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RESEARCH ARTICLE

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Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette

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Abstract In *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) multiple gene deletions are limited by the number of readily available selection markers. We have therefore constructed a blaster cassette which enables successive gene knock-outs in *H. jecorina*. This 3.5 kb *pyr4* blaster cassette contains the *H. jecorina pyr4* marker gene encoding orotidine-5′-monophosphate (OMP) decarboxylase flanked by two direct repeats of the *Streptoalloteichus hindustanus* bleomycin gene (*Sh ble*), which facilitate the excision of the blaster cassette by homologous recombination after each round of deletion. Functionality of this *pyr4* blaster cassette was demonstrated by deletion of the *glk1* encoding glucokinase and *hxk1* encoding hexokinase. 1.4–1.8 kb of the non-coding flanking regions of both target genes were cloned into the respective blaster cassettes and transformation of a *pyr4* negative *H. jecorina* strain with the two cassettes resulted in 10–13% of the transformants in the deletion of one of the two kinase genes. For excision of the *pyr4* blaster cassettes, Δ*glk1* strains were selected for growth in the presence of 5-fluoroorotic acid. Recombination between the two *Sh ble* elements resulted in uridine auxotrophic strains which retained their respective glucokinase negative phenotype. Subsequent transformation of one of these auxotrophic Δ*glk1* strains with the hexokinase blaster cassette resulted in *pyr4* prototrophic strains deleted in both *glk1* and *hxk1*. Δ*glk1* strains showed reduced growth on d-glucose and d-fructose whereas Δ*hxkl* strains showed reduced compact growth on d-glucose but were unable to grow on d-fructose as carbon source. The double Δ*glk1*Δ*hxk1* deletion strain was completely unable to grow on either d-glucose or d-fructose.

Keywords *Trichoderma reesei* · Hexokinase · Glucokinase · 5-FOA · Transformation · *Pyr4*

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Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers DQ068384 (*H. jecorina glk1*) and DQ068385 (*H. jecorina hxk1*).

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Introduction

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is industrially applied for the production of enzymes including a number of (hemi)cellulases and its strong cellobiohydrolase promoters are used for the expression of recombinant proteins (Penttilä 1998). Although a sexual cycle of *H. jecorina* has been described (Kuhls et al. 1996), most of the research and all of the industrial application are performed almost exclusively with a single asexual isolate *H. jecorina* QM6a from the Solomon Islands and its derivatives (Kubicek and Harman 1998). Functional genomic studies in *H. jecorina* depend on an efficient targeted gene manipulation system and the construction of defined mutants for the investigation of gene function. DNA mediated transformation in *H. jecorina* is integrative and relies on a limited number of dominant markers and auxotrophic markers. These include the *Escherichia coli hph* (hygromycin B phosphotransferase), the *E. coli* and *Strepotalloteichus hindustanus ble* (bleo/phleomycin resistance), the *Aspergillus nidulans amdS* (acetamidase) or the *H. jecorina pyr4* (for a review see Mach 2004).

Traditional DNA mediated transformations are limited in terms of the number of marker genes which can be inserted. This fact restricts studies of e.g. the function of orthologous and paralogous genes or of whole gene families. Therefore the development of a versatile transformation system independent on the number of available markers would be beneficial. In yeasts, so called blaster cassettes were developed which allow the repeated use of the *URA3* (the yeast *pyr4*

Table 1 Oligonucleotide list

Name	Sequence[a]
zeo1fw	5′-GATCTCTAGAACCATGGCCAAGTTGACCAG-3′
zeo1rv	5′-GATCCTCGAGTCAGTCCTGCTCCTCGG-3′
zeo2fw	5′-GATCCTCGAGACCATGGCCAAGTTGACCAG-3′
zeo2rv	5′-GATCGGATCCTCAGTCCTGCTCCTCGG-3′
Gluco5′F	5′-GATCGAATTCAAAGCAGCAGAACAACG-3′
Gluco5′R	5′-CTAGGGATCCTCCGAAAAGTCGAACTG-3′
Gluco3′F	5′-GATCTCTAGATAAGGTACTGACCATGTC-3′
Gluco3′R	5′-GATCTCTAGAGAATTCCAGCAGCACAACATATAC-3′
Hexo5′F	5′-GATCGAATTCTATGAGGTACGTATGTAG-3′
Hexo5′R	5′-GATCGGATCCATGGTGGTCAGTATTTTC-3′
Hexo3′F	5′-CTAGAAGCTTTAGATTTGGAACATGTTTGTC-3′
Hexo3′R	5′-GATCAAGCTTGAATTCAAGTTGGGCAG-3′

