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The NAD-dependent ligase encoded by *yerG* is an essential gene of *Bacillus subtilis*

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ABSTRACT

DNA ligases are grouped into two families, ATP-dependent and NAD-dependent, according to the cofactor required for their activity. A surprising capability of both kinds of ligases to complement for one another *in vivo* has been observed. *Bacillus subtilis* harbours one NAD-dependent ligase, YerG, and two ATP-dependent ligases, YkoU and YoqV, this last one being encoded by the 134 kb lysogenic bacteriophage SPβ and consisting of a single adenylation domain typical of ATP-dependent ligases. Because the genetics of ligases in *B.subtilis* had not been studied previously, the genes encoding for one ligase of each kind, *yerG* and *yoqV*, were investigated. We found that the *yerG* gene was essential in *B.subtilis*. This suggests that none of the ATP-dependent ligases was able to complement the *yerG* defect. In addition, the ATP-dependent ligase encoded by *yoqV*, when cloned on a plasmid under appropriate expression signals, was unable to rescue a *yerG* mutant strain. The two *B.subtilis* ligase genes *yerG* and *yoqV* were also introduced in an *Escherichia coli* strain encoding a thermosensitive ligase (*ligts*), and whereas *yoqV* did not complement the *ligts* defects, *yerG* fully complemented the growth and UV sensitivity defects of the *lig* mutant. We propose to rename the *yerG* and *yoqV* genes of *B.subtilis ligA* and *ligB* respectively.

INTRODUCTION

DNA ligases catalyse the joining of breaks in duplex DNA during DNA replication, repair and recombination. They are grouped into two families, ATP-dependent DNA ligases and NAD-dependent ligases, according to the cofactor required for their activity. Apart from a common KXDG motif, these two families share few amino acid similarities. However, the reactions catalysed by the two classes of DNA ligase are identical, except for the fact that the AMP group is derived from two different cofactors. This implies that first there is covalent attachment of AMP to the lysine residue of the KXDG motif, followed by its transfer to the 5′-phosphoryl group at a nicked site of duplex DNA. The AMP group is finally then released from the adenylated DNA as the phosphodiester bond is formed. The crystal structure of the 41 kDa ATP-dependent ligase of bacteriophage T7 has been solved (1), as well as the adenylation domain of the NAD-dependent DNA ligase of *Bacillus stearothermophilus* (2). This enabled Wigley and colleagues (2) to make a structural alignment of the adenylation domain of the two proteins, which prove to have a similar fold and to share several critical residues. More recently the three-dimensional structure of the complete 76 kDa NAD-dependent ligase of *Thermus filiformis* has been reported (3).

NAD-dependent ligases are of a uniform size (~70 kDa) and reveal extensive amino acid sequence conservation throughout the entire protein. ATP-dependent ligases are more diverse in size, but share a common ligase domain, ~270 residues in length, possibly flanked by different domains. Until recently, the common view was that NAD-dependent ligases are present in eubacteria only, whereas ATP-dependent ligases are common to all other living organisms, including bacteriophages, archeabacteria, viruses and eukaryotes. However, a different picture has emerged with the rising number of complete bacterial genomes that have been sequenced. Among the 17 complete genomes of eubacteria presently available, all contain at least one gene homologous to the NAD-dependent ligase family, but three contain one or several copies of genes homologous to the ATP-dependent ligase family as well: *Bacillus subtilis*, *Mycobacterium tuberculosis* and *Haemophilus influenzae* (Table 1).

Table 1. Distribution of DNA ligases in *B.subtilis*, *H.influenzae* and *M.tuberculosis*

Bacterium species	NAD-dependent ligase (length in amino acids)	ATP-dependent ligase (length in amino acids)
B.subtilis	YerG (668)	YoqV (270)
		YkoU[a] (611)
M.tuberculosis	LigA (691)	LigB (507)
		LigC (358)
		O28549 (759)
H.influenzae	DNLJ (679)	DNL1 (268)

[a]This protein is not mentionned as a putative ligase in data banks, however the Pfam algorithm for protein alignment includes it in its ATP-dependent ligase family. Indeed all conserved motives among ATP-dependent ligase are present in this protein (to see the alignment: http//www.sanger.ac.uk/Software/Pfam).

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In two eubacterial species, *Escherichia coli* and *Salmonella typhimurium*, the NAD-dependent ligase gene, encoded by the *lig* gene, is known to be essential (4–6). However in the three species where more than one ligase gene is present, nothing is known as to whether any of the genes are essential. This question is addressed here with regard to *B.subtilis*. The *B.subtilis* genome harbours one NAD-dependent ligase-like gene, *yerG*, and two ATP-dependent ligase-like genes, *yoqV* and *ykoU*. The *yoqV* gene is encoded by the 134 kb lysogenic bacteriophage SPβ, but *ykoU* has no apparent bacteriophage origin. YoqV is a small protein composed of 270 amino acids, which consist of a single domain, the adenylation domain typical of ATP-dependent ligases. The YkoU protein is 611 amino acids in length, and its adenylation domain encompasses the first 292 residues. Of these two, only the smaller one, YoqV, was studied here in detail. Both ATP-dependent ligase genes are dispensable, because a strain cured of the SPβ prophage is viable, as well as a *ykoU* mutant, which has been constructed in the frame of a systematic analysis of unknown genes of *B.subtilis* (Micado database: http://locus.jouy.inra.fr/cgi-bin/genmic/madbase_home.pl; strain constructed in the laboratory of Dr K. Devine).

