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Bacterial *abl*-like genes: production of the archaeal osmolyte $N^{\varepsilon}-$acetyl$-\beta-$lysine by homologous overexpression of the *yodP–kamA* genes in *Bacillus subtilis*

Stefan Müller · Tamara Hoffmann · Helena Santos · Stephan H. Saum · Erhard Bremer · Volker Müller

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Abstract $N^{\varepsilon}-$acetyl$-\beta-$lysine is an archaeal compatible solute whose synthesis is mediated by the sequential reactions of the lysine-2,3-aminomutase AblA and the acetyltransferase AblB. α-Lysine serves as the precursor and is converted by AblA to β-lysine, and AblB then acetylates this intermediate to $N^{\varepsilon}-$acetyl$-\beta-$lysine. The biochemical and biophysical properties of $N^{\varepsilon}-$acetyl$-\beta-$lysine have so far not been studied intensively due to restrictions in the supply of this compound. A search for *ablAB*-like genes in the genomes of members of the family *Bacillaceae* revealed the *yodP–kamA* genes that encode a AblA-related lysine-2,3-aminomutase and AblB-related putative acetyltransferase. In *Bacillus subtilis*, the *yodP–kamA* genes are part of a transcriptional unit (*yodT–yodS–yodR–yodQ–yodP–kamA*) whose expression is upregulated during sporulation and controlled by the mother-cell-specific transcription factor SigE. $N^{\varepsilon}-$acetyl$-\beta-$lysine was not detectable in vegetatively growing or osmotically stressed *B. subtilis* cells, and the deletion of the *yodT–yodS–yodR–yodQ–yodP–kamA* region had no noticeable effects on growth in rich or minimal media or osmotic stress resistance. However, when we expressed the *yodP–kamA* genes outside their natural genetic context from an isopropyl β-D-1-thiogalactopyranoside-inducible promoter on a plasmid in *B. subtilis*, the recombinant strain synthesized considerable amounts (0.28 μmol/mg protein) of $N^{\varepsilon}-$acetyl$-\beta-$lysine. The data reported here thus open the bottleneck for the large-scale production of $N^{\varepsilon}-$acetyl$-\beta-$lysine to investigate its properties as a compatible solute.

Keywords Archaea · Methanogens · Bacteria · Salt adaptation · Compatible solutes · Lysine-2,3-aminomutase

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S. Müller · S. H. Saum · V. Müller (✉)
Molecular Microbiology & Bioenergetics,
Institute of Molecular Biosciences, Goethe University,
Max-von-Laue-Str. 9,
60438 Frankfurt am Main, Germany
e-mail: vmueller@bio.uni-frankfurt.de

T. Hoffmann · E. Bremer
Laboratorium für Mikrobiologie, Philipps Universität Marburg,
Karl-von-Frisch Str. 8,
35043 Marburg, Germany

H. Santos
Instituto de Tecnologia Química e Biológica,
Universida de Nova de Lisboa,
2780-156 Oeiras, Portugal

Present Address:
S. H. Saum
Department of Microbiology and Molecular Genetics,
Harvard Medical School,
200 Longwood Avenue,
Boston, MA 02115, USA

Introduction

Microorganisms frequently have to cope with changing environmental conditions in their natural habitats. If no active counter measures would be taken, hypoosmotic as well as hyperosmotic conditions would inevitably lead to growth arrest and eventually to death of the bacterial cell (Csonka 1989; Kempf and Bremer 1998; Poolman and Glaasker 1998; Wood 1999; Roeßler and Müller 2001). To adapt to high osmotic conditions, most microorganisms utilize a "salt-out" cytoplasm strategy. It is widely used since it does not require the adaptation of the intracellular enzyme machinery to high ionic strength conditions. Instead of ions, organic osmolytes that do not interfere

with the cell physiology, the compatible solutes, are preferentially amassed (Galinski and Trüper 1994).

The nature of the compatible solutes accumulated by eukarya, bacteria, and archaea, their biosynthesis and uptake under hyperosmotic conditions, and their expulsion under hypoosmotic circumstances via mechanosensitive channels are actively studied. The pertinent findings have been the subject of a number of excellent reviews (Kempf and Bremer 1998; Roeßler and Müller 2001; Hohmann 2002; Saito and Tatebayashi 2004; Sheikh-Hamad and Gustin 2004; Grant 2004; Ziegler et al. 2010; Pflüger et al. 2005; Spanheimer and Müller 2008; Burg and Ferraris 2008; Hagemann 2011). The spectrum of compatible solutes used in nature comprises only a limited number of compounds, and these can be principally divided into two major groups: (1) sugars, polyols, and their derivatives and (2) α- and β-amino acids and their derivatives, including methylamines. The limited number of compounds used as biocompatible solutes reflects the biophysical constraints imposed on these compounds to be congruous with macromolecular and cellular functions (Le Rudulier et al. 1984; Street et al. 2006).

