Fructose operon mutants of *Spiroplasma citri*

Patrice Gaurivaud, Frédéric Laigret, Eric Verdin, Monique Garnier and Joseph M. Bové

Author for correspondence: Frédéric Laigret. Tel: +33 5 56 84 31 50. Fax: +33 5 56 84 31 59.  
e-mail: laigret@bordeaux.inra.fr

**Fructose-negative mutants of *Spiroplasma citri* wild-type strain GII-3 were selected by two methods. The first method is based on the selection of spontaneous xylitol-resistant mutants, xylitol being a toxic fructose analogue. Five such mutants were obtained, but only one, xyl3, was unable to use fructose and had no phosphoenolpyruvate:fructose phosphotransferase system (fructose-PTS) activity. Amplification and sequencing of the fructose permease gene of mutant xyl3 revealed the presence of an adenylic insertion leading to a truncated permease. The second method is based on inactivation of *fruA* and/or *fruK* by homologous recombination involving one crossing-over between the chromosomal genes and inactivated genes carried by replicative plasmids. Fructose-negative mutants were obtained at a frequency of about 10%. Fructose-PTS activity and 1-phosphofructokinase activity were not detected in four representative mutants that were characterized (H31, H45, E38 and E53). In strain H31, Southern blot analysis and PCR showed that the result of homologous recombination was, as expected, the presence in the chromosome of two mutated *fruA–fruK* copies with the plasmid sequence in between. Only the mutated copy, under control of the fructose operon promoter, was transcribed. This work describes for the first time the use of two methods to obtain fructose-auxotrophic mutants of *S. citri*. The method involving homologous recombination is a general procedure for gene disruption in *S. citri*.

**Keywords:** *Spiroplasma citri*, fructose operon, xylitol, gene disruption

**INTRODUCTION**

*Spiroplasma citri* is a phytopathogenic mollicute (Saglio *et al*., 1973). Although this spiroplasma was first isolated and cultured from sweet orange (*Citrus sinensis*) trees affected by ‘stubborn’ disease, the agent infects many plant species other than citrus, including periwinkle (*Catharanthus roseus*), where it induces stunting, leaf yellows and wilting, leading to death of the plant a few weeks after infection (Calavan & Bové, 1989). This wall-less bacterium is transmitted from plant to plant by phloem-feeding leafhoppers (Calavan & Bové, 1989). All phytopathogenic mollicutes are restricted to the sieve tube elements of the phloem tissue. The mechanisms involved in their phytopathogenicity are poorly understood. Recently, a so-called non-pathogenic mutant of *S. citri*, mutant GMT 553, was obtained by random insertion of transposon Tn4001 into the genome of the wild-type (wt) strain GII-3 (Foissac *et al*., 1997a, b). In mutant GMT 533, transposon Tn4001 was shown to be inserted in *fruR*, the first of the three fructose operon genes (Gaurivaud *et al*., 2000). *fruR* probably encodes the putative regulator protein of the operon. The putative protein deduced from the nucleotide sequence of *fruR* belongs to the DeoR family, which includes bacterial transcriptional regulators of carbohydrate catabolic operons (Reizer *et al*., 1996). The second gene, *fruA*, encodes the fructose permease of the phosphoenolpyruvate:fructose phosphotransferase system (fructose-PTS). The fructose permease allows the uptake and concomitant phosphorylation of fructose into fructose 1-phosphate. The third gene, *fruK*, encodes 1-phosphofructokinase (EC 2.7.1.56), which uses ATP to phosphorylate fructose 1-phosphate to fructose 1,6-bisphosphate. In the wt strain GII-3, the fructose operon is transcribed into two RNAs, one of 2.8 kb (*fruR* and
fruA) and one of 3.8 kb (fruR, fruA and fruK). Insertion of transposon Tn4001 at the 5′ end of fruR abolishes expression of all three genes of the operon (Gaurivaud et al., 2000). This explains why mutant GMT 553 is unable to use fructose as carbon and energy sources. Functional complementation of mutant GMT 553 with expression vectors harbouring different combinations of the fructose operon genes has shown that fruA–fruK or fruA alone are able to restore fructose utilization (Gaurivaud et al., 2000).