Various studies have shown that heterologous complementation is possible between ATP-dependent and NAD-dependent enzymes. Among examples of ATP-dependent ligases able to complement bacterial ligase mutants, it has been shown that the bacteriophage T4 ligase is able to rescue a *lig* mutant of *S.typhimurium* (4), and the catalytic domain of human ligase I restores the thermoresistance of an *E.coli* strain encoding a thermosensitive ligase (*ligts*) (7). Conversely, the NAD-dependent ligase of *E.coli* restores the thermoresistance of a *Saccharomyces cerevisiae* strain encoding a thermosensitive ligase (*cdc9ts*) (8).

Because of the high degree of complementation between both classes of enzymes reported in the literature, it was not possible to tell *a priori* whether the three ligase genes of the *B.subtilis* genome would have overlapping functions, or whether one of them was essential for viability. We therefore decided to investigate whether the NAD-dependent ligase encoding gene, *yerG*, was essential or dispensable in *B.subtilis*. We show here that this gene is essential in *B.subtilis*. In addition, we found that the ATP-dependent ligase encoded by *yoqV*, when cloned on a plasmid under appropriate expression signals, is unable to rescue a *yerG* mutant strain. This indicates that the two ligases cannot substitute for one another in *B.subtilis*. The two *B.subtilis* ligase genes *yerG* and *yoqV* were also introduced in an *E.coli ligts* strain, and we found that whereas *yoqV* does not complement the *ligts* defects, *yerG* fully complements the growth and UV sensitivity defects of the *lig* mutant.

MATERIALS AND METHODS

Strains and plasmids

Strains and plasmids are listed in Table 2. *Bacillus subtilis* and *E.coli* cells were grown on LB medium unless otherwise stated. When needed, antibiotics were added to a final concentration of 5 μg/ml for kanamycin and 0.5 μg/ml for erythromycin for *B.subtilis*, and 100 μg/ml ampicillin for *E.coli*. The chemically defined medium has been described elsewhere (9). Constructions were performed as described below. The integrity

Table 2. Strains and plasmids

Name		Relevant genotype, description	Reference, source
Strains	JJC75[a]	*lig7ts*	B.Michel
	168	*trpC2*	C.Anagnostopoulos
	HVS610	168 *ins*[b] pMAP149 in *yerH*	This work
	HVS609	168 *ins* pMAP52 in *yerG*	This work
	HVS611	168 *ins* pMAP59 in *yerG*, with plasmid pMAP141	This work
Plasmids	pDG148	pBR322-pUB110 *KanR lacI Pspac*	(15)
	pMUTIN2	pBR322 *lacZ lacI EmR Pspac*	(16)
	pMAP52	pMUTIN2 *Pspac*:(nt –20[c] to 275 of *yerG*)	This work
	pMAP59	pMUTIN2MCS *Pspac*:(nt 330–666 of *yerG*)	This work
	pMAP65	pUB110 *KanR lacI*	(9)
	pMAP138	pDG148 *Pspac*:*yoqV*	This work
	pMAP140	pDG148 *Pspac*:*yerG*	This work
	pMAP149	pMUTIN2MCS *Pspac*:(nt 540–854 of *yerH*)	This work
	pIL253	EmR	(17)
	pMAP139	pMAP138Δ(pBR322 *lacI*) from *Aat*II to *Pst*I, KanR	This work
	pMAP141	pMAP140Δ(pBR322 *lacI*) from *Aat*II to *Pst*I, KanR	This work

[a]AB1157 background, *hsdR* thr+ pro+.
[b]*ins* indicates the insertion of a given plasmid in the *B.subtilis* chromosome.
[c]Gene numbering starts at the start codon.

