Arch Microbiol (2002) 177 : 410–419
DOI 10.1007/s00203-002-0406-6

ORIGINAL PAPER

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Two class II D-tagatose-bisphosphate aldolases from enteric bacteria

Received: 6 September 2001 / Revised: 10 January 2002 / Accepted: 30 January 2002 / Published online: 16 March 2002

Abstract *Escherichia coli, Salmonella enterica, Klebsiella pneumoniae* and *Klebsiella oxytoca* were found to contain two D-tagatose 1,6-bisphosphate (TagBP)-specific aldolases involved in catabolism of galactitol (genes *gatY gatZ*) and of *N*-acetyl-galactosamine and D-galactosamine (genes *kbaY kbaZ*, also called *agaY agaZ*). The two aldolases were closely related (≥53.8% identical amino acids) and could substitute for each other in vivo. The catalytic subunits GatY or KbaY alone were sufficient to show aldolase activity. Although substantially shorter than other aldolases (285 amino acids, instead of 358 and 349 amino acids), these subunits contained most or all of the residues that have been identified as essential in substrate/product recognition and catalysis for class II aldolases. In contrast to these, both aldolases required subunits GatZ or KbaZ (420 amino acids) for full activity and for good in vivo and in vitro stability. The Z subunits alone did not show any aldolase activity. Close relatives of these new TagBP aldolases were found in several gram-negative and gram-positive bacteria, e.g., *Streptomyces coelicolor*.

Keywords D-Tagatose 1,6-bisphosphate aldolases · Class II aldolases · Enteric bacteria

Introduction

Two forms of the enzyme fructose-1,6-bisphosphate aldolase (E.C.4.1.2.13) and of other ketose-1,6-bisphosphate aldolases are known. Class I aldolases form an imine between an essential lysine residue in the catalytic center and the substrate. These enzymes, formerly considered to be typical for eukaryotes, have been found in a rapidly increasing number of prokaryotes (references in Thomson et al. 1998). They have subunit molecular masses ranging from 27 to 40 kDa and occur in monomeric to decameric complexes. Their apparent K_m values are in the millimolar to micromolar range. The substrates usually include, besides fructose 1,6-bisphosphate (BP), the corresponding ketose isomers, i.e., L-sorbose-, D-tagatose- and D-psicose-bisphosphate, and even D-fructose 1-phosphate (1P) (Stribling and Perham 1973). The genes for class I aldolases are often members of operons for the catabolism of specific carbohydrates and inducible by the corresponding substrates, e.g., those involved in lactose and galactose metabolism in gram-positive bacteria (Anderson and Bissett 1982; Bissett and Anderson 1980; Rosey and Stewart 1992; Van Rooijen et al. 1991), or the class I fructose bisphosphate (FruBP)-aldolase FbaB (formerly DhnA) from *Escherichia coli* inducible by pyruvate and lactate (Stribling and Perham 1973; Thomson et al. 1998).

Class II aldolases, in contrast, are homodimeric (subunit M_r ca. 40,000) and require a divalent metal ion in the active site. They normally represent a key enzyme in the Embden-Meyerhoff-Parnass pathway, e.g., the major fructose-bisphosphate aldolase FbaA (formerly Fda), of *E. coli*, and are synthesized constitutively. Despite these differences, class I and class II aldolases share characteristic similarities at the primary amino acid level and at the structural level (Anderson and Bissett 1982; Anderson and Markwell 1982; Plater et al. 1999; Stribling and Perham 1973).

Growth of enteric bacteria on galactitol (Gat, formerly dulcitol) generates D-tagatose 1,6-bisphosphate (TagBP) and requires an aldolase for further catabolism (Lengeler 1977; Markwell and Anderson 1981; Markwell et al. 1976; Wolff and Kaplan 1956). This aldolase appears to be temperature-sensitive in *E. coli*, thus causing a Gat (Ts) phenotype. Temperature-resistant derivatives able to grow at 42 °C on galactitol have been isolated and the corresponding *kba* (Tr) mutations were mapped near the *argG* locus at 70.5 min (Lengeler 1977; Nobelmann and Lengeler 1996). Subsequent cloning and sequencing of the *gat* operon from wild-type *E. coli*, strain EC3132 and from *E. coli*, strain K-12, however, revealed a gene *gatY*,

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located at 46.7 min, whose product GatY shows TagBP aldolase activity as revealed by SDS-PAGE and enzyme tests (Blattner et al. 1997; Nobelmann and Lengeler 1995, 1996). The genome sequencing project of *E. coli* K-12 also revealed a second gene, *agaY*, with high similarity to *gatY* (Reizer et al. 1996). The new gene is a member of the *aga* regulon for *N*-acetyl-galactosamine and D-galactosamine degradation which, similar to *kba* (Tr), also maps at 70.5 min (Brinkkötter et al. 2000). Here we report that GatY and KbaY indeed correspond to two different but highly similar TagBP aldolases. Unexpectedly for class II aldolases, both require for full activity and stability a second protein encoded by genes *gatZ* and *kba*Z, respectively.

Materials and methods

Bacterial strains and plasmids

Mutants and derivatives of *E. coli* C and K-12 as well as the plasmids isolated and constructed are listed in Tables 1 and 4 or in Ausubel et al. (1990). Strain JWL150 (formerly L150) is a derivative of *E. coli* K-12 (F^- *thiA1 metB1 arg*$^+$ *mtlA50 gutA52 gatR49 gat*$^+$ Kba(Ts) *rpsL104*) with the Gat(Ts) phenotype characteristic for this strain (Lengeler 1977). Due to genotypic exclusion, the *gat* genes from *E. coli* K-12 can be deleted completely by P1 transduction and selecting by growth on D-arabitol or ribitol for the *atl-rtl* genes as substitutes (Heuel et al. 1998; Link and Reiner 1983; Woodward and Charles 1983). Strain LAB107 is a P1 transductant from NP315 (Böck and Neidhardt 1966) carrying the *zgf-3156*::Tn*10 kan* insertion from CAG18604 (Berlyn et al. 1996) near its *fbaA*(Ts) allele. This allele confers a temperature-sensitive D-fructose 1,6-BP aldolase or Fba(Ts) phenotype, e.g., during growth on glucose or fructose. The allele can be transduced by means of the *zgf* insertion (cotransduction >30%) selecting for Kan^r Glc(Ts) transductants. Strain JLV350 carries a Tn*10* insertion in the *kdp* genes and can be used to transduce *nag* alleles (cotransduction 30%) for *N*-acetyl-glucosamine-degrading enzymes (Vogler et al. 1989). The construction and mapping of other mutants and mutations will be described in the Results section. P1 transduction was done as described by Lengeler (1975).

