Characterization and Cloning of Two *Rhizobium leguminosarum* Genes Coding for Glutamine Synthetase Activities

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We have demonstrated that *Rhizobium leguminosarum* strain LPR1105 contains a heat stable and a heat labile glutamine synthetase (EC 6.3.1.2) activity similar to those described for other *Rhizobiaceae*. Most of the activity is heat stable when this strain is grown on glutamine as sole nitrogen source, but most is heat labile when grown on nitrate. Using a gene bank of *R. leguminosarum* DNA we have isolated two clones, which code for heat stable (p7D9) and heat labile (p4F7) glutamine synthetase activity, by complementing the glutamine auxotrophy of *Klebsiella pneumoniae* *glnA* mutants. Cross-hybridization of p7D9 with a fragment of the *glnA* gene of *K. pneumoniae* was observed, but no cross-hybridization between p7D9 and p4F7 was found. Since these two regions hybridize to genomic DNA of *R. leguminosarum* they are probably the structural genes for GSI and GSII, and the availability of these genes will make it possible to test this hypothesis. Clone p4F7 complements an *ntrC*+ but not an *ntrC* *K. pneumoniae* *glnA* mutant, suggesting that the *ntrC* gene is required for the complementation of the glutamine auxotrophy by this plasmid.

INTRODUCTION

In enteric bacteria, the activity of glutamine synthetase (encoded by the *glnA* gene) is influenced by a variety of regulatory proteins [such as the *ntrB* (*glnL*) and the *ntrC* (*glnG*) gene products] which act both to control the rate of synthesis of glutamine synthetase and to convert this enzyme from an inactive to an active form in response to the nitrogen requirements of the cell (Ginzburg & Stadtman, 1973; Magasanik, 1982; Merrick, 1982). Two forms of glutamine synthetase (EC 6.3.1.2), GSI and GSII, have been demonstrated in all but one of the tested species of the family *Rhizobiaceae* (Darrow, 1980; Darrow et al., 1981; Donald & Ludwig, 1984). The role of these two enzymes in symbiotic nitrogen fixation is not clear. Glutamine auxotrophs have been isolated in cowpea *Rhizobium* (Ludwig, 1978) and in *Rhizobium meliloti* (Kondorosi et al., 1977). These mutants have little or no glutamine synthetase activity, but the nature of the mutations has not been established. In both species the mutants induced nodules which were not capable of fixing nitrogen (Fix-). Revertants of the cowpea *Rhizobium* mutant were of two types (Ludwig, 1980): some were GSI+ GSII− and regained the ability to fix nitrogen, whereas the others were GSI− GSII+ and remained Fix−. GSI insertion mutations of *R. meliloti* showed no effect on both nodulation and fixation (Somerville & Kahn, 1983).

GSI has been shown to be similar to the single glutamine synthetase of enteric bacteria and of *Rhizobium* sp. strain ORS571. It is a polymeric enzyme consisting of 12 identical subunits of M, 59000, is relatively heat stable and its activity is affected by a reversible adenyllylation cascade system (Darrow, 1980; Darrow & Knotts, 1977; Donald & Ludwig, 1984). In contrast, GSII is

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or phenotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>pro leu thi</td>
<td>Maniatis et al. (1982)</td>
</tr>
<tr>
<td>M5a</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>UNF1827†</td>
<td>glnA201 lac-2001 hsd</td>
<td>Espin et al. (1982)</td>
</tr>
<tr>
<td>UNF1848†</td>
<td>(glnA-ntrBC) hisD2 hsd</td>
<td></td>
</tr>
<tr>
<td>R. leguminosarum (biovar viceae)</td>
<td>Rif&quot; derivative of RCC1001</td>
<td>Hooykaas et al. (1982)</td>
</tr>
<tr>
<td>LPR1105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMMB34</td>
<td>Km' IncQ</td>
<td>Frey et al. (1983)</td>
</tr>
<tr>
<td>pRK2073</td>
<td>Tm' Mob+</td>
<td>C. K. Kennedy‡</td>
</tr>
<tr>
<td>pAM51§</td>
<td>glnA (internal fragment)</td>
<td>M. J. Merrick‡</td>
</tr>
<tr>
<td>p7D9</td>
<td>Gln+(GSI) Km' IncQ</td>
<td>This work</td>
</tr>
<tr>
<td>pMG10</td>
<td>Gln+(GSI) Km' IncQ</td>
<td></td>
</tr>
<tr>
<td>pSC11</td>
<td>Tc' Ap'</td>
<td></td>
</tr>
<tr>
<td>p4F7</td>
<td>Gln+ (GSIII) Km' IncQ</td>
<td></td>
</tr>
</tbody>
</table>