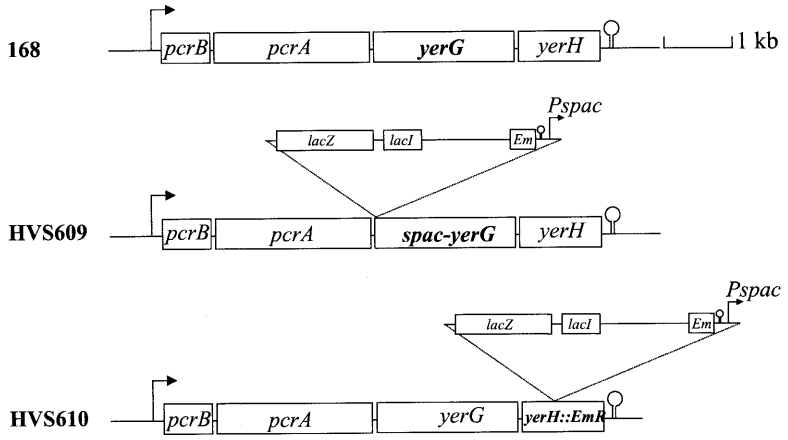

Figure 1. Chromosomal structure of wild-type strain 168 and mutant strains. Strain 168 is shown at the top of the figure. In strain HVS609, plasmid pMAP52 is integrated in *yerG*, so as to fuse *yerG* to the *Pspac* promoter. In strain HVS610, plasmid pMAP149 is disrupting the *yerH* gene.

of the sequence of all DNA fragments generated by PCR was verified by automated dideoxysequencing.

yerG transcriptional fusion strain HVS609. To fuse *yerG* to an inducible promoter, plasmid pMUTIN2 was used. This is a pBR322 derivative containing a *Pspac* IPTG inducible promoter upstream of a multiple cloning site flanked by *Bam*HI and *Eco*RI. The promoter is preceded by two transcription terminators. Further downstream on the same plasmid are the *lacI* gene and a selectable erythromycin resistance marker (Em^R) for *B.subtilis*. A DNA fragment starting 20 bp upstream of the *yerG* start codon, so as to include a ribosome binding site 296 bp in length, and flanked by artificially introduced *Eco*RI and *Bam*HI sites, was generated by PCR and cloned into pMUTIN2. The resulting plasmid, pMAP52, contained a transcriptional fusion between *Pspac* and the first few hundred nucleotides of *yerG*. To generate the fusion between *Pspac* and the complete ORF of *yerG*, plasmid pMAP52 was introduced by single crossing over into the chromosome of *B.subtilis* wild-type 168 strain. The resulting strain, HVS609 (Fig. 1), harbors a single copy of the *yerG* gene under the control of the *Pspac* promoter.

yerH mutant strain HVS610. An internal fragment of the *yerH* gene, 350 bp in length, was generated by PCR and cloned between the *Eco*RI and *Bam*HI sites of pMUTIN2 to give plasmid pMAP149. To disrupt *yerH*, plasmid pMAP149 was introduced by a single crossing over into the chromosome of *B.subtilis* to give strain HVS610 (Fig. 1).

Plasmid pMAP59. To disrupt the *yerG* gene, a 314 bp internal segment of *yerG* was generated by PCR and cloned between the *Eco*RI and *Bam*HI sites of pMUTIN2 to give plasmid pMAP59.

Plasmids pMAP138 to 141. To clone *yoqV* under strong expression signals, plasmid pDG148 was used as a vector. It is a pBR322-pUB110 shuttle vector, which replicates both in *E.coli* and *B.subtilis*. It contains a strong promoter active both in *B.subtilis* and *E.coli* and a canonical ribosome binding site immediately upstream of an *Xba*I site followed by a *Sal*I site. A DNA fragment containing the *yoqV* ORF flanked by artificially introduced *Xba*I and *Sal*I sites was generated by PCR and cloned into pDG148 in *E.coli*. The resulting plasmid, pMAP138, was used for complementation tests in *E.coli*. For *B.subtilis* a smaller derivative, pMAP139, deleted for the pBR322 sequences and the *lacI* gene, was made by an *Aat*II–*Pst*I deletion of pMAP138. Using the same strategy, the *yerG* ORF was cloned into pDG148 to give pMAP140, and a smaller derivative devoid of pBR322 and *lacI* sequences, pMAP141, was obtained by an *Aat*II–*Pst*I deletion of pMAP140.

Adenylation test

Bacillus subtilis and *E.coli* crude extracts were prepared in the following way. A 100 ml overnight culture was centrifuged, and the pellet stored at –80°C. To lyse cells, the frozen pellet was resuspended in 1 ml of 50 mM Tris pH 7.5, 5 mM DTT, 1 mM EDTA, 2 mg/ml lysozyme, complemented with a cocktail of protease inhibitors (Roche). The suspension was incubated for 20 min in ice. Sonication was applied for 5 s using 1 s pulse/9 s pause cycles, with a small probe (Vibracell apparatus). The soluble fraction (2 ml) was collected after a 30 min ultracentrifugation at 100 000 *g*, its concentration was ~4 mg/ml of total protein.

To test the adenylation activity of crude extracts, 4 µl of extract was incubated in buffer A (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM ATP), in a final volume of 20 µl, in the presence of 5 µCi of [α-^{32}P]ATP, for 15 min at 20°C (the ratio between cold- and radiolabelled ATP was critical for optimal detection of the adenylation activity in crude extracts). Proteins were then denatured in a SDS buffer and separated by polyacrylamide gel electrophoresis (PAGE). The gel was fixed, colored and dried prior to exposition. Radioactivity was detected using a Storm apparatus (Molecular Dynamics).

