Oxolinic Acid-resistant Mutants of *Bacillus subtilis*

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Two mutants of *Bacillus subtilis* have been independently selected for resistance to oxolinic acid, an inhibitor of DNA gyrase. The mutations, designated *oxr-1* and *oxr-2*, map very close to one another but are clearly separated from mutations in the genes for DNA gyrase. Many of the phenotypic properties of the mutants differ from those of a strain containing the *gyrA* mutation described by other workers. In particular, the *oxr* strains are as sensitive as the wild-type to inhibition by nalidixic acid on solid medium. In addition, experiments with DNA synthesis in toluenized cells show that the enzyme of the *gyrA* mutant is resistant to oxolinic acid, whereas DNA synthesis in the *oxr* mutants is as sensitive as it is in wild-type preparations. It is concluded that resistance to oxolinic acid is not due to an alteration in the DNA gyrase, but is more probably the result of an impaired uptake of the inhibitor.

Although growth of the mutants on agar plates is inhibited at high concentrations of oxolinic acid, lower concentrations (1-2 μg ml⁻¹) can be used to distinguish them from the wild-type. The *oxr-1* and *oxr-2* mutations define a new genetic locus and can be used as genetic markers in *B. subtilis*.

**INTRODUCTION**

Oxolinic acid and nalidixic acid are two closely related inhibitors which prevent synthesis of DNA by interacting with the *gyrA* subunit of the enzyme DNA gyrase. This is a topoisomerase which controls the extent of DNA supercoiling (Sugino *et al.*, 1977; Gellert *et al.*, 1977) and its action is necessary for DNA replication.

Sugino *et al.* (1977) have shown that DNA gyrase isolated from nalidixic acid-resistant strains of *Escherichia coli* is resistant to both oxolinate and nalidixate in in vitro assays. Gyrase has also been isolated from *Micrococcus luteus* (Liu & Wang, 1978) and from *Bacillus subtilis* (Sugino & Bott, 1980). However, in neither study was it shown that DNA gyrase isolated from nalidixic acid-resistant strains was resistant to both inhibitors.

In this paper we describe the independent isolation of two oxolinic acid-resistant strains which differ genetically and physiologically from the previously described nalidixic acid-resistant strains. The results presented show that the resistance to oxolinate is not due to an alteration of DNA gyrase. Instead, the evidence indicates that the permeability of the cells to oxolinate in these strains has been altered.

**METHODS**

*Organisms.* *Bacillus subtilis* Nil, a derivative of strain 168 *trpC2*, was used; it will be referred to as the wild-type. It is auxotrophic for thymine and tryptophan and carries a defective PBSX phage in its genome which does not induce lysis of the cells when DNA synthesis is inhibited (Karamata, 1968). Strains PB1706

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(trpC2 hisB2 tyrA araB2 nal3) and PB1707 (pyrA16 leu8 nal3) were kindly donated by Dr Mazza and are resistant to high concentrations of nalidixic acid (300 µg ml⁻¹).

**Growth.** Casein hydrolysate (CH) medium was prepared as described by Sterlini & Mandelstam (1969). CH medium was inoculated with a colony from a plate and grown overnight with shaking at 30 °C. The culture was then transferred to 37 °C and allowed to grow at this temperature for at least two generations. Growth was followed by measuring the turbidity of the cultures at 600 nm using a Unicam SP600 spectrophotometer. Readings were converted to dry wt ml⁻¹ with a calibration curve relating turbidity to dry weight.

