**The Bacillus subtilis lipoprotein LplA causes cell lysis when expressed in Escherichia coli**

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**INTRODUCTION**

Many species in the genus Bacillus synthesize one or more extracellular amylases (Priest, 1977). Bacillus subtilis synthesizes an α-amylase (AmyE) that is one of the major extracellular enzymes (Yang et al., 1983). This work was originally oriented to the isolation of new amylolytic enzymes from B. subtilis; we therefore constructed a genomic library from this organism and expressed it in E. coli. To our surprise, one of the clones initially selected as amylolytic carried a B. subtilis gene whose expression in *Escherichia coli* caused cell lysis. We suggest that this gene codes for a putative lipoprotein-like protein, since it behaves in a similar way to the Streptococcus pneumoniae lipoprotein genes *malX* and *amiA* (Martin et al., 1989) when expressed in *E. coli*. In this paper we describe the isolation of the *B. subtilis* *lplA* gene and the phenotypic characterization of the protein that it encodes.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. In general, *B. subtilis* and *E. coli* strains were grown in LB broth or plates. Ampicillin was used at a concentration of 50 μg ml⁻¹ and chloramphenicol was used at 5 μg ml⁻¹ for both *E. coli* and *B. subtilis*; erythromycin was used at a concentration of 10 μg ml⁻¹ for *B. subtilis* and 100 μg ml⁻¹ for *E. coli*.

The GenBank accession number for the sequence reported in this paper is 0001-8743.

**General cloning procedures.** We followed the techniques described in Sambrook *et al.* (1989). Restriction enzymes, T4 DNA ligase, Klenow polymerase and calf alkaline phosphatase were from Promega.

**Construction of the genomic library.** Chromosomal DNA from *B. subtilis* and plasmid DNA from *E. coli* were purified following standard procedures (Marmur, 1961; Sambrook *et al.*, 1989). The DNA from *B. subtilis* was partially digested with the endonuclease *Sau3A* and electrophoresed in a preparative agarose gel. Fragments ranging from 3 to 9 kb were recovered by electroelution. The purified DNA was ligated to pBR322, previously cut with *BamHI* and dephosphorylated with calf alkaline phosphatase. The ligation mixture was used to transform *E. coli* DH5α. Around 25000 transformants containing inserts of the *B. subtilis* chromosome were obtained. From those, around 2400 transformants giving tiny colonies were initially selected and plated on selective media (LB supplemented with \(50 \mu g\ ampicillin ml⁻¹\) and \(1\%\, (w/v),\) insoluble starch).

**Transformation methods.** *E. coli* cells were made competent and transformed following the method described in Hanahan (1983), with some modifications. *B. subtilis* cells were made competent as described in Bron & Luxen (1985), and transformed with 1–5 μg DNA for 45 min at 37 °C; then the cells were diluted twofold with LB broth and incubated at 30 °C (erythromycin selection) or 37 °C (chloramphenicol selection) for 90 min to allow expression of antibiotic resistance markers. Transformants were selected on LB plates supplemented with antibiotics at appropriate concentrations.