When the compatible solutes accumulated by archaea and bacteria are compared, important differences become apparent. Most archaeal compatible solutes resemble in their chemical structure their bacterial counterparts, with the striking difference that the majority of them carry a negative charge (Martin et al. 1999; Roeßler and Müller 2001). In addition, some compatible solutes are unique to bacteria and archaea. One example is the archaeal osmolyte $N^{\varepsilon}-$acetyl$-\beta-$lysine.

So far, the occurrence of $N^{\varepsilon}-$acetyl$-\beta-$lysine is restricted to methanogenic archaea and is synthesized by every methanogen investigated so far for compatible solute production (Sowers et al. 1990; Lai et al. 1991; Robertson et al. 1992; Roberts et al. 1992; Sowers and Gunsalus 1995; Pflüger et al. 2003; Spanheimer and Müller 2008).

Biosynthesis of $N^{\varepsilon}-$acetyl$-\beta-$lysine starts with α-lysine that is first converted to β-lysine by a lysine-2,3-aminomutase (AblA), and this intermediate is then acetylated to $N^{\varepsilon}-$acetyl$-\beta-$lysine by the action of an acetyltransferase (AblB) (Fig. 1) (Roberts et al. 1992). In *Methanosarcina mazei* Gö1, the *ablA* and *ablB* genes constitute an operon whose expression is drastically increased under high salinity growth conditions, indicating that $N^{\varepsilon}-$acetyl$-\beta-$lysine production significantly contributes to salt adaptation of the cells (Spanheimer and Müller 2008). Indeed, a *Δabl::pac* mutant of the metabolically restricted, hydrogenotrophic methanogen *Methanococcus maripaludis* JJ was no longer able to synthesize $N^{\varepsilon}-$acetyl$-\beta-$lysine and exhibited a salt-sensitive growth phenotype (Pflüger et al. 2003). Likewise, a *Δabl::pac* mutant in the well-studied model methanogen *M. mazei* Gö1 also no longer produced $N^{\varepsilon}-$acetyl$-\beta-$lysine but, surprisingly, was still able to grow in high salt media. However, it was found that the loss of $N^{\varepsilon}-$acetyl$-\beta-$lysine was compensated for by the osmotically stressed cells by increased glutamate production. In addition, a novel solute, alanine, was synthesized (Saum et al. 2009).

In addition to their well-studied function as water-attracting osmolytes, compatible solutes possess stabilizing properties for proteins, membranes, and even entire cells, and they also effect melting of double-stranded nucleic acids. These features have attracted interest to use these compounds biotechnologically, medically, and in consumer health care products (Lentzen and Schwarz 2006). There is an increasing interest to explore commercial applications of different types of compatible solutes (Vyrides et al. 2010). The biotechnological potential of $N^{\varepsilon}-$acetyl$-\beta-$lysine has so far not been explored. This is primarily due to the laborious cultivation of methanogens that require anaerobic growth conditions and their low growth yields. Heterologous, functional expression of the *ablAB* genes from *M. mazei* Gö1 in genetically readily tractable *Escherichia coli* and *B. subtilis* host strains has failed so far (V. Müller, unpublished data). Interestingly, homologues of the structural genes of the archaeal AblA and AblB proteins are present in various members of the Bacteria, but their physiological function has not yet been elucidated. Here, we show that the homologous overexpression of the *ablAB*-related genes *kamA* and *yodP* from *B. subtilis* lead to the synthesis of the archaeal compatible solute $N^{\varepsilon}-$acetyl$-\beta-$lysine in *B. subtilis*. Our findings open a possible route to produce $N^{\varepsilon}-$acetyl$-\beta-$lysine in a well-studied and easy to grow and manipulate industrial workhorse.

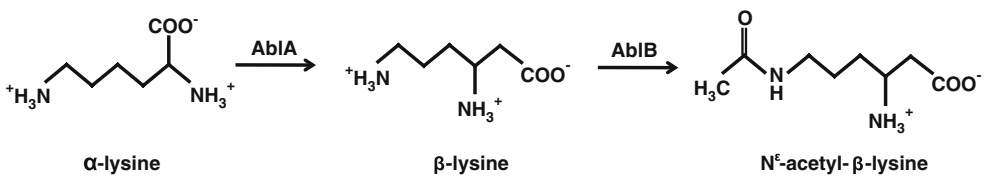

Fig. 1 Pathway of N^{ε}-acetyl-β-lysine synthesis in methanogenic archaea. α-Lysine is converted to β-lysine by a lysine-2.3-aminomutase (AblA) that is subsequently acetylated to $N^{\varepsilon}-$acetyl$-\beta-$lysine by action of an acetyltransferase (AblB)