Table 1 Bacterial strains and plasmids. The Δ*gat* symbol indicates that the *gat* genes were deleted and replaced with the *atl/rtl* genes from *Escherichia coli* C. For the *aga/kba* genes, the (*o*) allele corresponds to an internal deletion which prevents their expression in *E. coli* K-12;+designates an intact and inducible regulon, (*Con*) a constitutive mutation, and (*Tr*) an unidentified mutation that allows their expression during growth on galactitol (*Gat*) at 42 °C. The allele *nag-30* enables cells to take up *N*-acetyl-galactosamine (*Aga*) through the enzyme II^{Nag} and to grow on Aga. In the plasmids, expression of the plasmid-encoded *gat* or *kba* genes was either from *gatYp* (in pBNL6) or from *lacZp* of the vector (all other constructs)

E. coli strains or plasmids	Relevant genotype or cloned genes	Construction or vector	Reference
Strains			
JWL150	*gat*$^+$ *kbaYZ*° *nag*$^+$ *fbaA*$^+$		Lengeler (1975)
LAB107	*gat*$^+$ *kbaYZ*° *nag*$^+$ *fbaA*(Ts)	P1.CAG18604×NP 315	This study
LAB103	Δ*gat kbaYZ*° *nag*$^+$ *fbaA*$^+$	P1.*E. coli* C×JWL150	This study
LAB108	*gat*$^+$ *kbaYZ*° *nag*$^+$ *fbaA*(Ts)	P1.LAB107×JWL150	This study
LAB111	Δ*gat kbaYZ*° *nag*$^+$ *fbaA*(Ts)	P1.LAB107×LAB103	This study
JWL153 (JWL183)	*gat*$^+$ *kbaYZ*(Tr) *nag*$^+$ *fbaA*$^+$		Lengeler (1975)
JWL4001	Δ*gat kbaYZ*(Tr) *nag*$^+$ *fbaA*$^+$	P1.*E. coli* C×JWL153	Heilenmann (1986)
LAB88	*gat*$^+$ *kbaZ*::*kan*(Tr) *nag*$^+$ *fbaA*$^+$	JWL153 Gat(Ts) mutant	This study
LAB89	Δ*gat kbaZ*::*kan*(Tr) *nag*$^+$ *fbaA*$^+$	P1. *E. coli* C×LAB88	This study
LAB109	*gat*$^+$ *kbaYZ*(Tr) *nag*$^+$ *fbaA*(Ts)	P1.LAB107×JWL153	This study
LAB110	Δ*gat*$^+$ *kbaYZ*(Tr) *nag*$^+$ *fbaA*(Ts)	P1.LAB107×JWL4001	This study
LAB30	*gat*$^+$ *kbaYZ*° *nag-30 fbaA*$^+$	*E. coli* K-12 Aga$^+$ mutant	Brinkkötter et al. (2000)
LAB31	*Δgat kbaYZ*° *nag-30 fbaA*$^+$	P1.*E. coli* C×LAB30	This study
LAB56	*gat*$^+$ *kbaYZ*° *nag-30 fbaA*$^+$	P1.JLV350×LAB30	This study
LAB92	*gat*$^+$ *kbaZ*::*kan*(Tr) *nag-30 fbaA*$^+$	P1.LAB56×LAB88	This study
LAB94	*Δgat kbaYZ*° *nag-30 fbaA*$^+$	P1.LAB56×LAB103	This study
LAB95	Δ*gat kbaYZ*(Tr) *nag-30 fbaA*$^+$	P1.LAB56×JWL4001	This study
LAB151	*gat-75 aga/kba*$^+$ *nag*$^+$ *fbaA*$^+$ *arg*$^+$	P1.*E. coli* C×LAB150	Brinkkötter et al. (2000)
LAB152	*gat*$^+$ *aga/kba*$^+$ *nag*$^+$ *fbaA*$^+$ *arg*$^+$	P1.LAB150×JWL184–1	Brinkkötter et al. (2000)
LAB204	*gat*$^+$ *agaR(Con) nag*$^+$ *fbaA*$^+$ *arg*$^+$	LAB152 Gat(Tr) mutant	Brinkkötter et al. (2000)
Plasmids			
pBNL6	*gatYZABCDR'*	pSU18	Nobelmann and Lengeler (1996)
pLAB10	GatY	pSU19	This study
pLAB22	GatZ	pSU19	This study
pLAB30	GatYZ	pSU19	This study
pLAB35	KbaZ	pSU18	This study
pLAB47	GatY	pUC19	This study
pLAB400	KbaY	pSU19	This study
pKBA403	KbaY	pUC19	This study

Culture media and growth conditions

Lennox broth (LB), phosphate-buffered minimal medium (MM), and MacConkey agar plates containing 1% (w/v) of the carbohydrate to be tested have been described before (Lengeler and Lin 1972). In minimal media, the following were added at the concentrations indicated: L-amino acids and nucleosides, 20 mg l^{-1}; vitamins, 5 mg l^{-1}; carbohydrates, 10 mM. Sterile-filtered antibiotics were used at the following final concentrations (mg l^{-1}): rifampicin, 400; ampicillin and spectinomycin, 100; streptomycin, 50; chloramphenicol and kanamycin, 25; and tetracycline, 10.

For transport and enzyme assays the bacteria were grown exponentially to about 5×10^8 cells/ml, harvested and washed three times in 1% NaCl. For transport tests, the cells were resuspended in minimal medium at 25 °C to 5×10^8 cells per ml and tested as described (Heuel et al. 1997, 1998). For aldolase tests, the cells were washed at 4 °C, resuspended to 1.6×10^9 cells per ml in 20 mM Tris-HCl/1 mM $ZnCl_2$, pH 7.5, and treated either with toluene (10 µl per ml cells for 1 min) and vortexed, or shaking in a Retsch mill with zirconium beads for 20 min (Retsch, Haan, Germany). Cell extracts were centrifuged for 15 min at 15,000×*g* and the supernatants used for enzyme tests.