**Detection of starch hydrolysis on plates.** Recombinant clones from the *B. subtilis* library were tooth-picked on LB medium supplemented with 50 μg ampicillin ml⁻¹ and 1% (w/v) insoluble starch (Sigma). Incubation of plates was carried out at 37 °C.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Material</th>
<th>Relevant features</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5a</td>
<td>supE44 ΔlacU169 hisD17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type strain</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>MB11</td>
<td>amyB3 metB16 hisH2</td>
<td></td>
</tr>
<tr>
<td>DB104</td>
<td>amyB3 hisH1 nap18 napR2 apaA3</td>
<td></td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBR322</td>
<td>E. coli vector</td>
<td></td>
</tr>
<tr>
<td>pC194</td>
<td>B. subtilis vector</td>
<td></td>
</tr>
<tr>
<td>pGA14</td>
<td>B. subtilis vector</td>
<td></td>
</tr>
<tr>
<td>pUC13, pUC18</td>
<td>E. coli vector</td>
<td></td>
</tr>
<tr>
<td>pUC</td>
<td>pC194 + pUC18</td>
<td></td>
</tr>
<tr>
<td>pAMY</td>
<td>pUC + B. licheniformis a-amylase gene</td>
<td></td>
</tr>
<tr>
<td>pLPLA</td>
<td>pUC + 4.5 kb EcoRI-EcoRI insert*</td>
<td></td>
</tr>
<tr>
<td>pEm1</td>
<td>pUC13 + ermC gene</td>
<td></td>
</tr>
<tr>
<td>pEm2</td>
<td>pEm1 + 536 bp central fragment of lplA</td>
<td></td>
</tr>
<tr>
<td>pA-1</td>
<td>pBR322 + 6.7 kb Sau3A-Sau3A insert</td>
<td></td>
</tr>
<tr>
<td>pAE-1</td>
<td>pUC13 + 4.5 kb EcoRI-EcoRI insert</td>
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</tr>
<tr>
<td>pAE-1</td>
<td>pUC13 + 2.1 kb EcoRI-PvuII insert</td>
<td></td>
</tr>
<tr>
<td>pAE-2</td>
<td>pUC13 + 2.1 kb EcoRI-PvuII insert</td>
<td></td>
</tr>
<tr>
<td>pAES-1</td>
<td>pUC13 + 1.6 EcoRI-SacI insert</td>
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</tr>
<tr>
<td>pAES-1A1</td>
<td>See details in the text</td>
<td></td>
</tr>
<tr>
<td>pAES-1A2</td>
<td>See details in the text</td>
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</table>

* The insert refers to DNA fragments containing the complete or partial sequences of the lplA gene.

37 °C; after 24 h, clones that showed a halo of starch hydrolysis were selected.

**Southern blot hybridization.** In each case, chromosomal or plasmid DNA was digested with the desired restriction enzymes and subjected to electrophoresis on 0.7% (w/v) agarose gels. The separated fragments were visualized by staining with ethidium bromide and transferred to a Hybond-N membrane (Amersham), following the standard procedure. DNA probes were labelled with [α-32P]dCTP using the random primed labelling kit from Boehringer Mannheim. Hybridizations were carried out by standard procedures (Sambrook et al., 1989).

**DNA sequencing.** DNA sequencing was done by the dideoxynucleotide chain termination procedure (Sanger et al., 1977) with the Sequenase enzyme (USB). Unidirectional, sequential deletions in plasmid pAES-1 were obtained by the exonucleaseIII–S1 nuclease digestion protocol (Henikoff, 1984), using a kit from Pharmacia. In some cases specific restriction fragments cloned into pUC vectors were used as templates in the sequencing reactions.

**Analysis of sequence data.** Analysis of DNA and protein sequence data was carried out with the DNASIS, PROSIS (Pharmacia) and GCG (Devereux et al., 1984) packages. The National Biomedical Research Foundation-Protein Identification Resource (NBRF-PIR), SwissProt, EMBL and GenBank were the databases used for protein and DNA homology searches.

**Isocitrate dehydrogenase assay.** Isocitrate dehydrogenase was measured as described by Smith et al. (1987).

**Incorporation of [3H]palmitic acid.** E. coli cells carrying different plasmids and growing exponentially were incubated with [9,10(n)-3H]palmitic acid (20 μCi ml⁻¹) (Amersham) at 37 °C for 1 h. Cells were harvested and washed four times with distilled water to remove unincorporated radioactive precursor. Finally, cells were boiled in SDS-loading buffer and the extracted proteins analysed by SDS-PAGE and fluorography.

**Construction of the Bacillus subtilis integrative plasmids pEm1 and pEm2.** pEm1 was obtained by ligating an EcoRI–SacI fragment containing the erythromycin resistance gene ermC* from plasmid pGA14, in pUC13 previously digested with the same set of enzymes. A HindIII–EcoRV fragment from the sequencing deletion clone t7.12 containing 536 bp of the central sequence of lplA (Fig. 1) was subcloned into pEm1 previously digested with HindIII and EcoRV, giving the plasmid pEm2.