[a] Respective restriction sites are underlined

homologue) marker to construct multiple disrupted strains (Alani et al. 1987; Fonzi and Irwin 1993). Such blaster cassettes consist of the *URA3* encoding the orotidine-5′-decarboxylase flanked by two direct repeats. Mutants which are defective in *URA3* are auxotrophic for uridine (uracil), but are—in contrast to prototrophic strains—resistant to 5-fluoroorotic acid (5-FOA; Boeke et al. 1984), which is converted by orotidine-5′-monophosphate (OMP)-decarboxylase to the toxic intermediate 5-fluoro-UMP. Integration of the blaster is therefore selected via Ura3 function and excision of the *URA3* marker is then forced in the presence of 5-FOA by recombination between the two direct repeats. As a consequence, this blaster cassette can be reused for successive rounds of gene deletions, allowing multiple deletions with a single cassette. This cassette has permitted successive disruption of *C. albicans* alleles (reviewed in Pla et al. 1996) and even families of genes (Mio et al. 1996; Muhlschlegel and Fonzi 1997; Sanglard et al. 1997) with a single auxotrophic marker. In filamentous fungi, a similar blaster cassette was successfully applied for the deletion of *rodA* in the opportunistic pathogen *A. fumigatus* (d'Enfert 1996) and *aroC* in *A. nidulans* (Krappmann and Braus 2003).

We developed a blaster cassette for multiple gene deletions in *H. jecorina* based on the *H. jecorina pyr4* flanked by direct repeats of the *S. hindustanus ble*. The functionality of the blaster cassette for successive gene deletion is demonstrated by the construction of stable *H. jecorina* strains deleted in the gluco- or hexokinase encoding genes and the reuse of the *pyr4* blaster to construct double knock-out strains.

Materials and methods

Strains and culture conditions

Hypocrea jecorina strain QM9414 (ATCC 26921) and its uridine auxotrophic *pyr4* mutant TU-6 (ATCC MYA-256) (Gruber et al. 1990b) were maintained on malt extract agar (Merck, VWR International, Austria) or potato dextrose agar (Difco, BD Biosciences, Schwechat, Austria) supplemented with 10 mM uridine when necessary. Fungal cultures were grown at 30°C in a medium described by (Mandels and Andreotti 1978). Fungal growth on different carbon sources was determined by placing a small piece of agar ($d = 0.5$ cm) in the centre of each agar plate. *Escherichia coli* strain JM109 (Promega, Madison, WI,USA.) was used for plasmid propagation.

Identification and sequence analysis of the *H. jecorina glk1* (encoding glucokinase) and *hxk1* (encoding hexokinase)

A tblastn search of the *T. reesei*/*H. jecorina* QM6a genome sequence (http://gsphere.lanl.gov/trire1/trire1.home.html) with the *Aspergillus niger* glucokinase (GenBank accession no. CAA67949) and hexokinase (GenBank accession no. CAA08922) proteins as query identified single orthologues for each gene. The deduced aa sequence of the *H. jecorina* glucokinase encoding gene (*glk1*) and the hexokinase encoding gene (*hxk1*) showed 58% sequence identity to the *A. niger* GlkA, and 73% to the *A. niger* HxkA, respectively. The two kinase genes were amplified by PCR with oligonucleotide pair Gluco5′F and Gluco3′R, as Hexo5′F as Hexo3′R respectively from *H. jecorina* QM9414 genomic DNA and sequenced (Table 1).