* Rif, rifampicin; Km, kanamycin; Tm, trimethoprim; Tc, tetracycline; Ap, ampicillin.
† Strain carries recA56 cotransducible with sbl-300::Tn10.
‡ Address: AFRC Unit of Nitrogen fixation, University of Sussex, Brighton, UK.
§ EcoRI fragment (0.86 kb) in pACYC184.
|| EcoRI fragment of p7D9 (1.3 kb, hybridizing to pAM51) in pBR328.

made of smaller subunits, $M$, 36000, which are heat labile and not known to be modified after translation (Darrow, 1980; Darrow & Knotts, 1977). These proteins are apparently products of different genes (Carlson et al., 1985; Darrow, 1980; Somerville & Kahn, 1983) and are differentially regulated in response to changes in nitrogen source (Ludwig, 1980a, b), carbon source (Darrow, 1980) and oxygen concentration (Darrow et al., 1981; Rao et al., 1978).

To study the mechanism of glutamine synthetase regulation during nodule development and nitrogen fixation we isolated glutamine synthetase genes from *Rhizobium leguminosarum* (biovar *viceae*, which nodulates *Pisum*, *Vicia*, *Lens* and *Lathyrus*). We report here the isolation of two different genes of *R. leguminosarum* coding for glutamine synthetases of apparently different $M$, than when in the native state; (c) one of them appears to require the *K. pneumoniae* ntrC gene for its expression, when present in this micro-organism.

**METHODS**

*Bacterial strains, plasmids and media.* Bacterial strains and plasmids are listed in Table 1. *R. leguminosarum* was grown either in YMB medium or RMM medium (Hooykaas et al., 1977) with mannitol as a carbon source. Potassium nitrate in RMM medium was substituted in some experiments by either glutamine, glutamate or ammonium sulphate (all at 1 pg ml$^{-1}$). *Escherichia coli* or *K. pneumoniae* strains were grown in TY (Lennox, 1955) or minimal (Vogel & Bonner, 1956) medium. Nitrogen-free minimal medium contained sodium phosphate instead of sodium ammonium phosphate. Nitrogen sources were added as specified in the text. Antibiotics and amino acid supplements were used at the following concentrations: chloramphenicol, 30 pg ml$^{-1}$; kanamycin sulphate, 30 pg ml$^{-1}$; rifampicin, 100 pg ml$^{-1}$; spectinomycin, 200 pg ml$^{-1}$; tetracycline, 15 pg ml$^{-1}$; trimethoprim, 20 pg ml$^{-1}$; glutamine, 200 pg ml$^{-1}$; histidine, 20 pg ml$^{-1}$.

*Microbiological procedures.* To isolate glutamine synthetase clones a complete gene bank was crossed from *E. coli* HB101 into *K. pneumoniae* UNF1827 and UNF1848 (Gln$^{-}$) by means of the helper plasmid pRK2073 carried by strain HB101. Donor strains were grown overnight in microtitre plates containing TY plus kanamycin, recipient and helper strains overnight in 10 ml TY and TY plus trimethoprim respectively. Donors were replicated by means of a multiprobe onto TY plates and a 1:1 (v/v) mixture of recipient and helper was replicated on top of them. The mating plates were incubated for 7 h at 37°C, replicated onto selective plates (minimal-kanamycin, without glucose) and incubated for 7 d at 37°C. Transconjugants were streaked for single colonies for further analysis. In histidine inhibition experiments single colonies of Gln$^{+}$ transconjugants were resuspended in 50 μl minimal medium and replicated onto minimal plates containing either histidine (1 mg ml$^{-1}$) or histidine plus glutamine.
**Glutamine synthetase genes of R. leguminosarum**

Fig. 1. Rate of heat inactivation of transferase activity in crude extracts of *R. leguminosarum* grown in glutamine (●), glutamate (○) and nitrate (△) as the sole nitrogen source. Specific activities at time 0 are given in Results. Portions of the extracts were heated at 55 °C for the stated times. They were rapidly cooled in ice, centrifuged and assayed as described in Methods.