**RESULTS**

**Cloning of B. subtilis DNA fragments conferring starch hydrolytic ability**

Initially, the cloning strategy was designed to isolate E. coli transformants that were able to express amylolytic enzymes. In this way, a B. subtilis DNA genomic library was constructed in pBR322. Since the expression of heterologous a-amylases in E. coli is sometimes toxic (Willemot & Cornelis, 1983; Yang et al., 1983), of 25000 transformants originally obtained, 2400 were initially selected as giving tiny colonies, and plated on selective media (LB supplemented with 50 μg ampicillin ml⁻¹ and 1%, w/v, insoluble starch). From these, only 15 transformants showed a clear starch hydrolysis phenotype.

Plasmid DNA isolated from these clones showed an insert size that ranged from 2.7 to 6.7 kb. A 2.1 kb EcoRI–PvuII fragment from the insert of one of these clones was
The subcloning of the insert contained in pA-1 demonstrated that the EcoRI–PvuII fragment of 2.1 kb was capable of conferring the starch hydrolysis phenotype, suggesting that this fragment contained the gene and its own promoter that allowed expression in E. coli. This fragment was cloned in pUC13 and pUC18, giving the plasmids pAEP-1 and pAEP-2 respectively.

To determine the nucleotide sequence of the cloned gene, partial digestion with exonuclease III was used to generate overlapping deletion clones. The 2.1 kb EcoRI–PvuII fragment was sequenced from both strands (Fig. 2); it revealed an ORF of 1509 bp. We called the gene lplA (see below for the explanation). The lplA gene coded for a 502 amino acid (56 kDa) protein with several interesting properties: (i) a putative signal sequence at the N-terminus (amino acids 1–21); (ii) a putative transmembrane anchor domain (amino acid 201–225) according to the hydropathy analysis plot (Hoop & Wood, 1981) (data not shown); and (iii) a putative leucine zipper (amino acid 384–433) with eight Leu or Ile residues.

In the 5' upstream untranslated region, typical B. subtilis vegetative promoter regions were found (a -35 region and a -10 TATA box) and also a ribosome binding domain close to the ATG. However, in the 3' downstream region we could not find any transcriptional terminator related sequence.

Surprisingly, neither the protein nor the nucleotide sequence showed homologies with any of the amylases whose sequences have been published, or to any protein or nucleotide sequence in the GenBank, EMBL, SwissProt or NBRF databases.

The B. subtilis lplA gene does not code for any amylase

To determine whether the cloned gene encoded an amylase, we expressed it in different Amy- B. subtilis strains. A 45 kb EcoRI–EcoRI fragment from pA-1 containing the whole gene and the regulatory sequences was subcloned in pUC13 and the resulting plasmid, named pAE-1, was cut with HindIII and ligated with HindIII-digested pC194, a typical B. subtilis vector. The resulting plasmid was named pLPLA. A negative control was constructed by digesting pC194 with HindIII, blunting with Klenow polymerase and then ligating with pUC18 previously digested with ScaI. This plasmid was named pCUC. A positive control, named pAMY, was constructed by introducing in pCUC a 2 kb EcoRI–HindIII fragment from plasmid pGA14, which contained the a-amylase gene from B. licheniformis. The three plasmids were used to transform two Amy- strains of B. subtilis (MB11 and DB104). Only when pAMY was used were amylolytic transformants obtained. Using pLPLA, none of the transformants showed any amylolytic phenotype.