**DNA synthesis.** This was followed by measuring incorporation of [methyl-³H]thymidine into DNA. Cells were grown in CH medium containing radioactive thymidine (0.2 µCi ml⁻¹) and carrier thymidine (20 µg ml⁻¹). Samples (1 ml) were taken at intervals and fixed in trichloroacetic acid (TCA) (1 ml; 10%; w/v). The samples were filtered by suction through glass-fibre filters (Whatman GF/C, 2-1 cm diam., 1-2 µm pore size). The filters were then washed with TCA (10 ml; 5%, w/v) containing thymidine (300 µg ml⁻¹) and then with ethanol (10 ml; 96%, v/v). They were dried in a vacuum oven at 80 °C for 60 min and then transferred to plastic scintillation vials containing 5 ml scintillation fluid [2-(4'-tert-butylphenyl)-5-(4"'-biphenylyl)-1,3,4-oxadiazole (butyl-PBD); 0.5%, w/v, in toluene]. Radioactivity was counted in a Wallace LKB 1215 Rack Beta scintillation counter, using the module for ³H.

**Degradation of DNA.** Cells were grown from a small inoculum in CH medium containing [methyl-³H]thymidine (0.5 µCi ml⁻¹) and carrier thymidine (20 µg ml⁻¹) to a density of about 0-06 mg dry wt ml⁻¹. They were harvested, washed once with sporulation medium (Sterlini & Mandelstam, 1969) and then resuspended in CH medium containing excess unlabelled thymidine (100 µg ml⁻¹). Nalidixic acid or oxolinic acid was then added and samples (1 ml) were collected at intervals and added to tubes containing TCA (1 ml; 10% w/v). The suspensions were centrifuged and portions (0-1 ml) of the supernatant were put on to glass-fibre filters (Whatman GF/B, 2-1 cm diam). The filters were dried and counted.

**Short-term viability.** This term refers to viability in liquid medium over a period of a few hours. Cultures were grown in CH medium to a cell density of 0-06-0-10 mg dry wt ml⁻¹. Nalidixic acid or oxolinic acid was then added and samples (0-1 ml) were taken at intervals for up to 4 h. The samples were then diluted with sporulation medium and suitable dilutions (10³, 10⁴ and 10⁵) were plated in duplicate on nutrient agar plates.

**Long-term viability.** This is what is generally referred to as 'viability', i.e. the ability of a cell to form a colony on solid medium. Brain heart infusion broth (BHIB; Oxoid) was inoculated with a small colony and grown at 37 °C for 48 h, when more than 95% of the cells had been killed, the culture was diluted 10-fold and 100-fold in fresh CH medium and incubated overnight; the treatment with oxolinic acid was then repeated. After eight cycles, the predominant population consisted of a resistant strain which was designated Nil (oxr-2) and Nil (oxr-1) was obtained after repeated cycles of short-term killing of Nil cells in liquid medium. An exponential-phase culture of Nil in CH medium was treated with oxolinic acid (10 µg ml⁻¹) when the density had reached 0-140 mg dry wt ml⁻¹. After 5 h, when more than 95% of the cells had been killed, the culture was diluted 10-fold and 100-fold in fresh CH medium and incubated overnight; the treatment with oxolinic acid was then repeated. After eight cycles, the predominant population consisted of a resistant strain which was designated Nil (oxr-2).

Nil (oxr-2) was obtained by mutagenesis of Nil with ethyl methanesulphonate (EMS). Nil cells were grown in BHIB to a density of 0-12 mg dry wt ml⁻¹. EMS (0-2 M final concn) was then added to the cells and the culture was incubated for 30 min. The cells were washed with sporulation medium and resuspended in BHIB, and the culture was grown overnight at 37 °C. The following day the cells were spread on to plates containing oxolinic acid (5 µg ml⁻¹). Nil (oxr-2) was the genetically most stable colony of several picked.

Nil (gyrA) was obtained by introducing the gyrA gene of strain PB1707 into Nil. The procedure used was transformation by congression with Trp⁺ by means of the Ephrati-Elizur technique (see above).