Mutant GMT 553 has drawn our attention to the possible involvement of fructose metabolism in the phytopathogenicity of S. citri and also the possible involvement of other sugars such as glucose and trehalose in its virulence and insect transmission. However, no method has been described to specifically obtain sugar-auxotrophic mutants of S. citri because the culture media contain animal serum and have complex compositions. To produce such targeted fructose operon mutants, we have explored two different strategies. The first is based on the fact that xyitol, a fructose analogue, is toxic for many bacteria, including S. citri (Labarère & Barroso, 1989). Therefore, xyitol-resistant mutants might be mutated in the fructose operon. The second strategy involves disruption of the fructose operon by homologous recombination involving one crossing-over.

**METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* strain XL-1 Blue MRK® Kan (Stratagene) was used as host strain for cloning experiments and plasmid propagation. It was grown in LB medium (Sambrook et al., 1989) or on LB plates containing 10 µg kanamycin ml⁻¹ and 50 µg ampicillin ml⁻¹. For plasmid transformation, preparation of *E. coli* XL-1 Blue competent cells and electroporation were performed as described by Dower et al. (1988) using a gene pulser (Bio-Rad). S. citri wt strain GII-3 was originally isolated from *Circulifer haematoceps*, the S. citri leafhopper vector (Vignault et al., 1980). Strains used in complementation experiments were obtained previously (Gaurivaud et al., 2000). S. citri was grown in SP-4 medium, which contains 0.5% (w/v) added glucose (Tully et al., 1977). Mutant GMT 553 was grown in the presence of 100 µg gentamicin ml⁻¹. Plasmid pBS was purchased from Stratagene. Plasmid pBOT carrying the S. citri oriC and tetracycline-resistance gene (tetM) was described elsewhere (Renaudin et al., 1995). Transformation of *S. citri* by electroporation with pBOT and its derivatives was as previously described (Stamburski et al., 1991). S. citri transformants were selected by plating on SP-4 solid medium supplemented with 2 µg tetracycline ml⁻¹. Each mutant selected was cloned as described elsewhere (Tully, 1983).

**DNA and RNA methods.** Total DNA from the *S. citri* wt strain GII-3 and mutants was isolated as follows. Thirty millilitres of spiroplasma culture were collected by centrifugation and resuspended in 270 µl PBS buffer. Cells were lysed by adding 30 µl 10% (w/v) SDS and incubated for 30 min at 37 °C. The DNA was further purified by phenol/chloroform deproteinization and ethanol/acetate precipitation. Restricted DNA fragments were separated by agarose gel electrophoresis, and blotted onto charged nylon membranes by the alkali transfer procedure. Total RNA from *S. citri* cells, extracted by the guanidine thiocyanate/caesium chloride method (Chirgwin et al., 1979), was separated by electrophoresis on 1% (w/v) agarose formaldehyde 16:6% (v/v), MOPS 1× gel, and blotted onto C-extra membrane (Amersham). Hybridization with [α-32P]dATP-labelled probes was performed using standard stringency conditions (Sambrook et al., 1989). The fruR probe was the 0.4 kb HindII fragment obtained from plasmid pRAK (Gaurivaud et al., 2000). Expression of fructose operon genes was monitored using a fruA–fruK-specific probe (the 2.4 kb Hpal fragment from plasmid pRAK). DNA sequencing was performed with the T7 DNA sequencing kit (Pharmacia), [α-32P]dATP and appropriate primers. Topology prediction of membrane proteins was done by the HMM method (Sonnhammer et al., 1998). The sequence of the fructose operon has been deposited in GeneBank (accession number AF202663). The ProDom database was used to study protein-domain arrangements (Corpet et al., 1999). Multiple sequence alignment was done with the Multalin program (Corpet, 1989). Primers FruAI (AATTC-GCTTCTCGTTCGG) and FruK (CCCTTTCCACCAATC-ACC), corresponding to nucleotides 2054–2071 and 4350–4368, respectively, in the sequence of the fructose operon, and primers FruAI and Rev (GGAAACAGCTATGACCATG), corresponding to nucleotides 979–997 in the sequence of the pBS plasmid, were used in PCR amplification with 100 ng genomic DNA of *S. citri* in a 50 µl mixture containing 20 mM Tris/HCl pH 8.5, 10 mM (NH4)2SO4, 2 mM MgCl2, 100 µg BSA ml⁻¹, 0.1% (v/v) Triton X-100, 200 µM (each) deoxynucleoside triphosphate, 0.5 µM (each) of primers and 2.5 U *Tag* DNA polymerase (Gibco-BRL). Thirty-five cycles were performed using three steps (1 min/92 °C, 30 s/52 °C and 4 min/72 °C) in a thermocycler (Eurogenetec).