Another way to determine if the isolated gene coded for an amylolytic enzyme was to disrupt the wild-type allele of the gene and then test whether the disrupted strain was able to show the original amylolytic phenotype. From a sequencing deletion clone, a 536 bp fragment corresponding to the central domain of the gene (Fig. 1a) was subcloned in pEm1, an integrative vector containing the B. subtilis erythromycin resistance marker. The resulting plasmid (pEm2) was used to transform the B. subtilis wild-type strain, and the disrupted transformants were selected by their ability to grow on erythromycin. The disruption was checked by Southern blot analysis (Fig. 1b). Transformants showed the two expected HindIII bands of 6.8 and 3.7 kb as predicted by the integration of the pEm2

![Fig. 1.](attachment:image.png)
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Fig. 2. Nucleotide sequence of the lplA gene and its deduced amino acid sequence. The typical vegetative promoter regions and the ribosome binding domain are underlined. The putative signal sequence is overlined and the arrow shows the putative cleavage site. The transmembrane anchor domain is boxed and the amino acids involved in the putative leucine zipper are also boxed.

plasmid into the B. subtilis chromosome. The disrupted strain showed the same level of amylolytic activity as the wild-type strain, in both solid and liquid culture. This confirmed that the cloned gene was not involved in starch degradation and did not code for an amylase. Also, the disrupted transformant showed no differences with respect to the wild-type strain in colony morphology, the profile of sugar assimilation (tested using a API 50 CH strip), growth kinetics, and sporulation capability (data not shown). Thus, the cloned gene appeared not to be involved in any vital cell function.

The expression of the lplA gene in E. coli causes cell lysis

If the B. subtilis lplA gene was not involved in the amylolytic system of this micro-organism, why did we isolate a plasmid containing it from E. coli cells that showed a clear amylolytic phenotype? The most plausible explanation was that the expression of the gene caused cell lysis and that the periplasmic or cytoplasmic α-amylases and the cytoplasmic amylomaltase of E. coli (Schwartz, 1987; Raha et al., 1992) were released to the medium, producing the characteristic amylolytic phenotype. To investigate this, we followed the appearance of a cytosolic marker, isocitrate dehydrogenase activity, in the culture medium. E. coli DH5α was transformed with pAE-1 and pUC13, and the transformants were grown on LB with ampicillin. An increase in the amount of isocitrate dehydrogenase was detected in the culture media of cells
containing the lplA gene (0.4 mU l⁻¹ in pUC13 transformed cells versus 20 mU l⁻¹ in pAE-1 transformed cells). Although this amount was only around 13% of the total enzyme found when the cells carrying pAE-1 were broken with glass beads, it seemed that this percentage of cell lysis was enough to release cellular contents and cause visible haloes of starch hydrolysis.

The growth kinetics were also studied in E. coli DH5a transformed with regular (pBR322 and pA-1) or high copy (pUC13 and pAE-1) number plasmids. As shown in Fig. 3, the growth curves of the strains carrying the lplA gene were delayed with respect to those of the strains carrying the vector alone. The delay was higher in the strain carrying the pAE-1 high copy number plasmid. After 6 h growth in selective media, we studied the presence of the 'amyloytic phenotype' in the cells and found that in the case of pA-1, the proportion of cells that showed an 'amyloytic phenotype' was 100%. However, in the case of pAE-1, only 13% of the population remained showing the 'amyloytic phenotype'. These results indicated that the E. coli lysis phenotype was related to the expression of the foreign gene, and that the higher the number of copies of the gene the more toxic the phenotype was. During growth, cells with low or no levels of expression of the lplA gene could be selected; this may be the explanation for the delay found in the growth curves.

**Only the N-terminal domain of LplA is involved in cell lysis**

To test the minimal sequence required to cause cell lysis, several clones were constructed that lacked different portions of the gene. Plasmid pAES-1 was constructed by eliminating the 0.5 kb SalI-PradI fragment from pAEP-1, and then religating. Plasmid pAES-1Δ1 was a 3' deletion clone from pAES-1 which lacked the leucine zipper and the transmembrane domain. Finally, pAES-1Δ2 was constructed by fusing in frame a 5' deletion clone from pAES-1 and an EcoRI-BsmI fragment containing the regulatory sequences and the ATG codon of the gene; the fusion was checked by sequencing. These constructs were used to transform E. coli, and in each case, the appearance of the lysis phenotype was tested by the starch hydrolysis test (Fig. 4).