UV sensitivity test

Exponentially growing cells were diluted serially and plated on LB. Plates were irradiated at a fluence of 2 J/m^2/s for different times, and then incubated for growth at 30°C (for strain JJC75) or 42°C for the other two strains.

Table 3. A *yerG*, but not a *yoqV* overproducing strain allows the selection of *B.subtilis* transformants harbouring a *yerG* disruption

	Proportion of disrupted *yerG* clones[a] among transformants[b] of *B.subtilis* 168 strain containing:		
Transforming DNA	no plasmid	pMAP141 (*yerG*)	pMAP139 (*yoqV*)
Plasmid DNA pMAP59 (disrupts *yerG*)	N.R.	5/15	0/2
Total DNA of strain HVS611 (*yerG* disrupted)	0/48	18/18	0/18

[a]The proportion of transformants effectively containing a *yerG* disruption but not resulting from a congression is indicated (see text for details).
[b]Competent cells of the indicated strains were transformed to EmR with the indicated DNA. The three strains tested had a similar level of competence, estimated by transformation with the plasmid pIL253 (between 1.3×10^5 and 3.7×10^5 transformants per µg of DNA were obtained).
N.R., not relevant (no transformants obtained).

RESULTS

yerG is an essential gene

The NAD-dependent ligase gene is located at 55° on the *B.subtilis* chromosome, as the third gene of a putative operon encoding *pcrB*, *pcrA*, *yerG* and *yerH* (Fig. 1). Among these four genes, only *pcrA*, encoding a DNA helicase of the SF1 family, has been characterised and found to be an essential gene (9). The *pcrB* and *yerH* genes are unknown and do not match with any known protein from other organisms.

A *yerG* mutant strain was constructed to examine whether the encoded NAD-dependent ligase enzyme is essential *in vivo*. To disrupt the *yerG* gene, *B.subtilis* was transformed with pMAP59, a non-replicative plasmid containing an internal fragment of the gene. No transformants were obtained on rich media, nor on chemically defined media.

The impossibility of disrupting *yerG* could result from a polar effect of the insertion on the downstream gene of the operon (*yerH*), or from the requirement of *yerG* for viability. To test for the first alternative, a *yerH* mutant was constructed. As for the *yerG* gene, a non-replicative plasmid containing an internal fragment of *yerH* was used to transform *B.subtilis*. Transformants were obtained, which contained the appropriate gene disruption, and the resulting strain (strain HVS610, Fig. 1) did not show any growth defect. We concluded therefore that *yerH* was not essential, and in turn, that the impossibility to disrupt *yerG* was likely to be due to its requirement for viability.

To confirm this conclusion, strain HVS609 was constructed, in which *yerG* was fused to an IPTG inducible promoter. We investigated whether strain HVS609 was able to grow in the absence of IPTG. Almost no sign of lethality was observed after approximately 30 generations of growth in the absence of inducer. This could be due to a low concentration of LacI in the cell (only one copy of the *lacI* gene had been inserted into the chromosome, together with the *Pspac–yerG* fusion), allowing leaky *yerG* expression. When plasmid pMAP65, a pUB110 derivative encoding LacI, was introduced into strain HVS609, arrest of growth was observed in the absence of inducer, as shown in Figure 2. After 2 h, the number of viable cells ceased to increase but optical density continued to increase for one additional hour, as a result of cell filamentation, before leveling off. Therefore, strain HVS609 containing pMAP65 is IPTG-dependent for growth. This confirms that *yerG* is essential.

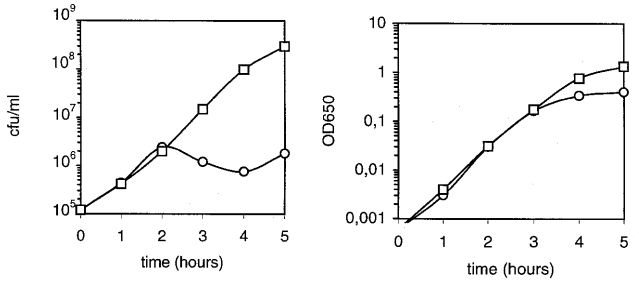

Figure 2. Growth curves of strain HVS609 harbouring plasmid pMAP65. Colony forming units (cfu, left) and optical density (OD650, right) of the strain grown either in the presence (squares) or absence (circles) of IPTG are reported as a function of time.

Overexpression of the *yoqV* encoded ATP-dependent ligase does not rescue a *yerG* mutation

Two potential ATP-dependent ligases are encoded by the *B.subtilis* genome, *ykoU* and *yoqV*, neither of which is essential. Since *yerG* is essential in a wild-type strain of *B.subtilis*, this suggests that neither ATP-dependent ligase can complement *yerG* activity. Because the *yoqV* gene belongs to SPβ prophage, we reasoned that its transcription was likely to be repressed in the chromosome. The possibility existed therefore that the ATP-dependent ligase once fused to appropriate expression signals would be able to complement a *yerG* mutation. To test this possibility, a plasmid was constructed, pMAP139, in which the *yoqV* ORF was cloned into a pUB110 derivative, under strong expression signals, including the *Pspac* promoter and a canonical ribosome binding site. As a control, the *yerG* gene was cloned similarly on the pUB110 derivative, to give plasmid pMAP141.