Aldolase assays

Assays for aldolase activity were done in 50 mM KC1 and 50 mM Tris-HC1, pH 7.5, with 2,4 mM D-tagatose 1,6-BP or 4,0 mM D-fructose 1,6-BP as a substrate, 0.2 mM NADH, 20 units of triosephosphate isomerase and 1 unit of glycerol-3-phosphate isomerase. After equilibration of the assay mixture at the desired temperature, the reaction was started by adding extract from exponentially growing cells. The decrease of NADH absorbance was measured photometrically at 340 nm and compared to a control without substrate (Nobelmann and Lengeler 1996). Protein concentrations were determined according to Bradford (1976). Activities are given in nmol min^{-1} (mg protein)$^{-1}$.

Genetic and cloning techniques

Cloning and isolation of DNA followed standard methods (Sambrook et al. 1989; Ausubel et al. 1990). Restriction enzymes and other enzymes used in recombinant DNA technology were used according to the specifications of the manufacturers. The genes *kbaZ* and *kbaY* were cloned by PCR from chromosomal DNA of *E. coli* JWL153 (Saiki et al. 1985). With the primers Agz1 (5′-ATGGATCCATGACGCAGTATCAACTGAAAC-3′) and Agz2 (5′-TATTTGGCATGCGTGTTCCTCTTTGTTTTGCTTA-3′) the gene *kbaZ* was amplified. The *Bam*HI-/ and *Sph*I-digested PCR product was cloned into the *Bam*HI- and *Sph*I-digested vector pSU18. The gene *kbaY* was amplified with the primers Kex1 (5′-CATGGACTGAAGAGATC-3′) and Kex3 (5′-TCCTGAATTCTTAAGGCACTCCCCGATATTAATCG-3′). The *Bam*HI- and *Eco*RI-digested PCR product was cloned into the *Bam*HI- and *Eco*RI-digested vector pSU19. The same fragment was also cloned into a *Bam*HI- and *Eco*RI-digested pUC19 (pKBA403). Plasmids with *gatY* and/or *gatZ* genes, shown in Table 1, are subclones from plasmid pBNL6 (Nobelmann and Lengeler 1995) which carries genes *gatZYABCDR'*. Thus, pLAB10 is a pSU19 derivative and pLAB47 a pUC19 derivative which carry *gatY* on a 1.7-kb fragment.

Plasmid pLAB30 (*gatY gatZ*) contains a 2.7-kb *Pst*I-*Eco*RI fragment from pBNL6 in pSU19. By deleting a 0.7-kb *Sph*I fragment from pLAB30, plasmid pLAB22 (*gatZ*) was constructed. Plasmid pLAS1, containing the *gat* genes from *Klebsiella oxytoca* M5a1, is a shotgun clone selected in strain JM109 (Ausubel et al. 1990) on galactitol plates starting with chromosomal DNA from M5a1. The genes *kbaY* and *kbaZ* from *E. coli* C were amplified by PCR (Saiki et al. 1985) and cloned into the pGEM-T vector (Promega, Madison, Wis., USA) to obtain plasmids pAGA11 (*kbaY*) with the primers AgaA14 (5′-CCGCAACCGGTACCTGGACTG-3′) and AgaA15 (5′-CTCCCCGATATTAATCGATAA-3′), and pAGA14 (*kbaZ*) with the primers AgaA23 (5′-GCTGAATGATCTGTTCTCGTCCGT-3′) and AgaA24 (5′-CGTTGGCTACCAGCACCAGATTTG-3′). To sequence these four genes, known restriction sites were used to obtain smaller clones, the ends of which could be sequenced after subcloning. Finally, specific DNA primers were used to complete the sequences flanking the restriction sites.

DNA sequencing and analysis

DNA was sequenced using the dideoxynucleotide chain-termination method of Sanger et al. (1977) with the T7 sequencing kit from Pharmacia according to the instructions supplied by the manufacturer. The sequences obtained were analyzed by the BLAST family of programs (Altschul et al. 1990) or the DNAsis for Windows DNA and protein analysis system (Hitachi Software Engineering America, San Francisco, Calif., USA).

Chemicals

D-Tagatose 1,6-bisphosphate (TagBP) was a kind gift of W.-D. Fessner (Darmstadt, Germany), and [^{3}H]-galactitol was obtained from Amersham Life Sciences (Buckinghamshire, UK). All other chemicals were of commercial origin.

Results

There are at least three class II ketose-bisphosphate aldolases in *E. coli*

The *gat* genes and enzymes for galactitol degradation are expressed constitutively in *E. coli* K-12. This did not include the TagBP aldolase, whose activity could only be seen after overexpressing the *gat* genes from *E. coli*, strain EC3132 on pBNL6 (Nobelmann and Lengeler 1996). In strain JWL150, however, a constitutively expressed low aldolase activity was seen which was eliminated in Δ*gat* mutants (e.g., LAB103 and LAB110 in Table 2). This TagBP aldolase did not correspond to the well-known FruBP-aldolase (FbaA; gene locus *fbaA* at 66.1 min) because strains carrying an *fbaA*(Ts) allele, (e.g., LAB108 in Table 2) had unchanged TagBP aldolase activities and stabilities.