**DNA manipulation.** Total DNA was prepared by the alkaline lysis method (Birnboim & Doly, 1979) and plasmid DNA was prepared by the boiling method (Maniatis *et al.*, 1982). Hybridization experiments were all carried out at 60 °C. Other procedures used were according to Maniatis *et al.* (1982). For the construction of the gene bank, total DNA from *R. leguminosarum* LPR1105 was partially digested with the restriction endonuclease Sau3A1, loaded on a sucrose gradient (10-40% in 10 mM-Tris/HCl pH 8.0, 1 mM-EDTA, 1 M-NaCl) and run for 16 h at 32000 r.p.m. in an SW41 rotor. Fractions containing fragments of about 30 kb were treated with calf intestine phosphatase, ligated to the cosmid pMMB34 previously digested with BamHI, extracted with phenol and precipitated with ethanol. The ligation reaction contained 3-6 μg pMMB34 DNA and 1 μg *R. leguminosarum* DNA fragments per 15 μl. A portion of the ligated DNA was packaged with a phage lambda packaging extract (Amersham) and used to transduce strain HB101 onto TY-kanamycin plates.

**Glutamine synthetase assays and preparation of cell extracts.** Bacteria grown under the desired conditions were harvested by centrifugation at 4 °C and then frozen as a pellet at −20 °C. To 100–150 mg of frozen pellet, 1 ml of cold extraction solution (Ferguson & Sims, 1971) was added, and the cells were resuspended. The cell suspension was treated twice for 45 s, with a 1 min interval for cooling, with an MSE sonic oscillator, and then it was centrifuged for 20 min at 15000 r.p.m. in a Sorvall SS34 rotor. Portions of the supernatant were assayed for protein concentration (Groves *et al.*, 1968) and either for biosynthetic (Bender *et al.*, 1977) or transferase glutamine synthetase activity (Ferguson & Sims, 1971). Glutaminase (EC 3.5.1.2.) activity was assayed by omitting ADP from the transferase assay.

**Gel electrophoresis.** Native protein polyacrylamide gel electrophoresis (PAGE) was done as described by Ludwig (1978).

**RESULTS**

**Two glutamine synthetase activities in *R. leguminosarum***

We assayed glutamine synthetase activity in crude extracts of *R. leguminosarum* strain LPR1105 grown in the presence of different nitrogen sources. Specific activities in the transferase assay [nmol min⁻¹ (mg protein)⁻¹] were 860, 270, 100 and <5 when the bacteria were grown on nitrate, glutamate, glutamine and NH₄⁺ respectively. The growth rate in NH₄⁺ was lower than in glutamate or glutamine, indicating poor NH₄⁺ assimilation, probably as a consequence of the low glutamine synthetase activity. Fig. 1 shows the rate of inactivation of transferase activity in crude extracts, preincubated at 55 °C, from bacteria grown on nitrate, glutamate and glutamine. Activity was mostly heat labile (98%) when the bacteria were grown in nitrate, whereas it was mostly heat stable (>80%) when they were grown in glutamine. When
Table 2. Growth rates and glutamine synthetase activity of UNF1827(p7D9) and UNF1827(p4F7)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimal medium</th>
<th>Glutamine synthetase activity†</th>
<th>Transferase - ADP</th>
<th>Ratio of biosynthetic to transferase (+ADP) activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Glutamine</td>
<td>+Glutamine</td>
<td>+ADP</td>
<td></td>
</tr>
<tr>
<td>M5al</td>
<td>70</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>UNF1827(p7D9)</td>
<td>100</td>
<td>100</td>
<td>1450</td>
<td>47</td>
</tr>
<tr>
<td>UNF1827(p4F7)</td>
<td>100</td>
<td>70</td>
<td>327</td>
<td>1891</td>
</tr>
</tbody>
</table>