Materials and methods

Media and growth conditions The various *B. subtilis* strains were grown either in complex medium [Luria–Bertani (LB)] or in Spizizens' minimal medium (SMM) with 0.5% (*w*/*v*) glucose as the carbon source and a solution of trace elements (Harwood and Archibald 1990). The salinities of growth media were adjusted with NaCl as required. For derivates of the *B. subtilis* strain JH642 (*trpC2* and *pheA1*) (strain 1A96, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA), SMM was supplemented with L-tryptophan (20 mg/l) and L-phenylalanine (18 mg/ml); growth of *B. subtilis* 168 (*trpC2*) (strain 1A1, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA) required the addition of L-tryptophan (20 mg/l) to SMM. Cells were grown aerobically at 37°C in 100-ml Erlenmeyer flasks with a culture volume of 50 ml in a shaker set at 125 rpm. The growth of the bacterial cultures was monitored spectrophotometrically at a wavelength of 578 nm (OD_{578}). To select for the presence of plasmids pDG148 (kindly provided by P. Stragier) and its recombinant derivative pBS02 (*kamA*$^+$–*yodP*$^+$) in *B. subtilis* strains, kanamycin was added to the growth medium at a final concentration of 10 μg/ml. In order to induce the expression of the cloned *kamA*$^+$–*yodP*$^+$ genes in *B. subtilis* under the control of the *spac* promoter, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures of the *B. subtilis* 168 (pBS02) strain at a final concentration of 1 mM. To determine the sporulation frequency of the *B. subtilis* wild-type strain JH642 and its derivative TMB121 [Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1], cells were cultured in Difco sporulation medium (DSM) for 30 h. Serial dilutions of the cells were then plated on DSM solid medium to determine the total viable cell numbers; agar plates were incubated at 37°C. Duplicate cell samples were heated to 80°C for 30 min, and appropriate dilutions were plated on DSM agar plates to enumerate the spore count. Sporulation efficiency was recorded as the percentage of the colony count from the 80°C treated cell samples relative to the total viable cell number.

Bacterial strains and construction of B. subtilis mutants The *B. subtilis* strains used in this study, for the mutational analysis of the *kamA* and *yodP* chromosomal region, are all derivatives of strain JH642 (*trpC2 pheA1*). The following JH642-derived mutant strains were used: (1) strain JSB8 [Δ(*proHJ::tet*)1] (Brill et al. 2011), (2) TMB121 [Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1] (this study), and (3) TMB124 [Δ(*proHJ::tet*)1 Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1] (this study). To delete the *yodT–yodS–yodR–yodQ–yodP–kamA–yokU* region from the *B. subtilis* chromosome of strain JH642, flanking regions of this genomic segment were amplified by PCR and joined by PCR (Kuwayama et al. 2002) to the DNA sequence of a spectinomycine resistance cassette (amplified from plasmid pDG1726) (Guerout-Fleury et al. 1995). The resulting PCR product was then transformed into competent cells of strain JH642 by selecting for spectimomycine resistant colonies (100 μg/ml). The integration of the [Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1] deletion mutation via a double cross-over event into the *B. subtilis* chromosome was verified by PCR. This genetic construction resulted in strain TMB121. Chromosomal DNA of strain TMB121 was then prepared and used to transform the [Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1] deletion mutation into strain JSB8 [Δ(*proHJ::tet*)1], a JH642-derived *B. subtilis* strain that is defective in the osmoadaptive synthesis of the compatible solute proline (Brill et al. 2011). The resulting double mutant strain was TMB124. The *B. subtilis* 168 (*trpC2*) strain carrying the *yodP*$^+$–*kamA*$^+$ expression plasmid pBS02 (Kan^R) was used for the production of $N^{\varepsilon}-$acetyl$-\beta-$lysine. For general cloning procedures, the *E. coli* strain DH5α was used. In order to obtain non-methylated DNA of plasmid pDG148 for cloning procedures, the *E. coli* strain JM110 was employed. The strains used in this study are summarized in Table 1.