A Gat (Tr) mutant JWL153 isolated from strain JWL150 (Lengeler 1977) expressed a slightly increased aldolase activity which was increased further after growth at 42 °C in the presence of galactitol. This increase of activity depended on galactitol transport and metabolism (Table 2). A Δ*gat* derivative JWL4001 showed a lowered aldolase activity (Table 2) but with an increased temperature resistance (see below). These results suggested that *gat*$^+$ strains of *E. coli* express only a temperature-sensitive GatY aldolase. The Kba(Tr) phenotype, however, seemed to be caused by the expression of a second and temperature-resistant aldolase, Kba(Tr), encoded in the *kba*(Tr) locus, which was expressed in mutant JWL153 during growth on galactitol at 42 °C. KbaY-negative derivatives of mutant JWL153 should consequently show the original Gat(Ts) phenotype. Such a mutant (LAB88) no longer expressed the temperature-resistant aldolase but retained a temperature-sensitive activity (Table 2). The Tn insertion

Table 2 Specific activities of D-tagatose 1,6-bisphosphate (*TagBP*) and D-fructose 1,6-bisphosphate (*FruBP*) aldolases in cell extracts from various mutant strains of *E. coli* K-12 during the exponential growth phase. The cells were grown on glycerol (*Gly*) or on galactitol (*Gat*) at 30 °C or 42 °C as indicated, and on glycerol with 0.1 mM glucose 6-phosphate at 42 °C for mutants LAB108 to LAB111. For aldolase tests, 2.4 mM D-tagatose 1,6-bisphosphate or 4 mM D-fructose 1,6 bisphosphate were used and activities expressed in nmol min $^{-1}$ (mg protein)$^{-1}$. The genetic symbols are as described in Table 1;+indicates growth, and – indicates no growth on the media and carbohydrates indicated at 30 °C or 42 °C

Strains	Genotype			Growth on galactitol		Tag BP aldolase activity			Fru BP aldolase activity	
	gatYZ	*kbaYZ*	*fbaA*	30 °	42 °	Gly 30 °C	Gat 30 °C	Gat 42 °C	30 °	42 °
JWL150	+	0	+	+	–	4	4	–	85	80
LAB103	Δ	0	+	–	–	≤1	–	–	82	86
LAB108	+	0	Ts	+	–	3	4	–	19	≤1
LAB110	Δ	0	Ts	–	–	≤1	–	–	17	≤1
JWL153	+	Tr	+	+	+	11	17	44	78	83
JWL4001	Δ	Tr	+	–	–	5	–	–	87	85
LAB88	+	*Z*::*kan*	+	+	–	4	4	–	77	78
LAB89	Δ	*Z*::*kan*	+	–	–	≤1	–	–	83	88
LAB109	+	Tr	Ts	+	(+)	5	7	–	20	≤1
LAB111	Δ	Tr	Ts	–	–	3	–	–	19	≤1

present in mutant LAB88 was mapped by P1 transduction to the *kba* locus. As expected, a double mutant (LAB89; Δ*gat kba*::*kan*) lacked all TagBP aldolase activities. Where present, as in strain LAB109 and LAB111, these activities were not affected by the *fbaA*(Ts) mutation.

Further proof for this contention came from the analysis of derivatives of strain *E. coli* K-12, able to grow on *N*-acetyl-galactosamine (Aga). The complete *aga/kba* locus of *E. coli* comprises 12 genes for *N*-acetyl-galactosamine and D-galactosamine degradation, and a repressor AgaR responding to these amino sugars. The genes are organized in two adjacent operons, including a gene *agaR* for the repressor, and cannot be induced in *E. coli* K-12 (Brinkkötter et al. 2000). After cloning and sequencing, the Tn insertion of mutant LAB88 was mapped to the gene *kbaZ* (or *agaZ*). This *kbaZ*::*Tn10* insertion not only inactivated gene *kbaZ*, but through polarity effects also prevented induction and hence expression of gene *kbaY* (*agaY*) which apparently encodes the second temperature-resistant TagBP aldolase.

Two types of mutants able to take up *N*-acetyl-galactosamine, and hence to be induced for the *aga/kba* genes, can be isolated (Brinkkötter et al. 2000). The first class carries mutations in the transporter (enzyme II^{Nag}) for *N*-acetyl-glucosamine which allow uptake of *N*-acetyl-galactosamine through enzyme II^{Nag} (*nag-30* mutants in Table 3). Such mutants expressed a temperature-resistant TagBP aldolase which was induced during growth on *N*-acetyl-galactosamine (e.g., strain LAB30 in Table 3). Because this activity remained even after deletion of the *gat* genes (strain LAB94), it must correspond to the KbaY activity. An Aga$^+$ derivative of strain JWL4001, strain LAB95, also expressed this inducible Kba aldolase, in contrast to a *kbaZ*::Tn*10* derivative LAB92 (Table 3). The second mutant class carried an intact *aga/kba* regulon from *E. coli* strain C and also expressed the KbaY al-

Table 3 Specific activities of D-tagatose 1,6-bisphosphate aldolases in cell extracts from various mutant strains of *E. coli* K-12 during the exponential growth phase. The cells were grown on glycerol (*Gly*), *N*-acetyl-D-galactosamine (*Aga*), or galactitol (*Gat*) at 30 °C or 42 °C and tested with 2.4 mM D-tagatose 1,6-bisphoshate (*TagBP*). Activities are in nmol min^{-1} (mg protein)$^{-1}$. The genetic symbols are as in Table 1;+indicates growth, and – no growth on the carbohydrates and at 30 ° or 42 °C; nt not tested

Strain	Genotype			Growth on galactitol on *N*-acetyl-D-galactosamine			TagBP aldolase activity			
	gat	*aga/ kba*	*nag*	Gat 30 °	Gat 42 °	Aga 42 °	Gly 30 °	Aga 30 °	Gat 30 °	Gat 42 °
JWL150	+	0	+	+	–	–	4	–	4	–
LAB30	+	0	*–30*	+	–	+	4	67	nt	4
LAB94	Δ	0	*–30*	-	–	+	≤1	64	–	–
JWL153	+	(Tr)	+	+	+	–	11	15	17	44
LAB92	+	*Z*::*kan*	*–30*	+	–	+	5	5	nt	–
LAB95	Δ	(Tr)	*–30*	–	–	+	6	71	–	–
LAB151	*–75*	+	+	–	–	+	≤1	26	–	–
LAB152	+	+	+	+	–	+	4	24	3	–
LAB204	+	(Con)	+	+	+	+	32	28	28	nt

