The Helicobacter pylori ureC Gene Codes for a Phosphoglucosamine Mutase

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The function of UreC, the product of a 1,335-bp-long open reading frame upstream from the urease structural genes (ureAB) of Helicobacter pylori, was investigated. We present data showing that the ureC gene product is a phosphoglucosamine mutase. D. Mengin-Lecreulx and J. van Heijenoort (J. Biol. Chem. 271:32–39, 1996) observed that UreC is similar (43% identity) to the GlmM protein of Escherichia coli. Those authors showed that GlmM is a phosphoglucosamine mutase catalyzing interconversion of glucosamine-6-phosphate into glucosamine-1-phosphate, which is subsequently transformed into UDP-N-acetylgalactosamine. The latter product is one of the main cytoplasmic precursors of cell wall peptidoglycan and outer membrane lipopolysaccharides. The present paper reports that, like its E. coli homolog glmM, the H. pylori ureC gene is essential for cell growth. It was known that growth of a lethal conditional glmM mutant of E. coli at a nonpermissive temperature can be restored in the presence of the ureC gene. We showed that complete complementation of the glmM mutant can be obtained with a plasmid overproducing UreC. The peptidoglycan content and the specific phosphoglucosamine mutase activity of such a complemented strain were measured; these results demonstrated that the ureC gene product functions as a phosphoglucosamine mutase. Homologs of the UreC and GlmM proteins were identified in Haemophilus influenzae, Mycobacterium leprae, Clostridium perfringens, Synechocystis sp. strain PCC6803, and Methanococcus jannaschii. Significant conservation of the amino acid sequence of these proteins in such diverse organisms suggests a very ancient common ancestor for the genes and defines a consensus motif for the phosphoglucosamine mutase active site. We propose renaming the H. pylori ureC gene the glmM gene.

Helicobacter pylori is a microaerophilic, gram-negative bacterium which is an etiologic agent of chronic gastritis; it is associated with peptic ulceration and some cases of gastric carcinoma (15). Various virulence factors have been identified, but the pathogenic mechanisms of H. pylori are still poorly understood (7). One of the identified factors, a very active urease, is common to all H. pylori isolates. The genetic organization of a large H. pylori DNA region comprising the urease structural (ureAB) and accessory (ureIEFGH) genes has been determined (2, 8) (see Fig. 1). The presence of a 2.2-kb-long DNA region immediately upstream from the ureAB genes is required to confer urease activity on a Campylobacter jejuni recipient strain (8). However, nothing was known about the importance of this region containing two open reading frames (ORFs), designated ureC and ureD, for urease production in H. pylori. No role has been attributed to the ureD ORF and, similarly, no function has been defined for the product of the ORF ureC (1,335 bp), which maps 820 bp upstream from the start codon of ureA and is transcribed in the same direction (Fig. 1). Downstream from the ureC gene, there is a palindromic sequence followed by a run of eight T residues showing all of the characteristics of the Esherichia coli rho-independent terminators, suggesting that ureC and ureA are not co-transcribed. Sixty H. pylori isolates have been investigated by PCR analysis and DNA sequencing for the presence of ureC; the gene was found in every isolate, and the UreC protein encoded is highly conserved (reference 6 and unpublished results).

A previously published (13) search of protein databases for homologs of the E. coli phosphoglucosamine mutase GlmM identified a protein with 43% identity: the ureC gene product. Herein, we present data showing that the ureC gene product is a phosphoglucosamine mutase, an enzyme which catalyzes the interconversion of GlcN-6-phosphate (GlcN-6-P) and GlcN-1-P isomers (13). In E. coli, GlcN-1-P is acetylated to GlcNAc-1-P and then UDP ribosylated to produce UDP-GlcNAc. UDP-GlcNAc is one of the main cytoplasmic precursors of bacterial cell wall peptidoglycan (20) and in E. coli (and other gram-negative bacteria) is also a precursor of outer membrane lipopolysaccharide (16).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The E. coli strains used in this work are listed in Table 1. pILL394, carrying a 5.1-kb DNA fragment from H. pylori including the ureC gene, has been previously described (8), and the pTrc99A vector was purchased from Pharmacia (Uppsala, Sweden). Unless indicated otherwise, 2YT, a rich medium, was used to grow cells (14). Growth was monitored by measuring optical density at 600 nm (OD600) with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). For strains carrying drug resistance genes, antibiotics were used at the following final concentrations (in micrograms per milliliter): ampicillin, 100; spectinomycin, 100; kanamycin, 30; chloramphenicol, 25.