The mutation oxr-1 was placed in an isogenic background by transferring it by congression with His⁺ into strain GSY1021 (His⁻ Pur⁻) and then back into Nil by congression with Trp⁺.
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Effect of oxolinic acid and nalidixic acid on DNA synthesis

Cells grown in CH medium in the presence of [methyl-3H]thymidine were treated with either oxolinic acid (10 μg ml⁻¹) or nalidixic acid (50 μg ml⁻¹). In the wild-type, nalidixic acid largely prevented incorporation for about 2 h; synthesis of DNA was then resumed at a reduced rate (Fig. 1a). Oxolinic acid not only prevented net synthesis (Fig. 1a), but also produced degradation of DNA; this is more clearly shown in the experiments described in the following section. In NiI (gyrA), both inhibitors reduced DNA synthesis by about 50% (Fig. 1b). NiI (om-I) and NiI (om-2) behaved identically, exhibiting a considerably reduced rate of synthesis with both inhibitors after an initial lag (Fig. 1c).

Effect of oxolinic acid and nalidixic acid on degradation of DNA

Degradation of DNA in NiI, NiI (oxr-I), NiI (oxr-2) and NiI (gyrA) was compared by measuring the release of previously incorporated [methyl-3H]thymidine into the acid-soluble fraction. The wild-type control culture did not degrade any DNA during 4 h, nor did any of the untreated mutants (Fig. 2). Both inhibitors produced appreciable degradation of DNA (35–65%) in the wild-type; oxolinic acid caused some degradation (15–20%) in all three mutants, but nalidixic acid did not (Fig. 2).

Short-term viability

Killing of cells by the inhibitors was measured in liquid medium over a period of 4 h (see Methods). In the wild-type culture, nalidixic acid killed about 85% of the cells in the first hour but there was no further fall in the next 3 h (Fig. 3a). In the three mutants treated with nalidixic acid the viable numbers remained stable at first and then began to increase (Fig. 3b, c, d). Oxolinic acid reduced the viability of the wild-type by 99% in less than 3 h (Fig. 3a). In oxolinic acid-treated NiI (gyrA) cultures, the numbers of viable cells remained constant for the duration of the experiment. This was in contrast to the behaviour of NiI (oxr-I) and NiI (oxr-2): in both these strains, there was an initial killing of about 70% followed by a slow recovery, which was reproducibly slower in NiI (oxr-2) than in NiI (oxr-I) (Fig. 3b, c, d).
Incubation time (h)

Fig. 2. Effect of oxolinic acid or nalidixic acid on degradation of DNA in strains Nil (gyrA), Nil (oxr-1), Nil (oxr-2) and Nil (wild-type). Each strain was grown in CH medium (60 ml) containing \( ^{3}H \) thymidine (0.5 \( \mu \)Ci ml\(^{-1} \)) and carrier thymidine (20 \( \mu \)g ml\(^{-1} \)). When the cells reached a density of about 0.06 mg dry wt ml\(^{-1} \), the cultures were centrifuged, and the cells were washed once with sporulation medium containing thymidine (100 \( \mu \)g ml\(^{-1} \)) and resuspended in CH medium containing thymidine in excess (100 \( \mu \)g ml\(^{-1} \)). Each culture was split into three portions with additions as follows: oxolinic acid (10 \( \mu \)g ml\(^{-1} \)), nalidixic acid (50 \( \mu \)g ml\(^{-1} \)) or none (control). Samples were taken at intervals and the radioactivity in the TCA-soluble fraction was measured. ●, Nil + oxolinic acid; ●, Nil + nalidixic acid; ▲, Nil (oxr-1) + oxolinic acid and Nil (oxr-2) + oxolinic acid (results identical); □, Nil (gyr) + oxolinic acid. ■, Controls of the four strains and also the mutants Nil (oxr-1), Nil (oxr-2) and Nil (gyrA) treated with nalidixic acid.

Long-term viability

Again, there was a clear phenotypic difference between Nil (oxr-1) and Nil (oxr-2) on the one hand and Nil (gyrA) on the other, when the strains were exposed to the inhibitors. Growth of the oxolinic acid-resistant strains was completely inhibited on plates containing nalidixic acid (50 \( \mu \)g ml\(^{-1} \)). However, with Nil (gyrA) the reduction in number of cells caused by nalidixic acid was much less (about 50%), as expected (Table 1). Oxolinic acid caused viability to fall by three to four orders of magnitude in all three mutants.