Plasmid constructions

The *pyr4* blaster cassette was constructed by inserting the *H. jecorina pyr4* (Gruber et al. 1990a) gene between two *S. hindustanus Sh ble* fragments orientated as direct repeats. Therefore the *Sh ble* was amplified twice from the plasmid pPICZB (Invitrogen, Vienna, Austria) using two primer pairs and introducing the

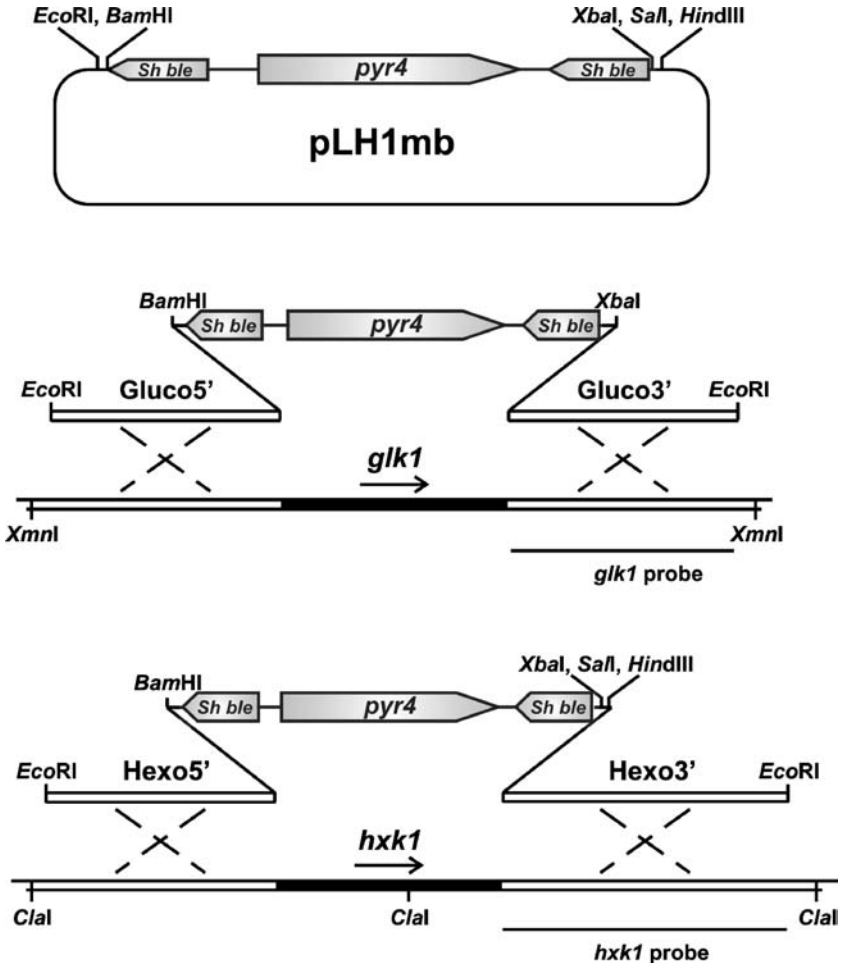

Fig. 1 Schematic representation of the *pyr4* blaster cassette pLH1mb and gene replacement at the *H. jecorina glk1* and *hxk1* loci. pLH1mb contains the *H. jecorina pyr4* gene flanked by two direct repeats of a *S. hindustanus ble* fragment. Orientation of the different genes is indicated by *arrows*. Important restriction enzyme sites which are useful for cloning of the up and downstream regions of the target genes into pLH1mb, for the release of the blaster cassettes from the vector or for the Southern analyses are also indicated. Positions of the probes for *glk1* and *hxk1* are indicated

following restriction sites (given in brackets): zeo1fw (*Xba*I) and zeo1rv (*Xho*I), respectively zeo2fw (*Xho*I) and zeo2rv (*Bam*HI). The resulting 380 bp amplicons were digested with *Xba*I/*Xho*I and *Bam*HI/*Xho*I respectively and ligated into an *Xba*I/*Bam*HI digested pUC19 (Yanisch-Perron et al. 1985). The resulting vector containing the two *Sh ble* gene fragments as direct repeat was digested with *Xho*I to insert a 2.7 kb *Sal*I *H. jecorina pyr4* fragment resulting in the 6.2 kb blaster plasmid pLH1mb.

About 1.4 kb of the 5′ and 3′ region of *glk1* were amplified using the primer pairs which introduced the following restriction sites: gluco5′F (*Eco*RI) and gluco5′R (*Bam*HI), gluco3′F (*Xba*I) and gluco3′R (*Xba*I and *Eco*RI). The *Eco*RI/*Bam*HI restricted 5′ region fragment was ligated into the *Eco*RI/*Bam*HI sites of pLH1mb. Next, the *Xba*I restricted 3′ region of *glk1* was inserted into the *Xba*I site resulting in the final vector pΔ*glk1*.