General DNA techniques. E. coli cell transformation, and complementation of the E. coli glmM thermosensitive mutant. The alkaline lysis method was used for both small- and large-scale plasmid preparations. Plasmid DNA was further purified on cesium chloride-ethidium bromide gradients (18). Standard procedures for endonuclease digestions, ligation, and agarose electrophoresis were used (18). E. coli cells were made competent and transformed with plasmid DNA either by the method of Dagert and Ehrlich (3) or by electroporation. Thermosensitive mutant strain GPM83 was transformed with the plasmids to be tested as follows. Competent cells (300 µl), mixed with plasmid DNA, were kept on ice for 3 h and then heated for 3 min at 42°C. A 400-µl volume of 2YT

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medium was then added, and the cells were incubated at 30°C for over 2 h to allow expression of plasmid-encoded genes. One-hundred-microliter aliquots of the cell suspension were plated onto two prewarmed 2YT-antibiotic plates, one incubated at 30°C and the other incubated at 42°C. Growth was observed after 24 h of incubation.

Construction of plasmids. A plasmid suitable for high-level overproduction of UreC was constructed as follows. PCR primers were designed to incorporate a BspHI site (in boldface) 5’ to the initiation codon (underlined) of ureC (5’-TAAATCTGGAATTTTGGACTATGTT-3’) and a PstI site (in boldface) 3’ to the gene after the stop codon (5’-TATAGCGATTTGGCAAAAATGGCCCTTT-3’). These primers were used to amplify the ureC gene from plasmid pILL594. The isolated DNA was then treated with BspHI and PstI, and the two resulting fragments (ureC contains an internal BspHI site) were then ligated together between the compatible NcoI and PstI sites of vector pTrc99A. The ligation mixture was used to transform (by electroporation) strain GPM83, and transformants were selected for both ampicillin resistance and growth at 42°C. About 20 such transformants were isolated, all carrying the desired construct, named pMLD106, allowing expression of the reconstituted ureC gene under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter.

TABLE 1. E. coli strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM83</td>
<td>F- ara D (lac-proAB) rpsL thi [F80 d lac (lacZ M15)]</td>
<td>21</td>
</tr>
<tr>
<td>GPM83</td>
<td>JM83 glmM::kan (pGMM)</td>
<td>13</td>
</tr>
<tr>
<td>URE832</td>
<td>JM83 glmM::kan (pILL594)</td>
<td>This work</td>
</tr>
<tr>
<td>URE832</td>
<td>JM83 glmM::kan (pMLD106)</td>
<td>This work</td>
</tr>
</tbody>
</table>

Preparation of crude enzyme. Cells (0.5-liter cultures) were grown exponentially at 30 or 42°C in 2YT medium to an OD600 of 0.7 (250 mg of bacteria [dry weight] per liter of culture). URE832 cells (0.5-liter cultures) were grown exponentially at 30 or 42°C in 2YT medium to an OD600 of 0.5 (250 mg of bacteria [dry weight] per liter of culture). The isolated DNA was then treated with BspHI and PstI, and the two resulting fragments (ureC contains an internal BspHI site) were then ligated together between the compatible NcoI and PstI sites of vector pTrc99A. The ligation mixture was used to transform (by electroporation) strain GPM83, and transformants were selected for both ampicillin resistance and growth at 42°C. About 20 such transformants were isolated, all carrying the desired construct, named pMLD106, allowing expression of the reconstituted ureC gene under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter.