Effect of different concentrations of oxolinic acid on DNA synthesis and long term viability of Nil (oxr-1) and Nil

From the experiments already described it was apparent that in mutants carrying oxr the capacity for DNA synthesis and long-term viability were both still seriously affected by the inhibitors. For the mutations to be genetically useful markers it was necessary to establish the conditions in which their phenotype could be clearly distinguished from that of the wild-type. Accordingly DNA synthesis and long-term viability were measured in the presence of lower concentrations of oxolinic acid, to determine whether such a distinction could be made.

Nil (oxr-1) and Nil strains were exposed to different concentrations of oxolinic acid up to 10 \( \mu \)g ml\(^{-1} \), and DNA synthesis was measured. In the wild-type Nil, oxolinic acid at 5 and 10 \( \mu \)g ml\(^{-1} \) completely stopped DNA synthesis and also induced the degradation of DNA. Even at 2.0 \( \mu \)g ml\(^{-1} \), DNA synthesis was greatly reduced (Fig. 4a). This concentration did not significantly affect DNA synthesis in Nil (oxr-1) (Fig. 4b). Higher concentrations (5.0 and 10 \( \mu \)g ml\(^{-1} \)) retarded the onset of synthesis of DNA but recovery then occurred (Fig. 4b). A similar result was obtained with Nil (oxr-2) (not shown).

The effect of different concentrations of oxolinic acid on long-term viability was then determined. Suitable dilutions of cultures were plated on agar plates containing oxolinic acid at 1.0, 2.0 and 3.5 \( \mu \)g ml\(^{-1} \). With both oxr strains, concentrations below 2.0 \( \mu \)g ml\(^{-1} \) gave viable counts that were 30 to 50% of those in the controls, while 3.5 \( \mu \)g ml\(^{-1} \) reduced viability
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Fig. 3. Short-term viability in strains NiI (oxr-1), NiI (oxr-2), NiI (gyrA) and NiI (wild-type). Each strain was grown in CH medium (30 ml) to a cell density of 0.06-0.10 mg dry wt ml⁻¹. Each culture was then split into three portions and received: no inhibitor (control) (○), oxolinic acid (10μg ml⁻¹) (△) or nalidixic acid (50 μg ml⁻¹) (□). Samples (0-1 ml) were taken at intervals for up to 4 h and dilutions plated for viable counts (see Methods).

Table 1. Long-term viability in strains NiI (oxr-1), NiI (oxr-2), NiI (gyrA) and NiI (wild-type)

The four strains were grown in BHIB (10 ml) to a cell density of 0.140 mg dry wt ml⁻¹. Various dilutions were made of these cultures (10² to 10⁷) and plating was done on nutrient agar plates containing oxolinic acid (3.5 μg ml⁻¹) or nalidixic acid (50 μg ml⁻¹). Values are given as colony-forming units ml⁻¹.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No inhibitor</th>
<th>Oxolinic acid</th>
<th>Nalidixic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>NiI (oxr-1)</td>
<td>9.3 x 10⁸</td>
<td>8.1 x 10⁸</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>NiI (oxr-2)</td>
<td>1.2 x 10⁸</td>
<td>1.2 x 10⁶</td>
<td>&lt;10²</td>
</tr>
<tr>
<td>NiI (gyrA)</td>
<td>6.0 x 10⁸</td>
<td>7.5 x 10⁵</td>
<td>3 x 10⁵</td>
</tr>
<tr>
<td>NiI</td>
<td>1.6 x 10⁹</td>
<td>&lt;10²</td>
<td>&lt;10²</td>
</tr>
</tbody>
</table>