In order to investigate whether this new strain, in which the *yoqV* ATP-dependent ligase was constitutively expressed, could survive in the absence of the *yerG* encoded NAD-dependent ligase, an attempt at disrupting *yerG* with the non-replicative plasmid pMAP59 (see first paragraph) was repeated. Table 3, lane 1, shows the proportion of transformants in which the *yerG* gene was effectively interrupted in various *B.subtilis* strains.

In the *B.subtilis* strain harbouring the *yerG* encoding plasmid pMAP141, only a few transformants were obtained (50/µg of plasmid DNA). This was expected and resulted from the

reduced homology between recipient and transforming DNA: plasmid pMAP59 shares a 337 bp homology with the *yerG* gene, and also a 293 bp homology with both plasmids pMAP139 and pMAP141 in a segment encompassing the *Pspac* promoter. To distinguish between the transformants in which pMAP59 had recombined with the chromosomal copy of *yerG*, and those in which pMAP59 had recombined with the resident plasmid pMAP141, total DNA was extracted from 15 transformants. Five of the 15 clones contained a disrupted copy of *yerG* (Table 3, lane 1) as determined by Southern blot hybridisation (not shown). The other 10 clones contained a recombinant plasmid between pMAP141 and pMAP59. From this analysis we concluded that it is possible to construct a *yerG* disruption in a *B.subtilis* strain containing a plasmidic copy of the gene. The resulting strain was named HVS611.

In the *B.subtilis* strain harbouring the *yoqV* encoding plasmid pMAP139, transformation with plasmid pMAP59 yielded two transformants only, and none of these two transformants contained a disrupted copy of *yerG*. Rather, both of them had a mixture of two plasmids, the resident pMAP139, and a 14 kb plasmid recombinant between pMAP139 and pMAP59. The low transformation efficiency, and the absence of transformants harbouring a *yerG* disruption, suggested that a *yerG* mutation was not tolerated in a strain expressing *yoqV*. However, given the low number of transformants, the possibility that such a disruption is indeed tolerated could not be completely excluded.

To increase the chances to get a *yerG* disruption in strain 168 harbouring pMAP139, another attempt at disrupting *yerG* was made. Instead of pMAP59 as a source of DNA, total DNA of strain HVS611, in which pMAP59 is already integrated into *yerG*, was used (Table 3, lane 2). In such a case, the homology between resident and transforming DNA is greatly increased, and higher transformation frequencies are expected. However, because the total DNA of strain HVS611 also contains pMAP141 plasmid DNA, the possibility exists to obtain 'false positive transformants', resulting from a congression, i.e. the simultaneous acquisition of two independent markers, *yerG*::EmR and plasmid pMAP141. In strain 168 harbouring the *yerG* encoding plasmid, a high yield of transformants (3.5×10^4/µg of DNA) were obtained as expected, and of among 18 clones analysed, all contained an interrupted copy of yerG (Table 3, lane 2) as determined by a PCR analysis. With strain 168, 10 times less transformants (2×10^3/µg of DNA) were obtained, and among 48 clones analysed, all were resistant to kanamycin, the marker of plasmid pMAP141. This showed that these transformants were 'false positives'. In strain 168 harbouring pMAP139, 6×10^3 transformants per µg of DNA were obtained, a result similar to strain 168. This suggested that all transformants also resulted from a congression of *yerG*::EmR and pMAP141 DNA. Unlike recipient strain 168, the strain containing plasmid pMAP139 was already resistant to kanamycin and as such congression could not be tested phenotypically. To check for the presence of plasmid pMAP141 in the transformants, total DNA of 18 clones was extracted, and the plasmid and chromosomal DNA was analysed by Southern blotting. In all clones, plasmid pMAP141 was found effectively, either alone or in combination with plasmid pMAP139. Therefore a proportion of 0 among 18 clones contained a *yerG* disruption (Table 3, lane 2).

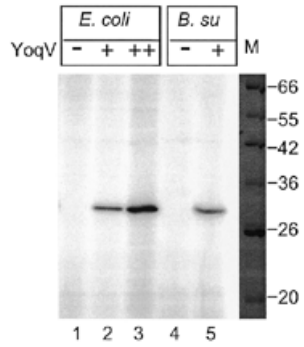

Figure 3. Presence of an adenylate activity specific of *E.coli* and *B.subtilis* crude extracts from strains containing a plasmid overproducing YoqV. Crude extracts of *E.coli* strains JM105 (lane 1), JM105 + pMAP138 grown without IPTG (lane 2), JM105 + pMAP138 grown with IPTG (lane 3), and *B.subtilis* strains 168 (lane 4) and 168 + pMAP139 (lane 5), were incubated with radiolabelled ATP, separated by PAGE, stained with Coomassie blue (right, only the marker lane is shown), and exposed (left). Lane M, molecular weight markers, relevant sizes are indicated in kDa.