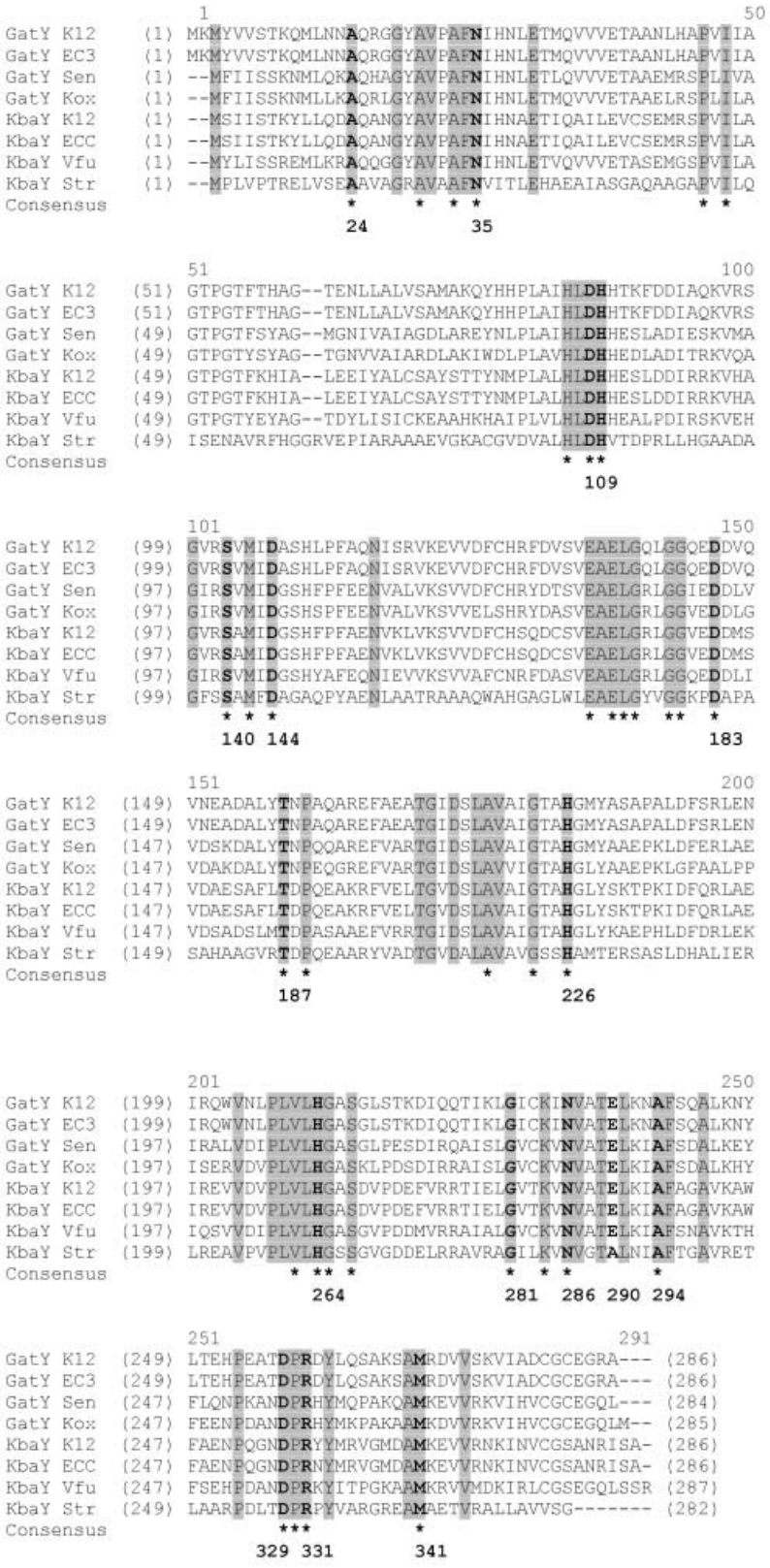

Fig. 1 Sequence alignments of GatY-like proteins from various bacteria. The sequences for various GatY-like proteins were aligned. Residues identical in all proteins are *shaded*. In the consensus sequence all residues also present in the fructose-1,6-bisphosphate aldolase FbaA of *Escherichia coli* are marked. To facilitate comparison, numbered residues from FbaA recognized as essential are highlighted. The origins of the sequences are: the aldolase subunits GatY from *E. coli* strains K-12 (K12) and EC3132 (EC3), from *Salmonella enterica* serovar typhi (Sen) and from *Klebsiella oxytoca* strain M5a1 (Kox); the aldolase subunit KbaY from *E. coli* strains K-12 (K12) and C (ECC); the ManF protein from *Vibrio furnisii* (Vfu) and AgaY from *Streptomyces coelicolor* (Str) of unknown function. The accession number of the *K. oxytoca gatY* sequence is AF416702

dolase after growth on *N*-acetyl-galactosamine (mutants LAB151, LAB152 in Table 3). Final proof of our hypothesis that the Gat(Tr) phenotype was caused by expression of the temperature-resistant KbaY aldolase is based on the observation that *aga/kba*$^+$ strains with an inducible phenotype (mutant LAB152) could not grow on galactitol at 42 °C while mutants (e.g. LAB204) expressing these genes constitutively had the expected Gat(Tr) phenotype. We