by three orders of magnitude (Fig. 5). Wild-type NiI did not grow at any of the concentrations tested. This established that oxolinic acid at concentrations between 1 and 2 μg ml⁻¹ could be used as a selective agent.
Fig. 4. Effect of different concentrations of oxolinic acid on DNA synthesis in strains NiI (wild-type) and NiI (oxr-I). Each strain was grown in CH medium (100 ml) containing $[^{3}H]$thymidine (0.2 μCi ml$^{-1}$) and carrier thymidine (20 μg ml$^{-1}$), to a cell density of about 0.06 mg dry wt ml$^{-1}$. Each culture was split into four portions receiving: no oxolinic acid (control) (O), 2.0 μg ml$^{-1}$ (●), 5.0 μg ml$^{-1}$ (△) and 10 μg ml$^{-1}$ (▲). Samples were taken at intervals and DNA synthesis was followed (see Methods).

DNA synthesis in toluenized cells

The experiments described, together with the finding that the oxr mutations do not map at the gyrA locus (see below), suggested that the mutations in the oxolinic acid-resistant strains had not affected the DNA gyrase but might have conferred a decreased permeability to
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Fig. 6. DNA synthesis in toluenized cells of strains NiI (oxr-1), NiI (oxr-2), NiI (gyrA) and NiI (wild-type). Each strain was grown in CH medium (50 ml) to a cell density of about 0.06 mg dry wt ml⁻¹. The cultures were centrifuged and the cells were washed with potassium phosphate buffer (10 ml, pH 7.4) and then suspended in 1-0 ml of the same buffer. Toluene (10 μl) was added and the cells were agitated at 1 rev. s⁻¹ for 10 min at room temperature. Then the cells were cooled at 4 °C for 5 min. The temperature for measuring DNA replication was 37 °C; replication was measured as follows. A 0.1 ml sample of toluenized cells was added to 0.4 ml of incubation mixture to form a final reaction mixture containing 70 mM-potassium phosphate buffer (pH 7.4), 13 mM-MgSO₄, 1-3 mM-ATP, 33 μM-dGTP, 33 μM-dCTP, 33 μM-dTTP, 2 mM-dithiothreitol and 2 μCi [³H]TTP. No ATP was added to samples in which 'repair' DNA synthesis was to be determined. Samples (50 μl) were removed at 15 min intervals for 1 h, mixed with herring DNA (2 mg ml⁻¹) and precipitated with TCA (10%, 2 ml). The samples were chilled in ice for 30 min and then filtered through glass-fibre filters (Whatman GF/C, 2-1 cm diam.), and washed with TCA (10%, 10 ml) and ethanol (96%, 10 ml). The filters were dried in a vacuum oven (80 °C, 60 min) and counted. □, Control; △, oxolinic acid (100 μg ml⁻¹); □, no ATP.

NiI (oxr-1), NiI (oxr-2) and the wild-type NiI were equally inhibited by oxolinic acid.

Oxolinic acid. To test this hypothesis, DNA synthesis was followed in vitro using cells which had been made permeable by treatment with toluene.

The concentration of oxolinic acid necessary to stop DNA synthesis in wild-type cells under these conditions was 10-fold higher than that needed in vivo. A similar effect in toluenized cells of E. coli has been reported by Staudenbauer (1976), who measured the inhibition of DNA synthesis by oxolinic acid. Strains NiI, NiI (oxr-1), NiI (oxr-2) and NiI (gyrA) were tested. To show that DNA synthesis was due to the action of DNA polymerase III and not to repair synthesis, controls were included in which either ATP was excluded from the reaction mixture or 6-(p-hydroxyphenylazo)uracil (HPUra), a specific inhibitor of DNA polymerase III (Gass et al., 1973) was added at a concentration of 50 μg ml⁻¹. Both treatments prevented the synthesis of any significant amount of DNA. The result clearly showed that NiI (gyrA) could synthesize DNA in the presence of a high concentration of oxolinic acid, and the amount synthesized was similar to that in control cells (Fig. 6a). The oxolinic acid-resistant strains and the wild type NiI were equally inhibited by oxolinic acid (Fig. 6b).