* The doubling time of strain UNF1827 in the presence of glutamine was 70 min.
† Strains were grown in minimal containing glutamate (1 mg ml⁻¹) as sole nitrogen source. Values are nmol min⁻¹ (mg protein)⁻¹.

the bacteria were grown in glutamate, heat stable (40%) and heat labile (60%) fractions were present. However, the heat stable fraction was not as stable as the activity found in glutamine-grown bacteria, suggesting that these two heat stable activities might be different. Since several interpretations are possible for the results shown in Fig. 1, we decided to discriminate among them by first cloning gene(s) coding for glutamine synthetase activity.

Isolation of the *R. leguminosarum* glutamine synthetase genes by complementation of *K. pneumoniae* strains

We constructed a gene bank of *R. leguminosarum* LPR1105 DNA using pMMB34 as the vector. This cosmid vector, derived from plasmid RSF1010, confers resistance to kanamycin, and is mobilized to, and replicates in, any Gram-negative species (Frey et al., 1983). We crossed the complete gene bank (1056 clones) from *E. coli* into *K. pneumoniae* UNF1827 (glnA) (transfer frequency 10⁻²) and found 28 transconjugants complementing the Gln⁻ phenotype after 7 d growth. After isolation of single colonies, these transconjugants grew faster, perhaps because of the DNA rearrangements, as shown below for one of them (p4F7).

Since histidine and other metabolites inhibit glutamine synthetase activity (Stadtman et al., 1968), we attempted to subdivide these transconjugants into classes by differential inhibition experiments in the presence of histidine, as a result of which we found three classes. Class I (12 clones) showed no inhibition by histidine (1000 µg ml⁻¹); class II (10 clones) showed inhibition by histidine, which was relieved by glutamine; class III (six clones) showed inhibition by histidine, but the relieving effect of glutamine reversion occurred with a 2 d delay.

In order to confirm that interspecific complementation was due to glutamine synthetase activity, we analysed representatives of each class using the transferase assay. Heat stable transferase activity was found in strains carrying class III clones, while class II clones gave rise to a heat labile activity (see below). Therefore class III clones appeared to code for an activity analogous to that of GS1 described in other *Rhizobiaceae*, while class II clones probably code for an activity analogous to GSII. Class I clones showed an intermediate level of heat stability and two glutamine synthetase bands on native protein PAGE (data not shown). They were not studied further because their analysis will be simpler when more is known about the clones of the other classes and their gene product(s).

The duplication time of strain M5al (wild-type *K. pneumoniae*) was 70 min at 37 °C in minimal medium, while that of UNF1827(p7D9) (class III) and of UNF1827(p4F7) (class II) was 100 min (Table 2). Addition of glutamine had no effect on UNF1827(p7D9), while it reduced the duplication time of UNF1827(p4F7) to 70 min. The specific activity of glutamine synthetase assayed by the biosynthetic and the transferase assays in UNF1827(p7D9) and UN1827(p4F7) showed that these values are different in the two strains. Apparent glutaminase activity was 1–2% of total transferase activity in the wild-type strain M5al and in UNF1827(p7D9), while it was 40% in UNF1827(p4F7). The ratio of the biosynthetic to total transferase assay was 0-045 for UNF1827(p7D9) and 0-85 for UNF1827(p4F7).
Glutamine synthetase genes of *R. leguminosarum*

Fig. 2. Rate of heat inactivation of transferase activity in crude extracts of UNF1827(p7D9) (●) and UNF1827(p4F7) (△) grown in minimal medium with glutamate as the sole nitrogen source. The protein concentration of each extract was adjusted to 7 mg ml⁻¹. Preincubation at 0 °C for 3 h did not change the specific activity, which was 3200 and 1400 nmol min⁻¹ (mg protein)⁻¹ for UNF1827(p7D9) and UNF1827(p4F7) respectively. For further details see Fig. 1.