**Gene disruption of the fructose operon.** Plasmids H and E used for gene disruption of the fructose operon of wt strain GII-3 were constructed as follows. Plasmid pRAK (Gaurivaud et al., 2000) was partially digested with *Hpa*I. The 2.9 kb *Hpa*I fragment was cloned into plasmid pBS. The recombinant plasmid (pHinc2.9.1) was digested with BamHI and SpeI and the 2.9 kb fragment was cloned in plasmid pBOT digested with the same enzymes. The plasmid was named plasmid H. Plasmid pHinc2.9.1 was digested with BsrXI and treated with exonuclease III for different times, extremities were blunt-ended with S1 nuclease, and the plasmid was recircularized with T4 DNA ligase, yielding plasmid pEXO35. This plasmid is characterized by a deletion of the intergenic region between fruA and fruK, and a deletion of the 3′ extremity of fruA and the 5′ extremity of fruK. This plasmid was digested with BamHI and SpeI, and the resulting 2.5 kb fragment was cloned in pBOT, yielding plasmid E. The wt strain GII-3 was transformed with plasmids E and H. After selection on SP-4 plates supplemented with 2 µg tetracycline ml⁻¹ and multiplication of colonies in SP-4 liquid medium during 15 passages, integration of the plasmids into the chromosome was followed by Southern blots.

**Carbohydrate fermentation.** Catabolism of carbohydrate in *S. citri* results in lactic acid production. Acidification of the growth medium was used as an indicator of carbohydrate utilization. *S. citri* growth requires complex media supplemented with calf fetal or horse serum, the latter bringing carbohydrates and enzymes such as invertase which may interfere with fermentation of sugar added to the medium. For these reasons we used HSI medium [mycoplasma broth base 15 g l⁻¹, PPLO serum fraction 1% (v/v), sorbitol 7% (w/v), phenol red 30 mg l⁻¹, penicillin 2 l×10⁶ U ml⁻¹] (Whitcomb, 1983) for monitoring carbohydrate fermentation. In HSI, a sugar-free serum fraction (PPLO serum fraction) replaces the...
normal serum; thus, without sugar addition no fermentation is observed. *S. citri* cells growing exponentially in SP-4 medium were harvested by centrifugation (20 min, 12000 g, 20 °C), washed twice and resuspended with 8 mM HEPES pH 7-4, 10% (w/v) sorbitol. HSI medium supplemented with glucose, fructose or sorbitol (0-5% w/v) was inoculated with the washed cells. Decrease of pH was followed by the colour change of phenol red from red to yellow.

**Determination of the minimal inhibitory concentration of xylitol.** The MIC of xylitol was determined in HSI medium with glucose as carbon source. *S. citri* cells grown in SP-4 medium were harvested by centrifugation (20 min, 12000 g, 20 °C), washed twice and resuspended in 8 mM HEPES pH 7-4, 10% sorbitol. HSI medium supplemented with 0-5% glucose and xylitol at concentrations ranging from 0 to 8% (w/v) was inoculated with the washed cells and incubated at 32 °C for 1 week. The MIC of xylitol was the lowest concentration which prevented acidification of the medium.

**Enzyme assays.** Protein concentration was determined by the procedure of Bradford (1976), using the Bio-Rad protein assay kit. The activity of PTS was measured as described by Navas-Castillo et al. (1993). The 6-phosphofructokinase (6-PFK) and 1-Phosphofructokinase (1-PFK) activities were determined as described by Pollack (1995).

