Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2010;19:134–139
DOI: 10.1159/000321499

Published online: October 6, 2010

Interactions between Glycolytic Enzymes of *Mycoplasma pneumoniae*

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Key Words
Protein-protein interaction · Substrate channeling · Glycolysis · Mollicutes

Abstract
With only 688 protein-coding genes, *Mycoplasma pneumoniae* is one of the smallest self-replicating organisms. These bacteria use glycolysis as the major pathway for ATP production by substrate-level phosphorylation, suggesting that this pathway must be optimized to high efficiency. In this study, we have investigated the interactions between glycolytic enzymes using the bacterial adenylate cyclase-based two-hybrid system. We demonstrate that most of the glycolytic enzymes perform self-interactions, suggesting that they form dimers or other oligomeric forms. In addition, enolase was identified as the central glycolytic enzyme of *M. pneumoniae* due to its ability to directly interact with all other glycolytic enzymes. Our results support the idea of the formation of a glycolytic complex in *M. pneumoniae* and we suggest that the formation of this complex might ensure higher fluxes through the glycolytic pathway than would be possible with isolated non-interacting enzymes.

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Introduction

Mycoplasma pneumoniae, a cell wall-less pathogenic bacterium, is the best-studied representative of the class mollicutes. These bacteria are derived from Gram-positive ancestors that are related to *Bacillus subtilis* and lactic acid bacteria. The evolution of the mollicutes is characterized by substantial genome degeneration that reflects adaptation of the bacteria to a life in close association with eukaryotic hosts [Razin et al., 1998; Stülke et al., 2009].

M. pneumoniae causes usually mild diseases such as pneumonias, however implication of this bacterium in arthritic, cutaneous or autoimmune diseases as well as in encephalitis has been reported [Atkinson et al., 2008; Domenech et al., 2009; Jacobs, 1997; Schalock and Dinulos, 2009; Waites and Talkington, 2004]. Although *M. pneumoniae* is highly adapted to a life on mucosal surfaces, the bacteria are able to live independently of host cells. With fewer than 700 genes, *M. pneumoniae* belongs to the smallest organisms that are capable of autonomous life [Himmelreich et al., 1996].

The small genome of *M. pneumoniae* is also reflected by its limited metabolic capabilities. The bacteria can utilize glucose, fructose or glycerol as carbon sources [Halbedel et al., 2004; Yus et al., 2009]. These carbohydrates are metabolized via the glycolytic pathway, leading to the concomitant generation of ATP by substrate-level phosphory-

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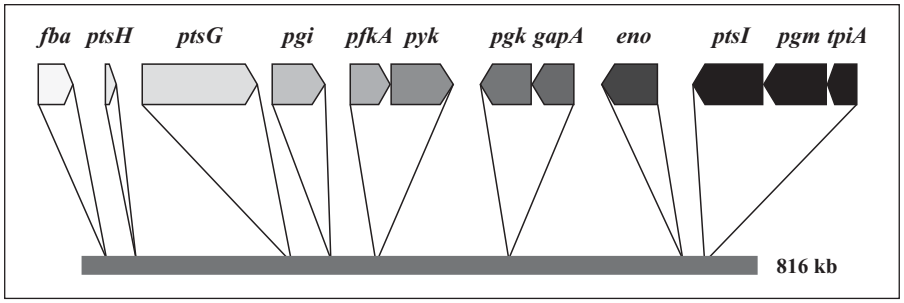

Fig. 1. Genomic localization of glycolytic genes in *M. pneumoniae*. The 816 kb genome of *M. pneumoniae* is shown true to scale in grey. Genes coding for glycolytic enzymes are illustrated relative to their position on the genome.

lation. However, *M. pneumoniae* does not possess an active citric acid cycle; similarly the pentose phosphate shunt is incomplete. While *M. pneumoniae* can produce ATP, nearly all anabolic pathways have been lost during degenerative evolution. Thus, *M. pneumoniae* depends heavily on the host cells or on organic nutrient supply from the artificial medium [Halbedel et al., 2007; Miles, 1992].