Construction of the plasmid pBS02 To generate an *yodP*$^+$–*kamA*$^+$ overexpression plasmid, the *yodP* and *kamA* genes were amplified by PCR from the genomic DNA from *B. subtilis* 168 using the oligonucleotides Bs_*abl*_for (5′-GCT

Table 1 Strains used in this study

B. subtilis strains	Description or genotype	Source or reference
JH642	*trpC*2 *pheA*1	BGSC 1A96
168	*trpC*2	BGSC 1A1
JSB8	JH642 (Δ*proHJ*::*tet*)1	Brill et al. 2011
TMB121	JH642 Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1	This study
TMB124	JH642 (Δ*proHJ*::*tet*)1 Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1	This study

tet Tetracycline resistance; *spec* spectinomycin resistance; *BGSC Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH, USA

CTA GAA TGC TCA AGT CAA TAA AGA GTA G-3′) and Bs_*abl*_rev (5′-ACG CGT CGA CTC ATG AAG AAT CCC CTC CGC-3′). The resulting 2,290 bp PCR fragment was cleaved with *Xba*I–*Sal*I and cloned into the vector pUC19, thereby yielding plasmid pBS01. This genomic segment was subsequently recovered from plasmid pBS01 by restriction digestion and inserted into the multiple cloning site of plasmid pDG148, a vector that can replicate both in *E. coli* and in *B. subtilis*, in such a way that the expression of the *yodP*$^{+}$–*kamA*$^{+}$ genes can be triggered from the IPTG-inducible *spac* promoter present on plasmid pDG148. This construction yielded plasmid pBS02.

NMR analyses The pool of compatible solutes was analyzed by nuclear magnetic resonance spectroscopy. Cells were grown aerobically at 37°C in 1-l Erlenmeyer flasks with a culture volume of 200 ml in a shaker set at 125 rpm. The growth of the bacterial cultures was monitored spectrophotometrically at a wavelength of 578 nm (OD_{578}). Cells were harvested either in exponential growth phase (OD_{578} of about 0.8) for studying different stress factors or in stationary phase when heterologously overproducing N^{ε}-acetyl-β-lysine (OD_{578} of about 2.5–3.0). Subsequently, cells were lyophilized, and the concentration of intracellular solutes was measured.

Quantification of intracellular solutes The pool of compatible solutes was analyzed by nuclear magnetic resonance spectroscopy (NMR). The extraction and quantification of solutes was performed according to previously established protocols (Santos et al. 2006). The assignment of resonances was done by comparison with literature data and our previous work (Sowers et al. 1990; Saum et al. 2009).

Analytical methods High-performance liquid chromatography (HPLC) analysis was performed as previously described by (Saum et al. 2006).

Results

abl-Related genes in Bacteria Aminomutases and acetyl transferases are common to the metabolism of many archaea and bacteria. Therefore, it was not surprising that database searches revealed the widespread occurrence of *ablA*- or *ablB*-related genes in the genomes of many microbes from different phylogenetic groups when the amino acid sequences of the AblA and AblB proteins from *M. mazei* Gö1 were used as search templates. We focused our analysis on gene clusters that possess *ablA*- and *ablB*-like genes positioned adjacent to each other, a genetic configuration found in methanogenic archaea (Fig. 2)

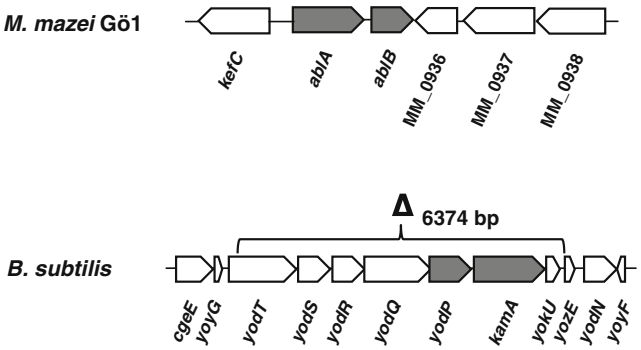

Fig. 2 Genomic contexct of the *ablA-ablB* region in the *M. mazei* Gö1 genome and the *yodP-kamA* region in the *B. subtilis* 168 and JH642 genomes. The genetic organization of the genes coding for the lysine-2,3-aminomutase and the acetyltransferase (marked in *gray*) and their flanking regions are shown. The location of the deletion introduced into the *B. subtilis* genome is indicated. This region was replaced by a spectinomycin resistance cassette in strain TMB121. Flanking genes in *M. mazei* Gö1 are functionally not related to the *ablA-ablB* operon and are transcribed in the opposite direction. Their tentative assigned functions are: *kefC*, glutathione-regulated potassium-efflux system protein; MM_0936, hypothetical protein MM_0936; MM0937, putative flagellar related protein J; MM_0938, putative flagellar related protein *Fla*I

(Pflüger et al. 2003), to make our database searches more stringent for the discovery of proteins that might be involved in the production of $N^{\varepsilon}-$acetyl$-\beta-$lysine. Interestingly, the Gram-positive soil bacterium *B. subtilis* strain 168 as well as strain JH642 (both members of the 168 group) turned out to possess a homologue of AblA, KamA, and a protein related to AblB, YodP, whose structural genes are located next to each other on the genome (Fig. 2). The KamA protein is also known as YodO, a biochemically characterized L-lysine-2,3-aminomutase, which has been previously shown to convert L-lysine into beta-lysine in the presence of *S*-adenosylmethionine and sodium dithionite.