To test whether a GlmM deficiency in E. coli could be compensated by the H. pylori UreC protein, we used the E. coli glmM lethal conditional mutant GPM83 (13). This strain is a JM83 derivative and contains (i) an inactivated glmM copy on the chromosome and (ii) a wild-type glmM allele on plasmid pGMM, a derivative of vector pMAK705 which bears a thermosensitive replicon (Table 1). At a nonpermissive temperature for plasmid replication (42°C), GPM83 failed to grow on 2YT plates because of the loss of plasmid-borne glmM. At a permissive temperature (30°C), JM83 and GPM83 were identical in growth rate and cell morphology. Strain GPM83 was transformed with pILL594 (Fig. 1), a pILL570 derivative (itself derived from pBR322) carrying part of the urease region and the entire ureC gene under control of its own promoter (9). As previously described (13), this strain, designated URE831 (Table 1), grew on plates at the nonpermissive temperature (42°C). However, strain URE831 formed smaller colonies than did strain GPM83 harboring a pUC18 derivative, pMLD99, carrying glmM (13) under the same growth conditions. The loss of thermosensitive plasmid pGMM in strain URE831 was verified by screening for loss of chloramphenicol resistance conferred by pGMM. During liquid growth at 42°C, the URE831 cells bulged slightly and were ovoid in shape as assessed by phase-contrast microscopy, suggesting some defect in cell wall synthesis. The complementation of the E. coli glmM mutant by ureC on pILL594 was presumably, therefore, only partial. Consequently, we constructed a plasmid overproducing UreC in E. coli.

Assay for phosphoglucomamine mutase activity. A coupled assay was routinely used in which the GlcN-1-P synthesized from GlcN-6-P by the mutant was quantitatively converted to UDP-GlcN-ac in the presence of the purified bifunctional GlmU enzyme (12). The standard assay mixture contained 50 mM Tris-HCl buffer (pH 8.0), 3 mM MgCl2, 1 mM GlcN-6-P, 0.4 mM [14C]acetate (37 Ci/mmol, 400 Bq), 10 mM IPTG, 0.1 mM Glc-1,6-diP, 1 μg of the pure GlmU enzyme, and the crude enzyme (0.1 to 10 μg of protein, depending on overexpression and purification factors) in a final volume of 100 μl. Mixtures were incubated at 37°C for 30 min, and reactions were terminated by addition of 10 μl of acetic acid. Reaction products were separated by high-voltage electrophoresis on Whatman 3MM filter paper in 2% formic acid (pH 1.9) for 90 min at 40 V/cm with an LT36 apparatus (Savant Instruments, Hicksville, N.Y.). The only two radioactive spots (acetate-CoA and UDP-GlcN-ac) were located by overnight autoradiography with type B2 films (3M, St. Paul, Minn.) or with a radioactivity scanner (Multi-Tracker LB285; Berthold, Elancourt, France). Spots were cut out and counted in an Intertechnique SL 30 liquid scintillation spectrophotometer with a solvent system consisting of 2 ml of water and 13 ml of Aqualyte mixture (J. T. Baker Chemicals, Deventer, The Netherlands). One unit of enzyme activity was defined as the amount which catalyzed the formation of 1 μmol GlcN-1-P per min.

Chemicals. [14C]Acetate-CoA (1.85 GBq · mmol−1) was purchased from ICN (Irvine, Calif.), GlcN-1-P, GlcN-6-P, Glc-1,6-diP, UTP, and UDP-GlcN-ac were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Complementation of an E. coli glmM conditional mutant by the H. pylori ureC gene. In E. coli, the glmM gene, like the other genes involved in peptidoglycan synthesis, is essential for bacterial growth. Attempts to inactivate ureC on the H. pylori chromosome by allelic exchange with a copy of the gene interrupted by a kanamycin resistance cassette were repeatedly unsuccessful, indicating that ureC is also an essential gene in H. pylori.