We are interested in the glycolytic pathway. *M. pneumoniae* encodes all glycolytic enzymes. Interestingly, some of these enzymes have multiple functions: the glycolytic kinases are also active in nucleotide phosphorylation thus replacing the nucleoside diphosphate kinase [Pollack et al., 2002]. Moreover, several glycolytic enzymes are subject to phosphorylation. However, it is not known how these phosphorylation events are catalyzed and how they affect the enzymatic activities [Schmidl et al., 2010]. For the regeneration of NAD, *M. pneumoniae* possesses a lactate dehydrogenase; alternatively the NAD oxidase may directly oxidize $NADH_2$, and the latter reaction is coupled to the production of hydrogen peroxide, the major virulence factor of *M. pneumoniae* [Cole et al., 1968; Halbedel et al., 2007; Low and Zimkus, 1973].

For a long time it was thought that metabolic pathways take place in an unorganized chaotic way in bacterial cells. However, at the same time there have been data indicating that, e.g. tryptophan metabolism in *Escherichia coli* involves protein complexes [Yanofsky and Rachmeler, 1958]. Similarly, glycolytic protein complexes were reported for *E. coli* as well as for eukaryotic cells [Campanella et al., 2005; Mowbray and Moses, 1976]. The accumulating recent evidence suggests that enzymes of one pathway may be clustered to allow efficient metabolism. This was shown for purine biosynthesis and branched-chain amino acid catabolism in human cells as well as for glycolysis in *B. subtilis* [An et al., 2008; Commichau et al., 2009; Islam et al., 2007]. Moreover, a recent in silico study supports the idea that glycolytic flux is much more efficient if the enzymes form a complex as compared to free floating enzymes [Amar et al., 2008]. The analysis of protein complexes in *M. pneumoniae* suggests that glycolytic enzymes might also interact in this organism [Kühner et al., 2009].

The analysis of primary protein-protein interactions in *M. pneumoniae* and other mollicutes is hampered by an alteration of the genetic code in these organisms: they use the UGA codon for the incorporation of tryptophan, whereas it specifies a stop in most other organisms. Due to this problem, the interactions between *Mycoplasma* proteins have so far never been studied using a two-hybrid system. We have developed a method for the quick replacement of stop codons [Hames et al., 2005]. Based on this method, we have studied the interactions between all glycolytic enzymes of *M. pneumoniae* using a bacterial two-hybrid (B2H) system. Our results support the idea that these enzymes interact, and that metabolism is well structured even in a seemingly primitive organism.

Results

Organization of Glycolytic Genes in M. pneumoniae

In *B. subtilis* and other firmicutes, genes encoding glycolytic enzymes from PTS proteins for glucose transport and phosphorylation to the pyruvate kinase are clustered in five operons [Ludwig et al., 2001]. In contrast, the corresponding *M. pneumoniae* genes are scattered on the chromosome (fig. 1). This is even the case for the PTS genes in *M. pneumoniae* that are clustered in most bacteria with a functional PTS sugar transport system [Barabote and Saier, 2005]. In *M. pneumoniae*, three gene clusters with glycolytic genes are present: The *pfkA pyk* cluster encodes the two glycolytic kinases. These genes also form an operon in *B. subtilis*. The second cluster is made up of the *pgk* and *gapA* genes encoding phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, respectively. These genes are clustered in most bacteria, and

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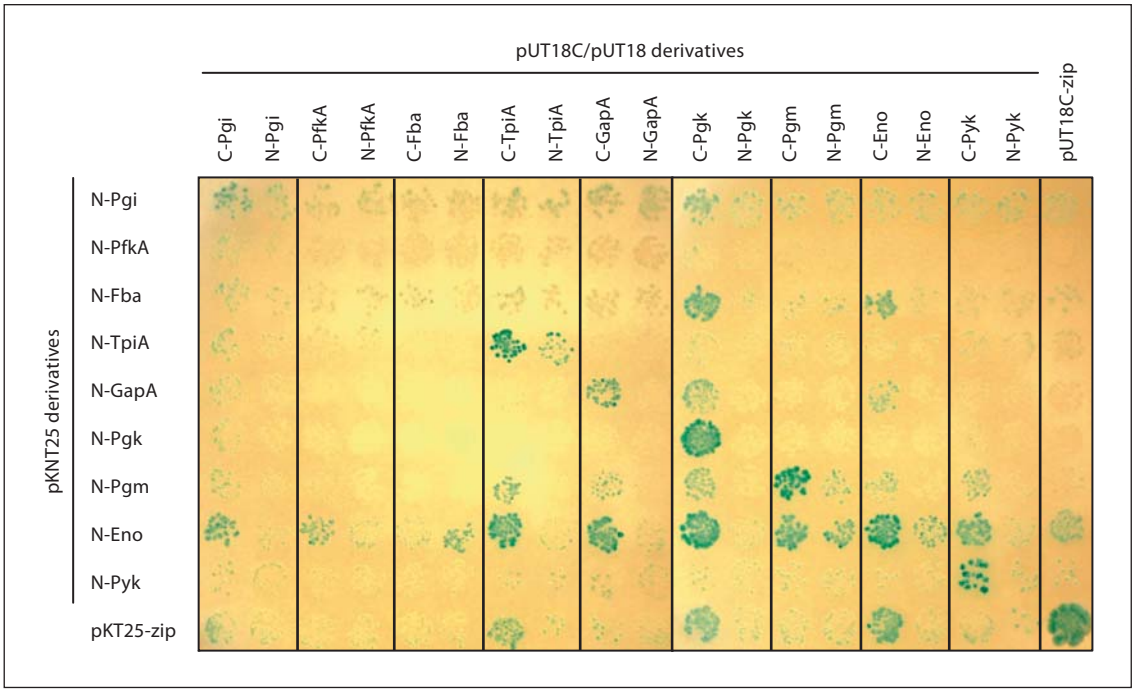