In conclusion, when plasmid pMAP141 was present in the recipient wild-type strain of *B.subtilis*, the chromosomal copy of *yerG* could be disrupted. However, when the *yoqV* encoding plasmid pMAP139 was present, no transformants containing an disrupted copy of *yerG* were obtained.

To verify that the ATP-dependent ligase cloned into pMAP139 was active, an adenylation test was performed on *B.subtilis* crude extracts. The first step of the ligation process consists of the covalent attachment of AMP to the lysine residue of the ligase, which can be visualised easily on a protein gel if the AMP is radiolabelled. Extracts treated with RNase were incubated in the presence of [α-^{32}P]ATP for 15 min at room temperature and analysed by denaturing PAGE. As shown in Figure 3, lanes 4 and 5, a radioactive signal was detected in samples extracted from the YoqV-containing strain, and was absent in the *B.subtilis* wild-type extract. This signal corresponded to a protein of ~30 kDa, which is close to the expected size of 31 kDa for YoqV. Therefore the YoqV ligase was present and active in the strain containing plasmid pMAP139. The absence of any signal in crude extracts of *B.subtilis* wild-type extracts suggests that neither the YoqV nor the YkoU ATP-dependent ligases were expressed at a detectable level in this strain.

The *yoqV* ligase does not complement a *ligts* mutant of *E.coli,* but the *yerG* ligase does

In *E.coli*, the C-terminal part of the human ATP-dependent ligase I is able to restore the thermoresistance to a *ligts* strain (7), and in *S.typhimurium* the bacteriophage T4 ATP-dependent ligase, which contains only the minimal adenylation domain, like the *yoqV* gene product, allows growth of the NAD-dependent ligase disrupted strain (4). We therefore investigated whether YoqV was able to complement a *ligts* strain of *E.coli*. For this, plasmid pMAP138, a hybrid composed of pBR322, *lacI* and pMAP139, was constructed. The plasmid was transformed into strain JJC75, containing the *lig7 ts* allele, and the resulting strain was grown at various temperatures. Plates incubated at 42 and 30°C are shown in Figure 4. As with the *ligts*, the strain

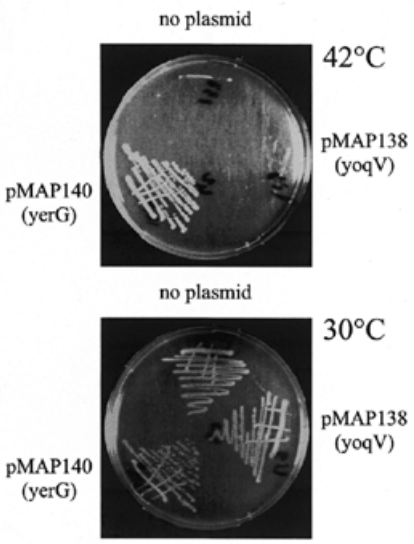

Figure 4. *yerG*, but not *yoqV*, restores thermoresistance to the *E.coli ligts* strain. Petri plates showing streaks on LB of the *E.coli ligts* strain harbouring no plasmid (no plasmid), plasmid pMAP138 [pMAP138 (yoqV)] or pMAP140 [pMAP140 (*yerG*)], grown either at 42 (top) or 30°C (bottom).

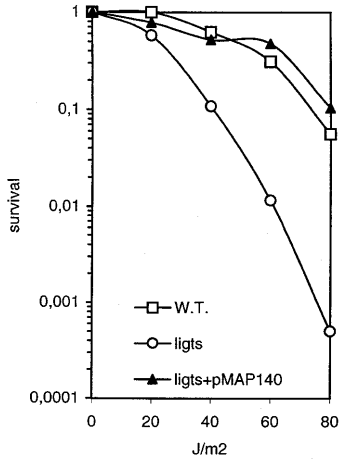

Figure 5. UV sensitivity of wild-type *E.coli* strain JJC40 grown at 42°C (squares), its *ligts* derivative JJC75 grown at 30°C (circles) and JJC75 harbouring the *yerG* encoding plasmid pMAP140, grown at 42°C (triangles). The fraction of surviving clones is reported as a function of the UV dose.

harbouring plasmid pMAP138 did not grow at 42 or 37°C (not shown), both in the presence and absence of IPTG. To further confirm this defect, cells were grown exponentially at 30°C and the plating efficiency at 42°C relative to 30°C was determined. Only 10^{-5} survivors were found at 42°C with the *ligts* strain, with or without pMAP138. This shows that the *B.subtilis* ATP-dependent ligase does not complement the growth defect of the *ligts* strain.