About 1.4 kb of the 5′ and 1.8 kb of the 3′ region of *hxk1* were amplified using the primers hexo5′F (*Eco*RI) and hexo5′R (*Bam*HI), respectively hexo3′F (*Hin*dIII) and hexo3′R (*Hin*dIII plus a natural *Eco*RI site). The *Eco*RI/*Bam*HI digested 5′ region was ligated into the respective sites in pLH1mb following the insertion of the 3′ region of *glk1* into the *Hin*dIII site resulting in pΔ*hxk1*.

Transformation of *H. jecorina*

Protoplast preparation and DNA mediated transformation was described by (Gruber et al. 1990b). For deletion of the *glk1* and *hxk1* the blaster cassettes (about 6.2 and 6.6 kb) were (1) excised from pΔ*glk1 and* pΔ*hxk1* with *Eco*RI. Fragments were purified from agarose gels (QIAquick Gel Extraction Kit, VWR International, Vienna, Austria). After transformation protoplasts were stabilized and regenerated on minimal medium plates containing d-sorbitol (1 M). d-glucose as carbon source was replaced by glycerol or l-arabinose to prevent a negative selection for strains deleted in one of the kinase genes. After 4–5 days colonies were transferred to minimal medium without d-sorbitol for sporulation. Conidia were usually obtained after 3–4 days and purified on minimal medium plates containing the colony restrictor Triton X-100 (0.1% v/v) and peptone (0.1% w/v) which accelerates germination. After 1.5 days single colonies were picked and transferred to minimal medium for sporulation.