**RESULTS AND DISCUSSION**

**Isolation and characterization of spontaneous xylitol-resistant mutant xyl3 of *S. citri***

It is well known that xylitol enters the bacterial cell as xylitol 5-phosphate, via the same PTS as fructose or via a xylitol-specific PTS. As xylitol phosphate is not catabolized, it accumulates and a xylitol-specific PTS. As xylitol phosphate is not catabolized, it accumulates and undergoes a futile phosphorylation–dephosphorylation cycle and becomes toxic for the cell (Hausman et al., 1984; Trahan et al., 1991). The following experiments show that in *S. citri* xylitol sensitivity is directly related to fructose utilization. MICs of xylitol were found to be 0-5% for the wt strain GII-3 and at least 8% for mutant GMT 553, which is unable to use fructose. When mutant GMT 553 was complemented with fructose operon genes or gene combinations unable to restore fructose utilization (*fruR, fruR–fruA or fruK*) (Gaurivaud et al., 2000), the xylitol MIC remained higher than 8%. However, with the gene combination that fully restored fructose utilization, i.e. with the whole fructose operon (*fruR–fruA–fruK*), the MIC was as low as 0-5%, and it was 4% with the genes that only partially restored fructose utilization (*fruA* or *fruA–fruK*), the rate of fructose utilization being half that shown by the wt strain GII-3.

In order to select spontaneous xylitol-resistant mutants, wt strain GII-3 was inoculated into HSI medium containing 0-5% glucose and 2% xylitol, and incubated at 32 °C for 3 weeks, at the end of which time acidification of the medium eventually occurred. Spiroplasma cells were plated on solid SP-4 medium, and five colonies (xyl1 to xyl5) were tested for their resistance to xylitol. They showed a xylitol MIC higher than 8%. Fermentation of glucose or fructose, in HSI medium, showed that mutants xyl1, xyl2, xyl4 and xyl5 used glucose and fructose, whereas mutant xyl3 used glucose but not fructose. Fructose-PTS activity was not detected in mutant xyl3 whereas 1-PFK activity remained unchanged (Table 1). In these experiments Fru− mutants could be obtained in HSI medium in the presence of glucose and xylitol because the fructose operon of *S. citri* is constitutively expressed and is not catabolically repressed by glucose (F. Laigret and others, unpublished results). This is also evidenced by the fact that in SP-4 medium, which contains 0-5% added glucose, spiroplasma cells can be shown to possess an active fructose PTS (Table 1).

It is well established that the PTS is composed of two general energy-coupling proteins (Enzyme I and HPr) and a sugar-specific permease (Postma et al., 1993). The fact that glucose was used by mutant xyl3 indicated that enzyme I and HPr were functional, but the fact that fructose was not used suggested that the fructose permease, encoded by *fruA*, was probably mutated.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-PFK*</td>
</tr>
<tr>
<td>GII-3 (wt)</td>
<td>150</td>
</tr>
<tr>
<td>GMT 553</td>
<td>–</td>
</tr>
<tr>
<td>xyl3</td>
<td>111</td>
</tr>
<tr>
<td>H11</td>
<td>107</td>
</tr>
<tr>
<td>H31</td>
<td>–</td>
</tr>
<tr>
<td>H45</td>
<td>–</td>
</tr>
<tr>
<td>E38</td>
<td>–</td>
</tr>
<tr>
<td>E53</td>
<td>–</td>
</tr>
</tbody>
</table>

–, Not detected; nd, not done.
* nmol min⁻¹ (µg protein)⁻¹.
† nmol phosphorylated fructose (30 min)⁻¹ (mg protein)⁻¹.

† E53 shown that mutants xyl1, xyl2, xyl4 and xyl5 used glucose and fructose, whereas mutant xyl3 used glucose but not fructose. Fructose-PTS activity was not detected in mutant xyl3 whereas 1-PFK activity remained unchanged (Table 1). In these experiments Fru− mutants could be obtained in HSI medium in the presence of glucose and xylitol because the fructose operon of *S. citri* is constitutively expressed and is not catabolically repressed by glucose (F. Laigret and others, unpublished results). This is also evidenced by the fact that in SP-4 medium, which contains 0-5% added glucose, spiroplasma cells can be shown to possess an active fructose PTS (Table 1).
different from the corresponding region of the wt permease (Fig. 1) because of the frameshift introduced by the adenylic insertion. The hydrophobic domain IIC is involved in carbohydrate binding and penetration (Postma et al., 1993). The C-terminal extremity of domain IIC is important for the stability of the protein and/or its optimal insertion into the membrane as well as for carbohydrate binding, as demonstrated by deletion of the last transmembrane α-helix of the E. coli mannitol permease (Briggs et al., 1992). It is therefore not surprising that, in xyl3, alteration of the permease results in inactivation of the fructose-PTS. Unlike mutant xyl3, the four other xylitol-resistant mutants were able to use fructose. This could be due to a modification of the fructose permease, leading to discrimination between fructose and xylitol, or to a mutation in one of the several ABC transporter genes known to be present in S. citri (F. Laigret and others, unpublished result).