To test whether a GlmM deficiency in E. coli could be compensated by the H. pylori UreC protein, we used the E. coli glmM conditional mutant GPM83 (13). This strain is a JM83 derivative and contains (i) an inactivated glmM copy on the chromosome and (ii) a wild-type glmM allele on plasmid pGMM, a derivative of vector pMAK705 which bears a thermosensitive replicon (Table 1). At a nonpermissive temperature for plasmid replication (42°C), GPM83 failed to grow on 2YT plates because of the loss of plasmid-borne glmM. At a permissive temperature (30°C), JM83 and GPM83 were identical in growth rate and cell morphology. Strain GPM83 was transformed with pILL594 (Fig. 1), a pILL570 derivative (itself derived from pBR322) carrying part of the urease region and the entire ureC gene under control of its own promoter (9). As previously described (13), this strain, designated URE831 (Table 1), grew on plates at the nonpermissive temperature (42°C). However, strain URE831 formed smaller colonies than did strain GPM83 harboring a pUC18 derivative, pMLD99, carrying glmM (13) under the same growth conditions. The loss of thermosensitive plasmid pGMM in strain URE831 was verified by screening for loss of chloramphenicol resistance conferred by pGMM. During liquid growth at 42°C, the URE831 cells bulged slightly and were ovoid in shape as assessed by phase-contrast microscopy, suggesting some defect in cell wall synthesis. The complementation of the E. coli glmM mutant by ureC on pILL594 was presumably, therefore, only partial. Consequently, we constructed a plasmid overproducing UreC in E. coli.

Concentration of an E. coli strain overexpressing the H. pylori ureC gene. To overproduce UreC in E. coli, the ureC gene was put under control of the IPTG-inducible trc promoter of vector pTrc99A to give pMLD106 (see Materials and Methods and Fig. 1). Introduction of pMLD106 into E. coli glmM mutant GPM83 resulted in strain URE832 (Table 1). Strain URE832 formed normal colonies (i.e., comparable to those of strain GPM83 carrying pMLD99) at the nonpermissive temperature, even in the absence of IPTG, indicating that the repression by the plasmid lacIq gene was not complete.

Strain URE832 was grown in the presence of 1 mM IPTG at the nonpermissive temperature. The ureC gene was strongly overexpressed. SDS-PAGE was used to analyze crude extracts (prepared as detailed in Materials and Methods) of strain URE832 carrying grown in the absence or presence of 1 mM IPTG (lanes B and C of Fig. 2, respectively) and of parental strain JM83 carrying the vector pTrc99A as a control (lane A of Fig. 2). A large amount of an approximately 50-kDa protein was detected in strain URE832 grown in the presence of IPTG. This protein corresponds to UreC; its molecular mass is in
FIG. 1. Localization of the ureC gene near the urease region of H. pylori and restriction maps of the H. pylori DNA insert of pILL594 (8) and the entire pMLD106 plasmid constructed to overexpress the ureC gene. The genes are shown as boxes with arrows indicating the direction of their transcription. The boxed region of pMLD106 corresponds to the vector pTrc99A with the IPTG-inducible promoter P_{trc}, the lacP8 gene, the pBR322 origin of replication, and the gene conferring ampicillin resistance. ureB indicates that this gene is truncated in pILL594.

Phosphoglucomannose mutase

Genes coding for the urease structural subunits

Accessory genes required for nickel incorporation into the apoenzyme

urease region of H. pylori

H. pylori DNA insert of plasmid pILL594

pMLD106

1 Kb

According to the 49,191 Da calculated from the deduced amino acid sequence of ureC.

In strain URE832 grown with IPTG, about two-thirds of the UreC protein was overexpressed and was thus not retained in the soluble fraction obtained after sonication. Aggregates, presumably of UreC, were also detected by microscopic examination of these cells as intracellular refringent nodules. In addition, these cells presented an unusual morphology: they were curved and presented a clear defect in cell septation.