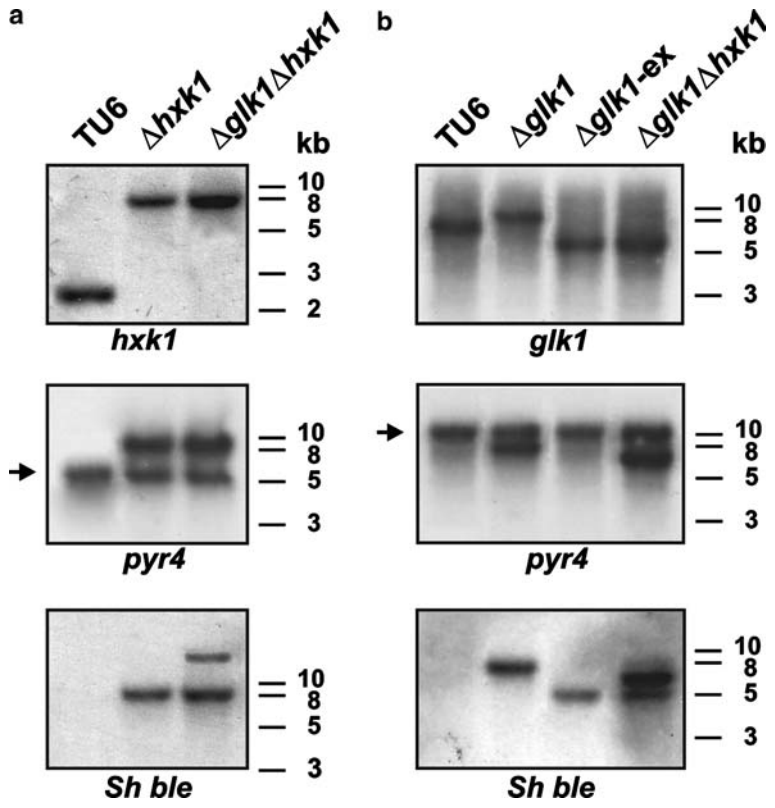

Fig. 2 Southern analyses of *H. jecorina* gluco- and hexokinase negative strains. The endogenous non-functional copy of the *pyr4* is marked by an arrow. **a** Genomic DNA of the parental strain TU-6, a Δ*hxk1* and a Δ*glk1*Δ*hxk1* strain were digested with *Cla*I and probed with *hxk1*, *pyr4* and *Sh ble* fragments. Insertion of the blaster cassette at the *hxk1* locus leads to an increase of the hybridizing band from 2.7 kb in strain TU-6 to 8.7 kb in the Δ*hxk1* and Δ*glk1*Δ*hxk1* using the *hxk1* fragment as probe. This 8.7 kb band is also detected with the *pyr4* or *Sh bl* fragments as probe. In the Δ*glk1*Δ*hxk1* strain an additional weaker hybridizing fragment is found with the *Sh ble* fragment as probe which corresponds to a single *Sh ble* fragment resulting from the excision of the glucokinase blaster cassette. **b** Genomic DNA of strain TU-6, a Δ*glk1*, a glucokinase blaster excised strain Δ*glk1*-ex and a Δ*glk1*Δ*hxk1* strain were digested with *Xmn*I and probed with the respective fragments. Homologous insertion of the glucokinase blaster cassette leads to an increase of the hybridizing band from 7.1 kb in the TU-6 strain to 8.8 kb in the Δ*glk1* strain when probed with the *glk1* fragment. This 8.8 kb band is also detected with the *pyr4* or *Sh ble* probe. In strain Δ*glk1*-ex the *glk1* and *Sh ble* hybridizing band is reduced to 5.5 kb due the excision of the glucokinase blaster. In strain Δ*glk1*Δ*hxk1* the *glk1* band is also reduced to 5.5 kb, but an additional stronger *Sh ble* hybridizing band resulting from two *Sh ble* fragments of the hexokinase blaster and a *pyr4* hybridizing band are found

Excision of the *pyr4* blaster cassette

Two to three day old spores were suspended in 0.9% (w/v) NaCl and 0.05% (w/v) Tween 80, filtered through glass wool to remove residual hyphae. 0.9×10^7 –1.5×10^7 conidia were plated on minimal medium plates containing 5-FOA (1.5 g/l; Fermentas, St. Leon-Rot, Germany), peptone (0.1 g/l) and 10 mM uridine. 5-FOA resistant colonies were obtained after 3–4 days and transferred to minimal medium containing uridine for sporulation. Purified conidia were then tested for uridine auxotrophy on minimal medium plates.

Fungal DNA isolation and hybridization

DNA was prepared from *H. jecorina* strains grown for about 24–30 h in 100 ml flasks on a rotary shaker (250 rpm) at 30°C. Mycelia were harvested by filtration, washed with cold sterile tap water, blotted dry between paper towels, and ground to a fine powder under liquid nitrogen. Powdered mycelia was suspended in buffer A (0.1 M Tris–HCl, pH 8.0, 1.2 M NaCl, 5 mM EDTA), incubated for 20 min at 65°C, cooled down on ice, mixed with 0.5 v phenol and 0.5 v chloroform and centrifuged (12,000 rpm, 15 min). Following a chloroform (1 v) extraction, DNA was precipitated with 1 v of isopropanol and washed with 70% (v/v) ethanol. Standard methods (Sambrook and Russel 2001) were used for DNA electrophoresis, blotting, and hybridization of DNA. Probes labelled with [α^{32} P]dCTP by random priming were: a 1.4 kb *Xba*I *glk1*, a 1.8 kb *Hin*dIII *hxk1*, a 2.7 kb *Sal pyr4* fragment and a 380 bp *Sh ble* amplicon.

Results

Construction of a *pyr4* blaster cassette for sequential targeted gene deletions

A blaster cassette containing the *H. jecorina pyr4* gene flanked by two gene fragments of the *S. hindustanus*