To verify that the ATP-dependent ligase cloned into pMAP138 was active, an adenylation test was performed on *E.coli ligts* crude extracts (Fig. 3, lanes 1–3). A radioactive signal was detected in samples extracted from the YoqV containing strain, and was absent in the extract that did not contain YoqV. It was 10-fold more intense in the sample in which expression of *yoqV* had been derepressed upon addition of IPTG. This showed that the ATP-dependent ligase encoded by *yoqV* was active in *E.coli* crude extracts.

Similar heterologous complementation experiments were carried out in parallel with the *yerG* encoded NAD ligase. Plasmid pMAP140, a hybrid of pBR322, *lacI* and pMAP141, which provides IPTG-dependent expression of the *B.subtilis* NAD-dependent ligase in *E.coli*, was constructed and introduced in the *ligts* background. In contrast to the *yoqV* experiment, this strain did form individual colonies at 42°C (Fig. 4), and it plated at an efficiency close to 100% at this temperature, even in the absence of IPTG. This shows that the *B.subtilis* NAD-dependent ligase complements the growth defect of the *ligts* strain.

Because the YerG ligase was able to restore growth of the *ligts* strain at 42°C, its ability to complement the UV sensitivity of the *ts* strain was further investigated. Whereas the *ligts* strain was UV sensitive at 30°C (Fig. 5, circles), this defect disappeared in the presence of *yerG*, even at 42°C (triangles), and the strain became as UV resistant as the original wild-type strain (squares). In conclusion, the *yerG* gene product allowed the full restoration of the growth and UV resistance defects of the *ligts* strain.

DISCUSSION

In *B.subtilis*, we have shown that the *yerG*-encoded NAD-dependent ligase, which we propose to rename *ligA*, is essential for growth. This was somewhat unexpected, given the presence of two other potential ligase genes in its chromosome. These two other ligases, *ykoU* and *yoqV*, belong to the family of ATP-dependent ligases. Observations have shown that some ATP-dependent ligases, although originating from organisms completely unrelated to bacteria, such as the T4 bacteriophage and *Homo sapiens*, were able to restore the viability of a *S.typhimurium* or a *E.coli lig* mutant (4,7), therefore suggesting a surprisingly broad range of activity and compatibility for these enzymes.

Because the *yoqV* gene is part of the SPβ prophage, the possibility existed that the absence of complementation was simply due to a lack of a sufficient level of expression. To increase the expression of the *yoqV* encoded ATP-dependent ligase, which we propose to rename *ligB*, this gene was cloned on a plasmid under appropriate expression signals. Even in this case, the *yerG* gene could not be disrupted. To further investigate the activity of *yoqV*, the gene was cloned in *E.coli* and its ability to complement the growth defect of the *lig7ts* strain was tested. Again, at 42°C *yoqV* failed to restore growth of the *ligts* mutant. To exclude the possibility that the ligase encoded by *yoqV* was in fact an inactive enzyme, its adenylation activity in *B.subtilis* and *E.coli* crude extracts was tested and found to be present. It appears therefore that, in *B.subtilis* as well as in *E.coli*, not all ATP-dependent ligases provide sufficient activity for maintaining alive bacterial cells devoid of their native NAD-dependent ligase.

In cases where a complementation has been reported, it should be noted that not all phenotypes were restored. Neither

the Chlorella virus ATP-dependent ligase nor the *E.coli* NAD-dependent ligase, although restoring growth of a *S.cerevisiae* cdc9Δ strain, restored complete UV resistance or MMS resistance (8). It has been suggested that DNA ligases perform different functions, some of which are generic and can be achieved by an NAD-dependent ligase as well as by an ATP-dependent ligase, the others being enzyme specific. The ligation of Okasaki fragments during DNA replication was suggested to be a generic, as well as an essential function. Here we report, however, that the SPβ ATP-dependent ligase is not able to provide this essential function, whichever it may be. What properties of the bacteriophage T4 ATP-dependent ligase, which are not present in the SPβ ligase, make it a good substitute to the bona fide ligase of *S.typhimurium*? Both enzymes are small in size and do not contain additional domains supposedly involved in protein–protein interactions, so it is tempting to speculate that the difference lies in the intrinsic enzymatic activities of these proteins, one having a higher specific activity, or a higher stability than the other. As a point of comparison, the adenylation activity of YoqV in *B.subtilis* crude extracts was equivalent to the signal obtained with 0.25 U of commercial T4 ligase mixed with a wild-type *B.subtilis* crude extract (not shown). Reported to the total amount of protein, and converted into the units as defined by Modrich and Lehman (10), it corresponded to 4 U/mg of protein. The NAD-dependent ligase of *E.coli* gives between 0.4 and 1.7 U/mg of protein, and this amount was reported to be much more than actually needed in the cell (11). Therefore, in terms of quantity and adenylation activity, YoqV does not appear to be defective. A reasonable possibility is that the second step of the ligation reaction, the transfer of the AMP moiety to the DNA and subsequent ligation, is inefficient in YoqV.