Peptidoglycan content of the E. coli glmM conditional mutant complemented by the ureC or glmM gene product. When a culture of strain GPM83 was shifted from exponential growth at 30°C (at a cell density of approximately 6 \times 10^{8} \text{ ml}^{-1}) to the nonpermissive temperature of 42°C, the growth rate rapidly slowed and finally stopped 5 h later (after approximately 12 doubling times) at a cell density of approximately 3 \times 10^{8} \text{ ml}^{-1}. At that stage, cells entered into a prococious stationary phase and progressively lysed, as shown by a slow decrease of the culture OD_{600}. In addition, the cell morphology progressively changed from rods to enlarged ovoids whereas that of parental cells grown under the same conditions was unaltered. This altered morphology was a consequence of progressive depletion of the GlmM protein, an enzyme essential for peptidoglycan synthesis, which was itself due to the dilution of plasmid pGMM by cell division (13). To evaluate the effects on peptidoglycan synthesis of the glmM mutation alone or complemented by the wild-type glmM allele or by the H. pylori ureC gene, the murein contents of sacculi of various E. coli transformants were measured (Table 2 and Materials and Methods).

The phosphoglucomannose concentration in strain GPM83 was 9,000 nmol \cdot g^{-1} (dry weight) of bacteria^{-1} during growth at 30°C. At 42°C, it reached the critical value of 5,500 nmol \cdot g^{-1}, at which the cells stop growing and start to lyse (Table 2). Strain URE831, grown at 42°C, contained only 7,000 nmol \cdot g^{-1}, confirming the partial complementation by the ureC gene expressed from plasmid pILL594 (Table 2). However, this peptidoglycan level was sufficient to allow growth. It is thus possible to reduce the E. coli peptidoglycan content by about 30 to 40% without seriously affecting cell wall integrity. This suggested that about 50% of the total murein content of the cell is in excess, possibly allowing maintenance of cell integrity if growth conditions change.

In strain URE832 grown at 42°C with or without IPTG, sufficient amounts of UreC protein were produced to fully replace the E. coli GlmM protein for peptidoglycan synthesis since the murein level was equivalent to that of a wild-type strain (Table 2).

Levels of phosphoglucomannose mutase of the E. coli glmM conditional mutant complemented by the ureC or glmM gene product. To measure phosphoglucomannose mutase activity, a coupled assay in the presence of the purified bifunctional enzyme GlmU was used (for details, see Materials and Methods). Strain GPM83 grown at 42°C presented negligible phosphoglucomannose mutase activity, 2% of that of the wild type (Table 2). The reduced growth rate and subsequent lysis of this strain are thus, indeed, a consequence of progressive GlmM depletion. Measurement of the phosphoglucomannose mutase activity in strain GPM83 complemented by the ureC gene thus allowed direct evaluation of H. pylori mutase activity with no significant interference from the host (E. coli) GlmM activity.

In strain URE831, the phosphoglucomannose mutase activity of UreC was low, only 6% of wild-type GlmM activity (Table 2). This was, however, sufficient to allow growth of the corresponding cells, despite an apparently imperfect cell wall. In strain URE832 carrying the ureC gene under control of the P_{Trc} promoter on pMLD106, in the absence of IPTG, the H. pylori phosphoglucomannose mutase activity was only 12% of wild-type GlmM activity (Table 2). Interestingly, this low activity level is apparently sufficient for E. coli to show normal...
by SDS-PAGE analysis of crude extracts. This excess phosphogluco-
mucase activity also seems to be deleterious for cell wall synthesis, since these cells presented abnormal shapes and an apparent septation defect.