Fig. 2 shows the rate of inactivation of transferase activity in crude extracts prepared from UNF1827(p7D9) and UNF1827(p4F7) preincubated at 55 °C. A dramatic difference in heat stability can be seen. UNF1827(p7D9) shows a heat sensitive fraction for which we have no obvious explanation. UNF1827 itself did not have any detectable activity (<1%). A mixture of UNF1827(p7D9) and UNF1827(p4F7) extracts incubated at 55 °C in a 1:17 (v/v) ratio gave a result superimposable to the sum of the two curves in Fig. 2, thus excluding the possibility that factors independent of glutamine synthetase activity were the cause of heat lability.

Crude extracts of UNF1827(p7D9) and UNF1827(p4F7) yielded protein bands with different relative electrophoretic mobility that exhibited transferase activity on non denaturing acrylamide gels (Fig. 3). The lower mobility band corresponded to the glutamine synthetase coded for by p7D9, the higher mobility band to the glutamine synthetase coded for by p4F7. These results suggested that the glutamine synthetases coded for by the two clones had different $M_r$ values. The difference in intensity between the two bands may be interpreted as either due to GSI having a lower activity when assayed *in situ* than in the tube assay, or due to inactivation of glutamine synthetase during electrophoresis.

*Lack of homology between two glutamine synthetase clones*

The restriction maps of p7D9 and p4F7 are presented in Fig. 4. The insert of p7D9 is 27 kb in length, and hybridization to *R. leguminosarum* genomic DNA confirms both the size of the insert as well as its colinearity. The bands present in an EcoRI digest of p7D9 are all visible when this DNA is used as a probe in a hybridization to EcoRI-digested total *R. leguminosarum* DNA, except for the uppermost band containing the vector and the low $M_r$ bands because of inefficient blotting (Fig. 5). This shows that at least two-thirds of the insert is colinear with genomic DNA. Hybridization of p7D9, using the *K. pneumoniae glnA* DNA of plasmid pAM51 as a probe, is positive with the 1.3 kb EcoRI fragment (Fig. 4). A 6-4 kb fragment of p7D9, containing the *glnA* cross-hybridizing region was subloned, together with a portion of the vector, into pACYC184 (pMG10; Fig. 4).

The insert of p4F7 is only 2–3 kb (Fig. 4). This is as a consequence of a DNA deletion which ends in a non-defined site of the vector. The HindIII site shown in Fig. 4 is deduced to be a vector site because of comparison with the restriction map of pMMB34, while the PstI site is not present in the vector in the position shown. Therefore, the endpoint of the deletion must be between HindIII and PstI. Hybridization of p4F7 to *R. leguminosarum* genomic DNA digested
Fig. 3. Glutamine synthetase activities of (a) UNF1827(p7D9) and (b) UNF1827(p4F7) transconjugants. Strains were grown in minimal medium containing glutamate as the sole nitrogen source. Portions (700 μg protein) of crude extracts, prepared as described in Methods, were loaded onto a 5% polyacrylamide slab gel at 4 °C for electrophoresis. Assays were conducted for 30 min at 37 °C, as described in Methods. The sample origin is at the top of the figure.

Fig. 4. Restriction maps of p7D9, pMG10 and p4F7. Cosmids p7D9 and p4F7 are made of Sau3AI fragments of R. leguminosarum DNA inserted between the EcoRI and SsrI sites of the vector pMMB34. Plasmid pMG10 contains a HindIII fragment of p7D9 (made of the 6-4 kb HindIII/EcoRI fragment shown in the figure plus a 3-5 kb EcoRI/HindIII fragment of pMMB34) cloned into pACYC184. ■, pMMB34 DNA; ○, pACYC184 DNA; - - - - - - , fragment cross-hybridizing to the specific K. pneumoniae glnA probe: - - - - - - - - - - , R. leguminosarum DNA. The dotted line in p4F7 indicates that it is not known where vector DNA ends and insert DNA begins. The insert of p7D9 contains no HpaI sites; it contains seven HindIII sites, but only the one used for pMG10 construction is shown. The two rightmost EcoRI sites have not been ordered.
with EcoRI and BamHI reveals three bands of 3.2, 2.8, and 1.3 kb; hybridization to genomic DNA digested with PstI reveals two bands, of 8 and 2.5 kb, showing that the insert is *R. leguminosarum* DNA and is colinear to it. The inserts of pMG10 and p4F7 have different restriction sites, indicating that they code for different genes. This is confirmed by the lack of cross-hybridization with either p7D9 or with its 1.3 kb EcoRI fragment as a probe, and by lack of extra bands in the hybridizations to genomic DNA reported above (see Fig. 5).