The gene located next to *kamA* on the *B. subtilis* genome is *yodP* (Fig. 2). The encoded protein (YodP) is 34% identical to AblB from *M. mazei* Gö1 and other GNAT-type (GCN-5- related *N*-acetyltransferase) acetyltransferases (Suppl. Table 1), indicating that it might function as an acetyltransferase. Enzymes belonging to this superfamily contain in their primary structure four poorly conserved motifs (A–D). The best conserved one is motif A, which contributes most of the contacts to the acetyl-group donor acetyl-CoA with the sequence Q/R-x-H/K-G-x-G/A-K/R (Dyda et al. 2000). The YodP protein possesses a related motif, R–G–H–S–L–T–S. Hence, the YodP enzyme could thus potentially acetylate beta-lysine, the reaction product of the KamA enzyme (Chen et al. 2000), and thereby form $N^{\varepsilon}-$acetyl$-\beta-$lysine (Fig. 1).

Genomic context of the yodP–kamA genes in B. subtilis The genomic context of the *yodP–kamA* genes in the *B. subtilis*

chromosome are shown in Fig. 2. Previous transcriptional profiling studies have demonstrated that the *yodT–yodS–yodR–yodQ–yodP–kamA* genes apparently form a transcriptional unit since the expression of all these genes is upregulated during sporulation (Fawcett et al. 2000; Steil et al. 2005). Transcription of this entire gene cluster is under the genetic control of SigE, a mother-cell-specific alternative sigma factor (Errington 2003), and a SigE-dependent promoter was detected by a bioinformatic approach upstream of *yodT* (Steil et al. 2005). The putative products of this gene cluster are currently annotated in the UniProt database as an aminotransferase (YodT), as a CoA-transferase (alpha subunit) (YodS), as a CoA-transferase (beta subunit) (YodR), as a metallohydrolase/deacylase (YodQ), as an *N*-acetlytranferase (YodP), and as a L-lysine 2,3-aminomutase (KamA). Collectively, these annotations suggest that these proteins have a role in L-lysine metabolism and $N^{\varepsilon}-$acetyl$-\beta-$lysine formation.

A genetic organization identical to that of *B. subtilis* is found in the genomes of other bacilli, such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus megaterium*. In the genome sequences of the pathogens *Bacillus weihenstephanensis*, *Bacillus cereus*, *Bacillus pseudomycoides*, and the marine *Bacillus* sp. NRRL B-14911, a gene encoding a putative regulatory protein containing a PAS domain, separates the *yodP* and *kamA* genes. This protein is annotated as an L-lysine aminomutase regulator with similarities to the RocR activator, a regulatory protein that is involved in the control of arginine utilization genes in *B. subtilis* through interactions with a Sigma-54 transcription factor (Gardan et al. 1997).

Possible functions of the YodP and KamA proteins in osmotic stress adaptation of B. subtilis strain JH642 The genomic context of the *yodP–kamA* genes in the *B. subtilis* genome (Fig. 2) might indicate a role in L-lysine degradation/metabolism and hence $N^{\varepsilon}-$acetyl$-\beta-$lysine might be produced as an intermediate. However, the *B. subtilis* strain JH642 did not grow on L-lysine as the only carbon, nitrogen, or energy source in SMM. In contrast, *B. subtilis* was able to readily grow on arginine, ornithine, citrulline, glutamate, and proline when these compounds were provided as sole carbon and energy sources (final concentration of 50 mM). As expected, no $N^{\varepsilon}-$acetyl$-\beta-$lysine was produced in any of these cultures (data not shown). To address a possible physiological function of the *yodP–kamA* genes of *B. subtilis* further, we deleted a 6,374-bp genomic region encompassing the entire *yodT–yodS–yodR–yodQ–yodP–kamA* gene cluster and replaced it by a spectinomycin resistance cassette (Fig. 2). The resulting strain TMB121 had no noticeable growth defect either in rich (LB) or minimal (SMM) medium. Since the expression of the *yodT–yodS–yodR–yodQ–yodP–kamA* gene cluster is under sporulation control via the SigE transcription factor, we wondered whether the deletion of this operon would affect sporulation. We therefore determined the sporulation frequency of the wild-type strain JH642 and its derivative TMB121 [Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1] in DSM medium. Both strains sporulated with a frequency of 64%, demonstrating that the *yodT–yodS–yodR–yodQ–yodP–kamA* gene cluster is not critical for the sporulation of *B. subtilis*.