Fig. 2. B2H analysis to identify interactions among glycolytic enzymes. All genes were cloned in the plasmids pUT18, pUT18C, and pKNT25. Plasmids pUT18 and pUT18C allow the expression of the selected enzymes fused either to the N- or the C-terminus of the T18 domain of the *B. pertussis* adenylate cyclase, respectively. Plasmid pKNT25 allows the expression of the selected enzymes fused to the N-terminus of the T25 domain of the adenylate cyclase. The plasmids pKT25-zip and pUT18C-zip served as positive controls for complementation. The *E. coli* transformants were incubated for 48 h at 30°C. The level of protein-protein interaction was analyzed by observation of the blue coloration of the colonies.

it has been suggested that the clustering of these genes might reflect physical interaction of the encoded enzymes which catalyze consecutive reactions [Dandekar et al., 1998]. The third glycolytic cluster is formed by the *ptsI*, *pgm*, and *tpiA* genes encoding Enzyme I of the PTS, phosphoglycerate mutase and triose phosphate isomerase, respectively. The *pgm* and *tpiA* genes are also part of one operon in *B. su*btilis [Ludwig et al., 2001].

Interactions between Glycolytic Enzymes

In order to identify possible primary interactions between glycolytic enzymes, we made use of the B2H system. In this system, the T25 and the T18 fragments of the catalytic domain of the *Bordetella pertussis* adenylate cyclase were fused to full-length copies of all glycolytic enzymes of *M. pneumoniae*. The leucine zipper of the yeast GCN4 transcription factor served as a control [Karimova et al., 1998]. The results of the B2H analysis are shown in figure 2. As expected, the leucine zipper of GCN4 showed strong self-interaction.

As shown in figure 2, we observed self-interactions for all glycolytic enzymes with the exception of phosphofructokinase and fructose 1,6-bisphosphate aldolase. In addition to self-interaction, we detected several interactions between different glycolytic enzymes (fig. 2, 3). Of all glycolytic enzymes, enolase is involved in the largest number of interactions with other enzymes. We observed interactions of enolase with all other glycolytic enzymes. The strongest interactions were detected between enolase and triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase and pyruvate kinase. A strong interaction was also detected between phosphoglycerate kinase and fructose 1,6-bisphosphate aldolase. Weaker interactions were observed between enolase and phosphoglucose isomerase, phosphofructokinase and fructose 1,6-bisphosphate aldolase. Moreover, we observed weak interactions between glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase as well as between triosephosphate isomerase and