**Complementation analysis**

Complementation analysis of p7D9, pMG10 and p4F7 was done in UNF1827 (*glnA*) and UNF1848 (*glnA-ntrC*). Plasmids p7D9 and pMG10 complemented both UNF1827 and UNF1848, whereas p4F7 could complement UNF1827 only. This suggests that the *K. pneumoniae ntrC* gene is involved in the complementation of the Gln+ phenotype (unpublished observations). With respect to p7D9 and pMG10, we cannot exclude that complementation could be the result of read-through from the vector.

**DISCUSSION**

Regulation of glutamine synthetase is of special interest in the *Rhizobiaceae*, since assimilation of NH₃ is thought to be inhibited in bacteroids. Newly fixed nitrogen diffuses to the plant cytoplasm in the form of ammonia, where it is assimilated by plant-encoded glutamine synthetase (Lara *et al.*, 1983). To initiate the study of glutamine synthetase regulation at the molecular level, we have isolated *gln* genes from *R. leguminosarum*. While a gene coding for GSI
has been previously isolated from *R. meliloti* (Somerville & Kahn, 1983) and *Bradyrhizobium japonicum* (Carlson et al., 1985), this is the first report of two different genes coding for glutamine synthetase activity from *Rhizobiaceae*. Our data suggest that there are two distinct glutamine synthetase isozymes in *R. leguminosarum*, but confirmation of this awaits their isolation. Since the 28 clones complementing Gln" mutants of *K. pneumoniae* fall into three classes based on histidine inhibition experiments, we cannot exclude that there might be more than two glutamine synthetase isozymes in *R. leguminosarum*. Results reported in this paper will make it possible to construct *R. leguminosarum* mutant strains, allowing the glutamine synthetase structural genes and their products to be analysed.

Histidine inhibits complementation of *glnA* mutants by p7D9 and p4F7. Glutamine relieves this inhibition in strains carrying p4F7, but only partially in strains carrying p7D9. Growth rate experiments show that the GlnA" mutation is partially complemented by both p7D9 and p4F7. Glutamine can bypass this lack of complementation in strains carrying p4F7 but not in strains carrying p7D9. This might be interpreted as an effect of protein(s) coded for by p7D9 on either glutamine uptake and/or ammonia assimilation.

Restriction and hybridization analysis of p7D9 and p4F7 demonstrated that the DNA inserts on these clones carry different genes. The glutamine synthetases detected in UNFI827(p7D9) and UNFI827(p4F7) may correspond to GSI and GSII respectively because of the difference in heat stability, although this difference might result from the expression of a *R. leguminosarum* gene in *K. pneumoniae*. Glutaminase activity is present in UNFI827(p4F7) but not in UNFI827(p7D9) and wild-type *K. pneumoniae*. Such an activity has been reported for GSII by Ludwig (1978). However, Ludwig interpreted this result as an artefact: GSII of cowpea *Rhizobium* had glutaminase activity on polyacrylamide gels, which disappeared when the enzyme was eluted from the gel and dialysed. Since the insert on p4F7 is between 2 and 3 kb only (equivalent to a 66 000–100 000 M, encoded protein), glutaminase activity is either coded for by a gene linked to the GSII gene, or is a second activity of GSII itself. This activity of GSII could be the reason for its lower ratio of biosynthetic to transferase activity when compared to GSI.

Transferease activity assays of *R. leguminosarum* grown on different nitrogen sources indicated that the presence of active GSI and/or GSII depends on the nitrogen source. Mostly, GS activity is detected in strains grown on glutamine and only GSII activity in strains grown on nitrate. Both activities are absent when grown in NH4+. Different signals regulating glutamine synthetase expression and/or activity induced by different nitrogen sources might be important for efficient symbiosis.

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