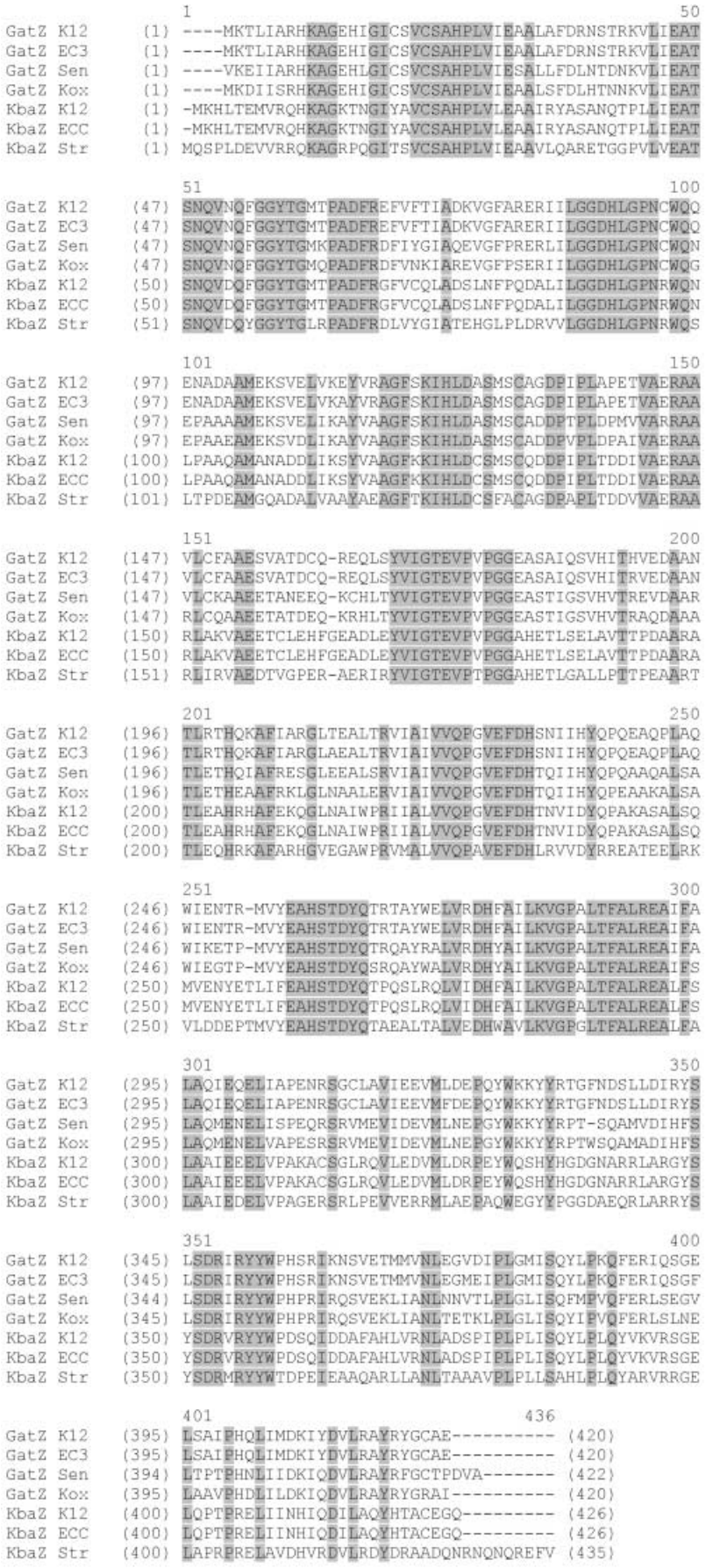

Fig. 2 Sequence alignments of GatZ-like proteins from various bacteria. The sequences for various GatZ-like proteins were aligned. Identical residues are *shaded*; these are: GatZ from *E. coli* strains K-12 (K12), and EC3132 (EC3), *S. enterica* serovar typhi (Sen), and from *K. oxytoca* strain M5a1 (Kox); KbaZ from *E. coli* strains K-12 (K12) and C (ECC); AgaZ from *S. coelicolor* (Str) of unknown function. The accession number of the *K. oxytoca gatZ* sequence is AF416702

speculated consequently that the Gat(Tr) phenotype of the original mutant JWL153 was due to a regulatory mutation in the *aga/kba* locus, most likely the repressor, which allowed increased expression of the KbaY aldolase during growth on galactitol at 42 °C, as shown in Table 2.

The GatYZ and KbaYZ TagBP aldolases from various eubacteria constitute a new type of ketose-bisphosphate aldolases

The *gatY* and *kbaY* genes from various bacteria were sequenced and compared (Fig. 1). Based on these alignments, the GatY sequences from *E. coli* strains K-12 and EC3132 are identical and closely related to the GatY from *Salmonella enterica* serovar typhi (and the identical serovar typhimurium), and from *Klebsiella oxytoca* strain M5a1 (65.3% and 64.2% identical residues, respectively). A second group of highly related proteins comprises the KbaY aldolases from *E. coli* strains K-12 and C (1 exchange), one putative aldolase from *Vibrio furnissii* involved in amino sugar metabolism (Bouma and Roseman 1996; Charbit and Autret 1998), and a protein from *Streptomyces coelicolor* (Redenbach et al. 1996). This group shares ca. 45% identical residues with the GatY group, and the different Y peptides have almost identical length (282–287 residues).

Besides their high similarity which identifies the GatY and KbaY aldolases as members of one group, these enzymes share a second very characteristic property. As will be shown in the next section, all require for full activity and good stability the presence of a second protein, i.e., GatZ for the GatY and KbaZ for the KbaY aldolases. The Z-type proteins originating from *E. coli, S. enterica, K. oxytoca*, and *S. coelicolor* as well as from orthologous and paralogous genes of these organisms are remarkably similar in size and sequence (Fig. 2).

Peptides Y and Z belong to the new type of aldolases

It has been speculated that proteins GatZ and KbaZ (alternative name AgaZ) correspond to phosphotagatose kinases (Reizer et al. 1996). However, we have shown for *E. coli* (Brinkkötter et al. 2000; Lengeler 1977), and others for *K. oxytoca* (Markwell and Anderson 1981; Markwell et al. 1976), that only the phosphofructokinase I (PfkA) is required for growth on galactitol, *N*-acetyl-galactosamine and D-galactosamine. Z-type proteins neither resembled phosphoketo-kinases nor any other protein in the gene databases (Daldal 1983). Their unusually high conservation, however, hinted at an essential role of these proteins.