Since the sequential action of the YodP and KamA enzymes could possibly lead to the formation of the archaeal compatible solute $N^{\varepsilon}-$acetyl$-\beta-$lysine (Fig. 1), we explored a potential role of the *yodP–kamA* genes in the acclimatization of *B. subtilis* to a high salinity environment. To test whether *B. subtilis* produces $N^{\varepsilon}-$acetyl$-\beta-$lysine during vegetative growth under osmotic stress conditions, cells were grown in minimal medium at different salinities ranging from 0 to 1.2 M NaCl. Their solute pools were subsequently assessed by HPLC and NMR analysis. As observed before (Whatmore et al. 1990), an increase in the salinity of the growth medium triggered a raise in the internal glutamate and proline concentrations (data not shown), but no $N^{\varepsilon}-$acetyl$-\beta-$lysine was detected. This compound was also not produced by *B. subtilis* cells as a response to either cold or heat stress (16°C and 52°C, respectively).

We then tested if the Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1 mutation would influence either the growth properties of *B. subtilis* at high salinity or would influence the compatible solute pool of osmotically stressed cells. Growth of the deletion strain TMB121 was not impaired at high salinity since it grew with a comparable growth rate to its parent strain JH642 in SMM with 1 M NaCl. *B. subtilis* synthesizes large amounts of the compatible solute proline when it is exposed to high salinity (Whatmore et al. 1990). The Δ(*proHJ::tet*)1 mutation present in strain JSB8 abolishes the osmoadaptive proline biosynthesis and causes an osmotically sensitive growth phenotype in minimal media such as SMM (Brill et al. 2011). The introduction of the Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec*)1 deletion into the Δ(*proHJ::tet*)1 strain JSB8 did not further increase the osmotic sensitivity of this strain in SMM with 1.0 M NaCl (data not shown). We also did not detect an influence on the compatible solute pool of osmotically stressed cells of the Δ(*yodT–yodS–yodR–yodQ–yodP–kamA–yokU::spec)*1 deletion strain TMB121 relative to its parent (data not shown). We conclude from these combined data that $N^{\varepsilon}-$acetyl$-\beta-$lysine is not synthesized by *B. subtilis* as part of its cellular defense against high salinity.

Cloning and functional expression of yodP–kamA genes from B. subtilis The *yodP–kamA* genes were amplified by PCR from chromosomal DNA of the *B. subtilis* strain 168 and inserted into the expression vector pDG148 in such a way that their expression could be triggered from the IPTG-inducible *spac* promoter. The resulting plasmid pBS02 was transformed into the *B. subtilis* strain 168. Cells harboring pBS02 were grown in LB medium, *yodP–kamA* expression was induced by the addition of IPTG to the cultures, and the cells were harvested after 24 h of further growth. Their compatible solutes pools were then analyzed by HPLC. Only two peaks were observed (Fig. 3). The retention time of the first peak corresponded to that of glutamate, the dominant amino acid in the solute pool of *B. subtilis* (Whatmore et al. 1990). The second peak had the same retention time as $N^{\varepsilon}-$acetyl$-\beta-$lysine present in a solute extract prepared from *M. mazei* Gö1, suggesting that the cloned *yodP–kamA* genes under the control of the *spac* promoter were responsible for its production. Indeed, when *yodP–kamA* expression in the pBS02-carrying *B. subtilis* 168 strain was not induced with IPTG, glutamate was detected but the second solute, putatively $N^{\varepsilon}-$acetyl$-\beta-$lysine, was not detectable.

To confirm the identification of the second substance, NMR analyses were performed. The proton NMR spectrum of ethanolic cell extracts revealed the presence of the resonances characteristic of $N^{\varepsilon}-$acetyl$-\beta-$lysine, a solute

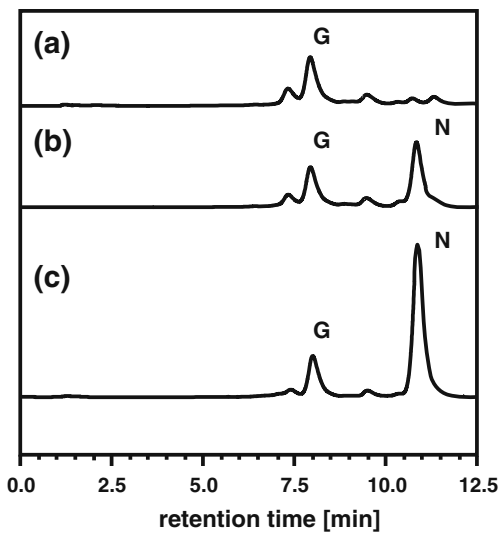

Fig. 3 HPLC profile of compatible solutes extracted from *B. subtilis* 168 [pBS02]. Compatible solutes were isolated from *B. subtilis* carrying the N^{ε}-acetyl-β-lysine synthesis genes *kamA* and *yodP* on the IPTG-inducible plasmid pBS02. Without induction (**a**), only glutamate (G) can be detected as a compatible solute, whereas upon addition of IPTG (**b**), a second compound can be found that co-elutes with a compound (N) that can also been found in *M. mazei* Gö1 (**c**) and which was identified to be N^{ε}-acetyl-β-lysine

earlier identified in several methanogens (Fig. 4) (Sowers et al. 1990).