Plasmids pAES-1 and pAES-1Δ1 still showed a clear lysis phenotype, so the absence of the putative leucine zipper in the former (pAES-1), or the absence of the putative transmembrane anchor domain in the latter (pAES-1Δ1), did not eliminate the lysis phenotype; therefore, they were not responsible for this phenotype. On the other hand, plasmid pAES-1Δ2, which lacked the N-terminal domain but still synthesized most of the protein, showed no lysis. Taking all these results together, we concluded that a domain located at the N-terminus of the LplA protein was responsible for the lysis phenotype.

Recently it has been shown that the signal sequence of the S. pneumoniae amiA and malX gene products was responsible for the lytic phenotype when these genes were expressed in E. coli (Martin et al., 1989). As shown in Fig. 5, there was a great deal of homology between the signal sequence cleavage sites of the E. coli Lpp lipoprotein, MalX and AmiA (from S. pneumoniae), other lipoproteins from Bacillus species and LplA (B. subtilis).

Table 1: Deletion analysis of the domain of LplA involved in cell lysis.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>E SP</th>
<th>TM</th>
<th>LZ</th>
<th>P</th>
<th>Lytic phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAES-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>pAES-1Δ1</td>
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<td>pAES-1Δ2</td>
<td></td>
<td></td>
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<td>-</td>
</tr>
</tbody>
</table>

Table 1: Homology between the N-terminal domains of different Gram-positive lipoproteins, LplA and the major E. coli lipoprotein (Lpp). Residues shown in lower-case letters correspond to the mature N-terminal part of the proteins. Similarities between LplA and Lpp are shown: identical amino acids are indicated by asterisks (*); similar amino acids are indicated by colons (:). Dots indicate gaps introduced in one sequence to give maximum correspondence. References: LplA, this work; Lpp, Martin et al. (1989); AmiA, Martin et al. (1988); MalX, Martin et al. (1988); PrsA, Simonen & Palva (1993); PAL, Simonen & Palva (1993); PenP, Simonen & Palva (1993).

It has also been described that the expression of S. pneumoniae AmiA and MalX lipoproteins in E. coli resemble some phenotypes of the E. coli lpp mutants, as sensitivity to EDTA (Martin et al., 1989). In order to demonstrate the EDTA sensitivity of E. coli cells containing the lplA gene, E. coli DH5α was transformed with pAES-1 or with the vector alone (pUC13). pAES-1 was
divided into two aliquots of 25 ml. EDTA was added to a final concentration of 10 mM to one aliquot and then all the cultures were grown at 37 °C. At different times, samples were taken from each culture and the c.f.u. per ml determined. As shown in Fig. 6, cells carrying pAES-1 were sensitive to EDTA, as has been observed in many mutants defective in the outer membrane. From these results, we inferred that the B. subtilis lplA gene may work in E. coli in the same way as do other lipoproteins. For this reason we named it lplA (lipoprotein like).

LplA behaves as a lipoprotein in E. coli

Further confirmation of the lipoprotein nature of LplA was its lipophilic modification by the addition of radio-labelled palmitic acid. As shown in Fig. 7, when E. coli cells carrying pAES-1 were incubated with [9,10(n)-3H] palmitic acid for 1 h, we were able to recover a lipoprotein band that corresponded to the expected molecular mass of the truncated LplA protein (42 kDa). This lipoprotein was absent in E. coli cells transformed with the vector alone (pUC13) and also in cells transformed with the construction that lacked the N-terminal domain of LplA (pAES-1Δ2), confirming that this part of the protein was completely essential for the lipophilic modification of LplA.