**Mutants with a disrupted fructose operon**

Functional complementation experiments with mutant GMT 553 showed homologous recombinations to occur between the fructose operon of the GMT 553 chromosome and homologous genes inserted in plasmid pBOT (Gaurivaud et al., 2000), in spite of the fact that this organism is RecA− (Marais et al., 1996). Complementation experiments also showed that strains of mutant GMT 553 complemented with fruK or fruK>fruA were unable to use fructose (Gaurivaud et al., 2000). Hence, inactivation of fruK or fruA>fruK by homologous recombination should be sufficient to abolish fructose utilization.

**Production of fructose-negative mutants by homologous recombination.** Two pBOT-derived plasmids carrying mutated versions of fruA>fruK were constructed (Fig. 2). In plasmid H, fruA carries a deletion of the first 99 bases, and fruK, a deletion of the last 105 bases. In plasmid E, fruA and fruK carry deletions of both 5′ and 3′ ends, and the intergenic region is deleted. After transformation of the wt strain GII-3 with plasmid E or H, 60 colonies from each transformation experiment were propagated during 15 passages in SP-4 medium supplemented with 2 μg tetracycline ml−1. The culture from each colony was screened for fructose fermentation and glucose fermentation in HSI medium, and for resistance to 2% xylitol.

---

**Fig. 1.** Sequence alignment between domain IIC of protein FruA deduced from the fruA gene of wt strain GII-3 (FruAWT) and mutant xyl3 (FruAxyl3). Putative transmembrane domains (TMD1 to TMD8) are indicated as black boxes. Putative intracellular (I1, I2 and I3) and extracellular (E1, E2, E3 and E4) loops are underlined. Asterisks show amino acid residues different between the two sequences. The arrow indicates the position of the mutation from glycine in FruAWT or FruAxyl3 to glutamic acid in FruA of strain H31.
Fructose-negative mutants of Spiroplasma citri

Fig. 2. Disruption of the fructose operon by homologous recombination with one crossing-over, using plasmid E (a) or plasmid H (b). The putative fructose operon promoter (PFRU) and terminators (T) are indicated. Hp, HpaII restriction site.

Fig. 3. Southern blot hybridization between probe fruR and Hpall-digested genomic DNA from wt strain GII-3 (lanes 1 and 7), and strains E38 (lane 2), H31 (lane 3), E53 (lane 4), H45 (lane 5) and H11 (lane 6).
The following experiment conducted with strain H31 demonstrated that, in addition, integration has resulted in duplication of fruA–fruK with the pBOT sequence in between the two copies, as represented in Fig. 2. DNA from strain H31 was digested with Bsp119i or Bsp119i + SmaI and hybridized with the fruA–fruK probe. Since there are no Bsp119i restriction sites within the fructose operon nor in pBOT, only one restriction fragment was obtained with Bsp119i alone (data not shown). SmaI has no restriction site in the fructose operon and only one in pBOT. Therefore two fragments were obtained when both Bsp119i and SmaI were used together (data not shown). These experiments indicate that the two copies of fruA–fruK are separated by pBOT.

Transcription of the fructose operon genes in the recombinational mutant H31. As shown in Fig. 2, the recombinational mutants contain potentially active fruA and/or fruK genes. For instance, in mutant H31 (Fig. 2b) a complete fruA gene is present downstream of the fructose operon promoter FPRU, and a complete fruK gene occurs downstream of the pBOT plasmid sequences. However, as indicated above (Table 1) no fructose-PTS activity and no 1-PFK activity could be detected in any of the E or H mutants studied. To confirm this result, expression of fruA and fruK was assessed by Northern hybridization experiments. Hybridization of total RNAs from the wt strain GII-3 with a fruA–fruK-specific probe revealed the two typical 2.8 kb and 3.8 kb RNA messengers of the fructose operon (Fig. 2b, wt, and Fig. 4, lane 2). With strain H31 (Fig. 2b), a 2.8 kb transcript was also detected, but the 3.8 kb message was replaced by a 5.2 kb RNA (Fig. 4, lane 1), corresponding to fruK, fruA, fruK and part of the pBOT plasmid, since there is no transcription terminator at the end of the truncated fruK gene. No evidence was obtained indicating that transcription of the fruA–fruK copy downstream of pBOT occurred.