Cells grown in LB medium in the absence of NaCl had a content of 0.13 μmol glutamate/mg protein, 0.17 μmol/mg protein glycine betaine (imported from components of the LB medium), and 0.28 μmol/mg protein $N^{\varepsilon}-$acetyl$-\beta-$lysine. Proline was not detectable under these conditions. Assuming an internal volume of 1 μl cell water/mg of protein this corresponds to an $N^{\varepsilon}-$acetyl$-\beta-$lysine concentration of 0.28 M. Hence, a *B. subtilis* strain overexpressing its own *yodP–kamA* genes from a multicopy number plasmid under the transcriptional control of a heterologous promoter (P*spac*) synthesizes considerable amounts of the osmolyte $N^{\varepsilon}-$acetyl$-\beta-$lysine.

Discussion

Spectroscopic analysis revealed that the YodO (KamA) protein contains an $[4Fe–4S]^{3+}$ cluster (Chen et al. 2000). Although the purified YodO protein has been carefully studied by Chen et al. (2000), the function of this enzyme in the context of the physiology of the *B. subtilis* cell has remained unexplored. The $KamA_{Bs}$ and $AblA_{Mm}$ proteins are 51% identical and 71% similar. The KamA aminomutase has conserved domains, such as an *S*-adenosylmethionine (SAM) binding-site and a cysteine-rich region, which is essential for the formation of three [4Fe–4S] clusters similar to those found in the $AblA_{Mm}$ protein. The $AblA_{Mm}$ possesses three cysteine residues near its C-terminus, and this domain might constitute a zinc-binding site and could therefore be critical for enzymatic activity (Pflüger et al. 2003). In contrast, the *B. subtilis* $AblA_{Mm}$ homologue KamA lacks these cysteine residues, and Chen et al. (2000) could not detect any zinc in the purified *B. subtilis* YodO (KamA) protein. These authors have suggested that the YodO protein of *B. subtilis* constitutes a novel type of lysine 2,3-aminomutase. The aminomutases-like KamA proteins from *Clostridium subterminale* SB4 are pyridoxal-5′-phosphate (PLP) dependent enzymes (Chirpich et al. 1970). $KamA_{Cs}$ possesses a PLP-binding motif (L–V–K–I–R–V), and this cofactor-binding motif can also be found in the KamA (YodO) protein from *B. subtilis* but is not present in the $AblA_{Mm}$ protein.

The previous biochemical characterization of the YodO (KamA) protein of *B. subtilis* (Chen et al. 2000), and our bioinformatic analysis suggests that the combined enzymatic reactions of the YodP and KamA (YodO) proteins should lead to the synthesis of $N^{\varepsilon}-$acetyl$-\beta-$lysine (Fig. 1). Indeed, the expression of the cloned *yodP–kamA* genes from a heterologous promoter (p*spac*) led to the synthesis of $N^{\varepsilon}-$acetyl$-\beta-$lysine in *B. subtilis*. This