Cell extracts from strain LAB89 lacking the *gatYZ* genes and *kbaYZ* expression had no detectable TagBP aldolase activity. This contrasts with derivatives expressing from plasmids either all *gat* genes (pBNL6) or the *gatYZ* genes alone (pLAB30) which had very high aldolase activities (100% and 108%, respectively; Table 4). Derivatives expressing either *gatY* (pLAB10) or *kbaY* (pKBA400) alone had lower and temperature-labile activities (11% and 10%, respectively), while derivatives expressing *gatZ* (pLAB22) or *kbaZ* (pLAB35) alone had no detectable activity. In apparent agreement with these results, it was observed that strain JWL150 with the Gat(Ts) phenotype retained this phenotype after transformation with pLAB10 or pKBA400, but grew rapidly on galactitol at 42 °C after transformation with pBNL6 or pLAB30. Suppression of a temperature-sensitive phenotype through overexpression of the temperature-labile enzyme has been described repeatedly. Surprisingly, however, strain JWL150 transformed with *gatZ* (pLAB22) or even with *kbaZ* (pLAB35) alone also grew rapidly on galactitol at 42 °C (Table 4). The corresponding cell extracts showed high and heat-stable TagBP aldolase activities which resembled the activities found in strains LAB89/pBNL6 and LAB89/pLAB30 (Table 4). Due to plasmid instability, the genes *kbaY* and *kbaZ* could only be expressed together in

Table 4 Specific D-tagatose 1,6-bisphosphate aldolase activities expressed in nmol min^{-1} (mg protein)$^{-1}$ in cell extracts of mutant LAB89 during the exponential growth phase on Luria Broth at 30 °C. LAB89 lacked the *gat* and *kbaYZ* genes, and expressed the subunits indicated from plasmids. Cells of mutant JWL150, which did not express the *kbaYZ* genes but were *gat*$^+$, and expressed from the same plasmids the subunits indicated, were tested for growth on galactitol at 28 ° and 42 °C. +Growth, – no growth

Plasmid	Subunit	Growth on galactitol		TagBP aldolase activity	
		28 °	42 °		
–	–	+	–	≤1	
pBNL6	GatYZABCDR'	+	+	115	±5
pLAB10	GatY	+	(+)	13	±2
pLAB22	GatZ	+	+	≤1	
pLAB30	GatYZ	+	+	124	±6
pKBA400	KbaY	+	(+)	11	±2
pLAB35	KbaZ	+	+	≤1	

Table 5 Specific activities, temperature stabilities, and apparent K_m values (in mM) of D-tagatose 1,6-bisphosphate (*TagBP*) aldolase activities in cell extracts of strain LAB89 (Δ*gat kbaZ*::*kan*) with various plasmids expressing the subunits indicated during the exponential growth phase on Luria Broth at 30 °C. $T_{1/2}$ Temperature at which 50% activity was lost during a 10 min incubation. Enzyme activities from the crude cell extracts expressed in nmol min^{-1} (mg protein)$^{-1}$, using total protein concentrations, were used to estimate the apparent K_m values for TagBP

Plasmid	Enzyme or peptide	$T_{1/2}$	Apparent K_m (mM) for Tag BP	Activity	%
–	–	–	–	<1	–
pLAB47	GatY	44 °C	1.0	12.7±2	22.0
pLAB22	GatZ	–	–	<1	–
pLAB47+pLAB22	GatY GatZ	44 °C	0.3	58±5	100.0
pLAB47+pLAB35	GatY KbaZ	80 °C	0.2	47.3±4	82.0
pKBA403	KbaY	44 °C	1.2	6.7±2	29.0
pLAB35	KbaZ	–	–	<1	–
pKBA403+pLAB35	KbaY KbaZ	80 °C	0.5	23.3±3	100.0
pKBA403+pLAB22	KbaY GatZ	44 °C	0.6	10±2	43.0

strain LAB89 when located on different plasmids. Such extracts also showed good though varying activities (data not shown, and Table 5).

From these results we concluded that GatY and KbaY were the catalytic subunits of two aldolases which required in vivo, but especially in vitro a second protein or subunit, GatZ and KbaZ, respectively, for full activity and stability. Of the two aldolases, the KbaYZ pair appeared to be more stable thus allowing growth on galactitol (strain JWL153) and *N*-acetyl-galactosamine (strain LAB30) at 42 °C (Table 3). Because the corresponding peptide pairs resembled each other closely (Figs. 1, 2) and apparently could be used in vivo as substitutes, we expressed them alone and in cross-wise pairs in the Δ*gat kbaZ*::Tn*10* mutant LAB89 (Table 5). The cell extracts were then tested for aldolase activity and heat stability. As before, cells containing only GatZ or KbaZ did not have activity, cells expressing GatY or KbaY had intermediate activities (22% and 29% activities, respectively), while cells expressing the homologous or heterologous pairs had always the highest activities (100%). In addition, the two pairs containing KbaZ showed an increase in temperature stability, from $T_{1/2}$=44 °C to $T_{1/2}$=80 °C. Thus, the presence of the Z-peptides, and in particular of KbaZ, increased the aldolase activity and temperature stability of the Y subunits.

Attempts to isolate a TagBP aldolase activity from extracts of strain JWL150 grown on galactitol at 30 °C (our unpublished results) and from a typical *E. coli* K-12 strain (Eyrisch et al. 1993) showed a severe loss of activity during each of the purification steps. These authors consequently used strain JWL183, an isogenic derivative of our temperature-resistant mutant JWL153, to isolate an aldolase. This purified enzyme has an at least five-fold increase in thermal stability compared to the wild-type enzyme. It was shown to be a Zn^{2+}-requiring class II aldolase with a high activity for TagBP [apparent K_m ~0.33 mM; V_{max} 7.5 μmol min^{-1} (mg protein)$^{-1}$] and a low activity (ca. 1%) for FruBP. This apparent K_m value is close to the value observed by us for the various YZ protein pairs in crude cell extracts, and three times lower than the values for the two Y peptides expressed alone (Table 5). Finally, all our preparations had a high aldolase activity for TagBP (100%) and a low one (≤4%) for FruBP (data not shown).