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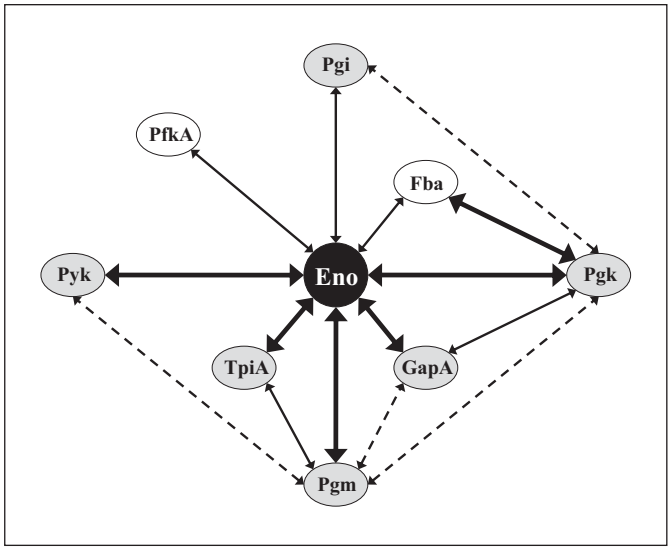

Fig. 3. Schematic summary of the outcome of the B2H analysis. The interaction network of glycolytic enzymes is depicted with the enolase (Eno) as the core protein. Proteins that are able to form homomultimeric complexes are highlighted. Dashed arrows indicate weak interactions, whereas regular or bold black arrows point out moderate and strong interactions, respectively.

phosphoglycerate mutase. These latter interactions reflect the genomic organization with *pgk-gapA* and *pgm-tpiA* gene clusters (fig. 1). Finally, faint interactions were observed between phosphoglycerate mutase on the one hand and glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase on the other. A very weak interaction was also detectable between phosphoglycerate kinase and phosphoglucose isomerase.

Thus, all glycolytic enzymes are involved in interactions with other enzymes of the pathway, suggesting that glycolysis proceeds in a well-organized and structured manner in *M. pneumoniae*.

Discussion

Glycolysis is the major pathway for the generation of energy by substrate-level phosphorylation in *M. pneumoniae* and most other mollicutes. In good agreement with both the importance for cellular metabolism and the ancient origin of the glycolytic pathway is the observation that the genes encoding glycolytic enzymes are essential in many organisms and that these enzymes have additional functions that are completely unrelated to the biochemical pathway [Canback et al., 2002; Commichau et al., 2009; Kim and Kang, 2005].

In this study, we have addressed the interactions between the glycolytic enzymes themselves. A recent proteome-level analysis of protein complexes in *M. pneumoniae* [Kühner et al., 2009] as well as experiments with the Gram-positive model bacterium *B. subtilis* [Commichau et al., 2009] provided indications for such interactions. In order to identify the primary interactions between the glycolytic enzymes, we applied the technique of two-hybrid analysis for the first time to proteins from a *Mycoplasma* species. Our studies revealed self-interactions that may correspond to the formation of dimers or other homo-oligomers for all glycolytic enzymes except phosphofructokinase and fructose 1,6-bisphosphate aldolase. This observation is in good agreement with the known structures of glycolytic enzymes that indicate the formation of dimers or tetramers [Erlandsen et al., 2000].

Our study indicates that enolase is of central importance in the organization of glycolysis in *M. pneumoniae* since this is the only enzyme that is capable of interacting directly with any other glycolytic enzyme (fig. 3). Moreover, most of the strongest interactions involve enolase. Another interesting conclusion from this work is that the enzymes of the lower part of glycolysis are much more strongly embedded in the interaction network than those of the hexose phosphate part (fig. 3). This reflects that the triose phosphate interconversion is the key module of the whole pathway in the living world, whereas the first intermediates of this part, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate may be obtained from very different sources including the upper part of glycolysis, the pentose phosphate pathway, the Entner-Doudoroff pathway, or from glycerol phosphate dehydrogenase/oxidase.

In their proteome-level analysis, Kühner et al. [2009] proposed two glycolytic complexes in *M. pneumoniae*. One of these complexes consists of glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and enolase. Our results are in support of this complex and provide compelling evidence that these three enzymes are capable of interacting directly with each other. The second complex was suggested to be composed of glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and fructose 1,6-bisphosphate aldolase. We did not observe primary interactions between any couple of these three proteins. However, glyceraldehyde 3-phosphate dehydrogenase, as a member of the first complex, does directly bind to enolase, and we observe direct interaction between enolase and the two other enzymes. In any case, it seems difficult to name distinct complexes from in vivo

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pull-down studies that have the same members. Finally, Kühner et al. [2009] suggested a complex made up of enolase, triose phosphate isomerase, a metalloprotease (MPN569) and an unknown protein (MPN316). The interaction between the two glycolytic enzymes is supported by our analysis. It is interesting to note that enolase may be involved in interactions that are not related to glycolysis. This has also been observed in *E. coli* and *B. subtilis*. In both organisms, enolase is part of the RNA-degrading multiprotein complex called RNA degradosome [Carpousis, 2007; Commichau et al., 2009].