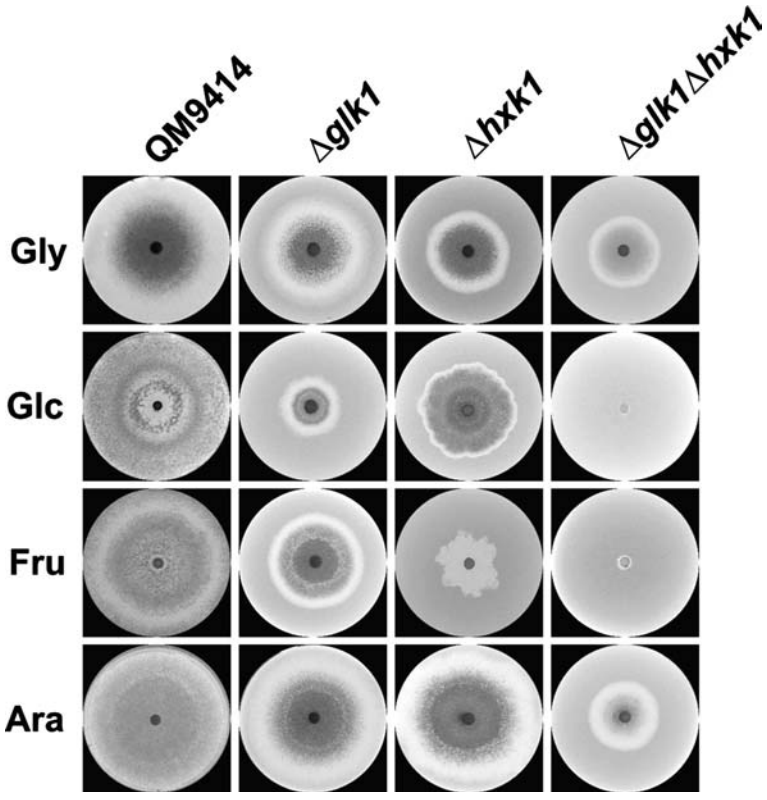

Fig. 3 Growth comparison of QM9414, a Δ*glk1*, a Δ*hxk1* and a Δ*glk1*Δ*hxk1* strain after 3.5 days on different carbon sources. The growth behaviour of QM9414 or TU-6 strains with an ectopically integrated hexokinase or glucokinase cassette was essentially the same. Abbreviations:*Gly* glycerol,*Glc* d-glucose, *Fru* d-fructose and *Ara* l-arabinose

Sh ble was constructed (Fig. 1). The two *Sh ble* elements were orientated as direct repeats to facilitate the excision of the *pyr4* marker by loop-out between the homologous regions after a successful gene deletion. On each side of the blaster cassette unique restriction sites (*Eco*RI, *Bam*HI, *Xba*I, *Sal*I and *Hin*dIII) were located to enable the insertion of the up- and downstream regions of the target genes. Starting from this *pyr4* blaster cassette we constructed two different blaster cassettes for deletion of the *H. jecorina* glucokinase (*glk1*) and hexokinase (*hxk1*) encoding genes by amplification of their noncoding regions by PCR. Appropriate restriction sites were introduced at the end of each fragment by PCR to facilitate the ligation of these fragments into the *pyr4* blaster cassette. The final glucokinase blaster pΔ*glk1* contained 1.4 kb of each flanking regions of the *glk1* coding region, while the hexokinase blaster pΔ*hxk1* contained 1.4 kb of the up- and 1.8 kb of the downstream region of the *hxk1* coding region. In addition, the final blaster cassettes for the two genes were constructed in such a way that they could easily be excised by a single *Eco*RI digest. Alternatively, the respective cassette can be amplified by PCR using the primer pair located in the up- and downstream regions.

Deletion of *glk1* encoding glucokinase and *hxk1* encoding hexokinase in *H. jecorina*

The functionality of the blaster approach for *H. jecorina* was tested by deletion of two genes encoding hexose phosphorylating enzymes in the uridine auxotrophic *pyr4* negative strain TU-6. Although *A. nidulans* strains lacking hexokinase or glucokinase grew well on d-glucose containing media (Flipphi et al. 2003), we replaced d-glucose in the protoplast regeneration plates by either glycerol or l-arabinose to avoid any possible negative selection for homologous integrated blaster cassettes. Glycerol was chosen because it is channelled into glycolysis after the hexose phosphorylation steps while the pentose l-arabinose is catabolized by a path not involving glycolytic enzymes (Chiang and Knight 1961). *H. jecorina* TU-6 was transformed with the two blaster fragments and the resulting transformants were selected for uridine prototrophy on minimal medium. Purified transformants were tested for growth on a number of carbon sources including d-glucose, d-fructose, glycerol, and l-arabinose to select for putative gene knock-outs. Southern analysis confirmed the *glk1* or *hxk1* deletions (Fig. 2a, b). 13% of the total number of glucokinase and 10% of the hexokinase transformants showed a deletion at the respective gene locus. Hybridization with the coding region of the respective genes confirmed their complete removal. Growth tests on a number of carbon sources showed that Δ*glk1* strains showed reduced growth on d-glucose and d-fructose, whereas Δ*hxk1* strains showed reduced compact growth on d-glucose and were unable to grow on d-fructose (Fig. 3). However, growth of both deletion strains was also affected on glycerol or l-arabinose indicating a pleiotropic effect resulting from these deletions.