DISCUSSION

Cloning of genes encoding extracytoplasmic proteins in E. coli is sometimes difficult or even impossible, presumably because it leads to cell death (Henning et al., 1979). When the protein to be expressed is targeted to the membrane, the effects of its expression may be more deleterious since the protein may be anchored, altering the properties of these membranes. This seems to be the case when the LplA protein is expressed. The protein has a consensus N-terminal signal sequence that may allow it to be translocated through the inner membrane of E. coli. LplA also contains a typical transmembrane anchor domain that may enable the protein to be anchored in the inner membrane, and a putative leucine zipper domain at the C-terminus, that may be involved in the oligomerization of the protein at the level of the membrane (Fig. 2).

From our results (Fig. 4), we know that both the transmembrane anchor domain and the leucine zipper are not responsible for the lytic phenotype observed when the protein is expressed in E. coli. Only the N-terminal domain of the protein seems to play a crucial role in cell lysis. Recently, it has been shown that signal sequences of other Gram-positive bacteria proteins are responsible for a lytic phenotype when they are expressed in E. coli (Gilson et al., 1988; Martin et al., 1989). It was suggested that the deleterious effects of these proteins could be directly related to the sequence of their signal peptides because they corresponded to the consensus cleavage sequence of E. coli lipoprotein precursors: Leu-Y-Z-[cleavage site]-Cys-y-z; where Y is Ala, Ser, Val, Gln or Thr; Z is Gly or Ala; y is Ser, Gly, Ala, Asn, Gln or Asp; and z is Ser, Ala, Asn or Gln [see Yamaguchi et al. (1988) for a review]. Normal lipoproteins in E. coli are exported through the cytoplasmic membrane, where the signal

chosen since it showed greater phenotype stability than pAE-1. After 6 h growth, more than 90% of the cells still showed an amylolytic phenotype. Perhaps the downstream piece of DNA contained in plasmid pAE-1 could account for the different stability of the phenotype. Cells were grown overnight in 5 ml LB with 100 µg ampicillin ml⁻¹. A 250 µl portion of the overnight culture was used to inoculate 50 ml of fresh LB/ampicillin medium. Cells were grown at 37 °C for 150 min and then the culture was

Fig. 6. EDTA sensitivity of E. coli transformed with different plasmids (see text for details). ○, pUC13-EDTA; ●, pUC13 + EDTA; △, pAES-1-EDTA; ▲, pAES-1 + EDTA.

Fig. 7. Lipophilic modification of LplA. E. coli cells were labelled with [3H]palmitic acid as described in Methods and the protein extracts analysed by SDS-PAGE and fluorography. Molecular size markers are on the left (kDa).
sequence is cleaved, and the N-terminal cysteine is then transformed into a lipo-amino acid. This lipophilic modification is thought to be responsible for membrane anchorage of the lipoproteins. Due to the homology between the signal sequence of the \textit{E. coli} lipoprotein Lpp and the \textit{B. subtilis} LplA protein we suggest that the \textit{E. coli} export machinery would recognize the LplA signal sequence as a normal lipoprotein: the LplA N-terminal cysteine would be lipophilically modified and the protein would be anchored to the membrane by this lipophilic modification. Such a mechanism has been suggested for the export of MalX and AmlA lipoproteins (Gilson et al., 1988; Martin et al., 1989). In addition, the second amino acid of the LplA mature protein is a Ser, which seems to be a specific signal for targeting proteins to the outer membrane in \textit{E. coli} (Gennity & Inouye, 1991; Gennity et al., 1992). Sensitivity to EDTA (Fig. 6) suggests that the lytic phenotype was related to a depletion in regular lipoproteins, as has also been suggested for other Gram-positive bacteria lipoproteins such as MalX and AmlA (Martin et al., 1989). The lipophilic modification of LplA with palmitic acid (Fig. 7) clearly demonstrates that the protein behaves as a lipoprotein in \textit{E. coli}.

During the selection procedure, only clones carrying plasmids with different insert length but always containing the \textit{lplA} gene were picked up. Perhaps other \textit{B. subtilis} lipoproteins could escape from the initial selection procedure because they gave a different colony phenotype or they were so toxic that cell growth was inhibited.

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**REFERENCES**


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