In conclusion, our results support the idea that glycolytic enzymes interact in *M. pneumoniae*. However, in contrast to previous findings, we suggest the presence of one complex of glycolytic enzymes that is organized around enolase. It is tempting to speculate that such a complex may be important for the efficiency of the glycolytic pathway, and thus for the efficiency of energy production in *M. pneumoniae*.

Experimental Procedures

Bacterial Strains and Growth Conditions

E. coli XL1-Blue (Stratagene) and BTH101 [Karimova et al., 1998] were used for cloning experiments and B2H analyses, respectively. *E. coli* was grown in LB medium. LB plates were prepared by the addition of 18 g Bacto agar/l (Difco) to LB medium.

DNA Manipulation and Transformation

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures [Sambrook et al., 1989]. Restriction enzymes, T4 DNA ligase and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using the Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). *Phusion* DNA polymerase was used for the polymerase chain reaction (PCR) as recommended by the manufacturer. All primer sequences are available upon request. DNA sequences were determined using the dideoxy chain termination method [Sambrook et al., 1989]. All plasmid inserts derived from PCR products were verified by DNA sequencing. *E. coli* transformants were selected on LB plates containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

Plasmid Constructions

The genes encoding the glycolytic enzymes from *M. pneumoniae* were first subcloned as follows. The coding sequence of each gene was amplified by PCR with gene-specific primers using chromosomal DNA of *M. pneumoniae* M129 as the template. The PCR products were digested as appropriate with BamHI and HindIII or with BamHI and PstI and cloned into the appropriately linearized expression vector pWH844 [Schirmer et al., 1997]. These plasmids allowed the expression of the glycolytic enzymes carrying an N-terminal His_6-tag. The resulting plasmids are listed in the supplementary material. In *M. pneumoniae* the UGA specifies a tryptophan, however in *E. coli* it is a stop codon. With the exception of *pfkA* and *pgm*, the glycolytic genes contain one to three UGA codons that were replaced by the multiple mutation reaction [Hames et al., 2005] using phosphorylated mutagenic oligonucleotides and the former plasmids as template. The resulting final plasmids are listed in the supplementary material.

To obtain the plasmids for the B2H analyses, the coding sequences of the glycolytic genes were amplified by PCR using the latter plasmids. The PCR products were digested as shown in the supplementary material, and the resulting fragments were cloned into each of the three plasmids – pKNT25, pUT18 and pUT18c [Claessen et al., 2008; Karimova et al., 1998], digested with the same enzymes. The resulting plasmids used for the B2H analyses are listed in the supplementary material. All plasmid inserts were verified by DNA sequencing.

B2H Analysis

Primary protein-protein interactions were identified by B2H analysis [Karimova et al., 1998]. The B2H system is based on the interaction-mediated reconstruction of adenylate cyclase (CyaA) activity from *B. 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-CAP-dependent promoter of the *E. coli lac* operon. Thus, a high β-galactosidase activity reflects the interaction between the hybrid proteins. Plasmids pUT18 and pKNT25 allow the expression of proteins fused to the N-terminus of the T18 and T25 fragments of the CyaA protein, respectively, and the plasmid pUT18C allows the expression of proteins fused to the C-terminus of the T18 fragments of the CyaA protein, respectively [Claessen et al., 2008; Karimova et al., 1998]. 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 due to the leucine zipper motifs resulting in an active CyaA enzyme and a high β-galactosidase activity. The plasmids constructed for the B2H assay (see supplementary material) 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), X-Gal (40 μg/ml) (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (0.5 mM) (isopropyl-β-D-thiogalactopyranoside). The plates were incubated for a maximum of 60 h at 30°C. The B2H assays were performed in triplicate.

Supplementary Material

Information on the plasmids and oligonucleotides used in this study is available as supplementary material (http://genmibio.uni-goettingen.de/index.php?id=120).

Acknowledgements

We are grateful to Julia Busse, Henrike Pförtner, Martin Arnold, and Frederik Meyer for helpful discussions. This work was supported by Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. S.R.S. was supported by a personal grant from the Studienstiftung des Deutschen Volkes.

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