Mapping of oxr-1, oxr-2 and gyrA

Strain PB1706 is a nalidixic acid-resistant strain with high resistance to the inhibitor (up to 300 μg ml⁻¹). Its gyrA mutation has been mapped between purA (39% cotransduction) and cysA (52% cotransduction) by Canosi & Mazza (1974). In our experiments the values were roughly similar: 30% and 42-44% respectively (Table 2).

To compare the locations of oxr-1 and oxr-2 with that of gyrA, these strains were also mapped by PBS1-mediated transduction. Table 2 shows the strains used as recipients and the linkage values obtained with different auxotrophic markers. The oxr mutations were
Table 2. Linkage of gyrA, oxr-1 and oxr-2 to different markers

PBS1-mediated transduction (see Methods) was employed to map the different strains. As recipients, two cysA and two purA auxotroph strains were used.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Selected marker</th>
<th>No. of gyrA or oxr transductants/total</th>
<th>Linkage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB1706 (gyrA)</td>
<td>QB944 (cysA purA trpC2)</td>
<td>cys*</td>
<td>38/90</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pur*</td>
<td>34/113</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cys* pur*</td>
<td>43/49 and 68/70</td>
<td></td>
</tr>
<tr>
<td>BD112 (cysA)</td>
<td>QB944 (cysA purA trpC2)</td>
<td>cys*</td>
<td>38/86 and 59/133</td>
<td>44 and 44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pur*</td>
<td>21/88</td>
<td>24</td>
</tr>
<tr>
<td>Nil (oxr-1)</td>
<td>BD112 (cysA)</td>
<td>cys*</td>
<td>16/127</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>GSY1021 (purA hisB)</td>
<td>pur*</td>
<td>1/127</td>
<td>0</td>
</tr>
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<td></td>
<td></td>
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<td>0/58</td>
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<td>cys*</td>
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<td>19 and 18</td>
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<tr>
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<td></td>
<td>pur*</td>
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<td>0</td>
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</table>

Fig. 7. Linkage of oxr-1 and oxr-2 to cysA. PBS1-mediated transduction was used to locate oxr-1 and oxr-2 in relation to the previously mapped cysA, purA and gyrA markers. The numbers represent transduction map units (100 - percentage co-transduction). The linkage of purA to cysA was obtained from Dubnau et al. (1967).

completely unlinked to purA and had low linkage to cysA. When selection was made simultaneously for cys+ and pur+, the oxr mutations were excluded. They clearly did not lie between the cysA and purA markers but were close to each other. Figure 7 shows the position of oxr-1 and oxr-2 in the map and their distance from cysA. They are clearly separated from gyrA.

DISCUSSION

The phenotypic and genetic properties of strains resistant to oxolinic acid and those resistant to nalidixic acid can be summarized as follows.

1. The mutations oxr-1 and oxr-2 (which seem to be close together) on the one hand and gyrA on the other have quite different chromosomal locations.

2. In short-term viability experiments, gyrA mutants are even more resistant to oxolinic acid than oxr mutants.

3. The oxolinic acid-resistant strains show no resistance to nalidixic acid in long term viability experiments.

4. Strains containing gyrA are able to synthesize DNA in vitro in the presence of a high concentration of oxolinic acid, whereas DNA synthesis in the oxolinic acid-resistant strains under the same conditions is effectively inhibited.
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These results appear to rule out the possibility that the oxr mutations have altered the structure of DNA gyrase. Instead, the evidence obtained suggests that the oxr mutations have reduced the permeability of cells to oxolinic acid so that the cells become resistant to low concentrations of the inhibitor (i.e. below 5 \( \mu \)g ml\(^{-1} \)). Since there is also some cross-resistance to nalidixic acid, mainly observed when degradation of DNA or short-term viability are measured, it is possible that oxolinic acid and nalidixic acid share a common transport mechanism.

Although the degree of resistance to oxolinic acid is not very high, it is sufficient to allow the oxr mutations to be used as genetic markers in genetic analysis.

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