytic proteins for which no function had been annotated. We could demonstrate that this protein is required for the processing of the *gapA* operon mRNA. So far, RNases J1 and J2 have been described as the functional equivalents of *E. coli* RNase E (36). However, neither RNase J1 nor RNase J2 is involved in the processing of the *gapA* mRNA (37). Therefore, other endoribonucleases must exist in *B. subtilis*. Our data are in good agreement with the idea that Rny is this RNase. However, we were so far unable to demonstrate this function *in vitro*. This may be difficult because of the presence of Rny in a large protein complex (this work) and because of the requirement for membrane insertion of Rny for proper activity.[2]

RNA degradation by large multiprotein complexes seems to be a general rule. In archaea and eukaryotes, the exosome forms a macromolecular cage for RNA degradation (38, 39). In several proteobacteria such as *E. coli* and *Rhodobacter capsulatus* as well as in the actinobacterium *Streptomyces coelicolor*, RNase E is the key component of the RNA degradosome. In addition to RNase E, the degradosome may contain an RNA helicase, the phosphonucleotide phosphorylase, and other factors such as RNase R, the transcription factor Rho, or the glycolytic enzyme ENO (15, 40–42). In *B. subtilis*, RNases J1, J2, and Rny might play the role of RNase E. The two-hybrid analysis revealed the formation of a complex of these three proteins with PnpA as well as with the glycolytic enzymes PFK and ENO. This composition is very reminiscent of the degradosomes in the other organisms. It should be noted that *E. coli* and *B. subtilis* are so far the only bacteria with glycolytic enzymes in their degradosomes. The function of these enzymes in the *B. subtilis* degradosome will be the subject of further studies. The observation of an RNA degradosome with key players different from RNase E supports the striking importance of protein complexes for mRNA processing and degradation. It is tempting to speculate that such complexes may be present in all bacteria. It has not escaped our attention that the interaction pattern of the RNases J1 and J2 immediately suggests why the *rnjA* gene encoding RNase J1 is essential in *B. subtilis* whereas *rnjB* (RNase J2) is not. RNase J1 is part of the core of the degradosome. In contrast, RNase J2 is connected to the degradosome only via J1. In an *rnjA* mutant, no J-type RNase could assemble with the other

degradosome components, whereas functional degradosomes containing RNase J1 can assemble in *rnjB* mutants.

The analysis of proteins encoded by essential genes has recently attracted much attention. Several studies have been devoted to the identification and functional characterization of essential genes and proteins in *B. subtilis* (5, 6, 32). The study of these proteins helps to eliminate uncharted territories on the map of our knowledge. In addition, these proteins are excellent candidates for being novel drug targets (43). This is specifically true for essential proteins that do not have counterparts in eukaryotes. However, it is difficult to design assays for essential proteins of unknown function. Essential unknown proteins are therefore regarded as low priority candidates for drug development (44). The identification of a biological activity for Rny makes it an excellent candidate. With the exception of some *Mycoplasma* species, Rny is present in all firmicutes including many severe pathogens such as *Staphylococcus aureus* that are often resistant to many common antibiotics. Moreover a recent study has shown that Rny is also essential in *Mycoplasma pulmonis* (45), suggesting an important role in those mollicutes that retained the *rny* gene.

Another interesting result of this study is the observation of a complex of glycolytic enzymes. Such a complex, called the glycosome, has been reported previously for some protozoans of the genera *Trypanosoma* and *Leishmania* (46). Similarly clustered association of glycolytic enzymes was also observed in human and murine erythrocytes and in yeast (47–49). Our work provides compelling evidence for a complex composed of PFK, PGM, and ENO. However, we cannot rule out the possibility that other proteins are present in this complex. In the two-hybrid screen, the GAPDH did not interact with any protein. However, GAPDH was found to be present in a complex with PFK, PGK, and ENO in the SPINE analysis. It is possible, that the GAPDH carrying protein tags is unable to participate in productive interactions. The observation of a complex between glycolytic enzymes raises the question of the physiological significance of such a complex. It has been suggested that the glycolytic intermediates can be directly transferred from one enzyme to the next in the complex. This metabolic or substrate channeling may be advantageous for glycolytic fluxes as compared with free diffusion of the intermediates. The relevance of metabolic channeling in glycolysis has been controversially discussed (50, 51). Alternatively the complexes of glycolytic enzymes might facilitate fine tuning of the activities of the enzymes. Clearly new experimental approaches to address this important issue are urgently required. Compartmentalization of enzymes of one metabolic pathway has also been observed for purine biosynthesis (the "purinosome") and branched-chain amino acid catabolism in human cells (52, 53). The analysis of branched-chain amino acid utilization supports the idea of a specific metabolic unit with substrate channeling between the components (53). A recent *in silico* study suggests that flux through glycolysis in terms of pyruvate production is more efficient if the glycolytic

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[2] F. M. Rothe, M. Lehnik-Habrink, F. M. Commichau, and J. Stülke, unpublished results.

enzymes form a complex as compared with non-associated glycolytic enzymes (54).

The work presented here demonstrates that glycolytic enzymes are involved in many protein-protein interactions in *B. subtilis*. This observation opens many new fields for further research. Does the complex of glycolytic enzymes contribute to metabolic efficiency? What is the biochemical activity of Rny? Can Rny be useful to isolate new drugs against multiresistant staphylococci? What are the functions of the glycolytic enzymes in the RNA degradosome? What is the relevance of those interactions that were observed but not further addressed in this work? The answers to these questions are not only important for our understanding of the biology of *B. subtilis* but also for the general understanding of metabolic and regulatory networks.

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