Identification of a new family of proteins: the phosphoglu-
cosamine mutases. Searches of the data banks identified pro-
tein similar to GlmM in the following organisms that have been the subjects of partial or total genome sequencing projects (Fig. 3): *Haemophilus influenzae* (4), 73% identity along 445 residues (accession no. P45164); *Mycobacterium leprae*, 47% identity along 463 residues (accession no. U00020); *Clostridium perfringens* (partial sequence): 45% identity along 140 residues (accession no. X86494); *Synechocystis* sp. strain PCC6803 (5), 40% identity along 481 residues (accession no. D90908); *Methanococcus jannaschii* (1), 31% identity along 448 residues (accession no. U67553). No function has previously been attributed to these putative proteins, although relatedness to phosphoglucomutases has been suggested for some of them. Six sequences significantly similar to that of GlmM, including the *H. pylori* UreC sequence (43% identity along 445 residues), are compared to the *E. coli* GlmM se-
quency in Fig. 3. There are highly conserved regions, possibly those involved in the enzymatic specificity of these proteins. The presence of GlmM homologs in these organisms also suggests that they have a metabolic pathway leading to UDP-
GlcNAc analogous to that described in *E. coli*.

A sequence similar to the putative active site of hexosephos-
phate mutases has been identified in the GlmM sequence (13).

The following consensus motif for the phosphogluco-
mutases has been identified in the GlmM sequence (13). A sequence similar to the putative active site of hexosephosphate mutases has been identified in the GlmM sequence (13). The following consensus motif for the phosphogluco-
mutases has been identified in the GlmM sequence (13).

<table>
<thead>
<tr>
<th>Strain (condition)</th>
<th>Temp (°C)</th>
<th>Relevant genotype</th>
<th>Peptidoglycan content (mmol \cdot g [-1 \cdot dry wt] of bacteria)</th>
<th>Sp act (U \cdot mg of protein)</th>
<th>Sp act amplification factor</th>
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<tbody>
<tr>
<td>JM83</td>
<td>30</td>
<td>Wild-type <em>glmM</em></td>
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<td>0.05</td>
<td>1</td>
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<td>JM83</td>
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<td>Wild-type <em>glmM</em></td>
<td>9,000</td>
<td>0.04</td>
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<td>9,000</td>
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<td>1.16</td>
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<td><em>glmM</em> Ts mutant</td>
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<td>9,000</td>
<td>0.006</td>
<td>0.12</td>
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<tr>
<td>URE832 (with 1 mM IPTG)</td>
<td>42</td>
<td><em>glmM</em> Ts mutant carrying pMLD106</td>
<td>9,000</td>
<td>0.95</td>
<td>19</td>
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</table>

* Cells were grown exponentially in 2YT medium at 30°C (permissive temperature) or first at 30°C and then for 5 h at 42°C (nonpermissive temperature), the time at which the growth rate of mutant strain GPM83 began to decrease (13).

* The peptidoglycan content of sacculi was quantified as detailed in Materials and Methods and expressed in terms of muramic acid content.

* The peptidoglycan content of sacculi was quantified as detailed in Materials and Methods and expressed in terms of muramic acid content.

* Phosphoglucozymic specific activity was measured as detailed in Materials and Methods. One unit of enzyme activity was defined as the amount which catalyzed the synthesis of 1 μmol GlcN-1-P per min.

* Ts, temperature sensitive.
No UreC or GlnM homologs were found in the complete genomic sequences of Mycoplasma genitalium and Mycoplasma pneumoniae, which are known to be cell wall-less bacteria. One could propose either that these organisms possess no phosphoglucomutase, possibly because this function was lost during evolution, or that the amino acid sequence of the Mycoplasma phosphoglucomutase had considerably diverged and could not be distinguished, by data bank analysis, from those of the other hexosephosphate mutases.

In conclusion, although little data about the H. pylori cell wall is available, our finding of a phosphoglucomutase suggests the existence of a pathway similar to that described in E. coli for the synthesis of cell wall peptidoglycan and lipopolysaccharide. That ureC is an essential gene in H. pylori supports this hypothesis and might open new perspectives in the search for antimicrobial agents against H. pylori. Finally, the role of the product of ureC does not include involvement in the expression of urease activity in C. jejuni (8). Consequently, we favor the hypothesis that, in C. jejuni, the 2.2 kb-long DNA region upstream from ureA might be a putative transcriptional activator for full expression of the ureAB genes in this organism. For these reasons, we decided to rename the H. pylori ureC gene the glmM gene.

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REFERENCES