The NAD-dependent ligase of *B.subtilis* is able to complement a *ligts* strain of *E.coli*. This is interesting in view of the growing evidence that DNA ligases of a large size, apart from their ligase domain, possess several other domains that may be involved in protein–protein interactions. For instance, the mammalian DNA ligase I has been shown to bind to PCNA (12), and mammalian DNA ligase IV to XRCC4 (13,14). No interaction has yet been reported for the bacterial NAD-dependent ligases, but it has been suggested that their BRCT domain may serve this function (3). If a NAD-dependent ligase from a distantly related bacterium like *B.subtilis* (the two proteins share 49% of amino acid identity) is able to complement the ligase defect in *E.coli*, this suggests either that this interaction is conserved between the two organisms, or that the *B.subtilis* protein does not need to interact with any *E.coli* protein to work efficiently.

On a practical level, the observations reported here encourage the search for antibiotics directed specifically against NAD-dependent ligases, since these enzymes prove to be essential for bacterial growth and specific for this reign. Although some bacteria like *B.subtilis*, *M.tuberculosis* and *H.influenzae*, harbour more than one ligase gene, the NAD-dependent ligase gene is always unique, and at least in the case of *B.subtilis* it proves to be essential.

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REFERENCES

1. Subramanya,H.S., Bird,L.E., Brannigan,J.A. and Wigley,D.B. (1996) Crystal structure of a DExx box DNA helicase. *Nature*, **384**, 379–383.
2. Singleton,M.R., Hakansson,K., Timson,D.J. and Wigley,D.B. (1999) Structure of the adenylation domain of an NAD+-dependent DNA ligase. *Structure Fold Des.*, **7**, 35–42.
3. Lee,J.Y., Chang,C., Song,H.K., Moon,J., Yang,J.K., Kim,H.K., Kwon,S.T. and Suh,S.W. (2000) Crystal structure of NAD(+)-dependent DNA ligase: modular architecture and functional implications. *EMBO J.*, **19**, 1119–1129.
4. Park,U.E., Olivera,B.M., Hughes,K.T., Roth,J.R. and Hillyard,D.R. (1989) DNA ligase and the pyridine nucleotide cycle in *Salmonella typhimurium*. *J. Bacteriol.*, **171**, 2173–2180.
5. Konrad,E.B., Modrich,P. and Lehman,I.R. (1973) Genetic and enzymatic characterization of a conditional lethal mutant of *Escherichia coli* K12 with a temperature-sensitive DNA ligase. *J. Mol. Biol.*, **77**, 519–529.
6. Gottesman,M.M., Hicks,M.L. and Gellert,M. (1973) Genetics and function of DNA ligase in *Escherichia coli*. *J. Mol. Biol.*, **77**, 531–547.
7. Kodama,K., Barnes,D.E. and Lindahl,T. (1991) *In vitro* mutagenesis and functional expression in *Escherichia coli* of a cDNA encoding the catalytic domain of human DNA ligase I. *Nucleic Acids Res.*, **19**, 6093–6099.
8. Sriskanda,V., Schwer,B., Ho,C.K. and Shuman,S. (1999) Mutational analysis of *Escherichia coli* DNA ligase identifies amino acids required for nick-ligation *in vitro* and for *in vivo* complementation of the growth of yeast cells deleted for CDC9 and LIG4. *Nucleic Acids Res.*, **27**, 3953–3963.
9. Petit,M.A., Dervyn,E., Rose,M., Entian,K.D., McGovern,S., Ehrlich,S.D. and Bruand,C. (1998) PcrA is an essential DNA helicase of *Bacillus subtilis* fulfilling functions both in repair and rolling-circle replication. *Mol. Microbiol.*, **29**, 261–273.
10. Modrich,P. and Lehman,I.R. (1970) Enzymatic joining of polynucleotides. IX. A simple and rapid assay of polynucleotide joining (ligase) activity by measurement of circle formation from linear deoxyadenylate-deoxythymidylate copolymer. *J. Biol. Chem.*, **245**, 3626–3631.
11. Gellert,M. and Bullock,M.L. (1970) DNA ligase mutants of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **67**, 1580–1587.
12. Levin,D.S., Bai,W., Yao,N., O'Donnell,M. and Tomkinson,A.E. (1997) An interaction between DNA ligase I and proliferating cell nuclear antigen: implications for Okazaki fragment synthesis and joining. *Proc. Natl Acad. Sci. USA*, **94**, 12863–12868.
13. Grawunder,U., Zimmer,D. and Leiber,M.R. (1998) DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. *Curr. Biol.*, **8**, 873–876.
14. Critchlow,S.E., Bowater,R.P. and Jackson,S.P. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Curr. Biol.*, **7**, 588–598.
15. Stragier,P., Bonamy,C. and Karmazyn-Campelli,C. (1988) Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. *Cell*, **52**, 697–704.
16. Vagner,V., Dervyn,E. and Ehrlich,S.D. (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology*, **144**, 3097–3104.
17. Simon,D. and Chopin,A. (1988) Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. *Biochimie*, **70**, 559–566.