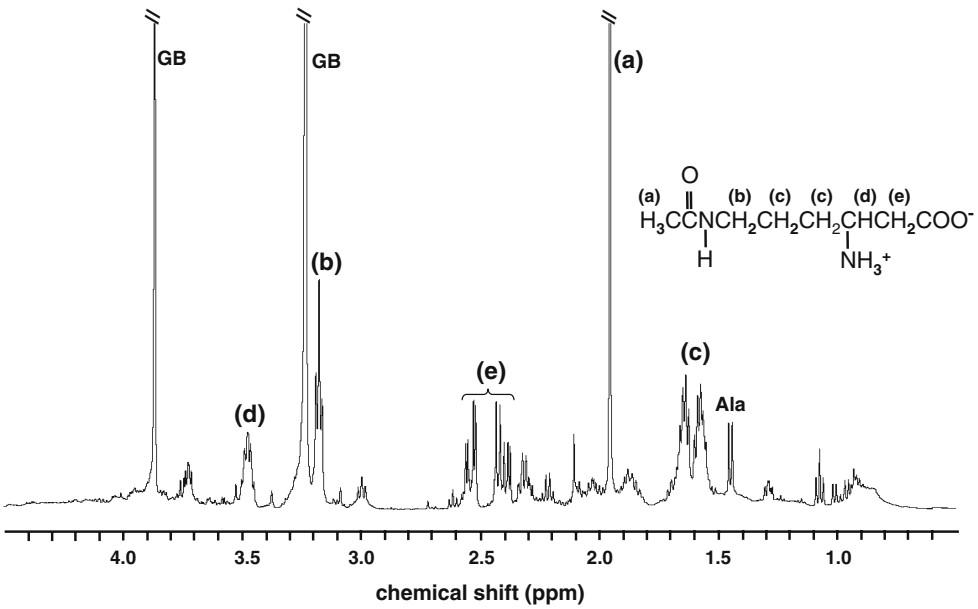

Fig. 4 ^{1}H-NMR (500 MHz) spectrum of an ethanolic extract of *Bacillus subtilis* 168 [pBS02] grown in complex medium with 0.3 M NaCl. Resonances of N^{ε}-acetyl-β-lysine are labeled with *a–e*; *GB* glycine-betaine; *Ala* methyl resonance of alanine

finding supports the functional annotation of the KamA protein as a L-lysine 2,3-aminomutase and of the YodP protein as a *N*-acetlytranferase whose joint enzymatic activities lead to the formation of $N^{\varepsilon}-$acetyl$-\beta-$lysine.

$N^{\varepsilon}-$acetyl$-\beta-$lysine is a compatible solute frequently synthesized by osmotically stressed methanogenic archaea (Roeßler and Müller 2001). However, we could not detect $N^{\varepsilon}-$acetyl$-\beta-$lysine in osmotically stressed *B. subtilis* cells, and the deletion of the YodP and KamA structural genes did not cause osmotic sensitivity or influenced the compatible solute pool of this soil bacterium. Hence, the production of $N^{\varepsilon}-$acetyl$-\beta-$lysine is clearly not an important facet of the cellular acclimatization reactions of *B. subtilis* to high osmolarity environments (Bremer 2002). Consistent with this conclusion is the observation that the expression of the *yodP–kamA* genes is not up-regulated in response to high salinity (Steil et al. 2003; Hahne et al. 2010). In contrast, the transcription of the *proHJ* operon, which encodes two enzymes for the production of the major compatible solute (proline) synthesized by *B. subtilis* (Whatmore et al. 1990), are strongly induced in high salinity challenged cells (Steil et al. 2003; Hahne et al. 2010). Likewise, in archaea that use $N^{\varepsilon}-$acetyl$-\beta-$lysine as an osmostress protectant, the expression of the *ablAB* operon is strongly upregulated in response to increases in the external salinity (Pflüger et al. 2003; Pflüger et al. 2007; Saum et al. 2009), highlighting the role of $N^{\varepsilon}-$acetyl$-\beta-$lysine as an osmostress protectant in these microbes.

The SigE-dependent upregulation of the *yodT–yodS–yodR–yodQ–yodP–kamA* gene cluster during sporulation (Fawcett et al. 2000; Steil et al. 2005) suggests that the encoded gene products play a so far not understood role in the mother cell during the sporulation process. However, this does not necessarily imply that $N^{\varepsilon}-$acetyl$-\beta-$lysine is produced as an end product in the mother cell or somehow incorporated into spores since it could be an intermediate in a sporulation-specific biosynthetic or degradative process.

As described in this communication, from the finding of *ablAB*-like genes in bacteria, it is dangerous to hypothesize that these bacteria produce $N^{\varepsilon}-$acetyl$-\beta-$lysine as a compatible solute. Very recently, the presence of *ablAB*-like genes was reported for some green sulfur bacteria and *B. cereus* CECT 148 (Triadó-Margarit et al. 2011) and $N^{\varepsilon}-$acetyl$-\beta-$lysine has been detected by NMR but its role in osmoadaptation remains to be confirmed.

Compatible solutes find increasing biotechnological applications (Roberts 2005; Pastor et al. 2010), but the protective properties of $N^{\varepsilon}-$acetyl$-\beta-$lysine for cells or macromolecules are largely unexplored. This is primarily due to the restrictions in the supply of this compound for bioassays due to the difficulties in the cultivation of natural $N^{\varepsilon}-$acetyl$-\beta-$lysine producers. We report here a way to open this bottleneck with a recombinant *B. subtilis* strain expressing the *yodP–kamA* genes independent of their natural genetic context from a plasmid-encoded synthetic promoter. Since *B. subtilis* can easily be cultivated and is amenable to sophisticated molecular and genetic techniques, our recombinant strain should become a good starting point for the large-scale production of $N^{\varepsilon}-$acetyl$-\beta-$lysine.

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