Discussion

E. coli contains a class I aldolase FbaB (gene *fbaB* or *dhnA*) (Stribling and Perham 1973; Thomson et al. 1998) and a typical class II aldolase FbaA (gene *fbaA*) which is active as a homodimer (M_r 78,980) and has a low activity for ketose phosphates and bisphosphates other than fructose BP (Stribling and Perham 1973). Based on our data, *E. coli* contains two additional aldolases which are involved in the metabolism of galactitol, *N*-acetyl-galactosamine and D-galactosamine (Brinkkötter et al. 2000; Lengeler 1977; Nobelmann and Lengeler 1996). Mechanistically these new aldolases belong to the class II aldolases, i.e., the purified enzymes from *E. coli* strain JWL183 (isogenic to JWL153) (Eyrisch et al. 1993) and *K. oxytoca* strain PRL-R3 (Anderson and Markwell 1982) are EDTA-sensitive and BH_4^--resistant. In contrast to, e.g., FbaA they require, besides a catalytic subunit (peptides GatY and KbaY), a second peptide (GatZ and KbaZ) for full activity and stability. Although the Y catalytic subunits are shorter than, e.g., FbaA (285 instead of 358 residues), they comprise most or all essential residues as identified from crystal structure and localized mutagenesis studies of FbaA (Packmann and Berry 1995; Plater et al. 1999; Stribling and Perham 1973). These are, in particular, D109, D144, N286, D329 and R331 (Fig. 1; numbering as in FbaA) which are involved directly or indirectly in catalysis and substrate binding; H110, H226, and H264 involved in Zn^{2+} binding; as well as A24, N35, and D290 (which may be an E) with unknown functions. Thus, of the 62 residues conserved within the eight TagBP aldolase sequences, 36 residues, or 59%, are also found in FbaA. The alignment predicts the existence of four linkers in FbaA (residues 1–23, 111–136, 230–256, 299–324) whose increased lengths compared to the GatY and KbaY aldolases fully account for the 73 additional residues found in FbaA.

Compared to the Y subunits (62 identical residues or 21.7% for the eight sequences shown in Fig. 1) the conservation of the Z subunits (164 identical residues or 38.5% for the seven sequences shown in Fig. 2) is very high considering the phylogenetic position of the host bacteria. In contrast, the aldolases involved in galactose/tagatose metabolism of gram-positive bacteria (Rosey and Stewart 1992; Van Rooijen et al. 1991) are class I ketose-BP aldolases (homo-tetramers; M_r 148,000) with a high activity for TagBP (100%) and FruBP (47%) (Anderson and Bissett 1982; Bissett and Anderson 1980), but no significant similarity at the amino acid level to the new aldolases.

Class II TagBP aldolases have been purified from *E. coli* strain JWL183 and *K.oxytoca* strain PRL-R3. Although they should resemble each other closely, their published properties deviate rather drastically. Thus, the heat-stable enzyme from *K. oxytoca* was claimed to be a "homodimer" with an M_r of 157,000 for the complex as estimated by Sephadex G150 chromatography (Anderson and Markwell 1982). Based on the physiological data of Markwell et al. (1976), only GatYZ is expressed in that organism, during growth at 30 °C on galactitol, the conditions used during growth of the producer organism (Anderson and Markwell 1982). This result agrees with our sequencing results which showed that *K. oxytoca* lacks genes *kbaY* and *kbaZ* (our unpublished results). As a putative two-fold heterodimer ($GatY_2$ $GatZ_2$), its calculated M_r would be $(31{,}350+46{,}200)_2$ or 155,100, close to the observed value. This aldolase has a high specificity for TagBP (K_m 0.4 mM; 100% activity), and a low one for FruBP (K_m 0.9 mM; 4% activity). In contrast, the enzyme isolated from JWL183 allegedly is a "homotetramer" with an M_r of 110,000 and high specificity for TagBP

(K_m 0.3 mM; 100% activity) compared to FruBP (K_m 0.5 mM; 1% activity) (Eyrisch et al. 1993). Unfortunately, strain JWL183 expressed during growth at 42 °C on galactitol, i.e., the growth conditions used for production, both GatYZ and KbaYZ (Tables 2, 3) and furthermore seemed to form mixed enzymes (i.e., GatY/KbaZ or KbaY/GatZ) under these conditions (Tables 4, 5). The increased temperature stability of the purified enzyme, which contrasts with the inherent lability of the GatYZ complex, and the deviations in the observed (M_r 110,000) compared to the calculated (M_r 125,400) molecular mass for a GatY homotetramer indicate perhaps that either KbaY and/or (fractions of) mixed heterodimers have been purified by Eyrisch et al. (1993). Our data support the hypothesis that the original Gat(Ts) phenotype of *E. coli* was caused primarily by the lability of the GatYZ aldolase (Lengeler 1977) while all mutants, e.g. strains JWL153 (JWL183) and LAB204 (Table 3) which comprise the heat-stable KbaYZ aldolase, showed a Gat(Tr) phenotype.

It remains to be shown why these TagBP aldolases require a second subunit Z for full activity and stability when the catalytic proteins Y closely resemble the FbaA-type aldolases, and when the low-GC gram-positive bacteria rely on simple class I aldolases for the same function. In this context it is perhaps interesting to point out that cells of *E. coli* strain K-12 grown under acid stress (Blankenhorn et al. 1999) or overproducing foreign proteins show increased amounts of GatY, which is frequently found in inclusion bodies (our unpublished results). In a recent study (Houry et al. 1999) it was shown that GatY belongs to a structurally unstable class of proteins that require GroEL for conformational maintenance, especially under the conditions of heat stress. These proteins consist preferentially of several domains with αβ-folds and extensive hydrophobic surfaces, and they are predicted to fold slowly and to be prone to aggregation. Upon immunoprecipitation with GroEL-specific antibodies, GatY co-precipitates with GatD, also containing the αβ-domain structure, and with GatZ. Because GatZ lacks the required αβ-domain structure, it seems merely to co-precipitate with GatY in the form of stable GatY/GatZ complexes. Consequently, GatZ (and in analogy the other Z proteins) could have a chaperone-like function for the proper and stable folding of GatY, and when titrated out, would leave free labile GatY to be incorporated into inclusion bodies. To date, no direct experimental support can be given for this hypothesis, except for the fact that Y subunits synthesized separately in cells and mixed only after cell lysis with Z proteins retained low and labile activities (our unpublished results). This contrasts with the stabilizing effect GatZ had when synthesized simultaneously with GatY in one cell (Tables 4, 5).

Acknowledgements We would like to thank C.-A. Alpert, Paris, for help during the initial phase of this work; W.-D. Fessner for the gift of D-tagatose 1,6-bisphosphate; K. Schmid, Osnabrück, for critical reading of the manuscript; and E. Placke for help in preparing the manuscript. We also would like to thank the Deutsche Forschungsgemeinschaft for grants given to A. Brinkkötter and A. Shakeri-Garakani through the Graduiertenkolleg "Molekulare Zellbiologie mit Schwerpunkt Membranbiologie".

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