Only the copy under the control of the fructose operon promoter was transcribed and thus both the 2.8 kb and the 5.2 kb transcripts contain the fruA sequences. However, no fructose-PTS activity could be detected (Table 1), suggesting that the fruA gene was inactivated by mutation. Indeed amplification of fruA in strain H31 with primers FruA1 and Rev and sequencing revealed that glycine at position 410 of the wt fructose permease was replaced by glutamic acid. This substitution corresponds to a single mutation of GGA to GAA. Glycine at position 410 is well conserved among fructose permeases and is located in the putative transmembrane domain TMD3 (Fig. 1). Probably the presence of a charged and polar residue such as glutamic acid strongly modifies the TMD3 domain and must prevent fructose permease from functioning.

Conclusion

We have used two approaches to obtain fructose operon mutants. In the first, we produced spontaneous XylR mutants and selected those which were Fru−. Mutant xyl3 is such a XylR Fru− strain. In xyl3, the fructose-PTS was undetectable, confirming that in S. citri fructose and xylitol have the same PTS. This result was already known from our first fructose operon mutant, transpositional mutant GMT 553, where xylitol resistance was also associated with lack of fructose-PTS activity. This situation is similar to that in E. coli (Reiner, 1977) and Streptococcus mutans (Trahan et al., 1983), but different from that in Lactobacillus casei Cl-16 (London & Hausman, 1982; Hausman et al., 1984) where a xylitol-specific PTS occurs.

The second approach consisted in fructose operon gene disruption by homologous recombination between fruA–fruK of the chromosomal fructose operon and partially deleted fruA–fruK carried by replicative plasmid pBOT. Recombinational mutants were obtained in which two copies of disrupted fruA–fruK genes were present in the sriplasmal chromosome in the following order: fructose operon promoter/copy 1/pBOT plasmid/copy 2 (Fig. 2). This is characteristic of homologous recombination involving one crossing-over. Only copy 1 is transcribed. This copy may contain a complete fruA gene (see Fig. 2) which can thus be transcribed. A complete fruK gene can be present in copy 2, but copy 2, and thus fruK, is not transcribed. Therefore, in certain mutants, where fruA, but not fruK, can be transcribed and translated, fructose-PTS activity could occur in the absence of 1-PFK activity. This activity would result in accumulation and toxicity of fructose 1-phosphate in the sriplasmal cell as no 1-PFK activity is present to remove fructose 1-phosphate. Toxicity of fructose 1-phosphate has been described for E. coli (Ferenci & Kornberg, 1973) and Bacillus subtilis (Gay & Rapoport, 1970), precisely in strains lacking 1-PFK activity. In fact with our recombinational mutants, we have not observed such toxicity. In the case of mutant H31, which has been studied in more detail, a complete fruA gene was transcribed from copy 1 but in spite of this no active...
fructose permease occurred as judged from the absence of fructose-PTS activity. Interestingly, it could be shown that a mutation affected the permease gene, rendering the enzyme inactive. Probably, mutation of the *fruA* gene is a way for the spiroplasma cell to prevent fructose-1-phosphate toxicity. We believe that such *fruA* mutations are not restricted to strain H31, but probably occur in other mutants with a complete *fruA* gene in copy 1 in the absence of a complete *fruK* gene.

ACKNOWLEDGEMENTS

We are grateful to J. G. Tully for the gift of PPLO serum fraction. This work was financially supported in part by grants from the Conseil Régional d’Aquitaine (no. 960307003), Ministère de l'Enseignement Supérieur et de la Recherche and by the AIP Microbiologie Fondamentale grant from INRA (no. P00188). Support for P. Gaurivaud was provided by the Ministère de l'Enseignement Supérieur et de la Recherche. General support from CIRAD–FHLOR is gratefully acknowledged.

REFERENCES


Received 5 May 2000; accepted 30 May 2000.