Excision of the *pyr4* blaster in the Δ*glk1* strain

A successful re-use of the blaster cassette depends on the excision of the *pyr4* marker by recombination between the flanking *Sh ble* direct repeats. We chose three Δ*glk1* strains and plated their conidiospores on 5-FOA plates to force and select for the excision of the *pyr4* blaster. 5-FOA resistant colonies appeared after 3–5 days of incubation with a frequency of about $1–2\times10^{-4}$. As these colonies did not sporulate, 5-FOA resistant colonies were transferred to minimal medium plates containing uridine which allowed also the growth of strains in which the blaster cassette did not loop out. Purified colonies were then tested for uridine auxotrophy on minimal medium. About 90% of the 5-FOA resistant strains picked were found to be uridine auxotroph while the remaining 10% were uridine prototroph. A Southern analysis showed that only a single copy of the *Sh ble* fragment was left in the auxotrophic strains and that the *pyr4* from the blaster cassette was completely removed, but the Δ*glk1* genotype retained (Fig. 2b). Sequencing of a PCR fragment comprising the disrupted *glk1* locus confirmed that only a single *Sh ble* fragment was left in these strains. The *Sh ble* fragment was bordered by two restriction enzyme sites for *Xba*I and *Bam*HI which could only originate from a recombination between the two original *Sh ble* fragments from the blaster cassette.

Deletion of *hxk1* in the Δ*glk1* strain

Following the successful excision of the blaster cassette, we demonstrated its reuse in a second round of gene deletion. We chose to construct a Δ*glk1*Δ*hxk1* strain and transformed therefore five of the uridine auxotrophic Δ*glk1* strains – obtained after the blaster cassette excision – with the hexokinase blaster. All five Δ*glk1* strains could be transformed to uridine prototrophy, indicating that their auxotrophy was indeed a result of the excision of the *pyr4* blaster and not due to other mutations. l-arabinose was used in the protoplast regeneration medium since we expected the double mutant to be unable to grow on d-glucose. Transformants were purified and subjected to growth tests and Southern analyses (Figs. 2, 3). About 12% of the transformants turned out to be double deleted Δ*glk1*Δ*hxk1* strains. They were completely unable to grow on d-glucose and d-fructose, and showed a stronger reduced growth on glycerol and l-arabinose.

Discussion

Complementation of uridine auxotrophic *pyr4* mutants to prototrophy is probably the most successful strategy for gene manipulation in filamentous fungi but is limited by the one-time use of the *pyr4* as marker gene. Here, we successfully overcame this limitation by applying a blaster approach for successive gene knock-outs in *H. jecorina* using a single marker. The *pyr4* blaster cassette was successfully excised by selection for resistance to 5-FOA in Δ*glk1* strains and could be re-used to construct Δ*glk1*Δ*hxk1* strains. The frequency of recombination between the two 380 bp *Sh ble* fragments was with $1–2\times10^{-4}$ in about the same range as reported for *A. fumigatus* (4×10^{-4}; d'Enfert 1996) and for *A. nidulans* (2×10^{-4}; Krappmann and Braus 2003), which allowed a straight forward selection of *pyr4* negative strains resulting from the looping out of the blaster cassette. 5-FOA resistance can in principle result from mutations in at least two genes: orotate phosphoribosyltransferase (*pyr2*) and OMP decarboxylase (*pyr4*). 90% of the obtained 5-FOA resistant colonies were uridine auxotrophic, while the remaining 10% were prototrophic. This was most probably the result of the transfer of the colonies to non-selective medium which was done to facilitate their sporulation. Transformation of five randomly chosen auxotrophic strains with the hexokinase blaster showed that all five strains could be complemented with the *pyr4* gene proving that the uridine auxotrophy was the result of the *pyr4* excision.

In *H. jecorina*, research is focused on its anamorph form *T. reesei*. The lack of research with the sexual form prevented characterization of auxotrophic mutants, and therefore transformation strategies that involve the conversion of auxotrophic mutants to prototrophy are only poorly developed in *H. jecorina*, while they are well established in other fungal species including *S. cerevisiae* or *A. nidulans*. The successful application of the blaster cassette system to the anamorph of *H. jecorina*, provides therefore an interesting opportunity to accelerate functional genomics in this fungus especially in the view of the recent release of a draft version of the *H. jecorina* genome (http://gsphere.lanl.gov/trire1/trire1.home.html). Although it is at the moment illusive to target all putative genes in *H. jecorina*, we think that our approach is especially valuable for the investigation of the function of fungal, specifically, *H. jecorina* specific genes. Our interest is directed towards paralogous genes which have developed during evolution from their ancestral genes by gene duplication and often tend to evolve toward functional diversification. It may also aid in the investigation of the function of whole gene families: *H. jecorina* is an excellent producer of extracellular enzymes secreting a high number of e.g. cellulases or xylanases most of which have not yet been functionally characterized. A search of the *H. jecorina* genome sequence database reveals the presence of a high number of additional biomass degrading enzymes including genes encoding for cellulases, xylanases, pectinases or chitinases.

The blaster system offers also an application for the construction of industrial *Hypocrea*/*Trichoderma* strains. As a producer of low cost enzymes and recombinant

proteins for a number of applications, genetic transformation systems are desired which do not lead to the accumulation of antibiotic resistance marker. Although the strains used in this study still carry a single antibiotic resistance marker after excision of the *pyr4* blaster, it should be possible to replace the *Sh ble* direct repeat by an autochthonous *H. jecorina* sequence.

Here, we applied the blaster system to *H. jecorina*, but it can easily be adapted to other *Trichoderma* or fungal species in general, especially for those in which classical genetic approaches are not practicable. The range of organisms seems to be limited only by the availability of OMP decarboxylase negative strains. Such strains can be obtained by classical mutagenesis approaches and selection on 5-FOA (Gruber et al. 1990b). Fungal OMP decarboxylase genes are highly conserved and work therefore also in heterologous systems (cf: Gruber et al. 1990b; d'Enfert 1996; Punt et al. 2001). It is therefore possible to construct multiple disrupted strains with this blaster cassette in any fungal species which is efficiently transformed by the *H. jecorina pyr4* gene.

In *A. nidulans*, glucose-, hexokinase mutants and double mutants were obtained by classical mutagenesis (Roberts 1963; Flipphi et al. 2003). Although the growth phenotype of *A. nidulans hxk*A1 (hexokinase deficient, formerly designated *fr*A1 for fructose non-utilizing) mutant (Roberts 1963; Ruijter et al. 1996) is comparable to the *H. jecorina* Δ*hxk1* by being unable to grow on d-fructose and that both *H. jecorina* and *A. nidulans* double mutants are unable to grow on d-glucose and d-fructose, we noted differences in the utilization of the other carbon sources tested. While the *A. nidulans* glucokinase and hexokinase single mutants exhibit no other nutritional deficiencies, we found that in *H. jecorina* both kinase genes are necessary for fast growth on a number of carbon sources in *H. jecorina* including l-arabinose or glycerol.

d-fructose inhibited the growth of the *hxk*A1 mutant on other sugars (Roberts 1963) and Ruijter et al. 1996) showed that d-fructose and d-mannitol inhibited growth of this mutant on l-arabinose. This observation could be explained by repression of enzymes involved in l-arabinose catabolism by d-fructose and d-mannitol: Although both are not metabolized in the absence of hexokinase, their accumulation might be able to at least partially repress the synthesis of enzymes necessary for metabolism of other carbon sources.

The availability of the three isogenic mutants constructed in this study will also allow the study of the role of the two hexose phosphorylating enzymes in the signalling of carbon catabolite repression and in a second path of d-galactose utilization besides the classical Leloir pathway in *H. jecorina* (Seiboth et al. 2004). In *A. nidulans* only the double mutant is impaired in d-glucose and d-fructose repression for ethanol and acetate catabolism and xylan degradation (Flipphi et al. 2003). In *H. jecorina*, so far only a single carbon catabolite derepressed mutant which has a truncated *cre1* gene was described (Ilmen et al. 1996). With respect to d-galactose utlization in *A. nidulans* Fekete et al. (2004) showed that a double mutant in the galactokinase (which catalyzes the first step in the Leloir pathway of d-galactose) and hexokinase is unable to grow on d-galactose as single carbon source. Although differences in the catabolic pathways for d-galactose in these two fungi might exist, it is likely that the second pathway of d-galactose utlization in *H. jecorina* proceeds also via d-fructose involving hexokinase.

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